Regulation of cell growth in Multiple Myeloma: a role for the HGF/MET and WNT signaling pathways

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CHAPTER 1
B CELL DEVELOPMENT AND CANCER

B CELL DEVELOPMENT

B cells represent the humoral component of the adaptive immune system. In the bone marrow (BM), committed lymphoid progenitors will differentiate into immature B cells, a process that is antigen independent. During this development, B cells acquire the ability to express surface-bound immunoglobulins constituting the B cell antigen receptors (BCR). During the first stages of B cell development, expression of the pre-B cell receptor (pre-BCR)(1), formed as a result of the rearrangement of variable (V), diversity (D) and joining (J) regions of the Ig heavy chain gene (IGH) locus located on chromosome 14 (2,3) is indispensable for B cell survival. Signaling via the pre-BCR and will induce rearrangement of the V and J segments of the Ig light chains (the x or λ locus) to produce a mature BCR of the IgM isotype (1).

Immature B cells expressing a mature BCR will migrate to the secondary lymphoid organs (i.e. spleen, mucosa associated lymphoid tissue (MALT), or lymph nodes). These peripheral lymphoid tissues provide the structures that facilitate antigen presentation. Two basic structures can be recognized in all peripheral lymphoid organs; a T-cell rich interfollicular area and a B cell rich follicle consisting mainly of naïve B cells. The antigen-presenting cells in the T cell areas are dendritic cells of hematopoietic origin, whereas the B cell follicles mainly harbor follicular dendritic cells (FDC), which are of mesenchymal origin (4,5). Antigens will be bound to surface expressed BCRs and internalized. This event, in combination with additional stimuli provided by T cells ("T cell help"), can activate the B cells, leading to direct differentiation into plasma cells (fig. 1). These plasma cells secrete low-affinity antibodies, that provide a rapid, early immune response. Other B cells migrate into the primary follicles, initiating a series of events known as the germinal center (GC) reaction. (4,6). Based on histological architecture, GCs are comprised of a dark and light zone (fig. 2). B cells that undergo a GC reaction will first develop into rapidly proliferating centroblasts. As a result, the naïve cells will be pushed to the periphery of the follicle and form a mantle zone (fig. 2). Expanding centroblasts will

![Figure 1. Normal B cell differentiation and the related stages of B cell neoplasia. Schematic representation of B cell differentiation. The malignant counterparts are indicated in parenthesis. See text for further detail. BM=bone marrow, PEL=primary effusion lymphoma, MM=multiple myeloma, DLBCL=diffuse large B cell lymphoma, FL=follicular lymphoma, MZL=marginal zone lymphoma, CLL=chronic lymphatic leukemia/lymphoma, MALT=mucosa associated lymphoid tissue, IC=immunocytoma, MCL=Mantle cell lymphoma, ALL=acute lymphoblastic leukemia.](image-url)
undergo mutation of their IGV genes, a process called somatic hypermutation (SHM) (7). SHM of IGV genes requires transcription of the IGV gene and occurs at an extremely high rate compared to genomic somatic mutation (>10⁶X).

After clonal expansion and somatic hypermutation, the centroblasts migrate to the basal part of the GC light zone and differentiate into centrocytes. These centrocytes will express mutated surface Igs and re-encounter antigen, presented by the FDCs (8). In the light zone, B cells are selected based on the affinity of their BCRs for antigen, a process called affinity maturation (9). Whereas low affinity mutants and auto-reactive mutants die by apoptosis, high affinity mutants will internalize antigen and process it during their migration to the api cal light and outer-zones of the GC. This stringent selection allows the generation and diversification of cells, while rigorously controlling their specificity. During this process, the affinity-selected B cells present antigen to antigen-specific GC T cells (10-12). These T cells, which have been activated by dendritic cells (13), will provide the B cells with stimulatory signals (e.g. CD40-CD40 ligand interaction, production of cytokines) (10-12,14), leading to class switch recombination and differentiation into either memory B cells or plasma cells (4,15).

Figure 2. The germinal center (GC) reaction. Schematic representation of the developmental steps that take place during the T cell-dependent B cell differentiation in secondary lymphoid organs. See text for further detail. B=B cell, T=T cell, FDC=folicular dendritic cell.

**B CELL NON HODGKIN’S LYMPHOMA**

The classification of human lympho-proliferative disorders has steadily evolved since their recognition by Thomas Hodgkin in 1832 (16). The characteristics displayed by B cell tumors are often reminiscent to that of normal B cells, allowing classification based on morphology, configuration of the BCR, expression of membrane associated molecules, and recently, genetic profiling (17,18). The current classification of B cell tumors is based on the concept that B cells, when transformed into a malignant cell, preserve their pattern of genetic rearrangement and mutational status of the IGH and IGL genes. In normal lymphoid tissues, both class switch recombination and SHM of the IGV genes take place within the GC. Extensive single-cell studies performed by the Rajewski lab, have shown that normal mantle zone B cells are devoid of mutated IGV genes, whereas GC B cells undergo continuous hypermutation (9). Therefore, the configuration of the IGV genes of a B cell tumor, i.e. the status of rearrangement of the IGV genes, the presence of somatic mutations, and the status of the switching isotype, can be used to determine the cellular origin of B cell tumors (table 1).

Errors in B cell-specific DNA remodeling processes that take place in the IGV genes, may lead to translocations, including the IGV locus (table 1). As a result, the powerful enhancers which normally control BCR expression, are juxtaposed to genes that play important roles in apoptosis, cell cycle regulation, or proliferation. Examples of translocations that involve IGV sequences are t(1;14), t(11;14), and t(8;14), effecting the expression of BCL2, CCND1, and MYC, respectively (19-21). The resulting aberrant expression of the affected genes may thus lead to the repression of apoptosis, enhancement of proliferation, susceptibility to genomic damage, or blockage of differentiation. Translocations were initially thought to arise solely during D-J or V-D-J recombination in BM precursor B cells (22). However, it has become clear that some of the translocations found in B cell tumors do not show a typical VDJ rearrangement "signature" but instead places the effected genes adjacent to switch regions, or areas that are targeted during the SHM process (23,24). SHM involves mostly single base pair substitutions; however, also small insertions and deletions are found, albeit at a very low frequency (25). Even though it is currently not clear whether these insertions and deletions are normal by products or unwanted side effects of SHM (26), it suggests that SHM might be associated with the occurrence of double strand breaks.

SHM was originally thought to be a process specific for rearranged IGV genes. However, the finding that SHMs are present in BCL6 in DLBCL, on translocated as well as normal alleles, initiated the analysis of the of BCL6 in normal B cells (27). Intriguingly, BCL6 mutations were detected in human GC and memory B cells, but not in naïve B cells, suggesting that non-IG genes could also be affected by somatic hypermutation (28). Also, the TNFRSF6 gene (FAS) has been identified as a target of the SHM machinery in normal B cells (29). These findings suggest that the hypermutation process might also promote tumorigenesis in B cells by targeting regulatory or coding sequences of genes, resulting in deregulated expression. In diffuse large B cell lymphoma (DLBCL), several other
**INTRODUCTION**

Table 1. Examples of translocations mediated by B cell-specific mechanisms

<table>
<thead>
<tr>
<th>Gene anomaly</th>
<th>Genes involved/effected</th>
<th>B cell tumor</th>
<th>cause</th>
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<tbody>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>CCND1 and IGH</td>
<td>MCL</td>
<td>V(D)J recombination</td>
</tr>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>CCND1 and IGH</td>
<td>MM</td>
<td>V(D)J recombination</td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>BCL2 and IGH</td>
<td>FL, MM</td>
<td>V(D)J recombination</td>
</tr>
<tr>
<td>miscellaneous involving 3q27</td>
<td>BCL6</td>
<td>DLBCL</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>t(2;8)(p12;q24)</td>
<td>MYC and IGH or IHL</td>
<td>Burkitt’s lymphoma</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>t(8;14)(q24;q32) and variants</td>
<td>Switch recombination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(14;16)(q32;p33)</td>
<td>MAF and IGH</td>
<td>MM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>t(4;14)(p16;q32)</td>
<td>FGFR3 and IGH</td>
<td>Switch recombination</td>
<td></td>
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</tbody>
</table>

MCL=mantle cell lymphoma, FL=follicular lymphoma, DLBCL=diffuse large B cell lymphoma

Non IG genes have been found affected by somatic hypermutation, among which are PAX5, MYC, RHO and PIM1 (30). Interestingly, the aforementioned mutated oncogenes are also often involved in translocations in B cell malignancies (31), thus providing support for the hypothesis that hypermutated genes are more susceptible for translocation events. Recently, an enzyme termed activation-induced deaminase (AID), has been shown to play a pivotal role in both class switch recombination and SHM. Whereas disruption of the AID gene leads to absence of class switch recombination and SHM (32,33), ectopic expression of AID induces these processes (34), even in non-lymphoid cells (35). These findings demonstrate clearly that SHM is not restricted to IG sequences.

B cell tumors often exhibit a great deal of heterogeneity, showing multiple morphological presentations, dissemination patterns, and genetic aberrations. Especially in DLBCL, the genetic abnormalities are diverse and multiple, consisting of translocations, numerical chromosomal abnormalities, amplifications, deletions, and point mutations (36). A recent study by Alizadeh et al remarkably improved the detection of clinically relevant subtypes of DLBCL, by using genetic expression profiling (37). The authors identified two molecularly distinct forms of DLBCL that showed gene expression patterns indicative of different stages of B cell differentiation. One type of expressed genes showed characteristics of GC B cells (BCL6, CD38, CD10), while the other group expressed a more “activated-like” B cell group of genes (CD44, BLC2, FLIP).

**MULTIPLE MYELOMA**

Multiple myeloma (MM) is a malignant plasma cell neoplasm that is characterized by clonal expansion of terminally differentiated B cells in the BM. This disease is often preceded by a pre-malignant expansion of plasma cells called monoclonal gammopathy of undetermined significance (MGUS). Despite intense efforts and a dramatic increase in our understanding of the molecular processes that are deregulated in MM, the 5-year survival rate remains unchanged at 28%, and there is currently little progress in the identification of the parameters that direct the transition from normal plasma cell to MGUS, and, ultimately, MM.

Translocations involving the IGH locus are present in ~47% of MGUS tumors, in 60-70% of BM-localized MM, and in >80% of extramedullary MM (38). Translocations in MM are multiple and diverse (table 1), and to what extent these changes correspond to clinical outcome remains to be determined. However, deletion of chromosome 13, which is identified in approximately 20% of newly diagnosed patients, is associated with very poor prognosis (39). Despite the limited amount of data available for MGUS, it is clear that processes leading to karyotypic abnormalities begin at the MGUS stage, and progress gradually through the evolution of MM.

The cell whose transformation ultimately leads to the malignant population in MM, is most probably an activated B memory cell or a plasma blast, generated during the T-cell dependent antibody response in secondary lymphoid tissues, and programmed to home specifically to the BM. In the BM, a fine-tuned network of growth factors,
cytokines, and cell surface receptor molecules govern the proliferation, differentiation and function of hematopoietic cells. Stromal cells within the BM microenvironment fulfill a major role in this process; they represent an important source of growth factors and adhesion molecules. The pathogenesis of MM within the BM strongly depends on the availability of these molecules that guide growth and survival-related processes.

By far the most studied cytokine in the pathogenesis of MM is IL6. Under normal physiological conditions, IL6 regulates the induction of differentiation from B cell to Ig-secreting plasma cell (40). Several studies have established a role for IL6 in MM by showing that; (i) IL6 induces in vitro growth of freshly obtained MM cells, (ii) MM cells show concomitant expression of both IL6 and IL receptor (IL6R), (iii) anti-IL6 antibodies induce anti-tumor effects in MM cells both in vitro and in vivo (41,42). Whether IL6 is produced through autocrine or paracrine means remains controversial. However, most evidence suggests that osteoclasts, osteoblasts, and stromal cells of the BM micro-environment are the paracrine source of IL6 production during MM pathogenesis (43,44).

The IL6R contains an α-chain, and a β-chain (aka GP130), which is the signal transducing subunit (45). Binding of IL6 to IL6R induces the formation of a complex composed of two IL6 and two IL6R molecules. Subsequently, GP130 is phosphorylated, resulting in the activation of downstream effector kinases such as Janus kinase (JAK) and SRC. Activation of JAK leads to initiation of a series of signal transduction cascade known as the JAK/STAT pathway (46). JAKs induce phosphorylation and dimerization of STATs, which will then translocate to the nucleus, inducing transcription of distinct target genes such as MYC, FOS, and JUN (47). Stimulation of MM cells with IL6 however, does not increase the activity of the STATs involved, which are constitutively phosphorylated in both IL6-responsive as IL6 non-responsive MM cells (48). However, expression of a dominant negative form of STAT3 induces apoptosis in MM cells, suggesting that constitutive activation of STATs is necessary to sustain a malignant MM population (49).

A major transducer of proliferative signals in MM is the RAS/MAPK pathway, a signal transduction cascade that will be discussed in more detail later. The RAS/MAPK pathway is activated after stimulating cells with IL6 (50), and treatment with MAPK anti-sense oligonucleotides inhibits the proliferation induced by IL6 (51). The tyrosine kinase(s) that activate the most proximal RAS/MAPK pathway members (e.g. GRB2) in response to IL6 are unknown. Prominent candidates are the JAKs, because JAK2 has been shown to interact with the SRC-homology 2 (SH2) domain of GRB2 in response to oncostatin M (52). The importance of deregulated RAS/MAPK signaling in MM pathogenesis is strengthened by the finding that activating RAS mutations occur in approximately 40% of newly diagnosed MM patients (53), and that the frequency of RAS mutations is increased during disease progression (54,55). Moreover, overexpression of both wild-type and constitutive active forms of RAS, induce the malignant transformation and plasmacytoid differentiation of human EBV-transformed B lymphoblasts (56), and renders the IL6-dependent cell line ANBL6 IL6-independent (57). Recently, it was shown that targeting of p38, a member of the MAPK family, inhibits MM cell growth in situ (58).

Growth of MM cells is not only established by induction of cell division, but also by preventing apoptosis. Anti-apoptotic signals in MM might be transduced through the PI3-K/PIPKB pathway (which will be discussed in greater detail later), which is activated by numerous cytokines and growth factors in MM cell lines and primary tumor cells (59,60). The most likely candidate for executing both the PI3K-mediated proliferation and survival signals is the PH domain-containing effector molecule PKB/AKT (61). Inhibition of constitutively activated PKB in MM cells by using pharmacological inhibitors or dominant negative constructs, have shown that PKB may control the anti-apoptotic signals in MM cells (59,62).

MM cells migrate to the BM where they interact with the BM microenvironment using a specific set of adhesion molecules (53). Important molecules in this context are molecules that mediate adhesion of MM cells to stromal cells (e.g. CD44, very late antigen 4 (VLA4), and lymphocyte function-associate antigen 1 (LFA1)), a process that can induce anti-apoptotic responses in B cells (63), or molecules that are involved homotypic adhesion (e.g. CD56)(64,65). Adhesion of MM to stromal cells can trigger the production of multiple growth factors and cytokines such as IL6 (41), and vascular endothelial growth factor (VEGF)(66), resulting in inhibition of apoptosis and enhancement of proliferation. Several other cytokines/growth factors that are produced within the BM micro environment have also been shown to play a role in either growth, or migration of MM cells, such as IL10 (67), IL21 (68), insulin growth factor 1 (IGF1)(69), hepatocyte growth factor (HGF)(this thesis), and WNTs (this thesis). Moreover, cytokines can modulate the adhesion of MM cells in the BM. For example, tumor necrosis factor α (TNFα) can induce an up-regulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1), on both BM stromal cells as well as MM cells, leading to increased binding and the production of IL6 and VEGF by BM stromal cells (70).
**THE HGF/MET SIGNALING CASCADE**

**HEPATOCELLULAR GROWTH FACTOR**

Hepatocyte growth factor/scatter factor (HGF) was independently identified by several groups working in different fields of research. A factor present in the serum of partially hepatectomized rats and in rat platelet lysates, was found to have a strong mitogenic effect on hepatocytes (71-74), hence the name hepatocyte growth factor (HGF). Almost simultaneously, a fibroblasts-secreted molecule was identified, which caused dissociation or "scattering" of epithelial cell colonies, and was thus named scatter factor (SF)(75). Subsequent structural and functional studies showed HGF and SF to be identical (76-82).

Genomic studies have revealed that human HGF is encoded by a single gene localized on 7q21.1 (83). The gene spans about 70 kbp of DNA and contains 18 exons (84) (fig. 3a). The promoter region contains a number of regulatory sequences, including a TATA-like element, an IL6-responsive element (IL6RE), and a potential binding site for nuclear factor-IL6, a regulator of IL6 expression (84). Northern blotting revealed 3 HGF mRNA transcripts of 6, 3 and 1.5 kb, respectively (80,85,86). The 6 and 3 kb messages originate from differential polyadenylation (82), whereas the 1.5 kb mRNA represents a splice variant encoding the N-terminal domain of HGF in combination with the first 2 kringle domains (85,86). This variant, NK2, behaves as an HGF antagonist (85). The subsequently described one kringle domain variant, NK1, functions as a partial HGF agonist (87,88).

The full-length human HGF cDNA encodes a protein of 728 amino acids (fig. 3a) (89). Due to proteolytic cleavage at an Arg-Val cleavage site, HGF consists of an α and β-chain (fig. 3b). The α-chain of HGF contains 4 kringle domains, structures that play a role in protein-protein interaction. The β-chain shows high homology with the catalytic domain of plasminogen, but lacks proteolytic activity, due to the lack of 2 crucial amino acids from the active site.
The Hepatocyte Growth Factor Receptor, MET

MET, the receptor for HGF, was originally identified as the product of a chromosomal translocation, TPR-MET, fusing the sequence encoding the intracellular domain of MET to that of TPR oncogene (90,91). TPR-MET functions as a constitutively active homodimer with a strong transforming capacity (90,92). Cloning of the MET proto-oncogene indicated that this molecule is a cell-surface receptor tyrosine kinase for growth factors (93-95). Functional studies revealed that HGF is the ligand of MET (79,96). MET is located on 7q31, and harbors 21 exons, spanning a region of >130 kb of genomic DNA (90,93,97) (fig 4a). The p190MET HGF receptor is encoded by the most abundant 8 kb MET mRNA. Also, a 7 kb, non-functional transcript has been observed as a result of "skipping" of exon 2, which may represent a mechanism of regulating the amount of a functional MET product (97). The sequence of the MET promoter region revealed a number of binding sites for regulatory elements, including AP1, AP2, NF-κB, and, like the HGF promoter, IL6RE (98).

MET is synthesized as a single-chain 170 kDa precursor. After synthesis, the molecule is cleaved
and rearranged into a 190 kDa heterodimer linked by a disulfide bridge (fig. 4A) (99,100). The MET heterodimer is composed of a 50 kDa α-subunit, and an 145 kDa β-subunit (100) (fig. 4B). The extracellular part of the β-chain contains a SEMA-domain with which it can bind to Semaphorin-type proteins (101). The cytoplasmic tail contains the tyrosine kinase domain and a "docking site", which interacts with multiple signaling molecules (102).

**Expression and functions of HGF/MET**

During embryonic development of rodents, Hgf is prominently expressed in a multitude of tissues, mainly at sites where epithelial/mesenchymal interactions determine organogenesis (103). Throughout early organogenesis, overlapping expression of Hgf and Met is found in the heart, condensing somites, and neural crest cells. However, a second and distinct pattern of expression, characterized by the presence of the ligand in mesenchymal tissues and the receptor in the surrounding ectoderm, is seen in the bronchial arches and in the limb buds. At E13, only this second pattern of expression is observed in differentiated somites and several major organs, such as the lungs, the liver, and the gut (104). The expression of the Hgf and Met genes throughout embryogenesis suggests a shift from autocrine to paracrine signaling. Halfway gestation, Hgf is present in renal collecting tubes of the kidney, in the liver, in esophageal and skin squamous epithelium and in bronchial epithelium (105,106). Hgf is also detected in brain, somites, hematopoietic cells, and chondrocytes (105).

Similar patterns of Met and Hgf expression are found along human embryonic development. From the 5th week of gestation onwards, placental tissue highly expresses HGF and MET. HGF is secreted by amniotic epithelium, the placental villi and the villous core mesenchyme, whereas MET is present on the trophoblast and vascular endothelium (107-109). The absolute dependence of placenta maturation on HGF has been unequivocally shown in Hgf null mutant mouse embryos, whose placenta fail to develop properly and which die in utero (110,111). From week 6-13 of gestation, when major organogenesis takes place, Hgf and Met are co-expressed in liver, metanephric kidney, intestine, lung, gall bladder and spleen (112,113). In the digestive tract of 7-8 week old embryos, Met is localized in epithelia of the liver, pancreas, esophagus, stomach, the small and large intestine, and in smooth muscle layers, whereas HGF becomes concentrated in mesenchymal tissue and smooth muscle (114). Interestingly, Hgf expression has also been shown in epithelial tissues in the interval from week 9-17 of gestation, particularly in the crypt region of the small intestine, keratinizing epithelium of the tongue, skin and esophagus (113).

HGF can induce scattering of epithelial cells in vitro (115) through activation of MET (116). HGF-induced scattering in vitro reflects the first phase of epithelial morphogenesis through mesenchymal induction (secretion of HGF), underlying the complex, but coordinated formation of branched organs, such as the lungs, the kidney and mammary gland (117). In epithelial cells derived from the mammary gland, HGF treatment leads to the formation of branches and structures resembling mammary gland ducts when cultured in a 3-dimensional matrix (118-121).

HGF has initially been described as a mitogenic factor for cultured hepatocytes (71,72). In the liver, HGF is expressed in Ito cells, whereas MET is strongly expressed by hepatocytes (122). Apart from their placental phenotype described above, Hgf null mutant mouse embryos fail to develop a fully functional liver (110), demonstrating that the presence of Hgf is an absolute requirement during liver organogenesis. Thus, HGF acts as a paracrine factor for hepatocyte proliferation and differentiation.

HGF and MET are furthermore involved in the proliferation and migration of a wide variety of epithelial cells, and in the morphogenesis of epithelial tissue. In colon epithelial cells, a complete epithelial developmental program is enrolled upon treatment with HGF, including apical/basal polarization and the formation of crypt-like structures (118). Apart from the effects on epithelial/mesenchymal tissues and liver, HGF/MET can also induce angiogenesis in vivo when rabbit cornea is treated with HGF (123). In the nervous system, depending on the spatio temporal distribution pattern and the type of neurons involved, HGF may act as a neural inducer (124), a neuronal survival factor (125), or an axonal guidance factor (126). HGF is also an inducer of myogenic migration during embryonic development (127) and of satellite cell proliferation during muscle regeneration (128). Other implications of HGF/MET include the development of bone (especially of cartilage) (129), teeth (130), the (male and female) reproductive tract (131), and the regulation of hair growth (132). Contrarily to the mutually exclusive expression pattern generally found in mesenchymal/epithelial tissues, myoblast proliferation may be regulated by HGF/MET in an autocrine fashion (133).

**Expression and function of HGF/MET during hematopoiesis**

The HGF/MET pathway has also been implicated in hematopoiesis. Both HGF and MET are expressed in the yolk sac of the chicken embryo (134), and in the human and rodent fetal liver, primordial sites of hematopoiesis (122,135). Within the adult hematopoietic microenvironment, the BM, MET is expressed by a subset of hematopoietic
transduce the phosphorylation of tyrosine in the HGF/MET pathway. These proteins, such as PI3K and SH2, are constitutively associated with the HGF exchange factor, SOS, through its SH3 domain. By binding of GRB2 to MET, SOS is translocated to the plasma membrane, where it activates downstream MAPKs and SH2, resulting in the phosphorylation and activation of downstream effectors.

THE HGF/MET SIGNALING PATHWAY

The prototypic model for activation of all known receptor tyrosine kinases except the insulin receptor, is ligand-induced dimerization and oligomerization of monomers in the cell membrane (142). This usually leads to enzymatic activity, and transphosphorylation of regulatory tyrosines. Subsequently, additional phosphorylation of other tyrosine residues will occur, resulting in binding of downstream signaling effectors (142). In the case of activation of MET, these include phosphatidylinositol 3 kinase (PI3K)(143), the non-receptor tyrosine kinase SRC (102), and adapter proteins GAB1 (144), GRB2 (102), STAT3 (145), and SHC (146). These proteins can in turn bind phospholipase Cγ (PLCγ)(147) and SHP2 (148)(fig. 5). Most biological responses are transduced via the phosphorylation of tyrosine residues Y1349 and Y1356 of MET (102,149-151).

HGF/MET signaling via RAS

Activation of the RAS/Mitogen Activated Protein Kinase (MAPK) pathway has been implicated in a wide variety of cellular responses including adhesion, differentiation, and both proliferation and apoptosis. The RAS/MAPK pathway is triggered after activation of MET and subsequent binding of GRB2 to MET (102). GRB2 is an adaptor protein consisting of one SRC homology (SH)2 and two SH3 domains. GRB2 is constitutively associated with the RAS exchange factor, SOS, through its SH3 domain. By binding of GRB2 to MET, SOS is translocated to the plasma membrane, where it activates downstream MAPKs and SH2, resulting in the phosphorylation and activation of downstream effectors.

HGF/MET signaling via PI3K

PI3K can associate with MET via either direct or indirect interaction. Either interaction will promote PI3K activity and localize PI3K in the proximity of its substrates (143,159). When activated, PI3K can phosphorylate PIP2 (phosphatidylinositol bis(2)phosphate) in order to produce PIP3. Following this, PIP3 can bind and activate PH domain-containing target proteins, resulting in membrane localization and, indirectly, their activation. Protein kinase B (PKB, aka AKT) is a PH domain-containing effector molecule of PI3K, that is activated by a dual regulatory mechanism which requires translocation to the plasma membrane and phosphorylation by PDK1 (160). Downstream effector molecules for the PI3K-regulated kinase PKB include the BCL2 family member BAD, which can exert its pro-apoptotic activity by interacting with BCL2 (161,162), p70S6, involved in the regulation of protein synthesis and gene expression (163);forkhead transcription factors FKHR, FKHL1 (164); p27kip1, implied in the PKB-induced proliferative responses (165), and glycogen synthase kinase 3 β (GSK3β) which plays an important role in preventing the degradation of cyclin D1 (166).

Two additional mechanisms may account for the HGF-induced PI3K activation (fig. 5). First, the p85 subunit of PI3K was found to interact with GAB1 (167). GAB1 contains a PH domain as well as a serine/threonine kinase domain that can phosphorylate and activate MEK, resulting in the activation of downstream MAPKs and SH2. Subsequently, transcription factors like ETS2 and ELK1 will be activated, thereby regulating expression of immediate early genes, such as FOS, eventually leading to cell proliferation (155). Using a mutant MET that fails to bind GRB2 only, it was shown that GRB2 association to MET is required for branching morphogenesis, but not for scattering in MDCK cells (150,156,157). Interestingly, the unaffected scattering response was abolished by repression of MEK, suggesting that MET can activate RAS independently of GRB2 (158).
as a proline rich region, can be tyrosine-phosphorylated, and has the ability to directly associate with numerous signaling molecules (167). Although several studies imply that binding of GAB1 to MET in vivo is mediated by GRB2 (168-170), in vitro, GAB1 can interact directly with MET via a proline rich binding domain (144). Interestingly, besides being able to associate with PI3K, GAB1 requires PI3K activity for proper localization and induction of morphogenesis (171). Second, PI3K has been identified as an effector molecule for RAS, via direct interaction of its p110 catalytic subunit (152).

Activation of PI3K is required and sufficient for HGF-induced tubulogenesis, and required for scattering (172-174). Mutation of the docking site of MET, which results in the loss of PI3K and GAB1 association with MET upon HGF treatment, does not abrogate HGF-induced scattering or RAS activation (158). Whether PI3K activation alone is sufficient for HGF-induced scattering of MDCK cells is still a matter of debate. Potempa and Ridley reported that neither expression of an active mutant of PI3K, nor the combined expression of PI3K with active RAF or MEK, was sufficient for adherents junction disassembly, a prerequisite for scattering (173). Contrary to these findings, Khwaja et al. reported that expression of an active mutant of PI3K is sufficient to induce scattering, provided a basal level of MAP kinase activity is present (172).

Additional MET-associated signaling molecules

An additional MET associating protein is PLCγ (102). PLCγ mediates the production of IP3, which results in enhanced calcium and diacylglycerol release thereby activating PKC. Indeed, PKC has been implicated in MET signaling in a variety of cell types (175-179). Furthermore, both PKC and calcium can regulate MET signaling, by phos-
phorylation of residue S985 of MET, resulting in diminished kinase activity (180-182). HGF also activates STAT3 (183), and stimulates recruitment of STAT3 to the autophosphorylated Y1356 of MET (145). Upon phosphorylation, the STAT proteins can dimerize and translocate to the nucleus, where they act as transcription factors controlling the promoter activity of target genes (47). Inhibition of STAT-mediated transcription prevents HGF-induced tubulogenesis, whereas scattering and proliferation remain unaffected (145).

Furthermore, the SRC tyrosine kinase was shown to directly associate with MET (102). Both the association with MET, and the subsequent activation of SRC, play a critical role in carcinoma cell motility (184), and in HGF-induced phosphorylation of FAK (185). Although the tyrosine phosphatase SHP2 can also be indirectly recruited to MET via GAB1 (171), no functional data are available yet. In addition, BAG-1, a cell death suppressor gene product that binds the anti-apoptotic proto-oncogene product BCL2 in a cooperative fashion (186), interacts with MET, independent of phosphorylation of either Y1349 or Y1356 of MET (187). Accordingly, overexpression of BAG-1 enhances the anti-apoptotic effect of HGF on liver progenitor cells (187).

**ONCOGENIC AND METASTATIC PROPERTIES ELICITED BY MET**

Activation of MET without appropriate temporal regulation, or displayed in an aberrant cellular context, is oncogenic and can lead to the initiation or progression of malignancy. Under these conditions, MET can disturb the subtle balance between growth and apoptosis, and induce unrestricted growth and motility, accounting for cellular transformation, invasion and metastasis. Numerous stud-

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<th>Functional consequence</th>
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<td>P1009S</td>
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<td>Gastric cancer (199)</td>
<td>Tumor formation in nude mice, anchorage independent growth</td>
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<td>g</td>
<td>HPRC (204)</td>
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* homologous to mutations found in the KIT oncogene in mastocytosis (213). # homologous to mutations found in the RET oncogene in multiple endocrine neoplasia 2B (MEN2B)(214). Numbering is according to Schmidt et al (201). (Only mutations that have been analyzed for functionality are shown)
ies have investigated MET's oncogenic potential and linked this behavior with deregulated catalytic activity (reviewed in references (101,188)). Constitutive activation of tyrosine kinase receptors can be achieved via several mechanisms. (i) Autocrine activation, which induces independence of paracrine produced growth factors, and can render cells highly tumorigenic (189,190). Interestingly, MET overexpression can be induced by HGF itself, as well as by a number of other cytokines, including EGF, IL1, and IL6 (191). Accordingly, transgenic overexpression of HGF results in a multitude of MET-overexpressing tumors of both epithelial as mesenchymal origin (192). Moreover, overexpression of wild-type Met in mice induces hepatocellular carcinomas that regress following transgene inactivation (193). (ii) Overexpression of the receptor, which has been found in human tumor cells and can be caused by gene amplification, can result in receptor oligomerization and possible ligand-independent autophosphorylation of MET (99,194-196). (iii) Structural alterations of the receptor that lead to aberrant activation or degradation. Recent studies have demonstrated a direct role for MET in the genesis of hereditary papillary renal carcinomas (HPRC), by showing that these germline and somatic MET mutations are tumorigenic both in vitro and in vivo (197-201). Of note, tumors of the effected individuals often show a duplication of the chromosome bearing the mutated MET allele (202,203). Since then, similar mutations were found in a subset of sporadic papillary renal- (202,204,205), hepatocellular- (206), and gastric carcinomas (199)(table 2). These mutations result in enhanced kinase activity upon stimulation with HGF and were shown to mediate transformation, invasive growth, and protection from apoptosis (197,199,200,204,207,208) (table 2). The effected regions of MET are the catalytic domain and the juxtamembrane region, deregulating either the activation or degradation of MET. Recently, it has been hypothesized that somatic MET mutations are selected during metastatic spread of carcinomas, following the detection of mutations mainly in metastasizing cells (208,209).

In vitro studies have indicated that activation of mutant as well as wild type MET is strictly HGF-dependent, indicating that the availability of active HGF in vivo is a prerequisite for MET-induced tumorigenesis. This concept is strongly supported by several studies reporting high levels of HGF in serum or pleural effusion fluid in several human tumors (210-212). These data furthermore suggest that paracrine or autocrine stimulation of cancer cells by HGF promotes migration to metastatic sites. In conclusion, the HGF/MET pathway, together with its down-stream effector molecules and the genes it regulates, is a major accomplice in the genesis, progression and metastasis of cancer.

The HGF/MET pathway in B cell neoplasia

The HGF/MET pathway might also promote B cell tumorigenesis. MET is constitutively expressed by several Burkitt’s lymphoma cell lines, including Raji, BJAB, and EB4B (139,215), as well as by a subset of native Burkitt’s lymphomas (216) (chapter 4). On these tumor cells, which represent the malignant counterparts of GC centroblasts, HGF induces MET phosphorylation, as well as activation of downstream signaling molecules including MAP kinases ERK1 and -2 and PKB (139,217). Expression of MET has been also been observed in DLBCL (216)(chapter 4), MM (218,219)(chapter 2 and 3), primary effusion lymphoma (PEL) (220), and Hodgkin lymphoma (HL) (221) (chapter 4). In these malignancies, concomitant expression of MET and HGF is often observed, suggesting that the HGF/MET pathway may be activated by autocrine means (218-220). However, we have demonstrated HGF production by non-tumor cells in DLBCL (chapter 4), while BM stromal cells have been shown to produce HGF. Hence, paracrine activation may also take place within these tumors. This is supported by the finding that increased HGF levels are associated with unfavorable prognosis in both MM and HL (212,221). Using gene expression profiling, HGF was the only growth factor significantly up-regulated in MM compared to normal plasma cells (222), suggesting that HGF plays a role in the transformation to malignancy in MM. We have shown recently that in MM cells, phosphorylation of MET induces activation of the RAS/MAPKK and PI3K/PKB pathways (chapter 2 and 3), signaling routes that have been implicated in the control of proliferation and survival, respectively (155,161,162).

A role in MM dissemination is suggested by the fact that HGF has been shown to regulate integrin activity on GC B cells (139), an that HGF is able to promote adhesion and migration of Burkitt’s lymphoma cell lines (139,216). Key regulatory molecules implicated in inside-out signaling to integrins are PI3K and different RAS-like GTPases, the activity of which can be controlled by HGF/MET (102) (chapter 2).

Although these data present circumstantial evidence implicating the HGF/MET pathway in the pathogenesis of multiple myeloma, direct functional evidence is scarce and comes from studies from our laboratory showing that HGF mediates growth and survival in MM cell lines and primary MM (chapter 2 and 3). We also show that whereas the PI3K/PKB pathway is required for both HGF-induced proliferation and rescue from apoptosis, the RAS/MAPK pathway is required for proliferation (chapter 2). Taken together, these data strongly suggest a role for the HGF/MET pathway in the pathogenesis and progression of B cell neoplasia.
THE WNT SIGNALING PATHWAY

WNT signaling governs multiple developmental processes such as embryonic development, generation of cell polarity, and specification of cell fate in a wide range of organisms (223,224), whereas inappropriate activation of WNT signaling can lead to cancer in numerous tissue types (225,226).

Canonical WNT signaling

WNT genes encode a large family of at least 16 secreted glycoproteins, which promiscuously interact with several Frizzled (FZD) receptors (227,228), leading to activation of intracellular signaling (fig. 6). In the absence of a WNT signal, glycogen synthase kinase 3β (GSK3β) is active and forms a large complex together with β-catenin, the tumor suppressor protein adenomatous polyposis coli (APC), and axin, or its analogue conductin (229-232). WNT signaling is initiated by binding of WNT to FZD, leading to the phosphorylation of dishevelled (DVL) (233), which, through association with axin, prevents GSK3β from phosphorylating its substrates (232). Consequently, β-catenin is stabilized, and ubiquitination and proteosomal breakdown is prevented, resulting in the accumulation of active, non-phosphorylated β-catenin (234,235). Finally, β-catenin will translocate to the nucleus, where it will heterodimerize to one of the four T cell factor (TCF) family of HMG box proteins (236,237), driving transcription of target genes (238,239). In the absence of a WNT signal, the TCF/LEFs are associated with the co-repressors Groucho (240,241), CIBP (242), or the CREB-bind-

Figure 6. The canonical WNT signaling pathway. In the absence of a WNT signal, β-catenin forms a complex together with APC, GSK3β, and AXIN. As a consequence, β-catenin will be phosphorylated, tagged with poly ubiquitin (UB), followed by proteosomal degradation. In this scenario, TCF/LEF is associated with co-repressors, and gene transcription is inhibited (left). In the presence of WNT, the kinase activity of GSK3β is inhibited by phosphorylated dishevelled (DSH), resulting in the dissociation of the aforementioned complex. As a result of this, β-catenin will accumulate and translocate to the nucleus, where it will bind TCF/LEF, and potentiate activate transcription of target genes (right). DKK=dickkopf, LRP=LDL related protein.
ing protein CBP (243), which inhibit transcription of target genes.

**WNT SIGNALING AND CANCER**

The key event in WNT signaling, is the suppression of β-catenin degradation. Specific serine and threonine residues are involved in the inhibition of degradation of β-catenin (234,235). Deregulation and accumulation of β-catenin is typically due to truncating mutations in APC, or to mutations in the GSK-3β target-residues in CTNNB1. Mutations of APC or CTNNB1 have been found at high frequency in colorectal cancer, pilomatrixoma, and hepatoblastoma, but the mechanism of nuclear β-catenin accumulation in several other tumor types is, at present, unclear (226). The critical consequence of these mutations is the elevation of the levels of β-catenin leading to the formation of constitutive nuclear β-catenin/TCF complexes and altered expression of TCF target genes (226,244). Several downstream targets of WNT signaling such as MYC (238), CD44 (245), MET (246), and the urokinase-type plasminogen activator receptor (247), show a strong correlation with regards to tumor progression (188,247-249). Interestingly, during active WNT signaling in both normal and malignant intestinal epithelium, MYC is expressed, and blocks the expression of the cell cycle inhibitor p21CIP1/MAF1, leading to cell cycle progression (244).

Members of the TCF/LEF(Lymphoid enhancer factor-1) family of transcription factors were initially discovered in models of lymphocyte development (250-252). Although TCF/LEFs bind directly to DNA through their HMG domains, no direct independent gene transcription has been demonstrated (253,254). Within the immune system, TCF1 is restricted to the T cell lineage, whereas all T cells and pro-B lymphocytes express LEF1 (255). The biological relevance during T- and B cell development was demonstrated by genetic disruption of Tcf1 and Lef1, in which Tcf1 appeared to be essential for the sustentation of early thymocyte progenitor compartments, but dispensable for proliferation and function of mature T lymphocytes (256-258), whereas Lef1 displayed multiple non-immunological effects (259). Interestingly is the recent observation by Grosschedl and co-workers, who observed that WNT signaling regulates pro-B cell proliferation, a process that was shown to be Lef1-dependent (260).

The fact that transcription factors indispensable for WNT signaling play such important roles during lymphocyte development, suggests that WNT-signaling might also regulate lymphocyte growth and survival. The first direct link of a function for WNT signaling during hematopoiesis, came from a study showing that Wnt proteins (Wnt1, Wnt5a and Wnt10b) stimulated the survival and proliferation of hematopoietic progenitors (261). Furthermore, aberrant activation of components of the WNT signaling cascade might contribute to B cell neoplasia. Indeed, expression of β-catenin has been linked to adhesion, growth and survival of Jurkat T cells and myeloid leukemia cell lines (262). Also, the most recent progeny of the family of WNTs, WNT16, was found to be activated by the E2A-PBX1 fusion product in acute lymphoblastic leukemia (ALL) (263).

(For additional data on WNT signaling, and the most recent update on WNT-signaling target genes, visit the WNT pages hosted by the Nussle as well as the Moon lab:  http://www.stanford.edu/musse/wntwindow.html or http://faculty.washington.edu/rtmoon/)

**HEPARAN SULFATE PROTEOGLYCAN**

Heparan sulfate proteoglycans (HSPGs) are abundant membrane or matrix molecules that consist of a core protein to which heparan sulfate (HS) glycosaminoglycan (GAG) polysaccharides are attached (264). Heparin, a mammalian GAG, has the highest negative charge density of any known biological macromolecule, and is thus prone to interact with a variety of proteins (265). Despite extensive evidence showing specific functions of HSPG in vivo, the prevailing view of many biologists is that the anionic HSPGs interact at random with secreted growth factors and extracellular matrix (ECM) components, thereby functioning as a non-specific sequestering molecules. However, recent genetic studies in flies and mice have revealed complex and dedicated functions for HSPGs in regulating cell signaling pathways, thereby controlling multiple cellular processes (266-268).

**HSPG BIOSYNTHESIS**

Virtually all cells from invertebrates to humans have the capacity to produce HS. These polysaccharides are covalently bound to serine (Ser) residues in the core protein through a common GAG-protein linkage structure (GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser), and characterized by alternating uronic acid (D-glucuronic acid (GlcA), or L-iduronic acid (IdoA)), and D-glucosamine (GlcN) units (264)(fig. 7). The GlcN residues in heparin are mainly N-sulfated, whereas those in HS show a more varied composition of both N-sulfated, N-acetylated, and a small amount of N-unsubstituted GlcN units (269). These structures are generated through the formation of a [GlcA-GlcNAc] polymer (chain polymerization, fig. 7), that is subsequently modified by partial N-deacetylation/N-sulfation of GlcNAc units, C-5 epimerization of GlcA to IdoA residues, and finally, incorporation of O-sulfate.
**Figure 7. HS chain biosynthesis.** The HS side chains are attached to the core protein at specific Ser-Gly residues. A common tetrasaccharide linkage region \((-\text{GlcA-Gal-Gal-Xyl})\) is formed, followed by the addition of alternating GlcNAc residues. The chain is then enzymatically modified by the indicated enzymes. The symbols used are defined by the structures below the scheme. Xyl=xylose, Gal=galactose, PAPS=nucleotide sulfate, IdoA=L-iduronic acid, GlcA=D-glucuronic acid, GlcNAc=N-acetyl glucosamine. See text for further detail. (adapted from Esko and Lindahl, 2001 (132))

**Core proteins carrying HS**

A variety of proteins have been shown to carry HS side chains. These HSPGs are diverse and can be either transmembrane (syndecan and CD44); bound by a glycosyl phosphatidylinositol (GPI) linkage to plasma membrane lipids (glypicans), or secreted into basement membranes (agrin and perlecan) (264,265,268). Glypicans and syndecans represent the two main cell-surface HSPGs (270). In mammals, four different syndecan and six different glypican genes have been identified. Syndecans can, under certain conditions, be proteolytically cleaved near the cell surface, releasing the HS-bearing ectodomain, a process mediated by metalloproteinases, and referred to as “shedding” (271). Shedding of syndecans thus results in soluble effectors, often found in wound fluids (272), where they can control growth factor signaling (273).

As mentioned, a number of studies have provided evidence for an in vivo role of cell surface HSPGs in growth control and morphogenesis (265,274). Disruption of certain glypicans or enzymes required for the synthesis of HSPGs in *Drosophila melanogaster*, leads to major developmental defects (267). In humans, deletions or mutations in the glypican3 gene (GPC3) results in
the X-linked Simpson-Golabi-Behmel syndrome, characterized by pre- and postnatal overgrowth of multiple tissues and organs, and an increased susceptibility to the formation of tumors (275). Interestingly, studies in mice suggest that glypican3 may regulate bone morphogenetic protein 4 (Bmp4), because crosses between Gpc3<sup>−/−</sup> and haploinsufficient mutants of Bmp4, show skeletal abnormalities (276). Targeted disruption of the syndecan1 gene, the main syndecan expressed by epithelial and plasma cells, yields healthy and fertile mice, in spite of the abundant expression of syndecan1 during development (265). However, syndecan1 knock-out mice are remarkably resistant against Wnt1-induced tumorgenesis of mammary epithelium (277), and withstand infection induced by intra-nasally administered *Pseudomonas aeruginosa* (278). These data indicate that phenotypic abnormalities in syndecan1-deficient mice are only observed when their epithelia are perturbed. They furthermore suggest that redundancy plays an important role in HSPG-mediated developmental processes, but cannot replace the designated functions of syndecan1 during adult life.

In addition to playing a role in development, HSPGs have been implicated in cell adhesion and migration, angiogenesis, and in the regulation of blood coagulation (266,279,280). Binding of cytokines and chemokines to HS side chains often takes place in these processes, and may serve a variety of functions ranging from immobilization and concentration, to distinct modulation of their biological task (265,266,281). Upon binding to HS, proteins can also undergo a conformational change (282), as has been implied for HGF (283), which may facilitate ligand-receptor interaction. Furthermore, binding of growth factors to HS may result in their protection from degradation (284). Interactions between secreted proteins and HS shows a certain degree of selectivity. For example, fibroblast growth factor (FGF)2 requires an N-sulfated sequence with a single IdoA 2-O-sulfate group for binding to HS (285-287). By contrast, interactions with PDGF (288), HGF (289,290), and herpes simplex gC glycoprotein (291) all depend on the presence of one or more GlcN 6-O- sulfate groups. The importance of growth factor/HSPG interaction is well illustrated by FGF2; binding of FGF2 to HS is compulsory for its biological effects (282,292). HS side-chains can bind and oligomerize FGF2, thereby enhancing FGF receptor (FGFR) cross-linking and subsequent activation (293,294). A recent structural model of a FGF2:FGFR complex indicates that HS-induced dimerization yields the minimal structural unit required for FGFR activation, and suggests direct physical interaction between HS and FGFR (295). The modular structure of HGF has facilitated the identification of the domains responsible for binding to MET and heparin/HS. By using deletion mutants of HGF and examining their binding ability to immobilized heparin, Mizuno et al. identified the hairpin loop of the amino-terminal domain and the second kringle domain as sites essential for heparin binding (296), domains also critical for MET binding and signaling (297-300). In order to dissect the binding sites for MET and heparin/HS in HGF, three-dimensional models of the individual HGF domains were generated to help to design specific mutants (301). Based on the X-ray structures of antithrombin- (302) and FGF-heparin complexes (287), they predicted the heparin-binding sites to contain clusters of positively charged residues which make electrostatic contact with negatively charged groups in HS-chains. Indeed, three such clusters were identified on the surface of HGF, two in the hairpin loop and one in the kringle 2 domain (301). By introducing specific mutations at these sites, it was confirmed that these residues play a key role in heparin-binding (303). A study by Chirgadze et al. reported the crystal structure of NK1, a natural splice variant of HGF with agonistic activity, consisting of the N- and first kringle-domains (304). It was shown that NK1 assembles as an asymmetric homodimer in which the N-domain of one partner interacts with the kringle domain of the other. Short heparin fragments (14-mer) effectively dimerized NK1 in solution, suggesting that heparan sulfate chains expressed on cells or in the ECM may stabilize the NK1 dimers in vivo. Furthermore, binding to the primary site in the N-domain is essential for biological activity, whereas binding to the K domain reduces activity, demonstrating the complex role of HS in HGF/MET signaling (305). Interestingly, the structural specificity for binding to HS differs radically from that of FGF-2 (285), illustrating the importance of structural diversity of the HS-chain in selective growth factor binding. HSPGs are, analogous to FGF2, also required for WNT signaling (306,307). In this process, WNT signaling is regulated by heparin-specific N-acetyl glucosamine sulfatases that control desulfation of cell surface HSPGs (308).

**Syndecans**

The syndecan family of HSPGs consists of four members: syndecan1 (aka CD138) syndecan2 (fibroglycan), syndecan3 (N-syndecan), and syndecan4 (amphiglycan). Syndecan core proteins range in size from ~20 to ~90kDa, although their apparent molecular mass is much higher due to the 3 covalently attached HS side chains (309). In adult mammalian tissues, syndecan1, -2 and -3 are the major syndecans expressed by epithelial cells, fibroblasts and neuronal cells (265,270). The syndecan core proteins have several important domains, which may interact with (cell surface) receptors, downstream effector molecules, or trigger
distinct signal transduction pathways (fig. 8 & 9). The cytoplasmic domains of the syndecans contain two regions (C1 and C2) that are highly conserved among the four family members, flanked by a variable region (V) that is distinct for each family member (fig. 8). A common feature of the C2 region is binding to type II PDZ domain-containing proteins, a process that may mediate the correct localization of syndecans in the plane of the plasma membrane (309). For example, syndecan1 and syndecan2 can be found in complex with the PDZ domain-containing the guanylate kinase CASK/LIN2, the cytoskeletal protein 4.1, and cadherin-linked β-catenin (310). Furthermore, syndecan binding to CASK/LIN2, affects its nuclear translocation and transcription regulatory activity (311). In addition, the V-regions of the syndecans are likely to govern distinct roles specific for each syndecan and cellular context in which they are expressed. Examples of this include a signaling role within focal adhesion complexes and matrix assembly (see fig. 9A and C).

![Figure 8. Syndecan core protein structure.](image)

The extracellular domain of the syndecans contains the heparan sulfate (HS) glycosaminoglycan (GAG) attachment sites. The cytoplasmic region contains two domains (C1 and C2) that are highly conserved in each of the four syndecans. A variable (V) region, distinct for each syndecan family member, is located in between the conserved regions. See text for further detail.

![Figure 9. Regulation of signaling by syndecans.](image)

**A)** Cell-cell adhesion. Syndecans recruit to sites of cell-cell contact, where they may control cytoskeleton re-organization by distribution of CASK/LIN-2, β-catenin/cadherin complexes and protein 4.1. **B)** Growth factor signaling. Syndecans HS side chains may bind and cluster growth factors (GF), and present them to their cognate receptors, thereby regulating growth factor induced signaling. **C)** Cell-matrix adhesion. Syndecans may associate with integrins, leading to focal adhesion assembly. Simultaneously, binding of a disintegrin and metalloproteinase, like e.g. ADAM12, to HS, can promote interaction between the core protein, integrins or other unknown transmembrane proteins and trigger integrin activation (166). Indicated are several downstream effector molecules, regulating stress fiber and focal adhesion formation, e.g. syndesmos (SYN), protein kinase C (PKC), and focal adhesion kinase (FAK). (adapted from (163)).
Apart from mediating cell-cell interactions, binding growth factors and regulating their activity, syndecans can also contribute to cell matrix adhesion (fig. 9C)(309,312). Syndecans co-localize with integrins at focal adhesions in a range of cell types and on numerous substrates (313). Insertion into focal adhesions appears to be independent of the type of integrin involved, but requires activation of PKC (266,313,314). For example, the cytoplasmic tail of syndecan4 can interact with (and activate) PKCα, PIP2, and sydesmos, which are all implicated in syndecan4-controlled integrin-mediated focal adhesion formation and cell spreading (309,315). In contrast, the lymphoid syndecan1-expressing Raji and ARH77 cells, show adherence to fibronectin and anti-syndecan antibodies, independently of β1 integrins (316,317).

The majority of studies concerning HSPGs have focused on epithelial and mesenchymal type tissues. Given the fact that many immune-related cytokines and growth factors are heparin/HS binding, it is likely that HSPGs also play prominent roles in the immune system, and its malignancies. Chemokines and other heparin-binding cytokines have important functions in the regulation of inflammation, lymphocyte trafficking, -growth, -differentiation and -survival. Therefore, HSPGs might also be involved in the regulation of lymphocyte biology. Recently, studies from our laboratory have shown that stimulation of tonsillar B cells using BCR and CD40, triggers up-regulation of a HS-bearing CD44 isoform (CD44-HS)(141). Moreover, HGF strongly binds to CD44-HS, thereby promoting HGF-induced signaling in MET-expressing B cells (141,318). Within the immune system, data revealing expression of HSPGs are scarce. Mouse precursor B cells and plasma cells have been shown to express syndecan1 (319), whereas syndecan4 is expressed by Ig isotype-switched mature B cells (320). In humans, syndecan1 is expressed by plasma cells, and their malignant counterpart, MM (321,322)(chapter 3), as well as the Reed Sternberg cells of classical Hodgkin’s lymphoma (323), AIDS-related lymphoma’s (323), and a subset of B-CLL (324). Functional studies have shown that MM cells that express syndecan1, strongly bind to collagen, and do not show invasive behavior (325). In addition, syndecan1 may control HS side chains-mediated cell-cell adhesion (326). These findings are strengthened by data showing that syndecan1 accumulates at the leading edge of myeloma cells (lamellopodium), and at membrane protrusions called uropods (327,328). Interestingly, heparin binding growth factors such as HGF have been found to co localize with syndecan1 in uropods of MM cells (328). Indeed, we have substantiated a functional role for this interaction in MM by demonstrating that HGF binding to the HS side-chain of syndecan1 strongly promotes HGF-induced responses (chapter 3), an effect that may be modulated by soluble syndecan1 shed from the MM cell surface (329), outlining an important role for syndecan-1 in the pathogenesis of MM.

**Aims and outlines of this thesis**

The studies described in this thesis investigate the contribution of two potent oncogenic pathways to B cell tumor growth and survival, i.e. the HGF/MET- and WNT signaling pathways, and substantiate a functional role of heparan sulfate proteoglycans (HSPGs) in these malignancies. Chapter two reveals the functional impact of HGF/MET activation in B cell malignancies, by showing potent HGF-induced growth and survival in multiple myeloma.

HSPGs play a crucial role in binding and presenting growth factors to their cognate receptors. Expression of the HSPG syndecan-1 (CD138) is characteristic of terminally differentiated B cells, i.e., plasma cells, and their malignant counterpart, multiple myeloma. Chapter three studies the distribution of HSPGs in MM and investigates the influence of HSPGs on HGF-induced signaling in malignant plasma cells. In chapter four, we have examined expression patterns of HGF and MET on normal tonsillar B cells, and on a large panel of several B cell malignancies, and screened these tumors for chromosome 7 abnormalities and mutations in MET.

WNT signaling governs many cellular processes during embryonic development and postnatal life by regulating β-catenin/TCF-mediated transcription. Deregulation of this signaling route had been shown to play a major role in epithelial-type tumors. Even though TCF transcription factors are involved in early lymphocyte development, a function for β-catenin/TCF-mediated transcription in lymphoid malignancies has remained largely unexplored. Chapter five investigates the functional impact of WNT signaling in MM, by presenting data that suggest a role for the WNT pathway in MM tumor growth.

Chapter six summarizes and discusses the results presented in this thesis.
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CHAPTER 1


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