Regulation of Hematopoietic Stem Cells

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REGULATION OF HEMATOPOIETIC STEM CELLS

Abstract

This thesis is about the regulation of hematopoietic stem cells (HSCs) that represent rare cells residing in the bone marrow (BM) of adults. They are multipotent cells that have the capacity to differentiate to all mature cells of the blood system and have the capacity to self-renew, i.e. generate new HSCs. These cells have tremendous therapeutic potential to treat a variety of hematopoietic disorders through blood and marrow transplantation. Today, peripheral blood (PB) is the most commonly used source as HSCs can be mobilized from BM to PB. A third more convenient source for harvesting HSCs is cord blood (CB). However, the yield of HSCs in CB is too low for successful transplantation to most adult patients. Thus, ideally HSCs from CB needs to be increased in number (expanded) ex vivo before transplantation. Expansion of HSCs holds great promise but has been met with limited success due to incomplete knowledge regarding regulation of HSCs. Thus, deeper understanding of the regulatory mechanisms that govern HSCs fate is critical to allow expansion of HSC ex vivo and improve HSC-based therapies.

The studies presented in this thesis have identified factors involved in the regulation of HSC fate decisions. In summary, our results demonstrate that increased Smad4 expression, a key component in the transforming growth factor-β (TGF-β) signaling pathway, sensitizes human CB HSPCs to TGF-β. This leads to growth arrest and apoptosis in vitro and reduced HSPC reconstitution capacity in vivo with no effect on lineage distribution. Together, these findings demonstrate an important role for TGF-β signaling in the regulation of human HSCs in vivo (Article I). Furthermore, with the purpose to investigate and characterize a novel regulator, we have studied the role of pigment epithelium-derived factor (PEDF) in murine HSCs. Absence of PEDF leads to reduced HSC numbers and impaired engraftment following transplantation. Here, we report for the first time that PEDF is a critical regulatory factor for HSC function (Article II). Last, we have identified the cell surface antigen CD9 as a positive marker for HSCs that provides a simple alternative for stem cell isolation at high purity. Using CD9 as a tool we have dissected heterogeneity within the HSC pool as defined by CD9 expression (Article III).

Taken together, we have identified several molecules of human and murine HSCs that are important for stem cell function and fate outcome.

Key words: Hematopoiesis, hematopoietic stem cells, hematopoietic stem cell expansion, cord blood, transforming growth factor beta, Smad signaling, pigment epithelium derived factor, CD9

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“Stupidity has a certain charm - ignorance does not.”

- Frank Zappa

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ABBREVATIONS

BFU-E	burst forming unit-erythrocyte
BMP	bone morphogenic proteins
BMT	bone marrow transplantation
BrdU	5-bromo-2-deoxyoridine
CB	cord blood
CDKI	cyclin-dependent kinase inhibitor
CFU	colony-forming unit
CFU-GEMM	colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte
CFU-GM	colony-forming unit-granulocyte/macrophage
CFU-S	colony-forming unit spleen
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRU	competitive repopulation units
CXC12	chemokine motif ligand 12
dHSC	definitive-HSC
DNA	deoxyribonucleic acid
ECM	extra cellular matrix
FACS	fluorescence activated cell sorting
Flt3	fms-related tyrosine kinase 3
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony-stimulating factor
H2B-GFP	histone 2B-green fluorescent protein
hESC	human embryonic stem cell
HLA	human leukocyte antigens
HSC	hematopoietic stem cell
Il2rg	IL-2R common γ chain
iPS-cell	induced pluripotent stem-cell
LLC	large latent complex
lncRNA	long noncoding RNA
LR	non-integrin laminin receptor
LSK	$Lin^{-}Sca^{+}c\text{-}Kit^{+}$
LTC-IC	long-term culture initiating cell
M-CSF	macrophage colony stimulating factor
MPP	multipotent progenitor

MSC	mesenchymal stem cell
NOD	nonobese diabetic
NOG	NOD/Shi-scid/Il2rg$^{-/-}$
NSG	NOD/LtSz-scid/Il2gr$^{-/-}$
OB	osteoblast
OC	osteoclast
PB	peripheral blood
PEDF	pigment epithelium-derived factor
PGE2	prostaglandin E2
RPC	retinal progenitor cell
RSC	retinal stem cell
SCF	stem cell factor
SCID	severe combined immune-deficiency
SCR	scid-repopulating cell
sh-RNA	short hairpin RNA
SNS	sympathetic nerve system
TGF-β	transforming growth factor-β
TPO	thrombopoetin
VEGF	vascular endothelial growth factor
wt	wild type

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

II. Rörby E, Billing M, Dahl M, Andradottir S, Miharada K, Siva K, Blank U, Karlsson G, Karlsson S. *Pigment epithelium-derived factor regulates hematopoietic stem cell maintenance.* Manuscript 2014.

III. Karlsson G, Rörby E, Pina C, Soneji S, Reckzeh K, Miharada K, Karlsson C, Guo Y, Fugazza C, Gupta R, Martens JH, Stunnenberg HG, Karlsson S, Enver T. *The tetraspanin CD9 affords high-purity capture of all murine hematopoietic stem cells.* Cell Rep. 2013 Aug 4(4):642-648.

PREFACE

Since late 2009 I have been a PhD student in Stefan Karlsson's lab at the division of Molecular Medicine and Gene Therapy. When I started the day of dissertation seemed so far away. However, time has moved quickly and the journey has been filled with blood (a lot of blood actually), sweet and tears. But also with pure joy! My supervisor says, "*science is not only work, it is a life style"* and I have enjoyed having this life style for the past years and I'm proud to introduce you to the work I have done. As Winston Churchill said however, "*Now this is not the end. It is not even the beginning of the end. But it is, perhaps the end of the beginning*".

This thesis is all about blood stem cells, or hematopoietic stem cells (HSCs), these are rare cells that reside in the bone marrow (BM) of adults. HSCs are multipotent i.e. they have the capacity to differentiate to all the mature cells of the blood system but not to any other organ systems, they have also the capacity to self-renew i.e. make a copy of themself. Mature blood cells in our body are short-lived and without HSCs to replace them would be equal with death. The HSC was discovered about 50 years ago by Till and McCulloch, although the German scientist Ernst Haeckel used the word "stammzelle" as early as 1868 and the histologist Alexander Maximow postulated year 1909 that hematopoiesis is a cellular hierarchy derived from a common precursor (Haeckel, 1968; Maximow, 1909; Till and McCulloch, 1961). The field has evolved dramatically since Till and McCulloch reported their discovery and today HSCs are by far the most studied stem cell in our body. This is most likely because they are easy to access in the BM, but they are also connected to many blood disorders why insight to the field of hematopoiesis has great clinical relevance. However the exact mechanism governing HSC fate remain elusive. In this thesis we have investigated transforming growth factor beta (TGF-β) signaling in human HSCs (Article I), we can also for the first time report the pigment epithelium-derived factor (PEDF) is a regulator of HSC fate (Article II). Finally, this thesis also describes CD9 as a cell surface marker that captures all HSCs in murine BM and how this is a useful tool to dissect heterogeneity within the HSC pool (Article III).

Emma Rörby
Malmö, July 2014

BACKGROUND

Hematopoiesis

Blood and the system that forms it, the hematopoietic system, consists of many different cell types, white blood cells fight infections, red blood cells carry oxygen to the tissues and platelets help prevent bleeding (Table 1). Many blood cells are short-lived and need to be replenished continuously. In the human body the turnover of hematopoietic cells is close to 10^{12} cells per day (Ogawa, 1993). The production of these cells relies on a rare population of multipotent hematopoietic stem cells (HSCs). These are primitive, tissue-specific somatic stem cells that can *differentiate* (start the path towards becoming a mature cell) to generate all cells of the hematopoietic system. Importantly, they can also simultaneously *self-renew* (give rise to other HSCs), a property fundamental for life since it keeps the stem cell pool intact. Self-renewal and differentiation are under strict control in the bone marrow (BM) where the process of blood cell formation occurs and is referred to as hematopoiesis. Hematopoiesis can be described as hierarchical with the rare HSCs at the apex and the large numbers of terminally differentiated mature cells at the bottom. The cells that are in between HSCs and mature cells in this hierarchical tree are referred to as progenitor cells (Figure 1).

The hematopoietic system is divided into two lineages where distinct progenitor populations, common myeloid progenitor (CMP) and common lymphoid progenitor (CLP), are believed to divide the hematopoietic hierarchy into either myeloid or lymphoid restricted potential, respectively (Akashi et al., 2000; Kondo et al., 1997). However, the exact model for HSC and lineage commitment remains debated (Adolfsson et al., 2005; Arinobu et al., 2009; Ema et al., 2014; Pronk et al., 2007; Reya et al., 2001; Yamamoto et al., 2013).

Table 1. Mature blood cells and their functions

Type of cell	Main functions
White blood cells (leucocytes)	
Granulocytes	
Neutrophils	Phagocytose invading bacteria
Eosinophils	Destroy parasites and modulate allergic responses
Basophils	Release histamine
Monocytes	
Macrophages	Phagocytose invading microorganisms and cellular waste products
Lymphocytes	
B-cells	Make antigens
T-cells	Kill virus-infected cells and regulate other leucocytes
NK-cells	Kill virus-infected cells and some tumor cells
Red blood cells (erythrocytes)	Transport oxygen and carbon dioxide
Platelets (thrombocytes)	Initiate blood clotting

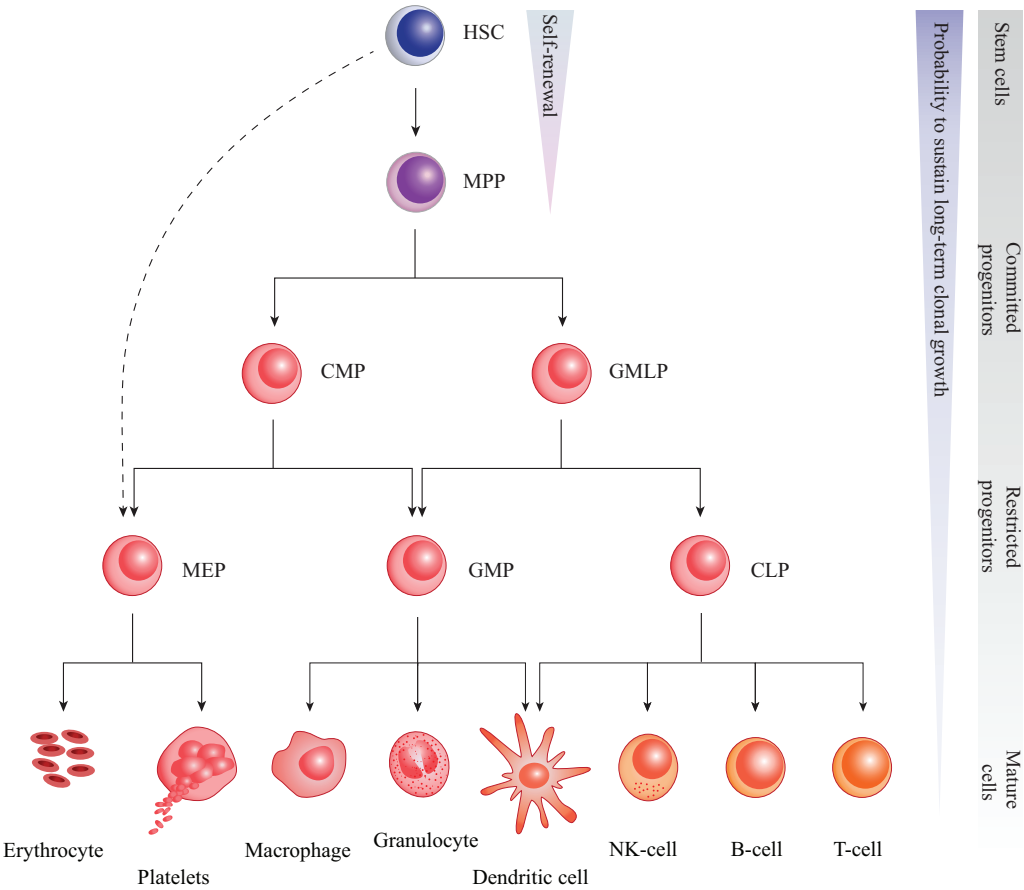

Figure 1. The hematopoietic hierarchy. HSCs produce progenitor cells with progressively decreased multipotency and self-renewal capacity as indicated. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common myeloid progenitor, GMLP, granulocyte/macrophage/lymphoid progenitors; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; CLP, common lymphoid progenitor.

Discovery of HSCs

In 1961 two pioneers of stem cell research, James Till and Ernest McCulloch provided evidence that multipotent HSCs exist. They found that a population of clonogenic BM cells could generate colonies in the spleen, named colony-forming unit spleen (CFU-S), of lethally irradiated mice (Becker et al., 1963; Till and McCulloch, 1961). When retransplanted into secondary hosts these colonies could reconstitute multiple blood cell lineages (Siminovitch et al., 1963). These ground breaking experiments defined the properties of stem cells (i) self-renewal capacity, (ii) multi-lineage potential, and (iii) extensive proliferation ability. Only a

true stem cell can give life-long reconstitution of an irradiated animal and retroviral marking studies further proved the existence of HSCs giving important insight into the enormous potential these cells exhibit. Retroviral DNA can be integrated into the genome of a host cell and because of this, the progeny of individual cells can be tracked. Identical retroviral sites were demonstrated to be found in all lineages of the hematopoietic system of primary and secondary hosts, thus identifying a common origin and the existence of HSCs (Dick et al., 1985; Jordan and Lemischka, 1990; Keller et al., 1985; Keller and Snodgrass, 1990). However, the ultimate proof for HSCs came in 1996 when Osawa *et al.* showed that single cells were able to reconstitute an irradiated host long-term with multilineage capacity (Osawa et al., 1996)

Methods to study the hematopoietic system

In vivo assays

The main advantage (and limitation) of Till and McCulloch´s classical *in vivo* CFU-S study was the short-term nature of the assay (typically 12 days) (Till and McCulloch, 1961). Methods that truly define HSCs are long-term reconstitution assays were mice are preconditioned by lethal irradiation making them acceptable to new HSCs after the recipient mice are injected with either a mixed population of cells containing HSCs or with purified HSCs (Domen and Weissman, 1999; Morrison et al., 1995). The injected cells (donor cells) of the new host (recipient) will migrate to the hematopoietic organs (BM, liver, spleen), a process named *homing*, and repopulate the hematopoietic system of the recipient. At different time point after transplantation the donor cells from the hematopoietic organs can be collected and analyzed by a method called fluorescence activated cell sorting (FACS). This technique is based on the expression of proteins on the cell-surface (e.g., isoforms of the pan-hematopoietic marker CD45) that can be recognized by specific monoclonal antibodies. Using FACS, the donor cells can be separated from recipient cells and lineage markers can detect skewing in lineage commitment. Working with HSCs is difficult because they are very rare, 0.004% of the total bone marrow cellularity, which is why the development of FACS has been crucial for the field of hematopoiesis, enabling recognition and quantification of very small numbers of cells (Osawa et al., 1996).

To study long-term reconstitution mice are analyzed 12-16 weeks after transplantation, but to extend the potential of this assay serial transplantations can be performed. In this case bone marrow from the primary recipients becomes the HSC source for second irradiated recipients. If this results in en increased repopulation capacity it could be an indication of enhanced self-renewal. In contrast, decreased repopulation capacity in secondary hosts could be a result of impaired self-renewal capacity, leading to HSC loss or excessive self-renewal resulting in exhaustion.

In an experimental setting to further investigate the reconstitution capacity of HSCs, they may be transplanted together with wild type (wt) HSC in an approach called *competitive bone marrow transplantation*. Moreover, a stringent method to quantify HSCs is using a *limiting-dilution assay,* where different doses of a test population are transplanted together with competitor cells (Micklem et al., 1972; Szilvassy et al., 1990). Recipients are considered positive for reconstitution if peripheral blood (PB) contains over 1% test cells in both myeloid and lymphoid lineage 12 weeks post transplantation. The number of recipients who regenerated hematopoietic tissues from the dilutions of test cells can then be used to calculate the frequency of competitive repopulating units (CRU) within the donor (based on Poisson statistics). The CRU-assay quantifies HSC numbers, however, it does not take into account homing and repopulation kinetics, which are important features for engrafting HSCs.

In vitro assays

There are also a number of different methods that have been developed to study HSCs *in vitro.* One of the most commonly used is colony-forming unit (CFU) assay that measures the frequency of hematopoietic progenitors, differentiation capacity and proliferative potential (Wognum and Szilvassy, 2013). In these experiments, semi-solid medium supplemented with cytokines supporting growth and differentiation of different lineages is being used. A progenitor responding to these cytokines will form a colony that is detected and analyzed. Since each progenitor is responsible for one colony, this allows for the quantification of the numbers of progenitors of the selected lineage present in a sample. The size and diversity of cell-types in the colony is a measurement of proliferation potential and primitiveness, respectively. For example, if a progenitor is restricted to the myeloid lineage, colony-forming units-granulocyte/macrophage (CFU-GM, CFU-G, CFU-M) will be formed. Current protocols also enable detection of burst/colony forming units-erythrocyte (BFU-E, CFU-E) as well as mixed colonies (CFU-GEMM) which are the largest colonies formed that originate from the most immature progenitors having multilineage potential (Wognum and Szilvassy, 2013).

One other *in vitro* assay is the long-term culture initializing cell (LTC-IC) assay allowing for the quantification of hematopoietic progenitor cells upstream of those giving rise to CFUs (Sutherland et al., 1990). Initially, test cells are seeded onto irradiated marrow feeders or a stromal cell line with HSC-supportive activity. Committed progenitors have limited proliferation capacity and will disappear during the initial 3-5 weeks of culture. In contrast, the more primitive cells present in the test cell population will maintain throughout the culture and generate new committed progenitors. At the end of the assay the remaining cells are plated in semi-solid medium to analyze their CFU capacity as discussed above. Finally, the number of primitive progenitors in the initial test population can be calculated (Liu et al., 2013). LTC-IC assays can be performed in a limiting dilution manner and can complement the CRU assays (described above). However, they do not truly reflect the *in vivo* situation and LTC-ICs are for this reason biologically distinct from cells engrafting in their natural environment in the BM.

Embryonic origin of hematopoiesis

Adult hematopoiesis occurs in the BM microenvironment called the HSC niche, where HSCs reside and progenitor cells are continuously formed. However, this is not the location of HSCs during ontogeny. During embryogenesis the process of blood cell development takes place at many sites and is characterized by two waves (Figure 2). *Primitive hematopoiesis* generates mainly primitive erythrocytes and certain myeloid cells, and later *definitive hematopoiesis* generates definitive-HSCs (dHSCs) which give rise to all mature blood cells (Medvinsky et al., 2011). The first wave and the origin of the hematopoietic system includes a common mesodermal precursor for both hematopoietic and endothelial cells, the hemangioblast and the first hematopoietic cells are thought to appear in the extra-cellular yolk sac (Choi et al., 1998; Moore and Metcalf, 1970).

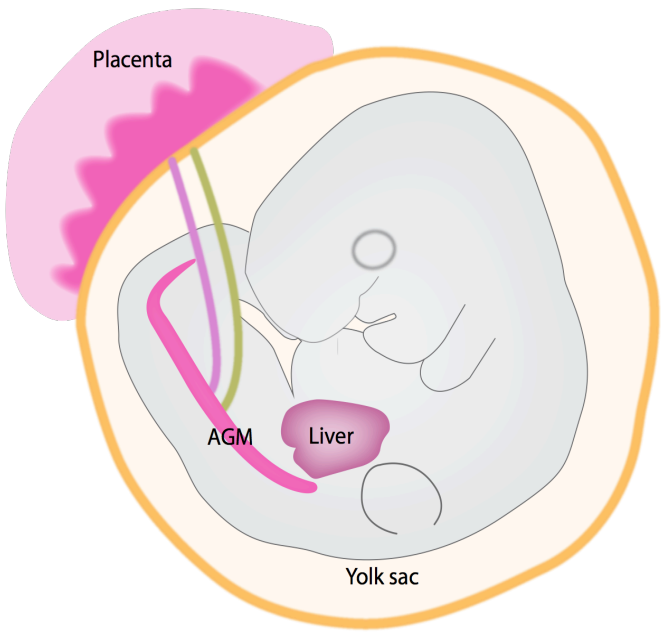

Figure 2. Hematopoietic developmental sites in the embryo. Figure modified from Dzierzak and Speck, 2008.

The second wave and the site of definitive hematopoiesis is the intra-embryonic aorta-gonad-mesonephros (AGM) region that has been shown to be a source of dHSC (Medvinsky and Dzierzak, 1996; Muller et al., 1994). However, the true origin of dHSC is a matter of disagreement. More recent reports have shown that the placenta contains definitive HSC activity before the onset of an established circulatory system, which suggests that this could be the site for dHSC development (Gekas et al., 2005; Rhodes et al., 2008). Upon establishment of the circulatory system the cells migrate to the fetal liver, which becomes the major site for dHSCs. Here, a robust expansion of the cells occurs, increasing the HSC reservoir (Ema and Nakauchi, 2000). Finally, HSCs migrate to the thymus and spleen and during the later stages of gestation HSCs will colonize the BM which in adults are the permanent site for hematopoiesis (Medvinsky et al., 2011).

Isolation of mouse HSCs

The flow cytometry technique has been fundamental in enabling the identification of HSCs. As mentioned earlier HSCs are very rare and FACS-based cell sorting makes it possible to isolate these cells with high purity. Monoclonal antibodies conjugated to fluorescent markers allows for the detection of antigens on the cell surface, and combination of monoclonal antibodies directed against cell surface markers expressed by HSCs must be used in order to isolate these cells. It has been well established that HSCs are negative for markers associated with mature blood cells (collectively referred to as lineage negative, Lin-) and positive for stem cells antigen (Sca)-1, which is very commonly used to enrich HSCs in the murine system (Spangrude et al., 1988). C-kit is another commonly used marker, with an important function as the receptor for stem cell factor (SCF) (Ikuta and Weissman, 1992). The combination of Lin-, Sca-1+ and c-Kit+ are referred to as LSK and mark a heterogeneous population of cells enriched in HSCs. Since only a fraction of the cells are stem cells, this population needs to be further subdivided and can be enriched by different additional marker combinations. For example, long-term (LT)-HSCs have been shown to be negative for CD34 and Flt3 ($LSKCD34^{-}Flt3^{-}$), the CD34 marker is associated with the activation state and has been shown to be reversible, since activated CD34 positive stem cells can return to a CD34 negative state after transplantation (Osawa et al., 1996; Sato et al., 1999). LT-HSCs have the capacity to sustain life-long hematopoiesis and exhibit extensive self-renewal potential, while short-term (ST)-HSCs ($LSKCD34^{+}Flt3^{-}$) have more restricted self-renewal capacity and can only contribute the hematopoietic system for a limited period of time (Yang et al., 2005). Furthermore, upregulation of Flt3 occurs in the multipotent progenitor (MPP) state and is associated with a loss of self-renewal potential (Figure 1) (Adolfsson et al., 2001). In addition, the signaling lymphocyte activating molecule (SLAM) family receptors CD150 and CD48 ($LSKCD150^{+}CD48^{-}$) can further enrich the stem cell population and a combination of CD34/Flt3 and SLAM markers is often used to purify LT-HSCs (Adolfsson et al., 2001; Kiel et

al., 2005; Yang et al., 2005). Metabolic dyes have also been used to isolate HSC, this method is based on the cells efflux activity. When Hoechst 33342 is analyzed at two-emission wavelength a distinct subset of cells termed side population (SP) is identified that are highly enriched in HSCs (Goodell et al., 1996). Regardless of which marker combination is used, heterogeneity still remains in the HSC population with respect to lineage output and repopulation capacity (Copley et al., 2012).

Isolation of human HSCs

The first marker used to enrich humans HSCs was CD34. In contrast to mouse cells the fraction of human stem cells can be found in the CD34 positive population (Civin et al., 1984). There are also other markers that lack congruence between the two species, for example human HSCs are positive for Flt3 while they do not express CD150 (Larochelle et al., 2011; Sitnicka et al., 2003). The fraction of CD34 positive cells is heterogeneous and therefore the purification of primitive human hematopoietic cells requires additional markers. Using a xenograft model, it was demonstrated that the CD90-marked cells (Thy1) together with CD34 enriched HSCs (the rare $Lin^-CD34^+CD90^+$ population in human fetal BM) were shown to contain pluripotent hematopoietic progenitors (Baum et al., 1992). Furthermore, exclusion of CD45RA and CD38 has been shown to purify HSCs as they are expressed on more differentiated progenitors (Bhatia et al., 1997; Lansdorp et al., 1990). Thus, the marker combination of $Lin\text{-}CD34^+CD38^-CD45RA^-CD90^+$ has been frequently used to purify HSCs over the past decade. Importantly, in a recent study Notta et al. showed that transplanted single $Lin^-CD34^+CD38^-CD45RA^-Thy1^+Rho^{lo}CD49f^+$ cells were highly efficient in generating long-term multilineage engraftment (Notta et al., 2011). In contrast, loss of the CD49f marker characterized transient engraftable cells capable of multilineage reconstitution, thus reflecting MPPs (Notta et al., 2011).

Interestingly, it is controversial whether all HSCs reside in the CD34 positive fraction. Although most human HSCs are associated with CD34 expression several studies have described the existence of primitive hematopoietic cells in the CD34 negative population (Anjos-Afonso et al., 2013; Bhatia et al., 1998; Goodell et al., 1997; Wang et al., 2003). In a recent study, Anjos-Afonso et al. reported $Lin^-CD34^-CD38^-CD93^{hi}$ cells to be at the apex of the human HSC hierarchy, placed above $CD34^+$ cells (Anjos-Afonso et al., 2013).

Xenograft models

Because HSCs are identified and studied through functional repopulation assays the majority of our understanding of hematopoiesis is based on mouse experiments. However, mouse and human differ and the direct relevance to develop therapies requires the complement of human-based studies. Xenotransplantation, i.e. transplantation from one species to another, and *in vitro*

clonal assays have made it possible to study human HSC biology. The major problem with xenotransplantation is that transplanted cells will activate an immune response eliminating these "invading" transplanted cells. Development of immunodeficient mice was the breakthrough that made it possible to study human HSCs *in vivo*. The first model was the severe combined immune-deficient (*Scid*) mice lacking T and B cells, later crossed with the non-obese diabetic (NOD) mice (Bosma et al., 1983). NOD-*Scid* mice were shown to have defects in innate immunity and for that reason supported higher level of human engraftment (Shultz et al., 1995). Primitive human hematopoietic cells capable of engrafting these mice are defined as Scid-repopulating cells (SCRs) (Larochelle et al., 1996). The development of NOD-Scid mice paved the way for scientist to study human hematopoiesis *in vivo*. A major disadvantage with the NOD-*Scid* model however, is that the mice have a short lifespan, making it difficult to follow HSCs long-term. Moreover the fact that they have NK-cell activity limits the human engraftment potential. To circumvent these problems NOD-Scid mice were crossed with a mouse strain either truncated in the IL-2R common γ chain (Il2rg), termed NOD/Shi-scid/Il2rg$^{-/-}$ (NOG), or with a strain that completely lacked the Il2rg, termed NOD/LtSz-scid/Il2rg$^{-/-}$ (NSG) (Ito et al., 2002; Shultz et al., 2005). Il2rg is a critical component in the immune response and deletion of this gene leads to loss of B, T and NK-cell activity, thus supporting engraftment of human hematopoietic cells.

There have been approaches to humanize the xenograft models by introducing expression of human cytokines in the murine system when the mouse counterpart has no cross-species activity of that particular cytokine. Thrombopoetin (TPO), IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) have been replaced by knock-in gene replacement thus mimicking human environment (Rongvaux et al., 2011; Willinger et al., 2011). However, even using humanized models one should always remember that some results might be artifacts of the surrogate xenograft model, demanding parallel studies of mouse and human cells.

HSCs in the clinic

HSCs have tremendous therapeutic potential and have been used clinically since 1959 to treat a variety of hematopoietic disorders e.g. leukemia and immunodeficiencies. The pioneer was E. Donnall Thomas who performed the first bone marrow transplantation (BMT) of two leukemic patients using donor BM from the patient's healthy identical twin (Thomas et al., 1959). Transplantations of BM and HSCs can be performed in different settings, autologous transplantation is when a patient's own BM tissue is re-infused, or in rare cases using identical twins' BM as donor sample (often referred to as syngeneic transplantation). Using donor BM between individuals who are not genetically identical is known as allogeneic transplantation and can result in graft-versus-host disease (GVHD). The immune system uses human leukocyte antigens (HLA) present on white blood cells, to distinguish between "self" and "non-self" and

the clinical successes of BMT did not come until the until late 1960's when the importance of HLA system was understood (Thomas, 1999).

In the beginning, transplantations were performed using BM as the sole source of HSCs. Today, PB is most commonly used as HSCs can be mobilized from BM to PB by the use of growth factors like granulocyte-colony stimulating factor (G-CSF) and/or the mobilizing agent AMD3100 (Korbling and Freireich, 2011; Motabi and DiPersio, 2012; To et al., 1997). A third more convenient source for harvesting HSCs discovered almost 30 years ago is cord blood (CB) (Broxmeyer et al., 1989). Umbilical CB that is normally discarded after delivery is a convenient source for harvesting HSCs (Sorrentino, 2004). Furthermore, cells from CB can more easily be transplanted across HLA barriers and could be stored in large banks with a spectrum of different HLA-types for medical use (Rubinstein et al., 1998). The yield of HSCs in CB however, is too low for successful transplantation to most adult patients, and thus ideally HSCs from CB needs to be increased in number (expanded) *ex vivo* before transplantation (Brunstein and Wagner, 2006; Doulatov et al., 2012; Sorrentino, 2004). Expansion of HSCs holds great promise, but has been met with limited success due to incomplete knowledge regarding regulation of HSC proliferation and self-renewal (Figure 3). Thus, deeper understanding of the regulatory mechanisms that govern HSC fate is fundamental to expand cell numbers *ex vivo* and improve HSC-based therapies (Article I-III). In addition to self-renewal, survival of stem cells and prevention of apoptosis has also emerged as an important mechanism to maintain HSCs *in vivo* and *ex vivo*. It was recently discovered that prevention of endoplasmic reticulum stress and improved protein quality control in HSCs prevents apoptosis of HSCs (Miharada et al., 2014).

The properties of HSCs make them very suitable for gene therapy. The idea behind gene therapy is to correct monogenetic disorders using viral vectors carrying the therapeutic gene of interest to correct the defect gene. Tailored and genetically modified HSCs could theoretically lead to life-long correction and would restore normal biological function of the whole hematopoietic system (Karlsson et al., 2002). Gene therapy has been used successfully to treat hematopoietic disorders, including sever combined immune deficiency-X1 (SCID) which provided proof that this advanced therapy works in practice (Cavazzana-Calvo et al., 2000). Unfortunately, insertional mutagenesis due to integration of the therapeutic gene near proto-oncogenes is a serious concern that results in leukemic transformation (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). The risks associated with gene integration temporarily halted some gene therapy trials and raised doubts concerning the safety of genetically engineered cells. However, gene therapy of ADA-SCID has been very successful with long-term efficacy of the treatment and without any adverse effects due to insertional mutagenesis (Aiuti et al., 2009). To overcome the problems of insertional mutagenesis in the future both vector design and knowledge regarding gene therapy needs to be improved. HSC expansion would further benefit gene therapy, since increased self-renewal of HSCs would result in increased HSC yield and more efficient transduction.

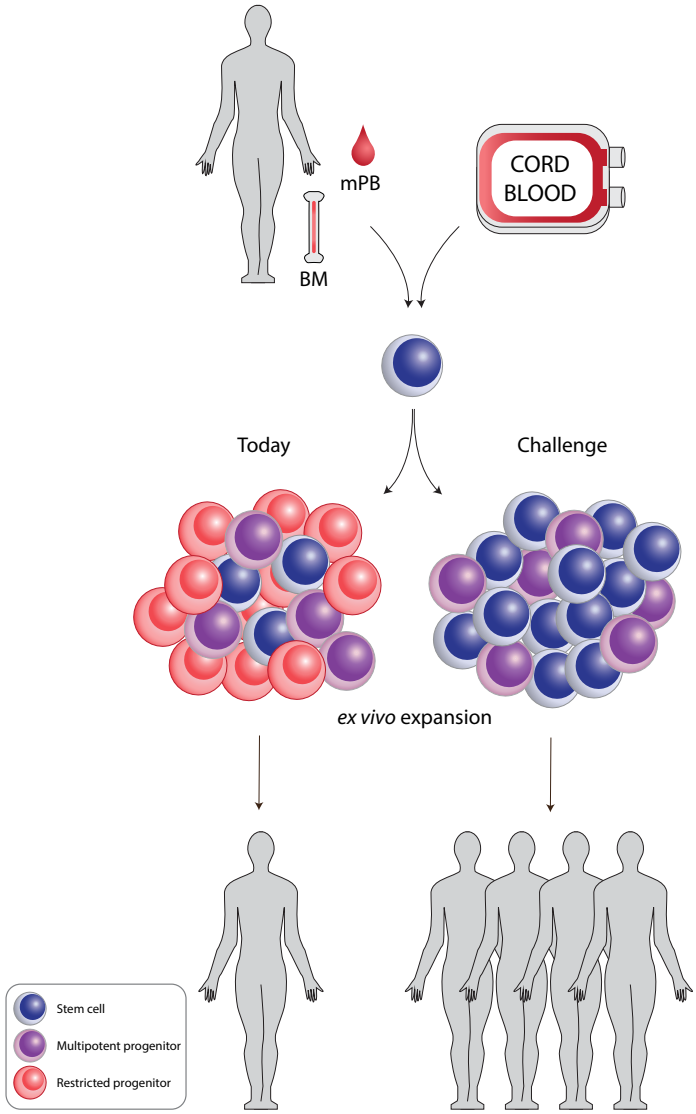

Figure 3. HSC expansion today and the challenge for future *ex vivo* protocols. Bone marrow (BM), mobilized peripheral blood (mPB) and cord blood contain HSCs. Future protocols requires expansion of undifferentiated HSCs for sufficient therapy of adult patients.

iPS cells

Hope for another advanced tailored cell therapy came in 2006 when Shinya Yamanaka and colleagues published an extraordinary paper describing induced pluripotent stem (iPS) cells. Dr. Yamanaka, who was awarded with the Nobel prize in 2012, showed that ectopic co-expression of four factors (c-Myc, Klf4, Oct4 and Sox2) could reprogram mouse fibroblast into

iPS cells (Takahashi and Yamanaka, 2006). These cells were almost indistinguishable from embryonic stem cells, multipotent stem cells able to form every type of cell in the human body apart from the placenta. One year later the same group reported the successful generation of iPS cells from human fibroblast cells (Takahashi et al., 2007). This discovery was groundbreaking and could theoretically result in a clinical scenario where a skin cell from a patient with a particular disease could be genetically modified and thereafter reprogrammed before re-infusion into the patient after gene correction, to replace the dysfunctional cells and cure the patient. A huge advantage with iPS-based technology is that the treatment would be autologous as the cells originate from the patient and would therefore not be rejected. This scenario was proven to work in the mouse system in 2007, when Hanna et al. successfully cured an animal model of sickle-cell anemia using this approach (Hanna et al., 2007). This proof of principle study showed that the cutting edge technology of iPS cells holds great clinical promise for the treatment of human diseases. However, the safety of iPS cell based therapy and realistic clinical potential have yet to be determined. (Riggs et al., 2013; Robinton and Daley, 2012; Yamanaka, 2012). Furthermore it has been challenging to generate engrafting HSCs from iPS cells, therefore plans to generate HSCs from iPS cells for blood cell therapies are not realistic unless the molecular mechanisms that control HSC generation from iPS cells can be identified and manipulated (Cherry and Daley, 2013; Wang et al., 2005).

Regulation of HSC fate

HSCs have a number of cell fate properties, these include the possibility to *self-renew*, *differentiate*, go through *apoptosis* (programmed cell death) or *migrate* from the BM (Figure 4) (Wagers et al., 2002). The balance between these activities determines the number of stem cells present in our body and deregulation of HSC fate could easily result in malignant diseases. The process of *self-renewal* (generation of an identical copy of the cell after division) is required to prevent depletion of the HSC pool and is important both during homeostasis as well as under physiologic stress, e.g. infection and injury. Too much self-renewal can lead to improper differentiation and exhaustion of HSC, while too little self-renewal can lead to insufficient number of HSCs and eventually hematopoietic failure. Self-renewal is a unique property that can either be symmetric or asymmetric in its nature. *Symmetric division* results in two HSC-daughter cells, leading to subsequent expansion of HSCs and is for this reason thought to be important after transplantation. *Asymmetric division* results in two daughter cells with different fate; one committed cell that has started the path towards becoming a mature blood cell and one qualitative HSC keeping the HSC pool intact (Morrison and Kimble, 2006). The differentiation property of HSCs is crucial since most mature blood cells are short-lived and need to be replaced continuously. Unbalanced differentiation however, can result in myeloproliferative disease and leukemia. Furthermore, induction of apoptosis is required to balance stem cell number and is an essential trait for the prevention of cancer (Reya et al., 2001). Stem cells can also migrate, a phenomenon that is particularly important during development when HSCs seed the fetal liver, spleen and BM, as previously discussed (Medvinsky et al., 2011). The migration of HSCs is also important later in life for mobilization of HSCs from the BM to the periphery (Wright et al., 2001).

One hallmark of the hematopoietic system is the ability to respond rapidly to situations of stress, such as acute blood loss or transplantation. HSCs are under strict control in the niche and thought to be controlled by a complex system of both cell-autonomous and niche-induced signals (Enver et al., 1998; Ogawa, 1999). The *cell-autonomous or stochastic model* proposes that HSC fate has a random nature of gene expression and that external signaling, like growth factors, only mediates viability and proliferating signals (Ogawa, 1999). Opposed to this, the niche-induced or *instructive/deterministic model* suggests that HSCs can be instructed by extrinsic signals that will induce fate decisions (Morrison and Weissman, 1994). It remains debatable whether stochastic and/or deterministic events regulate HSCs. Regardless, HSCs are most likely controlled by a complex interplay between these two systems and it is important to understand the circuitry of their interaction.

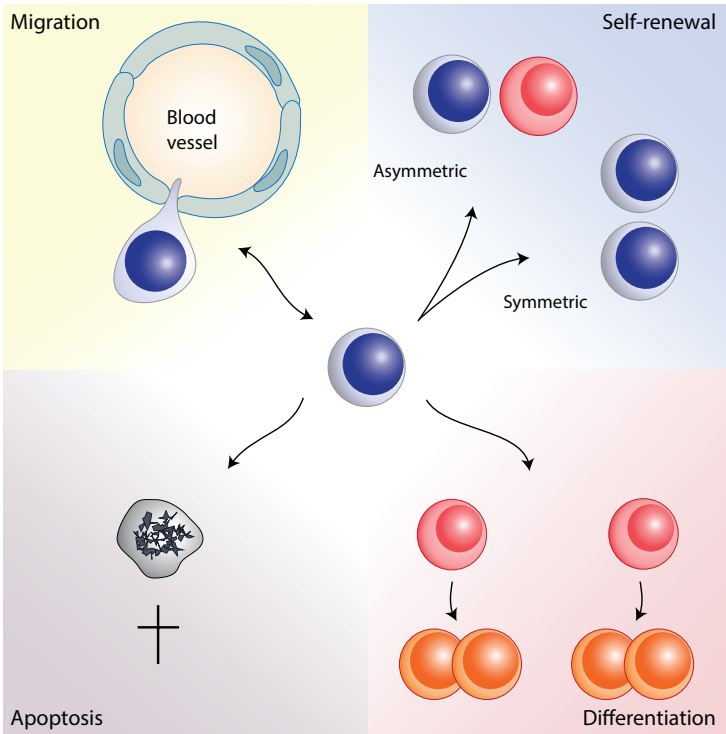

Figure 4. HSC fate options.

The HSC niche

The HSC niche is the microenvironment in the BM where HSCs reside. Schofield first proposed the term "niche" in 1978 as the location in BM that preserves the reconstituting ability of stem cells (Schofield, 1978). Since then, in order to understand more about HSCs biology, the niche has been well studied. However, like any interactive system there is a complex interplay between different cell types and factors and the understanding of the HSC niche is far from complete.

Two niches

Two distinct anatomical niches have been proposed, the *endosteal* and the *vascular niche* (Ehninger and Trumpp, 2011; Kiel et al., 2005; Lo Celso et al., 2009; Mendez-Ferrer et al., 2010; Xie et al., 2009). In the endosteal niche osteoblasts (OBs) and bone remodeling are important components of HSC maintenance. It has been shown that the number of OBs, calcium-release (released during active bone turnover), TPO, angiopoietin-1 (Ang-1) and osteopontin (expressed by OB) are all important for HSC regulation (Adams et al., 2006; Arai

et al., 2004; Calvi et al., 2003; Nilsson et al., 2005; Yoshihara et al., 2007; Zhang et al., 2003). Miharada et al. identified the developmental cue, Cripto, as a HSC regulator (Miharada et al., 2011). Cripto preserves stem cell properties *in vitro* and *in vivo* by signaling through its cell surface receptor, 78 kDa glucose regulated protein (GRP78). $GRP78^+$ HSCs that can respond to Cripto signaling are located in the endosteal region and were shown to be largely quiescent with high glycolytic activity (Miharada et al., 2011). The endosteal niche is thought to be low in oxygen level (hypoxic) and it has been suggested that an oxygen gradient exists in the BM, with HSCs residing in the most hypoxic environment (Cipolleschi et al., 1993; Parmar et al., 2007). A hypoxic niche protects HSCs from reactive oxygen species (ROS) since ROS can induce cycling, which in turn would lead to exhaustion of HSCs (Ito et al., 2006). However, this view has been challenged. In a resent study Spencer *et al.* measured oxygen levels in live animals and proposed a revised model where the oxygen concentration is highest near the endosteal region, thus leading to a reversed oxygen gradient (Spencer et al., 2014). The exact role for hypoxia in the niche remains an open question, however the oxygen level is an important feature in the regulation of HSCs.

HSCs are also associated with the vascular niche, composed of perivascular and endothelial cells (Kiel et al., 2005; Sugiyama et al., 2006). Importantly, HSCs are mobile cells that migrate from the BM to the circulation. In keeping with this, HSCs observed near vessels may not permanently reside at this location but could have been observed entering or exiting circulation. In any case, one important regulator for HSC self-renewal and maintenance is SCF expressed by the vascular niche cells, and at lower levels by the endosteal lining (Ding et al., 2012). Mesenchymal stem cells (MSCs) are also known to express SCF as well as chemokine (C-X-C) motif ligand 12 (CXCL12 a.k.a. stroma cell derived factor-1, SDF-1), shown to be critical in mobilization of HSC (Mendez-Ferrer and Frenette, 2007; Sugiyama et al., 2006). The presence of CXCL12-abundant reticular (CAR)-cells, associated with microvasculature, has been shown to be an important component of the HSC niche (Omatsu et al., 2010). In addition, Mendez-Ferrer *et al.* used mice expressing GFP under the Nestin promoter to show that BM $Nestin^+$ perivascular cells, that are associated with putative HSCs, express not only SCF and CXCL12 but also Ang-1, OPN and vascular cell adhesion molecule 1 (VCAM1), all documented HSC maintenance factors (Mendez-Ferrer et al., 2010). Furthermore, selective depletion of $Nestin^+$ cells reduced in numbers of HSCs, providing evidence that $Nestin^+$ cells are important for HSCs maintenance (Mendez-Ferrer et al., 2010). Importantly, approaches for allowing the conditional deletion of specific cells as well as factors in the niche environment make it possible to study niche functions. One should however, keep in mind that HSCs could be directly affected and/or alternatively indirectly affected by the environmental changes in the niche caused by the deletion.

Importantly, HSCs have been shown to be regulated by adrenergic nerve fibers of the sympathetic nerve system (SNS), responsible for the circadian oscillations of circulating HSCs (Ehninger and Trumpp, 2011; Katayama et al., 2006; Mendez-Ferrer et al., 2008). Moreover,

non-myelinating Schwann cells, associated with sympathetic nerves in the BM niche, have been shown by Yamazaki et al. to activate latent forms of transforming growth factor-β (TGF-β) and demonstrated to be located in close contact with HSCs (Yamazaki et al., 2011). TGF-β is a potent inhibitor of hematopoietic stem and progenitor cell proliferation and will be discussed in more detail in the next chapter.

Dormant HSCs

A switch from fetal to adult hematopoiesis occurs three weeks after birth when the HSCs undergo a major change from frequently cycling to a more inactive stage (Bowie et al., 2006). Adult HSCs are largely non-dividing and cell cycle analysis has revealed that most HSCs are in G_0 phase of the cell cycle (Cheshier et al., 1999; Goodell et al., 1996; Wilson et al., 2008). DNA-labeling agents can be used to investigate the existence of quiescent HSCs. A commonly used labeling agent is 5-bromo-2-deoxyuridine (BrdU), an analog to thymidine that is incorporated into the DNA. *In vivo* studies using BrdU have challenged the existence of quiescent HSCs. It was postulated that HSCs divide regularly and that the HSC pool enters the cell cycle on average every 57 days (Cheshier et al., 1999; Kiel et al., 2007) However, two more recent studies, using transgenic mice expressing the fusion protein histone 2B-green fluorescent protein (H2B-GFP) under the control of tetracycline-responsive regulatory element, revealed that about one seventh of the studied HSC population divided as seldom as every 145 days, equivalent to five times per mouse lifetime (Wilson et al., 2008). Furthermore, this dormant population of HSCs performed better upon serial transplantation compared to more frequently dividing HSCs and is thought to represent a subpopulation of HSCs that are triggered to proliferate in response to G-CSF or injury signals (Foudi et al., 2009; Wilson et al., 2008).

The role of the two aforementioned niches is a matter of dispute. It has been suggested that the endosteal niche contains the largest amount of bona fide stem cells that are dormant while the vascular niche is housing self-renewing, actively proliferating HSCs (Ehninger and Trumpp, 2011; Wilson et al., 2007; Wilson and Trumpp, 2006). However, recent reports have implicated a role for the endosteal niche as a location for early lymphoid restricted progenitors, thus being cellularly and functionally distinct from the vascular niche where putative HSCs are suggested to be located (Ding and Morrison, 2013; Greenbaum et al., 2013; Morrison and Scadden, 2014). Kiel and colleagues showed that a low fraction of HSCs (14%) were located near the BM endosteal surface, while the majority of HSCs were found in the center part near bone-marrow sinusoids (Kiel et al., 2005). As it is a complex system of cell types and factors that regulate stem cells in the BM, it is also possible that lineage-restricted niches exist (Chow et al., 2013; Morrison and Scadden, 2014). Previous work regarding the biology of the HSC niche has answered many questions, but niche related research is rapidly progressing making it likely that additional components will be defined in the near future.

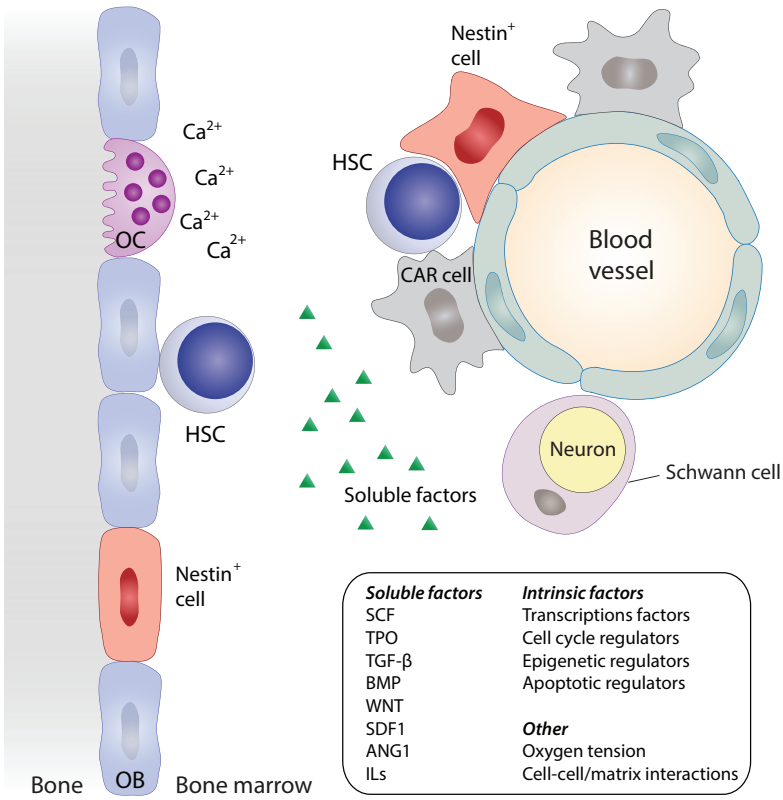

Figure 5. Location of HSC in the endosteal and the vascular niche. The endosteum is lined by osteoblasts (OB) and is remodeled by osteoclasts (OC). The vascular niche is composed of perivascular cells, endothelial cells and CXCL12-abundant reticular (CAR)-cells.

Intrinsic regulation

The microenvironment in the BM is clearly important to regulate the fate outcome of HSCs. In addition to this, a wide variety of intrinsic factors are also responsible for HSCs regulation, including transcription factors, transcriptional repressors, cell cycle regulators and anti-apoptotic signals (Domen, 2000; Ooi et al., 2010; Pietras et al., 2011; Sauvageau et al., 2004). To study the mechanisms of intrinsic factors, the approach of loss- or gain of function studies is often used. An example of this is a well-studied transcription factor, HoxB4, the overexpression of which resulted in increased HSC regeneration (Antonchuk et al., 2001, 2002; Sauvageau et al., 1995; Thorsteinsdottir et al., 1999). Surprisingly, deletion of HoxB4 using a

conditional knockout mouse model only mildly effected HSC function, perhaps due to redundancy of other Hox proteins (Bjornsson et al., 2003; Brun et al., 2004). Moreover, when the epigenetic regulator Bmi-1, a member of the polycomb group of transcriptional repressors, was deleted fetal liver HSCs isolated from Bmi-1 knockout animals exhibited impaired repopulation capacity, suggesting that Bmi-1 is important for functional HSCs (Park et al., 2003). In addition, elevation of Bmi-1 levels result in enhanced HSC function, with increased self-renewal potential both *in vitro* and *in vivo* (Iwama et al., 2004). Growth factor independent-1 (Gfi-1) has also been shown to be important for HSC maintenance, loss of which leads to increase cycling, followed by a decreased capacity to reconstitute irradiated recipients (Hock et al., 2004; Zeng et al., 2004). Gfi-1 knockout-HSCs expressed lower levels of cyclin-dependent kinase inhibitor (CDKI) and G_1 checkpoint regulator p21, which could in part explain the observed phenotype (Hock et al., 2004). CDKIs are central regulators of cell cycle and play a crucial role in HSC self-renewal. When p21 was deleted in a knockout mouse model, HSC proliferation and absolute number were increased. Serial transplantation experiments resulted in HSC exhaustion, leading to hematopoietic failure (Cheng et al., 2000). However, a more recent study demonstrated that p21 deletion in a different mouse background did not have major differences in cell cycle status or HSC number (van Os et al., 2007). These conflicting results demonstrate that the mouse background used can influence the results, which should be taken into account when drawing conclusions.

Anti-apoptotic regulators have also been linked to HSC maintenance. This has been demonstrated in studies where the expression of the apoptotic-suppressing gene Bcl-2 has been modified. HSCs with enforced Bcl-2 expression display a repopulative advantage over wt HSCs in competitive transplantations. Furthermore, they were protected from apoptotic stimuli, leading to increased an number of HSCs in the BM (Domen et al., 2000; Domen and Weissman, 2003). Recently, prevention of apoptosis through reduction in endoplasmic reticulum stress has been shown by our laboratory to improve HSC survival *in vitro* and *in vivo* and increase competitive repopulation compared to control cells after *ex vivo* culture (Miharada et al., 2014).

Epigenetic regulation

Over the past decade accumulating evidence has suggested that epigenetic mechanisms are involved in the regulation of critical biological processes. This type of gene modification, thought to be a higher level of intrinsic regulation includes DNA methylation, chromatin remodeling, covalent histone modification and non-coding RNAs. This type of regulation does not affect the DNA sequence but instead leads to modifications that change overall DNA and chromatin structure, which in turn determines the ability for transcriptions factors to get access to their target genes. Yamanaka and colleagues extraordinary work with iPS cells drew a lot of

attention to the field of epigenetic regulation as iPS cell technology relies on epigenetic changes (Takahashi and Yamanaka, 2006; Watanabe et al., 2013). This is because reprogramming factors are thought to not only to be able to regulate genes, but also to function as chromatin organizers rearranging chromatin architecture from a somatic to a pluripotent state (Apostolou and Hochedlinger, 2013). Furthermore, epigenetic regulation has been shown to be involved in hematopoietic development and altered epigenetic modification contributes to hematological malignancies. Importantly it has been shown that epigenetic modifiers can influence the balance between the two main fate options of HSCs, –self-renewal and differentiation, and their role as key instructors of lineage choice is now emerging.

DNA methyltransferases (Dnmts) are a family of enzymes that catalyze the transfer of methyl groups to DNA, more precisely to the 5' position of cytosine followed by guanine (CpG) (Attwood et al., 2002). CpG-rich regions in the genome are referred to as CpG-islands and are found in the promoter regions of about 60% of human genes (Antequera and Bird, 1993). Unmethylated CpG-islands are characterized by an open chromatin structure which correlates with active gene expression (Esteller, 2007). In mammals Dmnt1 primarily copies the cytosine methylation patterns from a hemimethylated substrate after DNA replication thus, serves as a maintenance enzyme (Song et al., 2011). Dmnt3a and Dmnt3b serves as *de novo* methyltransferases as they acts on unmethylated DNA substrates (Ooi et al., 2009). Dnmt-knockout mice die during early development of fetus or a few weeks after birth, while conditional deletion of Dnmt1 in the hematopoietic system results in impaired self-renewal capacity and altered differentiation of Dnmt1-null HSCs (Broske et al., 2009; Li et al., 1992; Okano et al., 1999; Trowbridge et al., 2009). Dnmt3a has also been shown to be important for HSC, with conditional knockout of Dnmt3a resulting in expansion of HSCs and impaired differentiation following transplantation (Challen et al., 2012). These results clearly show that DNA methyltransferases have different roles in HSCs and highlights their importance in HSC biology. Interestingly, genome-wide methylation mapping of hematopoietic progenitors revealed that myeloid commitment was associated with less global DNA methylation compared to lymphoid. This indicates that DNA methylation is critical for differentiation choice of myeloid versus lymphoid restriction (Ji et al., 2010).

DNA is spooled around histones in the form of an octamer that organizes DNA strands into higher orders of compaction called nucleosomes (Luger et al., 1997). Posttranscriptional modification occurs on histone proteins and two thoroughly studied modifications are *acetylation* and *methylation* of the N-terminal tail. Covalent modifications of histones, referred to as the "histone code", regulate the structure of chromatin and determine the transcriptional status for a specific genomic region (Bannister and Kouzarides, 2011). Deletion of the histone acetyltransferase (HAT) Moz leads to embryonic lethality and transplantation of fetal liver HSCs results in decreased reconstitution capacity (Katsumoto et al., 2006; Thomas et al., 2006). Furthermore, the Mll methyltransferases, members of the trithorax group family that methylates histone H3K4, are associated with gene activation (Bernstein et al., 2007). Since

Mll deficiency also results in embryonic lethality, Jude et al. used an inducible knockout model to demonstrate that adult HSCs deficient in Mll expression fail to reconstitute recipient mice because of defects in maintaining quiescence (Jude et al., 2007; Yu et al., 1995). Moreover, chromosomal translocations leading to a disrupted Mll gene have been observed in acute leukemias (Tkachuk et al., 1992). In murine embryonic stem cells the long noncoding RNA (lncRNA) Mistral has been shown to recruit Mll to chromatin, leading to activation of various Hox genes including Hox6 and Hox7 (Bertani et al., 2011). Non-coding RNAs are another class of epigenetic regulators, a diverse group of non-protein coding molecules that are involved in cellular processes. One example of these is microRNAs, regulating gene expression at a translational and post-transcriptional level e.g. though degradation of mRNA. There are more than 100 microRNAs expressed in the hematopoietic system, however the function of microRNAs in hematopoietic stem and progenitor cells remains to be completely understood (O'Connell et al., 2010). Han et al. showed in a recent report that microRNA-29a is expressed by HSCs and elevated levels lead to increased proliferation of hematopoietic progenitors and induced myeloproliferative disease in primary chimeras (Han et al., 2010).

The mechanisms involved in epigenetic regulation have become more evident during the past decade and clearly play key roles in hematopoietic development controlling self-renewal as well as differentiation. Epigenetic marks also appear to be involved in hematopoietic disorders which is why developing techniques to manipulate the epigenetic pattern holds great promise for novel approaches in the treatment of leukemia.

Extrinsic regulation

Cytokines such as SCF and TPO are traditionally included in the culture media of HSCs. These provide survival signals, promote proliferation *in vitro* and have been shown to be important for the maintenance of quiescent HSCs *in vivo* (Borge et al., 1996; Ema et al., 2000; Keller et al., 1995; Li and Johnson, 1994; Qian et al., 2007; Sitnicka et al., 1996a; Yoshihara et al., 2007). Using knockout mouse model approaches, SCF and TPOs respective receptors c-kit and c-mpl were disrupted leading to decreased HSC numbers and defects in long-term repopulation capacity (Kimura et al., 1998; Miller et al., 1996; Murone et al., 1998). Interestingly, Kent et al. used single cell *in vitro* cultures supplemented with different concentrations of SCF to demonstrate that SCF-signaling intensity can directly alter the transcription factor profile and long-term repopulating capacity of HSCs, thus providing further evidence for extrinsic regulation of HSCs (Kent et al., 2008). According to the stochastic model, external signaling only mediates viability and proliferating signals. However, in a recent study using video imaging and single-cell gene expression analysis, Mossadegh-Keller showed that stimulation of HSCs with macrophage colony stimulating factor (M-CSF) resulted in increased number of $PU.1^{+}$ cells with myeloid gene signatures and differentiation potential (Mossadegh-Keller et

al., 2013). These results clearly show that cytokines not only support survival and growth, but also have the capacity to direct HSCs fate, thus challenging the stochastic model.

In addition to cytokines that support HSCs growth there are also regulators that inhibit proliferation and a balance between positive and negative regulators is thought to be important for HSCs maintenance. One well established regulator for HSC growth is TGF-β, shown to inhibit HSC proliferation and discussed in more detail in the next chapter. Other factors inhibit hematopoietic progenitors include interferon-γ (IFN-γ) and tumor necrosis progenitors-α (TNF-α) (Bryder et al., 2001; Jacobsen et al., 1992; Maciejewski et al., 1995; Zhang et al., 1995).

Expansion of human HSCs ex vivo

Recently, increased engraftment of human hematopoietic cells has been reported following treatment with cytokines, developmental cues and chemical compounds. However, it is still challenging to expand human HSC for use in the clinic. The Notch signaling pathway ha been demonstrated to be highly involved in HSC regulation, with treatment of primitive CB cells with Notch ligand Delta-like 1 (DLK1) improving repopulation capacity in transplantation experiments (Delaney et al., 2010; Delaney et al., 2005; Ohishi et al., 2002). Delaney et al. reported a 6-fold increase in repopulating cells and a small clinical phase I trial, where patients with acute leukemia were transplanted with DLK1-treated CB paired with unmanipulated CB, showed rapid hematopoietic engraftment. However only two out of ten patients exhibited long-term persistence from the expanded CB (Delaney et al., 2010).

Another signaling pathway also involved in controlling HSC fate is the Wnt/β-catenin signaling pathway. Wnt proteins are crucial for HSC homeostasis and manipulation of Wnt signaling has been shown to affect HSCs expansion, although there is discrepancy between some of the findings (Austin et al., 1997; Baba et al., 2005; Baba et al., 2006; Reya et al., 2003; Staal and Luis, 2010; Willert et al., 2003). Nevertheless, prostaglandin E2 (PGE2) can increase the number of murine HSCs by 4-fold, the effect based on the stabilization of β-catenin and activation of Wnt target genes suggesting cooperation between Wnt and other signaling pathways (Goessling et al., 2009; North et al., 2007). In a recent preclinical rhesus macaque model, a 1-hour PGE2 treatment of $CD34^+$ CB cells augmented HSC frequency by 2-fold (Goessling et al., 2011). Furthermore, Cutler et al. demonstrated multilineage engraftment and accelerated neutrophil recovery of dimethyl-PGE2 treated CB units in a phase I trial (Cutler et al., 2013) (CTI: NCT00890500). This clinical trial demonstrated safety for use in the clinic, but increased engraftment of dimethyl-PEG2 treated cells was not observed.

Screening approaches can be used to identify novel regulators that target yet unknown pathways. One example of this is the approach that Boitano and colleagues employed based upon the sustained expression of CD34, indicating decreased differentiation in culture, which could be used to test potential compounds that promote HSC expansion (Boitano et al., 2010). They screened a library of 100 000 compounds and identified the purine derivate

StemReginin1 (SR1) which increased the number of HSCs by17-fold, determined by limiting dilution assay in NSG mice (Boitano et al., 2010). SR1 is an antagonist of the aryl hydrocarbon receptor (AhR) and in agreement with previous finding it was recently reported that deletion of AhR results in increased proliferation (Boitano et al., 2010; Singh et al., 2011). Clinical studies are currently ongoing using SR1-expanded CB cells for patients with hematological malignancies (CTI: NCT01474681).

Other compounds such as angiopoietin-like 5 (Angptl-5) and pleiotrophin have been shown to stimulate *ex vivo* expansion of HSCs (Himburg et al., 2010; Zhang et al., 2008). In a recent study Chaurasia et al. demonstrated that histone deacetylase inhibitor valproic acid (VPA) increased the number of SRCs by remarkable 36-fold compared to primary $CD34^+$ cells (Chaurasia et al., 2014). The authors conclude that VPA is responsible for the epigenetic reprogramming of cultured CB cells and that added cytokines are responsible for proliferation. These results highlights the power of epigenetic regulation, however further investigation is needed to prove the safety of epigenetic reprogrammed HSCs.

Molecular biology of TGF-β signaling pathway

Ligands, receptors and the Smad signaling pathway

Members of the transforming growth factor-β superfamily, including the TGF-βs, activins and bone morphogenetic proteins (BMPs), regulate different cellular processes from the very beginning of ontogeny, patterning tissues in the developing embryo, to later in life, controlling homeostasis and wound healing in the adult (Chang et al., 2002). The actions of TGF-β ligands are highly context-dependent based on cell type and environment, and can regulate apoptosis, proliferation as well as differentiation and serve a key role in HSC biology (Blank and Karlsson, 2011; Shi and Massague, 2003; Sporn and Roberts, 1988).

TGF-β ligands bring together serine/threonine kinase receptor I with receptor II on the cell surface, receptor II subsequently phosphorylates the kinase domain of receptor I and initiates signaling through phosphorylation of Smad proteins (Kitisin et al., 2007; Shi and Massague, 2003). TGF-β ligands convey signals intracellularly through the Smad signaling pathway (Figure 6). The Smad proteins can be divided into three functional classes depending on their role in the pathway; the receptor activated Smads (R-Smad; Smad1, 2, 3, 5, and 8), the common-mediator Smad (Co-Smad; Smad4) and the inhibitory Smads (I-Smad; Smad6 and 7). Ligand binding followed by receptor phosphorylation activates the intracellular R-Smads, which then form a complex with Smad4. The activated complex subsequently translocates into the nucleus, where it regulates transcription of target genes. R-Smad2 and 3 operate downstream of TGF-β and activin receptors, while R-Smad1, 5 and 8 primarily act downstream of BMP receptors. The I-Smads function to inhibit Smad signaling by interacting with the Co-Smad or competing with R-Smad receptor binding. Depending on the combination of ligands and receptors that interact, different Smad proteins are activated specifying the signaling outcome (Shi and Massague, 2003). Since R-Smads and Co-Smad have rather low affinity for DNA binding, activated Smad-complexes arriving in the nucleus are associated with DNA-binding partners ensuring high-affinity binding. Furthermore, co-activators, like CBP and p300, and co-repressors, such as Ski and SnoN, can influence the outcome of Smad signaling (Massague et al., 2005; Massague and Wotton, 2000; Shi and Massague, 2003). Thus depending on Smad-complex interactions with other proteins, as well as cell type and cell status, the gene response can be of great diversity.

In a variety of cell lineages TGF-β ligands have been shown to signal through a non-canonical pathway via TGFβ–activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase (MAPK) signaling pathway (Yamaguchi et al., 1995). Interestingly, TAK1 is expressed by HSC (Emma Rörby, Ulrika Blank, Stefan Karlsson, unpublished observation) and has been shown to be essential for HSC survival, as TAK1 deletion resulting in BM failure due to massive apoptotic death of hematopoietic cells (Tang et al., 2008).

TGF-β signaling in hematopoiesis

The TGF-β superfamily has been demonstrated to have different context dependent effects on HSCs. For example, BMP4 has been implicated as a positive regulator of proliferation and survival of human CB HSCs (Bhatia et al., 1999). Less is known about activins influence on the hematopoietic system, although Utsugisawa et al. showed that activin A has an inhibitory function on murine HSC growth *in vitro* (Utsugisawa et al., 2006). Moreover, low concentrations of TGF-β2 have been suggested to stimulate murine HSCs proliferation, conversely TGF-β1 is a well-established negative regulator of HSC growth, through effects on the cell cycle and apoptosis (Langer et al., 2004; Larsson and Karlsson, 2005).

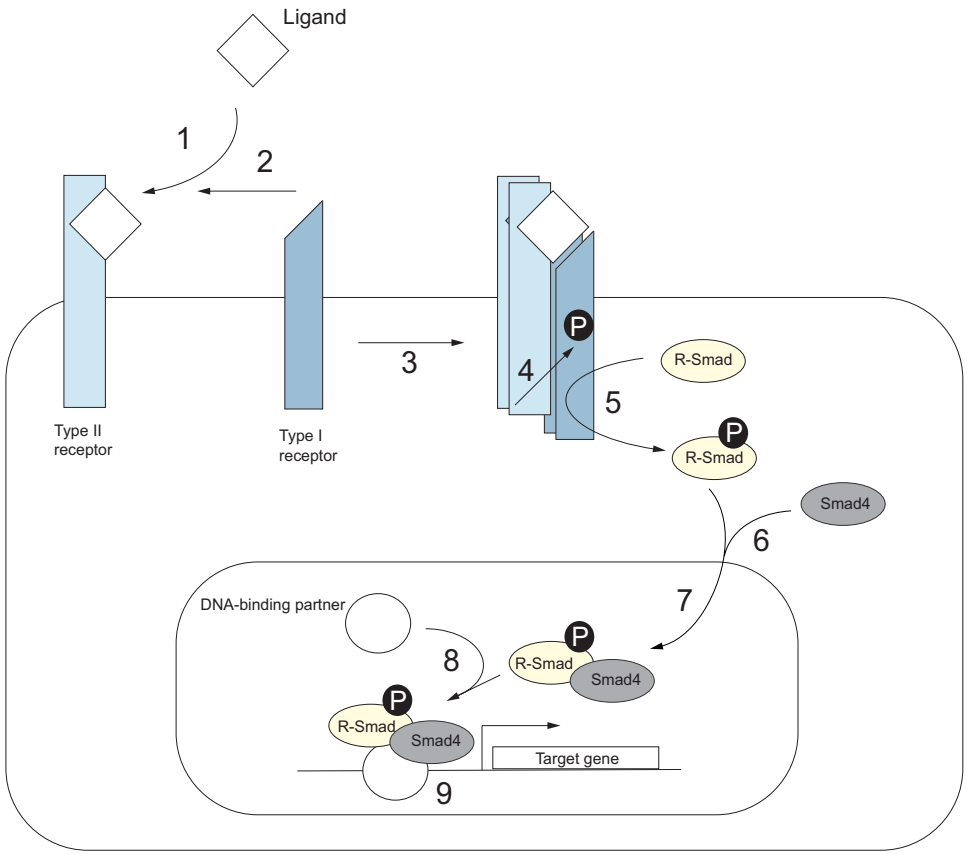

Figure 6. Signaling flow. Ligand binding brings together type II receptor (1) and type I receptor (2) on the cell surface. Formation of the receptor complex (3) leads to phosphorylation of type I receptor (4). The activated type I receptor subsequently phosphorylated a receptor-regulated Smad (R-Smad) (5) following binding of Smad4 (6). This complex will enter the nucleus (7), bind to DNA-binding partners (8) and regulate transcription og target gene (9). Adapted from Ulrika Blank, with permission.

TGF-β

In the adult, TGF-β has a very important function in regulating the immune system. All leukocytes produce and respond to TGF-β, playing a pivotal role in regulating the immune response with both stimulatory and inhibitory effects (Li et al., 2006). Consistent with this, TGF-β deficient mice develop lethal inflammatory disorders in both receptor and ligand knockout mouse models (Letterio et al., 1996; Leveen et al., 2002; Yaswen et al., 1996). When BM cells were transplanted from TGF-β1 deficient neonates, before the onset of inflammation, it resulted in impaired reconstitution activity due to defective homing (Capron et al., 2010). Yamazaki et al. generated a TGF-β type II receptor knockout in a immune-deficient Rag deficient background, to circumvent the development of lethal inflammatory disorder, and demonstrated that HSCs had increased cycling and reduced long-term repopulation capacity (Yamazaki et al., 2011). In contrast, HSCs from a TGF-β type I receptor knockout displayed normal regenerative functions *in vivo* when analyzing cell before the development of multifocal inflammatory disease, or when transplanted to immune-deficient mice (Larsson et al., 2003; Larsson et al., 2005). The discrepancies between these finding may be a result of redundant functions of other type I receptors and perhaps also other ligands. Interestingly, Iwata et al. recently reported that absence of TGF-β type II receptor induced non-canonical TGF-β signaling activating the TAK1/p38-MAPK pathway (Iwata et al., 2012).

TGF-β exists in three isoforms, TGF-β1-3, all signaling though the downstream Smad signaling pathway (Figure 7) (de Martin et al., 1987; Derynck et al., 1985; Derynck et al., 1988; Madisen et al., 1988; Piek et al., 1999; ten Dijke et al., 1988). The TGF-βs are secreted as latent proteins named the large latent complex (LLC) at the extra cellular matrix (ECM), which contains proteases that cleave TGF-β into its biologically active form (Annes et al., 2003; Gleizes et al., 1997). Recently, it was shown that a large proportion of dormant HSCs were in close contact to non-myelinating Schwann cells responsible for the activation of latent TGF-β in the BM (Yamazaki et al., 2011). As previously mentioned, TGF-β1, the most studied isoform, is a very potent inhibitor of murine and human HSCs and primitive progenitors (Batard et al., 2000; Fortunel et al., 2000; Garbe et al., 1997; Jacobsen et al., 1991; Sitnicka et al., 1996b). TGF-β has been hypothesized to be a key regulatory factor of HSC quiescence and, in agreement with this, administration of TGF-β1 into the femoral artery of mice resulted in inhibition of hematopoietic progenitors *in vivo* (Goey et al., 1989). In addition, several groups have shown that neutralization of TGF-β *in vitro* releases HSCs and hematopoietic progenitors from quiescence (Fortunel et al., 1998; Hatzfeld et al., 1991; Soma et al., 1996). Interestingly, accumulating evidence suggest that the adult HSC population consists of functionally distinct subsets of cells that differ in self-renewal and differentiation potential (Dykstra et al., 2007; Ema et al., 2014; Sieburg et al., 2006; Wilson et al., 2008). Consistent with this, Challen et al. reported that the response to TGF-β is different among discrete HSC subtypes (Challen et al., 2010). Low concentrations of TGF-β resulted in stimulation of myeloid-biased HSCs, while the opposite was observed for lymphoid-biased HSCs both *in vitro* and *in vivo* (Challen et al.,

2010). In a recent study the importance of TGF-β after myelosuppressive chemotherapy was demonstrated (Brenet et al., 2013). During *in vivo* stress, e.g. 5-FU treatment, blockade with TGFβ-neutralizing antibody resulted in prolonged cycling of hematopoietic progenitors and delayed the return to quiescence, thus increasing the hematopoietic reconstitution potential. However, blocking of TGF-β during homeostasis did not affect cycling of progenitor cells (Brenet et al., 2013). This suggests that TGFβ is important for the induction of dormancy after activation, but may not serve as a key regulator during steady state conditions.

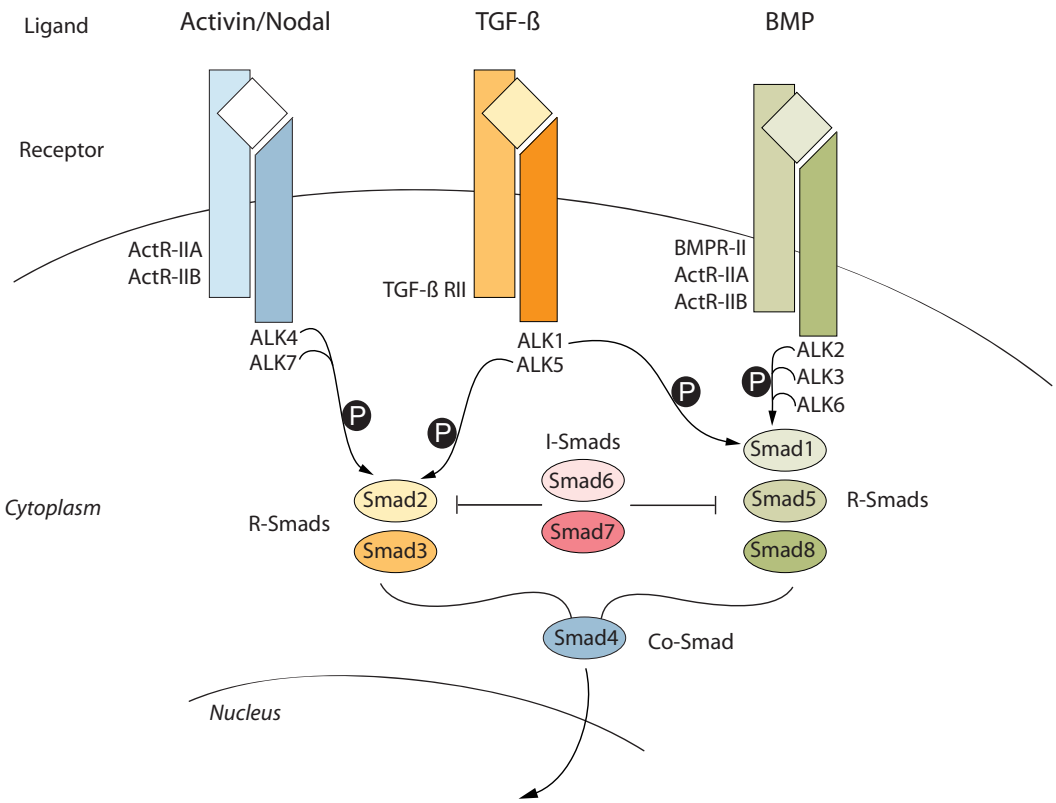

Figure 7. Divergence and convergence of Smad signaling. Commonly used alternative names include ALK2/Activin type I receptor, ALK3/BMP type IA receptor, ALK4/Activin type IB receptor, ALK5/TGF-β type I receptor, ALK6/BMP type IB receptor. Adapted from Ulrika Blank, with permission.

Mechanistically, TGF-β induces two classes of anti-proliferative responses. The first is downregulation of the growth stimulatory protein c-Myc and the second is upregulation of CDKIs, such as p15, p21 and p27 (Dao et al., 2002; Dao et al., 1998; Ducos et al., 2000; Massague et al., 2000). C-myc represses CDKIs and most cell types down-modulate c-myc in response to TGF-β. In contrast, CDKIs act in different combinations depending on cell type, for example inhibition of primitive hematopoietic cells has been suggested to be independent of p21 and p27 (Cheng et al., 2001). Another member of the CDKI family, p57, has been shown to be crucial for TGF-β-induced cell-cycle arrest in human hematopoietic cells (Scandura et al., 2004). Brenet et al. identified p57 as a key downstream mediator of TGF-β signaling response, as p57-knockout hematopoietic progenitors were unable to return to a quiescent state after 5-FU treatment, similarly to what was observed when adding TGFβ-neutralizing antibody (Brenet et al., 2013). In line with this, Yamazaki et al. demonstrated an enrichment of p57 in primitive HSCs ($LSKCD34^-$) in comparison to more mature $LSKCD34^+$ cells (Yamazaki et al., 2006). Furthermore, the same group showed that high level of p57 correlates with phosphorylation of Smad2 and Smad3 in freshly isolated $LSKCD34^-$, but not in $LSKCD34^+$ progenitors cells (Yamazaki et al., 2009). Interestingly, p57 and p27 were demonstrated to cooperate in order to maintain HSCs in a quiescence state and that the interaction with heat shock cognate protein 70 (Hsc70) was critically important to maintain HSC cell cycle kinetics (Matsumoto et al., 2011; Zou et al., 2011).

Disruption of the Smad pathway in HSCs

A recent study inhibited the entire Smad signaling pathway through the overexpression of the inhibitory Smad7 in murine HSCs by retroviral gene transfer. This resulted in increased self-renewal of HSCs *in vivo* with no abnormalities in differentiation of the myeloid and lymphoid lineages (Blank et al., 2006). However, Smad7 overexpression did not result in increased self-renewal of HSCs *in vitro*, indicating that the *in vivo* phenotype was dependent on the environment of the BM niche. In an MxCre inducible knockout model targeting Smad4, disrupting the entire Smad pathway, a loss of self-renewal was observed in murine HSCs *in vivo*, while *in vitro* studies showed that the proliferation capacity was normal (Karlsson et al., 2007). Importantly, Smad7 overexpression in Smad4 deficient HSCs demonstrated that the Smad7-induced regeneration capacity was dependent on Smad4, suggesting that the level at which the Smad pathway is disrupted is important for the effect on HSCs (Blank et al., 2006). In contrast, another Smad7 overexpression study showed that human SCID repopulating cells differentiated to myeloid progenitors at a higher frequency (Chadwick et al., 2005). It has also been reported that the transcriptional intermediary factor 1gamma (TIF1-γ) forms a complex with R-Smads and can direct human primitive hematopoietic cells toward erythroid differentiation (He et al., 2006).

When the Smad-mediated BMP signaling was disrupted upon knocking out Smad1 or Smad1/5, using a fetal liver-specific Cre-driver to induce deletion, hematopoiesis was normal

in transplantation experiments (Singbrant et al., 2010). Thus, these results clearly show that canonical BMP-signaling pathway is not critical for adult HSC maintenance. These different outcomes illustrate how complex the Smad signaling pathway is and it will be an important topic for future studies of HSC function (Article I).

Smad signaling in hematopoietic malignancies

Given the anti-proliferative effects of TGF-β signaling it is not surprising that mutational inactivation involving TGF-β pathway members occurs in several cancers, including pancreatic and colon cancer (Grady et al., 1999; Villanueva et al., 1998). Surprisingly, as TGF-β has been shown to be an important regulatory factor for HSC/progenitor proliferation and differentiation, alterations in genes encoding TGF-β ligand/receptors or Smads are uncommon in leukemias and other hematological malignancies (Kim and Letterio, 2003). A few cases of mutation in *SMAD4*, TGF-β type I and TGF-β type II-receptor have been reported in acute myelogenous leukemia (AML) and T-cell lymphoma (Imai et al., 2001; Knaus et al., 1996; Molenaar et al., 1998; Schiemann et al., 1999; Scott et al., 2003; Yang et al., 2006). Oncoproteins like TAX, EVI-1 and AML1-ETO can cause suppression of Smad-dependent transcriptional response to perturb TGF-β signaling in hematological malignancies (Jakubowiak et al., 2000; Kurokawa et al., 1998; Lee et al., 2002). Interestingly, Smad4 has been suggested to protect primitive hematologic cells from leukemia transformation (Quere et al., 2011). Specifically, Smad4 was shown to bind the oncoproteins HoxA9 and Nup98-HoxA9 and sequester them to the cytoplasm, with absence of Smad4 accelerating the progression of AML in mice (Quere et al., 2011). The fact that TGF-β-signaling-deficient mouse models studied in our laboratory do not develop of hematopoietic malignancies suggests that loss of TGF-β signaling is more important for progression rather than initiation of leukemogenesis (Blank et al., 2006; Karlsson et al., 2007; Larsson et al., 2005; Singbrant et al., 2010). In agreement with this, Wolfraim et al. found that SMAD3 protein could not be detected in fresh samples from T-cell acute lymphoblastic leukemia (ALL), although *SMAD3* mRNA was present and no mutations in the *SMAD3* gene were observed, importantly *SMAD3*-deficient mice require loss of p27 to initiate leukemia (Wolfraim et al., 2004).

In addition, TGF-β can paradoxically promote cancer, particularly in the later stage of disease. This is due to the effects on the microenvironment, impairing cytolytic T-lymphocyte activity, enhancing angiogenesis and rendering patient/animals with a reduced ability to reject tumor cells, due to the balance has tipping in the favor of pro-oncogenic activities of TGF-β (Hollsberg et al., 1994; Kim et al., 1991). Numerous of studies have described TGF-β inhibition as connected to leukemogenesis, but also the development of TGF-β resistance seems to be an important mechanism for oncogenic activity which further demonstrates the complexity of TGF-β/Smad signaling and its dual role in disease progression.

Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF; encoded by SERPINF1) is a 50 kDa secreted glycoprotein belonging to the superfamily of Serpins (serine protease inhibitors) but without inhibitory function (Becerra et al., 1995). PEDF research began in the 1990s, the protein was first purified from the conditioned media of human retinal pigment epithelial cells, hence its name (Steele et al., 1993; Tombran-Tink et al., 2005; Tombran-Tink and Johnson, 1989). Later the PEDF protein was discovered to have inhibitory functions in physiological and pathological angiogenesis, as Dawson and colleagues demonstrated that PEDF is among the most potent natural inhibitors of angiogenesis (Dawson et al., 1999).

Multiple functions of PEDF

PEDF is widely expressed throughout the body and has been detected in a broad range of tissues including eye, liver, brain, plasma, bone, heart and lung (Alberdi et al., 1998; Bilak et al., 1999; Quan et al., 2005; Sawant et al., 2004; Tombran-Tink and Johnson, 1989; Uehara et al., 2004). It is a multifunctional protein involved in several biological activities, the protein having been shown to be anti-angiogenic, anti-tumorgenic, anti-inflammatory and neuroprotective (Table 2) (Abe et al., 2004; Bilak et al., 1999; Cao et al., 1999; Dawson et al., 1999; Garcia et al., 2004a; Zhang et al., 2006). The anti-angiogenic signal induced by PEDF involves activating Fas-Fas ligand (Fas-L) and results in apoptosis of endothelial cells (Volpert et al., 2002). In addition, p38 MAP kinase has also been demonstrated to be a key mediator of the apoptotic effects induced by PEDF and human umbilical vein endothelial cells have been shown to induce PEDF mediated-apoptosis through induction of peroxisome proliferator-activated receptor γ (PPARγ) and p53 (Chen et al., 2006; Ho et al., 2007). One key factor that stimulates angiogenesis is the vascular endothelial growth factor (VEGF) and PEDF have been described to inhibit VEGF. An example of this is the overexpression of PEDF reducing VEGF levels in the eye, prevented ocular neovascularization (Haurigot et al., 2012; Park et al., 2011). These findings are important as neovascularization in the eye can cause blindness and, for this reason, PEDF is a potential therapeutic agent against vessel growth induced blindness. Furthermore, PEDF has also been shown to regulate VEGF in other tissues, for example exogenous PEDF was shown to downregulate VEGF expression in an osteosarcoma cell line, both at mRNA and protein levels (Takenaka et al., 2005). Moreover, Grippo and co-workers reported inverse levels of PEDF vs. VEGF in human pancreatic cancer (Grippo et al., 2012). Cai et al., demonstrated two mechanisms by which PEDF inhibits VEGF, first by enhancing γ-secretase-cleavage, resulting in cleavage of VEGF receptor 1 (VEGFR1), and second by inhibiting the phosphorylation of VEGFR1 (Cai et al., 2006a).

PEDF is suggested to be an important player in various cancers and decreased PEDF expression has been linked to increased metastasis and poor prognosis in pancreatic cancer, prostate cancer, breast cancer, melanomas, osteosarcomas and neuroblastomas among others (Cai et al., 2006b; Crawford et al., 2001; Doll et al., 2003; Garcia et al., 2004b; Halin et al., 2004; Takenaka et al., 2005; Uehara et al., 2004). Because of PEDFs anti-angiogenic properties it has the potential to be used as an anti-cancer peptide (Becerra and Notario, 2013). Importantly, PEDF has also been described as acting directly on tumor cells inducing apoptosis in various cancer cell types including; osteosarcoma cells, melanoma cells and prostatic xenograft induced cancer cells (Takenaka et al., 2005; Zhang et al., 2007). Mechanistically, PEDF upregulates PPARγ in prostate cancer cells leading to the suppression of nuclear factor-κB (NF-κB)-mediated transcriptional activation and reduced production of interleukin 8 (IL-8), followed by limited proliferation of cancer cells (Hirsch et al., 2011). Given the negative effects of PEDF on tumor cell function it is not surprising that expression of PEDF is downregulated during tumor progression. This has created an interest in PEDF as a therapeutic agent against cancer and future studies will likely focus on defining the mechanisms that are involved in PEDF regulation.

Tabel 2. Biological responses to PEDF

PEDF action	Target cell	References
Anti-angiogenic	Ocular endothelial and tumor endothelial cells	Becerra and Notario, 2013 Dawson et al., 1999 Haurigot et al., 2012 Park et al., 2011
Induces apoptosis	Endothelial cells and tumor cells	Chen et al., 2006 Ho et al., 2007 Takenaka et al., 2005 Volpet et al., 2002 Zhang et al., 2007
Anti-inflammatory	Retina	Zhang et al., 2006
Neuroprotective	Cerebellar granule cells, hippocampal neurons and spinal cord motor neurons	Bilak et al., 1999 Coster et al., 1999 Taniwaki et al., 1997
Self-renewal factor	Neural stem cells, retinal stem cells and human embryonic stem cells	Andreu-Agullo et al., 2009 Anisimov et al., 2010 De Marzo et al., 2010 Gonzalez et al., 2010

In addition, PEDF also has neuroprotective properties in several parts of the nervous system. In cerebellar granule cells (CGCs), the neuroprotective functions of PEDF are dependent on activation and translocation of NF-κB to the nucleus (Yabe et al., 2001). Nanogram amounts of PEDF have been demonstrated to provide protection of glutamate toxicity of several cells including CGCs, hippocampal neurons and spinal cord motor neurons (Bilak et al., 1999; DeCoster et al., 1999; Taniwaki et al., 1997). In a model system of light-dependent degeneration of rat photoreceptor cells intravitreal injections of PEDF resulted in neuroprotection (Cao et al., 2001). Since neurodegeneration is a common result of neovascular disease the neuroprotective properties adds to the value of PEDF as a therapeutic factor.

In summary, PEDF initiate both cell survival and cell death depending on which signaling cascade and cell type that is affected. While this seems contradictory, it emphasizes the potential dual activity of this protein.

PEDF receptors

Mapping for binding sites of PEDF to ECM components demonstrated a cluster of acidic amino acids involved in collagen binding and a cluster of basic amino acids necessary for heparin binding (Simonovic et al., 2001; Yasui et al., 2003). The biological activities of PEDF are thought to depend on its interactions with cell-surface receptors. The first receptor identified for PEDF was the protein encoded by patatin-like phospholipase domain containing protein (PNPLA2) on retinal epithelial and hepatocyte cells (Chung et al., 2008; Notari et al., 2006). This receptor has been shown to bind PEDF with high affinity and is for that reason called the PEDF receptor (PEDFR). Filleur et al. demonstrated that two epitopes of PEDF, the 34 mer and 44 mer peptides, were able to bind to the cell surface of endothelial and prostate cancer cells (Filleur et al., 2005). These peptides did not compete with each other for receptor binding, indicating binding to two distinct receptors. Indeed, it was later shown that several receptors bind PEDF, including non-integrin laminin receptor (LR), F1ATPase and low-density lipoprotein receptor related protein 6 (LRP6) (Bernard et al., 2009; Macaulay et al., 2013; Notari et al., 2010). A recent study, where short hairpin (sh)-RNA was directed towards either PEDFR or LR in cultured human melanoma cells, demonstrated that LR, but not PEDFR, is responsible for the PEDF mediated anti-inflammatory and anti-thrombogenic effects in these cells (Matsui et al., 2014). Ongoing studies will further clarify the details of PEDF receptor interactions and signaling cascade. Importantly, PEDF is generally regarded as a secreted protein but several immunohistochemical studies have reported significant quantities of PEDF in the nucleus of different cell types including retinal pigment epithelial cells (RPE), Y-79 retinoblastoma cells, NA neuroblastoma cells and hepatocarcinoma HepG2 cells (Karakousis et al., 2001; Sawant et al., 2004; Tombran-Tink et al., 1995). Furthermore, in a recent study PEDF was shown to bind importin-b family member transportin-SR2, associated with active nuclear transport (Anguissola et al., 2011). In the nucleus PEDF could perform specific functions regulation of cell cycle for example. This was described in human fibroblast

where PEDF has been associated with a G_0 growth arrest (Pignolo et al., 2003). It is likely that PEDF can act in both a cell-autonomous fashion as well as a secreted autocrine/paracrine fashion.

PEDF in stem cell biology

Human embryonic stem cells

Accumulating evidence demonstrates a role for PEDF in a variety of stem cells. Mainly, PEDF has been shown to support stem cell survival and maintaining multipotency. An example of this is that PEDF is highly expressed in fibroblasts that serve as feeder cells for human embryonic stem cells (hESCs) (Anisimov et al., 2011). Generally feeder cells lose their supportive capacity following long-term culture *in vitro,* using microarray analysis Anisimov and colleagues demonstrated that foreskin fibroblast cells (hFFs) have high expression level of PEDF during early passages and lose expression in intermediate and late passages. Based on these findings PEDF is thought to play an important role in the support of hESC growth. Additionally, in a recent study PEDF was established to support self-renewal of hESC (Gonzalez et al., 2010). Gonzalez et al. reported that PEDF promotes long-term growth of pluripotent hESC without bFGF or TGFβ/Activin/Nodal ligand supplementation, and that the effect seen was due to activation of ERK1/2 signaling. Moreover, knockdown of PEDFR in hESCs using sh-RNA triggered the loss of Oct4 expression and resulted in cellular differentiation (Gonzalez et al., 2010). However, contradictory result were reported by Kanemure et al. who recently showed that PEDF secreted from primary or iPSC-derived retinal pigment epithelium dramatically inhibits the growth of iPSCs and induces apoptotic cell death (Kanemura et al., 2013).

Retinal stem cells

Studies have shown that polarized retinal pigment epithelium derived from hESC secrete high levels of PEDF, supporting retinal progenitor cell (RPC) survival (Zhu et al., 2011). Upon addition of a neutralizing PEDF antibody to the culture media the increase in RPC proliferation and survival was diminished. A recent study reported that PEDF increased growth of retinal stem cells (RSCs) isolated from adult mouse eyes. When RSCs were culture in media supplemented with PEDF together with fibroblast growth factor (FGF) the yield of RSCs was enhanced and BrdU-incorporation increased, indicating that PEDF can increase growth of RSCs (De Marzo et al., 2010).

The limbus is anatomically located between the cornea and the conjunctiva on the ocular surface. Limbal epithelial stem cells (LSCs) are a special cell population that is enriched in the basal layer of limbal epithelium and proliferation of LSC has recently been shown to be enhanced by PEDF treatment *in vitro* (Ho et al., 2013). In addition, ointment supplemented

with PEDF was shown to accelerate the corneal wound healing process of mouse eyes (Ho et al., 2013).

Neural stem cells

PEDF is produced in the subventricular zone (SVZ) of mouse brain and Ramirez-Castillejo et al. demonstrated in 2006 that PEDF promotes self-renewal of neural stem cells (NSCs) *in vitro* (Ramirez-Castillejo et al., 2006). When PEDF was infused into the lateral ventricle of adult mice it stimulated expansion of the stem cell niche and PEDF-infused mice formed more secondary neurospheres indicating that PEDF increased the number of self-renewing NSCs also *in vivo*. The self-renewal effects seen after PEDF treatment were restricted to the N-terminal half of PEDF and resulted in increased expression of the Notch effector Hes1 (Ramirez-Castillejo et al., 2006). In addition, PEDF can modulate the balance between symmetric and asymmetric divisions in NSCs, via effects on Notch transcriptional activity (Andreu-Agullo et al., 2009). Activation of Notch receptors results in a movement of intracellular domain of Notch (NICD) to the nucleus, where it binds the repressor C promoters-binding factor 1 (CBF1) to initiate the transcription of genes e.g. *Hes1*. N-CoR is a co-repressor that binds CBF1 and it was postulated that CBF1 binding sites in *Hes1* and *Egfr* promoters are occupied by N-CoR, thus leading to repressed activation of these genes. Furthermore, Andreu-Agullo found that in the presence of PEDF and activation of a non-canonical NF-κB pathway, the N-CoR moves away from CBF1 binding sites, allowing NICD/CBF1-dependent transcription of *Hes1* and *Egfr* (Andreu-Agullo et al., 2009).

Mesenchymal stem cells

MSCs have the capacity to differentiate into mesodermal tissues including bone, cartilage and adipose (Si et al., 2011). Interestingly, PEDF is among the proteins that have been identified in MSC-conditioned media (Sarojini et al., 2008). It has been shown by immunofluoresent staining that PEDF is present at high levels in the rough endoplasmic reticulum/golgi areas in MSCs, indicating that the protein is destined for secretion (Sarojini et al., 2008). PEDF has recently been demonstrated, using sh-RNA or PEDF knockout animals in both human and mouse MSC, to direct MSC fate outcome inhibiting adipogenesis while promoting osteogenesis (Gattu et al., 2013). Interestingly, PEDF delays cellular senescence of human MSCs *in vitro,* this finding was associated with decreased p53 expression and reduced oxidative stress (Cao et al., 2013).

PRESENT INVESTIGATION

Aim

HSCs are by far the most studied stem cell in our body, but one of the remaining fundamental questions of stem cell biology is how self-renewal is regulated. To date, many factors have been found to expand stem cells. However, extensive *in vitro* amplification of HSCs without loss of their repopulation potential has not yet been achieved in a clinical setting. To be able to control cell fate in future *in vitro* protocols it is critical to understand how HSCs are regulated in their natural environment.

The studies presented in this thesis have identified factors involved in the regulation of HSC fate decisions. More precisely we aimed at delineating the role of TGF-β and Smad signaling in human hematopoiesis and HSC function by studying Smad4 overexpression in hematopoietic cells from CB. Furthermore, with the purpose to investigate and characterize a novel regulator we have studied the role of PEDF in murine HSCs. Lastly, we identified CD9 as a cell surface marker that captures all HSCs in murine BM and described how this is a useful tool to study the HSC molecular signature.

Summary of results

Article I

Here, we asked whether enforced expression of Smad4 could reveal a role for TGF-β in human hematopoietic stem/progenitor cell (HSPC) regulation *in vivo* or affect self-renewal and regenerative ability of HSCs *in vitro*. To elucidate the effect of Smad4 overexpression in hematopoiesis, full-length complementary (cDNA) of human Smad4 was cloned into a lentiviral vector carrying a GFP reporter gene, referred to as the Smad4 vector. As a control, a lentiviral vector carrying GFP alone was generated. Human CB HSPCs overexpressing Smad4 displayed increased sensitivity to TGF-β in colony assays and importantly, the addition of a TGF-β inhibitor targeting ALK4, 5 and 7 receptors (SB431542) rescued the colony forming capacity demonstrating the functional overactivity of the TGF-β pathway in Smad4 overexpressing cells. Since TGF-β is a well-known growth inhibitor of hematopoietic progenitors we further analyzed the cell cycle status of transduced cells (Batard et al., 2000; Jacobsen et al., 1995; Sitnicka et al., 1996b). Cells with enforced expression of Smad4 and increased TGF-β sensitivity were to a larger extent in the quiescent state of the cell cycle (G_0) compared to control cells when cultured for six days, but could be released from G_0 when treated with SB431542. Moreover, as TGF-β is also known to induce apoptosis we further investigated if enforced expression of Smad4 would affect apoptosis of cultured CB cells. After six days of culture Smad4 overexpressing cells had significantly higher AnnexinV binding compared to control cells, an effect that also was decreased when adding SB431542 to the culture. Furthermore, we transplanted transduced CB cells into NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice. Interestingly, despite having a similar transduction efficiency as the empty vector control (27 ±8.35% vs. 27 ±7.32% for Smad4 vector and control vector, respectively), CD34^{+} CB hematopoietic stem/progenitor cells (HSPCs) transduced with the Smad4 vector had impaired engraftment as measured by FACS analysis of bone marrow six months post transplantation (Smad4 vector 1.5 ±0.88% GFP vs. control vector 5.60 ±1.54% P=.0029). Expression of lineage surface markers (CD13, CD15 and CD19) in PB three months post transplantation was unaltered.

In summary, our results demonstrate that increased Smad4 expression sensitizes human CB HSPCs to TGF-β. This leads to growth arrest and apoptosis *in vitro* and reduced HSPC reconstitution capacity *in vivo,* with no effect on lineage distribution. Together, these findings demonstrate an important role for TGF-β signaling in the regulation of human HSCs in vivo.

Article II

PEDF was one of 62 genes identified by gene expression profile analysis as deregulated when multipotent, self-renewing hematopoietic cell line entered differentiation (Bruno et al., 2004). We analyzed expression of PEDF in primary cells by real time–PCR. In agreement with the previously published cell line data we found that PEDF is highly expressed in primary long-term-HSCs. Therefore, we decided to characterize a knockout mouse model for PEDF to investigate the role of PEDF in hematopoiesis and HSC function.

Interestingly, we could show that PB from these mice had reduced white blood cell (WBC) counts and BM cells had impaired capacity to form secondary myeloid colonies in methylcellulose culture. However, immunophenotypic HSCs and primitive progenitors in BM were similar to those in wt controls and competitive repopulation (CRU) assays resulted in similar frequency of HSC in BM. Interestingly, in competitive transplantation experiments PEDF-/- BM cells reconstituted the HSC population to a lesser degree than WT controls (LSKCD150+CD48- per femur of primary transplanted recipients: 0.0044% ±0.0012 vs. 0.0011% ±0.0003 for PEDF+/+ and PEDF-/-, respectively P=0.0088). This was accompanied with impaired repopulative capacity (engrafted donor cells in bone marrow of tertiary transplanted mice: 38% ±5.9 vs. 12% ±5.1 for PEDF+/+ and PEDF-/-, respectively P=0.0031). PEDF deficient cells also exhibited altered lineage distribution with impaired myeloid contribution. We are currently performing gene expression assays to obtain an insight into the changes that occur to the transcriptome in the absence of PEDF.

Taken together, we report that PEDF is a critical regulatory factor for HSC function. Our results strengthen the accumulating evidence of PEDF as an important regulator in several stem cell systems.

Article III

The ability to isolate progenitors and stem cells of the hematopoietic system to high purity is required for analysis of the hierarchical organization and to characterize the transcriptional programs regulating these cells. To date, the most commonly used methods for purification of HSCs involve flow cytometry techniques using combinations of antibodies directed against LSK further subdivided by either Flt3 and CD34 ($LSKFlt3^{-}CD34^{-}$) or SLAM markers ($LSKCD48^{-}CD150^{+}$) (Adolfsson et al., 2001; Adolfsson et al., 2005; Kiel et al., 2005; Osawa et al., 1996; Yang et al., 2005). Alternative strategies based on metabolic dyes have also been used to isolate HSCs and are based on the cells efflux activity (Goodell et al., 1996). Regardless of purification strategy, considerable heterogeneity remains in the stem cell compartment and the presence of contaminating cells within the putative HSC fraction hinder direct analysis of HSCs.

The tetraspanin CD9 was identified by gene expression analysis of hematopoietic cell lines to be deregulated when primitive cells entered differentiation (Bruno et al., 2004). Here we identified CD9 as a novel positive marker for murine HSCs. More specifically, CD9 enriches for long-term reconstituting cells in previously immunophenotypically-defined BM subpopulations demonstrated to contain LT-HSCs. CD9 also identifies rare long-term reconstituting cells in cell fractions isolated on the basis of having short-term reconstitution activity and could replace a number of currently used markers thereby simplifying isolation of HSCs. We used CD9 as a tool to separate contaminating non-reconstituting cells from HSCs and combined global RNA-seq and single cell gene expression analyses to study the HSC molecular signature. However, even after depletion of contaminating cells our results show that there is still considerable transcriptional noise and fate priming within the functionally largely homogenous population of LT-HSCs (Figure 8).

To summarize, we have identified the cell surface antigen CD9 as a positive marker for HSCs that provides a simple alternative for stem cell isolation at high purity. Using CD9 as a tool we have dissected heterogeneity within the HSC pool as defined by CD9 expression.

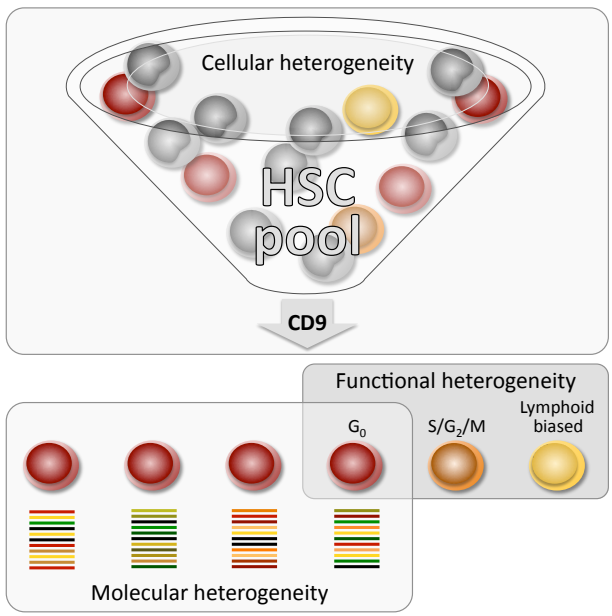

Figure 8. CD9 captures all HSCs in murine BM. CD9 is a useful tool for HSC isolation that dissect molecular heterogeneity within the $CD9^+$ HSC pool (red cells). Adapted from Christine Karlsson, with permission (Karlsson et al., 2013).

Conclusions

- HSPC cells from cord blood become oversensitive to TGF-β upon Smad4 overexpression (Article I).
- Increased TGF-β sensitivity results in growth arrest and increased apoptosis of human HSPC *in vitro* (Article I).
- Smad4 overexpression leads to reduced HSPC reconstitution capacity *in vivo* with no effect on lineage distribution (Article I).
- PEDF is a regulator of hematopoiesis (Article II).
- Absence of PEDF leads to reduced HSC numbers and impaired engraftment following transplantation (Article II).
- Our results suggest that loss of PEDF is not critical during homeostasis but important during hematopoietic regeneration. (Article II).
- CD9 is a novel positive marker for murine HSCs and enriches for long-term reconstituting cells (Article III).
- CD9 provides a simple tool for stem cell isolation that allows for analysis of molecular heterogeneity within the HSC pool (Article III).

GENERAL DISCUSSION

Identifying HSC regulators

It will be fundamental to develop methods for CB HSC expansion, making potential donor samples available to a larger number of patients and for advanced stem cell therapy to reach its full capacity. Therefore, continued investigation to gain deeper understanding of how HSCs are regulated and the identification of factors that are important for HSC fate decisions are imperative for HSC research. Relevant to this work has been the concept of HSC expansion and the use of *in vitro* and *in vivo* assays including genetically modified animal models to study HSCs biology that will be discussed in detail below.

Overexpression studies

Overexpression of genes using lentiviral vectors is a valuable method for studying gene function in the hematopoietic system. This approach enables stable integration of a gene into the genome of a stem cell, which leads to expression of the inserted gene in all progeny and the entire hematopoietic system. Furthermore, high transduction efficiencies can be achieved in slow cycling human hematopoietic cells, which preferably can be transplanted to xenograft mouse models to study repopulation capacity and lineage commitment over time. This approach makes it possible to study human cells in an *in vivo* setting, leading to increased knowledge of human HSC biology. However, overexpressing studies using viral vectors comes with some disadvantages. For example, high non-physiological levels of a protein could be related to unspecific effects. Such effects are not necessarily related to normal function of the protein and are for that reason not reflecting normal HSC regulation. Using strong transgene promoters in lentiviral constructs, such as spleen focus-forming virus (SFFV)-promoter, can lead to massive increase of the product from the transgene. In the case of overexpressing Smad4, the expression of Smad4 mRNA was approximately 5-fold and elevated levels of Smad4 increased sensitivity to TGF-β followed by effects on cell cycle and apoptosis, well-established TGF-β effects, which clearly shows that our observations are of physiological relevance (Article I) (Batard et al., 2000; Cashman et al., 1990; Jacobsen et al., 1995; Sitnicka et al., 1996b). Since the TGF-β type I receptor inhibitor could rescue the effects caused by the elevated Smad4 level it further shows that the overexpression per se does not cause toxicity. Furthermore, insertional mutagenesis is a serious concern when introducing genetic material via integrating viral vectors. This is because integration of the transgene near proto-oncogenes can result in leukemic transformation (Woods et al., 2003). Although, we had high transduction

efficiency in the Smad4 overexpression study, which would correspond to multiple copy numbers per GFP-positive cell, we did not observe any lineage skewing that would indicate leukemic transformation. Another concern is the time of culture before evaluating the cells, preferably the cells are prestimulated for 24h followed by transduction (incubation with the virus for 24-48h). During this time primitive stem cells start to proliferate and potentially also differentiate, which leaves us with less stem cell-material to investigate. However, as we analyzed the mice 25 weeks after transplantation we clearly manage to transduce and transplant HSCs capable of long-term engraftment in NSG mice.

Taken together, in the human setting viral transfer for studying both robust silencing and increased transgene expression using shRNA and overexpression studies, respectively, are critical for exploring human HSCs biology *in vivo*. An additional approach is to study hematopoietic cells from patients with genetic disease or leukemia to identify the molecular pathways that create the disease phenotype.

New insight into TGF-β regulation

Blank et al., published the Smad7 overexpression study where the entire TGF-β signaling pathway was inhibited leading to increase HSC repopulation capacity (Blank et al., 2006). It is intriguing and important to speculate about the cause of this phenotype. Interestingly, this model leaves Smad4 to interact with non-canonical Smad pathways and importantly the phenotype seen was dependent on Smad4 expression. The Smad members have been demonstrated to interact with signaling molecules involved in both the Wnt and Notch pathway (Itoh et al., 2004; Labbe et al., 2000; Nishita et al., 2000). Thus, we hypothesized that overexpression of Smad4 might be able to mimic the phenotype seen in the Smad7 overexpression study and addressed this question in the human system. However, when we overexpressed Smad4 using lentiviral vectors in primitive cord blood cells we found that increased Smad4 levels made HSCs more sensitive to TGF-β which kept them in a low cycling state and induced apoptosis. Our result, together with previously published data from the Smad4 knockout mouse model, clearly shows that Smad4 protein levels can modulate the response to TGF-β (Article I) (Karlsson et al., 2007). This does not necessarily mean that Smad4 cannot be involved in non-canonical pathways, but it shows that Smad4 levels regulate the response to canonical TGF-β signaling. Interestingly, accumulating evidence demonstrate that miRNA regulates hematopoietic cells by targeting the TGF-β pathway, which provides new insight into the regulation of TGF-β signaling (Bhagat et al., 2013; Emmrich et al., 2014; Hager et al., 2011). The TGF-β signaling pathway is of great biological relevance for HSC regulation and regardless of the precise molecular mechanisms it is clear that the level of Smad signaling correlates with the sensitivity to TGF-β, which affects HSPC maintenance and survival (Figure 9).

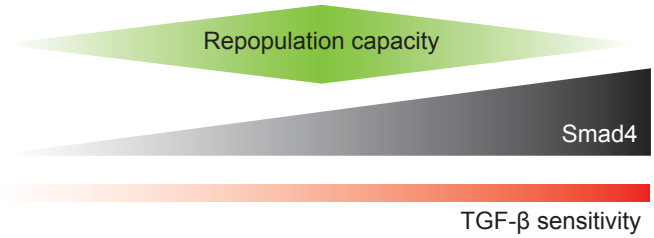

Figure 9. Model for the regulation of repopulative capacity by the experssion level of Smad4. The pricise level of Smad4 can modulate the sensitivity to TGF-β and affect repopulative capacity of HSPC *in vivo*. Smad4-deficient cells have decreased repopulation capacity and are insensitive to TGF-β. Overexpression of Smad4 leads to increased sensitivity to TGF-β, which translates into reduced repopulayive capacity.

TGF-β and HSC expansion

Clinical experience has shown that the number of transplanted stem cells is related to patient survival as well as time required for engraftment (Barker, 2007; Brunstein et al., 2010; Wagner et al., 2002). Thus expansion of HSCs and progenitor cells *ex vivo* is expected to be highly beneficial and of great clinical relevance. The promising potential of finding factors to enhance HSC self-renewal is the primary goal for advanced stem cell therapy. However, extreme expansion of HSC number is not needed, even modest expansion of HSCs would improve the number of potential donors. Furthermore, to shorten the time to neutrophil recovery (TNR), important for post-transplant survival, is equally important (Brunstein and Wagner, 2006). Since the balance between self-renewal, differentiation, growth arrest and apoptosis determines the fate outcome of a cell it is critical to find the right equilibrium. Therefore, to identifying negative regulators might be important to gain ideal culture conditions. For example, Csaszar et al. expanded HSCs based on the fed-batch culture strategy, a system that limits the endogenously produced inhibitory feedback signals produced in HSCs culture media (Csaszar et al., 2012). Moreover, they demonstrated that the inhibitory effect during early cord blood cultures primarily was driven by TGF-β secretion. Thus, it seems reasonable to argue that negative factors play an important role in HSC regulation *ex vivo*. In contrast however, it was shown that TGF-β is secreted from murine HSCs in a latent form and that HSCs themselves cannot activate latent TGF-β (Yamazaki et al., 2009). This might be a result of discrepancies between species since our results imply that the level of Smad4 is important for modulating the response to autocrine and/or paracrine TGF-β produced in serum-free human cell cultures. Importantly, addition of the TGF-β type I receptor inhibitor reversed this effect (Article I). Indeed, it has been shown in earlier studies that TGF-β has an autocrine/paracrine effect in

cultures, as addition of anti-TGF-β1 under serum-free culture conditions augmented proliferation of CD34+CD38−Lin− cells (Akel et al., 2003; Fan et al., 2002). Therefore, transient blocking of TGF-β signaling might be a tool for *ex vivo* expansion protocols that aim to increase HSC numbers. Given the functional heterogeneity within the HSCs and progenitors compartment, future expansion protocols will probably not contain one universal factor but ideally a combination of factors that enhance stem cell and progenitor numbers. Importantly, the challenge to generate fully functional HSCs *ex vivo* for future clinical goals remains.

Knockout mouse models

The mouse is a powerful model system for studying HSC biology. The most accurate assay for studying HSC function is *in vivo* transplantation into lethally irradiated recipients. Using this approach the true potential of a stem cell can be measured. For example, HSC reconstitution capacity and contribution to mature lineages can be analyzed in congenic mouse strains. Increased repopulation capacity could be an indication of enhanced self-renewal. While, decreased repopulation capacity in secondary hosts could be a result of impaired self-renewal capacity leading to HSC loss or excessive self-renewal resulting in exhaustion. It is very important to keep in mind that genetic background and homing potential can influence the results. Knockout models is probably the most stringent method to studying gene function in murine HSCs without adding additional parameters to the system like transduction and prestimulation discussed above. There are two approaches; *conventional knockouts* i.e. deletion of a genetic region where the targeted gene is functionally eliminated, or *conditional knockouts* i.e. temporal and tissue-specific gene deletion with controlled inactivation of the target gene. The conditional model is a powerful tool since the gene deletion is controlled, this can circumvent embryonic lethality caused by gene deletion during development. Frequently used conditional knockouts are depended on activation of Cre-recombinase to induce gene loss (Sauer, 1998). One criticism raised is the potential effect on HSCs from triggering interferon production, often used to induce gene deletion is Mx-Cre systems, as well as spontaneous deletion (Baldridge et al., 2010; de Bruin et al., 2013). Another disadvantage related to both types of knockout models is their time consuming feature coupled to high cost.

Several lines of evidence suggest that PEDF is an important regulatory factor for multiple stem cells (Andreu-Agullo et al., 2009; Anisimov et al., 2011; De Marzo et al., 2010; Gonzalez et al., 2010). Here, we analyzed expression of PEDF by real time–PCR and in agreement with the previously published cell line data found that PEDF is highly expressed in primary LT-HSCs (Bruno et al., 2004). The fact that PEDF is highly expressed in primitive cells and a secreted factor made PEDF a suitable gene for further investigations. We used a conventional model for PEDF knockout that was viable, thus indicating that PEDF is not crucial during development. In Article II we show that loss of PEDF in adult BM cells leads to decreased HSC frequency in competitive transplantations followed by impaired repopulative capacity

(Article II). On one hand, this could theoretically mean that loss of PEDF in the HSCs impair their repopulation capacity. On the other hand, systemic PEDF deletion might affect the niche that HSCs originate from and for that reason could be the cause of impaired HSC functions. As discussed previously, PEDF is expressed in most body tissues and many type of cells have been shown to express and secrete PEDF including osteoblast, mesenchymal cells and Schwann cells (Chiellini et al., 2008; Crawford et al., 2001; Quan et al., 2005; Sarojini et al., 2008). However, wild type cells transplanted into a PEDF deficient marrow have normal engraftment indicating that PEDF work in a cell-autonomously fashion. It would be interesting to transplant these wild type cells once again into a normal (wild type) niche in order to investigate if the exposure to the PEDF-/- environment had an impact on long-term regeneration capacity. Importantly, bone morphology of PEDF deficient mice did not show any apparent alteration in bone tissues (Emma Rörby, Mats Ehinger and Stefan Karlsson, unpublished observation). Interestingly, PEDF is generally regarded as a secreted protein but several immunohistochemical studies have reported significant quantities of PEDF in the nucleus of different cell types including retinal pigment epithelial cells (RPE), Y-79 retinoblastoma cells, NA neuroblastoma cells and hepatocarcinoma HepG2 cells (Karakousis et al., 2001; Sawant et al., 2004; Tombran-Tink et al., 1995). In a recent study PEDF was shown to bind importin-b family member transportin-SR2, which is associated with active nuclear transport (Anguissola et al., 2011). These findings suggest that PEDF might not only work as a secreted factor but could also have cell-intrinsic gene regulatory functions. Future experiments will aim to elucidate the mechanism involved in the discovered HSCs phenotype.

Heterogeneity within the HSC pool

Even if HSCs are the most characterized stem cells, their prospective isolation is an ongoing process of refinement. We have identified CD9 as a novel positive marker that captures all HSCs in murine BM. CD9 distinguishes long-term repopulation cells from ST-HSCs by immunophenotypic markers for LSKFlt3CD34, as well as LSKSLAM staining (Adolfsson et al., 2005; Kiel et al., 2005; Osawa et al., 1996; Yang et al., 2005). Furthermore, CD9 enables high purity cell isolation with reduced number of detector channels needed (Article III). Separating long-term repopulating cells from contaminating cells is important when investigating transcriptional signatures as was demonstrated with our single cell gene expression analysis. With the depletion of more committed progenitors from long-term stem cells using CD9 we could investigate the transcriptional program of primary HSCs. However, although homogeneity of the LSKCD48^{-}CD150^{+}CD34^{-}CD9^{high} population was seen on a functional level, heterogeneity was observed at the molecular level. This could be due to transcriptional noise but could also be of biological relevance. For example, the heterogeneity seen in the CD9^{high} population might not be related to additional HSC cell-surface markers for

increased purity, but could theoretically be a readout of the potentially different niches that the cells originate from in the marrow. The lack of methodology combining localization of HSCs with functional assays makes it difficult to assess where in the BM HSCs predominantly reside.

Difficulties in defining *ex vivo* conditions for HSC expansion are partly due to insufficient understanding of the molecular mechanisms controlling HSC fate. To understand the interaction networks and key molecules important for HSCs regulation might be the way to improve expansion. Nevertheless, future single cell analysis requires high stem cell purity to study molecular heterogeneity and lineage commitment that are critical for normal and malignant hematopoiesis.

Future directions

TGF-β ligands and the downstream Smad signaling pathway serve a key role in HSC biology and manipulations of this pathway at various levels have revealed important functions for HSC maintenance. In the future, more detailed mechanistic studies of human HSC are required to define if blockage of TGF-β/Smad signaling can contribute to faster regeneration following transplantation and how reduced TGF-β signaling can be exploited for expansion of HSCs in advanced cell therapy.

It is clear that PEDF is a critical regulatory factor for HSC function and our *in vivo* results suggest that PEDF works in an autocrine fashion. Importantly, since PEDF is a secreted factor we aim to investigate if recombinant PEDF can influence murine and/or human HSC growth *in vitro*. Furthermore, it is our goal to elucidate the mechanism involved in PEDF's regulation of HSCs. With this intention, ongoing research in our lab aims to analyze the gene expression profile of HSCs from 5-FU treated PEDF deficient mice to get an insight to the changes that occur to the transcriptome in the absence of PEDF under stress conditions. In addition, if recombinant PEDF has an effect on HSC growth in culture, PEDF treated cells will be obvious targets for gene expression analysis. Interestingly, as discussed above PEDF has been implicated in regulating p38/MAPK signaling, VEGF expression and Notch/Hes1 signaling, pathways known to be important for HSC survival and maintenance (Baudet et al., 2012; Gerber et al., 2002; Ito et al., 2006; Stier et al., 2002). Microarray technology could identify differentially expressed genes and unveil signaling pathway/s involved in the phenotype observed in HSCs. To my knowledge deregulated PEDF is not frequently seen in diseases relevant to the hematologic system, suggesting that PEDF is not involved in leukemogenesis. However, deeper understanding of PEDF signaling interactions could reveal findings important also for leukemic transformation. Based on the assays performed in Article II, steady state mice have a normal phenotype except for decreased WBC and colony forming capacity. Thus, these results strengthen the idea that loss of PEDF is not crucial under homeostasis but important during regeneration. Importantly, it cannot be excluded that the impaired engraftment capacity observed when transplanting PEDF deficient cells could be due to homing defects. In this respect it would be important to investigate whether the homing capacity is impaired in PEDF knockout cells. Worth mentioning is also that PEDF knockout mice are on a mixed background and therefore we have performed all experiments using littermate controls to eliminate possible differences due to genetic background alterations. In addition, we are now performing transplantation experiments with PEDF knockout animals backcrossed for >10 generations to confirm our findings.

We investigated PEDFR expression and found that PEDFR is expressed by ten percent of HSCs in the BM and it will be interesting to study if this receptor is responsible for PEDF signaling transduction of HSC. The literature describes additional receptors for PEDF, including LR and LRP6 receptor, and we aim to investigate if any of these receptors are

expressed on HSCs (Bernard et al., 2009; Macaulay et al., 2013; Notari et al., 2010). As a first step, cells will be sorted based on the above-mentioned receptor types and the response to PEDF will be determined in *in vitro* culture.

Another focus for future investigation would be CD9 biology and a more careful assessment of the signaling mechanism downstream of the receptor. Since CD9 distinguish long-term repopulation cells and the mRNA expression profile of CD9 is very distinct in the LT-HSC compared to more mature cells such project would hold promise to uncover signaling mechanisms important for HSC function.

SAMMANFATTNING PÅ SVENSKA

Det finns celler i vår kropp som har ett livslångt ansvar att tillverka alla blodceller. De kallas blodstamceller, eller hematopoetiska stamceller, och är en till antalet liten population celler som har den enorma kapaciteten att göra 10^{12} blodceller om dagen under hela vårt liv. Blodstamceller har multipotentiell differentieringsförmåga dvs. de kan bilda alla mogna blodceller från röda blodkroppar som transporterar syre, vita blodkroppar som ingår i immunförsvaret till blodplättar som stoppar blödning. Men de kan förutom att göra alla celler i blodet också göra kopior av sig själva, s.k. självförnyelse, där stamcellens egenskaper bibehålls i minst en dottercell efter celldelning. Denna unika förmåga gör att dessa celler kan användas i kliniskt syfte t.ex. vid benmärgstransplantation för att behandla blodsjukdomar som leukemi. Blodstamceller finns i benmärgen men också i navelsträngsblod varifrån de kan renas fram och användas för att transplantera patienter. Navelsträngsblod, som i vanliga fall är en restprodukt från födseln, är en lättåtkomlig källa för att skörda blodstamceller som sedan kan lagras i blodbanker. Navelsträngsblod innehåller dock inte något stort antal blodstamceller och de skulle därför behöva expanderas (öka i antal) utanför kroppen för att möjliggöra transplantation av vuxna patienter. Men idag saknas tillfredställande kunskap om hur cellerna regleras vilket har lett till att försök att expandera blodstamceller har rönt liten framgång. Denna avhandling syftar till att få utökad kunskap om blodstamceller och framförallt hur dessa celler är reglerade och vad som kan påverka deras tillväxt.

Det är ett komplext system av olika faktorer och molekylära mekanismer som påverkar cellerna i benmärgen. Det kan vara både cellkomponenter i cellerna själva och faktorer utanför cellerna i den omkringliggande miljön som reglerar dem. Transforming growth factor-β (TGF-β) är en känd extern faktor som påverkar tillväxten. TGF-β i sin tur aktiverar en signalväg inuti cellerna, kallad Smad. I tidigare studier där man i möss hämmat denna signalväg har det visat sig att blodstamcellernas tillväxt påverkas. Det är idag okänt vilken roll Smad-signalering spelar i humana stamceller i en transplantationskontext. Därför har vi studerat Smad-signaleringen i humana celler, för att försöka förstå hur den påverkar cellerna. Genom att, med virus, överföra en viktig komponent i Smad-signaleringen kallad Smad4 har vi studerat effekten av ökad Smad-signalering i humana blodstamceller från navelsträngsblod. Med ökat uttryck av Smad4 har vi lyckats skapa celler som är överkänsliga mot TGF-β. För att studera hur detta påverkar cellernas förmåga att nybilda det hematopoetiska systemet, transplanterades cellerna till möss. Resultaten visar att en ökad känslighet för TGF-β försämrar förmågan hos stamcellerna att nybilda sig själva. Därför har denna studie bidragit till ökad förståelse för hur humana blodstamceller från navelsträngsblod är reglerade (Arbete I).

Vi har även försökt hitta, för det hematopoietiska systemet, nya faktorer som påverkar och reglerar blodstamcellerna genom att studera en musmodell som saknar uttryck av en viss

gen, Pigment epithelium derived factor (PEDF). Denna faktor har visat sig vara viktig för andra stamcellstyper t.ex. embryonala och neuralastamceller och nu har vi upptäckt att blodstamceller som saknar denna gen har försämrad förmåga att återbilda blodsystemet. Dessa resultat visar att PEDF är nödvändig även för blodstamcellernas funktion och nu försöker vi komma fram till varför PEDF verkar vara så viktig och framför allt hur det påverkar stamcellerna (Arbete II).

Slutligen har vi också upptäckt att cellytemarkören CD9 uttrycks av stamcellerna. Blodstamceller kan studeras och sorteras ut med hjälp av avancerad teknik som baserar sig på cellytemarkörer. Ju renare stamcellspopulationen kan bli desto lättare kan vi studera funktioner och mekanismer som är unika för just blodstamcellerna. Detta projekt har inte endast bidragit till att en renare population av blodstamceller kan studeras vilket är av oerhörd vikt i jakten på ökad kunskap om dessa celler, utan också påvisat en molekylär heterogenitet inom stemcellspopulationen (Arbete III).

Sammanfattningsvis har vår forskning ökat förståelsen för hur blodstamceller är reglerade vilket har lett till ytterligare insikt i stamcellsbiologin, denna kunskap kan komma att bli viktig för effektivare behandling av blodsjukdomar i framtiden.

ARTICLES NOT INCLUDED IN THIS THESIS

Miharada K, Karlsson G, Rehn M, Rörby E, Siva K, Cammenga J and Karlsson S. *Cripto regulates hematopoietic stem cells as a hypoxic niche related factor through cell surface receptor GRP78.* Cell Stem Cell, 2011 Oct 4;9(4):330-44.

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APPENDICES (ARTICLES I-III)