DEFINING REGULATORS OF HUMAN HEMATOPOIETIC STEM CELLS

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Abstract

Bone marrow transplantation (BMT) is a conceptual and elegant example of stem cell therapy, rendered possible by the dual capacity of hematopoietic stem cells (HSCs) to self-renew and differentiate. These defining features ultimately ensure the production of all blood cell lineages and simultaneous maintenance of the stem cell pool, thereby maintaining lifelong homeostasis. Banked umbilical cord blood (CB) is an abundant and readily available stem cell resource. However, the relatively low numbers of hematopoietic stem- and progenitor cells (HSPCs) present in a typical single CB unit and the associated delay in engraftment restrict its routine applicability to primarily children. Despite intense global efforts to find strategies that would enable the *ex vivo* amplification of transplantable stem cells, the culture of HSCs outside their natural environment has proven difficult. It is therefore important to understand how HSC self-renewal, proliferation, and differentiation are integrated, which molecules participate in their regulation and how these could be modified for clinical benefit.

With the aim to discover new growth factors supporting stem cells in culture, we have assessed 276 extrinsic signaling molecules for their effect on CB-derived HSPCs. We identified the immunoregulatory chemokine (C-C motif) ligand 28 (CCL28) as a novel growth- and survival factor for primitive hematopoietic cells. CCL28 strongly supported the proliferation and clonogenic potential of hematopoietic progenitors from different ontogenetic origins, and significantly enhanced the ability of cultured putative HSCs to long-term reconstitute immunodeficient mice. Thus, CCL28 represents one of the few cytokines that can maintain the primitive properties and functional integrity of cultured human HSPCs. Furthermore, we identified myostatin propeptide, a naturally occurring inhibitor of the transforming growth factor-ß (TGF-ß) family member myostatin, as a novel promoter of HSPC proliferation during *ex vivo* culture.

Based on the limited self-renewal capacity of HSCs *in vitro*, we have developed a forward RNA interference (RNAi)-based screening method that allows the discovery of novel genes implicated in stem- and progenitor cell proliferation. Using pooled lentiviral short hairpin RNA (shRNA) libraries transduced into CB cells, we have identified short hairpins designed against exostoses 1 (shExt1), phospholipase C zeta 1 (shPLCZ1) and serine threonine kinase 38 (shSTK38) as new fate determinants for HSPC differentiation, proliferation, and self-renewal, respectively. However, first-generation RNAi screening in primary cells yielded a considerable amount of target gene-unrelated, yet shRNA-specific events (so-called 'off-target effects'), exemplified by shSTK38. Tracking of library-transduced HSPCs by next-generation sequencing significantly improved the resolution and feasibility of the screening approach and identified inhibition of MAPK14/p38α as means to promote expansion of undifferentiated cells, as shown by both RNAi and pharmacological modification of p38 using small molecule inhibitors.

Taken together, in this thesis we employed different screening approaches to identify novel regulators of primitive human hematopoietic cells. We conclude that systematic growth factor screenings as well as forward RNAi-based technologies are powerful tools to detect and subsequently define novel mediators of HSPC fate decisions.

Key words

Hematopoietic stem cell, cord blood, expansion, high-throughput screening, RNA interference, extrinsic signaling molecules

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To my family

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ABBREVIATIONS

AGM	aorta-gonad-mesonephros
AML	acute myeloid leukemia
Angpt	angiopoietin
Angptl	angiopoietin-like
BFU-E	burst forming unit erythroid
BM	bone marrow
BMP	bone morphogenetic protein
BMT	bone marrow transplantation
BrdU	bromodeoxyuridine
CB	cord blood
CBT	cord blood transplantation
CCL28	chemokine (C-C motif) ligand 28
CD	cluster of differentiation
CDC	cell division cycle
CDKI	cyclin-dependent kinase inhibitor
CFC	colony forming cell
CFU-E	colony forming unit erythroid
CFU-GM	colony forming unit granulocyte/macrophage
CFU-S	colony forming unit spleen
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CRU	competitive repopulation unit
CXCR4	C-X-C chemokine receptor type 4
DNA	deoxyribonucleic acid
E	embryonic day
ECM	extracellular matrix
EPO	erythropoietin
ESC	embryonic stem cell
EXT1	exostoses 1
FACS	fluorescence activated cell sorting
FA	Fanconi Anemia
FDR	false discovery rate
FGF	fibroblast growth factor
FL	fetal liver
FLT3	fms-related tyrosine kinase 3
FNBP1L	forming binding protein 1-like
G-CSF	granulocyte colony-stimulating factor
GDF	growth and differentiation factor
GFP	green fluorescent protein
GMP	granulocyte/macrophage progenitor
HCT	hematopoietic cell transplantation
HLA	human leukocyte antigen
HPRT	hypoxanthine guanine phosphoribosyl transferase
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
HSPC	hematopoietic stem- and progenitor cell
IDDb	insertional dominance database
IGF	insulin growth factor
IGFBP2	IGF-binding protein 2
IL	interleukin
KO	knockout

Lin	lineage
LSK	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{+}$
LTC-IC	long-term culture initiating cell
LT-HSC	long-term HSC
LTR	long terminal repeats
MAPK	mitogen-activated protein kinase
MEP	megakaryocyte/erythrocyte progenitor
MHC	major histocompatibility complex
miRNA	microRNA
MP	myostatin propeptide
MPP	multipotent progenitor
MSTN	myostatin
NOD	non-obese diabetic
NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ
OTE	off-target effect
PB	peripheral blood
PLCZ1	phospholipase C zeta1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RTCGD	retrovirus-tagged cancer gene database
SAM	significance analysis of microarrays
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCID	severe combined immunodeficiency
SD	standard deviation
SDF-1	stromal derived factor-1, also called CXCL12
SEM	standard error of the mean
shRNA	short hairpin RNA
SIN	self-inactivating
siRNA	short interfering RNA
SRC	SCID-repopulating cell
ST-HSC	short-term HSC
STK38	serine/threonine kinase 38
TGF-β	transforming growth factor β
Tie2	tyrosine kinase receptor 2
TPO	thrombopoietin

LIST OF PUBLICATIONS

This thesis is based on the articles listed below, which are referred to in the text by their roman numerals (I-IV).

I **Karlsson C**, Baudet A, Miharada N, Soneji S, Gupta R, Magnusson M, Enver T, Karlsson G, and Larsson J. *Identification of the chemokine CCL28 as a growth and survival factor for human hematopoietic stem- and progenitor cells*. Blood (2013), 121(19):3838-42.

II **Karlsson C**, Miharada N, Galeev R, Baudet A, Karlsson G, and Larsson J. *The myostatin antagonist myostatin propeptide promotes proliferation of human hematopoietic stem- and progenitor cells ex vivo*. Manuscript 2013.

III Ali N, **Karlsson C**, Aspling M, Hu G, Hacohen N, Scadden DT, and Larsson J. *Forward RNAi screens in primary human hematopoietic stem/progenitor cells*. Blood (2009), 113(16):3690-5.

IV Baudet A, **Karlsson C**, Talkhoncheh MS, Galeev R, Magnusson M, and Larsson J. *RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion*. Blood (2012), 119(26):6255-8.

Articles and manuscripts not included in this thesis:

Baudet A, **Karlsson C**, and Larsson J. *The SCID repopulating activity of cultured human hematopoietic cells is exclusively contained within a small population expressing CD90*. Manuscript 2013.

Karlsson C, Larsson J, Baudet A. *Forward RNAi screens in human stem cells*. Methods Mol Biol (2010), 650:29-43.

Glanz C, Rebetz J, Stewénius Y, Persson A, Englund E, Mandahl N, Mertens F, Salford LG, Widegren B, Fan X, Gisselsson D. *Genetic intratumour heterogeneity in high-grade brain tumours is associated with telomere-dependent mitotic instability*. Neuropath Applied Neuro (2007), 33(4):440-54.

PREFACE

Stem cells with high regenerative potential exist in a variety of organs, but their clinical use in tissue regeneration has been limited as retrieving and successfully delivering the stem cells poses a great challenge in most tissue types. The inherent ability of intravenously infused hematopoietic stem cells (HSCs) to seed and engraft their tissue, the bone marrow (BM), is an exception in this regard and forms the basis for a more than four decades-long clinical application of bone marrow transplantation (BMT). However, early in the history of BMT it was obvious that finding a suitable donor within an acceptable time frame would be a limitation for this treatment modality. Since alternative sources of stem cells were desired to increase the donor pool, it was proposed that umbilical cord blood (CB) collected at birth might contain sufficient amounts of HSCs for clinical use. Initial attempts to grow and characterize these cells *in vitro* had shown sufficient success to attempt the use of CB in a clinical transplantation, which then became reality in October 1988 (reviewed in Rubinstein, 2006). Combining Dr. Hal Broxmeyer's expertise in handling and storage of CB with Dr. Arleen Auerbach's skills in prenatal diagnosis of genetic disorders, the team around the leading hematologist Dr. Elaine Gluckman transplanted a 5-year-old boy with CB from his sister and consequently cured him from a life-threatening disease called Fanconi anemia (Gluckman et al., 1989). This, the world's first successful cord blood transplantation (CBT), proved that CB indeed contained pluripotent HSCs and ultimately transformed the traditionally regarded waste-product following childbirth into a clinically beneficial source of life-saving stem cells. Since then, distinct criteria for collection, quality control and cryopreservation of CB, the establishment of CB banks, and our advanced understanding of the biological properties of CB cells have turned the field of CBT into a fast developing area of translational research. To date, it is estimated that more than 30,000 CB transplants have been performed worldwide. As anticipated from the very beginning of CBT, during the years it has become clear that the number of cells present in a typical single CB unit is the crucial determinant for transplantation success or graft failure. Intense global efforts are therefore undertaken to overcome the major roadblock of limiting numbers of transplantable cells to further extend the clinical application of CB cells. These options include expansion of CB cells in a laboratory milieu prior to transplantation, efforts to enhance the engraftment capacities of the transplanted cells, and the use of multiple CB units. In this thesis we have used different screening approaches to identify and characterize (i) novel HSC-supportive growth factors, and (ii) new intrinsic regulators of CB-derived stem cells with the ultimate goal of *ex vivo* stem cell expansion.

Christine Karlsson
London, Spring 2013

HEMATOPOIESIS

Hematopoiesis is the continuous and dynamic process of blood cell formation. Blood is one of the most highly regenerative adult tissues, with one trillion new blood cells being produced every day to ensure a variety of functions that are pivotal for life (Ogawa, 1993). This astonishing number can be further outranged as infections arise or upon acute blood loss, where rapid yet controlled production of enormous numbers of specialized blood cells is crucial to sustain homeostasis. White blood cells battle invading pathogens by recognizing and killing material that is foreign to the body. Platelets prevent bleeding by the formation of blood clots, and red cells, the most abundant blood cell type, deliver oxygen from the lungs to all peripheral tissues (Table 1). With the exception of some specialized lymphocytes, mature blood cells are short-lived and thus need to be replenished throughout life to maintain steady state levels. Their persistent generation directly depends on a rare population of HSCs, which primarily reside in the BM of adult individuals. HSCs ensure lifelong hematopoiesis due to their ability to generate at least one daughter cell of equal identity as the parent during cell division, and the capacity to differentiate into all mature blood cell lineages. These HSC-defining properties of *self-renewal* and *multipotential differentiation* serve as a measure that putative HSCs are evaluated against in functional assays when determining whether they qualify as true stem cells or not. To pass this quality control, cells must (i) possess the ability to self-renew, (ii) hold multipotential capacity, and (iii) restore the blood system of an ablated recipient. Importantly, cells should do so at the single cell level, emphasizing the clonal origin of hematopoiesis.

Table 1. Mature blood cells and their specialized functions

Blood cell type	Main function
Platelet	Blood clotting
Erythrocyte	Transport of oxygen and carbon dioxide
Granulocyte	
Neutrophil	Protection of the host against bacterial infection
Eosinophil	Destruction of parasites; immune and allergic reactions
Basophil	Histamine release in inflammatory reactions
Monocyte	
Macrophage	Phagocytosis of pathogens and cellular waste products
Osteoclast	Bone resorption
Dendritic cell	Antigen presentation; link between innate and adaptive immune responses
NK cell	Cytotoxicity against certain tumor types and microbial infections
B cell	Antibody production, regulation of humoral immunity
T cell	Destruction of virus-infected cells, regulation of cellular immunity

NK, natural killer; “B” stands for bursa of fabricius; “T” stands for thymus

Hematopoietic stem cells

The concept of a common precursor capable of generating the entire spectrum of hematopoietic cells in a cellular hierarchy was first postulated at the transition from the 19th to the 20th century (reviewed in Ramalho-Santos and Willenbring, 2007). This hypothesis was experimentally proven in a series of groundbreaking experiments performed by Till, McCulloch, and colleagues during the early 1960s, providing definite evidence that a multipotential HSC existed in a population of mouse BM cells (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961). Retroviral marking studies further proved the existence of a single common cell-of-origin for all hematopoietic lineages. Since identical retroviral integration sites were identified in lymphoid and myeloid cells of both primary and secondary hosts, these primitive progenitors were supposedly equipped with self-renewal capacity (Dick et al., 1985; Jordan and Lemischka, 1990; Keller et al., 1985; Keller and Snodgrass, 1990; Lemischka et al., 1986). Single cell transplantation experiments formally demonstrated the ability of individually isolated HSCs to long-term reconstitute irradiated recipients and provided the ultimate proof for the self-renewal and multilineage differentiation capacity of HSCs (Osawa et al., 1996). The prospective identification of HSCs by cell surface markers, their isolation by advanced cell sorting technology, and the ability to clonally analyze their hematopoietic potential by means of functional *in vitro* and *in vivo* assays shaped today's view of hematopoiesis as a differentiation hierarchy with multipotent HSCs on top and mature blood cells at the bottom. Self-renewal capacity is gradually lost as HSCs enter the differentiation path through a cascade of distinct progenitor stages, eventually generating terminally differentiated cells restricted to a certain fate (Figure 1). While an early separation into either the lymphoid or the myeloid lineage is the essence of the so-called 'classical model' of hematopoiesis, recent mouse and subsequent human studies indicate that this separation is not as stringent by demonstrating that potential for both cell fates exists downstream the HSC stage, thereby refining the hematopoietic branching points (Ceredig et al., 2009; Dorshkind, 2010; Kawamoto and Katsura, 2009).

While researchers have been able to isolate functional murine HSCs for many years (Bryder et al., 2006), purification strategies for human HSCs have been delayed by the obvious barrier to identify these cells based on their functional properties (as discussed in the next chapter). However, recent technological progress has substantially facilitated the identification of human HSCs and enabled their functional characterization at the single cell level (Notta et al., 2011). The CD34 antigen was the first and is yet the most commonly used marker to enrich for human hematopoietic stem- and progenitor cells (HSPCs) (Civin et al., 1984). Although virtually all *in vitro* clonogenic potential resides within the $CD34^+$ compartment, this population is highly heterogeneous and can be further enriched by exclusion of CD38 and CD45RA as

markers for more differentiated cells (Bhatia et al., 1997b; Krause et al., 1996; Lansdorp et al., 1990; Larochelle et al., 1996). While the combination of CD34 and CD90 (Thy1) detects human HSPCs within lineage negative (Lin^-) mobilized peripheral blood (PB) and human fetal BM cells, Majeti et al. used this marker combination together with CD38 and CD45RA on CB cells to prospectively isolate a candidate human multipotent progenitor (MPP) population (Baum et al., 1992; Majeti et al., 2007; Murray et al., 1995). Recently, human HSC activity has been resolved to the single cell level by means of CD49f expression (Notta et al., 2011). Transplantation of single CB $Lin^-CD34^+CD38^-CD45RA^-Thy1^+Rho^{lo}CD49f^+$ cells enabled long-term multilineage reconstitution in immunodeficient mice, while loss of CD49f identified transiently engrafting MPPs (Notta et al., 2011). The phenotypic and functional discrimination of human HSCs and MPPs provides means to compare these populations at a molecular level with the aim to identify pathways and developmental programs inherent to putative human HSCs (Doulatov et al., 2012).

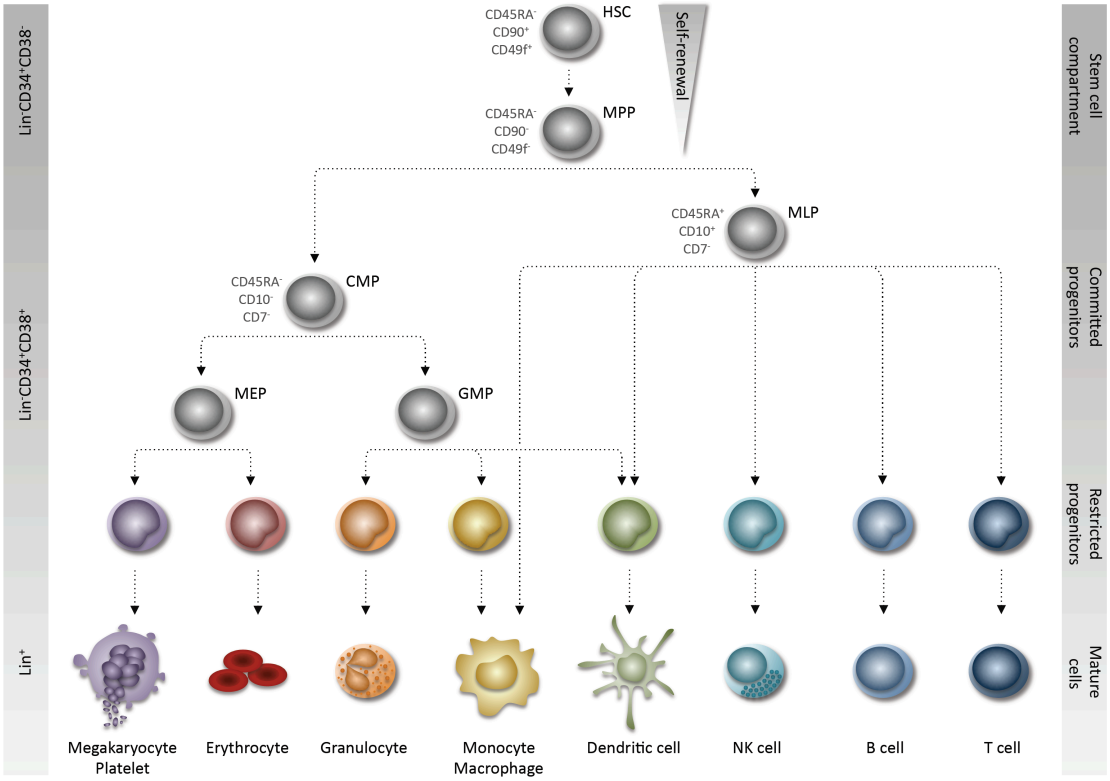

Figure 1. The hematopoietic hierarchy. Simplified schematic representation of lineage determination during human hematopoiesis, whose detailed structure is under constant refinement. Self-renewal capacity decreases progressively as HSCs enter the differentiation path, as indicated by the triangle. Stages of progenitor cell differentiation and consecutive fate restriction are defined by changes in cell surface marker expression. The phenotype of the major classes of primitive stem- and progenitor cells is listed to the left and next to each population. HSC, hematopoietic stem cell; MPP, multipotent progenitor; MLP, multilymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; Lin^-, lineage negative (lack of cell surface markers expressed by mature cells).

The dynamics of how individual HSCs contribute to lifetime hematopoiesis at any given time has been a topic of controversy and provoked two general models of clonal kinetics in blood cell formation. The 'clonal succession' theory refers to the sequential activation of a small number of HSC clones (Abkowitz et al., 1990; Lemischka et al., 1986; Snodgrass and Keller, 1987), while the 'clonal maintenance' model advocates the averaged contribution of the entire HSC pool during steady-state hematopoiesis (Gerrits et al., 2010; Harrison et al., 1988; McKenzie et al., 2006). Given that results from divisional tracking studies with a fluorescent cell staining dye did not fit either of the above models, Takizawa and colleagues proposed the 'dynamic repetition model'. Herein, the clonal contribution of HSCs to hematopoiesis fluctuates with respect to the unique cycling characteristics of the clones (Takizawa et al., 2011). With regard to the methodological constraints and the potential bias of clonal kinetic analyses, Bystrykh et al. emphasize the complexity of HSC behavior and question the need to fit such into pre-assembled models (Bystrykh et al., 2012). Nevertheless, along with these studies, a picture of highly heterogeneous HSC populations has emerged with respect to their proliferation and self-renewal capacity, lineage bias, and turnover rate (Copley et al., 2012; McKenzie et al., 2006; Muller-Sieburg et al., 2012; Schroeder, 2010).

Methods to study hematopoietic cells

More than 50 years ago, Till and McCulloch showed that the clonal capacity of primary cells could be analyzed based on their ability to form discrete nodules in the spleens of irradiated mice (Till and McCulloch, 1961). The advent of this so-called colony-forming-unit spleen (CFU-S) assay launched the functional study of hematopoiesis and established the concept of multipotential HSCs. Since then, a variety of *in vitro* and *in vivo* methods have further founded the clonal hierarchy of hematopoietic cells. Common to all is to experimentally test the functional potential of the cells. Initially, these assays were limited to the mouse as a model system. However, the step-wise introduction of appropriate xenotransplantation models and the continuous improvement of clonal *in vitro* assays tailored to read-out human HSPCs have significantly moved the study of human hematopoiesis forward.

While HSCs from murine BM were initially enriched by size and density, nowadays stem- and progenitor cell populations are prospectively isolated and characterized by cell surface phenotype and fluorescence-activated cell sorting (FACS) (Weissman and Shizuru, 2008). FACS integrates the physical properties of the cells and the ability to label them with fluorochrome-conjugated antibodies specific to cell surface antigens distinctly expressed by certain cell types, enabling the phenotypic but not the functional characterization of the test cells. Since HSCs are exceptionally rare cells (estimated 1 in 3 x 10^6 human BM cells) (Wang et al., 1997), and indistinguishable from their descendents based on e.g. morphological properties, their HSC identity and potential can only be assessed retrospectively by *in vitro* and *in vivo* functional assays.

The commonly used *in vitro* colony-forming unit (CFU) assay measures the frequency of hematopoietic progenitors and their ability to proliferate and differentiate in response to hematopoietic cytokines (Wognum and Szilvassy, 2012). CFU assays are established by plating single-cell suspensions into semi-solid medium supplemented with certain combinations of cytokines. The responsiveness, differentiation and proliferation capacity of the seeded cells is measured based on their ability to generate discrete hematopoietic colonies after a defined culture period. The gel-like consistency of semi-solid media (usually methylcellulose) ensures that the clonal progeny of a single progenitor stay closely together, attributing individual colonies to one single progenitor cell. Progenitors restricted to the erythroid and myeloid lineages form morphologically distinct burst-/colony-forming units-erythroid (BFU-E, CFU-E) and colony-forming units-granulocyte/macrophage (CFU-GM), respectively. In general, the most immature progenitors give rise to the biggest colonies which are composed of a mixture of cells (colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte, CFU-GEMM), thus having multilineage potential (Wognum and Szilvassy, 2012).

Hematopoietic progenitors upstream of those giving rise to CFUs can be identified and quantified in long-term culture-initiating cell (LTC-IC) assays (Sutherland et al., 1990). LTC-IC assays are performed by culturing a test population of cells on a layer of HSPC-supportive primary BM-derived stromal cells or genetically modified cytokine-secreting stroma (Sutherland et al., 1991). After a culture time of at least 5 weeks, the CFU potential of the culture is determined as described above. The long culture period ensures that mature differentiated cells, or any cell equipped with CFU potential present in the initial population, will disappear while the most primitive progenitors will persist. Consequently, the detected colonies must be derived from a cell that maintained the functional properties of an immature progenitor during the long-term culture. Ideally, LTC-IC assays are performed at limiting dilution, allowing the quantification of primitive progenitors in the initial test population (Miller and Eaves, 2002; Wognum and Szilvassy, 2012). Although regarded as a surrogate measure of multipotent cells and *in vitro* self-renewal, LTC-ICs are biologically distinct from cells that can contribute to human engraftment in immunodeficient mice (as discussed below) (Gan et al., 1997; Larochelle et al., 1996).

The development of humanized mouse models in the late 1980s marked the beginning of the functional *in vivo* study of human hematopoiesis (Shultz et al., 2007). In general, humanization is achieved by intravenous or intrafemoral injection of human hematopoietic cells into immunodeficient mice. Accordingly, premises for successful xenograft models are (i) a lack of a functional immune system in the mouse host, enabling the transplantation of human cells, and (ii) an appropriate environment to support human cell engraftment (Willinger et al., 2011a). As for the hematopoietic system, these features have step-wise been modified, aiming to provide a foundation to measure human HSCs based on their capacity to restore long-term multilineage hematopoiesis in irradiated hosts *in vivo*. While early xenograft models such as the

severe combined immunodeficiency (*Scid*) (Bosma et al., 1983) and the "gold standard" non-obese diabetic (NOD)-*scid* mice (Shultz et al., 1995) supported human engraftment, they were limited by their relatively short life span and the overall low engraftment levels, likely attributed to residual mouse NK cell activity (Christianson et al., 1996; Lapidot et al., 1992; McCune et al., 1988; Mosier et al., 1988; Pflumio et al., 1996). Nevertheless, these mice have been used extensively in the study of human hematopoiesis, and provided means to identify and characterize SCID-repopulating cells (SRCs), which are primitive human hematopoietic cells capable of proliferation and differentiation in the BM of immunodeficient mice (Larochelle et al., 1996). Thus, the advent of the SRC xenotransplantation assay provided a powerful tool to measure human HSC activity.

A major step forward was the generation of immunodeficient mouse strains with targeted mutations in the Il-2 receptor common γ chain (*Il2rg*) (Cao et al., 1995; Ohbo et al., 1996). Due to insufficient cytokine signaling (Sugamura et al., 1996), mice with either a truncation (NOD/Shi-*scid*/*Il2rg*$^{-/-}$, NOG), or a complete absence (NOD/LtSz-*scid*/*Il2rg*$^{-/-}$, NSG) of the *Il2rg* gene lack B, T, and NK cell activity and thus support higher levels of engraftment of human cells (Ishikawa et al., 2005; Ito et al., 2002; Shultz et al., 2005). Besides reproducible engraftment and differentiation of human cells, NOG and NSG strains enable quantitative assessment of limiting HSC numbers and secondary transplantation, and thus provide adequate tools to measure human HSC activity *in vivo*. Importantly, sex-specific factors appear to affect the engraftment and proliferation of HSCs in this xenograft setting, emphasizing the need to carefully monitor the experimental design of such studies (Notta et al., 2010).

Table 2. Hematopoietic stem cell assays

Assay	Method	Measurement		Comment
		Progenitor potential	Reconstitution potential	
Phenotypic analysis	FACS-based characterization of stem cell markers	No	No	Fast Not functional
Clonogenic assays	*In vitro colony assay*			
CFU	Quantification of multipotential HPCs in semi-solid media	Yes	No	Fast Reproducible
	In vitro liquid culture			
LTC-IC	Quantification of HSPCs capable of sustaining hematopoiesis for several weeks on stromal feeders	Yes	No	Semi-fast Complex
Repopulation assays	*In vivo*			
SRC	Evaluation of engraftment, proliferation, and differentiation of HSPCs in immune-deficient mice	Yes	Yes	Slow Stringent Variability
Limiting dilution	Quantification of SRC numbers by transplanting different cell doses into immune-deficient mice	Yes	Yes	Slow Quantitative Number of mice

The table summarizes commonly used methods to study human hematopoietic cells. HPC, hematopoietic progenitor cell; HSPCs, hematopoietic stem- and progenitor cell; FACS, fluorescence-activated cell sorting; LTC-IC, long-term culture-initiating cell; SRC, SCID-repopulating cell.

An additional approach to optimize xenograft models is the delivery of human cytokines to the mouse host by either injection of recombinant proteins (Huntington et al., 2011; Huntington et al., 2009; van Lent et al., 2009), transgenic overexpression (Billerbeck et al., 2011; Miller et al., 2013; Nicolini et al., 2004), or knock-in gene replacement (Rongvaux et al., 2011; Willinger et al., 2011b), thus providing a nurturing environment for the engrafted human cells by mimicking human hematopoiesis. Furthermore, modification of background-specific genetic factors, such as polymorphism of the transmembrane protein SIRP-α, represents a strategy to improve the engraftment of human cells (Strowig et al., 2011; Takenaka et al., 2007; Yamauchi et al., 2013).

HSC fate options in the HSC niche

HSCs ensure the continuous production of mature blood cells throughout life. An intricate balance of their *'cell fate decisions'* is essential to maintain steady-state hematopoiesis, and to respond rapidly to situations of hematopoietic stress, such as acute blood loss, injury, and infection. The options HSCs are confronted with are controlled by a complex interplay of cell-autonomous (intrinsic/stochastic) and cell non-autonomous, niche-induced (extrinsic/deterministic) signals (Enver et al., 1998; Morrison and Weissman, 1994; Ogawa, 1999). Self-renewal, quiescence, differentiation, apoptosis, and migration are all possible fates stem cells can adopt, with self-renewal as their defining feature (Figure 2) (Wagers et al., 2002).

Self-renewal is essential to prevent depletion of the HSC pool, and can be defined as a process where at least one daughter cell maintains stem cell properties upon cell division. The result of a *symmetric* self-renewing division is two daughter cells which both conserve their HSC characteristics. This outcome theoretically expands the stem cell pool and is therefore crucial in situations of temporary hematopoietic stress and during HSC ontogeny. Conversely, in *asymmetric* self-renewal divisions the progenies take on different fates, generating one qualitative HSC and one committed cell, thereby maintaining steady-state hematopoiesis (Morrison and Kimble, 2006). Self-renewal as a separate incident may reflect the integration of several cellular responses such as proliferation, survival, and suppression of alternative molecular stages thus preventing lineage-specification (Enver et al., 2009). In keeping with that, prevention of *differentiation* and *apoptosis* is essential to maintain the stem cell pool, whereas signals instructing HSCs to differentiate or commit apoptosis are required to balance stem- and progenitor cell numbers, thereby ensuring homeostasis and the prevention of cancer (Blank et al., 2008; Reya et al., 2001).

Following the 'fetal to adult switch' occurring three to four weeks into mouse development, it is generally assumed that HSCs are mostly in an inactive, non-dividing state (Arai and Suda, 2007; Bowie et al., 2006; Orford and Scadden, 2008). This relative proliferative *quiescence* is important to prevent HSCs from premature exhaustion and DNA damage in physiological conditions (Wang et al., 2012; Yahata

et al., 2011). Using 5-bromo-2'-deoxyuridine (BrdU), it has been demonstrated that murine HSCs are recruited into the cell cycle at low frequencies, dividing on average once a month during steady-state hematopoiesis (Bradford et al., 1997; Cheshier et al., 1999). A close association between slow proliferation kinetics and functional HSC activity could further be proven by refined *in vivo* labeling techniques (Nygren and Bryder, 2008). Label retaining studies using drug-inducible histone 2B-GFP expression suggest the existence of two HSC populations; one homeostatic which divides on a more frequent basis, and one dormant subpopulation that divides at much lower frequencies than earlier observed, i.e. every 145 days or five times per life span. These cells were able to undergo a switch from dormancy to self-renewal in response to injury signals. Moreover, they returned back to a dormant state after the re-establishment of homeostasis, thus playing a predominant role during stress hematopoiesis (Foudi et al., 2009; Wilson et al., 2008). Similarly, the existence of a fast cycling and a quiescent HSC population was demonstrated using *in vivo* tracking of carboxyfluorescein succimidyl ester (CFSE)-labeled HSCs (Takizawa et al., 2011). Applying single-cell staining and imaging analysis, Yamazaki and colleagues have demonstrated the significance of lipid raft clustering in the maintenance of HSC dormancy/hibernation. Here, lipid raft clustering as a result of cytokine stimulation induces HSC cell cycling, whereas the lack of lipid rafts characterizes a dormant HSC state (Yamazaki et al., 2006). Using mathematical modeling of stem cell behavior, human HSC were suggested to divide once every 40 weeks, which is considerably lower than the rates observed for mice employing comparable methods (Catlin et al., 2011).

Depending on the developmental stage of the organism, HSC niches (see below) vary in location and sequentially shift from the yolk sac via the placenta and fetal liver to the adult BM (Kiel and Morrison, 2008). The ability of HSCs to *migrate* in and out of their respective niches is therefore crucial during embryonic development, but also during the mobilization of HSPCs from the BM to the periphery in adult life (Goodman and Hodgson, 1962; Wright et al., 2001). Circulating HSPCs are partly regulated by rhythmic circadian signals released by the sympathetic nervous system (SNS), which tightly control the expression of chemokine (C-X-C) motif ligand 12 (CXCL12) in the BM. Here, HSC fluctuation inversely correlates with CXCL12 expression, which is also known as stromal cell derived factor-1 (SDF-1) and has key functions in HSC migration (Mendez-Ferrer and Frenette, 2007; Mendez-Ferrer et al., 2008).

Initially proposed by Schofield in 1978, the concept of *stem cell niches* refers to cellular and molecular microenvironments that house and maintain HSCs. While interaction of a HSC with “other cells which determine its behavior” ensures survival, contact loss was postulated to trigger differentiation (Schofield, 1978; reviewed in Wang and Wagers, 2011). Although these specialized microenvironments are spatially and temporally flexible, two distinct anatomical niches have been proposed,

the endosteal and the vascular niche (Figure 2) (Ehninger and Trumpp, 2011; Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009). Osteoblasts line the endosteal bone surface and are the major components of the *endosteal niche*. They are suggested to provide crucial factors for HSC maintenance such as thrombopoietin (TPO), CXCL12, and angiopoietin 1 (Ang1) (Arai et al., 2004; Calvi et al., 2003; Xie et al., 2009; Yoshihara et al., 2007). Dynamic bone remodeling by osteoclasts, and chemical factors such as Ca^{2+} gradients as well as oxygen levels and reactive oxygen species (ROS) further play a role in endosteal niche regulation (Adams et al., 2006; Eliasson and Jonsson, 2010; Ito et al., 2006; Kollet et al., 2006; Mansour et al., 2012; Parmar et al., 2007; Wang and Wagers, 2011).

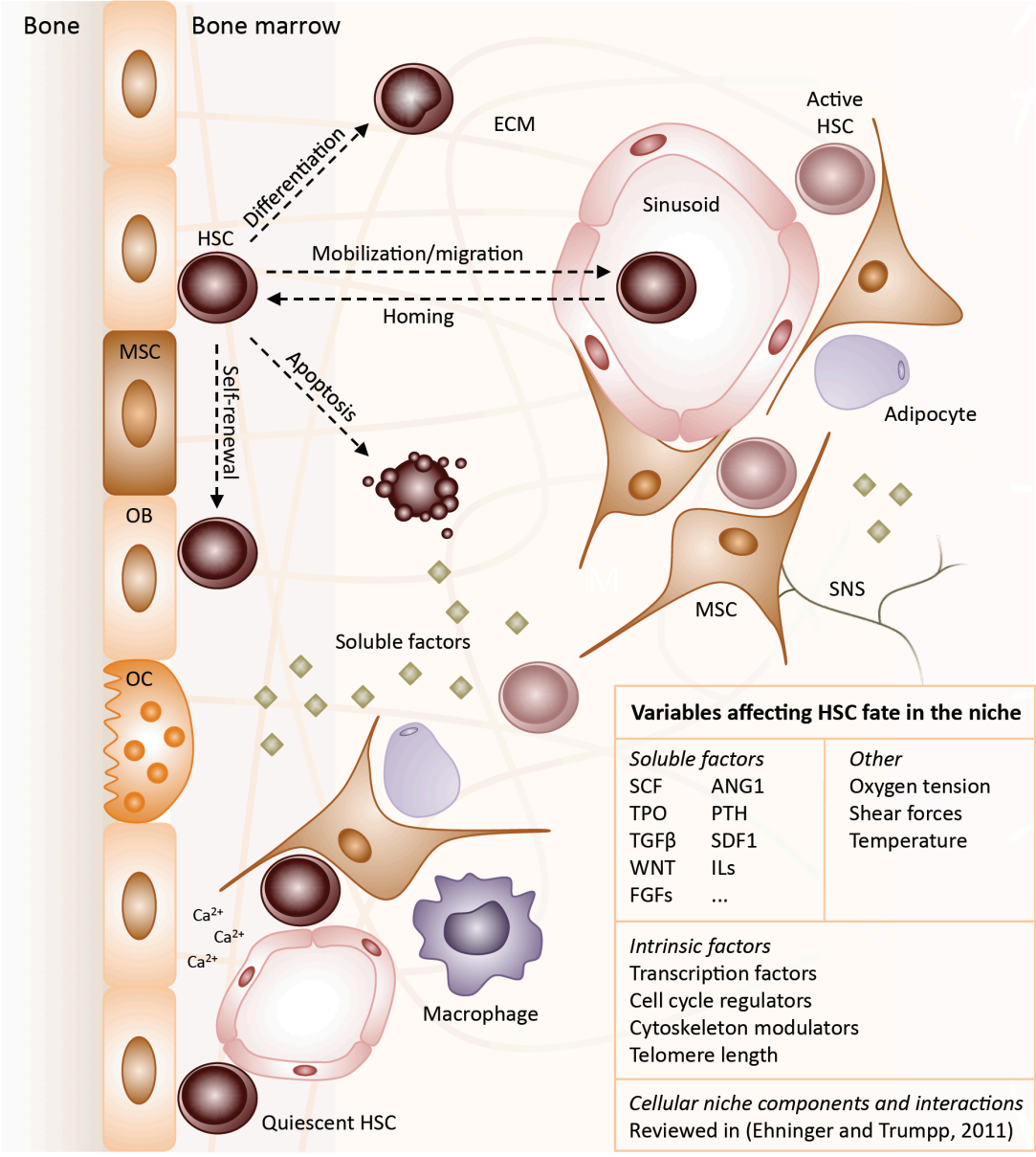

Figure 2. HSC fate decisions in the HSC niche. Supposedly, dormant HSCs reside in niches at the endosteal bone lining, whereas activated HSCs are in close proximity to vascular sinusoids. ECM, extracellular matrix; MSC, mesenchymal stem cell; OB, osteoblast; OC, osteoclast; SNS, symphathetic nervous system.

In addition to the trabecular regions of the bones, HSCs associate with the BM microvasculature in a proposed *vascular niche*, comprised of sinusoidal endothelial cells, perivascular stromal cells, and neural cells (Ding et al., 2012; Kiel et al., 2005; Mendez-Ferrer et al., 2010; Sugiyama et al., 2006; Yamazaki et al., 2011). The cellular niche components producing stem cell factor (SCF), an essential HSC maintenance factor (see next chapter), have recently been determined. Using SCF-green fluorescent protein (GFP), Scf^{gfp}, knock-in mice, Ding et al. detected Scf expression in perivasular and endothelial cells throughout the BM (Ding et al., 2012). Importantly, Gfp and hence Scf expression could not be seen at the endosteal lining of the bones. In line with these findings, conditional deletion of Scf from osteoblasts and HSCs did not alter HSC frequency or function, whereas its deletion from endothelial and perivascular cells depleted HSCs (Ding et al., 2012).

Although the detailed location, function, and integration of the two separate HSC niches is a matter of ongoing illumination, it has been suggested that the endosteal niche promotes HSC maintenance, whereas the vascular niche provides an environment for active HSCs (Ehninger and Trumpp, 2011; Wilson et al., 2007). However, two recent reports further fuel this debate as they revealed the existence of separate niches for restricted hematopoietic progenitor cells (HPC) and putative HSCs. Using selective deletion of CXCL12 in different subsets of niche-embedded support cells, HSCs were shown to reside in the perivascular niche, whereas early lymphoid progenitors occupied the endosteal niche (Ding and Morrison, 2013; Greenbaum et al., 2013). While these findings may provide means to model environments distinct to either HSCs or HPCs *in vitro*, they also revive the fundamental question whether osteoblasts provide a bona fide niche for HSCs or not (Ding and Morrison, 2013; Ding et al., 2012).

Regulation of HSCs

In the BM environment, HSCs are exposed to a wide variety of both intrinsic and extrinsic signals. Their fine-tuned interplay directs the fates HSCs can adopt, and illustrates the resilient nature of the hematopoietic system. However, little is known whether or how precisely cell autonomous and non-autonomous signals interact with each other in networks (Enver and Jacobsen, 2009).

Intrinsic factors important for HSC regulation include transcription factors, transcriptional repressors, cell cycle regulators, and anti-apoptotic signals (Domen, 2000; Pietras et al., 2011; Sauvageau et al., 2004). To a large extent, their significance has been proven by analyses of knockout mice (Rossi et al., 2012). An additional regulatory network was recently revealed with the discovery that microRNAs (miRNAs), small noncoding RNAs which affect gene expression at the post-transcriptional level, control HSC fate determination and tumorigenesis (Han et al., 2010; O'Connell et al., 2010; Ooi et al., 2010).

Besides cell autonomous regulation, *extrinsic signals* such as growth factors and developmental regulators can influence HSCs. Using video imaging and single cell gene expression, it has just recently been suggested that macrophage colony-stimulating factor (M-CSF) directly instructs mouse HSCs to commit to the myeloid lineage (Mossadegh-Keller et al., 2013). These findings propose cytokines as genuine instructors of HSC fate, thereby contrasting the stochastical assumption that cytokines directly influence lineage specification of progenitors but not of HSCs (Cross and Enver, 1997; Enver et al., 1998).
The classical hematopoietic cytokines SCF and TPO provide survival and proliferation signals for primitive hematopoietic cells *in vitro* and are crucial for the maintenance of quiescent adult 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., 1996; Yoshihara et al., 2007). Importantly, the intensity of SCF signaling can directly alter the transcription factor profile and reconstitution ability of mouse HSCs maintained in single-cell *in vitro* cultures, providing evidence of directed extrinsic alteration of HSC self-renewal (Kent et al., 2008). The physiological importance of SCF and TPO has further been demonstrated by means of mutant and knockout mouse models for their respective receptors c-kit and c-mpl, which exhibit reduced HSC numbers and restricted repopulation ability (Kimura et al., 1998; Miller et al., 1996). TPO has emerged as a genuine regulator of megakaryocyte and HSC biology (Chou and Mulloy, 2011). Rather than promoting HSC expansion, TPO was shown to support viability and counteract apoptosis (Borge et al., 1996; Pestina et al., 2001). In fact, administration of a single TPO dose prevented the death of myelosuppressed mice as a consequence of p53-dependent apoptosis inhibition (Pestina et al., 2001). Using *Mpl*$^{-/-}$ mice, de Laval et al. demonstrated a novel role for TPO in irradiation damage by stimulation of the DNA repair machinery (de Laval et al., 2013). Importantly, TPO administration before irradiation protected HSCs from injury and mutagenesis, implying a potential role for TPO agonists in the treatment of patients receiving radiotherapy (de Laval et al., 2013).

Over the last few years, it has become evident that the *metabolic activity* of stem cells can be correlated with their functional behavior (Folmes et al., 2012). The regulation of HSC self-renewal and differentiation has recently been connected to a change in the metabolic states of stem cells and their differentiated progeny (Simsek et al., 2010). Corresponding to the postulation that HSCs reside in hypoxic regions within the BM, quiescent HSCs use anaerobic glycolysis and have lower oxygen consumption than the remaining BM cells (Simsek et al., 2010; Takubo et al., 2013). Instead, cells switch to mitochondrial oxidative phosphorylation to meet the energy demands of proliferating and differentiating cells (Simsek et al., 2010; Yu et al., 2013). Genes and pathways involved in this regulation of energy homeostasis in steady state and stress conditions include for example the PTEN-like mitochondrial phosphatase PTPMT1, pyruvate dehydrogenase kinase (Pdk)-dependent mechanisms,

the tumor suppressor liver kinase B1 (Lkb1), and peroxisome proliferator-activated-gamma coactivator-1alpha (PGC-1alpha) (Basu et al., 2013; Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010; Takubo et al., 2013; Yu et al., 2013). Importantly, maintenance of HSC quiescence through modification of the metabolic status enables cells to adapt to a given environmental situation, as shown by the cross-regulation of Hif-1α signaling/hypoxia and energy metabolism (Takubo et al., 2010; Takubo et al., 2013). Furthermore, lipid metabolism, i.e. the promyelocytic leukemia (PML)-peroxisome proliferator-activated receptor delta (PPAR-δ)-fatty acid oxidation (FAO) pathway, was recently discovered to regulate HSC maintenance (Ito et al., 2012).

The fact that quiescent HSCs rely on glycolysis instead of oxidative phosphorylation may protect them from ROS produced by the mitochondria (Turrens, 2003). ROS can induce oxidative damage of DNA with genomic instability as a possible consequence, and excessive ROS levels have been implicated in aging and senescence (Balaban et al., 2005; Ergen and Goodell, 2010; Naka et al., 2008). Using a mouse model deficient in a gene controlling genomic integrity, namely the 'ataxia telangiectasia mutated' (*Atm*) gene, it has been shown that elevated ROS levels inhibit the self-renewal capacity of HSCs (Ito et al., 2004; Shiloh, 2003). Interestingly, antagonism of p38/mitogen-activated protein kinase (MAPK) signaling reversed the ROS-induced HSC exhaustion in serial transplantation experiments, indicating that the ROS-p38/MAPK pathway can modify the lifespan of HSCs (Ito et al., 2006). Apart from these negative effects, physiological ROS levels could maintain the genomic stability in other stem cell systems, and were shown to play a role in the lodging of HSCs after transplantation (Lewandowski et al., 2010; Li and Marban, 2010). Thus, the balance of intracellular ROS levels may be a critical denominator for HSC integrity.

HSC ontogeny

HSC develop during embryogenesis at multiple anatomical sites in successive yet overlapping waves (Figure 3) (Dzierzak and Speck, 2008; Medvinsky et al., 2011). The first hematopoietic cells emerge in the blood islands of the extra-embryonic yolk sac from the hemangioblast, a mesodermal precursor shared by endothelial and hematopoietic cells. Yolk sac hematopoiesis generates a large amount of primitive erythrocytes which ensure oxygen supply to the embryo, but also gives rise to a few primitive macrophages and megakaryocytes (Choi, 1998; Cumano and Godin, 2007; Moore and Metcalf, 1970; Palis and Yoder, 2001; Tober et al., 2007). These early hematopoietic progenitors are transient in nature and the initial wave of *primitive hematopoiesis* is followed by the generation of *definitive* hematopoietic cells in the intra-embryonic aorta-gonad-mesonephros (AGM) region and in the placenta (Cumano et al., 1996; Gekas et al., 2005; Ivanovs et al., 2011; Medvinsky and Dzierzak, 1996; Mikkola et al., 2005; Muller et al., 1994; Ottersbach and Dzierzak,

2005; Robin et al., 2009). Provided their full hematopoietic potential, these 'adult-type' HSCs can reestablish the blood system of an irradiated host (Medvinsky and Dzierzak, 1996). With the onset of circulation, they migrate to and colonize the fetal liver (FL), where they substantially expand to increase the HSC reservoir of the developing embryo in preparation for postnatal life (Ema and Nakauchi, 2000). Thereafter, hematopoiesis relocates to the BM prior to birth and remains the major site of blood cell production throughout adult life, now in turn seeding hematopoietic organs such as the thymus and the spleen (Medvinsky et al., 2011).

The 'true' origin of the adult hematopoietic system is a matter of disagreement, since definitive HSCs coexist at diverse sites (AGM, yolk sac, and placenta) during blood cell manifestation and specification in the mouse embryo (Medvinsky et al., 2011). Transplantable hematopoietic activity has also been reported from early stage mouse umbilical vessels (de Bruijn et al., 2000a; de Bruijn et al., 2000b; Medvinsky et al., 2011). However, a recent study in the human embryo indicates a clearly timed resolution of HSC appearance, suggesting the AGM as the sole first site of definitive hematopoiesis (Ivanovs et al., 2011).

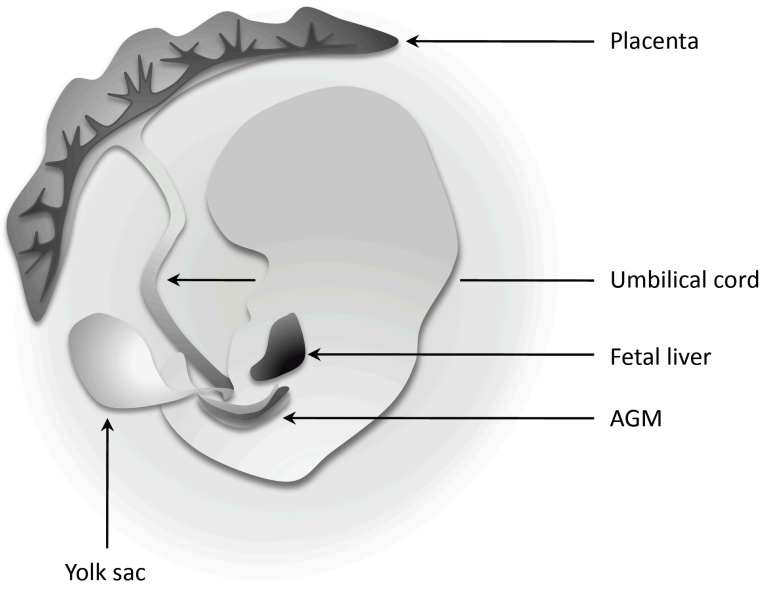

Figure 3. Multisite hematopoietic development. Simplified illustration of a human embryo and sites with reported definitive hematopoietic activity.

Beyond controversy, HSCs from different ontogenetic origins are biologically distinct with respect to their functional properties (Lessard et al., 2004; Mayani, 2010). Neonatal HSCs obtained from the umbilical cord differ from their adult counterparts, which can be derived from the BM or mobilized peripheral blood (mPB) (Cord blood (CB) as HSC source will be discussed in the next chapter). Despite the notion that CB contains higher frequencies of immature $CD34^{+}CD38^{-}$ cells compared to BM, ontogeny-related differences in proliferation and expansion potential characterize their distinct stem cell activity (Mayani, 2010; Ueda et al., 2001; Weekx et al., 1998). A gradual decline in stem- and progenitor cell function from fetal to neonatal to adult has been well established during murine and human ontogeny (Pawliuk et al., 1996; Rebel et al., 1996a; Rebel et al., 1996b; Weekx et al., 1998). Several studies have outlined the higher proliferative capacity of CB cells compared to BM and mPB, which manifests in increased CFC potential, engraftment capacity in immunodeficient mice, and SRC frequency (Coulombel, 2004; Hao et al., 1995; Lansdorp et al., 1993; Noort et al., 1998; Ueda et al., 2001; Wang et al., 1997). Moreover, cytokine requirements and cytokine responses change during development, with the general view that ontogenetically more mature cells need higher cytokine stimulation during *in vitro* culture (Weekx et al., 1998; Zandstra et al., 1998). Whether these ontogeny-related differences reflect the heterogeneity of the cells or rather distinct molecular mechanisms operating during fetal, neonatal, and adult life is not entirely understood. Several alternative aspects have been discussed, such as telomere length, cell cycle kinetics, and gene expression patterns (Mayani, 2010). The latter has recently been assessed in a great effort to map the molecular networks during mouse HSC ontogeny, where McKinney-Freeman et al. uncovered three major transcriptional states of developing HSCs and identified their putative regulators (McKinney-Freeman et al., 2012).

Stem cell-based therapy

Given their role in tissue maintenance and repair, stem cells are prime targets for regenerative medicine. The liquid nature of the hematopoietic system and underlying easy delivery of HSC to their host tissue has made HSCs the prototype of organ-regenerating stem cells and the paradigm for other stem cell-based therapies. Transplantation of HSCs is a conceptual and elegant example of stem cell therapy, and provides means to treat hematopoietic malignancies such as leukemias, lymphomas and immunodeficiencies. The beginning of hematopoietic cell transplantation (HCT) dates back to the late 1950's but clinical success and subsequent breakthrough came with the discovery of the human leukocyte antigen (HLA) system and the selection of immunologically compatible donors in the beginning of the 70's (Thomas, 1999). The basis for this prototype of cellular therapy is the treatment with radio- and/or chemotherapy to breakdown the patient's own hematopoietic system, followed by infusion of new stem- and progenitor cells which

gradually reestablish hematopoiesis. The time to donor engraftment, also called time to neutrophil recovery (TNR) or immune reconstitution, is a critical period as it leaves the patient susceptible to infection with increased risk of transplant-related mortality. HCT can either be autologous, re-infusing the patients own HSCs; or allogeneic, involving a donor, which in turn can be related or unrelated. The success of allogeneic transplantations greatly depends on the degree of immunological compatibility between donor and recipient tissues as mediated by the HLA genes, and the related occurrence of graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) effect (Copelan, 2006; Kolb, 2008). Traditionally, all stem cell transplants performed used BM as sole source of HSCs. Today, HSCs obtained from mPB are more commonly used (Korbling and Freireich, 2011). HSC mobilization can be achieved with e.g. chemotherapy, the cytokine granulocyte colony-stimulating factor (G-CSF) and/or AMD3100, a drug that inhibits binding of the homing molecule CXCL12 to its receptor CXCR4 (Broxmeyer et al., 2005; Donzella et al., 1998; Flomenberg et al., 2005; Liles et al., 2003; Motabi and DiPersio, 2012). CB as a third stem cell source was introduced in 1988 and will be discussed in detail below. Whenever possible, the choice of HSC source is tailored to meet the needs of each individual patient, as they differ with respect to engraftment potential and kinetics, immunogenic characteristics, as well as development and severity of GVHD (Anasetti et al., 2012a; Anasetti et al., 2012b; Korbling and Anderlini, 2001).

Safe and efficient genetic engineering of HSCs is a central yet intricate goal of stem cell therapy and the potential solution to a variety of hereditary and acquired disorders (Riviere et al., 2012). Gene therapy aims at the correction of a defective gene and thus the underlying monogenetic disease, and is achieved by means of *ex vivo* transduction of patient cells with viral vectors carrying the therapeutic gene and subsequent autologous HCT. The ability of retroviral vectors to stably integrate into the hosts genome and the hierarchical nature of the hematopoietic system allows genetically modified HSCs to transmit their alteration to all mature blood cell types and theoretically provides life-long correction of the disease (Karlsson et al., 2002). Gene therapy as treatment modality for severe combined immunodeficiencies (SCID) and thalassemia has shown compelling success with regard to therapeutic efficacy (Aiuti et al., 2007; Aiuti et al., 2002; Boztug et al., 2010; Cavazzana-Calvo et al., 2010; Gaspar et al., 2011). However, genotoxicity of the so called 'first generation' retroviral vectors was observed and resulted in leukemic transformation due to insertional activation of protooncogenes (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b; Ott et al., 2006). These shortcomings seriously halted research and clinical trials in the beginning of the 21st century and raised doubts as to the merits of HSC gene therapy.

It is now clear that genetic engineering of stem cells implicates complex biological challenges. These include collection of efficient amounts of patient cells for *ex vivo* manipulation, effective transduction of self-renewing HSCs to ensure gene delivery as well as polyclonal hematopoiesis, and appropriate vector design to minimize the

universal risk of insertional mutagenesis. The awareness of limitations in current technologies and associated collective efforts to overcome these problems mainly focus on improvements in vector design and technologies for targeted gene delivery, and will gradually re-establish the promising potential of gene therapy (Riviere et al., 2012). Given the need to modify HSPCs outside of their natural environment to achieve gene modification, successful *ex vivo* expansion of these cells would further benefit gene therapy approaches.

Direct reprogramming of somatic cells to induced pluripotent stem (iPS) cells has recently spurred the field of regenerative medicine and offers hope for individually tailored cell therapies. Pluripotent cells, with embryonic stem cells (ESCs) as their prototype, have the ability to differentiate into any of the three embryonic germ layers. Thus, they retain the capacity to constitute all cell types of the body, and accordingly hold great potential for tissue regeneration. In 2006, the manipulation of cell fate through reprogramming was illustrated for the first time in the groundbreaking work of 2012's Nobel prize laureate Shinya Yamanaka, who demonstrated that ectopic co-expression of four transcription factors (c-Myc, Klf4, Oct4, and Sox2) was sufficient to reprogram terminally differentiated murine fibroblasts into cells with ESC-like properties (Takahashi and Yamanaka, 2006). Reprogramming technology has experienced an explosive development in the years thereafter and iPS cell lines from a variety of adult and neonatal tissues in the murine and human system have been generated (Broxmeyer et al., 2011; Maherali and Hochedlinger, 2008; Takahashi et al., 2007a; Yu et al., 2007). Steps towards personalized medicine followed with the creation of patient-specific iPS cells, enabling disease modeling and *in vitro* drug screening (Robinton and Daley, 2012). The combination of reprogramming, gene repair, and tissue replacement therapy has proven successful in animal models of sickle-cell anemia and Parkinson's disease (Hanna et al., 2007; Wernig et al., 2008). These seminal proof-of-principle studies show that, even though far from clinical practice, reprogramming and iPS cell technology hold potential to model and eventually treat human disease. However, the realistic therapeutic potential and safety of iPS cells and their subtle differences to ESCs have yet to be determined (Anasetti et al., 2012b; Daley and Scadden, 2008; Robinton and Daley, 2012; Yamanaka, 2009).

CORD BLOOD – FROM BENCH TO BEDSIDE

History of cord blood transplantation and clinical relevance

Stem cell transplantation is a standard treatment for hematological disorders, and immunological compatibility between donor and transplant recipient, HLA-matching, plays an important role in engraftment and incidence of GVHD (Kawase et al., 2007; Morishima et al., 2002; Petersdorf et al., 2001). The genes encoding HLA antigens are inherited as a set known as haplotype from either parent, and mediate self-recognition and immune defense against everything regarded as non-self. They are highly polymorphic and may be unique to a person. With generally low birth rates in the US and Europe (www.prb.org), only 30% of the patients in need of a transplantation have an HLA-matched sibling donor (Barker and Wagner, 2003; Li and Sykes, 2012). Despite efforts to improve the donor search process by international registries operated by e.g. the National Marrow Donor Program (NMDP; www.marrow.org), the challenge to find a suitable HLA-matched unrelated donor in an acceptable time frame leaves a number of patients ineligible for allogeneic transplantation. Also, genetic heterogeneity and rare HLA-haplotypes of certain ethnic minorities potentiate the problem of insufficient donor pools (Laver et al., 2001; Sonnenberg et al., 1989).

Since the first successful cord blood transplantation (CBT) in 1988 (Gluckman et al., 1989), 25 years of extensive research have established umbilical CB as a valuable and safe alternative stem cell source for HCT (Figure 4) (Gluckman, 2011). Interestingly, the first indications of CB as clinical source of HSCs date back to the late 1960s (Wagner and Gluckman, 2010), and in 1972, Ende and Ende reported a series of CB transfusions from multiple donors to treat a child with leukemia (Ende and Ende, 1972). However, initially both scientists and clinicians were attentively skeptical as to the merit of CB in clinical HCT (Wagner and Gluckman, 2010).

Although several researchers had shown that CB contained cells capable of multilineage differentiation *in vitro* (Fauser and Messner, 1978; Knudtzon, 1974; Leary et al., 1984; Nakahata and Ogawa, 1982), the experimental evidence of long-term reconstituting HSCs in near-term and neonatal blood was first provided in a mouse model (Boyse et al., 1987), prompting the investigators to propose the therapeutic use of CB (Broxmeyer et al., 1989). This suggestion was soon followed by the pivotal work of Hal Broxmeyer and colleagues, who systematically evaluated the effect of parameters such as temperature-dependent storage time on the *in vitro* potential of CB-derived hematopoietic progenitors. Based on these findings, they established efficient and practical guidelines for collection, handling, and storage of CB (Broxmeyer et al., 1989). Importantly, cryopreservation of CB cells does not significantly affect viability and proliferation capacity of hematopoietic progenitors (Koike, 1983), and highly efficient recovery of functional CB progenitors has been

reported up to 23 years post freezing (Broxmeyer et al., 2011). The ability to long-term store CB was the prerequisite for the establishment of CB banks (CBB), which in turn made CBT per se accessible to the public. To date, approximately 600,000 CB units are distributed in more than 100 CBB worldwide (Gluckman, 2011), although this number only reflects a fraction of potential collections given the continuously increasing amount of private/family banks. Pioneered by a series of initial clinical studies (Gluckman et al., 1997; Kurtzberg et al., 1994; Kurtzberg et al., 1996; Wagner et al., 1996), umbilical CB has emerged as a commonly used alternative source of HSCs for the treatment of malignant and non-malignant disorders, with more than 3000 CBT performed annually worldwide (Foeken et al., 2010; Rocha and Broxmeyer, 2010). Global networks of transplant centers and regulatory agencies promote international collaborations, providing comprehensive standards for quality control, donor/recipient selection, and distribution of CB units (Gluckman, 2011). Despite these universal efforts, concrete frameworks for more efficient CB unit search, selection, and acquisition are required for the logistic implementation of these standards (Barker et al., 2011).

A distinct advantage of CB is its prompt "off-the-shelf" availability, particularly important for the treatment of fast progressive acute leukemias. CB collection is a non-invasive process and puts both mother and child at no risk. A major disadvantage is the limited number of stem- and progenitor cells per CB unit, typically about 1/10 of that in a BM allograft. Since the content of infused $CD34^+$ cells per kilogram of the recipient's body weight correlates with engraftment and overall transplantation outcome, low HSPC numbers in CB units hold back the outright potential of CBT. (Barker and Wagner, 2003; Wagner et al., 2002). Strategies to increase the number and potency of CB cells to enhance the efficiency of CBT are discussed in the subsequent chapter.

Several studies retrospectively comparing the influence of graft source, i.e. BM, mPB or CB, on the outcomes of unrelated HCT in both children and adults indicate no significant differences between the treatment modalities (Eapen et al., 2010; Eapen et al., 2007; Gluckman, 2011; Hwang et al., 2007; Laughlin et al., 2004; Rocha et al., 2004; Takahashi et al., 2007b). However, a recent comprehensive meta-analysis of 7 studies totaling more than 3000 patients concluded that CBT led to inferior outcomes than BMT in patients with acute leukemia (Zhang et al., 2012). Atsuta et al. strengthened the importance of disease-specific comparison of unrelated CBT and BMT, as multivariate analysis revealed different outcomes of mortality for acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). CB recipients with AML had lower overall survival rates, while no significant difference was found between CB and BM recipients with ALL (Atsuta et al., 2009). General consensus of these clinical studies is that (i) CBT is associated with a lower incidence of GVHD and thus allows less strict HLA-matching, (ii) CBT results in decreased engraftment and delayed immune reconstitution, (iii) CB should be considered as alternative stem cell source for patients lacking an HLA-matched BM donor (Li and Sykes, 2012;

Rocha et al., 2000; Smith and Wagner, 2009). However, the ultimate choice of stem cell source for HCT depends on donor availability, patient age, disease status, and the preferences of the respective transplantation center, and must eventually be individualized for each patient (Ballen et al., 2012b).

Beyond hematological malignancies, CBT is being evaluated, or discussed, as treatment option for inherited metabolic diseases, hemoglobinopathies, immune deficiencies, and cardiac repair (Boelens et al., 2013; Escolar et al., 2005; Henning et al., 2010; Kamani et al., 2012; Knutsen and Wall, 2000; Prasad et al., 2008; Staba et al., 2004). Given that CB was traditionally regarded as a birth waste product, the history of CBT, from studies of CB biology to clinical practice, represents an exceptional example for translational medicine.

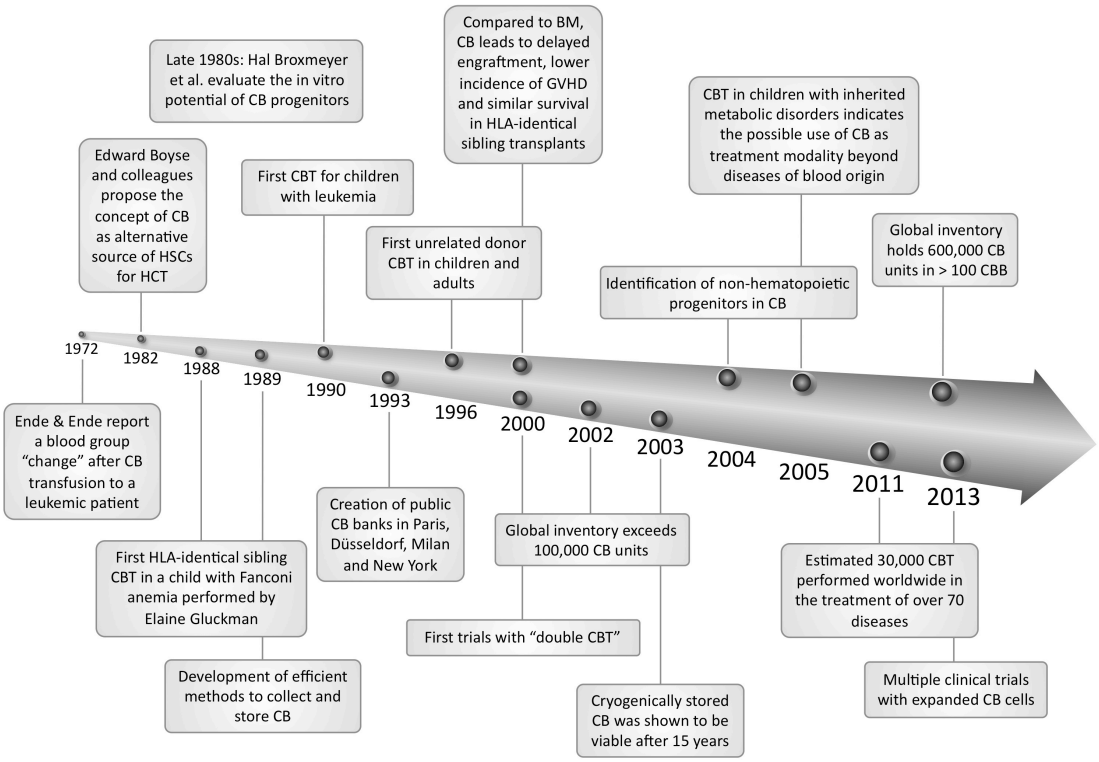

Figure 4. Milestones in CBT. The timeline is based on a summary of the first 20 years of CBT reviewed by Wagner and Gluckman (Gluckman, 2011; Wagner and Gluckman, 2010) and the references stated therein as well as information provided by CORD:USE (http://cordbloodbank.corduse.com). CB, cord blood; CBB, cord blood bank; CBT, cord blood transplantation; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen.

Strategies to improve cord blood transplantation

Despite the undisputed role of CB as an abundant and readily available alternative stem cell source, the relatively low numbers of stem- and progenitor cells in a single unit limit the use of CB in HCT. Given the low cell numbers, prime targets for CBT are pediatric patients. According to the Center for International Blood and Marrow Transplant Research (CIBMTR), CB accounts for more than 40% of allogeneic unrelated HCT in children, while the corresponding number in adults is only approximately 10% (Pasquini and Wang, 2012). There is a clear association between infusion of low cell numbers and delayed immune reconstitution, which in turn increases the risk of early transplant-related mortality (Gluckman et al., 2001; Laughlin et al., 2001; Migliaccio et al., 2000; Rubinstein et al., 1998; Wagner et al., 2002). Consequently, the overall goal is to overcome the cell-dose limitation. Simplified, the more cells transplanted, the faster recovery, the better outcome. Substantial clinical benefit may be achieved through generation of sufficiently high numbers of progenitors that ensure faster hematopoietic recovery and thus counteract the transplantation-induced neutropenia, while at the same time providing stem cells for stable long-term engraftment. To make CB as a donor choice more feasible for adult patients, extensive research has been devoted to find strategies that would improve the numbers, potency, and engraftment capabilities of transplantable HSPCs prior to transplantation.

Efforts to enhance starting cell numbers

Since the number of recovered total nuclear cells is strongly correlated to collection volume, the yield of CB cells can be increased by efficient isolation procedures (Yang et al., 2011). Collection from both the cord and by perfusion of placental blood vessels substantially enhances the numbers of recovered HSPCs (Bornstein et al., 2005; Broxmeyer et al., 1991). Considering the need for trained personnel and the expenditure of time, the feasibility of this method for banking purposes remains to be evaluated.

Based on the assumption that engraftment of a single CB unit may be potentiated by addition of a second, Barker and colleagues pioneered the use of double cord blood transplantation (dCBT) to overcome the cell dose limitations in single CB units (Barker et al., 2001). Safety and clinical benefit was subsequently shown in larger cohorts of patients (Barker et al., 2005; Brunstein et al., 2007), and since 2007, more adults with hematological disorders receive dCBT instead of single units (Rocha et al., 2010). A recent retrospective comparison of single vs. double CBT confirmed the benefits of dCBT for patients with acute leukemia, thus extending the use of CBT to virtually all patients lacking sufficiently dosed single CB units (Scaradavou et al., 2013). Interestingly, only one ‘winning’ CB unit is responsible for long-term hematopoiesis after dCBT in the majority of patients. Although the biological basis

for this outcome is incompletely understood, immunological reactivity of the dominant against the non-engrafting unit is suggested to mediate single-donor dominance after dCBT (Gutman et al., 2010; Milano et al., 2013; Ramirez et al., 2012).

Ex vivo expansion of hematopoietic stem- and progenitor cells

Although dCBT can overcome the limitation of low cell numbers in single CB units and improve sustained donor engraftment, delayed hematopoietic recovery during the first weeks post transplant still remains (Barker et al., 2005; Scaradavou et al., 2013). Furthermore, once a CB unit is used it is gone, leaving no cells available in case of a graft failure. Thus, the establishment of culture conditions under which transplantable HSCs can be amplified *in vitro* to sufficient amounts prior to transplantation has been a longstanding, yet elusive, goal in transplantation therapy. *Ex vivo* expansion is not only relevant to increase the numbers of HSPCs derived from umbilical CB, but may also benefit patients undergoing autologous transplantations, as in the case for e.g. multiple myeloma (Matsui et al., 2012).

A major roadblock is the difficulty to culture HSCs outside their natural *in vivo* environment. Typically, the relative quiescence of primitive CB cells is soon relieved when these start to proliferate under *in vitro* culture conditions, resulting in an engraftment defect and a significant decline of SRC frequency subsequent to the cell cycle entrance (Alvarado-Moreno et al., 2007; Bhatia et al., 1997a; Glimm et al., 2000; Gothot et al., 1998; Mazurier et al., 2004; McKenzie et al., 2007; Rebel et al., 1999; Summers et al., 2001). Moreover, dissociation between phenotype and SRC function does not permit prompt conclusions as to the functionality of the cultured cells (Dorrell et al., 2000).

Numerous attempts have been made to mimic the HSC microenvironment in a culture dish. These efforts aim to identify *ex vivo* conditions that may stimulate proliferation and self-renewal of HSCs, and/or restrict their differentiation into more mature progenies. Nonexclusive strategies to enhance HSPC numbers via cytokine-mediated expansion methods, or modification of intrinsic and environmental factors are exemplified below.

Cytokine-driven *ex vivo* expansion methods

Initial attempts to amplify human BM, mPB, and CB-derived stem- and progenitor cells through extrinsic control of fate decisions focused on the supplementation of the culture media with “classical” hematopoietic cytokines. Various combinations of early- and late-acting growth factors such as SCF, TPO, FLT3L, interleukins, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been extensively studied for more than two decades. The inter-laboratory variation of *in vitro* experimental conditions and subsequent functional read-outs does not allow the direct

comparison of the obtained results. However, the general conclusion from these preclinical studies is that expansion of progenitor cells can be achieved, while the used growth factors showed little or no expansion effect on more rigorously defined HSCs. In fact, it is generally assumed that multiple cytokine stimulation may contribute to lineage commitment and subsequent differentiation at the expense of HSC maintenance (Chou et al., 2010). Furthermore, the sole use of cytokines in *ex vivo* expansion strategies has not significantly improved clinically relevant parameters such as speed of hematopoietic recovery when translated into the clinic (Dahlberg et al., 2011; Norkin et al., 2012; Sauvageau et al., 2004; Walasek et al., 2012b). Thus, the proper combination of cytokines used in *ex vivo* culture conditions has yet to be determined, and identification of novel extrinsic factors stimulating HSCs self-renewal programs may provide tools for successful *ex vivo* expansion in the future.

Developmental factors

The *Notch signaling pathway* determines cellular identity by regulation of cell fate, cell proliferation, and cell death (Pajcini et al., 2011). Given the conserved role of Notch function during development, and expression of Notch receptors and ligands by HSPCs and their microenvironment, a potential implication for Notch in hematopoiesis was raised (Karanu et al., 2000; Kojika and Griffin, 2001; Milner et al., 1994; Pereira et al., 2002; Varnum-Finney et al., 1998). Activation of Notch signaling by means of retrovirus-mediated expression of a constitutively active form of Notch, or activation of endogenous Notch receptors, influences the self-renewal capabilities of murine and human HSPCs in culture as measured by reconstitution analysis (Karanu et al., 2000; Ohishi et al., 2002; Varnum-Finney et al., 2003; Varnum-Finney et al., 1998; Varnum-Finney et al., 2000). On the basis of studies suggesting a 100-fold increase in transplantable $CD34^+$ CB progenitors after cytokine-supplemented *ex vivo* culture in the presence of immobilized Notch ligand Delta-1, Delaney et al. developed a method to increase stem- and progenitor cell numbers prior to transplantation (Delaney et al., 2010a; Delaney et al., 2005; Ohishi et al., 2002). Delta-1-mediated *ex vivo* expansion of CB progenitors resulted in a 6-fold increase in repopulating cells capable of accelerated myeloid reconstitution in NSG mice compared to those receiving uncultured control cells. Importantly, safety and feasibility of this approach was proven in a subsequent phase I clinical trial, where infusion of Delta-1-expanded CB progenitors to patients with acute leukemia demonstrated rapid hematopoietic engraftment and no signs of GVHD (Delaney et al., 2010a). Long-term benefit and risk assessment will be evaluated in a randomized multi-center phase II trial (ClinicalTrials.gov Identifier (CTI): NCT01690520).

Differential requirements for *WNT signaling* during *in vitro* and *in vivo* hematopoiesis and varying experimental approaches by different laboratories have resulted in divergent interpretations regarding the role of WNT signaling in hematopoiesis (Staal

and Luis, 2010). Nevertheless, different WNT proteins promote *in vitro* proliferation and self-renewal in fetal and adult HSCs and their interplay is crucial for normal HSC homeostasis (Austin et al., 1997; Reya et al., 2003; Willert et al., 2003). Pharmacological inhibition of the negative WNT regulator glycogen synthase kinase-3β (GSK-3β) by means of the synthetic compound 6-bromoindirubin 3'-oxime (BIO) resulted in delayed cell cycle progression and enhanced engraftment of *ex vivo*-cultured human CB $CD34^+$ cells. BIO-treatment did not alter expression of canonical WNT target genes but modulated genes implicated in Notch signaling (Ko et al., 2011). This finding confirms earlier reports suggesting that WNT and Notch pathways act synergistically to maintain the stem cell state (Duncan et al., 2005; Trowbridge et al., 2006). Cooperations between WNT and PTEN/PI3k/Akt or WNT and mTOR signals have further been shown to induce expansion of primitive cells and provide examples of how interaction of multiple signals determines HSC fate (Huang et al., 2012; Perry et al., 2011). Combined inhibition of GSK-3 and the mTOR-dependent nutrient sensing pathway preserved the functional integrity of cultured mouse and human HSCs in cytokine-free conditions, as tested by limiting dilution analysis in mice. Although a net expansion of HSCs was not achieved, Huang et al. provide the first evidence that putative stem cells can be cultured in the absence of exogenous hematopoietic growth factors, which may have major impact on basic research and stem cell-based therapy (Huang et al., 2012).

Novel cytokines

Using a gene expression profiling approach, Zhang et al. demonstrated that HSC-supportive mouse fetal liver cells specifically express insulin-like growth factor 2 (IGF-2), its binding protein IGFBP-2, and several angiopoietin-like (ANGPTLs) proteins. Addition of these factors, or combinations of them, to culture media supplemented with saturating doses of other cytokines resulted in a substantial net expansion of both mouse and human HSCs capable of long-term multilineage reconstitution in mice (Zhang et al., 2006; Zhang et al., 2008; Zhang and Lodish, 2004). Recently, the immune-inhibitory receptor human leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue paired immunoglobulin-like receptor (PIRB), expressed on human and murine hematopoietic progenitors, respectively, were shown to mediate the ANGPTL-induced *ex vivo* expansion of HSCs (Zheng et al., 2012). The fact that PIRB supported leukemia development in a MLL-AF9 mouse model may raise concerns as to the use of ANGPTLs in clinical *ex vivo* expansion protocols (Zheng et al., 2012).

Pleiotrophin (PTN) is a neurotrophic growth factor that has recently been recognized as a secreted component of the BM vascular niche (Himburg et al., 2012; Istvanffy et al., 2011). Besides its potential to robustly induce *ex vivo* expansion of murine HSCs, and modestly enhance the engraftment capabilities of cultured human CB $CD34^+CD38^-Lin^-$ cells, it has recently been identified as a regulator of self-renewal,

homing, and retention of HSCs in the BM microenvironment (Himburg et al., 2012; Himburg et al., 2010). These findings point to a predominant *in vivo* effect of PTN on human HSCs and suggest a potential clinical application of PTN or anti-PTN administration for enhanced HSC regeneration or HSPC mobilization, respectively (Himburg et al., 2012).

Co-culture of HSPCs with MSCs

Mesenchymal stem cells (MSCs) are stromal components of the BM niche closely associated with HSCs, which they provide crucial maintenance and survival factors for *in vivo* (Mendez-Ferrer et al., 2010). Such HSC-supportive factors are missing in *in vitro* suspension culture systems, which exclusively rely on the support of selected cytokines. Therefore, co-culture of HSPCs with MSCs has been proposed as means to increase cell numbers and thus improve CB engraftment. Employing this approach pre-clinically, McNiece et al. showed that co-culture of CB-derived mononuclear cells (MNCs) on MSCs resulted in a robust expansion of hematopoietic progenitors compared to fresh input cells (McNiece et al., 2004; Robinson et al., 2006). Robinson et al. compared suspension culture of CD133-selected CB cells with MSC co-cultured unfractionated CB cells and concluded that CB-MSC co-culture had a superior effect on the progenitor output (Robinson et al., 2006). Co-culture of CB MNCs with allogeneic MSCs in a dCBT setting significantly improved engraftment compared to historical controls, offering a rationale for direct comparison of unmanipulated and MSC-expanded dCBT in future studies (de Lima et al., 2012) (CTI: NCT00498316). Interestingly, MSCs can be cultured as self-renewing non-adherent mesenchymal spheres, "mesenpheres", which provide three-dimensional structures that may better resemble the *in vivo* environment than plastic-adherent MSCs (Mendez-Ferrer et al., 2010). Such human mesenpheres were recently shown to support the *ex vivo* HSC activity of CB-derived $CD34^+$ cells, and may provide means to identify novel HSC-supportive factors (Isern et al., 2013).

Alternative culture systems

Zandstra and colleagues apply stem cell bioengineering approaches to develop clinically relevant growth conditions based on the assumption that properties of a entity as a whole, rather than an individual component (such as an individual cell), account for the robustness of a system. Dynamic changes in the composition of cells and their secreted factors during *in vitro* culture negatively affect HSC output (Madlambayan et al., 2005). Furthermore, primitive hematopoietic cells have both higher cytokine requirements and cytokine depletion rates compared to their more mature counterparts (Petzer et al., 1996; Zandstra et al., 1997). Continuous perfusion cultures with automated supply of fresh and withdrawal of spent media can partly overcome this problem. However, undesired inhibitory factors from mature

hematopoietic cells will quickly reappear (Csaszar et al., 2012; Jaroscak et al., 2003; Meagher et al., 1988). The newly described "Fed-Batch" strategy developed in Peter Zandstra's laboratory contains an input stream only, thus leading to a constant increase in culture volume. Applying this approach they could reduce the accumulation of inhibitory signaling factors, which yielded a 11-fold increase in HSC numbers and underscores the importance of global feedback control (Csaszar et al., 2012).

Intrinsic factors

Transcriptions factors belonging to the Homeobox (Hox) gene family are well-established regulators of hematopoiesis. Retrovirus-mediated *HOXB4* overexpression can induce a robust *in vitro* expansion of murine HSCs, characterized by a 40-fold net increase of multipotent HSCs (Antonchuk et al., 2002). However, the extent of expansion was considerably lower when the HOXB4 recombinant protein was delivered to either murine HSCs or human CB-derived $CD34^+$ cells, partly due to the short half-life of the HOXB4 protein (Amsellem et al., 2003; Krosl et al., 2003). Embarking on this approach, Lee et al. have recently reported that degradation-resistant HOXB4 protein confers a proliferation advantage over its natural counterpart, and future studies will clarify the potential expansion effect of the stable HOXB4 protein (Lee et al., 2013).

The zinc finger transcription factor *SALL4* was shown to stimulate human HSPCs and may present a new avenue for *ex vivo* expansion strategies (Aguila et al., 2011). Of interest, *SALL4* regulates self renewal and survival of both normal and leukemic stem cells, and targeting the *SALL4* oncogenic complex has been proposed as a new approach in treating leukemia (Gao et al., 2013; Yang et al., 2008; Yang et al., 2007). Interfering with negative regulators of cell growth by modulation of the cell cycle machinery may represent an option to overcome the relative quiescence of HSCs, provided that proliferation can be induced without loss of the stem cell potential. Therefore, attempts have been made to expand HSCs *ex vivo* by post-transcriptional suppression of cyclin-dependent kinase inhibitors (CDKIs). Using a lentiviral vector containing full-length p21-antisense transduced into CB $CD34^+$ cells, Stier et al. reported a release of cell cycle inhibition and a relative expansion of the stem cell pool compared to controls (Stier et al., 2003).

Small molecules

Aryl hydrocarbon receptor antagonists

Using a screening approach based on CD34 expression as preliminary indicator of progenitor- and stem cell content in cultured cells, Boitano et al. evaluated the effect of 100,000 chemical compounds on blood from mobilized donors. As a result, they

identified the purine derivate StemReginin1 (SR1), which efficiently increased $CD34^+$ cell generation (Boitano et al., 2010). In the presence of hematopoietic cytokines, SR1 maintained a HSC phenotype and expanded multipotent progenitors, as tested by CFC assays. Importantly, SR1 increased the functional HSC pool of *ex vivo* cultured CB-derived $CD34^+$ cells 17-fold as determined by limiting dilution analysis in NSG mice. Given this remarkable expansion, a 'First-in-human' study evaluating the safety of infusing SR1-expanded CB (HSC835) in patients with hematological malignancies is currently being conducted by Novartis (CTI: NCT01474681). Mechanistically, the effects of SR1, and biochemically similar compounds, were mediated by direct binding and inhibition of the aryl hydrocarbon receptor (AhR) (Boitano et al., 2010; Bouchez et al., 2011). Further studies are needed to define the role of AhR in HSC biology, and whether the SR1-induced expansion of primitive cells reflects an effect on putative HSCs, or the expansion of 'facilitator' cells (Boitano et al., 2010).

Copper chelation

Based on studies indicating that the cellular copper (Cu) balance regulates the proliferation and differentiation of human stem- and progenitor cells, the copper chelator tetraethylenepentamine (TEPA) has been evaluated for its potential positive effect on HSPCs in preclinical and clinical settings (Peled et al., 2005; Peled et al., 2002). Although culture of CB-derived primitive cells with TEPA in the presence of cytokines supported expansion of $CD34^+$ cells and NOD/SCID engraftment compared to fresh input equivalents, TEPA-treatment hasn't proven its clinical benefit in a phase I study performed by Shpall and colleagues (de Lima et al., 2008; Peled et al., 2004). An international multi-center historical cohort-controlled phase II/III study further evaluating the efficacy of this approach ("StemEx") is currently ongoing (CTI: NCT00469729).

Histone deacetylase inhibitors

Stem cells are characterized by a distinct epigenetic signature, which notably changes during differentiation and commitment (Meissner, 2010). Since HSCs are regarded to be transcriptionally permissive, epigenetic chromatin modifications such as DNA methylation and histone acetylation can influence cell fate decisions during hematopoietic development (Cedar and Bergman, 2011; Weishaupt et al., 2010). In view of this, chromatin-modifying agents such as histone deacetylase inhibitors (HDI) and demethylating compounds may alter the behavior of primitive hematopoietic cells and favor stem cell self-renewal over differentiation. The HDI valproic acid (VPA) enhances stem cell proliferation during *ex vivo* culture of human and murine HSPCs (Bug et al., 2005; De Felice et al., 2005; Seet et al., 2009). The maintenance of murine HSPCs in culture could further be promoted by the synergistic effect of VPA and lithium, which potently delayed HSPC differentiation at the cellular and molecular

level (Walasek et al., 2012a). If adaptable to human cells, this approach may prove beneficial in *ex vivo* expansion protocols designed to inhibit stem cell differentiation.

Efforts to enhance homing and engraftment

CXCL12-CXCR4 axis

Due to the liquid nature of the hematopoietic system, HSC can migrate in and out from their natural BM niches through the blood (Wright et al., 2001). Hence, homing and engraftment are flexible processes that can be modified. The CXCL12-CXCR4 axis has been implicated in migration, retention, and mobilization of HSCs (Cottler-Fox et al., 2003). The chemokine CXCL12 is secreted by BM stromal cells and, based on its chemotactic activity and CXCR4 receptor expression on HSPCs, traffics cells to the BM after transplantation and actively helps to keep them there (Beider et al., 2003; Dar et al., 2005; Deichmann et al., 1997; Lapidot et al., 2005; Sugiyama et al., 2006). Effective engraftment of HSCs in the BM is therefore dependent on the level of CXCL12 expression in the BM microenvironment, and the CXCR4-mediated ability of HSCs to respond to a CXCL12 gradient (Delaney et al., 2010b). The membrane-bound cell surface protein CD26/Dipeptidylpeptidase IV (DPPIV) truncates CXCL12 and thereby inhibits its chemotactive activity. Consequently, endogenous expression of CD26 on donor cells negatively regulates homing and engraftment (Christopherson et al., 2002; Christopherson et al., 2004). Enzymatic inhibition of CD26 activity by means of small peptides, or by complete disruption of CD26 signaling in $CD26^{-/-}$ mice, was shown to enhance the homing/engraftment capacity of both murine and human HSCs and hence the overall efficiency of transplantation (Campbell et al., 2007; Christopherson et al., 2004; Christopherson et al., 2007). Due to these results, the effect of systemic CD26 inhibition using the drug Sitagliptin are underway at Indiana University School of Medicine (CTI: NCT00862719, NCT01720264). Recently, a general role for CD26 in regulating the potency of colony-stimulating factors (CSFs) in stress hematopoiesis was revealed. In line with predicted CD26 truncation sites on other growth factors, the applicability of CD26 inhibitors may extend to other organ systems (Broxmeyer et al., 2012).

Furthermore, CXCL12/CXCR4-priming factors, molecules that enhance the responsiveness of $CXCR4^{+}$ stem- and progenitor cells to a CXCL12 gradient, have been identified. These include e.g. constituents of innate immunity such as C3 complement cleavage fragments, and components of leukapheresis products from mobilized patients, including fibrinogen, fibronectin, and adhesion molecules (Ratajczak et al., 2004; Reca et al., 2003; Wysoczynski et al., 2005). These preclinical observations were soon translated into a phase I study demonstrating that successive co-infusion of unmanipulated and C3a-primed CB to patients was a safe and feasible approach (Norkin et al., 2012). Whether C3a-treatment of one CB unit facilitates engraftment and overall transplantation outcome is currently under investigation at

the Masonic Cancer Center, University of Minnesota (Brunstein et al., 2012) (CTI: NCT00963872).

Recently, bioactive lipids such as sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) have emerged as homing factors involved in stem cell trafficking (Kim et al., 2012; Ratajczak et al., 2012; Ratajczak et al., 2010). Their potential clinical benefit has yet to be determined (Ratajczak et al., 2012).

Prostaglandin E2

The multifunctional eicosanoid prostaglandin E2 (PGE2) regulates several aspects of HSC homeostasis including survival, proliferation and stem cell homing (Pelus and Hoggatt, 2011). Limiting dilution analysis demonstrated increased HSC frequency and a stable competitive advantage upon short *ex vivo* exposure of murine HSCs to dimethyl-PGE2 (dmPGE2). Homing to either *in vitro* SDF-1 gradients or to BM *in vivo* was promoted and shown to underlie the PGE2-induced upregulation of CXCR4 surface expression. Thus, PGE2-mediated enhanced engraftment is attributed to both increased HSC self-renewal and homing (Hoggatt et al., 2009). Modulation of the BM microenvironment and expansion of the phenotypic short-term HSC pool has further been demonstrated by *in vivo* administration of PGE2 to mice (Frisch et al., 2009). On the molecular level, PGE2 functions via modification of the Wnt signaling pathway and control of apoptosis through reduction of caspase-3 levels (Goessling et al., 2009; Hoggatt et al., 2009). Goessling et al. evaluated the therapeutic potential of dmPGE2 treatment in xenotransplantation settings using human and nonhuman primate HSCs. Stimulation of CB-derived $CD34^+$ cells, and clinically more relevant unmanipulated whole CB samples, enhanced HSC function *in vitro* and *in vivo*. Furthermore, stable multilineage engraftment of dmPGE-treated mobilized peripheral blood stem cells demonstrated the safety of this approach in a nonhuman primate autologous transplantation setting (Goessling et al., 2011). In a phase I study, Cutler et al. validated the feasibility and safety of dmPGE2-treatment of one CB unit in a dCBT setting. Given the fast neutrophil recovery and dominance of the PGE2-modulated CB unit in the majority of the recipients, the potential benefit of this strategy to improve engraftment in patients undergoing CBT is subject to further clinical testing (Cutler et al., 2011) (CTI: NCT00890500).

Additional means to enhance engraftment

Direct *intrabone injection* as means to bypass the homing problem and/or to limit cell loss during circulation confers an engraftment advantage over intravenously injected cells in xenotransplantation settings (Castello et al., 2004; Mazurier et al., 2003). This new route for transplantation has been adopted by some European transplant centers (Rocha and Broxmeyer, 2010), based on results from a phase I/II study suggesting

beneficial effects of intrabone CBT on transplantation outcome even when low, HLA-mismatched CB cells were used (Frassoni et al., 2008) (CTI: NCT00696046).

Other approaches to enhance HSC homing and to manipulate the BM niche include *fucosylation of CB* cells prior to transplantation (Robinson et al., 2012; Xia et al., 2004) (CTI: NCT01471067), and administration of *parathyroid hormone (PTH)* to patients following dCBT (Ballen et al., 2012a). However, despite its stimulatory effects on BM-embedded osteoblastic cells in mice (Calvi et al., 2003), PTH treatment did not influence hematopoietic recovery in a cohort of 13 patients (Ballen et al., 2012a) (CTI: NCT00393380).

Table 2. Examples of recent approaches to improve stem cell transplantation

Expansion strategy	Rationale	Examples[§,†]	Effect[‡]			References
			Fold over control	Fold over input	SRC expansion	
Cytokines	Simulation of *in vivo* conditions by extrinsic addition of growth factors	Angptl-5, IGFBP-2	↑ (NOD/SCID)	↑ (NOD/SCID)	~20-fold	Zhang et al., 2008
		Pleiotrophin	↑ (CFU) ↔ (NOD/SCID)	↑ (CFU) ↑ (NOD/SCID)	N/D	Himburg et al., 2010
Conserved pathways	Modulation of developmentally conserved pathways	Delta1*	↑ (NSG)	↑ (NSG)	6-fold	Delaney et al., 2010
		WNT	↑ (CFU) ↑ (NOD/SCID)	↓ (NOD/SCID)	N/D	Ko et al., 2010
Transcription factors	Intrinsic regulation of HSC self-renewal	SALL4	↑ (LTC-IC) ↑ (NSG)	N/D N/D	N/D	Aguila et al., 2011
Stromal co-culture	Mimic the *in vivo* environment	MSC co-culture*	N/A	↑ (CFU)	N/A	de Lima et al., 2012
Chelators	Reduction of free Cu ions modulates differentiation	TEPA*	↑ (CFU)	N/D	N/D	Peled et al., 2005
Aryl hydro-carbon receptors	Antagonism of AhR signaling	SR1*	↑ (CFU) ↑ (NSG)	↑ (NSG)	17-fold	Boitano et al., 2010
Feedback inhibition	Reduction of inhibitory feedback signals	Fed-Batch Strategy	↑ (CFU, LTC-IC) ↑ (NSG)	↑ (NSG)	11-fold	Csaszar et al., 2012
Engraftment	Modulation of homing and engraftment	CD26/DPPIV*	↑ (NOD/SCID)	N/D	N/D	Campbell et al., 2007
		PGE2*	↑ (CFU) ↑ (NOD/SCID)	N/D N/D	N/D N/D	Goessling et al., 2011

HSPCs, hematopoietic stem- and progenitor cells; SRC, SCID-repopulating cell; Angptl, angiopoietin-like protein; IGFBP, insulin-like growth factor binding protein; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; Cu, copper; CFU, colony-forming unit; TEPA, tetraethylenepentamine; SALL, Sal-like; LTC-IC, long-term culture-initiating cell; NSG, NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ; MSC, mesenchymal stem cell; AhR, aryl hydrocarbon receptor; SR1, StemRegenin 1; PGE2, prostaglandin E2; N/D, not determined; N/A, not applicable.

[§]Selected strategies are listed.

[†]Test molecules are listed, complete culture conditions and cytokine supplementation can be found in the stated references.

[‡]Functionally assessed by CFU-assays and/or transplantation.

*Clinical trial ongoing/completed.

↑ ↓ ↔ signify increased, decreased or maintained engraftment levels in immunodeficient mice, respectively.

SCREENING STRATEGIES TO IDENTIFY HSC REGULATORS

General aspects

Approaches to correlate a gene with a certain phenotype can proceed in two opposite directions, either from 'gene to phenotype' or vice versa (Figure 5). *Reverse genetics* ascribes a phenotype/function to a defined given gene. Conversely, the concept of *forward genetics* is based on the selection of mutants/clones performing well in a phenotypic assay and the retrospective identification of the gene responsible for the phenotype. Traditional reverse genetic methods in the field of hematopoiesis include loss- and gain-of-function studies using genetically engineered mouse models and virally mediated insertion of foreign genes into cells of interest. While a majority of research relies on this kind of observational and hypothesis-based evaluation of genes, forward genetic screens enable collection of large sets of unbiased data in a systematic manner.

Retroviral and transposon-based insertional mutagenesis have been valuable tools for cancer gene discovery (Copeland and Jenkins, 2010; Kool and Berns, 2009). Based on the ability of retroviral vectors to stably integrate into the host cell genome and the hierarchical organization of the hematopoietic system, the specific integration pattern of the proviral DNA is passed on to all HSC progeny. Random integration of retroviruses has been associated with activation of oncogenes or deactivation of tumor suppressor genes, thus leading to cancer. Such "common insertion sites" (CIS) are summarized in the retrovirus-tagged cancer gene database (RTCGD) and the insertional dominance database (IDDb) that represent useful tools for the identification of genes regulating HSC turnover as well as malignant clonal expansion (Akagi et al., 2004; Kustikova et al., 2007).

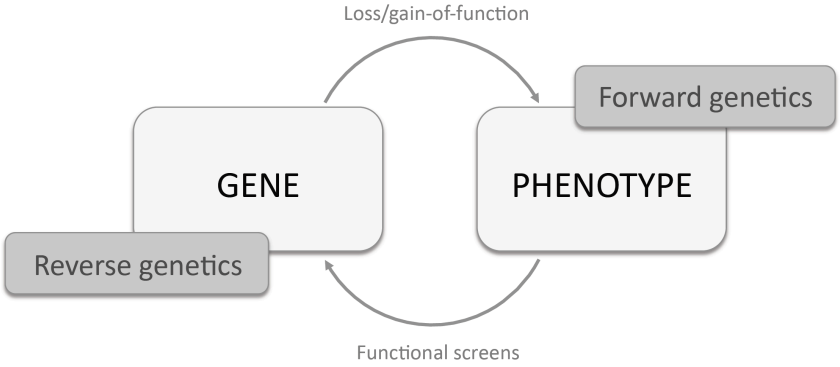

Figure 5. From gene to phenotype and back. Based on the succession of correlating a gene with a phenotype, approaches to identify biologically relevant genes may be referred to as either reverse or forward genetics.

RNAi-based forward genetic screens

RNA interference (RNAi) is an endogenous cellular process that ultimately results in the post-transcriptional, sequence-specific down-regulation of target genes. The RNAi machinery can be turned on by exogenous and endogenous triggers of double-stranded RNA (dsRNA), which are further processed into small interfering RNAs (siRNA) or microRNAs (miRNA) by the ribonuclease Dicer. These short dsRNA molecules are subsequently incorporated and unwound in the RNA-induced silencing complex (RISC) where they serve as guides for substrate selection and subsequent mRNA degradation and/or translational repression (Figure 6) (Hannon, 2002; Meister and Tuschl, 2004; Sharp, 1999). In contrast to siRNA, miRNA are naturally expressed small noncoding RNAs. Since they are suggested to alter the expression of a wide range of target mRNAs, miRNAs have the potential to change complex biological pathways and biological states by affecting cellular gene expression profiles (Bartel, 2009).

Originally discovered in *Caenorhabditis elegans* in 1998, RNAi has emerged as a feasible tool for the relatively fast evaluation of reduced gene expression on a cell or organism scale *in vitro* and *in vivo* (Brummelkamp and Bernards, 2003; Dickins et al., 2007; Fire et al., 1998; Hannon and Rossi, 2004). Furthermore, the creation of large RNAi libraries allows for genome-wide loss-of-function screening and provides powerful tools to perform high-throughput forward genetics (Moffat and Sabatini, 2006). The basis of a successful RNAi screen is a robust assay that specifically reflects the biological process being investigated. Factors such as event frequency, library size and coverage as well as analysis and validation of the screening results need to be considered to avoid unbalanced false positive and false negative event rates and thus low efficiency of the screen (Campeau and Gobeil, 2011; Echeverri et al., 2006). Standard screening formats are arrayed library screens where each siRNA is monitored individually using e.g. morphological readouts, survival, or biochemical assays. Complex mixtures of short hairpin RNAs (shRNA) can also be assayed simultaneously in pools using a selection-based screening approach that filters cell populations based on phenotypic criteria, such as growth selection. The development of vector-based shRNA libraries combined with viral transduction methods enables high-throughput screens in both short-term and long-term assays, providing means to accomplish functional genetic screens in primary mammalian cells, including stem cells (Echeverri and Perrimon, 2006; Moffat and Sabatini, 2006). Early pioneering examples for the feasibility of pooled large-scale screening approaches in mammalian cells are the identification of new components of the p53 tumor-suppressor pathway, the identification of RE1-silencing transcription factor (REST) as a novel human tumor suppressor, and the mapping of candidate regulators involved in mitotic progression and proliferation (Berns et al., 2004; Moffat et al., 2006; Paddison et al., 2004; Westbrook et al., 2005).

Importantly, forward genetic screens can be accomplished at the stem cell level. Using an *in vivo* RNAi screening approach, Hope et al. identified fate determinants implicated in polarity and asymmetric cell division in murine HSCs (Hope et al., 2010). Employing a similar strategy, Wang et al. uncovered a differentiation checkpoint limiting HSC self-renewal in response to DNA damage (Wang et al., 2012). Furthermore, RNAi screens provide tools to spot disease-relevant genes and tumor-suppressors, successfully demonstrated by e.g. the identification of RPS14 as a 5q- syndrome gene, and shRNA-targeting of chromatin regulators in an AML mouse model and subsequent identification of bromodomain-containing 4 (Brd4) inhibition as potential therapeutic option in AML (Bric et al., 2009; Ebert et al., 2008; Meacham et al., 2009; Zuber et al., 2011).

Apart from RNAi-based screening technology, gain-of-functions screens as well as compound-based screening offer valuable tools to unravel novel regulators of vertebrate hematopoiesis and potential modifiers of *ex vivo* HSC expansion (Boitano et al., 2012; Boitano et al., 2010; Deneault et al., 2009; North et al., 2007).

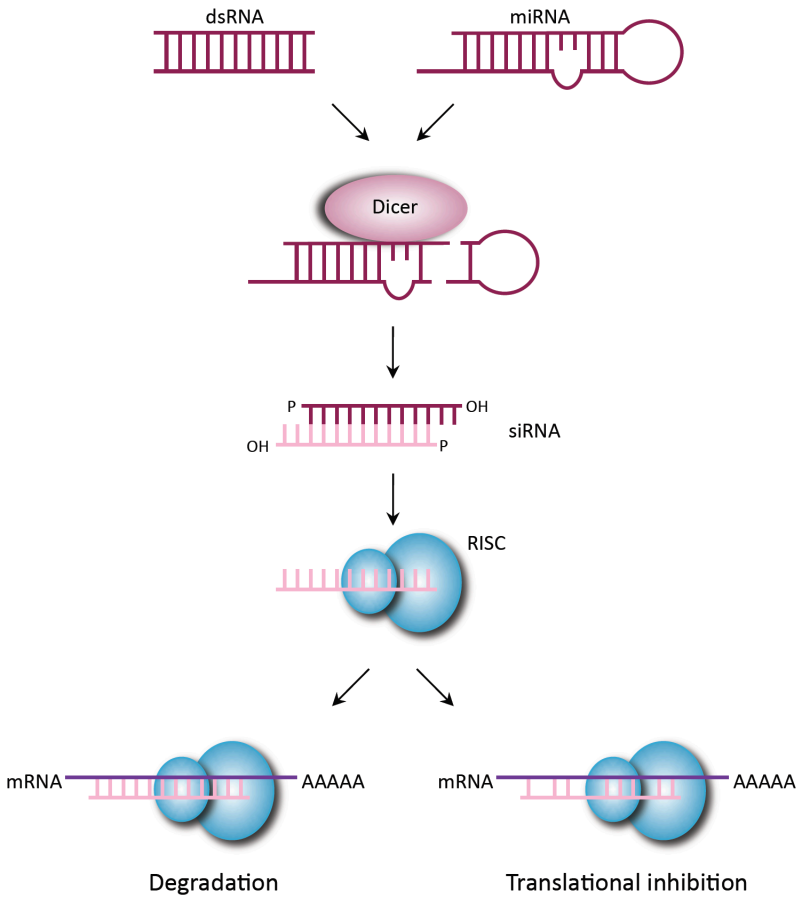

Figure 6. Simplified schematic representation of the RNAi pathway. Exogenous and endogenous RNAi triggers are processed into siRNA or miRNA molecules by Dicer, subsequently leading to target gene mRNA degradation or translational repression, respectively. ds, double-stranded; mi, micro; si, small interfering; RISC, RNA-induced silencing complex.

PRESENT INVESTIGATION

Focus and aim

Given their pronounced regenerative ability, HSCs are ideal targets for cell- and gene therapy of hematological disorders. Although CB provides an abundant and readily available stem cell resource, the limited numbers of stem- and progenitor cells present in a single CB unit has restricted its clinical application to a broad patient spectrum. Despite intense global efforts to find strategies that would enable the *ex vivo* amplification of transplantable stem cells, the culture of HSCs outside their natural *in vivo* microenvironment remains difficult, posing a major hurdle for both stem cell expansion protocols and basic HSC research. Since *in vitro* culture of HSCs typically results in differentiation or apoptosis and an overall loss of HSC integrity, identification of culture conditions that maintain the primitive properties of HSCs has been a longstanding goal of regenerative medicine. To this end, numerous attempts have been undertaken to manipulate HSC fate *ex vivo* by extrinsic and intrinsic regulators with the aim to find conditions that would promote HSC proliferation in the absence of differentiation and/or apoptosis. Some of these have proven beneficial in preclinical and clinical settings. Nonetheless, the stimulation of self-renewal *in vitro* is a continuing ambition of stem cell biology. It is therefore important to understand how HSC self-renewal, proliferation, and differentiation are integrated, to identify novel molecules participating in their regulation, and how these could be modified for clinical benefit as the ultimate goal.

This thesis was intended to systematically identify and subsequently characterize new regulators of human stem- and progenitor cells. For this purpose we have developed, optimized, and applied a growth factor screen as well as forward RNAi-based screening approaches to retrieve distinct factors capable of affecting the fate decisions of cultured HSPCs.

Summary of results

Paper I – Identification of the chemokine CCL28 as a growth and survival factor for human hematopoietic stem- and progenitor cells

In an attempt to discover novel positive regulators of cell proliferation and possible mediators of expansion, we performed a comprehensive assessment of growth factor responses in human CB-derived HSPCs. Theoretical essence of this screen is the restricted ability of HSCs to preserve their functional identity upon *in vitro* culture. Here, we screened 276 extrinsic signaling molecules for expansion of primary CB-derived $CD34^+$ cells based on high-throughput FACS analysis of CD34 expression following a 7-day culture period. We identified the immunoregulatory chemokine (C-C motif) ligand 28 (CCL28) as novel growth factor for primitive hematopoietic cells from different ontogenetic origins with the capacity to expand the functional progenitor content of their cultured progeny. CCL28 supported proliferation by recruiting hematopoietic progenitors into cell cycle and by suppressing apoptosis. When added alone, CCL28 failed to induce a net proliferation of $CD34^+$ cells, but was able to maintain progenitor activity, suggesting that it provides crucial survival signals for HSPCs during *ex vivo* culture. Moreover, addition of CCL28 to cultures of HSC-enriched $CD34^{hi}CD38^{lo}CD90^+CD45RA^-$ cells significantly enhanced the ability of the cells to long-term repopulate immunodeficient mice compared to equivalent input numbers of fresh cells. Together, our findings identify CCL28 as a novel growth and survival factor for primitive human hematopoietic cells.

Paper II – The myostatin antagonist myostatin propeptide promotes proliferation of human hematopoietic stem- and progenitor cells *ex vivo*

In conjunction with the growth factor screening described in paper I, we identified myostatin propeptide (MP), a naturally occuring inhibitor of the TGF-ß superfamily member myostatin, as a potent inducer of *ex vivo* proliferation of primitive human hematopoietic progenitors. MP promoted proliferation of FL-, CB-, and BM-derived $CD34^+$ cells and expanded the clonogenic potential of CB progenitors as assessed by CFC assays. Beyond the bulk $CD34^+$ fraction, MP stimulated putative human HSCs in a direct manner with effects similar to those of TPO. In the presence of SCF, MP significantly improved short- and long-term reconstitution of immunocompromised mice compared to cultures treated with SCF alone, indicating that MP supports HSCs *ex vivo* following a 7-day culture period. Interestingly, myostatin expression was absent across diverse fractions of mature and primitive hematopoietic progenitors from different ontogenetic sources, suggesting that MP may exert its growth-promoting effects through mechanisms other than inhibition of myostatin. This notion was further supported as addition of the known myostatin inhibitors follistatin (FST) and GDF-associated serum protein-1 (GASP-1) could not recapitulate the growth-

promoting activity of MP. Collectively, we have identified MP as a novel modifyer of *ex vivo* HSPC proliferation. Future work is aimed at determining whether the growth promotion is mediated through effects within or outside the canonical TGF-ß family signaling pathway.

Paper III – Forward RNAi screens in primary human hematopoietic stem/progenitor cells

In paper III, high-throughput RNAi screening based on the limited persistence of HSPCs under *ex vivo* culture conditions was used to unravel novel modulators of human hematopoiesis in CB-derived $CD34^+$ cells. Briefly, a pooled version of a lentiviral shRNA library targeting kinases, phosphatases and proteases was transduced into CB $CD34^+$ cells. Subsequently, cells were passaged in long-term *in vitro* cultures followed by a functional CFC readout to positively select for clones that exhibited an enhanced proliferation ability combined with a sustained primitive potential. Functional validation of dominant hits retrieved from the screen enabled identification of novel target genes or shRNA constructs capable of altering HSPC proliferation and differentiation, respectively. Specifically, knockdown of exostoses 1 (shEXT1) affected HSPCs along the erythroid differentiation path resulting in increased production of BFU-E/CFU-Es. Cells transduced with either shPLCZ1 (phospholipase C zeta1) or shSTK38 (serine/threonine kinase 38) exhibited a >1000-fold increased expansion of total hematopoietic progenitors during culture. While shPLCZ1 did not score in subsequent stem cell assays, shSTK38 amplified the LTC-ICs content of one week cultured cells, and expanded candidate human HSCs as determined by a 50-fold increased NOD/SCID repopulation ability over 26 days of *ex vivo* culture. However, its specific molecular targets remained undefined as additional shRNA constructs against STK38 did not trigger a similar expansion effect despite similar suppression of the STK38 transcript, suggesting off-target effects on one or several other genes. Despite gene expression profiling of shSTK38-transduced CB $CD34^+$ cells and subsequent functional *in vitro* and *in vivo* validation of microarray-retrieved target genes (data not shown), we have to date not been able to discover the actual shSTK38 gene target. Nevertheless, in this proof-of-principle study we conclude that RNAi screening of genetic modifiers against a functional outcome of interest holds great potential to identify novel genes directing cell fate in rare populations such as stem- and progenitor cells.

Paper IV – RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion

In paper IV we employed next generation sequencing to track shRNA-transduced HSPCs as a versatile tool to optimize the resolution and feasibility of the RNAi-screening approach described above. We selected an shRNA library that included a

high proportion of druggable genes, with the aim to explore the applicability of retrieved hits from the screen by e.g. small molecules and pharmacological inhibition. Upon transduction of CB $CD34^+$ cells with a pooled lentiviral library targeting mainly kinases and phosphatases, relative changes in contribution of shRNAs during a culture period of 20 days were determined by high-throughput sequencing. Among several genes involved in cell proliferation and tumor suppression, we observed an enrichment of three independent shRNAs targeting the mitogen-activated protein (MAP) kinase family member *MAPK14*/p38α in the primary screens. Lentiviral silencing of *MAPK14* and pharmacological inhibition of p38α recapitulated the screening results, i.e. enhanced frequency of $CD34^+$ cells throughout the culture period, and suggested *MAPK14*/p38α targeting as potential means to modulate *ex vivo* HSPC activity. To this end, we tested four independent p38 inhibitors for their capacity to promote expansion of undifferentiated cells over a culture period of five days. Treatment with any of the four inhibitors increased the frequency and numbers of cells co-expressing CD34 and CD90 compared to control conditions, and further resulted in higher functional HSPC content as measured by long-term multilineage reconstitution in immunodeficient mice. Furthermore, inhibition of p38 decreased the intracellular levels of reactive oxygen species (ROS) in treated cells, suggesting reduced oxidative stress as one possible mechanism behind the enhanced stem cell activity of cultured p38α-inhibited HSPCs. Together, these findings implicate that functional forward genetic screens in conjunction with next generation sequencing are feasible tools to retrieve relevant insights into HSC biology in general and stem cell maintenance in particular. In addition, we suggest that p38 inhibitors should be considered in strategies that aim at expanding HSCs for clinical benefit.

Conclusions from present studies

I. Primary human hematopoietic progenitors are suitable targets for empirical and genetic screening approaches (Papers I - IV).

II. The limited persistence of cultured HSPCs provides an attractive basis to functionally select for perturbations promoting the maintenance or expansion of undifferentiated cells (Papers I - IV).

III. The chemokine CCL28 is a novel growth factor for human HSPCs (Paper I).

IV. CCL28 maintains the primitive properties and functional integrity of cultured human hematopoietic cells (Paper I).

V. Myostatin propeptide, a naturally occurring inhibitor of the TGF-ß superfamily member myostatin, represents a new hematopoietic modifier with the ability to induce HSPC proliferation (Paper II).

VI. Pooled RNAi screening is a feasible tool to discover novel factors that directly alter the fate of CB-derived human $CD34^+$ hematopoietic progenitors (Paper III and IV).

VII. Targeting gene-unrelated events, so called 'off-target-effects', introduces an undesired complexity to first-generation RNAi screening in primary cells (Paper III).

VIII. Second-generation RNAi-based screens in conjunction with next generation sequencing optimize the resolution and specificity of the screening approach (Paper IV).

IX. Inhibition of p38 enhances the stem cell activity of HSPCs during *ex vivo* culture (Paper IV).

GENERAL DISCUSSION

Establishing culture conditions under which large numbers of fully functional HSPCs can be generated *in vitro* has been a central goal in transplantation medicine. In this thesis we studied novel extrinsic and intrinsic regulators and investigated their biological effects on HSC characteristics with the ultimate goal to find determinants of HSC maintenance and/or expansion. Relevant to this work have been the use of *in vitro* and *in vivo* HSC assays, the concept of stem cell expansion, and the use of high-throughput screening assays to retrieve candidate HSC modifiers, which will be discussed in detail below.

Modeling human hematopoiesis

Humanized mice have emerged as the gold standard to experimentally predict the functionality and behavior of human hematopoietic cells. Still, to date no mouse model can completely measure true human HSC behavior *in vivo* (Takenaka et al., 2007; Willinger et al., 2011a). This is illustrated by the general decline of human cell engraftment over time, myeloid-lymphoid lineage skewing in recipient mice, the relative absence of erythroid and megakaryocytic cells, and the low efficiency of serial transplantations (Rongvaux et al., 2013). These observations demonstrate that the microenvironment of immunocompromised mice cannot entirely support/maintain human HSCs, and yet-to-be-identified niche determinants may account for this apparent limitation. Attempts to provide a 'niche in a dish' via co-culture techniques or extramedullary bone models may represent alternative means for human cells to engraft in a human environment, although such strategies ultimately rely on their experimental validation in mouse models (Chen et al., 2012; Corselli et al., 2013). Larger animal models such as dogs or nonhuman primates enable assessment of long-term engraftment and resemble certain aspects of human HSC biology, e.g. cell cycle kinetics, better than mice, and are generally the prerequisite for clinical translation of experimental evidence (Horn et al., 2003; Shepherd et al., 2007). Given their size, these models may also provide a basis to put more similar demands on the transplanted cells compared to a relatively small host like the mouse.

Heterogeneity and limitations with current HSPC assays

In contrast to cells from inbred, congenic mouse strains, human stem- and progenitor cell populations, and primary human cells in general, are characterized by their heterogeneity and genetic diversity. These features introduce an intrinsic constraint into all experimental models of human hematopoiesis. Bulk populations of CB CD34-enriched- but also more primitive $CD34^{+}CD38^{lo/-}$ cells are functionally heterogeneous with regard to their ability to proliferate in response to cytokine stimulation *in vitro,*

and their self-renewal capacity *in vivo* (Guenechea et al., 2001; Hao et al., 1995; Hogan et al., 2002; Lemischka and Jordan, 2001; Mazurier et al., 2004; McKenzie et al., 2006; Summers et al., 2001). This intrinsic biological heterogeneity, characterized by differences in proliferation potential of distinct CB units of up to one log, can be challenging when evaluating the effects of novel hematopoietic growth factors (Delaney et al., 2010a).

In this thesis we have used clonogenic *in vitro* assays as a first measurement of stem- and progenitor cell activity followed by transplantation of defined cell populations into immunocompromised mice (Papers I-IV). While colony assays measure multipotent and committed progenitors, LTC-IC assays detect the most primitive progenitors assessable *in vitro*. Ultimately, NSG transplantations enable the functional *in vivo* study of human hematopoiesis. The *in vivo* data generated in paper I indicate that low doses of SCF are superior to multiple cytokine stimulation in regard to sustaining the long-term repopulation ability of cultured cells. Conversely, the opposite was true for the potential to maintain *in vitro* self-renewal decisions as determined by the LTC-IC content. Similarly, an inverse correlation between LTC-IC and SRC activity was observed following addition of TPO to HSPCs maintained in low doses of SCF (Paper I). Such dissociation between SRC and LTC-IC has previously been observed in different experimental culture systems (de Wynter et al., 2001; Gan et al., 1997). The relative discrepancy between *in vitro* and *in vivo* read-out therefore reflects the capability of the different assays to support hematopoietic progenitors or stem cells, respectively. However, it may also highlight the remaining limitations with current human HSC assays, questioning how precisely these assays predict true human HSCs. The moderately poor translation of promising experimental findings into clinically relevant treatments further supports this notion.

Stem cell expansion

Identification of novel hematopoietic cytokines

The defining ability of stem cells to self-renew can be elegantly modeled in the mammalian hematopoietic system both *in vitro* and *in vivo*. However, when cultured without support from stroma cells, HSPCs can maintain an undifferentiated state only for a short time. Thus, defining the *in vitro* culture conditions which best mimic the natural HSC microenvironment would substantially impact current stem cell-based therapies. Initial growth factor-mediated expansion attempts have only moderately translated into beneficial clinical outcomes, partly due to the gradual decline of engraftment abilities in response to cell cycle entry following cytokine stimulation (Dahlberg et al., 2011; Glimm et al., 2000). Attempts to reinforce a quiescent state cannot bypass this engraftment defect (Glimm et al., 2000; Wiesmann et al., 2000). In fact, few cytokines are capable to maintain the primitive properties of transplantable cultured HSCs. In this context, it should be of value to revisit the potential of

individual growth factors in a systematic and unbiased manner with the aim to identify those that can maintain the functional integrity and repopulation capability of cultured human stem- and progenitor cells. To this end, we have performed a systematic survey of 276 recombinant soluble growth factors for their ability to augment human HSPCs after *ex vivo* culture. Given the high intrinsic proliferation and expansion potential of CB cells, we hypothesized that the power of each factor would be best distinguished in low SCF concentrations (Gammaitoni et al., 2004; Lansdorp et al., 1993; Ueda et al., 2001). In doing so we also attenuated presumable adverse effects of multiple cytokine stimulation on long-term HSC function (Huang et al., 2012). By applying this approach, we have identified CCL28 and MP as potent new HSC-supportive factors (Paper I and II). However, while several known growth factors, such as TPO and FLT3L, scored high in the screens using these restricted culture conditions, other well-known cytokines, e.g. IL6 did not. IL6 is a factor that mainly has been shown to support HSPCs in the context of multiple cytokine stimulation (Boitano et al., 2010; Conneally et al., 1997; Piacibello et al., 1999; Ueda et al., 2000). Identification of factors in that category will require a refinement of the screen and a more stringent readout of HSPC content. Our growth factor screen, and the entire arsenal of *in vitro* culture systems in general, was influenced by variables such as composition and purity of the starting cell population, cytokine concentrations and combinations, culture medium and culture time, and finally the choice of assay to analyze the cells (Figure 7). As the optimal interplay of these parameters varies, it should be pointed out that all growth factors in the library might not have been assessed in an optimal way, again exemplified by IL6 (Paper I).

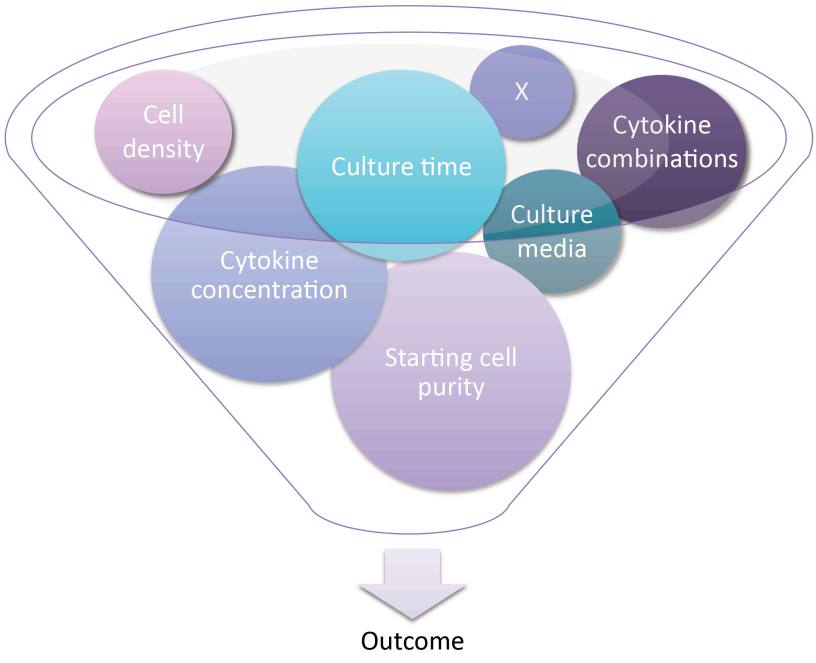

Figure 7. A complex interplay of variables determines the outcome.

In line with our findings of differential effects of low and high cytokine stimulation on the repopulation ability of cultured human HSPCs, several recent studies employing culture conditions similar to those used in paper I (i.e. STF) report reduced levels of HSC engraftment relative to starting cell numbers as a consequence of multiple cytokine stimulation (Boitano et al., 2010; Huang et al., 2012). Since we have not performed a direct comparison between stringent SCF combined with TPO or CCL28 vs. multiple cytokine stimulation, we cannot exclude that variables such as feedback inhibition from mature cells may contribute to these distinct outcomes (Csaszar et al., 2012). Nonetheless, restricted and preferably targeted cytokine support may provide means to specifically maintain the functional characteristics of cultured human HSCs. In fact, limited cytokine stimulation improved engraftment of *ex vivo* cultured rhesus $CD34^+$ cells (Takatoku et al., 2001). This notion is supported by the fact that culture of CB $CD34^+$ cells in SCF at 100 ng/mL is sufficient to maintain SRC activity in primary and secondary recipients to a similar extent as unstimulated input cells (Christine Karlsson and Jonas Larsson unpublished observation). However, given the functional heterogeneity within the HSPC population and the correlated distinctive cytokine requirements of different cells, it might be challenging to find one universal factor that sufficiently enhances both progenitor- and stem cell numbers *in vitro* (Petzer et al., 1996; Zandstra et al., 1997).

Intrinsic control

In addition to extrinsic regulation, identification and characterization of intrinsic cell fate determinants will contribute to a better understanding of the molecular mechanisms regulating HSC self-renewal and differentiation. Applying an RNAi-based screening approach (discussed in greater detail below), we have discovered three distinct HSC fate determinants of self-renewal, proliferation, and differentiation, as exemplified by shSTK38, shPLCZ1, and shEXT1, respectively (Paper III). While shPLCZ1 and shEXT1 do not qualify as potential candidates in *ex vivo* expansion protocols aiming to influence HSC numbers, shSTK38 increases the NOD/SCID repopulation activity of cultured cells. However, since quantification of HSC numbers using the NOD/SCID mouse model is technically challenging, precise conclusions about its expansion potential cannot be drawn. Using a refined screening strategy, we identified MAPK14/p38α as a regulator of HSPC proliferation and further showed that its pharmacological inhibition enhances functional stem- and progenitor cell activity *ex vivo* compared to controls grown in standard cytokine conditions (Paper IV). This approach elegantly illustrates the feasibility to target cell intrinsic factors that can be modified by small molecules or pharmacological intervention.

Prospects to HSC expansion

Development of optimal HSC expansion protocols will presumably involve a combination of the currently studied and yet-to-be-identified approaches to modulate self-renewal and survival of HSPCs in culture. Integration of cytokines, small molecules, and developmental factors with variables such as feedback inhibition and improved culture systems may provide a basis to achieve the amplification of transplantable HSCs in the future. Since the balance between proliferation, self-renewal, differentiation, and apoptosis determines the outcome, it will be crucial to fine-tune their interaction. Ultimately, the success or extent of *ex vivo* expansion may depend on the intrinsic biological properties of each individual CB unit.

A relevant question is whether we really need *ex vivo* stem cell expansion to achieve clinical benefit. For example, approaches to enhance the engraftment capabilities of the cells rather than to affect their numbers have shown promising results in preclinical settings and are currently being translated to the clinic (CD26 inhibition via Sitagliptin: NCT00862719 and NCT01720264; dmPGE2 treatment: NCT00890500). In addition, progenitor expansion with concomitant HSC maintenance may be sufficient to overcome the delayed hematopoietic recovery during the first weeks of transplantation.

Still, we believe that the ability to expand HSCs would have benefits for a number of clinical applications, such as gene therapy, and could promote CB as an off-the-shelf product tailored to meet the demands of the recipients. Provided that safe and effective expansion is achieved, CB units could be used for several patients and also be stored as back up in situations of inadequate donor engraftment.

Stem- or progenitor cell 'expansion' is sometimes 'casually' used as an increase over experimental controls (also by us in paper III). However, it should be noted that true HSC expansion refers to an increase over unstimulated input cell numbers. A vague nomenclature can sometimes overestimate the actual experimental finding.

High-throughput screening in HSPCs

In this thesis, we used the limited ability of HSPCs to sustain their immature properties under *ex vivo* culture conditions as a basis for high-throughput genetic and empirical screens. Applying this approach, we have identified novel modulators of human HSPCs (Paper I-IV). In contrast to the systematic growth factor screen, which represents a more direct approach to high-content screening, RNAi-based genetic approaches entail a higher inbuilt complexity. The success of such screens is largely dependent on the ability of shRNA vectors to infect sufficient numbers of target cells as well as the specificity and sensitivity of assays to read out an altered phenotype. HSPCs have clear advantages in both respects since they can be readily transduced in large numbers with lentiviral vectors, and further tested in quantitative and qualitative assays for stem and progenitor cell function (Woods et al., 2000).

We chose to assay mixtures of lentiviral-delivered shRNAs simultaneously in pools using a selection-based screening approach that filters cell populations according to their proliferation potential. Briefly, library-transduced cells were passaged in long-term liquid cultures and analyzed in CFC assays allowing positive selection of clones with sustained primitive potential. To retrospectively identify the silencing triggers responsible for the phenotype, the proviral insert was subcloned into bacteria followed by sequencing of a given number of clones per pool (Paper III). We considerably improved the feasibility of the screening approach by next generation sequencing in paper IV. Here, the distribution of shRNAs was determined directly after transduction and at the end of the culture period, estimating the relative abundance of shRNAs during culture. The functional validation of the screening results by reassessment of individual candidate shRNAs is discussed in a separate section below.

Based on the heterogeneity of $CD34^+$ cells, the library coverage in the most primitive populations such as LTC-ICs and SRCs was comparably low, possibly skewing the screening readout towards a progenitor cell phenotype (Paper III). This limitation has partly been addressed in paper IV by increasing the starting cell numbers. Although RNAi-screening in putative human HSCs (Notta et al., 2011) would be a desired and elegant approach, its practical realization is challenging given the immense cell numbers required to adequately cover the library (Aurélie Baudet and Jonas Larsson, unpublished observation).

Challenges in target validation

Once the initial screen is accomplished, the validation process begins. To functionally validate the selection strategy, individual candidate shRNAs are subcloned into the lentiviral vector and reintroduced into HSPCs. shRNAs showing a positive score in the validation experiment are further examined for their target specificity (Figure 8). This seemingly straightforward process harbors a number of challenges.

Validation of the screening strategy

One general consideration when introducing genetic material via integrating viral vectors is the possibility that functional outcomes are associated with insertional mutagenesis events (Woods et al., 2003). While the results from the human screens described in papers III and IV can consistently be repeated with individual shRNAs across several independent experiments, thus arguing against any involvement of lentviral integration effects, we obtained virtually different outcomes from a retroviral-based RNAi screen performed in murine LSK $CD150^+$ cells. Here, *in vitro*- and *in vivo* selection assays were used followed by functional readout for HSC activity in serial transplantations. Although we observed a substantial amount of dominant hits across 295 clones from 35 mice analyzed as a result of the primary screen, none of these scored positive in the following validation assays (Christine

Karlsson and Jonas Larsson, unpublished observation). These differences between the human and mouse screen may be attributed to the different viral vector systems used, with a higher reported oncogenic potential of retroviral vectors and increased risk of insertional mutagenesis, as well as different transformation requirements for normal human and mouse primary cells, respectively (De Palma et al., 2005; Montini et al., 2006; Rangarajan et al., 2004). Thus, in our hands, human HSPCs assayed *in vitro* represent a more robust cell source for selection-based forward genetic screens. Also, we nowadays only consider genes for which at least two individual shRNAs showed enrichment in the initial screen. Together with the development of knockdown-validated shRNA libraries (www.sigmaaldrich.com), the inclusion of these criteria has effectively increased the resolution of our current screening approach (Paper IV).
Furthermore, the *in vivo* selection of individual clones regardless of the perturbation mediated by the shRNA may potentially influence the screening outcome. In that case, the stochastic selection according to the clonal succession theory would render attempts to validate distinct screening results extremely difficult (Lemischka et al., 1986). Relying on our experience, we conclude that a robust screening system is crucial for experimental success or failure, with special emphasis on the necessity to perform replicate screens before putting time and effort into target validation (Paper IV).

Validation of the target identity

Upon deconvolution of the screening pools and confirmation of the screening results, the levels of the target gene transcripts are evaluated. Once sufficient suppression of target gene transcript levels is achieved, additional hairpins are tested to further confirm the gene specificity for the observed phenotype. shRNA redundancy, i.e. multiple shRNAs target the same gene and yield the same phenotype, should be demonstrated with at least two individual shRNAs (Echeverri et al., 2006).
The identity of an RNAi-induced phenotype is the sum of the shRNA-mediated on- and off-target effects. We and others have shown that shRNAs have a high tendency to generate false-positive hits (Paper III) (Echeverri and Perrimon, 2006). Silencing triggers that are in part complementary to an unintended mRNA can either lead to mRNA degradation of so-called off-targets, or act as miRNA and block translation of unintended transcripts. Moreover, siRNA can stabilize the genome and affect chromatin remodeling (Figure 8). Finally, transcript suppression and thus functional outcomes may differ in each cell depending on its differentiation stage, vector copy number and integration site, illustrating the challenge of performing knockdown studies in primary cells such as HSPCs.
The target gene specificity or non-specificity is exemplified by shEXT1 and shSTK38, respectively (Paper III). While we observed a clear correlation between knockdown and phenotype for two individual shEXT1 constructs, no such relationship was apparent in case of shSTK38. Considering the highly interesting phenotype mediated by shSTK38, we aimed at deciphering its molecular target. Gene expression profiling

identified forming binding protein 1-like (FNBP1L), a regulator of cytoskeleton dynamics (Ho et al., 2004; Takenawa and Suetsugu, 2007), as one of the two most down-regulated transcripts. However, functional *in vitro* and *in vivo* assays evaluating FNBP1L knockdown in CB-derived HSPCs did not recapitulate the shSTK38 phenotype, leaving the molecular target of shSTK38 undefined (Christine Karlsson and Jonas Larsson, unpublished data). These findings illustrate that RNAi-mediated off-target effects occur at the expense of RNAi specificity, and can substantially complicate the interpretation of RNAi-mediated phenotypes. Moreover, this example demonstrates some of the challenges accompanied with high-throughput screening, the labor-intensive follow-up work once a distinct biological event cannot be correlated to its intended target, and questions the overall convenience of pursuing off-target effects. The earlier mentioned inclusion criteria of at least two hairpins per gene and the availability of knockdown-validated shRNA libraries have considerably reduced the likelihood to come across off-targets during the screening process (Paper IV). However, knowing the target by which a drug conducts its effect is desirable, but not essential for the drug to be of value. Although it is of greatest interest to identify the molecular target of shSTK38, the hairpin itself, regarded as a chemical modifier, represents a rather unique tool to affect HSPCs activity while preserving their differentiation capability.

Ultimately, conclusions from RNAi experiments should be supported by data from non-RNAi approaches such as chemical modifiers (Paper IV) and/or rescue experiments. The latter involves the elegant yet technically demanding strategy to rescue the RNAi effect by expression of a 'siRNA-resistant' version of the gene of interest (Echeverri et al., 2006).

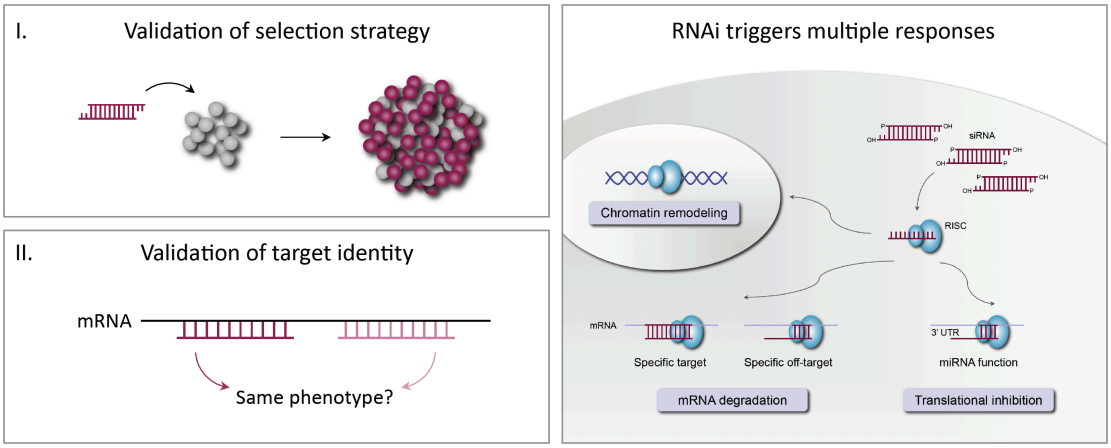

Figure 8. The validation of candidate shRNAs at various levels. (I) Validation of the selection strategy by individual assessment of shRNAs retrieved from the screen. (II) Validation of the target identity by shRNA redundancy. The identity of an RNAi-induced phenotype is the sum of the shRNA-mediated on- and off-target effects.

Future directions

Optimization of culture conditions for human HSPCs

Having established CCL28 and MP as growth factors for primitive human HSPCs, the following step will be their assessment to improve HSC *ex vivo* culture conditions aiming at stem cell expansion. This should be conducted in a systematic manner in the context of other growth factors and recently discovered molecules for HSC expansion (Boitano et al., 2010; Buehler et al., 2012; Delaney et al., 2010a; Himburg et al., 2010; Zhang et al., 2008). Moreover, it will be interesting to evaluate p38 inhibitors, combined with different HSC-supporting molecules, for their potential further clinical implementation. In parallel, establishment of good manufacturing practice (GMP)-approved expansion methods will be essential.

The functional quantification of HSC numbers in relation to fresh unstimulated controls will be important. Incorporated into this approach should be the direct comparison of multiple vs. restricted cytokine stimulation. As mentioned earlier, Zandstra and colleagues have identified parameters that limit the cytokine-mediated expansion of primitive cells (Madlambayan et al., 2005; Zandstra et al., 1997). In that respect, frequent cytokine supplementation is feasible, whereas the implementation of automated closed culture systems controlling global feedback mechanisms would be desired, yet may be difficult to accomplish (Csaszar et al., 2012).

With the growth factor library at hand, it would be interesting to modify our experimental settings to (i) evaluate the effects of each individual cytokine without the support of baseline SCF levels; and (ii) adjust the cytokine screen to a, currently regarded, more clinically relevant setting with the commonly used growth factor combination 'STF'. Furthermore, adjustment of the culture period, from short cytokine pulses to exposure times of days to weeks, may substantially influence the screening outcome.

Improving the applicability of shSTK38 poses an interesting but challenging avenue for future studies. Revised attempts to identify the molecular target(s) of shSTK38 may shed lights onto its identity. Since off-target effects have been attributed to partial or complete complementarity between the siRNA seed region (nucleotides 2-8 at the 5' end of the siRNA duplex) and the 3'UTR sequence of unintended transcripts, computerized seed region analysis may be useful to determine the shSTK38 target(s) (Nakayama et al., 2003). Applying Sylamer analysis to the existing gene expression data, it may be possible to systematically determine which seed sequences are enriched in shSTK38 (van Dongen et al., 2008). Briefly, this method enables the identification of miRNA or siRNA off-targets from gene expression data, based on the overrepresentation of certain nucleotides in a ranked list, similarly to gene set enrichment analysis (GSEA) (Subramanian et al., 2005; van Dongen et al., 2008). In the best-case scenario, that seed region might match an existing miRNA (Eugen Buehler, personal communication). Given that the seed region of any given siRNA

will match with a large number of genes, it will be substantially more difficult to identify one distinct off-target.

Furthermore, the expansion potential of shSTK38 should be revisited in a quantitative fashion by limiting dilution assays in NSG mice, preferably in combination with different baselines of cytokine stimulation. Although the actual shSTK38 target(s) remain elusive, the hairpin itself may be used to modify HSPCs *ex vivo* in a more clinically relevant setting. However, considering the potential transformation risk accompanied with lentiviral transduction methods, it will be essential to assess the feasibility of non-viral shRNA delivery in this regard, for example nucleofection.

MP may present an exciting new opportunity to induce HSPC proliferation via modification of molecules within or outside the canonical TGF-ß signaling pathway. To clarify whether canonical signaling is activated, studies evaluating the Smad2/3 response upon MP, myostatin, and TGF-ß are currently underway in our laboratory. Given the absence of myostatin expression on HSPCs, MP action via modification of TGF-ß family ligands other than myostatin seems more likely than myostatin suppression itself. It will be of considerable interest to identify these potential novel MP interaction partners.

The role of CCL28 in hematopoiesis

CCL28 is mainly known for its role in mucosal immunity, guiding the homing of lymphocytes to their sites of action (Pan et al., 2000). Several directions could be taken to extend this knowledge to the hematopoietic system. With regards to the expression of CCL28 in the BM microenvironment (Nakayama et al., 2003) (Paper I), a compelling approach for future studies is the potential role of CCL28 in regulating HSPCs *in vivo* as a niche-secreted factor. Using a murine model of sublethal irradiation, it would be interesting to see whether CCL28 secretion of niche components, e.g. osteoblasts, is increased in response to injury, according to the underlying hypothesis that damaged BM cells release CCL28 as a chemoattractant, thereby recruiting HSCs to injured sites (Porter et al., 2013). In line with that, *in vivo* modification of the HSPC pool via CCL28 administration would be of significant value and may answer the question whether the observed *in vitro* survival effect of CCL28 can be translated to the *in vivo* setting (Paper I). A more technically appealing approach would be the genetic modification of existing murine stromal cell lines to overexpress CCL28 and the subsequent evaluation whether such a modification can alter the *in vitro* self-renewal potential of HSPCs as measured by LTC-IC assays (Sutherland et al., 1991). Ultimately, the most elegant strategy to determine the physiological expression and relevance of CCL28 would be the generation of CCL28-GFP knock-in mice, and its conditional deletion from different subsets of candidate niche cells and HSCs, respectively (Ding and Morrison, 2013; Ding et al., 2012).

A direct connection between hypoxia and CCL28 secretion has recently been established in a model of human ovarian cancer (Facciabene et al., 2011). It is

tempting to speculate whether such a correlation would also apply to our experimental setting. In that regard, CCL28 secretion of BM-derived MSCs should be tested in different oxic and hypoxic conditions, while the level of reactive oxygen species (ROS) should be examined after culture of HSPCs with or without CCL28.

Finally, the molecular mechanism of CCL28 action needs to be elucidated. Although our data indicate that CCL28 may operate through CCR10 on early hematopoietic progenitors, CCR10 knockdown and subsequent CCL28 stimulation may provide a rationale to proof this assumption. CCL28 treatment of $CCR10^{+}$ and $CCR10^{-}$ HSPCs could also address this question. It will be of considerable interest to test whether CCL28 can facilitate chemotaxis of hematopoietic progenitors, and whether it elicits a similar intracellular response as other known chemokine/chemokine receptor interactions (Youn et al., 2000).

SAMMANFATTNING PÅ SVENSKA

All blodbildning utgår från blodbildande, s.k. hematopoetiska stamceller (HSC) i benmärgen, som har förmågan att dels skapa blodets alla mogna celler men också möjligheten att dela sig i två identiska nya stamceller. Processen där HSC skapar en eller två exakta kopior av sig själv kallas *självförnyelse*. Celldelning som leder till att stamcellens egenskaper ej bibehålls anses som *differentiering* och ger upphov till mogna blodceller. Tillsammans innebär dessa egenskaper att stamcellerna, utan att förbrukas, kan förse kroppen med ca 10^{12} nya blodceller varje dag genom hela livet. Bland dessa finns röda blodceller som transporterar syre, vita blodkroppar som garanterar vårt immunförsvar och blodplättar som hejdar blödning. Självförnyelse och differentiering regleras noggrant och kräver ett samspel mellan signaler och tillväxtfaktorer från den omgivande benmärgen och molekyler i stamcellerna själva för att inte livshotande sjukdomar, t.ex. leukemi skall utvecklas. Trots att HSC är de mest studerade av alla typer av stamceller så är vår kunskap om mekanismerna bakom sådan reglering begränsad. Därför har man än idag inte lyckats att odla HSC utanför sin kroppsliga miljö.

Transplantation av stamceller är en etablerad behandlingsmetod vid leukemi och andra blodsjukdomar. Stamceller kan utvinnas antingen ur benmärg eller perifert blod från en donator, men också från navelsträngsblodet. Navelsträngsblod är en restprodukt från födseln, och därmed en lättåtkomlig källa för blodstamceller. HSC från navelsträngsblodet har med framgång använts för behandling av barn, men innehåller inte ett tillräckligt stort antal stamceller för att möjliggöra rutinmässig transplantation av vuxna patienter. Målet i vår forskargrupp är att etablera metoder för att i cellkultur öka antalet navelsträngs-HSC till sådana nivåer att de skall kunna användas till behandling av alla patienter.

Den här avhandlingens specifika syfte var att testa tusentals biologiska signaler för deras förmåga att öka antalet omogna celler i kultur genom genetiska och empiriska screens. För att uppnå detta mål använde vi först en systematisk screeningmetod där vi testat närmare 300 tillväxtfaktorer för deras förmåga att styra HSC tillväxt. Stamcellernas funktion prövades sedan med olika metoder, t.ex. genom att transplantera dem till bestrålade möss. Dessa representerar ett experimentellt system för att mäta de förändrade stamcellernas förmåga att bistå långtidsblodbildning. På det här sättet lyckades vi upptäcka flera nya faktorer som kan öka antalet omogna celler i kultur. En av dem, chemokine ligand 28 (CCL28) har visat sig bevara flera av stamcellernas funktionella egenskaper och motverka den annars oundvikliga utmognaden som sker när stamceller odlas i laboratoriemiljö *(Arbete I)*. Ytterligare studier krävs för att fastställa CCL28's specifika roll i blodbildning och dennas användbarhet för att etablera effektivare cell- och genterapi av blod- och immunsjukdomar.

I samband med tillväxtfaktorscreeningen upptäckte vi även att myostatin propeptide (MP) är ett potent verktyg för att inducera tillväxt hos stamceller från navelsträngsblodet *(Arbete II)*. MP är en naturlig antagonist av myostatin, som i sin tur är ett protein tillhörande den stora familjen av TGF-ß signaleringsmolekyler som tidigare visats vara viktiga för normal stamcellsfunktion. Vi håller just nu på att undersöka exakt hur MP framkallar tillväxt hos stamcellerna.

För att dessutom upptäcka HSC-reglerande molekyler inuti cellen, har vi använt så kallade virusbibliotek, som kan infektera stamceller och därefter hämma uttrycket av tusentals gener samtidigt *(Arbete III och IV)*. Genom denna genetiska screeningstrategin har vi lyckats identifiera ett flertal molekyler som påverkade stamcellernas tillväxt, bl.a. MAPK14 vars farmakologiska hämning avsevärt ökade HSC funktion.

Sammanfattningsvis har vi undersökt olika strategier för att identifiera nya tillväxtfaktorer för stamceller från navelstängsblodet, och har på så sätt upptäckt nya signaler som styr HSC tillväxt.

ZUSAMMENFASSUNG AUF DEUTSCH

Stammzellen sind weitgehend unspezialisierte Zellen, die nach ihrer Herkunft und ihrem Differenzierungspotential in embryonale und adulte Stammzellen unterteilt werden. Im Gegensatz zu den *totipotenten* embryonalen „Alleskönnern", die die Fähigkeit haben, sämtliche Gewebezellen eines neuen Organismus hervorzubringen, sind adulte Stammzellen in ihrem Differenzierungsvermögen beschränkt. Diese *multipotenten*, gewebespezifischen Stammzellen findet man beispielsweise im Knochenmark, im Gehirn, Herz, Leber und in der Haut, wo sie während der gesamten Lebensdauer des Organismus Nachschub an allen Körperzellen der jeweiligen Gewebeart gewährleisten. Bei der Blutbildung, die auch *Hämatopoese* (griechisch; haima = Blut und poïesis = „zu machen") genannt wird, entwickeln sich aus Blutstammzellen in mehreren Teilungsschritten die verschiedenen Blutzellen, die neben Blutplasma den Grundbestandteil unseres Blutes ausmachen. Da reife Blutzellen nur eine begrenzte Lebensdauer von wenigen Tagen bis zu mehreren Monaten haben, und pro Sekunde ca. 2 Millionen von ihnen verbraucht werden, müssen diese ständig von so genannten *hämatopoetischen Stammzellen* (HSZ) im Knochenmark neu gebildet werden. Diese zeichnen sich durch zwei Grundeigenschaften aus: während der Zellteilung können sie einerseits durch *Selbsterneuerung* ein oder zwei identische Kopien ihrer selbst erstellen und sichern somit das Fortbestehen des HSZ Reservoirs; andererseits können sie sich durch *Differenzierung* in jede beliebige Zelle des hämatopoetischen Systems entwickeln und bedingen so die Produktion aller korpuskulären Bestandteile des Blutes. Veranschaulicht wird dies durch ihr Vermögen, täglich mehrere Milliarden neue Blutzellen im Knochenmark bilden zu können; unter ihnen rote Blutkörperchen (Erythrozyten), die Sauerstoff transportieren, weiße Blutkörperchen (Leukozyten), die als Teil der Immunabwehr Infektionen bekämpfen, und Blutplättchen (Thrombozyten), die eine unerlässliche Rolle bei der Blutgerinnung spielen. Die Aufrechterhaltung dieses dynamischen Systems erfordert das geregelte Zusammenspiel von Signalen im umliegenden Knochenmark mit Wachstumsfaktoren, Zell-Zell Kontakten und intrinsischen Molekülen der Blutstammzellen selbst, um das Auftreten von lebensbedrohenden Krankheiten wie z.B. Leukämie zu verhindern. Da die zugrundeliegenden molekularen Mechanismen dieser Regulierung nur bedingt bekannt sind, ist es trotz intensiver Forschung bislang noch nicht möglich, Stammzellen außerhalb ihres natürlichen Milieus zu expandieren.

Blutstammzelltransplantation, kombiniert mit Chemo- und/oder Strahlentherapie, ist eine etablierte Behandlungsmethode bei bestimmten Krebserkrankungen wie beispielsweise Leukämie. Blutstammzellen können aus dem Knochenmark und peripherem Blut eines Spenders gewonnen werden, jedoch ebenso aus Nabelschnurblut. Dies ist ein „Abfallprodukt" der Geburt und stellt eine leicht

zugängliche Quelle von Blutstammzellen dar. Seit Beginn der 90er Jahre werden Nabelschnurbluttransplantationen zunehmend in der Behandlung von Kindern eingesetzt. Ihre routinemäßige Anwendung bei der Transplantation erwachsener Patienten ist jedoch aufgrund der vergleichsweise geringen Zahl der Stammzellen, die in einer Nabelschnurblutkonserve enthalten sind, beschränkt. Ziel unserer Forschung ist es, Blutstammzellen zu ausreichenden Mengen im Labor zu vermehren, um somit ihre therapeutische Verwendungsmöglichkeit zu erweitern. Wichtige Ansatzpunkte hierbei sind beispielsweise die Identifizierung neuer Wachstumsfaktoren oder die Manipulation zell-intrinsischer Moleküle.

In meiner Dissertation haben wir eine Vielzahl biologischer Signale in empirischen und genetischen Screens auf ihre Fähigkeit getestet, die Anzahl unspezialisierter Stammzellen in Kultur zu erhöhen. In *Arbeit I* haben wir hierzu systematisch das Potential hunderter Moleküle untersucht, das Stammzellwachstum zu erhöhen, ohne die Zellen zu differenzieren. Der direkte Effekt verschiedener Wachstumsfaktoren auf HSZ wurde durch Transplantation der kultivierten Stammzellen in bestrahlten Mäusen untersucht. Diese dienen als experimentelles Modellsystem, um die Funktionalität der manipulierten Stammzellen zu untersuchen. Mithilfe dieses Ansatzes haben wir mehrere neue Faktoren entdeckt, welche die Anzahl primitiver Zellen in Kultur steigern. Einer dieser Faktoren ist das Zytokin *chemokine-ligand 28* (CCL28), welches der ansonsten unvermeidlichen kultur-bedingten Reifung durch Erhaltung wesentlicher funktioneller Stammzelleigenschaften entgegenwirkte. Weitere Studien sind erforderlich, um CCL28's Eignung zur Etablierung effektiverer Therapien hämatologischer Erkrankungen zu bestimmen.

Im Zusammenhang mit dem Wachstumsfaktorscreening konnten wir zudem in *Arbeit II* nachweisen, dass *myostatin propeptide* (MP) als Werkzeug zur Wachstumsinduktion primitiver Stamm- und Vorläuferzellen dienen kann. MP ist ein natürlich vorkommender Antagonist von Myostatin, welches wiederum als Mitglied der TGF-ß Familie essentiell für normale Stammzellfunktion ist. Momentan untersuchen wir die zugrundeliegenden Mechanismen dieser Wachstumsförderung.

Im Gegensatz zur obigen Strategie verwendeten wir in *Arbeit III* und *IV* sogenannte Virusbibliotheken, welche das Vermögen haben, die Funktion tausender Gene zu unterdrücken. Ziel war dementsprechend durch Hemmung bestimmter Gene das Zellwachstum zu stimulieren. Nabelschnurblutstammzellen wurden mit diesen Virusbibliotheken behandelt und anschließend kultiviert, worauf die Selbsterneuerungsfähigkeit der manipulierten Stammzellen in murinen Transplantationsmodellen untersucht wurde. Mithilfe dieser Methode haben diverse wachstumsfördernde Faktoren identifiziert, beispielsweise *MAPK14*/*p38α*, deren pharmakologische Hemmung die Stammzellfunktion verbesserte.

Zusammenfassend haben wir verschiedene Strategien untersucht, um neue Wachstumsfaktoren für Nabelschnurblutstammzellen zu entdecken. Wir kommen zu dem Schluss, dass sowohl empirische als auch genetische Methoden geeignete Werkzeuge zur Identifizierung neuer Wachstumssignale von HSZ darstellen.

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APPENDICES (ARTICLES I-IV)