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THE ROLE OF BMP SIGNALING AND ENDOGLIN IN REGULATION OF HEMATOPOIESIS

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THE ROLE OF BMP SIGNALING AND ENDOGLIN IN REGULATION OF HEMATOPOIESIS

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Abstract

Hematopoiesis, the formation of blood cells, ultimately depends on a rare population of hematopoietic stem cells (HSCs), which can both self-renew to maintain the HSC pool, and differentiate into all mature blood lineages. HSC fate decisions are governed by a complex combination of numerous signals, although the molecular mechanisms are not fully understood. This thesis describes the role of two important regulatory candidates, Bone Morphogenetic Proteins (BMP) and Endoglin in adult hematopoiesis. Several publications have described the importance of BMP signaling in specification of hematopoietic tissue in developing embryos. In Article I and II, we investigate the full role of BMP signaling in adult mice, using single and double Cre-based conditional knockout mice for the BMP signaling mediators Smad1 and Smad5. We demonstrate that BMP signaling deficient bone marrow (BM) competes normally with wild-type cells and display unperturbed self-renewal and differentiation capacity when transplanted into lethally irradiated recipients. Thus, despite its crucial role in initial patterning of hematopoiesis, BMP signaling is not required to maintain adult hematopoiesis. Endoglin is a TGF-β accessory receptor recently demonstrated to identify functional long-term HSCs, although little is known regarding its function in these cells. We have investigated the role of endoglin in hematopoiesis by both knocking down, and over-expressing its levels using viral vectors. Interestingly, our study suggests a pivotal role for endoglin in adult erythropoietic development. However, transplantation of transduced BM stem and progenitor cells reveals that neither endoglin suppression nor over-expression affects the ability of HSCs to long-term repopulate recipient marrow.

Key words: Smad-signaling, BMP, Endoglin, Hematopoiesis, Hematopoietic Stem Cell, Conditional Knockout, Viral-mediated gene expression

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Wisdom is humble that he knows no more"
William Cowper

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Article I **Smad5 is dispensable for adult murine hematopoiesis.** Singbrant S, Moody J.L, Blank U, Karlsson G, Zwijsen A, Umans L, Karlsson S. *Blood* 108(12): 3707-12 (2006)

Article II **BMP signaling is essential to preserve colon architecture, but is dispensable for hematopoietic stem cell function in adult mice.** Singbrant S, Karlsson G, Ehinger M, Jaako P, Zwijsen A, Stadtfeld M, Graf T, Karlsson S. *Manuscript* (2008)

Article III **Endoglin is not critical for hematopoietic stem cell engraftment and reconstitution but regulates adult erythroid development.** Moody J.L, Singbrant S, Karlsson G, Blank U, Aspling M, Flygare J, Bryder D, Karlsson S. *Stem Cells* 25(11): 2809-19 (2007)

ABBREVIATIONS

5-FU	5-fluorouracil
ActRII	Activin receptor II
AGM	aorta-gonad-mesonephros
ALK	activin receptor-like kinase
AML	acute myeloid leukemia
Angptl	angiopoietin-like
BAMBI	BMP Activin receptor membrane bound inhibitor
BFU-E	burst forming unit-erythroid
BM	bone marrow
BMP	bone morphogenetic protein
BMPRII	BMP receptor II
BMT	bone marrow transplantation
CB	cord blood
CDKI	cyclin-dependent kinase inhibitor
cDNA	coding DNA
CFU-E	colony-forming unit-erythroid
CFU-GM	colony-forming unit-granulocyte-macrophage
CFU-S	colony-forming unit-spleen
CLP	common lymphoid progenitor
CMP	fluorescence-activated cell sorting
CRU	competitive repopulating unit
DNA	deoxyribonucleic acid
E	embryonic day
ECM	extra-cellular matrix
EMT	epithelial to mesenchymal transition
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
Floxed	flanked by loxP
G-CSF	granulocyte colony-stimulating factor

GDF	growth and differentiation factor
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HA	hyaluronic acid
HLA	human leukocyte antigen
HOX	homeobox
HSC	hematopoietic stem cell
IL	interleukin
iPS	induced pluripotency
I-Smad	inhibitory Smad
KD	knock-down
LMPP	lymphoid primed multipotent progenitor
LSK	lineage- Sca-1^+ c-kit^+
LTC-IC	long-term culture initiating cell
LT-HSC	long-term HSC
MAD	mothers against decapentaplegic
MAPK	mitogen-activated protein kinase
MBP	mobilized peripheral blood
MFI	mean fluorescent intensity
MH	mad homology
MIG	MSCV-IRES-GFP
MIS	muellerian inhibiting substance
MPP	multipotent progenitor
mRNA	messenger RNA
MSCV	murine stem cell based vector
NK-cell	natural killer-cell
NOD/SCID	non-obese diabetic/SCID
NOG	NOD/SCID/Cγnull
OPN	osteopontin
PB	peripheral blood
PCR	poly chain reaction

PI3K	phosphatidylinositol-3-kinase
pSP	para-aortic splanchopleura
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNAi	RNA interference
R-Smad	receptor-regulated Smad
RU	repopulating unit
SARA	smad anchor for receptor activation
SBE	smad binding element
SCF	stem cell factor (Kit ligand)
SCID	severe combined immunodeficiency
SCL	stem cell leukemia
SCNT	somatic cell nuclear transfer
SDF	stromal cell derived factor
Shh	sonic hedgehog
shRNA	short hairpin RNA
SIN	self-inactivating
siRNA	small interfering RNA
Smad	the mammalian version of MAD
Smurf	smad ubiquitination-related factor
SP	side population
SRC	SCID repopulating cell
ST-HSC	short-term HSC
TβRII	TGF-β receptor II
TGF-β	transforming growth factor-β
TIF	transcriptional intermediary factor
TNF-α	tumor necrosis factor-α
TPO	thrombopoietin
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	very late antigen
wt	wild-type
QRT-PCR	quantitative reverse transcriptase PCR

SVENSK SAMMANFATTNING

Blodet innehåller flera olika sorters celler som har till uppgift att förse kroppen med syre (röda blodkroppar), levra blodet för att förhindra att vi förblöder vid skada (blodplättar), och skydda oss mot bakterier och virus (vita blodkroppar som utgör immunförsvaret). Dessa celler är kortlivade, och varje dag genom hela livet byts 1 trillion blodceller ut. Denna process kallas blodbildning eller hematopoes, och är beroende av ett litet antal blodstamceller som finns i benmärgen. Blodstamceller kan per definition både ge upphov till nya stamceller för att säkerställa en stamcellpool som varar livet ut, och dessutom bilda alla typer av celler som finns i blodet, och därmed bilda ett helt nytt blodsystem (**Figur 1-2**). Denna process kräver noggrann reglering för att inte livshotande sjukdomar som t.ex. leukemi skall utvecklas. För att öka förståelsen för mekanismerna bakom dessa cellers reglering har jag under min doktorandtid studerat proteiner involverade i signalering som styr blodstamcellerna.

Leukemi, eller blodcancer, behandlas idag framgångsrikt genom att ta bort patientens sjuka blodsystem med cellgifter och/eller strålning, och sedan transplantera patienten med friska blodstamceller. Dessa celler hittar sen sin väg tillbaks till benmärgen, varifrån de bygger upp ett nytt blodsystem med kapacitet att generera nya blodceller livet ut. Många viktiga kontrollsystem i kroppen är väl bevarade mellan olika arter. Därför studerar man ofta funktionen av proteiner som man tror är viktiga för olika organfunktioner eller sjukdomsbilder i djurmodeller, t.ex. i möss. Det mest noggranna och rättvisande sättet att studera funktionen av ett protein är att ta bort genen som kodar för det specifika proteinet och se vad som händer. Detta är tekniskt möjligt att göra i så kallade ”knockout-möss”, där möss saknar en eller flera gener som man vill studera. Ett problem med den här tekniken är att många gener är livsnödvändiga under fosterutvecklingen, och alltså gör det svårt att studera funktionen av dessa proteiner i vuxna möss. Det finns dock inducerbara ”knockout-möss” som inte saknar genen, utan har den ”inom parantes”. Forskaren kan sedan själv bestämma när, och i vilken typ av celler, genen ”inom parantes” ska tas bort. Andra sätt är att över- eller underuttrycka en gen i celler utanför kroppen (*ex vivo*) med hjälp av virala vektorer (baserade på virus som kan ta sig in i arvsmassan). I den här avhandlingen har samtliga metoder använts.

Denna avhandling är baserad på tre studier där vi undersöker rollen av två viktiga kandidater för reglering av vuxen blodbildning, ***BMP*** och ***Endoglin***, vilka båda är medlemmar av TGF-β-familjen. TGF-β-familjen

består av flera olika tillväxtfaktorer som t.ex. TGF-β och BMP, vilka reglerar en mängd viktiga funktioner i kroppen, bl.a. blodbildning. Då flera av dessa faktorer är viktiga under fosterutvecklingen, dör knockout-mössen innan födseln om de saknas. Därför är kunskapen om deras funktion ofta baserad på experiment utförda i väldigt tidig fosterutveckling, eller i en konstgjord laboratoriemiljö (*in vitro)*, vilket ofta inte speglar deras faktiska roll i en levande organism (*in vivo*). Ett stort antal artiklar har beskrivit vikten av fungerande BMP-signalering för att blod ska bildas under tidig fosterutveckling (**Figur 9**), medan väldigt lite är känt om dess roll i ett befintligt blodsystem. Vi har använt oss av inducerbara knockout-möss och slagit ut hela BMP-signalvägen i blodstamceller genom att ta bort generna som kodar för Smad1 och Smad5, vilket avbryter BMP-signalernas väg från cellens yta till cellkärnan (**Figur 7**). För att studera blodbildning transplanterades sedan dessa modifierade blodceller till möss, som genom strålningsbehandling mist sina egna funktionella blodstamceller. Våra resultat avslöjar att blodstamceller som saknar BMP-signalering ändå kan bilda ett nytt fungerande blodsystem innehållande alla typer av blodceller (**Artikel I och II**). Detta antyder att BMP-signalering inte är viktigt för vuxen blodbildning, även om det spelar en livsavgörande roll för uppkomsten av blod i det utvecklande embryot.

Endoglin är en hjälpreceptor till TGF-β som finns uttryckt på blodstamcellernas yta, och kan användas för att identifiera dessa väldigt ovanliga celler. Endoglin fyller en viktig funktion vid bildandet av blodkärl, men dess roll för reglering av blodstamceller har tills nu varit okänd. Vi har studerat detta genom att både öka och minska mängden Endoglin som finns på ytan av blodstamcellerna med hjälp av virala vektorer. I **Artikel III** visar vi att mängden Endoglin är viktig för bildandet av röda blodkroppar. Däremot påverkades inte blodstamcellernas förmåga att nybilda ett fullt fungerande blodsystem av vare sig upp- eller nedreglering av Endoglin.

Sammantaget har våra studier bidragit till ökad förståelsen för hur blodstamceller regleras. Resultaten är av klinisk relevans då lämpliga donatorer av friska stamceller för transplantation är en bristvara, och mycket av dagens forskning strävar efter att lära sig kontrollera dessa celler för att förbättra möjligheten att behandla fler patienter och fler typer av sjukdomar.

PREFACE

Stem cells are undifferentiated cells defined by their ability to both self-renew and differentiate to produce mature progeny. The most primitive form is the *embryonic stem cell* (ESC) that is derived from the very early blastocyst stage of a developing embryo. ESCs are pluripotent, hence can give rise to all of the specialized embryonic tissues. Additionally, there are adult stem cells, or *tissue specific stem cells*. These are multipotent, and act as a repair system for the body, replenishing specialized cells within a certain type of tissue, and maintain normal turnover of regenerative organs such as blood, skin or intestinal tissues.

Hematopoietic stem cells (HSCs), or blood stem cells, are by far the best characterized multipotent stem cells, in part owning to that they are relatively easy to access where they reside in the bone marrow (BM). More importantly, the existence of primitive hematopoietic multipotent progenitor cells was discovered almost half a century ago (Till and McCulloch, 1961) allowing for immense progress in isolation of these cells, as well as development of assays to characterize them. However, although much is known, the exact mechanisms that govern HSC fate still remain elusive. This thesis describes the role of the two important regulatory candidates ***Bone Morphogenetic Protein*** **(BMP)** ***signaling*** and ***Endoglin*** in adult hematopoiesis.

BACKGROUND TO THIS THESIS

HEMATOPOIESIS

The word hematopoiesis means formation of blood, and stems from the ancient Greek words "haima" meaning blood, and "poiesis", which means to make. The blood system is a highly complex tissue that is constantly renewed and adapted to face the surrounding demands. Around 1 trillion (10^{12}) new blood cells are made in the human body every day throughout life under steady state conditions (Ogawa, 1993). Under stressed situations, such as massive bleeding, the production of blood cells is even higher.

The Function of the Blood & Immune System

The blood consists of several different cell types, all with specific functions (reviewed in (Orkin, 2000)). Most frequent are the *red blood cells*, or erythrocytes, which transport oxygen from the lungs to the rest of the body. *Platelets*, or thrombocytes, are derived from giant megakaryocytes residing in the BM. One megakaryocyte can give rise to thousands of platelets, which migrate into the blood stream and in cooperation with a number of coagulation factors form blood clots to prevent bleeding at sites of injury. The remaining blood cells are *white blood cells*, or leukocytes, which compose the immune system defending the body against invading bacteria, virus, parasites and fungi, as well as cancer cells.

The immune system can be divided into innate immunity and adaptive immunity. *Innate immunity* is referred to as the first line of host defense, and is mediated by phagocytes including *macrophages* derived from monocytes, *granulocytes* (neutrophils, eosinophils, basophils/mast cells), and *dendritic cells* (Akira et al., 2006). This first response to invading pathogens is fairly non-specific, and the innate immune system is primarily able to discriminate between self-antigens and a variety of pathogens by recognizing a limited number of microbial components. Phagocytes ingest and degrade pathogens and subsequently present antigens from the foreign microorganism on their surface, thereby activating the adaptive immune system. Macrophages also release cytokines and chemokines that further stimulate other cells of the immune system. *Natural killer (NK)-cells* do not phagocytose, but rather lyse susceptible targets by releasing enzyme-containing granules, and play an important role in tumor immune-surveillance and in combating infections. Like macrophages, NK-cells also produce substances to recruit and amplify the inflammatory response (Di

Santo, 2006). *Adaptive immunity*, described as the second line of defense, consist of the lymphoid *B- and T-cells*, and is involved in the late phase elimination of pathogens (Germain, 2001). The adaptive immunity is highly specific due to clonal selection from an immense range of lymphocytes bearing antigen-specific receptors. When B- and T-cells are activated by antigen presenting cells they rearrange their antigen receptor genes, and mature into effector cells producing antibodies and inflammatory cytokines respectively. Most lymphocytes generated in response to infection die, but some remain through out life and generate an immunological memory, which enable a fast response the second time a microorganism invade the body.

The Hematopoietic Hierarchy

The majority of the mature effector cells described above are non-dividing and have a limited life span ranging from days to months, with the exception of memory B- and T-cells. It is therefore crucial to have a continuous production of new blood cells. Ultimately, life long renewal of the blood system is maintained by a rare population of *HSCs*, which reside primarily in the BM of adult mammals. The HSC can per definition both self-renew, and thereby maintain the stem cell pool, as well as differentiate into all mature lineages of the hematopoietic system. In between the primitive HSC and the large numbers of mature blood cells, exist several maturation stages of increasingly lineage-restricted progenitors with concurrent decreasing self-renewal capacity. The hematopoietic system is therefore often described as a hierarchical structure depicted in **Figure 1** (further discussed under "Identification of hematopoietic stem and progenitor cells"). What was initially thought to be the HSCs population (Spangrude et al., 1988) has today been further subdivided into long-term HSCs (LT-HSC), short-term repopulating HSCs (ST-HSC) and multipotent progenitors (MPP) (Morrison et al., 1997a), where LT-HSC are the only true stem cells able to give rise to an infinite functional blood system. ST-HSCs can give rise to all mature lineages, but have a diminished ability to self-renew and can therefore only reconstitute transiently (Yang et al., 2005). Further downstream follow oligo-potent progenitors dividing into a lymphoid branch (common lymphoid progenitor; CLP) (Kondo et al., 1997)) and a myeloid branch (common myeloid progenitor; CMP) (Akashi et al., 2000)). CLPs can differentiate into mature B-cells, T-cells and NK-cells, while CMP give rise to monocytes/macrophages, granulocytes, megacaryocytes/platelets, and erythrocytes. Dendritic cells have been reported to derive from both CLPs and CMPs (Manz et al., 2001). However,

the branching point between the myeloid and lymphoid restricted cell populations remains controversial (Adolfsson et al., 2005; Pronk et al., 2007; Reya et al., 2001). Adolfsson et al. recently presented evidence for an adult BM progenitor population termed LMPP for lymphoid primed multipotent progenitor. In contrast to the classical MPP, LMPPs display sustained lympho-myeloid differentiation potential, but lacks the ability to become erythroid and megakaryocytic cells (Adolfsson et al., 2005), and hence challenges the classical hematopoietic hierarchy (Kondo et al., 1997).

Interestingly, Enver and colleagues have shown that single multipotent hematopoietic cells co-express genes normally found at high levels in mature progenitors, such as the erythroid β-globin gene and the myeloid gene myeloperoxidase (Hu et al., 1997). Commitment of HSCs and progenitor cells to a certain lineage, so called "lineage priming", is therefore believed to occur by stepwise extinction of all but one lineage-specific transcriptional program from an initial co-existence of several transcriptional programs. However, gene expression programs of lymphocytes does not seem to be represented in HSCs (Miyamoto et al., 2002), suggesting that HSCs have to go uphill to become lymphoid.

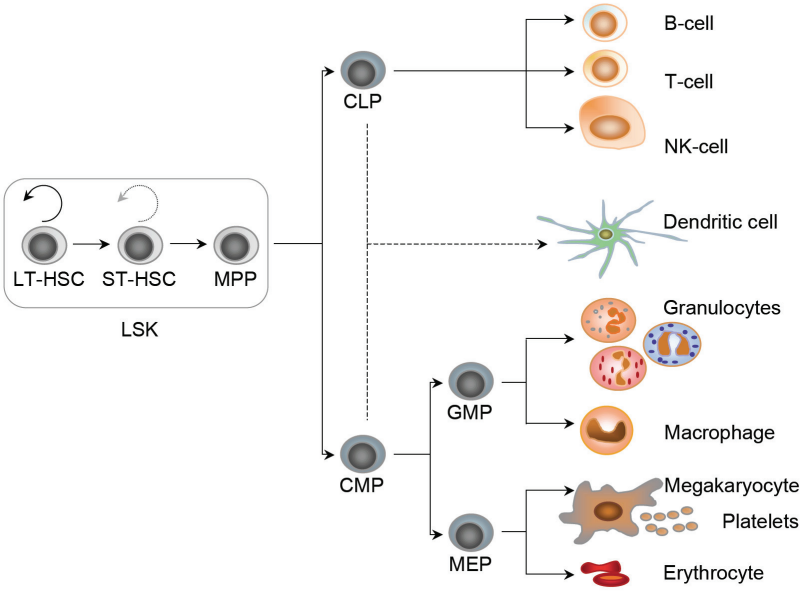


Figure 1: The hematopoietic hierarchy. The hematopoietic system is often described as a hierarchical structure with a few primitive HSCs at the top, followed by several maturation stages of increasingly lineage-restricted progenitors with concurrent decreasing self-renewal capacity (marked by the curved arrow), and large numbers of mature blood cells at the bottom. The primitive LSK compartment (Lineage$^-$ Sca1$^+$ c-Kit$^+$) can be further subdivided into long-term HSCs (LT-HSC), short-term repopulating HSCs (ST-HSC) and multipotent progenitors (MPP), where LT-HSC are the only true stem cells. Further downstream follow oligo-potent progenitors dividing into a lymphoid branch (common lymphoid progenitor; CLP) and a myeloid branch (common myeloid progenitor; CMP), although this branching point is debated. CMPs are subsequently restricted towards a Granulocytic/Macrophage fate (GMP) or a Megacaryocytic/Erythroid fate (MEP). Modified from a figure by Dr. Ann Brun with permission.

The Discovery of Hematopoietic Stem Cells

In the late 1950s, it was shown that BM contains cells capable of rescuing hematopoiesis in lethally irradiated mice (Ford et al., 1956). Soon BM transplantation was also applied in the clinic as treatment for leukemia (Thomas et al., 1959), which was rewarded with the Nobel Prize in 1990. However, it was in 1961 that Till and McCulloch first demonstrated a functional definition of HSCs (Till and McCulloch, 1961). When transplanting BM into lethally irradiated mice, they discovered that a proportion of the injected BM gave rise to macroscopic colonies in the recipient spleens. These so-called colony-forming units spleen (CFU-S) were found to contain cells of multiple blood lineages (Till and McCulloch, 1961). A subset of cells derived from CFU-S also resulted in new CFU-S when transplanted into secondary recipients, thereby displaying self-renewal capacity (Siminovitch et al., 1963). By introducing chromosome aberrations through irradiation of the BM giving rise to CFU-S, subsequent experiments established that every CFU-S originated from one cell, and were hence clonal (Becker et al., 1963; Wu et al., 1968). Although later studies demonstrated that CFU-S are not actual LT-HSCs, but rather primitive multipotent progenitors (Magli et al., 1982), these ground braking experiments defined the properties of stem cells that are still valid today: 1) self-renewal capacity, 2) multi-lineage differentiation potential, and 3) extensive proliferation ability. HSCs have been demonstrated to maintain stem cell function through multiple serial transplantations (Harrison et al., 1978).

Identification of Hematopoietic Stem & Progenitor Cells

HSCs cannot be selected for by morphology or *in vitro* culturing conditions. Isolation of these primitive cells therefore rely on the expression of specific cell-surface antigens, which can be identified by staining with a combination of specific monoclonal antibodies conjugated to fluorescent markers, and subsequent detection by fluorescence-activated cell-sorting (FACS). Technical advances have recently made it possible to identify, or at least isolate populations highly enriched for various stages of maturation. All HSC activity is found within the cell population negative for mature lineage markers, and positive for Sca-1 and c-kit (LSK), which constitute approximately 0.1% of total BM cells (Ikuta and Weissman, 1992; Okada et al., 1992; Okada et al., 1991; Spangrude et al., 1988). However, only a fraction of this infrequent LSK population comprises true LT-HSCs, which can be further identified by the use of additional surface markers. LT-HSCs are found to also express low levels of Thy1/CD90 (Spangrude et al., 1988),

be negative for CD34 (Osawa et al., 1996) and Flt3/Flk2 (Adolfsson et al., 2001), and positive for the SLAM family receptor CD150 (Kiel et al., 2005). Another marker defining LT-HSCs is ***Endoglin*** (Chen et al., 2002) (further discussed under "The Role of the TGF-β Family Signaling in Hematopoiesis and Disease" and **Article III**). Furthermore, HSCs can be isolated by their ability to efficiently efflux Hoechst 33342 dye, and form a small and distinct subset referred to as the side population (SP) when Hoecht fluorescence is analyzed at two emission wavelengths simultaneously (Goodell et al., 1996). Although human stem and progenitor cells are not as well characterized as the murine HSCs, human HSCs have been demonstrated to be comprised within the lineage$^-$ CD34$^+$CD38$^-$ CD90/Thy1$^+$ population of both BM and cord blood (CB) (Majeti et al., 2007).

It should be kept in mind that some of these HSC-enriching surface markers change with cell cycle status, and can therefore not be used for a certain period of time after transplantation or other stress situations. One example is the expression of CD34, which is reversibly increased upon activation of HSCs (Ogawa, 2002). Furthermore, LT-HSCs down-regulate c-kit and up-regulate Mac-1 expression after 5-fluorouracil (5-FU) treatment, making it hard to distinguish them from more differentiated progenitors (Randall and Weissman, 1997). Additionally, the expression of Endoglin, Mpl and Tie-2 is down-regulated upon culturing of LT-HSCs, although this difference in cell surface phenotype did not significantly alter their repopulation ability (Zhang and Lodish, 2005). However, the SLAM family receptors are conserved both in old, reconstituted and cytokine-mobilized cells (Yilmaz et al., 2006a).

Fate Options

The indefinite contribution of HSCs to the hematopoietic system is partly attributed to their ability to remain ***quiescent***, demonstrated by that HSCs are spared after treatment with cytotoxic drugs, which selectively depletes proliferating cells (Lerner and Harrison, 1990). However, studies have shown that 3-5% of LT-HSCs are found in active cell cycle (S/G2/M phase) at any given time (Cheshier et al., 1999; Morrison and Weissman, 1994), and virtually all HSCs have cycled within 30 days, as shown by BrdU incorporation (Bradford et al., 1997; Cheshier et al., 1999). This demonstrates that HSCs divide regularly rather than remaining quiescent for long periods of time. However, in a very recent study Wilson et al. demonstrate the existence of a very rare population of HSCs in mice, so-called dormant HSCs, that only divide about every 145 days, as measured

by BrdU retention in vivo and computational modeling (Wilson et al., 2008). When a stem cell divides the daughter cells commit to one of several cell fate options (**Figure 2**). HSCs can per definition both ***self-renew*** to keep the stem cell pool intact, and also ***differentiate*** into all mature blood lineages to replenish the blood system upon demand. HSCs can also undergo ***apoptosis*** to regulate HSCs numbers. Additionally, the cells can either remain in the BM or ***migrate*** through the blood stream to distinct BM sites, or to extra-medullary hematopoietic organs. The importance of migration is especially clear during embryonic development (see "Development of the Hematopoietic System"). The regulation of these fate decisions is discussed in more detail below.

While *symmetric division* generates identical progeny, *asymmetric division* yields daughter cells with different fates, giving three possible scenarios: Asymmetric divisions resulting in maintenance (one HSC and one committed daughter cell), symmetric division giving rise to two HSCs, thereby leading to expansion, and symmetric division resulting in two committed daughter cells, which in the long run would result in exhaustion of the stem cell pool (**Figure 2**). Asymmetric division can depend on both intrinsic and extrinsic mechanisms, where intrinsic cues results in differential segregation of cell fate determinants along an axis of polarity, and subsequent orientation of the mitotic spindle along this axis. The other condition is that the two daughter cells following a symmetric division get positioned in diverse locations in the microenvironment, and hence get exposed to different extrinsic factors (Congdon and Reya, 2008; Morrison and Kimble, 2006).

The decision to self-renew or differentiate can be modeled as either a random *stochastic* process governed by intrinsic determinates that cannot be influenced, or an instructive *deterministic* process, in which exogenous cues influence the cell fate decision (reviewed in (Muller-Sieburg and Sieburg, 2006; Viswanathan and Zandstra, 2003)). Though early studies mainly support the stochastic model, an increasing body of evidence has demonstrated the important role of cytokines in fate determination. The controversy has therefore shifted to debate whether cytokines play an *instructive* role (determining the fate decision of a cell) or *permissive* role (allowing for a cell to commit to a predestined fate) (Kondo et al., 2000; Ogawa, 1993). It is likely that the initial divisions of HSC are stochastic to secure maintenance of the HSC pool also in substantial demand for one particular lineage, but that deterministic processes become increasingly important as the cells commit to a certain lineage, and have to respond to immediate changes in the microenvironment (Enver et al., 1998).

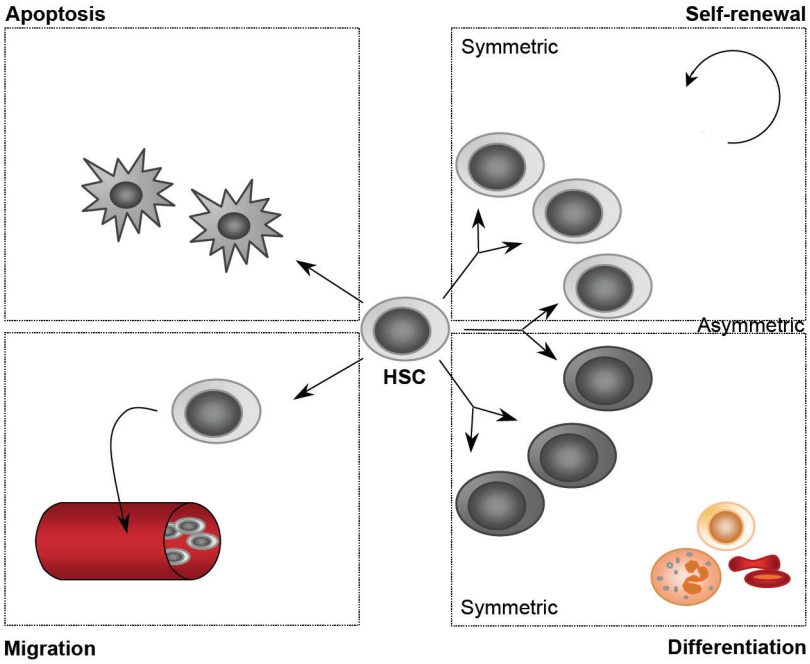


Figure 2: Fate options of HSCs. HSCs can except for remaining quiescent also (1) *self-renew* to keep the stem cell pool intact, (2) *differentiate* into all mature blood lineages to replenish the blood system upon demand, (3) undergo *apoptosis* to regulate HSCs numbers, and finally either remain in the BM or (4) *migrate* through the blood stream to distinct BM sites, or to extra-medullary hematopoietic organs.

Regulation of Hematopoietic Stem Cells

Because of the high turnover rate of blood cells it is absolutely crucial with functional homeostatic control mechanisms, assuring a proper balance between the different cell fate options to not develop hematological diseases like leukemia. Although the molecular mechanisms behind this regulation are not fully understood, it is well recognized that cell fate decisions depend on a complex combination of cell-intrinsic and cell-extrinsic signals (reviewed in (Akala and Clarke, 2006; Blank et al., 2008)) (**Figure 3**). The importance of these signals are underlined by the fact that deregulation of many of the factors implicated in HSC self-renewal also have been reported to promote tumorigenesis (Reya et al., 2001).

i) Intrinsic Factors

Transcription Factors

Transcription factors are sequence-specific DNA binding proteins that control the transcription of genetic information from DNA to RNA. The very early initiation of hematopoiesis during embryonic development is dependent on the transcription factor *stem cell leukemia* (*SCL)/tal1*. Homozygous deletion of SCL results in complete absence of yolk sac hematopoiesis, as determined by cultures of yolk sac cells from these mice (Robb et al., 1995). By over-expressing the SCL homolog in Zebrafish, it was later determined that SCL specifies the formation of the "hemangioblast", the common precursor for the hematopoietic and endothelial lineages (Gering et al., 1998). Likewise, *acute myeloid leukemia (AML)1/Runx1*, which is the most frequent target of chromosomal rearrangement in human leukemias, is also critical for the generation of hematopoiesis in the embryo. Embryos deficient for AML1 display normal yolk sac derived erythropoiesis, but lack definitive hematopoiesis (Okuda et al., 1996). Although both SCL and AML1 have more recently been shown to be dispensable for adult HSC function when conditionally deleted in adult mice, they both remain important for proper differentiation of blood cells in adult hematopoiesis (Ichikawa et al., 2004; Kunisato et al., 2004; Mikkola et al., 2003). In contrast, the transcription factor *Tel/Etv6,* which is also frequently targeted in translocations in human leukemias (Golub et al., 1996), seems to be redundant in embryonic hematopoiesis but crucial for HSC function in adults. Whereas, Tel deficient embryos exhibit largely unperturbed blood formation (Wang et al., 1997), conditional deletion of the gene in adult mice results in almost complete HSC depletion, as determined by transplantation experiments (Hock et al., 2004b).

An additional set of transcription factors that are crucial for embryonic body patterning and organogenesis, are the *homeobox (Hox) genes*, many of which are highly expressed in HSCs and then down-regulated upon differentiation. Most extensively studied is HoxB4, which has been shown to greatly expand HSCs both *in vitro* and *in vivo* when over-expressed (Antonchuk et al., 2002; Miyake et al., 2006; Sauvageau et al., 1995). It was therefore surprising when it was shown that HoxB4 is not required for the generation of HSCs or the maintenance of steady state hematopoiesis *in vivo*, using a conditional HoxB4-knockout mouse (Brun et al., 2004). This discrepancy can possibly be explained by compensatory mechanisms from related Hox-genes. To address this, a triple knockout of

HoxB3, HoxB4 and HoxA9 was created in our laboratory. Although triple knockout HSCs display a significantly reduced repopulative capacity, it was not more severe than the repopulation defect seen when HoxA9 was deleted alone (Lawrence et al., 2005), suggesting that that even though these transcription factors play a role in regulating HSCs, they are not absolutely essential for formation of all major blood lineages (Magnusson et al., 2007). Additionally, the Hox cofactor *Pbx1* has been implied in HSC regulation, as conditional inactivation of the gene results in progressive loss of LT-HSCs (Ficara et al., 2008). Interestingly, HoxB4-induced self-renewal of HSCs is accompanied by increased expression of another transcription factor, the proto-oncogene *c-Myc* (Satoh et al., 2004). c-Myc is known to induce a number of target molecules involved in regulating cell cycle progression (Eilers et al., 1991), and has recently been reported to control the balance between HSC self-renewal and differentiation. Conditional deletion of c-Myc results in severe cytopenia of all blood lineages and accumulation of self-renewing HSCs in the BM due to failure to initiate differentiation (Wilson et al., 2004). This retention of HSCs in the niche might be explained by the concurrent increase in expression of stromal adhesion molecules (see "Adhesion Molecules") (Wilson et al., 2004).

Yet another protein important for HSC maintenance is *Gfi-1*, a transcription factor previously implicated in regulation of lymphopoiesis. HSCs deficient in Gfi1 display increased proliferation rates and are functionally compromised in their competitive repopulation ability in mice. Hence, Gfi1 was shown to prevent exhaustion of HSCs by suppressing proliferation (Hock et al., 2004a; Zeng et al., 2004). Furthermore, a recent study demonstrates a pivotal role for the transcription factor *FoxO3a* in maintaining the HSC pool. Deletion of FoxO3a in mice was accompanied with impaired long-term reconstitution, elevated levels of reactive oxygen species and defective maintenance of quiescence, suggesting that FoxO3 also plays a role in regulating stress resistance of HSCs (Miyamoto et al., 2007).

Epigenetic Factors

Bmi-1 is a transcriptional repressor from the Polycomb group of genes, which control gene silencing by epigenetic modifications including histone methylation and deacetylation. Bmi-1 deficient mice display normal numbers of fetal liver HSCs while the number of HSCs in BM after birth is reduced, and when transplanted both fetal liver and BM HSCs possess only transient repopulative capacity (Park et al., 2003). Gene expression analysis revealed that Bmi-1 target the cell cycle inhibitors p16 and p19 to allow

proliferation and inhibit apoptosis respectively (see below) (Oguro et al., 2006; Park et al., 2003). In agreement with the loss-of-function studies, over-expression of Bmi-1 enhances symmetric cell division and promotes HSC expansion (Iwama et al., 2004).

Cell Cycle Regulators

The cell cycle consists of the resting G0/G1 stage, and the actively dividing S/G2/M stage. Cyclin-dependent kinase inhibitors (CDKIs) are central components of the cell cycle control system, and serve as checkpoints restricting transition through the cycle, mainly during the G1-phase. CDKIs are composed of two families, the early acting lnk4 family with members like p15, p16, p18 and p19, and the late acting Cip/Kip family including p21, p27, and p57 (reviewed in (Cheng, 2004)). *p21* governs the cell cycle entry of stem cells. In the absence of p21 HSCs are more prone to proliferate under normal homeostatic conditions, but demonstrate impaired serial transplantation capacity and exhaustion under conditions of stress (Cheng et al., 2000b). The related *p27* does not affect HSC function or numbers on its own, but alters the proliferation of more mature hematopoietic progenitors, as assessed by colony-forming assays (Cheng et al., 2000a). However, concurrent deletion of p27 and the Myc-antagonist MAD1 results in an expanded pool of quiescent HSCs, which exhibit an enhanced proliferative response in conditions of stress (Walkley et al., 2005). In sharp contrast, HSCs deficient in the early acting p18 display improved long-term engraftment, largely by increased self-renewal divisions of the transplanted HSCs (Yu et al., 2006; Yuan et al., 2004). Early acting CDKIs interact with the retinoblastoma protein (*Rb*), which plays a central role in the regulation of the G1-S transition. It was therefore quite surprising when it was discovered that Rb is not intrinsically required for self-renewal and multi-lineage differentiation of adult HSCs (Walkley and Orkin, 2006).

Adaptor Proteins

Adaptor proteins are accessory to main proteins in a signal transduction pathway, and generally drive the formation of protein complexes. One example is *Pten*, a tumor suppressor that negatively regulates signaling through the phosphatidylinositol-3-kinase (PI3K)-Akt pathway, which is important in cell proliferation, differentiation and migration (Stiles et al., 2004). Conditional deletion of Pten leads to a transient expansion and subsequent depletion of the HSC pool, mainly due to increased HSC activation. Moreover, Pten deficient HSCs are unable to long-term

reconstitute recipients (Yilmaz et al., 2006b; Zhang et al., 2006b). Intriguingly, deletion of Pten also results in generation of transplantable leukemia, indicating different self-renewal mechanisms for maintenance of normal HSCs and leukemic stem cells (Yilmaz et al., 2006b).

Anti-apoptotic Proteins

A significant body of evidence suggests that suppression of apoptosis, a form of programmed cell death, is required for HSC maintenance. This is supported by studies using mice with modified expression of the apoptosis suppressing genes Bcl2 and Mcl-1. Forced expression of *Bcl2* protects HSCs from several apoptotic stimuli such as growth factor deprivation and chemotherapeutic agents, and lead to increased numbers of HSCs in the BM (Domen et al., 2000; Domen and Weissman, 2003). Furthermore, these HSCs display a repopulative advantage compared to wt HSCs when transplanted in a competitive setting (Domen et al., 2000). In contrast, deletion experiments demonstrate that Bcl2 is not essential for HSCs self-renewal, although critical for the formation of lymphoid development (Matsuzaki et al., 1997). The related *Mcl-1* gene is, however, required for HSC survival, as assessed by induced deletion of the gene and subsequent transplantation experiments (Opferman et al., 2005).

ii) Extrinsic Factors

Extrinsic cues consist of membrane-bound and secreted factors provided by cells and extra-cellular matrix (ECM) components of the surrounding microenvironment (further discussed under "The Hematopoietic Stem Cell Niche"), which promote HSC maintenance and regulate HSC migration, quiescence and differentiation.

Cytokines & Growth Factors

Cytokines are signaling molecules extensively involved in cellular communication, which are capable of stimulating or inhibiting cellular growth, proliferation and differentiation. Since finding means to efficiently expand HSCs *ex vivo* would be very beneficial for clinical purposes, there is a great search for the optimal combination of cytokines for HSC expansion. Although single or combinations of cytokines can promote a few rounds of self-renewing division, there is to this date no effective way to expand HSCs *ex vivo* ensuring that the expanded cells maintain their stem cell properties. The most commonly used cytokines for maintaining HSC viability in culture are different combinations of Stem Cell Factor (SCF),

Thrombopoietin (TPO) and Flt3 ligand (Ema et al., 2000; Goff et al., 1998; Miller and Eaves, 1997; Yagi et al., 1999). *SCF*, which is also known as Kit ligand or Steel factor, has repeatedly been shown to promote survival and proliferation of HSCs in *vitro* (Domen and Weissman, 2000; Li and Johnson, 1994). Furthermore, it was recently shown that the SCF receptor *c-Kit* regulates maintenance of HSC quiescence *in vivo*, as c-Kit mutants ($Kit^{W41/W41}$) have an almost two-fold reduction of long-term HSCs. Additionally, self-renewing $Kit^{W41/W41}$ HSCs were progressively depleted when transplanted, suggesting a role for c-Kit in promoting HSC survival (Thoren et al., 2008). Similarly, deletion studies have demonstrated a vital and non-redundant role for *TPO* and its receptor *c-Mpl* in self-renewal and expansion of HSCs (Kaushansky, 2005; Kimura et al., 1998). BM cells from c-Mpl deficient mice generate almost ten-fold fewer spleen colonies than wt BM, and fail to long-term reconstitute irradiated recipients when transplanted in a competitive setting (Kimura et al., 1998). The importance of TPO in HSC regulation was further high-lighted when it was recently connected to *LNK*, a cytokine signaling inhibitor of several cytokine signaling pathways including TPO and SCF. LNK-deficient mice display increased numbers of HSCs, which additionally possess elevated self-renewal capacity (Ema et al., 2005). A phenotype that was later demonstrated to be TPO dependent (Buza-Vidas et al., 2006). The third growth factor commonly used when culturing HSCs *in vitro* is *Flt3 ligand*. It has, however, been shown that the receptor Flt3 is not expressed on long-term HSCs, and that up-regulation of Flt3 expression on murine primitive LSK cells is accompanied with loss of self-renewal capacity, suggesting that Flt3 ligand may not contribute to survival of the actual LT-HSCs *in vitro* (Adolfsson et al., 2001). These survival cytokines are often combined with Interleukins (IL) like IL-3, and IL-6, which promotes growth (Bryder and Jacobsen, 2000) (Audet et al., 2001). However, because the magnitude of expansion of HSCs *ex vivo* has been rather modest so far, additional strategies are needed.

Some promising factors and signaling pathways that have proven important for regulating HSC self-renewal include Fibroblast Growth Factor-1 (FGF1), Angiopoietin-like (Angptl) proteins, and Notch and Wnt signaling, which are reviewed below. De Haan et al. have demonstrated that prolonged cultures of BM cells in serum-free medium supplemented only with *FGF1* result in robust expansion of HSCs capable of multi-lineage long-term repopulation (de Haan et al., 2003). Intriguingly, Lodish and colleagues have established a further 24- to 30-fold net expansion of long-term HSCs, when HSCs were cultured with *Angptl protein* 2 or 3, in

combination with SCF, TPO, IFG2, and IGF1 for 10 days, as determined by reconstitution of irradiated mice (Zhang et al., 2006a). *Notch signaling* is highly active in HSCs and is down-regulated upon differentiation. Inhibition of Notch signaling in adult murine HSCs results in accelerated differentiation *in vitro* and depletion of HSCs *in vivo* (Duncan et al., 2005). Accordingly, enforced activation of Notch1 lead to decreased differentiation and enhanced self-renewal of HSCs *in vivo* (Stier et al., 2002). Notch signaling has also been reported to play an indirect role in HSC expansion via the niche. Constitutive activation of osteoblasts in transgenic mice results in increased numbers of osteoblasts, which display enhanced expression of the Notch-ligand Jagged1. This lead to increased self-renewal capacity of wt primitive hematopoietic cells, as measured by long-term culture initiating cell (LTC-IC) assay (Calvi et al., 2003). However, Notch1-deficient HSCs were later shown to be able to reconstitute mice with Jagged1-deficient BM stroma (Mancini et al., 2005), suggesting that Notch signaling through these molecules is dispensable for HSC function *in vivo*.

Wnt signaling is another pathway shown to be involved in HSC self-renewal (Staal and Clevers, 2005). Exposure of BM HSCs to purified Wnt3a protein was found to expand HSCs in vitro, while maintaining their ability to reconstitute recipients (Willert et al., 2003). In agreement, Wnt3a deficiency leads to a decrease in HSCs derived from fetal liver, and severely reduces their reconstitution capacity, as measured by secondary transplantation assays (Luis et al., 2008). Furthermore, constitutive activation of β-catenin, a down-stream component of Wnt signaling, results in expansion of HSCs *in vitro*, as well as increased reconstitution potential *in vivo* (Reya et al., 2003). Accordingly, enhanced degradation of β-catenin by the expression of Axin inhibits HSC growth *in vitro*, and reduces reconstitution *in vivo* (Reya et al., 2003). In sharp contrast, Cobas et al. show that conditional deletion of β-catenin in murine adult HSCs does not impair their ability to self-renew or reconstitute all hematopoietic lineages, even in a competitive setting (Cobas et al., 2004). Furthermore, another study reports that constitutive activation of Wnt signaling through conditional expression of a stable form of β-catenin, causes multi-lineage differentiation block and compromised HSC maintenance (Kirstetter et al., 2006). Thus, although Wnt may prove useful for *ex vivo* expansion of HSCs, the full role for Wnt signaling *in vivo* remains to be determined. Interestingly, Duncan et al showed that intact Notch signaling is required for Wnt-mediated maintenance of undifferentiated HSCs, but not for survival or entry into the cell cycle *in vitro* (Duncan et al., 2005). These data provide a model for how HSCs may integrate multiple signals to

maintain the stem cell state. This phenomenon has also been demonstrated for other HSC regulatory pathways. For example, stimulation with *Sonic hedgehog* (*Shh*) was shown to induce expansion of human CB HSCs *in vitro* in a Bone Morphogenetic Protein (BMP) dependent manner (Bhardwaj et al., 2001). In turn, ***BMP signaling*** plays a major role in the specification of hematopoiesis during embryonic development (Sadlon et al., 2004), and high concentration of BMP4 has also been shown to maintain human HSCs derived from CB in culture (Bhatia et al., 1999). However, we have now demonstrated that BMP signaling does not seem to be important in adult hematopoiesis (**Article I** and **Article II)**, which will be discussed in greater detail later in this thesis.

Much less is known about *negative regulators*. Apart from Transforming Growth Factor-β (TGF-β), which will be further discussed under "The role of the TGF-β Signaling Family in Hematopoiesis", Tumor Necrosis Factor-α (TNF-α), and Interferon-γ (IFN-γ) have been reported to negatively regulate hematopoietic stem and progenitor cells *in vitro* (Bryder et al., 2001; Sato et al., 1995).

Adhesion Molecules

Although the vast majority of HSCs reside in the BM, at any given moment a few HSCs can be found in the blood stream (Wright et al., 2001). The fate options to either remain quiescent in the BM, proliferate, or migrate out into the periphery are largely affected by expression of various adhesion molecules on cells surrounding the HSCs. This is a possible explanation why stromal cell environments can maintain HSCs in a primitive state better than combinations of soluble cytokines and growth factors *in vitro*. In response to BM injury HSCs migrate from the BM to the blood stream. This can also be induced by treatment with mobilizing agents like cyclophosphamide or granulocyte colony-stimulating factor (G-CSF) (reviewed in (Cottler-Fox et al., 2003)). Both scenarios involve the release of neutrophil proteases that degrade niche-retention signals and adhesive connection. Examples of adhesion molecules that are important for retaining HSCs in the niche are N-cadherins (Zhang et al., 2003), Angiopoietin-1 (Ang-1) exclusively found on osteoblasts and its receptor tyrosine kinase Tie2 expressed on HSCs (Arai et al., 2004), and Stromal cell Derived Factor SDF-1/CXCL12 and its receptor CXCR4 (Sugiyama et al., 2006), which will all be further discussed below. The cytoskeleton also plays a role in regulating migration and adhesion. *Rho GTPases* are critical regulators of actin assembly and motility in mammalian cells, and have also been shown to regulate adhesion and migration in a wide variety of hematopoietic cells.

Concurrent deletion of the Rho GTPase Rac1 and Rac2 leads to a massive egress of hematopoietic stem and progenitor cells to the blood from the BM niche (Gu et al., 2003), suggesting that Rac1 and Rac2 play essential roles in lodging and retention of HSCs in the niche.

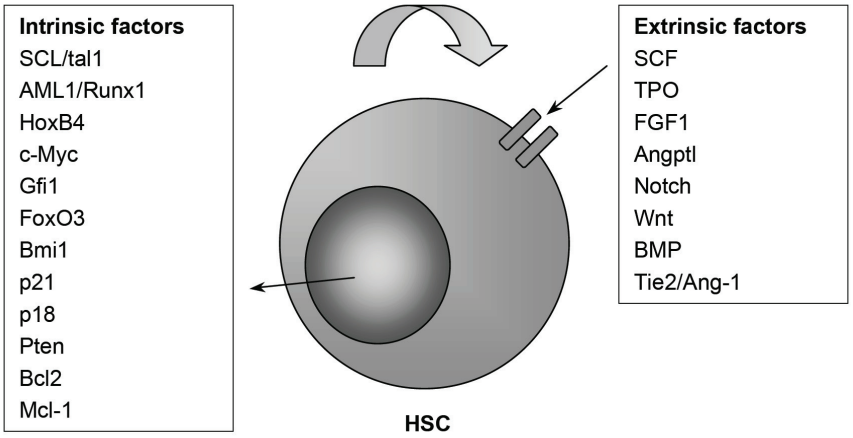


Figure 3: Regulation of HSCs. HSC fate decisions depend on a complex combination of cell-intrinsic and cell-extrinsic signals.

The Hematopoietic Stem Cell Niche

The concept of a stem cell niche was first proposed by Schofield in 1978, who suggested that HSCs reside in a distinct three dimensional structure within a specific location in the BM (Schofield, 1978) **(Figure 4)**. This specialized microenvironment has later been further localized to the *endosteal surface* of trabecular bone, which is lined with supporting stromal cells (Haylock et al., 2007; Nilsson et al., 1997; Nilsson et al., 2001; Zhang et al., 2003). Recent studies have demonstrated the importance of interactions between HSCs and osteoblasts at the endosteal surface (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003), although other environmental cell types and ECM proteins also contribute to the niche. Osteoblasts can activate independent signaling pathways to either expand the HSC pool by producing hematopoietic growth factors like G-CSF and IL-6 (Taichman and Emerson, 1994; Taichman et al., 1997) and activate Notch-1 (Calvi et al., 2003) (see "Cytokines & Growth Factors), or promote HSC quiescence by expressing Ang-1 (Arai et al., 2004), activate PI3K/Akt-mediated regulation of p21 (Hirao et al., 2004), and express the glycoprotein osteopontin (OPN) (Nilsson et al., 2005) (further discussed below).

Recently, an increasing body of evidence shows that HSCs also reside adjacent to *sinusoidal blood vessels* in the BM (Kiel et al., 2005; Watchman et al., 2007), where perivascular cells secrete HSC maintaining factors like SDF1/CXCL12 and_Ang-1(Sacchetti et al., 2007; Sugiyama et al., 2006). In addition, endothelial cells can promote the maintenance of HSCs in culture (Ohneda et al., 1998). Furthermore, the BM is richly innervated with *sympathetic nerve fibers*, which terminate on perivascular stromal cells (Yamazaki and Allen, 1990). Although there is some controversy regarding the role for direct neural input in controlling hematopoiesis, several studies have shown that chemical and/or mechanical denervation of these nerves results in decreased femoral cellularity and increased mobilization of cells into the peripheral circulation, suggesting that BM innervation may play a role in regulating cellular retention in the HSC niche during steady state (Afan et al., 1997; Benestad et al., 1998; Maestroni, 1998; Miyan et al., 1998). Interestingly, a study using mice with defective adrenergic neurotransmission recently indicated that norepinephrine signaling controls G-CSF-induced osteoblast suppression, bone CXCL12 down-regulation, and mobilization of hematopoietic stem and progenitor cells, thus indicating that the sympathetic nervous system regulates the attraction of HSCs to their niche (Katayama et al., 2006).

The exact role of the endosteal versus the vascular niche remains elusive. One theory is that endosteal niche is more quiescent than the vascular niche, and that activated HSCs might be recruited to the vascular niche in response to injury (Wilson et al., 2007).

Engraftment & Homing To the Niche

The niche not only plays a regulatory role for HSC, but also influences the attraction, lodging, retention and release of the residing stem cells (Haylock and Nilsson, 2005; Lapidot et al., 2005). Some examples of critical players in the lodgment of HSCs to their niche are Hyaluronic acid (HA), OPN, and the membrane-bound form of cytokine SCF. *HA*, is a component of the ECM within the BM microenvironment, but is also expressed on both human and murine HSCs. Enzymatic removal of HA from HSCs prior to transplantation results in a significant alteration in spatial distribution of HSCs, demonstrating a critical role for HA in the lodgment of transplanted HSCs within the BM (Nilsson et al., 2003). The glycoprotein *OPN* is expressed by osteoblasts and plays a dual role in regulating HSCs in the endosteal niche. First it contributes to HSC trans-marrow migration towards the niche via interaction with β1-intgrins on the HSCs cell surface. Within the niche OPN is subsequently involved in anchoring of HSCs to the osteoblasts (Haylock and Nilsson, 2006). Mice deficient in OPN display massively increased cycling, as determined by BrdU incorporation, and a concurrent two-fold increase in the number of HSCs (Nilsson et al., 2005). Additionally, the same effect was seen when wt HSCs were transplanted into OPN-deficient recipients, suggesting that the OPN production of osteoblasts regulates HSCs in a negative manner (Stier et al., 2005). Both HA and OPN bind to the cell surface glycoprotein CD44, which is expressed on HSCs (Wilson and Trumpp, 2006). The *membrane bound form of SCF* is expressed by osteoblasts, and has an increased capacity to activate c-Kit on the cell surface of HSCs as compared to the soluble form, thereby contributing to controlling HSCs in the niche (Driessen et al., 2003; Miyazawa et al., 1995).

A number of recent publications have discovered several important molecules involved in HSC migration to and localization in the BM. One such critical component is *N-cadherin*, which has been shown to anchor HSCs to the osteoblasts in the niche (Zhang et al., 2003). Supporting this, ectopic expression of N-cadherin by OP9 stromal cells significantly increased the ability to maintain murine HSCs *in vitro* (Arai et al., 2004). The latter study also demonstrated that interaction of *Tie2* found on HSCs with its ligand *Ang-1* produced by osteoblasts maintains long-term

repopulating activity of HSCs *in vivo*. Furthermore, Ang-1 enhances the ability of HSCs to become quiescent and induces adhesion to bone, resulting in protection of the HSC compartment from myelosuppressive stress. Together these data suggest that the Tie2/Ang-1 signaling pathway plays a critical role in the maintenance of HSCs in a quiescent state in the BM niche (Arai et al., 2004). Furthermore, *SDF-1/CXCL12* and its receptor *CXCR4* have been shown to regulate HSC migration to and localization in the BM (Petit et al., 2002). Another study demonstrated that most HSCs in the niche are found in close contact with reticular cells abundant in CXCL12 production (so-called CAR cells), which reside both surrounding sinusoidal endothelial cells and near the endosteum (Sugiyama et al., 2006). Induced deletion of CXCR4 lead to severe reduction in HSC numbers and increased sensitivity to myelotoxic drugs (Sugiyama et al., 2006). Additionally, CXCL12 plays a crucial role in colonization of the BM by HSCs during ontogeny (Ara et al., 2003). Other cell-surface adhesion molecules, like integrins, are also important in regulating HSC migration. Pretreatment with antibodies against either the *β1-integrin* Very Late Antigen (VLA) 4, or its ligand Vascular Cell Adhesion Molecule (VCAM) before transplantation both result in significantly reduced homing to the BM (Papayannopoulou et al., 1995). In addition administration of the same antibodies to normal mice causes mobilization of hematopoietic progenitors into the blood stream (Papayannopoulou et al., 1995), conclusively indicating a crucial role for the VLA4-VCAM interaction in retaining HSCs in their niche.

When HSCs are transplanted into the blood, they find their way back into the BM niche. Wright et al. elegantly demonstrated that transplanted cells are cleared from the blood within just minutes (Wright et al., 2001). About 0.1-1% of all HSC niches are open at any given time and available for HSC engraftment (Bhattacharya et al., 2006). However, in both experimental and clinical settings the absolute majority of recipients of bone marrow transplants (BMT) undergo a preconditioning myeloablative treatment with irradiation and/or cytotoxic drugs to obtain improved engraftment of the transplanted cells and suppress host immunity. Several groups have shown that long-term engraftment is dependent on that the transplanted cells remain quiescent (Bowie et al., 2006; Glimm et al., 2000; Passegue et al., 2005). Morrison et al. showed that mobilization of HSCs using cyclophosphamide and G-CSF drove both ST and LT multipotent progenitors into cycle. However, although an increased percentage of HSCs isolated from BM and spleen were cycling, HSCs released into the blood tended to be in G0/G1 (Morrison et al., 1997b). This is in agreement with

previous publications (Donahue et al., 1996; Roberts and Metcalf, 1995; Uchida et al., 1997). Nygren et al further stress the importance of quiescence for successful engraftment by showing that although fetal liver HSCs are expanding rapidly, they too display a prolonged G1 transit and passage through a state of relative G0 quiescence (Nygren et al., 2006). How efficiently single HSCs can engraft and long-term reconstitute a host is highly debated, and range between 21% and 96% depending on what cell population are used, and how much competition the single HSC is faced with (Benveniste et al., 2003; Camargo et al., 2006; Matsuzaki et al., 2004; Osawa et al., 1996).

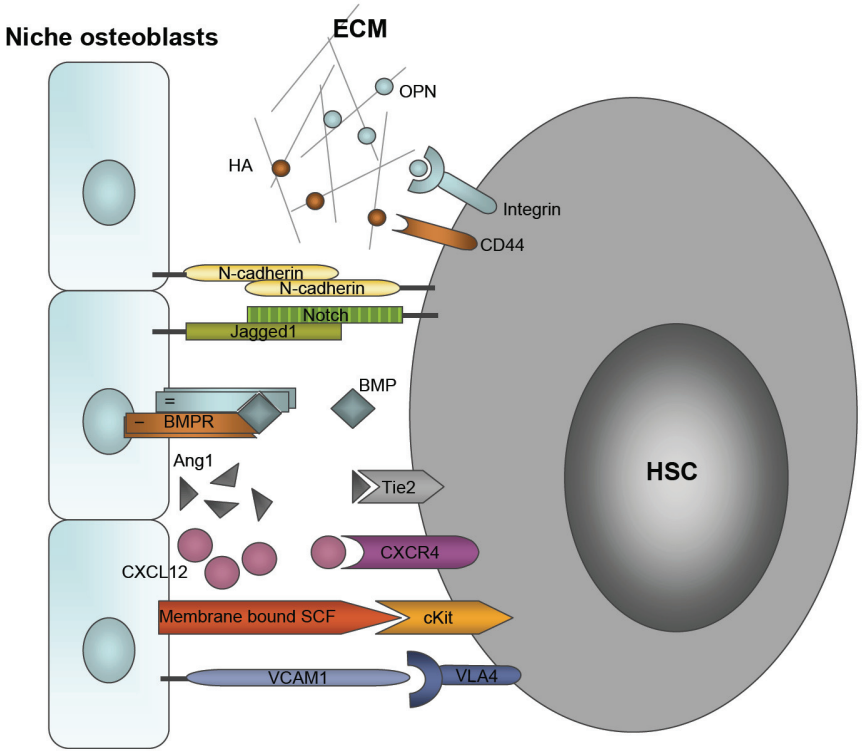


Figure 4: The endosteal niche. HSCs reside in the BM in close contact with specialized osteoblasts, extra-cellular matrix (ECM) and other stromal cells. Depicted are a number of factors and adhesion molecules that influences the attraction, migration, and lodging to the niche, as well as retention or release of residing HSC.

DEVELOPMENT OF THE HEMATOPOIETIC SYSTEM

The hematopoietic system develops early during embryogenesis in a complex manner involving multiple anatomical sites **(Figure 5)**. This shift in location during hematopoietic development is necessary since the embryo changes during organogenesis. Moreover, it might allow for different regulating signals from the various niches to support development of primitive HSCs in one site, while generating lineage-committed cells in other locations.

Hematopoietic tissue arises from the *mesoderm* germ layer, via a common mesodermal precursor for endothelial and hematopoietic lineages, the so-called "hemangioblast" (Dieterlen-Lievre, 1998). The first visible hematopoietic cells are the primitive erythrocytes, which are formed in close proximity to developing vasculature as blood islands in the extra-embryonic *yolk sac* between embryonic day (E)7 and E7.5 (Palis and Yoder, 2001; Sabin, 2002). Definitive erythroid progenitors arise in the yolk sac one day later, as determined by colony formation *in vitro* of primitive erythroid burst forming units (BFU-E), and the more mature erythroid colony forming units (CFU-E) (Palis and Yoder, 2001). Furthermore, progenitors of several myeloid lineages originate and expand in the yolk sac (Palis and Yoder, 2001), while progenitors with both lymphoid and myeloid potential are generated *de novo* from E7.5 in the para-aortic splanchopleura (pSP) region (that later becomes aorta-gonad-mesonephros (AGM)) within the embryo proper, as determined by explant cultures (Cumano et al., 1996). Multiple studies have demonstrated that definitive HSCs originate from sites in the embryo proper (reviewed in (Dzierzak and Speck, 2008) and (Mikkola and Orkin, 2006)). HSCs able to engraft and confer long-term multi-lineage hematopoietic reconstitution of irradiated adult recipients mice first appear at E10 in the *AGM region* in the embryonic body (Medvinsky and Dzierzak, 1996), and in the viteline and umbilical arteries (de Bruijn et al., 2000). Shortly thereafter HSCs are also found in the placenta, yolk sac and fetal liver. The fetal liver does not generate hematopoietic cells, but gets colonized by cells formed in other locations, beginning at E9 (Johnson/Moore 1975). However, both yolk sac and *placenta* contribute to the HSC pool in the fetal liver, either through *de novo* generation of HSCs, or by expansion of pre-existing cells (Alvarez-Silva et al., 2003; Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). Proving where a specific population of hematopoietic cells is actually generated is complicated by the circulation, which is initiated on E8.5 (Delassus and Cumano, 1996). Two studies have elegantly provided

evidence for *de novo* production of definitive myeloid progenitors in the yolk sac by preventing the formation of circulation. This was created by either deleting the endothelial marker VE-cadherin, thereby preventing the yolk sac to get connected to the vasculature of the embryo (Rampon and Huber, 2003), or by deleting the Ncx1 gene (Lux et al., 2008). Ncx1-deficient embryos fail to initiate a heartbeat on E8.25, but continue to develop until E10, revealing that fairly all primitive and definitive progenitors that seed the fetal liver at 9.5 are generated in the yolk sac (Lux et al., 2008). Since HSCs are evaluated by their ability to long-term reconstitute irradiated recipients, one explanation for the lack of HSC activity before E10 could be that true HSCs exist, but are unable to engraft in the adult hematopoietic microenvironment. In fact, several reports demonstrate that true HSCs exist as early as E8, as shown by transplantation of different subsets of cells into busulfan-treated embryos (Yoder and Hiatt, 1997) and Rag2-γc mice, which are depleted of B- T- and NK-cells (Cumano et al., 2001). Contradicting previous reports, a tracking study recently demonstrated that yolk sac cells that were marked at E7.5 contribute to hematopoiesis of all lineages, including lymphoid cells, in adult mice (Samokhvalov et al., 2007).

The *fetal liver* is the main hematopoietic organ in the embryo, and the main site for HSC expansion and differentiation. The first wave of seeding to the fetal liver initiates at E9.5-10.5 and consists of myeloerythroid progenitors, probably derived from the yolk sac. The first HSCs appear at E11.5, and are likely derived from the AGM and placenta (Mikkola and Orkin, 2006). The number of HSCs reaches a maximum of about 1000 HSCs at E15.5-16.5 (Ema and Nakauchi, 2000), where after fetal liver cells start to seed to the BM, which is the major hematopoietic organ after birth. Interestingly, HSCs in the fetal liver expands rapidly, while HSCs in the BM are mainly quiescent, indicating that there are inherited differences between fetal and adult HSCs. However, the fetal liver niche may also provide different signals than the BM to promote symmetric self-renewing divisions (Martin and Bhatia, 2005). Eaves and colleges recently showed that HSCs remain cycling until 3 weeks after birth, and then within 1 week become quiescent (Bowie et al., 2006). This transition from a proliferating fetal HSC to a dormant adult HSC is accompanied with a change in surface markers (reviewed in (Mikkola and Orkin, 2006)). As opposed to quiescent HSCs, cycling fetal HSCs express CD34 (Ogawa et al., 2001), and also certain lineage markers like the monocyte/macrophage marker Mac1 (Morrison et al., 1995), and the B-cell marker AA4-1 (Jordan et al., 1995).

Like adult HSCs, fetal HSCs also express Sca1 (Morrison et al., 1995), Tie2 (Hsu et al., 2000), and CD150 (Kim et al., 2006).

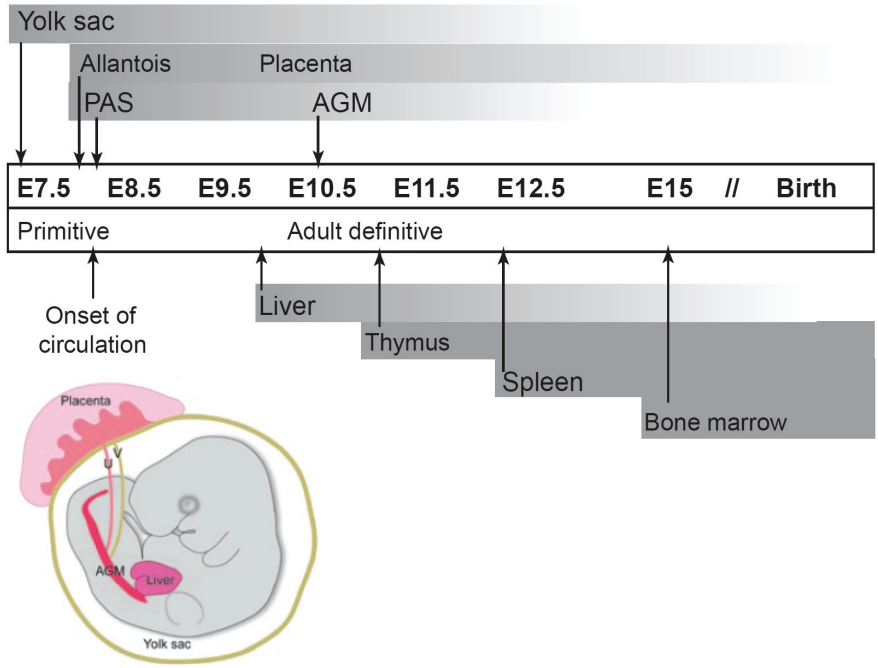


Figure 5: Development of the hematopoietic system in the embryo.

The figure is modified from Dzierzak & Speck 2008.

CLINICAL USE OF HEMATOPOIETIC STEM CELLS

Bone Marrow Transplantations

HSCs have been used clinically since 1959 when Dr. Thomas performed the first successful bone marrow transplantation (BMT) (Thomas et al., 1959), which since then has become a routine treatment mainly for *hematopoietic malignancies* like leukemias and lymphomas. BMTs are either autologous, transplanting back the patients/hosts own BM, or allogenic, where the BM graft comes from an appropriate human leukocyte antigen (HLA)-matched donor. HLAs are expressed by white blood cells and are used by the immune system to distinguish between "self" and "foreign". Although the donor and host are HLA-matched, lymphoid cells from an allogenic graft can mount an immune response against the host-cells. This syndrome is known as graft-versus-host disease (GVHD), and constitutes a great risk that can even be lethal (Copelan, 2006). Since autologous BMT present perfect compatibility between the graft and the recipient, this does not result in GVHD. However, for the same reason an autologous graft also do not present a graft-versus-leukemia (GVL) effect that helps to fight the cancer, which is the main disadvantage of using this setting. Additionally, autologous grafts also risk being contaminated with remaining leukemic cells (Kondo et al., 2003).

Another use of allogenic BMT is to cure diseases that involve genetic or acquired *BM failure* such as aplastic anemia, thalassemia, and sickle cell anemia (Copelan, 2006), but also inborn metabolic disorders including osteopetrosis (Askmyr et al., 2008). Autologous BMT is increasingly used in treatment of *autoimmune diseases* like type-1 diabetes (Beilhack et al., 2005) and rheumatoid arthritis, where the BMT replaces an autoimmune blood system with one that lacks the autoimmune risk gene.

Gene Therapy

Autologous BMT can also be used for *gene therapy*. Gene therapy means to correct a defective gene or insert a missing gene into cells of a patient to treat a disease. Because of the unique ability of HSCs to long-term reconstitute hematopoiesis following myeloablation, genetically modified HSCs are expected to last a lifetime in the recipient following transplantation, and can therefore potentially cure hematological disorders. Oncoretroviral vectors have been the main vehicles used for HSCs due to their ability to integrate into the DNA (Kay et al., 2001). However, since oncoretroviral vectors require dividing target cells for efficient integration

of the provirus, and only a small fraction of HSCs is dividing at any given time, the transduction efficiency of oncoretroviral vectors of human HSCs in clinical trials has been low (Karlsson et al., 2002). To ensure that the small set of transduced HSCs are not out-competed, it is possible to add a selective marker (Neff et al., 2006), or to reduce the mass of untransduced HSCs with chemotherapy and/or irradiation. However, if the genetically corrected cells obtain a selective advantage, low transduction efficiency is enough to successfully treat a disease. One such example is the treatment of patients with severe combined immunodeficiency (SCID) (Cavazzana-Calvo et al., 2000), which constitutes a great breakthrough for the gene therapy field. However, the success is hampered by the adverse effect seen in some patients, where the gene transfer resulted in clonal T-cell proliferation due to insertional mutagenesis into the proto-oncogene LMO2 (Hacein-Bey-Abina et al., 2003).

The risk associated with gene integration strongly demonstrates the need for safer vectors. Lentiviral vectors have recently emerged as promising vehicles for human HSCs since they also transduce non-dividing cells. The need to manipulate the HSCs *ex vivo* to get them into cycle, which also affects their differentiation and repopulation ability (Glimm et al., 2000; Passegue et al., 2005), is thereby abolished. Additionally, lentiviral vectors are safer since they do not appear to preferentially integrate into promotor-proximal regions like oncoretroviral vectors do (Neschadim et al., 2007). Another way to improve the safety of genome integrating vectors is the use of self-inactivating (SIN) vectors with an internal weak or tissue-specific promoter (Cavazzana-Calvo and Fischer, 2007; Neschadim et al., 2007).

Cell Sources

Donor cells used for hematopoietic transplantations were originally derived from the BM. Standard clinical practice today is, however, to use mobilized peripheral blood (MBP), where hematopoietic stem and progenitor cells are recruited from the BM into the blood using mobilization factors, such as cyclophosphamide and/or G-CSF (Kondo et al., 2003). Unrelated umbilical CB was discovered as a potential source of transplantable HSCs almost 20 years ago (Broxmeyer et al., 1989) and is now increasingly being used. As a banked source CB HSCs are rapidly available for transplantation. Additionally, for a given HLA match the risk of GVHD is lower with CB than with HSCs derived from BM or MPB, possibly due to that the immune system of a newborn baby has not been primed against foreign antigens yet (Barker and Wagner, 2003).

Additionally, CB contains a higher proportion of primitive hematopoietic cells with superior proliferative response *in vitro* and engraftment capacity *in vivo* compared to adult BM and MPB (Barker and Wagner, 2003). The main disadvantage is the limited cell dose per graft. Although the number of HSCs in CB is often enough to efficiently engraft children, it is generally not sufficient to repopulate adult patients, as demonstrated by delayed engraftment and aberrant immune reconstitution (Barker and Wagner, 2003). As for adult HSCs, attempts to expand CB derived HSCs *ex vivo* have failed to improve engraftment because of concurrent differentiation of the expanded cells (Hofmeister et al., 2007).

To provide a wider access to cell therapy and to avoid the risk of GVHD, attempts have been made to *produce pluripotent stem cells* from adult tissue specific cells (reviewed in (Daley and Scadden, 2008)). This can be achieved in two ways; (1) by somatic cell nuclear transfer (SCNT), in which a cell nucleus from an adult somatic cell is injected into an enucleated oocyte (Markoulaki et al., 2008), or (2) by direct reprogramming of an adult somatic cell back to a pluripotent state by over-expressing specific transcription factors, so-called induced pluripotency (iPS) (Takahashi et al., 2007). The latter groundbreaking work by Yamanaka and colleagues demonstrated that retroviral transduction of only four genes encoding for the transcription factors c-Myc, Klf4, Oct4 and Sox2 is enough to induce pluripotency in both adult murine and human fibroblasts (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). By reprogramming adult somatic patient cells to iPS, and subsequently differentiate them into the tissue type that is needed, the goal is to generate patient-specific tissue for regenerative medicine in the future.

ASSAYS & METHODS

Functional Biological Assays Evaluating HSCs

As previously described, it is possible to select for cell populations highly enriched for HSCs using FACS. However, functional HSCs cannot be measured solely based on immunophenotypical analysis. The only assay truly defining HSCs is the *long-term repopulating assay* (**Figure 6**). In brief, a source of HSCs with unknown features is transplanted with or without competitor cells into recipients, which have usually undergone myeloablative treatment such as irradiation. When preformed in a competitive manner, this evaluates the quality of the test cells in relation to normal wt cells, and can be measured as repopulating units (RU) (Harrison et al., 1993). The outcome of the transplantation is subsequently assessed by taking out and analyzing PB or BM at different time-points. Multi-lineage donor reconstitution observed later than 12 weeks post transplant is considered to be derived from LT-HSCs, while earlier readouts measure the activity of more mature progenitors. To distinguish the donor cells from endogenous recipient or competitor cells, congenic mouse strains are often used that differ only in the pan-hematopoietic gene CD45 (CD45.1, 5.2, or 5.1/5.2). Furthermore, the frequency of HSCs can be measured using the *limited dilution assay* (reviewed in (Purton and Scadden, 2007)). A series of dilutions of repopulating cells are transplanted together with a fixed number of competitor cells into groups of lethally irradiated recipients. Mice with both donor derived myeloid and lymphoid cells 10 to 16 weeks post transplant are scored as positive. Readout at 37% negative mice gives the number of cells containing 1 competitive repopulating unit (CRU), as calculated using Poisson statistics (Szilvassy et al., 1990). To further evaluate the self-renewal capacity of HSCs, BM from primary recipients can be re-transplanted into secondary recipients. Only the most primitive HSCs are able to sustain hematopoiesis through serial transplantations, presumably through self-renewing divisions.

Primitive human hematopoietic cells can be assayed on the basis of their ability to repopulate immune-deficient non-obese diabetic (NOD)/SCID mice, and have been termed SCID repopulating cells (SRCs). Since NOD/SCID mice lack a functioning immune system, they are unable to reject the human graft (Dick et al., 2001). A limiting factor is that human cells engraft with rather low efficiency, possibly due to biological differences between mouse and human in the BM niche. Furthermore, transplanted NOD/SCID mice die early, making it hard to evaluate long-

term repopulation. However, a new strain of mice used for xenotransplantations has been developed, called the NOD/SCID/Cγnull (NOG)-mouse, which seems to greatly improve the ability to study the function of human HSCs *in vivo* (Ito et al., 2008).

The original quantitative stem cell assay is the *CFU-S assay* developed by Till & McCulloch in 1961 (Till and McCulloch, 1961). This short-term *in vivo* assay measures the ability of early engrafting progenitors to form colonies in the spleen after transplantation. Colonies formed after 12 days represent more primitive cells than the ones scored on day 8, although none of the readouts measure true stem cells. Although *in vivo* assays are preferable when studying stem cell function, these assays are generally time consuming and expensive, and are not always available. Additionally, transplantation assays rely on that the transplanted cells display unperturbed homing and engraftment ability, which is not always the case. Two assays that are used to predict HSC frequency *in vitro* are the *Cobble-stone-forming cell* (CAFC) assay, and the *long-term culture-initiating cell* (LTC-IC) assays, which both employ co-culturing of primitive cells with stroma for more than five weeks. The LTC-IC assay is based on the ability of HSCs, as opposed to more mature cells, to maintain progenitors with clonogenic potential in long-term cultures. In the CAFC assay, cell populations including HSCs are plated onto a monolayer of stromal cell. When grown, stem and progenitor cells form "cobble stone areas" under the stromal layer, and the longer they maintain growth, the more primitive are the cells (van Os et al., 2004).

Assays Evaluating Committed Progenitors

Limitations of the CFU-S assay, such as the difficulty to follow CFU-S formation in the spleen and that it cannot be used to study human cells, led to the development of colony assays *in vitro* allowing for growth of hematopoietic progenitors derived from BM and spleen (Bradley and Metcalf, 1966). The *colony-forming unit (CFU)-assay* measures the ability of progenitor cells to form colonies in semi-solid methylcellulose supplemented with various cytokines. The more primitive the initiating cell is, the more diverse is the formed colony. The most immature colony, the CFU-GEMM, is scored on day 12 in the murine setting and contains granulocytes, erythroid cells, macrophages and megakaryocytes. However, the majority of CFUs consist of lineage-restricted colonies such as the myeloid CFU-GMs or the erythroid CFU-Es. Using the CFU-assay, it is also possible to measure the proliferation potential of the commencing cell by assessing the size of the colony (Purton/Scadden 2007). Furthermore,

proliferation capacity of progenitors can be measured using *in vitro proliferation assays*, where the cells are plated either as single cells or in bulk in medium supplemented with maintaining cytokines (see Cytokines & Growth Factors). The cell number per well and number of proliferating clones is commonly scored on day 12.

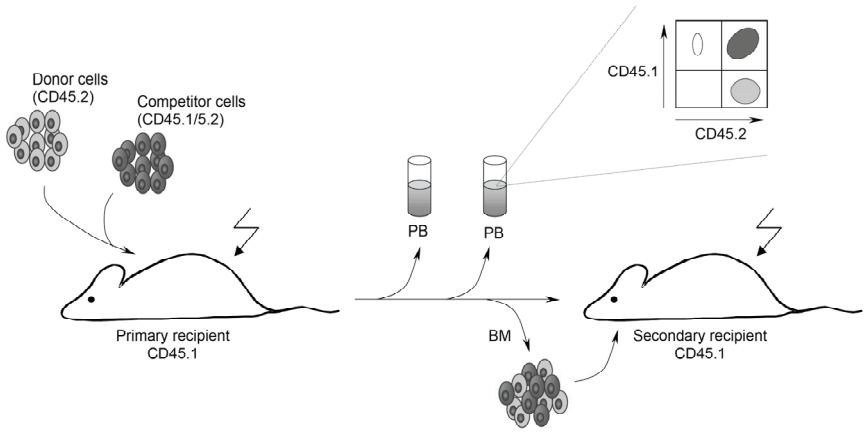


Figure 6: Competitive transplantation assay. A source of HSCs with unknown features (donor cells) is transplanted together with competitor cells from congenic mice into irradiated recipients. The outcome of the transplantation is subsequently assessed by analyzing peripheral blood (PB) at different time-points. Multi-lineage donor reconstitution observed later than 12 weeks post transplant is considered to be derived from LT-HSCs, while earlier readouts measure the activity of more mature progenitors. To further evaluate the self-renewal capacity of HSCs, bone marrow (BM) cells from the primary recipient are re-transplanted into secondary recipients. The donor cells are distinguished from endogenous recipient or competitor cells, by staining for the different versions of the pan-hematopoietic gene CD45 (Donor = CD45.2, Recipient = CD45.1, and competitor = CD45.1/5.2) and analyze by FACS.

MOLECULAR BIOLOGY OF TGF-β SIGNALING

Ligands, Receptors & Smads

As described above HSCs are governed by an intricate network of signaling pathways. One pathway that has proven important in hematopoiesis is the one including the TGF-β superfamily of growth factors, which can be subdivided into the TGF-β/Activin/Nodal family, and the BMP/Growth and Differentiation Factor (GDF)/Muellerian Inhibiting Substance (MIS) family, based on sequence similarity and the specific signaling pathway that they activate **(Figure 7)**. Signaling by these growth factors is initiated when binding of the ligand induces the assembly of a heteromeric complex of type I and type II serine/threonine kinase receptors. Type II receptors for TGF-β and activin (TβRII, ActRII), bind their ligands with high affinity, while type II receptors for BMP (BMPRII) bind BMPs only weakly in the absence of type I receptors (Kawabata et al., 1998). Type II receptors, which are constitutively active kinases, recruit and activate type I receptors, (also known as activin receptor-like kinases (ALKs)), by phosphorylating its cytoplasmic Gly-Ser (GS) domain (Wrana et al., 1994). The activated type I receptor in turn activates specific intracellular receptor-regulated Smads (R-Smads: Smad1-3, 5, and 8) by phosphorylation (Shi and Massague, 2003; ten Dijke and Hill, 2004). Dimers of activated R-Smads subsequently form complexes with the common-partner Smad4 and translocate into the nucleus, where they participate in transcriptional regulation of more than 500 target genes in a cell-specific, ligand dose-dependent manner (Massague et al., 2005; Shi and Massague, 2003; ten Dijke and Hill, 2004). To provide a balanced response to ligand-induced activation, R-Smads are constantly dephophorylated, resulting in dissociation of the Smad complex and export of inactive Smads to the cytoplasm, which are then available for reactivation. Generally, Smad2 and 3 act downstream of the TGF-β and activin receptors, while Smad1, 5, and 8 primarily mediate BMP signals. However, in endothelial cells TGF-β signals via both ALK5 and ALK1, inducing phosphorylation of Smad2/3 and Smad1/5 respectively, which plays an important role in balancing processes of angiogenesis (Goumans et al., 2003; Goumans et al., 2002).

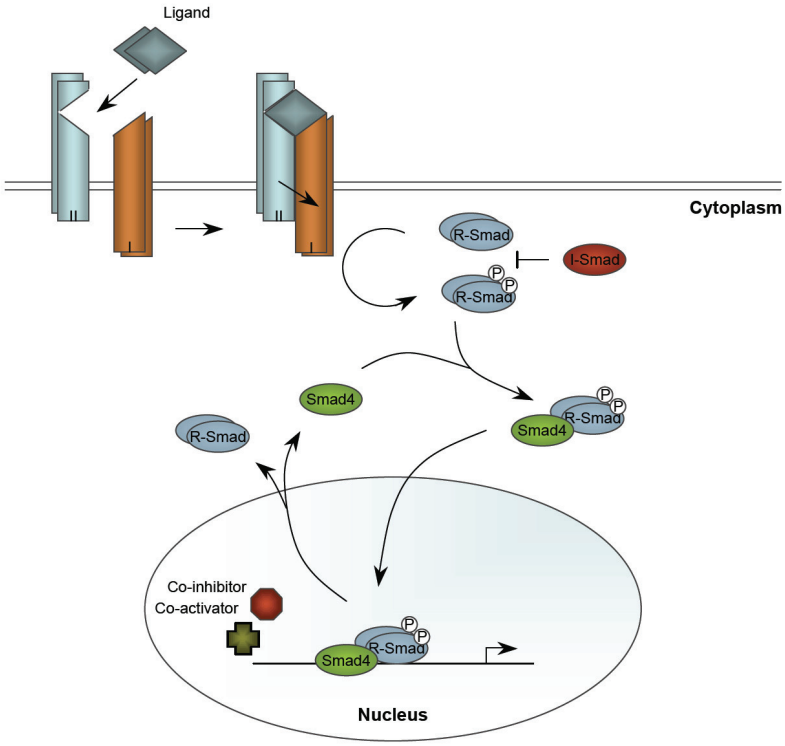
TGF-β family signaling is carefully regulated at several levels. The majority of the *ligands* are dimeric proteins produced as large precursor proteins that require proteolytic cleavage to become active (Derynck et al., 1985). Moreover, ligand traps such as Decorin antagonizing TGF-β, Follistatin inhibiting Activin and BMP binding, as well as Noggin and

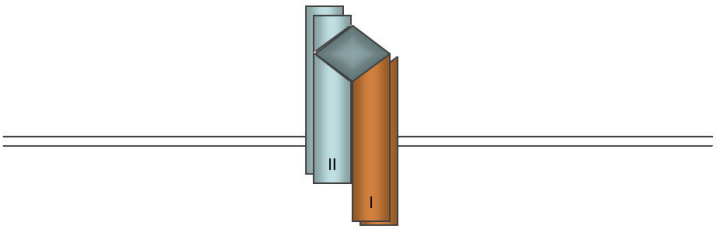
Chordin that prevent BMP activation, sequester ligands and control their accessibility to the receptor (Kawabata et al., 1998; Shi and Massague, 2003). The serine/threonine kinase *receptors* comprises seven type I receptors and five type II receptors, which can form complexes in different combinations. A ligand can thus induce different responses depending on the composition of the receptor complex (Derynck and Zhang, 2003). Additionally, the specificity of the signaling is modulated by accessory receptors such as Betaglycan, which promotes the binding of TGF-β to TβRII. The Betaglycan-related co-receptor ***Endoglin*** is capable of binding several superfamily ligands, but only when it is associated with a typeI/typeII receptor complex (reviewed in (Fonsatti et al., 2001)). Its function is best characterized in endothelial cells, where it serves to modulate TGF-β signaling through ALK1 and ALK5, balancing activating and inhibitory signals regulating cell migration and proliferation respectively (Blanco et al., 2005; Lebrin et al., 2004; Pece-Barbara et al., 2005). Conversely, BMP and Activin receptor membrane bound inhibitor (BAMBI) inhibit receptor activation by competing with the type I receptor for incorporation into ligand-induced receptor complexes (Shi and Massague, 2003). Receptor activation is also regulated by *intracellular proteins*. The protein Smad anchor for receptor activation (SARA) facilitates Smad presentation to the activated receptor complexes, and also tethers Smads in the cytoplasm in the basal state (Shi and Massague, 2003). Moreover, inhibitory Smads (I-Smad: Smad6 and 7) negatively regulate TGF-β signaling by competing with the R-Smads for receptor or Smad4 interaction, and by marking the receptors for degradation by recruiting E3-ubiquitin ligases (Smad ubiquitination-related factor; Smurf) (Shi and Massague, 2003).

Target Gene Selection & Transcriptional Control

Activated Smad complexes bind to DNA of target genes at the Smad binding element (SBE, 5'-CAGAC-3') (Dennler et al., 1998; Johnson et al., 1999). Both activation and repression of target genes use the same set of activated Smad proteins. The gene response depends on the cell-type, and the cell-status at the time of stimulation (Massague, 2000). This diversity in biological response to the same signaling pathway is partly explained by the recruitment of different accessory proteins. Since individual Smad proteins have a rather low DNA binding affinity, transcriptional co-activators and co-repressors are needed to obtain a specific transcriptional response. Some of these co-factors are restricted to certain cell-types, thereby providing a cell-specific response. Examples of co-activators are CBP and p300, while

Ski and SnoN regulate Smad-dependent transcription in a negative manner (Massague et al., 2005; Shi and Massague, 2003).





Ligand	Type II	Type I	R-Smad
TGF−β	TβRII	ALK5 (TβRI)	Smad2, 3
		ALK1	Smad1, 5, 8
Activin	ActRIIA, ActRIIB	ALK4 (ActRIB)	Smad2
		ALK7	Smad2
BMP	BMPRII	ALK3 (BMPRIA)	Smad1, 5, 8
		ALK6 (BMPRIB)	Smad1, 5, 8
	ActRIIA, ActRIIB	ALK2 (ActRIA)	Smad1, 5

Figure 7. Signaling of the TGF-β family. Signaling is initiated when a ligand bind to a constitutively active type II serine/threonine kinase receptor, which subsequently recruits and activates a type I receptor/ALK by phosphorylating its cytoplasmic Gly-Ser (GS) domain. This induces the assembly of a heteromeric complex of two type I, and two type II receptors. The activated type I receptor in turn activates specific intracellular receptor-regulated Smads (R-Smads: Smad1-3, 5, and 8) by phosphorylation. Dimers of activated R-Smads subsequently form complexes with the common-partner Smad4 and translocate into the nucleus, where they take part in transcriptional regulation of more than 500 target genes in a cell-specific, ligand dose-dependent manner. To provide a balanced response to ligand-induced activation, R-Smads are constantly dephophorylated, resulting in dissociation of the Smad complex and export of inactive Smads to the cytoplasm, which are then either degraded or available for reactivation. The table summarizes the different combinations of typeI/typeII receptors and R-Smads that are activated by the different ligands. The figure is modified from Derynck & Zhang 2003.

Features of the Smad Proteins

The Smad proteins are, as mentioned above, the intracellular mediators of TGF-β family signaling. The Smads are well conserved between species, and the name is a combination of the two homologs of the drosophila protein, mothers against decapentaplegic (MAD), and the C. elegans protein SMA. A curious detail is that it was found in drosophila that a mutation in the "mad-gene" in the mother, repressed the gene decapentaplegic in the embryo. The phrase "Mothers against" was added since mothers often form organizations opposing various issues e.g. Mothers Against Drunk Driving.

Smad proteins consist of two conserved globular domains, the Mad homology (MH)1 and MH2 domain, and a variable linker region **(Figure 8)**. The *MH1 domain* is highly conserved among R-Smads and Smad4, and contains a β-hairpin structure responsible for DNA-binding and sites involved in nuclear import, while I-Smads lack the MH1 domain. The *linker* region of R-Smads encloses multiple phosphorylation sites for mitogen-activated protein kinase (MAPK) and CDKs allowing for crosstalk between different pathways. The R-Smad and I-Smad linker also contain a PY motif recognized by Smurf ubiquitin ligase for degradation. The *MH2 domain* of R-Smads has a conserved C-terminal motif, Ser–X–Ser, that is phosphorylated by activated type I receptors. In both R-Smads and Smad4 it also includes a basic pocket for oligomerisation with R-Smads. Additionally, the R-Smad MH2 domain interacts with different co-activators and co-repressors, as well as SARA for cytoplasmic retention (reviewed in (Massague et al., 2005)).

Smad-dependent & Smad-independent Signaling Crosstalk

Apart from Smad-mediated transcription, TGF-β can also activate other signaling pathways, including the Erk, JNK and p38 MAPK pathways, as well as Wnt and Notch signaling (review in (Derynck and Zhang, 2003; Guo and Wang, 2008)). A growing number of studies indicate that this crosstalk is more extended than initially thought, and although the biological consequences of non-canonical TGF-β signaling remains quite elusive, it has been shown that activation of other pathways in addition to the Smads and can result in both cooperating and counteracting outcomes (Derynck and Zhang, 2003; Kimura et al., 2000).

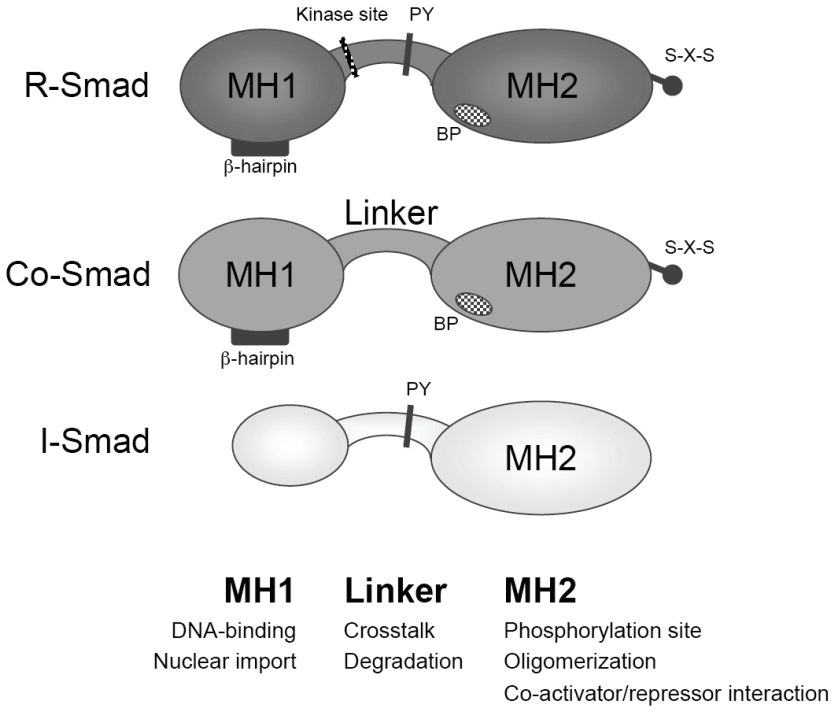


Figure 8. Structure of the Smad proteins, including a β-hairpin for DNA-binding, multiple kinase sites for crosstalk, a PY motif for degradation, a Ser–X–Ser (S-X-S) motif that is phosphorylated by activated type I receptors, and a basic pocket (BP) for oligomerisation with R-Smads.

THE ROLE OF THE TGF-β FAMILY SIGNALING IN HEMATOPOIESIS AND DISEASE

The TGF-β superfamily of growth factors regulates a wide variety of biological functions such as proliferation, differentiation, migration and apoptosis in a highly context dependent manner. The outcome of the signaling depends on environmental features, the differentiation stage of the cell, expression of assisting and opposing factors, the concentration and isoform of the cytokine, as well as if the action of the growth factor is paracrine of autocrine (Ruscetti et al., 2005). Therefore studies performed *in vitro* and *in vivo* often result in dissimilar conclusions. Members of the TGF-β signaling family have been shown to be important regulators of hematopoiesis (Larsson and Karlsson, 2005; Ruscetti et al., 2005), which will be discussed in detail below (**Figure 9**).

TGF-β

TGF-β is the founding member of the signaling family, and exists in 3 isoforms in mammals: TGFβ1 (Derynck et al., 1985), TGFβ2 (de Martin et al., 1987; Madisen et al., 1988) and TGFβ3 (Derynck et al., 1988; ten Dijke et al., 1988). These isoforms share 70-80% identity, and signal through the same receptors (TβRII, TβRI/ALK5 and ALK1). While they have similar actions *in vitro*, the isoforms display different expression patterns and *specific functions in vivo* (Fortunel et al., 2000; Larsson and Karlsson, 2005). A significant number of studies have demonstrated that TGF-β1, which is the best studied isoform, inhibit proliferation of both human and murine hematopoietic stem and primitive progenitor cells *in vitro* (Batard et al., 2000; Jacobsen et al., 1991a; Keller et al., 1990; Lu et al., 1993; Sitnicka et al., 1996), whereas further differentiated progenitors are more resistant to TGF-β inhibition (Jacobsen et al., 1991a; Keller et al., 1990). However, the effect on mature cells seems to be more complex and depend on the presence of other growth factors (Ruscetti and Bartelmez, 2001). Furthermore, hematopoietic stem and progenitor cells derived from fetal liver, which are more actively cycling than BM cells, are less sensitive to TGF-β growth inhibition (Weekx et al., 1999). Accordingly, transient blockage of autocrine TGF-β signaling by expression of a dominant negative TβRII enhances the proliferation kinetics and survival of human HSCs *in vitro* (Fan et al., 2002). In agreement with the studies performed *in vitro*, injection of TGF-β1 into the femoral artery of mice effectively inhibits multipotent hematopoietic progenitors in the BM, establishing an inhibitory role for TGF-β1 also *in vivo* (Goey et al., 1989). This suggests

that TGF-β1 is important for controlling quiescence of HSCs, and both autocrine production of TGF-β by HSCs and TGF-β produced by stromal cells in the niche can contribute to this HSC maintenance (Kim and Letterio, 2003; Ruscetti et al., 2005). As opposed to TGF-β1, TGF-β2 has been demonstrated to also function as a positive regulator of HSCs, while TGF-β3 only works inhibitory on primitive hematopoietic cells (Jacobsen et al., 1991a; Langer et al., 2004). When administered to liquid cultures of primitive murine LSK cells, TGF-β2 lead to a biphasic response with stimulatory effect at low concentrations, whereas high concentrations inhibit proliferation (Langer et al., 2004). The molecular mechanisms behind these conflicting properties of TGF-β1 and TGF-β2 remain to be established.

The exact *mechanisms* behind TGF-β-mediated effects exercised on hematopoietic stem and progenitor cells are not fully understood, but are believed to in part be due to modifications of cytokine receptor expression. Keller and colleagues have shown that TGF-β down-regulates the expression of the receptors for granulocyte macrophage (GM)-CSF, G-CSF, IL-3, IL-1, and SCF, which are all cytokines involved in regulating hematopoiesis (Dubois et al., 1990; Dubois et al., 1994; Jacobsen et al., 1991b). Growth inhibition is exerted by the ability of TGF-β to induce two classes of anti-proliferative gene responses that effectively inhibit cell cycle progression. The first is down-regulation of the growth stimulating protein c-Myc, and the second is the induction of CDKIs like p15, p21 and p27 (Massague et al., 2000). As c-Myc antagonizes TGF-β signaling by acting as a repressor of CDKIs almost all cells inhibited by TGF-β down-regulates this protein, while different cell types have different combinations of CDK-inhibitory responses. Neither p21 nor p27 appears to be required for TGF-β-mediated proliferation inhibition of primitive hematopoietic cells (Cheng et al., 2001). Recently, a study using primary human CB cells and microarray analysis demonstrated that p57 is the only CDKI induced by TGF-β. Furthermore, by inhibiting p57 with siRNA they showed that p57 is crucial for TGF-β-mediated cell cycle arrest in hematopoietic cells (Scandura et al., 2004). In most cases this arrest is believed to be reversible, supported by several studies (Batard et al., 2000; Sitnicka et al., 1996), but in some cases it leads to terminal differentiation or apoptosis. TGF-β induced apoptosis is an essential component of proper development of some tissues and organs, like mammary gland involution, and is also involved in tumor suppression (Massague et al., 2000). Additionally, TGF-β has been reported to induce apoptosis in primitive murine hematopoietic cells (Jacobsen et al., 1995).

Targeted disruption of the TGF-β1 gene results in embryonic lethality at E10.5 due to defective yolk sac vasculogenesis and hematopoiesis in about 50 % of the mice (Dickson et al., 1995; Kulkarni et al., 1993). The other mice survive until 3 weeks after birth, probably due to maternal transfer of TGF-β1 (Letterio et al., 1994), and then succumb to a severe multi-focal inflammatory disease (Shull et al., 1992). This lethal inflammatory disorder is transplantable, and is hence due to intrinsic TGF-β signaling deficiency (Leveen et al., 2002). Mice lacking TβRII also die at E10.5 and display very similar defects in yolk sac hematopoiesis and formation of blood vessels (Oshima et al., 1996). Together these studies indicate a critical role for TGF-β signaling in embryonic hematopoietic development. Mice lacking TβRI also display severe yolk sac anemia (Larsson et al., 2001). However, clonogenic assays on yolk sac-derived hematopoietic precursors *in vitro* revealed that TβRI-deficient mice exhibit normal hematopoietic potential, and even display an increased number of erythroid colonies (Larsson et al., 2001). Thus, even though TGF-β seems to inhibit early hematopoietic progenitors (Larsson et al., 2001; Park et al., 2004), TGF-β signaling does not appear to be a crucial for the specification of hematopoiesis in the developing embryo.

Since deletion of components of TGF-β signaling result in embryonic lethality, our laboratory has assessed its role in *adult hematopoiesis* using conditional knockout mice. Conditional deletion of the receptor TβRI or TβRII both result in complete block of TGF-β signaling, and display indistinguishable phenotypes including the inflammatory disease reported in conventional receptor-knockouts (Larsson et al., 2003; Leveen et al., 2002). Intriguingly, before onset of disease all hematopoietic parameters are normal (Larsson et al., 2003). By competitively transplanting BM from mice lacking the TβRI to immune-deficient nude mice, thereby bypassing the inflammatory disease, it was revealed that HSCs deficient in TGF-β signaling still possess normal proliferation kinetics and multi-lineage repopulation capacity, both in primary and secondary recipients (Larsson et al., 2003). This also held true when TGF-β signaling deficient HSCs were further challenged with repetitive myeloablative treatment that selectively kills cycling cells, or serial transplantations (Larsson et al., 2005). Conclusively, this suggests that TGF-β is not essential for regulation of HSC quiescence or self-renewal *in vivo*. The sharp contrast discovered between the role of TGF-β *in vitro* and *in vivo* probably depends on dissimilar microenvironments, but also on possible crosstalk and compensatory mechanisms in the *in vivo* setting. This further stresses the immense context dependency that regulates the outcome of TGF-β

signaling. To address the issue of compensatory mechanisms within the TGF-β signaling family, our group has employed two separate approaches to eliminate the entire TGF-β family induced Smad-specific signaling, namely by conditionally knocking out the common Smad4 (Karlsson et al., 2007), or by over-expressing the inhibitory Smad7 (Blank et al., 2006). Surprisingly, targeted deletion of Smad4 in HSCs results in impaired self-renewal and repopulative capacity, while over-expression of Smad7 leads to increased self-renewal in a Smad4 dependent manner (Blank et al., 2006; Karlsson et al., 2007). One possible explanation for the discrepancy between the two phenotypes could be that Smad4 and/or Smad7 may interact with regulatory circuits outside of the canonical Smad pathway. As mentioned above, a growing body of evidence suggests that crosstalk exists between the Smad and MAPK pathways, as well as with Wnt and Notch signaling (Derynck and Zhang, 2003; Guo and Wang, 2008). Additionally, a recent study showed that the nuclear protein Transcriptional Intermediary Factor1γ (TIF1γ) could bind to phosphorylated Smad2/3 in competition with Smad4 in CB cells, and direct the primitive hematopoietic cells toward erythroid differentiation, while Smad2/3 in complex with Smad4 mediate an anti-proliferative response (He et al., 2006).

Due to the importance of TGF-β signaling for a wide range of biological functions, deregulation of many of these factors are involved in a variety of *diseases*, including developmental disorders and various types of cancers (reviewed in (Massague et al., 2000)). Interestingly, TGF-β plays a dual role in carcinogenesis. On one hand, TGF-β works as a tumor suppressor by inhibiting cell growth as described above. This function is lost in many tumor-derived cell lines, and mutations that disable components of the TGF-β pathway are commonly encountered in human cancers including pancreatic, colon and breast cancer. On the other hand, TGF-β can promote cancer by suppressing immune-surveillance and stimulating production of angiogenic factors. Since many tumor cells selectively lose the response to TGF-β-mediated growth inhibition, but still answer to migratory signals, TGF-β can also promote invasive behavior of tumor cells (Massague et al., 2000). Although TGF-β plays an important role in regulating the balance between proliferation and differentiation in hematopoietic cells, inactivating mutations in the TGF-β signaling pathway is uncommon in leukemias and other hematological malignancies (reviewed in (Kim and Letterio, 2003)).

Activins

Activins are pleiotropic dimeric proteins that signal through different combinations of the receptors ActRIIA, ActRIIB, ActRIA/ALK2 and ActRIB/ALK4. Best studied is Activin A, which has been shown to be important for the differentiation of mesodermal precursors to the hemangioblast fate during embryonic development (Johansson and Wiles, 1995; Park et al., 2004; Pearson et al., 2008). In adults Activin A is constitutively expressed by stromal cells in the hematopoietic microenvironments (Shav-Tal and Zipori, 2002), while its expression is low to undetectable in HSCs and subsequently induced during hematopoietic differentiation (Maguer-Satta et al., 2001; Utsugisawa et al., 2006). Never the less, Activin A demonstrates an obvious inhibitory effect on proliferation of murine HSCs when cultured *in vitro* (Utsugisawa et al., 2006). Activin A also negatively regulates B-cell lymphopoiesis by inducing cell cycle arrest, mediating apoptosis and antagonizing cytokines required for normal B-cell development (Shav-Tal and Zipori, 2002; Shoham et al., 2003). Furthermore, Activin A is involved in regulation of erythropoiesis, and has been shown to enhance erythroid differentiation of primitive human hematopoietic cells *in vitro* (Maguer-Satta et al., 2003; Shav-Tal and Zipori, 2002).

BMP

BMPs were originally identified as molecules that induce bone and cartilage formation when implanted at ectopic sites in rats (Urist, 1965), but have later been shown to be multi-functional proteins that are crucial regulators of early embryogenesis and subsequent organogenesis (Hogan, 1996; Wozney, 2002). More than 15 different BMPs have been identified in mammals, out of which BMP2 and BMP4 are the best studied. BMPs generally signal through different combinations of three types of type II receptors (BMPRII, ActRIIA and ActRIIB), and two kinds of type I receptors (BMPRIA/ALK3 and BMPRIB/ALK6). However, some BMPs including BMP7 can also bind to the activin type I receptor ALK2 (Kawabata et al., 1998).

Over the last two decades a significant number of studies have revealed an important role for BMP signaling in mesoderm patterning, and subsequent *specification of hematopoiesis* in developing embryos (reviewed in (Sadlon et al., 2004; Snyder et al., 2004)). The necessity of BMP signaling in hematopoietic initiation is largely conserved through evolution from Zebrafish and Xenopus to mouse and human (Bhatia et al., 1999;

Chadwick et al., 2003; Gupta et al., 2006; Huber et al., 1998; Maeno et al., 1996; Mishina et al., 1995; Winnier et al., 1995). Targeted disruption of murine BMP4 (Winnier et al., 1995), BMP2 (Zhang and Bradley, 1996), BMPRI/ALK3 (Mishina et al., 1995) or BMPRII (Beppu et al., 2000) all result in early embryonic lethality and reduced formation of mesoderm. Hence, much of the initial knowledge about its role in blood formation is generated from studies in lower vertebrates and from the use of *in vitro* systems. BMP4 has been established as a potent inducer of ventral mesoderm in Xenopus (Dale et al., 1992), and when BMP4 was ectopically expressed using a Xenopus explant assay, it was able to induce large numbers of erythroid cells from non-mesodermal tissue in cooperation with FGF or Activin (Huber et al., 1998). Additionally, further hematopoietic commitment induced by the transcription factors GATA-1 and GATA-2 requires intact BMP signaling (Huber et al., 1998; Maeno et al., 1996). Moreover, BMP4 has also been reported to induce hematopoietic differentiation in both murine and human ES cells *in vitro* (Chadwick et al., 2003; Johansson and Wiles, 1995). This transition was recently defined as a stepwise specification driven by sequential exposure to BMP4, Activin A, FGF and VEGF (Park et al., 2004; Pearson et al., 2008). By studying murine ES cells under serum-free conditions *in vitro*, BMP4 was shown to promote very efficient formation of mesoderm, while Activin A and FGF induced differentiation into the hemangioblast fate. VEGF was required for the production of fully committed hematopoietic progenitors (Pearson et al., 2008).

In concordance with BMP ligand and receptor mutants, homozygous deletion of the BMP signaling mediator ***Smad5*** is also embryonic lethal. Smad5 deficient mice die between E9.5 and E11.5, mainly due to defects in angiogenesis (Chang et al., 1999; Yang et al., 1999). Mice lacking the related Smad1 also die around E10.5 because of failure to connect to the placenta (Tremblay et al., 2001). Smad5 has been shown to negatively regulate the proliferation and self-renewal of early multipotent hematopoietic progenitors derived from yolk sac and embryonic bodies *in vitro* (Liu et al., 2003). Although there are some controversies regarding its role in differentiation (Liu et al., 2003; Yang et al., 1999), it can be concluded that Smad5 plays a role in murine embryonic hematopoiesis, while the role of the related Smad1 and Smad8 is largely unknown. In Zebrafish, however, loss of either *Smad1* or *Smad5* causes failure in generating definitive hematopoietic progenitors (McReynolds et al., 2007).

The fact that disruption of Smad5 leads to increased self-renewal of hematopoietic progenitors proposes that Smad5 plays an opposite role of

BMP4. This is astonishing considering that Smad5 primarily is thought to mediate BMP signaling. Interestingly, the phenotype of Smad5 deficient yolk sac (Chang et al., 1999; Yang et al., 1999) resembles the phenotypes described for mice deficient in TGF-β signaling components (Dickson et al., 1995; Larsson et al., 2001; Oshima et al., 1996), suggesting that *Smad5 might mediate TGF-β signals* in the developing embryo. In addition it has been shown that Smad5, but not Smad 1 or 8, can bind to the Smad binding element (SBE) at similar levels as the TGF-β-specific Smad3 and Smad4, further suggesting that Smad5 may play a role in TGF-β signaling (Li et al., 2001). Accordingly, Smad5 has been reported to transduce inhibitory signals from TGF-β1 on proliferation of hematopoietic progenitors from human BM (Bruno et al., 1998) and primitive multipotent progenitors derived from murine embryonic bodies (Liu et al., 2003). However, in both studies loss of Smad5 was unable to reverse inhibition caused by higher doses of TGF-β1, suggesting a predominating role for other Smads or pathways in the transmission of its inhibitory effects at high concentrations. As described in **Article I**, our study reveals unaltered TGF-β sensitivity of both proliferation and colony formation of adult hematopoietic progenitors lacking Smad5, demonstrating that Smad5 is not necessary for TGF-β1 inhibition of adult murine hematopoietic progenitors *in vitro* (Singbrant et al., 2006).

While the importance of BMP signaling in embryonic hematopoiesis is well defined, very little is known about its role in *adult hematopoiesis*. A recent study demonstrated an indirect effect of BMP on the number of adult murine HSCs. By conditionally knocking out BMPRIA/ALK3 in adult mice Zhang et al. showed that impaired BMP signaling increases the niche size, and thereby enhances the number of HSCs (Zhang et al., 2003). The same study established normal reconstitution ability of ALK3 deficient cells in wt recipients, although this did not adequately address the loss of BMP signals in HSCs intrinsically, as ALK3 is not expressed by these cells (Utsugisawa et al., 2006; Zhang et al., 2003). High concentration of BMP4 has also been reported to maintain proliferation of human CB HSCs *in vitro*, while BMP2 and BMP7 and low concentrations of BMP4 inhibit proliferation (Bhatia et al., 1999). Furthermore, Shh has been shown to induce expansion of human CB HSCs *in vitro* in a BMP-dependent manner (Bhardwaj et al., 2001). In sharp contrast, when we cultured adult murine LSK $CD34^-$ cells with and without BMP4, neither high nor low concentrations of BMP4 had any effect on the proliferation of HSCs (Utsugisawa et al., 2006). These diverging results could possibly be explained by biological differences between mouse and human, or by ontogenic differences in the BMP response

(further discussed under "General discussion"). Although the effects of BMP4 on the differentiation of human BM $CD34^+$ hematopoietic progenitors are inconclusive, reports have suggested a stimulatory effect of BMP4 on both erythroid and myeloid colony formation *in vitro* (Detmer and Walker, 2002; Fuchs et al., 2002; Maguer-Satta et al., 2003). In the murine setting hematopoietic progenitors derived from BM has remained unresponsive to BMP4 stimulation, while BMP seems to play a role in stress induced erythropoiesis in the spleen (Lenox et al., 2005). As the entire BMP signaling pathway converge on the intracellular signaling transducers Smad1 and Smad5, we have investigated the full role of BMP signaling in adult murine hematopoiesis by using conditional knockout mice for Smad1, Smad5 and Smad1/5 to circumvent the embryonic lethal phenotypes of the conventional knockouts. As described in **Article I** and **Article II**, BM HSCs deficient in both Smad1 and Smad5, thereby completely blocking the BMP signaling, compete normally with wt BM and display unaffected self-renewal and differentiation capacity *in vivo*. Thus, we can conclude that BMP signaling is not required to maintain adult hematopoiesis despite its crucial role in the initial patterning of hematopoiesis in early embryonic development (Singbrant et al., 2006) (Manuscript Singbrant et al, 2008).

Endoglin

Endoglin, also known as CD105, is as mentioned earlier a TGF-β co-receptor that is crucial for regulation of *angiogenesis* (Lebrin et al., 2004). Studies in the murine system have revealed that complete deletion of endoglin in mice leads to embryonic lethality at approximately E10 due to abnormal yolk sac vasculature and cardiac defects (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). Additionally, mutations in endoglin in both humans and mice are associated with hereditary hemmoragic telangiectasia, an inherited disease characterized by malformations of the blood vessels and bleedings (Bourdeau et al., 1999; McAllister et al., 1994). Since angiogenesis is a crucial feature for tumor development, anti-angiogenic therapy is a promising cancer treatment. Interestingly, assessment of neovascularization by endoglin staining, as well as levels of soluble endoglin, has been found to represent a potential predictor of prognosis in several solid and myeloid malignancies respectively (Calabro et al., 2003; Duff et al., 2003; Fonsatti and Maio, 2004).

Endoglin has also been suggested to play a role in *hematopoiesis*. The abnormal yolk sac angiogenesis in endoglin knockout mice was in on study accompanied by anemia in the embryo proper (Arthur et al., 2000).

However, pools of red blood cells could occasionally be found in distended yolk sac vessels, suggesting that hematopoiesis per se may not have been completely impaired. Endoglin is differentially expressed on hematopoietic cells, and was recently identified as a marker defining functional long-term repopulating stem cells (Chen et al., 2003; Chen et al., 2002). The potential relevance of endoglin in hematopoiesis can be insinuated from its expression on various human hematopoietic populations, including early B-cell progenitors and erythroblasts in fetal BM (Rokhlin et al., 1995), pro-erythroblasts in adult BM (Buhring et al., 1991; Rokhlin et al., 1995), $CD34^+$ CB cells (Pierelli et al., 2000), macrophages (Lastres et al., 1992), and $CD4^+$ T-cells (Schmidt-Weber et al., 2005). Additionally, studies assessing hematopoietic differentiation of murine ES cells *in vitro* have shown that deletion of endoglin results in profound reduction in the formation of hemangioblasts (Perlingeiro, 2007), as well as impaired erythroid and myeloid differentiation (Cho et al., 2001). However, the function of endoglin in primary adult hematopoietic cells has not previously been characterized. As described in **Article III**, we have utilized viral vectors to both knock down and over-express endoglin in murine HSC. Our results suggest that the engraftment and reconstituting capacities of HSC are not critically dependent on endoglin. However, altered endoglin expression impacts erythroid proliferation and differentiation at distinct stages (Moody et al., 2007).

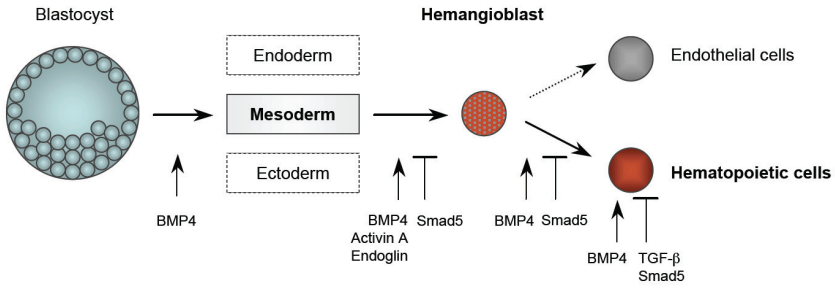


Figure 9: The role of TGF-β family signaling in early hematopoiesis. While BMP4, Activin A and Endoglin have been reported to induce the formation of hematopoietic tissue during embryonic development, TGF-β and Smad5 are indicated to regulate early hematopoiesis in an inhibitory manner. The figure is modified from Larsson & Karlsson 2005.

SPECIFIC AIMS OF THIS THESIS

The over all aim of the work included in this thesis has been to delineate the previously unknown role of two important candidates in the regulation of HSC in adult hematopoiesis, namely *BMP signaling* and *Endoglin*. More explicitly we sought the answers to the following question:

1. Does Smad5 work as a negative regulator of adult hematopoiesis as has been described in the early developing embryo?

2. Does Smad5 mediate TGF-β signals in addition to BMP signals in adult murine hematopoietic cells?

3. Is there redundancy among the Smad proteins that mediate BMP signaling in adult hematopoiesis?

4. Are there ontogenic differences in the BMP response between HSCs derived from fetal liver and bone marrow?

5. Does the cell surface marker Endoglin that identifies functional long-term repopulating HSCs play a regulatory role for these cells?

SUMMARY OF RESULTS

ARTICLE I

Smad5 is dispensable for adult murine hematopoiesis. Singbrant S, Moody J.L, Blank U, Karlsson G, Zwijsen A, Umans L, Karlsson S. *Blood* 108(12): 3707-12 (2006)

Smad5 is known to transduce intracellular signals from BMPs, which belong to the TGF-β superfamily. Recent findings suggest that BMP4 stimulates proliferation of human CB HSCs *in vitro*, while Smad5 has been reported to regulate early progenitors in murine embryonic hematopoiesis in a negative manner. In this study, we evaluate the role of Smad5 in the regulation of HSC fate decisions in adult mice. To circumvent the embryonic lethal phenotype of the conventional Smad5 knockout, we have used an inducible *MxCre*-mediated conditional knockout model. Surprisingly, analysis of induced mice reveals unperturbed cell numbers and lineage distribution in peripheral blood (PB), BM and spleen. Furthermore, phenotypic characterization of the stem cell compartment reveals normal numbers of primitive LSK cells in Smad5 deficient BM. When transplanted in a competitive fashion into lethally irradiated primary and secondary recipients, Smad5 deficient BM cells compete normally with wt cells, are able to long-term reconstitute the hosts and display normal lineage distribution. Taken together, Smad5 deficient HSCs from adult mice show unaltered differentiation, proliferation, and repopulating capacity. Therefore, in contrast to its role in embryonic hematopoiesis, Smad5 is dispensable for hematopoiesis in the adult mouse.

ARTICLE II

BMP signaling is essential to preserve colon architecture, but is dispensable for hematopoietic stem cell function in adult mice. Singbrant S, Karlsson G, Ehinger M, Jaako P, Zwijsen A, Stadtfeld M, Graf T, Karlsson S. *Manuscript* (2008)

Numerous publications have described the importance of BMP signaling in the specification of hematopoietic tissue in developing embryos. We demonstrate in **Article I** that Smad5, an intra-cellular mediator of BMP signaling, is dispensable for normal hematopoiesis in the adult mouse. In this study we further examine the possibility that the lack of phenotype in Smad5 deficient HSCs could be due to redundancy between the related Smads mediating BMP signaling, or to ontogenic differences in BMP response. We therefore investigated the full role of BMP signaling in both adult and fetal liver hematopoiesis by generating conditional knockout mice for Smad1, Smad5 and Smad1/5. Our findings demonstrate that concurrent deletion of Smad1 and Smad5 results in lethality due to extra-hematopoietic pathological changes in the colon. However, when Smad1/5 deficient BM cells are transplanted into lethally irradiated recipients, they compete normally with wt BM and display unaffected self-renewal and differentiation capacity. Moreover, even though BMP receptor expression is increased in fetal liver, Smad5 deficient fetal liver cells could still long-term reconstitute lethally irradiated recipients in a multi-lineage manner. Thus, we can conclude that canonical BMP signaling is not required to maintain adult hematopoiesis despite its crucial role in the initial patterning of hematopoiesis in early embryonic development.

ARTICLE III

Endoglin is not critical for hematopoietic stem cell engraftment and reconstitution but regulates adult erythroid development. Moody J.L, Singbrant S, Karlsson G, Blank U, Aspling M, Flygare J, Bryder D, Karlsson S. *Stem Cells* 25(11): 2809-19 (2007)

Endoglin is a TGF-β accessory receptor recently identified to define long-term repopulating HSC. However, little is known regarding its function in these cells. We have employed two complimentary approaches towards understanding endoglin's role in HSC biology; one that efficiently knocks down expression via lentiviral driven shRNA, and another utilizing retroviral-mediated over-expression. Altering endoglin expression had functional consequences for hematopoietic progenitors *in vitro* such that endoglin-suppressed myeloid progenitors display a higher degree of sensitivity to TGF-β-mediated growth inhibition, while endoglin over-expressing cells are partially resistant. However, transplantation of transduced BM enriched in primitive hematopoietic stem and progenitor cells reveals that neither endoglin suppression nor endoglin over-expression affect the ability of HSC to short-term or long-term repopulate recipient marrow. Furthermore, transplantation of cells altered in endoglin expression yields normal white blood cell proportions and PB platelets. Interestingly, decreasing endoglin expression increases the clonogenic capacity of early erythroid progenitors, while over-expression compromises erythroid differentiation at the basophilic erythroblast phase, suggesting a pivotal role for endoglin at key stages of adult erythropoietic development.

GENERAL DISCUSSION

LOSS OF FUNCTION

Conditional Knockout Mice

Complete deletion of a gene is probably the most stringent method to study its functional role *in vivo*. A limitation of conventional knockout models is that many genes are critical during embryonic development, and hence result in embryonic lethality if the gene is deleted. Although offering a good model to study development, this greatly aggravates the ability to study the role of several genes in an adult setting. Recent techniques have made it possible to conditionally knock out genes using Cre site-specific DNA recombinase (Sauer, 1998). This enzyme recognizes a specific short sequence, and efficiently deletes genes that are flanked by these so-called loxP sites ("floxed" genes) in a highly specific manner. By mating mice that carry a "floxed" gene of interest with a mouse strain having the Cre recombinase under an inducible tissue specific promoter, it is possible to control both when, and in which type of cells the gene will be deleted.

Because conventional knockouts of Smad1 and Smad5 both result in embryonic lethal phenotypes we used Cre-mediated conditional knockout mice in **Article I** and **Article II**, which have been previously described (Huang et al., 2002; Umans et al., 2003). By crossing mice with "floxed" Smad1 and Smad5 alleles respectively, we also generated double knockouts, thereby disrupting the entire canonical BMP signaling pathway. In the adult setting we utilized mice with the Cre-recombinase gene under control of the *Mx1-promoter* (*MxCre*) (Kuhn et al., 1995), which is an interferon-inducible promoter that has been shown in multiple studies to very efficiently delete "floxed" genes in hematopoietic stem and progenitor cells (Larsson et al., 2003; Singbrant et al., 2006; Wilson et al., 2004). Since deletion of Smad5 results in embryonic lethality at E10.5 due to defective angiogenesis and the *Mx1*-promoter is active also in endothelial cells, *MxCre* cannot be used to study fetal liver hematopoiesis at E14.5. We therefore generated homozygously "floxed" Smad1/5 mice with the Cre-recombinase gene under control of the *Vav*-promoter *(VavCre*). *Vav* is a pan-hematopoietic gene, and *Cre*-expression driven from the *Vav*-promoter has been reported to cause recombination in essentially all hematopoietic cells including HSCs with an efficiency of 93-96% in fetal liver, while leaving endothelial cells unaffected (Stadtfeld and Graf, 2005).

To confirm efficient deletion of Smad1 and Smad5 respectively, individual hematopoietic colonies were screened using PCR analysis. Consistent with previous reports testing *MxCre*-mediated deletion of single genes in hematopoietic colonies, exon 2 was deleted in 100 % of the colonies tested from Smad1 and Smad5 single knockout mice (Larsson et al., 2003; Singbrant et al., 2006). However, when concurrently knocking out Smad1 and Smad5, the deletion efficiency was sometimes slightly lower of one or both of the genes. Although the vast majority of the screened hematopoietic progenitors were completely lacking both Smad1 and Smad5, it can be argued that the lack of phenotype could be due to the very few cells expressing wt Smad1 or Smad5. Although we cannot entirely exclude this, we find it highly unlikely since we could not detect any differences in any hematopoietic parameters analyzed between mice having 100% deletion of both Smad1 and Smad5 and mice with slightly lower deletion efficiency. Additionally, Arnold et al recently demonstrated that concurrent heterozygous deletion of Smad1 and Smad5 is enough to result in embryonic lethality, even though single heterozygotes of Smad1 or Smad5 are normal (Arnold et al., 2006).

shRNA

One disadvantage of using conventional or conditional knockout mice is that the method is very time consuming and expensive. A much faster approach to reduce the expression of a gene is the usage of RNA interference (RNAi), which has emerged as a powerful tool to gain insight into gene function. In brief, RNAi is a sequence-specific post-transcriptional gene silencing mechanism, which is mediated by small interfering RNAs (siRNAs). Delivery of siRNAs into mammalian cells by transfection with synthetic siRNAs or DNA plasmids expressing short hairpin RNAs (shRNAs) has been shown to successfully mediate RNAi. Therefore, lentiviral vectors that express shRNA represent a powerful tool to achieve stable knock-down of gene expression in HSCs (reviewed in (Dykxhoorn et al., 2003; Shi, 2003)).

To knock down endoglin expression in **Article III**, BM enriched for stem and progenitor cells were transduced with lentivirus driving the expression of a specifically designed shRNA against endoglin (endoglin KD), or a control virus containing a scrambled shRNA (scrambled KD) that does not recognize any target in the mouse genome. The shRNA used knocks down both of the two reported isoforms of endoglin (Perez-Gomez et al., 2005). The degree of reduction in surface endoglin was typically 70-80 %. However, it should be noted that the majority of endoglin KD GFP^+

cells were negative for surface expression, while cells that were "positive" expressed very low levels. Correspondingly, quantitative reverse transcriptase (QRT)-PCR analysis of RNA from GFP^+ sorted cells demonstrated endoglin expression levels that averaged 20% of scrambled levels. We therefore estimated that we could achieve approximately 80% knockdown of endoglin expression using our shRNA strategy. Furthermore, protein suppression was sustained long-term throughout *in vivo* transplantation, thereby demonstrating that an shRNA strategy can efficiently suppress gene expression in studies of murine HSC.

GAIN OF FUNCTION

Retroviral Over-expression

As discussed under "Gene Therapy", over-expression of genes can be accomplished using viral vectors. The ability of retroviral vectors to integrate into the genome, in combination with the capacity of HSCs to long-term reconstitute hematopoiesis following myeloablation, makes it possible to study the effect of genetically modified HSCs throughout life in transplanted recipients. Additionally, the transplantation-approach limits the over-expression to the hematopoietic system. Moreover, as opposed to genetic transfer into human cells, introduction of genes into murine HSCs using retroviral vectors has proven to be both efficient and stable, and has been widely used for the purpose of evaluating gene function at the stem cell level (Dick et al., 1985). It should be kept in mind that the high levels of gene expression accomplished using this method are non-physiological. Effects revealed might hence not be relevant for the normal function of the protein. Another adverse feature of retroviral over-expression is that efficient transduction of the gene of interest is accompanied with the risk of insertional mutagenesis in the transduced cells, as observed in the SCID trials. However, as evident by our study discussed in **Article III**, the very mild phenotype seen in HSCs over-expressing endoglin is unlikely to depend on any such effect. Furthermore, we did not detect any signs of malignant proliferation, strongly supporting that the vector has not been inserted into a proto-oncogene in our case.

In **Article III** we used the MIG vector, which is a murine stem cell-based vector (MSCV) containing the reporter gene Green Fluorescent Protein (GFP). To over-express endoglin we transduced cells with the murine cDNA corresponding to murine L-endoglin, a full-length splice variant that is predominantly expressed in mouse tissues (Perez-Gomez et

al., 2005). Sustained high levels of endoglin protein were evident by western analysis of lysates from MIG endoglin cells expanded in culture for 8 days post-transduction, while endogenous levels dropped below detectable levels in differentiating MIG cells. Furthermore, QRT-PCR analysis of MIG endoglin GFP^+ cells assessed 48 hours post-transduction revealed an average 70-fold increase of endoglin RNA levels compared to MIG transduced cells. Two weeks post-transduction, at the point where MIG cells have lost endoglin surface expression in culture, FACS analysis revealed that MIG endoglin GFP^+ cells had a mean fluorescent intensity (MFI) of endoglin expression 2-fold higher than that observed for control transduced cells. Taken together, these data established the feasibility of our approach to alter endoglin expression in transduced hematopoietic cells.

REDUNDANCY IN THE SMAD SIGNALING PATHWAY

In **Article I**, we demonstrate that Smad5 is dispensable for adult murine hematopoiesis, in sharp contrast to its role as a negative regulator of primitive hematopoietic cells in the developing embryo (Liu et al., 2003). A likely explanation for this lack of effect is that the related Smad1 may functionally compensate for the deficiency of Smad5 (**Figure 10**). This theory is supported by the fact that mutants lacking BMP2, BMP4, or the upstream ALK3 receptor display earlier and/or more severe phenotypes than the Smad1 or the Smad5 knockouts (Mishina et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996). Even though Smad1 and Smad5 possess inherent specificities, demonstrated by different expression and distribution patterns (Flanders et al., 2001; Monteiro et al., 2004; Tremblay et al., 2001) as well as dissimilar knockout phenotypes (Chang et al., 1999; Lechleider et al., 2001; Tremblay et al., 2001; Yang et al., 1999) it is conceivable that the absence of effects in our earlier study is due to redundancy with Smad1. Redundant mechanisms between Smad1 and Smad5 were recently demonstrated as $Smad1^{+/-}$; $Smad5^{+/-}$ murine mutants, in contrast to Smad1 or Smad5 single heterozygotes, are embryonic lethal and display defects that closely resemble those seen in Smad1 or Smad5 homozygous mutants (Arnold et al., 2006). Additionally, Smad1 is able to rescue the $Smad5^{-/-}$ phenotype in Zebrafish (McReynolds et al., 2007). Thus, Smad1 and Smad5 share equivalent functional activities in the early embryo.

To address the issue of functional redundancy between related Smads in adult hematopoiesis in **Article II**, we created a conditional double knockout mouse for Smad1 and Smad5, enabling us to analyze the distinct

versus overlapping roles of these related Smads. To our surprise, HSCs with disrupted BMP signaling could still long-term repopulate lethally irradiated mice in a multi-lineage manner in both primary and secondary recipients, demonstrating that BMP signaling is not required for HSC self-renewal, proliferation, differentiation or engraftment. Although Smad8 is not expressed in primitive hematopoietic cells in the BM (Bhatia et al., 1999; Utsugisawa et al., 2006) we examined whether the expression levels of Smad8 was altered when knocking out Smad1 and Smad5. QRT-PCR analysis on sorted LSK cells from induced *MxCre;Smad1*$^{fl/fl}$*Smad5*$^{fl/fl}$ and wt mice demonstrated that Smad8 remained undetectable, suggesting that redundancy between related Smads is not accountable for the lack of phenotype observed in BMP signaling deficient adult hematopoiesis *in vivo*.

ONTOGENIC DIFFERENCES

Numerous publications have described the importance of BMP signaling in the formation of hematopoietic tissue in developing embryos (Snyder et al., 2004). Additionally, BMP4 has been shown to promote hematopoietic differentiation of ESCs (Chadwick et al., 2003), and to stimulate proliferation and colony formation of human primitive progenitors purified from CB *in vitro* (Bhatia et al., 1999). However, BMP4 does not have a stimulatory effect on either proliferation or colony formation of adult hematopoietic progenitors from murine BM (Utsugisawa et al., 2006). Accordingly, our results from **Article I** and **Article II** clearly demonstrates that BMP signaling is dispensable for adult hematopoiesis in the mouse, even when assessing the HSCs under stressed conditions, such as after serial transplantation. Conclusively, this indicates that the developmental context is an important factor and that this discrepancy in response to BMP may depend on ontogenic differences (**Figure 10**). In fact, Bhatia et al. demonstrated significant expression of ALK3 and ALK6 in HSCs derived from human CB, while the expression level of these BMP type I receptors in BM HSCs was very low to undetectable (Bhatia et al., 1999). The absence of these receptors has also been documented in murine BM (Utsugisawa et al., 2006).

Because targeted disruption of different components of the BMP signaling pathway all result in early death of the embryo (Beppu et al., 2000; Chang et al., 1999; Mishina et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996), the majority of the studies of BMP function in hematopoiesis are based on *in vitro* experiments using very primitive hematopoietic cells derived from murine or human ESC (Chadwick et al.,

2003; Johansson and Wiles, 1995), murine yolk sac or embryonic bodies (Liu et al., 2003; Yang et al., 1999). We were therefore interested in studying the role for BMP signaling in fetal liver hematopoiesis, a developmental phase in between the adult hematopoiesis in the BM and the primitive hematopoiesis found in yolk sac when the HSCs are massively expanded. To address the question of possible biological differences between these two ontogenic stages, we analyzed the expression level of receptors involved in BMP signaling by QRT-PCR in sorted primitive fetal liver cells (**Article II**). Intriguingly, the expression of ALK3 was markedly increased compared to levels in BM HSCs, suggesting that there may be a discrepancy in response to BMP signaling between BM and fetal liver HSCs. However, in spite of differential expression of BMP receptors, Smad5 remains dispensable in fetal liver hematopoiesis. Together this implies that cells of early ontogeny may reflect a unique population of HSCs with a distinct response to growth factors.

BMP SIGNALING IN THE COLON

Concurrent deletion of Smad1 and Smad5, but not of Smad1 or Smad5 alone, results in death due to pathology in the colon (**Article II**). Most of the BMP signaling deficient mice died within 4 weeks after induction, and all of them were anemic and tested positive for hemoglobin in the feces. Accordingly, histological examination of the gastrointestinal organs from these mice revealed pathological changes in the colon submucosa, including dilated crypts and occasional infiltration of inflammatory cells. Several studies have previously shown that BMP signaling is crucial for maintenance of normal intestinal function (He et al., 2004; Shroyer and Wong, 2007). Additionally, when we conditionally knocked out the common Smad4 in a previous study, this resulted in a dramatic colon phenotype and intestinal hemorrhage, which was hypothesized to be due to disrupted BMP signaling (Karlsson et al., 2007). Hence, the phenocopy we now see in the Smad1/5 knockout clearly confirms this hypothesis. Mice with disrupted BMP signaling also exhibit a compensatory enlargement of the spleen, and display a decrease in B-cells and increase in myeloid cells in the BM. The latter findings indicate that simultaneous deletion of Smad1 and Smad5 may affect the differentiation capacity of hematopoietic progenitors. However, the disturbed lineage distribution in the Smad1/5 knockout mice at steady state might also be explained by that these mice are moribund and are in a bad general condition. Accordingly, none of the mice transplanted with Smad1/5 deficient BM display any of the described

symptoms, supporting that the phenotype is of extra-hematopoietic origin. Interestingly, since knocking out only Smad1 or Smad5 did not result in disruption of the colon architecture, these related Smads seem to play a redundant role in maintaining colon homeostasis.

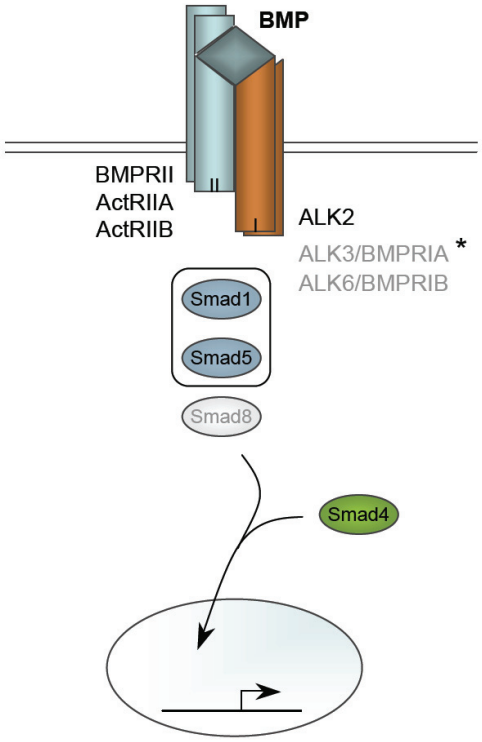


Figure 10. Ontogenic differences and redundancy in BMP signaling. ALK3, ALK6 and Smad8 (depicted in grey) are not expressed in BM derived HSCs. However, ALK3 expression is detected in fetal liver (*), and hence constitutes a possible ontogenic difference in BMP response between BM and fetal liver HSCs. Furthermore, it is possible that the related Smad1 might functionally compensate for deficiency of Smad5.

ENDOGLIN IN HEMATOPOIESIS

Endoglin is one of a number of genes expressed by endothelial cells that recently have been described as identifying markers for HSCs (Balazs et al., 2006; Chen et al., 2002; Matsubara et al., 2005). The expression of these molecules in endothelial and hematopoietic cells is intriguing given their intertwined origin (Jaffredo et al., 2005). While the role for endoglin in HSCs until now has been unknown, it is well defined in endothelial cells, where it balances TGF-β signals that promote stimulatory effects through the TGF-βRII/ALK1 complex, with inhibitory signals transduced by TGF-βRII in complex with ALK5 (Lebrin et al., 2004). Likewise, proliferation and quiescence must be carefully balanced in HSCs. However, QRT-PCR on purified $LSKCD34^-$ cells revealed undetectable levels of ALK1(Utsugisawa et al., 2006), suggesting that an analogous function for endoglin involving ALK1 in HSCs is unlikely. Accordingly, our results indicate that modulating endoglin expression has no impact on the proliferative response of hematopoietic stem and progenitor cells, as assessed *in vivo* by their reconstituting ability and *in vitro* using proliferation and colony forming assays.

In endothelial cells endoglin expression is in part regulated by hypoxia (Sanchez-Elsner et al., 2002), and endoglin has been implicated in the protection from hypoxia-induced apoptosis in these cells (Li et al., 2003). Interestingly, HSC localize to the endosteal surface in the BM, an area that is also considered to be hypoxic. It was therefore a possibility that endoglin could play a role in protecting HSCs from factors in the niche. However, the sustained engraftment levels of HSC seen in our experiments suggest that endoglin suppression does not confer a disadvantage for cells in their microenvironment, nor does over-expression of endoglin afford any advantage to these cells. It is curious that altering expression of a marker that is relatively definitive for long-term repopulating stem cells does not affect any of the measured functions of transplanted HSCs. Endoglin is thought to modulate signaling initiated by the TGF-β signaling family. Of these, the TGF-β pathway is the most thoroughly characterized especially with respect to its negative regulation of murine HSC function *in vitro* (as previously described). However, *in vivo* its role is less important to HSC function, as proven by the normal HSC reconstitution kinetics and differentiation capacity when TGF-β signaling was abolished by deleting TβRI/ALK5 (Larsson et al., 2003). It remains a dilemma if these and our results indicate a lack of importance for TGF-β and endoglin signaling in

HSC regulation *in vivo*, or if compensatory mechanisms resulting from redundancy within the TGF-β superfamily are at play.

Several lines of evidence have suggested a potential role for endoglin in erythropoiesis. Endoglin expression has been described on various human erythroid populations (Rokhlin et al., 1995). Additionally, a study using hematopoietic differentiation of endoglin deficient ESC suggests that complete deficiency of endoglin allows for progression to the $Flk1^{+}$ stage, representative of definitive hematopoiesis, but compromises differentiation towards the myeloid lineage 5-8 fold, and towards the erythroid lineage 16-fold (Cho et al., 2001). Furthermore, endoglin is found on primitive murine erythroid progenitors, and is down-regulated as cells progress from basophilic erythroblasts to polychromatophilic erythroblasts (D. Bryder, unpublished observations). Accordingly, our results suggest that endoglin negatively regulates the proliferation of primitive erythroid progenitors. Furthermore, our data reveal that differentiation through the basophilic erythroblast stage is negatively impacted by over-expression of endoglin, suggesting a critical role for its down-regulation in progression throughout this phase of maturation. The lack of altered red blood cell counts in the transplanted recipients is likely due to compensation by either un-transduced cells and/or the 5-10 % of endogenous cells that remain. Our system does not allow us to track transduced cells past this stage, as vector-driven GFP becomes undetectable in later erythroid progeny (J. Moody and D. Bryder, unpublished observations).

CONCLUSIONS

From the work included in **Article I-III** we have drawn the following conclusions:

- Smad5 is not required for TGF-β1 inhibition of hematopoietic progenitors from adult mice *in vitro* (I)

- **Smad5 deficient BM HSCs can long-term repopulate lethally irradiated mice in a multi-lineage fashion in both primary and secondary recipients. Hence, in contrast to its role in embryonic hematopoiesis, Smad5 is dispensable for hematopoiesis in the adult mouse (I)**

- **BMP signaling is not essential for HSC function in adult mice (II)**

- Redundancy between related Smads is not accountable for this lack of phenotype observed in BMP deficient adult hematopoiesis (II)

- **Smad5 remains dispensable in fetal liver hematopoiesis in spite of increased expression of BMP receptors (II)**

- Endoglin negatively regulates early erythroid proliferation, while persistence of endoglin expression at the basophilic erythroblast stage negatively impacts erythroid differentiation (III)

- **Neither inhibition, nor over-expression of Endoglin affects the ability of HSCs to engraftment and long-term reconstitute lethally irradiated recipients (III)**

FUTURE DIRECTIONS

Double Knockout for Smad1 and Smad5 in Fetal Liver Hematopoiesis

As previously described in this thesis, considerable amounts of data demonstrate that BMP signaling is crucial for the initial specification of hematopoiesis in the developing embryo. It was therefore surprising to discover that HSCs completely lacking BMP signaling display unperturbed ability to long-term reconstitute mice in a multi-lineage manner in the adult system. When comparing the expression profiles from HSCs derived from BM and fetal liver, we discovered that ALK3, which is missing in BM HSCs, is expressed in primitive fetal liver cells, suggesting that BMP may affect these cells in a different manner. Since Smad5 is the only Smad mediating BMP signals that has been implicated in regulating hematopoiesis in mammals, we investigated its role in fetal liver hematopoiesis using conditional knockout mice. Unexpectedly, Smad5 was shown to be dispensable for normal HSC function also in the fetal liver. Together this implies that cells of early ontogeny may reflect a unique population of HSCs with a distinct response to growth factors. However, it cannot be excluded that the related Smad1 is able to functionally compensate for the lack of Smad5 in the fetal liver. Therefore we have also created a Smad1/5 double knockout on *VavCre*-background, hence completely blocking canonical BMP signaling in these cells. Analysis of BMP deficient fetal liver hematopoiesis is in progress. The fact that HSCs in the fetal liver are greatly expanding, as compared to the mainly quiescent HSCs found in adult hematopoiesis, speaks in favor of that these cells might respond differently to various growth factors, such as BMP.

The Role of BMP Signaling in Human Cord Blood Hematopoiesis

As in the case of murine investigations, the majority of the human studies evaluating the role of BMP in hematopoiesis have also been carried out in embryonic cells, or in CB cells. CB represents a source of stem cells that is quite distinct from adult BM. In fact CB HSCs have been shown to be more readily cycling than the HSCs derived from BM (Lansdorp et al., 1993). Additionally, when kept in serum-free cytokine-supplemented long-term cultures *in vitro,* primitive CB cells were able to expand the number of CD34+ cells, while the number of CD34+ cells derived from primitive BM remained constant (Lansdorp et al., 1993), suggesting that these HSCs respond differentially to growth factors. In accordance, Bhatia et al.

demonstrate significant expression of ALK3 and ALK6 in CB HSCs, while the expression level of these BMP type I receptors in BM HSCs is very low to undetectable (Bhatia et al., 1999). The same group has also shown that a high dose of BMP4 is able to maintain human CB HSCs *in vitro* (Bhatia et al., 1999), and more importantly, that Shh is able to induce expansion of functional human CB HSCs *in vitro* in a BMP-dependent manner, as verified by transplanting the expanded cells into NOD/SCID mice (Bhardwaj et al., 2001). This strongly suggests that, although BMP signaling is not important for adult hematopoiesis, it may still play an essential role in *ex vivo* expansion of CB cells. Evaluation of human HSCs has been hampered by the lack of good functional assays. However, as discussed under "Assays & Methods", technical advances during recent years including the new NOG-mouse and improved vector design have provided more reliable tools to functionally study these cells. As expansion of CB cells is of utmost clinical importance, being an upcoming source for HSC transplantations (further see "Clinical Use of Hematopoietic Stem Cells"), it would be interesting to further study the role of BMP signaling in CB cells using the new improved xenograft models available. One experiment would be to culture CB cells enriched for primitive stem and progenitor cells in serum-free medium with and without BMP4 in combination with maintaining cytokines (such as SCF, TPO, Flt3L and IL-6), and subsequently assess their long-term reconstitution ability in NOG-mice. In addition, it would be interesting to concurrently knock down the expression of Smad1 and Smad5 to get a total block of BMP signaling, using the shRNA approach described in Article III, to further delineate its role in these cells.

Interactions Between BMP Signaling and Other Regulatory Pathways

We have in Article II ruled out the possibility of redundancy within the Smad-signaling pathway by knocking out both Smad1 and Smad5, and controlling for that Smad8 is not expressed. It is however possible that crosstalk with other pathways might influence the readout of our experiments. The diverging result seen when blocking the entire TGF-β family signaling by over-expressing the inhibitory Smad7 (Blank et al., 2006) or knocking out the common Smad4 (Karlsson et al., 2007) suggests that this might be an option. It has for example been shown that crosstalk between BMP and Wnt signaling is vital during early embryonic development in Xenopus (Niehrs, 2004). To address this question it would be interesting to perform an expression profile analysis of Smad1/5 deficient LSK CD34- cells to look for changes in other regulatory pathways.

Complete Knockout of Endoglin

It is unexpected that altering expression of a marker that selectively marks long-term repopulating HSCs like endoglin does not affect any of the measured functions of transplanted HSCs. We are confident that our dual approach to both over-express and knock down endoglin expression, in combination with using stringent and correct assays to assess HSC self-renewal and differentiation ability, would have revealed a potential role for endoglin in regulating hematopoiesis if there was one. Using an shRNA strategy we achieved an approximate 80% knock-down of endoglin expression, which was sustained long-term throughout *in vivo* transplantations at the protein level. However, it could be argued that the remaining 20 % of normal endoglin expression potentially might rescue a hypothetical phenotype. To address this, one could repeat our study in an endoglin knockout mouse. A conditional knockout mouse for endoglin is available, and could be crossed onto the MxCre or VavCre background to study the role of complete endoglin deficiency in adult murine hematopoiesis.

PUBLICATIONS NOT INCLUDED IN THE THESIS

1. **Smad4 is critical for self-renewal of hematopoietic stem cells.** Karlsson G, Blank U, Moody J.L, Ehinger M, Singbrant S, Deng CX, Karlsson S. *Journal of Experimental Medicine* 19;204 (3):467-74 (2007)

2. **Smad7 promotes self-renewal of hematopoietic stem cells in vivo.** Blank U, Karlsson G, Moody J.L, Utsugisawa T, Magnusson M, Singbrant S, Larsson J, Karlsson S. *Blood* 15;108 (13):4246-54 (2006)

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