Pathways that govern hematopoietic stem cell fate

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DOCTORAL DISSERTATION

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Abstract

Hematopoietic stem cells (HSCs) compose a rare population of undifferentiated cells, residing in the bone marrow of adult individuals, ensuring life-long maintenance and replenishment of the blood system. This fantastic achievement is possible owing to two special characteristics of the HSCs: their ability to make copies of themselves (self-renew), and their capacity to differentiate to all lineages of the blood system. The process of blood formation, hematopoiesis, is a dynamic and complicated process reliant on the strict balance between a large number of regulatory factors. Hematopoietic stem cell transplantation (HSCT) is currently used to treat hematological disorders such as leukemia. Cord blood is an easily accessible source of stem cells, however the number of HSCs extracted from one cord are not enough to successfully transplant adult patients. This limitation could be circumvented if we were capable of expanding stem cells outside the body. However, to reach this goal it is crucial to first understand how these cells are regulated in their natural environment. More knowledge is required to understand the interplay between different intrinsic and extrinsic factors participating in governing HSCs. *Ex vivo* HSC expansion would not only be beneficial for making HSCT accessible to a larger number of patients, but would also enable profound studies of HSC function and regulation.

In this thesis we have identified and evaluated factors involved in the regulation of HSC fate decisions. Transforming growth factor-β (TGFβ) is one of the most potent inhibitors of hematopoietic stem and progenitor cell (HSPC) proliferation *in vitro*. However, the complete mechanism behind the growth inhibitory effect and the precise function of this signaling pathway *in vivo*, is still to be unraveled. Our results in Article I suggest that Smad4 is a limiting factor for TGFβ-mediated Smad signaling critical for long-term HSC function and demonstrate that the level of Smad4 can modulate the response to TGFβ in human cells. Furthermore, we describe a negative regulatory role of the Smad signaling pathway on human HSPCs during regeneration after transplantation - affecting self-renewal capacity but not lineage choice. In Article II, we identify a transcriptional network, consisting of important stem cell regulators, TGFβ(Smad4)/GATA2/p57, that is critical in controlling the proliferation of primitive hematopoietic cells. We further generate a database of genes that become deregulated following TGFβ stimulation, and demonstrate that GATA2 is involved in a large part of the TGFβ response. At last, in Article III, we have studied the role of Pigment epithelium-derived factor (PEDF) in murine hematopoiesis. Our findings demonstrate that PEDF is an important regulatory factor for HSC regeneration and that PEDF *in vivo* works in a cell-autonomous fashion. For the first time, we propose a role of PEDF in HSC biology.

Taken together, the work in this thesis has contributed to the field by increased understanding of mechanisms and factors involved in the regulation of HSC fate decisions.

Key words: Hematopoiesis, hematopoietic stem cells, TGF beta, Smad signaling, GATA2, p57, PEDF

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”To be absolutely certain about something, one must know everything or nothing about it.”

- Olin Miller

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ABBREVIATIONS

AGM	aorta-gonad-mesonephros
ALK	Activin receptor-like kinase
AML	acute myeloid leukemia
Ang-1	angiopoietin-1
BFU-E	burst-forming unit-erythroid
BM	bone marrow
Bmi-1	B lymphoma Mo-MLV insertion region 1
BMP	bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
CAR	CXCL12-abundant reticular
CB	cord blood
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitors
CFU	colony-forming unit
CFU-GEMM	colony-forming unit -granulocyte/erythrocyte/macrophage/ megakaryocyte
CFU-S	colony-forming unit spleen
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CRA	competitive repopulation assay
CRU	competitive repopulation unit
CXCL12	C-X-C motif ligand 12
CXCR4	C-X-C chemokine receptor 4
dHSC	definitive hematopoietic stem cell
Evi1	ecotropic viral integration site-1
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
FL	fetal liver
Flt3	Fms-like tyrosine kinase 3
GFP	green fluorescent protein
GM	granulocyte macrophage
GMLP	granulocyte-monocyte-lymphoid progenitor
GVHD	graft-versus host disease

hESCs	human embryonic stem cells
HLA	human leukocyte antigen
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HSPC	hematopoietic stem/progenitor cell
iPS cells	induced pluripotent stem cells
KO	knockout
LAP	latency-associated peptide
LMPP	lymphoid-primed multipotent progenitor
LR	laminin receptor
LSK	Lin-Sca-1+c-kit+
LT	long-term
LTBP	latent TGFβ binding protein
LTC-IC	long-term culture-initiating cell
MAPK	mitogen-activated protein kinase
MDS	myelodysplastic syndrome
MegE	megakaryocyte and erythroid
mPB	mobilized peripheral blood
MPP	multipotent progenitor
MSC	mesenchymal stem cell
NOD	non-obese diabetic
NSC	neural stem cell
OPN	osteopontin
PEDF	Pigment epithelium-derived factor
RPEs	retinal pigment epithelial cells
RSC	retinal stem cell
SBE	Smad binding element
Sca-1	stem cell-associated antigen 1
SCF	stem cell factor
SDF-1	stromal derived factor 1
SCID	severe combined immune-deficient
SP	side population
SRC	SCID-repopulating cell
SVZ	subventricular zone
TAK1	TGFβ-activated kinase 1
TF	transcription factor
TGFβ	transforming growth factor-β
TPO	thrombopoietin
VEGF	vascular endothelial growth factor
wt	wildtype

LIST OF PUBLICATIONS

Articles included in this thesis:

This thesis is based on the articles listed below. The articles are referred to in the text by their roman numbers (I-III).

I. **Human hematopoietic stem/progenitor cells overexpressing Smad4 exhibit impaired reconstitution potential in vivo.** Rörby E, Hägerström MN, Blank U, Karlsson G, Karlsson S. *Blood.* 2012 Nov 22;120(22):4343-51. doi: 10.1182/blood-2012-02-408658.

II. **A network including TGFβ/Smad4, GATA2 and p57 regulates proliferation of mouse hematopoietic stem/progenitor cells.** Billing M, Rörby E, May G, Tipping A.J, Soneji S, Brown J, Salminen M, Karlsson G, Enver T, Karlsson S. *Exp Hematol.* 2016 May;44(5):399-409.e5. doi: 10.1016/j.exphem.2016.02.001. Epub 2016 Feb 10.

III. **Pigment epithelium-derived factor regulates hematopoietic stem cell maintenance.** Rörby E, Billing M, Dahl M, Andradottír S, Miharada K, Siva K, Blank U, Karlsson G, Karlsson S. *Manuscript*

Article not included in this thesis:

Signaling via Smad2 and Smad3 is not crucial for adult hematopoiesis. Billing M, Rörby E, Dahl M, Blank U, Andradottír S, Matzuk M, Ehinger M, Karlsson G, Karlsson S. *Manuscript.*

TGIF1 is a negative regulator of MLL-rearranged acute myeloid leukemia. Willer A, Jakobsen JS, Ohlsson E, Rapin N, Waage J, Billing M, Bullinger L, Karlsson S, Porse BT. *Leukemia.* 2015 May;29(5):1018-31. doi: 10.1038/leu.2014.307.

The anemia and lethal bone marrow failure in a mouse model for Diamond Blackfan anemia is cured by bone marrow transplantation without myeloablative conditioning. Dahl M, Debnath S, Jaako P, Warsi S, Billing M, Siva K, Flygare J, Richter J, Karlsson S (2015). *Manuscript*.

Loss of Scd1 affects the steady state hematopoietic progenitor cell compartment but is dispensable for hematopoiesis in bone marrow transplants. Dahl M, Warsi S, Rörby E, Billing M, Siva K, Karlsson G, Karlsson S. *Manuscript.*

PREFACE

Stem cells are unspecialized cells with the remarkable ability to give rise to many different cell types. Stem cells are discriminated from other cell types by two important characteristics. First, they have self-renewal capacity, and secondly, they can be induced to differentiate into mature specialized cells. The most primitive stem cell, the embryonic stem cell, derived from an early stage of the developing embryo, can give rise to all cells of the body. In addition, there are tissue-specific adult stem cells that have the capacity to maintain and repair a set type of tissue or organ system. Today, adult stem cell populations have been identified in a wide range of tissues including brain, eye, intestine, lung and skeletal muscle. Yet, the blood forming stem cells, hematopoietic stem cells, are by far the most studied adult stem cells. Although hematopoietic stem cell biology has been under intensive study for more than 50 years, there are many things that remain to be explored and understood.

Hematopoietic stem cell transplantation is currently used to treat hematological disorders such as leukemia. However, this treatment option is limited by the difficulty of finding genetically matched donors as well as the shortage of cells when using cord blood as the stem cell source. These problems could be circumvented if we were capable of expanding stem cells outside the body. This would not only be beneficial for making stem cell transplantation accessible to a larger number of patients, but also for the development of future cell-based therapies, as well as for deeper investigation of the disease progress in various hematological malignancies. In fact, the stem cells themselves may represent the source of origin of several diseases. Hence, it is fundamental to understand the underlying biology of the disease and how stem cells are balanced between different fate options.

In this thesis we investigate different factors involved in hematopoietic stem cell regulation. In addition to the scientific contribution of my PhD studies, I here summarize three lessons learned: 1) only a fraction of the work ever end up in publications; 2) when estimating time, include a thorough prediction of unexpected events and their duration; 3) the importance of clear communication.

Matilda Billing
Lund, April 2016

”Science never solves a problem without creating ten more.”

- George Bernard Shaw

BACKGROUND

Hematopoiesis

The hematopoietic (blood) system consists of several different cell types, all with specific functions, and is often described as hierarchical with a common precursor at the top and the mature blood cells at the bottom. The mature cells display a variety of functions that are fundamental for life. For instance, the red blood cells facilitate the transport of oxygen from the lungs to all tissues of the body, while platelets play a critical role in blood coagulation and wound healing. Another very important component in the blood are the white blood cells, constituting parts of the immune system acting as a defense barrier against pathogens, recognizing and killing foreign substances.

The process of blood formation is referred to as *hematopoiesis* and takes place in the adult bone marrow (BM). Since most mature blood cells are short lived, with a life span ranging from a few days to a few months, there is a need for constant replacement of these cellular components of the blood to maintain homeostasis (Morrison et al., 1995; Orkin, 2000). In adult humans, blood is one of the most highly regenerative tissues, producing by estimate one trillion (10^{12}) new cells every day (Ogawa, 1993). To fulfill the high demand of blood cell production throughout life, the hematopoietic system is dependent on a rare cell type called hematopoietic stem cells (HSCs).

Hematopoietic stem cells

HSCs have self-renewal capacity (can generate daughter stem cells) and multi-lineage differentiation potential, meaning that they have the capacity to give rise to cells of all the different lineages of the blood system, and at the same time maintain the stem cell pool throughout the lifetime of the organism. To prevent proliferative exhaustion of the HSC pool, the production of mature blood cells occurs in a hierarchical manner, involving many intermediate stages of progenitor cells (Figure 1). Self-renewal capacity is gradually lost as the HSCs start to differentiate towards progenitors, with an increasingly restricted differentiation potential but with immense proliferation potential. Eventually, mature blood cells restricted to a certain fate are generated.

The term *stem cell* has been used since 1896 (by Pappenheim) to describe a precursor cell in the hematopoietic system, capable of giving rise to both red and white blood cells. Although, it was not until in the early 1960s that the concept of a self-renewing, pluripotent HSC was experimentally confirmed using the spleen colony-forming assay (Till and Mc, 1961). Mouse BM cells isolated and intravenously injected into lethally irradiated hosts gave rise to colonies on the spleens of recipient mice in a dose-dependent manner. These colonies consisted of myeloid and erythroid cells and were termed colony-forming unit spleen (CFU-S) (Becker et al., 1963; Till and Mc, 1961). If re-transplanted, CFU-S could rescue irradiated mice and some of them gave rise to secondary colonies also containing T cells (Siminovitch et al., 1963). Thus, the CFU-S assay was thought to reflect HSC activity, however it was later established that it is primarily the more committed progenitors, rather than HSCs, which possess the colony forming capacity (Jones et al., 1990; Magli et al., 1982; Schofield, 1978). By retroviral marking of the BM cells prior to transplantation, making it possible to track the original integration sites in the progeny, one could confirm that all mature cells within a colony had originated from the same ancestor, since the same integration was found present in all blood lineages (Dick et al., 1985; Keller et al., 1985; Lemischka et al., 1986). Together, these initial experiments clearly demonstrated the existence of a cell in the BM that could self-renew and functionally reconstitute the entire blood system of an irradiated host, setting the groundwork for further studies of the hematopoietic system with more and more fine-tuned techniques and assays. The isolation of prospective stem cells using unique markers was subsequently required to investigate them further. The development of fluorescence activated cell sorting (FACS) made the characterization and isolation of HSCs, and other cell fractions in the hematopoietic system, possible (see section: Isolation of HSCs by FACS). Finally, in the 1990s the existence of long-term multilineage repopulating cells was undoubtedly proven, when single transplanted cells were sufficient to rescue irradiated host mice (Osawa et al., 1996; Smith et al., 1991).

Illustrating the hematopoietic hierarchy, the self-renewing, multipotent HSCs are placed at the top and give rise to multipotent progenitors (MPPs) with lost self-renewal capacity, as a result of initial differentiation events (Morrison et al., 1997). The first steps of lineage commitment remain controversial. For a long time it was believed that the MPPs could give rise to two lineages with restricted differentiation potential, represented by common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), respectively (Akashi et al., 2000; Kondo et al., 1997). However, the CLP population was found to be highly heterogeneous (Pronk et al., 2007) and the identification of a lymphoid-primed multipotent progenitor (LMPP) (and the GMLP, granulocyte-monocyte-lymphoid progenitor, with similar cell surface profile), instead suggested a common ancestry of the

granulocyte, macrophage (GM) and lymphoid lineages, separating them from the megakaryocyte and erythroid (MegE) lineages (Adolfsson et al., 2005; Arinobu et al., 2007). However, this model has also been challenged (Forsberg et al., 2006) and recent observations suggest the existence of alternative paths of commitment (Yamamoto et al., 2013). Downstream the GMLP stage, progenitors become restricted to the GM lineages or the lymphoid lineages via CLPs, where the latter holds the capacity to generate B, T and NK cells (Kondo et al., 1997). To conclude, the hematopoietic system is very complex and despite joint efforts to resolve the hematopoietic hierarchy over the years, any attempt to model this system will probably still provide a somewhat simplified view of reality. One should keep this in mind and not view the hierarchal model as a fixed map of differentiation paths.

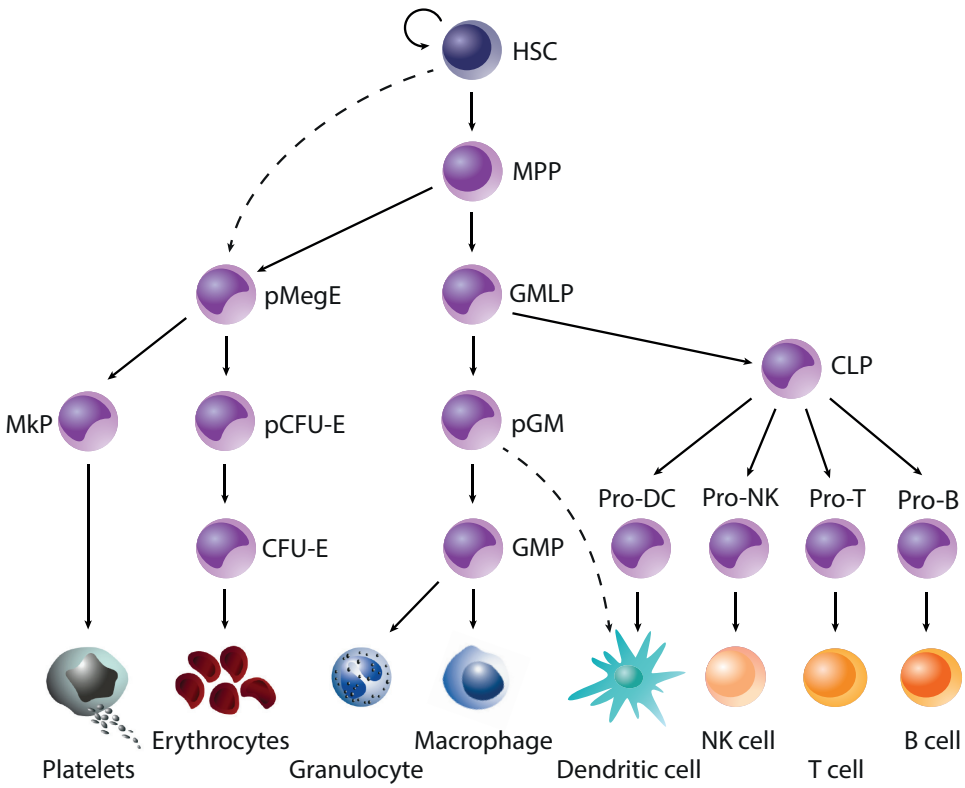


Figure 1. The hematopoietic hierarchy. Schematic depiction of the proposed hematopoietic hierarchy with differentiation paths from HSCs to mature cells. HSCs (with self-renewal capacity) reciding at the top of the hierarchy give rise to intermediate progenitors, with progressively restricted differentiation potential, which in turn generate various mature cell types at the bottom of the hierarc hy. For details see text.

Blood cell development

Adult hematopoiesis takes place in the BM, however this is not where the hematopoietic system has its origin. During ontogeny the process of blood formation is characterized by two waves and takes place at different locations in the embryo. The first wave, *primitive* hematopoiesis, gives rise to transient hematopoietic cells that are not thought to contribute to adult hematopoiesis (for a review see (Medvinsky et al., 2011)). These first hematopoietic cells arise in the blood island of the extra-embryonic yolk sac from a mesodermal precursor called the hemangioblast, at around embryonic day 7-7.5 (E7-7.5) (Choi, 1998; Moore and Metcalf, 1970). Large amounts of erythroid progeny are generated to ensure oxygen supply to the embryo, but also some primitive myeloid cells arise in the yolk sac (Medvinsky et al., 2011; Tober et al., 2007). However, in the second wave, *definitive* hematopoiesis, cells with HSC characteristics, giving rise to all mature blood cells, are generated (Medvinsky et al., 2011). One of the best described sites for definitive hematopoiesis is the aorta-gonad-mesonephros (AGM) region of the dorsal aorta (Medvinsky and Dzierzak, 1996; Muller et al., 1994), although other studies have indicated that definitive HSCs (dHSCs) can also arise from the placenta (Gekas et al., 2005; Mikkola et al., 2005; Ottersbach and Dzierzak, 2005) as well as the yolk sac (Samokhvalov et al., 2007; Yoder et al., 1997). Irrespective of their origin, HSCs start to migrate to the fetal liver (FL) at around E11 with the onset of circulation (Yokota et al., 2006). In the FL a robust expansion occurs to increase the HSC reservoir (Ema and Nakauchi, 2000) before the cells finally migrate to the BM, thymus and spleen prior to birth (Medvinsky et al., 2011). From here the BM is the major site of hematopoiesis throughout adult life. Until 3-4 weeks after birth the HSCs in the mouse BM display FL characteristics, but at this time point a developmental switch from fetal to adult HSC behavior occurs (Bowie et al., 2007).

Therapeutic potential of HSCs

Hematopoietic stem cell transplantation

Given the liquid nature of the hematopoietic system and the relative ease of HSC delivery to their host tissue, transplantation of HSCs (HSCT) has been performed successfully in humans since the beginning of the 70's (Thomas, 1999) and paved the way for other stem cell-based therapies. HSCT can be used to treat hematopoietic malignancies such as leukemias, lymphomas and immune-deficiencies. The basis for this therapy is the treatment with radio- and/or chemotherapy to eradicate the patient's blood system, enabling reestablishment of a new hematopoietic system by healthy infused HSPCs. Autologous HSCT is the re-infusion of the patients own HSCs, while allogeneic HSCT instead is carried

out with cells from a donor. A severe side effect of allogeneic HSCT is that T cells in the donor population recognize the host as non-self and cause immunological reactions, so called graft-versus host disease (GVHD), against the tissue. To avoid severe GVHD it is important to use genetically matched donors (human leukocyte antigen, HLA, compatible) and to give the patients immunosuppressive drugs (Copelan, 2006; Hardy and Ikpeazu, 1989).

Three different cell sources can be used for HSCT; aspirated BM, mobilized peripheral blood (mPB) stem cells and umbilical cord blood (CB). Traditionally, all HSCT were carried out with BM cells, but today HSCs from mPB are generally utilized (Korbling and Freireich, 2011). Since 1988, CB has also been used as an alternative cell source for HSCT (Gluckman, 2011; Gluckman et al., 1989). The advantages of using CB are that it is easily collected without any risks for the mother or baby and that it is less immunologically active, meaning that a higher degree of HLA-mismatch can be tolerated without developing severe GVHD (Rubinstein et al., 1998). Importantly, CB progenitors can also be cryopreserved in banks for long periods of time, without losing their function, making them readily accessible when required (Broxmeyer et al., 2011). However, a limitation with CB is that the number of cells isolated from one cord is usually not enough to successfully transplant adult patients (Brunstein and Wagner, 2006; Doulatov et al., 2012). To circumvent this problem one of the major goals in HSC research is to be able to expand HSCs *ex vivo*. This means that by creating a culture environment that stimulates proliferation and at the same time maintains the self-renewal characteristics of the cells, it would be possible to increase HSPC numbers prior to transplantation.

Gene therapy

In patients with various monogenic disorders of the hematopoietic system, correction of the mutation within the HSC population will theoretically provide a life-long cure of the disease (Karlsson et al., 2002; Riviere et al., 2012). Gene therapy can be performed by isolating HSCs from the patient, transduce them *ex vivo* with a vector carrying a corrected version of the gene in question, and finally re-inject the corrected cells into the patient. Protocols involving retroviral vectors to introduce the therapeutic gene have been successful in curing patients, one example being the monogenic X-linked SCID disorder (Cavazzana-Calvo et al., 2000). The long term effects of such treatment were however not completely explored and tragically some patients developed acute leukemia as a result of insertional activation of proto-oncogenes (Hacein-Bey-Abina et al., 2003). Development of safer vectors and technologies for targeted gene delivery is now crucial to re-establish the promising therapeutic potential of gene therapy (Riviere et al., 2012). Recently, our group was able to cure mice with type 1 Gaucher disease with gene therapy using safe self-inactivating lentiviral vectors, with the

corrected gene under the control of two different human cellular promoters, supporting the use of such vectors in future clinical gene therapy protocols (Dahl et al., 2015). Clinical gene therapy directed against an inherited immunodeficiency using a similar approach with self-inactivating lentiviral vectors has also been carried out successfully (Aiuti et al., 2013).

Methods of studying the hematopoietic system

One major reason why the hematopoietic system has been so well defined in the mouse is the development of advanced cell sorting techniques (FACS) making it possible to isolate functional HSCs. To evaluate whether putative HSCs are true stem cells they have to fulfill the defining criteria of HSCs in functional assays. This means that cells need to have the ability to self-renew, possess multipotential differentiation capacity as well as be able to establish a complete new blood system in an ablated host. To verify these properties at the clonal level, single cell assays are required.

Isolation of HSCs by FACS

This method is based on the recognition of cell-surface proteins that can be labeled by specific monoclonal antibodies conjugated to fluorescent proteins. The fact that cell surface molecules are expressed in different combinations depending on the maturation stage of the cells, makes it possible to isolate populations with different surface marker profiles and thereafter test their function to narrow down which population that contains the HSCs. By combining several markers expressed by HSCs, this rare population can now be isolated with high purity.

Mouse HSCs

It is well established that murine HSCs can be purified as being negative for lineage markers associated with mature cells (Lin^-) and positive for stem cell-associated antigen 1 ($Sca\text{-}1^+$) as well as c-kit ($c\text{-}kit^+$), the receptor for stem cell factor (SCF) (Ikuta and Weissman, 1992; Muller-Sieburg et al., 1986; Okada et al., 1992; Okada et al., 1991; Spangrude et al., 1988). Although being highly enriched in HSCs, the $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) population displays a strong heterogeneity and only a fraction of these cells are true long-term (LT)-HSCs (Bryder et al., 2006). The LSK population can be further enriched by the use of different additional marker combinations. For example, CD34 and Fms-like

tyrosine kinase 3 (Flt3) (LSKCD34$^-$Flt3$^-$) or signaling lymphocyte activating molecule (SLAM) family receptors CD150 and CD48 (LSKCD150$^+$CD48$^-$) are commonly used discretely, or in combination, to purify HSCs (Adolfsson et al., 2001; Kiel et al., 2005; Morita et al., 2010; Osawa et al., 1996; Yang et al., 2005). HSCs express membrane pumps involved in export of toxic compounds. This efflux activity in HSCs made it possible to isolate HSCs based on their ability to exclude the DNA binding dye Hoechst 33342 and the mitochondrial binding dye Rhodamine 123 (Wolf et al., 1993). Even stronger purification of HSCs can be obtained in a distinct subset of cells termed the side population (SP), observed if Hoechst 33342 is analyzed at two-emission wavelengths simultaneously (Goodell et al., 1996). Yet, despite great advances in this field enabling single cell transplantations, regardless of what purification strategy is used the isolated HSC compartment is still markedly heterogeneous (Copley et al., 2012). In the continuous work trying to further purify HSCs, recent identification of new markers have contributed to modest improvements of the current isolation protocols (Balazs et al., 2006; Fathman et al., 2014; Karlsson et al., 2013; Ooi et al., 2009), and the cellular basis for HSC heterogeneity remains elusive.

Human HSCs

Human stem cells were first enriched by CD34 positive selection (Civin et al., 1984). However the CD34$^+$ population is still highly heterogeneous and additional markers are crucial for better purification. Using a xenograft model, Baum et al. demonstrated that the rare Lin$^-$CD34$^+$Thy1/CD90$^+$ population in human fetal BM contained pluripotent hematopoietic progenitors (Baum et al., 1992). Additionally, the exclusion of CD45RA and CD38 was found to purify HSCs, as these markers are expressed on differentiated progenitors (Bhatia et al., 1997; Lansdorp et al., 1990). In a more recent study two additional markers (Rho and CD49f) were used to sort out Lin$^-$CD34$^+$CD38$^-$CD45RA$^-$Thy1$^+$Rho^{lo}CD49f$^+$ single cells highly efficient in generating long-term engraftment, while absence of CD49f characterized transient engraftable cells reflecting multipotent progenitors (Notta et al., 2011). Even though CD34 expression has long been used to define human HSPCs, it is controversial whether all HSCs are CD34$^+$. For example, a recent study reported Lin$^-$CD34$^-$CD38$^-$CD93^{hi} cells as placed above CD34$^+$ cells in the human HSC hierarchy (Anjos-Afonso et al., 2013).

In vivo transplantation assays

To evaluate putative HSCs, different variants of long-term repopulation assays can be used. In such a procedure recipient mice are exposed to a lethal irradiation dose, making them able to accept new HSCs, and subsequently transplanted with either a mixed population of cells containing HSCs or with purified HSCs from a donor mouse (Domen and Weissman, 1999; Morrison et al., 1995). In the new

host, donor cells will migrate to the hematopoietic organs, a process called *homing*, and start establishing a new blood system. The proof for the existence of an HSC in the transplanted test population is that multilineage donor engraftment can be detected for more than 12 weeks. At different time points after transplantation, peripheral blood can be collected and at the experimental end point BM can be analyzed for donor contribution and lineage distribution with FACS technology. By using a combination of antibodies, donor cells can be separated from recipient cells (Ly5/CD45 system; see below) and the mature blood cells can be evaluated for potential lineage skewing. At 12-16 weeks after transplantation the BM is preferably analyzed and serial transplantations can be carried out to demonstrate significant self-renewal of the stem cells in the original test population, by transferring a sample of the BM from the initially transplanted mouse to new lethally irradiated recipients (Lemischka et al., 1986).

The golden standard test for HSC function, the *competitive repopulation assay (CRA),* is a variant of the before-mentioned long-term repopulation assay where the test population is transplanted together with a competitive population, usually unfractionated normal BM (Figure 2) (Harrison, 1980).

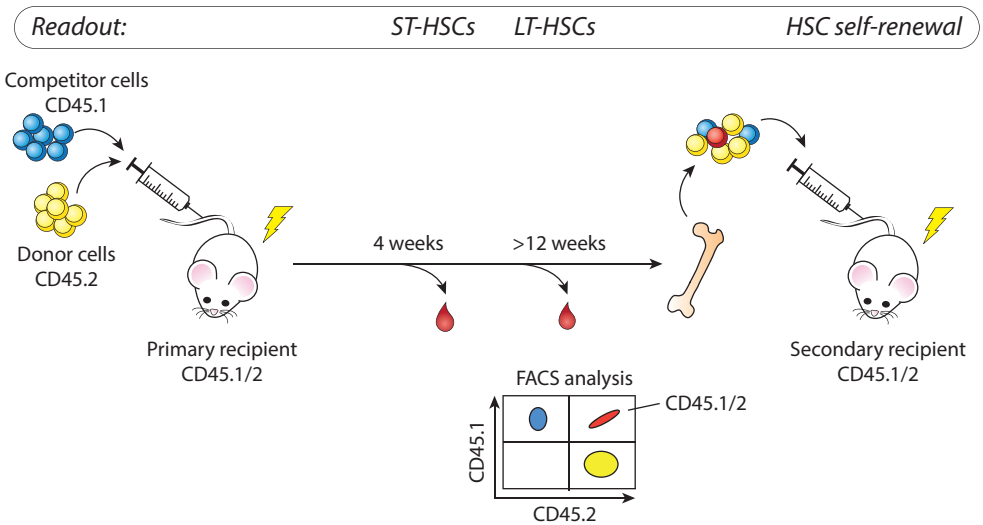


Figure 2. Competitive repopulation assay. HSCs with unknown characteristics (donor cells) are transplanted together with competitor cells from congenic mice into lethally irradiated recipients. The outcome is assesed by FACS analysis of peripheral blood at different time points. Staining for the different versions of the pan-hematopoietic gene CD45 makes it possible to distinguish between donor cells (CD45.2; yellow), competitor cells (CD45.1; blue) and recipipent cells (CD45.1/2; red). LT-HSCs give rise to multi-lineage reconstitution later than 12 weeks post transplant, while earlier readouts demonstrate the activity of less primitive ST-HSCs. To evaluate the self-renewal capacity of HSCs, bone marrow from the primary recipient are re-transplanted into secondary recipients.

Today this assay is performed using congenic mouse strains genetically identical apart from one locus, the Ly5/CD45, which is expressed on the surface of all hematopoietic cells (Shen et al., 1986). This gene exists in two isoforms making it possible to distinguish between donor, competitor and recipient cells allowing for the analysis of the relative reconstitution of these different populations. It is also possible to calculate the frequency of functional HSCs, so called competitive repopulation units (CRU), by applying limiting dilution to the CRA. Different cell doses are then transplanted together with competitor cells, and recipients with >1% LT donor chimerism in both myeloid and lymphoid lineages are considered positive for reconstitution (Szilvassy et al., 1990). Readout at 37% negative mice gives the number of cells containing one CRU, as calculated by Poisson statistics (Szilvassy et al., 1990).

Xenograft models

Due to differences in mouse and human biology, human studies complementary to mouse experiments are needed to develop relevant therapies. Human HSPCs can be functionally analyzed *in vivo* using xenograft assays. To avoid issues like immune rejection due to species differences, putative human HSPCs can be transplanted into and evaluated in immune-deficient mice. The first such model was the severe combined immune-deficient (SCID) mouse lacking B cells and T cells, later crossed with the non-obese diabetic (NOD) mouse to support higher levels of human engraftment (Bosma et al., 1983; Shultz et al., 1995). Primitive human hematopoietic cells capable of repopulating these mice are referred to as SCID-repopulating cells (SRCs) (Larochelle et al., 1996). Major limitations with this model are that the mice have a relatively short lifespan, making it difficult to assess LT HSC engraftment, and that the residual NK-cell activity limits the human engraftment potential. To circumvent these problems the NOD-SCID strain was crossed with mice displaying either a truncation or the complete absence of the IL-2R common γ chain (Il2rg) (termed NOG and NSG mice respectively) (Ito et al., 2002; Shultz et al., 2005), leading to the loss of B, T and NK cell activity and thereby improved levels of human engraftment.

In vitro assays for HSPCs

The most common *in vitro* assay, referred to as the colony-forming unit (CFU) assay, measures the frequency of hematopoietic progenitors, their proliferative capacity, as well as differentiation potential in response to hematopoietic cytokines (Wognum, 2015). Single-cell suspensions are plated in semi-solid medium containing different combinations of cytokines and are cultured a defined period of time to generate distinct colonies. Each individual colony originates from a single progenitor cell, with either erythroid, myeloid or multilineage potential giving rise to morphologically different burst-/colony-forming units-erythroid (BFU-E, CFU-

E), colony-forming units-granulocyte/ macrophage (CFU-GM) or colony-forming units-granulocyte/erythrocyte/macrophage/ megakaryocyte (CFU-GEMM), respectively.

The long-term culture-initiating cell (LTC-IC) assay is used to predict HSC frequency *in vitro* (Sutherland et al., 1990). To measure LTC-ICs a test population is kept on a layer of irradiated primary stromal cells or a stromal cell line with HSC-supportive activity. Progenitors and mature cells with limited proliferation capacity will die during the first weeks of culture, and after six to eight weeks only the most primitive cells are maintained. The remaining cells are plated in a CFU assay and the number of seeded primitive cells can be calculated based on the amount of colonies generated (Liu et al., 2013). The LTC-IC assay can be used as a complement to the CRU assay, and is regarded as a surrogate measure of multipotent cells and *in vitro* self-renewal. However, the stromal cells do not truly reflect the natural HSC environment and therefore the LTC-ICs are biologically distinct from cells that can contribute to BM engraftment *in vivo*.

HSC regulation

Fate options

HSCs are responsible for maintaining steady state hematopoiesis and additionally have to be able to respond rapidly to potential crises including acute blood loss, injury and infection, facilitating the recovery of normal blood levels. To accomplish this, a complex balance of different cell fate decisions is taking place in the natural microenvironment of HSCs. These fate options are controlled by fine-tuned interactions of intrinsic (cell-autonomous) and extrinsic (cell non-autonomous) signals (Enver et al., 1998; Morrison and Weissman, 1994; Ogawa, 1999) (described in more detail in the next sections) and include the possibility of *quiescence*, *self-renewal*, *differentiation*, *migration* from the BM or undergoing *apoptosis* (Figure 3) (Wagers et al., 2002). Still, relatively little is known about how (and if) these different intrinsic and extrinsic cues interact in networks (Enver and Jacobsen, 2009).

The process of self-renewal is a defining feature of HSCs essential to prevent depletion of the HSC pool. When a cell divides, the outcome can be either self-renewal, where at least one daughter cell preserves the stem cell properties, or differentiation, generating committed cells. A *symmetric* division will generate two identical daughter cells, either with HSC characteristics and thus expanding the HSC pool, or it will give rise to two progenitor cells destined for differentiation (Morrison and Kimble, 2006). While expansion is important during

development, and in situations of hematopoietic stress, too much self-renewal can lead to exhaustion of HSCs and improper differentiation. Balanced differentiation is critical to ensure enough production of all short-lived mature blood cells. Maintenance of the HSC pool takes place through *asymmetrical* division, resulting in one stem cell and one committed progenitor cell (Morrison and Kimble, 2006). HSCs can also leave the BM and migrate to the periphery (Wright et al., 2001). Finally, stem cells have the possibility of undergoing programmed cell death, apoptosis, to regulate stem cell numbers and to deplete clones with genetic alterations (Reya et al., 2001).

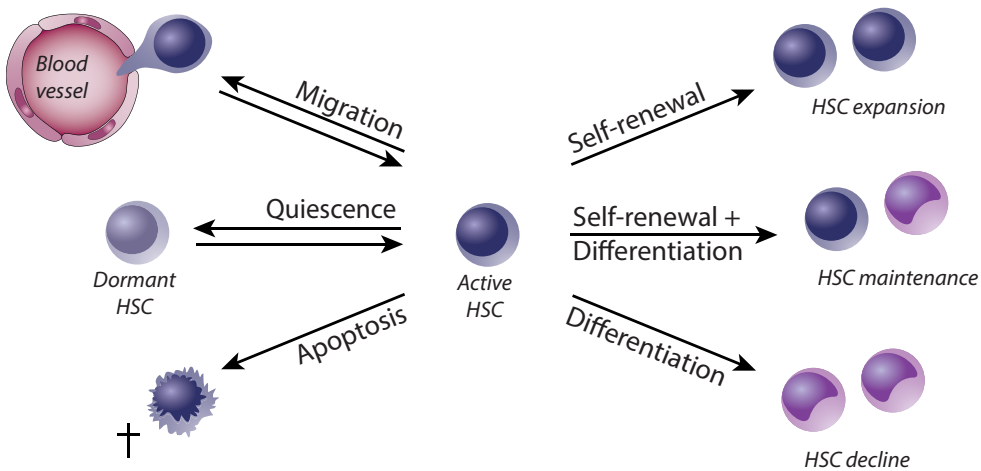


Figure 3. HSC fate options.

Cell cycle/ quiescence

Around three weeks after birth mouse development undergoes a major switch from fetal to adult hematopoiesis, completely changing the cycling rate of HSCs from highly active to an inactive hibernation state known as quiescence (Arai and Suda, 2007; Bowie et al., 2006; Pardee, 1974). It is believed that quiescence is a protective mechanism intended to prevent DNA damage and premature exhaustion of HSCs, thereby ensuring long-term performance of the otherwise stress sensitive HSC (Orford and Scadden, 2008). If the body is exposed to hematopoietic stress and urgently needs more blood cells, HSCs can be (reversibly) activated and enter the cell cycle, facilitating fast reestablishment of hematopoietic homeostasis (Essers et al., 2009; Wilson et al., 2008). Somatic cells divide as they go through the different phases of the cell cycle, G_1 (interphase), S (DNA synthesis phase) G_2 (interphase) and M (mitosis phase) (Sisken and Morasca, 1965). Fluctuations in cyclin-dependent kinases (CDKs) and cyclins control progression through the cell cycle (Lundberg and Weinberg, 1999). Inhibitory effects on CDK-cyclin

complexes may lead to cell cycle arrest, differentiation, quiescence, or even apoptosis (Lundberg and Weinberg, 1999). Important negative regulators of CDKs are the CDK inhibitors (CDKIs), divided into two families, the Ink4 and the Cip/Kip family. The Ink4 members (p15, p16, p18 and p19) cause G_1 arrest through competition with cyclin D. At low levels, the Cip/Kip family members p21, p27 and p57 bind to cyclin-CDK complexes and promote their assembly, whereas at high levels they abrogate CDK activity, thus negatively regulating cell cycle progression (Sherr and Roberts, 1999).

The DNA-labeling thymidine analog 5-bromo-2-deoxyuridine (BrdU) has been used to study the phenomenon of quiescent HSCs. *In vivo* BrdU experiments demonstrated that although 75% of the HSCs are quiescent at any given moment, they are all regularly recruited into the cell cycle, dividing on average every 57 days (Cheshier et al., 1999). However, more recent label retaining studies, using drug-inducible histone 2B-GFP (green fluorescent protein) expression, discovered that a subpopulation of the HSC population divided only every 145 days, equivalent to five times per lifetime. This suggested the presence of two populations of HSCs, where the more dormant one is thought to be triggered upon hematopoietic stress (Foudi et al., 2009; Wilson et al., 2008). Approaches to label cell surface or intracellular proteins have been taken be able to trace cell divisions by tracking the retention/loss of the label (Nygren and Bryder, 2008; Takizawa et al., 2011). For example, analysis of HSCs labeled with the fluorescent dye 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) lead to the estimation that HSCs divide on average every 39 days (Takizawa et al., 2011). Moreover, quiescent HSCs use anaerobic glycolysis and consume less oxygen than other BM cells (Simsek et al., 2010; Takubo et al., 2013). Interestingly, it has been reported that the metabolic profile of stem cells can be correlated to their functional behavior (Folmes et al., 2012; Ito and Suda, 2014)

The HSC niche and extrinsic regulation

Adult HSCs reside in a specialized three-dimensional microenvironment in the BM known as the HSC *niche*, where numerous intrinsic and extrinsic regulatory factors combine to determine HSC fate (Figure 4) (Wilson and Trumpp, 2006). The concept of the niche as the BM location that preserves the reconstituting ability of stem cells was first proposed by Schofield in 1978 (Schofield, 1978), and has been extensively explored ever since. Today, the niche is described as comprising different cell types, anatomical locations, soluble factors, signaling cascades and gradients as well as physical factors, exemplified below. The two anatomical sites that have been suggested to house HSCs include the endosteal surface, covering trabecular and compact bone at the inside of the BM space, and the perivascular area of the sinusoidal blood vessels, representing the *endosteal*

and the *vascular niches* (Ehninger and Trumpp, 2011; Kiel et al., 2005; Lo Celso et al., 2009; Mendez-Ferrer et al., 2010; Xie et al., 2009). The endosteal niche has been proposed to mediate maintenance of HSC quiescence, while the vascular niche provides an environment for active HSCs (Ehninger and Trumpp, 2011; Wilson et al., 2007). However, recent studies instead suggest that lymphoid progenitors occupy the endosteal niche, while the HSCs reside in the perivascular niche (Ding and Morrison, 2013; Greenbaum et al., 2013). Furthermore, it has been proposed that the sinusoidal vessels reside in close proximity to the endosteum (Lo Celso et al., 2009; Xie et al., 2009), insinuating that these candidate niches may actually be connected and both play important roles in the regulation of HSCs. Clearly, this issue is highly controversial and remains to be delineated with further studies. The current lack of methods to combine locational imaging with functional analysis of HCSs is however limiting the definitive determination of where in the BM the HSCs are mainly housed.

The major component of the endosteal niche is the bone-synthesizing osteoblasts (Calvi et al., 2003). Lining the endosteal bone surface, they provide factors that are important to maintain HSCs, such as thrombopoietin (TPO), osteopontin (OPN) and angiopoietin-1 (Ang-1) (Arai et al., 2004; Ehninger and Trumpp, 2011; Nilsson et al., 2005; Yoshihara et al., 2007). Osteoblasts express high levels of the chemotactic agent C-X-C motif ligand 12 (CXCL12) that interacts with C-X-C chemokine receptor 4 (CXCR4) on the surface of HSCs and other hematopoietic cells (Shahnazari et al., 2013). This interaction is not only affecting chemotaxis and homing to the BM, but has also been shown to be involved in HSC proliferation and survival (Sugiyama et al., 2006). Gradients of Ca^{2+} and oxygen also play crucial roles in HSC regulation (Adams et al., 2006; Eliasson and Jonsson, 2010; Parmar et al., 2007; Spencer et al., 2014; Suda et al., 2011). Additionally, the bone-resorbing osteoclasts have been shown to be important for HSC mobilization into circulation, by cleavage of the stromal derived factor 1 (SDF-1 or CXCL12) (Kollet et al., 2006). The developmental cue Cripto was identified as a HSC regulator since HSCs responsive to Cripto signaling was shown to be located in the endosteal region, largely quiescent with high glycolytic activity (Miharada et al., 2011).

The vascular niche is described as being composed of sinusoidal endothelial cells, perivascular stromal cells and cells of the peripheral nervous system (Kiel et al., 2005; Sugiyama et al., 2006; Yamazaki et al., 2011). Ding et al. showed that the essential HSC maintenance factor stem cell factor (SCF) is produced by perivascular as well as endothelial cells, and its deletion in those cell types leads to depletion of HSCs. However, deletion of SCF in osteoblasts and the HSCs themselves did not lead to alterations in HSC frequency or function (Ding et al., 2012). The mesenchymal stromal cells are known to express the chemokine CXCL12 in addition to SCF (Mendez-Ferrer et al., 2010; Sugiyama et al., 2006).

The presence of such CXCL12-abundant reticular (CAR) cells, in association with the microvasculature in the BM, has been shown to be an important component of the niche, since deletion of CAR cells resulted in a reduction of the HSC population (Omatsu et al., 2010). Furthermore, selective deletion of Nestin^{+} mesenchymal stem cells (MSCs), expressing several documented HSC maintenance factors, also lead to a decrease in HSC numbers, proving their important role in HSC maintenance (Mendez-Ferrer et al., 2010). Importantly, MSCs can differentiate into osteoblasts and are thereby involved in the regulation of osteoblast numbers, contributing to HSC maintenance at an additional level (Mendez-Ferrer et al., 2010). Since most perivascular cells types have been identified based on their expression of cell surface markers, there may be an overlap between different cell subsets (Wang and Wagers, 2011).

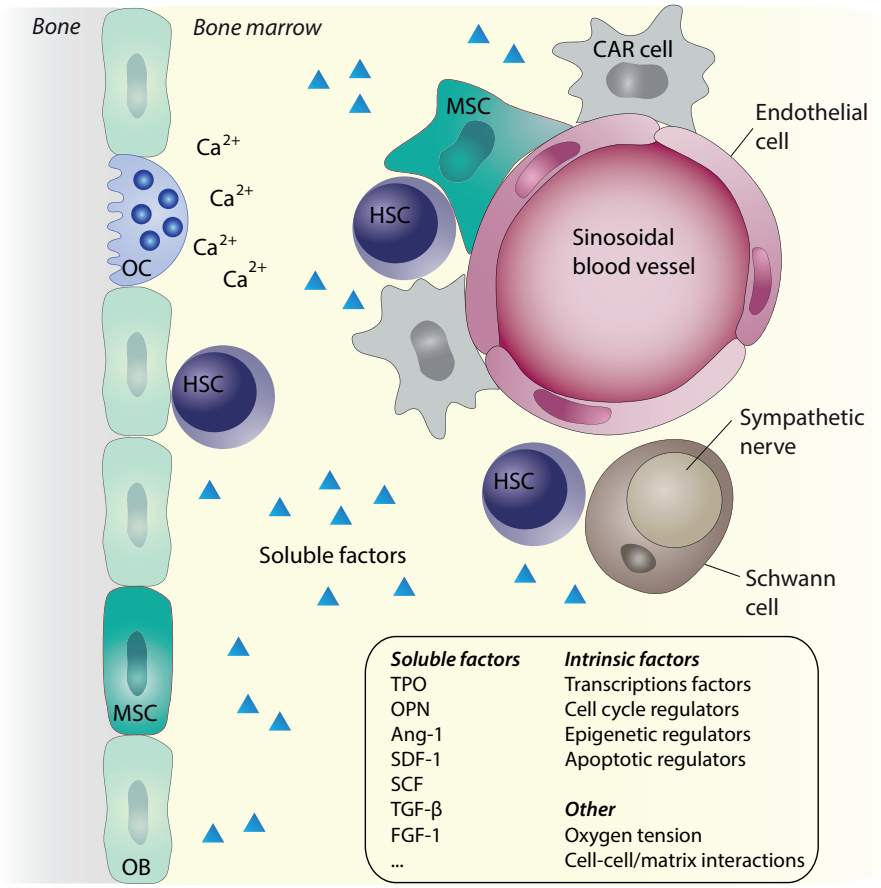


Figure 4. The HSC niche. The figure highlights the location of hematopoietic stem cells (HSCs) in the suggested endosteal and vascular niche and factors important for HSC regulation. OC, osteoclast; MSC, mesenchymal stem cell, OB, osteoblast; CAR cell, CXCL12-abundant reticular cell. Adapted from Emma Rörby, with permission.

Adrenergic fibers of the sympathetic nervous system have been shown to regulate circulating HSCs, by releasing rhythmic circadian signals controlling bone cells and mobilization of HSCs (Ehninger and Trumpp, 2011; Katayama et al., 2006).

Importantly, Yamazaki et al. showed that non-myelinating Schwann cells (associated with sympathetic nerves in the BM niche) were located in close contact with HSCs and could activate latent forms of transforming growth factor-β (TGFβ) (Yamazaki et al., 2011). TGFβ is an evolutionarily conserved growth factor with a potent inhibitory effect on hematopoietic stem and progenitor cell (HSPC) proliferation *in vitro* (Batard et al., 2000; Keller et al., 1990; Sitnicka et al., 1996), as well as a role in HSC self-renewal *in vivo* (Blank et al., 2006; Karlsson et al., 2007) (more details in the next chapter). Another growth factor, the fibroblast growth factor (FGF)-1, has been shown to expand transplantable long-term repopulating HSCs after 4 weeks in culture, being the only supplement to serum-free medium (de Haan et al., 2003). The FGF receptors (FGFR-1, -3, and -4, but not FGFR-2) were shown to be exclusively expressed on long-term HSCs, separating them from short-term HSCs and more committed progenitors (de Haan et al., 2003). In another study, LSK cells with constitutively active FGFR2 (under the Tie2 promoter) possessed increased multilineage reconstitution and decreased apoptosis after transplantation into wildtype (*wt*) mice (Shigematsu et al., 2010). To explain the role of FGF in hematopoiesis completely, it will be necessary to update our knowledge with better defined HSC populations and the usage of todays gold standard assays (Coutu and Galipeau, 2011).

Intrinsic regulation

In addition to all cell non-autonomous signals affecting HSC fate there are also several intrinsic players involved in HSC regulation, including transcription factors, transcriptional suppressors, cell cycle regulators and apoptotic signals (Domen, 2000; Ooi et al., 2010; Pietras et al., 2011; Sauvageau et al., 2004).

The homeobox (Hox) genes are well-studied transcription factors that have been implicated in HSC regulation. For example, overexpression of HoxB4, HoxA9 and HoxA10 has been shown to lead to expansion of HSCs (Antonchuk et al., 2002; Magnusson et al., 2007; Sauvageau et al., 1995; Thorsteinsdottir et al., 2002). In the absence of HoxA9 HSCs display a severely compromised reconstitution capacity (Lawrence et al., 2005). Surprisingly, HoxB4-deficient mice do not dramatically affect HSC function, which could be explained by redundancy of other Hox proteins (Bijl et al., 2006; Bjornsson et al., 2003; Brun et al., 2004).

Several cell cycle regulators, including CDKIs, have been proven to have a role in HSC biology. The G1 checkpoint regulator p21, belonging to the Cip/Kip family, is important for HSCs to be able to re-enter quiescence after cell cycle activation, as well as for maintaining the cells in dormancy (Cheng et al., 2000). This was

concluded from studies in a knockout mouse model, where deletion of p21 lead to increased proliferation, resulting in HSC exhaustion and hematopoietic failure (Cheng et al., 2000). However, deletion of p21 in another mouse strain did not result in any substantial differences in either cell cycle status or HSC number (van Os et al., 2007), demonstrating that the mouse background is important to take into consideration when drawing conclusions, since it can clearly impact the results of the study. When analyzing the mRNA expression of different Cip/Kip family members in LSKCD34$^-$ cells, Yamazaki et al. observed low levels or both p21 and p27, while a third member, p57 was highly expressed (Yamazaki et al., 2006). p57 has been suggested to play a role in HSC quiescence (Matsumoto et al., 2011; Scandura et al., 2004; Zou et al., 2011), discussed further in a later section "The role of p57 in hematopoiesis").

In the case of overexpression of the apoptotic–suppressing gene Bcl-2 the HSC population increased in size and reconstituted better than *wt* HSCs (Domen, 2000; Domen et al., 2000; Domen et al., 1998). This indicates the importance of apoptosis in the modulation of HSC numbers.

Epigenetic control, including DNA methylation, chromatin remodeling, histone modifications and non-coding RNAs, is also a key player in HSC regulation. These modifications affect the overall chromatin or DNA structure and thereby determine the transcription factor accessibility to target genes. The polycomb group members are examples of transcriptional repressors exerting such epigenetic regulation. One of them, B lymphoma Mo-MLV insertion region 1 (Bmi-1), is needed to generate self-renewing adult HSCs and loss of this protein led to impaired repopulation capacity of fetal liver HSCs (Park et al., 2003). Furthermore, enforced expression of Bmi-1 resulted in increased self-renewal *in vitro* and expansion of the stem cell pool both *in vitro* and *in vivo* (Iwama et al., 2004), confirming an important role of Bmi-1 for functional HSCs. MicroRNAs (small non-coding RNAs) offer another way of controlling HSC fate determination by affecting gene expression at the translational and post-transcriptional level (Han et al., 2010; O'Connell et al., 2010; Ooi et al., 2010).

The field of epigenetic regulatory mechanisms and their importance for cell identity has been a hot research topic in recent years. Interest in the field was stimulated by the pioneering work of Yamanaka and colleagues, demonstrating reprogramming of somatic cells to induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). The induced expression of (four) transcription factors (TF) associated with embryonic stem cells could re-specify somatic fibroblast cells to become iPS cells with the potential to differentiate into all cell types of an organism (Takahashi and Yamanaka, 2006). In addition to their regulation of genes, the reprogramming factors are thought to convert a somatic cell to a pluripotent state by rearranging chromatin architecture (Apostolou and Hochedlinger, 2013).

TGFβ signaling pathway

Transforming growth factor-β (TGFβ) is an evolutionarily conserved growth factor and the founding member of a large family of structurally related growth factors. Members of the TGFβ superfamily regulate a variety of different cellular processes including proliferation, differentiation and apoptosis, from the very beginning of life and throughout adulthood (Chang et al., 2002; Ruscetti et al., 2005). A hallmark of the TGFβ superfamily ligands is that their actions are highly context-dependent varying with dose, target cell type, and environment (Blank and Karlsson, 2011; Massague, 2012; Sporn and Roberts, 1988).

Ligands, receptors and Smads

The ligands of the TGFβ superfamily can be divided into families of bone morphogenetic proteins (BMPs), TGFβs and Activin/Nodals (Shi and Massague, 2003). The mammalian genome encodes three different isoforms of TGFβ (TGFβ-1 to 3), four Activins, and over 20 BMPs (Figure 5) (Derynck and Feng, 1997; Massague, 1998). The TGFβs are secreted as large precursor molecules that interact with two other polypeptides, latency-associated peptide (LAP) and latent TGFβ binding protein (LTBP) (ten Dijke and Arthur, 2007). LAP is responsible for the physiological inactivity by masking the receptor-binding domains of TGFβ, while binding to LTBP allows for storage of this complex in the extracellular matrix (Lawrence et al., 1985; Miyazono et al., 1991). Cleavage of the TGFβ precursor to its biologically active form relies on several proteases, including plasmin and elastase, as well as the glycoprotein thrombospondin present in the extracellular space (Crawford et al., 1998; Gleizes et al., 1997; Taipale et al., 1994; ten Dijke and Arthur, 2007). In addition, it was recently shown that non-myelinating Schwann cells in the BM are responsible for activation of latent TGFβ (Yamazaki et al., 2011).

The transduction of TGFβ-related signals is initiated upon ligand binding to type II serine/ threonine kinase receptors. The constitutively active type II receptors form a heterotetrameric receptor complex with type I receptors (also known as Activin receptor-like kinases (ALKs)) and activates them by phosphorylation of their cytoplasmic domains (Wrana et al., 1994). This initiates a phosphorylation cascade in which the intracellular receptor-activated Smads (R-Smads: Smad1-3, 5 and 8) are activated, allowing binding to the common (co)-Smad4 and translocation to the nucleus. It is here that gene targets are regulated in cooperation with other nuclear cofactors (Figure 5) (Massague et al., 2005; Mullen et al., 2011; Schmierer and Hill, 2007; Shi and Massague, 2003; ten Dijke and Hill, 2004). The inhibitory Smads (Smad6 and Smad7) inhibit the whole signaling pathway by competing

with the R-Smads to bind activated type I receptors (Hayashi et al., 1997; Itoh and ten Dijke, 2007; Nakao et al., 1997). Moreover, Smad7 can target activated receptors for degradation by the recruitment of ubiquitin ligases (Ebisawa et al., 2001; Kavsak et al., 2000) and Smad6 can associate with Smad4, creating a non-functional complex (Hata et al., 1998; Ishisaki et al., 1999).

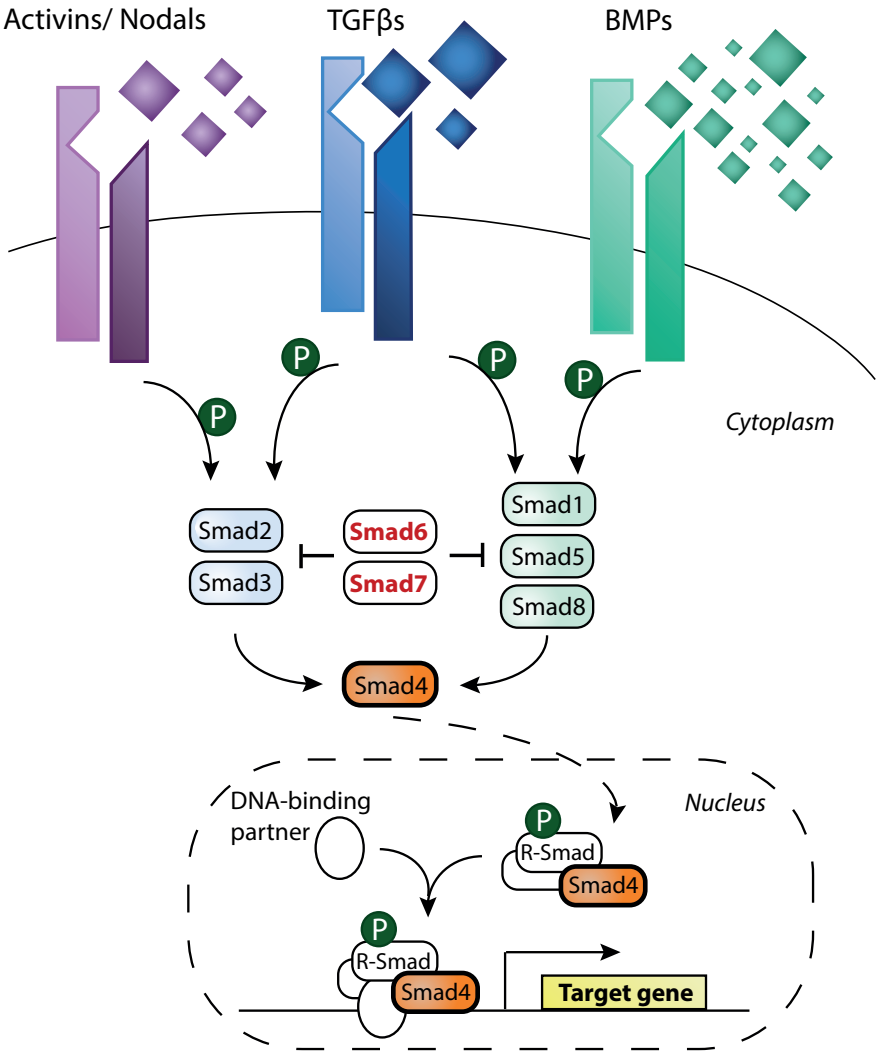
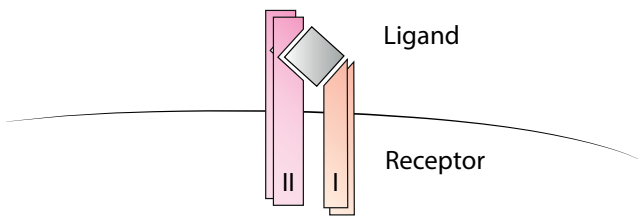


Figure 5. The TGFβ superfamily and Smad signaling pathway. Ligand binding brings together type I and type II receptors on the cell surface. Formation of the receptor complex leads to phosphorylation of the type I receptor and subsequent phosphorylation of a receptor-regulated Smad (R-Smad). The R-Smad binds to Smad4 and this complex translocates to the nucleus, where it associates to DNA-binding partners and regulate transcription of target genes.

Smad6 and Smad7 are involved in a negative feedback mechanism regulating Smad signaling, demonstrated by their accumulation in the cytoplasm upon activation of Smad signaling (Ishisaki et al., 1999; Ishisaki et al., 1998; Itoh and ten Dijke, 2007; Nakao et al., 1997). The receptors involved in the TGFβ signaling pathway include seven type I receptors and five type II receptors. Different receptor combinations will induce diverse responses, meaning that the same ligand can mediate a variety of downstream effects depending on the composition of the receptor complex (Figure 6) (Derynck and Zhang, 2003; Groppe et al., 2008).

TGFβ and Activin signaling progress mainly through Smad2 and 3, while BMP signaling is mediated by Smad1, 5 and 8 (Heldin et al., 1997; Massague, 1998). Smad proteins contain two conserved regions known as the N-terminal Mad homology domain-1 (MH1) and the C-terminal (MH2), joined by a poorly conserved linker region (Moustakas and Heldin, 2009; Shi and Massague, 2003). The MH1 domain assigns the Smads (except Smad2) with DNA-binding properties, while the MH2 domain is responsible for Smad-Smad interactions during complex formation and integration with other TFs (Massague et al., 2005). The linker region contains several phosphorylation sites for mitogen-activated protein kinase (MAPK) and CDKs, enabling crosstalk between different pathways (reviewed in (Massague et al., 2005)).



Ligand	Type II	Type I	R-Smad
Activin	ActRIIA, ActRIIB ActRIIB	ALK-4 (ActRIB) ALK-7	Smad2 Smad2
TGFβ	TβRII	ALK-5 (TβRI) ALK-1	Smad2, 3 Smad1, 5
BMP	BMPRII ActRIIA, ActRIIB	ALK-3 (BMP-RIA), ALK-6 (BMP-RIB), ALK-2 (ActRIA) ALK-3, ALK-6	Smad1, 5, 8

Figure 6. Summary of the best-documented receptor combinations and their R-Smads. Illustration modified from (Derynck and Zhang, 2003).

Specificity and transcriptional regulation

Smads bind to the DNA of the target genes at a specific sequence known as the Smad binding element (SBE) or CAGA box (Dennler et al., 1998; Johnson et al., 1999; Mullen et al., 2011; Shi et al., 1998). The R-Smads, as well as Smad4, have rather low DNA-binding affinity and therefore associate with various DNA-binding partners in the nucleus to ensure high-affinity binding (Massague and Wotton, 2000). In addition, the binding partners can be activators, such as CBP and p300, or repressors, like Ski and SnoN, and thus influence the outcome of the signaling response by positive or negative regulation of gene transcription (Massague et al., 2005; Shi and Massague, 2003). Over fifty known proteins have been reported to be interacting with either Smad2 and/or Smad3 in the nucleus (Brown et al., 2007). Additional co-factors are constantly being identified and since each co-factor mediates R-Smad-Smad4 complex binding to unique gene promoters, this leads to a vast diversity of cellular responses.

TGFβ in growth control and malignancies

The growth inhibitory effect of TGFβ has been observed in multiple cell types including hematopoietic, endothelial, epithelial, and neural cells, likely constituting its most predominant function (Massague et al., 2000). This anti-proliferative effect is exerted by the induction of two types of gene responses that effectively inhibit cell cycle progression. One response is the downregulation of c-Myc, occurring in almost all cells inhibited by TGFβ (Alexandrow and Moses, 1995). c-Myc is repressing TGFβ-induced transcriptional activation of CDKIs, thereby antagonizing TGFβ signaling (Claassen and Hann, 2000; Warner et al., 1999). The other response is the induction of CDKIs like p15, p21, p27 and p57, which inhibit the cell cycle by binding to and inactivating cyclin-CDK complexes (Massague et al., 2000; Sherr and Roberts, 1999). Different cell types have different combinations of CDK-inhibitory responses and interestingly, in primitive hematopoietic cells it seems like all Cip/Kip family members are induced by TGFβ, but only p57 is required for the induced growth arrest (Dao et al., 1998; Ducos et al., 2000; Scandura et al., 2004) (more discussed in the section “The role of p57 in hematopoiesis”).

Smad signaling has been shown to be important for the regulation of an array of physiological processes like patterning of tissues, wound healing, angiopoiesis and hematopoiesis (Chang et al., 2002). Thus, alterations in components of the TGFβ superfamily signaling lead to imbalances in the system and have consequently been shown to frequently underlie a range of human malignancies, including developmental and vascular disorders, as well as various types of cancers (reviewed in (Massague et al., 2000)). Interestingly, TGFβ plays a dual role in carcinogenesis. TGFβ commonly works as a tumor suppressor by inhibiting cell

growth, since mutations that disable components of the signaling pathway are found in a diverse set of human cancers. For example *TβRII*, *Alk5*, *SMAD2* and *SMAD4* are frequently mutated in gastric-, pancreatic- and colorectal cancers (Derynck et al., 2001; Levy and Hill, 2006). Contrarily, TGFβ can also work to promote cancer, particularly in the later stages, by stimulating angiogenesis and suppressing immune-surveillance (Hollsberg et al., 1994; Kim et al., 1991). Surprisingly, Smad signaling components are rarely found mutated in human leukemias, although some cases have been reported (Imai et al., 2001; Kim and Letterio, 2003; Scott et al., 2003; Yang et al., 2006). However, other mechanisms leading to loss of TGFβ responsiveness have been observed in leukemic cells, demonstrating an important role for TGFβ in leukemogenesis. For instance SMAD3 protein levels were undetectable in cells from T cell acute lymphoblastic despite normal mRNA levels (Wolfraim et al., 2004). In addition, a number of oncoproteins have been observed to associate with Smads and abolish the TGFβ response by for example perturbing transcription (Jakubowiak et al., 2000; Mitani, 2004). Moreover, Quere et al. showed that Smad4 sequestered the oncoproteins HoxA9 and Nup98-HoxA9 to the cytoplasm and thereby protected primitive hematopoietic cells from leukemia transformation (Quere et al., 2011).

TGFβ/Smad signaling in hematopoietic cells

Eight Smad family members have been identified in mammals (Smad1-8), of which Smad6 and Smad8 are not expressed in hematopoietic cells (Utsugisawa et al., 2006). The three isoforms of TGFβ appear to have similar actions *in vitro*, whereas *in vivo* they have been found to display different expression patterns and mediate specific functions (Fortunel et al., 2000; Larsson and Karlsson, 2005). The most studied isoform, TGF-β1, has a well-documented potent inhibitory effect on both murine and human primitive hematopoietic cell proliferation *in vitro* (Batard et al., 2000; Fortunel et al., 2000; Jacobsen et al., 1991; Keller et al., 1990; Larsson and Karlsson, 2005; Lu et al., 1993; Sitnicka et al., 1996), while more differentiated progenitors are more resistant to TGFβ inhibition (Jacobsen et al., 1991; Keller et al., 1990). Furthermore, the effect on mature cells depends on the presence of other growth factors and is even more complex (Ruscetti and Bartelmez, 2001). TGF-β3 displays only an inhibitory effect on primitive hematopoietic cells, while administration of TGF-β2 to LSK cells *in vitro* leads to a stimulatory effect at low concentrations, but inhibitory at high concentrations (Jacobsen et al., 1991; Langer et al., 2004). Interestingly, accumulating evidence suggest that the HSC compartment is comprised of distinct subsets of cells that differ in functionality as well as self-renewal and differentiation potential (Dykstra et al., 2007; Ema et al., 2014; Sieburg et al., 2006; Wilson et al., 2008). In line with this, it was recently reported that discrete HSC subtypes respond differently to TGFβ (Challen et al., 2010). TGF-β1 induced a proliferative response in

myeloid-biased HSCs, while being inhibitory to lymphoid-biased HSCs (Challen et al., 2010).

In the adult, TGFβ is a central regulator of immune function (Li et al., 2006). Consequently, mice deficient of TGF-β1 either die during embryogenesis or develop a lethal wasting syndrome and multifocal inflammatory lesions, leading to death as early as three to four weeks after birth (Kulkarni et al., 1993; Letterio et al., 1994; Shull et al., 1992). Accordingly, conditional deletion of the TGFβ receptors *Alk5* and *TβRII* in BM cells lead to a similar pathologic phenotype as the one observed in *TGFβ*$^{-/-}$ mice (Larsson et al., 2003; Leveen et al., 2002). Importantly, Leveen et al. demonstrated that the BM cells were causing the disorder, since the inflammation was transplantable (Leveen et al., 2002). To circumvent the inflammatory phenotype, Yamazaki et al. generated a TβRII knockout in an immune-deficient mouse background (Rag). In this model they demonstrated increased cycling and reduced long-term repopulation capacity of HSCs (Yamazaki et al., 2011). However, when transplanting TβRI-deficient HSCs into immune-deficient mice the regenerative function was unaltered (Larsson et al., 2005). These conflicting results could perhaps be explained by redundancy or by crosstalk mechanisms with non-canonical signaling pathways.

The importance of the TGFβ signaling pathway in HSC biology was highlighted anew after observations of the Smad pathway being specifically activated in HSCs, in combination with studies where deletion of TGFβRII or Smad4 in genetic mouse models results in increased proliferation and decreased self-renewal of HSCs (Karlsson et al., 2007; Yamazaki et al., 2011; Yamazaki et al., 2009). Thus, this strongly indicates that TGFβ plays a critical role in maintaining HSCs in quiescence. Accordingly, recent work suggests that the physiological relevance for TGFβ in HSC biology is to keep HSCs quiescent and re-establish HSC homeostasis following hematopoietic stress (Brenet et al., 2013). Surprisingly, our group has previously shown that TGFβ receptor type I (TGFβRI/ALK5) KO mice do not have any HSC phenotype (Larsson et al., 2003; Larsson et al., 2005) and studies on the BMP pathway similarly showed that mice deficient in all BMP signaling in adult hematopoiesis exhibit normal HSC function (Singbrant et al., 2010). Furthermore, overexpression of Smad7 lead to the opposite phenotype of deletion of Smad4, with increased self-renewal (Blank et al., 2006; Karlsson et al., 2007). The fact that both Smad4 and Smad7 phenotypes are lost *in vitro* indicates that Smad signaling regulates the most primitive HSCs *in vivo* through mechanisms that are dependent on the niche. To conclude, our previous studies demonstrate that deletion of the BMP signaling or single molecules of the TGFβ pathway, do not result in an effect on the HSC compartment. This may be due to redundancy by other molecules in the TGFβ signaling pathway, since disruption of the whole signaling pathway alters HSC function.

While the mechanism mediating TGFβ signaling is well characterized, the molecular mechanisms underlying the downstream effector response in hematopoietic progenitors are poorly understood. In **Article II** we identified a network of genes involved in TGFβ-induced proliferation arrest in hematopoietic progenitor cells.

The role of p57 in hematopoiesis

As previously discussed, the expression of CDKIs is induced in most cell types whose growth is inhibited by TGFβ. When it comes to hematopoietic cells it has been suggested that p21 and p27 are dispensable for the TGFβ-induced growth arrest (Cheng et al., 2001). Interestingly, TGFβ has been reported to induce expression of the cell cycle inhibitor p57 in hematopoietic progenitor cells (HPCs) (Scandura et al., 2004) and HSCs (Yamazaki et al., 2009), this activation being crucial for TGFβ-induced cell cycle arrest *in vitro* (Scandura et al., 2004). Accordingly, long-term HSCs have been reported to express high levels of p57, while short-term HSCs do not (Passegue et al., 2005; Umemoto et al., 2005; Yamazaki et al., 2006; Zou et al., 2011). In addition, p57 is a likely downstream effector molecule of TGFβ signaling in HSPCs *in vivo*, since $p57^{-/-}$ HSCs from a conditional knockout model lose quiescence and possess severely impaired self-renewal capacity (Matsumoto et al., 2011), similar to phenotypes observed in TGFβ/Smad loss-of-function models. The colony-forming ability of HSCs was further reduced with additional ablation of p21 in the p57-null background (Matsumoto et al., 2011). Of note, p27 was reported to be able to compensate for p57 loss, however deletion of both proteins altered cell cycle kinetics. These observations suggested that regulation of cytoplasmic localization of the heat shock cognate protein 70 by p27 and p57 is a critical mechanism in controlling HSC quiescence (Matsumoto et al., 2011; Zou et al., 2011). Furthermore, active TGFβ signaling, demonstrated by phosphorylation of Smad2 and Smad3, was found to correlate with high level of p57 in freshly isolated HSCs, whereas progenitor cells had neither phosphorylated Smad2/Smad3 nor detectable p57 levels (Yamazaki et al., 2009). This suggests that TGFβ has a role in keeping HSCs in a quiescent state through a mechanism involving the transcriptional activation of p57 (Figure 7).

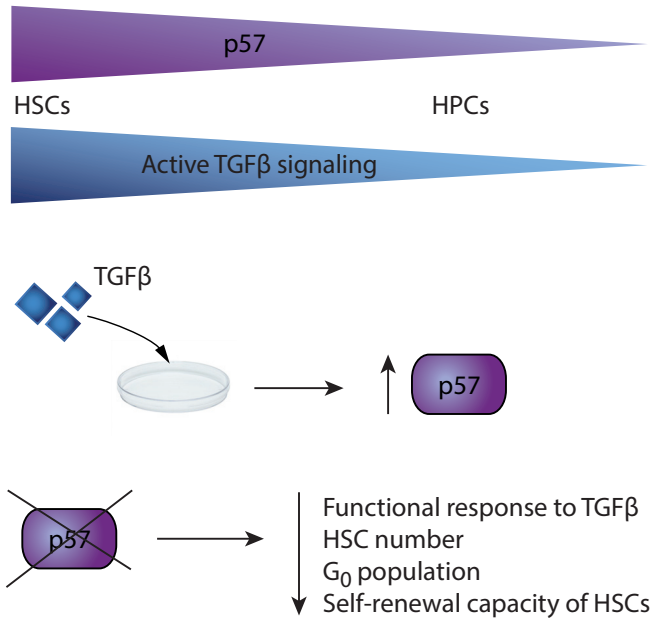


Figure 7. Summary of observations demonstrating a connection between p57 and TGFβ signaling in primitive hematopoietic cells. See text for details.

Non-canonical TGFβ signaling

In addition to the canonical Smad signaling, TGFβ can activate other non-canonical pathways such as Erk, JNK and p38 MAPK pathways (Derynck and Zhang, 2003). In the MAPK pathway TGFβ has been shown to signal through TGFβ-activated kinase 1 (TAK1) (Yamaguchi et al., 1995). In a more recent study, deletion of TAK1 resulted in apoptotic death of hematopoietic cells and subsequent BM failure, demonstrating its importance for HSC survival (Tang et al., 2008). Furthermore, unpublished observations from our lab demonstrate that BMP signaling is required for hematopoietic regeneration following serial BMT, via mechanisms separated from the canonical Smad pathway (Blank, 2016). Using a conditional BMP type II receptor (BMPR-II) knockout model, indirect reduction of p38 phosphorylation and importantly excessive expression of the tight junction protein *TJP1* was observed in BMPR-II$^{-/-}$ HSCs (Blank, 2016). Considering the documented role of *TJP1* as a self-renewal regulator in other stem cells types (Bruno et al., 2004; Ramalho-Santos et al., 2002; Xu et al., 2012), this suggests that the disruption of self-renewal in BMPR-II$^{-/-}$ HSCs is mediated via the upregulation of *TJP1*.

GATA transcription factor family

The GATA family of nuclear regulatory factors is constituted of evolutionary conserved proteins that play important roles in the development and differentiation of vertebrates (Ko and Engel, 1993). They bind the consensus DNA sequence (A/T)GATA(A/G) through two zinc finger domains, hence their name (Ko and Engel, 1993; Merika and Orkin, 1993). In mammals, the GATA family is composed of six members, whereof GATA1-3 are primarily expressed in hematopoietic cells (Weiss and Orkin, 1995). GATA1 and GATA3 are recognized as lineage-specific TFs, GATA1 affecting erythroid cells (Pevny et al., 1991; Simon et al., 1992), megakaryocytes (Shivdasani et al., 1997; Vyas et al., 1999), and eosinophils (Hirasawa et al., 2002), while GATA3 has an impact on T cells (Pandolfi et al., 1995). GATA2 is predominantly expressed in early progenitors as well as in megakaryocytes and mast cell lineages (Jippo et al., 1996; Minegishi et al., 1998; Orlic et al., 1995; Tsai et al., 1994; Tsai and Orkin, 1997). However, these factors are also present in other tissues, where they are important for development (Vicente et al., 2012a). GATA4-6 are mainly regulating tissue-specific gene expression in tissues of mesodermal and endodermal origin such as the heart, liver, lung, gut and gonads (Vicente et al., 2012a). The absence of either of the GATA genes, with the exception of GATA5 (Molkentin et al., 2000), results in embryonic lethality in mice, showing that these transcriptional regulators are essential during development (Brewer et al., 1999; Fujiwara et al., 1996; Kuo et al., 1997; Ling et al., 2004; Pandolfi et al., 1995; Pevny et al., 1991; Tsai et al., 1994; Tsai and Orkin, 1997). *In vivo* replacement experiments indicate that GATA factors can functionally substitute each other with regard to erythropoiesis, whereas the GATA1 protein is indispensable for megakaryopoiesis (Vicente et al., 2012a). Thus, the redundancy between GATA factors is limited and dependent on the cellular context.

The role of GATA2 in hematopoietic stem cells

GATA2 is required for HPC maintenance, proliferation and/or survival during definitive hematopoiesis. More specifically, GATA2 null embryos are anemic, have somewhat reduced numbers of primitive erythroid cells and die at day E10-11, the time of HSC induction and expansion (Tsai et al., 1994; Tsai and Orkin, 1997). Additionally, GATA2 null embryonic stem cells do not contribute to any hematopoietic tissue in chimeric mice (Tsai et al., 1994). In a later study, Ling et al. showed that GATA2 plays at least two functionally distinct roles during ontogeny (Ling et al., 2004). Through analyzing $GATA2^{+/-}$ embryos a quantitative reduction of HSCs specifically in the AGM was observed. However, although quantitatively normal, adult $GATA2^{+/-}$ HSCs performed worse than their *wt*

counterpart in a competitive transplantation setting, demonstrating a qualitative defect in those cells. In addition, regeneration of the hematopoietic system after cytotoxic treatment was delayed in GATA2$^{+/-}$ HSCs, suggesting that the level of GATA2 is involved in the regulation of proliferation. Thus, these results implicate that GATA2 plays a role in the production and expansion of HSCs in the AGM region, as well as in the proliferation of HSCs in the adult BM (Ling et al., 2004). Haploinsufficiency of GATA2 in adult mice resulted in reduced number of functional HSCs and notably the HSC compartment was more quiescent and apoptotic (Rodrigues et al., 2005). However, estradiol-induced GATA2 was shown to inhibit growth in progenitor-enriched primary cells, via a mechanism including accumulation of p21 and p27, suggesting that GATA2 contributes to quiescence by controlling the levels of cell cycle regulators (Ezoe et al., 2002). In line with this, enforced expression of GATA2 in human HPCs lead to increased quiescence (Tipping et al., 2009). Moreover, constitutive expression of GATA2 in mouse BM blocked both amplification and differentiation of pluripotent cells (Persons et al., 1999). Enforced GATA2 expression in a multipotent hematopoietic progenitor cell line also abrogates self-renewal by inhibiting growth but at the same time promotes differentiation, contradictory to other results (Heyworth et al., 1999). These conflicting results may be due to differences between the cell types used in the experiments, variation in effect *in vivo* and *in vitro* or a potentially different behavior of the artificially generated GATA2 proteins in the overexpression models (Kitajima et al., 2002).

Furthermore, transcription of GATA2 is regulated by several loci including BMP4, CEPBA, EVI1, HOXA9, NOTCH1 and PU.1 (Vicente et al., 2012a). The oncogenic TF ecotropic viral integration site-1 (Evi1) has been reported to regulate HSC proliferation through GATA2 (Yuasa et al., 2005). Evi1 is predominantly expressed in HSCs and Evi1$^{-/-}$ embryos contained fewer HSCs, which in addition showed defective self-renewing proliferation and repopulating capacity, and died at day E10.5 similarly to GATA2 null embryos. GATA2 expression was profoundly reduced in HSCs derived from Evi1$^{-/-}$ embryos, and restoration of either GATA2 or Evi1 expression in this population could revert the *in vitro* phenotype (Yuasa et al., 2005).

To better understand the role of GATA2, downstream targets mediating its effects have been investigated in mature cell types such as megakaryocytes and erythroid cells (Fujiwara et al., 2009; Huang et al., 2009). However, the targets in primitive hematopoietic cells are not well studied. Recently, it was suggested that GATA2 regulates HSC proliferation through TGFβ signaling (Dong et al., 2014) and in **Article 2** we report additional findings of a network between GATA2 and TGFβ.

GATA2 in human pathophysiologies

More evidence for the important role of GATA2 in HSCs arises from recent reports of the involvement of GATA2 in various hematological malignancies. Around 100 GATA2 mutations have been documented either as germ-line genetic defects or somatic mutations along with other drivers, as for example biallelic CEBPA mutation in AML (reviewed in (Collin et al., 2015)). Despite the wide variety of mutations, including whole gene deletions, single nucleotide deletions, frame-shift and nonsense mutations, the functional effects of heterozygous mutations are primarily due to haploinsufficiency of GATA2 (Collin et al., 2015). In addition to mutations, post-transcriptional regulation of GATA2, such as hypermethylation, can also lead to loss of expression and be the cause of disease (Celton et al., 2014). Four human clinical syndromes harboring germline mutations in GATA2, uncovered by independent research groups, are associated with increased incidence of myeloid neoplasia, either myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) (Collin et al., 2015). Three of the syndromes, monocytopenia/ mycobacterium avium complex (MonoMAC) (Hsu et al., 2011; Vinh et al., 2010), dendritic cell, monocyte, B- and natural killer lymphoid (DCML) deficiency (Dickinson et al., 2011) and Emberger's syndrome (Ostergaard et al., 2011) also share the feature of immune dysfunction, while the forth, familial MDS/AML (Hahn et al., 2011) is not associated with immune dysfunction. Although individuals with defective GATA2 alleles are often healthy at childhood they bear a 90% risk of developing clinical complications by 60 years of age (Collin et al., 2015). Two recent studies highlight that carriers with GATA2 mutations are hematologically normal at birth with mononuclear cytopenia or DCML deficiency developing over time and finally that a feature common for all patients with symptoms is the loss of mononuclear cells (Dickinson et al., 2014; Spinner et al., 2014). The hypothesis that dysregulation of GATA2 expression may contribute to leukemogenesis arising more than 10 years ago (Wieser et al., 2000) was recently confirmed in 3q rearrangement involving removal of the GATA2 distal hematopoietic enhancer and simultaneous *cis*-activation of the neighbouring locus EVI1 (Groschel et al., 2014; Yamazaki et al., 2014). GATA2 has also been found to be upregulated in AML and this predicts a poor prognosis (Shimamoto et al., 1995; Vicente et al., 2012b). Additionally, Zhang et al. identified a gain-of-function mutation occurring specifically in the accelerated phase of blast crisis of chronic myeloid leukemia (CML) (Zhang et al., 2008; Zhang et al., 2009). Together, these results emphasize the importance of a strict regulation of the level of GATA2.

Pigment epithelium-derived factor

The functions of PEDF

Pigment epithelium-derived factor (PEDF; encoded by *SERPINF1*), a non-inhibitory member of the serine protease inhibitor (serpin) superfamily, is well conserved in evolution and widely expressed in many tissues of the body including brain, liver, bone, adipose tissue, eye, ovary, testis, plasma, heart, kidney and lung (Becerra et al., 1995; Steele et al., 1993; Tombran-Tink et al., 1996). PEDF was originally identified and isolated from conditioned medium of cultured human retinal pigment epithelial cells (RPEs) (Tombran-Tink and Johnson, 1989). Over the years, several important functions of this protein, such as anti-angiogenic, anti-tumorogenic, anti-inflammatory and neuroprotective properties, have been described (Bilak et al., 1999; Cao et al., 1999; Dawson et al., 1999; Garcia et al., 2004; Zhang et al., 2006). A small selection of the reported effects of PEDF in various cell types and diseases will be discussed below.

In 1999, Dawson et al. observed that PEDF was a very potent inhibitor of angiogenesis, exhibiting a greater anti-angiogenic effect than any other known endogenous factor (Dawson et al., 1999). Both Fas-Fas ligand and p38 MAP kinase have been found to mediate PEDF-induced apoptosis of endothelial cells that have been activated by various angiogenic inducers, thereby restraining angiogenesis (Chen et al., 2006; Volpert et al., 2002). One key pro-angiogenic factor is vascular endothelial growth factor (VEGF), which has been shown to be inhibited/downregulated by PEDF in several tissues including the eye and osteosarcoma cells (Haurigot et al., 2012; Takenaka et al., 2005). Clearly, the balance between these factors is essential for angiogenic homeostasis. PEDF has also been reported to act as a potent neurotrophic factor inducing cell differentiation and protecting neurons in the brain, eye and spinal cord against a series of neurodegenerative insults such as ischemia, glutamate excitotoxicity, axotomy and oxidative stress (Bilak et al., 2002; Cao et al., 1999; DeCoster et al., 1999; Houenou et al., 1999). The major intracellular mediator in these processes is nuclear factor kappa-B (NFκB). Additionally, a reduction of PEDF expression has been coupled to increased metastasis and poor prognosis in a range of cancers such as pancreatic (Uehara et al., 2004), prostate (Halin et al., 2004), breast (Cai et al., 2006), osteosarcomas (Takenaka et al., 2005) and neuroblastomas (Crawford et al., 2001). Besides to its anti-angiogenic property, PEDF can act directly on tumor cells by limiting proliferation and inducing apoptosis (Hirsch et al., 2011; Takenaka et al., 2005; Zhang et al., 2007). Mice deficient in PEDF show a wide range of defects such as morphological alterations and increased microvessel density in the retina, loss of ganglion cells, increased stromal vessel growth as

well as pancreatic and prostatic epithelial cell hyperplasia (Doll et al., 2003). These results confirm the data reported from multiple studies that PEDF regulates angiogenesis as well as neuronal cell survival and cell differentiation.

Two epitopes of PEDF, the 34 mer and the 44 mer peptides, were shown to bind to the cell surface of endothelial and prostate cancer cells without competition for receptor binding, indicating the existence of several receptors (Filleur et al., 2005). Soon thereafter the first receptor to be activated upon PEDF binding, a lipase-linked cell membrane protein given the name PEDFR, was identified (Notari et al., 2006). Later studies have reported findings of additional receptors for PEDF: 1) the laminin receptor (LR), responsible for mediating the anti-angiogenic function of PEDF in epithelial cells (Bernard et al., 2009); 2) F_1 ATP synthase, expressed on endothelial and tumor cells (Notari et al., 2010) and; 3) low-density lipoprotein receptor related protein 6 (LRP6) which blocks Wnt signaling in RPEs (Park et al., 2011). Using a short-hairpin approach, Matsui et al. recently reported that LR, but not PEDFR, is responsible for PEDF-mediated anti-inflammatory and anti-thrombogenic effects in cultured human melanoma cells (Matsui et al., 2014) indicating different biological outcome depending on receptor type. Further studies are needed to elucidate the role of the diverse receptor interactions in PEDF signaling.

Taken together PEDF is involved in many important biological processes and could be a potential therapeutic agent against various diseases including cancer, neurodegenerative diseases and neovascular disorders. Since it exists in most normal human tissues, it is unlikely to give rise to toxic effects. Nevertheless, additional studies are needed to define the mechanisms involved in PEDF regulation throughout the body before testing its potential in clinical trials.

PEDF in stem cell biology

PEDF has been described to have a role in a variety of stem cells, mainly a supportive role for stem cell survival as well as for sustaining the multipotent capacity of the cells. For instance, fibroblasts that serve as feeder cells for human embryonic stem cells (hESCs) highly express PEDF during early passages but later the expression level is dropped (Anisimov et al., 2011). Since feeder cells generally lose their supportive capacity following long-term culture *in vitro*, these observations suggest that PEDF has an important role in the growth supportive property of fibroblasts. Furthermore, PEDF was recently reported to promote long-term growth of hESCs through activation of ERK1/2 signaling, while loss of PEDFR resulted in cellular differentiation (Gonzalez et al., 2010). However, conflicting results were recently published indicating that PEDF manifestly suppresses growth and induces apoptosis in iPSCs (Kanemura et al., 2013).

In the adult mouse eye, PEDF has been shown to increase growth of retinal stem cells (RSCs) (De Marzo et al., 2010). In addition, proliferation of limbal epithelial stem cells (located in the eye surface, between the cornea and the conjunctiva) was stimulated by PEDF-treatment *in vitro*. Also, by applying PEDF-containing cream on disrupted cornea, an accelerated wound healing process was observed (Ho et al., 2013).

In the mammalian brain neurogenesis persists in two areas, the subventricular zone (SVZ) and the hippocampus. Ramirez-Castillejo and colleagues found that PEDF is secreted in the murine SVZ and that it can promote self-renewal of adult neural stem cells (NSCs) in neurosphere assays (Ramirez-Castillejo et al., 2006). In addition, they determine that the effects on self-renewal are restricted to the N-terminal part of PEDF, where the neurotropic but not the anti-angiogenic activity resides. Interestingly, the self-renewal effects documented by Ramirez-Castillejo et al. as well as PEDF-mediated influence on the balance between symmetric and asymmetric cell divisions later reported by Andreu-Agullo et al. both involve regulation of the Notch transcriptional activity (Andreu-Agullo et al., 2009; Ramirez-Castillejo et al., 2006).

MSCs are able to differentiate into various mesodermal tissues including bone, cartilage and adipose tissue (Si et al., 2011). Noteworthy, PEDF was one of the most abundant proteins found in the murine MSC-conditioned medium (Sarojini et al., 2008). By reducing PEDF levels *in vitro* and *in vivo* (human and mouse), Gattu et al. were recently able to show that PEDF inhibit adipogenesis while promoting osteogenesis, thus demonstrating a role for PEDF in governing MSC fate (Gattu et al., 2013). PEDF has also been found to delay senescence of human MSCs *in vitro* through mechanisms involving reduction of p53 expression and reduced oxidative stress, (Cao et al., 2013). Furthermore, PEDF was reported to play a critical role in the regulatory effects of MSCs against myocardial infarction injury (Liang et al., 2013).

PRESENT INVESTIGATION

HSCs are ideal targets for cell- and gene therapy, largely due to their distinguished regenerative capacity and the liquid nature of the blood system. However, to be able to keep and expand HSCs *in vitro*, it is crucial to first understand how these cells are regulated in their natural environment *in vivo*. The fine-tuned balance between different cell fate options must be controlled in culture to make it possible to preserve the repopulation potential, while at the same time stimulating self-renewing divisions. Although being well studied for many years, the mechanisms behind the regulation of self-renewal are still not completely understood. More knowledge is needed to understand the interplay between different intrinsic and extrinsic factors participating in governing HSCs. In this thesis we have identified and evaluated factors involved in the regulation of HSC fate decisions.

Specific aims

Article I

Evaluate the role of TGFβ signaling in human HSPC regulation *in vivo* and *in vitro* by Smad4 overexpression.

Article II

Delineate effector mechanisms downstream of TGFβ signaling, including p57 involvement, in primitive hematopoietic cells.

Article III

Investigate and characterize the role of PEDF in murine HSC biology.

Summary of results

Article I

Given the reported inhibitory role of TGFβ on HSPC growth *in vitro* but the lack of knowledge regarding similar effects *in vivo*, this study aimed to explore whether enforced expression of Smad4 could reveal a role for TGFβ in human HSPC regulation *in vivo*. To this end, we used lentiviral vectors carrying a GFP reporter gene, together with full-length Smad4 cDNA (Smad4 vector) or GFP alone (control vector). Overexpression of Smad4 in human CB HSPCs led to an impaired proliferative capacity, whereas the proportion of primitive cells was unaltered after *in vitro* culture. Moreover, enforced expression of Smad4 increased the sensitivity to TGFβ in colony assays, an effect that could be rescued by the addition of a TGFβ type I receptor inhibitor (SB431542), demonstrating a functional over-activity of the TGFβ pathway in these cells. We further analyzed the cell cycle status of the transduced cells and found that after six days in culture, cells with enforced Smad4 expression exhibited a 2-fold increase in the proportion of cells in G_0 state (quiescence) compared to control cells. The fact that these cells could be released from quiescence when treated with SB431542, propose that the small amount of autocrine TGFβ present in the culture caused the observed cell cycle alteration. Since TGFβ has also been described to induce apoptosis we further investigated if enforced Smad4 expression would affect apoptosis of cultured CB cells. Indeed, we could establish that Smad4 overexpression resulted in an increased proportion of cells in early apoptosis after six days of culture, an effect that also could be reversed upon addition of the inhibitor SB431542. To evaluate how TGFβ functions *in vivo* we transplanted transduced CB cells into NSG mice. Lineage distribution appeared to be normal, as determined by PB analysis three months post transplant. However, HSPCs transduced with the Smad4 vector exhibited impaired engraftment already 7 weeks post transplant and this phenotype was sustained in BM after six months. Together with the fact that engraftment 3 weeks after transplantation was similar for both control and Smad4-transduced cells, this indicates that Smad4 overexpression has no impact on short-term HSCs, while the long-term stem cells are affected.

Taken together, these results suggest that Smad4 is a limiting factor for TGFβ mediated Smad signaling critical for long-term HSC function and demonstrate that the level of Smad4 can modulate the response to TGFβ in human cells. Furthermore, we describe a negative regulatory role of the Smad signaling pathway on human HSPCs during regeneration after transplantation affecting self-renewal capacity but not lineage choice.

Article II

In this study, we aimed to elucidate how TGFβ exerts its antiproliferative effect on HSCs through mechanisms involving the cell cycle regulator p57. After treating HPCs with recombinant human TGF-β1 (referred to as TGFβ), we observed a robust induction of p57 mRNA expression in a primitive hematopoietic cell line. However, the response was delayed and dependent on *de novo* protein synthesis, indicating that p57 is not an immediate effector molecule in the TGFβ response. Using a global gene expression profiling approach to discover early targets, we could identify the TF and known HSC regulator GATA2 as a direct target of TGFβ signaling in HPCs. Furthermore, we were able to confirm TGFβ-induced upregulation of both GATA2 and p57 in fresh primary HPCs and also establish that this regulation is dependent on the presence of Smad4.

Interestingly, we observed a significant overlap between GATA2 targets identified by ChIP sequencing and the downstream signature of TGFβ signaling, as determined by microarray. We could confirm binding to the p57 promoter using qPCR of GATA2-bound chromatin in primary cells. Together, this data implies that GATA2 binds to a large number of TGFβ targets, one of them being the cell cycle regulator p57. Importantly, we also found that Smad4 binds to an upstream enhancer of the GATA2 gene, suggesting a regulatory network involving Smad4, GATA2 and p57 downstream of TGFβ signaling. In an attempt to investigate the relevance of this network in the HSC niche, we found that GATA2 expression was unaltered in highly purified HSCs from Smad4 KO mice, while p57 expression was significantly reduced. Still, deletion of GATA2 lead to a strong reduction of p57 baseline levels in HPCs as well as a failed p57-induction after stimulation with TGFβ. Most importantly, progenitor-enriched GATA2-deficient cells were desensitized to TGFβ-induced proliferation arrest and showed a strong reduction in colony-forming ability along with a decreased sensitivity to TGFβ-inhibition of colony formation. Together these observations suggest that maintenance of physiological p57 levels requires the presence of both GATA2 and TGFβ signaling (Smad4) and that GATA2 is critical for TGFβ-induced upregulation of p57 and subsequent proliferation arrest.

To summarize, our results reveal a transcriptional network, involving GATA2, p57 and members of the TGFβ signaling pathway, active in HSCs and downregulated upon differentiation. Importantly, GATA2 levels are critical for TGFβ-induced growth arrest demonstrating the functional importance for the TGFβ/Smad/GATA2/p57 axis in the regulation of hematopoietic progenitor cell proliferation. Thus, this study provides a detailed mechanistic insight to TGFβ-induced growth arrest in the context of hematopoietic progenitor cells.

Article III

PEDF has been found to promote self-renewal of adult NSCs in neurosphere assays (Ramirez-Castillejo et al., 2006), as well as being suggested to be involved in regulating the balance between symmetric and asymmetric cell divisions (Andreu-Agullo et al., 2009). By measuring PEDF expression in murine primary cells, we found that PEDF is highly expressed in LT-HSCs and downregulated in more restricted populations. This proposed a role of PEDF also in hematopoiesis and HSC function. Hence, we decided to characterize the hematopoietic parameters in a knockout mouse model for PEDF.

Interestingly, PB analysis revealed a reduction in white blood cell counts in PEDF knockouts. *In vitro*, PEDF null BM cells showed a reduced capacity to form secondary myeloid colonies, although the overall proportions of the different progenitor cell populations were similar in *wt* and PEDF-deficient animals. Further analysis revealed that the frequency of HSCs, both phenotypical and functional (CRUs), was unaltered in PEDF null mice. However, in competitive transplantation assay PEDF-deficient BM cells contributed to the HSC population to a lesser extent than *wt* cells. In fact, the overall engraftment of PEDF null donor cells in BM of primary and secondary transplanted mice was impaired compared to *wt* donor engraftment. In tertiary recipients the engraftment of PEDF null donor cells was further decreased, the LSK compartment undetectable, and lineage distribution skewed towards a decrease in the myeloid lineage, demonstrating an important role of PEDF for HSC maintenance.

Furthermore, we found that around 10% of HSCs express PEDF receptor (PEDFR). Since PEDF has previously been reported to be expressed and secreted by cell types in the HSC niche, we speculated that the presence of PEDF in recipient mice would be important for the repopulative capacity of HSCs. However, in reverse transplantation experiments we observed normal engraftment of *wt* cells in irradiated $PEDF^{-/-}$ recipients. Moreover, lineage distribution and the frequency of stem cells were normal in these recipients, indicating that PEDF in the niche is dispensible for HSC engraftment and functions in an autocrine fashion in HSCs.

In conclusion, our results demonstrate that PEDF is an important regulatory factor for HSC regeneration and that PEDF *in vivo* works in a cell-autonomous fashion. For the first time, these findings propose a role of PEDF in HSC biology and strengthen the accumulating evidence of PEDF as an important regulator in several stem cell systems.

Conclusions

Article I

- Overexpression of Smad4 in CB HSPCs increases their sensitivity to TGFβ.
- TGFβ hypersensitivity results in growth arrest and increased apoptosis of human HSPCs *in vitro*.
- Enforced expression of Smad4 leads to impaired HSPC reconstitution capacity with unaltered lineage potential.

Article II

- GATA2 is a direct target of canonical TGFβ signaling in murine HSPCs.
- p57 is an indirect target of canonical TGFβ signaling largely regulated via GATA2.
- GATA2 mediates a large part of the molecular programs downstream of TGFβ signaling.
- Three factors involved in HSC self-renewal form a functionally relevant network regulating proliferation of hematopoietic progenitor cells.

Article III

- PEDF is involved in the regulation of hematopoiesis.
- Mice deficient of PEDF show overall normal hematopoietic parameters during homeostasis, while presence of PEDF is critical for hematopoietic regeneration.
- Loss of PEDF results in a decreased HSC population as well as reduced engraftment following transplantation.

GENERAL DISCUSSION

Unraveling the HSC regulatory network

For clinical purposes it would be beneficial to maintain and expand HSCs in culture, without losing their stem cell properties. However, to achieve this goal, increased knowledge regarding the natural microenvironment of HSCs and the factors that regulate their fate decisions is required. The work in this thesis has contributed to the field by 1) adding knowledge regarding the impact of the level of Smad4 signaling on human HPCs *in vitro* as well as in a transplantation setting, 2) identifying a functional network between TGFβ/GATA2/p57 relevant for HPC proliferation, and generating a database of genes differentially expressed by TGFβ in HPCs, including a list of 110 genes directly bound by GATA2 and 3) identifying of a novel regulator, of HSC biology, PEDF.

The use of mouse models

Overexpression studies

In order to evaluate the role of a "novel" gene in the blood system, overexpression of the gene of interest is commonly a valuable tool. Lentiviral vectors can be used to enable stable integration of the transgene into the genome (Singer and Verma, 2008). By introducing the construct to the genome of a hematopoietic stem cell, the transgene will be overexpressed in every progeny of the entire hematopoietic system. This approach can be used in human cells, making it possible to study human HSC biology in an *in vivo* setting, by transplanting the transduced cells into a xenograft mouse model. However, exposure to non-physiologically high levels of a protein could result in effects unrelated to the normal function of the protein. The use of strong transgene promoters can lead to a massive increase of the transgene product, although in the case of overexpressing Smad4 (Article I) the increase in mRNA level was modest (approximately 5-fold). Smad4 overexpression in human CB HPCs resulted in hypersensitivity to TGFβ, followed by well-established TGFβ effects on cell cycle and apoptosis (Batard et al., 2000; Jacobsen et al., 1995; Sitnicka et al., 1996). In addition, these effects were rescued in the presence of a TGFβ type I receptor inhibitor, altogether demonstrating that

our observations are of physiological relevance, and that the overexpression does not cause toxicity in itself.

A severe side-effect when using integrating viral vectors is the risk of leukemic transformation that can occur if the transgene integrates near proto-oncogenes (Woods et al., 2003). In our experiments we did not observe any lineage skewing that would indicate the induction of leukemia.

Furthermore, it is important to be aware that the time the cells are kept in culture before evaluation can have impact on the outcome. Commonly, cells are pre-stimulated for 24h, and incubated with virus for additional 24-48h. Even if we start with primitive cells, they will begin to proliferate and perhaps also enter various differentiation programs during the culture period, leaving us with a heterogeneous population with less stem cell potential than our starting material. In spite of all this, we clearly managed to transduce and transplant LT-engrafting HSCs, as human reconstitution was observed in NSG mice 25 weeks after transplantation.

In situations where the gene of interest is already present in abundant levels, further enforcement of its expression may not reveal the function of this gene. In such cases loss-of-function models are preferred.

Knockout mouse models

Methods of analyzing loss-off-function include knockdown approaches or the use of knockout mice, and can provide valuable information regarding the normal function of a gene. *Conventional* knockout models are based on the functional elimination of the targeted gene in every cell of the body, whereas *conditional* knockout models allow for a tissue-specific gene deletion and temporal control of induction. In the case where the gene of interest is crucial during development and a conventional knockout model leads to embryonic lethality, a conditional and inducible approach based on the Cre-loxP system can be used, enabling studies of the gene function in the adult system. This technique is based on the specific excision of DNA segments, flanked by two short sequences known as loxP sites, by induction of Cre recombinase (Sauer, 1998; Sauer and Henderson, 1989). In Article II, we used transgenic mice with loxP sites inserted around exons of either the Smad4 gene or the GATA2 gene, and crossed them with a strain that carries the Cre recombinase gene under the control of the Mx1 promoter (MxCre) (Kuhn et al., 1995). The Mx1 promoter is responsive to interferon or the interferon-inducing synthetic RNA molecule poly IC, and is suitable for studies of the hematopoietic system since gene deletion has been shown to be highly efficient in BM cells, including HSCs (Brakebusch et al., 2002; Kuhn et al., 1995; Larsson et al., 2003; Wilson et al., 2004). However, it is important to be aware that Mx1 promoter-activation is not restricted to cells of hematopoietic origin. To rule out

that phenotypic observations are not due to gene deletion in other organs, transplantation of BM cells into *wt* recipient mice is a relevant strategy to sequester the effect of the KO to the hematopoietic system.

In the GATA2 KO mouse model, we used multiple injections of poly IC to efficiently induce the expression of Cre recombinase. Remarkably, even when harvesting BM directly after the final injection, we observed an almost complete loss of the LSK compartment in GATA2-deficient BM. This complicated our analysis, since we were limited to investigate progenitor cells. Although we would expect the effect of the gene deletion to be most pronounced in the most primitive cells, the fact that we observed functional effects such as desensitization to TGFβ-induced inhibition of colony-forming capacity and proliferation arrest in GATA2 KO HPCs, strongly suggest an important role of GATA2 downstream the TGFβ signaling pathway. Additionally, the lack of an HSC population in the absence of GATA2 suggests that GATA2 is critical for HSC maintenance/survival.

In Article III, we used a conventional knockout model for PEDF, with the aim of investigating the role of PEDF in the hematopoietic system. PEDF null offspring were viable, demonstrating that PEDF is dispensable during development. Steady state hematopoiesis was essentially normal in PEDF-deficient adult mice, however knockout BM cells contributed to a reduced HSC frequency, and overall impaired reconstitution potential, in competitive transplantation assays. As discussed earlier, several groups have reported findings of PEDF expression and secretion in cell types implicated in the BM niche environment (Crawford et al., 2001; Quan et al., 2005; Sarojini et al., 2008). To resolve whether the effect on HSC function was due to loss of PEDF in the actual HSCs, or due to a niche defect introduced by systemic PEDF deletion, we performed reverse BM transplantations, injecting *wt* cells into PEDF-deficient recipients. We could conclude that PEDF likely regulates HSCs in a cell-autonomous fashion, since *wt* cells engrafted normally in PEDF knockout BM.

Effector molecules of TGFβ signaling

While the mediators of TGFβ signaling are well characterized, the downstream effector molecules in hematopoietic progenitors are poorly understood. Therefore, in Article II we set out to elucidate the effector mechanisms involving p57, one known target of TGFβ signaling (Scandura et al., 2004; Yamazaki et al., 2009), in primitive hematopoietic cells. However, as our initial experiments indicated that p57 was not an immediate effector molecule in the TGFβ response, we decided to carry out a global gene expression analysis to reveal direct targets of TGFβ. From the database generated we selected one top candidate, the TF GATA2, for further investigation. Interestingly, haploinsufficiency of GATA2 in a mouse model leads to similar effects on HSCs as a total blockage of TGFβ signal transduction

(Karlsson et al., 2007; Rodrigues et al., 2005; Yamazaki et al., 2011). Moreover, enforced GATA2 expression in human CB progenitor cells mimics the effects seen after TGFβ stimulation of HSPCs, with inhibited cell cycle both *in vitro* and *in vivo* (Batard et al., 2000; Sitnicka et al., 1996; Tipping et al., 2009). The resulting quiescent state of primitive hematopoietic cells in these two seemingly contradictory scenarios of deregulated GATA2 expression, could perhaps be explained by differences between mouse and man, or by that the precise level of GATA2 is determining the downstream effect. We provide evidence for GATA2 transcriptional regulation of the quiescence marker p57, which however does not exclude a role for GATA2 as a positive regulator of cell cycle downstream of other pathways. Importantly, we found GATA2 to bind to a large number of additional TGFβ targets, opening up for deeper investigation of the mechanism behind the previously reported divergent phenotypes. Based on our observations, we suggest that even though the regulation of GATA2 and p57 expression is likely governed by multiple pathways, maintenance of physiological p57 levels requires the presence of both GATA2 and TGFβ signaling (Smad4), and that GATA2 is critical for TGFβ-induced proliferation arrest (Figure 8).

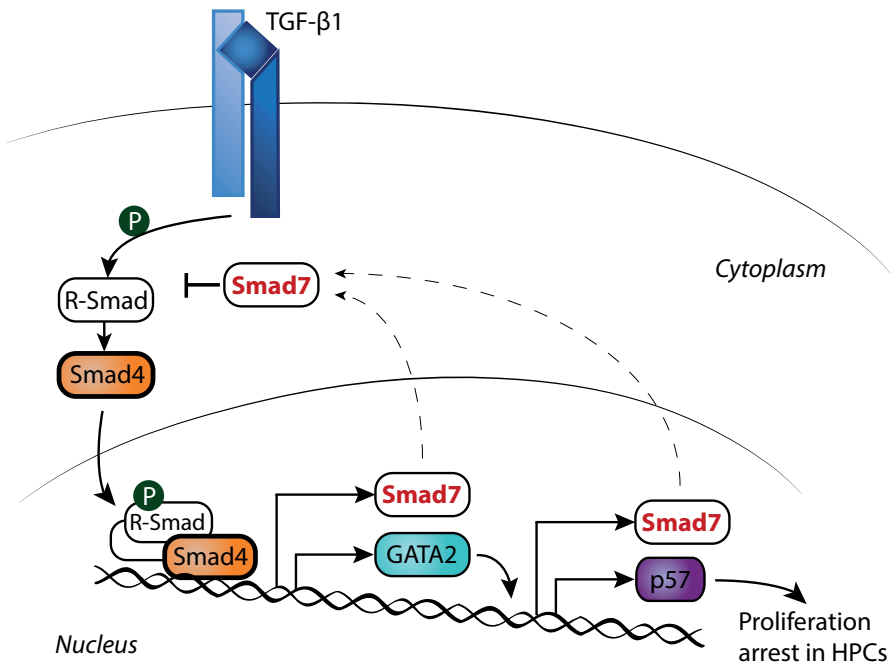


Figure 8. Model of the molecular mechanism behind the response to TGFβ signaling in hematopoietic progenitor cells (HPCs).

Additionally, our findings indicate that GATA2 directly regulates the main negative feedback molecule of TGFβ signaling, Smad7. The Smad7 genomic region was highly enriched by GATA2 in primitive hematopoietic cells, and TGFβ-induced upregulation of Smad7 was significantly dampened in GATA2 KO progenitor cells. This made us speculate of the existence of a negative feedback mechanism between these genes, that remains to be further investigated.

New insight into TGFβ regulation

TGFβ is one of the most potent inhibitors of HSPC proliferation *in vitro*. However, the mechanism behind the growth inhibitory effect and the precise function of this signaling pathway *in vivo,* are still to be unraveled. TGFβ is believed to maintain stem cells in a quiescent state through negative regulation of self-renewal. Thus, inhibition of the entire TGFβ signaling pathway would theoretically release HSCs from dormancy, and lead to increased proliferation. This was shown to be the case as a result of overexpression of the inhibitory Smad7, in that Smad7 overexpressing HSCs were increased in number, and exhibited a significantly improved regenerative capacity over control transduced cells upon secondary transplantation (Blank et al., 2006). Forced expression of Smad7 in Smad4-deficient HSCs failed to reproduce the effect seen in *wt* cells, implying a Smad4-dependent mechanism. Intriguingly, loss of Smad4 in the BM resulted in the opposite phenotype, with impaired donor contribution of Smad4 mutant BM (Karlsson et al., 2007). This finding allowed for the speculation that the effects of Smad7 overexpression could be due to excessive Smad4 protein available, resulting in enhanced interaction of Smad4 with mediators of signaling pathways distinct from the canonical Smad pathway. To investigate this idea in more detail, we set out to overexpress the level of Smad4 in human CB cells with the hypothesis that this would mimic the phenotype seen after Smad7 overexpression. However, instead of revealing connections to positive regulators of self-renewal, we observed an increased sensitivity to TGFβ signaling of CB progenitor cells *in vitro* translating into impaired LT engraftment in transplantation assays. This demonstrates that the level of Smad4 determines the response to canonical TGFβ signaling, but does not exclude that Smad4 could be involved in other pathways as well. Therefore, it is important to further investigate the interaction of Smad4 with other proteins, to gain insight into the mechanism behind the contrasting phenotypes observed upon modulation of the Smad pathway. In Article II, we identify a network of known stem cell regulators, to be involved in TGFβ-induced growth inhibition in a Smad4-dependant manner. We further generate a database of genes that become deregulated following TGFβ stimulation, presumably containing additional, yet unexplored, important factors underlying this mechanism. Moreover, we demonstrate that GATA2 is involved in a large part of the TGFβ response, suggesting that profound future studies of the

GATA2-bound factors we present may be of great importance for a more detailed understanding of this long-studied pathway.

One obstacle along the road to understanding the mechanism behind the inhibitory effect of TGFβ, may be the discrepancy between species. In Article I, Smad4 overexpression results in TGFβ-dependent effects on human HSPCs under serum-free culture conditions, implying that the level of Smad4 is important for modulating the response to autocrine and/or paracrine TGFβ *in vitro*. In line with our data, an autocrine/paracrine effect of TGFβ has been noted in human cultures before (Akel et al., 2003; Fan et al., 2002). In the mouse system however, it has been shown that TGFβ is secreted in a latent form and that HSCs themselves are incapable of activating it (Yamazaki et al., 2009). These results emphasize the importance of accumulating information from several species.

Regardless of the precise molecular mechanism, it is clear that the TGFβ signaling pathway is of great biological relevance for HSC regulation, and the level of Smad signaling correlates with the sensitivity to TGFβ. Although, based on the complexity of the system regulating HSCs *in vivo*, a combination of factors is most likely necessary to control the HSC fate decisions in desired direction *in vitro*, to reach clinical implication.

Therapeutic relevance

From clinical experience, it is apparent that patient survival is related to the number of transplanted cells, in addition to the time to immune reconstitution (Barker, 2007; Brunstein et al., 2010; Brunstein and Wagner, 2006; Wagner et al., 2002). Successful expansion of CB cells, even a modest increase in cell numbers, would have a large impact on the number of patients susceptible for transplantation. It would also increase the diversity of the pool of available donor samples, making transplantation treatment accessible to a larger number of patients.

Furthermore, when it comes to human malignancies, both GATA2 haplo-insufficiency as well as enforced expression of GATA2 has lately been detected in association with a variety of hematological diseases (Collin et al., 2015; Vicente et al., 2012a). Our results in Article II strengthen the recent therapeutic hypothesis that inhibiting TGFβ signaling is a potential strategy for novel treatments of hematopoietic disorders with altered GATA2 expression (Herbertz et al., 2015; Migliaccio, 2015; Valcarcel, 2015). In addition, since correction of GATA2 haploinsufficiency is predicted to lead to a competitive survival advantage for HSCs in the niche, familial GATA2 mutation qualifies as a good candidate to target with gene therapy strategies (Collin et al., 2015).

Future directions

Members of the TGFβ superfamily and the downstream Smad signaling pathway have, through diverse manipulations at various levels, been established to be liable for important functions in the regulation of HSCs. The only part of the ”Smad puzzle” that has not yet been extensively studied in the murine hematopoietic system involves signaling through the R-Smads Smad2 and Smad3. Considering that knocking out those two genes would contribute to a better understanding regarding the opposite phenotypes observed in the Smad4/TGFβRII and Smad7 models, we carried out this investigation in parallel with the work leading to this thesis. Our hypothesis was that either the loss of both TGFβ and Activin pathways lead to increased HSC self-renewal, or these two signaling pathways are critical to keep HSC stemness and therefore these knockout mice will show impaired self-renewal. Surprisingly, we observed no critical role for Smad2 and Smad3 signaling in adult hematopoiesis (unpublished observations). This leads to the conclusion that the complex network of factors involved in TGFβ signaling, besides the Smad pathway, requires additional investigation to fully elucidate the downstream effects of the TGFβ signaling pathway. The database of genes differentially expressed by TGFβ in HPCs generated in Article II, can be of great value when designing such future studies. As an example, upregulation of Hes1, a target of Notch signaling, was induced by TGFβ. Considering that the Notch signaling pathway has been implicated as directly interacting with the Smad pathway (Blokzijl et al., 2003; Itoh et al., 2004; Larrivee et al., 2012), along with reports suggesting a role for Notch signaling in the regulation of HSC self-renewal (Duncan et al., 2005; Stier et al., 2002; Varnum-Finney et al., 2000), Hes1 would be an interesting candidate for further evaluation in the context of TGFβ signaling in HSCs. Additionally, top candidates selected among the overlapping targets between the GATA2 bound genes and the downstream signature of TGFβ signaling as determined by microarray, could be investigated to delineate their potential involvement in the TGFβ/GATA2/p57 network and TGFβ-induced growth arrest of HSPCs.

Moreover, detailed mechanistic studies of human HSCs are needed to assess if modulation of TGFβ signaling could be utilized in clinical objectives. More specifically, it remains to be determined whether blockage of TGFβ/Smad signaling can contribute to faster regeneration following transplantation and whether TGFβ signaling can be adapted *in vitro,* with the purpose of expanding HSCs to be utilized in cell therapies.

Our findings in Article II suggest a transcriptional network, consisting of important stem cell regulators (TGFβ(Smad4)/GATA2/p57) that is important in controlling the proliferation of primitive hematopoietic cells. We hypothesize that

HSCs lose GATA2 expression as they start to proliferate (leave quiescence). To address this idea, a future experiment could be to sort out cells in different cell cycle stages (G_0, G_1, $S/G_2/M$) and compare their mRNA level of GATA2. By including analysis of p57 expression in these populations it would be possible to determine if the expression of GATA2 and p57 correlates with HSC quiescence. Furthermore, given that FL HSCs are more proliferative than adult HSCs, it would be interesting to investigate the role of TGFβ in FL HSCs. By measuring the basal levels of GATA2 and p57 in FL HSCs and compare them to the levels in adult HSCs, we would get an indication whether the TGFβ/GATA2/p57 network is activated in HSCs during development. Based on the active cell cycle status of the FL HSCs it is likely that the TGFβ signaling pathway is not activated in these cells. Furthermore, treating FL HSCs with TGFβ, to observe potential alterations in GATA2 and p57 expression levels, would reveal if the response to TGFβ and downstream functions differ between proliferating FL HSCs and adult HPCs.

Another unanswered question is whether GATA2 KO mice lose the HSC compartment or if the absent LSK population in these mice is due to a change in surface marker phenotype. This could be determined by transplantation of unfractionated BM cells, or sorted SP cells, and subsequent analysis of LT engraftment and multilineage reconstitution, revealing if the donor population contained cells with HSC potential.

In article III we suggest a crucial role for PEDF in HSC function and our results imply that PEDF exerts its effect on HSCs via an autocrine mechanism. Since PEDF is a secreted protein we aim to examine what influence recombinant PEDF might have on murine and human HSC growth *in vitro*. The analysis of steady state mice in our study display a normal blood phenotype, except for decreased white blood cell counts and colony forming capacity, supporting the idea that PEDF is not crucial to keep homeostasis but important under stress conditions like hematopoietic regeneration. Without further investigation, it cannot be excluded that the impaired engraftment capacity of transplanted PEDF deficient cells, is due to a homing deficiency.

Several receptors have been described to mediate PEDF signal transduction (Bernard et al., 2009; Notari et al., 2010; Park et al., 2011). We aim to investigate whether it is PEDFR, which we found to be expressed by ten percent of the HSCs, or any of the other reported PEDF receptors that are responsible for mediating the observed effects on HSC function.

Another point worth mentioning is that the experiments in Article III are performed using PEDF knockout mice on a mixed genetic background. To avoid any influence on the results due to background differences, we have used littermate control mice in all our assays. In addition, we are currently performing transplantation experiments using PEDF knockout animals backcrossed for >10

generations to confirm our findings. So far, preliminary results from these transplantations indicate that the observed phenotype of PEDF null HSCs is lost in backcrossed mice. This would suggest that PEDF is not a critical HSC regulator. Clearly, further experiments are needed to draw the final conclusions regarding the role of PEDF in hematopoiesis.

"How insidious Nature is when one is trying to get at it experimentally."

- Albert Einstein

POPULÄRVETENSKAPLIG SAMMANFATTNING

Blodsystemet består av flera olika typer av celler med livsviktiga funktioner såsom att förse kroppen med syre, stoppa blödningar och bekämpa infektioner. Dessa mogna blodceller är kortlivade och måste kontinuerligt ersättas med nya. Förutom de mogna cellerna innehåller blodet även en liten samling stamceller, hematopoietiska stamceller (HSC), som är ansvariga för att dagligen ge upphov till en trillion nya blodceller, utan att själva förbrukas. HSC har två egenskaper som gör dem mycket speciella; 1) de har förmågan att bilda alla typer av blodceller och 2) de kan vid celldelning skapa exakta kopior av sig själva genom så kallad självförnyelse. Denna självförnyelse är avgörande för att organismen ska bibehålla tillräckligt med stamceller för att kunna upprätthålla ett funktionellt blodsystem livet ut. HSC är belägna i benmärgen, där de regleras av en mängd faktorer, inräknat proteiner i stamcellerna själva och signaler från den omgivande miljön. Ett noggrant kontrollerat samspel mellan kända och ännu okända faktorer i olika kombinationer och koncentrationer, bidrar till stamcellernas slutgiltiga öde. Störningar i detta system kan leda till utvecklandet av livshotande sjukdomar som leukemi och immunbristsjukdomar.

Transplantation av HSC är en effektiv metod för att behandla allvarliga blodsjukdomar. Vid leukemi behandlas patienten med strålning och/eller cellgifter, vilket tar död på patientens blodsystem, inklusive cancercellerna. Därefter injiceras färska friska donatorceller intravenöst. De transplanterade cellerna kan nu återskapa ett blodsystem i mottagaren. Begränsande faktorer såsom brist på matchande donatorer och för lågt antal stamceller i donatorns benmärg eller blod, innebär att många patienter inte kan erbjudas benmärgstransplantation som behandlings-alternativ. Förutom benmärg och cirkulerande blod är navelsträngsblod en alternativ källa för stamceller. Fördelarna med navelsträngsblod, som annars är en restprodukt från födseln, är att det är lättillgängligt och kan förvaras en lång tid i blodbanker. Dessvärre är antalet stamceller som kan utvinnas från en navelsträng för få för att effektivt kunna behandla en vuxen individ. Om vi kunde manipulera stamcellerna till att föröka sig själva utanför kroppen så skulle stamcellstransplantation, oavsett cellkälla, bli tillgänglig för fler individer. Idag är vår kunskap om stamcellerna otillräcklig för att uppnå detta mål. Därför syftar denna avhandling till att få en djupare förståelse

för hur stamcellerna regleras i kroppen med målsättningen att kunna efterlikna detta system i labbet. Sådan kunskap är inte bara viktig i avsikt att kunna bota fler patienter genom stamcellstransplantation, utan skulle också gynna utvecklingen av genterapi som behandlingsmetod för flera medfödda sjukdomar samt kunna användas i forskningssyfte för att utöka kunskapen inom stamcellsbiologi.

En sedan länge känd faktor att påverka tillväxten av HSC är Transforming growth factor-β (TGFβ). Det är emellertid relativt okänt genom vilka mekanismer och förmedlare TGFβ utövar sin effekt. Cellcykelhämmaren p57 har dock visats vara involverad i TGFβ-styrd reglering av tillväxt genom att hämma celldelning. I Arbete II ville vi undersöka kopplingen mellan TGFβ och p57 mer i detalj, men fann till vår förvåning att p57 inte var direkt reglerat av TGFβ i primitiva blodceller från möss. I stället upptäckte vi att nivån av en annan viktig stamcellsfaktor, GATA2, blev direkt förhöjd när dessa celler behandlades med TGFβ i laboratoriemiljö, och att GATA2 i sin tur var ansvarigt för att nivån av p57 ökade och tillväxten minskade. Våra resultat antyder dessutom att GATA2 är involverat i regleringen av en stor del av de gener som påverkades i cellerna som vi behandlade med TGFβ. Identifieringen av denna typ av nätverk och förmedlare i TGFβ-signaleringen tillför ett viktigt bidrag för förståelsen av mekanismerna bakom regleringen av celldelning.

När celler stimuleras med TGFβ aktiveras en signalväg, kallad Smad, inuti cellerna. Om denna signalväg hämmas i möss påverkas HSC tillväxt. Det är dock inte känt exakt vilken roll Smad-signalering har i humana celler. Detta undersökte vi i Arbete I genom att studera effekten av ökad Smad-signalering i humana HSC från navelsträngsblod. Våra resultat visar att celler med förhöjda nivåer av Smad4, en viktig komponent i Smad-signaleringen, blir överkänsliga mot TGFβ i cellkultur. Genom att transplantera dessa celler till möss med nedsatt immunförsvar (för att de inte ska stöta bort de humana cellerna) kunde vi dra slutsatsen att ökad känslighet för TGFβ har negativ påverkan på cellernas förmåga att självförnya sig.

Slutligen har vi upptäckt att HSC som saknar genen Pigment epithelium derived factor (PEDF) har försämrad förmåga att återbilda blodsystemet (Arbete III). PEDF har tidigare visats vara viktig för andra stamcellstyper, men detta är första gången PEDF studeras i blodsystemet. Nu försöker vi komma fram till hur PEDF påverkar stamcellerna för att förstå varför detta protein är viktigt för stamcellernas funktion.

Sammanfattningsvis så har resultaten av vår forskning lett till en mer detaljerad bild av hur HSC är reglerade. Denna kunskap skulle kunna bidra till utvecklandet av effektivare behandling av blodsjukdomar i framtiden.

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“There is nothing better than a friend, unless it is a friend with chocolate.”

- Linda Grayson

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"As history has shown, pure science research ultimately ends up applying to something. We just don't know it at the time."

- Neil deGrasse Tyson

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