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Heparan sulfate proteoglycans: key moderators of the interaction of multiple myeloma with the bone marrow niche

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CHAPTER

1

General introduction

CHAPTER 1

General introduction

1.B cell development and differentiation

The adaptive immune system, which plays an important role in the fight against pathogens, consists of two types of branches: cell-mediated immunity and humoral immunity. Humoral immunity is mediated by B cell-derived antibodies. B cells are characterized by the expression of a cell-surface B cell receptor (BCR), which in principle consists of a membrane-bound immunoglobulin. B cells undergo a complex differentiation process to plasma cells, which secrete immunoglobulin by deletion of the membrane-associated portion of the BCR. In mammals, B cells differentiate from hematopoietic stem cells (HSCs), which reside in the bone marrow (BM)¹. This process is tightly regulated by complex developmental pathways. HSCs can differentiate into two lineages: the lymphoid lineage and the myeloid lineage. In the lymphoid lineage, B cells arise from the common lymphocyte progenitor (CLP). The expression of the transcription factors PAX5 and E2A commits differentiation toward the B cell lineage^{2,3}. At the pro-B cell stage, the immunoglobulin heavy chain is assembled, in a process called V(D)J rearrangement as the immunoglobulin heavy chain gene locus consists of V(variable), D (diverse) and Joining (J) segments^{4,5}. This process is regulated by the recombination activating gene (RAG)-1 and RAG-2⁶. Subsequently, the heavy chain will pair to a surrogate light chain to form a precursor BCR (pre-BCR)^{7,8}. The survival and further differentiation of pre-B cells is highly dependent on the expression of a functional pre-BCR¹. After the successful expression of pre-BCR, pro-B cells differentiate to large pre-B cells, which undergo proliferation induced by pre-BCR signaling. At this stage, RAG1 and RAG2 expression are downregulated, which inhibits further V(D)J recombination. After several rounds of proliferation, large pre-B cells differentiate to small pre-B cells. RAG1 and RAG2 expression are upregulated again, which activates immunoglobulin light-chains recombination^{6,9}. Then pre-B cells differentiate to immature B cells. From this stage onwards, the mature BCR (IgM) is successfully expressed on the cell surface. Immature B cells leave the BM as transitional B cells and subsequently migrate to the peripheral lymphoid organs such as lymph nodes and spleen. In the secondary lymphoid organs, immature B cells finalize their early development and become mature naïve B cells¹⁰.

Upon encountering antigen in the secondary lymphoid organs spleen and lymph nodes, naïve B cells undergo antigen-specific B cell activation, which can be subclassified into T cell-dependent and T cell-independent activation. In T cell-independent activation, upon encountering antigen, the BCR undergoes clustering, which activates downstream signaling without T cell help. During this T cell-independent activation, B cells generate a massive wave of IgM producing plasma cells as a quick response to the antigen¹¹. Most antigens cannot crosslink multiple BCRs. In order to activate B cells recognizing these antigens, help from T cells is needed. This is called T cell dependent activation. After

recognition by the BCR, antigens will be internalized and processed into small peptides by B cells. Subsequently, they will be presented to T cells by major histocompatibility complex (MHC) class II molecules. Then T cells will stimulate the B-cells via CD40L-CD40 interaction, which activates NF- κ B signaling in the B cells. In addition, the T cells will secrete stimulatory cytokines, such as IL-21 and IL-4, which induce B cell proliferation¹².

After antigen-induced activation, B cells can differentiate to memory B cells or to antibody-producing plasma cells. This takes place in the germinal center (GC), is a specialized microenvironment in the secondary lymphoid organs. Histologically, the GC can be divided into a dark zone and a light zone¹³. In the dark zone of the GC, B cells undergo somatic hypermutation, a process during which mutations are introduced in the immunoglobulin-V genes, producing B cells expressing BCRs with different antigen-binding affinity^{14,15}. These B cells migrate to the light zone where they re-encounter antigens presented by the follicular dendritic cells (FDCs) and undergo selection based on their BCR affinity. B cells with a high-

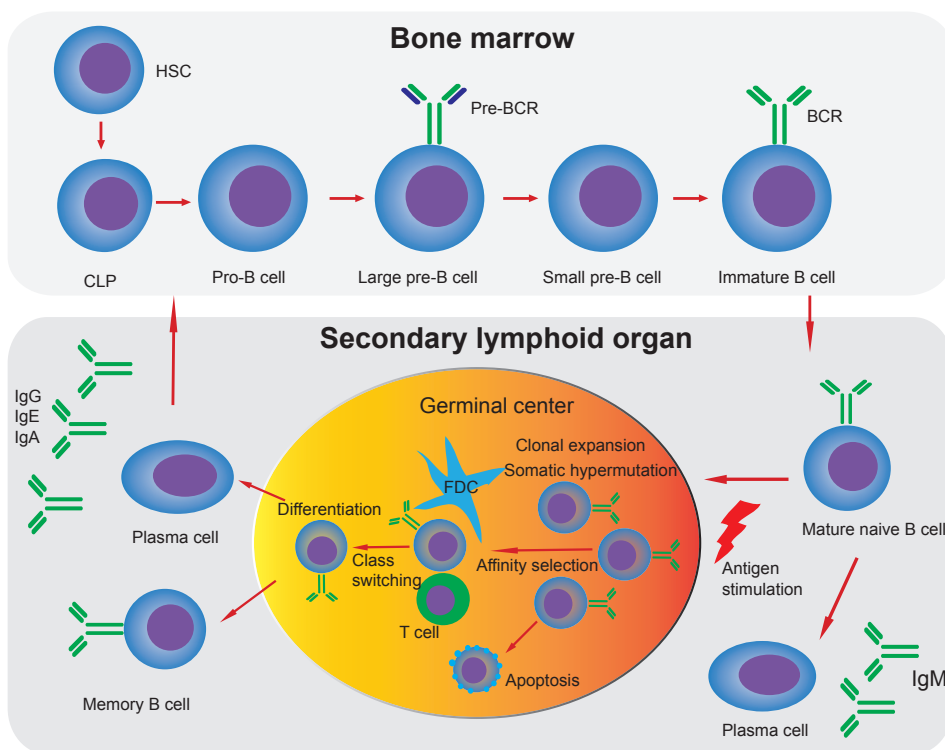


Figure 1. B cell development, activation, and differentiation. B cells differentiate from HSCs in the BM. After successful expression of the BCR, B cells exit the BM and migrate to secondary lymphoid organs like spleen and lymph nodes, where they finalized their maturation. Upon encountering antigen, mature naïve-B cells undergo antigen-specific B cell activation. Some of the activated B cells directly differentiate to IgM producing plasma cells. Most B cells undergo a germinal center reaction, which is T-cell dependent, and differentiate to antibody-producing plasma cells or memory B cells.

affinity BCR will receive survival and proliferation signals, while B cells with a low-affinity BCR will undergo apoptosis^{16,17}. In the GC-light zone, B cell may also undergo immunoglobulin class-switch recombination (CSR), changing the immunoglobulin isotype. For example, IgM positive B cells, which have a low antigen affinity and are normally at the beginning of an infection, can switch to high-affinity IgG B cells which are normally generated later during infection. The CSR allows B cells to produce diverse isotypes of antibodies, *i.e.* IgM, IgG, IgA, and IgE. The different immunoglobulin isotypes have distinct properties and functions^{9,18}. After class-switch recombination, the centrocytes in the GC can differentiate to either memory B cells or antibody-producing plasma cells. Upon a secondary infection, memory B cells rapidly differentiate to plasma cells, which assures a quick response against the infection. After differentiation to plasma cells, some of the plasma cells home to the BM to become long-lived plasma cells. The survival of these long-lived plasma cells is independent of antigen, due to loss of BCR expression. Instead, they require survival signals from the BM microenvironment^{19,20}.

2. Multiple Myeloma

Multiple Myeloma (MM) is a hematologic malignancy characterized by a clonal expansion of malignant plasma cells in the BM. It is the second most common hematologic malignancy²¹. Despite the fact that major progress has been made over the past two decades, MM still is largely incurable. Myeloma can affect many organs, and the symptoms vary greatly among different patients. The common symptoms of MM are summarized as CRAB (C: hypercalcemia, R: renal failure, A: anemia, B: bone lesions)²². On the basis of the chromosomal studies, MM can be classified into two groups: the hyperdiploid group and the non-hyperdiploid group. Approximately half of MM are hyperdiploid, usually with extra copies of the odd-numbered chromosomes (3,5,7,9,11,15,19, and 21). In the non-hyperdiploid MM group, most MMs carry gene translocations involving the immunoglobulin heavy-chain locus on chromosome 14q32. Commonly translocated genes are *CCND1*, *CCND3*, *MAFC*, *MAFB* and *NSD2* (MMSET) / *FGFR3*²³. Generally, MM with a primary immunoglobulin translocation has a worse prognosis than hyperdiploid MMs^{24,25}. Genetic alterations associated with disease progression in MM include *MYC* dysregulation, mutations in *KRAS*, *NRAS*, *TP53*, *BRAF*, *CCND1*, *LTB*, *IRF4*, and *FAM46C*^{26,27}. Most MMs are preceded by a premalignant disease, named monoclonal gammopathy of undetermined significance (MGUS)^{28,29}. Approximately 1% of MGUS patients progress to MM per year. MGUS patients generally have no obvious symptoms; they mostly are diagnosed during routine laboratory blood tests. Despite the presence of a variety of chromosomal aberrations, translocations, and mutations in essential growth control genes, oncogenomic studies have identified only a few differences distinguishing MGUS from MM³⁰⁻³². As a matter of fact, it was shown that the genetic difference between MGUS and MM are much smaller than the differences between healthy plasma

cells and MGUS³³. In addition, whole-exome sequencing analysis of paired MGUS–MM samples demonstrated that most somatic mutations are already present at the MGUS stage^{27,34}. This suggests extrinsic factors in the BM microenvironment also play an important role in progression to MM^{27,35}.

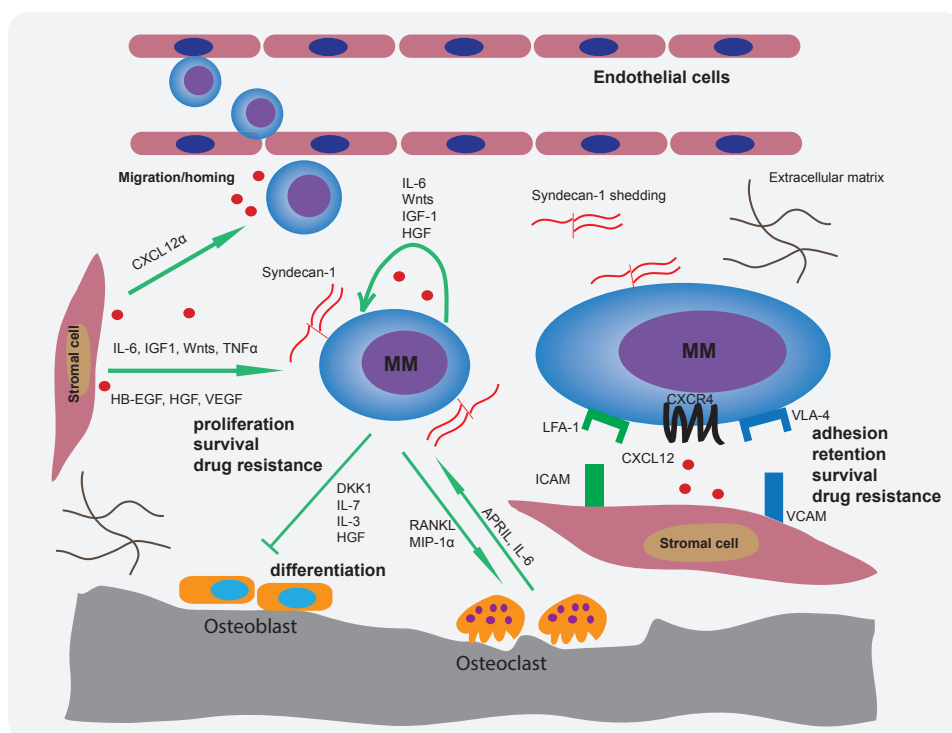


Figure 2. A schematic overview of the interaction between MM cell and the BM niche. BMSCs secrete high level of CXCL12, which induces MM cells migration/homing to the BM. In BM, the niche cells including BMSCs, osteoblasts, osteoclasts and MM cells themselves produce many growth factors and cytokines, which play an important role in MM proliferation, survival and drug resistance. Many of these soluble factors can bind to the heparan sulfate chains of syndecan-1 on the MM cell surface. In addition, MM cells can interact with the BM niche via direct contact. CXCL12/CXCR4 induced integrin-dependent adhesion to BMSC and extracellular matrix plays an important role in MM BM retention, survival and drug resistance. Besides, MM cells produce factors that induce osteolytic bone disease by inhibiting osteoblast differentiation and stimulating osteoclast activity.

3. Multiple Myeloma bone marrow microenvironment and drug resistance

Numerous studies have shown that MM cells, similar to the normal long-lived plasma cells, are highly dependent on the BM niche for survival and proliferation. The lack of unequivocal genetic difference between MM and MGUS further underscores the crucial role of BM niche in MM progression and proliferation. The MM BM niche is composed of many different cell types including MM cells, hematopoietic cells, bone marrow stromal cells (BMSCs), endo-

thelial cells, osteoclasts, osteoblasts, myeloid cells, granulocytes, and megakaryocytes³⁵⁻³⁷. The niche cells and MM tumor cells secrete a large variety of soluble factors including cytokines, chemokines, and growth factors, such as IL-6, insulin-like growth factor (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), Wnts, heparin-binding epidermal growth factor (HB-EGF), and a proliferation-inducing ligand (APRIL), which have been shown to promote MM survival and/or proliferation³⁸⁻⁴⁵. The HSPG syndecan-1 which is highly expressed on MM cell surface can bind to many of these factors and thereby plays an important role in the interaction of MM cells with BM niche^{36,46}.

Apart from these soluble factors produced by niche cells, MM cells can also interact with the BM microenvironment via the direct contact. MM cells express high-level integrins $\alpha 4\beta 1$ (also known as VLA4) and $\alpha L\beta 2$ (also known as LFA-1)^{47,48}. Integrins are heterodimeric transmembrane receptors, which functions as the major cellular receptors for extracellular matrix proteins such as fibronectin and collagen, to mediate cell-extracellular matrix adhesion^{49,50}. In addition, integrins can also bind to the immunoglobulin-superfamily member adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1), which are highly expressed on BMSC^{51,52}. Cell-cell contact between BMSCs and myeloma cells is mediated by integrin/VCAM-1 and/or ICAM-1 dependent adhesion⁵³⁻⁵⁵.

Over the past two decades, the prognosis of MM has been greatly improved, particularly by the introduction of proteasome inhibitors (bortezomib and carfilzomib), immunomodulatory agents (thalidomide and lenalidomide), and autologous stem-cell transplantation. Bortezomib is a selective and potent inhibitor of the 26S proteasome⁵⁶, a large protease complex responsible for the degradation of ubiquitinated proteins. By blocking the proteolysis normally performed by the proteasome, bortezomib induces cell-cycle arrest and apoptosis in MM cells^{57,58}. Given the fact that I κ B α is a substrate of the proteasome, bortezomib-induced cell death in MM was initially attributed to inhibition of NF- κ B signaling, which has been shown to increase MM cell survival^{59,60}. However, PS-1145, a specific I κ B kinase inhibitor, showed much less cytotoxicity effect than bortezomib⁶¹, suggesting that inhibition of NF- κ B signaling cannot explain the potent and selective effect of bortezomib in MM. Instead, malignant plasma cells produce extremely high levels of immunoglobulin protein and this continues production subjects MM cells to high levels of unfolded proteins, causing strong ER stress^{62,63}, explaining the high and selective sensitivity of MM to proteasome inhibition.

Despite the significant impact of these novel therapies on MM, there still are many patients who do not benefit from these treatments due to either primary resistance or relapse. However, the molecular mechanism underlying drug resistance in MM remains elusive^{64,65}. To investigate the mechanism of bortezomib drug resistance in MM, Oerlemans *et al.* have developed bortezomib resistance MM cells by culturing MM cell lines in stepwise

increasing concentrations of bortezomib. They found that bortezomib resistance is associated with selective overexpression of a mutant proteasome beta5-subunit protein⁶⁶. However, this mutation has thus far not been found in MM cells from bortezomib resistant patients⁶⁷.

As discussed previously, the BM microenvironment plays an important role in MM-cell survival. Previous studies have shown that various growth factors and cytokines in the BM, such as IL-6, IGF-1, IL-8, and HGF, are involved in bortezomib resistance of MM⁶⁷⁻⁷¹. This growth factors and cytokine-induced drug resistance is called soluble factor mediated-drug resistance (SFM-DR)^{72,73}. In addition, the interaction of MM cells with the extracellular matrix and with BM niche cells through integrin adhesion molecules can also mediate drug resistance⁷⁴, which is called cell adhesion mediated drug resistance (CAM-DR)^{72,73}. Targeting the interaction between MM cells and the BM microenvironment has been suggested as a promising strategy for the treatment of MM^{74,75}.

Heparan sulfate proteoglycans

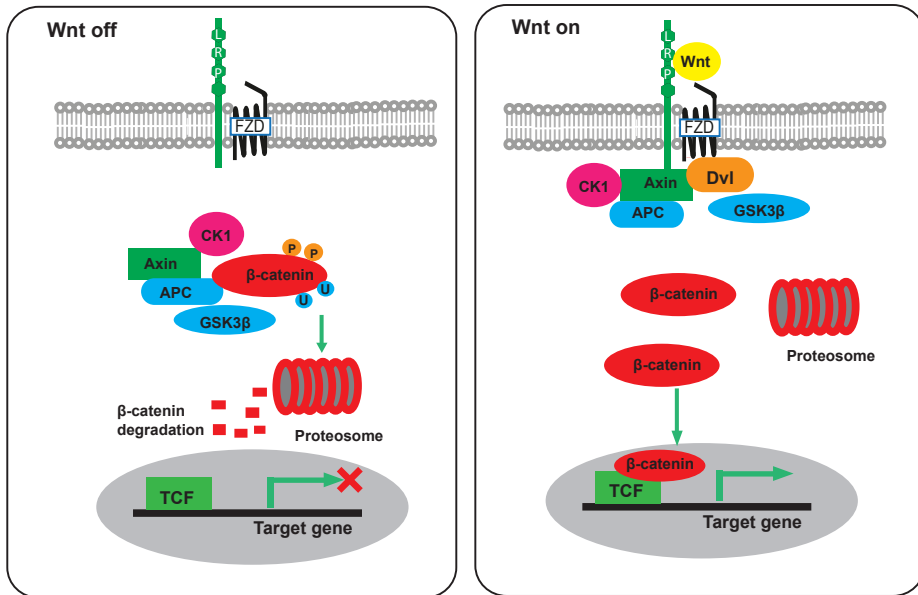
Heparan sulfate proteoglycans (HSPGs) are a class of extracellular matrix or cell-membrane-bound glycoproteins consisting of a protein core with covalently attached heparan sulfate (HS) glycosaminoglycan (GAG) chains, which in the native form contain alternating N-acetylated glucosamine (GlcNAc) and D-glucuronic acid (GlcA)^{36,76-78}. Due to their unique capacity to regulate many growth factor-mediated signaling pathways, HSPGs have emerged as key molecules in a variety of cellular processes, thereby affecting many physiological and pathological processes such as angiogenesis, fibrosis, immunity, cancer, and infectious diseases^{79,80,81}. For more details about HSPG structure, biosynthesis, modification, and functions, see chapter 5.

Wnt/ β -catenin signaling

Wnt signaling is one of the crucial signaling pathways regulating development, homeostasis, and disease. Wnt signaling was first implicated in cancer by the discovery of Wnt1 (previously known as int1) as a locus associated with mouse mammary tumor virus driven tumorigenesis⁸². In humans, there are 19 genes that encode Wnts⁸³. These ligands couple to various receptors and co-receptors and thereby activate different downstream pathways. These pathways have been classified into three categories: the Wnt/ β -catenin signaling pathway (canonical Wnt pathway), the Wnt/planar cell polarity signaling pathway, and the Wnt/calcium signaling pathway. Wnt/ β -catenin signaling is the best-characterized Wnt pathway. Briefly, Wnt/ β -catenin pathway activation starts with binding of a Wnt ligand to its receptor Frizzled (FZD), in complex with the co-receptors LRP5/6, which activate the Dishevelled (Dvl) proteins. Then Dvl binds to C-terminus of FZD and recruits Axin from the β -catenin destruction complex, which mainly consists of Axin, adenomatous polyposis (APC), casein kinase 1 α (CK1 α) and glycogen synthase kinase-3 β (GSK-3 β). As a result, the β -catenin de-

struction complex cannot be formed, causing an accumulation of β -catenin in the cytoplasm and, eventually, translocation into the nucleus⁹⁴. In the nucleus, β -catenin, in cooperation with T cell factor/lymphoid enhancing factor (TCF/LEF) family transcription factors, induces

A



B

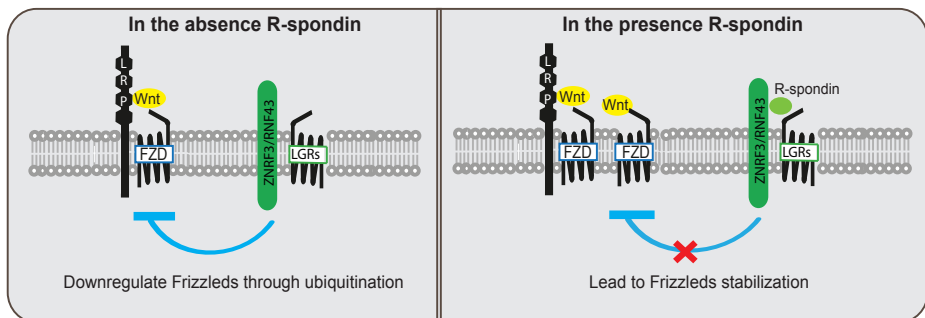


Figure 3 A. Canonical Wnt signaling. (Left) In the absence of Wnt, a destruction complex consisting of CK1, Axin, APC, and GSK3 β binds to β -catenin. β -catenin is then phosphorylated and ubiquitinated. Subsequently, β -catenin is degraded by the proteasome. In the nucleus, T cell factor (TCF) is in an inactive state, and Wnt target genes are not expressed. (Right) In the presence of Wnt, binding to its receptors induces the association of Axin with lipoprotein receptor-related protein (LRP), which leads to the disruption of β -catenin destruction complex. Consequently, β -catenin can no longer be degraded, instead, will accumulate and, subsequently, translocate to the nucleus. In the nucleus, β -catenin binds to TCF, promoting target genes expression. **B. The mechanism of action of R-spondin in Wnt signaling.** Wnt ligands bind to the Frizzled (FZD) and LRP receptors, activating downstream signaling. In the absence of R-spondin, ZNRF3 and RNF43, which are membrane ubiquitin ligases, continually ubiquitinate FZD, leading to down-regulation of FZD. In the presence of R-spondin, R-spondin binds to the LGR4/5 receptor relieves ZNRF3 and RNF43 activity, leading to FZD stabilization thereby enhanced Wnt signaling activation.

target gene transcription^{85,86}, including transcription of c-Myc and Cyclin D1, which play crucial roles in the pathogenesis of many cancer^{87,88}. By contrast, in the absence of Wnts, the β -catenin destruction complex targets β -catenin for phosphorylation, ubiquitination, and proteasome degradation⁸⁴ (Figure 3A). Over the past decades, our understanding of Wnt/ β -catenin signaling has increased enormously by the identification of cell surface receptors and co-receptors for Wnts, various secreted antagonists, including Dickkopf-related protein 1 (DKK1), Sclerostin, Cerberus and secreted Frizzled-related proteins (sFRPs), and Wnt agonists such as the R-spondin family and Norrin⁸⁹. Notably, R-spondin family proteins have been identified as important positive regulator of Wnt signaling in stem cells and many cancers^{90,91}. R-spondins act through their receptors leucine-rich repeat-containing G-protein coupled receptors (LGRs). Binding of R-spondins to LGR4 or LGR5 alleviates the inhibitory effect of two homologous membrane-bound E3 ubiquitin-ligases RNF43 and ZNRF3, which ubiquitinate and thus degrade the FZD receptor from the cell surface^{90,92-95} (Figure 3B). R-spondins can act as an oncogene and have tumorigenic properties^{96,97}. Therapeutic targeting of tumor-derived R-spondin attenuates Wnt/ β -catenin signaling-dependent tumorigenesis in many cancers^{96,98}.

Aberrant Wnt/ β -catenin signaling activation in cancer usually results from mutations in *APC*, β -catenin (*CTNNB1*), or *Axin*, which drive constitutive, ligand-independent pathway activation⁹⁹. However, in some cancers such as MM and breast cancer, such mutations are rare. The activation of Wnt signaling in these cancer involves autocrine and/or paracrine Wnts emanating from the tumor microenvironment^{100,101}. In addition, oncogenic Wnt pathway activity in MM involves epigenetic silencing of the feedback inhibitors DKK1 and sFRPs^{102,103}, aberrant expression of the transcriptional co-activator BCL9¹⁰⁴, and mutational inactivation of CYLD, a deubiquitinating enzyme that negatively regulates Wnt¹⁰⁵.

The roles of heparan sulfate proteoglycans in Wnt signaling

HSPGs play an important role in a wide range of biological processes, including development, angiogenesis, and cancer^{106,107}, regulating a variety of growth factor signaling pathways. The first evidence for the involvement of HSPGs in Wnt signaling came from a study by Bradley and Brown (1990), which showed that Wnt-1 can bind heparin *in vitro*¹⁰⁸. Model organisms, including *Drosophila* and *Xenopus*, have been used to further explore the roles of HSPGs and their synthesis and modification enzymes in Wnt signaling. In *Drosophila*, the abrogation of HS by mutation of exostin (EXT) family genes, which are named tout velout (tv) and sister of tout velout (stv) in *Drosophila*, leads to reduced Wnt signaling and loss of Wnt target gene expression¹⁰⁹. In *Xenopus*, glypican 4, which is a major member of HSPG family, mediates Wnt signaling by physically interact with Wnt11 during gastrulation¹¹⁰ and axis formation¹¹¹, while in mice, HS increases the cell-surface binding affinity of Wnt ligands to intestinal epithelium, thereby promoting activation of canonical Wnt signaling and facili-

tating regeneration of small intestinal crypts after epithelial injury¹¹². Furthermore, HSPGs mediate Wnt signaling during in embryonic stem cell self-renewal and regulating pluripotency¹¹³.

The first study indicating that HSPGs play a role in Wnt signaling-mediated cancer was done by Alexander *et al*, who showed that syndecan-1 is required for Wnt-1-induced mammary tumorigenesis. Syndecan-1 deficient mice were found to be resistant to Wnt-1-induced mammary gland tumorigenesis. In addition, *in vitro* studies demonstrated that soluble syndecan-1 ectodomain strongly stimulates Wnt pathway activation. These results demonstrate that HSPGs stimulates Wnt signaling pathway, and this stimulation effect is HS-dependent¹¹⁴. Glypican-3 (GPC3), a member of glypican family proteins, is expressed by most hepatocellular carcinomas. GPC3 promotes the growth of hepatocellular carcinomas by stimulating Wnt/ β -catenin signaling, an effect attributed to the ability of GPC3 to increase the binding of Wnts to their FZD receptors^{115,116}.

The mechanisms by which HSPG regulates Wnt-signaling have been explored in several studies. Apart from direct interaction between Wnt proteins and HSPGs at the cell surface to increase the concentration of Wnt proteins and their access to specific receptors^{112,117}, HSPGs can stabilize Wnt protein activity in the extracellular matrix¹¹⁸. Wnts are secreted proteins, which are modified by the addition of palmitic acid on the first conserved cysteine and palmitoleic acid on a highly conserved serine residue^{119,120}. These modifications render Wnt proteins hydrophobic, which causes them to aggregate in the extracellular environment and prevents them to diffuse freely to cells distant from their site of production. The hydrophobicity also is the reason why CHAPS has to be added to Wnt proteins to prevent the aggregation *in vitro*, thereby maintaining their biological activity^{118,121,122}. Fuerer *et al.* have demonstrated that binding of Wnt3a to HSPGs can prevent its aggregation that normally occurs in an aqueous environment and thereby maintaining the biological activity of Wnt3a¹¹⁸.

CXCL12/CXCR4 signaling

Chemokines are a family of small cytokines (8–14 kDa) secreted by many cell types. Chemokines induce migration of leukocytes to sites of inflammation and hence play a crucial role in the immune system. In addition, some chemokines are also involved in hematopoiesis, development, and cancer^{123,124}. To date, more than 40 different chemokines have been identified in humans. They can be classified into four main subfamilies: CXC-chemokines, CC-chemokines, CX3C-chemokines, and C-chemokines^{125,126}. CC chemokines can induce migration of monocytes, dendritic cells, NK cells, T cells, and B cells¹²⁷. CXC-chemokines are further sub-divided to ELR- and ELR+ chemokines, based on the absence or presence of the tripeptide glutamic acid-leucine-arginine (ELR motif) preceding the CXC domain. ELR+ chemokines induce migration of neutrophils via chemokine receptors CXCR1 and CXCR2. ELR-

chemokines, such as CXCL13 and CXCL12, are more like to be chemoattractants for lymphocytes^{127,128}. All of these chemokine ligands exert their biological functions by interacting with chemokine receptors which are seven transmembrane G-protein-coupled receptors (GPCR). Thus far, 19 human chemokine receptors have been identified¹²⁵. Upon binding to their cognate receptors, chemokines can trigger a rapid change in the receptor conformation, which leads to the separation of the intracellular subunits G α i and G β γ , resulting in the activation of downstream signaling^{123,125,127}.

CXCL12/CXCR4 signaling is the best studied chemokine pathway. CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a potent chemotactic factor for many immune cells and tumor cells. The role of CXCL12 in lymphocyte trafficking is well established. Its cognate receptor CXCR4 is widely expressed by many cell types including lymphocytes, monocytes, and CD34+ hematopoietic progenitor cells^{129,130}. CXCR4 is well known for its important role in HIV entry in T cells¹²⁹. In mice, knockout of either CXCL12 or CXCR4 is embryonic lethal, due to the defects in heart and brain development¹³¹. CXCL12 is highly expressed by BMSCs and play an important role in maintaining hematopoietic progenitor cells in BM¹³². As discussed previously, after B cells differentiate to plasma cells, CXCL12 induces plasma cells to migrate to the BM, where they give rise to long-lived plasma cells. CXCL12/CXCR4 signaling is also involved in many types of cancer, including several solid tumors and hematological malignancies such as MM, B cell chronic lymphocytic leukemia (CLL), B cell acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML), which all express functional CXCR4 receptor^{131,133}. CXCL12 secreted by BMSCs or lymphoid tissues induces migration of these malignant cells to the BM or to lymphoid tissues, where they receive survival signals from the niche cells¹³³.

Apart from inducing MM cell homing to the BM, CXCL12 also modulates integrin-mediated MM cell adhesion to the ECM protein fibronectin as well as to VCAM-1 and ICAM-1, which are highly expressed on BMSCs. This adhesion can be inhibited by pertussis toxin, indicating the involvement of receptor-linked G α i protein downstream signaling⁵⁴. Pharmacological targeting of CXCR4 has been reported by many studies. The small molecular inhibitor AMD3100 (also known as plerixafor) was developed to specifically targeting CXCR4¹³⁴. In clinical practice, AMD3100 in combination with granulocyte-colony stimulating factor (G-CSF), is used to mobilize hematopoietic stem cells from BM to peripheral blood, which enables safe and effective collection of stem cells for transplantation^{135,136}. In MM, AMD3100 inhibits CXCL12 induced cell adhesion and migration^{74,137}. Previous studies have shown that inhibiting CXCR4 by AMD3100 disrupt the interaction between MM cells and BMSCs, resulting in enhanced MM sensitivity to therapies^{35,74,75}.

Due to alternative splicing, 6 CXCL12 isoforms can be generated, *i.e.*, CXCL12 α , CXCL12 β , CXCL12 γ , CXCL12 δ , CXCL12 ϵ , and CXCL12 ϕ ¹³⁸ (figure 4). To date, almost all *in vitro* functional studies, including studies on MM adhesion and migration, have exclusively used

the CXCL12 α isoform. Furthermore, *in vivo* functional studies do not allow discrimination between the different isoforms, since the KO mice reported either had a total deletion of CXCL12 or a deletion of CXCR4, which is the cognate receptor for all the CXCL12 isoforms. CXCL12 α contains three exons and encodes an 89 amino acid protein. Other alternative CXCL12 splice variants all share the same first three exons with CXCL12 α and differ in the fourth exon^{138,139}. CXCL12 β is highly similar to CXCL12 α , but has a fourth exon consisting of four amino acid residues attached to a C-terminus, and shows similar biological activity both *in vitro* and *in tissues*¹³⁹. CXCL12 γ differs from other CXCL12 isoforms by a fourth exon which is highly enriched in basic amino acids, containing 4 overlapped BBXB HS binding motifs. This unique structure causes CXCL12 γ to bind to HSPGs with an unprecedentedly high affinity^{140,141}. Interestingly, CXCL12 γ has been shown to promote *in vivo* leukocyte accumulation and angiogenesis with a much higher efficiency than CXCL12 α . In murine BM, CXCL12 γ was reported to be the dominant CXCL12 isoform^{140,142}. The expression and roles of CXCL12 γ in the human BM are currently unknown.

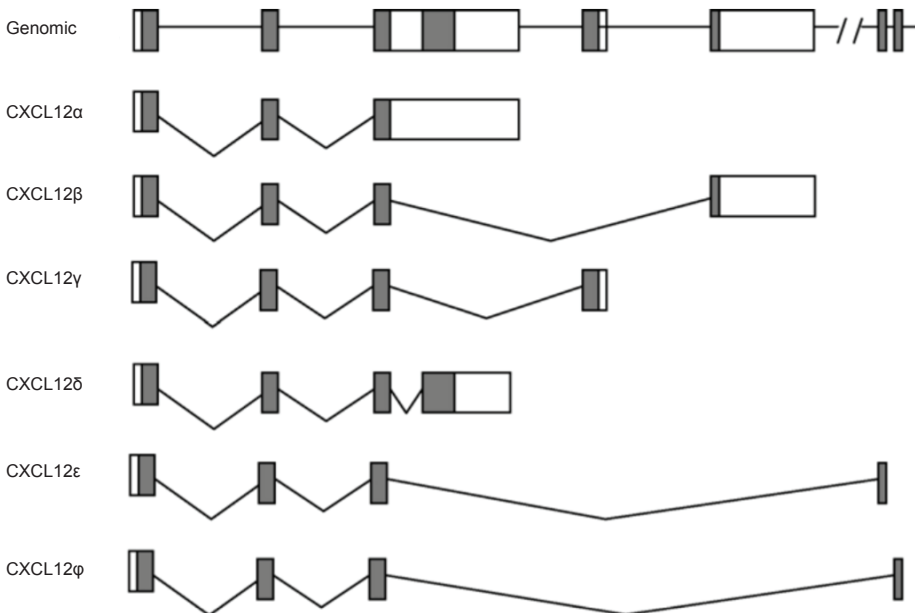


Figure 4. A schematic overview of the different CXCL12 isoforms. Boxes represent exons. The coding region is indicated in black boxes. Lines represent introns. All six isoforms share the same first three exons. (Adapted and modified from Yu et al. 2006, Gene)

Aim and outline of this thesis

MM cells are highly dependent on the BM microenvironment for survival and proliferation. The aim of the studies described in this thesis was to investigate the role of HSPGs in the interaction of MM with the BM niche.

Previously, we and others have shown that aberrant Wnt signaling activation plays an important role in MM proliferation. However, classical Wnt pathway mutations are rare in MM, indicating the aberrant activation of Wnt signaling in MM is mainly ligand-dependent. Recently, R-spondins have emerged as a positive regulator of Wnt signaling. **In chapter 2**, we show that LGR4, a cognate receptor for R-spondins, is expressed in primary MM cells and human MM cell lines, but not in normal plasma cells or their B cell precursors. This aberrant expression of LGR4 allows MM cells to respond to R-spondins, which are secreted by osteoblasts in the BM niche, resulting in a dramatically enhanced sensitivity to autocrine and paracrine Wnts, inducing Wnt signaling activation. MM cells are characterized by expressing a high level of cell surface HSPG syndecan-1. **In chapter 3**, we investigated the role of the HSPG syndecan-1 in Wnt signaling in MM. We show HS-deficient MM cells display a strongly decreased autocrine Wnt pathway activity. Inducible knockdown of EXT1, a co-polymerase of HS, inhibits Wnt signaling-dependent MM cell growth. In addition, we demonstrate that loss of HS also impairs Wnt pathway activation induced by paracrine Wnts and R-spondins. Mechanistically, we demonstrated HS chains decorating syndecan-1 bind Wnts and R-spondins to promote aberrant Wnt signaling activation and cell growth in MM. **In chapter 4**, we describe an important role for the BMSC-derived CXCL12 γ isoform and HSPGs in the interaction of MM with BMSCs. We show that CXCL12 γ is expressed *in situ* by stromal cells in both the normal and MM bone marrow microenvironment. Importantly, upon secretion, CXCL12 γ is retained on the surface of BMSCs by HSPGs. Functionally, deletion of the CXCL12 γ or of HS causes a strongly reduced MM cell adhesion and impaired BMSC-mediated protection of MM cells from bortezomib-induced cell death. **In chapter 5**, we review the important roles of HSPGs in the interaction of normal and malignant plasma cells and their bone marrow niche. In addition, we discuss the current therapeutic strategies and clinical trials, targeting HSPGs and their modification enzymes. Lastly, **chapter 6** summarized and briefly discussed the results presented in this thesis.

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