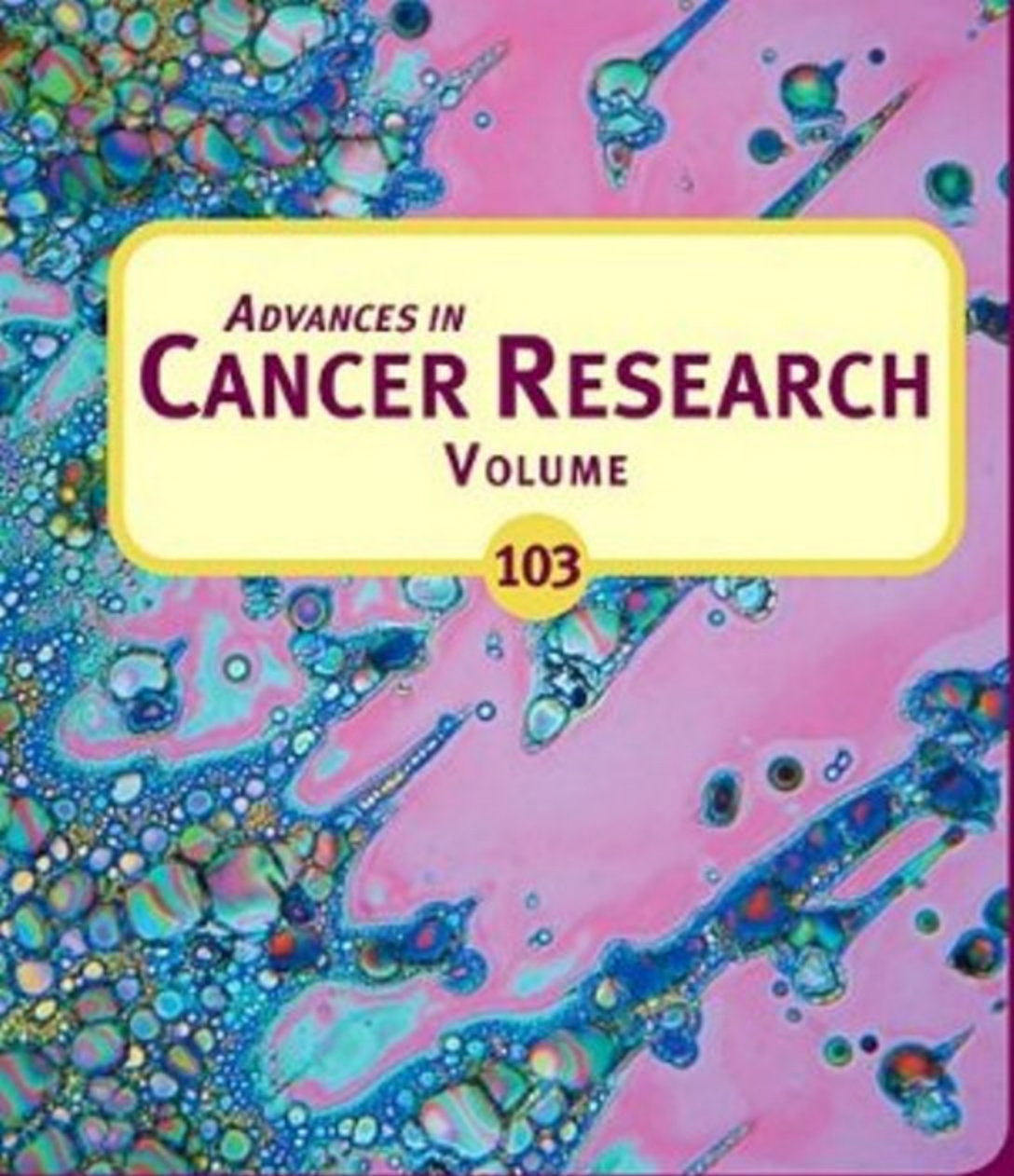

Advances in Cancer Research

Volume 103

Edited by

George F. Vande Woude

George Klein

Advances in CANCER RESEARCH

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525 B Street, Suite 1900, San Diego, CA 92101-4495, USA
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
32 Jamestown Road, London, NW1 7BY, UK
Linacre House, Jordan Hill, Oxford OX2 8DP, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2009

ISBN: 978-0-12-374773-0
ISSN: 0065-230X

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Printed and bound in USA

09 10 11 12 10 9 8 7 6 5 4 3 2 1

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The Function, Proteolytic Processing, and Histopathology of Met in Cancer

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The hepatocyte growth factor (HGF) and its receptor, the Met receptor tyrosine kinase, form a signaling network promoting cell proliferation, invasion, and survival in normal and cancer cells. Improper regulation of this pathway is attributed to many cancer types through overexpression, activating mutations, or autocrine loop formation. Many studies describe the localization of Met as membranous/cytoplasmic, but some studies using antibodies targeted to the C-terminal domain of Met report nuclear localization. This chapter seeks to highlight the histopathology and expression of Met in cancer and its association with clinicopathological characteristics. We also discuss recent studies of the proteolytic processing of Met and effects of the processing on the subcellular localization of Met. Finally, we comment on Met as a therapeutic target for cancer treatment.

Advances in CANCER RESEARCH
0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)03001-2

I. INTRODUCTION

The hepatocyte growth factor receptor (Met) is a transmembrane receptor tyrosine kinase (RTK) primarily expressed in both epithelial and endothelial cells. Met is produced as a single-chain 170 kDa precursor, which is then proteolytically cleaved at a furin site to produce its α (45 kDa) and β (150 kDa) subunits linked by a disulfide bond. The α subunit is highly glycosylated and entirely extracellular. The β subunit has a large extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular portion of the Met receptor, including the entire α subunit, shares homology to semaphorins and is therefore termed the Sema domain. It is this Sema domain that is responsible for ligand binding. The intracellular domain of Met contains three functionally important regions, the juxtamembrane domain, the tyrosine kinase domain, and the multisubstrate docking site at the C-terminal tail. The juxtamembrane region contains a serine (985) that can be phosphorylated by PKC to downregulate the kinase activity of the receptor as well as a tyrosine (1003) where the ubiquitin ligase Cbl can bind and lead to Met polyubiquitination and subsequent degradation (Birchmeier *et al*., 2003; Gentile *et al*., 2008).

Met is activated by the binding of its ligand, hepatocyte growth factor/scatter factor (HGF/SF), which then leads to the dimerization and autophosphorylation of the tyrosine residues (1230, 1234, 1235) within the activation loop of the tyrosine kinase domain. Subsequent phosphorylation of the C-terminal docking sites (tyrosines 1349 and 1356) of Met allows binding of downstream signaling molecules (many of which contain SH2 domains), including Grb2, Shc, Src, p85 subunit of PI3K, and Gab1. This leads to signal transduction through a number of pathways essential for an invasive growth program. In epithelial cells *in vivo*, this invasive growth program orchestrates cell spreading, cell–cell dissociation and an increase in motility. These processes together are known as cell "scattering," and are morphologically similar to features of cells undergoing an epithelial–mesenchymal transition (Birchmeier *et al*., 2003). In addition the cells then migrate and settle in a new environment where they proliferate and generate new tubular structures (Gentile *et al*., 2008). All of these features of Met activation *in vivo* can be simulated *in vitro* by stimulating MDCK cells with HGF. Classical Met/HGF signaling promotes this invasive growth phenotype of cell survival and proliferation; however, a recent study has also demonstrated that caspase cleavage leads to the formation of a 40 kDa intracellular fragment of Met that was also proapoptotic through an unknown mechanism (Tulasne and Foveau, 2008).

II. ONCOGENIC PROPERTIES OF MET

Under physiological conditions HGF secreted by mesenchymal cells acts on epithelial cells expressing the Met receptor. Both HGF and Met are essential for controlling processes during mammalian embryogenesis and as a result transgenic mice lacking either HGF or Met die by embryonic day 16.5 with defects in liver, tongue, and diaphragm, failure of skeletal muscle progenitor cells to migrate to limbs, as well as defects in branching morphogenesis of the lungs and kidneys (Birchmeier *et al*., 2003; Schmidt *et al*., 1995). In the adult, upregulated HGF and Met is observed after injury to liver, kidney, or heart and is important in wound healing of the skin as well as liver regeneration (Birchmeier *et al*., 2003; Borowiak *et al*., 2004; Chmielowiec *et al*., 2007). In addition to Met's functions in these normal processes, its ability to induce proliferation, motility, and invasion can also contribute to the development of cancer. Some tumors express both HGF and Met leading to an autocrine loop where secreted HGF causes the constitutive activation of Met and as a consequence, enhances tumor cell growth and metastasis. Met can also be activated independent of HGF stimulation as a result of overexpression, abnormal processing, absence of negative regulators such as Cbl, expression of the *TPR–MET* gene fusion product formed due to chromosomal rearrangement, or a number of activating mutations in the juxtamembrane and kinase domains that have been identified in renal papillary carcinoma, lung cancer, hepatocellular carcinoma, and gastric cancer (Danilkovitch-Miagkova and Zbar, 2002; Gentile *et al*., 2008; Lee *et al*., 2000; Ma *et al*., 2003; Park *et al*., 1986; Peschard *et al*., 2001).

III. RECEPTOR CROSS TALK

Met is known to interact and cross talk with several membrane proteins, including a number of RTKs (Fig. 1). One of the first RTKs identified to interact with Met was the recepteur d'origine nantais (Ron). Ron is a RTK with significant homology to Met and is activated by binding of its ligand macrophage stimulating protein (MSP) (Thomas *et al*., 2007). Met and Ron have been shown to interact before ligand induced dimerization and are able to transphosphorylate each other. In addition, the expression of an inactive Ron receptor was able to suppress the transforming capabilities of activating Met mutants suggestive of a dominant negative role (Follenzi *et al*., 2000). In a cohort of ovarian cancers, Ron and Met were found to be coexpressed in 42% of the specimens. In addition, coactivation of both receptors in ovarian

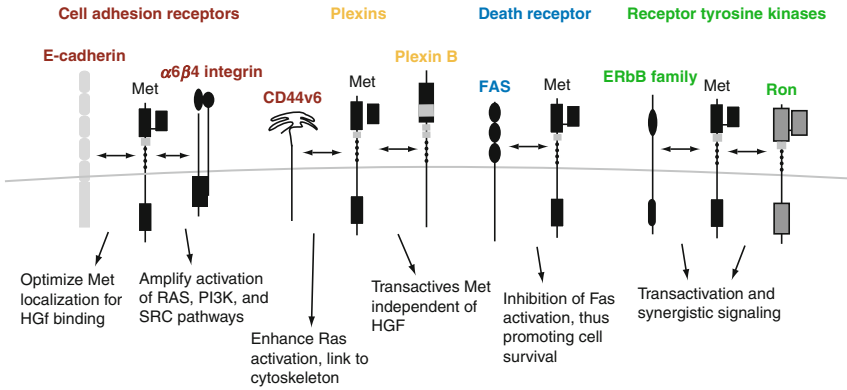

Fig. 1 Met cross talk with other membrane receptors. Met interacts with the cell adhesion receptors E-cadherin, CD44v6, $\alpha 6\beta 4$ integrin, members of the Plexin B family, the death receptor Fas, and other receptor tyrosine kinases such as Ron and ErbB family members.

cancer cell lines synergistically enhanced the motility and invasiveness of the cells (Maggiora *et al.*, 2003). Ron and Met coexpression associate with shorter survival in cancer implying that the interaction and subsequent activation of both Ron and Met may be involved in promoting distant metastasis and recurrence in many tumor types (Cheng *et al.*, 2005; Lee *et al.*, 2005).

Met and Ron also share many structural similarities in the extracellular domain with the Plexin B family of semaphorin receptors. They all contain the ~500 amino acid conserved Sema, the ~80 amino acid cysteine rich Met-related sequence, and four copies of an Ig domain (Gherardi *et al.*, 2004). Giordano *et al.* first reported the ability of Plexins of the B family to transactivate Met and Ron in the absence of HGF/MSP when stimulated with their semaphorin ligands as a mechanism to activate the invasive growth program (Conrotto *et al.*, 2004; Giordano *et al.*, 2002). This interaction was also found to have proangiogenic properties in endothelial cells (Conrotto *et al.*, 2005).

Met also interacts with the v6 splice variant of CD44 to associate Met with the actin cytoskeleton via the Ezrin, radixin and moesin (ERM) proteins, and for the proper assembly and activation of the downstream Ras/MAPK pathway (Orian-Rousseau *et al.*, 2002, 2007). Met interaction with the laminin receptor, $\alpha 6\beta 4$ integrin, leads to phosphorylation of $\alpha 6\beta 4$ integrin which then recruits and amplifies signaling of the Ras–Src signaling cascade (Bertotti *et al.*, 2005, 2006; Trusolino *et al.*, 2001). Met interacts with the death receptor Fas in a ligand independent manner and prevents Fas ligand binding, thereby inhibiting Fas activation and induction of Fas promoted apoptosis (Wang *et al.*, 2002). HGF binding to Met however

displaces Met from Fas which can then induce downstream Met signaling promoting cell curvival. Alternatively, HGF-induced disassociation of Fas from Met may provide a proapoptotic effect allowing the FasL to bind the free Fas. In addition, Met is shown to play an additional proapoptotic role in a caspase dependent manner (Foveau *et al*., 2007). Finally, we and others have shown that E-cadherin interacts with Met at the plasma membrane to optimize the localization of the receptor for ligand stimulation (Hiscox and Jiang, 1999; Reshetnikova *et al*., 2007).

Met also interacts with the EGF family of receptors. Met was found to coimmunoprecipitate with EGFR in human epidermoid carcinoma cell line, but not normal hepatocytes (Jo *et al*., 2000). Met is transactivated by EGFR and G-protein coupled receptors in pancreatic and hepatocellular carcinoma cell lines (Fischer *et al*., 2004). Engelman *et al*. recently reported that MET amplification may contribute to gefitinib resistance in EGFR-activated (via activating mutation or deletion) NSCLC through Met driven ErbB3 (Her3) activation (Engelman *et al*., 2007). Recently, we have shown that in a wild-type EGFR and Met overexpressing NSCLC cell line, H441, Met cross talks with EGFR, Her2, and Her3. This Met overexpression enhances the wild-type EGFR downstream signaling to the levels that are seen with constitutively activated EGFR (via mutation or deletion). In this context, maximal growth inhibition is achieved with combined use of a dual Her2/EGFR and Met tyrosine kinase inhibitors (TKIs) (Agarwal *et al*., 2009). This is especially relevant clinically as only a small percent of NSCLC patients have activating mutations or deletions in EGFR while the majority of patients have wild-type EGFR that mostly do not respond to EGFR targeted inhibitors. Our data suggests that Met overexpression may be useful for selecting patients that may benefit from combination therapy against Met and EGF family of receptors. Other recent studies also document the cooperation of Met and EGFR family members in cancer and suggest combinatorial inhibition of both may be needed to effectively abrogate tumor growth (Guo *et al*., 2008; Mueller *et al*., 2008; Shattuck *et al*., 2008; Tang *et al*., 2008; Yano *et al*., 2008).

IV. PROTEOLYTIC PROCESSING OF MET

Most studies on Met have focused on the conventional full-length receptor signaling and downregulation mechanisms. However, several recent studies have revealed the importance of the proteolytic processing of Met as a means not only for the downregulation of Met, but also to generate biologically active fragments with novel functions (Fig. 2).

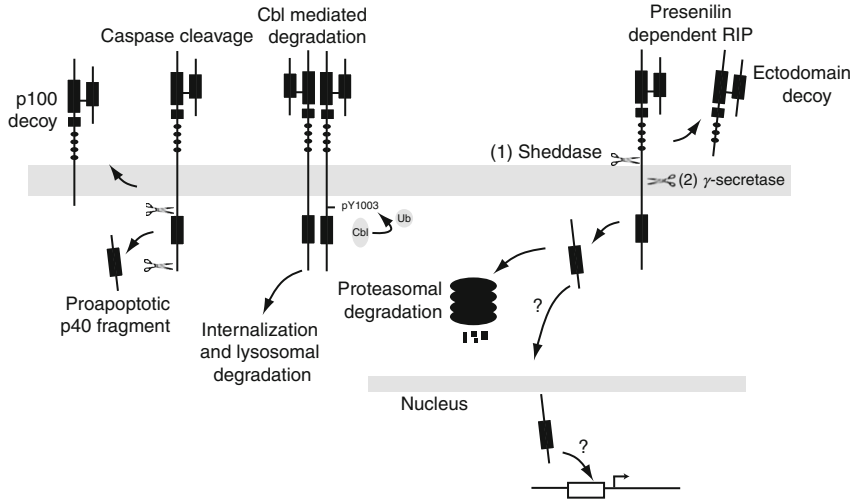

Fig. 2 Proteolytic processing of Met. Met is a substrate of sequential caspase cleavages to generate a proapoptotic p40 Met fragment and a p100 Decoy fragment. Met is targeted for internalization and lysosomal degradation after activation and recruitment of the Cbl ubiquitin ligase. Finally, Met is a substrate of presenilin-dependent RIP where shedding of the ectodomain is followed by cleavage by the γ-secretase complex.

A. Cbl Mediated Ubiquitination

Met is rapidly targeted for ubiquitination, endocytosis, and transport to endosomal compartment for lysosomal degradation by the recruitment of the E3 ubiquitin Ligase, Cbl to phosphorylated tyrosine 1003 (Jeffers *et al.*, 1997; Peschard *et al.*, 2001, 2004). This process is mediated by a complex formation of endophilins, CIN85 and Cbl mediating Met internalization and downregulation (Petrelli *et al.*, 2002). The prevention of Cbl mediated downregulation by mutation or deletion of Met Y1003, as seen with the TPR–Met fusion leads to oncogenic transformation of cells through constitutively active Met signaling (Abella *et al.*, 2005; Mak *et al.*, 2007; Peschard *et al.*, 2001).

B. Caspases and p40

While classical Met/HGF signaling promotes cell survival and proliferation, an intracellular 40 kDa fragment (p40) resulting from sequential caspase cleavages of Met is shown to be proapoptotic in stress conditions (Foveau *et al.*, 2007; Tulasne *et al.*, 2004). Although the exact mechanism of

apoptosis amplification is unclear, kinase activity of the p40 fragment is required as a kinase dead mutant did not induce apoptosis (Tulasne *et al.*, 2004). The membrane bound extracellular counterpart p100 is then able to act as a decoy receptor and sequester HGF and inhibit downstream signaling (Deheuninck *et al.*, 2008). The complex regulatory role Met plays in cell survival signaling is further complicated by its association and inhibition of the Fas death receptor and the activation of downstream signaling molecules PI3K/Akt that further promote cell survival. This balance of proapoptotic and cell survival signaling roles for Met may provide cells with a system of checks and balances for proper regulation.

C. RIP and Ectodomain Shedding

The ectodomain shedding of membrane proteins is a proteolytic processing event resulting in a membrane bound stub containing the intracellular domain and the release of the ectodomain into the extracellular space. The known sheddases consist of metalloproteases, ADAMs, and aspartic proteases (BACE). While the ectodomain shedding of Met is not completely understood, it has been shown to be metalloprotease dependent and downstream of EGF stimulation, G-protein coupled receptors, and integrins (Nath *et al.*, 2001). ADAM 17 and ADAM 10 have been implicated to be involved in this process (Foveau *et al.*, 2009; Kopitz *et al.*, 2007). The ectodomain shedding rate of Met correlates with the malignant potential of a variety of cultured cancer cells, and the overall tumor burden of mice harboring subcutaneous human tumor xenografts (Athauda *et al.*, 2006). Additionally, deletion of the ectodomain of Met increases the tumorigenic potential of NIH3T3 cells compared to NIH 3T3 cells transduced with WT Met (Merlin *et al.*, 2009). Shedding may also provide an endogenous decoy receptor that is capable of sequestering HGF thereby inhibiting HGF dependent signaling.

Ectodomain shedding is often the first step of regulated intramembrane proteolysis (RIP) in which a second cleavage of the membrane bound stub occurs within its transmembrane domain, releasing a soluble intracellular protein. The intramembrane proteases consist of the γ-secretase complex for type I membrane proteins and the site 2 protease (S2P) or the signal peptide peptidase like (SPPL) family for type II membrane proteins. Rhomboid proteins have also been shown to act as intramembrane proteases, but they do not require a preceding ectodomain shedding step. RIP is a tightly controlled processing event required not only for the degradation of membrane proteins, but also for signal transduction mechanisms in organisms including animal cells, yeast, viruses, and bacteria (Brown *et al.*, 2000). Numerous membrane proteins undergo RIP and translocate to the nucleus

such as ErbB4, Notch, APP, CSF-1, E-cadherin, and CD44 (Carpenter and Liao, 2009). Recently, Foveau *et al.* provided the first evidence that Met undergoes presenilin-dependent RIP independent of HGF activation to generate a labile 50 kDa fragment. The authors use a TRK-Met (tropomyosin-related kinase receptor) fusion construct that is unable to undergo ectodomain shedding and RIP to show that the RIP of Met may be an alternative mechanism for the downregulation of Met signaling (Foveau *et al.*, 2009). The cytoplasmic domain of Met has been localized to the nucleus in a number of studies, but it is not known if that fragment is the product of presenilin cleavage. Although the cytoplasmic domain of Met may have a novel function in the nucleus similar to what has been ascribed to other substrates of presenilin-dependent RIP (e.g., ErbB4), this has not yet been proved.

V. NUCLEAR LOCALIZATION OF MET

Several reports have described the immunohistochemical expression of Met as not only membranous and cytoplasmic, but also at times nuclear. In melanoma, basal melanocytes, melanocytic nevi, and a few dermal nevus cells almost always showed nuclear expression (Saitoh *et al.*, 1994). Similarly, cultured melanoma cells almost always show nuclear staining. Nuclear Met expression and an increase in Met expression are observed at the invasive front of breast carcinoma cells (Edakuni *et al.*, 2001). HGF treatment can induce the nuclear localization of Met in a uveal melanoma cell line (Ye *et al.*, 2008). One study has found that when SkHep1 cells are treated with HGF the full-length Met rapidly translocates into the nucleus (Gomes *et al.*, 2008). Furthermore, full-length Met translocation to the nucleus was dependent on the adaptor protein Gab1 that has a nuclear localization signal (NLS) and importin β1 which guides importin-α/cargo complexes through the nuclear pore complex.

Our lab observed that antibodies against the cytoplasmic but not the extracellular domain of Met were prognostic for poor patient outcome in lymph node-negative breast carcinomas, suggestive of a cleavage event where the C terminus is present in the absence of the N terminus (Kang *et al.*, 2003). Cell fractionation studies revealed a unique 60 kDa fragment in the nuclear fractions by several commercially available antibodies against the C-terminal domain of Met (Pozner-Moulis *et al.*, 2006). However, the lack of a smaller transcript in Northern blots suggests that this fragment is derived from a posttranslational cleavage event. Using GFP-tagged recombinant Met, N-terminal deletions of the cytoplasmic domain identified a region of the juxtamembrane domain (P1027-I1084) required for nuclear

translocation. The nuclear localization of Met was also found to depend on cell density where cells at low density expressed Met in the nucleus and cytoplasm, but at high density Met was predominately expressed in the membrane and cytoplasm (Pozner-Moulis *et al*., 2006). Other reports using cytoplasmic domain antibodies have also described the nuclear localization of phospho-Met (Y1003) suggestive of activated Met localizing to the nucleus (Ma *et al*., 2005). Phospho-Met (Y1230/1234/1235) also colocalized in a punctate nuclear compartment with the transcription factor, PAX5 in SCLC cells upon treatment with HGF (Kanteti *et al*., 2009). Finally, Matteucci *et al*. described the nuclear localization of a 60 kDa fragment and provided the first *in vitro* evidence for a function of Met in the nucleus. Using N-terminal deletion constructs of Met fused to a Gal4 DNA-binding domain cotransfected with a Gal4-Luciferase reporter they showed the transactivating activity of nuclear Met suggesting Met fragments in the nucleus may act as transcription factors (Matteucci *et al*., 2009).

VI. HISTOPATHOLOGY AND EXPRESSION OF MET IN CANCER

The level of expression of Met is associated with poor prognosis in many cancers. However, we and others have reported that the detection of Met in formalin-fixed paraffin-embedded (FFPE) tissues is difficult due to a limited number of monoclonal antibodies to Met that are reproducible and properly validated (Knudsen *et al*., 2009; Pozner-Moulis *et al*., 2007). Nonetheless, many studies have documented its overexpression in several cancer types and association with clinical outcome as discussed below.

A. Breast

Met is expressed in normal ductal and lobular epithelium of the breast and HGF is expressed primarily by mammary fibroblasts (Niranjan *et al*., 1995). One of the first immunohistochemical studies evaluating Met expression in breast cancer found that higher or lower Met expression when compared to adjacent normal epithelium was associated with poor prognosis in lymph node-negative breast cancer patients (Tsarfaty *et al*., 1999). In addition, Met expression and HGF expression have been shown to increase from normal epithelia, benign hyperplasia, DCIS with highest expression in invasive carcinoma (Jin *et al*., 1997). Furthermore, we reported that Met overexpression identifies a unique subset of patients independent of Her2, EGFR, and hormone receptors ER/PR (Tolgay Ocal *et al*., 2003). The increased

expression of HGF and Met at the front of breast tumors concomitant with Ki67 staining is suggestive of the Met pathway to be especially active at the invading front of tumors (Edakuni *et al.*, 2001; Tuck *et al.*, 1996). Edakuni *et al.* (2001) also describe a few cases of the nuclear localization of Met and the autocrine expression pattern of Met and HGF in almost half of their cohort (41/88 breast cancer cases). Furthermore, they found 45 cases of high Met expression at the cancer front with most of these tumors also expressing HGF. Met and Ron coexpression also associated with shorter survival in a cohort of lymph node-negative patients (Lee *et al.*, 2005). Interestingly, our lab has noted a difference between antibodies against the cytoplasmic domain of Met compared to the extracellular domain where the cytoplasmic domain antibodies but not the extracellular were prognostic in a lymph node-negative cohort of 330 breast cancer cases for total Met expression (Kang *et al.*, 2003; Tolgay Ocal *et al.*, 2003). Using automated quantitative analysis AQUA® system, we showed that the expression of Met in the nucleus is associated with shorter 5-year survival in a cohort of 688 breast cancer cases comprising of half node-negative and half node-positive cases (Pozner-Moulis *et al.*, 2007).

B. Lung

In normal lung development, Met is expressed in tubular epithelium and HGF is expressed in the lung mesenchyme. This appositional expression allows HGF to play a critical role in the mesenchymal–epithelial interaction during lung development. In organ culture, HGF stimulated the branching morphogenesis of the fetal lung (Ohmichi *et al.*, 1998). Met is expressed at very low levels in the normal adult lung (Ma *et al.*, 2005) and overexpressed in lung cancer and mesothelioma. The overexpression of Met in NSCLC correlates with higher tumor differentiation and is associated with poor prognosis (Ichimura *et al.*, 1996; Takanami *et al.*, 1996; Tsao *et al.*, 1998). Concomitant Met and HGF expression also has a significantly lower survival rate than patients positive or negative for either (Masuya *et al.*, 2004). This study also found a correlation between Met and HGF expression and a high Ki67 index. In another NSCLC study Met was expressed in 100% ($n = 23$) tumor tissue examined with 61% showing strong expression and phospho-Met Y-1003 was observed preferentially at the invasive fronts (Ma *et al.*, 2005). In addition, the phospho-Met localization in squamous and carcinoid tumors was predominantly nuclear, further suggestive of a role for Met in the nucleus. However, it should be noted that no clinicopathological features were analyzed in this cohort. A similar study by Nakamura *et al.* (2007) showed that Met expression correlated with pathological stage and lymph node metastasis, but had no association with survival in adenocarcinomas. This group also showed the expression of phospho-Met Y1235 in

21.5% of cases that correlated with HGF expression, but also found 12 phospho-Met positive cases with no expression of HGF, suggestive of a ligand independent activation of Met. They also noted an interesting expression pattern, where phospho-Met was localized to the apical portion of cells and total Met preferentially localized in the basolateral surface of cells suggesting that the apical phospho-Met positive cells represent HGF stimulated cells (Nakamura *et al*., 2007).

Met is amplified in NSCLC cell lines and patients treated with and resistance to gefitinib (Bean *et al*., 2007; Engelman *et al*., 2007). However, amplification seems to be a rare event in pretreated biopsies and patients not exposed to EGFR TKIs, but is a negative prognostic factor (Cappuzzo *et al*., 2009a,b). Similar results were observed in a Japanese NSCLC study where amplification of Met was again rare (5.6%, $n = 213$), but the 12 patients identified with increased copy number associated with worse prognosis (Okuda *et al*., 2008).

In addition, Met/HGF is expressed and active in SCLC. Ma *et al*. described the expression of phospho-Met (Y1003 and Y1230/1234/1235) preferentially at the invasive front of the tumor in several SCLS patients similarly to the expression pattern in NSCLC (Ma *et al*., 2007). Furthermore, serum HGF is significantly higher in SCLC patients than normal and high levels are associated with shorter survival (Bharti *et al*., 2004). Met/HGF has also been shown to be active and expressed in 82% of malignant pleural mesothelioma (MPM) patients and the serum HGF levels were twice as high in MPM patients as controls (Jagadeeswaran *et al*., 2006).

C. Gastric Carcinoma

Met expression correlates with progression and prognosis in gastric carcinoma (Taniguchi *et al*., 1998). Additionally, Met expression was higher in stage IV gastric cancers with liver metastasis versus no metastasis with Met localization primarily cytoplasmic with some nuclear and plasma membrane staining (Amemiya *et al*., 2002). TPR–Met was detected in 12 of 22 patient biopsies of human gastric mucosa with precursor lesions and carcinoma (Soman *et al*., 1991). Met has also been shown to be amplified and mutated in gastric carcinomas (Hara *et al*., 1998; Lee *et al*., 2000; Smolen *et al*., 2006).

D. Melanoma

The first study in melanoma was reported by Natali *et al*. (1993). They detected only four of 23 primary melanomas were positive for Met, but 17 of 44 metastatic lesions scored positive and multiple metastases from the same

patient were homogeneously positive for Met (Natali *et al.*, 1993). In another study, the nuclear localization of Met was described extensively in basal melanocytes, melanocytic nevi, and a few dermal nevus cells when using a C-terminal antibody (Saitoh *et al.*, 1994). The same study however found no significant difference in Met expression between benign and malignant melanocytic lesions.

Another group found Met expression in all cases of primary cutaneous malignant melanoma and found no nuclear expression, only membranous and cytoplasmic (Cruz *et al.*, 2003). However, they used an antibody to the extracellular β-chain. The neoplastic cells primarily expressed Met in the cytoplasm and occasionally found strong paranuclear expression, suggesting accumulation of Met in the golgi complex compartment. Thus, for analysis they qualitatively and semiquantitatively evaluated membranous and cytoplasmic expression and found membranous expression associates with aggressive clinicopathological parameters, metastasis and overall survival (Cruz *et al.*, 2003). Mutations in the juxtamembrane domain of Met have also been identified in melanoma in a cohort of 20 nevi, 16 primary melanomas, and 24 metastatic melanomas (Puri *et al.*, 2007). This study also found that 85% of nevi had no expression of Met, but 88% of malignant melanomas were positive. Additionally, the staining was described as cytoplasmic in primary melanomas and cytoplasmic and membranous in metastatic melanomas. This group also found 21% of the melanomas were phospho-Met positive and the phsopho-Met (Y1003) was again preferentially expressed in the invasive front of the tumor. The MITF transcription factor controls melanocyte differentiation and also regulates Met expression, further indicative of Met's role in melanocyte differentiation and melanoma progression (Beuret *et al.*, 2007; McGill *et al.*, 2006). High Met expression also correlates with poor survival in uveal melanoma, where nuclear Met has also been described upon HGF treatment in a uveal melanoma cell line (Mallikarjuna *et al.*, 2007). Recently, Met gene amplification was reported in 47% cases of metastatic melanoma in frozen tissue samples from 19 patients (Moore *et al.*, 2008).

E. Prostate Cancer

Met is expressed in basal and luminal cells of the normal prostate epithelia, and HGF from the prostate stroma activates Met in a paracrine mechanism primarily (Knudsen and Edlund, 2004). Met expression is highest in androgen receptor (AR) negative prostate cancer cell lines, and only slightly expressed in the AR positive cell lines (Humphrey *et al.*, 1995; Knudsen *et al.*, 2002). Furthermore, castrated rats have an increase in Met expression in prostatic epithelium (Humphrey *et al.*, 1995; Nishi *et al.*, 1996). Recently,

AR was found to negatively regulate the transcription of Met suggesting the loss of AR upon androgen ablation therapy may allow prostate cancers to progress to androgen insensitive through the upregulation of Met (Verras *et al*., 2007).

Met expression is reported in 45–84% of localized prostate carcinomas (Humphrey *et al*., 1995; Knudsen *et al*., 2002; Pisters *et al*., 1995; Watanabe *et al*., 1999). Some reports indicate a correlation between Met expression and higher grade (Pisters *et al*., 1995; Watanabe *et al*., 1999). Interestingly, all of these studies found Met to be highly expressed in most metastatic lesions (Humphrey *et al*., 1995; Knudsen *et al*., 2002; Pisters *et al*., 1995; Watanabe *et al*., 1999), and Knudsen *et al*., 2002 observed higher Met expression in bone metastases than lymph node metastases. Additionally, Watanabe *et al*. (1999) reported more intense staining of Met along the invasive fronts similar to what has been described in melanoma, breast, lung, and colon cancer. These studies all described the Met localization as membranous and cytoplasmic using C-terminal antibodies to Met.

F. Hepatocellular Carcinoma

Met plays a key role in liver development as knockout mice die *in utero* with underdeveloped liver (Bladt *et al*., 1995). Met/HGF is critical for efficient liver regeneration and repair upon partial hepatectomy and induced injury (Borowiak *et al*., 2004; Hu *et al*., 2007) Met is overexpressed in HCCs compared to adjacent normal tissue and normal livers (Ke *et al*., 2009; Ueki *et al*., 1997). In a recent study, high Met expression correlates with vascular invasion, tumor size, TNM stage, and tumor differentiation (Ke *et al*., 2009). In their cohort, Met expression alone did not stand as an independent prognostic factor, but concomitant high expression of Met and CD151 was an independent prognostic predictor of overall survival and recurrence. This study used an antibody against the N-terminal domain of Met and detected cytoplasmic and membranous expression of Met, but no nuclear localization. A Similar observation was made when Met expression was assessed by Western blotting and patients with high Met protein levels had a significantly shorter 5-year overall survival (Ueki *et al*., 1997).

G. Colon Cancer

Ginty *et al*. (2008) show that the relative membrane to cytoplasmic expression is a significant predictor of survival in stages I and II colon cancer using an N-terminal antibody and fluorescent based IHC. The membrane expression alone was not predictive, suggesting that activated internalized

Met may contribute to tumorigenesis (Ginty *et al.*, 2008). Using the same cohort no correlation of Met expression with survival was found using conventional DAB staining. In a different cohort, Met mRNA expression level correlated well with protein expression and high expression was associated with tumor depth of invasion and lymph node metastasis (Takeuchi *et al.*, 2003). Additionally, patients with high Met expression combined with high HGF mRNA expression were also shown to have poor prognosis (Kammula *et al.*, 2007). Finally, Met and Ron expression were evaluated in a cohort of 135 colorectal cancer patients by IHC (Lee *et al.*, 2008). The localization of Met with a C-terminal directed antibody was described as diffusely cytoplasmic with occasional membranous staining. It also showed intense staining in regions of deep invasion and an increase in expression at the invasive front of the tumors. Furthermore, this study reported that high Met or Ron associates with shorter survival and patients with high Met/high Ron were 11 times more likely to recur than patients low for both (Lee *et al.*, 2008).

H. Glioblastoma

Glioblastoma is the most frequent and deadly brain tumor in adults. Both Met and HGF expression increase with the malignancy and grade of primary brain tumors (Koochekpour *et al.*, 1997; Nabeshima *et al.*, 1997). Nabeshima *et al.* (2007) described the localization of Met as predominantly cytoplasmic in tumor cells with occasional membranous and nuclear staining, but patchy and heterogeneous using a C-terminal antibody. Met was also positive in endothelial cells in the perivascular and vascular areas of glioblastoma. In addition, Met has been reported to be amplified in 4% of glioblastomas and a rare somatic Met mutation has been described in 1 out of 11 glioblastoma patients (Moon *et al.*, 2000; TCGA Research Network, 2008).

VII. MET AS A THERAPEUTIC TARGET

Extensive evidence implicates Met as a major component employed by cancer cells to progress the disease, making it an excellent target of cancer drug development. It is also a rather versatile target in that there are several strategies available for Met inhibition. First, the interaction between HGF and Met can be targeted using HGF antagonists or decoys to stoichiometrically compete with the ligand or receptor. Indeed, HGF fragments NK2/NK4 and uncleavable HGF have been shown in *in vitro* and *in vivo* models to bind Met without inducing dimerization and activation (Comoglio *et al.*, 2008). Additionally, a soluble recombinant Met decoy corresponding to the extracellular domain of Met is reported to not only bind and sequester HGF, but also bind full-length Met impairing dimerization (Michieli *et al.*, 2004).

Alternatively, antibodies to HGF or Met can be employed to prevent ligand/receptor binding as well as induce downregulation of Met by increasing its shedding from the cell surface. A fully humanized IgG2 monoclonal antibody AMG102 (Amgen, Inc.) shown to inhibit HGF/Met dependent glioma cell xenograft growth in mice (Burgess *et al*., 2006) is currently in a phase II clinical trial in patients with glioblastoma (Burgess *et al*., 2006; Reardon *et al*., 2008). Targeting Met with antibodies has been difficult due to partial agonistic effects of many antibodies allowing Met dimerization and activation (Prat *et al*., 1998). However, a one–armed 5D5 monovalent antagonist Met antibody shown to inhibit growth of intracranial orthotopic xenografts of glioma cells is currently in Phase I clinical studies, with indications that it is safe and well tolerated (Martens *et al*., 2006; Salgia *et al*., 2008). Another monoclonal antibody, DN30 reportedly functions to downregulate Met by inducing the ectodomain shedding of Met providing an excracellular decoy Met and rapid degradation of the intracellular fragment (Petrelli *et al*., 2006). Finally, the kinase activity of Met can be inhibited with the use of small molecule TKIs. Several specific Met TKIs are currently in early phases of clinical development. In addition multitargeted TKIs are also in development with activity against Met and other RTKs such as VEGFR, Ron, FGFR, Flt-3, PDGFR, and Kit (Comoglio *et al*., 2008; Eder *et al*., 2009). There are several clinical trials currently underway evaluating various Met inhibitors including a combination of Met inhibitor (XL184, a small molecule TKI) with an EGFR inhibitor (Tarceva).

VIII. PERSPECTIVE

Although this review highlights years of work analyzing the role of Met in cancer prognosis and progression, we still have a long way to go. The difference in prognosis when examining expression of different regions of the protein and the recent studies revealing the proteolytic processing of Met highlight the need for development of reproducible monoclonal antibodies suitable for immunohistochemistry, against both intracellular and extracellular domains of Met. Many questions remain to be answered about the importance of the RIP of Met. Are these cleaved fragments responsible for the nuclear expression observed in many of these studies, and more importantly are they contributing to cancer progression and metastasis independent of Met's traditional signaling pathways? In an age where there is a continued push for development of effective targeted therapies, Met has emerged as a promising candidate, and future clinical studies combining Met TKIs with other small molecule inhibitors, like EGFR TKIs in NSCLC may provide further evidence of the importance of such targeted therapies.

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Managing Tumor Angiogenesis: Lessons from VEGF-Resistant Tumors and Wounds

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It is now well established both experimentally and clinically, that new blood vessel growth is required for tumors to grow beyond a few millimeters and metastasize [Folkman, J. (1995). In: Mendelsohn, L., Howley, P., Israel, A. (Eds.), The Molecular Basis of Cancer, WB Saunders Company, Philadelphia, pp. 206–225]. Angiogenesis, the process of forming new blood vessels from preexisting vessels, provides the tumor with additional oxygen and nutrients for its continued growth. In addition, the proximity and increase in vascular density enhance the likelihood of tumor cells entering the bloodstream to eventually metastasize. Since the initial observations of Dr. Folkman in the late 1970s, research over the past 30 years has focused intensely on identifying points in which the angiogenic cycle can be disrupted and has become an important component of current therapies to limit tumor progression.

ABBREVIATIONS

BMDC, bone marrow-derived cells; EC, endothelial cell; EPC, endothelial progenitor cell; HIF, hypoxia inducible factor; MMP, matrix metalloproteinase; MMTV, mouse mammary tumor virus; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)03002-4

I. ANGIOGENESIS

Whereas angiogenesis is absolutely essential for embryonic growth, its occurrence in adults is highly restricted and limited to the female reproductive cycle or during pathological conditions including rheumatoid arthritis, wound healing, diabetic retinopathy, and tumor growth (Carmeliet, 2003).

During sprouting angiogenesis, endothelial cells (ECs) first degrade the vascular basement membrane, and subsequently adhere to, proliferate and migrate into adjacent stroma, and then resynthesize a new basement membrane to form functional conduits to transport blood and nutrients. The angiogenic process requires coordinated changes in the expression of proteases, adhesion molecules, cell cycle regulators as well as factors that direct the appropriate morphological response of EC to their immediate microenvironment. Tumors and the activated tumor stroma produce an array of proangiogenic factors that act on the adjacent endothelium to induce sprouting angiogenesis. In addition to resident cells of the tumor stroma, infiltrating immune cells (leukocytes) recruited to the tumor also produce angiogenic factors and bone marrow-derived endothelial progenitor cells (EPCs) or immature myeloid cells can also directly incorporate into the growing vasculature. While sprouting angiogenesis is a local response involving existing blood vessels, the *de novo* formation of new blood vessels that arises through the mobilization and recruitment of EPC from the bone marrow or other sites is often referred to as vasculogenesis (Carmeliet, 2000). More recently, it has been shown that in addition to endothelial precursor cells, several different bone marrow-derived cell (BMDC) population, notably immature myeloid Cd11b+Gr1+ cells also directly contribute to angiogenesis either by residing in a perivascular location and providing additional angiogenic stimuli or becoming directly incorporated into the new sprouts (Crosby *et al.*, 2000).

During physiological angiogenesis, once the target tissue has been vascularized, the expression of angiogenic growth factor ceases. EC migration, proliferation, and proteolysis come to a halt and the newly formed vessels undergo maturation where tight cell–cell connections are reestablished and a continuous basement membrane is formed, and perivascular support cells are recruited to further stabilize and maintain quiescence in the newly formed vessels. This maturation process, however, is often absent in tumor-induced angiogenesis. The continuous input of angiogenic factors prevents tumor-induced capillaries from maturing and the resulting tumor vasculature is irregular, leaky, and tortuous and is constantly being remodeled (Hashizume *et al.*, 2000). Chronic recruitment of BMDC also contributes to the sustained proteolytic activity, angiogenesis, and leakage of the tumor vasculature.

II. VEGF AND TUMOR ANGIOGENESIS

Vascular endothelial growth factor (VEGF), the most extensively characterized angiogenic factor, is produced and secreted by a number of normal cell types and its expression is markedly increased in tumor cells as well as in the immediate tumor stroma (Bergers *et al.*, 2000; Chen *et al.*, 2009; Coussens *et al.*, 1999; Kerbel, 2008; Lin *et al.*, 2007; Murdoch *et al.*, 2008). Expression of VEGF is induced by low oxygen tension or hypoxia that arises from masses of tightly packed, rapidly growing cells that lack access to an adequate supply of nutrients. In the hypoxic tumor microenvironment, the usually labile Hif1α and Hif2α transcription factors, which bind directly to the VEGF promoter, are stabilized resulting in increased transcription (Forsythe *et al.*, 1996; Gerber *et al.*, 1997). Increased HIF expression in a wide variety of tumors correlates with poor prognosis, resistance to radiotherapy and chemotherapy, and increased patient mortality (Harris, 2002; Unruh *et al.*, 2003).

In addition to hypoxia, VEGF can also be induced in the tumor microenvironment by other factors such as low pH, inflammatory cytokines (such as interleukin-6), growth factors (such as basic fibroblast growth factor or FGF), and chemokines (like stromal cell-derived factor-1, SDF-1) (Kerbel, 2008). VEGF can also be induced under normoxic conditions when epithelial cell organization and/or polarity is disrupted (Chen *et al.*, 2009). In addition to transcriptional activation, VEGF protein can also be bound and stored in the tumor matrix, and tumor-mediated activation of matrix metalloproteinase, MMP9, liberates the sequestered VEGF enabling it to initiate angiogenesis (Bergers *et al.*, 2000).

III. VEGF AND RECRUITMENT OF EPC IN TUMOR ANGIOGENESIS

In addition to direct activation of sprouting angiogenesis by VEGF, VEGF also mediates recruitment and trafficking of select subpopulations of BMDC to tumors that are necessary for sustained tumor angiogenesis (Lyden *et al.*, 2001). Specifically subsets of BMDC bearing surface VEGFR receptors and identified as EPCs are recruited from the bone marrow and directly contribute to angiogenesis by being incorporated into growing angiogenic sprouts; a process that could be blocked by addition of VEGF-neutralizing antibodies (Murdoch *et al.*, 2008). Subsequent studies showed that the exact contribution of EPC to tumor angiogenesis is variable and can range from 5% to even 50% of tumor associated vasculature depending on the tumor type and the

markers used to detect EPC (Bertolini *et al.*, 2006; Peters *et al.*, 2005; Shaked *et al.*, 2006). Nonetheless, these seminal studies defined a critical role for recruited BDMC in tumor angiogenesis and progression (Gao *et al.*, 2009; Lyden *et al.*, 2001; Shojaei *et al.*, 2007; Yang *et al.*, 2004).

IV. ROLE OF BONE MARROW-DERIVED IMMUNE CELLS IN ANGIOGENESIS AND TUMOR PROGRESSION

Subsequent studies have also demonstrated that other BMDC subpopulations recruited to growing tumors may also directly contribute to angiogenesis. Specifically, bone marrow-derived Cd11b+Gr1+ immature myeloid cells and monocytes may directly incorporate into vessels (Yang *et al.*, 2008) or locate perivascularly and support growth of resident EC (Grunewald *et al.*, 2006). While the evidence showing direct incorporation of BMDC into the vasculature is limited, strong experimental evidence underscores the contribution of these and numerous other recruited BDMC cells in driving and sustaining tumor angiogenesis via increased production of proteases and VEGF. Transgenic *de novo* models of carcinogenesis have been particularly valuable in demonstrating the role of immune cells in driving tumor angiogenesis that have not previously been appreciated in studies using immune compromised animals and tumor xenograft tumor models. For example, in the K14-HPV16 transgenic mouse model of *de novo* skin carcinogenesis, mice reproducibly develop skin hyperplasia (1 mo.), dysplasia (3–6 mo.), and by 1 year of age 50% develop invasive carcinomas (Hanahan, 1985). Early hyperplasia is accompanied by increased mast cell recruitment and degranulation to activate angiogenesis in the dermis (Coussens *et al.*, 1999). B lymphocytes initiate a series of immune reactions culminating in both mast cell activation and release of proteases which promote angiogenesis and progression to dysplasia and ultimately carcinogenesis (de Visser *et al.*, 2005). Attenuation of either mast cell influx, B cell-mediated immune responses, and/or recruitment of immature myeloid cells blocks angiogenesis and tumor progression. With progression to overt carcinogenesis, tumor epithelium also produces VEGF and basic fibroblast growth factor (bFGF) and thus, both tumor cells and recruited inflammatory cells drive angiogenesis in this model of skin carcinogenesis (Coussens *et al.*, 1999).

The MMTV-PyMT transgenic mouse model develops mammary adenocarcinomas with 100% penetrance in females in a relatively short time span of 90 days (Lin *et al.*, 2003). MMTV-PyMT tumors also develop in an angiogenic-dependent manner with reproducible stage-wise progression

with premalignant, hyperplastic lesions (5–6 weeks) followed by adenoma/mammary intraepithelial neoplasia (MIN) (7–8 weeks), early carcinogenesis at 9–10 weeks and late carcinogenesis at >12 weeks. By 16 weeks, metastasis to lungs is reproducibly observed (Lin *et al.*, 2003). The transition from dysplasia to adenoma/MIN between 5 and 8 weeks coincides with activation of angiogenesis, in large part mediated by an influx of macrophages into the tumor tissue (Lin *et al.*, 2006). Macrophages are a rich source of VEGF to drive tumor angiogenesis and depletion of macrophages severely reduces tumor progression and angiogenesis whereas replenishing VEGF in the absence of macrophages leads to resumption of tumor progression (Lin *et al.*, 2007). In addition, Cd11b+Gr1+ immature myeloid cells as well as a subset of TIE-2-expressing monocytes from the bone marrow are also recruited to tumors in the PyMT model, and produce multiple angiogenic cytokines including VEGF as well as proteases that promote sprouting angiogenesis (for review see Murdoch *et al.*, 2008). Thus, in both these dynamic models, the stepwise progression to carcinoma is dependent upon angiogenesis driven by an influx of immune cells and recapitulates key aspects of human tumor progression and VEGF-mediated angiogenesis.

V. LIMITATIONS IN TARGETING VEGF

The above summarizes the various means by which tumor microenvironment consisting of epithelial cells, activated stromal cells, and recruited immune cells can induce or liberate stored VEGF and in turn stimulate sprouting angiogenesis or vasculogenesis to enhance tumor progression. While VEGF is derived from many sources in the tumor microenvironment, expression of its cognate receptor, VEGFR2 is largely restricted to EC or EPC, and thus inhibiting VEGF has become an attractive approach to selectively inhibit tumor angiogenesis. Indeed interfering with VEGF or VEGFR2 to selectively target tumor endothelium is supported by encouraging clinical data showing reduced tumor progression in solid tumors including colon, breast, and nonsmall cell lung cancers (Kesisis *et al.*, 2007).

Despite these encouraging findings, targeting VEGF alone is not proved universally effective in inhibiting angiogenesis induced by tumors in different organs or at various stages of neoplastic progression (Carmeliet, 2000; Crosby *et al.*, 2000; Hashizume *et al.*, 2000). Moreover, two highly publicized recent studies showed that transient VEGF blockade in fact promoted development of more aggressive, metastatic tumors (Ebos *et al.*, 2009; Paez-Ribes *et al.*, 2009). While the clinical shortcomings of anti-VEGF treatment are disappointing, managing tumor angiogenesis remains a viable and critical step in controlling tumor progression, and further analysis of the

limitations of targeting VEGF alone have yielded fundamental insights into the complex and dynamic nature of tumor angiogenesis that may provide additional avenues for future therapies.

VI. TUMOR STAGE-DEPENDENT RESPONSES TO VEGF

Much of the early experimental evidence that demonstrated a potent role for VEGF in tumor progression was obtained using xenografted human tumors implanted into immunocompromised mice (Yoshiji *et al.*, 1997). In this model, established tumors initiate a rapid and robust angiogenic response that is almost entirely dependent upon VEGF (Yoshiji *et al.*, 1997). In many cases, chronic VEGF expression in the tumor microenvironment interferes with PDGFβ signaling to prevent smooth muscle cell recruitment and vessel maturation (Greenberg *et al.*, 2008). Thus, maturation of tumor vasculature is relatively limited. However, using genetic stepwise models of *de novo* tumor progression, Bergers and Hanahan noted that inhibition of VEGF was sufficient to delay early tumor growth and progression in the RIP1-Tag2 model of pancreatic cancer but not later stages (Bergers *et al.*, 2003). In the RIP1-Tag2 model, sustained expression of SV40 large T antigen in pancreatic islets results in the appearance of hyperplastic/dysplastic islets (with quiescent vasculature) within 5 weeks of age (Hanahan, 1985). By 9 weeks, approximately 25% of these islets have switched on angiogenesis, with histological features of high-grade dysplasias and are susceptible to inhibition by VEGF blockade (Folkman *et al.*, 1989). However, when VEGF inhibition is initiated in 12-week-old mice with established tumors, inhibition of VEGF alone was not sufficient to limit further angiogenesis and growth; instead, inhibition of both VEGF and PDGF was required as established tumor vessels had matured and the increased coverage with perivascular or mural cells reduced the dependence on VEGF for survival (Bergers *et al.*, 2003; Pietras *et al.*, 2008).

Previous studies also noted that inhibition of VEGF could "normalize" the early tumor-induced vasculature to reduce leakiness and consequently be exploited to improve drug delivery or sensitivity to radiation (Jain, 2005). This approach, however, is limited to specific therapeutic windows, usually with newly established vessels in early stage tumors and not effective at later stages and together emphasize the tumor stage dependence of the vasculature on VEGF.

Other recent studies have noted that the hyperactivated angiogenic state of the tumor environment can result in formation of a very dense but ultimately nonproductive vascular network that fails to support tumor growth.

For example, studies in mice lacking DLL4, a notch ligand induced by VEGF which functions as a negative regulator of angiogenesis, an extensive angiogenic vasculature develops yet, growth of tumors (including VEGF-resistant tumors) was impaired (Thurston *et al.*, 2007). Analysis of the neovasculture in these mice revealed that while the vascular network was accompanied by extensive sprouting, the vessels were very poorly perfused and essentially nonfunctional, likely due to a failure to undergo subsequent steps in vessel maturation and/or morphogenesis in the absence of Dll4.

A series of elegant studies examining the tumor angiogenesis induced by both tumor and myeloid cell-derived VEGF revealed extensive vascular sprouting but development of immature and nonproductive angiogenesis that limited tumor growth. Selective ablation of myeloid cell-derived VEGF resulted in reduced vascular density but also increased maturation of vessels as indicated by reduced tortuosity and increased pericyte coverage. The resulting maturation or normalization of the existing tumor beds by reducing the total amount of VEGF in the tumor microenvironment ultimately increased tumor growth and progression (Stockmann *et al.*, 2008). However, the studies subsequently showed that the resulting functional vascular network allowed for more efficient delivery of chemotherapeutic agents and thus rendered the tumors more susceptible to treatment. Thus, the relative state of the tumor vasculature, whether more mature, chronically leaky, or nonproductive may determine whether anti-VEGF therapy alone would be effective. Potentially even VEGF-resistant tumors, which produce nonfunctional vessels, may benefit from combining antiangiogenic and neoadjuvant treatments to limit new sprouting angiogenesis, induce maturation of existing vessels, and improve delivery of chemotherapeutic agents to tumors (Fig. 1).

VII. MULTIPLE ANGIOGENIC FACTORS PRODUCED BY THE TUMOR MICROENVIRONMENT

Tumors and recruited immune cells also produce a variety of angiogenic factors other than VEGF (Bergers *et al.*, 2000; Chen *et al.*, 2009; Kerbel, 2008) including PDGF, bFGF, HGF, and EGF that could potentially drive angiogenesis even with complete VEGF blockade (de Jong *et al.*, 1998). In addition, the tumor microenvironment produces several chemokines that not only recruit tumor promoting BMDC but are also directly angiogenic including CXCL12 (SDF-1) (Kryczek *et al.*, 2005) and CCL-2 (MCP-1) (Niu *et al.*, 2008).

A recent study of four VEGF-refractory tumors revealed a critical role for recruited Cd11b+Gr1+ immature myeloid in driving angiogenesis that did

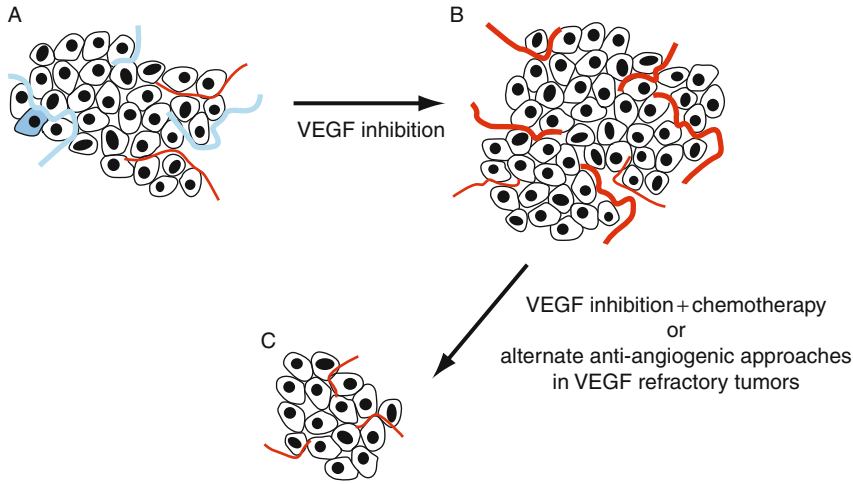

Fig. 1 Schematic representation of the fate of tumors that do not regress in response to anti-VEGF monotherapy. (A) Early stage tumors contain many immature or nonfunctional vessels (blue). (B) Inhibition of VEGF alone may (i) induce vessel maturation to improve perfusion and tumor growth; (ii) transient VEGF inhibition may promote development of more aggressive tumor behavior; or (iii) angiogenesis is maintained independent of VEGF and the tumor continues to expand. (C) Combining anti-VEGF with chemotherapeutics may normalize vessels and improve drug penetration/toxicity or the use of alternate angiogenesis inhibitors in VEGF-refractory tumors can also lead to vascular regression and reduction in tumor growth.

not respond to VEGF inhibition. Further analysis of angiogenic factors produced by these cells showed that the chemokine Bv8 produced by the immature myeloid cells was responsible for promoting angiogenesis when VEGF was blocked (Shojaei *et al.*, 2007, 2009). Thus, multiple angiogenic factors produced by tumor cells and recruited BDMC could prevail to drive angiogenesis when VEGF is inhibited. Designing effective antiangiogenic treatments then should also consider whether different tumors display temporal changes in reliance on different angiogenic mediators or whether tumors that induce distinct immune responses rely on unique angiogenic effectors.

VIII. VEGF INHIBITION AND INCREASED TUMOR AGGRESSIVENESS

One of the more potentially serious shortcomings of anti-VEGF monotherapy is the emergence of more aggressive metastatic tumors following transient VEGF blockade. Although the mechanisms responsible for this phenotypic switch have not been clearly defined, increased reliance on

recruited BMDC producing additional factors to further destabilize the tumor microenvironment is one possibility. Alternatively the reduced perfusion of tumors during transient VEGF inhibition has previously been shown to increase tumor aggressiveness (Paez-Ribes *et al.*, 2009). Recent work described a critical role played by the oxygen-sensing prolyl hydroxylase domain (PHD) proteins in vessel normalization and inhibition of metastasis. When oxygen tension dropped, PHDs become less active, and stabilized HIFs initiate VEGF transcription and the angiogenic response. However, under severe tumor hypoxia, such as that which may occur following treatment with VEGF, an excessive, perhaps compensatory release of angiogenic cytokines generates vessels characterized by hypermotile EC with protruding fillopodia in the lumen and perivascular stroma, EC with irregular cell borders, loosely attached layer, and even vessels with denuded areas (Jain, 2005; Mazzone *et al.*, 2009). However in PHD2+/− mice, the EC can upregulate (s)Flt1 and VE-cadherin to stabilize vessels, reduce junctional leakage, and improve vessel perfusion and oxygenation and thus impair metastasis (Mazzone *et al.*, 2009). Additional investigation into other emergent VEGF-refractory tumors and the responses of transiently hypoperfused tumors may reveal mechanisms that could be exploited to improve targets and/or combinations of effective antiangiogenic and chemotherapeutic therapies.

IX. WOUND ANGIOGENESIS

The increasing recognition of the critical role played by inflammation and the emergence of VEGF-resistant tumor angiogenesis underscores the dynamic, multifactorial nature of tumor-mediated angiogenesis. Although investigators have sought to identify a common angiogenic switch during tumor progression, the genetic instability and phenotypic plasticity of tumors, the number of potential angiogenic effector proteins, and the various cellular sources of these factors emphasize that rather than simple hypoxic activation of VEGF, tumor angiogenesis evolves from complex, temporal, spatial, and redundant mechanisms analogous to wound-induced angiogenesis. Indeed wound and tumors employ many similar mechanisms to recruit a new vascular supply and over 25 years ago Harold Dvorak proposed “Tumors are wounds that do not heal” based on the observations that chronic wounds are characterized by leaky vasculature, excessive proteolytic activity, sustained inflammation, and inability of activated endothelial and epithelial cells to exit proliferative state and mature to a quiescent stable phenotype (Dvorak, 1986). Thus, direct comparison of normal, chronic wounds and tumor angiogenic processes, may identify

distinguishing features or control points that could be exploited to develop new approaches to regulate excessive chronic angiogenesis (Schafer and Werner, 2008).

Following wounding, tissue repair takes place in a stepwise manner with defined yet overlapping phases. An immediate inflammatory phase is marked by influx of neutrophils (0–24 h) followed by increased infiltration of macrophages and other BMDC. The growth factors and other products secreted by the inflammatory cells initiate the proliferative phase, which is marked by increased proliferation of fibroblasts and migration of epithelial cells. Due to the high metabolic activity at the wound site, there is an increasing demand for oxygen and nutrients. Local factors in the wound microenvironment such as low pH and reduced oxygen tension actually initiate the release of factors needed to bring in a new blood supply (Knighton *et al.*, 1983). The angiogenic response is critical for appropriate repair as inhibition of angiogenesis or even delay in angiogenesis is linked to poor healing such as that seen in diabetic or wounds in aged individuals (Eming *et al.*, 2007).

Analogous to the tumor microenvironment, VEGF expression is highly increased in wound tissues and many studies have documented the contribution of VEGF to wound angiogenesis. Not surprisingly, wounds that fail to heal efficiently are directly linked to an impaired angiogenic response, including reduced expression of VEGF (Rossiter *et al.*, 2004). It is worth noting, however, that while reduced VEGF expression correlates with reduced wound angiogenesis, inhibition of VEGF alone is NOT sufficient to significantly delay wound healing in mice (Hong *et al.*, 2004). Although inhibiting VEGF reduced vascular density by >30%, this was not sufficient to delay healing and emphasizes the contribution of multiple angiogenic factors and processes that are utilized, possibly in temporally distinct phases to ensure sufficient vascularization and supply of nutrients to wounds (Knighton *et al.*, 1983). Indeed, analogous to later stage tumors with more mature vasculature, PDGF has been shown to play a major role in wound vascularization and is approved for clinical use in stimulating wound angiogenesis and healing (Goldman, 2004).

Whether factors such as immature myeloid cell-derived Bv-8, implicated in induction of angiogenesis in VEGF-refractory tumors (Shojaei *et al.*, 2009) play a significant role in wound angiogenesis has not been explored but the inflammatory response has long been recognized as a key initiator of wound angiogenesis. Numerous studies have documented both reduced angiogenesis and healing rates in wounds depleted of neutrophils, mast cells or with inhibitors of TNFα, IL-6 or other anti-inflammatory molecules (Martin and Leibovich, 2005). Analogous to the tumor stroma, the immediate wound microenvironment produces the factors responsible for inflammatory influx and subsequent activation of angiogenesis. The redundant nature of angiogenic programs arising from both the resident cells as well

as the recruited immune cells contributes to wound angiogenesis, analogous to the complex and dynamic tumor angiogenic microenvironment.

While the initial wound vasculature is similar to tumor vasculature in that is it leaky and immature, accompanying normal wound healing, most angiogenic vessels either mature via decreasing permeability and recruitment of mural cells or undergo regression (for review see Eming *et al.*, 2007). However in chronic or nonhealing wounds, sustained inflammation interferes with normal repair and vascular maturation (Wetzler *et al.*, 2000) and increased numbers of neutrophils and macrophages during late stages of repair in chronic wounds are indicative of chronic inflammation and a concomitant delayed reepithelialization and neovascularization (Wetzler *et al.*, 2000). Certainly, the treatment of sustained inflammation in chronic wounds has been well established but despite the growing evidence that immune cells play a significant role in tumor angiogenesis and progression, the use of anti-inflammatory or immune-modulating agents to control tumor angiogenesis has been relatively underexplored. Many earlier studies focused on harnessing the immune system to subsequently attack tumors, but more recent evidence has shown that tumor-induced immune cell activation results in expansion of immune cells with protumorigenic and proangiogenic phenotypes (Murdoch *et al.*, 2008). In the MMTV-PyMT model described earlier, DeNardo *et al.* (2009) showed that CD4+ T cells and production of IL-4 resulted in infiltrating breast tumor macrophages expressing a T_H2 phenotype marked by increased proteolytic and destructive activity leading to destabilization of the mammary acinar structure, activation of angiogenesis, and increased pulmonary metastasis. During normal wound healing T_H2 macrophages assist with healing (Mosser and Edwards, 2008) and although essential for normal repair and angiogenesis (Leibovich and Ross, 1975), their sustained presence during late stages of repair in problematic wounds contributes to poor healing and persistence of immature leaky vasculature (Wetzler *et al.*, 2000). Thus, targeted approaches to redirect macrophage phenotype rather than generalized depletion may prove useful in managing both healing and tumor angiogenesis and progression.

X. VASCULAR REGRESSION

During wound repair, many vessels formed during the early inflammatory and proliferative phases eventually undergo regression; marked by apoptosis of EC. While the underlying mechanisms are not well defined, further study of wound vessel regression may provide some additional insights and potentially identify mechanisms that could be therapeutically exploited to manage

persistent, immature tumor vessels. Regression of wound vasculature is reduced in mice lacking $\alpha2\beta1$ integrin and these animals exhibit robust wound angiogenesis that persists into the resolution/scar tissue formation phase, a time when many wound vessels either mature or undergo apoptosis (Grenache *et al.*, 2007). One explanation proposed is that lack of $\alpha2\beta1$ integrin prevents the EC from sensing the associated change from a provisional (angiogenic) matrix consisting largely of fibrin and fibronectin to that of a more mature collagenous matrix normally recognized by $\alpha2\beta1$ integrin (Eming *et al.*, 2007). The lack of signaling through $\alpha2\beta1$ integrin once in the collagenous matrix prevents activation of apoptotic signals and the ECs persist. Indeed, mice lacking $\alpha2\beta1$ integrin also exhibits an increase in adult tumor angiogenesis compared to their wild type counterparts but whether this arises through increased survival is not known. EPC also expresses high levels of $\alpha2\beta1$ integrin (Caplice and Doyle, 2005), however, the loss of $\alpha2\beta1$ would be predicted to reduce recruitment of EPC to growing tumor vessels. Whether reduced EPC recruitment via reduced $\alpha2\beta1$ integrin instead promotes maturation of existing vessels and survival via alternative pathways has not been investigated.

A number of factors are essential for EC survival, most notably VEGF. Interestingly, autocrine rather than paracrine VEGF signaling is essential for endothelial survival as selective deletion of EC produced VEGF, but not paracrine sources of VEGF disrupted EC homeostasis (Lee *et al.*, 2007). This raises the interesting possibility of whether wound vessel regression is mediated by a loss of autocrine VEGF expression and/or signaling rather than a general loss of all angiogenic factors in the healing wound environment.

XI. WOUND FIBROBLASTS

Recent studies have also shown that contraction by wound fibroblasts leads to increased tissue tension and translocation of existing large vessels into the granulation tissue (Kilarski *et al.*, 2009). This co-option of existing vasculature has been proposed to account for the rapid appearance of large functional vessels in granulation tissue. Again this mechanism is not unique to wounds, as earlier reports also demonstrated a role for co-option of existing vasculature by tumors (Holash *et al.*, 1999). Interestingly, the co-opted vasculature was subject to rapid regression and eventually the tumor induced new sprouting angiogenesis to sustain its growth. Thus, wounds and tumors both employ multiple means to establish a neovasculture (co-option, sprouting angiogenesis, vasculogenesis via recruitment of EPC) and further consideration of the dynamic, diverse programs used to ensure continued nutrient supply may help to refine approaches to limit tumor progression.

XII. *HOX* GENES IN WOUND AND TUMOR ANGIOGENESIS

Considering that tumor angiogenesis is initiated and sustained by multiple cells types and factors, understanding how vasculature normally differentiates and subsequently sustain a quiescent nonreactive state may prove to be a viable approach to limiting tumor angiogenesis. In other words, rather than attempting to block multiple source of angiogenic factors, understanding the mechanism which maintains differentiated tissue function may provide a means to prevent EC activation and angiogenesis.

During embryonic development, key morphoregulatory factors, namely *Homeobox* (*Hox*) genes guide organogenesis or act to maintain a differentiated homeostatic tissue function (Wang *et al.*, 2009). The *Hox* genes also act in adult EC to coordinately activate or suppress angiogenic programs in complex and dynamic wound or tumor microenvironments (Arderiu *et al.*, 2007; Botas, 1993; Chen and Gorski, 2008; Mace *et al.*, 2005; Rhoads *et al.*, 2005). *Hox3* genes play key roles in facilitating angiogenesis. For example, *HoxD3* increases expression of matrix degrading proteinases along with coordinate upregulation of integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ that mediate proliferation and migration in provisional matrices (Boudreau and Varner, 2004; Boudreau *et al.*, 1997). *HoxA3* is functionally similar in that it induces angiogenesis but activates distinct targets including urokinase-type plasminogen activator receptor (uPAR) and matrix metalloproteinase MMP-14 to promote EC migration, while *HoxB3* induces synthesis of angiogenic guidance molecules including ephrinA1 (Mace *et al.*, 2005; Myers *et al.*, 2000). Moreover, expression of these proangiogenic *Hox3* genes is upregulated in the tumor microenvironment, but subsequently suppressed in quiescent vessels. *Hox3* genes also can be applied therapeutically to induce angiogenesis and to accelerate healing of problematic wounds (Hansen *et al.*, 2003; Mace *et al.*, 2005, 2009). Importantly, *HoxA3* not only induces sprouting angiogenesis but also induces recruitment of proangiogenic BMDC while simultaneously attenuating the persistent inflammatory response in diabetic wounds (Mace *et al.*, 2009). The ability to coordinate the angiogenic and inflammatory response within a tissue by single morphoregulatory gene may have important implications in managing tumor angiogenic responses.

In addition, other *Hox* genes actively maintain EC in their quiescent or differentiated state and do so in a dominant manner to override both proangiogenic Hox programs as well as those activated by multiple angiogenic factors including VEGF, bFGF, and TNFα. For example, both *HoxA5* and *HoxD10* are expressed in resting quiescent vessels, while its expression is lost in tumor-associated vessels (Myers *et al.*, 2002). However, sustained ectopic expression of either *HoxA5* or *HoxD10* in angiogenic EC acts

dominantly to suppress the angiogenic phenotype (Myers *et al.*, 2002; Rhoads *et al.*, 2005). Notably, in contrast to inhibition of VEGF alone, sustained expression of either *HoxD10* or *HoxA5* is sufficient to impair wound-induced angiogenesis, suggesting that these genes may be sufficient to lock ECs in a nonreactive, quiescent state that could be potentially exploited to limit tumor angiogenesis.

Further evaluation of tumors that fail to respond to conventional anti-angiogenic treatments or analysis of factors that limit wound angiogenesis, or maintain quiescence of the resting vasculature may identify new approaches that can be used in combination with current neoadjuvant therapies to manage tumor progression.

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The TRAIL to Targeted Therapy of Breast Cancer

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Breast cancers can be classified into those which express the estrogen (ER) and progesterone (PR) receptors, those with HER-2 amplification, and those without expression of ER, PR, or amplified HER-2 (referred to as triple-negative or basal-like breast cancer). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) activates apoptosis upon binding to its receptors in many tumor types and the ligand and agonist antibodies are currently being studied in patients in clinical phases I and II trials. Cell line studies suggest that many breast cancer cell lines are very resistant to TRAIL-induced apoptosis. However, recent data suggest that a subset of triple-negative/basal-like breast cancer cells is sensitive to TRAIL as a single agent. In addition, many studies have demonstrated that resistance to TRAIL-mediated apoptosis in breast cancer cells can be overcome by combinations of TRAIL with chemotherapy, radiation, and various targeted agents. This chapter will discuss the current understanding of the mechanisms, which control TRAIL-mediated apoptosis in breast cancer cells. The preclinical data supporting the use of TRAIL ligands and agonistic antibodies alone and in combination in breast cancer will also be discussed.

I. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of cancer death for women. Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die from breast cancer annually (Garcia *et al.*, 2007). In the United States, approximately 180,000 cases of invasive breast cancer and

Advances in CANCER RESEARCH

0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)03003-6

60,000 cases of *in situ* breast cancer are diagnosed annually, and more than 40,000 women die from breast cancer each year—second only to lung cancer (Jemal *et al.*, 2008). The mortality due to breast cancer has been declining in the United States since 1990. The death rate was 32.69 per 100,000 women in 1991 but fell to 25.19 per 100,000 women in 2003 (Jemal *et al.*, 2007). The continuing decrease in mortality from breast cancer has been attributed to early detection due to screening, improved adjuvant therapy, and more recently to decreases in the incidence due to lowered rates of usage of hormone replacement therapy (Berry *et al.*, 2005; Ravdin *et al.*, 2007).

Breast cancer can be divided into several distinct subtypes that have prognostic and therapeutic implications. Clinically, breast cancer patients routinely have the expression of estrogen receptor (ER), progesterone receptor (PR), and amplification of HER-2 evaluated (Brenton *et al.*, 2005). This allows the classification of breast cancer as hormone receptor positive tumors, HER-2 amplified tumors (which may or may not express hormone receptors), and those tumors which do not express ER, PR, and do not have HER-2 amplification. The latter group is referred to as triple-negative breast cancer based on the lack of these three molecular markers. Generally, hormone receptor-expressing breast cancers have a more favorable prognosis than either those with HER-2 amplification or those that are triple-negative (Brenton *et al.*, 2005). While all breast tumor types may be treated with chemotherapy, therapeutic options in both early and late stage breast cancer are affected significantly by the expression of these three markers. Tumors that express ER and PR are treated with agents that interfere with hormone production or action. Tumors that have amplified HER-2 are treated with agents that inhibit HER-2. Triple-negative tumors are treated with predominantly chemotherapy (Brenton *et al.*, 2005).

Recent expression profiling of human breast cancers has allowed classification of the tumors based on clustering and the similarity of expression patterns between normal breast cells and tumors (Perou *et al.*, 2000; Sorlie *et al.*, 2001). The hormone receptor-expressing breast cancers resembled most closely the luminal cells of the breast ducts but could be further subdivided into several subgroups that have different prognoses and responses to hormonal therapy. The tumors with HER-2 amplification clustered together and were found to have a poorer prognosis than the luminal subtype. These data were compiled prior to the introduction of trastuzumab. The triple-negative tumors resembled most closely basal cells, cells found on the outside of the breast ducts, and had the worst prognosis (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Subsequent analyses have suggested that the clinical triple-negative classification and the array based basal classification significantly overlap but are not identical (Rakha *et al.*, 2009). Ongoing clinical trials are beginning to evaluate the use of these and other molecular classifications of breast cancer for making treatment decisions (Sotiriou and

Pusztai, 2009). While yet to be applied to the routine care of breast cancer patients, array based molecular classification is likely to allow more individualized treatment in the future.

Despite the advances made in the detection and treatment of early breast cancer that have contributed to the declining mortality in the United States, metastatic breast cancer remains an incurable disease. More efficacious treatments to prevent relapse in early stage patients and to treat metastatic disease are needed if a major impact is to be realized in the mortality of breast cancer. This review will focus on the potential use of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor agonists for the treatment of breast cancer.

II. TRAIL AND ITS RECEPTORS

There are several fundamental apoptosis pathways in cells which are defined by where the initial caspase activation occurs (Fig. 1). One pathway, referred to as the extrinsic pathway, mediates activation of caspase 8 or 10 by ligand binding to cell surface receptors (Ashkenazi, 2002; Danial and Korsmeyer, 2004). A second pathway, referred to as the intrinsic pathway, mediates caspase 9 activation by the mitochondrial release of proapoptotic proteins such as cytochrome *c* in response to a variety of stimuli such as the absence of growth factors, DNA damage, and viral infection (Danial and Korsmeyer, 2004). Cytosolic cytochrome *c* binds to apoptotic peptidase activating factor 1 (APAF-1) and activates caspase 9 in an ATP dependent reaction (Danial and Korsmeyer, 2004). Other pathways trigger the activation of caspase 2 in response to heat shock or DNA damage (Sidi *et al*., 2008; Tu *et al*., 2006) or caspase 12 in response to ER stress (Nakagawa *et al*., 2000). After the activation of the initiator caspases, the pathways converge on downstream caspases such as caspases 3, 6, and 7, so-called effector caspases (Danial and Korsmeyer, 2004). In addition, after the activation of the primary initiator caspases specific to each pathway, other initiator caspases can be activated downstream of these primary caspases (Fig. 1). For example, death receptor (DR) activated caspases 8 and 10 can cleave the BH3 only protein BID, leading to its translocation to the mitochondria where it activates the mitochondrial pathway leading to activation of caspase 9 (Ashkenazi, 2002; Suliman *et al*., 2001). Similarly, activated caspase 3 can directly activate the DR initiator caspase 8 (Slee *et al*., 1999; Sun *et al*., 1999). Thus, while each pathway is defined by the initiating stimuli and caspase that becomes activated, these pathways form an interconnected network within the cell.

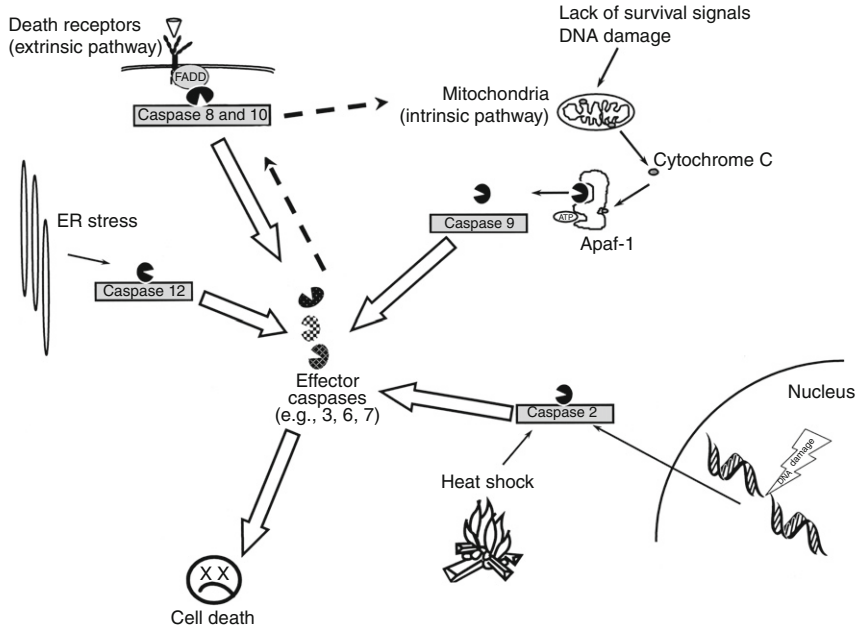

Fig. 1 Apoptosis pathways. Different stimuli and cell stresses result in activation of distinct initiator caspases (i.e., caspases 2, 8, 9, 10, and 12 shown in gray boxes) as discussed in the text. These in turn cleave and activate downstream effector caspases (e.g., caspases 3, 6, and 7). The primary initiator caspase can activate secondary initiator caspases as discussed in text (dashed arrows). The activation of effector caspases leads to apoptotic cell death.

The DRs belong to the tumor necrosis factor (TNF) family. The TNF family has more than 20 receptors of which six are DRs and activate apoptosis in response to binding of their respective ligands (Ashkenazi, 2002). The six proteins are TNFR1 (a.k.a. Death Receptor 1 or DR1), FAS (a.k.a. CD95, DR2), DR3, TRAIL-R1, TRAIL-R2, and DR6. These receptors are activated by their respective ligand: TNF for TNFR1, CD95 Ligand for FAS (a.k.a FAS Ligand), TL1A for DR3, and TRAIL for TRAIL-R1 and TRAIL-R2 (Ashkenazi, 2002). A ligand for DR6 has not been identified. All of these receptors are homotrimeric proteins which activate apoptosis via a cytoplasmic domain known as the death domain (DD) (Fig. 2). These domains serve as protein dimerization motifs that, upon ligand binding, recruit the DD containing adaptor FAS-associated via death domain (FADD) protein. FADD in turn recruits caspases 8 and/or 10 via a death effector domain (DED), a second protein dimerization motif. Recruitment of FADD and the caspases to the receptor forms the death-inducing signaling complex (DISC) and results in activation of the initiator caspases (caspases 8 and 10). The initiator caspases exist in the cell as inactive proenzymes

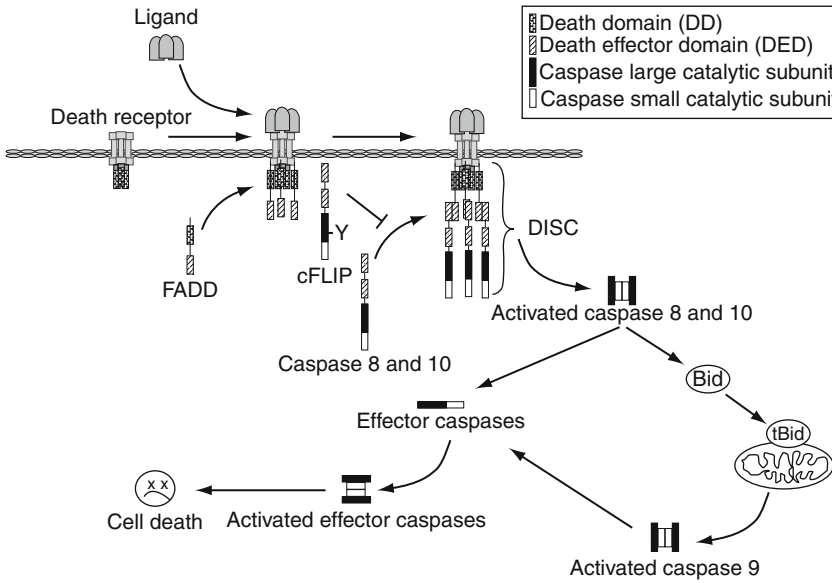

Fig. 2 Death receptor pathway. TNF family death ligands (e.g., TNF, FAS, TRAIL) bind to their cognate receptors and initiate the formation of the DISC. All of the TNF family receptors which induce apoptosis contain a highly conserved death domain (DD) in their cytoplasmic tails. The adaptor protein FADD contains an N-terminal DED and a C-terminal DD. FADD is recruited to the activated receptor by homotypic interactions between the C-terminal DD of FADD and the DD of the receptor. Inactive caspases 8 and 10 zymogens are recruited to the DISC by homotypic interactions between the N-terminal DED domains of the caspases and FADD. cFLIP can be recruited to the DISC and prevents recruitment of caspase 8 or 10. The recruitment of caspase 8 or 10 to the DISC results in activation of the caspases and autoprocessing into the active forms of the caspase (reviewed in Riedl and Shi, 2004). Activated caspase 8 or 10 can directly activate effector caspases (e.g., caspases 3, 6, and 7). Activated caspase 8 or 10 also can cleave the BH3 only protein Bid. Cleaved Bid (tBid) translocates to the mitochondria where it activates the extrinsic pathway.

which become activated upon dimerization at the DISC. They subsequently undergo autoprocessing resulting in the release of a large subunit and a small subunit from the precursor. The processed caspases form a tetramer composed of two large subunits and two small subunits and have markedly increased activity compared to the unprocessed enzyme (Ashkenazi, 2002; Riedl and Shi, 2004). Once activated the initiator caspases can directly cleave and activate the downstream effector caspases. Also, activated caspases 8 and 10 can cleave the BH3 only containing protein Bid, which then translocates to the mitochondrial membrane where it activates the intrinsic pathway (Fig. 2). Cellular FLICE inhibitory protein (cFLIP) is an important negative regulatory molecule in the DR pathway. cFLIP was identified by

homology to viral FLIP proteins which inhibit apoptosis by binding to the DR/FADD complex via DED domains and preventing recruitment of caspase 8 or 10 (Irmler *et al.*, 1997). cFLIP is similar in structure to the caspase 8 proenzyme, containing two N-terminal DED domains and caspase-related domains in the C-terminal. However, the active site cysteine required for caspase activity is replaced by a tyrosine in cFLIP. Thus, cFLIP can be recruited to the DISC and prevent the initiator caspases from being recruited and activated (Fig. 2).

Also, activation of DRs can result in signaling that does not induce apoptosis. For example, TNFR1 can, via recruitment of TRADD, regulate gene expression by activation of nuclear factor-kappa B (NF-κB) and AP1 transcription factors (Wilson *et al.*, 2009). Similarly, FAS can activate proimflammatory responses in addition to apoptotic signaling (Wilson *et al.*, 2009). Like TNFR1, TRAIL receptors can activate NF-κB. This appears to be mediated by the recruitment of RIP which leads to activation of the inhibitor of κB kinases, phosphorylation of the inhibitor of κB, and activation of NF-κB (Falschlehner *et al.*, 2007). Also, TRAIL can activate AKT and MAPK, but the links to these pathways are unclear (Falschlehner *et al.*, 2007). While the TRAIL receptors can signal to nonapoptotic pathways, this review will focus on the role of the TRAIL ligand and its DRs in inducing apoptosis in breast cancer cells.

TRAIL (a.k.a. Apo2L) was initially identified and cloned based on homology searches of EST databases for cDNAs related to TNF and Fas ligand (Pitti *et al.*, 1996; Wiley *et al.*, 1995). These studies identified a ligand that is highly homologous to FAS and TNF and that is able to induce apoptosis in a diverse range of tumor cell lines. Also, the receptors for TRAIL were identified based on homology searches for ESTs that were similar to TNFR1 (Pan *et al.*, 1997a,b). In humans, there are two receptors for TRAIL that can induce apoptosis upon ligand binding, TRAIL-R1 (a.k.a. DR4) and TRAIL-R2 (a.k.a. DR5, TRICK2, and KILLER) (Fig. 3) (MacFarlane *et al.*, 1997; Pan *et al.*, 1997a,b; Screaton *et al.*, 1997; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 1997). There are three receptors, TRAIL-R3 (a.k.a. Decoy Receptor 1, TRID, and LIT), TRAIL-R4 (a.k.a. Decoy Receptor 2 and TRUNND), and TRAIL-R5 (a.k.a. osteoprotegerin) which have incomplete DDs or lack DDs (Degli-Esposti *et al.*, 1997a,b; Emery *et al.*, 1998; Marsters *et al.*, 1997; Pan *et al.*, 1997a, 1998; Schneider *et al.*, 1997a; Sheridan *et al.*, 1997). These three receptors act as inhibitors of TRAIL-induced apoptosis by binding the ligand and sequestering it from the death-inducing receptors (Fig. 3). Expression of TRAIL and TRAIL receptors is found widely distributed throughout the organism (Spierings *et al.*, 2004). Animal studies implicate TRAIL and its receptor as negative regulators of immune responses. TRAIL-deficient mice have a

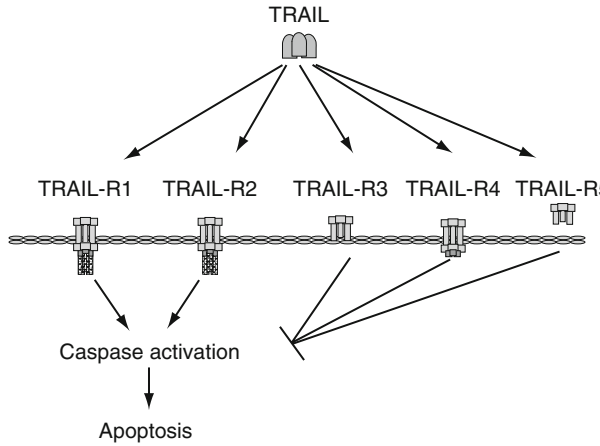

Fig. 3 TRAIL receptors. TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins that contain a DD. Ligand binding to these receptors results in activation of caspases. TRAIL-R3 is a glycophospholipid-anchored cell surface protein, TRAIL-R4 is a transmembrane protein lacking an intact DD, and TRAIL-R5 is a secreted protein. These proteins bind TRAIL but are unable to activate caspases. These receptors act as decoy receptors and can inhibit TRAIL-mediated apoptosis by competing with TRAIL-R1 and TRAIL-R2 for the ligand.

defect in thymocyte apoptosis and a concomitant hypersensitivity to the development of autoimmune T-cell-mediated responses in several experimental systems (Cretney *et al*., 2005; Lamhamedi-Cherradi *et al*., 2003). Also, there is evidence that TRAIL may enhance T-cell-mediated neural cell death in an animal model of autoimmune encephalomyelitis (Aktas *et al*., 2005). Thus, TRAIL may both inhibit and promote autoimmune disease. Mice have a homolog of TRAIL-R2 but do not have a TRAIL-R1 (Kelley and Ashkenazi, 2004). Loss of TRAIL-R2 results in enhanced immune responses to CMV infection, consistent with a role as a negative regulator of innate immune responses (Diehl *et al*., 2004). Importantly, animal studies suggest that TRAIL plays a role in tumor surveillance. Neutralization or deletion of TRAIL in several animal models demonstrates that the loss of TRAIL activity promotes the growth and metastasis of tumors in both transplanted and spontaneous tumors (Cretney *et al*., 2002; Sedger *et al*., 2002; Takeda *et al*., 2001; Zerafa *et al*., 2005). The antitumor effects of TRAIL in these studies appear to be mediated by NK cells (Cretney *et al*., 2002; Takeda *et al*., 2001). In addition, T-cell-mediated graft versus tumor activity appears to be mediated at least in part by TRAIL as allogeneic hematopoietic-cell transplantation from TRAIL-deficient animals resulted in less graft versus tumor activity (Schmaltz *et al*., 2002).

The interest in the antitumor activity of TNF family ligands is based in part on work done by William Coley over 100 years ago which found that bacterial toxins could induce hemorrhagic necrosis of tumors and induce meaningful responses in patients with inoperable tumors such as sarcomas (Coley, 1893, 1906). The search for the biological mediators of these responses led to the identification of TNF and its receptor (Balkwill, 2009; Carswell *et al.*, 1975). Inspired by the results reported by Coley, TNF was tested in patients with cancer but TNF causes severe toxicity and has little efficacy as systemic therapy for cancer (Balkwill, 2009). The second TNF family DR ligand, FAS ligand, has not been tested in clinical trials due to lethal hepatic apoptosis in animal studies (Ogasawara *et al.*, 1993). TRAIL is currently in clinical trials and has generated much excitement as a potential systemic cancer therapy. Early *in vitro* experiments suggested that TRAIL could kill tumor cells in culture but was not toxic to nontransformed cells (Ashkenazi *et al.*, 1999; Keane *et al.*, 1999, 2000). Subsequent experiments with TRAIL in mice, cynomolgus monkeys, and chimpanzees confirmed that TRAIL is well tolerated by animals (Ashkenazi *et al.*, 1999; Kelley *et al.*, 2001; Lawrence *et al.*, 2001). This has lead to phase I clinical trials of both TRAIL and agonistic TRAIL receptor antibodies which have demonstrated that these agents are well tolerated at doses that result in serum levels that are above the therapeutic concentrations that have been used in preclinical studies (Fig. 4) (Camidge *et al.*, 2007; Hotte *et al.*, 2008; Ling *et al.*, 2006; Plummer *et al.*, 2007; Tolcher *et al.*, 2007). These agents are undergoing further testing in clinical trials as single agents and in combination with chemotherapy.

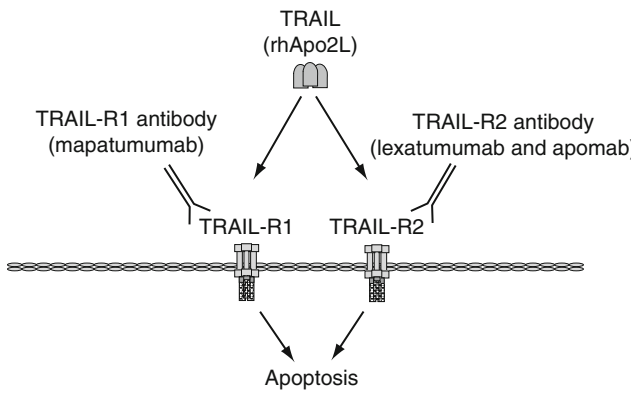

Fig. 4 *TRAIL agonists*. Clinical trials testing TRAIL (a.k.a. rhApo2L), an agonistic TRAIL-R1 antibody (mapatumumab), and agonistic TRAIL-R2 antibodies (lexatumumab and apomab) are currently ongoing.

III. TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

TNF and FAS agonists have been studied using *in vitro* models of breast cancer (e.g., Jaattela *et al*., 1995; Keane *et al*., 1996). However, the toxicity and lack of efficacy of TNF in clinical trials and the toxicity of FAS ligands in preclinical studies has precluded further clinical development of these ligands (Balkwill, 2009; Ogasawara *et al*., 1993). Initial studies of TRAIL-mediated apoptosis in breast cancer cell lines demonstrated that while TRAIL could induce apoptosis in the MDA-MB-231 breast cancer cell line, the majority of cell lines tested were very resistant to TRAIL-mediated apoptosis (Ashkenazi *et al*., 1999; Buchsbaum *et al*., 2003; Keane *et al*., 1999, 2000; Singh *et al*., 2003). These studies were able to establish that TRAIL induced caspase-mediated apoptosis in the sensitive cell line and that TRAIL activated caspases within minutes of addition to the cells (Keane *et al*., 1999, 2000). However, these studies did not systematically evaluate breast cancer cell lines with different phenotypes as defined above (e.g., hormone receptor positive, HER-2 amplified, or triple-negative cell lines). Recently, our laboratory reexamined TRAIL sensitivity in breast cancer cells using a panel of cell lines that included multiple cell lines of each phenotype (Neve *et al*., 2006; Rahman *et al*., 2009). This study found that TRAIL sensitivity varied with the phenotype of the breast cancer cell lines (Fig. 5) (Rahman *et al*., 2009). Strikingly, eight of 11 triple-negative breast cancer cell lines were very sensitive to TRAIL-induced apoptosis with the IC50 ranging from 10 to 250 ng/ml (~0.2–5.8 nM). By contrast, all five of the ER positive cell lines tested were resistant to TRAIL-induced apoptosis across a wide range of doses. Two of five cell lines with HER-2 amplification showed a modest sensitivity to TRAIL, only reaching an IC50 at approximately 1000 ng/ml (~20 nM). Other studies, although not designed to specifically look at TRAIL sensitivity based on the phenotype of the cell lines, found similar results. For example, Chinnaiyan *et al*. (2000) studied TRAIL sensitivity in 10 breast cancer cell lines. They found that three of five triple-negative breast cancer cell lines were TRAIL-sensitive. Two HER-2 amplified breast cancer cell lines and three ER positive cell lines were TRAIL-resistant (Chinnaiyan *et al*., 2000). Similarly, Buchsbaum *et al*. (2003) found that an agonistic anti-TRAIL-R2 antibody induced apoptosis in one of two triple-negative breast cancer cell lines but not any of four HER-2 amplified breast cancer cell lines nor in an ER positive cell line. Together the data from these three studies demonstrated that 10 of 14 triple-negative breast cancer cell lines were sensitive to TRAIL-induced apoptosis while only two of eight HER-2 amplified cell lines, and none of seven ER positive lines were sensitive to TRAIL-induced apoptosis.

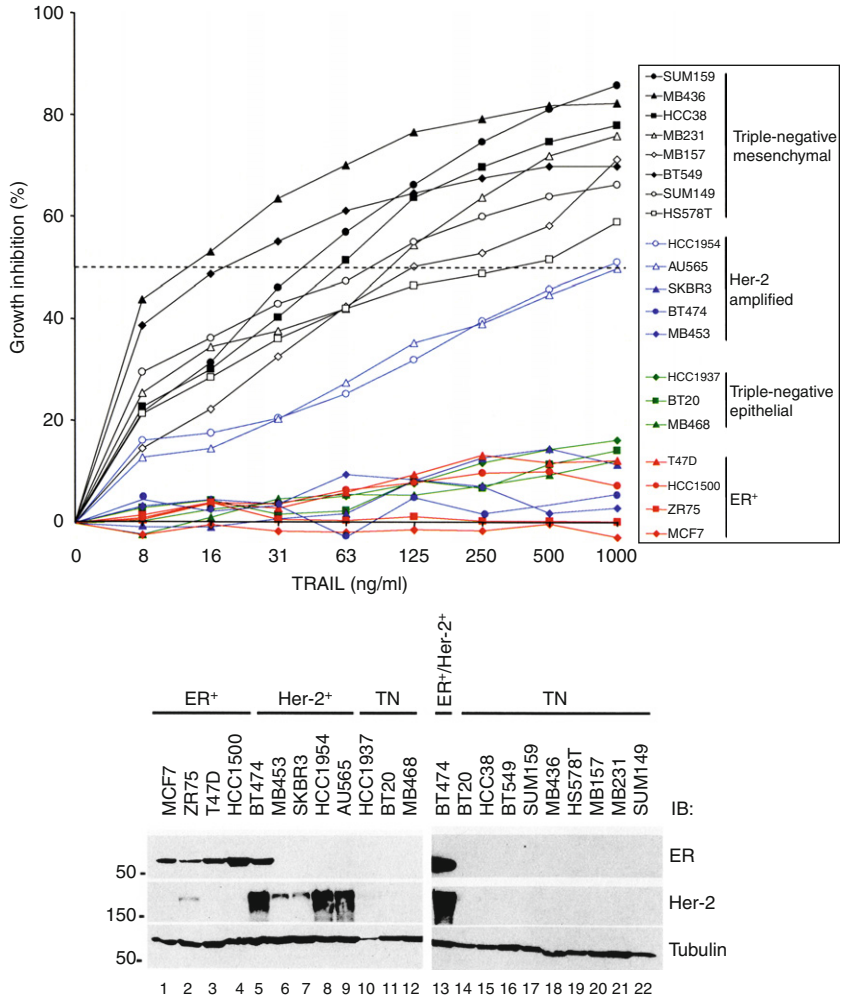

Fig. 5 TRAIL selectively kills mesenchymal triple-negative breast cancer cell lines. (*Top*) Growth inhibition of breast cancer cells incubated with TRAIL. Black lines represent mesenchymal triple-negative cell lines, green lines represent epithelial triple-negative cell lines; blue lines represent HER-2 amplified cell lines, and red lines represent ER positive cell lines. (*Bottom*) Characterization of ER and HER-2 expression in the breast cell lines. This figure is reproduced with kind permission of Springer Science and Business Media from Fig. 1 in Rahman *et al.* (2009).

In cancer cells, the mutation or absence of p53 renders cells resistant to chemotherapy or radiation therapy (Bunz *et al.*, 1999; Lee and Bernstein, 1993; Lowe *et al.*, 1993, 1994). In breast cancer specifically, primary

resistance to doxorubicin has been associated with p53 mutations (Aas *et al.*, 1996). Interestingly, recent work has found that p53 absence or mutation is frequent in triple-negative/basal-like breast cancers (Brenton *et al.*, 2005). The TRAIL-sensitive triple-negative breast cancer cell lines described above frequently have lost or mutated p53 (Neve *et al.*, 2006; Rahman *et al.*, 2009). The ability of TRAIL to kill tumors that are p53 mutant or deleted has been observed in cell lines from a wide variety of tumor types (Ashkenazi *et al.*, 2008). This suggests that TRAIL ligands may be particularly useful as a therapeutic agent in tumors deficient in p53.

Recent work has classified a large number of breast cancer cell lines based on transcriptional profiling (Neve *et al.*, 2006). Like the array based profiling of primary tumors described above (Perou *et al.*, 2000; Sorlie *et al.*, 2001), breast cancer cell lines could be classified into two main groups, luminal and basal. The triple-negative breast cancer cell lines were classified as basal by this analysis (Neve *et al.*, 2006). The basal group was further subdivided into basal "A" and basal "B" groups. The basal B cell lines were distinguished based on expression of mesenchymal markers such as the cytoskeletal protein vimentin. An independent group classified breast cancer cell lines by transcriptional profiling and similarly found that a subset of the triple-negative cell lines had mesenchymal features (Charafe-Jauffret *et al.*, 2006). Interestingly, we found that all of the TRAIL-sensitive triple-negative cell lines we tested have mesenchymal features based on these analyses (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). In contrast, the TRAIL-resistant triple-negative cell lines in our study were ones that are classified as epithelial by these analyses (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). A number of studies using the mesenchymal triple-negative breast cancer cell line MDA-MB-231 have demonstrated the efficacy of TRAIL ligands or agonistic antibodies in xenograft studies, confirming the sensitivity of this cell line *in vivo* (Buchsbaum *et al.*, 2003; Shankar *et al.*, 2004; Singh *et al.*, 2003; Thai le *et al.*, 2006). Together these results suggest that triple-negative/basal-like breast cancers with mesenchymal features are more likely to be sensitive to TRAIL-induced apoptosis.

The mesenchymal characterization of the triple-negative breast cancer cell lines which are sensitive to TRAIL was initially identified by transcriptional profiling and confirmed by immunoblotting for vimentin, a mesenchymal marker protein (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Rahman *et al.*, 2009). However, the mesenchymal subset of tumors was not identified in the early transcriptional profiling of primary breast cancer samples that defined the luminal and basal subsets of breast cancer (Sorlie *et al.*, 2001). More recently, immunohistochemical studies of primary breast tumors have identified a subset of tumors in which the cancer cells express vimentin, consistent with the existence of mesenchymal tumors (Livasy *et al.*, 2006;

Umemura *et al.*, 2005; Willipinski-Stapelfeldt *et al.*, 2005). The largest study of more that 2500 primary breast tumors found that approximately 14% of all of the tumors and 35% of the ER negative tumors expressed vimentin (Willipinski-Stapelfeldt *et al.*, 2005). In this study, approximately 7% of the ER positive tumors expressed vimentin. The enrichment of vimentin positive tumors within the ER negative samples is consistent with an enrichment within the triple-negative samples, but this study did not simultaneously evaluate HER-2 amplification so that vimentin positive tumors cannot be classified as triple-negative (Willipinski-Stapelfeldt *et al.*, 2005). The other two studies identified vimentin expression in 17 of 18 and 4 of 11 triple-negative breast cancer samples (Livasy *et al.*, 2006; Umemura *et al.*, 2005). In the study by Livasy *et al.* (2006), the tumors were categorized as luminal, basal, or HER-2 amplified by cDNA microarray expression profiling. This study found 17 of 18 triple-negative/basal-like tumors had strong and diffuse vimentin staining in the tumor cells (Livasy *et al.*, 2006). Only 1 of 16 ER positive/luminal cancers and 1 of 12 HER-2 amplified tumors expressed vimentin in the tumor cells. These studies suggest that a subset of triple-negative breast cancers have mesenchymal features.

As described above, in humans there are two receptors for TRAIL that induce apoptosis, TRAIL-R1 and TRAIL-R2. Previous work using mutants of TRAIL that bind selectively to either TRAIL-R1 or TRAIL-R2 has demonstrated that TRAIL induces apoptosis predominantly via TRAIL-R1 in some tumor types and via TRAIL-R2 in others (Kelley *et al.*, 2005; MacFarlane *et al.*, 2005; van der Sloot *et al.*, 2006). Both receptors are expressed at the mRNA and protein levels in the TRAIL-sensitive breast cancer cells (Keane *et al.*, 1999; Rahman *et al.*, 2009). A study using agonist antibodies to either TRAIL-R1 or TRAIL-R2 has shown that both can induce apoptosis in the MDA-MB-231 cell line (Zhang and Zhang, 2008). Interestingly, despite expression of both receptors on the breast cancer cells and the ability of the agonist anti-TRAIL-R1 antibody to induce apoptosis, experiments using RNA interference or receptor selective mutants of TRAIL indicate that TRAIL-R2 is the predominant mediator of apoptosis in the breast cancer cells exposed to TRAIL (Kelley *et al.*, 2005; Rahman *et al.*, 2009). The basis for the selective activity of TRAIL-R2 in the breast cancer cells is not clear. One possibility is that the absolute level of TRAIL-R2 at the cell surface is significantly greater than that of TRAIL-R1, but this has not been demonstrated. Alternatively, binding studies suggest that TRAIL-R2 has a significantly higher affinity ($K_d \leq 2$ nM) for TRAIL than TRAIL-R1 ($K_d = 70$ nM) when the binding studies are carried out at 37 °C (Truneh *et al.*, 2000). This observation could explain the discordant results described above, where the agonist anti-TRAIL-R1 antibody can induce apoptosis in MDA-MB-231 cells but the ligand utilizes preferentially TRAIL-R2.

Overall, these observations about breast cancer subtype and receptor selectivity will be important in planning clinical trials of TRAIL ligands or agonistic antibodies in breast cancer patients.

IV. MECHANISMS DETERMINING TRAIL SENSITIVITY IN BREAST CANCER CELLS

The underlying determinants of TRAIL sensitivity in the breast cancer cell lines have not been clearly established. While the experiments described above suggest a subset of breast cancer cells are intrinsically more sensitive to TRAIL (i.e., triple-negative breast cancer cells with mesenchymal features), no clear mechanistic basis for this was determined (Rahman *et al*., 2009).

Of the five receptors for TRAIL two receptors, TRAIL-R1 and TRAIL-R2, induce caspase activation and apoptosis upon ligand binding (Fig. 3) (MacFarlane *et al*., 1997; Pan *et al*., 1997a,b; Screaton *et al*., 1997; Sheridan *et al*., 1997; Walczak *et al*., 1997; Wu *et al*., 1997). In some tumor types, such as neuroblastoma, lack of surface expression of TRAIL-R1 or TRAIL-R2 has been found to correlate with the lack of TRAIL sensitivity (Yang *et al*., 2003). Expression of TRAIL receptors on breast cancer cells has been examined in a number of studies. The levels of receptor either by mRNA, total protein levels, or surface expression are not predictive of TRAIL sensitivity (Buchsbaum *et al*., 2003; Keane *et al*., 1999; Rahman *et al*., 2009). For example, we determined the surface expression of both TRAIL-R1 and TRAIL-R2 in seven sensitive and seven resistant breast cancer cell lines (Fig. 6). The expression of TRAIL-R1 overlapped significantly between the sensitive and resistant cell lines and did not allow discrimination of the sensitive and resistance cells. However, as described above, studies suggest that TRAIL-R1 may not contribute significantly to the induction of apoptosis by TRAIL in breast cancer cells (Kelley *et al*., 2005; Rahman *et al*., 2009). While the expression of TRAIL-R2 is generally higher on TRAIL-sensitive cells than on TRAIL-resistant cells there was again significant overlap between the surface level on sensitive and resistant cells (Fig. 6). In addition, many of the TRAIL-resistant cells expressed only marginally less surface TRAIL-R2 than the sensitive cells (Fig. 6). Three receptors, TRAIL-R3, TRAIL-R4, and TRAIL-R5, can bind TRAIL but do not have a functional DD (Fig. 3) (Degli-Esposti *et al*., 1997a,b; Emery *et al*., 1998; Marsters *et al*., 1997; Pan *et al*., 1997a, 1998; Schneider *et al*., 1997a; Sheridan *et al*., 1997). These receptors have been shown to inhibit TRAIL-induced apoptosis when overexpressed and have been called decoy receptors. However, the expression of these decoy receptors has not

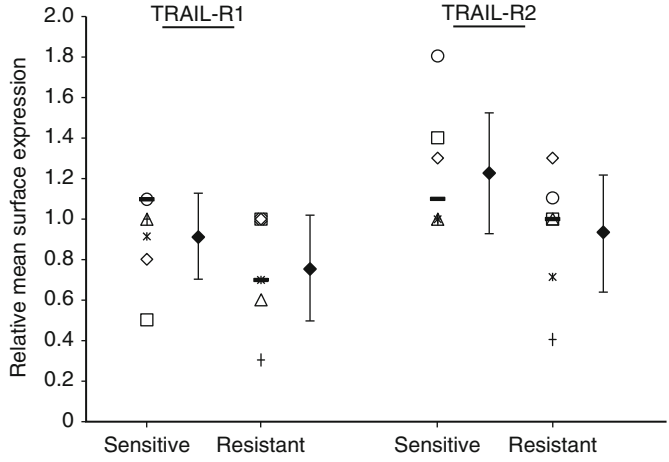

Fig. 6 Cell surface expression of TRAIL-R1 and TRAIL-R2 do not correlate with TRAIL sensitivity. Mean cell surface expression of TRAIL-R1 and TRAIL-R2 in seven TRAIL-sensitive and seven TRAIL-resistant breast cancer cells was measured by flow cytometry (see text for discussion).

been found to correlate with resistance to TRAIL-induced apoptosis in breast cancer cell lines or in other tumors (Griffith *et al.*, 1998, 1999; Keane *et al.*, 1999; Rahman *et al.*, 2009). Thus, expression levels of TRAIL receptors do not appear to be predictive of TRAIL sensitivity.

Examination for evidence of mutation in the death-inducing TRAIL receptors (TRAIL-R1 and TRAIL-R2) in breast cancer cells has given contradictory results. One study found that three of 57 (5.3%) primary breast cancers had mutations in the DD of TRAIL-R1 and four of 57 (7.0%) had mutations in the DD of TRAIL-R2 (Shin *et al.*, 2001). Interestingly, all of these mutations were found in the group of 34 breast cancers that were metastatic to the regional lymph nodes and none were found in the 23 samples from tumors that had not spread to regional nodes. In addition, these mutant receptors were impaired in their ability to induce apoptosis compared to wild-type receptors (Shin *et al.*, 2001). A second study looked at a series of primary breast cancers and found that two out of 50 had sequence variants in TRAIL-R1 and 11 of 95 tumors had sequence variants in TRAIL-R2. However, all of the sequence variants were found in matched normal tissue leading to the conclusion that they represented polymorphisms and not cancer-specific mutations. None of these sequence variants were in the DD but no functional studies were undertaken for these sequence variants. Overall, the second study concluded that there was no relationship between these polymorphisms and breast cancer. While no systematic sequence data for the cell lines has been reported, one study has sequenced TRAIL-R1 and

TRAIL-R2 from seven breast cancer cell lines but found no correlation of sequence variants with TRAIL sensitivity (Zhang and Zhang, 2008). Similarly, we have examined the mRNA sequence for TRAIL-R1 and TRAIL-R2 from a number of the TRAIL-sensitive and -resistant cells and have not found evidence for mutation or sequence variation that correlates with TRAIL sensitivity or resistance (unpublished data).

In general, studies have not identified individual components of either the TRAIL pathway (e.g., TRAIL receptors, FADD, caspase 8) or apoptosis modulators (e.g., cFLIP, IAPs, or Bcl-2 family members) whose expression is predictive of TRAIL sensitivity or resistance (Keane *et al*., 1999; Rahman *et al*., 2009). A number of studies have found that altering the levels or activity of antiapoptotic proteins such as Bcl-2 or Bcl-XL, FLIP, NFκB, or Survivin can alter the sensitivity of cells to TRAIL (Fulda and Debatin, 2004; Fulda *et al*., 2002; Guseva *et al*., 2008; Keane *et al*., 2000; Kim *et al*., 2003; Palacios *et al*., 2006). However, these studies do not demonstrate that the levels or activity of these proteins are the primary reason for TRAIL resistance in the breast cancer cells.

Recent analysis in pancreatic cancer, colorectal cancer, non-small-cell lung cancer, and melanoma cell lines has identified low expression of *O*-glycosylation genes as a potential mechanism of TRAIL resistance (Wagner *et al*., 2007). This study found that *O*-glycosylation of the TRAIL receptors promoted ligand-induced clustering of the receptors and subsequent recruitment and activation of initiator caspase 8. However, gene expression analysis in the breast cancer cell lines did not find a correlation between the genes which regulate *O*-glycosylation and TRAIL sensitivity (Rahman *et al*., 2009). Further experiments with inhibitors of *O*-glycosylation or overexpression of genes which mediate *O*-glycosylation did not affect TRAIL sensitivity in the breast cancer cell lines (unpublished observations). Thus, this mechanism does not appear to determine TRAIL sensitivity in breast cancer cells.

An intriguing observation is that the TRAIL-sensitive MDA-MB-231 breast cancer cell line (a triple-negative breast cancer cell line with basal and mesenchymal features) has low expression of the small heat shock protein, αB-crystallin, while several TRAIL-resistant cell lines (including a TRAIL-resistant triple-negative and an ER positive cell line) have high expression of αB-crystallin (Kamradt *et al*., 2005). Overexpression of αB-crystallin in MDA-MB-231 decreases the sensitivity to TRAIL and RNAi-mediated knockdown of αB-crystallin in one cell line increased sensitivity to TRAIL (Kamradt *et al*., 2005). No systematic evaluation of αB-crystallin and TRAIL sensitivity in a more extensive panel of breast cancer cells representing the different subtypes has been undertaken. Paradoxically, studies of primary breast cancer samples have demonstrated that αB-crystallin is expressed predominantly in triple-negative/basal-like breast cancers and not in ER positive or HER-2 positive tumors

(Moyano *et al.*, 2006; Sitterding *et al.*, 2008). These results are at odds with the cell line data described above which showed low expression in one triple-negative/basal-like breast cancer cell line (MDA-MB-231) but high expression in one triple-negative/basal-like and one ER positive cell line (MDA-MB-468 and MCF 7, respectively) (Kamradt *et al.*, 2005). Thus, further work will be needed to determine if αB-crystallin is predictive of TRAIL sensitivity in a wider sample of breast cancer cells.

Like many cell surface receptors, DRs undergo activation-induced internalization via the endocytic pathway (Austin *et al.*, 2006; Higuchi and Aggarwal, 1994; Kohlhaas *et al.*, 2007; Lee *et al.*, 2006; Schneider-Brachert *et al.*, 2004; Siegel *et al.*, 2004). Internalization appears required for optimal induction of apoptosis by TNFR and FAS (Lee *et al.*, 2006; Schneider-Brachert *et al.*, 2004). Studies with TRAIL receptors have shown that TRAIL-R1 and TRAIL-R2 undergo clathrin dependent endocytosis upon ligand activation (Austin *et al.*, 2006; Kohlhaas *et al.*, 2007; Zhang *et al.*, 2008). In contrast to TNFR and FAS, internalization is not required for effective apoptotic signaling by TRAIL receptors (Austin *et al.*, 2006; Kohlhaas *et al.*, 2007). Interestingly, activation of TRAIL-R2 results in caspase dependent cleavage of clathrin and this attenuates internalization (Austin *et al.*, 2006). These studies further suggest that endocytosis negatively regulates apoptotic signaling and that blocking endocytosis (e.g., by expression of a dominant negative dynamin mutant or by inhibition with chlorpromazine) potentiates TRAIL-induced apoptosis (Austin *et al.*, 2006; Zhang *et al.*, 2008). One study has found that the TRAIL receptors are localized predominantly in the cytosol in TRAIL-resistant breast cancer cells while they are localized on the plasma membrane on TRAIL-sensitive cells (Zhang and Zhang, 2008). Inhibition of clathrin-mediated endocytosis increased the cell surface expression of TRAIL receptors and the sensitivity to TRAIL-mediated apoptosis in these resistant breast cancer cells (Zhang and Zhang, 2008). This suggests that a defect in proper trafficking of the TRAIL receptors could account for TRAIL resistance. No mechanism was described to account for the preferential localization of the TRAIL receptors in the cytosol of resistant cells.

V. OVERCOMING TRAIL RESISTANCE

Many studies in the literature have investigated the combination of a wide range of drugs with TRAIL to potentiate cell death and/or overcome resistance. This has also been investigated in breast cancer cells and, as will be outlined below, the results of many studies suggest that TRAIL may have the widest use in treating breast cancer when used in combination with other agents.

Combinations of chemotherapy with TRAIL have been extensively studied in many cancer cell types (Ashkenazi *et al*., 2008). In breast cancer cells, the combination of TRAIL with chemotherapeutic drugs commonly used in the treatment of breast cancer can enhance the induction of apoptosis in the cancer cells (Buchsbaum *et al*., 2003; Keane *et al*., 1999; Singh *et al*., 2003). A wide range of drugs has been tested in these studies including camptothecin, doxorubicin, etoposide, 5-fluorouracil, melphalan, methotrexate, paclitaxel, vincristin, and vinblastin. While each of the drugs can enhance TRAIL-mediated apoptosis in some of the breast cancer cells tested, the most consistent finding across the three studies is that doxorubicin synergistically enhances TRAIL-mediated apoptosis (Buchsbaum *et al*., 2003; Keane *et al*., 1999; Singh *et al*., 2003). Importantly, the combination of TRAIL with chemotherapeutic drugs can overcome the intrinsic resistance to TRAIL in breast cancer cell lines (Buchsbaum *et al*., 2003; Keane *et al*., 1999; Singh *et al*., 2003). Similarly, tumor xenograft studies using the TRAIL-sensitive MDA-MB-231 cell line have shown that the combination of TRAIL and chemotherapeutic drugs more effectively inhibits the growth of tumors than either alone (Buchsbaum *et al*., 2003; Singh *et al*., 2003). Several mechanisms have been proposed by which chemotherapeutic drugs enhance TRAIL-mediated apoptosis in the resistant breast cancer cells. In one study, concurrent administration of TRAIL and the chemotherapeutic agent caused markedly increased caspase activation. Interestingly, drugs that themselves activated caspases interacted synergistically with TRAIL while those that did not activate caspases did not enhance TRAIL-mediated apoptosis (Keane *et al*., 1999). Caspase inhibition using the pan-caspase inhibitor ZVAD-FMK blocked the cell death induced by TRAIL, the caspase activating chemotherapeutic drugs, and the combination of the two. In this study, using simultaneous treatment with TRAIL and chemotherapeutic drugs, no consistent change in mRNA for TRAIL receptors or other apoptosis regulators was identified. This suggested that the independent activation of caspases by TRAIL and the chemotherapeutic drug accounted for the synergism. A second study found that a 24 h pretreatment of breast cancer cells with chemotherapeutic drugs resulted in an upregulation of TRAIL-R1 and TRAIL-R2 mRNA and protein and that this correlated with the increased sensitivity of the cells to TRAIL (Singh *et al*., 2003). This study found that simultaneous treatment of cells with TRAIL and chemotherapeutic drug or pretreatment with TRAIL followed by the chemotherapeutic drug was not as effective as preincubation of the cells with the chemotherapeutic drug. In addition, upregulation of the mRNA for proapoptotic Bcl2 family members (e.g., BAX and BAD) was observed in cells pretreated with chemotherapeutic drugs. Consistent with the first study, this second study also found that the chemotherapeutic drugs activated caspases and that caspase inhibition blocked the toxicity of the drugs, TRAIL, and the

combination (Singh *et al.*, 2003). As in cell lines from other tumor types, the interaction of TRAIL and chemotherapeutic drugs appears independent of p53 status in the cell lines studied (Ashkenazi *et al.*, 2008; Keane *et al.*, 1999; Singh *et al.*, 2003). The ability of chemotherapeutic drugs to enhance TRAIL-mediated apoptosis and overcome the intrinsic resistance of breast cancer cells to TRAIL provides a rationale for combining TRAIL with chemotherapeutic drugs in clinical trials.

Radiation also enhances TRAIL-mediated apoptosis in cell lines and in tumor xenografts (Buchsbaum *et al.*, 2003; Chinnaiyan *et al.*, 2000). Mechanistic studies suggest that radiation results in upregulation of TRAIL-R2 in a p53 dependent fashion (Chinnaiyan *et al.*, 2000). Interestingly, TRAIL-R2 was independently identified as a p53-regulated gene induced by DNA damage (Wu *et al.*, 1997). However, while the combination of radiation and TRAIL appears to be synergistic, the utility of this approach to treating breast cancer is limited by the systemic nature of the disease.

While chemotherapeutic agents have shown promise in combination with TRAIL, they also may increase toxicity. For example, while we found that chemotherapeutic drugs could enhance TRAIL-mediated apoptosis in resistant breast cancer cell lines, these combinations also resulted in increased death of normal mammary epithelial cells (Keane *et al.*, 1999). This has lead to an interest in combining TRAIL with targeted agents in breast cancer and in other tumor types in an effort to identify combinations that may potentiate the death of tumor cells without increasing toxicity (Ashkenazi *et al.*, 2008).

HER-2 is a member of the epidermal growth factor receptor (EGFR) family that is amplified and overexpressed in 15–30% of breast and ovarian cancers (Slamon *et al.*, 1987, 1989; Tyson *et al.*, 1991; Zhang *et al.*, 1989). The majority of breast cancer cell lines with HER-2 amplification are resistant to TRAIL-mediated apoptosis (Buchsbaum *et al.*, 2003; Chinnaiyan *et al.*, 2000; Rahman *et al.*, 2009). The humanized anti-HER-2 antibody, trastuzumab (Herceptin®), has clinical activity alone and in combination with chemotherapy in metastatic breast cancer, but only when HER-2 is amplified (Pegram and Slamon, 1999; Pegram *et al.*, 1998; Slamon *et al.*, 2001; Vogel *et al.*, 2002). TRAIL-induced apoptosis could be enhanced in some resistant breast and ovarian cancer cell lines with HER-2 amplification (e.g., SKBR3, MDA-MB453, and SKOV3) when the cells were pretreated with trastuzumab (Cuello *et al.*, 2001; Dubska *et al.*, 2005). There was no interaction between TRAIL and trastuzumab in cells without HER-2 amplification. Mechanistic studies demonstrated that trastuzumab induces downregulation of the HER-2 protein and that this results in inhibition of AKT kinase activity (Cuello *et al.*, 2001). Also, AKT inhibition resulted in increased TRAIL sensitivity in these cells and expression of constitutively active AKT inhibited both TRAIL-mediated apoptosis and its potentiation

by trastuzumab. In contrast to these results, investigation of another cell line (BT474) with HER-2 amplification showed that trastuzumab decreased TRAIL-mediated apoptosis (Dubska *et al*., 2005). As in the report by Cuello *et al*., this study found that incubating cells with trastuzumab resulted in decreased AKT kinase activity in the BT474 cells. However, in these cells this lead to a decrease in the surface expression of TRAIL-R1 and TRAIL-R2 and resistance to TRAIL-induced apoptosis. How a decrease in AKT activity can lead to such different results is unclear. Recent studies have demonstrated that different isoforms of AKT have different biological roles and different affects on apoptosis (Irie *et al*., 2005; Kim *et al*., 2009; Maroulakou *et al*., 2007). Thus, it is possible that the opposing effects of trastuzumab on TRAIL-induced apoptosis are mediated by different AKT isoforms. Interestingly, the BT474 cell line expresses ER in addition to amplified HER-2 (Neve *et al*., 2006; Rahman *et al*., 2009). This result suggests that within HER-2 amplified breast cancers, there may be distinct subgroups that will have very different outcomes if TRAIL and trastuzumab are combined. Thus, tumors with HER-2 amplification may benefit from combined molecularly targeted therapies of TRAIL and trastuzumab. However, more studies will be needed to identify which tumors do and which do not benefit.

EGFR activity can attenuate DR-mediated apoptosis and EGFR inhibition can increase sensitivity of cancer cells to TRAIL (Bremer *et al*., 2005; Gibson *et al*., 1999, 2002; Park and Seol, 2002; Shrader *et al*., 2007; Teraishi *et al*., 2005). High levels of EGFR expression are frequently seen in triple-negative/basal-like breast cancer cells (Korsching *et al*., 2002; Livasy *et al*., 2006; Neve *et al*., 2006; Rahman *et al*., 2009). EGFR inhibition can enhance TRAIL-mediated apoptosis in EGFR-expressing breast cancer cells that are already sensitive to TRAIL (i.e., the mesenchymal triple-negative/basal-like breast cancer cell lines) but not in TRAIL-resistant cancer cell lines (Rahman *et al*., 2009). This suggests that the expression of EGFR in breast cancer cells can modulate trail sensitivity but that it is not the primary reason for resistance to TRAIL-mediated apoptosis. The mechanisms by which EGFR inhibition enhances TRAIL-induced apoptosis in breast cancer cells are not known, but studies in other tumor cell types implicate the inhibition of AKT in mediating these effects (Bremer *et al*., 2005; Gibson *et al*., 1999, 2002; Henson *et al*., 2003; Shrader *et al*., 2007; Teraishi *et al*., 2005). A variety of possible mechanisms downstream of AKT inhibition have been suggested by these studies, including decreased cFLIP expression, decreased XIAP expression, inactivation of Bcl-xL, and decreased Mcl-1 expression. Overall, these data support the investigation of EGFR inhibitors in combination with TRAIL.

ER-expressing cell lines have all been resistant to the induction of apoptosis by TRAIL alone (Buchsbaum *et al*., 2003; Keane *et al*., 1999; Singh *et al*., 2003). These studies have shown synergistic interactions between TRAIL and chemotherapeutic drugs demonstrating that the TRAIL resistance can be overcome in

ER positive cells. ER positive breast cancers are treated with agents that inhibit the activity of the ER including the selective ER modulators tamoxifen (Jordan and Brodie, 2007). A recent study demonstrated that tamoxifen can enhance TRAIL-induced apoptosis in breast cancer cell lines *in vitro* and *in vivo* (Lagadec *et al.*, 2008). Surprisingly, tamoxifen enhanced TRAIL-mediated apoptosis in both ER positive (MCF 7 and T47D) and ER negative (MDA-MB-231 and BT20) breast cancer cell lines. Tamoxifen has been shown to induce apoptosis in an ER independent fashion (Mandlekar and Kong, 2001). Possible mechanisms include activation of Jun N-terminal kinase signaling, activation of p38 signaling, induction of oxidative stress, and induction of ceramide production (Mandlekar and Kong, 2001). Further work is necessary to understand the enhancement of TRAIL-induced apoptosis by tamoxifen.

TRAIL receptors can activate the antiapoptotic transcription factor NF-κB (Chaudhary *et al.*, 1997; Degli-Esposti *et al.*, 1997a; Schneider *et al.*, 1997b). NF-κB can protect cells from a variety of apoptotic stimuli by increasing expression of antiapoptotic proteins (Baeuerle and Baltimore, 1996; Beg and Baltimore, 1996; Mayo *et al.*, 1997; Ravi *et al.*, 2001; Van Antwerp *et al.*, 1996; Wang *et al.*, 1998). Both TRAIL-sensitive and TRAIL-resistant breast cancer cells have detectable NF-κB activity in nuclear extracts prior to treatment with TRAIL which increases upon TRAIL treatment (Keane *et al.*, 2000). In TRAIL-sensitive breast cancer cells, the activation of caspases occurs within minutes of ligand addition to the cells so that the induced NF-κB activity is not likely to have time via transcriptional activation to inhibit the apoptosis (Keane *et al.*, 2000). In TRAIL-resistant breast cancer cell lines, inhibition of NF-κB by over-expression of a genetic inhibitor increased TRAIL-mediated apoptosis (Keane *et al.*, 2000). Similar enhancement of TRAIL-mediated apoptosis has been seen in other tumor types (Jeremias and Debatin, 1998; Jeremias *et al.*, 1998). A recent paper, using the MDA-MB-435 cell line has demonstrated that aspirin can enhance TRAIL-mediated apoptosis *in vitro* and in xenografts (Lu *et al.*, 2008). They found that aspirin treatment resulted in proteasomal degradation of survivin and similarly that RNAi-based down-regulation of survivin enhanced TRAIL-mediated apoptosis (Lu *et al.*, 2008). Interestingly, salycilates have been shown to inhibit NF-κB at the concentrations used in this study (Yin *et al.*, 1998). However, the study by Lu *et al.* did not investigate whether inhibition of NF-κB contributed to the enhancement of TRAIL-mediated apoptosis (Lu *et al.*, 2008). One caveat in this work is that while the MDA-MB-435 cell line was originally derived from a patient with breast cancer, there is controversy in the literature as to whether the cell line in use by investigators originated from a breast cancer or melanoma (Chambers, 2009; Lacroix, 2009). Nonetheless, together these results suggest that small molecule inhibitors of NF-κB could be a means to enhance TRAIL-mediated apoptosis in breast cancer cells.

Histone deacetylase (HDAC) inhibitors are currently under investigation for the treatment of cancer (Carew *et al*., 2008). HDACs mediate deacetylation of histones, generally leading to chromatin compaction in histones and transcriptional repression. By altering the epigenetic regulation of gene transcription, HDAC inhibitors have been shown to induce cell cycle arrest, promote differentiation, and cause tumor cell death (Carew *et al*., 2008). One HDAC inhibitor, vorinostat, has been approved for the treatment of cutaneous T-cell lymphoma (Duvic and Vu, 2007). Preclinical studies combining HDAC inhibitors with TRAIL have shown synergistic induction of apoptosis in tumor cells (Fulda, 2008). Multiple mechanisms have been demonstrated for the enhancement of TRAIL in tumor cells by HDAC inhibitors including upregulation of TRAIL receptors, redistribution of TRAIL receptors to membrane lipid rafts, increased activation of the mitochondrial pathway, downregulation of antiapoptotic proteins (e.g., cFLIP, antiapoptotic Bcl-2 family members, and survivin), and upregulation of proapoptotic Bcl-2 family members (Fulda, 2008). In breast cancer cells, several HDAC inhibitors have been shown to enhance TRAIL-mediated apoptosis (Chopin *et al*., 2004; Singh *et al*., 2005). One study found that the synergistic enhancement of TRAIL-mediated apoptosis in several TRAIL-resistant cell lines by HDAC inhibition was secondary to p21 expression (Chopin *et al*., 2004). The second study described enhanced TRAIL-mediated apoptosis in both TRAIL-sensitive and -resistant cell lines by HDAC inhibitors (Singh *et al*., 2005). This study found that HDAC inhibition resulted in upregulation of TRAIL-R1, TRAIL-R2, and proapoptotic Bcl-2 family members. Interestingly, the upregulation of TRAIL-R1 and TRAIL-R2 by HDAC inhibitors is mediated by NF-κB (Shetty *et al*., 2005; Singh *et al*., 2005). This latter result suggests that NF-κB inhibition (described earlier) may either enhance or inhibit TRAIL-mediated apoptosis depending on the context.

Triterpenoids are naturally occurring compounds synthesized by many plants and two of the naturally occurring triterpenoids, oleanolic acid, and ursolic acid, have weak anti-inflammatory and antitumor effects *in vivo* (Liby *et al*., 2007). More potent synthetic derivatives 2-cyano-3,12-dioxooleana-1, 9(11)-diene-28-oic acid (CDDO) and its derivative 1-(2-cyano-3,12-dioxooleana-1, 9(11)-diene-28-oyl) imidazole (CDDO-Im) have been shown to enhance TRAIL-mediated apoptosis in TRAIL-resistant breast cancer cells both *in vitro* and *in vivo* (Hyer *et al*., 2005). These studies found that CDDO and CDDO-Im enhance apoptosis induced by TRAIL and by agonistic antibodies to either TRAIL-R1 or TRAIL-R2. These studies further showed that CDDO and CDDO-Im downregulate the expression of the antiapoptotic protein cFLIP and upregulate the mRNA and protein expression of TRAIL-R1 and TRAIL-R2. The mechanism by which these compounds regulate the expression of these proteins is unclear (Hyer *et al*., 2005).

VI. FUTURE DIRECTIONS

The data discussed above provide evidence that TRAIL or agonist antibodies directed at TRAIL-R2 could have clinical activity in the treatment of breast cancer. The underlying mechanisms that control TRAIL sensitivity in breast cancer cells have not been clearly defined. The phenotypic markers such as triple-negative/basal-like features and mesenchymal gene expression (e.g., vimentin) may act as surrogate biomarkers to predict the patients most likely to benefit from TRAIL treatment. Clinical trials aimed at these patients would be a logical first step in the clinic. However, further work is necessary to identify the true determinants of TRAIL sensitivity or resistance in breast cancer cells as these are more likely to be robust biomarkers. In addition, a large body of evidence suggests that resistance to TRAIL may be overcome in the other types of breast cancer by combinations of TRAIL ligands with various agents including chemotherapy and targeted therapies. Again, understanding the underlying molecular mechanisms that determine resistance may ultimately lead to more efficacious agents to combine with TRAIL in clinical studies.

ACKNOWLEDGMENTS

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. We thank Marion Nau for critical reading of this manuscript.

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Hepatitis B Virus X Protein: Molecular Functions and Its Role in Virus Life Cycle and Pathogenesis

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Despite the existence of effective vaccines, HBV infection remains a major health problem with 2 billion people infected worldwide. Among them, 350 million are chronically infected, a major risk factor for the development of hepatocellular carcinoma (HCC). There is a strong need to develop new and efficient treatments against chronic infection and HCC. It is therefore important to understand HBV replication and persistence as well as the role of HBV in liver carcinogenesis. This chapter focuses on the regulatory protein HBx which is thought to play a central role in HBV regulation and pathogenesis. HBx has been shown to modulate a myriad of viral and cellular functions, yet its role in virus replication and pathogenesis in infected individuals remains far from being completely understood. © 2009 Elsevier Inc.

I. INTRODUCTION

The human hepatitis B virus (HBV) is the prototype member of a family of small, enveloped DNA virus called hepadnaviruses. These viruses can infect mammals and birds, they display narrow host range, and they infect hepatocytes preferentially. Hepadnaviruses share similar virion structure and relaxed circular, partially double-stranded DNA genome (RC-DNA) that is

Advances in CANCER RESEARCH
0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)03004-8

replicated via an RNA intermediate (Wei and Tiollais, 1999). After entry into hepatocytes, HBV RC-DNA is transported to the nucleus and converted into a covalently closed circular molecule: cccDNA (Beck and Nassal, 2007; Mason *et al.*, 1980; Weiser *et al.*, 1983). cccDNA is the template for the transcription of subgenomic RNAs as well as the pregenomic RNA (pgRNA). In the cytoplasm, pgRNA is then selectively packaged into progeny capsids and reverse transcribed by the viral polymerase into relaxed circular DNA (RC-DNA). Capsids containing mature RC-DNA are either used for intracellular cccDNA amplification or for assembly with the viral envelope in the endoplasmic reticulum, leading to the formation of the viral particles that will be released from the cell (Wei and Tiollais, 1999) (Fig. 1).

Upon HBV infection, the majority of infected patients have subclinical disease and only one-third will experience acute hepatitis with 0–1%

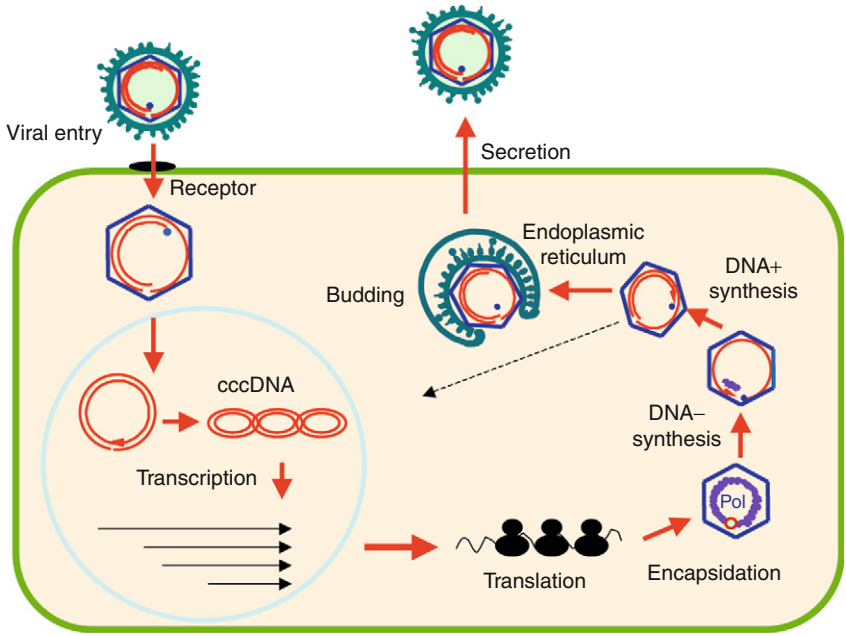

Fig. 1 *HBV life cycle.* After attachment, the nucleocapsid is released into the cytosol and the viral genomic DNA is transported to the nucleus where the virion DNA is repaired and converted to covalently closed circular DNA (cccDNA). The cccDNA is the template for transcription of all viral RNAs. The pregenome RNA is encapsidated into core particles, along with the HBV polymerase. The polymerase synthesizes a negative-strand DNA copy and degrades the RNA template. Positive-strand DNA synthesis begins within the intact core but is only partially completed. With completion of 50% or more of the plus strand, nucleocapsids are packaged into envelopes by budding into the endoplasmic reticulum. Alternatively, nucleocapsids may also migrate to the nucleus to facilitate production of additional cccDNA.

developing fulminant hepatitis. Whereas most patients will then clear the virus, a significant proportion will develop chronic hepatitis as a result of the failure of the host immune response against the virus. Despite the existence of effective vaccines, it has been estimated that 350 million people are chronically infected worldwide. Epidemiological studies have established that persistent HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) and HBV is now thought to be one of the most important environmental carcinogen for humans (Parkin *et al.*, 2001; Szmuness, 1978).

Because of the increasing number of chronic HBV carriers and the poor prognosis of HCC, there is an urgent need to fully understand the mechanism of HBV replication as well as the mechanism of cancer liver development. This chapter will focus on HBx, a regulatory protein that is essential for virus replication. HBx, in order to favor virus replication, has been shown to subvert cellular activities such as signal transduction, transcription, and proliferation. In doing so, HBx might induce the accumulation of dysfunctions and alterations in the cell ultimately leading, in the case of viral persistence, to cancer development.

II. IS HBx AN ESSENTIAL OR ACCESSORY REGULATORY PROTEIN FOR VIRUS REPLICATION?

Sequencing of the HBV genome, and then of woodchuck (WHV) and ground squirrel (GSHV) hepatitis virus genomes, allowed the identification of four open reading frames (Fig. 2). The pre-S/S ORF encodes three viral surface proteins; the pre-C/C ORF encodes the hepatitis B e antigen (HBeAg) and the structural protein of the core: hepatitis B core antigen (HBcAg); the P gene encodes the viral polymerase. Finally, the smallest ORF, which was named X because it shares no homology with any known gene, encodes a 154 amino acid polypeptide called HBx. This protein is produced at very low level during acute and chronic hepatitis and induces humoral and cellular immune responses (Chun *et al.*, 2003; Chung *et al.*, 1999; Hwang *et al.*, 2002; Malmassari *et al.*, 2005). Interestingly, this ORF is present in all mammalian hepadnaviruses but is absent in avian viruses, although HBx expression appears to be required for viral infection and replication. It was first suggested that the X gene product was essential for virus replication *in vivo* since WHV genomes deficient for the expression of WHx cannot replicate in the woodchuck host (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Using a similar model, others have found that such mutant viruses are still able to replicate, albeit at low level (Zhang *et al.*, 2001). However, WHV revertants expressing a wild-type WHx protein eventually emerged, pointing

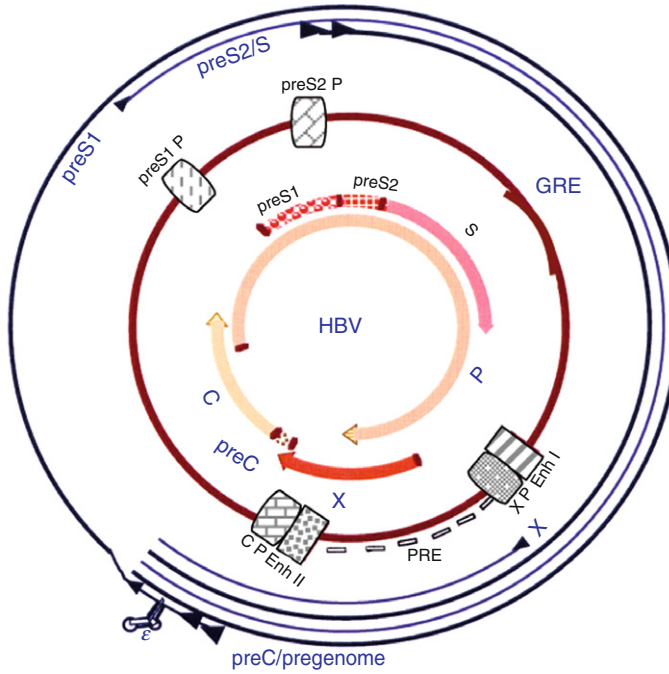

Fig. 2 *Genomic organization of HBV.* The four open reading frames encoding seven proteins are indicated by large arrows. The *cis* elements that regulate HBV transcription are represented by oval and rectangular symbols. PreS1 promoter (PreS1 P), PreS2 promoter (PreS2 P), core promoter (CP) and X promoter (XP), and Enhancer I (Enh I) and Enhancer II (Enh II) are shown. The viral transcripts are represented in the outer layers, with arrows indicating the direction of transcription.

out the importance of a wild-type WHx for full replication. This observation is supported by a recent study using hydrodynamic injection in mice showing that an HBx-deficient HBV genome is strongly compromised for HBV replication (Keasler *et al.*, 2007). HBx expression in this model was able to restore virus replication and viremia to wild-type levels. Similarly, transgenic mice constitutively expressing the wild-type HBV genome or a mutant genome that cannot express HBx shows that HBx increases virus replication even if it is not essential for the virus life cycle (Xu *et al.*, 2002). These results highlight the need to study HBx activity in a context of virus replication closely recapitulating the *in vivo* setting. This discrepancy could be due to a difference in virus transcription between an integrated genome versus the cccDNA or to a difference in the host immune response. The same difficulty in assessing the role of HBx in virus life cycle was encountered when studying replication in tissue culture. Indeed, it has been shown that

HBx-deficient HBV genomes are still able to replicate in the Huh7 cell line, while viral replication is strongly reduced in HepG2 cells using the same construct (Bouchard *et al*., 2001b; Keasler *et al*., 2007; Leupin *et al*., 2005; Melegari *et al*., 1998; Tang *et al*., 2005). Except for the study of WHV in the living host, the role of HBx during the complete virus life cycle has not been assessed, which impedes a comprehensive view of the role of this protein. Given this limitation, most studies however strongly support the importance of HBx in the virus life cycle. Yet the functions supplied by HBx in virus replication still need to be fully elucidated.

III. HBx: A POTENTIAL CANDIDATE IN HCC DEVELOPMENT

The evidence for a role of HBx in the development of HBV-associated pathogenesis and liver cancer came first from indirect proof. Indeed, anti-HBx antibodies are frequently detected in chronic HBsAg carriers showing markers of active viral replication and chronic liver disease, and in HCC patients (Hwang *et al*., 2003; Levrero *et al*., 1991; Vitvitski-Trepo *et al*., 1990; Zhu *et al*., 1993). In HBV-associated HCC, viral DNA sequences have been found in integrated state in 85–90% of cases. While these genomes are incomplete and often rearranged, the X gene is frequently conserved and HBx expression is preferentially maintained in HCC (Hwang *et al*., 2003; Paterlini *et al*., 1995; Peng *et al*., 2005; Su *et al*., 1998). Interestingly, different studies report the transcription of the X gene with a deletion in the C-terminal portion subsequent to the integration of the HBV genome (Iavarone *et al*., 2003; Sirma *et al*., 1999; Tu *et al*., 2001; Wang *et al*., 2004b; Wei *et al*., 1995; Wollersheim *et al*., 1988). While some studies concluded that truncated mutants retain transcriptional transactivation ability, others found that HBx mutants have lost most of the activities associated with wild-type HBx, in particular that they can enhance (instead of inhibit) the transforming activity of Ras and Myc (Tu *et al*., 2001). It remains unclear however whether these mutants play a role in HCC development during HBV infection. It will be interesting to determine if they play a role in the first stages of oncogenesis, or if they emerge later on during tumor progression, allowing full cellular transformation or providing an additional step in the transformation process.

The role of HBx in tumorigenesis has also been studied more directly using animal and cell culture models, but results remain controversial. It has been shown that HBx is able to transform several cell lines such as the NIH3T3 and Rev-2 rodent cell lines expressing the simian virus 40 large tumor antigen (SV40TAg) (Gottlob *et al*., 1998; Seifer *et al*., 1991). In agreement

with these reports, HBx has been reported to cooperate with Ras in the transformation of NIH3T3 and immortalized rodent cells (Kim *et al.*, 2001). In contrast, other laboratories have reported that HBx can suppress transformation of primary rat embryo fibroblasts or of NIH3T3 cells transformed by different oncogenes due to induction of apoptosis (Kim *et al.*, 1998; Schuster *et al.*, 2000). The oncogenic potential of HBx has also been assessed in transgenic mouse models, giving rise again to divergent results. These studies have been carried out in mice generated from different genetic background, and HBx expression was controlled either by its natural HBV enhancer/promoter sequences or by heterologous liver-specific promoters (Koike, 2002). Development of HCC associated with HBx expression has been essentially described for a transgenic mouse line generated in the outbred CD-1 background and expressing high level of HBx in the liver (Kim *et al.*, 1991; Koike *et al.*, 1994a). In other transgenic lineages, expression of HBx by itself does not lead to HCC development (Billet *et al.*, 1995; Perfumo *et al.*, 1992). It is thus possible that the X gene used to generate the transgenic mice as well as the lifelong expression of HBx could impact on the development of HCC (Koike, 2002). However, even if further studies are needed to confirm that HBx can directly induce transformation in mice, its role as a cofactor of carcinogenesis is well accepted. It has been shown, for example, that HBx cooperates with *c-myc* or with chemical carcinogens in hepatocarcinogenesis (Slagle *et al.*, 1996; Terradillos *et al.*, 1997). It has also been reported that HBx expression induces the development of HCC in p21-deficient mice (Wang *et al.*, 2004a). How HBx operates in cellular transformation remains however unclear. HBx is a multifunctional protein exhibiting numerous activities affecting gene transcription, intracellular signal transduction, cell proliferation, apoptotic cell death, and DNA repair that are described in this chapter (Fig. 3). Any or all of these multiple activities could contribute to hepatocarcinogenesis.

IV. HBx: STRUCTURAL AND BIOCHEMICAL FEATURES

Little is known about the three-dimensional structure and the biochemical features of the HBx protein due to the difficulty to produce sufficient amounts of soluble protein. The X ORF codes for a protein of approximately 17 kDa that shows only weak sequence homology with known structural motives or proteins. Sequences analysis of HBx proteins from mammalian hepadnaviruses allowed the identification of highly conserved regions (residues 1–20, 58–84, and 120–140) interspaced by less conserved regions (21–57, 85–119, and 141–154) (Kumar and Sarkar, 2004). Although the domain 85–119 seems to be poorly conserved, it contains the minimal

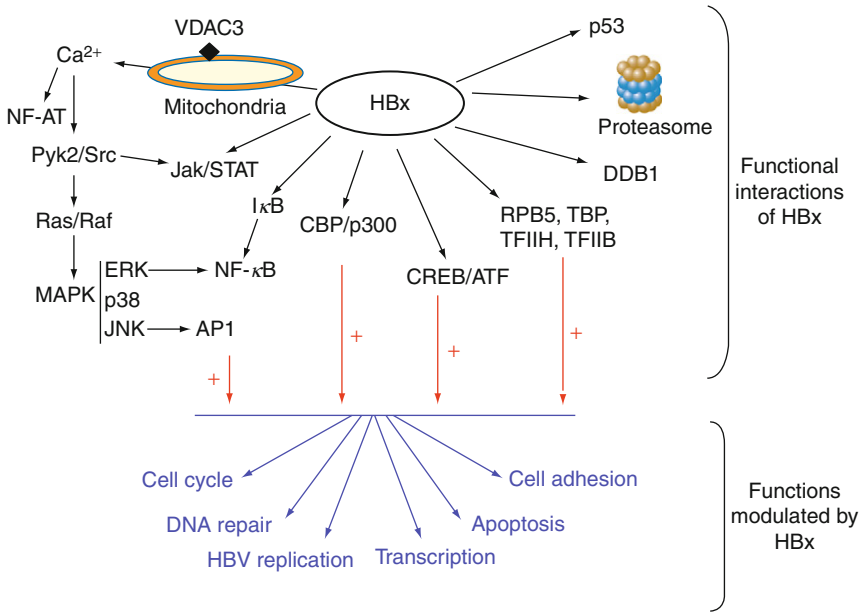

Fig. 3 *Multiple functions of the regulatory protein*. The figure illustrates the complexity of the biological activities of HBx. HBx activates transcription through direct binding to transcription factors, coactivators, and components of the basal transcription machinery. HBx transcriptional activity is also linked to its capacity to stimulate MAPKs and JAK/STAT signaling pathways. Activation of these pathways is indirect and HBx is thought to trigger the release of calcium into the cytosol, which in turn activates the Pyk2/FAK and Src kinase families. Activated Src kinases stimulate in turn a variety of signaling pathways leading, for example, to the activation of transcription factors. HBx interacts with different cellular partners such as CRM1, p53, mitochondria, proteasome, and DDB1 that are involved in HBx activities and could stimulate both HBV replication and be relevant to cell transformation.

domain required for DDB1 binding. This minimal domain is relatively well conserved and seems to adopt a helix structure found in HBx and WHx, as well as in DDB2, a known DDB1-interacting partner (Bergametti *et al.*, 2002a; Scrima *et al.*, 2008). In an attempt to define the structure of HBx by spectroscopic assays, Rui *et al.* (2005) used a version of HBx deleted of its N-terminal cysteines and concluded that HBx appears as an unstructured protein that can gain secondary structure under specific conditions. HBx might be folded and acquire specific function through its interaction with target proteins, and this flexibility could account for the large array of HBx activities (Rui *et al.*, 2005). However, analysis of the structure of HBx produced either from *E. coli* or insect cells, suggested that the cysteines present in HBx form internal disulfide bonds *in vivo* (Lin and Lo, 1989;

Urban *et al.*, 1997). Finally, a report suggests that cysteine residues present in the N-terminal third of HBx could rather be involved in the dimerization of HBx (Gupta *et al.*, 1995). However, the homodimerization domain has been mapped by Murakami *et al.* (1994) in the Ser/Pro-rich region at amino acids 21–50, as part of the negative regulatory domain. The question of whether the N-terminal part is involved in oligomerization, and the role of dimerization in HBx activity warrants further studies.

The localization of HBx remains also a matter of debate. Some studies showed a cytoplasmic localization, whereas others found that the protein is preferentially nuclear, or present both in the cytoplasm and the nucleus (Doria *et al.*, 1995; Schek *et al.*, 1991; Sirma *et al.*, 1998a; Weil *et al.*, 1999). Divergent data were also obtained when studying the expression of HBx in infected hepatocytes (Dandri *et al.*, 1998; Hoare *et al.*, 2001; Su *et al.*, 1998). As a possible explanation to these seemingly contradictory findings, studies from different laboratories have shown that HBx expressed at very low level is predominantly nuclear, whereas high levels of HBx lead to cytoplasmic accumulation (Cha *et al.*, 2009; Henkler *et al.*, 2001). Interestingly, Cha and collaborators reported that both cytoplasmic and nuclear HBx participate in HBV replication (Cha *et al.*, 2009; Henkler *et al.*, 2001). A recent publication from Keasler *et al.* (2009) using a cell culture and a mouse models confirmed the importance of nuclear HBx for restoring the replication of HBx-deficient virus. Altogether, these results argue in favor of a dual localization of HBx consistent with its pleiotropic activities. The localization of HBx during virus replication could fluctuate depending on HBx concentration, but also on the accessibility of cellular partners involved in its nuclear import and export. Indeed, HBx has been shown to bind IkB-α that is, in turn, involved in its nuclear translocation (Weil *et al.*, 1999). Another group reported that HBx interacts with CRM1 and that the cytoplasmic localization of HBx is sensitive to drugs inhibiting CRM1 activity (Forgues *et al.*, 2001).

The dual localization of HBx is consistent with the finding that HBx turnover follows a bimodal kinetic of 20 min and 3 h. The pool of HBx associated with the cytoplasmic fraction appears to decrease much more rapidly than the nuclear and cytoskeleton-associated fraction (Dandri *et al.*, 1998; Schek *et al.*, 1991). To date, the reason for the slower decay of the nuclear fraction remains unknown. A work of Bergametti *et al.* (2002b) showed that the binding of HBx to DDB1 protects the viral protein from degradation. This interaction occurs preferentially in the nucleus and might account for the prolonged half-life (Bontron *et al.*, 2002). HBx turnover has been shown to be both ubiquitin-dependent and ubiquitin-independent (Hu *et al.*, 1999; Kim *et al.*, 2008a). It is however not known whether the two phases of HBx degradation are both ubiquitin-dependent. The E3 ubiquitin ligase regulating HBx turnover remains also unidentified. Id-1 has

been shown to stimulate HBx degradation by facilitating the interaction of HBx with the proteasome (Ling *et al.*, 2008). However, it is not clear whether this process is mediated via the ubiquitination of HBx. The HBV core protein and the cellular protein p53 have also been shown to increase HBx degradation in an indirect fashion (Kim *et al.*, 2003; Park *et al.*, 2009).

Finally, HBx has been shown to be subjected to other posttranslational modifications, such as phosphorylation and acetylation (Schek *et al.*, 1991; Urban *et al.*, 1999). Acetylation occurs at the amino terminus and has been observed only in insect cells (Urban *et al.*, 1999). Phosphorylation of HBx has been described in human hepatoblastoma cells as well as in insect cells and it has been shown that HBx can be phosphorylated *in vitro* by protein kinase C and mitogen-activated kinase (Lee *et al.*, 2001b; Schek *et al.*, 1991; Urban *et al.*, 1999). The relevance of these modifications for HBx activities remains however poorly understood. Noh *et al.* (2004) proposed that HBx is phosphorylated by the extracellular–response kinase 1 and 2 (ERK1/2), which induces its nuclear translocation. It has recently been shown that the peptidyl propyl isomerase Pin1 interacts specifically with phosphorylated HBx and increases its stability, which correlates with enhanced HBx transcriptional activity and HBx-induced tumor development (Pang *et al.*, 2007). The significance of this interaction for HBx activities remains unclear since a mutant that cannot interact with Pin1 does activate transcription and cell proliferation to the same extent as wild-type HBx. However, the finding that this mutant has reduced capacity to induce tumor development compared to the wild-type protein argues for a role of Pin1 in HBx-induced carcinogenesis *in vivo*. Whether Pin1 is only involved in HBx stability or involved in other functions will need to be clarified.

V. HBx ACTIVITIES

A. Transactivation Mechanism of HBx

Transcriptional activation was one of the first functions attributed to HBx (Tiollais *et al.*, 1981).This activity is believed to be crucial for the development of liver cancer because it is involved in HBV transcription/replication (Chou *et al.*, 2005; Tang *et al.*, 2005), as well as in upregulation of a large number of cellular genes involved in oncogenesis, proliferation, inflammation, and immune response (Avantaggiati *et al.*, 1993; Balsano *et al.*, 1991; Cougot *et al.*, 2007; Kim *et al.*, 1996; Lara-Pezzi *et al.*, 1998b; Mahe *et al.*, 1991; Majano *et al.*, 2001; Menzo *et al.*, 1993; Robinson *et al.*, 1993; Twu *et al.*, 1993; Wu *et al.*, 2001; Yen, 1996; Zhou *et al.*, 1990). HBx has been shown to activate the expression of genes involved in cellular proliferation,

such as c-jun, c-fos, PCNA, cyclin D1, or in angiogenesis, such as the vascular endothelial growth factor (VEGF) and IL8 (Avantaggiati *et al.*, 1993; Cougot *et al.*, 2007; Mahe *et al.*, 1991; Park *et al.*, 2006; Robinson *et al.*, 1993; Twu *et al.*, 1993; Yoo *et al.*, 2003). HBx induces TGF-β, a cytokine that plays a major role in hepatic fibrosis and cirrhosis (Yoo *et al.*, 1996). Accordingly, Martin-Vilchez *et al.* (2008) have shown that HBx induces activation of hepatic stellate cells and subsequent amplification of fibrosis through the induction of TGF-β. Interestingly, HBx is able to amplify TGF-β signaling by increasing Smad4 transcriptional activity (Lee *et al.*, 2001a). HBx also increases hepatic steatosis through the induction of SREBP1 and PPARγ expression and transcriptional activation of hepatic adipogenic and lipogenic target genes (Kim *et al.*, 2007a). HBx could also participate in hepatocarcinogenesis by modifying gene expression through epigenetic mechanisms. HBx upregulates the DNA methyltransferases DNMT1, DNMT3A1, and DNMT3A2 leading to an increase in their enzymatic activity. HBx might thus act at the epigenetic level, inducing regional hypermethylation leading to inactivation of genes such as E-cadherin (Lee *et al.*, 2005) or tumor suppressor genes such as $p16^{INK4A}$ (Jung *et al.*, 2007; Park *et al.*, 2007). Recently, HBx has also been shown to be involved in autophagy through the induction of beclin 1 expression (Tang *et al.*, 2009). Global approaches using cDNA microarray, serial analysis of gene expression (SAGE) or combination of chip-based chromatin immunoprecipitation (ChIP on chip) techniques clearly illustrate the ability of HBx to promiscuously activate a myriad of promoters and thus deregulate a large number of cellular genes (Hu *et al.*, 2006; Wu *et al.*, 2001, 2002; Zhang *et al.*, 2009).

Although HBx is described as a weak transactivator, it is capable of activating a wide range of cellular and viral promoters dependent on Polymerase I, II, or III, including the HBV promoters and enhancers (Kumar and Sarkar, 2004; Rossner, 1992; Wang *et al.*, 1995, 1997, 1998; Yen, 1996). HBx activates transcription via several DNA sites such as the binding sites for NF-κB, AP-1, c-EBP, ATF/CREB, SP1, HIF-α, E2F, and NF-AT (Choi *et al.*, 2001; Kumar and Sarkar, 2004; Rossner, 1992; Wang *et al.*, 1995, 1997, 1998; Yen, 1996). HBx does not directly bind DNA and thus, various mechanisms have been described to explain this pleiotropic transcriptional activation, including direct interaction with nuclear transcriptional regulators and activation of cytosolic signal transduction pathways.

HBx has been shown to interact with components of the basal transcriptional machinery (TFIIB, TFIIH, RPB5, and TBP) (Cheong *et al.*, 1995; Haviv *et al.*, 1998a,b; Lin *et al.*, 1997; Qadri *et al.*, 1995) or with transcription factors (CREB/ATF, ATF2, C/EBPα, ATF3, NF-IL-6, Oct1, SMAD4, and SREBP1) (Barnabas and Andrisani, 2000; Choi *et al.*, 1999; Kim *et al.*, 2007a; Lee *et al.*, 2001a; Maguire *et al.*, 1991; Natoli *et al.*, 1994a), as well

as coactivators (Cougot *et al*., 2007). The activation of CREB/ATF transcriptional activity by HBx appears to result from dual mechanisms, since HBx has been shown to increase CREB/ATF DNA-binding affinity and to enhance the recruitment of CBP/p300 to CREB/ATF bound to endogenous cellular DNA (Barnabas *et al*., 1997; Cougot *et al*., 2007). The modulation of CREB/ATF activity by HBx might represent an important aspect of HBx activities since the CREB/ATF family members play an essential role in liver metabolism and proliferation. Recently, CREB has also been implicated in hepatocarcinogenesis (Abramovitch *et al*., 2004). Moreover, this activity could also be involved in the activation of HBV transcription mediated by HBx, since a CREB-binding site-like sequence (CRE) is present in the HBV enhancer 1 and in PreS2 (Tacke *et al*., 2005; Trujillo *et al*., 1991). Further studies will be needed to investigate the respective role of CREB and of coactivators such as CBP/p300 in the activation of HBV replication by HBx. CBP/p300 are known to bind and activate a large variety of cellular transcription factors such as c-Jun, c-Fos, and NF-κB. In a recent analysis of hepatic steatosis, Na *et al*. (2009) showed that HBx increased liver X receptor (LXR) transcriptional activity through direct binding and suggested that HBx could act by increasing the recruitment of CBP to LRX bound to its target promoter. Interaction of HBx with coactivators could thus explain partially the broad activity of HBx on transcription. Kong *et al*. (2003) reported an interaction between HBx and the cancer-amplified transcription coactivator (ASC-2), suggesting a role of HBx in the assembly of the enhanceosome and its activity. Binding sites for some of the HBx-interacting partners have been identified, and the domain necessary for transactivation has been mapped between amino acids 52 and 148, with the last 13 C-terminal amino acids (149–154) of HBx being dispensable and the first 50 N-terminal amino acids behaving as a negative regulatory region (Kumar and Sarkar, 2004).

A second important mechanism for HBx transcriptional activity is linked to its capacity to activate signal transduction pathways. This function is mediated by the cytoplasmic pool of HBx (Bouchard and Schneider, 2004). HBx has been shown to activate mitogen-activated protein kinase pathways including the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases/NH2-terminal jun kinases and the p38 kinase, and janus family of tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Benn and Schneider, 1994; Benn *et al*., 1996; Cross *et al*., 1993; Doria *et al*., 1995; Klein and Schneider, 1997; Lee and Yun, 1998; Natoli *et al*., 1994b; Su and Schneider, 1996b; Tarn *et al*., 2001, 2002). Activation of these pathways by HBx is dependent on the activation of nonreceptor tyrosine kinases of the Src family, since inhibition of Src kinases prevents the activation of the Ras–Raf–MAP kinase, JNK, p38 MAPK, or JAK/STAT pathways (Klein and Schneider, 1997; Tarn

et al., 2002). However, alternative activation of the JAK/STAT signaling pathway mediated through a direct interaction between HBx and JAK1 has also been described (Lee and Yun, 1998). NF-κB, an important mediator of the cellular stress responses that control the expression of several acute phase response proteins, cytokines, and adhesion molecules, is among the factors or functions modulated by HBx through the activation of the MAPK pathways (Doria *et al.*, 1995; Ghosh *et al.*, 1998; Su and Schneider, 1996a). Activation of NF-κB is Src- and Ras-dependent and results from phosphorylation and degradation of the NF-κB inhibitor IkB-α as well as downregulation of p105 NF-κB1 inhibitor, leading to nuclear translocation of NF-κB (Su and Schneider, 1996b). Ras-independent pathways are also suspected to be involved in NF-κB activation, such as sequestration of newly synthesized IkB-α by HBx leading to the sustained activation of NF-κB (Chirillo *et al.*, 1996; Weil *et al.*, 1999). Expression of the hypoxia-inducible factor 1α (HIF-1α) as well as its target gene VEGF has been shown to be increased in HBx-transgenic mice. Yoo *et al.* (2003) showed that HBx stabilizes and activates HIF-1α through the activation of the MAPK pathways. Recently however, the same authors reported that HBx also activates HIF-1α via the induction of MTA1 and HDAC1, two proteins known to modulate HIF-1α activity. Moreover, they reported that HBx interacts with HIF1-α in the nucleus, though the role of this interaction in the formation of the HIF-1α/MAT1/HDAC1 complex and the deacetylation of HIF-1α was not assessed (Yoo *et al.*, 2008). HIF-1α has been shown to be upregulated in a large number of tumors and is associated with tumor progression. Its upregulation in HBx-transgenic mice suggests that HIF-1α plays a role in HBx-associated hepatocarcinogenesis. Another mechanism triggered by HBx-induced Src activation might be transcriptional activation of the androgen receptor (AR) (Chiu *et al.*, 2007; Yang *et al.*, 2009). The effect of HBx on AR transcriptional activity was found to be dependent on androgen concentration. This interesting finding sheds light on the observed predominance of HCC in HBV-infected males. Of note, HBx also enhances AR dimerization and activation by inhibiting glycogen synthase kinase-3β (GSK-3β). Inactivation of GSK-3β by HBx and subsequent activation of the Wnt/β-catenin pathway have been previously reported. In this case, HBx has been shown to activate Wnt/β-catenin signaling through the activation of Src kinase or ERK (Cha *et al.*, 2004; Ding *et al.*, 2005). This finding could be of significant importance for hepatocarcinogenesis. Indeed, abnormal activation of the Wnt signaling pathway is associated with the development of different tumors such as HCC (de La Coste *et al.*, 1998; Polakis, 2000). It has also been shown that HBx causes activation of the transcription factor AP-1 through the Ras–RAF–MAPK and JNK pathways (Benn and Schneider, 1994; Benn *et al.*, 1996; Bouchard *et al.*, 2006; Cross *et al.*, 1993; Natoli *et al.*, 1994b). Some studies have reported that activation of diacylglyerol-dependent protein kinase C is responsible for HBx induction of AP1 and NF-κB activity

(Kekulé *et al.*, 1993; Luber *et al.*, 1993), but it was not confirmed in other studies (Lucito and Schneider, 1992). Finally, Ras signaling is also involved in the stimulation of RNA pol I- and pol III-dependent transcription (Johnson *et al.*, 2000; Wang *et al.*, 1997, 1998). Importantly, HBx activation of MAPKs and JNKs has been demonstrated in HBx-transduced mouse liver. Such constitutive activation is associated with increased activity of AP-1 (Nijhara *et al.*, 2001).

Additionally, activation of Src kinases by HBx has been involved in HBx activities that are not linked to its transcriptional function, such as HBx effects on destabilization of cellular adherent junctions through Src activation (Lara-Pezzi *et al.*, 2001). Disruption of intercellular adhesion might represent a mechanism by which HBx contributes to the development of liver cancer. Finally, activation of Src kinases by HBx has been shown to stimulate HBV replication at the level of DNA replication (Bouchard *et al.*, 2003, 2006; Klein *et al.*, 1999). Notably, these studies emphasized that HBx might stimulate the viral polymerase activity rather than acting at a transcriptional level. Recently, Melegari *et al.* (2005) showed that HBx stimulates core phosphorylation, which correlates with an increase of HBV DNA synthesis. Whether this activity needs Src activation awaits being determined. Using a similar construct, other studies have however shown that HBx also acts at the transcriptional level, suggesting a complex role of HBx on virus replication (Chou *et al.*, 2005; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Melegari *et al.*, 2005).

HBx does not interact directly with Src kinases and recent studies from Bouchard and colleagues made a significant contribution to our understanding of Src activation by HBx. They showed that HBx induces the activation of upstream activators of Src kinases: the focal adhesion kinase (FAK) and the proline-rich tyrosine kinase (Pyk2) through the modulation of cytosolic calcium (Bouchard *et al.*, 2001b, 2006). Direct measurement of cytosolic calcium in HBx-expressing cells confirmed that HBx expression correlates with an increase in cytosolic calcium (Chami *et al.*, 2003; McClain *et al.*, 2007). HBx might mediate this activity through its association with mitochondria (Clippinger and Bouchard, 2008; McClain *et al.*, 2007). Studies from different groups showed that HBx associates with cell mitochondria, disrupts their architecture, and induces depolarization (Kim *et al.*, 2007b; Shirakata and Koike, 2003; Takada *et al.*, 1999). Interaction with mitochondria occurs in part via components of the mitochondrial permeability transition pore (MPTP) such as the voltage-dependent anion channel (VDAC3), which in turn deregulates MPTP function and influences cytosolic calcium level (McClain *et al.*, 2007; Rahmani *et al.*, 2000). A role of HBx and mitochondrial alterations in the deregulation of cytoplasmic calcium is supported by the results of Chami and collaborators showing that deregulation of calcium signaling is due both to a reduced uptake by mitochondria and to inactivation of the plasma

membrane calcium ATPase (PMCA). Moreover, interaction of HBx with mitochondria can lead to apoptosis (see Section V.D). The role of calcium as a mediator of HBx activities has been confirmed for the activation of the MAPK pathways, as well as for the activation of transcription factors, such as nuclear factor of activated T-cells (NF-AT) (Lara-Pezzi *et al.*, 1998a; Tarn *et al.*, 2002). Interestingly, activation of NF-AT by HBx is dual. On one hand it is involved in the dephosphorylation and the nuclear translocation of NF-AT by a calcium/calcineurin-dependent mechanism. On the other hand, HBx acts at the nuclear level as a coactivator by interacting directly with the acidic transactivation domain of NF-AT, increasing its transcriptional activity (Canettieri *et al.*, 2003). The importance of calcium signaling in virus replication has also been confirmed (Bouchard *et al.*, 2001b, 2003). However, although calcium-mobilizing agents can rescue HBx-deficient HBV DNA replication to about 50% of the wild-type level, these compounds do not increase polymerase activity as HBx does (Bouchard *et al.*, 2003). It is not known, for example, whether these compounds increase pgRNA encapsidation that could compensate for the defect of polymerase activity, since calcium has been shown to increase core assembly (Choi *et al.*, 2005). These data argue that even if HBx needs calcium signaling for its activity, it probably has additional functions on virus replication.

Transactivation activity of HBx could therefore lead to the modulation of a large number of functions, such as virus replication, cell cycle regulation, angiogenesis, apoptosis, and DNA repair that could be relevant for cellular transformation.

B. Additional Cellular Partners and Functions of HBx

Besides its interaction with transcription factors, coactivators or components of the basal transcription machinery, a myriad of HBx partners, including mitochondrial molecules, have been described that could be relevant for virus replication or oncogenesis, or both.

To evade apoptosis, viruses have evolved strategies allowing to circumvent this cellular response. Thus, viral transforming protein such as E6 from oncogenic papillomaviruses or large T from SV40 can suppress p53 function, allowing virally infected cells to evade apoptosis. HBx has been shown to interact *in vitro* and *in vivo* with the tumor suppressor p53 (Feitelson *et al.*, 1993; Truant *et al.*, 1995). Although this interaction remains controversial, it is thought to be involved in the inactivation of critical p53 activities. HBx has been reported to inhibit p53 sequence-specific DNA-binding (Wang *et al.*, 1994). Chung *et al.* (2003) reported the downregulation of the tumor suppressor PTEN by HBx through repression of p53 transcriptional activity. Interestingly, p53 can bind to and repress HBV

enhancer leading to the inhibition of HBV replication, and such repression can be relieved by HBx expression (Doitsh and Shaul, 1999; Ori *et al*., 1998). It has been proposed that HBx might interact with and sequester p53 in the cytoplasm, leading to its functional inactivation (Elmore *et al*., 1997b; Ueda *et al*., 1995), but other studies have failed to detect colocalization of p53 and HBx (Su *et al*., 2000). Functional inhibition of the tumor suppressor gene p53 is a common abnormality in human cancer cells. It is thus tempting to speculate that HBx, through p53 inactivation, participates to HCC development and to the high chromosomal instability of HBV-related tumors.

It has also been reported that HBx interacts with components of the proteasome such as PSMA7, an α subunit of the 20 S proteasome complex, or PSMC1, a subunit of the 19 S regulatory factor (Hu *et al*., 1999; Sirma *et al*., 1998b; Zhang *et al*., 2000). However, it remains unclear whether HBx inhibits proteasome activity or whether the proteasome is needed for HBx activity. Indeed, one study reported that inhibition of the proteasome impairs HBx transcriptional activity. In the same work, the authors showed that HBx inhibits proteasome-mediated proteolysis (Hu *et al*., 1999). A second study suggested that HBx might enhance HBV replication through proteasome inhibition (Zhang *et al*., 2004). The authors showed that proteasome inhibitors restored the replication of X-negative virus to the wild-type level, whereas they had no effect on the replication of the wild-type virus. In the context of virus replication, the effect of HBx as well as of proteasome inhibitors seems to be exerted at the posttranscriptional level. Finally, HBx has been shown to interfere with the ubiquitin degradation pathway and to block the degradation of c-Myc through a direct interaction with the F box region of Skp2 (Kalra and Kumar, 2006). Interestingly, dysregulation of protein degradation pathways is a common strategy used by viruses to provide a favorable environment for their replication, and to escape protective mechanisms developed by the host cell (Barry and Fruh, 2006). HBx interaction with DDB1, a core subunit of the Cul4A-based ubiquitin E3 ligase complex, has been very well documented. It has been shown that the HBx/DDB1 interaction is essential for virus replication and for the maintenance of HBx activities (Lee *et al*., 1995; Leupin *et al*., 2005; Lin-Marq *et al*., 2001; Rui *et al*., 2006; Sitterlin *et al*., 1997, 2000b). The precise role of DDB1 in HBx activities remains however unknown. DDB1 was first described as a protein involved in DNA repair (Chu and Chang, 1988). Thus, it was proposed that HBx impairs DNA repair through its interaction with DDB1. *In vitro* as well as *in vivo* studies led to conflicting results and the role of DDB1 in the inhibition of DNA repair by HBx has not been confirmed (Becker *et al*., 1998; Bergametti *et al*., 1999; Capovilla and Arbuthnot, 2003; Madden *et al*., 2000). Further studies will be needed to determine the function of HBx/DDB1 interaction in virus replication and in HBx activities at the molecular level.

Interestingly, several publications report the interaction of HBx with histone deacetylase 1 (HDAC1). A role of HDAC1 in HBx functions has been first described for the inhibition of ER alpha-dependent transcriptional activity by HBx and then for the activation of HIF-1α (Han *et al.*, 2006; Yoo *et al.*, 2008). Finally, HBx has been shown to repress the transcription of the insulin-like growth factor-binding protein 3 (IGFBP-3) through the interaction and the recruitment of HDAC1 on the promoter and the formation of a Sp1/HDAC1 complex, which results in the inhibition of Sp1 (Shon *et al.*, 2009). Of note, HBx has been previously shown to negatively regulate XPD and XPC transcription through the inhibition of Sp1 (Jaitovich-Groisman *et al.*, 2001). Interaction with HDAC1 could be a way for HBx to regulate a broad range of viral and cellular functions.

HBx has been shown to interact with and to sequester the nuclear export receptor CRM1, leading to the nuclear localization of NF-κB and to aberrant centriole replication as well as formation of multipolar spindles (Forgues *et al.*, 2001, 2003). Deregulation of mitotic spindle assembly by HBx is associated with aneuploidy, which can lead to genomic instability and contribute to cancer development (Forgues *et al.*, 2003).

C. HBx and Cell Cycle Regulation

Dysregulation of the cell cycle is a common feature of transformed cells. In this regard, many viral oncoproteins, such as adenovirus E1A, HTLV-I Tax, and HPV-16, deregulate cell cycle progression. Actively replicating cells are believed to provide a favorable environment for virus replication (Neuveut and Jeang, 2002; Op De Beeck and Caillet-Fauquet, 1997). Many studies have focused on the impact of X gene expression on the cell cycle. It was found that activation of signal transduction pathways (described earlier) such as MAPK, JNK, and Src kinases by HBx stimulate cell cycle progression, accelerating the progression of quiescent G0 cells through the G1- to S-phase, as well as from the G2- to M-phase (Benn and Schneider, 1995; Koike *et al.*, 1994b). The consequences of HBx expression on the cell cycle depend on the presence of stimulatory factors. Indeed, Bouchard *et al.* (2001a) have demonstrated that serum-starved HBx-expressing cells exited G0 but stalled at the G1/S boundary. Similar findings have been reported by Chirillo *et al.* (1997) in serum-starved cells, where HBx induces DNA synthesis followed by apoptosis. The question remains open as to whether HBx induces cell cycle progression or apoptosis. Similarly, some studies have shown that HBx induces the expression of the cell cycle regulators p21 and p27 and the subsequent arrest of the cell at the G1/S boundary (Park *et al.*, 2000; Qiao *et al.*, 2001). Others studies have reported

a repression of p21 expression leading to cellular growth (Ahn *et al*., 2001, 2002). These conflicting data on HBx activity might result from the models used and/or from the expression level of HBx. HBx might differentially regulate cell cycle progression depending on the differentiation state of a hepatocytic cell line (Lee *et al*., 2002). Studies performed with HBx-transgenic mice reflect the *ex vivo* conflicting results. Madden *et al*. (2001) reported that expression of HBx is associated with a significant increase in S-phase hepatocytes in liver of young animals but not in adult mice. Another study reported increased apoptosis in the liver of HBx-transgenic mice. However, using the same model it was shown that HBx cooperates with *myc* in oncogenesis, arguing that HBx behaves differentially depending on the cellular context (Terradillos *et al*., 1997, 1998). Finally, HBx impairs hepatocyte regeneration after partial hepatectomy (Tralhao *et al*., 2002; Wu *et al*., 2006). One study describes the same complicated pattern as observed in tissue culture: HBx promoted the transition of quiescent hepatocytes from G0 to G1, but cells stalled at the G1/S boundary and underwent apoptosis (Wu *et al*., 2006).

It is important to point out that there is a consensus on the fact that HBx induces mitotic aberrations such as multipolar spindle formation, amplification of centrosome, chromosome segregation defects and formation of multinucleated cells (Forgues *et al*., 2003; Fujii *et al*., 2006; Kim *et al*., 2008b; Martin-Lluesma *et al*., 2008; Rakotomalala *et al*., 2008; Wen *et al*., 2008; Yun *et al*., 2004). The molecular mechanisms leading to such abnormalities seem however diverse. Indeed, HBx is thought to exert this function through its interaction with either DDB1 or proteins that interact with the centrosome such as HBXIP and CRM1, or with BubR1, a component of the mitotic checkpoint (Forgues *et al*., 2003; Fujii *et al*., 2006; Kim *et al*., 2008b; Martin-Lluesma *et al*., 2008; Wen *et al*., 2008). Other studies incriminate the activation of the Ras–MEK–mitogen-activated protein kinase signaling pathway by HBx (Yun *et al*., 2004). Finally, Rakotomalala *et al*. (2008) reported recently that HBx increases simultaneously the expression of the replication initiation factors Cdc6 and Cdt1, while inhibiting the expression of geminin, the inhibitor of replication licensing. Modulation of Cdt1/geminin ratio will thus lead to uncontrolled DNA rereplication (Rakotomalala *et al*., 2008). Downregulation of geminin is in agreement with centrosome duplication and mitotic defects (Lu *et al*., 2009; Tachibana *et al*., 2005). However, others failed to observe any DNA rereplication or modulation of Cdt1 or geminin expression in HBx-expressing cells (Martin-Lluesma *et al*., 2008). Whether these discrepancies may be related to the models used needs further demonstration. The exact molecular mechanisms by which HBx induces mitotic defects still await elucidation.

D. HBx and Apoptosis

As mentioned before, several studies have shown that HBx can modulate both cellular proliferation and viability. Again reflecting its seemingly contrasting and complex functions, HBx has been shown to either mediate apoptosis, sensitize cells to proapoptotic stimuli, or to prevent apoptosis. In chronic HBV infection, liver cell injury is believed to be mediated mostly by the cellular immune response. However, several studies suggest that HBx might contribute to liver disease by modulating pathways controlling apoptosis. HBx exerts a spontaneous proapoptotic effect in cultured primary hepatocytes and in the liver of HBx-transgenic mice (Koike *et al.*, 1998; Pollicino *et al.*, 1998; Terradillos *et al.*, 1998, 2002). Induction of cell death by HBx has been described to be both p53-mediated as well as p53-independent and could be mediated through interaction with c-FLIP or by causing loss of mitochondrial membrane potential (Chami *et al.*, 2003; Clippinger and Bouchard, 2008; Koike *et al.*, 1998; McClain *et al.*, 2007; Pollicino *et al.*, 1998; Terradillos *et al.*, 1998, 2002). The role of mitochondria in HBx-induced apoptosis is supported by the fact that direct interaction has been reported between HBx and the mitochondria as mentioned earlier (Kim *et al.*, 2007b; Rahmani *et al.*, 2000; Shirakata and Koike, 2003). HBx-induced apoptosis can be blocked by permeability transition pore (PTP) inhibitors, reactive oxygene species (ROS) scavenger, caspase inhibitors or by overexpression of BCL-2 or BCl-xl (Chami *et al.*, 2003; Shirakata and Koike, 2003). Moreover, the decrease of intracellular calcium level using calcium chelators or calcium-free media significantly decreases apoptosis induced by HBx (Chami *et al.*, 2003). Whether HBx interaction with mitochondria results in cytochrome *c* release and caspase activation remains under debate (Chami *et al.*, 2003; Shirakata and Koike, 2003; Takada *et al.*, 1999). This interaction has also been shown to be responsible for the production of ROS and lipid peroxide (Lee *et al.*, 2004; Waris *et al.*, 2001). Interestingly, such dysfunction seems to sensitize cells to apoptotic signals rather than inducing apoptosis *per se* (Lee *et al.*, 2004). This finding is supported by the work of Waris *et al.* (2001), showing that ROS production in HBx-expressing cells leads to STAT-3 and NF-κB activation. It is important to note that replication of HBV at high levels in transgenic mouse liver has not been associated with pathological death of hepatocytes (Guidotti *et al.*, 1995). Furthermore, HBx expressed from a replicating HBV genome might not directly induce apoptosis but act as a "sensitizer" to other proapoptotic stimuli. More specifically, HBx might provide hypersensitivity to killing by tumor necrosis factor (TNF-α) through a particular set of conditions, involving activation of JNK and Myc pathways (Su and Schneider, 1997; Su *et al.*, 2001). This finding has been confirmed by

different groups (Kim and Seong, 2003; Lee *et al.*, 2004). In striking contrast, HBx has been found to inhibit apoptosis induced by p53, transforming growth factor β (TGF-β), or Fas (Elmore *et al.*, 1997a; Pan *et al.*, 2001; Shih *et al.*, 2000). The antiapoptotic activity of HBx could be mediated through its interaction with the survivin–HBXIP complex or through the activation of the PI-3-K or NF-κB signaling pathway (Marusawa *et al.*, 2003; Shih *et al.*, 2000; Su *et al.*, 2001). From the study of Su *et al.* (2001), it seems that HBx's effect on cell viability might be highly dependent on the cellular context. This idea is supported by the work of Clippinger *et al.* (2009), showing that expression of HBx alone or in the context of HBV replication in primary rat hepatocytes induces or protects from apoptosis depending of the NF-κB status. In this study, the authors link apoptosis to the modulation of the MPTP. To date, there is no direct evidence that HBV is able to modulate the apoptotic pathways, especially under *in vivo* conditions, nor that apoptosis could provide any advantage to virus replication. A reasonable scenario is that HBx would inhibit apoptosis during early hepatocyte infection, favoring viral replication, and that it would activate apoptosis at later stages to facilitate viral spread and immune evasion. A consequence of HBx-induced apoptosis could be the enhancement of the regeneration process providing a larger reservoir of hepatocytes for virus spreading. Alternatively, apoptosis could be a consequence of other activities of HBx that are deleterious for the cell, such as the deregulation of cell cycle.

E. HBx and DNA Repair

Active mechanisms protect the genome of human cells from endogenous or exogenous substances that damage cellular DNA. The DNA repair enzymes constantly scan the global genome to detect and remove DNA damage. Five DNA repair pathways have been identified such as homologous recombinational repair (HRR), nonhomologous end joining (NHEJ), mismatch repair (MMR), nucleotide excision repair (NER), and base excision repair (BER) (Bernstein *et al.*, 2002). NER affects the repair of different type of lesions. In particular, it eliminates highly promutagenic DNA lesions induced by UV irradiation or by DNA-adducting carcinogens such as aflatoxin B1 (a liver-specific carcinogen), lesions that are known to block transcription. Dysregulation of this function leads to accumulation of mutations that predispose cells to transformation. Several groups have investigated whether HBx could interfere with this process. It has been described that HBx inhibits the repair of DNA damage in cell culture (Becker *et al.*, 1998; Groisman *et al.*, 1999; Jia *et al.*, 1999; Prost *et al.*, 1998). The mechanism by which HBx inhibits NER is unknown, but is thought to occur through the interaction of HBx with proteins or protein complexes

involved in DNA repair such as TFIIH and p53 (Feitelson *et al.*, 1993; Jia *et al.*, 1999; Prost *et al.*, 1998; Wang *et al.*, 1994, 1995). HBx could also modulate NER activity through downregulation of the XPB and XPD components of TFIIH (Jaitovich-Groisman *et al.*, 2001). A recent report shows that HBx interferes with DNA interstrand crosslink (ICL) repair, leading to an increase in DNA breaks. The authors suggested that HBx interferes with the intra-S-phase checkpoint (Wu *et al.*, 2008). Madden and colleagues have developed a transgenic mouse model to measure the action of HBx on DNA repair *in vivo*. They showed that HBx did not significantly increase the accumulation of spontaneous mutations, suggesting that inhibition of NER by HBx may lead to an increase in mutation frequency only after exposure to exogenous mutagenic agents (Madden *et al.*, 2000). A report from the same group failed to detect any obvious increase in mutations in the liver of HBx-transgenic mice treated with the hepatocarcinogen diethylnitrosamine, and they proposed that HBx might act as a tumor promoter by increasing the proliferation rate, allowing the proliferation of hepatocytes containing unrepaired DNA damage (Madden *et al.*, 2001). Using the same model, they assessed the impact of HBx expression on the frequency of aflatoxin B1-induced DNA mutation. They observed a modest increase in mutation frequency in HBx mice, associated however with an increase in the incidence of transversion mutations (Madden *et al.*, 2002). The interference of HBx with the cellular DNA repair system provides yet another potential mechanism by which HBx contributes to liver carcinogenesis.

VI. CONCLUSION

Despite extensive and significative studies, the precise role of HBx in HBV replication and in the development of liver cancer remains an opened question. The difficulty to assess its role comes first from the lack of a convenient and clinically relevant model to study virus replication. Moreover, the use of different models and immortalized or transformed cell lines to study HBx activities contributes unquestionably to the emergence of seemingly contradictory results. However, in light of all the activities described above on virus replication and on the modulation of the cellular environment by acting on transcription, cycle regulation, apoptosis, and DNA repair, HBx plays without doubt an important role in the replication of HBV, but also in cancer development. This idea is further supported by the fact that the duck HBV that lacks an X open reading frame does not induce cancer in duck. Thus, HBx represents an attractive therapeutic target. Indeed, interfering with HBx expression or activity could allow controlling not only virus load but

also tumor growth, since HBx could be expressed both by the integrated virus and by the episomal DNA found in some tumors. Control of HBx expression could be done through the use of anti-HBx antibodies. Attempts have already been made and resulted in significant tumor regression (Kumar and Sarkar, 2004). On the other hand, HBx may be targeted by the mean of ribozymes or small interfering RNAs. Such approaches led to the inhibition of HBV replication as well as to a potent suppression of tumor growth (Cheng *et al*., 2007; Kumar and Sarkar, 2004; Nash *et al*., 2005; Tang *et al*., 2008). Finally, an alternative approach will be to target HBx activities. Its interaction with its cellular partner DDB1 appears a promising therapeutic target since the integrity of the interaction between HBx and DDB1 has been shown to be required in tissue culture for HBx activities, but also *in vivo* for virus replication in the woodchuck model (Leupin *et al*., 2005; Sitterlin *et al*., 2000a).

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Drosophila Myc

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Myc genes play a major role in human cancer, and they are important regulators of growth and proliferation during normal development. Despite intense study over the last three decades, many aspects of Myc function remain poorly understood. The identification of a single Myc homolog in the model organism *Drosophila melanogaster* more than 10 years ago has opened new possibilities for addressing these issues. This review summarizes what the last decade has taught us about Myc biology in the fruit fly. © 2009 Elsevier Inc.

ABBREVIATIONS

BHLHZ, basic region-helix-loop-helix-leucine zipper; CNS, central nervous system; dm, diminutive (=*Drosophila melanogaster* Myc gene); Dpp, Decapentaplegic (a *Drosophila melanogaster* TGFβ homolog); FRT, FLP-recombinase target; GMC, ganglion mother cell; GSC, germline stem cell; H3K4me3, histone H3, trimethylated on lysine 4; Hh, Hedgehog; Inr, Insulin receptor; MB1/2/3, Myc box 1/2/3; SID, Sin3-interaction domain; TOR, target of rapamycin; UAS, upstream activating sequence; Wg, Wingless (a *Drosophila melanogaster* Wnt homolog); ZNC, zone of nonproliferating cells.

I. INTRODUCTION: THE MYC/MAX/MXD NETWORK IN VERTEBRATES

Myc is amongst the most intensely studied genes in biomedicine—more than 19,000 articles dealing with Myc can be found in PubMed (Meyer and Penn, 2008). Several recent publications have extensively reviewed different

Advances in CANCER RESEARCH
0065-230X/09 $35.00

DOI: 10.1016/S0065-230X(09)03005-X

aspects of Myc function (Cole and Cowling, 2008; Cowling and Cole, 2006; Dang *et al.*, 2006; Eilers and Eisenman, 2008; Meyer and Penn, 2008; Pirity *et al.*, 2006; Vita and Henriksson, 2006). Therefore, I will only briefly summarize some key features of vertebrate Myc proteins. The main part of this review is dedicated to the characterization of Myc in the fruit fly *Drosophila melanogaster*: what this protein does in insects, how it does it and how its activity is controlled.

The "Myc saga" began more than 30 years ago with the identification of the first Myc genes as the transforming principles of different avian retroviruses. Subsequent research identified the cellular homologs c-, N-, and L-Myc in vertebrates. The corresponding proteins were found to be frequently overexpressed in human and animal tumors and to causally contribute to the development of cancer, as demonstrated in numerous animal models. The transforming power of Myc could be traced back to Myc's ability for influencing a variety of cellular processes, most notably growth, cell cycle progression, apoptosis, cell migration, cell adhesion, and stem cell behavior. Most of these processes are also controlled by Myc proteins in physiological situations and during normal development. Myc's versatility is explained by its molecular activity as a transcription factor that controls hundreds if not thousands of target genes, including genes transcribed by RNA polymerases I, II, and III. However, each of these targets is only moderately affected by Myc, typically by two- to threefold.

Myc proteins consist of an N-terminal transcription regulatory domain containing the highly conserved "Myc boxes" 1 and 2 (MB1 and MB2), an ill-defined central region with another conserved sequence called Myc box 3 (MB3), and a C-terminal basic region-helix-loop-helix-leucine zipper (BHLHZ) domain, that mediates heterodimerization with another BHLHZ-domain protein, Max ("Myc-associated protein X"), as well as binding to DNA. Myc:Max heterodimers recognize so-called E-boxes (CACGTG, and variants thereof), and activate the expression of nearby genes. In addition to binding to all members of the Myc family, Max also homodimerizes, and it interacts with the Mxd proteins (Mxd1–4, formerly known as Mad1, Mxi1, Mad3, Mad4, respectively), with Mnt and with Mga. All these Max-partners contain BHLHZ domains and their heterodimers with Max control similar genes as Myc:Max dimers, but in contrast to Myc:Max heterodimers, they repress the corresponding targets. Accordingly, these Max partners function as antagonists of Myc. Besides activating many target genes, Myc:Max dimers also repress a distinct set of targets; Myc:Max does not recognize these Myc-repressed genes by directly binding to DNA at E-boxes, but indirectly via the interaction with other DNA-bound transcription factors. Finally, Myc has recently also been shown to control DNA replication independently of transcription.

As diverse as the transcriptional targets of Myc are the cofactors recruited by Myc to control the expression of these targets. They include the histone acetyltransferases GCN5, Tip60, and CBP, the INI1 chromatin remodeling complex, the P-TEFb protein kinase that phosphorylates the C-terminal domain of RNA polymerase II, and several proteins that have no known enzymatic functions or that participate in different multiprotein complexes. For most target genes, it is currently unclear to which extent individual cofactors contribute to their Myc-dependent regulation.

II. THE MYC/MAX/MNT NETWORK IN FLIES

The search for a Myc/Max/Mxd network in invertebrates was initially motivated by the need for a simple model system—a system that contains less gene redundancy than vertebrates, that is genetically tractable, and that is more easily accessible at all stages of development. Widely used models such as yeasts and worms turned out to lack Myc genes (although *Caenorhabditis elegans* contains two Max genes and one gene coding for a Mxd-like protein; Yuan *et al.*, 1998), but *D. melanogaster* fit the bill: fruit flies carry one gene each coding for Myc, Max, and for a Mxd-family member protein. *Drosophila* Myc has even been known to biologists long before the vertebrate Myc genes. In 1935, a mutation was described that results in a small adult body size, disproportionally small bristles and female sterility (Bridges, 1935). Based on these phenotypes, the affected gene was dubbed "*diminutive*," abbreviated as "*dm*." Many years later, molecular cloning revealed the identity of *diminutive* with the *Drosophila* Myc gene (Gallant *et al.*, 1996; Schreiber-Agus *et al.*, 1997). According to *Drosophila* conventions this gene should therefore be called *diminutive/dm*; to minimize confusion I will refer to the gene and protein as "Myc" in the following text and to the mutant alleles as "dm^X" (where X is the allele identifier).

A. Basic Properties of the Myc/Max/Mnt Proteins in Flies

Drosophila Myc was identified in yeast 2-hybrid screens with human Max as the bait (Gallant *et al.*, 1996; Schreiber-Agus *et al.*, 1997). Subsequent 2-hybrid screens used first *Drosophila* Myc as the bait to clone *Drosophila* Max (Gallant *et al.*, 1996) and then *Drosophila* Max as the bait to fish out *Drosophila* Mnt (Loo *et al.*, 2005); Mnt was also identified independently based on the published *Drosophila* genome sequence (Peyrefitte *et al.*, 2001). All three proteins show clear sequence similarity to their vertebrate

counterparts. Thus, Myc is 26 % identical in its overall amino acid sequence to human c-, N-, and L-Myc, and it contains the conserved sequence motifs MB2 (whose role in transactivation and repression was demonstrated for vertebrate Myc) and MB3 (of unknown function), as well as a BHLHZ domain at its C terminus (Fig. 1). Furthermore, vertebrate and insect Myc genes have an identical genomic organization: in all cases the major open reading frame starts at the beginning of the second exon and ends in the third exon, and the second intron interrupts the open reading frame at the same codon within the conserved MB3 (reviewed by Gallant, 2006).

Drosophila Mnt also shares the functionally identified domains with the vertebrate Mnt and Mxd proteins (although the sequence similarity is higher to vertebrate Mnt): an N-terminally located SID ("Sin3-Interaction Domain" that mediates binding to the transcriptional corepressor Sin3) and a centrally positioned BHLHZ (Fig. 1). Interestingly, two Mnt splice variants have been identified that lack either the SID or the leucine zipper, suggesting the existence of protein variants that either do not repress transcription (MntΔSID) or do not bind to Max and DNA (MntΔZ), and thereby might act as antagonists of the full-length variant of Mnt (Loo *et al.*, 2005). Finally, Max is the most highly conserved component of the whole network, with 52% overall amino acid sequence identity to human Max protein, and an identical genomic organization (reviewed by Gallant, 2006).

The *Drosophila* Myc, Max and Mnt proteins also share biochemical similarities with their vertebrate homologs: in both vertebrates and *Drosophila*, Myc and Mnt only interact with Max, whereas Max is also able to homodimerize (in addition, Myc also has certain functions that are independent of its dimerization with Max, see below). Furthermore, in band shift assays all possible types of dimers (Myc:Max, Mnt:Max, Max:Max)

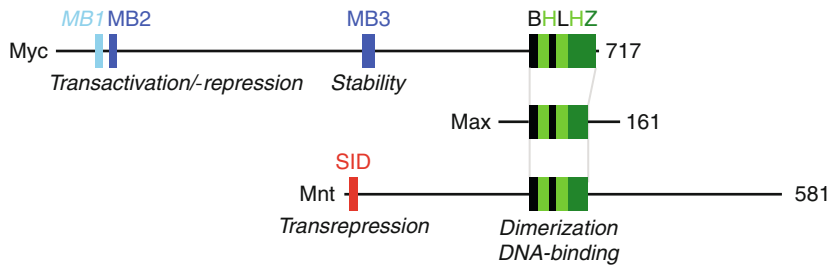

Fig. 1 Domain structure of the *Drosophila* Myc, Max, and Mnt proteins. Domain names are explained in the main text. MB1 is only tentatively indicated as it shows low sequence similarity to the corresponding domain in vertebrate Myc proteins. The exact extents of the regions involved in transactivation, transrepression, and protein stability are not known. The BHLHZ domains mediate dimerization with Max and DNA-binding. The numbers to the right show the the protein lengths (in amino acids).

bind to the same E-box sequence that is also recognized by the corresponding vertebrate complexes (and Myc has also been shown to bind an E-box in a target gene promoter in tissue culture cells; Hulf *et al.*, 2005). Myc:Max dimers activate, and Mnt:Max dimers repress, transcription from artificial reporters (Gallant *et al.*, 1996; Hulf *et al.*, 2005; Loo *et al.*, 2005). Finally, *Drosophila* and vertebrate Myc proteins can even functionally substitute for each other: *Drosophila* Myc can collaborate with activated Ras to transform rat embryo fibroblasts (Schreiber-Agus *et al.*, 1997), and it overcomes the proliferation block in mouse embryonic fibroblasts that lack the endogenous c-Myc gene (Trumpp *et al.*, 2001). Conversely, human c-MycS (a translation variant of c-Myc with a truncated N terminus) rescues the development of flies carrying the lethal Myc-allele dm^{PG45} (Benassayag *et al.*, 2005).

These observations show that the Myc/Max/Mnt network has been conserved during evolution, and they suggest that whatever we learn about Myc function in flies is relevant for our understanding of vertebrate Myc biology. What then is the function of *Drosophila* Myc?

B. Biological Functions

As is the case for its vertebrate homologs, overexpression, or downregulation of *Drosophila* Myc affects several cellular processes (Fig. 2). Some of these processes may be dependent on each other, but the molecular nature of such putative connections is as yet unknown, and therefore, the individual activities of Myc will be treated separately below. However, if there is any unifying theme behind Myc's different biological activities, it is the control of size. Most of the individual activities listed below somehow conspire to control the size of cells, of organs, and of the whole animal.

1. *DROSOPHILA* AS AN EXPERIMENTAL SYSTEM

Before delving into the biological properties of Myc and consorts, I need to briefly introduce the model system and some of the principal experimental techniques that made these analyses possible in the first place. For a more detailed description of the biology and experimental analysis of *D. melanogaster* the reader is referred to several excellent treatises (e.g., Ashburner *et al.*, 2005; Dahmann, 2008; Greenspan, 2004).

The fruit fly develops in about 10 days from the egg to the adult (under optimal growth conditions at 25 °C). Along the way, the fly spends 1 day in embryogenesis, 4 days in larval stages (three different larval stages, or "instars"), and the last 5 days immobilized in a pupal case where it metamorphoses into an adult. Of particular interest for scientists studying growth

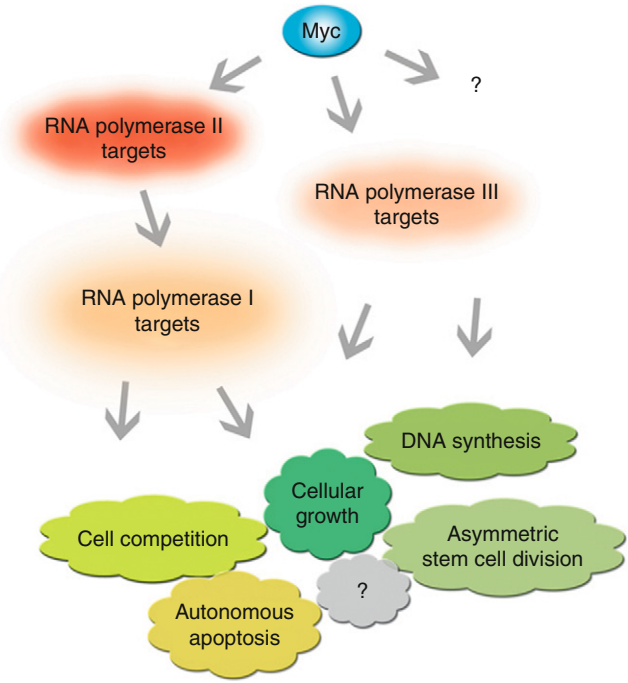

Fig. 2 Activities of Myc. Myc controls the activity of RNA Polymerases II and III, and (indirectly) of RNA Polymerase I. Their targets (together with possible transcription-independent activities of Myc) affect the indicated cellular processes.

and proliferation is the larval phase, since this period is characterized by a massive, 200-fold increase in weight, but as yet little cellular differentiation. Most of the larval mass is found in different polyploid tissues, for example, fat body, salivary gland, and muscles. These tissues attain their final cell number already during embryogenesis and afterward only endoreplicate their genomes without undergoing cell division, reaching ploidies of up to 2000 N and accordingly large nuclear volumes. During metamorphosis, most of these polyploid tissues are histolysed and their contents used by diploid imaginal tissues (abdominal histoblasts and imaginal discs that give rise to adult appendages and body wall structures) for their own growth. These imaginal discs consist of an epithelial monolayer of columnar cells that proliferate near-exponentially during larval phases and are subject to similar regulatory mechanisms as typical vertebrate cells.

A large number of experimental techniques have been developed to manipulate these different cell types. For example, by expressing the yeast recombinase FLP (from a heat-shock inducible or a tissue-specifically

expressed transgene) mitotic recombination can be induced between two homologous chromosomes that each carry an FRT site ("FLP-recombinase target"), resulting in two daughter cells that are homozygous for either the corresponding paternal or maternal chromosome, including any mutation that is located on these chromosomes (or more precisely: the part of the chromosome that is distal to the FRT site). By following the descendants of such homozygous mutant cells (i.e., clones), the properties of mutations can be determined *in vivo*, even if such mutations are lethal at the organismic level and do not allow the animals to develop to a stage where they can be analyzed (reviewed in Xu and Harrison, 1994). A large number of reagents also exist that allow controlled overexpression of transgenes. Many of these rely on the temporally or spatially controlled expression of the yeast transactivator GAL4 (by transgenes where specific artificial or endogenous enhancers control the expression of GAL4) together with transgenes containing a cDNA under the control of GAL4-responsive UAS elements ("upstream activating sequences"). Many hundreds of different GAL4 lines and even more different UAS lines currently exist. Hence, by crossing such flies together, an enormous variety of transgene expression patterns can be achieved (reviewed in Brand *et al.*, 1994). The GAL4/UAS- and the FLP/FRT-systems can also be combined such that heat-shock induced FLP expression triggers FRT-mediated recombination within a GAL4-expressing transgene, leading to the constitutive expression of GAL4 (Pignoni and Zipursky, 1997). By keeping the heat-shock conditions mild (i.e., incubating the larvae for only a few minutes at the inducing temperature) FLP is induced in only a few random cells per animal, and hence GAL4 can drive the expression of UAS-transgenes in only these few cells. Such cells then go on to form clones, and the behavior of these clones (most typically size, shape, cell number) can be assayed at freely chosen times after their induction. Such timed induction of GAL4 can also be used for polyploid tissues, although the "clones" in these tissues only consist of one polyploid cell each (if the heat-shock is given after the end of embryogenesis).

This is only a small selection from the vast and ever-growing "*Drosophila* toolkit," but I hope that it facilitates the understanding of the following text.

2. CELLULAR GROWTH

The observation of the small adult flies carrying the hypomorphic Myc-allele dm^1 immediately revealed Myc's involvement in size control (see above, Bridges, 1935). In more detailed studies it was later shown that reduction of Myc levels decreases the size of larval diploid cells (Johnston *et al.*, 1999) and of Schneider S2 cells grown in culture, while at the same time slowing down passage through G1 phase (Hulf *et al.*, 2005). As a consequence, cells depleted of Myc accumulate to lower numbers than

untreated cells (Boutros *et al.*, 2004). Conversely, overexpression of Myc in clones of diploid wing imaginal disc cells increases the size of the clones and of the cells constituting these clones, without affecting cell number (i.e., division rates). Myc overexpression is able to accelerate passage through G1 phase, but these cells compensate by extending their G2 phase. When the cell cycle regulator Cdc25/String (which is limiting for entry into M-phase) is coexpressed with Myc, both gap phases are shortened and cell division times are significantly reduced. Such Myc + Cdc25/String coexpressing clones are equally large as clones expressing Myc alone, but the former consist of an increased number of normally sized cells, whereas the latter contain the same number of cells as control clones, albeit these cells are much bigger in size (Johnston *et al.*, 1999). These properties of Myc contrast with those of a typical cell cycle regulator such as Cyclin E: downregulation of Cyclin E also impairs progression into S-phase and leads to accumulation of G1-phase cells, but at the same time allows growth to continue unabated, thus resulting in bigger than normal cells (Hulf *et al.*, 2005). This demonstration that Myc controls cellular growth in flies was echoed by similar findings in vertebrates, revealing another evolutionary conservation of Myc function (Iritani and Eisenman, 1999; Schuhmacher *et al.*, 1999).

Thus, in addition to its (in vertebrates) long-accepted role in influencing passage from G1- to S-phase, Myc also controls the increase in cellular mass. This effect is likely to be explained by the nature of Myc's transcriptional targets. Like its vertebrate homologs, *Drosophila* Myc controls the expression of a large number of genes, possibly many hundreds of them (Hulf *et al.*, 2005; Orian *et al.*, 2003). These genes fall into different functional categories, but many of them play a role in ribosome biogenesis, such as the RNA helicase Pitchoune whose vertebrate homolog MrDb/DDX18 is also a Myc target (Grandori *et al.*, 1996; Zaffran *et al.*, 1998) and Modulo, a putative homolog of the vertebrate Myc target Nucleolin (Greasley *et al.*, 2000; Perrin *et al.*, 2003). Myc also contributes to ribosome biogenesis by stimulating RNA polymerases I and III (Grewal *et al.*, 2005; Steiger *et al.*, 2008), as do its vertebrate counterparts (Arabi *et al.*, 2005; Gomez-Roman *et al.*, 2003; Grandori *et al.*, 2005). In contrast to vertebrates, however, the activation of RNA polymerase I by Myc occurs indirectly, presumably via the RNA polymerase II-dependent activation of RNA polymerase I cofactors such as TIF-1A (Grewal *et al.*, 2005). Thus, activation of Myc presumably leads to a general increase in cellular translational capacity, resulting in increased growth.

Interestingly, the different proteins that have been shown to promote an increase in cell size (i.e., "growth") do so in qualitatively different ways. Thus, the Insulin receptor (Inr) pathway differs from Myc in that it has a prominent effect on the cytoplasmic volume of polyploid cells and on the

level of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Britton *et al.*, 2002; see below). Also, unlike Myc, the growth-promoting Cyclin D/Cdk4 complexes stimulate and are critically dependent on, mitochondrial activity (Frei *et al.*, 2005). These differences emphasize the different molecular mechanisms that underlie different types of "growth," and they suggest ways how growth regulators could collaborate even though all ultimately control the rate of cellular size increase.

3. DNA SYNTHESIS

Myc also strongly influences the nuclear size of polyploid cells in larvae (fat bodies, salivary glands, muscles) and in adult egg chambers (somatic follicle cells and germline-derived nurse cells). In these cells Myc predominantly controls the rate of endoreplication and hence DNA content: whereas overexpression increases DNA content in polyploid larval cells by up to eightfold (Berry and Baehrecke, 2007; Demontis and Perrimon, 2009; Pierce *et al.*, 2004), mutation of Myc strongly reduces the ploidy of such larval or ovarian cells (Demontis and Perrimon, 2009; Maines *et al.*, 2004; Pierce *et al.*, 2004, 2008; Steiger *et al.*, 2008). Myc does not seem to affect the onset of endoreplication, since Myc overexpression does not induce premature endocycles (at least in follicle cells; Shcherbata *et al.*, 2004), although forced Myc expression can extend the duration of endoreplication (Pierce *et al.*, 2004). It is not clear whether Myc is also required for sub-genomic polyploidization, that is, the amplification of specific genes. Thus, chorion genes are amplified in wild-type follicle cells after they have become polyploid, and this chorion gene amplification was reported to occur normally in follicle cell clones that are homozygous for a strong Myc-allele dm^2 and that are surrounded by phenotypically wild-type tissue (Maines *et al.*, 2004). In contrast, females that are homozygous for the weak Myc-allele dm^{P1} show reduced chorion gene amplification in their follicle cells (Quinn *et al.*, 2004). The reason for these differences is unclear, but the dm^{P1} mutant flies clearly suffer from reduced growth rates throughout their body, and it is conceivable that this systemically impacts the behavior of follicle cells (e.g., via reduced levels of circulating growth factors).

Myc activity has less dramatic effects on DNA replication in diploid cells. On one hand, Myc overexpression does not trigger polyploidization in diploid cells (and only shortens the duration of G1 phase). On the other hand, the loss of Myc slows down G1 phase and overall cell division rates, but has a comparatively mild effect on the structure of diploid tissues (imaginal discs). This can be seen in *Myc Mnt* double mutant animals, where polyploid tissues remain severely stunted as compared to wild-type animals (and, as a consequence, such double mutant larvae are considerably smaller than the control). In contrast, diploid imaginal discs show normal

patterns of proliferation and differentiation, and they develop to comparable sizes as wild-type discs, although they do so more slowly and require several days more for this process (Pierce *et al.*, 2008). The same analysis cannot be carried out in *Myc* single mutant animals, since they die before the third larval instar when most of the size increase of imaginal discs takes place. However, a genetic trick allows the generation of *Myc*-mutant eye imaginal discs within an animal that is otherwise functionally wild type for *Myc*. Such flies develop to fully viable adults with surprisingly normal-looking eyes and heads that are composed of Myc-mutant cells, although these organs are clearly smaller than in the control (Schwinkendorf and Gallant, 2009; Steiger *et al.*, 2008).

There are two reports, though, showing dramatic effects of forced Myc expression on cellular proliferation. Ectopic expression of different transcription factors in developing eye-antennal imaginal discs strongly interferes with their development, and often results in flies lacking heads altogether (Jiao *et al.*, 2001). This defect can be largely overcome by coexpression of Myc, but also by coexpression with Cyclin E which specifically controls cell cycle progression, suggesting that in such an artificial situation Myc is able to stimulate the proliferation of diploid imaginal disc cells (Jiao *et al.*, 2001). Similarly, certain mutations in the transcription factor Prd produce male flies with strongly reduced cellularity in their accessory glands, and these deficits can be overcome by ectopic expression of Myc or of Cyclin E (Xue and Noll, 2002). The molecular basis of these effects has not been analyzed, and it is therefore not known whether Myc directly stimulates the cell cycle machinery or whether the effect is more indirect.

The effects of Myc on DNA replication could be mediated by different transcriptional targets. In genome-wide and directed expression analyses several cell cycle regulators have been found to respond to changes in Myc levels, for example, dE2F1, RBF, different cyclins, Stg/Cdc25, but it is unclear whether these constitute direct Myc targets (Duman-Scheel *et al.*, 2004; Hulf *et al.*, 2005; Orian *et al.*, 2003). A better characterized, presumably directly Myc activated gene is the "DNA-replication element binding factor" DREF that itself controls the expression of DNA-replication related genes such as dE2F, dPCNA, and Cyclin A (Thao *et al.*, 2008). Interestingly, the DREF-binding site (DRE) is significantly enriched in the promoters of Myc activated genes, raising the possibility that Myc might also cooperate with DREF in controlling the expression of S-phase specific targets (Orian *et al.*, 2003). In addition to directly controlling DNA replication specific genes, Myc may also influence endoreplication rates indirectly, via the same targets that promote growth and overall cell size increases in diploid cells. For example, the S-phase regulator Cyclin E (which is also essential for endoreplication) has been shown to be controlled posttranscriptionally by

Myc (at least in imaginal disc cells, but the same may hold true for polyploid cells as well; Prober and Edgar, 2000), possibly via Myc's effect on ribosome biogenesis and hence protein synthesis (Grewal *et al.*, 2005). Finally, it is conceivable that Myc influences DNA replication directly in a transcription-independent manner, as has been shown for vertebrate Myc (Dominguez-Sola *et al.*, 2007). However, such an activity has not been demonstrated in *Drosophila* so far.

Interestingly, Myc has little (if any) effect on cytoplasmic and overall size in polyploid cells. This contrasts with Myc's command on the size of diploid cells (see above), but also with the ability of another growth regulator, the insulin signaling pathway, to control polyploid cell size (e.g., Demontis and Perrimon, 2009). It is conceivable that Myc's effect on diploid and on polyploid cells are mediated by different sets of targets and constitute separate biological activities of Myc. Alternatively, the same downstream effectors of Myc control both diploid cell and polyploid cell behaviors, but the two cell types are wired differently to respond either with cytoplasmic growth or with endoreplication, respectively.

4. APOPTOSIS

We have seen that overexpression of Myc increases the size of the affected cells and organs, but there are limits to this growth-stimulating activity. Excessive Myc activity triggers apoptosis that can overcome the gain in tissue mass caused by Myc-induced growth (with the definition of "excessive" depending on tissue and developmental stage). Thus, high-level Myc overexpression in eye imaginal discs is accompanied by different hallmarks of apoptosis, such as activation of Caspase 3 and DNA fragmentation as revealed by TUNEL- and acridine orange-staining (Montero *et al.*, 2008). The resulting adult eyes are disorganized and rough, they all but lack a particular cell type (pigment cells), and their ommatidia are smaller than those of flies expressing more moderate levels of Myc—attributes that presumably reflect the death of some cells during ommatidial differentiation, and hence the absence of these cells from the mature ommatidia (Steiger *et al.*, 2008). Signs of apoptosis are also seen upon Myc overexpression in wing imaginal discs (Benassayag *et al.*, 2005; de la Cova *et al.*, 2004; Montero *et al.*, 2008), and expression of a mutant form of Myc (with a presumably slightly higher activity than wild-type Myc) in clones of cells leads to their elimination from the wing disc as a consequence of apoptosis (Schwinkendorf and Gallant, 2009). In contrast, Myc overexpression does not stimulate or inhibit the autophagic cell death of third instar larval polyploid salivary gland cells, nor does a Myc mutation induce autophagy, indicating that some tissue types and some modes of cell death are not affected by Myc (Berry and Baehrecke, 2007; Scott *et al.*, 2004).

Importantly, this ability of Myc to induce cell death is not only observed upon overexpression. In hypomorphic Myc mutants, where Myc activity is reduced by three- to fivefold (but not completely eliminated), some forms of cell death are impaired, as would be expected if Myc has a normal role in controlling this process. Thus, dm^{P0} homozygous females do not show the nurse cell death that normally occurs in late-stage egg chambers, and this presumably contributes to the sterility of these flies (Quinn *et al.*, 2004). Also, dm^{P0}- and dm^{P1}-mutant wing imaginal discs show a significantly reduced incidence of apoptosis upon exposure to low doses of X-rays (up to 10 Gy), although higher doses (50 Gy) evoke similar apoptotic responses in wild type and Myc-mutant cells (Montero *et al.*, 2008).

The molecular pathway by which Myc influences apoptosis is poorly understood. Myc overexpression leads to the upregulation of p53 mRNA within 1 h of Myc induction, raising the possibility that Myc directly activates transcription of p53. However, p53 is not required for the Myc-dependent apoptosis, since Myc equally efficiently triggers cell death in p53 null mutant wing imaginal disc cells (Montero *et al.*, 2008). In contrast, heterozygosity for chromosomal deletions that simultaneously eliminate the four proapoptotic genes *hid*, *grim, reaper*, and *sickle* (or only three of them) strongly reduces Myc-induced apoptosis in wing discs, indicating that these proteins are important for this process (de la Cova *et al.*, 2004; Montero *et al.*, 2008). These four proteins have previously been shown to bind and inactivate the caspase-inhibitor dIAP1, resulting in caspase activation and cell death (Steller, 2008). Their expression is induced by a variety of proapoptotic stimuli, including Myc—and the kinetics of induction of *reaper* and *sickle* by Myc is comparably rapid as that of p53. Thus, Myc might transcriptionally activate these genes, presumably by direct binding of Myc:Max heterodimers to E-boxes located in their regulatory regions (Montero *et al.*, 2008). However, Myc can also induce cell death through other pathways that do not involve E-box containing target genes. This was shown in experiments where Myc's partner Max was knocked down (Steiger *et al.*, 2008). Myc requires Max for binding to E-boxes, and downregulation of Max abrogates Myc's ability to induce E-box dependent targets and promote overgrowth in the eye—but it leaves intact the ability of overexpressed Myc to trigger apoptosis. This suggests that Max-independent activities such as the activation of RNA polymerase III (see below) contribute to Myc's proapoptotic actions, but the relative contributions of E-box dependent and independent targets, and possible differences between different tissues and different developmental stages, have not been explored in detail. Furthermore, it is not known whether physiological levels of Myc (that are required for the normal apoptotic response to DNA damage, as described above) affect apoptosis via the same pathways as overexpressed Myc.

5. CELL COMPETITION

The notion that Myc affects apoptosis cell-autonomously is familiar to scientists studying Myc in vertebrates. In addition, *Drosophila* Myc also influences cell death nonautonomously in neighboring cells, in a process called "cell competition."

"Cell competition" was first described 30 years ago in a study of a class of mutants called *Minutes* (Morata and Ripoll, 1975; Simpson and Morata, 1981). There are more than 50 different *Minute* loci in flies, and we now know that most (perhaps all) of them code for ribosomal proteins (Lambertsson, 1998). Homozygous *Minute* mutations are cell-lethal, as would be predicted; even heterozygosity for a *Minute* mutation reduces cellular proliferation rate and extends the overall duration of development, but ultimately such *Minute*/+ animals eclose with a normal morphology, although their bristles are more slender than those of wild-type flies (Lambertsson, 1998). The process of cell competition is observed when cell clones are generated during imaginal disc development such that *Minute*/+ cells are juxtaposed to +/+ cells. While it would be expected that the former grow more slowly than the latter and ultimately occupy an accordingly smaller area, the growth defect of *Minute*/+ cells has more dramatic consequences: these cells are killed by the contact with their faster growing, healthier neighbors and tend to disappear altogether from the wing tissue—even though such *Minute*/+ cells would have the potential to give rise to a complete adult animal as we have seen above. The demise of these *Minute*/+ cells is prevented if the growth rate of the surrounding cells is also decreased (e.g., by heterozygosity for a different *Minute* mutation), or if they are separated from the competing cells by a compartment boundary; that is, slow-growing cells in the posterior compartment of a wing imaginal disc are not affected by adjacent wild-type cells in the anterior compartment. The final size of the resulting wing is not changed by the cell competition taking place during larval wing development, and it has been proposed that cell competition serves as a quality control mechanism to replace "unfit" cells by their healthier neighbors (de la Cova *et al.*, 2004).

Cell competition is thought to arise from differences in growth rates between adjacent cells, and additional growth regulators have been proposed to affect cell competition, for example, components of the Hippo tumor suppressor pathway (Tyler *et al.*, 2007) and most notably Myc. A moderate reduction of Myc levels still allows for the development of phenotypically normal (albeit small) animals, but the same reduction of Myc levels in clones triggers their elimination if they are surrounded by phenotypically wild-type cells (Johnston *et al.*, 1999). Conversely, overexpression of Myc leads to the death of surrounding *wild-type* cells, even though they are perfectly healthy, making these Myc-overexpressing cells

"super competitors" (de la Cova *et al.*, 2004; Moreno and Basler, 2004). This process can be triggered by remarkably small differences in Myc levels between adjacent cells (presumably twofold or even less), which distinguishes cell competition from the cell-autonomous apoptosis that is induced by comparatively high-level Myc overexpression only (Moreno and Basler, 2004). The study of Myc-dependent cell competition also suggested an additional biological function for this process: when apoptosis (and hence cell competition) was blocked during the development of wing imaginal discs, the resulting adult wings showed considerably higher variability in their sizes, although the average size was the same as in control. Thus, cell competition might also serve to reduce the consequences of "developmental noise" (de la Cova *et al.*, 2004).

The mechanism that senses the subtle differences in Myc activity is currently under investigation. Some effector components of the "cell competition pathway" have been identified. For example, engulfment of competed Minute/+ cells by their wild-type neighbors was shown to be essential not only for the removal of the dead cells, but also for allowing these cells to die in the first place (Li and Baker, 2007). In the case of Myc-induced competition the proapoptotic gene *hid* also plays an important role: competed cells upregulate *hid*, and heterozygosity for this gene virtually eliminates Myc-dependent cell competition and allows wing disc compartments containing competed cells to overgrow (de la Cova *et al.*, 2004). However, neither *hid* nor the engulfment factors explain how differences in cellular growth rate are sensed in the first place, and the question remains how the competition process is initiated. A candidate upstream factor is the signaling pathway activated by the TGFβ-homolog Dpp. In a competing environment *Minute*/+ cells transduce the Dpp signal with reduced efficiency as compared to their surviving neighbors, leading to excessive expression of the Dpp-repressed gene *Brinker*, followed by activation of the kinase Jnk and subsequent apoptosis (Moreno *et al.*, 2002). The involvement of Jnk signaling downstream of Myc-dependent competition remains controversial, though (de la Cova *et al.*, 2004), and it has been suggested that it is the experimental heat-shock treatment that leads to the activation of Jnk, rather than cell competition *per se* (Tyler *et al.*, 2007). Consistent with a possible involvement of Dpp signaling in cell competition, different mutants that prevented the competition of *Minute*/+ cells also reestablished Dpp signaling activity (Tyler *et al.*, 2007). Conversely, upregulation of the Dpp-pathway in cells suffering from Myc-dependent competition also rescued their survival (Moreno and Basler, 2004), as did the elimination of the Dpp-effector Brinker or its putative transcriptional cofactor dNAB (Ziv *et al.*, 2009). The defect in Dpp signaling in the competed cells has been suggested to result from impaired endocytosis (Moreno and Basler, 2004), but it is still enigmatic which signals could mediate the slight initial differences in Myc

activity between neighboring cells and subsequently lead to reduced endocytosis and presumably additional defects that induce a cell to die. Such signals are likely to be diffusible, since cell competition was observed at a distance of up to eight cell diameters between the competed and the competing cell (de la Cova *et al.*, 2004). To find these signals a cell-culture based system was developed where Myc-overexpressing *Drosophila* Schneider cells induce apoptosis in naïve Schneider cells (Senoo-Matsuda and Johnston, 2007). This system mimics several aspects of the cell competition observed in the animal (e.g., the ability of Myc-expressing "super competitors" to induce apoptosis without direct cell–cell contact), and there is hope that this approach, or a genetic screen similar to the one recently published (Tyler *et al.*, 2007), will soon unravel the molecular basis of cell competition. Investigations of cell competition are fuelled by an interest for its role during normal insect development, but in part also by the speculation that an analogous process might contribute to human cancers that are characterized by overexpression of one of the Myc oncoproteins, although currently no data exist to support this notion (Moreno, 2008).

A discussion of cell competition would be incomplete without mentioning the phenomenon of "compensatory proliferation" (reviewed by Fan and Bergmann, 2008). The term originates from the observation that different types of abuse (e.g., strong irradiation, prolonged heat-shock) will kill the majority of imaginal disc cells, but nevertheless allow the eclosion of normally shaped adults, since the surviving cells increase their proliferation rate and thus replace the dead cells. Before they die, such mortally wounded cells synthesize different patterning factors (Wg, Dpp, Hh, depending on the tissue type) that might induce the compensatory proliferation of the surrounding cells. Whereas the connections between compensatory proliferation and cell competition have not been extensively investigated, it is tempting to speculate that (while they are dying) the competed cells feed back on the competing cells and further stimulate their growth, thus helping to reinforce the "fitness difference" between the "winners" and the "losers." To date there is no evidence for a specific involvement of Myc in compensatory proliferation, but it is interesting to note that larvae carrying a hypomorphic Myc mutation are more sensitive to ionizing irradiation than control animals (Jaklevic *et al.*, 2006), even though their wing disc cells show a reduced rate of apoptosis (Montero *et al.*, 2008). One possible explanation for this observation is that these animals might suffer from a defect in compensatory proliferation. However, the increased sensitivity to irradiation is not restricted to *Myc* mutations, as disruption of other growth regulators (e.g., Cdk4, the Insulin pathway) results in a similar defect (Jaklevic *et al.*, 2006). Given the current interest in cell competition, compensatory proliferation and Myc, it is likely that any missing molecular links between these three will soon be uncovered.

6. ASYMMETRIC STEM CELL DIVISION

Another similarity between vertebrates and *Drosophila* resides in the involvement of Myc in stem cell biology. One tissue where this function of Myc has been studied is the female germline. Oogenesis in *Drosophila* takes place in about 18 ovarioles per ovary (reviewed in Bastock and St Johnston, 2008; Fuller and Spradling, 2007). At one end of each of these ovarioles resides a stem cell niche harboring 2–3 germ-line stem cells (GSCs). These stem cells undergo asymmetric divisions, producing another GSC and a differentiating cystoblast, which will divide four more times to form an egg chamber that then develops into an oocyte. Myc protein is highly expressed in the GSCs, but drops to low levels in their daughter cystoblasts (by a poorly defined mechanism involving the protein Mei-P26), before it rises again during later stages of oogenesis (Neumuller *et al.*, 2008; Rhiner *et al.*, 2009). When Myc levels are kept artificially high by means of a constitutively expressed transgene, the differentiating cystoblasts maintain a stem cell-like morphology and retain the ability to efficiently transduce the Dpp signal (emanating from the stem cell niche), suggesting that the drop in Myc levels contributes to the differentiation of these cells, although it is not clear how (Rhiner *et al.*, 2009). Interestingly, GSCs can also compete with each other for niche occupancy, similar to the cell competition in imaginal discs that was discussed above. The involvement of Myc in this type of competition is controversial, though—two recent publications came to opposite conclusions in this regard. The group of Moreno found hypomorphic Myc-mutant GSCs to be driven from the niche by adjacent wild-type GSCs, whereas GSCs with higher than normal Myc levels behaved as "super competitors" and chased away the neighboring wild-type GSCs (Rhiner *et al.*, 2009). In contrast, Xie and coworkers observed no competitive disadvantage in Myc-null mutant GSCs as compared to their wild-type neighbors, nor any competitive advantage of Myc-overexpressing GSCs (Jin *et al.*, 2008). It is conceivable that differences in overexpression regimes and in the examined *Myc*-mutant alleles are responsible for this discrepancy. For now, the jury is still out whether Myc is also involved in GSC competition.

However, Myc is likely to play a role in other stem cell divisions as well. Similar to GSCs, larval neuroblasts contain high levels of Myc protein (Betschinger *et al.*, 2006). These cells divide in a stem cell-like manner, producing another neuroblast and a ganglion mother cell (GMC), which then gives rise to differentiated neurons. As in the germline, Myc levels are considerably lower in the differentiating GMCs than in their stem cell mothers. Both the asymmetric neuroblast division and the downregulation of Myc in GMCs require the protein Brat (brain tumor). During the neuroblast division Brat localizes to the GMC where it downregulates

Myc posttranscriptionally. In *Brat* mutants neuroblasts divide to produce two additional neuroblasts, and the levels of Myc protein remain high in both of these daughter cells. Interestingly, Brat and Mei-P26 have a similar domain architecture (both containing a "B-Box" and an "NHL domain") and they share at least one interaction partner (the RNase Argonaute1, which is a key component of the miRNA-producing RISC complex), suggesting that both proteins might control Myc levels by a similar mechanism.

These studies did not address a functional requirement for Myc in neuroblast divisions, but two other reports revealed an effect of Myc on neurogenesis. First, the Myc gene was identified as a quantitative trait locus for adult bristle number—a hypomorphic mutation in Myc reduced the number of abdominal and sternopleural bristles (Norga *et al.*, 2003). Second, overexpression of Myc in the embryonic CNS increased the number of neuroblasts, consistent with the idea that Myc might promote neuroblast self-renewal at the expense of producing differentiating daughter cells (Orian *et al.*, 2007). Myc is normally expressed in these embryonic neuroblasts, where it was proposed to act by binding to the transcriptional corepressor Groucho and thereby antagonizing Groucho's repressive activity. Some of the common target genes of Myc and Groucho have an established role in the development of the CNS, but interestingly, they lack the typical Myc: Max-binding sites (E-boxes) and they have also not been identified as Max or Mnt targets (Orian *et al.*, 2003), suggesting that Myc's action on Groucho and on these targets might be independent of Max (Orian *et al.*, 2007). This is most probably not the only mechanism by which Myc influences stem cell fate. Brat-mutant, Myc-overexpressing larval neuroblasts are characterized by larger nucleoli (Betschinger *et al.*, 2006), as are Myc-overexpressing imaginal disc and salivary gland cells (Grewal *et al.*, 2005), raising the possibility that Myc's general growth-stimulating activity might contribute to "stemness."

7. OTHER FUNCTIONS

The enumeration of *Drosophila* Myc's biological activities is necessarily incomplete. Several abstracts or short descriptions have been published that suggest additional functions for Myc that are not obviously connected to any of the processes described above. For example, during oogenesis Myc presumably controls the migration of follicle cells, in particular of a subpopulation called "border cells" (King, 1970; King and Vanoucek, 1960). It is to be expected that we will learn more about additional Myc activities in the future.

C. Molecular Mechanism of Myc Action: The Partners

The genetic tractability of *Drosophila* holds great promise for the functional analysis of proposed transcriptional cofactors of Myc and the identification of novel such cofactors, and hence for the characterization of the mechanism by which Myc controls the expression of its target genes. To date, studies have been published that address the function of the DNA helicases Tip48 and Tip49, of Max, the corepressor Groucho, several Trithorax- and Polycomb-group proteins, as well as the Myc-antagonist Mnt (Fig. 3).

1. MAX

The first identified Myc partner, and arguably the best characterized, is the BHLHZ protein Max. Different studies in vertebrate tissue culture cells have convincingly demonstrated that Myc requires the association with Max in order to bind to E-boxes and control the activation of the corresponding targets (Amati *et al.*, 1992; Kretzner *et al.*, 1992), but also for the repression

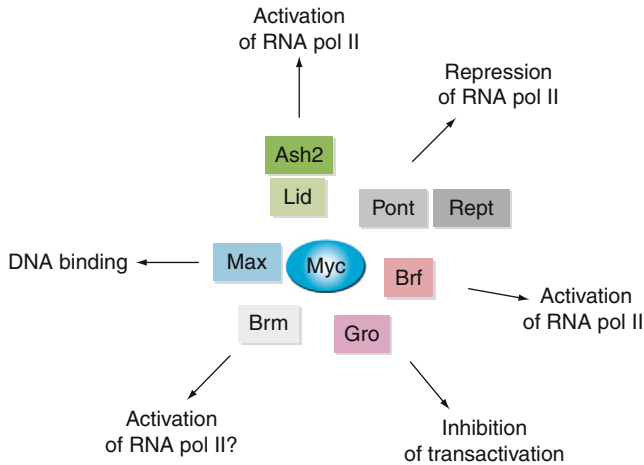

Fig. 3 Myc-interacting proteins. The depicted proteins have been shown to (directly or indirectly) bind to Myc. Ash2 and Lid are thought to contact Myc:Max complexes, whereas Brf and Gro are believed to interact with Myc independently of Max; no pertinent information exists for the other proteins. Some putative functions of the interacting proteins are also shown. Full protein names are (in parentheses: vertebrate homologs): Ash2/"absent, small, or homeotic discs 2" (ASH2L), Brf (BRF1), Brm/Brahma (Brg1, hBrm), Gro/groucho (TLE), Lid/"little imaginal discs" (Rbp-2/JARID1A, PLU-1/JARID1B), Pont/Pontin (TIP49/RUVBL1), Rept/Reptin (TIP48/RUVBL2).

of genes lacking E-boxes (Facchini *et al.*, 1997; Mao *et al.*, 2003). A mutated form of vertebrate c-Myc that cannot associate with Max is incapable of transforming cultured rat embryo fibroblasts, or of stimulating cell cycle progression or inducing apoptosis in established rat fibroblasts (Amati *et al.*, 1993a,b). Based on these and similar observations it was speculated that all functions of Myc might depend on Max, because Myc might require the dimerization with Max for its correct folding (Adhikary and Eilers, 2005). It therefore came as surprise that *Drosophila* Myc retains substantial activity even in the absence of Max (Steiger *et al.*, 2008). This is most strikingly demonstrated by the phenotypic differences between *Myc*- and *Max*-mutant animals: flies lacking Myc altogether fail to grow and mostly die as small larvae, whereas up to a third of *Max*-null mutant flies initiate metamorphosis and many of them even reach the pharate adult stage (i.e., they develop all adult body structures but they do not manage to leave the pupal case and die at this stage).

Part of this difference can be explained by the Myc antagonist Mnt, whose activity is also lost in *Max* mutants but not in *Myc* mutants: *Myc Mnt* doubly mutant animals survive for longer and grow larger than *Myc* singly mutant animals, presumably because typical Myc-activated genes are expressed at higher levels in *Myc Mnt* larvae than in *Myc* mutants (although still substantially lower than in control animals). This indicates that Myc functions in part to derepress Mnt-repressed genes (Pierce *et al.*, 2008), as has been shown in vertebrate studies (Hurlin *et al.*, 2003; Nilsson *et al.*, 2004). However, Myc retains substantial activity in the absence of Max, and *Myc Mnt* doubly mutant animals clearly do not grow as well and do not develop as far as *Max* mutants. Thus, endoreplication is only partially impaired by the loss of Max but strongly by the loss of Myc, overexpressed Myc is capable of inducing cell-autonomous apoptosis in the absence of Max, and differences in Myc levels still trigger cell competition in *Max*-mutant animals. These observations point to the existence of substantial Max-independent activities of Myc. At least some of these may reside in Myc's interaction with RNA polymerase III (Steiger *et al.*, 2008). It has previously been found that vertebrate Myc can activate RNA polymerase III, and that Myc does so by physically interacting with the polymerase III cofactor Brf (Gomez-Roman *et al.*, 2003). This activity of Myc was shown to be conserved in flies, that is, *Drosophila* Myc activates RNA polymerase III targets and is required for their full expression, and *Drosophila* Myc physically and genetically interacts with Brf (Steiger *et al.*, 2008). Importantly, both Myc's effect on Pol III targets and its interaction with Brf are also observed in the absence of Max. Thus, this effect on polymerase III may explain some of the observed differences between *Myc* (or *Myc Mnt*) and *Max* mutants, but there are likely to be additional functions of Myc that do not rely on the association with Max.

2. GROUCHO

One of these may be mediated by the transcriptional corepressor Groucho (Orian *et al.*, 2007). Groucho was found to associate with several genes that are also bound by Myc but lack known Myc:Max-binding sites (E-boxes). It is possible that Myc and Groucho are recruited to these genes together in the absence of Max, since Myc and Groucho also physically associate *in vivo* and *in vitro*. Several of these common targets play a role in neurogenesis and mitosis, and it was proposed that Groucho and Myc antagonistically control these genes and thereby affect the neuronal development: Groucho mediates the activity of the Notch-signaling pathway in repressing these genes, whereas Myc acts downstream of the EGF-receptor in activating them and promoting neuronal specification (Orian *et al.*, 2007). While this observation suggests an interesting new role for Myc, the mechanistic details of the Myc:Groucho interaction still need to be worked out. In particular, the additional components of the Myc:Groucho complex need to be identified, that determine how the complex gets recruited to its target genes and how it controls their expression.

3. TIP48 AND TIP49

In contrast to Groucho, the DNA helicases Tip48 and Tip49 have already been identified in studies in vertebrate tissue culture cells as putative coactivators for Myc (Wood *et al.*, 2000). The analysis of their *Drosophila* homologs (called Pontin and Reptin, respectively) confirmed their physical interaction with Myc and the existence of a ternary Myc:Pontin:Reptin complex, and further showed that Pontin (and to a lesser extent Reptin) is essential for Myc-dependent growth *in vivo* (Bellosta *et al.*, 2005). Unexpectedly, Pontin could not be shown to play a role in Myc-dependent gene activation, but instead in Myc-dependent gene repression. An analogous repressive function was investigated in greater detail for the *Xenopus* homologs of Pontin (and Reptin). Both proteins were demonstrated to be essential for the ability of *Xenopus* Myc to repress the transcriptional activator Miz-1 and prevent it from activating the cell cycle inhibitor p21 (Etard *et al.*, 2005). These observations further confirm the similarity between insect and vertebrate Myc. The mechanistic basis for the action of Pontin and Reptin remains open, though, as both proteins can act in several different transcription-associated complexes and it is not clear which of them is responsible for the observed repressive effects (Gallant, 2007).

4. POLYCOMB- AND TRITHORAX-GROUP PROTEINS

The identification of Polycomb- and Trithorax-group genes in genetic screens emphasizes the potential of *Drosophila* for the discovery of novel Myc cofactors. The Trithorax-group genes ash2 ("Absent, small, or

homeotic discs 2"; the homolog of vertebrate ASH2L), brahma (the homolog of human hBrm and Brg1) and lid ("Little imaginal discs"; the homolog of vertebrate Rbp-2/JARID1A and PLU-1/JARID1B) were found to be required for overexpressed Myc to promote overgrowth (Secombe *et al.*, 2007). The three proteins physically interact with Myc in two separate complexes, one containing Ash2 and Lid, the other one containing Brahma. Lid was further shown to be required for the full activation of at least one direct Myc-activated gene. Such a role in gene activation is consistent with Lid's classification as a Trithorax-group protein (as Trithorax proteins generally play a positive role in transcription), but appears at odds with Lid's molecular activity as a histone H3 lysine 4 trimethyl (H3K4me3) demethylase, as trimethylation on H3K4 is generally associated with active transcription. However, this demethylase activity does not seem to be required for Lid's ability to cooperate with Myc *in vivo*, since a mutant form of Lid lacking the demethylase domain also enhanced a Myc-overexpression phenotype, and since binding to Myc inhibits this demethylase activity. This does not explain how Lid helps Myc in the activation of its targets, but an answer might be found in the recent observation that Lid can associate with, and inhibit, the histone deacetylase Rpd3 in a potentially demethylase-independent manner, and thereby promote the transcription of certain target genes (Lee *et al.*, 2009). The roles of Ash2 and Brahma can more easily be rationalized, as Ash2 is known from other studies to be associated with H3K4 trimethyltransferases and Brahma is a component of the SWI/SNF chromatin remodeling complex, and hence both have a documented function in transcriptional activation.

In an independent screen, Pc ("Polycomb"; the homolog of human CBX2/4/8), Psc ("Posterior sex combs"; the homolog of vertebrate Bmi1), Pho ("Pleiohomeotic"; the homolog of vertebrate YY1), and Ash1 ("Absent, small, or homeotic discs 1"; the homolog of vertebrate ASH1L) were found to affect the expression of some Myc targets during embryogenesis (Goodliffe *et al.*, 2005, 2007). Some of these targets were activated by Myc and by these other proteins, others (including the *Myc* locus itself) were repressed by both, and yet others were repressed by Pc and Pho, but activated by Myc. However, none of these proteins has been shown to physically associate with Myc so far, and it is possible that their influence on Myc target gene expression is indirect. For example, it has been suggested that Ash1 functions as an H3K4 mono- and dimethyltransferase, thereby creating a substrate for the subsequent H3K4 trimethylation by an Ash2-containing complex (Byrd and Shearn, 2003). It is conceivable that Myc (in conjunction with an Ash2-complex) is involved in such a H3K4 trimethylation, and thereby (indirectly) depends on the prior activity of Ash1. Alternatively, Ash2 might help recruit Myc to genes that are already trimethylated on H3K4, as this posttranslational modification has been shown to predate

Myc recruitment to its targets in vertebrates (Guccione *et al.*, 2006). It is currently unclear how Pc and Pho (which are both found in the same complex, PRC1; Schuettengruber *et al.*, 2007) affect Myc targets.

Finally, a close functional connection between Myc and Trithorax-/Polycomb-group proteins was also suggested by the recent comparison of Myc targets with those of Trx ("trithorax," homolog of vertebrate MLL proteins). Many of these genes were found to be arranged in clusters, and most of these target clusters were shared between Myc and Trx (Blanco *et al.*, 2008). Whereas the molecular mechanisms of the interactions between Myc and these Polycomb-/Trithorax-proteins still need to be worked out, there is a good chance that (some of) this mechanism is conserved in vertebrates, since the vertebrate homologs of Lid (Secombe *et al.*, 2007), Ash2 (Luscher-Firzlaff *et al.*, 2008), Brahma (Cheng *et al.*, 1999), Psc/Bmi1 (e.g., Jacobs *et al.*, 1999), and Pho/YY1 (Austen *et al.*, 1998; Shrivastava *et al.*, 1993) all were shown to physically and/or functionally interact with vertebrate Myc.

5. THE MYC PROTEIN

The sections above have addressed different trans-acting factors that collaborate with Myc in the control of gene expression. In addition, the fruit fly has also been used to analyze the requirement of parts of the Myc protein itself for transcriptional regulation (Schwinkendorf and Gallant, 2009). Previous work in vertebrate tissue culture systems had identified Myc box 2 (MB2) as important for transactivation and repression, and as generally essential for all biological activities of Myc proteins. This domain is highly conserved in *Drosophila* Myc, and it therefore came as surprise that it is partially dispensable for Myc function *in vivo*. A mutant Myc protein lacking MB2 can rescue the lethality of a substantial fraction of flies lacking all endogenous Myc, indicating that MB2 only modulates Myc activity, but is not essential for it. The cofactors contacting MB2 in *Drosophila* (that are therefore partially dispensable for Myc function *in vivo*) still need to be identified (Schwinkendorf and Gallant, 2009).

It is to be expected that future experiments in *Drosophila* will result in the identification of additional transcriptional cofactors for Myc. It will be important to explore the possible connections between the different Myc partners mentioned above (as well as between these proteins and the sequence motifs within Myc itself). It is likely that Myc recruits different enzymatic activities to control the expression of its target genes, and hence that some of these factors associate separately with Myc, but it is also conceivable that some of these proteins that have been analyzed separately so far are located in the same multiprotein complexes.

D. Control of Myc Activity

A large variety of inputs controls Myc activity in vertebrates (reviewed in Liu and Levens, 2006; Spencer and Groudine, 1991). In *Drosophila*, fewer such signals have been reported to date, simply because this subject has not yet been investigated to the same depth, but the short half-life of *Drosophila* Myc raises the possibility of an equally tight regulation: whereas the stability of *Drosophila* Myc mRNA has not been determined yet, *Drosophila* Myc protein decays with a half-life of 30–60′, comparable to that of its vertebrate counterparts (Galletti *et al.*, 2009; Schwinkendorf and Gallant, unpublished data). The pathways currently known to affect this protein stability or Myc's expression are summarized below (Fig. 4).

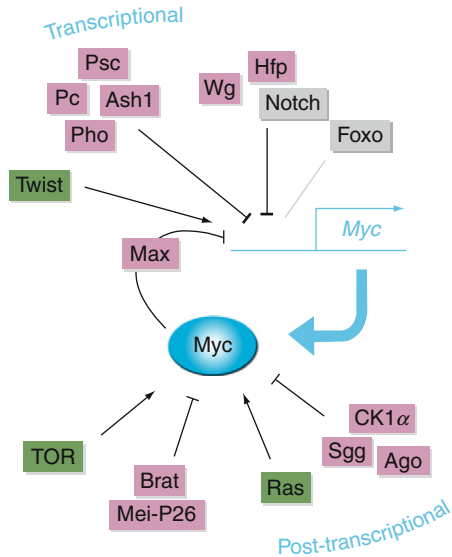

Fig. 4 Upstream regulators of Myc. Proteins in the top half affect Myc mRNA abundance (presumably transcriptionally), whereas the proteins in the lower half act posttranscriptionally on Myc protein levels. Proteins that are thought to act in the same pathway (e.g., Wg, Hfp, and Notch) or use the same molecular mechanism (e.g., Brat and Mei-P26) are grouped together. The directionality of the effect (increase versus decrease of Myc levels) is reflected in the shape of the arrows and the color of the proteins (red or green, respectively). The effects of Notch and Foxo are ambiguous. Full protein names are (in parentheses: human homologs): Ago/archipelago (FBXW7), Ash1/"absent, small, or homeotic discs 1" (ASH1L), Brat/"brain tumor" (similar to TRIM32), CK1α/"Casein kinase 1α," Foxo/"forkhead box, sub-group O" (FOXO3), Hfp/pUf68 = "poly U binding factor 68 kD" (FIR/PUF60), Mei-P26 (similar to TRIM32), Pc/Polycomb (CBX2/4/8), Pho/pleiohomeotic (YY1), Psc/"Posterior sex combs" (Bmi1, Mel-18), Sgg/shaggy (GSK3β), TOR/"Target of rapamycin" (mTOR), and Twist (TWIST), Wg/Wingless (Wnt).

1. CONTROL OF MYC EXPRESSION

During early embryogenesis, maternally deposited Myc mRNA is ubiquitously distributed in all cells (Gallant *et al.*, 1996). Fertilization destabilizes this maternal message (as is the case for 21% of all maternal transcripts), such that its levels are significantly reduced in 4–6 hr old embryos (Tadros *et al.*, 2007). Zygotic Myc transcripts then accumulate in the presumptive mesoderm, presumably under the control of the mesoderm specifying transcription factor Twist, which has been shown to bind to the Myc gene (Sandmann *et al.*, 2007). Later, Myc is induced (by some as yet unknown mechanism) in the cells of the gut and salivary placodes (Gallant *et al.*, 1996).

During larval development, Myc transcripts can be broadly detected in diploid and polyploid cells. However, in the second half of the third larval instar, a stripe of cells along the future wing margin, called the "zone of nonproliferating cells" (ZNC), exits from the cell cycle and downregulates Myc expression. This Myc repression is mediated by the Wingless signaling pathway, as the expression of dominant-negative Pangolin/TCF (the transcription factor at the end of the Wingless cascade) prevents this downregulation and the cell cycle exit of the ZNC cells (as does forced expression of Myc; Duman-Scheel *et al.*, 2004; Johnston *et al.*, 1999). It is not clear, though, whether TCF directly represses Myc expression. According to one report, Wingless signaling upregulates a protein called Half-pint (Hfp), which in turn represses Myc (Quinn *et al.*, 2004; interestingly, Hfp is also repressed by the molting hormone ecdysone via the zinc-finger transcripton factor Crooked Legs/Crol, indicating that ecdysone can also positively regulate Myc expression: Mitchell *et al.*, 2008). Mutation of Hfp leads to increased Myc mRNA levels in imaginal disc clones (including clones that extend into the ZNC) and in egg chambers. Consistent with this, heterozygosity for Hfp suppresses the female sterility associated with hypomorphic Myc alleles. Hfp is the *Drosophila* homolog of vertebrate FIR ("FBP interacting protein"), which was shown to repress vertebrate c-Myc through the "far upstream sequence element" (FUSE) (Liu *et al.*, 2000), raising the possibility that Hfp directly binds to and represses the Myc gene—although no FUSE has been identified in *Drosophila* Myc so far (Quinn *et al.*, 2004).

A separate report showed that Wingless signaling (and TCF) acts by repressing the Notch pathway, which in turn represses Myc (Herranz *et al.*, 2008). An opposite effect of Notch on Myc expression was observed in larval neuroblasts, where a mutation of Aurora A kinase leads to upregulation of Notch and subsequent induction of Myc (Wang *et al.*, 2006). The molecular basis for either of these Notch effects is currently unknown, but it is interesting to note that a genetic interaction between the Notch pathway

and Myc has been reported (Muller *et al.*, 2005; Orian *et al.*, 2007). It remains possible that Notch also affects Hfp expression, or that Hfp, Notch (and possibly TCF) provide separate and parallel inputs into Myc expression.

As might be expected, Myc expression is also affected by the major growth-regulating axis in *Drosophila*: the Inr/TOR pathway. This pathway monitors the fly's nutrient status: when food is copious, Inr signaling stimulates protein synthesis and induces the phosphorylation and inactivation of the transcription factor Foxo; at the same time, TOR activity increases translation rates and the transcription of growth-activating genes. On the other hand, upon starvation Inr and TOR are reduced in their activity, Foxo is dephosphorylated, enters the nucleus and binds its target genes—including Myc (Teleman *et al.*, 2008). The consequences of Foxo binding for Myc expression are ambiguous, though, as shown by either site-directed mutation of the Foxo-binding site in the Myc promoter or by mutational inactivation of Foxo itself. Both treatments increase Myc expression in the fat body of fed larvae (i.e., in a situation where Foxo is normally kept inactive by Inr signaling), but they reduce Myc expression in starved larvae (where Foxo is normally active). The situation is different again in larval muscles, where the deletion of the Foxo-binding site has no effect on Myc mRNA levels, but a Foxo mutation increases Myc levels specifically in starved larvae. These observations show that the action of Foxo on Myc levels depends on tissue type and nutritional status of the animal, although the basis for these differences is currently not known. Taking into consideration that TOR signaling also controls Myc protein levels (see below), and that Foxo was also proposed to affect Myc *activity* independently of Myc levels (Demontis and Perrimon, 2009), it is difficult to predict how Inr, TOR, and Myc actually cooperate in the control of growth at the organismal level.

Growth is also controlled by the evolutionarily conserved Hippo/Yorkie signaling pathway. One of the upstream regulators of this pathway is the transmembrane protein Fat (reviewed by Reddy and Irvine, 2008). Mutations in Fat induce tissue overgrowth. This overgrowth is accompanied by increased expression of Myc and hypomorphic mutations in Myc strongly reduce the growth-promoting effect of Fat (Garoia *et al.*, 2005). These observations suggest that the Hippo/Yorkie pathway also controls Myc transcription.

Finally, *Drosophila* Myc has been shown to autorepress its own expression (Goodliffe *et al.*, 2005). Like in vertebrates, this autorepression requires dimerization of Myc with Max (Facchini *et al.*, 1997; Steiger *et al.*, 2008), and it involves the Trithorax- and Polycomb-proteins discussed above (Pc, Pho, Psc, Ash2; Goodliffe *et al.*, 2005, 2007), but the relevant cis-acting sequences in the Myc gene have not been analyzed yet.

Thus, Myc transcript levels might be as tightly regulated in flies as they are in vertebrates. Surprisingly, though, such a tight control does not seem to be essential for *Drosophila* development. A transgene directing ubiquitous expression of a Myc cDNA (under the control of the α-*Tubulin* promoter) is able to fully rescue the development of *Myc*-null mutant flies, although these rescued animals suffer from a slight growth deficit (Schwinkendorf and Gallant, 2009). This suggests either that the physiological pattern of Myc activity is not required for development, or that (partially redundant) mechanisms control Myc activity at the posttranscriptional stage. Indeed, several such pathways have been identified in recent years, and they are summarized below.

2. CONTROL OF MYC PROTEIN LEVELS

The stability of vertebrate Myc is regulated by the ubiquitin proteasome pathway. Briefly, the Ras/Raf/ERK kinase cascade leads to the phosphorylation of serine 62 (S62, located within Myc box 1/MB1). This phosphorylation has a stabilizing effect on Myc, but it is also a prerequisite for the phosphorylation of threonine 58 (T58, also within MB1) by GSK3β. The doubly phosphorylated (T58 S62) protein is then dephosphorylated on S62 by the consecutive actions of the prolyl isomerase Pin1 and protein phosphatase 2A (PP2A), which in turn leads to Myc's ubiquitination by the E3 ubiquitin ligase Fbw7 and subsequent degradation. These different reactions are facilitated by the scaffolding protein Axin, which binds several of the involved proteins, including Myc (reviewed by Sears, 2004; Schulein and Eilers, 2009).

This pathway is (at least partially) conserved in flies. Thus, Myc levels are posttranscriptionally increased in imaginal disc cells expressing activated Ras (RasV12) (Prober and Edgar, 2002; note, though, that a different publication observed no such upregulation of Myc upon overactivation of the EGF-receptor that acts upstream of Ras: Parker, 2006). On the other hand, the kinase GSK3β (called Shaggy/Sgg in *Drosophila*) triggers ubiquitination of Myc in cultured cells and, as a consequence, decreases Myc stability in tissue culture and in imaginal discs *in vivo* (Galletti *et al.*, 2009). An involvement of Axin has not been demonstrated yet. Interestingly, though, another kinase known to associate with Axin, Casein Kinase 1α (CK1α) (Huang and He, 2008), has similar effects on Myc as GSK3β in cultured cells (and to some extent *in vivo* as well). MB1 and hence the phosphorylation site for GSK3β in vertebrate c-Myc is not well conserved in *Drosophila* Myc, but two other putative targets for phosphorylation by GSK3β and CK1α have been identified, and their mutation strongly increases Myc stability. One of these sites is located within an acidic stretch that is highly conserved across Myc proteins from different species and that has been dubbed Myc box 3 (MB3), the function of which has remained

mysterious in the past (Galletti *et al.*, 2009). Another conserved player in the degradation pathway is the F-box containing E3 ubiquitin ligase Ago ("Archipelago"; homolog of vertebrate Fbw7; Moberg *et al.*, 2004). Ago physically interacts with Myc and targets it for degradation. Loss of Ago in cell clones increases Myc protein levels and the size of these clones; heterozygosity for Ago in entire animals reduces the growth deficit of hypomorphic Myc-mutant flies and increases their fertility. It is not known which sequence in the Myc protein contacts Ago, since the Fbw7 interaction site in vertebrate c-Myc (MB1) is only poorly conserved. However, *Drosophila* Myc contains several suboptimal Ago binding sites, and one of them coincides with MB3, suggesting that the phosphorylation of this domain by CK1α and GSK3β triggers recognition by Ago and subsequent degradation of Myc (Galletti *et al.*, 2009; Moberg *et al.*, 2004).

Having identified these proteins that regulate Myc stability, it will be of obvious interest to characterize the upstream inputs that feed into this degradation pathway. GSK3β is known to be controlled by the Inr signaling pathway, but so far no effects of this pathway on Myc stability have been reported. On the other hand, the TOR kinase has been shown to feed back on components of the Inr pathway, including GSK3β (e.g., Sarbassov *et al.*, 2005; Zhang *et al.*, 2006). Since rapamycin-mediated inhibition of TOR has been shown to reduce Myc protein levels posttranscriptionally (Teleman *et al.*, 2008), it is conceivable that this effect is mediated by the pathway outlined above. This report also identifies Myc as a downstream mediator of TOR's growth-promoting effects. Consistent with this observation, reduced TOR activity (caused by expression of either the negative upstream regulators TSC1 and TSC2 or a dominant-negatively acting TOR itself) can be overcome by ectopic expression of Myc (Hennig and Neufeld, 2002; Tapon *et al.*, 2001).

TOR is certainly not the only regulator that affects the levels of Myc protein. One additional family of proteins that control Myc levels has been identified in asymmetrically dividing stem cells. As mentioned above, in neuroblasts mutation of Brat posttranscriptionally elevates Myc protein levels (Betschinger *et al.*, 2006), and in female germline stem cells, the loss of Mei-P26 has a similar effect (Neumuller *et al.*, 2008; Rhiner *et al.*, 2009). Brat and Mei-P26, as well as a third *Drosophila* protein called Dappled, are related in domain structure, suggesting that they might affect Myc levels through a common mechanism. This mechanism appears to be evolutionarily conserved, as a vertebrate homolog of these proteins, TRIM-32, was recently shown to mediate ubiquitination and subsequent degradation of c-Myc (Schwamborn *et al.*, 2009).

These different observations suggest the existence of several mechanisms that control Myc levels. It will be interesting to determine the molecular details of these pathways, as well as possible connections to the "core degradation machinery" described above.

E. Outlook

Myc proteins have fascinated biomedical researchers for 30 years. This interest is largely explained by the enormous impact of *Myc* mutations on human health. In addition, Myc's central role in coordinating growth during normal development has become increasingly obvious in recent years. The discovery of the Myc/Max/Mxd network in *Drosophila* has opened a new experimental window for addressing these physiological functions of Myc. Research in the fruit fly has already contributed significantly to our understanding of pathological and physiological Myc function in vertebrates, for example, by pinpointing the control of cellular growth as an essential, evolutionarily conserved role of Myc. Additional findings made in *Drosophila* are likely to be valid for the vertebrate system as well, such as the realization of Max-independent functions of Myc and the identification of alternative mechanisms of transcriptional control by Myc. Similarly, I expect the results of the genetic screens in *Drosophila* to play an important role in shaping our molecular understanding of the Max network, in flies as well as in vertebrates.

Beyond the molecular dissection of Myc's transcriptional function, *Drosophila* will be increasingly used to uncover systemic interactions with the different pathways controlling organismal development. These include the Insulin, TOR, and Hippo/Salvador/Warts signaling pathways, which have been defined as the major determinants of body size. In addition, the effect of extrinsic factors, such as food availability, on Max network activity need to be addressed. *Drosophila* offers an ideal experimental system for investigating such issues, and we can expect significant advances in the near future. Stay tuned!

ACKNOWLEDGMENTS

Many thanks to Laura Johnston and Hugo Stocker for carefully reading the manuscript and making good suggestions. The work in my lab was supported by the Swiss National Science Foundation (SNF).

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