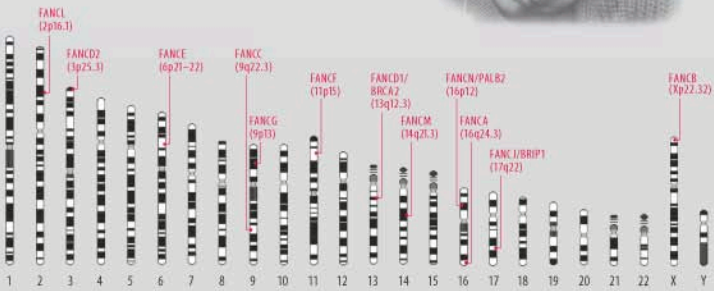
Monographs in Human Genetics
Editor: M. Schmid
Vol. 15

Fanconi Anemia

A Paradigmatic Disease for the Understanding of Cancer and Aging

Editors
D. Schindler
H. Hoehn



KARGER

. . . . . .

Fanconi Anemia

Monographs in Human Genetics

Vol. 15

Series Editor

Michael Schmid, Würzburg

Fanconi Anemia

A Paradigmatic Disease for the Understanding of Cancer and Aging

Volume Editors

Detlev Schindler, Würzburg
Holger Hoehn, Würzburg

59 figures, 34 in color, and 14 tables, 2007

KARGER

Basel · Freiburg · Paris · London · New York ·
Bangalore · Bangkok · Singapore · Tokyo · Sydney

Holger Hoehn, M.D.
Professor of Human Genetics
Department of Human Genetics
University of Würzburg
Am Hubland
D–97074 Würzburg

Detlev Schindler, M.D.
Professor of Human Genetics
Department of Human Genetics
University of Würzburg
Am Hubland
D–97074 Würzburg

Library of Congress Cataloging-in-Publication Data

Fanconi anemia : a paradigmatic disease for the understanding of cancer and aging / volume editors, Detlev Schindler, Holger Hoehn.
p. ; cm. – (Monographs in human genetics, ISSN 0077-0876 ; v. 15)
Includes bibliographical references and indexes.
ISBN-13: 978-3-8055-8277-3 (hard cover : alk. paper)
1. Fanconi's anemia–Genetic aspects. I. Schindler, Detlev. II. Hoehn, Holger. III. Series.
[DNLM: 1. Fanconi Anemia–genetics. W1 M0567P v. 15 2007 / WH 175 F1995 2007]
RC641.7.F36F34 2007
616.1′52042–dc22

2007011109

Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

www.karger.com
Printed in Switzerland on acid-free paper by Reinhardt Druck, Basel
ISSN 0077–0876
ISBN 978–3–8055–8277–3

Dedicated to George M. Martin

Contents

Editorial

Fourteen volumes of *Monographs in Human Genetics* were published between 1966 and 1992. Since then a plethora of experimental data obtained by the new molecular techniques has led to paradigmatic changes in understanding the mechanisms operating in human heredity. Currently human genetics presents as a highly diversified field covering an ever increasing number of topics, ranging from basic research to medical practice. This calls for a specialized forum where the rapid advances in human genetics are reviewed by experts of different fields. In response to these developments, the traditional book series *Monographs in Human Genetics* will be revived with two volumes per year, focusing on important hereditary diseases, their molecular basis, their clinical impact and their eventual treatment. With its concise but highly informative reviews, *Monographs in Human Genetics* provides essential reading not only for researchers but also for physicians and students interested in specific genetic diseases. All articles published in this book series are reviewed according to classical standards.

The present volume is devoted to 'Fanconi Anemia', a chromosome instability disorder whose molecular basis has been all but elucidated in recent years. By their very nature, monograph-types of publications are more comprehensive and synthetic rather than hot-off-the-press reports. A case in point is the topic 'Fanconi anemia' where the pace of gene discovery has accelerated during recent years. While this volume was in preparation three new disease causing genes have been discovered, the most recent of which (FANCI) was too recent to be included. Even though monographs may not cover the very last developments in a given field, their undisputed value arises from their unhurried and in

depth treatment of a given subject that can hardly be achieved with the usual publish-or-perish types of publications. As such, monographs fulfill the important task to remind the reader that any type of scientific progress has its roots in a multifaceted landscape of prior insights and achievements that need to be collected and preserved in order to provide fertile ground for future growth.

I would like to thank all the authors for their interesting contributions, the Editors Holger Hoehn and Detlev Schindler for their invaluable support and help in the organization of this book, and the Publisher Thomas Karger for having offered the opportunity to reinitiate this book series.

Michael Schmid
Würzburg, January 2007

Preface

The revival of the series 'Monographs in Human Genetics' starts with a volume on Fanconi anemia, a rare inherited disease that was discovered 80 years ago by an eminent Swiss pediatrician, Guido Fanconi, then working in Geneva. The Editors of the volume gratefully acknowledge the initiative by Karger Publishers (Basel, Switzerland), and by Michael Schmid of the University of Würzburg, Germany, in getting this new series started. The volume not only pays tribute to Guido Fanconi's discovery of a disease that teaches us much about the connection between genetic instability, cancer and premature aging, but also pays tribute to Traute Schroeder-Kurth who in 1964 discovered the chromosome instability in Fanconi anemia, and who has contributed numerous important observations on the clinical and genetic aspects of the disease. On the occasion of Traute Schroeder-Kurth's 75th birthday we convened a small meeting in Würzburg in order to honor her seminal contributions to the diagnosis and pathophysiology of Fanconi anemia. The talks presented at the Schroeder-Kurth symposium provide the basis for this volume. We are very grateful to the contributing authors for complementing and updating their previous oral presentations. Following introductory chapters that include a historical account, exemplatory case reports, and a summary of the current status of FA genes and their mutations, there are two chapters on neoplasia in FA, three chapters reviewing diagnostic approaches in FA, including prenatal diagnosis, and one chapter each on the phenomenon of revertant mosaicism or 'natural gene therapy' and on hematopoietic stem cell transplantation as the only curative approach in FA. The final three chapters deal with evolutionary aspects of the FA genes with special emphasis on the avian genome, with recombinational

types of DNA repair in FA, and with the establishment of a test system for the Rad51 recombinase in homology-directed DNA repair. Even though it is impossible to cover all aspects of Fanconi anemia within the space available for such a volume, we hope that it may serve as useful introduction to a disease that provides unique insights into the complex network of genomic maintenance systems which protect us from cancer and premature aging.

We dedicate this volume to George M. Martin, M.D., Professor Emeritus of Pathology and Genetics at the University of Washington, Seattle, USA, on the occasion of his 80th birthday. Like Traute Schroeder-Kurth with respect to Fanconi anemia, George Martin was one of the first scientists to recognize the intimate relationship between genetic instability, cancer and aging by studying the Werner progeria syndrome. Very early on he was convinced of the model character and unique value of the rare human chromosomal breakage syndromes. He emphasized that these rare experiments of nature would provide unprecedented insights into the complex mechanisms of DNA damage recognition and repair which are essential for long lived, warm-blooded species such as ours. George Martin encouraged us to study the human caretaker gene syndromes, including Fanconi anemia, and we owe him much in terms of motivation, mentorship, and guidance.

Detlev Schindler
Holger Hoehn
Würzburg, January 2007

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 1–8

Why, What and How Can We Learn from a Rare Disease Like Fanconi Anemia?

Traute Schroeder-Kurth

Department of Human Genetics, University of Würzburg, Würzburg, Germany

Abstract

In a field that embraces multiple aspects of both clinical and basic research and that moves impressively fast, any answers to the questions why, what and how can we learn from a rare disease like Fanconi anemia (FA) must remain tentative and preliminary. However, there are very encouraging advances, most notably at the level of understanding the molecular basis of FA and at the level of treatment via hematopoietic stem cell transplantation. For the sake of our patients we clearly need to arrive at meaningful genotype-phenotype correlations and individualized risk profiles. This requires prospective and longterm studies carried out in close cooperation among patients, clinicians and basic scientists. There are a number of open questions, for example relating to the mechanisms of chromosomal breakage and DNA repair, to the spectrum of genetic changes that herald and promote the emergence of leukaemia and solid tumors, and to the emergence of genetically reverted cells in blood and bone marrow of FA patients. Researchers in the fields of cancer and aging should be encouraged to and are likely to benefit from the study of Fanconi anemia, as are our patients from the welcome results of such studies.

The year 2007 marks the 80th anniversary of the original description by Guido Fanconi of ‘Familial infantile pernicious-like anemia’, a rare genetic disease that since carries the name of this eminent physician-scientist [1]. Why should we study such a rare disease, what can we learn from its myriad clinical and molecular manifestations, and how should we go about it? I will try to give some brief answers to these questions even though by necessity these answers are tentative and preliminary. Despite undoubted progress, there still are many aspects of this enigmatic disease that we do not fully understand.

Why Study Fanconi Anemia?

First of all, for the sake of the affected patients. Even though there is no definite cure for FA on the horizon, there is encouraging progress regarding diagnosis and treatment. Timely diagnosis is crucial both for the institution of adequate treatment and for the prevention of inadequate medical management that may result from failure to recognize the hereditary and specific nature of the disease. If one looks back at the last monograph on Fanconi anemia that I had the pleasure to edit with my colleagues Arleen Auerbach and Gunter Obe in the year 1989 [2], the progress that has since been made is impressive and encouraging. Two major areas stand out: molecular genetics, and hematopoietic stem cell transplantation. Progress in the understanding of the molecular basis of Fanconi anemia is astounding indeed: even though genetic heterogeneity of FA had been convincingly documented prior to 1989 [3, 4], it was not until 1992 that the first FA gene had been cloned and, as of this writing, 12 FA causing genes have been identified. Several more are in the offing, as there are a number of patients who apparently do not belong to any of the known complementation groups [5]. There is also increasing understanding of how the FA proteins work, how they work together and how they might interact with other proteins in maintaining a stable genome [6, 7].

Although less spectacular than the gene discovery itself, the virtual explosion of knowledge concerning the molecular basis of Fanconi anemia during the past 10–15 years translates into immediate benefits to FA patients and their families. For example, precise knowledge of the affected gene and the specific type of mutation in a given patient will increasingly be of prognostic value and serve as a rational guide for optimized medical management. We clearly need to collect many more data in order to arrive at clinically useful genotype-phenotype correlations. We should strongly oppose arguments that deem the collection of such additional data unnecessary once the clinical diagnosis of FA has been established and confirmed by a chromosome breakage or cell cycle test. The ultimate goal of such efforts is to arrive at individualized risk profiles for FA patients similar to what has already been achieved with congenital abnormality scores [8]. Biallelic mutations in two of the FA genes (*FANCD1/BRCA2* and *FANCN/PALB2*) have so far been associated with very early childhood cancers [9, 10], and awareness of such correlations are of undisputed value in the clinical management of these young tumor patients. Precise knowledge of the disease causing mutations also improves and facilitates prenatal diagnosis that remains an unwelcome but compromise option for some of the FA families. Last but not least, participation in gene therapy trials requires knowledge of underlying genes and mutations.

Although one may question the extent to which the vastly improved molecular knowledge has so far been of immediate benefit to the majority of

FA patients, such reservations do least apply to the progress in the field of hematopoietic stem cell transplantation (HSCT). The outcomes of matching donor sibling transplantations have continuously improved such that today early HSCT is highly recommended in such families [11]. Unrelated donor transplantation still carries a higher risk, but modified conditioning protocols using fludarabine and T-cell depletion, reduction or complete absence of whole body irradiation, the introduction of minitransplants and improved post-transplant care have contributed to much better longterm survival [12]. The serious problem of posttransplant squamous cell carcinomas remains to be solved [13]. The message from all this progress is to consider the option of HSCT in each patient at a much earlier stage of the disease and with much more optimism than we could have had in the year 1989.

What Can We Learn from Fanconi Anemia?

We have learned that introducing a gene into something as accessible as a bone marrow stem cell does not necessarily result in improvement [14]. There are many reasons for the slow progress in gene therapy, but there is no reason to give up. Improvements in stem cell collection and improved gene transfer protocols are on the horizon [15, 16]. Notwithstanding all previous failures and disappointments, the replacement of a defective gene copy by an intact gene remains an attractive option which should and needs to be further explored.

Looking back at the recent progress of FA research it seems to me a kind of miracle that at first chromosome instability showed up and initiated the search for possible molecular mechanisms causing breaks, gaps and reunions which we saw in the chromosome preparations. Our very first observations reported in 1964 taught us that chromosomal instability was present not only in patients presenting with FA symptoms, but also in their siblings without any clinical manifestation of the disease [17]. We also quickly learned that chromosome instability occurs in vivo as it was observed in direct preparations of bone marrow cells [18]. If dividing bone marrow cells exhibit chromosome aberrations, this surely must reflect a fundamental and intrinsic defect of genomic maintenance with severe consequences for the renewal of blood cell lineages. The more we learned about the complex structure that makes up a chromosome, the more naive appeared the simple notion of a DNA lesion that was not properly repaired and therefore manifests as a chromosome break. Why do breaks reunite if there is a repair defect, and what are the prerequisites for a reunion to take place? Why do crosslink-induced radials preferentially form between nonhomologous autosomes and are virtually absent from gonosomes? [19]. What is the role of low copy repeats that are increasingly recognized as focal points for

non-allelic homologous recombination? [20]. Does the chromosomal position within the nucleus influence the opportunity for the type of radial formation? There are many unsolved questions, there still is much to be learned.

One of the most impressive lessons we learned from patients with Fanconi anemia is the intimate relationship between genetic instability and cancer. FA patients carry a high risk for acute leukaemia, squamous cell carcinomas and other tumors [21]. Young FA patients with certain gene mutations have a higher risk to develop leukaemia. The older a FA patient gets the higher is the risk to develop a solid tumor. There are impressive case histories describing adult patients in whom a leukaemia or a solid tumor were the first manifestations of FA [22, 23]. With the exception of *FANCF* that is frequently inactivated by an epigenetic mechanism in various types of tumors, the other FA genes seem to be relatively intact in most neoplasias as if they were needed for tumor cell growth [24]. However, monoallelic truncating mutations in at least three of the FA genes (*FANCD1/BRCA2*, *FANCJ/BRIP1*, and *FANCN/PALB2*) have been shown to confer variant degrees of breast cancer susceptibility in non-FA patients [25, 26]. Those are important new insights, connecting a rare with a common disease.

In 1971 we postulated that in vivo chromosomal instability leads to multiple somatic mutations which finally may confer a selective advantage to a single mutated cell, giving rise to malignant cell growth [27]. By careful bone marrow studies, this process has been directly observed in FA patients. Specific chromosomal changes in bone marrow cells appear to herald progression to malignancy even prior to any clinical manifestation of leukaemia [28]. It was quite clear in the 1970s that aberrant clones may also appear in peripheral blood lymphocyte cultures. Over time, these cytogenetically aberrant clones were subject to clonal attenuation and clonal succession, but they undoubtedly were bad news for the patient. One of these patients I followed for more than six years with repeated chromosome studies [29]. He died at age 35 of bronchial cancer (unpublished). These types of longterm observations are important for our patients and should be supported as much as possible. There is much to be learned about the close relationship between FA and cancer for the sake of our patients, and for our understanding of the origin of cancer [30].

Even though a fundamental change of the high cancer risk in FA is not in sight, we have already learned that predictive and preventive measures are beneficial for our patients. As already mentioned, regular monitoring for aberrant bone marrow clones, particularly those involving monosomy 7 and 3q duplications, is of great value for both physicians and patient in their difficult decision as to whether and when to proceed with HSCT [28]. Likewise, non-invasive measures for the early detection of oral cavity and genital area lesions as currently developed by the group of Ruud Brakenhoff in Amsterdam [31] will gain even greater importance with more and more patients undergoing and surviving HSCT.

Another lesson we are beginning to appreciate from Fanconi anemia is the discovery of reverse mutations in blood cells [32]. One in four or five patients displays MMC resistant cells among the original MMC sensitive cells. These patients are mosaic FA patients who have a chance to escape BM failure and, possibly, the development of leukaemia. Much research has to be done to clarify the underlying mechanisms. The likelihood of 'natural gene therapy' leading to revertant mosaicism appears to be higher in patients belonging to certain complementation groups and in the presence of compound heterozygosity. Longterm observations of these mosaic FA patients will ultimately teach us whether there is more than a temporary benefit of this type of 'natural gene therapy' to the individual, and what the prerequisites are for somatic reversions to occur. And, of course, could there be ways to enhance the occurrence of such reversions for the benefit of our patients?

How Should We Go About It?

First and foremost, FA research needs the cooperation of the affected families and patients as well as the unrelenting interest and commitment of their doctors. A well established (and well funded!) FA registry such as pioneered by Arleen Auerbach in New York is essential for both clinical care and basic research [33, 34]. It should include regular follow-ups with complete information on the natural history of the disease in each individual family. For practical purposes, such registries should be organized at the regional or national levels, but they should and must communicate with each other. Today we have every reason to believe that most FA families are aware of the importance of FA research for the sake of their children and thus are willing to cooperate with such registries. Many research projects require the participation and involvement of the patients themselves, and there is little doubt that the affected patients and families can still teach us a lot about their disease. On the technical side, new tools such as retroviral complementation, knockdown of FA genes via RNA interference, or MLPA for the detection of large deletions have contributed both to diagnosis and research. With such technical advances at hand the time might have come to tackle the old question of what really causes the oxygen sensitivity of FA cells [35, 36].

We now know that at least some of the FA genes are highly conserved during evolution and that they play a major role during DNA replication and recombination in premeitotic stages and meiosis. Some of the FA genes have been recognized as very ancient 'caretaker' genes that have emerged even prior to the vertebrate lineage [37]. In addition to valuable insights gained from vertebrate model organisms such as zebrafish, mouse and birds, scientists working

on ancient model organisms like yeast, *C. elegans* and *Drosophila* should be invited and encouraged to participate in FA-oriented basic research. Last but not least, Fanconi anemia should be introduced as a human model system into basic cancer research. It has already been shown that targeted inactivation of FA genes in cancer cells may offer specific therapeutic options [38, 39]. I am convinced that both, our patients and our colleagues involved in cancer research will benefit from inclusion of Fanconi anemia into current research paradigms.

Concerning the premature aging phenotype of Fanconi anemia, it is obvious that truly progeroid features (such as in the Hutchinson-Gilford or Werner syndromes) are far less conspicuous in FA. The decline of bone marrow function in FA clearly is a much more progressive and devastating event compared to what happens during normal aging. This is the likely reason why Martin and Oshima did not include FA in their presentation of human genetic instability syndromes with progeroid features [40]. However, squamous cell carcinomas of the oral cavity and genital area are typical tumors of advanced age that occur very prematurely in FA patients. Likewise, endocrine abnormalities including hyperinsulinemia, growth hormone deficiency and hypothyroidism as typical endocrine abnormalities of older individuals affect more than 80% of FA patients during young adulthood [41]. Impaired gametogenesis and premature reproductive aging are additional features of FA patients that are reminiscent of an accelerated aging process. Collectively, the premature manifestation, in FA patients, of clinical features also encountered during normative aging seems to indicate that genetic instability per se may be one of the most significant factors that contributes to and promotes aging. From this point of view, scientists involved in aging research might benefit from the study of Fanconi anemia. Conversely, Fanconi anemia patients may ultimately benefit from the insights gained from the ongoing efforts of serious and scientifically sound anti-aging research.

References

1 Lobitz S, Velleuer E: Guido Fanconi (1892–1979): a jack of all trades. Nat Rev Cancer 2006;6: 893–898.

2 Schroeder-Kurth TM, Auerbach AD, Obe G (eds): Fanconi Anemia. Clinical, Cytogenetic and Experimental Aspects. Berlin, Heidelberg, New York, Springer-Verlag, 1989.

3 Zakrzewski S, Sperling K: Genetic heterogeneity of Fanconi's anemia demonstrated by somatic cell hybrids. Hum Genet 1980;56:81–84.

4 Duckworth-Rysiecki G, Cornish K, Clarke CA, Buchwald M: Identification of two complementation groups in Fanconi anemia. Somat Cell Mol Genet 1985;11:35–41.

5 Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30: 410–420.

6 Taniguchi T, D'Andrea AD: Molecular pathogenesis of Fanconi anemia: recent progress. Blood 2006;107:4223–4233.
7 Gurtan AM, D'Andrea AD: Dedicated to the core: understanding the Fanconi anemia complex. DNA Repair (Amst) 2006;5:1119–1125.
8 Rosenberg PS, Huang Y, Alter BP: Individualized risks of first adverse events in patients with Fanconi anemia. Blood 2004;104:350–355.
9 Alter BP, Rosenberg PS, Brody LC: Clinical and molecular features associated with biallelic mutations in *FANCD1/BRCA2*. J Med Genet 2007;44:1–9.
10 Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2006;39:162–164.
11 Rosenberg PS, Alter BP, Socie G, Gluckman E: Secular trends in outcomes for Fanconi anemia patients who receive transplants: implications for future studies. Biol Blood Marrow Transplant 2005;11:672–679.
12 Wagner JE, Eapen M, Macmillan ML, Harris RE, Pasquini R, et al: Unrelated donor bone marrow transplantation for the treatment of Fanconi anemia. Blood 2007;109:2256–2262.
13 Alter BP: Fanconi's anemia, transplantation, and cancer. Pediatr Transplant 2005;9:81–86.
14 Williams DA, Croop J, Kelly P: Gene therapy in the treatment of Fanconi anemia, a progressive bone marrow failure syndrome. Curr Opin Mol Ther 2005;7:461–466.
15 Jacome A, Navarro S, Casado JA, Rio P, Madero L, et al: A simplified approach to improve the efficiency and safety of ex vivo hematopoietic gene therapy in Fanconi anemia patients. Hum Gene Ther 2006;17:245–250.
16 Kelly PF, Radtke S, Kalle C, Balcik B, Bohn K, et al: Stem cell collection and gene transfer in Fanconi anemia. Mol Ther 2007;15:211–219.
17 Schroeder TM, Anschütz F, Knopp A: Spontane Chromosomenaberrationen bei familiärer Panmyelopathie. Humangenetik 1964;1:194–196.
18 Schroeder TM: Cytogenetische und cytologische Befunde bei enzymopenischen Panmyelopathien und Pancytopenien. Humangenetik 1966;2:287–316.
19 Newell AE, Akkari YM, Torimaru Y, Rosenthal A, Reifsteck CA, et al: Interstrand crosslink-induced radials form between non-homologous chromosomes, but are absent in sex chromosomes. DNA Repair (Amst) 2004;3:535–542.
20 Erdogan F, Chen W, Kirchhoff M, Kalscheuer VM, Hultschig C, et al: Impact of low copy repeats on the generation of balanced and unbalanced chromosomal aberrations in mental retardation. Cytogenet Genome Res 2006;115:247–253.
21 Rosenberg PS, Greene MH, Alter BP: Cancer incidence in persons with Fanconi anemia. Blood 2003;101:822–826.
22 Liu JM, Auerbach AD, Young NS: Fanconi anemia presenting unexpectedly in an adult kindred with no dysmorphic features. Am J Med 1991;91:555–557.
23 Huck K, Hanenberg H, Gudowius S, Fenk R, et al: Delayed diagnosis and complications of Fanconi anaemia at advanced age – a paradigm. Br J Haematol 2006;133:188–197.
24 Neveling K, Kalb R, Schindler D: Cancer in Fanconi anemia and Fanconi anemia genes in cancer; in Schindler D, Hoehn H (eds): Fanconi Anemia, Basel, Karger-Verlag, 2007, pp 59–78.
25 Seal S, Thompson D, Renwick A, Elliott A, Kelly P, et al: Truncating mutations in the Fanconi anemia J gene *BRIP1* are low-penetrance breast cancer susceptibility alleles. Nat Genet 2006;38: 1239–1241.
26 Rahman N, Seal S, Thompson D, Kelly P, Renwick A, et al: *PALB2*, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 2006;39:165–167.
27 Schroeder TM, Kurth R: Spontaneous chromosomal breakage and high incidence of leukemia in inherited disease. Blood 1971;37:96–112.
28 Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, Neitzel H: Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood 2003;101:3872–3874.
29 Schroeder TM, Drings P, Beilner P, Buchinger G: Clinical and cytogenetic observations during a six-year period in an adult with Fanconi′s anemia. Blut 1976;34:119–132.
30 Mathew CG: Fanconi anaemia genes and susceptibility to cancer. Oncogene 2006;25:5875–5884.

31 Bremmer JF, Braakhuis BJ, Ruijter-Schippers HJ, Brink A, Duarte HM, et al: A noninvasive genetic screening test to detect oral preneoplastic lesions. Lab Invest 2005;85:1481–1488.
32 Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, et al: Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 1997;5:137–148.
33 Auerbach AD, Schroeder TM: First announcement of the Fanconi Anemia International Registry. Blood 1982;60:1054.
34 Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, et al: A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–1256.
35 Pagano G, Degan P, d'Ischia M, Kelly FJ, Nobili B, et al: Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. Eur J Haematol 2005;75:93–100.
36 Mukhopadhyay SS, Leung KS, Hicks MJ, Hastings PJ, Youssoufian H, Plon SE: Defective mitochondrial peroxiredoxin-3 results in sensitivity to oxidative stress in Fanconi anemia. J Cell Biol 2006;175:225–235.
37 Nanda I, Buwe A, Wizenman A, Takata M, Haaf T, Schartl M, Schmid M: Fanconi anemia genes in vertebrates: evolutionary conservation, sex-linkage, and embryonic expression of *FANCC* and *FANCG* in avian cells; in Schindler D, Hoehn H (eds): Fanconi Anemia, pp 183–199. Basel, Karger-Verlag, 2007.
38 van der Heijden MS, Brody JR, Dezentje DA, Gallmeier E, Cunningham SC, et al: In vivo therapeutic responses contingent on Fanconi anemia/BRCA2 status of the tumor. Clin Cancer Res 2005;11:7508–7515.
39 Gallmeier E, Calhoun ES, Rago C, Brody JR, Cunningham SC, et al: Targeted disruption of *FANCC* and *FANCG* in human cancer provides a preclinical model for specific therapeutic options. Gastroenterology 2006;130:2145–2154.
40 Martin GM, Oshima J: Lessons from the human progeroid syndromes. Nature 2000;408:263–266.
41 Wajnrajch MP, Gertner JM, Huma Z, Popvich J, Lin K, et al: Evaluation of growth and hormonal status in patients referred to the International Fanconi Anemia Registry. Pediatrics 2001;107: 744–754.

Prof. Dr. med. Traute Schroeder-Kurth
Wilhelm-Doles-Str. 7
D–97246 Eibelstadt (Germany)
Tel./Fax +49 9303 8762

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 9–22

Fanconi Anemia: A Disease with Many Faces

R. Dietrich[a], *E. Velleuer*[b]

[a]Deutsche Fanconi-Anämie-Hilfe e.V., Unna; [b]Zentrum für Kinder- und Jugendmedizin, Universitätsklinikum Düsseldorf, Germany

Abstract

We present the clinical case histories of 7 patients with Fanconi Anemia (FA) in order to illustrate the widely divergent phenotypes and clinical courses of the disease. Moreover, these case histories demonstrate that androgen therapy and hematopoietic stem cell transplantation are therapeutic options that have proven their worth despite side effects that can be severe or even fatal. We show that mild forms and severe forms of the disease can occur in individuals with identical mutations, as in siblings from a single family. We wish to convince the reader that there is no 'typical' FA patient, even though there are a number of congenital malformations and clinical manifestations whose patterns and combinations should alert the physician to the diagnosis of Fanconi anemia. Each individual case history teaches us certain facets but never the entire spectrum of a disease that has as many faces as affected individuals. Fanconi anemia is a disease physicians must think of. We will soon reach a point where parents and patients need be less discouraged and less concerned by the diagnosis of Fanconi anemia since there is tremendous progress in understanding the causes and treating the consequences of the disease. There is no definitive cure on the horizon, but there is continuous improvement of the quality of life for many patients. Early diagnosis, up-to-date information, closely knit surveillance, participation in family support groups, and a mutually trustful alliance with expert physicians and scientists are the cornerstones of optimal care.

Since FA is a rare entity, family support groups play an important role in the collection and dissemination of information concerning the disease. In addition to the families themselves, physicians and researchers benefit from the vast amount of knowledge and personal experience that accumulate within the realm of daily family life. Much of this experience has been summarized and is updated regularly in special publications and newsletters edited by the Fanconi Anemia Research Fund (FARF) and its German equivalent, the Deutsche

Fanconi-Anämie-Hilfe e.V. [1–3]. Family support groups organize annual meetings where families have the opportunity to meet each other and to establish personal contacts to specialized physicians and FA researchers. Family support groups explain the aims and importance of research projects to their lay members and encourage participation. As pointed out repeatedly by one of the leading researchers in the field, Professor Hans Joenje of the Free University Amsterdam, the close interaction and mutual trust between family support groups and FA researchers has been instrumental in the deciphering of the genetic, cellular and molecular basis of the disease.

Members of academic institutions frequently approach family support groups asking whether they could provide photographs of a 'typical' FA patient for the purpose of teaching or publication. No one knows better than the affected families themselves that there is no 'typical' FA patient but rather a highly variable spectrum of phenotypes and clinical courses. There are patients born with multiple congenital anomalies and severe growth retardation, and patients without any congenital anomalies and normal growth. There are patients with neonatal onset of thrombocytopenia and early bone marrow failure, and there are adult patients with close to normal or even completely normal blood counts. There are patients who succumb to solid tumors or leukemia as newborns or toddlers, and patients whose first manifestations of the disease are malignancies arising during adulthood. Recent anecdotal evidence may illustrate this point: The authors showed photographs of 30 proven FA patients to a group of experienced FA specialists who came to the conclusion that they could not recognize any features of FA in 8 of these 30 pictures, and only minimal signs in further five. Thus, there were no 'typical' clinical features in almost half of the 30 patients.

In non-consanguineous, random populations there are only between 2 and 5 affected individuals per million births such that the average physician has no personal experience with the manifold manifestations and highly variable clinical course of the disease. Given this pronounced variability of phenotypes and variable onset, severity and course of hematopoietic failure, the correct diagnosis may be missed or delayed. However, in the interest of optimal patient care, there is urgent need for a timely and correct diagnosis. This will become even more important in future years as the outcome of alternative donor hematopoietic stem cell transplantation (HSCT) continues to improve. Since FA is a recessive disorder with a 25% risk to siblings, early diagnosis is also mandated by considerations of family planning.

There are numerous clinical problems which require special attention and special treatments in FA patients. For example, conventional chemotherapy containing DNA crosslinking agents is poorly tolerated by these highly sensitive individuals. In case of solid tumors, surgery and subsequent radiotherapy

are the treatments of choice. For FA patients with incipient leukemia, the only valid option is immediate HSCT. In order to detect leukemic changes as early as possible, FA patients should be monitored at regular intervals for clonal chromosome changes arising in their bone marrow cells. There is solid evidence that a specific pattern of chromosome changes emerges prior to the onset of clinically overt leukemia (see chapter by Neitzel et al.). The emergence of such aberrant cells is a warning sign that requires immediate action in terms of preparation for HSCT. Four German patients have recently been transplanted after positive bone marrow findings thus sparing them the additional (and often fatal) complications of overt leukemia.

The following case reports are meant to illustrate the high degree of variability of phenotype and clinical course encountered among FA patients. Since by now there are 12 and possibly more genes whose mutational changes lead to the development of FA, much of the clinical variability can be attributed to this tremendous amount of genetic and, at the single gene level, mutational heterogeneity. In the absence of prospective studies we still are fairly ignorant about genotype-phenotype correlations, even though tentative evidence for such correlations exists for some of the FA genes (see chapter by Kalb et al.). Despite recent progress in our understanding of the molecular basis of the disease, there are many open questions relating to gene and protein function that need to be answered before we can explain, for example, why manifestations of the disease can be so varied and different, even among siblings carrying identical mutations. In presenting these case reports, the authors wish to point out that even though these descriptions are kept in the usual neutral and medical style, these individuals are exceptional human beings by instructing us not only about medical facts, but also by teaching us humility towards the manifold facets, pleasant and unpleasant, of human life.

Philipp

Philipp (fig. 1) was delivered via C-section in the 37th week of pregnancy after sonography at 32 weeks had revealed a hydrocephalus. He weighed 2125 g, his height was 44.5 cm, and his head circumference was 32 cm. In addition, there were multiple structural and functional defects, including persisting ductus Botalli, cleft lip and palate (Pierre-Robin sequence), bilateral radius aplasia, bilateral thumb aplasia, anal stenosis, aqueduct stenosis with hydrocephalus, hypospadia, cryptorchism, pulmonary hypertension due to atelectasis, strabism, multiple café-au-lait spots, horseshoe kidney, rib anomalies.

Intubation and assisted ventilation were required during the first week of life. Subsequent pneumonia was controlled with antibiotics. Philipp had to be

Fig. 1. (from left to right) Philipp at 6, Dominik at 7 and Michael at 25 years.

fed via gastric tube, and the anal stenosis had to be corrected surgically. From day 3 of his life, Philipp received Vojta-type of physical therapy which was very exhausting for him, possibly due to the at that time still unknown persistence of the duct of Botalli. His parents felt that Philipp's declining health was due to these stressful exercises. Because of increasing intracranial pressure, Philipp had to be re-intubated at the age of 2 weeks, and a shunting procedure was performed. There was an episode of bloody liquor four days after shunting, but Philipp's condition gradually improved. After four weeks the shunt became infected and had to be removed. An attempt to close the persisting ductus Botalli by drug therapy failed, but surgical closure was finally successful. At the age of 4 months, a plate was installed in his palate in preparation for the later surgical correction of the large cleft. Except for low thyroid function (for which he was treated with thyroid hormone) laboratory parameters were within normal limits, including peripheral blood counts. He was discharged from the hospital at age 5 months with the preliminary diagnosis of VACTERL association. There was an additional episode of increased intracranial pressure at age 8 months which required treatment. Growth retardation became apparent around age 1. Because of the bilateral atresia of his auditory canals, Philipp received a hearing device, and glasses were prescribed to improve strabism and poor eye sight. Also at age 1 his cleft palate was closed.

During the second and third years of his life, his pronounced statomotoric retardation was treated with physiotherapy. Developmental milestones were severely retarded. When he entered kindergarten at age 3 Philipp could neither speak, nor feed himself, nor walk, and he required assistance for upright sitting and standing. During the course of recurrent infections his thrombocyte counts were noted to decrease such that at age 5 the possibility of FA was considered

and quickly confirmed by cell cycle and chromosome breakage studies. Philipp belongs to the rare X-linked complementation group FA-B.

At the time of his diagnosis hemoglobin was 5.7 g/dl, thrombocytes were 20,000/μl, leukocytes were 3,300/μl. His blood counts continued to decrease, requiring regular thrombocyte and erythrocyte transfusions. A venous port catheter was implanted, but became infected and had to be replaced. Until age 6, Philipp received a total of 15 erythrocyte concentrates and 35 units of thrombocytes. Even though his thrombocyte counts returned to normal shortly after the transfusions, a week later his counts could be as low as 8,000/μl. Further complications were esophagitis and esophageal bleeding between ages 5 and 6. Because of his multiple impairments and poor health status, neither his parents nor the attending physicians considered HSCT as a viable option for Philipp, who requires full time care. In a recent issue of the German FA family news letter his parents write: 'If he feels well, Philipp likes to laugh and conveys the impression of alertness, happiness and interest in his environment. Despite all the physical, mental and emotional energy it takes to care for him, we get so much back from this very special child and we would not want to miss him'.

Dominik

Dominik (fig. 1) was born at 36 weeks of an uneventful pregnancy to healthy, non-consanguineous parents. Following spontaneous delivery, numerous congenital abnormalities were noted: partial right radial ray aplasia, aplasia of the right thumb, hypoplasia of the left thumb, membranous duodenal atresia, hypospadia, hyperopia and strabismus, renal dysplasia with vesico-urethral reflux, severely stunted growth (weight and length under the 3rd percentile). Because of normal peripheral blood counts, the child was thought to be affected by a sporadic developmental disturbance (VATER-association). During the following years, Dominik was repeatedly hospitalized for the surgical repair of his duodenum, thumbs, kidneys and urethra. Despite recurrent urinary tract infections and progressive renal insufficiency, he continued to grow along the 3rd percentile. Low thrombocyte and erythrocyte counts were first noted at age 3, and the clinical suspicion of FA was confirmed by cell cycle and chromosome breakage studies. Dominik carries the mutation c.1650delT (exon 8) in *FANCB* on his X-chromosome.

He was started on oxandrolone therapy (25 mg/day) with excellent response of his blood counts. Side effects were signs of virilization and sonographic changes of liver parenchyma, but liver enzymes and AFP remained within normal limits. After one year of androgen therapy his blood counts began to decline such that, starting at age 6, he required weekly thrombocyte transfusions.

Erythrocyte transfusions were required every third week. Because of obvious non-response, oxandrolone was discontinued and briefly replaced by erythropoietin, however without any success. Bone marrow biopsy showed high grade hypoplasia with myelodysplastic changes indicative of MDS. There were 4–5% blasts, and bone marrow cytogenetics revealed monosomy 7.

Since his older sister was available as HLA-compatible donor, Dominik underwent bone marrow transplantation. Because of his renal insufficiency he had to be continuously dialyzed, but there were no other complications. Two years after HSCT he received a renal transplant that was likewise successful. With the help of a tutor, Dominik was able to complete 4 years of elementary school. Since he always was last in his class, he transferred to a school for the handicapped where he belonged to the top students. At 6 years after HSCT and 4 years after renal transplant, Dominik had stable renal function and normal blood counts. Growth hormone therapy was started at age 12. Today, at age 13, Dominik still is on immunosuppressive medication but leads a nearly normal life.

Michael

Even though Michael (fig. 1) was born at term, he was small for date, weighing 2,250 g and measuring 45 cm. He displayed a number of birth defects, including bilateral radial ray and thumb aplasia, coloboma of the right eye, and rightsided inguinal hernia. Feeding was difficult with frequent regurgitation requiring a gastric tube with individual meals lasting up to 2 h. A membranous narrowing of his esophagus was finally diagnosed as cause of his problems which improved after surgical correction. But because of continuous difficulties to swallow, Michael required mainly liquid or semi-solid foods until the age of 23. Failure to thrive prompted an investigation of his growth hormone levels at age 3, but results were normal. The diagnosis of FA was considered at age 4 and confirmed by chromosome breakage studies but his complementation group was never determined. Because of poor vision he required glasses from age 6. A tendency to bleed and cytopenia became apparent at age 9. Michael was treated with cortisol for two weeks which he did not tolerate well. There were multiple episodes of esophageal bleedings which were thought to be due to his low thrombocyte counts, but esophageal varices were diagnosed at age 23 together with 'fibrosis' of his liver. Attempts to stop the bleeding episodes by sclerosing the varices were only partly successful. Despite these recurrent medical problems, Michael did well in school and obtained his baccalaureate and his driving license. At age 24 Michael was diagnosed with pituitary dysfunction, hypogonadism and hypothyroidism. From there on, he was treated with anabolic steroids, growth hormone and thyroxin. At age 28 his height was 152 cm and he

weighed 46 kg. He developed diabetes mellitus which was controlled with oral medication. Under continuous androgen therapy his blood counts remained rather stable, with normocytic anemia (hemoglobin 10.9 g/dl) and thrombocytes the order of 93,000/μl. He required no more than 12 erythrocyte transfusions during his entire lifetime.

At age 31, Michael had to undergo bilateral cataract extraction, laser coagulation of the retina of his right eye, and surgical resection of a malignant bone tumor of his right jaw. He nevertheless finished his apprenticeship, obtained a license as a motor boat pilot, and joined the administrative staff of the local state opera. A year later he experienced increasing pains of the neck and was diagnosed with advanced squamous cell carcinoma of the lateral and posterior oropharyngeal wall extending to the base of his skull that had escaped detection on prior routine examinations. He underwent a heroic 14 h operation with subsequent radiotherapy. After he had received a total dose of only 8 Gy, the radiotherapy had to be discontinued because of severe thrombocytopenia which could not be controlled. Michael died at age 32 from the complications of surgery, radiotherapy and pneumonia.

Marleen B.

Marleen (fig. 2) was born two weeks prior to her due date with hypoplastic thumbs and a number of fairly large café-au-lait spots. After her first feeding, she suffered from intestinal tract obstruction due to annular pancreas requiring immediate surgical correction. Because of her anomalies, a chromosome analysis was ordered yielding a normal female karyotype. All additional investigations were non-contributory. A first low blood count was observed at age 6, but, because of a recent episode of infection, was considered transitory and not followed up with additional tests. However, upper respiratory tract infections increased in frequency and severity over the next year, and repeated blood tests showed a decrease in all three blood cell lineages such that the diagnosis of FA was finally considered and confirmed by the appropriate laboratory tests. Marleen B. belongs to complementation group FA-A and is heterozygous for the *FANCA* mutation c.4010+(1-18)del. The other mutation is unknown.

Marleen quickly became transfusion dependent, and attempts to prevent her bone marrow failure by androgen and cortisol treatments were unsuccessful. To make things worse, AML was diagnosed at age 8.5. Although treatment with thioguanin was successful in eradicating her blast cells, it led to rapid deterioration of her bone marrow function. Aspergillosis of her lungs was treated with granulocyte transfusions (collected from her relatives), but her condition remained critical. A matching donor could not be found within the available

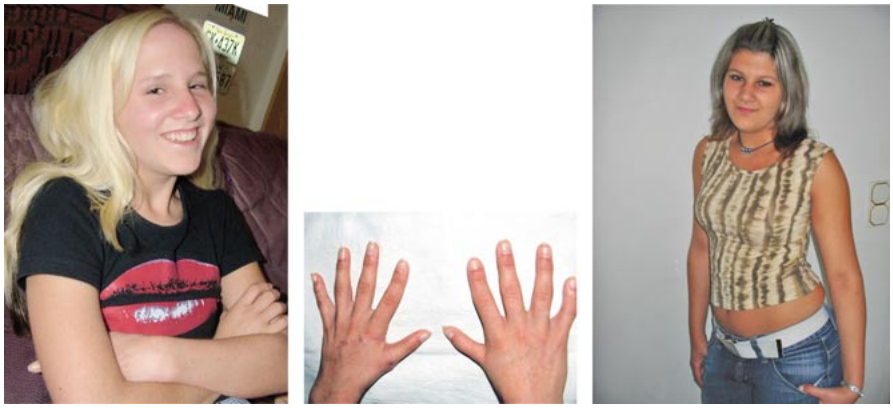


Fig. 2. (left) Marleen B. at age 13; (center) her hands after surgery (left thumb and index finger); (right) Marleen S. at age 15.

time such that her physicians were forced to use her father despite 2 mismatches of his HLA surface antigens. Surprisingly, the bone marrow of her father engrafted rapidly and without any complication. Much sooner than anticipated she could be discharged with stable blood counts and in good general health. Today, seven years after transplant, she is still healthy and lives a full life. She opted for surgical correction of one of her thumbs (fig. 2) and trained the other so well that she has excellent manual function. Because of proven growth hormone deficiency, she was started on growth hormone therapy at age 12 which led to a regular adolescent growth spurt. A very satisfying story for all involved.

Marleen S.

Marleen (fig. 2) was delivered three weeks past her due date via C-section. She weighed 2,750 g. During her second day of life she underwent surgery for esophageal atresia type IIIb that was successfully corrected. There were no complications, and there was no evidence for any other congenital defect. Her developmental milestones were normal, and she started to use her first words at the age of 18 months. However, she was difficult to understand and further investigations revealed a hearing impairment due to middle ear malfunction. A tympanoplastic procedure as unilateral correction (on her left side) was attempted, but without notable success. She therefore received bilateral hearing aids which she still uses today. Supportive logopedic training improved her speech and hearing.

Somewhat lower than normal blood counts were first noted during preparation for ear surgery at age 4. A control investigation at age 5 yielded the following results: hemoglobin 10.4 g/dl, leukocytes 3,300/μl, thrombocytes 70,000/μl. Neutrophils were low with 10%. During the following months, hemoglobin rose to 12 g/dl and neutrophils to 35%. There were no infections such that no measures were taken. Chromosome analysis at that time revealed a normal female karyotype, but there were no chromosome breakage studies.

In the meantime a horseshoe kidney and low-grade microcephaly had been additionally diagnosed, but it took until age 6 before the possibility of FA was considered and confirmed by cell cycle studies. Marleen turned out to belong to group FA-A, carrying the *FANCA* mutation c.1567-1G>C in a homozygous or heterozygous state. In addition to the findings described above, a thorough physical examination at age 6.75 years noted a brownish skin color of her trunk and freckling of her left axilla. Except for slightly decreased blood counts, all other investigations were within normal limits, including bone marrow cytology. At that time her height was 119 cm (50th percentile), her weight was 23 kg (between the 50th and 75th percentile), and the final report emphasizes her excellent health. A heart murmur was noted at age 12 which turned out to be due to a persisting ductus arteriosus Botalli. Angioplastic closure was uneventful.

Contrary to most other FA patients, Marleen's blood counts remained stable over the years such that she needed no further intervention. Annual bone marrow biopsies gave only normal results without evidence for cytogenetic changes. At ages 13 and 14 three hundred ml of bone marrow were obtained under general anesthesia, frozen and deposited as reserve for future autologous transplantation and/or gene therapy. Interestingly, there was further improvement of her blood parameters in the wake of these procedures. Neither cell cycle nor chromosome breakage studies provided evidence for somatic mosaicism. Now at age 15, Marleen leads a normal teenage life. Her only handicap is her hearing impairment which she controls with her hearing aids. She is an example of a very mild clinical course despite several congenital anomalies.

Sarah Ninja and Valeska

One of the unsolved questions in FA relates to the observation of discordance of phenotype and clinical course among siblings with identical mutations. In this context we summarize the clinical histories of two sisters belonging to complementation group FA-A, both compound heterozygous carriers of the mutations: c.1360_1826del467 pat (genomic deletion including exons 15–20), and c3788_3790delTCT mat (p.F1263del). Table 1 compares phenotypes and clinical course of the two sisters who were born at term with similar birth

Table 1. Divergent course of the disease among siblings with identical mutations

Clinical parameter	Sarah Ninja	Valeska
Adult height	138 cm	154 cm
Adult weight	41 kg	65 kg
Radial ray anomalies	both thumbs, moderately severe	single thumb, mild
Café au lait spots	+++	++
Diagnosis of FA	age 7.5	age 2 (because of sister)
Start of androgen treatment	age 8	age 8 (following an episode of osteomyelitis)
Duration of androgen treatment	12 years (due to large size adenomas at age 9 stepwise reduction to 0.2 mg/kg/day after age 15)	12 years (2 mg/kg/day for 3 years, 0.5 mg/kg/day for 2 years and 0.1 mg/kg/day for 7 years)
Liver adenomas	+++++	none
Transfusion dependency (erythrocytes)	age 5.5	age 19
Transfusion dependency (thrombocytes)	age 9	none
Duration of transfusion dependency	13 years	<1 year
Lifetime total of transfusions	>700 (300 ery./400 thrombo.)	<15 (erythrocytes only)
Profuse bleeding episodes	multiple, for many years (nose and gingiva)	few
Episodes of physical weakness, forced inactivity	multiple and severe	none
Infections	multiple and severe	few
G-CSF treatments	starting at age 15 (neutrophils below 500)	none
Clonal bone marrow changes	from age 15, multiple clones, chromosome 3q affected	from age 19, single monosomy 7 clone
Age at and cause of death	21 years, multiorgan failure due to irreversible pancytopenia	20 years, severe infections and brain hemorrhage after alternate donor HSCT

weights (2.5 kg Sarah Ninja, 2.2 kg Valeska), similar low head circumference (32.5 and 31 cm, respectively) and similar body length (46 and 47 cm, respectively). Overall, the sisters' phenotypes were relatively mild with short stature, moderate microcephaly, thumb anomalies, and pigment spots as the only manifestations pointing towards FA (fig. 3).

Sarah Ninja experienced a severe clinical course with repeated life-threatening episodes of epistaxis, very severe anemia, thrombocytopenia and neutropenia, leading to early and life-long transfusion dependency and recurrent infections requiring antibiotics and, from age 15, regular G-CSF treatments. She received

Fig. 3. Sarah Ninja at ages 6 (left) and 19 (center); Valeska at age 17 (right).

the diagnosis of aplastic anemia at age five, and the diagnosis of FA at age 7.5. Her younger sister Valeska showed a much milder course. Other than Sarah Ninja, who had been treated with transfusions for 2.5 years prior to starting her on androgens, Valeska received androgen therapy prior to the onset of transfusion dependency. After two years, her androgens (oxymetholon, 2 mg/kg/day) were reduced stepwise over a period of several years to a maintenance dose of 0.1mg/kg/day. Although there were signs of virilization, Valeska never experienced any severe complications of androgen therapy (large liver adenomas) that forced a stepwise reduction of androgen therapy to a minimum dose of 0.1 mg/kg/day in Sarah Ninja. Until her final year of life when the appearance of a monosomy 7 clone in her bone marrow mandated preparation for HSCT, Valeska had sufficient bone marrow function to lead a normal and very active teenage life. In contrast, from her childhood years on, Sarah Ninja's quality of life was impaired, with many intermittent episodes of severe distress and near-fatal medical complications due to severe bleedings and/or infections. Without the dedicated care of her family and her physicians she would have fallen victim to her refractory bone marrow failure at a much younger age. It is very tragic indeed that the younger sister Valeska, having been much less affected by the disease throughout her lifetime, succumbed to the complications of alternate donor HSCT and thus died at an even younger age than her more severely affected older sister.

The medical histories of these two siblings illustrate several points: (1) clinical course and quality of life can be very different among affected individuals, even among siblings carrying identical mutations, (2) in the case of the two sisters, the quality of life depended above all on the degree of residual bone marrow function and/or responsiveness to androgen therapy, (3) whereas bone marrow failure is, within obvious limits, amenable to dedicated medical and family care, the appearance of cytogenetically aberrant bone marrow clones, in particular trisomy 3q and/or monosomy 7, may herald leukemia which leaves HSCT as the only but decidedly high risk option.

Concluding Remarks

The first patient among our case reports illustrates the most severe form of Fanconi anemia, with severe pre- and postnatal growth retardation, failure to thrive, a multitude of malformations and malfunctions, including severe cognitive impairment which otherwise is rare. Such a child requires 24 h medical and family care, and his or her life expectancy is severely limited by the multisystem disease. Despite these severe handicaps, his family felt that caring for Philipp is a demanding but likewise rewarding experience. The other patients were far less affected, but the majority nevertheless required life-saving surgery as newborns or at some point later in their lives. Surgery included correction of esophageal atresia, esophageal membranes, pancreas annulare, duodenal atresia, urethral obstruction, anal atresia, heart, eyes (cataract extraction), middle ear, and thumbs. Without these surgical interventions, either these patients would not have survived or their quality of life would have been severely impaired.

Pharmacological intervention consisted mainly of androgen therapy which stabilized blood counts in half of the patients, in the case of Valeska as long as 12 years. Growth hormone was helpful in three of the patients with proven growth hormone deficiency, and G-CSF was evidently useful in cases of severe granulocytopenia. With the exception of Marleen S. who never required transfusions, blood product substitution became mandatory in the majority of patients at some point, amounting to more than 700 transfusions in the case of Sarah Ninja or to only 13 transfusions in the case of Michael who died at age 32 from squamous cell carcinoma, the most feared solid tumor in FA patients. Programs and methods for early detection and prevention of SCC must be one of the highest priorities in FA medical research. Michael is also an example of endocrine deficiencies which seem to develop much sooner in FA patients than in ‘normal’ aging, including pituitary dysfunction, hypothyroidism, hypergonadotrophic hypogonadism and diabetes mellitus.

The case history of Dominik illustrates that HSCT is the therapy of choice if a matching sibling donor is available, and that additional medical complication, like kidney failure, can be overcome once bone marrow function is restored. AML as the most frequent hematopoietic malignancy in FA struck Marleen B. at age 8.5, and despite many concerns a partially mismatched transplant from her father engrafted well, a perfect example of the beneficial nature of a ‘graft versus leukemia reaction’. With additional growth hormone therapy and hand surgery her quality of life is excellent. This is also the case for Marleen S. despite the fact that she had to undergo surgery for esophageal atresia, for middle ear problems and for persisting ductus Botalli. Marleen S. is an example of FA patients whose low blood counts remain stable over many years

without any specific therapy, and without evidence for somatic reversion of one of her constitutional mutations.

That siblings with identical mutations can show divergent phenotypes and a divergent course of their disease has been noted and reported before [e.g. reference 4]. In the case of Sarah Ninja and Valeska this divergence may in part have been due to the protracted diagnosis of Sarah Ninja who had to be treated for aplastic anemia with blood products very early in life, 2.5 years prior to starting her own androgen therapy. Being aware of the diagnosis in the family when the younger sister Valeska began to show signs of incipient bone marrow failure, she was treated with androgens right away and she did not respond with the massive adenomatous growth in the liver that forced drastic reduction of androgen therapy in Sarah Ninja's case. We still are largely ignorant why adult height and weight, bleeding episodes, infections, medical emergencies and overall quality of life were so different between the two sisters carrying identical mutations. As postulated for the divergence of ages-of-onset of chorea Huntington [5] polymorphisms in genes that interact or are associated with the affected FA gene might provide some explanation but remain to be identified in the case of FA. Finally, the case history of Valeska reminds us that despite undoubted progress (see Chapter by Eyrich et al.) alternate donor HSCT remains a high risk procedure, particularly if coincident with clonal bone marrow changes, preexisting chronic infections, and adulthood.

Altogether, we hope this brief presentation of case histories will introduce the non-specialist reader to the impressive spectrum of phenotypic manifestations and the widely divergent clinical courses of FA. There is no doubt that medical interventions and devoted parental care have improved the quality of life for the great majority of FA patients. There is a clear bias in the literature and in physicians' minds in favor of severe cases such as represented by Philipp among our case histories, because children with severe growth deficits and malformations are easily recognized and diagnosed. Likewise, our sample of case histories by necessity lacks the description of cases that lead a normal or close to normal life as adults, and who may be diagnosed only in case of malignancy, late onset bone marrow failure, or a sibling affected with FA [e.g. references 6 and 7]. We personally know of at least two adult individuals without any congenital malformations and completely or close to normal blood counts who carry the diagnosis of FA (based upon family history and chromosome breakage studies) but lead a normal working and family life. Working out genotype-phenotype correlations in such extreme cases, either mild or severe, possibly by studying polymorphisms in FA-related genes, will remain one of the great challenges in FA research in the years to come.

Acknowledgements and Dedication

The authors and editors are deeply grateful to the patients and their families who consented to the publication of their first names, medical histories and photographs with the explicit wish to inform the readers of this volume about the many faces of Fanconi anemia. They would like to dedicate this paper to the memory of Michael, Sarah Ninja, Valeska and all the other FA patients who lost their heroic struggle against a deadly disease but who remain alive in our hearts.

References

1 Joyce O: Fanconi Anemia. Standards for Clinical Care. Fanconi Anemia Research Fund Inc., Eugene, Oregon, 1999.
2 Frohnmayer L, Frohnmeyer D: Fanconi Anemia. A Handbook for Families and Their Physicians. ed 3. Eugene, Oregon, Fanconi Anemia Research Fund Inc., 2000.
3 Fanconi Anämie. Ein Handbuch für Eltern, Patienten und ihre Ärzte. Translated version and extension of reference 2. Deutsche Fanconi-Anämie-Hilfe e.V., Unna, 2005.
4 Schoof E, Beck JD, Joenje H, Doerr HG: Growth hormone deficiency in one of two siblings with Fanconi's anemia complementation group FA-D. Growth Horm IGF Res 2000;10:290–294.
5 Metzger S, Bauer P, Tomiuk J, Laccone F, Didonato S, et al: The S18Y polymorphism in the *UCHL1* gene is a genetic modifier in Huntington's disease. Neurogenetics 2006;7:27–30.
6 Kwee ML, van der Kleij JM, van Essen AJ, Begeer JH, Joenje H, et al: An atypical case of Fanconi anemia in elderly sibs. Am J Med Genet 1997;68:62–66.
7 Huck K, Hanenberg H, Gudowius S, Fenk R, Kalb R, et al: Delayed diagnosis and complications of Fanconi anemia at advanced age – a paradigm. Br J Haematol 2006;133:188–197.

Ralf Dietrich
Deutsche Fanconi-Anämie-Hilfe e.V.
Böckenweg 4
D–59427 Unna (Germany)
Tel. +49 23 08 21 11 or 23 24, E-Mail ralf.dietrich@fanconi.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 23–38

Milestones in Fanconi Anemia Research

M. Digweed[a], *H. Hoehn*[b], *K. Sperling*[a]

[a]Institute of Human Genetics, Charité – Universitätsmedizin Berlin, Berlin,
[b]Department of Human Genetics, University of Würzburg, Würzburg, Germany

Abstract

The recessive disease Fanconi anemia (FA) is a prototype chromosome instability syndrome which shows a high level of spontaneous and induced chromosomal aberrations in combination with a significantly increased cancer risk. Thus, the underlying defect must be directly or indirectly involved in a fundamental cellular task of long-lived mammalian cells, the maintenance of genomic integrity. In addition to the delineation of the FA clinical and cellular phenotypes, prominent milestones in FA research include proof of extensive genetic heterogeneity, identification of (so far) 12 disease genes, and elucidation of the FA pathway involved in the repair of crosslinks at arrested replication forks. What is referred to as the FA/BRCA pathway represents only part of a complex network of protein-protein interactions which is far from being understood. The ultimate milestone in FA research would be the achievement of an individualized and curative therapy. Currently, hematopoietic stem cell transplantation is a promising but still high-risk therapy, and gene therapy is still at the experimental stage. Nonetheless, in a significant proportion of patients, a kind of 'natural gene therapy' can be observed which results from intragenic recombination or from compensating second site mutations. The elucidation of these somatic events and their underlying mechanisms can be considered a milestone in genetic research. The remarkable progress in FA research during the last 10–15 years was fostered by the foundation of FA patient support groups in many countries. These support groups are gratefully acknowledged as important motivational milestones in FA research.

'Many phenomena that basic research tries to explain would simply be unknown had they not been uncovered by the study of diseases. Phenomena such as spontaneously enhanced chromosome instability in Fanconi anemia or Bloom syndrome with all their consequences for somatic mutation and cancer formation were discovered accidentally in the process of examining certain patients for diagnostic reasons'. This statement in the introductory chapter of Vogel and Motulsky's famous textbook, 'Human Genetics – Problems and Approaches' [1]

is still valid today. Currently, following the identification of 12 different genes leading to the Fanconi anemia (FA) phenotype, the statement can be extended to include the understanding of basic biological principles related to the maintenance of genome stability, the elucidation of relevant pathways and their interconnection in genetic networks. Here, a brief and necessarily subjective review is given of what most researchers in the field might consider milestones in FA research. More details are presented in subsequent articles of this volume and in a number of excellent reviews [2–9].

The Foundations of FA Research: Clinical Observations

In 1927, the Swiss pediatrician Guido Fanconi (1892–1979) first described 3 brothers with a specific combination of bone marrow failure (pancytopenia) and various physical anomalies, such as short stature, hypogonadism and hyperpigmentation, pointing to a new autosomal recessive disorder [10]. Generally, the substantiation of a new genetic disease is more likely to be based on particularly severely affected individuals, as these individuals receive preferential medical attention. As a consequence, the disease in FA index cases is regularly more severe than in their affected sibs [11]. Only around 70–75% of FA patients display the 'classical' FA phenotype which consists of short stature in combination with a typical spectrum of congenital defects, including radial ray defects, pigmentary changes, urogenital malformations, atresia of the external ear canal, and many others [12]. Endocrine abnormalities reminiscent of premature aging occur in nearly all FA patients with developmental defects. They include hyperinsulinemia, growth hormone insufficiency and hypothyroidism [13]. Reproduction is severely impaired due to failure of spermatogenesis in males and a low pregnancy rate in females [14].

Following the initial and follow-up descriptions by Fanconi himself [10, 15], a landmark paper in FA research was the publication by Gmyrek and Syllm-Rappoport in 1964 who reviewed phenotypic features and clinical course of 129 patients [16]. Much of what we know today about the spectrum of congenital malformations and course of the disease is already mentioned in this paper. From a European perspective, another landmark publication was the 1976 paper by Traute Schroeder and colleagues, entitled 'Formal Genetics of Fanconi's Anemia' [17]. Based upon an extensive collection of pedigrees, the authors provided unequivocal proof of autosomal recessive inheritance despite the surprising fact that none of the families examined showed evidence for consanguinity. There was a slight preponderance of affected males which retrospectively would be compatible with a small proportion of X-linked recessive cases which we now know are due to mutations in the *FANCB* gene, the only FA

gene located on the X-chromosome. Because the authors noted a high intrafamilial correlation for age of onset and for the severity of malformations, they correctly predicted genetic heterogeneity and stated that 'apart from the standard type, an especially mild type with late onset, few malformations, and a relatively benign course seems to exist' [17]. They could not have been more correct, since today we are well aware that 'mild' mutations exist in some of the FA genes, and that the phenomenon of revertant mosaicism may mitigate the clinical course. Around 1982, Arleen Auerbach started the Fanconi Anemia International Registry in the United States which has made a number of landmark contributions to our understanding of the clinical and genetic heterogeneity of FA so far [18–22].

A definitive clinical milestone in FA research was the realization that patients exhibit a high risk for myelodysplastic syndrome (MDS), and for developing overt malignancies [23]. Leukemia is found in about 10% of younger patients (especially acute myeloblastic leukemia, AML), and solid tumors, particularly squamous cell carcinomas of the oral cavity and genital area have been reported in almost 10% of older patients. This, in combination with bone marrow failure, explains the significantly reduced life expectancy of FA patients. The cumulative incidence of solid tumors reaches 30% by the age of 45 years and remains the major threat to older FA patients [24, 25]. Since malignant growth is one of the possible endpoints of somatic mutations, the high cancer risk reflects the genetic defect in FA, which is related to the defective maintenance of genomic integrity. The discovery of spontaneous chromosomal instability as cytogenetic expression of the (at that time still elusive) genetic defect marks the first of the many experimental milestones of FA research.

Chromosomal Instability and Sensitivity to DNA Crosslinking Agents

On the time scale shown in figure 1, the first milestone following Fanconi's original description of three patients was the discovery of chromosomal instability. In 1964, Traute Schroeder and colleagues, then working at the University of Heidelberg, reported increased spontaneous chromosomal instability in blood lymphocytes of two affected brothers [26]. Less than a year later, this key observation was confirmed by the Zurich group of Werner Schmid, with Guido Fanconi himself as senior author of the paper [27]. The chromosomal aberrations described by the Schroeder and Schmid groups were mainly of the chromatid type and manifested at metaphase as chromatid breaks or chromatid interchanges. Obviously, these originate during the S-phase of the cell

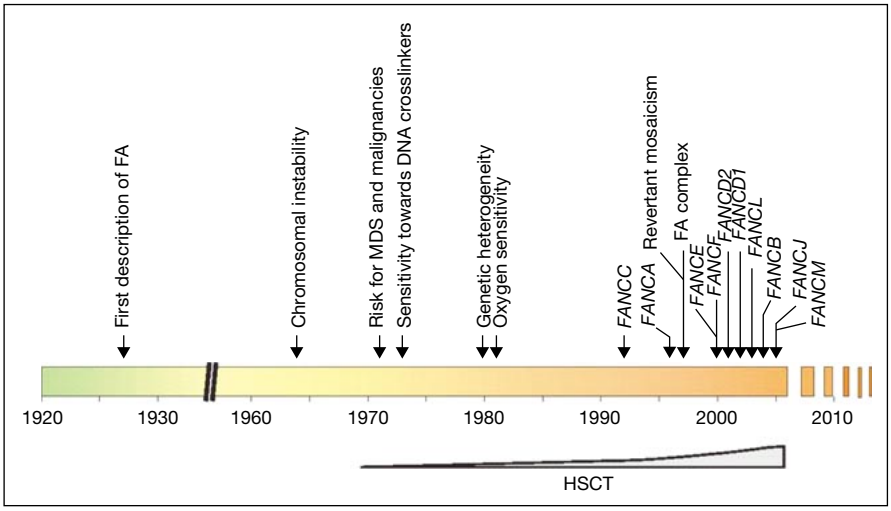


Fig. 1. Milestones in FA-research. The figure does not yet include *FANCN* as the most recently identified FA gene whose product proved identical to PALB2, a protein that functions downstream of FANCD2 as 'partner and localizer of BRCA2' [26, 77].

cycle and involve newly replicated DNA. Since each chromatid break is the visible manifestation of a DNA double-strand break, the characteristic chromosomal instability of FA indicates that the underlying defect in this genetic disease is directly or indirectly involved in a fundamental cellular process, the repair of DNA double-strand breaks. Almost at the same time, spontaneous chromosomal instability was also observed by James German and colleagues in Bloom syndrome [28]. While in FA cells the breakpoints of the interchanges are almost randomly distributed, in Bloom syndrome they preferably involve homologous regions, indicating the impairment of different DNA repair pathways [29]. Looking back at these early data from today's vantage point it is quite remarkable how careful observations at the cytogenetic level were able to predict molecular differences.

About 10 years after the discovery of spontaneous chromosomal instability as a cytogenetic hallmark of FA, Sasaki and Tonomura showed that the spontaneous chromosome instability in FA is associated with a high rate of induced chromosomal aberrations after treatment with DNA crosslinking agents [30]. The determination of cellular sensitivity towards crosslinking agents such as diepoxybutane, mitomycin C, cisplatinum or nitrogen mustard still serves as gold standard for the confirmation of the clinical diagnosis of FA [31]. Because of the highly variable clinical phenotype, each putative patient requires confirmation via the demonstration of increased in vitro sensitivity towards crosslinking

agents. As an alternative to chromosome breakage studies, crosslink sensitivity can also be assessed via cell cycle analysis [32].

The term chromosome instability syndrome now covers an increasing number of recessive disorders which share a high level of spontaneous and induced chromosomal aberrations. However, the type of chromosome anomalies and the specificity of the clastogens vary considerably among the different disorders. For instance, in Ataxia telangiectasia and Nijmegen breakage syndrome, the break points of the spontaneous translocations and inversions in lymphocytes preferentially involve the T-cell receptor and immunoglobulin gene loci. Other than FA, these patients are highly sensitive to ionizing radiation, but they share a common characteristic of all chromosome instability syndromes which is a sharply increased risk of malignancy [8].

Analysis of Genetic Heterogeneity

In most organisms, the existence of (intergenic) heterogeneity in recessive traits can easily be studied by crossing pairs of mutants and analyzing their offspring. In the case of heterogeneity the progeny of such crossings has a normal phenotype due to complementation of the parental defects. In our species, family studies have only rarely provided evidence for the presence of distinct genes. The arrival of somatic cell genetics allowed researchers to fuse different human cell lines and thus perform experimental complementation studies in vitro. In FA, normalization of the characteristic hypersensitivity to crosslinkers in proliferating cell hybrids should indicate complementation, and thus prove the existence of genetic heterogeneity. This was first demonstrated by Zakrzewski and Sperling in 1980 after fusion of an SV40 transformed FA cell line with diploid fibroblasts derived from another patient. In contrast to the parental cells, the resulting cell hybrids did not show hypersensitivity to DNA cross-linkers proving mutual complementation of distinctive gene defects [33]. However, due to the inherent chromosomal instability and frequent chromosome loss caused by the SV40 genome, using SV40 transformed cell lines has severe shortcomings. These problems were overcome by using Epstein-Barr virus immortalized B lymphoblasts from different FA patients. EBV transformed lymphoid cell lines remain mostly diploid and can be sustained with different selectable markers for the isolation of cell hybrids [34]. Such cell fusion studies using lymphoid cell lines were first performed in the laboratory of Manuel Buchwald in Toronto and later on, most extensively, in the laboratory of Hans Joenje in Amsterdam. These tedious cell fusion studies were of fundamental importance for the ensuing progress of FA research, and the Amsterdam laboratory of Hans Joenje deserves credit for having provided evidence for at least 12 and possibly even

more FA complementation groups [9]. Reference cells lines for each newly described complementation group were generously provided by the Amsterdam group to laboratories all over the world, boosting FA research.

The genes underlying 12 complementation groups have been identified so far. Their cDNAs are extremely useful for assigning unclassified patients to a specific complementation group. For this, the relevant cDNAs are inserted into retroviral or episomal expression vectors, which are then transferred into the patient's cell line and tested for complementation [35]. This was first exemplified in 1997 by using a recombinant retroviral vector which stably integrates into the host genome [36]. In the following years, vector design and transfection efficiencies were substantially improved by the laboratories of David Williams and Helmut Hanenberg such that assignment of FA patients to the respective complementation groups has become part of diagnostic routine [37, 38]. Interestingly, a number of patient cell lines are not complemented by any of the known FA cDNAs rendering the actual figure of complementation groups higher than 12. Clearly, experimental demonstration and molecular proof of extensive genetic heterogeneity has been another major milestone in FA research.

Identification of FA Genes

As illustrated on the time scale of figure 1, the early nineties of the 20th century marked the beginning of a new, molecular area in FA research. The first FA gene identified in 1992 belonged to complementation group C and was termed *FACC* (FA complementation group C complementing). Since then, the nomenclature has changed and today is based on a truncation of the name Fanconi and the letter of the respective complementation group. Therefore the 12 FA genes are: *FANCA, FANCB* (syn. *FAAP95*), *FANCC, FANCD1* (syn. *BRCA2*), *FANCD2, FANCE, FANCF, FANCG* (syn. *XRCC9*), *FANCJ* (syn. *BRIP1*), *FANCL* (syn. *FAAP43, PHF9*), *FANCM* (syn. *FAAP250, KIAA1596*) and *FANCN* (syn. *PALB2*). Figure 2 shows the human chromosome map with the location of the 12 known FA genes. With the exception of *FANCC* and *FANCG*, which are both located on chromosome 9, and *FANCA* and *FANCN*, which are both located on chromosome 16, the other FA genes are spread over different chromosomes, including *FANCB* on the X.

The advent of the Human Genome project paved the way for the identification of human disease genes by the technique of positional cloning, reported for the first time in 1986 [39]. Positional cloning is based on the knowledge of a gene's location in the genome, the isolation of candidate genes from the critical region and the identification of mutations in a candidate gene in patient DNA. A prerequisite for the success of this approach is the availability of many

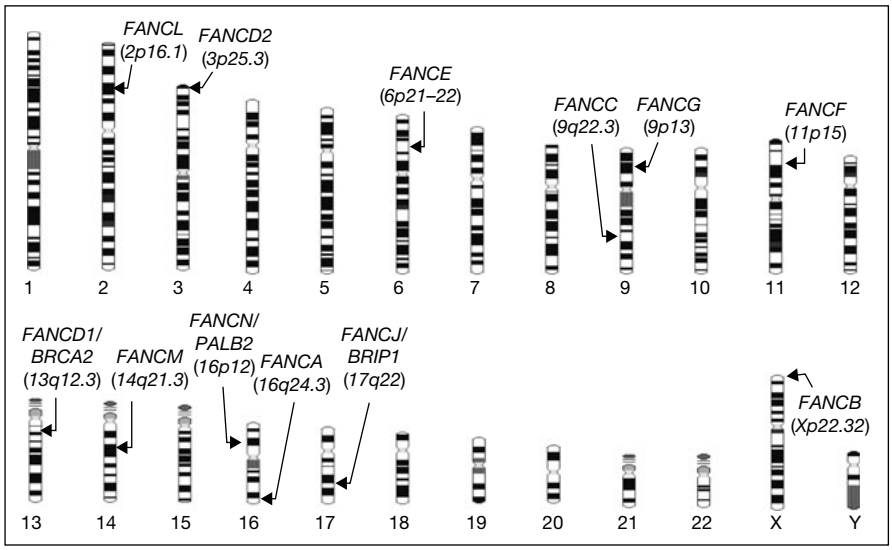


Fig. 2. Human karyotype map with locations of the 12 known Fanconi anemia genes. Figure taken from reference [75], with permission.

affected individuals, usually from different families, but belonging to the same complementation group. Thus, due to the extensive genetic heterogeneity, this approach has obvious limitations in FA. In contrast, the older technique of 'functional cloning' of a disease gene depends on fundamental information about the basic biochemical defect and – due to our ignorance – is rarely applicable. In the pre-genomic era, many different biochemical lesions have been attributed to FA cells, including alterations affecting DNA ligase activity, the intracellular distribution of topoisomerase, UV excision repair or cellular NAD^+ levels. Today we know that these were only secondary phenomena.

Expression cloning is a form of functional cloning which does not require knowledge of the primary defect. In the case of FA it is based on the transfection of cells with a normal cDNA library or, alternatively, fusing patient cells with so called mini cells containing different human chromosomes/chromosomal regions. Successfully complemented cells are not longer sensitive to cross-linkers since they now contain a functional FA cDNA. The next step is the identification of the complementing cDNA/genomic DNA. This approach led to the cloning of the first gene underlying Fanconi anemia (*FANCC*) in 1992 by Manuel Buchwald's group in Toronto, opening up the molecular avenues in FA research [40]. Most of the other FA genes were identified by the expression cloning approach (*FANCA, FANCE, FANCF, FANCG,* and *FANCD2*). *FANCA* was also independently identified by positional cloning, as was *FANCJ*.

Based on the observation that many FA proteins form a complex together with other unidentified proteins, FAAPs (Fanconi Anemia Associated Proteins) that could be isolated by immunoprecipitation, three additional FA genes were identified. Using this biochemical approach, Weidong Wang collaborating with the Joenje group showed that FAAP43, a protein with a molecular weight of 43 kDa, was absent in the only patient belonging to complementation group L, thereby defining FAAP43 as the elusive *FANCL* gene [41]. Subsequently, the FA-associated proteins FAAP95 and FAAP250 were shown to be defective in patients belonging to complementation groups B (X-linked) and M [9].

Altogether, these studies confirmed that each complementation group corresponds to a distinct FA gene – with one exception. The exception is group D, which comprises the *FANCD1* and the *FANCD2* genes. This heterogeneity is not well understood, but might be due to the fact that *FANCD1*, in contrast to almost all other FA genes, acts downstream of *FANCD2* which might have affected the results of the initial complementation studies. Most importantly, *FANCD1* was subsequently shown to be identical to *BRCA2* by systematic screenings of FA patients for mutations in *BRCA2* [42]. It came as a great surprise, of course, that *FANCD1* corresponds to *BRCA2*, a prominent cancer gene known to be involved in homology directed DNA repair via its association with the RAD51 recombinase. A single patient initially assigned to complementation group H was later shown to carry biallelic mutations in *FANCA* such that the reference cell line had to be reassigned to group FA-A [43].

In many of the FA genes no functional domains are apparent in the protein sequences and no strong homologies exist in nonvertebrate species. Consequently, their biochemical function has remained obscure. One exception was the *FANCG* gene which was shown to be identical to *XRCC9*, a gene known to be involved in DNA repair [44]. The situation changed completely when in 2001 the groups of Markus Grompe and Alan D'Andrea discovered the *FANCD2* gene which is highly conserved in plants (*A. thaliana*), nematodes (*C. elegans*), and insects (*Drosophila*), indicating the possible conservation of a 'basic' FA pathway in lower organisms [45]. In addition, two of the most recently identified FA genes, *FANCJ* and *FANCM*, are identical to conserved genes with known DNA maintenance functions. FANCJ is identical to the BRIP1/BACH1, a DNA helicase, which interacts with BRCA1. FANCM has homology with both helicases and endonucleases, including the archaeal Hef protein. Another conserved FA gene, *FANCL*, has a ring finger motif that is typical for E3 ubiquitin ligases [9].

Most FA patients belong to the complementation groups A, C and G. However, there are also ethnic differences, which are mainly due to founder mutations. Thus, most FA patients in the Afrikaans-speaking population in South Africa belong to group A [46], whereas in the Ashkenazi-Jewish population,

group C is most frequent [47]. Interestingly, there is no strong correlation between a given complementation group and a given clinical phenotype, simply because different mutations in the same FA gene can lead to strikingly different phenotypic consequences. As a rule, null mutations lead to more severe and earlier disease manifestation than mutations that result in a partially functional gene product [5]. There is no doubt that identification of each single FA gene represents a milestone in FA research, both with respect to the practice of medical genetics and the understanding of FA gene and protein function.

Elucidation of FA Pathways and Networks

Elucidation of the FA pathway should link the clinical and cellular FA phenotype to the mutations in the affected genes. However, recent insights into the molecular pathophysiology of FA have indicated an enormous degree of complexity in which individual pathways are only parts of a network of protein-protein interactions. Nonetheless, the most important advances in medical research are often obtained by reducing complexity to basic principles. In the case of FA, one major unanswered question is how chromosome instability is linked to the susceptibility of FA cells to DNA crosslinking agents.

Some important milestones in this research were:

- The proof in 1997 that the FA proteins, A and C, form a nuclear complex and the subsequent realization that the FA proteins B, E, F, G, L, and M are also part of this core complex [48, 49].
- The discovery that the FANCD2 protein takes a central position in the FA pathway. The FANCD2 protein is activated by the addition of a ubiquitin molecule in response to DNA damage [50].
- The finding that the FANCD2 protein directly interacts with known DNA repair proteins such as BRCA1 and BRCA2/FANCD1, linking DNA double-strand-break (DSB) repair with DNA cross-link repair [50, 51].

It is now well established that each of the proteins that are assembled to the FA core complex is needed for monoubiquitination of FANCD2. The catalytic function is obviously provided by the FANCL protein with its E3 ubiquitin ligase domain. Thus, all these 8 proteins act upstream of the evolutionarily conserved FANCD2 protein. The posttranslational modification of FANCD2 appears to be crucial for its activity. Proficient monoubiquitination is easily recognized by the appearance of a larger FANCD2 isoform (FANCD2-L). Western blotting thus permits convenient subtyping of FA cell lines, since patients with defects in any of the core complex genes fail to monoubiquitinate FANCD2 and display only the small (FANCD2-S) isoform [52]. Activated, intact FANCD2 protein is targeted to discrete nuclear foci. The combination of proteins

comprising these foci varies with the cell cycle and the type of DNA damage. FANCD2 co-localizes in these foci with FANCD1/BRCA2 and RAD51 (proteins involved in error-free repair) or BRCA1 and RAD50 (proteins involved in error prone repair). FA foci are found in S-phase cells and after treatment with crosslinkers and ionizing radiation. Thus, the association with proteins involved in DNA repair indicates that the FA pathway is directly involved in the DNA-damage-response during S-phase [5, 9].

Following DNA damage, the FA core complex directly binds to chromatin, whereby the FANCM protein with its helicase motif seems to be essential for recognizing crosslinked DNA [53]. It has been speculated that the activated core complex stabilizes arrested replication forks blocked by crosslinks, thereby promoting repair via DNA double-strand-break intermediates, either by translesion synthesis or by homologous recombination. FANCD1/BRCA2, FANCJ, and FANCN are involved in these repair processes and act downstream of FANCD2 in the FA pathway(s). Clearly, we are only just beginning to understand the complexity of FA mediated crosslink repair, and confirmation of theoretical models by biochemical studies is still very limited.

Moreover, it is still unknown how the high sensitivity of FA cells to chromosomal breakage by atmospheric oxygen is linked to the FA pathway [54]. Reducing oxygen tension in tissue culture incubators from 20% to 5% normalizes the cellular FA phenotype with respect to chromosomal breakage and G2-phase delay [55]. The molecular explanation of the obvious oxygen sensitivity of FA cells will represent another milestone in FA research. It also needs to be clarified whether, in the patients themselves, oxidative stress is a primary or secondary phenomenon, and to which extent oxidative stress contributes to the features of the FA clinical phenotype [56, 57].

What is the reason for the excessive apoptosis of bone marrow stem cells in FA patients resulting in hematopoietic failure? What is the functional relevance of the interaction of FANCC with STAT1, hsp70, NADPH cytochrome p450 reductase, FAZF, GRP94 and cdc2 [7], and in how many additional pathways are the other FA proteins involved? Clearly, the complexity of protein-protein interactions is bewildering and, in addition, depends on the specific stage of development and the individual tissue. The combination of reductionistic approaches with systems biology might lead to insight into these networks. It is safe to predict that the way to this goal will be paved with future milestones in FA research.

The Difficult but Promising Road to Therapy

Deeper insight into the molecular pathogenesis of FA will hopefully result in a better understanding of its pathophysiology, which is the prerequisite for a

rational, individualized therapy. From the patient's point of view, this would clearly be the ultimate milestone in FA research. Currently, there is no causal therapy for the genetic instability underlying the clinical manifestations of FA. In this situation, prenatal diagnosis is requested by couples at risk [58]. Both functional studies, e.g. determination of sensitivity towards DNA crosslinking agents, and direct FA gene mutation analysis can be performed with fetal cells. Although prenatal diagnosis is an important by-product of FA research, it merely represents a makeshift solution rather than a genuine therapeutic milestone.

A number of conventional treatments are available to combat bone marrow failure, including steroid and cytokine medications, but the first line of therapy is hematopoietic stem cell transplantation (HSCT), pioneered in FA among others by Elaine Gluckman and her coworkers [59]. As indicated graphically in figure 1, both the number of patients transplanted and the success of HSCT have greatly increased during recent years, mostly due to improvements of conditioning regimens and graft T-cell depletion [60]. Even with suitable donors HSCT still carries a relatively high risk. In this context, a key observation made in the laboratory of Heidemarie Neitzel has great impact on the difficult decision whether and when to subject a patient to transplantation. Neitzel and colleagues found that specific clonal chromosomal aberrations, most significantly gains of 3q, precede the onset of myelodysplasia and/or acute myelocytic leukemia [61]. Knowing whether a potentially devastating conversion to malignant cell growth is in the offing is an important step forward in the medical management of FA patients. In combination with the establishment of a rapid and sensitive FISH-assay [62], this has important consequences for early intervention and treatment of FA patients. Given a low a priori chance of 1 in 4, and given the small sizes of present day families, matching sibling donors are available in only a minority of affected families. In such a precarious situation, preimplantation genetic diagnosis (PGD) for the selection of an HLA identical embryo as potential stem cell donor has been proposed and performed [63, 64]. The first case involving an FA family made headlines worldwide, both positive and negative ('designer baby'). Clearly, PGD is neither a desirable milestone in FA research nor a breakthrough for patient care but rather an expression of the desperate situation of parents faced with limited therapeutical possibilities. Both the economical and psychological burden of PGD are tremendous and the success rate is, for biological reasons, very low such that PGD is unlikely to have a major impact on the medical care of FA families.

Despite considerable efforts, gene therapy is still at the experimental stage [65]. Nonetheless, in a significant proportion of FA patients a kind of 'natural gene therapy' can be observed. This is based on the observation that some patients have two sets of peripheral blood cells: MMC sensitive and

MMC insensitive [66, 67]. In some cases, the wildtype cells completely replace the defective ones. Skin fibroblasts, however, remain MMC sensitive. This self-correction, which ideally should take place in a hematopoietic stem cell [68] may more often than not lead to improvement of the hematological status [67, 69, 70]. In recessive diseases, such as FA, different molecular mechanisms have been shown to be instrumental in somatic reversion, including intragenic recombination, gene conversion, or back-mutation. In addition, partial restoration of protein function can be due to compensating mutations *in cis* of one of the affected alleles [66, 71]. The elucidation of these mechanisms can surely be considered a milestone in genetic research. A dogma of classical genetics was that recombination occurred only between genes but not within a gene. In the fifties of the last century, this dogma was rejected for prokaryotes and fungi, in the sixties, for *Drosophila*, and, in the nineties, for our species. Intragenic recombination is highly increased in compound heterozygotes with the chromosomal instability syndrome Bloom syndrome [72]. In FA the occurrence of reverted cells in peripheral blood indicates a likely proliferative advantage of reversed cells within the FA bone marrow. In addition, analysis of the clonal expansion of these stem or progenitor cells is expected to give insight into their proliferating potential, which is of relevance for somatic gene therapy in general. Unfortunately, in the murine FA knockout mouse models, hematopoiesis is almost unaffected and tumor incidence is not increased [73, 74]. Thus, with respect to the development of somatic gene therapy and the understanding of FA pathophysiology, these models are only of limited value.

The transition from the cellular to the (molecular) genetic approach about 15 years ago opened completely new perspectives for FA research and gave exciting new insights into basic problems in cell biology. We now know that the FA family of genes plays an important role in the maintenance of genomic stability, and thereby in the prevention of cancer and premature aging. This progress was accompanied and promoted by the foundation of FA patient support groups in many countries. Their goal is to catalyze and support scientific research on Fanconi anemia. In the US, under the guidance of Lynn and Dave Frohnmayer, the Fanconi Anemia Research Fund has raised considerable sums for FA research. In addition, the FARF, as it is known, has edited several brochures for FA patients and their physicians, provides newsletters and information on research projects, and organizes annual meetings both for scientists and FA families. Parallel to this development in the USA, Ralf Dietrich founded a patient support group in Germany with aims and activities similar to those of FARF. The enormous contribution of the Deutsche Fanconi-Anämie-Hilfe e.V. to progress in the understanding of FA can hardly be overstated. As scientists we greatly appreciate the support and motivation provided by the family

support groups. As such, each of these groups clearly represents a most welcome milestone in the common quest to improve the life expectancy and quality of life of FA patients.

Note Added in Proof

The elusive gene underlying complementation group FA-I (Levitus et al., 2004) has been identified as a paralog and binding partner of FANCD2, bringing the total number of identified FA genes to 13. FANCI was detected as an ATM/ATR kinase target protein required for resistance to mitomycin C. Like FANCD2, the newly detected FANCI protein is monoubiquitinated at a highly conserved lysine residue. In the chain of events leading to DNA crosslink repair, FANCI and FANCD2 appear to form an interdependent complex ('ID-complex') required for mutual ubiquitination, activation and association with chromatin (Smorgorzewska et al., 2007).

References

Levitus M, Rooimans MA, Steltenpool J, Cool NF, Oostra AB, et al: Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood 2004;103:2498–2503.

Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald ER III, Hurov KE, et al: Identification of the FANCI Protein, a monoubiquitinated FANCD2 Paralog required for DNA repair. Cell 2007; doi:10.1016/j.cell.2007.03.009.

References

1 Vogel F, Motulsky AG: Human genetics; in Vogel F, Motulsky AG (eds): Human Genetics 'Problems and Approaches', ed 3. Heidelberg-Berlin, Springer, 2002.
2 Digweed M, Sperling K: Molecular analysis of Fanconi anemia. Bioessays 1998;18:579–585.
3 Joenje H, Ketan JP: The emerging genetic and molecular basis of Fanconi Anemia. Nat Rev Genet 2001;2:446–457.
4 D'Andrea AD, Grompe M: The Fanconi Anemia/BRCA pathway. Nature 2002;3:23–34.
5 Kennedy RD, D'Andrea AD: The Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes Dev 2005;19:2925–2940.
6 Niedernhofer LJ, Lalai AS, Hoeijmakers HJ: Fanconi Anemia (Cross) linked to DNA repair. Cell 2005;123:1191–1198.
7 Bagby GC, Alter BP: Fanconi Anemia. Semin Hematol 2006;43:147–156.
8 Digweed M: Genomic instability in Fanconi anemia and Nijmegen Breakage Syndrome. Genome Dyn Stab 2007;1:363–380.
9 Levitus M, Joenje H, de Winter JP: The Fanconi anemia pathway of genomic maintenance. Cell Oncol 2006;28:3–29.
10 Fanconi G: Familiäre infantile perniziosartige Anämie (perniziöses Blutbild und Konstitution). Jahrb Kinderh 1927;117:257–280.
11 Glanz A, Fraser FC: Spectrum of anomalies in Fanconi anemia. J Med Genet 1982;19:412–416.
12 Gillio AP, Verlander PC, Batish SD, Giampietro PF, Auerbach AD: Phenotypic consequences of mutations in the Fanconi anemia FAC gene: an International Fanconi Anemia Registry study. Blood 1997;90:105–110.

13 Wajnrajch MP, Gertner JM, Huma Z, Popovic J, Lin K, et al: Evaluation of growth and hormonal status in patients referred to the International Fanconi Anemia Registry. Pediatrics 2001;107: 744–754.
14 Alter BP, Frissora CL, Halperin DS, Freedman MH, Chitkara U, et al: Fanconi's anemia and pregnancy. Br J Haematol 1991;77:410–418.
15 Fanconi G: Familial constitutional panmyelocytopathy, Fanconi's anemia (F.A.). I. Clinical aspects. Semin Hematol 1967;4:233–240.
16 Gmyrek D, Syllm-Rapoport I: On Fanconi's Anemia. Analysis of 129 Cases. Z Kinderheilkd 1964;91:297–337.
17 Schroeder TM, Tilgen D, Kruger J, Vogel F: Formal genetics of Fanconi's anemia. Hum Genet 1976;32:257–288.
18 Auerbach AD, Rogatko A, Schroeder-Kurth TM: International Fanconi Anemia Registry: relation of clinical symptoms to diepoxybutane sensitivity. Blood 1989;73:391–396.
19 Auerbach AD, Allen RG: Leukemia and preleukemia in Fanconi anemia patients. A review of the literature and report of the International Fanconi Anemia Registry. Cancer Genet Cytogenet 1991;51: 1–12.
20 Giampietro PF, Adler-Brecher B, Verlander PC, Pavlakis SG, Davis JG, Auerbach AD: The need for more accurate and timely diagnosis in Fanconi anemia: a report from the International Fanconi Anemia Registry. Pediatrics 1993;91:1116–1120.
21 Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD: Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood 1994;84: 1650–1655.
22 Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, et al: A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–1256.
23 Schroeder TM, Kurth R: Spontaneous chromosomal breakage and high incidence of leukemia in inherited disease. Blood 1971;37:96–112.
24 Alter BP, Caruso JP, Drachtman RA, Uchida T, Velagaleti GV, Elghetany MT: Fanconi anemia: myelodysplasia as a predictor of outcome. Cancer Genet Cytogenet 2000;117:125–131.
25 Rosenberg PS, Greene MH, Alter BP: Cancer incidence in persons with Fanconi anemia. Blood 2003;101:822–826.
26 Schroeder TM, Anschütz F, Knopp A: Spontane Chromosomenaberrationen bei familiärer Panmyelopathie. Humangenetik 1964;1:194–196.
27 Schmid W, Scharer K, Baumann T, Fanconi G: Chromosomal fragility in familial panmyelopathy (Fanconi type). Schweiz Med Wochenschr 1965;95:1461–1464.
28 German J, Archibald R, Bloom D: Chromosomal breakage in a rare and probably genetically determined syndrome of man. Science 1965;148:506–507.
29 Schroeder TM, German J: Bloom's syndrome and Fanconi's anemia: demonstration of two distinctive patterns of chromosome disruption and rearrangement. Humangenetik 1974;25:299–306.
30 Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 1973;33:1829–1836.
31 Auerbach AD: Fanconi anemia diagnosis and the diepoxybutane (DEB) test. Exp Hematol 1993;21: 731–733.
32 Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, et al: Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 1995;85: 2233–2237.
33 Zakrzewski S, Sperling K: Genetic heterogeneity of Fanconi's anemia demonstrated by somatic cell-hybrids. Hum Genet 1980;56:81–84.
34 Strathdee CA, Duncan AM, Buchwald M: Evidence for at least four Fanconi anemia genes including *FACC* on chromosome 9. Nat Genet 1992;1:196–198.
35 Pulsipher M, Kupfer GM, Naf D, Suliman A, Lee JS, et al: Subtyping analysis of Fanconi anemia by immunoblotting and retroviral gene transfer. Mol Med 1998;4:468–479.
36 Fu KL, Foe JR, Joenje H, Rao KW, Lui JM, Walsh CE: Functional correction of Fanconi anemia group A hematopoietic cells by retroviral gene transfer. Blood 1997;90:3296–3303.
37 Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30: 410–420.

38 Chandra S, Levran O, Jurickova I, Maas C, Kapur R, et al: A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 2005;12: 976–984.
39 Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, et al: Cloning the gene for an inherited human disorder – chronic granulomatous disease – on the basis of its chromosomal location. Nature 1986;322:32–38.
40 Strathdee CA, Gavish H, Shannon W, Buchwald M: Cloning of cDNAs for Fanconi's anemia by functional complementation. Nature 1992;356:763–767.
41 Meetei AR, Levitus M, Xue Y, Medhurst AL, Zwaan M, et al: X-linked inheritance of Fanconi anemia complementation group B. Nat Genet 2004;36:1219–1224.
42 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
43 Joenje H, Levitus M, Waisfisz O, D'Andrea A, Garcia-Higuera I, et al: Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. Am J Hum Genet 2000;67: 759–762.
44 De Winter JP, Waisfisz Q, Rooimans MA, van Berkel CGM, Bosnoyan-Collins L, et al: The Fanconi anemia group G gene *FANCG* is identical with *XRCC9*. Nat Genet 1998;20: 281–283.
45 Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, et al: Positional cloning of a novel Fanconi anemia gene *FANCD2*. Mol Cell 2001;7:241–248.
46 Tipping AJ, Pearson T, Morgan NV, Gibson RA, Kuyt LP, et al: Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Afrikaner population of South Africa. Proc Natl Acad Sci USA 2001;98:5734–5739.
47 Whitney MA, Saito H, Gibson RA, Moses RE, Grompe M: A common mutation in the *FACC* gene causes Fanconi anemia in Ashkenazi-Jewish individuals. Nat Genet 1993;4:202–205.
48 Kupfer GM, Naf D, Suliman A, Pulsipher M, D'Andrea AD: The Fanconi anemia proteins, FAA and FAC, interact to form a nuclear complex. Nat Genet 1997;17:487–490.
49 De Winter JP, van der Weel L, de Groot J, Stone S, Waisfisz O, et al: The Fanconi anemia protein FANCF forms a nuclear complex with FANCA, FANCC and FANCG. Hum Mol Genet 2000;9: 2665–2674.
50 Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, et al: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 2001;7:249–262.
51 Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD: S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 2002;100:2414–2420.
52 Shimamura A, D'Andrea AD: Subtyping of Fanconi anemia patients: implications for clinical management. Blood 2003;102:3459.
53 Wang X, Andreassen PR, D'Andrea AD: Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol 2004;24:5850–5862.
54 Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB: Oxygen-dependence of chromosomal aberrations in Fanconi's anemia. Nature 1981;290:143–144.
55 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
56 Pagano G, Youssoufian H: Fanconi anemia proteins: major roles in cell protection against oxidative damage. Bioessays 2003;25:589–595.
57 Pagano G, Degan P, d'Ischia M, Kelly FJ, Nobili B, et al: Oxidative stress as a multiple effector in Fanconi anemia clinical phenotype. Eur J Haematol 2005;75:93–100.
58 Auerbach AD, Adler B, Chaganti RS: Prenatal and postnatal diagnosis and carrier detection of Fanconi anemia by a cytogenetic method. Pediatrics 1981;67:128–135.
59 Gluckman E, Auerbach AD, Horowitz MM, Sobocinski KA, Ash RC, et al: Bone marrow transplantation in Fanconi anemia. Blood 1995;86:2856–2862.
60 Boulad F, Gillio A, Small TN, George D, Prasad V, et al: Stem cell transplantation for the treatment of Fanconi anemia using a fludarabine-based cytoreductive regimen and T-cell-depleted related HLA-mismatched peripheral blood stem cell grafts. Br J Haematol 2000;111: 1153–1157.

61 Tönnies H, Huber S, Kühl JS, Gerlach A, Ebell W, Neitzel H: Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood 2003;101:3872–3874.
62 Tönnies H, Huber S, Volarikova E, Gerlach A, Neitzel H: Interphase FISH-assay for the detection of MDS- and AML-associated chromosomal imbalances on uncultivated bone marrow and peripheral blood cells; in Schindler D, Hoehn H (eds): Fanconi Anemia, pp 95–109. Karger, Basel, 2007.
63 Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE: Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. Blood 2004;103:1147–1151.
64 Rechitzky S, Kuliev A, Tur-Kaspa I, Morris R, Verlinsky Y: Preimplantation genetic diagnosis with HLA matching. Reprod Biomed Online 2004;9:210–221.
65 Croop JM: Gene therapy for Fanconi anemia. Curr Hematol Rep 2003;2:335–340.
66 Kwee ML, Poll EH, van de Kamp JJ, de Koning H, Eriksson AW, Joenje H: Unusual response to bifunctional alkylating agents in a case of Fanconi anemia. Hum Genet 1983;64:384–387.
67 Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, et al: Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 1997;5: 137–148.
68 Gregory JJ Jr, Wagner JE, Verlander PC, Levran O, Batish SD, et al: Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci USA 2001;98:2532–2537.
69 Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, et al: Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res 2002;98: 126–135.
70 Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, et al: Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 2005;105:1329–1336.
71 Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, et al: Spontaneous functional correction of homozygous Fanconi anemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet 1999;22:379–383.
72 Ellis NA, Lennon DJ, Proytcheva M, Alhadeff B, Henderson EE, German J: Somatic intragenic recombination within the mutated locus *BLM* can correct the high sister-chromatid exchange phenotype of Bloom syndrome cells. Am J Hum Genet 1995;7:1019–1027.
73 Chen M, Tomkins DJ, Auerbach W, McKerlie C, Youssoufian H, et al: Inactivation of *Fac* in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anemia. Nat Genet 1996;12:448–451.
74 Cheng NC, van de Vrugt HJ, van der Valk MA, Oostra AB, Krimpenfort P, et al: Mice with a targeted disruption of the Fanconi anemia homolog *Fanca*. Hum Mol Genet 2000;9:1805–1811.
75 Kalb R, Neveling K, Nanda I, Schindler D, Hoehn H: Fanconi anemia: causes and consequences of genetic instability; in Volff J-N (ed): Genome and Disease. Genome Dynamics. Karger, Basel, 2006, vol 1, pp 218–242.
76 Xia B, Dorsman JL, Ameziane N, de Vries Y, Rooimans MA et al: Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet 2007;39:159–161.
77 Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2007;39:162–164.

Karl Sperling
Institute of Human Genetics, Charité – Universitätsmedizin Berlin
Augustenburger Platz 1
D–13353 Berlin (Germany)
Tel. +49 30 450566081, Fax +49 30 450566904, E-Mail karl.sperling@charite.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 39–58

Fanconi Anemia Genes: Structure, Mutations, and Genotype-Phenotype Correlations

R. Kalb[a], *K. Neveling*[a], *S. Herterich*[b], *D. Schindler*[a]

[a]Department of Human Genetics, and [b]Department of Clinical Chemistry and Pathobiochemistry, University of Würzburg, Würzburg, Germany

Abstract

This article is meant to introduce the reader to the FA/BRCA pathway, the currently known human FA genes, and their mutations. We review structure and mutational profiles of the 12 FA genes identified to date. The FA genes display striking variability of size and mutational load which partly reflect their genomic composition and functional constraints. Biallelic mutations in three genes (*FANCA*, *FANCC* and *FANCG*) account for over 80% of all FA patients worldwide, and there are only single or very few patients with mutations in the more recently discovered FA genes. *FANCA* as the most frequently affected gene displays the entire spectrum of genetic alterations, including at least 32% large deletions correlated to *Alu*-mediated recombination. Viability of FA-D2 patients appears to depend on the presence of hypomorphic mutations and residual protein. Gene frequencies in excess of 1:100 have been observed, mainly for mutations in *FANCA* and *FANCC*, in isolated populations with defined founder mutations. With the exception of complementation groups FA-D1 and FA-N whose patients overwhelmingly present with malignancies in early childhood, most FA genes lack strict genotype-phenotype correlations. Rather, the particular type of mutation in a given gene appears to determine the severity of congenital malformations, the age of onset of bone marrow failure, and the length of survival. FA patients who reach adulthood may carry mild mutations or may have developed somatic reversions. Prospective studies are needed for a more definitive exploration of genotype-phenotype correlations.

Around 80% of all patients suffering from Fanconi anemia (FA) belong to one of three prevalent complementation groups (FA-A, FA-C and FA-G). According to the International Fanconi Anemia Registry (IFAR), 57% of the FA patients have mutations in *FANCA*, 15% in *FANCC* and 9% in *FANCG*, followed by 4% in *FANCD1* and 3% in *FANCD2* [1]. Mutations in the other FA

genes are rare, and only single patients have been described for *FANCL* and *FANCM*. The estimated overall incidence of FA is 1 in 200,000 with a carrier frequency of 1 in 300 [2]. However, much higher patient and carrier frequencies are encountered in certain populations due to founder and inbreeding effects (see below).

The initial FA-complementation groups were defined via somatic cell fusion [3, 4], but a variety of strategies were applied for the identification of the underlying genes. Expression cloning was used for the identification of *FANCA* [5], *FANCC* [6], *FANCE* [7], *FANCF* [8] and *FANCG* [9], while positional cloning independently led to the identification of *FANCA* [10], but also to the identification of *FANCD2* [11] and *FANCJ* [12, 13]. *FANCD1* was identified by a classical candidate gene approach [14], as was *FANCN* [15]. Biochemical isolation of the respective proteins led to the identification of *FANCB* [16], *FANCL* [17] and *FANCM* [18]. The identification of the first FA genes (*FANCC, FANCA* and *FANCG*) provided only few hints to their molecular function. Except for *FANCG*, which is identical with *XRCC9*, the initially discovered FA genes shared neither significant homologies to other proteins nor to known functional domains. This changed when Howlett and coworkers reported that the breast cancer associated protein 2 (*BRCA2*) carries biallelic mutations in FA patients belonging to complementation group D1 [14]. This finding implicated the likely involvement of at least some of the FA proteins in DNA repair via homologous recombination. In addition, some of the subsequently discovered genes contain protein domains with defined biochemical functions. For example, FANCL or PHF9/Pog has a RING domain with ubiquitin E3 ligase activity [17], FANCJ (identical to BRIP1/BACH1) has a DNA helicase domain [13], and FANCM (homolog to Hef) shows homologies to DNA translocases [18]. Specific functional properties can also be deduced for two of the most recently discovered FA proteins, FANCJ and FANCN, since these proteins interact with and contribute to the functions of the well known breast cancer proteins BRCA1 and BRCA2 [12, 19]. The current view is that FA proteins participate in a complex molecular crosstalk with a large number of other proteins involved in the regulation and execution of DNA maintenance functions, including homology directed DNA repair [20, 21].

The FA/BRCA Pathway

As a specific but not exclusive function, FA genes are thought to play an important role in the removal of DNA interstrand crosslinks (ICL). Cells of all FA subgroups display a characteristic hypersensitivity against DNA damage induced by bifunctional alkylating agents implying cooperation of FA proteins in a common pathway, which is referred to as FA/BRCA pathway. The current

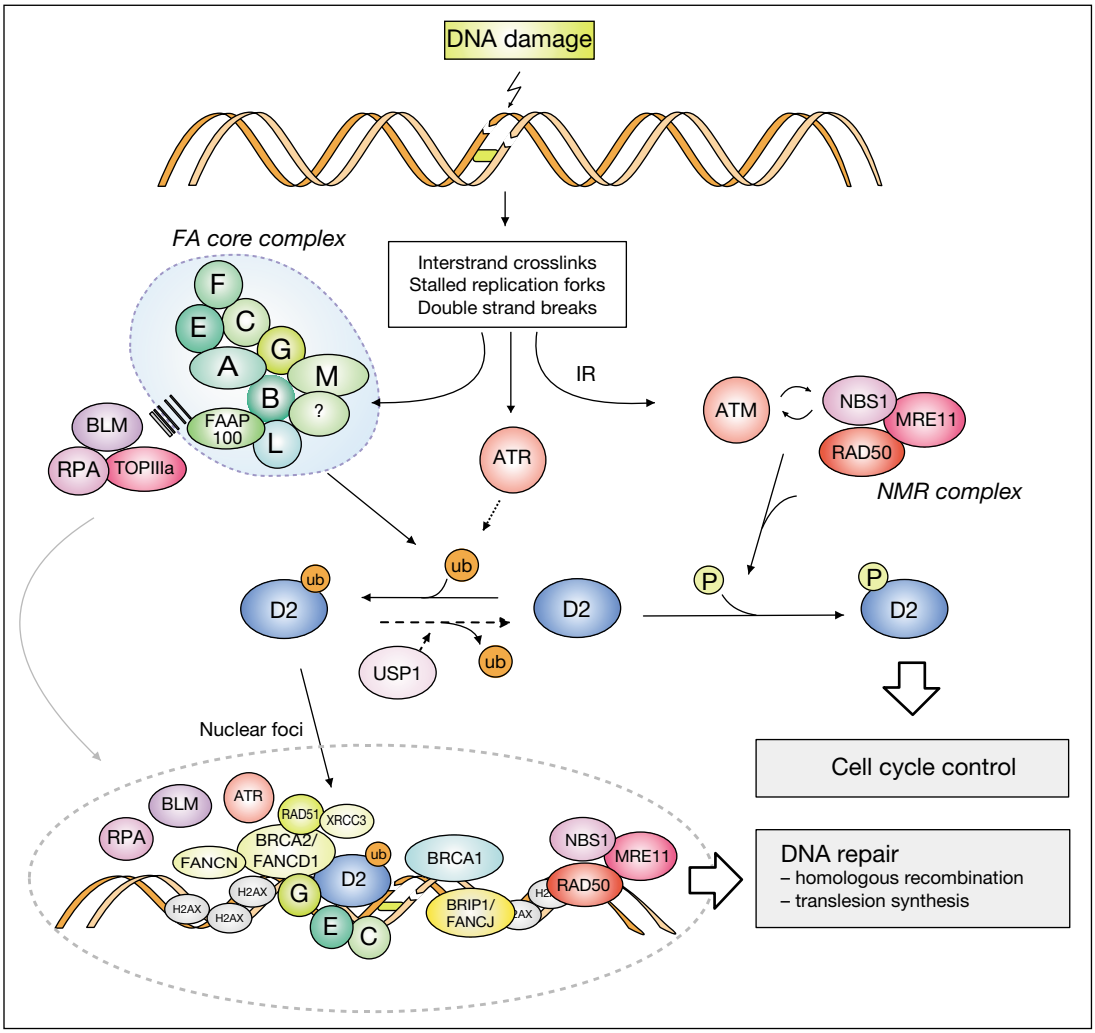


Fig. 1. Current concept for the involvement of FA proteins in the response to DNA damage. For explanations see text.

model of FA protein function (cf. fig. 1) includes direct links to recombinational types of DNA repair and translesion synthesis [22, 23]. As shown in figure 1, specific types of DNA damage result in the assembly of at least 8 FA proteins (FANC-A, B, C, E, F, G, L and M) into a nuclear multisubunit complex (FA core complex) that is required for FANCD2 monoubiquitination at lysine 561 [24]. A member of the complex, the RING finger containing protein FANCL,

provides the presumptive ubiquitin E3 ligase activity [18]. An extended form of the FA core complex is the so called 'BRAFT complex' (BLM, RPA, FA, Topo III) in which other proteins like BLM helicase, topoisomerase III alpha and replication protein A (RPA) participate [25], underlining the close association if not partial overlap of DNA replication and DNA repair functions.

A central step in the FA/BRCA pathway is the monoubiquitination of FANCD2. This post-translational modification causes a dynamic nuclear relocalization of FANCD2 from a state of diffuse nuclear distribution into discrete nuclear foci containing many other proteins involved in DNA repair [24, 26]. Colocalization with FANCD2 was found for a number of proteins involved in DNA damage signaling including ATR kinase [27], components of homologous recombination (RAD51, BRCA1, BRCA2) [28], proteins of the non-homologous end joining pathway such as NBS1 [29, 30], the BLM helicase, and partially other FA proteins (FANCE, FANCC, FANCJ) [1, 31]. FANCM (together with FAAP24) and FANCD2 are able to bind directly to DNA with a structure specific affinity [32, 33] similar to that observed for FANCJ/BRIP1 and FANCD1/BRCA2, the only known FA proteins that are not required for FANCD2 monoubiquitination.

In addition to the proteins of the FA core complex, the ATR kinase is required for DNA damage induced monoubiquitination and relocation of FANCD2 [27]. ATR-mediated activation of FANCD2 takes place in response to DNA-ICLs or stalled replication forks. It appears to depend on the intact FA core complex but also on NBS1, linking FA to the non-homologous end-joining (NHEJ) pathway of DNA repair [29, 34]. Depletion of the ATR-interacting protein, ATRIP, in *Xenopus* egg extracts results in defective chromatin binding of xFANCD2 [35]. ATR functions either by direct phosphorylation of FANCD2, as indicated by in vitro experiments, or indirectly by phosphorylation of components participating or regulating the FA/BRCA pathway [27].

As shown in figure 1, following exposure to ionizing radiation, FANCD2 is phosphorylated at serine 222 in an ATM-kinase dependent manner which activates an intra S phase checkpoint [36]. ATM-mediated phosphorylation depends on NBS1, implying a regulatory connection between the NBS1/MRE11/RAD50 complex and the FA proteins [30]. More detailed descriptions and discussions of the increasingly complex and manifold interactions of the Fanconi anemia proteins can be found in series of recent reviews [20, 22, 23, 37].

Fanconi Anemia (FA) Genes

The FA genes are scattered widely throughout the human genome and vary considerably in both size and structure, as summarized in table 1. Sequence

Table 1. Summary of Fanconi anemia (FA) complementation groups, corresponding genes and proteins

Complementation group		Gene	Chromosome location	Number of exons	Protein size, kDa	Protein domains[b]	Reference
Name	Frequency, %[a]						
FA-A	57	*FANCA*	16q24.3	43	163	partial leucine zipper, NES, NLS	[5]
FA-B	0.3	*FANCB/FAAP95*	Xp22.3	10	95	NLS	[16]
FA-C	15	*FANCC*	9q22.3	15	63	–	[6]
FA-D1	4	*FANCD1/BRCA2*	13q12.3	27	384	BRC repeats, RAD51-/DSS1-/ssDNA binding, NLS	[14]
FA-D2	3	*FANCD2*	3p25.3	44	155 (162)	–	[11]
FA-E	1	*FANCE*	6p21–22	10	58	–	[7]
FA-F	2	*FANCF*	11p15	1	42	homology to ROM	[8]
FA-G	9	*FANCG/XRCC9*	9p13	14	68	tetratricopeptide repeat	[9]
FA-I	rare	–	–	–	–	–	–
FA-J	1.6	*FANCJ/BRIP1/BACH1*	17q22	20	140	DEAH-helicase, BRCA1-binding	[12, 13]
FA-L	0.1	*FANCL/PHF9*	2p16.1	14	43	WD40 repeats, PHD finger	[17]
FA-M	rare	*FANCM/Hef*	14q21.3	23	250	homology to helicase and endonuclease domains	[18]
FA-N	rare	*FANCN/PALB2*	16p12	13	131	C-terminal WD40-like repeats	[15]

[a]Based on the International Fanconi Anemia Registry (IFAR), reviewed in reference [1].
[b]NES: nuclear export sequences; NLS: nuclear localization signals.

comparisons indicate that some of the FA genes must have arisen fairly recently in evolution, lacking homologs beyond the vertebrate kingdom. However, there are also FA genes/proteins that are more ancient and highly conserved. For example, FANCM is closely related to the archael protein Hef (helicase-associated endonuclease for fork-structured DNA) [19], and FANCJ belongs to the group of recQ-like helicases that share highly conserved domain structures. In addition, homologs have been found for FANCD2 in *D. melanogaster, C. elegans* and *A. thaliana* [11], and for FANCL in *D. melanogaster* and *A. gambiae* [18]. This might reflect a basic function of the conserved FA proteins, most likely in the area of DNA repair [38], which has been complemented by the recruitment of the other FA genes later in evolution. Since oxidative stress and oxidative damage appear to activate the FA/BRCA pathway, an attractive hypothesis posits that warm-blooded and long-lived species require the extended FA family of genes as additional protection against DNA damage caused by reactive oxygen species.

Figure 2 provides a graphic summary of the known FA genes, depicting relative size and intron/exon structure. In addition, numbers and types of mutations are indicated above and below the respective genes. As such, figure 2 illustrates the impressive degree of structural and mutational variation among the known FA genes, both at the genomic and the cDNA levels. One of the smallest FA-specific genomic regions, comprising less than 5 kb and only a single exon, contains the entire *FANCF* gene, whereas *FANCC* and *FANCJ* are relatively large genes spanning 218 kb and 180 kb, albeit with widely differing intron/exon structures.

The occurrence of genetic alterations in a given gene depends on a variety of factors related to gene structure, including the presence of repetitive elements and base pair composition such as CpG dinucleotides. Physical size by itself clearly cannot be correlated with the number of mutations in a given FA gene. A multitude of mutations have been described in relatively small or medium sized genes like *FANCG* and *FANCA*, while genes with large transcripts like *FANCM* and *FANCD1* appear to be affected by relatively few alterations. With respect to type, quantity and distribution of mutations, *FANCA* clearly stands out. It is so far the only FA gene with a high proportion of large deletions, amounting to at least 32% [39, 40] of all observed mutations in *FANCA*. The prevalence of large deletions has been correlated with the presence of numerous *Alu*-type sequences, promoting *Alu*-mediated recombination [41]. With the advent of the MLPA (multiplex ligation dependent probe amplification) technique, it is very likely that the proportion of deletions detected in *FANCA* and perhaps in other FA genes will increase in the future. As illustrated by the example shown in figure 3, MLPA uncovers deletions that escape detection with standard PCR-based techniques of mutation analysis.

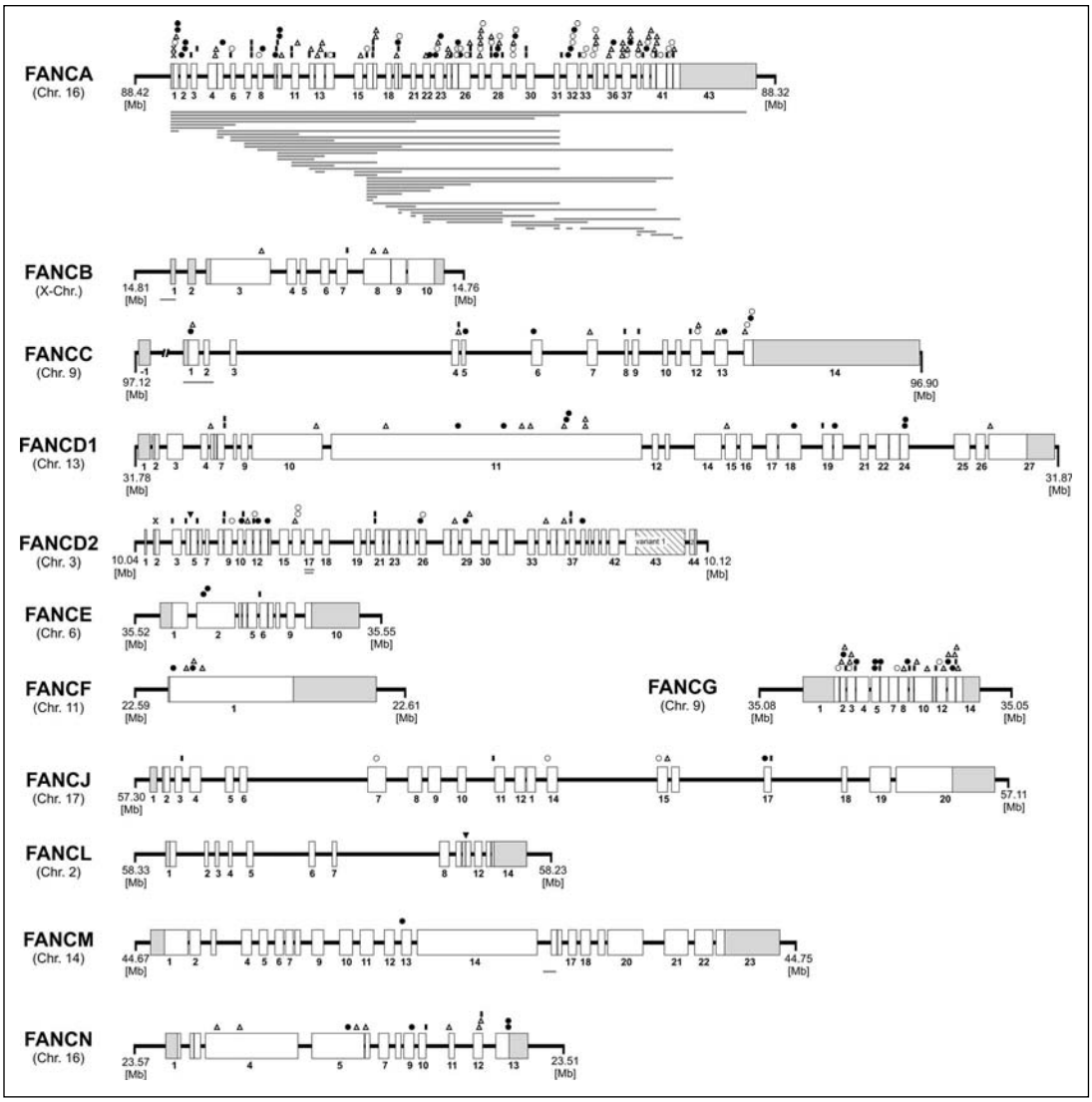


Fig. 2. Relative size, exon/intron structure and mutational spectra of the known FA genes. In order to accommodate and compare the distribution of mutations, exons were drawn 50-fold larger than corresponding introns. Mutational changes are shown above and below the respective genes. Key to symbols: ○ missense mutation, ● nonsense mutation, △ small deletion/insertion or duplication, ▮ aberrant splicing/splice site mutation, ▼ large insertion, ▬▬ large deletion, X translation initiation (unpublished data and references for: *FANCA* [45], *FANCB* [16, 49], *FANCC* [2, 6, 88], *FANCD1* [51, 52], *FANCD2* [11], *FANCE* [7], *FANCF* [8], *FANCG* [86], *FANCJ* [12, 13], *FANCL* [17], *FANCM* [18] and IFAR (Rockefeller University)).

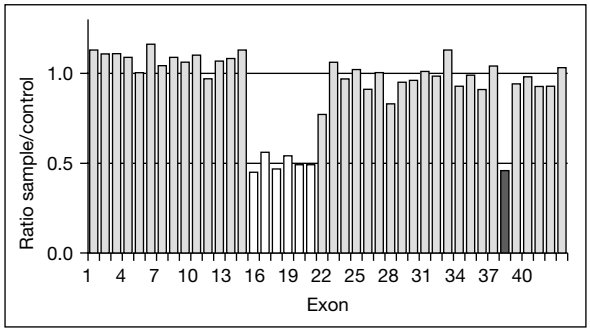


Fig. 3. Multiplex ligation dependent probe amplification (MLPA) assay for the detection of deletions in *FANCA*. A reduction of the sample to control ratio to approximately 0.5 indicates a deleted region. The example shown is from a compound heterozygous FA-A patient. One allele carries a large deletion involving exons 15 to 20; the other allele carries a small deletion in exon 38 that involves only 3 bp (c.3788-3790delTCT) and obviously affects the primer binding site.

FANCD2, a gene nearly of the same size and structure as *FANCA*, also contains a high number of repetitive elements including many *Alu* repeats but so far there are only two deletions involving a single exon. The mutational record of the FA genes indeed suggests that evolutionarily conserved genes like *FANCD2*, *FANCL* or *FANCM* may be more important than the more recent *FANCA* or *FANCG* genes which seem to be highly tolerant of mutations. In addition to serving as catalytic subunit of the FA core complex and as such responsible for the monoubiquitination of FANCD2 there may be other, as yet unknown function(s) of FANCL that might explain the relative paucity of respective patients and mutations. Much more in depth analysis of FA gene structure and function will be required to clarify whether the less frequently affected genes truly have more important functions, or whether their underrepresentation among FA patients has other, possibly structural reasons. A case in point is the highly conserved *FANCD2* gene. All FA-D2 patients examined to date have at least one hypomorphic or leaky mutation resulting in residual levels of protein which implicates lethality of biallelic null mutations [42]. However, patients with mutations in some of the evolutionarily novel genes like *FANCE* and *FANCB* also are exceedingly rare, suggesting that mutational inactivation of these genes might not be as permissive as mutational inactivation of *FANCA*, *FANCC* or *FANCG*. To add to the complexity, several functions other than its participation in the FA core complex have been suggested for *FANCA*, and yet biallelic null mutations in *FANCA* are compatible with viability and defects in this gene are impressively numerous.

Annotations of Individual FA Genes (in Alphabetical Order)

FANCA codes for a protein of 163 kDa which contains a leucine zipper-like motif, two overlapping bipartite nuclear localization signals (NLS) [43] and five functional leucine-rich nuclear export sequences (NES) that contribute to CRM1 (chromosome region maintenance 1)-dependent nuclear export [44]. Since the first identification in 1996 more than 200 different mutations of all possible types have been described (see fig. 2) (reviewed in reference [45]). Large deletions are notably frequent in *FANCA* with a striking overlap of the affected exons. Even though the exact breakpoints have not been determined in all cases, a number of reports indicate a direct correlation between the presence of *Alu* repeats and deletion breakpoints [39, 46, 47]. In addition to large deletions, small deletions and insertions/duplications are also prevalent [48]. These changes often are associated with short direct repeats, homonucleotide tracts, and hotspot consensus sequences like CpG dinucleotides. Base substitutions account for less than half of the mutations in *FANCA* and cause premature termination, amino acid exchanges, or affecting translation initiation or normal exon recognition.

FANCB is the only X-linked FA gene. Approximately 60% of the genes located at Xp22.3 exhibit biallelic expression in females, which however is not the case for *FANCB* which undergoes X-chromosomal inactivation (NCBI, OMIM, *300515). To date, mutations in *FANCB* have been found in five male FA patients [16, 49] including a deletion affecting the promotor region and portions of the 5′ untranslated regions (exon 1), three microdeletions/-insertions, and a single splice site mutation. The analysis of three healthy female *FANCB* carriers revealed the preferential inactivation of the X-chromosome carrying the mutated allele in both peripheral blood cells and fibroblasts, suggesting a proliferative advantage for cells expressing the wildtype allele early in embryogenesis [16].

FANCC was the first FA gene identified in 1992 [6]. It gives rise to two alternative transcripts differing in usage of the first untranslated exon, denoted as –1 or –1a [41]. At the genomic level, *FANCC* is one of the larger FA genes spanning more than 218 kb, but coding for a protein with a molecular weight of 63 kDa. Two putative p53 binding sites of unknown functional significance have been described in *FANCC* [50]. Mutations in *FANCC* are responsible for 10–15% of all FA cases, but surprisingly few private mutations have been reported. As shown in figure 2, the mutational spectrum is heterogeneous, and alterations are scattered widely throughout the gene with the exception of a possible cluster in the C-terminal region.

FANCD1 is identical to the gene coding for the breast cancer associated protein 2 (BRCA2) [14] whose monoallelic mutations predispose to breast, ovarian, pancreatic and possibly other cancers. Biallelic mutations in

FANCD1/BRCA2 result in a severe form of FA with a high and very early risk for the development of malignancy [51–53]. At the mRNA level, *FANCD1/BRCA2* is the largest of the FA genes with an open reading frame of 10254 nt. There are numerous functional domains which have been extensively reviewed [54, 55]. Crucial for FANCD1/BRCA2 function are 8 BRC repeats, a helicase domain followed by 3 oligonucleotide/-saccharide binding (OB1, OB2, OB3) folds, and a tower domain inserted in OB2. The FANCD1/BRCA2 protein shows structure-specific DNA binding activity and interacts directly with several other proteins some of which have been implicated in DNA repair like RAD51, FANCG and FANCD2 [56–58]. FANCD1/BRCA2 appears to function in multiple and diverse cellular processes including stabilization of stalled replication forks, homologous recombination (via interaction with the RAD51 recombinase), and regulation of cytokinesis [59]. Mutations in *FANCD1/BRCA2* are distributed over the entire gene, but monoallelic preservation of a functional BRC-repeat domain has been observed for the majority of mutations in FA-D1 patients. Compared to the prevalence of gene carriers in different populations, the frequency of FA-D1 patients appears low, suggesting nonviability of most biallelic types of mutations [52, 60].

FANCD2 is expressed in alternatively spliced variants, one with a large continuous exon 43, and one with an additional exon 44, the latter giving rise to a functional protein [11]. *FANCD2* is a highly conserved gene that is flanked by two distinct pseudogene regions *FANCD2-P1* and *FANCD2-P2* showing homologies to the middle portion of the gene [42]. FANCD2 protein is recruited to chromatin after DNA damage-induced monoubiquitination at lysine 561 [24, 61] where it colocalizes with other proteins involved in DNA repair including BRCA2, RAD51 and BRCA1 [56, 57]. Purified FANCD2 has DNA-binding activity with structure-specific affinity to branch points and free DNA ends such as Holliday junctions and DNA double strand breaks [33], supporting its active participation in DNA repair pathways. Based on the analysis of more than 30 patients and disregarding recurrent mutations, there is an almost random distribution of mutations (cf. fig. 2). In fact, more than 50% of the mutations observed in *FANCD2* affect splicing, a figure that is much higher than in any of the other FA genes. Residual levels of FANCD2 protein were detected in all patient-derived cell lines, which underline the functional importance of FANCD2 [42].

FANCE was identified by expression cloning and mutation analysis of three families assigned to complementation group FA-E [7]. Except for the original families, no further mutations of FA-E patients have been reported.

FANCF consists of only a single exon. The encoded protein shares homologies to the prokaryotic RNA-binding protein (ROM) [8], which is without known significance [62]. To date, only 5 nonsense mutations and microdeletions

have been found in FA-F patients [8]. These alterations appear to cluster in the 5′ portion of the gene. However, *FANCF* has gained special prominence as a gene that is frequently silenced by hypermethylation of its promotor region in various types of malignancies [63–65] (see chapter by Neveling et al.).

FANCG is identical to *XRCC9* which was identified in Chinese hamster cells as a gene involved in DNA repair [9, 66]. FANCG has seven tetratricopeptide repeat motifs (TRPs) [67] thought to function as a scaffold for mediating protein-protein interaction. The molecular function of FANCG is not restricted to the FA core complex, since it has been shown to interact with BRCA2 and XRCC3 directly and independently of other FA proteins [68]. Mutations in *FANCG* are of all types, with the exception of deletions. They are scattered throughout the gene, with possible preference for the N- and C-terminal regions (cf. fig. 2).

FANCJ is identical to the BRCA1-interacting protein 1 (BRIP1), also denoted as BRCA1-associated C-terminal helicase 1 (BACH1) [12, 13]. FANCJ/BRIP1 was originally found as a BRCA1 interacting protein containing two functional domains, a DNA-dependent ATPase and a 5′- to 3′-DNA helicase [69]. In vitro, BRIP1 has DNA unwinding activity with a high preference for forked duplex substrates and apparently executes efficient strand displacement from a D loop substrate representing a likely intermediate in DNA repair via homologous recombination [70]. On the genomic level, *FANCJ* belongs to the larger FA genes, but only 8 different mutations have been described to date with most of the affected patients being homozygous or heterozygous for c.2392T>C (R798X) [12, 13].

FANCL was identified by mass spectroscopy of a previously isolated 43-kDa FA-associated polypeptide [17]. Sequence analysis revealed identity to the WD40-repeat containing PHD finger protein-9 (PHF9) which is highly conserved. FANCL is thought to serve as the catalytic subunit of the FA core complex during monoubiquitination of FANCD2. Consistent with this important role in the FA/BRCA pathway, there is only a single patient known to date whose disease is caused by a homo- or hemizygous insertion of 177 bp into a pyrimidine-rich sequence between intron 10 and exon 11, leading to skipping of exon 11.

FANCM also belongs to the highly conserved FA genes [18]. It shows similarities to the archael protein Hef which displays a helicase and an endonuclease domain. However, the biological relevance of these domains for the current function of FANCM has yet to be established. Using specific antibodies lack of FANCM was discovered in a single cell line from a patient previously excluded from most of the known complementation groups [18]. Genetic work up revealed compound heterozygous mutations, a maternal nonsense mutation in exon 13 and a paternal deletion within exon 15.

FANCN was recently found to be identical to *PALB2* (partner and localizer of BRCA2) [15]. Originally, PALB2 was identified as a BRCA2-interacting protein that promotes BRCA2 stability and localization in nuclear structures [19]. Using a candidate gene approach, biallelic mutations were found in the *PALB2* gene in a cohort of FA patients that could not be assigned to any of the other known complementation groups. Cells from FA-N patients show intact FANCD2 monoubiquitination which localizes the function of FANCN to the group of proteins acting downstream of FANCD2, including FANCD1/BRCA2 and FANCJ/BRIP1. Early childhood cancer appears to be a common denominator of mutational inactivation of the downstream genes *FANCD1/BRCA2* and *FANCN/PALB2* [15].

Founder Mutations and Ethnically Associated Mutations

A number of FA causing mutations are recurrently prevalent in certain populations due to founder or inbreeding effects. Examples of such 'marker' mutations are shown in table 2. A prominent example is the *FANCA* mutation c.295C>T in the Spanish Gypsy population where it is encountered with a carrier frequency of 1/64 to 1/70 [71]. The occurrence of the mutation is restricted to the Iberian peninsula where it presumably emerged less than 600 years ago in a Gypsy family that migrated to Spain. Carrier frequencies higher than 1/100 have also been observed in the Ashkenazi Jewish population for the *FANCC* mutation c.456+4A>T (previously IVS4+4A>T or c.711+4A>T) [2]. This mutation must have originated in the Israelite population that left Palestine during the Roman Empire and settled in Europe, since it is not encountered among Sephardic Jews [72].

A classical founder effect was demonstrated for the large intragenic deletion of exons 12–31 in the *FANCA* gene which has been shown to account for 60% of FA chromosomes in 46 unrelated Afrikaner FA patients [73]. This mutation was apparently introduced to the Cape region of South Africa at the end of the 17th century by a French Huguenot couple. In the black South African population, including individuals from Swaziland, Mozambique, and Malawi, 82% of all FA patients were found to carry the deletion c.637_643delTACCGCC in *FANCG* [74]. The distribution of this mutation in diverse geographic regions and tribes was used to date the origin to the arrival of the Bantu speakers in South Africa around 400 AD.

In regions of high historical migration like Europe or North America some mutations were found recurrently among patients of the same ethnic origin (cf. table 2). However, due to the obvious heterogeneity of the populations there is little or no increased allele frequency, even if haplotype analysis suggests a common ancestry. Notable exceptions are mutations found in geographically

Table 2. Recurrent/founder mutations in Fanconi anemia genes

Gene	Mutation	Ethnicity	Allele frequency[a]	Other populations	Reference
FANCA	c.295C>T	Spanish Gypsy	1/64–1/70	Portuguese	[71]
	c.890_893del	Tunisian Jewish	rare	–	[82]
	c.894_1359del	Irish	n.d.	–	[76]
	c.894_1626del	South African	n.d.	–	[73]
	c.1007_3066del	South African	n.d.	German (Western Ruhr), French Huguenot ancestry	[73]
	c.1115_1118del	variable	n.d.	–	[48]
	c.2574C>G	Indian Jewish	1/53	–	[82]
	c.2172_2173dupG	Moroccan Jewish	1/200	–	[82]
	c.3398delA	South African	n.d.	–	[73]
	c.3788_3790del	Brazilian	n.d.	–	[48, 83]
	c.4275delT	Moroccan Jewish	rare	–	[82]
FANCC	c.67delG (c.322delG)	Northern European	n.d.	Dutch	[2]
	c.456+4A>T (c.711+4A>T)	Ashkenazi Jewish	1/89	Sephardic Jews (with Ashkenazi ancestry)	[2, 84]
	c.456+4A>T (c.711+4A>T)	Japanese	n.d.	–	[81]
FANCD2	c.1948-16T>G	Turkish	n.d.	Turkish North Americans, Czech	[42]
	c.1948-6C>A	German	n.d.	North American	[42]
	c.2444G>A	Spanish	n.d.	Italian, North American	[42]
FANCG	c.307+1G>C	Japanese	n.d.	Korean	[85, 86]
	c.313G>T	German	n.d.	–	[87]
	c.637_643del	black South African	1/35–1/475	Bantu-speakers: Swaziland, Mozambique, Malawi	[74]
	c.1066C>T	Japanese/Korean	<1/156	Korean	[85]
	c.1077-2A>G	Portuguese-Brazilian	n.d.	Portuguese	[86]
	c.1183_1192del	German	n.d.	–	[87]
	c.1480+1G>C	French-Acadian	n.d.	–	[86]
	c.1649delC	Turkish	n.d.	–	[87]
	c.1794_1803del	Northern European	n.d.	–	[86]
FANCJ	c.2392C>T	Inuit	n.d.	different ethnic groups	[12]

[a]n.d.: not determined.

isolated regions. In Italy, for example, two mutations in *FANCA*, c.790C>T and c.3559insG were detected in otherwise unrelated families within the Campania region [75]. Founder mutations or mutations sharing the same haplotype have been reported for several FA complementation groups (FA-A, C, D2, G and J) among different ethnic backgrounds, and independent of the overall mutation frequencies of the FA genes.

Not all recurrent mutations can be sufficiently explained by founder effects, but may represent examples of gene regions vulnerable to genetic change ('mutational hotspots'). A case in point is the mutation c.2392C>T in *FANCJ* which was detected in a few unrelated Inuit patients that share the same haplotype. However, the same mutation was also found in patients from diverse populations and ethnical backgrounds where it was correlated with at least three different haplotypes [12]. The combination of different haplotypes and the rarity of FA-J patients imply an independent recurrence of the mutation c.2392C>T.

Genotype-Phenotype Correlations

Clinical course and severity of FA vary strongly between and even within families, suggesting relatively weak, if any, genotype-phenotype correlations. In particular, the onset of hematological abnormalities and longterm survival appear to depend on additional factors such as number and severity of infections, nutritional status, and access to and quality of medical care. Any definite conclusion about genotype-phenotype correlations must await the results of prospective studies which are not available to date. A retrospective study comparing the haematological profiles of complementation groups FA-A, -C and -G failed to reveal statistically significant differences in the onset of aplastic anemia [76]. However, the manifestation of cytopenia was more severe in patients belonging to complementation group FA-G. In addition, FA-G patients may develop AML or MDS earlier than patients of the other complementation groups. Congenital abnormalities were comparable among FA-A and FA-G patients, but less severe than in certain FA-C patients. The clinical data of patients belonging to complementation group D2 suggests certain differences to other FA groups [42]. Compared to other patients listed in the IFAR database, FA-D2 patients exhibit a higher average frequency of congenital abnormalities. Their clinical course is additionally marked by early average onset of bone marrow failure and early dependency on hematopoietic stem cell transplantation. It should be noted, however, that this FA-D2 patient cohort contained a number of patients with identical mutations which complicates the statistical comparison. An undoubtedly severe phenotype is caused by biallelic mutations in *FANCD1/BRCA2*. Many of these patients succumb to their malignancies

(Wilms tumor, medulloblastoma and leukemias) prior to the onset of pancytopenia and thus may never be diagnosed as FA [51, 52]. Because of the unusually severe and early onset of neoplasia in FA-D1 patients, their phenotype appears to be distinctive and may be more appropriately labeled FA-like rather than genuine FA [51, 77].

Genotype-phenotype correlations are further complicated by the obvious heterogeneity of the mutational spectrum within each FA gene, the private character of mutations and, of course, the high prevalence of compound heterozygosity increasing the possibility of somatic reversion. Any missense change involving a non-conserved position within the gene should be formally tested for its functional significance. The availability of site-directed mutagenesis, retroviral vectors with high transduction efficiencies, and loss of MMC sensitivity as a functional marker of complementation facilitate the performance of such 'confirmatory' tests. Adachi and coworkers [78] have published an exemplary study in which the functional significance of a large number of *FANCA* mutations has been tested. In terms of cost and labor such confirmatory studies tend to exceed the capacity of the average diagnostic laboratory and might best be performed by specialized centers. Notwithstanding these difficulties, empirical studies have already shown that some mutations can be correlated with distinct phenotypes. The mutation c.1007_3066del in *FANCA*, for example, is associated with an increased rate of AML or MDS and a higher frequency of severe congenital abnormalities [76]. In contrast, the *FANCC* c.67delG alteration is associated with milder hematologic symptoms and a lower rate of somatic abnormalities when compared to the majority of other FA patients. Another example of a mild mutation is the missense mutation c.2444G>A in *FANCD2* which seems to be associated with a relatively mild clinical course [42].

A mild clinical course may either be due to mutations that retain some degree of protein function or to reverse mosaicism. Mild mutations and/or reverse mosaicism will be preferentially found among older FA patients or patients who have escaped the diagnosis of FA because of lack of clinical symptoms. A prominent example of such a cryptic clinical course has recently been described by Huck et al. [79] whose patient lacked somatic abnormalities and was diagnosed as FA, at the remarkable age of 49 years, only because of an unusually severe response to chemotherapy for bilateral breast cancer. Concerning somatic reversion, there are numerous examples of reversions in the *FANCA*, *FANCC* and *FANCD2* genes. Somatic reversions preferentially involve compound heterozygous patients and, depending on the cell lineages affected, may lead to improved blood counts and a mild or protracted clinical course (see chapter by Hoehn et al.).

An additional level of complexity is introduced by the genetic background of the respective patients or patient group. A case in point is the mutation

c.456+4A>T in *FANCC* which shows a high prevalence in patients of both Ashkenazi Jewish and Japanese origin [80, 81]. The Ashkenazi Jewish patients are severely affected whereas the Japanese patients have a much milder phenotype both in terms of hematologic onset and pattern of congenital abnormalities.

In summary, there is wide variation of phenotypes and clinical course among the most prevalent FA subgroups FA-A, -C and -G. The cumulative record of genotype-phenotype correlations clearly indicates that the nature of the underlying mutation is more important than the underlying complementation group. An exception to this rule are biallelic mutations in *FANCD1/BRCA2* and *FANCN/PALB2* which cause unusually severe phenotypes with early childhood leukemia and solid tumors, setting FA-D1 and FA-N patients apart from the majority of FA patients.

References

1 Taniguchi T, D'Andrea AD: The molecular pathogenesis of Fanconi anemia: recent progress. Blood 2006;107:4223–4233.
2 Verlander PC, Kaporis A, Liu Q, Zhang Q, Seligsohn U, Auerbach AD: Carrier frequency of the IVS4 + 4 A→T mutation of the Fanconi anemia gene *FAC* in the Ashkenazi Jewish population. Blood 1995;86:4034–4038.
3 Joenje H, Oostra AB, Wijker M, di Summa FM, van Berkel CG, et al: Evidence for at least eight Fanconi anemia genes. Am J Hum Genet 1997;61:940–944.
4 Levitus M, Rooimans MA, Steltenpool J, Cool NF, Oostra AB, et al: Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. Blood 2004;103:2498–2503.
5 Lo Ten Foe JR, Rooimans MA, Bosnoyan-Collins L, Alon N, Wijker M, et al: Expression cloning of a cDNA for the major Fanconi anaemia gene, *FAA*. Nat Genet 1996;14:320–323.
6 Strathdee CA, Gavish H, Shannon WR, Buchwald M: Cloning of cDNAs for Fanconi's anaemia by functional complementation. Nature 1992;356:763–767.
7 de Winter JP, Leveille F, van Berkel CG, Rooimans MA, van Der Weel L, et al: Isolation of a cDNA representing the Fanconi anemia complementation group E gene. Am J Hum Genet 2000;67:1306–1308.
8 de Winter JP, Rooimans MA, van Der Weel L, van Berkel CG, Alon N, et al: The Fanconi anaemia gene *FANCF* encodes a novel protein with homology to ROM. Nat Genet 2000;24:15–16.
9 de Winter JP, Waisfisz Q, Rooimans MA, van Berkel CG, Bosnoyan-Collins L, et al: The Fanconi anaemia group G gene *FANCG* is identical with *XRCC9*. Nat Genet 1998;20:281–283.
10 The Fanconi anemia/breast cancer consortium: Positional cloning of the Fanconi anaemia group A gene. Nat Genet 1996;14:324–328.
11 Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, et al: Positional cloning of a novel Fanconi anemia gene, *FANCD2*. Mol Cell 2001;7:241–248.
12 Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, et al: The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 2005;37:931–933.
13 Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, et al: The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 2005;37:934–935.
14 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
15 Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2007;39:162–164.

16 Meetei AR, Levitus M, Xue Y, Medhurst AL, Zwaan M, et al: X-linked inheritance of Fanconi anemia complementation group B. Nat Genet 2004;36:1219–1224.
17 Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, et al: A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet 2003;35:165–170.
18 Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, et al: A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet 2005;37:958–963.
19 Xia B, Sheng Q, Nakanashi K, Ohashi A, Wu J, et al: Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell 2006;22:719–729.
20 Bagby GC, Alter BP: Fanconi anemia. Semin Hematol 2006;43:147–156.
21 Surralles J, Jackson SP, Jasin M, Kastan MB, West SC, Joenje H: Molecular cross-talk among chromosome fragility syndromes. Genes Dev 2004;18:1359–1370.
22 Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: Replication, recombination, repair, and recovery. Environ Mol Mutagen 2005;45:128–142.
23 Kennedy RD, D'Andrea AD: The Fanconi Anemia/BRCA pathway: new faces in the crowd. Genes Dev 2005;19:2925–2940.
24 Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, et al: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 2001;7:249–262.
25 Meetei AR, Sechi S, Wallisch M, Yang D, Young MK, et al: A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol 2003;23:3417–3426.
26 Wang X, Andreassen PR, D'Andrea AD: Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol 2004;24:5850–5862.
27 Andreassen PR, D'Andrea AD, Taniguchi T: ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 2004;18:1958–1963.
28 Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M, D'Andrea AD: S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 2002;100:2414–2420.
29 Pichierri P, Rosselli F: The DNA crosslink-induced S-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. EMBO J 2004;23:1178–1187.
30 Nakanishi K, Taniguchi T, Ranganathan V, New HV, Moreau LA, et al: Interaction of FANCD2 and NBS1 in the DNA damage response. Nat Cell Biol 2002;4:913–920.
31 Pace P, Johnson M, Tan WM, Mosedale G, Sng C, et al: FANCE: the link between Fanconi anaemia complex assembly and activity. EMBO J 2002;21:3414–3423.
32 Ciccia A, Ling C, Coulthard R, Yan Z, Xue Y, et al: Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. Mol Cell 2007;25:331–343.
33 Park WH, Margossian S, Horwitz AA, Simons AM, D'Andrea AD, Parvin JD: Direct DNA binding activity of the Fanconi anemia D2 protein. J Biol Chem 2005;280:23593–23598.
34 Stiff T, Reis C, Alderton GK, Woodbine L, O'Driscoll M, Jeggo PA: Nbs1 is required for ATR-dependent phosphorylation events. EMBO J 2005;24:199–208.
35 Sobeck A, Stone S, Costanzo V, de Graaf B, Reuter T, et al: Fanconi anemia proteins are required to prevent accumulation of replication-associated DNA double-strand breaks. Mol Cell Biol 2006;26:425–437.
36 Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, et al: Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002;109:459–472.
37 Cantor SB, Andreassen PR: Assessing the link between BACH1 and BRCA1 in the FA pathway. Cell Cycle 2006;5:164–167.
38 Marek LR, Bale AE: *Drosophila* homologs of FANCD2 and FANCL function in DNA repair. DNA Repair (Amst) 2006;5:1317–1326.
39 Morgan NV, Tipping AJ, Joenje H, Mathew CG: High frequency of large intragenic deletions in the Fanconi anemia group A gene. Am J Hum Genet 1999;65:1330–1341.
40 Callen E, Tischkowitz MD, Creus A, Marcos R, Bueren JA, et al: Quantitative PCR analysis reveals a high incidence of large intragenic deletions in the *FANCA* gene in Spanish Fanconi anemia patients. Cytogenet Genome Res 2004;104:341–345.
41 Savoia A, Centra M, Ianzano L, de Cillis GP, Zelante L, Buchwald M: Characterization of the 5′ region of the Fanconi anaemia group C (*FACC*) gene. Hum Mol Genet 1995;4:1321–1326.

42 Kalb R, Neveling K, Hoehn H, Schneider H, Linka Y, et al: Hypomorphic mutations in the gene encoding a key Fanconi anemia protein, FANCD2, sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet 2007, in press.
43 Lightfoot J, Alon N, Bosnoyan-Collins L, Buchwald M: Characterization of regions functional in the nuclear localization of the Fanconi anemia group A protein. Hum Mol Genet 1999;8: 1007–1015.
44 Ferrer M, Rodriguez JA, Spierings EA, de Winter JP, Giaccone G, Kruyt FA: Identification of multiple nuclear export sequences in Fanconi anemia group A protein that contribute to CRM1-dependent nuclear export. Hum Mol Genet 2005;14:1271–1281.
45 Levran O, Diotti R, Pujara K, Batish SD, Hanenberg H, Auerbach AD: Spectrum of sequence variations in the *FANCA* gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat 2005;25:142–149.
46 Levran O, Doggett NA, Auerbach AD: Identification of Alu-mediated deletions in the Fanconi anemia gene *FAA*. Hum Mutat 1998;12:145–152.
47 Centra M, Memeo E, d'Apolito M, Savino M, Ianzano L, et al: Fine exon-intron structure of the Fanconi anemia group A (*FAA*) gene and characterization of two genomic deletions. Genomics 1998;51:463–467.
48 Levran O, Erlich T, Magdalena N, Gregory JJ, Batish SD, et al: Sequence variation in the Fanconi anemia gene *FAA*. Proc Natl Acad Sci USA 1997;94:13051–13056.
49 Holden ST, Cox JJ, Kesterton I, Thomas NS, Carr C, Woods CG: Fanconi anaemia complementation group B presenting as X-linked VACTERL with hydrocephalus syndrome. J Med Genet 2006;43:750–754.
50 Liebetrau W, Buhner M, Hoehn H: Prototype sequence clues within the Fanconi anaemia group C gene. J Med Genet 1995;32:669–670.
51 Wagner JE, Tolar J, Levran O, Scholl T, Deffenbaugh A, et al: Germline mutations in *BRCA2*: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood 2004;103:3226–3229.
52 Reid S, Renwick A, Seal S, Baskcomb L, Barfoot R, et al: Biallelic *BRCA2* mutations are associated with multiple malignancies in childhood including familial Wilms tumour. J Med Genet 2005;42:147–151.
53 Alter BP: The association between *FANCD1/BRCA2* mutations and leukaemia. Br J Haematol 2006;133:446–448; author reply 448.
54 Shivji MK, Venkitaraman AR: DNA recombination, chromosomal stability and carcinogenesis: insights into the role of BRCA2. DNA Repair (Amst) 2004;3:835–843.
55 West SC: Molecular views of recombination proteins and their control. Nat Rev Mol Cell Biol 2003;4:435–445.
56 Hussain S, Wilson JB, Medhurst AL, Hejna J, Witt E, et al: Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Hum Mol Genet 2004;13:1241–1248.
57 Hussain S, Witt E, Huber PA, Medhurst AL, Ashworth A, Mathew CG: Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. Hum Mol Genet 2003;12: 2503–2510.
58 Tarsounas M, Davies D, West SC: BRCA2-dependent and independent formation of RAD51 nuclear foci. Oncogene 2003;22:1115–1123.
59 Daniels MJ, Wang Y, Lee M, Venkitaraman AR: Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science 2004;306:876–879.
60 Popp H, Kalb R, Fischer A, Lobitz S, Kokemohr I, et al: Screening Fanconi anemia lymphoid cell lines of non-A, C, D2, E, F, G subtypes for defects in BRCA2/FANCD1. Cytogenet Genome Res 2003;103:54–57.
61 Montes de Oca R, Andreassen PR, Margossian SP, Gregory RC, Taniguchi T, et al: Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. Blood 2005;105:1003–1009.
62 Leveille F, Blom E, Medhurst AL, Bier P, Laghmani el H, et al: The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem 2004;279:39421–39430.
63 Tischkowitz M, Ameziane N, Waisfisz Q, De Winter JP, Harris R, et al: Bi-allelic silencing of the Fanconi anaemia gene *FANCF* in acute myeloid leukaemia. Br J Haematol 2003;123:469–471.

64 Narayan G, Arias-Pulido H, Nandula SV, Basso K, Sugirtharaj DD, et al: Promoter hypermethylation of *FANCF*: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res 2004;64:2994–2997.
65 Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, et al: Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med 2003;9:568–574.
66 Liu N, Lamerdin JE, Tucker JD, Zhou ZQ, Walter CA, et al: The human *XRCC9* gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. Proc Natl Acad Sci USA 1997;94:9232–9237.
67 Blom E, van de Vrugt HJ, de Vries Y, de Winter JP, Arwert F, Joenje H: Multiple TPR motifs characterize the Fanconi anemia FANCG protein. DNA Repair (Amst) 2004;3:77–84.
68 Hussain S, Wilson JB, Blom E, Thompson LH, Sung P, et al: Tetratricopeptide-motif-mediated interaction of FANCG with recombination proteins XRCC3 and BRCA2. DNA Repair (Amst) 2006;5:629–640.
69 Cantor SB, Bell DW, Ganesan S, Kass EM, Drapkin R, et al: BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell 2001;105:149–160.
70 Gupta R, Sharma S, Sommers JA, Jin Z, Cantor SB, Brosh RM Jr: Analysis of the DNA substrate specificity of the human BACH1 helicase associated with breast cancer. J Biol Chem 2005;280:25450–25460.
71 Callen E, Casado JA, Tischkowitz MD, Bueren JA, Creus A, et al: A common founder mutation in *FANCA* underlies the world's highest prevalence of Fanconi anemia in Gypsy families from Spain. Blood 2005;105:1946–1949.
72 Peleg L, Pesso R, Goldman B, Dotan K, Omer M, et al: Bloom syndrome and Fanconi's anemia: rate and ethnic origin of mutation carriers in Israel. Isr Med Assoc J 2002;4:95–97.
73 Tipping AJ, Pearson T, Morgan NV, Gibson RA, Kuyt LP, et al: Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Afrikaner population of South Africa. Proc Natl Acad Sci USA 2001;98:5734–5739.
74 Morgan NV, Essop F, Demuth I, de Ravel T, Jansen S, et al: A common Fanconi anemia mutation in black populations of sub-Saharan Africa. Blood 2005;105:3542–3544.
75 Savino M, Borriello A, D'Apolito M, Criscuolo M, Del Vecchio M, et al: Spectrum of *FANCA* mutations in Italian Fanconi anemia patients: identification of six novel alleles and phenotypic characterization of the S858R variant. Hum Mutat 2003;22:338–339.
76 Faivre L, Guardiola P, Lewis C, Dokal I, Ebell W, et al: Association of complementation group and mutation type with clinical outcome in Fanconi anemia. European Fanconi Anemia Research Group. Blood 2000;96:4064–4070.
77 Hirsch B, Shimamura A, Moreau L, Baldinger S, Hag-alshiekh M, et al: Association of biallelic *BRCA2/FANCD1* mutations with spontaneous chromosomal instability and solid tumors of childhood. Blood 2004;103:2554–2559.
78 Adachi D, Oda T, Yagasaki H, Nakasato K, Taniguchi T, et al: Heterogeneous activation of the Fanconi anemia pathway by patient-derived *FANCA* mutants. Hum Mol Genet 2002;11:3125–3134.
79 Huck K, Hanenberg H, Gudowius S, Fenk R, Kalb R, et al: Delayed diagnosis and complications of Fanconi anaemia at advanced age – a paradigm. Br J Haematol 2006;133:188–197.
80 Kutler DI, Auerbach AD: Fanconi anemia in Ashkenazi Jews. Fam Cancer 2004;3:241–248.
81 Futaki M, Yamashita T, Yagasaki H, Toda T, Yabe M, et al: The IVS4 + 4 A to T mutation of the fanconi anemia gene *FANCC* is not associated with a severe phenotype in Japanese patients. Blood 2000;95:1493–1498.
82 Tamary H, Bar-Yam R, Shalmon L, Rachavi G, Krostichevsky M, et al: Fanconi anaemia group A (*FANCA*) mutations in Israeli non-Ashkenazi Jewish patients. Br J Haematol 2000;111:338–343.
83 Magdalena N, Pilonetto DV, Bitencourt MA, Pereira NF, Ribeiro RC, et al: Frequency of Fanconi anemia in Brazil and efficacy of screening for the *FANCA* 3788-3790del mutation. Braz J Med Biol Res 2005;38:669–673.
84 Whitney MA, Saito H, Jakobs PM, Gibson RA, Moses RE, Grompe M: A common mutation in the *FACC* gene causes Fanconi anaemia in Ashkenazi Jews. Nat Genet 1993;4:202–205.

85 Yagasaki H, Oda T, Adachi D, Nakajima T, Nakahata T, et al: Two common founder mutations of the fanconi anemia group G gene *FANCG/XRCC9* in the Japanese population. Hum Mutat 2003;21:555.
86 Auerbach AD, Greenbaum J, Pujara K, Batish SD, Bitencourt MA, et al: Spectrum of sequence variation in the *FANCG* gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat 2003;21:158–168.
87 Demuth I, Wlodarski M, Tipping AJ, Morgan NV, de Winter JP, et al: Spectrum of mutations in the Fanconi anaemia group G gene, *FANCG/XRCC9*. Eur J Hum Genet 2000;8:861–868.
88 Gibson RA, Morgan NV, Goldstein LH, Pearson IC, Kesterton IP, et al: Novel mutations and polymorphisms in the Fanconi anemia group C gene. Hum Mutat 1996;8:140–148.

Reinhard Kalb
Department of Human Genetics, University of Würzburg
Biozentrum, Am Hubland
D–97074 Würzburg (Germany)
Tel. +49 931 8884089, Fax +49 931 8884069, E-Mail R.Kalb@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 59–78

Cancer in Fanconi Anemia and Fanconi Anemia Genes in Cancer

K. Neveling, R. Kalb, D. Schindler

Department of Human Genetics, University of Würzburg, Biocenter, Würzburg, Germany

Abstract

At the cellular level, defects in Fanconi anemia (FA) genes manifest themselves as hypersensitivity to DNA damaging agents which can be assessed by increased chromosome breakage and cell cycle changes. As long-term manifestations of cellular genetic instability, FA patients are at high risk for the development of a rather narrow spectrum of malignancies. Neoplasia occurs at a much younger age than in non-FA patients, and chiefly includes acute myeloid leukemia (AML) and squamous cell carcinomas (SCC). Given the role of FA genes in the maintenance of genetic stability, one would expect that FA genes are frequently altered in various kinds of tumors arising in non-FA patients. Much to our surprise and with the possible exception of pancreatic carcinoma, there appears to be no convincing evidence for a frequent association between either germline or somatic alterations in FA genes and malignancies arising in non-FA patients. Another notable exception is the *FANCF* gene which features prominently among the many genes that are silenced in cancer via promoter CpG island hypermethylation. However, the map location of *FANCF* adjacent to the 11p hotspot region of hypermethylation raises concerns about a strictly causal relationship between *FANCF* inactivation and tumorigenesis. Altogether, the available evidence suggests that tumor cells just like normal cells benefit from intact FA genes, possibly by keeping their replication machinery intact and by optimizing their defense against the adverse effects of reactive oxygen species.

The risk of neoplasia is increased in most genetic instability disorders caused by mutations in human caretaker genes. It is thought that this increased risk results from defects in the cellular response to DNA damage, including DNA damage recognition and DNA repair. Cell genetic evidence suggests that defective caretaker genes render the genome unstable and thereby promote the error rate in somatic cells. This would of course have the most devastating consequences in a somatic stem cell [1]. Since only a limited number of genetic

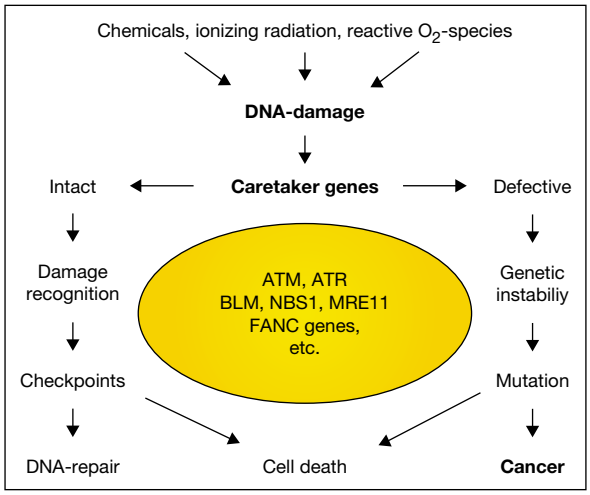


Fig. 1. Hypothetical role of human caretaker genes in the DNA damage response. For explanation see text.

changes appear to be required for the emergence of a clinically expressed malignancy, genetic instability due to defective caretaker genes is likely to speed up the process of malignant transformation [2]. Certain cellular proteins may represent crucial targets for the tumor initiating and tumor promoting effects of genetic instability. To these belong DNA polymerases, proteins which repair DNA, proteins which affect chromatin structure, kinetochore proteins, spindle proteins, and proteins which regulate apoptosis and cell cycle events in response to DNA damage [2]. Since FA proteins seem to be involved in various aspects of DNA maintenance including replication, recombination, repair and recovery [3], they themselves feature prominently among the 'at risk' proteins of genetic instability. A current concept depicting the role of caretaker genes in the DNA damage response is shown in figure 1. DNA damage can be induced in different ways, for example by chemicals or ionizing radiation or, endogenously, by reactive oxygen species. As long as caretaker genes are intact, DNA damage is recognized and, dependent on the kind of damage, checkpoints are activated, and either DNA repair or elimination via apoptosis will occur. In the case of defective caretaker genes, a regulated and orderly cell response is not guaranteed. DNA damage recognition and repair might be impaired, not occur at all, or be error-prone, resulting in genetic instability. As a result, mutations accumulate, ultimately leading either to cell death or to the emergence of malignant cell growth.

From a clinical point of view, there are two striking observations in support of a causal connection between the genetic instability phenotype of FA cells and

Table 1. Types of malignancies in human caretaker syndromes

Sensitivity	Syndrome	Gene	Malignancy
Chemicals like 4-NQO, BrdU	Werner syndrome	*WRN* helicase	soft tissue sarcomas
	Bloom syndrome	*BLM* helicase	all types of leukemias and solid tumors
	Rothmund-Thompson syndrome	*recQ-IV* helicase	soft tissue sarcomas
Ionizing radiation protection	Ataxia telangiectasia	*ATM* kinase	lymphoma
	Nijmegen breakage syndrome	*NBS1*	lymphoma
	AT-like syndromes	*MRE11, RAD50, LIG4*	lymphoreticular neoplasia
Reactive oxygen species, crosslinking agents	Fanconi anemia	*FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N*	AML, squamous cell carcinoma
(?) Reactive oxygen species	breast cancer ovarian cancer	*BRCA1, BRCA2*	breast cancer ovarian cancer

the emergence of neoplasia. The first observation concerns the age of onset of the neoplastic change which in contrast to the general population occurs in the teens [4] or, in the case of biallelic mutations in *FANCD1/BRCA2*, in infants less than 5 years of age [5, 6]. The second observation concerns the fact that the types of malignancies prevalent in FA neither mirror the spectrum of malignancies that is seen in the general population, nor do they mimic the kind of malignancies that are seen in the other caretaker syndromes (cf. table 1). AML and squamous cell carcinomas of the oropharynx and the genital area dominate in FA. These types of malignancies are rarely if at all encountered in other caretaker gene syndromes (cf. table 1). This suggests that susceptibility to certain types of neoplasms primarily reflects the type of caretaker gene that is defective in a given patient. Both the cellular and the clinical phenotype of FA indicate a close connection between the FA proteins, DNA repair and tumorigenesis. This article summarizes some basic information about the obvious FA–cancer connection and thus focuses on the role of cancer in FA as well as on what is currently known on disruptions of FA genes in cancer.

Neoplasia in Fanconi Anemia

Myelodysplastic Syndrome (MDS)

MDS is a frequent hematological disorder in FA. In MDS, bone marrow precursor cells fail to differentiate properly such that not enough mature

erythrocytes, leukocytes or thrombocytes arrive in the periphery. MDS occurs in around 7% of all patients with FA [4] amounting to around 50% of all hemato-proliferative changes seen in FA [7]. The presence of MDS affects the prognosis in FA: the estimated 5-year survival with MDS amounts to 0.09, versus 0.92 without MDS [8]. If at all, FA-related MDS progresses to leukemia more slowly compared to MDS in non-FA patients. FA patients may suffer from MDS for many years without developing overt leukemia. Among 69 cases with MDS described by Alter et al. [8], only 10 developed leukemia. MDS in FA obviously follows a different natural course than MDS in non-FA patients [8].

Leukemia

The most common malignancy arising in FA is leukemia. The overall cumulative incidence of leukemia is 37%, the median age of onset is 14 years [4]. The types of leukemia prevalent in FA differ substantially from what is seen in the general population: Acute myeloid leukemia (AML) dominates in FA (94% of all leukemias), whereas acute lymphocytic leukemia (ALL) amounts to only 6%. The reverse holds for non-FA patients (84% of leukemias in the general population are ALL) [4]. Hematological malignancies other than AML or ALL are relatively rare in FA. Chronic myelo-monocytic leukemia (CMMOL) or Burkitt lymphoma have been described as single cases [7].

It is important to point out that AML may be the first and only manifestation of FA in otherwise normal patients [9]. All defined FAB subclasses [10] are represented in FA-associated AML [4], albeit with different proportions compared to the general population. There are more FA patients in the M4 and M6 and less in the M1 and M2 subclasses of AML compared to non-FA patients [4]. Several recent studies show that certain clonal aberrations can affect the risk of developing AML [8, 11–13]. The most frequently reported acquired clonal aberrations in FA patients are trisomies of chromosome 1q and monosomies of chromosome 7. In addition, partial trisomies and tetrasomies of chromosome 3q are proven risk factors for the development of MDS and AML [14] (see Chapter by Neitzel et al.).

Solid Tumors

The risk for solid tumors in FA patients exceeds that of the general population by nearly the 50-fold [15]. In addition, cancer in FA strikes very early. The median age is 16 years, whereas it is 68 years in the non-FA population [4]. FA patients may have combinations of different malignancies [4, 7]. In 25% of FA cases with cancer, the malignancy was present prior to diagnosis of FA [4], an extreme example being a 49-year-old patient with bilateral carcinoma of the breast who developed MDS and AML following chemotherapy [16].

Squamous Cell Carcinoma

Five percent of all FA patients summarized by Alter [4] had solid tumors, the most typical tumors being squamous cell carcinomas of the head and neck (HNSCC) in both males and females, and of the genital area in females. HNSCC in FA include mostly tumors of the tongue, gingiva, upper esophagus, larynx and oropharynx [15]. The risk for HNSCC in FA patients is more than 700 times greater than in the general population [15]. In non-FA patients, HNSCC normally occurs in men older than 40 years who smoke and drink [17]. In contrast, FA patients with HNSCC are usually young, non-smokers and non-drinkers. Females are more often affected than males, especially among those patients in whom the diagnosis of FA was made after the diagnosis of cancer, but this may reflect the overall survival advantage of female FA patients [15].

The mucous epithelia are common routes for viral infections, especially for HPV (human papilloma virus) and HSV (herpes simplex virus). Immunosuppression associated with bone marrow failure might render patients vulnerable to the integration of the viral DNA [7]. While HPV infections are proven triggers for the development of cancer of the cervix uteri, their role in non-anogenital cancers is still unclear [18]. The expression profile of HPV-associated HNSCC differs from that in HPV-negative HNSCC: *TP53* is mutated in HPV-negative tumors, but remains functional in HPV-associated HNSCC because of rapid p53 degradation by the viral protein E6 [18]. The presence of HPV in tumors may affect the prognosis, as patients with HPV-associated HNSCC have a lower risk for death than HPV-negative HNSCC patients [18]. Concerning FA, it has been found that 54% of all patients with genital squamous cell carcinomas of cervix, vulva and anus had HPV-associated condylomas before occurrence of the tumor [7]. These findings were seemingly confirmed by a second study showing that 84% of FA-associated squamous cell carcinomas (SCC) had detectable HPV-DNA, while only 36% of non-FA SCC showed viral infection [19]. However, other investigators were unable to demonstrate such high rates of HPV contamination. For example, four cell lines freshly established from FA HNSCC tumors had no detectable HPV DNA. No differences concerning loss of heterozygosity pattern, *TP53* mutations and *TP53* polymorphisms were found between these FA cell lines and non-FA HNSCC cell lines leading the authors to conclude that FA-associated HNSCC are not fundamentally different from sporadic HNSCC, except for their sensitivity to crosslinking agents [20]. In addition, chromosomal changes in oral squamous cell carcinomas of two FA patients were very similar to what was seen in non-FA oral tumors [21]. Squamous cell carcinomas are aggressive tumors that initially present as minor mucosal lesions, chronic inflammations or red or white cell spots. Diagnosis is often delayed leading to poor therapeutic success and no better than 50% 2-year survival rates [17]. A sensitive brush method for obtaining cells from tongue

and oral cavity may facilitate monitoring of early cell changes and thereby contribute to the prevention of this devastating type of malignancy.

Due to immune suppression and/or conditioning measures, the risk for developing solid tumors in FA patients increases after hematopoetic stem cell transplantation (HSCT). Conditioning but also post-transplant complications (e.g. graft versus host disease and infections) sharply increase this risk for malignancies with poor survival. The cumulative incidence of head and neck carcinoma in FA patients is 20% and 53% by 10 and 15 years after transplantation, respectively [82]. The median age of FA patients who developed cancer following HSCT was 21 years [4].

Liver Tumors

Liver tumors are frequent in FA and in the great majority of cases arise in the context of androgen therapy for aplastic anemia. They mostly are adenomas and rarely hepatocellular carcinomas. The cumulative incidence for these tumors is 46% by age 50, and the median age of onset is 13 years [4]. Development of liver tumors due to androgen therapy is frequent in FA but not specific for FA patients. The risk for hepatic carcinomas may depend on the specific type of androgen used. Oxymetholone and methyltestosterone seem to be associated more frequently with the development of hepatocellular carcinomas (HCC), whereas patients treated with danazol develop adenomas but may be less affected by hepatic carcinomas [22]. Adenomas usually regress upon discontinuation of therapy. In rare cases, both HCCs and adenomas can be found in the same patient.

Other Tumors

Other tumors occasionally found in FA patients include brain and renal tumors, breast carcinoma, basal cell carcinoma, neuroblastoma, desmoid tumors, gonadoblastoma, melanoma, neurilemmona and osteogenic sarcoma [7]. A major factor thought to promote tumor development in FA is oxidative stress [23–26]. It should also be emphasized that biallelic mutations in two of the downstream FA genes (*FANCD1/BRCA2* and *FANCN/PALB2*) confer an exceptionally high risk for early childhood leukemia and a variety of soild tumors [6, 43, 84].

Fanconi Anemia Genes in Cancer

Disruption of the FA/BRCA pathway by a defect in one of the underlying genes results in a defective response to certain types of DNA damage. Defects in the FA/BRCA pathway may therefore contribute to non-FA tumorigenesis.

So far, this has been especially reported for the *FANCF* gene which was found to be epigenetically inactivated in a number of tumors in non-FA patients (reviewed in [27]). In order to find evidence for the involvement of any of the other FA genes in malignancies, a variety of tumors have been examined for sequence variations in FA genes in non-FA patients. Studies searching for germline changes including polymorphisms of FA genes as possible predisposing factors of tumorigenesis in non-FA patients are summarized in table 2, whereas the special case of *FANCF* will be discussed separately (see below).

Table 2 provides a survey of germline mutations in FA genes that were found in tumors of non-FA patients. Listed are the respective FA genes, the type of tumors investigated, the family history and the kind of mutations that were detected. The question whether a given mutation is likely to be causal for tumorigenesis is answered by 'yes' or 'no' depending on the respective authors' interpretation. The conclusion suggested by the data presented in table 2 is that there is no convincing evidence for a causal association between germline changes in FA genes and propensity to tumorigenesis in non-FA patients.

In contrast to table 2, table 3 summarizes studies looking for purely somatic changes in FA genes in tumor patients without FA germline mutations. Again, studies involving *FANCF* are not included but are discussed below. Table 3 shows the respective FA genes analyzed, the types of tumors investigated, the putative family history, and the kinds of mutations detected. Answers to the question whether a mutation is likely to be causal for tumorigenesis are indicated according to the respective authors' interpretation. It should be pointed out that in table 3 there are more 'yes' answers than 'no' answers to this question. However, most of the 'yes' answers had to be marked with a question mark which underlines the high degree of uncertainty that characterizes the interpretation of most of these studies. Final conclusions must await additional studies, but what is already clear at this point is that somatic mutations in FA genes, with the possible exception of pancreatic carcinoma, appear to be surprisingly infrequent events in various types of human malignancies.

As listed in tables 2 and 3, a number of malignancies in non-FA patients, most notably AML, pancreatic cancer, carcinoma of the breast and ovary, and squamous cell carcinoma, have been repeatedly investigated for possible associations with FA germline and/or somatic mutations. The following is a brief discussion of the most important findings of these studies (see also [28]).

AML

AML is the most common hematological malignancy in FA, and FA genes were investigated in several cases of familial and sporadic non-FA AML. Occasional mutations and polymorphisms were described in *FANCA*, *FANCC*, *FANCD1/BRCA2* and *FANCG*. Since more than 50% of FA patients belong to

Table 2. Germline sequence variations of FA genes in non-FA tumor patients

Gene	Cancer type[a]	Sporadic/ familial	Mutation type found	Likely to be causal	Reference
FANCA	AML	sporadic	deletions	yes	[30]
	breast cancer	familial	missense/polymorphism	no	[58]
		sporadic	polymorphism	no	[60]
		both	polymorphism	no	[59]
	ovarian cancer	both	polymorphism	yes?	[59]
	pancreatic cancer	familial	missense	no	[38]
FANCC	AML/ALL (childhood)	sporadic	polymorphism	yes?	[32]
	T-ALL	familial	frameshift	yes	[31]
	breast cancer	familial	polymorphisms	no	[58]
	pancreatic cancer	familial	polymorphisms	no	[37]
		sporadic	missense	yes?	[35]
			frameshift, missense	yes	[36]
FANCD1/ BRCA2	AML (childhood)	sporadic	no IVS7 splice site mutation	no	[33]
	esophageal SCC	familial	missense	no?	[61]
	ovarian cancer	both	frameshift, stop	yes?	[50]
FANCD2	breast cancer	familial	polymorphisms	no	[58]
		familial	polymorphisms, silent variants	no	[56]
		sporadic	polymorphism	yes	[60]
FANCE	breast cancer	familial	missense/polymorphisms	no	[58]
FANCF	breast cancer	familial	polymorphism	no	[58]
FANCG	AML (childhood)	sporadic	aberrant splicing, missense, deletion	no	[34]
	breast cancer	familial	–	no	[58]
	pancreatic cancer	familial	polymorphisms	no	[37]
		sporadic	stop	yes?	[35]
FANCJ	breast cancer	familial	missense	yes?	[53]
		sporadic	missense	?	[53]
		familial	missense, polymorphisms	no	[55]
		familial	missense, frameshift, silent	no	[56]
		familial	polymorphism	yes	[54]
		familial	polymorphism	no	[57]
		familial	frameshift/stop	yes	[83]
FANCL	breast cancer	sporadic	polymorphism	no	[60]
FANCN	breast cancer	familial	frameshift/stop	yes	[84]
	breast cancer	familial	missense/frameshift	yes	[85]
	breast cancer	familial	frameshift	yes	[85]

[a]ALL: Acute lymphocytic leukemia; AML: acute myeloid leukemia; SCC: squamous cell carcinoma.

Table 3. Somatic changes of FA genes in non-FA tumor patients

Gene	Cancer type[a]	Sporadic/ familial	Mutation type	Likely to be causal	Reference
FANCA	AML (adult)	sporadic	not found	yes?	[29]
FANCC	pancreatic cancer	sporadic	frameshift	yes?	[35]
FANCD1/BRCA2	breast cancer	?	frameshift	yes?	[48]
		sporadic	frameshift, missense, silent variants	yes?	[49]
	esophageal SCC	?	frameshift, missense, silent variants	no?	[61]
	oral SCC	sporadic	genomic alteration	yes?	[62]
	ovarian cancer	both	aberrant splicing, frameshift, stop	yes?	[50]
FANCD2	oral SCC	sporadic	genomic alteration	yes?	[62]
FANCG	oral SCC	sporadic	genomic alteration	yes?	[62]

[a]AML: Acute myeloid leukemia; SCC: squamous cell carcinoma.

complementation group FA-A, in many studies the *FANCA* gene has been the primary target of investigation. For example, a leukemic cell line of an adult non-FA AML patient was found to exhibit hypersensitivity towards mitomycin C (MMC) and diepoxibutane (DEB) combined with a decrease of FANCA, FANCG and FANCD2-L expression which could be corrected by transduction with a *FANCA* containing vector. However, the authors of this study failed to find any evidence for mutations in the *FANCA* gene. They therefore proposed the idea of unidentified factors affecting the cellular localization and binding activity of FANCA, leading to cytogenetic instability and clonal progression [29]. In another study four cases of heterozygous germline *FANCA* deletions were reported among 101 sporadic AML patients. In all four cases, there was no evidence for inactivation of the second allele either by mutation or promoter hypermethylation. Since the frequency of the detected deletions was 35-fold higher than the expected frequency for germline *FANCA* deletions, the authors concluded that deletions leading to reduced expression of *FANCA* may be involved in a subset of cases of sporadic AML [30]. The possibility of a connection between tumorigenesis and FA genes was also considered for *FANCC*: Rischewski and coworkers described a heterozygous *FANCC* frameshift mutation in two siblings with T-ALL [31]. No other mutations were found in these patients, leading the authors to the conclusion that heterozygous carriers of FANC mutations might have an increased risk for developing AML.

Subsequently, Barber and colleagues found 12 *FANCC* sequence variants in genomic DNA from sporadic AML/ALL cases [32]. However, there was no evidence for known pathogenic *FANCC* mutations, but one of the polymorphisms (S26F) was observed four times more frequently in AML patients than in random cord blood samples suggesting a possible contribution to the risk for childhood AML. Other researchers found no convincing evidence for frequent occurrence of FA gene mutations in AML. For example, *FANCD1/BRCA2* patients who develop AML frequently show certain exon 7 splice mutations. In contrast, such 'signature' mutations were not present in DNA from non-FA sporadic childhood AML patients [33]. In a subsequent study, 107 children with sporadic AML were investigated for germline sequence variants in *FANCG*. Different kinds of mutations were found in seven of these children. Two patients had biallelic *FANCG* variants, suggesting that they might have been undiagnosed FA patients. The overall message from this study was that FA carrier status does not predispose to sporadic AML [34]. Despite occasional positive findings, it must be emphasized that in the great majority of non-FA patients there is no convincing evidence for a clear association between defective FA genes and occurrence of AML. However, one should be aware of the possibility of undiagnosed FA cases among sporadic AML patients.

Pancreatic Cancer

FANC gene mutations in pancreatic cancer have been described for *FANCA*, *FANCC* and *FANCG*. Again, in some cases the authors of these studies consider a connection between detected mutations and tumorigenesis, but other studies fail to find evidence for such an association. In particular, *FANCC* mutations were found repeatedly in sporadic cases of pancreatic cancer, and inherited as well as somatic mutations were reported in patients with early onset pancreatic cancer. The mutations were hemizygous due to loss of one allele in the tumor. Additionally, a probably inherited nonsense mutation was found in *FANCG*. The authors concluded that their findings may point towards a general involvement of FA genes in cancer [35]. Mutations in *FANCC* and *FANCG* in sporadic pancreatic cancer were also described in another study, in which germline truncating and missense *FANCC* mutations were reported as well as a single somatic missense mutation in *FANCG*. The germline *FANCC* mutations were associated with loss of heterozygosity (LOH) in the tumor. Again, the authors suggested that inherited mutations in *FANCC* may predispose to pancreatic cancer [36]. However, in critically reviewing these and other data, Rogers et al. [37] concluded that germline and somatic changes in *FANCC* and *FANCG*, if at all, may have comparatively low penetrance for the pancreatic cancer phenotype. The same was suggested regarding *FANCA*: although several exonic *FANCA* variants, including disease-associated variants, were identified

in lymphocyte DNA from familial pancreatic cancer patients, these variants were either found in controls at the same frequency as in familial pancreatic cancer, or there were no convincing segregation data for these variants. Rogers et al. therefore maintain that germline *FANCA* mutations do not contribute to familial pancreatic cancer susceptibility [38]. In reviewing these studies we find that there is some degree of suggestive but no definitive evidence for a causal association between defective FA genes and pancreatic cancer.

Breast and Ovarian Cancer

BRCA2 (breast cancer susceptibility gene 2) is one of the most important caretaker genes associated with breast and ovarian cancer. *BRCA2* was identified as the second gene mutated in high risk breast cancer families in 1994 [39, 40]. In 2002, biallelic mutations in *BRCA2* were described to result in an FA-like phenotype [41]. Therefore, monoallelic inactivation of this caretaker gene causes breast and ovarian cancer predisposition, while biallelic mutations cause a severe form of FA [42, 43]. The role of heterozygous germline mutations in *BRCA2* as a predisposing factor for familial breast and ovarian cancer has often been described (for review see [44]) and germline mutations were also found to be responsible for some kinds of apparently sporadic forms of breast and ovarian cancer [45–47]. Somatic *BRCA2* mutations are rare. A first case of a somatic truncating mutation in a sporadic breast cancer involved a 1-bp deletion resulting in premature truncation. This change was detected in a primary ductual breast carcinoma with demonstrated LOH of *BRCA2*-flanking markers [48]. Interestingly, somatic *BRCA2* mutations were also described in rare cases of sporadic male breast cancer involving frameshift, missense and silent mutations in tumor tissues, all associated with allelic loss at the *BRCA2* locus [49]. Regarding ovarian cancer (sporadic as well as familial), germline as well as somatic *BRCA2* mutations were occasionally found, all being frameshift mutations or base substitutions resulting in premature termination and all associated with LOH of flanking markers in the tumor [50]. Taken together, it is firmly established that germline mutations of *BRCA2* are associated with breast and ovarian cancer. These alterations are accompanied by allelic loss in the tumor tissue, suggesting a tumor suppressor function, whereas somatic mutations in *BRCA2* seem to be rare events in breast and ovarian cancer.

FANCJ/BRIP1/BACH1 is one of the recently identified FA genes (BRIP1: BRCA1 interacting protein/BACH1: BRCA1 associated C-terminal helicase) [51, 52]. Because of its direct interaction with BRCA1 at the protein level, *FANCJ* is an obvious candidate for a breast cancer susceptibility gene. Several studies investigated this hypothesis but arrived at different conclusions. Cantor and colleagues described heterozygous germline missense mutations in two breast cancer patients (P47A and M299I), one being familial and the other sporadic

[53]. Co-segregation of the familial mutation with breast cancer could not be tested because DNA from the family members was not available. However, both mutations were not found in 200 controls. The authors concluded that mutant *BACH1/BRIP1* participates in breast cancer development. In a subsequent study, genomic DNA from familial breast cancer cases was investigated for single nucleotide polymorphisms (SNP) in *BACH1/BRIP1*. The polymorphism P919S (rs4986764) was thought to be associated with increased breast cancer risk [54]. In contrast, different heterozygous *BACH1*/*BRIP1* germline alterations (polymorphisms and missense mutations) were observed in a screen of *BRCA1/2*-negative breast cancer families, and none of these sequence alterations seemed to be associated with a detectable risk for breast cancer [55]. A subsequent study from Australia reported different heterozygous germline *BRIP1/FANCJ* variants in a similar screen of *BRCA1/2*-negative breast cancer families [56]. Investigation of relatives of the index patients provided no evidence for co-segregation of the sequence variants with the tumors, contradicting any causal association between *BRIP1* alterations and familial breast cancer. A comparable Scandinavian study found six germline alterations in *FANCJ*, none of them segregating with the cancer phenotype. Notably, the P919S polymorphism, previously reported to be associated with breast cancer, showed no connection to increased breast cancer risk in the Finnish study [57]. However, the two largest studies to date of BRCA1/BRCA2 negative familial breast cancer implicate both *FANCJ/BRIP1* and the newly discovered *FANCN*/*PALB2* gene as low penetrance breast cancer susceptibility genes [83–85].

In addition to *FANCD1/BRCA2* and *FANCJ/BRIP1*, other FANC genes have also been investigated for being putative breast and ovarian cancer susceptibility genes. For example, germline DNA from 88 *BRCA1/2*-negative breast cancer families was examined for mutations in *FANCA, FANCC, FANCD2, FANCE, FANCF* and *FANCG.* Altogether, 69 sequence variants were found, among them 25 exonic variants (including 14 polymorphisms), but no frameshift and no nonsense mutations. Only two conservative missense variants were observed, one in *FANCA* and one in *FANCE*. These data suggest that heterozygous FA gene mutations in genes other than *FANCD1*/*BRCA2* do not confer a high risk for breast cancer [58]. In a following study, a novel duplication-polymorphism in the promoter region of *FANCA* was reported, and germline DNA from breast cancer patients, ovarian cancer patients and controls was investigated for this polymorphism [59]. The authors found a non-significant decrease of the mutant allele compared to the common allele in breast cancer patients, and a significant decrease in ovarian cancer patients. Accordingly, this particular polymorphism may not influence the risk for breast cancer but might rather convey a protective function regarding ovarian cancer. Concerning *FANCD2*, a recent study reported lack of expression in 10–20% of sporadic

and BRCA1-related breast cancer, but it was not stated whether this was due to somatic mutations, defective processing, or epigenetic silencing [87]. Otherwise, there are contradictory data concerning genetic variation of *FANCD2* and breast cancer risk [56, 60]. The overall conclusion from these studies is that a number of germline mutations have been identified in FA genes of non-FA patients with early onset breast cancer. However, a likely role as low-penetrance susceptibility genes has been demonstrated only for alterations in the BRCA-associated genes *FANCJ* and *FANCN*.

Squamous Cell Carcinoma

Esophageal squamous cell carcinomas (ESCC) have been shown to have a high rate of allelic loss involving chromosome 13, including the locus of the *BRCA2* gene. These observations prompted the screening of DNA from ESCC patients for mutations in *BRCA2*. Altogether, three patients with germline mutations and two patients with somatic sequence variants were found. These included missense or silent variants. The authors suggested that *BRCA2* mutations do occur in ESCC, but are infrequent and of unknown consequence [61]. In another study, Sparano and colleagues searched for candidate genes responsible for oral squamous cell carcinoma (OSCC) by array comparative genomic hybridization (aCGH) [62]. They assessed 512 genes commonly altered in cancer. Among cancer related genes that were found to be changed in more than 25% of OSCC, the authors identified several FA genes, including *FANCD1*, *FANCD2* and *FANCG*. This might be an important clue. Given the high frequency of OSCC in FA it would come as no surprise if FA genes would play a role in the genesis of OSCC, but additional studies are needed to confirm these preliminary observations.

FANCF: A Genuine Tumor Gene?

Whereas the data presented in tables 2 and 3 overwhelmingly contradict the hypothesis of a non-random, causal association between altered FA genes and the occurrence of neoplasia in non-FA patients, an important exception is the *FANCF* gene. *FANCF* is the smallest of the presently known 12 FA genes. It consists of only a single exon, and the coding region comprises only 1124 nucleotides. The 42 kDa protein has homology to the prokaryotic RNA binding protein ROM, but no function similar to ROM. Known mutations in *FANCF* are deletions and nonsense mutations [63]. The only function of FANCF seems to be its role as a flexible adapter protein of the FA core complex [64]. Contrary to its small size and to its presumably minor function in the FA nuclear core complex, *FANCF* has been found silenced by hypermethylation of

its promoter CpG island in a variety of human neoplasias. Table 4 contains a list of different kinds of tumors in which *FANCF* hypermethylation has been detected.

The prevailing concept is that inactivation of a caretaker gene such as *FANCF* leads to genomic instability and therefore might be an early step in tumorigenesis [65]. This concept has received strong support by the recent observation of frequent epigenetic silencing of another caretaker gene, *WRN*, in human tumors [66]. As summarized in table 4, epigenetic silencing of *FANCF* has been investigated in a large number of different tumors, with positive results in many of them. However, it is not clear at present whether disruption of the FA/BRCA pathway by *FANCF* methylation is a causative factor in cancerogenesis or whether *FANCF* methylation occurs because of its location adjacent to a known hotspot region for hypermethylation at 11p15 [63, 67]. Silencing effects due to physical proximity to hotspot regions for hypermethylation have been described for other genes. For example, altered expression of the *CDKN1C* gene by promoter hypermethylation has been reported for the Beckwith-Wiedemann syndrome and for several other human cancers [68]. Therefore, the contribution of *FANCF* silencing to tumorigenesis might be a coincidental rather than primary event [86].

General Conclusions

Individuals with biallelic mutations in any of the 12 known FA genes have a strongly elevated risk for neoplastic disease at comparatively young age. The types of neoplasias that predominate in FA patients include AML and squamous cell carcinomas of the oropharynx and the genital area. Given the high degree of genetic instability and elevated mutation rates seen in FA patients, there is little doubt about a causative relationship between FA-specific defects of genome maintenance and the emergence of malignant cells. If the immune system was not relatively intact in most FA patients until the terminal stages of bone marrow failure we would most likely encounter even higher frequencies of malignant growth in these patients, and at even younger ages. In addition to their increased sensitivity towards DNA crosslinking agents, FA cells are exceedingly sensitive to reactive oxygen species which is unique for any of the human caretaker gene syndromes and may explain part of their susceptibility to malignant transformation. Ambient air and thus possibly harmful concentration of oxygen is present at the sites most frequently affected by squamous cell carcinomas in FA patients (oral cavity and genital area), but other factors like virus infections may also contribute to tumor development at these particular sites.

Table 4. Silencing of *FANCF* in human malignancies

Cancer type[a]	*FANCF* methylation	Proportion (%)	Reference
AML	• one cell line (CHRF-288) • 0/36 sporadic AML patients	0	[71]
Astrocytoma	0/1 gastrocytoma cell line	0	[72]
Sporadic childhood leukemia	0/81 diagnostic bone marrow aspitates	0	[73]
Biliary cancer	0/4 biliary cancer cell lines	0	[72]
Bladder cancer	0/2 bladder cancer cell lines • 1/23 cell lines • 1/41 bladder cancer tissues	0 4 2	[74] [86]
Breast cancer	13/75 primary breast tumors 0/1 breast cancer cell line 1/15 breast cancer cell lines (decreased expression, not tested for methylation)	17 0 6	[75] [74] [72]
Cervical cancer	• 27/91 primary tumors • 3/9 cell lines	30 33	[76]
Colon cancer	0/3 colon cancer cell lines	0	[74]
HNSCC	0/8 HNSCC cell lines 13/89 primary HNSCC 0/10 HNSCC cell lines	0 15 0	[72] [74] [77]
NSCLC	• 22/154 primary NSCLC • 0/20 NSCLC cell lines	14	[74]
LCLC	0/1 LCLC cell line	0	[72]
SCLC	0/10 SCLC cell lines	0	[74]
Osteosarcoma	0/1 osteosarcoma cell line	0	[74]
Ovarian cancer	• 2/25 ovarian tumor lines • 4/19 primary ovarian tumors 6/25 granulosa cell tumors 0/106 stage III/IV epithelial ovarian tumors • 1/7 ovarian cancer cell lines • 5/18 primary ovarian tumors	8 21 24 0 14 28	[65] [78] [79] [80]
Prostate cancer	0/1 prostate cancer cell line 0/6 prostate cancer cell lines	0 0	[74] [72]
Testicular germ cell tumor (non-seminoma)	4/70 germ cell tumors	6	[81]

[a]AML: Acute myeloid leukemia; HNSCC: head and neck squamous cell carcinoma; LCLC: large-cell lung carcinoma; NSCLC: non-small cell lung cancer; SCLC: small-cell lung cancer.

In contrast to the strongly elevated cancer risk in FA patients, there is little evidence so far that germline mutations and/or polymorphisms of FA genes play a major role in tumorigenesis of non-FA tumor patients. This is in good agreement with the observations that heterozygous parents and siblings of FA patients are not at increased risk for malignancies, indicating that FA genes, with the possible exception of *FANCD1/BRCA2*, are unlikely to function as classical tumor suppressor genes [69].

With respect to purely somatic mutations, the situation is less clear, but again the evidence in favor of a major contribution of somatic FA mutations to tumorigenesis in non-FA patients is tenuous at best. The only exception is the *FANCF* gene which has been found to be epigenetically silenced in a variety of tumors and tumor cell lines. However, *FANCF* maps to a known hotspot of hypermethylation in the human genome such that the possibility of a secondary rather than primary effect of *FANCF* silencing must be seriously considered.

As pointed out by van der Heijden and colleagues, there might well be selection *for* FA protein function in tumorigenesis in order to assure a certain DNA repair capacity of malignant cells [70]. These authors speculate that 'a limited level of genetic instability could be beneficial to tumorigenesis, but excessive DNA repair defect would not'. In reviewing the evidence for genetic changes in FA genes in non-FA tumors, we tend to arrive at a similar conclusion. The relative paucity of either germline or somatic mutations in FA genes in non-FA tumor patients, and the possibility of a 'bystander' rather than primary effect of *FANCF* silencing in a variety of human neoplasias strongly suggest that loss of FA gene function may be detrimental rather than beneficial for most tumor cells. It seems that a family of genes like the FA family that participates in diverse functions of DNA maintenance, including DNA replication and DNA repair, is essential for cells with proliferative capacity. Loss of FA gene function impairs proliferation of hematopoietic stem cells and might also jeopardize proliferation of cancer stem cells.

References

1 Park Y, Gerson SL: DNA repair defects in stem cell function and aging. Annu Rev Med 2005;56:495–508.

2 Beckman RA, Loeb LA: Genetic instability in cancer: theory and experiment. Semin Cancer Biol 2005;15:423–435.

3 Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 2005;45:128–142.

4 Alter BP: Cancer in Fanconi anemia, 1927–2001. Cancer 2003;97:425–440.

5 Meyer S, Fergusson WD, Oostra AB, Medhurst AL, Waisfisz Q, et al: A cross-linker-sensitive myeloid leukemia cell line from a 2-year-old boy with severe Fanconi anemia and biallelic *FANCD1/BRCA2* mutations. Genes Chromosomes Cancer 2005;42:404–415.

6 Reid S, Renwick A, Seal S, Baskcomb L, Barfoot R, et al: Biallelic *BRCA2* mutations are associated with multiple malignancies in childhood including familial Wilms tumour. J Med Genet 2005;42:147–151.
7 Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, et al: A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–1256.
8 Alter BP, Caruso JP, Drachtman RA, Uchida T, Velagaleti GV, Elghetany MT: Fanconi anemia: myelodysplasia as a predictor of outcome. Cancer Genet Cytogenet 2000;117:125–131.
9 Auerbach AD, Weiner MA, Warburton D, Yeboa K, Lu L, Broxmeyer HE: Acute myeloid leukemia as the first hematologic manifestation of Fanconi anemia. Am J Hematol 1982;12:289–300.
10 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, et al: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 1982;51:189–199.
11 Fundia A, Gorla N, Larripa I: Spontaneous chromosome aberrations in Fanconi's anemia patients are located at fragile sites and acute myeloid leukemia breakpoints. Hereditas 1994;120:47–50.
12 Ferti A, Panani A, Dervenoulas J, Raptis SA: Cytogenetic findings in a Fanconi anemia patient with AML. Cancer Genet Cytogenet 1996;90:182–183.
13 Oliveira NI, Ribeiro EM, Raimondi SC, Bittencourt MA, Pasquini R, Cavalli IJ: Two different karyotypes with 1q abnormalities in a patient with Fanconi anemia. Leuk Res 2002;26: 1047–1049.
14 Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, Neitzel H: Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood 2003;101:3872–3874.
15 Alter BP, Joenje H, Oostra AB, Pals G: Fanconi anemia: adult head and neck cancer and hematopoietic mosaicism. Arch Otolaryngol Head Neck Surg 2005;131:635–639.
16 Huck K, Hanenberg H, Gudowius S, Fenk R, Kalb R, et al: Delayed diagnosis and complications of Fanconi anaemia at advanced age – a paradigm. Br J Haematol 2006;133:188–197.
17 Kutler DI, Auerbach AD, Satagopan J, Giampietro PF, Batish SD, et al: High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg 2003;129:106–112.
18 Gillison ML, Lowy DR: A causal role for human papillomavirus in head and neck cancer. Lancet 2004;363:1488–1489.
19 Kutler DI, Wreesmann VB, Goberdhan A, Ben-Porat L, Satagopan J, et al: Human papillomavirus DNA and p53 polymorphisms in squamous cell carcinomas from Fanconi anemia patients. J Natl Cancer Inst 2003;95:1718–1721.
20 van Zeeburg HJ, Snijders PJ, Pals G, Hermsen MA, Rooimans MA, et al: Generation and molecular characterization of head and neck squamous cell lines of Fanconi anemia patients. Cancer Res 2005;65:1271–1276.
21 Hermsen MA, Xie Y, Rooimans MA, Meijer GA, Baak JP, et al: Cytogenetic characteristics of oral squamous cell carcinomas in Fanconi anemia. Fam Cancer 2001;1:39–43.
22 Velazquez I, Alter BP: Androgens and liver tumors: Fanconi's anemia and non-Fanconi's conditions. Am J Hematol 2004;77:257–267.
23 Pagano G, Youssoufian H: Fanconi anaemia proteins: major roles in cell protection against oxidative damage. Bioessays 2003;25:589–595.
24 Pagano G, Degan P, d'Ischia M, Kelly FJ, Nobili B, et al: Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. Eur J Haematol 2005;75:93–100.
25 Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB: Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature 1981;290:142–143.
26 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
27 Taniguchi T, D'Andrea AD: The molecular pathogenesis of Fanconi anemia: recent progress. Blood 2006;107:4223–4233.
28 Lyakhovich A, Surralles J: Disruption of the Fanconi anemia/BRCA pathway in sporadic cancer. Cancer Lett 2006;232:99–106.
29 Lensch MW, Tischkowitz M, Christianson TA, Reifsteck CA, Speckhart SA, et al: Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia. Blood 2003;102:7–16.

30 Tischkowitz MD, Morgan NV, Grimwade D, Eddy C, Ball S, et al: Deletion and reduced expression of the Fanconi anemia *FANCA* gene in sporadic acute myeloid leukemia. Leukemia 2004;18:420–425.
31 Rischewski JR, Clausen H, Leber V, Niemeyer C, Ritter J, et al: A heterozygous frameshift mutation in the Fanconi anemia C gene in familial T-ALL and secondary malignancy. Klin Padiatr 2000;212:174–176.
32 Barber LM, McGrath HE, Meyer S, Will AM, Birch JM, et al: Constitutional sequence variation in the Fanconi anaemia group C (*FANCC*) gene in childhood acute myeloid leukaemia. Br J Haematol 2003;121:57–62.
33 Barber LM, Barlow RA, Meyer S, White DJ, Will AM, et al: Inherited *FANCD1/BRCA2* exon 7 splice mutations associated with acute myeloid leukaemia in Fanconi anaemia D1 are not found in sporadic childhood leukaemia. Br J Haematol 2005;130:796–797.
34 Meyer S, Barber LM, White DJ, Will AM, Birch JM, et al: Spectrum and significance of variants and mutations in the Fanconi anaemia group G gene in children with sporadic acute myeloid leukaemia. Br J Haematol 2006;133:284–292.
35 van der Heijden MS, Yeo CJ, Hruban RH, Kern SE: Fanconi anemia gene mutations in young-onset pancreatic cancer. Cancer Res 2003;63:2585–2588.
36 Couch FJ, Johnson MR, Rabe K, Boardman L, McWilliams R, et al: Germ line Fanconi anemia complementation group C mutations and pancreatic cancer. Cancer Res 2005;65:383–386.
37 Rogers CD, van der Heijden MS, Brune K, Yeo CJ, Hruban RH, et al: The genetics of *FANCC* and *FANCG* in familial pancreatic cancer. Cancer Biol Ther 2004;3:167–169.
38 Rogers CD, Couch FJ, Brune K, Martin ST, Philips J, et al: Genetics of the *FANCA* gene in familial pancreatic cancer. J Med Genet 2004;41:e126.
39 Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, et al: Localization of a breast cancer susceptibility gene, *BRCA2*, to chromosome 13q12-13. Science 1994;265:2088–2090.
40 Wooster R, Bignell G, Lancaster J, Swift S, Seal S, et al: Identification of the breast cancer susceptibility gene *BRCA2*. Nature 1995;378:789–792.
41 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
42 Offit K, Levran O, Mullaney B, Mah K, Nafa K, et al: Shared genetic susceptibility to breast cancer, brain tumors, and Fanconi anemia. J Natl Cancer Inst 2003;95:1548–1551.
43 Wagner JE, Tolar J, Levran O, Scholl T, Deffenbaugh A, et al: Germline mutations in *BRCA2*: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood 2004;103:3226–3229.
44 Wooster R, Weber BL: Breast and ovarian cancer. N Engl J Med 2003;348:2339–2347.
45 Lancaster JM, Wooster R, Mangion J, Phelan CM, Cochran C, et al: *BRCA2* mutations in primary breast and ovarian cancers. Nat Genet 1996;13:238–240.
46 Miki Y, Katagiri T, Kasumi F, Yoshimoto T, Nakamura Y: Mutation analysis in the *BRCA2* gene in primary breast cancers. Nat Genet 1996;13:245–247.
47 Teng DH, Bogden R, Mitchell J, Baumgard M, Bell R, et al: Low incidence of *BRCA2* mutations in breast carcinoma and other cancers. Nat Genet 1996;13:241–244.
48 Weber BH, Brohm M, Stec I, Backe J, Caffier H: A somatic truncating mutation in *BRCA2* in a sporadic breast tumor. Am J Hum Genet 1996;59:962–964.
49 Kwiatkowska E, Teresiak M, Breborowicz D, Mackiewicz A: Somatic mutations in the *BRCA2* gene and high frequency of allelic loss of *BRCA2* in sporadic male breast cancer. Int J Cancer 2002;98:943–945.
50 Hilton JL, Geisler JP, Rathe JA, Hattermann-Zogg MA, DeYoung B, Buller RE: Inactivation of BRCA1 and BRCA2 in ovarian cancer. J Natl Cancer Inst 2002;94:1396–1406.
51 Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, et al: The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 2005;37:931–933.
52 Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, et al: The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 2005;37:934–935.
53 Cantor SB, Bell DW, Ganesan S, Kass EM, Drapkin R, et al: BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. Cell 2001;105:149–160.

54 Sigurdson AJ, Hauptmann M, Chatterjee N, Alexander BH, Doody MM, et al: Kin-cohort estimates for familial breast cancer risk in relation to variants in DNA base excision repair, BRCA1 interacting and growth factor genes. BMC Cancer 2004;4:9.
55 Luo L, Lei H, Du Q, von Wachenfeldt A, Kockum I, et al: No mutations in the *BACH1* gene in *BRCA1* and *BRCA2* negative breast-cancer families linked to 17q22. Int J Cancer 2002;98:638–639.
56 Lewis AG, Flanagan J, Marsh A, Pupo GM, Mann G, et al: Mutation analysis of *FANCD2, BRIP1/BACH1, LMO4* and *SFN* in familial breast cancer. Breast Cancer Res 2005;7:R1005–R1016.
57 Vahteristo P, Yliannala K, Tamminen A, Eerola H, Blomqvist C, Nevanlinna H: *BACH1* Ser919Pro variant and breast cancer risk. BMC Cancer 2006;6:19.
58 Seal S, Barfoot R, Jayatilake H, Smith P, Renwick A, et al: Evaluation of Fanconi Anemia genes in familial breast cancer predisposition. Cancer Res 2003;63:8596–8599.
59 Thompson E, Dragovic RL, Stephenson SA, Eccles DM, Campbell IG, Dobrovic A: A novel duplication polymorphism in the *FANCA* promoter and its association with breast and ovarian cancer. BMC Cancer 2005;5:43.
60 Barroso E, Milne RL, Fernandez LP, Zamora P, Arias JI, et al: *FANCD2* associated with sporadic breast cancer risk. Carcinogenesis 2006;27:1930–1937.
61 Hu N, Li G, Li WJ, Wang C, Goldstein AM, et al: Infrequent mutation in the *BRCA2* gene in esophageal squamous cell carcinoma. Clin Cancer Res 2002;8:1121–1126.
62 Sparano A, Quesnelle KM, Kumar MS, Wang Y, Sylvester AJ, et al: Genome-wide profiling of oral squamous cell carcinoma by array-based comparative genomic hybridization. Laryngoscope 2006;116:735–741.
63 de Winter JP, Rooimans MA, van Der Weel L, van Berkel CG, Alon N, et al: The Fanconi anaemia gene *FANCF* encodes a novel protein with homology to ROM. Nat Genet 2000;24:15–16.
64 Leveille F, Blom E, Medhurst AL, Bier P, Laghmani el H, et al: The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem 2004;279:39421–39430.
65 Taniguchi T, Tischkowitz M, Ameziane N, Hodgson SV, Mathew CG, et al: Disruption of the Fanconi anemia-BRCA pathway in cisplatin-sensitive ovarian tumors. Nat Med 2003;9: 568–574.
66 Agrelo R, Cheng WH, Setien F, Ropero S, Espada J, et al: Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 2006;103: 8822–8827.
67 de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB: The short arm of chromosome 11 is a 'hot spot' for hypermethylation in human neoplasia. Proc Natl Acad Sci USA 1988;85:5693–5697.
68 Hoffmann MJ, Florl AR, Seifert HH, Schulz WA: Multiple mechanisms downregulate *CDKN1C* in human bladder cancer. Int J Cancer 2005;114:406–413.
69 Tischkowitz M, Dokal I: Fanconi anaemia and leukaemia - clinical and molecular aspects. Br J Haematol 2004;126:176–191.
70 Van der Heijden MS, Brody JR, Elghalbzouri-Maghrani E, Zdzienicka MZ, Kern SE: Does tumorigenesis select for or against mutations of the DNA repair-associated genes *BRCA2* and *MRE11*?: considerations from somatic mutations in microsatellite unstable (MSI) gastrointestinal cancers. BMC Genet 2006;7:3.
71 Tischkowitz M, Ameziane N, Waisfisz Q, De Winter JP, Harris R, et al: Bi-allelic silencing of the Fanconi anaemia gene *FANCF* in acute myeloid leukaemia. Br J Haematol 2003;123:469–471.
72 Van Der Heijden MS, Brody JR, Kern SE: Functional screen of the Fanconi anemia pathway in cancer cells by Fancd2 immunoblot. Cancer Biol Ther 2004;3:534–537.
73 Meyer S, White DJ, Will AM, Eden T, Sim A, et al: No evidence of significant silencing of Fanconi genes *FANCF* and *FANCB* or Nijmegen breakage syndrome gene *NBS1* by DNA hyper-methylation in sporadic childhood leukaemia. Br J Haematol 2006;134:61–63.
74 Marsit CJ, Liu M, Nelson HH, Posner M, Suzuki M, Kelsey KT: Inactivation of the Fanconi anemia/BRCA pathway in lung and oral cancers: implications for treatment and survival. Oncogene 2004;23:1000–1004.
75 Olopade OI, Wei M: *FANCF* methylation contributes to chemoselectivity in ovarian cancer. Cancer Cell 2003;3:417–420.
76 Narayan G, Arias-Pulido H, Nandula SV, Basso K, Sugirtharaj DD, et al: Promoter hypermethylation of *FANCF*: disruption of Fanconi Anemia-BRCA pathway in cervical cancer. Cancer Res 2004;64:2994–2997.

77 Snyder ER, Ricker JL, Chen Z, Waes CV: Variation in cisplatinum sensitivity is not associated with Fanconi Anemia/BRCA pathway inactivation in head and neck squamous cell carcinoma cell lines. Cancer Lett 2006;245:75–80.
78 Dhillon VS, Shahid M, Husain SA: CpG methylation of the *FHIT, FANCF, cyclin-D2, BRCA*2 and *RUNX3* genes in granulosa cell tumors (GCTs) of ovarian origin. Mol Cancer 2004;3:33.
79 Teodoridis JM, Hall J, Marsh S, Kannall HD, Smyth C, et al: CpG island methylation of DNA damage response genes in advanced ovarian cancer. Cancer Res 2005;65:8961–8967.
80 Wang Z, Li M, Lu S, Zhang Y, Wang H: Promoter hypermethylation of *FANCF* plays an important role in the occurrence of ovarian cancer through disrupting Fanconi Anemia-BRCA pathway. Cancer Biol Ther 2006;5:256–260.
81 Koul S, McKiernan JM, Narayan G, Houldsworth J, Bacik J, et al: Role of promoter hypermethylation in cisplatin treatment response of male germ cell tumors. Mol Cancer 2004;3:16.
82 Guardiola P, Socié G, Li X, Ribaus P, Devergie A, et al: Acute graft-versus-host disease in patients with Fanconi anemia or aquired aplastic anemia undergoing bone marrow transplantation from HLA-identical sibling donors: risk factors and influence on outcome. Blood 2004;103:73–77.
83 Seal S, Thompson D, Renwick D, Elliott A, Kelly P, et al: Truncating mutations in the Fanconi anemia J gene *BRIP1* are low-penetrance breast cancer susceptibility alleles. Nat Genet 2006;38: 1239–1241.
84 Rahman N, Seil S, Thompson D, Kelly P, Renwick A, et al: *PALB2*, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 2007;39:165–167.
85 Erkko H, Xia B, Nikkila J, Schleutker J, Syrjakoski K, et al: A recurrent mutation in *PALB2* in Finnish cancer families. Nature 2007;446:316–319.
86 Neveling K, Kalb R, Florl AR, Herterich S, Friedl R, et al: Disruption of the FA/BRCA pathway in bladder cancer. Cytogenet Genome Res 2007, in press.
87 Van der Groep P, Hoelzel M, Buerger H, Joenje H, de Winter JP, et al: Loss of expression of FANCD2 protein in sporadic and hereditary breast cancer. Breast Cancer Res Treat 2007; Epub ahead of print.

Kornelia Neveling
Department of Human Genetics, University of Würzburg, Biocenter
B107, Am Hubland, 97074 Würzburg (Germany)
Tel. +49 931 888 4089, Fax +49 931 888 4069
E-Mail kornelia.neveling@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 79–94

Clonal Chromosomal Aberrations in Bone Marrow Cells of Fanconi Anemia Patients: Results and Implications

H. Neitzel[a], *J.-S. Kühl*[b], *A. Gerlach*[a], *W. Ebell*[b], *H. Tönnies*[a]

[a]Institute of Human Genetics and the [b]Department of General Pediatrics, Bone Marrow Transplant Unit, Charité – Universitätsmedizin Berlin, Berlin, Germany

Abstract

Fanconi anemia patients have a high risk for bone marrow failure, aplastic anemia, myelodysplastic syndrome, and acute myeloid leukemia. Many FA patients acquire chromosomal aberrations in their bone marrow (BM) cells. The significance and predictive value of these somatic aberrations for hematopoietic function and malignant progress are not fully understood. Therefore, we initiated in cooperation with the 'Deutsche Fanconi-Anämie-Hilfe e.V.' a prospective cytogenetic follow-up in BM cells of FA patients utilizing systematically molecular cytogenetic techniques. The most frequent clonal aberrations are gains of material of the long arm of chromosome 3, loss of most of the long arm of chromosome 7 or loss of one entire copy of chromosome 7, and gains of the long arm of chromosome 1. The available data suggest that these clonal chromosome aberrations in bone marrow cells of FA patients represent an important step in the initiation of MDS and AML. Our data of patients with 3q aberrations revealed that gains of 3q are an adverse risk factor. The overall survival in the 3q group was extremely poor compared to FA patients without such aberrations. None of the FA patients with 3q gains survived without undergoing HSCT. Because of the high MDS/AML risk and the significantly higher mortality in the group of FA patients with 3q aberrations, we recommend a systematic evaluation of all FA patients by molecular cytogenetics in order to detect aberrations, including subtle aberrations, as early as possible. Such clonal aberrations are a very strong clinical warning sign. All available evidence suggests that the finding of any chromosomal abnormality in bone marrow cells, especially abnormalities involving chromosomes 3 and 7, warrant very close clinical follow-up, as they may signal the development of MDS or AML.

Fanconi anemia patients have a high risk for bone marrow failure, aplastic anemia (AA), myelodysplastic syndrome (MDS), acute myeloid leukemia

(AML), and, later in life, for epithelial malignancies. The most life-threatening early event in most complementation groups is bone marrow failure (patients with bi-allelic *BRCA2* mutations, who seem to have early onset solid tumors, may be exceptions). The hematologic complications of FA have been extensively reviewed [1]. Patients with FA develop bone marrow (BM) failure, typically during the first decade of life. The actuarial risk of developing BM failure is 90% by 40 years of age. The actuarial risk of developing hematologic neoplasms is 33% by 40 years of age [2]. The median age of patients who develop cancer is 14 years for acute myelogenous leukemia (AML). Many FA patients acquire chromosomal aberrations in their bone marrow cells. The significance and the predictive value of such clonal alterations with respect to hematopoietic function and malignant progress are not fully understood but are important to define. This is the aim of our studies.

Cytogenetic Studies in Bone Marrow Cells of Fanconi Anemia Patients

The first cytogenetic studies of bone marrow cells in FA were published more than 30 years ago, most of them as single case reports (table 1). A review of 1991 [3] summarized these early reports and updated them with data from the International Fanconi Anemia Registry (IFAR). Out of those patients who had a cytogenetic analysis 26 of 32 FA patients with AML had clonal aberrations detectable by conventional cytogenetics and all patients with MDS ($n = 5$) and AA ($n = 13$) presented with aberrations. The most frequent aberrations detected were duplications of 1q, complete monosomy 7, deletions of 7q, and deletions of 5q [3].

In a later study of MDS and AML cases in FA patients, a high incidence of monosomy 7, deletions of 7q, and rearrangements involving 1q24–q34 were reported [4] (table 1). The cytogenetic findings of further 20 patients with Fanconi anemia were added in 1996 [5]. Out of these, seven patients had a monosomy 7 or a deletion in 7q. Alter et al. reported that approximately half of the patients with clonal aberrations had chromosome 7 abnormalities, while a quarter had chromosome 1 abnormalities [6]. Data of 16 FA patients with clonal aberrations and a cytogenetic follow up were presented in 2000 [7]. Out of these, two had a monosomy 7, one a partial monosomy 7q, and four additional material of 1q. All studies performed until 2000 demonstrated that among the chromosomal aberrations observed in bone marrow cells of FA patients the following stand out: monosomy 7, deletions of 7q, gains of 1q, monosomy 5 or deletions of 5q.

Since the first publications of clonal aberrations in BM cells of FA patients there is a dispute about the relevance of clonal aberrations in BM cells of FA

Table 1. Overview of clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients reported in the literature

Hematological status[a]	Clonal aberration	Reference
Non-malignant	46,XX,Dq+	[17]
Non-malignant	47,XX,+21	[12]
AML-M4	45,XY,t(5;18)(q?;q?)	[18]
Preleukemia	46,XX,dup(3)(q12q27)/add(12)(q24)	[12]
AML	46,XX,add(1)(p36)dup(3)(q12q27),add(7)(p22),add(12)(q24)	[12]
AML	der(14)t(1;14)(q12 or q21;q23),4q−,add(6)(q27),7q−,add(13)(q34),20q−	[19]
Acute leukemia	46,XY,der(6)t(1;6)(q32;p25)/46,idem,der(11)t(11;?)(q25;?)	[20]
Acute leukemia	der(6)t(1;6)(q22 or 23;pter)/idem,5q−	[19]
Erythroleukemia	46,XY,dup(1)(p32p34),trp(3)(q12q27),del(7)(q11)	[11]
Preleukemia	46,XX,dup(2)(q24q36)/47,idem,+?4p	[11]
AML	46,XX,−7,−8,+mar1,+mar2/complex −7,+mar/complex mar	[21]
Erythroleukemia	add(1)(p36),t(13;15)	[22]
AML	45,XY,−7	[23]
Preleukemia	46,XX,t(10;?)(q26;?),del(12)(p11),−17,der(17)t(1;17)(q21;p13)	[24]
AML	46,XX,7q−,9q−	[25]
Non-malignant	46,XX,i(7)(q10)	[26]
Preleukemia-AML	46,XY,dup(1)(q24q44),del(6)(p15),del(7)(q11)	[27]
Non-malignant	46,XY,trp(1)(q12q32)	[28]
MDS	46,XY,der(6)t(1;6)(q12;p25)/46,idem,del(5)(q21q23)/46,t(1;2)(q12;q37),del(5)	[29]
AML	47,XY,+20,−22,dup(1)(q22q34),del(22)(q11)	[30]
Preleukemia	der(2)t(2;3)(q35?;p21?),der(6)t(1;6)(q22;p25)	[31]
MDS	47,XY,+8	[32]
AML	46,XX,der(1)t(1;?)(p36;?)	[33]
MDS	46,X,−Y,+der(Y;1)(q12;q21)	[34]
AML-M4	del(7)(p11),6p+,1q+	[3]
AML-M6	18q+,dup(12)(q),5q+,−16	[3]
NS	del(7)(q)	[3]
NS	−7	[3]
NS	2q+,6p+	[3]
MDS	2q+,+mar	[3]
MDS	t(1;5)(p34;q31)	[3]
AA	−13,t(1q;13q),del(7)(q32)	[3]
AA	dup(1)(q24q32),t(17;?)(p12;?)	[3]
AML	45,XY,−7	[35]
AML	47,XY,trp(1)(q32q44),+mar	[35]
AML	46,XY,add(1)(q34),del(7)(q13)	[35]
Non-malignant	46,XX,−5,+8	[35]
Non-malignant	46,XX,+5,−21	[35]
MDS	46,XY/47,XY,+21	[4]
AML	46,XX,−21,t(3;11)(q27;q21)/46,XX,t(3;11;21)(q27;q21;p11)	[4]
MDS	45,XY,−7	[4]

Table 1. (continued)

Hematological status[a]	Clonal aberration	Reference
BMF	46,XY,del(7)(q22q36)	[4]
MDS	46,XY,del(7)(q22q36)/46,XY,idem,del(6)(p23)	[4]
BMF	46,XX,t(1;?)(p36;?)/46,XX	[4]
MDS	46,XX,t(1;?)(p36;?)/46,XX,t(1;6)(q21;p21)	[4]
NS	add(6)(p22),t(1;17)(q21;p11)	[4]
NS	add(1)(p36),t(1;6)(q21;p21)	[4]
NS	del(6)(p21q25),dup(12)(q21q24)	[4]
NS	del(13)(q14q32)	[4]
MDS	46,XX,add(1)(p34),del(5)(q31q35),inv(9)(p11q11)x2,add(16)(q24)	[36]
NS	46,XY,der(2)t(1;2)(q12;q32)	[36]
AML	47,XY,der(6)t(1;6)(q23;p22),+mar	[37]
MDS	der(1)dup(1)(q12 or q21q24)	[6]
AML	der(18)t(1;18)(q12;p11),del(12)(p12.1)	[6]
MDS	del(3)(q22q24)	[6]
MDS	dup(1)(q23q44)	[6]
AML	47,XY,der(6)t(1;6)(q23;p22),+mar	[38]
AML	46,XX,trp(1)(q23q42)/46,XX,idem,+13,−20	[39]
NS	46,XX,del(13)(q12q14)/46,XX	[7]
MDS	46,XX,add(21)(q22)/46,idem,del(13)(q21q22)	[7]
MDS	46,XX,add(1)(p11),add(2)(q33),add(6)(p11)/46,XX	[7]
MDS	46,XY,der(11)t(1;11)(q23;q23)/46,idem,del(6)(p21)/46,XY	[7]
AML	46,XX,t(q?;q?)	[7]
AML	46,XX,add(14)(p11.2)/46,XX	[7]
MDS	46,X,der(X)t(X;3)(p22.2;q13),+3	[7]
MDS	46,XX,del(7)(q31.2)/48,idem,add(1)(p36.1)/46,XX	[7]
MDS	47,XY,+i(1)(q10)/46,XY	[7]
MDS	47,XY,+i(1)(q10)/46,XY	[7]
normal	49,XXX,+8,+21	[7]
AML	45,XY,−7/46,XY	[7]
MDS	46,XX,dup(1)(q21q42)/46,XX,del(7)(q31)/ 46,XX,del(11)(q21q25)/46,XX,der(20)t(1;20)(q10q13.3)	[7]
AML	46,XY,del(1)(q32)	[40]

[a]AML: Acute myeloid leukemia; BMF: bone marrow failure; NS: not specified; MDS: myelodysplastic syndrome.

patients. Auerbach and Allen mentioned that ‘considerable evidence shows that the presence of chromosomally abnormal clones in the BM heightens the risk for development of leukemia’ [3]. This is in agreement with Butturini et al. [8] who demonstrated that the detection of a clonal cytogenetic abnormality correlates with decreased survival of FA patients. In 1993, a provocative question was

formulated [9]: 'Clonal chromosomal abnormalities in Fanconi's anemia: what do they really mean?' Alter et al. [9] presented three of 11 FA patients with clonal abnormalities after adequate cytogenetic analysis. The fact that some of the clonal aberrations displayed transient appearance led those authors to reason that 'changing cytogenetic patterns may not be related to leukemic evolution in patients with a DNA repair defect'. In contrast, in 2000 it was demonstrated that the five-year probability of survival after detection of a clone is 0.40 compared to 0.94 without a clone [7]. Notwithstanding these observations, those authors stated that the morphological changes in the direction of MDS may be more important than clonal aberrations with respect to the prediction of an adverse outcome. Along these lines, Maarek et al. emphasized: 'The significance of chromosomal changes without apparent evolution to overt AML or MDS will only be understood when more FA patients are cytogenetically studied and followed over a sufficient period of time' [5]. However, up to now long term follow-up data are sparse and there is urgent need to collect such data.

All cytogenetic studies in the 70s to 90s have in common that the chromosomal aberrations could not be sufficiently defined by conventional cytogenetics alone in a considerable number of patients. Karyotype descriptions denoted as '+', 'add', or 'mar' are common, indicating that additional material of unknown origin was detected either as translocation to another chromosome (+; add) or as marker chromosomes (+mar) of unidentified origin (table 1).

The results from these early cytogenetic studies in bone marrow cells of FA patients illustrate the problem of all studies which were based solely on conventional cytogenetics. There is a limited number of analyzable BM metaphases, especially in patients with progressive bone marrow failure, and there is a limited resolution of GTG-banded chromosomes of BM metaphases resulting in the inability to identify and assign chromosomal changes correctly, especially when these are subtle and/or mosaic. However, the correct assignment of the karyotypic changes in BM cells is a prerequisite for any predication with respect to hematopoietic function and progression to malignancy.

A Prospective Study of Bone Marrow Cells of FA Patients with Systematic Application of Molecular Cytogenetic Techniques

The ongoing debate about the relevance of clonal aberrations in patients with Fanconi anemia prompted us to initiate a systematic, prospective cytogenetic follow-up in BM cells of FA patients in Germany starting in 1996. The study was supported by the German Patient Organisation 'Deutsche Fanconi-Anämie-Hilfe e.V.'. In our study, we utilized for the first time comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) in

Table 2. Characteristics and outcome of FA patients with or without trisomies or tetrasomies of chromosomal segment 3q26–3q29 as published 2003 by Tonnies et al. [10]. FANCA indicates Fanconi anemia complementation group A; FANCC, Fanconi anemia complementation group C; FANCG, Fanconi anemia complementation group G; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; and HSCT, hematopoietic stem cell transplantation

	Total	With chromosome 3q gain	Without chromosome 3q gain	p[d]
Patients[a,b,c]	53	18	35	
Age				
Median (months)	141	149	125	
Range (months)	34–463	95–463	34–442	
Gender				
Male	28	11	17	
Female	25	7	18	
Complementation group				
FANCA	28	10	18	
FANCC	4	4	0	
FANCG	8	3	5	
unknown	13	1	12	
genotypic reversion[a]	3	1	2	
Outcome				
MDS[b]	9	9	0	<0.001
AML[c]	5	4	1	
MDS+AML	14	13	1	<0.001
Alive	44	11	33	0.005
HSCT	20	12	8	

[a]Spontaneous reversion of the cellular FA phenotype in lymphocytes by back mutation or gene conversion in compound heterozygotes.

[b]MDS was defined according to the FAB classification; 8/9 MDS patients presented 3–26% blasts in the marrow.

[c]AML was defined by more than 30% blasts in the marrow.

[d]Fisher's exact test (2-sided).

a systematic fashion. The investigation of all BM samples by CGH allows the comprehensive analysis of the entire genome in just one experiment. Our approach provides information not only about the chromosomal assignment but also about the size of the chromosomal imbalances. All CGH results were validated by FISH with specific probes hybridized onto BM metaphases. The results of this very first molecular cytogenetic study of clonal aberrations were published in 2003 [10] yielding an unexpected finding: a high prevalence of gains for chromosome 3q (table 2).

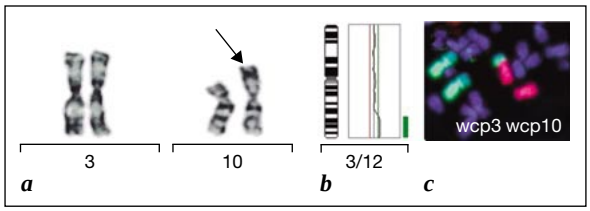


Fig. 1. Conventional and molecular cytogenetic analysis in bone marrow cells of an FA patient with a gain of 3q. (***a***) GTG-banding of chromosomes 3 and 10; the conventional analysis revealed additional material on the short arm of one chromosome 10. (***b***) CGH indicated a gain of material of 3q. (***c***) The result of the CGH was confirmed with whole chromosome paints for chromosome 3 (green) and chromosome 10 (red) indicating that additional material of chromosome 3 was translocated to 10p.

Out of 53 FA patients, 28 had a normal karyotype in BM cells after conventional cytogenetics and CGH, while 25 patients had clonal aberrations. Out of these, 18 (72%) revealed partial trisomies or tetrasomies of the long arm of chromosome 3 indicating an extremely high prevalence of 3q aberrations in our FA cohort. The 3q aberrations of almost all FA patients were mosaics with subtle aberrations due to unbalanced translocations of distal 3q to various other chromosomes. Prior to our study, only very few other FA patients with additional material of chromosome 3q in BM cells have been described in the literature, and all had trisomies or tetrasomies for almost the entire long arm from 3q13 to 3qter permitting the identification by conventional cytogenetics [6, 11–13]. Considering the prevalence of 3q gains in our FA patient cohort compared to the very low occurrence in the published cases, we assumed that other studies might have failed to detect the more subtle partial trisomies and tetrasomies of 3q. An example of such a subtle chromosomal change is shown in figure 1 demonstrating that a translocation of additional material to the short arm of chromosome 10 can be recognized by conventional cytogenetics. However, the identification of the additional material is extremely difficult by conventional cytogenetics alone. Using CGH the additional material could be unambiguously assigned to 3q. Subsequent fluorescence in situ hybridization using whole chromosome paints (wcp) for chromosomes 3 and 10 confirmed this interpretation.

Evidently, the interpretation of chromosomal aberrations in bone marrow cells of FA patients may be misleading when recognition is based solely on conventional cytogenetics. Such a case is illustrated in figure 2. Conventional cytogenetic analysis of an FA patient with progressive bone marrow failure yielded poor banding resolution and only few metaphases were available for analysis which showed a deletion of 6q and additional material at 2q. Thus, based on

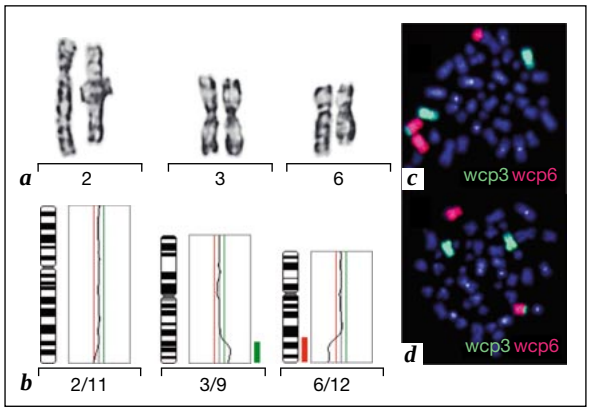


Fig. 2. Conventional and molecular cytogenetic analysis in bone marrow cells of another FA patient with a gain of 3q. (***a***) GTG-banding of chromosomes 2, 3 and 6; from conventional cytogenetics the interpretation of the karyotype is t(2;6)(q37;q23) indicating a balanced translocation of 6q material to 2q. (***b***) CGH showed a more complex situation revealing a deletion of 2q37, additional material of 3q25–qter and a deletion of 6q23–qter. (***c***) FISH for chromosome 3 and 6 displayed that in fact 6q is translocated to 2q but only in some of the cells while others had two normal chromosomes. Additional 3q material is translocated to the long arm of chromosome 6 leading to a partial trisomy of 3q.

conventional cytogenetics, the interpretation of the karyotype would be t(2;6)(q37;q23) indicating a balanced translocation of 6q material to 2q. However, the application of CGH showed a more complex situation revealing a deletion of 2q37, additional material of 3q25–qter, and a deletion of 6q23–qter. Subsequent FISH analysis using whole chromosome paints of chromosomes 3 and 6 revealed that in fact 6q was translocated to 2q, but only in some of the cells while others had two normal chromosomes 2. Furthermore and most importantly, additional 3q material was translocated to the q-arm of chromosome 6 resulting in partial trisomy 3q. The combined data of the CGH and FISH procedures imply that the first step of the aberration was a translocation of 6q to 2q that was accompanied by a deletion of 2qter, and an unbalanced translocation of 3q to 6q with a gain of 3q. As a second event, the translocation chromosome t(2;6) was lost and the normal chromosome 2 was duplicated resulting in a uniparental disomy (UPD) for chromosome 2.

In this context, we should mention that another case of UPD was observed in bone marrow cells of another patient who had an unbalanced translocation der(19)t(3;19)(q23;p13.3) (fig. 3). During subsequent clonal evolution, the derivative of chromosome 19 was duplicated and the normal chromosome 19 was lost. There is at least one further report in the literature describing a duplication of an

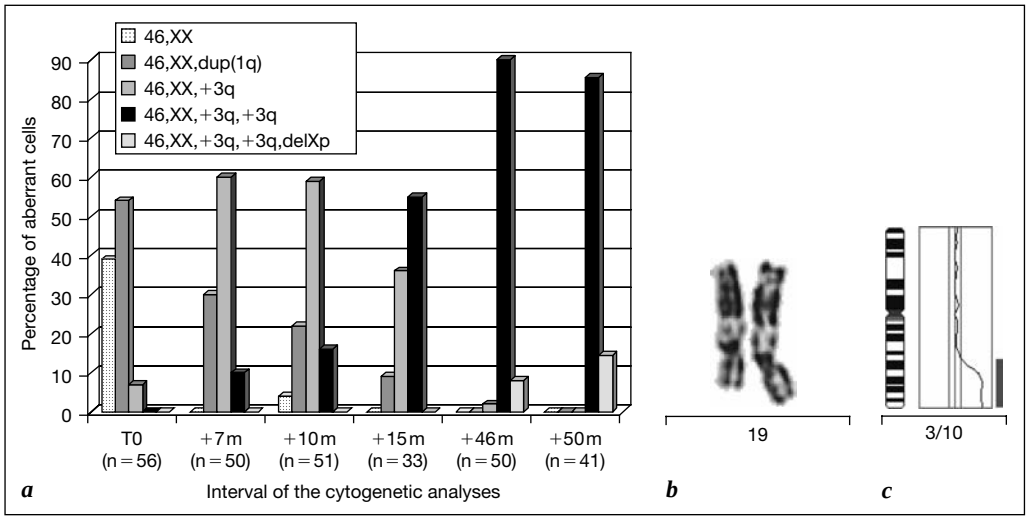


Fig. 3. Increase of a clonal 3q gain over time as determined by conventional cytogenetics. (***a***) The conventional cytogenetic analyses revealed first an outgrowth of a clone with a trisomy of 3q. After 7 months a tetrasomic clone appeared due to a duplication of the aberrant chromosome 19 which carries 3q material on the short arm and the loss of the normal chromosome 19 as depicted in GTG-banding (***b***) of the derivative chromosomes 19. (***c***) CGH indicates a gain of 3q. In this patient, the tetrasomic clone replaced the trisomic clone over time indicating either a higher proliferative advantage or an increased survival of bone marrow cells with the aberration.

aberrant derivative chromosome [47,XX,add(13)(q34), +add(13) (q34)] [5]. This suggests that the formation of UPD might not be such a rare event in BM cells of FA patients.

In 16 out of 18 FA patients with 3q gains, serial bone marrow analyses were performed, amounting to an average of 5.6 analyses per patient. By conventional cytogenetics, we observed in all of these patients a considerable expansion over time of the clone with additional 3q material (figs. 3 and 4). Furthermore, three patients developed a tetrasomic 3q clone, presumably derived from the trisomic clone by a subsequent duplication event or by UPD. In this patient group, the tetrasomic clones gradually replaced the trisomic clones. Thus, our data strongly suggest that gains of 3q confer either a proliferative advantage or an increased survival to bone marrow cells. This notion is supported by the fact that there were no transient 3q gains as described for other clonal aberrations in FA patients.

Out of 18 patients eight had an additional monosomy 7. In two of these patients the clone with the 3q gain and the monosomy 7 was already present at

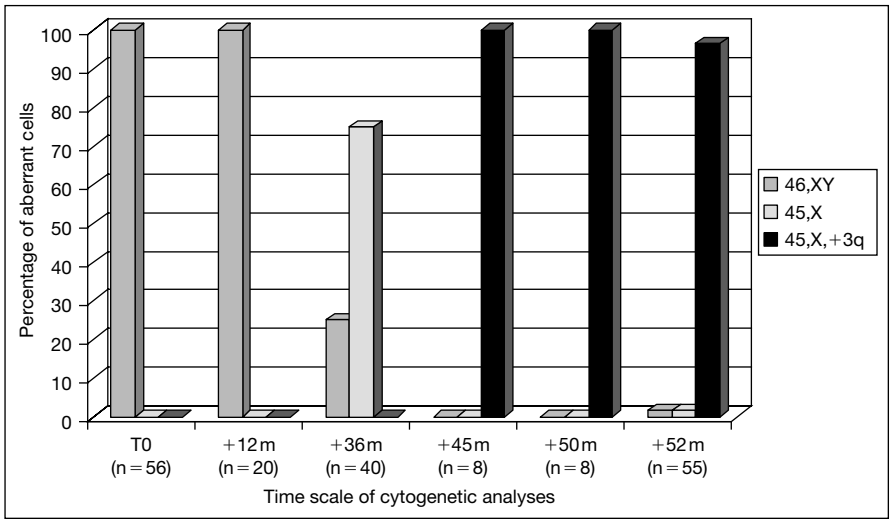


Fig. 4. Another example for the outgrowth of cells with a clonal 3q aberration. The first aberrant clone was detected 3 years after the first cytogenetic analysis of bone marrow cells in this FA patient. At this time, more than 70% of the cells revealed the karyotype 45,X indicating a loss of the Y chromosome. Only 9 months later, all cells carried additional material of 3q. The analyses 5 and 7 months later indicated that the clone persisted.

the time of the first BM analysis while in the other 6 patients the monosomy 7 had developed in the 3q aberrant clone as a secondary event. Even though the number of patients affected with both, a gain of 3q and monosomy 7, is fairly small, our data imply that gains of 3q might increase the risk for the subsequent development of monosomy 7.

In order to determine the percentage of aberrant cells in non-dividing BM and peripheral blood mononuclear cells (PBMC), we performed interphase FISH analyses using a YAC from 3q27–28. Our results demonstrate that FA patients with 3q aberrations in up to 70–80% of their BM and PBMC cells have more than two signals as compared to a maximum of 2–3% in normal control subjects. Since there was a normal karyotype in their T- and B-lymphocytes, our results suggest that almost all circulating granulocytes must carry the 3q aberration in these patients. In three of the FA patients the 3q aberration was detected only by screening PBMC by interphase FISH prior to conventional cytogenetic analysis of BM cells. This proves the high sensitivity and specificity of interphase FISH as a screening tool for aberrant clones in PBMC (for details regarding interphase FISH screening of PBMC see the chapter by Tönnies et al.).

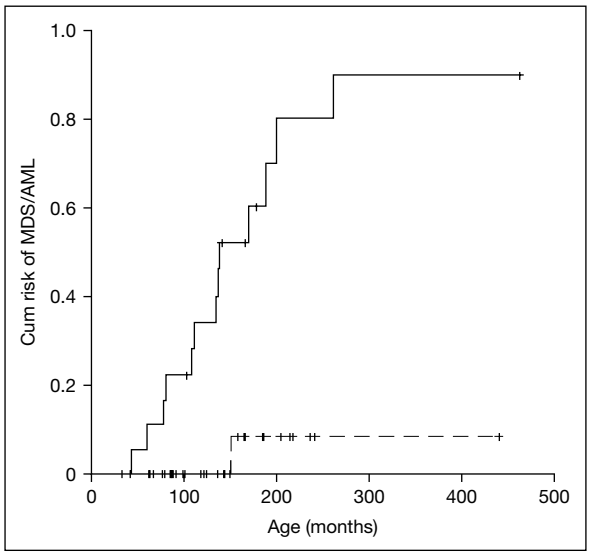


Fig. 5. Risk of developing MDS or AML in Fanconi anemia with (—) or without (----) chromosome 3 aberration (Log Rank: $p < 0.001$) as published 2003 by Tonnies et al. [10]. (—) n = 18, MDS/AML 13, risk 0.90 ± 0.09. (----) n = 35, MDS/AML 1, risk 0.08 ± 0.08.

Clonal Chromosomal Aberrations in Bone Marrow Cells and Clinical Course

In order to determine the clinical relevance of our findings, we compared cytogenetic data, morphologic features of bone marrows, and the clinical course of 18 FA patients carrying chromosome 3 aberrations to 35 FA patients without any evidence of clonal aberrations involving 3q. Both groups did not differ significantly with respect to age, gender, complementation group or genotypic reversion. There was a slight preponderance of males and of individuals belonging to complementation group FA-C in the cohort with 3q aberrations (table 1). Despite the fact that more hematopoietic stem cell transplants (HSCT) had been performed in this cohort there was a significant survival advantage of patients without abnormalities of chromosome 3q. Even more pronounced was the increased risk of patients with gains of 3q material with respect to the development of morphologic MDS and AML (table 2; fig. 5). Thus, our data of 18 patients with 3q aberrations revealed that gains of 3q are strongly associated with a poor prognosis and represent an adverse risk factor in FA. An actual update of our data is presented in table 3. Another five patients have been identified with 3q gains. The overall survival in the 3q group is extremely poor

Table 3. Update of the data of table 2 in 2006

	With chromosome 3q gain	Without chromosome 3q gain
Patients	23	35
Male	16	17
Female	7	18
Overall survival	8/23 (35%)	27/35 (77%)
HSCT	17/23 (74%)	10/35 (29%)
Survival after HSCT	7/17 (41%)	7/10 (70%)
Survival without HSCT	0/5 (0%)	20/25 (80%)

Table 4. Correlation of clonal chromosomal aberrations and hematological status (MDS/AML) as reported by Tonnies et al. [10] and by Hirsch et al. [14]

		Karyotype (%)	Mean age (year)	MDS/AML (%)
Patients (n = 53) [10]	Normal	66	10.4	3
	Aberrant	34	12.4	72
Patients (n = 99) [14]	Normal	56	9.2	6
	Aberrant	44	14.2	66

compared to FA patients without such aberrations. None of the FA patients with 3q gains (n = 5) survived without undergoing HSCT. Furthermore, there is a clear prevalence of males in the cohort carrying 3q aberrations.

Our data of the high prevalence of 3q gains in FA patients were confirmed at the 15th Annual Fanconi Anemia Research Scientific Symposium by the group of Hirsch et al. [14] from the University of Minnesota School of Medicine (table 4). The data of Hirsch et al. [14] were based on the cytogenetic investigation of 99 FA patients. Fifty-six percent of the patients had normal chromosome findings, 44% had one or more clonal abnormalities. The most frequently seen clonal aberrations were gains of material of the long arm of chromosome 3, loss of most of the long arm of chromosome 7 or loss of one entire copy of chromosome 7, and gain of material of the long arm of chromosome 1. Like in our data set there was a significant correlation between the degree of hematopathology and the presence of clonal abnormalities. Only 6% of patients with normal cytogenetics had MDS or AML, while two-thirds of patients with clonal aberrations had apparent MDS or AML.

At present, the data of Hirsch et al. [14] and our investigations are the only studies utilizing molecular cytogenetic techniques for the investigation of altogether more than 150 FA patients. Both groups report a high incidence of 3q gains which clearly stands out as the most frequent clonal aberration. The second frequent clonal aberration in FA is monosomy 7 (partial 7q or complete) followed by 1q gains.

The high frequency of monosomy 7 and the occasional occurrence of monosomy 5 have led to the suggestion that AML in FA is similar to secondary AML which is induced by alkylating agents in non-FA patients [15]. Various alkylating agents are carcinogenic and augment the incidence of cancer in the general population, and the incidence of AML is dramatically increased in cancer patients secondary to chemotherapy with alkylating agents. These patients often develop nonrandom clonal chromosomal changes in the preleukemic phase, especially monosomy 7, deletion of 7q, and monosomy 5. There is little doubt that the presence of such chromosomally abnormal BM clones heightens the risk for the development of leukemia in non-FA patients with secondary AML. It therefore appears unlikely that these clonal aberrations should be predictors for the leukemic process in secondary AML of non-FA patients, but only innocent bystanders in the malignant progress in patients with DNA repair deficiencies. Thus, the statement made by Alter et al. in 1993 that 'changing cytogenetic patterns may not be related to leukemic evolution in patients with a DNA repair defect' may no longer be valid [9].

While monosomy 5 and monosomy 7 are aberrations found in non-FA patients with secondary myelodysplastic syndrome (MDS), gains of 3q have not been described in secondary MDS. In AML of adults, 3q abnormalities are seen in only 2% of patients and they are thought to be extremely rare in primary childhood AML of non-FA patients. Common chromosomal aberrations in childhood AML of non-FA patients include a number of balanced translocations like t(8;21), t(9;11), inv(16), t(11;19). These aberrations have not been described in FA patients, indicating that AML in FA patients may indeed be similar to secondary AML. With regard to the obvious rarity of 3q aberrations in primary childhood AML a recent publication is of special interest. Haltrich et al. [16] investigated 28 childhood AML cases with FISH and found aberrations of chromosome 3 in nine out of the 28 patients. Four patients had a trisomy 3, one patient a tetrasomy of 3q26.3–q28, one patient loss at 3q13–q21, and three patients had structural rearrangements involving the 3q26 and/or 3q21 site. All pediatric AML patients with 3q aberrations had a poor outcome [16].

The data of Haltrich et al. [16] thus suggest that it might turn out that 3q aberrations are risk factors for the development of primary AML in both FA and non-FA patients. It is noteworthy that the 3q chromosomal region is also involved in multiple solid tumors indicating that its tumor promoting potential

may not be cell line or tissue specific but may be generally involved in tumor initiation and/or progression. Four genes known to be involved in MDS and/or AML have been identified in the critical region 3q that are shared by our FA patients: myelodysplasia-myeloid leukemia factor 1 (*MLF1*), myelodysplasia syndrome-associated sequence 1 (*MDS1*), murine myeloid leukemia-associated gene *EVI1*, and Epstein-Barr associated protein *EAP*. Obviously, further data on the role of these candidate genes located at 3q26–q29 are needed to elucidate the pathomechanisms of MDS and AML in FA- and non-FA patients.

Conclusions

Because of a high MDS/AML risk and a significantly higher mortality in the group of FA patients with 3q aberrations, we recommend a systematic evaluation of all FA patients by molecular cytogenetics in order to detect aberrations, including subtle aberrations, as early as possible. The available data suggest that specific clonal chromosome aberrations in bone marrow cells of FA patients represent an important step in the initiation of MDS and AML. Consequently, these aberrations seem to be of high prognostic value and might thus serve as important criteria for the initiation of therapeutic measures such as HSCT. At the moment, they are a very strong clinical warning sign. All available evidence suggests that the finding of any chromosomal abnormality in bone marrow cells, especially abnormalities involving chromosomes 3 and 7, warrant very close clinical follow-up, as they may signal the development of MDS or AML.

Acknowledgement

Supported by grants from the Deutsche Fanconi Anämie Hilfe e.V. and the Charité Research Fund (No.2000-627).

References

1 Alter BP, Greene MH, Velazquez I, Rosenberg PS: Cancer in Fanconi anemia. Blood 2003;101: 2072.
2 Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, et al: A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–1256.
3 Auerbach AD, Allen RG: Leukemia and preleukemia in Fanconi anemia patients. A review of the literature and report of the International Fanconi Anemia Registry. Cancer Genet Cytogenet 1991;51: 1–12.
4 Butturini A, Gale RP: Long-term bone marrow culture in persons with Fanconi anemia and bone marrow failure. Blood 1994;83:336–339.

5 Maarek O, Jonveaux P, Le Coniat M, Derre J, Berger R: Fanconi anemia and bone marrow clonal chromosome abnormalities. Leukemia 1996;10:1700–1704.
6 Alter BP: Fanconi's anemia and malignancies. Am J Hematol 1996;53:99–110.
7 Alter BP, Caruso JP, Drachtman RA, Uchida T, Velagaleti GV, Elghetany MT: Fanconi anemia: myelodysplasia as a predictor of outcome. Cancer Genet Cytogenet 2000;117:125–131.
8 Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD: Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood 1994;84: 1650–1655.
9 Alter BP, Sealise A, McCombs J, Najfeld V: Clonal chromosomal abnormalities in Fanconi's anaemia: what do they really mean? Br J Haematol 1993;85:627–630.
10 Tonnies H, Huber S, Kuhl JS, Gerlach A, Ebell W, Neitzel H: Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood 2003;101:3872–3874.
11 Berger R, Bernheim A, Le Coniat M, Vecchione D, Schaison G: Chromosomal studies of leukemic and preleukemic Fanconi's anemia patients: examples of acquired 'chromosomal amplification'. Hum Genet 1980;56:59–62.
12 Berger R, Bussel A, Schenmetzler C: Somatic segregation and Fanconi anemia. Clin Genet 1977;11: 409–412.
13 Schaison G, Leverger G, Yildiz C, Berger R, Bernheim A, Gluckman E: Fanconi's anemia. Incidence of its development into leukemia. Presse Med 1983;12:1269–1274.
14 Hirsch B, Dolan M, Brandt K, MacMillan ML, Wagner J, et al: Recurring clonal chromosomal abnormalities in Fanconi Anemia: characterization and association with bone marrow pathology. 15th Annual Fanconi Anemia Research Scientific Symposium, 2004.
15 Bagby GC, Alter BP: Fanconi Anemia. Semin Hematol 2006;43:147–156.
16 Haltrich I, Kost-Alimova M, Kovacs G, Klein G, Fekete G, Imreh S: Multipoint interphase FISH analysis of chromosome 3 abnormalities in 28 childhood AML patients. Eur J Haematol 2006;76: 124–133.
17 Lisker R, Cobo de Gutierrez A: Cytogenetic studies in Fanconi's anemia. Description of a case with bone marrow clonal evolution. Clin Genet 1974;5:72–76.
18 Bourgeois CA, Hill FG: Fanconi anemia leading to acute myelomonocytic leukemia: cytogenetic studies. Cancer 1977;39:1163–1167.
19 Tanzer J, Frocrain C, Desmarest MC: Anomalies du chromosome 1 dans 3 cas de leucémie compliquant une aplasie Fanconi (FA). Nouv Rev Fr Hématol 1980;22(suppl):XCIII.
20 Obeid DA, Hill FG, Harnden D, Mann JR, Wood BS: Fanconi anemia. Oxymetholone hepatic tumors, and chromosome aberrations associated with leukemic transition. Cancer 1980;46: 1401–1404.
21 Auerbach AD, Weiner MA, Warburton D, Yeboa K, Lu L, Broxmeyer HE: Acute myeloid leukemia as the first hematologic manifestation of Fanconi anemia. Am J Hematol 1982;12:289–300.
22 Rotzak R, Kaplinsky N, Chaki R, Blei F, Berkowitz M, et al: Giant marker chromosome in Fanconi's anemia transforming into erythroleukemia in an adult. Acta Haematol 1982;67: 214–216.
23 Stivrins TJ, Davis RB, Sanger W, Fritz J, Purtilo DT: Transformation of Fanconi's anemia to acute nonlymphocytic leukemia associated with emergence of monosomy 7. Blood 1984;64: 173–176.
24 Nowell P, Bergman G, Besa E, Wilmoth D, Emanuel B: Progressive preleukemia with a chromosomally abnormal clone in a kindred with the Estren-Dameshek variant of Fanconi's anemia. Blood 1984;64:1135–1138.
25 Woods WG, Nesbit ME, Buckley J, Lampkin BC, McCreadie S, et al: Correlation of chromosome abnormalities with patient characteristics, histologic subtype, and induction success in children with acute nonlymphocytic leukemia. J Clin Oncol 1985;3:3–11.
26 Huret JL, Benz E, Guilhot F, Brizard A, Tanzer J: Fluctuation of a clone 46,XX,i(7q) in bone marrow in a Fanconi anaemia. Hum Genet 1986;74:98–100.
27 Barton JC, Parmley RT, Carroll AJ, Huang ST, Goodnough LT, et al: Preleukemia in Fanconi's anemia: hematopoietic cell multinuclearity, membrane duplication, and dysgranulogenesis. J Submicrosc Cytol 1987;19:355–364.

28 Schindler D, Kubbies M, Hoehn H, Schinzel A, Rabinovitch PS: Confirmation of Fanconi's anemia and detection of a chromosomal aberration (1q12–32 triplication) via BrdU/Hoechst flow cytometry. Am J Pediatr Hematol Oncol 1987;9:172–177.
29 Huret JL, Tanzer J, Guilhot F, Frocrain-Herchkovitch C, Savage JR: Karyotype evolution in the bone marrow of a patient with Fanconi anemia: breakpoints in clonal anomalies of this disease. Cytogenet Cell Genet 1988;48:224–227.
30 Berger R, Bussel A, Schenmetzler C: Cytogenetic studies in four cases of Fanconi's anemia. Nouv Rev Fr Hematol 1975;15:539–550.
31 Baron F, Sybert VP, Andrews RG: Cutaneous and extracutaneous neutrophilic infiltrates (Sweet syndrome) in three patients with Fanconi anemia. J Pediatr 1989;115:726–729.
32 Standen GR, Hughes IA, Geddes AD, Jones BM, Wardrop CA: Myelodysplastic syndrome with trisomy 8 in an adolescent with Fanconi anaemia and selective IgA deficiency. Am J Hematol 1989;31:280–283.
33 Bessho F, Mizutani S, Hayashi Y, Moriwaki K, Yokota S, Inaba T: Chronic myelomonocytic leukemia with chromosomal changes involving 1p36 and hepatocellular carcinoma in a case of Fanconi's anemia. Eur J Haematol 1989;42:492–495.
34 Thompson PW, Standen GR, Geddes AD: Transient t(Y;1)(q12;q21) in a patient with Fanconi anemia and myelodysplastic syndrome. Cancer Genet Cytogenet 1991;52:201–202.
35 Berger R, Le Coniat M, Schaison G: Chromosome abnormalities in bone marrow of Fanconi anemia patients. Cancer Genet Cytogenet 1993;65:47–50.
36 Mozziconacci MJ, Arnoulet C, Stoppa AM, Mattei MG, Simonetti J, et al: Anomalies cytogénétiques clonales dans l'anémie de Fanconi. Nouv Rev Fr Hématol 1995;37:52–53.
37 Ferti A, Panani A, Dervenoulas J, Raptis SA: Cytogenetic findings in a Fanconi anemia patient with AML. Cancer Genet Cytogenet 1996;90:182–183.
38 Auerbach AD, Verlander PC: Disorders of DNA replication and repair. Curr Opin Pediatr 1997;9: 600–616.
39 Verbeek W, Haase D, Schoch C, Hiddemann W, Wormann B: Induction of a hematological and cytogenetic remission in a patient with a myelodysplastic syndrome secondary to Fanconi's anemia employing the S-HAM regimen. Ann Hematol 1997;74:275–277.
40 Oliveira NI, Ribeiro EM, Raimondi SC, Bittencourt MA, Pasquini R, Cavalli IJ: Two different karyotypes with 1q abnormalities in a patient with Fanconi anemia. Leuk Res 2002;26: 1047–1049.

Heidemarie Neitzel
Institute of Human Genetics, Charité, Medical University of Berlin
Augustenburger Platz 1
D–13353 Berlin (Germany)
Tel. +49 30 450566411, Fax +49 30 450566933, E-Mail heidemarie.neitzel@charite.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 95–109

Interphase FISH-Assay for the Detection of MDS- and AML-Associated Chromosomal Imbalances in Native Bone Marrow and Peripheral Blood Cells

H. Tönnies, S. Huber, E. Volarikova, A. Gerlach, H. Neitzel

Institute of Human Genetics, Charité, Universitätsmedizin Berlin, Berlin, Germany

Abstract

Bone marrow (BM) failure in Fanconi anemia (FA) patients followed by myelodysplastic syndrome or acute myeloid leukemia is frequently associated with the occurrence of specific clonal chromosomal imbalances in BM cells. Our previous data of patients with 3q aberrations revealed that gains of 3q are strongly associated with a poor prognosis and represent an adverse risk factor in FA. In routine cytogenetic diagnostics, these clonal aberrations can be detected after BM aspiration, culturing of bone marrow cells, and karyotyping of GTG-banded metaphase chromosomes. However, some unbalanced mosaic aberrations in bone marrow cells are too subtle to be detected by conventional cytogenetics alone. In order to develop a sensitive and fast detection protocol for routine use, we studied FA cells by interphase fluorescence in situ hybridization (I-FISH) using a YAC panel. We established a sensitive interphase FISH assay for the early detection of prognostically poor clonal chromosome aberrations in bone marrow and peripheral blood interphase cells of FA patients as a useful complement to metaphase chromosome analysis of bone marrow cells. The analysis on the single cell level allows the early and sensitive detection and the monitoring of chromosomal imbalances as prerequisite for a timely intervention and treatment of affected patients.

Common hematological findings in Fanconi anemia (FA) patients are thrombocytopenia, aplastic anemia (AA), and myelodysplastic syndrome (MDS), as well as an increased rate of malignancies, including transformation into acute myeloid leukemia (AML) [1, 2]. Furthermore, FA patients have an increased risk for the development of solid tumors [3]. Only relatively few studies and mostly single case reports investigating cytogenetic aberrations in FA bone marrow cells are available to date [4–10]. Many FA patients with MDS

and AML develop clonal chromosome aberrations in their bone marrow cells, and clonal cytogenetic abnormalities are often detected in close temporal proximity with or at the time of diagnosis of MDS or AML [5, 7, 11, 12]. Even though clonal chromosome changes may persist in FA patients for many years without conversion to full-blown MDS or AML, these alterations clearly are of concern since chromosomal imbalances or chromosome rearrangements are frequent manifestations of the conversion of normal to malignant cell growth. More than 30 years ago, Traute Schroeder discovered the spontaneous chromosome instability in FA cells and postulated that the underlying defect in DNA repair might itself initiate and promote, together with extrinsic factors, chromosomal and genetic instability and thus contribute to the emergence of malignancy in FA patients [13, 14].

Recently, we could show that the most frequent chromosome imbalances detected in a group of 53 FA patients with clonal bone marrow aberrations were partial trisomies and tetrasomies for the long arm of chromosome 3 [12]. Our follow-up data of eighteen patients with 3q aberrations, including eight patients with additional monosomy 7, revealed that these aberrations were strongly associated with a high risk for the development of MDS and AML.

This previous study suggests that the emergence of 3q aberrations is an important warning sign that should have immediate consequences in the medical care of FA patients. It is therefore extremely important to identify the relevant aberrations with high diagnostic sensitivity. However, the detection of these chromosome imbalances by classical metaphase cytogenetics alone may be hampered by insufficient numbers of metaphases, and by the typically low quality of morphology and banding resolution of bone marrow chromosome preparations. Therefore, a fast and sensitive interphase fluorescence in situ hybridization (I-FISH) assay operating at the single cell level would be highly desirable. In the present study, we established such an interphase FISH assay for the systematic detection of adverse clonal aberrations in mononuclear cells from uncultured bone marrow and peripheral blood cells.

Materials and Methods

Conventional Cytogenetics

The diagnosis of Fanconi anemia was confirmed in all patients by a standard chromosome breakage test after mitomycin C treatment. Cell culture and high-resolution chromosome preparations from peripheral blood lymphocytes and synchronized bone marrow metaphases for the detection of numerical and structural chromosome aberrations employed standard techniques and GTG-banding [15]. Computer-assisted karyotyping of GTG-banded chromosomes was performed with the Ikaros system (MetaSystems, Altlussheim, Germany).

Direct Cell Preparations

For interphase cell preparation, native bone marrow (BMD: bone marrow direct preparation) and peripheral blood (PBD: peripheral blood direct preparation) specimens (0.5 ml) were incubated without prior culturing in hypotonic potassium chloride solution (0.4%; 10 min at 37°C) and fixed in ice-cold methanol/acetic acid (v/v, 3:1) three times. After dropping on slides, cells were aged overnight at room temperature. For long time storage, slides were kept at –20°C. Peripheral blood and bone marrow smears were prepared by standard protocols.

Molecular Cytogenetics

In addition to conventional cytogenetics where up to 50 bone marrow metaphases were analyzed, we utilized comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) with whole chromosome painting probes for the detection and characterization of chromosomal abnormalities.

Comparative Genomic Hybridization (CGH)

CGH was performed as described previously [16] with slight modifications. In brief, test and control DNAs were differently labeled by nick translation with SpectrumGreen®-dUTP and SpectrumOrange®-dUTP (VYSIS) respectively. 200 ng of labeled test DNA, 200 ng reference DNA, and 12.5 μg Cot-1 DNA were co-precipitated, denatured, and hybridized to denatured metaphase spreads derived from a clinically normal male. After incubation at 37°C for 72 h, standard post-hybridization washes were performed. Metaphase images were evaluated using an epifluorescence microscope (Axioskop, ZEISS, Germany) fitted with a cooled CCD-camera (Hamamatsu) and appropriate single band pass filter sets. Image analysis and karyotyping were performed using the ISIS analysis system (Metasystems, Germany). Positive CGH results were validated using the patients' bone marrow metaphase spreads for metaphase FISH with commercial whole chromosome painting probes (Oncor; VYSIS) according to the manufacturers' instructions.

Interphase FISH with YAC Clones

YAC clones for the critical regions of chromosome 3 and 7 (3q: 909d10; 7q: 942g09) and a control region on the short arm of chromosome 3 and 7 (3p: 808b10; 7p: 956e01) were selected from the MCN Reference Center at the Max-Planck Institute for Molecular Genetics, Berlin, Germany (for detailed YAC-data see http://www.mpimg-berlin-dahlem.mpg.de/~cytogen/probedat.htm). YAC-DNA was amplified (1st PCR) and labeled (2nd PCR) by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) as described by Telenius et al. [17] with minor modifications.

Interphase FISH with the labeled probe (e.g. chromosome 3: 808b10, red; 909d10, green) was performed according to standard protocols. In brief, after treatment with RNase solution (100 μg/ml) at 37°C for 1 h and denaturation for 5 min at 73°C in 70% formamide/2× SSC, slides were dehydrated in cold ethanol series. YAC-probes were denatured at 73°C for 5 min and placed on denatured cells. After overnight hybridization at 37°C, slides were washed in 0.4× SSC/0.3% Igepal at 73°C for 2 min and rinsed in 2× SSC/0.1% Igepal. Slides were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and mounted with antifading solution (Vectashield, Vector Laboratories, Inc. Burlingame, CA). Image capturing and analysis were performed as described for CGH. At least 500 nuclei were analyzed by manual inspection of each hybridized aliquot.

Determination of Positive Cut-off Values

The cut-off values for positive I-FISH results (e.g. three FISH signals for 3q) were determined by statistical evaluation of I-FISH results of mononuclear cells (BMD and PBD) from normal control subjects: the mean ±3 SD of false-positive nuclei was taken as the cut-off level for diagnosing a duplication or deletion of chromosomal material.

Results

Sensitivity of I-FISH

To circumvent selective outgrowth of clonal aberrations in vitro and to gain insights into the in vivo situation of the patient, we decided to analyze uncultured interphase cells. As a first technical result, comparative analyses of bone marrow or peripheral blood smears versus directly prepared cells showed that hybridization quality and success rate were superior when cell suspension slides (BMD and PBD) rather than smears were used (data not shown). Therefore, we determined the positive cut-off levels of our YAC-clone probe sets on bone marrow and peripheral blood direct preparations by hybridizing 70 control samples (56 PBD; 14 BMD). An average of 500 nuclei for each hybridization were scored, which means that positive cut-off values were determined based on the evaluation of 70,000 interphase nuclei. The mean percentage of 3q positive cells (presence of three green signals; GGG) in BMD preparations of control subjects was 1% (mean +3 SD; SD = 0.25), whereas using PBD the cut-off level was 2.9% (SD = 0.69). The cut-off values for partial tetrasomy of chromosome 3q, which in FA specimens often results from clonal progression of a partial trisomy 3q clone, were 0.98 (SD = 0.27) for BMD and 0.26 (SD = 0.08) for PBD. The monosomy 7 cut-off values were 3.59 (SD = 0.99) in BMD and 0.46 (SD = 0.14) in PBD using the chromosome 7 probe set.

Comparison between Conventional Cytogenetic Data and I-FISH

The detection of chromosomal aberrations by classical cytogenetics after GTG-banding of bone marrow metaphase chromosomes was performed by routinely analyzing up to 50 metaphase spreads. In order to determine the sensitivity of the FISH assay, we performed I-FISH experiments with the probe sets on BMD and PBD of 3q-positive patients sampled at the same time as preparations for conventional cytogenetics (see fig. 1).

In most analyses of samples taken at comparable time points, the number of aberrant cells (partial trisomy 3q) was highest in metaphase preparations of cultured bone marrow cells (BM-CC). In contrast, the number of aberrations in non-cycling interphase cells, describing the in vivo situation, were lower in BMD, and lowest in PBD. However, in all cases the number of aberrant cells in

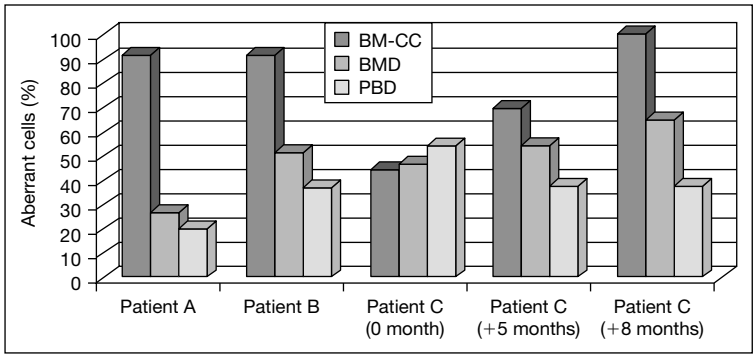


Fig. 1. Percentage of affected cells with partial trisomy 3q obtained by I-FISH with the chromosome 3 YAC probe on cultivated bone marrow cells (BM-CC), bone marrow direct preparations (BMD) and peripheral blood direct preparations (PBD) of three patients (A–C). For patient C, the follow-up period extended over eight months.

direct preparations was higher than the positive cut-off values for the chromosome 3 probe set on BMD (1%) and PBD (2.9%).

Comparison between Concomitant BMD and PBD I-FISH

To further evaluate the feasibility of analyzing easily accessible peripheral blood specimens (PBD) instead of BMD samples during the follow-up of FA patients, we compared I-FISH data from analyses performed on ten paired sets of BMD and PBD that had been collected from FA patients positive for partial trisomy of the chromosome 3q critical region (fig. 2). In the BMD preparations, percentages of partially trisomic cells ranged from 31.5 to 86.1%, whereas in PBD preparations 22.4 to 78.2% of the cells were found to be trisomic. In eight of these ten cases, the numbers of affected cells were lower in PBD cells compared to BMD preparations. Interestingly, in two cases (cases 3 and 10) the number of partially trisomic cells was higher in PBD compared to parallel BMD preparations. The mean difference between both types of specimens was 11.5%; we therefore conclude that the detection sensitivity is somewhat lower in PBD compared to BMD cells. However, an important point is that all positive cases were successfully detected using PBD preparations.

Monitoring Clonal Evolution by Chromosome 3 I-FISH

In the beginning of our I-FISH studies, the significance of partial tri- or tetrasomies of chromosome 3 was unknown. Therefore, we monitored patients frequently in PBD in between their yearly bone marrow aspirations in order to

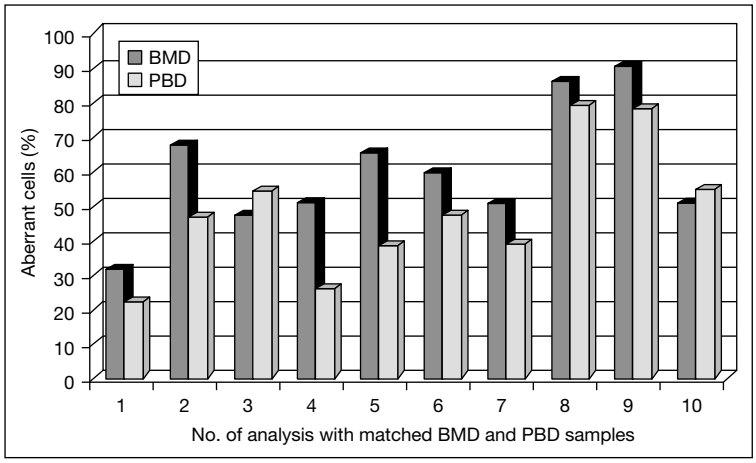


Fig. 2. Percentage of cells affected by chromosome 3q imbalance in native bone marrow (BMD) and peripheral blood nuclei (PBD) screened by I-FISH. Bone marrow and blood of the ten paired samples were taken at the same time. I-FISH was performed and 500 nuclei per hybridization were analyzed.

ascertain whether aberrations of 3q show transient fluctuations. Figure 3 illustrates the results of such long term monitoring of a single FA patient. Via repeated I-FISH examinations, information is gathered about quantitative (outgrowth of the clone) and qualitative (clonal evolution; evolution of a clone from partial trisomy to partial tetrasomy) changes over the time (fig. 3). Starting with approximately 7% tri- and tetrasomic cells in PBD at the time of the first investigation (month zero), the number of normal disomic cells decreased dramatically to less than 20% after 10 months of follow-up, whereas the tetrasomic clone gradually replaced the trisomic clone. BMD analyses seven and ten months after the first investigation also showed the clonal progression of the tetrasomic clone.

There is a good correlation between the I-FISH data and CGH analyses performed during the monitoring period. The imbalance of chromosome 3q was clearly detectable by CGH using PBD-DNA, starting at month 4 of the observation period; however at month zero the number of affected cells (around 14%) was too low to be reliably detected by CGH alone.

Early Detection of Secondary Monosomy 7 in Peripheral Blood Cells

One of the goals of the I-FISH assay is the early and sensitive detection of adverse chromosomal imbalances in FA cells by prospective monitoring of FA patients. In a second example of long-term follow-up of an FA patient

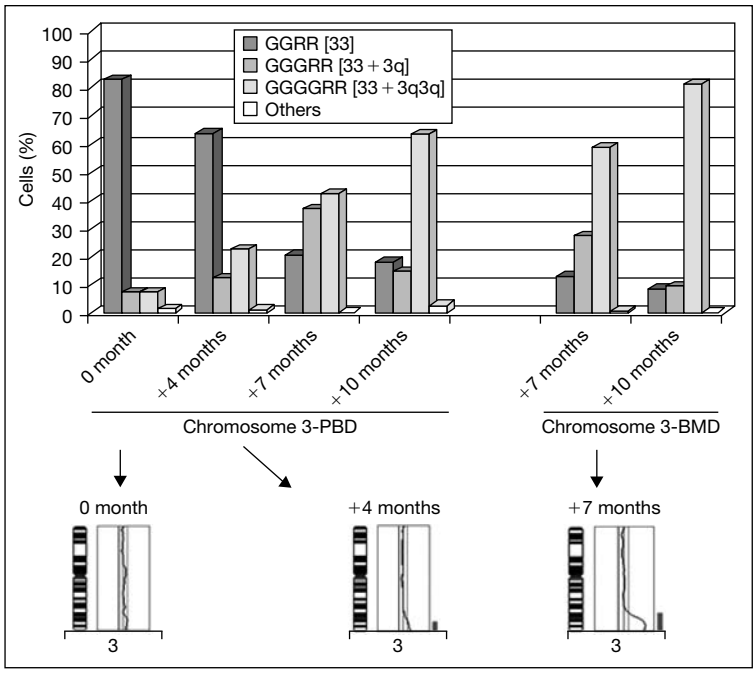


Fig. 3. Long term monitoring of the adverse imbalance of chromosome 3q in PBD (left) and BMD (right) cells together with chromosome 3 ratio profiles of CGH analyses (bottom) illustrating the outgrowth of two different chromosome 3q clones. GGRR[33], two green (G) I-FISH signals for the critical region and two red (R) I-FISH signals for the control region of chromosome 3; GGGRR[33+3q], three green I-FISH signals for the critical region (aberrant) and two I-FISH signals for the control region of chromosome 3; GGGGRR [33+3q3q], four green I-FISH signals for the critical region (aberrant) and two I-FISH signals for the control region of chromosome 3. Bottom: CGH ratio profile of chromosome 3 after analysis of 10–12 single chromosomes at different time points.

with chromosome 3 aberration, we observed remarkable fluctuations of the percentages of aberrant cells (partial trisomy 3q) over an 18-months interval in PBD cells (fig. 4), even though the aberrant clone did at no time disappear. At month 16 of monitoring, neither conventional cytogenetic analysis nor I-FISH of bone marrow yielded any evidence for monosomy 7 (data not shown). However, only two months later a complete monosomy 7 had emerged in the cell line marked by the 3q aberrations. This clone had been detected in PBD cells only two months after a routine BM standard cytogenetic analysis and BMD-I-FISH. The conventional cytogenetic analysis of bone marrow cells at month 18, immediately after its detection in PBD, confirmed

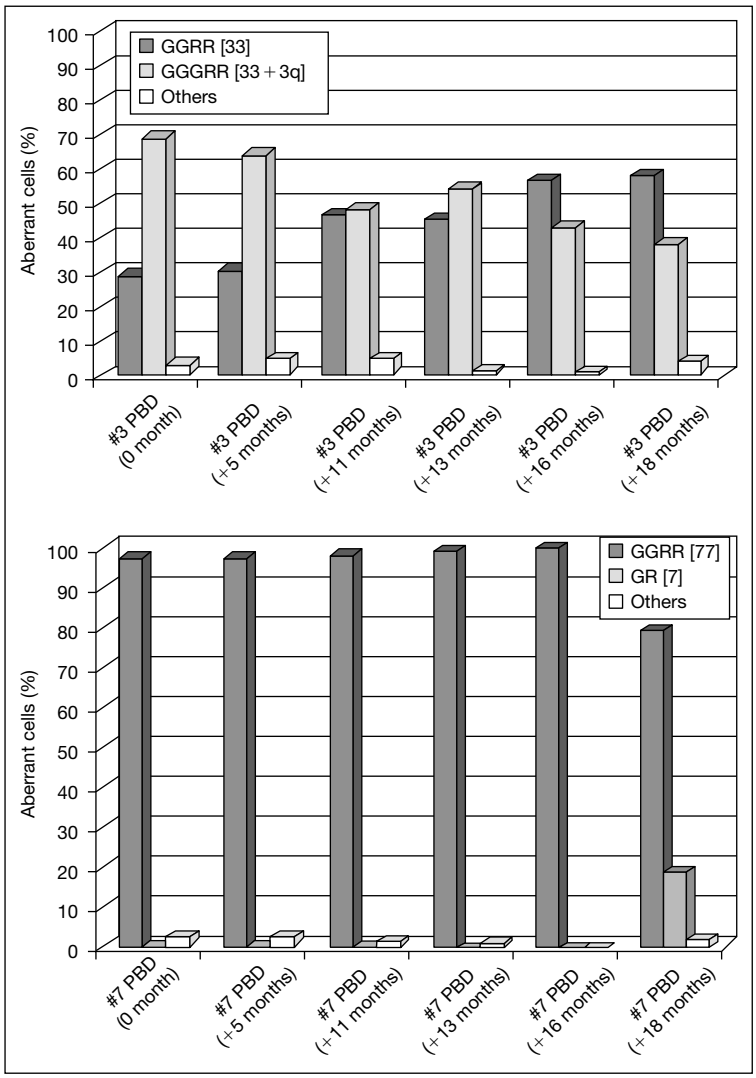


Fig. 4. Early detection of a secondary monosomy 7 in peripheral blood cells of a patient with clonal chromosome 3q imbalance during long term follow-up (18 months). GGRR[33], two green I-FISH signals for the critical region (G) and two red I-FISH signals for the control region (R) of chromosome 3; GGGRR[33+3q], three green I-FISH signals for the critical region (aberrant) and two red I-FISH signals for the control region of chromosome 3. GGRR[77], two green I-FISH signals for the critical region of 7q and two red I-FISH signals for the control region of chromosome 7; GR[7], one signal each for chromosome 7, indicating monosomy 7.

the monosomy 7 in cultured metaphase spreads (45% affected cells) and BMD interphase cells (68% affected cells). This long term follow-up clearly illustrates that I-FISH on PBD cells is a powerful tool for the early and sensitive detection of adverse clonal evolution in uncultured peripheral blood cells of FA patients.

Discussion

Common hematological findings in FA patients are thrombocytopenia, aplastic anemia, and MDS, as well as an increased rate of malignancies, most prominently AML [2] and squamous cell carcinomas [1]. Many FA patients with MDS and AML develop clonal chromosome aberrations in their bone marrow cells [4–10, 12]. Most clonal aberrations are unbalanced while balanced chromosome translocations such as t(8;21) and t(15;17) that are commonly associated with M2, M3, and M5 AML of childhood leukemia of non-FA patients have not been described in FA patients [5, 10]. The significance and the predictive value of most clonal changes found in FA bone marrow cells are far from being understood. However, in the last years, evidence has accumulated that there is a significant correlation between the presence of specific clonal abnormalities, the disease status of the patients (e.g. MDS), and an adverse outcome [11, 12].

In our cohort of 53 FA patients published in 2003 [12], more than 50% of patients had acquired clonal chromosome aberrations. The most frequent aberrations were partial trisomies and tetrasomies for the long arm of chromosome 3, duplications involving 1q, and monosomy 7. Moreover, our data of 18 patients with 3q aberrations and eight patients with monosomy 7 revealed that these specific aberrations are strongly associated with poor prognosis and thus represent adverse risk factors for FA patients.

Based on these observations, we decided to establish and test a fast and sensitive I-FISH assay. Such an assay could play an important role in the timely detection of suspicious clones in FA patients. Moreover, follow-up and prognosis might benefit from such a test. To establish the I-FISH assay, we performed the following analyses: (1) determination of sensitivity by testing the positive cut-off values for the different YAC clones on BMD and PBD, (2) comparison of conventional cytogenetic data of clonal aberrations with the BMD and PBD results at the same time points, (3) direct comparison of I-FISH results obtained with bone marrow versus peripheral blood specimens, (4) collection of quantitative data on clonal outgrowth and evolution, and (5) early sensitive detection of a secondary adverse chromosomal imbalance.

Determination of Positive Cut-off Values

For the definition of positive cut-off values of the YAC probes, we analyzed 500 hybridized nuclei each of 14 bone marrow and 56 peripheral blood direct preparations of control subjects. Cut-off levels (mean +3 SD) for the chromosome 3 YAC probe for the detection of a partial trisomy 3q were 1% for BMD and 2.9% for PBD, and 0.98% (BMD) and 0.26% (PBD) for a partial tetrasomy. The monosomy 7 positive cut-off values were 3.5% for BMD and 0.46% for PBD. These values are comparable to cut-off values reported by other groups using interphase FISH for the determination of chromosomal imbalances [18–20].

Comparison between Conventional Cytogenetic and I-FISH Data in 3q-Positive Patients

Traditionally, conventional cytogenetic analysis of bone marrow metaphase spreads is used for the detection of chromosome aberrations in routine diagnostics. However, karyotype analysis reflects genetic alterations only in the dividing cell population (metaphase spreads). Thus, in previous FA BM studies, many aberrations probably remained undetected by conventional cytogenetics due to the low average resolution of the bone marrow chromosome preparations [12]. To the best of our knowledge, there are only anecdotal reports of FA patients with MDS or AML showing additional material of chromosome 3q in BM cells [11, 21]. Considering the significant overrepresentation of 3q trisomies and tetrasomies in our series of FA patients, we assume that other studies might have failed to detect the more subtle aberrations of 3q because only conventional cytogenetics rather than CGH or I-FISH were applied.

The precise characterization of chromosome aberrations is important for the distinction between primary, pathogenetically relevant changes and secondary aberrations reflecting clonal evolution [22]. In addition to CGH, we routinely examined up to 50 metaphase spreads for the detection of chromosome aberrations in bone marrow specimens (data to be published elsewhere). To test the sensitivity of the I-FISH assay for the detection of unbalanced clones in uncultured cells we performed comparative FISH experiments with probe sets applied in parallel to bone marrow and peripheral blood cells (BMD, PBD), and concomitant with conventional cytogenetics. In these experiments, the highest numbers of aberrant cells with partial trisomy 3q were found in cultured bone marrow metaphase preparations (BM-CC), whereas the figures for aberrant interphase cells were lower in BMD preparations and still lower in PBD specimens. Similar differences have also been described by other authors [23–25]. It is generally assumed that cells with chromosome abnormalities in disorders like MDS and AML often acquire a higher capacity for division in culture than normal cells. Therefore karyotype analysis does not provide an accurate

estimate of the proportion of genetically altered cells in native bone marrow preparations (in vivo situation) [25].

The number of affected cells in our test samples was clearly higher than the positive cut-off values for the chromosome 3 probe set for BMD (1%) and PBD (2.9%). We therefore conclude that in the case of FA patients who show aneuploidy by conventional cytogenetics of BM cells, the application of I-FISH on uncultured BMD and PBD cells leads to the accurate confirmation of the chromosomal alterations.

Comparison between BMD and PBD I-FISH in 3q-Positive Patients

We compared I-FISH data from ten additional paired sets of BMD and PBD collected from FA patients with a partial trisomy for the critical region of chromosome 3q26q29. The percentages of aberrant cells varied among FA patients, but in a given case there was good agreement between the number of positive aberrant BMD and PBD cells. In the BMD cells, the percentages of abnormal cells ranged from 31 to 86%, whereas in PBD preparations 22 to 78% affected cells were detected. As expected, in most cases the number of affected cells was lower in PBD compared to BMD preparations. The mean difference between both analyses was 11%; indicating that the detection rate is slightly lower when PBD cells are analyzed. Nevertheless, all positive cases were detected successfully using PBD preparations. Significantly, using selective enrichment of granulocytes from peripheral blood and subsequent I-FISH, we could demonstrate that the chromosome aberrations are exclusively confined to the granulocytes which represent approximately 80% of the peripheral mononuclear cells (data not shown) while they were not detected in either B or T lymphocytes. This may explain the smaller number of affected cells in PBD in comparison to BMD.

Due to the small amount of blood needed and the fast results after I-FISH, this technique is ideally suited for screening during the intervening time between two bone marrow aspirations. As we have demonstrated, I-FISH is a reliable and rapid method for the detection and monitoring of premalignant clones in easily accessible cells of FA patients. Thus, a close follow-up is easily achieved, especially if blood counts are fluctuating which could be a sign for the appearance of cells with clonal aberrations. Despite these attractive attributes of the I-FISH method, it can only complement but not replace regular bone marrow based cytogenetic analysis which obviously has the highest sensitivity for the detection of aberrant clones.

Follow-up of Clonal Succession and Clonal Evolution by I-FISH

In the beginning of our I-FISH studies, the relevance of the partial tri- or tetrasomy of chromosome 3q was not known. We therefore followed all patients

closely and examined their peripheral blood cells between the yearly bone marrow aspirations. With the help of I-FISH, quantitative (e.g. outgrowth of the clone) and qualitative (e.g. clonal evolution from partial trisomy to partial tetrasomy) information can be gathered. The example presented in this report (fig. 3) demonstrates that even small numbers of aberrant cells with initially only 7% tri- and tetrasomic cells can be detected by I-FISH. The dramatic increase of the tetrasomic clone could be followed over time. Parallel analyses of BMD and PBD cells during this follow-up (at months 7 and 10) clearly showed that the number of cells with partial tetrasomy 3q is always higher in BM than in PB. None of our FA patients with 3q aberrations experienced the disappearance of the aberrant clone over time. The behavior of cell clones carrying 3q aberrations obviously follows a different pattern than is seen with other types of clonal changes in FA patients [10, 21, 26, 27].

Early Detection of a Secondary Adverse Cell Clone in PBD Cells

During the follow-up of a second FA patient with chromosome 3 aberrations, we observed substantial fluctuations of the proportion of aberrant cells in PBD over a period of 19 months. However, the aberrant clone did not disappear over the entire observation period. After 18 months, a second adverse aberration was detected by I-FISH in PBD cells: the cells with the 3q aberration acquired in addition a monosomy 7. Cells with both aberrations must have expanded rapidly, since a cytogenetic analysis and I-FISH of bone marrow two months prior to the positive PBD findings did not reveal any such cells (data not shown). Conventional cytogenetic analysis at month 18 on BM metaphase spreads confirmed the monosomic clone in 45% of all metaphase spreads. I-FISH was positive in 68% of BMD interphase cells.

Monosomy 7 has previously been reported in FA patients and is associated with poor prognosis [8, 12, 19, 28, 29]. Interestingly, Thurston et al. [19] described a patient, in whom they found two out of 30 bone marrow metaphase spreads with monosomy 7. Parallel I-FISH with an alphoid centromeric probe for chromosome 7 indicated that 40% of cells had a monosomy 7. Rescoring of two earlier samples by I-FISH demonstrated that the first sample, taken 19 months before, had 4% nuclei with monosomy 7 and the second sample, taken 12 months before, contained 21.5% of cells with a monosomy 7, whereas the cytogenetic analyses at this date showed no monosomy 7. These results indicate a slow evolution towards monosomy 7 in the patient's bone marrow, whereas in our patient, monosomy 7 could not be detected in bone marrow cells by I-FISH. These data demonstrate that the dynamics of clonal outgrowth can vary considerably among FA patients.

Thurston and coworkers concluded that the presence of a monosomy 7 clone in bone marrow aspirates can remain undetected by standard cytogenetic

analysis. Kearns et al. [20] examined bone marrow cells from 96 unselected patients with bone marrow failure syndromes to assess the frequency of undetected aneuploidy for chromosomes 7 and 8 by FISH. They identified 27 patients with cytogenetically undetected monosomy 7 or trisomy 8. According to these authors, undetected aneuploidy exists in bone marrow cells of a significant percentage of patients with bone marrow failure syndromes. As we have demonstrated in this report, the problem can be overcome by continued monitoring of FA patients using I-FISH on PBD and, if available BMD cells. This approach will assure the early detection of adverse clones with monosomy 7 or gain of 3q allowing earlier intervention, such as bone marrow transplantation, to prevent the onset of leukemia in these patients. We therefore have adopted I-FISH analysis with specific YAC clones for all FA patients as a standard practice. Using this strategy, 3q aberrations were detected by screening PBD cells in three other FA patients (data not shown). This observation emphasizes the high sensitivity and specificity of I-FISH as a screening assay for the detection of aberrant clones in native peripheral blood mononuclear cells.

Concluding Remarks

We have shown that continued monitoring of FA patients by I-FISH on both BMD and PBD detects abnormal clones with monosomy 7 or additional chromosome 3q material quickly and with high sensitivity. Whereas conventional cytogenetics of bone marrow metaphases permits the evaluation of the entire karyotype, only targeted aberrations are investigated by the I-FISH assay. Nevertheless, systematic screening for the most adverse clonal aberrations in Fanconi anemia patients can be recommended, and I-FISH has been shown to be very useful for this purpose. In addition to monitoring by I-FISH we recommend the application, in regular intervals, of conventional cytogenetics and CGH in bone marrow cells of FA patients, since only conventional cytogenetics is able to detect clonal aberrations affecting chromosomes other than 3q or 7.

Acknowledgements

We are indebted to all Fanconi anemia patients, their parents, and their clinicians who supported this study. YAC clones were provided by the Max-Planck Institute of Molecular Genetics, Berlin, Germany. Supported by grants from the Charité Universitätsmedizin Berlin and the Deutsche Fanconi-Anämie-Hilfe e.V. (www.fanconi.de).

References

1 Rosenberg PS, Greene MH, Alter BP: Cancer incidence in persons with Fanconi anemia. Blood 2003;101:822–826.
2 Tischkowitz M, Dokal I: Fanconi anaemia and leukaemia – clinical and molecular aspects. Br J Haematol 2004;126:176–191.
3 Alter BP, Greene MH, Velazquez I, Rosenberg PS: Cancer in Fanconi anemia. Blood 2003; 101:2072.
4 Berger R, Bernheim A, Le Coniat M, Vecchione D, Schaison G: Chromosomal studies of leukemic and preleukemic Fanconi's anemia patients: examples of acquired 'chromosomal amplification'. Hum Genet 1980;56:59–62.
5 Auerbach AD, Allen RG: Leukemia and preleukemia in Fanconi anemia patients: a review of the literature and report of the International Fanconi Anemia Registry. Cancer Genet Cytogenet 1991;51:1–12.
6 Berger R, Le Coniat M, Schaison G: Chromosome abnormalities in bone marrow of Fanconi anemia patients. Cancer Genet Cytogenet 1993;65:47–50.
7 Butturini A, Gale RP, Verlander PC, Adler-Brecher B, Gillio AP, Auerbach AD: Hematologic abnormalities in Fanconi anemia: an International Fanconi Anemia Registry study. Blood 1994;84:1650–1655.
8 Berger R, Jonveaux P: Clonal chromosome abnormalities in Fanconi anemia. Hematol Cell Ther 1996;38:291–296.
9 Alter BP: Fanconi's anemia and malignancies. Am J Hematol 1996;53:99–110.
10 Maarek O, Jonveaux P, Le Coniat M, Derre J, Berger R: Fanconi anemia and bone marrow clonal chromosome abnormalities. Leukemia 1996;10:1700–1704.
11 Alter BP, Caruso JP, Drachtman RA, Uchida T, Velagaleti GV, Elghetany MT: Fanconi anemia: myelodysplasia as a predictor of outcome. Cancer Genet Cytogenet 2000;117:125–131.
12 Tonnies H, Huber S, Kühl JS, Gerlach A, Ebell W, Neitzel H: Clonal chromosomal aberrations in bone marrow cells of Fanconi anemia patients: gains of the chromosomal segment 3q26q29 as an adverse risk factor. Blood 2003;101:3872–3874.
13 Schroeder TM, Kurth R: Spontaneous chromosomal breakage and high incidence of leukemia in inherited disease. Blood 1971;37:96–112.
14 Schroeder TM: Relationship between chromosomal instability and leukemia. Hematol Bluttransfus 1974;14:94–96.
15 Barch MJ, Knutsen T, Spurbeck JL: The AGT Cytogenetics Laboratory Manual. Philadelphia, Lippincott-Raven, 1997.
16 Tonnies H, Stumm M, Wegner RD, Chudoba I, Kalscheuer V, Neitzel H: Comparative genomic hybridization based strategy for the analysis of different chromosome imbalances detected in conventional cytogenetic diagnostics. Cytogenet Cell Genet 2001;93:188–194.
17 Telenius H, Carter NP, Nordenskjold M, Ponder BA, Yunnacliffe A: Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 1992;13:718–725.
18 Flactif M, Lai JL, Preudhomme C, Fenaux P: Fluorescence in situ hybridization improves the detection of monosomy 7 in myelodysplastic syndromes. Leukemia 1994;8:1012–1018.
19 Thurston VC, Ceperich TM, Vance GH, Heerema NA: Detection of monosomy 7 in bone marrow by fluorescence in situ hybridization. A study of Fanconi anemia patients and review of the literature. Cancer Genet Cytogenet 1999;109:154–160.
20 Kearns WG, Sutton JF, Maciejewski JP, Young NS, Liu JM: Genomic instability in bone marrow failure syndromes. Am J Hematol 2004;76:220–224.
21 Berger R, Bussel A, Schenmetzler C: Somatic segregation and Fanconi anemia. Clin Genet 1977;11:409–412.
22 Tonnies H: Modern molecular cytogenetic techniques in genetic diagnostics. Trends Mol Med 2002;8:246–250.

23 Wilkens L, Komminoth P, Nasarek A, von Wasielewski R, Werner M: Rapid detection of karyotype changes in interphase bone marrow cells by oligonucleotide primed in situ hybridization (PRINS). J Pathol 1997;181:368–373.
24 Velagaleti GV, Tharapel SA, Tharapel AT: Validation of primed in situ labeling (PRINS) for interphase analysis: comparative studies with conventional fluorescence in situ hybridization and chromosome analyses. Cancer Genet Cytogenet 1999;108:100–106.
25 Yan J, Zhang XX, Fetni R, Drouin R: Trisomy 8 and monosomy 7 detected in bone marrow using primed in situ labeling, fluorescence in situ hybridization, and conventional cytogenetic analyses. A study of 54 cases with hematological disorders. Cancer Genet Cytogenet 2001;125:30–40.
26 Huret JL, Benz E, Guilhot F, Brizard A, Tanzer J: Fluctuation of a clone 46,XX,i(7q) in bone marrow in a Fanconi anaemia. Hum Genet 1986;74:98–100.
27 Ferti A, Panani A, Dervenoulas J, Raptis SA: Cytogenetic findings in a Fanconi anemia patient with AML. Cancer Genet Cytogenet 1996;90:182–183.
28 Auerbach AD, Weiner MA, Warburton D, Yeboa K, Lu L, Broxmeyer HE: Acute myeloid leukemia as the first hematologic manifestation of Fanconi anemia. Am J Hematol 1982;12:289–300.
29 Stivrins TJ, Davis RB, Sanger W, Fritz J, Purtilo DT: Transformation of Fanconi's anemia to acute nonlymphocytic leukemia associated with emergence of monosomy 7. Blood 1984;64:173–176.

Dr. H. Tönnies
Institute of Human Genetics, Charité, Universitätsmedizin Berlin
Augustenburger Platz 1
D–13353 Berlin (Germany)
Tel. +49 30 450566807, Fax +49 30 450566933, E-Mail holger.toennies@charite.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 110–130

Applications of Cell Cycle Testing in Fanconi Anemia

D. Schindler[a], *R. Friedl*[a], *I. Gavvovidis*[a], *R. Kalb*[a], *K. Neveling*[a], *Y. Linka*[b], *H. Hanenberg*[b], *M. Kubbies*[c], *H. Hoehn*[a]

[a]Department of Human Genetics, University of Würzburg, Würzburg, [b]Department of Pediatric Hematology, Oncology and Clinical Immunology, Heinrich Heine University School of Medicine, Düsseldorf, [c]Roche Diagnostics, Department of Cell Analytics, Pharmaceutical Research Oncology, Penzberg, Germany

Abstract

Chromosome breakage analysis following exposure of cultured cells to DNA-crosslinking agents has long been considered the 'gold standard' for the confirmation or exclusion of Fanconi anemia (FA). Cells containing DNA damage are arrested and accumulate, with a 4c DNA content, near the S/G2-phase border of the cell cycle. As manifestation of their impaired DNA damage response, FA cells typically express elevated G2-phase cell fractions which can easily be measured by flow cytometry. In our experience with more than 3,000 such analyses, at most 1 in 10 blood samples submitted for the exclusion or confirmation of FA yields a positive result. Compared to traditional chromosome breakage analysis, cell cycle testing is less demanding and offers the advantage of speed and low cost. We prefer flow cytometric cell cycle testing for the initial screening of patients, in whom unexplained thrombocytopenia, macrocytic aplastic anemia or other clinical findings require the exclusion of FA. In addition to its application in diagnostic screening, we here show that cell cycle analysis has become a valuable tool for the determination of clastogen sensitivity (such as required within the context of complementation studies), for the precise definition of cell cycle checkpoints, and for the quantitative determination of compartment-specific cell cycle delay or cell cycle arrest. Cell cycle analysis not only provides a reliable test system for the initial confirmation or exclusion of FA, but also serves as a highly informative tool for the comprehensive characterization of the FA cellular phenotype.

Chromosome breakage analysis using diepoxybutane (DEB) or mitomycin C (MMC) as clastogenic agents has long been considered the 'gold standard' for the laboratory confirmation of Fanconi anemia (FA) [1, 2]. Other methods

have been regarded as less reliable, and laboratory strategies for the confirmation or exclusion of FA have largely followed longstanding experience with little need for alternatives.

Early studies such as summarized in the 1989 monograph by Schroeder-Kurth and colleagues [3] have established that FA cells are sensitive to a variety of DNA-crosslinking agents. In addition to DEB and MMC, diagnostic protocols have variously employed nitrogen mustard, cisplatinum, psoralen-UVA, and others. As pointed out by Hans Joenje on several occasions, any differences in the chromosome breakage response towards the various DNA-crosslinking agents are likely to reflect differences in drug stability and laboratory procedures rather than intrinsic, biological differences. From a theoretical point of view, there is little reason to assume genuine differences in the mode of action of any of these agents. Use of a certain drug by a given laboratory primarily reflects empirical preferences and practical considerations. For instance, safety concerns for the laboratory personnel would argue against the use of volatile agents. Likewise, the necessity for metabolic activation of a substance may imply uncertainty as to its efficacy, and the use of UV requires special equipment.

The observation by Traute Schroeder and co-workers [4] of increased spontaneous chromosome breakage in patient cells turned the attention of diagnostic efforts to chromosome techniques. With the advent of complementation assays, analysis of cell survival employing dose-response curves has been added as a standard procedure with both diagnostic and research applications [5]. Virtually all of the recent FA gene discovery reports include such cell survival assays. Most recently, FANCD2 immunoblotting has been advocated as a convenient tool for the rapid recognition of FA patients belonging to subtypes upstream of and including FA-D2 [6, 7]. The subject of the present chapter, cell cycle testing using flow cytometry, was introduced into the FA field after chromosome labeling techniques had suggested a cell cycle disturbance in FA [8]. G2 arrest, both spontaneous and enhanced by exposure to DNA-crosslinking agents, was recognized as a hallmark cell cycle lesion of FA cells that could be exploited for diagnostic purposes [9–12]. In 1995, it was shown that chromosome breakage studies and flow cytometric cell cycle analysis yield equivalent results, thus validating the diagnostic use of cell cycle analysis [13]. In recent years, flow cytometric cell cycle analysis has gained additional prominence as a convenient read-out system for complementation studies [14, 15].

Recent insights into the FA/BRCA pathway have provided a new rationale for the use of cell cycle analysis. DNA-interstrand crosslinks (ICLs) represent obstacles to the progression of DNA synthesis at replication forks. Facing an ICL, the DNA replication machinery is stalled. The barrier can be overcome by a variety of mechanisms, including translesion synthesis and homologous recombination, which both involve members of the FA pathway. Since FA cells

are defective in the removal of ICLs, stalled replication forks activate cell cycle checkpoint controls. These signaling cascades ensure that cell cycle progression occurs only after successful bypass or repair of a given lesion. Whereas chromosome breakage studies reflect the unsuccessful or faulty repair of DNA lesions, cell cycle studies indicate whether or not checkpoint controls and DNA repair function normally. Another advantage of cell cycle studies derives from the fact that the number of cells that are investigated exceeds those examined in chromosome studies by orders of magnitude.

MMC-Treated FA Cells Accumulate in G2 Phase in a Dosage-Dependent Manner

The DNA histograms in the left hand panel of figure 1 (shaded grey) represent 48-h cell cycle distributions of FA fibroblasts exposed to increasing concentrations of MMC as indicated on the right. In the range from 10 to 100 nM MMC, G2-phase accumulations increase from 21.9 to 72.8%, reflecting the innate MMC sensitivity of FA cells. Via retroviral complementation, the fibroblast strain shown in figure 1 had been typed as FA-G, since it was complemented by stable transfer of *FANCG* cDNA in a bicistronic construct with *GFP*. Gene transfer was achieved in 61.8–68.4% of cells. The series of cell cycle distributions shown in the right hand panel of figure 1 represent the isogenic, but complemented counterparts of the distributions shown on the left. Non-complemented cells remain GFP-negative (shaded grey) whereas complemented cells are GFP-positive (shaded green). The GFP marker permits the unambiguous discrimination between complemented and non-complemented cells via electronic gating on green-positive and exclusion of green-negative cells. The MMC response of complemented cells (green panels) is far less pronounced than that of FANCG-defective cells (grey panels). In the concentration range between 10 and 100 nM MMC, the respective G2-phase accumulations of complemented cells increase only from 12.0 to 34.4%. Since the cell cycle distributions shown in figure 1 are all derived from one and the same fibroblast culture of a single FA-G patient, the striking differences in the response to MMC between the left and right hand panels solely and uniquely reflect the functional competence of the *FANCG* gene in protecting cells from the adverse effects of MMC.

Figure 2 illustrates dose-response curves of the G2-phase fractions of non-complemented vs. complemented FA-G cells over a broader range of MMC concentrations. In the range of 33–100 nM, differences of the G2-phase fractions between non-complemented and complemented cells from these otherwise isogenic cultures amount to 30% of the total cell count. This interval indicates a diagnostic window allowing for optimal discrimination between

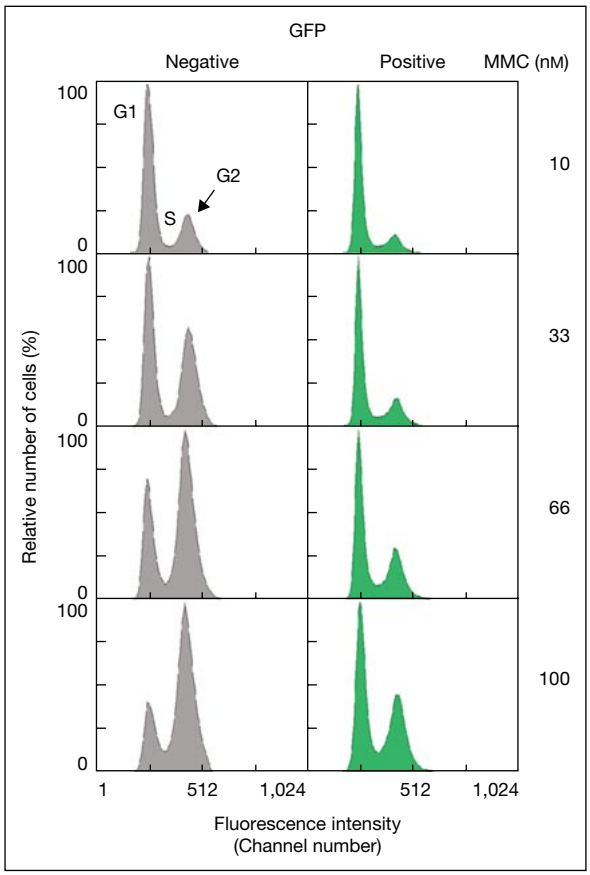


Fig. 1. MMC-induced G2-phase arrest of FA fibroblasts. Subcultures of fibroblasts derived from an FA-G patient were transduced with a retroviral vector containing wt*FANCG* cDNA in a bicistronic construct with *GFP*, and were exposed to different concentrations of MMC for 48 h. Cells without (left) and with (right) effective gene transfer were gated by flow cytometry according to the absence (left) or presence (right) of green fluorescence. At each MMC concentration, Hoechst-33342-stained cells that have retained the FA genotype (left) display much higher G2-phase peaks than cells, whose genotype was corrected by transduction with wt*FANCG* (right).

MMC-sensitive and MMC-resistant fibroblasts. Similar curves were obtained for other fibroblast strains and other cultured cell types such as primary blood lymphocytes and EBV-transformed lymphoblast cell lines. Whereas concentrations between 150 and 300 nM MMC (corresponding to 50–100 ng/ml) are routinely used for chromosome breakage studies, we here show that a diagnostically valid discrimination between MMC-sensitive and MMC-resistant cells can be

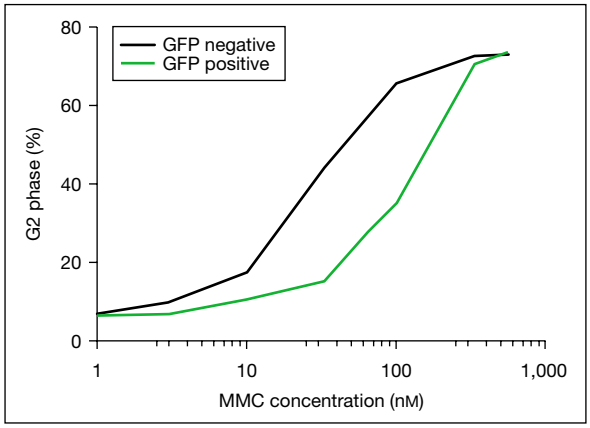


Fig. 2. Dose-response curves of G2-phase accumulations. Non-complemented (black curve) and complemented (green curve) FA-G fibroblasts from one and the same cell culture show differential G2-phase accumulations as a function of MMC concentration. Evaluation was with cell cycle distributions of the same type as in figure 1. Cells corrected by transfection with wt*FANCG* were gated by their green fluorescence due to GFP co-expression from a bicistronic vector.

achieved with much lower concentrations, if G2-phase arrest rather than chromosome breakage is measured. This proves the higher sensitivity of the cell cycle assay compared to chromosome breakage analysis.

ICL-Induced Cell Cycle Arrest Occurs after Completion of S Phase but prior to G2-M Transition

As shown above for fibroblasts, following exposure to MMC, EBV-transformed lymphoblast cell lines respond in the same way with an increase of their G2-phase cell fractions. In the experiments summarized in figure 3, we used a concentration of 45 nM and 48-h exposures for the discrimination between FA and non-FA lymphoblast cultures. The top row panels comparing untreated and treated control cultures show that there is only a very minor MMC-induced G2-phase increase in a non-FA (CON) lymphoblast cell line. In contrast, lymphoblast cultures of FA subtypes A, D2 and D1 (second, third and fourth row) show higher baseline (untreated) G2-phase fractions and respond to MMC with a more pronounced G2-phase increase, reflecting their innate MMC sensitivity.

The horizontal axes of the cell cycle distributions displayed in figures 1 and 3 indicate DNA content as measured by the fluorescence intensity of a dye that binds stoichiometrically to DNA. Thus, in each of these panels the distributions of cells within the G0/G1, S and G2/M compartments of the cell cycle are visualized from left to right. Cells in G0/G1 have a 2c DNA content, whereas cells in G2/M, having completed semiconservative DNA replication, have a 4c DNA content. G2- and M-phase cells cannot be discriminated on the basis of DNA content. In order to answer the question whether the cell cycle arrest in response to MMC treatments affects both the G2- and the M-phase compartments of the cell cycle, we included phospho-histone H3 (H3P)-staining as a cellular protein marker, whose expression is limited to the M phase of the cell cycle [16]. The simultaneous measurement of H3P and DNA content permits the discrimination between G2- and M-phase cells. As shown by the framed DNA histogram sections of figure 3, H3P-positive (mitotic) cells represent only a small proportion of the respective G2/M peaks. Following MMC treatments, the proportion of H3P-positive cells changes inversely to the increase of the G2/M peak. The higher the G2/M peak, the smaller the H3P-positive M fraction. In a series of eight different non-FA control cell lines, we observed a G2-phase increase of $5.2 \pm 1.6\%$ (mean $\pm$ 1 SD) and a H3P decrease of $0.06 \pm 0.14\%$. In contrast, the G2-phase increase for five different FA cell lines was $15.6 \pm 6.2\%$ (mean $\pm$ 1 SD) and the H3P decrease $0.49 \pm 0.23\%$. Thus, an approximately 3-fold increase of the G2/M peak resulted in an approximately 8-fold decrease of the H3P-positive cell population. These observations suggest that the MMC-induced accumulation of cells with a 4c DNA content must occur prior to entry into the M phase.

This interpretation was strengthened by the experiment shown in the bottom panels of figure 3: a non-FA control cell line was exposed to nocadazol, an agent that binds to tubulin and thereby prevents microtubule protofilament polymerization and spindle fiber formation. Nocadazol treatment resulted in a G2/M-peak increase of 23.5%, which is very similar to FA cells exposed to MMC. Strikingly however, the proportion of H3P-positive cells in the culture exposed to nocadazol showed an increase of 15.8%, accounting for 2/3 of the total G2/M-peak increase. In contrast to MMC, nocadazol clearly induces accumulations of cells that have completed G2 and entered the M phase of the cell cycle.

This series of experiments proves that the MMC-induced accumulation of cells occurs prior to the M phase, but after completion or near completion of DNA replication. Otherwise the accumulated cells would not display a 4c DNA content. These observations localize the putative crosslink-sensitive checkpoint into the early portion of the G2 phase, presumably close to the S/G2 border.

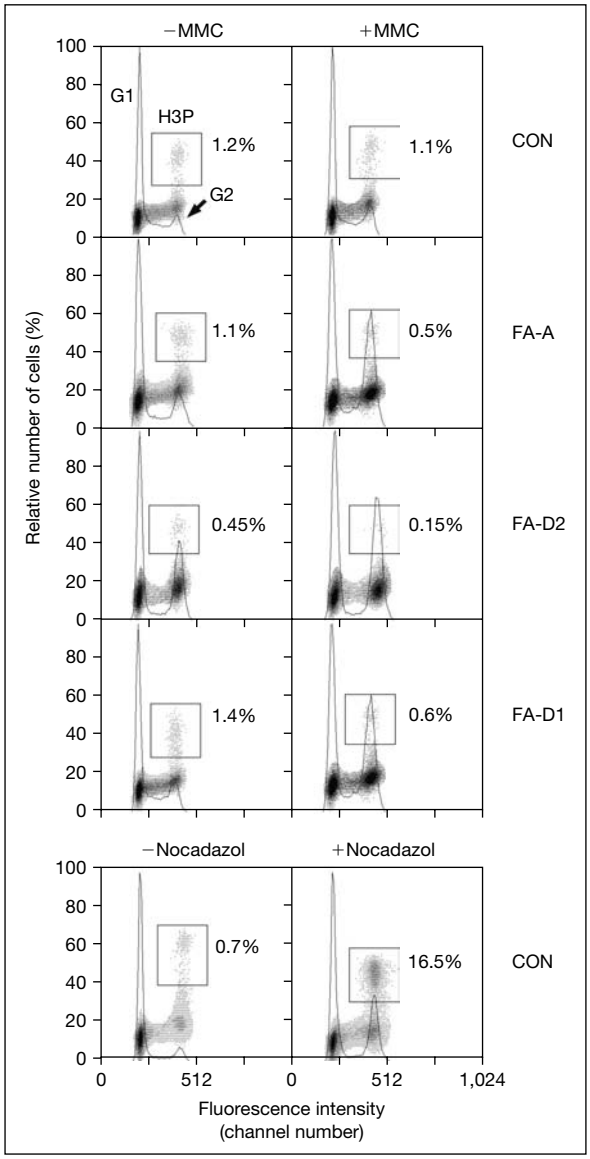


Fig. 3. Flowcytometric differentiation between G2- and M-phase cells reveals an inverse relationship between MMC-induced G2-phase arrest and H3P expression. FA lymphoblasts respond to exposure to 45 nM MMC for 48 h with increases of their G2-phase fractions to high levels (FA-A, row 2, 16.3–42.1%; FA-D2, row 3, 25.2–36.9%; FA-D1, row 4, 16.0–31.2%), shown in DNA histograms recorded after Hoechst-33342 staining. Simultaneously, H3P-positive (mitotic) cells (scatter plots boxed above the G2-phase cells) become substantially reduced as indicated. Normal control lymphoblasts show only modest G2-phase

Bivariate Flow Cytometry Enhances the Resolution of Cell Cycle Analysis in FA

Following the pioneering work of Peter Rabinovitch at the University of Washington in Seattle [17], we initiated studies with Hoechst-33258 fluorescence quenching following incorporation of the base analog 2-bromo-5′-deoxyuridine (BrdU) into DNA, a phenomenon first observed in cytogenetics. Added to cell cultures, BrdU is incorporated into newly synthesized DNA in place of thymidine. As a consequence, Hoechst-33258 fluorescence is suppressed in a DNA replication-dependent manner. In combination with a second DNA-specific dye, e.g., ethidium bromide (EB) emitting at a different wavelength, which does not show Hoechst-dependent fluorescence quenching, it is possible to resolve the replicative heterogeneity within a given cell culture. Figure 4 shows an example of the BrdU-Hoechst/EB double-staining technique as applied to a 96-h peripheral blood lymphocyte culture. In the presence of PHA, these cells start cycling between 30 and 35 h after initiation of the culture. The first replicating cells are represented by a signal track (S) trailing upward and to the left from the non-cycling cell cluster (G0/G1). As DNA content increases during replication, fluorescence due to EB staining also increases on the y-axis, but the intensity of Hoechst-33258 fluorescence on the x-axis decreases as result of Hoechst-fluorescence quenching by incorporation of BrdU into newly synthesized DNA. The first cells reaching the G2/M-phase compartment emerge at the 40-h time point. At 45 h, the first cycle is complete and, following mitosis, a cluster of cells emerges with half the Hoechst and EB fluorescence. These are cells that have reached the G1 phase of the second cycle (G1′). At 50 h, a subset of cells has entered the S phase of the second cycle (S′), whose signal track turns into a mirror image of that of the first cycle. This reflects the smaller increment of Hoechst quenching with BrdU substitution of second DNA strands as opposed to the substitution of first strands. After the 55-h time point, the second cycle is complete. Still cycling cells undergo division and enter the G1 phase of the third cycle (G1″), which appears to the left of the G1′ cluster. This cell fraction increases over time and at 60 h, some of these cells enter a new round of replication and their corresponding S and G2/M phase signals (S″ and G2″) run parallel to those of the

increase (row 1, 8.3–14.1%) and corresponding H3P decrease. In contrast, exposure of normal control lymphoblasts to 1.5 μM nocadazol for 24 h increases the G2/M phase (row 5, 8.3–31.8%) similar to FA lymphoblasts exposed to MMC, but most of this increase is due to H3P-expressing cells.

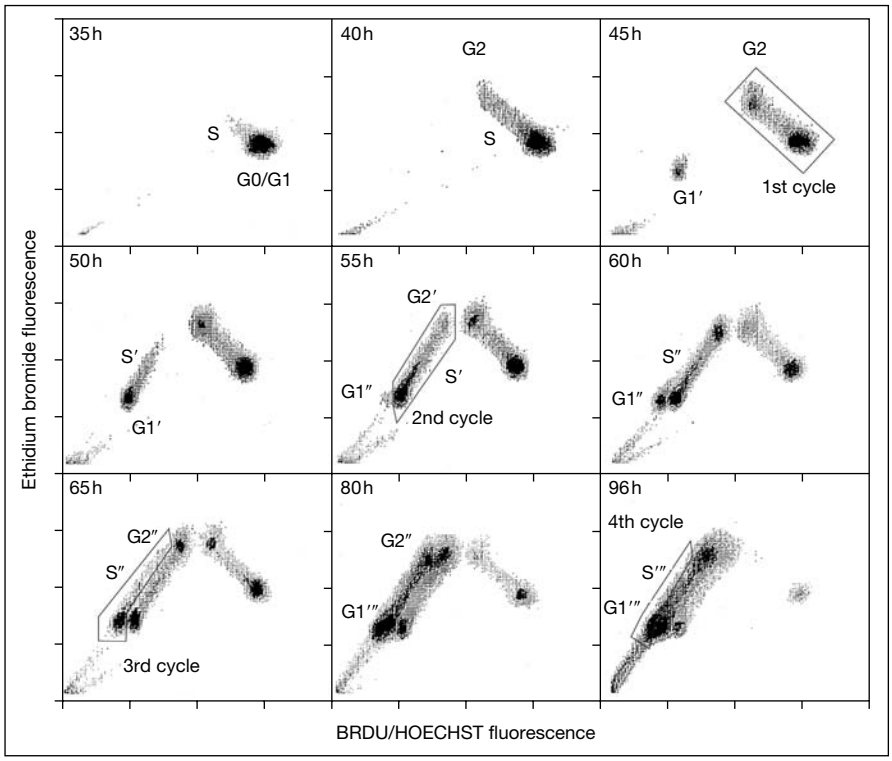


Fig. 4. Sequence of cell cycle distributions reveals entry and progression of PHA-stimulated lymphocytes throughout four consecutive cell cycles. Bivariate scatter plots of single cells, reflecting their Hoechst-33258- (x-axis) and EB- (y-axis) fluorescence intensities. Aliquots of BrdU-substituted PHA-stimulated lymphocytes from a healthy donor were harvested at intervals from 35 to 96 h as indicated, stained, and flow measurements were recorded to reveal static images of the dynamic progression through four consecutive cell cycles (cycle one, labeled G0/G1, S, G2; cycle two, G1′, S′, G2′; cycle three, G1″, S″, G2″; cycle four, G1‴, S‴). Modified from [28].

second cycle. The third cell cycle is almost complete after 65 h. At 80 h, cells have visibly progressed into the G1 phase of a fourth cycle (G1‴) and transition through this cycle is almost complete at 96 h. As the percentage increment of BrdU substitution of DNA decreases from cycle to cycle, the additional Hoechst-quenching effect becomes smaller with each cycle, which limits the resolution of the BrdU-Hoechst technique to about four rounds of replication. In addition to showing the distribution of cells throughout up to four cell cycles within a single cell culture, the BrdU-Hoechst technique permits the

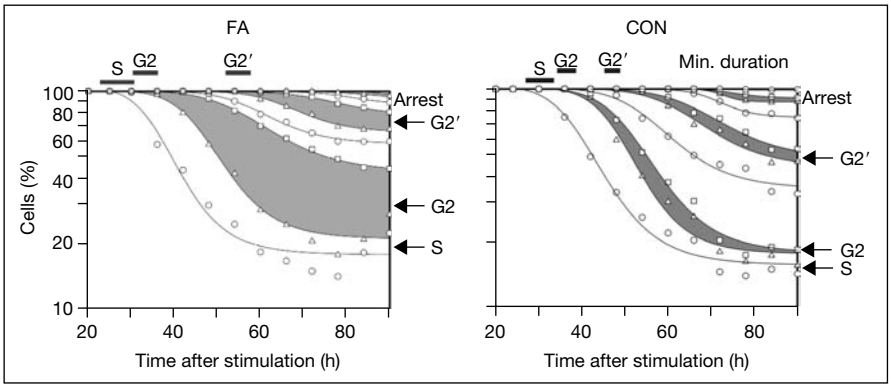


Fig. 5. Kinetic display of successive cell cycle compartment transitions. The curves represent fits of semi-logarithmic plots of the cell fractions in PHA-stimulated lymphocyte cultures remaining in any given compartment as a function of time after culture initiation (α-plots according to Smith and Martin, modified as proposed by Rabinovitch [17]). The left hand graph represents a PHA-stimulated lymphocyte culture derived from an FA patient, the right hand graph that of a non-FA control. The distances of intercepts of exit curves with the x-axis (representing minimum S, G2 and G2′ durations) are indicated by headline bars, the corresponding arrest fractions are represented as distances of intercepts of the same curves with the y-axis. The respective G2-phase compartments are shaded.

quantitative assessment of the fraction of cells that fails to respond to the mitogen (G0/G1 cell fraction). Unlike any other technique, the resolution of successive cell cycles permits the characterization of disturbances of cell cycle progression due to endogenous or exogenous influences. How this technique can be applied to the study of the FA cellular phenotype is illustrated in the following section.

The Cell Cycle Disturbance in FA Includes Delay and Arrest in S and G2

More than 20 years ago, Kubbies et al. [9] first described the cell kinetic behavior of FA cells as revealed by the BrdU-Hoechst technique. Because of the unique insights provided by this technique, we briefly summarize the essence of these types of experiments in figure 5. During the course of a 96-h study of PHA-stimulated lymphocytes, 12 harvests at 6-h intervals were performed, the first 24 h after initiation of the culture and the last at 90 h. Each harvest was recorded in the way shown in the previous section. Data on the fractions of cells

in the various cell cycle compartments were extracted and the percentages of cells remaining in each compartment were then plotted as function of time after stimulation. The resulting curves were fitted to the transition probability model of cell kinetics by Smith and Martin, modified to include a variable proportion of non-cycling cells in addition to the growth fraction with probabilistic rates of transition from phase to phase [17]. There are two advantages for FA studies of using the BrdU-Hoechst data in this manner: first, minimum compartment transit times can easily be derived by computing the distances between the x-axis intercepts of pairs of successive curves, each denoting the time course of cell exit from a given compartment. Second, the fraction of cells incapable of exit from a given compartment can be measured by comparing the plateau phases of two successive exit curves. If all cells that enter also leave a given compartment, then the extrapolated plateau levels of the two curves delimiting that compartment must coincide. If, in contrast, a proportion of cells are permanently halted after entry into such a compartment, then the two delimiting curves will plateau at different levels, the difference being the fraction of cells arrested within the respective compartment [9].

Single-time point, univariate flow cytometric measurements assign the disturbance of FA cell cycle progression to the G2 phase. The low resolution of such univariate and single-time measurements, however, does not permit to decipher FA cell cycle disturbances other than global G2 accumulations, nor do univariate measurements answer the question whether G2-phase accumulations result from increased G2-phase transit times (delay or prolongation), or from complete blockage of cells in G2 (arrest). Bivariate measurements and sequential analyses of untreated, PHA-stimulated lymphocyte cultures from an FA patient (fig. 5, left) reveal S- and G2-phase durations in the first cycle of 10.4 and 5.6 h (indicated by bars above the x-axis) compared to 9.4 and 3.4 h (likewise indicated by bars) in a normal control culture (fig. 5, right). The arrest of cells in the S and G2 phases of the first cycle, indicated on the y-axes, amounts to 4.2 and 16.2% in the FA patient (fig. 5, left) whereas the corresponding numbers are 1.2 and 1.5% in the non-FA control (fig. 5, right). Thus, the cell cycle disturbance in FA is more precisely described as a combination of S- and G2-phase delay and S- and G2-phase arrest with a clear predominance, however, of delay and arrest in the G2-phase compartment [9]. The relatively minor contribution of S-phase delay and S-phase arrest to the cell cycle disturbance of FA cell cultures is consistent with observations from univariate cell cycle assays showing preferential accumulations of cells with a 4c DNA content. The analysis of exit kinetic curves such as depicted in figure 5 also demonstrates that the cell cycle disturbance in FA is not confined to just a single cell cycle, but rather involves successive cell cycles consistent with the endogenous nature of the genetic defect underlying FA.

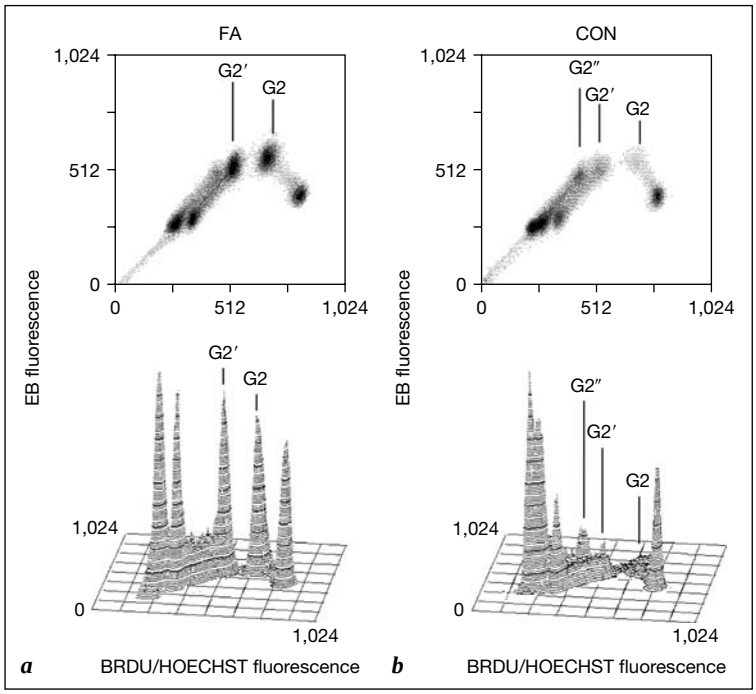


Fig. 6. Bivariate Hoechst-33258- and EB-fluorescence flow cytograms of PHA-stimulated 72-h primary lymphocyte cultures. The left hand panel (***a***) represents a patient with FA, the right (***b***) a normal control donor. Conspicuous are high G2-phase accumulations in successive cell cycles (G2, 28.0%; G2′, 12.7% and G2″, 1.5%) of the cytograms from the FA patient as opposed to only minor G2-phase cell fractions (G2, 4.4%; G2′, 6.1% and G2″, 3.9%) of the normal control. The differences are apparent both in signal-density (upper panel) and signal-height graphs (lower panel). The examples were selected for similar growth fractions to emphasize the different G2 phase accumulations of cells. Modified from [18].

The FA Cell Cycle Disturbance is Consistently Present and can be Exploited for Diagnostic Purposes

Figure 6 shows typical images of bivariate cell cycle distributions assayed by Hoechst-33258- and EB-fluorescence of 72-h PHA-stimulated FA (a) and non-FA (b) control cultures. Global G2-phase arrest observed in univariate cell cycle distributions such as shown in figures 1 and 3 corresponds in figure 6 to G2 arrest in each successive cell cycle (compare G2 and G2′ in a vs. b). This is evident in signal-density representations (fig. 6, upper panels), where densities reflect the cell numbers, but is also apparent in signal-height graphs, where

peak heights represent the respective cell numbers (fig. 6, lower panels). In the present example, the sum of G2 phases in the FA culture (fig. 6a) amounts to 42.2% of all cells as compared to 14.4% in the non-FA control (fig. 6b). Moreover, cell cycle progression encompasses four cycles in the control, but only three in the FA culture. On the other hand, 27.5% of all cells of the control and 20.7% of all cells in the FA culture did not respond to the mitogen within the 72-h culture period and thus remain in the G0/G1-phase compartment. This suggests that the mitogen response of the patient's PHA-responsive lymphocyte population is not systematically impaired. Vice versa, 72.5% of the control cells and 79.3% of the FA culture have entered the cell cycle. This cycling population is referred to as 'growth fraction' (GF). Whether or not G2 accumulations indicate the diagnosis of FA does not only depend on absolute G2-phase peak heights, but rather on G2-phase peak heights relative to the GF that may vary over a wide range among tested cultures. In order to assess this relationship numerically, we employ a ratio comprising the 'sum of all G2 compartments divided by the growth fraction' (sG2/GF). In the non-FA culture (fig. 6b), this ratio is close to 0.2. We would consider this ratio tentatively normal. The same sum of G2 compartments relative to a much smaller growth fraction, such as a GF of only 30% in a poorly growing culture, would result in an sG2/GF of about 0.5 similar to that illustrated by the FA-positive example of figure 6a.

The type of bivariate 72-h cell cycle distributions depicted in figure 6 is what our laboratory routinely uses for screening of blood samples in order to confirm or exclude the clinical suspicion of FA [18]. The sG2/GF parameter derived from the computer-assisted evaluation of such bivariate cytograms is subsequently plotted against the percentage of non-cycling (G0/G1) cells of a given culture. Figure 7 shows the results of altogether 394 FA and 630 non-FA 72-h peripheral blood lymphocyte cultures assessed in this way. It is important to note that these measurements did not include prior exposures to MMC. What we measure with obviously high sensitivity is the spontaneous, intrinsic cell cycle disturbance of FA cells. We believe that this spontaneous cell cycle disturbance, like spontaneous chromosome breakage, is a primary reflection of unrepaired spontaneous DNA damage and of the innate sensitivity of FA cell cultures to ambient air culture conditions with unphysiologically high oxygen concentrations. Lowering oxygen concentrations during cell culture to near physiologic conditions (5% v/v) all but eliminates the spontaneous cell cycle disturbance [19].

In the diagnostic series shown in figure 7, the majority of non-FA cultures has low sG2/GF ratios and their symbols (green circles) cluster within the left hand side, whereas the majority of FA cultures has high sG2/GF ratios and their symbols (red diamonds) come to lie to the right. With very few exceptions, there is a clear separation of the FA and non-FA genotypes with the discriminatory

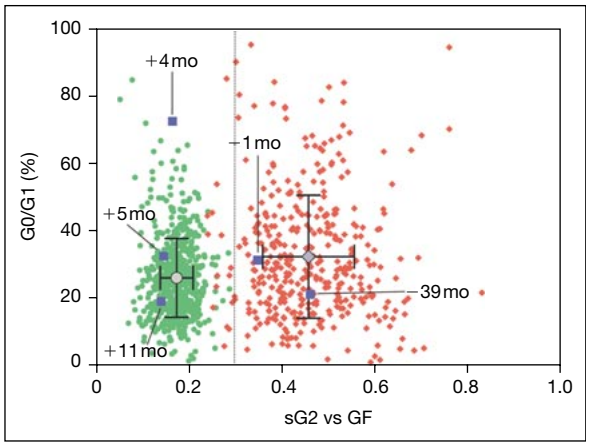


Fig. 7. Scattergram summarizing the results of flow cytometric cell cycle analyses of blood samples submitted to the Würzburg laboratory for the confirmation or exclusion of FA. 72-h, BrdU-substituted cultures of PHA-stimulated lymphocytes were subjected to Hoechst-33258- and EB-fluorescence intensity recording. Cell-cycle-resolved cytograms were electronically deconvoluted for quantitative assessment of each cell cycle compartment and the growth and resting cell fractions. The ratio 'sum of all G2 phases vs. growth fraction' (sG2 vs. GF) was calculated and plotted against the G0/G1 fraction, with the latter indicating the degree of lymphocyte activation. The panel represents 394 FA (red diamonds) and 630 non-FA (green circles) cases. Means of either group ± 1 SD are denoted by the large diamond and circle symbols and are given in the text. Blue squares denote successive cell cycle assays of an FA patient prior to and after successful HSCT.

threshold empirically set at an sG2/GF of 0.285. The few FA symbols that are scattered in the immediate vicinity below and above this threshold represent either proven or likely mosaic cases that consist of a mixture of MMC-sensitive and MMC-resistant (=reverted) cells, or they comprise cases with what we consider 'mild' mutations, i.e. retention of partial protein function. With respect to the sG2/GF parameter, the mean ± 1 SD amounts to 0.174 ± 0.035 for the non-FA samples, whereas the mean ± 1 SD of the FA samples is 0.456 ± 0.098 ($p < 0.001$). Again, these cultures were not treated with MMC such that these results would be comparable to the analysis of spontaneous chromosome breakage rates, which, in contrast, are known to vary greatly and which are generally judged to be of limited diagnostic value. Bivariate flow cytometry of cultures exposed to MMC provides even better separation with the FA and non-FA clusters drifting further apart (data not shown). Parallel exposure of cultures to MMC is always included in diagnostic studies, and such additional MMC testing is particularly helpful in cases of mild FA mutations or in mosaic cases (see contribution by Hoehn et al.).

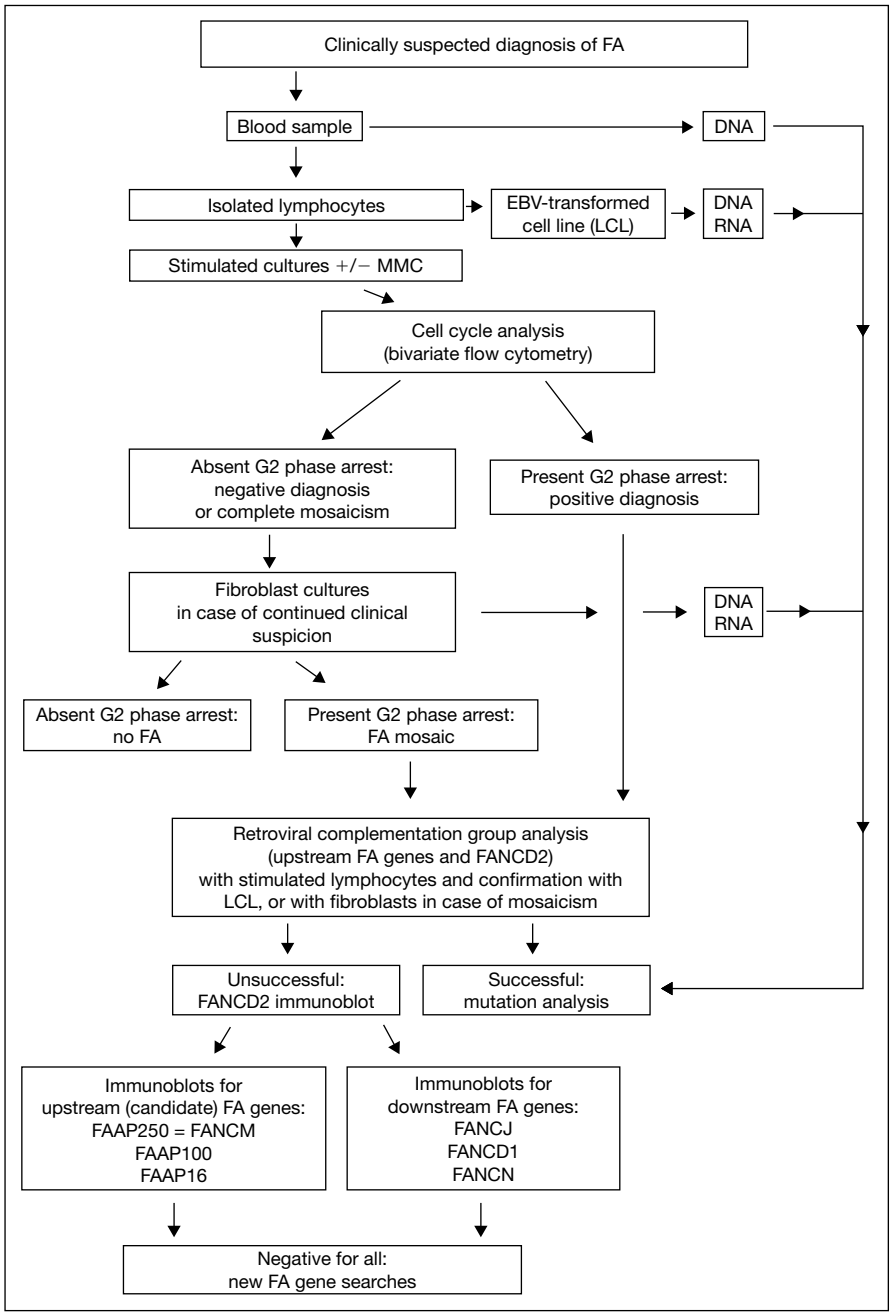
The FA and non-FA clusters depicted in figure 7 include cultures with a wide range of their non-cycling (G0/G1) cell fraction, reflecting considerable variability in their mitogen response. Empirically, cell samples with high proportions of non-cycling cells but normal sG2/GF parameters are overrepresented among patients with acquired non-FA types of aplastic anemia. However, as far as the comparison between our non-FA and FA cohorts is concerned, there is no significant difference of the mitogen response of lymphocytes of the non-FA (G0/G1 mean ± 1 SD, 25.9 ± 11.7%) vs. those of the FA cohort (G0/G1 mean ± 1 SD, 32.2 ± 18.3%).

How the FA genotype influences the outcome of the sG2/GF parameter is shown by the example of successful hematopoietic stem cell transplantation (HSCT) depicted by blue squares in the scattergram of figure 7. At 39 months prior to HSCT, cell cycle analysis clearly assigned this patient to the FA group of samples. This was confirmed at 1 month prior to HSCT, when the patient's sG2/GF had somewhat decreased but still remained within the cluster of FA cases. Following engraftment, and at 4, 5, and 11 months after HSCT, sG2/GF readouts of this patient are exclusively found among the non-FA cases, with continuously decreasing non-cycling cell fractions. This complete shift to normal values indicates establishment of complete lymphocyte chimerism. Immunosuppressive therapy most likely accounts for the rather high non-cycling cell fractions of the sample taken early after transplantation.

Cell Cycle Analysis as Starting Point for the Comprehensive Characterization of FA Patients

Figure 8 summarizes the diagnostic strategy currently adopted by the collaborating laboratories in Würzburg and Düsseldorf. Three steps follow each other: the first step involves the laboratory confirmation or exclusion of the clinical suspicion of FA via cell cycle analysis of 72-h cultures of peripheral blood lymphocytes. In cases of a normal flow cytometric result but persisting suspicion of FA on clinical grounds, MMC sensitivity testing of fibroblast cultures is performed in order to exclude revertant mosaicism [20]. If FA is confirmed by either of these studies, the second step involves the assignment of the patient to one of the known complementation groups. This may be performed by means of CD3/CD28/Il-2-stimulated lymphocytes set apart from the initial

Fig. 8. Current strategy for characterization of FA patients including flow cytometry-based cell cycle assays. The three-step procedure as currently performed in collaboration between the Universities of Würzburg and Düsseldorf comprises laboratory confirmation of the diagnosis, complementation group assignment and mutation analysis.


Clinically suspected diagnosis of FA
Blood sample
DNA
Isolated lymphocytes
EBV-transformed cell line (LCL)
DNA RNA
Stimulated cultures +/− MMC
Cell cycle analysis (bivariate flow cytometry)
Absent G2 phase arrest: negative diagnosis or complete mosaicism
Present G2 phase arrest: positive diagnosis
Fibroblast cultures in case of continued clinical suspicion
DNA RNA
Absent G2 phase arrest: no FA
Present G2 phase arrest: FA mosaic
Retroviral complementation group analysis (upstream FA genes and FANCD2) with stimulated lymphocytes and confirmation with LCL, or with fibroblasts in case of mosaicism
Unsuccessful: FANCD2 immunoblot
Successful: mutation analysis
Immunoblots for upstream (candidate) FA genes: FAAP250 = FANCM FAAP100 FAAP16
Immunoblots for downstream FA genes: FANCJ FANCD1 FANCN
Negative for all: new FA gene searches

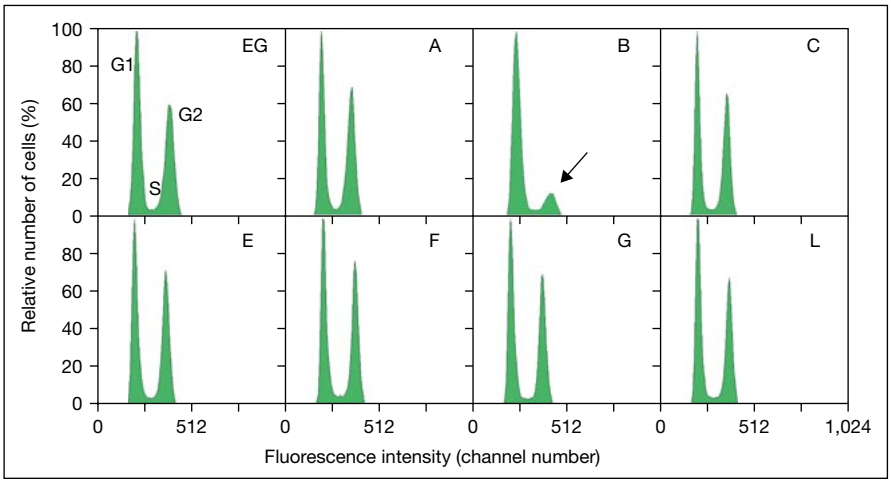


Fig. 9. Example of subgroup typing using retroviral transduction with FA cDNAs. EG = vector containing only the enhanced green-fluorescent protein cDNA insert; A, B, C, E, F, G, L = FA cDNA of the respective FA complementation groups in bicistronic constructs with EG. In this example, successful complementation was achieved with the vector containing *FANCB* cDNA as evidenced by the decrease of the G2 cell fraction (arrow).

blood sample, by means of an EBV-transformed lymphoblast line, and/or by means of cultured fibroblasts in case of proven mosaicism. As illustrated in figure 9, complementation analysis includes transfer of cDNA of the FA genes upstream of *FANCD2* by retroviral vectors and examination of MMC sensitivity of transduced cell cultures via univariate flow cytometry. The example shown in figure 9 involves the separate transfer of FANC genes A, B, C, E, F, G and L, with *FANCB* being the only cDNA that is capable of reducing the MMC-induced elevation of the G2 phase cell cycle fraction. This result proves that the tested cells belong to complementation group FA-B.

As outlined in figure 8, the complementation groups downstream of FA-D2 (FA-J, FA-D1 and FA-N) and FA-D2 itself are assayed by means of immunoblotting. Successful assignment of a patient to one of the known FA complementation groups will be followed by mutation analysis in the corresponding gene using DNA isolated from the initial blood sample and/or DNA and RNA from a lymphoblast line or a fibroblast strain. If an FA patient cannot be assigned to a known complementation group, FANCD2 immunoblotting will provide the information whether an unknown up- or downstream FA gene may be defective. This may then result in testing candidate genes or initiate other strategies to search for new FA genes.

Concluding Remarks

Cell kinetic studies such as originally performed by Kubbies et al. [9] have shown that the cell cycle disturbance of FA cells consists of both, delay and arrest within the S and G2 phases of the cell cycle. Flow cytometric measurements uniformly reveal a 4c DNA content of the accumulated cells. Since it has become clear that defective FA proteins cause accumulation of ICLs and replication-associated DNA-double strand breaks [21, 22], transient or permanent delay of cell cycle progression appears as the logical consequence for cells that have acquired such lesions. There are some seemingly contradictory findings as to where precisely within the cell cycle the accumulation of damaged cells takes place. When FA cells were treated with DNA-crosslinking agents after completion of DNA replication, i.e. during the G2 phase of the cell cycle, Akkari et al. [23] observed neither G2/M arrest nor increased chromosome breakage. The authors therefore concluded that crosslink-induced accumulations of FA cells take place in the late S phase, even though these cells have a 4c DNA content, which formally classifies them as having entered the G2 phase of the cell cycle. Since the early cell kinetic studies by Kubbies et al. [9] clearly showed delay and arrest during both, the S and G2 phases of the cell cycle, and since cell cycle studies uniformly prove the 4c DNA content of cells that accumulate in response to crosslinking agents, is comes down to a more or less semantic argument whether to call these accumulations late S or early G2. There is complete agreement, however, that the accumulations take place prior to entry into the M phase of the cell cycle, and the data summarized in figure 3 provide proof for this conclusion. Our demonstration that M-phase cells accumulate in response to a spindle damaging, but not in response to a DNA-crosslinking agent clearly separates the M- and S/G2-phase checkpoints in FA cells. These observations leave little doubt that the S/G2-phase checkpoint functions normally in FA cells, as previously demonstrated by the careful studies of Heinrich et al. [14] and Freie et al. [24]. As pointed out by these authors, spontaneous or crosslink-induced accumulations of FA cells in the G2 phase of the cell cycle do not reflect an abnormal cell cycle response per se, but rather represent a completely normal cellular response to unresolved DNA damage. Normal function of the S/G2 checkpoint is critical for the prevention of apoptosis or the erroneous re-replication of DNA [25]. Most importantly, S/G2 checkpoint-mediated arrest of damaged cells prevents their entry into mitosis, sets the stage for homology-directed DNA repair, and thereby minimizes the undesired perpetuation of genetic lesions.

In terms of diagnostic applications, we have outlined our approach and summarized our experience with cell cycle analysis as an alternative to traditional

chromosome breakage analysis for the laboratory confirmation or exclusion of FA. To the best of our knowledge, there were no false positives among our 394 cases that were classified as FA via cell cycle testing. However, we have identified a number of patients in whom the initial cell cycle screen gave a close to normal or completely normal result. Some of these false-negative patients were retested in fibroblasts because of persisting clinical suspicion of FA. With only few exceptions, the fibroblast cultures proved MMC-sensitive, classifying these patients as peripheral blood mosaics. As illustrated in the contribution by Hoehn et al., long-term monitoring of proven or presumptive mosaic patients via flow cytometry represents another highly informative application of cell cycle testing. This also holds for the use of cell cycle analysis in complementation assays. As illustrated in figure 9, failure or success of a given cDNA to complement the genetic defect in unclassified FA cells is easily recognized by the persistence or reduction of the G2-phase cell fraction. We wish to emphasize that retroviral complementation assays are not trivial, but with appropriate controls, and in experienced hands, the results of such assays are highly reliable [15, 26, 27]. In our protocol (cf. fig. 8), subtyping of newly diagnosed FA patients is regularly performed prior to mutation analysis.

There are, of course, many different protocols of how to optimize the diagnostic process in a rare and genetically heterogeneous disease such as FA. Most laboratories still rely on chromosome breakage analysis as the first diagnostic step, but FANCD2 immunoblotting has also been proposed as a primary diagnostic tool which however is limited to subtypes upstream of and including FA-D2 [5–7]. If mutation analysis is requested or important for clinical reasons, there again are different strategies. The Amsterdam laboratory, for example, starts with direct sequencing of the most frequent FA genes [2], whereas other laboratories, including our own, first attempt to identify the affected gene via retroviral complementation assays [15, 27]. As we have outlined in the flowchart of figure 8, it is important to secure DNA and/or cells at each level of the diagnostic procedure. In our opinion, cell cycle testing offers the opportunity for a fairly liberal, low cost screening of patients, in whom the diagnosis of FA may be considered [18]. Since the clinical manifestations of FA are so highly variable and frequently non-specific, the vast majority of the submitted blood samples will test negative. This probably is one of the strongest arguments in favor of cell cycle testing, which is less laborious and costly than conventional chromosome breakage studies. However, as we have shown in this chapter, there are many additional applications of cell cycle analysis, which go beyond mere diagnostic use and which have made major contributions to our understanding of the genetic and cell biological basis of the disease.

Acknowledgements

We are grateful to Birgit Gottwald and Gitta Emmert, Würzburg, for dedicated cell culture work. We recognize and appreciate the long-standing and invaluable support by Peter S. Rabinovitch, M.D., Ph.D., Seattle, with mathematical approaches to cell cycle models and software development.

References

1 Auerbach AD: A test for Fanconi's anemia. Blood 1988;72:366–367.
2 Joenje H, Oostra AB, Zwaan CM, Pals G: Fanconi anemia; in: Humana Press Series on Hereditary Cancer Syndromes. 2006, in press.
3 Schroeder-Kurth TM, Auerbach AD, Obe G (eds): Fanconi Anemia, Clinical, Cytogenetic, and Experimental Aspects. Berlin Heidelberg, Springer-Verlag, 1989, pp 1–378.
4 Schroeder TM, Anschütz F, Knopp A: Spontaneous chromosome aberrations in familial panmyelopathy. Humangenetik 1964;1:194–196.
5 Joenje H, Lo ten Foe JR, Oostra AB, van Berkel CG, Rooimans MA, et al: Classification of Fanconi anemia patients by complementation analysis: evidence for a fifth genetic subtype. Blood 1995;86:2156–2160.
6 Shimamura A, Montes de Oca R, Svenson JL, Haining N, Moreau LA, et al: A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood 2002;100:4649–4654.
7 Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, et al: Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 2005;105:1329–1336.
8 Dutrillaux B, Aurias A, Dutrillaux AM, Buriot D, Prieur M: The cell cycle of lymphocytes in Fanconi anemia. Hum Genet 1982;62:327–332.
9 Kubbies M, Schindler D, Hoehn H, Schinzel A, Rabinovitch PS: Endogenous blockage and delay of the chromosome cycle despite normal recruitment and growth phase explain poor proliferation and frequent endomitosis in Fanconi anemia cells. Am J Hum Genet 1985;37:1022–1030.
10 Kaiser TN, Lojewski A, Dougherty C, Juergens L, Sahar E, Latt SA: Flow cytometric characterization of the response of Fanconi's anemia cells to mitomycin C treatment. Cytometry 1982;2: 291–297.
11 Miglierina R, Le Coniat M, Berger R: A simple diagnostic test for Fanconi anemia by flow cytometry. Anal Cell Pathol 1991;3:111–118.
12 Fabio T, Crescenzio N, Saracco P, Leone L, Ponzio G, Ramenghi U: Cell cycle analysis in the diagnosis of Fanconi's anemia. Haematologica 2000;85:431–432.
13 Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, et al: Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 1995;85: 2233–2237.
14 Heinrich MC, Hoatlin ME, Zigler AJ, Silvey KV, Bakke AC, et al: DNA cross-linker-induced G2/M arrest in group C Fanconi anemia lymphoblasts reflects normal checkpoint function. Blood 1998;91:275–287.
15 Chandra S, Levran O, Jurickova I, Maas C, Kapur R, et al: A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 2005;12:976–984.
16 Hendzel MJ, Wei Y, Mancini MA, van Hooser A, Ranalli T, et al: Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 1997;106: 348–360.
17 Rabinovitch PS: Regulation of human fibroblast growth rate by both noncycling cell fraction and transition probability is shown by growth in 5-bromodeoxyuridine followed by Hoechst 33258 flow cytometry. Proc Natl Acad Sci USA 1983;80:2951–2955.

18 Schindler D, Hoehn H: Flow cytometric testing for syndromes with chromosomal instability, aplastic anemia and related hematological disorders; in: Wegner R-D (ed): Diagnostic Cytogenetics – Springer Lab Manual. Heidelberg, Springer, 1999, pp 269–281.
19 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
20 Joenje H, Arwert F, Kwee ML, Madan K, Hoehn H: Confounding factors in the diagnosis of Fanconi anaemia. Am J Med Genet 1998;75:228–229.
21 Sala-Trepat M, Rouillard D, Escarceller M, Laquerbe A, Moustacchi E, Papadopoulo D: Arrest of S-phase progression is impaired in Fanconi anemia cells. Exp Cell Res 2000;260:208–215.
22 Sobeck A, Stone S, Costanzo V, de Graaf B, Reuter T, et al: Fanconi anemia proteins are required to prevent accumulation of replication associated DNA double-strand breaks. Mol Cell Biol 2006;26:425–437.
23 Akkari YM, Bateman RL, Reifsteck CA, D'Andrea AD, Olson SB, Grompe M: The 4N cell cycle delay in Fanconi anemia reflects growth arrest in late S phase. Mol Genet Metab 2001;74: 403–412.
24 Freie BW, Ciccone SL, Li X, Plett PA, Orschell CMN, et al: A role for the Fanconi anemia C protein in maintaining the DNA damage-induced G2 checkpoint. J Biol Chem 2004;279:50986–50993.
25 Zhu W, Dutta A: An ATR- and BRCA1-mediated Fanconi anemia pathway is required for activating the G2/M checkpoint and DNA damage repair upon rereplication. Mol Cell Biol 2006;26: 4601–4611.
26 Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30:410–420.
27 Casado JA, Callen E, Jacome A, Rio P, Castella M, et al: A comprehensive strategy for the subtyping of Fanconi Anemia patients: conclusions from the Spanish Fanconi Anemia research network. J Med Genet 2006 [Epub ahead of print].
28 Poot M, Schmitt H, Seyschab H, Koehler J, Chen U, et al: Continuous bromodeoxyuridine labeling and bivariate ethidium bromide/Hoechst flow cytometry in cell kinetics. Cytometry 1989;10:222–226.

Detlev Schindler
Department of Human Genetics, University of Würzburg
Biozentrum, Am Hubland, D–97074 Würzburg (Germany)
Tel. +49 931 888 4089, Fax +49 931 888 4069
E-Mail schindler@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 131–148

Prenatal Diagnosis of Fanconi Anemia: Functional and Molecular Testing

A. Bechtold[a], *R. Kalb*[a], *K. Neveling*[a], *R. Friedl*[a], *B. Gottwald*[a], *S. Herterich*[b], *M. Schmugge Liner*[c], *C. Heilmann*[d], *H. Hanenberg*[e], *D. Schindler*[a]

[a]Department of Human Genetics, University of Würzburg, [b]Department of Clinical Chemistry and Pathobiochemistry, University of Würzburg, Würzburg, [c]Department of Pediatrics, University of Zürich, Zürich, Switzerland; [d]Department of Pediatrics, Rigshospitalet, Copenhagen, Denmark; [e]Department of Clinical Oncology and Hematology, University Children's Hospital, Heinrich-Heine-University, Düsseldorf, Germany

Abstract

There are two main approaches to the prenatal confirmation or exclusion of Fanconi anemia: functional testing and molecular testing. Functional testing involves the determination of crosslink sensitivity either by chromosome breakage analysis or cell cycle testing. Indications for functional testing include ultrasonographic findings of radial ray defects in the absence of a family history of FA, but also testing of at risk pregnancies in families with a prior affected child where for various reasons there is no information on complementation group and disease causing mutations. Since laboratories offering functional prenatal testing mostly use analysis of chromosome breakage, we here summarize our experience with flowcytometric testing of MMC-sensitivity in second trimester amniotic fluid cell cultures. We show that among a series of 20 pregnancies at risk three amniotic fluid cell cultures were highly sensitive to MMC as evidenced by their strong G2-phase elevations after exposure to 10 ng/ml of the drug. There were no false positives and no false negatives among our series suggesting single parameter flowcytometry as a speedy and reliable alternative to conventional chromosome breakage studies for the prenatal diagnosis of FA in situations where only functional testing can be performed. Molecular testing of course is the method of choice but requires prior knowledge of complementation group and mutations. Indirect genetic testing is possible if at least the complementation group is known and DNAs from both parents and an affected child are available. With the availability of retroviral vectors for rapid subtyping, and owing to advances in high-throughput mutation analysis including MLPA, direct molecular genetic testing is likely to replace functional testing for most but not all risk pregnancies in the near future. We illustrate the practice of direct prenatal genetic testing with examples from families belonging to complementation groups FA-A, FA-C, FA-G and FA-D2. Last but not least we comment on the implications of preimplantation genetic testing (PGD) as a high-tech but problematic procedure to preselect potential HLA-matched sibling donors.

Fanconi anemia (FA) is an inherited disease that is in principle amenable to symptomatic and curative treatment. However, present day small families often lack a matching sibling donor for lifesaving hematopoietic stem cell transplantation, and alternate donor transplants still carry a relatively high risk. Squamous cell carcinomas remain a lifelong threat. Despite remarkable therapeutic progress during recent years, Fanconi anemia still remains a serious and threatening disease. There are major impairments of the quality of life for the patients themselves, but also for their siblings and families [1]. Because of the severity of the disease, the demanding care, and the limitations of therapeutic interventions parents often feel overwhelmed and unable to care for a second or third affected child. Given a 25% recurrence risk and the possibility of prenatal diagnosis, a number of parents decide to use this option in their family planning. In contrast to many other medical interventions, prenatal diagnosis is not a routine procedure without impact on the physical and psychological well-being of patients [2, 3]. In the case of Fanconi anemia use of prenatal diagnosis is a reliable but desperate option in the face of our inability to provide a comprehensive cure for the disease.

The first prenatal diagnosis of FA was reported by Arleen Auerbach and her coworkers in 1979 who treated amniotic fluid cell cultures from at risk pregnancies with diepoxybutane (DEB) and studied chromosome breakage [4, 5]. Increased breakage rates were observed in affected fetuses and also in heterozygotes, but postnatal confirmation was obtained in only a single case [6]. Increased breakage rates of chromosomes prepared from amniotic fluid cell cultures that had been exposed to DEB were also reported in an affected fetus from South Africa [7]. In a subsequent series, Auerbach et al. did chromosome breakage studies in 30 pregnancies at risk for FA and were able to confirm the correctness of their prenatal findings by postnatal clinical and cytogenetic analyses [8]. This landmark study demonstrated that the chromosome breakage test for the confirmation or exclusion of FA could be used with amniotic fluid cell cultures much in the same way and with similar high degrees of sensitivity and specificity as had been shown and put into clinical practice for postnatally derived peripheral blood mononuclear cells.

The first prenatal diagnosis using fetal blood was reported by Shipley et al. [9]. The umbilical cord blood mononuclear cells of the affected fetus showed strongly elevated spontaneous and MMC-induced breakage rates. In 1985, Dallapiccola et al. [10] reported the first prenatal diagnosis using cells from a chorionic villus biopsy (CVS) with fetal trophoblast cells showing elevated chromosome breakage rates following DEB treatments. The usefulness of CVS-derived materials for the very early prenatal diagnosis of FA was quickly confirmed by French and American investigators [11, 12]. By that time, ultrasonography had become a standard monitoring procedure in prenatal care. A French group reported a family with three affected children one of whom had been diagnosed prenatally because of radial ray defects and elevated chromosome breakage rates

[13]. This article is the first to mention that families without matching sibling donors for their affected children might benefit from prenatal diagnosis and preselection of healthy children as potential hematopoietic stem cell donors. Prenatal identification of potential donors for umbilical cord blood derived stem cell transplantation was subsequently advocated by Auerbach et al. [14] and successfully applied, as an alternative to conventional bone marrow transplantation, by Elaine Gluckman and coworkers [15]. Despite obvious ethical problems, a number of children have since been conceived for the purpose of serving as stem cell donors for their affected siblings, culminating since 2001 in preimplantation-based selection of matched sibling donors (see below).

Most recently, flowcytometric determination of MMC-sensitivity was added to the spectrum of functional tests available for the prenatal confirmation or exclusion of FA [16]. It was shown that assessment of G2 phase cell blockage with and without prior exposure to mitomycin C (MMC) permits the distinction between affected and unaffected fetuses. However, this holds only for conventional single parameter flowcytometry of cultivated amniocytes, whereas bivariate BrdU-Hoechst/Ethidium bromide staining that is reliable with short term peripheral blood cultures proved unreliable with amniotic fluid cell cultures, the likely reason being variable degrees of BrdU sensitivity of these cells. In contrast, bivariate flowcytometry was shown to correctly predict affected fetuses using umbilical cord mononuclear cells after short term (72 h) culture, corresponding to what was known from the analysis of postnatally derived blood cell cultures. Given its speed and simplicity, single parameter flowcytometry was recommended by Bechtold et al. [16] as a screening procedure in pregnancies with low a priori risk of FA, e.g. ultrasonographic detection of radial ray abnormalities in the absence of any family history of FA.

After the first FA gene (*FANCC*) had been cloned in 1992, the first molecular prenatal diagnosis was reported in 1993 [17], when Murer-Orlando et al. confirmed the increased chromosome breakage rates found in amniocytes from an affected pregnancy by mutation analysis using DNA derived from chorionic villus materials. The fetus was found to carry biallelic mutations in the *FANCC* gene. The first molecular prenatal diagnosis of a twin pregnancy at risk for FA was carried out by Kwee et al. [18] in a family with a previous affected child identified with biallelic mutations in *FANCC*. The twins were shown to be heterozygous carriers of either the paternal or the maternal mutation and thus correctly predicted to be unaffected. The authors of these studies pointed out that prior knowledge of the complementation group and, most importantly, knowledge of the respective types of mutations assure a rapid and reliable diagnosis of FA during the 10th to 14th week of pregnancy, whereas functional tests (e.g. chromosome breakage studies using chorionic villus cells or amniocytes) were considered more time consuming and less reliable.

Categories of Prenatal Diagnosis

Depending on the family situation, there are two main categories of families requesting prenatal confirmation or exclusion of FA. The first category comprises pregnancies in which radial ray defects are observed as incidental findings on routine ultrasonographic examination. Even though such radial ray anomalies can be associated with a myriad of syndromes [19], this type of anomaly may of course be an important clinical sign of FA. Increased nuchal translucency as an ultrasonographic sign of an affected fetus has also been reported [20], as has been abnormal fetal motor behavior [21]. In these situations, there is no prior evidence for FA in the family, and the only practical way for confirmation or exclusion of FA is to carry out functional testing of fetal cells. Functional testing is also required in families with a prior affected child who had deceased or families who lack information on complementation group and mutation. To arrive at a timely molecular diagnosis may also be difficult in pregnancies where parents are not available for testing or in families where the disease causing genes are large and rather time consuming to type (e.g. *FANCA* or *FANCD2*). Subtyping and mutation analysis during the course of an ongoing pregnancy has so far only been reported in two families assigned to subtype FA-C, with *FANCC* being a relatively small gene where carrier testing and finding the disease causing mutations is relatively straightforward [22]. The second category, and of course optimal situation for the prenatal exclusion or confirmation of FA comprises families with prior affected children in whom the affected gene and both disease causing mutations are known. In such families, fetal DNA can be obtained via chorionic villus biopsy early in the course of pregnancy, and the results of the molecular analysis, including testing for maternal cell contamination, will be available within days [18, 23]. With the availability of rapid subtyping using retroviral vectors [24] and owing to technical advances of mutation analysis, including high throughput methods and MLPA, prenatal molecular genetic testing is expected to replace much of the present functional testing and thus be available for the majority of FA families in the near future.

Category 1: Functional Testing

Functional testing is based on the increased sensitivity of FA cells of all subtypes to DNA bifunctional alkylating agents such as diepoxybutane (DEB), mitomycin C (MMC), cisplatinum (CP) or nitrogen mustard (NM). Increased sensitivity to these agents can be assessed in different ways with identical results. The classical test assay is chromosome breakage [23, 25], but flowcytometric determination of the G2 phase cell fractions or simple cell growth assays

in the presence and absence of any of the damage inducing agents can also be employed [26–28]. In addition to carefully controlled cell culture conditions, monitoring of drug activities and testing of replicate cultures, an important requirement for the validity of functional testing is the concomitant analysis of both positive and negative control cells.

A potential drawback of chromosome breakage testing with amniotic fluid cell cultures is their heterogeneous growth pattern reflecting a variety of cell types of different fetal origins [29]. Under standard cell culture conditions that utilize incubators with a mixture of 5% CO_2 and ambient air, cells from affected fetuses may show poor growth since FA cells are known to be sensitive to ambient oxygen concentrations [30, 31]. For these reasons the yield of good quality metaphase spreads may be low or even insufficient for a timely and reliable diagnosis. The problem of variable to poor growth of amniotic fluid cell cultures is less critical and can largely be circumvented with the flowcytometric assay which determines the DNA content distributions of interphase cells that represent the vast majority of cells in amniotic fluid cell culture harvests. Additional advantages of the flowcytometric measurements are their relative speed, statistical accuracy and partial automation such that for some years our laboratory has given preference to this procedure. Whereas chromosome breakage testing is employed by most laboratories performing prenatal diagnosis of FA, flowcytometric testing is less well known such that our personal experience with this procedure will be briefly summarized below.

As shown in figure 1, ten-day amniotic fluid cell cultures established from a second trimester pregnancy were stained with the fluorescent dye DAPI and their DNA-content profiles were obtained using a flowcytometer of conventional design. Ultrasonographic examination had revealed moderate fetal growth retardation and bilateral radial ray abnormalities. Even without exposure to MMC, the fetal cells from the pregnancy at risk showed an elevated G2-phase fraction which became even more pronounced after treatment with 10 ng/ml MMC, indicating sensitivity to the crosslinking agent. Control cells from a normal pregnancy had much smaller G2-phase fractions and did not respond to MMC (negative control), whereas control cells from a pregnancy with proven FA showed a distinct response to MMC (positive control).

Figure 2 depicts the quantitative evaluation of MMC sensitivity in a series of 20 pregnancies belonging to category 1. For each of the pregnancies tested, the respective G2-phase fractions without prior MMC treatments are represented by light squares, and the G2-phase fractions after exposure to 10 ng/ml MMC are denoted by dark squares. For better visibility, the untreated and treated G2-phase fractions of each of the tested pregnancies have been connected by solid lines. Cases 5, 11, and 20 proved highly sensitive to MMC as evidenced by the large difference between the respective G2-phase fractions of

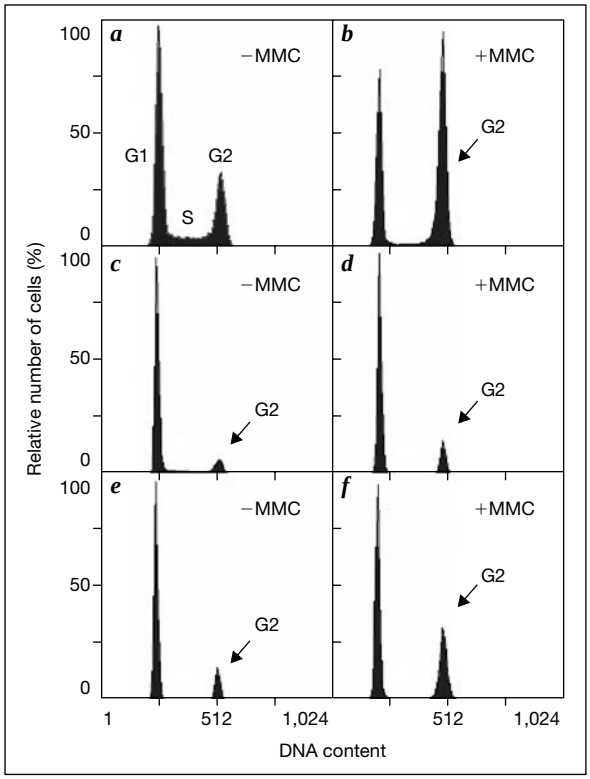


Fig. 1. Flowcytometric determination of MMC sensitivity of amniotic fluid cell cultures from a fetus at risk for FA (***a***, ***b***) together with negative (***c***, ***d***) and positive (***e***, ***f***) control cultures. Cells were grown without (left hand panels; −MMC) or with 10 ng/ml mitomycin C (right hand panels, +MMC) and analyzed after DAPI staining.

their untreated as opposed to their treated amniotic fluid cell cultures. These three cases were confirmed as affected either subsequent to termination of pregnancy or after birth. The data summarized in figure 2 illustrate two further points: (1) The G2-phase cell fractions of untreated cultures vary considerably, presumably due to different growth kinetics and variable amounts of G1-phase tetraploid cells that occur rather frequently in second trimester amniotic fluid cell cultures. (2) There is an impressive range of the degrees of MMC-response among FA-negative cell cultures, with cases number 8 and 10 showing no response at all, but cases 1 and 18 showing some minor degrees of MMC-sensitivity which, however, is far below of what is observed in affected cases. In the series of prenatal MMC sensitivity testing summarized in figure 2 there were no false positive and no false negative cases, suggesting that a reliable

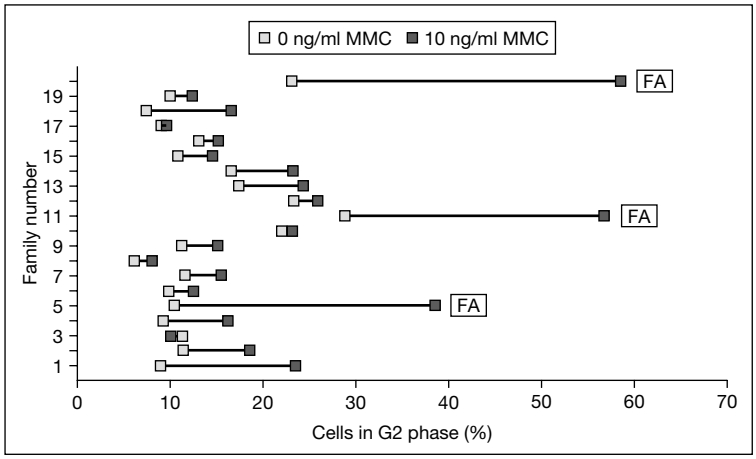


Fig. 2. Summary of flowcytometric MMC-sensitivity testing in 20 pregnancies at risk. Light squares: G2-phase fractions without exposure to MMC; dark squares: G2-phase fractions of parallel cultures after exposure to 10 ng/ml MMC. Cases 5, 11 and 20 were identified as FA due to strongly increased MMC sensitivity.

confirmation or exclusion of FA can be achieved with amniotic fluid cell cultures analyzed by conventional single parameter flowcytometry.

Category 2: Molecular Testing

As pointed out before, molecular testing is the method of choice for the prenatal diagnosis of FA, provided that the affected gene and both mutations are known. If only the complementation group is known, molecular testing can also be applied as long as DNA from both parents and an affected child is available. With the rapidly increasing catalogue of flanking and intragenic polymorphic markers, indirect genotyping has turned into a practical and accurate tool in situations in which the affected gene is known but the disease causing mutations remain to be detected. This is especially true for large and polymorphic genes such as *FANCA* [32].

Indirect Genotyping

Two children of family S. suffered from FA and their complementation group was determined as FA-A via retroviral gene transfer. The causal mutations in *FANCA*, however, were still unknown at the time when a third pregnancy was under way. Indirect genotyping with microsatellite markers flanking

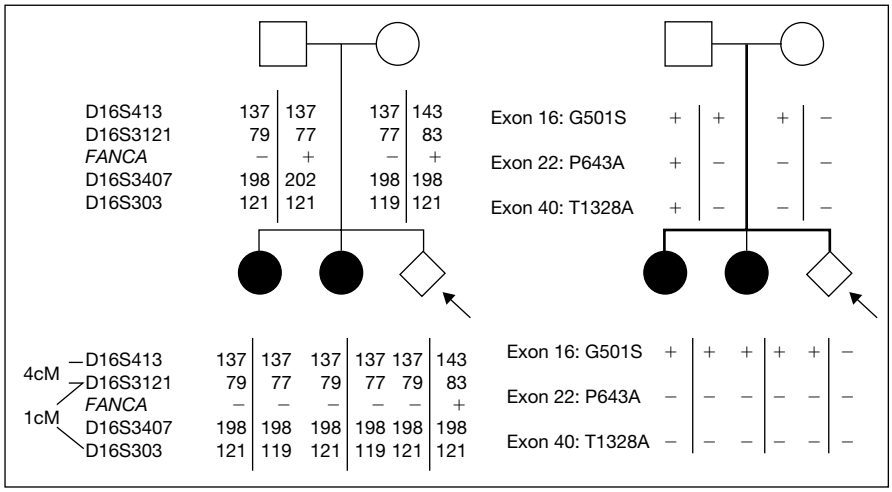


Fig. 3. Prenatal diagnosis via indirect genotyping in family S. with two affected girls. Left panel: genotyping using microsatellite markers flanking *FANCA*. Right panel: genotyping using intragenic single nucleotide polymorphisms. Fetus at risk marked by arrow.

the *FANCA* gene was carried out using DNA of both parents, the affected children, and fetal DNA obtained via chorionic villus biopsy. Using the markers D16S413, D16S3121, D16S3407, and D16S303 haplotypes were constructed encompassing the *FANCA* locus on chromosome 16. As shown in the left panel of figure 3, the fetus was found to carry the defective paternal but the wildtype maternal allele, identifying the fetus as heterozygous carrier not being affected by FA.

The prediction of an unaffected fetus via microsatellite haplotyping was confirmed by the analysis of intragenic single nucleotide polymorphisms of *FANCA* (fig. 3, right panel). Informative polymorphisms were G501S in exon 16, P643A in exon 22, and T1328A in exon 40. The parents were heterozygous for one respectively two of these variants. The fetus was shown to carry the wildtype maternal and the mutated paternal allele. Because of the concordance of the results of indirect genotyping with both flanking and intragenic markers, the fetus was confirmed as an unaffected carrier, and there were no signs of FA after birth.

Direct Genotyping

Prenatal Diagnosis Involving FANCA: The first child of family V. had been diagnosed with FA because of typical congenital malformations, aplastic anemia and elevated chromosome breakage. The child had already died by the

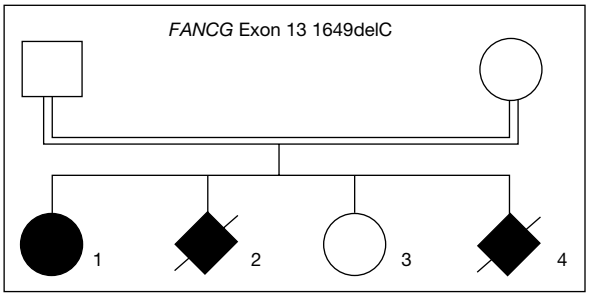


Fig. 4. Core pedigree of family A. Pregnancies 2, 3, and 4 underwent prenatal molecular testing as described in the text.

time of the second pregnancy, a dizygotic twin pregnancy, for which the parents requested prenatal diagnosis. Using parental blood and frozen materials from the deceased child, molecular analysis led to the diagnosis of compound heterozygosity for the following mutations in *FANCA*: a paternal 2-bp deletion at position 1165 in exon 13 leading to a frameshift, and a maternal 18-bp deletion within intron 40 affecting splicing (4010delG+18), resulting in skipping of exon 40. In the DNA prepared from chorionic villus biopsies of both twins there was no evidence for either of the parental mutations indicating that the twins were homozygous for the *FANCA* wildtype alleles. In order to exclude the risk of double puncture of the same chorion in dizygotic twins seven polymorphic DNA markers were analyzed. The twins were found to differ in six out of seven markers such that an erroneous puncture could be excluded. In summary, family V. could be told that neither of their unborn twins was carrier of a parental *FANCA* mutation, which excluded their being affected by FA. At birth, the fraternal twins were healthy and free of signs of FA.

Prenatal Diagnosis Involving FANCG: As shown in the family pedigree (fig. 4), the first child of family A. had been diagnosed with FA. During the second pregnancy amniotic fluid cells were analyzed in an outside laboratory and chromosome breakage rates were reported as suggestive of FA, even though the number and quality of the available metaphases was reported as too low for a reliable diagnosis. Complementation analysis with retroviral vectors assigned the amniotic fluid cells to complementation group FA-G, and mutation analysis detected a homozygous 1-bp deletion in exon 13 (1649delC). Homozygosity was expected because of consanguinity. The mutation leads to a frameshift and an unstable protein as described in the literature [33]. After induced abortion the diagnosis was confirmed via flow cytometric measurement of lymphocytes from

fetal heart blood: the response of mononuclear blood cells to PHA was reduced, there was increased cellular decay from the G0/G1 cell cycle fraction, and cell cycle blockage in the G2 phase was prominent. Even without MMC challenge, the ratio of sum of G2 phases vs. growth fraction was elevated to 0.405 which corresponds to the level seen in proven FA patients.

During the following pregnancy genomic DNA from chorionic villus cells was sequenced and the 1649delC mutation in *FANCG* exon 13 was detected next to the wildtype allele, showing that the fetus was heterozygous and not at risk of developing FA. Postnatally lymphocytes were analyzed via flow cytometry. The results of these functional studies confirmed the conclusion of the prenatal genotyping, as cell cycle distributions of neither untreated nor MMC-treated fetal cells revealed evidence for G2 phase arrest.

The fourth pregnancy in this family ended with intrauterine fetal death. DNA derived from the stillbirth was sequenced and the exon 13 mutation (1649delC) was detected in a homozygous state indicating that the fetus would have been affected by FA. Proving that the stillborn child would have suffered from FA relieved the grief of the parents over the pregnancy loss.

Prenatal Diagnosis Involving FANCC: Family I. has a child with FA assigned to complementation group FA-C. The parents requested prenatal diagnosis during their second pregnancy. cDNA from bone marrow cells of the first child was isolated and subjected to exon scanning sequencing of the entire *FANCC* gene. A homozygous frameshift mutation leading to premature termination of translation at seven codons downstream was found in exon 4 (454_455delAA; V152fsX159). The consanguineous parents were confirmed as heterozygous mutation carriers. There was no doubt about the pathogenicity of the mutation since it had been observed previously in another proven FA patient. DNA analysis from fetal chorionic villus materials revealed homozygosity for the mutation in exon 4, indicating an affected fetus. The results of the prenatal testing in family I. are illustrated in figure 5.

Prenatal Diagnosis Involving FANCD2: Family G. already had two children suffering from FA. The complementation group was determined via retroviral gene transfer and shown to be FA-D2. However, by the time of the third pregnancy the underlying mutations had not yet been detected. Functional testing using single parameter flow cytometry of cultured amniocytes showed a normal response to MMC, suggesting an unaffected fetus. In order to confirm the result of functional testing, an effort was made to detect the causal mutations in *FANCD2* via cDNA analysis of all family members. In case of the *FANCD2* gene, mutation analysis is complicated by the presence of pseudogenes, such that special primers had to be constructed [34]. The affected children showed skipping of *FANCD2* exon 22 resulting from the homozygous mutation c.1948-16C>T. This change could be shown to weaken the splice

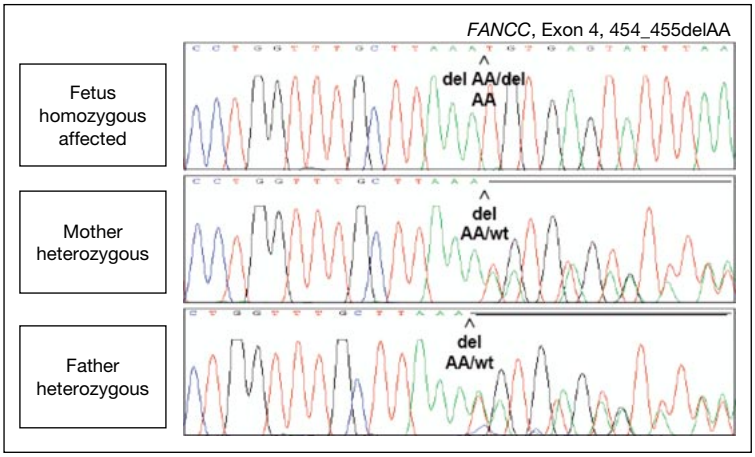


Fig. 5. Partial sequence of *FANCC* exon 4 of parents and fetus of family I. Both parents are heterozygous carriers of an identical 2-bp deletion within exon 4. The fetus was found to be homozygous for the defective parental allele and thus will be affected.

acceptor recognition of exon 21 by disruption of the pyrimidine-rich tract preceding the splice acceptor in intron 21 [34]. The consanguineous parents were heterozygous carriers, and the mutation was subsequently found in other FA families [34]. Like the parents the fetus in question turned out to be a heterozygous carrier, as evidenced by the simultaneous presence of exon 22 skipping and retention (fig. 6).

Since this was the first prenatal diagnosis in a *FANCD2* family that was carried out in our laboratory and given the slight background of aberrant exon 22 skipping that was observed in the affected sister (fig. 6) and even in healthy control individuals, it was decided to confirm the prediction of an unaffected, heterozygous carrier fetus by indirect genotyping. The affected children proved homozygous for three polymorphisms (IVS5-14delTT, IVS16+38A>C und IVS40+103C>T) whereas the paternal and the fetal cells showed heterozygosity at these loci. Unfortunately, neither of the above polymorphic markers nor six additional ones [1222A>G (V374V), 1509C>T (N503N), 2141A>C (P714L), 4098T>G (L1366L), 4453G>A and 4478A>G] were informative with regard to the maternal allele. They were homozygous in both mother and fetus indicating a high homogeneity of the maternal alleles. Since the mother clearly was not affected by FA, the observed constellation is not at variance with the presumed heterozygosity of the fetus. It was concluded that the results of prenatal molecular testing would be best explained by heterozygosity of the

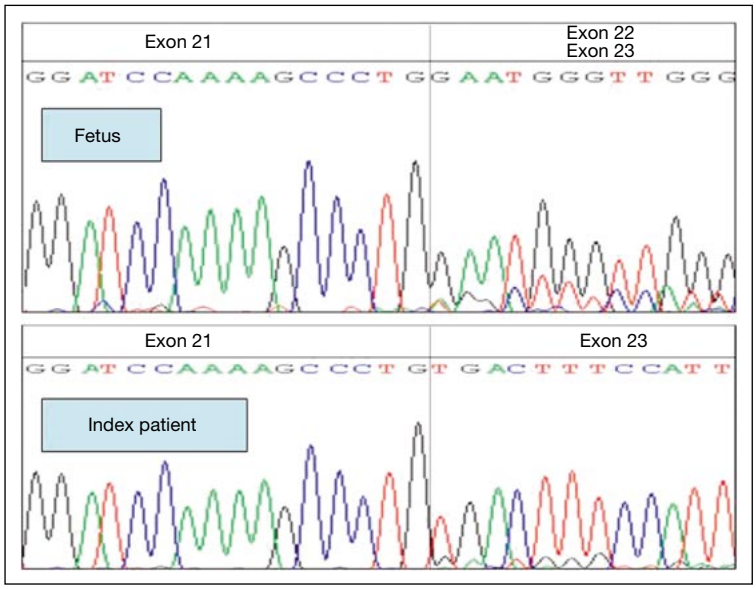


Fig. 6. Partial sequence of exons 21–23 of amniocytes (upper panel) and blood lymphoblasts (lower panel) showing homozygous skipping of exon 22 in the index patient of family G. and the heterozygous nature of the mutation in amniocytes of the tested fetus. Note slight background of exon 22 signals in the index patient.

FANCD2 mutation (c.1948-16C>T, r.1948_2021del74), identifying the fetus as not affected.

Another family (family S.) assigned to complementation group FA-D2 through an index patient was less fortunate. As shown in figure 7, testing of amniocyte DNA revealed the presence of both parental mutations, identifying the fetus as compound heterozygous and thereby affected. The index patient in that family was compound heterozygous for a 4-bp deletion at position c3453 and an *Alu* insertion in intron 4 leading to skipping of exon 5 due to insertional mutagenesis of the *Alu* element within the AT-rich target sequence of intron 4 [34]. Cultured amniocytes from the present pregnancy revealed exon 5 skipping together with the deletion c3453_3456delCAAA. The mother was shown to be a carrier of the *Alu* insertion in intron 4, whereas the father carried the 4-bp deletion within a poly A stretch at position 3453. Given that ultrasonographic examination revealed severe growth retardation, renal aplasia, and an unspecified heart defect, there was no doubt about the pathogenic nature of these

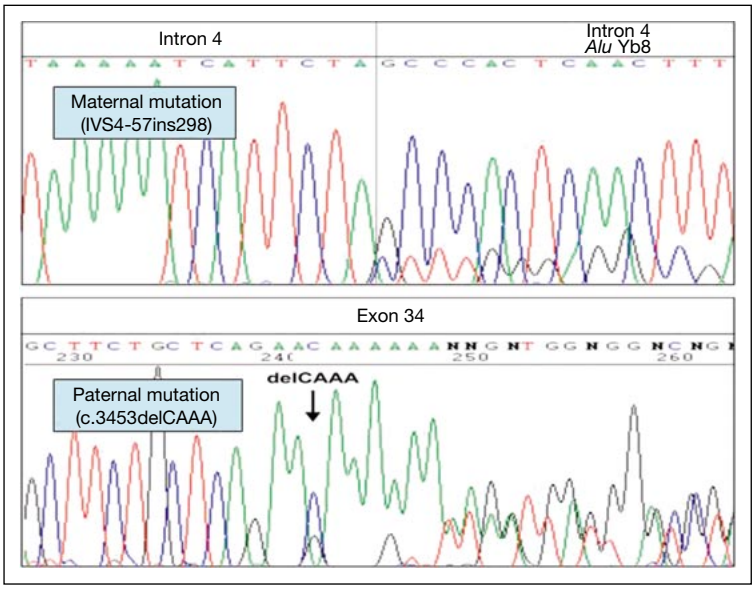


Fig. 7. Prenatal diagnosis in family S. Sequencing of *FANCD2* intron 4 and exon 34 DNA derived from fetal cells revealed the presence of both the maternal (top) and the paternal (bottom) defective allele, establishing the diagnosis of compound heterozygosity in the fetus.

mutations. In view of the result of the genetic testing the parents opted for termination of the pregnancy.

Comment on Molecular Testing in Pregnancies at Risk for FA

Even though prenatal molecular testing seems straightforward in families of category 2 in whom the affected gene, or better yet both mutations are known, our examples show that in practice the available molecular information may be incomplete or, conversely, too novel to be interpreted with sufficient confidence. This is the case with nearly all mutations that have not been previously associated with the disease phenotype, or where there is no possibility of validation of the pathogenic nature of a given mutation via the examination of an index patient. In the absence of comprehensive fetal pathology services in many hospitals, every effort should be made to confirm a pathologic prenatal result through functional testing of fetal cells. Although FA is a childhood disease, we still witness second and third pregnancies before a correct clinical

diagnosis has been made in the affected child. A timely and correct diagnosis is therefore urgent, and in families wishing to have more children diagnostic efforts should not be limited to the mere confirmation of the clinical suspicion of FA by way of a chromosome breakage or cell cycle test. Ideally, identification of the causal mutations should follow as soon as these tests have yielded a positive result. Knowledge of the causal gene and of the causal mutations not only provides valuable prognostic information (which will become more useful with each tested child), but also enables parents to request the optimal kind of prenatal diagnosis (i.e. direct genetic testing) in subsequent pregnancies if they wish to make use of this option.

Preimplantation Genetic Diagnosis (PGD)

Currently hematopoietic stem cell transplantation (HSCT) represents the only therapy for FA that is curative with respect to bone marrow function. A complete cure of the disease is not possible but HSCT can reconstitute normal bone marrow function and prevent hematological malignancies. The risk of solid tumors, however, persists in the patients since the transplanted cells only replace those in the bone marrow. If HSCT is considered and no HLA-matched sibling donor is available, PGD represents a novel option in many countries. Successful HSCT of FA patients following PGD for selection of an HLA-identical healthy donor has been reported from the US and Israel during the last few years [35–37]. Whereas prenatal HLA testing for the purpose of umbilical cord blood transplantation had been advocated and put into practice since the early nineties [14, 38], the first successful PGD-based combined HLA and FA testing was performed in 2001 by the Chicago group. Of 33 in vitro fertilized eggs blastomere biopsy and molecular testing identified 5 embryos suitable for transfer of which a single embryo resulted in the birth of a healthy child. His chord blood cells were harvested and used for HSCT of his affected 6-year-old sister [35, 36]. Her clinical and hematological condition improved dramatically and remained stable after the procedure. The worldwide second successful PGD involved the selection of an HLA compatible donor free of FA as umbilical blood cell donor for his 3.5-year-old affected brother [37].

One of the advantages of PGD as opposed to conventional prenatal diagnosis consists in the fact that couples are no longer confronted with the ethical dilemma of induced abortion. Main disadvantages are health risks implicit in the procedure, considerable financial and emotional burden for the families involved, and the relatively low success rate of the procedure. In the above mentioned successful cases 5 respectively 3 cycles of hormonal stimulation

and egg harvesting were necessary to achieve a single surviving child. In addition to these low rates of success, there are many unsolved ethical questions. For example, there is the questionable matter of parental motivation: parents might want to conceive a child with the intention of raising it just like any other of their children although the child was primarily conceived for the purpose of stem cell donation [39]. Transplantation associated risks may also play a role. If HSCT with umbilical cord blood cells fails (which is not infrequent due to the relatively low amount of putative stem cells that can be obtained from a single umbilical cord specimen), the donor child might be subjected to repeated stem cell harvests during the first months of life which always carries certain health risks. Some parents might even just want to obtain fetal stem cells and abort the fetus after fulfilling its 'purpose' as stem cell donor. Another major concern is the possible abuse of PGD for the purpose of sex selection.

At present, the overall success rate of PGD-based selection of HLA-matched sibling donors is still unknown, but certainly not as favorable as the publicity surrounding the procedure suggests. Out of 20 families participating in a recent meeting of the US American FA support groups 7 couples reported PGD attempts (Ralf Dietrich, personal communication, 2006). A number of these families have experienced repeated (3–5) unsuccessful PGD cycles. One woman went through nine consecutive pregnancy losses without achieving a liveborn child. Another family had attempted PGD 14 times without any success. The families voiced concerns about their physical and psychological strains, their disappointed hopes, their doubts and ambiguities, and last but not least about the high financial burden of the procedure. To simultaneously determine HLA type and to rule out FA in the minute amount of genetic material that is available from the nucleus of a single blastomere is an unprecedented technical challenge whose obvious limitations one tends to forget. This methodology clearly approaches the limits of what is biologically possible and technically feasible. The optimistic reports of the very few cases where PGD has been successful for the selection of suitable donors in FA families portray PGD as a safe and reliable procedure that can be routinely applied. Nothing could be further from the truth. In one of the US-families the first cycle resulted in a pregnancy with twin girls who were assumed to be FA-negative and HLA-identical to the affected child. However, molecular testing after birth revealed that both girls were in fact affected by FA. In addition, only one of the girls had the desired HLA characteristics. The family now faces the problem of having three affected children, and needing two instead of just one compatible HSCT donor. Due to the undisputable fact that in the case of FA most attempts of PGD-based matched sibling donor selection have ended with failures, there is increasing skepticism towards PGD in US-American families.

It is felt that the very few successful cases (at most 5 cases worldwide, of which only two have been published to date) might not justify the very high initial hopes of many families. An additional criticism voiced by the families is that adequate information concerning the risks, strains and limitations of the procedure have not been provided in many situations. Since allogeneic HSCT is increasingly successful in FA, it is doubtful whether such a high-risk, high-cost and low-success procedure like PGD will survive the test of time and practicability.

Acknowledgements

The authors wish to thank the Fanconi Anemia families requesting prenatal diagnosis for their patience and cooperation. Special thanks go to Ralf Dietrich of the German family support group for sharing information on the experiences and attitudes of American FA families with PGD.

References

1 Hutson SP, Alter BP: Experiences of siblings of patients with Fanconi anemia. Pediatr Blood Cancer 2006;48:72–79.
2 Schroeder TM: Ethical conflicts in genetic counselling during pregnancies (author's transl.). Monatsschr Kinderheilkd 1982;130:71–74.
3 Auerbach AD: Umbilical cord blood transplants for genetic disease: diagnostic and ethical issues in fetal studies. Blood Cells 1994;20:303–309.
4 Auerbach AD, Warburton D, Bloom AD, Chaganti RS: Preliminary communication: prenatal detection of the Fanconi Anemia gene by cytogenetic methods. Am J Hum Genet 1979;31:77–81.
5 Auerbach AD, Adler B, Chaganti RS: Prenatal and postnatal diagnosis and carrier detection of Fanconi anemia by a cytogenetic method. Pediatrics 1981;67:128–135.
6 Voss R, Kohn G, Shaham M, Benzur Z, Arnon J, et al: Prenatal diagnosis of Fanconi anemia. Clin Genet 1981;20:185–190.
7 Marx MP, Dawson B, Heyns AD: Prenatal diagnosis of Fanconi's anemia. S Afr Med J 1982;62: 348.
8 Auerbach AD, Sagi M, Adler B: Fanconi anemia: prenatal diagnosis in 30 fetuses at risk. Pediatrics 1985;76:794–800.
9 Shipley J, Rodeck CH, Garrett C, Galbraith J, Giannelli F: Mitomycin C induced chromosome damage in fetal blood cultures and prenatal diagnosis of Fanconi's anaemia. Prenat Diagn 1984;4: 217–221.
10 Dallapiccola B, Doria Lamba Carbone L, Ferranti G, Cristiani ML, Dagna Bricarelli F: Monitoring of pregnancies at risk for Fanconi's anemia by chorionic villi sampling. Acta Haematol 1985;73:157–158.
11 Boue J, Deluchat C, Nicolas H, Oury JF, Dumez Y: Prenatal diagnosis of genetic diseases using chorionic villi. J Genet Hum 1986;34:221–233.
12 Auerbach AD, Min Z, Ghosh R, Pergament E, Verlinsky Y, Nicolas H, Boue J: Clastogen-induced chromosomal breakage as a marker for first trimester prenatal diagnosis of Fanconi anemia. Hum Genet 1986;73:86–88.
13 Bonnet-Gajdos M, Vasmant D, Baruchel S, Mamou JF, Boue J, Lasfargues G: Prenatal diagnosis of Fanconi's anemia. Presse Med 1985;14:1612.

14 Auerbach AD, Liu Q, Ghosh R, Pollack MS, Douglas GW, Broxmeyer HE: Prenatal identification of potential donors for umbilical cord blood transplantation for Fanconi anemia. Transfusion 1990;30:682–687.
15 Gluckman E, Devergie A, Bourdeau-Esperou H, Thierry D, Traineau R, et al: Transplantation of umbilical cord blood in Fanconi's anemia. Nouv Rev Fr Hematol 1990;32:423–425.
16 Bechtold A, Friedl R, Kalb R, Gottwald B, Neveling K, et al: Prenatal exclusion/confirmation of Fanconi anemia via flow cytometry: a pilot study. Fetal Diagn Ther 2006;21:118–124.
17 Murer-Orlando M, Llerena JC Jr, Birjandi F, Gibson RA, Mathew CG: *FACC* gene mutations and early prenatal diagnosis of Fanconi's anaemia. Lancet 1993;342:686.
18 Kwee ML, Lo Ten Foe JR, Arwert F, Pals G, Madan K, et al: Early prenatal diagnosis of Fanconi anaemia in a twin pregnancy, using DNA analysis. Prenat Diagn 1996;16:345–348.
19 Goldfarb CA, Wall L, Manske PR: Radial longitudinal deficiency: the incidence of associated medical and musculoskeletal conditions. J Hand Surg 2006;7:1176–1182.
20 Tercanli S, Miny P, Siebert MS, Hosli I, Surbek DV, Holzgreve W: Fanconi anemia associated with increased nuchal translucency detected by first-trimester ultrasound. Ultrasound Obstet Gynecol 2001;17:160–162.
21 de Vries JI, Laurini RN, Visser GH: Abnormal motor behaviour and developmental postmortem findings in a fetus with Fanconi anaemia. Early Hum Dev 1994;36:137–142.
22 Merrill A, Rosenblum-Vos L, Driscoll DA, Daley K, Treat K: Prenatal diagnosis of Fanconi anemia (Group C) subsequent to abnormal sonographic findings. Prenat Diagn 2005;25:20–22.
23 Auerbach AD: Fanconi anemia: genetic testing in Ashkenazi Jews. Genet Test 1997;1:27–33.
24 Chandra S, Levran O, Jurickova I, Maas C, Kapur R, et al: A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 2005;12:976–984.
25 Joenje H, Oostra AB, Zwaan CM, Pals G: Fanconi anemia; in Humana Press Series on Hereditary Cancer Syndromes. Humana Press, 2006 in press.
26 Berger R, Le Coniat M, Gendron MC: Fanconi anemia. Chromosome breakage and cell cycle studies. Cancer Genet Cytogenet 1993;1:13–16.
27 Seyschab H, Friedl R, Sun Y, Schindler D, Hoehn H, Hentze S, Schroeder-Kurth T: Comparative evaluation of diepoxybutane sensitivity and cell cycle blockage in the diagnosis of Fanconi anemia. Blood 1995;85:2233–2237.
28 Schindler D, Hoehn H: Flow cytometric testing for syndromes with chromosomal instability, aplastic anemia and related hematological disorders; in Wegner R-D (ed): Diagnostic Cytogenetics. Berlin/Heidelberg, Springer Verlag, 1999, pp 269–281.
29 Hoehn H: Amniotic fluid cell culture; in Milunsky A (ed): Genetic Disorder and the Fetus, ed 4. Baltimore, The John Hopkins University Press, 1998, pp 128–149.
30 Joenje H, Arwert F, Eriksson AW, de Koning H, Oostra AB: Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. Nature 1981;290:142–143.
31 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
32 Levran O, Diotti R, Pujara K, Batish SD, Hanenberg H, Auerbach AD: Spectrum of sequence variations in the *FANCA* gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat 2005;25:142–149.
33 Demuth I, Wlodarski M, Tipping AJ, Morgan NV, de Winter JP, et al: Spectrum of mutations in the Fanconi anaemia group G gene, *FANCG/XRCC9*. Eur J Hum Genet 2000;8:861–868.
34 Kalb R, Neveling K, Hoehn H, Schneider H, Linka Y, et al: Hypomorphic mutations in the key Fanconi anemia gene *FANCD2* sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet 2007; in press.
35 Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A: Preimplantation diagnosis for Fanconi anemia combined with HLA matching. JAMA 2001;285:3130–3133.
36 Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE: Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. Blood 2004;103:1147–1151.
37 Bielorai B, Hughes MR, Auerbach AD, Nagler A, Loewenthal R, Rechavi G, Toren A: Successful umbilical cord blood transplantation for Fanconi anemia using preimplantation genetic diagnosis for HLA-matched donor. Am J Hematol 2004;77:397–399.

38 Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, et al: Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 1989;321:1174–1178.
39 Alby N: The child conceived to give life. Bone Marrow Transplant 1992;9:95–96.

Dr. Astrid Bechtold
Department of Human Genetics, Biozentrum
Am Hubland, University of Würzburg
97074 Würzburg (Germany)
Tel. +49 931 8884068, Fax +49 931 8884434
E-Mail astrid.bechtold@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 149–172

Revertant Mosaicism in Fanconi Anemia: Natural Gene Therapy at Work

H. Hoehn[a], *R. Kalb*[a], *K. Neveling*[a], *R. Friedl*[a], *A. Bechtold*[a], *S. Herterich*[b], *Y. Sun*[c], *B. Gruhn*[d], *H. Hanenberg*[e], *D. Schindler*[a]

[a]Department of Human Genetics, Biocenter, University of Würzburg, [b]Department of Clinical Chemistry and Pathobiochemistry, University of Würzburg, Würzburg, Germany; [c]Key Laboratory of Molecular and Medical Genetics, Nanjing Medical University, Nanjing, P.R. China; [d]Department of Pediatrics, University of Jena, Jena, [e]Department of Pediatric Hematology and Oncology, Children's Hospital, University of Düsseldorf, Düsseldorf, Germany

Abstract

One out of four to five patients with Fanconi anemia experience a reversion or attenuation of their constitutional mutations during their lifetime. If the reversion event takes place in a bone marrow stem cell or in an early precursor cell of hematopoiesis, peripheral blood cell counts may gradually recover, leading to improved quality of life. At the beginning of this process, MMC testing will reveal a mixture of MMC sensitive and MMC resistant blood lymphocytes, but after several years MMC sensitive cells (the original FA-cells) may be completely replaced by the progeny of the reverted progenitor cell such that the confirmation of FA requires testing of patient fibroblasts. Molecular analysis reveals the presence of the disease causing biallelic mutations in fibroblast-derived DNA whereas MMC-resistant blood cells show only a single defective allele, explaining their phenotypic reversion. The mechanisms leading to revertant mosaicism include intragenic mitotic recombination (crossing over and gene conversion), back mutation, and compensatory second site mutations. Evidence for each of these mechanisms has been obtained in mosaic FA-patients, but their molecular details are not fully understood. Compound heterozygosity facilitates some of these mechanisms, but reversions have also been observed in homozygous patients. Patients belonging to subtypes FA-A, FA-C, FA-D1, FA-D2 and FA-L have developed revertant mosaicism, with subtypes FA-A and FA-D2 being most frequently involved. Even though the phenomenon of revertant mosaicism has been well documented in FA, there still are many questions: we do not know whether the progeny of a single reverted blood stem or progenitor cell would be able to sustain lifelong hematopoiesis, whether revertant mosaicism provides protection against hematopoietic malignancy, or whether it would be possible to deliberately increase the rate of somatic reversions in order to improve chances for 'natural gene therapy'. Prospective and long-term follow-up studies are needed to answer these questions.

The restoration to normal (or close to normal) function of a gene previously inactivated by a constitutional mutation is referred to as 'reversion'. The coexistence of reverted and wildtype cells within otherwise isogenic organisms is referred to as 'reverse' or 'revertant' mosaicism. In contrast to Lo ten Foe et al. [1] we prefer the term 'revertant' which has been defined as 'a mutant which has regained, partially or completely, the wildtype phenotype by either a genetic or nongenetic mechanism of reversion' [2]. As pointed out by Jonkman et al. [3], the term 'reverse' simply implies a change in the opposite direction, but does not necessarily denote complete or partial restoration of a defective function. The phenomenon of revertant mosaicism has been observed in a number of inherited diseases, including a skin disease (epidermolysis bullosa), a metabolic disease (tyrosinemia type I), a number of primary immunodeficiency syndromes (Wiskott-Aldrich syndrome, X-linked severe combined immunodeficiency, ADA-deficiency), and in two chromosomal instability syndromes (Bloom syndrome and Fanconi anemia) (see reviews by Youssoufian and Pyeritz [4] and Hirschhorn [5]).

Revertant Mosaicism in FA: From Observations to Mechanisms

In the case of FA, revertant mosaicism has both clinical and theoretical implications [1, 6]. These implications relate to the altered in vivo behavior of the reverted cells and depend on the developmental stage, the blood cell lineage affected by the reversion event, and the clinical situation of the mosaic patient. In self-renewing tissues, the reversion event may convey a growth advantage to the reverted cells such that their progeny may outgrow and, ultimately, replace the defective cells. Such a positive outcome would suggest that gene therapy might be successful in FA and other stem cell diseases. Because of these positive prospects, revertant mosaicism has also been referred to as 'natural gene therapy' [3, 6–9].

As early as in 1983, the group of Hans Joenje in Amsterdam noted an unusual response to bifunctional alkylating agents in a 22-year-old patient with Fanconi anemia. Unexpectedly, only 40% of the patient's peripheral blood mononuclear cells proved highly sensitive to these agents as evidenced by strongly increased chromosome breakage while 60% of the patient's cells responded like cells from healthy individuals [10]. The patient's advanced age and the observation of two distinctive peripheral blood cell populations with striking differences in crosslink sensitivity leave little doubt that this patient represents one of the first well-documented cases of revertant mosaicism in Fanconi anemia.

At the same time, other authors noted that among clinically diagnosed FA patients between 1 to 3 out of 9 tested blood samples proved MMC- or DEB-resistant [11, 12]. There was some concern that loss of activity during storage of

the DEB or MMC stock solutions could give rise to false-negative chromosome breakage tests [13], but it was generally felt that a subgroup of patients with clinical manifestations of FA might lack the FA-typical pattern of crosslink sensitivity. The existence of such a subgroup was convincingly documented by Arleen Auerbach and Traute Schroeder-Kurth in their first report on the International Fanconi Anemia Registry (IFAR) [14]. Among a series of 162 FA patients Auerbach and colleagues noted 7 patients who appeared to have two populations of cells. 60–81% of DEB-treated cells from these patients displayed no evidence for chromosome breakage, while the remainder exhibited high numbers of breaks and exchanges typical of FA cells. Since three of these cases were from multiplex sibships, the authors came to the conclusion that the 'phenomenon of two-cell populations does not seem to be related to genetic heterogeneity in FA' [14]. Clearly, what the authors had observed but not recognized as such at that time was the phenomenon of revertant mosaicism.

Laboratories which routinely established lymphoid cell lines from FA patients noted that around 30% of such EBV transformed cell lines that originate from B-lymphocytes became crosslink resistant during the course of prolonged in vitro culture. However, it was not until 1997 when a landmark study by the Amsterdam group confirmed that between 20 and 30% of FA patients show evidence for a mixture of MMC-sensitive and MMC-resistant peripheral blood lymphocytes [1]. Molecular analysis of these two types of cells provided unambiguous proof of the concept of revertant mosaicism. In the study by Lo ten Foe and colleagues [1] the expected biallelic mutations in a given *FANC* gene were present in DNA from the MMC-sensitive subpopulation, whereas the MMC-resistant cells were found to carry only a single defective allele. Since in recessive diseases a single intact copy of a disease gene suffices for the maintenance of function, the study by Lo ten Foe et al. [1] provided molecular proof of rescue of defective gene function via somatic reversion of a constitutional mutation. In the same year, Jonkman and coworkers published their landmark study on somatic reversion as molecular cause of mRNA rescue in epidermolysis bullosa, suggesting that gene conversion might be the mechanism by which the defective function of one of the collagen XVII alleles had been restored in unaffected skin areas of their patient [15].

Detection of Somatic Reversion in FA

In FA patients, unexplained improvements of blood counts may herald the emergence of revertant mosaicism. A mosaic patient may also be older than the average FA patient, and the diagnosis of revertant mosaicism is frequently established at a time when these patients reach adolescence or adulthood without experiencing prior major medical problems [9, 10, 16–19]. However,

long before an improving hematological situation suggests the possibility of reversion, the diagnostic laboratory may have noted the co-existence of MMC-sensitive and MMC-resistant cell populations in peripheral blood mononuclear cell cultures as they are routinely used for the confirmation or exclusion of the clinical suspicion of FA. Likewise, FA research laboratories that routinely establish EBV-transformed lymphoid cell lines from peripheral blood cells may have noted the emergence of DEB or MMC resistance in a considerable proportion of these (B-lymphocyte derived) cell lines. Emergence of drug resistance signals loss of the FA cellular phenotype in these cell lines and precludes their use for complementation and other studies. Between 20 and 30% of lymphoblastoid cell lines derived from FA patients have been observed to undergo the transition from MMC sensitivity to MMC resistance [6, 20].

Chromosome Breakage Test for the Detection of Reverted Cells

Landmark studies by the Auerbach and Schroeder-Kurth laboratories (summarized in their common monograph on Fanconi anemia [21]) have defined culture conditions, clastogen types, clastogen concentrations, and numerous other criteria for evaluation of chromosome breakage rates. In particular, the parameter 'number of breaks per aberrant cell' was found to optimally define whether or not cells are sensitive to a given clastogen [22]. These criteria are still valid today and prove useful for the recognition of revertant mosaicism. In mosaic cases, laboratories using chromosome breakage analysis will typically detect a bimodal distribution of breakage rates whereas non-mosaic cases show unimodal breakage distributions. Figure 1 depicts chromosome breakage distributions of 72-h peripheral blood mononuclear cell cultures evaluated (from top to bottom) at 0, 50 and 100 ng/ml MMC. The panels of figure 1a and 1c illustrate examples of unimodal distributions. Such distributions classify the respective cell sample as either MMC-resistant (fig. 1a) or MMC-sensitive (fig. 1c). According to these criteria, the donor of blood sample 1a would be classified as non-FA, and the donor of blood sample 1c would be classified as FA-positive. Following exposure to MMC, FA positive cases typically exhibit cells with ten or more breaks per metaphase, reflecting the innate MMC-sensitivity of the FA genotype (panel 1c). MMC-treated cultures of exceptional individuals with a questionable diagnosis of FA may show intermediate types of MMC-sensitivity, such as shown in panel 1b. This pattern is in essence very similar to the pattern observed in genuine FA-mosaic cases (illustrated in panels 1d–f). However, the patient whose chromosome breakage study is shown in figure 1b developed B-cell lymphoma rather than AML, and he tolerated chemotherapy with alkylating agents rather well such that a diagnosis other than FA appeared more likely [23].

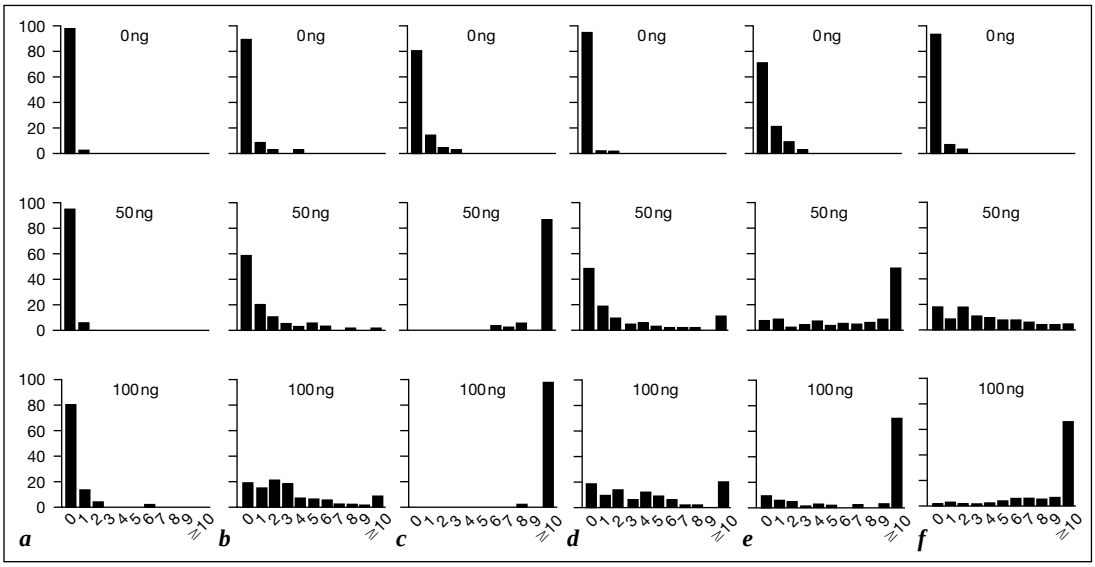


Fig. 1. Chromosomal breakage testing of 72-h peripheral blood mononuclear cell cultures without (top row) and with (middle row, 50 ng/ml; bottom row, 100 ng/ml) mitomcycin C. (***a***) MMC-resistant: non-FA; (***b***) intermediate type of MMC sensitivity: questionable diagnosis of FA; (***c***) MMC-sensitive: FA-positive; (***d***–***f***) intermediate types of MMC sensitivity: different grades of FA-mosaicism.

The panels shown in figures 1d–f illustrate three examples of revertant mosaicism in patients with clinical features of FA. The simultaneous existence of cells with zero and with ten or more breaks per metaphase at two different MMC concentrations indicates the presence of cell populations with striking differences in MMC sensitivity. Even if (such as shown in panel 1f) only a single metaphase without any breaks is identified among 50 cells studied after exposure to the highest clastogen concentration, the patient could be a mosaic, just like a single metaphase with more than 10 breaks/cell raises the suspicion of FA. In the examples shown in figure 1d–f one would classify the sample in figure 1d as a high-grade mosaic with predominance of MMC-resistant, i.e. reverted cells. The sample shown in figure 1f would qualify as a low-grade mosaic with a predominance of MMC-sensitive, i.e. non-reverted cells. The exclusive presence of reverted cells in a peripheral blood sample obscures the underlying disease genotype such that study of fibroblasts is required in order to arrive at the correct diagnosis (see below). However, because of the remarkable longevity of T-lymphocyte subsets, complete lack of MMC-sensitive cells is only rarely observed.

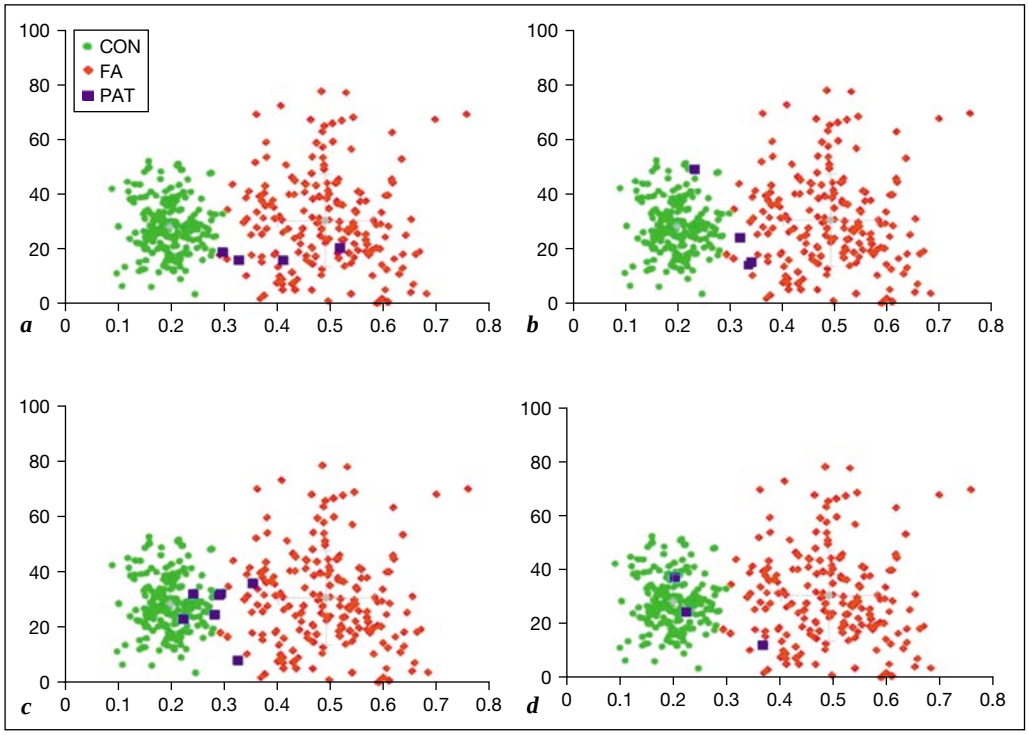


Fig. 2. Flowcytometric monitoring of 72-h peripheral blood mononuclear cell culture samples obtained from possible and proven mosaic FA patients. Horizontal axis: sum of G2/GF ratios as a measure of G2-phase arrest; vertical axis: G0/G1 cell fraction (%) as a measure of mitogen response [24]. Green symbols denote proven non-FA samples; red symbols denote proven FA-positive cases; blue square symbols denote repeat measurement over time of four different (***a***–***d***) mosaic cases. Years of the respective measurements are as follows (from right to left): (***a***) 2000, 2001, 2005, 2004; (***b***) 2005, 2004, 1998, 1997; (***c***) 5/2004, 2001, 1999, 1998, 2002, 2003, 1/2004; (***d***) 2004, 4/2005, 10/2005.

Cell Cycle Testing for the Recognition and Monitoring of Revertant Mosaicism

In order to see whether and how the two populations of MMC-sensitive and MMC-resistant cells evolve over time, mosaic patients should be monitored by repeated chromosome breakage or cell cycle studies. Examples of such long-term monitoring by way of cell cycle analysis are shown in figure 2. In this figure, the patient samples are marked by blue squares that are plotted against a background of laboratory cohorts comprising non-FA (green dots) and FA (red diamonds) samples. Using standardized procedures for bivariate cell cycle

analysis of 72-h peripheral blood mononuclear cell cultures [24] FA-negative samples display a sum of G2/GF ratios below 0.3 while FA-positive samples have a sum of G2/GF ratios higher than 0.3, reflecting their FA-typical G2-phase arrest (see contribution by Schindler et al.). As shown in figure 2a, mosaicism usually develops slowly over a time course of several years, as evidenced by the gradual shift of the sum of G2/GF parameter from above to below the 0.3 threshold. In the example shown in figure 2b, the first measurement in 1997 suggested nearly complete mosaicism, but subsequent measurement in 2004 and 2005 provided evidence for a persisting mixture of FA-positive and FA-negative cells. Such fluctuations over time are also documented in figure 2c, where the measurement 1/2004 yielded a majority of FA-negative, and the repeat measurement only 4 months later (5/2004) a majority of FA-positive cells.

The example illustrated in figure 2d implies progression of low-grade mosaicism to complete mosaicism within a time interval of little over a single year, with evidence for complete replacement of the original FA cells by reverted cells. Unless such a patient has been followed with repeat investigations over time, he or she can no longer be recognized as FA-positive by a one time chromosome breakage or cell cycle study. If in such instances the laboratory study yields a normal result but the possibility of FA persists on clinical grounds, examination of DEB- or MMC-sensitivity of patient fibroblast cultures is the only way to arrive at a correct diagnosis [25].

Skin Fibroblast Testing in Cases Discrepant for Clinical and Blood Findings

In order to resolve the discrepancy between a normal blood cell based chromosome breakage or cell cycle test and the persisting clinical suspicion of FA, a skin fibroblast culture needs to be established and tested. Because of the known oxygen sensitivity of FA fibroblasts, such cultures should ideally be propagated under hypoxic (i.e. 5% v/v oxygen) cell culture conditions [26, 27]. Figure 3 shows an example of MMC-sensitivity testing of a fibroblast culture derived from a patient with a completely normal cell cycle study of his peripheral blood cells. Daily cell counts reveal that patient fibroblasts grow normally in the absence of MMC, but fail to proliferate in the presence of the drug. Control fibroblasts from a non-FA donor also show lower cell counts after exposure to MMC, but this reduction is far less pronounced than in the FA positive sample. Using this simple test will assure the correct diagnosis despite the presence of 100% reverted peripheral blood lymphocytes. Similar results can of course be obtained by chromosome breakage testing or cell cycle studies of

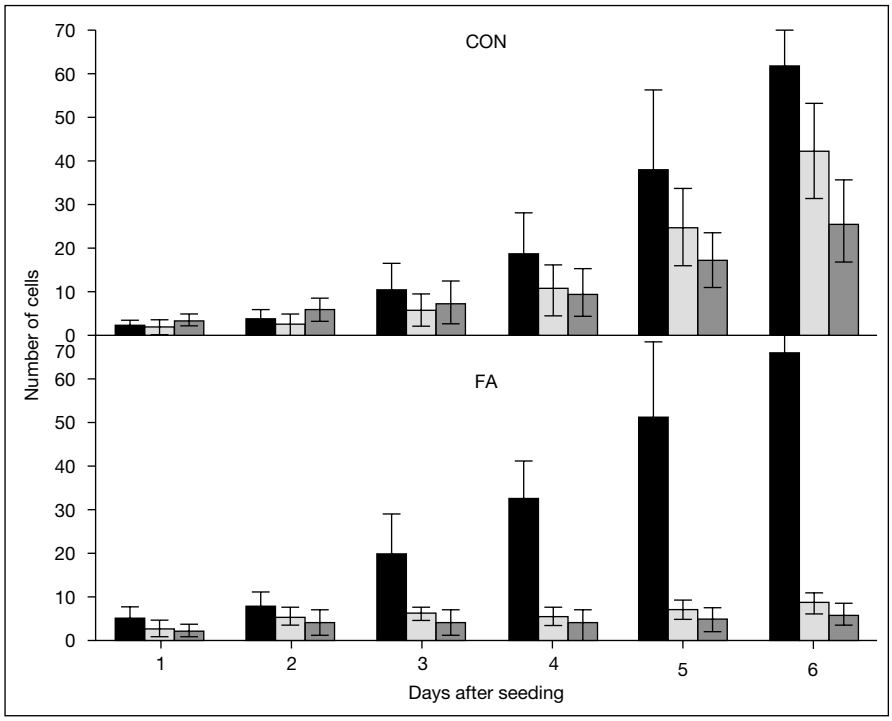


Fig. 3. Fibroblast growth assay without (black bars) and in the presence of 1μg/ml (light grey bars) or 3 μg/ml mitomcyin C (dark grey bars). Upper panel: control culture from a non-FA individual; lower panel: fibroblast culture derived from an FA patient. Bars indicate means and SD of daily triplicate cell counts.

patient derived fibroblast cultures, but simple cell counts such as shown in figure 3 are the easiest way to confirm or exclude an FA cellular phenotype.

Molecular Confirmation of Revertant Mosaicism in T- and B-Lymphocytes

Peripheral blood mononuclear cells (consisting mainly of subclasses of T- and B-lymphocytes) can be easily obtained by venipuncture. These cells are the most convenient and accessible source of cells for diagnostic and research purposes. Since bone marrow failure of FA normally progresses in the order of thrombopenia, erythropenia, leukopenia and lymphopenia, the lymphocytic cell compartment is relatively well preserved until the final stages of pancytopenia.

In most of the cases of revertant mosaicism reported in the literature, molecular confirmation of the somatic reversion event has been achieved by comparison of the respective types of mutations found in lymphocyte and fibroblast DNA of a given patient. This assumes that fibroblast-derived DNA reflects the constitutional pattern of mutations, whereas lymphocyte-derived DNA may have been altered by somatic mutations. So far there is no evidence that would contradict the assumption of relative stability of the fibroblast as opposed to the clonally expanding lymphocyte cell pool, notwithstanding the fact that in certain disease situations there can be clonal expansion of skin cell populations [3]. Molecular confirmation of revertant mosaicism has so far been documented mostly for the *FANCA* and *FANCC* genes (cf. table 1) which prompts us to illustrate the molecular confirmation of revertant mosaicism in two mosaic patients belonging to complementation group FA-D2 [28].

Examples of Somatic Reversions Involving FANCD2

Figure 4 shows the result of DNA sequencing of part of exon 29 of *FANCD2* in a patient whose EBV transformed lymphoid cell line proved resistant to MMC (patient FAD2-14 in table 1). One of the disease causing mutations of the compound heterozygous patient was a substitution of two cytidine by two thymidine residues within exon 29 (c.2775_2776CC>TT). Figure 4a and 4b shows this constitutional alteration both by gDNA and cDNA sequencing of fibroblast-derived DNA and RNA. As illustrated in figure 4c and 4d, the double base substitution present in fibroblasts had disappeared in DNA prepared from the patient's MMC-resistant lymphoblasts. The reemergence of exon 29 wildtype sequence at position c.2776 explains the phenotypic reversion of the patient's lymphoid cells to MMC-resistance. Both back mutation and gene conversion qualify as potential molecular mechanisms of reversion in this patient (see below).

A second example of a somatic reversion in *FANCD2* is illustrated in figure 5. This example involves a splice mutation which is the most common type of genetic alterations observed in FA-D2 patients [28]. The disease causing mutation in this patient is a homozygous T>G base change within intron 21 (panel 5a). This alteration affects splicing and results in skipping of exon 22 as shown in panel 5b. Genomic DNA derived from the patient's MMC-resistant lymphoid cell line (panel 5c) revealed the constitutional mutation within intron 21, but an additional base change (1954G>A) within exon 22 (panel 5c). This obviously somatic alteration within exon 22 resulted in the reappearance of exon 22 sequence at the cDNA level (panel 5d), indicating restoration of regular splicing of the respective allele transcript. The newly emerged 1954G>A within exon 22 therefore qualifies as a second site compensatory mutation that explains the conversion to MMC resistance of the patient's lymphoid cells.

Table 1. Molecular analysis of revertant mosaicism in FA patients reported until the end of year 2006, including cases from our own laboratories. Except for cases with mitotic crossover the allele targeted by a reversion event is listed as allele 2.

Patient code	*FANC* Genes	Allele 1	Allele 2 (target)	Reversion[a]	Probable mechanism[a]	Revertant cells[a]	Cellular phenotype[a]	Ref.[a]
URD	A	3976C>T	856C>T	856C (WT)	back mutation	PBLs, LCL	mild pancytopenia	[8]
STT	A	intragenic deletion	862G>T	862G (WT)	back mutation	PBLs, LCL	mild pancytopenia	[8]
MRB	A	IVS9-1G>T	971T>G	971T (WT)	back mutation	PBLs, LCL	mild pancytopenia	[8]
EUFA704	A	1615delG	1615delG	1637delA/ 1641delT	comp. mutation	PBLs, LCL	NR	[40]
FA67	A	3720-3724del	2546delC	2546C>T	comp. mutation	granulocytes, mononuclear phagocytes, LCL	mild pancytopenia	[18]
FA98	A	3720-3724del	2546delC	2546C>T+ 3720-3724del	comp. mutation	PBLs	progressive pancytopenia	[18]
NR	A	790C>T	2585delCT	2585CT	gene conversion	PBLs, LCL	normal CBC	[19]
PD839	A	2555ΔT	2670G>A	2927G>A	comp. mutation	PBLs, LCL	normal CBC	[9]
PD845	A	2555ΔT	2670G>A	2927G>A	comp. mutation	PBLs, LCL	normal CBC	[9]
IFAR557-2	A	genomic deletion at 3′ end of gene	2815-2816 ins19	2815-2816ins 19 (WT)	back mutation	PBLs, LCL stem cells	mild pancytopenia progression/ clonal evolution	[6]
EUFA393	A	3559insG	3559insG	3580insCG CTG or 3576 insGCTGC	comp. mutation	PBLs and LCL	NR	[40]

EUFA173	A	Del exon 17-31	2852G>A	2852G (WT)	back mutation	PBLs and LCL, respectively	mild pancytopenia	[8]
EUFA806	C	67delG	1806insA	heterozygote segregants	mitotic crossover	PBLs, LCL	normal CBC	[1]
EUFA449	C	L554P	67delG	67delG (WT)	gene conversion	PBLs, LCL	mild pancytopenia	[1]
RNT	C	67delG	IVS11-2A>G	heterozygote segregants	mitotic crossover	PBLs, LCL	progressive pancytopenia	[8]
EUFA506	C	1749T>G	1749T>G	1748C>T	comp. mutation	PBLs, LCL	NR	[40]
FA-AML1	D1	8415G>T	8732C>A	8731T>G	comp. mutation	Leukemic cells	resistance to MMC	[41]
FAD2-3	D2	1948-16T>G	1948-16T>G	c.1954G>A	comp. mutation	PBL, LCL, bone marrow	resistance to MMC	[28]
FAD2-14	D2	1948-6C>A	2775_2776 CC>TT	2776CC (WT)	back mutation/ conversion	PBL, LCL	resistance to MMC	[28]
FAD2-15	D2	1948-6C>A	2775_2776 CC>TT	(heterozygote segregants?)	(mitotic crossover?)	PBL	resistance to MMC	[28]
FAD2-26	D2	3803G>A	376A>G	c.376A (WT)	back mutation	PBL, LCL	resistance to MMC	[28]
FAL-1	L	891C>G	483delATCAC	c.472-2A>G	comp. mutation	PBL, LCL, BM	resistance to MMC	[28]
EUFA 1341 (R)	N	exon 4 deletion	exon 4 stop codon mutation	in-frame fusion of exons 3 and 5; elimination of exon 4	comp. mutation (Alu-mediated recombination)	LCL	resistance to MMC	[34]

[a]BM = bone marrow cells; CBC = comprehensive blood counts; Comp. mutation = compensatory or second site mutation; LCL = Lymphoblastoid cell line; MMC = mitomycin C; NR = not reported; PBL = peripheral blood lymphocytes; Ref. = reference; WT = wild type. Tentative interpretations are shown in parentheses.

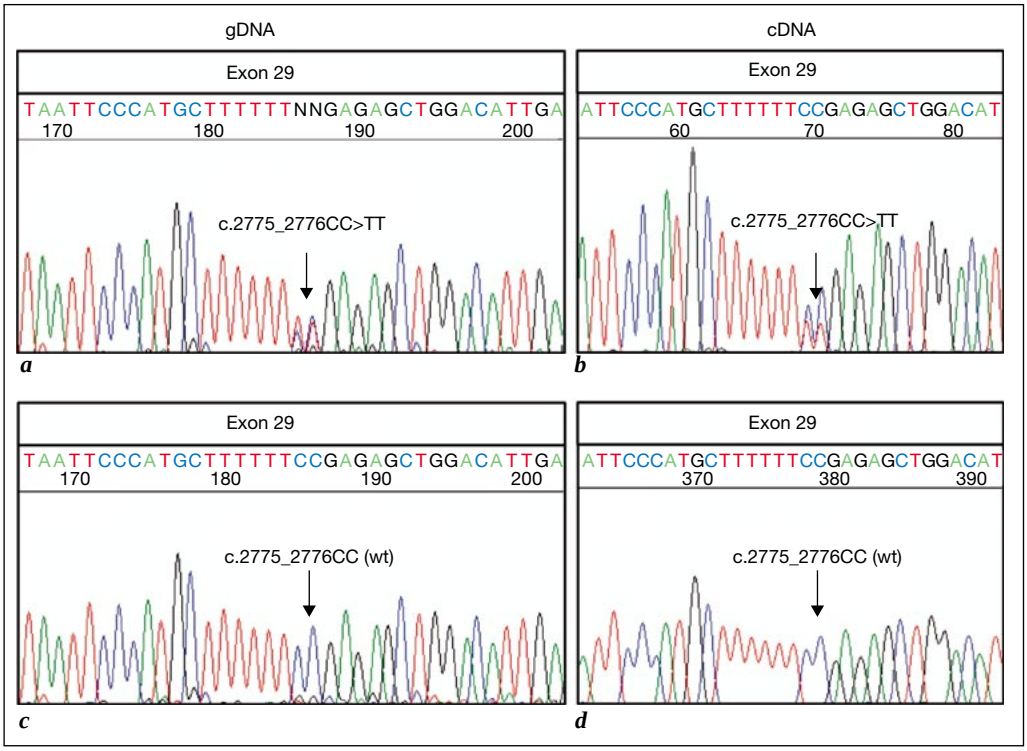


Fig. 4. Partial sequence of exon 29 of *FANCD2* at both gDNA and cDNA levels. Panels ***a*** and ***b*** show the sequence obtained from fibroblasts (constitutional mutations). Panels ***c*** and ***d*** depict the sequence obtained from lymphoid cells that had reverted to MMC-resistance.

Molecular Confirmation of Somatic Reversion in Hematopoietic Precursor Cells

Significant clinical improvement due to revertant mosaicism can only be expected if (1) the reversion event has taken place in a hematopoietic stem or early precursor cell, and if (2) the reversion event conveys a proliferative and functional advantage to the progeny of the reverted cell. A number of recent studies have successfully looked for somatic mutations in blood cell lineages other than peripheral blood T- and B-lymphocytes [6, 9, 18]. These studies have demonstrated multilineage somatic reversions of constitutional FA mutations that are consistent with a reversion event during early blood cell differentiation, or even at the stem cell level. A particularly instructive example is the observation by Mankad et al. [9] who detected, in lymphocyte DNA of twin sisters, an

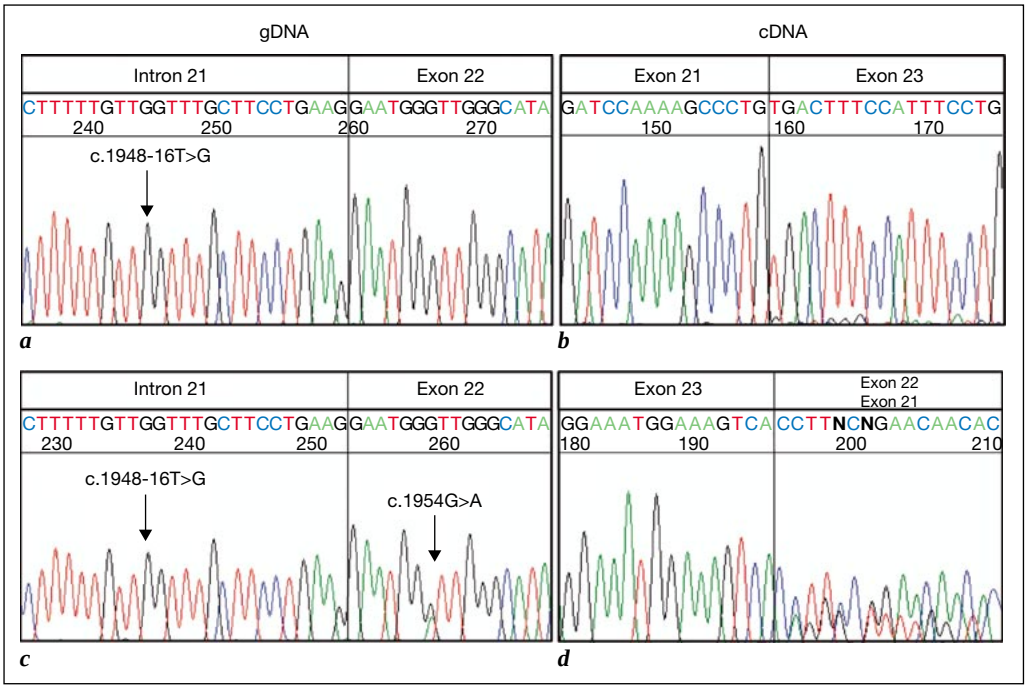


Fig. 5. Partial sequence of *FANCD2* extending from intron 21 through exon 23 illustrating heterozygous restoration of exon 22 sequence in patient lymphocytes. The best sequencing result showing reappearance of exon 22 sequence was obtained by use of reverse primers, which explains the reverse sequence orientation in panel ***d***.

identical compensatory missense mutation in exon 30 of *FANCA* that corrected a constitutional missense mutation in exon 28, restoring nuclear localization and function of the resulting protein. Both twin sisters had normal blood cell counts since birth, suggesting that the somatic reversion event may have occurred prenatally in a hematopoietic stem cell in one of the twins. The other twin may have acquired the corrected stem cell progeny via common intrauterine blood circulation, and the lifelong persistence of reverted cells supports the idea of a hematopoietic stem cell reversion [9].

Examples of Multilineage Somatic Reversions in FA-A Patients

In order to emphasize that multilineage somatic reversion is necessary for a sustained improvement of the hematological situation of mosaic patients, we review two examples in which in addition to DNA derived from lymphoblasts and fibroblasts, DNA from other cell lineages was analyzed as well. The first patient (patient EUFA173 in table 1) has been previously described by Joenje

et al. [29] and Gross et al. [8]. He manifested with severe pancytopenia at age 15. His maternal *FANCA* allele carries the base change 2852G>A and his paternal allele shows a large genomic deletion involving exons 17–31 [29]. As shown in figure 6a–c, the patient's blood counts unexpectedly improved over a time course of 3 to 8 years such that his hematological status was close to normal at age 22. The obvious improvement of more than a single cell lineage suggested a reversion event at the level of a hematopoietic precursor if not stem cell. Sequencing at age 26 revealed almost complete reversion to wildtype of the 2852G>A mutation in granulocyte DNA and in DNA from a newly established B-lymphoblast cell line, whereas a mixture of mutated and wildtype sequence was observed in the T-lymphocyte derived sample (fig. 6d–f). The persistence of a fraction of non-reverted cells in the T-lymphocyte sample may reflect mitogenic activation of long-lasting, quiescent subsets of T cells that were already present in vivo before the reversion took place [30]. Because of the remarkable longevity of certain T cell types, one may encounter traces of non-reverted DNA sequence in T-lymphocyte samples even decades after the initial reversion event has taken place in a lymphocyte precursor cell. Since the region corresponding to the 2852G>A alteration is deleted on the other allele and thus cannot serve as template for a gene conversion event, back mutation is the most likely explanation for the reversion to wildtype.

The second example of multilineage reversion involves a patient who developed severe thrombocytopenia and anemia at age 6 years but experienced unexpected improvements of his blood counts shortly thereafter (patient MRB in table 1). Using retroviral complementation analysis [31] the patient was assigned to complementation group FA-A and mutation analysis of fibroblast DNA revealed a maternally transmitted base change (971T>G) and a splice mutation (IVS9-1G>T) on the paternal allele. As illustrated in figure 7a–c, introduction of an empty vector containing only *EGFP* failed to resolve the G2 phase accumulation of the patient's cells, while transfection with the vector containing the wildtype *FANCA* sequence resulted in reduction of the G2 phase arrest, proving complementation. The patient's hemoglobin and thrombocyte counts started to improve at around three years after he had been diagnosed with FA such that androgen treatment could be reduced to a low maintenance level (cf. fig. 7g, h). Sequencing at that time revealed that the maternal mutation had all but disappeared in whole blood cell DNA (fig. 7e) while it was of course present in fibroblast DNA (fig. 7d). In order to prove multilineage reversion, leukocytes were separated into monocytes, T and B lymphocytes, and individual white cell (CFU-GM) and red cell (BFU-E) colonies were isolated using a standard bone marrow progenitor cell assay. A representative result of this study is shown in figure 7f which proves complete reversion to wildtype of the maternal 971T>G change in DNA derived from BFU-E colonies. A similar result

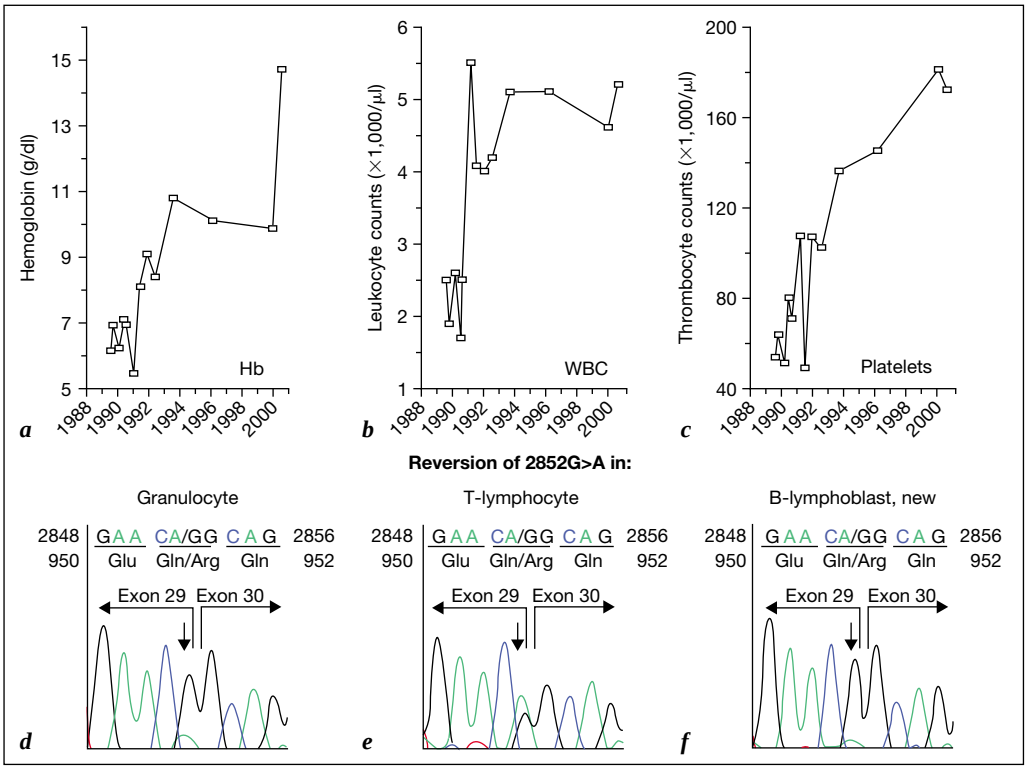


Fig. 6. Evidence for phenotypic (panels ***a***–***c***) and genotypic (panels ***d***–***f***) reversion of the constitutional mutation 2852G>A in patient EUFA173 (cf. table 1). The horizontal axis in panels ***a***–***c*** denotes the years 1988–2000 during which time the recovery of blood counts took place. Panels ***d***–***f*** illustrate *FANCA* sequence stretches containing the 2852G>A change.

was obtained for the other cell lineages tested, assigning the reversion event to at least an early hematopoietic precursor, if not to a stem cell.

Which FA Genes are Frequent Targets of Somatic Reversions?

Table 1 provides a summary of somatic reversions reported in the literature up to 2006, including cases from our own laboratories [28]. Of the total 23 reversions that have been analyzed at the molecular level, twelve were found in *FANCA*, four each in *FANCC* and *FANCD2*, and a single case each in *FANCD1/BRCA2*, *FANCL* and *FANCN*. The preponderance of *FANCA* might be explained by the fact that in most populations *FANCA* is the most frequently affected FA

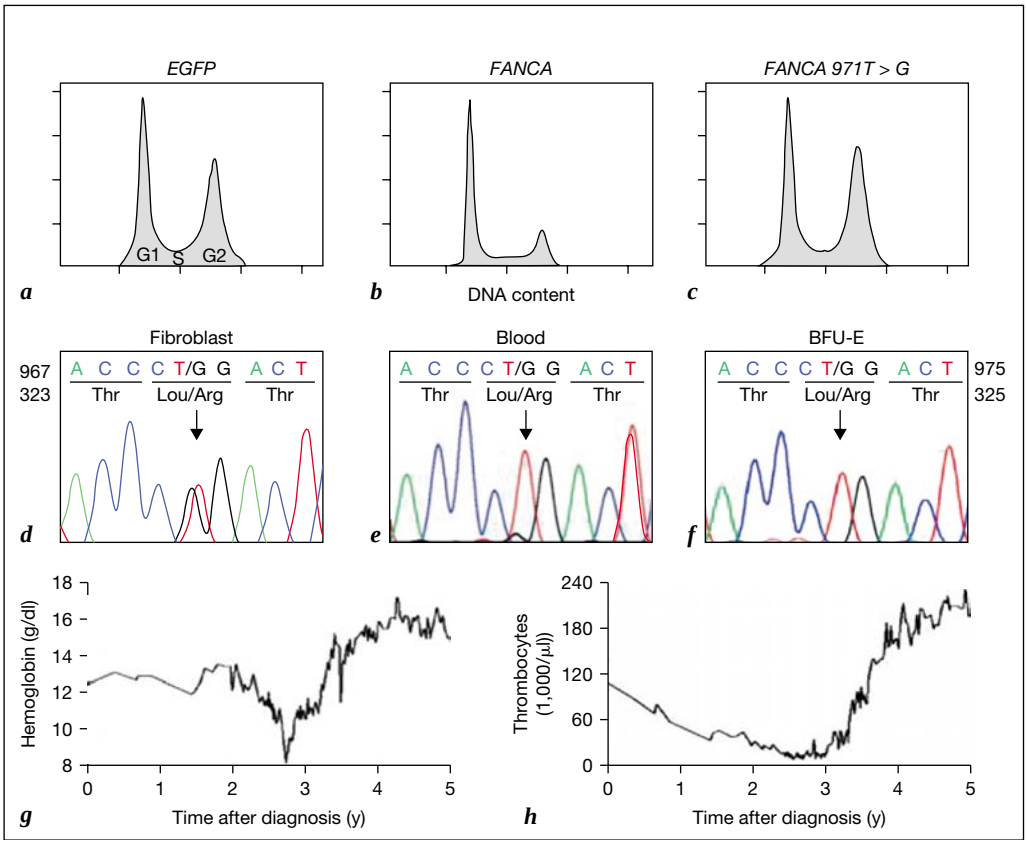


Fig. 7. Assignment of patient MRB to complementation group FA-A via retroviral complementation (panels ***a***–***c***). Panels ***d***–***f***: evidence for multilineage reversion (***e*** and ***f***) of the constitutional base substitution 971T>G present in fibroblast-derived DNA (***d***). Improvement of blood counts was noted at 3 years after the patient had been diagnosed with FA (panels ***g*** and ***h***).

gene. In addition, *FANCA* harbors a number of repetitive sequence elements that may facilitate reversion, for example by polymerase slippage [32]. Taking into account that Soulier et al. [33] reported additional 5 FA-D2 patients (not listed in table 1 due to lack of molecular confirmation) with evidence for revertant mosaicism, revertant mosaicism may also be a relatively frequent event in subtype FA-D2. Most recently, a somatic reversion event has been reported in the lymphoid cell line derived from a patient belonging to complementation group FA-N [34], adding this subtype to the FA genes that potentially exhibit somatic reversions.

Molecular Mechanisms of Somatic Reversion

As listed in table 1, there are four principal mechanisms that have been implicated in revertant mosaicism in FA: two of these involve mitotic recombination (mitotic crossover and gene conversion). The other two mechanisms are back mutation and compensatory or second site mutation. Different molecular types of reversion can give rise to compensatory mutations (see below). The mechanisms leading to revertant mosaicism have first been explored in a number of diseases other than FA, as described in the cited reviews [3–5]. A particularly instructive disease with respect to the molecular mechanisms of revertant mosaicism is the skin disease epidermolysis bullosa where areas of reverted skin can be easily recognized and probed as to their molecular constitution. The group of Professor Jonkman at the University of Groningen has published a number of key articles on this topic, including a recent report in which two patients are presented who each display multiple reversion events affecting different body parts [35]. In these two patients, the mechanisms underlying somatic reversion were studied side by side within the same individual. They were found to include back mutation, gene conversion, and second site (compensatory) mutations. Even though damaged skin and skin renewal in epidermolysis bullosa represents a very special situation, these impressive 'experiments of nature' suggest that, by analogy, multiple reversion events within a given bone marrow and possibly other cellular compartments of FA patients might occur. What we presently know about revertant mosaicism in FA may therefore represent only the literal tip of the iceberg. We strongly encourage the interested reader to study the instructive examples of revertant mosaicism presented by the Jonkman group, but limit ourselves to briefly review the molecular mechanisms of reversion that have been described in FA patients. Figure 8 summarizes the various proposed mechanisms in a simplified form.

Intragenic crossover is a conservative mechanism of mitotic recombination that was first shown in a mosaic FA-C patient by Lo ten Foe et al. [1]. By comparing the haplotypes of polymorphic markers flanking the *FANCC* gene on chromosome 9 between MMC-sensitive fibroblasts and MMC-resistant lymphoblasts, Lo ten Foe et al. provided evidence for haplotype switching in the lymphoblast-derived DNA sample, suggesting a mitotic recombination event as explanation for the phenotypic reversion to MMC-resistance. Indirect evidence for intragenic recombination was also provided by Gross et al. [8] who demonstrated the simultaneous presence, in their reverted lymphoid cell culture, of wildtype alleles and alleles carrying either a single or both mutations which would be the expected result after segregation of mitotic crossover recombinants. The simultaneous presence of these three allele types suggests an in vitro rather than in vivo reversion event. Both patients were compound heterozygotes,

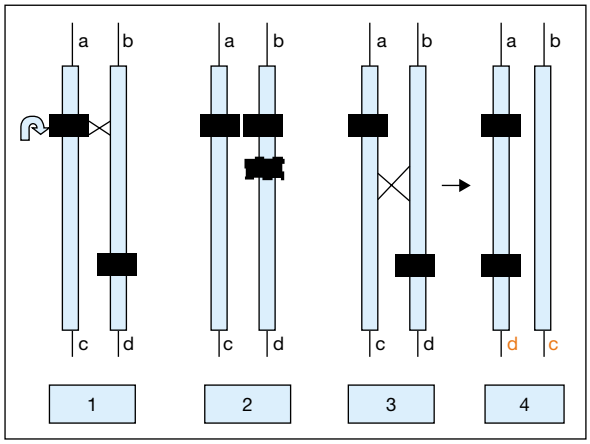


Fig. 8. Molecular mechanisms leading to somatic reversion in FA. Simplified scheme depicting pre-replication chromosome pairs carrying either compound heterozygous (1, 3) or homozygous (2) biallelic mutations (black boxes). (1) Either back mutation or gene conversion reverts one of the two alleles to wildtype. There is no evidence for strand exchange, and the original wildtype sequence is restored. (2) Compensatory second site mutation leading to restoration of the open reading frame upstream or downstream of a constitutional mutation. (3 and 4) Intragenic mitotic crossing over converts compound heterozygous biallelic mutations to (3) functional heterozygosity with the resulting alleles (4) carrying either two or none of the original mutations. Analysis of flanking markers (marked in red) provides proof of strand switching between homologous chromosomes (3).

and the mitotic recombination events might have been facilitated by the mutually distant locations of the respective paternal and maternal mutations.

Gene conversion is another conservative mechanism of mitotic recombination without, however, mutual strand exchange. Proof of such locus-restricted recombinational events requires the analysis of polymorphic markers flanking the mutation which retain their parent-of-origin specific patterns. An intact homologous copy of the mutated region is required in order to serve as template for the conversion of the mutated to wildtype sequence. Gene conversion can only function in compound heterozygous patients, and it does not function if the homologous gene region on the other allele is deleted. Gene conversion as a probable mechanism of revertant mosaicism was first suggested by Lo ten Foe et al. [1]. These authors observed two mosaic siblings harboring segregant alleles with only single mutations, but without evidence for haplotype switching. Alter et al. [19] also suggested gene conversion as mechanism for revertant mosaicism in their compound heterozygous patient even though there was no study of polymorphic markers within or flanking the presumptive site of gene

conversion. Given the relatively wide spacing of polymorphic markers, and given the presumptive shortness of conversion tracts in mammals (less than 58 bp; [36]), gene conversion as a causal mechanism of revertant mosaicism is still difficult to prove. An additional problem is the requirement for somatic pairing of homologous chromosomes which may be mediated, at least in the case of *FANCA*, by a large heterochromatin block on chromosome 16 [37], but which otherwise has been rarely observed in mitotic cells.

Back mutation is another possible mechanism underlying revertant mosaicism that, again, is difficult to prove since there is no change of polymorphic markers surrounding the back-mutated gene locus. Back mutation has been invoked to explain mosaicism for blood cells with either high and low SCE phenotypes in two patients carrying homozygous mutations in the Bloom syndrome gene [38]. In compound heterozygous FA-A patients, back mutation has been assumed as probable mechanism of reversion since (1) the homologous gene region opposite to the reverted mutation was deleted in some of these patients, and (2) the reverted allele displayed the original wildtype rather than any random sequence [8]. With two exceptions, all presumptive cases of back mutation listed in table 1 were observed in *FANCA*, a gene with a high number of small and large repetitive elements such that slipped-strand mispairing has been assumed as major mechanism of mutagenesis in this gene [32]. The first case of revertant mosaicism in an FA-A patient ascribed to back mutation was reported by Gregory et al. [6] who observed the reversion to wildtype of a maternally inherited 19-bp insertion within exon 29 of *FANCA*. The authors argued that the insertion creates a tandem repeat sequence in close proximity to a deletion hot spot consensus sequence with the possible consequence of formation of a loop structure. If this loop is deleted prior to a next round of replication, the wildtype sequence would be restored. A very similar line of arguments was followed by Gross et al. [8] who observed that the reversion in three of their mosaic FA-A patients affected the region of exons 10–11 of *FANCA* which is known as a highly mutable region due to an abundance of repetitive elements and sequence motifs, including palindromic sequences. Such structures are known to be involved in the breakage and rejoining of DNA. Gross et al. [8] therefore assumed that back mutation (via mechanisms such as slipped-strand mispairing and others) would be the most likely explanation for the fact that one of the mutated single base pair alleles in all of their FA-A patients had reverted precisely to wildtype. They further argued that random base alterations would have yielded mostly non-conservative amino acid exchanges such that the resulting protein may have been functionally impaired. As suggested by experiments in mice [39] selection would favor cells with reversions to the original nucleotide since only these would assure full restoration of protein function. Back mutation combined with selection for the

restored wildtype allele was therefore considered as most likely explanation for the surprisingly uniform pattern of reversion observed in these patients [8].

Compensatory or *second site mutations in cis* as first observed by Waisfisz et al. [40] turn out to be the most frequent type of mechanism underlying revertant mosaicism in FA (cf. table 1). Even though this type of reversion may not result in complete restoration of protein function, it is the prevailing mechanism that leads to at least partial functional rescue in the case of homozygous mutations. The original report by Waisfisz et al. [40] documents such rescue in the case of homozygous microdeletions, microinsertions and missense mutations in two FA-A siblings and an FA-C patient. A frameshift mutation (1615delG) in *FANCA* was compensated by two downstream single-base pair deletions, another frameshift mutation (3559insG) was compensated by a downstream five base pair insertion, and a homozygous missense mutation in *FANCC* (1749T>G) was altered by a base change in the preceding position. In all these cases the predicted proteins were different from wildtype, but their cDNAs were shown to complement the typical MMC sensitivity of mutant FA cells, explaining the phenotypic reversion of the patient's peripheral blood cells. The landmark study by Waisfisz et al. [40] makes it clear that compensating second site mutations can lead to partial or complete mRNA rescue without the necessity for elimination of the constitutional mutation. Even though the resultant protein may differ from wildtype, its function is likely to be sufficient for phenotypic reversion.

As already mentioned above, Mankad et al. [9] observed a most impressive example of a compensatory second site mutation in the *FANCA* gene that must have taken place early in embryogenesis since the FA twin siblings were both found to carry the compensatory change.

Compensatory mutations may not only be found upstream or downstream of the constitutional mutation, but may also affect the mutation itself [3]. Such an example was documented by Hamanoue et al. [18] who found the constitutional *FANCA* frameshift mutation 2546delC reverted to 2546C>T, resulting in expression of a functional missense protein. Compensatory second site mutations have also been observed in FA-D1 and FA-D2 patients (cf. table 1). In the case of the FA-D1 patient, a two-year-old boy affected by AML, the compensatory change emerged in a patient derived leukemic cell line [41]. The child had biallelic mutations in *FANCD1/BRCA2*. In the patient's leukemic cells the 8732C>A nonsense mutation was changed into the missense alteration 8731T>G, leading to restoration of the open reading frame and explaining the MMC-resistance of the leukemic cells [41]. An example of a compensatory second site mutation in an FA-D2 patient has been illustrated in figure 5. In this patient, a homozygous mutation in intron 21 of *FANCD2* caused skipping of exon 22 which was restored by a second site compensatory mutation [28]. Most

recently, the newly discovered *FANCN* gene has been added to the list of genes that exhibit somatic reversions, at least in cultured lymphoid cells [34]. The reversion in *FANCN* was shown to result from *Alu*-mediated recombination leading to an in-frame fusion of exons 3 and 5, thereby eliminating one of the disease causing mutations located in exon 4. *Alu*-mediated recombination can therefore be added to the list of mechanisms instrumental in compensatory mutations [34].

Clinical Consequences of Revertant Mosaicism

Improvement of the hematological situation of a given patient can be expected in cases of multilineage reversions, meaning that the reversion event has to take place in an early hematopoietic precursor cell, ideally in a hematopoietic stem cell. We have documented two such cases in detail (cf. figs. 6 and 7), and a number of other examples are listed in table 1. In fact, the majority of mosaic patients summarized in table 1 showed improvement of their hematological status, mostly characterized as mild or non-progressive pancytopenia. However, with few exceptions [6, 9, 18] the hematological consequences of revertant mosaicism have not been fully documented over time, such that definite statements concerning the clinical benefits of revertant mosaicism must await the results of prospective studies. The patients documented in figures 6 and 7 in whom multilineage reversion has been firmly established, and the case reports by Hamanoue et al. [18] and Mankad et al. [9] leave little doubt about a positive, long lasting effect on hematopoiesis if the reversion event has taken place in an early progenitor or stem cell. An overall beneficial effect of revertant mosaicism has also been documented by Soulier et al. [33] who compared the hematological status of 8 mosaic with that of 45 non-mosaic patients. As shown in figure 9, blood counts were on average higher in mosaic patients. P values comparing mosaic vs. non-mosaic patients were 0.00017 for white blood counts, 0.0001 for neutrophils, 0.001 for hemoglobin, and likewise 0.001 for platelets, whereas differences in MCV were not significant.

Whereas lack of hematological improvement is not surprising in cases where the reversion is limited to a precursor cell of the lymphoid cell lineage (e.g. patient RNT in table 1; [8]), the patient reported by Gregory et al. [6] demonstrates that despite proven multilineage reversion a clonal cytogenetic change may arise in the remaining fraction of non-revertant bone marrow cells such that the possibility of malignant cell growth cannot be entirely excluded in mosaic patients. A similar observation was made by Manoharan [42] whose patient developed clonal trisomy 1q despite longstanding and apparently

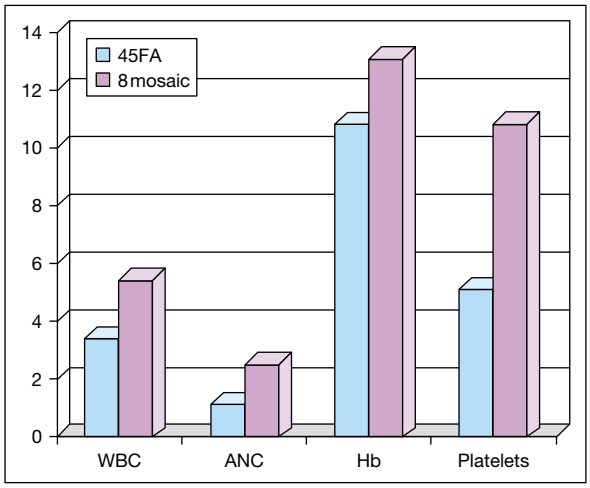


Fig. 9. Comparison of blood cell counts between mosaic and non-mosaic FA patients. Data plotted from table 3 of Soulier et al. [33]. ANC = Absolute neutrophil count; Hb = hemoglobin; WBC = white blood cell count; vertical axis in arbitrary units. See text for details.

multilineage revertant mosaicism [16]. In addition, our laboratory has observed at least two patients in whom the fraction of FA-negative (i.e. reverted) peripheral blood mononuclear cells has not, as expected, increased but decreased over time. It will be important to follow these cases closely in order to answer the question whether these are random fluctuations due to clonal expansion and clonal attenuation of lymphocytic cells, for example in response to infections, or whether these changes may have clinical significance. At this point we simply do not know whether reverted cell clones may in fact be limited in the number of progeny and lifespan. Assuming that reversion has taken place in a hematopoietic stem or early progenitor cell, we also do not know whether the clonal expansion of the reverted cell in a milieu of non-corrected stromal cells would in each instance be successful. Sensitivity to oxidative stress surely would remain a major obstacle for the stromal cell population [43] which may adversely impact the proliferative capacity of the reverted cell lineage. Finally, it is clear that mosaic patients need special attention during preparation for HSCT which still may be required if the reversion is limited to the lymphocytic cell lineage. Conversely, if there is proven multilineage reversion, HSCT may not be a prime therapeutic consideration. Clearly, much more has to be learned about the unique phenomenon of revertant mosaicism, its molecular mechanisms, and its clinical consequences.

References

1 Lo Ten Foe JR, Kwee ML, Rooimans MA, Oostra AB, Veerman AJ, et al: Somatic mosaicism in Fanconi anemia: molecular basis and clinical significance. Eur J Hum Genet 1997;5:137–148.
2 Rieger R, Michaelis A, Green MM: Glossary of Genetics and Cytogenetics: Classical and Molecular, ed 4. New York, Springer, 1996.
3 Jonkman MF, Castellanos Nuijts M, van Essen AJ: Natural repair mechanisms in correcting pathogenic mutations in inherited skin disorders. Clin Exp Dermatol 2003;28:625–631.
4 Youssoufian H, Pyeritz RE: Mechanisms and consequences of somatic mosaicism in humans. Nat Genet Rev 2002;3:748–758.
5 Hirschhorn R: In vivo reversion to normal of inherited mutations in humans. J Med Genet 2003;40:721–728.
6 Gregory JJ Jr, Wagner JE, Verlander PC, Levran O, Batish SD, et al: Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. Proc Natl Acad Sci USA 2001;98:2532–2537.
7 Youssoufian H: Natural gene therapy and the Darwinian legacy. Nat Genet 1996;13:255–256.
8 Gross M, Hanenberg H, Lobitz S, Friedl R, Herterich S, et al: Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. Cytogenet Genome Res 2002;98:126–135.
9 Mankad A, Taniguchi T, Cox B, Akkari Y, Rathbun RK, et al: Natural gene therapy in monozygotic twins with Fanconi anemia. Blood 2006;107:3084–3090.
10 Kwee ML, Poll EHA, Van de Kamp JJP, De Koning H, Eriksson AW, Joenje H: Unusual response to bifunctional alkylating agents in a case of Fanconi anaemia. Hum Genet 1983;64:384–387.
11 Cohen MM, Simpson SJ, Honig GR, Maurer HS, Nicklas JW, Martin AO: The identification of Fanconi anemia genotypes by clastogenic stress. Am J Hum Genet 1982;34:794–810.
12 Duckworth-Rysiecki G, Hulten M, Mann J, Taylor AM: Clinical and cytogenetic diversity in Fanconi's anemia. J Med Genet 1984;21:197–203.
13 Cervenka J, Hirsch BA: Cytogenetic differentiation of Fanconi anemia, 'idiopathic' aplastic anemia, and Fanconi anemia heterozygotes. Am J Med Genet 1983;15:211–223.
14 Auerbach AD, Rogatko A, Schroeder-Kurth TM: International Fanconi Anemia Registry: First Report; in Schroeder-Kurth TM, Auerbach AD, Obe G (eds): Fanconi Anemia, Clinical Cytogenetic and Experimental Aspects. Berlin/Heidelberg, Springer-Verlag, 1989, pp 3–17.
15 Jonkman MF, Scheffer H, Stulp RP, Pas HH, Nijenhuis M, et al: Revertant mosaicism in epidermolysis bullosa caused by mitotic gene conversion. Cell 1997;88:543–551.
16 Hopkins NC, Manoharan A, Lammi AT: Spontaneous improvement in Fanconi's anemia. Pathology 1991;23:254–255.
17 Kwee ML, van der Kleij JM, van Essen AJ, Begeer JH, Joenje H, et al: An atypical case of Fanconi anemia in elderly sibs. Am J Med Genet 1997;68:362–366.
18 Hamanoue S, Yagasaki H, Tsuruta T, Oda T, Yabe H, et al: Myeloid lineage-selective growth of revertant cells in Fanconi anaemia. Br J Haematol 2006;132:630–635.
19 Alter BP, Joenje H, Oostra AB, Pals G: Fanconi anemia: adult head and neck cancer and hematopoietic mosaicism. Arch Otolaryngol Head Neck Surg 2005;131:635–639.
20 Joenje H, Oostra AB, Zwaan CM, Pals G: Fanconi anemia; in Humana Press Series on Hereditary Cancer Syndromes. Totowa, Humana Press, 2006; in press.
21 Schroeder-Kurth TM, Auerbach AD, Obe G (eds): Fanconi Anemia, Clinical Cytogenetic and Experimental Aspects. Berlin/Heidelberg, Springer-Verlag, 1989, pp 1–378.
22 Schroeder-Kurth TM, Zhu TH, Hong Y, Westphal I: Variation in cellular sensitivities among Fanconi Anemia patients, non-Fanconi Anemia patients, their parents and siblings, and control probands; in Schroeder-Kurth TM, Auerbach AD, Obe G (eds): Fanconi Anemia, Clinical Cytogenetic and Experimental Aspects. Berlin/Heidelberg, Springer-Verlag, 1989, pp 106–136.
23 Bakhshi S, Joenje H, Schindler D, Oostra A, Mohamed AN, et al: A case report of a patient with microcephaly, facial dysmorphism, mitomycin-C-sensitive lymphocytes, and susceptibility to lymphoma. Cancer Genet Cytogenet 2006;164:168–171.
24 Schindler D, Hoehn H: Flowcytometric testing for syndromes with chromosomal instability, aplastic anemia and related haematological disorders; in Wegner RD (ed): Diagnostic Cytogenetics. Berlin/Heidelberg, Springer-Verlag, 1999, pp 269–281.

25 Joenje H, Arwert F, Kwee ML, Madan K, Hoehn H: Confounding factors in the diagnosis of Fanconi anemia. Am J Med Genet 1998;79:403–405.
26 Schindler D, Hoehn H: Fanconi anemia mutation causes cellular susceptibility to ambient oxygen. Am J Hum Genet 1988;43:429–435.
27 Kalb R, Duerr M, Wagner M, Herterich S, Gross M, et al: Lack of sensitivity of primary Fanconi's anemia fibroblasts to UV and ionizing radiation. Radiat Res 2004;161:318–325.
28 Kalb R, Neveling K, Hoehn H, Schneider H, Linka Y, et al: Hypomorphic mutations in the key Fanconi anemia gene *FANCD2* sustain a significant group of FA-D2 patients with severe phenotype. Am J Hum Genet 2007; in press.
29 Joenje H, Levitus M, Waisfisz Q, D'Andrea A, Garcia-Higuera I, et al: Complementation analysis in Fanconi anemia: assignment of the reference FA-H patient to group A. Am J Hum Genet 2000;67:759–762.
30 Globerson A, Effros RB: Ageing of lymphocytes and lymphocytes in the aged. Immunol Today 2000;10:515–521.
31 Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, et al: Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30:410–420.
32 Levran O, Diotti R, Pujara K, Batish SD, Hanenberg H, Auerbach AD: Spectrum of sequence variation in the *FANCA* gene: an International Fanconi Anemia Registry (IFAR) study. Hum Mutat 2005;25:142–149.
33 Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, et al: Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 2005;105:1329–1336.
34 Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, et al: Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet 2006;39:159–161.
35 Pasmooij AMG, Pas HH, Deviaene FCL, Nijenhuis M, Jonkman MF: Multiple correcting *COL17A1* mutations in patients with revertant mosaicism of Epidermolysis bullosa. Am J Hum Genet 2005;77:727–740.
36 Elliott B, Richardson C, Winderbaum J, Nickoloff JA, Jasin M: Gene conversion tracts from double-strand break repair in mammalian cells. Mol Cell Biol 1998;18:93–101.
37 Haaf T, Steinlein K, Schmid M: Preferential somatic pairing between homologous heterochromatic regions of human chromosomes. Am J Hum Genet 1986;38:319–329.
38 Ellis NA, Ciocci S, German J: Back mutation can produce phenotype reversion in Bloom syndrome somatic cells. Hum Genet 2001;108:167–173.
39 Battaile KP, Bateman RL, Mortimer D, Mulcahy J, Rathbun RK, et al: In vivo selection of wild-type hematopoietic stem cells in a murine model of Fanconi anemia. Blood 1999;94:2151–2158.
40 Waisfisz Q, Morgan NV, Savino M, de Winter JP, van Berkel CG, et al: Spontaneous functional correction of homozygous Fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. Nat Genet 1999;22:379–383.
41 Ikeda H, Matsushita M, Waisfisz Q, Kinoshita A, Oostra AB, et al: Genetic reversion in an acute myelogenous leukemia cell line from a Fanconi anemia patient with biallelic mutations in *BRCA2*. Cancer Res 2003;63:2688–2694.
42 Manoharan A: Fanconi anaemia. Br J Haematol 2006;135:139.
43 Cohen-Haguenauer O, Peault B, Bauche C, Daniel MT, Casal I, et al: In vivo repopulation ability of genetically corrected bone marrow cells from Fanconi anemia patients. Proc Natl Acad Sci USA 2006;103:3240–3245.

H. Hoehn
Department of Human Genetics, Biocenter
Am Hubland, 97074 Würzburg (Germany)
Tel. +49 931 888 4071, Fax +49 931 888 4434, E-Mail hoehn@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 173–182

Stem Cell Transplantation in Fanconi Anemia – Recent Advances with Alternative Donors

M. Eyrich, B. Winkler, P.G. Schlegel

Pediatric Stem Cell Transplant Program, Children's Hospital, University of Würzburg, Würzburg, Germany

Abstract

Bone marrow transplantation from an HLA-identical sibling donor is the treatment of choice for Fanconi anemia (FA) patients with bone marrow failure. However, with today's small size families less than 25% of FA patients have a matching sibling donor. The remaining patients can be treated with stem cell transplantations from alternative donors such as matched unrelated or haploidentical donors. While results with conventional bone marrow transplantation continually improved, outcome after alternative donor transplantation remained poor due to high rates of transplant-related complications, graft rejection and graft-versus-host disease (GvHD). Recently, advances with less myeloablative and more immunosuppressive conditioning regimens have demonstrated promising results. Insights into the mechanisms of immune reconstitution could show that at least in children immune recovery after haploidentical stem cell transplantation is fast and results in complete restoration of normal immune function within the first year after transplantation. Finally, innovations in the field of stem cell collection and processing have led to cellular graft compositions which now provide reliable engraftment in almost all patients with concomitant low incidence of GvHD. This review discusses these recent advances in alternative donor transplantations and concludes that this treatment option should be an early recommendation for FA patients with bone marrow failure who lack an HLA-identical sibling donor.

Although the name of the disease suggests a purely hematological disorder, Fanconi anemia (FA) is a systemic disease which is characterized by physical abnormalities, progressive bone marrow failure and increased risk of malignancies. A typical diagnostic feature, which might in part explain some of these symptoms, is increased chromosome breakage at baseline, and even more

dramatic upon exposure to clastogenic agents such as mitomycin C. Complete bone marrow failure is often not present at diagnosis. Mild to moderate thrombocytopenia or leukopenia may precede pancytopenia. However, if pancytopenia develops, 80% of patients would not survive more than two years without therapies other than blood transfusions [1]. Another threat for FA patients is the occurrence of acute leukemias which has been reported to be in the range of 10–30% [2]. Once overt leukemia has developed, patients suffer from a poor prognosis since conventional chemotherapy is difficult to implement. Therefore, it is important to treat bone marrow failure before complicating events such as aplastic anemia or leukemia can develop. Up to now, the only curative option to achieve this goal involves allogeneic bone marrow transplantation. However, it has to be kept in mind that this procedure will *not* correct the genetic background, physical abnormalities and the increased risk of solid tumors in older long-term survivors.

Early attempts to treat bone marrow failure in FA patients with allogeneic bone marrow transplantation demonstrated the feasibility of this approach, however with limited success [3]. High cyclophosphamide toxicity, severe graft-versus-host disease (GvHD), and graft rejection urged researchers to look for modified conditioning regimens and better GvHD prophylaxis. The use of low dose cyclophosphamide, limited field irradiation, plus antithymocyte globulin (ATG) for pretransplant conditioning and cyclosporine for GvHD-prophylaxis resulted in a 66% two-year survival after transplantation of bone marrow from a human leukocyte antigen (HLA-) identical sibling [4]. However, results with transplantations from alternative donors (unrelated or mismatched related) remained poor (29%) [4]. Taken together, these early reports about the use of bone marrow transplantation in FA patients suggested that this approach is feasible and may be extended to patients who lack an HLA-identical sibling donor. In order to improve results of alternative donor transplantations, less toxic conditioning regimens and novel techniques for tighter control of the balance between GvHD and graft rejection had to be developed.

Transplantation beyond HLA-Barriers

Until recently, transplantation of bone marrow from an HLA-identical sibling was considered the standard treatment regimen for children requiring a stem cell graft. Transplantation of complete bone marrow, however, has major limitations: first, the limited availability of an HLA-identical sibling donor, and second the potential risk of developing GvHD, which may attack skin, gut and liver of the transplant recipient. From 1990 on peripheral blood stem cells were increasingly used instead of bone marrow, unfortunately this did not result in a

decline of the incidence of acute or chronic GvHD, probably due to the even higher number of T cells in peripheral blood stem cell grafts. For years, it has been believed that T cells in the graft are indispensable to allow for a stable engraftment in an allogeneic environment. Only patients with an inherent lack of T cells like children with severe combined immunodeficiency were able to accept even T-cell depleted, haploidentical grafts without intensive pretransplant conditioning [5]. However, this view changed with introduction of the 'megadose concept': high numbers of $CD34^+$ stem cells without T-cell support are able to suppress anti-donor cytotoxic T lymphocyte (CTL) responses and ensure engraftment even after transplantation beyond HLA-barriers [6]. Innovations in the field of stem cell processing techniques made it possible to isolate the required large numbers of highly purified hematopoetic progenitor cells [7]. This approach was rapidly translated into clinical protocols, first in high-risk adult leukemia patients [8], later also in a pediatric cohort with childhood leukemia [9]. The first case report of a successful haploidentical transplantation in a girl with FA was published by Elhasid et al. in 2000 [10]. Interestingly, this report followed exactly the megadose concept with transplantation of large numbers of highly purified hematopoietic progenitor cells and only minimal T-cell content. Another study, published in the same year reported 29 FA patients transplanted with partially T-cell depleted bone marrow from alternative donors after receiving cyclophosphamide (40 mg/kg), ATG and total body irradiation (450–600 cGy) as conditioning regimen [11]. In this cohort, the probability of survival at 1 year was only 34%. Although a final assessment about the use of haploidentical stem cell transplantation based on these results is not possible so far, it is tempting to speculate that the megadose concept will result in superior engraftment rates with a concomitant low incidence of acute and chronic GvHD. In fact, Lang et al. reported a series of 25 patients with nonmalignant diseases (excluding FA) where transplantation of large numbers of positively enriched $CD34^+$ hematopoietic stem cells from related or unrelated donors resulted in a survival rate of 88% without acute GvHD greater than grade II and limited chronic GvHD in only 8% of the patients [12]. Future studies have to show whether these encouraging results will also apply to FA patients. If haploidentical stem cell transplantation in combination with the megadose concept proves to be sufficiently safe and effective in FA then this therapeutic option could be offered early on to all FA patients with deterioration of bone marrow function. Either parents or siblings qualify as potential haploidentical donors such that almost every patient with FA is likely to have at least one haploidentical donor. However, successful outcome of stem cell transplantation is not only based on selection of the best donor and appropriate stem cell processing techniques but also on a pretransplant conditioning regimen which has been optimized for the special needs of FA patients.

Breaking Resistance of Host T Cells – The Way to New Immunological Conditioning Regimens

The first conditioning regimens used for preparation of patients for bone marrow transplantation contained mainly total body irradiation (TBI) plus one or two alkylating agents. These regimens created 'marrow space' for the allogeneic graft by eradicating host hematopoiesis but also had a profound immunosuppressive effect. Initial reports with these conditioning regimens in FA patients showed an unacceptable high toxicity especially due to the use of cyclophosphamide and its metabolites [3]. Another problem was the high rate of graft rejection frequently seen in FA patients [13]. These data suggested that existing protocols for pretransplant conditioning were too toxic albeit not immunosuppressive enough for FA patients to allow for safe allogeneic engraftment. In 1991 Fischer et al. demonstrated that addition of a monoclonal antibody against leukocyte function-associated antigen (LFA-1, CD11a) to the conditioning regimen significantly reduced rates of graft rejection in patients with nonmalignant diseases [14]. Furthermore, it could be shown that patients with primary nonengraftment or graft rejection can be successfully retransplanted after a purely immunological reconditioning based on the anti-T-cell antibody OKT3 and methylprednisolone [15]. In 1997, the first fludarabine-based protocol with addition of low-dose cyclophosphamide and ATG was published [16]. Fludarabine monophosphate is a purine analogue and has a much more favorable toxicity profile for FA patients compared to alkylating agents or irradiation. Most of the conditioning regimens published thereafter used low-dose cyclophosphamide and ATG in combination with either limited thoracoabdominal irradiation [11] or fludarabine [17–19] or both [20–22]. All authors reported a remarkable primary engraftment rate of almost 100% with secondary graft rejections in 4–25% of patients [17, 19, 21–23]. Most of these latter patients could be successfully retransplanted. The rates of acute and chronic GvHD also decreased over the past decade. However, the incidence of GvHD seems to be more related to donor selection and the degree of T-cell depletion than to the use of TBI, cyclophosphamide, or fludarabine.

Taken together, over the last two decades considerable improvements in pretransplant conditioning regimens were achieved. The application of irradiation or alkylating agents which carry a major risk for secondary malignancies in FA patients could be dramatically reduced to the benefit of less toxic and highly T-cell suppressive regimens. These advances in patient conditioning not only resulted in higher rates of engraftment but also paved the way for the use of more sophisticated grafts like T-cell depleted stem cells from alternative donors. Recently, a study has been initiated which completely avoids irradiation and alkylating agents. Prepared by a conditioning regimen with fludarabine and

therapeutic antibodies against CD45 and CD52, FA patients in this study will be grafted with bone marrow from matched related or $CD34^+$ peripheral blood stem cells from haploidentical donors (Persis Amrolia, personal communication). This ongoing study surely forms the preliminary endpoint of a logical development, which was fostered by experiences with immunological conditioning regimens from the last 25 years.

Immune Reconstitution after Haploidentical Stem Cell Transplantation

The high expectations after the first successful stem cell transplantations from haploidentical donors were attenuated by a high rate of infectious complications, especially in adults [24]. This was due to the extremely low numbers of T cells in the graft and a subsequently delayed immune reconstitution. However, the patterns of immune recovery after transplantation of highly enriched, $CD34^+$ peripheral blood stem cells from haploidentical donors were initially only poorly understood. Our group has prospectively analyzed the patterns of immune reconstitution in children after haploidentical transplantations. In a first study, twenty children transplanted with high doses of purified $CD34^+$ hematopoietic stem cells were prospectively monitored for their immune reconstitution during the first post-transplant year [25]. T, B, and NK cells began to reconstitute (as a median) on day +72, +68, and +30 respectively (fig. 1). During extended follow-up, their numbers and proliferative capacity upon mitogen stimulation continually increased. Early reconstituting T cells were predominantly of a memory (primed, activated) phenotype. Naive T cells emerged after approximately 6 months post transplantation. All patients self-maintained sufficient immunoglobulin levels after day +200. This study demonstrated for the first time that at least pediatric recipients of highly purified, haploidentical stem cells are able to reconstitute functioning T-, B- and NK-cell compartments within the first post-transplant year.

In the subsequent follow-up study, we analyzed the factors governing the normalization of the initially restricted T-cell receptor repertoire [26]. Normalization of the initially restricted repertoire is of prime importance to the patient with regard to fighting off potentially life-threatening infections. We determined the relative contributions of naive and memory T-cell subsets within the $CD4^+$ and $CD8^+$ compartments to the evolution of overall TCR-repertoire complexity following transplantation of $CD34^+$ selected peripheral blood progenitors. During the first post-transplant year, sorted CD4/45RA, CD4/45RO, CD8/45RA and CD8/45RO subsets were analyzed at three monthly intervals for

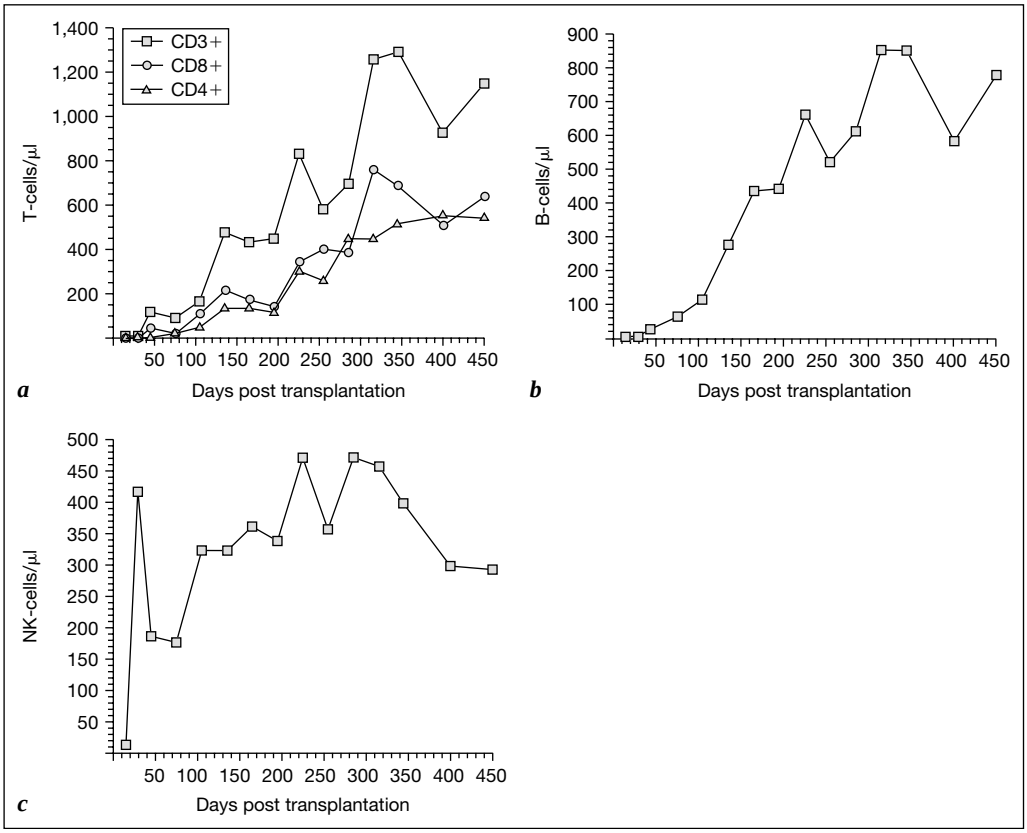


Fig. 1. Immune reconstitution after haploidentical stem cell transplantation in children [25]. (***a***) Increase in peripheral T-cell numbers ($CD3^+$, □) after haploidentical SCT (n = 20, median values). T-cell counts >100/µl were present after a median of 72 days (range 14–123). Cytotoxic, suppressor $CD8^+$ T-cells (◎) recovered faster than $CD4^+$ T-helper cells (△), resulting in an inverted CD4/CD8 ratio. For $CD4^+$ T-cells the median time to reach >100 cells/µl was 82 days (range 13–131), for $CD8^+$ T-cells the median time to reach >100 cells/µl was 74 days (range 15–123). (***b***) Increase in peripheral $CD19^+$ B-cell numbers (□) after haploidentical SCT (n = 20, median values). The median time to reach >100 $CD19^+$ cells/µl was 68 days (range 13–340). (***c***) Increase in peripheral $CD16^+/56^+$ NK-cell numbers (□) after haploidentical SCT (n = 20, median values). NK-cell values recovered more rapidly than T- and B-cell numbers and reached a median of 305 $CD16^+/56^+$ NK-cells/µl already on day 30 post-transplant.

their respective TCR-repertoire complexity by CDR3-size-spectratyping. Skewing of the repertoire was observed only in early memory-type T cells. With the help of sorting techniques, we found direct evidence that the emerging naive T cells were responsible for correcting the initially skewed TCR repertoire.

Thymus-dependent T-cell regeneration (de novo generation of naive T-cells) is a key pathway for immune reconstitution after stem cell transplantation. In a third study, we prospectively assessed T-cell dynamics and thymic function in 164 pediatric patients between 1 and 124 months post-transplant by measuring T-cell-receptor recombination excision circles (TRECs) as a measure for de novo thymic T-cell emigrants and spontaneous expression of Ki67 in peripheral T-cell subsets (as a measure for proliferation of T cells in the peripheral pool) [27]. We analyzed the impact of recipient age, conditioning regimen, type of donor and graft, stem cell dose, and graft-versus-host disease (GvHD) on the onset as well as on the plateau of thymic output. Multivariate analysis revealed that the onset of thymic recovery was inversely correlated only with recipient age ($p < 0.0002$), whereas the plateau of thymic output was higher in patients receiving increased ($>10^7$ $CD34^+$/kg BW) stem cell numbers ($p < 0.0022$). Interestingly, donor type, stem cell source and conditioning regimen influenced none of the analyzed parameters. Onset and plateau of thymic activity were found to be independently regulated by different transplant-related factors.

Taken together our data demonstrated that in children transplantation of highly purified stem cells results in fast reconstitution of NK-, T-, and B-cell compartments. These data are in contrast to results from adult patients after T-cell depleted haploidentical transplantations, whose T-cell reconstitution can take up to two years or more. They also correlate with the clinical observation that transplanted children have a lower incidence of infectious complications than adult patients. One of the major differences between adults and children with regard to T-cell reconstitution seems to be the enhanced thymic recovery in children [28]. Thus, transplantation of highly purified haploidentical stem cells should especially be considered in pediatric patients, since immune recovery in children is fast and no long-lasting immunodeficiency due to the transplant procedure has to be expected.

Future Directions

Bone marrow transplantation from an HLA-identical sibling donor is still the treatment of choice for FA patients with signs of bone marrow failure. However, recent advances in pretransplant conditioning regimens, stem cell processing techniques, and peritransplant supportive care have made transplantations from alternative donors an interesting option for FA patients lacking an HLA-identical sibling donor. To date, no randomized data exist about the preferential use of matched unrelated versus haploidentical donors, or cord blood versus peripheral blood stem cells. Several aspects point towards a beneficial

role of haploidentical stem cell transplantations in FA patients: hematologic and immune recovery are well documented and obviously fast, especially in children. Haploidentical donors are readily available for a second donation when graft rejection occurs or for immunotherapy in case of increasing mixed chimerism, which should be treated promptly in FA patients [11]. A number of innovations are currently under investigation, namely the use of conditioning regimens, which completely avoid irradiation and alkylating agents, and novel stem cell processing techniques such as CD3/CD19 depletion instead of CD34 selection which will further promote engraftment rates by cotransplantation of large numbers of graft-facilitating cells. These improvements in the field of alternative donor transplantation will hopefully ensure that each FA patient with bone marrow failure can be offered a safe and effective allogeneic stem cell transplantation before aplastic anemia, myelodysplasia or even leukemia develops.

Acknowledgements

This work was supported in part by IZKF Wuerzburg (Z-2/7) and by a grant from the Childhood Cancer Parent's Initiative Wuerzburg.

References

1 Bernard J, Mathé G, Najean Y: Contribution de l'étude clinique et physiopathologique de la maladie de Fanconi. Rev Fr Etudes Clin Biol 1958;3:599–612.
2 Schaison G, Leverger G, Yildiz C, Berger R, Bernheim A, Gluckman E: Fanconi's anemia. Incidence of its development into leukemia. Presse Med 1983;12:1269–1274.
3 Gluckman E, Devergie A, Schaison G, Bussel A, Berger R, et al: Bone marrow transplantation in Fanconi anaemia. Br J Haematol 1980;45:557–564.
4 Gluckman E, Auerbach AD, Horowitz MM, Sobocinski KA, Ash RC, et al: Bone marrow transplantation for Fanconi anemia. Blood 1995;86:2856–2862.
5 Friedrich W, Goldmann SF, Ebell W, Blutters-Sawatzki R, Gaedicke G, et al: Severe combined immunodeficiency: treatment by bone marrow transplantation in 15 infants using HLA-haploidentical donors. Eur J Pediatr 1985;144:125–130.
6 Rachamim N, Gan J, Segall H, Krauthgamer R, Marcus H, et al: Tolerance induction by 'megadose' hematopoietic transplants: donor-type human CD34 stem cells induce potent specific reduction of host anti-donor cytotoxic T lymphocyte precursors in mixed lymphocyte culture. Transplantation 1998;65:1386–1393.
7 Schumm M, Lang P, Taylor G, Kuci S, Klingebiel T, et al: Isolation of highly purified autologous and allogeneic peripheral $CD34^+$ cells using the CliniMACS device. J Hematother 1999;8:209–218.
8 Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, et al: Successful engraftment of T-cell-depleted haploidentical 'three-loci' incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood 1994;84:3948–3955.
9 Handgretinger R, Klingebiel T, Lang P, Schumm M, Neu S, et al: Megadose transplantation of purified peripheral blood CD34(+) progenitor cells from HLA-mismatched parental donors in children. Bone Marrow Transplant 2001;27:777–783.

10 Elhasid R, Ben Arush MW, Katz T, Gan Y, Shechter Y, et al: Successful haploidentical bone marrow transplantation in Fanconi anemia. Bone Marrow Transplant 2000;26:1221–1223.
11 MacMillan ML, Auerbach AD, Davies SM, Defor TE, Gillio A, et al: Haematopoietic cell transplantation in patients with Fanconi anaemia using alternate donors: results of a total body irradiation dose escalation trial. Br J Haematol 2000;109:121–129.
12 Lang P, Klingebiel T, Bader P, Greil J, Schumm M, et al: Transplantation of highly purified peripheral-blood $CD34^+$ progenitor cells from related and unrelated donors in children with nonmalignant diseases. Bone Marrow Transplant 2004;33:25–32.
13 Zwaan CM, Van Weel-Sipman MH, Fibbe WE, Oudshoorn M, Vossen JM: Unrelated donor bone marrow transplantation in Fanconi anaemia: the Leiden experience. Bone Marrow Transplant 1998;21:447–453.
14 Fischer A, Friedrich W, Fasth A, Blanche S, Le Deist F, et al: Reduction of graft failure by a monoclonal antibody (anti-LFA-1 CD11a) after HLA nonidentical bone marrow transplantation in children with immunodeficiencies, osteopetrosis, and Fanconi's anemia: a European Group for Immunodeficiency/European Group for Bone Marrow Transplantation report. Blood 1991;77:249–256.
15 Schlegel P, Eyrich M, Bader P, Handgretinger R, Lang P, et al: OKT-3 based reconditioning regimen for early graft failure in HLA-non-identical stem cell transplants. Br J Haematol 2000;111:668–673.
16 Kapelushnik J, Or R, Slavin S, Nagler A: A fludarabine-based protocol for bone marrow transplantation in Fanconi's anemia. Bone Marrow Transplant 1997;20:1109–1110.
17 Motwani J, Lawson SE, Darbyshire PJ: Successful HSCT using nonradiotherapy-based conditioning regimens and alternative donors in patients with Fanconi anaemia – experience in a single UK centre. Bone Marrow Transplant 2005;36:405–410.
18 Rossi G, Giorgiani G, Comoli P, Nobili B, Salvaneschi L, et al: Successful T-cell-depleted, related haploidentical peripheral blood stem cell transplantation in a patient with Fanconi anaemia using a fludarabine-based preparative regimen without radiation. Bone Marrow Transplant 2003;31:437–440.
19 George B, Mathews V, Shaji RV, Srivastava V, Srivastava A, Chandy M: Fludarabine-based conditioning for allogeneic stem cell transplantation for multiply transfused patients with Fanconi's anemia. Bone Marrow Transplant 2005;35:341–343.
20 Boulad F, Gillio A, Small TN, George D, Prasad V, et al: Stem cell transplantation for the treatment of Fanconi anaemia using a fludarabine-based cytoreductive regimen and T-cell-depleted related HLA-mismatched peripheral blood stem cell grafts. Br J Haematol 2000;111:1153–1157.
21 Boyer MW, Gross TG, Loechelt B, Leemhuis T, Filipovich A, Harris RE: Low risk of graft-versus-host disease with transplantation of CD34 selected peripheral blood progenitor cells from alternative donors for Fanconi anemia. J Pediatr Hematol Oncol 2003;25:890–895.
22 Yabe H, Inoue H, Matsumoto M, Hamanoue S, Koike T, et al: Allogeneic haematopoietic cell transplantation from alternative donors with a conditioning regimen of low-dose irradiation, fludarabine and cyclophosphamide in Fanconi anaemia. Br J Haematol 2006;134:208–212.
23 Torjemane L, Ladeb S, Ben Othman T, Abdelkefi A, Lakhal A, Ben Abdeladhim A: Bone marrow transplantation from matched related donors for patients with Fanconi anemia using low-dose busulfan and cyclophosphamide as conditioning. Pediatr Blood Cancer 2006;46:496–500.
24 Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, et al: Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N Engl J Med 1998;339:1186–1193.
25 Eyrich M, Lang P, Lal S, Bader P, Handgretinger R, et al: A prospective analysis of the pattern of immune reconstitution following transplantation of HLA-disparate hematopoietic stem cells from parental donors. Br J Haematol 2001;114:422–432.
26 Eyrich M, Croner T, Leiler C, Lang P, Bader P, et al: Distinct contributions of $CD4^+$ and $CD8^+$ naive and memory T-cell subsets to overall TCR-repertoire complexity following transplantation of T-cell depleted CD34 selected hematopoietic progenitor cells from unrelated donors. Blood 2002;100:1915–1918.

27 Eyrich M, Wollny G, Tzaribaschev N, Dietz K, Brugger D, et al: Onset of thymic recovery and plateau of thymic output are differentially regulated after stem cell transplantation in children. Biol Blood Marrow Transplant 2005;11:194–205.
28 Storek J, Joseph A, Dawson MA, Douek DC, Storer B, Maloney DG: Factors influencing T-lymphopoiesis after allogeneic hematopoietic cell transplantation. Transplantation 2002;73: 1154–1158.

Matthias Eyrich
University of Würzburg
Children's Hospital, Pediatric Stem Cell Transplantation Program
Josef-Schneider-Strasse 2, D30
D–97080 Würzburg (Germany)
Tel. +49 931 201 27640, Fax +49 931 201 27649
E-Mail Eyrich_M@klinik.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 183–199

Fanconi Anemia Genes in Vertebrates: Evolutionary Conservation, Sex-Linkage, and Embryonic Expression of *FANCC* and *FANCG* in Avian Cells

I. Nanda[a], *A. Buwe*[a], *A. Wizenman*[b], *M. Takata*[c], *T. Haaf*[d], *M. Schartl*[e], *M. Schmid*[a]

[a]Department of Human Genetics, University of Würzburg, Würzburg, [b]GSF- National Research Center for Environment and Health, Institute for Stem Cell Research, Neuherberg/Munich, Germany; [c]Department of Human Genetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; [d]Department of Human Genetics, University of Mainz, Mainz, [e]Department of Physiological Chemistry I, University of Würzburg, Würzburg, Germany

Abstract

Orthologs of the human Fanconi anemia (*FANC*) genes have been identified in several vertebrate and invertebrate model organisms, indicating variously conserved functions of the FANC protein complex. In particular, the analysis of chicken DT40 cells has made important contributions to the functional characterization of *FANC* genes. Orthologs for most human *FANC* genes have been found in the chicken genome which is considerably smaller than the human genome. Even though a Fanconi anemia-like phenotype has not been described in avian species, *FANC*-deficient chicken cells display the same sensitivity to DNA crosslinking agents as mammalian cells. In addition to a brief review of *FANC* gene orthologs in vertebrates and lower organisms, we here show that the chicken *FANCC* and *FANCG* genes are highly expressed in multiple avian embryonic tissues, whereas in adult birds strong expression was only observed in gonads which underlines the putative function of these genes during premeiotic DNA replication and meiotic recombination. We further show that the avian homologs of two important members of the *FANC* gene family, *FANCC* and *FANCG*, are located on the chicken Z sex chromosome. We tested the sensitivity of chicken embryonic fibroblasts from males (ZZ) and females (ZW) towards mitomycin C and observed a gender difference. This observation is consistent with the absence of Z chromosome dosage compensation in birds and supports the essential role of *FANCC* and *FANCG* in the cellular defense against DNA crosslinking agents.

Cells of all FA patients are highly susceptible to the induction of chromosome breaks with DNA crosslinking agents such as diepoxybutane (DEB) or mitomycin C (MMC). This indicates that mutations in the different *FANC* genes cause a similar DNA repair defect(s) [1–4]. Sensitivity to crosslinking agents is a cytological 'FA hallmark', which is widely used for diagnostic testing of FA. Somatic cell fusion and biochemical analyses have delineated 13 different FA complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, and N). With the exception of *FANCI*, the disease genes underlying these groups have already been cloned [5–7].

Orthologs of the centrally important FANCD2 protein have been detected in such diverged organisms as *Arabidopsis, Caenorhabditis, Drosophila*, fugu and zebrafish [8–10], indicating likely conservation of FANCD2 function. In this report we review recent findings on the FA pathway in different vertebrates. Special attention is given to birds which phylogenetically are closest to mammals. Since they can be easily manipulated by homologous recombination, chicken DT40 cells are increasingly used in FA research. An interesting additional aspect of the avian cell model in FA research is the increased susceptibility of FA cells to oxidative stress because, for various reasons, birds appear to be less or more resistant than mammals to the detrimental effects of reactive oxygen species, in general [11, 12].

Conserved and Divergent Functions of *FANC* Genes

Double-strand break (DSB) intermediates formed by interstrand crosslinks in the stalled replication fork can be resolved through different mechanisms such as homologous recombination (HR), non-homologous end joining (NHEJ), and error-prone translesion synthesis (TLS). The finding that FANCD1 (BRCA2) and the HR protein RAD51 directly interact provided the first evidence connecting the FA pathway with HR-mediated DNA repair [13]. Subsequent studies found HR-defects in *BRCA2*-deficient cells [14] whereas the in vitro interaction between BRCA2 and RAD51 stimulates RAD51-mediated recombination [15]. In meiotic cells, antibodies against FANCD2 specifically label the synaptonemal complexes which are required for recombination between homologous chromosomes [16]. This supports the hypothesis that many DNA repair proteins that are required for the detection and processing of damaged DNA in somatic cells perform related functions during meiotic recombination [17].

DSBs induced by crosslinking agents are preferentially repaired by HR. Experimental evidence suggests that monoubiquitinated FANCD2 promotes efficient HR repair, possibly by interacting with the BRCA2-RAD51 complex

and other HR proteins like FANCJ [18, 19]. So far, there are only few studies that directly test recombination activity, for example by introducing a plasmid substrate in normal and FA cell extracts [20] or in murine cells with a targeted mutation at the *Fanca* locus [21]. However, there are a number of studies that have evaluated FANC protein function using cell-free assays with *Xenopus* egg extracts [22], knockdown zebrafish embryos [10] and chicken DT40 knockout cells [23]. Collectively, these results suggest a role(s) for FANC proteins in DSB repair most likely through error-free HR. As such, FANC proteins appear to be important for maintaining genomic stability [24]. This guardian role is consistent with evolutionary conservation of at least part of the FA pathway.

Besides their crucial role in HR-mediated DSB repair, FANC proteins have been functionally linked to oxygen metabolism, cell cycle regulation, hematopoiesis, and apoptosis. Human FA cells show increased susceptibility to oxidative stress which suggests a role for FANC proteins in the protection against the endogenous production of reactive oxygen species (ROS). In spite of a higher metabolic rate, body temperature and blood sugar level, which all contribute to increased DNA damage after oxidative stress, birds produce relatively fewer ROS than mammals [11, 12]. This suggests that birds must be endowed with preventive or protective mechanisms that may also include the FA family of genes.

Evolutionary Conservation of *FANC* Genes

The phenotypes of *Fanca, Fancc*, and *Fancg* knockout mice and even double knockouts are very similar, indicating involvement of different *Fanc* genes in the same biochemical pathway(s). Although *Fanc*-deficient mouse fibroblasts are also sensitive to crosslinking agents, unlike humans KO mice are not affected by bone marrow failure or malignancies [25]. Interestingly, the different KO mice all showed hypogonadism and impaired fertility [26], indicating a functional role for FANC proteins during meiosis.

Fancd2-deficient mice and zebrafish embryos exhibit some malformations, i.e. microcephaly and microphthalmia that are also observed in FA patients. Increased apoptosis in these embryos can be partially corrected by injection of human FANCD2 protein [10]. The zebrafish orthologs of nine human *FANC* genes have been cloned and mapped to syntenic regions shared by the zebrafish and human genomes [27]. Evidently, the *FANC* gene network and most likely the FA pathway already existed in a common ancestor of teleost fish and tetrapods. Because of a genome duplication event in the ray fin fish lineage [28, 29] most zebrafish orthologs of human genes exist as two paralogous gene copies. However, none of the zebrafish *Fanc* genes has such a second copy,

suggesting loss of the duplicated *Fanc* genes after the genome duplication event. The high degree of evolutionary conservation of *FANC* genes in lower vertebrates is also documented by the identification of *FANCD2* and *FANCL* orthologs in the chordate *Ciona intestinalis* [27].

Similar to the situation in humans, *Xenopus* FANCF protein appears to be crucial for proper assembly of the FANC core complex [30]. Both FANCA and FANCD2 along with FANCL prevent the accumulation of DNA breaks that arise during unperturbed DNA replication [22]. Functional homologs of FANCD2 and its activator FANCL have been found in *Caenorhabditis* and *Drosophila* [8, 31]. Homozygous *C. elegans* mutants are sensitive to crosslinking agents but viable [9, 32]. The fungus *Ustilago maydis* is endowed with a *FANCD1* (*BRCA2*) ortholog [33], and a homolog of the recently identified *FANCJ* (*Hef*) gene is even present in archaebacteria where it is needed to process blocked replication forks. *FANCJ* (*Hef*) homologs are indeed highly conserved among single and multicellular organisms [34], whereas orthologs of the sex-linked human *FANCB* gene are only found in mouse, chicken, and zebrafish but not in worm and fly [35].

FANC Orthologs in Birds

During the course of evolution, the avian genome has been subject to extensive structural reorganization. Modern bird genomes are compartmentalized into macro- and micro-chromosomes. Although the size of the chicken genome amounts to only 40% of the human genome it is endowed with almost the same gene content [36]. It is therefore not surprising that orthologs of almost all human *FANC* genes were found in chicken.

The first well characterized *FANC* gene in chicken was *FANCD1* (*BRCA2*) [37, 38], which maps to GGA chromosome 1. Most other avian *FANC* genes have been isolated from the highly recombinogenic chicken B cell line DT40 [39, 40]. The overall amino acid sequence similarity between human and chicken FANC orthologs is not particularly high, amounting to 39% for FANCC, 40% for FANCD1 (BRCA1), 49% for FANCG, 54% for FANCJ (BRIP1) and 57% for FANCD2. Despite this overall low level of sequence conservation between avian and mammalian *FANC* genes, functionally important domains, including the PHD finger domain in FANCL, the helicase and degenerated nuclease domains in FANCM, the helicase domain in FANCJ, the RAD51-binding BRC repeats in FANCD1, and to some extent the tetratricopeptide motif in FANCG are highly conserved in the chicken genome. This is consistent with the view that these domains act as scaffolds to co-ordinate the functions of different FANC proteins. One remarkable species difference is the absence of a specific

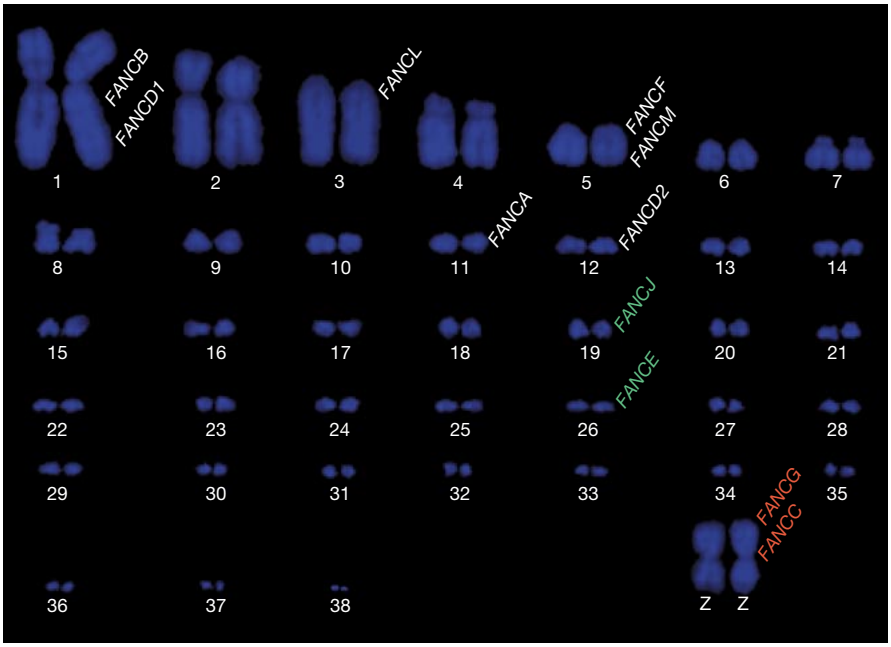


Fig. 1. DAPI-stained male chicken karyotype indicating the chromosomal localizations of human *FANC* gene orthologs. The prelimary assignments of *FANCE* to GGA26 and *FANCJ* to GGA19 are indicated in green. The newly mapped *FANCC* and *FANCG* in the Z chromosome are designated in red.

phosphorylation site in the chicken FANCJ protein. In human cells this site is critical for interaction of FANCJ with BRCA1 [39].

The availability of the complete chicken genome sequence [36] facilitates the in silico identification of avian orthologs of human disease genes. By BLAST searches of the chicken genome (http://www.ncbi.nlm.nih.gov/mapview/; http://www.ensembl.org/Gallus_gallus) with human *FANC* gene sequences, we have mapped orthologs of the human *FANC* genes to chicken chromosomes (fig. 1). With exception of *FANCE* and *FANCJ* which are represented by several contigs in the current version of the chicken genome, the chicken and human *FANC* orthologs lie in syntenic regions.

In order to confirm the in silico data, we localized *FANCC* and *FANCG* by fluorescence in-situ hybridization (FISH) on chicken metaphase spreads. Z-linkage of these genes is not unexpected because hemizygous (ZW) chicken DT40 cells are known to contain only single copies of *FANCC* and *FANCG* [23, 41]. Indeed, we found specific FISH signals for both genes (fig. 2a and b) on the short arm of the chicken Z sex chromosome with *FANCC* close to the

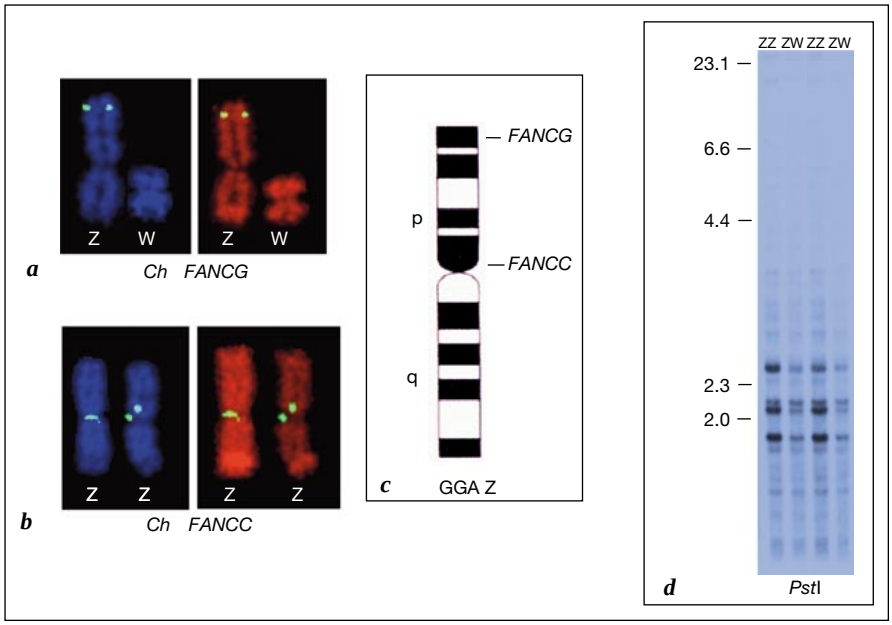


Fig. 2. Physical mapping of chicken *FANCG* and *FANCC* to the Z sex chromosome. (***a***) *FANCG* hybridization signals (green FITC fluorescence) on the Z short arm. The ZW cut-outs are counterstained with both DAPI (left) and propidium iodide (right). The bright PI fluorescence of terminal heterochromatin allows the distinction between the long and short arm of the metacentric Z. (***b***) *FANCC* signals appear close to the centromere of the Z short arm. (***c***) Ideogram of the chicken Z chromosome illustrating the location of both genes. (***d***) Southern hybridization of *FANCG* cDNA to *Pst*I-digested male and female chicken genomic DNA. Note the increased hybridization intensity of bands in males compared to females. Size markers in kb are indicated on the left.

centromere and *FANCG* at a telomeric position of Zp (fig. 2c). Z-linkage of these genes was also indicated from Southern hybridization of *FANCG* cDNA to male and female chicken genomic DNA (fig. 2d). The respective signals showed an approximately twofold intensity in males compared to females. Z-linkage, deduced from both the FISH and the Southern blot data is consistent with the position of *FANCC* and *FANCG* on human chromosome 9 which exhibits a high degree of synteny conservation with the chicken Z chromosome [42]. In contrast, the *FANCB* gene is X-linked in humans but autosomal in chicken, mapping to GGA1. Avian and mammalian sex chromosomes evolved independently from different ancestral autosome pairs [43, 44]. The localization of two *FANC* genes on the chicken Z which other than the mammalian X may not be subject to dosage compensation, is consistent with a higher gene dosage in male (ZZ) than in female (ZW) birds.

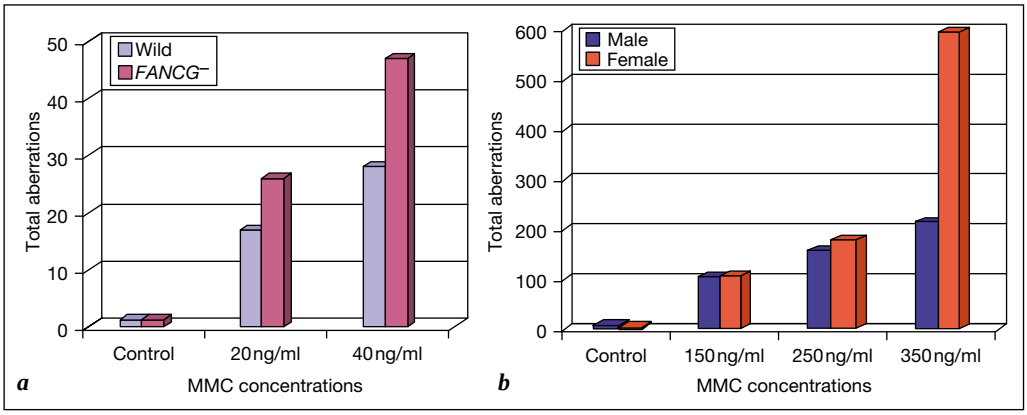


Fig. 3. (***a***) Frequency of aberrations in normal DT40 (blue bars) and *fancg*DT40 knockout (purple bars) cells after treatment with the indicated MMC concentrations (for details, see [23]). Note the increased level of MMC-induced DNA damage in *fancg* deficient cells. (***b***) Number of chromosome aberrations in diploid chicken embryonic fibroblasts of both sexes (male: blue bar and female: red bar) exposed to the indicated MMC concentrations. Note the significantly higher chromosome aberrations in female. 200 metaphases were screened for each experiment.

Lessons Learnt from Chicken DT40 Cells

One powerful approach to investigate FANC protein function is reverse genetics. The chicken B lymphocyte line DT40 has been widely used in different research areas such as receptor signaling, cell cycle regulation, gene conversion and apoptosis, because it can be much more easily manipulated than mammalian model systems. DT40 cells exhibit an exceptionally high integration rate of gene targeting constructs at homologous loci [45]. This high gene targeting efficiency facilitates the generation of null mutants through deletion of specific genomic regions. An added advantage compared to murine ES cells is that DT40 cells maintain a stable karyotype and cellular phenotype over prolonged culture periods. In contrast to the mouse, DT40 knockouts for many genes essential for recombinational types of DNA repair, including the Rad6 group of genes [46] are viable [47]. Another important but also critical aspect of these cells is their lack of functional p53 which enables DT40 cells to proliferate rapidly, with very short doubling times.

The disruption of different *FANC* genes in DT40 cells renders them sensitive to cross-linking agents and causes a high incidence of chromosome aberrations after MMC treatment (fig. 3a). Thus, the cellular phenotype (MMC sensitivity) of *fanc*DT40 cells is comparable to that of human FA cells, which

makes them an excellent model for molecular analyses of the FA pathway. The DNA repair defect in *fanc*DT40 cells has been examined with an elegant HR assay that measures repair of a genomic DSB induced at an *I-Sce*I restriction site, which then facilitates the expression of the reporter gene. So far five *fanc* genes have been disrupted in chicken DT40 cells and all knockouts displayed decreased HR activity [23, 41, 48, 49]. This is consistent with the homogeneity of the HR defect in human FA. Although *fancd2*DT40 cells show a 40-fold decrease in their HR activity compared to wild-type (which is much more pronounced than in human FA cells), the basic cellular defect is likely to be similar to that in human FA fibroblasts [21]. In mammals, cells deficient for bonafide HR repair enzymes (e.g. XRCC3) exhibit much more severe DNA repair defects than *FANC* mutants [49]. One plausible explanation is that human *FANC* genes are not directly involved in HR but rather modulate HR efficiency.

Matushita et al. [50] generated a chicken *fancd2*-ubiquitin fusion gene replacing the natural monoubiquitination site (*D2KR-Ub*). These cells reverse cisplatin hypersensitivity, and the fusion protein localized to chromatin in *FANCD2*-deficient DT40 cells. The observation that this fusion protein could not complement *FANCC*, *FANCG* or *FANCL* knockouts suggests that apart from FANCD2 monoubiquitination the core complex FANC proteins may have additional functions. Recent research using DT40 cells has contributed to the detection of a new down stream member of the FA pathway. FANCJ (BRIP1), which is a DEAH helicase that interacts with the BRCT domain of BRCA1, has an important function in BRCA1-dependent DNA repair and checkpoint control. *BRIP1*-deficient DT40 cells display the same sensitivity to DNA-crosslinking agents as *fancc*DT40 [39], but their cellular phenotype is clearly different from *BRCA1* knockouts. Their crosslink hypersensitivity is corrected by expression of human BRIP1 lacking the C-terminal BRCT-interacting domain. *BRIP1*-deficient DT40 cells therefore demonstrate that FANCJ (BRIP1) has a novel function in the FA pathway which is independent of BRCA1.

Another very interesting property of *FANC*-deficient DT40 cells is their increased rate of spontaneous sister chromatid exchanges (SCE) which is not typical for human FA mutant cells. Because several vertebrate cell lines with TLS repair defects including *RAD18*-deficient DT40 show increased SCE levels [51], it is assumed that TLS (possibly including the BLM helicase) may be impaired in *fancd2*DT40 cells. To elucidate the mechanism underlying elevated SCE levels, Hirano et al. [41] deleted *FANCC* in DT40 cells lacking the Rad51 paralog *XRCC3* and TLS factor Rad18. The spontaneous SCE rate was clearly decreased in *xrcc3*DT40 cells, but elevated in *fancc*DT40 cells. In *xrcc3/fancc* double mutants the SCE rate was similar to that of *xrcc3*-deficient cells. Thus, spontaneous SCE in *fancc*DT40 cells require XRCC3. The higher SCE rate in *fancc/rad18* double knockout DT40 cells compared to single

mutants supports a role for FA proteins in facilitating HR but not global TLS during crosslink repair. Taken together, *FANC*-deficient DT40 cells have clearly improved our understanding of the molecular mechanisms in the FA pathway.

Sex-Specific Sensitivity to DNA Crosslinking Agents in Birds

FA cells are characterized by increased sensitivity to DNA crosslinking agents such as MMC, DEB and others, but the molecular mechanism(s) underlying the various types of induced chromosome aberrations in FA in response to crosslinking agents are not fully understood. MMC appears to exert its main biochemical effects in the cytoplasm. Several lines of evidences suggest that chromosome damage in FA cells is associated with ROS generated by these crosslinking agents [52]. FA cells exhibit higher MMC sensitivity when grown at 20% ambient oxygen than at 5% oxygen [53, 54], indicating that ROS generated by MMC are toxic. Vice versa, expression of the anti-oxidant protein thioredoxin can decrease the MMC or DEB sensitivity of FA cells [55]. Additional evidence for a link between ROS and chromosome hypersensitivity comes from the interaction of FANCC with cytoplasmic enzymes that are associated with the production of ROS, i.e. NADPH cytochrome-P450 reductase [56], glutathione transferase [57], and FANCG with CYP2E1 [58]. Apart from their nuclear location, both FANCC and FANCG also appear to be present in the cytoplasm [59]. Collectively, these results suggest that FA cells exhibit increased susceptibility to oxidative stress induced by DNA crosslinking agents and that both FANCC and FANCG somehow interfere with the generation of such oxidative stress.

We have shown that both *FANCC* and *FANCG* are Z-linked in birds. Since there is no evidence for chromosome wide inactivation of the additional Z chromosome in the homogametic avian (ZZ) sex, males should express a higher dosage for the two Z-linked *FANC* genes. We treated male and female chicken cells with MMC to test whether *FANCC* and *FANCG* dosage influences the cellular response to oxidative stress. Whereas *FANCC*- and *FANCG*-deficient DT40 cells are known to be hypersensitive to MMC treatments [23, 41], it is important to note that DT40 cells lack the *TP53* tumor suppressor gene and are trisomic for GGA2 which carries the *c-myc* oncogene [60]. Thus, MMC hypersensitivity in DT40 cells may not fully reflect the situation in wild type avian cells. In our experiments, exposure of male versus female chicken embryonic fibroblasts to different MMC concentrations resulted in both chromosome and chromatid breaks (fig. 4) reflecting unresolved DNA damage sustained during the G_1 and G_2 phase of the cell cycle. As predicted, the frequency of chromatid and chromosome aberrations was significantly higher in heterogametic (ZW)

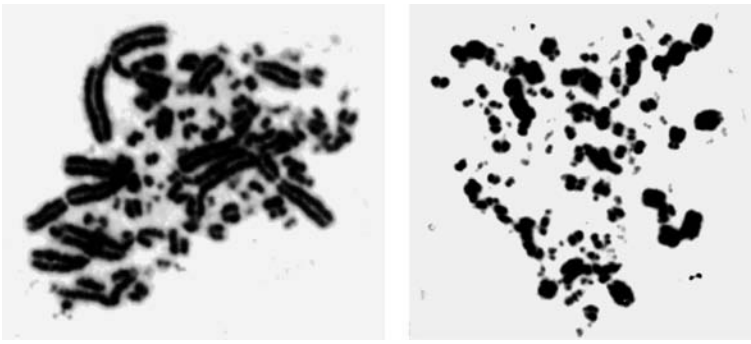


Fig. 4. Representative chicken metaphase spreads showing chromatid-type (G_2 phase) and chromosome-type (G_1 phase) aberrations and occasional chromosome pulverizations after exposure to the crosslinking agent MMC.

female cells than in homogametic (ZZ) male cells (fig. 3b). The increased sensitivity of female cells is particularly evident when the cells are exposed to relatively high MMC concentrations. At low MMC concentration the gender difference was less pronounced, however the SCE frequency was markedly increased in female compared to male chicken cells (data not shown).

As far as we can ascertain, the sex-specific cellular response to MMC treatments appears to be unique for birds. Although the *FANCB* gene is X-linked in mammals, this should not cause a sex-specific DNA damage response because of X-inactivation in females. It is tempting to speculate that because of their higher *FANCC* and *FANCG* gene dosage male birds may be better protected from oxidative damage through reducing ROS production. In most ratites (flight-less birds) the Z and W sex chromosomes are still very similar in size and gene content [61]. Thus, in contrast to modern birds female ratites most likely will have two copies of *FANCC* and *FANCG*. It will therefore be interesting to test whether sensitivity to MMC treatment and oxidative stress differs between male and female cells in ratites as we here show for modern birds.

Function of Avian *FANC* Genes during Embryogenesis and Gonadal Development

FA proteins are thought to interact in a common pathway for the repair of stalled replication forks which are common events in proliferating cells. Considering the high rate of cell proliferation in developing embryos, *FANC* genes are likely to play a critical role during early embryogenesis. The congenital defects in FA patients point towards impaired function(s) of FA genes

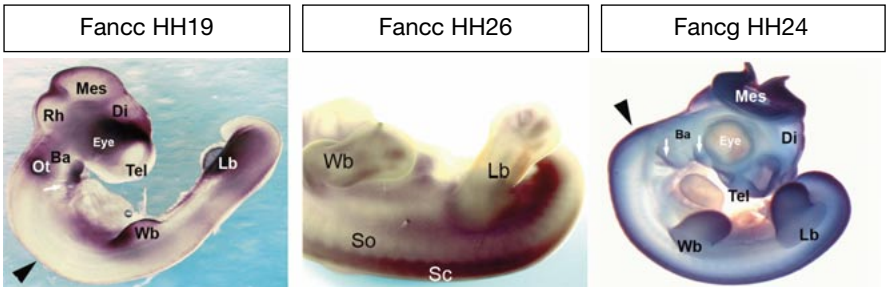


Fig. 5. Expression of *FANCC* and *FANCG* during chicken embryogenesis. Non-radioactive whole mount in situ hybridization with anti-sense RNA was performed on chicken embryos of HH stages 19, 24 and 26. Abbreviations used: Ba = Branchial arches, Di = Diencephalon, Lb = Limb bud, Mes = Mesencephalon, Ot = Otic placode, Rh = Rhombencephalon Sc = Spinal cord, So = Somites, Tel = Telencephalon, Wb = Wing bud.

during development. Mouse studies revealed that *Fancc, Fancg* and *Fanca* are preferentially expressed in highly proliferating and embryonic tissues [62–64]. The importance of *Fanc* genes during early development was also demonstrated in zebrafish embryos where Fancd2 prevented inappropriate apoptosis in neural cells and other highly proliferating tissues [10].

In order to explore the activity of *FANC* genes during avian development, we used whole mount RNA in situ hybridization on chicken embryos at different Hamburger Hamilton (HH) stages to delineate the expression patterns of chicken *FANCC* and *FANCG* genes. *FANCC* mRNA was first detected in embryos at HH19, whereas *FANCG* expression started at HH24 (fig. 5). At HH19 (fig. 5, left image) *FANCC* was highly expressed in the midline of the entire dorsal neural tube (including brain and spinal cord), in the eye and otic vesicle, at the tips of the branchial arches (white arrow), and in the mesenchyme around the diencephalon. *FANCC* was also expressed in both limb buds at HH19 and in the developing paws at later stages (fig. 5, middle image). In the limb buds, *FANCC* mRNA was enriched in the zone of polarizing activity (ZPA) and the apical epidermal ridge (AER). At HH26 and later stages *FANCC* was still expressed in the dorsal spinal cord and in the somites (fig. 5, middle image).

FANCG expression became detectable in HH24 embryos. Compared to *FANCC* it was expressed in a broader region of the dorsal neural tube with mRNA present in the entire dorsal midbrain and telencephalon (fig. 5, right image). In the branchial arches *FANCG* was only weakly expressed at the posterior edge of Ba1 and Ba3 (white arrows). In the limb buds *FANCG* mRNA covered the entire proximal-distal extent. In contrast to *FANCC, FANCG* was not

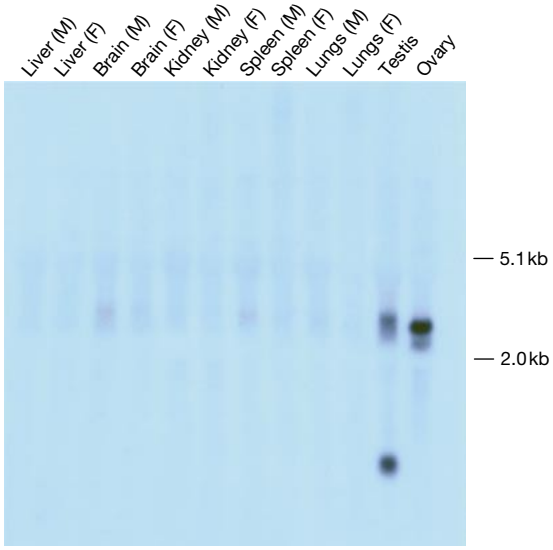


Fig. 6. Northern blot with total RNAs from different adult male and female chicken tissues hybridized with chicken *FANCG* cDNA. Note the prominent signals in the gonads. The two different bands in testis tissue may be due to alternative splicing.

detectable in eye and otic vesicle. At HH30 and later embryonic stages both *FANCC* and *FANCG* were expressed concomitantly in the urogenital region (data not shown), possibly indicating a function of these genes during gonad and kidney development. In contrast to our findings on chicken embryos, *FANCG* expression was not detectable by Northern blot analysis in adult somatic tissues. Only testis and ovary showed high *FANCG* mRNA levels on Northern blots (fig. 6). RT-PCR revealed low *FANCG* transcript levels in adult somatic tissues and high levels in gonads (data not shown).

The observed expression patterns support an important role for both *FANCC* and *FANCG* during chicken development. Clearly, expression of these genes is tightly regulated in a tissue- and developmental stage-specific manner. The *FANCC* and *FANCG* expression patterns are partially overlapping, but not identical. Abundant transcript levels in the developing eye, ear, spinal cord, and brain suggest a function for central nervous system development. This is consistent with the observation that in addition to other congenital malformations many FA patients suffer from eye and ear defects. The expression of chicken *FANCC* in limb and wing buds is consistent with the typical radial ray defects of FA patients.

Strong expression of *FANC* genes in chicken and mouse gonads, the impaired fertility of *Fanc* knockout mice, and the strongly reduced fertility of FA patients [64, 65] argue in favor of a germ-cell specific function of FA proteins. Interestingly, the function(s) of *FANC* genes and the FA pathway during germ cell development and meiotic recombination appear to be highly conserved throughout vertebrate evolution, whereas the effects of *FANC* gene deficiency on somatic development appear to vary widely among different species.

Perspective

Orthologs for almost all mammalian *FANC* genes have been identified in chicken. Because most *FANC* orthologs are not found in yeast, genetic analysis in this convenient eukaryotic model organism cannot be performed. In this light, the possibility to efficiently disrupt endogenous *FANC* genes in chicken DT40 cells has been extremely useful for the investigation of individual *FANC* genes, their specific functions, and the FA pathway.

In addition, birds have revealed interesting novel properties of *FANC* genes that are not evident in other vertebrates. With the exception of *FANCD1/ BRCA2*, monoallelic inactivation of *FANC* genes does not appear to cause serious health problems in humans, whereas compound heterozygotes are phenotypically affected. In birds we have the exceptional situation that the female (ZW) genome contains only one copy of both *FANCC* and *FANCG*. Consequently, female chicken cells exhibit a higher sensitivity to MMC treatments. Because male birds have a higher level of the Z-linked FANCC and FANCG proteins, they are likely to be more resistant to oxidative stress. This is consistent with observations that in many bird species males are longer-lived than females [66], and also have higher survival rates [67]. Whether the survival advantage of male birds results chiefly from their higher dosage of *FANC* caretaker genes or whether additional protective factors are involved remains to be elucidated.

The high expression of *FANC* genes in avian gonads, and the fact that fertility is compromised in *Fanc* knockout mice and in FA patients argues for an essential function of the *FANC* family of genes in germ cell development, most likely during recombination of homologous chromosomes during meiotic prophase I. The DNA replication process in avian gonads must be particularly efficient to ensure massive sperm production in male birds and ovary maturation in females. High levels of *FANC* gene and protein expression may be instrumental in repairing DSBs arising from DNA replication machinery slippage. However, FANC proteins may not only be involved in DSB repair by promoting homologous recombination, but may also contribute to the repair of base changes by translesion synthesis [68]. The study of the Fanconi anemia family of genes in vertebrate

model systems, including fish, amphibian and avian cells, contributes greatly to our understanding of the mechanisms of DNA maintenance without which the remarkable longevity of our species would not be possible.

Acknowledgements

This work was supported by a grant from German Research Foundation (DFG; Schm 484/20-2).

References

1 Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 1973;33:1829–1836.
2 German J, Schonberg S, Caskie S, Warburton D, Falk C, Ray JH: A test for Fanconi's anemia. Blood 1987;69:1637–1641.
3 Auerbach AD: A test for Fanconi's anemia. Blood 1988;72:366–367.
4 Grompe M, D'Andrea A: Fanconi anemia and DNA repair. Hum Mol Genet 2001;10:2253–2259.
5 Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, et al: The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 2005;37:934–935.
6 Meetei AR, Medhurst AL, Ling C, Xue Y, Singh TR, et al: A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet 2005;37: 958–963.
7 Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2007;39:162–164.
8 Timmers C, Taniguchi T, Hejna J, Reifsteck C, Lucas L, et al: Positional cloning of a novel Fanconi anemia gene, *FANCD2*. Mol Cell 2001;7:241–248.
9 Dequen F, St-Laurent JF, Gagnon SN, Carreau M, Desnoyers S: The *Caenorhabditis elegans FancD2* ortholog is required for survival following DNA damage. Comp Biochem Physiol B Biochem Mol Biol 2005;141:453–460.
10 Liu TX, Howlett NG, Deng M, Langenau DM, Hsu K, et al: Knockdown of zebrafish Fancd2 causes developmental abnormalities via p53-dependent apoptosis. Dev Cell 2003;5:903–914.
11 Herrero A, Barja G: H_2O_2 production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. Mech Ageing Dev 1998;103:133–146.
12 Ogburn CE, Carlberg K, Ottinger MA, Holmes DJ, Martin GM, Austad SN: Exceptional cellular resistance to oxidative damage in long-lived birds requires active gene expression. J Gerontol A Biol Sci Med Sci 2001;56:B468–B474.
13 Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL: RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene *brca2*. J Biol Chem 1997;272:31941–31944.
14 Tutt A, Bertwistle D, Valentine J, Gabriel A, Swift S, et al: Mutation in *Brca2* stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. EMBO J 2001;20:4704–4716.
15 Yang H, Jeffrey PD, Miller J, Kinnucan E, Sun Y, et al: BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. Science 2002;297:1837–1848.
16 Zickler D, Kleckner N: Meiotic chromosomes: integrating structure and function. Annu Rev Genet 1999;33:603–754.
17 Marcon E, Moens PB: The evolution of meiosis: recruitment and modification of somatic DNA-repair proteins. Bioessays 2005;27:795–808.

18 Mirchandani KD, D'Andrea AD: The Fanconi anemia/BRCA pathway: a coordinator of cross-link repair. Exp Cell Res 2006;312:2647–2653.
19 Kitao H, Yamamoto K, Matsushita N, Ohzeki M, Ishiai M, Takata M: Functional interplay between BRCA2/FancD1 and FancC in DNA repair. J Biol Chem 2006;281:21312–21320.
20 Thyagarajan B, Campbell C: Elevated homologous recombination activity in Fanconi anemia fibroblasts. J Biol Chem 1997;72:23328–23333.
21 Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, et al: Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 2005; 102:1110–1115.
22 Sobeck A, Stone S, Costanzo V, de Graaf B, Reuter T, et al: Fanconi anemia proteins are required to prevent accumulation of replication-associated DNA double-strand breaks. Mol Cell Biol 2006;26:425–437.
23 Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, et al: Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol Cell Biol 2003;23:5421–5430.
24 Levitus M, Joenje H, de Winter JP: The Fanconi anemia pathway of genomic maintenance. Cell Oncol 2006;28:3–29.
25 Noll M, Battaile KP, Bateman R, Lax TP, Rathbun K, et al: Fanconi anemia group A and C double-mutant mice: functional evidence for a multi-protein Fanconi anemia complex. Exp Hematol 2002;30:679–688.
26 Koomen M, Cheng NC, van de Vrugt HJ, Godthelp BC, van der Valk MA, et al: Reduced fertility and hypersensitivity to mitomycin C characterize *Fancg/Xrcc9* null mice. Hum Mol Genet 2002;11: 273–281.
27 Titus TA, Selvig DR, Qin B, Wilson C, Starks AM, et al: The Fanconi anemia gene network is conserved from zebrafish to human. Gene 2006;371:211–223.
28 Wittbrodt J, Meyer A, Schartl M: More genes in fish? Bioessays 1998;20:511–515.
29 Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y: Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res 2003;13:382–390.
30 Leveille F, Blom E, Medhurst AL, Bier P, Laghmani el H, et al: The Fanconi anemia gene product FANCF is a flexible adaptor protein. J Biol Chem 2004;279:39421–39430.
31 Meetei AR, de Winter JP, Medhurst AL, Wallisch M, Waisfisz Q, et al: A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet 2003;35:165–170.
32 Collis SJ, Barber LJ, Ward JD, Martin JS, Boulton SJ: *C. elegans* FANCD2 responds to replication stress and functions in interstrand cross-link repair. DNA Repair (Amst.) 2006;5:1398–1406.
33 Kojic M, Kostrub CF, Buchman AR, Holloman WK: BRCA2 homolog required for proficiency in DNA repair, recombination, and genome stability in *Ustilago maydis*. Mol Cell 2002;10:683–691.
34 Mosedale G, Niedzwiedz W, Alpi A, Perrina F, Pereira-Leal JB, et al: The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat Struct Mol Biol 2005;12: 763–771.
35 Meetei AR, Levitus M, Xue Y, Medhurst AL, Zwaan M, et al: X-linked inheritance of Fanconi anemia complementation group B. Nat Genet 2004;36:1219–1224.
36 International chicken genome sequence consortium: Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 2004;432:695–716.
37 Takata M, Tachiiri S, Fujimori A, Thompson LH, Miki Y, et al: Conserved domains in the chicken homologue of *BRCA2*. Oncogene 2002;21:1130–1134.
38 Warren M, Smith A, Partridge N, Masabanda J, Griffin D, Ashworth A: Structural analysis of the chicken *BRCA2* gene facilitates identification of functional domains and disease causing mutations. Hum Mol Genet 2002;11:841–851.
39 Bridge WL, Vandenberg CJ, Franklin RJ, Hiom K: The BRIP1 helicase functions independently of BRCA1 in the Fanconi anemia pathway for DNA crosslink repair. Nat Genet 2005;37:953–957.
40 Takata M, Kitao H, Ishia M: Fanconi anemia: genetic analysis of a human disease using chicken system. Cytogenet Genome Res 117 (2007, in press).
41 Hirano S, Yamamoto K, Ishiai M, Yamazoe M, Seki M, et al: Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM. EMBO J 2005;24:418–427.

42 Nanda I, Shan Z, Schartl M, Burt DW, Koehler M, et al: 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet 1999;21:258–259.
43 Fridolfsson AK, Cheng H, Copeland NG, Jenkins NA, Liu HC, et al: Evolution of the avian sex chromosomes from an ancestral pair of autosomes. Proc Natl Acad Sci USA 1998;95:8147–8152.
44 Marshall Graves JA, Shetty S: Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes. J Exp Zool 2001;290:449–462.
45 Buerstedde JM, Takeda S: Increased ratio of targeted to random integration after transfection of chicken B cell lines. Cell 1991;67:179–188.
46 Nojima K, Hochegger H, Saberi A, Fukushima T, Kikuchi K, et al: Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. Cancer Res 2005;65: 11704–11711.
47 Bemark M, Khamlichi AA, Davies SL, Neuberger MS: Disruption of mouse polymerase zeta (Rev3) leads to embryonic lethality and impairs blastocyst development in vitro. Curr Biol 2000; 10:1213–1216.
48 Yamamoto K, Hirano S, Ishiai M, Morishima K, Kitao H, et al: Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol Cell Biol 2005;25:34–43.
49 Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ: The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. Mol Cell 2004;15: 607–620.
50 Matsushita N, Kitao H, Ishiai M, Nagashima N, Hirano S, et al: A FancD2-monoubiquitin fusion reveals hidden functions of Fanconi anemia core complex in DNA repair. Mol Cell 2005;19: 841–847.
51 Yamashita YM, Okada T, Matsusaka T, Sonoda E, Zhao GY, et al: RAD18 and RAD54 cooperatively contribute to maintenance of genomic stability in vertebrate cells. EMBO J 2002;21: 5558–5566.
52 Ahmad SI, Hanaoka F, Kirk SH: Molecular biology of Fanconi anaemia – an old problem, a new insight. Bioessays 2002;24:439–448.
53 Clarke AA, Philpott NJ, Gordon-Smith EC, Rutherford TR: The sensitivity of Fanconi anaemia group C cells to apoptosis induced by mitomycin C is due to oxygen radical generation, not DNA crosslinking. Br J Haematol 1997;96:240–247.
54 Liebetrau W, Runger TM, Mehling BE, Poot M, Hoehn H: Mutagenic activity of ambient oxygen and mitomycin C in Fanconi's anaemia cells. Mutagenesis 1997;12:69–77.
55 Ruppitsch W, Meisslitzer C, Hirsch-Kauffmann M, Schweiger M: Overexpression of thioredoxin in Fanconi anemia fibroblasts prevents the cytotoxic and DNA damaging effect of mitomycin C and diepoxybutane. FEBS Lett 1998;422:99–102.
56 Kruyt FA, Hoshino T, Liu JM, Joseph P, Jaiswal AK, Youssoufian H: Abnormal microsomal detoxification implicated in Fanconi anemia group C by interaction of the FAC protein with NADPH cytochrome P450 reductase. Blood 1998;92:3050–3056.
57 Cumming RC, Lightfoot J, Beard K, Youssoufian H, O'Brien PJ, Buchwald M: Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. Nat Med 2001;7:814–820.
58 Futaki M, Igarashi T, Watanabe S, Kajigaya S, Tatsuguchi A, et al: The FANCG Fanconi anemia protein interacts with CYP2E1: possible role in protection against oxidative DNA damage. Carcinogenesis 2002;23:67–72.
59 Thomashevski A, High AA, Drozd M, Hunt DF, Grant PA, Kupfer GM: The Fanconi anemia core complex forms four complexes of different sizes in different subcellular compartments. J Biol Chem 2004;279:26201–26209.
60 Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, et al: *Rad51*-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998;17:598–608.
61 Ogawa A, Murata K, Mizuno S: The location of Z- and W-linked marker genes and sequence on the homomorphic sex chromosomes of the ostrich and the emu. Proc Natl Acad Sci USA 1998;95: 4415–4418.
62 Krasnoshtein F, Buchwald M: Developmental expression of the *Fac* gene correlates with congenital defects in Fanconi anemia patients. Hum Mol Genet 1996;5:85–93.

63 Abu-Issa R, Eichele G, Youssoufian H: Expression of the Fanconi anemia group A gene (*Fanca*) during mouse embryogenesis. Blood 1999;94:818–824.
64 Wong JC, Buchwald M: Disease model: Fanconi anemia. Trends Mol Med 2002;8:139–142.
65 Hölzel M, van Diest PJ, Bier P, Wallisch M, Hoatlin ME, et al: FANCD2 protein is expressed in proliferating cells of human tissues that are cancer-prone in Fanconi anaemia. J Pathol 2003;201: 198–203.
66 Holmes DJ, Thomson SL, Wu J, Ottinger MA: Reproductive aging in female birds. Exp Gerontol 2003;38:751–756.
67 Payevsky VA, Vysotsky VG, Yefremov VD, Markovets MY, Shapoval AP: Sex-specific survival rates in birds. Zh obshch Biol 1997;58:5–20.
68 Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 2005;45:128–142.

Indrajit Nanda
Department of Human Genetics, University of Würzburg
Biozentrum, Am Hubland
D–97074 Würzburg (Germany)
Tel. +49 931 888 4078, Fax +49 931 888 4058
E-Mail nanda@biozentrum.uni-wuerzburg.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 200–210

Studying Homologous Recombination in Fanconi Anemia

I. Demuth, M. Digweed

Institut für Humangenetik, Charité Universitätsmedizin Berlin,
Campus Virchow-Klinikum, Berlin, Germany

Abstract

Cells from patients suffering from the recessive syndrome Fanconi anemia (FA), are characterized by increased sensitivity to agents that induce DNA interstrand cross-links (ICLs). This hypersensitivity manifests as a dramatically elevated rate of chromosome breaks in FA cells when compared to controls and led, more than thirty years ago, to the suggestion that the repair of ICLs is disturbed in FA. Today, a DNA repair defect as the basis of FA is widely accepted, however, the exact role of the 12 known genes is still elusive. The past several years have brought growing evidence that FA cells are compromised in homology dependent DNA repair processes. This review will summarize these studies with a focus on integrated plasmid reporter assays which are used to investigate repair products after induction of a single defined DNA double-strand break (DSB).

Establishing a disturbance in repair of DNA damage as a possible explanation for the basic defect in Fanconi anemia (FA) was the merit of two papers: The discovery of an increased level of spontaneous chromosome aberrations in cells from FA patients published by Schroeder and colleagues in 1964 and, almost ten years later, Sasaki's and Tonomura's finding that the chromosomes of FA patients were extremely and exclusively sensitive to agents that produce interstrand cross-links (ICLs) in DNA [1, 2]. The authors of the latter paper concluded: '…, although the mechanism is unidentified, mammalian cells have an efficient repair mechanism to tolerate the DNA cross-links, and the FA cells are genetically handicapped in functioning the system'.

The mechanisms of ICL processing within the mammalian cell are still poorly understood but there is clear evidence that repair of these lesions utilizes proteins from different DNA repair pathways [3]. Most importantly, it has been shown that DNA double-strand breaks (DSBs) are generated as an intermediate of ICL repair [4, 5] so that the repair of this particular DNA lesion plays a critical role in the cellular response to ICLs.

There are two principle mechanisms by which DSBs are processed depending on the requirement for sequence homology. Non-homologous end-joining (NHEJ), as the name suggests, rejoins free DNA ends with no, or only limited, requirement for homology and is error prone (reviewed in [6]).

The repair mechanisms that involve sequence homology can be divided into two types based on whether homologous associations arise from strand exchange or strand annealing (fig. 1). The central protein of the pathway of homology directed repair (HDR) is RAD51. This evolutionarily conserved protein, with orthologues in prokaryotes and archaea, initiates strand exchange reactions between a broken sequence and its undamaged sister chromatid, or homologue, to allow restoration of the damaged region. As many as five RAD51 paralogues have been identified in mammals, all playing important roles in HDR. In addition, the genes *BRCA1* and *BRCA2* showed reduced HDR in experiments using integrated plasmid reporters (see below) indicating involvement in this repair pathway also.

The second homology dependent pathway, single strand annealing (SSA), uses homologies of the region flanking the DSB, e.g. repetitive sequences, which are present at high frequency within the mammalian genome. DNA end resection removes the sequence between the homologous elements and is followed by annealing of 3′ overhangs and local DNA synthesis to restore an intact DNA molecule.

During recent years, several important findings suggested a link between FA and homology-driven repair mechanisms. Most of the known FA proteins form the so called ‘FA core’ complex, which activates the FANCD2 protein by adding an ubiquitin molecule in S-phase or in response to DNA damage. Monoubiquitinated FANCD2-Ub then relocates to nuclear foci, where it colocalizes with the HDR proteins RAD51 and BRCA1 [7]. The identification of *BRCA2* as the gene mutated in FA patients of complementation group D1 [8], the finding of direct interaction of BRCA2/FANCD1 with the FA proteins FANCG and FANCD2 [9, 10] and the observation of attenuated RAD51 foci formation in FA cells [11] provided additional evidence for a connection between FA and HDR. These findings stimulated research on homology-driven repair in FA and much of the recent work in this field took advantage of integrated plasmid reporter assays originally introduced by Maria Jasin.

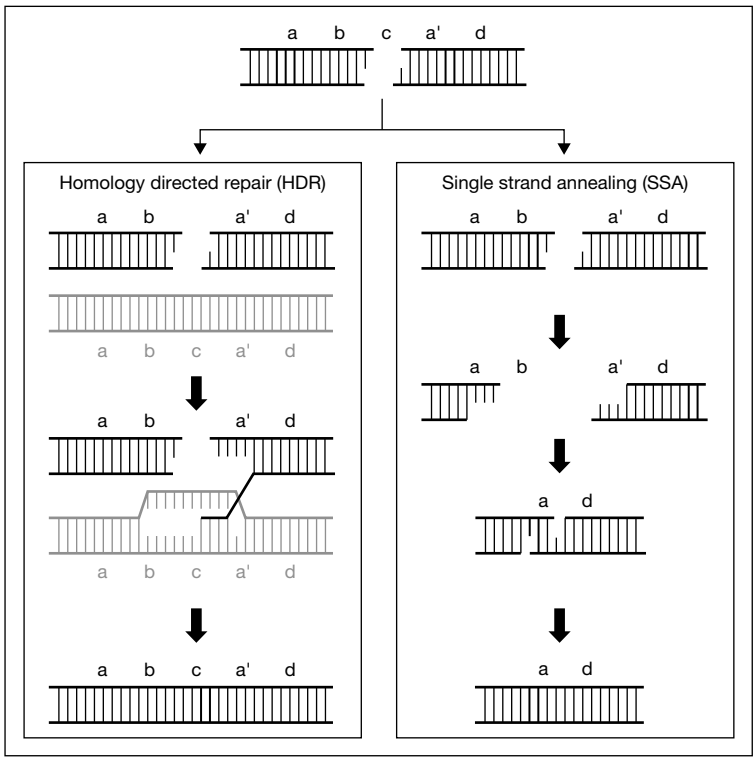


Fig. 1. DNA double-strand break repair mechanisms involving sequence homology**.** The graphic shows a highly schematic representation of two double-strand break repair pathways, homology directed repair (HDR) and single strand annealing (SSA). In HDR, a single strand, originating at the double-strand break, invades a homologous double helix (here in grey) and uses it as a template for DNA synthesis. In SSA, the DNA ends are processed back to the repetitive sequences a and a′. Annealing of the single stranded, complementary DNAs allows resynthesis of the missing DNA strands. Only in the HDR pathway is the original c-sequence restored.

Integrated Plasmid Reporter Assays

The reporter plasmids typically used to evaluate DSB repair contain two differently modified, non-functional reporter genes, one of these inactive because of the insertion of a recognition site for the rare cutting I-*Sce*I endonuclease and the other consisting of an internal fragment of the gene. Repair of a DSB induced in one copy of the gene, which exploits the second undamaged copy leads to the restoration of its function. The most commonly used reporter gene is the *green fluorescent protein* (GFP), but reporter plasmids with various drug resistance

genes have also been described. Expression of I-*Sce*I in cells containing a copy of the reporter introduces a single defined DSB. Subsequent analysis allows the efficiency of repair of the DSB to be quantified. For example, when GFP is used as the reporter, the HDR pathway restores the GFP function by recombination between the two non-functional GFP gene copies. Thus the fraction of cells that successfully employed HDR to repair the DSB can be directly assessed by counting green fluorescent cells. In addition, amplification and analysis of the sequence originally containing the I-*Sce*I recognition site can allow further determination of the exact repair pathway used (e.g. HDR, SSA or NHEJ).

Analysis of chicken DT40 cells with targeted mutations in FA genes has been shown to be a powerful strategy to investigate FA protein function and has been used by many researchers to analyse DNA repair with plasmid reporters (for references see table 1). Studies of DT40 cells with knocked-out FA genes, *fancg* or *fancd2*, revealed a mild but clear reduction in HDR whereas DT40 cells mutated in *fancj* and *fancm* were not affected in this pathway (table 1). Even the HDR analysis of the same *fancc* knock-out DT40 cell line yielded conflicting results [12–14].

Mouse ES cells, homozygous for a *Fanca* null mutation or for 'mild' hypomorphic mutations in the *Fancd1/Brca2* gene, displayed reduced HDR comparable to that of HDR-compromised DT40 FA cells [15–17].

Interestingly, in addition to the HDR defect, we found *Fanca*$^{-/-}$ mouse cells, as well as FA patient derived cells from complementation groups A, G and D2, to be deficient in a further DSB repair pathway, SSA, a finding also reported for *Brca2* mutated cells [17–19].

The relatively mild reduction in HDR capacity of human FA-A and FA-G cells stands in strong contrast to the more than 100-fold decrease in HDR of FANCD1/BRCA2-deficient CAPAN-1 tumour cells [15, 18]. In addition to FA-A and FA-G cells, we also found reduced HDR in FA-D2 cells, however in another report the same FA-D2 cell line (PD20) was not deficient in this pathway [18, 19]. MCF7 cells, depleted for FANCJ by siRNA mediated knockdown, showed a 7–10-fold reduction in HDR which is contradictory to the data obtained from the analysis of DT40 fancj cells [14, 20].

RAD51 Foci Formation

In response to ionizing radiation (IR) or treatment with other DNA damaging agents, RAD51 accumulates at the sites of DSBs, and this is visible, by immunofluorescence staining, as discrete nuclear foci. Attenuated RAD51 foci formation has been reported for a number of cell lines mutated in genes involved in HDR [16, 21–24]. Consequently, cell lines with knock-out mutations in FA

Table 1. Homology directed repair (HDR) in Fanconi anemia

FA gene disrupted	HDR (plasmid reporter assay)	Gene targeting[a]	Immunoglobulin gene conversion[a]	Sister chromatid exchanges[a]	Reference
Chicken DT40					
fancg	9-fold reduced	reduced	n.d.	normal (sp./MMC)	[41]
fancd2	reduced	reduced	deficient	sp. elevated; MMC, normal	[42]
fancj (bach1/brip1)	normal	n.d.	n.d.	sp. elevated	[14]
fancc[b]	3-fold reduced	reduced	n.d.	MMC/Cisplatin, defective induction	[12]
fancc[b]	normal	n.d.	n.d.	sp. elevated	[14]
fancc[b]	3-fold reduced	n.d.	deficient	sp. elevated; MMC, no further induction	[13]
fancm (hef1)	normal	n.d.	proficient	sp. elevated; MMC, no further induction	[13]
Mouse					
Fancd1 (Brca2)	5–6-fold reduced	reduced		n.d.	[15]
Fancd1 (Brca2)	reduced	n.d.		reduced (sp./MMC)	[16]
Fanca	reduced	n.d.		n.d.	[18]
Fanca	3-fold reduced	reduced		n.d.	[17]
Human					
FANCA	reduced			n.d.	[18]
FANCG	reduced			n.d.	[18]
FANCD1 (BRCA2)[c]	>100-fold reduced			n.d.	[15]
FANCD2[d]	reduced			n.d.	[18]
FANCD2[d]	normal			n.d.	[19]
FANCJ (BACH1/BRIP1)[e]	7–10-fold reduced			n.d.	[20]

[a]MMC: Mitomycin C; n.d.: not determined; sp.: spontaneous.
[b]Identical cell line.
[c]Tumour cell line.
[d]Identical cell line.
[e]siRNA mediated depletion.

Table 2. RAD51 foci formation in Fanconi anemia cells

FA gene(s) disrupted	RAD51 foci induction (treatment, time between treatment and analysis)[a]	RAD51 foci formation	Reference
Human			
FANC A/B/C/D1/D2/E/F/G	IR (12 Gy, 8 h)	reduced in all groups	[11]
FANC A/B/C/D1/D2/E/F/G	IR (12 Gy, 8 h), MMC (1 h/2.4 μg/ml, 24 h)	reduced in FANCD1 cells reduced in FANCD1 cells	[25]
FANC A/G/C	IR (5 Gy, 8 h) MMC (1 h/0.1 μg/ml, time course)	slightly reduced delayed	[43]
FANCA	IR (8 Gy, 2 h)	reduced	[44]
FANCD2	IR (12 Gy) MMC (40 ng/ml)	normal normal	[19]
FANC A/D1/F/I/J/L	IR (12 Gy, 7 h/24 h) MMC (1 h/2.4 μg/ml, 7 h/24 h)	reduced in FANCD1 cells reduced in FANCD1 cells	[26]
FANCJ (BACH1/BRIP1)	MMC HU (1 mM, 18 h)	normal normal	[20]
FANCD2	IR (2 and 15 Gy, 8 h)	reduced	[45]
Rodent			
CHO-*FANCG*	IR (8 Gy, 4 h)	normal	[46]
CHO-*BRCA2*	IR (12 Gy, 8 h) MMC (1 h/2.4 μg/ml, 8 h)	reduced reduced	[47]
Brca2	IR (10 Gy, 5 h)	reduced	[16]
Fanca	MMC (1 h/0.1 μg/ml, 9 h)	reduced	[17]
DT40			
fancd2	IR (8 Gy) MMC (1 h/0.5 μg/ml, time course)	normal normal	[40]
fancg	IR (8 Gy) MMC (1 h/0.5 μg/ml, time course)	normal normal	[41]

[a]HU: Hydroxyurea; IR: ionizing radiation; MMC: mitomycin C.

genes and FA patient cell lines have been analyzed for the integrity of RAD51 foci formation in response to IR or mitomycin C (MMC) (table 2).

As shown in figure 2, we found IR induced RAD51 foci formation was attenuated in FA cells of complementation groups FA-A, B, C, D1, D2, E, F and G [11]. This has been confirmed by several studies (table 2). However, the analysis of FA cells of the same complementation groups and cells of the newer complementation groups, FA-I, FA-J and FA-L, revealed a specific defect in RAD51 foci formation only in FA-D1 cells [25, 26]. Other reports in this context on single cell lines are also somewhat contradictory (table 2).

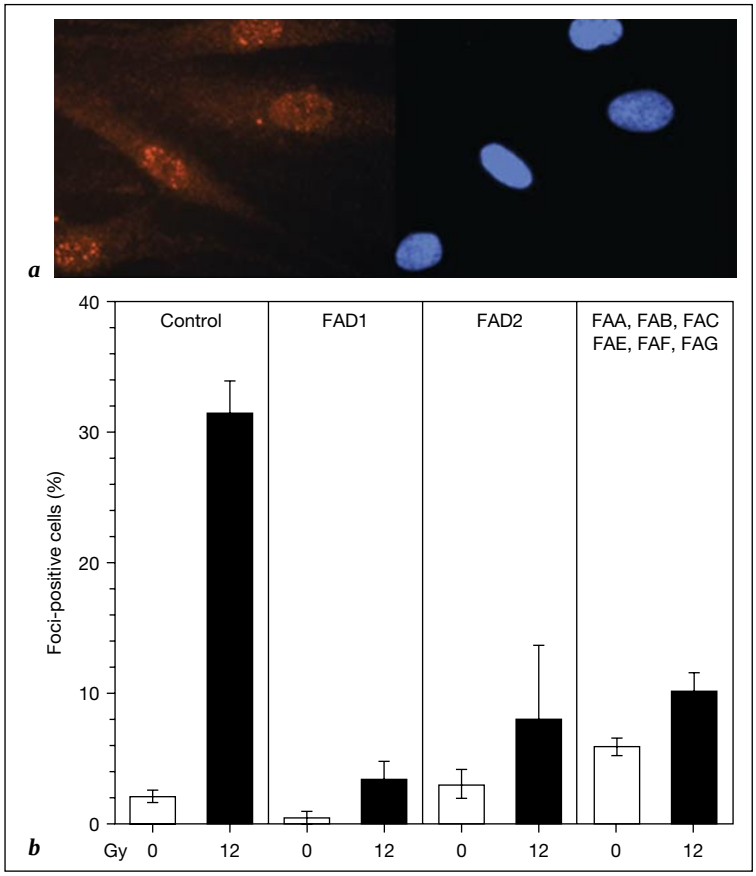


Fig. 2. ***a*** RAD51 nuclear foci observed in irradiated control cells. Primary fibroblasts were irradiated with 12 Gy and were fixed 8 h later, permeabilized and incubated with a primary rabbit antibody directed towards RAD51 followed by detection with a secondary Cy3-conjugated goat anti-rabbit Ig. Cells were counterstained with DAPI and examined microscopically using the appropriate filters. Representative digital images are shown. ***b*** Quantification of RAD51 foci in control and FA fibroblasts. The levels of RAD51 foci-positive cells are shown for control fibroblasts and fibroblasts from various FA complementation groups. Open columns represent unirradiated cells, filled columns are cells irradiated with 12 Gy and processed for immunofluorescence 8 h later.

Other Measures of HDR

The frequency with which plasmid-carried, homologous sequences are integrated into the mammalian genome, gene targeting efficiency, is a further sensitive method to analyze HDR [27]. Cell lines with mutations in FA genes

tested for this feature were concordant in targeting efficiency and HDR of a plasmid reporter (table 1).

The previously mentioned chicken DT40 cell line is derived from B lymphocytes and continuously undergoes immunoglobulin gene conversion in culture, a feature that can also be used to assess HDR at the sequence level. In those cases analyzed, results were in agreement with data from plasmid reporter assays, *fancc* and *fancd2* cells being deficient, and *fancm* cells showing proficiency, in immunoglobulin conversion (table 1).

Symmetrical exchanges between the two sister chromatids (sister chromatid exchanges, SCEs) can be visualized cytogenetically after DNA labeling with 5-bromodeoxyuridine (BrdU) during the preceding S-phase. SCEs occur spontaneously but their frequency can be enhanced by treatment with various DNA damaging agents. The exact mechanism by which SCEs are formed is not known, however, the interpretation of SCEs as the result of homologous recombination processes is widely accepted. Indeed, cells lacking key homologous recombination genes exhibit significantly reduced spontaneous and DNA-damage induced SCE levels [28]. Although spontaneous and induced SCE levels have been investigated in FA cells for more than thirty years, the reported studies are rather inconsistent (e.g. [29–31], see also table 1). However, cells from Bloom syndrome patients, deficient in a protein, BLM, which associates with the FA core complex [32], do show consistently excessive SCEs [33].

Concluding Remarks

Recent results have implicated at least a subset of FA proteins in HDR processes. FA proteins interact with HDR proteins in multiple direct and indirect ways: FANCD2 and FANCG interact physically with BRCA2/FANCD1, as shown in Yeast-Two-Hybrid analysis and in co-immunoprecipitations [9, 10]. Colocalizations in nuclear foci have been found in response to DNA damage for FANCD2, RAD51 and BRCA1 as well as for FANCG, BRCA2 and RAD51 [7, 9]. FANCD1/BRCA2 has been consistently reported to be essential for RAD51 foci formation [11, 25, 34]. The reason for contradictory reports on disturbed RAD51 foci formation in FA cells of complementation groups other than FA-D1 is not clear (table 2) but may possibly be related to cell cycle effects, or perhaps just reflect differences in foci formation kinetics.

The majority of studies on HDR based on the analysis of plasmid reporters after induction of a single defined DSB and tracking its repair, show reduced HDR, as summarized in table 1. In fact, for all analyzed FA genes, except *fancm*, there is at least one study reporting a deficiency in HDR. In two cases,

the analysis of the same cell lines (PD20, mutated in *FANCD2* and DT40, mutated in *fancc*) yielded contradictory results in different laboratories.

The observed deficiencies in HDR are relatively mild, especially when compared with the severe recombination defects seen in mutants of BRCA1, BRCA2, RAD51 and the Rad51 paralogues, XRCC2/XRCC3 [35–39]. This is in agreement with the viability of FA patients, and of mice mutated in FA genes, and suggests that the FA genes, except for *FANCD1/BRCA2*, are likely to be non-essential components of HDR.

In addition to recombination repair proteins, proteins from the excision repair pathway, FA proteins and components involved in translesion synthesis (TLS) have all been implicated in the repair of ICLs and there is growing evidence that FA proteins promote TLS [40]. Similarly, SSA is yet another pathway seemingly promoted by FA proteins [17, 18]. These recent discoveries suggest that FA proteins may be involved in early processing of DNA lesions or in stabilization of DNA repair intermediates before channeling them into an appropriate repair pathway. Future research will aim to define these FA protein functions more precisely by exploiting the powerful battery of experimental approaches already available.

References

1 Schroeder TM, Anschütz F, Knopp A: Spontane Chromosomenaberrationen bei familiärer Panmyelopathie. Humangenetik 1964;1:194–196.
2 Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. Cancer Res 1973;33:1829–1836.
3 McHugh PJ, Spanswick VJ, Hartley JA: Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. Lancet Oncol 2001;2:483–490.
4 De Silva IU, McHugh PJ, Clingen PH, Hartley JA: Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. Mol Cell Biol 2000;20:7980–7990.
5 Rothfuss A, Grompe M: Evidence for a DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway during the repair of DNA-interstrand crosslinks. Am J Hum Genet 2004;73:575.
6 Weterings E, van Gent DC: The mechanism of non-homologous end-joining: a synopsis of synapsis. DNA Repair 2004;3:1425–1435.
7 Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, et al: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. Mol Cell 2001;7:249–262.
8 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
9 Hussain S, Witt E, Huber PA, Medhurst AL, Ashworth A, Mathew CG: Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. Hum Mol Genet 2003;12:2503–2510.
10 Hussain S, Wilson JB, Medhurst AL, Hejna J, Witt E, et al: Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. Hum Mol Genet 2004;13:1241–1248.
11 Digweed M, Rothe S, Demuth I, Scholz R, Schindler D, et al: Attenuation of the formation of DNA-repair foci containing RAD51 in Fanconi anaemia. Carcinogenesis 2002;23:1121–1126.
12 Niedzwiedz W, Mosedale G, Johnson M, Ong CY, Pace P, Patel KJ: The Fanconi anaemia gene *FANCC* promotes homologous recombination and error-prone DNA repair. Mol Cell 2004;15: 607–620.

13 Mosedale G, Niedzwiedz W, Alpi A, Perrina F, Pereira-Leal JB, et al: The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat Struct Mol Biol 2005;2:763–771.
14 Bridge WL, Vandenberg CJ, Franklin RJ, Hiom K: The BRIP1 helicase functions independently of BRCA1 in the Fanconi anemia pathway for DNA crosslink repair. Nat Genet 2005;37:953–957.
15 Moynahan ME, Pierce AJ, Jasin M: BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell 2001;7:263–272.
16 Tutt A, Bertwistle D, Valentine J, Gabriel A, Swift S, et al: Mutation in *Brca2* stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. EMBO J 2001;20:4704–4716.
17 Yang YG, Herceg Z, Nakanishi K, Demuth I, Piccoli C, et al: The Fanconi anemia group A protein modulates homologous repair of DNA double-strand breaks in mammalian cells. Carcinogenesis 2005;26:1731–1740.
18 Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, et al: Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 2005; 102:1110–1115.
19 Ohashi A, Zdzienicka MZ, Chen J, Couch FJ: Fanconi anemia complementation group D2 (FANCD2) functions independently of BRCA2- and RAD51-associated homologous recombination in response to DNA damage. J Biol Chem 2005;280:14877–14883.
20 Litman R, Peng M, Jin Z, Zhang F, Zhang J, et al: BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. Cancer Cell 2005;8:255–265.
21 Bishop DK, Ear U, Bhattacharyya A, Calderone C, Beckett M, et al: Xrcc3 is required for assembly of Rad51 complexes in vivo. J Biol Chem 1998;273:21482–21488.
22 Takata M, Sasaki MS, Sonoda E, Fukushima T, Morrison C, et al: The Rad51 paralog Rad51B promotes homologous recombinational repair. Mol Cell Biol 2000;20:6476–6482.
23 O'Regan P, Wilson C, Townsend S, Thacker J: XRCC2 is a nuclear RAD51-like protein required for damage-dependent RAD51 focus formation without the need for ATP binding. J Biol Chem 2001;276:22148–22153.
24 Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK: The breast cancer susceptibility gene *BRCA1* is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. J Biol Chem 2000;275:23899–23903.
25 Godthelp BC, Artwert F, Joenje H, Zdzienicka MZ: Impaired DNA damage-induced nuclear Rad51 foci formation uniquely characterizes Fanconi anemia group D1. Oncogene 2002;21:5002–5005.
26 Godthelp BC, Wiegant WW, Waisfisz Q, Medhurst AL, Arwert F, et al: Inducibility of nuclear Rad51 foci after DNA damage distinguishes all Fanconi anemia complementation groups from D1/BRCA2. Mutat Res 2006;594:39–48.
27 Yamazoe M, Sonoda E, Hochegger H, Takeda S: Reverse genetic studies of the DNA damage response in the chicken B lymphocyte line DT40. DNA Repair 2004;3:1175–1185.
28 Sonoda E, Sasaki MS, Morrison C, Yamaguchi-Iwai Y, Takata M, Takeda S: Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. Mol Cell Biol 1999;19: 5166–5169.
29 Sperling K, Wegner RD, Riehm H, Obe G: Frequency and distribution of sister-chromatid exchanges in a case of Fanconi's anemia. Humangenetik 1975;27:227–230.
30 Fujiwara Y, Kano Y, Yamamoto Y: DNA interstrand cross-linking, repair, and SCE mechanism in human cells in special reference to Fanconi anemia. Basic Life Sci 1984;29(pt B):787–800.
31 Wilson JB, Johnson MA, Stuckert AP, Trueman KL, May S, et al: The Chinese hamster *FANCG/XRCC9* mutant NM3 fails to express the monoubiquitinated form of the FANCD2 protein, is hypersensitive to a range of DNA damaging agents and exhibits a normal level of spontaneous sister chromatid exchange. Carcinogenesis 2001;22:1939–1946.
32 Meetei AR, Sechi S, Wallisch M, Yang D, Young MK, et al: A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol 2003;23:3417–3426.
33 Chaganti RS, Schonberg S, German J: A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc Natl Acad Sci USA 1974;71:4508–4512.
34 Yuan SS, Lee SY, Chen G, Song M, Tomlinson GE, Lee EY: BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. Cancer Res 1999;59:3547–3551.

35 Westermark UK, Reyngold M, Olshen AB, Baer R, Jasin M, Moynahan ME: BARD1 participates with BRCA1 in homology-directed repair of chromosome breaks. Mol Cell Biol 2003;23: 7926–7936.
36 Moynahan ME, Cui TY, Jasin M: Homology-directed DNA repair, mitomycin-C resistance, and chromosome stability is restored with correction of a *Brca1* mutation. Cancer Res 2001;61: 4842–4850.
37 Stark JM, Pierce AJ, Oh J, Pastink A, Jasin M: Genetic steps of mammalian homologous repair with distinct mutagenic consequences. Mol Cell Biol 2004;24:9305–9316.
38 Johnson RD, Liu N, Jasin M: Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature 1999;401:397–399.
39 Pierce AJ, Johnson RD, Thompson LH, Jasin M: XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–2638.
40 Thompson LH, Hinz JM, Yamada NA, Jones NJ: How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. Environ Mol Mutagen 2005;45:128–142.
41 Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, et al: Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol Cell Biol 2003;23:5421–5430.
42 Yamamoto K, Hirano S, Ishiai M, Morishima K, Kitao H, et al: Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol Cell Biol 2005;25:34–43.
43 Pichierri P, Averbeck D, Rosselli F: DNA cross-link-dependent RAD50/MRE11/NBS1 subnuclear assembly requires the Fanconi anemia C protein. Hum Mol Genet 2002;11:2531–2546.
44 Djuzenova C, Flentje M, Plowman PN: Radiation response in vitro of fibroblasts from a Fanconi anemia patient with marked clinical radiosensitivity. Strahlenther Onkol 2004;180:789–797.
45 Wang X, Andreassen PR, D'Andrea AD: Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol 2004;24:5850–5862.
46 Tebbs RS, Hinz JM, Yamada NA, Wilson JB, Salazar EP, et al: New insights into the Fanconi anemia pathway from an isogenic *FancG* hamster CHO mutant. DNA Repair 2005;4:11–22.
47 Kraakman-van der Zwet M, Overkamp WJ, van Lange RE, Essers J, van Duijn-Goedhart A, et al: Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions. Mol Cell Biol 2002;22:669–679.

Ilja Demuth
Institut für Humangenetik, Charité Universitätsmedizin Berlin
Campus Virchow-Klinikum
Augustenburger Platz 1
13353 Berlin (Germany)
Tel. +49 30 450 566306, Fax +49 30 450 566904, E-Mail ilja.demuth@charite.de

Schindler D, Hoehn H (eds): Fanconi Anemia. A Paradigmatic Disease for the Understanding of Cancer and Aging. Monogr Hum Genet. Basel, Karger, 2007, vol 15, pp 211–225

Functional Knock-Down of Human *RAD51* for Testing the Fanconi Anemia-BRCA Connection

P. Rio[a,b], *H. Hanenberg*[a,c]

[a]Department of Pediatric Hematology, Oncology and Clinical Immunology, Children's Hospital, Heinrich-Heine-University, Düsseldorf, Germany; [b]Hematopoiesis and Gene Therapy Division, CIEMAT, Madrid, Spain; [c]Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Ind., USA

Abstract

One of the proteins essential in homologous recombination is RAD51, a recombinase involved in nucleoprotein filament formation. After DNA damage, RAD51 colocalizes in nuclear foci with other proteins involved in DNA repair. This foci formation is regulated by BRCA2/FANCD1. As *Rad51* deficiency is lethal in mice and a human disorder due to defects in *RAD51* does not exist, the aim of the current study was to develop a system to knock-down human *RAD51* in an inducible form. For this purpose, we took advantage of the regulatable shRNA delivery system developed by Wiznerovicz and Trono [1] based on the cotransduction of target cells with two lentiviral vectors. The first vector carries expression cassettes for the shRNA and for a marker gene. The second vector constitutively expresses the transcription suppressing protein tTRKRAB whose activity can be regulated via doxycycline (DOX). Here, we show the ability of lentiviral vectors expressing shRNAs designed against human *RAD51* to downregulate the protein expression in primary human fibroblasts and in HeLa cells. This effect on the protein levels of RAD51 was controllable by DOX and allowed to visualize RAD51 foci formation in cells via a gammaretroviral vector expressing RAD51 fused to the enhanced green fluorescent protein (EGFP). This inducible system for visualization of fluorescence-tagged essential cellular proteins might facilitate to study the role of RAD51 and its interaction with members of the FA/BRCA pathway in response to defined DNA damages.

As described in the preceding chapter by Demuth and Digweed, there is increasing evidence for the involvement of at least some of the FA-genes in

recombinational types of DNA repair. A central player in this type of repair is the *BRCA2* gene which recruits the RAD51 recombinase to the site of DNA damage. Formal proof for a direct connection between the FA and BRCA pathways was the identification of *BRCA2* as the gene mutated in patients belonging to the clinically most severe form of FA, the FA-D1 complementation group [2]. The recent discoveries of the BRCA1 interacting protein, BRIP1, as FANCJ [3, 4] and of the partner and localizer of BRCA2, PALB2, as FANCN [5] further strengthens the notion that FA genes acting downstream of FANCD2 are involved in homology directed types of DNA repair [6]. Although several interactions of upstream FA proteins with BRCA1 and BRCA2 have also been described [7, 8], the exact role of the FA pathway in the repair of DNA lesions induced by crosslinking agents such as mitomycin C (MMC) still remains elusive.

One of the proteins essential for the execution of DNA repair via homologous recombination is RAD51. RAD51 is a recombinase involved in the formation of the nucleoprotein filaments on single-stranded (ss) DNA mediating homologous pairing and strand exchange reactions between ssDNA and homologous double-stranded (ds) DNA [9, 6]. After DNA damage, RAD51 localizes in repair foci in the nucleus of cells together with other proteins essential for recombinational DNA repair [10]. In addition to BRCA1 and BRCA2/FANCD1, this also includes RPA which in close cooperation with RAD51 facilitates homologous pairing and DNA strand exchange [11]. The presence of RAD51 in these foci depends on BRCA2/FANCD1. The BRC repeats in the exon 11 of *BRCA2* directly interact with RAD51 and control the correct RAD51 oligomerization and filament formation [12, 13]. At least in murine cells, BRCA2 can also interact with RAD51 via a C-terminal motif [14]. In patients belonging to complementation group FA-D1, who have been shown to carry biallelic mutations in *BRCA2*, nuclear RAD51 foci are almost completely absent and not inducible by irradiation or DNA crosslinking agents [15]. In contrast to its counterpart RecA in *E. coli* (31) and scRad51 in yeast [16, 17], the mammalian RAD51 protein appears to be essential for cell survival, as targeted disruption of *Rad51* induces early embryonic lethality in mice [18]. In addition, there is neither a *RAD51* gene defect known in humans nor does a human cell line deficient in *RAD51* exist. Therefore, the aim of the current study was to develop a tool to conditionally knock down RAD51 levels in FA and normal primary cells, thereby facilitating functional studies on the role of RAD51 in the FA/BRCA pathway.

RNA interference (RNAi)-mediated gene silencing has emerged as a powerful approach to regulate levels of endogenous proteins and to analyze gene functions in many organisms [19]. In mammalian cells, RNAi-mediated gene silencing can be obtained by the delivery of chemically synthesized short (<30 nt) double-stranded siRNA molecules [20]. Alternatively, plasmid and viral vector-based systems have been developed for stable expression of short

hairpin RNAs (shRNAs) which then are processed to siRNA in the target cells [21]. In some instances, where the knock-out/down of a target gene is lethal for the cell, such as *RAD51*, alternative approaches for the controlled suppression of protein levels are needed. In the present study, we took advantage of the gene targeting system developed by Wiznerovicz and Trono [1] using lentiviral vectors for the production of siRNAs in a drug inducible system. In the targeting vector, the shRNA is expressed under the control of human H1 promoter located in the 3′ SIN LTR. Also included in the 3′ LTR are tet operator (tetO) sequences which directly bind the tetracycline repressor (tTR). Via a second vector, the KRAB domain of human *KOX1* fused to tTR is constitutively expressed in the target cell. In the absence of doxycycline (DOX), this tTRKRAB protein will bind to the tetO element and suppress any polymerase II and III promoters within 3 kb in an orientation independent manner. In the presence of DOX, tTRKRAB is sequestered away from tetO thereby allowing gene expression from both promoters, the H1 promoter driving the shRNA and the internal EF1α promoter driving EGFP as a marker gene for the easy detection of transduced cells and promotor activities. Using this targeting strategy, we established an inducible *RAD51* knock-down system that can be applied to the study of homologous recombination in human primary cells and cell lines, including cells derived from FA patients.

Materials and Methods

siRNA Transfection

Two different siRNAs against RAD51 were used (Invitrogen, Karlsruhe, Germany). A fluorescent oligo labeled with FITC was used as a control (Invitrogen). HeLa cells were transfected using lipofectamin 2000 following manufacturer's instructions (Qiagen, Hilden, Germany). Transfection efficiency was assessed by flow cytometry analysis of cells transfected with the fluorescent oligo.

Vector Construction

shRNAs against RAD51 were ordered as phosphorylated oligos with *Cla*I and *Mlu*I overhangs, subsequently annealed and then ligated into the LV-THM vector cut in the same way using standard protocols.

S11EGRAIN was constructed by introducing the EGFP cDNA into the retroviral vector S11RAIN (Velleuer and Hanenberg, unpublished) expressing human RAD51 linked via an EMCV IRES site to the neomycin phosphotransferase (*NEO*) gene under the control of the SFFV promotor. The presence of the *NEO* gene in the vector allowed us to select transduced cells using G418 (0.5 mg/ml, GIBCO/Invitrogen).

Supernatant Production

Lentiviral Vectors: HEK293T cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin

G and streptomycin (all from GIBCO/Invitrogen). Subconfluent HEK293T cells were cotransfected with 10 μg of the plasmid lentiviral vector, 10 μg of pCMV-DR8.91 and 10 μg of pMD2G-VSV-G using Fugene 6 (Roche Diagnostics, Mannheim, Germany). After 24 h, medium was changed and recombinant lentivirus containing supernatants were harvested 24 h later, filtered to 0.45 μm and then used freshly or stored at −80°C.

Retroviral Vectors: Ecotropic Phoenix (eFNX) cells were cultured under the same conditions as 293T cells. After seeding, eFNX cells were transfected with 20 μg of S11EGRAIN or S11EGIN using Fugene 6. Supernatants were harvested 48 h after transfection as described. 3.5×10^4 PG13 cells were seeded on a plate in complete DMEM medium and then transduced with supernatant obtained from eFNX cells. 48 h later, transduced PG13 cells were selected as bulk cultures with 0.5 mg/ml G418 (Gibco) and expanded. Supernatants were obtained as described above.

Primary Fibroblast and HeLa Cell Transduction

Lentiviral Vectors: 3.5×10^4 primary human fibroblasts were seeded per well of gelatinized 6-well plates and the next day transduced by LV-THM vectors expressing different shRNAs against human *RAD51*: shRNA5 5′-GCGCCAAAGAAGGAGCTAA-3′, shRNA6 5′-CCACCAGACCCAGCTCCTTTA-3′, shRNA7 5′-GCAGTGATGTCCTGGATAA-3′. HeLa cells were plated in 6-well plates (3.5×10^4 cells/well). After 24 h, 1 ml of medium containing LV-THM virus was added. The following day, supernatants were replaced by complete DMEM. To study the ability of the regulatable system to control EGFP expression, cells were cotransduced with 1:1 mixture of LV-THM and LV-tTRKRAB, respectively. Two days after transduction, doxycycline (DOX, Ratiopharm, Ulm, Germany) was added in different concentrations ranging from 2.5 μg/ml to 12.5 μg/ml. Five days after treatment, cells were analyzed by flow cytometry for the expression of EGFP. For experiments using 5 μg/ml of DOX, cells were analyzed every day for five days to determine the time period necessary for maximal EGFP expression.

Retroviral Vector: HeLa cells were plated in 6-well plates (3.5×10^4 cells/well). After 24 h, 1 ml of medium containing S11EGRAIN vector was added. The following day the supernatant was replaced by fresh DMEM supplemented with G418 for 7 days.

Flow Cytometry Analysis

HeLa cells were trypsinized, washed with PBS and then analyzed by flow cytometry using a FACScan (Becton Dickinson, Heidelberg, Germany) for EGFP fluorescence using standard filters. Cell quest software was used for analysis of the files containing 20,000–50,000 events.

Western Blot

Western blot analyses were performed using extracts of fibroblasts, collected by centrifugation, washed twice in PBS, lysed in 1× lysis buffer (50 nM Tris-HCl, 70 mM 2-mercaptoethanol, and 2% sodium dodecylsulfate (SDS), then boiled for 5 min, and subjected to 4–12% Nupage (Invitrogen). After electrophoresis, proteins were transferred to a nitrocellulose membrane using a submerged transfer apparatus (BioRad, Hercules, CA), filled with 25 mM Tris Base, 200 mM glycine, and 20% methanol. After blocking with 5% non-fat dried milk in TBS-T (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) the membrane was incubated with the primary antibodies (anti-RAD51, Calbiochem; anti-GFP or anti-β-actin, Abcam, Cambridge, MA) diluted in TBS-T. The detection was performed

with the Western Breeze Immunodetection Kit (Invitrogen). The concentration of protein was measured by the Bradford assay.

Immunofluorescence Studies of RAD51

For immunofluorescence studies, cells were fixed with 3.7% paraformaldehyde in PBS for 15 min followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. After 30 min in blocking buffer (10% FBS, 0.1% NP-40 in PBS) cells were incubated with anti-RAD51 anti-rabbit antibody (PC130) from Calbiochem and anti-GFP (Abcam) at 1/200 dilution. Cells were then washed three times in TBS and subsequently incubated with the TexasRed-conjugated anti-rabbit polyclonal antibody (Jackson Immunoresearch Laboratories, Cambridgeshire, CA). After 45 min, cells were washed three times with TBS and the slides were mounted in Vectashield (Vector Laboratories Burlingame, CA) with 4,6-diamidino-2-phenylindole (DAPI).

Results

Down-Regulation of RAD51 Protein Expression Using siRNA

HeLa cells were transfected with two different siRNAs designed against hRAD51 that are commercially available. In parallel, a fluorescent oligonucleotide labeled with FITC was utilized to verify by flow cytometry that 75% of the HeLa cells were transfected with the siRNA (fig. 1a). On these cells, western blot analysis was performed to analyze the kinetics of *RAD51* knock-down 24, 48 and 72 h after transfection. siRNA1 but not siRNA2 inhibited *RAD51* mRNA as evidenced by the absence of RAD51 protein (fig. 1b). When both siRNAs were transfected simultaneously, a similar inhibition of RAD51 protein translation was achieved. The downregulation of RAD51 protein was limited to the first 24 h interval after transfection, whereas there was no change of RAD51 protein levels at 48 h and 72 h. When we analyzed the ability of HeLa cells to form RAD51 foci 24 h after transfection, a marked depletion of MMC-induced RAD51 foci was only observed in cells transfected with siRNA1 (fig. 1c).

Stable Inhibition of RAD51 in Primary Fibroblast by Lentiviral shRNA

As the inhibition of RAD51 translation via siRNA1 was only transient, lasting no longer than 24 h, we subsequently explored a lentivirus-based stable shRNA system for RAD51 downregulation. Lentiviral vectors were constructed using the LV-THM as backbone kindly provided by Didier Trono [1]. Different shRNAs under the control of H1 promoter were cloned into the *Cla*I-*Mlu*I sites (fig. 2a) and tested for their ability to transduce primary human fibroblasts detectable by the simultaneous analysis of EGFP and knock-down of RAD51 protein.

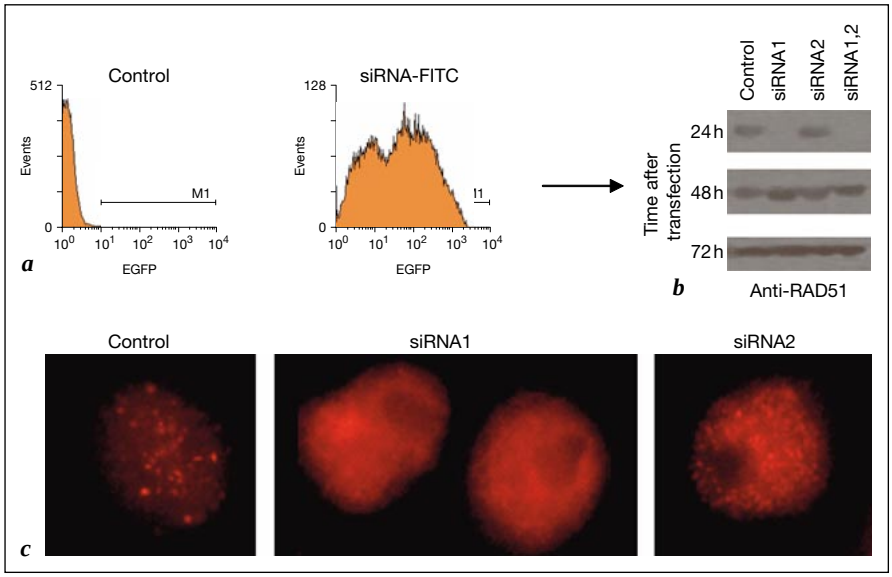


Fig. 1. Transfection of HeLa cells with two different siRNAs against RAD51. (***a***) HeLa cells were cotransfected with a fluorescent oligo (FITC) to analyze the efficiency of transfection. (***b***) Western blot of HeLa cells transfected with siRNA1, 2, or both. Western blot analyses were performed on cells harvested 24, 48 and 72 h after transfection. (***c***) Ability of HeLa cells transfected with RAD51 siRNAs to form RAD51 foci after induction of DNA damage with MMC. RAD51 foci appeared red due to staining with a Tx-Red labeled secondary antibody.

Lentivirus containing supernatant was produced in HEK293T cells with VSV-G as envelope protein and used to transduce primary human fibroblasts. Flow cytometric analysis revealed that more than 90% of the primary cells were EGFP positive, indicating a high rate of transduction (data not shown). As RAD51 protein appears in multiple discrete foci at sites of DNA damage, we next analyzed the ability of fibroblasts transduced with lentiviral vectors expressing different shRNA against RAD51 to form RAD51 foci after induction of DNA damage with MMC. Figures 2b and 2c demonstrate that cultures with untransduced cells (control) and cells transduced with a control vector (LV-THM) had similar percentages of cells with RAD51 foci after DNA damage. In contrast, cells transduced with LV-THM expressing RAD51 shRNA5, -6 and -7 displayed a distinctly reduced ability to form foci after DNA damage. When quantified as the number of foci per EGFP positive cell (fig. 2b and 2c), the effects are particularly pronounced for shRNA6 and -7, with the number of

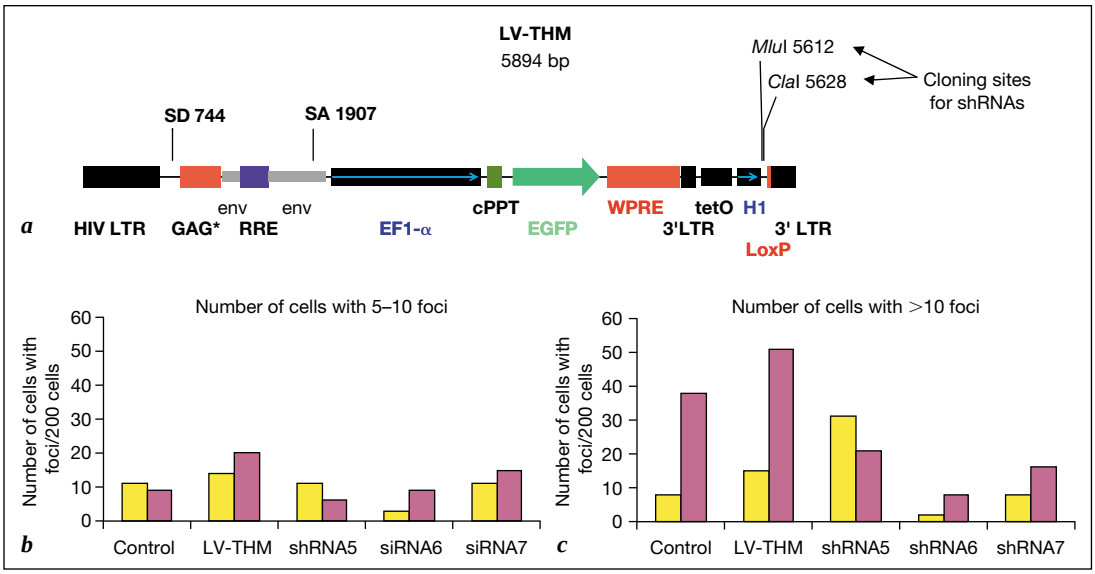


Fig. 2. (***a***) Map of the lentiviral vector LV-THM expressing shRNA. The shRNAs were cloned between the *Mlu*I and *Cla*I sites and expressed under control of the human H1 promoter. The EF1α promoter was used to express the enhanced green fluorescent protein (EGFP) as marker gene. (***b, c***) Number of RAD51 foci per 200 cells was counted in primary fibroblasts after transduction with LV-THM vectors expressing the shRNA5, -6 and -7 against RAD51. As control, nontransfected cells and cells transduced with LV-THM vector were utilized. (***b***) Number of cells with 5–10 foci per cell. (***c***) Number of cells with more than 10 foci per cell. Number of cells with foci after MMC treatment (purple column) and without MMC (yellow).

cells containing more than 10 foci per cell being strongly reduced compared to control cells. These results suggest that although formation of RAD51 foci is not completely abolished, shRNA6 and -7 expressing cells experience at least partial inhibition of RAD51 mRNA processing.

Doxycyclin-Regulated Activity of the LV-THM Lentiviral Vector

As there are no human cell lines deficient for RAD51, obviously due to the absolute requirement of the protein for long-term cellular survival [22], we next modified our lentiviral shRNA delivery system, aiming at an inducible interference with RAD51 expression via the DOX system. To this end, cells were transduced in parallel with the LV-tTRKRAB vector kindly provided by Didier Trono [1]. Expression of the tTRKRAB fusion protein inhibits the

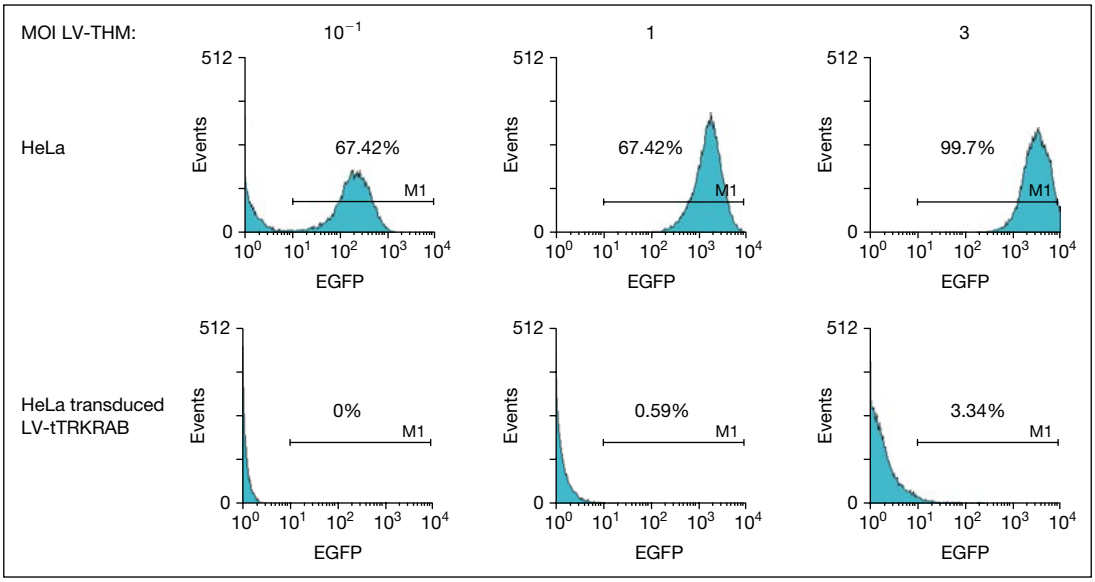


Fig. 3. Regulation of EGFP expression by LV-tTRKRAB vector. HeLa cells were transduced with LV-THM vector using different MOIs. The same cells were transduced with LV-tTRKRAB vector. The percentage of EGFP positive cells was determined by flow cytometry analysis.

transcription of both promotors, H1 and EF1α. Thus, without DOX in the medium, cells are neither green nor do they express the transfected shRNAs. In order to test the efficiency of this system, HeLa cells were infected with different multiplicities of infection (MOI) of the LV-THM vector while keeping the LV-tTRKRAB vector at a constant MOI. As shown in figure 3, LV-tTRKRAB can efficiently inhibit EGFP expression if the MOI for LV-THM is 1 or lower. At higher MOIs, the tTRKRAB mediated control of transcription appeared to be less stringent.

As a next step, we analyzed the optimal conditions for DOX dependent regulation of transcription in cells transduced with both the lentiviral shRNA vector and the LV-tTRKRAB vector. As read-out, the recovery of EGFP expression was analyzed by flow cytometry using 5 different concentrations of DOX ranging from 2.5 to 12.5 μg/ml (fig. 4). The results show that DOX concentrations of 2.5 and 5 μg/ml are sufficient to reexpress EGFP in all transduced cells. As concentrations of 7.5 μg/ml and higher markedly influenced cell growth kinetics (data not shown), 5 μg/ml DOX was used in all further experiments.

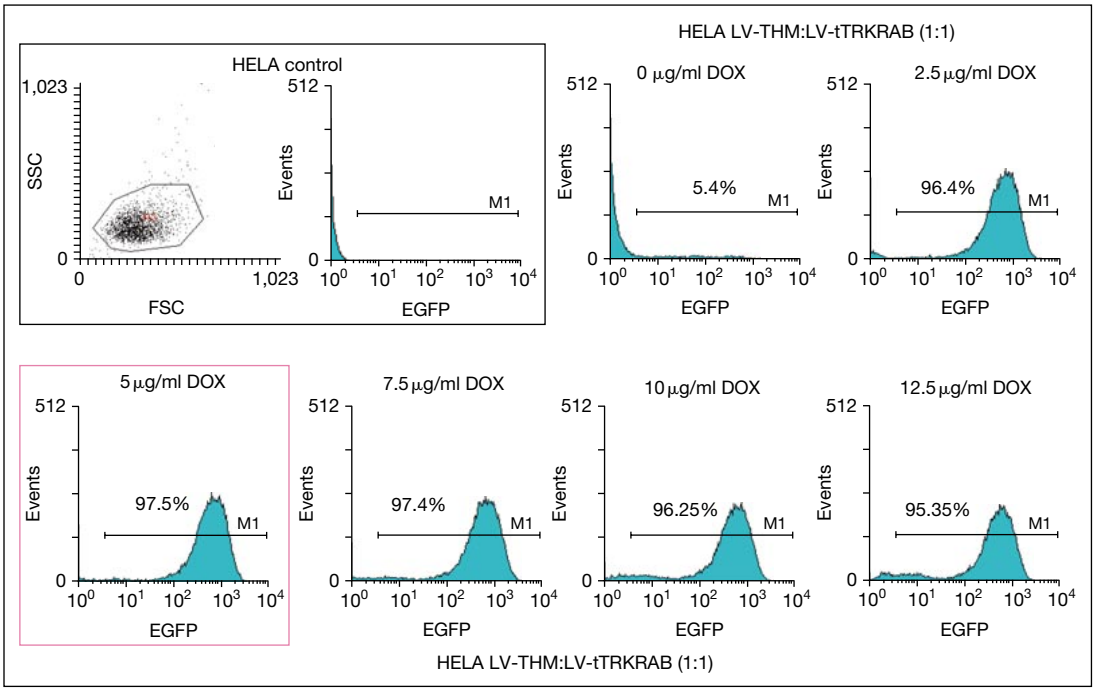


Fig. 4. Cells cotransduced with LV-THM and LV-tTRKRAB vector were incubated with different concentrations of doxycycline (DOX). HeLa cells were analyzed by flow cytometry five days after incubation with 0–12.5 μg/ml DOX.

We also tested the time period after adding DOX to LV-tTRKRAB expressing cells that would be required to completely abrogate the negative effect of the tTRKRAB protein on transcription from the H1 and EF1α promoters of the LV-THM vector. To this end, the percentage of cells transduced with both LV-THM and LV-tTRKRAB at a ratio of 1:1 was analyzed for the expression of EGFP in the presence of 5 μg/ml DOX. As shown in figure 5, flow cytometric analysis at 24 h intervals revealed that the tTRKRAB mediated inhibition of transcription starts to decrease at 24 h after incubation with DOX. It nevertheless took 72 h until the maximum EGFP expression occurred as determined by the percentage of EGFP positive cells and the mean fluorescence intensity (MFI) of cells in region M1 (data not shown).

DOX-Controlled Regulation of RAD51 and EGFP Protein Levels

With the above established conditions for optimal application of the LV-THM/LV-tTRKRAB system, we determined the time course of inhibition of

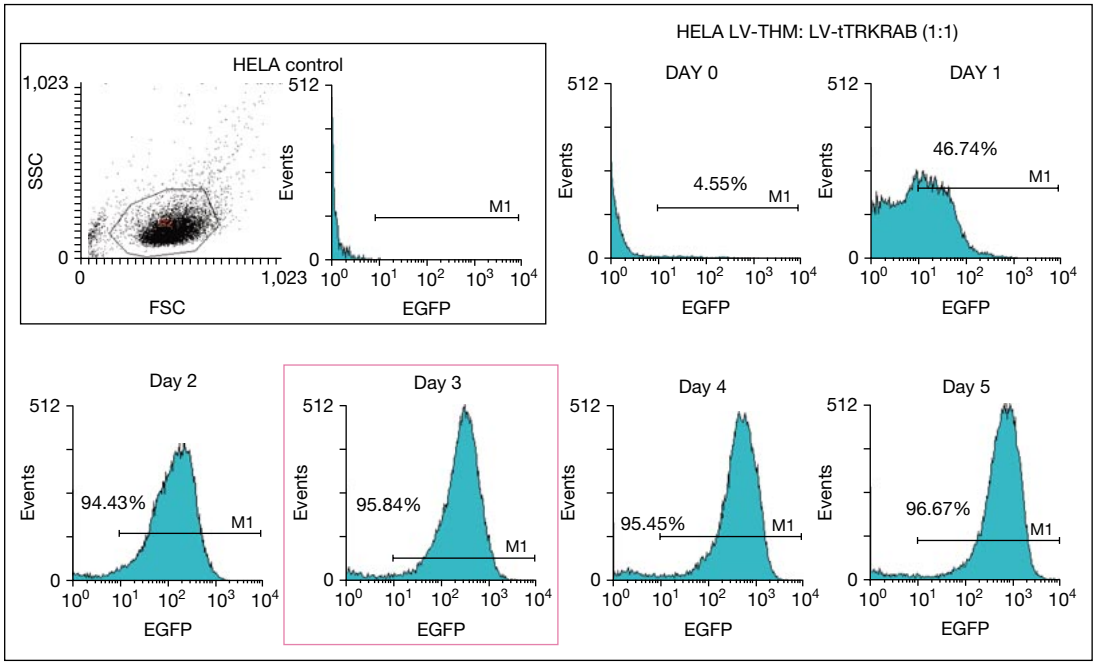


Fig. 5. Time for DOX to sequester tTRKRAB thereby inducing EGFP expression. Cells cotransduced with LV-THM and LV-tTRKRAB were treated with 5 μg/ml of DOX and the expression of EGFP was analyzed by flow cytometry between day 0 and day 5 after treatment.

RAD51 and of EGFP expression in HeLa cells cotransduced with LV-THM shRNA-6 and LV-tTRKRAB at a ratio of 1:1. Western blot analyses revealed that after three days of incubation with 5 μg/ml DOX, transduced HeLa cells achieved the maximal upregulation of EGFP in concert with downregulated RAD51 (fig. 6). The inverse parallel regulation of the two proteins by simple addition of DOX to the culture medium underlines the effectiveness of this lentiviral vector system for the induced expression of the shRNA cassette as well as for visualization of cells in which the shRNA cassette is actively expressed.

Expression of EGFP-Tagged RAD51

Controlled knockdown of endogenous RAD51 expression offers the unique possibility to visualize RAD51 protein trafficking in live cells by over-expression of a fluorescence tagged protein. For this purpose we constructed a retroviral vector expressing a fusion protein between RAD51 and EGFP (fig. 7a).

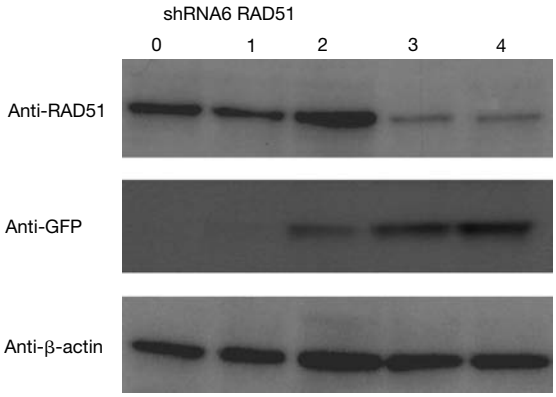


Fig. 6. Regulation of RAD51 and EGFP expression in primary fibroblasts cotransduced with the LV-THM/RAD51_shRNA6 and LV-tTRKRAB vectors. After transduction, cells were incubated with DOX (5 μg/ml) for 4 days. Samples were analyzed daily by western blot for RAD51 and EGFP expression. β-actin was used as loading control.

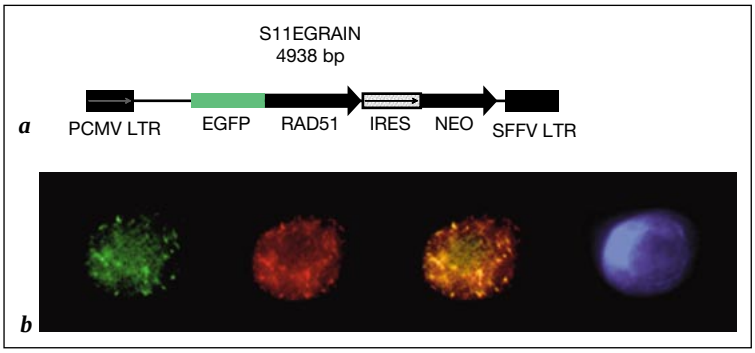


Fig. 7. Cotransduction of HeLa cells with LV-THM/RAD51_shRNA6 and S11EGRAIN. (***a***) The gammaretroviral vector S11EGRAIN expressing a fusion between *EGFP* and human *RAD51* genes under the control of the SFFV promoter linked via an EMCV IRES site to the neomycin phosphotransferase (*NEO*) was used to transduce HeLa cells also infected with LV-THM/RAD51_shRNA6. (***b***) The expression of RAD51 protein in cells was visualized by the stainings with antibodies directed against EGFP and hRAD51. The expression of the EGFP-RAD51 fusion protein after antibody staining is depicted in green. RAD51 foci formation is shown by staining with an anti-RAD51 antibody and a TxRed labeled secondary antibody. The overlap of both pictures revealed the normal distribution of EGFP-RAD51 fusion protein in cells. The DAPI staining of the nucleus is shown in blue.

In order to allow for selection of transduced cells, the vector constructs also coexpressed the neomycin phosphotransferase gene (*NEO*) via an encephalo myocarditis virus (EMCV) internal ribosomal entry site (IRES). To test the feasibility of this approach, HeLa cells stably expressing the shRNA6 directed against RAD51 were co-transduced with the S11EGRAIN vector and selected with G418. Using foci formation after staining with GFP and RAD51 antibodies as read-out (fig. 7b) demonstrates that exogenous EGFP-tagged RAD51, much like wildtype protein, is assembled into nuclear foci after DNA damage. These results suggest that the fusion protein retains its RAD51 specific function.

Discussion

The RAD51 recombinase is a key player for the execution of homologous recombination. Downregulation of RAD51 sensitizes cells to DNA-damaging agents [23], whereas increased RAD51 protein levels have been implicated in uncontrolled recombination, genome instability and increased resistance of tumor cells to radio- and chemotherapy [24]. In addition to its multiple interactions with proteins such as p53, BLM, c-Abl, stat 5, BRCA1 and others, the interaction between BRCA2 and RAD51 has revealed its crucial contribution to homology directed DNA repair [24]. As biallelic mutations in *BRCA2* cause a severe form of FA with an early tumor phenotype, the exploration of the precise role of FA-related proteins in homology directed DNA repair would benefit greatly from an experimental system in which the activity of the RAD51 recombinase could be fine-tuned and controlled. Our present results suggest that we are very close to this goal.

The elegant lentiviral shRNA system described by Trono's group [1, 21] has several advantages over siRNA transfection or other viral shRNA transfer systems such as AAV2 or gammaretroviral vectors. Recombinant lentiviruses especially when pseudotyped with the VSV-G glycoprotein are remarkably efficient for the delivery of expression cassettes of genes or shRNAs to adherent cells, usually resulting in transduction efficiencies in excess of 95% of cells. We here show that this high transfer efficiency is also observed in primary cells. Use in combination with the visualization of single transduced cells by simultaneous EGFP expression avoids artifact-prone selection or enrichment procedures which are essential in case the suppressed gene is critical for cell survival.

Our experiments have established suitable conditions for creating a regulatable RAD51 knock-down in HeLa cells. Maximum targeting effects were achieved after a 3-day exposure to 5 μg/ml DOX in cells simultaneously

transduced at a 1:1 ratio with shRNA and tTRKRAB expressing vectors. We also showed that knock-down using a single shRNA construct still allowed RAD51 foci formation in primary human cells albeit at reduced levels. We finally demonstrated that overexpression of a 5′ EGFP tagged human RAD51 protein in combination with knockdown of endogenous RAD51 protein led to unimpaired foci formation as detected in transduced cells through the EGFP tag.

Previous results showed that a vector expressing RAD51 fused to GFP was mainly expressed in the cytoplasm of cells. Translocation into the cell nucleus occurred only in response to DNA damage [25]. Although we still have to formally prove that our EGFP-RAD51 fusion protein functions normally and that its in vivo expression is stable over time, the normal distribution of EGFP positive RAD51 foci after DNA damage that we observed in cells with knock-down of the endogenous RAD51 suggests that the combination of the vectors we describe here could be an important approach for studying the role of RAD51 in cells of FA patients and normal controls. The possibility to induce local DNA damage employing a UV laser and confocal microscopy offers an exciting tool to describe the kinetics of RAD51 mobilization in living cells and to visualize the movement of FA proteins tagged with other fluorescent dyes to the site of damaged DNA. This approach might also be useful to study how homologous recombination (HR) takes place in cells of FA patients belonging to complementation group FA-D1, and how different mutations in *BRCA2* resulting in truncated proteins might still allow residual HR activity.

There is clear need for further improvement of our retroviral vectors as the shRNA against the RAD51 ORF will also influence the EGFP-tagged overexpressed protein. This problem can be addressed by introducing silent mutations in the *RAD51* cDNA thereby changing the particular sequences that are used for the shRNA targeting. In addition, the mutated and fluorescent tagged protein could be introduced into cells with the same lentiviral vector as the shRNA. With this design, it would be possible to utilize one construct to do both, overexpression of a tagged exogenous and knock-down of the endogenous protein. In addition, *EGFP* as a marker to visualize transduced cells should be replaced in the shRNA vector either by selectable genes such as *NEO* or by surface expressed molecules that enable selection of transduced cells by MACS or FACS. With these modifications, a multicolor approach using the same regulatable shRNA vector construct to target the endogenous protein and to overexpress a fluorescence-tagged version of the same protein appears to provide a fascinating starting point to visualize and better understand the complex mechanisms in FA cells undergoing HR following exposure to crosslinking agents.

Acknowledgements

We would like to thank Didier Trono for providing his inducible lentiviral shRNA vector system. We are deeply indebted to Juan Bueren for his continuous support and helpful discussions. This study was supported by the Fanconi Anemia Research Fund, (FARF), Inc., Portland, Oregon, U.S.A., and the Elterninitiative Kinderkrebsklinik e. V., Duesseldorf, Germany.

References

1 Wiznerowicz M, Trono D: Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J Virol 2003;77:8957–8961.
2 Howlett NG, Taniguchi T, Olson S, Cox B, Waisfisz Q, et al: Biallelic inactivation of *BRCA2* in Fanconi anemia. Science 2002;297:606–609.
3 Levitus M, Waisfisz Q, Godthelp BC, de Vries Y, Hussain S, et al: The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. Nat Genet 2005;37:934–935.
4 Levran O, Attwooll C, Henry RT, Milton KL, Neveling K, et al: The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. Nat Genet 2005;37:931–933.
5 Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, et al: Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2007;39:162–164.
6 Litman R, Peng M, Jin Z, Zhang F, Zhang J, et al: BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. Cancer Cell 2005;8:255–265.
7 Levitus M, Joenje H, de Winter JP: The Fanconi anemia pathway of genomic maintenance. Cell Oncol 2006;28:3–29.
8 Mirchandani KD, D'Andrea AD: The Fanconi anemia/BRCA pathway: a coordinator of cross-link repair. Exp Cell Res 2006;312:2647–2653.
9 Baumann P, Benson FE, West SC: Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. Cell 1996;87:757–766.
10 Haaf T, Golub EI, Reddy G, Radding CM, Ward DC: Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. Proc Natl Acad Sci USA 1995;92:2298–2302.
11 Golub EI, Gupta RC, Haaf T, Wold MS, Radding CM: Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. Nucleic Acids Res 1998;26:5388–5393.
12 Galkin VE, Esashi F, Yu X, Yang S, West SC, Egelman EH: BRCA2 BRC motifs bind RAD51-DNA filaments. Proc Natl Acad Sci USA 2005;102:8537–8542.
13 Pellegrini L, Yu DS, Lo T, Anand S, Lee M, et al: Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. Nature 2002;420:287–293.
14 Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, et al: Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking BRCA2. Nature 1997;386:804–810.
15 Godthelp BC, Wiegant WW, Waisfisz Q, Medhurst AL, Arwert F, et al: Inducibility of nuclear Rad51 foci after DNA damage distinguishes all Fanconi anemia complementation groups from D1/BRCA2. Mutat Res 2006;594:39–48.
16 Muris DF, Vreeken K, Carr AM, Broughton BC, Lehmann AR, et al: Cloning the RAD51 homologue of *Schizosaccharomyces pombe*. Nucleic Acids Res 1993;21:4586–4591.
17 Shinohara A, Ogawa H, Ogawa T: Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell 1992;69:457–470.
18 Tsuzuki T, Fujii Y, Sakumi K, Tominaga Y, Nakao K, et al: Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. Proc Natl Acad Sci USA 1996;93:6236–6240.
19 Sandy P, Ventura A, Jacks T: Mammalian RNAi: a practical guide. Biotechniques 2005;39:215–224.

20 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–498.
21 Wiznerowicz M, Szulc J, Trono D: Tuning silence: conditional systems for RNA interference. Nat Methods 2006;3:682–688.
22 Raderschall E, Bazarov A, Cao J, Lurz R, Smith A, et al: Formation of higher-order nuclear Rad51 structures is functionally linked to p21 expression and protection from DNA damage-induced apoptosis. J Cell Sci 2002;115:153–164.
23 Ohnishi T, Taki T, Hiraga S, Arita N, Morita T: In vitro and in vivo potentiation of radiosensitivity of malignant gliomas by antisense inhibition of the RAD51 gene. Biochem Biophys Res Commun 1998;245:319–324.
24 Henning W, Stuerzbecher HW: Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance. Toxicology 2003;193:91–109.
25 Kraakman-van der Zwet M, Overkamp WJ, van Lange RE, Essers J, van Duijn-Goedhart A, et al: Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions. Mol Cell Biol 2002;22:669–679.

Helmut Hanenberg, M.D.
Department of Pediatric Hematology, Oncology and Clinical Immunology
Children's Hospital, Heinrich-Heine-University, Moorenstr. 5
D–40225 Düsseldorf (Germany)
Tel. +49 211 811 6103, Fax 49 211 811 9436, E-Mail hanenberg@uni-duesseldorf.de

Author Index

Subject Index