Fine-tuning Hematopoiesis

Microenvironmental factors regulating self-renewal and differentiation of hematopoietic stem cells

Fine-tuning van Hematopoiese

Factoren uit de micro-omgeving reguleren zelfvernieuwing en differentiatie van hematopoietische stamcellen

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Cover: Detail of the tuning apparatus of a Portuguese guitar (original photo by Feliciano Guimarães, Portugal). Like the tension of the strings in a Portuguese guitar needs to be perfectly adjusted to produce the melodious sound characteristic of Fado, the signals received by the hematopoietic stem cells in the niche have to be fine tuned and strictly orchestrated, in order to sustain blood cell production throughout life.

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Tiago Cunha Luis

born in Lisbon, Portugal

Promoters: Prof.dr. J.J.M. van Dongen
Prof.dr. F.J.T. Staal

Other members: Prof.dr. R. Fodde
Prof.dr. W.E. Fibbe
Prof.dr. M.L.B.S. Parreira

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Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a Lua toda
Brilha, porque alta vive.

"To be great, be whole;
Exclude nothing, exaggerate nothing that is you.
Be whole in everything. Put all you are
Into the smallest thing you do.
So in each lake the whole moon gleams
Because it blooms up above."

Fernando Pessoa / Ricardo Reis (heteronym), In "Odes"

To my parents,

Fine-tuning Hematopoiesis

Microenvironmental factors regulating self-renewal and differentiation of hematopoietic stem cells

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Chapter 1

General introduction

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1.1 Hematopoietic stem cells and hematopoiesis

Hematopoietic stem cells (HSCs) have the ability to self renew and generate all lineages of blood cells. Although it is currently well established that hematopoietic stem cells (HSCs) regenerate the blood compartment, it was only in the 1960s that was introduced the notion that multipotent progenitors can be found in the adult bone marrow (BM) and are responsible for the continuous production of blood throughout life [1-3]. Since then, research from many different laboratories contributed to the phenotypic and functional characterization of HSCs and nowadays, the blood system constitutes a paradigm for understanding adult stem cell biology.

HSCs are located at the top of a hierarchy of lineage specific progenitors that differentiate in an ordered fashion towards fully mature blood cells thereby undergoing a stepwise loss of lineage potential, and becoming progressively committed to a single hematopoietic lineage [4] (Figure 1).

Hematopoietic Stem Cells

HSCs are functionally defined by their ability to mediate long-term (LT) repopulation after transplantation. The strictest version of this functional definition requires that HSCs have to be re-transplantable in serial recipients while retaining both self-renewal and multilineage differentiation capacity [5].

In the mouse, HSCs can be found in the BM within a rare population defined by the absence of lineage specific markers, and high expression levels of stem-cell antigen 1 (Sca1) and c-Kit. Therefore this BM subset is known as LSK (Lin$^-$Sca1$^+$Kit$^+$) [6,7]. Since only a small fraction of the LSK cells contain long-term repopulating (LTR) capacity additional markers have been introduced to subdivide this heterogeneous population. LT-HSC containing LTR activity are CD34$^-$, fms-related tyrosine kinase 3 (Flt3)$^-$ CD48$^-$ and CD150$^+$. Short-term (ST) -HSC which have only limited repopulation capacity are CD34$^+$ and Flt3$^-$, and multipotent progenitors (MPP) that lost self-renewal but retain multilineage differentiation potential are Flt3$^+$ and CD34$^+$ [8-10]. In addition to cell surface markers, HSCs can be identified by their ability to efflux fluorescent dyes such as Hoechst 33342. This property defines a subset named side-population (SP) which is highly enriched for LT-HSCs [11]. The basic principles of mouse HSCs seem to also apply to human HSCs. Nevertheless, due to the lack of efficient *in vivo* assays, the correct definition and isolation of human HSCs has been more difficult. In the human, HSCs are enriched in the Lin$^-$CD34$^+$CD38$^-$, as assessed in the SCID-repopulating cell (SRC) assay [12,13]. However, different studies revealed that expression of surface markers may change depending on developmental stage and cell cycle state of these cells [14]. Studies in the mouse predict that the majority of HSC in human cord blood and in the BM of young children are CD34$^+$. Despites

this, it is likely that, as in the mouse, a significant proportion of human adult HSCs do not express CD34. Thus, while CD34 selection constitutes a good method to purify human HSC from cord blood or after HSC mobilization, it may discard a sizable population of stem cells when applied to the BM of adult humans [14].

HSCs self-renewal and differentiation

All stem cells are characterized by having multilineage differentiation potential on a single-cell basis, which is defined by the capacity to give rise to a differentiated progeny comprising different cell types, and by their self-renewal capacity which allows them to replicate while

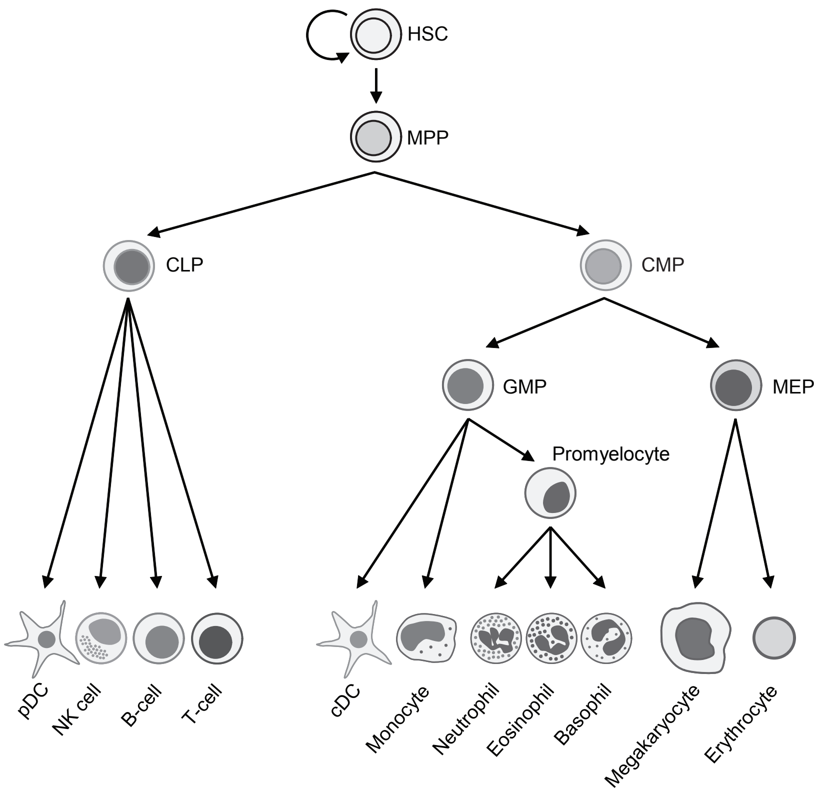

Figure 1. Schematic overview of the hematopoietic developmental hierarchy. Self-renewing HSCs reside at the top of the hierarchy, giving rise to a number of multipotent progenitors which develop in mature blood cells through a step wise process of lineage specification and commitment. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte-monocyte progenitor, MEP, megakaryocyte-monocyte progenitor, pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; NK, natural killer.

preserving their broad developmental potential. Precise regulation of these two processes is essential in order to sustain life-long blood production and to respond to external stresses like injury or infection [15].

A possible mechanism to regulate the balance between self-renewal and differentiation is by controlling symmetric and asymmetric cell divisions. Under homeostatic conditions the number of stem cells in a specific tissue remains relatively constant. It is commonly assumed that this balance is probably achieved by asymmetric cell division, through which one daughter cell maintains the stem cell identity and the other becomes differentiated. This asymmetry can be achieved by unequal distribution of cytoplasmatic cell-fate determinants before cell division (divisional asymmetry). Alternatively, the two identical daughter cells can be exposed to different environmental signals after cell division, preserving stem cell fate in one daughter cell and promoting differentiation in the other (environmental asymmetry)[16]. These processes have been elucidated most extensively in *Drosophila* and *Caenorhabditis elegans*. Although the molecules involved are highly conserved among species, these mechanisms still remain poorly understood in mammals and more specifically in HSCs. Initial studies in the hematopoietic system suggested that hematopoietic precursors in a pro-differentiation environment tend to divide asymmetrically while precursors in a pro-renewal environment preferentially divide symmetrically [17-19] in order to increase the HSC pool.

Adult HSCs are mostly quiescent with approximately 95% of them being in the G0 phase of the cell cycle. Based on the cycling behavior of the LT-HSCs two populations were defined, one actively cycling and one dormant, which may constitute a reservoir of the most potent HSCs and that, can be efficiently activated in response to injury. Indeed, the majority of multi-lineage long-term activity was found in dormant LT-HSCs [20,21]. Furthermore, the activation of dormant stem cells seems to be reversible as actively cycling HSCs return to dormancy upon re-acquisition of homeostasis [21]. The maintenance of a dormant pool of non-cycling and metabolic inactive HSCs was suggested to be a protective mechanism against exhaustion of a limited self-renewal potential [15]. In contrast to LT-HSCs, their progeny are actively cycling and are responsible for the majority of the cell expansion required for the daily production of billions of blood cells.

Ontogeny of the hematopoietic system

Blood is a mesodermally derived tissue and may have its origin in a common precursor for both endothelial and hematopoietic lineages, termed the hemangioblast [22-24]. Defining the origin of the hematopoietic system is difficult because blood is a mobile tissue and, hematopoietic cells emerge at different sites in the embryo and at different stages of the embryonic development, until the definitive hematopoietic organs are fully developed and ready to take over this function.

In the mouse, the first wave of hematopoiesis occurs in the extraembryonic yolk sac (YS) between embryonic days 7 (E7) and E8. This transient hematopoietic system is mainly oriented to red blood cell production. The erythrocytes produced in the YS at this stage do not get enucleated and therefore are called primitive. Besides this, myeloid progenitors are also detected in the YS before circulation is established (E8), and are therefore generated in this location[25,26].

After the first wave of primitive erythropoiesis, adult HSCs, functionally defined by their capacity to confer complete, long-term and multilineage repopulation of the hematopoietic system of adult recipient mice, are first generated in the para-aortic splanchnopleura- (pSp-) aorta-gonads-mesonephros (AGM) region and can be detected at around E10.5 [27,28]. These HSCs are located in the ventral region of the dorsal aorta, in association with the endothelium wall [29]. Based on this close proximity, it has been proposed that instead of a hemangioblast with both hematopoietic and endothelial potential, a "hemogenic endothelium" lining the ventral aorta from which the HSC emerge, could be the precursor of definitive HSCs [30-34]. Alternatively, a third model proposes that hematopoietic precursors located in subaortic patches migrate through the mesenchyme into the dorsal aorta [35].

Shortly after being generated in the AGM region, HSCs are detected in other tissues such as the YS, placenta and fetal liver [36-38]. Whether *de novo* generation or only expansion of HSCs occurs in the YS and placenta is still under debate. The fetal liver does not autonomously generate HSCs. Instead, hematopoietic cells migrating from the AGM, YS and placenta colonize the fetal liver, which becomes the major hematopoietic organ until birth when BM hematopoiesis is established [37,39,40]. Analogous to the mouse embryo, the hematopoietic development in the human embryo occurs in a similar fashion [41] (Figure 2).

Fetal versus Adult HSCs

The fetal liver is the main fetal hematopoietic site for HSCs expansion and differentiation [42]. Although hematopoietic progenitors with myelo-erythtroid potential can be detected in the liver rudiment as early as E9.5, the first definitive HSCs only colonize the fetal liver at around E11.5. In the liver, the HSC pool expands rapidly. Quantification of this expansion revealed a 38-fold increase in competitive repopulating units between E12 and E16. After E16 the number of repopulating units declines probably due to massive exit of HSC to either the spleen or the BM [43] (Figure 2). Whereas most adult HSCs are quiescent, fetal liver HSCs are actively cycling. Besides this, the self-renewal potential of fetal liver HSCs is also higher in comparison with BM HSCs, which are outcompeted in transplantation experiments [44-46]. Although this advantage can be explained by differences in the signals provided by the fetal liver and BM microenvironments, the differences in repopulation capacity observed when fetal and adult HSCs are transplanted in irradiated recipient mice also suggests the existence of inherent differences between them [42].

In addition, fetal and adult HSCs vary in phenotypic and functional characterization. Contrarily to adult long-term reconstituting HSCs, fetal HSCs express markers such as Mac1 (CD11b) [45,47] and AA4.1 (CD97) [48]. Functionally, mouse fetal HSCs are able generate $V\gamma3^+$ and $V\gamma4^+$ T-cells in response to fetal thymic environment, whereas adult HSCs cannot [49], and are more capable of generate B1 lymphocytes [50,51]. Moreover, several regulators were found to be essential for adult HSCs, such as *Bmi1*, *Tel/Etv6* and *Gfi1*, but not for fetal HSCs [52-54]. On the other hand, it was recently demonstrated that the transcription factor *Sox17* is required for fetal and neonatal but not adult HSC function [55]. Moreover, gene expression profiling demonstrated differences in adult versus fetal HSCs gene expression [56,57] which may underlie the functional differences in adult versus fetal HSC.

In the last stages of gestation, cells start migrating to the BM. In the mouse, the transition from fetal to adult HSC properties happens 3-4 weeks after birth, when HSCs abruptly become quiescent. [58] (Figure 2). The functional differences that distinguish fetal versus adult HSCs may be conferred by their unique microenvironments. Nevertheless, the mechanisms underlying these modifications are still largely unknown.

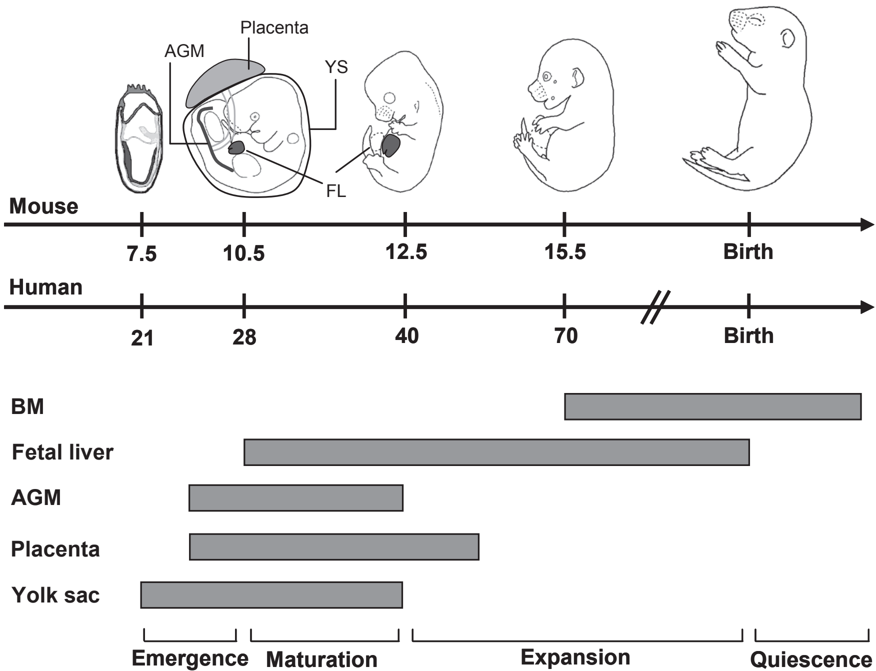

Figure 2. Establishment of the definitive hematopoietic stem cell compartment during embryonic development. Bars reperesent the ages at which mouse and human hematopoietic sites are active. AGM, aorta-gonad-mesonephros region; FL, fetal liver; YS, yolk sac.

Hematopoietic stem cell niche

The existence of a specialized niche or microenvironment that promotes HSCs maintenance was initially proposed in 1978 by Schofield et al. [59]. Already by this time it was suggested that stem cells are seen in association with other fixed-tissue cells that prevent stem cell differentiation and ensure its continuous proliferation. However, only recently advances have been made to define their exact location as well as the molecular mechanisms by which they regulate HSCs [5,60,61].

HSCs are mainly found in BM cavities in the trabecular region of long bones. Inside these cavities they are positioned at the interface of bone and BM, known as endosteum, in close proximity to bone-forming osteoblasts [62-64]. A direct implication of osteoblast in the regulation of HSCs was observed in studies in which an experimentally induced increase in the number of osteoblasts and trabecular bone area directly correlated with an increase in HSC pool size, but not in other hematopoietic cells in BM [64,65]. In agreement, depletion of osteoblasts *in vivo* was associated with reduced BM cellularity and extramedullary hematopoiesis [66]. Furthermore, osteoblasts secrete factors that regulate HSC maintenance such as angiopoietin, thrombopoietin and the chemokine Cxcl12 [61]. Despites these evidences it still remains uncertain whether the endosteal interface represents a niche or if it only secrets factors that diffuse to nearby niches.

Besides the endosteal niche a vascular niche in BM was also proposed upon observation of a large proportion of phenotypically defined $CD150^+$ HSCs attached to the endothelium of BM sinusoids [9]. This idea is not unexpected since both lineages share a common embryonic origin and during fetal development HSCs are maintained in niches that do not involve bone. Moreover, adult HSCs can also be found in extramedullary organs such as spleen and liver, and hematopoiesis can shift to these locations for long periods of time, in the absence of an endosteal niche. Although stem cells seem to localize close to sinusoids in spleen [9], these niches are still undefined and non-vascular cells may also be essential to regulate HSC function in these locations. Similarly to osteoblasts, perivascular cells also produce factors important for HSCs, such as Cxcl12 [67]. However, this dual niche hypothesis was recently challenged by new *in vivo* imaging techniques showing that osteoblasts and vasculature are in close proximity in the BM cavities indicating that distinct endosteal and vascular niches are not anatomically feasible [68]. In addition the fact that osteoblast depletion results in extrameduallary hematopoiesis [66] suggest that a vascular niche alone is not sufficient to maintain hematopoiesis. Therefore, it is possible that rather than 2 different niches, vascular cells may have important roles in regulating HSCs near the osteoblasts, constituting in this way a niche composed of different cell types [5,61].

The stem-cell-niche synapse

Many molecular and functional evidences suggest the existence of a complex molecular cross-talk between HSCs and the niche cells in their close vicinity, leading to the definition of one adhesion and signaling unit termed “stem-cell-niche synapse”, in analogy to the neuronal and immunological synapses [5]. A wide variety of factors are involved in this synapse, and they mediate mainly two types of interactions. They promote adhesion between both cells and to the extracellular matrix, in order to maintain both cells in close proximity and, they promote activation of specific signaling pathways that influence HSCs fate decisions, survival and proliferation. The activation of signaling pathways can be mediated by direct cell-cell interactions through binding of membrane associated ligands and receptors, such as Notch signaling, or by binding of soluble factors to specific receptors located both on the HSC and on the niche cell, such as Wnt, Smad/TGF/BMP/Activin and Hedgehog signaling, and also hematopoietic cytokines like stem cell factor that binds to the Kit receptor and, thrombopoietin which binds to the Mpl receptor [5,60].

Studies from many different laboratories implicated several evolutionary conserved signaling pathways in the regulation of HSC function. All these pathways regulate important aspects during development and have been shown to be involved in cell-fate decisions and in the maintenance of stem cells in different tissues. These include the Wnt, Notch, Smad and Hedgehog pathways. Because of the highly redundant nature of these signaling routes and due to complex synergistic and antagonistic interactions between them and with other signals, the precise function of these pathways has remained vague, and frequently highly controversial. While generally *in vitro* assays and *in vivo* gain-of-function assays showed that these pathways regulate HSC function, *in vivo* loss-of-function of key components of these pathways such as β-Catenin (Wnt), Rbpj (Notch) and Smoothened (Hedgehog) suggested that none of these pathways is essential for HSC function [60,69-71]. These issues will be further discussed in this chapter and later in this thesis.

Two models of Hematopoiesis

Upon receiving signals to undergo differentiation HSCs gradually lose self-renewal potential while become committed to the different hematopoietic lineages. Lineage commitment is the process by which a multipotent stem or progenitor cell becomes increasingly limited in its lineage fate options and develops into a progenitor fully restricted to a single lineage. However the cellular pathways of lineage commitment until progenitor cells are fully restricted are still under debate [72-74].

The classical model of hematopoiesis

The classical model of hematopoiesis initially proposed by studies from the Weissman laboratory, predicts that the first step of lineage commitment from HSC or MPPs consists in a

strict and symmetric separation between lymphoid and myeloid potentials (Figure 3). This model proposes that HSCs initially generate a common myeloid progenitor (CMP) containing both myeloid and erythroid potentials, and common lymphoid progenitor (CLP) containing all the potential for B-cell lineage and for the T-cell lineage upon migration to the thymus. Subsequently, CMP would than originate granulocyte/monocyte (GM) and megakaryocyte/erythrocyte (MkE) progenitors. This model was strongly supported by the prospective identification of these cell subsets [75,76]. A model in which CMPs and CLPs are obligatory intermediates for lineage commitment would imply that LT-HSCs, ST-HSCs and MPPs only differ in self-renewal capacity but retain full lineage differentiation potential. In addition, progenitors with combined lymphoid and myeloid potential without having the full hematopoietic lineage potential should not exist.

Alternative model for early HSC fate decisions

Different studies in both fetal and adult hematopoiesis have identified progenitor subsets with mixed myeloid and lymphoid potential, [77-81] thereby challenging the classical model of the myeloid-lymphoid dichotomy [82]. More recently, it was demonstrated that Flt3^{hi} LSKs or MPPs, when compared to HSCs, sustain at single cell level lymphoid (B and T) and GM potential but little or no MkE potential [83-85] (Figure 3). Due to the lack of MkE potential and maintenance of lymphoid and myeloid potential these progenitors were termed lymphoid-primed MPPs (LMPP). Further analysis at molecular level revealed that LT-HSCs are MkE and GM but not lymphoid transcriptionally primed, meaning that they co-express MkE and GM but not lymphoid programs. Furthermore, transcriptional lymphoid priming was only initiated at LMPP stage after down regulating the MkE transcriptional priming [86,87]. Importantly, combined lymphoid and MkE transcriptional priming at single cell level, was never observed in any of the HSC and LMPP subsets. In this way, distinct patterns of multilineage transcriptional priming provide molecular support for the existence of GM-MkE and GM-lymphoid restricted commitment pathways [74]. Thus, this model proposes a hierarchical organization of the different lineage programs within each of the HSC and LMPP compartments, with the GM program being probably a default lineage program. Although it implies that CMPs and CLPs are not obligatory or even main intermediates in early lineage fate decisions, this model is compatible with the existence of theses populations [86]. Finally, the recent finding that thymic progenitors retain myeloid potential at single-cell level further supports this alternative model of lineage commitment [88,89].

From Hematopoietic Stem Cells to T-cells

T-cell development occurs at a different anatomic site compared to all other lineages that develop in the BM. Hematopoietic progenitors, ultimately derived from the BM enter circulation

and migrate to the thymus where they definitively commit to the T-cell lineage and further mature to functional T lymphocytes. Since thymic progenitors lack self-renewal capacity, a continuous import of progenitors from the BM is required to sustain T-lymphopoiesis [90]. T-cell development proceeds through a series of discrete phenotypic stages that can be recognized by the expression of several important membrane molecules, most notably CD4 and CD8 (Figure 4). In both humans and mice, thymocyte differentiation occurs through successive $CD4^{-}CD8^{-}$ (double negative, DN), $CD4^{+}CD8^{+}$ (double positive, DP) and $CD4^{+}CD8^{-}CD3^{+}$ or $CD8^{+}CD4^{-}CD3^{+}$ (single positive, SP) stages. The DN subset can be further subdivided into four stages (DN1 till DN4) in mice and humans [91-93].

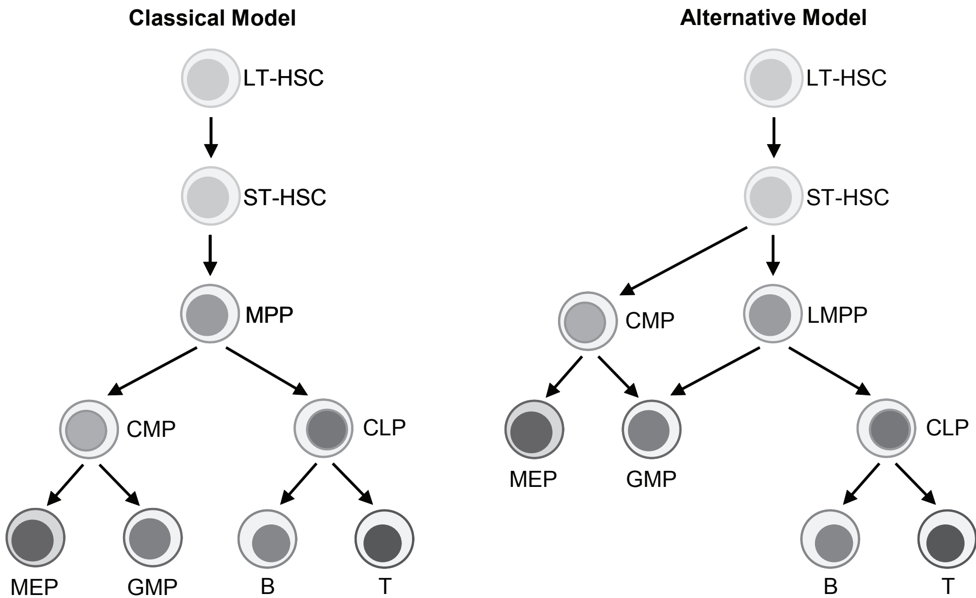

Figure 3. Classical and alternative model for hematopoietic stem cell commitment. The classical model postulates that the first lineage commitment step results in a strict separation of myelopoiesis and lymphopoiesis. This is supported by the prospective isolation of common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). The alternative model suggests that a pluripotent HSC upon loss of Mk and E potential develops into a lymphoid primed multipotent progenitor (LMPP) that upon loss of GM potential generates the CLP. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; GMP, granulocyte/macrophage pronenitor; MkEP, megakaryocyte /erythroid progenitor; B, B cell; T, T cell.

Early thymic progenitors and multilineage hematopoiesis in the thymus

The exact identity of the progenitors that seed the thymus is still elusive due to the fact that several different BM progenitors contain T-cell lineage potential and due to the extremely low number of cells required and participating in this process. These progenitors enter the thymus through the cortical tissue close to the corticomedullary junction, from which they migrate into the thymic tissue [94] (Figure 4). Although few progenitors migrate to the thymus, they extensively proliferate in response to the environmental cues they encounter, while starting a T-cell transcriptional program. These environmental signals include cytokines like SCF and Flt3L but also Wnt and Notch signals among many others [93].

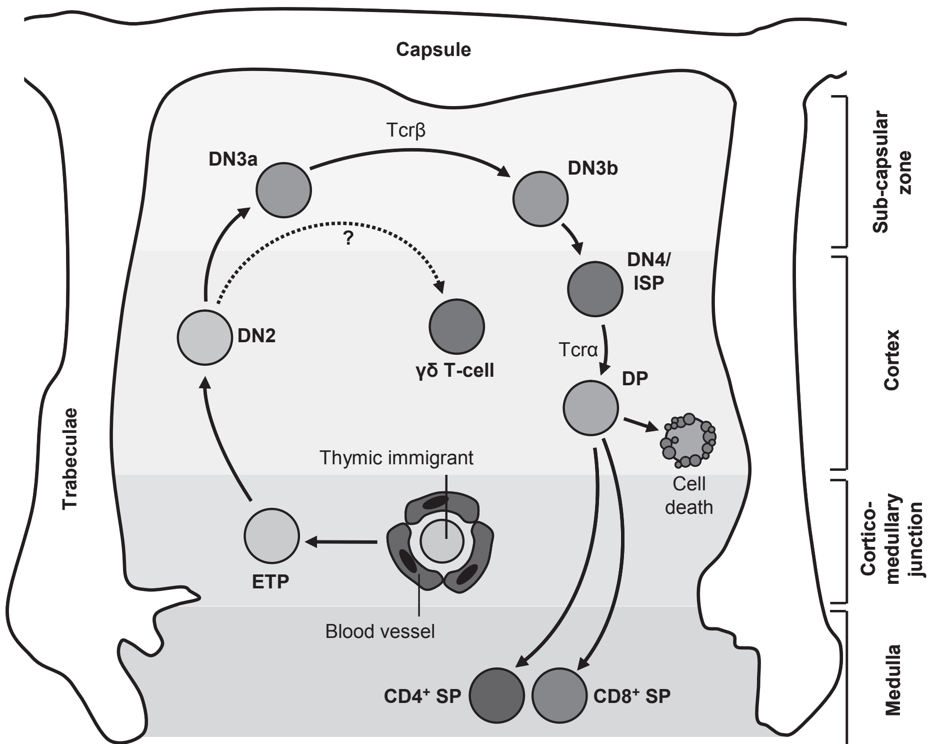

Figure 4. Stages in early T-cell development. Cross-section of an adult thymic lobule showing the migration path of T-cell precursors during development. Immigrant precursors enter the thymus through blood vessels near the cortico–medullary junction, the early T-cell precursors (ETP) subsequently migrate, and differentiate to double negative (DN), double positive (DP) and finally to single positive (SP) stages, through the distinct microenvironments of the thymus. β-selection occurs during the accumulation of the DN3 T-cells in the extreme outer portion of the thymus (sub-capsular zone). A directional reversal of migration back across the cortex towards the medulla occurs for the later stages of thymocyte development. ISP, immature single positive; TCR, T-cell receptor.

Many types of progenitors have been proposed to seed the thymus and most of them are known to circulate and express chemokine receptors and adhesion molecules, shown to be involved in thymus migration and settling. These include Ccr7, Ccr9, and the P-selectin ligand Psgl1, among others. Despites the several thymus-settling candidates, one major progenitor source consists of lymphoid-primed multipotent progenitors (LMPP) [95,96]. These progenitors are defined as Lin^{-} $Sca1^{+}$ $c\text{-}Kit^{+}$ $Flt3^{+}$ and, besides T-cell potential they can give rise to macrophages, dendritic cells, NK cells and B-cells, but not erythrocytes or mega-caryocytes [83,85]. After entering the thymus T-cell precursors develop through distinct stages. Progression through these stages involves gradual steps of lineage specification, characterized by the acquisition of a T-cell specific transcriptional program. Concomitantly to the lineage specification events, T-cell precursors gradually and irreversibly lose alternative non-T lineages potential till they are fully committed to the T-cell lineage. While B-cell potential is rapidly lost by the majority of the progenitors entering the thymus, the potential to develop into dendritic cells (DCs), natural killer (NK) cells and macrophages is maintained till later stages.

Initial studies identified cells with a $CD3^{-}CD4^{-}CD8^{-}CD25^{-}CD44^{+}$ surface phenotype (named DN1) as the most immature T-cell progenitors in the thymus. Further investigation demonstrated that this is still a highly heterogeneous population also containing mature NK, NKT-cells, γδ T-cells and also unrelated hematopoieitic cells. In addition efficient T-lineage progenitor activity was shown to reside in a small subset of DN1 cells expressing c-Kit, which was termed early thymic progenitors (ETP) [97-100]. ETPs are very efficient in the generation of DN2 cells (defined as $CD3^{-}CD4^{-}CD8^{-}CD25^{+}CD44^{+}$) but they still retain NK, DC, myeloid and at a lower extent also B-cell potential. Similarly to the LMPPs, a small fraction of ETPs also expresses Flt3 and CCR9. These are believed to be the most immature T-cell progenitors in the thymus.

The existence of alternative lineage potential in T-cell progenitors implies the existence of mechanisms to instruct a T-cell fate at the expense of other lineages. The most well known instructive signals to promote T-cell development are the Notch signals. Activation of the Notch signaling pathway by ligands of the Delta family was shown to be essential to induce T-cell development [101-105]. However, the precise role of Notch is still under debate. While it seems to be involved in the restriction of alternative lineage potentials by inhibiting B-cell and myeloid cell fates, it may alternatively promote survival and expansion of the T-cell progenitor populations [104,106-108]. Another signaling pathway shown to be essential for these early events in T-cell development is the Wnt/β-catenin/Tcf1 signaling pathway, which is currently seen as a rate-limiting positive regulator of the transition to the DN2 stage [109]. These topics will be further discussed in this chapter and later on in this thesis.

T-cell commitment in the thymus

Once in the thymus pro-T cells migrate through different anatomical zones which may provide different micrenvironmental signals that help the stablishment of a T-cell development program (Figure 4) [110,111]. As ETPs start migrating through the cortical region towards the sub-capsular zone they become more restricted to the T-cell lineage and start expressing genes critical for T-cell receptor (TCR) rearrangements, assembly and signaling, such as recombinase activating gene 1 (Rag1) and Rag2, CD3 chains and Lck, among others [91]. The progressive upregulation of T-cell identity genes is accompanied by the acquisition of a DN2 and DN3 ($CD3^-CD4^-CD8^-CD25^+CD44^-$) surface phenotypes.

Only at DN3 stage pro-T cells loose alternative potentials and become fully committed to the T-cell lineage. DN3 stage is characterized by an arrest in cell-cycle allowing in this way the rearrangement of the *Tcr* genes, which encode for the variable region of the antigen receptors in T-cells. These rearrangements occur through a process termed V(D)J recombination which allows the generation of a high diversity of antigen receptors. *Tcr* gene rearrangements occur in a fixed order. Efficient *Tcrd* and *Tcrg* genes rearrangement may result in a functional γδ-Tcr and the development of γδ-T cells. Alternatively a cell may develop through the αβ-lineage and rearrange the *Tcrb* gene. Successful rearrangement of the *Tcrb* gene is functionally tested by its expression on the cell membrane. Productively rearranged Tcrβ chains are coupled to an invariant pre-Tα chain to form the Pre-TCR complex. Signaling through the Pre-TCR induces proliferation, survival and differentiation, in a process called β-selection. Cells that pass β-selection are instructed to develop into the αβ-T cell lineage [112] and acquire DN4 ($CD3^-CD4^-CD8^-CD25^+CD44^-$), ISP ($CD3^-CD4^-CD8^+$ in mice or $CD3^-CD4^+CD8^-$ in humans) and later DP ($CD4^+CD8^+$) surface phenotypes. After these highly proliferative stages another arrest in proliferation is imposed once the cells reach the DP stage and start rearranging the *Tcra* gene. Efficient *Tcra* rearrangement leads to the expression of a TCRαβ complex on the cell surface. These TCRαβ complexes are then functionally tested for the recognition of self-MHC (major histocompatibility complex) molecules (positive selection) and absence of reactivity against self-antigens (negative selection) [113]. Therefore this stage is characterized by very high apoptosis in order to eliminate non-functional and auto-reactive T-cells [114]. Concomitantly with the positive and negative selection processes, cells with a functional T-cell receptor further maturate to $CD4^+$ T-helper cell or to $CD8^+$ cytotoxic T-cell lineages and migrate to the periphery.

Signaling transduction pathways in hematopoiesis

Developmental processes including hematopoiesis require a strict spatial and temporal coordinated gene expression program. The vast majority of patterning and cell-fate specification events in metazoans development are controlled by a small set of signaling pathways including

Wnt, Notch, TGF-β, Hedgehog, and receptor tyrosine kinases (RTK). Each pathway is used repeatedly in several different processes, activating different subsets of target genes in different developmental contexts. How this specificity is achieved is subject of extensive research. However, although extremely different concerning their biochemical mechanisms, three main common functional properties can be identified in all these signaling pathways. These are: activator insufficiency, cooperative activation and default repression and have been termed "The three habits of highly effective signaling pathways" [115].

Upon the binding of a ligand to the pathway receptor an intracellular signaling cascade is initiated involving intermediate activators (such as β-catenin in the Wnt pathway and N^{ICD} in the Notch pathway) that cannot initiate expression of target genes independently but rather require cooperation of other factors, which display specific patterns of expression [116]. In this way, "activator insufficiency" prevents the indiscriminate activation of all the target genes of a specific pathway in an inappropriate context. On the other hand, "cooperative activation" ensures specificity of the target genes induced depending on the specific combination of local activators and, thereby restricting target gene expression to the appropriate context. Cooperative activation may involve synergies at the level of DNA binding, chromatin remodeling and interactions with the basal transcription complex [116]. Nevertheless, a potential drawback of this elegant solution to increase specificity is the fact that co-activators can be partially sufficient to activate target genes in a signaling-independent fashion. This disadvantage is circumvented by "default repression" of target gene transcription, in the absence of pathway activating signals. Thus, activation of the pathway involves the switching of the target genes from a transcriptional repression to a transcriptional activated state, often using the very same signal-regulated transcription factors (such as Tcf/Lef in the Wnt pathway and Rbpj in the Notch pathway) [117-119]. Summarizing, these three functional properties explain the basic principles by which a high degree of specificity in the pattern, timing and level of expression of target gene is achieved [120].

The next two parts of this chapter will discuss the involvement of the Wnt and Notch signaling transduction pathways in the regulation of hematopoiesis.

1.2 Wnt signaling pathway

The term Wnt is derived from a contraction of the names for the *Drosophila melanogaste*r segment-polarity gene Wingless and Integrase-1 [121], a mouse protooncogene that was discovered as an integration site for mouse mammary tumor virus. Integrase-1 is the vertebrate homologue of *D. melanogaster* Wingless, thereby linking a key developmental gene to cancer [122]. There are 19 *Wnt* genes in the human and mouse genomes, all encoding lipid-modified secreted

glycoproteins (Table 1). Here, we use the term Wnt to refer to any of these family members. Wnt signaling is crucial for various basic developmental processes, such as cell-fate specification, progenitor-cell proliferation, establishment of the dorsal axis and control of asymmetric cell division. In hematopoietic cells, Wnt proteins not only function as proliferation-inducing growth factors, but might also affect cell-fate decisions, as morphogens do in other systems. The number of studies on Wnt signaling by immunologists and hematologists has increased sharply during the past few years. From a curious pathway that was investigated by a handful of developmental immunologists with interest in the thymus, where Wnt proteins initially were thought to act solely as cytokine-like growth factors for immature thymocytes, Wnt signaling has now entered mainstream immune-hematology. New and sometimes conflicting data potentially implicate Wnt signaling in the self-renewal of hematopoietic stem cells, the maturation of DCs, peripheral T-cell activation and migration, and the development of leukemias.

Wnt signaling: Multiple pathways

There are at least three different Wnt pathways: the canonical Wnt pathway, which involves β-catenin (also known as cadherin-associated protein-β) and members of the T-cell factor (Tcf)/ lymphocyte-enhancer binding factor (Lef) family; the planar cell polarity (PCP) pathway; and the Wnt-Ca^{2+} pathway.

Canonical Wnt signaling

Canonical Wnt signaling is also known as β-catenin/Wnt signaling and is the most well-known and best-characterized Wnt pathway. The vast majority of studies on immune and blood cells have focused on this pathway in which β-catenin is the central player. [134,135] (Figure 5) This 92 kD protein serves two roles in most cells: one as important component of cadherin-mediated cell adhesion, the other as the active signaling component of the canonical Wnt pathway.

In the absence of Wnt signaling free cytoplasmic β-catenin is kept at very low levels through proteosomal degradation. β-catenin degradation is accomplished through active phosphorylation at conserved regions by the ser/thr kinases glycogen synthase kinase 3β (Gsk-3β) and Casein Kinase 1 (Ck1). These proteins belong to the so called destruction complex, that also includes the scaffolding proteins axis inhibition protein 1 and 2 (Axin1 and Axin2), and the tumor suppressor protein adenomatous polyposis coli (Apc). β-catenin is phosphorylated first on Ser45 by Ck1, and then on Ser33, Ser37 and Thr41 by Gsk-3β to create recognition sites for the ubiquitin ligase β-transducin repeat-containing protein (β-Trcp), leading to its ubiquitylation and subsequent proteasomal breakdown [136] (Figure 5A).

At the cell membrane, upon binding of a Wnt protein to the Frizzled receptor and the co-receptor low density lipoprotein receptor-related protein 5 (Lrp5) or Lrp6, the signaling cascade

is initiated. Formation of the Frizzled–Lrp5/Lrp6 results in the ser/thr kinases inhibition. This inhibition, mediated via Dishevelled (Dlv), disrupts the destruction complex and allows the stabilization of β-catenin in the cytoplasm. Accumulation of β-catenin most probably in its amino-terminally dephosphorylated form [137] is followed by translocation to the nucleus where it binds to Tcf/Lef transcription factors. Tcf normally assembles a transcriptional repressor complex [138]. Formation of the active β-catenin/Tcf transcription-factor complex culminates in the activation of Wnt target genes. These genes have remained largely elusive in the haematopoietic system, with the exception of immature thymocytes, in which proliferation, cell adhesion and anti-apoptotic genes have been identified as Wnt targets [139] (Figure 5B).

New insights into the biochemistry of the canonical pathway have revealed dual roles for Gsk-3β and Ck1. Besides a negative involvement in the pathway by promoting β-catenin phosphorylation and degradation, they were also shown to be positively involved. By phosphorylation of specific residues of Lrp6 they allow docking of Axin which in this way is pulled away from the destruction complex. Thus, in contrast with their cytosolic forms, membrane associated Gsk-3β and Ck1 promote Wnt signaling upon activation of the pathway [140-142]. Recently Bilić *et al*. suggested a model in which Wnt ligands bridge Lrp6 and Frizzled transmembrane receptors leading to the subsequent formation of proteins aggregates by recruiting Dvl, Axin, Ck1 and Gsk-3β. The assembling of these large membrane-associated aggregates, called Lrp6-signalosomes, is mediated by Dvl through a still largely unknown mechanism. Axin recruitment in the signalosome may block β-catenin phosphorylation and degradation in the cytoplasm [143]. Later, these large complexes are internalized by Caveolin mediated endocytosis allowing recycling of the different Wnt components [143].

The canonical Wnt pathway is subject to strict regulation at different levels (reviewed in [144]). Binding of Wnts to the receptor complex can be actively prevented by naturally occurring soluble decoy receptors such as secreted Frizzled-related protein (sFrp) and Wnt inhibitory factor 1 (Wif1) [145]. This Wnt antagonist binds directly to Wnts, thereby altering their capacity to bind the Wnt receptor complex. Another class of soluble Wnt antagonist comprises the Dickkopf homologues (Dkk), which bind and block the Lrp5/Lrp6 co-receptor. In addition, Dkks can interact with another type of transmembrane receptors, the Kremens (Krm). Krm/Dkk1/Lrp6 form a ternary complex that disrupts Wnt/Lrp6 signaling by promoting endocytosis and removal of the Wnt receptor from the membrane [146].

In the nucleus, the cell autonomous inhibitor of β-catenin and Tcf (Icat) can inhibit interaction of β-catenin with Tcf and Lef molecules, thereby preventing assembly of the active bipartite transcription-factor complex [147]. In addition, at least eight isoforms of Tcf with different capacity to bind β-catenin are formed by alternative splicing and promoter usage, thereby influencing the responsiveness of cells to canonical Wnt signals [148]. In general, the longer Tcf isoforms have retained the amino-terminal catenin-binding domain, whereas the shorter

forms cannot bind catenins and function as naturally occurring repressors of the pathway.

The target genes activated by the canonical Wnt signaling pathway still remain highly unknown and differ between different tissues and probably between human and mouse (http://www.stanford.edu/~rnusse/pathways/targets.html). Nevertheless, *Axin2*, *c-Myc*, *CyclinD1*, *n-Myc*, *Lef1* and *Cd44* were shown to be regulated by Wnt signaling in different tissues [149-154].

Table 1. The 19 mammalian Wnt proteins and their role in hematopoiesis

Protein	Role in hematopoiesis	Signaling pathway	Refs
Wnt1	Growth factor for DN thymocytes; Produced by mouse macrophages.	Canonical/β-catenin	123,124
Wnt2b	Growth factor for human $CD34^+$ BM cells; Expressed in human BM B-cell progenitors.	Unknown	125,126
Wnt3	ND	Canonical/β-catenin	-
Wnt3a	Growth factor for mouse HSCs, CMPs, GMPs, pro-B cells and human $CD34^+$ prothymocytes.	Canonical/β-catenin	127-129
Wnt4	Growth factor for DN thymocytes	Wnt-$Ca2^+$	123
Wnt5a	Growth factor for mouse fetal liver HSCs, human $CD34^+$ BM and cord blood cells; Negatie regulator of mouse Pre- and Pro-B cells and thymocyte development.	PCP and Wnt-$Ca2^+$	126,130-132
Wnt5b	Expressed by human BM B-cell progenitors.	Wnt-$Ca2^+$	-
Wnt6	ND	Wnt-$Ca2^+$	-
Wnt7a	ND	Wnt-$Ca2^+$	-
Wnt7b	Produced by macrophages to induce cell death of vascular endothelial cells.	Wnt-$Ca2^+$	124
Wnt8a	Expressed by human BM B-cell progenitors.	Canonical/β-catenin	125
Wnt8b	ND	Canonical/β-catenin	-
Wnt9a	ND	ND	-
Wnt9b	ND	ND	-
Wnt10a	Expressed by human BM B-cell progenitors.	Canonical/β-catenin	125
Wnt10b	Growth factor for mouse fetal liver HSCs and human $CD34^+$ BM cells; Produced by mouse macrophages.	Canonical/β-catenin	126,130
Wnt11	ND	PCP and Wnt-$Ca2^+$	-
Wnt16	Target of E2A-PBX in Pre B-ALL; Expressed by human bone marrow B-cell progenitors.	ND	125,133

ALL, acute lymphoblastic leukemia; BM, bone marrow; CMP, common myeloid progenitor; DN, double negative; GMP, granulocyte-monocyte progenitor; HSC, hematopoietic stem cell; ND, not determined; PCP, planar cell polarity.

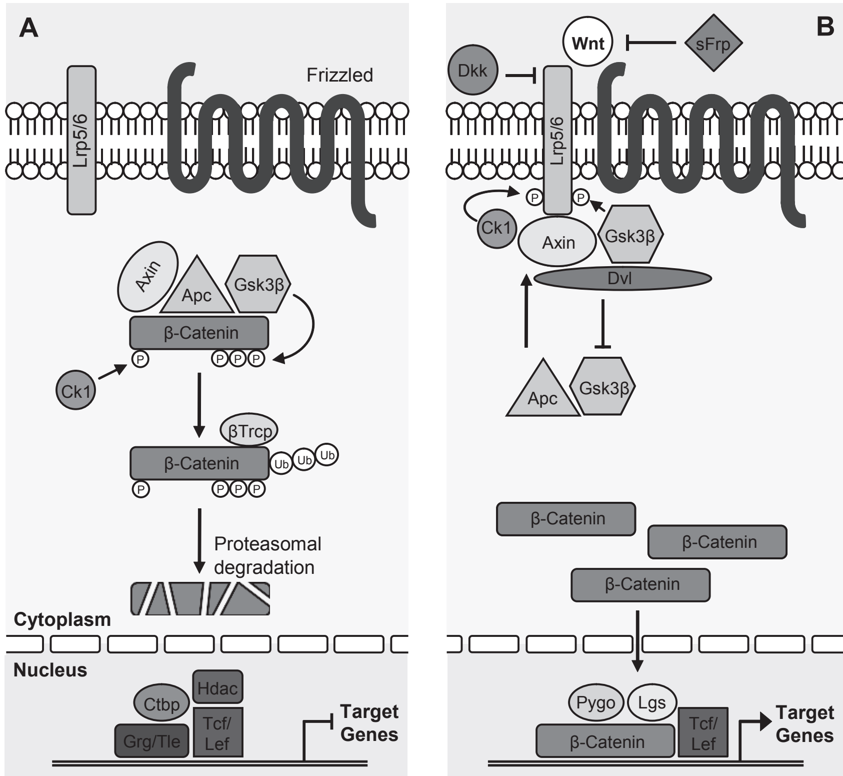

Figure 5. Canonical or Wnt-β-catenin-Tcf/Lef signaling. A) In the absence of Wnt signaling, β-catenin levels in the cytoplasm and nucleus are low as a result of continuous fosphorylation by the serine/threonine kinases Ck1 (casein kinase 1) and Gsk3β (glycogen synthase kinase 3β), leading to binding of β-transducin-repeat-containing protein (βTrcp) and to ubiquitylation and degradation by the proteasome. The destruction complex is composed of Ck1 and Gsk3β, as well as the anchor proteins Axin1 (axis inhibition protein 1) and Apc (adenomatous polyposis coli). In the nucleus, Tcf (T-cell factors) are bound by co-repressors such as Grg/Tle (Groucho/transducin-like enhancer) proteins that shut off expression of Wnt target genes. Other components of the repressor complex include CTbP (C-terminal binding protein) and Hdac (histone deacetylases). β-catenin in the nucleus is inhibited from binding Tcf by Icat (cell autonomous inhibitor of β-catenin and Tcf). The Frizzled receptor complex (composed of Frizzled and Lrp5 (Ldl receptor related protein 5) or Lrp6) can also be actively inhibited by receptor-bound soluble inhibitors such as Dkk1 (Dickkopf homologue 1). **B**) Upon binding of a lipid-modified Wnt protein to the receptor complex, a signaling cascade is initiated. LRP is phosphorylated by Ck1 and Gsk3β, and Axin1 is recruited to the plasma membrane. The kinases in the β-catenin destruction complex are inactivated and β-catenin translocates to the nucleus to form an active transcription factor complex with Tcf, leading to transcription of a large set of target genes. In the nucleus, β-catenin binds to Tcf and Lef factors and recruits co-factors such as legless (Lgs; also known as Bcl9) and Pygopus (Pygo), Cbp/p300, brahma and Med12/mediator to initiate transcription. Dvl, mammalian homologue of Drosophila Dishevelled.

These genes are generally involved in cell cycle regulation, apoptosis and in the transduction of the Wnt signals them-selves.

Non-canonical Wnt signaling

Lrp5 and/or Lrp6, and β-catenin are not involved in non-canonical Wnt signaling. Of the many non-canonical pathways that have been described in model organisms such as *D. melanogaster, Xenopus laevis* and *C. elegans*, the Planar Cell Polarity (PCP) and the Wnt-Ca^{2+} pathways are the best characterized and have been shown to have roles in hematopoiesis [131,132].

PCP signaling is often initiated by Wnt5a and Wnt11 through Frizzled and Dvl (but not Lrp) and might involve G proteins [155] (Figure 6). Dvl in association with Daam (Dishevelled-associated activator of morphogenesis) activates the Rhoa (Ras homologue gene-family member A)-Rock (Rho-associated coiled-coil-containing protein kinase) pathway, which mediates cytoskeletal re-organization [156]. Dvl also activates Rac1, and both of these small GTPases (Rhoa and Rac1) activate the Jun N-terminal kinase (Jnk, stress-response) pathway, which influences the cytoskeleton and thereby cell shape. The PCP pathway regulates positional signals in model organisms, is important in cell adhesion and migration by regulating changes in the cytoskeleton, and might inhibit canonical Wnt signaling in lymphocytes by downregulating β-catenin protein levels [131,132]. Several downstream pathways of DVL regulate the actin cytoskeleton and cell polarity, although this has not yet been shown in cells of the immune system.

One of the more recently discovered Wnt pathways is the Wnt-Ca^{2+} pathway, which can influence the activity of both non-canonical and canonical Wnt signaling. Wnt5a and Frizzled-2 are the most probable candidates to initiate this pathway [157,158]. The Wnt-Frizzled complex, through a G protein, activates phospholipase C (Plc), leading to the cleavage of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Figure 6), which activates protein kinase C (Pkc). InsP3 binding to its receptor on intracellular calcium stores leads to an increase in the cytoplasmic level of Ca^{2+} ions, which are normally kept low. This increased Ca^{2+} concentration activates the phosphatase calcineurin and several calcium-dependent kinases, including Pkc. Pkc in turn has a wide variety of effects, as does the increased activity of calcineurin, which activates Nfat (nuclear factor of activated T cells). In *Xenopus* spp., this pathway has been shown to regulate ventral patterning, which is mediated in part by Xenopus Nfat. An intriguing possibility is that the Wnt-Ca^{2+} pathway activates Nfat [159,160], which is involved in T-cell receptor (TCR)-mediated activation and interleukin-2 (IL-2) production in T cells. Another connection between Wnt and Nfat signaling has been described involving Gsk3β, which functions in exporting Nfat out of the nucleus in T cells [161]. So, there may be many levels at which the Nfat and Wnt pathways interact.

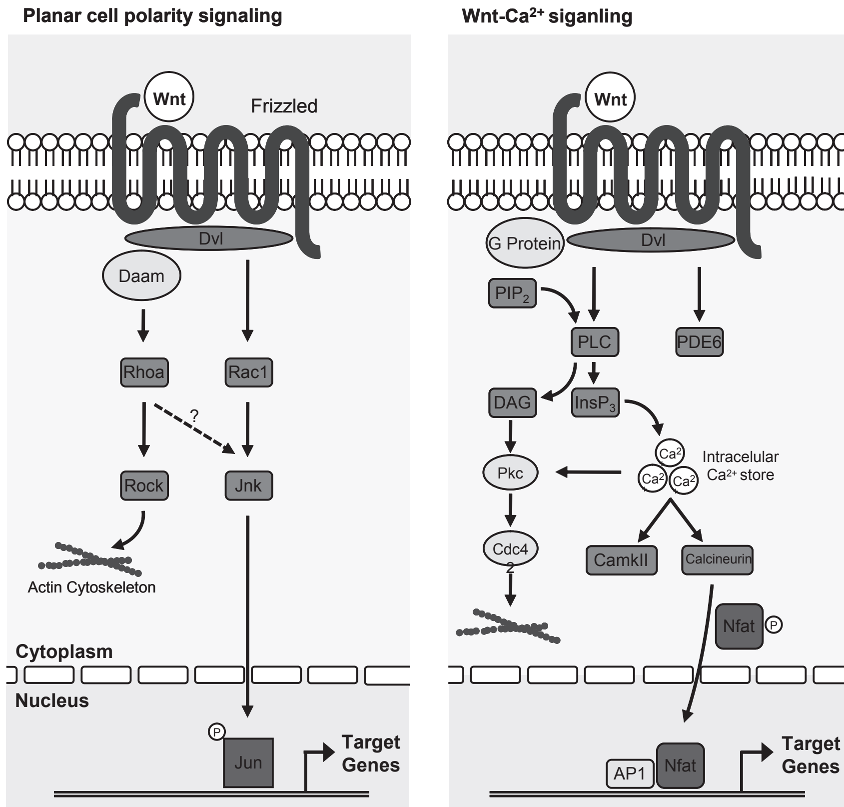

Figure 6. Non-canonical Wnt signaling. A) Planar cell polarity (PCP) signaling does not involve β-catenin, Lrp (LDLreceptor related protein) or Tcf (T-cell factors), but leads to the activation of the small GTPases Rhoa (Ras homologue gene-family member A) and Rac1, which activate the stress kinase Jnk (Jun N-terminal kinase) and Rock (Rho-associated coiled-coil-containing protein kinase 1) and leads to remodeling of the cytoskeleton and changes in cell adhesion and motility. Through largely unknown mechanisms, canonical β-catenin signaling can be inhibited by the PCP pathway. **B**) Wnt-Ca2^{+} signaling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the kinases Pkc (protein kinase C) and CamkII (calcium calmodulin mediated kinase II) and the phosphatase calcineurin. The activation of Plc (phospholipase C) by Dvl (mammalian homologue of *Drosophila* Dishevelled) leads to the cleavage of PtdIns(4,5)P2 (phosphatidylinositol bisphosphate) into InsP3 (inositol trisphosphate) and DAG (diacylglycerol). DAG, together with calcium, activates Pkc, whereas InsP3 binding to receptors on the membranes of intracellular calcium stores leads to a transient increase in cytoplasmic free calcium, often also triggering an increase from extracellular stores. AP1, activator protein 1; Cdc42, cell-division cycle 42; Daam, Dishevelled-associated activator of morphogenesis; Nfat, nuclear factor of activated T cells; Pde6, phosphodiesterase 6.

Wnt signaling: Regulation of HSC fate

Hematopoietic stem cells (HSCs) are responsible for the continuous production of blood cells and consequently help sustain immune function. This property is conferred by the capacity to self-renew and to differentiate into all blood lineages. The regulation of HSC fate depends on signals provided by the surrounding microenvironment, the so called HSC niche. Signals from the HSC niche form a complex network in which Wnt might have a role [60].

Evidence of Wnt signaling in HSCs

Several important studies have addressed a possible role for canonical Wnt signaling in HSC biology. Although collectively most reports indicate a role for Wnt signaling in the self-renewal of HSCs and proliferation of progenitor cells, this issue is not fully settled. Exactly how important Wnt signaling is, under which conditions (for example, steady-state compared with regenerating cells), at which dosage and using which transcriptional activator, currently remain controversial topics.

The first evidence for the involvement of Wnt signaling in HSC function stems from studies showing that Wnt ligands and receptors are produced not only by HSCs and primitive progenitors, but also by the cells constituting the surrounding microenvironment, both in mice [130] and humans [126], and in adult as well as in fetal hematopoietic organs. These patterns of expression indicated both paracrine and autocrine effects of Wnt ligands [162] (Figure 7). Wnt5a, Wnt2b and Wnt10b were shown to promote the *in vitro* proliferation of human HSCs and primitive progenitors and to maintain an immature phenotype in these cells [126]. It will be important to determine which *Wnt* genes and which corresponding Wnt receptors are expressed by HSCs and by stromal cells in the HSC niche, to determine the potential ligand-receptor combinations that might affect HSC function.

Mouse HSCs in their normal microenvironment respond to Wnt signals through the canonical Wnt-β-catenin-Tcf/Lef pathway, as determined by reporter assays 127 (Table 2). *In vitro* stimulation with purified Wnt3a led to increased reporter activity in Bcl2 (B-cell lymphoma 2)-transgenic LSK cells [127] and increased self-renewal capacity [128]. Correspondingly, inhibition of this pathway by ectopic expression of Axin1 or with a truncated form of Frizzled resulted in decreased *in vitro* proliferation and *in vivo* repopulation capacity [127]. Of interest, expression of constitutively active β-catenin in lymphoid or myeloid progenitors generated uncommitted cells with multilineage differentiation potential [173], which indicates that Wnt signaling has a role in maintaining an undifferentiated state.

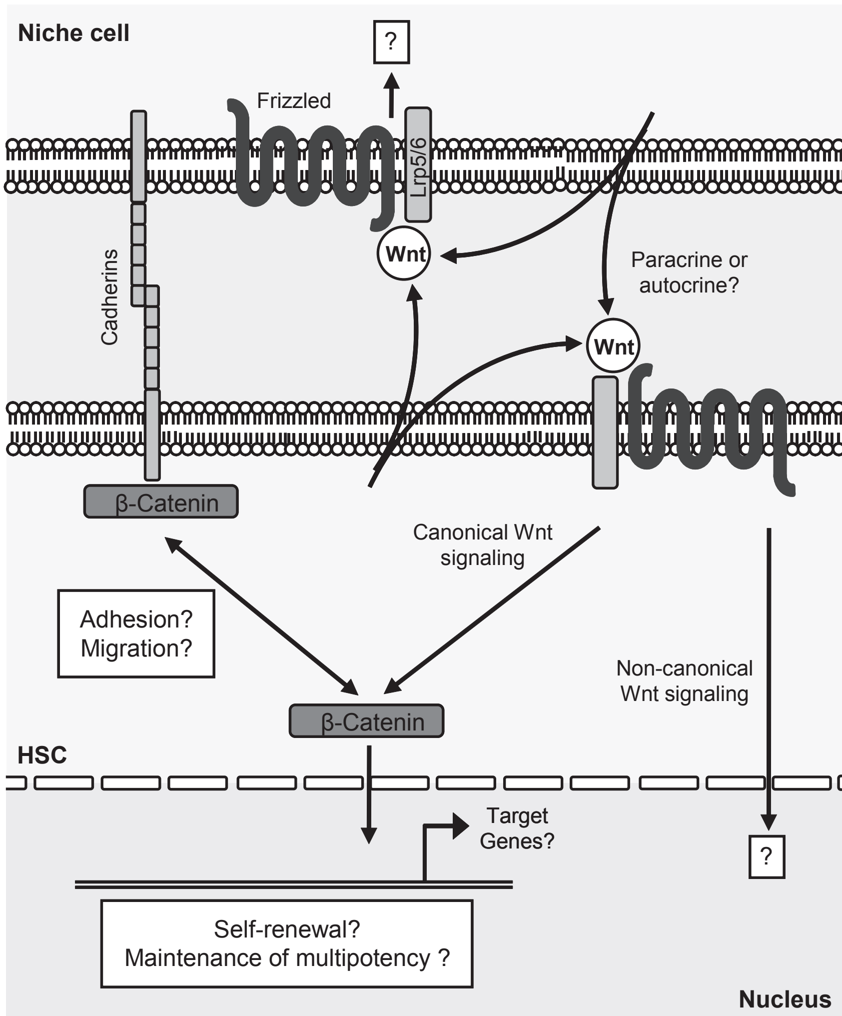

Figure 7. The potential role of Wnt in the stem-cell niche. Wnt ligands are probably produced both by haematopoietic stem cells (HSCs) and the niche environment, thereby influencing HSC function in an autocrine or paracrine manner. Through activation of the canonical pathway, Wnt proteins might influence the self-renewal and/or differentiation of HSCs. However, the target genes activated by this pathway are still elusive and controversial. B-catenin might also have a secondary role in regulating cell adhesion and mobility by connecting cadherins (in adherens junctions) to components of the cytoskeleton. Both N-cadherin and β-catenin were found to localize asymmetrically at the osteoblast–HSC synapse, which indicates that they might be important in the interaction with the niche. By keeping β-catenin at the cell membrane, cadherin interactions might decrease the level of cytoplasmic β-catenin, functioning in this way as a negative regulator of Wnt signaling. A possible function for Wnt signaling through activation of the non-canonical pathway in HSCs is not clear. Wnts might also regulate HSC fate indirectly by influencing the stromal cells that constitute the HSC niche. The niche might also merely provide secreted factors that are important for HSCs, such as Wnt proteins. Lrp, Ldl-receptor related protein.

Table 2. The Tools to identify and visualize Wnt signaling

Aproach	Principle	Aplication	Caveat	Refs
TOP/FOP reporter	Reporter gene(often luciferase) for β-catenin/ Tcf1 transcrptional activation containing Tcf/ Lef binding sites. TOP contains the optimal binding sites and FOP contains mutated sites to use as control	Transfection of cell, mostly cell lines; Transduction of primary cells (T-cells, HSCs, DCs)	Depending on minimal promoter used different results can be obtained. Not only reporting Wnt signaling	127,163-166
Wnt reporter mice	Similar TOP reporter constructs linked to LacZ or GFP	Measurment of in vivo Wnt signaling	No direct control (for example FOP mice) available	167-169
High levels of β-catenin	Wnt signaling stabilizes β-catenin at protein level	Histological slides, western blot and fluorescent microscopy	Do not always correlate with actual Wnt activity	170
Dephosphorilated β-catenin	Only dephosphorilated β-catenin migrates to the nucleus and is signaling competent	Histological slides, western blot and fluorescent microscopy	Confusing reports owning to wrong reagents. Antibody against activated β-catenin is reagent of choice.	137,171
Target genes	Measurment of direct Wnt target genes activity	PCR, Q-PCR, sometime western blot	Target genes are controversial (for example CyclinD1 may not be a target gene and Axin2 is a good candidate)	167,172

Axin2, axis inhibition protein 2; DC, dendritic cell; GFP, green fluorescent protein; Lef, lymphocyte-enhancer-binding factor; Tcf, T-cell factor.

Controversies

Two reports [174,175] using an *in vivo* mouse model for the expression of stabilized β-catenin have given different results to the work described above. A previous study had shown increased multilineage reconstitution of recipient mice using retroviral vectors to overexpress a stabilized form of β-catenin in stem/progenitor cells [127]. However, these results could not be reproduced using the inducible Mx–Cre system to achieve stabilization of β-catenin [174,175] and, instead, a block in multilineage differentiation was observed. In the latter studies, stabilized β-catenin forced the quiescent HSCs to enter into the cell cycle, leading to a transient expansion of the HSC population and, consequently, to exhaustion of long-term HSCs, as shown by failure to reconstitute lethally irradiated mice [174,175]. As the former study [127] was carried out on a Bcl2-transgenic background to minimize pro-differentiation stimuli and protect against the

deleterious, apoptotic effects of β-catenin overexpression, this might partially explain the differences observed with the different approaches. Furthermore, different *in vitro* conditions [176] did not result in the same effect of Wnt3a on Bcl2-transgenic LSK cells, which might reflect the complex interactions of Wnt signaling with other pathways, like anti-apoptosis pathways. It has been shown that Wnt signaling can reverse the fate of lineage-committed cells and might help to maintain a stem-cell pool [173]. Therefore, one would expect an even stronger Wnt stimulus, such as provided by Mx-Cre mediated expression of stabilized β-catenin [173-175], to arrest differentiation completely and eventually lead to bone marrow failure, as was indeed seen. So, these results suggest that the level of Wnt signaling differentially influences HSC biology.

Disparate results on the importance of canonical Wnt signaling came from studies using the inducible Mx-Cre system, in which deletion of β-catenin in HSCs did not affect self-renewal and multilineage differentiation capacity [177]. It was postulated that a β-catenin homologue, γ-catenin (also known as plakoglobin), might compensate for the lack of β-catenin. However, when both β- and γ-catenin were absent, no phenotype was observed in the hematopoietic system [163,178]. Interestingly, one of these studies showed that in the absence of both β- and γ-catenin, the Wnt signaling pathway was still active in HSCs, thymocytes and peripheral T cells [163]. These authors suggested an additional factor might exist that can transduce canonical Wnt signals in the hematopoietic system. Indeed, several candidate factors have been proposed in other developmental systems [179], although in most other cell types and tissues where canonical Wnt signaling is important, deletion of β-catenin leads to a detectable phenotype. However, recent work using the conditional Vav-Cre system to drive deletion of β-catenin in HSCs showed that β-catenin-deficient HSCs had reduced self-renewal capacity, indicating a role for Wnt signaling in the long-term growth and maintenance of HSCs [180]. The conflicting results obtained by using different approaches to conditionally delete β-catenin in HSCs (for example, Mx–Cre or Vav-Cre) warrant a critical look at the deletion methods used.

While Mx-Cre mediated deletion is activated upon stimulation with the synthetic double-strand RNA Poli(I:C) and consequent production of Interferon-α, which was shown to affect HSC function [181,182]. Vav-Cre allows deletion in the all hematopoietic system and endothelial cells both in the adult and during embryonic development [183,184]. A similar discrepancy between Mx-Cre-mediated and Lck-Cre-mediated deletions has also been observed in thymic phenotypes after conditional deletion of β-catenin (see below). Differences in the completeness of deletion and whether adult or fetal hematopoiesis is targeted by Cre-mediated deletion of β-catenin might in part explain differences between these approaches.

Gene activation by Wnt in HSCs

Although collectively these suggests a role for canonical Wnt signaling in HSC biology, the genetic program activated by this pathway is still largely unknown. The upregulation of components of the Notch signaling pathway, such as *Notch1* and the Notch target genes *Hes1* (hairy and enhancer of split 1) and *Dtx1* (Deltex homologue 1), by Wnt-β-catenin signaling in LSK cells 127,185 indicated that the Notch and Wnt pathways might cooperate to maintain HSC properties. In addition, the genes homeobox B4 (*Hoxb4*) [127,185] and Bmi1 [174,175], which were shown to be important for HSC function, were found to be regulated by Wnt signaling. The relevance of the Wnt pathway was further highlighted by studies showing that crucial factors for HSC function interfere with the responsiveness to and activation of the Wnt signaling pathway. For example, one crucial factor for mouse fetal HSCs, Sox17 (sex-determining region Y-Box 17), was shown to negatively regulate the expression of Dkk1, a negative regulator of the Wnt signaling pathway. In this way, Sox17 could maintain HSCs by promoting Wnt signaling, but this possibility still needs to be experimentally verified [55]. In conclusion, these recent studies indicate that involvement of the Wnt signaling pathway in HSC function can be regulated at many different levels.

Wnt signaling in developing T and B cells

The first reports of the importance of Wnt signaling in the immune system arose from studies of Wnt signaling in the context of T-cell development in the thymus. Using soluble Frizzled receptors as decoys for Wnt proteins, it was shown that such receptors inhibit thymocyte differentiation in fetal thymic organ cultures (FTOC), an *in vitro* assay for T-cell development, apparently mainly by negatively affecting thymocyte proliferation [123]. In accordance with this, the thymi of mice deficient in both Wnt1 and Wnt4 had low cellularity [186]. These first reports on Wnt signaling in the immune system were foreshadowed by seminal work showing defects in T- and B-cell development in mice deficient for the Wnt-responsive transcription factors Tcf1 and Lef1, respectively [129,187,188]. Young Tcf1-deficient mice have an incomplete block at the DN1, DN2 and immature SP stages of thymocyte development, whereas older mice have a complete block most likely at the DN1 stage [188] (Figure 8). Although Lef1$^{-/-}$ mice have normal T-cell development, mice deficient in both Lef1 and hypomorphic Tcf1 have a complete block in T-cell differentiation at the immature SP stage, which indicates redundancy between these factors during thymocyte development [187]. The key demonstration that Tcf1 and Lef1 interact with the Wnt mediator β-catenin, which converts them from repressors to transcriptional activators [148,189,190], fitted well with the notion that canonical Wnt signaling provides crucial proliferative signals to immature T and B cells [123,186,191].

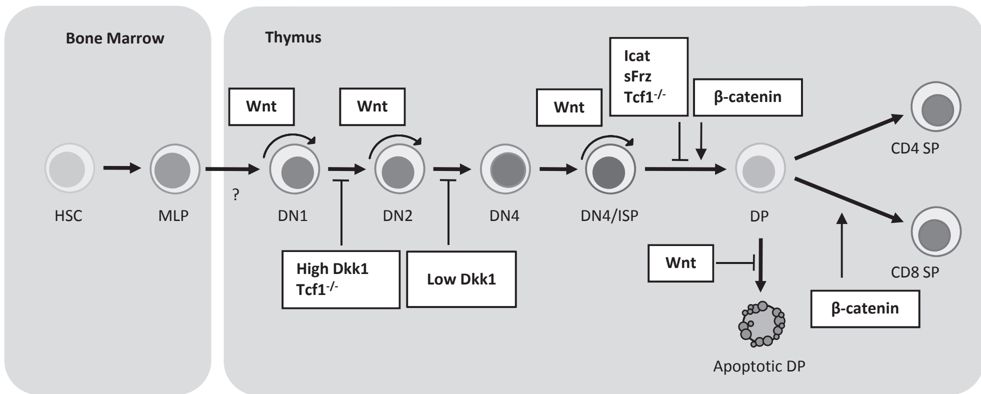

Figure 8. Wnt signaling in the thymus. The first cells to appear in the thymus are rare progenitor cells commonly referred to as ETPs (early thymic progenitors), which are part of the DN ($CD4^{-}CD8^{-}$ double negative (DN)) compartment. DN cells proliferate at a rapid rate, in part mediated by Wnt signaling. Inhibition of the Wnt pathway, by ectopic expression of soluble Frizzled receptor (which acts as a decoy receptor), Dickkopf homologue 1 (Dkk1; which inhibits binding to Ldl receptor related protein (Lrp) co-receptors) or the cell autonomous inhibitor of β-catenin and Tcf (Icat; which disrupts the β-catenin–Tcf interaction) leads to inhibition of T-cell development at various points in the DN pathway. Similarly, incomplete blocks in T-cell development are observed at DN1, DN2 and ISP (immature single positive) stages of development in Tcf1 (T-cell factor 1)-deficient mice. Wnt signaling also regulates the survival of double positive (DP; $CD4^{+}CD8^{+}$) thymocytes by inducing expression of the anti-apoptotic protein Bcl-Xl, and stabilized β-catenin influences positive selection and interleukin-7 receptor signaling, leading to increased numbers of $CD8^{+}$ SP (single positive) thymocytes. In addition, the levels of CD4 on both DP and $CD4^{+}$ SP cells are regulated in part by Tcf1 (not shown in the figure). HSC, haematopoietic stem cells; MLP, multilineage progenitor.

DN to DP thymocyte development

As the number of progenitors that enter the thymus is limited, an enormous expansion of the population takes place during the DN1 and DN2 stages of T-cell development. Cytokines, such as SCF, which binds Kit, IL-7 and Flt3 ligand, and also Wnt proteins, are responsible for the initial proliferation of thymocytes before β-selection. Indeed, it was previously shown that Wnt signaling occurs in the thymus and is most active in the immature DN stages [164]. Differential sensitivity to Wnt signaling at different thymocyte stages was not caused by increased expression of Frizzled or Wnt proteins on the most immature thymocytes, as Frizzled and Wnt seem to be expressed at comparable levels in all thymocyte subsets. Instead, increased expression of positively acting canonical Wnt factors (such as β-catenin) and decreased expression of inhibitory molecules (such as Axin1) was observed in DN thymocytes. So, responsiveness to Wnt signaling in thymocytes is regulated by the differential expression of intracellular signal transduction molecules [164]. Several studies have shown that Wnt signaling is important at the DN to DP transition of thymocyte development. Expression of Icat [192], which inhibits Wnt signaling by preventing binding of β-catenin to Tcf and Lef factors, blocks

this transition, but does not affect later stages of development (Figure 8). Similarly, the secreted Wnt inhibitor Dkk1, which blocks binding of Wnt to the required Lrp co-receptor, inhibits thymocyte differentiation at the DN stage in a dose-dependent manner, such that high levels of Dkk1 lead to complete inhibition of T-cell development at the DN1 stage [164] (Figure 8). Activation of the Wnt pathway by overexpressing activated forms of β-catenin led to the generation of more thymocytes [193], could bypass the requirement for pre-TCR signals in mice lacking a pre-TCR [194,195], and activated proliferation-associated genes in immature thymocytes[139]. Importantly, conditional T-cell-specific deletion of β-catenin, using the proximal Lck promoter to control Cre expression, impaired T-cell development at the β-selection checkpoint, leading to a marked decrease in the number of peripheral T cells [196]. Other important evidence for the role of Wnt signaling through β-catenin and Tcf is provided by studies using transgenic or retroviral reconstitution of Tcf1-deficient mice with various forms of Tcf1 that can or cannot interact with β-catenin [123,197]. Only those forms of Tcf1 that can interact with β-catenin can restore T-cell development in Tcf1-deficient mice, which is consistent with an important role for canonical Wnt signaling through β-catenin in the early stages of T-cell development in the thymus. Finally, conditional deletion of the Apc tumor-suppressor gene, which is part of the β-catenin destruction complex, also disrupts T-cell development in the thymus, at least in part by affecting β-catenin signaling [198].

DP to SP thymocyte development

Several lines of evidence indicate that Wnt signaling also regulates aspects of positive and negative selection and the DP to SP transition, although the canonical Wnt cascade is less active in signaling at this stage of development. Expression of CD4 by DP thymocytes is regulated in part by β-catenin-Tcf signaling [199], and *Tcf1*-deficient mice express lower levels of CD4 on DP and $CD4^+$ SP cells (Figure 8). Furthermore, a series of elegant experiments showed that over-expressing stabilized β-catenin regulates the positive selection of thymocytes [200,201]. The generation of fully selected $CD4^+$ and $CD8^+$ SP thymocytes occurred simultaneously when stabilized β-catenin was overexpressed, in contrast to the normal situation whereby the generation of $CD8^+$ SP thymocytes lags behind that of $CD4^+$ SP thymocytes. In addition, IL-7R signaling during positive selection was augmented, thereby promoting the development of CD8+ SP thymocytes [201] (Figure 8). The thymic microenvironment generates signals that are required for T-cell development from HSCs and progenitor cells. Although the lymph-node microenvironment produces similar signals, lymph node-derived progenitors fail to generate mature T-cells when cultured with stromal cells that express the Notch ligand Delta1. Lymph node stromal cells can provide most of the important signals for T-cell development, including IL-7, SCF and the Delta1, but they do not express Wnt transcripts [202]. Lymph-node T-cell progenitors can generate mature T cells when they are cultured with stromal cells expressing

Wnt4. This study therefore indicates that Wnt and Notch signals are required to act together for the induction of T-cell development.

Non-canonical Wnt signaling in T-cells

The role of the non-canonical Wnt-Ca^{2+} pathway in T-cell development was investigated using mice deficient for Wnt5a, or by providing high levels of exogenous Wnt5a [132]. Mice deficient for Wnt5a die at birth due to severe anatomical defects; therefore, T-cell development was investigated in fetal thymic organ cultures derived from these mice. Wnt5a produced in the thymic stroma was not essential for the development of DN and immature SP thymocytes, but it regulated the survival of αβ-lineage thymocytes. Loss of Wnt5a downregulated expression of the pro-apoptotic gene Bax, promoted expression of the anti-apoptotic gene Bcl2 and therefore, inhibited the apoptosis of DP thymocytes. On the other hand, exogenous Wnt5a increased the apoptosis of fetal thymocytes [132]. Of interest, and similar to other developmental systems, Wnt5a-induced Ca2+ signaling regulated the survival of DP and mature SP thymocytes in a manner that was antagonistic to canonical Wnt signaling.

B-cell development

Several reports have indicated that canonical Wnt signaling has a similar, although perhaps less pronounced, role in B-cell progenitors in the bone marrow to that in T-cell progenitors in the thymus. Lef1-deficient mice, which die shortly after birth, have decreased numbers of $B220^{+}$ B-cells in fetal liver and neonatal bone marrow [129,187]. In accordance with this, Lef1-deficient pro-B.cells show decreased proliferation and increased apoptosis, whereas culturing wild-type mouse fetal pro-B cells in Wnt3a-conditioned medium leads to increased proliferation [129]. Frizzled-9-deficient mice also have defective B-cell lymphopoiesis involving depletion of developing B-cells in the bone marrow, particularly in the cycling pre-B -cell stage [203]. However, for human B-cell progenitors, Wnt3a stimulation leads to decreased proliferation despite increased levels of β-catenin [125]. The reason for this species difference is unclear, although other B-cell growth factors such as IL-7 show similar species differences in that IL-7 functions as a potent growth factor for mouse, but not human, pre-B cells. Similar to the antagonism between Wnt5a and canonical Wnt signaling in the thymus, Wnt5a signals through the non-canonical Wnt-Ca^{2+} pathway to negatively regulate B-cell proliferation in a cell-autonomous fashion [131]. In addition, Wnt5a hemizygous mice develop clonal myeloid and B-cell leukemias, in after losing the heterozygosity for the Wnt5a allele, in the leukemic cells. Thus, Wnt5a inhibits both B- and T-cell development by counteracting canonical Wnt signaling and functions as a tumor suppressor in developing B cells.

Wnt signaling in hematological malignancies

In most solid tumors, constitutively active Wnt signaling is an important contributing or even initiating event for the development of such cancers [135]. The prototypical example is colon carcinoma, in which carcinogenesis is induced by inactivating mutations in the tumor suppressors Apc or Axin1 or activating mutations in β-catenin [204]. Over the past few years, it has become apparent that deregulated Wnt signaling is also important in the development of hematological malignancies. Although the underlying mechanism is not completely resolved, mutations leading to the overexpression of Wnt genes or β-catenin and γ-catenin seem to be important.

Acute Myeloid Leukemia (AML)

AML is a clonal malignancy that originates in HSCs or myeloid progenitors cells. AML is frequently associated with chromosomal translocations resulting in abnormal fusion proteins (such as AML1–ETO, PML–RARα, PLZF–RARα and CBF1–MYH11) or with activating mutations in the receptor tyrosine kinase Flt3, which is the receptor for the cytokine Flt3L. Target genes of these fusion proteins have been found to be associated with Wnt signaling, in particular γ-catenin [205]. In addition, high levels of β-catenin expression in AML cells indicate poor prognosis [206]. Finally, to underscore the importance of Wnt signaling in myeloid cells, myeloid progenitor cells of patients with severe congenital neutropenia had a marked downregulation of expression of Lef1 and its target genes. So, Lef1 has an important role in normal human myelopoiesis [207].

Chronic Myeloid Leukaemia (CML)

Almost all cases of CML carry the classical Philadelphia chromosome, which is caused by a t(9,22) translocation leading to the production of the abnormal BCR-ABL fusion protein. It has been shown that Wnt signaling is activated in the so-called 'blast crisis' of the disease, the terminal phase of CML in which the disease resembles acute leukemia [208], but is inactive at other stages, although the reason for this is still unclear. In a subset of patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), BCR-ABL translocations are also found. Interestingly, whereas β-catenin deletion in mice markedly inhibits the development of BCR-ABL-induced CML, the development of BCR-ABL-induced BCP-ALL was not altered [180]. As CML and ALL might arise from different cells of origin, these studies indicate that the use of Wnt signaling might depend on the cell of origin of a tumor.

Acute Lymphoblastic Leukemia (ALL)

Given the importance of Wnt signals for normal T-cell development, by analogy with Notch signaling, constitutively active Wnt signaling should lead to ALL [209]. Experimental evidence

for this notion has been provided by making use of conditional deletion of exon 3 in β-catenin, which leads to a constitutively active form of β-catenin that can no longer be phosphorylated and broken down in the proteossome [210]. This results in aggressive T-cell lymphomas that can invade the bone marrow and are transplantable into irradiated recipient mice. These tumors arise independently from Notch signals, indicating that although the Notch and Wnt pathways cooperate during the initial stages of T-cell development in the thymus, they can act independently during leukemogenesis. Another study indicated yet another mechanism by which oncogenic forms of β-catenin can cause thymic lymphomas; constitutively active forms of β-catenin were shown to cause p53-independent oncogene-induced-senescence, growth arrest and finally lymphoma development [211].

1.3 Notch signaling pathway

The Notch phenotype was initially described in *Drosophila* by Morgan in 1917 upon observation of a mutant strain with a phenotype resembling notches at the end of their wings [212]. Later, Notch was found to encode a highly evolutionary conserved type I single-pass transmembarne receptor that can be activated by two distinct cell surface ligands called Delta and Serrate. Since then, Notch signals were shown to regulate a broad range of cellular functions including cell proliferation, differentiation, migration and apoptosis in many developmental contexts, and have been implicated in several different developmental processes and human pathologies, such as leukemia and Alzheimer disease [213,214].

Notch receptors and their ligands

In mammals, 4 different Notch receptors (Notch1-4) highly homologous to the *Drosophila* Notch receptor, were identified. These receptors can be activated by 5 different ligands: 3 Delta (Delta 1, 3 and 4) and 2 Jagged (Jagged 1 and 2) ligands homologous to the *Drosophila* ligands Delta and Serrate, respectively [213] (Figure 9). The first mammalian homologue of the *Drosophila* Notch receptor was described in patients with T-cell acute lymphoblastic leukemia (T-ALL). These patients harbor a chromosomal translocation that juxtaposes a truncated form of the *NOTCH1* gene to the TCRB locus, resulting in the expression of an activated form of NOTCH1, termed TAN1 (translocation-associated Notch homolog) [215].

After being synthesized, Notch receptors are cleaved in the trans-Golgi network by a furin-like protease, generating a heterodimeric receptor, composed of a N-terminal extracellular subunit (N^{EC}) and a C-terminal intracellular subunit (N^{IC}) that also comprises the transmembrane domain (Figure 9). Both subunits of the Notch receptor are noncovalently linked to each other. The N^{EC} contains an array of 29-36 epidermal growth factor (EGF)-like repeats that interacts

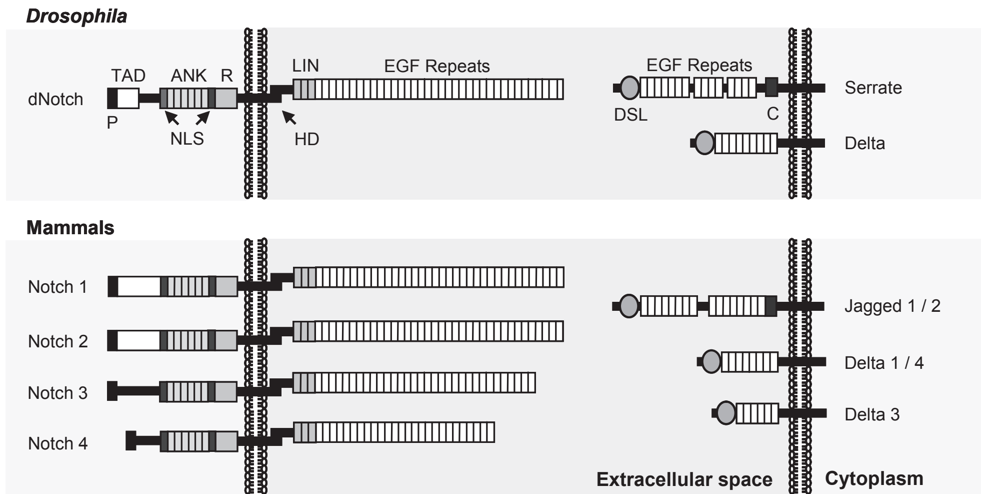

Figure 9. Notch Ligands and Receptors. Four Notch receptors are described in vertebrates (Notch1–Notch4) homologous of the *Drosophila* Notch receptor. The extracellular domain of the receptors contains EGF-like repeats (36 in Notch1 and Notch2, 34 in Notch3, and 29 in Notch4) followed by three cysteine-rich LIN domains that prevent ligand-independent activation and the heterodimerization domain (HD). The cytoplasmic domain contains a RAM domain followed by six ankyrin repeats (ANK) that bind to the CSL transcription factor, two nuclear localization signals (NLS), a transactivation domain (TAD; present in N1 and N2), and a PEST sequence (P) involved in regulating protein stability. Five conventional Notch ligands are known: Jagged1 and Jagged2 homologous of the *Drosophila* ligand Serrate, and Delta1, Delta3 and Delta4 homologous of the *Drosophila* Delta ligand. A common structural feature of all ligands is an amino-terminal domain called DSL (Delta, Serrate, and Lag-2) involved in receptor binding followed by EGF-like repeats. A cysteine-rich domain (C) is located downstream of the EGF-like repeats of Jagged1 and Jagged2 close to the plasma membrane (PM).

with the ligand, a heterodimerization domain and 3 Lin repeats which function is to prevent ligand-independent signaling [216]. Recent studies suggested that Notch receptors present an auto-inhibited conformation that is only released when the interaction with a ligand cause a conformational change [217]. The N^{IC} contain several signal transduction domains, including the RAM and Ankyrin repeats which promote protein-protein interactions with downstream members of the Notch signaling pathway, nuclear localization sequences (NLS) and, a C-terminal PEST domain that regulates the stability of the protein. Some Notch receptors also include a transactivation domain in the N^{IC}, required for optimum signaling. Besides the initial cleavage to form the heterodimeric receptor, Notch receptors are subject to several other post-translational modifications. Still during the trans golgi network the N^{EC} undergoes extensive N-linked and O-linked glucosylation and fucosilation. Although these glycosilations are not essential for expression of the receptor at the cell membrane, they are required for proper function and signal transduction [71,218].

Notch ligands are characterized by a highly conserved N-terminal DSL (Delta, Serrate and Lag) domain and varying numbers of EGF-like tandem repeats in the extracellular domain. Both domains are thought to be important for the binding to the receptor [216,219] (Figure 9).

Transduction of Notch signals

Activation of the Notch signaling pathway requires cell-cell interaction. This is mediated by the binding of a ligand to the extracellular domain of a Notch receptor on the cell surface

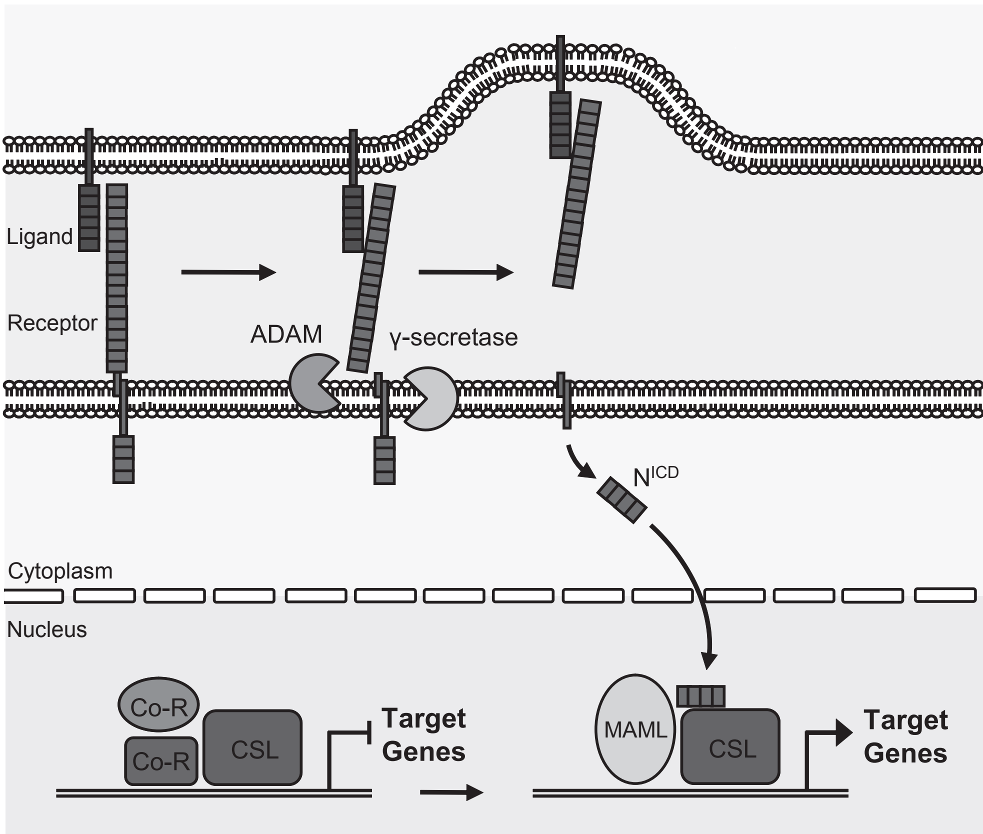

Figure 10. The Notch signaling pathway. Binding of a Notch ligand on one cell to the Notch receptor on another cell results in two proteolytic cleavages of the receptor. The ADAM10 or TACE (TNF-α-converting enzyme; also known as ADAM17) metalloprotease catalyses the first cleavage, generating a substrate for the second cleavage by the γ-secretase complex. This proteolytic processing releases the Notch intracellular domain (N^{ICD}), which enters the nucleus and interacts with the DNA-binding CSL (CBF1, Su(H) and LAG-1) protein. The co-activator Mastermind (Maml) and other transcription factors are recruited to the CSL complex, whereas co-repressors (Co-R) are released.

of an adjacent cell, and triggers two sequential proteolytic cleavages of the receptor. The first cleavage is performed by ADAM (A-desintegrin and metalloprotease) proteases in the extracellular domain close to the transmembrane region. This cleavage releases the N^{EC} which is trans-endocytosed together with the ligand by the signal-sending cell [220]. The ligand/receptor endocytosis process seems to be critical to induce a conformational change that allows the cleavage of the receptor [221]. Subsequently, a second cleavage in the intracellular domain is mediated by a protein complex with γ-Secretase activity. Inhibition of the γ-Secretase complex activity results in the inhibition of Notch signaling (Figure 10).

After the second proteolytic cleavage the Notch intracellular domain (N^{ICD}) is released in the cytoplasm and consequently migrates to the nucleus and regulates the expression of target genes. N^{ICD} does not bind directly to DNA. Instead, N^{ICD} binds to the basic helix-loop-helix (bHLH) transcription factor CSL (named after CBF-1 in humans, Su(H) in *Drosophila* and Lag-1 in *C.elegans*), also known as Rbpj in the mouse, converting it from a transcriptional repressor into a transcriptional activator [214]. On its basal state, CSL/Rbpj forms a complex with co-repressors that also recruit histone deacetylases (HDACs) in order to efficiently repress transcription of target genes. The composition of this complex vary between species and cell types but, in mammals includes proteins such as Smrt, Sharp and CtBP. Whether all co-repressors are simultaneously recruited or spatial and/or temporal differences exist, is still unclear. Interaction with N^{ICD}, upon cleavage of the Notch receptor, displaces co-repressors and recruits co-activators like the proteins of the Mastermind family which stabilize the binding between N^{ICD} and CSL/Rbpj [222-224]. In addition, Mastermind recruits other proteins like the histone acetylase p300 and other chromatin remodeling factors, which collaborate in transcription initiation and elongation [214] (Figure 10). Members of the Hairy enhancer of split (*Hes*) or Hairy related (*Hey* or *Hrt*) genes have been identified as Notch targets in many different tissues, while other genes are more tissue restricted. Besides these genes, the target genes of Notch signaling still remain largely unknown [225]. Other genes such as the negative regulators of *Notch Deltex1* and *Nrarp*, and the Pre-TCR component *Preta* were also shown to be regulated by Notch [226]. Further research will be required to identify tissue specific Notch target genes as well as their precise function.

The assembly of the co-activator complex also results in turnover of the N^{ICD}. The C-terminal PEST domain of N^{ICD} contains several phosphoylation sites that regulate its half-life [71,214]. Phosphorylation by the CdK8 and Gsk-3β kinases renders N^{ICD} as a substrate for the nuclear Fbw7 and Sel10 ubiquitin ligases, which is consequently targeted for proteasomal degradation [227]. This degradation critically regulates the duration of Notch mediated transcriptional activation and also explains why mutations within the PEST domain result in hyper-activation of the Notch pathway and leads to T-ALL, as do mutations in the Fbw7 ligase [228-231].

Regulation of Notch signaling pathway

Expression of Notch ligands and receptors is very dynamic during development thereby contributing to differential activity of the pathway. However, although controlling the availability of receptors and ligands constitutes a simple way to regulate Notch function, it is not sufficient to explain the heterogeneity of the activation of the pathway and the different functional outcomes of Notch signaling. Post-translational modification and trafficking regulation have recently emerged as important mechanism to control ligand and receptor availability as well as their productive interaction [214,221,232].

Besides the initial cleavage to form the heterodimeric receptor, Notch receptors are subject to several other post-translational modifications. During assembly in the Trans Golgi Network the N^{EC} undergoes extensive N-linked and O-linked glucosylation and fucosylation at specific EGF repeats by Rumi and Pofut1 glycosyltransferases. Although not essential for proper function and signal transduction, these glycosilations were shown to play an important role in the regulation of receptor activity [221,233]. In both flies and mammals, these O-fucose moieties can be further extended by Fringe glycosyltransferases [232]. In *Drosophila* fringe proteins enhance Delta-mediated activation of Notch while decreases activation by Serrate ligands. Three different Fringe protein variants have been identified in vertebrates: Lunatic, Manic and Radical. Lunatic Fringe is essential for an oscillatory mechanism of Notch activation during somitogenesis in the vertebrate embryo [234]. In the immune system, it has an important role in modulating Notch signaling during T-cell development [235-237] and, in cooperation with Manic fringe regulates marginal zone (MZ) B-cell development in the spleen [238].

Notch activity has also been showed to be regulated by endocytosis and vesicular trafficking. At the ligand level, endocytosis as a critical role in enhancing their signaling activity. Ligand endocytosis is triggered by ubiquitinylation by the ubiquitin ligases Neuralized and Mindbomb. Endocytic trafficking of Notch ligands may result ligand clustering, post-translational modification or recycling to specific membrane regions, leading in these ways to enhance ligand activity [239,240]. Notch receptors are also highly subjected to endocytosis through processes tightly regulated in time and space and that can result in positive or negative regulation of signaling activity, in a context dependent fashion. The availability of Notch receptors is controlled by several ubiquitin ligases such as Deltex and Nedd4, which can mediate notch receptors endocytosis and traffic towards lysossomal degradation or recycling, influencing in this way receptors half-life. Furthermore, the activity of the well characterized Notch inhibitor Numb also involves endocytosis. A possible mechanism involves endocytosis of other membrane associated factors essential for efficient Notch receptor activity. Mammalian Numb can also promote ubiquitinylation of the Notch receptor and consequent targeting of Notch for degradation [214,239-241].

Another mechanism to regulate Notch activity is based on the modulation of the extracellular concentration of Ca^{2+}, which binds to the EGF repeats of both receptors and ligands. Depletion

of Ca^{2+} leads to a strong ligand-independent activation of the Notch receptor, probably by inducing conformational changes in the N^{EC} domain[242].

In this way, different ligand-receptor combinations, post-translational modifications, ligand and receptor trafficking as well as several modulators of Notch activity contribute to a strict regulation of the strength and duration of the signals, in a highly temporal and spatial controlled fashion. The high diversity of signals and contexts in which Notch signaling occurs probably explain the wide range of functional outputs observed in many different biological systems.

Notch signaling in hematopoiesis

Notch signaling has been implicated in several stages of hematopoietic development. Besides a potential role in the regulation of HSC function, this pathway has been shown to induce commitment to the T-cell lineage and to regulate later stages of B-cell differentiation and myeloid development. In addition deregulated Notch signaling was observed in several leukemias, with mutations leading to the activation of the Notch pathway occurring in a large proportion of T-ALL[229,243].

Notch signaling: regulation of HSC fate

Notch signaling has been extensively studied in the hematopoietic stem cell compartment. While it was clearly demonstrated that this pathway is essential to generate definitive HSC during embryonic development, a potential role for Notch in adult HSC function is more controversial[71,225].

Germline mutant mice for Notch1 and for the downstream effector of all Notch receptors Rbpj, showed impaired generation of hematopoietic progenitors in the AGM region[244,245], accompanied by the lack of expression of the hematopoietic transcription factors Aml/Runx1, Gata2 and Scl/Tal1 by the aortic endothelium[245]. In contrast, Notch2 germline mutation did not affect the generation of HSC[244]. In addition, Jagged1 but not Jagged2 null embryos also failed to generate HSCs in the AGM, indicating that Notch signaling mediated by Notch1-Jagged1 interaction is required for the onset of definitive hematopoiesis during the mouse embryonic development[246].

A role for Notch signaling in the regulation of adult HSC function was initially proposed by *in vitro* studies in which both human and mouse hematopoietic progenitors cultured in the presence of Notch ligands could be expanded without losing *in vivo* reconstitution capacity[108,247-251]. In line with these results, over-expression of N^{ICD} as well as the Notch target gene *Hes1* in hematopoietic progenitor cells, enhanced self-renewal capacity of long-term HSCs [107,252,253]. Osteoblasts expressing Jagged 1 were identified as being part of the HSC niche[65]. Using an *in vivo* approach to activate osteoblasts by constitutive activation of the parathyroid hormone (PTH) receptor resulted in increased numbers of these cells in BM, which showed increased

expression of Jagged1. Such PTH-activated osteoblasts supported an increase in the number of HSC but not other hematopoietic progenitors consistent with expansion of HSCs through enhanced self-renewal. Importantly, HSCs in the BM of mice with enlarged osteoblast niche showed increased Notch1 activation *in vivo* and Notch inhibition with γ-secretase inhibitor reduced the supportive capacity of transgenic stroma to wild-type levels, in *in vitro* long-term co-cultures [65]. In agreement with these observations transgenic Notch reporter mice showed Notch signaling activity in prospectively defined HSCs and downregulated Notch signaling upon differentiation. Furthermore, inhibition of Notch signaling by over-expression of dominant negative forms of XSu(H) (the Xenopus homologue of SCL/Rbpj) and Mastermind resulted in increased differentiation *in vitro* and HSC depletion *in vivo* [254]. In contrast, loss-of-function assays resulted in controversial results, in showing that both Notch1 and Jagged1 are dispensable for HSC self-renewal and differentiation [255]. Conditional deletion of Notch1 or Jagged1 in HSCs and BM stromal cells did not affect HSC repopulation capacity, even when Notch1 deficient stem cells were transplanted into Jagged1 deficient recipient mice. This unexpected lack of phenotype may be explained by functional redundancy with other Notch receptors and ligands also expressed in HSC and in BM. Therefore experimental approaches to fully delete Notch activity will be necessary to clarify weather Notch signaling plays an essential role in HSC function [60].

Notch in developing T- and B-cells

The best characterized function of Notch in hematopoiesis is in the commitment of hematopoietic progenitors to the T-cell lineage [216,225,226]. The role of Notch signaling in T-cell commitment was initially observed using both gain and loss of function assays. Constitutive expression of the Notch activated form N^{ICD} in mouse hematopoietic progenitor cells resulted in thymic independent T-cell development in the BM when these cells were transplanted into recipient mice. Activation of Notch signaling allowed robust T-cell development till DP stage, concomitantly with a failure to generate B-cell progenitors [104]. Later, these findings were also extended to the human system, with N^{ICD} expression in cord blood $CD34^+$ cells promoting T-cell development at the expense of B-cell development, both *in vitro* and in transplanted NOD-SCID mice [256]. An essential role from Notch signaling in T-cell commitment was also confirmed in complementary loss-of-function studies. Conditional deletion of Notch1 in BM progenitors resulted in blocked T-cell development in the thymus at the most early intrathymic stage. Importantly Notch signaling inhibition resulted in ectopic development of $Notch1^{-/-}$ immature B-cells in the thymus [105,257]. In agreement, similar results were observed with conditional deletion of *Rbpj* gene [258] and with overexpression of the Notch antagonist Deltex in BM progenitor cells[259]. The similar phenotype observed with deletion of Notch1 and Rbpj indicates that Noch1 signals through CSL in a non-redundant way to specify the T-cell lineage [226]. This idea is consistent with the absence of a T-cell development defect in mice deficient for Notch2 [260] and Notch3 [261].

Initial studies to define which ligands could mediate activation of Notch1 to specify the T-cell lineage showed that Delta1 but not Jagged1-expressing stromal cells induce T-cell commitment in human [102] and mouse [262] hematopoietic progenitors. However, it was later demonstrated that conditional deletion of Delta1 on thymic epithelial cells (TEC) does not disturb T-cell development *in vivo*, indicating that Delta1 is dispensable for physiological T-cell lineage commitment. Other Delta ligands such as Delta4 which is also able to induce T-cell commitment *in vitro*, may play this role *in vivo* [263]. In agreement with a role for Delta ligands in T-cell commitment, ectopic expression of Lunatic Fringe in the thymus results in a phenotype similar to Notch1 conditional deletion [235]. The definitive involvement of Delta4 in this process was demonstrated in studies where conditional deletion of Delta4 in TECs resulted in a complete block in T-cell development and ectopic B-cell development in the thymus [101,178]. Thus, all these studies provide compelling evidence that Delta4-Notch1 mediated activation of Notch signaling is essential and sufficient to induce T-cell lineage commitment from hematopoietic progenitors. The target genes induced by Notch and whether Notch directly activates a T-cell specific gene program is still largely unknown.

Since Notch ligands are expressed in the BM there are probably mechanisms to prevent hematopoietic progenitors to develop into the T-cell lineage in this location. Indeed, activation of the Notch pathway is actively down-regulated by the ubiquitously expressed lymphoma-related factor (Lrp, formerly known as Pokemon). In this way, Lrf opposes Notch signals from the BM stroma that expresses moderate levels of Notch ligands. In the thymus, the abundant expression of Notch ligands seems to overrule the repressive role of Lrf on Notch function allowing the instruction of a T-cell fate on hematopoietic progenitor cells [264]. The mechanism by which Lrf represses Notch signaling is still unknown.

Later during T-cell development, Notch signaling is still required. While Notch seems to be dispensable for γδ-T-cell commitment [265,266], development of αβ-T-cells continuously requires Notch signals up to the DN3 stage, when cells have to pass the β-selection checkpoint. Lck-Cre mediated Notch1 deletion, starting at DN2 stage, results in an arrest at DN3 stage and impaired Tcrβ rearrangements [265]. In addition, *in vitro* studies demonstrated that successful transition through β-selection requires cooperative signaling of both Notch and pre-TCR, with Notch providing survival signals to thymocytes with productive *Tcrb* rearrangements 106,266. After passing β-selection checkpoint thymocytes down-regulate expression of Notch1 [267].

While Notch has an inhibitory effect on early B-cell development, it is later on required for the specification of marginal zone (MZ) versus follicular B-cell fate in the spleen. MZ B-cell fate specification non-redundantly depends on the activation of Notch2 by Delta1. Conditional deletion of either Notch2 or Delat1 results in a severe reduction in MZ B-cells [260,263]. In agreement, reciprocal studies in which the negative regulator of Notch signaling MINT was deleted led to an increase on MZ B-cells in the spleen [268].

Notch in T-cell Acute lymphoblastic leukemia (T-ALL)

A hallmark of human T-ALL is the presence of Notch mutations leading to aberrant and ligand-independent activation of the Notch signaling pathway. Indeed, human NOTCH1 gene was discovered as a partner in a chromosomal translocation t(7;9)(q34;q34.3) resulting in aberrant expression of a dominant active truncated N^{ICD} protein called TAN1 [215]. A causative effect of this translocation for disease development was obtained later in experiments showing that mice transplanted with BM progenitors expressing TAN1 developed T-cell leukemia as early as 2 weeks after transplantation [228]. Truncated forms of Notch2 and Notch3 were subsequently shown to also induce T-cell leukemias when expressed in BM progenitors or immature thymocytes [269,270].

Although translocations involving Notch1 are only found in less than 1% of T-ALLs, it was later discovered that more than 50% of pediatric T-ALL patients had activation mutations in the *NOTCH1* gene. These mutations involved the extracellular HD domain and the C-terminal PEST domain of NOTCH1 [229]. Mutations or deletions in exon 34, encoding the PEST domain, result in intracellular stabilization and consequently increased levels of N^{ICD} due to reduced proteasomal degradation. Mutations in exons 26 and 27 encoding the HD domain allow for ligand-independent proteolytic cleavage of the NOTCH receptor resulting in signaling in the absence of ligand-receptor interaction [229,271].

Notch in myeloid development

The role of Notch signaling in myeloid differentiation is still controversial [71,272]. Several *in vitro* studies with expression of active forms of Notch1 resulted in a block or delay in myeloid terminal differentiation, while expanding the stem/progenitor cell compartment [273-276]. While some studies using a similar approach *in vivo* corroborate these findings [107,277], other studies failed to show a similar effect on myelopoiesis [104]. In addition, conditional deletion of Notch1 or Rbpj in hematopoietic cells did not apparently affect myeloid lineages [105,258,278]. On the other hand, studies in which Notch activation was achieved by culturing of hematopoietic stem/progenitor cells with Delta1 ligand-expressing stromal cell lines showed a reduction in myelopoiesis [279,280]. A differential effect of Jagged and Delta ligands was observed in one of these studies. While Delta1-mediated activation of Notch led to a reduction in the number bipotent granulocyte-monocyte progenitors (CFU-GM, colony forming unit-granulocyte monocyte) and differentiated monocytic cells, Jagged1 increases clonogenicity as observed by increased numbers of CFU-GM -M and -G, without quantitatively affecting terminal cell differentiation [279]. Although several studies suggest a role of Notch signaling in the inhibition of myeloid potential in hematopoietic progenitors, the exact role of Notch in myelopoiesis is still controversial. Differences between studies may be explained by the context in which Notch activation was performed, with other signaling pathways and other factors such as cytokines

interfering with Notch signals. In one study in which mice were transplanted with BM progenitors expressing an active form of Notch1, increased terminal differentiation of non-transduced cells was observed suggesting that Notch may also affect hematopoiesis in a non-cell-autonomous fashion [281].

1.4 Aims of the thesis

Blood stem cells constitute one of the best characterized adult tissue-specific stem cells and the first to be clinically applied in the treatment of diseases such as hematological malignancies and primary immunodeficiencies. The potential to maintain, expand and manipulate HSCs for therapeutic purposes is currently challenged by the rapid loss of their ability to engraft and self-renew *in vivo* after culture. The capacity of HSCs to self-renew and differentiate is tightly regulated by signals from the specialized microenvironment or niche where they reside. In this way, defining the correct microenvironmental signals that support HSC function has been a major goal in stem cell biology. This thesis describes studies aimed to understand the role of two key signaling pathways, namely Wnt and Notch, involved in the regulation of HSC self-renewal and differentiation in the different hematopoietic lineages.

Chapther 2 and **3** describe the role of the Wnt signaling pathway in HSC function. In **Chapter 2** a loss-of-function approach based on the study of a mouse deficient for Wnt3a was used and HSC function was accessed by *in vivo* serial transplantation assays, *in vivo* reporter assays and *in vitro* differentiation systems. The studies on the role of Wnt signaling in HSC function were further extended in **Chapter 3** by means of a reciprocal gain-of-function approach. Using different mouse models carrying hypomorphic mutations on the negative regulator of the Wnt signaling pathway Apc, a gradient of Wnt signaling activation was obtained. The dosage dependent effect of Wnt signaling activation on HSCs and hematopoietic differentiation was studied *in vivo* and *in vitro*. **Chapter 4** and **5** describe the role of Notch signaling pathway in HSC self-renewal and differentiation with particular emphasis on the induction of a T-cell lineage program. In **Chapter 4** a mouse model for conditional deletion of the transcription factor downstream of all Notch receptors Rbp-Jk is used to study the effects of Notch deficiency on HSC function. In **Chapter 5** a genome wide gene expression profile approach was used to identify direct target genes of the Notch signaling pathway, involved in the induction of a specific T-cell lineage program. Finally, **Chapter 6** discusses the significance and implications of the studies described, and provide directions for future research.

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Chapter 2

Wnt3a is essential for hematopoietic stem cells self-renewal in fetal liver

Chapter 2.1

Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation

Tiago C. Luis[1], Floor Weerkamp[1], Brigitta A.E. Naber[1], Miranda R.M. Baert[1], Edwin F. E. de Haas[1], Tatjana Nikolic[2], Sjanneke Heuvelmans[1], Ronald R. De Krijger[3], Jacques J.M. van Dongen[1], Frank J.T. Staal[1]

[1]Department of Immunology, [2] Pulmonary medicine, [3]Pathology, Erasmus MC, Erasmus University Medical Center, 3015GE Rotterdam, The Netherlands

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ABSTRACT

Canonical Wnt signaling has been implicated in various aspects of hematopoiesis. Its role is controversial due to different outcomes between various inducible Wnt signaling loss-of-function models and also compared to gain-of-function systems. We therefore studied a mouse deficient for a Wnt gene that seemed to play a non-redundant role in hematopoiesis. Mice lacking Wnt3a die prenatally around E12.5, allowing fetal hematopoiesis to be studied using *in vitro* assays and transplantation into irradiated recipient mice. Here we show that Wnt3a deficiency leads to a reduction in the numbers of hematopoietic stem cells (HSC) and progenitor cells in the fetal liver (FL) and to severely reduced reconstitution capacity as measured in secondary transplantation assays. This deficiency is irreversible and cannot be restored by transplantation into Wnt3a competent mice. The impaired long-term repopulation capacity of Wnt3a$^{-/-}$ HSCs could not be explained by altered cell cycle or survival of primitive progenitors. Moreover, Wnt3a deficiency affected myeloid but not B-lymphoid development at the progenitor level, and affected immature thymocyte differentiation. Our results show that Wnt3a signaling not only provides proliferative stimuli, such as for immature thymocytes, but also regulates cell fate decisions of HSC during hematopoiesis.

INTRODUCTION

Hematopoietic stem cells (HSCs) are responsible for the continuous production of blood cells and consequently help to sustain immune function. This is achieved by their unique capacity to self-renew and ability to differentiate into all blood lineages. Several studies have implicated the Wnt signaling pathway in the regulation of these processes but its exact role is still not completely understood[1,2].

Upon binding of a Wnt protein to a Frizzled receptor and to a LRP5/6 co-receptor, an elaborate signaling route leads to cytoplasmatic accumulation and subsequent nuclear translocation of β-catenin, the key mediator of the Wnt signaling pathway. In the absence of a Wnt protein the levels of β-catenin are kept very low by the action of the so called destruction complex consisting of the casein kinase I (CKI) and glycogen synthase kinase 3β (GSK-3β) serine/threonine kinases, the tumor suppressor protein adenomatous polyposis coli (APC) and the scaffolding protein Axin. Phosphorylation of β-catenin by CKI and GSK-3β leads to its ubiquitination and subsequent breakdown in the proteossome. Activation of the Wnt pathway by a Wnt ligand results in inactivation of GSK-3β and consequent translocation of β-catenin to the nucleus. In the nucleus β-catenin binds to members of the Tcf/Lef transcription factors family, thereby converting these proteins from transcriptional repressors into transcriptional activators[3].

The first evidence for a role of Wnt proteins in hematopoiesis was reported in studies showing that stromal cell lines transduced with *Wnt1*, *Wnt5a* and *Wnt10b* have an *in vitro* stimulatory effect on mouse[4] and human[5] hematopoietic progenitors.

Using Tcf1/Lef-GFP reporter assays Wnt signaling was shown to be active in the highly HSCs enriched $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) population, both *in vivo* as well as *in vitro* after stimulation with purified Wnt3a[6]. Furthermore Wnt3a treatment *in vitro*, resulted in increased proliferation of LSK cells along with the maintenance of an immature phenotype, and led to increased self-renewal as determined by transplantation assays[7]. Retroviral expression of a constitutively active form of β-catenin in Bcl2 transgenic LSK cells resulted in augmented multilineage repopulation capacity. In agreement, ectopic expression of the Wnt signaling inhibitor Axin yielded opposite results[6]. However subsequent gain- and loss-of-function approaches to further elucidate the role of Wnt signaling in HSCs yielded contradictory results. Two independent studies using a conditional mouse model to express a stabilized form of β-catenin showed impaired multilineage differentiation and a transient increase in stem cell numbers, followed by exhaustion of the HSC pool, observed by failure to repopulate lethally irradiated recipients[8,9]. Therefore, the role of Wnt signaling in HSC biology is currently controversial. Besides this, reciprocal approaches to conditionally inactivate β-catenin in HSCs also showed contradictory results and brought further controversy, with some of these studies

showing normal hematopoiesis and stem cell activity in the combined absence of β-catenin and its homologue plakoglobin[10-13]. These issues are reviewed in more detail elsewhere[14].

The functions of several Wnt genes have been investigated by targeted mutations in the mouse. These led to specific developmental defects, which are lethal early during embryonic development or shortly after birth. Mice carrying a null allele of *Wnt3a* have been generated by McMahon and co-workers. *Wnt3a* homozygous mutant embryos die around day E12.5 of the embryonic development. These embryos lack caudal somites, have disrupted notochord and exhibit anomalies of the central nervous system[15]. The hematopoietic system of these embryos has not been studied yet. However, the fact that Wnt3a promotes proliferation of HSCs[7] and pro-B cells[16], and the high expression in the thymic stroma[17] make the *Wnt3a* gene of interest. In addition Wnt3a$^{-/-}$ embryos are morphologically highly similar to *Tcf1/Lef1* double deficient embryos[18,19], suggesting comparable defects in the hematopoietic system[20] of both strains. For these reasons we decided to investigate the hematopoietic system of Wnt3a$^{-/-}$ embryos.

Here we demonstrate that Wnt3a deficiency leads to a reduction in the numbers of hematopoietic stem and progenitor cells in the fetal liver (FL). Besides this, HSCs are functionally affected as purified LSK cells show severely reduced reconstitution capacity measured by secondary transplantation assays. This deficiency is irreversible since transplantation into wild type (Wt) recipient mice could not restore HSC function, suggesting a role for Wnt3a in the establishment of a HSC specific genetic program. Moreover Wnt3a deficiency affected myeloid but not lymphoid development at the progenitor level and early stages of T lymphoid development in the thymus.

Our data provide genetic evidence for the involvement of a specific Wnt protein in HSC self-renewal, suggesting that modulation of Wnt signaling in stem cells or in their environment may be beneficial for stem cell therapies, transplantation and regenerative medicine applications.

MATERIALS AND METHODS

Mice

Wnt3a heterozygous were bred and maintained in the Erasmus MC animal facility, according with the legal regulations in The Netherlands, and with approval of the local ethical committee.

Mice heterozygous for the Wnt3a mutation were kindly provided by T. Yamaguchi (National Cancer Institute-Frederick, Frederick, MD) and were backcrossed to C57Bl/6. Genotyping was performed as described[15]. To obtain Wnt3a$^{-/-}$ embryos, Wnt3a heterozygous mice were put

together in the afternoon and checked for vaginal plug the next mornings. The time at which the vaginal plug was observed was considered E0.5. Embryos were analyzed at E12.5. C57Bl/6-Ly5.1 mice were purchased from the Charles River Laboratories.

Flow cytometry and cell sorting

For flow cytometric analyses and cell sorting, cells were stained with monoclonal antibodies against the following molecules: B220 (Ra3-6B2), CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b/Mac1 (M1/70), CD11c (HL3), CD19 (ID3), CD43 (S7), CD45.1/Ly5.1 (A20), CD45.2/Ly5.2 (104), CD71 (C2), CD117/c-Kit (2B8), CD127/IL-7Rα (SB/199), CD135/Flt3 (A2F10.1), F4/80 (BM8), GR1 (RB6-8C5), IgM (R6-60.2), NK1.1 (PK136), Sca1 (E13-161.7) and Ter-119 all from Becton Dickinson/Pharmingen; CD34 (RAM34) and FcγRII/III (93) from eBioscience; Activated Notch1 (mN1A) from Abcam. Antibodies were used either directly conjugated or biotinylated. For secondary detection streptavidin conjugated with PE-Cy7 or APC-Cy7 was used. 7AAD (Pharmingen) or DAPI (Invitrogen) were used for dead cells exclusion. Stained cells were measured with a FACS-Calibur and/or a FACS-Canto, sorted on a FACS-Aria (Becton Dickinson), and analyzed with FlowJO software (Treestar). To analyze LSK in FL, antibodies for lineage markers included B220, CD4, CD11c, GR1, NK1.1 and Ter-119. When LSKs were analyzed in adult bone marrow (BM) antibodies against CD11b (Mac1) and CD3 were added to the lineage mix.

Cell cycle and apoptosis

Cell cycle analysis of FL stem and progenitor cells was performed by staining for 60 min 37ºC with Hoechst33342 (Invitrogen/Molecular probes, 10µg/ml) in DMEM medium supplemented with Verapamil inhibitor (Sigma, 10µM), 2% FBS and 1mM HEPES (Gibco). Hoechst33342 stained cells were than stained with flow cytometry antibodies. To analyze the percentage of apoptotic cells by flow cytometry, FL cells were stained with markers to define the LSK population (see above) and subsequently stained with FITC-conjugated AnnexinV and 7AAD (Becton Dickinson/Pharmingen) according to manufacturer's instructions.

Transplantation experiments

For competitive transplantation assays, C57Bl/6 Ly5.1 recipient mice (8-11 weeks old) were lethally irradiated (9.5 Gy). 400 sorted LSK (Ly5.2) from Wt or Wnt3a$^{-/-}$ E12.5 FLs were mixed with 2.5×10^5 total Ly5.1 FL cells (E12.5) in order to obtain 1:1 competition and intravenously injected in recipient mice. Repopulation, calculated as (percentage donor cells) x100/(percentage donor cells+percentage recipient cells), was evaluated at 4, 8, 10 and 12 weeks post transplantation in peripheral blood by flow cytometry. The mice were sacrificed 12 weeks after transplantation. For secondary transplantation, equal numbers of total BM cells

from primary recipients that received Wt or Wnt3a$^{-/-}$ LSKs were pooled and transplanted into lethally irradiated Ly5.1 secondary recipients. Peripheral blood from secondary transplanted mice was analyzed at 4, 8 and 12 weeks post transplantation. 13 weeks after transplantation mice were sacrificed and analyzed for reconstitution in BM, thymus, and spleen.

***In vitro* methylcellulose colony assays**

For methylcellulose colony assays FL cell suspensions were prepared in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2%FBS. CFU-C and CFU-E assays were performed with MethoCult M3434 (containing rmSCF 50ng/mL, rmIL-3 10ng/mL, rhIL-6 10ng/mL and rhEPO 3U/mL) or M3334 (containing rhEPO 3U/mL), respectively, (from StemCell technologies) in 35mm-dish, accordingly to manufacturer's instructions. All assays were done in triplicate. Colonies (>30 cells) were counted after 7 days (CFU-C) or 3 days (CFU-E) of incubation at 37oC, 5%CO_2 and humidified atmosphere. CFU-Mix were defined as colonies containing at least 3 different cell types. May-Grunwald Giemsa stainings were performed to confirm identification of the colonies by morphology.

Co-cultures of FL cells with OP9 bone marrow stromal cell line

Total FL cells were cultured in RPMI-1640 medium supplemented with 10%FBS, mrIL-7 (10ng/ml) and mrSCF (50ng/ml) (both from R&D), for 24 hrs in order to separate FL stroma from hematopoietic cells. 10,000-50,000 cells were then cultured on confluent layers of the OP9 BM stromal cell line in αMEM medium, also supplemented with FBS, mrIL-7 and mrSCF. After 7-16 days incubation at 37oC, cells were harvested and stained for flow cytometry.

Histology and Immunohistochemistry

6μm tissue sections of frozen E12.5 embryos were fixed in acetone. For visualization of the different organ structures, sections were stained with haematoxylin-eosin (HE). To envisage thymic epithelium, sections were incubated with concentrated supernatant from a hybridoma producing the ER-TR4 antibody for thymic stromal cells, followed by a biotinylated rabbit anti-rat antibody (DakoCytomation) and finally with avidin-biotin complex-horseradish peroxidase (DakoCytomation). Sections were incubated with 3-amino-9-ethylcarbazole (AEC) substrate (DakoCytomation) to detect peroxidase.

Fetal thymic organ culture (FTOC)

Fetal thymic lobes from Wt and Wnt3a$^{-/-}$ E12.5 embryos were isolated and cultured on the top of a filter (Whatman) floating on medium (IMDM, 10%FBS) . After 9-17 days of culture, cells were harvested and analyzed by flow cytometry.

RESULTS

Reduced numbers of HSC in Wnt3a$^{-/-}$ E12.5 embryos

The loss of Wnt3a leads to embryonic lethality around embryonic day (E) 12.5. Wnt3a$^{-/-}$ embryos harbor a severe phenotype with little or no caudal development posterior to forelimbs, disrupted notochord and abnormal formation of ectopic neural tubes[15]. After E12.5, recovery of viable Wnt3a$^{-/-}$ embryos dramatically decreases and therefore heterozygous mice were bred and the embryos analyzed at this age.

At E12.5 the FL is the major hematopoietic organ, which is colonized by progenitors from aorta-gonad-mesonephros (AGM) region, yolk sac and placenta, starting by late E9[21]. The FLs from Wnt3a$^{-/-}$ and Wt embryos did not differ in the total cell numbers ($2.86x10^6 \pm 1.28x10^6$ in Wnt3a$^{-/-}$ and $3.32x10^6 \pm 1.25x10^6$ in Wt FLs; p=0.54) and the total percentage of leukocytes (as determined by CD45 expression) did also not differ between Wt and Wnt3a$^{-/-}$ FLs (data not shown). Besides this, FLs were red suggesting that the embryos were not anemic.

Murine HSC are found in a rare population characterized by the absence of lineage markers and expression of Sca1 and c-Kit, the so-called LSKs (Lin$^-$ Sca1$^+$ c-Kit$^+$)[22]. Analysis of this population in the FLs from Wnt3a$^{-/-}$ embryos showed a marked reduction in the percentage of these cells (3-5 fold) comparing to Wt (Figure 1a,b). While, in agreement with previous reports the Wt FLs contained 0.51% LSK, the Wnt3a$^{-/-}$ FLs contained only 0.15% LSKs in average.

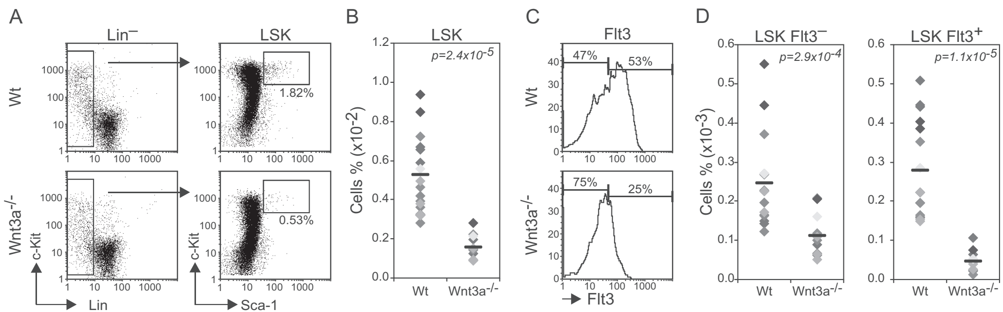

Figure 1. Wnt3a deficiency leads to a severe reduction in HSC (LSK Flt3-) and multipotent progenitor (MPP, LSK Flt3$^+$) numbers. (A) Flow cytometry analysis of LSK (Lin$^-$ Sca1$^+$ c-Kit$^+$) population in Wt and Wnt3a$^{-/-}$ FLs from E12.5 embryos. Lineage negative cells were electronically gated and analyzed for Sca1 and c-Kit expression. Percentage of Sca1$^+$c$^-$Kit$^+$ cells is indicated. (B) Percentage of LSK cells in total FL is strongly decreased. Data are from 16 Wt and 11 Wnt3a$^{-/-}$ FLs, belonging to 6 different litters identified by different colors. Mean values are presented as a dash. (C) Flow cytometry analysis of the Flt3 subsets in the LSK compartment. LSKs were electronically gated and analyzed for Flt3 expression. Percentages of FltL3$^-$ (HSCs) and Flt3$^+$ (MPPs) cells are indicated. (D) Frequency of Flt3$^-$ and Flt3$^+$ LSK cells in total FL. Data are from 16 Wt and 11 Wnt3a$^{-/-}$ FLs, belonging to 6 different litters identified by different colors. Mean values are presented as a dash. "*p*" values are indicated in all graphs. See page 212 for a full-color representation of this figure.

Different studies have shown that upregulation of Flt3/Flk2 expression is accompanied by loss of HSCs long-term reconstitution capacity in BM and FL[23-25]. The analysis of the Flt3^{-} (HSCs) and Flt3^{+} (multipotent progenitors; MPP) subsets within the LSK population revealed that, although the Flt3+ subset was more severely affected, both populations were strongly decreased indicating a HSC deficiency (Figure 1c,d). This quantitative difference led to a difference in actual stem cell dependent repopulation of recipient mice. That is, when different numbers of E12.5 FL cells from Wt or *Wnt3a*$^{-/-}$ embryos were transplanted into sub-lethally irradiated recipients (6Gy), only the highest number of *Wnt3a*$^{-/-}$ cells (4x10^{6}) could repopulate (>1% donor contribution in peripheral blood) 3 out of 3 recipient mice. By contrast, a 16-fold

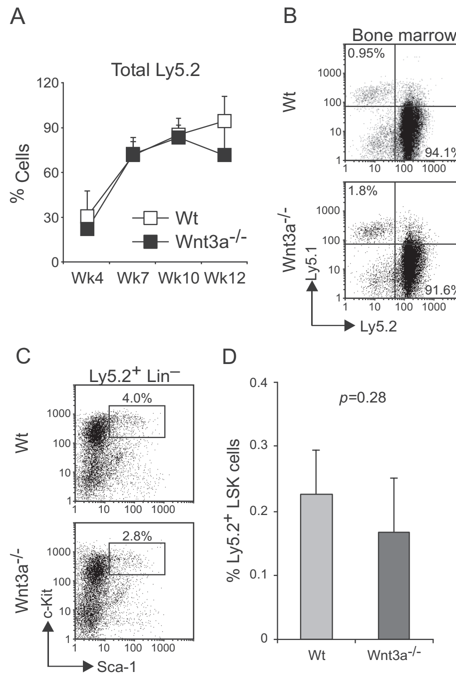

Figure 2. Wnt3a$^{-/-}$ LSKs efficiently repopulate primary recipient mice in competitive assays. Lethally irradiated Ly5.1 mice were transplanted with sorted LSK cells (Ly5.2), from Wt or Wnt3a$^{-/-}$ FLs, together with unfractionated FL cells (Ly5.1), in order to have a competition of approximately 1:1. (A) Repopulation efficiency (percentage of Ly5.2 cells) was analyzed in peripheral blood at the indicated time points. Graph shows mean ± SD of 7 and 3 mice that received Wt and *Wnt3a*$^{-/-}$ LSKs, respectively. (B) Repopulation efficiency (percentage of Ly5.2 cells) was analyzed in BM of recipient mice at 12 weeks. Numbers in quadrants indicate the percentage of total Ly5.1 or Ly5.2 cells (C) Analysis of the HSC compartment. Lin^{-} Ly5.2^{+} cells were electronically gated and analysed for Sca1 and c-Kit to define LSK population. (D) Percentage of Ly5.2 LSK cells in total BM of the recipient mice. Data are mean ± SD of 7 mice repopulated with Wt cells and 3 mice repopulated with *Wnt3a*$^{-/-}$ cells. Results are representative of 3 independent experiments.

lower number of Wt cells transplanted ($0.25x10^6$ cells) was able to repopulate 2 out of 6 recipient mice (Table1), while none of the mice (0 out of 6) that received *Wnt3a*$^{-/-}$ cells showed donor-derived reconstitution.

HSC self-renewal is irreversibly impaired

To test whether besides a severe quantitative reduction in HSC, these cells were also affected in their function, we performed competitive transplantation assays. For this, 400 LSKs isolated by cell sorting from Wt or Wnt3a$^{-/-}$ FLs (Ly5.2) were mixed with $2.5x10^5$ Wt total FL cells (Ly5.1), which were measured to also contain arround 400 LSK, in order to have an approximately 1:1 competition. The cell mixtures were intravenously injected into Wt Ly5.1 recipient mice which were lethally irradiated. The chimerism was followed in peripheral blood at four, eight, ten and twelve weeks after transplantation. Both Wt and Wnt3a$^{-/-}$ cells were able to repopulate the primary recipients with comparable efficiency (7/8 mice repopulated (>1%) with Wt cells and 3/4 mice repopulated with *Wnt3a*$^{-/-}$ cells)(Figure 2a). Besides this, twelve weeks after the beginning of the experiment, the analysis of BM of repopulated mice showed no significant difference in the percentage of total Ly5.2 (Figure 2b,c) and in the contribution towards the different lineages (data not shown). Analysis of the HSC compartment revealed a non statistically significant reduction in the frequency of Ly5.2 LSKs in the BM of mice receiving *Wnt3a*$^{-/-}$ cells (Figure 2d). Flt3$^-$ and Flt3$^+$ subsets within LSK compartment did not differ between the mice receiving Wt or Wnt3a$^{-/-}$ LSKs (data not shown).

However, when total BM cells from the primary recipient mice were re-transplanted into lethally irradiated Wt secondary recipients, the repopulation capacity measured in peripheral blood was severely reduced in mice receiving *Wnt3a*$^{-/-}$ cells, as early as 4 weeks post transplantation (Figure 3a). Furthermore, the contribution towards different lineages was strongly affected in the secondary recipients receiving Wnt3a$^{-/-}$ cells (Figure 3b). Analysis of the repopulation efficiency in BM 13 weeks after transplantation, showed an average contribution of 60% in the mice that received re-transplanted Wt cells whereas in the mice receiving re-transplanted Wnt3a$^{-/-}$ cells this was >15-fold reduced (4% chimerism in average) (Figure 3c,d). Moreover, although the total percentage of LSKs did not differ in the mice receiving Wnt3a$^{-/-}$ cells, this population mainly consisted of cells expressing the congenic marker Ly5.1, while around 70% (average) of the LSK from the Wt mice group expressed the Ly5.2 marker (Figure 3e,f). The analysis performed in other hematopoietic organs, such as thymus and spleen showed similar differences (data not shown). Thus, secondary transplantations revealed strongly reduced reconstitution by Wnt3a$^{-/-}$ HSC, showing that these cells have extremely limited self-renewal capacity. Importantly, although being transplanted into Wt recipient mice, in which Wnt3a is abundantly expressed in BM[16], the LSK cells had very low repopulation efficiency, indicating a permanent and irreversible self-renewal deficiency.

Wnt3a$^{-/-}$ HSC have normal differentiation capacity

At E12.5, FL hematopoiesis is mainly directed towards erythrocyte production, with around 95% of the cells constituting erythroid progenitors. Analysis of the different stages of erythroid development using the erythroid-specific marker Ter-119 and the transferrin receptor CD71[26] together with the progenitor marker c-Kit, showed no significant differences between Wt and Wnt3a$^{-/-}$ FLs, indicating normal erythroid development (data not shown). Analysis of the non-erythroid lineages (CD71^{-}Ter119^{-}) showed that in the Wnt3a$^{-/-}$ livers the B220^{+} cells were also present in a similar frequency as in Wt embryos. However, total CD11b^{+} myeloid cells were

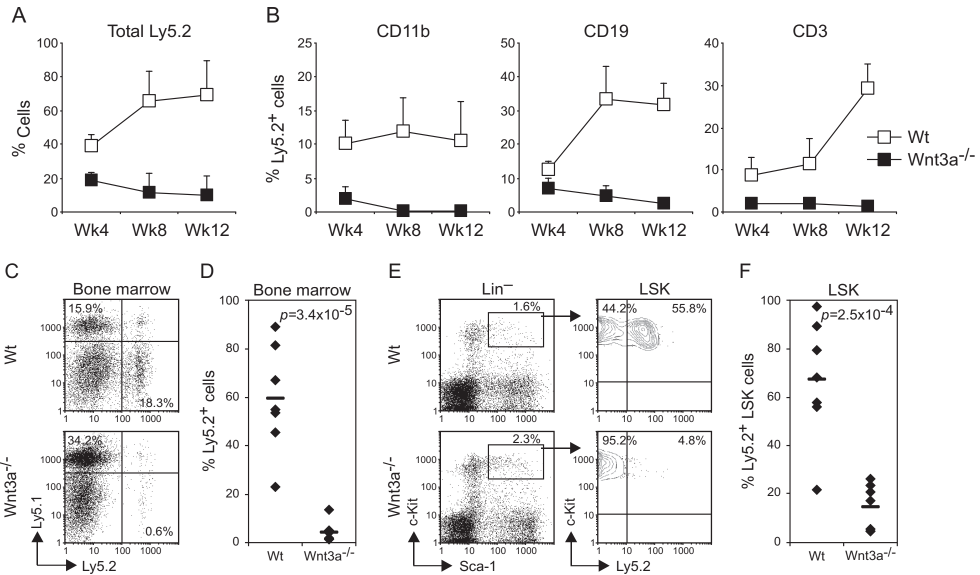

Figure 3. Wnt3a$^{-/-}$ HSC have severely reduced long-term repopulation capacity as revealed by failure to repopulate secondary recipients. Total BM cells from primary recipient mice were re-transplanted into lethally irradiated secondary Ly5.1 recipients. (A) Repopulation efficiency was analyzed in peripheral blood at the indicated time points. (B) Ly5.2 LSKs contribution towards myeloid (CD11b^{+}), B-lymphoid (CD19^{+}) and T-Lymphoid (CD3^{+}) lineages measured in peripheral blood at the indicated time points. (A, B) Graphs show mean ± SD of 7 mice in each group (Wt and Wnt3a$^{-/-}$). (C) Thirteen weeks after transplantation repopulation was analyzed in the hematopoietic organs. Numbers in quadrants indicate the percentage of total Ly5.1 or Ly5.2 cells in the BM of the recipient mice. CD45.1^{-}CD45.2^{-} double negative cells constitute erythrocytes and megakaryocytes. (D) Percentage of total Ly5.2 cells in the BM of the recipient mice. The averages are indicated by a dash. (E) HSC compartment analysis in the secondary recipient mice. LSK cells were gated and analyzed for Ly5.2 expression. (F) Frequency of Ly5.2 LSK cells in the BM of the recipient mice. The averages are indicated by a dash. (C-F) Data represent 7 mice in each group. “*p*” values are indicated in the graphs. Results are representative of 3 independent experiments.

slightly reduced (p=0.01) in the livers from Wnt3a$^{-/-}$ embryos (Figure 4a,b). Nevertheless this reduction was not observed in the more mature CD11b^{+}F4/80^{+} subset of myeloid cells, but it was due to a marked reduction in more immature CD11b^{+}F4/80^{-} myeloid cells (Figure 4a,b), suggesting a progenitor deficiency that does not impair terminal differentiation.

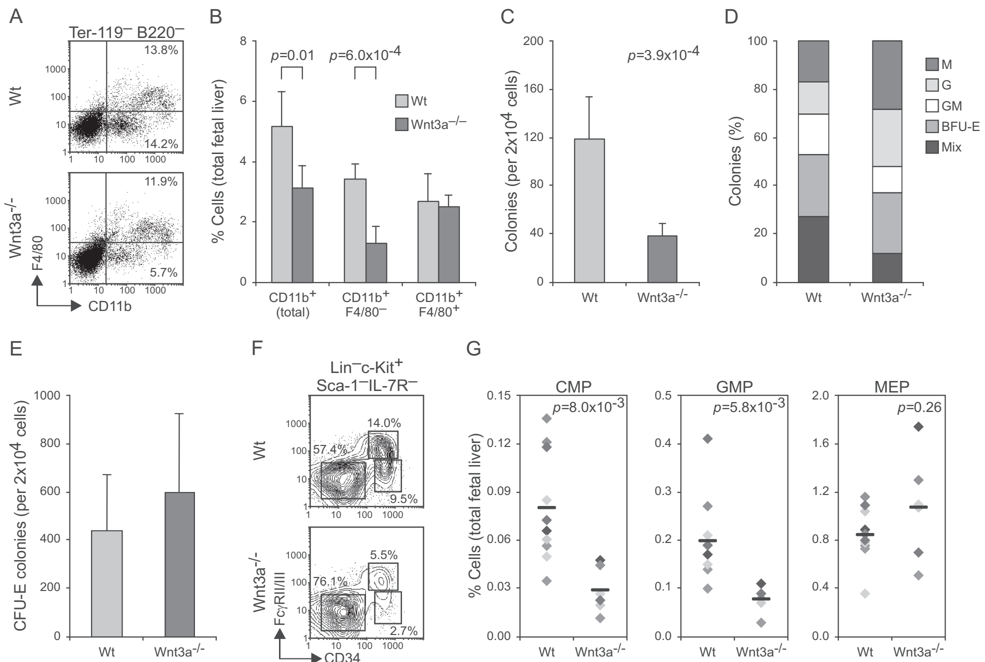

Figure 4. Wnt3a deficiency affects myeloid progenitors without impairing terminal differentiation. (A) E12.5 Wnt3a$^{-/-}$ livers show decreased frequency of myeloid CD11b^{+} (Mac1) and CD11b^{+} F4/80^{-} immature subsets, accessed by flow cytometry analysis. Numbers in quadrants denote frequencies of cells gated as indicated. (B) Frequency of CD11b^{+} myeloid cells and F4/80 ($^{+/-}$) subsets in total FL. Data are mean ± SD of 5 Wt and 4 Wnt3a$^{-/-}$ FLs, from 3 different litters. (C) Numbers of colonies yielded by Wt and Wnt3a$^{-/-}$ FLs in methilcelulose colony assays. Data are mean ± SD of 6 Wt and 3 Wnt3a$^{-/-}$ FLs, from 3 different litters, done in triplicate. p = 3.9×10-4 (D) Relative frequency of the different colonies determined by morphologic analysis and confirmed by staining with May-Grunwald Giemsa. Data are representative of 4 Wt and 3 Wnt3a$^{-/-}$ FLs, from 3 different litters. M, Macrophage; G, Granulocyte; BFU-E, Burst Forming Unit-Erythrocyte; Mix, Mixed (containing at least 3 different types of cells). (E) Erythroid colony-forming-unit (CFU-E) assay of Wt and Wnt3a$^{-/-}$ FLs cells. Data are mean ± SD of 5 Wt and 4 Wnt3a$^{-/-}$ FLs, from 3 different litters, done in triplicate. (F) Flow cytometry analysis of myeloid progenitor subsets in the FL of Wt and Wnt3a$^{-/-}$ embryos. Lineage negative cells were electronically gated and analyzed for c-Kit, Sca1 and IL-7R expression. c-Kit^{+} Sca1^{-} IL-7R^{-} cells were then analyzed for CD34 and FcγR II/III expression. Numbers next to outlined areas indicate the percentage of cells in each progenitor subset (CMP - FcγR^{lo} CD34^{+}; GMP - FcγR^{hi} CD34^{+}; MEP - FcγR^{lo} CD34^{-}). (G) Frequency of CMPs, GMPs and MEPs in total FL. The averages are indicated by a dash. Data from 11 Wt and 6 Wnt3a$^{-/-}$ FLs, belonging to 6 different litters identified by different colors. "*p*" values are indicated. See page 213 for a full-color representation of this figure.

Reduced numbers of myeloid but not B-lymphoid progenitors

The decreased frequency of more immature myeloid cells, together with the strong reduction of MPPs indicated that Wnt3a deficiency is affecting not only HSC self-renewal but also primitive progenitors subsets.

To further investigate a possible deficiency in myeloid progenitors, *in vitro* methylcellulose colony-forming assays were performed. Interestingly in the presence of multiple cytokines, compared to wild-type cells, the *Wnt3a$^{-/-}$* FL cells consistently yielded >3-fold fewer colonies (Figure 4c). Of interest, the relative frequency of the different types of colonies was also different between the Wt and Wnt3a$^{-/-}$ cells (Figure 4d). Wnt3a$^{-/-}$ FL cells yielded a lower number of CFU-Mix leading to a 6-fold reduction in the absolute numbers of these colonies. Besides this, also CFU-GM and BFU-E colonies appeared slightly reduced. In agreement with what was previously observed, absolute numbers of CFU-G and -M were not significantly different, indicating that more mature myeloid cells were produced at normal frequencies. Total number of CFU-E colonies was not significantly altered (Figure 4e). This was confirmed by flow cytometric analysis of the different myeloid progenitor subsets[27]. Both common myeloid progenitors (CMPs; FcγR^{lo}CD34^{+}) and granulocyte-monocyte- progenitors (GMPs; FcγRh^{i}CD34^{+}) frequencies in Wnt3a$^{-/-}$ FLs were severely reduced. Megakaryocyte-erythrocyte progenitors (MEPs; FcγR^{lo}CD34^{-}) were not affected (Figure 4f,g).

Wnt3a has been proposed to function as a growth factor for pro-B cells when provided as a prototype Wnt *in vitro*[16]. Moreover, the severe reduction in the numbers of MPPs suggested that Wnt3a$^{-/-}$ primitive progenitors might have reduced lymphoid potential.

Surprisingly, the analysis of the FL counterpart of adult common lymphoid progenitor (CLP), which is phenotipically similar (Lin^{-}IL^{-}7R^{+}c-Kit^{lo}Sca1^{lo}) and is present as a distinct population at E12.5[28], did not reveal any difference between Wt and Wnt3a$^{-/-}$ FLs, although there is a non-significant trend towards lower numbers of CLP in *Wnt3a$^{-/-}$* embryos (Figure 5a,b). Furthermore, after 7 days of culture on OP9 BM derived stromal cells, both Wt or *Wnt3a$^{-/-}$* E12.5 FL cells efficiently developed into CD19^{+} B-cell progenitors (Figure 5c). Most cells were in the pro-B cell stage, but after prolonged culturing intracellular IgM could be detected both in the cultures using Wt or Wnt3a$^{-/-}$ cells (data not shown). After 7 days of culturing, the percentage of CD19^{+} cells was significantly higher in the Wnt3a$^{-/-}$ cultures, but this increase was not reflected by an absolute increase in cell numbers. Rather it was attributed to the marked reduction of the CD11b^{+} population, confirming that Wnt3a deficiency negatively affects myeloid differentiation.

We were also interested in the capacity of *Wnt3a$^{-/-}$* progenitors to seed the thymus and differentiate into the T-cell lineage. In the developing mouse embryo, the thymic rudiment is first colonized by immature cells at approximately E11[21]. Since thymic lobes at E12.5 are not yet well-defined structures, sections were prepared and stained with HE to visualize different

tissues. Presence of a thymic rudiment could be demonstrated in Wnt3a$^{-/-}$ embryos and was confirmed by staining with ER-TR4, which identifies thymic epithelial cells[29] (Figure 5d). Although the thymic rudiment is seeded by hematopoietic cells, in *Wnt3a$^{-/-}$* embryos it shows a noticeable reduction in size indicating that T-cell development is affected with respect to cell numbers. To further characterize this deficiency, explant cultures of E12.5 thymic lobes (FTOC) were performed. In general, Wnt3a$^{-/-}$ thymi were found to contain fewer thymocytes (usually

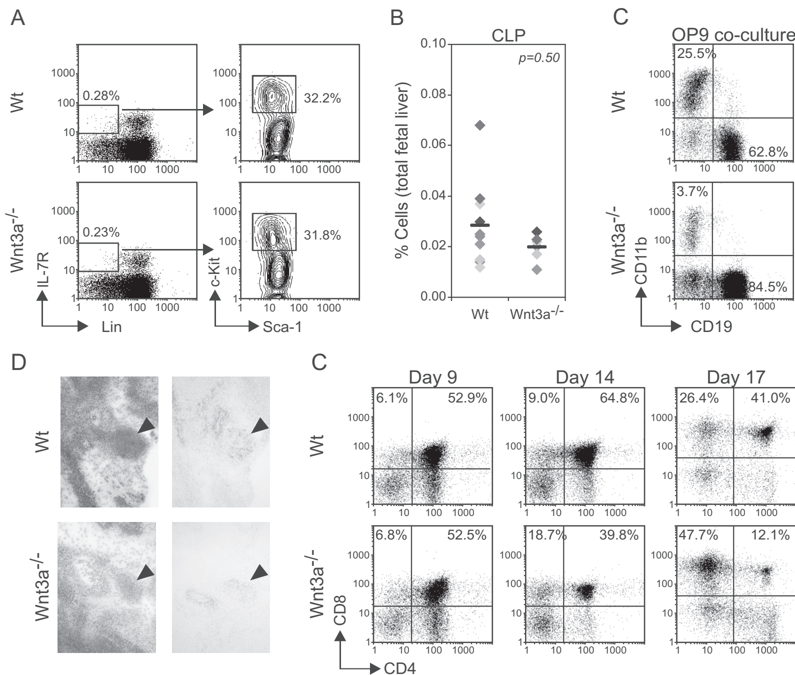

Figure 5. Lymphoid potential of Wnt3a-/- hematopoietic progenitors. (A) Flow cytometry analysis of common lymphocyte progenitors (CLPs). Lineage negative IL-7R^{+} cells were gated and analyzed for c-Kit and Sca1 expression. Numbers indicate the percentage of cells in each gate. (B) Percentage of CLPs in total FLs from Wt and *Wnt3a$^{-/-}$* embryos. The averages are indicated by a dash. Data from 11 Wt and 6 Wnt3a$^{-/-}$ FLs, belonging to 6 different litters identified by different colors. (C) To evaluate B-cell potential Wt and Wnt3a$^{-/-}$ FL cells were cultured for 7 days on confluent layers of OP9 BM derived stromal cells. Cells were harvested and presence of cells from different lineages was analysed by flow cytometry. Data are representative of 4 independent experiments. (D) Reduced cellularity in Wnt3a$^{-/-}$ thymic anlage revealed by immunohistochemistry analysis. Tissue sections of E12.5 embryos were stained with HE to visualize different tissues (left panels). Thymic epithelial cells stained with the thymic stroma antibody ER-TR4 (right panels) to confirm identification of thymic anlage in *Wnt3a$^{-/-}$* embryos. Staining with an isotype control for ER-TR4 was completely blank. Thymic anlage is indicated by an arrow head. Stainings shown are representative of four Wt and four Wnt3a$^{-/-}$ embryos examined. (E) Thymic lobes from Wt and *Wnt3a$^{-/-}$* E12.5 embryos were cultured in FTOC to allow T-cell development to proceed, and harvested at the indicated time points. Thymocytes subsets were analysed by flow cytometry. Data are from 5 Wt and 3 *Wnt3a$^{-/-}$* embryos analyzed. See page 214 for a full-color representation of this figure.

about 3-fold lower) than Wt embryos, irrespective of the duration of culturing (data not shown). After 9 days of culturing, all stages of T-cell development could be distinguished, including mature single positive (SP) $CD4^+$ and $CD8^+$ cells, both in Wt and $Wnt3a^{-/-}$ embryos (Figure 5e). At this time-point, no differences were detected in subset distribution between Wt and $Wnt3a^{-/-}$ thymi. However, when thymic lobes were cultured for prolonged times (14-17 days), T-cell development in $Wnt3a^{-/-}$ thymi rapidly deteriorated. This presented mainly as a decrease in the percentage of double positive (DP) cells and an accumulation of $CD8^+$ thymocytes, revealing a block at the ISP stage (Figure 5e). The progressively impaired T-cell development at the ISP stage phenocopies mice deficient in *Tcf1*[30], suggesting that the Wnt3a effects on immature thymocytes are mediated by signaling through Tcf1. To investigate whether this defect is intrinsic to the hematopoietic cells that seeded the thymus, we cultured Wt and $Wnt3a^{-/-}$ E12.5 FL cells in irradiated thymic lobes from Wt E14.5 embryos. After 14-21 days of culture, lobes were stained for flow cytometry. No differences were observed between FTOCs using Wt or $Wnt3a^{-/-}$ FL cells, with respect to cell numbers or thymocyte subset distribution (data not shown). The reduced T-cell development in $Wnt3a^{-/-}$ embryos must therefore be attributed to absence of Wnt3a production by the thymic stroma.

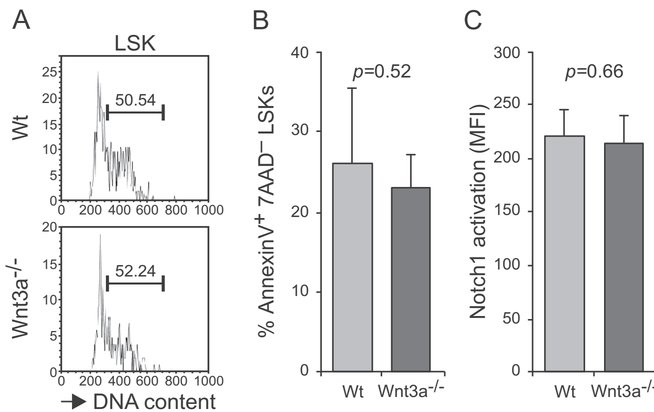

Figure 6. long-term repopulation deficiency is not caused by altered cell cycle or survival. (A) Cell cycle analysis (Hoechst 33342) of E12.5 FL LSKs from Wt and *Wnt3a*$^{-/-}$ embryos. Values represent percentage of LSK cells in S/G2/M phases of cell cycle. Results are representative of 3 *Wnt3a*$^{-/-}$ and 6 Wt embryos from 3 different litters. (B) Determination of percentage of apoptotic cells by staining with AnnexinV and 7AAD. Apoptotic cells were defined as $AnnexinV^+$ $7AAD^-$. Results are representative of 6 *Wnt3a*$^{-/-}$ and 6 Wt embryos from 2 different litters (C) Activation of Notch1 in E12.5 FL LSKs from Wt and $Wnt3a^{-/-}$ embryos. Results are representative of 4 *Wnt3a*$^{-/-}$ and 8 Wt embryos belonging to 4 different litters.

Wnt3a deficiency does not alter survival or cell cycle of hematopoietic stem and progenitor cells

Wnt3a$^{-/-}$ HSCs have impaired long-term repopulation capacity but have largely retained the ability to develop into all hematopoietic lineages. We next sought to study the cellular basis underlying this self-renewal defect.

HSC function depends on a tight regulation of the cell cycle. It was recently shown that activation of β-catenin leads to an increased proliferation followed by loss of stem cell activity and exhaustion of the HSC pool[8,9]. We therefore analyzed the cell cycle status of the HSC compartment. A high proportion of the LSK cells in the Wt FL are in cycle[22] in agreement with FL being an organ where expansion of the HSC compartment occurs. No significant differences were found in the frequency of LSK cells in S/G2/M suggesting that Wnt3a deficiency has no effect on the HSCs proliferation (Figure 6a). Furthermore, to investigate whether LSKs from Wnt3a$^{-/-}$ embryos have decreased survival capacity, we analyzed apoptosis in these cells. In Wt and *Wnt3a$^{-/-}$* LSK compartments, the frequency of AnnexinV^{+} cells did not differ (Figure 6b) indicating comparable apoptosis levels.

We also wanted to analyze weather Wnt3a deficiency differentially affected activation of the Notch signaling pathway since this has been shown to be important for HSC function and has been proposed to collaborate with Wnt signaling to maintain stem cell properties[31]. However using a specific antibody against activated Notch1 no differences were detected in the activation of this pathway in *Wnt3a$^{-/-}$* comparing to Wt LSKs (Figure 6c).

Thus, neither alteration in cell cycle nor in survival of stem/progenitor cells explains the reduced numbers of cells and decreased long-term repopulation capacity. Furthermore, differential activation of Notch1 was also not observed.

DISCUSSION

Using a mouse model that lacks expression of Wnt3a, we show here that the deficiency of one specific Wnt protein leads to markedly reduced numbers of HSCs *in vivo*. These HSCs were not affected in multilineage differentiation potential but they had an irreversible and severely decreased self-renewal and long-term repopulation capacity, as determined by secondary transplantation assays. The absence of Wnt3a also strongly affects the primitive MPP progenitors and more mature myeloid progenitors such as CMPs and GMPs which were decreased in number. By contrast CLPs and precursor B-cells were not affected.

Since MPPs retain both myeloid and lymphoid potentials but little or no erythroid-megakaryocyte potential[24,32,33], one could expect both myeloid and lymphoid progenitors to be affected. The fact that CMPs are severely reduced while CLP appeared not to be affected shows

a stronger dependency on Wnt3a for myeloid versus B lymphoid development. This is also supported by the strict dependence on Wnt-β-catenin signaling for the progression of chronic myelogenous leukemias whereas B-cell acute lymphoblastic leukemias can develop independently of β-catenin expression[13]. Despite this deficiency at the progenitor level, mature myeloid cells were present at normal numbers. This can be explained by the fact that the defects observed do not completely block differentiation and because lineage specific progenitors can probably compensate for the early incomplete differentiation block by rapid and extensive proliferation supported by cytokines that act in a lineage specific fashion, such as GM-, G- and M-CSF, as well as IL-7.

By means of co-culture of FL progenitors with a BM derived stromal cell line, no defects in B-cell development were observed. It was previously shown that B-cell development in Lef1$^{-/-}$ mice is partially blocked at the pro-B cell stage due to lack of Wnt dependent proliferation[16]. In agreement with this, Wnt5a was shown to negatively regulate proliferation of early B-cells, possibly by inhibiting canonical-Wnt target genes[34]. Although recombinant Wnt3a was shown to induce this proliferation *in vitro*[16], it is likely that Wnt3a is not the physiologically relevant Wnt for pro-B cell proliferation or other Wnt proteins are compensating this function, as our data do not show a block in B-cell development. This is consistent with the idea that HSCs and precursor B-cells occupy different niches.

In contrast, the T-cell lineage shows clear defects, probably reflecting the dependency of the early thymic emigrants on MPP as a major physiological precursor cell to the first cell that seeds the thymus[35]. The progressively impaired T-cell development at the ISP stage observed phenocopies mice deficient in Tcf1[30] suggesting that in these mice T-cell development is blocked because of lack of a nuclear response to proliferative Wnt signals, as has also been suggested by previous loss-[36,37] and gain-of-function studies[38]. This phenotype appears to be environmentally determined, because *Wnt3a*$^{-/-}$ cells develop normally in Wt FTOCs. This is supported by the expression of Wnt3a in thymic epithelium, but not in the developing thymocytes[17]. The similarities with Tcf1 deficiency suggest that Wnt3a is directly regulating thymocyte development, but it is possible that Wnt3a is also important for the thymic stroma and consequently affect T-cell development. Nevertheless, there appears to be an important difference in the function of Wnt3a for immature thymocytes, where it seems to be mainly a proliferation factor, versus HSCs where it influences self-renewal and differentiation cell fates.

The Wnt3a deficiency does not only lead to a quantitative defect in HSC numbers, but *Wnt3a*$^{-/-}$ HSCs are also functionally affected. Although they are able to efficiently repopulate lethally irradiated primary recipients, long-term reconstitution capacity is markedly reduced when HSCs are re-challenged by transplantation into secondary recipient mice, demonstrating a lack in self-renewal capacity.

Because Wnt3a is a secreted factor it might affect HSCs directly or indirectly by signaling through the stromal cells[39] that constitute the HSC niche. Thus, this environmentally determined deficiency turns into a cell-autonomous defect as Wnt3a in BM of the Wt recipient mice is not able to restore normal HSC function. This indicates that lack of Wnt3a during a critical period in embryonic development irreversibly impairs the self-renewal capacity of HSCs, suggesting that Wnt3a is important for the establishment of a specific genetic programme in HSCs. A similar phenotype was observed in adult HSCs when they transiently occupied a niche that overexpressed the Wnt inhibitor Dkk1[40]. Furthermore, the increased B-cell proliferation observed in Wnt5a-deficient FLs also could not be complemented by physiologic levels of Wnt5a expressed by Wt BM stroma[34]. The irreversible *Wnt3a$^{-/-}$* phenotype could possibly be explained by epigenetic alterations induced by Wnt signals. Indeed, different members of the Polycomb complex factors were shown to be essential for HSCs function[41,42]. More specifically, Rae28 deficiency results in reduced fetal HSC and progenitor numbers, while FL cellularity and mature cell subsets remained unaffected[42], similarly to what we observed here.

Several lines of evidence suggest that Wnt activity is context dependent. Wnt proteins have different effects depending on the target cell[34], receptor availability[43] and probably other signals or pathways activated at the same time[6,44]. In this way approaches to generally activate or ablate Wnt signaling may lose the regulatory mechanisms between different Wnts. Indeed different β-catenin gain-of-function studies yielded different results probably reflecting the effect of different dosages of activation of the pathway[6,8,9]. The notion that correct levels of Wnt signaling are critical in inducing a self-renewal type of expansion in HSC[45] is also supported by experiments from Baba et al[46,47], who showed that it is possible to induce some stem cell characteristics in committed progenitors by ectopic expression of stable β-catenin. However such progenitors did not reconstitute T-cells when transplanted to immunodeficient mice showing again that a very strong signal that bypasses all negative regulatory steps of the Wnt signaling cascade such as given by stabilized β-catenin cannot mimic events triggered by tightly regulated physiological Wnt signals. Although initial studies failed to show a phenotype in the absence of β-catenin[10-12], two recent loss of function approaches by knocking-out β-catenin[13] or overexpressing Dkk1[40], thereby inhibiting canonical Wnt signaling, showed reduced self-renewal capacity of HSCs. Here we were able to point one specific Wnt protein in this function, at least during embryonic development.

The reduced number of HSC and reduced long term repopulation capacity observed could not be explained by alterations in cell cycle or survival of stem/progenitor cells. In this way, HSCs from *Wnt3a$^{-/-}$* embryos seem to be biased to a non self-renewing type of division leading to depletion of the HSC pool. Several developmentally conserved signaling pathways, including Notch, Hedgehog and Smad pathways, appear to be important in the regulation of HSC function[48] and may normally collaborate with Wnt signaling in establishing a self-renewal

genetic program. One such candidate is Notch signaling. However the levels of activated Notch1 did not differ between Wt and *Wnt3a*$^{-/-}$ LSK cells, making it unlikely that notch1 plays this role.

Interestingly, besides the similarities with Tcf1$^{-/-}$Lef$^{-/-}$ embryos[18], the posterior patterning defects observed in the *Wnt3a*$^{-/-}$ embryos also resemble the ones described for the *Sox17*$^{-/-}$ embryos. Sox17 was recently shown to be a critical regulator of fetal HSCs maintenance. Deletion of *Sox17* leads to increased levels of Dkk1 implying that it maintains fetal HSCs by promoting Wnt signaling[49].

We and others[4] did not find *Wnt3a* expression at detectable levels in the FL, probably because of *Wnt3a* expression is only present a very restricted subset of niche cells. Nevertheless, this might indicate that the deficiency we report here is determined earlier during the embryonic development or in extra-hepatic HSC sources. Since the numbers of definitive HSCs in E12.5 FL results from the cumulative HSC activity from the aorta-gonad-mesonephros (AGM) region, yolk sac and placenta[21,50], these tissues warrant future study.

Given the differences between fetal and adult hematopoiesis, it is also important to investigate whether Wnt3a plays similar roles in the self-renewal of BM HSC. In view of the fact that Wnt3a is expressed in BM[16], such a functional role would not be unexpected. Finding specific Wnt proteins that regulate hematopoietic stem cell fate may have important implications for *in vitro* manipulation and expansion of the HSC compartment for transplantation and gene therapy applications as well as for the understanding of cancer stem cells in hematological malignancies.

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AUTHORSHIP

TCL and FW designed and performed experiments and wrote the manuscript, BAEN, MRMB, EFEdH, TN, SH performed experiments, RdH contributed with essential analytical tools, JJMvD wrote manuscript, FJTS design experiments, wrote manuscript and supervised project. The authors declare no competing financial interests.

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Chapter 2.2

Wnt3a non-redundantly controls hematopoietic stem cell function and its deficiency results in complete absence of canonical Wnt signaling

Tiago C. Luis[1,2], Brigitta A.E. Naber[1,2], Willem E. Fibbe[2], Jacques J.M. van Dongen[1] and Frank J.T. Staal[1,2]

[1]Department of Immunology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands. [2]Department of Immunohematology and Blood Transfusion, Leiden University medical Center, Leiden, The Netherlands.

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ABSTRACT

The canonical Wnt signaling pathway has been implicated in the regulation of HSC fate decisions but the role of specific Wnt proteins has remained elusive. We recently showed that Wnt3a deficiency leads to reduced numbers of HSCs in fetal liver (FL) and to severely impaired repopulating capacity as studied by serial transplantation assays. Our previous work did not address several important questions, namely to what extend Wnt signaling was affected in HSC, whether other Wnt genes could take over the role of Wnt3a and whether its action was autocrine or paracrine. Here we show that Wnt3a deficiency results in complete absence of canonical Wnt signaling in mutant HSC *in vivo*, that Wnt3A acts non-redundantly and in a paracrine fashion. Together these findings indicate the potential use of Wnt3a for *ex vivo* manipulation of HSC for transplantation and stem cells based therapies.

INTRODUCTION

HSC reside in specialized niches which provide signals that regulate stem cell function and fate decisions. A wide variety of signaling pathways has been proposed to regulate HSC function, among which the Wnt signaling pathway[1]. Canonical Wnt signaling is initiated upon binding of a soluble Wnt protein to a membrane-associated receptor (Frizzled-LRP5/6 complex). This binding ultimately leads to the stabilization and accumulation of β-catenin in the nucleus where it endows the transcription factors TCF/LEF with a potent transactivation domain and consequent expression of Wnt target genes[2]. Different approaches to activate the pathway in HSCs resulted in either enhancement of HSC function or exhaustion of the HSC pool[3-9], probably due to different levels of activation. On the other hand, initial attempts to inactivate the pathway with conditional deletion of β-catenin or both β-catenin and its homolog γ-catenin, did not alter HSC function[10-12]. These negative results were likely caused by the fact that canonical Wnt signaling remained intact as observed with different Wnt reporter assays[11], and therefore these approaches did not generate a null-mutant for Wnt signaling[13].

We recently studied the hematopoietic system of mice deficient in Wnt3a. Due to early embryonic lethality, this analysis was performed in FL at day 12.5 of the embryonic development. Remarkably, Wnt3a deficiency leads to reduced numbers of long-term HSCs and multipotent progenitors and these are severely and irreversibly impaired in long-term reconstitution capacity as observed in serial transplantation assays[14].

Nineteen different Wnt proteins with overlapping expression patterns have been described both in human and mouse, raising the possibility of functional redundancy. In the current study we set out to investigate the consequence of Wnt3a deficiency for canonical Wnt signaling *in vivo*, the expression patterns of Wnt3a and other hematopoietic Wnt genes to understand Wnt regulated self-renewal. Here we demonstrate that despite expression of several Wnt proteins in FL, Wnt3a plays a unique and non-redundant role in the activation of canonical Wnt signaling and, therefore, in the regulation of HSC self-renewal.

MATERIALS AND METHODS

Mice

Wnt3a heterozygous were bred and maintained in the Erasmus MC animal facility, according to the legal regulations in The Netherlands, and with approval of the local ethical committee.

Mice heterozygous for the Wnt3a mutation were kindly provided by T. Yamaguchi (National Cancer Institute-Frederick, Frederick, MD)[15] and backcrossed to C57Bl/6. Genotyping was performed as described. To obtain Wnt3a$^{-/-}$ embryos, Wnt3a heterozygous mice were put

together in the afternoon and checked for vaginal plug the next morning. The time at which the vaginal plug was observed was considered E0.5. Embryos were analyzed at E12.5. Bat-GAL Wnt reporter mice were described previously[16] and were kindly provided by R. Fodde (Erasmus MC, Rotterdam, The Netherlands).

Flow cytometry and FDG staining

For flow cytometric analyses cells were stained with monoclonal antibodies against the following molecules: B220(Ra3-6B2), CD3(145-2C11), CD4(L3T4), GR1(RB6-8C5), NK1.1(PK136), and Ter-119 all biotinilated from Becton Dickinson/Pharmingen; CD117/c-Kit(2B8) conjugated with APC from Becton Dickinson/Pharmingen and Sca1(D7) conjugated with PE-Cy7 from eBiosciences. For secondary detection streptavidin conjugated with PerCP-Cy5.5 was used.

Intracelular β-galactosidase activity was measured by staining cells with 1mM Fluorecein di-β-D-galactopyranoside (FDG) substrate (Molecular Probes). FDG was loaded into the cells by hypotonic shock at 37oC for 1 minute, prior to cell surface antibody staining[17]. β-galactosidase reaction was stopped with 1mM Phenylethyl β-D-thiogalactopyranoside (PETG, Molecular probes). Stained cells were measured with a FACS-Canto and analyzed with FlowJO software (Treestar).

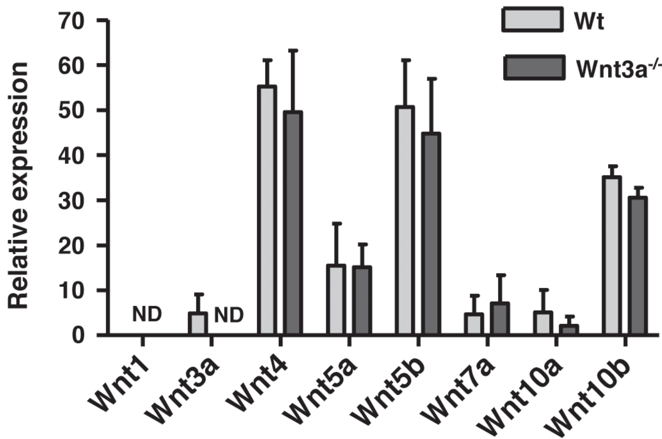

Figure 1. Wnt expression profile in E12.5 Wt and Wnt3a-/- fetal livers. Fresh Wt or Wnt3a$^{-/-}$ FLs were isolated and Ter119^{+} erythrocytes were depleted prior to mRNA isolation. Gene expression was analyzed by qPCR for the indicated Wnt genes and expression levels normalized for GAPDH. Results correspond to mean ± SEM of 2 independent experiments. In each experiment a pool of 4-8 fetal livers from each genotype was used. Measurements were performed in duplicate for each sample. ND, not detectable.

Gene expression analysis

Gene expression analysis was performed as described previously[18]. RNA was isolated using the Micro RNeasy kit (Qiagen) from fresh Ter119 depleted E12.5 Wt or Wnt3a$^{-/-}$ FLs. Ter119-depletion was performed by Magnetic Cell Sorting and Separation (MACS) using anti-Ter119 biotinilated antibody followed by magnetic beads-conjugated Streptavidin (Miltenyi Biotec). Taqman Gene Expression assay for Wnt3a (mM00437337) was purchased from Applied Biosystems. Other primer/probe sets were previously described in[18].

Statistical analysis

Statistical significance was determined by standard two-tailed t-test.

RESULTS AND DISCUSSION

Wnt expression profile in E12.5 fetal liver

We[14] and others[19,20] have presented evidence strongly suggesting that Wnt signaling is essential for HSC self-renewal. The severe phenotype observed in the Wnt3a$^{-/-}$ embryos suggested that Wnt3A is the most prominent Wnt gene for regulation of HSC function. Therefore, we determined the expression profile in FL of several Wnt genes previously shown to regulate hematopoiesis[2]. In addition, we also analyzed whether Wnt3a deficiency affected expression of those Wnt genes. For this, RT-qPCR analysis was performed on Ter119 depleted wild-type (Wt) or Wnt3a$^{-/-}$ FL cells. From the panel of Wnt genes analyzed Wnt4, Wnt5a and 5b and Wnt10b were expressed at higher levels while Wnt3a, Wnt7a and Wnt10a showed a comparatively lower expression in the Wt FLs (Figure1). Despite the abundant expression of other Wnt genes in the FL, HSC from Wnt3a$^{-/-}$ embryos showed a strong impairment in repopulation capacity as observed in secondary transplanted recipients[14]. The expression of Wnt3a by FL cells but not by the HSCs themselves[14] also indicates that Wnt3a acts in a paracrine fashion. Of interest, Wnt3a deficiency did not significantly influence the expression pattern of the other Wnt genes (Figure1) indicating that the lack of self-renewal by the HSCs in Wnt3a$^{-/-}$ FL was not due to an effect on the expression of other Wnt genes. Nevertheless, it remained important to study the effect of the Wnt3a mutation in the activation status of the canonical Wnt pathway.

Wnt3a deficiency completely abolishes activation of the canonical Wnt signaling pathway

In order to determine the effect of Wnt3a deficiency in the activation of canonical Wnt signaling pathway we used a Wnt reporter mouse model (Bat-Gal). In this model a LacZ gene

is under control of a regulatory sequence containing TCF/LEF-binding motifs, which results in LacZ expression when active β-catenin/Tcf complexes are present[16]. Analysis of reporter activity in E12.5 FL LSK ($Lin^-c\text{-}Kit^+Sca1^+$) cells showed that approximately 7% of the LSKs undergo active Wnt signaling (Figure 2a). To test the specificity of the reporter, total FL cell suspensions were incubated with LiCl (10mM) in order to activate canonical Wnt signaling pathway. Incubation with LiCl showed a 3 fold increase in the activity of the reporter in the LSK population, in comparison with the the KCl (10mM) treated control (data not shown), showing that this reporter is functional in FL HSCs and responsive to activation of Wnt pathway.

The analysis of Wnt3a deficient embryos carrying the reporter transgene in heterozygozity showed a profound reduction in the frequency of LacZ positive LSKs (Figure 2a, b), which was not significantly higher than background levels of LacZ staining (Figure 2c) in non-reporter mice. Thus, $Wnt3a^{-/-}$ LSKs show a complete abolishment of the activation of canonical Wnt signaling in comparison with littermate Wt embryos.

Activation of the Wnt signaling pathway by means of transgenic approaches, recombinant Wnt proteins or inhibitors of negative regulators of the pathway has been used in order to expand HSC and/or enhance HSC function[4,6,8,9,21,22]. Interestingly, Wnt3A was shown to preserve HSCs with an immature phenotype *in vitro* or to induce stem cell characteristics on hematopoietic

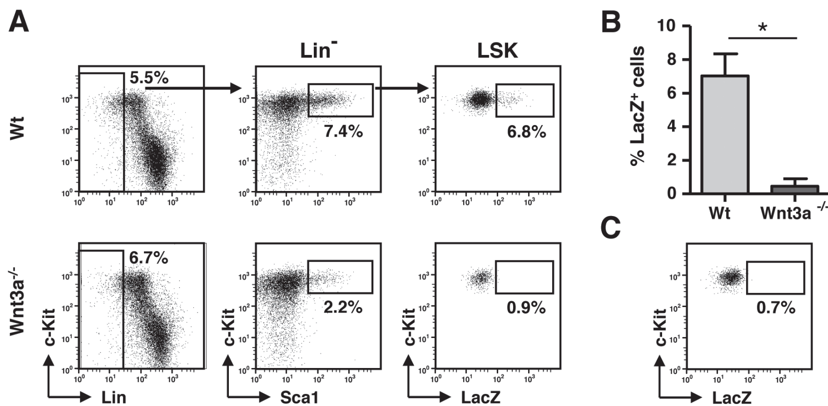

Figure2. Canonical Wnt signaling is completely abolished in HSCs from Wnt3a-/- embryos. A) Wnt3a mice were crossed with Bat-Gal Wnt reporter mice. Activation of the pathway was determined by LacZ staining on individual FL from $Bat\text{-}Gal^{Tg/+}$ Wt or $Wnt3a^{-/-}$ littermate embryos. LacZ expression was analyzed by flow cytometry on electronically gated LSK cells. B) Frequency of $LacZ^+$ cells inside LSK population. Results correspond to mean ± SEM of 2 independent experiments with 2 Wt and 1 $Wnt3a^{-/-}$ littermate embryos each. *,p=0.03. C) Littermate Wt embryos not carrying the reporter transgene were used as negative controls to determine the background staining of LacZ. LSK cells were electronically gated and frequency of $LacZ^+$ cells was determined in this population.

progenitors[9,23]. Besides this, treatment with Wnt3A resulted in increased proliferation and expansion of transplantable Bcl2-transgenic HSC[9]. Here we show *in vivo* that Wnt3a plays a non-redundant role in the regulation of FL HSCs function. Despite expression of other Wnt proteins and although being one of the lowest expressed Wnt proteins in total FL, Wnt3A is the only Wnt protein being able to activate canonical Wnt signaling in HSCs. This may be explained by the requirement of specific ligand/receptor combinations in HSC or by compartmentalization of Wnts expression in specialized niches in FL. Although Wnt3a may also indirectly regulate HSCs by influencing the niche microenvironment, our data indicate that HSCs do not produce Wnt3a themselves, but are directly affected by Wnt3a deficiency. This unanticipated non-redundant role of one specific Wnt protein in the regulation of HSCs self-renewal specifically points to Wnt3A as the Wnt protein of choice to expand HSCs *ex vivo* for transplantation and stem cell based therapies.

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AUTHORSHIP

TCL designed and performed experiments and wrote the manuscript, BAEN performed experiments, WEF and JJMvD wrote manuscript, FJTS designed experiments, wrote manuscript and supervised the project. The authors declare no competing financial interests.

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Chapter 2.3

Wnt Proteins: Environmental factors regulating HSC fate in the niche

Tiago C. Luis[1,2], Frank J.T. Staal[1, 2]

[1]Department of Immunology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands. [2]Department of Immunohematology and Blood Transfusion, Leiden University medical Center, Leiden, The Netherlands

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ABSTRACT

The Wnt signaling pathway has been implicated in regulation of haematopoiesis through a plethora of studies from many different laboratories. However, different inducible gain- and loss-of-function approaches retrieved controversial and some times contradictory results. Different levels of activation of the pathway, dosages of Wnt signaling required and the interference by other signals in the context of Wnt activation collectively explain these controversies. Gain-of-function or *in vitro* exposure to Wnt proteins and more specifically Wnt3a was shown to enhance hematopoietic stem cell (HSC) activity but its exact role was still not completely understood. In a recent study we analyzed the hematopoietic system of mice deficient for this specific Wnt gene. Wnt3a deficiency results in early embryonic lethality around embryonic day 12.5 (E12.5), precluding analysis in adult mice, but allowing hamatopoiesis to be studied in fetal liver and in the just colonized thymic rudiment. Notably, we showed that long-term HSCs and multipotent progenitors are reduced in fetal liver and have severely reduced long-term reconstitution capacity as observed in serial transplantation assays. Of interest, deficiency in Wnt3a leads to complete abolishment of canonical Wnt signaling in fetal liver hematopoietic stem and progenitor cells. This HSC deficiency is not explained by altered cell cycle or survival and is irreversible since it cannot be restored by transplantation into Wnt3a competent mice. In addition, Wnt3a deficiency differentially affects myeloid and B-lymphoid lineages with myeloid cells being affected at the progenitor level, while B lymphopoiesis is apparently unaffected. Immature thymocytes however were reduced in cell numbers due to lack of Wnt3a production by the thymic micro environment. Our results show that while in the thymus Wnt3a provides cytokine-like, proliferative stimuli to developing thymocytes, Wnt3a regulates cell fate decisions of fetal liver HSC in a non-redundant way.

INTRODUCTION

Wnt proteins are secreted lipid-modified molecules that bind to specific receptor complexes located at the cell membrane, activating a complex signaling transduction pathway which is ultimately responsible for the induction of specific genetic programs. Wnt signaling is essential for several basic developmental processes and has been implicated in the regulation of hematopoietic stem cell (HSC) function as well as other stages during hematopoiesis. In the absence of Wnt ligands bound to the Wnt receptor complex, the cytoplasmatic levels of β-catenin, the key player in this pathway, are kept very low through the action of a protein complex (the so-called destruction complex) that actively targets β-catenin for degradation. However, activation of the pathway by a Wnt protein leads to the inactivation of the β-catenin destruction complex allowing its accumulation in the cytoplasm and consequently migration to the nucleus. In the nucleus β-catenin binds to members of the Tcf/Lef transcription factors family, thereby converting them from transcriptional repressors into transcriptional activators (reviewed in[1]).

The role of the Wnt signaling pathway in HSCs has been studied using different gain and loss of function approaches, which yielded some controversial results.[2] Initial attempts to retrovirally overexpress a constitutively active form of β-catenin in Bcl2-transgenic hematopoietic stem/progenitor cells led to an increase in proliferation of HSCs and repopulation capacity upon transplantation into lethally irradiated mice.[3] However, two subsequent studies using a mouse model to conditionally overexpress a stabilized form of β-catenin led to a block in multilineage differentiation, and a transient expansion of the HSC pool which was followed by the exhaustion of the long-term HSCs[4,5]. The differences between these studies are likely due to different levels of activation of the Wnt pathway achieved with the two different approaches. Indeed, in many developmental systems Wnt factors are known to form gradients and in this way, the exact concentration of Wnts at a specific place, and consequently the resultant levels of Wnt signaling, may influence the effect of this pathway. Of note, various Wnt reporter mice show very different levels of Wnt signaling in various tissues with high levels of Wnt-β-catenin dependent transcription in gut and skin, lower levels in breast and central nervous system (CNS) and mostly modest levels in hematopoietic organs (Reviewed in [6,7], and references therein). In addition, the different genetic backgrounds of the mice used in these experiments (Bcl2-transgenic in the Reya et al study) may also account for these differences.

On the other hand, the reverse approaches to conditionally delete β-catenin showed contradictory results. Using the Mx-Cre system to drive deletion of β-catenin,[8] or both β-catenin and its homologue γ-catenin,[9,10] no defects were observed neither in HSC function nor in the hematopoietic system and of note in the thymus. Interestingly in one of these

studies[9] it was showed, using reporter assays, that the Wnt signaling pathway was still active in HSC, thymocytes and peripheral T-cells, in the combined absence of β- and γ-catenin. This could imply the existence of an alternative factor with the ability to transduce Wnt signals in the hematopoietic system, or alternatively, a hypomorphic allele of β-catenin may have been generated by the targeting approach that could theoretically permit low levels of Wnt signaling which may rescue a hematopoietic defect. However, a more recent study using the Vav-Cre system to achieve deletion of β-catenin[11] in HSCs showed reduced self-renewal capacity suggesting the requirement of Wnt signaling for the long-term growth and maintenance of HSCs.

WNT3A REGULATES HEMATOPOIETIC STEM CELL FATE

Nineteen different Wnt proteins were described in both human and mouse genomes and their patterns of expression overlap both spatially and temporarily during development, raising the possibility of functional redundancy between Wnt proteins. Wnt proteins were for the first time discovered as normal growth/differentiation factors for hematopoietic progenitors in *in vitro* assays (Austin et all, 1997, Blood; Van Den Berg, 1998, Blood). However further investigation showed that Wnts could have a role in maintaining or inducing an undifferentiated phenotype in hematopoietic cells. Exposure of Bcl2-transgenic HSCs to Wnt3a resulted in maintenance of an immature phenotype *in vitro* and enhanced the repopulation capacity of lethally irradiated mice.[12] In these studies Wnt3a treatment led to activation of the Wnt-β-catenin canonical pathway, as observed by TCF/LEF-GFP reporter assays.[3] Furthermore, in a recent study Wnt3a was showed to promote *in vitro*, the reacquisition of some stem-cell like characteristics in committed lymphoid progenitors,[13] in agreement with another study from the same group showing that expression of constitutively active β-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors.[14]

Besides Wnt3a, other Wnt proteins such as Wnt5a and Wnt4 were recently implicated in regulation of hematopoietic stem/progenitor cells. Wnt4 was showed to expand early hematopoietic progenitors through activation of non-canonical Wnt signaling.[15] Intraperitoneal administration of Wnt5a into NOD/SCID mice[16] and *in vitro* Wnt5a exposure prior to transplantation17 enhanced HSCs repopulation capacity. Of interest, the non-canonical Wnt5a signals may antagonize canonical Wnt signaling in HSCs.[17] However the results do not discriminate whether this antagonistic effect is due to interaction of the intracellular pathways or just to competition between Wnt ligands for the same Frizzeled receptor. Furthermore it will be important to investigate if this *in vitro* system reflects the *in vivo* situation.

In a recent study we analyzed the hematopoietic system of Wnt3a null embryos and found

reduced numbers of HSCs in situ which were functionally impaired in their long-term repopulation capacity, while multilineage differentiation capacity was in general not affected.[18] Wnt3a deficiency results in early embryonic lethality around embryonic day 12.5 (E12.5) and these embryos have a severe phenotype with little or no caudal development posterior to forelimbs, disrupted notochord, and abnormal formation of ectopic neural tubes[19] (Figure 1). This phenotype highly resembles the gross anatomical abnormalities seen in Tcf1$^{-/-}$ Lef1$^{-/-}$ embryos[20] suggesting lack of redundancy between Wnt3a and other Wnt proteins for at least some developmental functions.

At E12.5 the fetal liver is the main hematopoietic organ. In the fetal liver, HSCs and progenitors derived from aorta-gonad-mesonephros (AGM) region, yolk-sac and placenta, expand to generate the adult hematopoietic system. The analysis of the Lin^{-} Sca1^{+} Kit^{+} (LSK) compartment, which contains the multipotent HSCs, revealed a 3 to 5 fold reduction in the numbers of these cells, in comparison to littermate wild-type embryos. In addition, both Flt3- (HSCs) and Flt3^{+} (multipotent progenitors, MPPs) were reduced indicating a true HSC deficiency (Figure 2). This phenotypic reduction in HSCs numbers was also reflected in functional assays i.e., in non-competitive transplantation assays in which Wnt3a$^{-/-}$, in comparison to equal numbers of wild-type total fetal liver cells showed a severely reduced capacity to repopulate sub-lethally irradiated mice.

Since other Wnt proteins are expressed in fetal liver, Wnt3a animals were crossed with the Bat-Gal Wnt reporter mice21 to investigate the impact of absence of Wnt3a for the activation of the canonical Wnt signaling pathway in HSCs. In comparison to littermate Wt embryos in

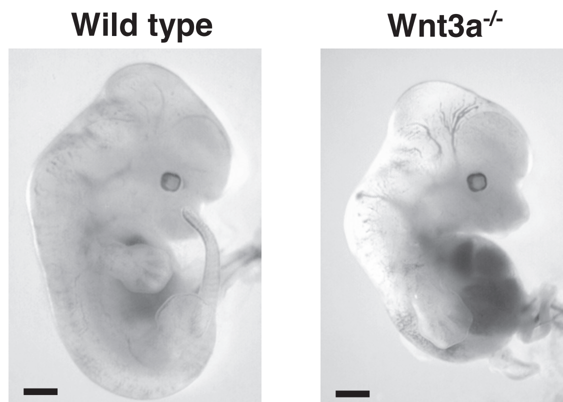

Figure 1. Wnt3a deficient embryos at 12.5 day of embryonic development. Wnt3a$^{-/-}$ embryos have a severe phenotype with little or no caudal development posterior to forelimbs, disrupted notochord, and abnormal formation of ectopic neural tubes, the so called vestigial tail. Bar represents 1 mm. See page 215 for a full-color representation of this figure.

which approximately 10% of the cells within the LSK population showed active Wnt signaling, the activation of this pathway was completely abolished in Wnt3a$^{-/-}$ fetal liver LSKs (Luis and Staal, unpublished observations). Thus, Wnt3a has a non-redundant role in the regulation of the HSC compartment and is the sole Wnt protein responsible for inducing canonical Wnt signaling in FL HSC.

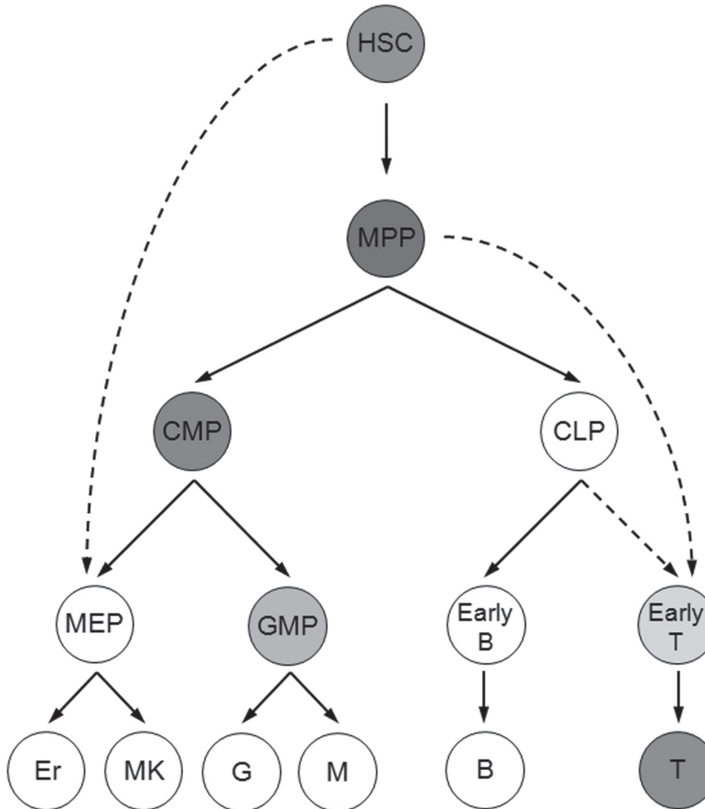

Figure 2. Hematopoietic subsets affected by Wnt3a deficiency. Absence of Wnt3a leads to reduced number of hematopoietic stem cells (HSC), which have lost long-term repopulation capacity and to reduced numbers of multipotent progenitors (MPP). Moreover, Wnt3a deficiency affected myeloid but not B-lymphoid development at the progenitor level, which resulted in reduced levels of common myeloid progenitors (CMP) and granulocyte-monocyte progenitors (GMP) but not common lymphoid progenitors (CLP). Although myeloid progenitors were reduced mature myeloid cells appear at normal frequencies by later expansion in response to myeloid growth fcators. Immature thymocyte differentiation is also impaired at immature single positive stage laeding to a block at the CD4-CD8- DN to CD4+ CD8+DP transition. Colored circles in the figure identify the subsets affected. Other abbreviations: MEP, Megakaryocyte-erytrhocyte progenitors; Er, Erythrocytes; MK. Megakaryocytes; G, Granulocytes; M, Monocytes/macrophages; B, B-cell; T, T-cell.

Besides being reduced in numbers, Wnt3a$^{-/-}$ HSCs were also affected in self-renewal capacity as observed in competitive and serial transplantation assays.[18] That is, equal numbers of purified Wnt3a$^{-/-}$ or wild-type LSKs (expressing the congenic marker Ly5.2) were transplanted, together with equal numbers of competitor cells (expressing the congenic marker Ly5.1), into lethally irradiated mice. Although both Wnt3a$^{-/-}$ and wild-type cells showed overall similar efficiency to repopulate primary recipients as observed 12 weeks after transplantation, when the bone marrow from primary recipients was re-transplanted into secondary recipient mice (also lethally irradiated), a dramatic impairment of repopulation capacity was observed as early as 4 weeks after transplantation. This was confirmed 12 weeks post transplantation in bone marrow where the competitor Ly5.1 HSCs almost completely over-compete the Wnt3a$^{-/-}$ HSCs, contrary to what was observed in the control mice receiving wild type cells. Thus, Wnt3a deficient HSC were almost completely devoid of repopulation capacity. Furthermore, the contribution of Wnt3a$^{-/-}$ Ly5.2 HSCs to the different lineages in peripheral blood was severely affected and thymus repopulation was completely abolished.

Although an indirect influence of Wnt3a on HSC, by modifying the niche where these cells reside cannot be excluded, this environmentally determined signal turned into a cell-autonomous defect since these cells loose long-term repopulation capacity of recipient mice. Indeed, Wnt3a expressed in the bone marrow of recipient mice[12,22] was not able to rescue HSC function indicating a permanent and irreversible self-renewal deficiency. Therefore, lack of Wnt3a during a critical period of embryonic development irreversibly impairs self-renewal of HSCs, suggesting that Wnt3a is necessary for the establishment of a specific genetic program in HSCs.

A similar phenotype was also observed when HSCs transiently occupied a niche that overexpresses the naturally occurring canonical Wnt signaling inhibitor Dkk1.[23] In our studies we show that a specific Wnt protein is responsible for this phenotype, at least during embryonic development, and that it acts in a non-redundant fashion with other Wnts. Together these studies suggest that HSCs require canonical Wnt signals to maintain stemness properties. This irreversible phenotype can possibly be explained by epigenetic modifications as a result of the absence of Wnt activation. Indeed, several epigenetic factors belonging to the polycomb complex such as Bmi1 and Rae28[24,25] were shown to be essential for both adult and fetal HSCs, respectively. More specifically, Rae28 deficiency results in reduced numbers of hematopoietic stem and progenitor cells without affecting fetal liver cellularity and more mature hematopoietic subsets,[25] similarly to the Wnt3a phenotype.

In our studies, Wnt3a deficiency did not influence the proliferation or survival of fetal liver LSKs, suggesting that Wnt signaling is regulating HSC fate decisions (self-renewal versus differentiation) and not promoting expansion of the HSC compartment. Furthermore, studies from Malhotra et al and Fleming et al suggest that canonical Wnt signals may slow HSCs proliferation. Since these studies were performed with bone marrow derived HSCs which are

highly quiescent cells, the difference to our own results might be explained by the highly proliferative status that fetal liver HSCs already present (approximately 50% of the cells are in S-G2-M phases of cell cycle). In contrast, exposure of Bcl2-transgenic HSCs to purified Wnt3a resulted in increased proliferation.[12] This likely is explained by the genetic background of these cells and to the culture conditions used in these experiments. Concluding, Wnt signals seem to regulate HSC function in the stem cell niche in a context dependent way. Many interactions with other signaling transduction pathways are described[26] and, the final outcome is dependent on different synergistic and antagonistic environmental signals that form a complex network. The Notch signaling pathway was proposed to collaborate with Wnt signaling to maintain stem cells properties.[27] Using an antibody specific for activated/cleaved Notch1, no difference were observed between wild-type and Wnt3a$^{-/-}$ in the signaling activity through this receptor. Therefore, it is unlikely that differential Notch signaling explains the lack of self-renewal of Wnt3a-defcient HSCs.

WNT3A REGULATES MYELOID AND T CELL PROGENITORS, BUT NOT B LYMPHOCYTE DEVELOPMENT

In addition to a role in regulation of HSC fate, Wnt3a also appeared to play other roles during hematopoiesis. Although mature myeloid cells were present at normal frequencies, a severe reduction in myeloid progenitors at the level of common myeloid progenitor (CMP) and granulocyte-monocyte progenitor (GMP) was observed (Figure 2), prospectively by flow cytometry and also functionally by *in vitro* methylcellulose colony assays.[18] The fact that there is not a complete block in myeloid differentiation probably allows myeloid progenitors that overcome this block to extensively proliferate and generate a normal mature myeloid compartment in response to other myeloid growth factors. Surprisingly despite a reduction in MPPs, which retain both myeloid and lymphoid potential but little or no erythroid-megakaryocyte potential,[28,29] common lymphoid progenitors (CLP) were not affected. These findings indicate that myeloid cells have a stronger dependence on Wnt3a in comparison to B-lymphoid cells (Figure 2). The higher requirement of Wnt3a by the myeloid cells is in agreement with a higher dependence on β-catenin signaling for the progression of chronic myelogenous leukemias while B-cell acute lymphoblastic leukemias can still develop in the absence of β-catenin.[11] In addition, B-cell development was also not impaired in cocultures of Wnt3a$^{-/-}$ fetal liver progenitors with OP9 stromal cells.[18] Although Wn3a was previously shown to induce proliferation of early B-cells and Lef1$^{-/-}$ mice have a partial block at Pro-B cell stage,[22] our results indicate that Wnt3a is not the physiologically relevant Wnt in this function, at least in fetal liver. Furthermore, in a more recent study, exposure to stromal cell-produced Wnt3a blocked B-cell development at an early stage.[13]

Despite normal lymphocyte development in fetal liver, histological analyzes of the thymic rudiment showed that, although this was colonized by hematopoietic progenitors it was clearly reduced in size demonstrating that T-cell development could be impaired at least in respect to cell numbers. Besides this, explant cultures of E12.5 Wnt3a$^{-/-}$ thymic lobes, the so called fetal thymic organ cultures (FTOC) showed a deterioration of T-cell development over time, observed by accumulation of immature single positive (ISP) thymocytes and reduction of double-positive ($CD4^{+}CD8^{+}$) cells. This progressive impairment of T-cell development at the ISP stage is highly similar to the phenotype of Tcf1$^{-/-}$ mice, suggesting that Tcf1 mediates the effects of Wnt3a in immature thymocytes and that the major phenotype in Tcf-1$^{-/-}$ mice is caused by lack of transduction of Wnt signals to target gene expression. In agreement with Wnt3a being produced by the thymic stroma but not by developing thymocytes,[30] Wnt3a$^{-/-}$ thymocytes developed normally in wild-type FTOCs. Therefore the defects in T-cell development must be attributed to absence of Wnt3a production by the thymic stroma. In this way, while Wnt3a is an important regulator of HSC fate decisions, influencing the choice between self-renewal and differentiation without affecting proliferation, it provides merely proliferative stimuli to developing thymocytes.

Since our analysis was limited to fetal hematopoietic stem cells due to the early lethality of Wnt3a$^{-/-}$ embryos and, given the differences between fetal and adult hematopoiesis it will be important to further study whether Wnt3a has also an essential role for bone marrow HSCs. Besides this, although the effect of Wnt signaling on HSCs has extensively been studied using different mouse models, it will be important to investigate whether this also applies to human HSCs. Activation of canonical Wnt signaling by inhibition of the Glycogen synthase kinase-3β (Gsk-3β) increased the repopulation potential of human CD34^{+} cells[31] and preserved a stem cell phenotype,[32] suggesting that Wnt proteins or pharmaceutical manipulation of the Wnt signaling cascade, may be useful for *ex vivo* manipulation of HSCs for transplantation and gene therapy applications. Understanding the regulatory mechanism of the Wnt signaling pathway will also contribute to increase our knowledge on leukemic stem cells which may aberrantly activate Wnt signaling,[33] and to find potential drug targets for therapy directed to disrupt the leukemogenic process.

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Chapter 3

Canonical Wnt signaling regulates hematopoiesis in a dosage-dependent fashion

Tiago C. Luis[1,2], Brigitta A.E. Naber[1,2], Edwin F.E. de Haas[1],
Mehrnaz Ghazvini[3], Willem E. Fibbe[2], Jacques J.M. van Dongen[1],
Riccardo Fodde[3] and Frank J.T. Staal[1,2]

[1]Department of Immunology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands. [2]Department of Immuno-Hematology and Blood Transfusion, Leiden University medical Center, Leiden. The Netherlands. [3]Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Erasmus University Medical Center, Rotterdam. The Netherlands

Under revision

SUMMARY

Canonical Wnt signaling has been implicated in the regulation of hematopoiesis. By employing a Wnt-reporter mouse, we observed that Wnt signaling is differentially activated during hematopoiesis, suggesting an important regulatory role for specific Wnt signaling levels. To investigate whether canonical Wnt signaling regulates hematopoiesis in a dosage-dependent fashion, we analyzed the effect of different mutations in the Adenomatous polyposis coli gene (*Apc*), a negative modulator of the canonical Wnt pathway. By combining different targeted hypomorphic alleles and a conditional deletion allele of A*pc*, a gradient of five different Wnt signaling levels was obtained *in vivo*. We here show that different, lineage-specific, Wnt dosages regulate hematopoietic stem cells (HSC), myeloid precursors and T lymphoid precursors during hematopoiesis. Differential, lineage-specific optimal Wnt-dosages provide an unifying concept that explains the differences reported among inducible gain-of-function approaches, leading to either to HSC expansion or depletion of the HSC pool.

INTRODUCTION

Hematopoiesis, i.e. the production of blood cells, is fully dependent on a rare population of hematopoietic stem cells (HSCs) which have the capacity to self-renew and differentiate into all blood cell lineages. This process is strictly regulated by signals provided by the surrounding microenvironment of the different organs where hematopoiesis occurs [1]. Although canonical Wnt signaling is recognized as one of the signaling cascades implicated in the regulation of HSC function as well as in other stages during hematopoiesis, its specific functional role has not been completely understood and is to date a matter of controversy [2,3]

Activation of the Wnt pathway occurs upon binding of a soluble Wnt protein to a membrane-associated receptor and leads to the disruption and inhibition of a protein complex responsible for the phosphorylation and breakdown of β-catenin. Inhibition of this so-called destruction complex, composed of the tumor suppressor Adenomatous Polyposis Coli (*Apc*), the Ser-Thr kinases Glycogen Synthase Kinase 3β (Gsk-3β) and Casein Kinase I (CK-I), and the scaffold and tumor suprresor protein Axin, results in stabilization and accumulation of β-catenin. Upon its intracellular stabilization, β-catenin subsequently translocates to the nucleus where, together with the Tcf-Lef family of transcription factors, it activates a Wnt-controlled gene expression program. Besides the canonical Wnt pathway which is mediated via β-catenin, other non-canonical Wnt signaling routes have been described, for instance those mediated by Calcium signaling or c-Jun N-terminal kinases (JNK) (Staal *et al*., 2008).

Canonical Wnt signaling has been implicated in the self renewal of various stem cell compartments in the gut, mammary gland, skin and embryonic stem (ES) cells. Notably, Wnt signaling influences the capacity of ES cells to differentiate into the three main germ layers: ectoderm, mesoderm and definitive endoderm, in a dosage-dependent fashion [4]. In addition, different levels of activation of the pathway confer different degrees of tumor susceptibility in different tissues [5,6]. These Wnt dosage-dependent effects also seem to hold true for adult self-renewing tissues such as gut and skin, although the underlying cellular and molecular mechanisms still remain poorly understood [7].

In the hematopoietic system, a role for Wnt signaling was first demonstrated during T-cell development in the thymus where it provides proliferation signals to immature thymocytes [3,8]. Indeed, *Tcf1* deficiency affects the highly proliferative stages double-negative 2 (DN2) and DN4 [9,10], and conditional deletion of β-catenin inhibits T-cell development at β-selection checkpoint (DN3) 11. Furthermore, activation of the pathway by *in vivo* stabilization of β-catenin resulted in thymocyte development without the requirement of pre-TCR signaling and impaired transition from double-negative (DN) to double-positive (DP) stages of T-cell development [12]. Besides regulating T-cell development in the thymus, Wnt signaling was also shown to play a role in the regulation of HSC function. We and others recently showed that

Wnt is necessary for normal HSC function by employing either Wnt3a deficient mice [13], or by overexpressing the Wnt negative regulator DKK1 in osteoblastic stem cell niche [14], or by Vav-Cre-mediated conditional deletion of β-catenin [15]. Besides these two approaches to inhibit Wnt signaling, gain-of-function approaches to activate the pathway in HSCs were perfomed with conflicting results. Stabilized forms of β-catenin resulted in either enhancement of HSC function and maintenance of an immature phenotype [16-19], or exhaustion of the HSC pool followed by failure of repopulation in transplantation assays [20,21]. These differences may be explained by the different levels of Wnt pathway activation [22,23], resulting from the different approaches employed and/or the interference of other signals in the context of Wnt activation [24-26].

Here, we studied the effect of different dosages of Wnt signaling activation on HSC self-renewal and differentiation capacity. To this aim, we used mouse models carrying specific hypomorphic mutations at the *Apc* gene resulting in specific Wnt signaling dosages [27,28]. The *Apc* gene encodes for a multifunctional protein which is most known for binding and down-regulating β-catenin. By employing this *in vivo* approach, we show that differential, lineage-specific, optimal Wnt dosages regulate HSC, myeloid precursors and T lymphoid precursors during hematopoiesis.

RESULTS

The canonical Wnt signaling pathway is differentially activated during hematopoiesis

In order to determine the activation status of the canonical Wnt signaling pathway in the HSC compartment and in the different hematopoietic lineages we employed the well-established $Axin2^{LacZ}$ Wnt reporter mice. Here, a *LacZ* cassette is inserted in-frame with the start codon of the ubiquitous Wnt target gene Axin2/Conductin [29-31], allowing analysis of the activation status of the Wnt/β-catenin pathway by measuring β-galactosidase (LacZ) activity *in vivo*. As Axin2 is also a negative regulator of the Wnt signaling pathway we also verified whether *Axin2* haploinsufficiency would affect the hematopoietic system, as the *LacZ* insertion effectively inactivates the targeted *Axin2* gene. In line with data from other developmental systems, the hematopoietic system of the $Axin2^{+/LacZ}$ mice was found to be overall normal when compared to mice not carrying the reporter transgene ($Axin2^{+/+}$) (Sup. Figure 1).

Canonical Wnt signaling was active in most of the subsets analyzed (Figure1). A remarkable difference was observed in the different hematopoietic subsets in bone-marrow (BM) (Figure1a, c) compared to the thymus (Figure1b, d). Thymocytes displayed higher levels of Wnt activation than progenitor cells in BM. Notably, in the bone-marrow long-term (LT) -HSCs showed enhanced Wnt activity when compared with short-term (ST) -HSCs and multipotent progenitors (MPP). Despite the absence or very reduced Wnt signaling levels in more mature myeloid and

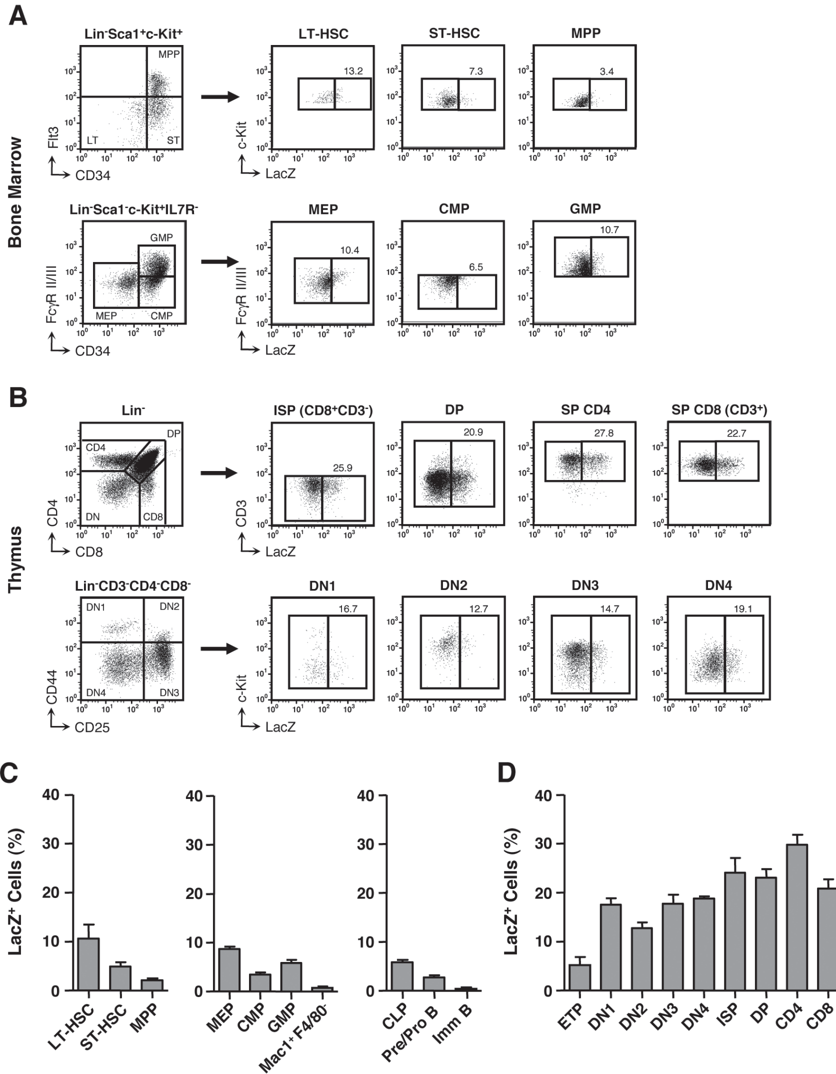

Figure 1. *In vivo* measurement of canonical Wnt signaling activity throughout hematopoietic development. Activation of the canonical Wnt signaling pathway was measured using the Axin2/conductin$^{LacZ/+}$ Wnt-reporter mice by FACS. A) β-galactosidase (LacZ) activity in different BM stem/progenitor subsets. HSCs, MPPs and myelo-erythroid progenitors were electronically gated and analyzed for LacZ expression. B) β-galactosidase activity in the thymus. Thymocyte subsets were electronically gated and analyzed for LacZ expression. Numbers represent frequency of LacZ^{+} cells in each subset. C, D) Quantification of the frequency of cells undergoing active Wnt signaling (LacZ^{+}) in different BM (c) and thymocyte (d) subsets. Littermate mice not carrying the reporter transgene (Axin2/Conductin$^{+/+}$) were used to define the LacZ^{-} population and determine the background staining of LacZ, for each population analyzed. Background staining frequencies were subtracted from the total frequency of LacZ^{+} cells. Data represents results from 6 Axin2/conductin$^{LacZ/+}$ mice and 3 Axin2/Conductin$^{+/+}$ control mice, from 2 independent experiments. Error bars represent standard error of the mean (SEM).

B-cell subsets, the most immature progenitors from both these lineages showed detectable Wnt activation. In the thymus, besides the generally higher activation levels, immature single positive (ISP), DP and SP stages showed a further signaling increase, in comparison to the double negative (DN) subsets. Of notice, early thymic progenitors (ETP) showed a significantly lower Wnt activity, suggesting that only after entering the thymus the T-cell progenitors receive stronger Wnt signals. This is in agreement with the reported high expression of several Wnt genes in thymic stromal cells [32,33]. Quantification of the mean fluorescence intensities of the LacZ positive populations provided generally similar results as by determining the percentages of positive cells (Sup.Figure 2).

Hypomorphic Apc mutations allow modulation of canonical Wnt signaling levels in the hematopoietic system

The differential Wnt signaling activity in distinct hematopoietic lineages suggested that specific levels of pathway activation could differentially affect hematopoiesis. In order to investigate whether Wnt signaling regulates HSC self-renewal and differentiation in a dosage-dependent fashion, we analyzed both *in vivo* and *in vitro* the effect of different targeted mutations in the *Apc* gene. Using a combination of 2 different hypomorphic alleles and a conditional deletion allele of *Apc*, a gradient of five different levels of Wnt signaling *in vivo* was obtained. Apc^{1572T} and Apc^{1638N} alleles were previously generated by targeting amino-acid residues 1572 and 1638 on exon-15, which encodes nearly all Wnt regulatory sequences in the Apc gene [6,34]. The Apc^{1572T} allele encodes for a truncated protein encompassing all β-catenin binding domains, 3 of the 7 β-catenin degradation repeats but none of the Axin/conductin binding (SAMP) motifs. The *Apc*^{1638N} allele encodes for only residual amounts (≈2%) of a slightly longer protein (encompassing one SAMP motif) (Figure 2a). Hence, these targeted mutations result in different levels and lengths of truncated Apc proteins with consequently leading to different levels of Wnt pathway activation [7,27]. As homozygosity for these Apc mutations leads to early embryonic lethality [34,35], we combined them with a conditional Apc-mutant allele (*Apc*^{15lox}) where A*pc* exon 15 is flanked by *LoxP* sequences which, upon Cre-mediated recombination, lead to an *Apc* null allele 28. The two hypomorphic alleles, the conditional deletion allele and the wild type Apc allele were then bred in order to obtain different compound heterozygous mouse lines providing a gradient of Wnt signaling activation. Deletion of *Apc* exon 15 within the Apc^{15lox} allele was performed *ex vivo* by using the retroviral Cre gene transduction system [36,37] (Figure 2b). $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$ (LSK) cells, which are highly enriched for HSCs, were isolated from the BM of mice carrying different *Apc* alleles combinations, and transduced with pMSCV-Cre-IRES-enhanced GFP retroviruses (Sup Figure 4). LSKs cells from wild type mice (*Apc*$^{+/+}$) transduced with the same viral construct were employed as control for all experiments. Two and a half days after transduction deletion was

virtually complete in GFP positive cells, as shown both at the DNA (Figure 2c) and mRNA (Figure 2d) levels.

To test whether the different combinations of *Apc* alleles lead to different levels of Wnt signaling activation in HSCs, *Axin2* expression was determined by RTq-PCR on GFP^+ cells. The Apc^{15lox} allele in heterozigozity ($Apc^{15lox/+}$) or in combination with the Apc^{1572T} allele ($Apc^{15lox/1572T}$) leads to a mild (2-fold) and intermediate (4-fold) increase of activation of the Wnt signaling pathway, respectively, when compared with wild type levels (Figure 2e). The combination of Apc^{15lox} and Apc^{1638N} alleles ($Apc^{15lox/1638N}$) results in a high activation level of the pathway (22-fold), which is in the same range of activation obtained with conditional deletion of *β-catenin* exon-3 ($Ctnb1^{\Delta ex3}$;20-fold), leading to its constitutive stabilization [20,21,38] (Figure 2e, f). Finally, the Apc^{15lox} allele when bred to homozygozity ($Apc^{15lox/15lox}$) results in an extremely high (72-fold) activation of the Wnt signaling pathway. Hence, by combining 3

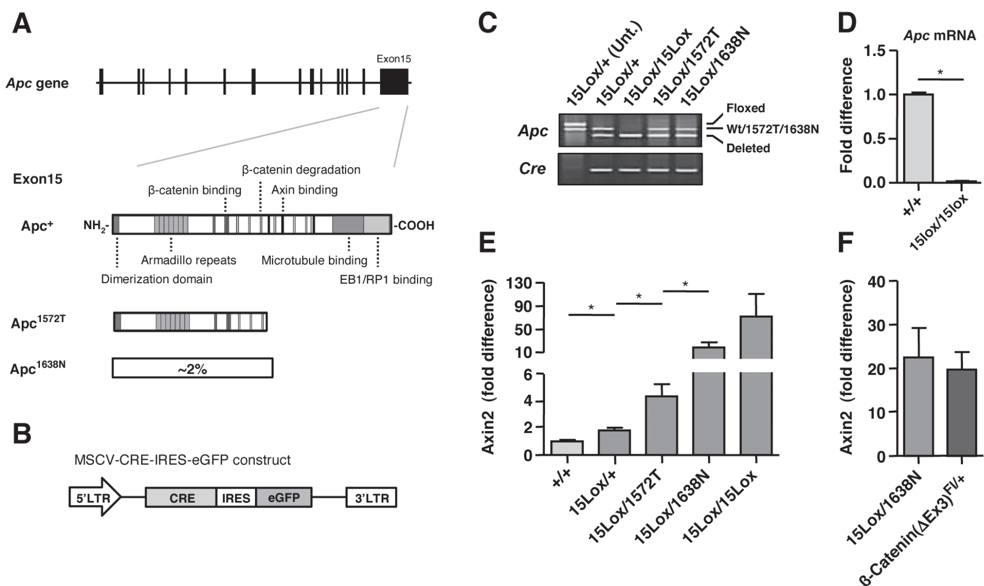

Figure 2. Targeted mutations of Apc allow modulation of canonical Wnt signaling in the hematopoietic system. A) Schematic representation of the *Apc* gene and the proteins expressed by the mutated *Apc* alleles. Different functional domains inside *Apc* exon 15 are depicted. B) Retroviral construct encoding for the Cre recombinase and GFP reporter genes, used for *ex vivo* deletion of the APC^{15lox} allele. C, D) Analysis of deletion efficiency of the Apc^{15lox} allele at DNA (C) and mRNA (D) levels. Analysis was performed on GFP^+ sorted cells, 3 days after transduction, by PCR and RTq-PCR respectively. Floxed/non-deleted band is no longer detected after transduction indicating that deletion was virtually complete. Data represents 3 independent experiments. Unt, Untrasnduced. E) Expression analysis of the Wnt-reporter gene *Axin2*, carried out in transduced (GFP^+) LSKs from mice with the indicated combinations of mutated and normal *Apc* alleles. Results represent 4 independent experiments. F) Expression analysis of Axin2 in transduced LSKs from $Ctnb1(\Delta ex3)^{Fl/+}$ mice. Results from 3 different mice. Error bars represent SEM.

different targeted *Apc* mutant alleles, a gradient of five different levels of pathway activation was obtained, enabling us to study Wnt dosage-dependent effects on HSC self-renewal and differentiation, as well as effects of Wnt signaling strength in other hematopoietic lineages. To this aim, deleted GFP^+ LSKs from mice carrying the above genotypes were employed for *in vivo* and *in vitro* assays.

Intermediately increased levels of Wnt signaling activation enhance clonogenicity and myeloid differentiation

We used methylcellulose colony-forming assays to determine clonogenic and differentiation potential of LSK cells from the different Apc mouse lines. *Apc*$^{15lox/+}$ and *Apc*$^{15lox/1572T}$ GFP^+ LSKs caused an increase of approximately 2 fold in the number of colonies, in comparison to *Apc*$^{+/+}$ GFP^+ LSKs. This was mainly due to an increase in Granulocytic-monocytic (GM) and monocytic (M) colony-forming units (CFU) suggesting that mild to intermediate levels of Wnt signaling activation results in increased clonogenicity and myeloid differentiation potential. Notably, a strong reduction in the number of colonies was observed with the higher levels of Wnt activation characteristic of *Apc*$^{15lox/1638N}$ and *Apc*$^{15lox/15lox}$ GFP^+ LSKs (Figure 3a). Importantly, analysis of c-Kit expression in the colonies from all genotypes analyzed revealed that increasing Wnt signaling activity results in increased frequency of c-Kit^+ cells and, therefore, leads to the maintenance of an immature phenotype (Figure 3b). PCR analysis on genomic DNA from day 9 colonies revealed complete deletion of the floxed exon 15 in all genotypes. Moreover, the gradient of Wnt signaling activation was maintained during culture as determined by Axin2 expression (data not shown).

Only mildly increased Wnt signaling levels enhance HSC repopulation capacity

To quantitatively determine the effects of increasing levels of Wnt signaling on HSC function, we performed competitive limiting-dilution transplantation assays using the Ly5.1/ Ly5.2 system. Notably, a mildly increased activation of the Wnt signaling pathway, corresponding to the haploinsufficient *Apc*$^{15lox/+}$ genotype, resulted in a 6-fold increase in the frequency of repopulating cells, in comparison to the wild type condition (Apc$^{+/+}$ GFP^+ LSKs) (Figure 4a). The frequency of competitive repopulating units (CRU) increased from 1/40371 (*Apc*$^{+/+}$) to 1/6717 (*Apc*$^{15lox/+}$), 12 weeks after transplantation (p=0.009). In contrast, intermediate (*Apc*$^{15lox/1572T}$) and higher (*Apc*$^{15lox/1638N}$ and *Apc*$^{15lox/15lox}$) levels of Wnt signaling activation resulted in a total impairment in repopulation capacity (Figure 4a, 4b). The increased repopulation capacity conferred by mildly increased Wnt levels was also observed by an approximately 3-fold increase in the level of chimerism in mice transplanted with *Apc*$^{15lox/+}$ cells, when compared to *Apc*$^{+/+}$ cells, 12 weeks after transplantation (p=0.011) (Figure 4c, 4d). Analysis of the HSC compartment in the BM of the recipient mice also showed an increase in

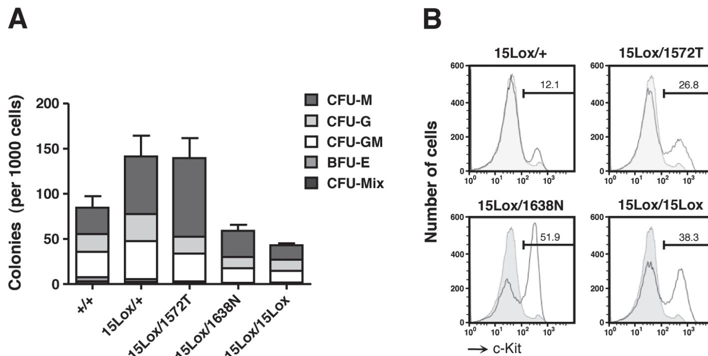

Figure 3. Mild to intermediate increase of Wnt signaling activity enhances myeloid differentiation and confers an immature phenotype. Methylcellulose colony-forming assays performed with sorted GFP+ LSKs from the different genotypes. A) Total number and relative frequency of the different colonies counted after 9-10 days of culture. B) c-Kit expression in total colonies from each condition. Numbers represent frequency of c-Kit+ cells. Shaded histogram represent $Apc^{+/+}$ (5.6% c-Kit+ cells). Results are representative of 3 independent experiments. Error bars represent SEM. CFU, colony forming unit; M, macrophage; G, granulocyte; GM, granulocyte-macrophage; Mix, mixed (containing at least 3 different types of cells); BFU-E, burst forming unit-erythrocytes.

the number of GFP+ LSKs (p=0.06, Figure 4e, 4f). Furthermore, secondary transplantation of these cells showed efficient repopulation of lethally irradiated Ly5.1 recipients. Twelve weeks after transplantation in secondary recipients the advantage showed by the *Apc*$^{15lox/+}$ cells could still be observed thus indicating that mildly increased levels of activation enhance long-term repopulation capacity (data not shown). In order to obtain insight into the molecular mechanism underlying the different outcomes obtained with different dosages of Wnt activation, we assessed expression of a number of HSC self-renewal related genes which may also represent downstream Wnt targets by quantitative RT-PCR on sorted GFP+ LSKs (Sup.Figure 4). Activation of β-Catenin was previously shown to lead to the downregulation of the self-renewal genes *Hoxb4* and *Cdkn1a* [20,21]. Accordingly, intermediate and high levels of Wnt activity resulted in the transcriptional downregulation of these genes. Notably, the same *Hoxb4* and *Cdkn1a* genes displayed normal expression levels in cells with a mildly increased Wnt pathway activation (*Apc*$^{15lox/+}$) and enhanced HSC function, suggesting that, in contrast to higher Wnt dosages, relatively low levels preserve self-renewal capacity. As Myc activity was recently shown to be critical for HSC function [39], we also analyzed expression of *c-Myc* and *N-Myc* genes. While no differences in *c-Myc* expression were observed, increasing levels of Wnt signaling resulted in a proportional down-regulation of *N-Myc* in GFP+ LSKs (Sup. Figure 4).

In addition to enhanced HSC function and in agreement with the *in vitro* methylcellulose colony assays, an increase in myeloid differentiation was also observed (Sup.Figure 5). The

myeloid compartment in BM of mice transplanted with *Apc*$^{15lox/+GFP+}$ was significantly enlarged (p=0.041), and this was mainly due to an effect in the immature CD11b^{+}F4/80^{-} myeloid cells (p=0.035; Sup.Figure 5a, b). Analysis of the early myeloid progenitor subsets showed that in the *Apc*$^{15lox/+GFP+}$, the GMP compartment was expanded (p=0.08) while CMPs appeared with a frequency comparable to the wild type control (Sup.Figure 5c). Despite the impairment in repopulation observed with intermediate levels of Wnt activation (*Apc*$^{15lox/1572T}$), the remaining detectable donor cells at 8 weeks post-transplantation were skewed towards myeloid development (68% in *Apc*$^{15lox/1572T}$ condition comparing to 46% in *Apc*$^{15lox/+}$; p=0.029; Sup.

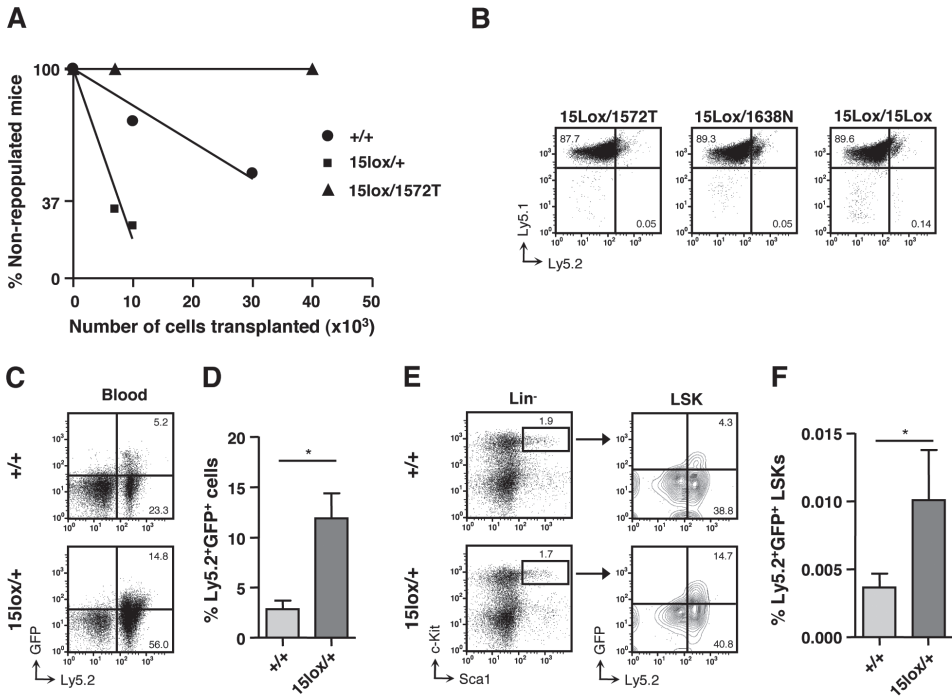

Figure 4. Mildly increased Wnt signaling enhances HSC function. A) Limiting dilution analysis of the repopulating ability of transduced (GFP^{+}) LSKs from the different genotypes specified. 4 to 6 mice were transplanted for each dosage of cells (7, 10, 30 and 40x10^{3}) per genotype in a total of 49 initial recipients. The CRU frequency increased from 1/40371, in the *Apc*$^{+/+}$ condition (95% confidence interval for mean: 1/22388-1/72798), to 1/6717 in the *Apc*$^{15lox/+}$ condition (95% confidence interval for mean: 1/4486-1/10059) (p=0.009). Dosages resulting in 100% of mice repopulated were not plotted.. B) Repopulation efficiency analyzed in the peripheral blood of mice transplanted with Apc$^{15Lox/1572T}$, Apc$^{15Lox/1638N}$ and Apc$^{15Lox/15lox}$, 12 weeks after transplantation. C-F) Cre-IRES-GFP transduced Apc$^{+/+}$ and Apc$^{15Lox/+}$ LSK cells were transplanted into lethally irradiated Ly5.1 recipients (without sorting of GFP^{+} cells). C, D) Repopulation efficiency analyzed in peripheral blood of recipient mice 12 weeks after transplantation (p=0.023). E) Analysis of the HSC compartment of recipient mice. LSKs were gated and analyzed for Ly5.2 and GFP expression. F) Frequency of Ly5.2^{+}GFP^{+} LSK cells in the BM of the recipient mice (p=0.05). C-F) Data represent 6 mice in each group. Error bars represent SEM.

Figure 5d, e). This indicates that intermediately increased levels of Wnt signaling activation also confer *in vivo* advantage to myeloid development.

T-cell development is enhanced by higher Wnt signaling levels

As HSC with the highest levels of Wnt signaling fail to reconstitute mice, this approach does not allow the study of the effect of these same levels on T cell development *in vivo*. In order to study Wnt dosage-dependent effects on T-cell development *in vitro*, we co-cultured GFP⁺ LSK together with the OP9 BM derived stromal cell line expressing the Notch ligand Delta1 [40] meant to induce a T-cell differentiation program [41]. After 13 days of co-culture, both DP and SP T-cells could be detected in wild type cultures (Figure 5a, b). Mild Wnt signaling increase (*Apc*$^{15lox/+}$) had no additional effect on T-cell development over wild type levels.

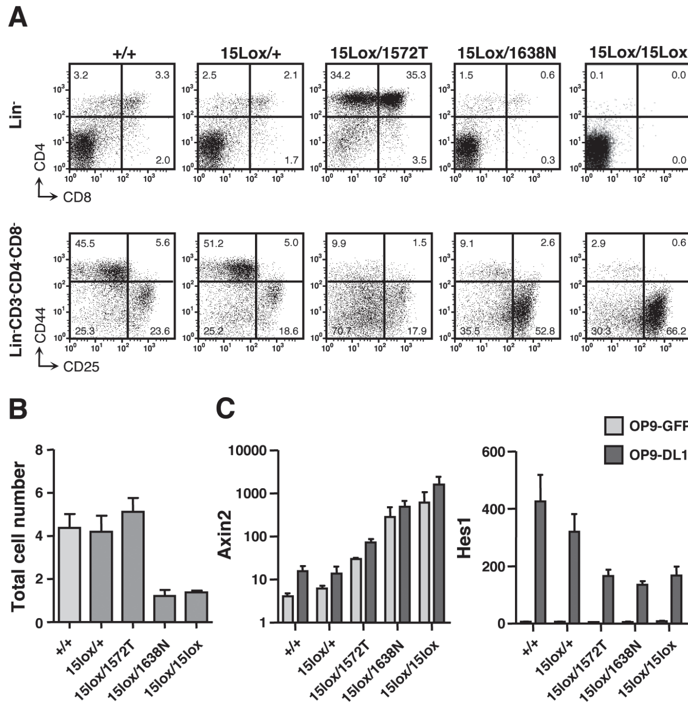

Figure 5. Intermediately increased Wnt activation enhances T-cell development. A) GFP⁺LSKs with different levels of Wnt activation were cultured for 13 days on OP9-DL1 . Analysis was performed inside a CD45 gate in order to exclude stromal cells. B) Total cell numbers obtained after 13 days of co-culture with OP9-DL1. Results are from 3 independent experiments. C) Transduced LSK cells from mice with the different Apc alleles combinations were co-cultured for 24 hrs with OP9-DL1 or OP9-GFP as control. RTq-PCR analysis of *Axin2* and *Hes1* expression was performed on sorted GFP⁺CD45⁺ cells. Results are from 2 independent experiments. Error bars represent SEM.

Notably, intermediate levels of Wnt activation (*Apc*$^{15lox/1572T}$) result in enhanced T-cell development, as observed by an approximately 3 fold increase in the frequency of DN4 cells and a 10 to 15 fold increase in DP and SP-CD4 T-cells (Figure 5a, b). In contrast, very high Wnt signaling activation results in a partial arrest of T-cell development at DN3 stage (Figure 5a), consistent with the reduced total cell numbers in culture (Figure 5b).

Previously, the Wnt and Notch signaling pathways were shown to cross-talk [42]. Therefore, we analyzed the effect of Notch signaling activation on Wnt activity by determining Axin2 expression levels on GFP^{+}CD45^{+} cells after 24hrs of co-culture on OP9-DL1 stromas, in comparison to cells cultured on OP9-GFP stromas. RTq-PCR analysis showed a slightly increased *Axin2* expression in all Wnt signaling conditions, (Figure 5c). Nevertheless, the gradient of Wnt signaling activation conferred by the different *Apc* alleles combinations was maintained in the presence of Notch signals. Notably, increasing levels of Wnt activation result in a proportional reduction in the activation of the Notch signaling pathway, as determined by expression analysis of the Notch target gene *Hes1* (Figure 5c).

In order to investigate whether the same Wnt dosage-dependent effects are observed *in vivo*, the different *Apc*-mutant mouse lines were crossed with Lck-Cre mice. Lck-Cre allowed conditional deletion of the *Apc*^{15lox} allele starting at the DN2 stage of thymocyte development. Deletion was virtually complete at the DN3 stage as observed by PCR analysis on genomic DNA (Figure 6a). In agreement with the *in vitro* data, only intermediate Wnt signaling activity (*Apc*$^{15lox/1572T}$) enhanced T-cell development. Furthermore, while mild levels of activation (*Apc*$^{15lox/+}$) had no effect on T-cell development, high (*Apc*$^{15lox/1638N}$) and very high (*Apc*$^{15lox/15lox}$) Wnt signaling activity resulted in accumulation of CD44^{-}CD25^{+} and CD44^{-}CD25^{int} DN3 cells (Figure 6b, c). Similarly to what was reported for the conditional stabilization of β-Catenin [12], high and very high Wnt activity resulted in impaired T*cr*β gene rearrangements (Figure 6d) and cells without T*cr*β rearrangements could escape the β-selection checkpoint and progress to the DP and SP stages. In contrast, intermediate levels of Wnt signaling (*Apc*$^{15lox/1572T}$) allowed cells to progress through DN3 stage and form DN4 thymocytes with normal expression levels of Tcrβ in the majority of the mice (Figure 6b, c, d). Only 1 out 6 mice showed a slightly reduced frequency of cells with Tcrβ expression (56% Tcrβ^{+} cells in comparison to 75.3% in the wild-type DN4 cells). Notably, an accumulation of CD44^{+}CD25^{-} cells (usually defined as DN1) was also observed. An increase in DN1 cells is unexpected since Lck-Cre mediated deletion only occurs at the DN2-DN3 stages. Further characterization of this population revealed that they do not express c-Kit, TCR-γδ or other lineage markers, thus excluding the possibility that these cells represent thymic progenitors or other alternative lineages, such as γδ-T cells or NK cells. Instead, these cells express intracellular Tcrβ (iTcrβ) and show deletion of the *Apc*^{15lox} allele (data not shown), suggesting that they are DN4 cells with CD44 up-regulation phenotipically reminiscent of DN1 cells. CD44 is indeed a well established Wnt downstream

target gene [43]. Therefore, for absolute quantification purposes, DN4 cells were defined as CD25^{-} iTcrβ^{+} in the *Apc*$^{15lox/1572T}$ mice. In this way, a 5-fold increase in the numbers of DN4 cells was observed in the thymus of Lck-Cre:*Apc*$^{15lox/1572T}$ mice, in comparison to the Lck-Cre:*Apc*$^{+/+}$ control mice (Figure 6b, c), thus indicating that intermediate levels of Wnt signaling favor early stages of thymocyte development. Nevertheless, thymi with intermediate, high and very high levels of Wnt signaling showed decreased numbers of DP thymocytes (2-, 4- and 38-fold respectively). This reduction was more pronounced with the higher levels of activation of the Wnt signaling pathway (Figure 6c). These results indicate that within the T-cell lineage, differential effects of Wnt signaling strength can be observed, namely the expansion of the DN subpopulations favoring higher levels of Wnt signaling than the DP and SP stages.

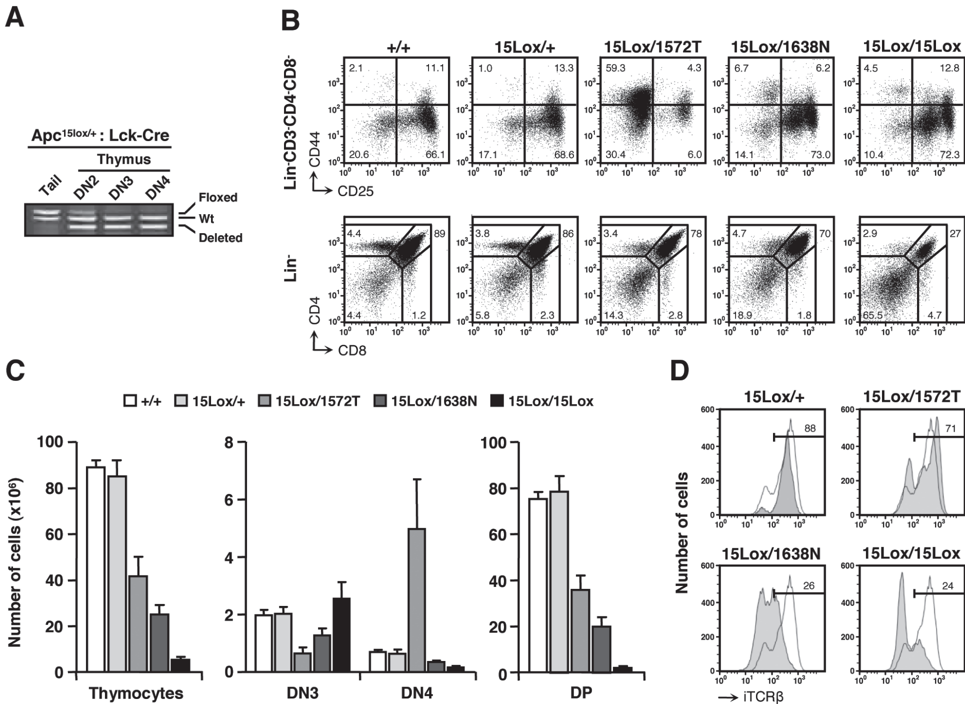

Figure 6. *In vivo* analysis of Wnt signaling dosage dependent effects on T-cell development using Lck-Cre mediated deletion. A) DN2, DN3 and DN4 thymocytes from Apc$^{15lox/+}$:Lck-Cre mice were sorted and the DNA was analyzed for deletion of the *Apc* exon15 allele by PCR. Deletion was virtually complete at DN3 stage. B) Immunophenotypical analysis of the different thymocyte populations. C) Thymic cellularity and absolute quantification of different thymocyte subsets. Data represent the mean from 3 independent experiments with a total of 6 to 7 mice per group. Error bars represent SEM. D) Analysis of intracellular Tcrβ expression on electronically gated DN4 thymocytes. Non-shadowed histograms represent Tcrβ expression on Apc$^{+/+}$:Lck-Cre mice (75.3% iTcrβ+ cells). Data is representative of 3 independent experiments.

DISCUSSION

In this study, we made use of combinations of different Apc-mutant mouse strains resulting in a gradient of five different levels of Wnt signaling *in vivo*. A differential optimum of Wnt signaling activation was observed in HSCs, myeloid progenitors and early thymocytes (Figure 7). Moreover, analysis of the $Axin2^{LacZ}$ Wnt-reporter mouse showed that the activity of the Wnt signaling pathway is regulated during hematopoiesis, with thymocytes exhibiting higher levels of pathway activation when compared with HSC and other BM progenitor subsets.

Canonical Wnt signaling was previously shown to regulate HSC function [13,14,18,20,21]. However, different strategies to achieve activation of the pathway resulted in contrasting outcomes, leading to either enhanced repopulation capacity or depletion of the HSC pool [3]. Here, by using the different Apc-mutant mouse lines, we observed that only a mild increase in Wnt signaling activation enhanced HSC function. Importantly, this mild Wnt activation leads to a remarkable increase in the number of CRUs and a subsequent increase in chimerism. In contrast, and in agreement with previous studies where *in vivo* conditional approaches to either stabilize β-catenin [20,21] or to delete Apc [44] in hematopoietic cells were employed, intermediate and higher levels of activation of the pathway impaired HSC repopulation capacity. Furthermore, while intermediate and higher levels of Wnt activity lead to down-regulation of the HSC self-renewal genes *Hoxb4* and *Cdkn1a* in agreement with a reduced repopulation capacity, a mild activation of the Wnt pathway does not influence the expression of these genes. This suggests that, at lower activity levels, Wnt signaling does not impair HSC function. Therefore, we here provide experimental evidence for a correlation between Wnt signaling strength and different outcomes in HSC function, as previously speculated to exist[22,23].Given that HSCs appear to strictly require lower levels of Wnt activity, it is likely that the remaining Wnt signaling still present in β-catenin/γ-catenin conditional deletion mouse models[29] is sufficient to sustain normal hematopoiesis. In accordance with a requirement of Wnt signaling for normal HSC function, Wnt3a deficiency [13] and overexpression of the Wnt inhibitor Dkk1 in the HSC niche [14], both resulting in the absence of canonical Wnt signaling[45], lead to impaired LT-HSC repopulation capacity. Recent studies using $Apc^{Min/+}$ mice [24,46] or a shRNA approach to knockdown Gsk3β [25] showed similarly increased HSC repopulation efficiency. However, decreased LT repopulation capacity in secondary transplantation assays was observed in two of these studies. In our studies a mild Wnt activation also favored LT reconstitution in secondary recipients. These differences may be explained by slight variations in the different Wnt levels achieved or, alternatively, by a sustained activation of the pathway having a harmful effect on HSCs.

Accordingly with the Wnt signaling levels observed during myeloid development, mild and intermediately increased Wnt activity enhanced myelopoiesis at progenitor level.

Concerning the B-cell lineage, despite the observed low Wnt activity in early B-cell progenitors, B-cell development was not affected by any of the Wnt dosages (data not shown). This is in agreement with our previous observations that Wnt3a deficiency only affects myeloid development at the progenitor level, but not B lymphocyte development [13].

Early thymocyte subsets showed an even stronger requirement for Wnt signaling. Canonical Wnt signaling was previously shown to regulate the DN3 to DP transition during thymocyte development [12,47,48]. Lck-Cre mediated stabilization of β-Catenin, starting at DN2-DN3 stages results in impaired *Tcrβ* rearrangements but allowed thymocytes to progress to DN4, DP and SP stages without pre-TCR signaling, thus bypassing the β-selection checkpoint. Accordingly, high levels of activation of the Wnt pathway showed a comparable phenotype which was more pronounced in the mice with the highest activation. Importantly, we show here that intermediate Wnt activation results in reduced number of DN3 cells and in a burst of DN4 thymocytes with normal Tcrβ expression. These findings suggest that intermediate Wnt activity enhances early stages of T-cell development while preserving physiological processes such as TCR rearrangements and maintaining developmental checkpoints. However, the numbers of DP cells and consequently the total thymus cellularity in mice with intermediate Wnt activity was still reduced which may indicate that later stages of T-cell development have different Wnt signaling requirements. Wnt signaling also regulates aspects of negative and positive selection and the DP to SP transition [3]. In agreement, in *in vitro* co-cultures with OP9 stromal cells expressing the Notch ligand Delta1, where positive and negative selection processes are less stringent [49], an increase in both DP and SP cells with intermediate but not higher levels of Wnt activation was observed.

Different studies demonstrated the interaction between Wnt and Notch signaling pathways in hematopoiesis [42,47]. Notably, increasing levels of Wnt signaling counteract the activation of the Notch signaling pathway in a dosage dependent fashion suggesting that Wnt regulates the responsiveness to Notch signals. Lck-Cre mediated deletion of Notch1 also leads to a DN3 arrest and impaired Tcrβ rearrengements [50]. Although reduced Notch activity may partially explain the phenotype observed with highly increased Wnt signaling [51], it cannot represent the sole reason as intermediate increases also lead to reduced Notch activation and these cells are able to rearrange Tcrβ genes. While early T-cell development was increased with intermediate Wnt activity, a milder Wnt activation had no effect, in contrast to what was observed for the HSCs compartment and myeloid differentiation in BM.

An alternative interpretation of our data is that the observed consequences of different combinations of *Apc* mutant alleles are not Wnt- but rather Apc-dependent. Although its major tumor suppressing function is known to reside in the capacity to negatively regulate the canonical Wnt/β-catenin signaling pathway, *Apc* does encode for a multifunctional protein involved in a broad spectrum of cellular functions ranging from cell adhesion and motility,

organization of the actin and microtubule networks, chromosomal segregation at mitosis, and cell cycle regulation [52]. From this perspective, one could argue that the effects on hematopoiesis result from the interference with one or more functions of Apc rather than the control of Wnt signaling. However, previous analyses of Apc function indicate that this scenario is highly unlikely. To date, most *Apc*-mutant mouse models are characterized by tumor phenotypes, the organ-specificity and severity of which depend on Wnt signaling dosage. *Apc*1638T, the only targeted *Apc* mutation that does not affect Wnt signaling, results in homozygous viable and tumor-free animals, notwithstanding the deletion of the C-terminal third of the protein containing the above mentioned functional domains [6]. Deletion of only few aminoacids encompassing crucial Axin-binding motifs from the C-terminus of Apc^{1638T} results in Wnt signaling activation and tumor formation. Finally, mutations affecting other members of the Wnt pathway like *Gsk3β* and *β-catenin* result in levels of signaling activation and hematopoietic defects which are fully in agreement with our results, as discussed above. Hence, we conclude that the most likely explanation is that specific levels of Wnt signaling are the major determinant of the observed differential effects on hematopoiesis.

In conclusion, we report differential Wnt signaling requirements and optimum levels of activity in HSCs, myeloid progenitors and early T-cell progenitors (Figure 7). In APC-driven tumorigenesis in both mouse and man [53,54], 'just-right' levels of Wnt/β-catenin signaling are selected to provide advantages to nascent tumor cells in a context- and dosage-dependent fashion. Our results indicate that the same is true for normal hematopiesis. It is well documented that morphogens like Wnt proteins determine different cell fates through concentration gradients that modulate basic cellular processes during development. We speculate that this also holds true for a strictly regulated process as hematopoietic development. The different cellular environments where hematopoiesis occur, e.g. the regions inside the thymus through which

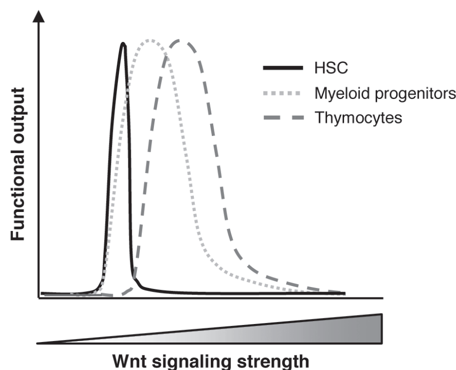

Figure 7. Differential optimum of Wnt signaling strength in HSCs and early myeloid and T-cell progenitors.

thymocytes migrate during T-cell development, are likely to provide different environmental signals that can vary in type, strength and duration of the signals, to ensure that different processes happen in the correct order and time. Such considerations are of great relevance towards the translation of basic findings on the molecular and cellular mechanisms underlying the regulation of hematopoiesis into clinical protocols for *ex vivo* expansion of HSC or thymic reconstitution. These applications are likely to be significantly improved and tailored by exposing cells to the "just right" dosage of canonical Wnt signaling to expand specific lineages and provide specific therapeutic options.

EXPERIMENTAL PROCEDURES

Mice

Mice were bred and maintained in the animal facilities of Erasmus Medical Center or Leiden University Medical Center, in accordance with legal regulations in The Netherlands and with the approval of the Dutch animal ethical committee. Mice carrying targeted mutations on Apc were previously described [6,28,34] and continuously backcrossed to C57Bl/6 background. Axin2/Conductin mice were generated and kindly provided by B. Jerchow and W. Birchmeier (Max Delbrueck Center for Molecular Medicine, Berlin, Germany) [31]. Ctnb$^{1\Delta ex3}$ mice were previously described [38] and kindly provided by B. Clausen (Erasmus Medical Center, Rotterdam, The Netherlands). C57Bl/6-Ly5.1 mice were purchased from Charles River Laboratories.

Flow cytometry

For flow cytometric analyses cells were stained with monoclonal antibodies against the following molecules: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b/Mac1 (M1/70), CD19 (ID3), CD25 (PC61), CD44 (IM7), CD45.1/Ly5.1 (A20), CD45.2/ Ly5.2,(104), CD117/c-Kit (2B8), CD127/IL-7R (SB/199), CD135/Flt3 (A2F10.1), B220 (Ra3-6B2), F4/80 (BM8), GR1 (RB6-8C5), IgM (R6-60.2), NK1.1 (PK136), TCRβ (H57-597) and Ter119 from Becton Dickinson/Pharmingen; CD34 (RAM34) and FcγRII/III (93) and Sca1 (D7) from eBioscience. Antibodies were used conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7, APC-AF780 and Biotin. For secondary detection of biotinylated antibodies, streptavidine conjugated with PE, PerCP, PE-Cy7 or APC-Cy7 (from Becton Dickinson/Pharmingen) were used. The different hematopoietic populations were defined as described in table1. Stained cells were measured with a FACS-Canto II or a LSRII, sorted on a FACS AriaII (Becton Dickinson) and analyzed with FlowJO software (Treestar).

Table 1. Phenotypic characterization markers for each hematopoietic subset analyzed

Subset	Markers
LT-HSC	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ CD11b$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^+$ c-Kit$^+$ Flt3$^-$ CD34$^-$
ST-HSC	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ CD11b$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^+$ c-Kit$^+$Flt3$^-$CD34$^+$
MPP	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ CD11b$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^+$ c-Kit$^+$Flt3$^+$CD34$^+$
MEP	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ IgM$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^-$ FcγR^{lo} CD34$^-$
CMP	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ IgM$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^-$ FcγR^{lo} CD34$^+$
GMP	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ IgM$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) Sca1$^-$ FcγR^{hi} CD34$^+$
CLP	Lin$^-$ (CD3$^-$ CD4$^-$ B220$^-$ CD11b$^-$ NK1.1$^-$ GR1$^-$ Ter-119$^-$) IL-7R$^+$ Sca1^{lo} c-Kit^{lo}
Pre/Pro-B	CD11b$^-$ F4/80$^-$ B220^{lo} IgM$^-$
Immature B	CD11b$^-$ F4/80$^-$ B220$^+$ IgM$^+$
Mature B	CD11b$^-$ F4/80$^-$ B220^{hi} IgM$^{+/lo}$
ETP	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^-$ CD25$^-$ CD44$^+$ c-Kit$^+$
DN1	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^-$ CD25$^-$ CD44$^+$
DN2	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^-$ CD25$^+$ CD44$^+$
DN3	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^-$ CD25$^{+/lo}$ CD44$^-$
DN4	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^-$ CD25$^-$ CD44$^-$
ISP	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^-$ CD4$^-$ CD8$^+$
DP	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD4$^+$ CD8$^+$
CD4 SP	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^+$ CD4$^+$ CD8$^-$
CD8 SP	Lin$^-$ (B220$^-$ CD11b$^-$ NK1.1$^-$ Ter-119$^-$) CD3$^+$ CD4$^+$ CD8$^-$

Quantification of β-galactosidase activity

Intracellular β-galactosidase activity was measured by staining cells with 1mM Fluorecein di-β-D-galactopyranoside (FDG) substrate (Molecular Probes). FDG was loaded into the cells by hypotonic shock at 37°C for 1 minute, prior to cell surface antibody staining [55]. β-galactosidase reaction was stopped with 1mM Phenylethyl β-D-thiogalactopyranoside (PETG, from Molecular probes).

Retroviral production and transduction

MSCV-Cre-IRES-GFP plasmid was kindly provided by Dr. Hiromitsu Nakauchi (Institute of Medical Science, University of Tokyo, Japan). This vector was transfected into Phoenix virus-packaging cell line in combination with (PGK-Puro) plasmid in a 10:1 ratio in order to obtain a stable virus producer cell line. Transfection was performed with FuGENE6 (Roche) according to manufacturer's instructions and Transfected cells were selected with 1μg/ml puromycin and by cell sorting of GFP$^+$ cells.

40,000-70,000 sorted LSK cells were stimulated over-night in serum-free medium (StemCell Technologies) supplemented with cytokines (100ng/ml rmSCF, 10ng/ml rmTPO and 50ng/ml rmFlt3L; all from R&D) and transduced with titrated amounts of virus using Retronectin (Takara Bio Inc.). Cells were cultured for 2 additional days and GFP^{+} cells were sorted and used for *in vitro* and *in vivo* assays.

Competitive transplantation assays

Competitive transplantation assays were performed using the Ly5.1/Ly5.2 congenic system. For the limiting dilution experiments the specified numbers of sorted GFP+ LSKs from mice with the different genotypes (Ly5.2) were transplanted into lethally irradiated (9.5Gy) Ly5.1 (9-12 weeks) mice together with $1.5x10^{5}$ Ly5.1 bone-marrow competitor cells and $5x10^{5}$ Ly5.1 spleen support cells. Chimerism was analyzed at 4, 8 and 12 weeks after transplantation in peripheral blood, and mice were sacrificed for analysis at 12 weeks post-transplantation. Mice were considered repopulated when ≥1% multilineage Ly5.1 cells could be detected in nuclecleated peripheral blood cells 3 months after transplantation. Calculation of CRUs was conducted using L-Calc software (StemCell Technologies).

Methilcelulose Colony-forming assays

For methylcellulose colony assays sorted GFP^{+} cells were cultured in MethoCult M3434 (containing 50ng/mL rmSCF, 10ng/mL rmIL-3, 10ng/mL rhIL-6, 3U/mL rhEPO; from StemCell Technologies) supplemented with 20ng/ml rmTPO (R&D), accordingly to manufacturer's instructions. All assays were done in triplicate. Colonies (>30 cells) were counted after 9-10 days. CFU-Mix was defined as colonies containing at least 3 different cell types. Colonies were identified by morphology and confirmed with May-Grunwald Giemsa staining.

Co-culture of LSK cells with OP9 stromal cell lines

5.000 sorted GFP^{+} LSKs were cultured on confluent layers of OP9-GFP or OP9-DL1 cells, with 50ng/ml rmSCF, 5ng/ml rmFlt3L and 20ng/ml rmIL-7 (all cytokines from R&D). After 14 days of culture cells were harvested and stained for flow cytometry analysis.

Gene expression analysis

Gene expression analysis was performed as described previously [33]. RNA was isolated using the Micro RNeasy kit (Qiagen) from sorted GFP^{+} cells 3 days after transduction. Taqman Gene Expression assay for Apc-exon15 (Mm00545882), Axin2 (Mm_00443608), c-Myc (Mm_00487804), N-Myc (Mm_00627179), Cdkn1a/p21 (Mm_00432448), HoxB4 (Mm_01307004) and Hes1 (Mm00468601) were purchased from Applied Biosystems.

Statistical analysis

Statistical significance was determined by standard t-test. Limiting-dilution transplantation assays results were analyzed with L-Calc software (StemCell Technologies)

ACKNOWLEDGMENTS

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SUPPLEMENTAL FIGURES

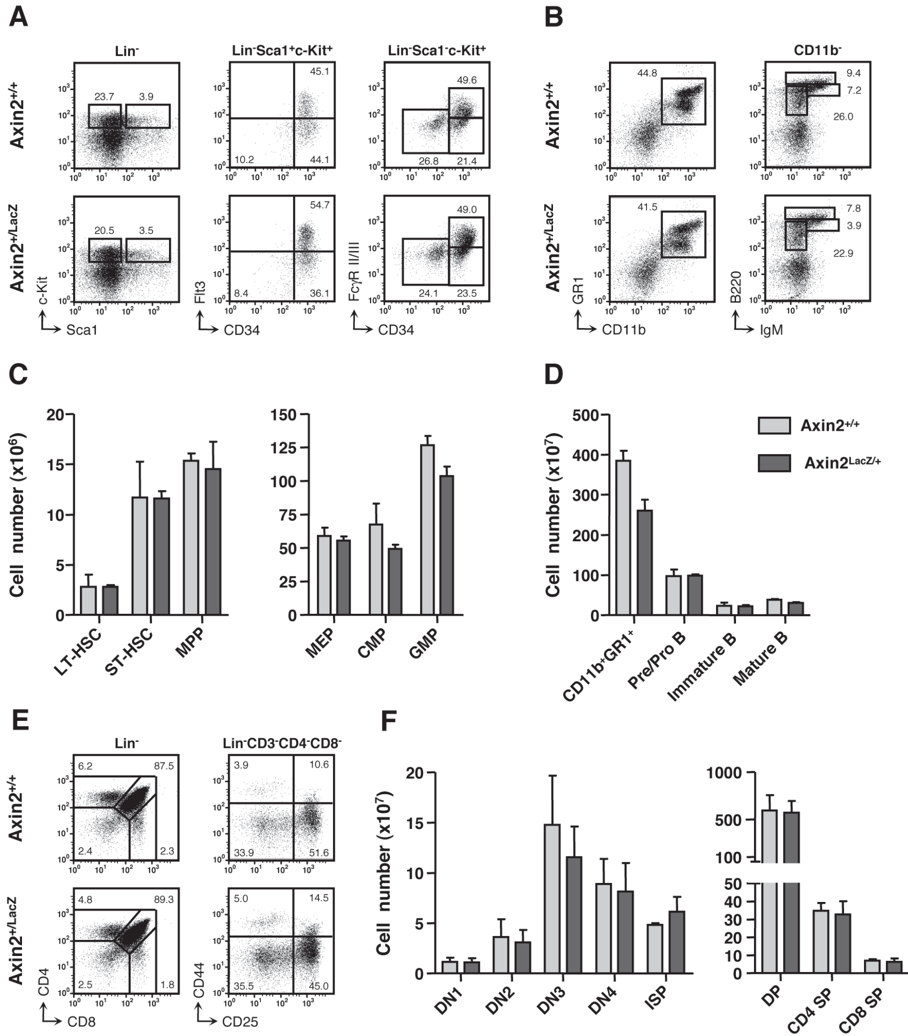

Figure S1. Normal hematopoietic system in Axin2/ConductinLacZ/+ Wnt reporter mice. Flow cytometric analysis of the hematopoietic stem/progenitors compartment (A, C) and mature myeloid and B-lymphoid compartments (B, D) in the BM. E, F)Analysis of the T-cell development in the thymus. Data from one representative experiment out of 2, with 3 Axin2/conductin$^{LacZ/+}$ mice and 3 Axin2/Conductin$^{+/+}$ control mice. Error bars represent standard error of the mean (SEM).

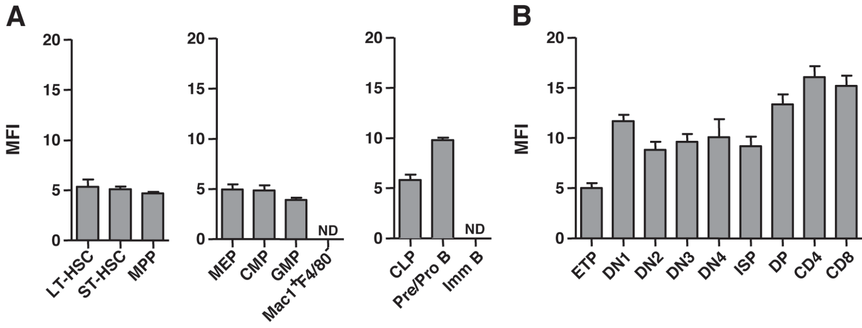

Figure S2. *In vivo* measurement of canonical Wnt signaling activity throughout hematopoietic development. Activation of the canonical Wnt signaling pathway was measured using the Axin2/conductin$^{LacZ/+}$ Wnt-reporter mice by FACS. Quantification of the mean fluorescence intensity (MFI) of the LacZ^{+} populations for each subset in the BM (A) and in the thymus (B). Littermate mice not carrying the reporter transgene (Axin2/Conductin$^{+/+}$) were used to define the LacZ^{-} population. MFI of the LacZ^{+} population was normalized for the MFI of corresponding LacZ^{-} population, for each subset. Data represents results from 6 Axin2/conductin$^{LacZ/+}$ mice and 3 Axin2/Conductin$^{+/+}$ control mice, from 2 independent experiments. Error bars represent standard error of the mean (SEM).

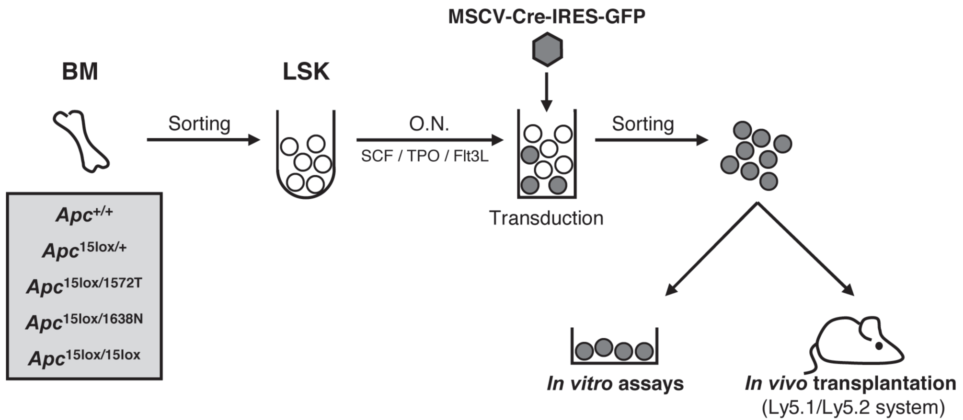

Figure S3. Experimental design to test the effects of Wnt signaling strength on HSC function. LSKs were FACS sorted from the BM of mice with the indicated *Apc*-mutant alleles combinations. Sorted LSKs were stimulated overnight with SCF, TPO and Flt3L and transduced with retrovirus encoding the Cre recombinase in order to achieve deletion of the *Apc*^{15Lox} allele. Two days after transduction GFP+ cells were FACS sorted and used for *in vitro* and *in vivo* transplantation assays (see also Experiment Procedures section).

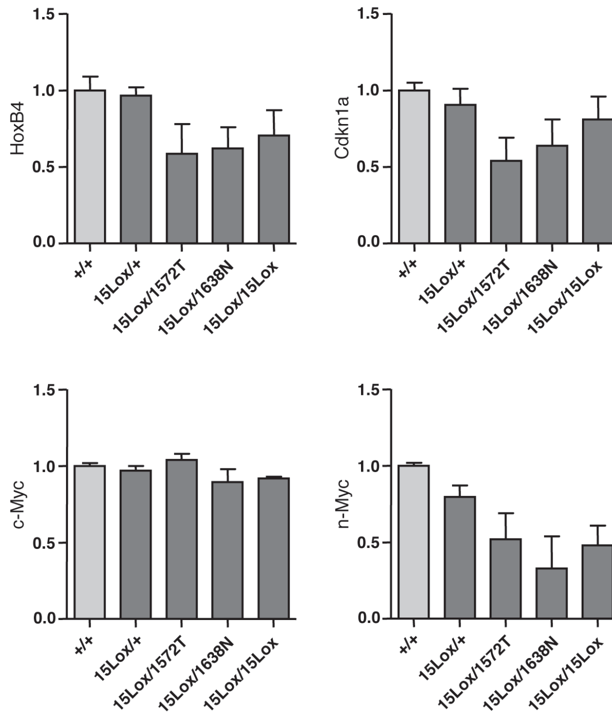

Figure S4. Gene expression analysis in LSK with different levels of Wnt signaling activation. Real-time RT-PCR analysis of expression of *Hoxb4*, *Cdkn1a*, *c-Myc* and *N-myc*, on RNA isolated from sorted GFP^+ LSKs from the different *Apc*-mutant mouse lines. Expression levels were normalized for *Gapdh* expression and presented as fold-induction (mean ± SEM) relative the expression of the $Apc^{+/+}$ cells. Results were obtained from two independent sets of sorted cells.

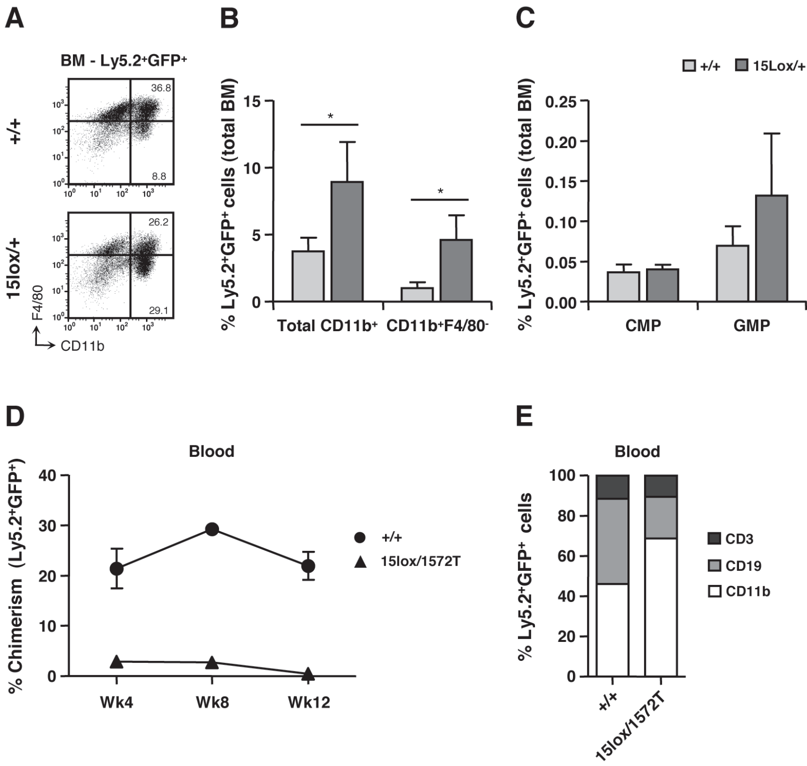

Figure S5. Enhanced myeloid differentiation in mice transplanted with *Apc*$^{15Lox/+}$ and *Apc*$^{15Lox/1572T}$ GFP$^+$ cells. A-C) Analysis of the myeloid compartment of recipient mice transplanted with Apc$^{+/+}$ or Apc$^{15Lox/+}$ GFP$^+$ LSK. A, B) Ly5.2$^+$GFP$^+$ cells were electronically gated and analyzed for expression of the pan-myeloid marker CD11b and the myeloid maturation marker F4/80. Total CD11b$^+$ ($p=0.041$) and CD11b$^+$F4/80$^-$ ($p=0.035$) populations were significantly increased in the mice transplanted with Apc$^{15Lox/+}$ GFP$^+$ cells. C) Frequencies of Ly5.2$^+$ GFP$^+$ CMPs and GMPs in the BM of transplanted mice. Data represent 6 mice in each group. D-E). Analysis of the chimerism in peripheral blood from mice transplanted with Apc$^{+/+}$ or Apc$^{15Lox/1572T}$ GFP$^+$ LSK. D) Repopulation efficiency was analyzed at the indicated time points. E) Ly5.2$^+$GFP$^+$ donor cells contribution toward myeloid (CD11b$^+$), B-lymphoid (CD19$^+$) and T-Lymphoid (CD3$^+$) lineages, 8 weeks after transplantation. Myeloid compartment was significantly increased ($p=0.029$) in mice that received Apc$^{15Lox/1572T}$ GFP$^+$ cells. Data from 2 independent experiments with a total of 6 (Apc$^{+/+}$) and 10 (Apc$^{15Lox/1572T}$) recipient mice. Error bars represent SEM.

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Chapter 4

Evidence for the role of canonical Notch signaling in hematopoietic stem cell function

Tiago C. Luis[1,2], Brigitta A.E. Naber[1,2], Edwin F.E. de Haas[1], Jennifer Veth[1], Jacques J.M. van Dongen[1] and Frank J.T. Staal[1,2]

[1]Department of Immunology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands. [2]Department of Immuno-Hematology and Blood Transfusion, Leiden University medical Center, Leiden. The Netherlands.

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ABSTRACT

Notch signaling has been extensively studied in the hematopoietic stem cell compartment but a comprehensive understanding of its role remains to be established. While initial gain-of-function studies showed expansion of repopulating-capable hematopoietic progenitors, most loss-of-function studies failed to demonstrate an essential role for this pathway in HSC function. Here we studied the role of Notch signaling in HSC self-renewal and differentiation capacities using a conditional deletion approach to inactivate the common downstream factor to all Notch receptors, the DNA binding protein *Rbpj*. *Rbpj* works as a molecular switch by either actively repressing gene transcription in the absence of Notch signaling or promoting transcriptional activation upon triggering of this same signaling pathway by physical binding to the intracellular part of Notch receptors. We show that deletion of *Rbpj* enhances the repopulation capacity of HSC, suggesting an important role for the repressor function of *Rbpj* in the regulation of HSC function.

INTRODUCTION

Hematopoietic stem cells (HSC) are responsible for the continuous production of blood cells. This is dependent on their unique capacity of self-renewal and differentiation into all blood lineages, and is strictly regulated by the specific microenvironment or niches, where HSCs reside. Several studies have implicated the canonical Notch signaling pathway in the regulation of these processes but its exact role is controversial and not completely understood[1-3].

Notch proteins are highly conserved cell surface receptors that regulate developmental decisions. Besides a role in normal development they are also implicated in several cancers including leukemia[4,5]. In mammals, 4 different Notch receptors (Notch1-4) have been described. These receptors can be activated by 5 different ligands belonging to the Delta (Delta 1, 3 and 4) and Jagged (Jagged 1 and 2) families[6]. Activation of the Notch signaling pathway requires cell-cell interaction. Binding of a ligand to the extracellular domain of a Notch receptor results in two sequential proteolytic cleavages of the Notch receptor and subsequent release of the Notch intracellular domain (N^{ICD}) in the cytoplasm, which migrates to the nucleus and binds to the transcription factor CSL (named after CBF-1 in humans, Su(H) in *Drosophila* and Lag-1 in *C.elegans*), also known as *Rbpj* in the mouse. In its basal state, CSL/*Rbpj* forms a complex with co-repressors in order to efficiently repress transcription of target genes. Interaction with N^{ICD} displaces co-repressors and recruits co-activators such as proteins of the Mastermind family, thereby converting *Rbpj* from a transcriptional repressor into a transcriptional activator, and subsequently initiating expression of target genes[7]. Members of the Hairy enhancer of split (Hes) or Hairy related (Hey or Hrt) genes have been identified as Notch targets in many different tissues, while other genes are more tissue restricted. Nevertheless most target genes of Notch signaling still remain largely unknown, although several studies on target genes in the T cell lineage have been performed[8,9].

The best characterized function of Notch in hematopoiesis is in the commitment of hematopoietic progenitors to the T-cell lineage. Several studies over the past 20 years provide compelling evidence that Delta4-Notch1 mediated activation of Notch signaling is essential and sufficient to induce T-cell lineage commitment from hematopoietic progenitors, at the expense of B-cell development[10-14]. In addition, Notch signaling has been critically implicated in the specification of the marginal zone B-celle in the spleen[15,16].

Notch signaling has been extensively studied in the hematopoietic stem cell compartment. Germline mutant mice for Notch1, Rbp-Jk, and for the ligand Jagged1 showed impaired generation of hematopoietic progenitors in the AGM region[17-19]. A role for Notch signaling in the regulation of adult HSC function was initially proposed by *in vitro* studies in which both human and mouse hematopoietic progenitors cultured in the presence of Notch ligands could be expanded without losing *in vivo* reconstitution capacity[20-25]. In agreement, over-expression

of N^{ICD} as well as the Notch target gene Hes1 in hematopoietic progenitor cells, enhanced self-renewal capacity of long-term HSCs[26-28]. Of interest, osteoblasts expressing Jagged 1 were identified as being part of the HSC niche[29]. Using an *in vivo* approach to activate osteoblasts by constitutive activation of the parathyroid hormone (PTH) receptor resulted in increased numbers of these cells in BM, which showed increased expression of Jagged1. Such PTH-activated osteoblasts supported an increase in the number of HSC but not other hematopoietic progenitors, consistent with HSC expansion by enhanced self-renewal. Importantly, HSCs in BM of mice with an enlarged osteoblast niche showed increased Notch1 activation *in vivo* and Notch inhibition with γ-secretase inhibitor in *in vitro* long-term co-cultures reduced the suppotrtive capacity of transgenic stroma to wild-type levels[29]. In agreement with these observations transgenic Notch reporter mice showed Notch signaling activity in prospectively defined HSCs. Reporter activity was downregulated when HSCs differentiate. Furthermore, inhibition of Notch signaling by over-expression of dominant negative forms of XSu(H) (the Xenopus homologue of *Rbpj*) or Mastermind resulted in increased differentiation *in vitro* and HSC depletion *in vivo*[30]. In contrast, loss-of-function assays retrieved some controversial results, by showing that both Notch1 and Jagged1 are dispensable for HSC self-renewal and differentiation[31]. The unexpected normal hematopoiesis could be explained by functional redundancy with other Notch receptors and ligands also expressed in HSC and in BM. However a recent approach to block Notch signaling independently of receptor or ligand usage, by expressing a dominant-negative form of MAML1 and subsequently inhibiting the formation of a functional transactivation complex in HSCs, also failed to show a role of Notch signaling in HSC function[32]. Concluding, while gain of function studies have shown the potential for Notch signaling to expand HSC *in vivo*, loss of function studies have indicated contradictory roles with a current consensus that Notch signaling may be dispensable for adult hematopoiesis.

We here studied the role of Notch signaling in hematopoietic stem cell and progenitor cell function using a loss-of-function approach based on conditional deletion of the DNA binding factor *Rbpj*. Since Rbpj is essential for signaling from all Notch receptors this approach should allow complete inactivation of the Notch pathway and circumvent possible redundancy between Notch receptors.

RESULTS

Conditional deletion o*f Rbpj*

In order to study the role of the Notch signaling pathway in HSC self-renewal and differentiation, we used conditional mutant mice for *Rbpj* ($Rbpj^{Fl/Fl}$), the downstream effector of all Notch receptors. This approach allowed for study of Notch signaling while avoiding

possible redundancy between Notch receptors and ligands. Deletion of the conditional Rbpj allele was performed *ex vivo* using the retroviral *Cre* gene transduction system[33,34] (Figure 1A). Lin$^-$c-Kit$^+$ (LK) or Lin$^-$Sca1$^+$c-Kit$^+$ (LSK) cells, which are highly enriched for HSCs, were isolated from the BM of Rbp*j*$^{Fl/Fl}$ mice, and transduced with pMSCV-Cre-IRES-enhanced GFP retroviruses. LSKs cells from littermate wild-type mice (*Rbpj*$^{+/+}$) transduced with the same viral construct were used as control for all experiments. 1.5 days after transduction deletion was virtually complete in GFP positive cells (Figure 1B), as observed by PCR on genomic DNA (Figure 1C). Transduced hematopoietic LK/LSK cells were subsequently used for *in vivo* and *in vitro* assays.

Rbp-J deletion reduces clonogenicity of hematopoietic progenitors and affects myeloid development

Activation of the Notch signaling pathway has been shown to promote expansion of hematopoietic stem/progenitor cells, while these retain *in vivo* reconstitution capacity. In order to see whether complete ablation of canonical Notch signaling affects the clonogenicity of hematopoietic progenitors, we performed methylcellulose colony-forming assays. For this, transduced (GFP$^+$) LSK cells were sorted, and seeded on semi-solid methylcellulose medium. Of notice, Rbpj deletion resulted in a reduction of more than 2-fold in the number of colonies, in comparison to transduced wild-type LSKs from wt littermate controls, (Figure 2A).

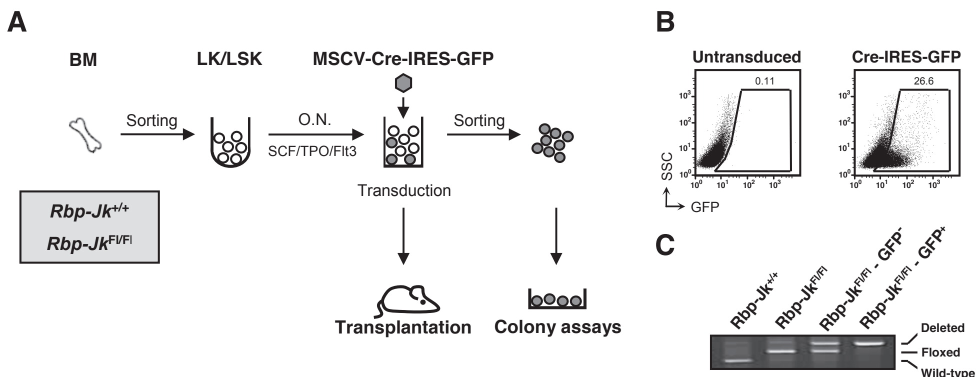

Figure 1. Study experimental approach. A) LK/LSK cells were FACS sorted from the BM of *Rbpj*$^{Fl/Fl}$ or *Rbpj*$^{+/+}$ mice. Sorted cells were stimulated over-night with SCF, TPO and Flt3L and transduced with a retrovirus encoding the Cre recombinase in order to achieve deletion of the conditional *Rbpj* allele. Two days after transduction GFP$^+$ cells were FACS sorted and used for *in vitro* and *in vivo* transplantation assays (see also materials and methods section). B) Transduction efficiency of LSK cells with MSCV-Cre-IRES-GFP retroviruses, 2 day after transduction. C) Analysis of deletion efficiency of the *Rbpj*^{Fl} allele. Analysis was performed on GFP$^+$ sorted cells, 2 days after transduction, by PCR on genomic DNA. Virtually complete (100%) deletion in GFP$^+$ cells was observed.

This reduction in the number of colonies was mainly due to a reduction in Mixed, granulocytic (G) and granulocytic-monocytic (GM) colony-forming units (CFU). The absolute number of monocyte (M) CFUs and burst forming units-erythrocyte (BFU-E) were not affected (Figure 2B). Together, these results suggest that Notch signals are required for hematopoietic progenitor cells and, that deficiency of Notch signaling affects myeloid differentiation.

Increased repopulation capacity in Rbpj deleted hematopoietic stem/progenitor cells

To determine the effects of complete ablation of Notch signaling in HSCs we performed competitive transplantation assays using the Ly5.1/Ly5.2 system. Sorted LK cells from $Rbpj^{Fl/Fl}$ and wild-type control mice (Ly5.2) were transduced and transplanted into lethally irradiated Ly5.1 recipient mice (Figure 1A). 15,000 GFP^{+} ($Ly5.2^{+}$) cells were transplanted along with $2x10^{5}$ Ly5.1 whole bone marrow (BM) competitor cells. Surprisingly, monitoring of the repopulation efficiency in the peripheral blood of recipient mice showed consistently a significantly increased frequency of donor cells in the mice receiving Rbpj deleted cells (Figure 3A). The significantly increased repopulation efficiency of the $Rbpj^{-/-}$ cells was also evident in the BM of the recipient mice, where a higher than 3-fold increase in the frequency of $Ly5.2^{+}GFP^{+}$ could be observed (Figure 3B, C). Interestingly, and due to the fact that $GFP^{-/low}$ also showed partial deletion of the conditional Rbp-j allele (Figure 1C), an expansion of the $Ly5.2^{+}GFP^{-}$ compartment was also observed (Figure 3B). The analysis of the HSC compartment of the

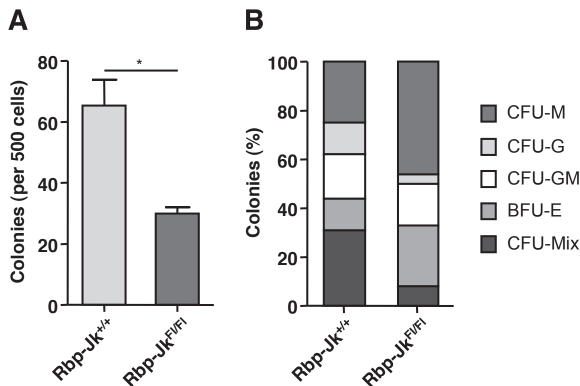

Figure 2. *Rbpj* deletion reduces clonogenicity of hematopoietic progenitors . Methylcellulose colony-forming assays performed with sorted GFP+ LSKs from $Rbpj^{Fl/Fl}$ and $Rbpj^{+/+}$ mice. Total number (A) and relative frequency of the different colonies (B) counted after 9-10 days of culture. Results are representative of 2 independent experiments. Error bars represent SEM. CFU, colony forming unit; M, macrophage; G, granulocyte; GM, granulocyte-macrophage; Mix, mixed (containing at least 3 different types of cells); BFU-E, burst forming unit-erythrocytes. *, p=0.008.

recipient mice showed similarly a significant increase in Ly5.2^{+}GFP^{+} LSK cells (Figure 3D, E) indicating that Rbp-j deletion confers increased repopulation capacity to HSC and/or early hematopoietic progenitors. As expected and in agreement with the strong requirement of Notch signaling for T-cell development,[14,35] analysis of the thymus of the recipient mice that received Rbpj$^{Fl/Fl}$ cells showed almost no contribution of Ly5.2^{+}GFP^{+} cells to the T-cell compartment and ectopic B-cell development in the thymus (Figure 4). This is consistent with efficient *Rbpj* inactivation and little or no selection for cells that might have escaped Cre-mediated deletion.

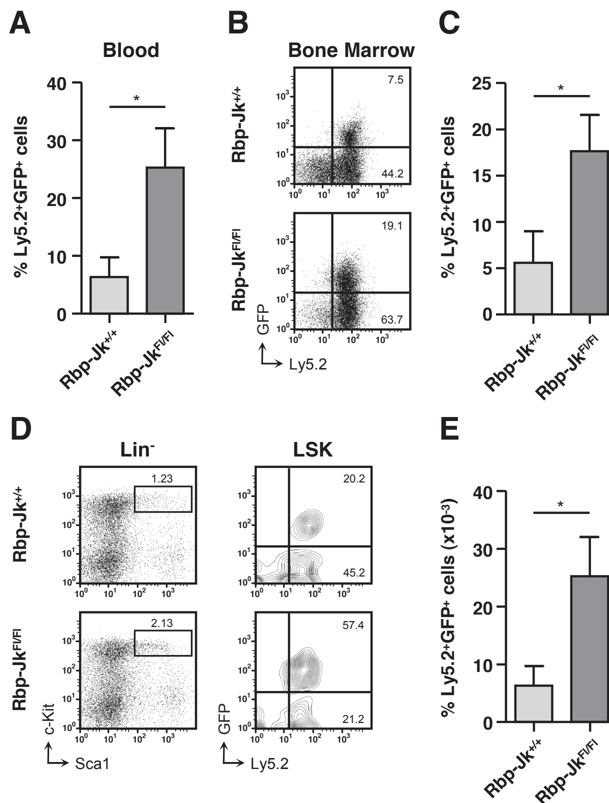

Figure 3. *Rbpj* deletion enhances repopulation capacity of HSCs. Cre-IRES-GFP transduced *Rbpj*+/+ and *Rbpj*$^{Fl/Fl}$ LK cells were transplanted into lethally irradiated Ly5.1 recipients. Repopulation efficiency analyzed in peripheral blood (A, p=0.021) and total bone marrow (B, C; p=0.023) of recipient mice 12 weeks after transplantation. D, E) Analysis of the HSC compartment of recipient mice. LSK cells were electronically gated and analyzed for expression of Ly5.2 and GFP (p=0.032). Graphs show mean ± SEM of 6 mice per group. Data is representative of 2 independent experiments.*, p<0.04.

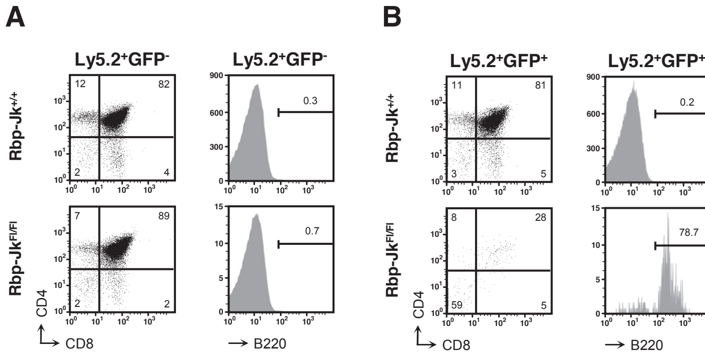

Figure 4. *Rbpj* deletion blocks T-cell development in the thymus of recipient mice. Flow cytometric analysis of thymocyte subsets and the presence of B220+ B-cells of donor (Ly5.2+GFP+) in mice transplanted with *Rbpj*+/+ and *Rbpj*Fl/Fl LK cells. A) Ly5.1 competitor BM progenitors cells efficiently repopulate the thymus and develop through the T-cell lineage. Ly5.1 B-cells are not present in the thymus. B) Strong inhibition of T-cell development and ectopic B-cell development in the thymus derived from *Rbpj*-/- cells, in comparison to the *Rbpj*+/+ controls, indicating efficient deletion of the conditional *Rbpj* allele.

DISCUSSION

The role of Notch signaling in hematopoiesis has been extensively studied. While Notch plays an essential role in commitment to the T-cell lineage and specification of marginal zone B-cells in the spleen, its role in HSC function and myelopoiesis is still controversial. Nevertheless several studies have implicated Notch signaling in expansion of the hematopoietic progenitor compartment while inhibiting terminal myeloid differentiation (reviewed in [3,36]). In agreement with this view, in methylcellulose colony-forming assays *Rbpj* deletion resulted in reduced clonogenicity with a strong decrease in immature CFU-Mix colonies. The differences to a previous study showing no effect of *Rbpj* inactivation on colony formation[35] may be due to cytokine composition and culture conditions as well as the conditional deletion approach used, since Mx-Cre mediated deletion involves stimulation with Poly(I-C)/Interferon-α which was shown to dramatically influence HSC differentiation and self-renewal. Besides this, *Rbpj* inactivation also leads to a reduction in more mature progenitors such as CFU-G and bipotent CFU-GM, while CFU-M numbers were not affected. The exact manner how Notch influences myelopoiesis is still highly controversial. Different studies report distinct effects that may be explained by different ligand-specific effects[37] and other signals such as cytokines in the context of Notch activation[38]. Furthermore, transduction of active forms of Notch (N^{ICD}) led to increased terminal differentiation of nontransduced cells. This suggests that Notch1 may affect hematopoiesis in a non-cell-autonomous manner[39], meaning that activation of Notch signaling in one cell may subsequently influence the cross-talk with neighboring cells.

Gain-of-function studies by different laboratories showed that activation of the Notch signaling pathway by expression of active forms of Notch (N^{ICD}) or exposure to Notch ligands resulted in the expansion of the HSC pool and increased self-renewal capacity. Importantly, HSCs were shown to undergo active Notch signaling *in vivo* using a transgenic reporter based on multimerized *Rbpj* binding sites[30] and ostoblasts expressing the Notch ligand Jagged1 were identified as being part of the HSC niche[29]. All together, these studies suggest an important role for Notch signaling in the regulation of HSC function. If Notch signaling plays an essential role in HSCs it would be expected that inactivation of the pathway results in impairment of self-renewal and repopulation capacity. Surprisingly, we here observed that deletion of the common downstream effector of all Notch receptors leads to a significant increase in repopulation capacity of lethally irradiated mice. The increase in repopulation capacity was observed in total chimerism in blood and BM and also in the HSC compartment where the number of *Rbpj*$^{-/-}$ LSK was increased. In order to see whether this also holds true for LT-HSC it will be important to perform serial transplantations to see if this increase is maintained in secondary transplanted recipient mice. Similarly, two recent independent studies report a trend to increased repopulation capacity after conditional deletion of *Rbpj* in the hematopoietic system[32,40]. However, initial Notch signaling loss-of-function studies in which a dominant-negative form of *Rbpj* that interacts with N^{ICD} but cannot bind DNA was over-expressed in HSCs, showed that transduced HSCs display a strong reduction in repopulation capacity[30]. Indeed, it is becoming clear that the phenotypes resultant from CSL depletion are not necessarily identical to loss-of-Notch function7. CSL/*Rbpj* is responsible for the activation of gene expression upon binding to NICD. However it also has a repression function in the absence of Notch signaling, which is also impaired by deletion of this factor. Therefore, while a dominant-negative form of CSL only impairs the activation function of *Rbpj*, complete deletion may also lead to the derepression of target genes. This “molecular Switch” property between repressor and activator of target genes was first appreciated in studies in Drosophila where repression by Su(H) was found to be essential in several cell-fate specification processes during embryonic development[41]. In one of these studies derepression was sufficient to partially rescue the wing primordium defects in γ-secretase mutants[42]. In this way, *Rbpj* deletion may to some extent phenocopy Notch activation and therefore may explain the increased repopulation capacity displayed by *Rbpj*$^{-/-}$ HSCs. It will be important to perform gene expression profiling in *Rbpj*$^{-/-}$ and dnRbpj transduced HSC in order to test this hypothesis and to see whether functional differences are reflected in differential expression of Notch target genes.

The widely expressed lymphoma related factor (Lrf, formerly known as Pokemon) was shown to down-regulate Notch signals in BM progenitors[43]. Thus, and in agreement with the low expression of Notch target genes such as Hes1 in HSC[32], it is possible that HSC require low levels of activation of this pathway. This view is in agreement with the hypothesis we

suggest here since derepression of Notch targets is not likely to yield very high transcriptional activity due to the absence of co-activators, normally recruited upon true activation of the pathway[7]. Consistent with this view, despite the advantage observed in repopulation capacity, *Rbpj*-/- BM progenitors, failed to differentiate into T-cells in our experimental setup suggesting that the transcriptional levels of Notch target genes required for T-cell development may be higher, compared to HSC. Down-modulation of Notch signals in hematopoietic progenitors probably has an essential role to prevent both ectopic development of T-cells and suppression of B-cell development.

Another approach to inactivate Notch signaling in HSCs was used by Maillard et al.[32]. This consisted of over-expressing a dominant-negative form of the co-activator Mastermind-like1 (dnMaml) in HSCs. The dnMaml interferes with the Notch transcriptional activation complex, leading to inhibition of Notch1-4 signaling[44,45]. Over-expression of dnMaml did not significantly affect HSC function as observed by normal reconstitution capacity in serial transplantation assays. Based on these results the authors suggested that canonical Notch signaling is dispensable for the maintenance of adult HSCs. However, the consequence of dnMaml expression for the activation status of the Notch pathway was not investigated. Since dnMaml inhibits Notch signaling by competing with the endogenous wild-type Maml, it remains possible that a low activity of the pathway persists in this approach. If the results we present here are explained by derepression of target genes, a hypothesis that still requires further investigation, a low level of activation of the pathway is in this situtaion sufficient to sustain normal HSC function. Although extremely informative as a strong positive control, the capacity to differentiate into the T-cell lineage should not be used alone as read-out for the activation status of the pathway since different lineages and hematopoietic subsets may have differential requirement in the strength of Notch signaling. A dosage dependent regulation of different hematopoietic lineages may also explain the apparent discrepancy between the *in vivo* and *in vitro* data we obtained. The *in vivo* analysis of Notch reporter mouse models in combination with the different loss-of-function approaches discussed here will be vital to understand the role of Notch signaling pathway in HSCs.

The results we present here suggest that the repression function of *Rbpj* may play an important role in the regulation of HSC function and raises the possibility of a dosage dependent regulation of hematopoiesis by Notch. Further research will be necessary to confirm these hypotheses as well as whether Notch plays an essential role in the maintenance of adult HSCs.

MATERIALS AND METHODS

Mice

Mice were bred and maintained in the animal facilities of Erasmus Medical Center or Leiden University Medical Center, in accordance with legal regulations in The Netherlands and with the approval of the Dutch animal ethical committee. Mice carrying the conditional deletion allele for the Rbp-Jk gene were previously described[35] and kindly provided by Prof. Tasuku Honjo (Graduate School of Medicine, Kyoto University,Kyoto, Japan). C57Bl/6-Ly5.1 mice were purchased from Charles River Laboratories. Deletion of the *Rbpj* conditional allele was analyzed by PCR on genomic DNA using the following primer combinations: m*Rbpj*-Lox-F, TTGGTTTGTTGTTTGGGTTG; m*Rbpj*-Lox-R, GCTCCCCACTGTTGTGAACT; m*Rbpj*-Del-F, GCGGATTTCTGAGTTTGAGG.

Flow cytometry

For flow cytometric analyses cells were stained with monoclonal antibodies against the following molecules: CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD11b/Mac1 (M1/70), CD45.1/Ly5.1 (A20), CD45.2/ Ly5.2,(104), CD117/c-Kit (2B8), B220 (Ra3-6B2), GR1 (RB6-8C5), NK1.1 (PK136), and Ter119 from Becton Dickinson/Pharmingen; Sca1 (D7) from eBioscience. Antibodies were used conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), Peridinin Chlorophyll-a Protein (PerCP), PE-Cy7, allophycocyanin (APC), APC-Cy7, APC-AF780 and Biotin. For secondary detection of biotinylated antibodies, streptavidine conjugated with PE, PerCP, PE-Cy7 or APC-Cy7 (from Becton Dickinson/ Pharmingen) were used. To sort or analyze LSKs in BM, antibodies for lineage markers included CD3, CD4, CD11b, B220, GR1, NK1.1 and Ter119.

Stained cells were measured with a FACS-CantoII or a LSRII, sorted on a FACS AriaII (Becton Dickinson) and analyzed with FlowJO software (Treestar).

Retroviral production and transduction

MSCV-Cre-IRES-GFP plasmid was kindly provided by Dr. Hiromitsu Nakauchi (Institute of Medical Science, University of Tokyo, Japan). This vector was transfected into Phoenix virus-packaging cell line in combination with (PGK-Puro) plasmid in a 10:1 ratio in order to obtain a stable virus producer cell line. Transfection was performed with FuGENE6 (Roche) according to manufacturer's instructions and Transfected cells were selected with 1µg/ml puromycin and by cell sorting of GFP^+ cells. Viral supernatants were harvested at 24 and 48hrs after replacement with fresh medium (IMDM, 10%FBS, 1%P/S) and stored at -80°C.

40-70000 sorted LSK cells were stimulated over-night in serum-free StemSpan medium (StemCell Technologies) supplemented with cytokines (100ng/ml rmSCF, 10ng/ml rmTPO and 50ng/ml rmFlt3L; all from R&D) in round-bottom 96-well plates. Stimulated cells were transduced in 96 well plates with titrated amounts of virus using Retronectin (Takara Bio Inc.) and the retronectin-bound virus infection method according to manufacturer's instructions. Cells were cultured for 1 additional day and used for *in vitro* and *in vivo* assays. A subsequent purification of GFP+ cells was performed for some of the experiments.

Competitive transplantation assays

Competitive transplantation assays were performed using the Ly5.1/Ly5.2 congenic system. $Rbpj^{Fl/Fl}$ and wild-type LSKs (Ly5.2) were trasnduced with MSCV-Cre-IRES-GFP retroviruses and transplanted (15.000 GFP^+ cells per mouse) into lethally irradiated (9.5Gy) Ly5.1 (9-12 weeks) mice together with $2x10^5$ Ly5.1 bone-marrow competitor cells. Chimerism was analyzed at 4, 8 and 12 weeks after transplantation in peripheral blood, and mice were sacrificed for analysis at 12 weeks post-transplantation. Mice were considered repopulated when ≥1% multilineage Ly5.1 cells could be detected in nuclecleated peripheral blood cells 3 months after transplantation.

Methilcelulose Colony-forming assays

For methylcellulose colony assays sorted GFP^+ cells were prepared in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2%FBS. CFU-C assays were performed with MethoCult M3434 (containing 50ng/mL rmSCF, 10ng/mL rmIL-3, 10ng/mL rhIL-6, 3U/mL rhEPO; from StemCell Technologies) supplemented with 20ng/ml rmTPO (R&D), in 35mm-dish accordingly to manufacturer's instructions. All assays were done in triplicate. Colonies (>30 cells) were counted after 9-10 days of incubation at 37oC, 5%CO2 and humidified atmosphere. CFU-Mix was defined as colonies containing at least 3 different cell types. Colonies were identified by morphology and confirmed with May-Grunwald Giemsa staining.

Statistical analysis

Statistical significance was determined by standard t-test.

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Chapter 5

Identification of Notch target genes in uncommitted T-cell progenitors: no direct induction of a T-cell specific gene program

Floor Weerkamp[1], Tiago Luis[1], Brigitta A.E. Naber[1], Esther E.L. Koster[1], Lucie Jeannotte[2], Jacques J.M. van Dongen[1], Frank J.T. Staal[1]

[1]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, The Netherlands, [2]Centre de recherche en cancérologie de l'Université Laval, Centre Hospitalier Universitaire de Québec, L'Hôtel-Dieu de Québec, Canada

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ABSTRACT

Deregulated Notch signaling occurs in the majority of human T-ALL. During normal lymphoid development, activation of the Notch signaling pathway poses a T-cell fate on hematopoietic progenitors. However, the transcriptional targets of the Notch pathway are largely unknown. We sought to identify Notch target genes by inducing Notch signaling in human hematopoietic progenitors using two different methods: an intracellular signal through transfection of activated Notch and a Notch-receptor dependent signal by interaction with its ligand Delta1. Gene expression profiles were generated and evaluated with respect to expression profiles of immature thymic subpopulations. We confirmed *HES1*, *NOTCH1* and *NRARP* as Notch target genes, but other reported Notch targets, including the genes for Deltex1, pre-T cell receptor α and E2A, were not found to be differentially expressed. Remarkably, no induction of T-cell receptor gene rearrangements or transcription of known T-cell specific genes were found after activation of the Notch pathway. A number of novel Notch target genes, including the transcription factor *TCFL5* and the *HOXA* cluster, were identified and functionally tested. Apparently, Notch signaling is essential to open the T-cell pathway, but does not initiate the T-cell program itself.

INTRODUCTION

One of the most important signaling cascades involved in T-cell development is the Notch pathway [1,2]. The Notch signal transduction pathway is an evolutionary conserved mechanism that regulates cell fate determination during developmental processes [3]. Four family members of the transmembrane Notch receptor are recognized, named Notch1 to 4. Signaling is initiated when the large extracellular domain of the Notch receptor binds a membrane-bound ligand on a neighboring cell. The five Notch ligands in mammals are Delta1, 3 and 4 and Jagged1 and 2. Binding of a ligand induces proteolytic cleavage of the intracellular part of the Notch protein (IC-Notch), which translocates to the nucleus and binds to the transcription factor CSL (in human referred to as CBF1, in mouse as Rbp-Jκ), activating transcription of Notch target genes[4].

T cells develop from multipotent progenitor cells that seed the thymus from the bone marrow or fetal liver [1]. The thymus is rich in expression of Notch ligands [5] and after entering the thymic microenvironment, progenitors immediately start expressing Notch target genes [6]. These newly generated thymocytes do not express CD4 and CD8 and are therefore called double negative (DN). In humans, these most immature DN thymocytes are characterized as $CD34^+CD1a^-CD38^-$ and are homologous to murine DN1 cells 7. In the next DN stage, thymocytes are $CD34^+CD1a^-CD38^+$ (DN2). This subset still contains (at the population level) potential to develop into all hematopoietic lineages [8]. In the subsequent stage ($CD34^+CD1a^+$, DN3), thymocytes become irreversibly committed to the T-cell lineage [7]. Finally, thymocytes lose expression of CD34 and proceed into the immature single positive (ISP) stage.

The importance of Notch signaling for the induction of a T-cell fate was first demonstrated in mice in which the Notch1 gene was conditionally deleted, resulting in a complete block in T-cell development at the DN1 stage [9] and the emergence of ectopic B-cell development in the thymus [10]. Conversely, overexpression of IC-Notch in the bone marrow instructed a T-cell fate in bone marrow progenitors and inhibited B-cell development [11]. The same effect ensued when Delta4 was overexpressed in the bone marrow [12]. *In vitro*, this phenomenon can be mimicked by expression of Delta1, but not Jagged1, in bone marrow stromal cell lines. Using this co-culture system, T-cell precursors can be efficiently generated from murine and human hematopoietic progenitors [13-16]. The significance of Notch signaling is underscored by studies on T-ALL leukemogenesis. At least half of all human T-ALL carry activating mutations in the intracellular part of Notch1 [17,18].

To develop into a T cell, Notch signaling is clearly indispensable, but the downstream mechanisms by which a Notch signal is translated into a T-cell program are still largely unclear. The best known Notch target genes are Hairy-Enhancer of Split (*HES*)1 and *HES5* and Hes-related repressor protein (*HERP*). Hes and Herp are basic-helix-loop-helix (bHLH) proteins

that function as transcriptional repressors [19,20]. Indeed overexpression of Hes1 and Hes5 in the bone marrow partly inhibits B-cell development [21]. But although proliferation of early thymocytes is severely affected by Hes1 deficiency [22,23], thymocytes still develop in these mice. Hes1 can therefore not be the sole target of Notch signaling responsible for inducing a T-cell fate.

PTCRA (the gene for pre-T cell receptor α (pTα)) was found as a Notch target using Representational Difference Analysis (RDA) in murine thymoma cell lines with or without retroviral transduction of IC-Notch [24]. The pTα promoter was demonstrated to contain a CSL binding site and could be activated by IC-Notch *in vitro* [25]. Nevertheless, the physiological role of Notch signaling for pTα expression during T-cell development remains controversial, as pTα expression was not affected in mice in which Notch1 was conditionally deleted from the DN3 stage onwards [26]. A role for Notch1 in opening of the T-cell receptor-β (*TCRB*) locus has been shown, but only for the complete TCRB rearrangements (V to DJ) [26,27].

Other Notch target genes identified in the thymoma cell line were *DTX1* (gene for Deltex1), *Ifi-202*, *Ifi-204*, *Ifi-D3*, *ADAM19* (*Meltrinβ*) [24]. A number of other genes have been reported as being Notch targets, including *NOTCH1* itself [28], *NRARP* in Xenopus embryos [29], *BCL2* in thymoma cells [30], *CCND1* (gene for cyclin D1) in a kidney cell line [31], *CDKN1A* (gene for cyclin-dependent kinase inhibitor 1A (p21, Cip1)) in keratinocytes [32] and *TCF3* (gene for E2A) [11,33]. It is unknown whether these genes are *in vivo* targets in the earliest thymocytes and whether they function in T-cell commitment. In this study we investigated the downstream mechanisms of Notch signaling in human hematopoietic progenitor cells.

MATERIALS AND METHODS

Isolation of cells

Human umbilical cord blood (UCB) material was obtained according to the informed consent guidelines of the Medical Ethical Committee of Erasmus MC, Rotterdam. UCB mononuclear cells were isolated using Ficoll density centrifugation and frozen down until further use. For each experiment, frozen cord UCB cells of at least four different donors were used. $CD34^+$ progenitor cells were purified using immunomagnetic beads (Miltenyi Biotec). Purity of the recovered subpopulations was checked by flow-cytometry (FACS Calibur, BD Biosystems) and was always greater than 95%.

Transfection of $CD34^+$ UCB cells

Human cDNA encoding the intracellular domain of Notch1 (amino acids 1770-2555 [34]) was cloned into the multiple cloning site of eukaryotic expression vector pcDNA3 (Invitrogen). $CD34^+$ UCB cells were cultured for one hour in the presence of 10 ng/ml recombinant human

IL-7 and 50 ng/ml recombinant human SCF (both from R&D Systems). Subsequently, $4*10^6$ cells were transfected with 4 µg pcDNA3-IC-Notch or empty pcDNA3 using a human CD34 Nucleofector kit (AMAXA) and an AMAXA Nucleofector Device, according to the manufacturers protocol. 0.1 µg of pEGFP-C1 (BD Clontech) was co-transfected with each sample. After transfection, cells were cultured on confluent layers of the murine bone marrow stromal cell line S17, in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 6 and 24 hours, cells were carefully harvested without disturbing the S17 monolayer and RNA was isolated. An aliquot of cells was taken from each sample to monitor transfection efficiency (GFP expression) by flow-cytometry.

Co-culture of CD34$^+$ UCB cells on S17

S17 bone marrow stromal cells retrovirally transduced with either Delta1-IRES-eGFP (S17-DL) or control LZRS-IRES-eGFP (S17-GFP) were kindly provided by dr. L. Parreira (Faculdade de Medicina de Lisboa, Portugal) and have been described previously [13]. S17 cells were grown in confluent monolayers in 24 wells plates. $1*10^5$ CD34$^+$ UCB cells were co-cultured with S17 in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 3, 6 and 24 hours cells were carefully harvested and processed for RNA isolation.

Microarray analysis

Affymetrix microarray analysis of the samples transfected with pcDNA or pcDNA-IC-Notch was performed as described previously [35]. For the samples co-cultured on S17, an extra cycle of cDNA and cRNA synthesis was performed. The starting amount of RNA for the first round of cDNA synthesis was 30-60 ng.

For the transfected samples, Affymetrix U133A and B microarrays were used, for co-cultured samples only U133A arrays. In comparison experiments, care was taken that the 5'/3' GAPDH ratio, scaling factor, noise, and percentage of presence calls were comparable. Scanned images were analyzed using Affymetrix Microarray Suite 5.1 software, and pair-wise comparisons were generated using the appropriate control samples (without induced Notch signaling) as baseline. Differential expression was considered significant when the gene received a 'present call' for at least one of the microarrays used for the comparison, the fold change was >1.8 and change-p value <0.003 by MAS and F-test [38]. Differential expression of genes after Notch induction was compared to expression profiles of CD34$^+$ UCB cells and the relevant thymocyte subsets: comparisons of CD34$^+$ UCB cells versus CD34$^+$CD1a$^-$CD38$^-$ (DN1) and CD34$^+$CD1a$^-$CD38$^-$ versus CD34$^+$CD1a$^-$CD38$^+$ (DN2)) (data generated previously 7).

Probes for *DTX1* and *NRARP* were present only on the U133B array. *HES5* was not represented on the microarray used (U133). Of the Ifi family, only *Ifi16* was present on the microarrays.

Pharmacological inhibition of Notch signaling

4×10^5 human CD34$^+$ UCB cells were cultured on confluent layers of the S17-DL1 stromal cell line, in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 16 hr incubation, DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma) was added to the cells to the final concentrations of 1 µM or 5µM. Alternatively, medium or DMSO alone was added as control. The cells were harvested before addition of DAPT and after 24 hours incubation, and used for RNA isolation and cDNA synthesis. *TCFL5* and *Hes1* expression was analyzed by Real-time quantitative-PCR. The vehicle alone control (DMSO) did not have any effect.

Real-time quantitative-PCR (RQ-PCR)

The expression of several target genes was tested using TaqMan-based RQ-PCR. 1 µg cRNA generated in the microarray analysis procedure was treated with 1U of DNase I and subsequently reverse transcribed to cDNA with avian myeloblastosis virus-reverse transcriptase (5 U), oligo(dT) and random hexanucleotide primers.

Mixes containing the appropriate pre-designed primer/probe sets were obtained from Applied Biosystems. For *TCFL5*, *Hes1*, *HoxA9* and *HoxA10* these were Assays-on-Demand, for *HoxA5* an Assay-by-Design. A 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The RQ-PCR reaction was performed using TaqMan Universal mastermix (Applied Biosystems) and was run on a PRISM 7700 sequence detection system containing a 96 well thermal cycler (Applied Biosystems). RQ-PCR results were normalized to GAPDH expression (kit from Applied Biosystems) in the same sample.

Functional testing of TCFL5 in fetal thymic organ culture (FTOC)

Full-length cDNA for human TCFL5 was obtained from Invitrogen, cloned into the retroviral vector LZRS-IRES-eGFP and high titer retrovirus was produced in the Phoenix packaging cell line. CD34$^+$ UCB cells were cultured for one day and retrovirally transduced for two days in the presence of 50 ng/ml SCF, 10 ng/ml recombinant human TPO and 50 ng/ml recombinant human Flt3L (all from R&D Systems). The percentage of GFP positive cells was determined using flow cytometry and cells were transferred into irradiated murine embryonic day 14 thymic lobes. After two days, lobes were cultured on filters floating on medium. After an additional nine days, lobes were harvested, single cell suspensions were made and T-cell development was analyzed using a FACS Calibur flowcytometer (BD Biosystems). Monoclonal antibodies against human antigens were CD1a-RD1 (Beckman Coulter) and TCRγδ-PE, CD3-PerCP, CD8-PerCP, CD4-APC and CD34-APC (all from BD Pharmingen). At least 50,000 events were acquired and analyzed by flow cytometry.

HoxA5 mutant mice

Mice deficient for HoxA5 function have been described in detail [36]. Hoxa5 heterozygous mice from an outbred genetic background were bred to generate homozygous, heterozygous and wild type littermates. Surviving HoxA5 homozygous mice were viable and did not display evident abnormalities [36]. In mice of 2.5, 4 and 25 weeks old, bone marrow, spleen and thymus were analyzed for presence of different hematopoietic lineages using flow cytometry. Embryonic thymic lobes of day 16 were directly stained for flow-cytometry. Embryonic thymic lobes of day 13 and 14 were cultured on a filter for 16 days, after which T-cell development was analyzed using flow-cytometry. Fetal livers of embryonic day 13 and 14 were either directly used for flow-cytometry to analyse progenitor populations or transferred into irradiated wild type day 14 thymic lobes for FTOC experiments. Chimeric thymic lobes were cultured for 14 days on a filter, after which T-cell develoment was analyzed using flow-cytometry.

HoxA5 deficient BM cells were assayed for the presence of granuloctye-macrophage colony-forming units (CFU-C) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium containing cytokines: for BFU-E 4U/ml human erythropoietin (EPO, Behringwerke AG) and 100 ng/ml murine SCF (R&D); for CFU-C 30 ng/ml murine IL-3, 100 ng/ml SCF and colony stimulating factor (CSF, 300x dilution of ConA adsorbed fraction of pregnant mouse uterus extract). 40,000 and 100,000 cells were plated in duplicate dishes. The number of colonies was determined after 10 days of culture.

HoxA5 deficient BM cells were assayed for their ability to reconstitute the immune system of sublethally irradiated mice. 8-11 week-old C57Bl/6 mice harboring the CD45.1 (Ly5.1) allele as a congenic marker, received a sublethal dose of 6 Gy total body irradiation. Mice were intravenously injected with $2*10^6$ thawed bone marrow cells ($CD45.2^+$). Ten weeks after transplantation, mice were killed and BM, spleen and thymus were isolated. In each organ, total cell numbers were determined and the percentage of $CD45.2^+$ cells and their subset distribution was assayed by flow cytometry.

Monoclonal antibodies against mouse antigens were ER-MP20-FITC (anti Ly6c, own culture), CD25-FITC, IgM-FITC, Sca1-FITC, CD45.2-FITC, CD4-PE, CD43-PE, TER119-PE, NK1.1-PE, CD127-PE, Sca-1-PE, CD3-PerCP, CD8-PerCP, Lin-PerCP (CD3-biotin, B220-PerCP, NK1.1-biotin, MAC1-PerCP, GR1-biotin, TER119-biotin, Streptavidin-PerCP), CD44-APC, B220-APC, CD19-APC, MAC1-APC and c-kit-APC (all from BD Pharmingen).

RESULTS

Induction of Notch signaling

To identify target genes of Notch signaling, gene expression profiles were generated of human hematopoietic progenitors that did or did not undergo Notch signaling. We provided Notch signals in two different ways: by transfection with IC-Notch or by co-culturing cells on Delta1 expressing stromal cells.

It has been shown previously that human CD34$^+$ cells expressing IC-Notch develop into T/NK progenitors when co-cultured on bone marrow stroma [37]. Human CD34$^+$ UCB cells were transfected with the *IC-NOTCH* gene, together with a separate GFP vector in order to monitor transfection efficiency. Six hours after transfection, the percentage of GFP expressing cells was high and comparable between IC-Notch transfected and control cells (Suppl. Fig 1). 24 hours after transfection GFP expression had slightly decreased (Suppl. Fig 1).

Another way to induce the Notch signaling cascade is to allow progenitor cells to interact with their cognate ligand responsible for inducing T-cell development. To this end, human CD34$^+$ UCB cells were cultured on the bone marrow stromal cell line S17, which was retrovirally transduced with either human Delta1 in combination with GFP (S17-DL) or with GFP only (S17-GFP) [13]. Other groups have shown that CD34$^+$ UCB cells cultured in this and similar systems develop into double positive (DP) thymocytes [13,16] and also in our hands CD34$^+$ progenitor cells efficiently developed into CD7$^+$ T/NK precursors cells after 2 weeks of culturing on S17-DL (data not shown). CD34$^+$ progenitor cells were cultured on S17-DL for 3, 6 and 24 hours, as kinetics of translocation of Notch-IC to the nucleus and induction of target RNA transcription are unknown.

From all samples RNA was isolated and gene expression profiles were generated using Affymetrix technology. Pair-wise comparisons were made between samples with or without Notch signaling. Numbers of significantly differentially expressed genes (≥ 2 fold difference, $p<0.003$ by MAS and F-test [38] are listed in Table 1. Both up- and downregulated genes were found, indicating that Notch functions as an activator as well as a repressor of transcription in hematopoietic progenitors.

Expression profiles of human thymocyte subsets have been previously generated and described in detail [7]. The differential expression of genes induced by Notch signaling in hematopoietic progenitors was compared to the relevant UCB and thymocyte subsets: CD34$^+$ UCB versus DN1 (CD34$^+$CD1a$^-$CD38$^-$) and DN1 versus DN2 (CD34$^+$CD1a$^-$CD38$^+$). DN1 cells have just entered the thymus and are therefore expected to upregulate Notch target genes [6]. Indeed, the development of murine early thymic progenitors (ETP) into subsequent thymic stages is strictly Notch-dependent [39]. DN2 cells should also express Notch target genes, as they have contacted Notch ligands for prolonged periods and as Notch-ligand interactions throughout

Table 1. Numbers of significantly up- or downregulated genes (≥2 fold, p<0.003)

Array	6 hr ICN[1]	24 hr ICN[1]	3 hr S17-DL[2]	6 hr S17-DL[2]	24 hr S17-DL[2]
U133A	70↑ 12↓	17↑ 44↓	10↑ 87↓	13↑ 14↓	137↑ 162↓
U133B	35↑ 5↓	13↑ 0↓	nt[3]	nt[3]	nt[3]

[1]ICN: progenitor cells from human UCB transfected with pcDNA-IC-Notch versus control pcDNA. [2]S17-DL: progenitor cells from human UCB cultured on S17-DL versus S17-GFP. nt: microarray not performed.

the DN1 and 2 stage are necessary for irreversible T-cell commitment [40]. In addition, during the DN2 stage, commitment to the T-cell lineage is initiated and cells start rearranging T-cell receptor β (TCRB) genes [7].

Expression of previously reported Notch target genes

Comparison files were mined for the expression of known Notch target genes. In all samples, transcription of *HES1*, the universal Notch target [3], was clearly increased after Notch signaling (Figure 1A). This was most pronounced in the progenitor cells transfected with IC-Notch for 6 hours, reflecting the strong Notch signal induced in this way. 24 hours after transfection, *HES1* was still upregulated, but to a lesser extend. This might indicate negative feed-back mechanisms that are initiated after receiving a strong Notch signal. Alternatively it may reflect the fact that transfection was transient, consistent with the slightly diminished GFP expression found after 24 hours (Suppl Figure 1).

In the cells cultured on S17-DL, *HES1* expression was most prominent after 24 hours (Figure 1A). *HES1* was also highly upregulated in the DN1 thymocytes as compared to $CD34^+$ UCB cells (Figure 1A), demonstrating that the artificially induced Notch signals in our experiments mimic the relevant processes *in vivo*.

HERP1 and 2 were not expressed in any of the samples, neither before nor after inducing a Notch signal (Figure 1A). *NOTCH1* itself is also a Notch target [28] and was found to be significantly upregulated after 6 hours of transfection and after 24 hours of culturing on S17-DL (Figure 1A). This was reflected by high expression in the DN1 subset as compared to UCB (Figure 1A). Also *NOTCH3* was transcribed at high levels in the DN1 cells as compared to UCB, but Notch signaling *in vitro* induced *NOTCH3* transcription to a lesser extend and with slower kinetics than *NOTCH1* (Figure 1A). *NOTCH2* (Figure 1A) and NOTCH4 (not shown) were not differentially expressed in any of the samples.

Of the other previously reported Notch target genes mentioned above, only *NRARP* was significantly upregulated after induction of Notch signaling (3.5 fold increased 6 hours after transfection of IC-Notch (not shown)). The other genes were either not differentially expressed or not expressed at all (Figure 1B and data not shown), indicating that they are not Notch target

genes in early hematopoietic progenitor cells. This finding is especially remarkable for Deltex1 and pTα, which have been reported as Notch targets during T-cell development [14,24,25]. In the thymocyte subsets, significantly increased mRNA expression of *PTCRA* was only found at the DN2 stage, but not at the DN1 stage (Figure 1B), while genuine Notch target genes (*HES1*, *NOTCH1*) were expressed already in DN1 thymocytes (Figure 1A). PTα may be a Notch target gene at later stages of T-cell development, but is not immediately upregulated when cells enter the thymus.

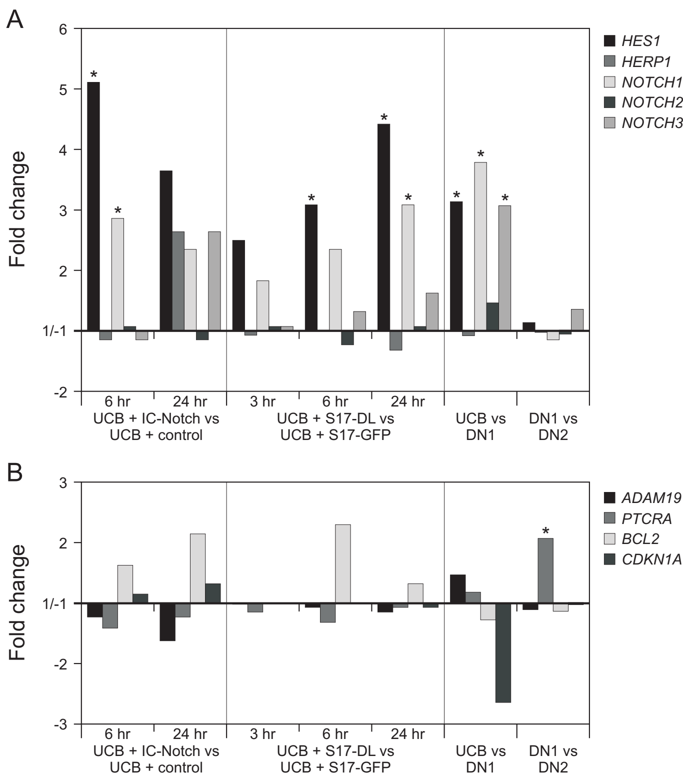

Figure 1. Differential expression of putative Notch target genes. (A and B) Fold changes in expression levels extracted from gene expression profiles generated from hematopoietic progenitor cells with or without induced Notch signaling, as well as previously performed gene expression profiles from human UCB and thymocyte subsets [7]. ICN vs control: comparisons of progenitors transfected with pcDNA-IC-Notch versus control pcDNA. DL vs GFP: comparisons of progenitors cultured on S17-DL versus S17-GFP. CB vs DN1: comparisons of $CD34^+$ UCB cells versus $CD34^+CD1a^-CD38^-$ (DN1) thymocytes. DN1 vs DN2: comparisons of $CD34^+CD1a^-CD38^-$ (DN1) thymocytes versus $CD34^+CD1a^-CD38^+$ (DN2) thymocytes. Asterisks indicate a significant difference in expression levels (fold change >1.8, p<0.003).

For *BCL2* a consistent, but not significant, trend of upregulation was observed in most comparisons, which was nevertheless not reflected in the thymocyte subsets (Figure 1B). Conversely, *CDKN1A* (p21) was significantly decreased in the DN1 subset, but did not show any differential expression after Notch activation (Figure 1B).

Notch does not directly induce a T-cell program

The mechanisms by which Notch signaling induces or promotes a T-cell fate still warrant elucidation. To investigate whether Notch signaling can trigger T-cell commitment by directly inducing TCR gene rearrangements or transcription of 'classical' T-cell specific genes, we mined our dataset for such genes.

No induction of *RAG1*, *RAG2* and *DNTT* (gene for TdT) and germline transcripts of *TCRB*, *TCRD* and *TCRG* were found after Notch signaling (Figure 2A and not shown), suggesting that Notch has no direct function in opening of the TCR loci or the initiation of TCR gene rearrangements.

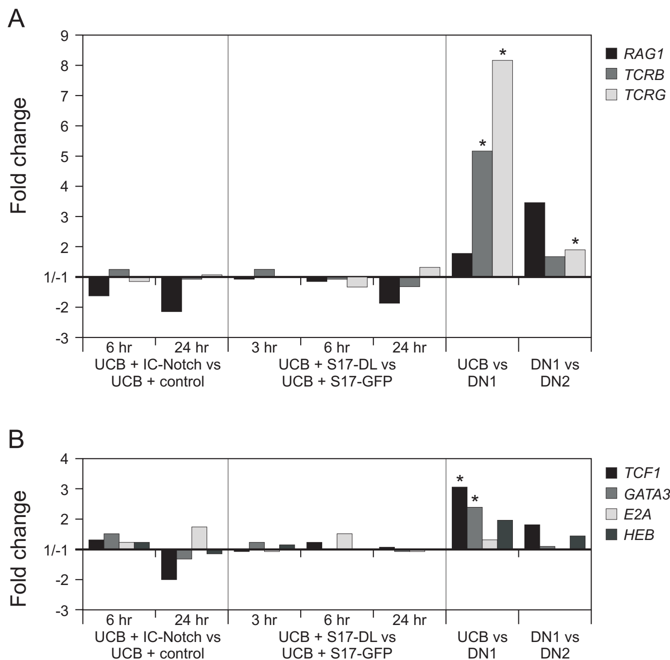

Figure 2. Differential expression of T-cell associated genes. Determined by microarray analysis as indicated for Figure 2. (A) TCR gene rearrangement-associated genes. (B) Transcription factors involved in T-cell development.

In addition, we found no differential expression (mostly no expression at all) of T-cell specific genes after activation of Notch signaling, while these genes were highly upregulated in the DN1 thymocytes (including *CD7* and *CD2*) or in the DN2 subset (including *CD1a*, *LAT* and *CD3γ*) (data not shown).

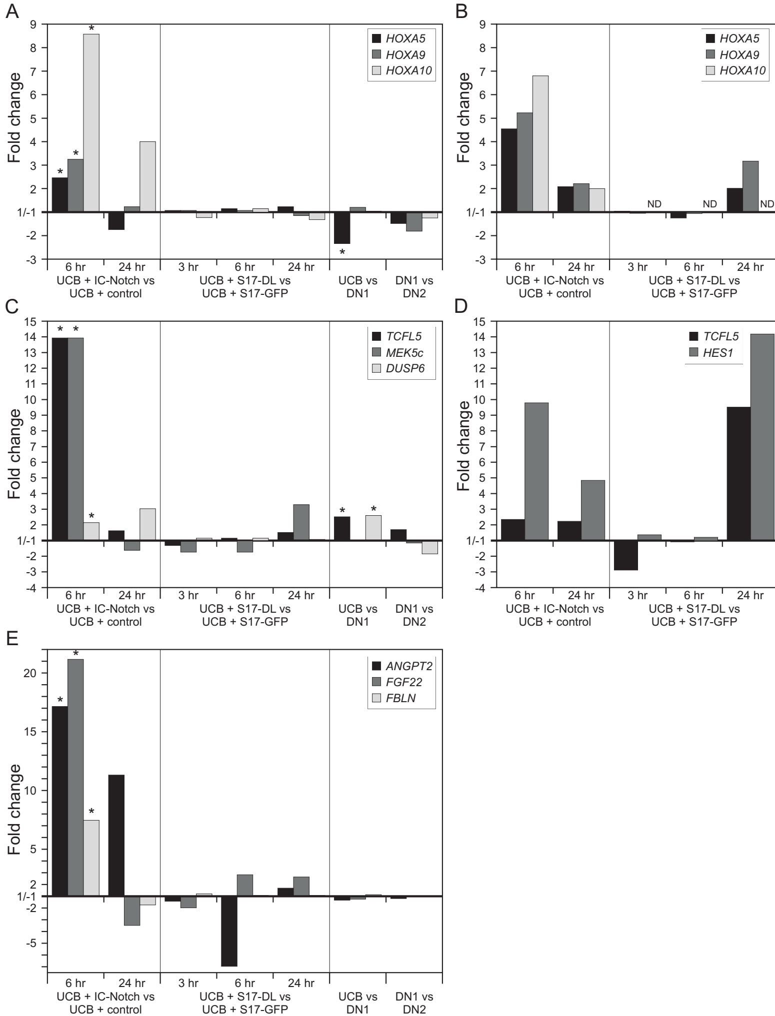

Figure 3. Differential expression of novel Notch target genes. (A) *HoxA* transcripts determined by microarray analysis as indicated for Figure 2. (B) *HoxA* transcripts determined by RQ-PCR. ND: not detected. (C) *TCFL5*, *MEK5c* and *Dusp6* transcripts determined by microarray analysis. (D) *Hes1* and *TCFL5* transcripts determined by RQ-PCR. (E) Transcription of angiogenesis genes determined by microarray analysis.

Next, we investigated differential expression of a number of transcription factors that are known to be important for T-cell development [1]. Tcf1 and GATA3 are among the first transcription factors to show up when a cell initiates a T-cell program [41], which we confirmed in our DN1 subset (Figure 2B). Although it has been suggested that Notch1 acts upstream of Tcf1 and GATA3 [27], we did not find induction of these genes after Notch signaling (Figure 2B).

Some reports have proposed that Notch inhibits B-cell development through inhibition of bHLH factor E2A [11,33], although E2A is clearly important for T-cell development as well, both as a homodimer and as dimerization partner for HEB [1]. In our experiments, we found no change in expression of either *E2A* or *HEB* after Notch signaling (Figure 2B).

Novel Notch target genes in hematopoietic progenitors

Next to the known Notch target genes *HES1* and *NOTCH1*, we discovered significant differential expression of a number of previously unknown Notch target genes. Six hours after transfection with IC-Notch, high induction of *HOXA5*, *HOXA9*, *HOXA10* and to a lesser extend *HOXA7* mRNA was detected (Figure 3A and B). Upregulation was markedly decreased after 24 hours. In S17-DL cultures, a small increase in HOXA transcripts could be detected after 24 hours by RQ-PCR but not by microarray analysis (Figure 3A and B). *HOXA5*, *A9* and *A10* were previously shown to be expressed in the human thymus [42], but we did not observe upregulation of these genes in DN1 and DN2 thymocytes (Figure 3A).

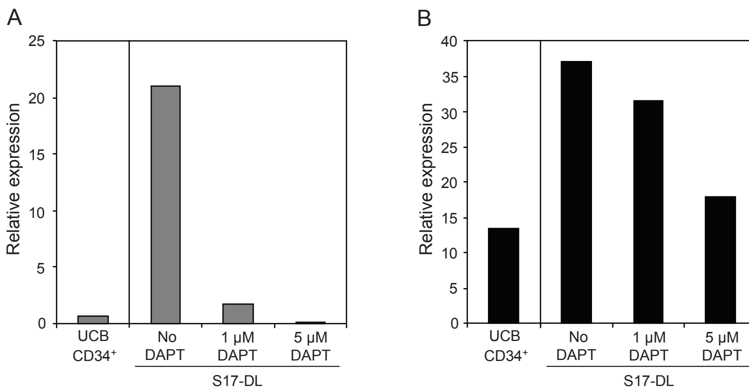

Figure 4. Pharmacological evidence for TCFL5 as Notch target gene. $CD34^+$ cells were cultured on S17-DL1 to induce Notch signaling, as evidenced by upregulation of Hes1 expression. Subsequently, cells were cultured in medium containing DAPT to inhibit Notch signaling. Expression of Hes-1 and TCFL5 were downregulated in a dose-dependent fashion Data shown are the average of duplicate measurements by RQ-PCR. Duplicate values always varied less than one Ct. Experiment shown is representative out of two done.

Among the highest upregulated genes 6 hours after IC-Notch transfection were *TCFL5* (Transcription Factor Like 5), *MEK5c* (Mitogen-activated protein kinase kinase) and *DUSP6* (Dual-specificity phosphatase 6, also termed MAP kinase phosphatase 3) (Figure 3C). TCFL5 is a transcription factor of the bHLH family, first identified in spermatocytes [43,44]. Both MEK5c and Dusp6 can influence MAP kinase activity and interactions between the Notch and MAP kinase pathways have been described [45]. Our microarray results showed limited upregulation of *MEK5c* and *TCFL5* transcripts after 24 hours of culturing on S17-DL, while for unknown reasons RQ-PCR validation of *TCFL5* showed high induction in this condition as well (Figure 3D). *TCFL5* and *DUSP6* were significantly increased in the DN1 thymocytes as compared to CD34$^+$ UCB (Figure 3C), supporting a functional role of these genes in T-cell differentiation.

Lastly, we found high upregulation of a number of genes associated with vasculogenesis, including *ANGPT2* (gene for angiopoietin), *FGF22* and *FBLN* (gene for fibulin) (Figure 3E). These genes were detected only after transfection with IC-Notch and not after co-culture on S17-DL or in thymocyte subsets, suggesting that their induction is not related to thymic function, but rather to other biological processes. These vasculogenesis genes may be physiologically induced by Notch ligands other than Delta1, for instance by Jagged. Interestingly, Notch signaling has been implicated in vasculogenesis and angiogenesis [46-48]. Furthermore, hemangioblasts (bipotent progenitors for endothelial and hematopoietic cells) are present among CD34$^+$ UCB cells [49]. This intriguing finding warrants further research, but is beyond the scope of this report.

Pharmacological inhibition of Notch signaling

Before starting on functional experiments to elucidate the role of TCFL5 in T cell development, we sought to obtain other experimental evidence that TCFL5 is a Notch target gene. For this, we have induced Notch signaling in CD34$^+$ uncommitted progenitor cells and subsequently inhibited Notch signaling with a γ-secretase inhibitor (DAPT). Direct addition of DAPT to cells cultured on S17-DL led to decreased Hes-1 and TCFL5 expression in some experiments, but this was inconsistent (data not shown) presumably due to the strong Notch signal delivered by S17-DLand the complex competition between activating (Delta) and inhibitory (DAPT) signals. Therefore we chose to first have cells undergo a Notch signal, followed by inhibition of that signal by DAPT. CD34$^+$ progenitor cells were cultured over night (16 hours) on S17-DL to induce Notch signaling. DAPT was added for the indicated periods of time, after which cells were harvested, RNA was isolated and used for RQ-PCR to determine levels of *GAPDH*, *TCFL5* and *HES1*. CD34$^+$ UCB cells from most donors do not express significant levels of *HES1* nor *TCFL5*, but after inducing Notch signaling *HES1* is abundantly expressed and *TCFL5* moderately. Both the levels of *HES1* and *TCFL5* were reduced by 50-70 % of controls (Figure 4), after inhibition of Notch signaling by the well-known Notch

inhibitor DAPT. Interestingly, both *HES1* and *TCFL5* decreased by incubation with DAPT. (Figure 4) in a dose-dependent fashion. This pharmacological approach therefore provides additional evidence that *TCFL5*, similar to *HES1*, is a true Notch target gene showing similar kinetics of induction and pharmacological inhibition, albeit with lower absolute expression.

Functional validation of novel Notch target genes *TCFL5* and *HOXA5*

Among the most striking novel Notch target genes identified in our studies were *TCFL5* and the *HOXA* genes. The relevance of these genes is substantiated by the finding of putative CSL binding sites upstream of *TCFL5* exon 1 and in the human *HOXA5-HOXA4* intergenic region (data not shown). Nevertheless, functional studies are necessary to understand the significance of TCFL5 and HoxA proteins for hematopoiesis and T-cell development in particular.

First we data-mined previously performed microarrays of all major thymocyte subsets [7] for the expression of *TCFL5*. We found a progressive increase in *TCFL5* mRNA from DN1 up to the early DP stage (Figure 5A). Levels sharply dropped when thymocytes started expressing CD3, suggesting that TCFL5 functions mainly during the early stages of T-cell development (Figure 5A).

To test whether TCFL5 is functionally important during T-cell development, we retrovirally expressed the *TCFL5* gene together with the GFP gene in human $CD34^+$ UCB hematopoietic progenitors. As a control, $CD34^+$ cells were transduced with the same construct containing only the *GFP* gene. After transduction, cells were cultured in fetal thymic organ cultures (FTOC) to allow them to differentiate into T cells. After 9 days in FTOC, thymic lobes were harvested and stained for flow-cytometric analysis. The TCFL5 transduced GFP^+ cells showed an acceleration of T-cell development: the percentage of DP cells was almost doubled as compared to control transduced GFP^+ and GFP^- cells and TCFL5 transduced GFP^- cells. (Figure 5B and data not shown). In addition, the percentage of $TCR\gamma\delta^+$ cells was markedly increased in the TCFL5 transduced GFP^+ cells (Figure 5C).

We found a remarkable increase in HoxA5, A9 and A10 in progenitor cells transfected with IC-Notch (Figure 4A), suggesting a role for these genes in hematopoiesis. Mice deficient for HoxA9 have previously been shown to display defects in myeloid, B-cell and T-cell development [50,51], while on the other hand overexpression of HoxA10 inhibited lymphoid development [52]. HoxA5 mutant mice were generated some years ago [53], but their hematopoietic system has not been studied. We now analyzed the bone marrow, spleen and thymus of HoxA5 homozygous, heterozygous and wild type littermates of 2.5, 4 and 25 weeks old. No consistent abnormalities were detected in populations of B, T, NK and myeloid cells in the thymus, bone marrow and spleen of HoxA5 deficient mice (data not shown). As the thymic defects of HoxA9 deficient mice were more pronounced in fetal mice than in adults, we also analyzed HoxA5

deficient embryos. We found normal T-cell development in fetal thymic lobes of HoxA5 deficient embryos and in FTOCs of HoxA5 deficient fetal liver cells (Figure 6A). Furthermore, no differences were found in numbers of Lin$^-$Sca-1$^+$c-Kit$^+$ cells between the fetal livers of HoxA5 deficient and normal embryos (Figure 6B), indicating that hematopoietic stem cells are normally present in these mice. Colony assays were performed to determine the frequency of erythroid and myeloid progenitors in HoxA5 deficient bone marrow: no abnormalities were

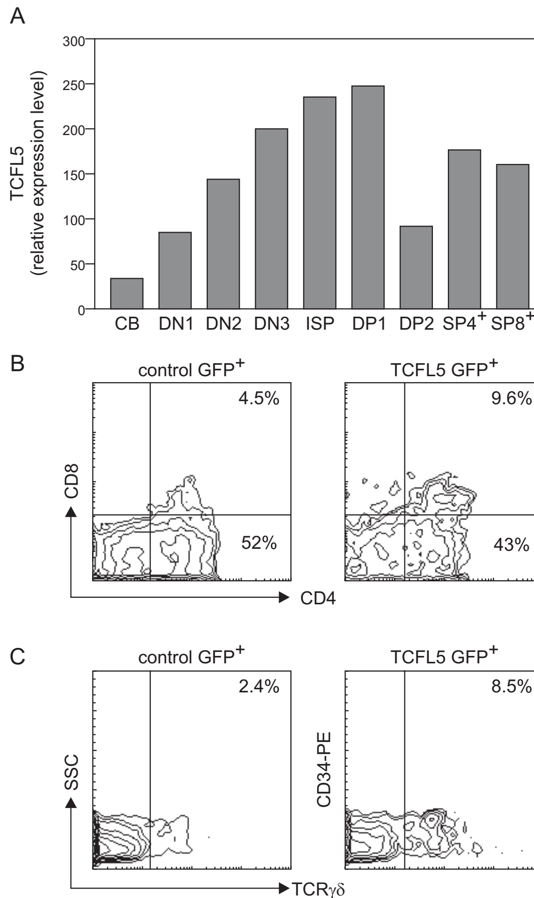

Figure 5. Functional validation of a role for TCFL5 in T-cell development. (A) Relative amounts of *TCFL5* mRNA in thymocyte subpopulations. Values extracted from previously performed gene expression profiles [7]. CB: CD34$^+$ UCB, DN1: CD34$^+$CD1a$^-$CD38$^-$, DN2: CD34$^+$CD1a$^-$CD38$^+$, DN3: CD34$^+$CD1a$^+$, ISP: CD4$^+$CD3$^-$, DP1: CD4$^+$CD8$^+$CD3$^-$, DP2: CD4$^+$CD8$^+$CD3$^+$, SP4+: CD4$^+$CD3$^+$, SP8+: CD8$^+$CD3$^+$. (B and C) Human CD34$^+$ UCB cells were retrovirally transduced with a vector containing only the gene for *GFP* (left panels) or *TCFL5* and *GFP* (right panels) and allowed to develop into T-cells in FTOC. After 9 days of culture, cells were harvested and examined by flow cytometry. (B) Expression of CD4 and CD8 within the GFP$^+$ gate. (B) Expression of TCRγδ within the GFP$^+$ gate.

detected (data not shown). To investigate whether HoxA5 deficient bone marrow progenitors can reconstitute the immune system under competitive conditions, bone marrow cells were transplanted into sublethally irradiated recipients. After five, eight and ten weeks, the percentage of donor cells in the blood was identical between mice transplanted with wild type or HoxA5 deficient bone marrow cells (Figure 6C). Furthermore, all hematopoietic lineages were normally present among donor cells in thymus, bone marrow and spleen of the recipients

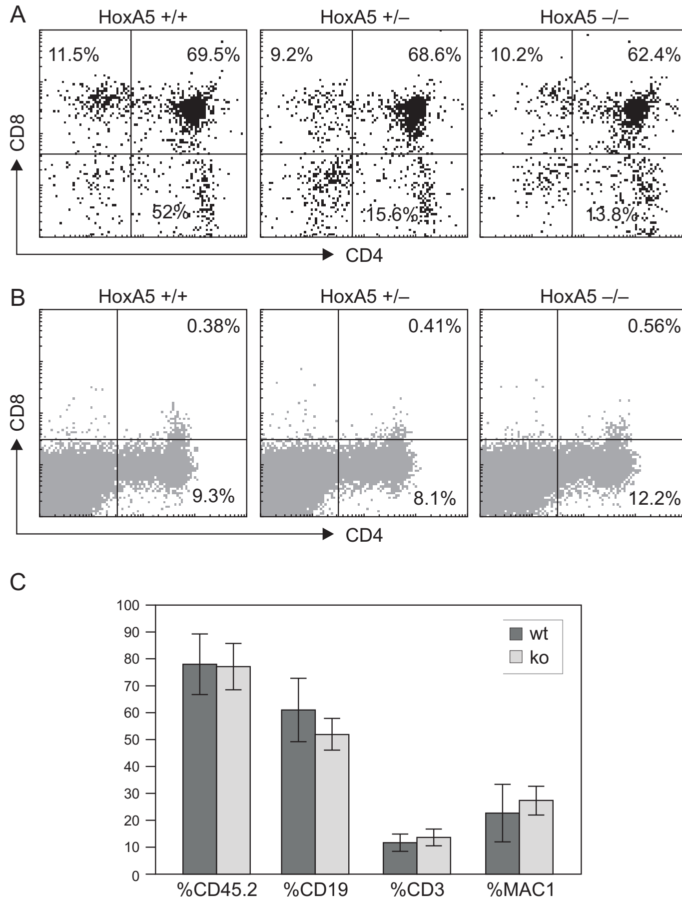

Figure 6. Hematopoiesis in HoxA5 deficient mice. (A) Thymic lobes of normal (left and middle panels) and HoxA5 deficient (right) embryos of day 14 of gestation (E14) were cultured on filters. After 16 days, T-cell development in the lobes was analysed using flow cytometry. (B) Hematopoietic progenitor populations were determined in fetal livers of normal (left and middle panels) and HoxA5 deficient (right) E12 embryos. Percentages of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells are shown in the upper right quadrant. (C) Sublethally irradiated mice were transplanted with wild type or HoxA5 deficient bone marrow cells. After ten weeks, the percentage of $CD45^{+}$ donor cells and the percentage of $CD19^{+}$ B cells, $CD3^{+}$ T cells, and $MAC1^{+}$ myeloid cells within a $CD45^{+}$ gate were determined in the blood of the recipients.

DISCUSSION

Despite the unmistakable role of Notch signaling for T-cell lineage specification, studies to identify downstream Notch target genes in relevant primary progenitors have not been undertaken.

In the present study we took advantage of the recently introduced AMAXA nucleofection system, which allows transient transfection of primary hematopoietic progenitor cells with a high efficiency and relatively low mortality. Using this technique, we were able to induce a rapid and strong Notch signal, as shown by high *HES1* expression after 6 hours. Furthermore, the S17-DL stromal cell co-culture system allowed us to more physiologically mimic the Notch signal encountered by progenitor cells that enter the thymus. We chose to use human $CD34^+$ UCB cells as source of progenitor cells, as these are readily available, unactivated, multipotent, and efficiently develop into T-cells when cultured in murine fetal thymic lobes or on bone marrow stroma expressing Delta1 [13,14,37]. Progenitor cells start expressing Notch target genes only after entering the thymus [6], which made them preferable over $CD34^+$ thymocytes which have already contacted Notch ligands. Using $CD34^+$ UCB cells and either IC-Notch transfection or the S17-DL co-culture system in combination with Affymetrix microarray technology, we found upregulation of the generally acknowledged Notch target genes *HES1*, *NOTCH1* and *NRARP,* which validated our procedures. The delayed upregulation of target genes in cells cultured on S17-DL as compared to transfection with IC-Notch indicates that cells need prolonged or multiple contacts with the Delta1 ligand to get accumulation of significant amounts of IC-Notch.

Our assays did not show induction of a number of genes previously reported to function as Notch targets during T-cell development, including *PTCRA*, *E2A* and *DTX1* [11,24,33]. This is especially critical for Deltex1, as many studies use expression of Deltex1 as the read-out for active Notch signaling, mostly in combination with Hes1 [14,54], but in some cases as sole evidence [55]. The main evidence for *PTCRA* and *DTX1* as Notch targets in T-cell development comes from studies by Deftos et al., in which IC-Notch was retrovirally transduced into a murine DP thymoma cell line [24,30]. Apart from the fact that Notch signaling and downstream events may be dysregulated in malignantly transformed cells, retroviral transduction takes several days and identified genes may represent indirect targets or may have resulted from compensatory mechanisms. Therefore Deltex expression may strongly depend on the context in which the Notch signal is given. Upregulation of genes identified in the thymoma line was confirmed in the thymi of mice transgenic for IC-Notch [24], but may again result from indirect activation. Nevertheless, it is possible that *PTCRA* is a Notch target gene in more differentiated T-cell precursors. This would fit with our finding that *PTCRA* is upregulated at the DN2 stage (Figure 2B) and with the fact that Notch signaling continues throughout the DN2 stage [40].

We did not find any evidence for direct induction of a T-cell program by Notch signaling. This is in contrast with a recent study by Höflinger et al., in which a Notch signal was induced by culturing multipotent progenitor cells on OP9-DL [27]. These cells rapidly upregulated *PTCRA*, *DTX1*, *GATA3* and *TCF1*, but not *HES1* transcripts. However, these results should be interpreted with caution, as this study used murine $Pax5^{-/-}$ pro-B cells expanded on the ST2 cell line as source of uncommitted progenitors: non-physiological cells that express many B cell specific genes and have already started TCR gene rearrangements (Vγ-Jγ, Vδ-Jδ and Dβ-Jβ) [27]. It remains however possible that sustained activation of Notch signaling (for at least several days), in addition to other signals and special microenvironments, is required to induce a T cell specific gene program. We cannot exclude that some of the target genes induced by Notch in turn can activate T cell specific genes (either alone or in concert with the right other signaling routes). In addition, some of the genes identified here may be indirect Notch targets. Our findings are consistent with experiments of Taghon et al. in which murine fetal liver progenitors were cultured on OP9-DL [54]. This study showed that *GATA3*, *TCF1* and *PTCRA* began to be expressed after three days of culture on OP9-DL, while high *HES1* transcription was detected already after one day. These findings indicate that either Notch signaling induces T-cell genes in more differentiated thymocytes, or Notch signaling stimulates the expression of other transcription factors, which then in turn activate or repress lineage differentiation genes. TCFL5 is a likely candidate for such a function.

TCFL5 is a member of the group B bHLH proteins and presumably binds to the same non-canonical E-box as Hes1 [43]. It was first found in spermatocytes, but was not detected in the thymus by Northern-blot analysis [43,44]. This might be explained by the fact that *TCFL5* levels decrease in the more mature stages of T-cell development (Figure 5A), which comprise the larger part of the thymus. Also during spermatogenesis, TCFL5 is expressed in a highly cell-type and stage-specific pattern [43,44].

By overexpressing TCFL5 in FTOC, we obtained initial evidence that TCFL5 positively regulates T-cell development. The observed effects were mild, presumably because TCFL5 is expressed already during the early stages of T-cell development (Figure 5A), and enhanced expression is not likely to induce remarkable changes. We have attempted loss of function studies using siRNA for TCFL5. These have not been successful in a setting (lentiviral vector) that allows sustained siRNA expression required in T cell developmental studies, while in transient assays downregulation of TCFL5 was readily obtained (data not shown). The gain–of-function experiments, however, give an indication that TCFL5 plays a functional role during T cell development in the thymus. Thus, TCFL5 may be negative regulatory bHLH factors that function in concert with or alternative to Hes1. The similar expression pattern during subsequent stages of thymic T cell development (not shown) further suggests such a role.

The finding of the *HOXA* cluster genes as Notch targets is novel and intriguing. Notch

induced *HOXA* transcripts may be important for T-cell development, as HoxA9 deficient mice have a partial block at the DN1 stage of thymocyte development [51]. Furthermore, overexpression of *HOXA* genes is observed in human T-ALL [56]. We did not find upregulation of *HOXA* genes in DN1 cells. Possibly, *HOXA* genes are induced only in a tiny subset of DN1 cells that have just contacted Delta, and are rapidly downregulated afterwards. However, we did not detect any defects in T-cell differentiation or hematopoiesis in general in HoxA5 deficient mice. The high functional redundancy of the different *HoxA* genes probably obscures the importance of HoxA5 for hematopoiesis. Other than a role in T-cell development, the Hox genes may be involved in alternative Notch-regulated processes in hematopoietic stem cells. Notch signaling is clearly important for self-renewal of hematopoietic progenitors (reviewed in [57]). Interestingly, *HOXA5*, *A9* and *A10* were found to be part of the 'stem cell profile' [58]. Without doubt, the relation between Notch signaling and expression of the HoxA cluster should be studied in greater detail.

The progenitor population we have used contains a very small population of apparently T cell committed $CD34^{+}CD7^{+}$ cells, as described by Haddad et al[59]. It is possible that activation of the Notch pathway leads to differentiation of $CD34^{+}CD7^{-}$ progenitors into $CD34^{+}CD7^{+}$ progenitors, as activation of Notch signaling by culturing on S17-DL1 leads to up regulation of CD7 (data not shown, but see also [60]). Alternatively, Notch signaling leads to selective outgrowth of the $CD45^{hi}CD34^{+}CD7^{+}$ cells described by Haddad et al, but this seems less likely, as the $CD34^{+}$CD7+ population expresses TCRB genes, whereas in our micro array experiments these were undetectable. Therefore, uncommitted $CD34^{+}$ cells (most likely $CD34^{+}CD38^{-}$ HSC) are induced to start T cell commitment, while losing myeloid potential, which is retained when cultured on S17-Jagged1 [60]).

The mechanism by which Notch signaling initiates a T-lineage differentiation program in hematopoietic progenitors remains a fascinating issue. Here we demonstrate that Notch does not directly activate a T-cell specific transcription program, nor does it directly induce TCR gene rearrangements. Apparently, other factors are needed besides Notch to induce T-cell commitment. Perhaps Wnt signaling, by delivering essential proliferative factors to immature thymocytes undergoing Notch signaling is important in this respect[61]. For sure, Hes1 executes part of the downstream Notch effects. In addition, TCFL5 and HoxA proteins may prove to be major player in Notch-regulated mechanisms during T-cell development. Furthermore, these novel Notch target genes may contribute to the pathogenesis of T-ALL with gain-of-function mutations in Notch1.

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Chapter 6

Discussion

A strict balance between self-renewal and differentiation of hematopoietic stem cells (HSCs) is required in order to maintain the homeostasis of the hematopoietic system, as well as to efficiently respond to stress situations like injury and infection. This balance is strictly regulated by signals from the specialized microenvironment or niche where HSCs reside, in the different blood forming organs throughout development.

It has been a long-standing challenge to mimic the niche signals HSCs receive, in order to expand and manipulate them for clinical purposes. The possibility of achieving these goals by stimulating HSCs with signaling ligands such as Wnt and Notch ligands is very attractive since this would constitute a much safer approach, comparing to methods requiring permanent genetic modifications. Indeed, initial studies showed that both Wnt and Notch ligands could efficiently promote expansion of the hematopoieitic progenitor compartment *in vitro* while maintaining an immature phenotype and/or retaining repopulation capacity [1-11]. However, further investigation to understand how the Wnt and Notch pathways regulate stem cell function failed to essentially implicate them in this process.

The findings described in this thesis provide new insights in how two different evolutionary conserved signaling transduction pathways, Wnt and Notch, regulate HSC self-renewal and differentiation. Importantly, this thesis provides evidence for an essential role of Wnt signaling in HSC function. It also shows that Wnt signaling regulates hematopoiesis in a dosage-dependent fashion, conciliating in this way several previous, contradictory findings.

Essential role of Wnt in HSC function

The role of several Wnt genes has been investigated by targeted mutations in the mouse, which lead to very specific developmental defects [12]. However, none of these Wnt proteins has been definitively implicated in HSC function. The striking morphological similarities between the Tcf1/Lef1 double deficient embryos and the Wnt3a deficient embryos [13,14] suggested that Wnt3a could play a non-redundant role in several biological processes during development [15,16]. Using a mouse model with a germline mutation specifically in the *Wnt3a* gene, we showed in Chapter 2 that Wnt signaling and more specifically Wnt3a is essential for self-renewal of fetal liver HSCs. Importantly, Wnt3a deficiency could not be compensated by any other Wnt protein expressed in fetal liver and resulted in the complete inhibition of the canonical Wnt signaling pathway, thereby confirming that Wnt3a plays a non-redundant role in the regulation of fetal liver HSC function. The fact that other Wnt proteins, also expressed in fetal liver, are not able to compensate for Wnt3a deficiency suggests that either only Wnt3a is present specifically in the niche or precise ligand-receptor interactions may be required. Evidence for an essential requirement of canonical Wnt signaling for adult HSCs was also obtained by studies from Fleming et al. In these studies a transgenic approach to over-express the Wnt inhibitor Dkk1 specifically in the osteoblastic niche showed, similarly

to what we present in Chapter 2, that in the absence of Wnt signaling HSCs loose long-term self-renewal capacity [17].

Wnt signaling has also been implicated in the regulation of constituent cells of the stem cell niche [18] and more specifically in maintaining osteogenic development [19-22]. Although in our results an indirect influence of Wnt3a on HSCs by affecting the niche cannot be excluded, Wnt reporter analysis demonstrated that HSCs are directly affected by Wnt3a deficiency. Moreover, given that Wnt3a is not expressed by the HSCs themselves, this environmentally determined deficiency turned into a cell-autonomous defect since these cells lost long-term reconstitution capacity of wild-type recipient mice, where Wnt3a is available [23]. This indicates that Wnt signaling deficiency permanently and irreversibly impairs the self-renewal capacity of HSCs (Chapter 2). The same was true for adult HSCs temporarily occupying a niche over-expressing Dkk1 [17]. Together these studies show that temporarily inhibition of the Wnt pathway during development or in the adult bone marrow niche irreversibly impairs HSC self-renewal. A possible explanation for this irreversibility may involve epigenetic modifications as a result of the absence of Wnt activation. Several epigenetic factors belonging to the polycomb complex, such as Rae28 and Bmi1 were shown to be essential for fetal and adult HSCs, respectively [24,25]. More specifically, Rae28 deficiency closely resembles the Wnt3a deficiency phenotype [25]. Additionally, several components of the Wnt signaling pathway are epigenetically silenced in leukemia leading to aberrant activation of the pathway [26,27]. It would be interesting to investigate whether epigenetic modifications could also result in permanent silencing of this pathway. Genome wide expression profiling on Wnt3a deficient HSCs will be essential to test this hypothesis and to unravel the molecular mechanisms underlying the self-renewal defect resulting from Wnt3a deficiency. Initial experiments to address this question have been undertaken.

The data presented in Chapter 2 show that maintenance of HSCs in fetal liver depends on Wnt3a availability. However, despite the fact that definitive HSCs are able to emerge in Wnt3a$^{-/-}$ embryos, since they can be found in fetal liver and repopulate primary recipient mice, a role for Wnt3a in the onset of definitive hematopoiesis was not ruled out. Importantly, Wnt3a is required for the formation of paraxial mesoderm in the gastrulating embryo [14] and Wnt3a deficiency results in the ectopic formation of neural tubes at the expense of mesoderm suggesting that Wnt3a regulates the balance between mesodermal and neural fates [28]. Given that the hematopoietic tissue has a mesodermal origin it would not be surprising if Wnt3a also plays an important role in the generation of definitive HSC in the aorta-gonads-mesonephros (AGM) region. Preliminary experiments suggest that HSCs in AGM and yolk sac at embryonic day 11.5 (E11.5) already display severely defective self-renewal capacity. Further experiments will be necessary to elucidate a possible role of Wnt3a in these processes. Defining the necessary factors for the emergence of HSCs during embryonic development

will be essential to instruct formation of multipotent hematopoietic tissue from embryonic stem (ES) cells or induced pluripotent stem cells (iPSCs)[29].

Although the studies we present in Chapter 2 together with the work by Fleming et al. essentially implicate canonical Wnt signaling in HSC function, previous loss-of-function studies with conditional inactivation of the key intermediate activator of the Wnt signaling pathway β-catenin, or both β-catenin and its homologue γ-catenin, did not show impaired stem cell function[30-32]. In one of these studies, Wnt reporter analysis showed remaining Wnt signaling activity after the combined inactivation of β-catenin and γ-catenin[31]. This suggested that the remaining residual Wnt activity was sufficient to sustain normal HSC function.

The unanticipated and non-redundant role of one specific Wnt protein points specifically to Wnt3a as the protein of choice to maintain and expand HSCs *ex vivo* for therapeutic applications. Although Wnt3a has already been shown to maintain stem cells properties in adult HSCs *in vitro*[3,4,33], it will be important to investigate whether it is also the physiologically relevant Wnt protein for adult HSCs and if, similarly to its function in fetal liver, it also has a non-redundant role in bone marrow HSC.

HSCs require mild levels of Wnt signaling activity

In Chapter 3, by making use of different transgenic mouse lines carrying different combinations of targeted mutations of the negative Wnt signaling regulator Apc, a gradient of Wnt signaling activation was generated[34] allowing the study of Wnt dosage dependent effects on HSCs. Strict limiting-dilution competitive transplantation assays demonstrated enhanced HSC activity with activation of this pathway but, importantly, HSCs only tolerate mild levels of Wnt signaling activation, approximately 2 fold higher than the normal physiological levels of Wnt signaling. Both intermediate and high levels of Wnt signaling activity resulted in HSC failure to repopulate recipient mice (Figure 1).

Several studies using different gain-of-function approaches retrieved contradictory results, showing either enhanced repopulation capacity or HSC failure. Of notice, previous studies reporting *in vivo* activation of this pathway by conditional stabilization of β-catenin in HSCs, which results in high Wnt activity, showed similar to what we present in Chapter 3, impaired reconstitution capacity due to exhaustion of the HSC pool and a block in multilineage differentiation[35,36]. However, other studies using retroviral vectors to express stabilized forms of β-catenin, reported enhanced self-renewal and repopulation capacity[4] or reacquisition of stem cell properties by lineage committed progenitors[37]. Although Wnt signaling activity was not measured in these two studies, the methods used are likely to lead to a lower Wnt activation as compared to the *in vivo* conditional β-catenin stabilization mouse models. Taking into account the findings we report in Chapter 3, different levels of activation of the pathway may explain the apparently contradictory results. Furthermore recent studies with Apc

haploinsufficiency [38], Apc$^{Min/+}$ [39] mice or a shRNA approach to knockdown Gsk-3β activity [40], which are expected to generate lower levels of Wnt activity showed enhanced HSC repopulation activity. Some of these reports describe reduced long-term reconstitution capacity, despite initial increased repopulation efficiency in primary recipients [38,40]. In our studies (Chapter 3) a mild Wnt activation also favored LT reconstitution in secondary recipients. These differences may be explained by slightly different levels achieved or alternatively, it cannot be excluded that a sustained activation of the pathway in all cells may have a harmful effect on HSCs. Indeed, Wnt reporter analysis showed that in all hematopoietic subsets analyzed only a fraction of the cells displayed reporter activity (Chapter 3) suggesting that the Wnt signaling pathway may be intermittently activated.

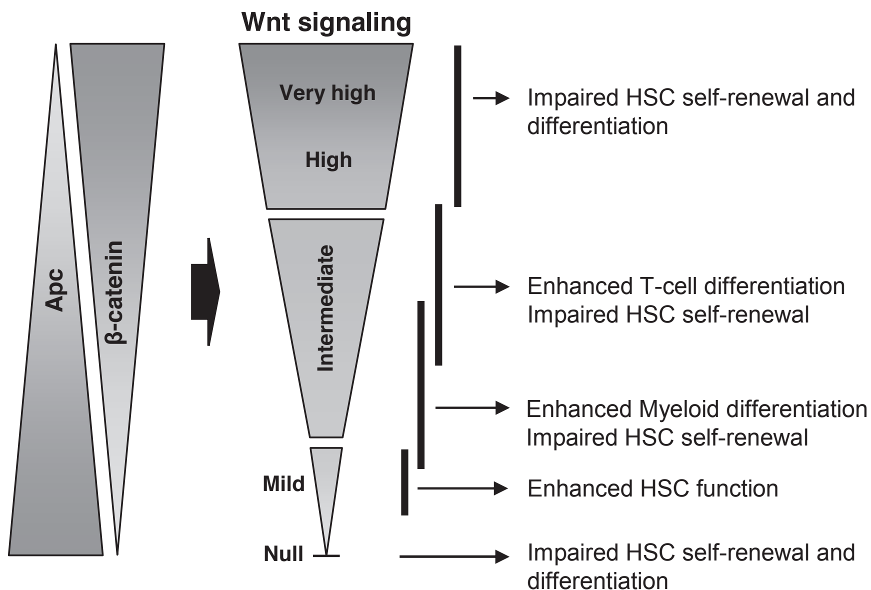

Figure 1. Canonical Wnt mediated regulation of HSC self-renewal and differentiation. There is a differential, lineage specific, optimum Wnt dosage in the hematopoietic system that differs between HSC, myeloid precursors and T lymphoid precursors. While HSCs specifically require mild levels of Wnt signaling activation, both mild and intermediate levels enhance myeloid differentiation. Early T-cell development is exclusively enhanced with intermediate levels of Wnt signaling activation. Both high and very high Wnt signaling activity impairs HSC self-renewal and differentiation through all hematopoietic lineages. The gradient of different levels of Wnt signaling activation was obtained by using different mouse models carrying distinct targeted hypomorphic mutations in the negative regulator of β-catenin, Apc (Chapter 3). The canonical Wnt signaling null situation was studied using a Wnt3a deficient mouse model (Chapter2).

Given that HSCs strictly require and can sustain only mild levels of Wnt activity (Figure 1), it is then to be expected that the remaining Wnt signaling still present in β-Catenin/γ-Catenin conditional deletion mouse models [31] is sufficient to support normal hematopoiesis. Interestingly, impaired HSC function was observed when deletion of β-catenin was mediated by *Vav-Cre* [41]. *Vav* mediated expression of *Cre* allows deletion not only in the hematopoietic system but also in the endothelium, starting already during embryonic development [42,43]. In light of the irreversibility of the HSC impairment resulting from Wnt inhibition (Chapter 2) it is very possible that the phenotype observed in these mice is a consequence of the inhibition of Wnt signaling in the embryo. The fact that β-catenin deletion impairs HSC function in the embryo but not in the adult suggests that fetal and adult HSCs may have different requirements of Wnt signaling strength. In this way, the remaining Wnt activity still present in this model after inactivation of β-catenin is than sufficient to sustain normal stem cell function in the adult where the required levels are low, but not in the embryo where a higher stem cells activity may involve higher levels of Wnt signaling activation. This explanation still requires experimental confirmation. Furthermore, β-catenin deletion was shown to impair *in vivo* the progression of Bcr-Abl-induced chronic myelogenous leukemia (CML) [41] and, of MLL-AF9 or HoxA9 and Meis1a induced acute myelogenous leukemia (AML) [44]. These leukemias are characterized by aberrant Wnt activation which may be related to the acquisition of stem cell properties by the leukemic stem cell (LSC). Therefore, similarly to the HSCs in the embryo, LSCs may have a higher requirement of Wnt activity comparing to adult HSC in bone marrow.

Wnt signaling regulates hematopoiesis in a dosage dependent fashion

Besides a role in the regulation of HSC function Wnt signaling has also been implicated in differentiation through the different hematopoietic lineages and more specifically in T-cell development, where its role was first described [45]. In line with the idea that fetal and leukemic stem cells may require higher Wnt activity than normal adult HSCs, the impaired T-cell development found with conditional deletion of β-catenin in the T-cell lineage [46] also suggests that thymocyte development requires higher levels of Wnt signaling. Supporting this idea, measurement of Wnt signaling activity with *in vivo* reporter assays (Chapter 3) showed a remarkable difference between bone marrow stem/progenitor cells and thymocytes (approximately 4 fold higher in thymocytes).

Using different combinations of targeted mutations in Apc in order to obtain a gradient of Wnt signaling activation, the Wnt dosage dependent effects in the differentiation potential of HSCs were also studied (Chapter 3). While HSC function was enhanced specifically with mild levels of Wnt signaling activity, only intermediate Wnt activation confers advantage to the early stages of T-cell development. High and very high levels of Wnt signaling activation (Chapter 3) similarly to stabilization of β-Catenin [47], result in accumulation of DN3 thymocytes

and in impaired *Tcrb* gene rearrangements (Figure 1). Despite the severe reduction in *Tcrb* gene rearrangements, Tcrβ$^-$ DP and SP cells could be detected in the thymus of these mice, although in reduced numbers, indicating that high Wnt signaling allows a bypass of the β-selection checkpoint. The reduced numbers of DP and SP thymocytes are probably due to the lack of proliferation and survival stimuli from a functional pre-TCR [47,48]. In contrast, an intermediate activation of the Wnt pathway enhanced early stages of T-cell development while preserving *Tcrb* rearrangements and maintaining developmental checkpoints. However the numbers of DP cells was still reduced which may indicate that later stages of T-cell development have different Wnt signaling requirements. In fact, Wnt signaling also regulates aspects of positive and negative selection and the DP to SP transition [49,50]. In agreement, in *in vitro* co-cultures with OP9 stromal cells expressing the Notch ligand Delta1, in which positive and negative selection processes are less stringent [51] an increase in both DP and SP cells with intermediate but not higher levels of Wnt activation was observed.

In agreement with a role of Wnt signaling in thymocyte development, Wnt3a deficiency also leads to the impairment of T-cell development. Despites an overall reduced cellularity of the thymic rudiment *in vitro* studies showed a developmental arrest in the ISP to DP transition (Chapter 2). The defect observed is due to lack of Wnt3a production by the thymic stroma since Wnt3a$^{-/-}$ progenitors develop normally in wild-type thymic lobes. This is in agreement with Wnt3a expression in the mouse thymus being restricted to the thymic epithelium [52]. Wnt signaling was also showed do be important for thymic stroma [53]. Although we cannot exclude that Wnt3a may play a role in the thymic stroma and only indirectly affects thymocyte development, the similarities with Tcf1 deficiency suggest that Wnt3a is directly regulating thymocytes development. A previous study with combined inactivation of Wnt1 and Wnt4 showed similarly to the Wnt3a$^{-/-}$ and Tcf1$^{-/-}$ mice, reduced thymic rudiment cellularity, although no changes in the differentiation pattern were observed [54]. The partially overlapping phenotype suggests the existence of high functional redundancy between these Wnt proteins in the thymus. In agreement with this, a much stronger phenotype, with complete absence of mature thymocytes was observed in the Tcf1/Lef1 double deficient mice [15]. This redundant role of Wnt3a for thymocytes is in contrast to its function in the regulation of the HSC compartment where it acts in a non-redundant fashion with other Wnt proteins. In addition, while in the thymus Wnt3a seems to have mainly a proliferative effect, in HSCs it influences self-renewal and differentiation cell-fate decisions (Chapter 2). Finally, HSCs and thymocytes require different levels of Wnt activation with the thymocytes displaying higher Wnt activity. Of interest, Wnt3a expression is much higher in fetal thymic lobes, in comparison to fetal liver (data not shown).

The studies with Wnt reporter mice (Chapter 3) showed that both early myeloid and B-cell progenitors display detectable levels of Wnt signaling activity, which is down-regulated as these cells differentiate. In agreement, Wnt3a deficiency affected hematopoietic progenitor

populations with multipotent progenitors (MPPs) and early myeloid progenitors being reduced in fetal liver, without affecting terminal myeloid differentiation. Importantly, common lymphoid progenitors as well as early B-cell progenitors were not affected suggesting a stronger dependency on Wnt3a for myeloid versus B-lymphoid development (Chapter 2). Although Wnt3a was shown to induce Pro-B-cell proliferation *in vitro* [23] it is possible that it is not the physiologically relevant Wnt in this process or other Wnts may act redundantly in early B cell development. Nevertheless, while myeloid development was favored with mild to intermediate levels of canonical Wnt activation, also at progenitor level, none of the Wnt activity dosages tested seemed to affect B-cell development (Chapter 3). This is also supported by the strict dependency on canonical Wnt signaling for the progression of CML, whereas precursor B-cell acute lymphoblastic leukemia (ALL) can develop independently of β-catenin [41] indicating that precursor B-ALL can develop independent of canonical Wnt signaling. Furthermore, Lef1 was shown to be essential for human granulocyte development [55] and its over-expression results in a skewing in differentiation towards the myeloid lineage at expense of lymphoid lineages in mice [56].

Although, the data we presented may suggest that Wnt signaling is not important for B-cell development, an essential role in this process cannot be excluded. Indeed, Lef1 deficiency was shown to result in reduced proliferation and survival of pro-B cells [23] and aberrant expression of this transcription factor was also observed in chronic lymphocytic leukemia (CLL) [56,57]. Importantly, the non-canonical Wnt5a was shown to inhibit B-cell development and to function as tumor suppressor in developing B-cells, probably by counteracting canonical Wnt signaling [58]. A more comprehensive analysis using an *in vivo* approach to achieve B-cell specific deletion of the conditional *Apc*^{15lox} allele will be essential to study Wnt signaling dosage dependent effects in B-cell development.

The studies presented in Chapters 2 and 3 provide compelling evidence for an essential and dosage-dependent regulation of hematopoiesis by Wnt. A differential optimum of Wnt signaling activation was observed in HSCs, myeloid development and early thymocytes, with HSCs having the lower requirements and early thymocytes having the highest ones. Importantly, very high Wnt signaling impaired both HSC self-renewal and differentiation through the different hematopoietic lineages. Canonical Wnt signaling has also been implicated in the self-renewal of other stem cell compartments in the gut, mammary gland, skin and ES cells. Notably, Wnt signaling influences the capacity of ES cells to differentiate into the three main germ layers: ectoderm, mesoderm and definitive endoderm, in a dosage-dependent fashion [34]. These Wnt dosage-dependent effects also seem to hold true for adult self-renewing tissues such as gut and skin, though the underlying cellular and molecular mechanisms still remain poorly understood [59,60]. In addition, different levels of activation of the pathway confer different degrees of tumor susceptibility in different tissues [61,62]. It will be important to study whether the different

hematopoietic malignancies where Wnt signaling is involved also have different and specific requirements of Wnt signaling strength. Finally as a specifically Wnt3a could be physiologically implicated in some of these processes, Wnt3a is the prime candidate for use in clinical applications.

Notch signaling in the regulation of HSC function and commitment to the T-cell lineage

Another signaling pathway extensively studied in hematopoiesis is the Notch signaling pathway. While Notch plays an essential function in commitment to the T-cell lineage and specification of marginal zone B-cells in the spleen, its role in HSC function is still controversial. In Chapter 4 we studied the consequences of deletion of the common downstream effector of all Notch receptors, the DNA binding factor *Rbpj*. Surprisingly, since *Rbpj* deletion is expected to block Notch signals from all Notch receptors, a significant increased in repopulation capacity by Rbpj deficient HSCs was observed. This is in contrast with a previous study, in which over-expression of a dominant-negative form of Rbpj that interacts with N^{ICD} but cannot bind DNA in HSCs, showed a strong reduction in repopulation capacity [63]. What can than explain the differences between these two different approaches to inactivate Rbpj? Rbpj works as a "molecular switch", alternating between a transcriptional repressor activity in the absence of Notch signaling and transcriptional activation activity upon interaction with N^{ICD}. Studies in *Drosophila* show that the phenotypes resulting from CSL depletion are not necessarily identical to loss-of-Notch function. While dominant-negative forms of Rbpj only inactivate the transcriptional activation function, complete deletion of this factor results in the impairment of both activation and repression activities [64,65]. Indeed, in some contexts losing the default repression of Notch target genes, in the absence of Notch signaling activation, may to some extent mimic the activation of this pathway [66]. Thus, similar to several Notch gain-of-function studies showing increased repopulation capacity of HSCs upon Notch signaling activation, Rbpj deletion may result in de-repression of Notch target genes, explaining the enhanced repopulation capacity observed in our studies. An increase in repopulation capacity upon *Rbpj* deletion was previously observed [67,68], however this dual role of Rbpj as regulator of Notch target genes transcriptional activity was not appreciated.

Loss of Rbpj default repression is not likely to result in a very high transcriptional activity due to the absence of co-activators, normally recruited upon activation of the Notch pathway [65,66.] Together with the results we presented in Chapter 4, this suggests that similarly to what we observed for the canonical Wnt signaling pathway, HSCs may require mild levels of Notch signaling activation. A recent study in which a dominant negative form of Mastermind-like1 (dnMamal) was over-expressed in hematopoietic stem cells showed no defective repopulation capacity while T-cell development was completely impaired [68]. However the activation status of the Notch signaling pathway was not analyzed. If a low activity of the

Notch pathway persists with this approach, it may be sufficient to allow normal HSC function. Furthermore, the capacity to differentiate into the T-cell lineage should not be used alone as read-out for the activation status of the Notch pathway since, similarly to what we observed for the Wnt signaling pathway, Notch may regulates hematopoiesis in a dosage dependent fashion. Indeed, the fact that T-cell development was impaired with Rbpj deletion suggest that thymocytes may require higher levels of Notch activity since the hypothetical de-repression of Notch target genes was not sufficient to rescue T-cell development in the thymus. Supporting these ideas, the higher expression of Notch ligands in the thymus, in comparison to the bone marrow microenvironments, may result in different signaling intensities. Furthermore, the Notch negative regulator lymphoma related factor (Lrf, formerly known as Pokemon) was shown to be essential to keep Notch signaling levels low in the bone marrow [69]. Down-modulation of Notch signals in hematopoietic progenitors probably has an essential role to prevent both ectopic development of T-cells and suppression of B-cell and myeloid development. The use of accurate Notch signaling reporter tools in combination with these loss-of-function approaches will be essential to unravel whether Notch plays an essential role in the regulation of HSC self-renewal. Furthermore, it will be important to perform genome-wide gene expression analysis to investigate whether Rbpj deletion indeed leads to de-repression of genes important in self-renewal. Although Notch signaling was shown to be active in HSCs [63], its target genes are still unknown. Although we could not yet essentially implicate Notch in the HSC self-renewal, one can hypothesize that such a “dangerous” signaling pathway, which leads to leukemia when deregulated [70], would have been negatively selected during evolution if it does not play an essential role in HSC function.

Despite the fact that it is well established that Notch signaling is required to promote a T-cell fate on hematopoietic progenitors migrating from the bone marrow, the mechanisms by which Notch induces a T-cell program are still elusive. As soon as thymic emigrants enter the thymus they receive Notch signals. During T-cell development Notch signaling activity increases till DN3 stage and is subsequently down-regulated after β-selection [71]. In Chapter 5, the target genes of Notch signaling in human hematopoietic progenitor/stem cells were investigated. Surprisingly, although *Hes1* which was shown to be essential for early stages of T-cell development [72] was quickly upregulated, no induction of T-cell specific genes and transcription factors was observed up to 24 hours upon Notch activation. This suggested that Notch is not directly inducing T-cell commitment in early thymic progenitors. In agreement with this, work from Taghon et al., reached similar conclusions [73] using mouse hematopoietic progenitors. Indeed, this study shows that Notch triggers a synchronized and delayed cascade of Notch responses that reversibly specify progenitors to the T-cell lineage. In this way, although indispensable, Notch does not seem to be sufficient to induce commitment to the T-cell lineage. In addition, in Chapter 5, novel factors, whose expression is quickly induced after Notch

signaling in uncommitted progenitors, were identified. These include genes from the *HoxA* cluster and *Tcfl5*. Interestingly, Tcfl5 is expressed in thymocytes with a pattern similar to *Hes1*. Furthermore, *Tcfl5* overexpression enhanced thymocyte differentiation in fetal thymic organ cultures. Further gain-of-function as well as loss-of-function experiments will be necessary to elucidate the role of this bHLH transcription factor in early T-cell development.

Integration of Wnt and Notch signaling in hematopoiesis

Wnt and Notch signaling pathways share very similar basic functional properties despite the use of different biochemical mechanism. Both β-catenin and N^{ICD} are not capable of directly activate expression of target genes, but rather require cooperative function of transcriptional co-activators, which also confer cell-type specificity. Moreover, in the absence of signaling activity both downstream transcription factors Tcf/Lef and Rbpj actively repress the induction of their target genes to avoid signaling-independent expression [66]. The results presented in this thesis indeed suggest that they regulate hematopoiesis using similar mechanisms. Here we demonstrated that Wnt plays an essential role in the regulation of HSC function and other aspects of hematopoiesis, in a dosage-dependent fashion. Furthermore we raise the intriguing possibility that, similarly to Wnt, Notch signaling activity levels differentially regulate hematopoiesis. Importantly, the results suggest that for both pathways signaling levels in the thymus and developing thymocytes are noticeably higher in comparison to HSCs in the BM. Of interest, the existence of a specialized organ devoted exclusively to T-cell development probably allowed such differences in signaling activity to be used in the regulation of hematopoiesis. Indeed, both Wnt and Notch ligands are much more abundant in the thymus than in BM [52,69].

Despites the regulation of hematopoiesis by means of signal levels, timing and duration, the outcome of such signals has also been shown to be critically determined by the cellular context in which these signals are received. The differences in cellular context may partially be explained by other signaling pathways activated at the same time. Indeed Wnt and Notch, as well as other signaling pathways such as Hedgehog, were previously shown to synergistically or antagonistically regulate each other [63,74-76]. Importantly, in Chapter 3 we show that Wnt signaling has a modulating effect on Notch activity. Increasing levels of Wnt signaling resulted in decreased induction of the Notch target gene *Hes1*, upon Notch activation on hematopoietic stem/progenitor cells. However, the molecular and/or biochemical mechanisms underlying these processes are still poorly understood. Gsk-3β is a potential candidate to mediate this crosstalk since it regulates the stability/degradation of both β-catenin and N^{ICD} [77,78] and its inhibition affects HSC function through mechanisms involving regulation of both Wnt and Notch target genes [79]. Additionally, *Jagged1* is a Wnt/β-catenin target gene in other systems [80], implying that a cross-talk involving these pathways may occur between neighboring cells.

The use of various transgenic and knock-out mouse models for components of these signaling pathways has evolved as a valuable tool to understand the *in vivo* functions of Wnt and Notch signaling. However, a critical interpretation of the different models and results will be essential to fully understand the role of these pathways. The high complexity underlying these processes, low levels of signaling activity required, and previously unexpected levels of regulation, may explain the controversies that have been masking an essential role for these signaling pathways in hematopoiesis. The work described in this thesis contributes to solve some of these controversies. Whether Notch plays an essential role in hematopoiesis still has to be fully addressed and, it will be essential to identify the target genes of both these pathways and their specific function in hematopoiesis.

HSC self-renewal, specification, commitment and differentiation are complex processes regulated by intricated networks of signals and transcription factors that have to be tightly orchestrated and "fine tuned" to correctly drive these processes. Changes in the delicate balances between these factors may lead to leukemia, immunodeficiencies or autoimmunity. Thus, unraveling these mechanisms will be essential in order to fully explore the potential of HSC and translate this basic knowledge into clinical applications. How to deal with such high complexity will be one of the major challenges in the near future.

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Summary
Samenvatting

Summary

Hematopoietic stem cells (HSCs) are characterized by their capacity to differentiate into all blood lineages and to self-renew, thereby sustaining blood cell production throughout life. These properties are strictly regulated by signals from the specialized microenvironment or niche where HSCs reside. In this way, defining the correct microenvironmental signals that support HSC function has been a major goal in stem cell research in order to increase clinical applications for the treatment of diseases such as hematological malignancies, primary immunodeficiencies and autoimmune disorders. The studies in this thesis are aimed at understanding the role of two key signaling pathways involved in the regulation of HSC self-renewal and differentiation., namely Wnt and Notch.

The role of the Wnt signaling pathway in the regulation of HSC function was studied using different approaches to either inactivate (Chapter 2) or activate (Chapter 3) this pathway. In Chapter 2 a loss-of-function study based on the analysis of a mouse deficient for Wnt3a showed that loss of Wnt3a leads to reduced numbers of HSCs and multipotent progenitors in fetal liver and to severely reduced long-term reconstitution capacity as observed in serial transplantation assays. Importantly, Wnt3a was shown to non-redundantly regulate fetal liver HSCs suggesting that Wnt3a may be the protein of choice to expand HSCs ex vivo for stem cell-based therapies. In addition, Wnt3a was shown to differentially regulate myeloid progenitors and B-cell progenitors. The studies on the role of Wnt signaling in HSC function were further extended in Chapter 3 by using different mouse models carrying hypomorphic mutations in Apc, which acts as a negative regulator of the Wnt signaling pathway. Using this genetic tool, a gradient of Wnt signaling activation was obtained allowing the study of dosage dependent effects of Wnt signaling activation on HSCs and hematopoietic differentiation. Wnt signaling was shown to regulate hematopoiesis in a dosage-dependent fashion with different, lineage-specific, Wnt dosages regulating HSCs, myeloid precursors and T lymphoid precursors.

Chapter 4 and 5 describe the role of Notch signaling pathway in HSC self-renewal and differentiation with particular emphasis on the induction of a T-cell lineage program. In Chapter 4 the role of Notch signaling in HSC function was studied using a conditional deletion approach to inactivate the common downstream factor to all Notch receptors, the DNA binding protein Rbpj. Deletion of Rbpj enhances the repopulation capacity of HSC while impairing T-cell development, suggesting an important role for the repressor function of Rbpj in the regulation of HSC function. The results may suggest that similarly to the Wnt signaling pathway, also Notch signaling may regulate hematopoiesis in a dosage-dependent fashion. In Chapter 5 a genome-wide gene expression profiling approach was used to identify direct target genes of the Notch signaling pathway, involved in the induction of a specific T-cell lineage program on hematopoietic stem and progenitor cells. Apparently Notch signaling does not directly induce

a T-cell lineage program. Different novel Notch target genes, such as TCFL5 were identified by this approach.

Finally the results of the different chapters are integrated in Chapter 6. The fine tuning of an intricated network of signals and transcription factors is essential to efficiently drive normal hematopoietic development. Defining which signals are important and how they are orchestrated will be critical to fully explore the potential of HSC and translate this basic knowledge into clinical applications.

Samenvatting

Bloedvormende stamcellen (ook wel hematopoietische stamcellen, HSC genoemd) worden gekenmerkt door het vermogen om te kunnen differentiëren in alle typen bloed cellen en door het vermogen tot zelfvernieuwing, waardoor de bloedproductie het hele leven door kan gaan. Deze eigenschappen worden strikt gereguleerd door signalen uit de micro-omgeving of niche waar de HSC zich bevinden. Daarom is het uitzoeken van de juiste signalen uit de micro-omgeving een belangrijk doel in stamcel onderzoek ten behoeve van klinische toepassingen bij hematologische maligniteiten, primaire immuundeficiënties en auto-immuunziekten. Het doel van dit proefschrift is de rol te begrijpen van twee signaaloverdrachtroutes, Wnt en Notch, in de regulatie van zelfvernieuwing en differentiatie van HSC.

De rol van Wnt signalering in HSC is bestudeerd door gebruik te maken van technieken die de route of inactiveren (hoofdstuk 2) of juist activeren (hoofdstuk 3). In hoofdstuk 2 laat een verlies-van-functie onderzoek gebaseerd op een Wnt3a deficiënte muis zien, dat het verlies van Wnt3a leidt tot verminderde aantallen HSC en voorloper cellen in de foetale lever en tot sterk verminderde langetermijns reconstitutie na seriële transplantatie. Bovendien is Wnt3a het enige eiwit dat foetale lever HSC reguleert, hetgeen suggereert dat Wnt3a het beste Wnt eiwit is voor ex vivo vermeerdering van HSC. Verder reguleert Wnt3a op verschillende wijzen myeloide en B lymfocitaire voorloper cellen. Het onderzoek naar Wnt signalering in HSC werd voortgezet in hoofdstuk 3 waarin verschillende hypomorfe mutanten van het Apc gen, een negatieve regulator van de Wnt route, werden gebruikt. Op deze manier werd een gradiënt in Wnt signaal sterkte verkregen, zodat dosis- afhankelijke effecten van Wnt signalering op HSCs konden worden bestudeerd. Wnt signalering reguleert bloedvorming op een dosis afhankelijke manier waarbij er per celtype (HSC, myeloide cel, T cel) een specifiek optimaal signaal bestaat.

Hoofdstuk 4 en 5 beschrijven de rol van de Notch route in HSC zelfvernieuwing en differentiatie, met speciale aandacht voor het aanzetten van een T cel specifiek genprogramma. In hoofdstuk 4 werd de rol van Notch in HSC bestudeerd door een conditionele deletie van het DNA bindende eiwit Rbpj, de gemeenschappelijke transcriptie factor waar alle typen Notch signalen op uit komen. Deletie van Rbpj verhoogt reeconstitutie door HSC, maar vermindert de T cel ontwikkeling, hetgeen suggereert dat met name de repressor functie van Rbpj belangrijk is in regulatie van HSC functie. Deze resultaten suggereren dat, net als de Wnt route, ook de Notch route HSCs reguleert op en dosis afhankelijke manier.

In hoofdstuk 5 werden m.b.v. een genoom-brede expressie analyse, de doelwit genen geïdentificeerd die betrokken zijn bij de inductie van een T cel specifiek genprogrmma in HSC en voorloper cellen. Het blijkt dat Notch signalering niet direct een T cel specifiek programma aanzet. Wel werden diverse nieuwe Notch doelwit genen, zoals TCFL5, gevonden.

Tenslotte werden de bevindingen van de verschillende hoofdstukken samengebracht in hoofdstuk 6. Het “fine-tunen” van een gecompliceerd netwerk van signalen en transcriptiefactoren is essentieel voor normale hematopoiese. Het bepalen welke signalen belangrijk zijn en hoe ze samenwerken is cruciaal om de mogelijkheden van HSC volledig te benutten en deze kennis te vertalen naar klinische toepassingen.

Abreviations

AGM	Aorta-gonads-mesonephros
ALL	Acute lymphoblastic leukemia
APC	Adenomatous polyposis coli
BM	Bone marrow
CD	Cluster of differentiation
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
E	Day of embryonic development
ETP	Early thymic progenitor
FACS	Fluorescence activated cell sorting
FDG	fluorescein di-β-D-galactopyranoside
FL	Fetal liver
FLT3L	FMS-related tyrosine kinase 3 ligand
FTOC	Fetal thymiic organ culture
GFP	Green fluorescent protein
HSC	Hematopoietic stem cell
IL	Interleukin
ISP	Immature single positive
Lin	Lineage
LSK	Lin^- $Sca1^+$ $c\text{-}Kit^+$
LT	Long-term
MEP	Megakaryocyte-erythrocyte progenitor
MPP	Multipotent progenitor
NK	Natural killer
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RQ-PCR	Real-time quantitative polymerase chain reaction
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SP	Single positive
ST	Short-term
TCR	T-cell receptor
TPO	Thrombopoietin
UCB	Umbilical cord blood
WT	Wild-type

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To all of you “Obrigado”

Tiago

About the Author

Personal details

Name	Tiago Cunha Luis
Age / Birth date	29 / 14 Jun 1981
Nationality	Portuguese

Education

Sep 2000 - Oct 2005	Licenciature (5 years degree) in Microbiology and Genetics Faculty of Sciences, University of Lisbon, Portugal.
Sep 1999 - Sep 2000	First year of the Licenciature in Chemistry Faculty of Sciences, University of Lisbon, Portugal.

Research activity

Nov 2005 - Nov 2010	PhD student at Department of Immunology, Erasmus University Medical Center (Erasmus MC), Rotterdam, The Netherlands. After February 2009 part of the research was performed at Department of Immunohematology and Blood Transfusion, Leiden University Medical Center (LUMC), Leiden, The Netherlands.
Sep 2004 - Oct 2005	Undergraduate student at Hematopoiesis unit, Instituto de Medicina Molecular (IMM), Faculty of Medicine, University of Lisbon, Portugal & Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal. Scientific supervisor: Prof.dr. Leonor Parreira.
Sep 2001 - Nov 2002	Undergraduate student at Department of Plant Biology, Faculty of Sciences, University of Lisbon, Portugal. Scientific supervisor: Prof. dr. Ana Cristina Figueiredo.

Publications

1. **Luis TC**, Naber BA, de Haas EF, Ghazvini M, Fibbe WE, van Dongen JJ, Fodde R, Staal FJ. Canonical Wnt signaling regulates hematopoiesis in a dosage-dependent fashion. **Under review**.

2. **Luis TC**, Naber BA, Fibbe WE, van Dongen JJ, Staal FJ. Wnt3a non-redundantly controls hematopoietic stem cell function and its deficiency results in complete absence of canonical Wnt signaling. **Blood**, 2010 Jul 22;116:496-7.

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8. Ferreira N. J., I. G. Meireles de Sousa, **T. Cunha Luís**, A. J. M. Currais, A. C. Figueiredo, M. M. Costa, A. S. B. Lima, P. A. G. Santos, J. G. Barroso, L. G. Pedro, J. J. C. Scheffer Pittosporum undulatum Vent. grown in mainland Portugal: secretory structures, seasonal variation and enantiomeric composition of the essential oil. **Flavor and Fragrance Journal**. 2007

PhD portfolio summary

Name PhD student:	Tiago Cunha Luis
Erasmus MC Department:	Immunology
Research School:	Molecular Medicine
PhD period:	November 2005 to November 2010
Promoter(s):	Prof.dr. Jacques J.M. van Dongen & Prof.dr. Frank J.T. Staal
Supervisor:	Prof.dr. Frank J.T. Staal

PhD training	**Year**
In-depth courses	
- 5th International Workshop on Innovative Mouse Models (Leiden).	2009
- Basic Data Analysis on Gene Expression Arrays Workshop.	2009
- Browsing Genes and Genomes with Ensemble – Bioinformatics workshop.	2008
- In Vivo Imaging Course: From molecule to Organism.	2007
- Introduction to Confocal Microscopy.	2007
- 4th International Workshop on Innovative Mouse Models (Leiden).	2007
- Course of Laboratory Animal Science (Article 9, Equivalence to FELASA C; Utrecht)	2006
- Molecular Immunology Course.	2006
National conferences	
- 4th Congress of the Dutch Society of Hematology (NVVH), Arnhem. *Oral presentation.*	2010
- 2nd Dutch Stem Cell meeting, Rotterdam.	2009
- 13rd Molecular Medicine Day, Rotterdam.	2009
- 2nd Congress of the Dutch Society of Hematology (NVVH), Arnhem. *Oral presentation.*	2008
- 12nd Molecular Medicine Day, Rotterdam. *Poster presentation.*	2008
- Mini-Symposium: Biology of Hematopoietic Stem cells, Rotterdam. *Oral Presentation.*	2007
- 11th Molecular Medicine Day, Rotterdam. Oral presentation.	2007
- International Symposium on Health and Evolution, Rotterdam.	2006
- 10th Molecular Medicine Day, Rotterdam.	2006

International conferences

- 39th Meeting of the International Society of Experimental Hematology (ISEH), Melbourne, Australia. *Oral presentation*. 2010
- 3rd International Symposium on Stem Cells, in Development and Regulation. Amsterdam, The Netherlands. *Poster presentation*. 2009
- 38th Meeting of the International Society of Experimental Hematology (ISEH), Athens, Greece. *Poster presentation* 2009
- 2nd International Symposium on Stem Cells, in Development and Regulation. Amsterdam, The Netherlands. 2008
- 4th International Conference on Gene Regulation in Lymphocyte Development (Aegean Conferences), Rhodos - Greece. *Oral presentation*. 2008
- Gene Expression and Signaling in the Immune System (Cold Spring Harbor meeting), Cold Spring Harbor, New York, USA. *Poster presentation*. 2008
- 36th Meeting of the International Society of Experimental Hematology (ISEH), Hamburg, Germany. *Oral presentation*. 2007
- 3rd International Conference on Gene Regulation in Lymphocyte Development (Aegean Conferences), Corfu - Greece. *Poster presentation*. 2006

Fellowships and Individual awards

- 1st Price, Young Investigator Awards at the 39th Meeting of the International Society of Experimental Hematology (ISEH), Melbourne, Australia. 2010
- Travel award. 39th Meeting of the International Society of Experimental Hematology (ISEH), Melbourne, Australia. 2010
- Best Poster Presentation. Annual meeting of the Molecular Medicine Postgratuate School – Erasmus MC, Rotterdam, The Netherlands. 2008
- PhD Fellowship (4 years). Attributed by “Fundação para a Ciência e a Tecnologia” (FCT), Lisbon, Portugal. 2006

Supervised students

- Jennifer Veth - Jan 2008 - May 2008; Project title: “Wnt dosage effects in hematopoiesis”. 2008

Appendix

Color figures

Color figures

Chapter 2.1

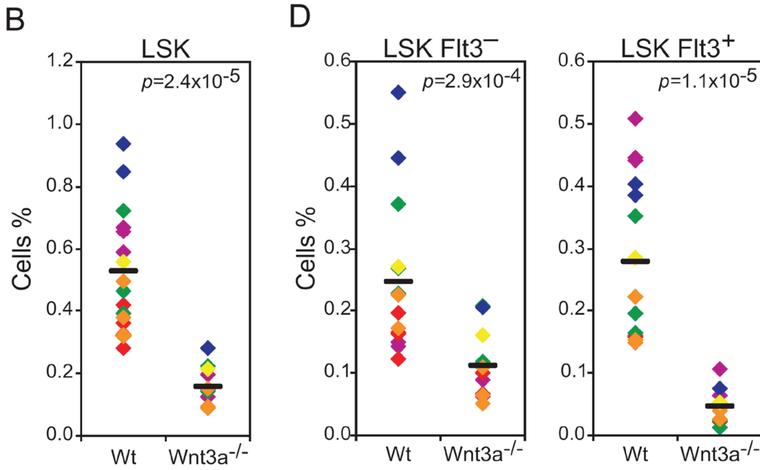

Figure 1. Wnt3a deficiency leads to a severe reduction in HSC (LSK Flt3-) and multipotent progenitor (MPP, LSK Flt3$^+$) numbers. (**B**) Flow cytometry analysis of LSK (Lin$^-$ Sca1$^+$ c-Kit$^+$) population in Wt and *Wnt3a$^{-/-}$* FLs from E12.5 embryos. Percentage of LSK cells in total fetal liver (FL) is strongly decreased. Data are from 16 Wt and 11 *Wnt3a$^{-/-}$* FLs, belonging to 6 different litters identified by different colors. (**D**) Flow cytometry analysis of the Flt3 subsets in the LSK compartment. Frequency of HSC (LSK Flt3$^-$) and multipotent progenitor (MPP, LSK Flt3$^+$) cells in total FL. Data are from 16 Wt and 11 *Wnt3a$^{-/-}$* FLs, belonging to 6 different litters identified by different colors. Mean values are presented as a dash. "*p*" values are indicated in all graphs.

Chapter 2.1

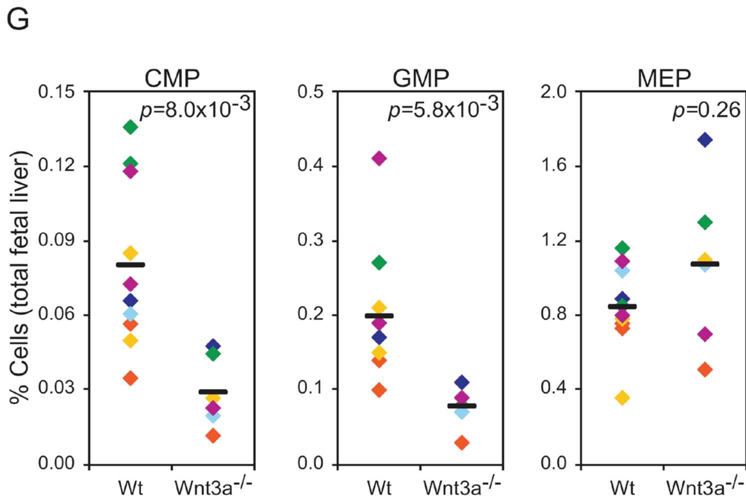

Figure 4. Wnt3a deficiency affects myeloid progenitors without impairing terminal differentiation. (**G**) Flow cytometry analysis of myeloid progenitor subsets in the FL of Wt and *Wnt3a*$^{-/-}$ embryos. Frequency of CMPs, GMPs and MEPs in total FL are depicted. The averages are indicated by a dash. Data from 11 Wt and 6 *Wnt3a*$^{-/-}$ FLs, belonging to 6 different litters identified by different colors. "*p*" values are indicated.

Chapter 2.1

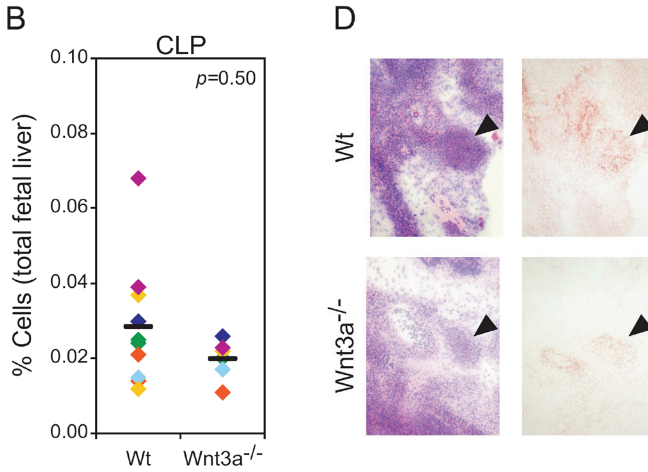

Figure 5. Lymphoid potential of Wnt3a-/- hematopoietic progenitors. (**B**) Flow cytometry analysis of common lymphocyte progenitors (CLPs). Percentage of CLPs in total FLs from Wt and *Wnt3a*$^{-/-}$ embryos. The averages are indicated by a dash. Data from 11 Wt and 6 Wnt3a-/- FLs, belonging to 6 different litters identified by different colors. (**D**) Reduced cellularity in *Wnt3a*$^{-/-}$ thymic anlage revealed by immunohistochemistry analysis. Tissue sections of E12.5 embryos were stained with HE to visualize different tissues (left panels). Thymic epithelial cells stained with the thymic stroma antibody ER-TR4 (right panels) to confirm identification of thymic anlage in *Wnt3a*$^{-/-}$ embryos. Staining with an isotype control for ER-TR4 was completely blank. Thymic anlage is indicated by an arrow head. Stainings shown are representative of four Wt and four Wnt3a$^{-/-}$ embryos examined.

Chapter 2.3

Figure 1. Wnt3a deficient embryos at 12.5 day of embryonic development. Wnt3a$^{-/-}$ embryos have a severe phenotype with little or no caudal development posterior to forelimbs, disrupted notochord, and abnormal formation of ectopic neural tubes, the so called vestigial tail. Bar represents 1 mm.