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Tjin, E.P.M.

Publication date
2005

Document Version
Final published version

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Esther Tjin

Thesis

The HGF/MET and WNT signaling pathways in B cell differentiation and neoplasia, by Esther Tjin

ISBN 90-9019896-2

Prepared at:

Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

This work was supported by grants from the Netherlands Organization for Scientific Research and the Dutch Cancer Society

Printing of this thesis was financially supported by:

Tebu-bio

The University of Amsterdam

Printed by PrintPartners Ipskamp, Enschede

The HGF/MET and WNT signaling pathways in B cell differentiation and neoplasia

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus prof.mr. P.F. van der Heijden
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit
op dinsdag 25 oktober 2005, te 10.00 uur

door **Esther Pit Mien Tjin**

geboren te Paramaribo (Suriname)

Promotiecommissie

Promotor:	Prof. dr. S.T. Pals
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CHAPTER **1**

General introduction

1 B cell development and cancer

1.1 B cell development and differentiation

1.1.1 B cell development

B cells represent about 5-15% of the circulating lymphocyte pool and play an important role in the adaptive immune response. During B cell development, B-lineage precursors in the bone marrow (BM) proliferate and undergo a highly regulated maturation process that culminates in the production of immature, surface immunoglobulin (Ig)-expressing B cells [1]. During the initial stages of development, expression of the pre-B cell receptor (pre-BCR), which is formed after succesfull rearrangement of the variable (V), diversity (D) and joining (J) gene segments of the Ig heavy chain (IgH) gene, is required for B cell survival. Signaling via the pre-BCR induces rearrangements of the V and J segments of the Ig light chain, resulting in cell-surface expression of a mature BCR of the IgM isotype. The IgM-expressing immature B cells migrate to the spleen, where they differentiate into naïve, mature B cells, which enter the recirculating lymphocyte pool. Upon antigen encounter in peripheral lymph nodes, gut-associated lymphatoid tissue or spleen, they can undergo Ag-specific differentiation (figure 1).

1.1.2 B cell differentiation

Ag-specific B cell differentiation takes place in the germinal centers (GC) of the secondary lymphoid tissues. The GC reaction starts after Ag-specific naive B cells are activated in the T cell areas of the lymphoid tissues, and migrate into the primary follicle. Here, these cells become centroblasts, which form the GC dark zone [1], and undergo rapid clonal expansion and somatic hypermutation (SHM). The SHM machinery introduces mutations at a very high rate in specific regions of the rearranged Ig variable (IgV) genes [2] to increase antibody diversity. Subsequently, the GC B cells, now centrocytes, migrate to the GC light zone. At this stage, the cells are selected on the basis of the affinity of their BCR for antigen presented on the surface of FDC. Only the GC B cells with high-affinity BCR receive survival- and proliferation signals from FDCs and T cells, whereas low affinity and self-reactive GC B cells undergo apoptosis [3-5]. The rescued GC B cells process the antigen from the FDCs and present the Ag-derived peptides to Ag-specific T cells. These cells provide survival signals to the B cells involving the cell surface molecules including the T cell receptor/CD3-MHC class II, the co-stimulatory molecules CD40 ligand-CD40 and CD80/CD86-CD28, and several cytokines [6-9]. These survival signals, including the interaction with FDCs, are necessary to sustain the expression of the long isoform of cellular $FLIP_L$ ($cFLIP_L$), resulting in the inhibition of caspase 8 activity and protection of GC B cells from FAS-induced cell death [10]. Cognate T-B cell interaction further may result in class-switch recombination (CSR), which involves replacing of the $C\mu$ constant region of IgM for other constant region exons, to vary the antibody repetoire. After expansion, the Ig isotype-switched B cells mature to either plasma cells or memory B cells. The former facilitates the eradication of infectious pathogens by secreting high-affinity Igs,

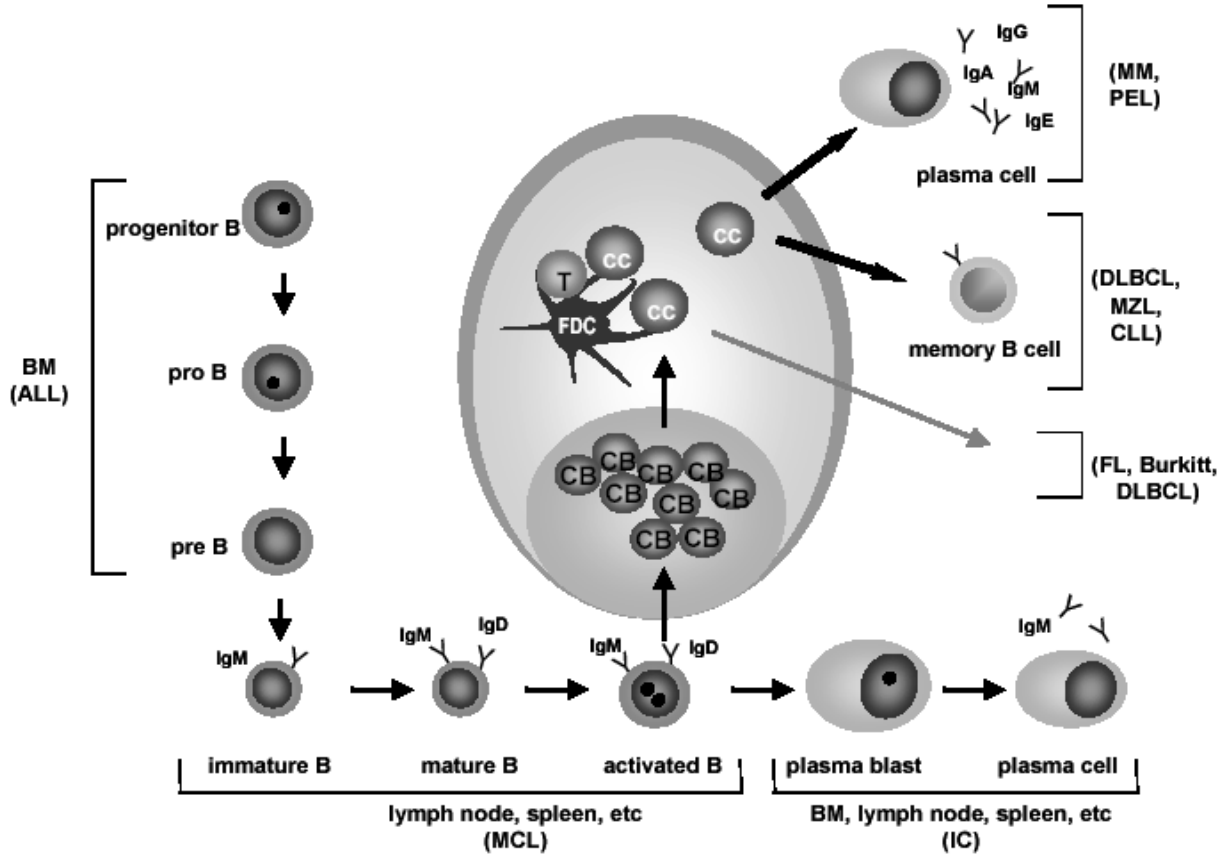


Figure 1 **Normal B cell differentiation and the related stages of B cell malignancy.**

Schematic representation of B cell differentiation. The malignant counterparts are indicated in parenthesis. See text for further detail. PEL= primary effusion lymphoma; MM= multiple myeloma; DLBCL= diffuse large B cell lymphoma; IC= immunocytoma; FL= follicular lymphoma; MZL= marginal zone lymphoma; CLL= chronic lymphocytic leukemia/lymphoma; MALT= mucosa-associated lymphoid tissue; MCL= mantle cell lymphoma; ALL= acute lymphoblastic leukemia.

whereas the latter allows rapid response to antigen recall (figure 2).

1.1.3 Transcriptional regulation of B cell differentation

B cell differentiation is a tightly controlled process. The transcription factors B cell lymphoma-6 (BCL-6), PAX-5, interferon regulatory factor 4 (IRF-4), X-box binding protein-1 (XBP-1), and B lymphocyte-induced maturation protein-1 (BLIMP-1) form a regulatory circuit that determines the crucial developmental decisions in GC B cell differentiation and plasma cell formation[11,12]. BCL-6 and PAX-5 are necessary for the commitment of bone marrow progenitors to the B cell lineage 13 and for GC B cell development. Furthermore, BCL-6 and PAX-5 block plasmacytic differentation by repressing the expression of BLIMP-1 and XBP-1, respectively [11,12]. After the termination of the GC reaction, BLIMP-1 is relieved from the BCL-6-dependent repression and induces plasma cell differentiation. BLIMP-1 ensures that the commitment to plasma cell fate is irreversible by repressing BCL-6 and PAX-5, and by upregulating XBP-1, which is also

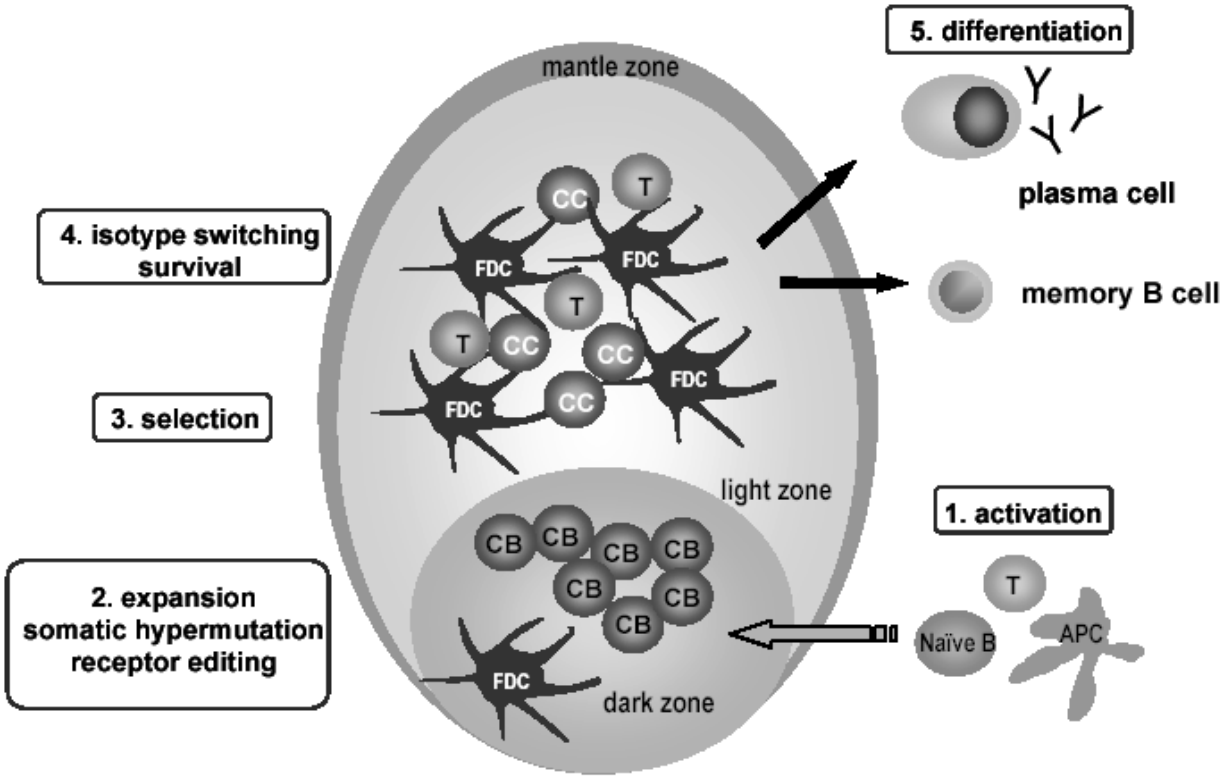


Figure 2 **The germinal center (GC) reaction.**

Schematic representation of the differentiation steps that take place during antigen (Ag)-specific differentiation in secondary lymphoid organs. See text for further detail. CC= centrocyte; CB= centroblast; FDC= follicular dendritic cells.

essential for plasma cell differentiation.

Cytokines, produced by T cells and FDCs, have an important role in B cell differentiation and plasma cell development [9]. In general, whereas IL-2 and IL-4 are required for the proliferation of GC B cells and favour memory B cell development, IL-10 interrupts memory B cell expansion [14] and drives plasma cell development [14]. Furthermore, IL-6 supports the proliferation of plasmablasts and the development of plasmablasts into mature plasma cells. A number of recent studies have demonstrated that cytokines regulate the expression of transcription factors. IL-4 represses *BLIMP-1* expression [11], thereby preventing plasma cell differentiation. Notably however, IL-4 has also been reported to induce *XBP-1* transcription [15], which contradicts its ability to drive memory B cell but not plasma cell development. Furthermore, in some B cell lines, IL-6 or the combination of IL-2 and IL-5 also induce *BLIMP-1* and *XBP-1* expression [11,12]. The precise mechanisms by which cytokines regulate transcription factors involved in B cell differentiation remain to be elucidated.

Chemokines and integrins controlling Ag-specific B cell differentiation

Chemokines and integrins can control multiple cell functions, including lymphocyte development, cell survival and homing during B cell differentiation. A small subset of the chemokine family helps to guide the movements of recirculating lymphocytes within the GC, the exit from the GC and the migration to the BM. The most potent

homeostatic chemokines that induce B- and T cell migration include CXCL-12/SDF1 [16], CXCL-13/BLC [17], CCL-19/ELC [18] and CCL-21/SLC [19]. The by far best-studied chemokine, CXCL-12, is essential for B lymphopoiesis and homing [16]. Mice deficient for CXCR4, the receptor for CXCL-12, and CXCL-12-/- mice show haematopoietic defects, which are restricted to the B cell- and myeloid lineage [20,21]. Importantly, CXCL-12 is involved in the localization of centroblasts to the dark zone [22], suggesting a role in the organization of the GC dark- and light zones [22]. Furthermore, CXCL-12 plays an important role in the homing of plasma cells to the BM [12,23].

B cells express the integrins $\alpha4\beta1$, $\alpha5\beta1$, $\alpha L\beta1$ and $\alpha4\beta7$ [24]. Their expression is dependent on the differentiation state and the site of origin of the B cell [25]. Integrins bind to several extracellular matrix components like fibronectin (FN) and laminin, or to cell surface-expressed members of the Ig superfamilly, including vascular cell adhesion molecule 1 (VCAM1) and intercellular cell adhesion molecules (ICAM1-3). Intracellular signals evoked by cell surface-expressed receptors can lead to integrin activation, a process known as inside-out signaling [26]. We have previously demonstrated that the BCR controls $\alpha4\beta1$-mediated adhesion of B cells to VCAM1 and FN [27,28], which involves cytoskeletal reorganization and integrin clustering [29]. Alternatively, outside-in signaling, i.e. integrin-mediated signals, can influence many biological responses like proliferation, cytokine production, and apoptosis [26,30,31]. In this context, the interaction between B cells and FDCs, which is strengthened by $\alpha L\beta1$/ICAM1 and $\alpha4\beta1$/VCAM1 [24,30], presumably contributes directly to the B cell selection process itself, as signaling through the $\alpha4\beta1$/VCAM1 rescues GC B cells from apoptosis [30,32]. These data clearly show that integrin activation or -mediated cell interactions within the lymphoid tissue microenviroment play a crucial role in Ag-specific B cell differentiation.

1.2 B cells and cancer

B cell malignancies

The development and progression of B cell malignancies is a multi-step process. The initial step in lymphomagenesis is the acquisition of a genetic abnormality, most often a chromosomal translocation involving a proto-oncogene, causing an increased lifespan and/or enhanced proliferation [33]. Chromosomal translocations often arise in the BM, during the rearrangement of the Ig genes of precursor B cells that is required to form a mature BCR. During the GC reaction, SHM and CSR are associated with double-strand DNA breaks, dangerous events that predispose to chromosomal translocations [34-36]. Consequently, the most common chromosomal translocations in B cell malignancies involve the Ig heavy chain (chromosome 14) or light chain gene loci (λ, chromosome 22; κ, chromosome 2), and oncogenes encoding proteins involved in proliferation, cell cycle control, differentiation or survival. These include *c-MYC* in Burkitt's lymphomas, *BCL-1* in mantle cell lymphomas (MCL), *BCL-2* in follicular lymphomas (FL), and *BCL-6* in most of the diffuse large B cell lymphomas (DLBCL) [33,37]. However, most of these chromosomal translocations are not tumorigenic as such and additional genetic alterations, e.g. mutations and loss of tumor suppressor genes, are required for the development of a fully malignant phenotype. Indeed, in general B cell malignancies exhibit multiple genetic abnormalities. A large proportion of DLBCLs (50%) show aberrant SHM, leading to mutations in multiple genes, including proto-oncogenes *c-MYC, PIM-1, PAX-5* and RhoH/*TTF* [38]. It has been demonstrated that both SHM and CSR are dependent on the activity of activation-induced cytidine deaminase (AID), which is a RNA-editing enzyme [39]. Recently, AID has been identified as a DNA mutator in man and has been implicated in the initiation and progression of GC-derived malignancies [40-42].

B cell malignancies are classified by the World Health Organization (WHO) into disease

categories based on histological characteristics, cell surface markers, and cytogenetic- and clinical features [33,37]. A useful parameter to identify the developmental stage of a B cell lymphoma is the variable region sequence of its Ig genes. Lymphomas that lack somatic mutations in the Ig variable chain region genes most likely are derived from pre-GC stage B cells, e.g. MCL. If the somatic mutations in a lymphoma vary among individual lymphoma cells suggesting ongoing SHM, the lymphoma is believed to be derived from GC stage cells, e.g. FL. When the somatic mutations are fixed among the lymphoma cells, the lymphoma is post-GC stage related, e.g. marginal zone B cell lymphoma and multiple myeloma (MM).

Diffuse large B cell lymphoma

DLBCL, which comprise the largest subgroup (30-40%) of B-NHL, particularly display marked phenotypic, molecular and clinical heterogeneity. They show a diverse scale of genetic abnormalities, including translocations, amplifications, deletions and point mutations involving genes controlling proliferation, apoptosis and differentiation [43]. For example, a subgroup (35-40%) shows translocation of *BCL-6* [44]. Furthermore, approximately 25% of the DLBCL cases show translocations of *BCL-2*, leading to abnormal survival of B cells, while amplification of the *Rel* gene, encoding a member of the NFκB/Rel familiy transcription factors, is involved in cell activation and survival [45]. Patients with DLBCL often show single or multiple rapidly enlarging, symptomatic masses at nodal or extranodal sites, e.g. the intestinal tract or central nervous system. By gene-expression profiles, recent studies indeed revealed that DLBCLs are actually a mixture of distinct cancers [46,47]. DLBCLs could be separated into at least 3 subgroups [47], associated with different prognosis. About half of all DLBCLs have a gene-expression profile that closely resembles that of normal GC B cells (GC DLBCLs) [46]. These lymphomas have highly mutated Ig genes and SHM is ongoing in the malignant clones [48]. The second largest subgroup of DLBCLs, representing about 30% of cases, show an expression profile similar to activated B cells. These activated B cell-like DLBCLs (ABC DLBCLs) have a high number of Ig somatic mutations but do not show ungoing SHM [48]. Of note, patients with GC DLBCL have a more favorable clinical course, with a 5-year survival rate of 60% compared with 35% for patients with ABC DLBCL [47]. The third subgroup, the Type 3, reveals an intermediate gene-expression profile and survival rate [47]. The different subgroups suggest that DLBCLs arise through distinct pathogenetic mechanisms and have dissimilar molecular abnormalities.

Multiple myeloma

MM is a lymphoproliferative disorder of post-GC B cells that accounts for approximately 1% of all cancer-related deaths in Western countries [49]. This plasma cell tumor is incurable and represents the second most common haematological malignancy with an annual incidence of approximately 5 per 100.000 inhabitants. Each year, there are 19.000 new cases and over 14.000 deaths in the European Community States. MM is preceded by a pre-malignant expansion of plasma cells called monoclonal gammopathy of undetermined significance (MGUS) (figure 3). MGUS does not grow progressively but is stable and asymptomatic, and is a fairly common disease (3.4% of the population over the age of 50). It has been estimated that 25% of the MGUS patients will finally progress to MM. MM is characterized by the accumulation of malignant plasma cells in the bone marrow (BM) and the aberrant production of Ig, usually monoclonal IgG or IgA. MM is distinguished from MGUS by having a greater intramedullary (i.e. within the BM) tumor cell content (>10%), osteolytic bone lesions and/or an increasing tumor mass. Common clinical features of MM further include susceptibility to bacterial infections, anemia, and renal insufficiency. Almost all of the genetic aberrations identified in MM,

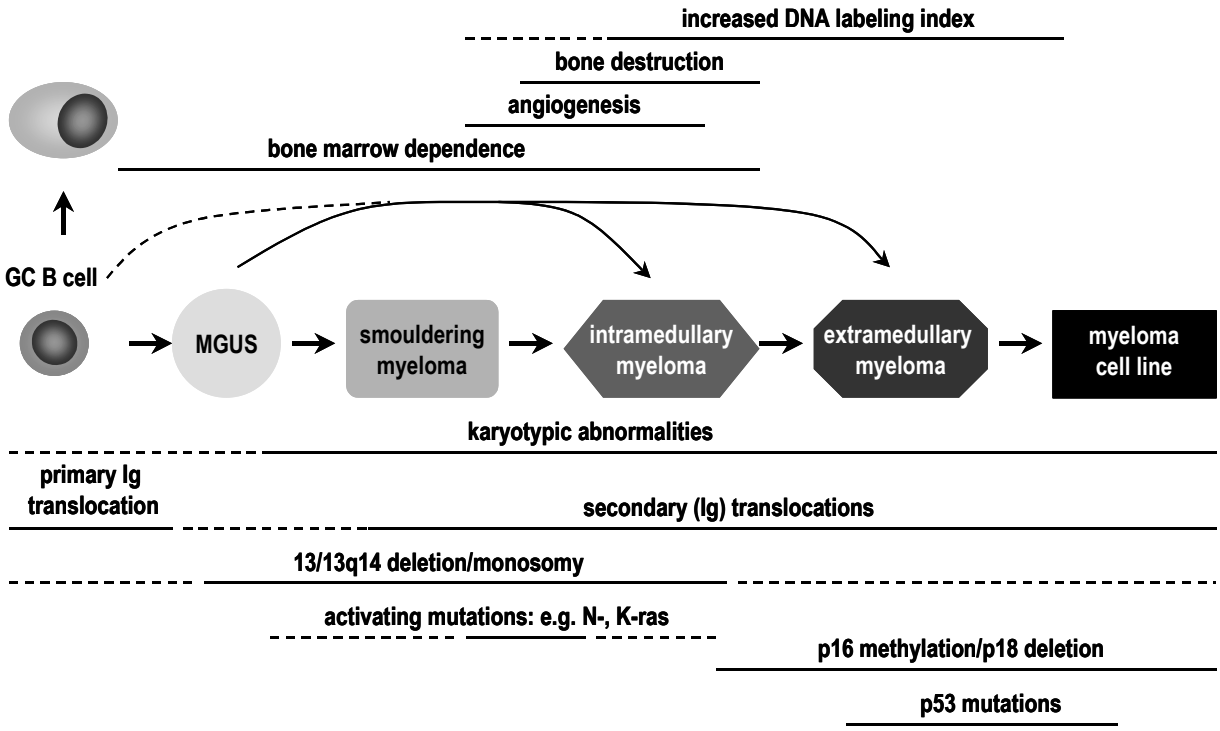


Figure 3 **Model for multi-step molecular pathogenesis of multiple myeloma (MM).**

Defined stages of MM pathogenesis are depicted with arrows indicating potential pathways. Several clinical features and oncogenic changes are depicted as horizontal lines, dashed regions reflecting the uncertainty at which time these changes occur. GC= germinal center; Ig= immunoglobulin; MGUS= monoclonal gammopathy of undetermined significance.

including aneuploidy, monosomy 13, and 14q32 chromosome translocations, are also present in MGUS [50]. Although, genetic changes are similar in MGUS and MM, the latter is distinguished by the presence of activating mutations of *N-* or *K-RAS*, and also by a higher incidence of monosomy 13, indicating a possible tumor suppressor gene on chromosome 13. *RAS* mutations occur in approximately 30-40% of early MM, and the frequency of *RAS* mutations is increased during disease progression [51]. Translocations in MM cells often fuse the Ig heavy chain locus to *cyclin D1*, *cyclin D3* [52,53], *c-maf*, fibroblast growth factor receptor-3 (*FGFR-3*), multiple myeloma SET domain *(MMSET)*, interferon regulatory factor-4 (*IRF-4*), or *MYC* genes. Furthermore, there is evidence that the retinoblastoma (Rb)/cyclin D pathway is disrupted in MM by inactivation of *Rb*, *p15*, *p16* and *p18*, which may contribute to dysregulation of cell cycle control.

MM and the microenviroment

Since MM cells only grow within the confines of the bone [54], MM cells appear to be critically dependent on factors that are present in the BM microenvironment. The BM provides a unique environment consisting of paracrine interactions between the tumor cells and other cells, including fibroblasts, osteoblasts and osteoclasts. A fine network of cytokines and growth factors in the BM microenviroment supports the growth and survival of MM cells (figure 4). These soluble factors are secreted primarily by BM stromal cells (BMSC) and MM cells themselves, and include IL-6, IL-10, IL-

15, IL-21, tumor necrosis factor (TNF), receptor activator of nuclear factor κB ligand (RANKL), stromal derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) [51]. Like in different B lymphomas [55-57], increased production of several cytokines and growth factors is detected in MM, which correlate with the progression of the disease [58-61]. These cytokines and growth factors can activate distinct signaling pathways both in MM cells as well as stromal cells. These pathways include extracellular signal-related kinase (ERK), phosphatidylinositol-3 kinase (PI3K)/AKT, Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3), and nuclear factor κB (NFκB) signaling cascades, involved in proliferation, cell cycle control, anti-apoptosis and migration. Notably, in BMSCs, activation of the NFκB signaling pathway results in the upregulation of the adhesion molecules ICAM1 and VCAM1, thereby further enhancing the binding of MM cells to BMSCs [51]. Binding of MM cells to BMSC triggers transcription and secretion of cytokines, including IL-6, IGF-1, SDF-1, and VEGF [51].

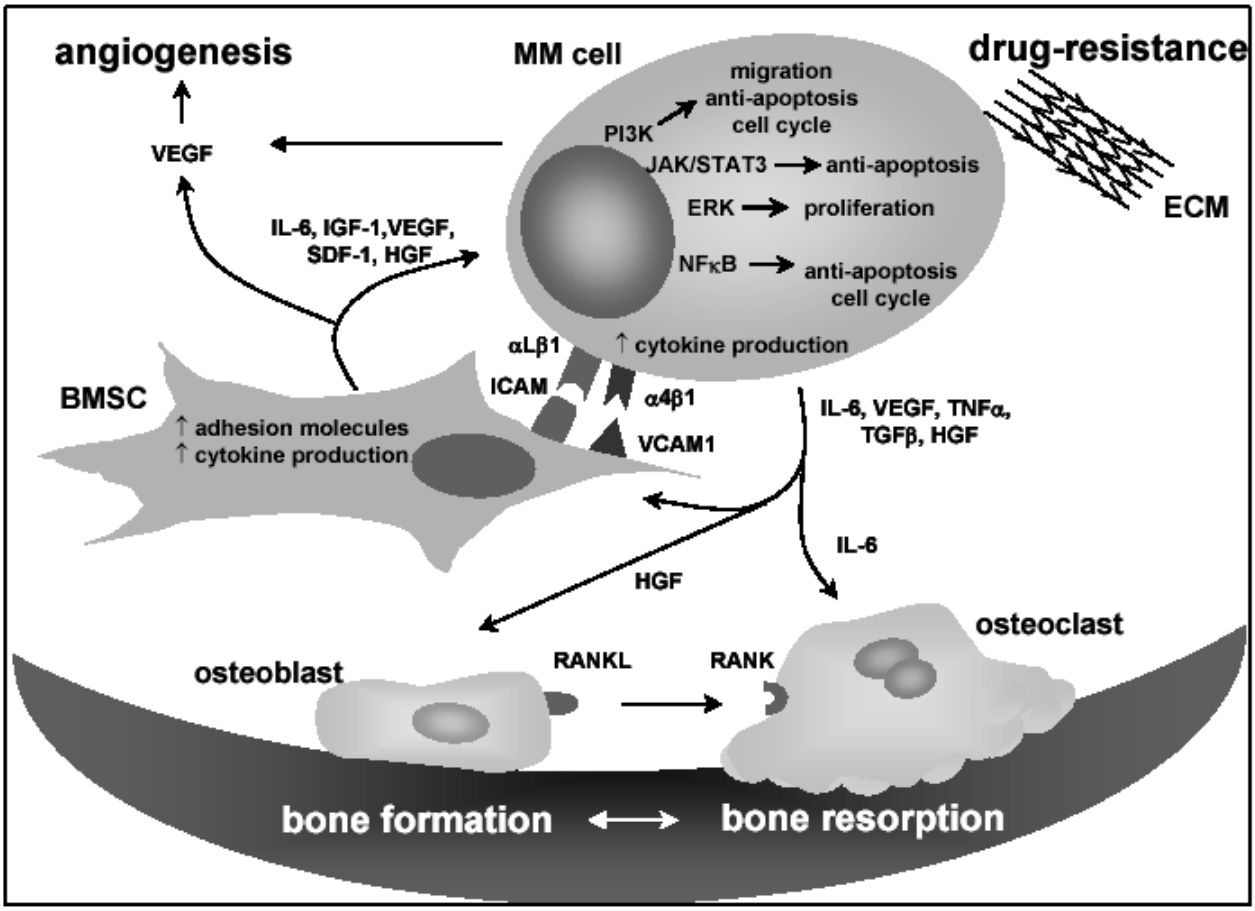


Figure 4 **Interaction of MM cells and their microenvironment.**

Binding of MM cells to bone marrow stromal cells (BMSC) triggers both adhesion- and cytokine-mediated MM cell growth, survival, migration and drug resistance. MM cell binding to BMSCs upregulates cytokines (e.g. IL-6, IGF-1, VEGF, SDF-1) secretion from both BMSCs and MM cells. These cytokines subsequently activate 3 major signaling pathways (ERK, JAK/STAT3, PI3K/AKT) and their downstream targets in MM cells. Adhesion-mediated activation of NFκB upregulates adhesion molecules ICAM1 and VCAM1 on both MM cells and BMSCs, further enhancing adhesion of MM cells to BMSCs.

Some of these cytokines were shown to induce secretion of other cytokines that promote MM growth and survival (e.g. SDF-1 induces IL-6 secretion), stimulate angiogenesis in the BM (e.g. VEGF) or protect MM cells against drug-induced apoptosis (e.g. IL-6, IGF-1) [51]. Moreover, adhesion of MM cells to BMSCs (e.g. $\alpha 4\beta 1$ to VCAM1) or extracellular matrix (ECM) proteins not only localizes tumor cells in the BM microenvironment but also contributes to cell adhesion-mediated drug resistance [51]. Taken together, these findings clearly show that the microenvironment, by providing growth- and survival factors, and mediating drug resistance, is indispensable for the development of the tumor. It is conceivable that B cells that have acquired genetic aberrations may become fully malignant through interaction with their microenvironment. The consequent massive tumor cell expansion may result in the gain of additional aberrations, which further promote cell transformation. Therefore, recent studies are focussing on gaining a better understanding of the pathogenesis of B cell malignancies, by defining the tumor host interaction, in order to identify novel molecular targets. Targeting both MM cells and the interaction of MM cells with the BM microenvironment has already provided some very promising novel therapeutic strategies and results [51]. This thesis will focuss on the role of two types of growth factors, i.e. hepatocyte growth factor (HGF) and WNTs, and their corresponding signaling pathways, in the pathogenesis of B cell malignancies.

2 The hepatocyte growth factor/MET signaling pathway

2.1 Hepatocyte growth factor and MET

Hepatocyte growth factor (HGF)

HGF is a pleiotrophic growth factor with a domain structure and a proteolytic mechanism of activation similar to that of the blood serine protease plasminogen. It was initially identified as a growth factor for hepatocytes [62]. Independently, it was isolated as a secreted product of fibroblasts that dissociates epithelial cells and increases their motility, designated scatter factor (SF). Genomic studies revealed that the human *HGF* is encoded by a single gene localized on 7q21.1 [63]. The gene spans about 70 kb of DNA and contains 18 exons [64]. The promoter region contains a number of regulatory sequences, including a TATA-like element, an IL-6 responsive element (IL-6RE) and a potential binding site for nuclear factor-IL-6, a regulator of IL-6 expression. The full-length human *HGF* cDNA encodes a protein of 728 amino acids. HGF is a heterodimeric protein consisting of a heavy chain and a light chain held by a disulfide bond. The α-chain consists of a putative hairpin loop and four triple-disulfide structures called kringle domains (NK4). The β-chain has homology to the catalytic domain of serine proteases but lacks enzymatic activity [65]. Nothern blotting revealed three *HGF* mRNA transcripts of 6, 3 and 1.5 kb. The 6 and 3 kb messages originate from differential polyadenylation [66], whereas the 1.5 kb mRNA represents a splice variant encoding the N-terminal domain of HGF in combination with the first two kringle domains [67,68]. This variant, NK2, acts as a HGF antagonist [67]. Subsequently, a kringle domain variant, NK1, has been described. This variant functions as a partial HGF agonist[69] (figure 5).

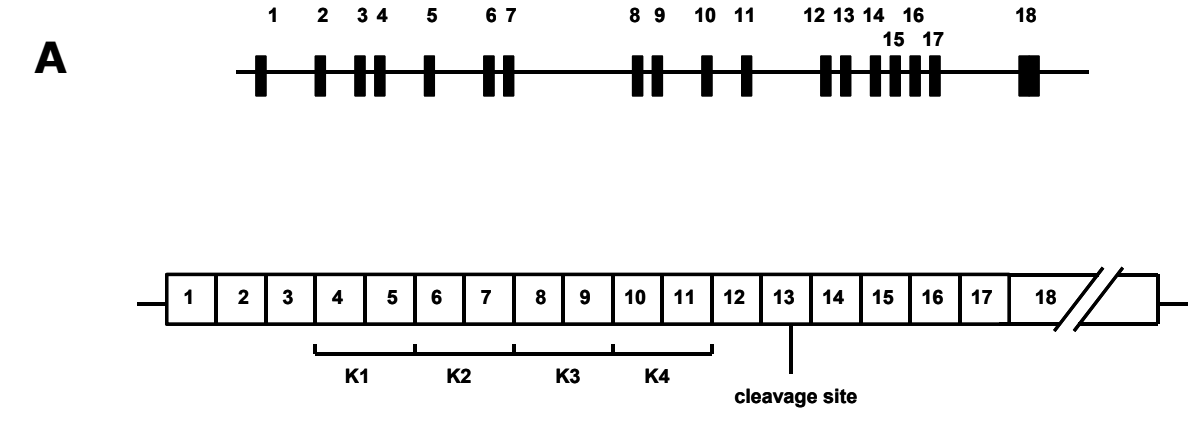
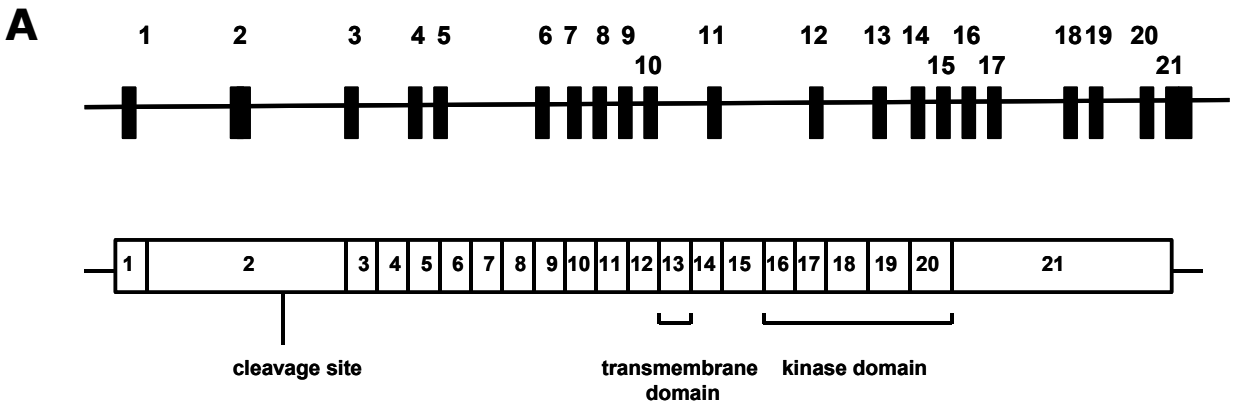


Figure 5 **Schematic representation of HGF.**
A) Structural organization of HGF. Shown are the genomic organization (top) and the intron/exon boundaries and functional domains (bottom). HGF contains 18 exons that are numbered accordingly. The four-kringle domains (K1-K4) and the cleavage site are indicated. B) Schematic representation of the functional domains of HGF. The α-chain contains the N-terminal domain, which harbors the hairpin loop and the four-kringle domains. The β-chain contains the catalytically inactive serine protease domain. The 60 kDa α- and the 30 kDa β-chain are linked via a disulphide bond (S-S).



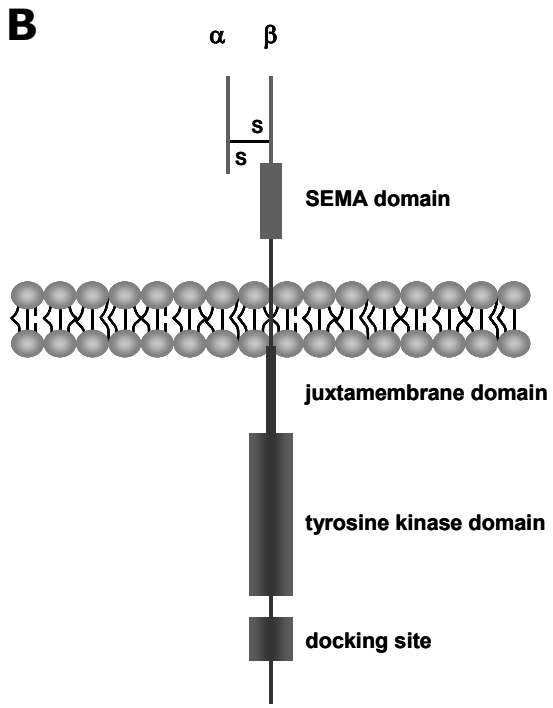


Figure 6 **Schematic representation of the receptor tyrosine kinase MET.**

A) Structural organization of MET. Shown are the genomic organization (top) and the intron/exon boundaries and functional domains (bottom). MET contains 21 exons coding for an extracellular-, transmembrane- and tyrosine kinase domain. B) Schematic representation of MET. The receptor is composed of two disulphide (S-S)-linked chains: a 50 kDa α-chain, and a 145 kDa β-chain that are formed by proteolytic processing. The extracellular region of MET contains a 500 amino acids encompassing SEMA-domain. The intracellular domains harbors four key tyrosine residues, either regulating enzymatic activity, or forming docking sites for several signal transducers. See text for further detail.

HGF is synthesized and secreted as a 90-kDa inactive single-chain (sc) precursor (pro-HGF). The precursor can bind to the HGF receptor MET, but with low affinity and without triggering receptor kinase activity [70,71]. To activate MET and elicit biological responses, HGF has to be activated by proteolytic cleavage at an Arg-Val site located in exon 13. This activation will be discussed later in more detail.

The receptor tyrosine kinase MET

The receptor for HGF is the receptor tyrosine kinase MET. The MET proto-oncogene was originally identified in a chromosomal translocation that generated the Tpr-MET fusion gene in cultured tumor cells treated with a chemical carcinogen [72,73]. The human *MET* gene is located on chromosome 7q21-q31, and harbors 21 exons, spanning more than 120 kilobases. The sequence of the *MET* promoter region contains a number of binding sites for regulatory elements, including AP1, AP2, NFκB and, like the *HGF* gene, the IL-6RE [74]. The *MET* gene encodes a 170-kDa-precursor protein, which is further glycosylated and cleaved into a 190-kDa heterodimer, consisting of a 50-kDa α– and a 140-kDa β-chain linked via disulfide bonds [75]. The extracellular part of the β-chain contains a SEMA domain to which semaphorin-type proteins can bind [76], which mediate cell

scattering, a phenomenon that is also transduced by MET. Recent findings have demonstrated that the SEMA domain of MET is necessary for ligand binding and receptor dimerization [77], suggesting an important role in receptor activation. The cytoplasmic tail contains a tyrosine kinase domain as well as a multifunctional docking site, which interacts with various signaling molecules [78] (figure 6).

2.2 Expression and function of HGF/MET

Expression and function of HGF/MET in mammalian development

MET and *HGF* are expressed 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, the limb bud- and the villous core mesenchyme, whereas MET is present on the trophoblast, vascular endothelium and myogenic precursor cells [79-82]. The absolute dependence of placenta and liver maturation on HGF has been clearly shown in *HGF* null mutant mice, which show embryonic lethality due to placenta and liver defects [83,84]. From week 6-13 of human gestation, when major organogenesis takes place, *HGF* and *MET* are co-expressed in liver, metanephric kidney, intestine, lung, gall bladder and spleen [85,86]. In the digestive tract of 7-8 week old embryos, *MET* is localized in epithelia of the liver, pancreas, esophagus, stomach, small and large intestine, and in smooth muscle layers, whereas HGF is concentrated in mesenchymal tissue and smooth muscle [87]. *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 [86]. In conclusion, expression of HGF/MET is found in a wide variety of organs and its expression pattern changes during development. Most important, HGF/MET plays a vital role in embryogenesis.

Expression and function of HGF/MET in haematopoiesis and B cell differentiation

HGF/MET has also been implicated in haematopoiesis. Both HGF and MET are expressed in the yolk sac of the chicken embryo [88], and in the human and rodent fetal liver, primordial sites of haematopoeisis [89]. In the adult haematopoietic microenvironment, MET is expressed by a subset of haematopoietic precursor cells (HPC), whereas HGF is expressed by BMSCs, suggesting that HGF functions as a paracrine growth factor [90-92]. It has been shown that HGF promotes differentiation and proliferation of HPCs. In the presence of IL-3, HGF stimulates the formation from $CD34^+$ progenitors of burst forming units erythroid, as well as colony forming units granulocyte macrophage, but not colony forming units granulocyte monocyte. Furthermore, HGF stimulation of $CD34^+$ cells leads to integrin-induced survival of haematopoietic cells [92].

In B cells, MET is predominantly expressed on $CD38^+CD77^+$ tonsillar B cells (centroblasts) localized in the dark zone of the GC and on plasma cells (this thesis). Expression of MET on tonsillar B cells can be transiently upregulated by concurrent CD40 and BCR stimulation [28]. Upregulation of MET may lead to enhanced adhesion, since HGF induces integrin β1-mediated adhesion of B cells to both VCAM-1 and fibronectin [28]. These data imply a role for the HGF/MET pathway in haematopoietic cells, and during antigen-dependent B cell differentiation.

2.3 HGF/MET signaling cascade

Activation of HGF/MET signaling requires phosphorylation of multiple residues on MET. Phosphorylation of the tyrosine residues Y1230, Y1234 and Y1235 located within the activation loop of the tyrosine kinase domain activates the intrinsic kinase activity of MET, while auto-phosphorylation of the tyrosine residues Y1349 and Y1356 of MET induces most of the biological responses. Mutational analysis of the multisubstrate docking site of

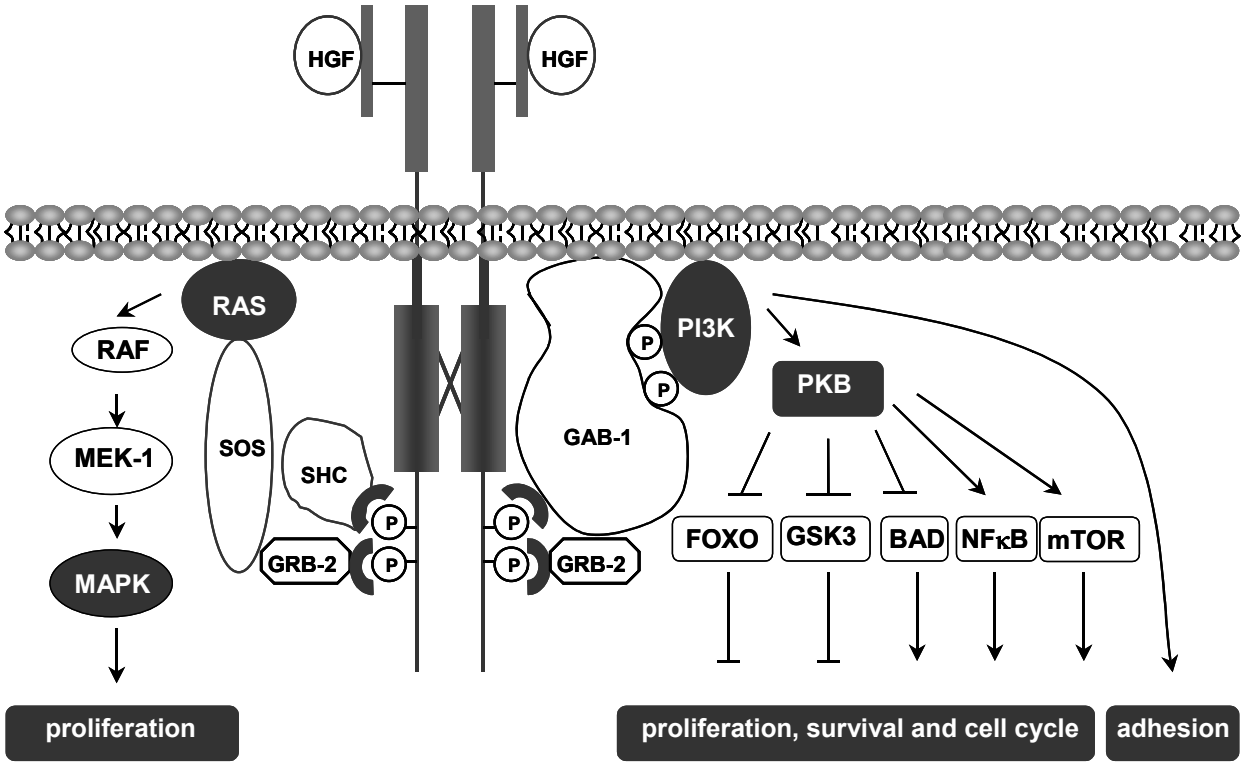


Figure 7 **The HGF/MET signaling pathway.**

Schematic representation of HGF-induced signaling. For reasons of clarity, only the RAS/MAPK, the PI3K/PKB pathway, and several additional important signal transducers are shown. Arrows represent activation, blunted arrows point out inhibition. See text for explanation of abbreviations and further details.

MET revealed that Y1349 and Y1356 mediate the interactions with SHC, SRC and GAB-1, while recruitment of GRB-2, PI3K, PLCγ and SHP2 is mediated by Y1356 [78,93,94]. Activation of the HGF/MET pathway stimulates cell growth, survival, adhesion and migration (figure 7).

HGF/MET signaling via RAS/MAPK pathway

The RAS/mitogen activated protein kinase (MAPK) pathway is triggered after activation of MET and subsequent binding of GRB-2 to MET [78]. GRB-2 is an adaptor protein consisting of one SRC homology (SH)2 and two SH3 domains, and is constitutively associated with the RAS exchange factor, SOS, through its SH3 domain. Binding of GRB-2 to MET translocates SOS to the plasma membrane where RAS is located. As a consequence, RAS will undergo transition from a GDP- to a GTP-bound state, thereby activating downstream effector molecules such as PI3K, RalGDS and RAF-1 [95]. The serine/threonine kinase RAF-1 can phosphorylate and activate MEK, resulting in the phosphorylation and activation of downstream MAPKs ERK1 and-2. Activation of these MAPKs leads to phosphorylation of transcription factors like ELK1 and ETS2, which mediates the expression of immediate early genes such as *FOS*, leading to cell proliferation [96]. The RAS/MAPK pathway has been implicated in a wide variety of biological responses, including differentiation, proliferation and apoptosis.

HGF/MET signaling via PI3K/PKB pathway

Activation of protein kinase B (PKB)/AKT by HGF involves recruitment of PI3K to the docking site of MET, either by a direct interaction, or indirectly by binding to the

docking protein GAB-1 [97,98]. Upon PI3K-dependent membrane localization, PKB is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK1) [99]. PKB controls anti-apoptosis by means of indirect effects on two regulators, i.e. nuclear factor of κB (NFκB) and p53 [99]. PKB excerts its survival effects via NFκB by phosphorylation and activation of IκB kinase (IKK), a kinase that induces degradation of the NFκB inhibitor IκB. Furthermore, PKB influences the activity of the pro-apoptotic tumor suppressor p53, through phosphorylation of the p53-binding protein MDM2. The downstream effector molecules that mediate pro-apoptotic signals, including the BCL-2 family member BAD and caspase 9, are inactivated through phosphorylation by PKB. PKB-induced phosphorylation also inhibits transcriptional activity of forkhead transcription factors, including FOXO4 (AFX), FOXO1 (FKHR) and FOXO3a (FKHRL1). This phosphorylation prevents their nuclear translocation and thereby the expression of FOXO target genes, including the pro-apoptotic genes *FasL* and *Bim*, and anti-proliferative genes like $p27^{KIP}$, *Rb2* and *cyclin D1/2*. In addition, FOXOs were shown to enhance transcription of *BCL-6*, which act as a transcriptional repressor of the anti-apoptotic gene $BCL\text{-}X_L$ [100]. PKB can further affect proliferation via the cell cycle machinery, including phosphorylation of glycogen synthase kinase-3 (GSK3), mammalian target of rapamycin (mTOR) and FOXOs [101]. In conclusion, the PI3-K/PKB pathway is an important regulatory pathway in HGF/MET-induced proliferative and anti-apoptotic responses.

2.4 Regulation of HGF activity

Activation of HGF by serine proteases

Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling [102]. To date, seven proteases with a potential role in the activation of HGF have been identified (table 1). These include plasma kallikrein [103], matriptase [104], plasminogen activators of urokinase-type (uPA) [105] and tissue-type (tPA) [106], blood coagulation factors XIa (FXIa) [103], XIIa (FXIIa) [107] and HGF activator (HGFA) [107,108]. Activation of HGF requires proteolytic cleavage at the Arg^{494}-Val^{495} peptide bond to convert the sc-HGF (pro-HGF) into the heterodimeric form, consisting of a 60-kDa α-subunit and a 30-kDa β-subunit [102]. In contrast to the other activators, plasma

Table 1 **Serine proteases with HGF-converting activity**

Enzyme	*activity* (μg/ml)*	*activator*	*endogenous inhibitors*
HGFA	0.02	thrombin	HAI-1, HAI-2
Factor XIIa	0.7	HW kininogen, plasma kallikrein	antithrombin III, C1-inhibitor, α2-antiplasmin
Matriptase	(>0.5)	?	HAI-1
Factor XIa	NA	HW kininogen, factor XIIa	HAI-2
Plasma kallikrein	NA	?	HAI-2
uPA	> 100	plasma kallikrein, factor XIIa, matriptase	PAI-1, protease nexin I
tPA	> 100	tissue kallikrein	PAI-1, PAI-2

* Concentration required for the activation of 50% of 200 μg/ml scHGF in 2 hours.

UPA: urokinase-type plasminogen activator; tPA: tissue-type plasminogen activator; HAI-1: HGFA inhibitor type 1; HAI-2: HGFA inhibitor type 2; PAI-1: plasminogen activator inhibitor type 1; PAI-2: plasminogen activator inhibitor type 2. HW= high molecular weight. NA= not available. *(References: Kataoke et al, Human Cell, Vol. 14, No. 1, 2001; Kataoka et al, Pathol Int; 52:89-102, 2002; Ichinose, J Biol Chem, Vol. 261, No.8 1986)*

kallikrein and FXIa also process pro-HGF at a second cleavage site, i.e. Arg^{424}-His^{425}, located in the K4 domain of the α-chain [103]. This results in an extra α-subunit of 50 kDa. Although, cleavage at the second site of HGF had no functional consequences for the activation of MET [103], the unusual cleavage might elicit other, yet unknown effects.

Previously, uPA, a serine protease known to convert plasminogen into plasmin, was believed to represent a major cellular activator of pro-HGF [105]. However, HGF conversion by uPA and tPA is very inefficient and shows the kinetics of a stoichiometric rather than a catalytic reaction [109]. Therefore, the activation of pro-HGF by uPA or tPA is only conceivable in environments where high concentration of uPA or tPA are available. Interestingly, upregulation of uPA and its receptor have been found in injured tissues and in many tumors [110], suggesting that it might play a role in the activation of pro-HGF during tissue repair and in cancer. The HGF-converting enzyme, matriptase, which is a membrane-type serine protease, is exclusively expressed on epithelial cells [104] and can also activate protease-activated receptor (PAR), as well as uPA [111]. This indicates that matriptase is an epithelial membrane localized activator for different proteases and growth factors.

The serine proteases FXII and its relative HGFA circulate in the plasma as inactive zymogens [107]. These zymogens are converted to fully functional proteases by limited proteolysis after activation of the intrinsic and/or extrinsic blood coagulation pathways, which are initiated by damage to the vessel wall or by tissue injury, respectively. Both pathways converge to a common pathway, i.e. factor X, leading to activation of thrombin and clot formation. FXII, which is linked to the intrinsic pathway, is activated by the high-molecular weight kininogen and plasma kallikrein [112], whereas HGFA is specifically activated by thrombin [113]. Interestingly, whereas the serum protease inhibitors antithrombin III, C1-inhibitor and α2-antiplasmin can inhibit the blood clotting- and HGF-converting activity of FXIIa, the HGF-converting activity of HGFA is unaffected [107]. This suggests that the active form of HGFA is not receptive for serum protein inhibitors. Compared to the other serine proteases, FXIIa and HGFA activate HGF more efficiently. Both serine proteases follow classic enzyme kinetics and cleave pre-HGF at enzyme:substrate ratios of <1:1000 [107]. However, the specific activity of FXIIa is lower than that of HGFA [107], showing that among the other serine proteases, HGFA is the most potent activator of HGF [107,114]. Taken together, these findings show that serine proteases involved in the blood coagulation pathway as well in tissue injury can activate HGF, but that HGFA is by far the most potent activator.

Structure of HGFA

HGFA was originally discovered in fetal bovine serum [115]. Human HGFA was purified from serum with a concentration of approximately $8x10^{-5}$ gram/liter [116]. The complete sequence of the *HGFA* gene covers about 7,5 kb of DNA, consisting of 14 exons. The nucleotide sequence of the *HGFA* precursor shares 39% homology with the blood coagulation factor XII, including several characteristic structural domains: a type-II fibronectin homology region (FnII), two epidermal growth factor (EGF) domains, a type-I fibronectin homology region (FnI), a kringle domain and a catalytic domain (figure 8). Although, the structural similarity suggests that the *HGFA* and *FXII* genes are derived from a common ancestral gene, the genes are mapped on 4p16 [114] and 5q33 [117], respectively.

HGFA is secreted by the liver and by injured tissues [108,118], as a 98-kDa inactive zymogen (pro-HGFA) in the blood [113] (figure 9). Activation of HGFA requires cleavage at the bond between Arg^{407} and Ile^{408} by thrombin, resulting in two fragments of 66-kDa and 34-kDa, linked by a disulfide bond. The 66-kDa fragment represents the inactive NH_2-terminal region and is possibly involved in binding HGFA to the cell surface. The 34-kDa fragment, composed of the COOH-terminal region, represents the active form of HGFA

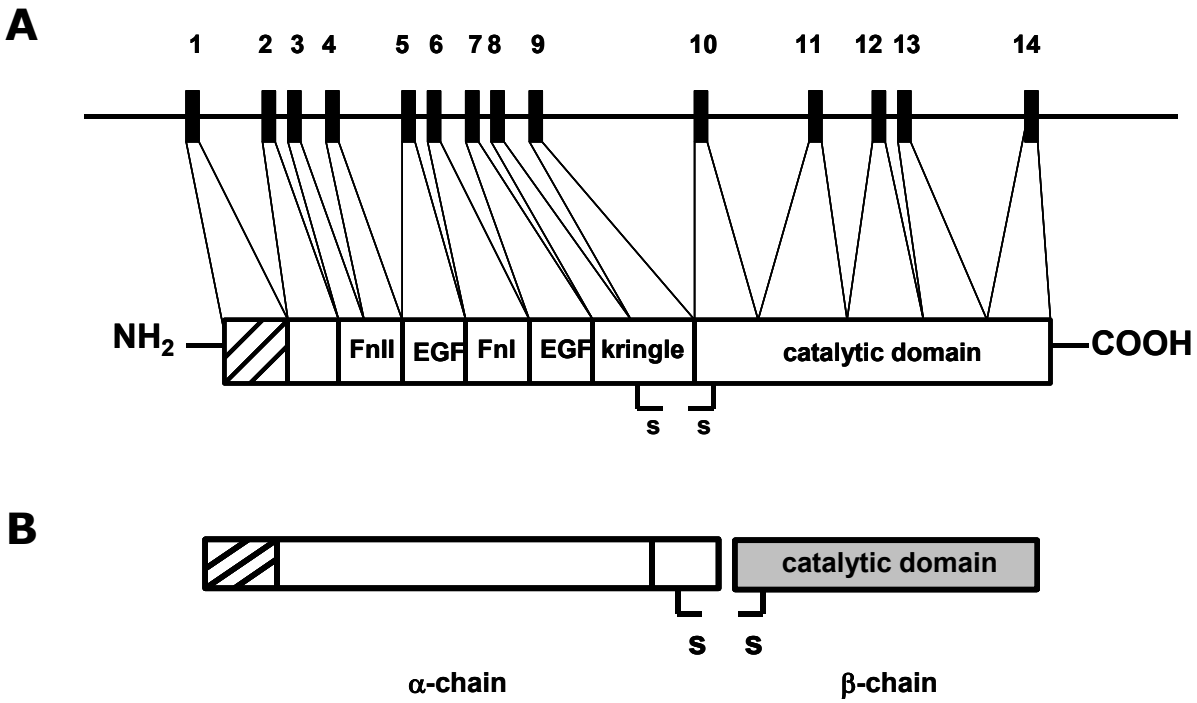


Figure 8 **Schematic representation of HGF activator (HGFA).**

A) Structural organization of HGFA. Shown are the genomic organization (top) and the intron/exon boundaries and functional domains (bottom). HGFA contains 14 exons. The hatched box represents the putative signal peptide. B) Schematic representation of HGFA. HGFA is composed of two disulphide (S-S)-linked chains: a 66 kDa α-chain, and a 34 kDa β-chain that are formed by proteolytic processing. See text for explanation of abbrevations and further details.

necessary for the activation of HGF [113]. In the absence of negative charged surfaces, thrombin can also cleave pro-HGFA at the Arg^{88}-Ala^{89} site [113] located in the inactive NH_2-region. This results in an extra band of 41-45-kDa. Furthermore, plasma kallikrein is able to cleave pro-HGFA at the bond between Arg^{372}-Val^{373} [113], generating an inactive 34-kDa form. These additional cleavages have no functional consequences for the activity of HGFA [113] (figure 10).

It has been suggested that HGFA needs to bind to the cell surface in order to be activated by thrombin. However, binding of pro-HGFA to the cell surface, presumably to heparan sulfate proteoglycan (HSPG) moieties [108], appears to be of low affinity, resulting in free diffusion of inactive HGFA. Interestingly, however, activation of HGFA results in a strongly increased heparin-binding capacity [113], suggesting that the active form of HGFA may bind to HSPGs on the cell surface where it can capture and activate HGF, which is also a HSPG-binding protein. Immunocytochemical stainings indeed revealed that the active forms of HGFA (34- and 96-kDa) are present on the cell surface [119]. The HSPG binding capacity of HGFA will locally restrain the activity of HGFA.

Expression and function of HGFA

Although the HGFA in the serum is believed to be largely produced by the liver [116], expression is also found in a variety of normal and pathological cell types/tissues, including white matter astrocytes and cells of the brain [120,121], uretic bud epithelial cells [122], normal intestinal mucosa, adenomas and carcinomas of the colorectum [123], normal and malignant breast cells [124] and a subset of B cells (this thesis).

Interestingly, HGF has been implicated in

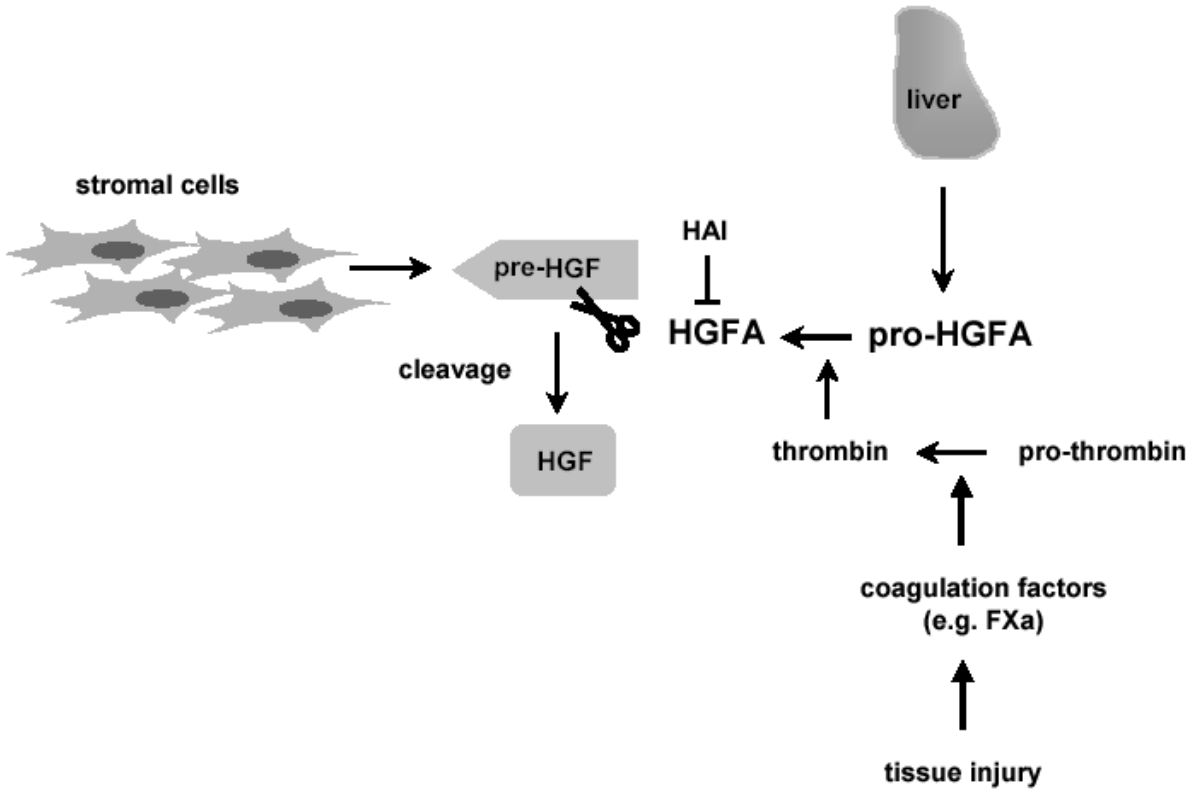


Figure 9 **Schematic representation of the activation of HGFA and HGF.**

Stromal fibroblasts synthesize and secrete pre-HGF. Pro-HGFA produced by the liver or derived form plasma can be activated by thrombin and/or unknown proteases. Upon activation, HGFA acquires the ability to efficiently activate pre-HGF.

the regeneration of damaged tissues [125,126]. Tissue injury is associated with strongly increased HGF activation [127]. Kitamura and colleagues demonstrated that activation of HGF by injured tissue-derived homogenates is completely dependent on HGFA [108], indicating a major role for HGFA in injured tissues. Recently, Kataoka and colleagues have established the biological relevance of HGFA *in vivo* by generating *HGFA*-deficient mice [128]. In contrast to *HGF*-deficient mice, which show embryonic lethality due to liver and placental defects [83,84], *HGFA*-deficient mice are viable and fertile without obvious abnormalities [128]. However, these mice showed delayed regeneration of injured intestinal mucosa. Since activation of HGF by HGFA was completely lost, proteases other than HGFA must have been responsible for the HGF activation required for the normal development of these mice. These findings show that HGFA is most likely the key enzyme involved in injured tissues but not essential for normal development during embryogenesis or after birth.

Inhibiton of HGFA by HGFA inhibitor (HAI)

Recently, two serine protease inhibitors of HGFA were purified from the conditioned medium of the human stomach cancer cell line MKN-45. These inhibitors were designated HGFA inhibitor type 1 (HAI-1) and type 2 (HAI-2) [129,130]. The genes of *HAI-1* and *HAI-2* are located on different chromosomes, 15q15 and 19q13.11, respectively [131]. Both inhibitors contain two Kunitz-type domains, originally discovered in the bovine pancreatic trypsin

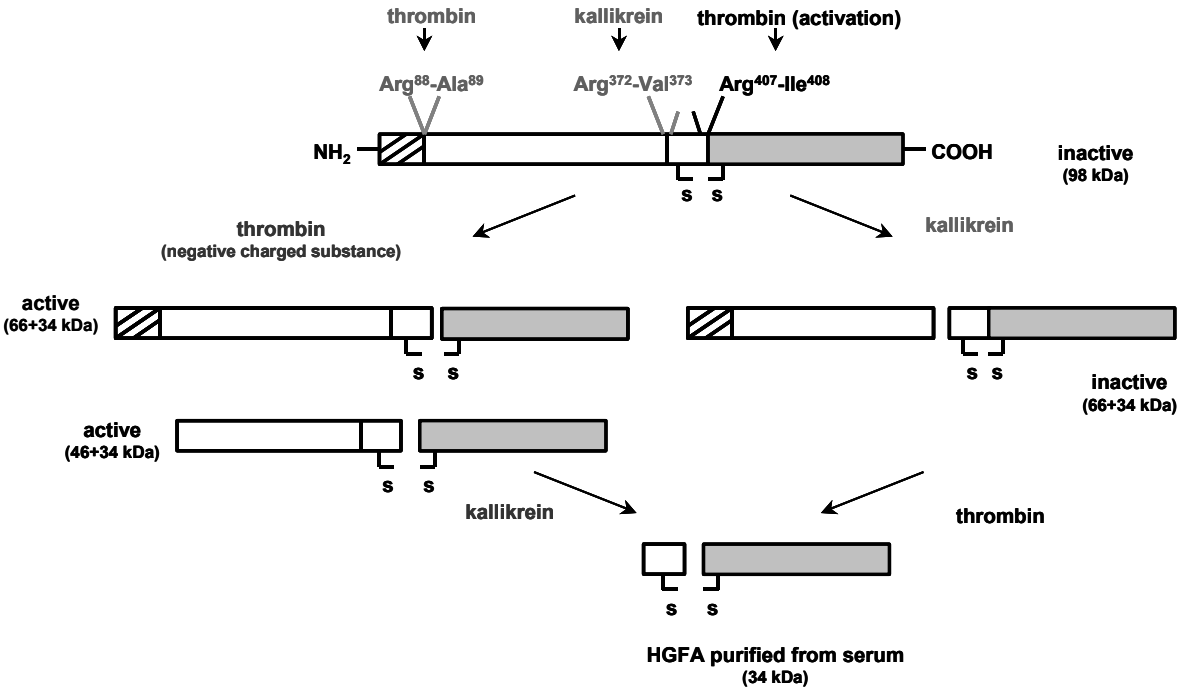


Figure 10 **Schematic representation of conversion of HGFA precursor by limited proteolysis.**

Activation of HGFA requires cleavage at the bond between Arg^{407} and Ile^{408} by thrombin, resulting in two fragments of 66-kDa and 34-kDa, linked by a disulfide bond. The 34-kDa fragment represents the active form of HGFA necessary for the activation of HGF. Additional cleavages by thrombin and plasma kallikrein are shown in grey. See text for further detail.

inhibitor and responsible for the inhibitory activity [132], and a single putative transmembrane domain. HAI-1 and HAI-2 show great structural homology but the low-density lipoprotein (LDL) receptor-like domain in HAI-1 is lacking in HAI-2 [129], suggesting distinctive functions between the HAIs.

HAI-1 is an integral-membrane protein of 66-kDa that, after processing, results into two secreted forms of 40- and 58-kDa. The 40-kDa form has one Kunitz domain and shows strong HGFA inhibitory activity, whereas the 58-kDa form, containing two Kunitz domains, shows very weak inhibitory activity [133]. Expression of HAI-1 is found in the normal and malignant colon mucosa, small intestine, liver, lung, placenta, pancreas, prostate, kidney and in breast carcinomas [134]. Studies have shown that membrane bound form of HAI-1 can function as a receptor for HGFA, localizing the active form of HGFA to the cell surface [119]. The interaction between HAI-1 and HGFA is reversible, suggesting a regulatory role for HAI-1 in HGFA activity. HAI-2 is a membrane-associated protein with a mature form of 14-kDa, which shows potent HGFA inhibitory activity [130,135]. HAI-2 is ubiquitously expressed in human tissues. However, in contrast to HAI-1, HAI-2 is not upregulated during regeneration of damaged mucosa [136]. Unlike the *HGFA*-deficient mice, disruption of the *HAI-1 or HAI-2* gene resulted in embryonic lethality in mice [128,137], showing a crucial role for both HAIs during embryonic development. This may be related to the fact that both HAIs can inhibit other serine proteases than HGFA. These data demonstrate the complexity of HAI in the regulation of HGFA activity.

2.5 Dysregulation of the HGF/MET signaling pathway

Aberrant MET signaling in cancer is common and can be caused by gene amplification, increased transcription/translation, or mutations in *MET* that promote auto-phosphorylation [138-142]. Most of the mutations are located within the tyrosine kinase domain [142]. These mutations, which result in enhanced kinase activity, mediate transformation, invasive growth and protection from apoptosis [76,143,144]. Missense mutations have been described in sporadic- and hereditary papillary renal carcinoma (HPRC) [145-148], hepatocellular carcinoma [149], and gastric carcinomas [150].

MET signaling is negatively regulated via at least two distinct mechanisms, i.e. tyrosine dephosphorylation by tyrosine phosphatases [151], and ubiquitination by ubiquitin ligases causing receptor endocytosis and, subsequently, receptor degradation [152]. Impaired deactivation of receptor tyrosine kinases may lead to aberrant signaling. Direct involvement of the JM region in the negative regulation of MET, was established by the identification of several negative regulatory sites in this region. These include a protein kinase C phosphorylation site (Ser 985), which negatively regulates MET kinase activity [153]; a PEST sequence (aa 986-1001), which can confer susceptibility to intracellular proteolysis; a sequence (aa 993-1007) associated with phosphatase activity [154], and a c-Cbl binding site (Y1001) responsible for MET ubiquitination and proteasomal degradation [155,156]. Several studies have shown that the E3 ubiquitin ligase c-Cbl binds to receptor tyrosine kinases, including epidermal growth factor receptor (EGFR)[157], colony stimulating factor-1 receptor (CSF-1R)[158], and platelet derived growth factor receptor (PDGFR)[159] and MET [160] (this thesis). By recruiting the endophilin-CIN85 complex, c-Cbl regulates receptor internalization and consequently, receptor degradation. Mutation in the Y1001 residue leads to a gain-of-function resulting in constitutive scattering and fibroblastoid morphology of epithelial cells [161]. The germline JM mutation P1009S (exon 14), detected in a patient with gastric carcinoma [150], shows increased and persistent phosphorylation after HGF stimulation. Furthermore, this mutation induces increased tumor formation and anchorage independent growth [150]. In contrast, the JM domain mutation T1010I (exon 14), which was found in HPRCC, breast cancer and in a lung cell line, did not show increased constitutive phosphorylation or increased focus formation. However, tumor cells with the T1010I mutation in athymic nude mice developed faster compared to cells with the P1009S mutation [150]. The precise mechanism by which these JM mutants activate the transforming potential of MET remains to be investigated.

Tumor formation via autocrine HGF/MET activation has been demonstrated by the generation of an autocrine signaling loop in nude mice by using MET-expressing human mammary tumor cells that was stably transfected with HGF [162], or by introducing human or mouse MET in NIH-3T3 cells, which express HGF endogenously [140,163]. Interestingly, co-expression of HGF and MET has been described in several primary human tumors [55,164-168], suggesting a role for autocrine activation of the HGF/MET signaling pathway. Taken together, activation of MET without appropiate regulation, can result in aberrant signaling, which is oncogenic and can lead to the initiation or progression of malignancy.

HGF/MET signaling pathway in B cell malignancies

Recent studies, including studies from our laboratory, have implicated the HGF/MET pathway in the development and progression of B cell malignancies [169-171]. Expression of MET has been found in several Burkitt's lymphoma [171] and cell lines [28,169], as well in MM [164,170,172], primary effusion lymphoma (PEL) [165], HL [55] and DLBCL [56,173]. Recently, a gene-profiling study showed significantly enhanced expression of MET upon transformation of low-grade FLs into DLBCLs within the same patient [173]. Furthermore, HGF/MET overexpression was associated with

poor survival of patients with DLBCL [174], suggesting that HGF/MET is a valuable clinical marker of prognosis for patients with DLBCL. High serum HGF levels were reported to correlate with poor prognosis in MM [58], HL [55] and DLBCL [56,57]. Interestingly, simultaneous expression of MET and HGF has been reported for MM [164,172], PEL [165] and HL [55,165], whereas HGF, in addition, can also be produced by (BM) stromal cells [91,175]. This suggests that both paracrine- and autocrine activation of the HGF/MET pathway contributes to tumorigenesis in these B cell malignancies.

Recently, we demonstrated that HGF functions as a potent MM growth- and survival factor [170]. Furthermore, we have obtained evidence that MM cells can further promotethese effects by expressing syndecan-1 (CD138) [176], a proteoglycan rich in heparan sulfate, on their surface. In this way, MM cells can bind and concentrate growth factors on the cell surface for consequential stimulation. In addition, MM cells as well as DLBCL cells express HGFA (this thesis), thereby regulating the bioavailability of active HGF in the tumor microenvironment. These data outline an important role for the regulatory components of the HGF/MET pathway, including HGFA, in the pathogenesis and progression of B cell malignancies.

3 The WNT signaling pathway

3.1 The WNT/β-catenin pathway

WNT proteins

WNTs proteins are a family of 19 cysteine-rich, secreted glycoproteins, which control gene expression, cell growth, motility, and differentiation during both embryonic development and postnatal life [177]. WNT1, the first member of the family, was discovered as a proto-oncogene that induces mammary tumors when overexpressed in mice [178]. WNT ligands act in a paracrine fashion by interacting with Frizzled (FZ) receptors, activating signaling cascades inside target cells. WNTs activate at least three distinct intracellular signaling cascades: the WNT/β-catenin pathway, the WNT/calcium pathway or the WNT/planar polarity pathway. By far the best-defined, and perhaps the most important, pathway is the β-catenin-TCF pathway, which is commonly referred to as the canonical WNT pathway.

The canonical WNT pathway

The canonical WNT pathway affects cellular functions by regulating β-catenin levels and subcellular localization. The key event in this signaling pathway is the stabilization of β-catenin. In the absence of WNT ligands, β-catenin is recruited into a destruction complex that contains the tumor suppressor gene product adenomatous polyposis coli (APC), axin, and glycogen synthase kinase-3β (GSK3β)□. This complex controls phosphorylation of specific serine and threonine residues in the N-terminal region of β-catenin by GSK3β. This GSK3β–mediated phosphorylation leads to the ubiquitination and proteasomal degradation of β-catenin. The binding of WNT to FZ leads to activation of the phosphoprotein dishevelled (DSH/DVL). The activation and membrane recruitment of DSH recruits axin and the destruction complex to the plasma membrane, where axin directly binds to the cytoplasmic tail of LRP5/6. Axin is then degraded, which decreases β-catenin degradation [179]. In addition, activation of DSH also leads to the inhibition of GSK3β. This further reduces the phosphorylation and degradation of β-catenin, resulting in the accumulation of non-phosphorylated β-catenin, which will translocate to the nucleus. In the nucleus, β-catenin interacts with transcription factors of the T-cell factor and lymphoid enhancer-binding protein (TCF/LEF) family to activate the transcription of target genes [180] (figure 11).

Secreted WNT antagonists

Several extracellular and intracellular proteins negatively regulate the canonical WNT signaling pathway. To date, five classes of secreted proteins including Dickkopfs (DKK), frizzled related proteins (sFRP), Cerberus, WNT inhibitory factor-1 (WIF1) and WNT modulator in surface ectoderm (WISE) [181], antagonize vertebrate WNTs and their activities. DKKs and WISE limit the availability of LRP5/6 receptors to WNTs by sequestering LRP5/6 into complexes with Kremens (Krm), and thereby inhibiting signals that emanate from the FZ-LRP complex [182]. sFRP are naturally occuring secreted forms of FZs, which contain the cysteine-rich domain of FZs but have no transmembrane region. They can bind WNT proteins in solution, prevent their association with LRP and FZ receptors, and thereby change the activity of WNTs [181]. Like sFRP, Cerberus and WIF-1 also bind to WNTs to interfere with interaction with FZ receptors. However, data about Cereberus and WISE are limited and are predominantly derived from the *Xenopus* model. Interestingly, DKK, sFRP and WIF1 contribute to regulation of WNT signaling in bone formation and disease including MM [183-185], which will be discussed later.

3.2 WNT signaling and cancer

It is now well established that unrestrained β-catenin/TCF activity plays a major role in many human cancers [180,186]. WNT signal transduction components, in particular *adenomatous polyposis coli* (*APC*) and

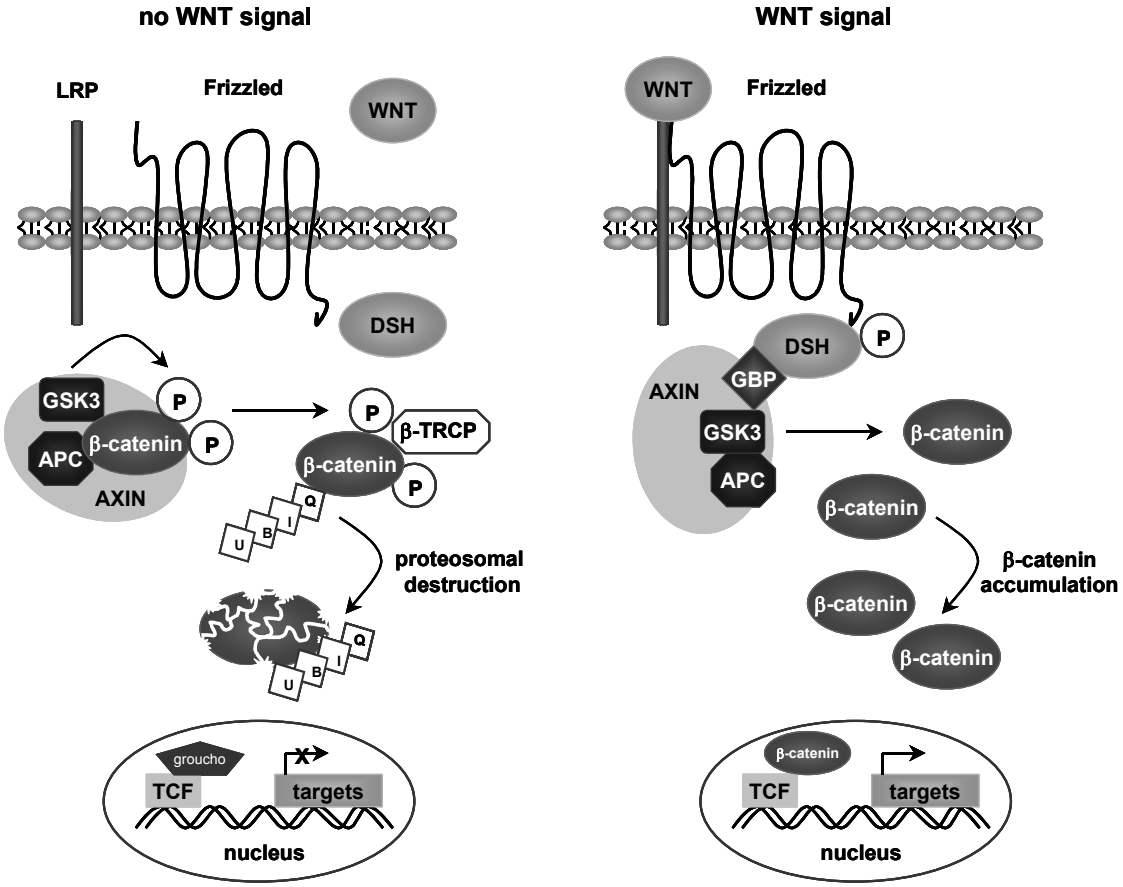


Figure 11 **The canonical WNT signaling pathway.**

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 (UBI), 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 complex. As a result, β-catenin will accumulate and translocate to the nucleus, where it binds TCF/LEF, and activates transcription of target genes (right). APC= adenomatous polyposis coli; GSK3β= glycogen synthase kinase-3β; GBP= GSK3β binding protein; β-TRCP= β-transducin repeat protein; TCF= T cell factor; LEF= lymphoid enhancer factor –1; LRP= LDL-related protein.

β-catenin are often mutated in cancers and sustained overexpression of *WNT* genes can cause cancer [180,186]. Mutations of the *APC* tumor suppressor gene or of the *β-catenin* sequences encoding the crucial GSK3β phosphorylation sites in the N-terminal domain have been found in the vast majority of colorectal cancers as well as many other cancer types [180,186]. 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 [180]. Target genes, which likely cooperate in neoplastic transformation, include *CCND1* (cyclin D1) [187], *uPAR* [188], *CD44* [189], *MET* [190] and *c-MYC* [191].

3.3 WNT signaling in haematopoietic cells and lymphocytes

Members of the TCF/LEF family of transcription factors were initially discovered in models of early lymphocyte development [192]. Within the immune system, TCF1 is restricted to the T cell lineage, whereas pro-B

cells and most cells of the T cell lineage express LEF1. The biological relevance of TCF1 and LEF1 during T- and B cell development was demonstrated by knockout mice studies. Genetic disruption of *TCF1* showed that TCF1 is crucial for the maintenance of early thymocyte progenitors, but is dispensable for proliferation and function of mature T cells [192]. *LEF1*-null mice did not show abnormalities in T cell development but displayed B cell defects and other abnormalities [192]. Interestingly, Reya *et al* demonstrated that WNT signaling regulates pro-B cell proliferation through a LEF-1 dependent mechanism [193].

Evidence for a function of WNT during haematopoiesis came from a study showing that WNT1, WNT5a and WNT10b stimulate survival and proliferation of haematopoietic progenitors [194]. Several studies confirmed and extended the role for WNT signals in early haematopoiesis, showing that WNT signals induce proliferation of immature haematopoietic progenitor cells including $CD34^+$ bone marrow cells and haematopoietic stem cell (HSC) [195-198], indicating that WNT signals might be involved in HSC self-renewal. In case of lymphocytes, WNT3a stimulates proliferation of pro-B cells [193], whereas WNT1 and WNT4 has similar effects on developing T cells [199]. So, WNT proteins can function as growth factors for progenitor cells and for both B- and T cell lineages.

3.4 WNT signaling in leukemias and lymphoproliferative disease

Since WNT signals promote the self-renewal and expansion of lymphocyte progenitors, aberrant activation of WNT signaling might contribute to the pathogenesis of lymphoproliferative disease. A number of recent studies suggest that activation of the WNT pathway indeed plays an important role in lymphomagenesis. Interestingly, in different types of lymphoproliferative disease, distinct mechanims of activation, including para/autocrine-, viral-, and mutational activation may be involved.

WNT signaling has been implicated in pre B-acute lymphoblastoid leukemia (ALL), where *WNT16* expression was induced by the transcription factor E2A-pre-B-cell leukemia transcription factor 1 (E2A-PBX1) fusion product [200]; in chronic lymphocytic leukemia (CLL) [201] and acute myeloid leukemia (AML) [202], which overexpress several *WNT* genes; and in chronic myeloid leukemia (CML) [203], where activated WNT signaling was found in the terminal phase of CML that is often associated with additional chromosomal changes. The first functional evidence for the involvement of the WNT pathway in a human lymphoproliferative disease, however, came from our laboratory showing that the canonical WNT pathway is activated an regulates proliferation of MM cells (this thesis). Here, the WNT activation appears to be ligand-dependent. Interestingly, it has been reported that MM cells overexpress the WNT antagonist DKK1 [184], suggesting a feedback loop in WNT signaling. DKK1 has also been implicated in the development of lytic bone lesions in MM by inhibiting osteoblast differentiation [184].

In immunodeficiency-related lymphoproliferative disease, activation of WNT signaling by gamma-herpes viruses may play a pathogenetic role. Kaposis-sarcoma-associated herpes virus (KSHV), latency-associated nuclear antigen (LANA) was shown to stabilize β-catenin, presumably by binding to the negative regulator GSK3β, resulting in increased cell proliferation. This could promote cell growth in PEL [204]. Also, Epstein-Barr virus (EBV) was shown to stabilize β-catenin and activate β-catenin/TCF-mediated transcription in type III latently infected B cells by sequestering deubiquitinating enzymes and/or by inhibiting GSK3β activity [205,206]. Although these studies were performed in EBV-transformed cell lines, they suggest a possible role for WNT pathway activation in EBV-related lymphomagenesis.

Recently, a study from Liang *et al* showed that inhibition of the non-canonical WNT signaling may play a role in lymphomagenesis. These authors demonstrated that WNT5a negatively regulates B cell proliferation, presumably through the WNT/Ca^{2+} pathway

and by downregulation of cyclin D1 expression [207]. Furthermore, *WNT5a* heterozygous mice developed clonal myeloid leukaemias and B cell lymphomas [207], whereas loss of WNT5a expression and/or deletion of the *WNT5a* gene were observed in the majority of human primary leukemias. These data imply that WNT5a functions as a tumor suppressor.

Aims and outlines of this thesis

The studies described in this thesis investigated the contribution of two potent oncogenic pathways to B cell tumor growth and survival, i.e. the HGF/MET and WNT/β-catenin signaling pathways. Considering important roles of HGF and MET in tumors, possible deregulation mechanisms, including defective receptor degradation, mutations in *MET* and autocrine activation, were investigated in B cell malignancies. Since extracellular activation of HGF is a critical limiting step in controlling the biological effects of the HGF/MET pathway, we studied the expression and role of HGFA in cells within normal tonsillar microenviroment, including B cells and FDCs (chapter 2), and B cell lymphomas, i.e. DLBCL (chapter 3) and MM (chapter 4). Also, a large panel of several B cell malignancies is examined for the expression of HGF and MET and screened for amplifications and mutations of *MET* (chapter 3). In addition, the functional role for the HGF/MET pathway in DLBCL, the most common type of B-NHL is investigated (chapter 3). In chapter 5, we identified c-Cbl as a downstream target of the HGF/MET signaling pathway, and analyzed its role in HGF-induced receptor ubiquitination and degradation in B cells. WNT signals form another class of paracrine growth factors that could influence MM cell growth. In chapter 6, we investigated the functional impact of WNT signaling in MM.

References

1. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19:595-621.
2. Klein U, Goossens T, Fischer M et al. Somatic hypermutation in normal and transformed human B cells. Immunol Rev. 1998;162:261-280.
3. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-139.
4. Liu YJ, de Bouteiller O, Fugier-Vivier I. Mechanisms of selection and differentiation in germinal centers. Curr Opin Immunol. 1997;9:256-262.
5. Dubois B, Barthelemy C, Durand I et al. Toward a role of dendritic cells in the germinal center reaction: triggering of B cell proliferation and isotype switching. J Immunol. 1999;162:3428-3436.
6. Cosgrove D, Gray D, Dierich A et al. Mice lacking MHC class II molecules. Cell. 1991;66:1051-1066.
7. Kawabe T, Naka T, Yoshida K et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity. 1994;1:167-178.
8. Renshaw BR, Fanslow WC, III, Armitage RJ et al. Humoral immune responses in CD40 ligand-deficient mice. J Exp Med. 1994;180:1889-1900.
9. Liu YJ, Arpin C. Germinal center development. Immunol Rev. 1997;156:111-126.
10. van Eijk M, Defrance T, Hennino A, de Groot C. Death-receptor contribution to the germinal-center reaction. Trends Immunol. 2001;22:677-682.
11. Calame KL, Lin KI, Tunyaplin C. Regulatory mechanisms that determine the development and function of plasma cells. Annu Rev Immunol. 2003;21:205-230.
12. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. Nat Rev Immunol. 2005;5:230-242.
13. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell. 1994;79:901-912.
14. Choe J, Choi YS. IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. Eur J Immunol. 1998;28:508-515.
15. Iwakoshi NN, Lee AH, Vallabhajosyula P et al. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nat Immunol. 2003;4:321-329.
16. Ansel KM, Cyster JG. Chemokines in lymphopoiesis and lymphoid organ development. Curr Opin Immunol. 2001;13:172-179.
17. Cyster JG, Ansel KM, Reif K et al. Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev. 2000;176:181-193.
18. Ngo VN, Tang HL, Cyster JG. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. J Exp Med. 1998;188:181-191.
19. Nagira M, Imai T, Yoshida R et al. A lymphocyte-specific CC chemokine, secondary lymphoid tissue chemokine (SLC), is a highly efficient chemoattractant for B cells and activated T cells. Eur J Immunol. 1998;28:1516-1523.
20. Nagasawa T, Hirota S, Tachibana K et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature. 1996;382:635-638.
21. Ma Q, Jones D, Borghesani PR et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in C. Proc Natl Acad Sci U S A. 1998;95:9448-9453.
22. Allen CD, Ansel KM, Low C et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol. 2004;5:943-952.
23. Hargreaves DC, Hyman PL, Lu TT et al. A coordinated change in chemokine responsiveness guides plasma cell movements. J Exp Med. 2001;194:45-56.
24. Koopman G, Parmentier HK, Schuurman HJ et al. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways. J Exp Med. 1991;173:1297-1304.
25. Butcher EC, Williams M, Youngman K, Rott L, Briskin M. Lymphocyte trafficking and regional immunity. Adv Immunol. 1999;72:209-253.
26. Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol. 1995;11:549-599.
27. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. J Exp Med. 2003;198:1539-1550.
28. van der Voort R, Taher TE, Keehnen RM et al. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. J Exp Med. 1997;185:2121-2131.
29. Arroyo AG, Yang JT, Rayburn H, Hynes RO. Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. Immunity. 1999;11:555-566.
30. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways

prevents apoptosis of germinal center B cells. J Immunol. 1994;152:3760-3767.
31. van Kooyk Y, Lub M, Figdor CG. Adhesion and signaling mediated by the cytoplasmic tails of leucocyte integrins. Cell Adhes Commun. 1998;6:247-254.
32. Koopman G, Keehnen RM, Lindhout E et al. Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. Eur J Immunol. 1997;27:1-7.
33. Kuppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med. 1999;341:1520-1529.
34. Goossens T, Klein U, Kuppers R. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci U S A. 1998;95:2463-2468.
35. Bross L, Fukita Y, McBlane F et al. DNA double-strand breaks in immunoglobulin genes undergoing somatic hypermutation. Immunity. 2000;13:589-597.
36. Papavasiliou FN, Schatz DG. Cell-cycle-regulated DNA double-stranded breaks in somatic hypermutation of immunoglobulin genes. Nature. 2000;408:216-221.
37. Shaffer AL, Rosenwald A, Staudt LM. Lymphoid malignancies: the dark side of B-cell differentiation. Nat Rev Immunol. 2002;2:920-932.
38. Pasqualucci L, Neumeister P, Goossens T et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature. 2001;412:341-346.
39. Muramatsu M, Kinoshita K, Fagarasan S et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-563.
40. McCarthy H, Wierda WG, Barron LL et al. High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. Blood. 2003;101:4903-4908.
41. Heintel D, Kroemer E, Kienle D et al. High expression of activation-induced cytidine deaminase (AID) mRNA is associated with unmutated IGVH gene status and unfavourable cytogenetic aberrations in patients with chronic lymphocytic leukaemia. Leukemia. 2004;18:756-762.
42. Smit LA, Bende RJ, Aten J et al. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype. Cancer Res. 2003;63:3894-3898.
43. Rao PH, Houldsworth J, Dyomina K et al. Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood. 1998;92:234-240.
44. Pasqualucci L, Bereschenko O, Niu H et al. Molecular pathogenesis of non-Hodgkin's lymphoma: the role of Bcl-6. Leuk Lymphoma. 2003;44 Suppl 3:S5-12.
45. Houldsworth J, Mathew S, Rao PH et al. REL proto-oncogene is frequently amplified in extranodal diffuse large cell lymphoma. Blood. 1996;87:25-29.
46. Alizadeh AA, Eisen MB, Davis RE et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503-511.
47. Rosenwald A, Wright G, Chan WC et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:1937-1947.
48. Lossos IS, Alizadeh AA, Eisen MB et al. Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas. Proc Natl Acad Sci U S A. 2000;97:10209-10213.
49. Bataille R, Harousseau JL. Multiple myeloma. N Engl J Med. 1997;336:1657-1664.
50. Avet-Loiseau H, Facon T, Daviet A et al. 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myelome. Cancer Res. 1999;59:4546-4550.
51. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood. 2004;104:607-618.
52. Chesi M, Bergsagel PL, Brents LA et al. Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines. Blood. 1996;88:674-681.
53. Shaughnessy J, Jr., Gabrea A, Qi Y et al. Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. Blood. 2001;98:217-223.
54. Anderson KC, Shaughnessy JD, Jr., Barlogie B, Harousseau JL, Roodman GD. Multiple myeloma. Hematology (Am Soc Hematol Educ Program). 2002;214-240.
55. Teofili L, Di Febo AL, Pierconti F et al. Expression of the c-met proto-oncogene and its ligand, hepatocyte growth factor, in Hodgkin disease. Blood. 2001;97:1063-1069.
56. Hsiao LT, Lin JT, Yu IT et al. High serum hepatocyte growth factor level in patients with non-Hodgkin's lymphoma. Eur J Haematol. 2003;70:282-289.
57. Giles FJ, Vose JM, Do KA et al. Clinical relevance of circulating angiogenic factors in patients with non-

Hodgkin's lymphoma or Hodgkin's lymphoma. Leuk Res. 2004;28:595-604.
58. Seidel C, Borset M, Turesson I et al. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood. 1998;91:806-812.
59. Iwasaki T, Hamano T, Ogata A et al. Clinical significance of vascular endothelial growth factor and hepatocyte growth factor in multiple myeloma. Br J Haematol. 2002;116:796-802.
60. Sezer O, Jakob C, Eucker J et al. Serum levels of the angiogenic cytokines basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) in multiple myeloma. Eur J Haematol. 2001;66:83-88.
61. Kyrtsonis MC, Dedoussis G, Zervas C et al. Soluble interleukin-6 receptor (sIL-6R), a new prognostic factor in multiple myeloma. Br J Haematol. 1996;93:398-400.
62. Zarnegar R, DeFrances MC, Oliver L, Michalopoulos G. Identification and partial characterization of receptor binding sites for HGF on rat hepatocytes. Biochem Biophys Res Commun. 1990;173:1179-1185.
63. Saccone S, Narsimhan RP, Gaudino G et al. Regional mapping of the human hepatocyte growth factor (HGF)-scatter factor gene to chromosome 7q21.1. Genomics. 1992;13:912-914.
64. Miyazawa K, Kitamura A, Kitamura N. Structural organization and the transcription initiation site of the human hepatocyte growth factor gene. Biochemistry. 1991;30:9170-9176.
65. Nakamura T, Nishizawa T, Hagiya M et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342:440-443.
66. Weidner KM, Arakaki N, Hartmann G et al. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc Natl Acad Sci U S A. 1991;88:7001-7005.
67. Chan AM, Rubin JS, Bottaro DP et al. Identification of a competitive HGF antagonist encoded by an alternative transcript. Science. 1991;254:1382-1385.
68. Miyazawa K, Kitamura A, Naka D, Kitamura N. An alternatively processed mRNA generated from human hepatocyte growth factor gene. Eur J Biochem. 1991;197:15-22.
69. Jakubczak JL, LaRochelle WJ, Merlino G. NK1, a natural splice variant of hepatocyte growth factor/scatter factor, is a partial agonist in vivo. Mol Cell Biol. 1998;18:1275-1283.
70. Hartmann G, Naldini L, Weidner KM et al. A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis. Proc Natl Acad Sci U S A. 1992;89:11574-11578.
71. Lokker NA, Mark MR, Luis EA et al. Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. EMBO J. 1992;11:2503-2510.
72. Cooper CS, Park M, Blair DG et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature. 1984;311:29-33.
73. Gonzatti-Haces M, Seth A, Park M et al. Characterization of the TPR-MET oncogene p65 and the MET protooncogene p140 protein-tyrosine kinases. Proc Natl Acad Sci U S A. 1988;85:21-25.
74. Liu Y. The human hepatocyte growth factor receptor gene: complete structural organization and promoter characterization. Gene. 1998;215:159-169.
75. Giordano S, Ponzetto C, Di Renzo MF, Cooper CS, Comoglio PM. Tyrosine kinase receptor indistinguishable from the c-met protein. Nature. 1989;339:155-156.
76. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer. 2002;2:289-300.
77. Kong-Beltran M, Stamos J, Wickramasinghe D. The Sema domain of Met is necessary for receptor dimerization and activation. Cancer Cell. 2004;6:75-84.
78. Ponzetto C, Bardelli A, Zhen Z et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 1994;77:261-271.
79. Somerset DA, Li XF, Afford S et al. Ontogeny of hepatocyte growth factor (HGF) and its receptor (c-met) in human placenta: reduced HGF expression in intrauterine growth restriction. Am J Pathol. 1998;153:1139-1147.
80. Wolf HK, Zarnegar R, Oliver L, Michalopoulos GK. Hepatocyte growth factor in human placenta and trophoblastic disease. Am J Pathol. 1991;138:1035-1043.
81. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature. 1995;376:768-771.
82. Yang XM, Vogan K, Gros P, Park M. Expression of the met receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in Splotch mice. Development. 1996;122:2163-2171.
83. Schmidt C, Bladt F, Goedecke S et al. Scatter factor/hepatocyte growth factor is essential for liver development. Nature. 1995;373:699-702.
84. Uehara Y, Minowa O, Mori C et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature. 1995;373:702-705.

85. Kolatsi-Joannou M, Moore R, Winyard PJ, Woolf AS. Expression of hepatocyte growth factor/scatter factor and its receptor, MET, suggests roles in human embryonic organogenesis. Pediatr Res. 1997;41:657-665.
86. Wang Y, Selden C, Farnaud S, Calnan D, Hodgson HJ. Hepatocyte growth factor (HGF/SF) is expressed in human epithelial cells during embryonic development; studies by in situ hybridisation and northern blot analysis. J Anat. 1994;185 (Pt 3):543-551.
87. Kermorgant S, Walker F, Hormi K et al. Developmental expression and functionality of hepatocyte growth factor and c-Met in human fetal digestive tissues. Gastroenterology. 1997;112:1635-1647.
88. Thery C, Sharpe MJ, Batley SJ, Stern CD, Gherardi E. Expression of HGF/SF, HGF1/MSP, and c-met suggests new functions during early chick development. Dev Genet. 1995;17:90-101.
89. Hu Z, Evarts RP, Fujio K, Marsden ER, Thorgeirsson SS. Expression of hepatocyte growth factor and c-met genes during hepatic differentiation and liver development in the rat. Am J Pathol. 1993;142:1823-1830.
90. Kmiecik TE, Keller JR, Rosen E, Vande Woude GF. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood. 1992;80:2454-2457.
91. Takai K, Hara J, Matsumoto K et al. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood. 1997;89:1560-1565.
92. Weimar IS, Miranda N, Muller EJ et al. Hepatocyte growth factor/scatter factor (HGF/SF) is produced by human bone marrow stromal cells and promotes proliferation, adhesion and survival of human hematopoietic progenitor cells (CD34+). Exp Hematol. 1998;26:885-894.
93. Zhu H, Naujokas MA, Fixman ED, Torossian K, Park M. Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. J Biol Chem. 1994;269:29943-29948.
94. Furge KA, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene. 2000;19:5582-5589.
95. Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signaling. Oncogene. 1998;17:1395-1413.
96. Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci. 1998;23:213-216.
97. Graziani A, Gramaglia D, Cantley LC, Comoglio PM. The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. J Biol Chem. 1991;266:22087-22090.
98. Ponzetto C, Bardelli A, Maina F et al. A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. Mol Cell Biol. 1993;13:4600-4608.
99. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2:489-501.
100. Tang TT, Dowbenko D, Jackson A et al. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem. 2002;277:14255-14265.
101. Burgering BM, Kops GJ. Cell cycle and death control: long live Forkheads. Trends Biochem Sci. 2002;27:352-360.
102. Naka D, Ishii T, Yoshiyama Y et al. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J Biol Chem. 1992;267:20114-20119.
103. Peek M, Moran P, Mendoza N, Wickramasinghe D, Kirchhofer D. Unusual proteolytic activation of pro-hepatocyte growth factor by plasma kallikrein and coagulation factor XIa. J Biol Chem. 2002;277:47804-47809.
104. Lee SL, Dickson RB, Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. J Biol Chem. 2000;275:36720-36725.
105. Naldini L, Tamagnone L, Vigna E et al. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J. 1992;11:4825-4833.
106. Mars WM, Zarnegar R, Michalopoulos GK. Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. Am J Pathol. 1993;143:949-958.
107. Shimomura T, Miyazawa K, Komiyama Y et al. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem. 1995;229:257-261.
108. Miyazawa K, Shimomura T, Kitamura N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem. 1996;271:3615-3618.
109. Naldini L, Vigna E, Bardelli A et al. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. J Biol Chem. 1995;270:603-611.
110. Mazar AP, Henkin J, Goldfarb RH. The urokinase plasminogen activator system in cancer: implications

for tumor angiogenesis and metastasis. Angiogenesis. 1999;3:15-32.
111. Suzuki M, Kobayashi H, Kanayama N et al. Inhibition of tumor invasion by genomic down-regulation of matriptase through suppression of activation of receptor-bound pro-urokinase. J Biol Chem. 2004;279:14899-14908.
112. Citarella F, Misiti S, Felici A et al. Estrogen induction and contact phase activation of human factor XII. Steroids. 1996;61:270-276.
113. Shimomura T, Kondo J, Ochiai M et al. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem. 1993;268:22927-22932.
114. Miyazawa K, Wang Y, Minoshima S, Shimizu N, Kitamura N. Structural organization and chromosomal localization of the human hepatocyte growth factor activator gene--phylogenetic and functional relationship with blood coagulation factor XII, urokinase, and tissue-type plasminogen activator. Eur J Biochem. 1998;258:355-361.
115. Shimomura T, Ochiai M, Kondo J, Morimoto Y. A novel protease obtained from FBS-containing culture supernatant, that processes single chain form hepatocyte growth factor to two chain form in serum-free culture. Cytotechnology. 1992;8:219-229.
116. Miyazawa K, Shimomura T, Kitamura A et al. Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. J Biol Chem. 1993;268:10024-10028.
117. Royle NJ, Nigli M, Cool D, MacGillivray RT, Hamerton JL. Structural gene encoding human factor XII is located at 5q33-qter. Somat Cell Mol Genet. 1988;14:217-221.
118. Okajima A, Miyazawa K, Naitoh Y, Inoue K, Kitamura N. Induction of hepatocyte growth factor activator messenger RNA in the liver following tissue injury and acute inflammation. Hepatology. 1997;25:97-102.
119. Kataoka H, Shimomura T, Kawaguchi T et al. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. J Biol Chem. 2000;275:40453-40462.
120. Yamada T, Yoshiyama Y, Tsuboi Y, Shimomura T. Astroglial expression of hepatocyte growth factor and hepatocyte growth factor activator in human brain tissues. Brain Res. 1997;762:251-255.
121. Moriyama T, Kataoka H, Tsubouchi H, Koono M. Concomitant expression of hepatocyte growth factor (HGF), HGF activator and c-met genes in human glioma cells in vitro. FEBS Lett. 1995;372:78-82.
122. van Adelsberg J, Sehgal S, Kukes A et al. Activation of hepatocyte growth factor (HGF) by endogenous HGF activator is required for metanephric kidney morphogenesis in vitro. J Biol Chem. 2001;276:15099-15106.
123. Kataoka H, Hamasuna R, Itoh H, Kitamura N, Koono M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. Cancer Res. 2000;60:6148-6159.
124. Parr C, Watkins G, Mansel RE, Jiang WG. The hepatocyte growth factor regulatory factors in human breast cancer. Clin Cancer Res. 2004;10:202-211.
125. Schmassmann A, Stettler C, Poulsom R et al. Roles of hepatocyte growth factor and its receptor Met during gastric ulcer healing in rats. Gastroenterology. 1997;113:1858-1872.
126. Watanabe S, Hirose M, Wang XE et al. Hepatocyte growth factor accelerates the wound repair of cultured gastric mucosal cells. Biochem Biophys Res Commun. 1994;199:1453-1460.
127. Miyazawa K, Shimomura T, Naka D, Kitamura N. Proteolytic activation of hepatocyte growth factor in response to tissue injury. J Biol Chem. 1994;269:8966-8970.
128. Itoh H, Naganuma S, Takeda N et al. Regeneration of injured intestinal mucosa is impaired in hepatocyte growth factor activator-deficient mice. Gastroenterology. 2004;127:1423-1435.
129. Shimomura T, Denda K, Kitamura A et al. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272:6370-6376.
130. Kawaguchi T, Qin L, Shimomura T et al. Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. J Biol Chem. 1997;272:27558-27564.
131. Itoh H, Yamauchi M, Kataoka H et al. Genomic structure and chromosomal localization of the human hepatocyte growth factor activator inhibitor type 1 and 2 genes. Eur J Biochem. 2000;267:3351-3359.
132. Laskowski M, Jr., Kato I. Protein inhibitors of proteinases. Annu Rev Biochem. 1980;49:593-626.
133. Shimomura T, Denda K, Kawaguchi T et al. Multiple sites of proteolytic cleavage to release soluble forms of hepatocyte growth factor activator inhibitor type 1 from a transmembrane form. J Biochem (Tokyo). 1999;126:821-828.
134. Parr C, Jiang WG. Hepatocyte growth factor activators, inhibitors and antagonists and their implication in cancer intervention. Histol Histopathol. 2001;16:251-268.
135. Qin L, Denda K, Shimomura T, Kawaguchi T, Kitamura N. Functional characterization of Kunitz domains in hepatocyte growth factor activator inhibitor type 2. FEBS Lett. 1998;436:111-114.
136. Itoh H, Kataoka H, Tomita M et al. Upregulation of HGF activator inhibitor type 1 but not type 2 along

with regeneration of intestinal mucosa. Am J Physiol Gastrointest Liver Physiol. 2000;278:G635-G643.
137. Mitchell KJ, Pinson KI, Kelly OG et al. Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet. 2001;28:241-249.
138. van der Voort R, Taher TE, Derksen PW et al. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. Adv Cancer Res. 2000;79:39-90.
139. Giordano S, Zhen Z, Medico E et al. Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. Proc Natl Acad Sci U S A. 1993;90:649-653.
140. Rong S, Segal S, Anver M, Resau JH, Vande Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A. 1994;91:4731-4735.
141. Jeffers M, Rong S, Vande Woude GF. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol. 1996;16:1115-1125.
142. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915-925.
143. Jeffers M, Fiscella M, Webb CP et al. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A. 1998;95:14417-14422.
144. Jeffers MF. Activating mutations in the Met receptor overcome the requirement for autophosphorylation of tyrosines crucial for wild type signaling. Oncogene. 1999;18:5120-5125.
145. Schmidt L, Duh FM, Chen F et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16:68-73.
146. Fischer J, Palmedo G, von Knobloch R et al. Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. Oncogene. 1998;17:733-739.
147. Olivero M, Valente G, Bardelli A et al. Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family. Int J Cancer. 1999;82:640-643.
148. Schmidt L, Junker K, Nakaigawa N et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene. 1999;18:2343-2350.
149. Park WS, Dong SM, Kim SY et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res. 1999;59:307-310.
150. Lee JH, Han SU, Cho H et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene. 2000;19:4947-4953.
151. Tonks NK, Neel BG. From form to function: signaling by protein tyrosine phosphatases. Cell. 1996;87:365-368.
152. Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem. 2001;70:503-533.
153. Gandino L, Longati P, Medico E, Prat M, Comoglio PM. Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. J Biol Chem. 1994;269:1815-1820.
154. Villa-Moruzzi E, Puntoni F, Bardelli A et al. Protein tyrosine phosphatase PTP-S binds to the juxtamembrane region of the hepatocyte growth factor receptor Met. Biochem J. 1998;336 (Pt 1):235-239.
155. Peschard P, Fournier TM, Lamorte L et al. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell. 2001;8:995-1004.
156. Taher TE, Tjin EP, Beuling EA et al. c-Cbl is involved in Met signaling in B cells and mediates hepatocyte growth factor-induced receptor ubiquitination. J Immunol. 2002;169:3793-3800.
157. Levkowitz G, Waterman H, Zamir E et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. 1998;12:3663-3674.
158. Lee PS, Wang Y, Dominguez MG et al. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J. 1999;18:3616-3628.
159. Miyake S, Lupher ML, Jr., Druker B, Band H. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. Proc Natl Acad Sci U S A. 1998;95:7927-7932.
160. Petrelli A, Gilestro GF, Lanzardo S et al. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature. 2002;416:187-190.
161. Weidner KM, Sachs M, Riethmacher D, Birchmeier W. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proc Natl Acad Sci U S A. 1995;92:2597-2601.
162. Lamszus K, Jin L, Fuchs A et al. Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice. Lab Invest. 1997;76:339-353.

163. Rong S, Bodescot M, Blair D et al. Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. Mol Cell Biol. 1992;12:5152-5158.
164. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996;88:3998-4004.
165. Capello D, Gaidano G, Gallicchio M et al. The tyrosine kinase receptor met and its ligand HGF are co-expressed and functionally active in HHV-8 positive primary effusion lymphoma. Leukemia. 2000;14:285-291.
166. Ferracini R, Di Renzo MF, Scotlandi K et al. The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. Oncogene. 1995;10:739-749.
167. Tuck AB, Park M, Sterns EE, Boag A, Elliott BE. Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. Am J Pathol. 1996;148:225-232.
168. Koochekpour S, Jeffers M, Rulong S et al. Met and hepatocyte growth factor/scatter factor expression in human gliomas. Cancer Res. 1997;57:5391-5398.
169. Jucker M, Gunther A, Gradl G et al. The Met/hepatocyte growth factor receptor (HGFR) gene is overexpressed in some cases of human leukemia and lymphoma. Leuk Res. 1994;18:7-16.
170. Derksen PW, de Gorter DJ, Meijer HP et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003;17:764-774.
171. Weimar IS, de Jong D, Muller EJ et al. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins. Blood. 1997;89:990-1000.
172. Borset M, Lien E, Espevik T et al. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem. 1996;271:24655-24661.
173. Elenitoba-Johnson KS, Jenson SD, Abbott RT et al. Involvement of multiple signaling pathways in follicular lymphoma transformation: p38-mitogen-activated protein kinase as a target for therapy. Proc Natl Acad Sci U S A. 2003;100:7259-7264.
174. Kawano R, Ohshima K, Karube K et al. Prognostic significance of hepatocyte growth factor and c-MET expression in patients with diffuse large B-cell lymphoma. Br J Haematol. 2004;127:305-307.
175. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology. 2001;102:506-514.
176. Derksen PW, Keehnen RM, Evers LM et al. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002;99:1405-1410.
177. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004;5:691-701.
178. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell. 1982;31:99-109.
179. Tolwinski NS, Wieschaus E. Rethinking WNT signaling. Trends Genet. 2004;20:177-181.
180. Polakis P. Wnt signaling and cancer. Genes Dev. 2000;14:1837-1851.
181. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. J Cell Sci. 2003;116:2627-2634.
182. Mao B, Wu W, Li Y et al. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature. 2001;411:321-325.
183. Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. Gene. 2004;341:19-39.
184. Tian E, Zhan F, Walker R et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349:2483-2494.
185. Bodine PV, Zhao W, Kharode YP et al. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol. 2004;18:1222-1237.
186. Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell. 2000;103:311-320.
187. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature. 1999;398:422-426.
188. Mann B, Gelos M, Siedow A et al. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc Natl Acad Sci U S A. 1999;96:1603-1608.
189. Wielenga VJ, Smits R, Korinek V et al. Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol. 1999;154:515-523.
190. Boon EM, van der NR, van de WM, Clevers H, Pals ST. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res. 2002;62:5126-5128.
191. Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. Nat Rev Cancer. 2002;2:764-776.

192. van de Wetering M, de Lau W, Clevers H. WNT signaling and lymphocyte development. Cell. 2002;109 Suppl:S13-S19.
193. Reya T, O'Riordan M, Okamura R et al. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity. 2000;13:15-24.
194. Austin TW, Solar GP, Ziegler FC, Liem L, Matthews W. A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood. 1997;89:3624-3635.
195. Van Den Berg DJ, Sharma AK, Bruno E, Hoffman R. Role of members of the Wnt gene family in human hematopoiesis. Blood. 1998;92:3189-3202.
196. Reya T, Duncan AW, Ailles L et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423:409-414.
197. Murdoch B, Chadwick K, Martin M et al. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proc Natl Acad Sci U S A. 2003;100:3422-3427.
198. Staal FJ, Clevers HC. WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol. 2005;5:21-30.
199. Staal FJ, Meeldijk J, Moerer P et al. Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. Eur J Immunol. 2001;31:285-293.
200. McWhirter JR, Neuteboom ST, Wancewicz EV et al. Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A. 1999;96:11464-11469.
201. Lu D, Zhao Y, Tawatao R et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2004;101:3118-3123.
202. Simon M, Grandage VL, Linch DC, Khwaja A. Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia. Oncogene. 2005.
203. Jamieson CH, Ailles LE, Dylla SJ et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med. 2004;351:657-667.
204. Fujimuro M, Wu FY, ApRhys C et al. A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. Nat Med. 2003;9:300-306.
205. Shackelford J, Maier C, Pagano JS. Epstein-Barr virus activates beta-catenin in type III latently infected B lymphocyte lines: association with deubiquitinating enzymes. Proc Natl Acad Sci U S A. 2003;100:15572-15576.
206. Morrison JA, Klingelhutz AJ, Raab-Traub N. Epstein-Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells. J Virol. 2003;77:12276-12284.
207. Liang H, Chen Q, Coles AH et al. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. Cancer Cell. 2003;4:349-360.

CHAPTER **2**

Follicular dendritic cells catalyze hepatocyte growth factor (HGF) activation in the germinal center microenvironment by secreting the serine protease HGF-activator

Esther P.M. Tjin, Richard J. Bende, Patrick W. B. Derksen, Anne-Pauline van Huijstee, Hiroaki Kataoka*, Marcel Spaargaren, Steven T. Pals

Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

*Second Department of Pathology, Miyazaki Medical College, Kiyotake, Japan

Antigen (Ag)-specific B cell differentiation, the process that gives rise to plasma cells and memory B cells, involves the formation of germinal centers (GC). Within the GC microenvironment, multiple steps of B cell proliferation, selection and maturation take place, which are controlled by the B cell receptor (BCR), in concert with cytokines and contact dependent signals from follicular dendritic cells (FDC) and T cells. Signaling by the multifunctional cytokine hepatocyte growth factor (HGF) and its receptor MET has been shown to induce integrin-mediated adhesion of B cells to vascular cell adhesion molecule-1 (VCAM-1), which is expressed by FDCs. In the present study, we have examined the expression of regulatory components of the HGF/MET pathway, including HGF activator (HGFA), within the secondary lymphoid organ microenvironment. We show that MET is expressed by both centroblasts and plasma cells, and that HGFA is expressed by plasma cells. Since we have shown that HGF is a potent growth and survival factor for malignant plasma cells, HGF may also serve as a survival factor for normal plasma cells. Furthermore, we demonstrate that FDCs are the major source for HGF and its activator within the GC microenvironment. Both HGF and HGFA are expressed by FDCs in the GC dark zone ($CD21^{high}/CD23^{low}$) but not in the light zone ($CD21^{high}/CD23^{high}$). These findings suggest that HGF and HGFA provided by the dark zone FDCs help to regulate the proliferation, survival and/or adhesion of MET-positive centroblasts. ***In press 2005, The Journal of Immunology.***

The germinal center (GC) comprises a dynamic microenvironment in which antigen (Ag)-specific B cell differentiation takes place. Morphological studies have shown that the GC consists of two distinct compartments, the dark and light zones, associated with important separate functions [1-4]. B cells in the dark zone, called centroblasts, undergo rapid clonal expansion and somatic hypermutation of their immunoglobulin (Ig) variable region

genes. They subsequently move to the light zone to become centrocytes, which undergo selection based on the affinity of their BCR for antigen presented by follicular dendritic cells (FDC) [3,5]. Centrocytes with low affinity receptors and centrocytes that do not receive T cell help undergo apoptosis, whereas centrocytes with a high affinity BCR receive survival signals from FDCs and T cells and differentiate into plasma cells or memory B cells [1-3,5,6].

Notwithstanding ample insight into the anatomy and function of the GC, the mechanisms orchestrating GC polarity and guiding B cell movement and differentiation within the distinct GC compartments are as yet incompletely understood. Molecules that play a crucial role are chemokines and adhesion receptors. Chemokines, including CXCL13 (BCA/BLC) [7,8] and CXCL12 (SDF-1), recruit B cells into the GC and regulate their positioning in the dark and light zones [9]. The integrin adhesion molecules lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) mediate attachment of GC B cells to FDCs [10,11]. The integrin activation required for this adhesion presumably is controlled by high affinity interactions of the BCR with antigen presented by FDCs [12]. Apart from establishing physical contact with FDCs, integrin signaling can rescue GC B cells from apoptosis [13,14], and hence may directly contribute to B cell selection. Once positioned within the GC microenvironment, B cell proliferation, survival and differentiation is supported by cytokines produced by T cells, FDCs and other GC accessory cells. An FDC-produced cytokine that may play a role in GC organization as well as in promoting GC B cell proliferation, survival and differentiation is hepatocyte growth factor/scatter factor (HGF) [15]. HGF is a multifunctional cytokine with a domain structure and a proteolytic mechanism of activation similar to the blood serine protease plasminogen, but lacks protease activity [15]. Via the tyrosine kinase receptor MET, HGF induces complex biological response in target cells, including growth, survival and motility. Whereas a functional HGF/MET pathway is indispensable for mammalian development, uncontrolled MET signaling is oncogenic, and has been implicated in the development of a variety of human cancers [16,17]. Within the GC microenvironment, MET is expressed by centroblasts while FDCs express and secrete HGF [18]. Upon CD40 and BCR stimulation naive B cells acquire MET expression and functional studies have shown that HGF induces integrin-dependent adhesion of MET-positive B cells to vascular cell adhesion molecule-1 (VCAM-1), expressed by FDCs [18-20]. Together, these data suggest a role for the HGF/MET pathway in regulating B cell-FDC interaction.

Upon secretion, HGF normally retains its 90-kDa single-chain (sc) precursor form, which is probably cell surface or extracellular matrix associated. For biological function however, proteolytic conversion of scHGF to the heterodimeric active form is essential [21]. Plasminogen activators, particularly uPA and factor XIIa have been shown to activate scHGF, although at low rates [22,23]. More recently, hepatocyte growth factor activator (HGFA), a factor XIIa-related serine protease with an efficient HGF-activating activity, was identified [21,23]. HGFA has been implicated in HGF activation at sites of inflammation and tissue repair [24], as well as by tumor cells [25], including multiple myeloma (MM), a malignancy of plasma cells [26]. Here, we have studied the mechanism of HGF activation within the GC microenvironment. We show that FDCs derived from the GC dark zone, in contrast to those from the GC light zone, produce HGFA, and in this way are able to catalyze HGF activation. In addition, we demonstrate that normal plasma cells express HGFA.

Materials and methods

Antibodies and reagents

The monoclonal antibodies used were: fluorescein isothiocyanate (FITC)-conjugated anti-human IgD (DAKO, Glostrup, Denmark); allophycocyanin (APC)-conjugated anti-human

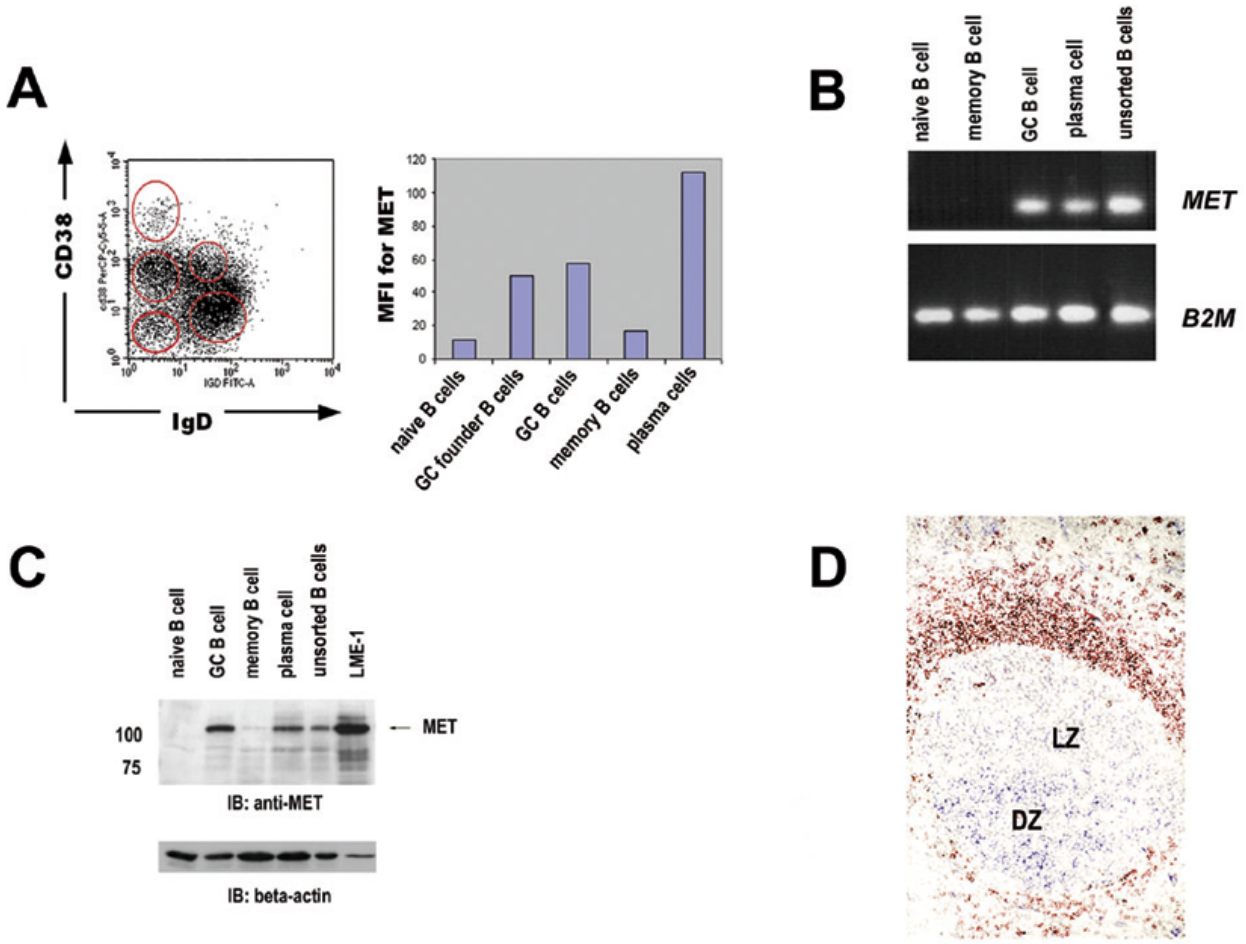


Figure 1 **Expression of MET by B cell subsets.**

A. B cell subsets were identified by IgD and CD38 staining as being naïve B cells (IgD+/CD38-), GC founder B cells (IgD+/CD38+), GC B cells (IgD-/CD38+), plasma cells (IgD-/CD38++) and memory B cells (IgD-/CD38-) (left panel). Unsorted B cells were analyzed for the expression of MET using a CD38/IgD/MET triple FACS staining. Results are shown as the mean fluorescence intensity (MFI) minus the MFI of the isotype control (right panel). **B.** MET mRNA is expressed in human GC B cells and plasma cells. Tonsillar B cell subsets were isolated by FACS sorting using a CD20/CD38/IgD triple staining. After RNA isolation and cDNA synthesis, RT-PCR for MET was performed. β2-microglobulin was used as housekeeping gene control. **C.** Expression of MET protein in human GC B cells and plasma cells. B cell subsets were collected by FACS sorting and the expression for MET was analyzed using immunoblotting. MM cell line LME-1 was used as positive control. **D.** MET expression in human tonsil. Immunohistochemical double staining of tonsillar sections for MET (blue) and IgD (brown), showing MET expression on GC B cells. DZ= dark zone. LZ= light zone.

CD38 (IgG1) (BD Biosciences, Erembodegem, Belgium); phycoerythrin (PE)-conjugated anti-human CD20 (DAKO); anti-human MET DO-24 (IgG2a) (Upstate Biotechnology, Lake Placid, NY); anti-HGFA (A1 and P1-4, IgG1) [24]; anti-factor XIIa, OT-2 (IgG1) (Sanquin, Amsterdam, The Netherlands); anti-human CD21L (DRC-1); anti-human CD21 (clone 1F8); FITC-conjugated anti-human CD21 (clone 1F8); anti-CD20 (L26) (all from DAKO); anti-CD14 (Leu M3, IgG2b); anti-CD3 (Leu 4, IgG1) (BD); isotype controls anti-IgG1, anti-IgG2a, and anti-IgG2b (DAKO). Secondary antibodies used were: post-antibody for powervision (Immunovision Technologies, Duiven, The Netherlands), horseradish peroxidase (HRP)-conjugated goat-anti-mouse/rat IgG and HRP-conjugated anti-FITC (DAKO).

Purification of B cell populations and FDCs

B cells were purified from human tonsils obtained from children undergoing routine tonsillectomy as described [13]. Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation. Monocytes and T cells were depleted by plastic adherence and sheep red blood cells rosetting, respectively. The total B cells fraction was > 97% pure as determined by FACS analysis. To obtain the different B cell populations (naive B cells, memory B cells, GC B cells and plasma cells), total B cells were stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20 and APC-conjugated anti-CD38 and sorted using a FACS aria (BD).

FDC clusters (enriched FDC preparation) were isolated from human tonsils as described previously by Liu et al. [27]. Tonsils were cut into pieces and treated with collagenase/DNase mix (200 U/ml collagenase IV, 10 U/ml DNase I, Boehringer Mannheim, Mannheim, Germany), followed by Ficoll-Isopaque density gradient centrifugation and density sedimentation on a Bovine Serum Albumin (BSA) gradient (1.5% BSA).

FDCs (CD21high) were stained with FITC-conjugated anti-CD21 and sorted using FACS aria (BD). PE-conjugated anti-CD23, together with anti-CD21, were used to obtain the CD23 FDC populations.

For immunocytochemical staining, FDCs were isolated from enriched FDC preparation

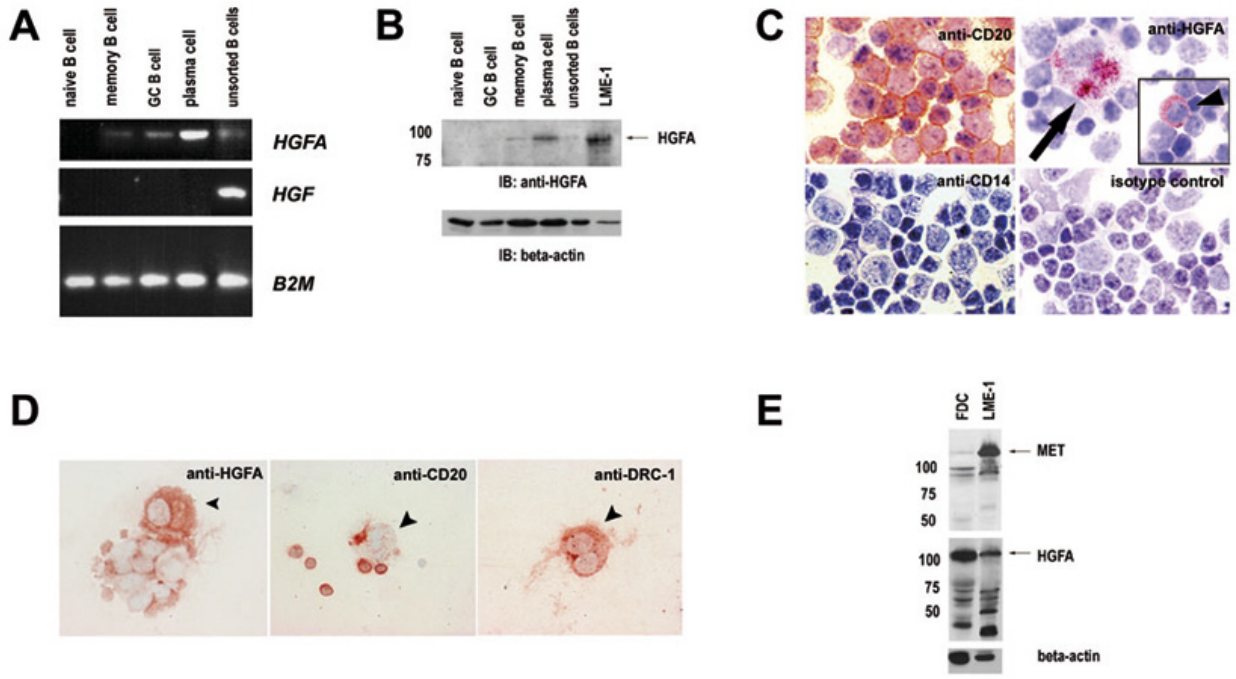


Figure 2 **Expression of HGFA in B cell subsets.**

A. Tonsillar B cell populations were isolated by FACS sorting using a CD20/CD38/IgD triple staining. After RNA isolation and cDNA synthesis, RT-PCR for HGFA and HGF was performed. β2-microglobulin was used as housekeeping gene control. **B.** Plasma cells express HGFA. Highly purified B cell populations were collected after FACS sorting and the expression for HGFA was analyzed using immunoblotting. MM cell line LME-1 was used as positive control. **C.** HGFA expression in total tonsillar B cell preparations. Immunocytochemical staining of unsorted B cell preparations for HGFA, the B cell marker CD20 and the monocyte marker CD14. Arrowhead indicates plasmacytoid cell (inset) whereas arrow indicates a FDC. **D.** HGFA is expressed by FDCs. FDCs were isolated from enriched FDC preparation by cell sorting of large cells. The cell purity was determined by staining for the FDC marker DRC-1 and B cell marker CD20. The monoclonal antibody A-1 against HGFA was used to detect HGFA expression. Arrow indicates a FDC. **E.** MET and HGFA expression in sorted FDC preparations. Purified FDC preparations were analyzed by immunoblotting for the expression of MET and HGFA.

by cell sorting of large cells. Cell purity was approximately 60-80%, as judged by staining for DRC-1, a FDC specific marker.

Assay for HGF activation

HGF activation was assayed as described previously [25]. In brief, single chain HGF (R&D Systems) was incubated with FDC conditioned medium. Conditioned medium was obtained as described previously [28].

For HGF activation, 20 μl conditioned medium was pretreated with 1 unit of thrombin and added to 0.1 μg scHGF. Inhibitor studies were done in the presence of leupeptin (500 μg/ml), neutralizing antibody against FXII (OT-2) (kindly provided by E. Hack, Sanquin, Amsterdam, The Netherlands) or neutralizing antibody against HGFA (P1-4) (40 μg/ml).

FDC sarcoma

The FDC sarcoma, which is a rare neoplasm that arises from lymph nodes as well as extra-nodal regions [29], was obtained from a 38-year old male patient, and diagnosed at the Department of Pathology, Academic Medical Center Amsterdam, The Netherlands. The tumor showed more than 95% CD21 and DRC-1 positive cells, indicating almost complete replacement of the normal follicle structure by the neoplasm.

Immunocytochemistry

Immunocytochemical stainings were performed on aceton-fixed cytospins. The cytospins were preincubated with 1% BSA in PBS for 15 minutes. After incubating with the primary antibody (overnight at 4 °C), endogenous peroxidase was blocked with 0.1% NaN_3 and 0.3% H_2O_2 in PBS for 10 minutes. Subsequently, the cytospins were stained with post-antibody of Powervision (Immunovision Technologies) for 15 minutes, followed by poly-HRP conjugated goat anti-mouse/rabbit IgG for 30 minutes. Substrate was developed with 3,3-amino-9-ethyl-carbazole (AEC) (Sigma, Bornem, Belgium).

RNA isolation, cDNA synthesis and reverse transcriptase-PCR

RNA from B cell fractions and cultures was isolated with nucleospin RNA 11 (Macherey-Nagel). For sorted fractions of plasmablasts and FDCs, Pico Pure RNA isolation kit (Arcturus Bioscience inc, CA) was used. After RNA isolation, cDNA was synthesized using 2 nmol of Pd(N)6 primer (Pharmacia Biotech, Roosendaal, The Netherlands) and 160 U of mouse moloney leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Breda, The Netherlands). The reaction mixture further contained 8 mmol/L dithiothreitol, 1 mmol/L of each dNTP, 1x first strand buffer (50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$) and 24 U of RNase inhibitor (Boehringer Mannheim). The reaction was performed for 15 minutes at 37°C after which the enzyme was inactivated during 10 minutes at 95°C.

PCR was performed using Taq DNA polymerase (Life Technologies), 10 uM dNTPs (Pharmacia Biotech) and 1,5 mM $MgCl_2$ in 1xPCR buffer (Life Technologies). Primers used were: HGFA forward (5'-AGGACACAAGTG CCAGATTG-3); HGFA reverse (5'-GTTGATCCA GTCCACACATAGT-3'); MET forward (5'-GAGAC TCATAATCCAACTG-3'); MET reverse (5'-AGCA TACAGTTTCTTGCAG-3'); HGF forward (5'-CAG CATGTCCTCCTGCATCTCC-3') and HGF reverse (5'-TCGTGTGGTATCATGGAACTCC-3').

Results

MET is expressed in human GC B cells and plasma cells

We investigated the expression of MET mRNA (Fig. 1B) and protein (Fig. 1A) in human tonsillar B cell subpopulations by using purified B cell subsets isolated by FACS sort ing or by FACS analysis of unsorted B cell fractions, respectively. As shown in Figure 1, we detected MET mRNA (Fig. 1B) and protein expression by GC B cells (Fig. 1A, C) but not by naive- (IgD+, CD38-) and memory B (IgD-, CD38-) cells. In addition, we observed that

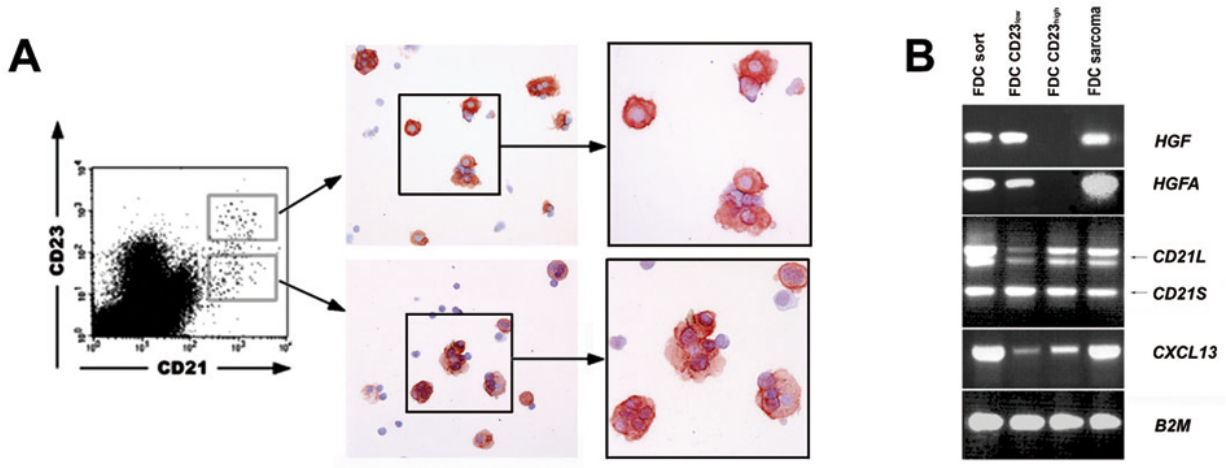


Figure 3 **Dark zone FDCs express HGF and HGFA.**

A. Highly purified FDCs were collected from enriched FDC preparation after FACS sorting by using the markers CD21 and CD23. The purity of both sorted FDC fractions was determined by restaining the cells with FITC- conjugated anti-human CD21, followed by HRP-conjugated anti-FITC and detection with AEC. Inset: high magnification of the cells. **B.** CD21+ FDCs, CD23low FDCs (GC dark zone), CD23high (GC light zone) and FDC sarcoma were analyzed for the expression of HGF, HGFA, CD21, and CXCL13 using RT-PCR. β2-microglobulin was used as housekeeping gene control.

MET is expressed by plasma cells (Fig. 1A-C), which represent a small (~1-4%) subset of tonsillar cells, characterized by a high expression of CD38 [30]. Consistent with the results obtained by studying isolated B cell subsets, immunohistochemical studies on tissue sections of human tonsils and lymph nodes also demonstrated MET expression on GC B cells (Fig. 1D). This expression was most prominent in the GC dark zones, indicating preferential expression of MET on centroblasts. Taken together, these data confirm our previous observation that MET is expressed by centroblasts [18] and, moreover, demonstrate that plasma cells also express MET.

Plasma cells and FDCs express the serine protease HGF-activator

We have previously shown that FDCs express and secrete HGF, the ligand of MET [18]. However, for biological function, proteolytic conversion of single-chain HGF (scHGF) to the active heterodimeric form is essential [21]. In a recent study, we demonstrated that MM plasma cells produce HGFA and in this way may activate HGF in the bone marrow microenvironment [26]. To assess whether HGFA is expressed and mediates HGF conversion in normal lymphoid tissue, we first assessed the expression of HGFA mRNA and protein by B cells and plasma cells. Interestingly, purified plasma cells strongly expressed HGFA mRNA (Fig. 2A) and protein (Fig. 2B), whereas HGFA expression in naive-, memory- and GC B cells was either weak or absent. In contrast to HGFA, HGF mRNA was neither detected in the purified B cell subsets nor the plasma cells. The observed expression of HGF mRNA in the unsorted B cell fractions is explained by the presence of FDCs (see below).

To confirm and extend these observations, we also performed immunocytochemistry on human tonsillar B cells depleted of T cells and monocytes (Fig. 2C). This population consisted of >97% CD20 positive cells and did not contain monocytes (CD14+) or T cells (CD3+). Of note, scattered cells within this population showed a strong expression of HGFA (Fig. 2C). Apart from a few cells with a plasmacytoid morphology, these HGFA positive cells showed morphological features of FDCs, i.e., large oval cells with an eccentric or double nucleus (Fig. 2C). Indeed, immunocytochemical stainings on FACS-sorted FDCs showed a distinct granular HGFA

expression pattern (Fig. 2D), while immunoblotting confirmed the expression of HGFA (Fig. 2E). Hence, both plasma cells and FDCs express HGFA.

HGF and HGFA are specifically expressed by GC dark zone FDCs

GCs consist of dark and light zones. The FDCs within these distinct functional compartments show marked phenotypic differences: whereas both dark- and light zone FDCs express the long isoform of CD21 (CD21L), FDCs in the light zone express additional molecules, including CD23, FcγRII, VCAM-1, and CXCL13 [1,8,9,31,32]. To assess whether HGF and HGFA are also differentially expressed in dark- and light zone FDCs, these cells were separated based on differential CD23 expression (Fig. 3A) [33]. Whereas no expression of HGF and HGFA mRNA was detected in the CD23high FDCs, the expression of both transcripts was prominent in the CD23low FDCs (Fig. 3B). Hence, HGF and HGFA expression is a feature of dark zone FDCs. In addition to differential HGF and HGFA expression, we also observed differential expression of CXCL13 mRNA in the CD23 sorted FDCs, confirming the study by Allan et al. [9] showing that this chemokine is predominantly expressed by GC light zone FDCs.

Expression of HGF and HGFA by FDC sarcoma

To corroborate our finding that FDCs express both HGF and HGFA, we also studied the expression of HGF and HGFA mRNA in FDC sarcoma, a rare neoplasm composed of malignant FDCs [29]. Like in normal dark zone FDCs, both HGF and HGFA were prominently expressed by this tumor (Fig. 3B). We were unable to detect MET expression on the FDC sarcoma cells (data not shown), indicating the absence of an autocrine HGF/MET loop in this tumor.

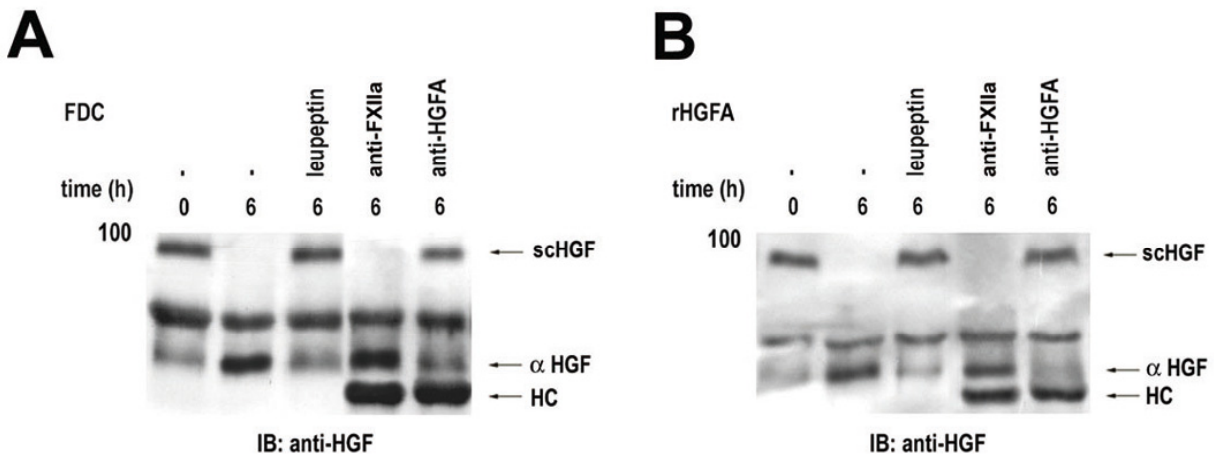


Figure 4 **FDCs autocatalyze HGF activation by producing HGFA.**

A. Conditioned medium of enriched FDC preparation was incubated with scHGF for 6 hours in the presence of thrombin, combined with either the serine protease inhibitor leupeptin, neutralizing antibody against HGFA (PI-4) or against Factor XII, as indicated. **B.** As positive control, HGF conversion by recombinant HGFA is shown. HGF conversion was determined by immunoblotting with anti-HGF. scHGF= single chain of HGF; αHGF= α chain of HGF; HC= heavy chain of immunoglobulin.

FDCs convert HGF by producing HGFA

Subsequently, we investigated whether FDCs are able to process scHGF to its active form. Conditioned media of FDCs effectively converted scHGF (Fig. 4). This required thrombin, while the conversion was completely inhibited by the serine protease inhibitor leupeptin (Fig. 4A). Since proteases other than HGFA are, although with low efficiency, capable of activating scHGF in vitro, we explored whether the conversion of scHGF by FDCs could be inhibited by specific interference with HGFA activity. We observed that the anti-HGFA monoclonal P1-4, which blocks HGFA function, effectively inhibits scHGF conversion by FDCs (Fig. 4A). These findings identify HGFA as the (major) serine protease responsible for the conversion of scHGF by FDCs and identify FDCs as important regulators of HGF activity in the GC microenvironment.

Discussion

Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling [21], In this study, we present data indicating an important role of the serine protease HGFA in regulating HGF activation in normal lymphoid tissue. We demonstrate that HGFA is expressed by plasma cells but not by other B-lineage populations. Furthermore, we show that HGFA is strongly expressed by FDCs and is confined to FDCs in the GC darkzone. These cells co-express HGF and are in close contact with MET-positive centroblasts. Our findings suggest specific functions of HGF/MET pathway in Ag-specific B cell differentiation, affecting plasma cell- and centroblast function.

Previous studies from other and our own laboratories have implicated the HGF/MET pathway in the pathogenesis of the plasma cell malignancy MM [34-37]. Our current study represents the first study of HGF/MET pathway components in normal plasma cells. We observed that normal plasma cells express the receptor tyrosine kinase MET, but do not express its ligand HGF (Fig. 1, 2A). In addition, we show that HGFA is expressed by plasma cells but not by other B-lineage populations (Fig. 2A, B). The co-expression of MET and HGFA in plasma cells is of interest since it indicates that plasma cells are well equipped to receive paracrine HGF signals. Indeed, tonsillar- as well as bone marrow stromal cells have been reported to produce HGF, and it is hence conceivable that plasma cells receive paracrine stimulation from the microenvironment [18,20,38]. Contrary to their malignant counterparts [35], normal plasma cells do not express HGF, and hence do not possess an autocrine HGF/MET loop. Although the functional consequences of HGF/MET signaling in plasma cell have not yet been explored, like in B cells [18,20,39], the pathway might regulate integrin-mediated adhesion and promote migration. HGFA expression by plasma cells could thus play a role in their homing to the bone marrow and control their integrin-mediated interaction with bone marrow stromal cells. These stromal cells produce cytokines that support plasma cell survival, including IL5, IL6, TNFα, and SDF-1. In view of the potent effects of HGF on the survival of MM plasma cells [37], it is conceivable that HGF produced by bone marrow stromal cells also contributes to plasma cell survival.

Differential protein expression within the GC dark- and light zone reflects the distinct processes that take place within these compartments. One of the key findings of our study is that FDCs are able to autocatalyze HGF activation by producing both HGF and HGFA (Fig. 4) and that HGFA and HGF expression by FDCs is confined to the CD23high subset, which resides in the GC dark zone (Fig. 3B). Combined with our previous observation that MET is selectively expressed by B cells in the GC dark zone, i.e. by centroblasts, these observations suggest specific functions for the HGF/MET pathway in the GC dark zone. HGF stimulation of B cells has been shown to mediate integrin activation, promoting B cell adhesion to

VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) [11,19], two important integrin ligands on FDCs [10,11]. Hence, active HGF in the GC dark zone could play a role in initiating physical contact of MET-positive centroblast, which have downregulated their BCR, with FDCs. Upon transition to the light zone, the B cells, now centrocytes, re-express their BCR, which engages in interactions with antigen presented by FDCs. At this stage, high affinityinteractions of the BCR with antigen could take over the regulation of the integrin activity required for B cell-FDC interaction [12]. Alternatively, MET-mediated growth and survival signals might directly contribute to the expansion of centroblasts. These signals could be crucial at a differentiation stage at which the cells are largely devoid of BCR expression, and may not receive sufficient growth and survival signals.

Cell migration within the lymphoid organs is directed by chemokines and is essential during GC reaction. The chemokine CXCL13 produced by follicular stromal cells is required for recruiting Ag-activated B cells to the GC [8]. We observed that CD23high FDCs expressed high levels of CXCL13 mRNA compared to CD23low FDCs (Fig. 3B). This observation confirms that of Allen et al, who by immunohistochemical staining of the GC and by microdissection of

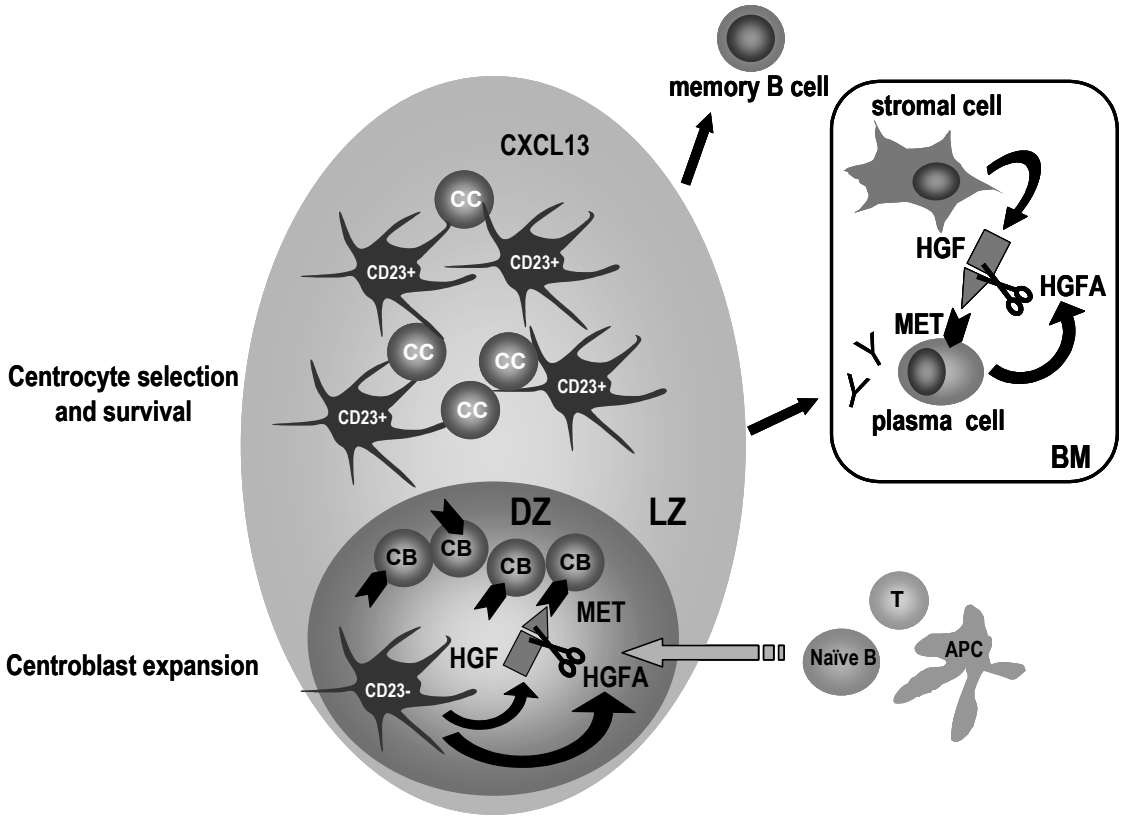


Figure 5 **Components of the HGF/MET pathway in the GC- and bone marrow microenvironment.**

During B cell differentiation, HGF and HGFA produced by the dark zone (DZ) FDCs ($CD23^-$) may act as proliferative- and survival factors for the MET-positive centroblasts (CB). In the light zone (LZ), where the FDC network is more extensive, the centrocytes (CC) are found in tight association with FDCs ($CD23^+$) and via direct physical contact, the CCs receive survival signals. After B cell differentiation, the plasma cells home to the bone marrow (BM), where BM stromal cells secrete HGF. The MET-positive plasma cells autoactivate HGF by producing HGFA, which may contribute to plasma cell survival.

the different GC regions, also demonstrated that CXCL13 is more abundantly expressed in the GC light zone than in the dark zone. Mice lacking CXCL13 (BLC) or its receptor, CXCR5, show defects in GC localization and size, indicating an important role in GC organization [8,40]. CXCR4 and its ligand SDF-1 also contribute to GC organization, specifically to sorting of centroblasts into the GC dark zone [9].

Several studies have implicated the HGF/MET pathway in the pathogenesis of B cell neoplasia [18,19,34-37,39]. Expression of MET has been demonstrated in MMs and a subset of non-Hodgkin's lymphomas. Activation of the pathway in these tumors may involve autocrine stimulation since co-expression of HGF and MET has been observed [35,41-43]. In MMs, MET activation promotes proliferation and survival [37]. We observed that MMs, compared to normal plasma cells, strongly expressed HGFA as well as MET protein (Fig. 2B). Overexpression of HGFA by tumor cells may enhance HGF/MET signaling, promoting cell growth and survival, and may contribute to disease progression. In the FDC sarcoma tested (Fig. 3B), we observed strong expression of both HGF and HGFA, suggesting that the tumor was related to GC dark zone FDCs. However, the tumor cells did not express MET, excluding a role for autocrine HGF/MET signaling in this tumor.

In summary, our study indicates that FDCs regulate the bioavailability of HGF within the GC microenvironment, a function that may contribute to the control of B cell growth, survival and adhesion during normal B cell differentiation within lymphoid tissue (Fig. 5).

Acknowledgements

We thank Berend Hooibrink for sorting the B cells and FDCs, and the co-workers of the Department of Otolaryngology of the Sint Lucas/Andreas Hospital Amsterdam for their kind support.

Reference list

1. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-139.
2. Liu YJ, Arpin C. Germinal center development. Immunol Rev. 1997;156:111-126.
3. Kelsoe G. The germinal center reaction. ImmunolToday. 1995;16:324-326.
4. Manser T. Textbook germinal centers? J Immunol. 2004;172:3369-3375.
5. McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG. Germinal center reaction. Curr Opin Hematol. 2001;8:52-59.
6. Lindhout E, Koopman G, Pals ST, de Groot C. Triple check for antigen specificity of B cells during germinal centre reactions. Immunol Today. 1997;18:573-577.
7. Legler DF, Loetscher M, Roos RS et al. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. J Exp Med. 1998;187:655-660.
8. Ansel KM, Ngo VN, Hyman PL et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature. 2000;406:309-314.
9. Allen CD, Ansel KM, Low C et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol. 2004;5:943-952.
10. Freedman AS, Munro JM, Rice GE et al. Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110. Science. 1990;249:1030-1033.
11. Koopman G, Parmentier HK, Schuurman HJ et al. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways. J Exp Med. 1991;173:1297-1304.
12. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. J Exp Med. 2003;198:1539-1550.
13. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J Immunol. 1994;152:3760-3767.
14. Koopman G, Keehnen RM, Lindhout E et al. Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. Eur J Immunol. 1997;27:1-7.
15. Nakamura T, Nishizawa T, Hagiya M et al. Molecular cloning and expression of human hepatocyte growth factor. Nature. 1989;342:440-443.

16. van der Voort R, Taher TE, Derksen PW et al. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. Adv Cancer Res. 2000;79:39-90.
17. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915-925.
18. van der Voort R, Taher TE, Keehnen RM et al. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. J Exp Med. 1997;185:2121-2131.
19. Weimar IS, de Jong D, Muller EJ et al. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins. Blood. 1997;89:990-1000.
20. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology. 2001;102:506-514.
21. Naka D, Ishii T, Yoshiyama Y et al. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J Biol Chem. 1992;267:20114-20119.
22. Naldini L, Tamagnone L, Vigna E et al. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J. 1992;11:4825-4833.
23. Shimomura T, Miyazawa K, Komiyama Y et al. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem. 1995;229:257-261.
24. Miyazawa K, Shimomura T, Kitamura N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem. 1996;271:3615-3618.
25. Kataoka H, Hamasuna R, Itoh H, Kitamura N, Koono M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. Cancer Res. 2000;60:6148-6159.
26. Tjin EP, Derksen PW, Kataoka H, Spaargaren M, Pals ST. Multiple myeloma cells catalyze hepatocyte growth factor (HGF) activation by secreting the serine protease HGF-activator. Blood. 2004;104:2172-2175.
27. Liu YJ, Xu J, de Bouteiller O et al. Follicular dendritic cells specifically express the long CR2/CD21 isoform. J Exp Med. 1997;185:165-170.
28. van Adelsberg J, Sehgal S, Kukes A et al. Activation of hepatocyte growth factor (HGF) by endogenous HGF activator is required for metanephric kidney morphogenesis in vitro. J Biol Chem. 2001;276:15099-15106.
29. Nakashima T, Kuratomi Y, Shiratsuchi H et al. Follicular dendritic cell sarcoma of the neck; a case report and literature review. Auris Nasus Larynx. 2002;29:401-403.
30. Terstappen LW, Johnsen S, Segers-Nolten IM, Loken MR. Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. Blood. 1990;76:1739-1747.
31. Camacho SA, Kosco-Vilbois MH, Berek C. The dynamic structure of the germinal center. Immunol Today. 1998;19:511-514.
32. Balogh P, Aydar Y, Tew JG, Szakal AK. Appearance and phenotype of murine follicular dendritic cells expressing VCAM-1. Anat Rec. 2002;268:160-168.
33. Hardie DL, Johnson GD, Khan M, MacLennan IC. Quantitative analysis of molecules which distinguish functional compartments within germinal centers. Eur J Immunol. 1993;23:997-1004.
34. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996;88:3998-4004.
35. Borset M, Lien E, Espevik T et al. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem. 1996;271:24655-24661.
36. Derksen PW, Keehnen RM, Evers LM et al. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002;99:1405-1410.
37. Derksen PW, de Gorter DJ, Meijer HP et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003;17:764-774.
38. Takai K, Hara J, Matsumoto K et al. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood. 1997;89:1560-1565.
39. Jucker M, Gunther A, Gradl G et al. The Met/hepatocyte growth factor receptor (HGFR) gene is overexpressed in some cases of human leukemia and lymphoma. Leuk Res. 1994;18:7-16.
40. Voigt I, Camacho SA, de Boer BA et al. CXCR5-deficient mice develop functional germinal centers in the splenic T cell zone. Eur J Immunol. 2000;30:560-567.
41. Seidel C, Borset M, Turesson I et al. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood. 1998;91:806-812.
42. Zhan F, Hardin J, Kordsmeier B et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and

normal bone marrow plasma cells. Blood. 2002;99:1745-1757.
43. Capello D, Gaidano G, Gallicchio M et al. The tyrosine kinase receptor met and its ligand HGF are co-expressed and functionally active in HHV-8 positive primary effusion lymphoma. Leukemia. 2000;14:285-291.

CHAPTER **3**

Aberrant HGF activator expression and functional analysis of HGF/MET signaling in diffuse large B cell lymphoma

Esther P.M. Tjin, Richard Groen, Irma Vogelzang, Patrick W. B. Derksen, Melanie D. Klok, Helen P. Meijer, Susanne van Eeden, Steven T. Pals, Marcel Spaargaren

Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

Inappropriate activation of MET, the receptor tyrosine kinase for hepatocyte growth factor (HGF), has been implicated in tumorigenesis. Although we have previously shown that HGF/MET signaling controls survival and proliferation of multiple myeloma (MM), its role in the pathogenesis of other B cell malignancies has remained largely unexplored. Here, we have examined a panel of 110 B cell malignancies for MET expression, which, apart from MM (48%), was found to be largely confined to diffuse large B cell lymphomas (DLBCL) (30%). No amplification of the *MET* gene was found, however, mutational analysis revealed two germline missense mutations: R1166Q in the tyrosine kinase domain in one patient, and R988C in the juxtamembrane domain in four patients. The R988C mutation has recently been shown to enhance tumorigenesis. In MET-positive DLBCL cells, HGF induces MEK-dependent activation of ERK and PI3K-dependent phosphorylation of PKB, GSK3 and FOXO3a. Furthermore, HGF induces PI3K-dependent $\alpha 4\beta 1$ integrin-mediated adhesion to VCAM-1 and fibronectin. Within the tumor-microenvironment of DLBCL, HGF is provided by macrophages, whereas DLBCL cells themselves produce the serine protease HGF activator (HGFA), which autocatalyzes HGF activation. Taken together, these data indicate that HGF/MET-signaling, and secretion of HGFA by DLBCL cells contributes to lymphomagenesis in DLBCL. *Submitted.*

B cell lymphomas represent the malignant counterparts of normal B cells, arrested at specific maturational stages. They are classified into distinct disease categories based on their stage-specific morphological features, molecular profile, and B cell receptor (BCR) configuration [1]. The initial step in lymphomagenesis is the acquisition of a genetic aberration, most often a chromosomal translocation involving a proto-oncogene, causing an increased lifespan and/or enhanced proliferation [2]. In general, this event *per se* is not tumorigenic but further (multiple) genetic alterations are required for the development of a fully malignant phenotype. In addition to these oncogenic events, B cell malignancies require signals from the microenvironment for their growth, survival and progression. These

signals, which include B cell receptor (BCR) stimulation by antigen [3], direct physical contact of (malignant) B cells with stromal cells via integrin adhesion receptors [4-7], as well as a number of cytokine/growth factors [8], activate intracellular signaling cascades and present potential targets for therapeutic intervention. One of the candidate growth factors in B cell malignancies is hepatocyte growth factor (HGF) [9-11].

HGF induces complex biological responses in target cells, including adhesion, motility, growth, survival and morphogenesis by activating the tyrosine kinase receptor MET. HGF/MET signaling is indispensable for mammalian development, while uncontrolled activation of MET is oncogenic, and has been implicated in the growth, invasion, and metastasis of a variety of tumors [12,13]. Several distinct mechanisms may underlie uncontrolled MET activation. These include translocation, amplification or mutation of the MET gene [12,14-19], and autocrine- or paracrine HGF production [14,20,21].

In B cells, the HGF/MET pathway has been implicated in differentiation, specifically in the regulation of adhesion and migration [13,22]. We have previously demonstrated that the MET protein is expressed on GC B cells [22], whereas follicular dendritic cells (FDC) and stromal cells express HGF [22,23]. Furthermore, we and others have identified the HGF/MET pathway as a potentially important signaling route in lymphomagenesis [9,13,24,25]. In several B cell malignancies, including multiple myeloma (MM) [24,26,27], primary effusion lymphoma (PEL) [28] and Hodgkin's lymphoma (HL) [9], co-expression of HGF and MET has been observed, suggesting autocrine activation of HGF/MET signaling. Furthermore, in MM, HL and diffuse large B cell lymphoma (DLBCL), elevated serum HGF levels correlate with unfavorable prognosis [9-11]. Moreover, we have recently shown that HGF induces a potent proliferative and anti-apoptotic response in MM cell lines and primary MMs [24,29]. Together with our observation that MM cells themselves produce the serine protease HGF activator (HGFA), and thereby are able to autocatalyze HGF activation [30], the above data suggest an important role for the HGF/MET pathway in the pathogenesis of MM. To examine the possible role of HGF/MET in the pathogenesis of other B cell malignancies, we have studied the expression of MET and HGF in a large panel of B cell malignancies, including all major B-cell non-Hodgkin lymphoma (B-NHL) subtypes, and we analyzed the MET gene in MET-positive lymphomas for the presence of amplification and mutations. Furthermore, we have defined the role of the HGF/MET pathway, including HGFA, in the most common type of B-NHL, i.e. DLBCL.

Materials and Methods

Antibodies and reagents

Mouse monoclonal antibodies used were: anti-CD68 (IgG1); anti-CD21L (DRC-1, IgM); FITC-conjugated anti-IgD (all DAKO, Carpinteria, CA); anti-MET, DO24 (IgG2a)(Upstate Biotechnology, Lake Placid, NY)(IgG1); APC-conjugated anti-CD38 (IgG1)(BD Biosciences, Erembodegem, Belgium); anti-HGFA A-1 (IgG1) and P1-4 (IgG1) [31]; anti-factor XIIa, OT-2 (IgG1) (Sanquin, Amsterdam, The Netherlands); anti-CD20 (L26) (DAKO, Glostrup, Denmark); fluorescein isothiocynate (FITC)-conjugated anti-human IgD (DAKO); allophycocyanin (APC)-conjugated anti-human CD38 (IgG1) (BD Biosciences); phycoerythrin (PE)-conjugated anti-human CD20 (DAKO); and antibodies against the integrin subunit α4 (CD49d) (HP2/1, IgG1) (Immunotech, Marseille, France) and $\alpha 4\beta 7$ (Act-1, IgG1) (a gift from A. Lazarovits, University of Western Ontario, London, Canada). Polyclonal antibodies used were: goat anti-human HGF (R&D Systems, Abindong, UK), rabbit anti-MET (C12), rabbit anti-PKB, rabbit anti-ERK1 and -2 (all from Santa Cruz, Biotechnology, Santa Cruz, CA); AP-conjugated goat anti-mouse; biotin-conjugated rabbit anti-mouse (both DAKO); RPE-conjugated rabbit anti-mouse IgG2a (BD Biosciences); AP-conjugated anti-digoxygenin (Roche, Almere, The Netherlands) HRP-conjugated swine anti-goat

(Biosource, Camarillo, CA); HRP-conjugated rabbit anti-mouse (DAKO). Antibodies against phoshorylated MET pY1230/1234/1235 (Biosource, Camarillo, CA); phospho-FOXO3a (FKHRL1, Thr32) (Upstate, Charlottesville, Virginia); rabbit anti-phospho-GSK3 α/β (Ser21 and Ser9); rabbit anti-phospho PKB/AKT (Ser 473); rabbit anti-phospho p44/42 MAP kinase (Thr 202/Tyr 204) (all from Cell Signaling, Beverly, MA). Reagents used were: recombinant HGF (ReliaTech GmbH, Braunschweig, Germany); recombinant single chain HGF (R&D Systems); the PI3K inhibitors LY294002 and Wortmannin (both Biomol, Plymouth Meeting, PA); the MEK inhibitor PD98059 (Alexis).

B cell tumors and DLBCL cell lines

Tissue samples of 89 cases of B-NHL and 21 cases of MM were obtained were obtained during standard diagnostic procedure at the Academic Medical Center Amsterdam, The Netherlands and the University Medical Center Utrecht (UMCU, Utrecht, The Netherlands), and frozen at –80 °C until further use. Mononuclear cells from BM-derived MM samples were obtained by standard Ficoll-Paque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden). All B cell malignancies were classified according to the WHO classification 1. DLBCLs cell lines OCI-LY-1, -3, -7 and -18 were cultured in Iscove's medium (Life Technologies, Breda, The Netherlands) supplemented with 10 % fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT), penicillin (50 U/ml), streptomycin (50 μg/ml) (both from Life Technologies). DLBCLs cell line OCI-LY-10 was cultured in the presence of 20% FCS, penicillin, streptomycin and β-mercaptoethanol (55 μM). OCI-LY-1, -7 and –18 were kindly provided by Dr. U. Klein (Institute for Cancer Genetics, Colombia University, NY); OCI-LY-3 and –10 were kindly provided by Dr. R. Küppers (Institute for Genetics and Department of Internal Medicine, University of Cologne, Germany). B cells were purified from human tonsils obtained from children undergoing routine tonsillectomy as described [7]. Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden). Monocytes and T cells were depleted by plastic adherence and sheep red blood cells rosetting, respectively. The B cell fraction was >97% pure and contained ≈ 60% naïve B cells and ≈ 35% GC B cells as determined by FACS analysis using FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20 and APC-conjugated anti-CD38. To obtain GC B cells, total B cells were stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD20 and APC-conjugated anti-CD38 and sorted using a FACS aria (BD).

Single stranded conformation polymorphism (SSCP)

High molecular weight genomic DNA was obtained employing standard methods by lysis in SDS, proteinase K digestion, phenol-chloroform extraction and ethanol precipitation (all Sigma, Bornem, Belgium). PCR was performed by amplifying exon 14, and 16 to 19 of MET, using intron-specific primers as described [16]. Integrity of the DNA was confirmed through amplification of the β-globin gene using primers pair GH20/PCO4. The radioactive PCR amplification was carried out in a 30 μl reaction mixture containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 10 mM of dATP, dTTP, and dGTP, 15 pmol of primer, 1.5 mCi [α32P] dCTP (Amersham Pharmacia) and 0.3 U of Taq DNA polymerase (Life Technologies). Following PCR, samples were diluted 1:7 in loading buffer (10 mM EDTA, pH 8.0, 0.05% SDS, 95% de-ionized formamid, 0.25% bromophenolblue, and 0.25% xylene cyanodie FF (all Sigma), denatured for 3 min. at 95 °C, and slowly cooled to 4 °C (1 °C /sec). Samples were loaded onto a 8% non-denaturing, 1x TBE acrylamide:bisacrylamide gel (50:1) (Life Technologies), containing 10% glycerol (Sigma), and run in for 16 hours at 8 Watts. Aberrant migrating amplicons were excised from the gel, re-amplified using Pfu

DNA polymerase (Stratagene, La Jolla, CA), cloned into EcoRV-digested pZeRO (Life Technologies), and sequenced using M13 primers and big-dye terminators (Amersham Pharmacia). Nucleotide and amino-acid numbering of MET was done according to the MET sequence as described by Schmidt *et al* [16].

Immunohisto- and cytochemistry

Immunohistochemical stainings were performed as described previously [32] on acetone-fixed cryostat sections (MET and DRC-1) or formalin fixed paraffin embedded sections (CD68). Substrate was developed with either 3,3-diaminobenzidine (Sigma) (anti-MET and CD68 staining), or fast blue BB (DAKO) (anti-CD21L, DRC-1) staining. Immunocytochemical stainings were performed on aceton-fixed cytospins. The cytospins were preincubated with 1% bovine serum albumin (BSA) (Sigma) in PBS for 15 minutes. After incubating with the primary antibody (overnight at 4 °C), endogenous peroxidase was blocked with 0.1% NaN3 and 0.3% H2O2 in PBS for 10 minutes. Subsequently, the cytospins were stained with post-antibody of Powervision (Immunovision Technologies, Duiven, The Netherlands) for 15 minutes, followed by poly-HRP conjugated goat anti-mouse/rabbit IgG for 30 minutes. Substrate was developed with 3,3-amino-9-ethylcarbazole. The immunohistochemical stainings were examined by use of an Olympus BX51 microscope (Olympus Optical, Hamburg, Germany) with a 40/0.85 objective. Images were acquired by an Olympus DP11 camera and processed with Adobe Photoshop 7.

Assay for HGF activation

HGF activation was assayed as described previously [33]. Conditioned medium was obtained as described previously [34]. In brief, 20 μl DLBCL conditioned medium was pretreated with 1 unit of thrombin (Sigma Aldrich Chemie GmbH, Germany) and added to 0.1 μg single chain HGF. Inhibitor studies were done in the presence of leupeptin (500 μg/ml) (Sigma), or neutralizing antibody against HGFA (P1-4) (40 μg/ml).

ELISA for HGF

Conditioned medium of DLBCL cell lines or, as positive controls, of MM cell line UM-3 and follicular dendritic cells was used to detect the production of HGF. The ELISA kit for HGF was used according to the manufacturer's instructions (R&D Systems). The lower detection limit was 125 pg/ml.

Immunoblot analysis

Immunoblotting was performed as described [32]. For signaling experiments, cells were serum-starved for 2 hours and lysed after the indicated treatments. The samples were analyzed by SDS-PAGE. The phosphorylation of MET, PKB, FOXO3a, GSK3 and ERK1 and –2 was examined by phosphorylation-state specific antibodies. After stripping, the same blot was restained with antibodies against MET, PKB and ERK. The samples of the HGF conversion assay or DLBCL cell lines were also analyzed by SDS-PAGE. The immunoblots were stained with anti-HGF or anti-HGFA (A-1) and detected with HRP-conjugated swine anti-goat and HRP-conjugated rabbit anti-mouse, respectively.

Cell adhesion assay

Adhesion assays were done essentially as described [35]. Briefly, 96-well plates were coated with 1 μg/ml soluble vascular cell adhesion molecule-1 (VCAM-1) (R&D Systems) or 10 μg/ml foreskin fibronectin (FN) (Sigma). 1.5 x 105 cells were pre-incubated for 30 min at 4°C in the presence or absence of monoclonal antibodies against integrins or inhibitors, and plated in the absence or presence of recombinant HGF (ReliaTech GmbH) or phorbol-12-myristate-13-acetate (PMA) (Sigma) in 100 μl/well, and incubated at 37 °C for 30 minutes. The adherent cells were stained with crystal violet, washed, the dye was eluted and absorbance was measured

on a spectrophotometer (Microplate Reader 450, Biorad). Background absorbance (no cells added) was subtracted. Maximal adhesion (= 100%) was determined by measuring non-specific adhesion to poly L-lysin-coated wells.

RNA isolation, cDNA synthesis and reverse transcriptase-PCR

RNA isolation and cDNA synthesis was done as described previously [22]. Primers used were: HGFA forward (5'-AGGACACAAGTGCCAGATTG-3); HGFA reverse (5'-GTTGATCCAGTCCACACA TAGT-3'); MET forward (5'-GAGACTCATAATCC AACTG-3'); MET reverse (5'-AGCATACAGTTTC TTGCAG-3'); HGF forward (5'-CAGCATGTCCTC CTGCATCTCC-3') and HGF reverse (5'-TCGTGT GGTATCATGGAACTCC-3').

HGF mRNA in situ hybridization

Snap-frozen tissues were collected and frozen at –80 °C until further use. 10 μm thick sections were cut, recovered on silianized slides, fixed for 15 minutes in 4% paraformaldehyde in PBS, dehydrated using ethanol, dried overnight and stored at –20 °C. Before use, tissue sections were re-hydrated, washed in PBS and incubated for 10 minutes in PBS containing 0.1 M glycine (Sigma). Sections were permeabilized for 15 minutes in 10 ug/ml proteinase K (Roche), after which the sections were washed three times with PBS, treated with 4% PFA/PBS for 10 minutes and washed with 4x SSC. Acetylation was done using acetic acid anhydride and triethanolamidehydrochlorid for 15 minutes and pre-hybridized in hybridization mixture (50% formamid, 5x SSC, 5x Denhardt's, 25 ug/ml baker's yeast tRNA, 500 ug/ml herring sperm DNA) for 1 hour. cRNA probes were synthesized as run-off transcripts using either T3 or T7 RNA polymerase and a digoxygenin RNA labeling kit (Roche). Probes were made from an 847 bp human HGF EcoR1 cDNA fragment (nt 186-1033, kindly provided by Dr W. Birchmeier, MDC, Berlin, Germany) in pBSSK+. Overnight hybridization was done at 58 °C. After hybridization, the slides were rinsed with 4x SSC/50% formamid, and subsequently washed in 4x SSC and 1x SSC, respectively. Sections were treated with 1% PFA/PBS for 30 minutes, washed in 0.1M glycine/PBS and equilibrated in TRIC buffer (100 mM Tris, 150 mM NaCl, pH 7.5). Blocking of non-specific binding was performed in TRICB buffer (TRIC buffer containing 1% blocking reagent (Roche). Slides were incubated with AP-conjugated antidigoxygenin antibody in TRICB buffer. Color development was done using nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT/ BCIP) (Roche) in color buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) containing 2.4 mg/ml levamisole (Sigma).

Table 1

Expression and mutational analysis of MET in B cell tumors

WHO classification	n	% positive (n)	Intensity*	# mutated examined
Precursor B lymphoblastic	3	0 (0)	-	0/3
Mantle Cell	5	0 (0)	-	0/3
Follicular	15	7 (1)	2	1/15
Burkitt's	12	8 (1)	3	1/12
DLBCL	43	30 (13)	2-3	2/39
Marginal Zone	3	0 (0)	-	ND
B-CLL	8	25 (2)	1	1/8
Multiple Myeloma	21	48 (10)	NA (Φ)	0/21

* 1=weak, 2=moderate, 3=strong staining with a monoclonal anti-MET antibody. N=number of samples; B-CLL=B cell chronic lymphocytic leukemia; DLBCL=diffuse large B cell lymphoma. NA=non applicable. (Φ)=expression of MET by multiple myeloma was determined by western blot analysis using a polyclonal anti-MET antibody. ND=not done.

Results

MET expression in B cell malignancies

To investigate the expression of MET in B cell malignancies, a panel of 110 B cell tumors of different subtypes, representing a broad spectrum of differentiaton stages ranging from precursor-B cell to plasma cell was analyzed by immunohistochemistry and/or immuno-blotting. A few cases of follicular- (FL), Burkitt's- (BL), and chronic lymphocytic lymphoma (CLL) showed MET expression (table 1), whereas MET was not detected on precursor-B cell lymphomas, mantle cell lymphomas and marginal zone lymphomas (table 1). MET expression was largely confined to MMs (48%) and DLBCLs (30%) (table 1). In several cases of DLBCL, which is the most common type of B cell non-Hodgkin lymphoma (B-NHL) (30-40% of cases), staining of MET was very strong (figure 1A, right panel), indicating overexpression relative to the

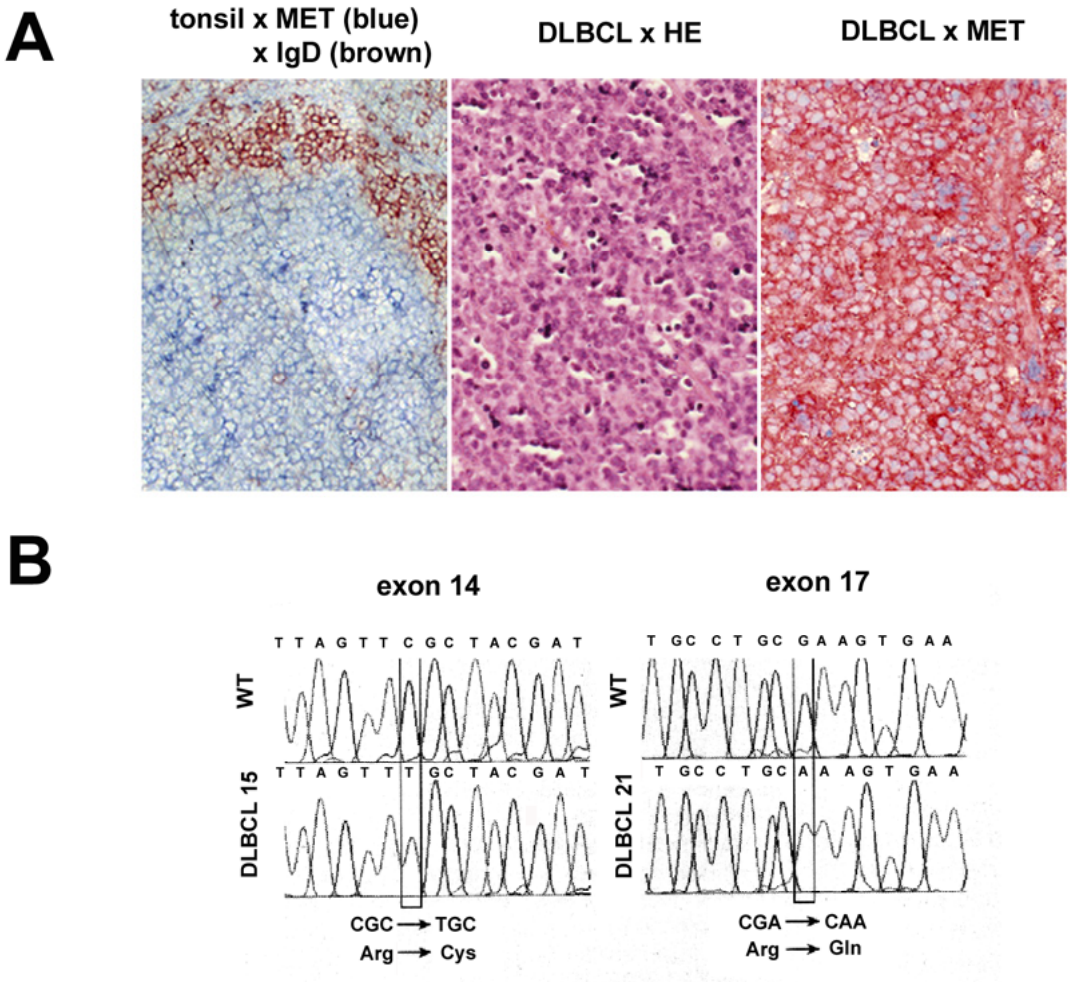


Figure 1

MET expression and missense germline MET mutations in DLBCL

A. MET expression in normal lymphoid tissue and primary DLBCL. Immunohistochemical double staining of tonsillar sections for MET (blue) and IgD (brown), showing MET expression on germinal center B cells (left panel). Frozen tissue sections of DLBCL were stained with haematoxylin-eosin (HE) (middle panel) or with haematoxylin and anti-MET (brown, right panel). Image magnification: 200x. **B.** Sequence analysis showing mutational transitions. PCR products were excised, re-amplified, cloned and sequenced. Shown are wild type (WT) and mutant sequences. Mutational transitions are boxed.

expression levels in normal GCs (figure 1A, left panel).

MET mutations in B cell lymphomas

Amplification of the MET gene resulting in MET overexpression has been described in several types of cancer [14,20,36]. However, Southern blot analysis using a MET-specific cDNA probe did not reveal MET gene amplification in the MET-positive lymphomas (data not shown). Furthermore, missense germline or somatic MET mutations have been found in HPRC [16,17], and in several other types of cancer [18,19,37]. The affected regions of MET are the catalytic- or the juxtamembrane (JM) domain, resulting in deregulated activation or degradation of MET, respectively [19,38,39]. Upon HGF stimulation, cells with MET mutations in these regions display enhanced or prolonged kinase activity, resulting in transformation, invasive growth and enhanced tumor cell survival [38-40]. To investigate whether MET mutations may also contribute to lymphomagenesis, we screened the tumor samples listed in table 1 for mutations in exon 14, encoding the JM region, and exon 16 to 19, encoding the tyrosine kinase and docking site regions of MET, by means of SSCP analysis. Using this approach, two distinct missense mutations were detected in MET (figure 1B). The first was at position 2961 (C to T) in exon 14, resulting in a nonconservative transition from arginine to cysteine at position 988 (R988C), and was found in 4 individual cases of CLL, FL, BL, and DLBCL. The second mutation (3496 G to A) in exon 17, resulting in a transition from arginine to glutamine at position 1166 (R1166Q), was detected in one case of DLBCL. Analysis of normal tissues from the affected individuals revealed that these mutations were germline mutations. Noteworthy, in none of the 5 DLBCL cell lines these mutations were found (data not shown). During the course of our study, the R988C mutation was also reported in several (non-) small cell lung cancer ((N)SCLC) cell lines [41,42], and in 2 lung cancer patients [41,43]. Interestingly, these studies revealed that R988C conveys enhanced in vitro tumorigenicity as well as lung tumor susceptibility in mice [42,43], indicating that R988C is a gain-of-function mutation.

HGF/MET signaling in DLBCL cells

Apart from MM, MET expression was largely restricted to DLBCLs (Table 1). Interestingly, in DLBCL high serum HGF levels were previously found, which were shown to be correlated with unfavorable prognosis [10,44]. In addition, a gene-profiling study showed significantly enhanced expression of MET upon transformation of low-grade FLs into DLBCLs 45, suggesting a pathogenic role for HGF/MET signaling in tumor progression. Therefore, we decided to explore the functionality of MET signaling in DLBCL cells and to examine which signaling pathways become activated upon HGF stimulation. For this purpose, we analyzed a panel of DLBCL cell lines for MET expression. In a subset of DLBCL cell lines (3/5), expression of MET was observed at mRNA (figure 2A) and protein level (figure 2B). HGF stimulation resulted in enhanced phosphorylation of MET in the strongly MET-positive DLBCL cell lines OCI-LY-3 and LY-10, whereas the weakly MET-positive cell line OCI-LY-1 showed a weak response (figure 2C). Moreover, HGF stimulation of the MET-positive cells leads to phosphorylation of the mitogen activated protein kinases ERK1 and –2, as well as to phosphorylation of PKB and of the PKB substrates GSK3 and the forkhead transcription factor FOXO3a (FKHRL1) (figure 2C). The unrelated PI3K inhibitors Wortmannin (WM) or LY294002 (LY) both completely abrogated the HGF-induced phosphorylation of PKB, GSK3 and FOXO3a, but hardly affected the phosphorylation of ERK-1/2 (figure 2D). Vise versa, the HGF-stimulated activation of ERK-1/2 was specifically blocked by the MEK inhibitor PD98059 (PD), which did not affect phosphorylation of PKB, GSK3 or FOXO3a (figure 2D). The RAS downstream effector components MEK and ERK-1/2 have been

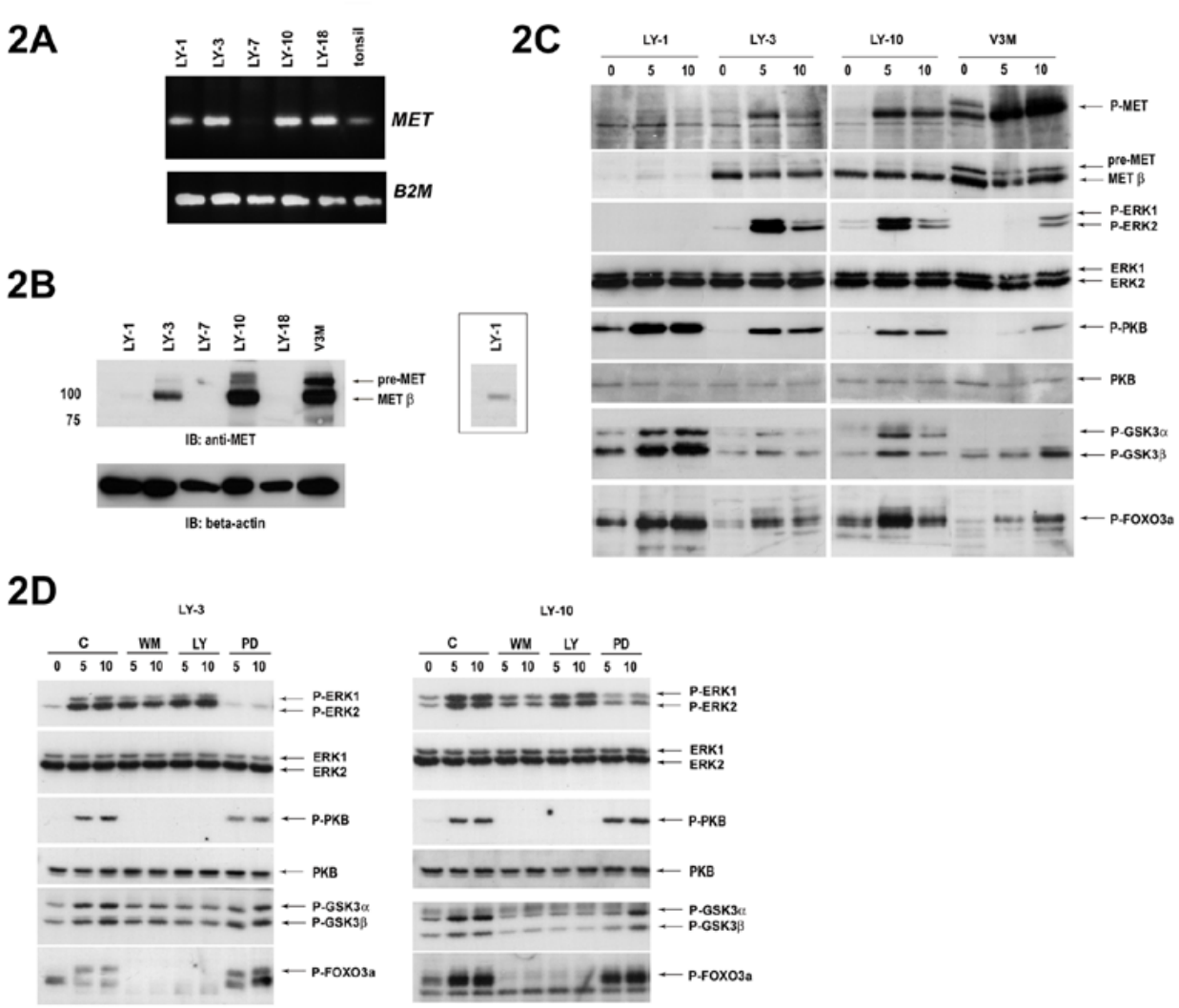


Figure 2

HGF induces phosphorylation of MET in DLBCL cells, and activates the RAS/MAPK and PI3K/PKB pathway.

A. mRNA expression of MET in DLBCL cell lines. After RNA isolation and cDNA synthesis, RT-PCR for MET was performed. β2-microglobulin was used as housekeeping gene control.

B. MET protein expression in DLBCL cell lines. DLBCL cell lines were analyzed by immunoblotting for the expression of MET. The (weak) expression of MET by OCI-LY-1 cells is clearly demonstrated by means of a 3 times longer exposure (see inset). Staining with anti-β-actin represents the loading control. **C.** HGF induces tyrosine phosphorylation of MET, PKB, FOXO3a, GSK3 and ERK. The DLBCL cells OCI-LY-1, -3 and −10 and MET transfected Namalwa cells (V3M) were stimulated with HGF for the indicated time periods. Cell lysates were immunoblotted with phosphorylation-specific antibodies against MET, FOXO3a, GSK3, PKB and ERK. The blots were stripped and restained with antibodies against MET, PKB and ERK.

D. HGF-induced phosphorylation of FOXO3a and GSK3 requires PI3K activity, whereas phosphorylation of ERK1 and -2 is MEK dependent. OCI-LY-3 and -10 cells were pretreated with the PI3K inhibitors wortmannin (WM) (50 μM) or LY294002 (LY) (20 μM), the MEK inhibitor PD98059 (PD) (50 μM), or DMSO (C) for 30 minutes, prior to incubation with HGF (200 ng/ml). Phosphorylation of ERK-1 and −2, PKB, GSK3 and FOXO3a was determined by immunoblotting with phosphorylation-specific antibodies. The blots were stripped and restained for ERK1 and −2, and PKB.

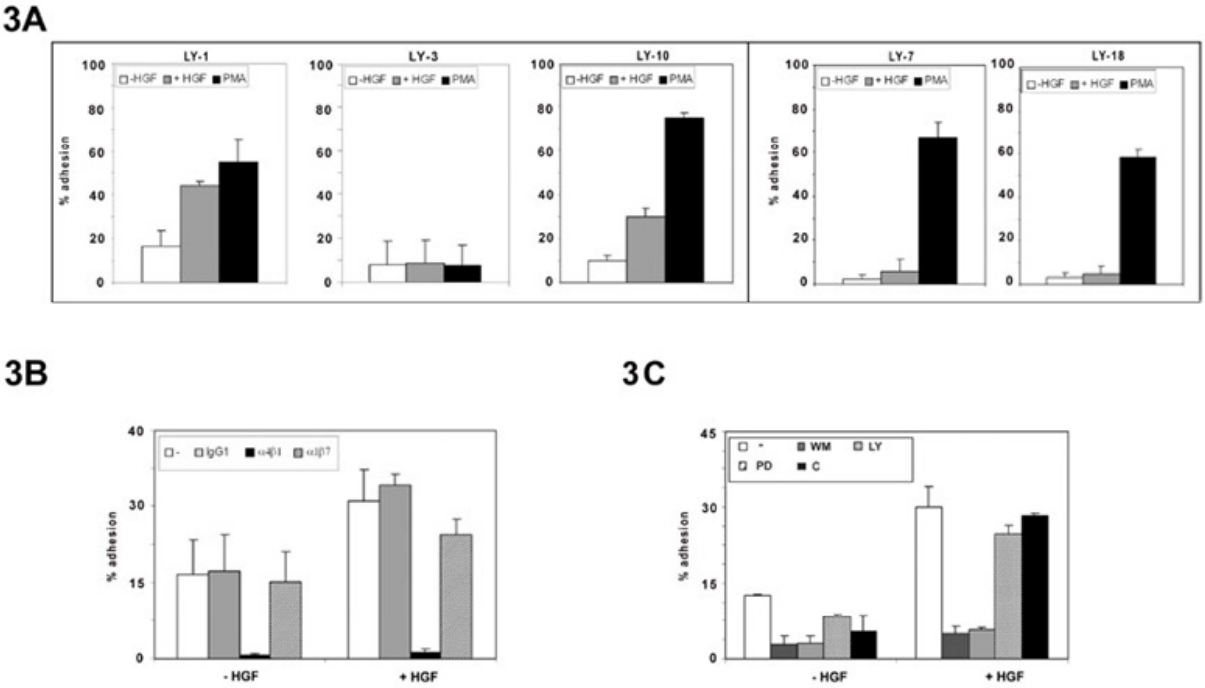


Figure 3

HGF induces α4β1-mediated adhesion of DLBCL cells in a PI3K-dependent fashion

A. HGF induces adhesion of DLBCL cell line OCI-LY-1 and LY-10 to VCAM-1. Cells were stimulated with 200 ng/ml HGF or 50 ng/ml PMA followed by adhesion to VCAM-1. The OCI-LY-3 cells displayed extensive (constitutive) cell aggregation. Neither HGF nor PMA could enhance adhesion of OCI-LY-3 (left panel). MET-negative OCI-LY-7 and LY-18 cells were used as negative controls (right panel). The results are expressed as a percentage of maximal adhesion. The bars represent the means ± the standard deviation of a triplicate experiment representative of at least three independent experiments.

B. HGF-induced adhesion involves α4β1 integrin. The effect of pre-incubation with anti-α4β1 (HP2/1) and anti-α4β7 (Act-1) integrin antibodies on the HGF-induced binding of DLBCL cell lines OCI-LY-10 to VCAM-1 was established. Cells were pre-incubated for 30 minutes in the presence or absence of anti-integrin monoclonal antibodies or isotype control antibody, as indicated. Next, adhesion to VCAM-1 in the presence of 200 ng/ml HGF was measured. Error bars represent the means standard deviation of a triplicate experiment representative of two independent experiments. **C.** HGF-induced adhesion requires PI3K activity. HGF-induced adhesion of OCI-LY-10 was determined after pretreatment with the PI3K inhibitors wortmannin (WM) (50 μM) and LY294002 (LY) (20 μM), the MEK inhibitor PD98059 (PD) (50 μM), or DMSO (C) for 30 minutes, followed by adhesion to VCAM-1 in the presence of 200 ng/ml HGF. The bars represent the means standard deviation of a triplicate experiment representative of two independent experiments.

directly linked to the regulation of cell proliferation [46,47], whereas PKB, GSK3 and FOXOs, targets of PI3K-derived signals, have been implicated in the both proliferation and survival [48,49].

HGF induces integrin-mediated adhesion of DLBCL cells in a PI3K-dependent fashion

Previous studies, including from our laboratory, revealed that HGF/MET signaling induces survival and proliferation of MM cells [24,29], and can control integrin-mediated adhesion of Burkitt's lymphoma cells [6,22] and MM cells [50]. Despite the activation of several survival- and proliferation-regulatory signaling proteins (figure 2C), we did not observe an effect of HGF on the survival or proliferation of DLBCL cells (data not shown). To further define the role of HGF/MET signaling in DLBCLs, we examined whether HGF may be able to control integrin-mediated adhesion. HGF stimulation of both OCI-LY-1 and LY-10 cells strongly augmented adhesion to both VCAM-1 (figure 3A) and FN (data not shown). In contrast, as expected, the MET-negative DLBCL cell lines OCI-LY-7 and –18 did not

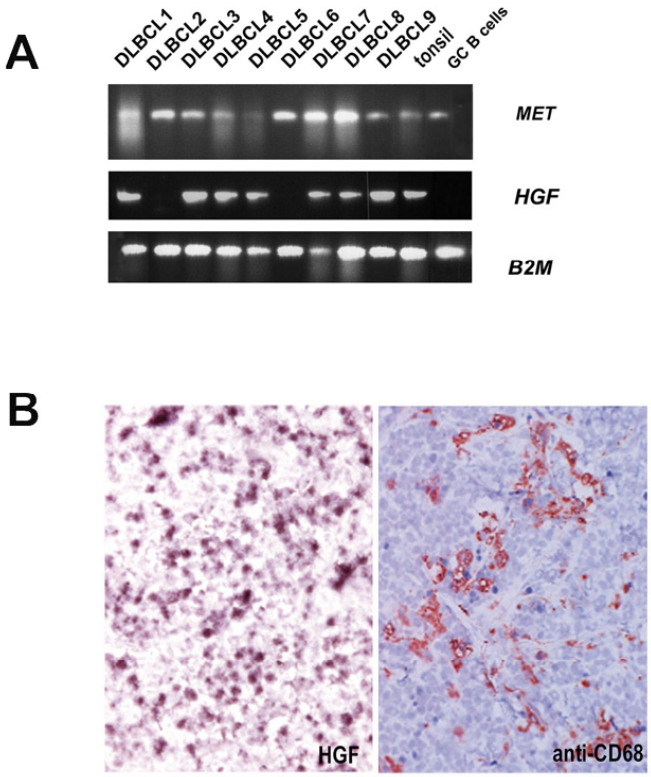


Figure 4 ***HGF*** **expression in DLBCL**

A. MET-positive primary DLBCLs were analyzed for HGF mRNA expression. After RNA isolation and cDNA synthesis, RT-PCR for MET and HGF was performed. β2-microglobulin was used as housekeeping gene control.

B. Frozen sections from a DLBCL case was analyzed for the presence of expression of HGF by mRNA *in situ* hybridization, using DIG-labeled anti-sense cRNA run-off transcripts. Serial sections were stained with anti-CD68 to identify macrophages. The section was counterstained with haematoxylin. Image magnification: 200x

exhibit any HGF-induced adhesion (figure 3A). Noteworthy, the OCI-LY-3 cells displayed extensive (constitutive) cell aggregation. As a consequence neither HGF nor PMA could enhance adhesion of OCI-LY-3, thus rendering this cell line useless for adhesion analysis (figure 3A).

To identify the adhesion receptors on the DLBCL cell lines responsible for the enhanced VCAM-1 binding, integrin expression analysis and antibody blocking experiments were performed. OCI-LY-1, -3 and –10 showed high expression of integrin α4β1 but do not express α4β7, as established by FACS analysis (data not shown). Notably, HGF treatment did not enhance α4β1 or α4β7 expression during the course of the adhesion assay (data not shown). The adhesion to VCAM-1 was completely blocked by the α4β1-blocking antibody HP2/1, whereas the α4β7-blocking antibody Act-1 or an isotype control antibody had no effect (figure 3B).

To investigate the functional importance of PI3K and MEK/MAPK in the HGF-induced adhesion of DLBCL cells, we analyzed the effect of the PI3K inhibitors WM or LY, and the MEK inhibitor PD. The specificity and effectivity of these inhibitors in DLBCL cells is shown in figure 2D. Both WM and LY completely abolished the HGF-induced adhesion of OCI-LY-10 cells, whereas hardly any effect by PD was observed (figure 3B). Hence, HGF-induced adhesion of DLBCL cells is dependent on PI3K, but not on MEK or MAPK activity. Taken together, these results show that the HGF/MET pathway controls α4β1 integrin-mediated adhesion of DLBCL cells in a PI3K-dependent manner.

HGF expression in the DLBCL microenvironment

In several epithelial tumors as well as in MM, autocrine HGF secretion by tumor cells as well as paracrine HGF production by fibroblasts and macrophages in the tumor stroma is crucial for the growth and invasion of MET-positive tumor cells [13]. To investigate whether autocrine or paracrine stimulation of MET by HGF takes place in DLBCLs and is responsible for the signaling and adhesion effects observed in the DLBCL cell lines, we

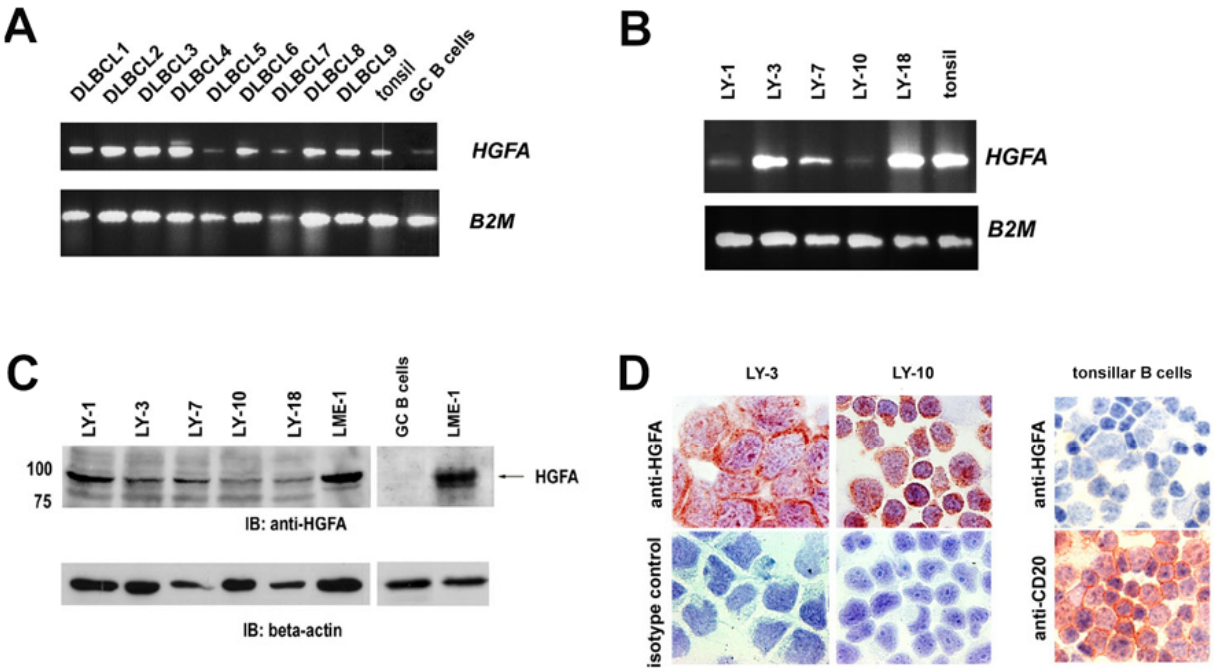


Figure 5 **HGFA expression in DLBCL cells.**

A. Expression of HGFA in GC B cells and primary DLBCLs at mRNA level. After RNA isolation and cDNA synthesis, RT-PCR for MET was performed. β2-microglobulin was used as housekeeping gene control. **B.** mRNA expression of HGFA in DLBCL cell lines. **C.** HGFA protein is expressed in DLBCL cell lines, but not in GC B cells. Cell lysates were immunoblotted using a monoclonal anti-HGFA antibody (A-1). MM cell line LME-1 was used as positive control. β-actin was used as loading control. **D.** Expression of HGFA protein in DLBCL lines and tonsillar B cells by immunocytochemical staining. DLBCL- and tonsillar B cells were immunocytochemically stained with mAb A-1 against HGFA (A-1), CD20 (L26) or isotype control, as indicated. Image magnification: 400x.

first studied the expression of HGF mRNA in a number of primary MET-positive DLBCL samples. RT-PCR showed HGF mRNA expression in most of the MET-positive primary DLBCL tumors (7/9) (figure 4A). By mRNA in situ hybridization, we found that HGF was localized in single cells and small cell clusters within the DLBCLs. Staining of serial sections with anti-CD68 showed a similar staining pattern, suggesting that these cells were (activated) macrophages (figure 4B). Furthermore, analysis of the conditioned medium of the DLBCL cell lines by means of HGF ELISA did not reveal any autocrine production of HGF (data not shown). Our findings suggest that DLBCL cells are stimulated via a paracrine rather than via an autocrine mechanism.

DLBCL cells activate HGF by secretion of HGFA

The serine protease HGFA has been shown to mediate proteolytic conversion of single-chain HGF (scHGF, HGF precursor) to its active heterodimeric form [31], which is essential for the activation and biological function of HGF [51]. HGFA, a factor XIIa-related serine protease has been identified as the most potent activator of HGF [31]. We have previously demonstrated that MM plasma cells produce HGFA, and in this way may activate HGF in the bone marrow microenvironment [30]. To assess whether HGFA is expressed and mediates HGF conversion in DLBCLs, we evaluated the expression of HGFA in MET-positive DLBCLs and cell lines. Interestingly, all primary DLBCLs and cell lines expressed HGFA mRNA (figure 5A+B). Moreover,

contrary to normal tonsillar naïve- or GC- or memory B cells, which do not or very weakly express HGFA (figure 5C, and data in press), the DLBCL cells express HGFA protein (figure 5C+D).

Subsequently, we examined whether DLBCL cells are able to process scHGF (precursor of HGF) to its active form. Indeed, we observed processing of scHGF to its α-chain by conditioned media from the DLBCL cell lines. This conversion was completely inhibited by addition of the serine protease inhibitor leupeptin (figure 6). Since proteases other than HGFA are, although with low efficiency, capable of activating scHGF in vitro, we explored whether the conversion of scHGF by DLBCLs could be specifically inhibited by interfering with HGFA activity. Indeed, we observed that the anti-HGFA monoclonal P1-4, which blocks HGFA function, effectively inhibits scHGF conversion by DLBCLs (figure 6). These findings demonstrate that HGFA is the serine protease responsible for the conversion of scHGF in DLBCLs and identifies the DLBCL cells themselves as an important source of HGFA, thereby regulating HGF activity within the tumor microenvironment.

Discussion

Uncontrolled activation of HGF/MET signaling pathway has been implicated in tumor growth, invasion and metastasis in both mice and human [13]. Here, we have investigated the expression of MET protein on a large panel of B cell malignancies. We have found that MET is frequently expressed on MM (48%) and DLBCL (30%) (table 1), and that MET is occasionally mutated in B cell malignancies, including DLBCL (figure 1). Furthermore, we have demonstrated that HGF is produced within the DLBCL microenvironment (figure 4), that DLBCL cells themselves produce HGFA thereby activating HGF (figure 5 and 6), and that HGF/MET signaling in DLBCL cells is functional and controls integrin-mediated adhesion (figures 2 and 3). Previously, overexpression of either HGF or MET in DLBCL tumor sections, as well as high levels of HGF in the serum of DLBCL patients, has been found to associate with poor prognosis [10,44,52]. Notably, the treatment response was associated with changes in serum HGF levels [10]. More recently, a gene-profiling study showed significantly enhanced expression of MET upon transformation of low-grade FLs into DLBCLs within the same patients [45]. Combined, these data strongly suggest a pathogenic role for HGF/MET signaling in DLBCL.

Overexpression of MET in tumor cells may be due to *MET* amplification, defective transcriptional regulation of the *MET* gene, *MET* amplification, or mutations affecting MET protein stability [14]. Although amplification of *MET* has been reported in several types of human cancer, this was not found in any of the MET-expressing lymphomas studied (data not shown).

Importantly, however, the high MET-expressing cell lines OCI-LY-3 and 10 are so-called activated B cell (ABC)-like DLBCL. These ABC-like DLBCLs are characterized by a nuclear factor κB (NFκB) expression profile, and indeed OCI-LY-3 and LY-10 exhibit constitutive NFκB activation [54]. This is particularly interesting, since the MET promoter contains several putative NFκB responsive elements.

Thus far, no MET mutations have been described for B cell malignancies. However, here we found two distinct germline missense mutations in *MET*: R1166Q in the kinase domain in one DLBCL patient; and R988C in the JM domain in four patients with either DLBCL, CLL, FL or BL (figure 2). Notably, no mutations in *MET* were found in the DLBCL cell lines (data not shown). In hereditary and sporadic papillary renal cell cancer, most missense mutations are located in the tyrosine kinase domain of *MET*, causing constitutive activity and/or a lower threshold for HGF-induced activation of the tyrosine kinase [38,40,53,54]. Thus, the R1166Q mutant may have a similar effect. Since the JM region of *MET* harbors important negative regulatory sites involved in receptor ubiquitination,

degradation and inhibition of kinase activity [55-58], the R988C MET mutation may affect these processes, leading to aberrant MET signaling. Recently, the R988C mutation has also been reported in 2 SCLC cell lines [42], and, during the preparation of this manuscript, in a NSCLC cell line and in 2 lung cancer patients [41,43]. Interestingly, upon transfection the R988C mutant promoted proliferation, motility and overall tyrosine phosphorylation of the pre-B cell line BaF3 [42]. Moreover, expression of the R988C mutant in a SCLC cell line resulted in enhanced focus-formation and soft-agar colony-formation [42]. These observations, combined with the recent demonstration that a mouse MET mutation homologous to R988C plays an important role in lung tumor susceptibility [43], strongly suggest that the R988C mutation is a true gain-of-function mutation. Since a recent study has shown that mice expressing oncogenic MET mutants develop lymphomas [59], it is conceivable that the R988C MET mutation can convey B cell lymphoma susceptibility in humans.

Within the DLBCL microenvironment, we found that HGF was localized in single cells and small cell clusters, most likely representing (activated) macrophages (figure 4B). This, combined with the lack of HGF expression by the DLBCL cell lines as measured in conditioned medium (data not shown), indicates a paracrine rather than an autocrine mechanism of MET activation in DLBCL. Noteworthy, since HGF itself has angiogenic properties [60], and has been demonstrated to induce expression of vascular endothelial growth factor (VEGF) as well [61,62], HGF might also stimulate angiogenesis in DLBCL, thereby promoting tumor growth. Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling. Here we have demonstrated that DLBCLs and cell lines express HGFA and are able to process scHGF to its active form (figure 6). This is in sharp contrast to tonsillar naïve-, GC or memory B cells, which do not or very weakly express HGFA (figure 5C, and data in press). Hence, autocrine production of HGFA by DLBCL cells may support tumorigenesis via autocatalyzation of HGF conversion, consequently providing a constant source of active HGF in the tumor microenvironment. HGF-induced activation of MET in DLBCL cells resulted in MEK-dependent phosphorylation of

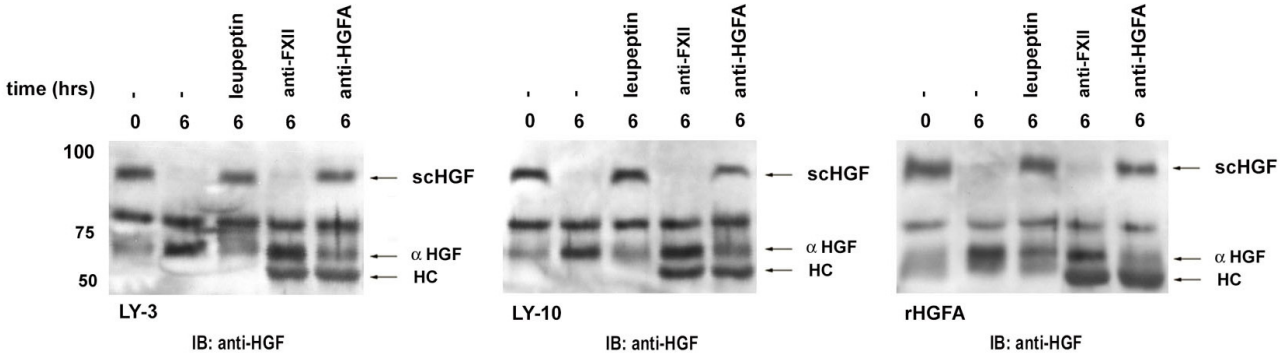


Figure 6 **DLBCL cells autocatalyze HGF activation by producing HGFA.**

Conditioned medium of DLBCL cell lines LY-3 (left panel) and LY-10 (middle panel) was incubated with scHGF for 6 hours in the presence of thrombin, combined with either the serine protease inhibitor leupeptin, neutralizing antibody against HGFA (PI-4) or factor XIIa (OT-2), as indicated. As positive control, HGF conversion by recombinant HGFA is shown (right panel). HGF conversion was determined by immunoblotting with anti-HGF. αHGF= active, α heavy chain of HGF; scHGF= inactive, single chain of HGF; HC= heavy chain of immunoglobulin.

the MAP kinases ERK-1 and –2 (figure 2C). The consecutive activation of MEK-1 and ERK-1/2, the phosphorylation of the transcription factors ELK1 and ETS2, and the expression of immediate early genes such as *FOS*, has been directly linked to regulation of cell proliferation [46,47]. Furthermore, upon HGF stimulation of DLBCL cells, we observed PI3K-dependent phosphorylation of PKB/Akt and its substrates GSK3 and FOXO3a (figure 2D). By direct phosphorylation, PKB can inhibit BAD and caspase 9, activate IKKα resulting in activation of NFκB, and inhibit GSK3 and forkhead transcription factors of the FOXO subfamily, including FOXO3a (FKHRL1), all of which contributes to its anti-apoptotic function [63]. Inhibition of GSK3 and FOXOs by PKB can also induce cell proliferation through enhanced cyclin D1 stabilization and transcription, respectively [63,64]. Noteworthy, overexpression of cyclin D1, often as a consequence of chromosomal translocations, is frequently observed in lymphomas [65]. Phosphorylation of FOXOs by PKB prevents their nuclear translocation and thereby the expression of FOXO target genes, which include the pro-apoptotic genes *FasL* and *Bim*, and the anti-proliferative genes *p27^{KIP}* and *Rb2* [66]. Indirectly, FOXOs can suppress expression of the anti-apoptotic gene *FLIP* [67] and the pro-proliferative genes *cyclin D1* and *D2* [63]. Recently, PI3K/PKB-mediated inactivation of FOXO3a was shown to be important for B cell proliferation [68]. The observed low expression of p27^{kip1} in GC B-type DLBCL, as well as the constitutive activation of NFκB and high expression of FLIP and cyclin D2 in ABC-type DLBCL [1,69], illustrates the relevance of these HGF/MET-controlled signaling cascades for DLBCL.

Similar to our previous studies with the GC B cell-like Burkitt's lymphoma cell line Namalwa [22], we have shown that HGF induces integrin-mediated adhesion of DLBCL cells to VCAM-1 and FN (figure 3A). Furthermore, this HGF-induced adhesion involved α4β1 integrin

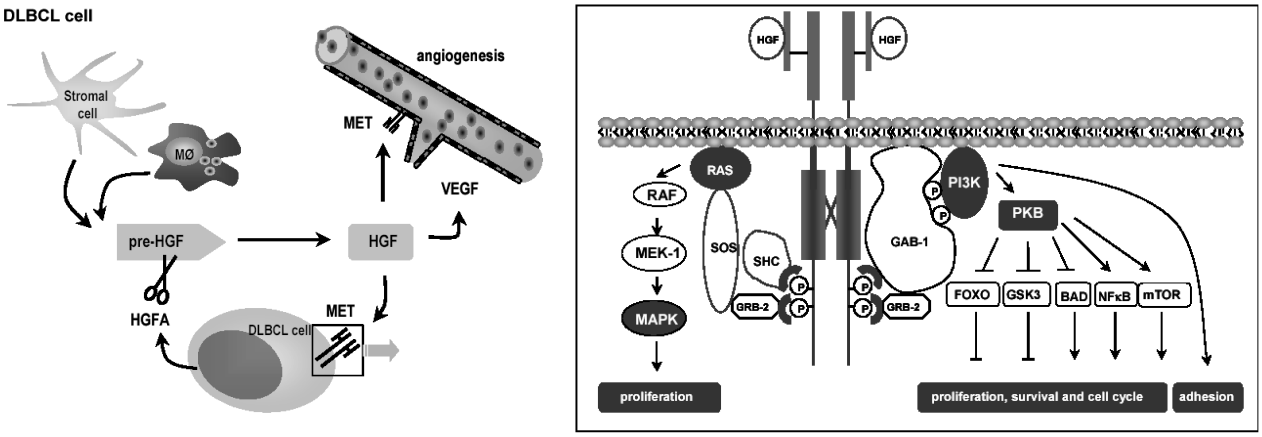


Figure 7 **Activation and biological actions of HGF in the DLBCL microenviroment.**

HGF is produced by macrophages (MØ) and/or stromal cells. Expression and secretion of HGFA by DLBCL cells regulates the bioavailability of active HGF in the DLBCL microenvironment. Catalyzation of HGF activation by DLBCL cells can directly stimulate HGF/MET signaling (insert), promoting DLBCL adhesion, growth and survival. In addition, HGF can directly or indirectly stimulate angiogenesis. Insert: Schematic representation of the HGF-induced signaling events in DLBCL cells. HGF-induced activation of MAPK and PKB is mediated by Grb2/SOS coupling to RAS and GAB-1 coupling to PI3K, respectively [71]. Activation of RAS/MAPK may lead to proliferation of DLBCL cells. Activation of PI3K/PKB leads to phosphorylation of FOXO3a and GSK3, which may control proliferation and survival (see Discussion for further details). Furthermore, PI3K mediates HGF-induced adhesion of DLBCL cells. pre-HGF= inactive, precursor of HGF.

(figure 3B) and required activation of PI3K (figure 3B). The HGF-induced integrin activation can control the interaction of the DLBCL cells with extracellular matrix, stromal cells and FDCs within the tumor microenvironment. By analogy to B cell antigen-receptor-controlled adhesion of GC B cells [7,35,70], this may provide important integrin-mediated outside-in, as well as paracrine, growth and survival signals.

Concluding remarks

A better understanding of the biology of B cell malignancies is needed in the development of potential therapeutic agents that target specific intracellular pathways and the crosstalk that occurs between malignant B cells and the microenviroment. Our data indicate that aberrant HGF/MET-signaling and conversion of HGF by DLBCL cell-secreted HGFA can play an important role in the control of adhesion, survival and proliferation of DLBCL cells, and thereby in the maintenance of DLBCL in the tumor microenvironment (figure 7). Thus, our data provide new insights into the pathogenesis of DLBCL and identify the HGF/MET pathway as a potential novel therapeutic target for the treatment of DLBCL.

Acknowledgements

The authors would like to thank Prof Dr H. Kataoka for the antibodies against HGFA and recombinant active HGFA.

References

1. Shaffer AL, Rosenwald A, Staudt LM. Lymphoid malignancies: the dark side of B-cell differentiation. Nat Rev Immunol. 2002;2:920-932.
2. Kuppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med. 1999;341:1520-1529.
3. Bende RJ, Aarts WM, Riedl RG et al. Among B cell non-Hodgkin's lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid factor reactivity. J Exp Med. 2005;201:1229-1241.
4. Patrick CW, Jr., Juneja HS, Lee S, Schmalstieg FC, McIntire LV. Heterotypic adherence between human B-lymphoblastic and pre-B-lymphoblastic cells and marrow stromal cells is a biphasic event: integrin very late antigen-4 alpha mediates only the early phase of the heterotypic adhesion. Blood. 1995;85:168-178.
5. Lindhout E, Koopman G, Pals ST, de Groot C. Triple check for antigen specificity of B cells during germinal centre reactions. Immunol Today. 1997;18:573-577.
6. Weimar IS, de Jong D, Muller EJ et al. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins. Blood. 1997;89:990-1000.
7. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J Immunol. 1994;152:3760-3767.
8. Klein B, Tarte K, Jourdan M et al. Survival and proliferation factors of normal and malignant plasma cells. Int J Hematol. 2003;78:106-113.
9. Teofili L, Di Febo AL, Pierconti F et al. Expression of the c-met proto-oncogene and its ligand, hepatocyte growth factor, in Hodgkin disease. Blood. 2001;97:1063-1069.
10. Hsiao LT, Lin JT, Yu IT et al. High serum hepatocyte growth factor level in patients with non-Hodgkin's lymphoma. Eur J Haematol. 2003;70:282-289.
11. Seidel C, Borset M, Turesson I et al. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood. 1998;91:806-812.
12. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer. 2002;2:289-300.
13. van der Voort R, Taher TE, Derksen PW et al. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B cell differentiation. Adv Cancer Res. 2000;79:39-90.
14. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915-925.
15. Fischer J, Palmedo G, von Knobloch R et al. Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. Oncogene. 1998;17:733-739.
16. Schmidt L, Duh FM, Chen F et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16:68-73.
17. Schmidt L, Junker K, Nakaigawa N et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene. 1999;18:2343-2350.

18. Park WS, Dong SM, Kim SY et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res. 1999;59:307-310.
19. Lee JH, Han SU, Cho H et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene. 2000;19:4947-4953.
20. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev. 2003;22:309-325.
21. Takayama H, LaRochelle WJ, Sharp R et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A. 1997;94:701-706.
22. van der Voort R, Taher TE, Keehnen RM et al. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. J Exp Med. 1997;185:2121-2131.
23. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology. 2001;102:506-514.
24. Derksen PW, de Gorter DJ, Meijer HP et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003;17:764-774.
25. Jucker M, Gunther A, Gradl G et al. The Met/hepatocyte growth factor receptor (HGFR) gene is overexpressed in some cases of human leukemia and lymphoma. Leuk Res. 1994;18:7-16.
26. Borset M, Lien E, Espevik T et al. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem. 1996;271:24655-24661.
27. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996;88:3998-4004.
28. Capello D, Gaidano G, Gallicchio M et al. The tyrosine kinase receptor met and its ligand HGF are co-expressed and functionally active in HHV-8 positive primary effusion lymphoma. Leukemia. 2000;14:285-291.
29. Derksen PW, Keehnen RM, Evers LM et al. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002;99:1405-1410.
30. Tjin EP, Derksen PW, Kataoka H, Spaargaren M, Pals ST. Multiple myeloma cells catalyze hepatocyte growth factor (HGF) activation by secreting the serine protease HGF-activator. Blood. 2004;104:2172-2175.
31. Shimomura T, Miyazawa K, Komiyama Y et al. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem. 1995;229:257-261.
32. van der Voort R, Taher TE, Wielenga VJ et al. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. J Biol Chem. 1999;274:6499-6506.
33. Kataoka H, Hamasuna R, Itoh H, Kitamura N, Koono M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. Cancer Res. 2000;60:6148-6159.
34. van Adelsberg J, Sehgal S, Kukes A et al. Activation of hepatocyte growth factor (HGF) by endogenous HGF activator is required for metanephric kidney morphogenesis in vitro. J Biol Chem. 2001;276:15099-15106.
35. Spaargaren M, Beuling EA, Rurup ML et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. J Exp Med. 2003;198:1539-1550.
36. Kuniyasu H, Yasui W, Kitadai Y et al. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun. 1992;189:227-232.
37. Lorenzato A, Olivero M, Patane S et al. Novel somatic mutations of the MET oncogene in human carcinoma metastases activating cell motility and invasion. Cancer Res. 2002;62:7025-7030.
38. Jeffers M, Fiscella M, Webb CP et al. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A. 1998;95:14417-14422.
39. Jeffers M, Schmidt L, Nakaigawa N et al. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A. 1997;94:11445-11450.
40. Michieli P, Basilico C, Pennacchietti S et al. Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. Oncogene. 1999;18:5221-5231.
41. Ma PC, Jagadeeswaran R, Jagadeesh S et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 2005;65:1479-1488.
42. Ma PC, Kijima T, Maulik G et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res. 2003;63:6272-6281.
43. Zaffaroni D, Spinola M, Galvan A et al. Met proto-oncogene juxtamembrane rare variations in mouse and humans: differential effects of Arg and Cys alleles

on mouse lung tumorigenesis. Oncogene. 2005;24:1084-1090.
44. Giles FJ, Vose JM, Do KA et al. Clinical relevance of circulating angiogenic factors in patients with non-Hodgkin's lymphoma or Hodgkin's lymphoma. Leuk Res. 2004;28:595-604.
45. Elenitoba-Johnson KS, Jenson SD, Abbott RT et al. Involvement of multiple signaling pathways in follicular lymphoma transformation: p38-mitogen-activated protein kinase as a target for therapy. Proc Natl Acad Sci U S A. 2003;100:7259-7264.
46. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer. 2003;3:11-22.
47. Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci. 1998;23:213-216.
48. Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation. Nat Rev Immunol. 2003;3:317-330.
49. Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell. 2004;117:421-426.
50. Hov H, Holt RU, Ro TB et al. A selective c-met inhibitor blocks an autocrine hepatocyte growth factor growth loop in ANBL-6 cells and prevents migration and adhesion of myeloma cells. Clin Cancer Res. 2004;10:6686-6694.
51. Naka D, Ishii T, Yoshiyama Y et al. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J Biol Chem. 1992;267:20114-20119.
52. Kawano R, Ohshima K, Karube K et al. Prognostic significance of hepatocyte growth factor and c-MET expression in patients with diffuse large B-cell lymphoma. Br J Haematol. 2004;127:305-307.
53. Jeffers MF. Activating mutations in the Met receptor overcome the requirement for autophosphorylation of tyrosines crucial for wild type signaling. Oncogene. 1999;18:5120-5125.
54. Chiara F, Michieli P, Pugliese L, Comoglio.P.M. Mutations in the MET oncogene unveil a 'dual switch' mechanism controlling tyrosine kinase activity. J Biol Chem. 2003.
55. Gandino L, Longati P, Medico E, Prat M, Comoglio PM. Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. J Biol Chem. 1994;269:1815-1820.
56. Villa-Moruzzi E, Puntoni F, Bardelli A et al. Protein tyrosine phosphatase PTP-S binds to the juxtamembrane region of the hepatocyte growth factor receptor Met. Biochem J. 1998;336 (Pt 1):235-239.
57. Peschard P, Fournier TM, Lamorte L et al. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell. 2001;8:995-1004.
58. Taher TE, Tjin EP, Beuling EA et al. c-Cbl is involved in Met signaling in B cells and mediates hepatocyte growth factor-induced receptor ubiquitination. J Immunol. 2002;169:3793-3800.
59. Graveel C, Su Y, Koeman J et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A. 2004;101:17198-17203.
60. Jiang W, Hiscox S, Matsumoto K, Nakamura T. Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. Crit Rev Oncol Hematol. 1999;29:209-248.
61. Wojta J, Kaun C, Breuss JM et al. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. Lab Invest. 1999;79:427-438.
62. Xin X, Yang S, Ingle G et al. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. Am J Pathol. 2001;158:1111-1120.
63. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2:489-501.
64. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 1998;12:3499-3511.
65. Nakamura S, Yatabe Y, Seto M. Cyclin D1 overexpression in malignant lymphomas. Pathol Int. 1997;47:421-429.
66. Coffer PJ, Burgering BM. Forkhead-box transcription factors and their role in the immune system. Nat Rev Immunol. 2004;4:889-899.
67. Skurk C, Maatz H, Kim HS et al. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. J Biol Chem. 2004;279:1513-1525.
68. Yusuf I, Zhu X, Kharas MG, Chen J, Fruman DA. Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. Blood. 2004;104:784-787.
69. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med. 2001;194:1861-1874.
70. Koopman G, Parmentier HK, Schuurman HJ et al. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very

late antigen 4/vascular cell adhesion molecule 1 pathways. J Exp Med. 1991;173:1297-1304.

71. Zhang YW, Vande Woude GF. HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem. 2003;88:408-417.

CHAPTER **4**

Multiple myeloma cells catalyze hepatocyte growth factor (HGF) activation by secreting the serine protease HGF-activator

Esther P.M. Tjin, Patrick W.B. Derksen, Hiroaki Kataoka*, Marcel Spaargaren, and Steven T. Pals

Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands

*Second Department of Pathology, Miyazaki Medical College, Kiyotake, Japan

Multiple myeloma (MM) is a common hematological neoplasm consisting of malignant plasma cells, which expand in the bone marrow. A potential key signal in the evolution of MM is hepatocyte growth factor (HGF), which acts as a potent para- and/or autocrine growth- and survival factor for MM cells. Proteolytic conversion of HGF into its active form is a critical limiting step in HGF/MET signaling. Here, we show that malignant MM plasma cells convert HGF into its active form and secrete HGF-activator (HGFA), a serine protease specific for HGF activation. By using serine protease inhibitors and neutralizing antibodies, we demonstrate that HGFA produced by the MM cells is responsible for their ability to catalyze HGF activation. We therefore suggest that autocatalyzation of HGF conversion by MM cells is an important step in HGF/MET-induced myeloma growth and survival, which may have implications for the management of this incurable form of cancer. ***Blood, 2004, Vol. 104 (7): 2172-2175.***

The unrestrained growth of tumor cells is generally attributed to mutations in essential growth control genes, but tumor cells are also influenced by signals from the environment. In MM, the factors and signals coming from the bone marrow (BM) microenvironment are possibly even essential for the growth of the tumor cells. As targets for intervention, these signals may be equally important as mutated oncogenes[1,2]. Recent studies have identified HGF as a potential key signal in the evolution of MM. HGF has a domain structure and proteolytic mechanism of activation similar to that of the blood serine protease plasminogen, but lacks protease activity. Instead, via its tyrosine kinase receptor MET, HGF induces complex biological responses in target cells, including motility, growth, and morphogenesis. Whereas a functional HGF/MET pathway is indispensable for mammalian development, uncontrolled MET signaling, provoked by *MET* activating mutations or *MET* amplification and overexpression, is oncogenic, and has been implicated in the development and progression of a variety of human cancers [3-5]. In MMs, HGF exerts strong proliferative and anti-apoptotic effects via the RAS/MAPK and PI3K/PKB pathways[6,7]. Within the BM microenvironment, stromal cells present a paracrine source of HGF[8], however, an autocrine HGF/MET loop has also been

reported in myeloma cells[9,10]. Furthermore, in a recent gene-profiling study HGF was the only significantly overexpressed growth factor in MM[11], while high serum HGF levels in MM patients predict unfavorable prognosis[12].

Upon secretion, HGF normally retains its 90-kDa single-chain (sc) precursor form, which is probably cell surface or extracellular matrix associated. For biological function however, proteolytic conversion of scHGF to the heterodimeric active form is essential[13]. Although the role of HGF in tumor progression has attracted much attention, the molecular mechanisms underlying HGF activation in tumor tissue remain largely unexplored. Plasminogen activators, particularly uPA and factor XIIa have been shown to activate scHGF, although at low rates [14,15]. More recently, hepatocyte growth factor activator (HGFA), a factor XIIa-related serine protease with an efficient HGF-activating activity, was identified[15-17]. This enzyme is secreted by the liver as an inactive zymogen[15-17] and has recently also been shown to be produced by colorectal cancer cells [18]. In this paper, we have studied the mechanism of HGF activation in MM. We show that myeloma cell lines as well as primary myelomas secrete HGFA and in this way are able to autocatalyze HGF activation.

Material and Methods

Antibodies

Monoclonal antibodies were: anti-HGFA, A-1 (IgG1) and P1-4 (IgG1)[15]; anti-factor XIIa, OT-2 (IgG1) (Sanquin, Amsterdam, The Netherlands); IgG1 control antibody (DAKO, Glostrup, Denmark); anti-hepatocyte growth factor activator inhibitor-1 (HAI-1) [18]. Polyclonal antibodies used were goat anti-human HGF (R&D Systems, Abington, UK); R-phycoerythrin-conjugated goat anti-mouse (Southern Biotechnology, Birmingham, AL); horseradish peroxidase (HRP)-conjugated rabbit anti-mouse (DAKO); HRP-conjugated goat-anti-rabbit (DAKO).

MM cells, cell cultures, transfectants and conditioned medium

MM cell lines UM1, UM3, UM6, L363, NCI-H929, OMP-1, LME-1, and XG-1 were grown as described previously[6,7]. COS7 cells were transiently transfected with the mammalian expression vector pCIneo-HGFA containing full-length HGFA[18] using the DEAE-dextran method. Conditioned medium was obtained as described previously [19].

Primary myeloma cells (PM) were obtained from the pleural effusion of a 67-year old male patient. FACS analysis showed > 95% $CD138^{high}$, $CD38^{high}$ cells. Mononuclear cells were harvested by standard Ficoll-Paque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden).

Immunoprecipitation and western blot analysis

Immunoprecipitation and western blotting was performed as described[6]. For the HGF activation assay, serum free cultured cells were lysed in the absence of protease inhibitors since these affect the function of HGFA. For immunoprecipitation of HGFA, the lysates were incubated with the monoclonal antibody A-1 pre-coupled to Protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). The precipitates were washed three times with lysis buffer and were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis under reducing conditions. The immunoblots were stained with anti-HGF or anti-HGFA and detected with HRP-conjugated swine anti-goat and HRP-conjugated rabbit anti-mouse respectively.

Assay for HGF activation

HGF activation was assayed as described previously[18]. In brief, single chain HGF (R&D Systems) was incubated with either intact MM cells, with MM conditioned medium or with HGFA immunoprecipitated from MM conditioned medium. To study activation by cells, these were washed thoroughly and

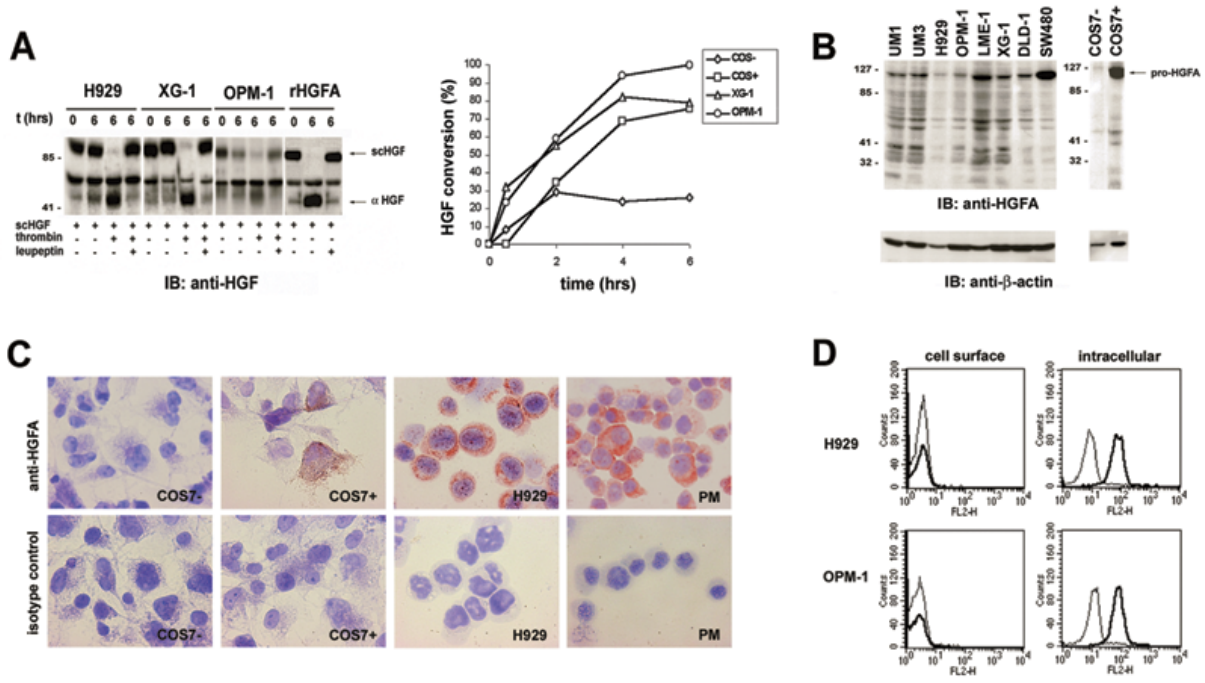


Figure 1

MM cells proteolytically convert HGF into its active form and express the serine protease HGFA.

A. MM cells convert HGF into its active form. MM cell lines NCI-H929, XG-1 and OMP-1 were incubated with scHGF for 6 hours in the presence or absence of thrombin and/or the serine protease inhibitor leupeptin, as indicated. HGF conversion was determined by immunoblotting with anti-HGF. As positive control, HGF conversion by recombinant HGFA is shown (left panel). The right panel shows the time kinetics of scHGF conversion by MM cells (in the presence of thrombin). As positive and negative controls, scHGF conversion by COS-7 cells transfected with either a plasmid containing *HGFA* or empty vector are shown. **B.** Expression of HGFA in MM cell lines. Cell lysates were immunoblotted using a monoclonal anti-HGFA antibody (A-1). COS-7 cells transfected with *HGFA* and the colorectal carcinoma cell lines DLD-1 and SW480 were used as positive controls. COS-7 cells transfected with empty vector were used as negative controls. β-actin was used as loading control (lower panel). **C.** Expression of HGFA in MM cell lines and primary myeloma cells. MM cell line NCI-H929, primary myeloma cells (PM), or COS-7 cells transfected with either empty vector or a plasmid containing *HGFA* were immunocytochemically stained with mAb A-1 against HGFA or isotype control. **D.** HGFA expression in MM cells is intracellular. The indicated MM cells, either permeabilized (right panel) or not (left panel), were stained with anti-HGFA mAb PI-4 (bold line) or isotype control antibody (grey line). Expression was measured by FACS analysis.

incubated serum free overnight. Subsequently, the cells were washed and 10^5 cells were incubated in 0.1 ml medium containing scHGF (1μg/ml) for the time indicated in the presence or absence of 4 units/ml thrombin (Sigma Aldrich Chemie GmbH, Germany). For HGF activation, 20 μl conditioned medium or sample containing immunoprecipitated HGFA were pretreated with 1 unit of thrombin and added to 0.1μg scHGF. Inhibitor studies were done in the presence of aprotinin (2TIU/ml), leupeptin (500μg /ml), C1-inhibitor (kindly provided by E. Hack, Sanquin, Amsterdam, The Netherlands) or neutralizing antibody P1-4 (40μg/ml).

Immunocytochemistry

HGFA expression in MM cell lines and primary myeloma cells was studied on aceton-fixed cytospins with mAb A-1 using biotin-conjugated rabbit as second step. The reaction was developed with 3,3-amino-9-ethylcarbazole (Sigma) and cytospins were counterstained with Haematoxylin. COS-7 cells transfected with a construct containing HGFA were used as positive control, and

appropriate isotype antibodies as negative controls.

Flow Cytometry

For the determination of HGFA expression, monoclonal antibody PI-4 and secondary antibody PE-conjugated goat anti mouse Ig (Southern Biotechnology) were used. For intracellular HGFA staining, the MM cell lines were fixed with 2% paraformaldehyde and permeabilized with saponin. Analysis was carried out on a FACScalibur flow cytometer (Becton Dickinson Biosciences) with CELLQuest TM software (BD).

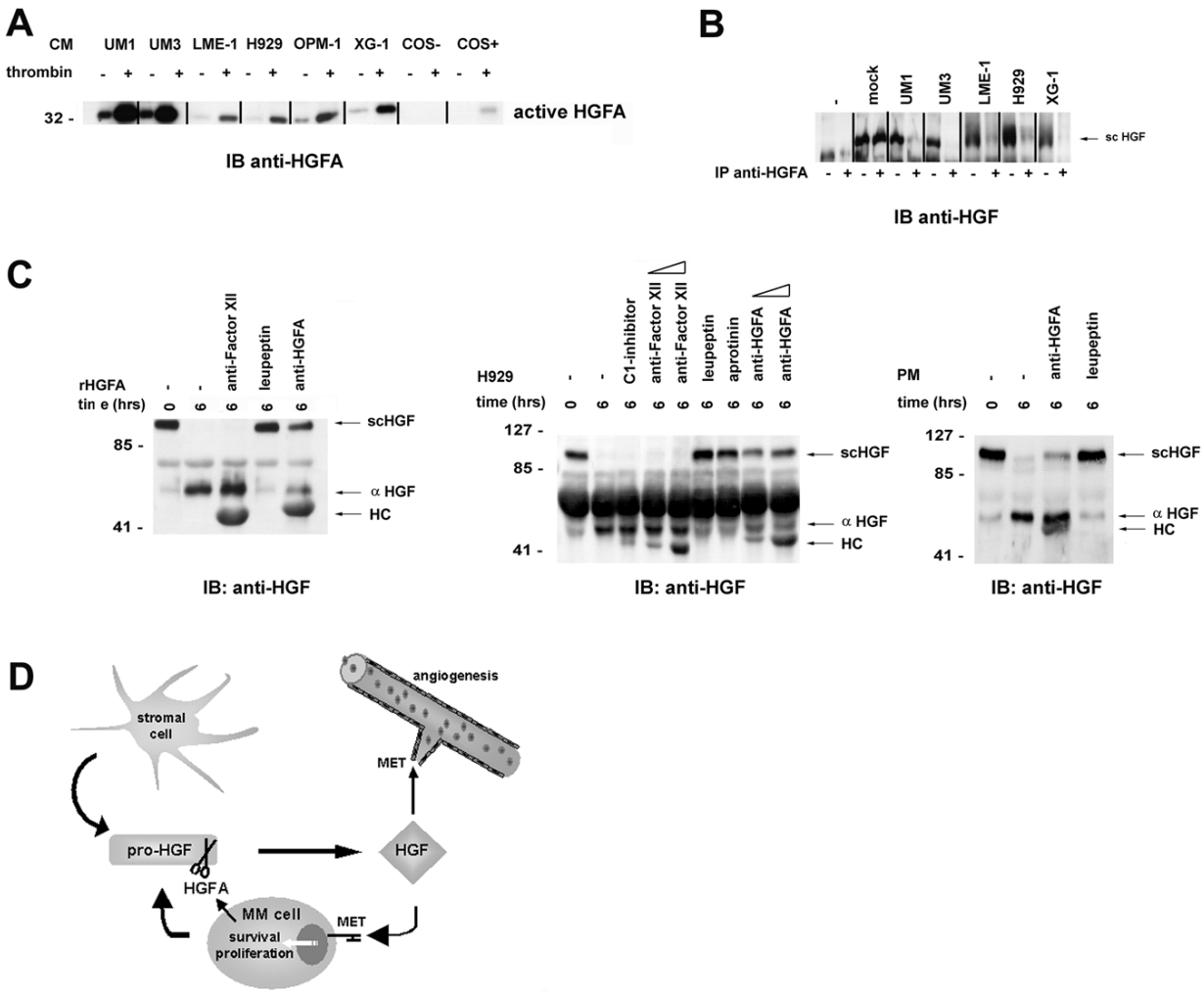


Figure 2 **HGFA mediates HGF conversion by MM cells.**

A. MM cells secrete HGFA. To detect the active (34 kDa) form of HGFA , MM conditioned medium (CM), either or not pre-treated with thrombin, as indicated, was immunoblotted with anti-HGFA antibody A-1. **B.** HGFA from MM conditioned medium converts HGF. HGFA (+) or IgG control (-) immunoprecipitates from MM conditioned medium were analyzed in a HGF conversion assay. **C.** HGFA mediates HGF activation by MM cells. The effects of neutralizing antibodies against HGFA (P1-4) and Factor XIIa (OT-2), protease inhibitors aprotinin and leupeptin, and C1-inhibitor on HGF activation by recombinant HGFA (left panel) and conditioned medium of MM cell line NCI-H929 (middle panel) and primary MM cells (right panel) were analyzed by the HGF conversion assay. HC= immunoglobulin heavy chain. **D.** Activation and biological actions of HGF in the myeloma microenvironment.

Results and discussion

Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling. We observed that the MM cell lines NCI-H929, XG-1 and OMP-1 cells were all able to process scHGF to its active form (Figure 1A). The processing of scHGF either required or was greatly enhanced by the addition of thrombin and was completely inhibited by the serine protease inhibitors leupeptin (Figure 1A) and aprotinin (not shown). Since HGFA is a serine protease specifically activated by thrombin [17,20], these observations suggested that the HGF processing activity might be due to HGFA. Indeed, RT-PCR analysis demonstrated the presence HGFA mRNA in all MM cells tested (data not shown), while a band of 96-kDa, which corresponds to the molecular mass of the proform of HGFA, was detected by immunoblotting (Figure 1B). This band was also detected in the lysates of the colorectal cancer cell lines DLD-1 and SW480, which express HGFA [18], and in the lysates of COS-7 cells transfected with a plasmid containing HGFA, but not in COS-7 cells transfected with empty vector (Figure 1B).

Immunocytochemistry showed a distinct granular intracytoplasmic HGFA expression pattern, which was present in all MM cell lines as well as in the HGFA transfected COS-7 cells (Figure 1C and data not shown). The same expression pattern was also found in all (n=8) primary MM samples analyzed as exemplified in figure 1C. FACS analysis confirmed the expression and intracellular localization of HGFA (Figure 1D).

The 34-kDa catalytically active form of HGFA [16] was not detected in the MM lysates (Figure 1B). By contrast, the conditioned media of the cell lines contained variable amounts of the 34-kDa form of HGFA indicating that MMs secrete and, to a certain extent, activate HGFA (Figure 2A). Indeed, HGFA immunoprecipitated from the MM conditioned media effectively converted scHGF (Figure 2B). Since proteases other than HGFA are, although with low efficiency, capable of activating scHGF *in vitro* [14,15], we explored whether the conversion of scHGF by MM cells could be specifically inhibited by interfering with HGFA activity. We observed that the anti-HGFA monoclonal P1-4, which blocks HGFA function [20] (Figure 2C, left panel), effectively inhibits scHGF conversion by MM cells (Figure 2C, middle + right panel). By contrast, scHGF conversion was not affected by interfering with factor XIIa function with either a blocking mAb (OT-2) or with the protease inhibitor C1-inhibitor [15] (Figure 2C). Hence, HGFA is the (major) serine protease responsible for the conversion of scHGF by MM cells. Most MM cell lines, including H929 and XG-1, also expressed HAI-1 (data not shown), but the presence of this HGFA-regulatory protein apparently did not block HGF conversion (Figure 1A). This seemingly contradictory finding may be explained by the complex effects of HAI-1 on HGF conversion. Thus, whereas the soluble Kunitz 1 form of HAI-1 can inhibit HGF conversion, the membrane bound form of HAI-1 is believed to concentrate active HGFA at the cell surface and, upon release, may promote activation of HGF [24]. Hence, it is not surprising that HAI expression per se does not predict inhibitory activity.

Our study identifies expression and secretion of HGFA by MM cells as a potentially important factor in regulating the bioavailability of active HGF in the MM microenvironment, while the activated BM stroma in MM may present an additional source of both HGF and HGFA. Catalyzation of HGF activation by MM cells may directly stimulate HGF/MET signaling in the tumor cells, promoting MM cell growth and survival[6,7]. In addition, since HGF is a potent angiogenic factor[2,3,21], it may also contribute to tumor angiogenesis which has recently been identified as an important process in the progression and prognosis of MM[22] (Figure 2D). Our study identifies the activation step of HGF as a promising new target in MM therapy.

References

1. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer. 2002; 2:175-187.
2. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat Rev Cancer. 2002; 2:927-937.
3. van de Voort, Taher TE, Derksen PW, Spaargaren M, van der Neut R, Pals ST. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. Adv Cancer Res. 2000; 79:39-90.
4. Zhang YW, Vande Woude G. HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem. 2003; 88:408-417.
5. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer. 2002; 2:289-300.
6. Derksen PW, Keehnen RM, Evers LM, van Oers MH, Spaargaren M, Pals ST. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002; 99:1405-1410.
7. Derksen PW, de Gorter DJ, Meijer HP, Bende RJ, van Dijk M, Lokhorst HM, Bloem AC, Spaargaren M, Pals ST. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003; 17:764-774.
8. Takai K, Hara J, Matsumoto K, Hosoi G, Osugi Y, Tawa A, Okada S, Nakamura T. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood. 1997; 89:1560-1565.
9. Borset M, Lien E, Espevik T, Helseth E, Waage A, Sundan A. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem. 1996; 271:24655-24661.
10. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996; 88:3998-4004.
11. Zhan F, Hardin J, Kordsmeier B, Bumm K, Zheng M, Tian E, Sanderson R, Yang Y, Wilson C, Zangari M, Anaissie E, Morris C, Muwalla F, van Rhee F, Fassas A, Crowley J, Tricot G, Barlogie B, Shaughnessy JJ. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. Blood. 2000; 99:1745-1757.
12. Seidel C, Borset M, Turesson I, Abildgaard N, Sundan A, Waage A. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. Blood. 1998; 91:806-812.
13. Naka D, Ishii T, Yoshiyama Y, Miyazawa K, Hara H, Hishida T, Kidamura N. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J Biol Chem. 1992; 267:20114-20119.
14. Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier W, Daikuhara Y, Tsubouchi H, Blasi F, Comoglio PM. Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J. 1992; 11:4825-4833.
15. Shimomura T, Miyazawa K, Komiyama Y, Hiraoka H, Naka D, Morimoto Y, Kitamura N. Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem. 1995; 229:257-261.
16. Miyazawa K, Shimomura T, Kitamura N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem. 1996; 271:3615-3618.
17. Miyazawa K, Shimomura T, Kitamura A, Kondo J, Morimoto Y, Kitamura N. Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. J Biol Chem. 1993; 268:10024-10028.
18. Kataoka H, Hamasuna R, Itoh H, Kitamura N, Koono M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. Cancer Res. 2000; 60:6148-6159.
19. Adelsberg J, Sehgal S, Kukes A, Brady C, Barasch J, Yang J, Huan Y. Activation of hepatocyte growth factor (HGF) by endogenous HGF activator is required for metanephric kidney morphogenesis in vitro. J Biol Chem. 2001; 276:15099-15106.
20. Shimomura T, Kondo J, Ochiai M, Naka D, Miyazawa K, Morimoto Y, Kitamura N. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem. 1993; 268:22927-22932.
21. Rosen EM, Goldberg ID. Scatter factor and angiogenesis. Adv Cancer Res. 1995; 67:257-279.
22. Rajkumar SV, Leong T, Roche PC, Fonseca R, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Kyle RA, Gertz MA, Greipp PR. Prognostic value of bone marrow angiogenesis in multiple myeloma. Clin Cancer Res. 2000; 6:3111-3116.

23. Rajkumar SV, Mesa RA, Fonseca R, Schroeder G, Plevak MF, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Gertz MA, Kyle RA, Russell SJ, Greipp PR. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res. 2002; 8:2210-2216.

24. Kataoka H, Shimomura T, Kawaguchi T, Hamasuna R, Itoh H, Kitamura N, Miyazawa K, Koono M. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. J Biol Chem. 2000; 275: 40453-40462.

CHAPTER **5**

c-Cbl is involved in MET signaling in B Cells and mediates hepatocyte growth factor-induced receptor ubiquitination

Taher E.I. Taher*, Esther P.M. Tjin*, Esther A. Beuling, Jannie Borst[§], Marcel Spaargaren and Steven T. Pals.

Department of Pathology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; [§]Department of Cellular Biochemistry, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

*T.E.I.T. and E.P.M.T. contributed equally to this manuscript

Hepatocyte growth factor/scatter factor (HGF) and its receptor tyrosine kinase Met are key regulators of epithelial motility and morphogenesis. Recent studies indicate that the HGF/MET pathway also plays a role in B cell differentiation, whereas uncontrolled MET signaling may lead to B cell neoplasia. These observations prompted us to explore HGF/MET signaling in B cells. Here, we demonstrate that HGF induces strong tyrosine phosphorylation of the proto-oncogene product c-Cbl in B cells and increases Cbl association with the Src–family tyrosine kinases Fyn and Lyn, as well as with PI-3 kinase and CrkL. In addition, we demonstrate that c-Cbl mediates HGF-induced ubiquitination of MET. This requires the juxtamembrane tyrosine Y1001 (Y2) of MET, but not the multifunctional docking site (Y14/15) or any additional C-terminal tyrosine residues (Y13-16). In contrast to wildtype c-Cbl, the transforming mutants v-Cbl and 70Z/3 Cbl, which lack the ubiquitin ligase RING finger domain, suppress Met ubiquitination. Our findings identify c-Cbl as a negative regulator of HGF/MET signaling in B cells, mediating ubiquitination and, consequently, proteosomal degradation of MET, and suggest a role for Cbl in MET-mediated tumorigenesis. ***The Journal of Immunology, 2002, 169: 3793-3800.***

Hepatocyte growth factor/scatter factor (HGF) is a multifunctional cytokine with a domain structure and proteolytic mechanism of activation similar to that of the serine protease plasminogen. By binding to the receptor tyrosine kinase MET, the product of the proto-oncogene *c-met*, HGF triggers intracellular signals regulating cell proliferation, migration, and survival [1-9]. In mice, HGF or MET deficiency results in embryonic death due to severe developmental defects in the placenta and liver, and disrupts the migration of myogenic precursors into the limb buds [6;9]. Other studies have provided evidence for an important role of HGF in angiogenesis and in the three-dimensional organization of epithelial tissues, including kidney tubules and mammary glands [3-5]. More recently, the HGF/MET pathway has also been implicated in B cell differentiation.

Specifically, HGF produced by follicular dendritic cells (FDC) or stromal cells has been shown to regulate integrin-mediated adhesion and migration of germinal center cells (GC) and plasma cells [10;11].

Apart from these physiologic functions, uncontrolled activation of MET is oncogenic and can promote tumor growth, invasion, and metastasis via several distinct mechanisms [3;12-19]. In hereditary papillary renal carcinoma (HPRC) MET mutations cause hyperactivation of the receptor in response to HGF stimulation. These receptor mutants can mediate transformation, invasive growth, and protection from apoptosis [20-24]. In B cell neoplasia, by contrast, auto- and/or paracrine stimulation of MET, rather than receptor mutation, appears to be the most important mechanism for transformation. It was recently demonstrated that HGF is a potent growth and survival factor for plasma cell myelomas [25]. These tumors frequently co-express HGF and MET, suggesting the presence of an autocrine loop [26]. For HGF negative myelomas, bone marrow stromal cells may present an alternative, paracrine source of HGF [27]. Similarly, HGF produced by follicular dendritic cells (FDC) and stromal cells in lymphoid tissues may stimulate the growth and survival of MET positive non-Hodgkin's lymphomas [10;11]. Consistent with a role for HGF/MET in myeloma progression, patients with high serum levels of HGF have an unfavorable prognosis [28].

HGF/MET signaling has been extensively studied in epithelial cells. These studies revealed a prominent role for the multifunctional docking site, consisting of tyrosine residues Y1349 (Y14) and Y1356 (Y15) [3;29]. Upon phosphorylation, this docking site mediates the interaction with Grb2, resulting in activation of the Ras-MAPK pathway. In addition, the docking protein Gab1 plays an important role in HGF/MET signaling as it is also able to interact directly with the docking site of MET, as well as with several signal transducing proteins, including PI-3K, CrkL and Shp2 [30-32]. Despite the role of HGF/MET signaling in normal B cell differentiation and malignancy [3;10;11;25;26], hardly anything is known about the underlying signal transduction mechanism in B cells. Recently, we have reported the presence of two prominent phosphoproteins of 110 and 120 kDa after HGF stimulation of B cells [33], of which the 110 kDa phosphoprotein was indentified as Gab1 [34]. In the present study, we identify the other major phosphoprotein as c-Cbl, which is a prominent target for B-cell antigen receptor signaling as well [35-38]. HGF induces a strong and transient tyrosine phosphorylation of c-Cbl, resulting in an increased association with Fyn, Lyn, PI-3 kinase, and CrkL. In addition, we demonstrate that c-Cbl, but not its oncogenic forms v-Cbl or 70Z/3 Cbl, negatively regulate MET by inducing ubiquitination of its cytoplasmic domain.

Materials and Methods

Antibodies

Monoclonal antibodies used were: anti-phosphotyrosine, PY20 (Affiniti, Nottingham, United Kingdom); and anti-hemagglutinin tag, 12CA5 (anti-HA) (Dr C. de Vries, Dept. of Biochemistry, AMC, Amsterdam, The Netherlands). The rabbit polyclonal antibodies used were: anti-ubiquitin (Dako, Glostrup, Denmark); anti-hMET (anti-human MET), C-12; anti-mMET (anti-mouse MET), SP260; anti-Fyn, FYN3; anti-Lyn, 44; anti-CrkL, C-20; and anti-Cbl, C-15 (all: Santa Cruz Biotechnology, Santa Cruz, CA); anti-regulatory subunit of PI-3 kinase (UBI, Lake Placid, NY).

Plasmids

The c-Cbl cDNA was a kind gift from Dr W.Y. Langdon (University of Western Australia, Nedlands, Australia). pMT2 encoding HA-tagged human c-Cbl, v-Cbl and 70Z/3 Cbl were generated from this cDNA by PCR. The constructs encoding Trk-MET (a chimeric receptor that consists of the extracellular domain of the NGF receptor, Trk A, and the cytoplasmic domain of c-MET), either wild

type (wt) or mutants of either tyrosine residue 1001 (Y2), 1232 and 1233 (KD), 1347 (Y14), 1354 (Y15), 1347 and 1354 (Y14/15), or 1311, 1347, 1354 and 1363 (Y13-16), were a kind gift from Dr W. Birchmeier (Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany) [39]. pMT2-encoding HA-tagged ubiquitin was kindly provided by Dr P. M. P. van Bergen en Henegouwen (Dept. of Molecular Cell Biology, Utrecht University, Utrecht, The Netherlands).

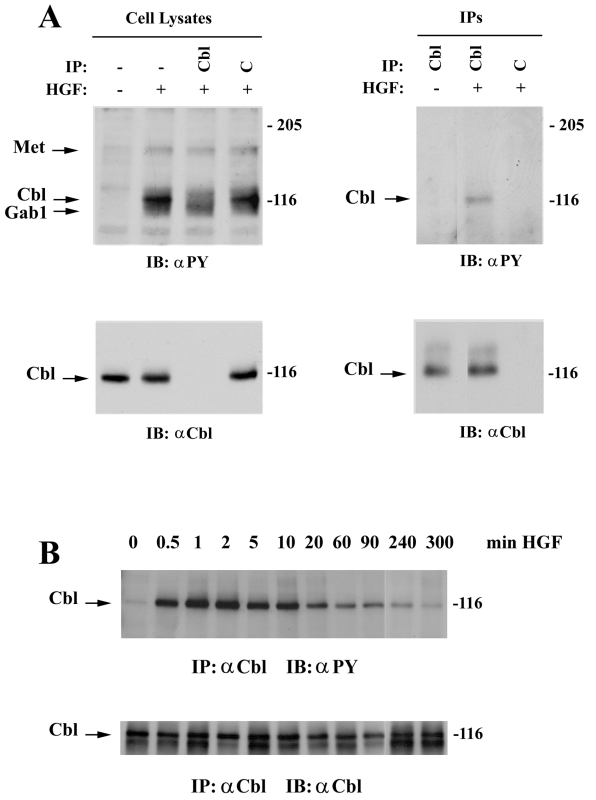


Figure 1

HGF induces tyrosine phosphorylation of Cbl.

A. Cbl is a prominent tyrosine phosphorylated protein in HGF-stimulated Namalwa cells. Cells were stimulated for 2 minutes with HGF. Total cell lysates of HGF-stimulated or control Namalwa B cells were immunodepleted or not with anti-Cbl (left), and their corresponding immunoprecipitates (right) immunoblotted with anti-phosphotyrosine (anti-PY20). The arrows indicate the tyrosine phosphorylated Cbl (upper panels). The blots were stripped and restained with anti-Cbl antibodies, confirming equal loading of the immunoprecipitates and successful immunodepletion of c-Cbl from the total cell lysates (lower panels). **B.** Time kinetics of HGF-induced tyrosine phosphorylation of Cbl. Cells were stimulated with HGF for the indicated time periods. Immunoprecipitation was performed with anti-Cbl antibodies and immunoblots were stained with anti-PY20. The arrow indicates the tyrosine phosphorylated Cbl (upper panel). Equal loading of the samples was confirmed by restaining the blot with anti-Cbl antibodies (lower panel).

Cell lines and transfectants

The Burkitt's lymphoma cell line Namalwa-V3M has been described [34]. The cells were cultured in RPMI 1640 in the presence of 10% Fetal Clone I serum (HyClone Laboratories, Logan, UT), 10% fetal calf serum (Integro, Zaandam, The Netherlands). Cos-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Using DEAE-dextran , Cos-7 cells were transiently transfected with 1 μg construct encoding Trk-MET, alone or together with 2 μg construct containing either HA-tagged c-Cbl, the oncogenic Cbl 70Z/3, and v- Cbl or ubiquitin.

Immunoprecipitation and western blot analysis

Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 2 mM sodium orthovanadate, 5 mM EDTA, and 5 mM sodium fluoride. The lysates were cleared by centrifugation at 10.000*g* at 4°C for 20 minutes, followed by preclearance using Protein A-Sepharose. The immunocomplexes were collected by adding the indicated antibodies, precoupled to Protein A-Sepharose, for at least 2 hours. The immunoprecipitates were washed three times with lysis buffer and the immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was performed using enhanced chemiluminescence lighting (ECL). For the immunodepletion experiments, the lysates were immunoprecipitated twice.

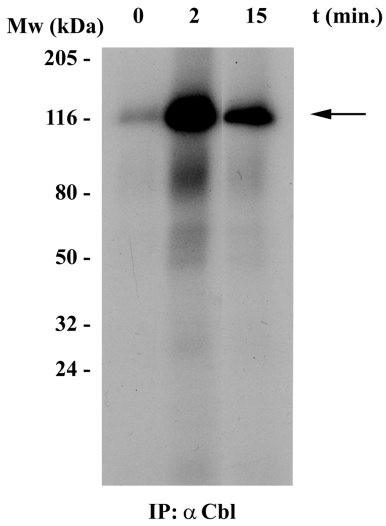


Figure 2

HGF stimulation induces an increase in the Cbl associated kinase activity.

Cbl immunoprecipitates collected from cells stimulated with HGF for the indicated time periods were phosphorylated in an *in vitro kinase* assay, as described in the materials and methods. The arrow indicates the *in vitro* phosphorylated Cbl protein. The positions of prestained molecular weight markers are indicated on the left side of the figure.

The lysates remaining after the second immunodepletion and the immunoprecipitates obtained from the first immunoprecipitation were analyzed by Western blotting. Densitometric quantification analysis of MET in figure 4C was carried out on directly scanned images using NIH Image 1.62 for Macintosh software. The densitometric quantification shows the MET protein at each time point expressed as a percentage of the initial Met protein at time zero. All values of MET have been adjusted for loading control protein ERK.

Immune Complex Kinase Assays

The Cbl immune complexes from unstimulated or HGF-stimulated cells were washed three times with lysis buffer followed by washing twice with kinase buffer (50 mM HEPES (pH 7.5), 10 mM MgCl, and 10 mM MnCl, 1 μM sodium orthovanadate), suspended in 20 μl of kinase buffer containing 10 μCi (γ-^{32}P)ATP, and incubated for 30 minutes at room temperature. The proteins were separated on 10 % SDS-PAGE, the gel was dried for 3 hours, and the dried gel was autoradiographed at -80°C overnight.

Results

Cbl is strongly phosphorylated on tyrosine residues following HGF stimulation

We have recently demonstrated that activation of MET in Namalwa B cells leads to strong tyrosine phosphorylation of two proteins with molecular weights of 110-120 kDa [33;34]. The smaller of these proteins was shown to represent the Grb2-associated

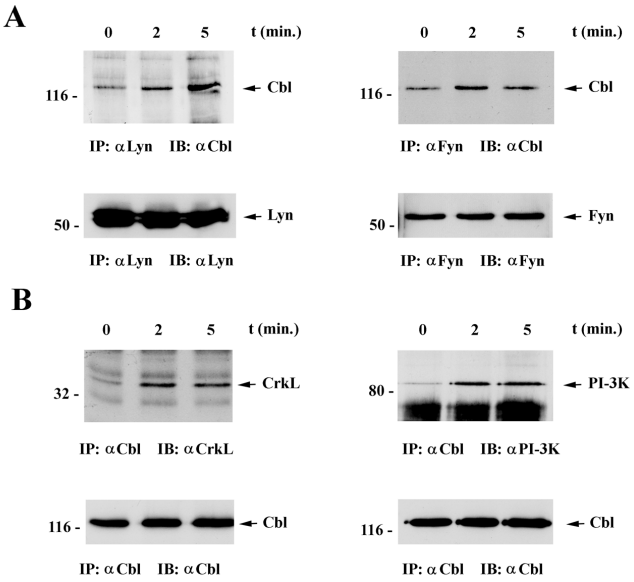


Figure 3

HGF stimulation leads to increased association of Cbl with Fyn, Lyn, CrkL and the p85 subunit of PI-3 kinase.

Namalwa B cells were stimulated with HGF for the indicated time. The cells were lysed and immunoprecipitates were collected using the indicated antibodies. **A.** Increased association with Lyn and Fyn. Lyn and Fyn immunoprecipitates were subjected to immunoblotting using anti-Cbl antibodies (upper panels). **B.** Increased association with CrkL and PI-3 kinase. Cbl immunoprecipitates were subjected to immunoblotting using anti-CrkL and anti-p85 antibodies (PI-3 kinase) (upper panels). Equal loading of the samples was confirmed by restaining the blots with the same antibodies used for immunoprecipitation. (lower panels).

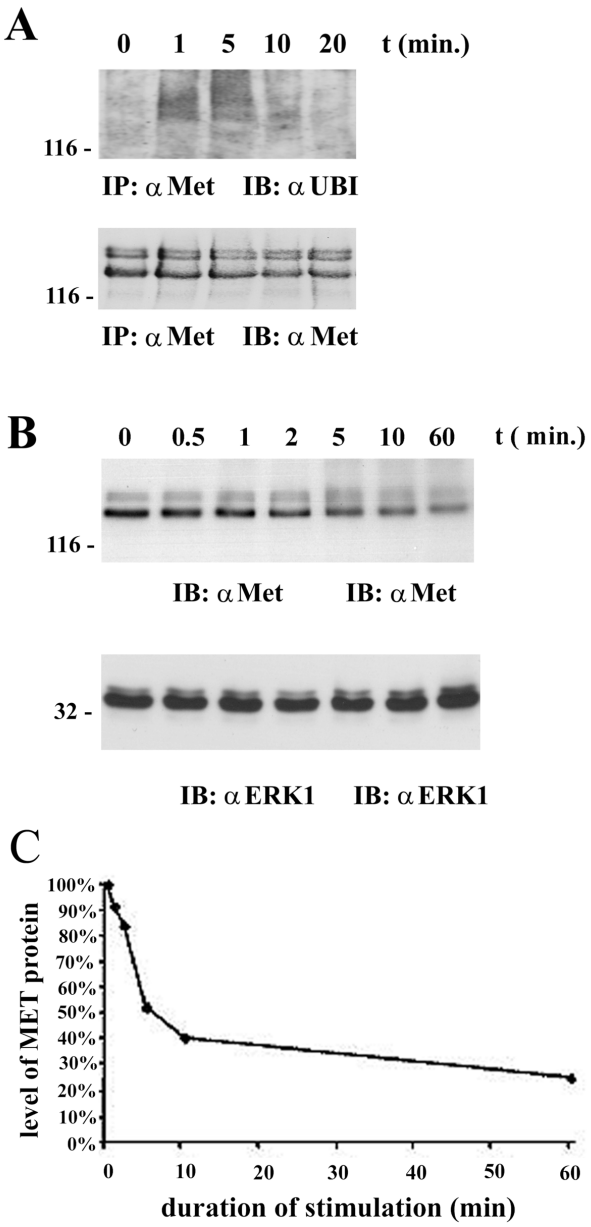


Figure 4

HGF stimulation induces ubiquitination and degradation of MET in B cells.

A. HGF induces ubiquitination of MET. Namalwa B cells were stimulated with HGF for the indicated time. Anti-MET (C12) immunoprecipitates were immunoblotted with anti-ubiquitin antibodies (upper panel) or, as a loading control, with anti-MET antibodies (lower panel). **B.** HGF induces degradation of MET. Namalwa B cells were incubated in the presence or absence of HGF for the indicated time. Cell lysates were immunoblotted with anti-Met antibodies (upper panel) or, as a loading control, with anti-ERK1 antibodies (lower panel). **C.** The protein levels of MET in 4B were analyzed by densitometric quantification and plotted as relative MET protein level. The Met level time zero was assigned as 100%.

binder 1 (Gab-1), an adaptor protein that can associate with the cytoplasmic docking site of MET [34]. By performing immunodepletion experiments, we now identified the larger prominent phosphoprotein in the lysates of HGF-stimulated cells as c-Cbl (Figure 1A). Immunoblotting of Cbl immunoprecipitates with antibodies against phosphotyrosine confirmed that HGF stimulation leads to a rapid and transient phosphorylation of Cbl on tyrosine residues, peaking at one minute and decreasing after 5 minutes (Figure 1B).

HGF stimulation leads to enhanced association of c-Cbl with Fyn, Lyn, PI-3 kinase, and CrkL

The above observations prompted us to explore the function of Cbl in MET signaling. Although Cbl itself lacks kinase activity, its characteristic modular structure enables it to act as a scaffold for various signaling molecules, including cytoplasmic tyrosine kinases [40]. To determine whether HGF stimulation leads to changes in the kinase activity associated with Cbl, we conducted *in vitro* kinase assays. We observed that a low level of kinase activity was associated with Cbl immunoprecipitated from unstimulated B cells. However, HGF stimulation greatly increased the Cbl-associated kinase activity (Figure 2). The 120 kDa *in vitro* phosphorylated protein present after stimulation with HGF represents c-Cbl itself, whereas the bands at 55-60 kDa may represent (auto-) phosphorylated Src-family tyrosine kinases associated with c-Cbl. These kinases presumably are involved in the *in vitro* phosphorylation of c-Cbl and associated proteins.

In order to identify signaling molecules that dock on Cbl following HGF stimulation, the effect of HGF stimulation on the physical interaction with several candidate partners of Cbl was explored. These included the Src-family tyrosine kinases Fyn and Lyn, the p85 regulatory chain of PI-3 kinase and CrkL. We observed that these molecules all show a weak basal interaction with Cbl. However, upon stimulation with HGF, these interactions

were either moderately (Fyn) or strongly (Lyn, CrkL, and PI-3K) enhanced (Figure 3). Hence, HGF stimulation does not only induce tyrosine phosphorylation of Cbl, but also enhances its ability to act as a docking protein for several important signaling molecules.

Cbl plays a critical role in the MET ubiquitination

The prominent phosphorylation of Cbl in response to HGF stimulation (Figure 1) combined with the recent observation that Cbl acts as an E3 ubiquitin ligase for the EGF and PDGF receptors [41;42], suggests that Cbl might be involved in the ubiquitination and degradation of MET. To adress this hypothesis, we first assessed whether Met on B cells is ubiquitinated in response to HGF stimulation. Hence, MET immunoprecipitates from HGF-stimulated cells were analyzed for ubiquitination by immunoblotting. We observed that HGF stimulation leads to a rapid ubiquitination of c-MET, which was maximal at 5 minutes (Figure 4A). Since the ubiquitination machinery adds multiple and variable numbers of ubiquitin moieties to a single target molecule, the poly-ubiquitinated MET species is detected as a smear rather than a distinct band (Figure 4A). In addition to inducing ubiquitination, HGF stimulation

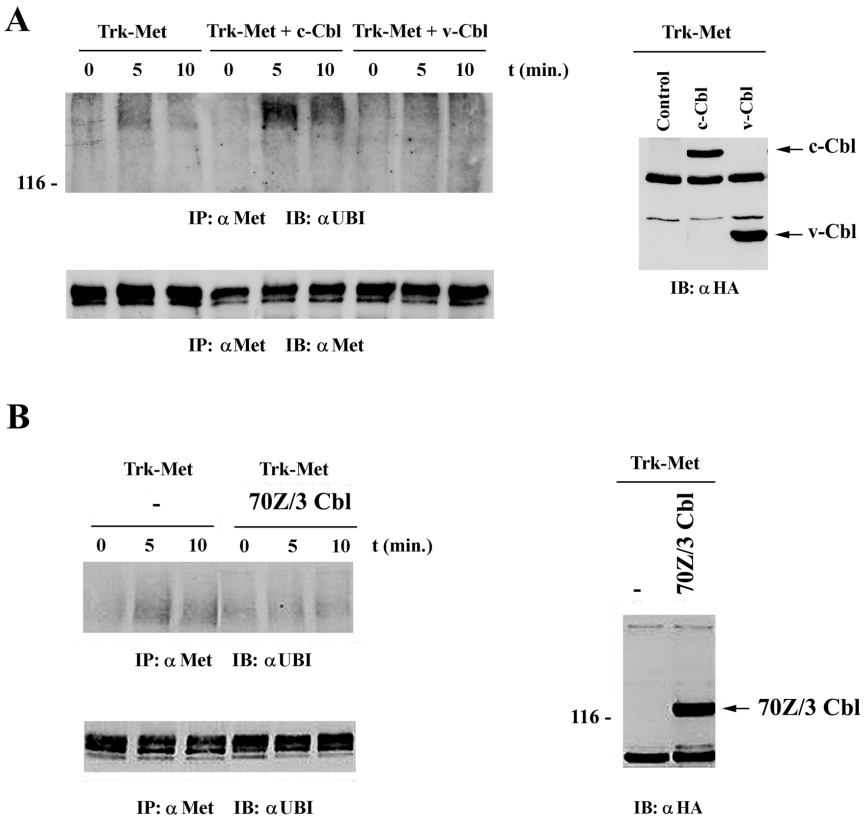


Figure 5

c-Cbl, but not the oncogenic Cbl variants v-Cbl and 70Z Cbl, mediates ubiquitination of MET.

A. c-Cbl, but not v-Cbl, mediates MET ubiquitination. Cos-7 cells were transfected with Trk-MET plus either c-Cbl or v-Cbl and stimulated with NGF for the indicated time periods. Anti-MET immunoprecipitates (SP260) were immunoblotted with anti-ubiquitin antibodies (upper panel). As a control for equal MET transfection and immunoprecipitation, the blot was re-stained with anti-MET antibodies (SP260) (lower panel). Right panel: The total cell lysates were immunoblotted with anti-HA antibodies to demonstrate equal expression of c-Cbl and v-Cbl. **B.** 70Z/3 Cbl is dominant negative in MET ubiquitination. Cos-7 cells were transfected with Trk-MET in the absence or presence of 70Z/3 Cbl and stimulated with NGF for the indicated time periods. Anti-MET immunoprecipitates (SP260) were immunoblotted with anti-ubiquitin antibodies (upper panel). As a control for equal MET transfection and immunoprecipitation, the blot was re-stained with anti-MET antibodies (SP260) (lower panel). Right panel: The total cell lysates were immunoblotted with anti-HA antibodies to demonstrate expression of 70Z/3 Cbl.

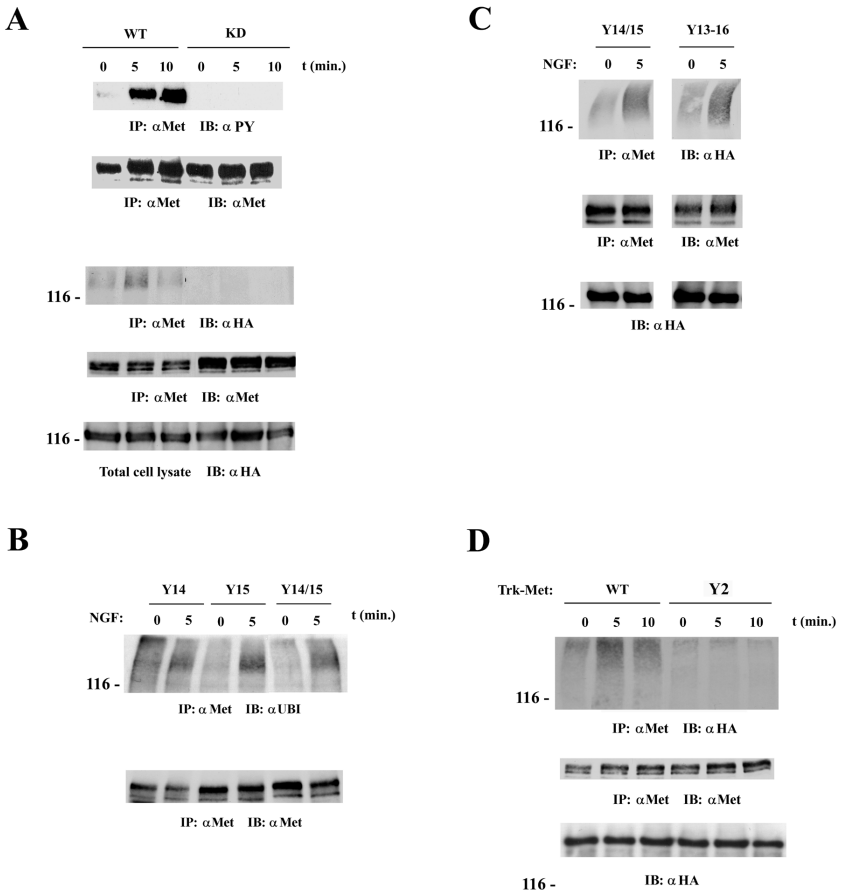


Figure 6

The juxtamembrane tyrosine residue 1001 (Y2) of MET, but not the docking site, is required for ligand-induced receptor ubiquitination by Cbl.

A. Tyrosine phosphorylation and ubiquitination of Trk-MET and kinase-dead Trk-MET (Trk-MET KD). Cos-7 cells, transfected with either wild type (WT) or kinase-dead (KD) Trk-MET were stimulated with NGF for 5 or 10 minutes. MET was immunoprecipitated with anti-MET (SP260) and the blot was stained with anti-phosphotyrosine (PY20) (top panel) and subsequently stripped and reprobed with anti-MET (B2) as a control (second panel). In parallel, Cos-7 cells were transfected with the indicated Trk-MET constructs together with HA-tagged ubiquitin and HA-tagged c-Cbl. Anti-MET immunoprecipitates were immunoblotted with anti-HA antibodies to detect MET ubiquitination (third panel), or as a control for equal MET transfection and immunoprecipitation, the blot was restained with anti-MET antibodies (fourth panel), and, as a control for the c-Cbl transfection, total cell lysates were blotted with anti-HA antibodies (bottom panel). **B.** Mutation of Y14, Y15 or both (Y14/15) of Trk-MET does not prevent ligand-induced receptor ubiquitination by Cbl. Cos-7 cells were transfected with the indicated Trk-MET mutant constructs together with c-Cbl. Unstimulated or NGF-stimulated cells were subjected to immunoprecipitation using anti-MET antibodies and immunoblotted with anti-ubiquitin antibodies (upper panel), and, as a control, the blot was restained with anti-MET antibodies (lower panel). **C.**Trk-MET mutated at tyrosines Y13-16 is ubiquitinated upon ligand stimulation. Immunoprecipitates of MET from Cos-7 cells, transfected with either Y14/15 or Y13-16 Trk-MET mutant together with HA-tagged ubiquitin and HA-tagged c-Cbl, were immunoblotted with anti-HA antibodies to detect MET ubiquitination (top panel), and the blot was reprobed with anti-MET antibodies to demonstrate equal transfection and immunoprecipitation (middle panel). In addition, total cell lysates were blotted with anti-HA antibodies to show equal transfection and expression of HA-tagged Cbl (bottom pannel). **D.** Y2 of MET is required for Cbl-mediated ubiquitination. Immunoprecipitates of MET from unstimulated or NGF-stimulated Cos-7 cells, transfected with either WT or an Y2 mutant of Trk-MET together with HA-tagged ubiquitin and HA-tagged c-Cbl, were immunoblotted with anti-HA antibodies to detect MET ubiquitination (top panel), and the blot was reprobed with anti-MET antibodies to demonstrate equal transfection and immunoprecipitation (middle panel). In addition, total cell lysates were blotted with anti-HA antibodies to confirm equal transfection and expression of HA-tagged Cbl (bottom pannel).

also resulted in degradation of MET, which was clearly detectable from 5-10 min of incubation onward (Figure 4B and C). Hence, HGF stimulation of B cells leads to both ubiquitination and degradation of MET.

To explore the role of Cbl in MET ubiquitination, COS-7 cells were transfected with Trk-MET, a chimeric receptor that consists of the extracellular domain of the NGF receptor (Trk A) and the intracellular domain of Met, either alone or in combination with c-Cbl. After NGF stimulation, Trk-MET was immunopreci-pitated and its ubiquitination was analyzed. As shown in Figure 5, cotransfection of c-Cbl clearly enhanced the ligand-induced ubiquitination of Trk-MET. By contrast, overexpression of the oncogenic Cbl variant v-Cbl, which only consists of the N-terminal 357 amino acids, did not enhance the ligand-induced ubiquitination of the transfected Trk-MET but rather suppressed the (weak) ubiquitination mediated by endogenous Cbl (Figure 5A). Moreover, similar results were obtained for the oncogenic mutant 70Z/3 Cbl, which only lacks a functional RING finger domain as a consequence of the deletion of amino acids 366-382 (Figure 5B) [40]. These findings demonstrate that c-Cbl is involved in Met ubiquitination, whereas the oncogenic v-Cbl and 70Z/3 Cbl are unable to mediate ubiquitination but, instead, act in a dominant negative fashion on endogenous c-Cbl.

The juxtamembrane tyrosine residue 1001 (Y2), but not the multisub-strate docking site of MET, is required for receptor ubiquitination by Cbl

Upon stimulation by HGF, the C-terminus of MET is strongly phosphorylated on tyrosine residues. Autophosphorylation of tyrosine residues 1349 (Y14) and 1356 (Y15) of MET is critical for most biological responses [29;43-47]. These tyrosine residues serve as a multisubstrate docking site for several proteins, including Gab-1, Grb-2, PI-3 kinase, PLC, Src, Shc, SHP-2, and Stat-3. To assess whether this site is also involved in transducing signals leading to MET ubiquitination, we employed Trk-MET mutated at Y14 and/or Y15. Whereas mutation of the kinase regulatory tyrosines 1234 (Y8) and 1235 (Y9), which gives rise to a kinase-dead Trk-MET, resulted in a total abrogation of ligand-induced autophosphorylation and ubiquitination (Figure 6A), NGF stimulation still resulted in a clear ubiquitination of the single (either Y14 or Y15) as well as double mutant (Y14/15) (Figure 6B). This demonstrates that Y14 and Y15 are not required for Cbl-mediated ubiquitination of MET (Figure 6B). This result was not due to functional redundancy by the presence of the tyrosines 1313 (Y13) and 1363 (Y16), as a Trk-MET mutant containing mutations in Y13-16, i.e., all four autophosphorylated residues of MET C-terminal of the kinase domain, was still readily ubiquitinated upon stimulation with ligand (Figure 6C). Given this unexpected result, combined with the observed gain-of-function effect as a consequence of its mutation, i.e. the transition of epithelial cells to a fibroblastoid phenotype [39], we hypothesized that the juxtamembrane tyrosine residue Y1001 (Y2) might play an important role in Met ubiquitination. Interestingly, mutation of Y2 indeed resulted in the complete loss of ligand-induced Cbl-mediated ubiquitination of MET (Figure 6D).

Discussion

We identified one of the most prominent phosphoproteins in lysates of HGF stimulated B lymphoma cells as Cbl (Figure 1), thus implicating Cbl in HGF/MET signaling. Recently, Cbl phosphorylation has also been observed upon HGF-stimulation of the epithelial MDCK and Hela cells [48;49] as well as in Tpr-MET transformed fibroblasts [50]. The multidomain docking protein p120 Cbl is the cellular homologue of the *v-cbl* oncogene from the murine Cas NS-1 retrovirus, which induces pre-B lymphomas and myeloid leukemias [51]. Cbl is prominently tyrosine phosphorylated upon stimulation of a number of receptors, resulting in its interaction with Src homology

2 (SH2) domain containing proteins such as the p85 subunit of the PI-3 kinase, the guanine nucleotide exchange factor Vav, and the Crk adaptor protein family [40]. Indeed, HGF stimulation led to an increase in the amount of kinase activity associated with Cbl (Figure 2), as well as an enhanced association between Cbl and Fyn, Lyn, the p85 chain of PI-3 kinase, and CrkL (Figure 3). Apart from binding to Cbl via their SH2 domains, these proteins may also interact with Cbl via their SH3 domains. This interaction with proline-rich regions on Cbl presumably is important for the stimulus independent part of their Cbl association (Figure 3) [52;53].

Our observation that HGF stimulation leads to an enhanced association of Cbl with PI-3 kinase as well with CrkL is of considerable interest. PI-3 kinase is a central regulator of different biological processes induced by HGF including adhesion and survival, and a specific PI-3 kinase docking site has been located on Y1349 of MET [34]. Our present findings suggest that association of PI-3 kinase with Cbl (Figure 3) might represent an alternative route for the regulation of PI-3 kinase activity by HGF. CrkL is an adaptor protein with two SH3 domains, which can specifically bind to the guanine exchange factor C3G, an activator of Rap-1 [54]. Formation of a Cbl-Crk-C3G complex may provide a mechanism for coupling MET with the Rap-1 pathway, which has been implicated in integrin activation [55]. Interestingly, we have recently shown that HGF induces activation of integrins in B cells [10]. Cbl may play a critical role in this HGF-induced integrin activation as suppression of Cbl expression by antisense Cbl resulted in a marked decrease in integrin activation [55-57].

Receptor ubiquitination and consequent degradation by the proteosomal/lysosomal pathway constitutes an integral part of the regulation of receptor PTK function [41;58-60]. Indeed, we observed that, following stimulation of B cells with HGF, MET is ubiquitinated and degraded (Figure 4). This observation confirms and extends observations by Jeffers and colleagues, who reported HGF-induced degradation and polyubiquitination of MET in epithelial cells [61]. Importantly, we now demonstrate that Cbl plays a key role in the negative regulation of MET signaling, by mediating receptor ubiquitination (Figure 5). A number of studies have identified Cbl as an important negative regulator of PTKs. In *C. elegans*, the Cbl homologue SLI-1 was shown to inhibit vulva development mediated by LET-23, a homologue of the mammalian epidermal growth factor (EGF) receptor [62], whereas overexpression of Cbl in mammalian cells inhibits activation of the EGF- and PDGF-receptors and JAK-STAT [63-65]. Recently, *in vitro* studies revealed that the c-Cbl has intrinsic E3 ubiquitin-protein ligase activity [60]. The RING finger domain of Cbl is critical for this regulatory function as mutants of Cbl, containing a complete (v-Cbl) or partial (70Z/3 Cbl) deletion, or a point mutation (Cys381-Ala) in the RING finger domain, are defective in promoting receptor tyrosine kinase ubiquitination. Indeed, also in our present study, the oncogenic mutants v-Cbl and 70Z/3 Cbl failed to induce ubiquitination of MET but rather had a dominant negative effect on the ubiquitination induced by endogenous Cbl (Figure 5). This is further supported by the recent finding that expression of 70Z/3 Cbl in MDCK cells results in an epithelial-mesenchymal transition, which resembles the effect of HGF stimulation [49]. Thus, expression of these oncogenic mutants of Cbl might result in overexpression and constitutive activation of MET, leading to MET-mediated tumorigenesis.

The tyrosine residues Y14 and Y15 play a critical role in virtually all MET-mediated biological responses. These residues serve as docking sites for multiple signaling molecules, including Gab-1, Grb-2, PI3-kinase, PLC, Src, Shc, SHP-2, and Stat-3 [29;43-47]. Interestingly, we observed that this multisubstrate docking site of MET is not required for ubiquitination by Cbl. Mutation of neither the tyrosines Y14 and/or Y15, nor of all autophosphorylated residues in the C-terminal domain of MET, *i.e.* Y13-16, interfered with NGF-induced ubiquitination of Trk-MET (Fig 6B and C).

By contrast, MET ubiquitination was dependent on the integrity of the juxtamembrane tyrosine residue Y2 (Fig 6D). These data support a recent study, which demonstrated a role for Cbl and Y2 in ligand-independent ubiquitination of MET [66]. In addition, here we have shown that Cbl and Y2 are also critical in MET ubiquitination induced by ligand (Fig 5 and 6D), that the oncogenic mutants 70Z/3 Cbl and v-Cbl act in a dominant negative fashion (Fig 5A and B), that ubiquitination of MET does not depend on its C-terminal tyrosine residues (Y13-16), which include the docking site of MET (Y14/15) (Fig 6B and C). Previously, it has been reported that mutation of residue Y2 of MET leads to a gain-of-function resulting in constitutive scattering and fibroblastoid morphology of epithelial cells [39]. Our data suggest that this may be due to a defect in Cbl-mediated MET ubiquitination. In addition, although most germline and sporadic MET mutations in human tumors involve the kinase domain and result in enhanced kinase activity upon stimulation with ligand [20-22], recently mutations have also been reported in the juxtamembrane portion of MET [24]. MET carrying such a missense mutation at P1009S (P989 in mouse) was not constitutively active but showed increased and persistent MET phosphorylation after HGF treatment. This activating mutation is localized in a PEST-like sequence, which has been implicated in ubiquitination [24]. Hence, tyrosine Y2 and adjacent sequences in the juxtamembrane domain of MET appear to play a critical role in the negative regulation of MET by Cbl. Taken together, these findings identify Cbl as negative regulator of MET and suggest that defects in this negative regulation, caused by mutations in either Cbl or MET may contribute to tumorigenesis.

Acknowledgements

We thank Lia Smit and Esther Schilder-Tol for technical assistance, and Gerda van der Horst for generation of the Cbl constructs. We are also grateful to Dr Martin Sachs and Dr Walter Birchmeier for kindly providing the Trk-MET constructs, and Dr Paul M. P. van Bergen en Henegouwen for the ubiquitin construct.

References

1. Bottaro, D.P., J.S. Rubin, D.L. Faletto, A.M. Chan, T.E. Kmiecik, W.G. Vande Woude, and S.A. Aaronson. 1991. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science 251*:802.
2. Naldini, L., E. Vigna, R.P. Narsimhan, G. Gaudino, R. Zarnegar, G.K. Michalopoulos, and P.M. Comoglio. 1991. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. *Oncogene 6*:501.
3. van de Voort, R., T.E. Taher, P.W. Derksen, M. Spaargaren, R. van der Neut, and S.T. Pals. 2000. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. *Adv Cancer Res 79* :39.
4. Brinkmann, V., H. Foroutan, M. Sachs, K.M. Weidner, and W. Birchmeier. 1995. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol 131 :1573.
5. Sachs, M., K.M. Weidner, V. Brinkmann, I. Walther, A. Obermeier, A. Ullrich, and W. Birchmeier. 1996. Motogenic and morphogenic activity of epithelial receptor tyrosine kinases. J Cell 133:1095.
6. Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 376:768.
7. Grant, D.S., H.K. Kleinman, I.D. Goldberg, M.M. Bhargava, B.J. Nickoloff, J.L. Kinsella, P. Polverini, and E.M. Rosen. 1993. Scatter factor induces blood vessel formation in vivo. Proc Natl Acad Sci U S A 90:1937.
8. Bardelli, A., P. Longati, D. Albero, S. Goruppi, C. Schneider, C. Ponzetto, and P.M. Comoglio. 1996. HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. EMBO J 15:6205.
9. Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda, and N. Kitamura. 1995. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature 373:702.
10. van de Voort, R., T.E. Taher, R.M. Keehnen, L. Smit, M. Groenink, and S.T. Pals. 1997. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. *J Exp Med 185*:2121.
11. Weimar, I.S., D. de Jong, E.J. Muller, T. Nakamura, J.M. van Gorp, G.C. de Gast, and W.R. Gerritsen.

1997. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins. *Blood 89*:990.

12. Giordano, S., Z. Zhen, E. Medico, G. Gaudino, F. Galimi, and P.M. Comoglio. 1993. Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc Natl Acad Sci U S A 90*:649.
13. Rong, S., S. Segal, M. Anver, J.H. Resau, and W.G. Vande Woude. 1994. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc Natl Acad Sci U S A 91*:4731.
14. Weidner, K.M., J. Behrens, J. Vandekerckhove, and W. Birchmeier. 1990. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J Cell Biol 111*:2097.
15. Ponzetto, C., S. Giordano, F. Peverali, V.G. Della, M.L. Abate, G. Vaula, and P.M. Comoglio. 1991. c-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. *Oncogene 6*:553.
16. Rusciano, D., P. Lorenzoni, and M.M. Burger. 1996. Constitutive activation of c-Met in liver metastatic B16 melanoma cells depends on both substrate adhesion and cell density and is regulated by a cytosolic tyrosine phosphatase activity. *J Biol Chem 271*:20763.
17. Di Renzo, M.F., M. Olivero, A. Giacomini, H. Porte, E. Chastre, L. Mirossay, B. Nordlinger, S. Bretti, S. Bottardi, and S. Giordano. 1995. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clin Cancer 1*:147.
18. Jeffers, M., S. Rong, and W.G. Vande Woude. 1996. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. *Mol Cell Biol 16*:1115.
19. Cooper, C.S., M. Park, D.G. Blair, M.A. Tainsky, K. Huebner, C.M. Croce, and W.G. Vande Woude. 1984. Molecular cloning of a new transforming gene from a chemically transformed human cell line. *Nature 311*:29.
20. Jeffers, M., L. Schmidt, N. Nakaigawa, C.P. Webb, G. Weirich, T. Kishida, B. Zbar, and W.G. Vande Woude. 1997. Activating mutations for the met tyrosine kinase receptor in human cancer. *Proc Natl Acad Sci U S A 94*:11445.
21. Michieli, P., C. Basilico, S. Pennacchietti, A. Maffe, L. Tamagnone, S. Giordano, A. Bardelli, and P.M. Comoglio. 1999. Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. *Oncogene 18*:5221.
22. Jeffers, M., M. Fiscella, C.P. Webb, M. Anver, S. Koochekpour, and W.G. Vande Woude. 1998. The mutationally activated Met receptor mediates motility and metastasis. *Proc Natl Acad Sci U S A 95*:14417.
23. Giordano, S., A. Maffe, T.A. Williams, S. Artigiani, P. Gual, A. Bardelli, C. Basilico, P. Michieli, and P.M. Comoglio. 2000. Different point mutations in the met oncogene elicit distinct biological properties. *FASEB J 14*:399.
24. Lee, J.H., S.U. Han, H. Cho, B. Jennings, B. Gerrard, M. Dean, L. Schmidt, B. Zbar, and W.G. Vande Woude. 2000. A novel germ line juxtamembrane Met mutation in human gastric cancer. *Oncogene 19*:4947.
25. Derksen, P.W.B., R.M.J. Keehnen, L.M. Evers, M.H.J. van Oers, M. Spaargaren, and S.T. Pals. 2002. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. *Blood 99*: 1405.
26. Borset, M., E. Lien, T. Espevik, E. Helseth, A. Waage, and A. Sundan. 1996. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. *J Biol Chem 271* :24655.
27. Takai, K., J. Hara, K. Matsumoto, G. Hosoi, Y. Osugi, A. Tawa, S. Okada, and T. Nakamura. 1997. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. *Blood 89*:1560.
28. Seidel, C., M. Borset, I. Turesson, N. Abildgaard, A. Sundan, and A. Waage. 1998. Elevated serum concentrations of hepatocyte growth factor in patients with multiple myeloma. The Nordic Myeloma Study Group. *Blood 91*:806.
29. Ponzetto, C., A. Bardelli, Z. Zhen, F. Maina, Z.P. dalla, S. Giordano, A. Graziani, G. Panayotou, and P.M. Comoglio. 1994. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell 77*:261.
30. Weidner, K.M., S. Di Cesare, M. Sachs, V. Brinkmann, J. Behrens, and W. Birchmeier. 1996. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature 384*:173.
31. Schaeper, U., N.H. Gehring, K.P. Fuchs, M. Sachs, B. Kempkes, and W. Birchmeier. 2000. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. *J Cell Biol 149*:1419.
32. Sakkab, D., M. Lewitzky, G. Posern, U. Schaeper, M. Sachs, W. Birchmeier, and S.M. Feller. 2000. Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. *J Biol Chem 275*:10772.

33. van de Voort, R., T.E. Taher, V.J. Wielenga, M. Spaargaren, R. Prevo, L. Smit, G. David, G. Hartmann, E. Gherardi, and S.T. Pals. 1999. Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. *J Biol Chem 274*:6499.
34. van de Voort, R., R.M. Keehnen, E.A. Beuling, M. Spaargaren, and S.T. Pals. 2000. Regulation of cytokine signaling by B cell antigen receptor and CD40-controlled expression of heparan sulfate proteoglycans. *J Exp Med 192*:1115.
35. Kim, T.J., Y.T. Kim, and S. Pillai. 1995. Association of activated phosphatidylinositol 3-kinase with p120cbl in antigen receptor-ligated B cells. *J Biol Chem 270*:27504.
36. Cory, G.O., R.C. Lovering, S. Hinshelwood, L. MacCarthy-Morrogh, R.J. Levinsky, and C. Kinnon. 1995. The protein product of the c-cbl protooncogene is phosphorylated after B cell receptor stimulation and binds the SH3 domain of Bruton's tyrosine kinase. *J Exp Med 182*:611.
37. Tezuka, T., H. Umemori, N. Fusaki, T. Yagi, M. Takata, T. Kurosaki, and T. Yamamoto. 1996. Physical and functional association of the cbl protooncogen product with an src-family protein tyrosine kinase, p53/56lyn, in the B cell antigen receptor-mediated signaling. *J Exp Med 183*:675.
38. Panchamoorthy, G., T. Fukazawa, S. Miyake, S. Soltoff, K. Reedquist, B. Druker, S. Shoelson, L. Cantley, and H. Band. 1996. p120cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. *J Biol Chem 271*:3187.
39. Weidner, K.M., M. Sachs, D. Riethmacher, and W. Birchmeier. 1995. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. *Proc Natl Acad Sci U S A 92*:2597.
40. Smit, L. and J. Borst. 1997. The Cbl family of signal transduction molecules. *Crit Rev Oncog 8*:359.
41. Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W.Y. Langdon, L. Beguinot, B. Geiger, and Y. Yarden. 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev 12*:3663.
42. Miyake, S., K.P. Mullane-Robinson, N.L. Lill, P. Douillard, and H. Band. 1999. Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. *J Biol Chem 274*:16619.
43. Fixman, E.D., M.A. Naujokas, G.A. Rodrigues, M.F. Moran, and M. Park. 1995. Efficient cell transformation by the Tpr-Met oncoprotein is dependent upon tyrosine 489 in the carboxy-terminus. *Oncogene 10*:237.
44. Nguyen, L., M. Holgado-Madruga, C. Maroun, E.D. Fixman, D. Kamikura, T. Fournier, A. Charest, M.L. Tremblay, A.J. Wong, and M. Park. 1997. Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional Grb2 binding site involving tyrosine 1356. *J Biol Chem 272*:20811.
45. Pelicci, G., S. Giordano, Z. Zhen, A.E. Salcini, L. Lanfrancone, A. Bardelli, G. Panayotou, M.D. Waterfield, C. Ponzetto, and P.G. Pelicci. 1995. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene 10*:1631.
46. Ponzetto, C., Z. Zhen, E. Audero, F. Maina, A. Bardelli, M.L. Basile, S. Giordano, R. Narsimhan, and P. Comoglio. 1996. Specific uncoupling of GRB2 from the Met receptor. Differential effects on transformation and motility. *J Biol Chem 271*:14119.
47. Zhu, H., M.A. Naujokas, E.D. Fixman, K. Torossian, and M. Park. 1994. Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. *J Biol Chem 269*:29943.
48. Garcia-Guzman, M., E. Larsen, and K. Vuori. 2000. The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene 19*:4058.
49. Fournier, T.M., L. Lamorte, C.R. Maroun, M. Lupher, H. Band, W. Langdon, and M. Park. 2000. Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. *Mol Biol Cell 11*:3397.
50. Fixman, E.D., M. Holgado-Madruga, L. Nguyen, D.M. Kamikura, T.M. Fournier, A.J. Wong, and M. Park. 1997. Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. *J Biol Chem 272*:20167.
51. Langdon, W.Y., J.W. Hartley, S.P. Klinken, S.K. Ruscetti, and H.C. Morse. 1989. v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. *Proc Natl Acad Sci U S A 86*:1168.
52. Feshchenko, E.A., W.Y. Langdon, and A.Y. Tsygankov. 1998. Fyn, Yes, and Syk phosphorylation sites in c-Cbl map to the same tyrosine residues that become phosphorylated in activated T cells. *J Biol Chem 273*:8323.

53. Hunter, S., E.A. Burton, S.C. Wu, and S.M. Anderson. 1999. Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. *J Biol Chem 274*:2097.
54. Miyake, S., M.L.J. Lupher, C.E. Andoniou, N.L. Lill, S. Ota, P. Douillard, N. Rao, and H. Band. 1997. The Cbl protooncogene product: from an enigmatic oncogene to center stage of signal transduction. *Crit Rev Oncog 8*:189.
55. Reedquist, K.A., E. Ross, E.A. Koop, R.M. Wolthuis, F.J. Zwartkruis, Y. van Kooyk, M. Salmon, C.D. Buckley, and J.L. Bos. 2000. The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J Cell Biol 148* :1151.
56. Meng, F. and C.A. Lowell. 1998. A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO J 17*:4391.
57. Zell, T., C.S. Warden, A.S. Chan, M.E. Cook, C.L. Dell, S.W. Hunt, and Y. Shimizu. 1998. Regulation of beta 1-integrin-mediated cell adhesion by the Cbl adaptor protein. *Curr Biol 8*:814.
58. Miyake, S., M.L.J. Lupher, B. Druker, and H. Band. 1998. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. *Proc Natl Acad Sci U S A 95*:7927.
59. Lee, P.S., Y. Wang, M.G. Dominguez, Y.G. Yeung, M.A. Murphy, D.D. Bowtell, and E.R. Stanley. 1999. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *EMBO J 18*:3616.
60. Joazeiro, C.A., S.S. Wing, H. Huang, J.D. Leverson, T. Hunter, and Y.C. Liu. 1999. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science 286*:309.
61. Jeffers, M., G.A. Taylor, K.M. Weidner, S. Omura, and W.G. Vande Woude. 1997. Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Mol Cell Biol 17*:799.
62. Yoon, C.H., J. Lee, G.D. Jongeward, and P.W. Sternberg. 1995. Similarity of sli-1, a regulator of vulval development in C. elegans, to the mammalian proto-oncogene c-cbl. *Science 269*:1102.
63. Ueno, H., K. Sasaki, K. Miyagawa, H. Honda, K. Mitani, Y. Yazaki, and H. Hirai. 1997. Antisense repression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathway but not the ras pathway in epidermal growth factor receptor signaling. *J Biol Chem 272*:8739.
64. Thien, C.B. and W.Y. Langdon. 1997. Tyrosine kinase activity of the EGF receptor is enhanced by the expression of oncogenic 70Z-Cbl. *Oncogene 15*:2909.
65. Bonita, D.P., S. Miyake, M.L.J. Lupher, W.Y. Langdon, and H. Band. 1997. Phosphotyrosine binding domain-dependent upregulation of the platelet-derived growth factor receptor alpha signaling cascade by transforming mutants of Cbl: implications for Cbl's function and oncogenicity. *Mol Cell Biol 17*:4597.
66. Peschard, P., T.M. Fournier, L. Lamorte, M.A. Naujokas, H. Band, W.Y. Langdon, and M. Park. 2001. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell 8*:995.

CHAPTER **6**

Illegitimate WNT signaling promotes proliferation of multiple myeloma cells

Patrick W.B. Derksen[1], Esther Tjin[1], Helen P. Meijer[1], Melanie D. Klok[1], Harold D. Mac Gillavry[1] Marinus H.J. van Oers[2], Henk M. Lokhorst[3], Andries C. Bloem[4], Hans Clevers[5], Roel Nusse[6], Ronald van der Neut[1], Marcel Spaargaren[1], and Steven T. Pals[1]

From the Departments of [1]Pathology and [2]Hematology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; the Departments of [3]Hematology and [4]Immunology University Medical Center, University of Utrecht, Utrecht, The Netherlands; [5]the Hubrecht Laboratory, Utrecht, The Netherlands; [6] Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University, Stanford, CA.

The unrestrained growth of tumor cells is generally attributed to mutations in essential growth control genes, but tumor cells are also influenced by signals from the environment. In multiple myeloma (MM), the factors and signals coming from the bone marrow (BM) microenvironment are possibly even essential for the growth of the tumor cells. As targets for intervention, these signals may be equally important as mutated oncogenes. Given their oncogenic potential, WNT signals form a class of paracrine growth factors that could act to influence MM cell growth. In this paper, we report that MM cells have hallmarks of active WNT signaling, whereas the cells have not undergone detectable mutations in WNT signaling genes such as *APC* and *β-catenin (CTNNB1)*. We show that the malignant MM plasma cells overexpress β-catenin, including its N-terminally un-phosphorylated form, suggesting active β-catenin/TCF-mediated transcription. Further accumulation and nuclear localization of β-catenin, and/or increased cell proliferation, was achieved by stimulation of WNT signaling with either Wnt3a, LiCl or the constitutively active S33Y mutant of β-catenin. In contrast, by blocking WNT signaling by dominant-negative T cell factor, we can interfere with the growth of MM cells. We therefore suggest that MM cells are dependent on an active WNT signal, which may have important implications for the management of this incurable form of cancer. ***Proceeding National Academy of Sciences, 2004, Vol. 101 (16): 6122-6127.***

Multiple myeloma (MM), one of the most common hematological malignancies in adults, is a neoplasm of terminally differentiated B cells, *i.e.* plasma cells. The tumor cells expand in the bone marrow (BM), ultimately leading to pancytopenia and osteolytic bone destruction. At present, the disease is still incurable with a median survival of ≈ 3-4

years. The transition of a plasma cell to a fully transformed, aggressive myeloma is a multistep process, which requires the acquisition of mutations in several proto-oncogenes and tumor suppressor genes [1]. Most of this evolution takes place in the BM, indicating that signals from the BM microenvironment, which may include paracrine growth factors, play a critical role in sustaining the growth and survival of MM cells during tumor progression [1, 2].

WNT signals form one class of paracrine growth factors that could act to influence MM cell growth. WNT signal transduction components, in particular adenomatous polyposis coli (APC) and β-catenin, are often mutated in cancers and sustained overexpression of *WNT* genes can cause cancer [3-6]. In addition, WNT proteins themselves are able to promote the proliferation of progenitor- or stem cells [7-10]. *WNT* genes encode a family of 19 secreted glycoproteins, which promiscuously interact with several Frizzled receptors. This interaction leads to intracellular signals that control gene expression, cell behavior, cell adhesion, and cell polarity, during both embryonic development and postnatal life [11, 12]. The key event in this signaling pathway is the stabilization of β-catenin. In the absence of WNT signals, a dedicated complex of proteins, including the tumor suppressor gene product APC, axin, and glycogen synthase kinase-3β (GSK3β) controls phosphorylation of specific serine and threonine residues in the N-terminal region of β-catenin. This GSK3β –mediated phosphorylation marks β-catenin for ubiquitination and degradation by the proteasome. Signaling by WNT factors blocks GSK3β activity, resulting in the accumulation of nonphosphorylated β-catenin, which will translocate to the nucleus. Here, it interacts with T cell factor (TCF) transcription factors [13, 14] to drive transcription of target genes [9, 15, 16]. It is now well established that unrestrained β-catenin/TCF activity plays a major role in many human cancers [4, 5]. Mutations of the *APC* tumor suppressor gene or of the sequences encoding the crucial GSK3β phosphorylation sites in the N-terminal domain of β-catenin have been found in the vast majority of colorectal cancers, as well as many other cancer types [4-6]. 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 [5, 9]. Target genes which likely cooperate in neoplastic transformation include *CCND1* (cyclin D1) [15], *MYC* [9, 15], *CD44* [17], and *MET* [18].

Members of the TCF/LEF family of transcription factors were initially discovered in models of early lymphocyte development. In mice and humans, T lineage cells express both TCF1 and LEF1 [7, 19-22], and studies in knockout mice have demonstrated that these factors are essential for the maintenance of progenitor T cell compartments [7, 23, 24]. Similarly, during early B cell development in the fetal liver and adult bone marrow, LEF1 is involved in the regulation of pro-B cell proliferation and survival [8]. By inference, these observations suggest a role for WNT signaling in the control of cell proliferation and survival during lymphocyte development. Indeed, recent *in vitro* and *in vivo* studies, demonstrating that WNT factors and β-catenin also affect lymphocyte progenitor fate as well as stem cell self-renewal, confirm this role [8, 10, 25, 26]. The involvement of the WNT pathway in the regulation of the survival and expansion of progenitor- and stem cells, in combination with its oncogenic potential in nonlymphoid cells, prompted us to test whether deregulation of the WNT pathway occurs in lymphoid neoplasia. Whereas the specificity of WNT signals with respect to target cells is relatively unknown, there are now powerful methods to examine whether cells are activated by a WNT signal. These tools include measuring the levels of the β-catenin protein, in particular a nonphosphorylated form of β-catenin, that is generated by active WNT signaling [27, 28]. In the nucleus, WNT signaling proceeds through the transcription factor TCF, and by interfering with TCF activity [using a dominant-negative form of TCF (ΔTCF4)], one

can examine to what extend the behavior and growth of cells depends on an active WNT signal. Here, we show that WNT signaling is active in MMs and that WNT signals are involved in the control of MM growth.

Materials & Methods

Antibodies

Mouse monoclonal antibodies used were: anti-CD138, BB4 (IgG1)(Instruchemie, Hilversum, The Netherlands); anti-β-catenin (clone 14; IgG1)(BD Biosciences, Erembodegem, Belgium), anti-active (non-phosphorylated) β-catenin (anti-ABC; IgG1) [27, 28]; anti-active (non-phosphorylated) β-catenin (clone 8E4; IgG1)(Alexis Biochemicals, Lausen, Switzerland); anti β-actin (clone AC15, IgG1)(Sigma, St Louis, MO); allophycocyanin (APC)-conjugated anti-CD19 (IgG1); FITC-conjugated anti-IgD (IgG1); FITC conjugated anti-CD45RA (IgG2b)(all BD Biosciences); biotin-conjugated anti-CD38 (IgG1) (Caltag, Burlingame, CA). Polyclonal antibodies used were: rabbit anti-human ERK2 (C14, Santa Cruz Biotechnology, Santa Cruz, CA); Dynabead-conjugated goat anti-mouse IgG (Dynal, Wirral, UK); HRP-conjugated rabbit anti-mouse; FITC-conjugated rabbit anti-mouse; and HRP-conjugated goat anti-rabbit (all DAKO, Carpinteria, CA).

Myelomas, cell cultures, and transfections

Primary myeloma samples and normal BM samples were obtained during routine diagnostic procedures. Mononuclear cells were harvested by standard Ficoll/Paque density gradient centrifugation (Amersham Biosciences, Roozendaal, The Netherlands). Plasma cells were sorted by positive selection using anti-CD138 (clone BB4, Instruchemie) and Dynabead-conjugated goat anti-mouse IgG (Dynal). Positive sorting yielded populations plasma cells that were >97% pure ($CD38^+$ and $CD45RA^-$) as defined by fluorescence-activated cell sorter (FACS) analysis using a FACScalibur flow cytometer (BD Biosciences). MM cell line XG1 [29], LME1 [2] and UM6 [30] were cultured in Iscove's medium (Invitrogen Life Technologies, Breda, The Netherlands) as described [2]. MM cell lines UM1 and UM3 [30], L363 [31], NCI H929 [32] and OPM1 [33] were cultured in RPMI medium 1640 (Invitrogen Life Technologies) containing 10% fetal clone I serum (HyClone Laboratories), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Conditioned medium from parental and Wnt3a-transfected L cells was prepared as described [34]. Wnt3a was purified as described by Willert *et al* [35]. Transient transfection was performed by electroporation of TOP/FOPFLASH [36], constructs expressing constitutively active β-catenin (β-catenin S33Y) [13], and ΔTCF4 [37], using the nucleofector technology in combination with solution V and program T-01 (Amaxa, Cologne, Germany). HeLa cell lysates were from BD Biosciences.

Isolation of B cell populations and plasma cells

B cells were isolated from human tonsils as described [38]. Total B cell fractions were >97% pure, as determined by FACS analysis. Subpopulations were identified according to Pascual *et al* [39], employing CD19, CD38 and IgD as disriminatory criteria. Analysis and sorting was performed by using a FACS Vantage flow cytometer (BD Biosciences).

Luciferase reporter assay

Cells were transfected, and efficiency was determined and normalized by transfection using pEGFP-N3 (Invitrogen Life technologies) and subsequent FACS analysis. After 24 hours, cells were stimulated with Wnt3a (100 ng/ml) for 18 hours, were harvested, were lysed, and luciferase activity was determined as described [18].

Immunohistochemistry and fluorescence microscopy

After the indicated stimulus, cells were washed in PBS, diluted to 1×10^6 cells per ml,

and 100,000 cells were spun onto slides before fixation. For detection of active, nonphosphorylated β-catenin and total β-catenin, mAb 8E4 (Alexis Biochemicals), and C14 (BD Biosciences) were used. In immunohistochemical studies, the slides were subsequently incubated with biotin-conjugated rabbit anti-mouse for 30 min, and horseradish peroxidase-conjugated avidin-biotin complex for 30 min. The substrate was visualized with 3,3-amino-9-ethylcarbazole (Sigma). In immunofluorescence studies, the primary antibodies were detected using biotin-conjugated goat anti-mouse, followed by streptavidin-FITC (both from DAKO) and analyzed by confocal laser scan microscopy. Nuclei were stained with propidium iodide (Molecular Probes, Leiden, The Netherlands).

Western blot analysis

A total of $5 \cdot 10^5$ cells were directly lysed in sample buffer, separated by SDS/10% PAGE, and were blotted. Equal loading was confirmed by staining the part of the blot <50 kDa with either anti-ERK2 or anti-β-actin. The upper part (>50 kDa) was stained for anti-active β-catenin (with either anti-ABC or 8E4), or anti-β-catenin. Primary antibodies were detected by horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibodies, followed by detection using an enhanced chemiluminescence kit (Amersham Pharmacia Biosciences).

Proliferation assay

24 hours after transfection, viable, GFP-positive cells were sorted by using a MoFlow flow cytometer (Cytomation, Freiburg, Germany). Either GFP-positive or nonsorted cells were plated in 96-wells flat bottom tissue culture plates (Costar, Cambridge, MA) at a density of 100,000 cells per ml. Stimuli were added, and cells were cultured in 2% serum for 2-3 days in a total volume of 200 μl. The cell culture was pulsed with 0.5 μCi (1 Ci = 37 GBq) methyl-[^{3}H] thymidine (87 μCi/mmol, Amersham Pharmacia Biosciences) during the last 4 hours of culture. Results are expressed as counts per minute (cpm).

RT-PCR Analysis of *WNT* expression

Total RNA was prepared using Trizol according to the manufacturer (Invitrogen Life technologies). 5 μg RNA was treated with 10 U DNAse I (Roche, Basel, Switzerland) for 1 hour at 37°C, after which reverse transcription was performed using random hexamers (Applied Biosystems, Foster City, CA) and MMLTV reverse transcriptase as recommended by the manufacturer (Invitrogen Life Technologies). Amplicons were size-separated by agarose electrophoresis in 1X TAE. PCR conditions were: denaturing at 96°C for 7 min., followed by 40 cycles of 50 sec, and a final extention step at 72 °C, for 10 min. Annealing was done at the temperature indicated. The *WNT* primer sets used are as follows: *WNT3a* : 5′ GGA ACT ACG TGG AGA TCA TGC 3′ and 5′ GCA CCT TGA AGT AGG TGT AGC 3′, annealing at 55°C; *WNT5a*: 5′ GGA CAG AAG AAA CTG TGC CAC 3′ and 5′ CG ATG TAG ACC AGG TCT TGT G 3′, annealing at 58°C; *WNT10b* : 5′ GCT CTA AGC AAT GAG ATT CTG GG 3′ and 5′ CTG TGT TGT GGA TTC GCA TTC 3′, annealing at 60°C; *WNT16* : 5′ CAG AAG GAG CTG TGC AAG AG 3′ and 5′ CTG ATC TTT TTC TCT CCT GCG 3′, annealing at 58°C. Amplicons were cloned into pCRII^{TOPO} (Invitrogen Life technologies), and 10 individual clones were sequenced to determine specificity of the amplified products using a big-dye terminator kit (Amersham Biosciences) and M13 forward and reverse primers. All primers were manufactured by Sigma.

Mutation analysis of *CTNNB1* and *APC*

The APC-binding domain of *CTNNB1* was screened for activating mutations as described [40]. For mutation analysis of *APC*, four overlapping regions were amplified spanning the mutation cluster region (MCR), using the following primers: fragment I; 5′ GAAATAGGATGTAATCAGACGA 3′ and 5′ CGCTCCTGAAGAAAATTCAAC 3′, for fragment

II; 5′ ACTGCAGGGTTCTAGTTTAT 3′ and 5′ GAGCTGGCAATCGAACGACT 3′, for fragment III; 5′ TACTTCTGTCAGTTCACTTGAT 3′ and 5′ ATTTTTAGGTACTTCTCGCTTG 3′, and for fragment IV; 5′ AAACACCTCCACCACCTCCT 3′, and 5′ GCATTATTCTTAATTCCACATC 3′. PCR conditions were: denaturing at 96°C for 7 min, followed by 35 cycles of 50 sec, and a final extention step at 72 °C, for 10 min. Annealing temperatures were: 52°C for fragments I and II, and 56°C for fragments III and IV. Amplicons were size-separated by agarose electrophoresis in 1X TAE, and sequenced to determine specificity using a big-dye terminator kit (Amersham Biosciences).

RESULTS

Myeloma cells overexpress β-catenin

Regulation of β-catenin levels plays a central role in WNT signaling. Our initial evidence that the WNT pathway might play a role in the pathogenesis of MM came from a screen of B lineage malignancies for expression of β-catenin. We used both a pan anti-β-catenin antibody and an antibody that specifically recognizes the nonphosphorylated N-terminal Ser-37 and Thr-41 epitope of β-catenin. Only these nonphosphorylated β-catenin species are able to transduce WNT signals to the nucleus [27, 28]. Interestingly, all the myeloma cell lines tested expressed β-catenin, whereas the majority of the myeloma cell lines also contained detectable levels of the active, nonphosphorylated form of the molecule (Fig. 1A). We then compared the β-catenin expression levels in MM to the levels in normal B cell subpopulations and plasma cells, isolated from human tonsils by FACS sorting, using CD19, CD38 and IgD as markers [39]. β-catenin levels were very low in all B cell subsets examined, *i.e.* in naive ($IgD^+/CD38^-$), germinal center ($CD38^+$), and memory ($IgD^-/CD38^-$) B cells, as well as in plasma cells ($CD38^{high}$). In these normal B cell populations, the nonphosphorylated form of β-catenin was undetectable (Fig. 1B).

To assess whether β-catenin accumulation also occurs in primary myelomas, we studied β-catenin expression in BM samples from MM patients. The samples studied were selected for extensive tumor infiltration and all contained >75% malignant plasma cells. β-catenin was expressed in 9 of 10 of these primary MM samples (MM1-10, Fig. 1C). Most cases also showed detectable expression of the active nonphosphorylated form of β-catenin (MM1-10, Fig. 1C). By contrast, β-catenin was not expressed in any of the nonneoplastic control BM samples examined (BM1-7, Fig. 1D). These results demonstrate that myeloma cell lines and most primary myelomas overexpress β-catenin, including the nonphosphorylated form of the molecule, an observation indicative for active WNT signaling.

WNT signaling regulates β-catenin levels and localization.

We did not detect *APC* or *CTNNB1* mutations in any of the MM cell lines used in this study (data not shown). We subsequently performed experiments to test whether MM cells could respond to WNT signaling. Initially, we tested the effects of lithium chloride (LiCl), which inhibits GSK3β and mimics WNT signaling by stabilizing β-catenin [41]. Stimulation with LiCl markedly increased the amounts of total and nonphosphorylated β-catenin in the myeloma cell lines tested, as measured by immunoblotting (Fig. 2A, *Left*). The accumulation was most pronounced in cells with relatively low baseline β-catenin levels. To substantiate this finding, we determined whether specific WNT proteins could also regulate β-catenin levels. To this end, we stimulated MM cells with conditioned medium derived from L cells secreting Wnt3a [34]. Indeed, this stimulation also resulted in β-catenin accumulation, including accumulation of nonphospho β-catenin (Fig. 2A, *Right*) [37]. Similar results were also obtained using purified Wnt3a (data not shown)

Apart from causing β-catenin accumulation, WNT signaling also affected the localization of

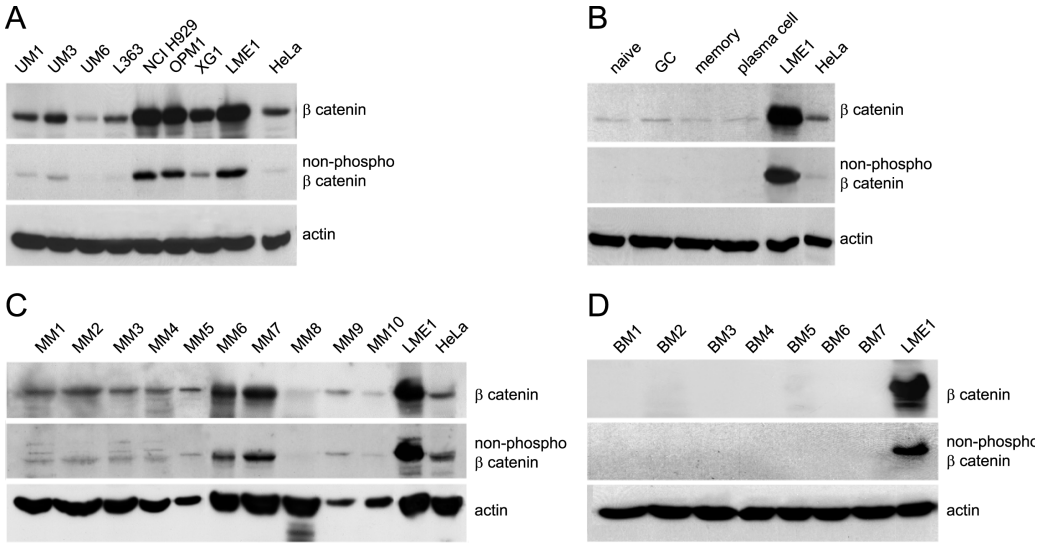


Figure 1 **Myeloma cell lines and primary myeloma cells overexpress β-catenin.**

A. β-catenin expression by myeloma cell lines. Cells were lysed and immunoblotted using a monoclonal anti-β-catenin antibody (top panel), or an antibody against non-phosphorylated β-catenin (8E4) (middle panel). **B.** β-catenin expression in normal B cell subsets and plasma cells. B cell subsets and plasma cells were sorted from human tonsils as described in the materials and methods section. Cells were lysed and immunoblotted as described above. **C.** β-catenin expression in primary MMs. BM mononuclear cells from myeloma patients were lysed and immunoblotted as described above. **D.** β-catenin expression in normal BM. Total BM-derived mononuclear cells were isolated from control individuals, lysed and immunoblotted as described above. **A-D.** Equal cell numbers of MM cell line LME1 were loaded as a reference for β-catenin expression. Stainings with anti-β-actin served as loading controls (bottom panels)

β-catenin. Before LiCl stimulation, low amounts of β-catenin were detected in the cytoplasm and nucleus of MM cells by confocal laser scan microscopy. Stimulation with LiCl led to an increase in the total amount of β-catenin, located to the plasma membrane at cell-cell contact sites, as well as in the nucleus (Fig. 2B). Stimulation with Wnt3a had similar effects (data not shown). Interestingly, most of the nonphosphorylated β-catenin was localized in the nucleus (Fig. 2B). Nonphosphorylated β-catenin was also present in the nucleus of primary myelomas (Fig. 2C). Nuclear localization of nonphosphorylated β-catenin was also detected in primary myelomas, but the levels varied among individual cells within a given tumor (Fig. 2C). This variation possibly reflects heterogeneity in the differentiation of individual tumor cells with higher WNT signaling in tumor stem cells [42].

WNT signaling controls proliferation of myeloma cells

We subsequently explored the effects of WNT signaling on MM proliferation. Myeloma cells responded to stimulation with Wnt3a-conditioned medium with a 2- to 4-fold increase in proliferation. This enhanced proliferation was already observed within the first 24 hours (Fig. 3A). During culture, cell numbers increased, whereas viability was not significantly affected by the presence of Wnt3a (data not shown). Importantly, similar results were also obtained with purified Wnt3a (Fig. 3B), which has recently become available [35], demonstrating the specificity of the effect of Wnt3a and ruling out indirect effects of other growth factors released as a result of autocrine stimulation of the Wnt3a-transfected L cells. Treatment with LiCl, which inhibits GSK3β resulting in β-catenin accumulation

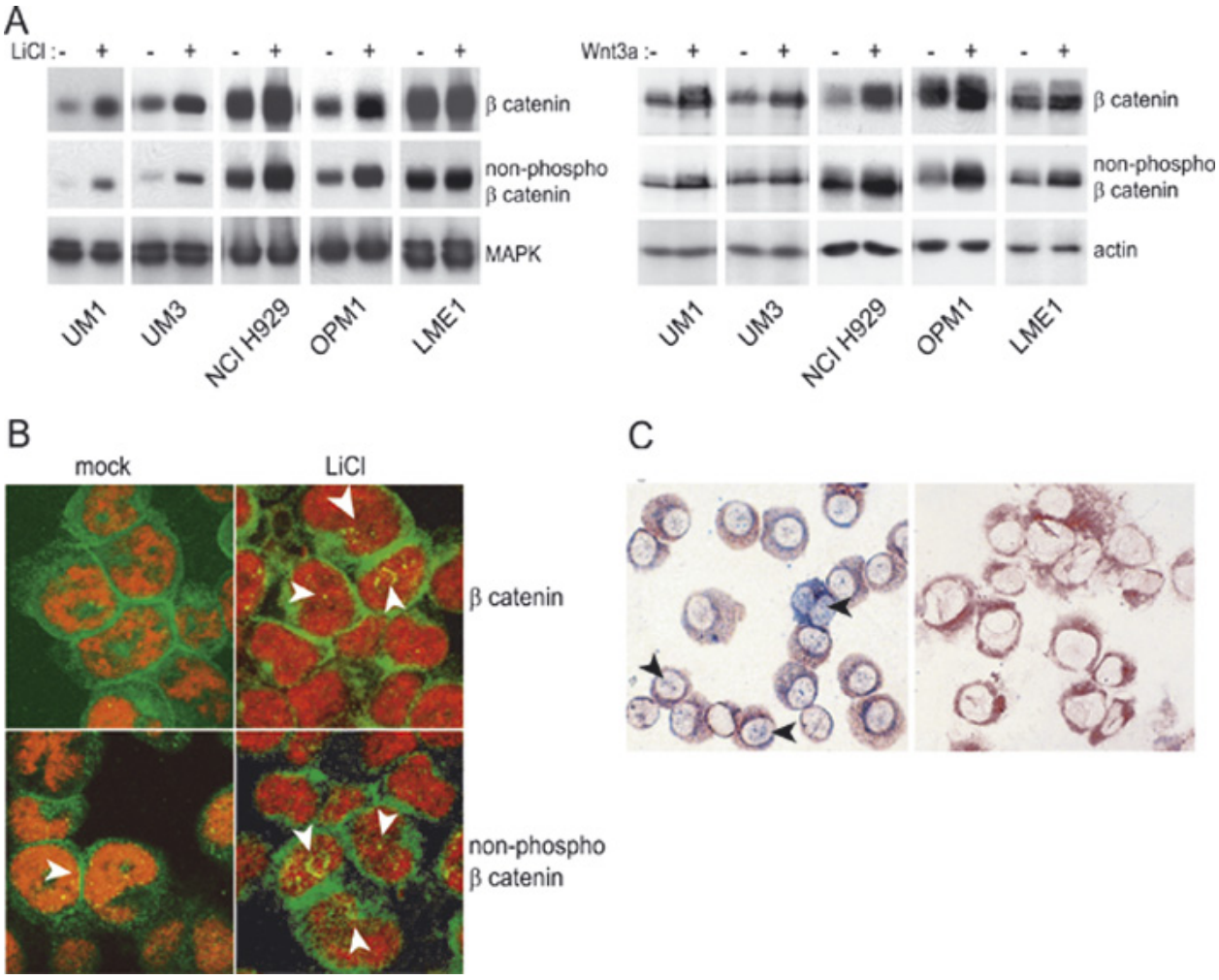


Figure 2

WNT signaling induces β-catenin accumulation and nuclear translocation in myeloma cells.

A. Accumulation of β-catenin in MM cells in response to LiCl, which mimics a WNT signal, or Wnt3a. Cells were incubated for two hours in the absence or presence of 20 mM LiCl (left panel) or in the absence or presence of Wnt3a conditioned medium (right panel). To assess β-catenin accumulation, cells were lysed and immunoblotted using a monoclonal anti-β-catenin antibody (top panel), or an antibody against non-phosphorylated β-catenin (8E4) (middle panel). The bottom part of the blot was stained with anti-ERK (MAPK), or anti-β-actin to verify equal loading. **B.** LiCl induces accumulation of β-catenin and increased nuclear localization of non-phosphorylated β-catenin. NCI H929 cells were incubated for two hours in the absence or presence of 20 mM LiCl. Cytospins were prepared and stained with an antibody against total β-catenin (top panels), or non-phosphorylated β-catenin (8E4)(bottom panels), followed by a FITC-conjugated secondary antibody and analyzed by CLSM. **C.** Expression and nuclear localization of β-catenin in primary MM. Immunohistochemical double staining of BM-derived mononuclear cells using antibodies against immunoglobuline(Ig) light chains (brown) and non-phosphorylated β-catenin (blue)(left panel). Note nuclear staining of β-catenin (arrows). As a control, an isotype-matched antibody in combination with the anti-Ig light chain antibodies, is shown (right panel).

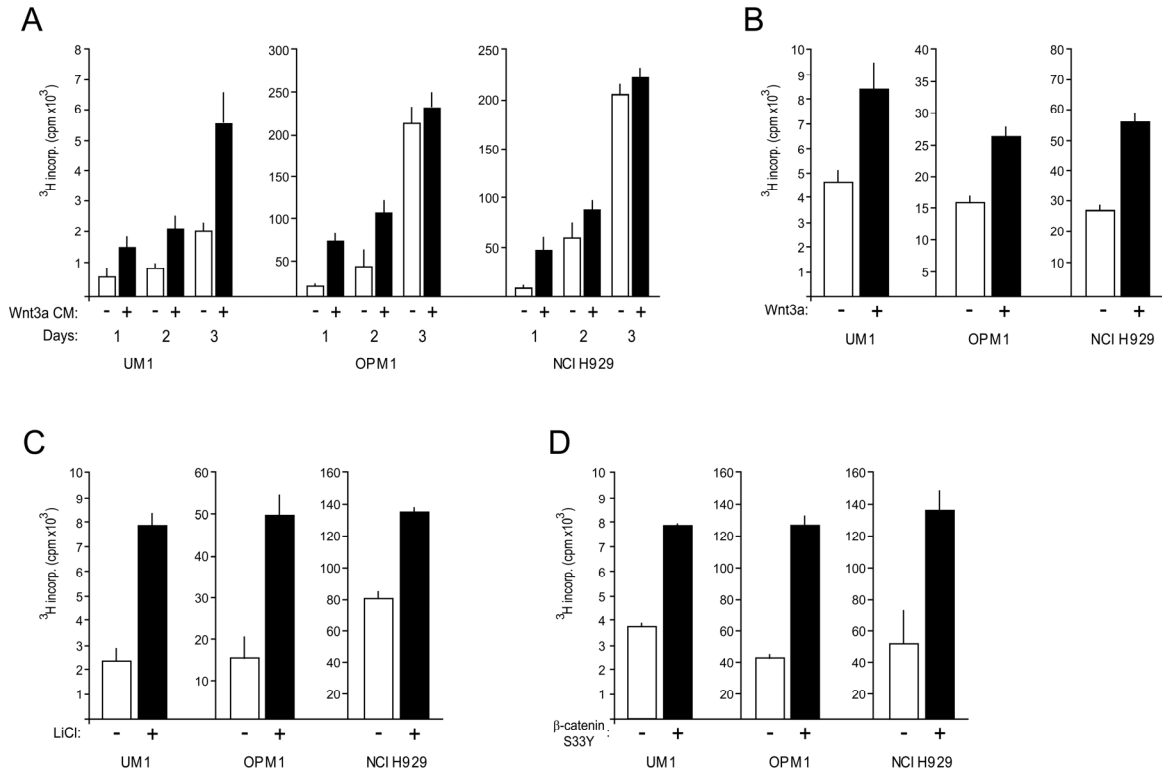


Figure 3

Stimulation of WNT signaling promotes proliferation of MM cells.

A. Exogenous Wnt3a promotes proliferation of MM cells. The MM cell lines UM1, OPM1, and NCI H929 were cultured in the presence of L-cell conditioned medium (white bars) or conditioned medium derived from Wnt3a-transfected L cells (Wnt3a CM, black bars) and [^{3}H]-thymidine incorporation was measured after 1, 2, and 3 days of culture. Error bars represent standard deviations of triplicate measurements. **B.** Purified Wnt3a promotes proliferation of MM cells. Cells were cultured in the absence (white bars) or presence (black bars) of purified Wnt3a (100 ng/ml) in serum free medium. [^{3}H]-thymidine incorporation was measured after two days of culture. **C.** LiCl stimulation promotes proliferation of MM cells. Cell were cultured in the absence (white bars) or presence (black bars) of 2 mM LiCl in serum free medium. [^{3}H]-thymidine incorporation was measured after three days of culture. **D.** Constitutively active β-catenin (β-catenin S33Y) promotes proliferation of MM cells. Cells were transfected with empty vector (white bars) or with β-catenin S33Y (black bars) and [^{3}H]-thymidine incorporation was measured after two days of culture.

(Fig. 2A), gave rise to a similar increase in proliferation (Fig. 3C). Furthermore, enhanced proliferation was also observed upon expression of the β-catenin mutant S33Y (Fig. 3D). Taken together, these data clearly demonstrate that activation of various components of the canonical WNT signaling pathway in myeloma cells induces proliferation. Finally, we investigated the effect of disruption of β-catenin/TCF activity on MM proliferation. As is shown in Fig. 4A, transfection with ΔTCF4 strongly inhibited proliferation of the MM cell lines OPM1 and NCI H929, which both contain large amounts of (active) β-catenin. These data demonstrate that WNT signaling regulates proliferation in MM cells.

Regulation of WNT signaling in MMs

Our observation that stimulation of WNT signaling by Wnt3a, LiCl, or mutant S33Y β-catenin promotes MM proliferation, whereas disruption of the pathway by ΔTCF4 inhibits proliferation, indicates that WNT signaling is constitutively active, but not maximally

activated and sensitive to regulation. To corroborate this conclusion, we directly monitored TCF transcriptional activity in the MM cell line OPM1 by transfecting a TCF reporter (pTOPFLASH). As a control, we used a reporter containing scrambled TCF-binding sites (pFOPFLASH). Consistent with our functional studies, OPM1 cells showed a moderate constitutive β-catenin/TCF activity. This activity was inhibited by cotransfection of ΔTCF4 (Fig. 4B). A strong reporter activity was obtained after cotransfection of the active β-catenin mutant S33Y. Moreover, TCF reporter activity was increased by stimulating MM cells with purified Wnt3a (Fig. 4C). pFOPFLASH activity was not affected by any of the applied stimuli (not shown). The above data imply that the WNT pathway in MM cells is intact and suggest changes in a regulatory component, *e.g.*, the presence of an autocrine activation loop. To explore this possibility, we assessed the expression of *WNT* genes previously demonstrated to be expressed within the hematopoietic environment, *i.e.*, *WNT3a, 5a*, *10b* and *16* [43-45]. Neither normal B cells nor plasma cells expressed these *WNTs* (Table 1). By contrast, in all myeloma cell lines tested, we found expression of *WNT5a*

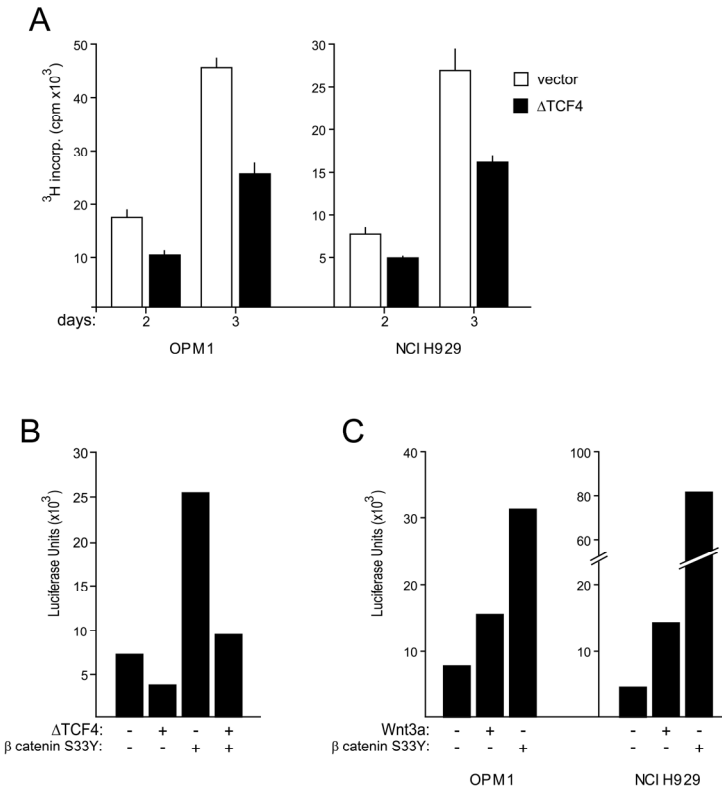


Figure 4

Dominant-negative TCF (ΔTCF4) inhibits MM proliferation and suppresses TCF reporter activity whereas both constitutively active (S33Y) β-catenin and Wnt3a stimulate TCF reporter activity

A. ΔTCF4 inhibits proliferation of MM cells. MM cell lines OPM1 and NCI H929 were transfected with either ΔTCF4 (black bars) or empty vector (white bars) both in combination with pEGFP constructs. After overnight culture, viable, GFP-positive cells were sorted and [^{3}H]-thymidine incorporation was measured after two and three days of culture. Error bars represent standard deviations of triplicate measurements. **B.** TCF reporter activity in MM cells. OPM1 cells were either transfected with luciferase reporter constructs (pTOPFLASH) alone, or in combination with a constitutive active β-catenin (β-catenin S33Y) and dominant negative TCF4 (ΔTCF4), and assayed for luciferase activity. **C.** Wnt3a stimulates TCF reporter activity in MM cells. OPM1 and NCI H929 cells were transfected with pTOPFLASH alone, or in combination with β-catenin S33Y. The cells were either or not stimulated with purified Wnt3a, and assayed for luciferase activity. As a control, a TCF reporter containing scrambled TCF binding sites (pFOPFLASH) was used. The activity of this control reporter remained unchanged (not shown).

and *WNT10b*, whereas W*NT16* transcripts were found in one MM cell line (Table 1). Also, in highly purified primary MM cells, obtained by positive selection with anti-CD138, we detected expression of *WNT5a* and/or *WNT10b* and, in one case, *WNT16* (Table 1). Interestingly, *WNT5A* and *WNT10b* expression was also found in BM stromal cells (Table 1), suggesting that these cells may function as a paracrine source of WNTs within the BM microenvironment.

DISCUSSION

In the present study, we identified the WNT pathway as a signaling route involved in MM growth control. This unexpected finding implies that the WNT pathway, which is essential for early T and B cell development and has recently been shown to play a role in the self-renewal of hematopietic stem cells [10], is activated in this tumor of terminally differentiated B cells.

Regulation of β-catenin levels plays a central role in WNT signaling. Our initial evidence for a role of the WNT pathway in the pathogenesis of MM came from the observation that all the MM cell lines, as well as most primary MM samples studied, expressed β-catenin (Fig.1). Compared to the very low or undetectable β-catenin expression in normal B cell subsets, plasma cells, and control BM, β-catenin was vastly over-expressed in most of these MMs (Fig.1). In addition to overexpressing β-catenin *per se*, the majority of MM cell lines and primary MMs also expressed detectable levels of β-catenin harboring nonphosphorylated N-terminal Ser/Thr residues. Recent studies [27, 28] have shown that only these nonphos-phorylated β-catenin species are signaling competent and can transduce WNT signals to the nucleus. Our observation that stimulation of MM cells with LiCl, which mimics WNT signaling by inhibiting GSK3β, or with Wnt3a, led to further accumulation and nuclear translocation of nonphosphorylated β-catenin (Fig. 2), implies that these tumors have an intact WNT pathway and suggests a functional role of the WNT pathway in the biology of MM. Indeed, stimulation of WNT signaling, either by LiCl, β-catenin S33Y, or by Wnt3a, promoted the proliferation of myeloma cells (Fig. 3), while disruption of β-catenin/TCF activity by ΔTCF4 inhibited MM proliferation (Fig. 4).

During normal lymphocyte development, WNT signaling plays an essential role in early lymphopoiesis by contributing to precursor cell survival and expansion independent of, or in parallel to, pre-antigen receptor signaling [8, 22-26]. Our present observation that the WNT pathway is active and controls proliferation in MM is unexpected, because this illegitimate activation involves the 'wrong', most terminal, end of the B cell differentiation spectrum. Our data present, to our knowledge, the first direct evidence for a role of the WNT pathway in the pathogenesis of lymphoid cancer. In the canonical cancers involving WNT signaling, such as colorectal cancer, pilomatricomas, and hepatoblastoma, 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* [5], but the mechanism of nuclear β-catenin accumulation in several other tumor types is,

Table 1

***WNT* expression* in myelomas and normal B lineage cells**

cell type	*WNT*			
	3a	**5a**	**10b**	**16**
naïve B cells	-	-	-	-
GC B cells	-	-	-	-
memory B cells	-	-	-	-
plasma cells	-	-	-	-
total B cells	-	-	-	-
fibroblast	-	+	+	+
BM stromal cells	-	+	+	-
MM cell lines	0/8	4/8	8/8	1/8
purified primary MMs	0/4	4/4	3/4	1/4

**WNT* expression was determined by RT-PCR as described in the Materials & Methods section.

at present, unclear. Although we have not yet identified the cause of β-catenin accumulation in MM, our current results make direct mutational activation of the WNT pathway unlikely. Firstly, we did not detect *APC* or *CTNNB1* mutations in any of the MM cell lines and primary MM samples studied. This finding does not exclude the presence of mutation in axin/conductin, or other WNT pathway components; however, such mutations appear to represent a rare cause of WNT activation in cancer. Secondly, whereas (*i*) the over-expression of (active) β-catenin, (*ii*) the spontaneous TCF reporter activity, and (*iii*) the growth inhibitory effects of ΔTCF4 all testify constitutive WNT activity in MM, the level of activation of the pathway in the MM cell lines clearly was suboptimal. This conclusion can be arrived at from the fact that exogenous WNT stimuli like Wnt3a and LiCl caused further β-catenin accumulation and translocation, and enhanced cell proliferation. The latter observations, specifically the growth-stimulatory effects of Wnt3a, point to an intact WNT pathway and therefore are difficult to reconcile with a direct mutational activation. Rather, the data suggests that the β-catenin accumulation and β-catenin/TCF activity results from changes involving a regulatory or upsteam component, *e.g.*, a member of the WNT family. Consistent with this idea, we observed that myeloma cell lines as well as primary myelomas, unlike normal B cell subsets and plasma cells, express *WNT5a*, *WNT10b*, and in some cases *WNT16*, mRNAs (Table 1), indicating an autocrine activation loop. Although the functionality of this loop needs to be explored, it might explain the observed constitutive, but still inducible, activation of the WNT pathway. In this context, it is of interest that WNT16-mediated autocrine growth has recently been proposed by Murre and coworkers to contribute to the development of t(1;19) positive pre-B-precursor acute lymphoblastic leukemia [45].

Although the above findings suggest an autocrine activation loop, paracrine stimulation of MM cells presumably also takes place within the BM microenvironment, because we observed that BM stromal cells express both *WNT5a* and *WNT10b* (Table 1). This observation corroborates studies by Austin *et al.* and Van den Berg *et al.*, who reported expression of WNT5a and 10b in mouse and human BM, respectively, and demonstrated that these factors function as hematopoietic growth factors, promoting expansion of mixed colony-forming units and burst-forming units-erythroid [43, 44]. It is conceivable that during progression of MM, a gain of *WNT* expression takes place, establishing an autocrine activation loop, thus leading to autonomous growth, and finally, allowing dissemination to extra-medullary sites. It will therefore be of great interest to assess the expression of β-catenin and WNTs at early stages of MM.

A key finding of our current study is that WNT signaling can control MM proliferation. Because normal plasma cells are terminally differentiated, nondividing, cells, activation of signaling route(s) promoting cell proliferation is a crucial step in their transformation to MM. Further studies are needed to establish whether deregulation of the WNT pathway is a general event in the initiation or progression of MM. The effects of WNT signaling on proliferation in our current study clearly involve the canonical WNT pathway, which regulates β-catenin/TCF-mediated transcription, since stimulation or inhibition by either Wnt3a, LiCl, S33Y β-catenin, or ΔTCF4, which affect the canonical WNT pathway at a number of distinct levels, had profound effects on proliferation (Figs. 3 and 4). During the preparation of this manuscript, a study by Qiang *et al.* [46] also reported WNT signaling in MM cell lines. Unlike our study, however, this study examined neither primary patient samples nor normal B cells and plasma cells. Moreover, the functional effects of Wnt3a stimulation reported by Qiang *et al.*, *i.e.* morphological changes and rearrangement of the actin cytoskeleton, were associated with a noncanonical WNT pathway dependent on RHO activation.

Our current study indicates that aberrant WNT signaling drives MM proliferation and could represent an important step in the

pathogenesis of MM. In intestinal epithelium, which presents the paradigm for the role of WNT signaling in tumorigenesis, the β-catenin/TCF complex constitutes the "master switch" that controls proliferation versus differentiation [9]. Interestingly, a recently study by Reya *et al.* [10] indicates that WNT signaling also controls the self-renewal of hematopoietic stem cells by the induction of proliferation and the prevention of hematopoietic stem cell differentiation. In intestinal epithelium, the proliferative effects of WNT signaling are mediated through its control over MYC. In the presence of active β-catenin/TCF complexes, MYC is expressed and blocks the expression of the cell cycle inhibitor $p21^{CIP1/WAF1}$, leading to cell cycle progression [9]. MYC expression is frequently deregulated in MMs [47, 48], but whether MYC also plays a central role in WNT-induced proliferation in MM remains to be determined. Alternative WNT target genes that may also contribute to the growth promoting effects of WNT signaling in MMs include the cell cyle regulator CYCLIN D1 [15] and MET [18], the receptor tyrosine kinase for HGF, a potent myeloma growth- and survival-factor [2, 49].

In summary, the data presented here implicate the canonical WNT signaling pathway in the pathogenesis of MM, by showing accumulation and nuclear localization of the active β-catenin and by demonstrating that these deregulated levels of active β-catenin contribute to MM proliferation. These findings indicate that a pathway that normally drives proliferation of hematopoietic stem cells may become illegitimately activated in MM cells and identify the WNT pathway as a potential novel target for therapy in MM.

Acknowledgements

We thank Niels van de Donk, Berris van Kessel, and Richard Bende for primary myelomas, cDNAs and helpful discussions. Frank van Diepen and Anita Pfauth for assistance with cell sorting. This work was supported by grants from the Dutch Cancer Society and the Association for International Cancer Research.

References

1. Kuehl, W. M. & Bergsagel, P. L. (2002) *Nat Rev* 2, 175-187.
2. Derksen, P. W., Keehnen, R. M., Evers, L. M., van Oers, M. H., Spaargaren, M. & Pals, S. T. (2002) *Blood* 99, 1405-1410.
3. Nusse, R. & Varmus, H. E. (1982) *Cell* 31, 99-109.
4. Bienz, M. & Clevers, H. (2000) *Cell* 103, 311-320.
5. Polakis, P. (2000) *Genes Dev* 14, 1837-1851.
6. Kinzler, K. W. & Vogelstein, B. (1996) *Cell* 87, 159-170.
7. Verbeek, S., Izon, D., Hofhuis, F., Robanus-Maandag, E., te Riele, H., van de Wetering, M., Oosterwegel, M., Wilson, A., MacDonald, H. R. & Clevers, H. (1995) *Nature* 374, 70-74.
8. Reya, T., O'Riordan, M., Okamura, R., Devaney, E., Willert, K., Nusse, R. & Grosschedl, R. (2000) *Immunity* 13, 15-24.
9. van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der, H. K., Batlle, E., Coudreuse, D., Haramis, A. P. *et al.* (2002) *Cell* 111, 241-250.
10. Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R. & Weissman, I. L. (2003) *Nature* 423, 409-414.
11. Wodarz, A. & Nusse, R. (1998) *Annu. Rev. Cell Dev. Biol.* 14, 59-88.
12. Moon, R. T., Bowerman, B., Boutros, M. & Perrimon, N. (2002) *Science* 296, 1644-1646.
13. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. & Clevers, H. (1996) *Cell* 86, 391-399.
14. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. & Birchmeier, W. (1996) *Nature* 382, 638-642.
15. Tetsu, O. & McCormick, F. (1999) *Nature* 398, 422-426.
16. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. & Kinzler, K. W. (1998) *Science* 281, 1509-1512.
17. Wielenga, V. J., Smits, R., Korinek, V., Smit, L., Kielman, M., Fodde, R., Clevers, H. & Pals, S. T. (1999) *Am J Pathol* 154, 515-523.
18. Boon, E. M., Van der Neut, R., van de Wetering, M., Clevers, H. & Pals, S. T. (2002) *Cancer Res.* 62, 5126-5128.
19. Travis, A., Amsterdam, A., Belanger, C. & Grosschedl, R. (1991) *Genes Dev* 5, 880-894.

20. van de Wetering, M., Oosterwegel, M., Dooijes, D. & Clevers, H. (1991) *Embo J* 10, 123-132.
21. Waterman, M. L., Fischer, W. H. & Jones, K. A. (1991) *Genes Dev* 5, 656-669.
22. van de Wetering, M., de Lau, W. & Clevers, H. (2002) *Cell* 109, 13-19.
23. Schilham, M. W., Wilson, A., Moerer, P., Benaissa-Trouw, B. J., Cumano, A. & Clevers, H. C. (1998) *J Immunol* 161, 3984-91.
24. Okamura, R. M., Sigvardsson, M., Galceran, J., Verbeek, S., Clevers, H. & Grosschedl, R. (1998) *Immunity* 8, 11-20.
25. Gounari, F., Aifantis, I., Khazaie, K., Hoeflinger, S., Harada, N., Taketo, M. M. & von Boehmer, H. (2001) *Nat. Immunol.* 2, 863-869.
26. Ioannidis, V., Beermann, F., Clevers, H. & Held, W. (2001) *Nat. Immunol.* 2, 691-697.
27. Staal, F. J., Noort Mv, M., Strous, G. J. & Clevers, H. C. (2002) *EMBO Rep* 3, 63-68.
28. van Noort, M., Meeldijk, J., van der Zee, R., Destree, O. & Clevers, H. (2002) *J Biol Chem* 277, 17901-17905.
29. Zhang, X. G., Gaillard, J. P., Robillard, N., Lu, Z. Y., Gu, Z. J., Jourdan, M., Boiron, J. M., Bataille, R. & Klein, B. (1994) *Blood* 83, 3654-3663.
30. Kuipers, J., Vaandrager, J. W., Weghuis, D. O., Pearson, P. L., Scheres, J., Lokhorst, H. M., Clevers, H. & Bast, B. J. (1999) *Cancer Genet Cytogenet* 109, 99-107.
31. Diehl, V., Schaadt, M., Kirchner, H., Hellriegel, K. P., Gudat, F., Fonatsch, C., Laskewitz, E. & Guggenheim, R. (1978) *Blut* 36, 331-338.
32. Gazdar, A. F., Oie, H. K., Kirsch, I. R. & Hollis, G. F. (1986) *Blood* 67, 1542-1549.
33. Katagiri, S., Yonezawa, T., Kuyama, J., Kanayama, Y., Nishida, K., Abe, T., Tamaki, T., Ohnishi, M. & S., T. (1985) *Int J Cancer* 36, 241-246.
34. Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M. & Takada, S. (1998) *Genes Cells* 3, 659-670.
35. Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R. & Nusse, R. (2003) *Nature* 423, 448-452.
36. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. *et al.* (1997) *Cell* 88, 789-799.
37. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* 275, 1784-7.
38. Koopman, G., Keehnen, R. M., Lindhout, E., Newman, W., Shimizu, Y., van Seventer, G. A., de Groot, C. & Pals, S. T. (1994) *J Immunol* 152, 3760-7.
39. Pascual, V., Liu, Y. J., Magalski, A., de Bouteiller, O., Banchereau, J. & Capra, J. D. (1994) *J Exp Med* 180, 329-39.
40. Voeller, H. J., Truica, C. I. & Gelmann, E. P. (1998) *Cancer Res.* 58, 2520-2523.
41. Stambolic, V., Ruel, L. & Woodgett, J. R. (1996) *Curr Biol* 6, 1664-8.
42. Rohrer, J. W., Vasa, K. & Lynch, R. G. (1977) *J Immunol* 119, 861-866.
43. Austin, T. W., Solar, G. P., Ziegler, F. C., Liem, L. & Matthews, W. (1997) *Blood* 89, 3624-35.
44. Van Den Berg, D. J., Sharma, A. K., Bruno, E. & Hoffman, R. (1998) *Blood* 92, 3189-3202.
45. McWhirter, J. R., Neuteboom, S. T., Wancewicz, E. V., Monia, B. P., Downing, J. R. & Murre, C. (1999) *Proc Natl Acad Sci U S A* 96, 11464-9.
46. Qiang, Y. W., Endo, Y., Rubin, J. S. & Rudikoff, S. (2003) *Oncogene* 22, 1536-1545.
47. Hallek, M., Leif Bergsagel, P. & Anderson, K. C. (1998) *Blood* 91, 3-21.
48. Zhan, F., Hardin, J., Kordsmeier, B., Bumm, K., Zheng, M., Tian, E., Sanderson, R., Yang, Y., Wilson, C., Zangari, M. *et al.* (2002) *Blood* 99, 1745-1757.
49. Derksen, P. W., De Gorter, D. J., Meijer, H. P., Bende, R. J., van Dijk, M., Lokhorst, H. M., Bloem, A. C., Spaargaren, M. & Pals, S. T. (2003) *Leukemia* 17, 764-774.

CHAPTER **7**

General discussion

Malignant transformation of B cells is caused by the gain of genetic aberrations that alter the proliferation, life span or differentiation of these cells. Signals from the microenvironment, e.g. growth factors, which control cell proliferation, survival, adhesion and differentiation during normal B cell development and differentiation, may equally contribute to tumor formation and growth.

Expression and function of the regulatory components of the HGF/MET pathway in normal and malignant B cells

Within the normal secondary lymphoid organ microenvironment, MET is expressed by both centroblasts and plasma cells, while HGFA, the most potent activator of HGF, is expressed by plasma cells but not by other B-lineage populations (chapter 2). Furthermore, FDCs are the major source of HGF and HGFA in the GC dark zone microenviroment (chapter 2). These findings indicate that centroblasts and plasma cells are well equipped to receive paracrine HGF signals. The observation that both HGF and HGFA are expressed by FDCs in the GC dark zone but not in the light zone, suggest that HGF and HGFA provided by the dark zone FDCs may control proliferation, survival and/or adhesion of MET-positive centroblasts. Functional studies have shown that HGF stimulation induces integrin-dependent adhesion of MET-positive B cells to ICAM-1 and VCAM-1, which are expressed by FDCs, and to FN present in the ECM [1-3]. Integrin-mediated interaction between B cells and FDCs [4,5] may directly contribute to the B cell selection process, since $\alpha 4\beta 1$/VCAM-1 and $\alpha L\beta 2$/ICAM-1 interaction suppresses GC B cell apoptosis [5,6]. These observations point out the potential relevance of the HGF/MET pathway for Ag-specific B cell differentiation. The restriced expression within the GC, as observed for HGF, MET and HGFA, has also been described for the chemokines CXCL-13 (BLC) and CXCL-12 (SDF-1). Whereas CXCL-13 is more abundantly expressed in the GC light zone than the dark zone [7,8], CXCL-12 is predominantly localized in the dark zone [9]. This differential distribution of chemokines reflects their role in the GC organization, specifically localizing the centroblasts to the dark zone and the centrocytes to the light zone. The significance of the confined expression of the regulatory components of the HGF/MET pathway in the GC dark zone has to be determined.

In notable contrast to GC B cells, plasma cells are quiescent and unable to proliferate. The co-expression of MET and HGFA in plasma cells is of interest since it indicates that plasma cells are well equipped to receive paracrine HGF signals. Indeed, tonsillar- as well as bone marrow stromal cells have been reported to produce HGF, implying that plasma cells receive paracrine stimulation from the microenvironment [1,3,10]. Contrary to their malignant counterparts [11], normal plasma cells do not express HGF, and therefore do not possess an autocrine HGF/MET loop. Although the functional consequences of HGF/MET signaling in plasma cell have not yet been explored, like in B cells [1,3,12], the pathway might regulate integrin-mediated adhesion and retention in the proper microenvironment, migration, and/or survival.

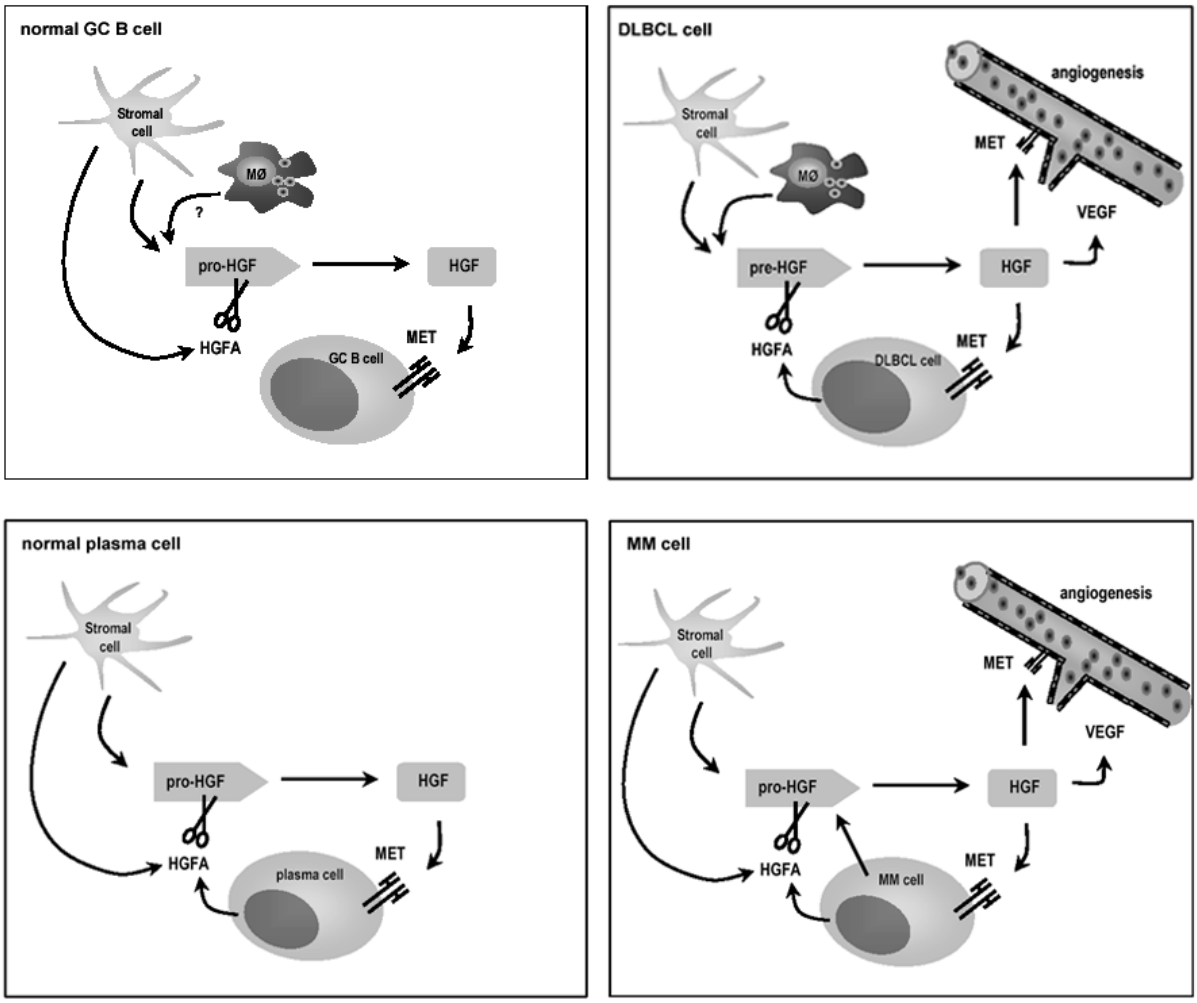


Figure 12 **Comparison between normal counterparts of DLBCL and MM.**

HGF/MET signaling can contribute to the pathogenesis of DLBCL and MM by influencing cell growth, survival, adhesion and angiogenesis. DLBCL and MM cells have acquired an additional mechanism for their autonomous growth. Contrary to their normal counterparts, DLBCL cells express HGFA and are able to autocatalyze HGF activation. In case of MM, malignant plasma cells but not the normal counterparts are able to produce HGF, resulting in an autocrine HGF/MET loop in MM cells.

Numerous studies have shown that uncontrolled MET signaling is oncogenic [13,14]. To explore the possible role of aberrant HGF/MET signaling in lymphomagenesis, we examined the expression of HGF/MET pathway components in a large panel of B cell malignancies, representing a broad spectrum of differentional stages ranging from precursor B cells to plasma cells (chapter 3). In concordance with other studies [2,11,15-17], we found MET expression in a subset of Burkitt's lymphomas and in a large percentage of MMs and DLBCLs. Previously, we have shown that HGF/MET signaling plays a key role in MM growth and survival [18]. In DLBCL cells, activation of MET by HGF, which is probably provided by activated macrophages (chapter 3) or stromal cells [1,3,10,19,20], results in phosphorylation and activation of ERK, PKB, GSK3 and FOXO3a (chapter 3). These components of the RAS/MAPK and PI3K/PKB pathway are linked to cell growth and survival. Furthermore, HGF induces integrin-mediated adhesion of DLBCL cells, which requires PI3K activity (chapter 3). In previous studies, overexpression of HGF/MET in patients with DLBCL has been associated with poor prognosis [17,21,22]. Our study is the first to show the functional consequences of active HGF/MET signaling in DLBCL cells and indicates that the HGF/MET signaling may contribute to the pathogenesis of DLBCL by

controlling cell adhesion, growth and survival. An important mechanism that may facilitate HGF/MET signaling in DLBCL as well in MM is uncontrolled expression of HGFA. Contrary to their normal counterparts, i.e. GC B cells (chapter 2), DLBCL cells express HGFA and are able to autocatalyze activation of HGF produced in the microenvironment (chapter 3), thus allowing their autonomous growth. Intriguingly, whereas normal plasma cells and their malignant counterparts MM cells express HGFA, only the latter cells display an autocrine HGF/MET loop [11,15,23]. It is conceivable that malignant B cells have acquired these self-supporting attributes through additional genetic aberrations during their cell expansion (figure 12). These findings strongly indicate that DLBCL- and MM cells regulate the HGF/MET signaling pathway themselves, emphasizing a key role for the HGF/MET pathway, including HGFA, in lymphomagenesis.

Besides autocrine activation, translocation, amplification, or mutations of the *MET* gene (chapter 3) can also result in inappropriate activation of MET. However, in the B cell malignancies studied, we did not detect *MET* amplification. This observation is in agreement with other studies showing that amplification of the *MET* locus is a rare event in non-lymphoid tumors as well [24]. Interestinlgy, we detected two germline missense mutations in MET: a R988C mutation (JM domain) was found in 4 patients with either DLBCL, CLL, FL or Burkitt's lymphoma, and a R1166Q (tyrosine kinase domain) in a DLBCL patient. Both mutations were also detected in normal tissues from the affected individuals, indicating that these mutations did not represent somatic mutations but were germline mutations. Germline mutations of *MET* have been found in HRPC and in patients with sporadic PRC [25,26], as well as in gastric cancer [27]. Recently, the R988C mutation has also been reported in two small cell lung cancer (SLCL) cell lines, a non-SLCL cell line and in two lung cancer patients [28,29]. Upon transfection, the R988C mutant reduced adhesion of the SLCL cell line H466 and promoted proliferation, motility and overall tyrosine phosphorylation of the pre-B cell line BaF3 [30]. Furthermore, expression of the R988C mutant in H466 cells resulted in enhanced focus-formation and soft-agar colony formation [30]. These findings, combined with recent observations that a mouse MET mutation homologous to R988C plays an important role in lung tumor susceptibility [29], strongly suggest that the R988C mutation is a gain-of-function mutation. Since a recent study has shown that mice expressing oncogenic MET mutations develop lymphomas [31], it is conceivable that the R988C MET mutation can convey B cell lymphoma susceptibility in humans.

Hyperactive signaling of MET can also be achieved by impaired inactivation of the receptor. We have identified the proto-oncogene product c-Cbl, an E3 ubiquitin ligase, as a downstream target of the HGF/MET signaling in B cells, and established its role in receptor downregulation (chapter 5). We show that HGF induces strong tyrosine phosphorylation of Cbl and increases association with the Src-family tyrosine kinases Fyn and Lyn, as well as with PI3K and CrkL. c-Cbl-mediated HGF-induced ubiquitination of MET requires the JM tyrosine Y1001 (Y2) of MET, but not the multi-substrate docking site residues Y1349/1356 (Y14/15) or the additional C-terminal tyrosine residues Y1313-1365 (Y13-16). Furthermore, the transforming mutants v-Cbl and 70Z/3 Cbl, which lack the ubiquitin ligase RING finger domain, suppress MET ubiquitination. Our data indicate that Cbl acts as a negative regulator in the HGF/MET signaling in B cells, mediating ubiquitination and, consequently proteosomal degradation and inactivation of MET. In concordance, several studies have established that Cbl-mediated ubiquitination plays a major role in RTK downregulation, including EGFR, CSF-1R and PDGFR [32-34], by targeting the receptors to lysosomes for proteasomal degradation [35]. However, it has become evident that RTKs can escape Cbl-mediated downregulation. In several human cancers, stabilization of EGFR is achieved by

deregulation of Src, which promotes Cbl ubiquitination and degradation [36]. Also, it has been shown that upon activation the GTPase CDC42 can sequester Cbl by binding to p85Cool-1/β-PIX [37], a protein that directly associates with Cbl [38]. This inhibits the binding of Cbl by the EGFR, resulting in defective Cbl-mediated EGFR degradation [37]. Alternatively, several oncogenic RTKs have lost the ability to recruit Cbl to the tyrosine kinase-binding (TKB) domain. Mutations of the Cbl binding site are frequently observed in CSF-1R in human myelodysplasia and acute myeloblastic leukemia, suggesting that loss of Cbl binding is implicated in oncogenic deregulation of CSF-1R in human cancer [39]. Furthermore, mutation in the Cbl binding site of MET (Y1001) resulted in impaired ubiquitination and degradation of MET (this thesis). The gain-of function mutation of Y1001 also results in consitutive scattering and fibroblastoid morphology of epithelial cells [40]. These data clearly identify Cbl as an important negative regulator for RTKs, including MET, and establish its role in RTK-mediated tumorigenesis.

WNT signaling in MM

Several studies have implicated the WNT/β-catenin signaling pathway in early lymphocyte development and in the self-renewal of haematopoietic stem cells. Our observation that the majority of the MM cell lines, in contrast to normal B cell and plasma cells, express large amounts of β-catenin, prompted us to investigate whether WNT signaling is involved in the pathogenesis of MM (chapter 6). Interestingly, most of the primary MM samples also expressed β-catenin, including the active, non-phosphorylated form of β-catenin. Most importantly, stimulation of WNT signaling by exogenous factors (LiCl or WNT3a) induced accumulation and nuclear localization of β-catenin and promoted the proliferation of myeloma cells, while disruption of β-catenin/TCF activity using ΔTCF4 inhibited MM proliferation (chapter 6). Numerous studies have shown that aberrant WNT signaling is involved in tumor formation in several cell types, and can be achieved by distinct mechanism of activation, including para/autocrine-, viral-, and mutational activation [41]. Mutations in *APC*, *CCNTB1* (β-catenin) or *AXIN* can result in the accumulation of β-catenin, and consequently in the activation of TCF target genes controlling cell proliferation and survival [41]. In our study, we have not detected mutations in either *APC* or *CTNNB1*, which makes direct mutational activation of WNT signaling in MM unlikely. Taken together, our findings strongly indicate that MM cells harbor an intact WNT signaling pathway, which can be further activated by exogenous stimuli, e.g. LiCl and WNT3a.

Our study is the first showing functional effects of the WNT signaling pathway in a human lymphoproliferative disease, i.e. MM. Our data suggest that activation of the canonical WNT pathway in MM is regulated by an upstream component, e.g. a member of the WNT family. Indeed, we detected *WNT5a*, *WNT10b*, and in some cases *WNT16*, in primary MM cells and cell lines, whereas normal B cell populations and plasma cells did not show expression, suggesting the presence of an autocrine activation loop (chapter 6). It has recently been suggested that aberrant WNT expression may contribute to the development of leukemias, including chronic lymphocytic leukemia (CLL), which overexpress several *WNTs* [42], and pre-B acute lymphoblastic leukemia (ALL), in which *WNT16* expression was transcriptionally regulated by the E2A-PBX1 fusion product [43]. WNT signaling has also been described in MM by Qiang *et al*, showing that WNT3a stimulation induces morphological changes and rearrangement of the actin cytoskeleton in MM cells [44]. The latter changes were not transduced by the β-catenin/TCF signaling, but were associated with a non-canonical WNT pathway, involving RHO activation. In agreement with our findings, Qiang *et al* showed that activation of the canonical WNT signaling pathway also occurs in MM cell lines, in contrast, however, they did not find an effect of WNT3a on the proliferation of MM cells [44]. Consistent with

our data, WNT3a stimulates the proliferation of pro-B cells [45]. Furthermore, WNT signaling can induce proliferation of purified haematopoietic stem cells, while inhibiting their differentiation [46,47], thereby resulting in functional self-renewal. Both events can be dangerous in a malignant situation.

The HGF/MET and WNT signaling pathways: implications in bone formation and MM-associated bone lesions

Osteolytic bone destruction in patients with MM can be caused by increased bone resorption by osteoclasts and, in more advanced stages, by a decreased bone formation by osteoblasts. A number of observations indicate that both the HGF/MET and WNT pathways play a crucial role in bone metabolism and MM-associated bone lesions. Expression of MET has been found on osteoblasts as well on osteoclasts, whereas osteoclasts secrete HGF [48], suggesting paracrine activation of osteoblasts and autocrine regulation of osteoclasts. In addition, high serum HGF levels were shown to correlate with advanced stage MM and extended bone lesions [49]. Hjertner *et al* showed in co-culture experiments with myeloma cells and osteoblasts, that myeloma-derived HGF can induce secretion of IL-11 from osteoblasts [50,51], which stimulates

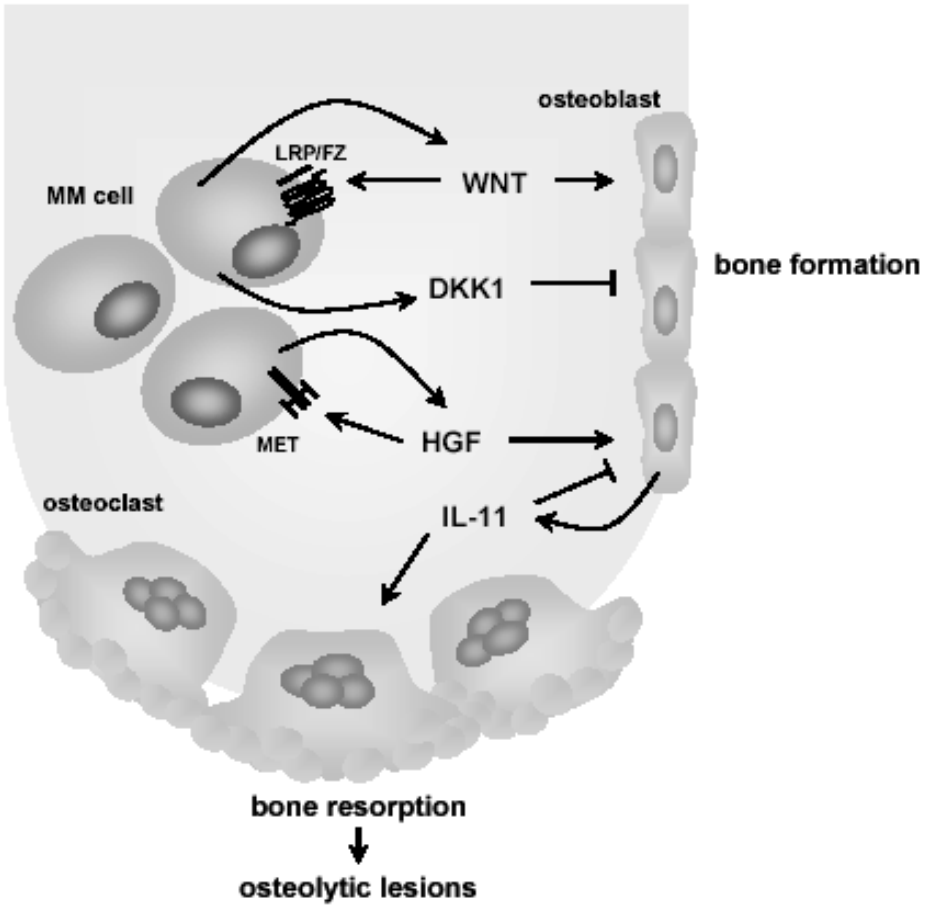


Figure 13 **The HGF/MET and WNT signaling pathways in MM-associated bone lesions.**

The HGF/MET and WNT pathways play a crucial role in bone metabolism and MM-associated bone lesions. Myeloma-derived HGF induces secretion of IL-11 from osteoblasts, which stimulates osteoclast formation and enhances bone resorption. WNTs, produced by MM cells, stimulate osteoblastogenesis, leading to increased bone mass, whereas the WNT antagonist DKK1, a WNT target, inhibits the differentiation of osteoblast precursor cells. Via both signaling pathways, MM cells can promote their outgrowth in the BM by suppressing osteoblast formation and by stimulating bone resorption by osteoclasts.

osteoclast formation [52] and suppresses bone formation [53]. On the other hand, in the absence of myeloma cells, HGF did not promote osteoclast function but, instead, inhibited bone resorption. This suggests that the HGF-mediated osteoclast formation and bone resorption require physical cell-cell contact between myeloma cells and osteoblasts, and likely involves additional factors, e.g. cytokines or growth factors.

It has been suggested that, under normal physiological circumstances, WNTs regulate the growth of osteoblast precursor [54] and the activity of alkaline phosphatase [54], which is an early event during osteoblast differentiation [55]. In mice, overexpression of WNT10b stimulates osteoblastogenesis and leads to an increased bone mass, while deletion of WNT10b results in loss of bone mass. These findings demonstrate that WNT10b is a regulator of bone formation [56]. Recently, Tian *et al* showed that elevated levels of DKK1, a WNT antagonist [57], in the BM plasma and peripheral blood of patients with MM were associated with the presence of bone lesions [58]. Most important, they showed that DKK1, produced by MM cells, inhibited the differentiation of osteoblast precursor cells *in vitro*. These findings suggest that MM cells can promote their outgrowth in the BM by suppressing osteoblast formation and stimulating bone resorption by osteoblasts. The production of DKK1 by MM cells themselves, suggests a negative feedback loop in MM cells, i.e. inhibition of WNT signaling. Paradoxically, our findings show that the WNT signaling pathway is active in MM (chapter 6). Remarkably, Niida *et al* found that the expression of DKK1 is upregulated by WNT signaling, showing that DKK1 is a target of the WNT pathway [59]. It is conceivable that WNT and DKK1 regulate the canonical WNT signaling pathway and bone metabolism in different ways depending on the physiological conditions in the microenvironment. WNTs can stimulate osteoblastogenesis [54,56], while DKK1 apparently inhibits this process [58], leading to bone destruction. A very attractive idea is that the WNT- and HGF/MET pathways act in concert to promote MM cell growth and bone destruction, and that DKK1 inhibits osteoblast maturation, whereas HGF promotes osteoclast formation (figure 13). Therefore, the functional consequences of the canonical WNT and the HGF/MET signaling pathway for osteoblasts as well for osteoclasts need to be clarified to better understand MM pathogenesis.

References

1. van der Voort R, Taher TE, Keehnen RM et al. Paracrine regulation of germinal center B cell adhesion through the c-met-hepatocyte growth factor/scatter factor pathway. J Exp Med. 1997;185:2121-2131.
2. Weimar IS, de Jong D, Muller EJ et al. Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins. Blood. 1997;89:990-1000.
3. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology. 2001;102:506-514.
4. Koopman G, Parmentier HK, Schuurman HJ et al. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways. J Exp Med. 1991;173:1297-1304.
5. Koopman G, Keehnen RM, Lindhout E et al. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J Immunol. 1994;152:3760-3767.
6. Koopman G, Keehnen RM, Lindhout E et al. Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. Eur J Immunol. 1997;27:1-7.
7. Voigt I, Camacho SA, de Boer BA et al. CXCR5-deficient mice develop functional germinal centers in the splenic T cell zone. Eur J Immunol. 2000;30:560-567.
8. Ansel KM, Ngo VN, Hyman PL et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature. 2000;406:309-314.

9. Allen CD, Ansel KM, Low C et al. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol. 2004;5:943-952.
10. Takai K, Hara J, Matsumoto K et al. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood. 1997;89:1560-1565.
11. Borset M, Lien E, Espevik T et al. Concomitant expression of hepatocyte growth factor/scatter factor and the receptor c-MET in human myeloma cell lines. J Biol Chem. 1996;271:24655-24661.
12. Jucker M, Gunther A, Gradl G et al. The Met/hepatocyte growth factor receptor (HGFR) gene is overexpressed in some cases of human leukemia and lymphoma. Leuk Res. 1994;18:7-16.
13. van der Voort R, Taher TE, Derksen PW et al. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. Adv Cancer Res. 2000;79:39-90.
14. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4:915-925.
15. Borset M, Hjorth-Hansen H, Seidel C, Sundan A, Waage A. Hepatocyte growth factor and its receptor c-met in multiple myeloma. Blood. 1996;88:3998-4004.
16. Elenitoba-Johnson KS, Jenson SD, Abbott RT et al. Involvement of multiple signaling pathways in follicular lymphoma transformation: p38-mitogen-activated protein kinase as a target for therapy. Proc Natl Acad Sci U S A. 2003;100:7259-7264.
17. Hsiao LT, Lin JT, Yu IT et al. High serum hepatocyte growth factor level in patients with non-Hodgkin's lymphoma. Eur J Haematol. 2003;70:282-289.
18. Derksen PW, de Gorter DJ, Meijer HP et al. The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia. 2003;17:764-774.
19. Kmiecik TE, Keller JR, Rosen E, Vande Woude GF. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood. 1992;80:2454-2457.
20. Weimar IS, Miranda N, Muller EJ et al. Hepatocyte growth factor/scatter factor (HGF/SF) is produced by human bone marrow stromal cells and promotes proliferation, adhesion and survival of human hematopoietic progenitor cells (CD34+). Exp Hematol. 1998;26:885-894.
21. Kawano R, Ohshima K, Karube K et al. Prognostic significance of hepatocyte growth factor and c-MET expression in patients with diffuse large B-cell lymphoma. Br J Haematol. 2004;127:305-307.
22. Giles FJ, Vose JM, Do KA et al. Clinical relevance of circulating angiogenic factors in patients with non-Hodgkin's lymphoma or Hodgkin's lymphoma. Leuk Res. 2004;28:595-604.
23. Derksen PW, Keehnen RM, Evers LM et al. Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. Blood. 2002;99:1405-1410.
24. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer. 2002;2:289-300.
25. Schmidt L, Duh FM, Chen F et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16:68-73.
26. Schmidt L, Junker K, Nakaigawa N et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene. 1999;18:2343-2350.
27. Lee JH, Han SU, Cho H et al. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene. 2000;19:4947-4953.
28. Ma PC, Jagadeeswaran R, Jagadeesh S et al. Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 2005;65:1479-1488.
29. Zaffaroni D, Spinola M, Galvan A et al. Met proto-oncogene juxtamembrane rare variations in mouse and humans: differential effects of Arg and Cys alleles on mouse lung tumorigenesis. Oncogene. 2005;24:1084-1090.
30. Ma PC, Kijima T, Maulik G et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res. 2003;63:6272-6281.
31. Graveel C, Su Y, Koeman J et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A. 2004;101:17198-17203.
32. Miyake S, Lupher ML, Jr., Druker B, Band H. The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. Proc Natl Acad Sci U S A. 1998;95:7927-7932.
33. Lee PS, Wang Y, Dominguez MG et al. The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. EMBO J. 1999;18:3616-3628.
34. Levkowitz G, Waterman H, Zamir E et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. 1998;12:3663-3674.

35. Shtiegman K, Yarden Y. The role of ubiquitylation in signaling by growth factors: implications to cancer. Semin Cancer Biol. 2003;13:29-40.
36. Bao J, Gur G, Yarden Y. Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors. Proc Natl Acad Sci U S A. 2003;100:2438-2443.
37. Wu WJ, Tu S, Cerione RA. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. Cell. 2003;114:715-725.
38. Flanders JA, Feng Q, Bagrodia S et al. The Cbl proteins are binding partners for the Cool/Pix family of p21-activated kinase-binding proteins. FEBS Lett. 2003;550:119-123.
39. Ridge SA, Worwood M, Oscier D, Jacobs A, Padua RA. FMS mutations in myelodysplastic, leukemic, and normal subjects. Proc Natl Acad Sci U S A. 1990;87:1377-1380.
40. Weidner KM, Sachs M, Riethmacher D, Birchmeier W. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proc Natl Acad Sci U S A. 1995;92:2597-2601.
41. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004;5:691-701.
42. Lu D, Zhao Y, Tawatao R et al. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2004;101:3118-3123.
43. McWhirter JR, Neuteboom ST, Wancewicz EV et al. Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A. 1999;96:11464-11469.
44. Qiang YW, Endo Y, Rubin JS, Rudikoff S. Wnt signaling in B-cell neoplasia. Oncogene. 2003;22:1536-1545.
45. Reya T, O'Riordan M, Okamura R et al. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity. 2000;13:15-24.
46. Reya T, Duncan AW, Ailles L et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423:409-414.
47. Willert K, Brown JD, Danenberg E et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 2003;423:448-452.
48. Grano M, Galimi F, Zambonin G et al. Hepatocyte growth factor is a coupling factor for osteoclasts and osteoblasts in vitro. Proc Natl Acad Sci U S A. 1996;93:7644-7648.
49. Alexandrakis MG, Passam FH, Sfiridaki A et al. Elevated serum concentration of hepatocyte growth factor in patients with multiple myeloma: correlation with markers of disease activity. Am J Hematol. 2003;72:229-233.
50. Romas E, Udagawa N, Zhou H et al. The role of gp130-mediated signals in osteoclast development: regulation of interleukin 11 production by osteoblasts and distribution of its receptor in bone marrow cultures. J Exp Med. 1996;183:2581-2591.
51. Hjertner O, Torgersen ML, Seidel C et al. Hepatocyte growth factor (HGF) induces interleukin-11 secretion from osteoblasts: a possible role for HGF in myeloma-associated osteolytic bone disease. Blood. 1999;94:3883-3888.
52. Girasole G, Passeri G, Jilka RL, Manolagas SC. Interleukin-11: a new cytokine critical for osteoclast development. J Clin Invest. 1994;93:1516-1524.
53. Hughes FJ, Howells GL. Interleukin-11 inhibits bone formation in vitro. Calcif Tissue Int. 1993;53:362-364.
54. Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. Gene. 2004;341:19-39.
55. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res. 2003;18:1842-1853.
56. Bennett CN, Longo KA, Wright WS et al. Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci U S A. 2005;102:3324-3329.
57. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. J Cell Sci. 2003;116:2627-2634.
58. Tian E, Zhan F, Walker R et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med. 2003;349:2483-2494.
59. Niida A, Hiroko T, Kasai M et al. DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. Oncogene. 2004;23:8520-8526.

CHAPTER **8**

Summary

B cell development and -differentiation are highly regulated multi-step processes that proceed through the maturation from a commited progenitor to a mature naïve B cell, and end with antigen (Ag)-specific B cell differentiation. A central part of Ag-specific B cell differentiation takes place within germinal centers of secondary lymphoid organs, which consist of two distinct compartments, i.e. the dark- and light zones. This GC reaction involves proliferation and selection of B cells leading to the generation of plasma cells and memory B cells with a diverse repetoire of B cell antigen receptors (BCR) that are capable of recognizing and eradicate pathogens. During several stages of B cell development and differentiation, genetic aberrations may occur that deregulate the expression and/or function of genes involved in cell proliferation, survival and differentiation. In addition, aberrant activation of growth factor signaling cascades by factors from the microenvironment contributes to the pathogenesis of lymphoproliferative diseases. In this thesis, we have explored the role of two potent oncogenic pathways, i.e. the HGF/MET and WNT/β-catenin signaling pathways in B cell malignancies, in particularly in DLBCL and MM.

In **chapter 2**, we examined the expression of regulatory components of the HGF/MET pathway, including the serine protease HGFA, within the normal secondary lymphoid organ microenvironment. We show that MET is expressed by both centroblasts and plasma cells, and that HGFA is expressed by plasma cells. Furthermore, we demonstrate that follicular dendritic cells (FDC) are the major source for HGF and its activator within the germinal center (GC) microenviroment. Both HGF and HGFA are expressed by FDCs in the GC dark zone but not in the light zone. Our findings suggest that HGF and HGFA provided by the dark zone FDCs control proliferation, survival and adhesion of MET-positive centroblasts. The co-expression of MET and HGFA in plasma cells is of interest since it indicates that plasma cells are able to receive paracrine HGF signals. In these cells, the HGF/MET pathway might regulate survival, integrin-mediated adhesion and migration during the process of homing to the bone marrow

Chapter 3 describes the expression of MET on a large panel of B cell malignancies, representing a broad spectrum of differentional stages ranging from precursor B cells to plasma cells. Expression of MET was found in a subsets of follicular- (FL), Burkitt's- and chronic lymphocytic leukemia (CLL) cases and in a large percentage of multiple myelomas (MM) and diffuse large B cell lymphomas (DLBCL). No indication of amplification of the MET gene was found in the tested B cell malignancies. However, we detected two germline missense mutations, located in the juxtamembrane- (JM) and tyrosine kinase domain in four and one lymphomas, respectively. Furthermore, we

detected HGF expression in most of the MET-expressing primary DLBCL tumors, which is probably produced by cells of the monocyte/macrophage lineage, suggesting that DLBCL cells are stimulated via a paracrine mechanism. HGF stimulation of DLBCL cells phosphorylated a range of downstream targets, including MAPKs ERK1 and –2, and PKB and its substrates GSK3 and FOXO3a, which are linked to proliferative- and survival functions. HGF also induced PI3K-dependent adhesion of DLBCL cells to VCAM-1 and FN. Finally, we demonstrated that DLBCLs themselves produce HGFA, which efficiently autocatalyzes HGF activation, thereby generating a constant source of active HGF in the microenvironment, potentially favouring tumor growth and progression.

Most of the evolution of MM takes place in the BM, indicating that signals from the BM microenvironment, which include growth factors and cytokines, play a critical role in sustaining the growth and survival of MM cells during tumor progression. A potential key signal in the evolution of MM is HGF, which acts as a potent para- and/or autocrine growth- and survival factor for MM cells. Since proteolytic conversion of HGF into its active form is a critical limiting step in HGF/MET signaling, we have studied in **Chapter 4** the mechanism of HGF activation in MM. We show that myeloma cell lines as well as primary myelomas secrete HGFA and in this way are able to autocatalyze HGF activation.

In **Chapter 5**, we identified proto-oncogene product c-Cbl, an E3 ubiquitin ligase, as a downstream phosphorylation target of the HGF/MET signaling pathway in B cells, and demonstrate that it mediates MET downregulation. We show that HGF induces strong tyrosine phosphorylation of Cbl and increases association with the Src-family tyrosine kinases Fyn and Lyn, as well as with PI3K and CrkL. Furthermore, c-Cbl mediates HGF-induced ubiquitination of MET, which requires the juxtamembrane tyrosine Y1001 (Y2) of MET, but not the multi-substrate docking site residues Y1349/1356 (Y14/15) or the additional C-terminal tyrosine residues Y1313-1365 (Y13-16). The transforming mutants v-Cbl and 70Z/3 Cbl, which lack the ubiquitin ligase RING finger domain, suppressed MET ubiquitination. Our data indicate that Cbl acts as a negative regulator of HGF/MET signaling in B cells, mediating ubiquitination and, consequently proteosomal degradation and inactivation of MET.

The WNT pathway has been implicated in the survival and expansion of lymphocyte progenitors. Dysregulation of the WNT signaling pathway may contribute to the pathogenesis of lymphoproliferative disease. In **Chapter 6**, we show that the malignant MM plasma cells overexpress β-catenin, including its N-terminally unphosphorylated form, suggesting active β-catenin/TCF-mediated transcription. Stimulation of WNT signaling with either WNT3a, lithium chloride (LiCl) or constitutively active S33Y mutant of β-catenin resulted in the accumulation and nuclear localization of β-catenin, and /or increased cell proliferation. Moreover, repression of β-catenin/TCF-mediated transcription led to decreased proliferative responses, implying that constitutive, endogenous WNT signaling potentiates MM cell growth. Combined with the absence of mutations in either APC or CCNTB1 (β-catenin), our findings illustrate that MM cells harbors an intact WNT signaling pathway. Finally, we detected WNT-5a, WNT-10b and WNT-16 transcripts in MM cells, whereas normal B cell populations and plasma cells did not show expression. This suggests an autocrine activation mechanism, which together with an intact WNT pathway, we consider 'illegitimate', because it involves induction of malignant plasma cell proliferation.

Samenvatting

Binnen het afweersysteem zijn B cellen verantwoordelijk voor de productie van antistoffen (= immunoglobulinen). Antistoffen zijn van belang om schadelijke indringers zoals micro-organismen (bijvoorbeeld bacteriën, virussen en parasieten) te verwijderen. Alvorens een B cel antistoffen kan produceren, moet de B cel zich ontwikkelen tot antistof-producerende plasma cel. In het beenmerg ondergaan voorloper B cellen een deel van de ontwikkeling (figuur 1). B cellen die het beenmerg verlaten, circuleren door het lichaam via bloed- en lymfebaan naar secundaire lymfoïde organen zoals milt. Op het moment dat zij daar in contact komen met een antigeen (= een component die een afweerreactie veroorzaakt), raken de B cellen geactiveerd, gaan delen en ontwikkelen zich verder tot plasma cellen en geheugen B cellen (= B cellen die snel kunnen reageren op een herinfectie van hetzelfde micro-organisme) (figuur 2).
Gedurende het leven en tijdens het doorlopen van de verschillende B cel ontwikkelingsstadia, kunnen fouten in het erfelijk materiaal van de cellen (bijvoorbeeld mutaties) ontstaan die onder normale omstandigheden worden hersteld. Niet-gerepareerde fouten in de genen die betrokken zijn bij de groei, overleving, adhesie, migratie van cellen, kunnen leiden tot ontregelde gen expressie en/of functie. Meerdere fouten in de genen zijn uiteindelijk nodig om kanker te veroorzaken.

In dit proefschrift hebben wij naar twee belangrijke B cel kankersoorten gekeken, namelijk multiple myeloma (MM, bekend als de ziekte van Kahler) en diffuus grootcellige B cel lymfoom (DLBCL, de meest voorkomende B cel-non Hodgkin lymfoom). MM is op een na de meest voorkomende bloedkanker (figuur 3). Het wordt veroorzaakt door woekering van kwaadaardige plasma cellen (= myeloom cellen) in het beenmerg. Door de massieve expansie van myeloom cellen en de abnormale productie van antistoffen, ontstaat er een pijnlijke tumor die gepaard gaat met botafbraak, nierafwijking, anemie (= bloed-armoede) en verstoorde afweer. Bij tijdige ontdekking kan deze kankersoort redelijk beheerst worden en wordt er gebruik gemaakt van stamcel- of chemotherapie. Momenteel is er geen genezing mogelijk. Er zijn aanwijzingen dat B cel maligniteiten zoals MM en DLBCL voor hun groei, overleving en ontwikkeling afhankelijk zijn van hun omgevingsfactoren zoals groeifactoren (figuur 4). Om de (her) uitgroei van de kanker aan te pakken is het van belang om de omgevingsfactoren in kaart te brengen en de rol daarin te begrijpen. Het doel van dit proefschrift was de rol van twee groeifactoren met bijbehorende signaleringsroutes, de hepatocyte groeifactor (HGF)/MET en de WNT/β-catenine, in de pathogenese van deze kankersoorten te bestuderen. Het ultieme doel is om een therapie te ontwikkelen die kan interfereren in die groeifactor-sigaleringsroutes om zo kankergroei te bestrijden.

Om de maligne B cel situatie (hoofdstuk 3) met de normale te kunnen vergelijken, hebben wij eerst de expressie van de componenten van de HGF/MET signaleringsroute waaronder HGF, zijn receptor MET en

activator, de serine protease HGF activator (HGFA), in een normaal secundair lymfoïd orgaan bestudeerd (hoofdstuk 2). Zoals beschreven vindt in het kiemcentrum van een secundaire lymfoide orgaan activatie en verdere ontwikkeling van B cellen plaats. Het kiemcentrum bestaat uit twee zones, de donkere en lichte zone, waar een B cel zich ontwikkelt van een kiemcentrum B cel (centroblast → centrocyt) tot een plasma cel (figuur 2). De aanwezige cellen in de zones zoals T cellen en folliculair dendritische cellen (FDC) spelen een belangrijke rol in het aansturen van B cel activatie, expansie en ontwikkeling. In hoofdstuk 2 laten wij zien binnen een normaal secundaire lymfoïd orgaan MET expressie te vinden is op centroblasten en plasma cellen, en HGFA op plasma cellen. FDCs blijken de belangrijkste bron van HGF en zijn activator te zijn. De gevonden co-expressie van MET en HGFA op plasma cellen is interessant omdat dit aangeeft dat plasma cellen in staat zijn om HGF signalen te ontvangen. De HGF/MET route kan de celoverleving, adhesie en migratie van plasma cellen aansturen die nodig zijn voor plasma cellen om naar het beenmerg te gaan. Verder hebben wij gevonden dat de FDCs die zich in de donkere zone van het kiemcentrum bevinden HGF en HGFA tot expressie brengen. Onze bevinding suggereert dat HGF en HGFA, die door de donkere zone FDCs geproduceerd worden, de proliferatie, overleving en adhesie van MET-positieve centroblasten kunnen bewerkstelligen. Onze bevindingen laten zien dat de HGF/MET route een rol kan spelen tijdens de B cel ontwikkelingsproces.

Hoofdstuk 3 beschrijft de expressie van MET in een groot aantal B cel maligniteiten die variëert in de ontwikkelingsstadium: van voorloper B cel tot plasma cel. Verhoogde MET expressie is gedetecteerd in een subgroep van folliculair-, Burkitt's en chronisch lymfocytische leukemie gevallen. Ook een hoog percentage van de MMs en DLBCLen brengt MET tot expressie. Wij hebben geen indicaties gevonden dat het MET gen in de bestudeerde B cel maligniteiten is geamplificeerd wat de oorzaak voor de overexpressie in deze tumoren zou kunnen zijn. Wel hebben wij twee missense mutaties in MET gevonden (in de juxtamembraan- en tyrosine kinase domein). HGF expressie is in veel van de MET-positieve primaire DLBCL tumoren gedetecteerd. De HGF wordt waar-schijnlijk geproduceerd door de aanwezige monocyten en/of macrofagen en suggereert dat DLBCL cellen via een paracriene activatie mechanisme worden gestimuleerd. In DLBCL cellen resulteert HGF stimulatie in de fosforylatie van een reeks onderliggende signaleringsmoleculen, waaronder MAPKs, PKB en zijn substraten GSK3 en FOXO3a. Deze moleculen zijn gekoppeld aan signaleringsroutes die biologische effecten als celgroei en –overleving induceren. HGF kan ook adhesie van DLBCL cellen induceren. HGF kan alleen in zijn actieve vorm MET activeren en biologische effecten veroorzaken. Activatie van HGF is dus de kritische en bepalende stap in de HGF/MET signalering. We laten zien dat DLBCL cellen, in tegenstelling tot zijn normale tegenhanger (de kiemcentrum B cellen), zelf HGFA produceren wat efficiënt de HGF activatie autocatalyseert. Daardoor is er een continu aanmaak van actieve HGF in de tumor omgeving wat de groei en progressie van DLBCL kan bevorderen. Onze bevindingen veronderstellen dat zowel de HGF/MET signalering als de secretie van HGFA door de DLBCL cellen een belangrijke bijdrage kunnen leveren in de pathogenese van DLBCLs.

Het grootste deel van de MM evolutie vindt plaats in het beenmerg wat impliceert dat signalen in het beenmerg, waaronder groeifactoren en cytokinen, een belangrijke rol spelen in het onderhouden van de groei en overleving van MM cellen. HGF induceert tumor angiogenese (= vorming van nieuwe bloedvaten) en wordt verhoogd aangetroffen in het serum van MM patienten wat tevens correleert met een slechte prognose. In tegenstelling tot normale plasma cellen die geen HGF produceren, brengen myeloom cellen zowel MET als HGF in verhoogde mate tot expressie. Onlangs hebben wij aangetoond dat HGF een sterke para- en/of autocriene groei- en overlevingsfactor voor MM cellen is.

Uit deze gegevens blijkt dat de HGF/MET signalering een mogelijke maligne route is in de evolutie van MM. Omdat MM cellen zelf in staat zijn om HGF te produceren met als gevolg dat ze onafhankelijk en autonoom kunnen groeien, hebben wij in hoofdstuk 4 de HGF activatie mechanisme in MM bestudeerd. We laten zien dat myeloom cel lijnen en primaire myelomas zelf HGFA, de meest potente activator van HGF, uitscheiden en via deze weg in staat zijn de activatie te autocatalyseren. Met deze studie hebben wij in MM de activatie stap van HGF geïdentificeerd wat als een nieuw uitgangs-punt kan dienen in het ontwikkelen van MM therapie.

Abnormale- en hyperactieve signalering van MET kan worden veroorzaakt door verstoorde negatieve regulatie en speelt een belangrijke rol bij de tumorgroei, invasie en metastasering. In hoofdstuk 5 hebben wij het proto-oncogeen product c-Cbl, een E3 ubiquitin ligase, geïdentificeerd als een interactie molecuul in de HGF/MET route in B cellen. We laten zien dat c-Cbl de down-regulatie van MET en dus de inactivatie van de HGF/MET signalering kan beïnvloeden. Verder hebben wij aangetoond welke domeinen in zowel MET als c-Cbl betrokken zijn bij de HGF-geïnduceerde downregulatie van MET. Dus samengevat: c-Cbl is een nega-tieve regulator van de HGF/MET signalering in B cellen.

Onder normale omstandigheden is de WNT signalering betrokken bij de overleving en expansie van lymfocyten voorloper cellen. Maar in het geval van een verstoorde WNT signalering kan dit juist een bijdrage leveren aan de pathogenese van B cel kanker als MM. In hoofdstuk 6 laten we zien dat maligne plasma cellen β-catenine tot overexpressie brengen. Stimulatie van de WNT signalering met WNT3a, lithium chloride of door gebruik te maken van constitutief actieve S33Y mutant van β-catenine resulteerde in de ophoping en nucleaire lokalisatie van β-catenine, en verhoogde celgroei. Het onderdrukken van de β-catenine/TCF cascade leidde tot inhibitie van de groei effecten wat impliceert dat constitutieve endogene WNT signalering MM celgroei kan ondersteunen. Doordat wij geen mutaties in de twee belangrijkste compenenten van de WNT route, te weten het APC of β-catenine gen, konden vinden, kan dit niet de oorzaak zijn van een actieve en intacte WNT signalering. In tegenstelling tot normale B cellen en plasma cellen, vonden wij verschillende WNT transcripten in MM cellen waaronder WNT-5a, WNT-10b and WNT-16. Deze bevinding suggereert dat MM cellen door zelf WNTs te produceren zich zelf kunnen activeren (= autocriene activatie route). Omdat onder normale omstandigheden B- en plasma cellen geen actieve WNT signalering hebben en geen WNTs produceren, beschouwen wij de gevonden intacte WNT signalering en autocriene activatie in MM cellen als 'niet legitiem'. Belangrijk is dat beide mechanismen de groei van maligne plasma cellen bevordert.

Het beter begrijpen van wat B cel tumoren nodig hebben voor hun progressie, is een eerste stap om betere therapeutische middelen te ontwikkelen. De interactie van de maligne cel met zijn omgeving speelt een cruciale rol bij hun groei en overleving. Zoals beschreven in dit proefschrift blijken MM- en DLBCL cellen de groeifactor-signaleringsroutes HGF/MET en WNT/β-catenine te gebruiken of zodanig te hebben aangepast ter bevordering van de tumorprogressie. Door niet alleen de maligne B cel aan te pakken maar ook in te grijpen op de groeifactor-signaleringsroutes en de activatie daarvan aan te pakken, kan een verbeterde therapie voor MM en DLBCL ontwikkeld worden.

Dankwoord

Deze bijna laatste pagina wil ik gebruiken om iedereen te bedanken die mij op een of ander manier heeft geholpen, gemotiveerd en/of geïnspireerd bij het verwezenlijken van dit proefschrift.

Steven, mijn promotor, wil ik bedanken voor zijn enthousiasme en kritische kijk gedurende het onderzoeksproject. Ik heb veel van jou geleerd.
Natuurlijk ook Marcel. Ik vind het leuk dat je mijn co-promotor bent. Het was wel wennen aan jouw breedsprakigheid en dus de ellenlange werkbesprekingen. Maar jouw enthousiasme en inzet maakte alles goed. Heel erg bedankt!
Veel dank ben ik verschuldigd aan Irma. Jij hebt mij het laatste jaar enorm geholpen en bergen werk verzet: het maken van de celklonen, en het uitvoeren van talloze signalerings- en adhesie proeven. Het was erg leuk werken met jou.
Richard Bende: jouw nieuwsgierigheid en 'wilde' proeven doen kon ik wel waarderen, maar ook jouw kennis op het gebied van B cellen en wat daar allemaal bij komt kijken. Hebben we toch nog leuk samen aan FDCs kunnen werken.
Ook dank aan Taher en Patrick voor de samenwerking; Elles, Laura en David voor jullie steun en de leuke gesprekken op de AIO kamer; de nieuwe generatie AIO's Rogier, Richard Groen (bedankt voor de mutatie analyse!), Febe en Jurrit voor de gezelligheid; en studente Anne-Pauline voor de vele immunohistochemische kleuringen.
Verder dank ik alle mensen van het lab en de lunch groep voor jullie behulpzaamheid, gezelligheid en leuke uitjes: Nike (superwomen!), Mirjam, het duo Esther Schilder-Tol/Monique, Alex, Folkert, Jitske, Wendy (zullen we nog een keer samen boetseren?), Thera, Annemieke, Liane, Inge, Sandra, Loes, Silvia, Danielle, Kasper, Geurt, Niels, Jose, Joris, Carla (gelukkig bleef je in de buurt werken), Anja, Cynthia, Surinder (altijd verrassend); Anand (wanneer ga je weer verhuizen?), Jelger, Onno en Peter Teeling voor de muziek/film tips of uitwisselingsprojecten; Peter van Amstel voor als ik weer een vraag had over de bestellingen, en bedankt voor de kastanjes; Wilfried voor de foto en poster zaken; Marjon en Lia van het secretariaat; de computerboys; en niet te vergeten (Cy-)Berend Hooibrink voor alle sorts.
Lieve Helen en Esther, tevens mijn paranimfen: ik dank jullie beiden voor de prettige sfeer en steun tijdens mijn AIO periode. We gaan gewoon gezellig door met restaurantjes in Amsterdam 'ontdekken'.
Als laatste wil ik mijn familie en vooral Hans bedanken dat jullie er altijd voor mij zijn.

List of publications

1. **Tjin EPM**, Groen R, Vogelzang I, Derksen PWB, Klok MD, Meijer HP, van Eeden S, Pals ST, and M Spaargaren. Aberrant HGF activator expression and functional analysis of HGF/MET signaling in diffuse large B cell lymphoma. *Blood, In press.*

2. **Tjin EPM**, Bende RJ, Derksen PWB, van Huijstee A, Kataoka H, Spaargaren M, and ST Pals. Follicular dendritic cells catalyze hepatocyte growth factor (HGF) activation in the germinal center microenvironment by secreting the serine protease HGF-activator. *J Immunol, In press.*

3. **Tjin EPM**, Derksen PWB, Kataoka H, Spaargaren M, and ST Pals. Multiple myeloma cells catalyze hepatocyte growth factor (HGF) activation by secreting the serine protease HGF-activator. *Blood, 2004, 104 (7): 2172-2175.*

4. Derksen PW, **Tjin EPM**, Meijer HP, Klok MD, Mac Gillavry HD, van Oers MHJ, Lokhorst HM, Bloem AC, Clevers H, Nusse R, van der Neut R, Spaargaren M, and ST Pals. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. *Proc Natl Acad Sci USA, 2004, 101 (16): 6122-6127.*

5. Taher TEI*, **Tjin EPM***, Beuling EA, Borst J, Spaargaren M, and ST Pals. c-Cbl is involved in MET signaling in B Cells and mediates hepatocyte growth factor-induced receptor ubiquitination. *J Immunol, 2002, 169(7): 3793-3800. * equal contribution*

6. Tekstra J, **Tjin EPM**, Tuk CW, Broekhuis-Fluitsma D, Havenith CE, and RH Beelen. Analysis of monocyte chemotactic protein-1 production in different major histocompatibility complex-restricted antigen presentation systems. *Clin Immunol, 2001, 101(1):77-85.*

7. van Lith M, van Ham M, Griekspoor A, **Tjin EPM**, Verwoerd D, Calafat J, Janssen H, Reits E, Pastoors L, and J Neefjes. Regulation of MHC class II antigen presentation by sorting of recycling HLA-DM/DO and class II within the multivesicular body. *J Immunol, 2001, 167(2): 884-892.*

8. van Ham M, van Lith M, Lillemeier B, **Tjin EPM**, Gruneberg U, Rahman D, Pastoors L, van Meijgaarden K, Roucard C, Trowsdale J, Ottenhoff T, Pappin D, and J Neefjes. Modulation of the major histocompatibility complex class II-associated peptide repertoire by human histocompatibility leukocyte antigen (HLA)-DO. *J Exp Med, 2000, 191(7): 1127-1136.*

9. van Ham SM, **Tjin EPM**, Lillemeier BF, Gruneberg U, van Meijgaarden KE, Pastoors L, Verwoerd D, Tulp A, Canas B, Rahman D, Ottenhoff TH, Pappin DJ, Trowsdale J, and J Neefjes. HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol, 1997, 7(12): 950-957.*