Abstract

Wnt proteins are known to signal via canonical β-catenin-mediated and non-canonical β-catenin-independent signaling pathways and are involved in various developmental processes. Both Wnt signaling pathways are involved in hematopoietic stem cell (HSC) maintenance, but how they regulate quiescence, proliferation and differentiation is not yet fully understood. In this thesis, I aim to gain insight in the role of Wnt signaling in HSC regulation.

HSCs have the capacity to self-renew, but can also be induced to differentiate into all cells of the hematopoietic compartment. Wnt proteins are secreted by the stromal cells that make up the bone marrow stem cell niche, but are also produced by HSCs themselves. Studies on how Wnt signaling relates to HSC function however yielded contrasting results, with the majority of evidence pointing to a role for Wnt signaling in proliferation and self-renewal. Surprisingly, deletion of β-catenin has no effect on HSC maintenance. In contrast, overexpression of β-catenin results in an increase of HSC proliferation, while stem cells retain an undifferentiated character. Deletion of Wnt3a impairs the self-renewal capacity of HSCs *in vivo* and consequently, overexpression of Wnt3a results in an increase of proliferation and a block in differentiation. Enforced activation of Wnt signaling pathways, achieved for example by inhibition of GSK-3β or deletion of Apc, results in expansion of the stem cell pool. Non-canonical Wnt signaling via Wnt5a or Wnt4 induces proliferation or provides a signal for quiescence, depending on the context in which signaling occurs.

Together, Wnt signaling appears to play an essential role in regulation of the hematopoietic stem cell pool. Most Wnt signaling pathways seem to promote self-renewal, as they induce proliferation and inhibit differentiation of HSCs. Therefore, Wnt signaling is a crucial factor in the maintenance of this adult stem cell pool.

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Introduction

Wnt signaling pathways

Wnt proteins

Wnt proteins are secreted lipid-modified glycosylated signaling molecules that are essential in various developmental processes. Wnt proteins contain an N-terminal signal sequence and are palmitoylated on a conserved cysteine residue. The palmitate is added in the endoplasmatic reticulum by the protein Porcupine (Porc) and is essential for signaling. Wnt proteins are morphogens; signaling is dependent on concentration gradients of the Wnt molecules. Wnt signaling pathways are not only involved in cell proliferation and tissue expansion, but also in stem cell maintenance, cell-fate determination and cell migration. In addition, abnormalities in the Wnt signaling cascade are notorious in certain types of cancer for causing aberrant proliferation.

The first Wnt protein was identified as a segment-polarity determinant in *Drosophila Melanogaster* and named Wingless (Wg). The mouse homologue was found to be a proto-oncogene and named Integrase-1 (Int1). Contraction of these two names eventually led to the term Wnt. So far 19 genes have been identified in the human and murine genome that all encode for Wnt proteins. Wnt proteins predominantly bind to and activate Frizzled (Fz) receptors; up to date ten different Fz receptors are identified (Clevers, 2006; Logan and Nusse, 2004; Staal et al., 2008).

Wnt/β-catenin-mediated signaling pathway

Multiple Wnt signaling pathways have been identified, of which the canonical Wnt/β-catenin-mediated signaling pathway is the best understood. β-catenin is the key player in this pathway and is degraded by the proteasome in the absence of an activating Wnt signal (*figure 1a*). The E3-ubiquitin-ligase β-transducin-repeat-containing protein (βTRCP) targets β-catenin for proteasomal destruction, but only recognizes its substrate in a phosphorylated state. β-catenin is phosphorylated by the destruction complex, which is composed of at least the Axis inhibition protein 1 (Axin1), adenomatous polyposis coli (Apc), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β). Axin1 interacts directly with all the other members of the destruction complex and functions as a scaffold protein. The two serine/threonine kinases CK1 and GSK3β phosphorylate β-catenin, which is then ubiquitinylated by βTRCP and degraded by the proteasome.

Signals induced by Wnt proteins interrupt the formation of the degradation complex, thereby preventing the phosphorylation and destruction of β-catenin (*figure 1b*). Wnt proteins bind to the extracellular N-terminal cysteine-rich domain of the Frizzled (Fz) receptor, which is in a complex with the low density lipoprotein receptor-related protein 5 or 6 (LRP5/6). After an activating Wnt signal the protein Dishevelled (Dvl) is recruited to the receptor complex and the cytoplasmic tail of LRP5/6 is phosphorylated by CK1 and GSK3β. This provides a docking site for Axin1, which is then recruited to the receptor complex. Axin1 is sequestered and assembly of the destruction complex is disrupted. β-catenin will accumulate in the cytoplasm and translocate to the nucleus, where it initiates transcription by activating T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors. In the absence of β-catenin TCF forms a transcriptional repressor complex with Groucho. Groucho is physically displaced by β-catenin and Pygopus and Legless are recruited to assemble a transcriptional activator complex (Clevers, 2006; Logan and Nusse, 2004; MacDonald et al., 2007; Mosimann et al., 2009; Staal and Clevers, 2005; Staal et al., 2008).

Modulators of Wnt/β-catenin-mediated signaling

The Wnt/β-catenin-mediated signaling pathway is negatively regulated by a number of inhibitory molecules that act at multiple levels in the signaling cascade, enhancing complexity of the pathway. There are multiple secreted extracellular soluble factors that are thought to inhibit Wnt signaling. Secreted Frizzled-related proteins (SFRPs) and Wnt Inhibitory Factors (WIFs) both contain parts that resemble the ligand binding domain of Frizzled receptors. They are able to directly bind Wnt proteins, thereby preventing activation of the receptor. Other extracellular factors do not act on Wnt proteins but on LRP5/6. For example, SOST and Wise both bind the LRP5/6 co-receptor and inhibit downstream signaling. The inhibitor Dickkopf (Dkk) crosslinks LRP5/6 with Kremen, leading to internalization and inactivation of the co-receptor (Staal and Clevers, 2005; Staal et al., 2008).

Other factors that are able to modulate Wnt signaling are present inside the cell. Protein Phosphatase 2 (PP2A) can be recruited to the destruction complex, where it is responsible for the

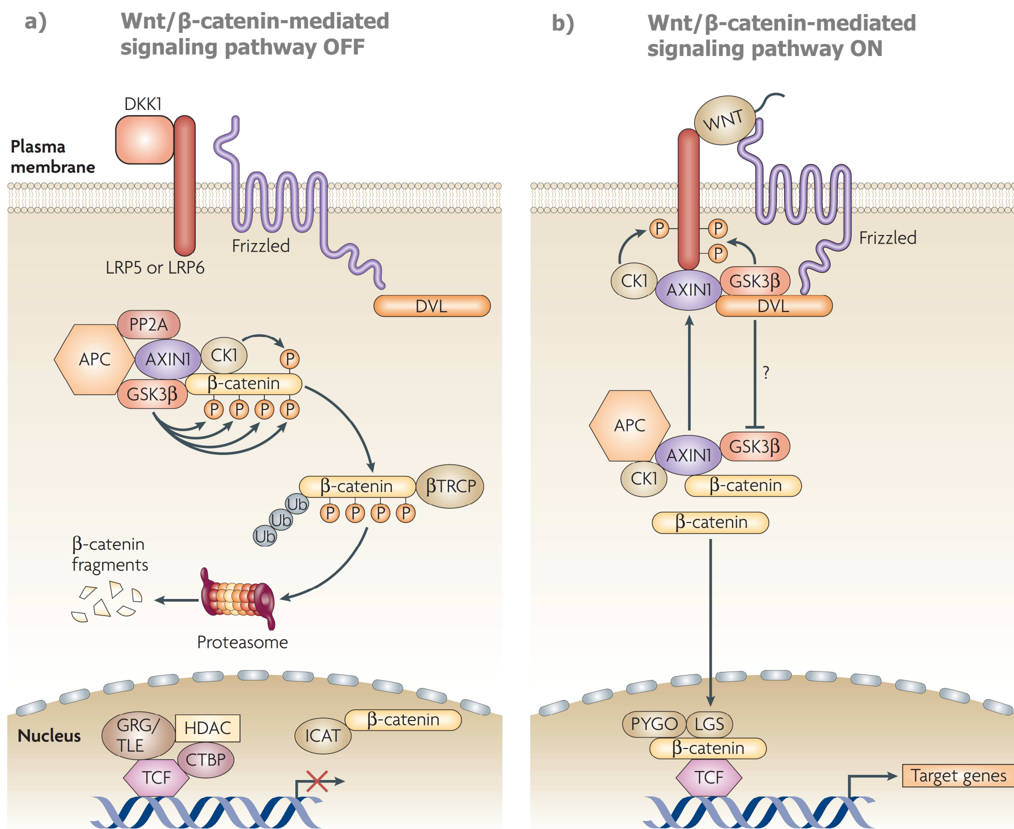

Figure 1: The canonical Wnt/β-catenin-mediated signaling pathway. ***a)*** *In the absence of a Wnt signal the β-catenin protein is phosphorylated by the destruction complex. It is then recognized and ubiquitinylated by βTRCP, targeting β-catenin for proteasomal destruction.* ***b)*** *When a Wnt protein binds the cysteine rich domain of a Frizzled receptor, formation of the destruction complex is disrupted. β-catenin is no longer degraded and accumulates in the cytoplasm. It translocates to the nucleus where it initiates transcription of target genes.* Figures derived from Staal *et al.* {{15 Staal,F.J.T. 2008;}}.

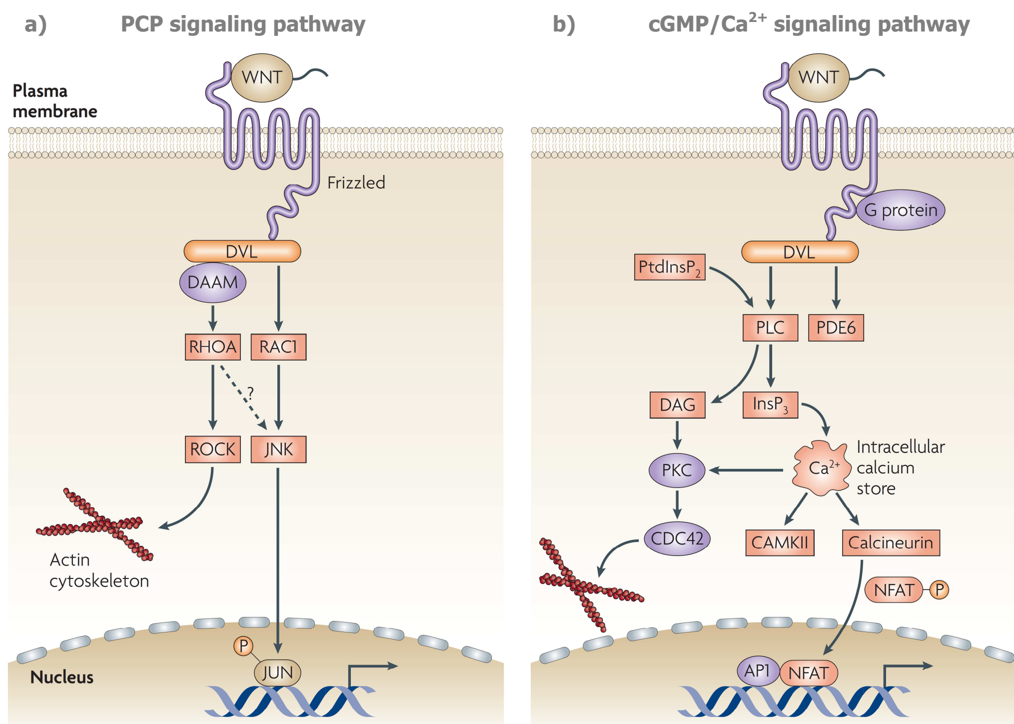

Figure 2: The PCP and cGMP/Ca^{2+} non-canonical signaling pathways. ***a)*** *Signaling via the planar cell polarity (PCP) signaling pathway is independent of β-catenin. Binding of Wnt11 or Wnt5a to a Frizzled receptor activates RHOA and RAC1, leading to cytoskeleton rearrangement and activation of the JNK pathway.* ***b)*** *Activation of the Frizzled receptor results in elevated levels of intracellular calcium, resulting in cytoskeleton rearrangement. Activation of Calcineurin in turn activates NFAT transcription factors and target genes are expressed.* Figures derived from Staal *et al.* {{15 Staal,F.J.T. 2008;}}.

dephosphorylation of β-catenin. In this manner, it regulates β-catenin stability as an antagonist of CK1 and GSK3β. Inside the nucleus inhibitor of β-catenin and TCF (ICAT) and Chibby bind directly to β-catenin and thereby prevent the formation of transcriptional activator complexes. NLK/Nemo decreases the DNA binding affinity of TCF transcription factors and thereby inhibits transcription. Besides these extra- and intracellular inhibitors, there are many other factors that modulate the canonical signaling pathway. Interestingly, not only Wnt proteins activate canonical signaling through Frizzled receptors. The cysteine-knot protein Norrin and R-spondins, proteins with a thrombospondin domain, are able to activate β-catenin signaling in a Fz-dependent manner (Staal and Clevers, 2005; Staal et al., 2008).

PCP and cGMP/Ca^{2+} signaling pathways

Besides the canonical β-catenin-mediated signaling pathway more than 10 non-canonical Wnt signaling pathways have been proposed, of which the planar cell polarity (PCP) pathway and the cGMP/Ca^{2+} pathway are the best described (Semenov et al., 2007). Signaling via these pathways is β-catenin-independent and does not involve LRP5/6. The planar cell polarity signaling pathway is presumably triggered by binding of Wnt11 or Wnt5a to the Frizzled receptor. Dishevelled (Dvl) is activated and assembles into a complex with DAAM (Dishevelled-associated activator of morphogenesis) and the G-protein RHOA (RAS homologue gene-family member A). This complex is responsible for the activation of ROCK (RHO-associated coiled-coil-containing protein kinase), resulting in actin cytoskeleton rearrangement. Dvl also activates the small GTPase RAC1 (RAS-related C3 botulinum toxin substrate 1), resulting in activation of the c-Jun N-terminal kinase (JNK) pathway. Interestingly, the non-canonical PCP signaling pathway seems to downregulate β-catenin expression, thereby inhibiting canonical β-catenin dependent signaling (*figure 2a*) (Kokolus and Nemeth, 2009; Malhotra and Kincade, 2009; Semenov et al., 2007; Staal et al., 2008).

The Wnt-cGMP/Ca^{2+} pathway can act via Wnt5a and Frizzled 2, and possibly Knypek or ROR2 co-receptors are involved. The Frizzled receptor is thought to operate via heterotrimeric G proteins to activate phosphodiesterase (PDE) and p38 kinase. PDE activation results in a decline in the intracellular levels of cyclic guanosine monophosphate (cGMP). The cGMP-dependent protein kinase G (PKG) is therefore inactive and the intracellular Ca^{2+} concentration increases. In addition, phospholipase C (PLC) is activated. PLC converts phosphatidylinositol-4,5-biphosphate ($PtdIns(4,5)P_2$) to inositol triphosphate ($InsP_3$) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) directly; InsP3 activates PKC indirectly by increasing the intracellular Ca^{2+} concentration. PKC activation ultimately results in changes in the actin cytoskeleton. The heightened Ca^{2+} levels also activate the protein phosphatase Calcineurin, which in turn activates the NFAT (nuclear factor of activated T cells) transcription factor (*figure 2b*) (Kohn and Moon, 2005; Kokolus and Nemeth, 2009; Semenov et al., 2007; Staal et al., 2008; Wang and Malbon, 2003).

Hematopoietic stem cells

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) predominantly reside in the bone marrow, but may also be found in peripheral blood and cord blood. A true HSC is described by its potential to give rise to all hematopoietic cell lineages and its ability to self-renew, a property extremely important in the maintenance of this adult stem cell population. Hematopoietic stem cells are believed to be quiescent in order to prevent exhaustion of the stem cell pool, as well as to prevent the occurrence of malignant mutations. Bone marrow HSCs are known for their capability to differentiate into various types of cells through a process termed hematopoiesis. Hematopoiesis is essential in maintaining and repairing homeostasis of blood and immune cells and has often been depicted as a hierarchical binary tree. After hematopoietic stem cell division a differentiating daughter cell commits to either the myeloid or the lymphoid lineage. A common lymphoid progenitor is eventually able to give rise to B cells, T cells and NK cells. A common myeloid progenitor will give rise to myeloid cells such as monocytes, neutrophils and erythrocytes. However, recently more complex hierarchical tree models have been proposed (Ceredig et al., 2009; Kindt et al., 2007; Sonoda, 2008).

During embryogenesis primitive hematopoiesis starts in the yolk sac and aorta-gonad-mesonephros (AGM), that are both derived from the mesoderm. Hematopoietic stem cells eventually migrate to the fetal liver, the main site for embryonic hematopoiesis. In the liver HSCs actively self-renew and hematopoiesis is skewed towards the erythroid lineage (Kindt et al., 2007).

Identification

The most convincing method to identify true hematopoietic stem cells is to perform single-cell transplantation assays in lethally irradiated mice. Long-term (> 16 weeks) reconstitution of all hematopoietic lineages in the recipient after transplantation of a *single* purified bone marrow cell, is currently regarded as a hallmark of HSCs (Nemeth and Bodine, 2007; Wilson and Trumpp, 2006). Hematopoietic stem cells in bone marrow can be visualized by staining with a fluorescent DNA-binding dye such as Hoechst 33342 or Rhodamine 123. HSCs actively transport the dye out of the cell at a higher rate compared to other bone marrow cells, therefore they will show low fluorescence. Hematopoietic stem cells are further identified by cell surface marker expression (Lanza and Klimanskaya, 2009). Hematopoietic stem cells do not express any differentiation markers, such as CD4/8 (T cells), B220 (B cells), Mac-1 (monocytes) or Gr-1 (granulocytes). Therefore, they are described as lineage-negative (Lin^-). In addition, HSCs are believed to express high levels of stem cell antigen-1 (Sca-1) and c-Kit (CD117). LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) cells comprise about 0,1% of the whole bone marrow and all functional hematopoietic stem cells are found in this subset (Wilson and Trumpp, 2006). Although this population is highly enriched for functional HSCs, less than 10% of LSK cells are able to long-term repopulate the hematopoietic compartment of lethally irradiated mice. Additional subsets are identified based on expression of CD34 (antiadhesive sialomucin), CD135 (FLT3 receptor) and the signal lymphocyte activating molecule (SLAM) receptors CD150 and CD48 (*see table 1*) (Essers et al., 2009; Wilson and Trumpp, 2006; Wilson et al., 2007).

The HSC population seems to be composed of both a dormant CD34-negative and an activated CD34-positive subset. About 70% of $LSKCD34^-CD150^+48^-$ long-term (LT-)HSCs reside in the G0 phase, while less than 2% are actively cycling. These LT-HSCs appear to possess the highest potential for long-term reconstitution, but are not believed to contribute to homeostasis. LT-HSCs only divide once every 145 days and might be activated in response to hematopoietic stress, upon which CD34 expression is acquired. Only $LSKCD34^+CD150^+48^-$ short-term (ST-)HSCs appear to actively self-renew and divide about every 36 days (Essers et al., 2009; Wilson et al., 2008; Wilson et al., 2007). Unlike mouse HSCs, human hematopoietic stem cells are commonly identified as $CD34^+CD38^-$. However, there is also evidence for a CD34-negative hematopoietic stem cell population in humans (Nemeth and Bodine, 2007; Sonoda, 2008). Clearly, identification of more and preferably unique HSC markers is required to facilitate the analysis of human HSC function.

LT-HSC	**ST-HSC**	**MPP1**	**MPP2**	**MPP3**	
LSK	LSK	LSK	LSK	LSK	
$CD34^-$	$CD34^+$	$CD34^+$	$CD34^+$	$CD34^+$	
$CD48^-$	$CD48^-$	$CD48^+$	$CD48^+$	$CD48^+$	
$CD150^+$	$CD150^+$	$CD150^+$	$CD150^-$	$CD150^-$	
$CD135^-$	$CD135^-$	$CD135^-$	$CD135^-$	$CD135^+$	
0,00125	*0,00425*	*0,0065*	*0,016*	*0,0225*	*% of total BM*

Table 1: Cell surface markers used to identify murine hematopoietic stem cells*. LT-HSC (long-term HSC), ST-HSC (short-term HSC), MPP (multipotent progenitor). CD34 is a sialomucin; CD135 is also known as Flk2/3; CD48 and CD150 are SLAM family receptors.* Table adapted from Wilson *et al.* {{25 Wilson,A. 2007;}}.

Asymmetrical cell division

The majority of cells in the human body undergo symmetrical division, resulting in the production of two identical daughter cells. HSCs are also believed to divide asymmetrically, thereby giving rise to one new stem cell and one daughter cell that will differentiate towards a hematopoietic lineage. A stable pool of hematopoietic stem cells is maintained, as asymmetric cell division ensures self-renewal. Two models for asymmetric cell division have been proposed. Divisional asymmetry is established when cell-fate determinants are distributed asymmetrically in the HSC prior to cell division. During environmental asymmetry two identical daughter cells develop differently depending on extrinsic factors and their location in the stem cell niche (*figure 3*) (Congdon and Reya, 2008; Wilson and Trumpp, 2006).

The hematopoietic stem cell niche

The bone marrow offers a nurturing three-dimensional micro-environment in which hematopoietic stem cells lodge. This stem cell niche provides both instructive and supportive

signals and has long been known to be essential for HSC maintenance. Stromal cells, such as fibroblasts, endothelial cells and adipocytes, make up this niche. Membrane-bound and secreted factors, such as cytokines, growth factors, matrix proteins and chemoattractants, are thought to contribute to regulation of HSC quiescence and differentiation. Deprived of these signals, HSCs die (Alberts et al., 2002; Kiel and Morrison, 2008; Kindt et al., 2007). Cell-extrinsic factors that have been proved to be essential for HSC survival *in vivo* include angiopoietin, osteopontin, stem-cell factor (SCF) and CXCL12 (reviewed in Kiel *et al.* (Kiel and Morrison, 2008)).

Within the bone marrow stromal micro-environment two types of stem cell niches are described (*figure 3*). One niche is located at the endosteum, that might provide both a quiescent-storage niche and a self-renewing niche. The endosteum is regarded as the border between the bone and bone marrow, consisting of osteoblasts, osteoclasts, stromal fibroblasts and CAR (CXCL12-abundant reticular) cells. Hematopoietic stem cells found at the endosteal region have the highest proliferative capacity and long-term reconstitution ability (Haylock et al., 2007). Osteoblasts are believed to the most dominant regulators at the endosteum. They secrete various HSC-affecting factors including angiopoietin, thrombopoietin and CXCL12. Interestingly, increasing osteoblast activity results in higher HSC numbers. However, osteoclast activity and calcium ions are also implicated in HSC regulation (Kiel and Morrison, 2008; Wilson and Trumpp, 2006; Wilson et al., 2007).

The other type of niche is located near bone marrow sinusoidal endothelial cells (BMECs) and named the vascular bone marrow stem cell niche. HSCs adhere to BMECs, which express cytokines and adhesion molecules. These sinusoidal endothelial cells are believed to play an important role in HSC engraftment, homing and mobilization. Perivascular reticular cells surrounding sinusoids have an exceptional high expression of the chemokine CXCL12 and thereby might control HSC movement. The vascular niche seems to provide an environment in which HSCs are able to self-renew and perform hematopoiesis. It is expected that there is intense cooperation between the endosteal and vascular stem cell niche, but these bone marrow hematopoietic stem cell niches are still not fully understood (Kiel and Morrison, 2006; Wilson and Trumpp, 2006).

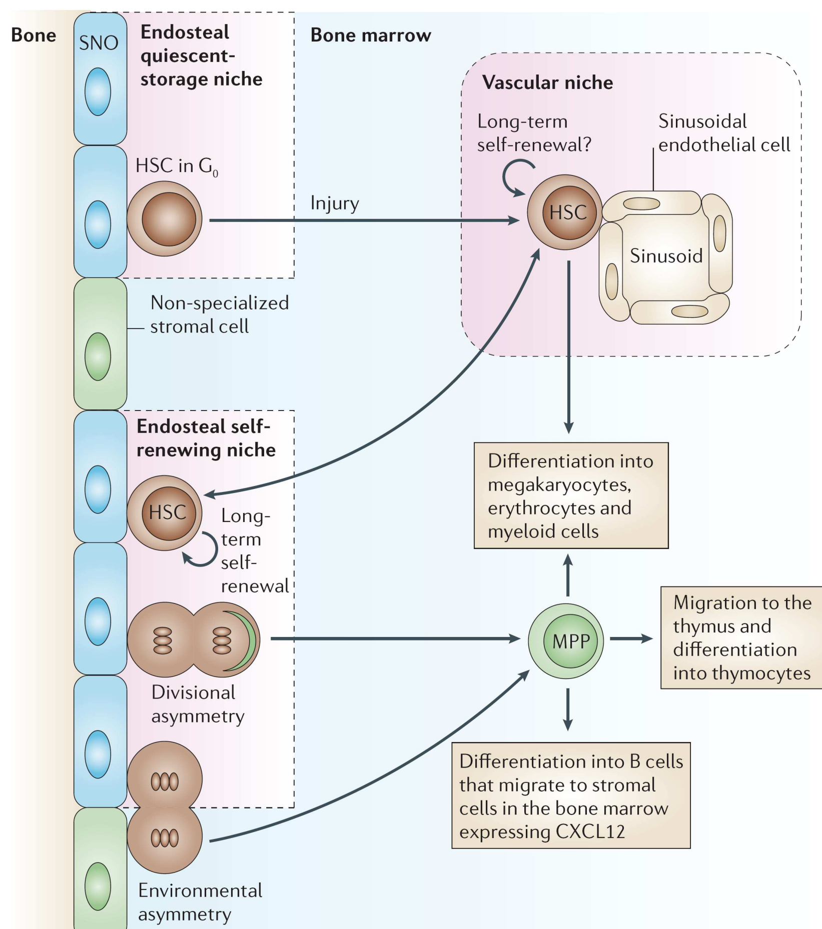

Figure 3: HSC niches in the bone marrow. *The bone marrow provides two distinct stem cell niches.* ***1)*** *The endosteal niche is located at the border between bone and bone marrow. The LT-HSCs that reside in this niche have the highest potential for proliferation and repopulation. The endosteal niche might provide both a quiescent-storage and a self-renewing niche. HSCs might divide asymmetrically. Divisional asymmetry is decided by distribution of intrinsic cell-fate determinants. Environmental asymmetrical cell division gives rise to two identical daughter cells that develop differently depending on localization.* ***2)*** *The vascular niche is situated around bone marrow sinusoidal endothelial cells. Presumably the vascular niche provides an environment in which HSCs are induced to self-renew and perform hematopoiesis.* Figure derived from Wilson *et al.* {{24 Wilson,A. 2006;}}.

Aim:
Gaining insight in the role of Wnt signaling in hematopoietic stem cell maintenance

It is still unclear how hematopoietic stem cells decide to self-renew or differentiate, but presumably the bone marrow micro-environment is involved in the regulation of HSC fate. Although numerous secreted factors, receptors and other molecules seem to be involved in HSC maintenance, in the last years much interest was directed to the Wnt signaling pathway. A role for Wnt proteins in hematopoiesis was first suggested in the nineties. Wnt signaling is involved in mesoderm development during embryogenesis; the adult hematopoietic system is derived from the mesoderm. Therefore, it seemed likely that Wnt proteins might also play a role in human hematopoiesis or HSC regulation. Indeed, Wnt proteins were found to be produced in hematopoietic stem cells and primitive progenitors. In addition, the stromal cells of the bone marrow micro-environment also express Wnt molecules (Austin et al., 1997; Reya et al., 2000; Van Den Berg et al., 1998).

Many studies have examined the role of these Wnt proteins in hematopoietic stem cell maintenance. Wnt proteins and other factors involved in Wnt signaling pathways have been well studied using both gain- and loss-of-function approaches, but research remains complicated due to conflicting results. To understand the function of Wnt signaling in HSC maintenance, it is important to keep in mind that Wnt signaling is known to act via activity gradients. Furthermore, the Wnt response is defined by context, as it is dependent on the type of target cell and the variety of Frizzled receptors it expresses. We also have to question whether Wnt proteins target hematopoietic stem cells directly or target the micro-environment to indirectly regulate HSC function.

Many studies have reported conflicting results. The majority of evidence seems to point to a role for Wnt proteins in proliferation of HSCs, while maintaining hematopoietic stem cells in an undifferentiated state. However, evidence for a quiescent signal has also been found. This thesis attempts to give a comprehensive review on the role of canonical and non-canonical Wnt signaling in hematopoietic stem cell maintenance.

Chapter I: β-catenin

Loss-of-function: β-catenin deletion *in vivo*

The Armadillo repeat protein β-catenin is the key player in the canonical Wnt/β-catenin-mediated pathway. It is specialized in protein-protein interactions and contains, in addition to 12 Armadillo repeats, binding domains for various factors (Mosimann et al., 2009). Both gain- and loss-of-function approaches have been developed to assess the role of β-catenin-mediated Wnt signaling in the hematopoietic system. Unfortunately, due to defects in gastrulation conventional β-catenin knock-out mice die during embryogenesis (Grigoryan et al., 2009). Therefore Cobas *et al.* created conditional Mx-Cre β-catenin$^{-/-}$ mice by flanking exons 2-6 of the β-catenin gene with loxP sites. Activation of the Cre recombinase results in recombination specifically at loxP sites, thereby excising the largest part of the β-catenin gene from the genome. By putting Cre under the control of the type I Interferon-inducible Mx promoter, deletion of β-catenin can be induced in adult mice with multiple injections of the synthetic dsRNA polyinosinic acid-polycytidylic acid (pI:pC). Using this conditional β-catenin$^{-/-}$ mouse model reconstitution experiments demonstrated that β-catenin deletion did not influence the self-renewal or differentiation capacity of HSCs. Even in the presence of competing wildtype bone marrow cells, β-catenin-deficient HSCs were perfectly able to reconstitute all blood cell lineages in lethally irradiated recipients (Cobas et al., 2004).

Nevertheless, in a similar model β-catenin deletion did have consequences for HSC growth and maintenance. In the Vav-Cre β-catenin$^{-/-}$ mouse the Cre recombinase is driven by vav regulatory elements, thereby allowing the deletion of β-catenin to begin during embryogenesis (Zhao et al., 2007). Expression of the adaptor protein Vav is supposed to be restricted to hematopoietic cells, but has however also been demonstrated in endothelial and germ cells (Georgiades et al., 2002). Compared to wildtype mice, Vav-Cre β-catenin$^{-/-}$ mice do not display any differences in number of long-term Lin$^{-/low}$Sca-1^{+}c-kit^{+}Flk2^{-} HSCs or progenitor cells. Transplantation assays with these β-catenin-deficient bone marrow cells in the presence of competing wildtype cells however exposed a defect in long-term growth and maintenance. Although β-catenin$^{-/-}$ HSCs are capable of multi-lineage differentiation, they have a reduced capacity for self-renewal. The difference between these two papers is claimed to be caused by the different stages of development at which deletion of β-catenin occurred (Zhao et al., 2007).

It was postulated that γ-catenin (or plakoglobin) compensates for β-catenin in these knock-out models, although this does not occur during other processes in embryogenesis. γ-catenin is structurally quite similar to β-catenin and is capable of binding to and activating TCF transcription factors. To create a double knock-out the Mx-Cre inducible β-catenin knock-out mouse was crossed with a conventional γ-catenin knock-out mouse. Surprisingly, two studies showed simultaneously that the combined deletion of β- and γ-catenin did not influence hematopoietic stem cell numbers, self-renewal or reconstitution capacity. Reconstitution capacity was not even affected in competitive assays with mixed chimaeras (Jeannet et al., 2008; Koch et al., 2008). Most unexpectedly, Jeannet *et al.* demonstrated that there was canonical signaling activity even after the combined deletion of β- and γ-catenin. Wnt-induced signaling activity was demonstrated with a β-galactosidase reporter for the Wnt target gene conductin/axin2 and also with a lentiviral reporter system under the control of TCF/LEF binding sites. The authors suggest that possibly an additional, not yet identified factor is involved (Jeannet et al., 2008). These studies indicate that deletion of β-catenin and even γ-catenin does not greatly influence hematopoietic stem cells *in vivo*. It is however wise to keep in mind that one single functional HSC is capable of reconstituting the entire hematopoietic compartment. It is therefore essential that in these knock-out experiments deletion of possibly involved factors such as β-catenin is verified and complete.

Gain-of-function: β-catenin overexpression

Reya *et al.* were amongst the first to propose a role for Wnt signaling in hematopoietic stem cell maintenance. As a gain-of-function approach they retrovirally transduced constitutively active β-catenin in Lin$^{-/low}$Sca-1^{+}cKit^{+}Thy-1.1^{low} (LSKT) HSCs isolated from H2K-BCL-2 transgenic mice. The decision for the use of Bcl2-transgenic mice has been questioned, but was taken to prevent HSC differentiation during retroviral transduction. Bcl2-transgenic cells namely only proliferate to stem cell factor (c-Kit ligand) and steel factor (SLF). β-catenin-transduced HSCs displayed an increase in proliferation in *in vitro* cultures, while maintaining an immature stem-cell-like LSKT phenotype. As HSCs were induced to self-renew, the stem cell pool vastly expanded. Importantly, these hematopoietic stem cells were still functional and capable of multi-lineage reconstitution in lethally irradiated mice (Reya et al., 2003).

Baba *et al.* employed a similar approach, but avoided the use of Bcl2-transgenic cells. Hematopoietic stem cells were retrovirally transduced with stabilized β-catenin under the control of an Internal Ribosomal Entry Site (IRES), resulting in rapid expansion of primitive progenitor cells. HSCs maintained an immature phenotype in co-cultures with stromal cells, as β-catenin induced proliferation of immature cells but restricted differentiation. The cells also maintained their multi-lineage differentiation capacity *in vitro*, but T lymphopoiesis did not occur after transplantation *in vivo* (Baba et al., 2006). The same group stated that constitutively active β-catenin bestows multi-lineage differentiation potential on lymphoid and myeloid progenitors. These progenitor cells seemed to have adopted hematopoietic stem-cell-like properties after retroviral expression of constitutively active β-catenin (Baba et al., 2005). Therefore, excessive β-catenin signaling *in vitro* seems to promote hematopoietic stem cell self-renewal, by inducing proliferation while maintaining an immature phenotype.

To assess the impact of β-catenin overexpression *in vivo*, an inducible knock-in mouse was created that expressed stabilized β-catenin in the entire adult murine hematopoietic compartment. Heightened levels of β-catenin resulted in a severe phenotype of hematopoietic failure, apparently promoting proliferation but inhibiting apoptosis of HSCs. Although LSK cell numbers increased in the bone marrow, these cells had lost all stem cell functionality. This was illustrated by a multi-lineage differentiation block and a complete loss of repopulation capacity (Kirstetter et al., 2006). Similar results were obtained by Scheller *et al.*, who also observed a vast expansion of the stem cell pool. Phenotypic HSCs lost their reconstitution and multi-lineage differentiation capacity due to excessive β-catenin expression (Scheller et al., 2006). These *in vivo* results suggest that disproportionate β-catenin-mediated signaling results in massive proliferation of hematopoietic stem cells, leading to exhaustion of the stem cell pool.

Gain-of-function: β-catenin overexpression in the stem cell niche

These studies did not include the β-catenin signals provided by the supporting stem cell niche, as stromal cells might have a great effect on HSC proliferation and differentiation. Surprisingly, there seems to be compartmentalization of Wnt signaling in the bone marrow. The canonical Frizzled receptors 1, 2, 7 and 8 are mainly expressed on stromal cells, as are molecules such as LRP5 and LRP6 (Kim et al., 2009). Expression of canonical Wnt proteins 1, 2b, 4 and 10b and inhibitors of canonical Wnt signaling is mainly established in hematopoietic cells. β-catenin accumulation was exclusively observed in stromal cells of the endosteal osteoblastic niche and particularly in conditions where the bone marrow was stimulated or stressed (Kim et al., 2009). Therefore, it was suggested that the stromal micro-environment produces a Wnt/β-catenin-signal that might regulate hematopoietic stem cell fate. *In vitro* retroviral β-catenin overexpression in stromal cells did not alter osteoblast composition, but the frequency of LSK HSCs increased in co-cultures. Stromal cells expressing high levels of β-catenin seem to maintain HSCs in an undifferentiated state and promote self-renewal, enhancing repopulation efficiency in transplantation experiments. This stroma-mediated signal was only exerted when HSCs and stromal cells made direct cell-to-cell contact (Kim et al., 2009). Therefore, increased β-catenin production by stromal cells seems to promote self-renewal.

Years before Yamane *et al.* established constitutive expression of β-catenin in a ST2 stromal cell line. These stromal cells were not able to support hematopoiesis normally, resulting in a partial block in formation of neutrophils, mast cells and osteoclasts. This reminds of the block in differentiation after induction of β-catenin overexpression *in vivo* as observed by Kirstetter *et al.* and Scheller *et al.* (Kirstetter et al., 2006; Scheller et al., 2006; Yamane et al., 2001).

Loss-of-function: β-catenin deletion in the stem cell niche

Overexpression of β-catenin in the stem cell niche maintains HSCs in an undifferentiated state. Nemeth *et al.* harvested bone marrow stromal cells from β-catenin knock-out mice and cultured them as confluent monolayers. Canonical signaling was significantly decreased and β-catenin nearly completely deleted. The stroma cultures were irradiated and wildtype Lineage-negative hematopoietic progenitors were added. β-catenin-deficient stroma cultures were not able to maintain hematopoietic progenitors and cell numbers decreased by half in 1 week. The *in vivo* model of wildtype hematopoietic progenitors transplanted into a β-catenin$^{-/-}$ HSC niche revealed a significant reduction in the percentage of LSK cells. However, HSC multi-lineage differentiation and self-renewal capacity were not affected. Interestingly, β-catenin deletion resulted in a decrease in osteoblast number and reduced expression of growth factors in the bone marrow micro-environment. β-catenin signaling in the micro-environment does not seem to directly regulate HSC

function, although LSK cell numbers decrease both *in vitro* and *in vitro.* β-catenin is essential in maintaining the composition of the environment (Nemeth et al., 2009).

Discussion

Many reports suggest that β-catenin is involved in the self-renewal of hematopoietic stem cells by promoting proliferation while blocking differentiation. However, β-catenin deletion does not seem to affect HSC maintenance or differentiation *in vivo*. One study was able to demonstrate a reduction in self-renewal capacity of HSCs that were derived from Vav-Cre inducible β-catenin knock-out mice (Zhao et al., 2007), but others using Mx-Cre inducible β-catenin$^{-/-}$ mice could not support this finding. Even combined deletion with γ-catenin, that might compensate for the loss of β-catenin, has no consequences for hematopoietic stem cell number, self-renewal of repopulation capacity (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). The major difference between these studies is the developmental stage at which β-catenin deletion occurs. Zhao *et al.* make use of Vav-Cre mice, in which deletion of β-catenin is induced by the Vav protein and begins early during embryonic life. The other three studies employ Mx-Cre mice, in which deletion of β-catenin is induced during adult life with pI:pC. Also, it is important to monitor whether deletion has been complete, as one single functional HSC is able to reconstitute the entire hematopoietic system of a lethally irradiated recipient.

Enforced β-catenin expression in Bcl2-transgenic cells induces proliferation *in vitro* while HSCs maintain an immature phenotype (Reya et al., 2003). β-catenin overexpression in wildtype cells yields similar results: HSCs are induced to proliferate while they maintain an immature phenotype (Baba et al., 2006). Therefore, elevated levels of β-catenin signaling *in vitro* induces self-renewal. An excess of β-catenin signaling *in vivo* however results in hematopoietic failure. HSCs massively proliferate but do not retain their stem cell function, resulting in exhaustion of the stem cell pool (Kirstetter et al., 2006; Scheller et al., 2006). There is a major difference in results derived from these *in vitro* and *in vivo* overexpression studies. *In vivo* various kinds of stimuli are present that are known to promote differentiation of HSCs and primitive progenitor cells. These factors are not necessarily present in *in vitro* cultures. Presumably β-catenin did induce proliferation *in vivo* but was not able to maintain HSCs in their primitive state, due to the presence of pro-differentiation stimuli. Taking other growth factors into account is essential in these kinds of models. In addition, there has been much debate about the use of Bcl2-transgenic hematopoietic stem cells by Willert *et al.*. This will be discussed in the next chapter.

In vitro retroviral β-catenin overexpression in stromal cells increases the frequency of LSK HSCs in co-cultures. Stromal cells expressing high levels of β-catenin seem to maintain HSCs in an undifferentiated state and promote self-renewal, enhancing repopulation efficiency in transplantation experiments (Kim et al., 2009). Retroviral transduction of a stromal cell line with constitutively active β-catenin resulted in a block in HSC differentiation (Yamane et al., 2001). Deletion of β-catenin in stromal cells results in a decline in HSC number in *in vitro* co-cultures and *in vivo* (Nemeth et al., 2009).

Together, these data suggest that β-catenin signaling is involved in the regulation of hematopoietic stem cell self-renewal. Interestingly, it has been reported that there is much β-catenin activity in LSK HSCs, while Lin$^-$Sca-1$^-$c-Kit^{hi} progenitor cells hardly signal via the β-catenin-mediated Wnt pathway. Similarly, cells bearing lineage markers express significantly less β-catenin (Baba et al., 2006; Reya et al., 2003). Therefore, it seems that β-catenin primarily acts on primitive hematopoietic stem cells.

Chapter II: Wnt3a

Wnt3a signaling in HSCs

Wnt3a is one of the ligands for the Frizzled receptor family and is involved in the canonical Wnt signaling pathway. It is known to stabilize cytoplasmatic β-catenin after binding to its Frizzled receptor. β-catenin then translocates to the nucleus, TCF/LEF transcription factors are activated and target genes expressed (Willert et al., 2003). Although there is not much evidence that HSCs themselves produce Wnt3a, Wnt3a mRNA is expressed in the bone marrow (Austin et al., 1997; Malhotra et al., 2008; Reya et al., 2000; Van Den Berg et al., 1998).

Reya *et al.* demonstrated that $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ HSCs respond to Wnt-signals *in vitro* and *in vivo*. HSCs were transduced with a lentiviral reporter construct consisting of destabilized GFP under the control of functional LEF-1/TCF binding sites. GFP expression was seen in the HSC population after transplantation into lethally irradiated mice, demonstrating that HSCs activate LEF-1/TCF transcription factors in response to signals provided by the micro-environment. Transduced HSCs were stimulated *in vitro* with purified Wnt3a protein and significant reporter activity was observed. As the reporter is turned on in response to Wnt3a, HSCs respond to Wnt3a signals via the canonical Wnt pathway (Reya et al., 2003). Another study supports that bone marrow hematopoietic cells are directly acted upon by Wnt3a and points out that stromal cells as well respond to Wnt signaling. Application of Wnt3a-conditioned medium to the ST2 stromal cell line resulted in expression of Frizzled receptors 1, 4, 5, 6, 7, 8, and an increase in cytoplasmatic β-catenin levels and nuclear translocation of β-catenin (Yamane et al., 2001).

Loss-of-function: Wnt3a deletion *in vivo*

Mice deficient in Wnt3a die around embryonic day (E) 12.5 and have morphological similarities with Tcf1/Lef1 double knock-out mice. Tcf1/Lef1 double-deficient mice die around E10.5, but show a defect in paraxial mesoderm differentiation similar to $Wnt3a^{-/-}$ mice. These similarities indicate a comparable defect and led Luis *et al.* to study fetal hematopoietic stem cells of $Wnt3^{-/-}$ mice around E12.5. The fetal liver is the major site for hematopoiesis at E12.5 and embryonic HSCs are found in this organ. Wnt3a-deficient mice had severely reduced LSK HSC numbers in the fetal liver. Besides the decline in number, hematopoietic stem cell function was impaired. Although $Wnt3a^{-/-}$ HSCs are able to reconstitute the hematopoietic system of lethally irradiated recipients in the presence of competing wildtype HSCs, they show a functional defect upon retransplantation into secondary recipients. Secondary competitive transplantation assays revealed a restricted capacity for self-renewal and long-term repopulation, but differentiation did not seem to be affected. HSC division seemed to be mostly non-self-renewing, leading to exhaustion of the stem cell pool. Together, these data suggest that Wnt3a is important for self-renewal (Luis et al., 2009; Luis and Staal, 2009).

Gain-of-function: Wnt3a stimulation and overexpression

Willert *et al.* isolated $Lin^{low/-}Sca\text{-}1^{low}c\text{-}Kit^{+}$ hematopoietic cells from Bcl2-transgenic mice and cultured them with purified Wnt3a protein in the presence of steel factor (SLF). Wnt3a-induced HSCs proliferated almost 6-fold more then control HSCs and seemed to be maintained in an undifferentiated state. These cells were not expressing lineage-determining cell-surface markers, suggesting that Wnt3a signaling induces self-renewal *in vitro*. Culturing HSCs with unfractionated Wnt3a-conditioned medium containing Wnt3a in a similar concentration, resulted in a significantly higher number of cells expressing lineage-markers. According to the authors, this indicates that other factors are present in Wnt3a-conditioned medium that are able to induce HSC differentiation. Transplantation experiments with Wnt3a-exposed HSCs revealed no defects in repopulation capacity. This suggests that Wnt3a promotes self-renewal of hematopoietic stem cells, while maintaining HSC function (Willert et al., 2003).

In the study of Yamane *et al.*, Wnt3a-conditioned medium was applied to a co-culture of HSCs with a ST2 stromal cell line. A block in hematopoiesis occurred, as Wnt3a signaling seemed to inhibit HSC differentiation. Wnt3a-induced stroma was not able to support the differentiation into various lineages, indicated by a reduced formation of B lymphoid cells and myeloid cells. The reduced differentiation capacity of HSCs induced by Wnt3a in this study was stroma-dependent, as the block in hematopoiesis did not occur in stromal cell-free cultures (Yamane et al., 2001).

Therefore, purified Wnt3a protein seems to induce self-renewal of HSCs in stromal cell-free cultures. Wnt3a-conditioned medium does not block hematopoiesis in stromal cell-free cultures, but does when HSCs are co-cultured with stromal cells.

LSK hematopoietic stem cells were transduced with a Wnt3a cDNA inserted into an IRES-GFP vector, GFP-positive cells were cultured under stromal cell-free conditions. Excessive autocrine production of Wnt3a protein prevented HSC differentiation *in vitro*, thereby blocking hematopoiesis (Malhotra et al., 2008). This is in accordance with the study of Willert *et al.*.

In a different study, transducing primary bone marrow cells with a bicistronic retroviral vector containing a Wnt3a-IRES-GFP cassette did not result in any changes in long-term reconstitution efficiency after transplantation into lethally irradiated recipients. Enforced autocrine Wnt3a production did not promote expansion of HSCs *in vivo* and even inhibited HSC expansion *in vitro*. In contrast to the report of Willert *et al.*, Wnt3a suppressed proliferation of wildtype lineage-negative Sca-1^{hi}c-Kit^{hi}IL-7Rα^{-} HSCs in stromal cell-free cultures, while the percentage of cells bearing primitive cell surface marker expression did not increase. This inhibition in proliferation is partly attributed to an increase in Bcl2-independent apoptosis. Transplanting Wnt3a-induced hematopoietic stem cells into lethally irradiated recipients resulted in no differences in either short- or long-term repopulation compared to control cells (Nemeth et al., 2007).

Two studies using a similar approach yielded different results. Malhotra *et al.* demonstrated inhibition of HSC differentiation after retroviral transduction of Wnt3a. Nemeth *et al.* were unable to demonstrate a block in hematopoiesis. In the latter study Wnt3a expression results in inhibition of proliferation. This is completely in contrast with previous results (Willert et al., 2003).

Gain-of-function: Wnt3a overexpression in the stem cell niche

OP9 stromal cells, that are unable to produce colony-stimulating factor, were retrovirally transduced to overexpress Wnt3a and co-cultured with primary control LSK HSCs. Stroma-produced Wnt3a resulted in a complete block of differentiation into lymphoid cells of the B lineage, as well as a block of differentiation into plasmacytoid dendritic cells. Although numbers of myeloid cells were reduced, differentiation into myeloid lineages did not seem to be affected. The authors state that they achieved very similar results with Bcl2-transgenic hematopoietic stem cells, but unfortunately do not show these data. Wnt3a signaling appeared to maintain HSCs in an undifferentiated state; a significant number of cells preserved their primitive cell surface marker expression and did not acquire lineage markers. This is in accordance with the study of Willert *et al.*, however Wnt3a did not promote stem cell proliferation. This block in hematopoiesis is comparable to the block in HSC differentiation observed by Yamane *et al.* in co-cultures with a stromal cell line. Although Wnt3a did not function as a growth factor, HSC primitive cell surface markers were retained and even reacquired by primitive progenitors (Malhotra et al., 2008). In conclusion, Wnt3a seems to be involved in inhibition of HSC differentiation.

Discussion

The Wnt3a protein is produced in the bone marrow micro-environment and is a known ligand for Frizzled receptors. Hematopoietic stem cells respond to Wnt3a via the Wnt/β-catenin-mediated signaling pathway. In addition, bone marrow stromal cells demonstrate elevated β-catenin levels upon stimulation with Wnt3a (Reya et al., 2003; Yamane et al., 2001). Deletion of Wnt3a *in vivo* results in decreased numbers of Lin^{-}Sca-1^{+}c-Kit^{+} in the fetal liver at embryonic day 12.5. These HSCs are impaired in long-term reconstitution capacity, as the loss of Wnt3a seems to negatively affect self-renewal (Luis et al., 2009).

Stimulation of Bcl2-transgenic HSCs with Wnt3a in stromal cell-free cultures results in an increase of HSC proliferation. The cells retain their primitive immunophenotypic character, as differentiation is inhibited. Therefore, Wnt3a promotes HSC self-renewal in these Bcl2^{+} Lin$^{low/-}$Sca-1^{low}c-Kit^{+} cells. In the same study the addition of Wnt3a-conditioned medium does not inhibit differentiation, therefore the authors suggest that other factors are present in Wnt3a-condioned medium that promote differentiation (Willert et al., 2003). However, in a co-culture of stromal cells and HSCs addition of Wnt3a-conditioned medium resulted in a block of hematopoiesis (Yamane et al., 2001). Willert *et al.* stimulated HSCs with purified Wnt3a protein in a stromal cell-free culture, resulting in promotion of proliferation and inhibition of differentiation. Addition of Wnt3a-conditioned medium to the same HSCs did not inhibit differentiation. Yamane *et al.* added Wnt3a-conditioned medium to HSCs and did not observe a block in hematopoiesis in their stromal cell-free culture, however in a co-culture of HSCs with a stromal cell line differentiation was inhibited. Therefore, Wnt3a seems to induce self-renewal of hematopoietic stem cells in its purified form in stromal cell-free conditions. In stromal co-cultures, but not in stromal cell-free conditions, Wnt3a-conditioned medium is able to block hematopoiesis. The context in which Wnt signaling occurs is essential. In addition, Willert *et al.* studied Lin$^{low/-}$Sca-1^{low}c-Kit^{+} cells, but it is questionable whether cells bearing this surface expression pattern are true hematopoietic stem cells.

In line with previous results, enforced autocrine production of Wnt3a in LSK HSCs prevents differentiation *in vitro* and blocks hematopoiesis (Malhotra et al., 2008). When stromal cells were transduced to overexpress Wnt3a, co-cultured HSCs were kept in an undifferentiated state and hematopoiesis was blocked. Wnt3a did not induce proliferation, but led to primitive progenitors to reacquire primitive cell surface markers (Malhotra et al., 2008).

In the study of Nemeth *et al.* retroviral transduction of a Wnt3a-expression cassette in HSCs suppressed proliferation in stromal cell-free *in vitro* cultures (Nemeth and Bodine, 2007). This is in contrast with the results obtained by Willert *et al.* (Willert et al., 2003). The difference in results is ascribed to the choice for Bcl2-transgenic cells in the study of Willert *et al.*, nevertheless Nemeth and colleagues performed additional experiments using $Bcl2^+$ HSCs. Also with these $Bcl2^+$ cells a Wnt3a-induced decrease in proliferation was observed *in vitro*. Thereby, wildtype and Bcl2-transgenic HSCs proliferated similarly in control or Wnt3a-stimulated cultures. There does not seem to be a big difference between wildtype and $Bcl2^+$ HSCs. However, stimulation with Wnt3a resulted in an inhibition of long-term repopulation after transplantation of $Bcl2^+$ HSCs in lethally irradiated recipients, where wildtype cells cultured with Wnt3a showed no difference with the control. It could be of importance that Willert *et al.* culture their HSCs with Wnt3a in the presence of SLF, whereas Nemeth and coworkers do not add any other stimulants. It is possible that other stimulants are required for Wnt3a to promote proliferation as it might act with other signals in a synergistic manner (Nemeth et al., 2007).

Nevertheless, Bcl2 overexpression in mice does have an effect on hematopoietic stem cells in the bone marrow. $Bcl2^+$ mice display increased HSC numbers and a higher percentage of Bcl2-transgenic HSCs resides in a quiescent state. In addition, reconstitution efficiency, as evaluated with competitive transplantation experiments, is enhanced (Domen et al., 2000). It is therefore important to keep this mind when evaluating studies that make use of Bcl2-transgenic HSCs.

Together, these data suggest that Wnt3a is mainly involved in the maintenance of primitive undifferentiated hematopoietic stem cells. Most reports demonstrate that Wnt3a signaling inhibits HSC differentiation. Whether Wnt3a may or may not act as a growth factor for wildtype HSCs, differentiation is restrained.

Chapter III: Wnt5a

Wnt5a signaling in HSCs

The Wnt5a protein is regarded as the model ligand for receptors that signal via non-canonical Wnt signaling pathways. It activates various β-catenin-independent Wnt signaling pathways, for example by binding to Frizzled receptor 2 or Orphan Receptor Tyrosine Kinase Ror2. Consequently it does not usually lead to β-catenin stabilization, but for instance to an increase in intracellular Ca^{2+} levels. The exact mechanism of activation of non-canonical pathways by Wnt5a signals is not completely understood (Sato et al., 2009). Initially expression of Wnt5a was found in human fetal stromal cell lines, as well as in hematopoietic progenitor cells in the fetal liver. Later it became apparent that Wnt5a is also produced by HSCs and stromal cells in the adult bone marrow (Austin et al., 1997; Kim et al., 2009; Liang et al., 2003; Reya et al., 2000; Van Den Berg et al., 1998).

Gain-of-function: Wnt5a stimulation and overexpression

Austin *et al.* were the first to examine the effect of Wnt5a on fetal liver derived hematopoietic stem cell progenitors (HSCPs). A synergistic effect of Wnt5a-conditioned medium and c-Kit ligand resulted in a dramatic increase in proliferation of HSCPs in stromal cell-free suspension cultures. Additionally, a larger percentage of cells displayed a less differentiated primitive morphology. HSCPs were retrovirally transduced with Wnt5a cDNA and stimulated with early acting cytokines IL-3, IL-6 and c-Kit ligand. Consequently, cell survival and proliferation was increased two-fold compared to the control (Austin et al., 1997). In accordance with these results, proliferation of human $CD34^{+}Lin^{-}$ adult bone marrow primitive progenitor cells is promoted in co-cultures with a stroma-like Wnt5a-transduced fibroblast cell line (Van Den Berg et al., 1998). Therefore, Wnt5a seems to promote proliferation of HSCs, while blocking differentiation.

However, neither proliferation nor differentiation of human HSCs was altered after stimulation with Wnt5a *in vitro* in the study of Murdoch *et al.* (Murdoch et al., 2003). Wnt5a-conditioned medium, derived from a Wnt5a-transduced stromal cell line, was applied to $CD34^{+}CD38^{-}Lin^{-}$ hematopoietic progenitors isolated out of human umbilical cord blood. Interestingly, these progenitor cells showed expression of Frizzled receptors 1 and 5, while more committed $CD34^{+}CD38^{+}Lin^{-}$ progenitors expressed Fz7. Wnt5a-conditioned medium however did not stimulate proliferation of $CD34^{+}CD38^{-}Lin^{-}$ cells. Proliferation and differentiation were not even affected in co-cultures of $CD34^{+}CD38^{-}Lin^{-}$ cells with a Wnt5a-transduced stromal cell line (Murdoch et al., 2003). An attempt was made to retrovirally transduce Wnt5a into human HSCs, but according to the authors the bicistronic retroviral vector MIEV could have failed to express Wnt5a in human hematopoietic cells. This might explain why autocrine Wnt5a production had no effect on HSC repopulation capacity after transplantation into NOD/SCID mice *in vivo* (Murdoch et al., 2003).

In another study, wildtype $lin^{-}Sca\text{-}1^{hi}c\text{-}kit^{hi}IL\text{-}7R\alpha^{-}$ (LSKI) cells were cultured under serum-free conditions with Wnt5a or a combination of Wnt5a and Wnt3a. After 6 days they were transplanted into lethally irradiated recipients. Significant 4-6 fold increases in both short-term and long-term repopulation was observed for cells stimulated solely with Wnt5a, while HSCs stimulated with Wnt3a alone had no enhanced repopulation capacity. Strangely, even a bigger effect on repopulation capacity was observed when HSCs were cultured with both Wnt5a and Wnt3a. It was demonstrated in LSKI cells that culturing with Wnt5a alone or both Wnt5a and Wnt3a results in a 2-fold increase in the percentage of cells in a quiescent state. Therefore, Wnt5a might promote quiescence and in this way might enhance repopulating capacity (Nemeth et al., 2007).

Culturing primary LSKI cells with both Wnt5a and Wnt3a resulted in inhibition of canonical signaling by Wnt5a, by destabilization of the β-catenin protein. However, incubating these cells with Wnt5a alone also seemed to increase β-catenin levels slightly compared to the control. Is this an indication that Wnt5a might also signal via the canonical pathway? Nevertheless, the non-canonical Wnt-ligand inhibits Wnt3a signaling in hematopoietic stem cells. Culturing LSKI cells with Wnt5a alone or with a combination of Wnt5a and Wnt3a resulted in a decrease in total cell expansion, in contrast to Willert *et al.*. The same experiment was repeated in Bcl2-transgenic LSKI cells that overexpress the human anti-apoptotic protein Bcl2. While $Bcl2^{+}$ HSCs that were stimulated with Wnt3a or cultured in control conditions did not show any difference to normal HSCs, $Bcl2^{+}$ cells that were stimulated with Wnt5a expanded more than wildtype cells (Nemeth et al., 2007). Wnt5a is also able to promote Bcl2 expression (Liang et al., 2007). Therefore, Wnt5a might act via Bcl2-coordinated pathways or act synergistically with another pathway stimulating survival.

Possibly, autocrine produced Wnt5a is unable to have an effect on HSCs. Murdoch *et al.* transduced murine hematopoietic cells with a Wnt5a-IRES-GFP cassette and were unable to demonstrate a change in long-term repopulation *in vivo*. Nemeth *et al.* used the same approach, and also did not observe a change in repopulation after transplantation of transduced HSCs into lethally irradiated recipient mice (Murdoch et al., 2003; Nemeth et al., 2007).

Wnt5a stimulation *in vivo*

Murdoch *et al.* were unable to demonstrate any changes in HSC differentiation or proliferation by stimulating with Wnt5a-conditioned medium *in vitro* or transducing HSCs with Wnt5a. However, injection of Wnt5a-conditioned medium into NOD/SCID mice 2-3 weeks after transplantation of human HSCs resulted in a striking enhancement in engraftment and reconstitution. The frequency of $CD34^+$ cells was significantly increased, especially the frequency of the more primitive $CD34^+CD38^-$ progenitors. Therefore Wnt5a might be involved in the HSC regulation and might promote self-renewal *in vivo*, but not *in vitro* (Murdoch et al., 2003).

Wnt5a deletion *in vivo*

Wnt5a knock-out mice die around the time of birth due to disrupted morphogenesis. They fail to develop multiple structures from the primary body axis, seemingly caused by insufficient cell proliferation. Previous reports have indicated increased Wnt5a signaling in the development of multiple types of cancer and stimulation with Wnt5a results in increased proliferation of hematopoietic stem cells (Austin et al., 1997; Van Den Berg et al., 1998). Therefore, Wnt5a appears to be involved in promoting proliferation. RT-PCR on hematopoietic tissues revealed that Wnt5a mRNA is expressed not only in fetal liver HSCs and bone marrow stromal cells, but also in various B cell progenitors and mature splenic B cells. Fetal liver cells of homozygous $Wnt5a^{-/-}$ mice were examined at E19 and surprisingly a significant increase in B progenitor cells was observed when compared to wildtype mice. Wnt5a deficiency results in increased numbers of B cells, therefore active Wnt5a signaling must inhibit B cell proliferation. Brdu incorporation assays revealed that this increase in B cell number is due to an increase in proliferation, B cells from $Wnt5a^{-/-}$ mice were not more resistant to apoptosis (Liang et al., 2003). In addition, heterozygous $Wnt5a^{+/-}$ mice spontaneously develop chronic myeloid leukemia or B cell lymphoma's, indicating that an absence of Wnt5a causes aberrant proliferation of B cells. Human leukemia tissues were also examined and a loss of Wnt5a function in these cancer cells was observed (Liang et al., 2003).

Unfortunately, completely different results were obtained when Malhotra *et al.* cultured $Lin^-c\text{-}Kit^{hi}Sca\text{-}1^-Thy1.1^{low/-}$ cells with a Wnt5a-transduced OP9 stromal cell line. Wnt5a production by stromal cells resulted in augmented numbers of $CD19^+$ B lymphoid cells. However, this was only a short-term effect. After 21 days in culture no difference in B cell numbers was observed when comparing Wnt5a-stimulated and control cultures. Therefore, Wnt5a primarily seems to act as a growth factor on primitive cells (Malhotra et al., 2008). In addition, Wnt5a is believed to play an important role in apoptosis of developing αβ thymocytes in the thymus (Liang et al., 2007).

Discussion

Wnt5a is regarded as the signature ligand of the non-canonical β-catenin-independent Wnt signaling pathways and is produced by the hematopoietic compartment in the bone marrow (Austin et al., 1997; Van Den Berg et al., 1998). Addition of Wnt5a-conditioned medium to fetal liver hematopoietic stem cell progenitors (HSCPs) in stromal cell-free cultures resulted in an increase in proliferation. HSCPs exposed to Wnt5a-conditioned medium proliferated more compared to the control, but were maintained in an undifferentiated state. In accordance with these results, Wnt5a-transduced HSCPs demonstrate a significantly increased proliferation (Austin et al., 1997). In addition, co-culturing human $CD34^+Lin^-$ adult bone marrow primitive progenitor cells with a stroma-like Wnt5a-transduced fibroblast cell line led to an increase in proliferation of these HSCs (Van Den Berg et al., 1998). Enhanced repopulation capacity was observed when lethally irradiated NOD/SCID mice were injected with Wnt5a-conditioned medium 2-3 weeks after transplantation of human HSCs. The frequency of $CD34^+$ cells was significantly increased (Murdoch et al., 2003).

Based on these results, it seems plausible that Wnt5a acts as a growth factor for immature hematopoietic stem cells and induces proliferation. There is evidence for a self-renewing signal, as Austin *et al.* observed a significant increase in the proportion of undifferentiated cells.

However, other studies do not agree. Murdoch *et al.* were unable to show any effect of Wnt5a on HSCs either directly by application of Wnt5a-conditioned medium or indirectly by retroviral

transduction of stromal cells. Transduction of HSCs with Wnt5a failed to lead to any differences in repopulation capacity *in vivo* (Murdoch et al., 2003). In contrast with previous reports, culturing LSKI cells with purified Wnt5a resulted in a significant decrease in cell expansion in the study of Nemeth *et al.* (Nemeth et al., 2007). In the same study, Wnt5a-exposed LSKI cells displayed augmented repopulation capacity. Although the number of HSCs decreased, the percentage of cells in a quiescent state increased (Nemeth et al., 2007). This is completely opposed to previous studies, which suggest that Wnt5a promotes proliferation.

It is impossible to draw one conclusion from the studies described above. It is still slightly unclear how Wnt5a activates non-canonical Wnt signaling pathways and which pathways exactly are initiated by this ligand. Therefore, it is difficult to interpret these results. Interestingly, incubating HSCs with Wnt5a results in a slight increase in β-catenin levels (Nemeth et al., 2007). Might Wnt5a also signal a little via the canonical β-catenin-dependent Wnt signaling pathway? Wnt5a is also known to inhibit canonical Wnt3a signaling. This only complicates the interpretation of the studies described, as one never exactly knows which pathways are activated. Nusse and colleagues recently proposed a model in which receptor context dictates whether Wnt5a protein activates or inhibits β-catenin-dependent signaling (Mikels and Nusse, 2006). They demonstrated that Wnt5a could initiate canonical signaling in cells overexpression Frizzled receptor 4, but not Fz6, 7 or 8. Therefore, if the appropriate receptors are present, Wnt5a can activate canonical signaling. In addition, presence of the orphan receptor ROR2 is required for the inhibition of canonical and activation of non-canonical signaling (Mikels and Nusse, 2006).

In conclusion, it is clear that Wnt5a does have an effect on HSCs. Most data point to a role in proliferation of primitive HSCs. Other studies suggest that Wnt5a provides a quiescent signal. Presumably, this depends on the context in which Wnt5a signaling occurs.

Chapter IV: Other Wnts

The greater part of research on Wnt signaling in hematopoietic stem cell maintenance has been directed to β-catenin and the Wnt3a and Wnt5a ligands. However, there is some evidence for a role for other Wnt proteins in the regulation of hematopoietic stem cell fate.

Wnt1, Wnt2b and Wnt10b

Wnt10b is expressed in the murine fetal liver micro-environment, as Wnt10b mRNA has been found in fetal liver hematopoietic stem cell progenitor cells (HSCPs), fetal bone marrow stromal cells, adult bone marrow and various hematopoietic cell lines. Wnt1 and Wnt2b mRNA is also expressed in the stem cell niche (Alberts et al., 2002; Reya et al., 2000; Van Den Berg et al., 1998). Conditioned medium derived from a stromal cell line transfected with Wnt10b was applied to fetal liver hematopoietic stem cell progenitors (HSCPs) in a stromal cell-free culture. In the presence of c-Kit ligand HSC proliferation increased 11-fold, the same effect was observed with Wnt1-conditioned medium. Wnt1- or Wnt10b-conditioned medium without the addition of c-Kit ligand did not promote much cell proliferation, so presumably c-Kit ligand acts with Wnt1 and Wnt10b in a synergistic manner (Austin et al., 1997). In a co-culture system with stromal-like fibroblast cells transduced with Wnt10b and $Lin^{-}CD34^{+}$ hematopoietic cells there was a vast increase in mixed colony-forming units. Wnt5a and Wnt2b increase colony-forming units of the erythroid lineage, but in this study Wnt1 and Wnt10b did not. In addition, cultures stimulated with Wnt10b and Wnt2b had a significant increase in $CD34^{+}$ hematopoietic cells, while maintaining the undifferentiated phenotype (Van Den Berg et al., 1998).

After injury of the bone marrow micro-environment, for instance induced by a combination of the drug cyclophosphamide and the cytokine G-CSF, HSCs have to regenerate quickly to produce all cells of the hematopoietic compartment. As the steady-state in the stem cell niche is disturbed, the bone marrow seems to improve at supporting hematopoietic stem cells. Wnt10b expression is enhanced in both stromal cells and hematopoietic cells in the bone marrow micro-environment, and Wnt10b is believed to act as a growth factor for hematopoietic precursors. In accordance with these results, increased activation of the Wnt signaling pathways is observed in regenerating HSCs (Congdon et al., 2008).

Wnt4

Louis *et al.* retrovirally transduced fetal liver hematopoietic stem cell progenitors with a Wnt4-encoding cassette and transplanted them in lethally irradiated mice. After 16 weeks long-term reconstitution was severely affected. The most striking result of Wnt4 overexpression was a 10-fold increase in early thymocyte progenitors in the thymus, especially of $Lin^{low/-}CD117^{hi}CD25^{-}$ cells and $Lin^{low/-}CD117^{hi}CD25^{+}$ thymocytes. Bone marrow cellularity was not altered, but numbers of LSK HSCs and $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{low}CD127^{+}$ common lymphoid progenitor cells were significantly increased in mice injected with Wnt4-transduced fetal liver cells. Wnt4 overexpression had no effect on the B lineage compartment, whereas higher numbers of myeloid progenitors cells were observed and production of erythroid cells was deteriorated. Interestingly, mice who received $Wnt4^{+}$ fetal liver cells had less actively cycling $Flt3^{+}$ and $Flt3^{-}$ HSCs in the bone marrow. Less cells were in the S and G_2/M phase of the cell cycle and less $Flt3^{+}$ cells were in apoptosis. Therefore, Wnt4a seems to induce cell-cycle arrest. $Wnt4^{-/-}$ neonates were examined just hours after birth, as $Wnt4^{-/-}$ mice usually die quickly after birth. Bone marrow cellularity appeared normal, but there was a selective reduction of $Flt3^{-}$ and $Flt3^{+}$ LSK HSCs. Thymic cellularity was markedly reduced (Louis et al., 2008).

In this report the Wnt4 protein did not signal via the canonical pathway, as β-catenin was not increased in co-cultures of fetal liver cells with fibroblasts producting Wnt4. In contrast, Wnt4 activated Jun-N-terminal kinase (JNK) in fetal liver cells, a kinase that is associated with various non-canonical Wnt signaling pathways (Louis et al., 2008).

Chapter V: Other factors

Besides Wnt proteins and β-catenin many other factors play major roles in the Wnt signaling pathways, including inhibitors complicating the action of Wnt signaling. We would expect that deletion or overexpression of these Wnt modulators has a great effect on HSCs, and these results would help to gain understanding in the role of Wnt signaling in hematopoietic stem cell maintenance.

Secreted Frizzled-related proteins

Secreted Frizzled-related proteins (SFRPs) are extracellular proteins that bind Wnt proteins directly, thereby competing with Frizzled receptors for their ligand and inhibiting both canonical and non-canonical Wnt signaling. Up to date four different SFRPs are identified (SFRP1-4). The N-termini of SFRPs presumably bind Wnt proteins, as they resemble the ligand-binding domain of Frizzled receptors and are rich in cysteines. SFRPs might also bind Fz receptors and form an inhibitory complex, although the exact mechanism of inhibition is unknown (Kawano and Kypta, 2003; Staal et al., 2008). SFRPs are however also implicated in the establishment of Wnt activity gradients, as they might act as Wnt protein transporters. In addition, they might activate Fz receptors directly (Mii and Taira, 2009; Renström et al., 2009).

SFRP1 and SFRP2 are expressed constitutively by osteoblasts of the endosteal stem cell niche in the bone marrow. The production of SFRP1 and SFRP2 increases in response to hematopoietic stress. This increase in SFRP1 and SFRP2 is due to the increase in osteoblasts in response to bone marrow injury, as SFRP expression per cell does not alter (Nakajima et al., 2009). Nakajima *et al.* isolated $CD34^-$LSK HSCs from murine bone marrow and stimulated them with either SFRP1 or SFRP2. In both conditions HSCs were induced to divide, as proliferation was amplified 1,5-fold compared to the control. Transduction of HSCs with a SFRP1 or SFRP2 expression cassette also resulted in increased proliferation in both SFRP1- and SFRP2-producing HSCs. There is also dissimilarity between these two proteins however, as SFRP1 seems to induce differentiation while SFRP2 partly blocks differentiation of HSCs. Excessive SFRP1 signaling *in vivo* results in an almost complete loss of repopulation ability, while SFRP2-cultured HSCs are believed to have a better repopulation capacity when transplanted into secondary recipients (Nakajima et al., 2009).

When SFRPs bind they act as Wnt inhibitors, high expression of SFRPs or stimulation with these proteins results in downregulation of the Wnt signaling cascade. The inhibition of Wnt signaling results in the promotion of HSC proliferation. In the case of SFRP1-mediated inhibition of the Wnt pathway, proliferation is induced and also differentiation, leading to exhaustion of the stem cell pool. SFRP2 blocks Wnt signaling while promoting proliferation and inhibits differentiation. In contrast with previous reports, the absence of Wnt signaling provides a signal for self-renewal.

SFRPs seem to be involved in the production of B and T cells and a link has been made with steroid hormones. For example, B and T cell lymphopoiesis is seriously declined during pregnancy when estrogen levels are high. Lymphoid precursor cells are known to express receptors for estrogens and androgens. However, inhibitors of lymphopoiesis might also be produced when stromal cells are stimulated with the hormone. In mice stimulated with estrogen, expression of SFRPs is upregulated as determined with RT-PCR. SFRP1 is expressed in the bone marrow particularly by osteoclasts and bone-lining cells in the endosteal region. The authors state that stimulation of stromal cells with estrogen results in an increased production of SFRPs, thereby inhibiting B cell and T cell lymphopoiesis (Yokota et al., 2008). Here, suppression of the Wnt signaling cascade inhibits differentiation of lymphoid progenitors.

Renström *et al.* set up a co-culture of SFRP1-knock-down stromal cells and Lineage-negative total bone marrow cells. Decreased expression of SFRP1 in the micro-environment resulted in an increased production of hematopoietic progenitors. The same was observed with stroma derived from $SFRP1^{-/-}$ mice and co-cultured with wildtype Lin^- bone marrow cells. The absence of SFPR1 supports the formation of colony-forming units. In $SFRP1^{-/-}$ mice there is no difference in HSC number or bone marrow cellularity. However, a higher frequency of LSK HSCs and multipotent progenitor cells seems to reside in the G0 or G1 phase of cell cycle. Transplantation of $SFRP1^{-/-}$ HSCs into lethally irradiated wildtype mice revealed no intrinsic defects. Transplantation of wildtype HSCs into lethally irradiated $SFRP1^{-/-}$ mice exposed an inability of $SFRP1^{-/-}$ stromal cells to support long-term hematopoietic stem cells (Renström et al., 2009). In this study, more active

Wnt signaling seemed to promote proliferation of hematopoietic progenitors, LSK HSCs were kept quiescent.

Knockdown of SFRP1 in hematopoietic stem cells abolishes the inhibition of Wnt signaling and the Wnt pathway will be more active. Therefore, active Wnt signaling seems to keep HSCs in a quiescent state, although long-term function is impaired. These results are however difficult to interpret as the action of Secreted Frizzled-related proteins is context- and concentration-dependent. Did these SFRPs act as an antagonist or agonist of Wnt signaling? In addition, these results seem contradictory. Stimulation of HSCs with SFRP1 or SFRP2 results in increased proliferation, respectively inducing differentiation and inhibiting differentiation. Deletion of SFRP1 from stromal cells however has a similar effect, resulting in increased hematopoietic progenitor production.

Dickkopf 1

The Dickkopf protein 1 (Dkk1) is a potent inhibitor involved in the canonical β-catenin-mediated Wnt signaling pathway. It contains cysteine residues and crosslinks the low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) to the Kremen receptor. Dkk1 antagonizes canonical signaling by promoting internalization of LRP5/6, thereby removing it from the cell membrane and inactivating it (Mao et al., 2002). Dkk1 is usually produced by cells of the osteoblastic lineage, while increased osteoblast activity has been shown to increase hematopoietic stem cell numbers in the bone marrow (Calvi et al., 2003). Therefore Fleming *et al.* created mice that overexpressed Dkk1 under the control of the osteoblast-specific collagen 1a promoter. Transduced mice showed a decrease in trabecular bone formation, but HSC numbers in the bone marrow were not different. Common lymphoid progenitor numbers appeared to have increased a little. HSCs derived from Dkk1-transduced mice were transplanted into lethally irradiated recipients, but were perfectly capable of reconstituting the entire hematopoietic system. However, serial transplants exposed an impaired long-term function of HSCs chronically exposed to Dkk1 and were no longer able to repopulate the bone marrow of the tertiary recipient (Fleming et al., 2008). Suppression of Wnt signaling in hematopoietic stem cells by Dkk1 results in impaired long-term function, as evaluated by serial transplantation experiments.

To evaluate the effect of a more temporary exposure to Dkk1, wildtype HSCs were transplanted into lethally irradiated Dkk1-transduced mice in the presence of competing wildtype HSCs. Complete reconstitution of the bone marrow occurred, but secondary transplantations of these short-term Dkk1-exposed HSCs into lethally irradiated wildtype mice failed to give rise to hematopoietic cells of all lineages. Interestingly, bone marrow of Dkk1-transgenic mice seemed to have become depleted of all LSK cells in a quiescent G0 phase. Therefore, Wnt signaling seems to be involved in maintaining quiescent HSCs (Fleming et al., 2008).

It is difficult to compare the *in vitro* study of Malhotra *et al.* with the *in vivo* study of Fleming and colleagues. An OP9 stromal cell line was transduced with Dkk1 and naturally did no longer respond to the canonical Wnt3a protein. Hematopoiesis was not significantly altered in co-cultures of these Dkk1-producing stromal cells and murine hematopoietic stem cells (Malhotra et al., 2008). It would be interesting to determine whether these HSCs are able to retain long-term function *in vivo*, but this was not examined. In the report of Fleming *et al.* initial rounds of hematopoiesis were not affected and a defect in long-term HSC maintaince was only exposed after serial transplantations.

Increased osteoblast activity is thought to increase HSC numbers in the bone marrow. So, contrary to the expectations, increased production of Dkk1 by osteoblasts does not result in elevated numbers of hematopoietic stem cells. HSCs exposed to Dkk1 for a longer period have defects in long-term function, while HSCs exposed to the Wnt inhibitor for a shorter period are forced out of their quiescent state. As increased inhibition of canonical Wnt signaling results in the depletion of quiescent HSCs, active Wnt signaling seems to play a role in maintaining quiescent HSCs.

Glycogen Synthase Kinase-3β

Glycogen Synthase Kinase-3β (GSK-3β) is present in the destruction complex that is responsible for β-catenin phosphorylation in the β-catenin-mediated Wnt signaling pathway. In the absence of an activating Wnt signal β-catenin is phosphorylated and targeted for proteasomal destruction. Casein Kinase 1 (CK1) is the priming kinase that phosphorylates β-catenin. After this priming event the serine/threonine kinase GSK-3β is able to recognize its target. It phosphorylates β-catenin on a couple of residues, thereby acting as a Wnt signaling pathway inhibitor. Besides

this Wnt pathway, GSK-3β is also involved in other signaling cascades such as the Hedgehog and Notch pathways (Staal et al., 2008; Trowbridge et al., 2006).

When wildtype hematopoietic stem cells are transplanted into lethally irradiated immunodeficient mice, reconstitution of the entire immune system occurs. Trowbridge *et al.* administered a GSK-3 inhibitor *in vivo* after transplantation of HSCs into NOD-SCID mice. The frequency and number of primitive hematopoietic cells originating from the transplanted stem cells was significantly higher and repopulation capacity increased 2-fold. The HSC pool was not significantly altered, but the progenitor cell population was vastly expanded. This increase in progenitor function was also observed with HSCs derived from human cord blood. Inhibition of GSK-3 results in higher levels of β-catenin, therefore active canonical Wnt signaling seems to be involved in inducing proliferation of primitive progenitor cells. As a result of an expanded progenitor pool, *in vivo* reconstitution ability is enhanced (Trowbridge et al., 2006).

In a different study, $CD34^+$ umbilical cord blood cells were isolated and cultured either in the presence or the absence of a GSK-3β inhibitor. In contrast to the previous study, GSK-3 inhibition did not induce proliferation of hematopoietic progenitor cells *ex vivo*. In fact, the percentage and number of $CD34^+$ slightly declined. When these cultured cells were transplanted into NOD-SCID mice, no difference was observed in repopulation capacity and total yield of cells between control and inhibitor-treated HSCs. Although numbers of $CD34^+$ cells declined after treatment with the inhibitor, hematopoietic stem cells capable of long-term reconstitution were still present. Surprisingly, mice transplanted with HSCs that were cultured with the GSK-3β inhibitor gave rise to a much higher number of $CD34^+$ cells, even though they had received less $CD34^+$ cells to begin with. Inhibition of GSK-3β *in vivo* promoted proliferation of primitive hematopoietic progenitor cells (Holmes et al., 2008).

It is generally accepted that administration of therapeutic lithium promotes the proliferation of hematopoietic stem and progenitor cells. As lithium targets the glycogen synthase 3β, GSK-3β might play an essential role in HSC maintenance. Bone marrow cells were depleted of GSK-3β using RNAi and transplanted into lethally irradiated mice. As GSK-3β mRNA was silenced, β-catenin levels increased. The pool of hematopoietic stem and progenitor cells was massively expanded four months after transplantation in the mice with depleted GSK-3β. BrdU incorporation assays revealed that the expansion of the HSC proliferation was due to proliferation. Due to this increased proliferation, the stem cell pool contained less functional HSCs as they were not as efficient in competitive reconstitution experiments. HSCs derived from RNAi-treated mice could not completely reconstitute the hematopoietic system of secondary and tertiary recipients after serial transplantation. Exhaustion of stem cell pool occurred and was most probably caused by the high level of proliferation (Huang et al., 2009).

These results are hard to interpret, as GSK-3β is involved in more pathways than the Wnt signaling pathway. Inhibition of GSK-3β is inhibition of a Wnt inhibitor and therefore leads to more active Wnt signaling and elevated β-catenin levels. Inhibition of GSK-3β *in vivo* appears to promote proliferation of primitive hematopoietic stem and progenitor cells. However, due to accelerated proliferation of quiescent HSCs it is possible that stem cells will not retain long-term function as the stem cell pool quickly becomes exhausted.

Adenomatous polyposis coli

The adenomatous polyposis coli (Apc) protein is a crucial scaffolding protein of the destruction complex and therefore an inhibitor of the β-catenin-mediated Wnt signaling pathway. It is described as a tumor supressor and is also involved in cellular processes such as migration and adhesion. RT-PCR on sorted LSK HSCs and $Lin^-Sca\text{-}1^-c\text{-}Kit^-IL\text{-}7R\alpha^-$ hematopoietic progenitor cells disclosed that Apc mRNA is expressed in the hematopoietic compartment. Apc was flanked by loxP sites and conditionally deleted in adult Mx-Cre mice. Apc depletion resulted in death of all mice within 2 weeks. Although mice displayed serious defects in hematopoiesis, it is not clear if this was the cause of death. HSCs derived from $Apc^{-/-}$ mice were impaired in their function as they were not able to completely repopulate the hematopoietic system of lethally irradiated wildtype mice. These HSCs were not able to give rise to hematopoietic cells of all lineages. In addition, exhaustion of the stem cell pool occurred as the proportion of quiescent stem cells was reduced. The loss of Apc resulted in the depletion of functional HSCs, leading to hematopoietic failure. Inhibition of the Wnt inhibitor Apc elevated the β-catenin protein levels. Therefore, active β-catenin signaling resulted in depletion of quiescent HSCs presumably due to increased proliferation (Qian et al., 2008).

Discussion

Canonical β-catenin-mediated Wnt signaling in HSCs

During the last decade, many studies have examined the role of Wnt signaling in hematopoietic stem cell maintenance. Unfortunately, even similar approaches yielded contrasting results. Wnt proteins are supposed to have a direct effect on hematopoietic stem cells; stimulation of HSCs with for example Wnt3a results in elevated β-catenin levels. Therefore we questioned what the effect was of activated Wnt signaling in HSCs. In general, β-catenin seems to provide a signal that promotes self-renewal of hematopoietic stem cells. Remarkably, β-catenin deletion itself did not result in a severe phenotype (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008; Zhao et al., 2007), but overexpression studies demonstrated that β-catenin induces HSC proliferation while inhibiting differentiation (Baba et al., 2006; Kim et al., 2009; Kirstetter et al., 2006; Reya et al., 2003; Scheller et al., 2006; Yamane et al., 2001).

Wnt3a is believed to mostly signal via the canonical β-catenin-mediated pathway and deletion of Wnt3a results in a defect in HSC self-renewal (Luis et al., 2009). Enforced expression of Wnt3a or stimulation with the protein generally increases proliferation of HSCs, while blocking differentiation (Malhotra et al., 2008; Willert et al., 2003; Yamane et al., 2001). In accordance with the previous results, stimulation with Wnt1, 2b or 10b induces proliferation and restrains differentiation of HSCs (Austin et al., 1997).

Studies on inhibitors of the canonical Wnt signaling pathway are in support of the previous results, as increased Wnt signaling in HSCs leads to proliferation. Both inhibition of GSK-3β and deletion of APC result in increased proliferation of the immature HSC pool (Holmes et al., 2008; Huang et al., 2009; Qian et al., 2008; Trowbridge et al., 2006). Overexpression of Dkk1 results in a defect in long-term self-renewal and the depletion of quiescent HSCs (Fleming et al., 2008). Together, these data suggest that the canonical Wnt/β-catenin-mediated pathway provides a proliferative signal for hematopoietic stem cells.

Overexpression of Secreted Frizzled-Related Proteins (SFRPs) inhibits both canonical and non-canonical Wnt signaling pathways, but studies employing this approach reported contrasting results. Reduced Wnt signaling by overexpression of SFRP1 or SFRP2 promotes HSC proliferation. However, SFRP1 induces differentiation while SFRP2 inhibits differentiation (Nakajima et al., 2009; Yokota et al., 2008). Knock-down of SFRP1, thereby augmenting Wnt signaling, results in increased production of hematopoietic progenitors (Renström et al., 2009). Strangely, SFRP overexpression and SFRP knock-down yielded similar results. SFRPs can inhibit both canonical and non-canonical pathways, therefore the discrepancy between these studies might be caused by a differential activation of Wnt pathways.

Non-canonical β-catenin-independent Wnt signaling in HSCs

Wnt5a, the hallmark ligand of non-canonical Wnt signaling, is also able to promote proliferation (Austin et al., 1997; Van Den Berg et al., 1998). However, it may support quiescence of hematopoietic stem cells (Murdoch et al., 2003; Nemeth et al., 2007). The effect of Wnt5a seems to depend on the type of receptors that are present on the surface of hematopoietic stem cells. It is now recognized that Wnt5a is able to stabilize β-catenin in the appropriate cellular context (Mikels and Nusse, 2006). Wnt4, that signals via the non-canonical JNK route, increases the number of lymphoid precursors, but provides a quiescent signal for immature HSCs (Louis et al., 2008).

Wnt signaling in the bone marrow stem cell niche

The bone marrow stem cell niche is essential in the maintenance of hematopoietic stem cells and produces Wnt proteins. In addition, bone marrow stromal cells respond to Wnt proteins themselves. Therefore, Wnt signaling might target HSCs indirectly by acting on stromal cells. Overexpression of β-catenin in stromal cells increases the number of HSCs *in vitro* and inhibits differentiation (Kim et al., 2009; Yamane et al., 2001). LSK HSC numbers decreased upon deletion of β-catenin from stromal cells *in vivo* (Nemeth et al., 2009). Wildtype HSCs are not able to long-term reconstitute the hematopoietic compartment of lethally irradiated SFRP1$^{-/-}$ mice. In addition, Wnt3a-induced stromal cells are not able to support differentiation of HSCs, resulting in a block in hematopoiesis (Yamane et al., 2001). Therefore, activation of Wnt pathways in stromal cells provides an indirect signal for HSCs. The composition of the stem cell niche might alter in

response to activated Wnt signaling, thereby impairing its ability to support hematopoietic stem cells (Nakajima et al., 2009; Nemeth et al., 2009).

Interpretation of the results described in this report is difficult, as some are contrasting. Some of these discrepancies could be caused by the methods that were used. In knock-out models it is extremely important that deletion of the factor is complete. It was demonstrated that in the β-catenin$^{-/-}$ mouse Wnt signaling was still continued. Was deletion not a 100%, or is there is an additional yet unidentified factor that also transmits Wnt signals? Other studies have used Bcl2^{+} HSCs, but it is clear that Bcl2-overexpression has great effects on the hematopoietic compartment of these transgenic mice (Domen et al., 2000). Discrepant results could be caused by different immunophenotyping of HSCs, as the cell surface markers of a true HSC are still not exactly identified. Some studies were unable to demonstrate a change in long-term repopulation after transplantation into primary lethally irradiated mice, but have not tried serial transplantation into secondary or tertiary recipients.

A complicating factor is that Wnt proteins are known to form activity gradients. Different concentrations of Wnt proteins lead to different amounts of signaling and will therefore lead to a different outcome. Different levels of activation of the Wnt signaling pathway caused by different approaches may result in completely different data (Staal et al., 2008). In addition, activation of different Wnt signaling pathways is context-dependent (Mikels and Nusse, 2006). The outcome of Wnt signaling depends on the type of target cell and the kind of Frizzled and co-receptors it expresses. It also depends on the presence of other signals, such as antagonists of the pathway but also cytokines. For example, The Notch and Sonic Hedgehog (shh) pathways have been shown to be active in HSCs and integrate with Wnt signaling (Blank et al., 2008; Duncan et al., 2005). Most probably, Wnt signaling pathways act in synergy with many other pathways. The combination of all these factors ultimately decides the fate of one hematopoietic stem cell.

In general, Wnt signaling seems to be involved in restraining hematopoietic stem cell differentiation, thereby maintaining the immature hematopoietic stem cell pool. In addition, it promotes proliferation while maintaining stem-cell-like properties.

Appendix I: Overview results

β-catenin deletion	
Reduced self-renewal capacity and defects in long-term maintenance	(Zhao et al., 2007)
No effect on self-renewal, differentiation or reconstitution	(Cobas et al., 2004)
No effect on HSC number, self-renewal or reconstitution	(Jeannet et al., 2008; Koch et al., 2008)
β-catenin overexpression	
Increased proliferation HSCs, inhibition of differentiation	(Reya et al., 2003)
Increased proliferation HSCs, inhibition of differentiation	(Baba et al., 2006)
Reacquisition multi-lineage differerentiation potential + stem cell like properties	(Baba et al., 2005)
Severe hematopoietic failure, promotion of proliferation, loss of all stem cell function	(Kirstetter et al., 2006; Scheller et al., 2006)
β-catenin deletion in stromal cells	
Decrease in HSC numbers, no effect differentiation and self-renewal	(Nemeth et al., 2009)
β-catenin overexpression in stromal cells	
Increased proliferation HSCs, inhibition of differentiation	(Kim et al., 2009)
Block in hematopoiesis, no support HSCs	(Yamane et al., 2001)

Wnt3a deletion	
Defect in self-renewal and long-term repopulation, decreased HSC numbers	(Luis et al., 2009; Luis and Staal, 2009)
Wnt3a stimulation or overexpression	
Increased proliferation HSCs, inhibition of differentiation	(Willert et al., 2003)
Inhibition of differentiation, block in hematopoiesis	(Malhotra et al., 2008)
No effect on reconstitution or expansion, inhibition expansion *in vitro*	(Nemeth et al., 2007)
Wnt3a stimulation of stromal cells	
Block in hematopoiesis, differentiation not supported	(Yamane et al., 2001)

Wnt5a stimulation or overexpression	
Increased proliferation HSCs, inhibition of differentiation	(Austin et al., 1997)
Increased proliferation of HSCs	(Van Den Berg et al., 1998)
No effect on proliferation, differentiation or repopulation Increased repopulation efficiency	(Murdoch et al., 2003)
No effect on long-term repopulation Inhibition HSC expansion Increased short- and long-term repopulation	(Nemeth et al., 2007)

Wnt1/Wnt2b/Wnt10b stimulation of HSCs	
Increased proliferation of HSCs, inhibition of differentiation	(Austin et al., 1997)

Wnt4 overexpression in HSCs	
Induction of cell-cycle arrest HSCs	(Louis et al., 2008)

SFRP stimulation or overexpression	
Increase in proliferation, either induction or inhibition of differentiation	(Nakajima et al., 2009)
Inhibition B and T lymphopoiesis	(Yokota et al., 2008)
SFRP knock-down	
Increased production hematopoietic progenitors	(Renström et al., 2009)

Dkk1 overexpression in stromal cells	
No effect on HSC number, but defect in long-term self-renewal Depletion of quiescent HSCs	(Fleming et al., 2008)
No effect on hematopoiesis	(Malhotra et al., 2008)

GSK-3β inhibition or knock-down	
Increased proliferation progenitor pool	(Trowbridge et al., 2006)
Increased proliferation primitive hematopoietic progenitor cells	(Holmes et al., 2008)
Increased proliferation HSCs, exhaustion of stem cell pool	(Huang et al., 2009)

Apc deletion	
Impaired function, exhaustion stem cell pool	(Qian et al., 2008).

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