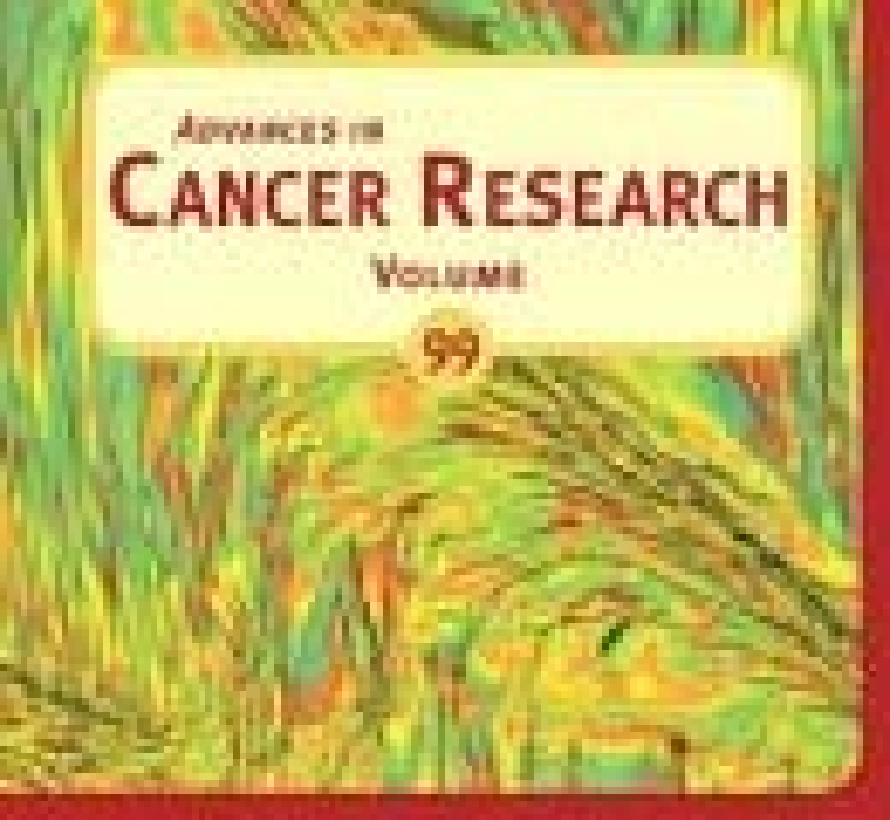

Edited by

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FOUNDATIONS IN CANCER RESEARCH
The Turns of Life and Science

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Comme Sodome et Gomorre puissez tomber en soulfre en feu & abysme, en cas que vous ne croyez fermement tout ce que je vous racompteray en ceste presente chronicque.
Rabelais, F.: Gargantua and Pantagruel

Like those of Sodom and Gomorrah, may you fall into sulphur, fire and bottomless pits, in case you do not firmly believe all that I shall relate unto you in this present Chronicle.
Rabelais, F.: Gargantua and Pantagruel (translated by Sir Thomas Urquhart of Cromarty and Peter Antony Morfeus)

This chapter provides a personal insight into the scientific and social atmosphere in former Czechoslovakia. It covers the period of the rise of Hašek's immunologic school and application of immunologic tolerance to *Rous sarcoma virus* (RSV) heterotransmission. These approaches permitted establishment of a new model of mammalian cells transformed by RSV (virogenic XC cells), where the noninfectious viral genome was kept indefinitely as new genetic information (provirus). RSV was rescued from nonpermissive mammalian cells by fusion (complementation) with permissive chicken fibroblasts; this opened the way to understanding virus nonpermissiveness. Mammalian cells transformed by the reverse transcript of v-*src* mRNA were characterized, and the resulting provirus was shown to be highly oncogenic for chickens and to carry tumor-specific transplantation antigen. Other areas covering epigenetic reversion of RSV-transformed cells and long-term persistence of chicken leucosis viruses in foreign avian species are discussed.

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I. TO WRITE OR NOT TO WRITE?

Since I received the invitation from George Klein to write my recollections, I have been postponing my final decision for 12 years. Several factors influenced my hesitation. I did not feel old enough to undertake such an enterprise, but unfortunately I had been learning about departures, either physical or mental, of my colleagues who were younger than me. The biological clock is ticking in everybody, and the longer the distance from past events, the worse their memory is kept.

In fact, I am going to write about a nonexistent country, Czechoslovakia, which was dissolved at the beginning of 1993, in the absence of any referendum, and which gave rise to the Czech Republic and Slovakia. However, strong ties among intellectuals of both newly established twin countries (who, according to my knowledge, would have preferred to stay together) remained viable and even formalized into joint scientific societies.

Encountering Western colleagues, we often discussed the unpredictable turns of the fate of this country, which at first glance appeared to be fatal for its culture and science. However, this was finally modified and regulated by our national sense of humor, heresy, and free thinking, traditions deeply rooted in our culture. I realized that despite the fact that some of the escapes from and solutions to absurd situations look strange to straightforward Anglo-American thinking, they bemused and sometimes even attracted the listeners. This aspect represents an additional excuse for writing further pages.

II. BOYS AND RESURRECTION OF CZECHOSLOVAKIA

Being a boy after the end of Second World War was a great privilege for several reasons. At the age of about 10 years, I was not deeply marked by the Nazi occupation like my older mates, who were expelled from the universities and in many cases sent to forced labor. The first 3 years after the liberation of the country in 1945 were extraordinarily fruitful and inspiring. There was general enthusiasm and willingness to catch up again with the civilized world. There was a plurality of views, a richness of information from both the East and West, and many opportunities to learn about and love nature—being a boy scout—or to try to understand human thinking by joining discussion clubs or the academic YMCA. To me it has been highly attractive to collect natural objects and to try to learn about their origin or function. I loved amphibians and to some degree also snakes (as viper) that I bred at home. These years formed my generation, which later had to live, for such long years, out of this crop of these seasons.

However, disaster was looming and fell in February 1948 as a communist coup d'état. Boys at the lyceum were not affected directly, but the freshly released high-school graduates who in spring tried to enter university often reappeared with pale faces, commenting on their nonadmission simply: "*La politique*."

However, for younger boys, the situation also became more difficult. Good teachers started to make far less comments and some of them invited selected students to their homes to discuss history and other topics freely. Having reached the last year of the lyceum, I learnt that I would not be recommended to enter any university. Being educated in the spirit of liberalism by my father and inclined to comment on what was going on, I finally faced real trouble. Several factors had been involved, but the most peculiar one stemmed from my friendship with one of my classmates. He had become seriously ill, and I taught him what he missed at school, for free of course, just on a friendly basis. By such occasion, I was also commenting critically public affairs. When he recovered, he converted to orthodox communism and became a leading person of the Party at school. Evaluating me, he postulated that although I was a good human being, I was an enemy of the working class. Such is, sometimes, the end of an open mind.

Again, the opportunity to study fortunately came about in 1951, when the political pressure was decreasing. Finally, I got notice that there was a chance for me to enroll, not in the humanities or at the highly desirable medical faculty, but with two provisions. The first was to get excellent notes in all subjects, which was against my mind and nature, so that I had to strongly force myself to comply with this unpleasant goal. The second condition was easier and involved labor work at the so-called Constructions of Youth, which included just digging and digging, however with a beautiful view of the hills of central Slovakia.

III. UNIVERSITY

> Vždyt' také je mnohem větší nebezpečenství při koupi nauk než při koupi jídel.
>
> Platon: Protagoras, aneb o výchově a občanské zdatnosti

> For there is far greater peril in buying knowledge than in buying meat and drink.
>
> Plato: Protagoras (translated by Benjamin Jowett)

> Avšak správná filosofie a věda žádá pro všecky obory, aby lidé myslili, aby nastřádali co nejrozsáhlejších zkušeností (indukce), aby pozorovali a srovnávali všecko, co dáno v přítomnosti a minulosti, a aby své výsudky ze zkušenosti ověřovali zkušeností další, aby se nedostali dedukováním z malé zkušenosti, dedukováním ukvapeným, do říše fantastiky.
>
> Masaryk, T. G.: Světová revoluce za války a ve válce 1914–1918

> However, the true philosophy and science requires of all its branches to make people think, to let them accumulate all possible experience (induction), to make them observe and compare all of the present and the past, and to make them verify the conclusions drawn from their experience by the new one, not to let them get, due to deducing from too little or too hasty experience, into the world of fantasy.
>
> Masaryk, T. G.: The World Revolution during and in the War, 1914–1918

Finally, I safely matriculated at the Faculty of Science of Charles University that offered my particular choices. In those days, biology was taken as a preferential subject, thanks to Lysenkoism, which for ideological reasons was implemented as the leading genetic teaching. However, in contrast to the humanities and social sciences, professors who did not fit with the forced ideological views were not removed but silenced. They were—even by their mere presence—a reminder that there was a wealth of genetic knowledge based on the Mendelian laws. I should add that in secondhand bookshops, one could still find books dealing in detail with classical genetics, and these became the source of solid information to any one wanting to pay attention to them.

From the beginning, I should have been interested in cytology, which, unfortunately, was viewed only as a morphological discipline. I got essential training in methods of plant anatomy; but in those days, there was almost no way to grow and influence living plant cells. Therefore, I requested and succeeded to get volunteer training in animal tissue culture in the laboratory of cell metabolism created by Dr. Keilová at the Academy of Sciences. This laboratory was interested in the study of possibly differing metabolic requirements between tumor and normal cells. I easily fell in love with tissue culturing and acquired my first experimental experience with the characterization of the growth properties and morphology of tumor cells. A great stimulus for my activity was represented by the arrival of a highly motivated, but to some degree eccentric, Ph.D. student Mojmír Brada. He came with new, sometimes too demanding, and almost unrealistic approaches, which spanned from single-cell biochemical analysis to *Rous sarcoma virus* (RSV) transformation of chick embryo explants. At this point, I should mention that I was amazed by the rapidity of RSV-induced cell transformation, which heralded the fact that the virus harbors the gene(s) responsible for such transformation. That is how I became attached to this model. Mojmír required my help with tissue culture and I learnt from him a series of new experimental techniques, including microinjection and individual cell isolation, which later became important. We became friends, and I was working with him usually from the afternoon until late at night. Once, leaving after midnight and being in a good mood, he asked me how Socrates departed life and if I would like to get his books. I still had to finish some work and therefore I did not pay much attention to these strange remarks, agreeing to borrow books from him. The next day, I learnt that Mojmír Brada

committed suicide using cyanic acid—faster than Socrates did. Learning about this news, I got the feeling that the world around me had collapsed, with no great chance to advance because of my position as an isolated student. Later, I was told that Mojmír attempted suicides in the past also.

All this happened at the time when Milan Hašek was taking over our department. With his arrival, the scientific atmosphere at the department changed profoundly because he brought with him the essential topic, immunologic tolerance, originally called by him immunologic approximation. Fortunately, I fitted within this field because some recent data of Morten Simonsen (1955) and Bob Harris (1956) pointed to the possibility that introduction of chicken blood in turkey embryos significantly increased their sensitivity to RSV. Together with Milan Hašek, we confirmed these results (Svoboda and Hašek, 1956) and extended them to ducklings (Svoboda, 1970). Because tolerance to RSV was elicited in both foreign avian species, I proposed that RSV triggers synthesis of a chicken antigen and therefore tolerance to chicken tissue favors growth of tumor cells (Svoboda, 1961).

It should be remembered that RSV-induced tumorigenesis in ducks was established as the first model of retrovirus heterotransmission. Work on it started in the thirties and was later elaborated by Duran-Reynals. It produced the first hint that retroviruses can overcome species barriers, as was exemplified several decades later.

The role of immunologic tolerance in retrovirus heterotransmission has not yet been clarified. However, the phenomenon is reproducible and is triggered by specific and less specific (Forssman) antigens. There might be involved occasional presence of *Avian leukosis virus* (ALV) in some samples of chicken blood, which should not play a role in duck experiment because they are resistant to common avian retroviruses. Immunologic tolerance in ducks lasts only for a fortnight after hatching, and older birds are fully resistant to RSV in spite of the fact that duck cells carry the RSV receptor (tvc). What is responsible for such powerful resistance is currently unknown. Factors in play might be natural immunity and mobilization of cell factors blocking virus replication. We might learn a lot from this phenomenon about the way to establish efficient resistance to a retrovirus.

As a student, I was assigned to Věra Hašková laboratory. She was a very nice person, efficient, and intelligent; we collaborated on the comparison of immunologic tolerance and enhancement and in attempts to genetically modify animals using DNA (Hašková and Svoboda, 1962; Svoboda and Hašková, 1959). Importantly, she also gave me space for RSV research.

Before proceeding further, I should deal with the year 1956, when anti-Stalinist revolts broke out in Eastern Europe. In our country, mainly students were involved in peaceful demonstrations as described by Jan Klein in this series (Klein, 1994). At the faculty, together with a few others, we

conceived a petition for the state and party organs in which we demanded democratic changes such as release of single-party domination, independent judiciary, and other demands. After various sudden changes of fortune, I was called before a special committee where, luckily, university professors also participated. I was accused of "antisocialist activity," but I defended myself by saying that I had not violated the Constitution. In this way, I survived the first storm, but I expected that the second one would follow at the Institute. However, Milan Hašek, after a few questions, let me go. One question was very tricky. Hašek asked me whether I was informed about the reforming movement in Poland (as we were referring to) from the Voice of America or from Polish resources. I, of course, answered that the Polish information service was the source of our knowledge, but there were also other sources not recommended to be mentioned. The only punishment was a year's delay in my admission to Ph.D. studies, which was not a big deal.

I wrote about Hašek's unique personality (Svoboda *et al.*, 2005). He had been devoted to his research, not forcing ideological influences on the laboratory. In daily communication, he behaved in a liberal way. Once we discussed Mendelian genetics, which he did not like. I put forward the argument that although it does not explain everything, Mendelian genetics still should be taught. Essentially, Milan did not object. I witnessed that he was sincerely interested in ways how to modify the genetic makeup of organisms and accentuated the somatic cell genetics and transplantation. He had always been broad-minded and supportive to his colleagues, defending them from external and even political pressure, and was on friendly terms with his collaborators. In his active period, he surpassed everybody in biological sciences in our country.

IV. VIROGENIC CELLS AND PROVIRUS INTEGRATION

My involvement in the immunologic tolerance to avian retroviruses in birds culminated in my first trip to Moscow on the occasion of the National Meeting of Transplantation. This event took place in 1959, in the period of the thaw. I still remember O. B. Lepeshinskaya, a pillar of Lysenkoist cytology, who had no idea about what was going on at the meeting. In fact, she asked the cochairman a control question—"Immunology?" On the other hand, bright young scientists such as George Svet-Moldavsky and Yuriy Vassilev raised their voices in favor of genetics, loudly opposing Lysenko's monstrous misconceptions.

The crowning event to our trip was a meeting with Lev Alexandrovich Zilber and his collaborators, namely with Igor Abelev. They were devoted to

the isolation and characterization of possible tumor-specific antigens in human malignancies using various available techniques.

I was associated with Zilber and his collaborator Kryukova as well as to George Svet-Moldavsky through their original discoveries of RSV pathogenic action in rats. What I learned indirectly was that their original aim had been induction of immunologic tolerance to this virus. In this way, there was a common denominator of our interests. Zilber was a magnificent personality, who pioneered modern virology and cancer research under incredibly oppressive conditions. Later, during his repeated visits in our country, he remembered his ordeals in a close circle of friends; we listened silently. An excellent account of his life and work was given by Kisselev *et al.* (1992).

We tried unsuccessfully to establish the role of immunologic tolerance in RSV-induced hemorrhagic disease in rats (Svoboda and Grozdanovič, 1960), but we were able to show that this disease was caused by the virus because it could be prevented by antiviral sera (Rychlíková and Svoboda, 1960). Of interest was the occurrence of late tumors in RSV-inoculated rats. This phenomenon remained enigmatic because no vestige of the presence of virus had been found in them. That was why Zilber interpreted these findings within the frame of his virogenetic theory, according to which a virus is responsible for initiation of tumor formation, but tumor cells need not produce the virus (Kisselev *et al.*, 1992; Zilber, 1961).

Overall, the question of RSV involvement in tumorigenesis in the mammalian host touched essential problems of oncology and attracted me fully. First I decided to monitor late tumors appearing after RSV infection for any viral activity. The simplest and more representative way turned out to be inoculation of chicks with minced tumor tissues. Using this approach, I found, in the case of tumor XC (from Latin cage No. 90), that after inoculation in chicks it produced RSV-containing sarcomas. Logically it indicated that the XC tumor contained the virus (Svoboda, 1960, 1961). After closer inspection, it became apparent that XC cells do not harbor any infectious virus. For its production, association of structurally intact XC cells with chicken cells was mandatory (Svoboda, 1962).

In the meantime, I was sent, against my will, by our Academy to Red China because at the beginning of the "Cultural Revolution," the Chinese liked to accept people involved in science and not in politics. The break between the Soviet Union and China was reaching its height. Visiting and lecturing at different institutions, I learnt that the scientific approaches that were followed those days in China were outdated and did not reflect what was going on in the world. I myself felt under scrutiny and was warned secretly by my interpreter that my views and comments were evaluated every evening. Life in that country was poor and grim. An appropriate comment I received from a Latin-American revolutionary granted asylum in China

was plainly that such sad communism would never fit with Latin-American sentiments.

The most important part of my trip in China was my way back, which included the flight over the immense green plains of Siberia until reaching Moscow. In the hotel, I liked to feed Chinese hamsters, which I carried as a gift with me on my seat. Unfortunately, some of them escaped when I opened the box, and it cost me quite an exercise to collect them and put them back into the box. This event delayed my calling Zilber, whom I was supposed to meet.

It was a warm meeting with Zilber's family, who also remedied my digestion problems caused by exotic meals with Armenian cognac. I then explained to Zilber what we knew about XC cells and virus rescue. He let me speak without interruption and finally concluded that this was the way to understand the interaction of an oncogenic virus with a cell and its transforming activity. Therefore, I returned from this big journey in a far better state of mind than I had before leaving. I then resumed work with enthusiasm. First, we verified the species origin of XC cells (Landa *et al.*, 1962) and the permanent presence of the RSV genome in them. Furthermore, we extended our XC model to rat cells transformed by cocultivation with chicken Rous sarcoma cells (Svoboda and Chýle, 1963). We had found that this cocultivation was an efficient way to produce transformation of mammalian cells by RSV, which was employed later as a useful tool for transformation of other cells of different species origin. There was another important finding for us that *in vitro* transformed rat cells behaved in the same way as XC and that the virus could be rescued from them only after association with chicken fibroblasts, which indicated that this phenomenon was of more general importance.

However, XC cells remained my principal interest. In order to extend this project, I invited Dušan Šimkovič as an experienced person in tissue culture to collaborate on long-term cultivation of XC cells. The goal was to establish monocellular clones, which we then successfully isolated (Šimkovič *et al.*, 1963). When individual XC clones were compared in their ability to rescue the virus by cell association, a comparable number of cells from different clones ranging from 10^5 to 10^6 cells per inoculum led to virus rescue. These numbers agreed with the cell number required for virus rescue from uncloned XC cell population, showing that the virus genetic information had been spread equally within the cell population. In separate experiments, we confirmed that in several grams of XC tumor tissue there was no infectious virus and its absence was confirmed serologically. This gave the final picture showing that XC cells harbor the viral genome (they are therefore virogenic), which is noninfectious but rescuable. This viral genome is indefinitely inherited in tumor cells as additional genetic information and is therefore integrated in them as a provirus (Svoboda *et al.*, 1963).

To this interpretation we were, of course, inspired by André Lvoff's concepts based on his bacteriophage studies.

All these studies raised the interest of my colleagues. Especially, Bob Huebner highly valued these results as opening of a new approach to tumor virology. In fact, he started his investigation using RSV-induced tumors in hamsters, where he detected viral-specific complement-fixing antigen. Howard Temin sent me a very enthusiastic letter mentioning that he was interested in provirus integration into XC cells and that he was going to publish similar data. There had been a disagreement between him and me about the mechanism of virus rescue. Temin, based on his chicken cell experience, proposed that virus rescue resulted from superinfection with avian or mammalian retrovirus. Contrary to that, I favored the interpretation that the virus is rescued by cell association between XC cells and chicken fibroblasts that makes possible fusion and complementation between both types of cells. As we shall see later, the latter explanation turned out to be right (relevant correspondence given in Svoboda, 2003).

Not until 1964 did I have a chance to meet a Western virologist. In that year, I was invited to attend the International Conference on Avian Tumour Viruses sponsored by NIH. Originally, as I was told, it should have taken place in New Orleans, but was then moved to Durham due to the fact that no hotel capable to arrange common accommodation of black and white persons was available in New Orleans.

The meeting was held in a positive spirit favoring an essential role of viruses in the genesis of tumors and was attended by virtually all scientists working in this field. For the first time, I could speak to Harry Rubin about virus defectiveness, to Howard Temin on provirus integration, and to Ludwig Gross, Peter Vogt, and many others whom I had known from the literature. What impressed me was a sense of cooperation in the absence of deadly competition. Possibly, this could have been associated with the fact that in those days only a handful of scientists were engaged in tumor virus research and that this research was not in the front line.

After the meeting, I lectured in various University and State institutions. Everywhere I noticed that laboratories were well equipped, including centrifuges, and were supplied with standard tissue culture media and plastic dishes. This had been a dream for us, but also gave us a warning that we could not work on a broad scale but must stick to our XC cells, which grew in the poor media available in our country. As my friend Bob Dougherty suggested to me, I visited plumbing stores, where I bought a pump for the CO_2 incubator. Therefore, we were able to construct in Czechoslovakia the first functional CO_2 incubator maintaining controlled pH. At this, my first visit to United States, I was struck by the flexibility of American organizations, the availability of resources, and the high standard of living. I felt that we had been lagging in many respects for at least 15 years.

There were additional occasions to return to the United States. On one of them, I visited Howard Temin in Madison, where he lived with his wife Rayla in a student's apartment. After a thorough discussion of my talk, we went to Vaclav Szybalski's department and engaged in discussion with his phage geneticist collaborators. They did not question our results but emphasized an important issue that virus rescue might be mediated by chicken cell inactivation of a repressor present in mammalian cells.

V. VIRUS RESCUE

After returning home, I felt strongly that the key problem to be solved was the mechanism of virus rescue. Assuming that the cell-to-cell contact allows spontaneous cell fusion and that around 10^5 to 10^6 virogenic cells are required for virus rescue mixed with chicken fibroblasts, an agent stimulating the fusion should augment the virus rescue. From papers by Okada, it was apparent that UV light-inactivated *Sendai virus* would be the best candidate for the fusogenic agent. After some problems with getting the right batch of virus, we succeeded in proving that it significantly increased the efficiency of virus rescue (Svoboda *et al.*, 1967). This, of course, opened the way for further studies.

Before these results had been accomplished, I went to the meeting celebrating the 70th birthday of L. A. Zilber. The conference was held in Sukhumi on the Black Sea, in the building originally belonging to Beriya and surrounded by a large park full of beautiful trees. Almost all Russian scientists appeared at that meeting, and I felt that they were unusually relaxed as a result of the de-Stalinization going on. There had also been an impressive attendance from the Western countries such as the Melnicks, the Kleins, H. Koprowski, R. Huebner, L. Sachs, and others. Some of them, who stemmed from Slavonic regions, communicated well in Russian, which pleased our hosts and contributed to an open atmosphere. At this meeting, I spoke about tumor-specific transplantation antigen (TSTA). This antigen in RSV-induced mouse tumors had been characterized *in extenso* by Bubeník *et al.* (1967) from our laboratory as producing rejection immunity against tumors of the same aetiology. As I had shown (Svoboda, 1965), TSTA was also present in RSV rat tumors—the immunity could have been transferred adoptively, by lymphoid cells, and there was clear antigenic cross-reactivity between rat and mouse tumors. This led to the speculation that TSTA appearance was related to the RSV provirus and its transformation activity, which, as we shall see later, was fully substantiated.

Surprisingly, there had been no comment on TSTA, but man questions about virus rescue, which I mentioned only peripherally. Repeated questions were

raised whether in our experiments a helper virus might be involved. As I had shown, it was not the case, and I stated again that for various reasons it was not. In extending the discussion to the analogy of our virogenic cells to SV40-transformed hamster cells, from which viruses can be rescued by cell association, I engaged in a confrontation between Hilary Koprowski and Albert Sabin; the latter stood in favor of virus rescue. There were obvious differences between RSV as an RNA virus and SV40 as a DNA virus, but I pointed out that theoretically "the question still remains whether the transforming part of RSV is DNA." However, as was shown several years later, the cytological basis of SV40 and RSV virus rescue was the same in both cases.

There were some adventures linked to the life in Sukhumi. One free afternoon we went, together with Pavel Koldovsky, for a public bus drive in the country. Everywhere, rhododendrons in bloom seemed unharmed by goats feeding on their leaves. On our way back, Pavel instructed me to be careful about my wallet. He felt safe because his pocket was buttoned. When we left the bus, he found out that his wallet, along with the cutoff button, was gone. We then reported the incident to the police. The "militia" men were not surprised and assured us that, according to local habits, the documents would be returned within 24 h—except the money, of course. This scenario took place as predicted.

Of the meeting participants, I was most impressed by Bob Huebner. At the reception dinner, he gave a highly stimulating talk, stressing the international collaboration in the cancer field. As I learnt later, he meant it seriously. In the next years, we met on various occasions. I keep in mind his arrival in Prague, joined with a dinner at a beer pub. We discussed in English when we were suddenly interrupted by a Czech soldier, who approached me and told me that it was scandalous to speak to our enemy. Bob noticed immediately that something went wrong and asked me to translate the soldier's comments to him, which I did. He then replied, reminding the soldier, that during the war he himself served as a doctor on a battleship in the Pacific and felt as an ally to Russia. This convinced our brave soldier, who shifted his mood to friendship.

Back in Prague, we wanted to reconcile the variable cytogenetic data obtained with RSV-transformed rat cells. There was no other way out than to employ another, more suitable model. Fortunately, we bred Chinese hamsters that I had previously brought from China (Hložánek *et al.*, 1966). They were easily adoptable for karyological experiments because of the low chromosomal number. In tackling the question whether virus-induced cell transformation is accompanied by karyological changes, we exposed primary cultures to the virus and evaluated the cells before, during, and after the transformation. It turned out that no noticeable karyological anomalies occurred at the stage of cell transformation. However, they appeared

gradually after repeated passages, indicating clearly that they were related to tumor progression. We thus abandoned karyology for a long time. It should be stressed that the issue of the role of karyological changes in tumorigenesis is now reemerging.

I had several chances to meet with French scientists and I highly valued G. Barski, who along with Soriel discovered somatic cell hybridization, and Philippe Vigier, internationally recognized "rousologist." Philippe was originally very critical of our findings but gradually became interested in them. Together with him, we obtained additional important data showing that virus rescue from XC cells was independent of any helper virus (Vigier and Svoboda, 1965).

I should also mention my ties with Germany. At the time of the Iron Curtain, East Germany's Berlin was the only place where we could illegally, but in a relatively safe way, reach the West. Together with Pavel Koldovský, we obtained permission to visit the Arnold Graffi Institute for Cancer Research in Berlin. In order to get to Berlin-Buch where the Institute was located, we had to employ urban transportation. We followed the available maps but confounded the connection, and at once we landed in the West without any complication, despite the fact that in our country a 20-year penalty existed for unofficial crossing of the Western borderline. At the Institute, we found a good scientific milieu, covering especially mouse leukemia viruses and their immunology, and Günter Pasternak took care of us.

Later on, I went in touch with the West Germany through the European Tumour Virus Group. This informal organization was set in the early sixties by Bob Harris and comprised only a few scientists. That was how I met George Klein, Willy Bernard, and some others. In a later meeting of this group participated Werner Schäfer from Tübingen, who became interested in avian retroviruses. Thus, we established a collaborative project on TSTA characterization. On the occasion of my visit to Tübingen, I gave a talk in which I mentioned our data pointing to the RSV genome integration in mammalian cells. The reaction of the audience was generally negative, with the exception of renowned virologist A. Gierer, who underlined the significance of our experimental approach and interpretation. This overrode the others.

I was very pleased by an invitation from Bob Harris, who also had attended the Sukhumi meeting and was head of the Mill Hill station belonging to the Imperial Cancer Research Fund. Bob's offer was attractive to me because he gave me total independence, a technician, and the possibility to invite my colleagues for a short stay. I was convinced that I would come to a laboratory where tissue culture was running routinely. How disillusioned I felt when I learnt that chicken fibroblasts did not grow! There was only one solution—to check every component of the culture medium. I made all

the possible exchanges between individual batches of different provenance, but this did not lead to any conclusions. The last factor in play was distilled water prepared in an apparatus equipped with rusty iron electrodes. My next move was to ask for glass distillation. My request was turned down with the note that the present apparatus was working well—a typical English approach. Finally, I persuaded my colleagues and bought a glass distiller. The newly prepared media made possible efficient growth of chicken fibroblasts, which opened the way to establishment of a quantitative assay detecting RSV cell transformation. I adjusted this assay for measurement of virus rescue from virogenic irradiated cells fused by *Sendai virus* with chicken fibroblasts. Under these conditions, the virus production was increased 100 times as compared to cocultivation. The rescue was proportional to the number of virogenic cells and correlated with the heterokaryon formation (Svoboda and Dourmashkin, 1969). All these results of tedious work convinced me that in the case of virus rescue, we were dealing with a phenomenon where the virus genome in a nonpermissive mammalian cell was not expressed fully until complemented by chicken cell machinery. Not many people believed it. Among the skeptics was Howard Temin, who engaged his pupil John Coffin in a virus rescue project, and John successfully confirmed our data (Coffin, 1972).

In the IRCF laboratory, I made friends with an excellent electron microscopist and cell morphologist Bob Dourmashkin, who helped me efficiently in these studies. I esteemed Bob Harris for his magnanimity and a very friendly approach to me. I should not forget the bright American Bob Bassin, who later went to John Moloney and with whom we discussed both science and politics. Also the external contacts were enjoyable and inspiring. They included Warren Levinson, whom we visited at London University and who gave us important suggestions about performing RSV assays. After returning to the United States, he introduced the RSV model into Mike Bishop's laboratory.

I was impressed by Tony Epstein, by his critical but constructive approach to tumor viruses, and made friends with gifted Ph.D. student Robin Weiss, who obtained the first data that in some chicken lines, genes encoding retroviral proteins are expressed, and opened the way to the definition of endogenous retroviral genomes. Very enjoyable was my visit in Glasgow at the Institute of Virology chaired by Michael Stoker. Retrovirology was represented there by Ian Macphearson, who discovered normally looking revertants in a population of RSV-transformed hamster cells. Because the virus was not rescuable from some of them, Ian interpreted this failure by loss of the provirus and proposed an episomal provirus state. Discussing this issue, I suggested increasing the number of virogenic cells used for virus rescue experiments, which worked, and the episomal hypothesis fell into oblivion. Ian had a great sense of humor, but he looked serious when a note

hit his Scottish heritage. For instance, he was ready to defend the existence of the Loch Ness monster until the last drop of his blood.

In England, we lived together with my wife happily—like a pair of squirrels, which by the way were plentiful around our house. My contacts with our country were irregular, but reading the German journal *Der Spiegel*, I got a feeling that deep changes in the direction of democratization and party power limitation were on the way. Fresh news reached us from Milan Hašek, who visited us in London. He was fully in favor of reforms and democratization, being fed up, similarly as most of party intellectuals, with inefficient bureaucracy and party reglementation.

Two important meetings took place before my leaving England. The first one I organized at the Wistar Institute in the United States and it was focused on virus induction by cell association. I tried to get together all the people who were contributing to this subject (Svoboda *et al.*, 1968). In this respect, the meeting was very successful. It enabled getting the information based on RSV in context with SV40 studies and showed that both were very close to each other. The greatest advancement was made by Mary Weiss, who studied the segregants of human–mouse (SV40-transformed) hybrids and demonstrated that the SV40 genome is preserved only in cells carrying certain mouse chromosomes. This indicated that the SV40 viral genome is integrated at least at the chromosomal level. Unfortunately, when working with rat–chicken hybrids, we were not in the position to analyze the hybrid progeny due to the nonviability of such hybrids. For me, the crowning event to our meeting at the Wistar Institute was Okada, a modest person who opened the field of artificial cell hybridization. Last evening, the participants were invited for an opulent dinner at which Hillary Koprowski gave a speech about science and literature. I was then called to speak. Not being notified in advance, I felt disoriented but fortunately Hillary whispered in my ear: sex among cells. Thus, I got an attractive subject for my talk.

The second meeting, convened by Michael Stoker, was devoted to the molecular biology of viruses and sponsored by the Society of General Microbiology. This was a unique session, which of course included traditional Franco-British confrontation. For our work, two talks were important. The first was given by Luc Montaignier, indicating the presence in the cell of double-stranded RNA that might be employed at certain stages of RSV replication. The existence of such RNA was criticized strongly by André Lwoff, who introduced the French Academy as l'Academie Française Royale, but Luc stuck to his conclusions. The second inspiring lecture was presented by Werner Arber, who dealt with the known and unknown mechanisms of bacteriophage host cell modifications and predicted restriction enzymes. We had already tried to detect some signs of RSV modification in mammalian cells, however, with a negative outcome. In my talk, I presented a classification of different types of RSV interactions with mammalian cells.

Of special interest was nonproductive (nonvirogenic) interaction characterized by tumor cells from which no virus was rescuable and in which no virus component was detected. However, such cells harbored TSTA specific for RSV-transformed cells. I concluded saying that "only the part of the viral genome responsible for transformation is present in these cells" (Svoboda, 1968).

This RSV transformation part, later called "oncogene," remained in the center of our interest also in later, more difficult years.

VI. PRAGUE SPRING 1968

> Under the Roman domination, the Greeks lost the self-confidence that belongs to political liberty, and in losing it acquired a paralysing respect for their predecessors.
>
> Russell, B.: A History of Western Philosophy

> Činí, co chtějí, nebot' jim nikdo nic říkati nesmí.
>
> Dačický, Mikuláš z Heslova: Paměti

> They do as they wish, for nobody may contradict them.
>
> Dačický Mikuláš z Heslova: Memories

I was eager to return to Prague and witness the unprecedented changes that were going on. However, I kept in mind that the freedom of information and criticism that I enjoyed so much in London could be far less in Prague and would eventually fade out. On one of my last evenings, I felt the need to see Pasternak's *Doctor Zhivago* and prompted my wife to go with me. This extraordinarily made film gave me a lot of warning about the cataclysms that may happen in a communist-ruled country. Essentially everything was possible! But I still believed that the flow of events in our country was irreversible and would be accepted by our Big Brother—USSR.

In the summer, the first World Congress of Virology was organized in Helsinki, in an optimistic spirit, also resulting from establishment of an independent virology organization. I traveled with Slovakian virologists, who were "well equipped." Later, a Swedish custom officer pointed without hesitation to a small smart luggage. When opened, five bottles appeared. Being asked by the officer what it was, my colleague answered simply: "Slivovitz." The officer did not ask more; in those days, Scandinavians were very friendly to us, probably being better informed than we were.

At the meeting, virus rescue was one of the important topics and I joined in actively. After my talk, I met Albert Sabin, who warned me that the Soviet Army was getting concentrated around our borderline, which was alarming. On our way back, we landed in East Berlin and, contrary to others, we were not allowed to leave the plane. It looked as if we were carrying an infection.

On August 21 at 3 a.m., I was woken up by a call of my colleague, who stated simply: "They're here." I was shocked, but soon ran to the streets to check what was going on. Prague was packed with Russian armaments and soldiers looking hungry and thirsty, but nobody offered them food or drink. I approached one soldier and asked him: "Where do you think you are?" His answer was simple: "In Germany." The most explosive situation was near the radio broadcasting station, where a Soviet tank went aflame lit by the demonstrators, and the horrified soldiers started to shoot into the crowd from their machine guns. Most of the people, including me, escaped in the corridors of the broadcasting station, but several were killed. Later I took part in street demonstrations and we were also joined by some American students. Surprisingly, they knew Harry Rubin's involvement in the civil right movement.

The invasion of the Warsaw Pact armies had disastrous consequences also for our work. Supplies were disorganized and our foreign contacts lost. I was especially waiting for Warren Levinson, with whom we planned a joint molecular hybridization study of our virogenic and nonvirogenic cells. Under such conditions, the solution for me would have been to emigrate. There was no obstacle to such a decision because I held a US visa and, in addition, an official invitation from Bob Huebner to join NIH as an independent scientist. However, shortly after the invasion, I received Milan Hašek's letter sent from Austria, in which he declared that he was not going to return until the Russians retreat and that for the time being he was handing over the directorship of the Institute to me. I was deeply shocked by this Danaian gift and for several days I felt torn apart by the question whether to stay or to go. Finally, I decided to stay, assuming that Milan wrote the letter in the impulse of the moment and that he would return. Actually, he came back about a year later and I handed the directorship back to him. Otherwise, the general situation in the country until 1971 had not been seriously oppressive. The Institute was further supervised by the Academy, which was not significantly interfering with the Institute's matters. There was also a strong public feeling that emigration of creative people would help the Russians to devastate our country. Finally, I was wrong in estimating that the occupation could not last more than 5 years. Later, Milan's estimate was 20 years, which was almost exact.

Therefore, without problems I attended several international meetings dealing with virus rescue by cell association. At a French meeting, I spent late hours discussing with Harry Rubin our experiments and general political and cultural problems. His insight was phenomenal and in answering the question about his preferences, he stated "Czech films and Kirkegaard." Fascinating was the Amsterdam meeting organized in honor of O. Mühlbock, who had retired. All scientists who had essentially contributed to retrovirus research and oncogenesis were given a chance to present their data and views.

In my talk, I provided an avian leukosis-sarcoma virus overview (Svoboda, 1972) and underlined data indicating the presence of viral-specific DNA in infected cells. Furthermore, I discussed cell association and phenotypic mixing as approaches to virus rescue.

The atmosphere of this meeting was, for several moments, highly confrontational. I remember Bob Huebner shouting at Howard Temin "You do not understand virology!" and the reply was "You do not understand oncology!" Also, I did not understand why such a heated debate. Behind these strong arguments were serious discrepancies in interpreting the origin of the cancer process. Temin (1972) proposed an original idea that a protovirus, simple provirus, integrates by reverse transcription and once integrated in the vicinity of potentially oncogenic genes it produces their activation resulting in cancer conversion. Huebner and Gilden (1972), on the other hand, stressed the role of endogenous retroviruses in triggering such a process and emphasized the role of derepression of viral oncogenes by other factors. Then, sitting in a bus I heard a discussion between two eminent scientists, indicating that the future Nobel Prize was at stake, which usually is accompanied by scientific fireworks. The competition at such a level was beyond my mind because I felt deeply disturbed by thinking about what was awaiting me back in Prague. An encouraging fact was that on my return to my country I was accompanied by Robin Weiss, the first and last international member of our Institute. A very positive event was that Milan Hašek had returned. I handed over the directorship to him. However, the worst was yet to come.

VII. DARK YEARS

> S řečí byli bychom ztratili i paměť, kdyby v naší moci bylo zapomínat tak jako mlčet.
> Tacitus, Z dějin císařského Říma

> With the faculty of speech we would have lost our memory, if it were in our power to forget in the same way as to be silent.
> Tacitus, From the History of Caesarean Rome

At the beginning of 1972, Husák's puppet government tightly closed the border. Then, purges started. I was called to a committee that asked me whether I agreed with the Soviet occupation. The answer was no, and I emphasized my argument by mentioning that the President of our Republic also did not agree. This was something they did not like to hear and consequences followed. I lost my department and remained solitary with no direct help. The same happened to Milan Hašek. It meant that we had to proceed by a slower path. I decided to visit Viliam Thurzo in his Institute in Bratislava, which in those days was also deserted. He behaved in a relaxed way, but

when I wanted to pay the dinner, he stopped me and gave me a Hungarian small gentry lesson, saying in Slovakian: "Pán sa neponáhlá, pán sa nečuduje, pán neplatí," which means: "A gentleman does not hurry, is never astonished, and does not pay." Thurzo was really helpful to us in the harsh years especially because his Institute was located in Slovakia and therefore protected by Husák. When later as a "reformist" I was banned from any publication activity, he opposed this party decision and argued that even former fascists sitting in editorial boards of our Journals were not punished in such a way.

Before all this happened, we undertook reexaminations of different types of RSV–mammalian cell interactions (Svoboda *et al.*, 1971), and using all the present-day knowledge, we eliminated any role of endogenous retroviruses in virus rescue, confirming the previous statement that chicken cell, not viral, factors were required for virus formation. Moreover, we characterized nonvirogenic cells as lacking the viral replicative gene product, but still were transformed and contained TSTA. This was a basis for my future plans aimed at the characterization of the virus-transforming part present in nonvirogenic cells. There was a visible hiatus between the great progress in retroviral oncology as performed on animal models and the lack of similar findings in humans. Therefore, we assumed that some human tumors might harbor cryptic proviral sequences as in the case of nonvirogenic cells. Such cryptic proviral oncogenic sequences could be occasionally transmitted by recombination with another virus. This hypothesis turned out not to be valid, but at least for a while helped sustain the interest in human retroviruses.

The field of retroviruses was moving fast as a result of the discovery of reverse transcriptase made by Temin and Mizutani (1970), and simultaneously by Baltimore (1970). Still, the question remained as to whether the whole retroviral genome becomes integrated into the cell DNA. Such a possibility might have been tested using transfection of chicken cells with DNA isolated from virogenic cells. We agreed on collaborative transfection experiments with Miroslav Hill on the occasion of the Czechoslovak Biological Society session in Brno in 1966. However, our ways had diverged—he went to France and I went to England. After the occupation, he established himself at Gustave Roussy in Paris and together with his wife they published a report describing the positive outcome of XC DNA transfection of chicken fibroblasts (Hill and Hillová, 1971). We were working on the same project in parallel. However, in the first step, I wanted to test whether the nuclei from XC cells could be transferred to chicken fibroblasts and there triggered virus rescue, essentially because we had already learned, using the enucleation procedure, that the nucleus is the seat of the provirus (Donner *et al.*, 1974). In spite of treating XC cells with hypertonic solution during nuclei isolation, where no cell should have survived, when I seeded a

suspension of nuclei in the culture flask, XC cell colonies later appeared. These unsuccessful experiments produced some delay in reaching the second step that involved DNA transfection. I should mention that I was strongly warned by our eminent virologist not to try such an approach as it was senseless, but I did not follow his advice. In independently designed experiments published about half a year after Hill, we obtained positive transfection with a single exposure of chicken fibroblasts to DNA from two lines of mammalian virogenic cells (Svoboda *et al.*, 1972). We also characterized the resulting viruses in the focus assay and assessed their efficient replication.

Things again turned in a wrong way, being even worse than the previous events. In the laboratory, there was a student who was taking drugs (as we learned later) and who deliberately destroyed most of tissue culture samples, trying to put the blame on somebody else. As the consequence of previous purges, I was powerless to stop such crazy behavior and I therefore called the police to clarify this case. In fact, the culprit was soon identified and I supposed that the case was finished, as the police officer assured me. However, the secret police smelled an open door to our Institute and started to search through the documents and to interrogate people. Somebody informed them—and it was no secret—that in 1967 I was awarded an American Jane Coffin Child Memorial Grant. *Quelle delicatesse* for the secret police in an occupied country! I became suspect number one because I was receiving American money without any obligation except for writing a progress report. The secret police officers came with a (for them) convenient story of my being involved in espionage. I then underwent long hours of interrogation, being asked stupid questions such as from where I knew Mrs. Ford, the grant administrator, and whether she was a relative of car builder Mr. Ford. After several months, I became exhausted and depressed. The last trump they drew from their pocket was their indignation that I bought several scientific books with the grant money. Because I kept these books in my office, there could not be any doubt of the money being misused. After that, I was relieved of interrogation, but my prospects remained grim. The US President launched the Virus-Cancer program, which should have joined the leading scientists in the field. Being invited, which I took as an honor, I asked the police whether I could take part at this highly important meeting. I was told that it was possible, but the final decision would be given at the airport. I thus prepared for the meeting and was waiting in the lounge. Close to the departure, I was informed that I was not allowed to go. Similarly, James Watson invited me for a Cold Spring Harbor symposium, but again I was not allowed to go, although Watson wrote a special letter to the then President of the Academy—there was no answer. Fortunately, the text of my talk was smuggled to the symposium by Jan Závada. We presented there the ultracentrifugation

analysis of XC DNA fragments efficient in transfection and concluded that only the DNA molecules that are of the provirus size or larger are active in transfection experiments. Furthermore, we documented profound differences in sensitivity to transfection among different avian cell strains (Svoboda *et al.*, 1975).

Of decisive importance was a "rescue" visit of British scientists led by Michael Stoker and based on the Academy agreement. They organized a scientific meeting with their Czech colleagues and strongly recommended our Academy to support research in virology and cancer. This was a turning point. In the dark years, additional visits of scientists from the West such as the repeated visits of Abraham Karpas helped us keep an optimistic spirit.

VIII. PARTIAL THAWING AND MOLECULAR BIOLOGY

> . . . freedom of thought in human history – not freedom in general, which has too many ambiguities, and may even be identified with the freedom of the strong to exploit the weak, but freedom to think and to speak.
>
> Stone, I. F.: The Trial of Socrates.

I am describing all these events because they forced me to accept the fact that I was not able to stay in the forefront of retrovirology any more, as well as to stay in contact with the dramatic development in this field. Retrovirology became technically demanding and dependent on the availability of special biochemicals such as labeled nucleoside triphosphates, restriction enzymes, and so on, which were out of reach for us. Our only resource, the American grant, was lost in the end. Looking for an open question within our reach, I became persuaded that of key importance were nonvirogenic cell lines, where we postulated the presence of only the RSV transforming part. I still kept in touch by mail with Marcel Baluda and he agreed to perform a collaborative study aimed at provirus definition in one nonvirogenic mouse cell line. Using liquid hybridization (labeled viral RNA annealed to cell DNA), it became apparent that in the mouse cell line employed, only about one-third of proviral sequences are present (Svoboda *et al.*, 1977). In the meantime, the molecular definition of RSV oncogene v-*src* was achieved by Stehelin *et al.* (1976). The question remained whether it corresponded to the partial provirus in nonvirogenic cells, which was likely, and how such a provirus might have arisen. Relying in those days on my own "working force," I decided to induce, by cloned RSV, a series of hamster tumors in order to obtain fresh defined material and to get an estimate of different virus–cell interactions. Very often, proviruses in hamster cells were defective, sometimes amplified, but the presence of inducible full proviruses was encountered regularly. One out of 24 tumors

behaved as nonvirogenic, and thus we obtained a proper model for further studies. The situation at the Institute started to improve, thanks to the new director, Josef Říman, who himself was a successful scientist and a clever and helpful person devoted to science. Many people, I included, owe him a lot for his support and understanding. Under these new circumstances at the end of the seventies, I was finally allowed to spend two months at Dominique Stéhelin's in Lille.

My visit in Lille had been totally concentrated on experimental work since the first day after my arrival. I brought with me representative samples of RSV-transformed cells and felt ready to become devoted to molecular hybridization procedures that for years had been escaping our technological possibilities. Dominique and his young enthusiastic collaborators were very helpful in introducing me to this fascinating field, and I spent days and nights in the laboratory in order to catch up with the time allocated to my visit. Soon I was able to analyze my cell lines. Shortly, it became apparent that the nonvirogenic line harbored a highly deleted provirus comprising about one-third of the full provirus present in virogenic cells. Such a simplified provirus retained only the expressed oncogene v-*src* and viral regulatory sequences (LTRs). Thus, finally nonvirogenic tumor cells were characterized structurally (Svoboda *et al.*, 1983). With good feeling, I was returning through Paris, where I was invited for a seminar at Collège de France and was pleased by a nice introduction given by Jean Dausset. To cross Germany by train, I was allowed a limit of 24 h. As I was in phone contact with Fritz Deinhardt at the Pettenkoffer Institute in Munich, he made an arrangement with the German control at the entry borderline not to stamp my passport—in this way, my journey would be uncontrollable. The policeman at the border was informed and assured me that he was not going to give me the stamp, but unfortunately, by habit he had done so. For me, this meant that after shaking hands with my friend in Munich I had to leave for Prague. Nothing dramatic happened, but this story illustrates some consequences of the divided Europe.

Returning home, I faced a new task in establishing retroviral molecular biology. Graciously, Dominique supplied me with most of the necessary components required for starting molecular analysis, which I did, and I also attracted some students. However, there were important items such as enzymes and nitrocellulose, which came from the West with long delays and irregularly and which were not distributed according to scientific criteria.

Together with my former student Josef Geryk, we attempted to rescue the highly simplified provirus consisting of LTR, v-*src*, LTR. After many fruitless attempts, we succeeded in transmitting this provirus using cell fusion combined with very efficient superinfection with a helper virus (Geryk *et al.*, 1986). Originally, I assumed that rescued viruses would originate only by recombination between the provirus and the helper virus.

The outcome of the experiments was more complex. In fact, we obtained clear evidence for the rescue and transmission of the LTR, v-*src*, LTR provirus without recombination (Svoboda *et al.*, 1986), but also for its recombination with the helper virus. Using different markers, we defined, together with Ram Guntaka, that recombination led to the acquisition of a part of the viral replication gene (*gag*), but the transforming gene v-*src* was kept intact (Svoboda *et al.*, 1990). Of major importance was the LTR, v-*src*, LTR nucleotide structure. This required more sequencing, which was made possible by a kind gift from NIH colleagues Bob Gallo and Mika Popovic, who provided me with the Sequenase kit that I decided to transport to Prague at any cost. I had to take with me two big boxes filled with dry ice and samples. After reaching Frankfurt, I was first watching for the police, and when the patrol disappeared, I slipped to a narrow corridor leading to the lavatory and transferred dry ice from a reserve box to the sample box. Clouds of evaporated CO_2 were produced as if I were a carbonieri preparing an explosion. Fortunately, my unusual behavior remained unnoticed and therefore I brought my treasures safely to my destination.

There appeared new problems related to provirus cloning that required phage packaging extracts. Finally, some had been homemade and some were kindly provided by Mariano Barbacid. We then obtained a clone that represented the first unique sequence cloned in our country and we established its primary structure. It became clear that the LTR, v-*src*, LTR provirus arose by regular reverse transcription of *src* mRNA, thus lacking any viral replicative gene sequence (Bodor and Svoboda, 1989). This finding provided confirmation of our original postulates and interpretations according to which the nonvirogenic cells carried only the virus transforming part.

I was happy about this progress, but it became obvious that we could not rely forever on scientific charity and gifts. In one way, we were in a preferential position because we had access to defined breeds of chickens. We therefore focused part of our effort on the definition of ALV pathogenesis. Furthermore, we asked ourselves whether the proto-oncogene c-*src* could be captured by ALV lacking any *src* gene sequences. This happened with very low frequency and led to formation of a new *Avian sarcoma virus* called PR2257 (Geryk *et al.*, 1989). As revealed by sequencing, this virus acquired full c-*src* and, in addition, a long stretch of the c-*src* untranslated region. The virus became oncogenic by a single nucleotide insertion resulting in an altered reading frame at the c-*src* carboxy end. We prepared more interesting mutations (Yatsula *et al.*, 1996), but the *src* field became so crowded that after a while we discontinued further work.

Deep changes triggered by Gorbatchov's Perestroika were on the move in the late eighties. This can be documented by the International Congress of Biochemistry held in 1988 in Prague, where my session on retroviruses was attended by eminent scientists such as H. Temin, H. Hanafusa, S. Hughes,

M. Lineal, R. Guntaka, and others. It became obvious that in certain aspects we were not lagging behind Americans, especially in the investigation of simple proviruses and their recombinations. There were excellent scientific discussions and open-minded comments on what was going on at our farm in Koleč and in my home. We succeeded in reestablishing confidence and collaboration with our colleagues, in which stayed especially Ramareddy Guntaka, Steve Hughes, and Howard Temin.

IX. SILENCING THE PROVIRUS AND THE RED POWER

Chacun pour soi et Dieu pour tous.
Everyone for himself and God for all.
French proverb cited by F. M. Dostoyevsky in his diaries

Hrajte, hoši, komedii,
bouda je dost veliká;
však je v této vaší boudě
věru chasa všeliká.
Z básně "Centralistická," Jan Evangelista Purkyně: Opera omnia

Get down to your comedy
Guys, your hut is spacious;
With those you have to play inside –
Of all the kinds, good gracious!
From poem "Centralistic," J. E. Purkynje, Opera Omnia

The year 1989 was very fruitful for me. From my past experience, especially from 1968, it indicated deep changes under way. This was heralded by disobedience to police orders, local demonstrations, finally culminating in a massive student demonstration. As usual, the police were ruthless, but in addition, horrifying news circulated that a student had been killed. Despite that it was untrue, it triggered an emotional reaction, and enforced persuasion that the rotten regime of the Party secretaries, which was linked to Soviet occupation, should be overthrown and replaced by a democratic government. I felt deep satisfaction that the demand of 1968 would be finally revived and substantiated. That was why I took part in street demonstrations attended mainly by youngsters, while a mob of adults looked reluctantly at what was going on. I became persuaded that the end of the communist era was close when we reached the Old Town Square and rhythmical shouting was heard "Here it had started and here it all ends" (the communist coup d'état in 1948 was announced just at this same place). Then I moved to the radio broadcasting station, where a group of people whom I joined were arguing with armed policemen and tried to persuade them that the end of their power had come. Later, I met by chance the wife of Ladislav Hejdánek, one of the main coauthors of *Charta 77*, and in their

home we celebrated the end of the Red era. Ladislav remarked pertinently that this end should have come earlier, but one cannot give orders to history.

The Academy soon became a boiling soup. The views on its future differed diametrically because a body of respectable and honest representatives was lacking. Finally, an assembly of members of the Academy (Academicians) was called together, supplemented, however, with representatives of Academy Institutions selected by scientists. A new governing body called Committee for Directing the Institutes had been elected and O. Wichterle, a highly respectable person, was assigned in charge of it. I was also elected as a member of this body. Many tasks had to be solved, but as a result of pseudoradicalism of a part of the members, the meetings of the Committee were not very fruitful. However, some important points had been reached. The dissolution of the body of Academicians dominated the discussions, as well as reshaping the Academy as a Confederacy of Research Institutes and establishing elected scientific councils. All this was incorporated into a law, which the Parliament approved. I had a good feeling about it because the main accent was on science and the power of directors was limited by elected scientific councils. However, public recognition of science remained low because of some misleading concepts such as that the communism identified itself with scientific progress. In reality, it was just the contrary; the Soviet block was not capable to ensure the required tempo of science development in its nonmilitary branches. I took advantage of Harold Varmus' stay in Prague, and, thanks to the contacts of Ladislav Hejdánek, I arranged his visit to President Havel. Harold had argued excellently in favor of science, but I do not think he persuaded our President.

Another burden was put on me because I was elected director of our Institute. I was showing quite a lot of resistance to this honor because I remembered taking over the directorship in 1968 and the consequences, but I was almost forced to take this responsibility. At the Institute level, we tried hard to equilibrate the budget because the allocated money was less than we had expected. The situation improved later with founding of the Grant Agency and with the availability of foreign grants, for which I strongly recommended to apply.

Another problem arose with the opening of the border. Understandingly, students wanted to acquire experience abroad and suddenly started to emigrate. My group lost three of them at once. Unfortunately, there was no chance to attract some of the colleagues who had emigrated previously because the conditions we were able to offer them were not comparable with those in their current position. Furthermore, I had to replace those group leaders who had signed collaboration with the secret police. Therefore, we had to reshape the scientific groups and finally succeeded in stabilizing the staff. In fact, people were allowed to go abroad for several years without losing their position in the Institute, which was the only attraction I was able to provide.

Being exposed to so many demanding tasks, only spare time remained for my scientific work. However, I still kept in close touch with my group. Of my continuous interest was the demonstration that the v-*src* oncogene induces specific tumor rejection immunity and contains the already mentioned TSTA. Based on my own experiments, I supposed that the LTR, v-*src*, LTR provirus would be a suitable tool for determining the structure of TSTA because its DNA produced fast growing sarcomas in chickens (Svoboda *et al.*, 1992). Together with Jiří Plachý, we made a series of experiments demonstrating that v-*src*-induced tumors tend to regress in one congenic line of chickens, but progress in another one. The difference between both chicken lines lay in the B (MHC) locus (Svoboda *et al.*, 1996). Then using further approaches, we demonstrated that in regressor chickens cell-mediated immunity is responsible for tumor rejection, that it is possible to elicit this immunity by DNA immunization (Plachý *et al.*, 2001), and that a specific antigenic epitope is localized at the RSV v-*src* carboxy end. Thus v-*src*, as a result of a stretch of 12 new amino acid incorporations, acquires a specific rejection antigen. All this enterprise was made possible by breeds of our congenic chicken lines in the Koleč farm.

Another phenomenon was puzzling, namely the lack of reversion in our virogenic cell lines. In some lines such as XC cells, as we learnt from our experience, the proviruses were amplified and therefore the likelihood of getting them all silenced was very low. How was it with a hamster cell line carrying only one incomplete LTR, v-*src*, LTR provirus? We tackled this problem together with Jiří Hejnar and found that revertants in this cell line arose with a high frequency far exceeding spontaneous gene mutation (Hejnar *et al.*, 1994). The provirus in revertants was highly and irreversibly methylated, as had been previously observed by John Wyke's group (Searle *et al.*, 1984). However, when proviruses from revertants were demethylated, they reacquired full oncogenicity, which proved that an epigenetic change was involved in the reversion. As suggested by some others' and our experiments, avian proviruses can be efficiently silenced in mammalian cells. Could such silencing be a consequence of provirus and especially RSV LTR methylation? A series of additional experiments agreed with this conclusion. Finally, we demonstrated that RSV LTR protected by an antimethylation CpG island expresses reporter genes in mammalian cells far better than unprotected constructs (Hejnar *et al.*, 2001). These studies are part of our long-term endeavor aimed at the definition of chicken cell factors facilitating RSV production in mammalian cells. The quest for identification of cell factor(s) modifying the course of retrovirus infection became an important issue having a great impact on finding new therapeutic strategies, including HIV. Separately, I summarized seven so far known blocks of RSV replication in the mammalian cell (Svoboda, 1998). However, in the more intensively studied HIV even a higher number of cellular factors, either

positively or negatively influencing virus replication, are in play. The long-term persistence provides retroviruses with a dangerous means to escape the defense activity of the organism. In pursuing the course of ALV infection in ducks lacking any endogenous viral chicken sequences, we established that ALV had persisted in infected ducks for 5 years, which was the period of observation (Nehyba *et al.*, 1990). This was corroborated by detecting proviral sequences in different organ tissues, especially during the early period after infection. Interestingly, we noticed periods of viraemia appearing and disappearing irregularly. This model of viraemia has been elaborated in detail (Trejbalová *et al.*, 1999) and should be useful for further studies in this direction. By this I have exhausted our main interests in the last turbulent period.

X. EPILOGUE

> Wer das angestrengte Nachdendken über wissenschaftliche Probleme kennt, der fühlt sich nie leer und einsam und zudern erlangt er einen festen Stand gegenüber dem Wechsel des Schicksals.
>
> Aus Albert Einstein's Brief

> Those who are familiar with intensive thinking about scientific problems never feel empty or lonely; in addition, they acquire a strong hold on the turns of fate.
>
> Facsimile of Albert Einstein's letter

> At' si bylo, jak si bylo, přece jaksi bylo, ještě nikdy nebylo, aby jaksi nebylo.
>
> Hašek, J.: Osudy dobrého vojáka Švejka za světové války

> Good times or bad times, they always were times of a kind; no times have ever been without being of a kind.
>
> Hašek, J.: The Good Soldier Svejk and His Fortunes in the World War

> Dobré pivo, dívka hezká, to ti dává země česká!
>
> Nápis v české hospodě

> Good beer and a pretty girl's cheer, that's what you get behind the Czech country frontier!
>
> Inscription on a Czech pub wall

It is hard to imagine that I am supposed to write an epilogue because in science, there is no epilogue. What I want to do is to comment on some crucial issues mentioned in these memoirs. People from the West might wonder how much space I dedicated to social life. Everybody who lived in Central Europe in the second half of the last century had to face the problem of how to cope with the disturbing flow of events without losing moral consciousness. Not surprisingly, individuals behaved in various ways. I keep in my mind the shock when I learnt that almost half of the group leaders of our Institute signed collaboration with the communist secret police. This I would never have imagined.

The mind of a scientist is generally obsessed by the vision that they are the "best and brightest" rulers in their field. In this respect, I would like to remember T. G. Masaryk, renowned thinker and first President of our Republic, who noticed that he never liked to be the first but the second. Finally, he became the first not by pushing himself, but being pushed by others. Scientific success is the dream of every scientist, but it remains risky and unpredictable. As many of us know, such success requires clear ideas, stubbornness, and hard work. Of great importance is speed of research ensured by the required resources and by the availability of trained students. The great majority of people reach the level of good science. As Howard Temin, who belonged to the wisest men I have ever met, commented: "Don't sell good for the best." I have never been selling out good and critically respected the best.

We are now getting to the issue of scientific collaboration. Fortunately, I had been repeatedly supported by international agencies and my Western colleagues. Nowadays, we have access to a series of American and European grants, which are still not used efficiently. However, with some nostalgia I remember the grounding period of retrovirology and cancer research in the sixties, when a handful of people involved were supportive to each other, knowing that the survival of these fields, which had been under heavy pressure from the side of classical disciplines, was in stake. At present, we have available international collaborative grants, which provide real stimulation to our laboratories and speed up the solution to problems by complementing technology and ideas. Recently, Mike Bishop raised his voice, favoring collaboration versus competition in science, and I agree with him. I quote his words: "The popular press dramatizes our competitions. But for each for these, there are countless collaborations" (Bishop, 2003).

Coming from a small country, I would like to comment on this fact briefly. In the sixties, Hašek put forward the idea that the Institute should create such a micromilieu that would protect it from external pressures. In that post-Stalinist era, the strategy provided a real shield. In spite of the fact that at present there is no need for such protection, a favorable cooperative and friendly atmosphere may contribute to attracting good people even from abroad. What kind of research can be performed in a small country? The answer is not simple, but preferentially such research should be based on outstandingly original observations and ideas.

I would also like to add a few words about loyalty. I was criticized by Howard Temin for not having left my country after the occupation. When I explained to him that the responsibility and probably the future of our Institute had been put on me, he agreed that mine was the right decision. I should underline that the Institute of Experimental Biology and Genetics represented a unique institution in our country and certainly was worth saving. Nowadays, the scientific institutes operate on the basis of selection of

staff members from different resources and sometimes it is hard to distinguish among them. However, there remain places with a traditionally high level of original-minded and cooperative science such as at Cambridge University and McArdle in Madison, which hopefully will be preserved and will keep their traditions.

Finally, how does the situation stand and will probably develop in the directions I covered in this chapter? The oncogene v-*src* and others, as well as their normal counterparts, were defined as crucial members of cell signaling and gene regulatory pathways, which represents essential information. However, we are still lacking the full picture about how the oncogenes accomplish their tumorigenic activity. As a special example, I would take v-*src*, transforming rodent but not human cells.

Retroviruses have become functionally characterized at the level of single nucleotide stretches. However, a new frontier of retrovirus control has appeared, represented by cell factors that either stimulate or inhibit virus replication. We were confronted years ago with such factors in mammalian RSV-transformed cells, which for virus production required complementary chicken cells (Svoboda and Dourmashkin, 1969). Using transfection and cloning, genes negatively regulating HIV entry (TRIM5α) and reverse transcription (APOBEC3G) were described (Bieniasz, 2004). There is good hope that they will be utilized in the battle against HIV. Thus, the past is contributing to the present. I am going to finish with a very appropriate statement by Peyton Rous (1965):

> Perhaps to-morrow some cleaving discovery on the causation of tumors by viruses will demolish the inferences of to-day; yet what we now know would be worth little if none was made.

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RUNX Genes in Development and Cancer: Regulation of Viral Gene Expression and the Discovery of RUNX Family Genes

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Mouse embryonal carcinoma (EC) cells, also called teratocarcinoma stem cells, are nonpermissive for polyomavirus growth, whereas differentiated derivatives of the cells are permissive. Mutant viruses capable of growing in EC cells can be isolated. They have genomic alterations within the viral enhancer, which is required for viral gene expression and DNA replication. This viral regulatory region was considered as a potential probe for mouse cell differentiation. The 24-bp-long A element within the enhancer was identified as a minimum element, which also shows a lower activity in EC cells compared with the differentiated cells. Transcription factors PEA1/AP1, PEA2/PEBP2, and PEA3/ETS were identified as A element-binding proteins. All of them are absent in EC cells and induced to be expressed when the cells are differentiated. Although PEBP2 has a weaker transactivation activity compared with other two, it is essential for the enhancer function of the A element. Purification and cDNA cloning revealed that PEBP2 has two subunits, DNA-binding α (PEBP2α) and non-DNA-binding β (PEBP2β). PEBP2α was found to be highly homologous to a Drosophila segmentation gene, *runt*, and a human gene *AML1* that was identified as a part of the fusion gene, AML1/ETO (MTG8) generated by t(8;21) chromosome translocation associated with acute myelogenous leukemia (AML). Core-binding factor (CBF), which interacts with a murine retrovirus enhancer, was found to be identical to PEBP2. runt, PEBP2α and AML1 are now termed RUNX family, which are involved in cell specification during development. There are three mammalian RUNX genes, *RUNX1*, *RUNX2*, and *RUNX3*. *RUNX1* is essential for generation of hematopoietic stem cells and is involved in human leukemia. *RUNX2* is essential for skeletal development and has an oncogenic potential. *RUNX3* is expressed in wider ranges of tissues and has multiple roles. Among others, *RUNX3* is a major tumor suppressor of gastric and many other solid tumors.

I. INTRODUCTION

The RUNX gene family has attracted broad interest in recent years because these genes encode transcription factors that are involved in cell lineage determination during development and various forms of cancer. The importance of *RUNX1* in hematopoiesis and leukemia is well established. *RUNX2* is required for skeletal development. *RUNX3* is ubiquitously expressed in different tissues and has been shown to have multiple roles in diverse biological systems. *RUNX3* regulates the development of dorsal root ganglion (DRG) neurons and is required for the axon path finding of proprioceptive neuron in the spinal cord. *RUNX3* is also primarily responsible for CD4 silencer activity in developing T cells. Recently, it is becoming increasingly acknowledged that *RUNX3*, which was first described as a tumor suppressor in gastric cancer, is involved in many distinct cancers in different tissues. Using the retrovirus insertional mutagenesis strategy in the mouse, *Runx1*, *Runx2*, and *Runx3* were shown to function as oncogenes in cooperation with *c-myc* to induce T-cell lymphoma. Thus, RUNX genes can

have both oncogenic and tumor-suppressive functions [see Ito and Bae (2004) for overview of the field].

The transcription factors encoded by the mammalian genes of the RUNX family were discovered by virologists. DNA and RNA tumor viruses have been characterized in landmark studies that defined many of the basic processes of carcinogenesis and established the foundation of modern molecular oncology. For example, research from our group led to the discovery and characterization of the middle T antigen of the polyomavirus (Py), which is the major oncoprotein encoded by this DNA tumor virus. One amazing accident is that the search for middle T antigen of a related virus *simian virus 40* (SV40) led to the discovery of a then unknown cellular protein p53, whose importance in cancer research was beyond anyone's imagination at the time of its initial characterization (Dilworth, 2002).

Before the technology for the molecular analysis of developing embryos was available, virologists used viruses as probes to analyze cellular processes. One line of research capitalized on the key observation that embryonal carcinoma (EC) cells have properties in common with early embryos. This line of research demonstrated that under specific conditions, EC cells that are introduced into blastocyst can differentiate into normal tissues in chimeric mice. Interestingly, undifferentiated EC cells are refractory to viral infection, but allow for viral growth when they are induced to differentiate. Importantly, mutant viruses that were capable of growing in undifferentiated cells were isolated and shown to contain mutations within the enhancer of the virus. Analysis of the Py enhancer led to the identification of a critical cellular transcription factor, the Py enhancer binding protein 2 (PEBP2).

Murine retroviruses induce tumors in various tissues. Several oncogenes and proto-oncogenes were identified by transduction into viral genomes or by viral integration into regulatory regions of cellular genes. Regulatory elements of retrovirus enhancers were also intensively examined because their activities affect the oncogenic potential of the viruses. Through these analyses, a cellular transcription factor named the core-binding factor (CBF) was identified. Subsequent studies revealed that PEBP2 and CBF are identical. Two other genes that had been identified by other groups were also found to be related to PEBP2/CBF. The *runt* gene was found to be important for the developmental regulation of early Drosophila embryos and the *AML1* gene was identified as a result of its involvement in chromosomal translocations in a type of human leukemia. Runt and *AML1* turned out to be the evolutionarily conserved genes encoding the DNA-binding subunit of PEBP2/CBF. In this chapter, I will summarize how our group discovered the RUNX gene family using the Py as a probe, and how our findings provided the fundamental basis for the development of many exciting research endeavors of the present day.

Note on the nomenclature: The three mammalian genes that are homologous to *runt* have been given a variety of names by different research groups, causing intractable confusions among novices and professionals alike. Thus, in 1999–2000, several researchers in the field, including Peter Gergen, Yorum Groner, Scott Hiebert, Paul Liu, James Neil, Misao Ohki, Nancy Speck, and myself, in coordination with Gary Stein and Andre van Wijnen, proposed a common naming system for these genes. The name that was adopted by this group was runt-related (RUNX) genes. The details of this discussion and choice are described by van Wijnen *et al*. (2004). Thus, each of the names within each of the following three clusters is identical to the RUNX nomenclature shown at the beginning of the cluster. The three RUNX genes are: *RUNX1(AML1/PEBP2αB/CBFA2)*, *RUNX2(AML3/PEBP2αA/CBFA1)*, and *RUNX3(AML2/PEBP2aC/CBFA3)*. To avoid unnecessary confusion, the RUNX nomenclature is used here unless the original names are necessary to describe the historical development of the RUNX field.

II. EC CELLS (TERATOCARCINOMA STEM CELLS): THE BIOLOGICAL MODEL THAT LED TO THE IDENTIFICATION OF PEBP2

Teratomas were first observed in mice by Stevens and Little (1954). Teratomas occur spontaneously in strains of 129 mice and can also be induced with high efficiency by grafting early embryos or genital ridges into the testes of many different strains of inbred mice. Whether spontaneous or induced, some teratomas are serially transplantable in syngeneic adult mice. Teratomas contain a wide variety of tissues that correspond to derivatives of the three germ layers. When transplantable, they contain embryonic-like cells called EC cells. EC cells are the stem cells of teratocarcinomas and are responsible for tumor induction following transplantation. EC cells are remarkably similar to the cells of early embryos. Both types of cells are pluripotent to differentiate into a variety of tissues from all three embryonic germ layers and also have self-renewal capacity. Cell lines can be relatively easily obtained from them that retain the fundamental properties of cells growing *in vivo*. Therefore, EC cells represented a useful alternative to studying early mouse embryo development (Martin, 1975; Nicolas *et al*., 1976). These properties of EC cells are particularly relevant in the context of our current knowledge of RUNX genes as cancer genes (Blyth *et al*., 2005). Because RUNX proteins are now known to be important in development and stem cell biology, their discovery in differentiating EC cells was in retrospective not accidental.

Under some conditions, EC cells develop into normal tissues when mixed with the inner cell mass of blastocysts (Mintz and Illmensee, 1975; Papaioannou *et al*., 1975). This observation suggested that a malignant phonotype could be reversed under some conditions. Furthermore, differentiated EC cells were found to lose their malignancy *in vivo*. These observations suggested that EC cells might be useful in studying the relationship between neoplasia and differentiation. Taken together, these properties of EC cells made them an attractive model for the study of embryogenesis and carcinogenesis, the same biological and pathological processes in which RUNX proteins are now known to participate.

In 1970, Stevens isolated transplantable teratomas that were derived from grafted embryos and designated them OTT followed by a number. Subsequently, Lehman *et al*. (1974) isolated cell lines from OTT6050 and characterized the *in vitro* differentiation of these cells. In addition to proliferating indefinitely *in vitro*, the isolated cell lines continually produce differentiated progeny in a manner similar to EC cells *in vivo*. Similarly, EC cells remain highly malignant *in vitro*, whereas the differentiated cells are usually benign (Pierce *et al*., 1960).

Lehman *et al*. (1974) had the foresight to pose the question, "…, is it possible that single or small numbers of HeLa cells may differentiate to a postmitotic, non-tumorigenic state and never be recognized because they are rapidly overgrown by the highly malignant cells?" This concept of highly malignant cells with self-renewal and differentiation capacities is exactly that of "cancer stem cells" recently being discussed intensely. This concept seems to be stemmed from the studies around that time. It is interesting to note that RUNX genes appear to have close relationship with cancer stem cells according to the recent studies.

The F9 cell line was isolated from OTT6050-970 by Grandchamp in Ephrussi's laboratory (Artzt *et al*., 1973). F9 cells do not differentiate and remain as ECs *in vivo* (nullipotent). Cultured F9 cells also remain undifferentiated; however, they can be induced to differentiate into endoderm-like cells *in vitro* by treating the cells with retinoic acid (Strickland and Mahdavi, 1978). These properties of F9 cells made them useful for several different investigative strategies.

III. INFECTION OF EC CELLS WITH RETROVIRUSES

Mouse EC cells were found to restrict the growth of Py, SV40, and murine retroviruses (Swartzendruber and Lehman, 1975; Teich *et al*., 1977). EC cell lines are refractory to murine retrovirus (MuLV) infection, whereas differentiated cells that are derived from EC cells are susceptible to viral infection.

Teich and Weiss found that deficits in postintegration and pretranslation were responsible for the lack of MuLV replication in undifferentiated PSA4 stem cells. The Moloney murine leukemia virus (MoMLV) does not normally replicate in EC cells; however, MoMLV replication is observed following fusion with permissive cells, suggesting that there is a unique host function that is vital to virus growth and is absent in EC cells. Jaenisch *et al.* (1982) showed that methylataion of the MoMLV genome in F9 cells prevented viral growth and that methylation will occur throughout the life of the mouse when the retrovirus is introduced prior to implantation of the mouse embryo, whereas MoMLV is not methylated (throughout the life of the host animal) when the retrovirus is introduced postimplantation (at day 8). As will be shown below, the mechanisms of nonpermissivity of EC cells for Py and murine retroviruses are entirely different, leading some researchers in the retrovirus field to study methylation and gene expression in viral and host genomes.

IV. INFECTION OF EC CELLS WITH Py

The natural host of the Py is the mouse. Differentiated cell lines derived from mouse teratocarcinoma cells, as well as normal mouse embryonal cells, are permissive for Py infection (Stewart *et al.*, 1960). Of the two related DNA tumor viruses, SV40 and Py, SV40 is a simian virus and does not grow in differentiated mouse cells, although it does induce early antigens (T antigen). Although the mechanisms of the restriction of viral growth in EC cells were studied using both SV40 and Py, the Py studies led to the development of the RUNX studies. Therefore, I will focus on the studies carried out using Py below.

Swartzendruber and Lehman (1975) studied the host–virus interaction of Py with cell lines established from a teratocarcinoma OTT6050. The teratocarcinoma cell lines used in these studies were multipotent stem cells (i.e., EC cells) and the differentiated cells that were derived from them. These studies showed that neither T antigen, V antigen, nor infectious virus could be detected in EC cells. Boccara and Kelly (1978) extended this study further by using heterokaryons of EC cells and differentiated cells. They found that the inhibition of the response to infection in the pluripotent teratocarcinoma stem cells was limited at a very early step of the lytic cycle, after penetration of the viral particle, but before synthesis of the T antigen. In addition, the fusion experiments indicated that the susceptibility of the differentiated cells to infection by Py was dominant over the resistance of the EC cells to infection, suggesting that "cellular factors required for viral expression are lacking in the EC cells and may appear or be activated during differentiation." These data suggested that presence of a diffusible repressor in the EC cells

was unlikely. RUNX and other genes that resulted in the viral phenotype that these investigators were observing were found later years to be quite consistent with this prediction (see below).

V. ISOLATION AND ANALYSIS OF PyEC MUTANTS

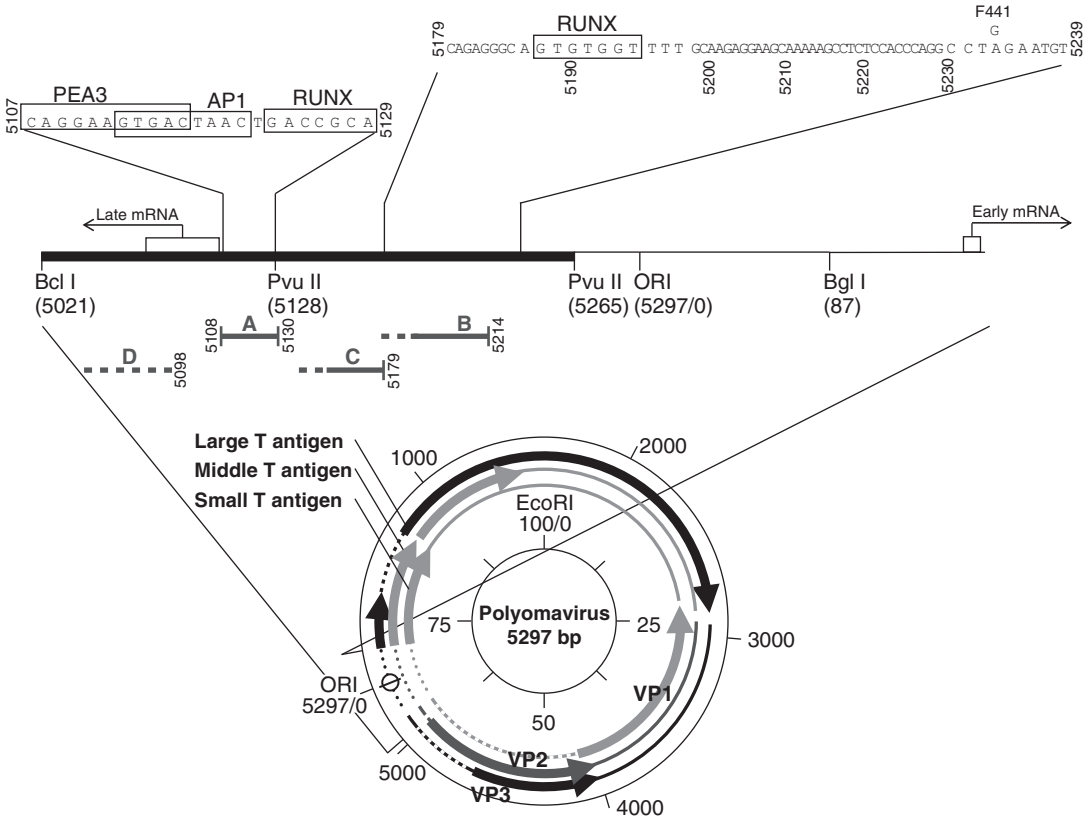
PyEC mutants of the virus that were capable of growing in EC cell lines, such as F9, PCC3, and PCC4, were isolated from the prolonged culture of EC cells infected with wild-type Py. These mutants were shown to grow well in both EC cells and differentiated derivatives. To understand the underlying mechanism of permissivity for viral infection, several laboratories isolated and characterized the PyEC mutants (Fujimura *et al.*, 1981; Sekikawa and Levine, 1981; Tanaka *et al.*, 1982; Vasseur *et al.*, 1980). The biological properties of the mutants, including oncogenicity, transforming ability, host range, and burst size, were similar to those of wild-type Py (Vasseur *et al.*, 1980).

The viral DNA of these mutants was cloned and the genomic differences were determined. The region of the viral genome that was altered in these mutants was identified as being between the origin of viral DNA replication and the initiation codon of a viral coat protein. This region was noncoding and considered to represent the regulatory region of viral replication and gene expression. Later, all the genomic differences in the mutant DNA were shown to be within the transcriptional enhancer that is contained in BclI (nt 5021) and PvuII (nt 5265) (Fig. 1).

The genomic alterations were very complex: a single nucleotide change of A to G at nt 5233 [PyF441 (Fujimura *et al.*, 1981)], a simple deletion of 23 nt [F9-5000 (Vasseur *et al.*, 1982)], duplication of various length of nearby enhancer sequences, and deletions of various sizes substituted with different size fragments representing the sequences within the enhancer. Presumably, sequence rearrangements would permit the expression of early and late functions on infection of EC cells (Katinka *et al.*, 1980, 1981). Following mixed infection of wild-type and PyEC mutants, growth of wild-type Py was not rescued by PyEC mutants in F9 cells (Fujimura and Linney, 1982). In a series of marker rescue experiments, viral genomes with mutant sequences in the backbone of the wild-type sequences were used to show that the altered nucleotides were responsible for the phenotype of the mutants (Fujimura *et al.*, 1981; Sekikawa and Levine, 1981).

VI. ISOLATION OF PyTr MUTANTS

Given the success of the studies on the PyEC mutants, we were curious to see if trophoblast cells would be resistant to Py infection. Indeed, trophoblast cells were resistant to Py infection; furthermore, like the PyEC mutants,


PEA3
AP1
RUNX
5107
C A G G A A G T G A C T A A C T G A C C G C A
5129
RUNX
5179
CAGAGGGC A G T G T G G T T T T GCAAGAGGAAGCAAAAAGCCTCTCCACCCAGG C C T A G A A TGT
5239
F441
G
5190
5200
5210
5220
5230
Late mRNA
Early mRNA
Bcl I
(5021)
Pvu II
(5128)
Pvu II
(5265)
ORI
(5297/0)
Bgl I
(87)
A
B
C
D
5108
5130
5214
5098
5179
Large T antigen
Middle T antigen
Small T antigen
1000
2000
EcoRI
100/0
Polyomavirus
5297 bp
75
25
3000
ORI
5297/0
50
VP1
VP2
VP3
5000
4000

mutant viruses capable of growing in trophoblast cells could be isolated. The results of our experience analyzing PyEC and Py trophoblast mutants are detailed below.

At about the 32-cell stage, the preimplantation blastocyst begins to form and the first morphologically detectable differentiation occurs. At this stage, the external cell layer forms the trophectoderm, from which the placental trophoblast is derived. The internal cells remain multipotent; the inner cell mass will develop into the embryo and extraembryonal endoderm (Jacob, 1977; Martin, 1975). Trophoblast cell lines were established from cultured midterm placentas of mice and were shown to produce transplantable tumors (Log *et al*., 1981). These cells exhibit several characteristics of the trophectoderm. These cells, when grown in mouse ascites, form a spherical structure that resembles the trophectoderm, that is, a blastocyst without the inner cell mass (Tanaka *et al*., 1982). The cultured trophoblast cells are unable to support the growth of wild-type Py. As in the EC cells, mutant viruses capable of growth in the trophoblast cells were isolated and designated PyTr mutants. We also isolated PyECF9 mutants in parallel. Both PyECF9 and PyTr mutants have genomic alterations within the same viral enhancer (Tanaka *et al*., 1982). This region is now known to contain several transcription factor-binding sites, including the RUNX elements. EC cells exhibit properties similar to cells of the inner cell mass. Thus, EC cells and trophoblast cells provide a model for studying the two types of cells present in the blastocyst: the trophectoderm, which emerges from the first differentiation event in murine development, and the multipotential inner cell mass.

The growth of the PyTr mutants were compared in EC cells, trophoblast cells, as well as 3T3 cells, which represent the cells of a somatic fibroblast. Mutants from less differentiated cells (such as EC cells) grow in more differentiated cells (such as trophoblast and 3T3 cells), but not vice versa. This unique growth property of the PyEC and PyTr mutants was observed in several EC cell lines representing successive stages of development (Georges *et al*., 1982). Thus, the genetic changes required to overcome restrictions in EC cells and trophectoderm cells are different and mechanisms that restrict the growth of the virus change as cells become more differentiated. This biological principle provided the basis for identifying the cellular factors that

Fig. 1 Circular map of Py genome. Coding regions for three tumor (T) antigens, or early antigens, and viral capsid proteins, VP1, VP2, and VP3 are shown. Noncoding region including the origin of viral DNA replication (ORI) is enlarged at the top. The start sites of early and late mRNAs are indicated. The thick bar between the restriction enzyme sites BclI at nt 5021 and PvuII at 5265 represents the enhancer for early and late transcription as well as for viral DNA replication. A, B, C, and D show the enhancer subelements. The binding sites for RUNX, AP1, and PEA3 are boxed. The position of a point mutant of Py capable of growing in EC cells, PyF411 (Fujimura *et al*., 1981), is indicated (see the text).

support viral replication at different developmental stages and eventually resulted in the discovery of PEBPs.

Differentiated cells derived from F9 cells after induction by retinoic acid (dF9) are permissive for wild-type Py growth. It was of interest to examine how many different stages of differentiation could be distinguished by examining the growth properties of Py mutants. From this point of view, it was noteworthy that mouse trophoblast cells obtained from the placenta at 13–15 days of gestation had properties of trophectoderm, which is formed at about the 32-cell stage. Although trophectoderm cells differentiate into several cell types, trophoblast and trophectoderm cells were equally resistant to wild-type Py infection and equally permissive for the growth of PyTr mutants. According to the criteria of permissivity to PyTr mutants, trophectoderm cells do not seem to differentiate extensively throughout their entire developmental pathway.

VII. THE MOLECULAR IMPLICATIONS OF PyFL AND PyNB MUTANTS

Friend erythroleukemia (FL) cells and neuroblastoma (NB) cells undergo differentiation in culture. When less differentiated cells are infected with Py, these cells give rise to persistently infected cultures. From these cultures, viral mutants (PyFL or PyNB) that show sequence alterations in the same noncoding region as PyEC and PyTr mutants can be isolated. When PyNB mutants are cultivated under the conditions in which cells assume different states of differentiation, the viral regulatory region changes accordingly. Sequence alterations in these mutants are deletions and direct repeats of certain parts of nearby viral sequences (Delli Bovi *et al*., 1984; Maione *et al*., 1985).

Throughout the studies that characterized the PyEC, PyTr, PyFL, and PyNB mutants, it was difficult to identify the unique genomic alterations within the viral enhancer that were specific to the cells from which the mutants were derived. Because the enhancer binds transcription factors, it seems that Py has a versatile enhancer that can adapt its regulatory region to support viral growth in different tissues at different stages of differentiation. It is presumed that the sequence alterations in Py mutants that are able to replicate create a binding site for available transcription factors or eliminate the binding of detrimental factors. However complex, detailed analyses of these sequences can reveal binding sites for transcription factors that function in specific cell types and during specific developmental stages. The recognition of this property of the Py enhancer drove researchers to explore the factors that regulate these developmental processes. To date, several of these Py mutants might still hold clues to developmental mechanisms that have not yet been explored.

VIII. DISSECTION OF THE Py ENHANCER

Transcriptional enhancers were originally identified within the genome of SV40 (Banerji *et al*., 1981; Moreau *et al*., 1981) and Py (de Villiers and Schaffner, 1981; de Villiers *et al*., 1982). Enhancers were conceived as DNA segments that stimulate the transcription of linked genes in an orientation- and distance-independent manner. The Py enhancer contains multiple short stretches of sequences that are homologous to other viral or cellular enhancer core sequences (Herbomel *et al*., 1984; Mueller *et al*., 1984; Veldman *et al*., 1985) and function as independent, functionally redundant elements (Bohnlein and Gruss, 1986; Fujimura, 1986; Ostapchuk *et al*., 1986; Piette and Yaniv, 1986). In the era before we knew of transcription factor consensus elements, motif analysis was difficult due to the lack of knowledge of what constitutes a functional unit.

de Villiers and Schaffner (1981) showed that a 244-bp fragment of DNA from the Py genome defined by the restriction enzyme sites from BclI at 5021, through PvuII at 5131, to PvuII at 5265 (and hence referred to as the BPP fragment) strongly enhances the level of correct rabbit β-globin gene transcripts over a distance of at least 1400 bp. This study demonstrated the phenomenon of enhanced gene expression (see Fig. 1 for the restriction enzyme sites and nucleotide numbers of the Py "enhancer" fragment). Interestingly, the Py transcriptional enhancer was also shown to be required for viral DNA replication (de Villiers *et al*., 1984). The necessity of a transcriptional enhancer for DNA replication in higher organisms remains to be determined. However, there is a distinct possibility that transcription factors are involved in the initiation of DNA replication in higher organisms. The BPP fragment was intensely studied by several groups. Of them, the group headed by Robert Kamen dissected the functional elements within this DNA segment using the Py DNA replication assay (Veldman *et al*., 1985). They adopted two approaches: one was to remove the BPP fragment from Py DNA to completely eliminate enhancer activity, replace it with BPP subfragments, and test the activity; the other approach was to make a series of internal deletions within the BPP fragment to determine the boundaries of the active elements. Using these approaches, they identified four elements within this BPP fragment: from origin distal to origin proximal, D (5021)–(5098); A, 5108–5130; C (5148)–5179; B (5179)–5214 (Veldman *et al*., 1985).

Through this series of studies, the most discrete boundary of the replication-activating element that was identified lies between nt 5129 and 5126. The deletion construct from the origin proximal PvuII site (nt 5265) toward BclI site (nt 5021) up to nt 5129 replicates at 15–30% of the wild-type level, whereas the deletion construct lacking 3 more bp does not replicate at all. Very interestingly, the former construct retains the RUNX-binding site in the A element, but the deletion extending to nt 5126

eliminates a part of the RUNX-binding site (Fig. 1). The boundary of the other side of this functional element was mapped to between nt 5109 and 5124 by using a different series of the internal deletions. From these results, the sequence from nt 5109 to 5130 (the A element) was deduced to have a minimal replication activator. However, some of the deletion constructs completely devoid of the A element also replicated efficiently. It was found that there are two elements located on either side of the PvuII site at nt 5128 that are critically involved in the activation of DNA replication. A construct containing a single copy of the A element was able to replicate, but only at 2–5% of the wild-type level, whereas a construct containing two copies of the A element restored replication activity to the wild-type level. The conclusion of that study was that a replication activator can be composed of two identical elements or a combination of different elements that are functionally equivalent. Herbomel *et al.* (1984) also described very similar results.

The consensus sequence (core sequence) proposed by Weiher *et al.* (1983), which is critical for SV40 enhancer activity and shared by Py and murine retrovirus, enhancers is found within the boundary between nt 5179 and 5214 (Herbomel *et al.*, 1984; Veldman *et al.*, 1985). As described below, a RUNX-binding site was found in the A element. After the RUNX protein was purified, a second RUNX-binding site was found between nt 5188 and 5194, which is well within this boundary (nt 5179–5214) (Kamachi *et al.*, 1990). It is interesting to note that Nancy Speck described a CBF that interacts with Weiher's enhancer core sequence in the MSV enhancer. The DNA-binding subunit of the CBF and RUNX are now known to be identical (see below). Therefore, the binding site of the CBF had been identified within the Py enhancer at that time. Then, why the A element was not detected as the one having homology with Weiher's enhancer core at that time? I shall come back to this question later.

IX. DEVELOPMENTAL REGULATION OF Py ENHANCER SUBFRAGMENTS IN F9 AND dF9 CELLS

Of the dissected subregions of the Py enhancer, which element(s) is involved in developmental regulation of Py growth? Herbomel *et al.* (1984) found that the A element (core of the BP fragment) resulted in a threefold higher enhancement of the α2-collagen promoter than the B element (core of the PP fragment). In mouse EC cells, the A element resulted in 3.5-fold lower activity than in fibroblasts, whereas the B element exhibited the same level of activity in both types of cells. Yaniv's group found that there are two factors, PEA1 and PEA2, that interact with the A element and are undetectable in

EC cells. PEA1 was shown to be induced after the cells are differentiated (Kryszke *et al*., 1987; Piette and Yaniv, 1987). PEA2, later shown to be RUNX, was also found to be synthesized after F9 cells were induced to differentiate (Furukawa *et al*., 1990). PEA1 was later shown to be identical to AP1, which was subsequently found to be a heterodimer of c-Jun and c-Fos. Over the course of these studies, we decided to focus on the A element of the Py enhancer.

Although the BP fragment was found to show differential activity in fibroblast cells and undifferentiated EC cells, there were genomic alterations in the PP region of the genome of the mutants that grew well in F9 and PCC4 cells. In particular, PyF441 that was isolated by Fujimura *et al*. (1981) had a single base change from A to G at position 5233. Therefore, it was of interest to identify and examine factors that differentially interact with this region of the mutant and wild-type enhancer in F9 cells and their differentiated derivatives. Indeed we found a protein that interacted with PyF441, but not with enhancers containing wild-type sequences (Kovesdi *et al*., 1987). However, this protein that interacted with PyF441 was expressed in a variety of cells, including F9 cells, and was not developmentally regulated. Furthermore, although this factor explained the phenotype of the PyF441 mutant, this mutation was not present in all the PyEC mutants and was, therefore, not further studied.

Another region of Py enhancer that interested us was the segment nt 5119–5142 that is deleted in the F9 mutant, F9-5000 (Vasseur *et al*., 1982). This deleted region spans the PvuII site in the middle of the BPP and overlaps with the deletions that we identified in PyTr-91 and PyTr-92 mutants (Tanaka *et al*., 1982). We hypothesized that ΔF9-5000, the region deleted in the F9-5000 mutant, might be the binding site for a repressor protein that is present in F9 cells. We identified a factor that interacts with ΔF9-5000, which we termed PEBP4, and found it to be present in F9 cells as well as differentiated cells (Furukawa *et al*., 1990). ΔF9-5000 also bound to two other factors, AP1 and RUNX. The binding site for RUNX overlapped with a part of the binding site for PEBP4 and the application of RUNX displaced PEBP4 from ΔF9–5000. In addition, RUNX was clearly shown to be undetectable in F9 cells, but was detectable in F9 cells that were induced to differentiate (Furukawa *et al*., 1990). These results suggested that there is an interplay of a ubiquitous negative factor and differentiation-induced positive factors, which might at least partially explain the differential activity of the Py enhancer in undifferentiated and differentiated cells. However, additional properties of purified PEBP4 suggested that its regulation is more complicated than initially believed and further characterization of PEBP4 was not pursued in our laboratory. Nevertheless, this study also suggested the importance of RUNX. Therefore, we continued to characterize the Py enhancer to identify the factors involved in developmental regulation.

X. RESPONSIVENESS OF Py ENHANCER A ELEMENT TO ACTIVATED Ras AND TPA

Another interesting feature of the Py enhancer was soon discovered: the function of the Py enhancer can be strongly modulated by viral or cellular oncogenes. This observation provided a very simple system to study the signal transduction pathways that regulate gene expression by oncogenes.

The transforming gene E1A of an adenovirus represses the activity of the enhancer in HeLa or L cells (Borrelli *et al*., 1984; Hen *et al*., 1986; Velcich and Ziff, 1985), whereas the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the Ha-ras oncogene activate the enhancer (Wasylyk *et al*., 1987). The target of repression by E1A was found to be left-hand (late) side of the A element. On the other hand, the target of stimulation by Ha-ras and TPA was found to be the AP1 and PEA3/PEBP5 sites (Satake *et al*., 1989; Yamaguchi *et al*., 1989) (Fig. 1). TPA also enhances viral DNA replication by activating the function of AP1 and PEA3/PEBP5 sites. Each of these two elements by themselves can activate DNA replication in response to TPA, suggesting the possibility that growth factors and oncogenes directly stimulate DNA replication (Asano *et al*., 1990). The RUNX site by itself is not able to stimulate DNA replication, but it functions as a positive element in the context of the A element (Murakami *et al*., 1990). These observations suggested that AP1, PEA3, and RUNX are induced to positively regulate gene expression after the differentiation of F9 cells. We wondered whether the induction of these factors would trigger the differentiation of F9 cells. Indeed, exogenous expression of activated Ha-ras increased the AP1 site DNA-binding activity in F9 cells. Ha-ras and c-Jun also induced the differentiation of F9 cells, suggesting that AP1 might play a key role in the initial stage of F9 cell differentiation (Yamaguchi-Iwai *et al*., 1990).

Around that time, Bob Tjian affinity-purified AP-1 using SV40 enhancer (Lee *et al*., 1987) and Michael Karin reported that AP-1 is responsive to TPA (Imagawa *et al*., 1987). Furthermore, two oncogene products, c-Jun identified by Peter Vogt (Maki *et al*., 1987) and c-Fos discovered by Curran *et al*. (1982), were found to form a heterodimer to function as AP-1 transcription factor (Bos *et al*., 1988). Soon, it was realized that PEA1 is identical to AP-1 and, later, PEA3 to be one of the family of Ets oncogene (Wasylyk *et al*., 1990).

XI. IDENTIFICATION OF PEA2/PEBP2

Using DNase I footprinting and electrophoretic mobility shift assays (EMSAs), PEA1/AP1, and PEA2/RUNX were identified as the A element-binding proteins using cell extracts from 3T6, F9, and dF9 (differentiated F9)

cells (Kryszke *et al*., 1987; Piette and Yaniv, 1987). These proteins are absent or present in very low amounts in F9 cells and become detectable when F9 cells are induced to differentiate (Furukawa *et al*., 1990; Kryszke *et al*., 1987). Thus, PEA1/AP1 and PEA2/RUNX were proteins that could account for the cellular competency for Py replication in 3T6 cells. Using 3T3 cell extracts, we identified proteins analogous to PEA1 and PEA2 (Satake *et al*., 1988). Our group used nomenclature that was different from Yaniv's group because we identified five proteins, PEBP1, 2, 3, 4, and 5 (Furukawa *et al*., 1990). PEBP1 and PEBP2 correspond to PEA1/AP1 and PEA2/RUNX, respectively, PEBP3 was detected in Ha-ras-transformed cells and shared a binding site with PEBP2, although PEBP3 was smaller than PEBP2 (Satake *et al*., 1988, 1989). Later, it was discovered that PEBP3 is a C-terminally truncated form of PEBP2. PEBP4 was identified as a factor that interacts with the ΔF9–5000 fragment and contains a complete binding site for PEBP2/RUNX (see above). The enhancer core-binding factor (CBF) that interacts with the MuLV enhancer core was independently identified by Speck and Baltimore (1987) using cell extracts from WEHI231. CBF was later found to be identical to PEA2/PEBP2/RUNX.

XII. WHY WE DECIDED TO STUDY PEBP2/RUNX FURTHER?

Analysis of the Py enhancer by Veldman *et al*. (1985) clearly showed that PEBP2/RUNX could profoundly influence the function of the Py enhancer. They found that the A element is the most discrete minimum functional element and that duplication of this element resulted in almost as much activity as the full-length enhancer. Most strikingly, deletion of 3 bp of the 3′ boundary of the A element, which eliminates 3 bp of the RUNX-binding site, completely abolished the enhancer activity. Base substitutions in the binding site also resulted in profound effects on replication enhancing activity (Murakami *et al*., 1990). These results firmly established the critically important role of RUNX in regulating enhancer activity. Although RUNX responded to Ha-ras and TPA more weakly than AP1 and PEA3 (Satake *et al*., 1989), these data suggested that RUNX must have a fundamental role in modulating the overall enhancer activity. That RUNX was a relatively new factor for which many characteristics were unknown, led us to focus our studies on the examination of RUNX structure and function.

An important feature of RUNX is that it is a differentiation-specific factor, which is detectable in 3T3 fibroblasts (Satake *et al*., 1988) and retinoic acid-induced F9 cells (Furukawa *et al*., 1990), but not in undifferentiated F9 cells (Furukawa *et al*., 1990; Kryszke *et al*., 1987). Furthermore, RUNX appears

to undergo molecular and functional modifications in association with cellular transformation. Although we know that PEBP3 is a C-terminally truncated form of PEBP2, we still do not understand the significance of the preferential conversion of PEBP2 to PEBP3 in Ha-ras-transformed cells. RUNX and AP1 can simultaneously bind to their adjacent recognition sites, suggesting the possibility of their functional cooperation (Martin *et al*., 1988; Piette and Yaniv, 1987; Satake *et al*., 1988). These discoveries suggested a synergism between RUNX and PEA3/Ets and AP1 factors. Through such interplay, it is possible that RUNX might contribute to the de-depression of Py enhancer activity on differentiation.

On the other hand, Wasylyk *et al*. (1988) described an opposing view that the PEA2(PEBP2) site might serve as negative transcriptional regulator because it inhibits the enhancer activity via the PEA1(AP1) motif in mouse L cells using F9 cells as hosts. It was reasonable to believe that the apparent regulatory influence of the RUNX site could depend on its context within the sequence tested as well as on the type of host cells employed; a presumption that we now know to be correct. However, to understand the overall role of the A element in Py enhancer activity during mouse EC cell differentiation and embryo development, we felt that it was essential to characterize PEBP2/RUNX and therefore decided to study RUNX by purifying the factor and cloning the cDNA.

XIII. PURIFICATION OF PEBP2 REVEALED THAT IT IS A HETERODIMER COMPOSED OF TWO SUBUNITS

Ha-ras-transformed NIH3T3 cells express a large amount of PEBP3. We knew that PEBP3 was a C-terminally truncated form of PEBP2. Therefore, we chose Ha-ras-transformed cells as a source of PEBP2/PEBP3 protein. We induced tumors in nude mice by inoculating them with these cells and the tumors were collected. PEBP3 was purified from the extracts of these tumors (Kamachi *et al*., 1990). The purification procedure involved three chromatographic steps: heparin-Sepharose chromatography, high-performance liquid chromatography, gel filtration on a TSK G3000SW column, and chromatography on specific DNA affinity columns with wild-type and mutated PEBP2 sequences. Final preparation using SDS-PAGE showed two groups of clustered polypeptide bands corresponding to 30–35 kDa (α-1 to α-4) and 20–25 kDa (β-1 and β-2). These two groups of polypeptides exhibited a distinctive color on silver staining, suggesting that they were chemically homologous within each group but different between groups (Fig. 2).

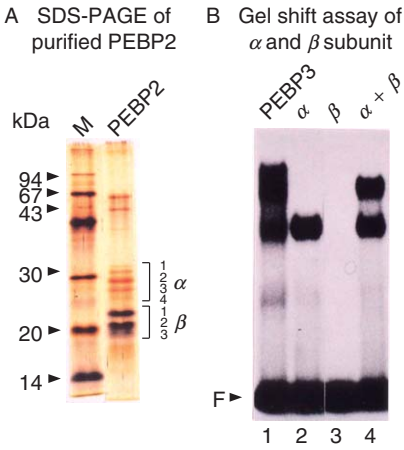


Fig. 2 Identification of α and β polypeptides in the purified PEBP2 preparations. (A) SDS gel electrophoresis pattern of purified PEBP2 as revealed by silver staining. Note that α polypeptides (representing the RUNX proteins) are stained light brown whereas β polypeptides (PEBP2β) are stained darker color. Distinct staining by different colors of two groups of polypeptides in the purified preparation of PEBP2 strongly suggested that PEBP2 is composed of two distinct proteins, later found to be RUNX and PEBP2β. (B) Electrophoresis mobility shift assay of PEBP3 (C-terminally truncated PEBP2), α polypeptide, β polypeptide, and the mixture of α and β polypeptides. Note that α polypeptide (subunit) migrates faster than PEBP3, β polypeptide (subunit) does not bind to DNA, and the mixture of the two subunits generated the band comigrating with PEBP3. The results established that PEBP2 is composed of the DNA-binding α subunit and the non-DNA-binding β subunit. (Kamachi *et al.*, 1990; Ogawa *et al.*, 1993a).

Each of the α and β polypeptides bound to the PEBP2 site as shown by EMSA. However, the position of the shifted bands generated by the β polypeptides was significantly slower than that of α polypeptides, despite the fact that the molecular size of β polypeptides (17–20 kDa) was significantly smaller than that of α polypeptides (23–30 kDa). Each of the shifted bands migrated faster than the PEBP3 band. However, when any α polypeptide was mixed with β polypeptide, the α–β complex was shifted to a position that was exactly the same as that of PEBP3. At that point, it was known that both α and β polypeptides were able to bind DNA, but it was not known that the β polypeptides represent the non-DNA-binding subunit of PEBP2. The reason that purified β polypeptides appeared to bind DNA was later found to be due to proteolytically degraded α polypeptides that comigrated with the β polypeptides in the SDS PAGE. On elution of the β polypeptides from the gel, the α and β polypeptides formed a heterodimer that exhibited DNA-binding activity (Ogawa *et al.*, 1993a).

These results established that PEBP2/PEBP3 is composed of two distinct polypeptides. Furthermore, footprint analyses revealed that the recognition

site of the α subunit is exactly the same as that of α–β heterodimer, suggesting that this transcription factor is unique because the subunits of previous heterodimers, such as AP-1, were shown to recognize a half site and not the recognition site of the heterodimer. This study also established that the consensus DNA-binding site for PEBP2/PEBP3 is PuACCPuCA. We termed the DNA-binding subunit PEBP2α and non-DNA-binding subunit PEBP2β.

PEBP2/PEBP3 was also found to bind to the B element, which is another equally important element of the Py enhancer (Kamachi *et al.*, 1990). As described above, a sequence homology search found Weiher's enhancer core sequence (common to SV40, Py, and MuLV; Weiher *et al.*, 1983) in the B element of the Py enhancer, but did not find the PEBP2 site in the A element (Herbomel *et al.*, 1984; Veldman *et al.*, 1985). The sequences of the PEBP2-binding site in the A and B elements are ACCGCA and ACCACA, respectively, whereas the original core sequence in the SV40 enhancer was CTTTCCA (opposite strand: TGGAAAG) (Weiher *et al.*, 1983). Weiher's SV40 core sequence was later modified to TGTGGA/T (opposite strand: T/ACCACA) due to a slight shift of the position (Herbomel *et al.*, 1984). The core sequence of the MoMLV is ACCACA (Speck *et al.*, 1990b). It turned out that purified PEBP2 hardly binds to the SV40 core sequence (Kamachi *et al.*, 1990); therefore, we proposed that the consensus PEBP2/RUNX-binding site was PuACCPuCA (later modified to Pu/TACCPuCA) (Kamachi *et al.*, 1990). Herbomel's consensus sequence for the SV40 homology was very similar to the sequence of the RUNX site, but the difference between having an A versus a T in the T/ACCACA sequence proved critical for PEBP2/RUNX binding. Figure 3 shows how close these investigators came to the real PEBP2/RUNX-binding site but with a lack of further information,

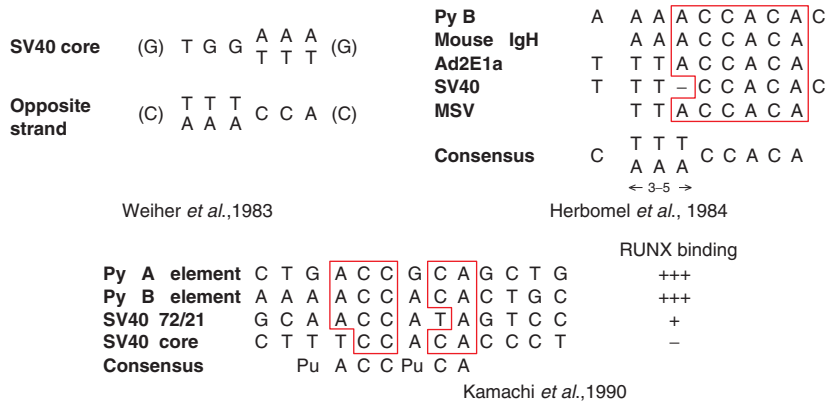


Fig. 3 Evolution of Weiher's enhancer core to RUNX-binding site. See the text for detail.

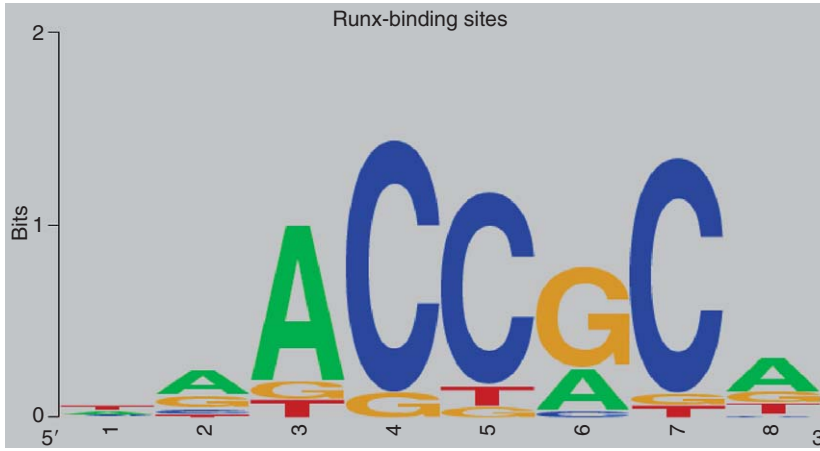


Fig. 4 Preferred RUNX-binding sequences as we know now (kindly provided by Katsuya Shigesada).

they missed the correct sequence. It is a historical irony that CBF (Speck and Baltimore, 1987), shown to be identical to PEBP2 (Satake *et al.*, 1992), does not bind to the original SV40 enhancer core sequence. Figure 4 shows the RUNX-binding site as we understand it now.

XIV. cDNA CLONING OF PEBP2αA[1]

The cDNA cloning of the RUNX protein was achieved by purifying the protein and obtaining partial peptide sequences and degenerate oligonucleotides (Ogawa *et al.*, 1993a,b). Degenerate oligonucleotides were prepared based on the partial amino acid sequences obtained from purified α and β polypeptides (Kamachi *et al.*, 1990), and cDNAs encoding these two polypeptides were cloned from a cDNA library prepared from RNA extracted from Ha-ras-transformed NIH3T3 cells (Ogawa *et al.*, 1993a,b). The cDNA containing 5496 nts, with a poly(A) tail starting from the beginning of protein-coding sequence (16th amino acid), and the shorter one containing 1060 nts longer toward the 5′ end including the first initiation codon were isolated. The combined sequence of the 6557-nt-long cDNA encoded a 513-amino acid-long polypeptide with long 5′ and 3′ noncoding regions. The 3′ noncoding region alone was 3957-bp long.

[1] Later this particular cDNA clone was identified as RUNX2. See van Wijnen *et al.* (2004) for nomenclature.

The cloned sequences revealed that the cDNA is homologous to two previously described cDNA sequences, Drosophila *runt* (Kania *et al.*, 1990) and human *AML1* (Miyoshi *et al.*, 1991). Drosophila *runt* is one of the segmentation genes belonging to the primary pair-rule class and this cDNA was the first to be reported among the family that would later be called the RUNX family or Runt domain transcription factor family. Runt was studied first as a segmentation gene, but was subsequently found to have independent functions in sex determination and neurogenesis in Drosophila. There was evidence to suggest that runt might regulate transcription, but at the time, runt did not have any known DNA-binding motif and therefore, the biochemical function of Runt was not clear. The second runt-related Drosophila gene, lozenge, was discovered and shown to be involved in the development of the eyes and hematopoietic cells (Daga *et al.*, 1996).

The N-proximal 128-amino acid region of PEBP2 cDNA showed 66% identity with the Runt protein. This highly evolutionarily conserved region was later designated as the Runt domain (see below). This observation immediately suggested that PEBP2 is likely to be involved in the regulation of early mammalian embryo development and that PEBP2 was one of the transcription factors that we intended to isolate using the Py system. Indeed, gene knockout studies performed later years confirmed that the gene is involved in the developmental regulation of mouse embryos.

The second member of the RUNX family, AML1 on chromosome 21q22, was first discovered as part of the fusion gene, AML1/MTG8(ETO). The t(8;21) (q22;q22) translocation is one of the most frequent karyotypic abnormalities in the M2 subtype [according to the French-American-British (FAB) classification] of acute myelogenous leukemia (AML). By isolating the chimeric transcripts, the gene termed AML1 was identified on chromosome 21. Break points of several cases revealed that they are clustered within the same intron and located adjacent to the last exon of the Runt domain on the C-terminal side. This means that the protein encoded by chromosome 8, termed MTG8 (ETO), is fused to the Runt domain at the C-terminal side to make AML1-MTG8 (ETO). The AML1 protein encoded by the cDNA described in this first report was later called AML1a, which encodes C-terminally truncated protein. The very fact that AML1 is involved in this recurrent chromosome translocation strongly suggested that the gene was likely to have oncogenic activity (Miyoshi *et al.*, 1991), which was later proven to be true. However, apart from its potential involvement in leukemogenesis, the normal function of AML1 was not clear. The observation that mouse PEBP2αA is homologous to human AML1 suggested that PEBP2αA also has carcinogenic potential, which was a satisfying notion to us, given the years of work that we had invested in identifying transcription factors involved in the multipotent and carcinogenic aspects of EC cells using the Py enhancer system.

XV. cDNA CLONING OF PEBP2β

Using degenerated oligonucleotide sequences based on the partial peptide sequences obtained from β polypeptides, three types of PEBP2β cDNAs were isolated that encoded 187, 182, and 155 amino acids, respectively. As suspected from the characteristics of purified PEBP2α and PEBP2β, PEBPαA and PEBP2β shared no sequence homology. Furthermore, PEBP2β polypeptides neither showed any significant homology with any other reported proteins nor did they have any known DNA-binding or dimerization domains. Thus, PEBP2 as a complex of PEBPαA and PEBP2β was suggested to constitute an entirely novel category of heterodimeric transcriptional regulators that included Runt and AML1. Of the three protein products, the former two (the 187- and 182-amino acid forms) were the predominant types and interacted with the α subunit, PEBP2αA. The latter type (the 155-amino acid form) showed very weak, if any, heterodimerization activity. Using an electrophoresis mobility shift assay, PEBP2β was shown to increase the DNA-binding activity of PEBP2αA. The details of the cDNAs of PEBP2αA and PEBP2β and the DNA-binding properties of PEBP2αA, AML1, and Runt have been described (Kagoshima *et al*., 1993; Ogawa *et al*., 1993a,b).

XVI. IDENTIFICATION OF THE RUNT DOMAIN: COMPARISON OF DROSOPHILA RUNT, HUMAN AML1, AND MURINE PEBP2αA

Peter Gergen, Misao Ohki, Katsuya Shigesada, and myself, together with the support of our research groups, wrote a brief report on the comparison of the three original members of this novel transcriptional regulator family (Kagoshima *et al*., 1993). Comparison of the amino acid sequences of Runt, AML1, and PEBP2αA revealed that they share a 128-amino acid region of high sequence homology without any insertions or deletions. This region was named the Runt domain after the first member of this family to be described at the molecular level [we specifically avoided naming it the Runt homology domain because, at that time, the Rel homology domain (RHD) had already been described]. The Runt domain of AML1 and PEBP2αA share 117 identical amino acids out of 128 (91% identity). The Runt domain of AML1 and that of murine homologue, which was isolated soon afterward and called PEBP2αB (Bae *et al*., 1993), are identical. The Runt domain in Drosophila exhibits 65% identity with its mammalian counterparts.

The highly conserved amino acid sequence of these three proteins immediately suggested that the Runt domain family is transcriptional regulator, since PEBP2 was known to be a transcription factor. We had shown that

PEBP2αA and PEBP2β form a heterodimer, which binds to the cognate DNA-binding site. We also reported that the evolutionarily conserved region of the Runt domain is the region that is required for its interaction with DNA and the β subunit (Ogawa *et al*., 1993a,b).

To confirm that AML1 and Runt interact with DNA and the β subunit, we tested whether AML1 and Runt would bind to the PEBP2-binding site and heterodimerize with PEBP2β. Indeed, both human and Drosophila proteins bound to PEBP2 site and interacted with murine PEBP2β. These results firmly established that the Runt domain is a novel evolutionarily conserved DNA-binding motif, as well as dimerization motif. Moreover, these data indicated that Runt, AML1, and PEBP2αA belong to the same family of heterodimeric transcription factors that function together with the β subunit (Kagoshima *et al*., 1993). Thus, the Runt domain transcription factor family would be an appropriate name for this family of heterodimeric transcription factors.

It was later discovered that there are three distinct genes in mammals that encode Runt, AML1, and PEBP2αA. Initially, these genes were designated with a 2 or 3 or with a B or C, which generated some confusion in the field and eventually led to a common nomenclature for the family. Since each of these proteins are related to Runt, which was the first gene to be described, a group of researchers in the field chose the name, Runt-related gene (RUNX) in accordance with the recommendation of the Human Genome Organization (see van Wijnen *et al*., 2004 for details). Thus, AML1 was designated RUNX1, PEBP2αA was designated murine RUNX2, and yet another gene was designated RUNX3 (see below).

In addition to the discovery of the Runt domain, comparison of the cDNAs of the Drosophila, human, and murine Runt genes revealed that there is a five-amino acid sequence, VWRPY, that is 100% conserved at the C-terminal end of each of the three gene products. [Note: The first report of the AML1 cDNA sequence by Miyoshi *et al*. (1991) was a splice variant, which was later termed AML1a, but the full-length cDNA that was later isolated and termed AML1b encodes the VWRPY sequence (Miyoshi *et al*., 1995)]. We noted an interesting similarity between runt/AML1/PEBP2αA and hairly/Hes. The product of Drosophila hairly, another segmentation gene of the primary pair-rule class, and its mammalian homologue Hes protein both contain the evolutionarily conserved four-amino acid sequence WRPW (Paroush *et al*., 1994) at the C-terminus that binds the transcriptional repressor Groucho or its mammalian homologue. The mammalian homologue of Groucho, TLE, was later found to interact with VWRPY (Imai *et al*., 1998; Levanon *et al*., 1998). *Caenorhabditis elegans* expresses a RUNX homolog that retains, in addition to a Runt domain, a variant C-terminal pentapeptide, IWRPF. More recently, the RUNX homolog of *Nematostella* was found to have a VWRPY sequence at its C-terminus (James C. Sullivan, personal communication).

XVII. IDENTIFICATION OF THE CBF AND cDNA CLONING OF CBFβ

The MoMLV induces thymomas when injected into newborn mice. The cell type specificity of the disease is mediated by the viral enhancer in the long terminal repeat (LTR). Of the multiple regulatory elements in the LTR, a sequence referred to as the conserved core motif, based on its homology with the SV40 and Py enhancers (Weiher *et al.*, 1983), was found to be primarily responsible for its cell type specificity. Of particular significance was the finding that point mutations in the core motif shifted the manifestation of the disease from thymic leukemia to erythroid leukemia (Speck *et al.*, 1990a,b).

The factor interacting with this core motif, CBF, was first identified in WEHI231 cells (Speck and Baltimore, 1987) and affinity-purified from calf thymus nuclei (Wang and Speck, 1992). The same factor was independently characterized by several other investigators that gave it a variety of different names that are reviewed by van Wijnen *et al.* (2004). CBF was found to be identical to PEBP2 and its isoform PEBP3 (Satake *et al.*, 1992). Purified CBF contains two major proteins, p24 and p19, both of which were shown to interact with the core motif. With a few exceptions, p24 and p19 primarily contain common tryptic peptides. A mouse thymus cDNA library was screened using degenerate oligonucleotides based on the amino acid sequences of the peptides. This cloning strategy yielded three cDNA clones that encoded isoforms of the same protein (p22, p21.5, and p17.6) due to alternative splicing of the same gene resulting in three distinct cDNA products. Quite unexpectedly, the protein products of these cDNAs did not bind to DNA, although the p24 and p19 proteins clearly copurified with CBF-binding activity. This finding, however unexpected, was in fact similar to results obtained with PEBP2.

As noted above, PEBP2/PEBP3 is composed of two distinct polypeptides, α and β, which are assembled into a heterodimer (Kamachi *et al.*, 1990). Kamachi and colleagues identified the α and β polypeptides as fundamentally distinct proteins by exploiting their serendipitous observation that these proteins have different colors when visualized by silver staining. Silver staining was used because only a small amount of purified protein was available. In contrast, the two major components of the CBF preparation studied by Speck and colleagues (p24 and p19) were visualized using Coomassie blue staining because they had purified a much larger amount of those proteins. It is likely that the α polypeptides, although more unstable than β polypeptides as we know now, were present at various steps in the protein purification of p24 and p19, but the use of Coomassie blue staining prevented the recognition of the presence of the two distinct classes of factors.

Another key technical difference that facilitated the characterization of PEBP2 was that some of the purified β polypeptides that were isolated during

PEBP2 affinity chromatography comigrated with proteolytically degraded α polypeptides. Unlike the β subunit, the α subunit has intrinsic DNA-binding activity and, as we now know, is allosterically activated by the β subunit to increase DNA binding (Ogawa *et al*., 1993a). While the studies on CBF had reached a temporary impasse, it is deemed that the findings of Kamachi *et al*. on the two distinct classes of polypeptides of PEBP2 allowed Speck *et al*. to perform experiments on CBF that were very similar to those described by Ogawa *et al*. (1993a). Purified CBF was separated by denaturing electrophoresis, bands were excised, and the DNA-binding ability of the recovered proteins was tested following renaturation. The DNA-binding activity of the α polypeptides that were identified by this procedure were combined with the p22 and p21.5 polypeptides (β subunits). The p22 and p21.5 proteins (but not p17.6) were shown to heterodimerize with the α polypeptides, which formed slower migrating protein complexes in EMSA. Comparison of the cDNA sequences revealed that p22 and p21.5 were identical to PEBP2β1 and PEBP2β2, respectively. In contrast, the sequence of a 23-amino acid-containing polypeptide obtained from purified CBF was not contained within the predicted amino acid sequences of CBFβ. This factor turned out to represent bovine AML1 (RUNX1) (Wang *et al*., 1993). Taken together, results of the studies from our group and those of the Speck laboratory provided independent validation of a key tenet in the field that is now taken as common knowledge. That is, PEBP2/CBF proteins are heterodimers that are composed of one of three α subunits that are encoded by three distinct genes and a distinct β subunit that is a product of alternative splicing of a single gene.

One can speculate how long it would have taken to solve the PEBP2/CBF puzzle if Kamachi had not used silver staining to detect the purified proteins. Undoubtedly, progress in the field would have occurred at a different pace. The competent guidance of Katsuya Shigesada, a superb biochemist, was instrumental in the purification of PEBP3, the subsequent isolation of PEBP2α and PEBP2β cDNAs, as well as the basic characterization of PEBP2/CBF as a heterodimeric transcription factor.

Only one PEBP2β/CBFβ gene is present in the mammalian genome. The official gene designate for the human gene is *CBFB*. The mouse *Pebpb2* gene has been registered (Bae *et al*., 1994) and the Drosophila β subunits, brother and big brother, were also subsequently identified (Golling *et al*., 1996).

XVIII. INVOLVEMENT OF PEBP2β/CBFβ IN HUMAN LEUKEMIA: cDNA CLONING OF THE inv(16) CHIMERIC GENE

One of the most frequently observed chromosome translocations in AML is the inversion of chromosome 16 [inv(16) (p13q22)]. When the fusion transcript was cloned, the results were a welcome surprise for researchers

working on PEBP2β/CBFβ. On 16q, inversion occurs near the end of the coding region for PEBP2β/CBFβ. On 16p, a smooth muscle myosin heavy chain (SMMHC) gene (MYH11) is broken. The isolated cDNA sequences showed that the first 165 amino acids of PEBP2β/CBFβ are fused to the tail region with coiled coil structure of SMMHC. This structure suggested that the chimeric protein might dimerize (Liu *et al*., 1993).

The discovery that the β subunit of the Runt domain transcription factor, PEBP2/CBF, is a part of a fusion protein suggested that both the α and β subunits of PEBP2/CBF are involved in leukemogenesis. Therefore, the importance of PEBP2 in leukemogenesis, and carcinogenesis in general, instantaneously increased. This discovery also strongly suggested that the α and β subunits of PEBP2 functioned together *in vivo*. Stronger evidence that the α and β subunits of Runt domain transcription factor functioned together *in vivo* came from gene knockout studies in later years. Results from these studies showed that the knockout phenotypes of Runx1 and PEBPb2/Cbfb knockout mice were nearly identical (Niki *et al*., 1997; Okada *et al*., 1998; Okuda *et al*., 1996; Wang *et al*., 1996a), which strongly supported the notion that they functioned together. Together, these results represent a biological validation of the biochemical evidence that the RUNX protein and PEBP2β/CBFβ form the heterodimeric transcription factor that was proposed by the laboratories of Katsuya Shigesada, Nancy Speck, and my own.

PEBP2β/CBFβ has also been shown to interact with RUNX2 and RUNX3. Thus far, all the *in vivo* evidence indicates that PEBP2β/CBFβ is required for the function of proteins from the RUNX family. It is possible, however, that RUNX proteins and β subunit could function in the absence of the partner subunit. It is worth noting that PEBP2β/CBFβ is expressed in embryonic stem (ES) cells (Liu *et al*., 2006), suggesting that β subunit functions in ES cells. On the otherhand phenotypes of Runx1, Runx2, and Runx3 knockout animals showed that the earliest phenotypic difference is a lack of definitive hematopoiesis resulting from a lack of Runx1 function at E9.5, suggesting that Runx genes do not have roles in the embryo earlier than E9.5. However the effects of a simultaneous disruption of all three Runx genes are not currently known; thus, it would be premature to conclude that RUNX genes do not have roles in ES cells.

XIX. DISCOVERY OF RUNX3

The third member of the Runt domain family, RUNX3, was first described by Levanon *et al*. (1994) and later by Bae *et al*. (1995) and Wijmenga *et al*. (1995). The Levanon/Groner group first used the 5′ and 3′ noncoding regions of AML1, which were described by Miyoshi *et al*. (1991), to isolate a fragment of AML1 cDNA. This cDNA fragment was used to screen a mouse leukocyte cDNA library to isolate a mouse clone containing the entire

cDNA region of PEBP2αB (murine Runx1) (Bae *et al.*, 1993). The mouse cDNA was used to screen a human HL60 cDNA library. A cDNA clone was found and designated human AML2 based on the map position (human chromosome 1p36) and the sequence homology within the Runt domain, but divergence from RUNX1 and RUNX2 outside of the Runt domain. This cDNA encoded a protein of 415 amino acids. A second cDNA from a human monocyte cDNA library was isolated and mapped onto chromosome 6p21. The sequence analysis revealed that this cDNA was a human homolog of the mouse PEBP2αA (initially, it was described as PEBP2α1). Almost at the same time, Bae *et al.* (1994) reported that PEBP2αA is on the region of mouse chromosome 17 that is syntenic to the human chromosome 6p21. Groner *et al.* (1994), in their paper in *Genomics*, described the chromosome map positions of the three members of the human Runt domain gene family, AML1 (RUNX1), AML2 (RUNX3), and AML3 (RUNX2) as 21q22, 1p36, and 6p21, respectively. The data on AML1 largely confirmed an earlier report by Miyoshi *et al.* (1991), although the encoded AML1 protein by their cDNA was larger than that of Miyoshi *et al.* and did not have the VWRPY motif at the C-terminal end. Their AML1a cDNA [different from Miyoshi's AML1a (Miyoshi *et al.*, 1991)] must have used an alternative exon that was transcribed in the cDNA around the region encoding the C-terminus of AML1. This was the first report of the chromosome locus and cDNA sequence of AML2 (RUNX3). The chromosome locus of AML3 (RUNX2) was also first reported here, although its cDNA sequence was not shown. Instead, mouse PEBP2αA cDNA was used to compare the three cDNA sequences.

RUNX3 (PEBP2αC) was independently described by Bae *et al.* (1995). The cDNA library from the human T-cell line KUT-2 was screened by using the Runt domain coding region of PEBP2αA1 (Runx2) (Ogawa *et al.*, 1993b) and PEBP2αB1 (Runx1) (Bae *et al.*, 1993) as probes under reduced stringency conditions. The deduced amino acid sequence of the cDNA was identical to AML2 (RUNX3). The chromosome locus was shown to be 1p36.11-p36.13 and the genomic structure contained five exons. Characteristics of PEBP2αC (RUNX3) were shown to be similar to the α subunit of PEBP2. PEBP2αA, PEBP2αB, and PEBP2αC all contained the VWRPY motif at the C-terminus, bound to the PEBP2 site, associated with the β subunit, and activated transcription. However, there was one important difference between RUNX1 and RUNX2 versus RUNX3. That is, in the RUNX3 gene locus, the exon that would be equivalent to exon 5 of RUNX1 and RUNX2 is missing (according to more recent numbering), the missing exon is now called exon 6 (Bangsow *et al.*, 2001). This missing exon encodes a part of the transactivation domain, TE3, and a part of negative regulatory domain for DNA binding (NRDB) (Kanno *et al.*, 1998). As mentioned above, full-length RUNX1 does not bind to DNA well. Both N-terminal and C-terminal regions flanking the Runt domain of RUNX1 are inhibiting the interfaces

of RUNX1 protein for interacting with DNA as well as the β subunit. When they dimerize with PEBP2β, the DNA-binding capacity of RUNX protein is fully activated.

There is an interesting case of cooperative binding of RUNX1 and Ets-1 that is worth mentioning here. The binding of Ets-1 to DNA is autoregulated by a region surrounding the DNA-binding domain called the Exon VII region (Lim *et al*., 1992). Surprisingly, the NRDB on the C-terminal side of RUNX1 and the Exon VII region of Ets-1 directly interact and mutually activate the DNA-binding ability of the two proteins in the absence of a β subunit. In other words, the presumably unrelated protein Ets-1 replaces the need for a β subunit when RUNX1 and Ets-1 bind side by side to the DNA of the T-cell receptor β (TCR-β) promoter (Kim *et al*., 1999). This is a remarkable case of the cooperation of two transcription factors for gene expression. RUNX3 lacks a part of the NRDB on the C-terminal side of the Runt domain and, as such, is unable to cooperate with Ets-1. It is interesting to note that PEBP2 (PEA2) and PEBP5 (PEA3, an Ets family protein) bind to the A element of the Py enhancer on each side of AP1 (PEA1). It would be interesting to examine whether these three transcription factors cooperate in binding to DNA as well as in transactivation. In any case, RUNX3 has the potential to serve as negative regulator of RUNX1 on TCR-β or any other promoters on which RUNX1 and Ets-1 function cooperatively. Since there are a large number of members of the Ets family that function in different tissues, it would be interesting to examine whether there are cases in which other members of the Ets family of proteins cooperate with RUNX1 or RUNX2, and if RUNX3 functions as a negative regulator in such cases.

A 228-bp fragment of mouse cDNA of Runx3 (Cbfa3) was described by Wijmenga *et al*. (1995). They used 2 five-amino acid regions within the Runt domain of PEBP2αA (Ogawa *et al*., 1993b) to obtain degenerate oligonucleotides and to screen a mouse thymus cDNA library. The sequencing revealed that the cDNA was a mouse homolog of AML2 (RUNX3). They determined that the locus of human CBFA3 (RUNX3) was 1p36-pter and deduced that the mouse homolog, Cbfa3 (Runx3), was on mouse chromosome 4. The locus of mouse Runx3 (Aml2, Cbfa3) was also determined to be on the distal region of mouse chromosome 4 (Avraham *et al*., 1995; Calabi *et al*., 1995).

Distal and proximal promoters were identified for all three RUNX genes. Transcripts from these two promoters encode essentially the same proteins except for the small N-terminal regions. The transcript from the distal promoter encodes 28 amino acids not present in the product from the proximal transcript. The proximal transcript has five amino acids (MRIPV) at the N-terminus that are not present in the transcript from the distal promoter. Specific details of the genomic structure and transcripts with various splicing are described by Levanon *et al*. (2001), Bangsow *et al*. (2001), and Terry *et al*. (2004).

XX. RUNX GENES IN DIFFERENT ORGANISMS: EVOLUTIONARY CONSERVATION

We now know that there are three RUNX genes in mammals. Four genes have been reported in zebra fish (Burns *et al*., 2002; Crosier *et al*., 2002; Kataoka *et al*., 2000), one in Xenopus (Tracey *et al*., 1998), four in Drosophila [runt, lozenge, RunxA, and RunxB (Daga *et al*., 1996; Kania *et al*., 1990; Rennert *et al*., 2003)], one in sea urchin (Coffman *et al*., 1996), and one in *C. elegans* [run (Nam *et al*., 2002)]. Drosophila lozenge gene was first described by Utpal Banerjee as a key regulator of eye development and cell fate determination (Daga *et al*., 1996). Later, lozenge was also found to be involved in the differentiation of Drosophila hematopoietic cells (Lebestky *et al*., 2000). As a vertebrate model system for hematopoiesis, the zebra fish has played a significant role in complementing the studies of mammalian hematopoiesis (Burns *et al*., 2005).

RUNX homologs were found in basal metazoans such as the starlet sea anemone (*Nematostella vectensis*) and sponge (*Oscarella carmela*) (James Sullivan, personal communication; Kagoshima *et al*., 2007). Since these organisms are the most primitive of all those so far described, RUNX genes appear to be invented with the advent of, and conserved throughout in metazoa. Examination of the function of RUNX genes in these organisms is expected to provide important clues as to what role(s) they play in early metazoan development and evolution.

XXI. DISRUPTION OF RUNX1 AND HEMATOPOIESIS

The involvement of the Runx gene family in the differentiation of cells is most clearly demonstrated in mouse targeting studies. Runx1 knockout mice completely lack definitive hematopoiesis in the fetal liver at embryonic day (E) 12.5 (Okada *et al*., 1998; Okuda *et al*., 1996; Wang *et al*., 1996a). Subsequent studies confirmed that Runx1 is essential for the generation of hematopoietic stem cells (HSCs) (North *et al*., 1999; Yokomizo *et al*., 2001). HSCs emerge from the endothelial cells of particular vessels at a specific embryonic stage in mammals. The ventral wall of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region around E10.5 in mice is one of such vessels where hematogenic endothelial cells are present. Runx1 is expressed in these hematogenic endothelial cells and Runx1-deficient endothelial cells are incapable of producing HSCs. These results suggest that Runx1 plays a critical role in the initiation of the hematopoietic system. Conditional targeting strategies in mice confirmed that Runx1 is involved in

hematopoiesis across multiple stages and lineages. Four different groups generated Runx1flox/flox mice crossed with MxCre or LckCre transgenic mice and induced Runx1-deficient alleles in hematopoietic cells at the adult stage (Growney *et al.*, 2005; Ichikawa *et al.*, 2004; Putz *et al.*, 2006; Taniuchi *et al.*, 2002). Runx1−/− mice showed defects in megakaryocyte and T-cell development. Surprisingly, Runx1 was not necessary for the maintenance of HSCs in the adult stage or expansion of HSC/progenitor cells (HSC/Ps). Runx1−/− mice did, however, develop myeloproliferative disease and T-cell lymphoma. It is therefore clear that Runx1 plays a pivotal role throughout hematopoiesis, from initiation to terminal differentiation. Runx1 appears to be a global regulator of hematopoiesis.

The involvement of Runx1 in hematopoiesis is supported by the fact that RUNX1 is one of the most frequently mutated genes in human leukemias (Osato, 2004; Speck and Gilliland, 2002). Mutations in RUNX1 is also associated with the human hereditary hematological disease, familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) (Song *et al.*, 1999).

XXII. DISRUPTION OF RUNX2 AND OSTEOGENESIS

Targeting studies in mice have also shown that RUNX2 is a key factor in osteogenesis. In 1997, two different groups simultaneously reported that Runx2 knockout mice die from asphyxiation soon after birth due to systematic lack of ossification, including a lack of ribs (Komori *et al.*, 1997; Otto *et al.*, 1997). Mutations in RUNX2 were also reported in the human congenital skeletal disorder, cleidocranial dysplasia (CCD) (Mundlos *et al.*, 1997; Otto *et al.*, 1997). Heterozygous Runx2+/− mice demonstrate phenotypes identical to those of human CCD patients. Since these landmark studies, RUNX2 has drawn much attention in the fields of osteopathic medicine and research. Given the growing number of individuals suffering from osteoporosis and aging-associated bone defects, RUNX2 has become more important than ever and a better understanding of its function will likely provide further insights into bone biology and novel therapeutics.

Runx2 has been described as an oncogene that cooperates with *c-myc* to induce T-cell leukemia (Stewart *et al.*, 1997). This oncogenic property of Runx2 is particularly interesting, given that RUNX3 is very well-documented tumor suppressor and induction of leukemia is associated with inactivation of RUNX1. Further examination of the oncogenic properties of RUNX genes will be important to understand the roles of these genes in carcinogenesis (Blyth *et al.*, 2005).

XXIII. KNOCKOUT PHENOTYPE OF *Runx3*: GASTRIC CANCER TUMOR SUPPRESSOR

RUNX3 is expressed in a broader range of tissues than the other two Runx genes. Therefore, the knockout phenotype of RUNX3 is observed in several different tissues.

A Runx3–/– mouse was first generated in the C57/BL6 strain in such a way that a chimeric protein containing the N-terminus to almost the end of the Runt domain of Runx3 was fused to the bacterial LacZ gene (Li *et al.*, 2002). Most of the Runx3–/– mice from this strain died within 24 h of birth due to starvation, the cause of which is not precisely known. Gastric epithelial cells of wild-type mice express Runx3, as evidenced by *in situ* hybridization and immunohistochemistry (IHC) with anti-Runx3 monoclonal antibodies. In embryos, the expression of Runx3 was much higher in the mesenchymal region of the stomach, but as mice grew there was a gradual but marked increase in the ratio of the expression in epithelial cells to mesenchymal cells as the embryos developed into adult organisms.

The gastric mucosa of Runx3–/– mice exhibits hyperplasias as a result of the stimulated proliferation and suppressed apoptosis in epithelial cells. The cells are resistant to the growth-inhibitory and apoptosis-inducing effects of TGF-β, indicating that Runx3 is a major growth regulator of gastric epithelial cells. Cell lines obtained from the gastric epithelium of p53−/−Runx3−/− mice are tumorigenic in nude mice, whereas cell lines obtained from p53−/−Runx3+/+ mice are not. Furthermore, some of the tumor cells that develop in nude mice become goblet cells, the hallmark of intestinal metaplasia associated with gastric cancer (Fukamachi *et al.*, 2004), which strongly suggests that the gastric hyperplasia and tumorigenicity of p53–/–Runx3–/– gastric epithelial cells are autonomous. Between 45% and 60% of human gastric cancer cells do not express substantial amounts of RUNX3 due to hemizygous deletion and hypermethylation of the RUNX3 promoter region. Tumorigenicity of human gastric cancer cell lines in nude mice has been shown to be inversely related to the level of RUNX3 expression. A mutation (R122C) occurring within the conserved Runt domain abolished the tumor-suppressive effect of RUNX3, suggesting that a lack of RUNX3 function is causally related to the genesis and progression of human gastric cancer.

Runx3 knockout mice were also generated in ICR strains of mice (Inoue *et al.*, 2002; Li *et al.*, 2002). In contrast to the C57/BL6 Runx3 knockout mice that died soon after birth, Runx3 knockout mice generated using the ICR strain survived until adulthood. Because the ICR strain is not an inbred strain, the Runx3−/− genotype was transferred to a Balb/c strain. Using the Balb/c strain, a significant proportion of Runx3−/− mice survived until adulthood when a "premalignant" phenotype of gastric epithelial cells was observed (K. Ito and Y. Ito, to be published).

Yoram Groner's laboratory has also generated Runx3 knockout mice using the ICR strain and observed the phenotype of the gastrointestinal (GI) tract. In this case, results from Groner's laboratory and ours are quite different; thus, it is worth exploring these differences in some detail. The first discrepancy relates to the question of whether Runx3 is expressed in the epithelial cells of GI tract (see Li *et al*., 2002, for evidence of expression; see Brenner *et al*., 2004, for a lack of expression). Recent observations made in our laboratory might help explain this discrepancy. We isolated a series of anti-Runx3 monoclonal antibodies, some of which detected Runx3 in the epithelial cells of GI tract by IHC. Several monoclonal antibodies that recognize the region of Runx3 spanning the C-terminus to amino acid 234 detected Runx3 in TrkC-expressing DRG neurons but, surprisingly, not in the epithelial cells of GI tract. It appears Runx3 is not detected in the GI tract because the binding of some antibodies is masked by a protein in the GI tract that binds to Runx3 at the C-terminal region or binding epitopes are altered by conformational change due possibly to tissue specific modification of Runx3 (K. Ito and Y. Ito, unpublished observation). The polyclonal antibody used by Brenner *et al*. (2004) primarily recognizes the surprisingly small region between amino acid 234 and amino acid 283, a region that is not "exposed" in the GI tract. Indeed, the polyclonal antibody used by Brenner *et al*. (2004) bound very little, if any, Runx3 in the GI tract despite marked binding to Runx3 in the DRG. While the molecular basis of this differential reactivity in the two tissues would be interesting to explore further, these current observations are sufficient to explain the apparent discrepancy.

A second discrepancy between the work of Groner's laboratory and my own centers around the hyperplasia and inflammation of the GI tract in Runx3 knockout mice. Groner's group reported that Runx3 knockout mice exhibit marked inflammation in the gut at about 4 weeks of age and substantial hyperplasia at 2 years of age, but no tumor formation. They concluded that the hyperplasia observed in the GI tract epithelium is secondary to the inflammation. As mentioned above, we concluded that the stomach hyperplasia of the Runx3 knockout mice is an autonomous epithelial cell phenomenon. Groner's group concluded that the hyperplasia is an autonomous phenomenon due to a lack of leukocytes. However, there is an important difference in the two strategies used to create the knockout mice. The knockout strategy used by Groner *et al*. allows the p33 splice variant of Runx3 (which lacks the N-terminal region to the middle of the Runt domain, but retains the C-terminal region) to be expressed (Fainaru *et al*., 2004). We know that the region retained in the p33 splice variant would bind to multiple cellular proteins, including the nuclear matrix. Therefore, the p33 splice variant could be an effective interfering molecule, possibly for all three Runx proteins. Consistent with this notion, Groner *et al*. observed an accelerated maturation of dendritic cells in bone marrow and spleen and the absence of Langerhans cells (a special type of dendritic cells) in the

skin of their knockout mice. Interestingly, the naturally occurring p33 splice variant is specifically expressed in dendritic cells during maturation (Puig-Kroger and Corbi, 2006). In contrast, the Runx3 knockout mice that we have used have Langerhans cells in the skin and the dendritic cells of the bone marrow are indistinguishable from those of wild-type mice. Furthermore, the knockout mice that we have used do not exhibit inflammation in the gut. Taken together, these results suggest that the different phenotypes of the wild-type and knockout mice used by Groner *et al.* could not be directly attributed to the absence of Runx3 without carefully considering the potential effects of p33. We feel that their report on accelerated dendritic cell maturation and massive inflammation, in particular, should be reevaluated to determine if these phenomena are direct result of knocking out the Runx3 gene.

XXIV. THE KNOCKOUT PHENOTYPE OF RUNX3: REGULATION OF AXONAL PROJECTIONS OF TRKC-EXPRESSING DRG NEURONS

DRG neurons specifically project axons to central and peripheral targets according to their sensory modality. Runx1 and Runx3 are expressed in TrkA- and TrkC-expressing DRG neuronal subpopulations, respectively, suggesting that they might regulate the trajectories of specific axons. Levanon *et al.* (2002) and Inoue *et al.* (2002) reported that Runx3-deficient (Runx3−/−) mice displayed severe motor discoordination and few DRG neurons synthesized the proprioceptive neuronal marker parvalbumin. In addition, proprioceptive afferent axons failed to project to their targets in the spinal cord and muscle. NT-3-responsive Runx3–/– DRG neurons also showed less neurite outgrowth *in vitro*. However, we found no changes in the fate specification of Runx3–/– DRG neurons or in the number of DRG neurons that expressed trkC. These data demonstrate that Runx3 is critical factor in regulating the axonal projections of a specific subpopulation of DRG neurons. Although there are some minor differences in the results from the two laboratories, the overall results are essentially the same.

XXV. KNOCKOUT PHENOTYPE OF RUNX3: CD4 SILENCER

T lymphocytes differentiate in well-defined stages within the thymus. Immature thymocytes lacking CD4 and CD8 co-receptors differentiate into double-positive cells ($CD4^+CD8^+$), which are selected to become either $CD4^+CD8^-$ helper cells or $CD4^-CD8^+$ cytotoxic cells. A stage-specific transcriptional silencer regulates the expression of CD4 in both immature

and $CD4^{-}CD8^{+}$ thymocytes. We described that binding sites for the Runx protein are essential for CD4 silencer function at both stages and that different Runx family members are required to fulfill unique functions at each stage. The thymopoietic phenotype observed in Runx3−/− mice has been examined by Taniuchi *et al.* (2002) and Woolf *et al.* (2003). Runx1 is required for active repression in $CD4^{-}CD8^{-}$ thymocytes, whereas Runx3 is required for establishing epigenetic silencing in thymocytes from the cytotoxic lineage. Runx3-deficient cytotoxic T cells, but not helper cells, have defective responses to antigen, suggesting that Runx proteins play a critical role in lineage specification and homeostasis of $CD8^{-}$ lineage T lymphocytes.

XXVI. KNOCKOUT PHENOTYPE OF CBFβ/PEBP2β

The PEBPb2 gene encoding PEBP2β/CBFβ has been disrupted and studied in at least two different laboratories. Exons 1–4 encoding amino acid 1–133 and the first 4 codons in exon 5 (amino acid 134–137) encode the heterodimerization domain for dimerization with α subunit (Runx1). Wang *et al.* (1996b) initially attempted to replace exons 4 and 5 with the neo^{r} gene, but this approach did not produce viable offspring. They subsequently made a hypomorphic Cbfb allele by allowing the synthesis of truncated Cbfb proteins lacking the exon 5-encoded sequences at low levels. Niki *et al.* (1997) successfully obtained PEBPb2-null mice that lack the first exon of the gene. In both cases, the homozygous mutation resulted in lethality around E12.5, due to massive hemorrhaging of the central nervous system. Additionally, definitive hematopoiesis in the liver was severely impaired. Interestingly, the phenotype of these knockout mice was strikingly similar to that of the Runx1 knockout animals (see above).

It is worth mentioning that the phenotypes of mice in which Runx1 or Pebpb2/Cbfb alleles were replaced with RUNX1-ETO (Yergeau *et al.*, 1997) or CBFB-MYH11 (Castilla *et al.*, 1996), respectively (knockin mice), turned out to be nearly identical to those of Runx1 or Pebpb2/Cbfb knockout mice. The result strongly suggests that these two fusion genes inactivate the remaining wild-type alleles of their respective genes by functioning in a dominant negative fashion. We find this to be a highly significant finding as this property of the fusion genes appears to be the basis of their leukemogenic potential.

XXVII. PERSPECTIVES

EC cells were identified as teratocarcinoma stem cells that can be transplanted to recipient cells. Only cells that remain undifferentiated within tumors are transplantable and will induce teratocarcinoma in recipient animals. Teratocarcinoma stem cells, therefore, have two fundamental

properties: multipotency and self-renewal capacity. In recent years, the concept of leukemia stem cells has emerged. Leukemia stem cells undergo differentiation *in vivo*, but only those that remain undifferentiated have the potential to generate leukemic cells. This concept has now been expanded to include solid tumors and so-called cancer stem cells have been identified in several tissues. Interestingly, RUNX genes appear to play critical roles in adult stem cells and very likely, in cancer stem cells as well.

Although RUNX3 was initially identified as a gastric cancer tumor suppressor, it is now known to be involved in many different cancers (Ito, 2004). Involvement of RUNX genes in various types of cancer is remarkable in three respects. First, RUNX is involved in cancers in many different tissues. Since mutations in RUNX3 gene are extremely rare and inactivation of RUNX3 often occurs as a result of promoter methylation, it is not easy to prove that the RUNX3 gene is responsible for any given cancer since the inactivation of genes by promoter methylation occurs in multiple genes in the same cancer cell simultaneously. In the cases of gastric (Li *et al.*, 2002) and bladder cancers (Kim *et al.*, 2005), however, loss-of-function mutations of RUNX3 were found (one and two cases, respectively), indicating that RUNX3 is indeed responsible for induction of these cancers. Nevertheless, it is remarkable to note that so many different cancers are suggested to exhibit inactivated RUNX3. Second, inactivation of RUNX3 in any given cancer type appears to occur in a high percentage of cases of that particular cancer. For example, RUNX3 is inactivated in more than 80% of the cases of gastric cancer by epigenetic silencing and protein mislocalization. Third, RUNX genes function as tumor suppressors in many cases, but they are also known to function as oncogenes in other types of cancer. The precise reason for the extensive involvement of RUNX genes in cancer is not yet known; however, these genes are likely to be involved in the fundamental process of carcinogenesis. We have preliminary evidence that Runx3 closely interacts with the signal transduction cascades that are known to be critical for the development and function of adult stem cells. This is a new and exciting area of research that has only just begun. Promising future directions of RUNX gene studies might include clarifying the potential roles of Runx genes in adult stem cells and cancer stem cells. I expect that one of the major directions of research in the RUNX field in the near future will be in the area of stem cell research.

RUNX genes also play critical roles in cell lineage specification. RUNX genes are epigenetically regulated, induce epigenetic silencing, and RUNX proteins have been shown to interact with a variety of chromatin-associated proteins. A close functional relationship of RUNX with polycomb genes is emerging. It is interesting to note that cancer cells often show a reduced stringency of cell lineage specificity. For example, some myeloid leukemia cells have rearranged immunoglobulin genes, which normally only happens in B cells. Gastric cancer cells often show both gastric and intestinal phenotypes. This loss of stringent lineage specificity might be related to a lack of RUNX gene function and carcinogenic potential.

Another interesting feature of RUNX proteins is their ability to interact with the nuclear matrix. Nuclear structure and its relation to a variety of nuclear function is still obscure, but this area is progressing steadily. The ability of the C-terminal side of the RUNX protein to tightly interact with the nuclear matrix is intriguing (Zeng *et al*., 1997). Using the Py DNA replication system, we observed that this property of the RUNX1 protein is essential for the viral DNA replication (Chen *et al*., 1998). It would be interesting to see if chromosomal DNA replication also requires RUNX and other transcription factors to recruit various components of the replication machinery to the site of the factory in the nuclear matrix. Interactions of RUNX proteins with some of the nuclear bodies have also been observed. This area might also reveal important aspects of roles of RUNX proteins in development and carcinogenesis.

XXVIII. CONCLUSION

EC cells offered the potential to study developmental regulation as well as deregulation of the normal processes that result in malignancy. It was attractive to study the interaction of Py with EC cells when it became known that Py is a useful probe to analyze some aspects of the differentiation of EC cells. EC cells are malignant, have self-renewal capacity, and are able to differentiate into many different tissues. Through this series of studies, we identified PEBP2. From studying PEBP2, we reached our current studies on the RUNX genes. Gene disruption studies revealed that RUNX genes are involved in cell specification in a variety of tissues and deeply involved in the cancers of many different tissues. Therefore, almost miraculously, RUNX genes turned out to be just the kind of genes that were initially sought to identify with the PyEC cell system.

With the number of key biological findings on RUNX (CBF/PEBP2) proteins increasing exponentially each year, it is remarkable that this field started with the identification of a few conserved nucleotides within the regulatory regions of DNA and RNA tumor viruses. Studies of the cellular machinery involved in development and carcinogenesis from the RUNX point of view, or "RUNX"ing (Zhong *et al*., 2006), seems to be promising approach to uncovering further details of the mysteries of the cell.

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The RNA Continent

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Recent progress in the analyses of the mouse transcriptome leads to unexpected discoveries. The mouse genomic sequences read by RNA polymerase II may be six times more than previously expected for human chromosomes. The transcript-abundant regions (named "transcription forests") occupy more than half of the genomic sequence and are divided by transcript-scarce regions (transcription deserts). Many of the coding mRNAs may have partially overlapping antisense RNAs. There are transcripts bridging several adjacent genes that were previously regarded as distinct ones. The transcription start sites appearing as cap analysis of gene expression (CAGE) tags are mapped on the mouse genomic sequences. Distributions of CAGE tags show that the shapes of mammalian gene promoters can be classified into four major categories. These shapes were conserved between mouse and human. Most of the gene has exonic transcription

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start sites, especially in the 3′ untranslated region (3′ UTR) sequences. The term "RNA continent" has been invented to express this unexpectedly complex and prodigious mouse transcriptome. More than a half of the RNA polymerase II transcripts are regarded as noncoding RNAs (ncRNAs). The great variety of ncRNAs in mammalian transcriptome implies that there are many functional ncRNAs in the cells. Especially, the evolutionarily conserved microRNAs play critical roles in mammalian development and other biological functions. Moreover, many other ncRNAs have also been shown to have biological significant functions, mainly in the regulation of gene expression. The functional survey of the RNA continent has just started. We will describe the state of the art of the RNA continent and its impact on the modern molecular biology, especially on the cancer research.

I. INTRODUCTION

In 1995, the mouse genome encyclopedia project (Hayashizaki, 2003a) was started. The firsthand goal of the project was the development of a comprehensive collection of full-length mouse cDNA clones and the nucleotide sequencing of these clones. The accumulated nucleotide sequence information of the full-length cDNA clones were annotated by an international consortium, the Functional ANnoTation Of Mouse cDNA (FANTOM) Projects 1 and 2 (Kawai *et al*., 2001; Okazaki *et al*., 2002). These efforts revealed the unexpected breadth and variety in the mammalian RNA expression. A new layer of complexity on mouse transcriptome was added within the FANTOM3 project (Carninci *et al*., 2005, 2006; Katayama *et al*., 2005). The cap analysis of gene expression (CAGE) tags and gene identification signature/gene signature cloning (GIS/GSC) ditags have been collected and mapped on the mouse genome (Carninci *et al*., 2005, 2006).

The concept of an "RNA continent" appears to describe the diversity of the mouse transcriptome (Hayashizaki and Kanamori, 2004; Suzuki and Hayashizaki, 2004). This RNA continent can be described with two different maps. One of the maps is the physical map of transcriptome on mouse genomic DNA sequences and the other one is the functional map, which is still hypothetical. The geography of the RNA continent in the first map can be described molecularly by mapping and manual annotations of cDNA clones and CAGE/GIS/GSC tags on the genomic sequences. In this chapter, we attempt to describe what were found in the mouse transcriptome and the biological significance of the discoveries in understandings of mammalian genome activity.

The map of the functional RNA continent is still fragmented and partial. Since establishment of the "central dogma" (Crick, 1958), scientists have tried to describe the functional interactions of proteins as a network flowchart or a map (Rual *et al*., 2005; Vidal, 2001). Today, it is known that many functional noncoding RNAs (ncRNAs), such as ribosomal RNAs and transfer RNAs, play essential roles in gene expression. Most of these ncRNAs

react as functional molecules in messenger RNA (mRNA) processing and translation machineries and are transcribed with RNA polymerase I or III (Paule and White, 2000). Hence, they are distinct from the mRNAs, which are considered as the templates for proteins synthesis and are transcribed with RNA polymerase II. The discovery of the many ncRNAs transcribed by RNA polymerase II in the FANTOM projects implies that many distinct ncRNA molecules can be placed as important regulatory factors in a variety of cellular functional pathways (Mendes Soares and Valcarcel, 2006). From now on, unless otherwise specified, we refer to ncRNA as an RNA transcribed by RNA polymerase II with a reading frame of less than 100 amino acid residues (Okazaki *et al*., 2002).

This chapter will introduce the known geography of the functional RNA continent in the light of cancer research. The adventure in the new continent has just been started. We will discuss the perspectives of our future journey. We hope to show a compass for our adventure in this chapter.

II. WHICH TECHNIQUE IS SUITABLE TO ANALYZE MAMMALIAN TRANSCRIPTOME?

How can we know the activity of the transcription in mammalian cells as a whole? One clear answer is the use of gene profiling analyses based on microarray technology. Recent progress in the generation of oligonucleotide lithography makes it possible to mount more than one hundred thousand probes on a chip, enough to cover most of the deduced protein-coding sequences in a mammalian genome (Chee *et al*., 1996). Since it is easily applicable to many samples, expression profiling of most of the human malignant neoplasias have been analyzed with oligonucleotide microarray technology for molecular classifications and diagnosis. Comprehensive reviews of gene profiling in cancer research are available (Bucca *et al*., 2004; Ciro *et al*., 2003; Panda *et al*., 2003). The meta-analysis of gene expression profiles of several different cell lines indicated that the similarity between tumors of distinct origins are restricted to the cell growth-related genes (Rhodes *et al*., 2004; Ross *et al*., 2000). On the other hand, a group of scientists pointed out the difficulty of defining a common set of genes for the prediction of prognosis of cancer patients by the gene profiling analysis using microarray technology (Ein-Dor *et al*., 2006).

The microarray technology is suitable for the analysis of dynamism of transcriptomes. For example, a systematic approach based on the gene expression profiling assays was employed to elucidate that a transcription factor, activating transcription factor 3 (ATF3), plays a key role in the lipopolysaccharide-mediated macrophage differentiation (Gilchrist *et al*., 2006).

In that study, transcriptomic data obtained from Toll-like receptor-activated macrophages by microarray gene profiling were analyzed. Cluster analyses revealed that chronological changes of expression profiles of several transcription factors including ATF3 showed a corresponding time course to the induction of cell differentiation. Prediction of protein–protein interaction by the use of database analysis and following experimental confirmation revealed that ATF3 is a suppressor of transcription induced by nuclear factor-κB and plays a critical role in macrophage differentiation. A combination of bioinformatics and expression profiling will be applied to the cancer research to find a new molecular mechanism of carcinogenesis and cancer malignancy (Bono and Okazaki, 2005; Bucca *et al*., 2004; Panda *et al*., 2003).

The gene expression profiling assay, however, has several intrinsic limitations regarding the analysis of transcriptome as the results of mammalian genome activity. One limitation is that a predesigned microarray chip cannot detect unknown transcripts such as antisense transcripts. Mapping of transcription start sites (TSSs) and identification of splice variants are also difficult to conduct with hybridization-based technologies. Another drawback of the microarray technology in transcriptome analyses is that it may not give any information for the whole structure of one transcript.

Cloning and nucleotide sequencing of full-length cDNAs is a direct method to overcome these limitations of microarray analyses. Nucleotide sequencing of full-length cDNAs will provide complete amino acid sequences of the corresponding proteins and provide a better expectation of the functions. Many signal sequences are known to localize at the N-terminal regions, which are coded at the 5′ end of the open reading frames of mRNAs. Full-length cDNA cloning can be used to identify ncRNAs which cannot be found when using the conventional computational exon identifier. Finally, isolation and cloning of cDNAs will provide invaluably important resources for further functional studies.

Even though the attempt to collect mouse cDNA clones won big successes, the cloning and sequencing approach obviously lacks the throughput (Shiraki *et al*., 2003). It is too expensive to analyze all the transcripts in a cell by such a one-by-one method. Moreover, highly expressed transcripts may be preferentially obtained by using cloning technology rather than rare transcripts. This potential bias in the full-length cloning method makes it difficult to apply to the quantitative gene expression-profiling assay in the transcriptome analysis.

To compensate the problems in those two completely different approaches (collection of full-length cDNA clones and gene expression profiling with microarray technologies), a tag-based technology to map the TSSs was established (Shiraki *et al*., 2003). The tag is named as CAGE. The essential point of the CAGE technology is the chemical targeting to the 5′ capped

RNAs and the formation of short stretches of the 5′ end of the cDNAs (tags). The RNA transcript is generated only from the 5′ to 3′ direction, so the regulatory sequences in the 5′ promoter regions have apparent biological importance to understand the regulatory mechanisms of gene expression. The mapping of CAGE tags will provide the clue to locate the critical regulatory sequences in the 5′ upstream regions of each transcript. The numbers of CAGE tags accumulated the same position in the genomic sequences roughly reflect the activity of the transcription initiation of the gene in the cells. The cumulative mapping of CAGE tags onto genomic sequences will give a different aspect of the transcriptome because the probes for mRNAs on oligonucleotide microarray are usually placed at the 3′ untranslated region (3′ UTR) of mRNA.

III. CAP TRAPPER AND FULL-LENGTH cDNA CLONING SYSTEM

Many technologies for high-throughput cloning and sequencing of mouse full-length cDNAs have been developed by the FANTOM project. One important step to collect full-length cDNA clones is the enrichment of fully synthesized cDNAs toward the 5′ end of mRNA. Just after the RNA polymerase II starts the transcription, the 5′ end of the nascent mRNA receive a stepwise modification: addition of a guanine monophosphate in 5′-5′ manner, followed by a methylation to the added guanine base (Gu and Lima, 2005). This structural modification is called capping and is believed to occur to almost all the mRNAs. Since the modification occurs just after the transcription initiation by RNA polymerase II, the 5′ cap structure (CAP) is a definite signature for mRNA.

Several methods were developed to enrich 5′-capped mRNA/cDNA from mammalian cells. The oligo-capping method is based on the enzymatic removal of CAP and the following ligation of the tag RNA to the 5′ end of full-length mRNA (Suzuki and Sugano, 2001, 2003). The ligated RNA is subjected to the reverse transcription-PCR (RT-PCR) amplification using the tag RNA sequences as upstream primer annealing sites in PCR. The CAP retention method enriches the 5′-capped mRNA/DNA hybrids by using CAP-binding protein and consequentially full-length cDNAs (Edery *et al.*, 1995). The CAP trapper is a unique method compared to the above-mentioned technologies (Carninci *et al.*, 1996, 1997, 2000). The method is based on chemical modification onto the CAP and the final enriched materials are not mRNAs but RNA/DNA hybrids. The procedures of the CAP trapper are shown in Fig. 1A.

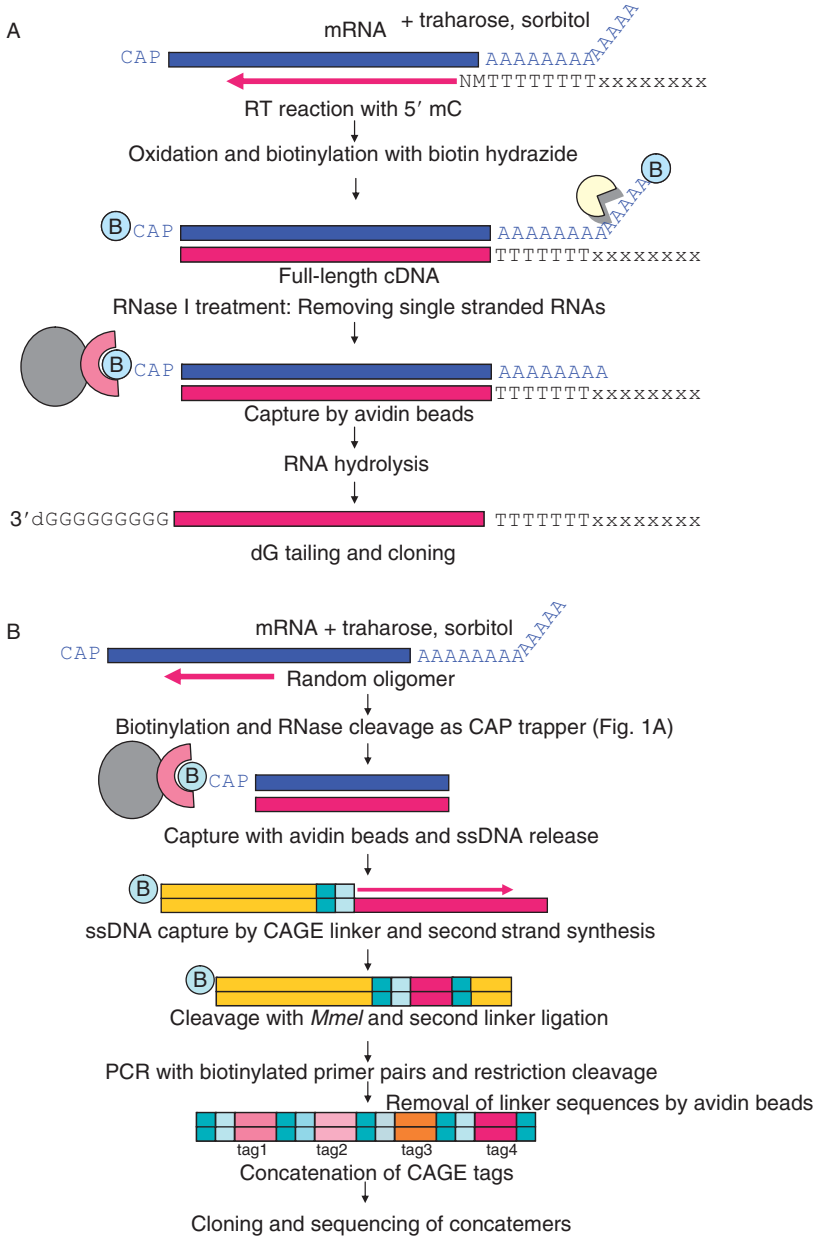

Fig. 1 CAP trapper method and CAGE tag formation. (A) A schematic diagram describing the outline of the CAP trapper method. The primer for reverse transcription has several restriction sites (shown as series of X) which do not anneal to the polyA stretches of the mRNA, indicated with blue rectangles (Mizuno *et al.*, 1999). When the full-length cDNA (red rectangles) synthesis

Since the mRNA secondary structures often block the elongation of cDNA synthesis, it is quite difficult to obtain cDNA containing the 5′ end of mRNA sequences. This obstacle can be circumvented by rising the reverse transcriptase reaction temperature in order to melt the mRNA secondary structures. Under such a harsh condition, the reverse transcriptase would be denatured and lose its activity. The solution of this problem is addition of protein stabilizing saccharides, trehalose (Kaushik and Bhat, 2003), and sorbitol (Gonzalez *et al*., 1995) in the reverse transcriptase reaction. The addition of these saccharides stabilizes the reverse transcriptase at a higher temperature (55 °C). This invention makes it possible for reverse transcriptase to generate full-length cDNAs without being blocked by the RNA secondary structure. It is reported, however, that a stable RNA secondary structure at 55 °C can be designed artificially and the full-length cDNA synthesis of the mRNA with this structure failed even with the addition of trehalose (Das *et al*., 2001). Moreover, the same study reports that the mRNA might be degraded at high temperature with the presence of divalent metal cations (Das *et al*., 2001). This particular problem may be insurmountable and considered as a "trade-off" to establish the comprehensive full-length cDNA libraries at maximum efficiency.

A further invention is the enrichment of the full-length cDNAs by the biotinylation of the CAP of the template mRNAs in the RNA/cDNA hybrid. The bioninylated mRNA/cDNA hybrids are easily collected by the streptavidine-conjugated beads. Addition of biotin hydrazine for CAP biotinylation can cause an incipient reaction between cytidine residue and biotin hydrazide (Hayatsu and Ukita, 1964). This incipient reaction between

is finished, RNA/DNA hybrids are formed with partially melted structure at the 3′ end of polyadenylated mRNA. Oxidation with $NaIO_4$ attacks the diol moieties in the riboses of the nucleotides at the CAP and the 3′ end of mRNAs. The oxidated reboses are subjected with biotinylation with biotin hydrazide. By RNase I cleavage, the 3′ end nucleotide of the mRNA labeled with biotin will be removed and only the 5′ end biotin of the RNA/DNA hybrid will remain (Shibata *et al*., 2001a,b). Collection of DNA/RNA hybrids by the use of biotin–avidin complex formation enables the enrichment of full-length cDNAs. (B) The schematic diagram describes the outline of the CAGE tag generation. The enrichment of biotinylated RNA/cDNA hybrids are done according to the same principle as the CAP trapper method. After the digestion of RNA with RNase, dsDNA linkers (yellow recangles) are ligated at the 3′ end of single-stranded cDNA and second strand synthesis follows. The dsDNAs are then cleaved with type IIS restriction enzyme, *MmeI*, to generate 20 base pair cDNA (CAGE tags). The CAGE tags are ligated to adapter oligomers at the 3′ end of their tags and the adapter sequences provide the following PCR primer annealing sites. The 20-base pair CAGE tags are amplified by PCR and the PCR products are subjected to restriction digestion and concatenation. Finally, the concatenated CAGE tags are subcloned into plasmid vectors and subjected to nucleotide sequencing.

the biotin hydrazide and cytidine base does not occur on double-stranded nucleic acids (Hayatsu, 1976) or 5′ methylated cytosine (Ohmori *et al.*, 1978). So, the order of the reactions is very critical: first strand cDNA synthesis must precedent to the diol oxidation and following biotinylation. The use of 5′ methylated cytosine deoxynucleotides for cDNA synthesis may have additional protective effects on the incipient reaction. The major reason of 5′ methylated cytosine is the removal of internal restriction sites for facilitating full-length cDNA cloning (Carninci *et al.*, 2000).

Related technologies have also been invented. The cloning vectors, λ-FLCs, are suitable to clone longer cDNAs than conventionally used vectors such as λ-ZAP or plasmids (Carninci *et al.*, 2001). The automation of plasmid purification was done by a filtration method (Itoh *et al.*, 1997, 1999). On the other hand, RIKEN developed its own capillary-based automatic sequencing system (RIKEN integrated sequence analyzer, RISA) (Shibata *et al.*, 2000). The establishment of a high-throughput cloning-sequencing system enabled to identify more than 110 thousand cDNAs in mouse cells. The resultant nucleotide sequence information of full-length cDNA was mapped on the mouse genomic sequences and annotated manually by the members of the FANTOM consortium (Furuno *et al.*, 2003; Maeda *et al.*, 2006). The detailed methods of mapping and annotation of those sequences were reported previously (Bono *et al.*, 2002; Kondo *et al.*, 2001; Konno *et al.*, 2001). Comprehensive reviews of the FANTOM projects are provided (Hayashizaki, 2003a,b; Hayashizaki and Kanamori, 2004).

IV. CAGE AND GIS/GSC DITAGS

The establishment of CAGE tag library was first reported in 2003 (Shiraki *et al.*, 2003). The aim of this method is to collect TSSs and to identify the localization and expression frequency of TSSs in the genome as much as possible. The methodological details were described in Kodzius *et al.* (2006). The technological point for enrichment of the 5′ end cDNA sequences in CAGE technology is the same as in the CAP trapper method. The CAGE tags are concatenated to each other so that multiple TSSs can be sequenced in one reaction. One can collect and characterize millions of TSSs from mammalian cells with the CAGE technology and analyze the activity of gene promoters in detail.

The mapping of short sequences like CAGE tags needs more elaborate procedures than the mapping of full-length cDNA sequences (Carninci *et al.*, 2005, 2006; Shiraki *et al.*, 2003). For example, some of the CAGE tags mapped multiple sites in the mouse genome and such tags were eliminated from the analysis (Carninci *et al.*, 2005).

Elucidation of the span of each transcript is critical to understand the transcriptome as genomic function. The GIS and GSC ditags are the technologies to reveal the span of each transcript without extensive cDNA sequencing. GIS analysis was established by Ng *et al.* (2005) and GSC is a variation of GIS that applies subtraction steps for collecting the rare transcripts (Carninci *et al.*, 2005). GIS/GSC technology is based on the full-length cDNA cloning and the generation of ditags that consists of 5′ and 3′ end of cDNA. The ditags are generated by cleavage of internal sequences of inserts after full-length cDNA cloning using type IIS restriction enzymes, *GsuI* and *MmeI*. The resultant vectors are self-ligated and the new inserts, consisting of only 5′ and 3′ end of the cDNA, are handled as a ditag. The ditags are cut out, concatenated, and amplified with PCR. The concatenated ditags are cloned into plasmid vectors and the inserts are subjected to the DNA sequencing. This technology is a bit more complicated than CAGE technology but still applicable to the high-throughput analysis.

V. THE STRUCTURE OF THE FANTOM DATASETS

Before going into detail of the physical geography of the RNA continent, we must clarify the structure of FANTOM datasets to be able to understand the biological significance of the mouse transcriptome. The initial attempt of the mouse encyclopedia project was to establish a complete set of cDNA clones representing whole mouse transcripts (Hayashizaki, 2003a). Since many transcripts express only limited time and tissue in an organism, it is essential to characterize a maximum number of cDNA libraries to cover all such variations as much as possible.

To construct the FANTOM2 dataset, tissue from more than 35,000 mice including embryos was extracted to establish an enormous number of distinct full-length cDNA libraries (Carninci *et al.*, 2003). The FANTOM3 dataset consists of the following number of libraries: 237 for the RIKEN full-length cDNA, 145 for the CAGE tag data, 4 each for the GIS and GSC ditag data, 266 for RIKEN 5′ EST data, 265 for RIKEN 3′ EST data, and 264 for 5′-3′ EST pair of RIKEN cDNAs (Carninci *et al.*, 2005). Some of the libraries were extensively subtracted to avoid redundant clones and the subtracted libraries were regarded as sublibraries. This extensive subtraction enabled us to collect very rare transcripts (Carninci *et al.*, 2000). For instance, the total number of the FANTOM3 cDNA collection is 102,281. Less than a half of this amount of cDNA (41,025) was reported by other groups in the GenBank and more than half of the clones were found only in the FANTOM3 collection (Carninci *et al.*, 2005). One problem of the subtraction procedure is that the tissue-specific or rare splice variants are

removed and lost from the libraries (Okazaki *et al.*, 2002; Suzuki and Hayashizaki, 2004). It has also been pointed out that the extensive normalization may introduce the potential contamination of pre-messenger RNAs in the libraries (Mattick and Makunin, 2006). These two arguments do not contradict each other. It is an open question whether these rare clones have biologically significant functions.

The landscape of the mouse transcriptome was described mainly with the whole mapping data of those cDNA/CAGE/GIS/GSC/EST sequence tags. This description was, in most cases, made without regard to the histological origins of the RNAs, the developmental stages, or the strain differences (Carninci, 2006; Carninci *et al.*, 2005). Moreover, most of the libraries were prepared with RNAs purified from a whole organ, which consists of a variety of cell lineages in which many genes can be differentially expressed. Therefore, each specific finding described here should be regarded as the results of genomic functions in whole animal body and these were not necessarily true to specific cell lineages. Of course, one can trace the histological origins of each cDNA or CAGE tag through the search of the FANTOM databases (http://fantom.gsc.riken.go.jp/), and accumulation of the tissue-specific CAGE library data will provide very important clues for understandings of the mechanism of transcription regulation in development and cell differentiation.

VI. THE LANDSCAPE OF THE MOUSE TRANSCRIPTOME: ncRNA, TRANSCRIPTION FOREST, AND TRANSCRIPTION DESERT

All the somatic cells in the body of a mouse essentially have the identical genomic information: a 2.5-Gb haploid genome (Waterston *et al.*, 2002). How much information is expressed from this huge genomic DNA? Full-length cDNA clones, GIS/GSC ditags, and ESTs provided 181,047 paired transcription start and stop sites. This is a much bigger number of transcripts than previously estimated for the mouse genome (28,097) (Waterston *et al.*, 2002). Hence, the collection should cover most of the expressed sequences in mouse cells.

The mapping of paired ends of transcripts (GIS/GSC ditags) revealed the span of genomic sequences read by the RNA polymerase II. Roughly speaking, through the mapping of these ditags, one can know the size of each transcribed gene. The gene size can be varied according to cellular context. One interesting example is the protein tyrosine phosphatase receptor type D, which was covered by six GSC ditags and its corresponding RIKEN EST clones. This gene has an mRNA length of 2475 bp and its gene

size is 2.2 Mb (Fig. 2). Because of the limitation of the insert size in the λ-FLC cloning vectors, very long mRNAs are not cloned as full-length cDNAs even with the CAP-trapper methods. For example, the 108-kb ncRNA, *Air* (Sleutels *et al*., 2002) and the 101-kb coding RNA, Titin (Bang *et al*., 2001) does not appear as single full-length cDNAs in the RIKEN FANTOM dataset (Furuno *et al*., 2006).

It is widely known that a "gene" can be transcribed in various ways; variation is caused by the alternative splicing, alternative TSSs, and alternative transcription termination sites. A term, transcription units (TUs), was defined to describe such overlapping, various transcripts as a unit. A TU is defined as a cluster of transcripts that contains a common core of genetic information such as protein-coding sequences (Okazaki *et al*., 2002). The FANTOM2 project identified 33,409 TUs and 17,594 (52.7%) were considered as protein-coding ones (Okazaki *et al*., 2002). In the new FANTOM3 dataset, the vast majority of mouse transcripts are ncRNAs. 44,147 TUs were identified and more than half of them (23,218, 52.6%) did not code proteins (Carninci *et al*., 2005). This large number of ncRNAs suggests that those RNAs may play some important roles in cellular functions.

Some TUs were interconnected to each other: we call such multigene TUs as "chains" (Engstrom *et al*., 2006). Figure 3 shows an example of chain. The definition of a chain is a group of three or more TUs interconnected with *cis*-antisense transcripts or bidirectional promoter arrangement or both (Engstrom *et al*., 2006). Based on the definition of chains, there were 1153 chains, containing 3987 TUs (11% of all TUs) in the mouse transcriptome and 13 chains showed complete structural conservation between human and mouse transcriptomes (Engstrom *et al*., 2006). The biological significance of this type of complex arrangement of transcripts remains to be elucidated.

The continuity of transcribed genomic sequences, regardless the direction of the transcripts, was determined by the mapping of full-length cDNA clones, GIS/GSC ditags, and EST. A genomic region that continuously transcribed either of the double strands, with at least one base pair overlap, was named "transcription forest" (TF) whereas a genomic region scarcely mapped with transcripts was named "transcription desert" (Carninci *et al*., 2005). Based on a conservative estimation, 62.5% of the genomic sequences were read by the RNA polymerase II and the estimated number of TFs is 18,461 in the mouse genome (Carninci *et al*., 2005). The coverage of the mouse genomic sequences by transcripts is approximately six times higher than that of the 10 human chromosomes estimated by the study using tiling array technology (Cheng *et al*., 2005; Kapranov *et al*., 2002). As mentioned earlier, it is necessarily not true that all the mouse cells can use 62.5% of the genomic sequences for their activities. So, it is quite possible that the difference of transcripts coverage between human and mouse is not as large as presently calculated.

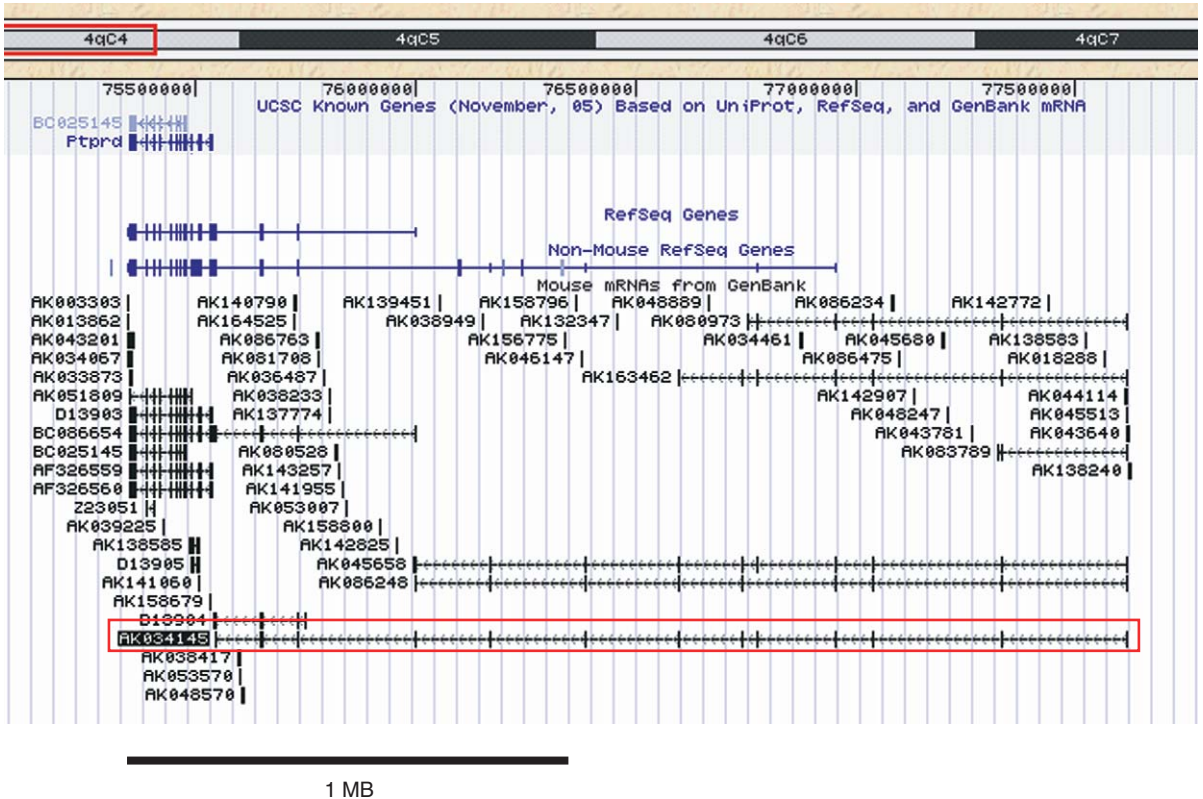

Fig. 2 An example of the mega transcript. The schematic diagram indicates the genetic mapping of a FANTOM clone drawn by the Genomic viewer at the web page of The University of California Santa-Cruz Genome Bioinformatics sites (http://genome.ucsc.edu/) (Kent *et al.*, 2002). The clone (DDBJ accession number: AK034145; in a red open box) spans 2.3 Mb in mouse chromosome 4.

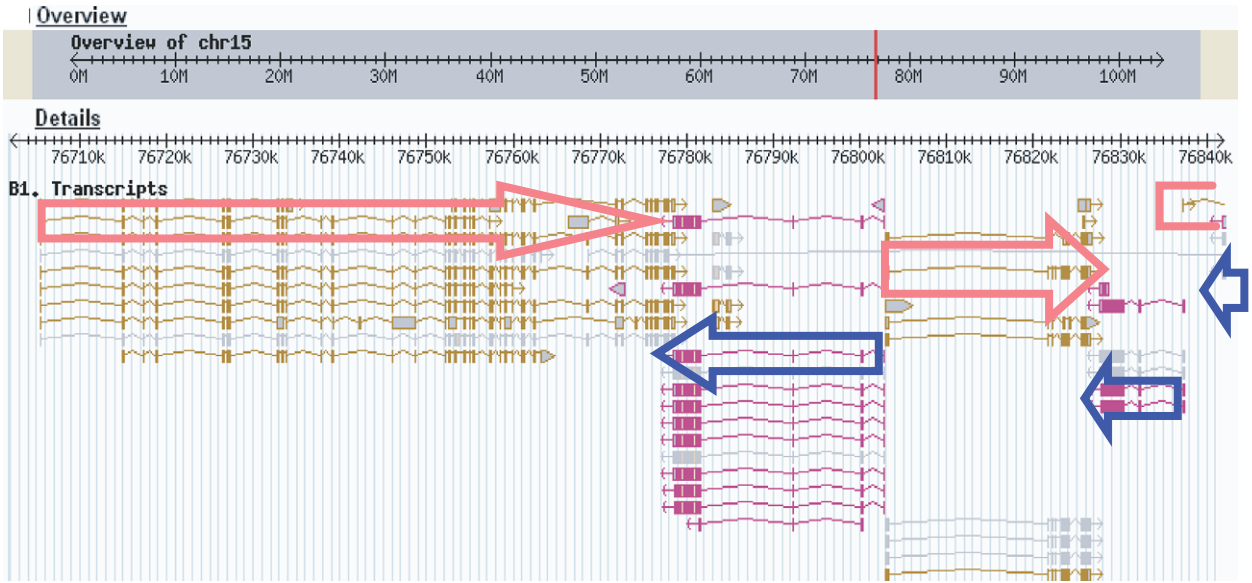

Fig. 3 An example of chain. The schematic diagram indicates that the genetic mapping of a chain drawn by the Genomic Elements Viewer (Kawaji *et al.*, 2006) at the web page of GSC RIKEN Yokohama Institute (http://fantom.gsc.riken.go.jp/). This chain consists of at least four distinct TUs at mouse chromosome 15. The TUs on the genomic plus and minus strands are shown in pink and blue arrows, respectively. From left to right, the aminoacyl tRNA transferase class II (D330001F17Rik, pink); BOP1, blue; Hsf1, pink; and Dgat1, blue.

VII. ABUNDANT NATURAL ANTISENSE TRANSCRIPTS AND THEIR BIOLOGICAL SIGNIFICANCE

Another important feature of the mouse RNA continent is the existence of abundant natural antisense transcripts. Previous reports suggested that up to 20% of the protein-coding transcripts have natural antisense RNA transcripts that can hybridize to the sense transcripts (Chen *et al*., 2004b; Kiyosawa *et al*., 2003; Yelin *et al*., 2003). The FANTOM3 study suggested that much more natural antisense transcripts exist in the mouse transcriptome (Katayama *et al*., 2005). When the TSS tags, such as CAGE tags, are taken into account the potential antisense transcripts increase dramatically. 31,422 out of 43,553 TUs (72.1%) have at least one transcript (or its tags) placed on the opposite strand (Katayama *et al*., 2005). The TSS-mapping results suggest that there is a possibility that many antisense transcripts still remain uncharacterized in the mouse transcriptome. Similar findings were reported for human transcriptome analyzed with tiling array technology (Cawley *et al*., 2004).

The number of confirmed pairs of overlapping sense–antisense transcripts was increased in the FANTOM3 dataset compared to the FANTOM2 dataset. In the FANTOM2 dataset, 2489 pairs of bidirectional transcripts that make sense–antisense overlapping and 899 pairs of bidirectional transcripts without overlapping regions in the pairs were found (Kiyosawa *et al*., 2003). The percentage of the sense–antisense pairs to the total loci was 14.1% in the FANTOM2 dataset (Kiyosawa *et al*., 2003). In the FANTOM3 dataset, the mapping data of the TUs were analyzed (Engstrom *et al*., 2006). In that study, which was conducted with a combination of FANTOM3 and GenBank datasets, they found that 5248 out of 9260 TUs (25.3%) have sense–antisense overlapping among the transcripts mapped in a same TU.

The idea that antisense transcripts can negatively regulate the expression of corresponding sense transcripts is widely accepted and supported experimentally (Dean, 2001; Delihas *et al*., 1997; Lavorgna *et al*., 2004). The mechanisms of the suppression can be various. It has been suggested that the RNA/RNA hybrid can provoke an RNAi reaction (Lavorgna *et al*., 2004). One study indicated, however, that such intermolecular hybridization is inefficient *in vivo* (Wang and Dolnick, 1993). Many natural antisense transcripts have been identified (Vanhee-Brossollet and Vaquero, 1998). Famous examples in the field of cancer research are the *N-myc* gene (Armstrong and Krystal, 1992; Krystal *et al*., 1990) and the *c-myc* gene (Spicer and Sonenshein, 1992). In the case of the *N-myc* gene, the sense–antisense pair of transcripts was coregulated during cell growth and differentiation, suggesting that the antisense transcripts may not be simply suppressive (Armstrong and Krystal, 1992). In 2003, one group of scientists reported about an

individual with α-thalassemia who has a chromosomal deletion that results in the expression of antisense RNA of a structurally normal α-globin gene (HBA2) (Tufarelli *et al*., 2003). Expression of the antisense RNA caused complete CpG island methylation of HBA2 in a transgenic model and in differentiating embryonic stem cells (Tufarelli *et al*., 2003). The relationship between the DNA methylation of sense promoter and the expression of antisense RNA is largely unknown (Mendes Soares and Valcarcel, 2006). A group of scientists reported that both sense and antisense strand transcriptions at the remote locus control region play an active role in the human growth hormone gene activation (Ho *et al*., 2006). Moreover, a report indicated that small double-stranded RNAs (dsRNAs) can induce transcriptional activation in human cells (Li *et al*., 2006). Interestingly, 21-nt dsRNAs targeting to a selected region of promoters can induce histone acetylation of the promoters and gene expression. Previous studies with a similar strategy indicated, however, contradictory results (Ting *et al*., 2005). It indicated that the positional sensitivity of transcription is regulated by the short dsRNAs (Li *et al*., 2006). The analyses of Katayama *et al*. (2005) using quantitative real time RT-PCR system revealed that some of the sense–antisense transcripts pairs seemed to be regulated reciprocally. There is no evidence that the reciprocal regulation is related to the interaction between sense and antisense transcripts.

VIII. INTERESTING FEATURES OF THE MOUSE TRANSCRIPTOME

An extensive collection of mouse full-length cDNA clones reveals that some RNA molecules are similar to the protein-coding mRNA but does not code full-length proteins. Frith *et al*. (2006) defined this type of mRNA as pseudo-messenger RNA (ψmRNA). More than 10% of the FANTOM3 mouse cDNAs (10,679 out of 102,801) were classified as ψmRNA. The ψmRNAs are further classified as mRNA expressed from pseudogenes and disrupted splice variants of coding mRNA. Nearly half of the ψmRNAs are transposon-associated ones. The biological significance of the existence of these ψmRNAs has not been deduced. The excess of ψmRNA transcripts only has the opal stop codon as a responsible termination codon for the disruption of long open reading frames. It suggests that more than expected number of selenoproteins can be expressed in the cells.

Moreover, some of the ncRNAs have extraordinary long trancripts without apparent open reading frames. The existence of these types of very long ncRNAs, called macro-ncRNA, is supported by the GSC/GIS ditags, the RT-PCR analysis, and the Northern blottings (Furuno *et al*., 2006).

Considering the limitation of the insert size of λ-FLC, there is no FANTOM clone covering such cDNA in full length. A series of shorter cDNAs covers most of the genomic sequences corresponding to the macroRNA (Furuno *et al.*, 2006). It is known that long ncRNAs such as the X chromosome inactivating gene, Xist, (Brockdorff *et al.*, 1992) and an imprinting gene, *Air* (Sleutels *et al.*, 2002), play a critical role in the epigenetic regulation. Xist is important for the gene dosage control of the X chromosomes in female cells, and *Air* for the control of imprinting. Therefore, it is possible that these macroRNAs function in the chromatin remodeling to regulate the expression of the adjacent genes.

IX. CAGE TAG AND PROMOTER ANALYSIS: THE DIVERSITY OF TRANSCRIPTION REGULATION

The mapping of each CAGE tag gives us a comprehensive picture of positions and usages of the TSSs in the genome. Analyses of 145 different mouse and 41 different human CAGE libraries identified 729,504 and 665,278 TSSs for mouse and human, respectively (Carninci *et al.*, 2006). Many CAGE tags overlapped each other and these overlapped tags were defined as CAGE tag clusters (Carninci *et al.*, 2006). Typically, the peaks of the CAGE tags were found in the 5′ upstream regions of the transcripts. 67% of the known protein-coding transcriptional units (13,767 of the 20,639) were supported with at least one or two CAGE tag clusters at ±20 nt of the known 5′ end of the cDNA. This means that two-third of the protein-coding transcripts are supported by multiple CAGE tags at those previously known promoters. This criterion may be a bit too strict for rare transcripts. Actually, most of the other protein-coding loci also were covered with individual CAGE tags (Carninci *et al.*, 2006).

Based on the analysis of frequently expressed CAGE tag clusters, which consist of more than 100 CAGE tags, the promoters of mammalian genomes were classified into four categories based on the shapes of the TSSs defined by CAGE tags (Fig. 4). In the single-peak (SP) class, a majority of the tags are concentrated to a peak, which spans four or less consecutive nucleotides. Others are classified as a broad distribution (BR), a broad distribution with a dominant peak (PB), and a bi- or multimodal distribution (MU). Similar observations have been reported by others (Kimura *et al.*, 2006; Suzuki *et al.*, 2001). Interestingly, the shape classes were highly conserved between orthologous mouse and human promoters. The evolutional conservation suggests that the classification of promoters by the shapes of CAGE tag cluster has some biological significance.

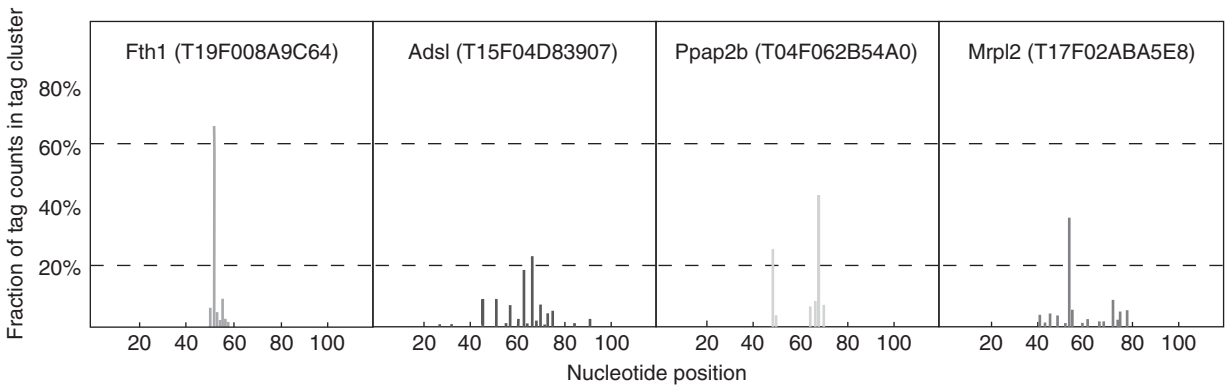

Fig. 4 Classification of promoter shapes by CAGE analysis. This figure is modified from Carninci *et al.*, 2006. Histograms of representative tag clusters for different shape classes are shown. The horizontal axis indicates the 120 bases window of promoter sequences. Vertical axis indicates that the percentage of the number of the tags at the position. From top to bottom rows, single peak (SP), broad (BR), bimodal/multimodal (MU), and broad with dominant peak (PB) are shown. The identifier of each tag clusters (e.g., HUGO gene names) is indicated at the top of the each panel.

It is indicated that the promoters with a highly "variable" amount of expression between cells are advantageous for the adaptation to the rapid changes of environment in yeast (Blake *et al*., 2006; Kaern *et al*., 2005). The rapid environment can induce the stress, to which one population in yeast can adapt better than other populations through the variation of expression of some transcripts. It is possible that the variation of TSSs observed in the CAGE mapping is a reflection of the "spatial" variations of promoter activities. This variation of TSSs may cause the better adaptation to the rapid changes of the environment.

X. ALTERNATIVE USAGE OF THE PROMOTERS: 5′ VARIATION AND 3′ UTR PROMOTER

One striking finding from the CAGE tag mapping is that multiple TSSs are found in one gene. This finding suggests that the regulation of the transcription initiation is much more sophisticated than it is commonly believed. The variation of TSSs in the 5′ UTR region often involves the changes of initiation codons (Carninci *et al*., 2006). Previous studies reported that 18–20% of the protein-coding genes use alternative promoters (Landry *et al*., 2003). The CAGE tag mappings revealed that 58% of the coding transcriptional units had two or more alternative promoters in mice (Carninci *et al*., 2006). A result similar to the one for human data: 52% of the human RefSeq genes used alternative promoters (Kimura *et al*., 2006). Moreover, 34–48% of the transcriptional units identified by CAGE have at least one overlapping alternative promoter in the coding sequences (Carninci *et al*., 2006).

The FANTOM3 datasets revealed that the usage of 3′ UTR promoters is unexpectedly common. The TSSs in the 3′ UTR show a distinct sequence motif. The high extent of interspecies conservation is found between +40 and +90 of the TSSs in the 3′ UTR (Carninci *et al*., 2006). Moreover, a sequence of GGG between −3 and −1 of the TSS is found in 59% of the TSSs in the 3′ UTR analyzed. The 3′ UTR transcripts may have functions distinct from the full-length transcripts. It is shown that 43% (168/391) of the representative transcripts shows the divergence of a CAGE tag distribution between the 5′ and the 3′ tags in at least one tissue. This finding suggests that the distinct regulation mechanisms should work between the 5′ and the 3′ promoters (Carninci *et al*., 2006).

There is a recent paper describing the relationship between two adjacent promoter functions in one gene (Martianov *et al*., 2007). The human dihydrofolate reductase (DHR) gene shows cell cycle–regulated expression and has two different promoters. One major, downstream, promoter works in proliferating cells and produce coding mRNA of DHR. The minor,

upstream, promoter works in quiescent cells and produces ncRNA and it can suppress the downstream major promoter. The suppression can be mediated with the ncRNA from the minor promoter. For efficient suppression of the major promoter transcription activity, the transcripts from the minor promoter should cover the DNA sequences corresponding to the major promoter of the DHR gene. Many of the intergenic TSSs found by CAGE mapping may be the targets of the promoter interference caused by the major 5′ promoter transcripts of the corresponding genes. Therefore, this promoter interference story in the DHR locus is very critical for the interpretation of the mapping data of CAGE.

XI. CHARACTERISTICS OF THE CpG ISLAND AND BIDIRECTIONAL PROMOTERS REVEALED BY CAGE ANALYSIS

Another interesting topic accessible by CAGE analysis is the characteristics of promoters with CpG islands. CpG islands are methylation-free CpG-rich regions and most of them are related to the promoters of housekeeping genes (Antequera *et al*., 1990). The multiple tissue-derived CAGE data suggested that many (34%) of the promoters with CpG islands functioned in a tissue-specific manner (Carninci *et al*., 2006). It is known that nearly half of the tissue-specific genes have CpG islands located downstream of the TSSs (Jones, 1999) and this observation with the CAGE data may fit to this story.

The CAGE tag mapping revealed that the CpG islands are commonly associated with bidirectional promoters. Some of the bidirectional promoters, such as Gabpa-Atp5j loci, are overlapping each other (Carninci *et al*., 2006). It is said that the overlapping bidirectional promoters may cause transcriptional interference in yeast (Prescott and Proudfoot, 2002). One possible explanation for this interfering effect is that these bidirectional promoters are not expressed simultaneously. It is an open question whether this transcriptional interference between two of the bidirectional promoters actually occurs or if they escape interference by the allelic or chronological differential expression of each promoter.

XII. SUMMARY OF THE PHYSICAL MAP OF THE RNA CONTINENT

In the mouse genome, the TFs, the genomic regions with abundant transcripts, occupy more than half of the genomic sequence and are divided by transcription deserts, genomic regions with few transcripts. The structures of

the TFs are very complex; a variety of sense and antisense RNAs are overlapped and interconnected to each other. Many ncRNAs are transcribed from the mouse genome although most of the ncRNAs found in the FANTOM project were functionally uncharacterized. Moreover, mammalian promoters can be classified into four major categories and the TSSs are distributed throughout the TU including 3′ UTR.

XIII. THE FUNCTIONAL ASPECT OF THE RNA CONTINENT IN CANCER RESEARCH

Accumulation of somatic genetic alterations and subsequent clonal expansions leads the cells to a malignant neoplasia formation. The pathway progresses in a stepwise manner and is called "multistep carcinogenesis" (Fearon and Vogelstein, 1990; Nowell, 1976; Sugimura, 1992; Weinberg, 1989). Cancer cells acquire a variety of growth-advantageous features such as suppression of apoptosis and loss of anchorage dependence during the process of carcinogenesis (Hanahan and Weinberg, 2000).

The major target of environmental carcinogens is the DNA. Many of the susceptible genes for hereditary cancer-prone diseases encode DNA repair genes. Therefore, the cancer is a disease of DNA. Somatic DNA alterations cause cancer. Because the "central dogma" is an almost ultimate paradigm in the molecular biology, cancer scientists mainly concentrated their attention to the protein-coding genes as susceptibility genes for cancer. Hence, they may not have paid enough attention to the possibility that RNAs are culprits of malignant human disorders. Before the discovery of microRNA (miRNA), the only major topics of ncRNAs in cancer research were the loss of imprinting (LOI) (Feinberg and Tycko, 2004) and the natural antisense transcripts for several protooncogenes (Vanhee-Brossollet and Vaquero, 1998). Nowadays the involvement of miRNA in carcinogenesis becomes apparent. In this section, we will describe the state of the art in cancinogenesis and ncRNA. This involves the topics miRNA, natural antisense transcripts, and the LOI. We also would like to discuss the recent discoveries on functional ncRNAs.

XIV. miRNA AND OTHER SMALL RNAs IN CANCER

The miRNA is a small, up to 22-base long, single-stranded RNA whose nucleotide sequences are evolutionally conserved from *Caenorhabditis elegans* to *Homo sapiens* (Pasquinelli *et al*., 2000) and that play critical

roles in biological functions (Bartel, 2004; Carthew, 2006). The miRNAs are expressed as hairpin-shaped double-stranded pre-miRNAs. Sequential processing by different RNase III enzymes, Drosha and Dicer, generates mature miRNA (Cullen, 2004).

The first characterized miRNA was the *lin-4* gene in *C. elegans* (Lee *et al*., 1993). The *lin-4* is known as a "heterochronic" gene: its mutant shows a disorder that affects the developmental timings, for example the first-stage larva could not proceed to the second stage. The *lin-4* negatively regulates LIN-14 protein in the first larval stage. Lee *et al*. revealed that the *lin-4* transcripts were small ncRNA of 22- and 61-nt lengths and that they contained sequences complementary to a repeated sequence element in the 3′ YTP of *lin-14* mRNA. A following study indicated that the *lin-4* suppresses translation of *lin-14* (Wightman *et al*., 1993). Similarly, another short RNA coding gene, *let-7* (Reinhart *et al*., 2000), is implicated in the heterochrony in worm development. The discovery of RNAi (Fire *et al*., 1998) and the identification of argonaute protein as a major player in the RNAi machinery (Tabara *et al*., 1999) led the way to connect the miRNA with the RNAi pathway (Grishok *et al*., 2001). The reduction of the gene expression of the factors in the RNAi pathway (*dcr*-1, Dicer homologue; *alg-1* and *alg*-2, Argonaute proteins) mimics the heterochronic phenotypes of the *lin-4* and *let-7* mutations. It is also shown that *dcr-1*, *alg-1*, and *alg*-2 are essential for the maturation of those miRNAs. The disruption of the RNAi pathway also caused the developmental disorders, some of which were rescued by an injection of mature miRNA, in zebrafish (Giraldez *et al*., 2005).

Although the genetics of *C. elegans* showed clearly that the miRNA can be a molecular switch through the suppression of translation, it has become evident that miRNAs can mediate cleavage of target mRNAs (Lim *et al*., 2005). It is shown that an efficient translation suppression by miRNA requires multiple miRNA binding sites in the 3′ UTR of the target mRNA (Doench and Sharp, 2004; Petersen *et al*., 2006). Moreover, the length of the target sequences of miRNA is only 8 bases so that one miRNA can have multiple targets in the cell (Lim *et al*., 2005).

Since miRNA has a broad range of targets in a cell (i.e., is less specific) and since the extent of suppression of the target expression by miRNA is generally moderate, the function of miRNAs should be considered as the "fine-tuning" of gene expression in mammalian cells (Bartel and Chen, 2004). The fine-tuning effect, however, is strong enough to be a selection pressure during the evolution (Farh *et al*., 2005; Stark *et al*., 2005). One example of this can be seen in the ubiquitously expressed housekeeping genes. These have relatively short 3′ UTRs and a relatively small number of miRNA target sites, indicating that such important genes in cellular functions lost those miRNA target sequences during the evolution.

It has become evident that RNAi pathway plays a critical role in the suppression of transposon (Tabara *et al*., 1999), in virus infection (Lecellier *et al*., 2005; Li *et al*., 2002), induction of differentiation (Chen *et al*., 2004a; Schratt *et al*., 2006), and in other biological regulations. Fazi *et al*. (2005) reported that the transcriptional regulation of miR-223 by the two transcription factors FNI-A and C/EBPα plays important roles in development. Interestingly, human miR-122 contributes to the *Hepatitis C virus* replication in human liver cells (Jopling *et al*., 2005). One study indicates that miRNAs can have critical functions in human hereditary disorders. Tourette's syndrome (TS) is a developmental neuropsychiatric disorder characterized by chronic vocal and motor tics. A genetic background has been suggested for the etiology. A frameshift mutation of the SLTRK1 gene, the candidate susceptible gene of the disease, was found in two independent cases of the disease. The disease-specific mutant transcript of SLTRK1 lost the target site of the miRNA miR-189. Both SLTRK1 and miR-189 are coexpressed in the brain region related to the Tourette's disease. Further, wild-type SLITRK1 overexpression induces dendritic growth in primary neuronal cultures whereas mutant does not (Abelson *et al*., 2005).

In terms of carcinogenesis, miRNA has been known as an important player (Hammond, 2006; Zhang *et al*., 2007). The first report suggesting the involvement of miRNA in malignant neoplasms appeared in 2002 (Calin *et al*., 2002). Calin *et al*. showed that two miRNAs, miR-15 and miR-16, are frequently deleted or downregulated in B cell chronic lymphocytic leukemia (CLL) (in 68% of the cases). Both miRNAs are located within a 30-kb region at chromosome 13q14, a region deleted in more than half of the CLL cases (Calin *et al*., 2002). It is also indicated that many human miRNA genes localize at fragile sites and cancer-related genomic regions, suggesting the involvement of change of expression of miRNA in carcinogenesis (Calin *et al*., 2004). One of the major topics of miRNA in cancer is that *let-7* miRNA may suppress the protein expression of the protooncogene RAS (Hayashita *et al*., 2005; Johnson *et al*., 2005). It is shown that the target of the *let-7* family, *let-60*, is a homologue of RAS in *C. elegans* and genetic evidence with *C. elegans* suggested that *let-7* is epistatic to the *let-60/RAS* (Johnson *et al*., 2005). The same epistatic relationship is conserved in human. Moreover, *let-7* expression is lower in lung tumors than in normal lung tissue, while RAS protein is significantly higher in lung tumors (Hayashita *et al*., 2005).

Biological significance of miRNA expression in cancer was also shown in other ways. The expression profiling analyses of miRNA in cancer tissue revealed that the miRNA profiles reflected the developmental lineage and differentiation state of the tumors. A general downregulation of miRNAs in tumors compared to miRNA in normal tissues were also observed (Lu *et al*., 2005). The study used multicolored fluorescent beads detection with a fluorescently activated cell sorting system and showed that the

miRNA expression profiles can be used more accurately than conventional mRNA expression profiles in cancer diagnosis. Similarly, the expression profiles of miRNAs showed association with the prognostic factors in CLL (Calin *et al*., 2005). A study indicated that a cluster of miRNAs, the miR-17–92 polycistron, can enhance the tumorigenicity of mouse B-cell lymphomas induced by the *Eu-myc* transgene (He *et al*., 2005). Moreover, the expression levels of the primary or mature miRNAs derived from the miR-17–92 locus are often substantially increased in these cancers in human B-cell lymphoma samples and in cell lines to normal tissues. Through functional screening, two miRNAs, miR-372 and miR-373, were identified as cooperators of oncogenic RAS in tumorigenesis of human testicular germ cell tumors (Voorhoeve *et al*., 2006). These miRNAs are supposed to neutralize p53-mediated CDK inhibition, possibly through direct inhibition of the expression of the tumor-suppressor LATS2. There are many other examples of miRNAs related to carcinogenesis (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006). Zhang *et al*. (2006) revealed that the copy numbers of miRNA containing chromosomal regions were very frequently changed in several human cancer cells.

Many small RNAs are characterized in various organisms (Kim, 2006). One important example is rasiRNA in *Drosophila* (Aravin *et al*., 2001, 2003, 2004). The function of the rasiRNA is implicated in the suppression of selfish genes in germ line cells (Vagin *et al*., 2006). Several scientists reported that similar small RNAs, such as piRNAs, are found in mammalian germ line cells (Aravin *et al*., 2006; Girard *et al*., 2006; Grivna *et al*., 2006; Watanabe *et al*., 2006). It is an open question whether such small RNAs play any critical role in carcinogenesis.

XV. EPIGENETICS AND ncRNA IN CARCINOGENESIS

Most of the cancers showed various amount of aberrant DNA methylation at the promoter regions of tumor suppressors (Ting *et al*., 2006). It is known that the chromatin-mediated abnormalities occur in early stage of carcinogenesis so the epigenetic changes may play an important role in the initiation of cancer (Feinberg *et al*., 2006).

It is widely accepted that the expression of ncRNAs is usually accompanied with the genome imprinting (O'Neill, 2005). On the other hand, no evidence is available that supports the expression of long ncRNAs can be related to the alteration of DNA methylation states in carcinogenesis or aging. LOI is the one of the earliest known epigenetic abnormalities found in cancer (Feinberg and Tycko, 2004). Imprinting is a phenomenon appearing as the monoallelic expression in some biallelic genes in the cells. Mammalian somatic cells have two copies of each autosomal gene: one from the

father and one from the mother. Such biallelic genes are usually expressed from both paternal and maternal alleles. The imprinted genes have been found to be expressed from only one of the two alleles. So, LOI appears as a disorder of this allelic restriction of the imprinted genes in cancer cells. It means that the biallelic gene expression occurs from the genes that are normally imprinted in noncancerous cells. LOI is shown to be important in cancinogenesis, although its mechanism is still largely unknown. One of the most characterized imprinted genes in terms of LOI is *IGF2*. The LOI of *IGF2* gene will cause increase of the dose of potent growth factor, IGF2. However, the RNA sequences of *H19* itself is not critical for the establishment of *IGF2* imprinting (O'Neill, 2005). Actually, the LOI state of *H19* and *IGF2* were not always correlated among different types of cancers (Kondo *et al*., 1995). Because a very long ncRNA, *Air*, plays a critical role in the establishment of *IGFR2* gene imprinting in mice (Sleutels *et al*., 2002), it is quite possible that unknown ncRNA plays a positive role in epigenetic disorders found in cancer cells.

XVI. THE TELOMERASE RNA COMPONENT AND SMALL NUCLEOLAR RNA

There are other types of functional ncRNAs. A unique group of such ncRNA is small nucleolar RNA (snoRNA). The snoRNAs are commonly coded in the intronic sequences of the ribosomal proteins and are important for the function in the modification (methylation and pseudouridylation) and processing of preribosomal RNA (Filipowicz and Pogacic, 2002). There is a subclass of snoRNA, small Cajal-body RNAs (scaRNA). Both scaRNA and snoRNA are involved in the methylation and pseudouridylation of RNA.

In the field of cancer research, an important ncRNA akin to the scaRNA is the telomerase RNA component (TERC). Telomerase holoenzyme is a reverse transcriptase that elongates telomere repeat and that is activated in most of the cancer cells. The TERC is a component of the holoenzyme and it functions as a template of telomeric repeat sequences (Chen and Greider, 2004). In yeast and mammals, TERC is transcribed by RNA polymerase II and has similar structure as scaRNA (Jady *et al*., 2004). TERC, scaRNA, and snoRNA share the same structural feature, the box H/CA domain. It was reported that the TERC was overexpressed in early esophageal cancers (Hiyama *et al*., 1999) and stomach cancers (Kuniyasu *et al*., 1997). Other scientists showed that TERC was essential for the malignant transformation mediated by the overexpression of the catalytic subunit of telomerase (Cayuela *et al*., 2005). These studies indicated that the overexpression of TERC may be important in human carcinogenesis.

XVII. RECENTLY CHARACTERIZED FUNCTIONAL ncRNAs IN MAMMALIAN CELLS

In this section, we will concentrate on another type of functional ncRNAs: mRNA-like ncRNAs. These RNAs are mainly transcribed by RNA polymerase II and some of them are spliced and polyadenylated as usual mRNA but do not code for proteins. Comprehensive reviews for such RNAs are available (Costa, 2005, 2007; Mattick and Makunin, 2006; Suzuki and Hayashizaki, 2004). Recent discoveries of functional ncRNAs were mainly related to the regulation of transcription.

Recently, an ncRNA, steroid receptor RNA activator (SRA), was isolated as a coimmunoprecipitant of the MyoD transcription factor. The SRA, accompanied by the RNA helicases p68/p72, behaved as a coactivator of MyoD. Reduction of SRA by siRNA caused the suppression of muscle gene expression and subsequent cell differentiation (Caretti *et al*., 2006). Similarly, an ncRNA, Evf-2, is reported as coactivator of Dlx-2 transcription factor (Feng *et al*., 2006).

There are other ncRNAs related to the cellular differentiation. A long ncRNA, pregnancy-induced ncRNA (PINC), is expressed in the regressed terminal ductal lobular unitlike structures of the mammary gland 4 weeks after withdrawal of hormonal stimuli (Ginger *et al*., 2006). PINC expression is temporally and spatially regulated in response to developmental stimuli *in vivo*. Moreover, PINC RNA is localized to distinct foci in either the nucleus or the cytoplasm in a cell cycle–specific manner. Reduction of PINC expression by siRNA suggests that the ncRNA has dual roles in cell survival and regulation of cell cycle progression. Similarly, the ncRNA Taurine Upregulated Gene 1 (TUG1) is identified as being upregulated by taurine in developing retinal cells (Young *et al*., 2005). In the newborn retina, reduction of TUG1 by RNAi caused malformations or defects of the outer segments of the photoreceptors. This loss of proper photoreceptor differentiation caused by the reduction of TUG1 with siRNA is considered to be a result of the dysregulation of photoreceptor gene expression (Young *et al*., 2005).

Comparison between human and chimpanzee genomic sequences picked up the genomic regions with accelerated evolution in the human genome (Pollard *et al*., 2006). The most significantly evolved region, HAR1, partly codes a novel RNA gene. The HAR1F is expressed specifically in Cajal–Retzius neurons in the developing human neocortex. Interestingly, the antisense transcript, HAR1R, is expressed differentially in human body including testes (Pollard *et al*., 2006). The function of HAR1F and HAR1R is unknown. The importance of these findings lies in the possibility of a rapid evolution occurring in the genes coding for ncRNA.

Another unique example of functional RNA is the one that can control the nuclear-cytoplasmic molecular transport, NRON (Willingham *et al.*, 2005). NRON was identified as a repressor of the nuclear factor of activated T cells (NFAT) by screening of evolutionarily conserved functional ncRNAs. NRON interacts with the importin-β superfamily proteins and is suggested as a specific regulator of NFAT nuclear trafficking.

XVIII. CONCLUSION: FUNCTIONAL RNA AND CANCER

The physical map of the RNA continent clearly indicates that the function of the genome is unexpectedly complex and unexpectedly various transcripts were generated from the mouse genome including ncRNAs and antisense

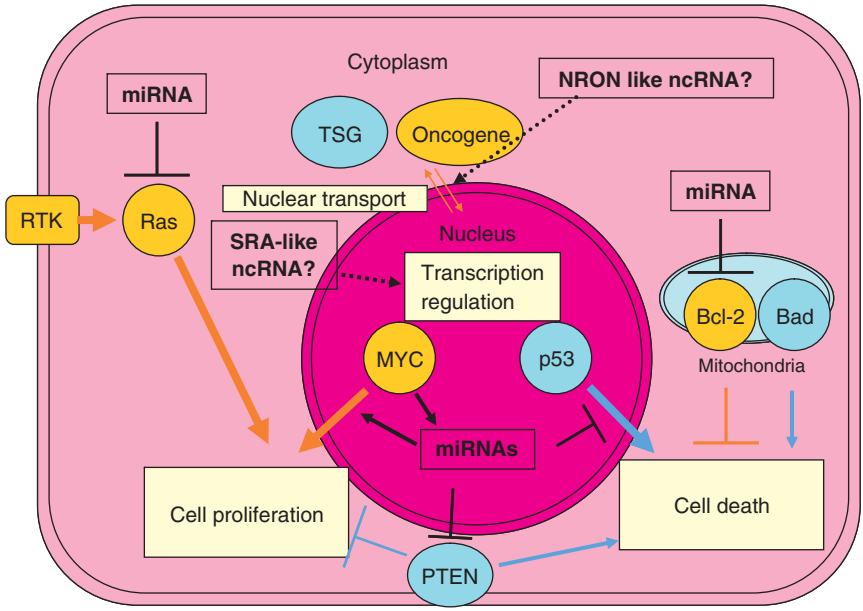

Fig. 5 Present map of the functional RNA continent in cancer cells. The schematic diagram represents mammalian cells with oncogenic pathways. Only the representative tumor-related gene products are shown. The oncogenic and tumor-suppressive proteins are indicated with orange and blue circles, respectively. The ncRNAs related to the functional regulation of these tumor-related gene products are indicated with rectangles. The arrows indicate positive regulation and the inverse-T shapes indicate negative regulation. Final direction in carcinogenesis is indicated in colors. The orange lines represent the tumorigenic pathways and the blue lines represent the tumor-suppressive pathways. The black lines indicate the regulatory effects by ncRNAs toward the tumor-related gene expression (Esquela-Kerscher and Slack, 2006).

transcripts. In cancer cells, the organized gene expression may be disrupted by the somatic genetic alterations. Such genetic disruption will cause the change of expression of functional ncRNAs in cells and contribute to the malignant transformation. A lot of miRNAs are known to be related to carcinogenesis (Fig. 5). It is possible that the expression profiling analysis of miRNAs in cancer cells provides critical information for differential diagnosis of the cancers and clues to find a new therapy of the patients. As mentioned above, the contribution of ncRNAs in transcription regulation of differentiation induction is obviously shown in several examples. These transcription cofactor ncRNAs, for example SRA (Caretti *et al*., 2006) and Evf-2 (Feng *et al*., 2006), might play a critical role in disorders of differentiation of cells and mediate carcinogenesis. It is important to investigate the specificity of such transcription cofactor ncRNAs. The NRON ncRNA is showing another possibility of ncRNA contribution in carcinogenesis. Many proteins, such as stabilized β-catenin, play critical roles in carcinogenesis only when they localize appropriate subcellular compartments (see Fig. 5). Therefore, it is quite likely that unidentified ncRNAs generated by cancer-specific gene alterations contribute to human carcinogenesis. Investigation of ncRNAs in cancer is clearly necessary for seeking new diagnostic and therapeutic modalities for cancers.

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The *c-myc* Promoter: Still *M*yster*Y* and *C*hallenge

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The transcription factor c-Myc is a key regulator of cell proliferation, cell growth, differentiation, and apoptosis. Deregulated *c-myc* expression possesses a high transformation potential and the proto-oncogene *c-myc* represents a promising target in anticancer therapy. This review on the *c-myc* promoter describes its organization, the different levels of its normal regulation (including initiation and elongation of transcription, the dual P1/P2 promoters, chromatin structure, c-Myc autosuppression) as well as its deregulation in Burkitt's lymphoma. Furthermore, it summarizes the many different transcription factors, signal transduction pathways, and feedback loops that activate or repress *c-myc* transcription. Finally, a concept for regulation of the *c-myc* promoter in different biological settings, for example, immediate-early induction, constant expression throughout the cell cycle in continuously cycling cells, repression during terminal differentiation and deregulation in cancer, is formulated.

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I. INTRODUCTION

c-Myc (MYC) biology is ***M***arvelous***l*** ***Y*** ***C***omplex (Oster *et al.*, 2002). Also the regulation of the *c-myc* promoter (Chung and Levens, 2005; Levens *et al.*, 1997; Liu and Levens, 2006; Marcu *et al.*, 1992, 1997; Potter and Marcu, 1997; Snyder and Miller, 1992; Spencer and Groudine, 1991) is very complex and yet poorly understood. Experimental results were often contradictory and confusing. Many signaling pathways, transcription factors, and *cis* regulatory elements were described to regulate *c-myc* transcription positively or negatively, but no simple model exists so far, which may explain how these diverse, dynamic, sometimes disparate and often context-dependent inputs are integrated and processed (Levens, 2002, 2003). Even the basal principles, rules, and patterns for transcriptional regulation of *c-myc* remained enigmatic. Thus, the *c-myc* promoter is still something of a black box (Eisenman, 2001b) and only one point is certain: regulation of the *c-myc* promoter is extremely complex with a lot of redundancy, many feedback loops, and several cross-regulatory circuits involved.

However, during the past years we have got many new data about regulation of the *c-myc* promoter by transcription factors, signaling pathways, and *cis* regulatory elements so that a pattern starts to emerge and important principles of its control are exemplified. The intention of this review is to emphasize what we already know about the control of the *c-myc* promoter. Nevertheless, obvious open questions will also be pointed out. This review tries to summarize the most important aspects of regulation of *c-myc* transcription although it is clearly impossible to cover each detail.

Readers who want to get a general survey of the regulation of the *c-myc* promoter without a detailed description of the transcription factors and signal transduction pathways involved are recommended to leave out Section IV, but instead to have a look at Figs. 4–7 and Tables I–III.

II. c-Myc: TRANSCRIPTION FACTOR AND ONCOGENE

A. c-Myc Function and Biology

The proto-oncoprotein c-Myc is a bHLHLZ (basic region/helix–loop–helix/leucine zipper) transcription factor that heterodimerizes with Max (Blackwood and Eisenman, 1991; Blackwood *et al.*, 1992) and then binds to specific E-boxes with the consensus core sequence 5′-CACGTG-3′ (Blackwell *et al.*, 1990, 1993; Grandori *et al.*, 2000; Lüscher and Larsson, 1999; Nair and Burley, 2006). c-Myc target genes are either activated via E-boxes (Amati *et al.*, 1992; Ayer *et al.*, 1993; Benvenisty *et al.*, 1992;

Kretzner *et al.*, 1992) or repressed via Inr (initiator)-dependent as well as Inr-independent mechanisms (Adhikary and Eilers, 2005; Amati *et al.*, 2001; Claassen and Hann, 1999; Cole and Nikiforov, 2006; Gartel and Shchors, 2003; Grandori *et al.*, 2000; Kleine-Kohlbrecher *et al.*, 2006; Oster *et al.*, 2002; Wanzel *et al.*, 2003). For activation, c-Myc recruits two TRRAP (transactivation/transformation domain associated protein)-containing HAT (histone acetyltransferase) complexes (GCN5, TIP60; Bouchard *et al.*, 2001; Frank *et al.*, 2001, 2003; McMahon *et al.*, 1998, 2000; Nikiforov *et al.*, 2002; Park *et al.*, 2001), an ATP-dependent chromatin remodeling complex (SWI/SNF; Cheng *et al.*, 1999b; Park *et al.*, 2002), the Pol II (RNA polymerase II) CTD (C-terminal domain) kinase P-TEFb (positive transcription elongation factor b; Eberhardy and Farnham, 2001, 2002; Kanazawa *et al.*, 2003), p300/CBP (CREB-binding protein; Vervoorts *et al.*, 2003), the mediator complex (Bouchard *et al.*, 2004; Eberhardy and Farnham, 2002), and the ubiquitin ligase component SKP2 (Kim *et al.*, 2003b; von der Lehr, 2003), which recruits components of the APIS (19S ATPase proteins independent of 20S) complex (Gonzalez *et al.*, 2002). For repression (Oster *et al.*, 2003), c-Myc recruits the DNA methyltransferase DNMT3a (Brenner *et al.*, 2005) and the ATPases TIP48 and TIP49 (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Wood *et al.*, 2000).

c-Myc is a key regulator of proliferation, differentiation, and apoptosis and plays a central role in cell growth control (Eisenman, 2001a; Grandori *et al.*, 2000; Hurlin and Dezfouli, 2004; Oster *et al.*, 2002). It drives cells through G_1-phase and induces S-phase entry. Ectopically expressed c-Myc is able to induce entry of quiescent cells into S-phase in the absence of mitogens (Eilers *et al.*, 1991). By activation (*cyclin D1*, *cyclin D2*, *cdk4*, *cdc25a*, *id2*, *cyclin E*, *cul1*, *cks2*) and repression (*p15*, *p21*, *p27*) of its target genes c-Myc activates cyclin D1(D2,D3)/Cdk4(6) as well as cyclin E/Cdk2 and inactivates RB (retinoblastoma protein) resulting in S-phase entry (Grandori *et al.*, 2000; Hurlin and Dezfouli, 2004; Lutz *et al.*, 2002; Nasi *et al.*, 2001; Oster *et al.*, 2002; Pelengaris *et al.*, 2002a; Steiner *et al.*, 1995; Zajac-Kaye, 2001). Since c-Myc potently stimulates proliferation and inhibits differentiation deregulated c-Myc possesses a high transformation potential. In addition, c-Myc drives cell growth, including RNA (Arabi *et al.*, 2005; Gomez-Roman *et al.*, 2003; Grandori *et al.*, 2005; Grewal *et al.*, 2005; Poortinga *et al.*, 2004) and protein synthesis as well as energy metabolism, reduces cell adhesion, stimulates angiogenesis, causes immortality, and promotes metastasis and genomic instability (Adhikary and Eilers, 2005; Boxer and Dang, 2001; Dang, 1999; Eisenman, 2001a; Gomez-Roman *et al.*, 2006; Grandori *et al.*, 2000; Lee and Dang, 2006; Levens, 2002; Lutz *et al.*, 2002; Mai and Mushinski, 2003; Nasi *et al.*, 2001; Oskarsson and Trumpp, 2005; Oster *et al.*, 2002; Pelengaris and Khan, 2003a; Pelengaris *et al.*, 2002a; Schmidt, 1999, 2004; Soucek and Evan, 2002;

Wade and Wahl, 2006). These properties potentiate c-Myc's high oncogenic potential so that deregulation of *c-myc* is associated with poor prognosis. However, in the absence of sufficient amounts of survival factors, c-Myc induces apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Nilsson and Cleveland, 2003; Prendergast, 1999). This represents a security mechanism against hyperproliferative signaling by c-Myc and limits its potent proliferation-stimulating effect to situations where proliferation is appropriate.

c-Myc is part of the Myc/Max/Mad network of bHLHLZ transcription factors that can be viewed as a functional module which integrates environmental signals and converts them into specific gene-regulatory programs for cell growth control (Eisenman, 2001a,b; Grandori *et al.*, 2000; Levens, 2002, 2003; Oster *et al.*, 2002). The ubiquitous and stable Max (Blackwood *et al.*, 1992) is the common heterodimerization partner for c-Myc and Mad proteins, the c-Myc antagonists, which both are very short lived. Mad proteins repress target genes via E-boxes (Ayer *et al.*, 1993; Hurlin *et al.*, 1995b; Zervos *et al.*, 1993) and recruit a Sin3-containing HDAC (histone deacetylase) complex (Alland *et al.*, 1997; Ayer *et al.*, 1995; Hassig *et al.*, 1997; Hurlin *et al.*, 1995a; Laherty *et al.*, 1997; Schreiber-Agus *et al.*, 1995). In contrast to c-Myc, Mad proteins inhibit proliferation, support differentiation, prevent apoptosis, and interfere with transformation (Baudino and Cleveland, 2001; Grandori *et al.*, 2000; Hooker and Hurlin, 2005; Hurlin and Dezfouli, 2004; Rottmann and Lüscher, 2006; Zhou and Hurlin, 2001). Accordingly, c-Myc, which is expressed in proliferating cells, and Mad proteins, which are expressed in non-proliferating cells, show opposite expression patterns (Ayer and Eisenman, 1993; Hurlin *et al.*, 1995a,b; Larsson *et al.*, 1994; Lee and Ziff, 1999; Schreiber-Agus *et al.*, 1994; Zervos *et al.*, 1993).

In summary, all crucial aspects of cell proliferation, cell growth, and tumorigenesis are positively regulated by c-Myc. Consequently, at the right time, at the right place, and in the right amount, c-Myc is essential for normal cell function, but its deregulation is extremely dangerous. This dualism requires the very tight control of *c-myc* expression so that it can be activated or repressed rapidly and precisely if appropriate. The essential importance of c-Myc for normal cell function is reflected by the estimation of 17,000–33,000 c-Myc binding sites in the human genome (Bieda *et al.*, 2006; Cawley *et al.*, 2004), by the identification of 4296 c-Myc binding sites in human P493 B cells (Zeller *et al.*, 2006) and by the finding that c-Myc binds to 10–15% of human and fly genes (Fernandez *et al.*, 2003; Levens, 2003; Li *et al.*, 2003; Orian *et al.*, 2003; Zeller *et al.*, 2006) although it seems to alter transcription levels of only a subset of them (Patel *et al.*, 2004; Zeller *et al.*, 2006).

B. *c-myc* Expression

c-myc mRNA and c-Myc protein are generally expressed at low levels in normal proliferating cells (Marcu *et al.*, 1992, 1997; Oster *et al.*, 2002; Spencer and Groudine, 1991). Corresponding to c-Myc's central role in cell growth control even slight changes in the amount of its mRNA and/or protein may have dramatic consequences for cell proliferation and cell fate so that both *c-myc* expression and c-Myc activity have to be tightly controlled (Chung and Levens, 2005; de la Cova *et al.*, 2004; Hanson *et al.*, 1994; Hooker and Hurlin, 2005; Liu and Levens, 2006; Moreno and Basler, 2004; Pirity *et al.*, 2006; Shichiri *et al.*, 1993; Trumpp *et al.*, 2001). Such a tight control of *c-myc* expression is achieved through fast regulation by many different signals on multiple levels, that is, transcription initiation and elongation, translation, and stability of mRNA and protein (both with extremely short half-life of 20–30 min) (Dani *et al.*, 1984, 1985; Hann and Eisenman, 1984; Hann *et al.*, 1988; Lemaitre *et al.*, 1996; Lüscher and Eisenman, 1990; Marcu *et al.*, 1992, 1997; Rabbitts *et al.*, 1985; Ryan and Birnie, 1996; Spencer and Groudine, 1991; Waters *et al.*, 1991). Furthermore, c-Myc activity is regulated by posttranslational modifications and interacting proteins (Facchini and Penn, 1998; Hann, 2006; Oster *et al.*, 2002; Sakamuro and Prendergast, 1999).

Controlling where, when, and how much c-Myc is made determines much of its action spectrum (Levens, 2002; Liu and Levens, 2006). Consequently, disentangling the regulation of the *c-myc* promoter is essential for the understanding of c-Myc biology. However, this is complicated by the fact that, for example, the Ras and PI3K (phosphatidyinositol 3-kinase) pathways, which are potent stimulators of cell proliferation (Massagué, 2004), target at least *c-myc* transcription, *c-myc* translation, and c-Myc protein stability to regulate the cellular c-Myc level (see Section IV.E). This strategy is biologically efficient, but makes it difficult to dissect effects on *c-myc* transcription from other regulatory effects in studies that do not address the question of *c-myc* promoter control.

Normal *c-myc* expression correlates strictly with cell proliferation (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Hooker and Hurlin, 2005; Lemaitre *et al.*, 1996; Marcu *et al.*, 1992; Oster *et al.*, 2002; Fig. 1; Spencer and Groudine, 1991). In proliferating cells the *c-myc* mRNA amount is 10- to 40-fold higher than in quiescent cells and during growth arrest and differentiation it drops about 90% (Spencer and Groudine, 1991). *c-myc* is virtually not expressed in quiescent cells. As typical immediate-early gene it is rapidly induced by mitogens during their reentry into the cell cycle (G_0/G_1 transition) independent of *de novo* protein biosynthesis (Iyer *et al.*, 1999). Then *c-myc* expression declines to a lower

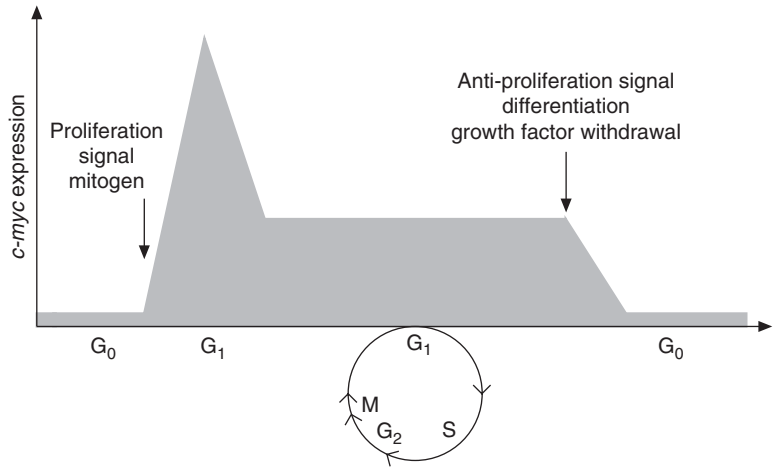

Fig. 1 *c-myc* expression. Adapted from Lemaitre *et al.* (1996, Fig. 8, p. 112).

level which persists during the whole cell cycle in continuously proliferating cells (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985). This constant *c-myc* expression depends on the permanent presence of growth factors. Their removal, stimuli of differentiation or other antiproliferative signals result in immediate downregulation of *c-myc*. In terminally differentiated cells *c-myc* is no longer expressed. Accordingly, in adults *c-myc* expression is restricted to tissues with proliferating cell types and areas of regenerative proliferation.

In summary, normal *c-myc* expression correlates strictly with cell proliferation and is tightly controlled by mitogens, stimuli of differentiation, proliferation and antiproliferation signals (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Marcu *et al.*, 1992; Oster *et al.*, 2002; Fig. 1; Spencer and Groudine, 1991). Thereby *c-myc* functions as integrator of extracellular signals and as central switch for a wide range of signaling pathways that regulate proliferation or differentiation (Eisenman, 2001a,b; Grandori *et al.*, 2000; Levens, 2002, 2003; Oster *et al.*, 2002).

III. REGULATION OF THE *c-myc* PROMOTER

A. The *c-myc* Locus

The *c-myc* locus is organized by two MARs (matrix attachment regions) and the MINE (*c-myc* insulator element) (Fig. 2). The MARs, which anchor the chromatin fiber to the nuclear matrix, separate the *c-myc* locus from

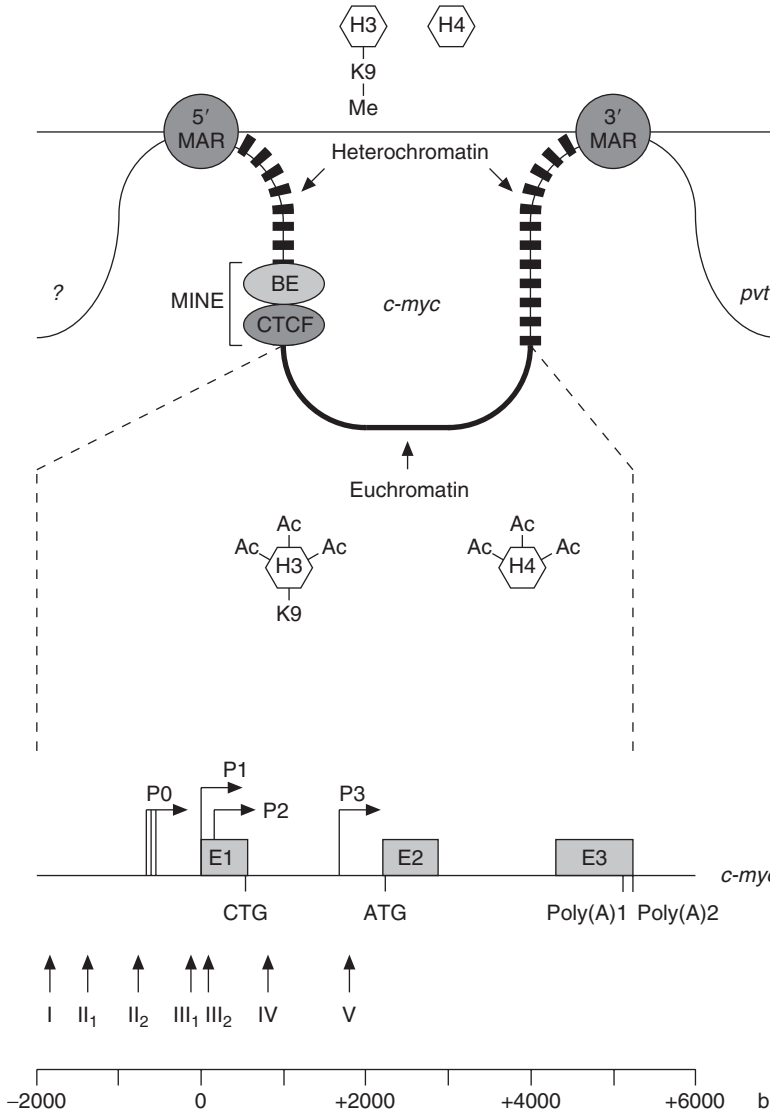

Fig. 2 Organization of the *c-myc* locus and structure of the human *c-myc* gene. Top panel: The *c-myc* locus. Not drawn to scale. The 5′ MAR (matrix attachment region) located 80 kb upstream and the 3′ MAR located 50 kb downstream define a 160-kb domain, which contains both hetero- and euchromatin, and separate this domain from the neighboring *pvt1* gene and another gene of unknown function (?) (Gombert *et al.*, 2003). Transcriptionally active euchromatin is characterized by hyperacetylation of histones H3 and H4 and lack of histone H3-K9-methylation. In contrast, transcriptionally inert heterochromatin is characterized by histone H3-K9-methylation and hypoacetylation of histones H3 and H4 (Gombert *et al.*, 2003). Euchromatin and heterochromatin are seperated by the 1.6-kb MINE (c-myc insulator element)

neighboring genes and may form a chromosomal loop that permits the association of the *c-myc* gene with transcriptionally active nuclear territories (Gombert *et al.*, 2003). Euchromatin, characterized by hyperacetylation of histones H3 and H4 and K9-nonmethylation of histone H3, covers the *c-myc* promoter region and the transcribed sequences (Farris *et al.*, 2005; Gombert *et al.*, 2003). This euchromatin is flanked on both sites by histone H3- and H4- hypoacetylated, histone H3-K9-methylated heterochromatin (Fig. 2; Gombert *et al.*, 2003). The *c-myc* gene is covered by such euchromatin in mitogen (IL-2)-induced CTLL2 cells expressing *c-myc* as well as in resting CTLL2 cells not expressing *c-myc*. However, the degree of histone H3-hyperacetylation is significantly higher in the former ones, while no change in histone H3-K9-methylation is observed (Gombert *et al.*, 2003). In the upstream region euchromatin and heterochromatin are separated by the MINE, which functions as efficient insulator and is composed of the BE (barrier element) and the CTCF (CCCTC-binding factor) binding element (Fig. 2). The MINE exhibits enhancer-blocking activity as well as barrier activity, which both are orientation dependent and contribute to its boundary function (Gombert *et al.*, 2003). The enhancer-blocking activity is mediated by CTCF (Gombert *et al.*, 2003; Lutz *et al.*, 2003) that is necessary and sufficient for vertebrate enhancer blocking (Bell *et al.*, 1999; Ishihara *et al.*, 2006). The CTCF binding element in the MINE is occupied by CTCF (Gombert *et al.*, 2003) and *in vivo* associated with the SNF2-like chromodomain helicase protein CHD8, which directly binds to CTCF (Ishihara *et al.*, 2006). shRNA-mediated knockdown of CHD8 resulted in CpG hypermethylation and histone H3-hypoacetylation in the vicinity and/or downstream of this CTCF binding element (Ishihara *et al.*, 2006). This suggests that CHD8 recruited by CTCF contributes to the barrier function

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that is composed of the 1-kb barrier element (BE) and the CTCF binding element (CTCF). The MINE is located approximately 2.5 kb upstream of the *c-myc* transcription initiation site and functions as an efficient insulator (Gombert *et al.*, 2003). The euchromatin extends over ca. 7–7.5 kb and includes the *c-myc* promoter region and the transcribed sequences (Gombert *et al.*, 2003). The dashed lines indicate this euchromatin region in the human *c-myc* gene. Bottom panel: The *c-myc* gene. Adapted from Spencer and Groudine (1991, Fig. 1, p. 3) and Marcu *et al.* (1992, Fig. 2, p. 832). Drawn to scale. Shown is the human *c-myc* gene with the three exons (gray boxes), the four promoters (P0, P1, P2, P3), the two major translation start codons (CTG, ATG), the two polyadenylation signals [poly(A)1, poly(A)2] and the DNAse I-hypersensitive sites (I, II_1, II_2, III_1, III_2, IV, V). The DNAse I-hypersensitive sites VI to IX downstream of exon 2 and in the 3′ flanking region (Murphy *et al.*, 1996) are not shown. The major protein product p64 (439 amino acids) starts with the ATG at the beginning of exon 2 (Hann and Eisenman, 1984; Hann *et al.*, 1988; Marcu *et al.*, 1992; Spencer and Groudine, 1991). The minor protein product p67 (14 additional N-terminal amino acids) starts with the CTG at the end of exon 1. P0 transcripts start at multiple initiation sites. The murine and rat *c-myc* genes possess the promoters P1, P2, and P3, but lack P0 (Marcu *et al.*, 1992; Spencer and Groudine, 1991).

of the MINE against the spread of adjacent transcriptionally inactive heterochromatin.

In addition, for the promoter and/or the transcribed region of *c-myc* the following histone modifications have been described: H3-phosphorylation (Chadee *et al.*, 1999; Dunn *et al.*, 2005), H4-K20-monomethylation (Talasz *et al.*, 2005), H3-K4-trimethylation (Sierra *et al.*, 2006), H3-K9-dimethylation versus H3-K9-acetylation (Cheng *et al.*, 2006), K4-di-, K36- and K79-methylation of histone H3 as well as incorporation of the histone variant H2A.Z (Farris *et al.*, 2005).

The *c-myc* gene is DNA hypermethylated and silenced for example in terminally differentiated human K562 erythroleukemia cells, induced to differentiate by ara-C (1-β-D-arabinofuranosylcytosine; Baker *et al.*, 1994), and in differentiated MEL (murine erythroleukemia) cells, induced to differentiate by DMSO (dimethyl-sulfoxide; Scarpa *et al.*, 2001). In contrast, the *c-myc* gene is DNA hypomethylated and overexpressed for example in hepatocellular carcinomas (Laird and Jaenisch, 1994; Tao *et al.*, 2000; Tsujiuchi *et al.*, 1999) and in gastric cancer (Fang *et al.*, 2004).

B. The *c-myc* Promoter Structure

The human *c-myc* gene possesses four promoters: P0, P1, P2, and P3 (Fig. 2; Battey *et al.*, 1983; Bentley and Groudine, 1986a,b; Marcu *et al.*, 1992; Ray and Robert-Lézènes, 1989; Spencer and Groudine, 1991; Spencer *et al.*, 1990; Stewart *et al.*, 1984; Taub *et al.*, 1984a,b; Watt *et al.*, 1983). In normal cells, the majority of transcripts initiate at the P1 and P2 promoters with a clear dominance of P2. Thus, 75–90% of *c-myc* transcripts initiate at P2, 10–25% at P1, less than 5% at P0 and also less than 5% at P3. The high strength of P2 is in part explained by its optimal TATA-box TATAAAAG (Bareket-Samish *et al.*, 2000; Hoopes *et al.*, 1998; Patikoglou *et al.*, 1999; Starr *et al.*, 1995; Wierstra and Alves, 2006d; Yean and Gralla, 1997) and the presence of two Inr elements (Facchini *et al.*, 1997; Krumm *et al.*, 1995; Marcu *et al.*, 1997) which synergize with the TATA-box in TFIID and TFIID/TFIIA binding (Chalkley and Verrijzer, 1999; Colgan and Manley, 1995; Emami *et al.*, 1997; Nakatani *et al.*, 1990; O'Shea-Greenfield and Smale, 1992; Smale, 2001; Smale and Kadonaga, 2003; Smale *et al.*, 1998; Verrijzer and Tjian, 1996; Verrijzer *et al.*, 1995). In contrast, P1 possesses the non-optimal TATA-box TATAATGC and lacks any Inr. Neither P1 nor P2, located 161 bp apart in the human *c-myc* gene, have a TFIIB recognition element (BRE), a downstream promoter element (DPE), or a motif ten element (MTE). Both P0 and P3 are TATA less.

Several DNAse I-hypersensitive sites were mapped in *c-myc* (Fig. 2; Ishihara *et al.*, 2006; Marcu *et al.*, 1992; Mautner *et al.*, 1995; Michelotti *et al.*, 1996b;

Murphy *et al.*, 1996; Spencer and Groudine, 1991 and references therein). They are presumed to result from perturbation of normal chromatin architecture and might reflect alterations of factor binding, nucleosome positioning, or DNA conformation. The intensity of cleavage at some sites parallels the synthesis of *c-myc* mRNA whereas other sites are constitutive (Levens *et al.*, 1997; Liu and Levens, 2006; references therein).

Some upstream segments of the *c-myc* gene are not B-DNA *in vivo*. Instead they are single-stranded, form Z-DNA and H-DNA (triple helix) or adopt G-quadruplex and *i*-tetraplex structures suggesting that DNA topology and DNA conformation may help to govern *c-myc* expression (Chung and Levens, 2005; Levens *et al.*, 1997; Liu and Levens, 2006; Marcu *et al.*, 1992; Michelotti *et al.*, 1996b; Siddiqui-Jain *et al.*, 2002; Simonsson *et al.*, 2000; Spencer and Groudine, 1991 and references therein) as evidenced for the FUSE (far upstream element) and the CT-element, also termed NHE (nuclease hypersensitive element) (see Sections IV.A.7, IV.A.8, and IV.A.12; Grand *et al.*, 2002, 2004; He *et al.*, 2000a; Kouzine *et al.*, 2004; Liu *et al.*, 2006a; Siddiqui-Jain *et al.*, 2002; Tomonaga *et al.*, 1998).

C. Regulation Levels of *c-myc* Transcription

So far, for the *c-myc* locus neither a well-defined LCR (locus control region) nor evidence for the assembly of an enhanceosome were found (Chung and Levens, 2005; Levens, 2003; Liu and Levens, 2006; Liu *et al.*, 2006a; Potter and Marcu, 1997). However, two independent distal tissue-specific enhancer elements are present in the 5′ upstream region of the *c-myc* gene, which are predicted to harbor conserved TCF-4 binding sites and to play a role in organ-specific growth control (Hallikas *et al.*, 2006). Moreover, two enhancer elements were identified 3′ of the third exon (Mautner *et al.*, 1995). The dual *c-myc* P1/P2 promoters offer the possibility for their independent and different regulation as well as for intimate linkage of their control. In addition, the transcription of *c-myc* is not only controlled at initiation, but also at elongation. The outcome of this scenario is very complex because a close connection exists between initiation and elongation of P1 transcripts, initiation and elongation of P2 transcripts, and the P1:P2 transcript ratio. Thus, the multiple positively and negatively acting transcription factors and signaling pathways that control the *c-myc* promoter may act simultaneously or sequentially during PIC (preinitiation complex) assembly or progression to promoter escape or regulate the holdback and release of paused Pol II complexes, Chung and Levens, 2005; Levens, 2003; Liu and Levens, 2006; Liu *et al.*, 2000, 2001; Weber *et al.*, 2005).

Regulation of *c-myc* expression by blockage of transcription elongation was first described 20 years ago (Bentley and Groudine, 1986a; Eick and

Bornkamm, 1986; Nepveu and Marcu, 1986). Since then this phenomenon, which was named block to elongation, attenuation, promoter proximal pausing, or holdback of Pol II, has been extensively studied. Pausing of *c-myc* transcription was found at three different sites: (1) P1-initiated transcripts between the P1 and P2 promoters, that is, immediately upstream of the P2 TATA-box or between the P2 TATA-box and the P2 transcription start site (Meulia *et al.*, 1992; Roberts *et al.*, 1992; Wright *et al.*, 1991), (2) P2-initiated transcripts at the P2 promoter, that is, immediately downstream of the P2 transcription start site (Albert *et al.*, 1997, 2001; Kohlhuber *et al.*, 1993; Krumm *et al.*, 1992, 1995; Strobl and Eick, 1992; Strobl *et al.*, 1993; Weber *et al.*, 2004; Wolf *et al.*, 1995), and (3) at the 3′ end of the first non-coding exon (Bentley and Groudine, 1988; Miller *et al.*, 1989; Spencer *et al.*, 1990; Wright and Bishop, 1989). Activation of the stalled Pol II complexes is thought to be mediated through transcriptional activators by increasing the elongation competence of Pol II (Krumm *et al.*, 1993, 1995; Liu *et al.*, 2006a; Madisen and Groudine, 1994; Yankulov *et al.*, 1994). The involvement of TFIIH, in particular its p89/XPB helicase, phosphorylation of the CTD of the largest Pol II subunit, acetylation signals, P-TEFb and/or the mediator complex was suggested (Chung and Levens, 2005; Krumm *et al.*, 1995; Liu and Levens, 2006; Madisen *et al.*, 1998; Marcu *et al.*, 1997; Schneider *et al.*, 1999; Weber *et al.*, 2005; Wolf *et al.*, 1995). CDK9 and cyclin T1, components of P-TEFb that phosphorylate the CTD, were shown to stimulate transcription from the *c-myc* P2 promoter (Majello *et al.*, 1999). Increasing or decreasing the promoter proximal pausing of Pol II complexes provide a rapid mode for positive and negative control of *c-myc* transcription in response to diverse stimuli (Marcu *et al.*, 1992, 1997; Potter and Marcu, 1997; Spencer and Groudine, 1991). An increase in the transcriptional pausing of Pol II could fully account for the fast reduction of steady-state *c-myc* mRNA levels during induction of terminal differentiation [e.g., by DMSO or RA (retinoic acid)], while the initiation rate of *c-myc* transcription was unaffected at first (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986; Krumm *et al.*, 1992; Mechti *et al.*, 1986; Nepveu and Marcu, 1986; Nepveu *et al.*, 1987; Salehi *et al.*, 1988; Siebenlist *et al.*, 1988; Strobl and Eick, 1992; Watson, 1988). Vice versa, the fast increase in steady-state *c-myc* mRNA levels during stimulation of quiescent cells with mitogens was due to a release of the block to transcription elongation (Cutry *et al.*, 1989; Eick *et al.*, 1987; Heckford *et al.*, 1988; Lindsten *et al.*, 1988; Nepveu *et al.*, 1987; Schneider-Schaulies *et al.*, 1987). Again transcription initiation was reported to remain constant at first. At later time points during induced terminal differentiation also the level of transcription initiation declined and finally initiation ceased (Siebenlist *et al.*, 1988). Repression of *c-myc* expression during differentiation seems to generally include two phases (Chen *et al.*, 2000b; Chung and Levens, 2005; Marcu *et al.*, 1992, 1997; Spencer

and Groudine, 1991; Zajac-Kaye *et al.*, 2000): The rapid early drop in transcriptional elongation is followed by a later decline in and a final loss of transcription initiation (Siebenlist *et al.*, 1988). Accordingly, at recently silenced *c-myc* genes most Pol II complexes reside in a paused configuration at the P2 start site while long-term repressed *c-myc* genes do not retain stalled Pol II complexes (Liu *et al.*, 2006a; Marcu *et al.*, 1997). In general, transcription from the *c-myc* P2 promoter is predominantly regulated at the level of elongation (Albert *et al.*, 1997, 2001; Kohlhuber *et al.*, 1993; Krumm *et al.*, 1992, 1995; Liu *et al.*, 2006a; Marcu *et al.*, 1997; Strobl and Eick, 1992; Strobl *et al.*, 1993; Wolf *et al.*, 1995): also repressed *c-myc* genes harbor paused Pol II complexes proximal to the P2 start site and even at highly expressed *c-myc* genes in rapidly proliferating cells most P2-initiated Pol II complexes reside in a paused configuration at the P2 promoter site while only a minor fraction of them actively transcribes *c-myc*. During DMSO-induced terminal differentiation of MEL cells repression of the P2 promoter occurs by promoter proximal pausing of Pol II whereas the P1 promoter is repressed by inhibition of transcription initiation (Kohlhuber *et al.*, 1993).

The element ME1a1 (mouse) or CT-I_2 (human), located between P1 and P2, is essential for the block to transcriptional elongation (Dufort *et al.*, 1993; Miller *et al.*, 1989; Wright *et al.*, 1991). In addition, ME1a1/CT-I_2 is essential for transcription from P2 (Table I), but functions as a negative element for transcription from P1 (Albert *et al.*, 2001; Asselin *et al.*, 1989; Bossone *et al.*, 1992; Carlberg *et al.*, 1999; DesJardins and Hay, 1993; Hall, 1990; Moberg *et al.*, 1991, 1992a; Wright *et al.*, 1991). Consequently, mutation of murine ME1a1 (Asselin *et al.*, 1989; Bossone *et al.*, 1992; Marcu *et al.*, 1997) and deletion of human CT-I_2 (DesJardins and Hay, 1993) resulted in a change of the P1:P2 transcript ratio from 1:5 to 10:1 or from 1:6 to 1:1, respectively. Also the element ME1a2 functions as positive element for transcription from P2 (Table I), so that mutation of murine ME1a2 (Bossone *et al.*, 1992; Marcu *et al.*, 1997) resulted in a change of the P1:P2 transcript ratio from 1:5 to 1:2. The *c-myc* promoter sequences between P1 and P2, in particular the element ME1a2/CT-I_2, play a central role for the complex regulation of initiation and elongation of P1 and P2 transcripts and the P1:P2 transcript ratio. ME1a1/CT-I_2 is also required for the maintenance of an open chromatin configuration at the dual *c-myc* P1/P2 promoters (Albert *et al.*, 2001; see Section III.D). In normal proliferating cells, the P1:P2 transcript ratio varies between 1:10 and 1:5 although P1 and P2 transcripts have similar half-lives (Bentley and Groudine, 1986a; Broome *et al.*, 1987; Marcu *et al.*, 1992; Nishikura and Murray, 1988; Spencer and Groudine, 1991; Spencer *et al.*, 1990; Taub *et al.*, 1984a,b; Yang *et al.*, 1985). Atypical cases, in which it approaches 1:1 or higher, have generally been associated with abnormal and deregulated

Table I Binding of Transcription Factors to the *c-myc* Promoter as well as Regulation of the *c-myc* Promoter and the Endogenous *c-myc* Expression by These Transcription Factors

		Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression		Regulation of *c-myc* promoter			
Section	Transcription factor (*or binding site*)	Method[a]	Comment[b]	Expression[c]	Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]	Manipulation of transcription factor[d]	References
III.C	(*ME1a1/CT-I*$_2$)[g]	F, M				T, IV S E	wt, del, P wt, del, P wt, P	↑ ↑ ↑	Asselin *et al.*, 1989; Moberg *et al.*, 1990, 1992a,b; Bossone *et al.*, 1992; DesJardins and Hay, 1993; Dufort and Nepveu, 1994; Komatsu *et al.*, 1997; Marcu *et al.*, 1997; Carlberg *et al.*, 1999; Izzo *et al.*, 1999; Albert *et al.*, 2001
	(*ME1a2*)[g]	F, M				T S	wt, del wt, del, P	↑ ↑	Asselin *et al.*, 1989; Moberg *et al.*, 1990, 1992a,b; Bossone *et al.*, 1992; Carlberg *et al.*, 1999; Izzo *et al.*, 1999

(*continues*)

Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter: Method[a]	Comment[b]	Regulation of endogenous *c-myc* expression: Expression[c]		Manipulation of transcription factor[d]	Regulation of *c-myc* promoter: Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	References
III.E	c-Myc	C		T, P, RO	↓	OE, del	T	wt, del, P	↓	OE, del	Lombardi *et al.*, 1987; Cleveland *et al.*, 1988; Grignani *et al.*, 1990; Penn *et al.*, 1990a,b; Lucas *et al.*, 1993; Facchini *et al.*, 1997; Mao *et al.*, 2003; Luo *et al.*, 2004; Wang *et al.*, 2006; Zeller *et al.*, 2006
	c-Myc/Max			T	↓	OE, del					Facchini *et al.*, 1997; Mao *et al.*, 2003
	Max	C					T	wt	↑	OE	Lee and Ziff, 1999; Mao *et al.*, 2003
IV.A.1	TCF-4 (*TBE1*, *TBE2*, *TBE3*)	E, D, C	S, C, P	T, P	↑	dn, del, RNAi, KO	T	wt	↑	dn, RNAi	He *et al.*, 1998; Kolligs *et al.*, 2000; Barker *et al.*, 2001; van de Wetering *et al.*, 2002; Sasaki *et al.*, 2003; Tong *et al.*, 2004; Hu and Rosenblum, 2005; Nateri *et al.*, 2005; Liu *et al.*, 2006a

	LEF-1	E, D, C	S	T	↑	OE, RNAi					Sasaki *et al.*, 2003; Sierra *et al.*, 2006; Skokowa *et al.*, 2006
	LEF-1			T	↓	KO					Reya *et al.*, 2000
	β-Catenin (*TBE1*, *TBE2*, *TBE3*)[b]	E, D, C	S, C, P	T, P	↑	OE, del, RNAi, ca in tg	T	wt, del, P, H	↑	OE, ca, del	He *et al.*, 1998; Kolligs *et al.*, 2000; Imbert *et al.*, 2001; Wolf *et al.*, 2002; Sasaki *et al.*, 2003; Teulière *et al.*, 2004; Hu and Rosenblum, 2005; Nateri *et al.*, 2005; Sierra *et al.*, 2006
	γ-Catenin[b]			T	↑	OE, tg, del in tg	T	wt, del	↑	OE	Kolligs *et al.*, 2000
	β-Catenin/TCF-4 (*TBE1*, *TBE2*, *TBE3*)			T, P	↑	OE, A/R	T	wt, del, P, H	↑	A/R	He *et al.*, 1998; Liu *et al.*, 2004; Hu and Rosenblum, 2005; Noubissi *et al.*, 2006; Sierra *et al.*, 2006
	TCF-1			T	↑	dn					Barker *et al.*, 2001
	Smad3 (*TBE3*)[i]	E, D	S				T	wt, del, P	↓	OE	Sasaki *et al.*, 2003
IV.A.2	E2F-1	E, C	IV, S, C				T	wt, del, P	↑	OE	Oswald *et al.*, 1994; Roussel *et al.*, 1994; Wong *et al.*, 1995; Campanero *et al.*, 2000; Albert *et al.*, 2001; Johansen *et al.*, 2001; Klappacher *et al.*, 2002; Ogawa *et al.*, 2002; Ren *et al.*, 2002; Iakova *et al.*, 2003; Wells *et al.*, 2003; Liu *et al.*, 2006a

(*continues*)

Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression		Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]	Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
	E2F-1/DP-1					T	wt, H	↑	OE	Majello *et al.*, 1995; Campanero *et al.*, 2000
	E2F-2	E, C	IV							Campanero *et al.*, 2000; Albert *et al.*, 2001
	E2F-3	E	IV							Campanero *et al.*, 2000;
	E2F-3/DP-1					T	H	↑	OE	Campanero *et al.*, 2000;
	E2F-4	E, C, D	IV, P							Campanero *et al.*, 2000; Albert *et al.*, 2001; Chen *et al.*, 2002; Klappacher *et al.*, 2002; Ogawa *et al.*, 2002; Ren *et al.*, 2002; Yagi *et al.*, 2002; Baek *et al.*, 2003; Iakova *et al.*, 2003; Wells *et al.*, 2003; Cam *et al.*, 2004; Frederick *et al.*, 2004; Sebastian *et al.*, 2005; Gomis *et al.*, 2006a; Liu *et al.*, 2006a; Rodriguez *et al.*, 2006
	E2F-4					T	del, P	↑	OE	Yagi *et al.*, 2002

	E2F-4/DP-1	E	IV, S, C, P					Yagi *et al.*, 2002; Frederick *et al.*, 2004
	DP-1	E, D	S, P					Wong *et al.*, 1995; Chen *et al.*, 2002
	E2F-5	E, C	IV					Campanero *et al.*, 2000; Chen *et al.*, 2002
	E2F-6	C						Ogawa *et al.*, 2002
	E2F[g]	E, M	IV, C, P	T, IV, M	wt, del, P, H	↑		Nishikura, 1986; Hiebert *et al.*, 1989; Thalmeier *et al.*, 1989; Mudryj *et al.*, 1990; Moberg *et al.*, 1990, 1992a,b; Hamel *et al.*, 1992; Batsche *et al.*, 1994; Ishida *et al.*, 1994, 1995; Watanabe *et al.*, 1995; Carlberg *et al.*, 1999; Albert *et al.*, 2001; Johansen *et al.*, 2001; Yagi *et al.*, 2002; Frederick *et al.*, 2004
	E2F[g,j]			E	wt, P	↓		Albert *et al.*, 2001
	E2F[g,j]			S	del	↑		Krumm *et al.*, 1995
IV.A.2, IV.A.3	Smad 2	D	P	T	wt	↓	OE	Chen *et al.*, 2001b; Yagi *et al.*, 2002; Suzuki *et al.*, 2004

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression		Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]	Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
	Smad3 (*TIE*)	E, D, F, M	IV, S, C, P	T, P ↓	OE, dn, del, KO	T	wt, P, H ↓		OE, del	Chen *et al.*, 2001b, 2002; Yagi *et al.*, 2002; Frederick *et al.*, 2004; Matsuura *et al.*, 2004; Suzuki *et al.*, 2004; Hu *et al.*, 2005; Buck *et al.*, 2006
	Smad2/3 (*TIE*)	C		T, P, RO, CH ↓	A/R	T	wt, del, P, H ↓		A/R	Zentella *et al.*, 1991; Iavarone and Massagué, 1997; Sun *et al.*, 1999; Chen *et al.*, 2001b, 2002; Yagi *et al.*, 2002; Kurisaki *et al.*, 2003; Frederick *et al.*, 2004; Matsuura *et al.*, 2004; Suzuki *et al.*, 2004; Hu *et al.*, 2005; Buck *et al.*, 2006; Gomis *et al.*, 2006a

	Smad4 (*TIE*)	D, C	P	T	↓	OE	T	wt	↓	OE, del, KO	Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Suzuki *et al.*, 2004
	Smad2 + Smad4 (*TIE*)						T	wt	↓	OE	Chen *et al.*, 2001b
	Smad3 + Smad4 (*TIE*)			T	↓	OE	T	wt	↓	OE	Chen *et al.*, 2001b; Frederick *et al.*, 2004
	Smad2 + Smad3 + Smad4 (*TIE*)						T	wt	↓	OE	Chen *et al.*, 2001b
IV.A.4	METS	E, C		P	↓	OE	T	wt	↓	OE, del	Klappacher *et al.*, 2002
IV.A.5	C/EBPα	C		T, P	↓	OE	T	wt, del, P	↓	OE, dn	Timchenko *et al.*, 1999; Johansen *et al.*, 2001; Iakova *et al.*, 2003
IV.A.6	STAT3	E, C	S, C	T, CH	↑	dn, ca, A/R	T	wt, del, P, H	↑	dn, A/R	Cressman *et al.*, 1996; Bromberg *et al.*, 1999; Kiuchi *et al.*, 1999; Bowman *et al.*, 2001; Kirito *et al.*, 2002; Barré *et al.*, 2003; Yin *et al.*, 2004; Vigneron *et al.*, 2005
	STAT1/3			T	↑	A/R	T	wt	↑	A/R	Kirito *et al.*, 2002
	STAT1	E	S, C				T	wt	↑	dn	Kiuchi *et al.*, 1999; Kirito *et al.*, 2002

(*continues*)

Table I (*continued*)

		Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter			
Section	Transcription factor (*or binding site*)	Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	References
IV.A.7	FBP	E, C, NMR, PP	IV, S, C, P	T, P	↑	OE, dn, RNAi	T	wt, del	↑	OE, dn, del	Avigan *et al.*, 1990; Duncan *et al.*, 1994, 1996; Bazar *et al.*, 1995a,b; Davis-Smyth *et al.*, 1996; Michelotti *et al.*, 1996b; He *et al.*, 2000a; Liu *et al.*, 2000, 2001, 2006a; Braddock *et al.*, 2002; Kim *et al.*, 2003a; Huth *et al.*, 2004; Kouzine *et al.*, 2004; Chung *et al.*, 2006
	FBP2	E	IV, C, P				T	wt	↑	OE	Davis-Smyth *et al.*, 1996; Chung *et al.*, 2006
	FBP3	E, C	IV, C, P				T	wt	↑	OE	Davis-Smyth *et al.*, 1996; Chung *et al.*, 2006
	FIR	E, C, PP	IV, S	T, P	↓	OE, del, RNAi	T	wt, del	↓	OE	Liu *et al.*, 2000, 2006a; Weber *et al.*, 2005; Matsushita *et al.*, 2006

IV.A.9	CNBP	E	IV, S, C	T	↑	KO	T	wt, H	↑	OE, KO	Michelotti *et al.*, 1995; Chen *et al.*, 2003; Shimizu *et al.*, 2003
IV.A.10	hnRNP K	E, C, PP	IV, C, P	T, P	↑	OE, RNAi	T, IV	wt, P, H	↑	P, OE, ca, del, RNAi	Takimoto *et al.*, 1993; Tomonaga and Levens, 1995, 1996; Michelotti *et al.*, 1996a,b, 1997; Mandal *et al.*, 2001; Ostrowski *et al.*, 2003; Huth *et al.*, 2004; Lynch *et al.*, 2005
IV.A.11	NM23-H2	E, M, FBA	IV, S, C, P	T, P	↑	tg	T, IV	wt, del	↑	P, OE	Postel *et al.*, 1989, 1993, 1996; Postel and Ferrone, 1994; Berberich and Postel, 1995; Hildebrandt *et al.*, 1995; Agou *et al.*, 1999; Postel, 1999; Arnaud-Dabernat *et al.*, 2004; Fournier *et al.*, 2005
IV.A.13	NF-κB	E, F, M, OC	C, P	T, P	↑	A/R	T	wt, del, P, H	↑	A/R	Duyao *et al.*, 1990a,b, 1992; Kessler *et al.*, 1992a,b; Ji *et al.*, 1994; Lee *et al.*, 1995a,b ; Wu *et al.*, 1996a; Kirillova *et al.*, 1999; Kim *et al.*, 2000a,b; Arcinas *et al.*, 2001; Jeay *et al.*, 2001

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
	RelA	E, C	S	P	↑	OE	T	wt, P, H	↑	OE	Ji *et al.*, 1994; La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Schauer *et al.*, 1996; Zou *et al.*, 1997a,b; Kim *et al.*, 2000a,b; Park and Wei, 2003
	c-Rel	E	S, C	T, P	↑	tg	T	wt, P, H	↑	OE	Ji *et al.*, 1994; La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Arsura *et al.*, 1996; Schauer *et al.*, 1996; Siebelt *et al.*, 1997; Grumont *et al.*, 2002; Romieu-Mourez *et al.*, 2003
	RelB			P	↑	RNAi					Demicco *et al.*, 2005
	p50	E, C	S, C				T	wt, P, H	↓	OE	Ji *et al.*, 1994; Lee *et al.*, 1995a; Schauer *et al.*, 1996; Siebelt *et al.*, 1997; Kim *et al.*, 2000a,b; Arcinas *et al.*, 2001; Grumont *et al.*, 2002; Park and Wei, 2003

RelA/p50	E	S, C, P				T	wt	↑	OE	La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Arsura *et al.*, 1996; Schauer *et al.*, 1996; Wu *et al.*, 1996a,b; Kim *et al.*, 2000a,b; Park and Wei, 2003; Demicco *et al.*, 2005
c-Rel/p50	E	S	T	↑	KO	T	P, H	↑	OE	La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Arsura *et al.*, 1996; Schauer *et al.*, 1996; Wu *et al.*, 1996a,b; Grumont *et al.*, 2002; Chandramohan *et al.*, 2004
p50/p50	E	S, C, P								La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Arsura *et al.*, 1996; Schauer *et al.*, 1996; Wu *et al.*, 1996a,b; Kim *et al.*, 2000a,b; Park and Wei, 2003; Chandramohan *et al.*, 2004; Demicco *et al.*, 2005
RelA/RelA	E									La Rosa *et al.*, 1994
c-Rel/RelA			T	↑	KO					Grumont *et al.*, 2004
c-Rel/c-Rel	E									La Rosa *et al.*, 1994; Arsura *et al.*, 1996; Schauer *et al.*, 1996; Wu *et al.*, 1996a,b

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
	RelB/p52	E					T	wt	↑	OE	Demicco *et al.*, 2005
IV.A.14	JunD	E, C	S, C								Iavarone *et al.*, 2003
	c-Jun	E, C	S, C	T	↑	dn	T	wt, H	↑	OE, dn, del, RNAi	Iavarone *et al.*, 2003; Toualbi *et al.*, 2006
	c-Fos	E, IB, IP	IV, S				T	wt	↑	OE, del, RNAi	Hay *et al.*, 1989; Takimoto *et al.*, 1989; Toualbi *et al.*, 2006
	c-Fos/c-Jun	E	IV				T	wt, H	↑	OE	Hay *et al.*, 1989; Iavarone *et al.*, 2003; Toualbi *et al.*, 2006
	AP-1	E	IV, C, P								Hay *et al.*, 1989; Takimoto *et al.*, 1989
IV.A.15	ETS-1/2	C									Albert *et al.*, 2001; Klappacher *et al.*, 2002
	ETS-1	E	C, P				T	wt, del, P	↑	OE	Roussel *et al.*, 1994
	ETS-2	E	IV, C, P	T, P	↑	dn, A/R					Langer *et al.*, 1992; Roussel *et al.*, 1994; Carbone *et al.*, 2004b
	PU.1			T	↓	OE	T	wt, del	↓	OE, del	Kihara-Negishi *et al.*, 2001

IV.A.16	Sp1	E, C, F, M	IV, S, C, P				T, IV, M	wt, del, P, H	↑	P, OE, del	Nishikura, 1986; Asselin *et al.*, 1989; Snyder *et al.*, 1991; DesJardins and Hay, 1993; Majello *et al.*, 1995, 1997; Geltinger *et al.*, 1996; Michelotti *et al.*, 1996a; Miller *et al.*, 1996; Sakatsume *et al.*, 1996; Vaquero and Portugal, 1998; Wittekindt *et al.*, 2000; Pei, 2001; Liu *et al.*, 2006a; Wierstra and Alves, 2007a
	Sp3[k]	E, F	IV, S, C				T	wt, del	↓	OE	Majello *et al.*, 1995; Geltinger *et al.*, 1996
IV.A.17	FOXM1c	E, C	IV, S, C	T	↑	tg	T	wt, del, P, H	↑	OE, dn, del	Ye *et al.*, 1999; Wang *et al.*, 2001a; Wierstra and Alves, 2006d, 2007a,b
IV.A.18	Blimp-1	E, C, M, OC	IV, S, C, P	T, P	↓	OE, dn, KO	T S	wt, del, P wt, del	↓ ↓	OE, del	Kakkis and Calame, 1987; Kakkis *et al.*, 1989; Numoto *et al.*, 1993; Lin *et al.*, 1997, 2000; Zou *et al.*, 1997b; Knödel *et al.*, 1999; Chang *et al.*, 2000; Yu *et al.*, 2000; Györy *et al.*, 2003; Tamura *et al.*, 2003; Horsley *et al.*, 2006

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
IV.A.19	PLZF	E, C	S, C, P	T	↓	OE	T	wt, del, P	↓	OE	McConnell *et al.*, 2003
IV.A.20	Ovol1	E, C	IV, S, P	T	↓	KO	T	wt, del, P	↓	OE, del, KO	Nair *et al.*, 2006
IV.A.21	MAZ[l] (*ME1a2*)	E, SW	S, C				T	wt, del, P	↓	OE	Pyrc *et al.*, 1992; Izzo *et al.*, 1999
	MAZ[l] (*ME1a1*)	E, F, M, FBA, SW	IV, S, C, P				T S	wt, P wt, del	↑ ↑	OE, dn OE	Bossone *et al.*, 1992; Pyrc *et al.*, 1992; DesJardins and Hay, 1993; Komatsu *et al.*, 1997; Marcu *et al.*, 1997
	MAZ[l] (*CT*)	E	IV								DesJardins and Hay, 1993
	MAZ[l] (*att*)	E	IV, C								Bossone *et al.*, 1992
IV.A.22	NFATc1	D	P	P	↑	OE, ca, RNAi	T	wt, del, P	↑	OE, RNAi	Neal and Clipstone, 2003; Buchholz *et al.*, 2006

IV.A.23	MBP-1	E, F, FBA, SW, FRET	IV, S, C, P	T, P	↓	OE	T S	wt, del wt	↓ ↓	OE, del OE, del	Ray and Miller, 1991; Chaudhary and Miller, 1995; Ray, 1995; Ray *et al.*, 1995; Ray and Steele, 1997; Ghosh *et al.*, 1999a,c, 2001; Feo *et al.*, 2000; Subramanian and Miller, 2000; Aoki *et al.*, 2006
	α-Enolase	E	IV, S, C, P								Feo *et al.*, 2000; Subramanian and Miller, 2000
IV.A.24	CTCF	E, C, M, MCA	IV, S, C, P	P	↓	OE, RNAi	T S	wt, P P	↓ ↓	OE, del	Filippova *et al.*, 1996, 2002; Burcin *et al.*, 1997; Pérez-Juste *et al.*, 2000; Klenova *et al.*, 2001; Gombert *et al.*, 2003; Lutz *et al.*, 2003; Qi *et al.*, 2003; Ishihara *et al.*, 2006
IV.A.25	TR/RXR	E	IV, S, C, P	T, CH	↓	A/R	T	wt, del, P, H	↓	A/R	Pérez-Juste *et al.*, 2000; Lutz *et al.*, 2003; Lemkine *et al.*, 2005
IV.A.26	MIBP1 *MIBP1* (*MIE1*)[m]	E	IV, S, C, P				T T	wt, del wt, del, P, H	↓ ↓	OE	Fukuda *et al.*, 2002 Reinhold *et al.*, 1995; Blake *et al.*, 1996; Chen *et al.*, 2000b; Itkes *et al.*, 2000; Zajac-Kaye *et al.*, 2000

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter: Method[a]	Comment[b]	Regulation of endogenous *c-myc* expression: Expression[c]		Manipulation of transcription factor[d]	Regulation of *c-myc* promoter: Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	References
	RFX1 (*MIE1*)[m]	E	S, C, P				T	wt, del, P, H	↓		Reinhold *et al.*, 1995; Blake *et al.*, 1996; Chen *et al.*, 2000b; Itkes *et al.*, 2000; Zajac-Kaye *et al.*, 2000
	HOXB4 (*MIE1*)[m]	E	IV, S, C				T	wt, del	↓		Pan and Simpson, 1999
	(*MIE1*)	E, F, EPA, SW	C, P								Zajac-Kaye *et al.*, 1988, Zajac-Kaye and Levens, 1990; Yu *et al.*, 1993; Pan *et al.*, 1996
	(*MIE2*)	E, EPA	C				T	H	↓		Yu *et al.*, 1993; Pan and Simpson, 1999; Itkes *et al.*, 2000
	(*MIE3*)	E, M, EPA	C, P				T	del	↓		Yu *et al.*, 1993; Pan and Simpson, 1999
	(*MIE2/3*)						T	del	↓		Yu *et al.*, 1993; Pan and Simpson, 1999
	MIBP1 (*5′MIF*)[m]	E	S, C								Itkes *et al.*, 2000
	RFX1 (*5′MIF*)[m]	E	S, C								Itkes *et al.*, 2000
IV.A.27	STAT1	E	S, C	T	↓	A/R, del	T	wt, del	↓	A/R	Ramana *et al.*, 2000
IV.A.28	STAT4	E, F	S, C, P				T	P, H	↑	A/R	Grigorieva *et al.*, 2000

IV.A.29	NFATc1	D	P	P	↑	OE, ca, RNAi	T	wt, del, P	↑	OE, RNAi	Neal and Clipstone, 2003; Buchholz *et al.*, 2006
IV.A.29	CSL	E, C	IV, C, P				T	wt, del, P, H	↑	ca	Satoh *et al.*, 2004; Klinakis *et al.*, 2006; Weng *et al.*, 2006
	Notch1[b]	E, C	IV, P	T, P, RO, CH	↑	ca, RNAi, ca in tg, A/R	T	wt	↑	ca	Rao and Kadesch, 2003; Satoh *et al.*, 2004; Klinakis *et al.*, 2006; Palomero *et al.*, 2006; Weng *et al.*, 2006
IV.A.30	KLF11	E, D	IV, P				T	wt, P, H	↓	OE	Buck *et al.*, 2006
IV.A.31	Smad1 (*SBE-A*)	E, C	S, C, P				T	wt, P	↓		Hu and Rosenblum, 2005
	Smad1 (*TBE-A*)[i]	E	S, C, P								Hu and Rosenblum, 2005
	Smad1 (*SBE-A*, *TBE-A*)[t]			T, P	↑	RNAi, A/R	T	wt, P	↑	RNAi, A/R	Hu *et al.*, 2003; Hu and Rosenblum, 2005
	TCF-4 (*SBE-A*)[i]	E	S, C, P								Hu and Rosenblum, 2005
	β-Catenin (*SBE-A*)[t]	E	S, C, P								Hu and Rosenblum, 2005
IV.A.32	Smad4 (*near TBE1*)[n]	D, C	P	T	↑	RNAi					Lim and Hoffmann, 2006

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter			References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]	Manipulation of transcription factor[d]	
IV.B.1	ER	C		T, P, RO	↑	RNAi, A/R	T	wt, del ↑	OE, del, A/R	Dubik *et al.*, 1988; Dubik and Shiu, 1992; Shang *et al.*, 2000; Shang and Brown, 2002; Jiang *et al.*, 2004; Liu and Bagchi, 2004; Zhang *et al.*, 2004; DeNardo *et al.*, 2005; Keeton and Brown, 2005; Laganière *et al.*, 2005; Park *et al.*, 2005; Carroll *et al.*, 2006; Cheng *et al.*, 2006; Oxelmark *et al.*, 2006
	AR	C		T	↑	A/R				Silva *et al.*, 2001; Amir *et al.*, 2003; Zhang *et al.*, 2004

IV.B.2	p53	C		T, P	↓	OE, dn, KO, A/R	T, IV	wt, del	↓	P, OE, del	Moberg *et al.*, 1992b; Levy *et al.*, 1993; Ragimov *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Frazier *et al.*, 1998; Kartasheva *et al.*, 2003; Ho *et al.*, 2005; Krieg *et al.*, 2006; Wei *et al.*, 2006
	Tumor-derived p53 mutants			T	↑	OE	T	wt, del, H	↑	OE, del	Frazier *et al.*, 1998
	p73			T	↓	OE					Kartasheva *et al.*, 2003
IV.B.3	GATA-1	C		T	↓	OE					Rylski *et al.*, 2003
IV.B.4	C/EBPβ	C		T	↓	OE, del	T	wt	↓	dn	Sebastian *et al.*, 2005; Berberich-Siebelt *et al.*, 2006; Gomis *et al.*, 2006a
IV.B.5	ID2	C									Rodriguez *et al.*, 2006
IV.B.6	ARID1A	C		T, P	↓	RNAi					Nagl *et al.*, 2006
IV.B.7	Pitx2	C					T	wt	↑	A/R	Baek *et al.*, 2003
IV.C.1	FOXO3a						T	wt	↓	ca	Dominguez-Caceres *et al.*, 2004
IV.C.2	Mxi1			T, P	↓	OE	T	wt, del, P	↓	OE, del	Lee and Ziff, 1999; Luo *et al.*, 2004
	USF						T	wt, del	↑	OE	Lee and Ziff, 1999
IV.C.3	HOXB4			T	↑	OE	T	wt	↑	OE	Satoh *et al.*, 2004
IV.C.4	BMAL1/NPAS2			T	↓	A/R	T	wt, del	↓	OE, A/R	Fu *et al.*, 2002

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Table I (*continued*)

Section	Transcription factor (*or binding site*)	Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
IV.C.5	STAT5			T, P, CH	↑	OE, dn, ca, del, A/R					Lord *et al.*, 2000; Nosaka *et al.*, 1999; Hoover *et al.*, 2001; Moon and Nelson, 2001; Moon *et al.*, 2004a; Sugimoto *et al.*, 2003
IV.C.6	ICAP-1						T	wt	↑	OE, del	Fournier *et al.*, 2005
IV.C.7	MAZR						T	wt, del	↑	OE, del	Kobayashi *et al.*, 2000
IV.C.8	Mel-18			T, P	↓	OE, RNAi, tg, KO	T	wt, del	↓	OE	Tetsu *et al.*, 1998; Guo *et al.*, 2007

[a] *Method (method used to demonstrate binding to the c-myc promoter):* E = EMSA = electrophoretic mobility shift assay; D = DNAP = DNA precipitation assay; C = ChIP = chromatin immunoprecipitation assay; F = (DNAse I) footprinting analysis; M = methylation interference analysis; NMR = NMR (nuclear magnetic resonance) structure; MCA = missing contact analysis; FBA = filter binding assay; EPA = exonuclease protection assay; SW = Southwestern blot analysis; FRET = real-time FRET (fluorescence resonance energy transfer) assay; PP = potassium permanganate modification; OC = OP/Cu (orthophenanthroline/copper) footprinting; IB = immunoblotting of protein–DNA complexes; IP = immunoprecipitation of labeled oligonucleotides.

[b] *Comment:* IV = *in vitro* = (partially) purified transcription factor; S = supershift experiments; C = competition experiments; P = binding site was point-mutated (or deleted).

[c] *Expression:* T = transcript = mRNA = endogenous mRNA level affected; P = protein = endogenous protein level affected; RO = nuclear run-on or run-off transcription; CH = effect was detected in the presence of cycloheximide.

[d] *Manipulation of transcription factor:* P = purified transcription factor; OE = overexpression of wild type; dn = dominant-negative form; ca = constitutively active form; del = analyzed with deletion (or/and point) mutants of the transcription factor; RNAi = RNA interference = knockdown (although mechanistically different siRNA, shRNA and antisense RNA approaches are listed as RNAi); tg = transgenic cells/mice; KO = knockout cells/mice (cancer cell lines deficient in transcription factor); A/R = activation/repression of the transcriptional activity of the transcription factor (see text for details).

[e] *Reporter contruct:* T = transiently transfected; S = stably transfected; E = episomal vector; IV = *in vitro* transcription; M = microinjection into *Xenopus laevis* oocytes.

[f] *Manipulation of binding site:* wt = "wild type" *c-myc* promoter; del = analyzed with deletion mutants of *c-myc* promoter; P = binding site was point-mutated; H = binding site upstream (or downstream) of heterologous core promoter.

[g] ME1a1/CT-I_2, ME1a2, and the "E2F" binding site each represent overlapping binding sites for several transcription factors.

[h] β-Catenin, γ-catenin, and Notch1, which do not bind to DNA, serve as coactivators for their DNA-binding partner transcription factors TCF-4, LEF-1, or CSL, respectively, that act as repressors in the absence of their coactivators.

[i] In addition to binding to their own genuine binding site, Smad1 and Smad3 were also found at a β-catenin/TCF-4 binding site (TBE3 = TBE-A) while vice versa TCF-4 and β-catenin were found at a Smad1 binding site (SBE-A), too. It is indicated in brackets, whether the data for Smad1, Smad3, TCF-4, and β-catenin were obtained at their own genuine binding site or at the additional site, where Smads and β-catenin/TCF-4/LEF-1 associate. Own genuine binding sites: SBE-A for Smad1; TIE for Smad3; TBE1, TBE2, TBE3 for TCF-4 and β-catenin. Additional sites: TBE-A for Smad1; TBE3 for Smad3; SBE-A for TCF-4 and β-catenin.

[j] Albert *et al.* (2001) point-mutated the E2F binding site in the context of the wild-type *c-myc* promoter with an otherwise intact P2 promoter region. Krumm *et al.* (1995) deleted the E2F binding site in the context of a deletion mutant of the *c-myc* P2 promoter already lacking the P2 TATA-box and both Inr of the P2 promoter.

[k] Sp3 slightly represses the human *c-myc* promoter in human HeLa cervix carcinoma cells, which contain endogenous Sp1 (Majello *et al.*, 1995). Sp3 was reported to transactivate the human *c-myc* promoter in Sp1-deficient *Drosophila* Schneider SL2 cells (Majello *et al.*, 1997), but coexpression of exogenous Sp1 restored the ability of Sp3 to repress the Sp1-mediated activity of the human *c-myc* promoter in these cells (Majello *et al.*, 1995, 1997).

[l] MAZ binds to ME1a1/CT-I_2, ME1a2, the CT-element (CT), and the *c-myc* attenuator (att) region within exon 1.

[m] MIBP1, RFX1, and HOXB4 bind to MIE1. In addition, MIBP1 and RFX1 bind also to 5′MIF, which is positioned 5′ of the P1 transcription start site.

[n] In addition to binding to the TIE, Smad4 binds also to a binding site adjacent to the TCF-4/LEF-1 binding site TBE1, where it associates with LEF-1. It is indicated in brackets whether the data for Smad4 were obtained at the TIE (TIE) or at this additonal Smad4 binding site adjacent to TBE1 (near TBE1).

transcriptional control (Marcu *et al.*, 1992; Siebenlist *et al.*, 1984). Well-characterized examples for deregulated *c-myc* transcription are Burkitt's lymphoma where a typical shift in promoter usage from P2 to P1 coincides with a loss of the block to transcriptional elongation (see Section IV.F).

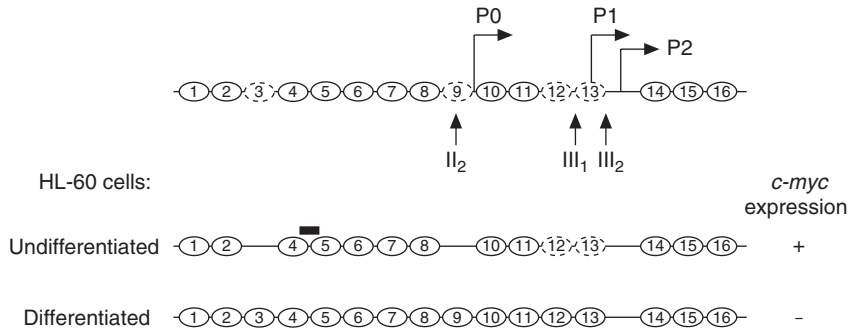
D. Chromatin Structure

The integrity of the long range chromosomal domain enveloping the *c-myc* gene appears to be necessary for correct control of *c-myc* transcription *in vivo* (Levens *et al.*, 1997; Liu and Levens, 2006; Potter and Marcu, 1997). So, reconstructed *c-myc* genes, containing 50 kb of continuous DNA sequences including the promoter, the three exons and approximately 20 kb of each 5′ and 3′ flanking sequences, failed to recapitulate many, if not most, features of the normal *c-myc* transcriptional control (Lavenu *et al.*, 1994; Mautner *et al.*, 1996).

Chromatin remodeling provides an additional level for control of the *c-myc* promoter. Several components of the mammalian ATP-dependent SWI/SNF nucleosome remodeling complexes BAF and PBAF, for example their ATPase subunit BRG-1 (Brahma-related gene-1), were found to be associated with the *c-myc* promoter indicating their implication in control of *c-myc* transcription. In addition, several transcription factors were reported to recruit different HAT and HDAC complexes to the *c-myc* promoter and to influence its histone acetylation status. Also association of several subunits of a MLL/SET1-type HMT (histone methyltransferase) complex, for example, of the HMT MLL2, with the *c-myc* promoter has been described suggesting its involvement in regulation of *c-myc* transcription. Regulation of *c-myc* transcription by chromatin remodeling is complex and not completely understood.

Mapping of nucleosomes on active *c-myc* genes in proliferating undifferentiated promyelocytic HL-60 leukemia cells and on inactive *c-myc* genes in DMSO-treated differentiated HL-60 cells revealed that the nucleosomes 3 (upstream region), 9 (P0 promoter region), 12 (directly upstream of P1 promoter), and 13 (at P1 promoter) were present only on the inactive *c-myc* genes (Fig. 3A; Pullner *et al.*, 1996). In contrast, the P2 promoter was never found to be occupied by a nucleosome (Albert *et al.*, 1997; Michelotti *et al.*, 1996b; Pullner *et al.*, 1996). This difference between the promoters P1 and P2 points to different modes of their repression during DMSO-induced HL-60 cell differentiation. Like the absence of the nucleosomes, the presence of the corresponding DNAse I-hypersensitive sites II_2 (P0 promoter region), III_1 and III_2 (P1 promoter region) correlated with the activity of the *c-myc*

A **Nucleosomal structure of active and inactive *c-myc* genes**



B **Hierarchal control of *c-myc* transcription**

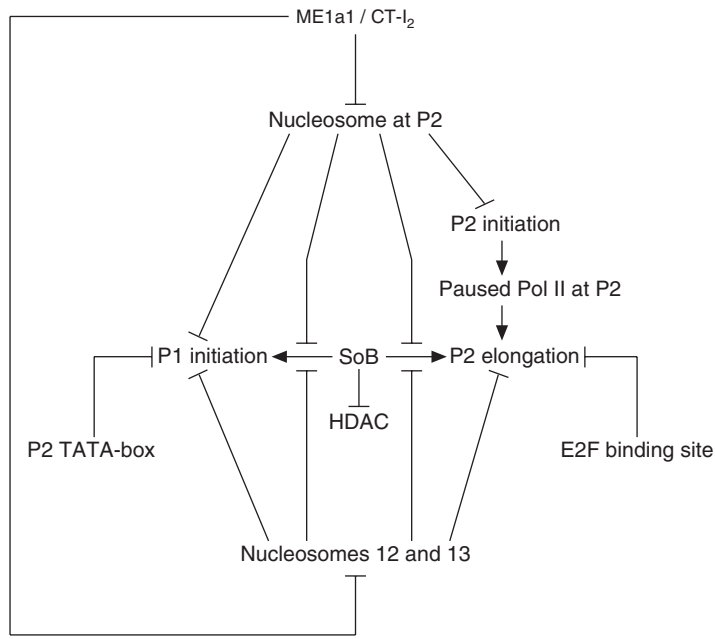


Fig. 3 (A) Nucleosomal structure of active and inactive human *c-myc* genes. Adapted from Pullner *et al.* (1996, Fig. 8, p. 31456). Shown are the nucleosomes 1–16, the promoters P0, P1, and P2, the DNAse I-hypersensitive sites II_2, III_1, and III_2 and the nuclease resistant nucleosomal spacer region (black box) between nucleosomes 4 and 5. Dashed nucleosomes were identified only in submolar amounts (Pullner *et al.*, 1996). Human promyelocytic HL-60 leukemia cells were differentiated toward granulocytes with DMSO (Pullner *et al.*, 1996). The activity of the *c-myc* gene is indicated (+ = expressed; – = not expressed). The DNAse I-hypersensitive sites II_2, III_1, and III_2 were only present on active *c-myc* genes of undifferentiated HL-60 cells (Pullner *et al.*, 1996). (B) Hierarchal control of transcription from the *c-myc* P1 and P2 promoters. See text (Section III.D) for a description. SoB = sodium butyrate.

promoter (Bentley and Groudine, 1986a; Dyson *et al.*, 1985; Eick and Bornkamm, 1986; Pullner *et al.*, 1996; Siebenlist *et al.*, 1988). The spacer region between nucleosomes 4 and 5 was found to be nuclease resistant only on active *c-myc* genes in undifferentiated proliferating HL-60 cells (Fig. 3A; Pullner *et al.*, 1996). Similarly, it was protected from nuclease cleavage in serum-stimulated *c-myc* expressing human Hs68 primary fibroblasts but cleaved by micrococcal nuclease in *c-myc* non-expressing IMR32 neuroblastoma cells (Liu *et al.*, 2006a). Furthermore, in two B-cell lines with stably transfected episomal *c-myc* genes it was nuclease resistant in the RF266C3 cells capable of expressing *c-myc* but nuclease sensitive in the MA76 cells, in which *c-myc* is silent (Michelotti *et al.*, 1996b). This nucleosomal spacer region colocalizes with the FUSE (Pullner *et al.*, 1996) that is single stranded, if *c-myc* is expressed, but duplex DNA, if *c-myc* is not expressed (Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b). The single-stranded FUSE, but not its duplex form, is bound by the single-strand-specific transcriptional activator FBP (FUSE binding protein), whose expression profile parallels the one of c-Myc so that expression of both FBP and c-Myc decreases with similar kinetics during induced HL-60 cell differentiation (Bazar *et al.*, 1995a,b; Braddock *et al.*, 2002; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b). Since FBP binds to the FUSE, only if *c-myc* is expressed (Avigan *et al.*, 1990; Bazar *et al.*, 1995a; Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b), binding of FBP to the FUSE could explain the nuclease resistance of the spacer region between nucleosomes 4 and 5 only on active *c-myc* genes (Pullner *et al.*, 1996). Alternatively, this nuclease resistance could reflect movement and heterogeneous positioning of nucleosome 5 (Liu *et al.*, 2006a).

In *c-myc* non-expressing IMR23 neuroblastoma cells, the double-stranded FUSE (Michelotti *et al.*, 1996b) is wrapped over nucleosome 5 and not occupied by FBP or FIR (FBP interacting repressor) (Liu *et al.*, 2006a). Vice versa, the FUSE is nucleosome free and occupied by FBP and FIR in continuously *c-myc* expressing U2OS osteosarcoma and SW13 adrenal cortical carcinoma cells (Liu *et al.*, 2006a). This chromatin remodeling at the FUSE is BRG-1-independent because SW13 cells are BRG-1-deficient.

A hierarchical control regulates P1 transcription, binding of paused Pol II complexes to the P2 promoter and processive transcriptional elongation by these P2-initiated Pol II complexes (Fig. 3B; Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996): At episomal *c-myc* promoters, P1 transcription and transcriptional elongation by P2-initated Pol II complexes are activated by the HDAC inhibitor SoB (sodium butyrate) and thus probably repressed by HDAC (Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996; Strobl *et al.*, 1993; Wolf *et al.*, 1995). This effect on transcription elongation at P2 depends at

least in part on the E2F-binding site strongly suggesting the involvement of E2F-pocket protein–HDAC complexes (Albert *et al.*, 2001; Harbour and Dean, 2000). In contrast, the effect on P1 transcription depends at least in part on the P2 TATA-box whereas the E2F-binding site has no influence on transcription from P1 (Albert *et al.*, 2001). It has to be noted that the E2F-binding site of the *c-myc* promoter colocalizes with the binding sites for ETS-1/2, STAT3 (signal transducer and activator of transcription 3), NFATc1 (nuclear factor of activated T cells c1), KLF11 (Krüppel-like factor 11), and METS (mitogenic Ets transcriptional suppressor) (Fig. 4) so that the effects of mutation of the E2F site could be caused by changes in E2F, ETS-1/2, STAT3, NFATc1, KLF11, or METS binding. The nucleosomes 12 and 13 are present at inactive, uninducible *c-myc* promoters whereas both active and inactive, but inducible *c-myc* promoters lack them (Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996). These two nucleosomes repress P1 transcription as well as transcription elongation by P2-initiated Pol II complexes and prevent induction of transcription from P1 and P2 by SoB (Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996). However, the absence of these two nucleosomes does not automatically result in activation of *c-myc* expression because both P1 transcription and transcriptional elongation by P2-initiated Pol II complexes still require induction by SoB at episomal *c-myc* promoters (Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996). Nevertheless, binding of paused Pol II complexes to the P2 promoter requires neither the absence of nucleosomes 12 and 13 nor induction by SoB so that Pol II complexes pausing at the P2 promoter are also found at inactive *c-myc* promoters (Albert *et al.*, 1997, 2001). The nucleosomes 12 and 13 seem to be under the control of the element ME1a1. Deletion of ME1a1 results in their appearance so that activation of *c-myc* transcription should depend on ME1a1 to eliminate them (Albert *et al.*, 2001). Moreover, ME1a1 seems to control an additional nucleosome that appears at the P2 promoter if ME1a1 is deleted. The presence of this additional nucleosome results in complete inhibition of the *c-myc* promoter, that is, inhibition of Pol II binding to the P2 promoter and thus inhibition of transcriptional elongation by P2-initated Pol II complexes, inhibition of P1 transcription and prevention of SoB-induced transcription from P1 and P2 (Albert *et al.*, 2001). Consequently, ME1a1 appears to function as a major regulator for the global chromatin structure at the *c-myc* promoter and thus for *c-myc* transcription (Albert *et al.*, 2001). In summary, activation of *c-myc* expression should include ME1a1-mediated elimination of the additional nucleosome at P2 allowing binding of promoter proximal paused Pol II complexes to P2 as well as ME1a1-mediated elimination of the nucleosomes 12 and 13 allowing SoB induction of both P1 transcription (at the level of initiation) and processive transcriptional elongation by P2-initiated Pol II complexes (Fig. 3B; Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996).

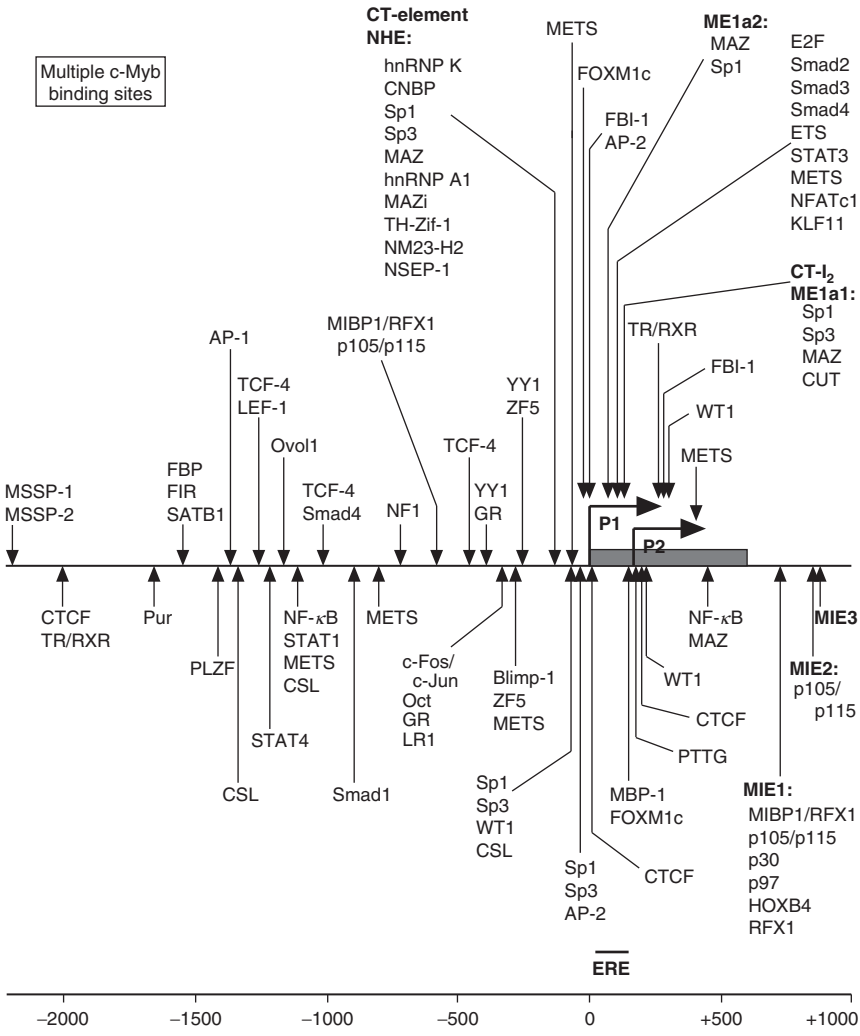


Fig. 4 Transcription factor binding sites in the *c-myc* promoter. Shown are transcription factors that bind to human and/or murine *c-myc* (normal), special sequence elements (bold), the promoters P1 and P2 (bold arrows) and exon 1 (gray box). For illustrative purposes and to give an overview neighboring binding sites are sometimes grouped at one arrow, but this does not necessarily indicate their overlap or adjacency. CT-I_2 is the human homolog of murine ME1a1. The transcription start site of the P1 promoter is indicated as +1. The estrogen response element (ERE) of the *c-myc* promoter was mapped to a 116-bp region positioned at +25 to +141 relative to the P1 transcription start (+1) (Dubik and Shiu, 1992). This region lacks any palindromic ERE consensus sequence (GGTCA-NNN-TGACC), but contains one or several imperfect ERE half sites (e.g. GGGCA) (Dubik and Shiu, 1992; Liu and Bagchi, 2004). FBP and FIR bind to the FUSE (see Section IV.A.7). E2F and Smad2/3 bind to the TIE (see Sections IV.A.2 and IV.A.3). The order of the three TCF-4 binding sites is 5′-TBE3-TBE1-TBE2–3′ (see Section

E. c-Myc Autosuppression

The protein c-Myc represses its own promoter in a concentration-dependent manner at the level of transcription initiation (Table I; Cleveland *et al.*, 1988; Facchini *et al.*, 1994, 1997; Grignani *et al.*, 1990; Lombardi *et al.*, 1987; Lucas *et al.*, 1993; Luo *et al.*, 2004; Mao *et al.*, 2003; Penn *et al.*, 1990a,b,). In accordance, the *c-myc* promoter is *in vivo* occupied by c-Myc itself (Mao *et al.*, 2003; Wang *et al.*, 2006). This c-Myc autosuppression provides an additional important mechanism for control of *c-myc* expression. It requires the heterodimerization of c-Myc with Max, but does not occur via a c-Myc/Max-specific E-box because the targeted *c-myc* promoter region lacks any such CACGTG sequence (Facchini *et al.*, 1997; Mao *et al.*, 2003). c-Myc represses the major *c-myc* P2 promoter by a mechanism that involves the Inr element(s) and the E2F-binding site because repression was completely lost if both the E2F site and the two Inr were mutated, whereas it remained normal if single P2 promoter elements were mutated or if only the intact Inr at the transcription start site plus the P2 TATA-box were present (Facchini *et al.*, 1997; Luo *et al.*, 2004). The same results were obtained for repression of the *c-myc* promoter by p107 (Dagnino *et al.*, 1995; Luo *et al.*, 2004). Since the c-Myc autosuppression was lost in p107-null cells, p107 is essential for c-Myc autosuppression, in contrast to the two other pocket proteins RB and p130 (Luo *et al.*, 2004). As p107 binds to both c-Myc and E2F (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994; Zhu *et al.*, 1995), it is suggested that the c-Myc autosuppression includes binding of c-Myc/Max to the Inr that may be mediated by an Inr binding protein, binding of E2F to the E2F site and interaction of p107 with both c-Myc and E2F (Luo *et al.*, 2004).

Lucas *et al.* (1993) reported that c-Myc can also repress the isolated *c-myc* P1 promoter.

This negative feedback regulation of *c-myc* represents a global homeostatic control mechanism, which seems to be critical for normal cell growth control as a "safety valve," because the c-Myc autosuppression is lost in many transformed or tumor-derived cell lines whereas it is operative in

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IV.A.1). The order of the five Sp1 binding sites is 5′-CT-element-distal–44-ME1a2-CT-I_2/ME1a1–3′ (see Section IV.A.16). The order of the two NF-κB binding sites is 5′-URE–IRE-3′ (see Section IV.A.13). The *c-myc* gene possesses multiple c-Myb binding sites (more than 10 high affinity sites and more than 10 low or intermediate affinity sites) that are scattered throughout the promoter region and exon 1 (Nakagoshi *et al.*, 1992; Zobel *et al.*, 1991). The *c-myc* promoter is occupied by the AhR (aryl hydrocarbon receptor) and possesses six potential AhR response elements (not shown; Yang *et al.*, 2005). In ChIP assays, the P2 promoter region, where YY1 may bind to the Inr, was found to be occupied by YY1 (not shown; Liu *et al.*, 2006a). Smad1 occupied also the proximal promoter region in ChIP assays (Hu and Rosenblum, 2005) suggesting that it may bind to the Smad binding site near the P2 transcription start, too.

primary and immortalized cell lines retaining contact inhibition of growth (Facchini and Penn, 1998; Facchini *et al.*, 1994, 1997; Grignani *et al.*, 1990; Penn *et al.*, 1990a; Potter and Marcu, 1997). This loss of autoregulation is one mechanism that contributes to oncogenic *c-myc* activation (Facchini *et al.*, 1994, 1997; Grignani *et al.*, 1990).

Autoregulation extends beyond c-Myc itself as the three members of the Myc family c-Myc (MYC), N-Myc (MYCN), and L-Myc (MYCL1) repress reciprocally their expression (Cleveland *et al.*, 1988; DePinho *et al.*, 1991; Potter and Marcu, 1997; Rosenbaum *et al.*, 1989).

Using an unbiased whole-genome mapping strategy called ChIP-PET (chromatin immunoprecipitation coupled with pair-end ditag sequencing analysis), Zeller *et al.* (2006) identified an additional c-Myc binding site in an intron of the human *c-myc* gene.

IV. CONTROL OF THE *c-MYC* PROMOTER

A. Transcription Factors That Directly Bind to the *c-myc* Promoter and Their Partners

1. TCF-4 AND LEF-1

The *c-myc* promoter possesses three TCF-4 (T-cell factor 4) binding sites that are named TBE1 (TCF-4 binding element 1), TBE2 (positioned most 3′), and TBE3 (positioned most 5′) (Fig. 4; Table I; He *et al.*, 1998; Hu and Rosenblum, 2005; Sasaki *et al.*, 2003). For TBE3 also binding of LEF-1 (lymphoid enhancer factor 1) was demonstrated (Sasaki *et al.*, 2003; Sierra *et al.*, 2006). TCF/LEF family transcription factors possess a HMG box as DBD (DNA-binding domain), but lack a TAD (transactivation domain) (Hurlstone and Clevers, 2002). β-catenin and γ-catenin (plakoglobin), which vice versa lack a DBD but possess a TAD, bind to and serve as coactivators for TCF/LEF transcription factors (Barker *et al.*, 2000). Consistently, β-catenin and γ-catenin activate the *c-myc* promoter through its TCF-4 binding sites (Fig. 5A; He *et al.*, 1998; Kolligs *et al.*, 2000; Sasaki *et al.*, 2003; Sierra *et al.*, 2006; Toualbi *et al.*, 2007; Wolf *et al.*, 2002). Induction of *c-myc* transcription by serum stimulation results in TCF-4 binding to the *c-myc* promoter, which is not occupied by TCF-4 in quiescent cells virtually not expressing *c-myc* (Fig. 6; Liu *et al.*, 2006a).

The *c-myc* promoter is a target of the Wnt signaling pathway (Fig. 5A; He *et al.*, 1998; Kolligs *et al.*, 2000; Pinto *et al.*, 2003; Sasaki *et al.*, 2003; Sierra *et al.*, 2006; van de Wetering *et al.*, 2002; Wolf *et al.*, 2002). This pathway is important for embryonic development and proliferation during adult tissue

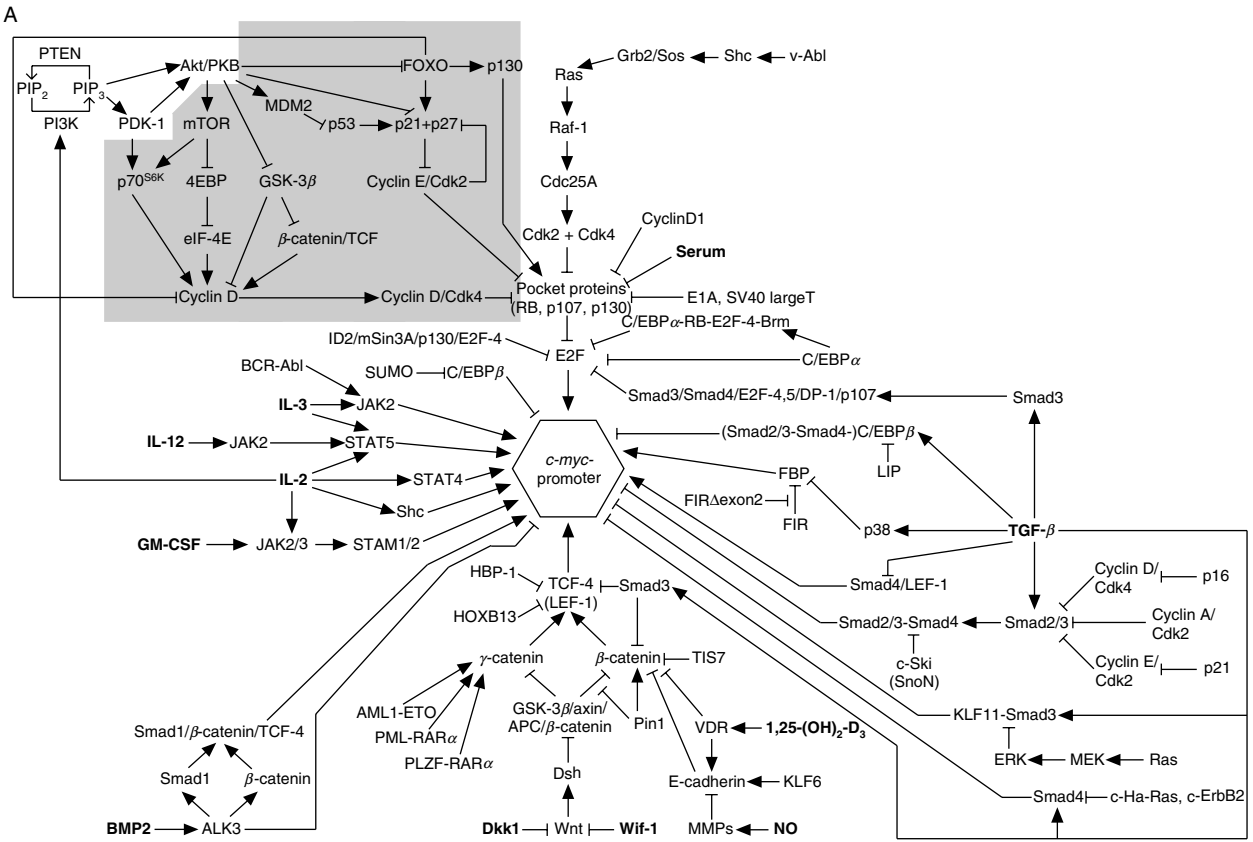
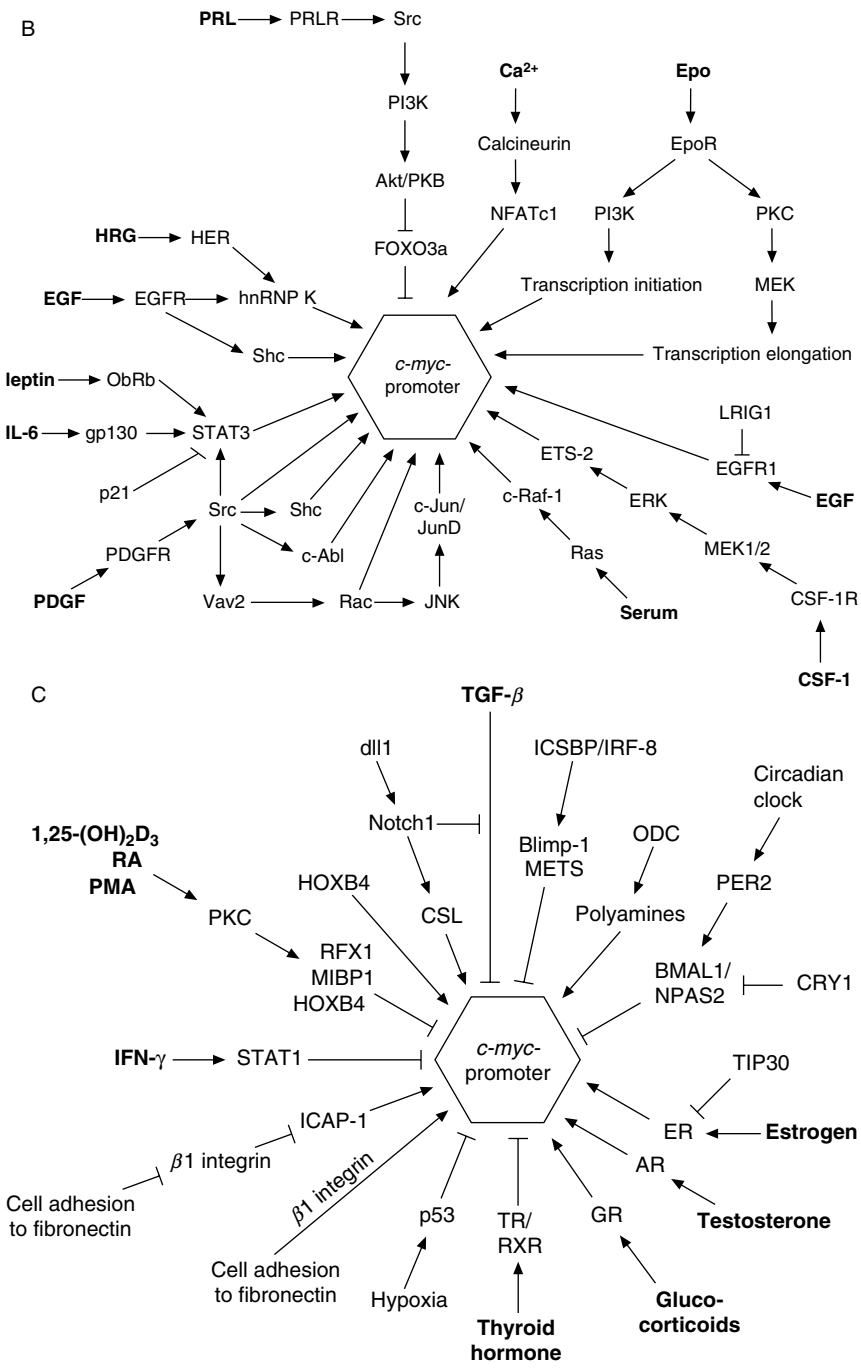


Fig. 5 (*continued*)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PRL
PRLR
Src
PI3K
Akt/PKB
FOXO3a
HRG
HER
EGF
EGFR
hnRNP K
Shc
leptin
ObRb
IL-6
gp130
STAT3
p21
Src
Shc
PDGFR
PDGF
c-Abl
Vav2
Rac
JNK
c-Jun/
JunD
c-Raf-1
Ras
Serum
ERK
MEK1/2
CSF-1R
CSF-1
ETS-2
LRIG1
EGFR1
EGF
c-myc-
promoter
Transcription initiation
Transcription elongation
Ca2+
Calcineurin
NFATc1
Epo
EpoR
PI3K
PKC
MEK
C
TGF-β
dll1
Notch1
CSL
ICSBP/IRF-8
Blimp-1
METS
ODC
Polyamines
Circadian
clock
PER2
BMAL1/
NPAS2
CRY1
1,25-(OH)2D3
RA
PMA
PKC
HOXB4
RFX1
MIBP1
HOXB4
IFN-γ
STAT1
ICAP-1
β1 integrin
Cell adhesion
to fibronectin
β1 integrin
Cell adhesion
to fibronectin
p53
Hypoxia
TR/
RXR
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hormone
GR
Gluco-
corticoids
AR
Testosterone
ER
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TIP30
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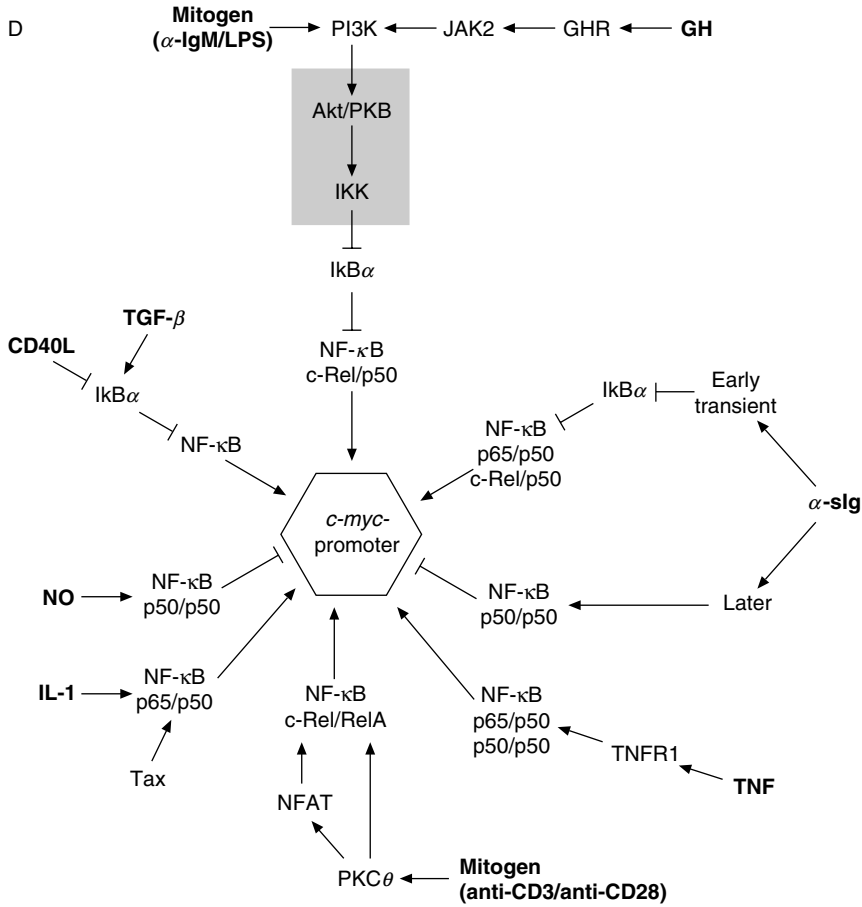

Fig. 5 Signaling pathways controling the *c-myc* promoter. Signaling pathways that regulate transcription of the human and/or murine *c-myc* promoter are shown as they were described so far. Thus some signaling routes, which are shown separately, may represent parts of the same signaling pathway whereas others may represent independent or alternative signaling pathways. The figure demonstrates that the complex regulation of the *c-myc* promoter implies many possibilities for crosstalk (not shown) between different signal transduction pathways. Redundancy in regulation of the *c-myc* promoter is obvious for TGF-β (A and D), IL-2 (A), PDGF (B), 1,25-$(OH)_2$-D_3 (A and C), and NO (A and D), which regulate *c-myc* transcription by targeting several different transcription factors that bind to the *c-myc* promoter. Growth factors, mitogens, cytokines, hormones, vitamines, ligands, etc. are depicted in bold type. (A) TGF-β-activated Smad3 inhibits transactivation of the *c-myc* promoter by β-catenin/TCF-4 (via TBE3). In contrast, transactivation of the *c-myc* promoter by LEF-1/β-catenin (via TBE3) is not inhibited by TGF-β-activated Smad3 (Sasaki *et al.*, 2003). The part of the signaling pathway shown in the gray box was not explicitely demonstrated for regulation of the *c-myc* promoter, but is based on a combination of the results of Ahmed *et al.* (1997), Brennan *et al.* (1997, 1999), Ghosh *et al.* (1999b), and Feng *et al.* (2000). It is supported by the current general knowledge

renewal (Barker *et al.*, 2000; Bienz and Clevers, 2000, 2003; Bienz, 2002, 2005; Brantjes *et al.*, 2002; Cadigan, 2002; Gregorieff and Clevers, 2005; Harris and Peifer, 2005; Henderson and Fagotto, 2002; Logan and Nusse, 2004; Moon *et al.*, 2004b; Nelson and Nusse, 2004; Polakis, 2000; Radtke and Clevers, 2005; Reya and Clevers, 2005; van Noort and Clevers, 2002; Willert and Jones, 2006; Xiong and Kotake, 2006). In the absence of Wnt signaling, β-catenin is targeted to a cytoplasmic multisubunit destruction complex, which includes APC (adenomatous polyposis coli), axin/conduction, GSK-3β (glycogen synthase kinase-3β), CK1α (casein kinase 1α), and PPA2 (protein phosphatase 2). Sequential phosphorylation of β-catenin within this complex first by CK1α and then by GSK-3β targets it for ubiquitination by the β-TrCP E3 ubiquitin ligase and subsequent proteolytic destruction by the 26S proteasome. APC triggers the phosphorylation of β-catenin by GSK-3β and thus the degradation of β-catenin. Wnt signaling, initiated by binding of Wnt ligands to transmembrane Frizzled receptors and mediated by Dsh (Disheveled), triggers a series of events which ultimately renders the APC/axin/GSK-3β destruction complex inactive so that β-catenin is no longer rapidly degraded, accumulates in the cytoplasm and translocates into the nucleus where it binds to TCF/LEF. Without β-catenin, TCF/LEF transcription factors act as transcriptional repressors, which recruit Groucho/TLE-1, HDAC1, CtBP, and other corepressors. Binding of β-catenin converts them into transcriptional activators. β-catenin that functions as coactivator of TCF/LEF transcription factors, binds to TBP as well as to other coactivators like p300/CBP, BRG-1, ISW1, CARM1, TIP49a/Pontin52, TIP49b/TIP48/Reptin, a MLL/SET1-type HMT complex, the PAF1 complex, and probably TRRAP/TIP60 and TRRAP/GCN5 HAT complexes. Dominant-negative forms of TCF-4 and TCF-1 and RNAi- or shRNA-mediated knockdown of TCF-4 and LEF-1 repressed the *c-myc* promoter and/or decreased the *c-myc* mRNA and/or protein expression in colon carcinoma (Chi *et al.*, 2003), non-small-cell lung cancer (Tong *et al.*, 2004), myeloid (Skokowa *et al.*, 2006), or kidney (inner medullary collecting duct; Hu and Rosenblum, 2005) cells, respectively. Downregulation (or absence) of LEF-1 correlated with reduced *c-myc* transcript levels in severe congenital neutropenia, a rare disorder of myelopoiesis (Skokowa *et al.*, 2006).

(see Section IV.E.5). Formation of a repressive complex of E2F-4, p130, mSin3A, and ID2 on the inactive *c-myc* promoter in quiescent cells is suggested by the results of Rodriguez *et al.* (2006; see text). PIP_3 = PtdIns(3,4,5)P_3 = phosphatidylinositol-3,4,5-triphosphate = lipid second messenger; PIP_2 = PtdIns(4,5)P_2 = phosphatidylinositol-4,5-bisphosphate. (B) Src = Src family tyrosine kinase. (D) The part of the signaling pathway shown in gray was not explicitely demonstrated for regulation of the *c-myc* promoter, but was suggested by the results of Jeay *et al.* (2001), Grumont *et al.* (2002), and Chandramohan *et al.* (2004). It is supported by the present general knowledge (Arsura *et al.*, 2000; Datta *et al.*, 1999; Kane *et al.*, 1999; Khwaja 1999; Ozes *et al.*, 1999; Romashkova and Makarov, 1999).

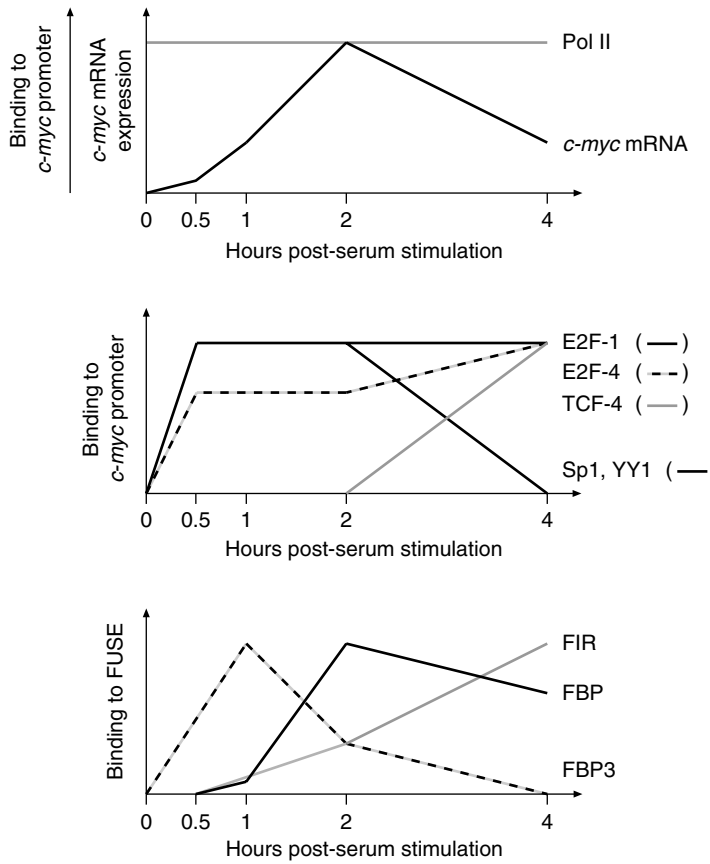

Fig. 6 *In vivo* occupancy of the *c-myc* promoter by different transcription factors in quiescent cells and during serum stimulation. Summarized are the results of sequential ChIP assays during serum stimulation of starved human Hs68 primary fibroblasts that have been performed by David Levens and coworkers (Chung *et al.*, 2006; Liu *et al.* 2006a). Note that the results of these two studies were combined into one figure on the basis that both studies revealed similar data for FBP (with the exception that each study lacked one time point).

Conversely, LEF-1 overexpression increased the endogenous *c-myc* mRNA expression in $CD34^+$ myeloid progenitor cells (Skokowa *et al.*, 2006). In contrast, *Lef1*$^{-/-}$ pro-B cells from fetal mouse liver displayed elevated *c-myc* mRNA levels (Reya *et al.*, 2000) indicating that TCF/LEF transcription factors are implicated in activation and repression of *c-myc* transcription. As target of the Wnt signaling pathway, the *c-myc* promoter is repressed by the tumor suppressor APC through its TCF-4 binding sites (Fig. 5A; He *et al.*, 1998; Liu *et al.*, 2004; Roose *et al.*, 1999; Shih *et al.*, 2000; Sierra *et al.*, 2006). The Wnt signaling pathway is often constitutively activated in

colon cancer by mutations that either inactivate APC and axin/conduction or stabilize and activate β-catenin (Fodde, 2002; Grady and Markowitz, 2002; Kinzler and Vogelstein, 1996; Morin, 1999; Polakis, 2000). Since APC is inactivated in most colorectal tumors the identification of *c-myc* as a target gene of Wnt signaling explained why *c-myc* is overexpressed in most colon cancers although genetic alterations of *c-myc* are rare in these tumors (He *et al.*, 1998; Kinzler and Vogelstein, 1996). Transgenic mice expressing stabilized, N-terminally truncated forms of β-catenin show upregulation of *c-myc* transcript levels compared to their wild-type littermates (Imbert *et al.*, 2001; Teulière *et al.*, 2004). Both β-catenin and γ-catenin are proto-oncogenes. c-Myc is required for transformation by γ-catenin while it is dispensable for transformation by β-catenin (Kolligs *et al.*, 2000).

Sierra *et al.* (2006) used ChIP (chromatin immunoprecipitation) assays to monitor the dynamic occupancy of the *c-myc* promoter, namely of TBE3, during induction of *c-myc* expression mediated by LiCl, a GSK-3β inhibitor, and during APC-induced repression of *c-myc* expression. Their study revealed constant occupancy of TBE3 by LEF-1 during both induction and repression of *c-myc*. LiCl-induced stabilization of β-catenin and thus activation of *c-myc* transcription resulted in association of β-catenin, RNAPII, Cdk9, Pygopus, Bcl-9/Lgs, p300, MLL2, Ash2, menin, APC and β-TrCP with the *c-myc* promoter and in dissociation of TLE-1 and GSK-3β from *c-myc* as well as in histone H3-K4-trimethylation at the *c-myc* promoter. Vice versa, APC-mediated degradation of β-catenin and thus shutoff of *c-myc* transcription led to dissociation of β-catenin, RNAPII, Cdk9, Pygopus, Bcl-9/Lgs, MLL2, Ash2, RbBP5 and Rpt6 from the *c-myc* promoter and to association of TLE-1, HDAC1, CtBP, YY1, APC, and β-TrCP with *c-myc* as well as to loss of histone H3-K4-trimethylation and histone H4-K8-acetylation at the *c-myc* promoter. The HMT MLL2, menin, Ash2, and RbBP5 are subunits of a MLL/SET1-type HMT complex that is recruited by β-catenin (Sierra *et al.*, 2006). MLL2 is important for *c-myc* mRNA expression and histone H3-K4-trimethylation by MLL2 might contribute to the β-catenin-mediated induction of *c-myc* transcription (Sierra *et al.*, 2006). Pygopus and Bcl-9/Lgs are required for nuclear retention of β-catenin and seem to function as its coactivators. TLE-1 and CtBP are corepressors of LEF-1 (Willert and Jones, 2006; Xiong and Kotake, 2006). This scenario, unexpected for APC, GSK-3β and β-TrCP, shows that APC does not only trigger the degradation of β-catenin but is also involved in the exchange of Wnt coactivator versus corepressor complexes at β-catenin/TCF/LEF target genes, which significantly precedes the drop in the β-catenin protein level that occurs as a result of its proteolytic destruction in the cytoplasm (Sierra *et al.*, 2006; Willert and Jones, 2006; Xiong and Kotake, 2006).

Human HT29 colorectal cancer cells, which express high levels of c-Myc, contain a truncated Class II mutant APC that is unable to degrade β-catenin

and to interact with CtBP (Sierra *et al.*, 2006). In these cells, the *c-myc* promoter was found to be constantly occupied by LEF-1, β-catenin, RNAPII, Cdk9, Pygosus, Bcl-9/Lgs, MLL2, Ash2, RbBP5 and Rpt6 as well as to be constantly H3-K4-trimethylated and H4-K8-acetylated (Sierra *et al.*, 2006). In contrast, TLE-1, HDAC1, CtBP, YY1, and β-TrCP were not observed at the *c-myc* promoter explaining at the molecular level the overexpression of *c-myc* in these colon cancer cells.

BRG-1, the ATPase of mammalian SWI/SNF complexes (Mohrmann and Verrijzer, 2005; Roberts and Orkin, 2004), interacts with β-catenin and its ATPase activity is required to enhance transcriptional activation by β-catenin/TCF-4 (Barker *et al.*, 2001). BRG-1 is recruited to the *c-myc* promoter in developing murine thymocytes, but not in peripheral, resting T cells (Chi *et al.*, 2003). BRG-1, and in particular its ATPase activity, is required for *c-myc* mRNA expression in developing T lymphocytes (Chi *et al.*, 2003) as well as in colon carcinoma cells with constitutive β-catenin/TCF-4 signaling resulting from a highly stable mutant form of β-catenin (Barker *et al.*, 2001). Since BRG-1 is recruited to the region of the *c-myc* promoter harboring TBE3 this BRG-1 requirement for *c-myc* expression was attributed to its function in transactivation by β-catenin/TCF-4 (Barker *et al.*, 2001; Chi *et al.*, 2003; Liu *et al.*, 2006a). Nevertheless, also other transcription factors binding to this region of the *c-myc* promoter (Fig. 4) could need BRG-1 to activate *c-myc* transcription. So, during serum stimulation of starved human Hs68 primary fibroblast TCF-4 occupies this region of the *c-myc* promoter clearly later than BRG-1 suggesting early recruitment of BRG-1 to this region by another transcription factor (Liu *et al.*, 2006a).

The role of the Wnt pathway and its target gene *c-myc* in promoting stem cell proliferation is particularly evident in the intestinal epithelium of the crypts of Lieberkühn (Korinek *et al.*, 1998; Muncan *et al.*, 2006; Pinto *et al.*, 2003; van de Wetering *et al.*, 2002). Pluripotent stem cells at the crypt bottom generate progenitors that occupy the lower third of the crypt and proliferate rapidly. In the midcrypt region, the cells differentiate into one of the functional cell types of the colon concomitant with cell cycle arrest. They migrate toward the tip of the villi, where they die within a few days (Gregorieff and Clevers, 2005; Potten and Loeffler, 1990; Radtke and Clevers, 2005; van de Wetering, 2002). The Wnt pathway, the TCF-4 target gene *c-myc*, and the c-Myc target gene *p21* constitute the dominant switch between proliferating undifferentiated precursor cells versus cell cycle-arrested differentiated intestinal cells (Korinek *et al.*, 1998; Pinto *et al.*, 2003; van de Wetering *et al.*, 2002). In the proliferative crypt compartment, Wnt signals result in accumulation of β-catenin so that β-catenin/TCF-4 complexes activate the *c-myc* promoter (van de Wetering *et al.*, 2002). c-Myc in turn represses the *p21* promoter so that high c-Myc levels in combination with lack of p21 expression drive progenitor proliferation.

In contrast, in the differentiation compartment, the absence of Wnt signaling leads to degradation of β-catenin so that the *c-myc* promoter is no longer activated by β-catenin/TCF-4 complexes (van de Wetering *et al.*, 2002). Release of the *p21* promoter from repression by c-Myc results in p21 expression so that low c-Myc levels in combination with p21 expression allow differentiation and cell cycle exit.

Colon crypts represent a good example for the dualism of *c-myc*, that is, on the one hand tightly controlled *c-myc* expression is essential for normal proliferation but on the other hand deregulated *c-myc* expression can result in tumorigenesis (Gregorieff and Clevers, 2005; Radtke and Clevers, 2005). In juvenile mice, the specific deletion of *c-myc* at the onset of crypt morphogenesis leads to a failure to form normal numbers of invaginated crypts in the small intestine although adult mutant mice recover from this insult and show an intestinal crypt morphology indistinguishable from control animals (Bettess *et al.*, 2005). In adult mice, conditional deletion of *c-myc* results in rapid loss (within weeks) of c-Myc-deficient crypts and their replacement by c-Myc-proficient crypts through a fission process of crypts that have escaped gene deletion (Muncan *et al.*, 2006). *Tcf-4* knockout mice and transgenic mice expressing the secreted Wnt inhibitor Dkk1 (Dickkopf-1) (Fig. 5A) lack the epithelial stem cell compartment in the intestinal crypts or/and show severe defects in crypt-villus organization demonstrating that maintenance of the proliferative colon crypt compartment is dependent on Wnt signaling resulting in activation of the *c-myc* promoter by β-catenin/TCF-4 complexes (Korinek *et al.*, 1998; Pinto *et al.*, 2003; van de Wetering *et al.*, 2002). Conversely, a constitutively active Wnt pathway leads to constitutive c-Myc expression and increases the pool of epithelial progenitor cells in the proliferative state (van de Wetering *et al.*, 2002) giving rise to overgrowths (polyps) that are primed for additional mutations and progression to carcinoma (Gregorieff and Clevers, 2005; Massagué, 2004; Radtke and Clevers, 2005).

Wif-1 (Wnt inhibitory factor-1) represses *c-myc* mRNA expression (Fig. 5A). Consistently, in bladder tumors its reduced expression (associated with promoter hypermethylation) correlated with increased *c-myc* mRNA levels (Urakami *et al.*, 2006).

Several other factors influence *c-myc* transcription via TCF-4/LEF-1:

1. Smad3 activated by ALK-5TD, an active form of the TGF-β (transforming growth factor-β) type I receptor, inhibits the activation of the *c-myc* promoter by β-catenin/TCF-4 complexes, but not its activation by β-catenin/LEF-1 complexes (Fig. 5A; Sasaki *et al.*, 2003). In accordance, in the presence of Smad3 activated by ALK-5TD, *c-myc* activation by β-catenin through TBE3 can be restored by overexpression of LEF-1, but not by overexpressed TCF-4. Smad3 activated by ALK-5TD disrupts the β-catenin/TCF-4 complex on TBE3 so that β-catenin is dissociated while TCF-4 and Smad3

bind to TBE3 (Table I; Sasaki *et al.*, 2003). In contrast, it does not destroy the β-catenin/LEF-1 complex on TBE3 so that a ternary complex of β-catenin, LEF-1, and Smad3 binds to TBE3 (Sasaki *et al.*, 2003). Yet it was not addressed whether Smad3 binds directly to TBE3 or whether Smad3 interacts with β-catenin, TCF-4, or LEF-1 at TBE3. Anyway, Smad3 has been described to bind directly to LEF-1 as well as to interact with β-catenin and both interactions could be stimulated by TGF-β (Jian *et al.*, 2006; Labbe *et al.*, 2000; Lei *et al.*, 2004; Letamendia *et al.*, 2001; Li *et al.*, 2006; Tian and Phillips, 2002). Repression of *c-myc* expression by TGF-β is essential for TGF-β-induced cell cycle arrest and growth suppression (Massagué and Chen, 2000; Massagué and Gomis, 2006; Massagué *et al.*, 2000; Siegel and Massagué, 2003). Conversely, constitutive *c-myc* expression renders cells resistant to TGF-β-induced cell growth arrest (Alexandrow *et al.*, 1995; Blain and Massagué, 2000; Claassen and Hann, 2000; Sun *et al.*, 1998; Warner *et al.*, 1999; Yagi *et al.*, 2002). Many cancer cells lose their growth-inhibitory response to TGF-β in the presence of intact TGF-β receptors and Smads (Sasaki *et al.*, 2003). Since enhanced expression of LEF-1 occurs frequently in colon cancer and metastatic melanoma (Filali *et al.*, 2002; Hovanes *et al.*, 2001; Murakami *et al.*, 2001) replacement of β-catenin/TCF-4 complexes by β-catenin/LEF-1 complexes on the *c-myc* promoter could explain how these tumor cells become refractory to down-regulation of *c-myc* expression by TGF-β and to the subsequent TGF-β-induced growth arrest (Sasaki *et al.*, 2003).

2. The acetyltransferase CBP acetylates β-catenin at lysine 49 that is frequently mutated in thyroid anaplastic carcinoma and its mutation to arginine (K49R) abolishes the acetylation of β-catenin by CBP (Wolf *et al.*, 2002). This unacetylatable mutant form of β-catenin is a more potent activator of the *c-myc* promoter than wild-type β-catenin suggesting that acetylation of β-catenin by CBP may negatively regulate *c-myc* transcription. Yet no differences between wild-type β-catenin and the K49R mutant with respect to protein stability, interaction with members of the Wnt signaling pathway or nuclear localization were found (Wolf *et al.*, 2002).

3. HBP1, which prevents DNA binding by TCF-4 probably through a physical interaction, inhibits *c-myc* mRNA expression (Fig. 5A; Sampson *et al.*, 2001). The cell cycle inhibitor and growth suppressor HBP1 is thought to block cell cycle progression until proliferative signals, as Wnt signaling, overcome this constitutive threshold. Thereby HBP1 should prevent inappropriate proliferation and maintain normal tissue homeostasis so that it is considered to be part of the normal barriers to proliferation in quiescent and differentiated cells (Sampson *et al.*, 2001; Shih *et al.*, 1998, 2001; Tevosian *et al.*, 1997). Accordingly, the candidate tumor suppressor HBP1 shows ubiquitous tissue distribution and *hbp*1 mRNA expression is decreased in proliferating cells.

4. HOXB13, which inhibits β-catenin/TCF-4-driven transcription, represses the *c-myc* promoter and decreases c-Myc protein expression (Fig. 5A; Jung *et al.*, 2004, 2005). It was described to reduce *tcf-4* expression at the mRNA and protein level but was not found to affect the activity of the TCF-4 promoter. HOXB13 suppresses cell growth and more than 70% of colorectal cancers have lost HOXB13 expression (Jung *et al.*, 2004, 2005).

5. TIS7 (TPA-induced sequence 7), which inhibits β-catenin/TCF-4-driven transcription in a HDAC-dependent manner, reduces the *c-myc* mRNA expression (Fig. 5A; Vietor *et al.*, 2005). It acts as a transcriptional corepressor and can interact with mSin3B, HDAC1, and other members of the HDAC-containing SIN3 complex (Vietor *et al.*, 2002). TIS7 enhances the interaction of β-catenin with HDAC4 and inhibits the p300-mediated stimulation of β-catenin/TCF-4-dependent transcriptional activation (Vietor *et al.*, 2005).

6. The second messenger NO (nitric oxide), a pleitropic regulator, is generated from L-arginine by NOS (nitric-oxide synthases) and modulates the etiology and phenotype of cancer cells (Xu *et al.*, 2002). The expression of iNOS (inducible NOS) is stimulated by cytokines and high expression levels of iNOS are found in tumor cells as well as in adenomatous polyps, precursor lesions for colorectal cancer (Lala and Chakraborty, 2001). NO treatment activates MMPs (matrix metalloproteinases), which cause the degradation of E-cadherin and the subsequent dissociation of β-catenin, so that β-catenin accumulates in the cytoplasm and relocates to the nucleus, where it forms β-catenin-TCF/LEF complexes that activate TCF/LEF target genes (Liu *et al.*, 2004; Mei *et al.*, 2000a,b, 2002; Takahashi *et al.*, 2000). Because of a mutation in APC IMCE cells ($Apc^{Min/+}$) have higher cytosolic and nuclear β-catenin levels than YAMC cells ($Apc^{+/+}$) (Liu *et al.*, 2004). In accordance, IMCE cells displayed higher c-Myc protein levels than YAMC cells. Several NO donors increased the c-Myc protein expression in YAMC cells so that Liu *et al.* (2004) suggested that NO may stimulate *c-myc* transcription via this pathway (Fig. 5A).

7. AML1-ETO, PML-RARα (retinoic acid receptor α), and PLZF (promyelocytic leukemia zinc finger protein)-RARα, the most frequent translocation products in AML (acute myeloid leukemia), encode aberrant transcription factors and induce a block in hematopoietic differentiation (Grignani *et al.*, 1993; Müller-Tidow *et al.*, 2004; Ruthardt *et al.*, 1997; Westendorf *et al.*, 1998). All three fusion proteins induce expression of γ-catenin on the mRNA and protein level as well as c-Myc protein expression (Müller-Tidow *et al.*, 2004). For AML1-ETO and PML-RARα activation of the *c-myc* promoter was shown. In addition, induction of ectopic AML-ETO1 expression results in occupancy of the *c-myc* promoter with γ-catenin in U937 cells (Müller-Tidow *et al.*, 2004). The study of Müller-Tidow *et al.* (2004) strongly suggest that AML1-ETO induces *c-myc* transcription via γ-catenin

and TCF-4/LEF-1 and that PML-RARα and PLZF-RARα may use this pathway, too (Fig. 5A). Nevertheless, the AML fusion proteins activate the γ-catenin promoter through an indirect mechanism where the direct effector protein is not known yet.

8. Coexpression of the large T antigen of the human polyomavirus JCV with β-catenin enhances *c-myc* transcription as the JCV large T antigen increases the stability of β-catenin, with which it interacts (Enam *et al.*, 2002; Gan and Khalili, 2004).

9. The peptidyl-prolyl *cis/trans* isomerase Pin1, which enhances β-catenin/TCF-4-driven transcription, activates the *c-myc* promoter and increases *c-myc* mRNA and protein expression (Fig. 5A; Chen *et al.*, 2006). Pin1 is overexpressed in cancers, enhances cell cycle progression, cell survival as well as tumor growth and has been implicated in cell transformation (Chen *et al.*, 2006; Lu, 2003; Wulf *et al.*, 2003). Pin1 stimulates β-catenin/TCF-4-driven transcription through several mechanisms: (1) Proline isomerization of β-catenin by Pin1 stabilizes β-catenin by preventing its binding to APC and thus its degradation (Ryo *et al.*, 2001). (2) Pin1 increases the expression of TCF-4 (Chen *et al.*, 2006). (3) AR (androgen receptor), which interacts with both β-catenin (Chen *et al.*, 2006; Song *et al.*, 2003; Yang *et al.*, 2002) and TCF-4 (Amir *et al.*, 2003), inhibits their interaction and thus represses β-catenin/TCF-4-driven transcription (Chen *et al.*, 2006). Pin1 prevents this AR-mediated repression of β-catenin/TCF-4-dependent transcription by preventing the disruption of β-catenin/TCF-4 complexes by AR and its interaction with β-catenin (Chen *et al.*, 2006). It is conceivable that stabilization of β-catenin and increased TCF-4 expression may account for activation of the *c-myc* promoter by Pin1. However, it is unknown whether AR represses transactivation of the *c-myc* promoter by β-catenin/TCF-4 and thus whether Pin1 counteracts such an AR-mediated repression of *c-myc*. In contrast, AR was shown to activate *c-myc* transcription in response to testosterone (Fig. 5C; Silva *et al.*, 2001; Zhang *et al.*, 2004; see Section IV.B.1). Nevertheless, the androgen 5α-dihydro-testosterone induces the occupancy of the *c-myc* promoter with AR (Amir *et al.*, 2003; Zhang *et al.*, 2004) and Amir *et al.* (2003) suggested that AR may be recruited to the *c-myc* promoter through β-catenin/TCF-4 complexes because of its interaction with both β-catenin (Chen *et al.*, 2006; Song *et al.*, 2003; Yang *et al.*, 2002) and TCF-4.

10. The tumor suppressor KLF6, a member of the Krüppel-like family of zinc finger transcription factors, transactivates and occupies the *E-cadherin* promoter (DiFeo *et al.*, 2006a). In accordance, siRNA to KLF6 reduces the *E-cadherin* mRNA and protein levels. The studies of DiFeo *et al.* (2006a,b) suggest that KLF6 downregulates *c-myc* expression via E-cadherin (Fig. 5A).

11. 1,25-$(OH)_2$-D_3 (1,25-dihydroxyvitamin D_3), the most active vitamin D metabolite, which represses β-catenin/TCF-4-driven transcription, downregulates *c-myc* mRNA expression with biphasic kinetics in VDR (vitamin D

receptor)-positive human SW480-ADH colon carcinoma cells: a rapid early reduction is followed by a later diminution (Palmer *et al.*, 2001). This early drop in *c-myc* mRNA level appears to be caused by 1,25-$(OH)_2$-D_3-stimulated direct binding of VDR to β-catenin, which leads to disruption of β-catenin/TCF-4 complexes. The later decrease in the *c-myc* mRNA level seems to be due to 1,25-$(OH)_2$-D_3-induced VDR-dependent E-cadherin expression resulting in binding of β-catenin to E-cadherin at the plasma membrane and thus to sequestration of β-catenin from the nucleus (Palmer *et al.*, 2001). 1,25-$(OH)_2$-D_3 has complex cell-specific antitumor properties. It induces cell cycle arrest, differentiation as well as apoptosis and inhibits invasion (Palmer *et al.*, 2001). Accordingly, synthetic vitamin D analogues are used in clinical trials as potential anticancer drugs.

Additionally, 1,25-$(OH)_2$-D_3 is supposed to repress the *c-myc* promoter also via the three intron 1 elements MIE1, MIE2, and MIE3 (*myc* intron elements 1–3) and through HOXB4, which binds to MIE1 (Figs. 4 and 5C; Pan and Simpson, 1999; see Section IV.A.26).

β-Catenin/TCF-4 increase *c-myc* expression not only through transactivation of the *c-myc* promoter but also through activation of their direct target gene *crd-bp* because the RNA-binding protein CRD-BP stabilizes *c-myc* mRNA and thus elevates the endogenous *c-myc* mRNA and protein levels (Noubissi *et al.*, 2006). CRD-BP (coding region determinant-binding protein), also known as IMP-1 [IGF2 (insulin-like growth factor 2) mRNA-binding protein], binds to the CRD (coding region determinant) of *c-myc* mRNA and shields the mRNA from attack by an endoribonuclease (Bernstein *et al.*, 1992; Doyle *et al.*, 1998; Prokipcak *et al.*, 1994). Thus, in studies that do not address control of the *c-myc* promoter by β-catenin/TCF-4 it is impossible to distinguish their effects on *c-myc* transcription from their CRD-BP-mediated effects on *c-myc* mRNA stability.

2. E2F

E2F binds to the *c-myc* promoter (Fig. 4; Table I; Albert *et al.*, 2001; Campanero *et al.*, 2000; Chen *et al.*, 2002; Frederick *et al.*, 2004; Hiebert *et al.*, 1989; Johansen *et al.*, 2001; Mudryj *et al.*, 1990; Roussel *et al.*, 1994; Thalmeier *et al.*, 1989; Wells *et al.*, 2003; Yagi *et al.*, 2002). E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5 were found to bind to the *c-myc* promoter *in vitro* (Campanero *et al.*, 2000; Chen *et al.*, 2002; Frederick *et al.*, 2004; Johansen *et al.*, 2001; Roussel *et al.*, 1994; Yagi *et al.*, 2002). *In vivo* binding of E2F-1, E2F-2, E2F-4, E2F-5, and E2F-6 to the *c-myc* promoter has been described (Albert *et al.*, 2001; Baek *et al.*, 2003; Cam *et al.*, 2004; Chen *et al.*, 2002; Gomis *et al.*, 2006a; Iakova *et al.*, 2003; Klappacher *et al.*, 2002; Liu *et al.*, 2006a; Ogawa *et al.*, 2002; Ren *et al.*, 2002; Rodriguez *et al.*, 2006;

Sebastian *et al.*, 2005; Wells *et al.*, 2003). The E2F-binding site of the *c-myc* promoter overlaps with those for ETS-1/2, STAT3, NFATc1, KLF11, and METS (Fig. 4; see Sections IV.A.4 , IV.A.6, IV.A.15, IV.A.22, and IV.A.30; Buchholz *et al.*, 2006; Buck *et al.*, 2006; Kiuchi *et al.*, 1999; Klappacher *et al.*, 2002; Roussel *et al.*, 1994). E2F-1 and E2F-3 were shown to transactivate the *c-myc* promoter (Campanero *et al.*, 2000; Johansen *et al.*, 2001; Majello *et al.*, 1995; Oswald *et al.*, 1994; Roussel *et al.*, 1994). Accordingly, the E2F-binding site functions as a positive element for the P2 promoter in transient transfection assays and *in vitro* transcription experiments (Albert *et al.*, 2001; Batsche *et al.*, 1994; Carlberg *et al.*, 1999; Frederick *et al.*, 2004; Hamel *et al.*, 1992; Johansen *et al.*, 2001; Moberg *et al.*, 1991, 1992a,b; Mudryj *et al.*, 1990; Nishikura, 1986; Thalmeier *et al.*, 1989; Yagi *et al.*, 2002). In contrast, at episomal *c-myc* promoters that establish a chromatin structure indistinguishable from the chromosomal *c-myc* gene the E2F-binding site acts as a negative element for the P2 promoter (Albert *et al.*, 2001). The episomal *c-myc* P1 and P2 promoters are repressed and transcription from both is strongly induced by the HDAC inhibitor SoB (see Section III.D; Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996; Strobl *et al.*, 1993; Wolf *et al.*, 1995). Mutation of the E2F-binding site results in constitutive transcription from the episomal P2 promoter so that its activity is only slightly additionally increased by SoB (Albert *et al.*, 2001). P2-iniated paused Pol II complexes are found at the P2 promoter independently from the integrity of the E2F-binding site. These findings suggest that the processivity of P2-initiated Pol II complexes is negatively regulated by HDAC recruited to the E2F-binding site, that is, that E2F-pocket protein–HDAC complexes inhibit transcriptional elongation (see Section III.D; Albert *et al.*, 2001). Phosphorylation of pocket proteins (RB, p107, p130) by cyclin D1/Cdk4 and cyclin E/Cdk2 results in the release of E2F from pocket proteins and HDAC (Harbour and Dean, 2000; Weinberg, 1995). Although E2F is not required for activation of the *c-myc* P2 promoter (Albert *et al.*, 2001) E2F, which directly binds to TFIIH (Pearson and Greenblatt, 1997), may stimulate the processive transcriptional elongation by P2-initiated Pol II complexes. In contrast, mutation of the E2F-binding site does not significantly affect the activity of the episomal P1 promoter or its inducibility by SoB (Albert *et al.*, 2001). Thus the E2F-binding site does not regulate the P1 promoter in the context of chromatin (see Section III.D).

The E2F-binding site 5′-GCGGGAAA-3′ of the *c-myc* promoter includes a CpG dinucleotide, the cytosine residue of which can be methylated. Methylation of this CpG motif inhibits the binding of E2F-1/DP-1 to the E2F site of *c-myc* whereas it minimally affects the binding of E2F-2/DP-1, E2F-3/DP-1, E2F-4/DP-1, or E2F-5/DP-1 (Campanero *et al.*, 2000). In accordance, methylation of the *c-myc* E2F-binding site prevents the transactivation of a heterologous minimal promoter through this site by E2F-1/DP-1 but does not alter its

transactivation by E2F-3/DP-1 (Campanero *et al.*, 2000). Thus, this example demonstrates how DNA methylation of the *c-myc* promoter can selectively influence its regulation by distinct transcription factors.

At their target genes E2F-1, E2F-2, and E2F-3 function as transcriptional activators in late G_1- and S-phase whereas E2F-4 and E2F-5 in association with p107 and p130 function as transcriptional repressors in G_0- and early G_1-phase as well as during cell cycle exit (e.g., terminal differentiation) (Attwooll *et al.*, 2004; Blais and Dynlacht, 2004; Bracken *et al.*, 2004; Cam and Dynlacht, 2003; Cobrinik, 2005; Dannenberg and te Riele, 2006; De Gregori, 2002; Dimova and Dyson, 2005; Du and Pogoriler, 2006; Frolov and Dyson, 2004; Giacinti and Giordano, 2006; Khidr and Chen, 2006; Macaluso *et al.*, 2006; Stevaux and Dyson, 2002; Stevens and La Thangue, 2003; Trimarchi and Lees, 2002; Zhu, 2005). An important unresolved aspect of this system is whether RB in association with E2F-1/2/3 or E2F-4 participates in promoter repression in early G_1- and G_0-phase (Attwooll *et al.*, 2004; Cobrinik, 2005; Macaluso *et al.*, 2006). In this respect, differences may exist among individual genes as well as between the G_0–G_1–S transition during stimulation of quiescent cells and the M–G_1–S transition in continuously cycling cells. RB binds and inactivates E2F-1/2/3 until it is phosphorylated by cyclin D1/Cdk4 and cyclin E/Cdk2 resulting in interruption of this interaction (Classon and Harlow, 2002; Ezhevsky *et al.*, 1997; Harbour and Dean, 2000; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Weinberg, 1995; Zhang and Dean, 2001). In addition, RB participates directly in the repression of a limited subset of E2F-responsive genes (e.g., *cyclin E*) in G_0 and early G_1 cells (Attwooll *et al.*, 2004; Cobrinik, 2005; Giacinti and Giordano, 2006).

In accordance with this general regulation of E2F target genes by different E2F family members and pocket proteins, ChIP analysis demonstrated the *in vivo* occupancy of the *c-myc* promoter as summarized in Table II.

Like other E2F target genes, the *c-myc* promoter was found to be occupied with E2F-6, HP-1γ, and Max in G_0 cells strongly suggesting that in G_0 cells also the *c-myc* promoter is repressed by a multimeric E2F-6 complex containing E2F-6/DP-1, Mga/Max, histone methyltransferases, HP1γ, and PcG (Polycomb group) proteins (Ogawa *et al.*, 2002). The *c-myc* promoter is not occupied by E2F-1 in quiescent cells virtually not expressing *c-myc* (Liu *et al.*, 2006a). Induction of *c-myc* transcription by serum stimulation results in binding of E2F-1 to the *c-myc* promoter suggesting that E2F-1 is involved in the serum-induced activation of *c-myc* transcription (Fig. 6; Liu *et al.*, 2006a). Surprisingly, Liu *et al.* (2006a) detected E2F-4 at the *c-myc* promoter only following serum stimulation, but not in quiescent human Hs68 primary fibroblasts, leaving the possibility that E2F-5–pocket protein–HDAC complexes or E2F-6 complexes may suppress the *c-myc* promoter in these cells during quiescence.

Table II *In Vivo* Occupancy of the *c-myc* Promoter by E2F Family Members and Pocket Proteins

	Proteins found at the *c-myc* promoter in ChIP analysis					
Cells	**E2F family members**		**Pocket proteins**			**References**
Asynchronously growing cells	E2F-1	E2F-4	RB	p107	p130	Wells *et al.*, 2003
Proliferating bone marrow progenitor cells expressing *c-myc*	E2F-1	E2F-4		*No p107*	*No p130*	Klappacher *et al.*, 2002
Terminally differentiated peritoneal macrophages (almost) not expressing *c-myc*	*no E2F-1*	E2F-4		p107	p130	
Adult rat livers not expressing *c-myc*	E2F-1	E2F-4	RB			Iakova *et al.*, 2003
Adult rat livers following PH (partial hepatectomy) expressing *c-myc*	E2F-1	E2F-4	*No RB*			
Early G_1-phase	E2F-1	E2F-4			p130	Ogawa *et al.*, 2002
Cell cycle arrest due to serum deprivation		E2F-4			p130	Cam *et al.*, 2004
p16-induced growth arrest		E2F-4			p130	
Cell cycle arrest due to contact-inhibition		E2F-4			p130	

RB represses whereas cyclin D1, adenovirus E1A, and SV40 large T activate the *c-myc* promoter through the E2F-binding site (Fig. 5A; Batsche *et al.*, 1994; Buchmann *et al.*, 1998; Dagnino *et al.*, 1995; Hamel *et al.*, 1992; Hiebert *et al.*, 1989; Lipp *et al.*, 1989; Moberg *et al.*, 1992a; Oswald *et al.*, 1994; Thalmeier *et al.*, 1989). In addition, E2F and pocket proteins have been reported to be involved in up- and downregulation of *c-myc* transcription in response to: (1) TPA (12-*O*-tetradecanoylphorbol-13-acetate) and RA, which repress the *c-myc* promoter and induce differentiation of HL-60 cells into the macrophage or granulocyte lineage, respectively. They decrease the amount of free E2F but

increase the amount of E2F–RB complexes bound to the E2F-binding site of the *c-myc* promoter (Ishida *et al.*, 1994, 1995). (2) IL (interleukin)-3, which activates the *c-myc* promoter. It decreases the amount of E2F–p107 complexes but increases the amount of free E2F bound to the E2F site of *c-myc* (Watanabe *et al.*, 1995). (3) The product of lipid peroxidation HNE (4-hydroxynenenal), which inhibits *c-myc* expression and cell proliferation. It decreases the amount of free E2F but increases the amount of E2F–pocket protein complexes bound to the E2F site of *c-myc* (Barrera *et al.*, 2002, 2004).

In wild-type MEFs (murine embryonic fibroblasts), the *c-myc* promoter is almost inactive in the absence of serum, but strongly induced by serum treatment (Klappacher *et al.*, 2002). In contrast, in TKO (triple knockout) MEFs lacking RB, p107, and p130 (Sage *et al.*, 2000), the *c-myc* promoter is already highly active in the absence of serum and only slightly more active after serum addition (Klappacher *et al.*, 2002). This finding indicates that pocket proteins are essential for repression of the *c-myc* promoter in quiescent cells while removal of pocket proteins from the *c-myc* promoter is central for induction of *c-myc* transcription by serum (Fig. 5A). Since pocket proteins recruit HDACs (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Frolov and Dyson, 2004; Harbour and Dean, 2000; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Zhang and Dean, 2001) this finding correlates with the result of Baek *et al.* (2003) that under serum-free conditions nuclear microinjection of α-HDAC1 causes a significant stimulation of the *c-myc* promoter similar to that obtained with serum. In accordance, Albert *et al.* (2001) showed that mutation of the E2F-binding site can for the most part substitute the induction of the episomal *c-myc* P2 promoter by the HDAC inhibitor SoB suggesting that inhibition of the pocket protein-mediated recruitment of HDACs to the E2F-binding site is sufficient for activation of the *c-myc* P2 promoter.

The major unresolved problem concerning the regulation of the *c-myc* promoter by E2F family members and pocket proteins is the contradiction that in continuously cycling cells *c-myc* transcription remains constant throughout the cell cycle while activation and repression by E2F and pocket proteins, respectively, oscillate during each cell cycle.

TGF-β, the quintessential growth-inhibitory cytokine (Bierie and Moses, 2006; Dumont and Arteaga, 2003; Massagué, 2000; Miyazawa *et al.*, 2002; Roberts and Wakefield, 2003; Wakefield and Roberts, 2002), is an important antagonist to the efficient stimulator of proliferation c-Myc and represses the *c-myc* promoter and thus the *c-myc* mRNA and protein expression (Buck *et al.*, 2006; Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Gomis *et al.*, 2006a; Hu *et al.*, 2005; Iavarone and Massagué, 1997; Kurisaki *et al.*, 2003; Matsuura *et al.*, 2004; Suzuki *et al.*, 2004; Yagi *et al.*, 2002; Zentella *et al.*, 1991). Downregulation of c-Myc expression is essential for the TGF-β-induced growth arrest (Alexandrow *et al.*, 1995;

Blain and Massagué, 2000; Chen *et al.*, 2001b; Claassen and Hann, 2000; Sun *et al.*, 1998; Warner *et al.*, 1999; Yagi *et al.*, 2002). TGF-β causes a G_1 cell cycle arrest by induction of *p15* and *p21* expression and by repression of *id1*, *id2*, *id3*, and *c-myc* expression (Derynck *et al*, 2001; Massagué and Chen, 2000; Massagué and Gomis, 2006; Massagué *et al.*, 2000; Siegel and Massagué, 2003). Since c-Myc represses both the *p15* and the *p21* promoters downregulation of c-Myc by TGF-β, which results in release of *p15* and *p21* from this repression, is required to allow activation of these two promoters by other aspects of TGF-β signaling (Claassen and Hann, 2000; Massagué and Gomis, 2006; Seoane *et al.*, 2004; Siegel and Massagué, 2003). Moreover, c-Myc activates cyclin D/Cdk4 and cyclin E/Cdk2 via its target genes (see Section II.A) so that it would counteract the inhibition of these G_1 cyclin/Cdk complexes by TGF-β-induced p15 and p21, respectively.

In brief, binding of TGF-β to its receptor, a serine/threonine kinase complex, induces phosphorylation of the TβRI (TGF-β type I receptor) by the TβRII (TGF-β type II receptor) and in turn TβRI phosphorylates Smad2 and Smad3 which results in their association with Smad4 (Fig. 5A; Derynck and Zhang, 2003; Feng and Derynck, 2005; Moustakas *et al.*, 2001; Shi and Massagué, 2003; ten Dijke and Hill, 2004). In the nucleus, these activated Smad2/3-Smad4 heterodimers or heterotrimers bind to Smad binding sites in target genes either alone or in complex with other transcription factors that bind to their own nearby binding sites (Attisano and Wrana, 2000; Massagué and Wotton, 2000; Massagué *et al.*, 2005; ten Dijke *et al.*, 2000, 2002).

TGF-β represses the *c-myc* promoter through the TIE (TGF-β inhibitory element) that is composed of the E2F-binding site (GCGGGAAA) and an 5′ adjacent imperfect Smad binding site (GGCTT) (Fig. 4), which both are required and together are sufficient for repression of the *c-myc* promoter by TGF-β (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). A complex containing Smad3, E2F-4/5, DP-1, and the corepressor p107 preexists in the cytoplasm (Chen *et al.*, 2002). In response to TGF-β, this complex associates with Smad4, moves into the nucleus, and recognizes the composite Smad-E2F site of *c-myc* for repression (Fig. 5A; Table I; Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Gomis *et al.*, 2006a; Yagi *et al.*, 2002). This TGF-β-induced repression of the *c-myc* promoter by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex through the TIE occurs fast (completed within 1 h after TGF-β addition) and can be triggered at any time point during the cell cycle so that it represents a rapid, cell cycle-independent mechanism for downregulation of *c-myc* transcription by TGF-β (Chen *et al.*, 2002; Zentella *et al.*, 1991). In this *c-myc*-repressing Smad3/Smad4/E2F-4,5/DP-1/p107 complex E2F-4 and E2F-5 are functionally redundant whereas p130 cannot substitute for p107 (Chen *et al.*, 2002). Smad3 was shown to bind directly and independently to p107, E2F-4, and E2F-5 (Chen *et al.*, 2002), the latter two of which are known to bind directly

to p107 (Classon and Dyson, 2001). Frederick *et al.* (2004) mapped the Smad binding site (TTGGCGGGGAA) of the TIE more 3′ so that it overlapped with the E2F-binding site.

A tumor-derived mutant Smad4 fails to repress the *c-myc* promoter in complex with Smad2 and/or Smad3 in response to TGF-β demonstrating how tumor cells can become resistant to repression of *c-myc* by TGF-β (Chen *et al.*, 2001b). Similarly, TGF-β is unable to repress the *c-myc* promoter in human MDA-MB-468 breast cancer cells which lack endogenous Smad4 (Chen *et al.*, 2001b).

In summary, these data suggest that E2F and pocket proteins may be important to determine whether the *c-myc* promoter is inducible by other signals (permissive) or whether it is kept in an uninducible state (unpermissive). Thus, repression of *c-myc* (in response to antiproliferative signals like TGF-β or growth factor withdrawal) by E2F-pocket protein complexes that recruit HDAC versus derepression of *c-myc* (in response to proliferation signals like serum or other mitogens) due to removal of pocket proteins and/or their associated HDAC may represent the dominant switch so that the E2F-binding site has a dual function for control of the *c-myc* promoter: repression and derepression. The actual activation of the *c-myc* promoter may then be achieved either by E2F-1/2/3 itself or by other transactivators, for example STAT3, NFATc1, and ETS-1/2 that bind to overlapping sites or NF-κB, AP-1, and β-catenin/TCF-4 that bind to distant sites (Fig. 4). E2F may cooperate with other transcription factors (Attwooll *et al.*, 2004; Bracken *et al.*, 2004; Cobrinik, 2005) in repression (e.g., METS; see Section IV.A.4) and activation (e.g., Sp1; see Section IV.A.16) of the *c-myc* promoter. However, the *c-myc* promoter cannot be regulated by E2F family members and pocket proteins in a cell cycle-dependent manner in continuously cycling cells because *c-myc* transcription remains constant throughout the cell cycle in these cells whereas E2F family members and pocket proteins oscillate depending on the cell cycle phase. Therefore, in such continuously proliferating cells, a mechanism has to operate at the *c-myc* promoter, which imposes its dominant control over the promoter to ensure the constant *c-myc* transcription required for homeostasis of normal cells. A model for this control is discussed in Section VI.B.

3. Smad2 AND Smad3

TGF-β represses the *c-myc* promoter by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex through the TIE (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). In addition, purified recombinant Smad3, comprising the MH1 domain, but lacking the MH2 domain, was shown to bind to the TIE directly in the absence of other transcription factors (Fig. 4; Table I; Frederick *et al.*, 2004; Yagi *et al.*, 2002).

TGF-β stimulates the Smad3 protein expression, which is accompanied by a decrease in the c-Myc protein level (Hu *et al.*, 2005).

Cyclin D/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2 phosphorylate Smad2 and Smad3, but not Smad4 (Fig. 5A; Matsuura *et al.*, 2004). Accordingly, p16 and p21 inhibit phosphorylation of Smad3 by cyclin D/CDk4 or cyclin E/Cdk2, respectively. Smad3 phosphorylation oscillates in a cell cycle-dependent manner with the maximal phosphorylation occurring at the G_1/S junction slightly before the peak of RB phosphorylation. Mutation of the Cdk2 and Cdk4 phosphorylation sites (serine 212, threonine 8 and 178) of Smad3 to valine or alanine increases the ability of Smad3 to repress the *c-myc* promoter in response to TGF-β (Matsuura *et al.*, 2004). Thus, phosphorylation of Smad3 by cyclin D/Cdk4 and cyclin E/Cdk2 should inhibit the TGF-β-induced Smad3-mediated repression of the *c-myc* promoter (Fig. 5A). Mutation of the Cdk phosphorylation sites in Smad3 augments its ability to inhibit G_1 cell cycle progression indicating that phosphorylation of Smad3 by G_1 cyclin/Cdk complexes facilitates cell cycle progression from G_1- to S-phase (Liu and Matsuura, 2005; Matsuura *et al.*, 2004). TGF-β and Smad3 target the *c-myc* promoter through several different pathways and transcription factors (Figs. 4, 5A and 5D). However, it was not examined which aspect is affected by cyclin/Cdk-mediated Smad3 phosphorylation. Like in the case of E2F and pocket proteins, the *c-myc* promoter cannot be regulated by G_1 cyclin/Cdk complex-mediated phosphorylation of Smad3 in a cell cycle-dependent manner in continuously cycling cells where *c-myc* transcription remains constant throughout the cell cycle.

Cancer cells often contain high levels of cyclin D/Cdk4 and cyclin E/Cdk2 because of frequent amplification, translocation or overexpression of *cyclin D1*, inactivation of p16, overexpression of *cyclin E* or decreases in p27 levels (Chau and Wang, 2003; Hall and Peters, 1996; Porter *et al.*, 1997; Sherr and McCormick, 2002; Sherr, 1996). Consequently, inactivation of Smad3 and presumably Smad2 by extensive cyclin/Cdk phosphorylation may provide an important mechanism for resistance of tumor cells to TGF-β growth-inhibitory effects (Liu and Matsuura, 2005; Matsuura *et al.*, 2004).

The oncoprotein c-Ski binds to the TGF-β-activated Smad2/3-Smad4 complex on DNA and thereby inhibits both activation and repression of TGF-β/Smad target genes (Frederick and Wang, 2002; Luo, 2004; Medrano, 2003; Miyazono *et al.*, 2003; Sun *et al.*, 1999; Wotton and Massagué, 2001). The oncoprotein SnoN, a Ski family member, exerts the same effects, at least in inhibition of TGF-β-induced Smad-mediated activation. Consistently, cancer cells with elevated levels of c-Ski or SnoN are insensitive to growth-inhibitory TGF-β signaling (Frederick and Wang, 2002).

In c-Ski transfected cells, which are resistant to TGF-β-induced cell growth inhibition (Luo *et al.*, 1999), reduction of *c-myc* mRNA expression by TGF-β (Sun *et al.*, 1999) and repression of TIE-driven

transcription by TGF-β are partially blocked (Suzuki *et al.*, 2004). Moreover, the TGF-β-induced deacetylation of histone H3 at the *c-myc* promoter (including the TIE) is blocked in these cells. c-Ski inhibits the TGF-β-induced repression of *c-myc* transcription by binding to the TIE together with Smad2-Smad4 or Smad3-Smad4 (Figs. 4 and 5A; Table I; Suzuki *et al.*, 2004). It strongly enhances the binding of Smad2-Smad4 to the TIE in the absence and presence of a constitutively active TGF-β type I receptor [ALK5(TD)]. Smad3-Smad4 binding to the TIE is strongly enhanced by c-Ski in the absence of ALK5(TD), but only weakly in its presence, in which Smad3-Smad4 bind already effectively without c-Ski. SnoN was shown to have the same effect on Smad2-Smad4 binding to the TIE (Fig. 5A; Suzuki *et al.*, 2004). It is unknown how this formation of Smad2/3-Smad4-c-Ski complexes blocks the TGF-β-induced repression of *c-myc* transcription or whether these complexes inhibit the formation of the Smad3/Smad4/E2F-4,5/DP-1/p107 complex and/or its binding to the TIE.

The TIE of the *c-myc* promoter is composed of an E2F-binding site bound by E2F–pocket protein complexes and a Smad binding site bound by Smad3–Smad4 (Fig. 4; Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). Strikingly, both pocket proteins (Ezhevsky *et al.*, 1997; Harbour and Dean, 2000; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Oswald *et al.*, 1994; Weinberg, 1995; Zhang and Dean, 2001) and Smad3 (Matsuura *et al.*, 2004) are phosphorylated by cyclin D/Cdk4 and cyclin E/Cdk2 resulting in inhibition of their ability to repress the *c-myc* promoter and thus in stimulation of *c-myc* transcription (Fig. 5A). (Yet it has to be noted that the cyclin/Cdk-dependent phosphorylation of Smad3 was not shown to target the TIE.) TGF-β represses the *c-myc* promoter by the Smad3–Smad4–E2F-4,5/DP-1/p107 complex through the TIE (Figs. 4 and 5A; Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). Altogether, these findings suggest that the TIE as well as Smad3–Smad4 and E2F–pocket protein complexes bound to it represent an important switch for control of the *c-myc* promoter. Proliferation signals that activate cyclin D/Cdk4 and cyclin E/Cdk2 and antiproliferative signals, like TGF-β, p16, or p21, could target them to turn on or off the *c-myc* promoter, respectively. This will be a general switch determining the state of the *c-myc* promoter, for example in terms of reentry into the cell cycle from quiescence (permissive) or exit from the cycle during the course of terminal differentiation (unpermissive), but no cell cycle-dependent regulation in continuously proliferating cells with constant *c-myc* transcription throughout the cell cycle.

TGF-β represses the *c-myc* promoter and thus the endogenous *c-myc* mRNA expression in HaCaT keratinocytes and in human MCF-10A mammary epithelial cells, but neither in MCF-10A(Ras/ErbB2) cells (MCF-10A

cells transformed by c-Ha-Ras oncogene and wild-type c-ErbB2) nor in human MDA-MB-231 breast cancer cells with a hyperactive Ras pathway (Chen *et al.*, 2001b). In accordance with Ras being able to prevent TGF-β growth inhibition (Calonge and Massagué, 1999; Filmus *et al.*, 1992; Houck *et al.*, 1989; Howe *et al.*, 1993; Longstreet *et al.*, 1992), this loss of *c-myc* repression by TGF-β in the oncogenically transformed MCF-10A(Ras/ErbB2) and MDA-MD-231 cells coincides with a loss of the TGF-β-induced cell cycle arrest whereas the normal MCF-10A and HaCaT cells are growth inhibited by TGF-β. TGF-β induces Smad4 binding to the TIE in the two normal cell lines whereas in the two transformed cell lines the association of Smad4 with the TIE in response to TGF-β is markedly decreased, which indicates that an activated Ras signaling pathway abolishes the repression of the *c-myc* promoter by TGF-β because it thwarts the TGF-β-induced formation of a repressive Smad complex on the TIE (Fig. 5A; Chen *et al.*, 2001b). Since these transformed MCF-10A(Ras/ErbB2) and MDA-MD-231 cells retain an otherwise active TGF-β/Smad pathway, such an activation of Ras signaling which is often found in cancer cells (McCormick, 1999), can render the *c-myc* promoter resistant to repression by TGF-β independent of inactivating mutations in the genes encoding TGF-β receptors or Smads (Chen *et al.*, 2001b).

Similarly, in Panc-1 pancreatic cancer cells with activated K-Ras, the Ras–MEK1/2c MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) kinase 1/2)–ERK1/2 cascade was reported to negatively affect TGF-β-induced Smad3 binding to the TIE (Fig. 5A; see Section IV.A.30; Buck *et al.*, 2006) and TGF-β failed to downregulate the *c-myc* transcript level in human SW480.7 colon carcinoma cells with an activated Ki-Ras oncogene (Calonge and Massagué, 1999).

TGF-β represses the *c-myc* promoter through the TIE by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002) so that Ras activation is likely to prevent repression of *c-myc* transcription by TGF-β by targeting this complex. Ras is known to interfere with the TGF-β/Smad pathway at multiple levels (Massagué, 2000, 2003; Massagué and Chen, 2000; Piek and Roberts, 2001). For example, Ras signaling decreases TGF-β receptor expression (Zhao and Buick, 1995) and ERK-mediated phosphorylation of Smad3 and Smad2 can inhibit their nuclear localization (Calonge and Massagué, 1999; Kretzschmar *et al.*, 1999), which each could explain the failure of TGF-β to repress *c-myc* transcription via the Smad3/Smad4/E2F-4,5/DP-1/p107 complex. In addition, p107 (Classon and Dyson, 2001) as well as Smad2 and Smad3 (Matsuura *et al.*, 2004), are inactivated by cyclin D/Cdk4 and cyclin E/Cdk2, which are activated by Ras that increases the level of cyclin D1 and reduces the level of p27 (Coleman *et al.*, 2004; Massagué, 2004; Takuwa and Takuwa, 2001). It appears conceivable that

such a Ras-stimulated inactivation of p107, Smad2, and Smad3 by cyclin D/Cdk4 and cyclin E/Cdk2 may inhibit the repression of the *c-myc* promoter by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex in response to TGF-β.

Smad3 targets at least two different sites in the *c-myc* promoter to mediate repression of *c-myc* by TGF-β: Through the TIE Smad3 represses the *c-myc* promoter via the Smad3/Smad4/E2F-4,5/DP-1/p107 complex in response to TGF-β (see Section IV.A.2; Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). At TBE3, Smad3 activated by ALK-5TD abrogates the transactivation of the *c-myc* promoter by β-catenin/TCF-4 by displacing β-catenin from TCF-4 and binding to TBE3 together with TCF-4 (see Section IV.A.1; Sasaki *et al.*, 2003). Thus, in response to TGF-β Smad3 not only represses the *c-myc* promoter through the TIE but also prevents at TBE3 its transactivation by β-catenin/TCF-4, an important activator of *c-myc* transcription (Fig. 5A). It is unknown whether Smad3 also disrupts the β-catenin/TCF-4 complexes at TBE1 and TBE3 to abolish the transactivation of the *c-myc* promoter by them.

4. METS

METS binds to six sites in the *c-myc* gene and represses the *c-myc* promoter (Fig. 4; Table I; Klappacher *et al.*, 2002). One METS binding site overlaps with the binding sites for E2F, ETS-1/2, NFATc1, KLF11, and STAT3 (Fig. 4), but is not required for repression of *c-myc*. METS possess an Ets-DBD and the METS consensus recognition site G-G-A-A-G/A-T/G includes the core ETS recognition sequence G-G-A-A. METS is (almost) not expressed in bone marrow progenitor cells, but highly expressed in terminally differentiated peritoneal macrophages. Thus its expression is strongly induced during M-CSF (macrophage colony stimulating factor)-stimulated differentiation of myeloid cells into macrophages, during which *c-myc* expression is repressed. ChIP analysis demonstrated occupancy of the *c-myc* promoter with ETS-1/2, E2F-1, and E2F-4, but not METS, p107, or p130, in proliferating bone marrow progenitor cells that highly express *c-myc* in contrast to occupancy with METS, E2F-4, p107, and p130, but not ETS-1/2 or E2F-1, in terminally differentiated peritoneal macrophages that do (almost) not express *c-myc* (Klappacher *et al.*, 2002). METS inhibits S-phase. It recruits the corepressor DP103, which interacts with NCoR, Sin3A, HDAC2, and HDAC5 and whose repression activity involves these four proteins as well as SMRT. Experiments in TKO MEFs lacking RB, p107, and p130 (Sage *et al.*, 2000) revealed that pocket proteins are required for both inhibition of S-phase and complete repression of the *c-myc* promoter by METS although they are dispensable for the intrinsic transrepression by GAL-METS and its corepressor GAL-DP103 (Klappacher *et al.*, 2002). Thus, induction of the ETS repressor METS during macrophage differentiation contributes to terminal cell cycle

arrest by repressing the transcription of cell cycle control genes including *c-myc*. The requirement of pocket proteins for both complete repression of the *c-myc* promoter and inhibition of S-phase by METS together with the ChIP results suggest that a combinatorial mechanism involving functional interactions between the METS/DP103 complex and E2F/pocket protein complexes is necessary to repress *c-myc* transcription and to achieve permanent cell cycle exit during terminal differentiation (Klappacher *et al.*, 2002).

5. C/EBPα

The balance between proliferation and differentiation is crucial for commitment of any cell type to a differentiation pathway. Since overexpression of c-Myc blocks terminal differentiation (Coppola and Cole, 1986; Dmitrovsky *et al.*, 1986; Freytag and Geddes, 1992; Freytag *et al.*, 1990; Iritani and Eisenman, 1999; Johansen *et al.*, 2001; Lin *et al.*, 2000; Miner and Wold, 1991; Prochownik and Kukowska, 1986) *c-myc* repression is a prerequisite for terminal differentiation (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Oster *et al.*, 2002). The differentiation program is so exquisitely sensitive to the level of c-Myc that in some cell systems downregulation of *c-myc* expression itself is sufficient to trigger differentiation (Facchini and Penn, 1998; Holt *et al.*, 1988; Johansen *et al.*, 2001; Oster *et al.*, 2002).

The transcription factor C/EBPα (CCAAT/enhancer binding protein α), which is induced in terminally differentiating cells and highly expressed in many differentiated cell types, is involved in late differentiation events and activates differentiation-specific genes (Johnson, 2005; Schuster and Porse, 2006). It plays a role in the differentiation of adipocytes, hepatocytes, and myeloid cells, which accordingly display high C/EBPα levels (Johnson, 2005; Lekstrom-Himes and Xanthopoulos, 1998). C/EBPα, a particularly potent regulator of cell cycle exit, blocks cell cycle progression at the G_1–S boundary and thus induces growth arrest (Johnson, 2005; Sebastian *et al.*, 2005; Schuster and Porse, 2006). As proliferation and differentiation are mutually exclusive, the proliferation factor c-Myc and the differentiation factor C/EBPα are antagonists, which show opposite expression patterns and reciprocally repress the transcription of their genes (Antonson *et al.*, 1995; Freytag and Geddes, 1992; Johansen *et al.*, 2001; Li *et al.*, 1994). In addition, c-Myc blocks the transactivation function of C/EBPα (Constance *et al.*, 1996; Mink *et al.*, 1996).

C/EBPα is essential for myeloblast commitment to and development into the granulocytic lineage (Lekstrom-Himes and Xanthopoulos, 1998). In order to impose cellular growth arrest and allow myeloid precursor cells to differentiate C/EBPα must repress *c-myc* expression, because c-Myc prevents cell cycle arrest and forces myeloblasts to remain in an

undifferentiated proliferative state whereas downregulation of c-Myc drives differentiation of myeloid cells into mature granulocytes (neutrophils) (Johansen *et al.*, 2001). C/EBPα represses the *c-myc* promoter that lacks any consensus C/EBPα-binding site through the E2F-binding site (Figs. 4 and 5A; Table I; Johansen *et al.*, 2001). It binds directly to E2F-1 and interacts also with E2F-2 and E2F-4, but does neither bind to the *c-myc* promoter nor disrupt E2F-1 binding to the *c-myc* promoter (Iakova *et al.*, 2003; Johansen *et al.*, 2001). Instead C/EBPα interferes with the transactivation of the *c-myc* promoter by E2F-1 (Fig. 5A). This inhibition occurs RB-independent because it was also observed in the RB-deficient SAOS osteosarcoma cells (Johansen *et al.*, 2001).

The liver is capable of completely regenerating itself in response to PH (partial hepatectomy), but this proliferative response is dramatically reduced in old animals (Fausto, 2000; Taub, 1996, 2004). *c-myc* is virtually not expressed in quiescent adult livers, but induction of *c-myc* plays a crucial role in regenerative liver proliferation after PH (Fausto, 2000; Taub, 1996). Accordingly, c-Myc expression is dramatically induced by PH in livers of young animals while no induction is observed in old animals (Iakova *et al.*, 2003). In livers of young mice, the *c-myc* promoter is occupied with E2F-1, E2F-4, and RB before PH whereas after PH E2F-1 and E2F-4 but not RB are found on the *c-myc* promoter (Iakova *et al.*, 2003). C/EBPα was never detected at the *c-myc* promoter in livers of young mice. In contrast, in livers of old mice, the *c-myc* promoter is occupied with E2F-1, E2F-4, RB, and C/EBPα both before and after PH. These findings indicate that in quiescent livers of young mice the *c-myc* promoter is repressed by an E2F-1/4-RB complex whereas after PH removal of RB allows its activation by E2F-1 or other transactivators, for example, STAT3 (see Section IV.A.6). However, in quiescent livers of old mice the *c-myc* promoter is repressed by a C/EBPα–RB–E2F-4–Brm complex (Fig. 5A) which blocks induction of *c-myc* transcription in response to PH as it remains on the promoter also after PH (Iakova *et al.*, 2003). This switch is due to (Iakova *et al.*, 2003; Timchenko, 2003): (1) a switch in the C/EBPα-induced growth arrest from Cdk inhibition (Wang *et al.*, 2001b) in young mice to repression of E2F-dependent transcription (Porse *et al.*, 2001) in old mice, (2) a dramatic reduction of C/EBPα expression after PH in young mice, but no alteration of C/EBPα levels in old mice (Timchenko *et al.*, 1998), and (3) a significant increase in Brm expression in livers of old mice. In quiescent livers of young animals, C/EBPα binds to and inhibits Cdk4 and Cdk2 whereas after PH strong induction of the cyclins D, E, and A leads to replacement of C/EBPα from these Cdks so that cyclin D/Cdk4 and cyclin E/Cdk2 phosphorylate RB, which releases E2F from RB as demonstrated for the *c-myc* promoter (Iakova *et al.*, 2003). In quiescent livers of old animals, the very high levels of Brm result in replacement of Cdk4 and Cdk2 from C/EBPα and in

formation of the C/EBPα–RB–E2F-4–Brm complex that inhibits E2F-dependent transcription. Since C/EBPα is not downregulated after PH in old animals this complex persists as demonstrated for the *c-myc* promoter (Iakova *et al.*, 2003).

C/EBPα binds via two different domains directly to RB and Brm (Chen *et al.*, 1996; Pedersen *et al.*, 2001; Porse *et al.*, 2001) and both of these domains are required for the interaction of C/EBPα with E2F-4 in the C/EBPα–RB–E2F-4–Brm complex (Iakova *et al.*, 2003). Since C/EBPα does not interact with E2F-4 in RB-deficient cells, RB is required for formation of this complex. Consistently, in RB-positive cells, where the *c-myc* promoter is occupied with E2F-1, E2F-4, and RB, induction of C/EBPα expression results in occupancy of the *c-myc* promoter with E2F-1, E2F-4, RB, and C/EBPα, whereas in RB-deficient cells the *c-myc* promoter remains occupied with only E2F-1 and E2F-4, but neither RB nor C/EBPα also after induction of C/EBPα expression (Iakova *et al.*, 2003). In accordance, induction of C/EBPα inhibits *c-myc* expression (Timchenko *et al.*, 1999) in RB-positive cells, but not in RB-deficient cells (Iakova *et al.*, 2003). Remarkably, this finding of Iakova *et al.* (2003) that repression of *c-myc* transcription by C/EBPα is RB-dependent is in clear contrast to the result of Johansen *et al.* (2001) that C/EBPα represses the *c-myc* promoter RB-independently, a discrepancy that cannot be attributed to use of different cell lines because RB-deficient SAOS cells were utilized in both studies.

6. STAT3

STAT3 is a mediator of cell proliferation and survival that participates in cellular transformation and tumorigenesis (Bromberg, 2002; Bromberg *et al.*, 1999; Haura *et al.*, 2005; Turkson, 2004; Yu and Jove, 2004). It is a latent transcription factor. In response to cytokine and growth factor signaling, STAT3 is rapidly activated by tyrosine phosphorylation resulting in its dimerization, translocation to the nucleus, DNA binding, and transactivation of target genes (Ihle, 2001; Levy and Darnell, 2002; O'Shea *et al.*, 2002; Rane and Reddy, 2002).

The STAT3 binding site of the *c-myc* promoter (Fig. 4; Kiuchi *et al.*, 1999) overlaps with the binding sites for E2F, ETS-1/2, NFATc1, KLF11, and METS. STAT3 was shown to bind to this site in response to leptin (Yin *et al.*, 2004), IL-6, and IL-2 as well as after stimulation of a chimeric receptor comprising the transmembrane and cytoplasmic domains of gp130 (Table I; Barré *et al.*, 2003; Kiuchi *et al.*, 1999). IL-6 and other cytokines of the IL-6 family that share the membrane glycoprotein gp130 as a signal transducing receptor component trigger homo- or heterodimerization of gp130, which leads to activation of associated cytoplasmic JAK (Janus kinase) family tyrosine kinases and subsequent phosphorylation and activation of STAT3

(Hirano *et al.*, 1997; Kamimura *et al.*, 2003; Taga and Kishimoto, 1997). In response to IL-6, also STAT1 binding to the STAT3 site of the *c-myc* promoter was observed (Kiuchi *et al.*, 1999).

IL-6 treatment activates the *c-myc* promoter via the pathway IL-6 → gp130 → STAT3 → *c-myc* promoter (Fig. 5B; Barré *et al.*, 2003; Kiuchi *et al.*, 1999). In accordance, IL-6 stimulation induces occupancy of the *c-myc* promoter with STAT3, Pol II, CBP, and SRC-1 (Barré *et al.*, 2003). Independent of IL-6 stimulation the TAD of STAT3 interacts with the HAT and coactivator p300/CBP, which is required for transcriptional activation by STAT3 (Nakashima *et al.*, 1999; Paulson *et al.*, 1999). In addition, IL-6 induces binding of STAT3 to the coactivator SRC-1, which potentiates transcriptional activation by STAT3 and directly binds to its TAD (Giraud *et al.*, 2001; Yin *et al.*, 2004). SRC-1/NcoA-1, a member of the SRC (steroid receptor coactivator) family, also recruits p300/CBP (Sheppard *et al.*, 2001).

Activation of *c-myc* transcription by IL-6 via STAT3 is essential for regenerative liver proliferation after PH (Cressman *et al.*, 1996): IL-6(–/–) mice had impaired liver regeneration and showed reduction of S- and M-phase hepatocytes. Hepatectomized IL-6(–/–) livers, which failed to activate STAT3, showed a significant reduction in expression of *c-myc* mRNA and other immediate-early genes. Preoperative IL-6 treatment of IL6(–/–) mice restored hepatocyte proliferation, STAT3 DNA binding and expression of *c-myc* mRNA and other immediate-early genes in livers after PH to near normal.

Serum stimulation induces occupancy of the *c-myc* promoter with CBP and Pol II (Barré *et al.*, 2003). Yet this could not only result from STAT3 binding to the *c-myc* promoter (Barré *et al.*, 2003) but also from binding of other transactivators, for example E2F-1, NFATc1, or ETS-1/2, which have overlapping binding sites (Fig. 4) and all are known to recruit CBP (Foulds *et al.*, 2004; Fry *et al.*, 1999; Kawamura *et al.*, 2004; Trouche and Kouzarides, 1996; Trouche *et al.*, 1996; Yang *et al.*, 1998).

Leptin, an adipocyte-derived cytokine, stimulates cell proliferation (Yin *et al.*, 2004). Upon binding to the long isoform of its receptor (ObRb), leptin activates STAT3 via JAK2 (Banks *et al.*, 2000) and induces binding of STAT3 to SRC-1 (Yin *et al.*, 2004). Leptin activates the *c-myc* promoter, which most likely occurs through STAT3, because leptin induces occupancy of the *c-myc* promoter with STAT3 and SRC-1 and because overexpression of SRC-1 augments stimulation of the *c-myc* promoter by leptin (Fig. 5B; Yin *et al.*, 2004).

A constitutively active mutant form of STAT3 that causes cellular transformation of rat 3Y1 immortalized fibroblasts increases the endogenous *c-myc* mRNA expression in 3Y1 cells (Bromberg *et al.*, 1999).

PDGF (platelet-derived growth factor) activates *c-myc* transcription via the pathway PDGF → PDGFR (PDGF receptor) → c-Src → STAT3 → *c-myc*

transcription, which plays an important role for PDGF-induced normal cell proliferation and in v-Src-induced oncogenic transformation (Fig. 5B; Bowman *et al.*, 2001). STAT3 exists in a complex with PDGF receptors and c-Src and is rapidly activated during PDGF stimulation in a c-Src kinase-dependent manner (Wang *et al.*, 2000). Consistently, PDGF and c-Src activate a *c-myc* promoter construct (–157 to +500 relative to the P1 transcription start site) (Chiariello *et al.*, 2001) that possesses the STAT3 binding site. Prior to delineation of this pathway (Bowman *et al.*, 2001), parts of it and their importance for normal PDGF-stimulated mitogenesis as well as for cell transformation by v-Src have been described (e.g., Barone and Courtneidge, 1995; Blake *et al.*, 2000; Bromberg *et al.*, 1998, 1999; Broome and Hunter, 1996; Cao *et al.*, 1996; Chiariello *et al.*, 2001; Kiuchi *et al.*, 1999; Shirogane *et al.*, 1999; Turkson *et al.*, 1998; Twamley-Stein *et al.*, 1993; Wang *et al.*, 2000; Yu *et al.*, 1995).

The oncogene Src activates the *c-myc* promoter and in accordance HT1080 fibrosarcoma cells stably expressing Src display elevated *c-myc* mRNA and protein levels compared to wild-type cells (Vigneron *et al.*, 2005). p21 inhibits this Src-dependent activation of the *c-myc* promoter and *c-myc* expression. p21 is recruited to the region of the *c-myc* promoter harboring the STAT3 binding site and associates with STAT3 on the *c-myc* promoter so that it does not affect the DNA binding by STAT3 (Vigneron *et al.*, 2005). Transcriptional activation by STAT3 is known to be suppressed by p21 that interacts with both STAT3 and p300/CBP (Coqueret and Gascan, 2000). Since p300/CBP, which interacts with STAT3, is required for transcriptional activation by STAT3 (Nakashima *et al.*, 1999; Paulson *et al.*, 1999) p21 is thought to interfere with the coactivator and HAT p300/CBP (Coqueret and Gascan, 2000). As CBP is recruited to the *c-myc* promoter together with STAT3 (Barré *et al.*, 2003), it seems conceivable that p21 may act similarly in repression of the *c-myc* promoter (Fig. 5B).

Kirito *et al.* (2002) suggested that EPO (erythropoietin), the major regulator of proliferation and differentiation of erythroid progenitors, may activate the *c-myc* promoter through the STAT3 binding site via the pathway EPO → EPOR (EPO receptor) → STAT1/3 → *c-myc* promoter. Kiuchi *et al.* (1999) reported that STAT1 can bind to the STAT3 binding site of the *c-myc* promoter (Table I).

7. FBP

FBP (FUSE binding protein) binds via its four KH [(hnRNP) K homology] domains to the FUSE (far upstream element) of the *c-myc* promoter and transactivates it (Fig. 4; Table I; Avigan *et al.*, 1990; Bazar *et al.*, 1995a,b; Braddock *et al.*, 2002; Chung and Levens, 2005; Chung *et al.*, 2006; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1994, 1996; He *et al.*, 2000a; Huth *et al.*,

2004; Kouzine *et al.*, 2004; Levens *et al.*, 1997; Liu and Levens, 2006; Liu *et al.*, 2000, 2001, 2006a; Michelotti *et al.*, 1996b). FBP binds to the non-coding single-strand of the FUSE, but not to its relaxed duplex form, and the FUSE embedded in supercoiled DNA is also bound by FBP (Bazar *et al.*, 1995b; Braddock *et al.*, 2002; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b). The FUSE is only single-stranded, if *c-myc* is expressed, but otherwise in duplex conformation, so that FBP binds to active, but not to inactive *c-myc* promoters (Avigan *et al.*, 1990; Bazar *et al.*, 1995a; Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b). In accordance, the FUSE retains single-stranded character throughout the cell cycle (Levens *et al.*, 1997). Negative supercoiling upstream of a promoter and DNA separation into single strands are known to be induced by transcription. Like *c-myc*, *fbp* is an immediate-early response gene, the expression of which does not require *de novo* protein synthesis, and both *fbp* mRNA (3 h) and protein (1.5 h) have brief half-lives (Bazar *et al.*, 1995a). FBP expression parallels that of c-Myc (Bazar *et al.*, 1995a; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1994): Both genes are expressed in proliferating cells during the whole cell cycle, but neither in quiescent nor in differentiated cells. The expression of FBP and c-Myc decline with similar kinetics during both PMA (phorbol 12-myristate 13-acetate)-induced and TPA-induced differentiation of HL-60 cells while during re-entry into the cell cycle of normal resting T lymphocytes and serum-starved fibroblasts a dramatic rise in *fbp* mRNA expression correlates with induction of *c-myc* transcription. The FUSE is nucleosome free and occupied by FBP in continuously *c-myc* expressing U2OS osteosarcoma and SW13 adrenal cortical carcinoma cells, whereas in *c-myc* non-expressing IMR23 neuroblastoma cells the double-stranded FUSE (Michelotti *et al.*, 1996b) is FBP free and wrapped over nucleosome 5 (Liu *et al.*, 2006a). In summary, FBP binding to the FUSE is limited to *c-myc* expressing proliferating cells by nucleosomal organization and DNA conformation of the FUSE as well as by FBP expression. FUSE binding by FBP is thought to be reflected by the appearance of a nuclease-resistant nucleosomal spacer region between nucleosomes 4 and 5 (see Section III.D; Fig. 3A; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b; Pullner *et al.*, 1996).

FBP binding to the FUSE of active *c-myc* promoters is essential for *c-myc* transcription because loss or inhibition of FBP function shut off transcription from both the P1 and P2 promoters and arrest cellular proliferation (He *et al.*, 2000a). Thus, the large set of other transcription factors that bind to and transactivate the *c-myc* promoter fails to sustain *c-myc* transcription in the absence of FBP (due to antisense mRNA) or if FBP function is extinguished (due to overexpression of a dominant-negative form). Consequently, FBP appears to function as a master regulator of the *c-myc* promoter (He *et al.*, 2000a). However, the single-stranded conformation of the FUSE represents

the prerequisite for FBP binding (Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b). The dynamic torsional stress resulting from ongoing transcription is sufficient to drive FUSE melting (Kouzine *et al.*, 2004). Thus, *c-myc* transcription needs to be started before its master regulator FBP can bind to the single-stranded FUSE (He *et al.*, 2000a; Levens *et al.*, 1997; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b).

The function of FBP as the master regulator of *c-myc* transcription correlates with the findings that it binds directly to the subunits p62, p80, and p89 of TFIIH and that its C-terminal TAD (Chung *et al.*, 2006; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1996) enhances the 3′–5′ helicase activity of p89, which is essential for transcription (Liu *et al.*, 2001; Tirode *et al.*, 1999). TFIIH is required at several steps during open complex formation, initiation, and promoter clearance (Coin and Egly, 1998; Egly, 2001; Svejstrup *et al.*, 1996). It is believed that by targeting TFIIH FBP is capable of governing the diverse inputs of the remainder of transcription factors to the basal transcription machinery (Chung and Levens, 2005; He *et al.*, 2000a; Liu and Levens, 2006; Liu *et al.*, 2000, 2001; Weber *et al.*, 2005).

This stimulation of the p89 helicase by FBP is counteracted by FIR (FBP interacting repressor), which binds directly to the p89 subunit of TFIIH and suppresses its 3′-5′ helicase activity (Chung and Levens, 2005; Liu and Levens, 2006; Liu *et al.*, 2000). FIR possesses tandem RRMs (RNA recognition motif), structural motifs binding single-stranded nucleic acids (usually RNA, but sometimes DNA), and a RRM-like U2AF homology motif (Kielkopf *et al.*, 2004). FIR directly binds to FBP, which is mediated by the two RRMs of FIR and a predicted amphipathic α-helix in the N-terminus plus (several possible) combinations of the four KH domains in the central DBD of FBP (Chung *et al.*, 2006; Liu *et al.*, 2000). FIR enhances the binding of FBP to the FUSE (Liu *et al.*, 2000) and the FUSE enhances the interaction between FBP and FIR (Chung *et al.*, 2006). Moreover, FIR represses the transactivation of an artificial promoter by the C-terminal TAD of FBP (Liu *et al.*, 2000). Consistently, FIR represses the *c-myc* promoter through the FUSE (Fig. 5A; Liu *et al.*, 2000) as well as the endogenous *c-myc* mRNA and protein expression (Matsushita *et al.*, 2006). FBP stimulates both initiation and promoter escape whereas FIR suppresses only the latter one (Liu *et al.*, 2000, 2001). FBP and FIR at the FUSE and TFIIH at the P2 (and P1) promoter are connected through a p89-dependent protein–protein bridge resulting in a FUSE-P2 loop or FBP/FIR-TFIIH loop that is lost in p89-mutant cells (Liu *et al.*, 2006a). FIR binds also directly to the FUSE (Fig. 4; Table I; Liu *et al.*, 2006a). Like FBP, FIR binds to the FUSE embedded in supercoiled DNA, but not to the relaxed duplex FUSE. If the FUSE is relaxed following its supercoiling FIR binding remains at least partially, while FBP binding is shut off (Liu *et al.*, 2006a). Like in the case of FBP, in continuously *c-myc* expressing U2OS and SW13 cells the nucleosome-free

FUSE is occupied by FIR whereas in *c-myc* non-expressing IMR23 cells the nucleosome-wrapped double-stranded FUSE (Michelotti *et al.*, 1996b) is not bound by FIR (Liu *et al.*, 2006a). Alone, much larger amounts of FIR than FBP are required to bind the FUSE *in vitro* (Chung *et al.*, 2006) and FBP is approximately five times more abundant than FIR in living cells (Liu *et al.*, 2006a) so that *in vivo* the melted FUSE is expected to be bound either by FBP alone or by FBP and FIR together, but not by FIR alone.

Since FBP as well as FIR target the p89 helicase of TFIIH both transactivation by FBP and repression by FIR are abolished in p89-mutant cells (Liu *et al.*, 2000, 2001) so that *c-myc* transcription, which is under antagonistic control by FBP and FIR, is affected in these cells (Liu *et al.*, 2001, 2006a; Weber *et al.*, 2005). In such continuously cycling cells, the mean steady-state levels of *c-myc* mRNA and c-Myc protein are only modestly elevated but the range of expression and its cell-to-cell variation are markedly increased indicating impaired fine tuning of *c-myc* (Weber *et al.*, 2005). In these cells, the zone of promoter proximal pausing of Pol II is diminished to the region between nucleotides +1 to +50 relative to P2 whereas reconstitution of wild-type p89 results in expansion of this zone until nucleotide +100 relative to P2 (Weber *et al.*, 2005).

In the XP (Xeroderma Pigmentosum) complementation group XPB inherited mutations of the TFIIH 3′–5′ helicase p89 (XPB, ERCC3) yield overlapping DNA repair and transcription syndromes. The high risk of cancer connected with this mutated p89/XPB seems to result from the combination of defects in NER (nucleotide excision repair) (Araujo *et al.*, 2000), p53-mediated apoptosis (Wang *et al.*, 1996a) and regulation of *c-myc* transcription (Liu *et al.*, 2001).

The FUSE–FBP–FIR–TFIIH system is thought to be important for fine tuning of *c-myc* transcription in order to suppress the intrinsic and extrinsic transcriptional noise and to ensure the steady *c-myc* output required for cellular homeostasis (Chung and Levens, 2005; He *et al.*, 2000a; Kouzine *et al.*, 2004; Liu and Levens, 2006; Liu *et al.*, 2000, 2001; Weber *et al.*, 2005): As the FUSE changes from duplex to single-strand conformation in response to the dynamic torsional stress from ongoing transcription FUSE melting has the capacity to serve as a real-time measure of *c-myc* promoter activity (Kouzine *et al.*, 2004). The single-strand-specific FBP and its antagonist FIR operate through TFIIH, and in particular through opposite effects on its p89 helicase, to activate or repress *c-myc* transcription, respectively (Liu *et al.*, 2000, 2001). Thereby the feedback loop of the FUSE–FBP–FIR–TFIIH system provides a fast mode for real-time regulation of *c-myc* transcription that is directly coupled to the current *c-myc* promoter activity. However, FBP can only modulate the expression of active *c-myc* genes whereas it is not suited for mediating the primary switch between silent and active states of the *c-myc* promoter, that is, FBP is unable to switch on a

silent *c-myc* gene (Chung and Levens, 2005; He *et al.*, 2000a; Liu *et al.*, 2006a).

FIR has no protein siblings (Liu *et al.*, 2006a), but exists in four splice variants that arise from alternative splicing of the two optional exons 2 and 5 (Matsushita *et al.*, 2006). FIR comprises exon 2, but not exon 5. The splice variant PUF60, which in contrast contains exon 5, is involved in pre-mRNA splicing (Kielkopf *et al.*, 2004). PUF60 represses *c-myc* transcription as efficiently as FIR (Matsushita *et al.*, 2006). In contrast, the splice variant FIRΔexon2, which lacks exon 2, fails to suppress *c-myc* mRNA and protein expression. Moreover, it abrogates *c-myc* suppression by FIR suggesting a dominant-interfering effect (Fig. 5A; Matsushita *et al.*, 2006). c-Myc is overexpressed in the majority of colon tumors due to deregulation of *c-myc* expression (Erisman *et al.*, 1985). Surprisingly, *fir* mRNA and protein expression were found to be upregulated in colorectal cancers and this high level of *fir* mRNA was positively correlated with a high level of *c-myc* mRNA (Matsushita *et al.*, 2006). *firΔexon2* mRNA expression was detected only in these human primary colorectal tumors (adenocarcinomas), but not in the adjacent normal tissue. FIRΔexon2 is suggested to prevent repression of *c-myc* transcription by FIR in colon cancers thereby disabling control of the *c-myc* promoter by the FUSE–FBP–FIR–TFIIH system and thus allowing deregulation of *c-myc* (Matsushita *et al.*, 2006). In tumors, which are addicted to a high c-Myc level, its reduction can cause apoptosis (Felsher and Bishop, 1999a; Shachaf and Felsher, 2005a,b; Shachaf *et al.*, 2004). In SW480 colon and HeLa cervix carcinoma cells enforced expression of FIR induces apoptosis whereas coexpression of FIRΔexon2, which alone fails to induce apoptosis, abrogates this FIR-driven cell death (Matsushita *et al.*, 2006). Also enforced expression of c-Myc prevents FIR-induced apoptosis in HeLa cells suggesting that repression of *c-myc* mediates this FIR-driven apoptosis. Thus the tumor-specific splice variant FIRΔexon2 may promote development of colorectal cancers by enabling cells to escape FIR-mediated repression of *c-myc* transcription and FIR-induced apoptosis thereby providing tumor cells with a growth advantage over their normal neighbors (Matsushita *et al.*, 2006). It would be interesting to analyze whether FIRΔexon2 is expressed in other tumors, too, and how the tumor-specific alternative splicing of FIR is regulated.

FBP is identical to HDH V (human DNA helicase V) (Vindigni *et al.*, 2001). Three FBP family members exist, namely FBP that is also termed FBP1, FBP2, and FBP3 (Chung *et al.*, 2006; Davis-Smyth *et al.*, 1996). FBP2, also known as KSRP (KH-type splicing regulatory protein), plays a role in mRNA splicing, RNA trafficking, RNA editing as well as mRNA stabilization and degradation (Min *et al.*, 1997). The three FBP family members are widely expressed but to varying absolute and relative amounts in different cells and tissues (Davis-Smyth *et al.*, 1996). Upon induced differentiation of

promonocytic U937 and promyelocytic HL-60 leukemia cells, *fbp* mRNA expression is rapidly shut off (Bazar *et al.*, 1995a; Duncan *et al.*, 1994) while *fbp2* and *fbp3* mRNA levels decline more slowly (Davis-Smyth *et al.*, 1996). All three FBP family members bind with high affinity only to the non-coding single-strand of the FUSE, but neither to its coding single-strand nor to the double-stranded FUSE (Table I; Davis-Smyth *et al.*, 1996). Like FBP (Duncan *et al.*, 1996), FBP2 and FBP3 possess tyrosine-rich C-terminal TADs and transactivate the *c-myc* promoter (Chung *et al.*, 2006; Davis-Smyth *et al.*, 1996). In general, FBP2 displays functional features similar to those of FBP whereas FBP3 deviates significantly from the former two (Chung *et al.*, 2006). (1) The strength of the C-terminal TADs of FBP and FBP2 is medium or weak, respectively, whereas FBP3 possesses by far the most potent TAD. (2) The N-terminal repression domains of FBP and FBP2 can repress the transactivation by the C-terminal TAD of FBP independent of FIR recruitment. In contrast, the N-terminal domain of FBP3 is unable to repress this transactivation by the FBP TAD and fails also to repress the transactivation by its own C-terminal TAD. (3) FBP and FBP2, but not FBP3 bind to FIR, which can be attributed to FBP3-specific amino acid substitutions in the predicted amphipathic α-helix in the N-terminus that is required for FIR binding. (4) Consequently, FBP3 is by far the most potent transactivator of the *c-myc* promoter (FBP2 is weaker than FBP). (5) In contrast to the nuclear localization of FBP (He *et al.*, 2000b) and FBP2, FBP3 is present in both cytoplasm and nucleus (Chung *et al.*, 2006). (6) Nuclear FBP3 has slower FRAP (fluorescence recovery after photobleaching) kinetics than FBP and FBP2 (Chung *et al.*, 2006), the former of which is already considerably slower moving than other transcription factors (Phair *et al.*, 2004). These differences of FBP3 versus FBP (and FBP2) offer the possibility for an additional control of the *c-myc* promoter by regulating the exchange of FBP (or FBP2) against FBP3 at the FUSE: FBP3 is a pure strong transactivator of the *c-myc* promoter, which cannot be repressed by FIR, and thus is equipped to drive *c-myc* transcription to high levels (Chung *et al.*, 2006), for example during the strong serum-induction of *c-myc* expression in quiescent cells that do virtually not express *c-myc* (see below). In contrast, FBP is a weaker transactivator of the *c-myc* promoter and can be repressed by FIR so that it is together with FIR well suited to define a narrow dynamic range of *c-myc* expression (Chung *et al.*, 2006), for example in continuously proliferating cells, which express *c-myc* mRNA at a constant low level throughout the cell cycle. Accordingly, in such continuously cycling cells, the FUSE is occupied by both FBP and FIR (Liu *et al.*, 2006a). The FUSE binding of individual FBP family members is determined by the ratio of their nuclear amounts (Chung *et al.*, 2006) and by the ratio of their affinities for the FUSE. The former parameter could be regulated through nuclear-cytoplasmic shuttling as well as through mRNA and protein synthesis and degradation

(Chung *et al.*, 2006). The latter parameter could be regulated through protein modifications and interaction partners [e.g., FIR enhances FBP binding to the FUSE (Liu *et al.*, 2000)]. So far, these pathways regulating the FBP family have remained largely unexplored.

Regulation of the *c-myc* promoter through exchange of FBP3 versus free FBP versus FIR-repressed FBP at the FUSE was nicely demonstrated by serial ChIP assays during serum stimulation of starved human Hs68 primary fibroblasts (Fig. 6; Chung *et al.*, 2006; Liu *et al.*, 2006a). Since the single-stranded conformation of the FUSE represents the prerequisite for binding of FBP (Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Michelotti *et al.*, 1996b), FBP2, and FBP3 (Davis-Smyth *et al.*, 1996) and since the dynamic torsional stress resulting from ongoing transcription is sufficient to drive FUSE melting (Kouzine *et al.*, 2004) *c-myc* transcription needs to be started before FBP can bind to the single-stranded FUSE (He *et al.*, 2000a; Levens *et al.*, 1997; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b). In quiescent fibroblasts virtually not expressing *c-myc*, the *c-myc* promoter is occupied by paused Pol II, but by none of the transcription factors examined (Fig. 6; Chung *et al.*, 2006; Liu *et al.*, 2006a). *c-myc* mRNA expression peaks 2 h after serum refeeding and then declines rapidly (Liu *et al.*, 2006a). Thirty minutes after serum addition, Sp1, YY1, E2F-1, and E2F-4, but neither FBP nor FIR are found on the *c-myc* promoter (FBP3 not examined; Liu *et al.*, 2006a). One hour post serum stimulation when the *c-myc* mRNA level rises the FUSE is maximally bound by FBP3, but (almost) not by FBP (FIR not examined; Chung *et al.*, 2006). Two hours after serum refeeding at the peak of *c-myc* mRNA expression Sp1, YY1, E2F-1, and E2F-4 still bind to the *c-myc* promoter, FBP binding to the FUSE is at maximum and FIR starts to appear while FBP3 has for the most part left (Chung *et al.*, 2006; Liu *et al.*, 2006a). Four hours post serum addition when the *c-myc* mRNA level drops FIR binding to the FUSE reaches its maximum, a reduced amount of FBP as well as E2F-1 and E2F-4 remain on the *c-myc* promoter, but FBP3, Sp1, and YY1 have disappeared (Chung *et al.*, 2006; Liu *et al.*, 2006a). At this time point also TCF-4 binds to the *c-myc* promoter, which is not detected earlier (Liu *et al.*, 2006a). In contrast, Pol II is present on the *c-myc* promoter before, throughout, and after serum stimulation (Liu *et al.*, 2006a) consistent with transcription from the P2 promoter being predominantly regulated at the level of elongation (see Section III.C). This succession (Fig. 6) suggests that Sp1, YY1, and E2F-1 induce *c-myc* transcription in response to serum so that the resulting torsional stress drives FUSE melting, which allows FBP and FBP3 binding (Liu *et al.*, 2006a). The strong transactivator FBP3 ensures an uninterrupted ascent to peak levels of *c-myc* transcription and then the weaker transactivator FBP replaces FBP3 to modulate the shape of the induced peak in *c-myc* transcription at its apex (Chung *et al.*, 2006). Finally, the late-recruited repressor FIR diminishes

c-myc transcription so that FBP3, FBP, and FIR act sequentially at the FUSE to generate the serum-induced pulse of *c-myc* transcription (Fig. 6; Chung *et al.*, 2006; Liu *et al.*, 2006a). However, they have to rely on Sp1, YY1, E2F-1, or other transactivators for the initial induction of *c-myc* transcription, which is required to mediate FUSE melting and thus to enable subsequent binding of FBP3 and FBP.

This finding that the consecutive function of FBP3, free FBP, and FIR-repressed FBP is necessary for the peak in *c-myc* transcription during serum stimulation of quiescent cells was verified by siRNA-mediated knockdown of FBP and FIR as well as in p89-mutant cells with combined loss of FBP/FBP3 activation and FIR repression (Liu *et al.*, 2006a): Starved p89-mutant cells respond to serum with a broad plateau of *c-myc* mRNA expression instead of the typical sharp peak in *c-myc* mRNA level followed by a rapid decline in normal human Hs68 primary fibroblasts. Thus in p89-mutant cells both the FBP3/FBP-mediated initial enhancement and the subsequent FIR-mediated repression of *c-myc* transcription seem to be abolished (Liu *et al.*, 2006a). Similar effects were observed in FIR depleted cells. Reduction of FBP in the presence of normal levels of the more potent transactivator FBP3 increases the amplitude of the serum-induced peak of *c-myc* mRNA expression (Liu *et al.*, 2006a), as expected if FBP is not available to delimit activation by FBP3 (Chung *et al.*, 2006). In contrast, it does not affect the subsequent drop of the *c-myc* mRNA level (Liu *et al.*, 2006a) probably because FBP2 substitutes for FBP in FIR recruitment. The finding that the FUSE–FBP–FIR–TFIIH system functions to intensify ongoing transcription as well as to limit its boost was also confirmed by reprogramming of transcription from a heterologous promoter by the *c-myc* FUSE (Liu *et al.*, 2006a): Insertion of the *c-myc* FUSE strongly enhanced transcription from this promoter resulting in a sharp peak followed by a rapid decline. This intensifying function of the FUSE depended on its position as well as on the strength of ongoing transcription, which needed to generate enough dynamic supercoiling to melt the FUSE and thus to allow binding of FBP, FBP3, and FIR. In summary, during serum stimulation of starved cells the FUSE–FBP–FIR–TFIIH system operates to govern the peak in *c-myc* transcription (Chung *et al.*, 2006; Liu *et al.*, 2006a).

p38, also known as JTV-1 and AIMP2, is a scaffold required for the assembly and stability of a multi-aminoacyl-tRNA-synthetase complex (Kim *et al.*, 2002). In addition, p38 directly binds to the C-terminal TAD of FBP, stimulates the ubiquitination of FBP and thus its 26S proteasome-mediated degradation resulting in reduced FBP protein levels that in turn lead to decreased *c-myc* mRNA and protein expression (Kim *et al.*, 2003a). p38 reduces cell proliferation and this antiproliferative activity is abolished by ectopic c-Myc expression implying that p38 controls cell proliferation mainly through *c-myc*. Accordingly, p38-deficient mice show severe

hyperplasia in various organs as well as defects in lung and thymocyte differentiation. TGF-β induces the expression of p38 and promotes its translocation into the nucleus. This TGF-β-dependent increase of p38 was shown to suppress the expression of FBP and c-Myc (Kim *et al.*, 2003a). During normal lung differentiation, *c-myc* expression is repressed (Chinoi *et al.*, 2001) and the study of Kim *et al.* (2003a) strongly suggests that repression of *c-myc* transcription by TGF-β via the pathway TGF-$\beta \rightarrow$ p38 —| FBP $\rightarrow$ *c-myc* transcription (Fig. 5A) is required to allow lung differentiation. p38 interacts also with FBP2 and FBP3, but less strongly than with FBP (worst with FBP3) (Chung *et al.*, 2006).

8. THE CT-ELEMENT/NHE: REGULATION BY TRANSCRIPTION FACTORS AND NON-B-DNA CONFORMATIONS

The CT-element (Figs. 4 and 7), positioned approximately at –150 to –100 relative to the transcription start site (+1) of the P1 promoter, encompasses five in part imperfect repeats of the sequence CCCTCCCC (CT-element). The CT-element is also termed NHE, as it correlates with the DNAse I-hypersensitive site III_1. Sometimes the NHE was defined as a part of the CT-element that is positioned at –142 to –115 relative to the P1 transcription start and comprises three runs of four cytosines and two runs of three cytosines. The CT-element/NHE is nucleosome free at active *c-myc* promoters, but is in nucleosomal conformation at inactive uninducible *c-myc* genes (Fig. 3A; see Section III.D; Albert *et al.*, 1997, 2001; Pullner *et al.*, 1996). The CT-element/NHE can form duplex as well as single-strand DNA (Levens *et al.*, 1997; Michelotti *et al.*, 1996b) or assume H-DNA conformation (Cooney *et al.*, 1988; Davis *et al.*, 1989; Wang and Vasquez, 2004). In addition, it can adopt paranemic DNA structures: Its polypurine antisense strand can form a G-quadruplex while its complementary polypyrimidine sense strand can form an *i*-tetraplex (see Section IV.A.12). These non-B-DNA conformations play important roles in regulation of the *c-myc* promoter (Grand *et al.*, 2002, 2004; Levens *et al.*, 1997; Liu and Levens, 2006; Michelotti *et al.*, 1996b; Siddiqui-Jain *et al.*, 2002; Tomonaga *et al.*, 1998). Many different transcription factors bind to the CT-element/NHE (Figs. 4 and 7; Chung and Levens, 2005; Levens *et al.*, 1997; Liu and Levens, 2006; Michelotti *et al.*, 1996b): For example, Sp1 binds to its duplex form (see Section IV.A.16), whereas hnRNP K (see Section IV.A.10) and CNBP (see Section IV.A.9) bind to its C-rich or G-rich single-strands, respectively. NM23-H2 (human nonmetastatic 23 isoform 2 protein) binds to its duplex form as well as to both single-strands (see Section IV.A.11).

9. CNBP

CNBP (cellular nucleic acid binding protein) binds to the purine-rich single-strand of the CT-element and activates the *c-myc* promoter (Figs. 4 and 7; Table I; Chen *et al.*, 2003; Michelotti *et al.*, 1995; Shimizu *et al.*, 2003). It stimulates cell proliferation (Chen *et al.*, 2003; Shimizu *et al.*, 2003). In the anterior region of CNBP$^{-/-}$ mouse embryos a substantial reduction in cell proliferation was found to correlate with the absence of *c-myc* expression (Chen *et al.*, 2003). CNBP plays a role in embryonic development, especially for anterior patterning, forebrain induction and specification and tissue patterning during anterior–posterior axis, craniofacial and limb development (Chen *et al.*, 2003; Shimizu *et al.*, 2003). It was suggested that *c-myc* may be an important CNBP target gene during embryonic development.

10. HNRNP K

hnRNP K (heterogeneous nuclear ribonucleoprotein K) binds via its KH motifs to the pyrimidine-rich single-strand of the CT-element, but not to its duplex form, and activates the *c-myc* promoter (Figs. 4 and 7; Table I; Davis *et al.*, 1989; Huth *et al.*, 2004; Mandal *et al.*, 2001; Michelotti *et al.*, 1996a,b; Takimoto *et al.*, 1993; Tomonaga and Levens, 1995, 1996; Tomonaga *et al.*, 1998). Accordingly, hnRNP K increases the endogenous *c-myc* mRNA and protein expression (Lynch *et al.*, 2005). It is present throughout the cell cycle (Dejgaard *et al.*, 1994) and interacts directly with TBP (Michelotti *et al.*, 1996a).

Serum stimulation of rat TGR and rat hepatoma HTC-IR cells induces binding of hnRNP K to the *c-myc* promoter strongly suggesting that it may be involved in the serum-induced activation of *c-myc* transcription (Lynch *et al.*, 2005; Ostrowski *et al.*, 2003). The finding that also the transcribed regions of *c-myc* in exons 2 and 3 were occupied with hnRNP K (Ostrowski *et al.*, 2003) could point to an additional involvement of hnRNP K in *c-myc* mRNA processing and transport (Michelotti *et al.*, 1996a). As component of the hnRNP (heterogeneous nuclear ribonucleoprotein) particle and as pre-mRNA-binding protein hnRNP K is known to facilitate various stages of mRNA biogenesis such as pre-mRNA splicing, nucleocytoplasmic mRNA transport, and cytoplasmic mRNA trafficking (Carpenter *et al.*, 2006; Carson *et al.*, 1998; Dreyfuss *et al.*, 1993; Expert-Bezancon *et al.*, 2002; Michael *et al.*, 1997; Nakielny and Dreyfuss, 1997; Nigg, 1997).

In MCF-7 breast cancer cells stably expressing hnRNP K, high hnRNP K protein levels correlated with an increase in *c-myc* promoter activity and c-Myc protein levels as well as with an enhancement of cell proliferation and anchorage-independent cell growth (Mandal *et al.*, 2001). EGF (epidermal growth factor) and HRG (heregulin-β1) induce *hnrnp k* mRNA and protein

expression. Furthermore, the anti-EGFR (EGF receptor) antibody C225 and the anti-HER2 (human epidermal growth factor receptor 2) antibody HCT (Herceptin, Trastuzumab) inhibit *hnrnp k* mRNA and protein expression as well as *c-myc* mRNA expression strongly suggesting that EGF and HRG induce *c-myc* transcription via the pathway EGF/HRG → EGFR/HER → hnRNP K → *c-myc* transcription (Fig. 5B; Mandal *et al.*, 2001).

11. NM23-H2

NM23-H2 binds to the NHE, namely to its duplex form as well as to both of its single-strands, and activates the *c-myc* promoter (Figs. 4 and 7; Table I; Agou *et al.*, 1999, 2000; Berberich and Postel, 1995; Fournier *et al.*, 2005; Hildebrandt *et al.*, 1995; Ji *et al.*, 1995; Postel and Ferrone, 1994; Postel *et al.*, 1989, 1993, 1996, 2000).

The ubiquitous NM23-H2, a hexamer of identical 17 kDa subunits, possesses DNA binding, DNA cleavage, and NDP (nucleoside diphosphate) kinase activity (Postel, 2003; Postel *et al.*, 2000). It is also known as PuF (purine-binding factor) and NDPK-B (nucleoside diphosphate kinase-B), a housekeeping enzyme catalyzing the transfer of γ-phosphoryl groups from nucleoside triphosphates to NDPs needed for maintenance of cellular nucleotide pools (Lascu *et al.*, 2000). NM23-H2 is able to break double-stranded DNA via a covalent protein-DNA interaction and was shown to cleave the NHE of the *c-myc* promoter (Postel, 1999).

12. REGULATION BY PARANEMIC DNA STRUCTURES: G-QUADRUPLEX AND i-TETRAPLEX

The G-rich antisense strand of the NHE can adopt a G-quadruplex structure while the complementary C-rich sense strand can adopt an *i*-tetraplex structure (Fig. 7; Ambrus *et al.*, 2005; Halder and Chowdhury, 2005; Hurley *et al.*, 2006; Kumar *et al.*, 2005; Mathur *et al.*, 2004; Olsen *et al.*, 2006; Phan *et al.*, 2004, 2005; Seenisamy *et al.*, 2004; Siddiqui-Jain *et al.*, 2002; Simonsson and Sjoback, 1999; Simonsson *et al.*, 1998, 2000; Yang and Hurley, 2006). Formation of these paranemic DNA structures results in repression of the *c-myc* promoter (Fig. 7; Grand *et al.*, 2004; Siddiqui-Jain *et al.*, 2002). The synthetic cationic porphyrin TMPyP4 binds to and stabilizes the G-quadruplex so that it decreases the activity of the *c-myc* promoter and consistently down-regulates *c-myc* mRNA and protein expression (Fig. 7; Freyer *et al.*, 2007; Grand *et al.*, 2002, 2004; Lemarteleur *et al.*, 2004; Phan *et al.*, 2005; Seenisamy *et al.*, 2004; Siddiqui-Jain *et al.*, 2002). As TMPyP4 can also interact with and stabilize *i*-tetraplex structures (Fedoroff *et al.*, 2000) its binding to the *i*-tetraplex at the NHE may additionally contribute to repression of

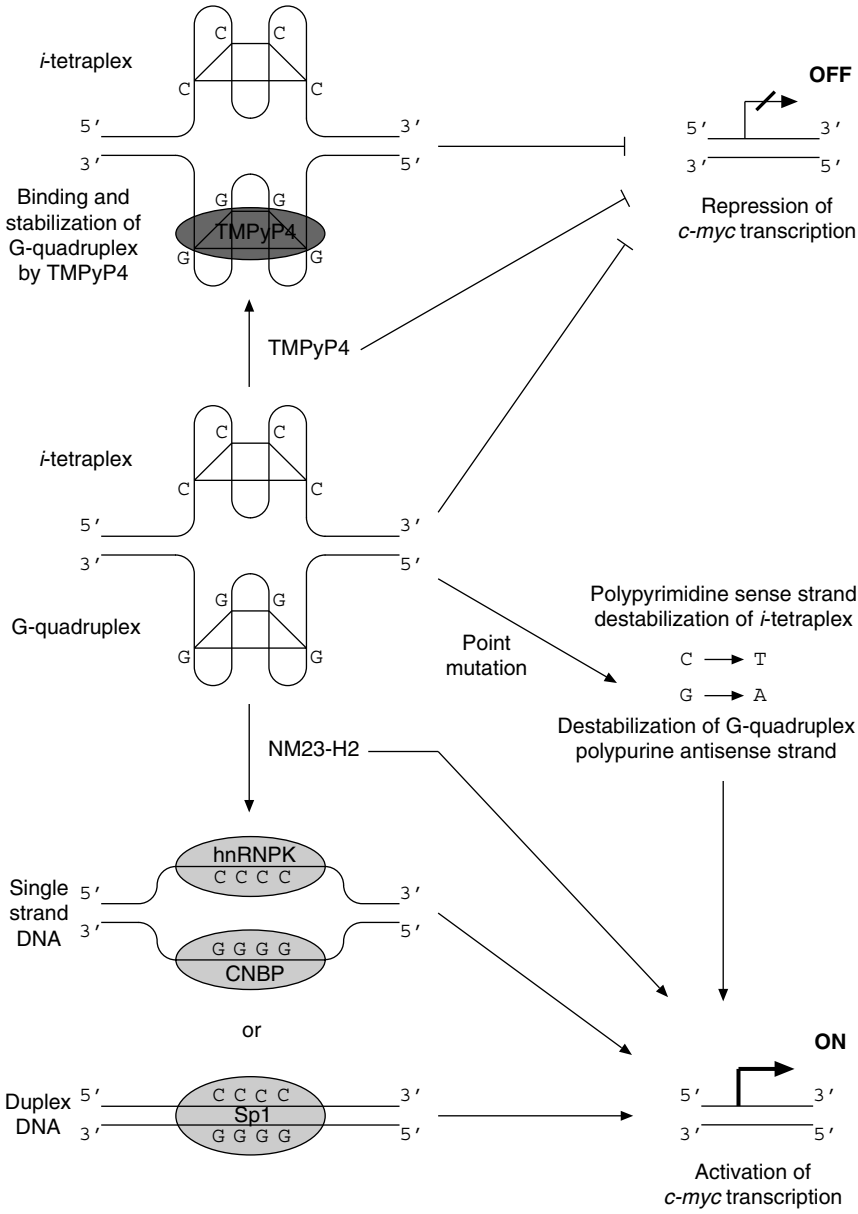

Fig. 7 The CT-element/NHE (nuclease hypersensitive element). The NHE can adopt paranemic DNA structures. Formation of a G-quadruplex in the polypurine antisense strand and an *i*-tetraplex in the polypyrimidine sense strand results in repression of *c-myc* transcription (see Section IV.A.12). The synthetic cationic porphyrine TMPyP4 binds to and stabilizes the G-quadruplex thereby additionally increasing this repression of the *c-myc* promoter

the *c-myc* promoter by TMPyP4. In order to activate the *c-myc* promoter the G-quadruplex and *i*-tetraplex have to be remodeled into duplex or single-strand DNA (Fig. 7). NM23-H2, which does not function as a conventional transcription factor at the NHE (Michelotti *et al.*, 1997), is thought to activate the *c-myc* promoter by converting these paranemic DNA structures to either duplex or single-stranded DNA (Grand *et al.*, 2004; Postel, 2003; Postel *et al.*, 2000; Siddiqui-Jain *et al.*, 2002; Simonsson *et al.*, 1998, 2000). Then Sp1 binding to the CT-element as duplex DNA or hnRNP K and CNBP binding to the pyrimidine- or purine-rich single-strand of the CT-element, respectively, can activate the *c-myc* promoter (Fig. 7). It was suggested that disruption of the G-quadruplex and *i*-tetraplex by NM23-H2 may include excision of part of the NHE DNA by NM23-H2 (Postel, 1999, 2003; Postel *et al.*, 2000). Alternatively, the torsional stress from ongoing transcription could separate the CT-element into single-strands (Levens *et al.*, 1997; Michelotti *et al.*, 1996b; Simonsson *et al.*, 2000).

Point mutation of one specific guanine–cytosine bp into an adenine–thymine bp destabilizes both the G-quadruplex and the *i*-tetraplex resulting in increased activity of the *c-myc* promoter (Fig. 7; Grand *et al.*, 2004; Halder *et al.*, 2005; Siddiqui-Jain *et al.*, 2002). Such point mutations were found at two positions in the NHE in colorectal tumor cell lines and colorectal cancer specimens, but neither in the surrounding normal tissue nor in colon adenomas indicating that G-quadruplex disruptive mutations in the *c-myc* promoter are a late event in colon tumorigenesis (Grand *et al.*, 2004). This could be explained by the finding that p53 inactivation is a late event in colorectal tumorigenesis, too (Kinzler and Vogelstein, 1996). Since high levels of *c-myc* expression will normally cause apoptosis in the absence of appropriate growth signals (Askew *et al.*, 1991; Evan *et al.*, 1992; Nilsson and Cleveland, 2003; Prendergast, 1999) these point mutations in the

(Grand *et al.*, 2002, 2004; Lemarteleur *et al.*, 2004; Seenisamy *et al.*, 2004; Siddiqui-Jain *et al.*, 2002). Point mutation of one specific guanine–cytosine bp into an adenine–thymine bp destabilizes both the G-quadruplex and the *i*-tetraplex resulting in activation of *c-myc* transcription (Siddiqui-Jain *et al.*, 2002; Grand *et al.*, 2004; Halder *et al.*, 2005). NM23-H2 (see Section IV.A.11), which binds to the NHE as duplex DNA as well as to both of its single-strands, converts the G-quadruplex and the *i*-tetraplex either in duplex DNA or in single-strand DNA thereby activating *c-myc* transcription (Grand *et al.*, 2004; Postel, 2003; Postel *et al.*, 2000; Siddiqui-Jain *et al.*, 2002; Simonsson *et al.*, 1998, 2000). Sp1 binds to the CT-element as duplex DNA and activates the *c-myc* promoter (see Section IV.A.16). hnRNP K (see Section IV.A.10) and CNBP (see Section IV.A.9) bind to the C-rich or G-rich single-strand, respectively, of the CT-element and both activate the *c-myc* promoter. The torsional strain of transcription could promote separation of the CT-element into single-strands (Levens *et al.*, 1997; Michelotti *et al.*, 1996b; Simonsson *et al.*, 2000). It was suggested that disruption of the G-quadruplex and *i*-tetraplex by NM23-H2 may include excision of part of the NHE by NM23-H2 (Postel, 1999, 2003; Postel *et al.*, 2000).

NHE which cause increased *c-myc* transcription should only be tolerated subsequent to disruption of the p53 pathway (Grand *et al.*, 2004).

TMPyP4 is studied as anticancer agent, which blocks telomerase activity by stabilization of telomeric G-quadruplexes leading to the so-called replicative senescence (Grand *et al.*, 2002; Lemarteleur *et al.*, 2004; Shammas *et al.*, 2003). Repression of the *c-myc* promoter by TMPyP4 clearly contributes to the effects of TMPyP4 in antitumor therapy, for example, by downregulation of the c-Myc target gene hTERT thereby additionally inhibiting telomerase function (Grand *et al.*, 2002). Like TMPyP4, other telomeric G-quadruplex interacting ligands were also shown to stabilize the G-quadruplex at the *c-myc* NHE (Lemarteleur *et al.*, 2004; Maiti *et al.*, 2003; Phan *et al.*, 2005; Rangan *et al.*, 2001). One aim is to design *c-myc*-specific ligands, for example expanded porphyrins (Seenisamy *et al.*, 2005). G-quadruplexes and *i*-tetraplexes are found in the promoter regions of other growth regulatory genes or proto-oncogenes, too (e.g., *c-ki-ras*, *c-src*, *c-yes*, *c-fgr*, *c-fos*, *c-jun*, *c-rel*, *c-ets*, *c-myb*, *c-sis*, *pdgf-a*, *vav*, *c-kit*, *c-mos*, *c-fes/fps*, *c-abl*) (Simonsson, 2001).

Decreasing and increasing *c-myc* transcription through sequestration of the NHE in or its release from the repressive G-quadruplex and *i*-tetraplex structures, respectively, could provide a fast mode for regulation of the *c-myc* promoter in response to antiproliferative signals and mitogens (Siddiqui-Jain *et al.*, 2002) that may be available in each cell cycle phase.

13. NF-κB

The NF-κB/Rel family of transcription factors orchestrates inflammatory and immune responses and regulates cell proliferation, survival, and differentiation (Chen and Greene, 2004; Ghosh and Karin, 2002). NF-κB is also involved in tumorigenesis (Demicco *et al.*, 2005; Karin, 2006; Karin *et al.*, 2002; Romieu-Mourez *et al.*, 2003; Sun and Xiao, 2003). The family members RelA (p65), RelB, c-Rel, p50, and p52 form different homo- and heterodimers, for example, classical NF-κB p65/p50. The characteristic N-terminal RHD (Rel homology domain) that is shared by all family members mediates DNA binding, dimerization, nuclear translocation, and interaction with the IκB family of NF-κB/Rel inhibitors. Only RelA, RelB, and c-Rel contain C-terminal TADs while p50 and p52 lack them so that p50 and p52 homodimers can function as repressors of NF-κB-specific transcription.

IκB binds to NF-κB and sequesters it in the cytoplasm (Baldwin, 1996; Ghosh *et al.*, 1998). Diverse stimuli, including cytokines and mitogens, induce the rapid translocation of NF-κB into the nucleus through activation of the IκB kinases IKK1 (IKKα) and IKK2 (IKKβ), which phosphorylate the IκB proteins thereby targeting them for ubiquitin-dependent proteasome-mediated degradation and thus liberating the NF-κB dimer (Bonizzi and

Karin, 2004; Ghosh and Karin, 2002; Hayden and Ghosh, 2004; Karin and Ben-Neriah, 2000; Li and Verma, 2002; Whiteside and Israel, 1997; Yamamoto and Gaynor, 2004). In addition, IκB is phosphorylated and thus inactivated by p38-activated CK2 (Viatour *et al.*, 2005).

The *c-myc* gene, which possesses the two NF-κB binding sites URE (upstream regulatory element; upstream of the P1 promoter) and IRE (internal regulatory element; within exon 1), is bound and regulated by several different NF-κB dimers (Fig. 4; Table I; Arcinas *et al.*, 2001; Demicco *et al.*, 2005; Duyao *et al.*, 1990a,b; Ji *et al.*, 1994; Kessler *et al.*, 1992a,b; Kim *et al.*, 2000a,b; La Rosa *et al.*, 1994; Lee *et al.*, 1995a,b; Park and Wei, 2003; Schauer *et al.*, 1996; Siebelt *et al.*, 1997; Wu *et al.*, 1996a,b, 1997; Zou *et al.*, 1997a,b). For example, RelB/p52 as well as p65/p50 and thus overexpression of p65 were shown to transactivate the *c-myc* promoter whereas p50/p50 and thus overexpression of p50 were shown to repress the *c-myc* promoter (Fig. 5D).

Several stimuli regulate the *c-myc* promoter through NF-κB:

1. Carcinogen-induced non-tumorigenic immortalization and malignant transformation of HMECs (human mammary epithelial cells) were shown to result in increased NF-κB binding to the URE of the *c-myc* promoter and in increased *c-myc* URE-driven transcription (Kim *et al.*, 2000b).
2. The pluripotent cytokine IL-1, which induces cell proliferation during wound healing, and the oncoprotein Tax of the HTLV-1 (human T cell leukemia virus type 1) activate the *c-myc* promoter through NF-κB via its NF-κB binding sites (Fig. 5D; Duyao *et al.*, 1992; Kessler *et al.*, 1992a,b).
3. The AhR (aryl hydrocarbon receptor), which mediates malignant transformation by environmental carcinogens, enhances the transactivation of the *c-myc* promoter by RelA dependent on the two NF-κB binding sites URE and/or IRE but independent of AhR-specific response elements (XREs) (Kim *et al.*, 2000a). The AhR interacts with RelA (Tian *et al.*, 1999) and forms a complex with RelA on the *c-myc* URE but does not bind to the URE itself (Kim *et al.*, 2000a).
4. The pleiotropic cytokine TNF (tumor necrosis factor) participates in a wide range of biological activities, including inflammation, growth, differentiation, and apoptosis. In growth-arrested LE6 rat non-transformed liver epithelial cells, TNF activates NF-κB and strongly increases DNA binding by p65/p50 and p50/p50. TNF causes a proliferative response of these growth-arrested cells and strongly induces *c-myc* mRNA expression (Kirillova *et al.*, 1999). This activation of *c-myc* is considerably diminished when NF-κB is inhibited by overexpression of a phosphorylation and degradation impaired IkBα mutant. Although binding of NF-κB to the *c-myc* promoter was not addressed the study of Kirillova *et al.* (1999) suggests that TNF activates *c-myc* transcription via the pathway TNF → TNFR1 → NF-κB → *c-myc* transcription (Fig. 5D). TNF activates also STAT3 via the

pathway TNF → TNFR1 → NF-κB → IL-6 → STAT3. However, the finding that *c-myc* mRNA accumulates before STAT3 activation, but after NF-κB activation argues against an involvement of STAT3 in induction of *c-myc* transcription by TNF in these cells (Kirillova *et al.*, 1999).

5. Mitogen (anti-CD3/anti-CD28) induces *c-myc* transcription in wild-type T cells, but not in rela$^{-/-}$c-rel$^{-/-}$ T cells, in which both proliferation and cell growth in response to anti-CD3/anti-CD28 are blocked (Grumont *et al.*, 2004). This demonstrates that c-Rel/RelA is essential for *c-myc* transcription, proliferation, and growth in T cells. In contrast, in pro-T cells (thymocytes) c-Rel and RelA are dispensable for *c-myc* transcription, cell division, and growth. The study of Grumont *et al.* (2004) strongly suggests that in T cells anti-CD3/anti-CD28 induces *c-myc* transcription via the pathway anti-CD3/anti-CD28 → PKCθ → RelA → *c-myc* transcription and anti-CD3/anti-CD28 → PKCθ → NFAT → c-Rel → *c-myc* transcription (Fig. 5D). Thereby PKCθ, the calcium-independent PKC (protein kinase C) isoform, is required for nuclear translocation of RelA and for NFAT-mediated *c-rel* transcription.

6. Mitogen (anti-IgM/LPS) induces *c-myc* transcription in wild-type B cells, but not in nfkb1$^{-/-}$c-rel$^{-/-}$ B cells, in which both proliferation and cell growth in response to anti-IgM/LPS are blocked (Grumont *et al.*, 2002). This demonstrates that c-Rel/p50 is essential for *c-myc* transcription, proliferation, and growth in B cells. Thus c-Rel and p50 are required for *c-myc* transcription in B cells, whereas c-Rel and RelA serve this role in mature T cells (Grumont *et al.*, 2002, 2004). The study of Grumont *et al.* (2002) strongly suggests that anti-IgM/LPS induces *c-myc* transcription in B cells via the pathway anti-IgM/LPS → PI3K —| IκBα —| c-Rel/p50 → *c-myc* transcription (Fig. 5D). Since the PI3K target Akt is known to associate with, phosphorylate and activate IKKα (Ozes *et al.*, 1999; Romashkova and Makarov, 1999) and the link PI3K → Akt → IKK —| IκB —| NF-κB is confirmed by several studies (Arsura *et al.*, 2000; Datta *et al.*, 1999; Kane *et al.*, 1999; Khwaja, 1999) it is conceivable that anti-IgM/LPS induces *c-myc* transcription via the pathway anti-IgM/LPS → PI3K → Akt/PKB (protein kinase B) → IKK —| IκBα — c-Rel/p50 → *c-myc* transcription (Fig. 5D). This pathway is supported further: in WEHI 231 cells, immature murine B lymphoma cells, BCR (B cell receptor) engagement due to α-IgM treatment PI3K-dependently increases binding of c-Rel/p50 to the URE of the *c-myc* promoter (Chandramohan *et al.*, 2004; Siebelt *et al.*, 1997). GH (growth hormone) induces *c-myc* transcription and PI3K-dependently increases binding of NF-κB to the URE of the *c-myc* promoter in Ba/F3 GHR cells, pro-B Ba/F3 cells stably transfected with the GHR (GH receptor) (Jeay *et al.*, 2001). The study of Jeay *et al.* (2001) strongly suggests that GH induces *c-myc* transcription via the pathway GH → PI3K —| IκBα —| NF-κB → *c-myc* transcription in Ba/F3 GHR cells (Fig. 5D). Since GH is known to

activate PI3K via JAK2 (Yamauchi *et al.*, 1998) it is conceivable that GH induces *c-myc* transcription via the pathway GH → GHR → JAK2 → PI3K → Akt/PKB → IKK —| IκBα —| NF-κB → *c-myc* transcription (Fig. 5D).

7. The cytokine CD40L (CD40 ligand) induces NF-κB (c-Rel/p50, p65/p50) binding to the IRE and URE of the *c-myc* promoter in WEHI 231 cells and accordingly increases the *c-myc* mRNA and protein expression (Schauer *et al.*, 1996; Siebelt *et al.*, 1997). The study of Schauer *et al.* (1996) strongly suggests that CD40L activates *c-myc* transcription via the pathway CD40L —| IκBα —| NF-κB → *c-myc* transcription (Fig. 5D). CD40L can activate IKKα via two different pathways, namely via the classical canonical pathway through the IKK complex, which includes IKKα, IKKβ and the scaffold protein NEMO (NF-κB essential modulator, IKKγ), as well as via the alternative non-canonical pathway through NIK (NF-κB-inducing kinase), which is independent of NEMO and IKKβ (Bonizzi and Karin, 2004; Hayden and Ghosh, 2004; Viatour *et al.*, 2005; Yamamoto and Gaynor, 2004).

8. TGF-β1, which represses the c-Myc expression in WEHI 231 cells, reduces significantly NF-κB binding to the IRE and URE of the *c-myc* promoter, but enhances the expression of IKBα by increasing its transcription (Arsura *et al.*, 1996). These findings strongly suggest that TGF-β1 represses *c-myc* transcription via the pathway TGF-β1 → IκBα —| NF-κB → *c-myc* transcription (Fig. 5D).

9. α-sIg (antiserum against the surface immunoglobulin) treatment of exponentially growing WEHI 231 cells results in a rapid transient increase in *c-myc* transcription followed by a drop below the initial level and later on *c-myc* transcription remains repressed. This is paralleled by an early transient increase in c-Rel/p50 and p65/p50 binding to the URE and IRE of the *c-myc* promoter followed by a decrease in NF-κB binding and subsequent appearance of p50/p50 that later on remains on the *c-myc* URE and IRE (Lee *et al.*, 1995a,b; Schauer *et al.*, 1996; Siebelt *et al.*, 1997; Wu *et al.*, 1996a). The c-Myc protein level parallels these changes in the *c-myc* mRNA level (Wu *et al.*, 1996a). The study of Lee *et al.* (1995a) strongly suggests that α-sIg treatment induces the early transient increase in *c-myc* transcription via the pathway α-sIg —| IκBα —| c-Rel/p50 + p65/p50 → *c-myc* transcription while the later repression of *c-myc* transcription is mediated through p50/p50 (Fig. 5D).

10. NO may regulate gene expression by directly modifying redox-sensitive residues of transcription factors and NF-κB is important for NO-elicited pathophysiological effects (Ghosh and Karin, 2002; Li and Verma, 2002). In P19 stem cells, an embryonal carcinoma cell line where the *c-myc* gene is constitutively active, the transactivating p65/p50 heterodimer is the major NF-κB species at both NF-κB binding sites of the *c-myc* promoter (Park and Wei, 2003). The NO donor SNP (sodium nitroprussid) rapidly represses

c-myc transcription in these cells. SNP decreases binding of the transactivator p65/p50, but increases binding of the repressor p50/p50 to both the URE and the IRE (Fig. 5D; Park and Wei, 2003). HDAC recruitment plays a major role for the repressive activity of p50/p50 (Ashburner *et al.*, 2001; Zhong *et al.*, 2002). In accordance, at the URE this replacement of p65/p50 by p50/p50 correlated with recruitment of gene-silencing HDAC1 and HDAC2 as well as with decreased histone H4 acetylation (Park and Wei, 2003).

Regulation of *c-myc* transcription by NF-κB (Fig. 5D) exemplifies: (1) how different members of a transcription factor family can contribute to positive or negative regulation of *c-myc* transcription, (2) that the individual family members, which are essential for *c-myc* transcription, can vary between different cell types, and (3) that the requirement of such particular family members for *c-myc* transcription can depend on the developmental stage of a certain cell type.

14. AP-1

AP-1 regulates cell proliferation, differentiation, apoptosis, angiogenesis, and invasion (Hartl *et al.*, 2003; Hess *et al.*, 2004; Karin *et al.*, 1997; Mechta-Grigoriou *et al.*, 2001; Ozanne *et al.*, 2007; Shaulian and Karin, 2001, 2002; van Dam and Castellazzi, 2001). It is implicated in transformation and tumorigenesis (Eferl and Wagner, 2003; Jochum *et al.*, 2001; Milde-Langosch, 2006; Vogt, 2001). The immediate-early AP-1 transcription factors and proto-oncoproteins c-Fos/c-Jun transactivate the *c-myc* promoter (Table I; Toualbi *et al.*, 2007).

AP-1 binds to a site in the *c-myc* promoter, which is positioned approximately 1.3 kb upstream of the P1 transcription start site (Fig. 4; Iavarone *et al.*, 2003). This AP-1 site is bound by c-Jun and JunD. c-Fos/c-Jun transactivate a heterologous promoter through this AP-1 site. In murine NIH3T3 cells, PDGF activates *c-myc* transcription via the pathway PDGF → JNK (c-Jun N-terminal kinase) → c-Jun → *c-myc* transcription (Iavarone *et al.*, 2003). PDGF was also shown to activate a heterologous promoter through this AP-1 site via c-Jun. Studies with Rac mutants suggest that Rac activates *c-myc* transcription via JNK in NIH3T3 cells (Iavarone *et al.*, 2003). The involvement of JNK in activation of *c-myc* transcription was confirmed by demonstrating that the MAPKKKs (MAP kinase kinase kinases) MEKK1 (MEK kinase 1) and MLK3 (mixed-lineage kinase 3), two upstream activators of the JNK cascade (Minden *et al.*, 1994; Teramoto *et al.*, 1996), activate *c-myc* transcription in NIH3T3 cells and a heterologous promoter through this AP-1 site (Iavarone *et al.*, 2003).

In addition, c-Fos/c-Jun bind to another site in the *c-myc* promoter, which is positioned approximately 300 bp upstream of the P1 transcription start site (Fig. 4; Hay *et al.*, 1989; Takimoto *et al.*, 1989). A *c-myc* promoter reporter

construct, which possesses this AP-1 site 300 bp upstream of P1 but lacks the other one 1.3 kb upstream of P1, is transactivated by ectopically expressed c-Fos/c-Jun as well as by each exogenous c-Jun and c-Fos alone (Toualbi *et al.*, 2007) indicating that c-Fos/c-Jun transactivate the *c-myc* promoter through the AP-1 site 300 bp upstream of P1. Consistently, the activity of this *c-myc* promoter reporter construct is diminished by siRNA- or antisense RNA-mediated knockdown of either c-Fos or c-Jun, respectively (Toualbi *et al.*, 2007).

Since it was previously shown that Src, Vav2, and Rac are involved in PDGF-induced activation of *c-myc* transcription in NIH3T3 cells (Chiariello *et al.*, 2001; see Section IV.E.1) Iavarone *et al.* (2003) suggested that PDGF may activate the *c-myc* promoter through the AP-1 site positioned 1.3 kb upstream of P1 via the pathway PDGF → Src → Vav2 → Rac → JNK → c-Jun/JunD → *c-myc* promoter (Fig. 5B). However, Chiariello *et al.* (2001) demonstrated the pathway PDGF → Src → Vav2 → Rac → *c-myc* promoter with a reporter construct carrying the *c-myc* promoter from position –157 to +500 (relative to the P1 transcription start site), which lacks the two AP-1 sites 1.3 kb and 300 bp upstream of P1. Thus, it remains to be elucidated whether the PDGF → Src → Vav2 → Rac pathway (Chiariello *et al.*, 2001) targets more than one site in the *c-myc* promoter.

15. ETS-1/2

The *c-myc* promoter possesses a combined E2F/ETS binding site which is perfectly conserved between human, murine and rat *c-myc* (Roussel *et al.*, 1994) and overlaps with the binding sites for STAT3, NFATc1, KLF11, and METS. This site is bound by ETS-1 and ETS-2 (Fig. 4; Table I; Roussel *et al.*, 1994). ETS-1 was shown to activate the *c-myc* promoter and ETS-2 stimulates *c-myc* expression, too (Carbone *et al.*, 2004b; Langer *et al.*, 1992; Roussel *et al.*, 1994). ETS transcription factors regulate both cellular proliferation and differentiation. They act as downstream effectors of multiple signaling pathways that mediate responses to growth factor receptor stimulation and are often activated during oncogenic transformation, for example the Ras/Raf MEK/MAPK cascade (Hsu *et al.*, 2004; Oikawa and Yamada, 2003; Seth and Watson, 2005; Sharrocks, 2001; Wasylyk *et al.*, 1998; Yordy and Muise-Helmericks, 2000). Overexpression of ETS-1 or ETS-2 increases proliferation, anchorage-independent growth, and tumorigenicity in nude mice (Seth and Papas, 1990; Seth *et al.*, 1989). In the hematopoietic system, ETS-1 and ETS-2 are thought to play critical roles in mediating both mitogenic and lineage-specific differentiation responses to colony stimulating factors (Klappacher *et al.*, 2002). In accordance, the *c-myc* promoter is bound by ETS-1/2 in unsynchronized bone marrow progenitor cells that express high amounts of *c-myc* mRNA, but not in terminally differentiated macrophages no longer

expressing *c-myc* (Klappacher *et al.*, 2002). Since it was shown that both MEK1/2 (Cheng *et al.*, 1999a) and ETS-2 (Langer *et al.*, 1992) are required for induction of *c-myc* transcription by CSF-1 (colony-stimulating factor 1) and since ETS-1/2 is activated by MEK1/2 via ERK1/2 (Cheng *et al.*, 1999a; Fowles *et al.*, 1998; McCarthy *et al.*, 1997; Waas and Dalby, 2001; Wasylyk *et al.*, 1997, 1998), it can be concluded that CSF-1 induces *c-myc* transcription via the pathway CSF-1 → MEK1/2 → ERK1/2 → ETS-1/2 → *c-myc* transcription (Fig. 5B).

PU.1, another member of the ETS family of transcription factors, represses the *c-myc* promoter (Table I; Kihara-Negishi *et al.*, 2001). This repression is independent from the ETS binding site of the *c-myc* promoter and from competition with ETS-1/2. However, it was not analyzed whether PU.1 binds to the *c-myc* promoter.

16. Sp1 AND Sp3

The ubiquitously expressed Sp1 (Black *et al.*, 2001; Bouwman and Philipsen, 2002; Chu and Ferro, 2005; Li *et al.*, 2004; Philipsen and Suske, 1999; Resendes and Rosmarin, 2004; Safe and Abdelrahim, 2005) transactivates the P1 promoter as well as the P2 promoter (Table I; Geltinger *et al.*, 1996; Majello *et al.*, 1995, 1997; Wierstra and Alves, 2007a; Wittekindt *et al.*, 2000). The *c-myc* promoter contains 5 Sp1-binding sites (Fig. 4): the CT-element, distal, and –44 (positioned at –44 relative to the P1 transcription start site) upstream of P1 as well as ME1a2 and ME1a1/CT-I_2 upstream of P2 (Asselin *et al.*, 1989; DesJardins and Hay, 1993; Geltinger *et al.*, 1996; Majello *et al.*, 1995; Michelotti *et al.*, 1996a; Miller *et al.*, 1996; Pei, 2001; Sakatsume *et al.*, 1996; Snyder *et al.*, 1991; Vaquero and Portugal, 1998; Wierstra and Alves, 2007a). The CT-element and –44 are high affinity Sp1-binding sites, whereas distal, ME1a2 and ME1a1/CT-I_2 display low Sp1 affinity (Asselin *et al.*, 1989; Geltinger *et al.*, 1996).

–44 is sufficient for strong transactivation of the P1 promoter by Sp1 while distal mediates modest transactivation of P1 by Sp1 (Geltinger *et al.*, 1996). Moreover, Sp1 activates *in vitro* transcription of a reporter construct bearing the CT-element in front of a heterologous minimal promoter (Michelotti *et al.*, 1996a). Sp1 transactivates the P1 promoter through its three Sp1-binding sites (–44, distal, and CT-element) synergistically or additively (Geltinger *et al.*, 1996).

CT-I_2 is essential for transactivation of the P2 promoter by Sp1 (Majello *et al.*, 1995).

The *c-myc* promoter is not occupied by Sp1 in quiescent cells that do virtually not express *c-myc*. Induction of *c-myc* transcription by serum stimulation results in binding of Sp1 to the *c-myc* promoter suggesting that

Sp1 is involved in the serum-induced activation of *c-myc* transcription (Fig. 6; Liu *et al.*, 2006a).

The ubiquitously expressed Sp3 (Suske, 1999) binds to four of the Sp1-binding sites in the *c-myc* promoter, namely to the CT-element, distal, –44, and CT-I_2 (Fig. 4; Table I; Geltinger *et al.*, 1996; Majello *et al.*, 1995). Sp3 slightly represses the *c-myc* promoter and abolishes its transactivation by Sp1 probably by competition for the same binding sites (Geltinger *et al.*, 1996; Majello *et al.*, 1995, 1997).

Sp1 and E2F-1/DP-1 together transactivate the *c-myc* promoter synergistically (Majello *et al.*, 1995). In contrast, Sp3 does not affect the transactivation of the *c-myc* promoter by E2F-1/DP-1.

17. FOXM1c

The forkhead/winged helix transcription factor FOXM1c binds to and transactivates both the *c-myc* P1 and P2 promoters (Fig. 4; Table I; Wierstra and Alves, 2006d). In accordance, the *c-myc* promoter was found to be *in vivo* occupied by FOXM1c in exponentially proliferating human promyelocytic leukemia HL-60 cells (Wierstra and Alves, 2006d) that express high levels of *c-myc*. Furthermore, *c-myc* expression was found to be upregulated in Foxm1b transgenic mice during liver regeneration following PH and CCl_4 injury and the liver regeneration phenotype of these mutant mice was most similar to that of *c-myc* transgenic mice (Wang *et al.*, 2001a; Ye *et al.*, 1999). The transactivator FOXM1c can function as a conventional transcription factor by binding to its consensus sequence 5′-A-T/C-AAA-T/C-AA-3′ (Wierstra and Alves, 2006a,b,c, 2007b). However, for transactivation of the *c-myc* P1 and P2 promoters it uses a new transactivation mechanism (Wierstra and Alves, 2006d): FOXM1c transactivates these two promoters via their TATA-boxes. It directly binds to the P1 (TATAATGC) and P2 (TATAAAAG) TATA-boxes as well as to TBP, TFIIA, and TFIIB, that is, to components of the basal transcription complex, which are positioned at or near the TATA-box. The transactivation of the *c-myc* P1 and P2 promoters by FOXM1c is significantly enhanced by the oncoprotein E7 of the transforming HPV16 (human papillomavirus 16; Wierstra and Alves, 2006d).

FOXM1c and Sp1, which interact directly, transactivate both the *c-myc* P1 and P2 promoters synergistically (Wierstra and Alves, 2007a). According to the definition of Herschlag and Johnson (1993), their synergisms in transactivation of *c-myc* belong to two different energetic classes (Wierstra and Alves, 2007a): positive cooperativity at the P1 promoter (i.e., transcription is greater than multiplicative) versus synergism with independent energetic effects at the P2 promoter (i.e., transcription is multiplicative). Since both the Sp1-binding site –44 and the P1 TATA-box are essential for activation of the P1 promoter by the Igκ enhancers κEi + κE3′ and for induction

of the promoter shift in transcription initiation from P2 to P1 by κEi + κE3′ (Geltinger *et al.*, 1996), this synergism of FOXM1c and Sp1 at the P1 promoter may be important for deregulation of *c-myc* in certain Burkitt's lymphoma with a translocation to the Igκ locus.

FOXM1, which like c-Myc shows a proliferation-specific expression pattern, stimulates proliferation by promoting S- and M-phase entry and regulates genes that control the G_1/S-transition as well as genes that control the G_2/M-transition (Costa, 2005; Costa *et al.*, 2003, 2005; Laoukili *et al.*, 2007; Wang *et al.*, 2001a; Wierstra and Alves, 2006d, 2007c; Ye *et al.*, 1999). In addition, it is assumed to be implicated in tumorigenesis. Many FOXM1-regulated genes are direct c-Myc target genes so that their indirect activation by FOXM1c via c-Myc seems to be conceivable (Wierstra and Alves, 2006d). Thus, transactivation of the *c-myc* promoter could explain stimulation of S-phase entry by FOXM1c and its implication in tumorigenesis.

18. Blimp-1

Blimp-1 (B lymphocyte-induced maturation protein-1), also known as PRF (plasmacytoma repressor factor), is the murine homolog of human PRDI-BF1 (Keller and Maniatis, 1991; Turner *et al.*, 1994). Blimp-1 binds to and represses the *c-myc* promoter (Fig. 4; Table I; György *et al.*, 2003; Horsley *et al.*, 2006; Kakkis and Calame, 1987; Kakkis *et al.*, 1989; Lin *et al.*, 1997; Numoto *et al.*, 1993; Yu *et al.*, 2000; Zou *et al.*, 1997b). Consistently, it decreases the endogenous *c-myc* mRNA expression (Chang *et al.*, 2000; Knödel *et al.*, 1999; Lin *et al.*, 2000; Tamura *et al.*, 2003) and both *c-myc* mRNA and protein expression are increased in *Blimp-1* knock-out skin of mice (Horsley *et al.*, 2006). Although Blimp-1 *in vitro* associates with YY1 (Lin *et al.*, 1997) and two YY1 binding sites are positioned adjacent and nearby the Blimp-1 binding site (Fig. 4) Blimp-1 and YY1 bind independently to the *c-myc* promoter (Yu *et al.*, 2000). Moreover, Blimp-1 represses and YY1 activates the *c-myc* promoter independently of one another (Yu *et al.*, 2000). Blimp-1 binds directly to HDAC-1 and HDAC-2 and HDAC activity is required for repression of the *c-myc* promoter by Blimp-1 (Yu *et al.*, 2000). Accordingly, Blimp-1 expression causes a significant deacetylation of histone H3 at the *c-myc* promoter. In addition, also HDAC-independent mechanisms seem to be necessary for Blimp-1-mediated repression of the *c-myc* promoter (Yu *et al.*, 2000).

Blimp-1 is a key regulator of terminal differentiation in two separate hematopoietic lineages: Its ectopic expression is sufficient to cause terminal differentiation of B lymphocytes into immunoglobulin-secretory non-dividing plasma cells (Lin *et al.*, 1997; Turner *et al.*, 1994) and terminal myeloid differentiation of U937 cells into macrophages (Chang *et al.*, 2000). In consistence, Blimp-1 expression is limited to mature or terminally

differentiated B cells, but not found in *c-myc* transcribing pro-B and pre-B cells (Kakkis and Calame, 1987; Kakkis *et al.*, 1989; Turner *et al.*, 1994). Correspondingly, Blimp-1 is present in peripheral blood monoycytes and granulocytes (Chang *et al.*, 2000). Blimp-1 suppresses cell growth (Lin *et al.*, 1997) and is required for both U937 differentiation and growth arrest during U937 differentiation (Chang *et al.*, 2000). As ectopic c-Myc expression blocks terminal B-cell differentiation of BCL-1 cells into plasma cells (Lin *et al.*, 2000) repression of *c-myc* expression is a prerequisite for their terminal differentiation (see Section IV.A.5). Consequently, repression of *c-myc* transcription by Blimp-1 is fully consistent with the role of Blimp-1 as a master regulator of terminal B cell and myeloid differentiation and represents an important step in these Blimp-1-induced differentiation programs (Chang *et al.*, 2000; Lin *et al.*, 1997, 2000; Yu *et al.*, 2000). In accordance, ectopically expressed c-Myc blocked the growth suppressing effect of high Blimp-1 expression (Lin *et al.*, 1997, 2000) and inhibition of endogenous *Blimp-1* expression abolished both repression of *c-myc* transcription and growth arrest (Chang *et al.*, 2000). Consistently, induction of *Blimp-1* mRNA expression was found to be correlated with repression of *c-myc* mRNA or protein expression during PMA- and DMSO-induced terminal myeloid differentiation of U937 and HL-60 cells into macrophages or granulocytes, respectively (Chang *et al.*, 2000), as well as during IL-2 + IL-5-induced terminal B-cell differentiation of BCL_1 cells into plasma cells (Lin *et al.*, 1997, 2000; Zou *et al.*, 1997b).

The oncogene BCL6 plays a role in B-cell activation and differentiation and is involved in cell cycle control. It represses the expression of *Blimp-1*. Inhibition of BCL6 function induces *Blimp-1* expression but downregulates *c-myc* mRNA and protein expression, causing cell cycle arrest in G_1 (Shaffer *et al.*, 2000). Therefore it is hypothesized that BCL6 may indirectly activate *c-myc* transcription through repression of *Blimp-1* expression.

ICSBP/IRF-8 (interferon consensus sequence binding protein/interferon regulatory factor 8), a hematopoietic cell-specific transcription factor, induces growth arrest and macrophage differentiation in myeloid progenitor cells. It indirectly downregulates the endogenous *c-myc* mRNA expression presumably by inducing the expression of Blimp-1 and METS, two repressors of the *c-myc* promoter that are expressed and active during macrophage differentiation (Fig. 5C; Tamura *et al.*, 2003). In accordance, ICSBP/IRF-8 seems to induce Blimp-1 binding to the *c-myc* promoter.

19. PLZF

PLZF binds to the *c-myc* promoter and represses the *c-myc* transcription (Fig. 4; Table I; McConnell *et al.*, 2003). PLZF binding to the *c-myc* promoter coincides with a decrease in *c-myc* occupancy with Pol II indicating that *c-myc* repression occurs via reduction of transcription initiation.

Ectopically expressed PLZF induces a cell cycle arrest in G_1-phase (McConnell *et al.*, 2003; Shaknovich *et al.*, 1998; Yeyati *et al.*, 1999), but ectopic c-Myc expression reverses this cell cycle arrest suggesting that PLZF prevents cell cycle progression and thus maintains cells in a quiescent state by repressing *c-myc* expression (McConnell *et al.*, 2003). This growth suppression, which is mediated by repression of the *c-myc* promoter by PLZF, may represent the major mode of action of PLZF in hematopoiesis (McConnell *et al.*, 2003). In t(11;17) APL (acute promyelocytic leukemia), the reciprocal translocation of PLZF to RARα results in fusion of seven of the nine zinc fingers of PLZF with the N-terminal activation domain of RARα in the translocation product RARα-PLZF (Chen *et al.*, 1993; Melnick and Licht, 1999). Therefore APL deregulation of *c-myc* transcription may result from loss of normal repression of the *c-myc* promoter by PLZF and possibly its additional activation by RARα-PLZF (McConnell *et al.*, 2003). The second translocation product PLZF-RARα increases the *c-myc* expression indirectly by inducing expression of γ-catenin (see Section IV.A.1; Müller-Tidow *et al.*, 2004).

20. Ovol1

Ovol1, a C_2H_2 zinc finger transcription factor, binds to and represses the *c-myc* promoter (Table I; Nair *et al.*, 2006). This repression requires the zinc fingers and the first 15 N-terminal amino acids of Ovol1, which resemble the SNAG (Snail/Gfi-1) repression domain. Two regions of the *c-myc* promoter were found to be occupied by Ovol1 and to mediate its repression by Ovol1. However, only the distal upstream site (Fig. 4) was directly bound by Ovol1, whereas no direct DNA binding of Ovol1 to the proximal *c-myc* P2 promoter could be detected (Nair *et al.*, 2006).

Ovol1 is expressed in embryonic epidermal progenitor cells that are passing from proliferation into terminal differentiation. It is thought to be required for growth arrest of committed epidermal progenitor cells (Nair *et al.*, 2006) and acts downstream of both the Wnt-β-catenin-LEF/TCF (Li *et al.*, 2002; Payre *et al.*, 1999) and TGF-β/BMP7-Smad4 (Kowanetz *et al.*, 2004) signaling pathways. Accordingly, the growth-inhibitory signals Ca^{2+}, TGF-β, and LiCl (which mimics activated Wnt signaling by inhibiting GSK-3β) failed to induce a cell cycle exit in *Ovol1*-deficient primary mouse keratinocytes whereas they efficiently growth-arrested wild-type keratinocytes, (Nair *et al.*, 2006). In addition, *Ovol1*-deficient primary keratinocytes showed increased endogenous *c-myc* transcript levels and increased *c-myc* promoter activity following differentiation-inducing Ca^{2+} treatment. Consistently, the developing epidermis of *Ovol-1*-deficient mouse embryos showed a failure to downregulate *c-myc* mRNA and protein expression

while both declined during development of wild-type skin (Nair *et al.*, 2006).

Downregulation of *c-myc* expression is important to maintain skin cells in a postmitotic state (Flores *et al.*, 2004; Pelengaris *et al.*, 1999; Waikel *et al.*, 2001) so that repression of the *c-myc* promoter by Ovol1 may be central for cell cycle exit of epidermal progenitor cells in answer to differentiation signals (Nair *et al.*, 2006). Nevertheless, in the absence of any differentiation or growth-inhibitory signals, actively proliferating *Ovol1*-deficient keratinocytes showed no alterations in endogenous *c-myc* mRNA expression or cell proliferation compared to wild-type cells (Nair *et al.*, 2006).

21. MAZ

MAZ (*MYC*-associated zinc finger protein), also known as ZF87 (zinc finger protein, 87 kDa), binds to the *c-myc* promoter, namely (1) to ME1a1/CT-I_2, (2) to ME1a2, (3) to the CT-element, and (4) to the *c-myc* attenuator region within the first exon (Fig. 4; Table I; Bossone *et al.*, 1992; DesJardins and Hay, 1993; Izzo *et al.*, 1999; Komatsu *et al.*, 1997; Pyrc *et al.*, 1992). MAZ binds ME1a1 with higher affinity than ME1a2 (Pyrc *et al.*, 1992). Overexpressed MAZ repressed the *c-myc* P2 promoter through ME1a2 in COS cells (Izzo *et al.*, 1999). In contrast, through ME1a1 overexpressed MAZ modestly activated the *c-myc* P2 promoter and had a less significant stimulating effect on the *c-myc* P1 promoter in BING cells (Marcu *et al.*, 1997). Accordingly, MAZ binding to CT-I_2 was suggested to play a key role for the transient increase in *c-myc* transcription after 48 h during RA-induced neuroectodermal differentiation of PC19 EC (embryonal carcinoma) cells (Komatsu *et al.*, 1997).

Marcu *et al.* (1997) proposed that ME1a1 may represent a molecular switch in *c-myc* transcriptional control targeting factors that promote PIC assembly and modulating the processivity of the assembled initiation complex and that MAZ may participate in both of these processes (Ashfield *et al.*, 1994; Bossone *et al.*, 1992; Duncan *et al.*, 1995; Parks and Shenk, 1996).

22. NFATc1

NFATc1 (nuclear factor of activated T cells c1, NFAT2) plays a central role in the T cell immune response and participates in the regulation of proliferation, differentiation, and apoptosis in non-immune cells (Hogan *et al.*, 2003; Viola *et al.*, 2005). NFATc1 is activated in response to an increase in the intracellular calcium concentration by the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin. It is overexpressed in pancreatic carcinomas and Ca^{2+}/calcineurin/NFATc1 signaling stimulates

cell proliferation and anchorage-independent growth of pancreatic cancer cells (Buchholz *et al.*, 2006). Constitutively, active NFATc1 induces a transformed phenotype in preadipocytes as well as tumor formation in nude mice (Neal and Clipstone, 2003).

NFATc1 binds to and activates the *c-myc* promoter (Table I; Buchholz *et al.*, 2006). In accordance, it stimulates the endogenous *c-myc* mRNA and protein expression in Panc-1 pancreatic cancer cells or preadipocyte 3T3-L1 fibroblasts, respectively (Buchholz *et al.*, 2006; Neal and Clipstone, 2003). The *c-myc* promoter contains a total of 15 putative NFAT-binding sites. NFATc1 was shown to bind to the most proximal one (Buchholz *et al.*, 2006), which overlaps with the binding sites for E2F, ETS-1/2, STAT3, KLF11, and METS (Fig. 4). This site mediates at least partially the activation of the *c-myc* promoter by NFATc1. The study of Buchholz *et al.* (2006) strongly suggests that the *c-myc* promoter is activated in response to Ca^{2+}/calcineurin signaling via the pathway Ca^{2+} → calcineurin → NFATc1 → *c-myc* promoter (Fig. 5B).

23. MBP-1

The ubiquitously expressed (Ray *et al.*, 1994) MBP-1 (*c-myc* promoter binding protein), whose binding site includes the *c-myc* P2 TATA-box, represses the *c-myc* promoter (Fig. 4; Table I; Aoki *et al.*, 2006; Chaudhary and Miller, 1995; Feo *et al.*, 2000; Ghosh *et al.*, 1999a, 2001; Ray, 1995; Ray and Miller, 1991; Ray and Steele, 1997; Subramanian and Miller, 2000). Like TBP (Starr and Hawley, 1991), MBP-1 binds to the minor groove and both proteins bind simultaneously to the minor groove in *c-myc* without competing with each other for DNA binding under *in vitro* conditions (Chaudhary and Miller, 1995). Since MBP-1 binding to the *c-myc* P2 promoter appears not to interfere with TBP binding to the P2 TATA-box, two modes for repression of the *c-myc* promoter by MBP-1 seem to be conceivable: first, MBP-1 could prevent PIC assembly subsequent to TBP (TFIID) binding or even disrupt the PIC (Chaudhary and Miller, 1995), and second, MBP-1 could inhibit transcriptional elongation without affecting PIC formation. The latter control mechanism may be especially important for the candidate tumor suppressor MBP-1 because transcription from the *c-myc* P2 promoter is predominantly regulated at the level of elongation (see Section III.C). Although MBP-1 can directly recruit HDAC1 for repression, it represses the *c-myc* promoter independent of HDAC recruitment (Ghosh *et al.*, 1999c). MBP-1, an alternative translation product of the ENO1 (α-enolase) gene (Feo *et al.*, 2000; Subramanian and Miller, 2000), is a potential tumor suppressor because the gene maps to a region of human chromosome 1 frequently deleted in cancers (Onyango *et al.*, 1998; White *et al.*, 1997). Accordingly, ectopic expression of MBP-1 induces apoptosis,

suppresses cell growth, reduces the invasive ability of carcinoma cells, results in loss of anchorage-independent growth and suppresses tumor formation in nude mice (Ghosh *et al*, 1999a, 2001, 2002, 2005; Ray, 1995; Ray *et al*., 1995; Ray and Steele, 1997). Surprisingly, shRNA-mediated knockdown of MBP-1 retarded the proliferation of human PC3 prostate cancer cells (Ghosh *et al*., 2006). The N-terminally extended translation product α-enolase, a glycolytic enzyme, can bind to the *c-myc* promoter (Table I), but is mainly retained in the cytoplasm whereas MBP-1 is preferentially localized in the nucleus (Feo *et al*., 2000; Subramanian and Miller, 2000).

MIP-2A (MBP-1 interacting protein-2A) binds directly to MBP-1 and antagonizes the MBP-1-mediated cell growth suppression. MIP-2A relieves the repression of the *c-myc* promoter by MBP-1, but does not affect *c-myc* transcription alone (Ghosh *et al*., 2001).

Upon serum stimulation of starved MCF-7 cells, the *mbp-1* mRNA expression increases strongly in parallel with the *c-myc* mRNA expression reaching its maximum at the peak of *c-myc* mRNA expression, but then the *mbp-1* transcript level remains at this maximum whereas the *c-myc* transcript level decreases to its thereafter constant rather low level (Fig. 1; Ray *et al*., 1994). This suggests that the ubiquitous repressor of the *c-myc* promoter MBP-1 may serve to set a security threshold for *c-myc* transcription so that a net activation of the *c-myc* promoter requires a signal that exceeds this threshold preventing the *c-myc* promoter from responding to inappropriate slight fluctuations of its transactivators.

24. CTCF

The ubiquitously expressed zinc finger protein CTCF can inhibit cell growth and displays also features that characterize tumor suppressor genes (El-Kady and Klenova, 2005; Klenova *et al*., 2001; Ohlsson *et al*., 2001; Qi *et al*., 2003; Rasko *et al*., 2001). CTCF, which is identical to NeP1 (negative protein 1), binds to the *c-myc* promoter, namely (1) to the CTCF binding element in the MINE, (2) to a binding site immediately downstream of the P1 transcription start site, and (3) to a binding site immediately downstream of the P2 transcription start site (Figs. 4 and 2; Table I; see Section III.A; Burcin *et al*., 1997; Filippova *et al*., 1996, 2002; Gombert *et al*., 2003; Ishihara *et al*., 2006; Klenova *et al*., 1993, 2001; Lobanenkov *et al*., 1990; Lutz *et al*., 2003). CTCF represses the *c-myc* promoter and accordingly diminishes the endogenous c-Myc protein level (Chernukhin *et al*., 2000; Filippova *et al*., 1996; Klenova *et al*., 2001; Qi *et al*., 2003). Preventing phosphorylation of CTCF by CK2 by substitution of all relevant serines results in enhanced repression of the *c-myc* promoter by CTCF without alteration of its *in vitro* binding to the *c-myc* promoter or its nuclear localization strongly suggesting that CK2, whose levels are often elevated in cancers (Litchfield, 2003), may

relieve *c-myc* repression by CTCF (El-Kady and Klenova, 2005; Klenova *et al.*, 2001). The two binding sites in the MINE and at the *c-myc* P2 promoter were found to be occupied by CTCF in resting as well as mitogen(IL-2)-induced murine CTLL2 cells and in proliferating as well as DMSO-induced differentiating human HL-60 cells although these treatments cause dramatic up- or downregulation of *c-myc* transcription, respectively (Gombert *et al.*, 2003). Thus, CTCF binds constitutively to the *c-myc* promoter independently of the level of *c-myc* transcription. Therefore, either CTCF has no direct role in repression of *c-myc* transcription (Gombert *et al.*, 2003) or instead of CTCF binding to *c-myc* repression of the *c-myc* promoter by CTCF is regulated, for example, by CK2 or by thyroid hormone (see Section IV.A.25). Different tumor-specific CTCF point mutations within its zinc finger domain from human breast, prostate, and Wilms' tumors were found to abrogate CTCF binding to its two binding sites immediately downstream of the *c-myc* P1 and P2 promoters (Filippova *et al.*, 2002).

25. TR/RXR

Thyroid hormones play important roles in metabolism, growth, and differentiation (Yen, 2001). Almost each cell type has the one or the other isoform of TR (thyroid hormone receptor). TR/RXR (retinoid X receptor) heterodimers, but not TR/TR or RXR/RXR homodimers, bind to two sites in the *c-myc* promoter (Fig. 4; Table I), namely to TRE(myc-N), positioned 5′ of the CTCF binding element in the MINE (Lutz *et al.*, 2003), and to TREmmyc (thyroid response element of *murine c-myc*), positioned 3′ of the CTCF binding site immediately downstream of the P2 transcription start site (Pérez-Juste *et al.*, 2000). Although TR/RXR interact directly with CTCF no cooperative DNA binding of them to TREmmyc or TRE(myc-N) was observed (Lutz *et al.*, 2003; Pérez-Juste *et al.*, 2000). T3 (thyroid hormone, triiodothyronine) treatment affects neither the interaction of TR/RXR with CTCF nor TR/RXR binding to TREmmyc or TRE(myc-N) (Lutz *et al.*, 2003; Pérez-Juste *et al.*, 2000). The composite element of the *c-myc* promoter comprising TRE(myc-N) and the neighboring CTCF binding element in the MINE mediates enhancer blocking dependently on the intact CTCF binding site and CTCF binding, but T3 abrogates this enhancer blocking (Lutz *et al.*, 2003). The enhancer-blocking potential was investigated by placing the respective DNA element between the β-globin enhancer and the promoter of a neomycin resistance gene and analyzing the number of G418-resistant colonies formed by K562 cells electroporated with these reporter plasmids.

Thyroid hormones are essential for brain development (Forrest, 2002). In N2a-β cells, a murine neuroblastoma cell line overexpressing the TR β1 isoform, T3 blocks proliferation by arresting cells in G_0/G_1-phase and induces differentiation (Lebel *et al.*, 1994; Perez-Juste and Aranda, 1999; Puymirat

et al., 1995). This T3-induced neuronal differentiation is preceded by a rapid decrease in *c-myc* mRNA expression that is independent of *de novo* protein synthesis (Pérez-Juste *et al.*, 2000). T3 represses the *c-myc* promoter dependent on TREmmyc (Fig. 5C; Lemkine *et al.*, 2005; Pérez-Juste *et al.*, 2000). A *c-myc* promoter fragment comprising TREmmyc and the neighboring CTCF binding site at the P2 promoter that both map within the region of Pol II pausing and release (Filippova *et al.*, 1996) confers repression by T3 to a heterologous promoter if placed downstream, but not if placed upstream, of this promoter and this repression by T3 depends on TREmmyc (Pérez-Juste *et al.*, 2000). Therefore, it is suggested that during early stages of neuronal differentiation T3 may repress *c-myc* transcription at the level of elongation and that TR/RXR binding to TREmmyc as well as possibly also CTCF binding to its adjacent site at the P2 promoter may be involved in this premature termination of *c-myc* transcription (Pérez-Juste *et al.*, 2000).

26. MIBP1, RFX1, AND HOXB4

Three protein binding sites located within intron 1 near the exon 1 boundary, named MIE1, MIE2, and MIE3 (Fig. 4) or MIF-1, MIF-2, and MIF-3 (*myc* intron factors 1–3), are implicated in negative regulation of *c-myc* transcription (Table I). This is underscored by the frequent finding of somatic point mutations in this region in Burkitt's lymphoma, which abolish protein binding to these sites (Yu *et al.*, 1993; Zajac-Kaye and Levens, 1990; Zajac-Kaye *et al.*, 1988). Furthermore, multiple copies of MIE1 and MIE2 were shown to repress transcription from a heterologous promoter (Blake *et al.*, 1996; Itkes *et al.*, 2000; Reinhold *et al.*, 1995). MIE1 is bound (1) by HOXB4, (2) by MIBP1/RFX1, which associate *in vivo*, and (3) by MIBP1 (*myc* intron binding polypeptide 1) independently by RFX1 (regulatory factor X 1) (Fig. 4; Table I; Blake *et al.*, 1996; Chen *et al.*, 2000b; Itkes *et al.*, 2000; Pan and Simpson, 1999; Reinhold *et al.*, 1995; Zajac-Kaye *et al.*, 2000). RFX1, the major histocompatibility complex (MHC) Class II promoter binding protein, appears to be ubiquitously expressed (Iwama *et al.*, 1999).

Protein binding to MIE1, MIE2, and MIE3 is suggested to be implicated in repression of *c-myc* transcription during differentiation of human promyelocytic leukemia HL-60 cells, which differentiate toward monocytes/macrophages in response to PMA or 1,25-$(OH)_2$-D_3 treatment but toward granulocytes following RA or DMSO application (Collins, 1987; Harris and Ralph, 1985). PKC plays a critical role in the differentiation response to PMA, RA, and 1,25-$(OH)_2$-D_3 (Chen *et al.*, 2000b; Savickiene *et al.*, 1995, 1997; Simpson *et al.*, 1998; Tonetti *et al.*, 1994).

Dependent on PKC, PMA treatment of HL-60 cells inhibits *c-myc* transcription and induces binding of two different protein complexes to MIE1, both of which contain RFX1 while only one contains MIBP1 (Chen *et al.*,

2000b; Pan *et al.*, 1996). Accordingly, repression of the *c-myc* promoter by PMA requires MIE1 and in particular the X box of MIE1, that is, the RFX1 binding site (Chen *et al.*, 2000b). PMA enhances the translocation of RFX1, but not MIBP1, into the nucleus without affecting the expression of these proteins. The study of Chen *et al.* (2000b) strongly suggests that during PMA-induced differentiation of HL-60 cells into monocytes/macrophages *c-myc* repression is established via the pathway PMA → PKC → RFX1 —| *c-myc* transcription (Fig. 5C).

In undifferentiated HL-60 cells, MIBP1 and RFX1 are neither bound to MIE1 nor found in nuclear extracts (Zajac-Kaye *et al.*, 2000). In contradiction, Chen *et al.* (2000b) detected both proteins in nuclear extracts of such cells. RA treatment of undifferentiated HL-60 cells inhibits *c-myc* transcription and induces binding of MIBP1/RFX1 to MIE1 (Fig. 5C; Zajac-Kaye *et al.*, 2000). Since PKC is important for the RA-induced differentiation of HL-60 cells (Savickiene *et al.*, 1995, 1997) and since the PMA-induced binding of MIBP1/RFX1 to MIE1 depends on PKC (Chen *et al.*, 2000b), one may speculate that PKC could also mediate the RA-induced binding of MIBP1/RFX1 to MIE1 (Fig. 5C).

Dependent on PKC, 1,25-$(OH)_2$-D_3 treatment of HL-60 cells inhibits *c-myc* transcription and induces protein binding to MIE1, MIE2, and MIE3 (Pan and Simpson, 1999; Pan *et al.*, 1996; Simpson *et al.*, 1989). In accordance, repression of the *c-myc* promoter by 1,25-$(OH)_2$-D_3 requires the region of *c-myc* intron 1, which comprises MIE1, MIE2, and MIE3 (Pan and Simpson, 1999). 1,25-$(OH)_2$-D_3 induces HOXB4 binding to MIE1 and increases the HOXB4 protein amount in nuclear extracts. The study of Pan and Simpson (1999) strongly suggests that during 1,25-$(OH)_2$-D_3-induced differentiation of HL-60 cells into monocytes/macrophages *c-myc* transcription is repressed via the pathway 1,25-$(OH)_2$-D_3 → PKC → HOXB4 —| *c-myc* transcription (Fig. 5C). Both RFX1 (Dikstein *et al.*, 1992) and HOXB4 (Shen *et al.*, 1997a,b) can heterodimerize with other transcription factors so that one may speculate that RFX1/HOXB4 heterodimers could bind to MIE1 (Chen *et al.*, 2000b; Pan and Simpson, 1999).

Repression of *c-myc* transcription during differentiation occurs in two phases (see Section III.C; Chen *et al.*, 2000b; Chung and Levens, 2005; Marcu *et al.*, 1992, 1997; Spencer and Groudine, 1991; Zajac-Kaye *et al.*, 2000): first by a block to transcriptional elongation (Bentley and Groudine, 1986a; Eick and Bornkamm, 1986; Nepveu and Marcu, 1986) and thereafter by loss of transcription initiation (Siebenlist *et al.*, 1988). RA, PMA, and 1,25-$(OH)_2$-D_3 are known to block transcriptional elongation of *c-myc* and for 1,25-$(OH)_2$-D_3 this was shown to be PKC dependent (Bading and Moelling, 1990; Bentley and Groudine, 1986a; Krumm *et al.*, 1992; Simpson *et al.*, 1989; Wilson *et al.*, 2002; Zajac-Kaye *et al.*, 1988). The PMA- and 1,25-$(OH)_2$-D_3-induced effects on protein binding to MIE1 were only

transient, as in response to PMA no difference in protein binding to MIE1 was detected between undifferentiated and fully differentiated HL-60 cells (Chen *et al.*, 2000b), and as in response to 1,25-$(OH)_2$-D_3 the HOXB4 protein levels were similar in undifferentiated and fully differentiated HL-60 cells (Pan and Simpson, 1999). Therefore it is suggested that binding of RFX1 and HOXB4 to MIE1 during PMA- or 1,25-$(OH)_2$-D_3-induced differentiation of HL-60 cells, respectively, may be involved in the early block to transcriptional elongation whereas later on other mechanisms may prevent transcription initiation (Chen *et al.*, 2000b; Pan and Simpson, 1999).

In addition to binding to MIE1, MIBP1 and RFX1 bind also to 5′MIF, which is positioned 5′ of the P1 transcription start site (Fig. 4; Table I; Itkes *et al.*, 2000). Surprisingly, Fukuda *et al.* (2002) reported that MIBP1 represses the *c-myc* promoter independent of MIE1, MIE2, MIE3, and intron 1 as well as independent of 5′MIF.

27. STAT1

STAT1 inhibits cell growth and acts as a proapoptotic factor (Calo *et al.*, 2003; Ihle, 2001; Levy and Darnell, 2002; O'Shea *et al.*, 2002). IFN-γ (interferon γ), which activates STAT1, is known to inhibit cell proliferation and to participate in tumor suppression (Farrar and Schreiber, 1993; Stark *et al.*, 1998). Its effect in limiting cell cycle progression depends on STAT1 (Ramana *et al.*, 2000). STAT1 binds as a homodimer to a GAS (gamma activated sequence) element in the *c-myc* promoter (Fig. 4; Table I). IFN-γ stimulates binding of STAT1 homodimers to the *c-myc* GAS element, represses the *c-myc* promoter dependent on this GAS element, and down-regulates the endogenous *c-myc* mRNA expression dependent on STAT1 (Fig. 5C; Ramana *et al.*, 2000).

28. STAT4

IL-2, a critical regulator of proliferation and differentiation in hematopoietic cells, stimulates *c-myc* transcription (Grigorieva *et al.*, 2000; Hatakeyama *et al.*, 1989; Miyazaki *et al.*, 1995; Shibuya *et al.*, 1992). In response to IL-2 treatment, STAT4 binds to the IL-2 responsive element of the *c-myc* promoter (Figs. 4 and 5A; Table I; Grigorieva *et al.*, 2000). Since IL-2 treatment was shown to cause tyrosine phosphorylation of both JAK2 and STAT4 in the NK (Natural Killer) cell line NK3.3 (Grigorieva *et al.*, 2000) and since it was reported that IL-2 is directly involved in the JAK2/STAT4 signaling pathway in this cell line as well as in primary NK cells (Wang *et al.*, 1999) Grigorieva *et al.* (2000) suggested that IL-2 may activate the *c-myc* promoter via the pathway IL-2 → JAK2 → STAT4 → *c-myc* promoter, at least in NK3.3 cells.

29. NOTCH1/CSL

Notch signaling regulates cell fate decisions and pattern formation during development (Allmann *et al.*, 2002; Artavanis-Tsakonas *et al.*, 1999; Lai, 2004; Maillard *et al.*, 2005). It mediates local cell–cell communication and maintains the self-renewal potential of some tissues (Wilson and Radtke, 2006). Notch signals can have diverse outcomes depending on dose, context, and cell type. Notch1 induces self-renewal of HSCs (hematopoietic stem cells) (Satoh *et al.*, 2004; Varnum-Finney *et al.*, 2000) and plays a role for lineage commitment in the lymphoid compartment, T cell development, and maturation of thymocytes (Allmann *et al.*, 2002; Maillard *et al.*, 2005; Wilson and Radtke, 2006). It regulates cell growth, proliferation, differentiation, and apoptosis. In general, Notch1 promotes cell growth, cell cycle progression, and proliferation (Palomero *et al.*, 2006; Rao and Kadesch, 2003; Satoh *et al.*, 2004; Weng *et al.*, 2006). It has also been implicated in oncogenesis (Hansson *et al.*, 2004; Klinakis *et al.*, 2006; Radtke and Raj, 2003; Wilson and Radtke, 2006).

Notch1 signaling is activated by binding of the transmembrane receptor Notch1 to its ligands (Delta, Serrate, Lag-2, and Jagged) on the surface of a neighboring cell, which induces two consecutive proteolytic cleavages in Notch1 resulting in release of its intracellular domain from the plasma membrane (Artavanis-Tsakonas *et al.*, 1999; Hansson *et al.*, 2004; Lai, 2004; Mumm and Kopan, 2000). This intracellular domain of Notch1 translocates to the nucleus where it binds to the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag-1; also known as RBP-Jκ), and converts the ubiquitous repressor CSL to a transcriptional activator (Lai, 2002). CSL/Notch1 complexes form a ternary complex with coactivators of the Mastermind-like (MAML) family to transactivate target genes.

CSL and CSL/Notch1 complexes bind to three sites in the *c-myc* promoter (Fig. 4; Klinakis *et al.*, 2006; Palomero *et al.*, 2006; Weng *et al.*, 2006), which is activated by the intracellular domain of Notch1 as well as by CSL-VP16 (RBP-VP16, CBF1-VP16), a fusion protein of CSL and the TAD of VP16 that functions as a transcriptional activator independently of Notch signaling (Fig. 5C; Table I; Klinakis *et al.*, 2006; Satoh *et al.*, 2004). In accordance, the endogenous *c-myc* mRNA and/or protein expression is increased by the intracellular domain of Notch1 or the Notch ligand dll1, respectively, but repressed by shRNA-mediated knockdown of Notch1, dominant-negative MAML1 or inhibition of the proteolytic release of the intracellular domain of Notch1, respectively (Klinakis *et al.*, 2006; Palomero *et al.*, 2006; Rao and Kadesch, 2003; Satoh *et al.*, 2004; Weng *et al.*, 2006).

c-Myc is involved in Notch1-mediated stimulation of cell growth (Weng *et al.*, 2006) and in Notch1-induced mammary tumorigenesis (Klinakis

et al., 2006). Furthermore, c-Myc is considered to be important for Notch1-induced self-renewal of HSCs (Satoh *et al.*, 2004).

Enforced expression of the intracellular domain of Notch1 prevents the repression of *c-myc* mRNA expression by TGF-β (Fig. 5C) and downregulation of the *c-myc* transcript level under low serum conditions in Mv1Lu epithelial cells (Rao and Kadesch, 2003). For TGF-β-induced growth arrest repression of *c-myc* expression is essential (see Section IV.A.2; Alexandrow *et al.*, 1995; Blain and Massagué, 2000; Chen *et al.*, 2001b; Claassen and Hann, 2000; Massagué and Chen, 2000; Massagué and Gomis, 2006; Massagué *et al.*, 2000; Siegel and Massagué, 2003; Sun *et al.*, 1998; Warner *et al.*, 1999; Yagi *et al.*, 2002). Consistently, the TGF-β-induced G_1 cell cycle arrest is abolished by overexpression of the Notch1 intracellular domain in Mv1Lu epithelial cells although TGF-β signaling is not affected per se (Rao and Kadesch, 2003). Thus, aberrant activation of Notch1 signaling, which is observed in various cancers (Hansson *et al.*, 2004; Maillard *et al.*, 2005; Radtke and Raj, 2003; Wilson and Radtke, 2006), could render the *c-myc* promoter resistant to repression by TGF-β, apparently without loss of TGF-β receptor or Smad function (Rao and Kadesch, 2003).

The effect of Notch1 on *c-myc* seems to be cell-type dependent (Rao and Kadesch, 2003; Weng *et al.*, 2006) in accordance with the outcome of Notch signals being highly context-specific.

Although not shown so far, one may speculate that the ubiquitous repressor CSL (Lai, 2002) could play a role for repression of the *c-myc* promoter until Notch1 signals arrive during development and for self-renewal of adult tissues.

30. KLF11

The tumor suppressor KLF11 (Krüppel-like factor 11), also termed TIEG2 (TGF-β-inducible early gene 2) or FKLF, inhibits cell growth and suppresses neoplastic transformation (Cook and Urrutia, 2001; Fernandez-Zapico *et al.*, 2003). Its expression is induced by TGF-β (Cook *et al.*, 1998) and it is required for TGF-β-induced cell cycle arrest (Buck *et al.*, 2006).

The ubiquitous zinc finger transcription factor KLF11 binds to the TIE and represses the *c-myc* promoter through it (Fig. 4; Table I; Buck *et al.*, 2006). KLF11 is required for repression of the *c-myc* promoter by a constitutively activated TGF-β type I receptor and thus for repression of *c-myc* mRNA expression by TGF-β. KLF11 binds directly to Smad3 that also binds to the TIE (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002) and they form a KLF11–Smad3 complex on the TIE (Buck *et al.*, 2006). Furthermore, KLF11 augments TGF-β-induced Smad3 binding to the TIE and they cooperate in repression through the TIE. TGF-β enhances KLF11 binding to the TIE and induces the interaction between KLF11 and

Smad3 indicating that TGF-β represses the *c-myc* promoter through the TIE via a KLF11–Smad3 complex (Fig. 5A; Buck *et al.*, 2006).

Ras-MEK1/2-ERK signaling, which can prevent TGF-β-induced cell cycle arrest (Calonge and Massagué, 1999; Filmus *et al.*, 1992; Houck *et al.*, 1989; Howe *et al.*, 1993; Longstreet *et al.*, 1992), inhibits repression of TIE-driven transcription by TGF-β and repression of *c-myc* mRNA repression by TGF-β (Buck *et al.*, 2006). Thereby Ras-MEK1/2-ERK signaling appears to target the KLF11–Smad3 complex on the TIE because it interferes with the interaction between KLF11 and Smad3 as well as with binding of Smad3 and KLF11 to the TIE (Fig. 5A). Thus, an activated Ras-MEK1/2-ERK pathway, which is often found in cancer cells (McCormick, 1999), can render the *c-myc* promoter resistant to repression by TGF-β, namely without alterations in TGF-β receptors or Smads (Buck *et al.*, 2006). In this scenario, several known effects of the Ras-MEK1/2-ERK cascade on Smad3 (Massagué, 2000, 2003; Massagué and Chen, 2000; Piek and Roberts, 2001) and KLF11 are conceivable because both are ERK substrates (Ellenrieder *et al.*, 2002, 2004; Kretzschmar *et al.*, 1999; Matsuura *et al.*, 2005), ERK-mediated phosphorylation of KLF11 disrupts its interaction with mSin3A (Ellenrieder *et al.*, 2002), a central component of mSin3-HDAC corepressor complexes (Ayer, 1999; Knoepfler and Eisenman, 1999) and prevents KLF11 from repressing the TGF-β-induced transcription of the inhibitory *smad7* (Ellenrieder *et al.*, 2004), ERK-mediated phosphorylation of Smad3 can inhibit its nuclear localization (Calonge and Massagué, 1999; Kretzschmar *et al.*, 1999), Ras signaling decreases TGF-β receptor expression (Zhao and Buick, 1995) and ERK-mediated phosphorylation of TGIF stabilizes this Smad corepressor (Lo *et al.*, 2001). However TGIF was not found to be associated with the TIE of *c-myc* (Chen *et al.*, 2001b).

Similarly, in breast cancer cells with a hyperactive Ras pathway and in Ras/ErbB2 transformed mammary epithelial cells markedly reduced Smad4 binding to the TIE in response to TGF-β was reported (Fig. 5A; see Section IV.A.3; Chen *et al.*, 2001b) and TGF-β failed to downregulate the *c-myc* transcript level in human SW480.7 colon carcinoma cells with an activated Ki-Ras oncogene (Calonge and Massagué, 1999).

31. Smad1

Smad1 binds to the *c-myc* promoter (Fig. 4; Table I). It is supposed to repress *c-myc* transcription because point mutating the Smad1 binding site increased the basal activity of the *c-myc* promoter although RNAi-mediated knockdown of Smad1 did not (Hu and Rosenblum, 2005). Surprisingly, β-catenin that interacts with Smad1 and Smad4 is also present at the Smad1 binding site (Table I; Hu and Rosenblum, 2005; Hu *et al.*, 2003).

Upon BMP2 (bone morphogenetic protein 2) binding, its type I receptor ALK3 [activin-like kinase 3; BMPR-IA (BMP receptor IA)] phosphorylates and activates Smad1, which in turn forms complexes with Smad4 that accumulate in the nucleus (Feng and Derynck, 2005; Massagué *et al.*, 2005).

BMP2 and ALK3 inhibit renal branching morphogenesis in embryonic kidney and in a transgenic mouse model overexpression of a constitutive active form of ALK3 caused renal medullary cystic dysplasia characterized by tissue malformation, decreased renal branching morphogenesis, increased cell proliferation in cystic tubules and epithelial de-differentiation (Hu *et al.*, 2003). BMP2 activates the *c-myc* promoter and thus increases the endogenous *c-myc* mRNA and protein expression in mIMCD-3 (inner medullary collecting duct) kidney cells (Hu and Rosenblum, 2005). Accordingly, kidney tissue from these ALK3 transgenic mice ($TgALK3^{QD}$) displays elevated c-Myc protein levels and increased histone H4 acetylation at the *c-myc* promoter (Hu and Rosenblum, 2005; Hu *et al.*, 2003). BMP2-induced *c-myc* promoter activity requires Smad1 and the Smad1 binding site (SBE-A) as well as TCF-4, β-catenin and a TCF-4 binding site (TBE-A = TBE3; Hu and Rosenblum, 2005). In accordance, in $TgALK3^{QD}$ kidney tissue Smad1 binding to SBE-A as well as β-catenin/TCF-4 binding to TBE-A are enhanced. Remarkably, in $TgALK3^{QD}$, but not wild-type kidney tissue, Smad1 is also found at TBE-A while TCF-4 is also associated with SBE-A (β-catenin is found at SBE-A in both wild-type and $TgALK3^{QD}$ kidney tissue) indicating the formation of Smad1/β-catenin/TCF-4 complexes at both SBE-A and TBE-A (Table I; Hu and Rosenblum, 2005). In consistence, in $TgALK3^{QD}$ kidney tissue as well as in BMP2-treated mIMCD-3 cells the interaction of Smad1 with β-catenin is enhanced (Hu and Rosenblum, 2005; Hu *et al.*, 2003). Moreover, the β-catenin protein level is increased. Thus, BMP2 signaling through ALK3 stimulates *c-myc* transcription via Smad1/β-catenin/TCF-4 complexes that target SBE-A as well as TBE-A (Fig. 5A; Hu and Rosenblum, 2005). This involvement of Smad1 in activation of the *c-myc* promoter by Smad1/β-catenin/TCF-4 complexes is in contrast to the assumption that Smad1 alone may repress the *c-myc* promoter.

It has to be noted that the constitutively activated form of the BMP2 receptor ALK3 ($ALK3^{QD}$) repressed the *c-myc* promoter in human HepG2 hepatocarcinoma cells (Fig. 5A) indicating that regulation of *c-myc* transcription by BMP2 and ALK3 is cell-type specific (Lim and Hoffmann, 2006).

32. Smad4

Smad4 binds to a site in the *c-myc* promoter adjacent to the TCF-4/LEF-1 binding site TBE1 (Fig. 4; Table I; Lim and Hoffmann, 2006). Exogenous Smad4 and exogenous LEF-1 together transactivate the *c-myc* promoter synergistically in Smad4-deficient MDA-MB-468 cells if the TIE is mutated

(Lim and Hoffmann, 2006) so that it is unable to mediate repression by TGF-β or to bind Smad4, Smad3, or E2F-4 (Chen *et al.*, 2002). Accordingly, shRNA-mediated knockdown of Smad4 decreases the endogenous *c-myc* transcript level in human HepG2 hepatocarcinoma cells, but expression of exogenous Smad4 alone does almost not affect the *c-myc* promoter (Lim and Hoffmann, 2006). LEF-1 is known to bind to Smad4 directly (Labbe *et al.*, 2000; Letamendia *et al.*, 2001; Nishita *et al.*, 2000). In addition, Smad4 interacts indirectly with β-catenin and their interaction is stimulated by both TGF-β and Wnt1 (Lei *et al.*, 2004; Nishita *et al.*, 2000; Tian and Phillips, 2002). The synergistic transactivation of the *c-myc* promoter by Smad4 and LEF-1 seems to require their interaction because the *c-myc* promoter and the endogenous *c-myc* mRNA expression are repressed by a peptide aptamer (Cui *et al.*, 2005) that interrupts their interaction in HepG2 and/or 4T1 mouse mammary gland cancer cells (Lim and Hoffmann, 2006). Thus, a Smad4/LEF-1 complex appears to transactivate the *c-myc* promoter through TBE1 and the adjacent Smad4 binding site (Fig. 5A; Lim and Hoffmann, 2006). It is unlikely that this complex contains Smad1, Smad2 or Smad3 because Smad1 and Smad3 did not bind to this Smad site and because two tumor-derived Smad4 mutants, which fail to bind phosphorylated Smad1 and Smad2 (Wu *et al.*, 2001), were capable of synergizing with LEF-1 in transactivation of the *c-myc* promoter (Lim and Hoffmann, 2006). TGF-β reduces Smad4 binding to this site adjacent to TBE1 (Lim and Hoffmann, 2006) suggesting that TGF-β, a potent inhibitor of *c-myc* transcription (see Sections IV.A.2 and IV.A.3), antagonizes transactivation of the *c-myc* promoter by the Smad4/LEF-1 complex (Fig. 5A).

Smad4 binds to two different sites in the *c-myc* promoter (Fig. 4), namely to the site adjacent to TBE1 (Lim and Hoffmann, 2006) and in response to TGF-β to the TIE (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Suzuki *et al.*, 2004). Remarkably, Smad4 exerts two opposite effects on *c-myc* transcription through these sites (Fig. 5A): In complex with Smad2/3 (Chen *et al.*, 2001b; Suzuki *et al.*, 2004) and E2F-4,5/DP-1/p107 (Chen *et al.*, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002) or/and C/EBPβ (Gomis *et al.*, 2006a) it represses the *c-myc* promoter through the TIE in response to TGF-β. In contrast, (probably) independent from Smad2/3 it activates the *c-myc* promoter synergistically with LEF-1 through the site adjacent to TBE1, which is (probably) counteracted by TGF-β (Lim and Hoffmann, 2006). TGF-β, which needs to repress the *c-myc* promoter in order to induce a G_1 cell cycle arrest (Alexandrow *et al.*, 1995; Blain and Massagué, 2000; Claassen and Hann, 2000; Massagué and Chen, 2000; Massagué and Gomis, 2006; Massagué *et al.*, 2000; Siegel and Massagué, 2003; Sun *et al.*, 1998; Warner *et al.*, 1999; Yagi *et al.*, 2002), switches Smad4 from a *c-myc* activator into a *c-myc* repressor by inducing its binding to the TIE (Chen *et al.*, 2001b, 2002;

Frederick *et al.*, 2004; Suzuki *et al.*, 2004; Yagi *et al.*, 2002), but inhibiting its binding to the site adjacent to TBE1 (Lim and Hoffmann, 2006).

Interestingly, two tumor-derived Smad4 mutants that fail to bind phosphorylated Smad2 (and possibly Smad3) due to substitutions of amino acids important for Smad complex formation (Shi *et al.*, 1997; Wu *et al.*, 2001) are still capable of transactivating the *c-myc* promoter synergistically with LEF-1 (Lim and Hoffmann, 2006). It is tempting to speculate that these Smad4 mutants may be unable to repress the *c-myc* promoter in complex with Smad2/3 through the TIE in response to TGF-β so that such tumor cells would escape Smad4-mediated repression of *c-myc* transcription by TGF-β, a major c-Myc antagonist and potent inhibitor of cell proliferation, but retain Smad4-enhanced activation of *c-myc* transcription by LEF-1 and thus remain responsive to Wnt signaling, a potent stimulus of cell proliferation (Fig. 5A).

Besides the Smad1/β-catenin/TCF-4 complexes at TBE3 and the Smad1 binding site (see Section IV.A.31; Hu and Rosenblum, 2005) this Smad4/LEF-1 complex at TBE1 represents another example for cooperation of Smad and TCF/LEF family members in transactivation of the *c-myc* promoter (Fig. 5A). In contrast, Smad3 antagonizes the transactivation of the *c-myc* promoter by β-catenin-/TCF-4 by displacing β-catenin from TCF-4 at TBE3 (Fig. 5A; see Section IV.A.1; Sasaki *et al.*, 2003). These findings suggest that cross talk between the Wnt pathway and signaling by TGF-β family members may be involved in regulation of *c-myc* transcription and that this cross talk may include synergistic as well as antagonistic effects on the activity of the *c-myc* promoter. Smads are well known to cooperate with other transcription factors in target gene regulation allowing for an extensive versatility and conferring cell type and target gene (group) specificity (Feng and Derynck, 2005; Massagué *et al.*, 2005). One could speculate that this cross talk between the Wnt pathway and TGF-β family members may manifest at the *c-myc* promoter in opposite outcomes in different biological settings, that is, cooperation in activation of *c-myc* transcription during development but antagonistic control of the *c-myc* promoter by proliferative Wnt versus antiproliferative TGF-β/Smad2/3 signaling during adult tissue homeostasis and renewal.

B. Transcription Factors That *In Vivo* Occupy the *c-myc* Promoter

1. ER (ESTROGEN RECEPTOR) AND AR (ANDROGEN RECEPTOR)

Steroid hormones, such as estrogen and testosterone, mediate their effects through binding to transcription factors that belong to the nuclear receptor superfamily, for example, ERα, ERβ and AR. Estrogen, an essential

regulator of female development and reproductive organ function, plays a central role in proliferation and differentiation of responsive cells, for example, breast and endometrial tissues (Nilsson *et al.*, 2001; Pettersson and Gustafsson, 2001) It has also been implicated as a causal factor in breast and endometrial cancers (Pearce and Jordan, 2004; Shao and Brown, 2004).

Estrogen (17β-estradiol) treatment rapidly induces *c-myc* transcription and occupancy of the *c-myc* promoter with ERα, which transactivates the *c-myc* promoter (Fig. 5C; Table I; Cheng *et al.*, 2006; DeNardo *et al.*, 2005; Dubik and Shiu, 1992; Dubik *et al.*, 1987; Jiang *et al.*, 2004; Keeton and Brown, 2005; Laganière *et al.*, 2005; Liu and Bagchi, 2004; Oxelmark *et al.*, 2006; Park *et al.*, 2005; Shang and Brown, 2002; Shang *et al.*, 2000; Zhang *et al.*, 2004). The ERE (estrogen-responsive element) of the *c-myc* promoter was mapped to a 116-bp region positioned at +25 to +141 relative to the transcription start site (+1) of the P1 promoter (Fig. 4; Dubik and Shiu, 1992). This ERE of *c-myc* lacks any palindromic ERE consensus sequence (GGTCA-NNN-TGACC) but harbors at least one imperfect ERE half site (Dubik and Shiu, 1992; Liu and Bagchi, 2004). Steroid receptors such as the ER and the AR can regulate gene transcription either by binding directly to the promoter or by binding indirectly through an as yet not fully characterized mechanism involving other transcription factors such as AP-1, NF-κB, and Sp1 (Nilsson *et al.*, 2001; Pettersson and Gustafsson, 2001). Because of the lack of a classical ERE and due the rapid induction of *c-myc* transcription by estrogen the ER is thought to bind indirectly to the *c-myc* promoter (Shang and Brown, 2002; Zhang *et al.*, 2004). The ERE of the *c-myc* promoter contains at least two functional Sp1-binding sites, namely CT-I_2/ME1a1 and ME1a2 (see Section IV.A.16), but no known binding sites for AP-1 or NF-κB (Fig. 4). Nevertheless, a dominant-negative form of c-Jun blocked the estrogen-induced expression of *c-myc* mRNA (DeNardo *et al.*, 2005).

Coactivators recruited by the ER include p300/CBP, P/CAF, BRG-1, CARM1, PRMT1, a DRIP/TRAP mediator complex, CIA, SRA, SNURF, a TRRAP-containing HAT complex, PGC-1, PELP1, and the three members of the SRC family (p160 family) SRC-1/NCoA-1, GRIP1/SRC-2/TIF2/NCoA-2, and AIB1/SRC-3/pCIP/ACTR/RAC3/TRAM1/NCoA-3, which serve as platforms to recruit HATs and protein methyltransferases (Barnes *et al.*, 2004; Belandia and Parker, 2003; Chen *et al.*, 2001a; Glass and Rosenfeld, 2000; Halachmi *et al.*, 1994; Klinge, 2000; Lemon and Freedman, 1999; Lonard and O'Malley, 2005, 2006; McKenna and O'Malley, 2002; McKenna *et al.*, 1999; Pearce and Jordan, 2004; Rosenfeld *et al.*, 2006; Sauve *et al.*, 2001; Shao and Brown, 2004). Corepressors recruited by the ER include NCoR, SMRT, and REA (Jepsen and Rosenfeld, 2002). Estrogen induces the occupancy of the *c-myc* promoter with ERα and its coactivators SRC-1, AIB1, GRIP1, p300, CBP, CARM1, and CIA (Cheng *et al.*, 2006; Jiang *et al.*, 2004; Park *et al.*, 2005; Shang and Brown, 2002; Shang *et al.*, 2000; Zhang *et al.*, 2004).

Furthermore, estrogen enhances the association of Pol II with the *c-myc* promoter (Cheng *et al.*, 2006).

Estrogen induces transcription cycles of ER target genes. These transcription cycles are mediated by cyclic association of the ER and its diverse coactivators with these ER target genes (Métivier *et al.*, 2003; Perissi and Rosenfeld, 2005; Shang *et al.*, 2000). Since p300/CBP, P/CAF, SRC-1, GRIP1 and AIB1 possess HAT activity ER-mediated transactivation is associated with histone acetylation at target gene promoters (Métivier *et al.*, 2003; Shang*et al.*, 2000). Consistently, estrogen was shown to induce cyclic transcription of *c-myc* (Shang *et al.*, 2000), cyclic or transient association of ERα, SRC-1, p300, CBP, CIA and CARM1 with the *c-myc* promoter (Jiang *et al.*, 2004; Zhang *et al.*, 2004) and histone acetylation at the *c-myc* promoter (Cheng *et al.*, 2006; Liu and Bagchi, 2004; Shang and Brown, 2002).

Acetylation versus dimethylation of lysine 9 of histone H3 (H3-K9) is correlated with gene activation versus repression, respectively (Kondo *et al.*, 2004; Peters *et al.*, 2003; Peterson and Laniel, 2004; Roh *et al.*, 2005). In accordance, during estrogen-induced *c-myc* expression the *c-myc* promoter acquires H3-K9 acetylation but looses H3-K9-dimethylation (Cheng *et al.*, 2006). Following estrogen stimulation of the *c-myc* promoter the peak (after 3 h) in its transient occupancy with ERα coincides with its maximal H3-K9-acetylation (Cheng *et al.*, 2006).

In response to estrogen, IKKα phosphorylates ERα and AIB1/SRC-3 on residues required for enhancing their transcriptional activity (Chen *et al.*, 2000c; Lannigan, 2003; Park *et al.*, 2005; Wu *et al.*, 2002, 2004). IKKα is important for induction of *c-myc* transcription by estrogen and estrogen induces occupancy of the *c-myc* promoter with IKKα (Park *et al.*, 2005).

The putative tumor and metastasis suppressor TIP30 (Tat interacting protein 30, CC3, Htatip2), which interacts directly with CIA, represses the estrogen-dependent transactivation of the *c-myc* promoter by ERα (Fig. 5C) and even more efficiently that by ERα plus CIA (Jiang *et al.*, 2004). In accordance, the estrogen-dependent transactivation of the *c-myc* promoter by ERα is enhanced in TIP30$^{-/-}$ MEFs and the endogenous *c-myc* mRNA expression is increased in mammary glands of TIP30$^{-/-}$ mice. Surprisingly, TIP30 was found at the *c-myc* promoter in the absence of estrogen when both ERα and CIA were absent from the *c-myc* promoter (Jiang *et al.*, 2004). TIP30 phosphorylates the CTD of Pol II (Xiao *et al.*, 2000) and a kinase defective mutant of TIP30 (Xiao *et al.*, 1999) failed to repress the estrogen-dependent transactivation of the *c-myc* promoter by ERα (Jiang *et al.*, 2004).

The SERMs (selective estrogen receptor modulators) tamoxifen and raloxifene are effective drugs for treatment of hormone-responsive breast cancer and function as estrogen antagonists in MCF-7 breast cancer cells (Fisher *et al.*, 1998; Jordan *et al.*, 2001). In these cells, both SERMs repress *c-myc* transcription by recruitment of corepressors to the SERM-liganded

ER (Liu and Bagchi, 2004; Shang and Brown, 2002): Tamoxifen induces the occupancy of the *c-myc* promoter with ERα, NCoR, SMRT, Mi2, TBL1, MTA1, HDAC1, HDAC2, HDAC3, and HDAC4 resulting in histone deacetylation and loss of Pol II at the *c-myc* promoter.

In a genome-wide analysis of ER binding sites using ChIP-on-chip, Carroll *et al.* (2006) identified an additional single ER binding site approximately 67 kb upstream from *c-myc*. Directed ChIP and real-time PCR validated estrogen-induced ER binding to this new site, which is in line with the evolving concept that distal enhancer elements together with the pioneer factor FoxA1 function to tether the ER to target gene promoters (Carroll and Brown, 2006; Carroll *et al.*, 2005). However, two predicted transcripts exist in the region between this upstream ER binding site and the *c-myc* gene although there was no evidence for their expression in the analyzed MCF-7 cells (Carroll *et al.*, 2006).

Like the ER in response to estrogen, also the AR in response to testosterone activates *c-myc* transcription (Fig. 5C; Table I) by binding probably indirectly to the *c-myc* promoter, which does not contain a classical HRE (hormone-responsive element) (Silva *et al.*, 2001; Zhang *et al.*, 2004). Testosterone induces the occupancy of the *c-myc* promoter with the AR and GRIP1 (Amir *et al.*, 2003; Zhang *et al.*, 2004).

2. p53 AND p73

In response to various stress signals, for example DNA damage and hypoxia, p53 induces in general either apoptosis or cell cycle arrest, the latter one presumably to allow for DNA repair (Cadwell and Zambetti, 2001; Harris and Levine, 2005; Vousden and Lu, 2002). The tumor suppressor p53 is the most frequently mutated gene in human cancer (in over 50% of all human cancers). Its mutation results in inactivation of its tumor suppressor function (loss of function). In addition, mutant p53 proteins often acquire also transforming activity (gain of function) so that they augment cell growth, inhibit apoptosis, enhance tumorigenicity and increase tissue invasiveness (Cadwell and Zambetti, 2001; Sigal and Rotter, 2000).

Wild-type p53 represses the *c-myc* promoter and targets the proximal P2 promoter including the E2F-binding site and/or ME1a2 (Table I; Frazier *et al.*, 1998; Moberg *et al.*, 1992b; Ragimov *et al.*, 1993). Accordingly, p53 represses the endogenous *c-myc* mRNA and protein expression in various human, murine and rat cell lines or tissues (Ho *et al.*, 2005; Levy *et al.*, 1993; Ragimov *et al.*, 1993; Yonish-Rouach *et al.*, 1993) indicating that this effect is not restricted to certain cell types. This p21-independent repression of *c-myc* expression is required for efficient induction of G_1 cell cycle arrest and differentiation by p53 (Ho *et al.*, 2005). The mechanism of p53-mediated repression remains a controversial area of p53 biology (Ho and Benchimol, 2003).

p53 was found to occupy at least two regions of the *c-myc* promoter, a proximal and a distal one that lack or include a consensus p53 binding site, respectively, but it is unclear whether it binds directly to the *c-myc* promoter (Ho *et al.*, 2005). p53 is known to recruit HDACs to its repressed target genes (Ho and Benchimol, 2003) and repression of *c-myc* mRNA expression by p53 is HDAC dependent, too (Ho *et al.*, 2005). In accordance, p53-mediated transcriptional repression of *c-myc* is accompanied by a decrease in the level of acetylated histone H4 at and an increase in recruitment of the corepressor mSin3A to the *c-myc* promoter (Ho *et al.*, 2005). In addition, p53 seems to interfere with PIC formation, but not transcriptional elongation at the *c-myc* P2 promoter (Ragimov *et al.*, 1993). It was shown that p53, which binds to TBP (Ko and Prives, 1996), prevents binding of TBP/TFIIA complexes to the adenovirus major late TATA-box that is identical to the *c-myc* P2 TATA-box (TATAAAAG) (Ragimov *et al.*, 1993). Like wild-type p53, also some p73 isoforms repress *c-myc* transcription (Table I; Kartasheva *et al.*, 2003).

Tumor-derived p53 gain-of-function mutants activate the *c-myc* promoter and the mutant p53-responsive region was mapped to the 3′ end of exon 1 or the exon 1/intron 1 junction (Table I; Frazier *et al.*, 1998). Since this mutant p53-responsive region maps near a site implicated in transcription attenuation (Bentley and Groudine, 1986a; Nepveu and Marcu, 1986) and since this region conferred mutant p53-responsiveness only at a downstream position and in the correct orientation it was suggested that mutant p53 may enhance the transcription elongation rate by allowing transcription to read through this region (Frazier *et al.*, 1998). However, it was not analyzed whether these tumor-derived p53 gain-of-function mutants or the 73 isoforms bind to the *c-myc* promoter.

In a whole-genome mapping approach that couples ChIP with the PET (paired-end ditag) sequencing strategy, Wei *et al.* (2006) identified an additional p53 binding site in the 3′ downstream region of the human *c-myc* gene at a distance of more than 50 kb.

In a global screening approach using ChIP followed by CpG island microarray hybridization to identify promoters bound by p53 under hypoxic stress Krieg *et al.* (2006) identified an additional p53 binding site in the second intron of the *c-myc* gene. Hypoxia, which stabilizes p53 (Hammond and Giaccia, 2005), increases the occupancy of this site with p53 and represses the c-*myc* mRNA expression (at least in part) dependent on p53 (Fig. 5C; Krieg *et al.*, 2006).

3. GATA-1

GATA-1, a lineage-instructive transcription factor essential for erythroid and megakaryocytic maturation, promotes terminal hematopoietic differentiation and inhibits cell proliferation (Pevny *et al.*, 1991; Rekhtman *et al.*, 1999; Rylski *et al.*, 2003; Shivdasani *et al.*, 1997; Weiss *et al.*, 1994, 1997).

Consistently, GATA-1 represses *c-myc* transcription and the *c-myc* promoter is occupied by GATA-1 (Table I; Rylski *et al.*, 2003). GATA-1 consensus motifs are present in the proximal *c-myc* promoter, but it was not analyzed whether GATA-1 binds directly or indirectly to the *c-myc* promoter. G1E cells, murine immortalized GATA-1 null (GATA-$1^{-/-}$) erythroid cells, proliferate continuously as immature erythroblasts, but undergo terminal maturation when GATA-1 expression is restored. In these cells, GATA-1 induces a G_1 cell cycle arrest and erythroid maturation (Rylski *et al.*, 2003; Weiss *et al.*, 1997). Enforced c-Myc expression prevents GATA-1-induced G_1 cell cycle arrest, but has minimal effects on GATA-1-induced erythroid maturation, indicating that repression of *c-myc* transcription by GATA-1 is required for the former process, but largely dispensable for the latter one (Rylski *et al.*, 2003).

4. C/EBPβ

The proximal *c-myc* promoter was found to be occupied by C/EBPβ (CCAAT/enhancer binding protein β) in HaCaT keratinocytes, EL-4 T cells and NIH 3T3 fibroblasts (Table I; Berberich-Siebelt *et al.*, 2006; Gomis *et al.*, 2006a; Sebastian *et al.*, 2005). Overexpression of LIP, a naturally occurring C/EBPβ inhibitory isoform that functions as a dominant-negative inhibitor of the functional C/EBPβ isoforms (LAP1, LAP2), increased the activity of the *c-myc* promoter (Gomis *et al.*, 2006a) but C/EBPβ failed to inhibit a *c-myc* promoter-driven reporter construct (Berberich-Siebelt *et al.*, 2006). Nevertheless, C/EBPβ impairs the endogenous *c-myc* mRNA expression (Gomis *et al.*, 2006a; Sebastian *et al.*, 2005), which requires the intact N-terminal TAD of C/EBPβ, but not its central regulatory domain (Berberich-Siebelt *et al.*, 2006). Sumoylation of the central domain of C/EBPβ inhibits this repression of *c-myc* mRNA expression by targeting C/EBPβ to pericentric heterochromatin (Fig. 5A; Berberich-Siebelt *et al.*, 2006).

C/EBPβ is expressed in T lymphocytes and induces a wide array of genes controlling cell differentiation, innate immunity, inflammatory and acute-phase responses (Berberich-Siebelt *et al.*, 2006; Lekstrom-Himes and Xanthopoulos, 1998). C/EBPβ arrests murine EL-4 thymoma cells in G_1-phase (Berberich-Siebelt *et al.*, 2006). Exogenous c-Myc is able to overcome this cell cycle arrest so that repression of *c-myc* expression by C/EBPβ seems to be important for inhibition of T cell proliferation by C/EBPβ and thus for C/EBPβ-mediated proliferation/differentiation control in T lymphocytes. Accordingly, this cell cycle arrest requires the same domains of C/EBPβ as repression of *c-myc* mRNA expression and is also suppressed by sumoylation of the central domain of C/EBPβ (Berberich-Siebelt *et al.*, 2006).

The bZIP (basic leucine zipper) transcription factor C/EBPβ can either promote or inhibit cell proliferation and displays tumor suppressor-like as well as prooncogenic properties (Gomis *et al.*, 2006a; Grimm and Rosen,

2003; Sebastian and Johnson, 2006; Zahnow, 2002). It can induce growth arrest and participates in terminal differentiation and senescence (Berberich-Siebelt *et al.*, 2006; Johnson, 2005; Lekstrom-Himes and Xanthopoulos, 1998; Ramij and Foka, 2002; Sebastian *et al.*, 2005).

C/EBPβ is required for repression of the *c-myc* promoter by TGF-β and thus for TGF-β-induced repression of *c-myc* mRNA expression, which are abolished by overexpression of LIP and/or shRNA-mediated knockdown of C/EBPβ in HaCaT keratinocytes (Fig. 5A; Gomis *et al.*, 2006a). C/EBPβ occupies the proximal *c-myc* promoter region including the TIE (Berberich-Siebelt *et al.*, 2006; Gomis *et al.*, 2006a; Sebastian *et al.*, 2005) in HaCaT cells also without TGF-β treatment and TGF-β causes a small increase in this association indicating that the occupancy of the *c-myc* promoter by C/EBPβ is partly constitutive (Gomis *et al.*, 2006a). It is unknown how TGF-β represses *c-myc* transcription dependent on C/EBPβ. C/EBPβ (LAP2) interacts with Smad2, Smad3, and Smad4 (Choy and Derynck, 2003; Coyle-Rink *et al.*, 2002; Gomis *et al.*, 2006a). TGF-β induces the interaction of Smad4 with C/EBPβ and with Smad2/3 (Gomis *et al.*, 2006a) as well as the formation of a C/EBPβ–Smad2/3 complex (Gomis *et al.*, 2006b). In addition, TGF-β strongly increases binding of Smad2/3 and E2F-4 to the TIE of the *c-myc* promoter (Gomis *et al.*, 2006a). These findings suggest that a Smad2/3–Smad4–C/EBPβ complex may mediate the C/EBPβ-dependent repression of *c-myc* transcription by TGF-β (Fig. 5A). Yet it is unknown whether C/EBPβ is involved in the TGF-β-induced repression of the *c-myc* promoter by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex through the TIE (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002).

C/EBPβ is expressed in three isoforms due to usage of different start codons. In contrast to the predominant LAP1 and LAP2, the truncated LIP lacks the TAD so that it functions as a dominant-negative inhibitor of the former two (Grimm and Rosen, 2003; Ramij and Foka, 2002; Zahnow, 2002). LIP abrogates the repression of the *c-myc* promoter by TGF-β (Fig. 5A; Gomis *et al.*, 2006a). In accordance, TGF-β is unable to repress *c-myc* mRNA expression in metastatic breast cancer cells with high LIP:LAP ratios but this ability of TGF-β can be restored by expression of exogenous LAP2 (Gomis *et al.*, 2006a). Because of such an increased LIP:LAP ratio the *c-myc* promoter can become resistant to repression by TGF-β in tumor cells that retain TGF-β receptor and Smad functions (Gomis *et al.*, 2006a; Massagué and Gomis, 2006).

Both repression of *c-myc* expression and activation of *p15* expression are essential for the cytostatic program of TGF-β (see Section IV.A.2; Massagué and Gomis, 2006; Siegel and Massagué, 2003). TGF-β inhibits the growth of cells that lack p15 or the c-Myc response, but breast cancer cells with a combined loss of these two gene responses evade the growth-inhibitory action of TGF-β (Chen *et al.*, 2001b, 2002; Gomis *et al.*, 2006a; Iavarone and Massagué,

1997; Latres *et al.*, 2000; Massagué and Gomis, 2006). C/EBPβ is required for both effects of TGF-β, *c-myc* repression as well as *p15* activation, and thus for TGF-β-induced growth arrest (Gomis *et al.*, 2006a,b). Accordingly, LIP overexpression abolishes not only repression of the *c-myc* promoter by TGF-β but also TGF-β-induced activation of the *p15* promoter and metastatic breast cancer cells with high LIP:LAP ratios have lost these two responses to TGF-β (Gomis *et al.*, 2006a). Consistently, the cytostatic effect of TGF-β is diminished in such breast cancer cells (Gomis *et al.*, 2006a) explaining why a high LIP:LAP ratio is implicated in breast cancer progression (Grimm and Rosen, 2003; Zahnow, 2002) and how tumor cells can evade TGF-β-induced growth arrest without loss of TGF-β receptor or Smad functions (Massagué and Gomis, 2006).

5. ID2

PH, the surgical removal of 70% of the liver, induces liver regeneration (Taub, 1996, 2004). It triggers proliferation of the remaining parenchyma cells that quickly restore the liver mass prior to returning to quiescence (Koniaris *et al.*, 2003). PH rapidly induces transcription of the immediate-early gene *c-myc* (Fausto, 2000). *c-myc* mRNA levels peak 1 h after PH and then return to control values by 6 h post-PH (Rodriguez *et al.*, 2006). Sequential ChIP assays monitoring occupancy of the rat *c-myc* promoter following PH revealed the presence of ID2 before PH and 6 h post-PH when *c-myc* mRNA levels are very low, but the absence of ID2 in between when the rate of *c-myc* transcription is high (Table I; Rodriguez *et al.*, 2006). A similar pattern was observed for mSin3A. In contrast, E2F-4 and p130 constantly occupy the *c-myc* promoter before PH and during the first 6 h after PH (Rodriguez *et al.*, 2006). BDL (bile duct ligation) is viewed as a model of liver cirrhosis that is regarded as a precancerous condition sharing some mechanistic aspects of liver regeneration (Findor *et al.*, 2002). Before BDL, when very low *c-myc* mRNA levels are expressed, the *c-myc* promoter is occupied by E2F-4, p130, mSin3A, and ID2 (Rodriguez *et al.*, 2006). The significant increase in *c-myc* transcription levels 28 days after BDL is accompanied by the disappearance of mSin3A and ID2 whereas E2F-4 and p130 remain on the *c-myc* promoter. These findings suggest a role for ID2, a direct c-Myc target gene, and mSin3A in repression of the *c-myc* promoter (Rodriguez *et al.*, 2006). ID2 binds to the pocket proteins RB, p107 and p130 (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996, 2000; Rodriguez *et al.*, 2006). Rodriguez *et al.* (2006) reported ID2 to co-immunoprecipitate E2F-4 and mSin3A in quiescent liver, which both also co-immunoprecipitated with p130, suggesting the formation of a repressive complex of E2F-4, p130, mSin3A, and ID2 on the inactive *c-myc* promoter, which looses its repressive activity on removal of mSin3A and ID2 (Fig. 5A). This is not surprising for mSin3A, a central component of mSin3-HDAC corepressor complexes

(Ayer, 1999; Knoepfler and Eisenman, 1999) that can associate with pocket proteins via RBP1 (retinoblastoma binding protein 1) (Lai *et al.*, 2001; Rayman *et al.*, 2002). However, this finding is unexpected for ID2 because ID (inhibitor of DNA binding) proteins are known to stimulate proliferation, inhibit differentiation, promote angiogenesis, invasion and migration and such are implicated in tumorigenesis (Lasorella *et al.*, 2001; Norton, 2000; Perk *et al.*, 2005). Yet in some settings, they act as positive regulators of differentiation, they downregulate expression of immediate-early genes (e.g., *c-fos*, *egr-1)* following mitogenic signaling and ID2 loss leads to enhanced proliferation of intestinal epithelia and development of intestinal tumors (Norton, 2000; Perk *et al.*, 2005; Yokota, 2001).

6. ARID1A (p270, BAF250a, hOSA1, SMARCF1)

ARID1A (p270, BAF250a, hOSA1, SMARCF1) is the non-catalytic unique signature subunit of the ATP-dependent chromatin remodeling complex BAF that distinguishes it from the second mammalian SWI/SNF complex PBAF (Mohrmann and Verrijzer, 2005; Roberts and Orkin, 2004). ARID1A contains an ARID (AT-rich interaction domain) as DNA-binding motif, by which it binds DNA without sequence specificity. ARID1A is required for proper differentiation-associated cell cycle arrest of murine MC3T3-E1 preosteoblasts (Nagl *et al.*, 2005, 2006). Its depletion results in impaired induction of $p21^{WAF1/CIP1}$ and failed repression of E2F-responsive genes in differentiating calvarial MC3T3-E1 cells.

c-myc mRNA and protein expression are downregulated during differentiation of MC3T3-E1 cells (Nagl *et al.*, 2006). In contrast, ARID1A-depleted cells fail to repress *c-myc* mRNA and protein expression during differentiation (Table I; Nagl *et al.*, 2006). The *c-myc* promoter is occupied by ARID1A in differentiating cells, but not in exponentially growing cells suggesting that ARID1A is required for repression of *c-myc* transcription during osteoblast differentiation (Nagl *et al.*, 2006).

Other components of both BAF and PBAF, namely the ATPase BRG-1 and the core subunits SNF5/INI1 and BAF155/170, are recruited to the *c-myc* promoter in both exponentially growing and differentiating cells (Nagl *et al.*, 2006) consistent with ATP-dependent chromatin remodeling complexes contributing to transcriptional activation as well as repression (Roberts and Orkin, 2004).

7. Pitx2

Pitx2, a cell-restricted bicoid-related homeodomain transcription factor, is required for effective cell-type-specific proliferation and serves as transcriptional regulator in early to late G_1-phase (Baek *et al.*, 2003; Chen *et al.*, 1997;

Kioussi *et al.*, 2002; Lin *et al.*, 1999). Pitx2 occupies the *c-myc* promoter (Table I), in which several Pitx2-binding sites were found, one of which precisely corresponding to the consensus CTAATCC bicoid recognition sequence (Baek *et al.*, 2003). In C2C12 myoblast cells and αT3–1 pituitary cells, Pitx2 could stimulate the *c-myc* promoter 2- to 2.5-fold requiring the presence of three defined Pitx2 sites and comparable to the effect of addition of LiCl, a selective GSK-3 inhibitor. Pitx2 may be required for serum-induced *c-myc* transcription because microinjection of αPitx2 IgG abolished the about three-fold stimulation of the *c-myc* promoter by serum (Baek *et al.*, 2003). Accordingly, under serum-free conditions and in the absence of LiCl Pitx2 was not detected on the *c-myc* promoter in C2C12 cells while it appeared on the promoter during serum treatment or LiCl addition. Also in the presence of serum, induction with LiCl resulted in stronger Pitx2 binding to the *c-myc* promoter (Baek *et al.*, 2003). This occupancy of the *c-myc* promoter correlates with the expression of Pitx2, which is downregulated in serum-starved C2C12 cells, but induced by both serum and the Wnt/β-catenin pathway (Baek *et al.*, 2003; Kioussi *et al.*, 2002).

C. Other Transcription Factors That Regulate the *c-myc* Promoter

1. FOXO3A

FOXO3a (FKHRL1) plays a role in maintenance of a cellular resting state. It is involved in mediation of cytostatic TGF-β signals as a Smad partner, but in response to mitogenic signals activation of the PI3K/Akt pathway leads to phosphorylation of FOXO3a by Akt barring it from the nucleus and thus from its target genes that include *p27*, *p21*, *p130*, *cyclin D1 and D2* (Accili and Arden, 2004; Brunet *et al.*, 1999; Greer and Brunet, 2005; Tran *et al.*, 2003; Vivanco and Sawyers, 2002). As an antiproliferation factor FOXO3a represents an antagonist to c-Myc, which potently stimulates proliferation including repression of *p21* and *p27* transcription as well as activation of *cyclin D1* and *cyclin D2* transcription (Massagué, 2004).

FOXO3a was shown to repress the *c-myc* promoter in W53 cells (Fig. 5B; Table I; Dominguez-Caceres *et al.*, 2004). Yet it was not analyzed whether FOXO3a binds to the *c-myc* promoter. Quiescent W53 cells, lymphoid cells expressing the PRLR (prolactin receptor), can be mitogenically stimulated by the pleiotropic cytokine PRL (prolactin) as the only growth factor (Fresno Vara *et al.*, 2001). PRL induces *c-myc* transcription in W53 cells and c-Myc seems to be essential for the mitogenic response of these cells to PRL (Dominguez-Caceres *et al.*, 2004; Fresno Vara *et al.*, 2001). The study of Dominguez-Caceres *et al.* (2004) strongly suggests that PRL induces

c-myc transcription in W53 cells via the pathway PRL → PRLR → Src → PI3K → Akt/PKB —| FOXO3a —| *c-myc* transcription (Fig. 5B).

2. Mxi1 AND USF

The bHLHLZ transcription factor USF (upstream stimulatory factor) activates the *c-myc* promoter (Table I; Lee and Ziff, 1999). In contrast, the Mad protein and c-Myc-antagonist Mxi1 represses the *c-myc* promoter and inhibits its activation by USF as well as the induction of *c-myc* transcription by serum (Table I; Lee and Ziff, 1999; Luo *et al.*, 2004). Mxi1 represses the *c-myc* P2 promoter by a mechanism that involves the Inr element(s) and the E2F-binding site (Lee and Ziff, 1999; Luo *et al.*, 2004) because this repression was completely lost if both the E2F-binding site and the two Inr were mutated, whereas it remained if either the Inr at the transcription start site or the E2F-binding site plus the other Inr were present (Luo *et al.*, 2004). Also USF targets the *c-myc* P2 promoter and Mxi1 inhibits this activation of the *c-myc* P2 promoter by USF (Lee and Ziff, 1999). USF is known to bind to the Inr of several genes and to stimulate core promoters through their Inr (Du *et al.*, 1993; Roy *et al.*, 1991). However, it was neither analyzed whether USF binds to the Inr element(s) of the *c-myc* promoter nor whether Mxi1 binds directly or indirectly to the *c-myc* promoter.

The ubiquitous and constitutive USF (Gregor *et al.*, 1990; Sirito *et al.*, 1994) may provide a relatively constant positive stimulus for the basal activity of the *c-myc* promoter under normal conditions (Lee and Ziff, 1999). In contrast, repression of *c-myc* transcription by Mxi1, whose expression is induced during terminal differentiation (Hurlin *et al.*, 1995b; Larsson *et al.*, 1994; Schreiber-Agus *et al.*, 1994; Zervos *et al.*, 1993), may be required to block *c-myc* expression in differentiating cells in order to allow terminal differentiation (Lee and Ziff, 1999). Thereby Mxi1 may overcome the constitutive stimulation of *c-myc* transcription by USF that may persist in differentiating cells.

3. HOXB4

HOXB4, a member of the HOX family of transcription factors, induces self-renewal of HSCs (Antonchuk *et al.*, 2002; Morgan *et al.*, 2004). It sustains the enduring proliferation of murine $Lin^{-}Sca\text{-}1^{+}$ bone marrow hematopoietic stem/progenitor cells and augments their cell cycle progression (Satoh *et al.*, 2004). Ectopically expressed c-Myc, which also enables these $Lin^{-}Sca\text{-}1^{+}$ HSCs to keep proliferating and stimulates their cell cycle progression, can probably also induce self-renewal of HSCs (Murphy *et al.*, 2005; Satoh *et al.*, 2004). Satoh *et al.* (2004) demonstrated that, HOXB4 activates the *c-myc* promoter and thus increases the endogenous *c-myc* mRNA expression (Fig. 5C; Table I), but they did not analyze whether HOXB4 binds to *the c-myc* promoter. c-Myc is considered to be important

for HOXB4-induced self-renewal of HSCs as a downstream mediator of HOXB4 signals (Satoh *et al.*, 2004).

Remarkably, HOXB4 was also suggested to be implicated in repression of the *c-myc* promoter through MIE1 in response to 1,25-$(OH)_2$-D_3 (Figs. 4 and 5C; Pan and Simpson, 1999; see Section IV.A.26).

4. BMAL1/NPAS2 AND THE CIRCADIAN CLOCK

Circadian rhythms, daily oscillations in various biological processes, are regulated by an endogenous clock (Fu and Lee, 2003). *c-myc* is a CCG (circadian clock-controlled gene) and is regulated by the core circadian regulators BMAL1, PER2 (Period2) and CRY1 (Cryptochrome1) (Fu *et al.*, 2002). BMAL1/NPAS2 heterodimers repress the *c-myc* promoter (Fig. 5C; Table I). Since BMAL1 and NPAS2 are bHLH (basic-helix-loop-helix) transcription factors and the BMAL1/NPAS2-responsive region of the *c-myc* promoter contains two E-box consensus sequences Fu *et al.* (2002) suggested that BMAL1/NPAS2 may repress the *c-myc* promoter through these E-boxes. However, it was not analyzed whether BMAL1/NPAS2 bind to the *c-myc* promoter. CRY1, which inhibits the activity of BMAL1/NPAS2, relieves the *c-myc* promoter from the repression by BMAL1/NPAS2 (Fig. 5C), but CRY1 alone does not affect *c-myc* transcription (Fu *et al.*, 2002). PER2, which stimulates *bmal1* transcription (Reppert and Weaver, 2001; Young and Kay, 2001), represses the *c-myc* promoter, probably indirectly through stimulation of *bmal1* transcription (Fig. 5C; Fu *et al.*, 2002). Accordingly, in $mPer2^{m/m}$ mice, which are deficient in the *mPer2* gene and in circadian clock function (Zheng *et al.*, 1999, 2001), deregulation of *bmal1* results in deregulation and overexpression of *c-myc*, that is, the *c-myc* mRNA expression oscillates with a phase shift and is significantly increased throughout the 24-h period (Fu *et al.*, 2002). The circadian clock and PER2 can be regarded as tumor suppressors (Fu and Lee, 2003). Consistently, $mPer2^{m/m}$ mice are cancer prone and show a neoplastic growth phenotype (Fu *et al.*, 2002). The high incidence of γ radiation-induced lymphoma in $mPer2^{m/m}$ mice is suggested to result from the combination of c-Myc overexpression with (partial) deficiency in p53-mediated apoptosis after γ radiation in $mPer2^{m/m}$ thymocytes (Fu and Lee, 2003; Fu *et al.*, 2002) because suppression of c-Myc-induced apoptosis in c-Myc overexpressing cells is sufficient to initiate tumor development without additional oncogenic mutations (Pelengaris *et al.*, 2002b).

5. STAT5

Like STAT3, STAT5 promotes cell proliferation and survival and contributes to cell transformation and oncogenesis (Buitenhuis *et al.*, 2004; Haura *et al.*, 2005; Ihle, 2001; Levy and Darnell, 2002; O'Shea *et al.*, 2002; Yu and Jove, 2004).

A constitutively active STAT5A mutant is able to induce *c-myc* mRNA expression in the absence of IL-3 in IL-3-dependent mouse pro-B Ba/F3 cells (Table I; Hoover *et al.*, 2001; Nosaka *et al.*, 1999).

IL-12 induces *c-myc* transcription and T cell proliferation (Sugimoto *et al.*, 2003). The results of Sugimoto *et al.* (2003) propose that IL-12 induces T cell proliferation via the pathway IL-12 → JAK2 → STAT5 → *c-myc* transcription → T cell proliferation (Fig. 5A). Nevertheless, an involvement of STAT3 in addition to STAT5 is not excluded by their results.

IL-2 and IL-3, which both induce tyrosine phosphorylation of STAT5 and thus DNA binding by STAT5 (Moon and Nelson, 2001; Moon *et al.*, 2004a; Mui *et al.*, 1996; Wang *et al.*, 1996b), each induce *c-myc* mRNA expression via STAT5 independent of *de novo* protein synthesis (Fig. 5A; Lord *et al.*, 2000).

IL-2, the principal mitogenic factor for activated T cells, induces heterodimerization of the β and γc subunits of the IL-2R (IL-2 receptor). This results in activation of JAK1 and JAK3 that phosphorylate tyrosine residues on the IL-2Rβ chain, which then serve as docking sites for recruitment of downstream signaling effectors (Leonard and O'Shea, 1998; Nelson and Willerford, 1998). Downstream of the IL-2R two distinct signaling pathways can independently generate a proliferative signal each including induction of *c-myc* transcription (Moon and Nelson, 2001; Moon *et al.*, 2004a). One is mediated by the adaptor molecule Shc, the other by the transcription factor STAT5 (Fig. 5A; Lord *et al.*, 1998, 2000). However, it was not analyzed whether STAT5 binds to the *c-myc* promoter. The Shc pathway is composed of at least two major branches: the Ras/ERK pathway, which is activated through a Shc/Grb2/Sos complex, and the PI3K pathway, which is activated through a Shc/Grb2/Gab2 complex (Gu *et al.*, 2000; McCormick, 1993; Ravichandran, 2001; Rozakis-Adcock *et al.*, 1992). The ERK pathway is dispensable for Shc-mediated induction of *c-myc* transcription whereas the PI3K pathway is required but not sufficient for maximal induction of *c-myc* transcription by Shc (Moon and Nelson, 2001). STAT5 induces *c-myc* transcription independent of *de novo* protein synthesis (Lord *et al.*, 2000; Moon and Nelson, 2001; Moon *et al.*, 2004a). In addition, dependent on *de novo* protein synthesis, STAT5 activation by the IL-2R results in a late wave of PI3K kinase activity that is required for optimal induction of *c-myc* transcription by STAT5 (Moon *et al.*, 2004a). This late STAT5-mediated PI3K activity is independent from the early activation of the PI3K pathway by Shc. The PI3K pathway is not necessary for optimal STAT5 phosphorylation, nuclear translocation or DNA binding, but it is required for optimal transcriptional elongation of the *c-myc* gene in response to IL-2 signaling (Moon *et al.*, 2004a). As the PI3K pathway is required for optimal binding of Pol II to the *cyclin D2* promoter one may speculate that this mechanism could also operate at the *c-myc* promoter. Thus these results suggest a permissive

role for the PI3K pathway in the STAT5-mediated induction of *c-myc* transcription by IL-2 (Moon and Nelson, 2001; Moon *et al.*, 2004a).

6. ICAP-1 AND β-INTEGRIN

Cell adhesion to the ECM (extracellular matrix) promotes cellular proliferation (Aplin *et al.*, 1999). Thereby binding of integrins to ligands in the ECM activates multiple signaling pathways (Hood and Cheresh, 2002; van der Flier and Sonnenberg, 2001). Cellular adhesion of epithelial cells to the ECM component fibronectin induces *c-myc* mRNA expression independent of growth factors or *de novo* protein synthesis and β1 integrins mediate this fibronectin-induced c-Myc protein expression (Fig. 5C; Benaud and Dickson, 2001). ICAP-1 (integrin cytoplasmic domain-associated protein 1), which interacts specifically with the β1 integrin chain (Chang *et al.*, 1997), activates the *c-myc* promoter (Table I; Fournier *et al.*, 2005). However, it was not analyzed whether ICAP-1 that interacts with NM23-H2 (Fournier *et al.*, 2002), binds to the *c-myc* promoter. During cell spreading on fibronectin ICAP-1 shuttles from the cytoplasm into the nucleus, but β1 integrin overexpression sequesters ICAP-1 in the cytosol (Fournier *et al.*, 2005). ICAP-1 overexpression increases cell proliferation. These results of Fournier *et al.* (2005) and Benaud and Dickson (2001) propose a pathway cell adhesion to fibronectin —| β1 integrin —| ICAP-1 → *c-myc* transcription → cell proliferation (Fig. 5C).

7. MAZR

MAZR (MAZ-related factor) activates the *c-myc* promoter (Table I; Kobayashi *et al.*, 2000). Yet it was not analyzed whether it binds to the *c-myc* promoter, which contains multiple potential MAZR binding sites. Activation of the *c-myc* promoter depends on the N-terminal region of MAZR that includes the BTB/POZ domain, but MAZR has no TAD. Bach2 binds to the BTB/POZ domain of MAZR, but also lacks any transactivation potential. Surprisingly, coexpression of MAZR and Bach2 leads to transactivation of the *fgf4* promoter and an artificial reporter construct. However, it was not shown that Bach2 is involved in activation of the *c-myc* promoter by MAZR. Both MAZR and Bach2 are highly expressed in pro- and pre-B-cell lines while only low expression is found in immature and mature B-cell lines. Therefore MAZR was suggested to cooperate with Bach2 in regulation of gene expression during early stages of B-cell differentiation. The high levels of MAZR expression in hematopoietic tissues

suggest that MAZR may activate *c-myc* transcription in hematopoietic cells (Kobayashi *et al.*, 2000).

8. Mel-18

The PcG protein Mel-18, also known as PCGF2 (polycomb group ring finger 2), plays a role in development and has tumor suppressive activity (Kanno *et al.*, 1995). It inhibits cell proliferation and induces premature senescence (Guo *et al.*, 2007; Tetsu *et al.*, 1998)

Mel-18 represses the *c-myc* promoter (Table I; Tetsu *et al.*, 1998). Accordingly, the endogenous *c-myc* mRNA and protein expression are downregulated by Mel-18 overexpression, but upregulated by shRNA-mediated knockdown of Mel-18 (Guo *et al.*, 2007). This downregulation of the c-Myc protein level requires the RING finger domain of Mel-18 (Guo *et al.*, 2007). Similarly, small resting B cells from *mel-18* transgenic and knockout mice exhibit reduced *c-myc* mRNA or elevated c-Myc protein expression, respectively (Tetsu *et al.*, 1998). However, binding of Mel-18 to the *c-myc* promoter was not addressed. Nevertheless, the 0.8 kb Mel-18-responsive region of the *c-myc* promoter was reported to contain a consensus Mel-18 binding site (Kanno *et al.*, 1995; Tetsu *et al.*, 1998).

Mel-18 was suggested to repress c-Myc expression during cellular senescence (Guo *et al.*, 2007).

BCR stimulation with α-IgM induces proliferation of splenic B cells, but fails to induce proliferation of cells from mel-18 transgenic mice (Tetsu *et al.*, 1998). BCR stimulation with α-IgM induces also a transient increase in the *c-myc* mRNA expression in splenic small resting B cells, which is abolished in cells from *mel-18* transgenic mice (Tetsu *et al.*, 1998). In splenic B cells from mel-18 transgenic mice, this proliferation in response to BCR stimulation is rescued by enforced c-Myc expression suggesting that Mel-18 suppresses cell proliferation through c-Myc (Tetsu *et al.*, 1998). In consistence, B cells from mel-18 knockout mice, which exhibit an increased c-Myc protein expression already in the absence of α-IgM, proliferate even without BCR stimulation (Tetsu *et al.*, 1998).

D. Additional Transcription Factors That Directly Bind to or/and *In Vivo* Occupy the *c-myc* Promoter

Table III summarizes additional transcription factors that directly bind to or/and *in vivo* occupy the human and/or murine *c-myc* promoter (Fig. 4). It is indicated whether these transcription factors were shown to activate or

Table III Additional Transcription Factors That Directly Bind to or/and *In Vivo* Occupy the *c-myc* Promoter

Transcription factor (*or binding site*)		Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
AhR	Aryl hydrocarbon receptor	C		T, P	↓	A/R	T	wt, P	↓	A/R	Yang *et al.*, 2005
AP-2		E, F	IV								Imagawa *et al.*, 1987; Moser *et al.*, 1995
CUT		E, FBA	IV, S, C				T	wt, del	↓	OE	Dufort and Nepveu, 1994
FBI-1	Factor binding to IST (inducer of short transcripts)	M	IV								Pessler and Hernandez, 2003
GR (*GRB1*)[g]	Glucocorticoid receptor	E, F, SW	IV, C				T	wt, del, H	↑	A/R	Ma *et al.*, 2000
GR (*A/G*)[g]		E, F, SW	IV, C				T	wt, H	↓	A/R	Ma *et al.*, 2000
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1	E	IV, C, P								Takimoto *et al.*, 1993
LR1		E, F	IV, P				T	wt, del, P	↑		Brys and Maizels, 1994

MAZi	Myc-associated zinc-finger protein of human islet	E, FBA	IV, C, P				T	wt, del	↑	OE, del	Tsutsui *et al.*, 1996
MSSP-1	*c-myc* gene single-strand binding protein-1	E, F, FBA, SW	IV, C, P								Negishi *et al.*, 1994
MSSP-2		E	IV, C, P								Takai *et al.*, 1994
c-Myb[j]		E, F, MPA, E III A	IV, C	T, P, CH	↑	OE, dn	T, CH	wt, del, P	↑	OE, dn, del	Evans *et al.*, 1990; Zobel *et al.*, 1991, 1992; Nakagoshi *et al.*, 1992; Cogswell *et al.*, 1993; Schmidt *et al.*, 2000
B-Myb							T	wt, del	↑	OE	Nakagoshi *et al.*, 1992
NF1		E	IV, C								Lang *et al.*, 1991
NSEP-1	Nuclease-sensitive element protein-1	E, FBA	IV, C, P								Kolluri and Kinniburgh, 1991; Kolluri *et al.*, 1992

(*continues*)

Table III (*continued*)

Transcription factor (*or binding site*)		Binding to *c-myc* promoter		Regulation of endogenous *c-myc* expression			Regulation of *c-myc* promoter				References
		Method[a]	Comment[b]	Expression[c]		Manipulation of transcription factor[d]	Reporter construct[e]	Manipulation of binding site[f]		Manipulation of transcription factor[d]	
Oct	Octamer binding protein	E	IV, C								Takimoto *et al.*, 1989
p30		CLA									Zajac-Kaye *et al.*, 2000
p97		CLA									Zajac-Kaye *et al.*, 2000
p105/p115		E	C				T	H	↓		Itkes *et al.*, 2000
PTTG	Pituitary tumor-transforming gene	E, F	IV, S, C, P	T, P	↑	OE, del	T	wt, del, P	↑	OE, dn, del	Pei, 2001
Pur		E, M, FBA	C, P								Bergemann and Johnson, 1992; Bergemann *et al.*, 1992
SATB1[h]	Special AT-rich binding protein 1	C		T	↓	KO					Alvarez *et al.*, 2000; Cai *et al.*, 2003
THZif-1	Triple helix-binding zinc-finger protein-1	E	IV, S, C, P				T, IV S	wt wt, del, H	↓ ↓	P, OE, del OE, del	Kawasaki *et al.*, 1996; Sakatsume *et al.*, 1996
WT1 (*human*)[i]	Wilms' tumor 1 gene	E, C	IV, C, P	T, P	↑	OE, NI	T	wt, del, P	↑	OE, NI	Han *et al.*, 2004
WT1 (*mouse*)[i]		E	IV, C				T	wt	↓	OE, NI	Hewitt *et al.*, 1995

YY1	Yin-yang 1	E, C, OC	IV, S, C	T	↑	OE	T	wt, del, P, H	↑	OE, del	Kakkis *et al.*, 1989; Riggs *et al.*, 1991, 1993; Lee *et al.*, 1994; Yu *et al.*, 2000; Kurisaki *et al.*, 2003; Rezai-Zadeh *et al.*, 2003; Favot *et al.*, 2005; Liu *et al.*, 2006a
ZF5		E, M, FBA, SW	C				T	wt	↓	OE	Numoto *et al.*, 1993

[a] *Method (method used to demonstrate binding to the c-myc promoter):* E = EMSA = electrophoretic mobility shift assay; C = ChIP = chromatin immunoprecipitation assay; F = DNAse I footprinting analysis; M = methylation interference analysis; FBA = filter binding assay; SW = Southwestern blot analysis; MPA = methylation protection assay; E III A = exenuclease III assay.

[b] *Comment:* IV = *in vitro* = (partially) purified transcription factor; S = supershift experiments; C = competition experiments; P = binding site was point-mutated (or deleted).

[c] *Expression:* T = transcript = mRNA = endogenous mRNA level affected; P = protein = endogenous protein level affected; CH = effect was detected in the presence of cycloheximide.

[d] *Manipulation of transcription factor:* P = purified transcription factor; OE = overexpression of wild type; dn = dominant-negative form; del = analyzed with deletion (or/and point) mutants of the transcription factor; KO = knockout cells/mice (cancer cell lines deficient in transcription factor); A/R = activation/repression of the transcriptional activity of the transcription factor (see text for details); NI = natural isoforms.

[e] *Reporter contruct:* T = transiently transfected; S = stably transfected; IV = *in vitro* transcription; CH = effect was detected in the presence of cycloheximide.

[f] *Manipulation of binding site:* wt = "wild type" *c-myc* promoter; del = analyzed with deletion mutants of *c-myc* promoter; P = binding site was point-mutated; H = binding site upstream (or downstream) of heterologous core promoter.

[g] The GR binds to two different sites in the *c-myc* promoter, which are designated GRB1 (positioned more 5′) and A/G (positioned more 3′).

[h] Although the *c-myc* transcript level is elevated in *Satb1*-null thymocytes compared to wild-type thymocytes before stimulation mitogenic stimulation (ionomycin plus PMA) strongly induces *c-myc* mRNA expression in wild-type thymocytes, but leads to a decrease of the *c-myc* transcript level in *Satb1*-null thymocytes.

[i] WT1 transactivates the human *c-myc* promoter, but represses the murine *c-myc* promoter.

[j] The *c-myc* gene possesses multiple c-Myb binding sites (more than 10 high affinity sites and more than 10 low or intermediate affinity sites) that are scattered throughout the promoter region and exon 1.

repress the *c-myc* promoter. It is also indicated whether they were shown to regulate the endogenous *c-myc* expression.

E. Signal Transduction Pathways

Virtually every major signal transduction pathway bearing proliferative or antiproliferative cues impacts directly or indirectly the *c-myc* promoter (Liu and Levens, 2006; Nasi *et al.*, 2001; Ponzielli *et al.*, 2005) so that a vast amount of signaling molecules has been implicated in regulation of *c-myc* transcription. Therefore this chapter on signal transduction pathways to the *c-myc* promoter is not intended to be comprehensive. Instead selected signaling pathways involved in control of *c-myc* transcription will be illustrated, which have not been covered in the preceding description of the transcription factors that regulate the *c-myc* promoter.

Before, some general aspects will be pointed out that should be considered:

1. The actual signaling route, the absolute requirement for a specific signal and the outcome of a particular signal may depend on cell type and cellular context as well as on the developmental or physiological state of a cell. In different cell types, ubiquitous factors could give rise to a uniform response whereas cell-specific factors could dictate differential responses.
2. Cross-talk is a central principle of signal transduction *in vivo*. There are two general classes of interconnections: Junctions are signal integrators. Nodes split the signal and route it to multiple outputs (Jordan *et al.*, 2000; Massagué, 2004). A major function of signaling networks is to place a value on the signal such that it is either converted into further biochemical event and subsequently a biological response or safely dissipated within the network (Jordan *et al.*, 2000). Two properties of the network emerge from this signal consolidation: The first is the setting of a threshold for the physiological response. The second is the ability to propagate responses across different time scales (Jordan *et al.*, 2000). The cell is confronted with many divergent, weak, fluctuating, subsaturating, pulsatile, contradictory signal inputs and cross-talk enables it to respond in an appropriate and coordinate fashion. Cross-talk between signaling pathways that regulate the *c-myc* promoter is especially important as c-Myc is on the one hand essential, but on the other hand dangerous. For simplification, the many obvious possibilities for cross-talk will be neither discussed nor shown in Fig. 5.
3. Many signaling components exist in several versions that are encoded by paralogous genes or products of alternative splicing of the same transcript. This variegation provides cell-type specificity as well as redundancy and robustness to signal transduction pathways (Massagué, 2004). For example, the Src family tyrosine kinases comprise c-Src, Fyn, Yes and Yrk,

which are ubiquitously expressed, as well as Lyn, Lck, Hck, Blk, and Fgr, which are restricted to hematopoietic tissues (Parsons and Parsons, 2004). The roles of these individual family members have for the most part not been addressed and they may have redundant functions (Bromann *et al.*, 2004) so that they are often referred to as Src (Fig. 5B; for Src family tyrosine kinase).

4. Experimental results were obtained under artificial conditions, which do not exist in organisms. For example, immortalized cell lines grow under optimized conditions and agents for their treatment are applied permanently in saturating concentrations for a long period.

5. Tumor cells, which are of special interest with respect to potential targets for anticancer therapy, are distinct from normal cells (Evan and Vousden, 2001; Green and Evan, 2002; Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000; Lowe *et al.*, 2004). The intracellular circuitry that regulates signal transduction and gene expression in cancer cells is very different, even bizarre, when compared to normal cells (Weinstein, 2000). Cancer cells are often dependent on the continued expression of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype (oncogene addiction) (Jonkers and Berns, 2004). Some cancer cells seem to be "hypersensitive" to growth-inhibitory effects of specific tumor suppressor genes (tumor suppressor gene hypersensitivity) as well as to interventions that reestablish pro-apoptotic pathways or disable survival programs. Both oncogene addition and tumor suppressor hypersensitivity provide Achilles heels for tumors that can be exploited in cancer therapy (Green and Evan, 2002; Jonkers and Berns, 2004; Lowe *et al.*, 2004; Weinstein, 2002).

6. Ras and PI3K networks power the G_1 engine of the cell cycle. Scores of mitogenic factors, acting through many different receptor tyrosine kinases and G-protein-coupled receptors, activate the Ras and PI3K pathways to stimulate cell proliferation, growth and survival (Massagué, 2004). These two potent proliferation pathways each increase the expression of c-Myc on at least three different levels: transcription, translation and protein stability. First, both PI3K and Ras are implicated in activation of the *c-myc* promoter (see Sections IV.E.2 and IV.E.5). Second, eIF-4E (eukaryotic initiation factor-4E) regulates cap-dependent translation and selectively enables translation of a limited pool of weak mRNAs, namely those encoding key proteins involved in cellular growth, angiogenesis, survival and malignancy (e.g., *c-myc* and *cyclin D1*) (Clemens, 2004; De Benedetti and Graff, 2004; Gingras *et al.*, 2004; Hay and Sonenberg, 2004; Mamane *et al.*, 2004). eIF-4E is bound by the inhibitory 4EBPs (eIF-4E binding proteins). Phosphorylation of the 4EBPs by mTOR (mammalian target of rapamycin) liberates eIF-4E from the 4EBPs. Phosphorylation of eIF-4E by the ERK- or p38MAPK-dependent kinase MNK activates eIF-4E (Holland *et al.*, 2004; Mamane *et al.*, 2004; Massagué, 2004; Rajasekhar *et al.*, 2003; Rosenwald, 2004). Thereby both

the PI3K-Akt/PKB-mTOR and Ras-Raf-MEK-ERK/MAPK-MNK pathways enhance ribosomal recruitment of *c-myc* mRNAs and thus increase *c-myc* translation. Third, phosphorylation of c-Myc at Ser-62 by ERK increases its stability whereas phosphorylation of c-Myc at Thr-58 by GSK-3 decreases its stability so that both activation of ERK via the Ras-Raf-MEK-ERK pathway and inhibition of GSK-3 via the PI3K-Akt/PKB-GSK-3 pathway result in higher c-Myc protein levels (Gregory and Hann, 2000; Gregory *et al.*, 2003; Sears, 2004; Sears and Nevins, 2002; Sears *et al.*, 1999, 2000). This multiple strategy of each Ras and PI3K to enhance c-Myc expression is biologically efficient, but makes it difficult to dissect effects on *c-myc* transcription from other effects in studies that do not address the question of *c-myc* promoter control. Thus, if a factor activates PI3K or Ras one has to keep in mind that it will (probably) also increase *c-myc* translation and c-Myc protein stability.

7. In Fig. 5 signaling pathways that regulate *c-myc* transcription are shown as they were described so far. In future studies some signaling routes, which are shown separately, will possibly turn out to be parts of the same signaling pathway while others will possibly be confirmed to be independent or alternative.

1. Src AND Shc

The classical experiments of Barone and Courtneidge (1995) demonstrated that PDGF-induced mitogenesis requires the two branches PDGF → PDGFR → Src → c-Myc → mitogenesis (Fig. 5B) and PDGF → PDGFR → Ras → c-Fos/c-Jun (AP-1) → mitogenesis (Abram and Courtneidge, 2000; Blake *et al.*, 2000; Eisenman and Cooper, 1995). PDGF activates the *c-myc* promoter via the pathway PDGF → PDGFR → Src → *c-myc* promoter (Fig. 5B; Barone and Courtneidege, 1995; Blake *et al.*, 2000; Chiariello *et al.*, 2001). This pathway is independent of the Ras/Raf/MEK/MAPK cascade. Downstream of Src PDGF-induction of *c-myc* transcription can occur through STAT3 (Bowman *et al.*, 2001) or Vav2 and Rac (Chiariello *et al.*, 2001) or c-Abl (Furstoss *et al.*, 2002) or Shc (Blake *et al.*, 2000) or possibly other factors (Fig. 5B) and it is unknown whether these four signaling routes are independent or parts of the same route/s (Bromann *et al.*, 2004). Also EGF and CSF-1 induce mitogenesis via Src and c-Myc (Barone and Courtneidge, 1995).

The pathway PDGF → PDGFR → c-Src → STAT3 → *c-myc* transcription (Fig. 5B; Bowman *et al.*, 2001) is supported by the finding that the pathway PDGF → PDGFR → Src → *c-myc* promoter was shown to target a *c-myc* promoter construct (–157 to +500 relative to the P1 transcription start site) (Chiariello *et al.*, 2001), which contains the STAT3 binding site (Fig. 4; see Section IV.A.6).

Vav2 can act as a tyrosine-phosphorylation-dependent GEF (guanine nucleotide exchange factor) for Rac (Abe *et al.*, 2000; Crespo *et al.*, 1997; Liu and Burridge, 2000). The pathway PDGF → PDGFR → Src → Vav2 → Rac → *c-myc* promoter was demonstrated for a *c-myc* promoter construct (–157 to +500 relative to the P1 transcription start site) (Chiariello *et al.*, 2001) that lacks both AP-1 binding sites of the *c-myc* promoter (Fig. 4). Nevertheless, Iavarone *et al.* (2003) suggested that PDGF may activate the *c-myc* promoter via the pathway PDGF → PDGFR → Src → Vav2 → Rac → JNK → c-Jun/JunD → *c-myc* promoter (Fig. 5B; see Section IV.A.14) through the AP-1 binding site, which is positioned approximately 1.3 kb upstream of the P1 transcription start site (Fig. 4).

The pathway PDGF → PDGFR → Src → *c-myc* promoter is well established and PDGF induces mitogenesis via Src and c-Myc (Fig. 5B; Barone and Courtneidge, 1995; Blake *et al.*, 2000; Chiariello *et al.*, 2001). The pathway PDGF → PDGFR → c-Abl → *c-myc* mRNA expression is also known and PDGF induces mitogenesis via c-Abl and c-Myc (Furstoss *et al.*, 2002). Consequently, the identification of the tyrosine kinase c-Abl as a Src substrate and the finding that PDGF induces mitogenesis via Src and c-Abl (Furstoss *et al.*, 2002; Plattner *et al.*, 1999) lead to the conclusion that PDGF may activate *c-myc* transcription via the pathway PDGF → PDGFR → Src → c-Abl → *c-myc* transcription (Fig. 5B). However, it was not demonstrated that c-Abl is downstream of Src in PDGF-induced *c-myc* transcription. Moreover, for activation of c-Abl by PDGFR the activity of Src kinases alone is not sufficient but functional PLC-γ1 (phospholipase C γ1) is required, too (Plattner *et al.*, 2003).

The two pathways PDGF → PDGFR → Src → Shc → *c-myc* transcription (Fig. 5B; Blake *et al.*, 2000) and EGF → EGFR → Shc → *c-myc* transcription (Fig. 5B; Gotoh *et al.*, 1997) are independent from the Ras/Raf/MEK/MAPK cascade, which was demonstrated with Shc mutants: EGF induces *c-myc* mRNA expression as well as MAPK activation. In EGF-stimulated cells expressing an autophosphorylation-minus mutant EGFR, the Shc double mutant Y239F/Y240F inhibits *c-myc* mRNA expression but not MAPK activation whereas vice versa the Shc single mutant Y317F suppresses MAPK activation but not *c-myc* mRNA expression (Gotoh *et al.*, 1997). In response to PDGF, Src phosphorylates Shc at Y239 and Y240 but not at Y317 and in the presence of an inhibitory Y239F/Y240F Shc mutant, additionally mutated in the SH2 domain, PDGF-induced mitogenesis is rescued by ectopic c-Myc expression, but not by ectopic c-Fos expression (Blake *et al.*, 2000).

In summary, from the available data it is quite clear that several growth factors (e.g., PDGF, EGF) can induce *c-myc* transcription through Src independent from the Ras/MAPK pathway (Abram and Courtneidge, 2000; Bromann *et al.*, 2004).

2. Ras

One major unresolved problem is the involvement of the classical Ras/Raf/MEK/MAPK pathway in activation of the *c-myc* promoter. Although several of its components have been described to stimulate *c-myc* transcription the importance of the Ras/Raf/MEK/MAPK cascade for activation of the *c-myc* promoter is still subject to conflicting debate (Abram and Courtneidge, 2000; Bromann *et al.*, 2004).

Since both MEK1/2 (Cheng *et al.*, 1999a) and ETS-2 (Langer *et al.*, 1992) are required for CSF-1-induced *c-myc* transcription and since ETS-2 binds to the *c-myc* promoter (Roussel *et al.*, 1994) the pathway CSF-1 → MEK1/2 → ERK1/2 → ETS-1/2 → *c-myc* transcription can be concluded (Fig. 5B; see Section IV.A.15). Constitutively active forms of Ras, c-Raf-1 and MEK1 efficiently activated *c-myc* promoter constructs (–157 to +500 or –140 to +340, respectively, relative to the P1 transcription start site) although an activated form of Src provoked a much greater response whereas vice versa a dominant-negative form of Ras repressed such a *c-myc* promoter construct (Chiariello *et al.*, 2001; Zou *et al.*, 1997a,b). Moreover, a MEK inhibitor slightly reduced the stimulation of such a *c-myc* promoter construct by PDGF and the PDGF-induced endogenous *c-myc* mRNA expression (Chiariello *et al.*, 2001). In addition, activation of c-Raf-1 induces *c-myc* mRNA expression (Kerkhoff *et al.*, 1998). Also activation of B-Raf activates the *c-myc* promoter and induces *c-myc* mRNA and protein expression (Aziz *et al.*, 1999). Serum-induced *c-myc* mRNA expression was shown to involve the pathway serum → Ras → c-Raf-1 → *c-myc* mRNA expression (Fig. 5B; Kerkhoff *et al.*, 1998). This pathway is supported by the finding that both B-Raf-induced and serum-stimulated *c-myc* mRNA expression are immediate-early responses, which show very similar kinetics (Aziz *et al.*, 1999). The kinetic of c-Raf-1-induced *c-myc* mRNA expression suggests that the isolated Raf/MEK/ERK signal transduction cascade is sufficient to induce *c-myc* expression because c-Raf-1 leads to a rapid and sustained induction of ERK kinases, but it is not able to directly activate during this time period other parallel signal transduction cascades, like the MEKK/SEK/JNK cascade (Kerkhoff and Rapp, 1997, 1998; Kerkhoff *et al.*, 1998; Minden *et al.*, 1994).

EGF activates the *c-myc* promoter through EGFR1. LRIG1 (leucine-rich repeats and immunoglobulin-like domains 1), a negative regulator of EGFRs, inhibits this activation of the *c-myc* promoter by EGF and thus reduces the endogenous c-Myc protein level (Fig. 5B; Jensen and Watt, 2006). The transmembrane protein LRIG1 interacts with EGFR1 and enhances its ubiquitylation and degradation resulting in downregulation of the EGFR1 level and thus in reduction of the EGF-induced ERK1/2 phosphorylation and the EGF-induced interaction of Ras with Raf1 (Gur *et al.*, 2004; Jensen and Watt,

2006). This suggests that the Ras → Raf1 → MEK → ERK1/2 cascade may be involved in activation of *c-myc* transcription by EGF.

Together these findings point to an involvement of the Ras → Raf → MEK → ERK → ETS pathway in activation of the *c-myc* promoter by mitogens, but its final proof is still outstanding. One important question is that of the downstream target of Raf (Chang *et al.*, 2003; Hindley and Kolch, 2002; Pearson *et al.*, 2000; Steelman *et al.*, 2004), which can for example activate MEK-1/2 (Dent *et al.*, 1992; Kyriakis *et al.*, 1992) or Cdc25A (Galaktionov *et al.*, 1995) or MEKK1 → IKKα/IKKβ (Arsura *et al.*, 2000; Baumann *et al.*, 2000; Lee *et al.*, 1997, 1998; Yamamoto and Gaynor, 2004). Transcription factors as potential targets of Ras/Raf signaling to the *c-myc* promoter are obvious, for example, ETS-1/2, AP-1, NF-κB, c-Myc, STAT3, and E2F (Fig. 4; Chang *et al.*, 2003; Decker and Kovarik, 2000; Hindley and Kolch, 2002; Lewis *et al.*, 1998; Rane and Reddy, 2002; Steelman *et al.*, 2004). Additionally, Ras/Raf signaling may affect *c-myc* transcription at the level of elongation by phosphorylating components of the basal transcription machinery.

So far, one can only speculate that Ras/Raf signaling or the classical Ras/MAPK pathway may play a role for activation of the *c-myc* promoter: (1) dependent on cell type and cellular context, (2) in response to particular proliferation signals, (3) in cooperation with other signaling pathways (e.g., Src) during high signal intensity to achieve the maximal induction of *c-myc* transcription, and (4) under abnormal circumstances when other pathways are blocked.

In mammary, colon and pancreatic tumor cells with an aberrant activation of Ras the Smad-mediated repression of the *c-myc* promoter by TGF-β is abolished (Fig. 5A; see Sections IV.A.3 and IV.A.30) (Buck *et al.*, 2006; Calonge and Massagué, 1999; Chen *et al.*, 2001b) suggesting that in normal cells cross talk between the Ras- and TGF-β/Smad pathways may be involved in regulation of *c-myc* transcription, too.

3. Abl

v-Abl, an oncogenic form of the c-Abl non-receptor tyrosine kinase, activates the *c-myc* promoter probably via the pathway v-Abl → Shc → Grb2/Sos → Ras → Raf1 → Cdc25A → Cdk2 + Cdk4 —| RB, p107, p130 —| E2F → *c-myc* promoter (Fig. 5A; Zou *et al.*, 1997a,b). However, the role of Cdc25A, which is phosphorylated and activated by Raf1 (Galaktionov *et al.*, 1995), is only implied but not proven in this pathway (Zou *et al.*, 1997a,b), parts of which were discovered in previous studies (e.g., Cleveland *et al.*, 1989; Raffel *et al.*, 1996; Sawyers *et al.*, 1992; Wong *et al.*, 1995). Studies on the oncogenic translocation product BCR-Abl strongly suggest that it can use the same pathway to activate *c-myc* transcription (e.g., Afar *et al.*, 1994; Goga *et al.*, 1995; Sawyers *et al.*, 1992, 1995; Stewart *et al.*, 1995). In addition, the study of Xie *et al.* (2002) strongly suggests that BCR-Abl

induces *c-myc* mRNA expression via the pathway BCR-Abl → JAK2 → *c-myc* mRNA expression (Fig. 5A).

4. CYTOKINES

A large variety of cytokines and molecules involved in cytokine signaling pathways have been described to activate *c-myc* expression so that only very few examples are mentioned here and the interested reader is referred to the original literature.

IL-2 and IL-3 stimulate *c-myc* mRNA expression (Miyazaki *et al.*, 1995; Shibuya *et al.*, 1992). Both IL-3 and GM-CSF (granulocyte/macrophage colony stimulating factor) activate the *c-myc* promoter via the pathway IL-3 or GM-CSF → JAK2 → *c-myc* promoter (Fig. 5A; Watanabe *et al.*, 1996). IL-2 induces *c-myc* mRNA expression via the pathway IL-2 → JAK3 → *c-myc* mRNA expression (Fig. 5A; Kawahara *et al.*, 1995). The studies of Takeshita *et al.* (1997), Endo *et al.* (2000) and Pandey *et al.* (2000) strongly suggest that both GM-CSF and IL-2 activate the *c-myc* promoter via the pathway GM-CSF or IL-2 → JAK2/3 → STAM1/2 (signal transducing adaptor molecule 1/2) → *c-myc* promoter (Fig. 5A). Minami *et al.* (1995) suggested that IL-2 induces *c-myc* expression via the pathway IL-2 → Syk → *c-myc* expression.

5. PI3K

IL-2 induces c-Myc protein expression via the pathway IL-2 → PI3K → Akt/PKB → c-Myc protein expression (Fig. 5A; Ahmed *et al.*, 1997). Of course, this induction of c-Myc protein could be achieved solely at the level of c-Myc protein stability through inactivation of GSK-3 by Akt/PKB (Gregory *et al.*, 2003; Sears and Nevins, 2002; Sears *et al.*, 2000) and the study of Ahmed *et al.* (1997) does not rule out this possibility. However, the combination of the results of Ahmed *et al.* (1997), Brennan *et al.* (1997, 1999), Ghosh *et al.* (1999b) and Feng *et al.* (2000) with the current general knowledge (Blume-Jensen and Hunter, 2001; Brazil *et al.*, 2004; Cantrell, 2001; Cully *et al.*, 2006; Greer and Brunet, 2005; Lawlor and Alessi, 2001; Liang and Slingerland, 2003; Massagué, 2004; McCormick, 1999; Rosenwald, 2004; Schmelzle and Hall, 2000; Sears and Nevins, 2002; and references therein) strongly suggests that PI3K activates the *c-myc* promoter via the hypothetical pathways shown in the gray box in Fig. 5A.

IL-2 induces E2F activity via the pathway IL-2 → PI3K → Akt/PKB → E2F activity (Fig. 5A; Brennan *et al.*, 1997). Thereby inhibition of PI3K inhibits phosphorylation of RB, induction of cyclin D3 and degradation of p27 (Fig. 5A; Brennan *et al.*, 1997). The tumor suppressor PTEN (phosphate and tensin homolog deleted on chromosome 10), which prevents Akt/PKB activation by PI3K (Cully *et al.*, 2006; Sulis and Parsons, 2003), represses *c-myc*

transcription (Fig. 5A; Ghosh *et al.*, 1999b). Akt/PKB phosphorylates p21 and p27 and inhibits them by causing their cytoplasmic retention (Liang *et al.*, 2002; Shin *et al.*, 2002; Viglietto *et al.*, 2002; Zhou *et al.*, 2001). Phosphorylation by Akt/PBK stabilizes MDM2 and causes its translocation to the nucleus so that it promotes degradation of p53 leading to a reduction in transcription of $p21^{Cip1}$ (Feng *et al.*, 2004; Murray *et al.*, 2003). Phosphorylation of p27 by its own target cyclin E/Cdk2 triggers its degradation (Sheaff *et al.*, 1997; Sherr, 2000). Akt/PKB phosphorylates FOXO transcription factors and inhibits them by causing their sequestration in the cytoplasm (Brunet *et al.*, 1999; Greer and Brunet, 2005). FOXO transcription factors activate transcription of *p27*, *p21* and *p130*, but repress transcription of *cyclin D1* and *D2* (Greer and Brunet, 2005; Kops *et al.*, 2002; Medema *et al.*, 2000; Nakamura *et al.*, 2000; Ramaswamy *et al.*, 2002; Schmidt *et al.*, 2002; Seoane *et al.*, 2004; Tran *et al.*, 2003; Vivanco and Sawyers, 2002). Akt/PKB phosphorylates and inactivates GSK-3β (Cross *et al.*, 1995), which phosphorylates both cyclin D1 and β-catenin resulting in their degradation (see Section IV.A.1; Bienz and Clevers, 2000; Diehl *et al.*, 1998). β-catenin-TCF/LEF complexes transactivate the *cyclin D1* promoter (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999). PI3K and Akt/PKB stimulate the rate of *cyclin D* translation (Muise-Helmericks *et al.*, 1998). Akt/PKB activates mTOR (Hay and Sonenberg, 2004; Thomas, 2006), which in turn phosphorylates the 4 EBPs thereby liberating eIF-4E that is particularly important for translation of mRNAs containing a highly structured 5′ UTR (untranslated region), such as the transcripts encoding cyclin D1 and c-Myc (Clemens, 2004; De Benedetti and Graff, 2004; Gingras *et al.*, 2004; Holland *et al.*, 2004; Mamane *et al.*, 2004; Rajasekhar *et al.*, 2003). $p70^{S6K}$ (p70 S6 kinase), the 40S ribosomal protein S6 kinase, which is phosphorylated and activated by mTOR and PDK-1 (3-phosphoinositide-dependent kinase-1) (Bjornsti and Houghton, 2004; Fingar and Blenis, 2004) increases E2F transcriptional activity (Brennan *et al.*, 1999). The studies of Brennan *et al.* (1999) and Feng *et al.* (2000) suggest that $p70^{S6K}$ upregulates cyclin D3 and cyclin D2 expression resulting in phosphorylation of RB and p130, which leads to enhanced transcriptional activity of E2F. Finally, phosphorylation of RB and the two other pocket proteins by cyclin D/Cdk4 and cyclinE/Cdk2 releases E2F (Fig. 5A; Ezhevsky *et al.*, 1997; Harbour and Dean, 2000; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Weinberg, 1995; Zhang and Dean, 2001).

6. Epo

Erythroid progenitor cell growth and differentiation are regulated by the hematopoietic growth factor Epo. Epo induces *c-myc* mRNA and protein expression in BaF3 cells stably transfected with EpoR cDNA (Chen and Sytkowski, 2001). The studies of Patel *et al.* (1992) and Chen and Sytkowski (2001) strongly

suggest that Epo activates *c-myc* transcription initiation via the pathway Epo → EpoR → PI3K → *c-myc* transcription initiation whereas Epo activates *c-myc* transcription elongation via the pathway Epo → EpoR → PKC → MEK → *c-myc* transcription elongation (Fig. 5B). Although PI3K-dependent phosphorylation of Akt/PKB as well as PKC- and MEK-dependent phosphorylation of ERK1/2 in response to Epo were shown (Chen and Sytkowski, 2001) it was not demonstrated that Akt/PKB and ERK1/2 are upstream of *c-myc* in Epo signaling. Chen and Sytkowski (2001) suggest that in hematopoietic cells activation of the MEK/ERK pathway by Epo may occur by either of two mechanisms depending either on Ras activation or on PKC.

7. BMP

BMPs, members of the TGF-β family of cytokines, play roles from early embryogenesis through the adult. BMP7 downregulates the *c-myc* mRNA expression in a Smad4-dependent manner in human mammary carcinoma MDA-MB-468 cells (Kowanetz *et al.*, 2004). Since BMP7 can activate Smad1, which forms complexes with Smad4 (Feng and Derynck, 2005; Massagué *et al.*, 2005) and binds to the *c-myc* promoter (Fig. 4; Hu and Rosenblum, 2005; see Section IV.A.31), one may speculate that BMP7 represses *c-myc* transcription through Smad1/Smad4 complexes.

8. POLYAMINES

Cellular polyamines (spermine, spermidine and their precursor putrescine) are essential for cell proliferation in the intestinal epithelium, which is a self-renewing tissue (Liu *et al.*, 2005, 2006b; McCormack and Johnson, 1991). Their regulation is thought to be the central convergence point for multiple signaling pathways driving epithelial cell decisions in control of intestinal mucosal homeostasis (Gerner and Meyskens, 2004). ODC (L-ornithine decarboxylase), a direct c-Myc target gene, is a rate-limiting enzyme in polyamine biosynthesis, necessary for DNA synthesis. ODC catalyzes the first step in the polyamine biosynthesis pathway forming putrescine, which is then converted into the higher polyamines spermidine and spermine. Cellular polyamines activate *c-myc* transcription and experiments with the specific ODC inhibitor DFMO (α-difluoromethylornithine) and rat intestinal IEC-6 cells stably expressing ODC strongly suggest that ODC activates *c-myc* transcription via cellular polyamines (Fig. 5C; Liu *et al.*, 2005, 2006b; Patel and Wang, 1997). This positive feedback loop (Fig. 8) exemplifies how *c-myc* transcription can be coupled to the metabolic state of the cell, a sensor for multiple cellular processes. c-Myc in turn exerts a strong impact on metabolism by regulating DNA, RNA and protein synthesis as well as

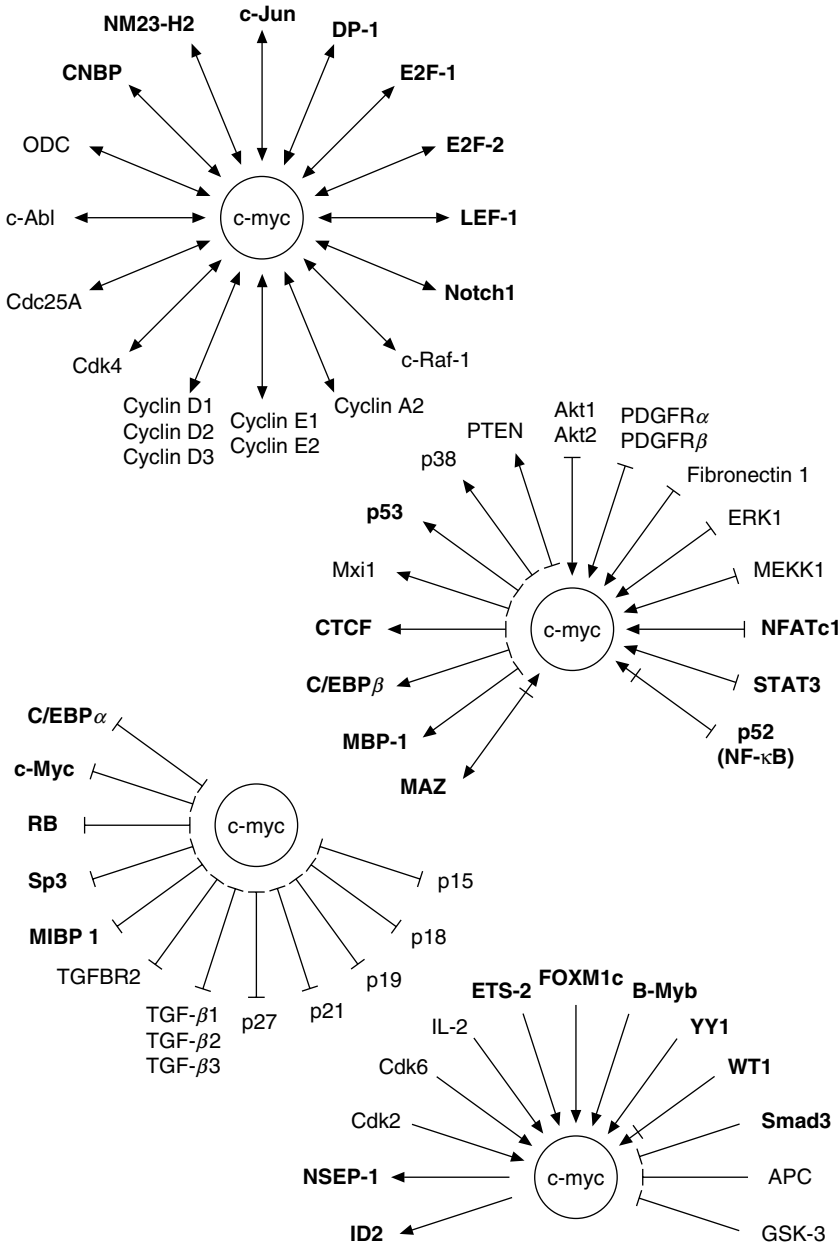

Fig. 8 Feedback loops between *c-myc* and its regulators. Shown are transcription factors that bind to the *c-myc* promoter (bold) and their direct interaction partners (RB and C/EBPα to E2F; Notch1 to CSL) (bold) as well as components of signaling pathways (normal) that regulate the *c-myc* promoter. ID2 (bold) seems to be part of a complex of ID2, mSin3A, p130, and E2F-4 at

energy metabolism (Adhikary and Eilers, 2005; Dang, 1999; Eisenman, 2001a; Gomez-Roman *et al.*, 2006; Grandori *et al.*, 2000; Lee and Dang, 2006; Oskarsson and Trumpp, 2005; Oster *et al.*, 2002).

9. HDAC INHIBITORS

In general, histone acetylation by HATs, which are components of diverse coactivator complexes, results in gene activation whereas histone deacetylation by HDACs, that are components of many corepressor complexes, leads to gene repression (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Accordingly, the recruitment of HATs and HDACs by different transcription factors to the *c-myc* promoter and their involvement in regulation of *c-myc* transcription have been described above. However, besides histones also a large variety of non-histone proteins, such as transcription factors and components of the basal transcription machinery, are substrates for acetylation and deacetylation by HATs or HDACs, respectively (Sterner and Berger, 2000). The outcome of these modifications of non-histone proteins is much more complicated so that acetylation as well as deacetylation events can result in both gene activation and gene repression.

The HDAC inhibitors butyrate and TSA (trichostatin A), which selectively up- or downregulate specific sets of genes (Chambers *et al.*, 2003; Mariadason *et al.*, 2000; Van Lint *et al.*, 1996), are well known to shut off the transcription of the endogenous *c-myc* gene and thus to repress the endogenous *c-myc* mRNA expression (Chambers *et al.*, 2003; Collins *et al.*, 1992; Herold and Rothberg, 1988; Heruth *et al.*, 1993; Koyama *et al.*, 2000; Krupitza *et al.*, 1995; Mariadason *et al.*, 2000; Souleimani and Asselin, 1993; Taylor *et al.*, 1992; Tong *et al.*, 2005; Van Lint *et al.*, 1996; Wilson *et al.*, 2002). This effect is in clear contrast to the general model of gene activation by histone acetylation. Nevertheless, this repression of *c-myc* correlates perfectly with the properties of these two HDAC inhibitors as promising anticancer drugs that induce growth inhibition, a G_0-G_1 cell cycle arrest, differentiation and apoptosis (Collins *et al.*, 1992; Koyama *et al.*, 2000; Lin *et al.*, 2006; Marks and Dokmanovic, 2005). Consistently, several HDAC inhibitors have entered clinical trials. Butyrate

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the *c-myc* promoter. p53 (bold) was found to occupy the *c-myc* promoter, but may possibly bind indirectly to it via other (general) transcription factors. Mxi1 (normal) was not shown to occupy the *c-myc* promoter so far, but may also indirectly bind to it via other transcription factors. The c-Myc regulated genes are either direct c-Myc target genes or may be indirectly regulated by c-Myc. [The Myc Target Gene Database (http://www.myccancergene.org/site/mycTargetDB.asp)] ↓ = activation; ⊥ = repression; | = c-Myc binds to this gene, but its regulation by c-Myc is so far unknown; | = ID2 occupies the *c-myc* promoter, but the supposed repression of the *c-myc* promoter by ID2 has not been demonstrated.

and TSA are thought to inhibit *c-myc* transcription at the level of elongation (Heruth *et al.*, 1993; Tong *et al.*, 2005; Wilson *et al.*, 2002). The mechanism for HDAC inhibitor-mediated repression of the endogenous *c-myc* promoter remains an unexplored area but a scenario appears to be conceivable, in which transcription factors that activate the *c-myc* promoter either may be inactivated by acetylation and thus by HDAC inhibitors or may require HDAC activity for activation of *c-myc*. Indeed, such effects were observed for some known transactivators of the *c-myc* promoter (see Sections IV.A.1, IV.A.13, IV.C.5 and Table III) although so far not at the *c-myc* promoter itself: (1) HDAC1 was required for transactivation of the MMTV (mouse mammary tumor virus) promoter by the GR (glucocorticoid receptor) (Qiu *et al.*, 2006). In this case, HDAC1, which was recruited by the GR and served as a coactivator for the GR, is supposed to deacetylate a non-histone protein in the immediate proximal promoter region (Mulholland *et al.*, 2003; Qiu *et al.*, 2006). (2) HDAC inhibitors, like TSA and butyrate, prevented induction of endogenous STAT5 target genes, but this inhibition was not due to effects on histone H3 and H4 acetylation or chromatin remodeling within the promoter region (Rascle *et al.*, 2003). Instead, following STAT5 DNA binding, they blocked transcription initiation by preventing recruitment of the basal transcription machinery (Rascle *et al.*, 2003). (3) The TNF-α-induced nuclear translocation of NF-κB and thus the TNF-α-stimulated DNA binding by NF-κB were inhibited by butyrate because it suppressed the TNF-α-induced degradation of IκB-α and increased the expression of an IκB that was not degraded during TNF-α treatment (Yin *et al.*, 2001). TSA had similar effects. (4) At least in *Drosophila*, acetylation of TCF by CBP interfered with its binding to β-catenin and thus repressed the TCF-mediated transactivation, which requires the recruitment of β-catenin by TCF (Waltzer and Bienz, 1998). Alternatively or additionally, at the *c-myc* promoter components of the basal transcription machinery may represent targets for HDAC inhibitors and/or transcription factors that repress the *c-myc* promoter either may be activated by acetylation and thus by HDAC inhibitors or may require HAT activity for repression of *c-myc*.

F. Deregulation of the *c-myc* Promoter in Burkitt's Lymphoma

1. RECIPROKE CHROMOSOME TRANSLOCATIONS AND THEIR CONSEQUENCES

Human Burkitt's lymphoma, especially aggressive B cell lymphoma often associated with EBV (Epstein-Barr virus), are characterized by a reciproke chromosome translocation between the *c-myc* locus (8q24) and one of the

immunoglobulin loci IgH (14q32), Igκ (2p12) or Igλ (22q11) that juxtaposes the translocated *c-myc* allele in the vicinity and thus under the influence of regulatory elements of the immunoglobulin genes (Klein, 1981, 1983). This translocation results in severe deregulation of the *c-myc* promoter in Burkitt's lymphoma because the positive regulatory elements of the permanently highly active immunoglobulin genes (i.e., enhancers, LCRs and MARs) prevent the normal silencing of the translocated *c-myc* gene and activate it so that it remains constitutively active (Ar-Rushdi *et al.*, 1983; Boxer and Dang, 2001; Cory, 1986; Hayday *et al.*, 1984; Hecht and Aster, 2000; Lindström and Wiman, 2002; Magrath, 1990; Marcu *et al.*, 1992; Sánchez-Beato *et al.*, 2003; Spencer and Groudine, 1991). Thereby they provide an open chromatin structure permissive for transcription at the *c-myc* locus and influence directly and dominantly *c-myc* transcription at initiation and elongation. Translocations to the immunoglobulin heavy (IgH) or light chain (Igκ and Igλ) loci occur in 80% or each 10% of cases, respectively, at varying break points. The *c-myc* coding region consisting of exons 2 and 3 remains always intact. Break points far 5′ of the *c-myc* gene leave the *c-myc* promoter intact while break points in exon 1 or intron 1 remove the normal P1 and P2 promoters as well as negative regulatory *c-myc* sequences so that transcription starts at P3 or cryptic promoters in intron 1 (Fig. 2). If the normal P1 and P2 promoters are present the block to transcriptional elongation is completely lost and a characteristic shift in promoter usage from P2 to P1 occurs resulting in P1:P2 transcript ratios of 1:1 to greater than 4:1 instead of the normal 1:10 to 1:5 (Boxer and Dang, 2001; Cesarman *et al.*, 1987; Eick *et al.*, 1988; Magrath, 1990; Marcu *et al.*, 1992; Taub *et al.*, 1984a,b; Spencer and Groudine, 1991; Spencer *et al.*, 1990; Strobl *et al.*, 1993). This constitutive and enhanced *c-myc* transcription is an important step in the pathogenesis of Burkitt's lymphoma because the permanently high c-Myc level forces the B cells to proliferative and maintains them in an undifferentiated state, in which a second mutation or deregulation event can easily propel them to full malignancy so that they are predisposed to develop lymphoid tumors (Magrath, 1990; Spencer and Groudine, 1991). Consistently, the rate of cell division in Burkitt's lymphoma is among the highest in any human tumor and typically more than 95% of tumor cells progress through the cell cycle (Hecht and Aster, 2000). Since high c-Myc levels induce apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Milner *et al.*, 1993) those cells are selected in Burkitt's lymphomas, which are protected from c-Myc-induced apoptosis by a second mutation (e.g., $p19^{ARF}$, p53, p73, MDM2, Ras, Bcl-6, Bax) or EBV infection (e.g., by Bcl-2 induction) (Eischen *et al.*, 1999; Hecht and Aster, 2000; Lindström and Wiman, 2002; Sánchez-Beato *et al.*, 2003; Takada, 2001; Wang and Boxer, 2005).

In addition, due to its vicinity to the immunoglobulin loci also the translocated *c-myc* allele is affected by somatic hypermutation (Bemark and

Neuberger, 2000; Boxer and Dang, 2001; Hecht and Aster, 2000; Lindström and Wiman, 2002; Sánchez-Beato *et al.*, 2003): Mutations in exon 1 and intron 1 contribute to the loss of the block to transcriptional elongation and eliminate binding sites for negative regulatory factors (Cesarman *et al.*, 1987; Zajac-Kaye *et al.*, 1988). Mutations in the coding sequence stabilize the c-Myc protein, which is degraded via the ubiquitin-proteasome pathway. Phosphorylation of S62 increases the stability of c-Myc whereas phosphorylation of T58 decreases its stability by marking it for ubiquitin-mediated proteolysis by the 26S proteasome (Amati, 2004; Chen *et al.*, 2000a; Sears, 2004). Consequently, Burkitt's lymphoma associated mutations at or surrounding T58 and S62 that result in more stable mutant c-Myc proteins additionally increase the high c-Myc level (Bahram *et al.*, 2000; Gregory and Hann, 2000; Salghetti *et al.*, 1999). Furthermore, some common Burkitt's lymphoma-derived c-Myc mutants show an increased tumorigenicity because they are specifically impaired in their ability to induce apoptosis but retain their ability to stimulate proliferation (Hemann *et al.*, 2005). In contrast to wild-type c-Myc, these mutants are defective at promoting apoptosis due to a failure to induce the pro-apoptotic protein Bim, which binds and inhibits the antiapoptotic protein Bcl-2 (Hemann *et al.*, 2005).

Some regulatory elements of the immunoglobulin genes and some transcription factors binding to them or to the translocated *c-myc* gene have been discovered which are involved in the severe deregulation of the *c-myc* promoter in Burkitt's lymphomas (Boxer and Dang, 2001; Hecht and Aster, 2000).

2. t(8;22)(q24;q11) TO THE Igλ LOCUS

A 12 kb fragment encompassing HuE λ (human Igλ enhancer), but not HuE λ alone, strongly activated *c-myc* expression and induced the promoter shift from P2 to P1 (Gerbitz *et al.*, 1999). For induction of this promoter shift and full activation by the Igλ enhancer sequences 1 kb upstream of the *c-myc* P1 promoter (−101 to −1056) are required.

3. t(2;8)(p12;q24) TO THE Igκ LOCUS

Three positive regulatory elements of the Igκ locus together, namely the MAR, κEi (κ intron enhancer) and κE3′ (κ3′ enhancer), are sufficient for maximal activation of the *c-myc* promoter, induction of the promoter shift in transcription initiation from P2 to P1 and loss of the block to transcription elongation (Geltinger *et al.*, 1996; Hörtnagel *et al.*, 1995; Polack *et al.*, 1991, 1993; Wittekindt *et al.*, 2000): κEi + κE3′ are sufficient to relieve the elongational block at P2 and to activate transcription from both P1 and P2 whereas the MAR is additionally required for maximal activation of the *c-myc* promoter. κEi + κE3′ seem to be sufficient also for induction of

the promoter shift from P2 to P1 as they induced a P1:P2 transcript ratio of about 1:1 and enhanced P1 transcription considerably stronger than P2 transcription.

Both the Sp1-binding site –44 of the *c-myc* promoter and the *c-myc* P1 TATA-box are essential for activation of the *c-myc* P1 promoter by κEi + κE3′ (Fig. 4; Geltinger *et al.*, 1996). In addition, either the Sp1-binding site distal or an upstream region of the *c-myc* promoter including the CT-element, which is bound by Sp1, are required. Both the Sp1-binding site –44 and the P1 TATA-box are also essential for induction of the promoter shift in transcription initiation from P2 to P1 by κEi + κE3′ so that in the presence of κEi + κE3′ deletion of either –44 or the P1 TATA-box reduced P1 transcripts about 90% and increased P2 transcripts about threefold resulting in a P1:P2 transcript ratio of 1:30 or 1:40 instead of 1:1 that is normally induced by κEi + κE3′ (Geltinger *et al.*, 1996). Interestingly, both the Sp1-binding site –44 and the P1 TATA-box are also required for the synergistic transactivation of the *c-myc* P1 promoter by Sp1 and FOXM1c, which interact directly (see Section IV.A.17; Wierstra and Alves, 2007a). Remarkably, FOXM1c transactivates the *c-myc* P1 promoter via the P1 TATA-box, to which it binds directly (Wierstra and Alves, 2006d). These findings suggest that the synergism of FOXM1c and Sp1 in transactivation of the P1 promoter may be important for deregulation of *c-myc* by κEi + κE3′.

Both the NF-κB binding site in κEi and the PU.1/Spi-1 binding site in κE3′ are essential for activation of transcription from the P1 and P2 promoters by κEi + κE3′ (Wittekindt *et al.*, 2000). Sp1 and p65 (RelA) interact directly (Perkins *et al.*, 1994). In the presence of κEi + κE3′, the *c-myc* P1 promoter is synergistically activated by Sp1 and p65, which bind to the Sp1-binding site –44 of *c-myc* and to the NF-κB binding site in κEi, respectively (Wittekindt *et al.*, 2000).

4. t(8;14)(q24;q32) TO THE IgH LOCUS

A 6.5-kb cassette comprising the 4 DNAse I-hypersensitive sites HS1234 derived from the 3′Cα regulatory region of the IgH locus functions as an enhancer-LCR (Madisen and Groudine, 1994). This HS1234 enhancer-LCR is sufficient to induce high-level *c-myc* transcription, the promoter shift from P2 to P1 and efficient transcriptional elongation (Kanda *et al.*, 2000; Madisen and Groudine, 1994). In consistence with the importance of the HS1234 enhancer for deregulation of *c-myc* expression, knock-in mice, in which HS1234 were integrated into the 5′ region of the *c-myc* locus, developed a Burkitt-like B-cell lymphoma (Wang and Boxer, 2005). The most active enhancer HS4 activates the *c-myc* promoter significantly and mutation of the NF-κB binding site in HS4 abolishes both its enhancer and promoter shift activity (Kanda *et al.*, 2000). Nevertheless, HS1, 2 and 3

contribute also to the maximal activation of the *c-myc* promoter and to the full promoter shift from P2 to P1 (Kanda *et al.*, 2000). The HS1234 enhancer-LCR mediates widespread histone hyperacetylation of the linked *c-myc* gene (Madisen *et al.*, 1998). It activates transcription from P2 through a mechanism that includes increased histone acetylation whereas an increase in histone acetylation had little effect on transcription from P1 strongly suggesting that the HS1234 enhancer-LCR activates the *c-myc* P1 and P2 promoters through different mechanisms (Madisen *et al.*, 1998).

On the translocated *c-myc* allele, but not on the untranslocated one, the NF-κB binding sites URE and IRE are protected in Raji or Ramos Burkitt's lymphoma cells, respectively, that each lack the other NF-κB site due to different positions of break points and deletions (Fig. 4; Ji *et al.*, 1994). The additional specific protection only on the translocated *c-myc* allele of the binding sites of NM23-H2 (Ji *et al.*, 1995) and the MAZ-related (MAZ-R) protein, a so far unidentified protein (Hu *et al.*, 2002), strongly suggests that binding of NF-κB, NM23-H2 and MAZ-R to the translocated *c-myc* gene is important for activation of *c-myc* transcription in Raji cells. Indeed, the MAZ-R site, located –523 upstream of the *c-myc* P1 transcription start site, was found to be important for activation of the *c-myc* promoter and induction of the promoter shift by the HS1234 enhancer (Hu *et al.*, 2002). The Sp1-binding site –44 of *c-myc* is also important for activation of the *c-myc* promoter by the HS1234 enhancer.

A super-repressor IkBα construct was capable of inhibiting IgH enhancer-driven *c-myc* promoter activity demonstrating that NF-κB/Rel transcription factors, binding to the *c-myc* promoter as well as to the IgH enhancer, play an important role in deregulation of the translocated *c-myc* gene and may represent a promising target in anticancer therapy of Burkitt's lymphoma (Kanda *et al.*, 2000).

Both NF-κB binding sites of the *c-myc* promoter were protected only on the translocated *c-myc* allele also in a transformed lymphoma, which in contrast to the less aggressive original follicular lymphoma had acquired a translocation of the *c-myc* gene into the IgH locus and showed increased *c-myc* mRNA expression (Arcinas *et al.*, 2001).

G. *c-myc* as Target for Anticancer Therapy

c-myc is one of the most frequently mutated genes in tumors and deregulation of *c-myc* is found in many, if not most, human tumors (Nesbit *et al.*, 1999). In most cases the expression of *c-myc* is deregulated whereas mutations in the c-Myc protein are rare. Such enhanced and/or constitutive *c-myc* expression can be the result of mutations in the *c-myc* locus (e.g., chromosomal translocations, gene amplifications, proviral insertions, retroviral

transductions) or in the signal transduction pathways that regulate *c-myc* expression (Marcu *et al.*, 1992; Spencer and Groudine, 1991). Since in normal cells c-Myc induces apoptosis in the absence of sufficient amounts of survival factors (Askew *et al.*, 1991; Evan *et al.*, 1992) activation of the oncogene c-Myc strongly selects for a second mutation that eliminates an apoptosis pathway (e.g., p53) or for activation of a second cooperating oncogene that inhibits apoptosis and stimulates cell survival (e.g., Bcl-2, Bcl-x_L, Ras) (Nilsson and Cleveland, 2003; Oster *et al.*, 2002). However, if c-Myc-induced apoptosis is suppressed (e.g., by coexpression of the anti-apoptotic proteins Bcl-x_L or Bcl-2 or by an excess of local survival factors) c-Myc activation alone is sufficient to trigger immediate carcinogenic progression in the absence of other cooperating oncogenic lesions (Luo *et al.*, 2005; Pelengaris and Khan, 2003a; Pelengaris *et al.*, 1999, 2002a,b). Vice versa, inactivation of the oncogene c-Myc alone can induce sustained tumor regression, reverse and revoke tumorigenesis and lead to the complete permanent loss of the neoplastic phenotype (Arvanitis and Felsher, 2005; Felsher, 2003, 2004; Giuriato and Felsher, 2003; Giuriato *et al.*, 2004; Pelengaris and Khan, 2003a; Pelengaris *et al.*, 2002a; Shachaf and Felsher 2005a,b):

In general, c-Myc inactivation in c-Myc-induced tumors results in proliferative arrest, re-differentiation, apoptosis, or/and vascular degeneration. Nevertheless, the outcome of c-Myc inactivation varies in different c-Myc-induced tumors depending on cell type, context, genetic, and epigenetic features. Thus, even brief c-Myc inactivation resulted in sustained tumor regression in osteogenic sarcoma (Jain *et al.*, 2002), whereas prolonged c-Myc inactivation failed to cause tumor regression in the majority of mammary adenocarcinomas (Boxer *et al.*, 2004; D'Cruz *et al.*, 2001). Generally, four different outcomes of c-Myc inactivation can be distinguished in c-Myc-induced tumors. (1) Tumor regression with initial differentiation of tumor cells followed by their complete permanent elimination through apoptosis (lymphoma, leukemia: Felsher and Bishop, 1999a; Karlsson *et al.*, 2003a). (2) Tumor regression with differentiation into normal mature quiescent tissue (osteogenic sarcoma: Jain *et al.*, 2002; skin papilloma: Pelengaris *et al.*, 1999; pancreatic islet β-cell carcinoma: Pelengaris *et al.*, 2002b) where the re-differentiated cells are permanently refractory to subsequent reactivation of c-Myc, which either results in apoptosis (osteogenic sarcoma: Jain *et al.*, 2002) or has no effect (skin papilloma: Flores *et al.*, 2004). (3) Tumor regression with differentiation (in part accompanied by apoptosis) where the re-differentiated normal-appearing cells remain in a reversible state of tumor dormancy so that subsequent c-Myc reactivation immediately restores tumorigenesis (hepatocellular carcinoma: Shachaf *et al.*, 2004; skin papilloma, pancreatic islet β-cell carcinoma (expressing Bcl-x_L): Pelengaris *et al.*, 2004).

(4) A failure to regress of the majority of mammary adenocarcinomas because they have become c-Myc-independent (in nearly 70% of cases due to activating point mutations in K-Ras2) coupled with spontaneous relapse of the majority of those breast tumors that fully regressed because they escape c-Myc-dependence through acquisition of additional genetic alterations (in a quarter of cases activating point mutations in K-Ras2) (Boxer *et al.*, 2004; D'Cruz *et al.*, 2001). Most if not all animals bearing fully regressed mammary adenocarcinomas harbor residual neoplastic cells, in which subsequent c-Myc reactivation results in rapid and full restoration of tumorigenesis (Boxer *et al.*, 2004). This worse outcome of hepatocellular carcinoma, which are generally refractory to clinical treatment, pancreatic β-cell carcinoma, skin papilloma and especially mammary adenocarcinomas is at least in part due to their epithelial origin (Felsher, 2006). The high frequency of c-Myc-independence in the latter one is caused at least partially by their tendency to acquire activating K-Ras2 (and N-Ras) mutations, apparently often already during c-Myc-induced breast tumor initiation (Boxer *et al.*, 2004; D'Cruz *et al.*, 2001). Some hematopoietic tumors were also reported to relapse because of novel chromosomal translocations that rendered them c-Myc independent (Karlsson *et al.*, 2003a). The acquisition of such new genetic alterations allowing tumor cells to escape c-Myc-dependence and thus to relapse may be promoted by c-Myc itself because it can cause genomic instability (Felsher and Bishop, 1999b; Karlsson *et al.*, 2003b; Mai and Mushinski, 2003; Soucek and Evan, 2002).

Since c-Myc overexpression is causative of full tumorigenesis whereas interference with c-Myc expression is effective in tumor treatment c-Myc represents an attractive target for anticancer therapy (Hermeking, 2003; Oster *et al.*, 2002; Ponzielli *et al.*, 2005; Vita and Henriksson, 2006). Besides strategies to interfere with *c-myc* mRNA and c-Myc protein or to exploit c-Myc activation (Felsher and Bradon, 2003; Oster *et al.*, 2002; Pelengaris and Khan, 2003b; Prochownik, 2004) several strategies to interfere with *c-myc* promoter activation are under intensive study (Ponzielli *et al.*, 2005): DNA-binding antibiotics (Portugal, 2003; Snyder *et al.*, 1991; Vaquero and Portugal, 1998), TFO (triplex-forming oligonucleotides) (Carbone *et al.*, 2004a; Napoli *et al.*, 2006; and references therein), small molecule ligands directed to block the DBD (FBP: Huth *et al.*, 2004) and decoy DNAs (Seki *et al.*, 2006) prevent transcription factor binding to the *c-myc* promoter. Small-molecule compounds that eliminate the interaction of β-catenin and TCF-4 (Lepourcelet *et al.*, 2004) as well as a peptide aptamer that interrupts the interaction of Smad4 and LEF-1 (Lim and Hoffmann, 2006) inhibit the c-Myc protein or mRNA expression, respectively. Cationic porphyrins are used and *c-myc*-specific (expanded) cationic porphyrins are designed to sequester the NHE in inhibitory paranemic DNA structures (G-quadruplex:

Grand *et al.*, 2002, 2004; Phan *et al.*, 2005; Seenisamy *et al.*, 2004, 2005; Siddiqui-Jain *et al.*, 2002). Prominent examples of antibodies directed against components of signal transduction chains and other kinase inhibitors (Sawyers, 2003, 2004) successfully used in clinical anticancer therapy are Herceptin (Trastuzumab), a humanized monoclonal antibody specific for the transmembrane tyrosine kinase receptor HER-2/neu (hnRNP K; Emens, 2005; Nahta and Esteva, 2006), and Gleevec (Imatinib, STI-571), a potent small-molecule tyrosine kinase inhibitor with relative selective activity against ABL proto-oncogene, BCR-ABL fusion protein, PDGFR and c-Kit receptor (Jones and Judson, 2005; Peggs, 2004). Inhibition of expression (ETS-2: Carbone *et al.*, 2004b) or function (NF-κB: Kanda *et al.*, 2000) of transcription factors (Darnell, 2002) that activate the *c-myc* promoter is interesting as they often regulate also antiapoptotic or/and other proliferation genes. A detailed understanding of *c-myc* promoter regulation is one essential prerequisite to benefit from progress in diagnosis of individual genetic lesions in individual tumors and in target-directed drug design.

V. THE *c-MYC* PROMOTER: BLACK BOX AND ENIGMA?

A. Feedback Loops

The *c-myc* promoter responds to numerous signals and integrates these diverse and dynamic inputs to set the *c-myc* mRNA output (Chung and Levens, 2005; Liu and Levens, 2006). Then the transcription factor c-Myc mediates specific gene expression programs that relate to cell cycle progression and cell growth (Eisenman, 2001a,b; Grandori *et al.*, 2000; Oster *et al.*, 2002). Thereby it seems likely that *c-myc* transcription will respond to feedback from most (if not all) subsystems regulated by c-Myc (Levens, 2002, 2003). Fig. 8 shows the feedback coupling of the *c-myc* promoter to c-Myc regulated genes that are either direct c-Myc target genes or may be indirectly regulated by c-Myc [The Myc Target Gene Database (http://www.myccancergene.org/site/mycTargetDB.asp); Adhikary and Eilers, 2005; Basso *et al.*, 2006; Claassen and Hann, 1999; Coller *et al.*, 2000; Dang, 1999; Eisenman, 2001a; Facchini and Penn, 1998; Fernandez *et al.*, 2003; Frank *et al.*, 2001; Frye *et al.*, 2003; Grandori *et al.*, 2000; Guo *et al.*, 2000; Huang *et al.*, 2003; Lee and Dang, 2006; Li *et al.*, 2003; Luoro *et al.*, 2002; Mao *et al.*, 2003; Menssen and Hermeking, 2002; Nasi *et al.*, 2001; Neiman *et al.*, 2001; Nesbit *et al.*, 2000; O'Connell *et al.*, 2003; O'Hagan *et al.*, 2000; Oster *et al.*, 2002; Schuhmacher *et al.*, 2001; Schuldiner *et al.*, 2002; Toyo-Oka *et al.*, 2006; Watson *et al.*, 2002; Yu *et al.*, 2002; Zeller *et al.*, 2003, 2006].

Four different groups of feedback mechanisms can be distinguished (Fig. 8):

1. Factors, which activate the *c-myc* promoter and whose expression is activated by c-Myc: In this group are prominent proliferation genes and notably many factors that activate E2F-dependent transcription. Since these factors and c-Myc will reciprocally enhance their expression they lead to a self-reinforcing transcription cycle, which drives cells through G_1-phase and induces S-phase entry. Such a positive feedback program is important to efficiently promote cell proliferation and to provide robustness against competing antiproliferative signals.
2. Factors, which repress the *c-myc* promoter and whose expression is repressed by c-Myc: In this group are prominent antiproliferation and differentiation genes, which act as antagonists to c-Myc in the proliferation/differentiation or proliferation/quiescence switch. In accordance, these factors and c-Myc reciprocally repress their expression. These antagonistic transcription programs are essential for cell fate determination. c-Myc itself represses the *c-myc* promoter. This negative feedback regulation of c-Myc autosuppression is important to ensure normal tissue homeostasis (see Section III.E; Facchini and Penn, 1998; Facchini *et al.*, 1994, 1997; Grignani *et al.*, 1990; Penn *et al.*, 1990a; Potter and Marcu, 1997).
3. Factors, which activate the *c-myc* promoter and whose expression is repressed by c-Myc: In this group are prominent proliferation genes. They induce *c-myc* expression and then repression of their expression by c-Myc serves to curtail the proliferation stimulus (Oster *et al.*, 2000). These negative feedback loops contribute to tight control of cell cycle progression (Facchini and Penn, 1998). This mechanism limits the time window of a particular mitogenic response and serves to maintain normal tissue homeostasis.
4. Factors, which repress the *c-myc* promoter and whose expression is activated by c-Myc: In this group are prominent antiproliferation genes and tumor suppressors. c-Myc activates their expression and then they repress *c-myc* expression. This feedback control represents a security mechanism against inappropriate hyperproliferative signaling by c-Myc. A well characterized example is p53. In the absence of sufficient amounts of survival factors, high c-Myc levels cause p53-dependent and p53-independent apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Nilsson and Cleveland, 2003; Prendergast, 1999).

There exist also many other feedback mechanisms that are not shown in Fig. 8. For example, the Ras/ERK and PI3K/Akt pathways increase c-Myc protein stability (see Section IV.E; Sears *et al.*, 1999, 2000; Gregory and Hann, 2000; Gregory *et al.*, 2003; Sears, 2004; Sears and Nevins, 2002), c-Myc blocks the transactivation function of C/EBPα (Constance *et al.*, 1996; Mink *et al.*, 1996) and the c-Myc target gene eIF-4E (Bush *et al.*, 1998; Jones *et al.*, 1996; Rosenwald *et al.*, 1993) enhances *c-myc* translation (see Section IV.E; Clemens, 2004; De Benedetti and Graff, 2004; Massagué, 2004; Rosenwald, 2004).

B. A Concept for Regulation of the *c-myc* Promoter

1. GENERAL ASPECTS OF *c-MYC* PROMOTER CONTROL

a. Mechanisms of *c-myc* Promoter Control

The control of the *c-myc* promoter is extremely complex and includes regulation at multiple levels:

1. A multitude of signaling pathways regulate the *c-myc* promoter (Fig. 5):
   a. For example, mitogens, growth factors, cytokines, interleukins, lymphokines, proliferation and antiproliferation signals, differentiation signals, hormones, oncogenes, tumor suppressors, cell adhesion, hypoxia, circadian clock
   b. Cross-talk between signal transduction pathways
   c. Redundancy (TGF-β, PDGF, IL-2, 1,25-$(OH)_2$-D_3, NO) (Fig. 5)
   d. Variability dependent on cell type and cellular context
2. Integrity of the long range chromosomal domain, that is, influence of far elements
3. Chromatin remodeling:
   a. presence or absence of particular nucleosomes (Fig. 3)
   b. ATP-dependent nucleosome remodeling
   c. Histone acetylation status
   d. Histone methylation
4. DNA methylation
5. DNA conformation and topology (single-strand regions, G-quadruplex, *i*-tetraplex, H-DNA, Z-DNA) (Fig. 7)
6. A multitude of transcription factors bind to the *c-myc* promoter (Fig. 4; Table III)
7. Repression, derepression, and activation
8. Transcription initiation and transcription elongation (Fig. 3B)
9. Interdependence as well as independence of the two major promoters P1 and P2
10. c-Myc autosuppression
11. Feedback loops to most (if not all) systems regulated by c-Myc (Fig. 8)
12. Integration of diverse and dynamic signal inputs and their processing to a *c-myc* mRNA output and thus finally to specific c-Myc-mediated gene expression programs

This complex regulation of the *c-myc* promoter (Liu and Levens, 2006) agrees completely with the biological properties of c-Myc as key factor in cell growth control. Since c-Myc positively regulates all aspects of proliferation, cell growth, and tumorigenesis it is essential for normal cell function, but its deregulation is very dangerous (see Section II.A). This dualism requires tight regulation of *c-myc* expression in time, place and quantity and thus tight control of the *c-myc* promoter in each cell, at each time point and under each

physiological condition. Furthermore, c-Myc is generally expressed at low levels in normal cells and controlling where, when and how much c-Myc is made determines much of its action spectrum (Levens, 2002; Liu and Levens, 2006) so that even slight changes in *c-myc* mRNA and protein expression can have severe consequences for cell proliferation and cell fate (Chung and Levens, 2005; de la Cova *et al.*, 2004; Hanson *et al.*, 1994; Liu and Levens, 2006; Marcu *et al.*, 1997; Moreno and Basler, 2004; Oster *et al.*, 2002; Pirity *et al.*, 2006; Shichiri *et al.*, 1993; Trumpp *et al.*, 2001). The Myc/Max/Mad network can be viewed as a functional module which acts to convert environmental signals into specific gene-regulatory programs (Eisenman, 2001a,b; Grandori *et al.*, 2000; Oster *et al.*, 2002). Consequently, the integration of a wide range of external and internal signals by the *c-myc* promoter (Fig. 5; Chung and Levens, 2005; Levens, 2002, 2003; Liu and Levens, 2006; Oster *et al.*, 2002) plays an important role for this network. Thus, the very complex regulation of the *c-myc* promoter reflects c-Myc's complex biological properties as a potent and essential, but also dangerous key factor for cell growth control. In other words: we cannot expect a simple *c-myc* promoter.

Prior to description of regulation of the *c-myc* promoter in different biological settings some general aspects of *c-myc* promoter control (Chung and Levens, 2005; Liu and Levens, 2006) will be discussed. Central for regulation of the *c-myc* promoter are (1) cross-talk, (2) redundancy, (3) integration of different signals at one transcription factor or one DNA element (Fig. 5), (4) branching of one signal to influence several transcription factors (Fig. 5), (5) feedback loops (Fig. 8), (6) non-canonical binding sites for several transcription factors, and (7) dependence on cell type and cellular context. These properties allow (1) very tight control, (2) flexibility and variability, (3) stability and robustness, (4) cooperation in order to achieve maximal *c-myc* transcription, (5) cell type specificity, (6) compensation for the lack of a single signal or factor, and (7) fast appropriate changes in *c-myc* transcription in response to the dynamic *in vivo* situation of each cell. Regulation of the *c-myc* promoter is often highly context-dependent: The same factors that exert dominant influence on *c-myc* transcription in one biological setting or cell line may be feeble or impotent under other circumstances (Levens, 2003; Liu and Levens, 2006). The same stimulus that upregulates *c-myc* in one circumstance or cell type may downregulate it in another (Chung and Levens, 2005). Although key components seem to operate in many cell types their actual importance for regulation of the *c-myc* promoter may depend on the particular cell type. For example, TCF-4 and Wnt signaling will be especially important for intestinal cells, NF-κB for B- and T-cells or the ER and estrogen for endometrial and breast cells. In addition, in different cell types ubiquitous factors may cooperate with different panels of tissue-specific factors. Similarly, constitutive and signal-dependent transcription factors may cooperate. Transcription factors, which are especially important for regulation of the *c-myc* promoter, for example, E2F, β-catenin/TCF-4, Smad3, FBP, NF-κB, and

STAT3 (Fig. 5A, B, and D), are targeted by multiple signaling pathways and such mediate regulation of *c-myc* transcription in response to various proliferation and antiproliferation signals. Consequently, these transcription factors serve as nodes for signal integration at the *c-myc* promoter.

b. Important *cis*-Elements for Control of the *c-myc* Promoter

A vast amount of deletion and mutation studies (Marcu *et al.*, 1992; Spencer and Groudine, 1991), studies on secondary DNA structures and nucleosome positioning (Albert *et al.*, 1997, 2001; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b; Pullner *et al.*, 1996) lead to the conclusion that the following *cis*-elements are important for control of the *c-myc* promoter (Fig. 4): (1) FUSE (FBP and FIR binding site), (2) CT-element or NHE, (3) ME1a1 or CT-I_2, (4) ME1a2, (5) the combined E2F/ETS/STAT3/NFATc1/KLF11/METS/Smad binding site [also including a consensus Sp1-binding site (GGCGGG)], and (6) the region from –60 to –38 relative to the transcription start of P1 (+1) (Nishikura, 1986), which harbors the Sp1-binding site –44, the Pitx2 binding site and part of one AP-2 binding site.

Three of these *cis*-elements seem to function as molecular switches for regulation of the *c-myc* promoter (Fig. 4): (1) the combined E2F/ETS/STAT3/NFATc1/KLF11/METS/Smad binding site, (2) the NHE or CT-element, and (3) the FUSE through which the FUSE–FBP–FIR–TFIIH system operates. The latter two elements are characterized by non-B-DNA structures, which they employ to control the *c-myc* promoter. The two former elements are (alternatively) bound by several different transcription factors and can turn from repressing to activating elements and vice versa. Thus, they act as nodes for signal integration.

At the combined E2F/ETS/STAT3/NFATc1/KLF11/METS/Smad binding site ETS-1/2, STAT3, NFATc1 and probably free E2F-1,2,3 serve as transactivators of the *c-myc* promoter whereas E2F–pocket protein complexes, the Smad3/Smad4/E2F-4,5/DP-1/p107 complex, C/EBPα, the C/EBPα–RB–E2F-4–Brm complex, the KLF11–Smad3 complex, a Smad2/3–Smad4–C/EBPβ complex as well as possibly METS and ID2 serve as repressors (Figs. 4 and 5A). The E2F-binding site appears to determine the general state of the *c-myc* promoter so that it is either inducible (permissive) or uninducible (unpermissive) by other signals. Consequently, the combined E2F/ETS/STAT3/NFATc1/KLF11/METS/Smad binding site plays an important role in repression, antirepression and activation of the *c-myc* promoter (see Sections IV.A.2–4, IV.A.6, IV.A.15, IV.A.22, and IV.A.30).

The NHE can be sequestered in repressive paranemic DNA structures (G-quadruplex, *i*-tetraplex). NM23-H2-mediated conversion of the NHE into single-stranded or duplex DNA allows binding of single-strand-specific (hnRNP K and CNBP) or duplex DNA-specific (Sp1) transactivators (Fig. 7). Sequestration of the NHE in or its release from these repressive paranemic

DNA structures is thought to provide a fast mode for regulation of *c-myc* transcription in response to antiproliferation and differentiation signals or to mitogens and other proliferation signals, respectively (see Section IV.A.12).

The FUSE becomes single-stranded because of torsional stress from ongoing transcription so that it provides a real-time measure for activity of the *c-myc* promoter. The FUSE–FBP–FIR–TFIIH system is thought to operate as a feedback loop for fine tuning of the *c-myc* promoter in order to ensure the steady *c-myc* transcription required for cellular homeostasis. In addition, FBP, which shows a parallel expression profile to c-Myc, functions as the master regulator of the *c-myc* promoter because in the absence of FBP the remainder of other transactivators that bind to the *c-myc* promoter is unable to activate *c-myc* transcription (see Section IV.A.7). Moreover, the FUSE–FBP–FIR–TFIIH system manages the sharp peak in *c-myc* transcription in response to serum stimulation of starved cells and the following rapid decline.

c. Levels of *c-myc* Promoter Regulation

Regulation of the *c-myc* promoter seems to include systems for rapid, transient reponses as well as for more slow, permanent responses. The former ones may serve to evaluate whether a signal or an altered condition persist before the final decision for the latter ones is made. So, down- and upregulation of *c-myc* transcription in response to differentiation agents or mitogens, respectively, is first achieved at the level of transcriptional elongation while later on changes in transcription initiation occur (see Section III.C). Similarly, sequestration of the NHE in or its release from repressive paranemic DNA structures provide an additional fast mode for modulation of *c-myc* transcription (see Section IV.A.12). In contrast, repression by differentiation-specific transcription factors, histone deacetylation and nucleosome remodeling represent more slow, permanent repression mechanisms.

Regulation of the *c-myc* promoter seems to include (1) the determination of its general state, that is, whether the *c-myc* promoter is responsive (permissive) or not responsive (unpermissive) for certain types of signals, and (2) the actual response to particular signals if they really arrive. E2F-pocket protein complexes and the master regulator FBP play important roles in determination of this general state of the *c-myc* promoter (see Sections IV.A.2 and IV.A.7). As a consequence, regulation of the *c-myc* promoter includes repression, anti-repression and activation.

Moreover, several repressors (e.g., MBP-1, CTCF, ZF5, KLF11, CSL) may set a security threshold so that a net activation of the *c-myc* promoter does only occur if an inducing signal exceeds this threshold. This security threshold may

be required to ensure that *c-myc* transcription is limited to appropriate situations.

Regulation at the level of transcription elongation is important for control of the *c-myc* promoter (see Section III.C). Candidate transcription factors that may influence transcriptional elongation of *c-myc* are for example FBP and FIR, which bind to and antagonistically influence the p89 helicase subunit of TFIIH (Liu *et al.*, 2000, 2001), E2F and ERα, which bind to TFIIH (Chen *et al.*, 2000c; Pearson and Greenblatt, 1997) as well as STAT3 and NF-κB, which bind to P-TEFb (Barboric *et al.*, 2001; Giraud *et al.*, 2004). Only after the paused polymerase exits the promoter the bound transcription factors can direct re-initiation so that the paused polymerase is a powerful check on overactivation (Chung and Levens, 2005; Liu and Levens, 2006; Liu *et al.*, 2006a).

All these mechanisms are supposed to serve the maintenance of normal tissue homeostasis.

d. Antagonistic Regulation of the *c-myc* Promoter by Proliferation and Antiproliferation Signals

c-Myc is a very potent stimulator of proliferation that drives cells through G_1-phase and induces S-phase entry (see Section II.A). Ectopic c-Myc expression is sufficient to drive quiescent cells into S-phase in the absence of mitogens (Eilers *et al.*, 1991). In consistence, the *c-myc* promoter is activated by most (if not all) major proliferation pathways, for example Wnt, Notch, interleukins (IL-2, IL-3, IL-6, IL-12), cytokines, lymphokines, growth factors (PDGF, EGF, CSF-1), hormones, PI3K/Akt, Ras/Raf, JAK/STAT and Src (Fig. 5; Liu and Levens, 2006). Negative regulators of these proliferation pathways, like the candidate tumor suppressor HBP-1 for Wnt/TCF-4 (Fig. 5A), may serve as normal security barriers so that activation of the *c-myc* promoter does only occur if the intensity of such proliferation signaling overwhelms this barrier.

c-Myc is central for the proliferation/differentiation switch and for the proliferation/quiescence switch (Baudino and Cleveland, 2001; Eisenman, 2001a; Grandori *et al.*, 2000; Oster *et al.*, 2002; Pelengaris *et al.*, 2002a; Zhou and Hurlin, 2001). Accordingly, the *c-myc* promoter is repressed by many differentiation and antiproliferation factors, like C/EBPα, C/EBPβ, Blimp-1, GATA-1, KLF11, IFN-γ, p21, p53 and TGF-β. (Fig. 5A and D).

Downregulation of c-Myc, an important TGF-β antagonist, is essential for TGF-β-induced cell cycle arrest. Repression of *c-myc* expression by TGF-β is required to allow induction of *p15* and *p21* expression by other aspects of TGF-β signaling and to deprive the cell of c-Myc's potent proliferation-stimulating functions so that *c-myc* downregulation plays an integrative role in the cytostatic program of TGF-β(see Section IV.A.2; Massagué and Gomis, 2006; Siegel and Massagué, 2003). In order to repress the *c-myc*

promoter TGF-β targets E2F through the Smad3/Smad4/E2F-4,5/DP-1/p107 complex (Chen *et al.*, 2001b, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002), β-catenin/TCF-4 through Smad3 (Sasaki *et al.*, 2003), LEF-1 probably through the Smad4/LEF-1 complex (Lim and Hoffmann, 2006), FBP through p38 (Kim *et al.*, 2003a) and NF-κB through IkBα (Arsura *et al.*, 1996) (Fig. 5A and D). By binding to the TIE, which is composed of the E2F-binding site and the adjacent Smad binding site, the Smad3/Smad4/E2F-4,5/DP-1/p107 complex should also block binding of STAT3, NFATc1 and ETS-1/2 because their binding sites overlap with the E2F site (Fig. 4). β-catenin/TCF-4, LEF-1, NF-κB, STAT3, ETS-1/2, NFATc1 and probably E2F-1,2,3 are important transactivators of the *c-myc* promoter and targets of potent proliferation pathways (Fig. 5A, B, and D). FBP is the positive master regulator of the *c-myc* promoter (He *et al.*, 2000a). Its ubiquitination and proteasome-dependent degradation is triggered by p38 (Kim *et al.*, 2003a). Thus TGF-β, a major c-Myc antagonist, represses *c-myc* transcription by targeting several important transactivators of the *c-myc* promoter as well as the master regulator of the *c-myc* promoter FBP. Thereby TGF-β blocks multiple proliferation pathways that activate the *c-myc* promoter, and prevents activation of the *c-myc* promoter by its transactivators because they all are unable to activate the *c-myc* promoter in the absence of FBP. This multiple strategy of TGF-β for repression of the *c-myc* promoter demonstrates how the biological antagonism of TGF-β and c-Myc manifests on the molecular level at the *c-myc* promoter.

2. REGULATION OF THE *c-myc* PROMOTER IN DIFFERENT BIOLOGICAL SETTINGS

Finally, transcription factors will be described, which are or may be involved in regulation of the *c-myc* promoter in different biological settings (Fig. 1).

a. Induction of *c-myc* Transcription during Re-entry of Quiescent Cells into the Cell Cycle

In quiescent cells, where *c-myc* is virtually not expressed, the *c-myc* promoter should be repressed by E2F–pocket protein–HDAC complexes (see Section IV.A.2; Albert *et al.*, 2001; Baek *et al.*, 2003; Klappacher *et al.*, 2002; Rodriguez *et al.*, 2006). Transcription of the typical immediate-early gene *c-myc* is rapidly induced by a wide variety of mitogens independent of *de novo* protein biosynthesis (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Iyer *et al.*, 1999; Lemaitre *et al.*, 1996; Marcu *et al.*, 1992; Oster *et al.*, 2002; Spencer and Groudine, 1991). Accordingly, immediate-early transcription factors, like AP-1, ETS-1/2, NF-κB, STAT3,

STAT4, and STAT5 (Fig. 4), should activate the *c-myc* promoter as an immediate-early response. Indeed, the *c-myc* promoter was found to be occupied by Sp1, YY1, E2F-1, and E2F-4, 30 min after serum refeeding of starved cells (Fig. 6; Liu *et al.*, 2006a). These transcription factors induce *c-myc* transcription that in turn drives FUSE melting so that first FBP3, then FBP and later on FIR can bind to the FUSE, which together help to generate the transient immediate-early pulse of *c-myc* transcription (Fig. 6; see Section IV.A.7; Chung *et al.*, 2006; Liu *et al.*, 2006a).

Reactivation of *c-myc* transcription in adults during regenerative proliferation should also be mediated by these immediate-early transcription factors as demonstrated for STAT3 after PH (Cressman *et al.*, 1996).

For immediate-early induction of *c-myc* transcription repression of the *c-myc* promoter by E2F–pocket protein–HDAC complexes has to be finished or overcome. However, removal of pocket proteins from E2F by cyclin D1/Cdk4- and cyclin E/Cdk2-mediated phosphorylation of the pocket protein would occur too late for immediate-early *c-myc* transcription because *cyclin D1* and *cyclin E* are expressed after *c-myc* during re-entry of quiescent cells into the cell cycle (Roussel, 1998). In addition, expression of *cyclin D1* and *cyclin E* would require *de novo* protein biosynthesis. The mechanisms involved in derepression and subsequent transactivation of E2F target genes that accumulate early in G_1-phase before accumulation of cyclin/Cdk activity are not well understood (Bracken *et al.*, 2004). Rodriguez *et al.* (2006) have shown that the *c-myc* promoter, which is occupied by E2F-4 and p130 in quiescent liver cells, remains so during the first 6 h after PH although PH strongly induces immediate-early *c-myc* transcription that reaches its maximum 1 h post-PH. This permanent occupancy of the E2F-binding site would exclude STAT3, NFATc1 and ETS-1/2 binding to their overlapping sites on the *c-myc* promoter (Fig. 4). Otherwise one may have speculated that their (possibly mass action driven) competition with E2F for the same binding site could replace E2F–pocket protein complexes from the *c-myc* promoter. Two (not mutually exclusive) scenarios seem to be conceivable for de-repression and activation of the *c-myc* promoter without displacement of either pocket proteins from E2F or E2F–pocket protein complexes from the *c-myc* promoter. First, potent activating immediate-early transcription factors that bind to distant sites, for example AP-1, NF-κB and STAT4 (Fig. 4), may overwhelm the repression imposed by E2F–pocket protein–HDAC complexes. Second, HDAC and other corepressors recruited by pocket proteins could be displaced from the pocket proteins, while the E2F–pocket protein complexes remain on the *c-myc* promoter. Interestingly, β-catenin was reported to interact with E2F-4, but not E2F-1, to inhibit HDAC1, to inhibit HDAC activity associated with E2F-4, p107, and p130 and to dismiss HDAC1 from the *c-myc* promoter (Baek *et al.*, 2003). Similarly, mSin3A, a central component of mSin3–

HDAC corepressor complexes (Ayer, 1999; Knoepfler and Eisenman, 1999), which occupies the *c-myc* promoter in quiescent liver cells, was found to have left the *c-myc* promoter 30 min after PH concomitant with immediate-early induction of *c-myc* transcription (see Section IV.B.5; Rodriguez *et al.*, 2006). ID2, whose presence at the *c-myc* promoter parallels that of mSin3A, is supposed to be involved in this displacement of mSin3A from the *c-myc* promoter (Rodriguez *et al.*, 2006).

b. Repression of the *c-myc* Promoter During Differentiation

During differentiation *c-myc* expression is quickly reduced and in terminally differentiated cells *c-myc* is no longer expressed (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Marcu *et al.*, 1992; Spencer and Groudine, 1991). This suppression of *c-myc* is mediated directly or indirectly by the "master regulator" of differentiation for that cell type (Oster *et al.*, 2002). Such differentiation-specific transcription factors, which repress the *c-myc* promoter during terminal differentiation, are Blimp-1, C/EBPα, C/EBPβ, METS, Ovol1, GATA-1, and Mxi1 (Figs. 4 and 5A). Also, E2F–pocket protein complexes should play a role in repression of the *c-myc* promoter in differentiated cells and they could cooperate with differentiation-specific transcription factors, like METS (Klappacher *et al.*, 2002). In particular, the multimeric E2F-6 complex that contains E2F-6/DP-1, Mga/Max, histone methyltransferases, HP1γ, and PcG proteins (Ogawa *et al.*, 2002) and the C/EBPα–RB–E2F-4–Brm complex, which was found in livers of old mice (Iakova *et al.*, 2003), should be involved in such long-term repression of the *c-myc* promoter.

c. Deregulation of the *c-myc* Promoter in Cancer

c-myc is frequently deregulated in tumors and in most cases its expression is deregulated (Dang *et al.*, 1999; Grandori *et al.*, 2000; Marcu *et al.*, 1992; Nesbit *et al.*, 1999; Oster *et al.*, 2002; Spencer and Groudine, 1991). This enhanced and/or constitutive *c-myc* expression is the result of mutations in the *c-myc* locus (e.g., Burkitt's lymphoma; see Section IV.F) or in the signal transduction pathways that regulate *c-myc* expression. The *c-myc* promoter is activated by a large variety of transcription factors that are associated with tumorigenesis (e.g., NF-κB, c-Myb, STAT3, STAT5, AP-1, ETS-1/2, LEF-1). Most (if not all) major proliferation pathways activate the *c-myc* promoter (see Section IV.E) and many of their components are proto-oncoproteins that are activated in cancers (Vogelstein and Kinzler, 2004). As these proliferation pathways and their target transcription factors are often deregulated in tumors the frequent deregulation of the *c-myc* promoter in tumors is the direct consequence of its normal regulation by them. In addition, *c-myc* transcription is regulated by many tumor suppressors (e.g., p53, RB, p107, APC, PTEN, KLF6, KLF11), cellular (e.g., c-Myb, STAT3, STAT5,

γ-catenin, c-Ski, PTTG, NF-κB, AP-1, ETS-1/2) or viral (e.g., Tax, E1A, SV40 large T, v-Src, v-Abl, EBNA2; Kaiser *et al.*, 1999; Schlee *et al.*, 2004; Zhao *et al.*, 2006) oncoproteins and oncogenic translocation products (e.g., BCR-Abl, AML-ETO1).

TGF-β inhibits the proliferation of divergent types of cells and the loss of the growth-inhibitory effect of TGF-β contributes to carcinogenesis (Bierie and Moses, 2006; de Caestecker *et al.*, 2000; Derynck *et al.*, 2001; Dumont and Arteaga, 2003; Massagué and Gomis, 2006; Massagué *et al.*, 2000; Roberts and Wakefield, 2003; Wakefield and Roberts, 2002). How the *c-myc* promoter can become resistant to repression by TGF-β has been exemplified or suggested: First, enhanced expression of LEF-1 in tumors results in loss of repression of the *c-myc* promoter by TGF-β-activated Smad3 because it cannot inhibit the activation of the *c-myc* promoter by β-catenin/LEF-1 complexes whereas it abolishes *c-myc* activation by β-catenin/TCF-4 complexes (Fig. 5A; Sasaki *et al.*, 2003). Second, the oncoprotein c-Ski (and probably the oncoprotein SnoN) blocks the TGF-β-induced repression of *c-myc* transcription by formation of Smad2/3-Smad4-c-Ski complexes on the TIE of *c-myc* (Fig. 5A; Suzuki *et al.*, 2004). Third, phosphorylation of Smad3 by cyclin D1/Cdk4 and cyclin E/Cdk2 decreases the repression of the *c-myc* promoter by TGF-β (Fig. 5A; Matsuura *et al.*, 2004). Since cancer cells often contain high levels of these cyclin/Cdk complexes because of diverse mutations and deregulation events (Chau and Wang, 2003; Hall and Peters, 1996; Porter *et al.*, 1997; Sherr, 1996; Sherr and McCormick, 2002) inactivation of Smad3 by extensive cyclin/Cdk phosphorylation may result in resistance of the *c-myc* promoter to TGF-β (Matsuura *et al.*, 2004). Fourth, TGF-β is unable to repress the *c-myc* mRNA expression in tumor cells with a high LIP:LAP ratio because the C/EBPβ inhibitory isoform LIP abolishes the repression of the *c-myc* promoter by TGF-β (Fig. 5A; Gomis *et al.*, 2006a). Fifth, aberrant activation of the Ras-MEK1/2-ERK pathway, which is often found in cancer cells (McCormick, 1999), prevents repression of *c-myc* transcription by TGF-β by probably targeting the KLF11–Smad3 complex on the TIE (Fig. 5A; Buck *et al.*, 2006) as well as by thwarting TGF-β-induced Smad4 binding to the TIE (Fig. 5A; Chen *et al.*, 2001b) and thus likely interfering with formation of the Smad3/Smad4/E2F-4,5/DP-1/p107 complex on the TIE. Sixth, overexpression of the Notch1 intracellular domain prevents the repression of *c-myc* mRNA expression by TGF-β (Fig. 5C; Rao and Kadesch, 2003) so that aberrant activation of Notch1 signaling, which is observed in various cancers (Hansson *et al.*, 2004; Maillard *et al.*, 2005; Radtke and Raj, 2003; Wilson and Radtke, 2006), could render the *c-myc* promoter resistant to TGF-β. Seventh, a tumor-derived mutant Smad4 fails to repress the *c-myc* promoter in complex with Smad2 and/or Smad3 in response to TGF-β (Chen *et al.*, 2001b). Eighth, TGF-β is unable to repress the *c-myc* promoter in

human MDA-MB-468 breast cancer cells which lack endogenous Smad4 (Chen *et al.*, 2001b).

If tumor cells, like Burkitt's lymphomas (see Section IV.F), have lost the block to transcriptional elongation of *c-myc* they miss an important mechanism for negative control of *c-myc* transcription.

At least in colorectal tumors, point mutation of a G-C bp to an A-T bp in the NHE was found to destabilize both the G-quadruplex and the *i*-tetraplex resulting in increased activity of the *c-myc* promoter (Fig. 7; Grand *et al.*, 2004; Halder *et al.*, 2005; Siddiqui-Jain *et al.*, 2002). Thus, with the ability to stably form these repressive paranemic DNA structures at the NHE these tumor cells have lost an important mechanism for negative control of *c-myc* transcription.

Mutations in the RB pathway are believed to occur in (nearly) all human cancers so that RB is thought to be functionally inactivated in (most) tumor cells either through mutation of the *RB1* gene itself or through dysregulation of the kinases that control its activity (Frolov and Dyson, 2004; Sherr, 1996; Sherr and McCormick, 2002). Most frequent are mutations in upstream regulators such as the Cdk inhibitor $p16^{INK4A}$, Cdk4, and cyclin D1 (Chau and Wang, 2003; Dimova and Dyson, 2005; Hall and Peters, 1996) which result in functional inactivation of all three pocket proteins. Consequently, the *c-myc* promoter should be released from repression by E2F–pocket protein complexes in most cancer cells so that most tumors should have lost this important mechanism for negative control of *c-myc* transcription.

The tumor-specific splice variant FIRΔexon2, which abrogates *c-myc* suppression by FIR, is expressed in colorectal tumors (Matsushita *et al.*, 2006). This suggests that the control of the *c-myc* promoter by the FUSE–FBP–FIR–TFIIH system is disabled in colon cancer because FIRΔexon2 prevents repression of *c-myc* transcription by FIR (Matsushita *et al.*, 2006). Like during immediate-early *c-myc* induction (see Section IV.A.7; Liu *et al.*, 2006a), FBP should drive *c-myc* transcription to maximum levels in the absence of repression by FIR in these tumor cells. The self-reinforcing cycle FBP binding → *c-myc* transcription → torsional stress → FUSE melting → FBP binding would result in maximal FUSE melting and thus in maximal binding of FBP (Kouzine *et al.*, 2004), which allows permanent transactivation of the *c-myc* promoter by the remainder of (now possibly deregulated) transcription factors (He *et al.*, 2000a). Thus, in cancer cells expressing FIRΔexon2 activation of the *c-myc* promoter by FBP, which is no longer counteracted by FIR, should further increase the deregulated *c-myc* transcription. So far, it is unknown whether FIRΔexon2 is also expressed in other tumors but it is intriguing to speculate that disabling of the FUSE–FBP–FIR–TFIIH system by FIRΔexon2 may represent a more general phenomenon in cancer, which allows deregulation of *c-myc*. It is also unknown whether FBP3, which is a by far more potent transactivator of the *c-myc*

promoter than FBP (Chung *et al.*, 2006), is expressed in these colon cancer cells. Anyway, a predominance of FBP3 over FBP at the FUSE in tumor cells would provide an additional mechanism to increase *c-myc* transcription.

Loss of c-Myc autosuppression has to be assumed to be a prerequisite for deregulation of *c-myc* transcription in cancer cells as otherwise enhanced *c-myc* expression would counteract itself. Accordingly, the c-Myc autosuppression is lost in many tumor-derived or transformed cell lines whereas it is operative in primary and immortalized (retaining contact inhibition of growth) cell lines (Facchini and Penn, 1998; Facchini *et al.*, 1994; Grignani *et al.*, 1990). One major unresolved question is how cancer cells can get rid of c-Myc autosuppression. It is thought that c-Myc autosuppression includes c-Myc/Max binding to the Inr that may be mediated by an Inr binding protein, binding of E2F to the E2F-binding site and interaction of p107, which is essential for c-Myc autosuppression, with both c-Myc and E2F (Luo *et al.*, 2004). The RB tumor suppressor pathway is perturbed in (most) human cancers (Chau and Wang, 2003; Dimova and Dyson, 2005; Frolov and Dyson, 2004; Hall and Peters, 1996; Sherr and McCormick, 2002; Sherr, 1996). The frequent mutations in its upstream components would eliminate the interaction between p107 and E2F (and possibly also the interaction between p107 and c-Myc). Mutations affecting the *p107* gene itself could have the same effect. Thus, although not elucidated so far, one may speculate that the c-Myc autosuppression is lost in tumor cells because the essential function of p107 is disabled.

In colon cancer cells, expression of a truncated mutant APC leads to the constant occupancy of the *c-myc* promoter with β-catenin/LEF-1 complexes and β-catenin coactivators (Pygosus, Bcl-9/Lgs, a MLL/SET1-type HMT complex with MLL2 as HMT) in correlation with the absence of TCF/LEF corepressors (TLE-1, HDAC1, CtBP) resulting in permanent deregulation of *c-myc* transcription (Sierra *et al.*, 2006).

Tumor-specific CTCF point mutations from breast, prostate and Wilms' tumors have lost their ability to bind to the *c-myc* promoter (Filippova *et al.*, 2002) suggesting that they should be unable to repress *c-myc* transcription.

In summary, tumor cells have acquired multiple alterations to evade the normal controls of *c-myc* transcription.

d. Control of the *c-myc* Promoter in Continuously Cycling Cells

The so far described immediate-early induction of *c-myc* transcription, permanent repression of the *c-myc* promoter during differentiation and deregulation of the *c-myc* promoter in cancer are quite well to explain whereas the regulation of the *c-myc* promoter in continuously cycling cells, where *c-myc* is constantly expressed throughout the cell cycle (Hann *et al.*, 1985; Rabbitts *et al.*, 1985; Thompson *et al.*, 1985), is poorly understood. Consequently, one major unresolved problem is how the *c-myc* promoter

is regulated in these latter cells. Based on the current knowledge we would like to discuss possibilities for *c-myc* promoter control in continuously proliferating cells, but we will also point to outstanding questions.

FBP (Fig. 4), which binds to the FUSE only at active, but not at inactive *c-myc* promoters and which shows a parallel expression profile to c-Myc (Avigan *et al.*, 1990; Bazar *et al.*, 1995a; Davis-Smyth *et al.*, 1996; Duncan *et al.*, 1994; Kouzine *et al.*, 2004; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b), functions as the master regulator of the *c-myc* promoter (He *et al.*, 2000a). In the absence of FBP or if FBP function is inhibited *c-myc* transcription from both the P1 and P2 promoters is abolished and cellular proliferation is arrested demonstrating that the large set of other transcription factors that bind to and transactivate the *c-myc* promoter fails to sustain *c-myc* transcription in the absence of (functional) FBP (He *et al.*, 2000a). However, since FBP binds only to the single-stranded FUSE but not to its relaxed duplex form *c-myc* transcription needs to be activated before FBP can bind to the FUSE (Duncan *et al.*, 1994; He *et al.*, 2000a; Kouzine *et al.*, 2004; Levens *et al.*, 1997; Liu *et al.*, 2006a; Michelotti *et al.*, 1996b). Consistently, immediate-early induction of *c-myc* transcription (Fig. 6) occurs independently of FBP (Liu *et al.*, 2006a). Then the torsional stress from ongoing *c-myc* transcription drives FUSE melting (Kouzine *et al.*, 2004) so that first FBP3 and then FBP (Chung *et al.*, 2006) bind to the now single-stranded FUSE (Liu *et al.*, 2006a) and thus allow activation of the *c-myc* promoter by the large variety of transactivators that bind to the *c-myc* promoter (Fig. 4). Thereby the self-reinforcing cycle FBP binding → *c-myc* transcription → torsional stress → FUSE melting → FBP binding ensures sustained FBP binding to the FUSE and thus drives the permanent *c-myc* transcription in continuously proliferating cells. Many of the transactivators that bind to the *c-myc* promoter are theirselves regulated in expression and/or activity so that the current actual transactivation of the *c-myc* promoter will vary (Chung and Levens, 2005; Liu and Levens, 2006). Consequently, for the constant *c-myc* expression throughout the cell cycle in continuously cycling cells a second mechanism is needed that holds *c-myc* expression constant on its rather low level. The c-Myc autosuppression is well suited to fulfill this function because the transcription factor c-Myc itself represses the *c-myc* promoter (Fig. 8) in a concentration-dependent manner at the level of transcription initiation (Facchini *et al.*, 1997; Grignani *et al.*, 1990; Luo *et al.*, 2004; Penn *et al.*, 1990a). Thereby, the very short half-lives of only 20–30 min of *c-myc* mRNA and c-Myc protein (Dani *et al.*, 1984, 1985; Hann and Eisenman, 1984; Hann *et al.*, 1988; Lüscher and Eisenman, 1990; Rabbitts *et al.*, 1985; Waters *et al.*, 1991) allow a fast feedback regulation. An increase in *c-myc* transcription is compensated by the resulting enhanced repression of the *c-myc* promoter by c-Myc while vice versa a decrease in *c-myc* transcription is counteracted by the resulting diminished repression of

the *c-myc* promoter by c-Myc. In this model the throughout the cell cycle constant rather low *c-myc* expression in continuously cycling cells results from the antagonism of the positive master regulator FBP, which allows permanent activation of the *c-myc* promoter by all of its transactivators, and the concentration-dependent c-Myc autosuppression, which holds *c-myc* transcription on a constant low level by repression of the *c-myc* promoter. Ubiquitous transcription factors that activate the *c-myc* promoter, like USF (Lee and Ziff, 1999), Sp1, YY1, hnRNP K and CNBP (Fig. 4), should provide relatively constant positive stimuli for *c-myc* transcription thereby ensuring that the *c-myc* promoter is permanently transactivated in continuously proliferating cells. In this model the fluctuation in the current actual activation of the *c-myc* promoter by its many diverse transactivators does not matter because the c-Myc autosuppression operates to limit *c-myc* transcription on a rather low level.

In addition, the FUSE–FBP–FIR–TFIIH system superimposes a dynamic real-time feedback onto the *c-myc* promoter to hold *c-myc* expression to appropriate tolerances and to control the intrinsic and extrinsic noise of transcription, which is especially pronounced for genes encoding short half-life, low abundance products like c-Myc (Chung and Levens, 2005; Liu and Levens, 2006). The FUSE–FBP–FIR–TFIIH system uses FUSE melting as a sensor for the intensity of ongoing transcription and FBP and FIR as effectors to provide positive or negative, respectively, feedback to TFIIH at the promoter. David Levens and coworkers suggested that the end-product feedback regulation via c-Myc autosuppression may be too slow to control the rapid stochastic fluctuations in activation of *c-myc* transcription so that the FUSE–FBP–FIR–TFIIH system should be needed as a real-time feedback, which anticipates how much c-Myc will be made before it is actually synthesized (Chung and Levens, 2005; Liu and Levens, 2006). It is obvious that this system, which is equipped to up- and downregulate transcription through the antagonists FBP and FIR, is intimately involved in control of *c-myc* transcription (He *et al.*, 2000a; Liu *et al.*, 2000; 2001; 2006a; Kouzine *et al.*, 2004; Weber *et al.*, 2005). However, the exact function of this system in continuously cycling cells remains still to be elucidated.

Although FBP-mediated stimulation of the 3′–5′ helicase activity of the p89 TFIIH subunit and FBP-stimulated promoter escape are counteracted by FIR (Liu *et al.*, 2000, 2001) this will only result in an invariant effect on *c-myc* transcription if this repression by FIR is not specifically regulated in response to the current rate of *c-myc* transcription or the current *c-myc* mRNA or protein levels. So far, no specific regulation of FIR activity or expression was described in continuously cycling cells, where the *c-myc* promoter is occupied by both FBP and FIR (Liu *et al.*, 2006a). If the activation by FBP is invariantly counteracted by FIR in continuously proliferating cells the FUSE–FBP–FIR–TFIIH system should operate as self-reinforcing cycle FBP binding → *c-myc* transcription → torsional stress → FUSE melting → FBP binding even in the

presence of FIR at the FUSE. Thus a major outstanding question is whether in continuously cycling cells activity and/or expression of FIR are invariant or specifically regulated, that is, whether the FUSE–FBP–FIR–TFIIH system operates as a self-reinforcing cycle or is anti-regulated in response to the rapidly varying current *c-myc* expression.

An alternative possibility to regulate FUSE-mediated activation and repression would be the dynamic exchange between the strong transactivator FBP3, the weaker transactivator FBP and FIR-repressed FBP. If instead of the expression or activity of FIR its recruitment to the FUSE would be controlled by regulated binding of either FBP, which can be repressed by FIR, or FBP3, which is unable to interact with FIR (Chung *et al.*, 2006), the repression of the *c-myc* promoter by FIR could be regulated in response to the current rate of *c-myc* transcription or the current *c-myc* mRNA or protein level in continuously cycling cells. However, such a required regulation of FBP and FBP3 in these cells, in terms of FUSE affinity, nuclear localization, synthesis or degradation (see Section IV.A.7), has remained largely unexplored. Furthermore and more important, the constant *c-myc* mRNA level in continuously proliferating cells is rather low whereas the potent transactivator FBP3 would lead to a high *c-myc* transcription so that an exchange of FBP3 versus free FBP versus FIR-repressed FBP at the FUSE should not be suited to control the *c-myc* promoter in these cells. In accordance, FBP3 is involved in the ascent of the serum-induced peak in *c-myc* transcription during stimulation of quiescent cells, but leaves the FUSE at its apex and has disappeared when the *c-myc* mRNA level declines (Fig. 6; Chung *et al.*, 2006). Therefore only FBP and FIR, but not FBP3 should regulate the *c-myc* promoter in continuously cycling cells because they define a narrow range of *c-myc* transcription (Chung *et al.*, 2006) that is conducive to the constant low *c-myc* mRNA expression throughout the cell cycle in these cells.

The exact contribution of the FUSE–FBP–FIR–TFIIH system to control of the *c-myc* promoter in continuously proliferating cells is an important open question. David Levens and coworkers suggested that for *c-myc* the influence of the FUSE–FBP–FIR–TFIIH system is greatest during physiological transitions and of lesser importance under steady-state conditions (Chung *et al.*, 2006).

Due to the parallel expression profiles of c-Myc and FBP, FBP is present when it is needed as positive master regulator of the *c-myc* promoter (e.g., throughout the whole cell cycle in continuously proliferating cells), but FBP expression is finished when the *c-myc* promoter must be repressed during terminal differentiation (Bazar *et al.*, 1995a; Duncan *et al.*, 1994). FBP2 and FBP3 are also downregulated during differentiation (Davis-Smyth *et al.*, 1996). If the master regulator FBP does no longer bind to the FUSE the activation of the *c-myc* promoter by the remainder of its transactivators should also be extinguished. FIR should not contribute to the enduring repression of the *c-myc* promoter in differentiated cells as it was undetectable at the FUSE after 7 days of serum starvation (Liu *et al.*, 2006a).

The c-Myc autosuppression is of course lost in terminally differentiated cells where instead differentiation-specific repressors suppress the *c-myc* promoter.

There are still many unanswered questions concerning the regulation of the *c-myc* promoter in continuously cycling cells, for example:

Pocket proteins as well as Smad3 are phosphorylated by cyclin D1/Cdk4 and cyclin E/Cdk2 and this affects the regulation of the *c-myc* promoter by E2F–pocket protein complexes (Oswald *et al.*, 1994) and Smad3 (Matsuura *et al.*, 2004) (Fig. 5A). However, phosphorylation of both pocket proteins and Smad3 by these G_1 cyclin/Cdk complexes is cell cycle-dependent whereas *c-myc* expression is constant throughout the cell cycle in continuously cycling cells (see Sections IV.A.2 and IV.A.3). Consequently, the question arises how this contradiction can be explained.

The constant *c-myc* expression in continuously proliferating cells depends on the permanent presence of growth factors so that their removal results in immediate downregulation of *c-myc* expression at any cell cycle time point (Facchini and Penn, 1998; Grandori *et al.*, 2000; Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Marcu *et al.*, 1992; Oster *et al.*, 2002; Spencer and Groudine, 1991). Antiproliferative signals lead also to immediate downregulation of *c-myc* expression independent from the cell cycle phase. Again cell cycle-dependently regulated cyclin D1/Cdk4 and cyclin E/Cdk2 and their targets at the *c-myc* promoter, that is, E2F–pocket protein complexes and Smad3, cannot account for these cell cycle-independent responses. Therefore the question arises how growth factor withdrawal and antiproliferative signals can affect the *c-myc* promoter independent from the cell cycle phase. The TGF-β-induced repression of the *c-myc* promoter by the Smad3/Smad4/E2F-4,5/DP-1/p107 complex through the TIE (Figs. 4 and 5A) provides one mechanism for rapid cell cycle-independent repression of *c-myc* transcription as it can be triggered at any point during the cell cycle (Chen *et al.*, 2002; Zentella *et al.*, 1991). In addition, increasing the transcriptional pausing of Pol II complexes should allow rapid downregulation of *c-myc* transcription at any cell cycle time point (see Section III.C). Furthermore, sequestration of the NHE in repressive paranemic DNA structures (Fig. 7) could provide a fast mode for regulation of the *c-myc* promoter in response to growth factor withdrawal and antiproliferative signals (Siddiqui-Jain *et al.*, 2002) that may be available in each cell cycle phase.

VI. SUMMARY AND PERSPECTIVES

In summary, the complex regulation of the *c-myc* promoter reflects the ***M***arvelous***l*** ***Y*** ***C***omplex biology of c-Myc (Oster *et al.*, 2002) and the dualism of c-Myc's essential importance for normal cell growth control versus the

dangerous high transformation potential of deregulated *c-myc* expression. This intimate correlation between c-Myc biology and regulation of the *c-myc* promoter is essential for development and normal tissue homeostasis.

This review summarized and tried to explain the regulation of the *c-myc* promoter by transcription factors, signaling pathways, and *cis*-regulatory elements. It showed that important principles of *c-myc* promoter control have been exemplified and that a pattern for its regulation in different biological settings starts to emerge so that the *c-myc* promoter has not to be considered as a complete enigma and a total black box. Nevertheless, many unanswered questions and outstanding problems remain so that the *c-myc* promoter is still ***M***yster***Y*** and ***C***hallenge. Like with c-Myc biology, we will certainly learn much about the *c-myc* promoter in the future and one should await some surprises.

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Designer Self-Assembling Peptide Nanofiber Scaffolds for Study of 3-D Cell Biology and Beyond

Dedicated to George and Eva Klein 80th Birthday Symposium

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"You should always ask questions, the bigger the better. If you ask big questions, you get big answers."

Francis Crick (1916–2004)

Biomedical researchers have become increasingly aware of the limitations of the conventional 2-D tissue cell cultures where most tissue cell studies including cancer and tumor cells have been carried out. They are now searching and testing 3-D cell culture systems, something between a petri dish and a mouse. The important implications of 3-D tissue cell cultures for basic cell biology, tumor biology, high-content drug screening, and regenerative medicine and beyond are far-reaching. How can nanobiotechnology truly advance the traditional cell, tumor, and cancer biology? Why nano is important in biomedical research and medical science? A nanometer is 1000 times smaller than a micrometer, but why it matters in biology? This chapter addresses these questions. It has become more and more apparent that 3-D cell culture offers a more

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Eva Klein

George Klein

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realistic local environment through the nanofiber scaffolds where the functional properties of cells can be observed and manipulated. A new class of designer self-assembling peptide nanofiber scaffolds now provides an ideal alternative system. Time has come to address the 3-D questions because quantitative biology requires *in vitro* culture systems that more authentically represent the cellular microenvironment in a living organism. In doing so, *in vitro* experimentation can become truly more predictive of *in vivo* systems.

PROLOGUE

Professors George and Eva Klein not only have inspired a few generations of leading biomedical researchers but also have had enormous influence in the fields of tumor and cancer biology, immunology, and virology around the world. They personally taught and mentored a very large number of influential biomedical and medical researchers and medical scientists not only at the Karolinska Institute, Stockholm, Sweden, but also in Europe and rest of the world. In addition to their pioneer research, either intentionally relentless pursuit or quickly recognizing the unexpected discoveries, they have had profound impact beyond their own fields; their contributions have also been felt far beyond the defined disciplines. Furthermore, because of their warm and open personalities, tireless traveling, keen interest in science, medicine and culture, as well as their impatience and intolerance for fools, their names have become synonymous with extremely high scientific standards and they have made a wide spectrum of friends, become close colleagues, and got to know a large number of acquaintances. They are highly respected beyond any geographic location, from Europe, Middle East, North and South America to China. Their names are legendary, and their legacy will have a lasting impact on many generations of scientists to come.

One might wonder how could a young researcher from China, outside of tumor and cancer biology, immunology, and virology fields, become very close to George and Eva Klein? The answer lies, not surprisingly, in their warm and open personality. Here is a moving story that illustrates the personality of George and Eva Klein.

When I was a graduate student studying *Tetrahymena* genetics at the University of California, Santa Barbara, in 1987, I never heard of George and Eva Klein. Although I have read literature widely, my own interest was in the detail structure of DNA, particularly the left-handed Z-DNA discovered by Alexander Rich and his colleagues at MIT in 1979. My interest was not only a scientific one but also a philosophical one. I asked why nature sometimes has remarkable symmetry and sometimes does not. Why most helices at molecular scales, for example, alpha-helix in proteins, DNA and RNA double helix in nucleic acids, and some helices in polysacchrides, are mostly right-handed.

My exposure to virology and immunology was kept at minimal, although I took two courses in these subjects. But in both the courses, the complex names, both in English and in non-English, as well as the endless abbreviated terms, Latin names, and hard-to-remember acronyms made these subjects less attractive as I was still struggling to learn English.

Then things changed dramatically in September 1987, when my son, Niklas, who had just turned 3, was diagnosed with childhood acute lymphoblast leukemia (ALL) with ~68% leukemia cells with double chromosomal translocations (7:9; 6:21) in his bone marrow. The doctors at several oncology clinics and at the Children's Hospital of Los Angeles refused to give a prognosis. This was devastating! Then I instantly read the latest literature about chromosomal translocations and their relationship in childhood leukemia. George Klein's name came up many times. At that time, I had never read or heard George Klein's name before, and had no idea that he is one of the most prominent tumor and cancer biologists and an authority on chromosomal translocation in the world. I wrote him asking for help for what was the most available treatment at that time. I wrote, with my cryptic English, to about 30 people who seem to be experts on childhood leukemia.

Not surprisingly, most people did not reply. Only three people replied to a totally unknown Chinese student, pleading for help; among them were Sharon Murphy, then at St. Jude Hospital; Janet Rowley of University of Chicago; and George Klein of Karolinska Institute, Sweden. George Klein not only wrote me a letter but also sent me a big package of his publications relevant to chromosomal translocation and other tumor biology. George probably does not remember my request anymore since he routinely replies requests, large and small. This is my first encounter with George Klein, not as a scientist but as a father trying to find the cause and cure for my son's mysterious disease.

Many years later, when I first visited Karolinska Institute in September 1999 to attend Dr. Bian Zhao's Ph.D. thesis defense, I met George Klein in person for the first time. I had no formal appointment with him nor had telephoned him in advance. I just walked into his office while he was very busy. However, he received me and had a chat with me. He not only signed his book *Atheist and Holy City*, recommended to me by Robert Horvitz, but also gave me another of his book, *Living Now*. He was very generous with his time and very kind to a stranger of foreign origin.

Subsequently, I invited George Klein to give a History of Biology Lecture at Massachusetts Institute of Technology (MIT) in September 2000. When I asked George to give me a title of his lecture, he did not just give me one but four titles! He came together with his son Dr. Peter Klein, a mathematician with a Ph.D. from Columbia University, New York. Peter was then interested in complex problems involving mathematics in biology. George not only met his old friends but also made new friends during his visit at MIT. Later I

visited Peter in his home in Greenwich near New York City, I had many visits with Peter Klein in his home and elsewhere since his daughter and son are about the same age as my son, Niklas.

I met Eva Klein in Peter's house in November 2002. Eva in her usual open-minded, direct, and warm manner immediately told me that I am now an honorary member of her family since I am of the same age as Peter Klein. I am very honored. During conversation, I learned that Eva is the person who, together with her postdoc, discovered the now extremely important and ubiquitous Natural Killer cells, a crucial advancement for immunology and tumor biology. I asked her why she gave the name Natural Killer cells. She told me that these cells were discovered from the control experiments and since these cells could be activated without external stimulations, they are the natural killers. Since this discovery was mostly either taken for granted or totally forgotten, I decided to invite Eva Klein to give a talk on the subject to inspire young researchers to make more discoveries, to do good controls, to make very careful observations, and to question unexpected results.

I also arranged Eva Klein to give the same History of Biology Lecture Series at MIT on March 24, 2003. She gave me a very unusual title: *Natural Killer cells: An unexpected discovery (met first as an annoying phenomenon)*. Her lecture was very well received. Robert Horvitz, Richard Hynes, Jack Buchanan, Gobind Khorana, Boris Magasanik, and many other faculty and students from the MIT Biology Department and elsewhere attended her lecture, full of interesting stories and current advancement of NK cells. She later told me that my insistence on her to give a lecture on the history of the discovery of NK cells has encouraged her to look into the active field much more closely.

Although their research areas are in tumor and cancer biology, virology, and immunology, they both recognize the importance of new findings outside their fields immediately. After my lecture, "Beyond Petri Dish," hosted by the late colleague and friend, structural biologist, Carl Brändén at Karolinska Institute in April 2003, Eva immediately asked me to edit a special focus volume for *Seminar on Cancer Biology* on 3-D cell culture. The issue came out in October 2005. George, likewise, suggested many experiments to study cancers using the designer biological scaffolds in 3-D systems.

Both George and Eva Klein have very little patience to tolerate fools. They are outspoken for many issues, including science, culture, and politics. George has written many books that are utterly refreshing. The ideas expressed and topics selected in his books are direct, sharp, intelligent, lucid, and eloquent. I wish there were more people who could write as direct as George Klein. Interestingly, he does not per se write the books; rather, he dictates his books, a special ability. When I stayed in their home, I found one morning, ~6 o'clock, he was busy dictating another book.

Both George and Eva survived the horrible holocaust, so they do not waste time for unimportant things. They are extraordinary people, both as

scientists and as humanists. They always ask big questions in medical science and other matters they consider important. They have made an enormous contribution not only to biomedical research but also for enriching our culture and politics. They are truly rare world-class citizens.

I. INTRODUCTION

Nearly all tissue cells are embedded in a 3-dimensional (3-D) microenvironment in the body surrounded by nanoscale extracellular matrix. On the other hand, nearly all tissue cells, including most cancer and tumor cells, have been studied in 2-dimensional (2-D) petri dish, 2-D multiwell plates, or 2-D glass slides coated with various substrata. How can one reconcile the apparent disparity? Likewise, although millions of cell biology papers have been published using the 2-D culture systems, one must ask how we can be so certain that the results obtained from the 2-D system truly reflect the *in vivo* conditions. Science, after all, is to constantly ask questions, big and small.

II. 2-D OR NOT 2-D

Although petri dish has had an enormous impact on modern biology, the petri dish culture system, including multiwell plates, glass cover slips, etc, is less than ideal to study tissue cells for several reasons: (1) It is a 2-D system that is in sharp contrast to the 3-D environment of natural tissues both in animals and in plants. (2) The petri dish surface without coating is rigid and inert, again in sharp contrast to the *in vivo* soft environment where cells intimately interact with the extracellular matrix and with each other. (3) The tissue cell monolayers on coated 2-D surface, such as poly-L-lysine, collagen gels, fibronectin, laminin and Matrigel (Kleinman and Martin, 2005; Kleinman *et al.*, 1986) as well as other synthetic materials containing segments of adhesion motifs, have only part of the cell surface attached to the materials and interact with neighboring cells. Often, the remaining parts are directly exposed to the culture media, unlike the tissue environment where every cell intimately interact with its neighbor cells and the extracellular matrix. Thus 3-D-matrix interactions display enhanced cell biological activities and narrowed integrin usage. (4) The transport phenomena of 2-D and 3-D are drastically different. In 2-D culture systems, cytokines, chemokines, and growth factors quickly diffuse in the media across the culture dish. This is again in sharp contrast to the *in vivo* environment where chemical and biological gradient diffusion systems play a vital role in signal transduction, cell–cell communications, and development. (5) Cells cultured on a 2-D petri dish are not readily transportable, that is, it is nearly impossible to move cells from one environment to another without incurring changes in the cell–material and cell–cell interactions. For example,

cell collections using trypsinization or mechanically using rubber policeman may have an adverse effect on cell–materials/environment interactions. In contrast, cells cultured on 3-D scaffolds are more readily transportable without significantly harming cell–material and cell–cell interactions, thus providing a significantly new way to study cell biology.

III. MICRO- AND NANOSCALES, WHY THEY ARE IMPORTANT?

The importance of length scales is apparent, for example, the scales of trees and grasses (Fig. 1). Both are made of the same basic building blocks, sugars that are polymerized by enzymes to produce cellulose fibers. Trees, usually 20–30 cm in diameter, are common in forests. If animals are in the forest, they can either go between the trees or climb onto the trees, they cannot go through the trees because they are in similar scales as the trees. On the other hand, grasses are usually 0.5 cm in diameter; grasses 0.3–1 cm in diameter are common. Although animals are embedded and surrounded in in grasses, they can move freely in the grasses. This analogy can be directly extended to scaffolds in various scales.

In the past three decades, several biopolymers, including PLLA, PLGA, PLLA-PLGA copolymers, and other biomaterials including alginate, agarose, collagen gels, and others, have been developed to culture cells in 3-D (Atala and Lanza, 2001; Hoffman, 2002; Lanza *et al.*, 2000; Palsson *et al.*, 2003; Ratner *et al.*, 1996; Yannas, 2001). These culture systems have significantly advanced our understanding of cell–material interactions and fostered a new field of tissue engineering and regenerative medicine. Attempts have been made to culture cells in 3-D using synthetic polymers or copolymers. However, these synthetic polymers are often processed into microfibers, ~10–50 μm in diameter, that are similar in size to most cells (~5–20 μm in diameter). Thus, cells attached to microfibers are still in a 2-D environment with a curvature depending on the diameter of the microfibers. Therefore, cells attached to microfibers are in fact in 2-D despite the various curvatures associated with the large diameter microfibers. Furthermore, the micropores (~10–200 μm cross) between the microfibers are often ~1000–10,000-fold greater than the size of bimolecular, including vitamins, amino acids, nutrients, proteins, or drugs, which as a consequence can quickly diffuse away, much like a car driving on highways. For a true 3-D environment, a scaffold's fibers and pores must be substantially smaller than the cells. In order to culture tissue cells in a truly 3-D microenvironment, the fibers must be significantly smaller than cells so that the cells are surrounded by the scaffold, similar to the extracellular environment and native extracellular

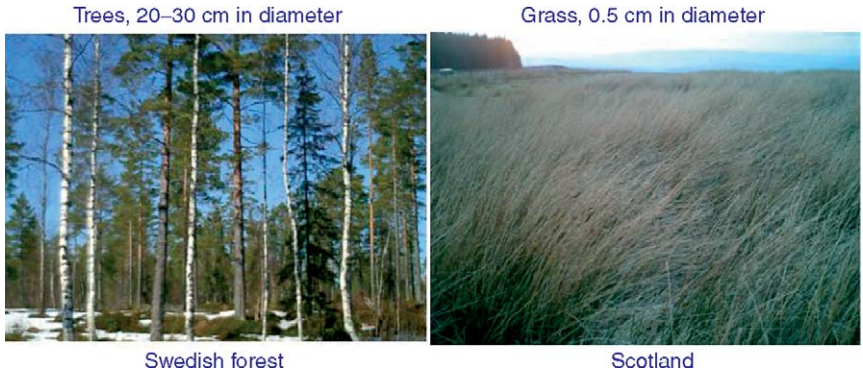

Fig. 1 The drastic difference in scales. Both trees and grasses are made of cellulose, or the same building blocks—sugars, but with very different scales. The trees shown on the left are 20–30 cm in diameter and the distance between the trees is in tens of meters. Animals cannot walk through the trees but between them. Some animals can climb on the trees (left panel). This is in analogy; cells ~5–20 μm can only attach to the microfibers. On the other hand, each grass is about 0.5 cm in diameter. When animals walk in the grass field, they are fully surrounded by the grasses, which do not hinder their movement. In this case, animals are embedded in 3-D (right panel). In analogy, cells in nanofibers are fully embedded in the nanofiber scaffolds, where they can still move freely without hindrance.

matrices (Ayad *et al.*, 1998; Kleinman *et al.*, 1986; Kreis *et al.*, 1999; Lee *et al.*, 1985; Oliver *et al.*, 1987; Timpl *et al.*, 1979).

Animal-derived biomaterials (e.g., collagen gels, polyglycosaminoglycans, and Matrigel) have been used as an alternative to synthetic scaffolds (Bissell *et al.*, 2002; Bissell 1981; Cukierman *et al.*, 2001, 2002; Kleinman *et al.*, 1986; Kubota *et al.*, 1988; Lee *et al.*, 1985; Oliver *et al.*, 1987; Schmeichel and Bissell, 2003; Weaver *et al.*, 1995; Zhau *et al.*, 1997). But while they do have the right scale, they frequently contain residual growth factors, undefined constituents, or nonquantified impurities. It is thus very difficult to conduct a completely controlled study using these biomaterials because they vary from lot to lot. This not only makes it difficult to conduct a well-controlled study but also would pose problems if such scaffolds were ever used to grow tissues for human therapies. Animal-derived biomaterials, for example, collagen gels, laminin, poly-glycosaminoglycans, and materials from basement membranes including Matrigel^{TM}, have been used as an alternative to synthetic scaffolds (Bissell *et al.*, 2002; Cukierman *et al.*, 2001, 2002; Kleinman *et al.*, 1986; Kubota *et al.*, 1988; Lee *et al.*, 1985; Oliver *et al.*, 1987; Schmeichel and Bissell, 2003; Weaver *et al.*, 1995; Zhau *et al.*, 1997). Although researchers are well aware of their limitations, it is one of the few limited choices. (Tables I and II).

An ideal 3-D culture system that can be fabricated from a synthetic biological material with defined constituents of truly biological origin is

Table I A Variety of Tissue Cells Cultured on the Designer Self-Assembling Peptide Nanofiber Scaffolds

Chicken embryo fibroblast	Bovine calf and adult chondrocytes
Mouse fibroblast	Bovine endothelial cells
Mouse embryonic stem cells	Mouse adult neural stem cells
Mouse cerebellum granule cells	Mouse and rat hippocampal cells
Mouse mesenchymal stem cells	Mouse cardiac myocytes
Rat adult liver progenitor cells	Rat liver hepatocytes
Rat pheochromocytoma	Rat cardiac myocytes
Rat neural stem cells	Rat hippocampal neural tissue slice
Bovine osteoblasts	Bovine endothelium cells
Chinese hamster ovary	Hamster pancreas cells
Horse bone marrow	Rat keratinocytes
Human cervical carcinoma	Human osteosarcoma
Human hepato-cellular carcinoma	Human neuroblastoma
Human embryonic Kidney	Human Hodgkin's lymphoma
Human epidermal keratinocytes	Human foreskin fibroblast
Human neural stem cells	human aortic endothelial cells

Note: These cells include stable cell lines, primary isolated cells from animals, progenitor, and adult stem cells.

Table II Animals that Have Been Exposed to Peptide Nanofiber Scaffolds

Mice
Rats
Hamsters
Rabbits
Goats
Monkeys
Pigs
Horses

Note: These animals were tested in various academic laboratories and commercial testing laboratories as well as biomaterials and medical device companies around the world.

thus required. Thus the molecular designer self-assembling peptide nanofiber scaffolds may be a promising alternative. We directly compared the Matrigel with the designer self-assembling peptide nanofiber scaffold (Fig. 2). They have similar nanoscales and similar porosity except Matrigel, which seems to have many particles, perhaps proteins that are contained in the Matrigel. On the other hand, the peptide nanofibers are very smooth, suggesting their purity and homogeneous structure (Fig. 2).

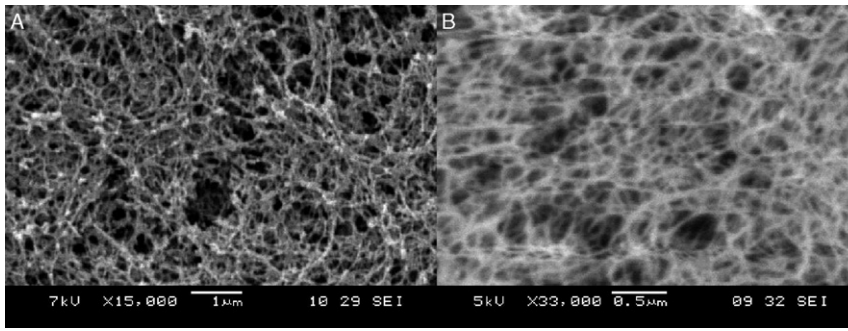

Fig. 2 SEM images of Matrigel and designed self-assembling peptide nanofiber scaffold. (A) Matrigel 1-μm scale bar (15,000X magnifications). (B) RADA16-I (33,000X magnifications) 0.5-μm scale bar. These peptides all form nanofiber scaffolds with nanopores (average 5–200 nm). It is worth noting that the nanopores may allow small molecular drugs (1–2 nm) and proteins (2–10 nm) to diffuse in the scaffolds slowly. This is in sharp contrast to many other biopolymer microfiber materials where the pores are also microns that drugs and proteins diffuse rather quickly. (Image courtesy of Fabrizio Gelain).

IV. THE IDEAL BIOLOGICAL SCAFFOLDS

Although there are a number of criteria to fabricate biological scaffolds, the ideal 3-D biological scaffolds should meet several important criteria: (1) the building blocks should be derived from true biological sources; (2) basic units should be amenable to design and modification to achieve specific needs; (3) exhibit a controlled rate of material biodegradation; (4) exhibit no cytotoxicity; (5) promote cell–substrate interactions; (6) afford economically scalable and reproducible material production, purification, and processing; (7) be readily transportable; (8) be chemically compatible with aqueous solutions and physiological conditions; (9) elicit no or little immune responses and inflammation if used in human therapies; and (10) integrate with other materials and tissues in the body.

V. DISCOVERY OF SELF-ASSEMBLING PEPTIDE SCAFFOLDS

The self-assembling peptide scaffold belongs to a class of biologically inspired materials. The first member, EAK16-II (AEAEAKAKAEAEAKAK), of the family was discovered from a segment in a yeast protein, Zuotin (Zhang *et al.*, 1992). The scaffolds consist of alternating amino acids that contain 50% charged residues (Caplan *et al.*, 2002; Gelain *et al.*, 2006; Holmes *et al.*, 2000; Horii *et al.*, 2007; Kisiday *et al.*, 2002; Zhang *et al.*, 1993, 1994, 1995). These peptides are characterized by their periodic repeats of alternating ionic

hydrophilic and hydrophobic amino acids with a typical β-sheet structure. Thus, these β-sheet peptides have distinct polar and nonpolar surfaces. The self-assembly event creating the peptide scaffold takes place under physiological conditions. They are like gel-sponge in aqueous solution and readily transportable to different environments. Individual fibers are $\sim$10 nm in diameter. A number of additional self-assembling peptides including RADA16-I (AcN-RADARADARADARADA-CNH2) and RADA16-II (AcN-RARADADAR-ARADADA-CNH2), in which arginine and aspartate residues substitute lysine and glutamate, have been designed and characterized for salt-facilitated nanofiber scaffold formation. The alanines form overlap hydrophobic interactions in water, a structure that is found in silk fibroin from silkworms and spiders. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. In general, these self-assembling peptides form stable β-sheet structures in water, which are stable across a broad range of temperature, wide pH ranges in high concentration of denaturing agent urea and guanidium hydrochloride. The nanofiber density correlates with the concentration of peptide solution, and the nanofiber retains extremely high hydration, $>$99% in water (5–10 mg/ml, w/v) (Fig. 3).

The peptide synthesis method uses conventional mature solid phase or solution peptide synthesis chemistry. Depending on the length of the motifs, highly pure peptides can be produced at a reasonable cost. Since the cost of peptide synthesis has decreased steadily in the past few years, it has become affordable for most people.

Many self-assembling peptides that form scaffolds have been reported and the numbers are still expanding (Zhang, 2002; Zhang and Altman, 1999). The formation of the scaffold and its mechanical properties are influenced by several factors, one of which is the level of hydrophobicity (Caplan *et al.*, 2002; Marini *et al.*, 2002]. That is, in addition to the ionic complementary interactions, the extent of the hydrophobic residues, Ala, Val, Ile, Leu, Tyr, Phe, Trp (or single letter code, A, V, I, L, Y, P, W), can significantly influence the mechanical properties of the scaffolds and the speed of their self-assembly. The higher the content of hydrophobicity, the easier it is for scaffold formation and the better for their mechanical properties (Caplan *et al.*, 2002; Kisiday *et al.*, 2002; Marini *et al.*, 2002).

VI. SELF-ASSEMBLING PEPTIDE NANOFIBER SCAFFOLDS

A single molecule of the ionic self-complementary peptide RADA16-I is shown in Figs. 3 and 4. Millions of peptide molecules self-assembled into individual nanofibers that further form the nanofiber scaffold (Fig. 3). The nanopores range from a few nanometers to a few hundred nanometers; the scales are similar in size as most biomolecules, so that these molecules or

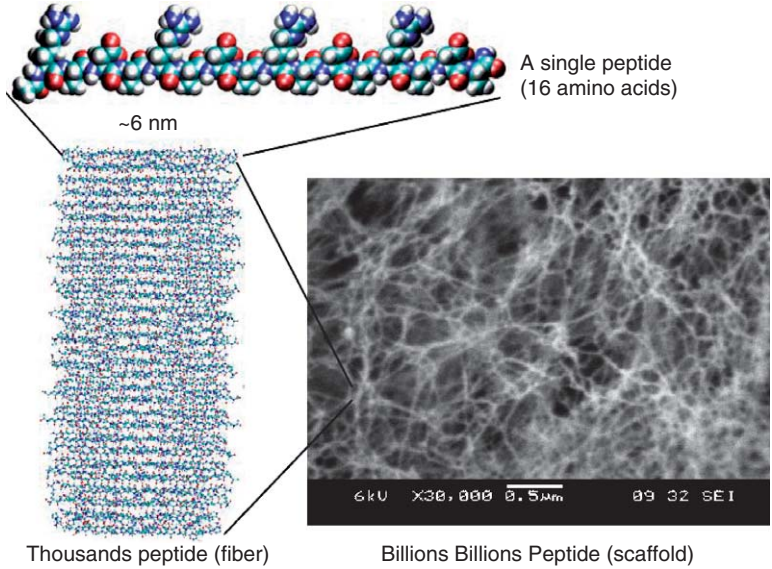

Fig. 3 Self-assembling peptide RADA16-I nanofiber scaffold hydrogel. (A) Amino acid sequence of RADA16-I, molecular model of a single RADA16-I nanofiber, the dimensions are ~6-nm long, 1.3-nm wide, and 0.8-nm thick; (B) tens and hundred thousands of individual peptides self-assemble into a nanofiber; and (C) SEM images of RADA16-I nanofiber scaffold. Note the scale bar, 0.5 μm or 500 nm. (SEM image courtesy of Fabrizio Gelain).

drugs may not only defuse slowly but also establish a molecular gradient in the scaffolds. Figure 4 shows the individual nanofibers ranging from a few hundred nanometers to a few microns. Peptide samples in aqueous solution, using environmental AFM examination, showed similar nanofiber results, suggesting that the nanofiber formation is independent of the drying process. It is interesting to observe that at high resolution the nanofibers appeared to have distinct layers, especially in some segments (Fig. 4D). The difference in height was about 1.3–1.5 nm, the similar dimension as a single thickness of a peptide. Figure 4E–H shows the peptide scaffold hydrogel at various concentrations, 0.6–3 mM (1–5 mg/ml, w/v, or 99.5–99.9% water content) (Yokoi *et al.*, 2005). The scaffold hydrogel is completely transparent, which is a very important requirement for accurate image collections for uses in 3-D tissue cell cultures.

VII. DYNAMIC REASSEMBLY OF SELF-ASSEMBLING PEPTIDES

The self-assembling process is reversible and dynamic (Fig. 5). Since these peptides are short and simple, numerous individual peptides can be readily self-organized through weak interactions including hydrogen bonds, ionic

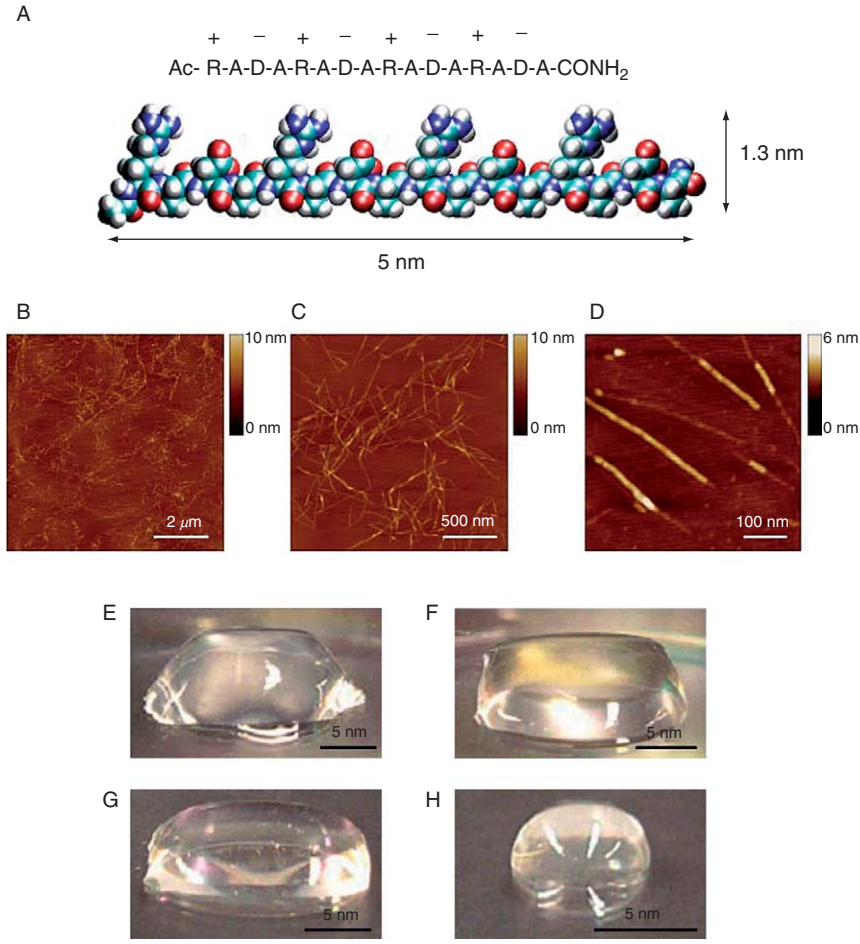

Fig. 4 Peptide RADA16-I. (A) Amino acid sequence and molecular model of RADA16-I, the dimensions are ~5-nm long, 1.3-nm wide, and 0.8-nm thick; (B) AFM images of RADA16-I nanofiber scaffold, 8 μm × 8 μm, (C) 2 μm × 2 μm (D) 0.5 μm × 0.5 μm. Note the different height of the nanofiber, ~1.3 nm, in D suggesting a double layer structure; Photographs of RADA16-I hydrogel at various conditions, (E) 0.5 wt% (pH 7.5), (F) 0.1 wt% (pH 7.5, Tris. HCl), (G) 0.1 wt% (pH 7.5, PBS) before sonication, (H) reassembled RADA16-I hydrogel after 4 times of sonication, respectively (images courtesy of Hidenori Yokoi). Reproduced from *Pnas* **102,** 8414–8419, 2005; Copyright (2005) National Acadamy of Sciences, USA.

bonds, hydrophobic and van der Waals interactions as well as water-mediated hydrogen bond formations. These nanofibers can be broken mechanically with sonication (Yokoi *et al.*, 2005). However, they can undergo dynamic reassembly repeatedly, similar as the material self-healing process (Fig. 5). Since the driving energy of the assembly in water is not only through hydrophobic van der Waals interactions but also through the arrays

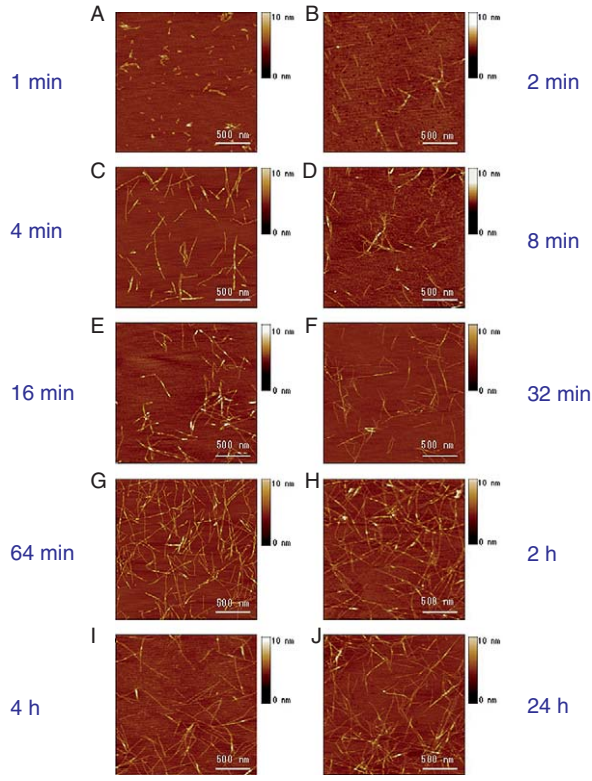

Fig. 5 AFM images of RADA16-I nanofiber at various time points after sonication. The observations were made using AFM immediately after sample preparation. (A) 1 min after sonication; (B) 2 min; (C) 4 min; (D) 8 min; (E) 16 min; (F) 32 min; (G) 64 min; (H) 2 h; (I) 4 h; and (J) 24 h. Note the elongation and reassembly of the peptide nanofibers over time. By~1–2 h, these self-assembling peptide nanofibers have nearly fully reassembled (images courtesy of Hidenori Yokoi). Reproduced from *Pnas* **102**, 8414–8419, 2005; Copyright (2005) National Acadamy of Sciences, USA.

of ionic interactions as well as the peptide backbone hydrogen bonds, this phenomenon can be further exploited for production and fabrication of many self-assembling peptide materials.

Unlike processed polymer microfibers in which the fragments of polymers cannot readily undergo reassembly without addition of catalysts or through material processing, the supramolecular self-assembly and reassembly event is likely to be widespread in many unrelated fibrous biological materials where numerous weak interactions are involved. Self-assembly and reassembly are very important properties for fabricating novel materials, and it is necessary to fully understand their detailed process in order to design better biological materials.

AFM images revealed that the nanofibers range from several hundred nanometers to a few microns in length before sonication. After sonication, the fragments were broken into ~20–100 nm. The kinetics of the nanofiber reassembly is followed closely at 1, 2, 4, 8, 16, 32, and 64 min as well as at 2, 4, and 24 h (Fig. 5). The nanofiber length reassembly is a function of time: by 2 h, the peptide nanofibers have essentially reassembled to their original length. This remarkable and rapid reassembly is interesting because there may be a little nucleation for regrowth of the nanofiber from the addition of monomers that could only be produced during sonication. It is plausible that a large population of the sonicated nanofiber fragments contains many overlap cohesive ends due to an undisrupted alanine hydrophobic side that may quickly find each other (Fig. 4D). The situation is analogous and commonly found in sonicated and enzymatic digested DNA fragments.

VIII. KINETICS OF NANOFIBER REASSEMBLY AND A PLAUSIBLE REASSEMBLY PROCESS

The reassembly kinetics is a function of time. Perhaps, similar to DNA reassembly, the reassembly largely depends on the concentrations of the short complementary fragments. In this case, the fragments are the sonicated peptide nanofibers with possible presence of sonicated monomers.

In order to understand the dynamic reassembly, we proposed a plausible sliding diffusion molecular model to interpret these observations of reassembly of the self-assembling RADA16-I peptides (Fig. 6). Unlike the left-handed helical structures observed in KFE8 (Marini *et al.*, 2002), a different self-assembling peptide, no helical structures were observed for RADA16-I using AFM and TEM (Gelain *et al.*, 2006; Holmes *et al.*, 2000).

For molecular modeling clarity, these RADA16-I β-sheets are presented as nontwisted strands. It is known that these peptides form stable β-sheet structure in water; thus they not only form the intermolecular hydrogen bonding on the peptide backbones but also have two distinctive sides, one hydrophobic with an array of overlapping alanines (Fig. 6, green color sandwiched inside), similar to that found in silk fibroin or spider silk assemblies (Pauling, 1961) and the other with negatively charged (−) aspartic acids, represented as red, and positively charged (+) arginines, represented as blue.

The alanines form packed hydrophobic interactions in water; during sonication the hydrophobic interaction could be disrupted mechanically. However, these hydrophobic cohesive ends could find each other quickly in water since the exposure of hydrophobic alanine arrays to water is energetically unfavorable. Since the hydrophobic alanines interaction is nonspecific, they can slide diffuse along the nanofiber, similar to trains on the train tracks. The same

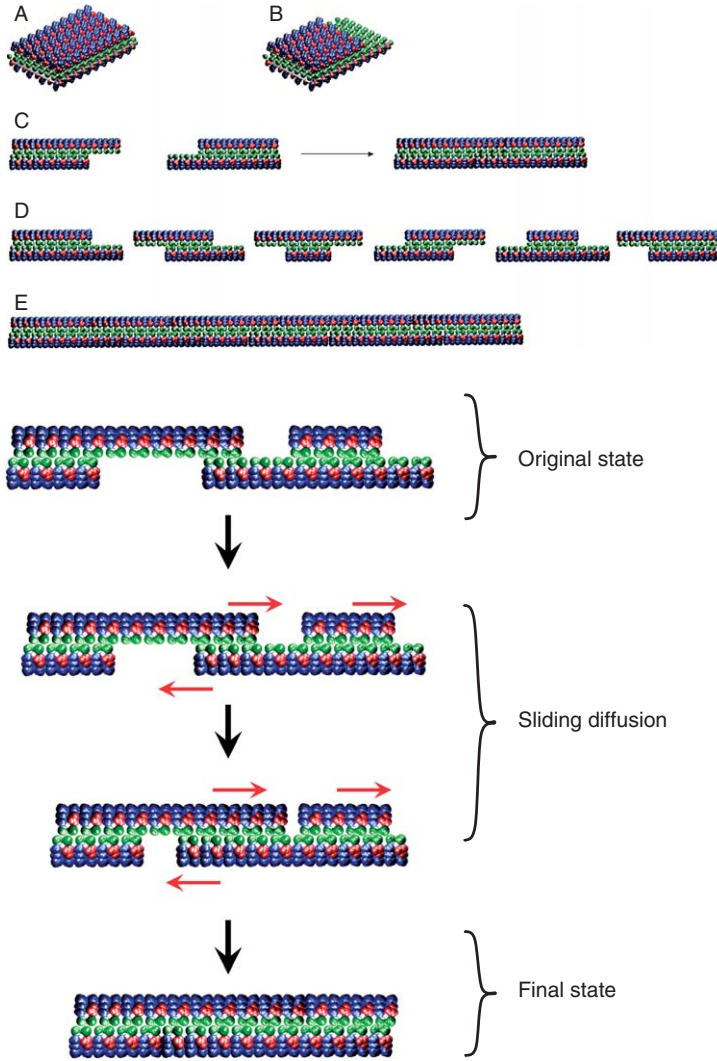

Fig. 6 A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling RADA16-I peptides. When the peptides form stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged aspartic acids and positively charged arginines. These peptides form antiparallel β-sheet structures. The alanines form overlap packed hydrophobic interactions in water, a structure that is found in silk fibroin from silkworms and spiders. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. These nanofiber fragments can form various assemblies similar to restriction-digested DNA fragments: (A) blunt ends; (B) semiprotruding ends. (C) These fragments with protruding ends could reassemble readily through hydrophobic interactions. (D) The fragments with semiprotruding

sliding diffusion phenomenon was also observed in nucleic acids where polyA and polyU form complementary base pairings that can slide diffuse along the chains (Felsenfeld *et al.*, 1957; Rich and Davies, 1956). If however, the bases are heterogeneous, containing G, A, T, and C, the bases cannot undergo sliding diffusion. Likewise, if the hydrophobic side of the peptides does not always contain alanine, such as valine and isoleucine, it would become more difficult for sliding diffusion to occur because of structure constraint.

On the charged side, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard manner (looking from the top). Likewise, collectively complementary + and − ionic interactions may also facilitate the reassembly. Similar to restriction-digested DNA fragments, these nanofiber fragments could form various assemblies: blunt, semiprotruding, and protruding ends. The fragments with semiprotruding and various protruding ends as well as those with blunt ends can reassemble readily through hydrophobic and ionic interactions.

IX. SELF-ASSEMBLING PEPTIDES NANOFIBER SCAFFOLD 3-D CELL CULTURE

The importance of nanoscale becomes obvious in 3-D cell culture. It is clearly visible in the SEM images that the cells are embedded in the self-assembling peptide nanofiber biological scaffolds in a truly 3-D culture (Fig. 7). Here, the cells and cell clusters intimately interact with the extracellular matrix where cells make on their own over time during cell growth and differentiation. Since the scaffolds are made of mostly water, ~99% water

and various protruding ends. (E) These fragments can reassemble readily. A proposed molecular sliding diffusion model for dynamic reassembly of self-assembling a single peptide nanofiber consisting of thousands of individual peptides. When the peptides form stable β-sheets in water, they form intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive sides, one hydrophobic with an array of alanines and the other with negatively charged aspartic acids and positively charged arginines. These peptides form antiparallel β-sheet structures. The alanines form overlap packed hydrophobic interactions in water, a structure that is found in silk fibroin from silkworms and spiders. On the charged sides, both positive and negative charges are packed together through intermolecular ionic interactions in a checkerboard-like manner. When the fragments of nanofiber first meet, the hydrophobic sides may not fit perfectly but with gaps. However, the nonspecific hydrophobic interactions permit the nanofiber to slide diffusion along the fiber in either direction, which minimizes the exposure of hydrophobic alanines and eventually fills the gaps. The sliding diffusion phenomenon was also proposed for nucleic acids of polyA and polyU in 1956 (Felsenfeld *et al.*, 1957; Rich and Davies, 1956). For clarity, these β-sheets are not presented as twisted strands. Color code: green, alanines; red, negatively charged aspartic acids; blue, positively charged arginines (images courtesy of Hidenori Yokoi). Reproduced from *Pnas* **102**, 8414–8419, 2005; Copyright (2005) National Acadamy of Sciences, USA.

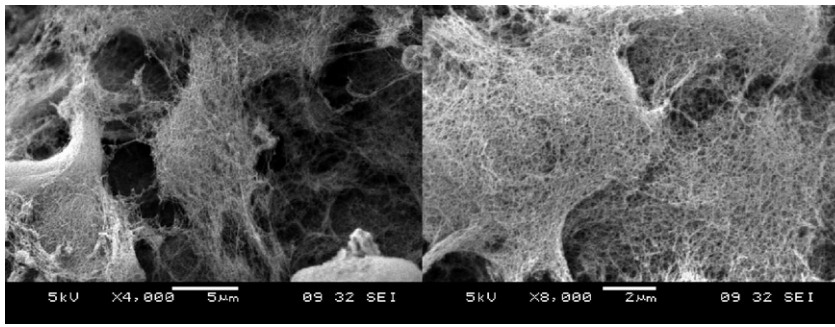

Fig. 7 Clusters of cells are fully embedded in the self-assembling peptide nanofiber scaffold. The scales of the nanofibers are similar to those of the extracellular matrices. Furthermore, the factors secreted from cells do not diffuse away quickly; thus a local concentration gradient could likely form, which is an absolute requisite for tissue development. Such 3-D cell clusters are nearly impossible to form on the 2-D petri dish and other 2-D culture systems. Likewise, several biopolymer microfibers commonly used in tissue engineering and regenerative medicine do not show such intimate cell–matrix interactions. Although there are similar cell–matrix interactions in Matrigel, it can never be used for human therapies. These cell clusters may be eventually form 3-D tissue over time under the appropriate conditions. There are few tissue examples that are shown in Fig. 5 (image courtesy of Fabrizio Gelain).

with 1% peptide, cells can migrate freely without hindrance, just as fish swim freely in a seaweed forest.

These new self-assembling peptide nanofiber biological scaffolds have become increasingly important not only in studying 3-D spatial behaviors of cells but also in developing approaches for a wide range of innovative medical technologies, including regenerative medicine (Fig. 8). One example is the use of peptide scaffolds to support neurite growth and differentiation, neural stem cell (NSC) differentiation, cardiac myocytes, and bone and cartilage cell cultures. The peptide scaffolds from RADA16-I and RADA16-II formed nanofiber scaffold in physiological solutions that stimulated extensive rat neurite outgrowth and active synapses formation on the peptide scaffold (Holmes *et al.*, 2000).

X. DESIGNER PEPTIDES SCAFFOLD 3-D CELL CULTURES

In a recent work, we directly and systematically compared NSC adhesion and differentiation on self-assembling RADA16-I scaffolds with other nature-based substrates including laminin, Collagen I, fibronectin, and some of the most commonly used synthetic biomaterials in tissue engineering such as poly-(DL-lactic acid), poly-(lactide-co-glycolide acid), and poly-(capro-lactone acid)

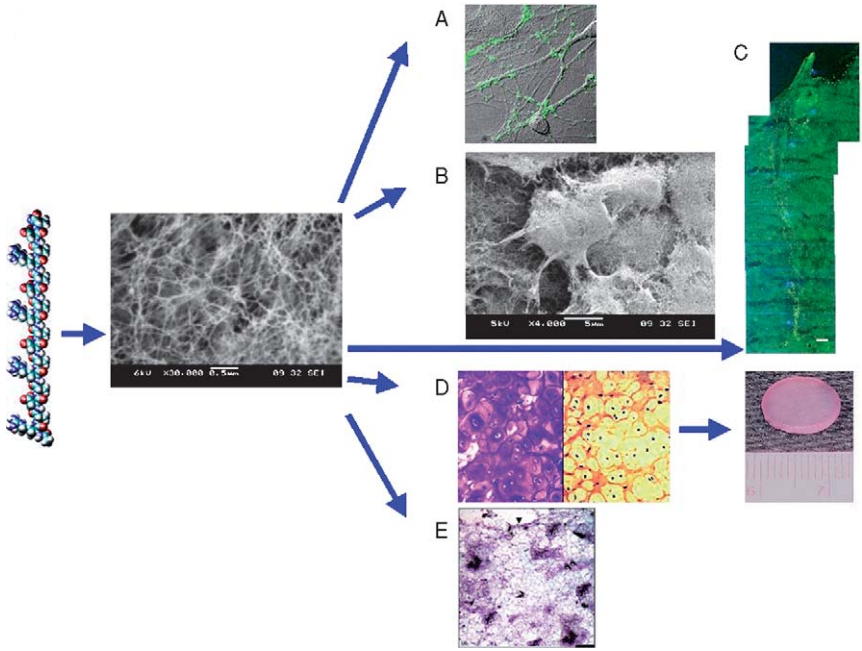

Fig. 8 From designer peptide to scaffold to tissues. (A) Active synapses on the peptide surface. Primary rat hippocampal neurons form active synapses on peptide scaffolds. The confocal images show bright discrete green dot labeling indicative of synaptically active membranes after incubation of neurons with the fluorescent lipophilic probe FM-143. FM-143 can selectively trace synaptic vesicle turnover during the process of synaptic transmission. The active synapses on the peptide scaffold are fully functional, indicating that the peptide scaffold is a permissible material for neurite outgrowth and active synapse formation. (B) Adult mouse NSC embedded in 3-D scaffold (image courtesy of Fabrizio Gelain). (C) Brain damage repair in hamster. The peptide scaffold was injected into the optical nerve area of brain that was first severed with a knife. The cut was sealed by the migrating cells after two days. A great number of neurons form synapses (image courtesy of Rutledge Ellis-Behnke). (D) Peptide KLD12 (KLDLKLDLKLDL), chondrocytes in the peptide scaffold and cartilage. The chondrocytes stained with TB showing abundant GAG production (left panel) and antibody to type II collagen demonstrating abundant type II collagen production (right panel). A piece of premolded cartilage with encapsulated chondrocytes in the peptide nanofiber scaffold. The cartilage formed over a 3–4-week period after the initial seeding of the chondrocytes (image courtesy of John Kisiday). (E) Von Kossa staining showing transverse sections of primary osteoblast cells on HA-PHP-RADA16-I self-assembling peptide nanofiber scaffold. Scale bar = 0.1 mm. The intensely stained black areas represent bone nodules forming (image courtesy of Maria Bokhari (Bokhari *et al.*, 2005)).

(Gelain *et al.*, 2007a). While nature-derived substrates showed the best performances, RADA16-I scaffold stimulated NSC differentiation and survival to a similar degree as did other synthetic biomaterials.

Although self-assembling peptides are promising scaffolds, they show no specific cell interaction because their sequences are not naturally found in

living systems. The next logical step is to directly couple biologically active and functional peptide motifs reported from a wealth of literature; accordingly the second generation of designer scaffolds will significantly enhance their interactions with cells and tissues.

The simplest way to incorporate the functional motifs is to directly synthesize them by extending the motifs onto the self-assembling peptides themselves (Fig. 9). The functional motifs are on the C-termini since peptide synthesis start from C-termini to avoid deletion during synthesis. Usually, a spacer comprising 2 glycines residues is added to guarantee a flexible and correct exposure of the motifs to cell surface receptors. Different functional motifs in various ratios can be incorporated in the same scaffold. Upon exposure to solution at neutral pH, the functionalized sequences self-assemble, leaving the added motifs on both sides of each nanofiber (Fig. 9). Nanofibers take part to the overall scaffold, thus giving microenvironments functionalized with specific biological stimuli (Fig. 9).

The self-assembling peptide scaffolds with functional motifs can be commercially produced at a reasonable cost. Thus, this method can be readily adopted for widespread use, including study of how cells interact with their local- and microenvironments, cell migrations in 3-D, tumor and cancer cells interactions with normal cells, cell processes and neurite extensions, cell-based drug screen assays, and other diverse applications.

We have produced different designer peptides from a variety of functional motifs with different lengths (Gelain *et al.*, 2006; Horii *et al.*, 2007). We showed that the addition of motifs to the self-assembling peptide RADA16-I did not inhibit self-assembling properties and nanofiber formations through mixing the modified peptides with the original RADA16-I. Although their nanofiber structures appear to be indistinguishable from the RADA16-I scaffold, the appended functional motifs significantly influenced cell behaviors.

Using the designer self-assembling peptide nanofiber system, every ingredient of the scaffold can be defined and, furthermore, can be combined with multiple functionalities, including the soluble factors. This is in sharp contrast with a 2-D petri dish where cells attach and spread only on the surface, whereas cells reside in a 3-D environment where the extracellular matrix receptors on the cell membranes can bind to the functional ligands appended to the peptide scaffolds. It is likely that higher tissue architectures with multiple cell types, rather than monolayers, could be constructed using these designer 3-D self-assembling peptide nanofiber scaffolds (A. Schneider *et al.*, unpublished results).

Even if only a fraction of functionalized motifs on the 3-D scaffold are available for cell receptor binding, cells may likely receive more external stimuli than when in contact with coated 2-D petri dishes or RGD-coated (or other motifs) polymer microfibers, which is substantially larger than the cell surface receptors and, in most cases, larger than the cell themselves. There cells are not in real 3-D; rather, they are in 2-D wrapping around the

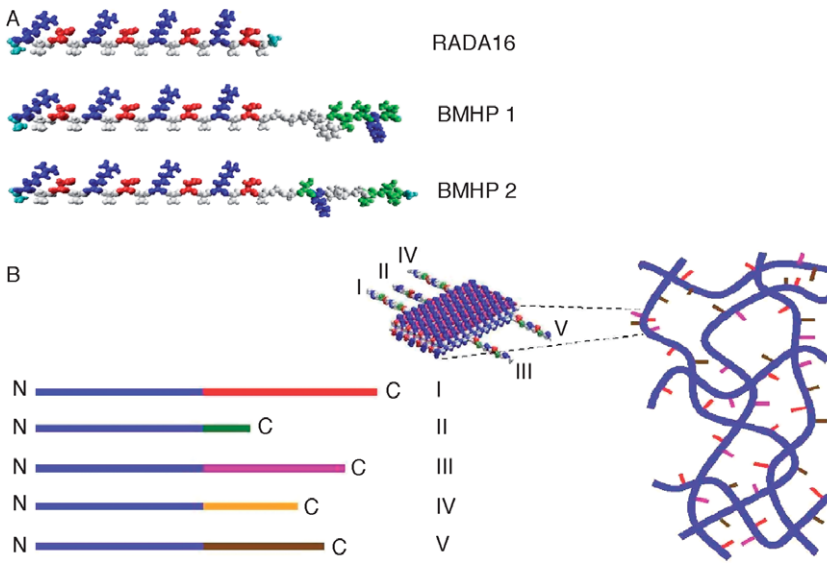

Fig. 9 Molecular and schematic models of the designer peptides and of the scaffolds. (A) Molecular models of RADA16, RADA16-Bone Marrow Homing Peptide 1 (BMHP1), and RADA16-Bone Marrow Homing Peptide 2 (BMHP2). RADA16 is an alternating16-residue peptide with basic arginine (blue), hydrophobic alanine (white), and aspartic acid (red). These peptides self-assemble once exposed to physiological pH solutions or salt. The alanines of the RADA16 providing hydrophobic interaction are on one side of the peptide, and the arginines and aspartates form complementary ionic bonds on the other. The BMHP1 and BMHP2 motifs were directly extended from RADA16 with two glycine spacers and are composed of a lysine (blue), serine and threonine (green), and different hydrophobic (white) residues. Neutral polar residues are drawn in green. (B) Schematic models of several different functional motifs (different colored bars) could be extended from RADA16 (blue bars) in order to design different peptides (I, II, III, IV, and V). They can be combined in different ratios. A schematic model of a self-assembling nanofiber scaffold with combinatorial motifs carrying different biological functions is shown.

micropolymers with a curvature depending on the diameter of the polymers. In a 2-D environment, where only one side of the cell body is in direct contact with the surface, receptor clustering at the attachment site may be induced; on the other hand, the receptors for growth factors, cytokines, nutrients, and signals are on the other sides that are exposed directly to the culture media. Thus cells may become partially polarized. In the 3-D environment, the functional motifs on the nanofiber scaffold surround the whole cell body in all dimensions and the factors may form a gradient in 3-D nano porous microenvironment.

In our search for additional functional motifs, we found that a class of bone marrow homing peptides (BMHP) (Gelain, *et al.*, 2006, 2007b) is one of the most promising active motifs for stimulating adult mouse NSC

adhesion and differentiation. This observation suggests a new class of designer self-assembling peptides for 3-D cell biology studies.

XI. DESIGNER PEPTIDE SCAFFOLDS FOR BONE CELLS AND 3-D MIGRATION

The designer self-assembling peptide nanofiber scaffolds have been shown to be an excellent biological material for 3-D cell cultures and capable of stimulating cell migration into the scaffold as well as repairing tissue defects in animals. We developed several peptide nanofiber scaffolds, designed specifically for osteoblasts (Horii *et al.*, 2007). We designed one of the pure self-assembling peptide scaffolds RADA16-I through direct coupling to short biologically active motifs. The motifs included osteogenic growth peptide ALK (ALKRQGRTLYGF), bone-cell secreted-signal peptide, osteopontin cell adhesion motif DGR (DGRGDSVAYG), and 2-unit RGD-binding sequence PGR (PRGDSGYRGDS). The new peptide scaffolds were made by mixing the pure RADA16-I and designer peptide solutions, and the molecular integration of the mixed nanofiber scaffolds was examined using AFM. Compared to pure RADA16-I scaffold, it was found that these designer peptide scaffolds significantly promoted mouse preosteoblast MC3T3-E1 cell proliferation. Moreover, alkaline phosphatase (ALP) activity and osteocalcin secretion, which are early and late markers for osteoblastic differentiation, were also significantly increased, thus demonstrating that the designer self-assembling peptide scaffolds promoted the proliferation and osteogenic differentiation of MC3T3-E1. Under the identical culture medium condition, confocal images unequivocally demonstrated that the designer PRG peptide scaffold stimulated cell migration into the 3-D scaffold (Fig. 10) (Horii *et al.*, 2007). Without the modified motif, cells did not migrate in 3-D.

XII. WHY DESIGNER SELF-ASSEMBLING PEPTIDE SCAFFOLDS?

One may ask why one should choose designer self-assembling peptide scaffolds while there are a large number of biomaterials on the market. The advantages of using the designer peptide nanofiber scaffolds are severalfold. (1) One can readily modify the designer peptides at the single amino acid level at will, inexpensively and quickly. This level of modification is impossible with Matrigel and other polymer scaffolds. (2) Unlike Matrigel, which contains unknown ingredients and quality that varies from batch to batch, the

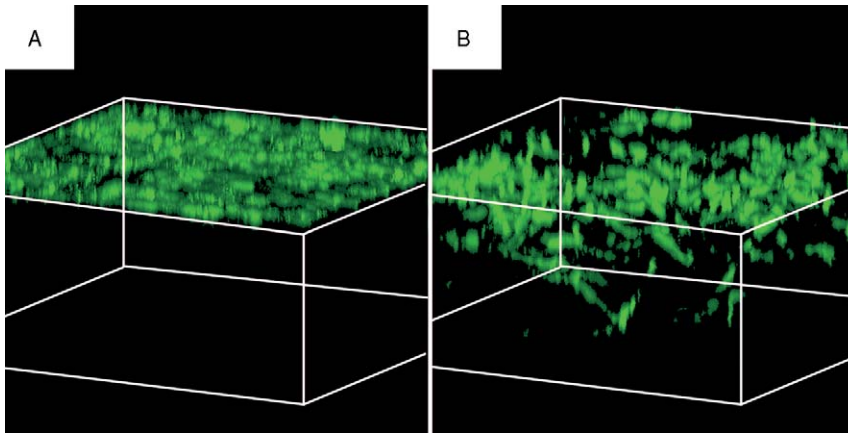

Fig. 10 Reconstructed image of 3-D confocal microscopy image of culturing on the different scaffolds consisting of different mix ratio of RADA16-I 1% (w/v) and PRG 1% (w/v) using calcein-AM staining. (A) PRG 10% and (B) PRG 70% of designer self-assembling peptide nanofiber scaffolds. The confocal images are horizontal view. There is a drastic cell migration into the scaffold with higher concentration of PRG motif (images courtesy Akihiro Horii).

designer self-assembling peptide scaffolds belong to a class of synthetic biological scaffolds that contains pure components and every ingredient is completely defined. (3) Because these designer peptide scaffolds are pure with known motifs, they can be used to study controlled gene expression or cell signaling process. Thus these new designer nanofiber scaffolds proved to be promising tools to study cell signal pathways in a selective way not possible with any substrates including Matrigel and collagen gels that result in confusing cell signaling activation. (4) The initiation of the self-assembly process is through the change of ionic strength at physiological conditions without temperature influence. This is again unlike collagen gels, for which gelation is through the change of temperature, which can sometimes induce unknown biological processes including cold or heat shocks. (5) These scaffolds provide the opportunity to incorporate a number of different functional motifs and their combinations to study cell behavior in a well-defined ECM-analog microenvironment, not only without any chemical cross-link reactions but also fully bio-reabsorbable scaffolds.

Although we have not studied cancer and tumor cells in our laboratory, others have carried out experiments for such studies. J. K. Park's group of Korea and colleagues reported using the peptide scaffold to study human hepatocellular carcinoma cells (Kim *et al.*, 2007). Ingemar Ernberg's laboratory in Karolinska Institute also used the peptide scaffold to study Human Hodgkin's lymphoma (Birgersdotter *et al.*, 2007). Lisa Spiro, then in Robert Weinberg's laboratory at the Whitehead Institute, also used the peptide scaffold to study

cancer cells [personal communication 2003]. So the time has come to study tumor and cancer cells using the designer self-assembling peptide nanofiber scaffold 3-D cell culture systems.

XIII. BEYOND 3-D CELL CULTURES

Researchers in neuroscience have a strong desire to study neural cell behaviors in 3-D and to fully understand their connections and information transmission (Edelman and Keefer, 2005). Beyond 3-D cell culture, since the building blocks of this class of designer peptide scaffolds are natural L-amino acids, the RADA16 has been shown not to elicit noticeable immune response nor inflammatory reactions in animals (Davis *et al.*, 2005, 2006; Ellis-Behnke *et al.*, 2006; Zhang *et al.*, 2005). The degraded products, amino acids, can be reused by the body and may also be useful as a bio-reabsorbable scaffold for neural repair and neuroengineering to alleviate and to treat a number of neurotrauma (Ellis-Behnke *et al.*, 2006) and neurodegeneration diseases.

In a recent work led by Richard Lee, mouse embryonic stem cells were suspended in RADA16-II peptide scaffold solutions and injected in the myocardium of 10-week-old mice (Davis *et al.*, 2005). In that study it has been demonstrated that self-assembling peptides can be injected into the myocardium to create a 3-D microenvironment. After 7, 14, and 28 days these microenvironments recruit both endogenous endothelial and smooth muscle cells, and exogenously injected cells survive in the microenvironments: self-assembling peptides can thus create injectable microenvironments that promote vascularization.

In addition, Lee's group also developed an appealing drug delivery strategy by using a biotinylated version of RADA-II to demonstrate a slow release of IGF-1 in infarctuated rat myocardia (Davis *et al.*, 2006). The biotin sandwich strategy allowed binding of IGF-1 and did not prevent self-assembly of the peptides into nanofibers within the myocardium. In conjunction with cardiomyocytes transplantation, the strategy showed that cell therapy with IGF-1 delivery by biotinylated nanofibers significantly improved systolic function after experimental myocardial infarction.

Ellis-Behnke and colleagues showed that self-assembling peptide material is a promising scaffold for neural regeneration medicine (Ellis-Behnke *et al.*, 2006). *In vivo* application to brain wounds was carried out using postnatal day-2 Syrian hamster pups. The optic tract within the superior colliculus (SC) was completely severed with a deep knife wound, extending at least 1 mm below the surface. At surgery, 10 animals were treated by injection of 10–30 μl of 1% RADA16/99% water, (w/v) into the wound. Control animals with the same brain lesion included 3 with isotonic saline injection (10 μl), numerous

additional cases, including 10 in which the dye Congo red was added into the peptide scaffold, and 27 earlier animals with knife cuts and no injection surviving 6–9 days. Animals were sacrificed at 1, 3, 6, 30, and 60 days for brain examinations. Histological specimen examinations revealed that only in the peptide scaffold-injected animals, but not in untreated animals, the brain tissue appears to have reconnected itself together at all survival times. Additionally, axons labeled from their retinal origin with a tracer molecule were found to have grown beyond the tissue bridge, reinnervating the SC caudal to the lesion. Most important, functional tests proved a significant restoration of visual function in all peptide scaffold-treated animals.

During the brain surgery experiments, Ellis-Behnke and colleagues found that the peptide nanofiber scaffold hydrogel could also stop bleeding in less than 15 s (Ellis-Behnke *et al.*, 2007). This is unlikely to be the conventional blood clogging mechanism because it takes place so rapidly. The molecular mechanism of speedily stopping bleeding remains to be uncovered. It is plausible that the nanofibers at the site quickly self-assembled into a dense mesh nanofiber network sponge that instantly blocked the rushing of the liquid. It may be perhaps nanomechanics rather than biochemistry.

XIV. CONCLUDING REMARKS

The development of new biological materials, particularly those biologically inspired nanoscale scaffolds mimicking *in vivo* environment that serve as permissive substrates for cell growth, differentiation, and biological function, is an actively pursued area that, in turn, could significantly advance regenerative medicine. These materials will be useful not only to further our understanding of cell biology in 3-D environment but also for advancing medical technology, regenerative biology, and medicine.

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Dendritic Cells in Cancer Immunotherapy

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Since their discovery, there has been significant progress in the understanding of dendritic cell (DC) biology. Their capacity for priming an immune response against pathogens and cancers has been exploited clinically. However, the objective responses obtained to date using DC cancer vaccines have been modest. Suboptimal DC preparations, limited tumor target antigens, and the essential need to initiate trials in immunocompromised patients with advanced disease, have all contributed to limited outcomes. The use of fully activated DCs, loaded with multiple, immunogenic, cancer-specific

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antigens, administered to patients with minimal residual disease and the manipulation of regulatory mechanisms underlying peripheral tolerance, may be the ingredients for future success.

I. DENDRITIC CELL BIOLOGY

Dendritic cells (DCs) are unique antigen presenting cells (APCs) that initiate and direct immune responses. They are present in virtually all tissues and interact with many cell types to link the innate and adaptive immune systems. There is an abundance of literature on murine DCs, with a number of distinct subsets that exert different functions. In contrast, there are relatively few studies on human DC subsets and their function. Mouse and human DC subsets are not homologous, making interspecies comparisons difficult. A greater understanding of human DC biology is essential to exploit these unique cells for immunotherapy.

A. Human DC Origin and Subsets

Human DCs are a heterogeneous cell population that originate from bone marrow precursors and are defined as leukocytes with high expression of major histocompatibility complex (MHC) antigens (notably Human Leukocyte Antigen (HLA)-DR) and a lack of other leukocyte lineage markers CD3 (T-cells), CD14 (monocytes) CD19/20 (B-cells), CD56 [natural killer (NK) cells] and CD34 (hematopoietic progenitors) (MacDonald *et al.*, 2002; O'Neill *et al.*, 2004). In human blood, DCs represent two major phenotypically and functionally distinct cell populations, the $CD11c^{+}$ myeloid population (MDC) and the $CD11c^{-}CD123^{high}$ $CD303^{+}$ (BDCA-2) plasmacytoid population (PDC). In the tissues, MDC include Langerhans cells that are generally found in the epidermis, oral-respiratory and genital mucosa, and the interstitial DCs present in other tissues. PDC reside mainly in the blood and lymphoid organs (Banchereau and Palucka, 2005; Radford *et al.*, 2005b).

Monocyte-derived DCs (MoDCs) can be generated *in vitro* by culturing monocytes in the presence of granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) or other cytokines. They exhibit many functional DC features, but there is only limited evidence that monocyte differentiation occurs in DC *in vivo*, although this may operate as an inflammatory boost pathway for APC production, as described in mice (Tacke and Randolph, 2006).

B. DC Function in Healthy Individuals

DCs are the most potent APCs of the body being the only cells capable of inducing naïve T-cell responses (Banchereau and Steinman, 1998; Hart, 1997; Steinman, 1991). Immature resident interstitial DCs migrate through

peripheral tissues and sample both foreign and self-antigenic material. The uptake of exogenous material can be achieved by (macro)pinocytosis or receptor-mediated phagocytosis or endocytosis (Banchereau and Palucka, 2005; O'Neill *et al.*, 2004). It is enzymatically degraded into peptides in endolysosomes and preferentially loaded onto membrane associated MHC class II molecules and recognized on the DC surface by $CD4^+$ T-cells. DCs can also process exogenously derived antigens through a cytosolic pathway, involving ubiquitination, cleavage into peptides by proteasomes and transport into the endoplasmic reticulum for binding to MHC class I molecules, a process called "cross-presentation" (Ackerman and Cresswell, 2004; Trombetta and Mellman, 2005). These complexes are recognized by $CD8^+$ T-cells. Endogenous antigens are also presented through this cytosolic pathway to MHC class I molecules.

Infection or local inflammation provides DC with the appropriate "danger" signals for further DC differentiation and activation. These signals include pathogen-associated molecular patterns (PAMPs) that are recognized by highly conserved pattern recognition receptors (PRR) on the DC surface such as toll-like receptors (TLR) and lectins. Other activating signals include components of dying cells and cytokines derived from activated macrophages, NK-cells, and T-cells. DCs will then acquire a more differentiated "mature" phenotype that involves a functional switch from antigen uptake to efficient interaction with and stimulation of various cells from the innate and adaptive immune system (Steinman and Hemmi, 2006). After an initial increase in antigen uptake and processing capacity, antigen uptake receptors and processing are downregulated and the DC chemokine receptors are modified.

Myeloid DCs migrate via the lymphatics to secondary lymphoid organs after upregulation of chemokine receptors CCR7 and CD62L, whereas PDC enter lymph nodes via lymphatic venules. Effective interaction with other cells of the immune system begins as DCs develop cytoplasmatic extensions and upregulate MHC class I and II molecules, T-cell costimulatory molecules (CD40, CD58, CD80, CD83, CD86, CD70, OX40L, 4-1BBL), and secrete cytokines and chemokines dependent on the DC subset (O'Neill *et al.*, 2004). Whereas MDCs produce a range of cytokines including IL-12, PDCs produce high amounts of type I interferon (IFN) and are often involved in antiviral immune responses (Banchereau and Palucka, 2005).

In secondary lymphoid organs, mature DCs will interact with both nonantigen-specific NK-cells, eosinophils and macrophages and antigen-specific B- and T-cells. Using cytokines and cell–cell-mediated molecular contacts, DCs can enhance NK-cell function including their antitumor cytotoxicity (Hamerman *et al.*, 2005; Munz *et al.*, 2005) and they enhance B-cell growth and differentiation (Dubois *et al.*, 1997). To prime naïve $CD8^+$ T-cells and induce cytotoxic T-lymphocytes (CTL), DCs must provide

three signals. First, the peptide–MHC complex on DCs must be recognized by the T-cell receptor (TCR); second, costimulatory molecules on DCs have to interact with their ligands on T-cells; and finally, activated DCs have to produce IL-12, or other stimuli, triggered by their interaction with $CD4^+$ T-helper cells, probably via CD40–CD40L interactions (Lee *et al.*, 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). The type of immune response generated is dependent on the type of DC and the signals it has encountered previously, the surface antigen concentration, the affinity of a TCR for the corresponding peptide–MHC, the duration of the DC/T-cell interaction and the inputs from the local lymph node (LN) environment (Gett *et al.*, 2003).

In the absence of microbial products or tissue damage, DCs will not differentiate equivalently after antigen uptake and probably undergo an "alternative activation" and as a result induce T-cell unresponsiveness and suppressive regulatory T-cells (Treg) (Matzinger, 1994; Rutella *et al.*, 2006). Natural cell death prevents DCs from inducing immune responses against self-antigens (Steinman *et al.*, 2000) and the majority of DCs migrate in an alternative "immature" state (Summers *et al.*, 2001), reinforcing peripheral tolerance or anergy. In the case of tumors, potential tolerance toward an evolving malignancy has to be overcome for effective immunotherapy.

C. DCs in Patients with Cancer

Patients with various malignancies, including breast cancer and multiple myeloma, show abnormalities in DC number and function (Fricke and Gabrilovich, 2006; Vuckovic *et al.*, 2004). Reduced DC counts in the peripheral blood of cancer patients have been associated with an accumulation of immunosuppressive immature myeloid cells (Almand *et al.*, 2001; Serafini *et al.*, 2004) or an alternative lineage negative, CD11c negative "gap" population of DC-like cells, as described first in our laboratory (Pinzon-Charry *et al.*, 2005), suggesting a defect in DC differentiation. Furthermore, functional deficiencies of tumor-infiltrating DCs (TIDCs) have been noted as well (Cochran *et al.*, 2006; Fricke and Gabrilovich, 2006). Through the production of cytokines and growth factors, tumor cells create an environment, which supports tumor growth and suppresses the host immune response. The latter effect operates at all levels but clearly compromises the initial events of DC antigen presentation and immune amplification, which can devastate the tumor immune response. For example, constitutive activation of Stat3, a common oncogenic signaling pathway in tumor cells, reduces the production of proinflammatory cytokines and chemokines (Wang *et al.*, 2004), but generates high levels of immunosuppressive cytokines like IL-10, transforming growth factor-beta (TGF-β) and

vascular endothelial growth factor (VEGF). These, in turn, may systemically influence DC hematopoiesis (Gabrilovich *et al.*, 1998), attract immature DCs and PDCs (Corinti *et al.*, 2001) to the tumor site, and influence DC function negatively by: (1) shortening DC survival through increased apoptosis, (2) impairing antigen uptake and presentation by inhibition of antigen processing machinery, (3) upregulating inhibitory molecules (indoleamine 2,3-dioxygenase (IDO), B7-H1 and B7-H4), (4) distorting DC differentiation, (5) downregulating costimulatory molecules (CD80/86 = B7.1 and 2), (6) impairing DC migration, and (7) cytokine production (Cochran *et al.*, 2006; Fricke and Gabrilovich, 2006). Taken together, these factors contribute to the lack of functionally activated MDCs and the presence of immature and "alternatively activated" DCs in the tumor environment and draining LNs.

A lack of activated DCs in breast (Iwamoto *et al.*, 2003) and prostate (Bigotti *et al.*, 1991) cancer patients has been associated with a poor clinical prognosis. Furthermore, spontaneously regressing melanomas in humans contain significantly more activated DCs than nonregressing tumors (Saleh *et al.*, 2005). It is therefore tempting to speculate that a lack of activated TIDCs results in insufficient CTL induction, which could partially explain the correlation between the number and functional state of TIDCs in cancer patients and clinical outcome (Bigotti *et al.*, 1991; Iwamoto *et al.*, 2003). Also the increased presence of immature DCs and "alternatively activated" DCs can further dampen antitumor immune responses by promoting the differentiation of naïve $CD4^+$ T-cells into immunosuppressive regulatory T-cells (Treg) (Rutella *et al.*, 2006). In contrast to the positive effect of activated MDCs, the presence of PDCs in ovarian cancer may be detrimental (Zou *et al.*, 2001) and their infiltration of primary localized breast tumors is associated with poor overall and relapse-free survival (Treilleux *et al.*, 2004). A possible underlying mechanism for this could be that PDCs upregulate inducible costimulator ligand (ICOS-L) during activation, resulting in the generation of IL-10 producing Treg, which adversely influence the antitumor immune response (Ito *et al.*, 2007).

Although tumor infiltrating and blood DCs from cancer patients have shown functional impairment, this seems to be reversible in multiple myeloma patients by the *ex vivo* addition of IL-12 or IFN-γ (Brown *et al.*, 2004) and functional blood DCs have been isolated from prostate cancer patients (Wilkinson *et al.*, 2006). Despite some contrary reports in hematological malignancies, MoDCs generated from cancer patients are functionally equivalent to those from healthy donors (Choi *et al.*, 1998; Fiore *et al.*, 2005; Vuillier and Dighiero, 2003) and they remain functionally active when injected back into the tumor environment (Triozzi *et al.*, 2000). However, steroids and other chemotherapeutics may compromise their function (Duperrier *et al.*, 2005).

In summary, DCs in cancer patients have both quantitative proportional and qualitative defects that are at least partially induced by tumor-derived factors. These can be overcome both *in vitro* and *in vivo*, making them very suitable for use in therapeutic cancer vaccines. It is a critical but underappreciated fact that this strategy requires that once activated appropriately *in vitro*, the injected DCs must retain their functional capacity *in vivo*.

D. The Immune Response in Cancer Patients

There is convincing evidence that both the innate and adaptive immune systems, particularly NK-cells and $CD8^+$ T-cells, but also $CD4^+$ T-cells, can recognize and kill tumor cells (Lanier, 2005; Nagorsen *et al.*, 2003). Clinically overt cancer indicates that the immune system has failed. Tolerance toward self-antigens, a dysfunctional immune system and tumor immunoselection all contribute to this failure. The paucity of danger signals from the tumor and the production of tumor-derived immunosuppressive cytokines, impair DC function and their ability to engage T-cells and other immune cells effectively. Even if CTL are induced, limited proliferation and migration to the tumor site and the presence of Treg and tumor cells expressing inhibitory factors may diminish their effectiveness. Defects in NK-cell function, as described in patients with advanced malignancies (Hadden, 1999), may also be part of the failed immune response against cancer. The failure to clear the tumor sets the stage for immunoselection, allowing tumor cells to become resistant to T-cell lysis by downregulating tumor antigens, molecules associated with antigen processing and presentation and death receptors (Zitvogel *et al.*, 2006).

The prospect of restoring DC function through selective tumor antigen loading and full activation motivated attempts to use DCs to boost antitumor immune responses for therapeutic benefit in cancer patients (Fig. 1). The balance between the ability of the stimulated immune system to recognize and eradicate tumor cells presenting overexpressed tumor-associated antigens (TAA), as opposed to damaging normal tissue, determines the "*therapeutic immune index*".

II. DCs AND THEIR USE IN IMMUNOTHERAPY

To generate an autologous DC vaccine *in vitro*, peripheral blood mononuclear cells (PBMC) are isolated from the patient by leukapheresis. Preformed blood DCs can then be selected directly or MoDCs can be generated from monocytes, loaded with TAA, and activated before injection back into the patient. Alternatively, mobilized $CD34^+$ progenitor cells can be used to

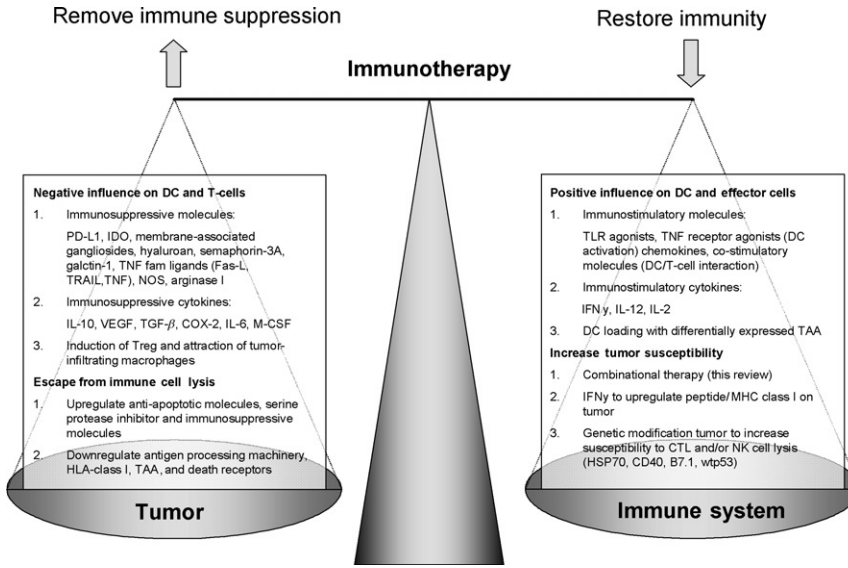

Fig. 1 The balance between the immune system and the tumor is influenced by immunotherapy. DCs play a key role in activating various effector cells of the immune system to recognize and kill tumor cells. However, tumor cells have evolved mechanisms to impair immune cell function and escape lysis through the expression and downregulation of certain molecules and cytokines. The goal of immunotherapy is to shift the balance to a functional immune system by fully activating DCs and effector cells, increasing tumor susceptibility to immune cell lysis and removing tumor-derived immunosuppressive factors.

generate DCs (Banchereau and Palucka, 2005; Banchereau *et al.*, 2005). Effective systemic CTL induction will only occur after DCs migrate to the draining LN to engage them in an appropriate microenvironment. CTL in their turn have to be able to find the tumor, penetrate the stroma, and survive the interaction with the tumor to destroy it (Fig. 2).

Extensive animal studies have shown that *ex vivo*-generated $CD34^{+}$-derived myeloid DCs loaded with TAA can induce both protective and therapeutic immunity against various malignancies (Banchereau and Palucka, 2005; Figdor *et al.*, 2004; Gilboa *et al.*, 1998). Furthermore, phase I clinical trials have shown that it is safe to inject autologous DCs loaded with overexpressed TAA and no severe autoimmune responses have been noted (Rosenberg *et al.*, 2004), apart from the anticipated vitiligo in melanoma patients (Mackensen *et al.*, 2000), validating the concept of a therapeutic immune index. However, the results make it clear that many variables need optimization before therapeutic human DC vaccines will be considered sufficiently efficacious and cost-effective for routine application.

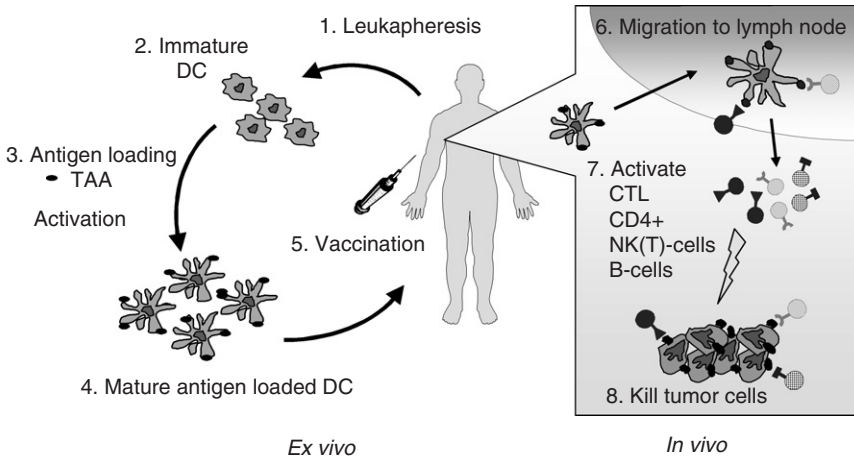

Fig. 2 Concept of DC immunotherapy. DCs are isolated or differentiated from leukapheresis products harvested from the patient (1, 2), loaded with TAA and activated *ex vivo* (3). They are then returned to the patient (5) where they migrate to the LNs (6). Here they induce the activation and proliferation of NK-cells and tumor-specific CTL, $CD4^+$, T-helper, and B-cells (7) that are capable of destroying tumor cells (8).

A. DC Type

MoDCs have been used most commonly in clinical trials to date. Traditionally, monocytes are differentiated in GM-CSF and IL-4, but recent data show that different cytokines like IL-13 (Morse *et al.*, 1999), IL-15 (Banchereau and Palucka, 2005; Mohamadzadeh *et al.*, 2001), IFN-α (Santini *et al.*, 2000), IL-3, and IFN-β (Buelens *et al.*, 2002) can skew DC differentiation toward a different phenotype and function that may increase their ability to induce CTL. Decreasing culture time (FastMoDC) from 5 to 2 days, may maintain their functional capacity (Tanaka *et al.*, 2006) but this approach has not been applied much in practice. A major concern regarding the use of MoDCs in vaccines is their limited migratory capacity *in vitro* (Luft *et al.*, 2002) and particularly *in vivo* (de Vries *et al.*, 2003a; Thomas *et al.*, 1999).

As an alternative, DCs can be generated from $CD34^+$ bone marrow or mobilized blood progenitors, but these, although very effective, also require *in vitro* culture in a cytokine mix for an extended period, limiting their widespread use (Hsu *et al.*, 2006; Palucka *et al.*, 2005). In direct comparison, $CD34^+$-derived DCs appear to be more effective than MoDCs in inducing CTL (Mortarini *et al.*, 1997).

In contrast to MoDCs and $CD34^+$-derived DCs, the "patient-manufactured" peripheral blood MDCs can be directly isolated in GMP-compatible

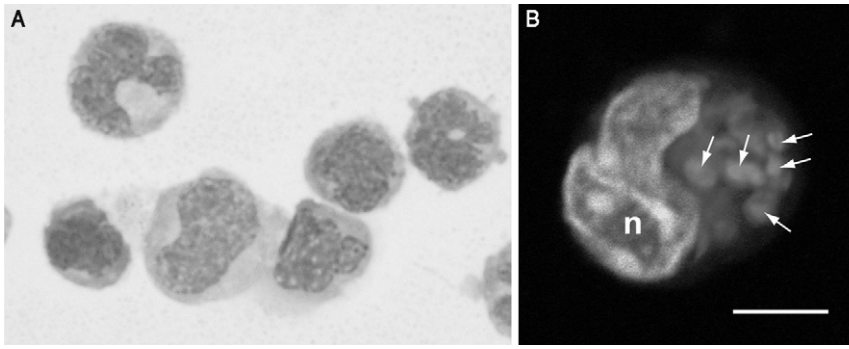

Fig. 3 (A) Clinical grade CMRF-56$^+$ selected MDCs. Cytospin preparation of CMRF-56 positive cells stained with Leishmans stain, visualized by oil immersion at 400×. (B) Liposome uptake by human MDCs. Human MDCs were exposed to DiI-labeled anti-huCD205 mAb liposomes and analyzed by laser scanning confocal microscopy. The nucleus (n) was stained with 4′,6-diamidino-2-phenylindole (DAPI) and the arrows indicate phagocytosed liposomes. The scale bar represents 5 μm. (DiI; fluorescent lipophilic dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.)

systems, which immunoselect on the basis of the DC surface antigens CD1c (BDCA-1) or CMRF56$^+$ (Fig. 3A) (Lopez *et al.*, 2003; Radford *et al.*, 2005a; Wilkinson *et al.*, 2006). *In vitro* data suggest that these cells have a higher migratory capacity and Th1-inducing capacity than MoDCs (Osugi *et al.*, 2002) and induce similar CTL responses (Radford *et al.*, 2006). We are currently treating prostate cancer patients with antigen-loaded CD1c$^+$ MDCs in a phase I clinical trial and the vaccine has now been administered without problems to six patients.

B. DC Differentiation and Activation

The necessity to use activated MoDCs in tumor vaccines was appreciated slowly. Immature DCs have been shown to turn off established CTL responses and only mature DCs migrate to secondary LN and stimulate naïve and memory T-cells compared to immature DCs (de Vries *et al.*, 2003a,b; Dhodapkar *et al.*, 2001). The most potent DC activation stimuli *in vivo* are pathogens or their components, but their safety for clinical use is still under debate (Jonuleit *et al.*, 2001). The current gold standard for *ex vivo* MoDC activation is a recombinant cytokine cocktail (CC), consisting of IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), and prostaglandin E_2 (PGE_2) (O'Neill *et al.*, 2004). PGE_2 increases CCR7 expression on MoDCs, thereby enhancing their migratory capacity toward draining LN. However, MoDCs matured with this cocktail did not secrete IL-12p70

(Kalinski *et al.*, 2001) and they upregulated IDO (Wobser *et al.*, 2006), which catalyzes the degradation of tryptophan, an essential amino acid for T-cells, thereby inhibiting their proliferation (Munn *et al.*, 2002). The addition of IFN-α, IFN-γ, and poly I:C, a synthetic mimic of viral RNA, resulted in increased IL-12p70 production and superior induction of anti-tumor immunity *in vitro* (Mailliard *et al.*, 2004). Advanced melanoma patients are presently being recruited in a clinical trial that compares the efficacy of maturing MoDCs in CC alone or combined with IFN-α, IFN-γ, and poly I:C (www.clinicaltrials.gov).

Only limited data address the optimal activation conditions for MDCs isolated from peripheral blood. Poly I:C was a potent stimulator of $CD11c^+$ MDCs *in vitro* in our hands (Radford *et al.*, 2006) and CMRF56 selected DCs induce CTL more effectively, when matured for 2 h with GM-CSF (Freeman *et al.*, in press). This also improves their migratory capacity and the short time required for their activation, reinforces the argument that once the logistics of their preparation are solved, their physiological responsiveness makes blood DCs attractive candidates for *ex vivo*-loaded DC vaccines.

An alternative approach is to mature DCs *in vivo* by treating the vaccination site with GM-CSF (Vuylsteke *et al.*, 2006) or a TLR agonist to enhance DC survival and their migratory capacity (Prins *et al.*, 2006). Imiquimod, a TLR7 agonist, is already applied topically in basal cell carcinoma and anogenital warts (Mizumoto *et al.*, 2005) and might be combined beneficially with DC vaccines.

When the tumor site is accessible, GM-CSF can be injected around the excised tumor site as well. This results in an increased number of mature MDCs in the draining LN of melanoma patients (Vuylsteke *et al.*, 2006). Also systemic GM-CSF administration during neoadjuvant chemotherapy was shown to increase the number of DCs in tumor draining LNs of breast cancer patients (Pinedo *et al.*, 2003). Alternatively, novel molecular adjuvants can, along with other strategies, improve DC activation status and will be discussed later in more detail.

C. Antigen Choice

Careful selection of the TAA on which to target a strong tumor-specific immune response is essential, not only for efficacy but also to minimize potential collateral tissue damage. An ideal TAA would only be expressed by tumor cells, its function should be essential for tumor cell survival and it should contain both $CD4^+$ T-helper cell and $CD8^+$ CTL epitopes for multiple MHC alleles. However to date, only cancer-testis antigens and a recently described group of phosphopeptides (Zarling *et al.*, 2006), generated by

altered signal transduction in malignant cells, approach these criteria. The recent discovery of cancer stem cells in hematological malignancies, brain and breast cancer (Jordan *et al.*, 2006) may help to identify specific markers for these cells and enable better targeting of therapy. For solid cancers such as breast and prostate cancer, only a few genuine TAA have been identified so far and the majority are overexpressed self-antigens as comprehensively summarized by Novellino *et al.* (2005) and on http://www.cancerimmunity.org/peptidedatabase/tumorspecific.htm. While it appears safe to inject autologous DCs loaded with these overexpressed self-antigens, it is difficult to induce high avidity CTL. Tissue specific breast and prostate autoantigens are also acceptable targets but these may not always generate high affinity CTL as deletional tolerance may have removed precursors from the patient's repertoire. Novel methods for TAA discovery are therefore needed urgently as current techniques are labor-intensive and success rates are very low (Viatte *et al.*, 2006). For this reason, our laboratory is developing a novel screening method that has the potential to identify new immunogenic TAA, irrespective of HLA-haplotype, from thousands of potential candidates.

The most commonly used approach in clinical trials has been to load DCs with short peptides (9–11 amino acids) predicted from whole TAA sequences with computer algorithms. These algorithms predict peptides sequences with high binding affinity for certain MHC alleles (HLA haplotype). The capacity of these peptides to induce an immune response has to be tested in the laboratory as well as their natural expression on tumor cells, before they can serve as useful antigens in a DC vaccine. Peptides are easy to generate in large quantities, their effectiveness has been proven in animal models and immune monitoring is easy with tetramers/pentamers. However, this "reverse immunology" approach to identify each TAA/HLA haplotype epitope is time-consuming and the use of HLA-restricted peptides limits the total number of patients that can be treated. Additional drawbacks are their potential limited efficacy *in vivo* due to transient expression of the MHC–peptide complexes on DCs and the absence of $CD4^+$ T-helper cell induction, required for maintaining $CD8^+$ T-cell responses (Muller *et al.*, 2003). In mice, vaccination with a longer peptide (35 amino acids) containing both a CD8- and CD4-epitope was more efficient in eradicating tumor than a conventional, short peptide (Zwaveling *et al.*, 2002). Ultimately, the production of whole TAA protein may even be seen worth the difficulties and cost involved, although methods to efficiently deliver protein to the cytosol will be necessary to ensure MHC class I presentation.

Bacterial or viral vectors, encoding a complete TAA, have been used in order to circumvent full TAA protein production and the necessity to identify both CD4 and CD8 epitopes. Incubating DCs with these vectors results in processing of whole TAA and DC activation is expected through TLR

triggering (Tan *et al.*, 2005). However, potential disadvantages are the risks associated with using infectious agents, the potential neutralizing effects of host viral or bacterial antibodies, and the upregulation of IDO on DCs after viral transduction (Su *et al.*, 2006).

Noninfectious methods have been developed to transfect DCs with whole tumor-derived antigens. DCs can be fused or incubated with apoptotic or necrotic tumor cells (Jenne *et al.*, 2000; Paczesny *et al.*, 2001), pulsed with tumor cell lysate (Fields *et al.*, 1998) or electroporated with tumor-derived whole tumor RNA (Nair *et al.*, 2000) or DNA (Tuting *et al.*, 1998) This allows selection of epitopes from a smorgasbord of potentially known and unknown TAA by host MHC class I and II molecules, which can be presented to $CD8^+$ and $CD4^+$ T-cells. Major restrictions are the limited amount of cancer tissue available from individual patients, the possible induction of autoimmune responses (Nestle *et al.*, 2005), and the preferential presentation of MHC class II epitopes by DCs predominantly inducing $CD4^+$ T-helper cells. The use of *in vitro* transcribed RNA for specific TAA (Gilboa and Vieweg, 2004), provides an opportunity to achieve defined GMP-compatible products from a minimal amount of cancerous tissue, in a less expensive GMP-compatible manner.

D. Antigen Loading

The methods for loading antigens into DCs depend in part on the antigen source and inevitably influence the vaccine's efficiency. The different techniques include passive pulsing, electroporation, and receptor-mediated uptake. The more efficient the loading technique, the higher the MHC–peptide density will be on the DC surface, which has in turn been correlated with the degree of CTL activation (Motta *et al.*, 1998; Wong *et al.*, 1998). However, excessive antigen density on DCs without sufficient costimulation can result in activation-induced T-cell death (Hopken *et al.*, 2005). Therefore, it is important that DCs are activated appropriately and the method and timing of activation need to be optimized for the particular loading technique used. Immature DCs are used when antigen uptake and processing are still required for passive pulsing with tumor lysate followed by an activation step, whereas DCs, which are to be pulsed with HLA class I binding peptides, can be activated first.

E. Vaccine Administration

The route of DC vaccine administration is critical because it will influence DC migration to draining LNs and therefore their ability to induce an immune response. Mice experiments showed that subcutaneously (s.c.) injected DCs

preferentially migrated to LN and induced effector and memory T-cells (Eggert *et al.*, 1999), whereas intravenously (i.v.) injected DCs migrated to liver and spleen (Mullins *et al.*, 2003). Moreover, in two clinical trials, superior T-cell responses were observed after intradermal and intranodal vaccine injection compared to i.v. injection (Bedrosian *et al.*, 2003; Fong *et al.*, 2001). Other clinical trials have shown that although T-cell responses are induced, only 0.5–2% of intradermally injected mature DCs reach the draining LN (de Vries *et al.*, 2003a; Martin-Fontecha *et al.*, 2003). This can be improved by treating the injection site with TNF-α (Martin-Fontecha *et al.*, 2003), TLR-agonists (Nair *et al.*, 2003b), or matrix metalloproteinases prior to vaccination (Figdor *et al.*, 2004).

Alternatively, antigen-loaded DCs can be injected directly into the tumor (Crittenden *et al.*, 2005). For example, genetically modified DCs significantly inhibited tumor growth and caused tumor protection in murine breast, prostate, and colon models when injected this way (Shurin *et al.*, 2006). Obviously, this approach is restricted to tumor sites that are accessible for injection and even then the ultrasound directed injection has proven fallible. This reason plus the associated logistical problems will limit its usefulness in clinical practice.

The DC administration regimen namely, cell numbers and schedule, is expected to play a role in the vaccine's clinical efficacy. Once a minimum cell dose is achieved, there is as yet no clear evidence of a cell dose effect. Likewise it is now assumed, but there is no formal data to support it, that ongoing boost injections may be required after the priming injections. Different vaccine formulations for priming and boosting may even be useful (Ali *et al.*, 2007). This highlights one of the main issues for the field. It is difficult to address the therapeutic significance of all variables scientifically in comparative small phase I studies, so preclinical humanized animal models deserve much more attention. Larger phase III studies will need to test well researched DC vaccine preparations or disappointing results are likely to occur (Schadendorf *et al.*, 2006).

F. Immunological Responses

Several techniques have been developed to monitor the immunological effects of DC vaccination in patients. Their aim is to measure the number and functionality of tumor-reactive T-cells by using either the pentamer assay, the IFN-γ ELISPOT, or the ^{51}Cr release assay (Comin-Anduix *et al.*, 2006; Michel *et al.*, 2002). However, these T-cell responses are only monitored readily in peripheral blood, whereas tumor-reactive T-cells may well be preferentially located in bone marrow (Feuerer *et al.*, 2001) or tumor-draining LN (Slingluff *et al.*, 2004). A possible way to overcome this

limitation may be to quantitate the tumor-specific T-cells in a skin biopsy after a delayed type hypersensitivity (DTH) test (de Vries *et al.*, 2005). Conventionally, this test consists of the intradermal injection of antigen-loaded DCs as used for prior vaccination followed by measuring skin induration. However, both loaded and unloaded autologous DC injections induce skin induration in melanoma patients after vaccination (de Vries *et al.*, 2005). Only when antigen-specific T-cells were isolated from these DTH biopsies, were specific responses documented, which correlated with clinical outcome (de Vries *et al.*, 2005). It will assist the field considerably, if surrogate immunological responses rather than clinical responses can direct vaccine development.

G. Clinical Responses

To date, more than 160 clinical trials have been reported using DC vaccines in patients with various hematological and solid malignancies, including multiple myeloma, leukemia, melanoma, renal cell carcinoma, gastrointestinal, breast and prostate cancer. Although surrogate immunological responses can be detected in many patients after DC vaccination, overall clinical response rates are much lower. Clearly, the detection of tumor-specific CTL in peripheral blood does not indicate whether these cells reach tumor sites and maintain their cytolytic function and as indicated earlier, tumor escape from the immune response is now a well documented phenomenon (Lee *et al.*, 1999; Offringa, 2006; Zippelius *et al.*, 2004). The latter phenomenon alone argues strongly for DC therapy to be applied early in states of minimal residual disease.

Rosenberg *et al.* (2004) reported a 3.3% objective clinical response rate in 1205 cancer patients treated with a variety of cancer vaccines. The majority of treated patients were diagnosed with melanoma. An objective response was defined as a minimal reduction of 50% in the sum of the products of the perpendicular diameters of all malignant lesions, without 25% growth of any lesion or the appearance of new lesions (WHO response criteria). Nine of 101 melanoma patients (8.9%) vaccinated with peptide- or lysate-loaded DCs had a clinical response compared to 7/196 (3.6%) patients vaccinated with peptide only and none after recombinant virus expressing a melanoma antigen. This might suggest a superior effect for antigen-loaded DC vaccination in melanoma patients. There are several summaries of vaccine clinical trials in solid cancers and hematological malignancies (Choudhury *et al.*, 2006) and our website contains an overview of all DC-based vaccine clinical trials (http://www.mmri.mater.org.au, navigate via the menu to “research” and “view clinical trials table”).

Of the 55 breast cancer patients treated with a DC vaccine to date, 11 showed (transient) regression of tumor metastasis. However, the percentage of tumor regression and appearance of new lesions has not been described in detail, making it difficult to evaluate objective clinical response rates according to WHO or RECIST criteria (Gehan and Tefft, 2000). Furthermore, it is debatable whether the clinical data can be combined, when different vaccine formulation and administration schedules have been used. From the 402 prostate cancer patients treated with a DC vaccine, 29 (7.2%) showed a partial response, defined as a minimal reduction in PSA of 50% or significant regression of bone metastasis or according to the National Prostate Cancer Project (NPCP) criteria. Small *et al.* showed a 4.5 month improvement of overall survival in metastatic prostate cancer patients treated with a DC vaccine (Small *et al.*, 2006).

Overall, DC vaccines are safe but require careful design and validation if they are to induce an efficient and long lasting immune response and justify the expense and the potential opportunity cost of a clinical trial. DCs must have the correct differentiation/activation status, a high migratory capacity, present sufficient, multiple, tumor-specific antigens, and induce high avidity, tumor-specific CD8 effectors and CD4 T-helper cells. Tumor-specific $CD8^+$ T-cells must be able to migrate to the tumor tissue, overcome the suppressive effects of the tumor microenvironment, and kill malignant cells. Persisting $CD4^+$ T-helper cells are also required to maintain a memory T-cell response.

III. FUTURE DC VACCINES

A greater understanding of DC biology will allow conventional DC vaccines to be improved. Undoubtedly, their activation and migration status will be enhanced with a combination of novel molecular adjuvants as described below. Additional cotransduction with immunostimulatory molecules (Kaufman, 2005) or the knockdown of genes encoding inhibitory molecules may advance their efficacy (Mao *et al.*, 2006). Ultimately, it may prove feasible and more practical to target DCs directly *in vivo* by fusing TAA to DC surface molecules (Proudfoot *et al.*, 2007). Besides intrinsic DC vaccine optimization and better TAA targets, patient selection and the timing of vaccination will play a crucial role in achieving better objective clinical response rates in cancer patients. Patients with minimal residual disease are not yet immunocompromised and the combination of a vaccine with other adjuvant treatments in these patients will be essential to overcome peripheral tolerance toward their tumor. Other immunotherapies and conventional adjuvant treatment regimes can potentiate DC vaccines by locally increasing target antigen availability, inducing inflammation, and

inhibiting immunosuppressive Treg and cytokines. As most prospects for improvement are evolved in mouse studies, the findings will need to be validated in human *in vitro* models, in humanized mice models and ultimately in phase I clinical trials before formal validation in phase III trials.

A. DC Activation

Superior activation and immunostimulatory cytokine profiles have been noted in DCs treated with various combinations of novel molecular adjuvants including TLR agonists, TNF receptor superfamily (TNFRS) agonists, and chemokine/cytokine receptor agonists, as reviewed by Kombluth *et al.* (Kornbluth and Stone, 2006). In mice, activation of peptide-loaded BMDC with a combination of TLR-3 and TLR-7 ligands induced superior CTL responses *in vivo* (Warger *et al.*, 2006) and combined TLR/CD40 stimulation resulted in superior $CD8^+$ T-cell expansion and memory T-cell formation in mice through upregulation of CD70 on DCs (Sanchez *et al.*, 2007). However, the majority of studies are performed on BMDCs from mice, which have a different TLR expression repertoire to human DCs. Even triggering the same receptor in different species can lead to different functional outcomes and preclinical evaluation in man is mandatory.

B. DC Cotransduction

A way to overcome the immature and tolerogenic state of DCs is to genetically modify them by transduction with viral vectors expressing both TAA and chemokines, immunostimulatory cytokines, costimulatory molecules, or a combination (Kaufman, 2005; Kikuchi, 2006). However, these vectors might need optimization as their use is associated with IDO expression on DCs (Tan *et al.*, 2005) and considerable GMP-related issues might complicate their clinical use.

Chemokine-transduced DCs have been shown to attract higher numbers of T-cells for interaction in the draining LN (Nukiwa *et al.*, 2006). Murine DCs virally transduced with TAA and lymphotactin/XCL1, secondary lymphoid tissue chemokine (SLC)/CCL21, monokine induced by IFN-γ (Mig)/CXCL19 or fractalkine/CX3CL1 induced more potent antitumor immune responses in tumor-bearing mice than DCs only presenting TAA (Kaufman, 2005; Kikuchi, 2006).

Costimulatory molecules and cytokines expressed by DCs are crucial for naïve T-cell activation. An increased expression of the human costimulatory molecules B7.1, a combination of B7.1, intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function-associated antigen (LFA-3) (TRICOM), or receptor activator of NF-kappaB (RANK-RANKL) by DC augmented T-cell responses in murine tumor models (Kaufman, 2005; Kikuchi, 2006).

Also intratumorally injected BMDCs painted with a combination of SLC, 4-1BBL (a member of the TNFRS), and tumor necrosis factor-related activation-induced cytokine (TRANCE) showed superior migration and systemic antitumor immune responses in mice (Liu *et al.*, 2007). DCs transfected with a fowlpox vector expressing CEA and TRICOM were safely administered to colon cancer patients (Morse *et al.*, 2005).

Cotransduction of DCs with IL-12 (Nishioka *et al.*, 1999), TNF-α (Chen *et al.*, 2002), IL-7, IFN-β (Sharma *et al.*, 2003; Shibata *et al.*, 2006), IL-23 (Hu *et al.*, 2006), or a combination of IL-12 and IL-18 or GM-CSF (Iinuma *et al.*, 2006; Ojima *et al.*, 2006; Tatsumi *et al.*, 2003) all showed enhancement of antitumor immune responses in various murine tumor models. Modification of DCs to express CD40L triggered their activation and IL-12 production, without the need for $CD4^+$ T-cell help, both in preclinical studies (Kikuchi, 2006; Kikuchi *et al.*, 2000) and a clinical study (Murphy *et al.*, 2005; Wierda *et al.*, 2000). Increased potency of DC vaccines in mice was also achieved when coadministered with cytokines such as IL-2, IL-15, and IL-21 (He *et al.*, 2006), but toxicity may limit cytokine use in patients (Blattman and Greenberg, 2004). However, systemic side effects might be reduced by the fusion of cytokines to tumor-specific monoclonal antibodies (mAb) (Nissim *et al.*, 2004). IFN-α has demonstrated efficacy in patients with renal cell carcinoma and chronic myeloid leukemia (Blattman and Greenberg, 2004) and IL-2 may be synergistic with vaccination in melanoma and breast cancer patients (Chianese-Bullock *et al.*, 2005; Svane *et al.*, 2007). One current clinical trial looks at the possible synergistic effect of combining antigen-loaded DC vaccination with IL-2 and TNF-α in patients with renal cell carcinoma (www.clinicaltrials.gov).

Besides the expression of appropriate molecules for T-cell attraction and stimulation, increased DC survival can also contribute to enhanced T-cell interactions. It has been reported that DC survival can be prolonged after transduction with antiapoptotic genes such as bcl-xl, bcl-2, X-linked inhibitor of apoptosis, and dominant-negative caspase-8/9 (Kim *et al.*, 2003). Mice injected intradermally with DNA encoding for the model antigen E7 and the antiapoptotic protein bcl-xl showed increased E7 specific DCs in draining LN and higher numbers of E7 specific CTL (Kim *et al.*, 2003).

Taken together, these data suggest that DC survival and immunostimulatory function can be improved in current vaccines by transducing them with a combination of immunostimulatory and antiapoptotic genes.

C. DC Gene Silencing

Another novel strategy is the use of small interfering RNA (siRNA) to silence gene expression in DCs. DCs transfected with siRNA encoding the proapoptotic proteins BAK and BAX, show prolonged survival and induce

strong antitumor immune responses in mice (Kang *et al.*, 2007). This technology may also be very promising for the knockdown of genes encoding inhibitory molecules or immunosuppressive cytokines thereby enhancing DC function (Mao *et al.*, 2006).

The suppressor of cytokine signaling 1 (SOCS 1) attenuates signals through the (Janus kinase) JAK/STAT (signal transducer and activator of transcription 3) pathway thereby controlling DC cytokine production and the extent of the antigen presentation process (Evel-Kabler and Chen, 2006). SOCS 1 silenced DCs pulsed with antigen are superior at inducing CTL in mice than untreated antigen-loaded DCs (Shen *et al.*, 2004) and they can break self-tolerance in combination with TLR-ligands by fully activating low-avidity, self-reactive T-cells (Evel-Kabler *et al.*, 2006). This may be useful for improving human DC vaccine efficacy, but antigens need to be chosen carefully to prevent severe autoimmune responses.

D. Targeting DCs *In Vivo*

Instead of *ex vivo* generation and loading of DCs, which can be complicated by regulations and is expensive on a large scale, DCs can be directly targeted *in vivo* by using TAA fused to DC-receptor specific antibodies like anti-FcγR or anti-CD205 (DEC205) (Bonifaz *et al.*, 2004; Proudfoot *et al.*, 2007). Antigens can also be loaded into liposomes expressing anti-DEC205 antibody and directly injected into patients for specific uptake by blood DCs (Fig. 3B. Badiee *et al.*, 2007).

An alternative strategy is to expand DCs *in vivo* with Flt3 ligand or G-CSF (Maraskovsky *et al.*, 2000; Pulendran *et al.*, 2000), followed by vaccination with TAA and a DC activating agent such as CpG oligodenucleotides. This strategy induced potent antitumor immune responses in three different mice tumor models (Okano *et al.*, 2005). *In vivo* expanded DCs can be attracted directly to the tumor site by genetically modifying tumors to express chemokines (Furumoto *et al.*, 2004) and/or DC maturing cytokines like GM-CSF (Hege *et al.*, 2006). An adenovirus expressing chemokines and cytokines can also be directly injected into the tumor. However, this approach is, once again, only suitable for tumors that are readily accessible for injection such as melanoma.

E. Patient Selection and Timing of Vaccination

Proper patient selection prior to treatment will also have a critical impact on clinical outcome. The general opinion is that DC vaccines will potentially be more effective in preventing disease recurrence in patients with minimal

residual disease than for eradicating bulk disease. A high tumor burden, an immunocompromised status and limited time for the generation of antitumor immune responses are all significant obstacles and in this context the clinical responses obtained to date are relatively encouraging.

Critically, a few phase III studies have shown improved progression-free survival rates for patients with minimal residual disease, who were vaccinated solely with peptide, killed tumor cells or shed antigens (Apostolopoulos *et al.*, 2006; Bystryn *et al.*, 2001; Jocham *et al.*, 2004). Recently, for the first time, early stage melanoma patients (no metastasis in LN or at distant sites) have been vaccinated with MART-1(MelanA)-loaded DCs shortly after surgery. The majority of patients still showed DTH reactivity against MART-1 in their skin 1 year after completing vaccination. They also had superior CTL responses compared to similarly treated stage IV melanoma patients in prior studies from the same research group (Tuettenberg *et al.*, 2006). At the time of publication, being 2–3 years after diagnosis, 10/13 vaccinated patients were still tumor free. Nevertheless, high patient numbers and long follow-up periods are needed to provide enough statistical power to determine efficacy of preventing cancer recurrence. However, regulations are strict and resources scarce for employing trained staff and setting up the GMP facilities necessary for the production of a cellular vaccine. Therefore, the majority of clinical trials can only include low patient numbers.

In two separate current clinical trials, patients with early stage breast cancer are being vaccinated with Her2-neu-loaded DCs prior to surgery or with a p53-loaded DC vaccine combined with adjuvant chemotherapy and radiotherapy (www.clinicaltrials.gov).

Apart from the potential benefit of treating patients with minimal residual disease, the timing of DC vaccination should be considered carefully. A rise of circulating tumor cells in peripheral blood has been reported when tumors are mechanically manipulated, for example, during breast cancer surgery (Galan *et al.*, 2002; Pachmann, 2005) or colonoscopy (Koch *et al.*, 2004). These data and the association of occult circulating tumor cells in the peripheral blood of various cancer patients with poorer overall survival (Guller *et al.*, 2002; Jotsuka *et al.*, 2004; Masuda *et al.*, 2005; Muller *et al.*, 2005), might suggest that patients need to receive their first DC vaccination even prior to surgery. Furthermore, it has been shown that currently used adjuvant therapies like chemotherapy and radiotherapy can increase the potency of DC vaccines in tumor-bearing mice (Casares *et al.*, 2005; Eralp *et al.*, 2004; Yu *et al.*, 2003). Combining conventional adjuvant therapy and other immunotherapies with DC vaccination might be more effective than treating patients sequentially.

Although still futuristic, screening cancer patients for their immune response genotype might be a consideration for directing individualized protocols in future clinical trials. Polymorphisms in genes encoding HLA

and cytokines have been associated with susceptibility for cancer and response to treatment (Baccar Harrath *et al.*, 2006; Basturk *et al.*, 2006; Gonzalez-Zuloeta Ladd *et al.*, 2007; Halma *et al.*, 2004; Liu *et al.*, 2005; McCarron *et al.*, 2002; Wu *et al.*, 2005). For example, improved overall survival was observed in patients expressing HLA-A2 and/or HLA-C3, receiving an allogeneic melanoma vaccine after melanoma resection (Sondak *et al.*, 2002). A subgroup analysis of HLA-$A2^+$/HLA-B44-melanoma patients revealed improved survival after autologous DC vaccination compared to patients with other haplotypes (Schadendorf *et al.*, 2006). This might reflect that certain HLA haplotypes present more immunogenic peptide epitopes than others. In addition, it has been reported that IFN-γ gene polymorphisms influence clinical outcome of melanoma patients, receiving chemotherapy combined with IL-2 and IFN-α (Liu *et al.*, 2005). However, further research is needed to establish underlying mechanisms for these findings and these results have to be interpreted with caution, given the limited sample sizes reported.

F. DC Vaccines Combined with Other Immunotherapies

1. DC VACCINE AND ADOPTIVE T-CELL TRANSFER

Adoptive transfer of autologous *ex vivo* expanded tumor-infiltrating T-cells into cancer patients has led to transient antitumor immune responses (Dudley *et al.*, 2005). When combined with nonmyeloblative lymphodepleting chemotherapy, 8.6% of metastatic melanoma patients showed complete responses and 43% showed partial responses with a mean duration of 11.5 months (Dudley *et al.*, 2005). This improvement was most likely achieved by the elimination of immunosuppressive cells such as Treg and myeloid suppressor cells and homeostatic proliferation of adoptively transferred T-cells due to decreased competition for important cytokines like IL-7 and IL-15 (Gattinoni *et al.*, 2006a). The maintenance of antitumor immune responses is clearly still limited and can potentially be improved when adoptively transferred T-cells are combined with a DC vaccine. In a few murine studies significantly higher proliferation and persistence of adoptively transferred T-cells was noted together with their selective migration to tumor sites when combined with DC vaccination (Jiang *et al.*, 2006; Lou *et al.*, 2004).

2. DC VACCINE AND REGULATORY T-CELL DEPLETION

Multiple regulatory mechanisms have evolved that induce peripheral tolerance to self-antigens and control autoimmunity including naturally occurring Treg. There is increasing evidence that naturally occurring Treg ($CD4^+CD25^+$CTLA-$4^+$$Foxp3^+$$GITR^+$) and *de novo*-generated tumor-specific Treg, induced by immature or alternatively activated DCs, play an

important and independent role in suppressing antitumor immune responses in cancer patients (Wang, 2006; Zhou and Levitsky, 2007). They inhibit the cytolytic function of antigen-specific $CD8^+$ T-cells through contact-dependant mechanisms and TGF-β signaling (Chen *et al.*, 2005; Kabelitz *et al.*, 2006). A few studies have shown an increased number of Treg in the peripheral blood and tumor environment of breast and prostate cancer patients (Liyanage *et al.*, 2002; Miller *et al.*, 2006) and their presence within the tumor is inversely correlated with patient survival (Curiel *et al.*, 2004; Hiraoka *et al.*, 2006; Kono *et al.*, 2006). Therefore, attempts have been made to systemically block or eliminate these Treg cells by the use of cyclophosphamide (Ghiringhelli *et al.*, 2004; Lutsiak *et al.*, 2005; North, 1984), anti-CD25 antibodies (Kohm *et al.*, 2006), IL-2-toxin chimeric proteins (Dannull *et al.*, 2005) or glucocorticoid-induced TNF-like receptor (GITR) (Calmels *et al.*, 2005; Ko *et al.*, 2005), and CD134/OX-40 (Takeda *et al.*, 2004; Valzasina *et al.*, 2005) ligands. Treg depletion with CD25-mAb is needed prior to vaccination, because CD25 is upregulated on all activated T-cells. In mice it was shown that Treg depletion prior to immunization with a DC vaccine significantly improved tumor protection (Sutmuller *et al.*, 2001; Van Meirvenne *et al.*, 2005) and transfer of these cells in an adoptive immunotherapy model for melanoma prevented CTL induced tumor destruction (Antony *et al.*, 2005). In a mouse model in which DC vaccination on its own was ineffective, tumors were completely eradicated when Tregs were depleted (Maksimow *et al.*, 2006). These findings were validated in a clinical trial involving patients with renal cell carcinoma, who showed a 16-fold increase in tumor-specific CTL when Treg were depleted with IL-2 diptheria toxin conjugate prior to DC vaccination (Dannull *et al.*, 2005). A new clinical trial is recruiting metastasized breast cancer patients, who will receive an IL-2 diphteria toxin to deplete Treg first, followed by multiple injections of DCs transduced with recombinant fowlpox virus expressing CEA and three costimulatory molecules (TRICOM). Another trial is treating metastasized melanoma patients with a combination of cyclophosphamide and DCs loaded with killed melanoma cells (www.clinicaltrials.gov).

The systemic elimination of all Treg can potentially result in autoimmune responses, so future Treg elimination studies may need to be refined by targeting tumor-specific Treg only.

3. DC VACCINE AND MONOCLONAL ANTIBODIES (mAbs)

a. Inhibitory T-Cell Receptor Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) (CD152) Antibody

CD80 (B7.1), one of the costimulatory molecules on activated DCs, can bind both CD28 and CTLA4. CD28 is constitutively expressed on T-cells and its engagement induces T-cell proliferation and IL-2 secretion. However, CTLA4, expressed on a subset of Treg and activated T-cells, has a higher

affinity for CD80 resulting in reciprocal DC IDO expression (Basu *et al.*, 2006). *In vivo* studies showed that CTLA4 blocking antibodies induced tumor regression in various murine cancer models (Gattinoni *et al.*, 2006b) especially when combined with peptide or DC vaccination (Prasad *et al.*, 2005; van Elsas *et al.*, 1999) and several clinical trials have shown increased clinical responses for patients receiving the CTLA4 antibody with or without peptide vaccination (Beck *et al.*, 2006; Hodi *et al.*, 2003; Phan *et al.*, 2003; Ribas *et al.*, 2005). The clinical responses in melanoma patients were obtained through increased T-cell activation and not through inhibition or depletion of regulatory T-cells (Maker *et al.*, 2005). Beck *et al.* showed a 14% clinical response rate in patients with melanoma or renal cell carcinoma (RCC) receiving the CTLA4 antibody with or without peptide vaccination. A clear correlation was noted between patients showing a clinical response and developing autoimmune enterocolitis. A recently initiated trial combines the CTLA4 antibody with MART-1-pulsed DCs in advanced melanoma patients (www.clinicaltrials.gov).

b. Agonistic TNF (Receptor) Superfamily (TNF(R)SF) Antibodies

Many members of the TNF(R)SF act as costimulatory molecules on T-cells and they can regulate DC function and survival via their interaction with the ligands on DCs (Tamada and Chen, 2006). Agonistic antibodies for 4-1BB, CD40, and OX40 have shown promising antitumor immune responses in mice (Tamada and Chen).

4-1BB (CD137) is selectively expressed on activated T- and NK-cells, as well as on DCs. Agonistic anti-4-1BB antibodies reversed T-cell anergy to soluble antigen in tumor-bearing mice (Wilcox *et al.*, 2004), increased infiltration of tumor-specific $CD8^+$ T-cells (Wilcox *et al.*, 2002), and blocked the immunosuppressive effects of Treg *in vivo* (Choi *et al.*, 2004). Improved survival of mice was noted when anti-4-1BB antibody was administered after tumor-lysate pulsed DC vaccination (Ito *et al.*, 2004).

c. Antitumor Antibodies

Antitumor efficacy of anti-CD20 (Rituximab) and anti-HER2/neu (Trastuzumab) therapeutic mAbs is dependent on the Fc portion of the mAb binding to FCγR receptors on monocytes, macrophages, and NK-cells resulting in antibody-dependent cellular cytotoxicity (ADCC) (Cassard *et al.*, 2006). DCs also express FCγR and ligation will result in phagocytosis of antibody–tumor immune complexes. Increased cross-presentation of TAA and antitumor cytotoxicity can be expected when these mAb are engineered to preferentially bind activating FcγRIII antibody receptors and ignore the inhibitory FcγRIIb receptor (Boruchov *et al.*, 2005; Clynes *et al.*, 2000).

Other mAbs, for example, the antideath receptor DR4 and DR5 antibodies that can induce tumor cell apoptosis, are currently being tested in phase I and II clinical trials (Cretney *et al.*, 2006). In mice, a combination of anti-DR5, anti-4-1BB, and anti-CD40 mAbs resulted in eradication of established fibrosarcoma and metastasis (Uno *et al.*, 2006).

Antitumor antibodies could be administered with DC vaccines to combine tumor destruction and the release of TAA with the generation of a tumor-specific immune response.

d. Programmed Death Receptor 1 (PD-1) and Ligand Antibodies

Various tumors and DCs express B7-H1 (PD-L1), which binds to programmed death receptor 1 (PD-1) on activated CTL and induces CTL apoptosis and hence inhibition of tumor cells lysis (Hirano *et al.*, 2005). Blockade of this interaction with B7-H1 and PD-1 mAbs increased tumor-specific T-cell proliferation and enhanced cytokine production and cytolytic activity *in vitro* and *in vivo* (Blank *et al.*, 2005, 2006; Brown *et al.*, 2003).

4. DC VACCINE AND NEUTRALIZATION OF IMMUNOSUPPRESSIVE CYTOKINES

a. Anti-IL-10, Anti-TGF-β

Increased levels of IL-10 and TGF-β in the tumor microenvironment have a negative effect on DC differentiation and CTL induction. A recent study showed that neutralizing IL-10 and TGF-β *in vitro* enhanced tumor-specific CTL responses (Jarnicki *et al.*, 2006). Neutralization of TGF-β in tumor-bearing mice using an antibody or DNA encoding for the soluble TGF-β type II/III receptor (TGFβRII/III) with a DC vaccine elicited strong antitumor immune responses and increased survival (Kim *et al.*, 2005; Kobie *et al.*, 2003; Kontani *et al.*, 2006). Patients vaccinated with autologous glioma cells transfected with antisense TGF-β in a phase I clinical trial showed an increased median survival compared to the historical value of patients treated conventionally (Fakhrai *et al.*, 2006).

b. Selective Cyclooxygenase-2 Inhibitors

Cyclooxygenase-2 (COX-2) overexpression along with increased PGE2 production by tumor cells has been associated with tumor growth, angiogenesis, and the induction of IDO expression on DCs. Recent studies in mice have demonstrated that celecoxib, a specific COX-2 inhibitor, in combination with a DC vaccine significantly increased efficacy by regression of primary tumors, prevention of metastasis, and prolonging mouse survival (Basu *et al.*, 2006; Hahn *et al.*, 2006).

G. DC Vaccines Combined with Conventional Treatment

The majority of patients with solid malignancies will undergo surgery as a first-line treatment to debulk tumor mass and depending on the tumor type and stage, they will subsequently receive adjuvant treatments. The majority of patients with hematological malignancies receive chemotherapy combined with hematopoietic stem cell transplantation as first-line treatment. With these treatments, destruction of tumor cells, vital tumor stroma or vasculature is achieved, resulting in the local release of TAA and proinflammatory cytokines potentially enhancing a DC vaccine when administered together.

1. DC VACCINE AND CHEMOTHERAPY

Traditionally it was thought that a combination of immunotherapy and chemotherapy would be unsuccessful, because chemotherapy would deplete T-cells. However, it has become clear that somewhat paradoxically, lymphodepletion prior to adoptive T-cell transfer leads to homeostatic T-cell proliferation with preferential expansion of infused T-cells (Dudley *et al.*, 2002). Furthermore, chemotherapy can cause massive tumor cell apoptosis, which results in increased availability of TAA in draining LN and the release of proinflammatory cytokines like TNF-α and heat shock proteins (HSP) inducing DC activation and their sensitization to CD40 signals (Lake and Robinson, 2005). Mucosal damage resulting in the release of TLR agonists and alkylating agents causing DNA damage, can further enhance DC activation (Gattinoni *et al.*, 2005) and IL-12 production. Finally, cyclophosphamide can specifically eliminate regulatory T-cells also attributing to a more effective CTL response (Ghiringhelli *et al.*, 2004; North, 1984). In a few murine models DC vaccines combined with chemotherapy have proven to be more efficient for tumor eradication (Casares *et al.*, 2005; Eralp *et al.*, 2004; Yu *et al.*, 2003). In one study, it was shown that DCs transduced with adenovirus expressing rat-Her2/neu and CD40L combined with intratumoral chemotherapy more efficiently suppressed tumor growth than both treatments separately (Akbulut *et al.*, 2006). In contrast to these findings, the majority of patients included in immunotherapy trials to date were treated first with chemotherapy until progressive disease developed and then subsequently treated with a vaccine.

Three recent clinical trials demonstrated an increased response rate to second-line chemotherapy in lung cancer and glioblastoma patients, who initially developed a tumor-specific immune response after DC vaccination (Antonia *et al.*, 2006; Gribben *et al.*, 2005; Wheeler *et al.*, 2004). It is tempting to suggest that the vaccines worked synergistically with chemotherapy.

A current clinical trial is recruiting breast cancer patients with locally recurrent or metastasized disease. They are receiving a multi-epitope DC vaccine combined with the chemotherapeutic agent vinorelbine and the her2-neu mAb trastuzumab. In another trial patients with renal carcinoma are receiving the chemotherapeutic agent fludarabine combined with a DC vaccine loaded with autologous lysate (www.clinicaltrials.gov).

It is important to note that, in contrast, certain cancer therapeutic agents, notably steroids and proteasome inhibitors, may have negative effects on DC function and combined therapy might not be beneficial (Duperrier *et al.*, 2005; Nencioni *et al.*, 2006; Xia *et al.*, 2005).

2. DC VACCINE AND RADIOTHERAPY

Similar to chemotherapy, radiotherapy also has important local effects resulting in changes to the tumor microenvironment that can influence antitumor immune responses (Antonia *et al.*, 2006). Exposure of tumor cells to radiation causes cellular stress resulting in the release of proinflammatory cytokines like IL-1β, TNF-α, HSP, and uric acid. Tumor cells also show upregulation of adhesion molecules, COX-2, peptide/MHC-class I molecules and death receptors (Fas, CD95). This will provide some of the required DC activation signals necessary for CTL induction and lead to sensitization of tumor cells for CTL killing (Hatfield *et al.*, 2005).

In various preclinical models of poorly immunogenic tumors therapeutic efficacy of DC vaccines was only evident when they were combined with local radiotherapy (Hatfield *et al.*, 2005; Nikitina and Gabrilovich, 2001). Significantly higher T-cell responses were noted when mice were first depleted of regulatory T-cells with a CD25 mAb and then treated with a DC vaccine and local radiotherapy (Kudo-Saito *et al.*, 2005). Also a significant increase in survival of mice was reported when B16 melanoma tumors were first injected with Ho-166 radiotherapy followed by the injection of immature MoDCs (Lee *et al.*, 2006). The effect of radioimmunotherapy has been tested in a few clinical trials. Prostate cancer patients treated with standard external beam radiotherapy, a poxvirus encoding for PSA, and IL-2 and GM-CSF showed immune responses against PSA and even against other TAA not included in the vaccine (Gulley *et al.*, 2005). Also intratumoral injection of DCs combined with conformal radiotherapy was safe and could induce tumor-specific and innate immunity in patients with refractory hepatoma (Chi *et al.*, 2005). In one study, currently open for enrolment, patients with high-risk sarcoma are being treated with both conventional external beam radiation and intratumoral DC injections prior to surgery and in another study immature DCs are being injected in metastasized skin lesions directly after local irradiation (www.clinicaltrials.gov).

3. DC VACCINE FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

Allogeneic HSCT has the capacity to cure hematological malignancies and the introduction of reduced intensity conditioning has reduced transplant related mortality and morbidity, extending its application. Now that it is realized that the graft versus tumor effect is crucial for the eradication of malignant disease in the host, donor leukocyte infusions are being used in the posttransplant period to maximize donor antitumor effects (Kolb *et al.*, 2005). This may be combined with or replaced by donor DC vaccination. If new anti-DC therapies are used to control early acute graft versus host disease (Sato *et al.*, 2003), then subsequent DC vaccination may be essential to preserve an antitumor effect. Autologous vaccination of the donor may also be used in the future to maximize antitumor CTL precursor frequency in the hematopoietic graft used for alloHSCT.

4. DC VACCINE AND HORMONAL THERAPY

The use of hormone deprivation therapy in patients with both prostate (Ryan and Small, 2006) and hormone receptor positive breast cancer (Leary and Dowsett, 2006) can lead to better clinical outcome. Androgen deprivation therapy (ADT) led to the infiltration of DCs and lymphocytes into prostate tissue (Mercader *et al.*, 2001) and in a mouse model ADT was able to attenuate tolerance to prostate antigens (Drake *et al.*, 2005) suggesting a possible synergistic effect when treatments are combined. One *in vitro* study showed that aromatase inhibitors sensitized breast tumor cells to monocyte-mediated killing in the presence of tumor-specific antibodies (Braun *et al.*, 2005). In contrast, negative effects on DC differentiation and immunostimulatory function were noted *in vitro* with the use of selective estrogen receptor modulators, tamoxifen and raloxifene (Nalbandian *et al.*, 2005). A subgroup analysis of patients with metastatic breast cancer showed a trend toward increased time to disease progression, when peptide vaccination was combined with aromatase inhibitors (Mayordomo *et al.*, 2004).

5. DC VACCINE AND ANGIOGENESIS INHIBITORS

Angiogenesis inhibitors restrict tumor growth by interfering with the normal tumor vascularization process (Nair *et al.*, 2003a) and were predicted to synergize with DC therapy. Bevacizumab, a mAb against VEGF, has proven efficacy in metastatic colon cancer and breast cancer (Lizee *et al.*, 2006), but has not yet been combined with a DC vaccine. A synergistic antitumor effect was achieved when mice were vaccinated with DCs loaded with both VEGF mRNA and tumor RNA without serious side effects

(Miyazaki *et al.*, 2005). These synergistic effects might be caused by blocking the immunosuppressive effect of VEGF and inhibiting angiogenesis. In a pancreatic cancer mouse model, the combination of TNP-470, an angiogenesis inhibitor, and DCs loaded with tumor cell lysate resulted in regression of tumor tissue. Intratumoral injection of an adenovirus expressing angiostatin and IL-12 also resulted in a total regression of breast tumor in 50% of the treated mice (Gyorffy *et al.*, 2001).

6. DCs AND PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) is used for the palliative treatment of surgically inaccessible tumors. In PDT, a systemically administered photosensitizer that preferentially accumulates in transformed cells is activated to generate cytotoxic intermediates after illumination with a laser beam. In one preclinical study, survival was significantly prolonged when mice with B16 melanoma or CT26 colon carcinoma first received PDT and then intratumoral DCs for four consecutive days. Tumor regression at distant sites was only noted in mice treated with both PDT and DCs suggesting the systemic induction of potent antitumor cytotoxic CTL (Saji *et al.*, 2006). These results confirm the findings from chemotherapy and radiotherapy studies indicating that therapy-induced tumor cell death and local inflammation can enhance the effects of a DC vaccine.

IV. CONCLUSION

The last decade has seen the ability of DCs to initiate and direct the immune response exploited in a variety of clinical applications. Their use as cancer vaccines in patients has proved safe and fears of major autoimmune side effects appear to be a lesser concern, perhaps as tumors have a "therapeutic immune index" of susceptibility compared to normal tissues. Although good immunological responses have been obtained with *ex vivo* generated TAA-loaded DCs, limited impact on clinical outcome has been reported in clinical trials. Data from *in vivo* studies in animal models and preliminary clinical trials point to important improvements that can be made in DC vaccine design including: optimizing DC preparations, DC activation, and the schedules used for their administration. It is now appropriate to contemplate vaccinating patients earlier in their disease, and this is expected to improve results. Thus, DC vaccination might be even started prior to surgery or induced early after conventional treatments have established a minimal residual disease state. Synergistic effects of DC vaccination and other immunotherapies and conventional cancer therapies are being

observed in mouse models and early clinical trials and this will be an exciting area for further investigation. Furthermore, DC vaccines will benefit from the addition of strategies to overcome immunoregulatory mechanisms such as the depletion of Tregs. The potential risk of inducing severe autoimmune responses, when such immunoregulatory mechanisms are inhibited systemically, requires an ongoing effort to improve the repertoire of candidate TAA. Thus, further characterization of cancer stem cells and the development of novel techniques to identify more specific TAA to improve the immune therapeutic index are essential. It is likely that novel molecules and methods for DC mobilization, recruitment, and activation will become available. Ultimately, "off the shelf pharmaceuticals" that target DCs *in vivo* may replace current cellular therapies but the ability of DC-based vaccines to deliver meaningful outcomes for patients in a range of settings over the next decade means that further convincing phase III studies are both necessary and expected.

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