Maintenance and expansion of hematopoietic stem cells in vitro using genetic methods

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**NANYANG
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UNIVERSITY**

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in vitro using genetic methods**

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School of Biological Sciences

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A thesis submitted to Nanyang Technological University

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A Thesis for the Degree of Doctor of Philosophy at Nanyang Technological
University

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Abstract

Chromosomal translocations encoding fusion proteins between N-terminal nucleoporin 98 (NUP98) and some members of the homeodomain protein superfamily have been observed recurrently in certain types of leukemia. Subsequent studies have shown that the ectopic expression of these leukemogenic or similar fusion genes in primitive bone marrow (BM) cells can induce hematopoietic stem cell (HSC) activity in these cells. We have extensively surveyed different branches of the homeodomain superfamily as partners to NUP98 in order to find fusion genes that could sustain HSC activity in BM cells in long-term continually expanding *in vitro* cultures. Remarkably, we found that CDX1, extended HOX subfamily member, when fused to NUP98, was able to sustain balanced HSC activity with high frequencies in massively expanding cultures as measured by robust long-term competitive *in vivo* transplantation assays. Interestingly, closely related NUP98-CDX4 induced leukemia with the shortest latency ever reported for NUP98-Homeodomain fusions unaided by MEIS1 transcription factor. Since HSC and leukemic stem cells (LSC) share similar self-renewal mechanisms, these pre-leukemic stem cells generated by the first genetic hits can provide useful models for elucidating the self-renewal mechanisms of HSC as well as LSC.

We also found that NUP98-HOXA10 was able to reprogram B220⁺ cells in the bone marrow and conferred them long-term reconstituting multipotentiality *in vivo*. Transcriptional profiling of NUP98-HOXA10 transduced BM cells revealed that the aryl hydrocarbon receptor (AhR) pathway was the most suppressed pathway. Inhibition of this pathway using small molecule AhR inhibitor in combination with cytokine cocktail

allowed 30-100 fold expansion of HSC *in vitro*. This provides an additional component to the elusive “magic” cocktail that allows maximal expansion of HSC *ex vivo*.

Abbreviation

5-FU	5-Fluorouracil
AGM	Aorta-gonad-mesonephros
AhR	Aryl hydrocarbon receptor
AML	Acute myeloid leukemia
BFU-E	Burst forming unit-erythrocyte
BM	Bone marrow
BMP	Bone morphogenetic protein
CB	Cord blood
CDKI	Cyclin-dependent kinase inhibitor
CFC-GEMM	Colony forming cell-granulocyte/erythrocyte/ megakaryocyte/macrophage
CFU-GM	Colony forming unit-granulocyte/macrophage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRU	Competitive repopulating unit
CXCL12	CXC chemokine ligand 12
CXCR4	CXC chemokine receptor 4
DEG	Differentially expressed genes
DOX	Doxycycline
ED	Embryonic day
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GMP	Granulocyte/Macrophage progenitor
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IL-6	Interleukin 6
IMDM	Iscoe's modified Dulbecco's medium

IRES	Internal ribosome entry site
LPS	Lipopolysaccharide
LSC	Leukemic stem cell
LTC-IC	Long-term culture-initiating cells
LT-HSC	Long term-hematopoietic stem cell
LTR	Long terminal repeat
MEIS1	Myeloid ecotropic viral integration site 1 homolog
MEP	Megakaryocyte/Erythrocyte progenitor
MPL	Myeloproliferative leukemia
MPP	Multipotent progenitor
MSCV	Murine stem cell virus
PBX1	pre-B-cell leukemia transcription factor 1
PREP1	PBX-regulating protein-1
PTEN	Phosphatase and tensin homologue
SCF	Stem cell factor
SDF-1	Stromal derived factor 1
STAT	Signal transducer and activator of transcription
ST-HSC	Short term-hematopoietic stem cell
TALE	Three amino acid loop extension
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TPO	Thrombopoietin
TRE	Tetracycline response element

Chapter 1 Introduction

1.1 Hematopoietic system and Hematopoietic stem cell

1.1.1 Hematopoietic hierarchy

Mammalian hematopoietic systems are extremely dynamic in almost every aspect. It is estimated that a daily replacement of more than 2×10^8 mature blood cells (mainly non-lymphoid cells) occurs in an adult mice (Cheshier, Morrison et al. 1999), and this number increases to 3×10^{11} in humans (Gordon, Lewis et al. 2002). While this high turn-over rate allows adequate replacement of short-lived cells and provides a swift and efficient defense system to combat infections, it also places a high divisional demand on blood precursors, which, if uncontrolled, would increase the likelihood of divisional mutations, a situation that would sabotage the advantages of having a dynamic blood system. Evolution has solved this problem by creating a hierarchical system characterized by differential cellular amplification abilities and longevities, with differentiated cells that are either short-lived or with limited divisional abilities at one end, and hematopoietic stem cells (HSC) that are capable of continuous self-renewal at the other end (Figure 1).

HSCs are multi-potent adult stem cells that at the clonal level can give rise to all lineages of the hematopoietic system by differentiation while maintaining their own numbers by self-renewal (Kondo, Wagers et al. 2003), two cardinal features that functionally define them and place them at the top of the hematopoietic hierarchy. As HSCs differentiate, they adopt sequentially new stable cellular states with characteristic changes in transcriptomes (Ivanova, Dimos et al. 2002; Forsberg, Prohaska et al. 2005; Kiel, Yilmaz et al. 2005; Kent, Copley et al. 2009), which are responsible for the associated diminishing self-

renewal ability, as well as the replacement of one stable set of cell surface markers by another. This process is so stereotyped that we can identify these different intermediate cellular states as short-term HSC (ST-HSC), multipotent progenitor (MPP), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP) and megakaryocyte/erythrocyte progenitor (MEP) (Figure 1) by using different combinations of markers and functional assays (Table 1). Accordingly, the most rigorous way to test the functions of HSC is to examine its ability to regenerate all these populations and their progenies *in vivo* for long term, an assay called long-term reconstitution.

Figure 1 Hematopoietic hierarchy

Hematopoietic stem cells are the most primitive cells in the hematopoietic system, which give rise to common lymphoid progenitors and common myeloid progenitors. Common lymphoid progenitors then differentiate into B cell, T cell, NK-cell, and dendritic cells. Common myeloid progenitors generate granulocyte/macrophage progenitors that differentiate into granulocytes and macrophages, and megakaryocyte/erythrocyte progenitor that differentiate into megakaryocyte and erythrocyte. Common myeloid progenitors can also differentiate into dendritic cells. LT-HSC, long-term hematopoietic stem cells; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor. Adapted from (Luis and Staal 2009; Söderberg, Karlsson et al. 2009).

Table 1 Phenotypes of different murine hematopoietic progenitors

Cell types	Phenotypes	References
LT-HSC	Lin ⁻ Sca ⁺ c-kit ⁺ Thy1 ^{lo} CD34 ⁻ CD150 ⁺ CD48 ⁻ EPCR ⁺	(Morrison and Weissman 1994; Osawa, Hanada et al. 1996; Kiel, Yilmaz et al. 2005; Balazs, Fabian et al. 2006; Kent, Copley et al. 2009)
ST-HSC	Lin ⁻ Sca ⁺ c-kit ⁺ CD34 ⁺ Flt3 ⁻ Thy1 ^{lo}	(Christensen and Weissman 2001; Yang, Bryder et al. 2005)
MPP	Lin ⁻ Sca ⁺ c-kit ⁺ Thy1 ⁻ Flt3 ⁺	(Adolfsson, Borge et al. 2001; Christensen and Weissman 2001)
CLP	Lin ⁻ Sca ^{lo} c-kit ^{lo} IL7R ⁺ Thy1 ⁻	(Kondo, Weissman et al. 1997)
CMP	Lin ⁻ Sca ⁻ c-kit ⁺ CD34 ⁺ FcγR ^{lo}	(Akashi, Traver et al. 2000)
GMP	Lin ⁻ Sca ⁻ c-kit ⁺ CD34 ⁻ FcγR ^{lo}	(Akashi, Traver et al. 2000)
MEP	Lin ⁻ Sca ⁻ c-kit ⁺ CD34 ⁺ FcγR ^{hi}	(Akashi, Traver et al. 2000)

1.1.2 HSC development and behavior

The first cohort of assayable HSCs appear at embryonic day 9 (ED9) at the aorta-gonad-mesonephros (AGM) region of the developing embryo, possibly via hemangioblast/hemagenic endothelium (Huber, Kouskoff et al. 2004; Lancrin, Sroczynska et al. 2009) intermediate stages. These cells then colonize the fetal liver (FL), where they undergo massive expansion before they seed the fetal bone marrow beginning at ED15 (Godin and Cumano 2002; Mikkola and Orkin 2006; Cumano and Godin 2007). After birth, HSCs still retain the active cell cycle status of the fetal state for a few weeks before they cease active division (Bowie, McKnight et al. 2006; Bowie, Kent et al. 2007). In adult mice, only 25% of HSCs are in active cycle at any moment (Cheshier, Morrison et al. 1999); these dividing HSCs can either self-renew, differentiate, or undergo apoptosis. The

rest of the 75% HSCs withdraw from the cell cycle and remain in a dormant G₀ phase until they are summoned, e.g., during hematopoietic stress or in response to physiological demands. In this sense, the HSC pool in adults serves as a hematopoietic rheostat that regulates the homeostasis of the hematopoietic system.

Hock et.al. in an elegant study used histone 2B-GFP fusion protein as a marker for cell division and demonstrated that HSCs with more extensive divisional history were impaired in their reconstituting abilities (Foudi, Hochedlinger et al. 2009), suggesting the need of placing a strict control over the divisional behavior of HSCs. Indeed, breach of these controls can be catastrophic, as seen in genetic mouse model with pancytopenia resulting in the exhaustion of HSC pool (Yilmaz, Valdez et al. 2006), and in acute myeloid leukemia (AML) with excessive production of nonfunctional myeloid cells from LSC.

Under steady-state, a small number of HSCs can leave the bone marrow and enter the periphery (a process called mobilization), or from the periphery return back to bone marrow (a process called homing) (Wright, Wagers et al. 2001; Bhattacharya, Czechowicz et al. 2009). The physiological reasons for such nomadic behavior are currently unclear. However, recent data showing that HSCs are responsive to toll like receptor stimuli (Nagai, Garrett et al. 2006; Massberg, Schaerli et al. 2007; Takizawa, Regoes et al. 2011) and type I interferon (Sato, Onai et al. 2009) suggest that there are physiological needs for such behavior. Nevertheless, the migratory behavior of HSCs has been extensively utilized in clinical settings. Various molecules have been used to encourage the egress of HSCs from the bone marrow, as a means of harvesting them from the peripheral blood for clinical purposes (Ross and Li 2009). On the other hand, the ability of HSCs to home to

their niches in the bone marrow is precisely why hematopoietic stem cell transplantation (HSCT) would ever work.

1.1.3 HSC and HSCT

As discussed above, the functional and behavioral properties of HSCs underlie the principles of bone marrow and cord blood transplantations (HSCT), whereby the diseased blood is replaced by progenies of HSCs from healthy donors. In fact, so far HSCT is the only successful adult stem cell therapy available, saving tens of thousands of lives every year (Sauvageau and Humphries 2010). The success of HSCT depends to a great extent on the number of HSCs available in the grafts (Wagner, Barker et al. 2002). However, in adult bone marrow, HSCs represent a rare population, present at a frequency of only 1 in 10,000 cells in the murine bone marrow (Kiel, Yilmaz et al. 2005); and human bone marrow is estimated to have even less frequent HSCs (Gordon, Lewis et al. 2002). Thus, the scarcity of HSCs is the major limiting factor for their clinical applications.

While *in vivo* HSC can expand as much as 8000 fold (Pawliuk, Eaves et al. 1996; Iscove and Nawa 1997; Benveniste, Cantin et al. 2003), under *in vitro* conditions, they undergo differentiation-biased divisions and gradually lose their stem cell identity. Therefore, the ability to expand robustly HSCs *ex vivo* would certainly benefit HSCT, as well as extend their usage beyond current clinical applications. These prospects have inspired a flurry of focused efforts over the past two decades to expand HSCs *ex vivo*. All these efforts are centered on steering HSC towards self-renewal instead of differentiation or cell death, by either manipulating extrinsic environmental factors or directly interfering with the transcriptome through gain or loss of function genetic methods. Therefore, a clear

understanding of the various extrinsic and intrinsic regulators of HSCs, as well as their interactions, is prerequisite for safe, relevant, *ex vivo* expansion of HSCs.

1.2 Extrinsic regulators of HSC and related methods for expansion

1.2.1 Extrinsic regulators of HSC

HSCs reside in the microenvironments of the bone marrow that consist of stromal cells (Mendez-Ferrer, Michurina et al. 2010), endothelial cells (Butler, Nolan et al. 2010), osteoclasts (Kollet, Dar et al. 2006), osteoblasts (Sugiyama, Kohara et al. 2006; Chan, Chen et al. 2009; Omatsu, Sugiyama et al. 2010), inorganic ions like Ca^{2+} (Adams, Chabner et al. 2006) and differentiated hematopoietic lineages like macrophages (Winkler, Sims et al. 2010; Chow, Lucas et al. 2011), which are collectively called the niche of HSCs. Extensive contacts between niche components and HSCs have been documented, the majority of which retain HSCs in their niche and maintain them in a quiescent state, e.g., interactions between Angiopoietin-1/2 and Tie-2 (Arai, Hirao et al. 2004), thrombopoietin (TPO) and myeloproliferative leukemia (MPL) proto-oncogene (Yoshihara, Arai et al. 2007), stem cell factor (SCF) and c-kit (Barker 1994; Bradfute, Graubert et al. 2005), CXC chemokine ligand 12 (CXCL12, also known as stromal derived factor-1, SDF-1) and its receptor CXC chemokine receptor 4 (CXCR4) (Sugiyama, Kohara et al. 2006), WNTs and WNT receptors (Fleming, Janzen et al. 2008; Nemeth, Mak et al. 2009), the controversial transforming growth factor β (TGF- β) and TGF- β receptors (Keller, McNiece et al. 1990; Hatzfeld, Li et al. 1991; Soma, Yu et al. 1996; Larsson, Blank et al. 2005; Yamazaki, Iwama et al. 2009; Challen, Boles et al. 2010), as well as various other cytokine and cytokine receptor pairs. The existence of multiple signaling pathways to ensure a dormant state of HSCs highlights the importance of doing

so. Indeed, awakening HSC at the wrong time or wrong place inadvertently causes exhaustion of the pool, as discussed in section 1.3. It seems that HSCs are exceptionally sensitive to differentiation signals, a property that may stem from the specific epigenome of HSCs, where many lineage-specific genes are epigenetically active or even expressed (Miyamoto, Iwasaki et al. 2002; Attema, Papathanasiou et al. 2007; Chung, Kim et al. 2009). However, under certain circumstances, e.g., hematopoietic stress mimicked by phosphamide/granulocyte-colony stimulating factor or 5-fluorouracil (5-FU) treatment, HSCs can re-enter cell cycle to meet the demands of the stressed hosts. It is believed that niche signals mediate this awakening process, but the specific initiating signals that stimulate division and the “safe-guard” signals that ensure their functional integrity are presently unknown; consequently, it is unclear how HSCs can be stimulated to divide safely *ex vivo*, i.e., without losing their HSC identity.

1.2.2 HSC expansion by manipulating extrinsic factors

As discussed above, HSCs do not exist in isolation. It has long been known that the cellular and molecular components in the bone marrow are important regulators of HSC homeostasis. Without a clear knowledge about these components, early attempts to maintain or expand HSCs *in vitro* made use of stromal cell lines that provided a “niche mimic”, largely in the form of stromal feeder layers (Dexter, Allen et al. 1977). Indeed, the first study demonstrating the feasibility of expanding long-term reconstituting HSC *in vitro* made use of bone marrow stromal cells as feeder layer (Fraser, Eaves et al. 1990; Fraser, Szilvassy et al. 1992); after 4-week culture on the feeder layer, certain clones from a retrovirally marked HSC pool were selectively expanded but still retain their *in vivo* long-term reconstituting ability. Although the roles of retrovirus in this study were largely

neglected and there was a net 6.5-fold decrease in total competitive repopulating units (CRU, *in vivo* readout for HSC), the fact that specific clones were selectively amplified nevertheless demonstrated that *bona fide* HSCs were expandable *ex vivo*.

Although stromal culture systems can maintain HSCs, sometimes over an extended period of time, they seldom enabled a net expansion of total HSCs and most often led to an expansion of more mature cell types (Table 2). Moreover, the undefined nature of stromal layers makes further improvement difficult. Subsequent studies shifted to more defined conditions with prescribed concentrations of different cytokines. Representative studies are also summarized in Table 2.

Table 2 Summary of representative studies that expand HSCs *in vitro* using stromal cells and cytokine cocktails

Culture system	Starting cells	Cytokine cocktails	Fold of expansion/reduction	References
Stromal layer	5-FU adult murine BM	Absent	6.5 fold reduction after 4 weeks	(Fraser, Szilvassy et al. 1992)
	Unenriched murine BM	Absent	No expansion after 3 weeks	(Wineman, Moore et al. 1996)
	Thy-1 ^{lo} Sca-1 ⁺ H-2K ^{hi} sorted from 5-FU treated murine BM	Leukemia inhibitory factor	2-3 fold reduction after 2 weeks	(Szilvassy, Weller et al. 1996)
	Primary human BM	Absent	6 fold reduction in frequency after 1 week	(Gan, Murdoch et al. 1997)
	Murine fetal liver AA4.1 ⁺ LSK and murine adult LSK BM	Absent	No expansion after 4-6 weeks	(Moore, Ema et al. 1997)
Classical cytokines	Murine Sca ⁺ Lin ⁻ BM	Serum-free, IL-11(100ng/ml), Flt3L(100ng/ml), SCF(50ng/ml)	3.5 fold after 9 to 10 days culture	(Miller and Eaves 1997)
	Human CD34 ⁺ CD38 ⁻ cord blood cells	Serum-free, SCF (300ng/ml), Flt3L (300ng/ml), G-	4 fold after 4 days culture, lost after 9 days culture	(Bhatia, Bonnet et al. 1997)

		CSF(50ng/ml), IL-3 (10ng/ml), IL-6 (10ng/ml)		
	Human CD34 ⁺ CD38 ⁻ CB cells	Serum free, human IL-3 (20ng/ml), human IL-6 (20ng/ml), human G-CSF (100ng/ml), human Flt3L (100ng/ml), human SCF (100ng/ml)	2 fold after 5-8 days culture	(Conneally, Cashman et al. 1997)
	Human CD34 ⁺ cord blood cells	Human TPO (10ng/ml), SCF (100ng/ml), IL-6 (100ng/ml), sIL-6R(1ug/ml), Flt3L (100ng/ml)	4.2 fold after 1 week culture	(Ueda, Tsuji et al. 2000)
	Murine Sca ⁺ Lin ⁻ BM	SCF (50ng/ml), Flt3L(100ng/ml), H-IL-6(40-100ng/ml, higher concentration is inhibitory) or IL-11(100ng/ml), or IL-6(20ng/ml) and sIL-6R(400ng/ml)	4 fold after 10 days culture	(Audet, Miller et al. 2001)
Non-classical cytokines	Unfractionated murine BMC	Serum free, FGF-1 (10ng/ml)	24 fold expansion (assessed at 2 month endpoint) after 4 week culture	(de Haan, Weersing et al. 2003)

	Murine fetal liver LSK cells and murine BMC side population cells	10% serum, SCF (50ng/ml), TPO(100ng/ml), <u>IGF-2</u> (500ng/ml)	2-3 fold expansion after 3 day culture	(Zhang and Lodish 2004)
	Murine bone marrow SP CD45 ⁺ Sca-1 ⁺	Serum free, SCF (10ng/ml), TPO (20ng/ml), <u>IGF-2</u> (20ng/ml), human <u>FGF-1</u> (10ng/ml), <u>Angptl2</u> (100ng/ml) or <u>Angptl3</u> (100ng/ml)	24-30 fold expansion after 10 days culture	(Zhang, Kaba et al. 2006)
	Human CB CD133 ⁺ cells	Serum free, Mouse SCF(10ng/ml), mouse TPO (20ng/ml), mouse <u>IGF-2</u> (20ng/ml), human <u>IGF-1</u> (10ng/ml), human <u>IGFBP2</u> (100ng/ml), human Angptl5(500ng/ml)	14-20 fold expansion after 10 days culture	(Zhang, Kaba et al. 2008)
	Human CB CD34 ⁺ CD90 ⁺ cells	30% serum, SCF(100ng/ml), Flt3L(100ng/ml), megakaryocyte growth and development factor (MGDF, 100ng/ml), IL-	9.6 fold expansion after 9 days culture	(Araki, Mahmud et al. 2006)

		3(50ng/ml), 5azaD (10 ⁻⁶ M), TSA (5ng/ml)		
	Human CB CD133 ⁺ cells	SCF(100ng/ml), TPO(10ng/ml), Flt3L(100ng/ml), IL- 6/sIL-6R (100ng/ml), IL-3(10ng/ml), immobilized delta1-Fc (10ug/ml)	5.8 fold after 3 week expansion	(Suzuki, Yokoyama et al. 2006)
	Murine CD34 ⁻ LSK Human CB Lin ⁻ CD34 ⁺ CD38 ⁻ cells	10% FBS, SCF(125ng/ml), Flt3L(50ng/ml), TPO (20ng/ml), Pleiotrophin (100ng/ml)	25 fold expansion for murine HSC; Increase engrafting frequency of human cells (not quantified)	(Himburg, Muramoto et al. 2010)

Studies which targeted late progenitor populations like colony-forming unit, long-term culture initiating cell were neglected from the summary. Highlighted molecules in the Non-classical cytokine section represent new additions to classical cytokine cocktails.

It is clear from these studies that significant expansion of HSCs requires the integration of multiple cytokine signals. However, most cytokines have pleiotropic effects on both primitive and more mature cells, very often with poorly understood mechanisms. This, together with the combinatorial nature of the cocktails, makes it difficult to predict or interpret the effects of a specific cocktail. For example, under certain circumstances, even the concentrations of the cytokines used can be the determining factor. This was exemplified by the gp130 family of cytokines—it was found that higher concentration of H-IL-6 (a hyperactive form of IL-6 which contains covalently linked IL-6 and soluble IL-

6 receptor) was inhibitory for HSCs expansion (Audet, Miller et al. 2001); in humanized mice constitutively expressing human IL-3, GM-CSF and SCF, the transplanted human primitive cell population was also compromised (Nicolini, Cashman et al. 2003). Moreover, with so many different cocktails having been tested, the magnitude of expansion achieved so far is still very limited. Thus, a “magic” cocktail that enables maximal expansion of HSCs has yet to be discovered. Systematic high-throughput methods that are capable of exhausting all the different combinations and “dosing” would be a great help to this end. Nonetheless, overall it seems that current cytokine combinations provide more of permissive signals rather than self-renewal signals.

Undoubtedly, a better understanding of niche-HSC interactions will help improve current culture conditions. The best candidate in this respect is the fetal liver. Fetal HSC and neonatal HSCs as described above are actively cycling and self-renewing, a cellular state that is very likely enforced by signals from their niche. Thus identification of these niche signals may suggest methods to evoke the self-renewal program. Studies by Lodish group (Zhang, Kaba et al. 2008) have illustrated well this rational. They first identified the CD3⁺Ter119⁻ population as the niche component that supports fetal HSC expansion; through transcriptional profiling, they then found that IGFBP2 and Anptl2/3 were highly expressed in this population, hence they devised a cocktail containing SCF, TPO, IGFBP2, FGF-1, Angptl5, which can expand human scid repopulating cells (SRC)--a surrogate readout for human HSCs in animals--as many as 20 folds, the highest achievable expansion with cytokine cocktails so far.

It is conceivable that continuous optimization will generate clinically relevant amount of expanded HSCs; however, this depends very much on our knowledge about the regulatory

networks that regulate HSC homeostasis, as well as the response of HSC transcriptomes to extracellular stimuli. Ironically, although much knowledge has been gained regarding the identities of different extracellular components, the molecular mechanisms that underlie their effects are, just like classical cytokines, largely obscure.

One class of niche components that have better defined signal transduction mediators are a class of developmental genes that specify the dorsal-ventral axis of the developing embryo. These include the sonic hedgehog, WNTs, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs). Signaling pathways elicited by these proteins as a result of engagement with their cognate receptors have been repeatedly used from ontogeny to adulthood (Dzierzak and Speck 2008). During development, they are involved in the specification of dorsal-ventral mesoderm, and may also initiate hematopoietic fates from ventral mesoderm (Sive 1993; Winnier, Blessing et al. 1995; Marshall, Kinnon et al. 2000). In adult hematopoietic system, pathway components for these factors are also expressed in the primitive hematopoietic compartment (Bhatia, Bonnet et al. 1999; Bhardwaj, Murdoch et al. 2001; Duncan, Rattis et al. 2005) and reinforcement of these signals can either influence or determine HSC fates. Overexpression of beta-catenin (Reya, Duncan et al. 2003), for example, has been shown to cause the expansion of HSCs; overexpression of the intracellular domain of Notch can create immortalized primitive cell lines (Varnum-Finney, Xu et al. 2000; Stier, Cheng et al. 2002), whereas overexpression of its downstream target HES1 can expand HSCs *in vivo* (Kunisato, Chiba et al. 2003). While these experiments demonstrated the potential of manipulating these pathways, e.g., by adding specific ligands, it is complicated by the fact that effects of these pathways can be influenced by many factors like the concentrations (they are morphogens by definition),

modes of presentation, presence of pathway signaling components, presence of other morphogens, developmental stages of the target cells, etc., making a clear assertion of their effects on HSC expansion difficult to make, as manifested by conflicting results in the literature (Malhotra and Kincade 2009).

Manipulations of *in vitro* HSCs cultures by optimizing different extrinsic factors have the advantages that they leave the genome intact, and that systematic high-throughput optimization, guided by mechanistic knowledge, can be conducted once the identities of the components are known. In this respect, they still hold the potential as the most ideal way of HSCs expansion, even though genetic modifications allows for much higher magnitude of expansion. However, the relatively “gentle” touch of the HSC self-renewal program by these methods, as reflected by the modest expansions reported so far, suggest that they are sub-optimal as the starting material for dissecting self-renewal mechanism. In contrast, gain-of-function or loss-of-function genetic modifications provide more powerful platforms for mechanistic studies.

1.3 Intrinsic regulators of HSC and related methods for HSC expansion

Apart from niche signals, HSCs themselves also house a plethora of intrinsic factors that maintain the balance between self-renewal and differentiation. These include cell cycle regulators, signal transducers, metabolic sensors and transcription factors, among others.

1.3.1 Cell cycle regulators

As mentioned above, although HSCs have to intermittently enter the cell cycle to replenish the loss of mature blood cells every day, under steady-state, most of them are

quiescent. This quiescent state is achieved by the integration of niche signals and reinforced by various cell cycle regulators, mainly cyclin-dependent kinase inhibitors (CDKI). There are two major classes of CDKIs. One is the INK4 family consisting of $p16^{\text{INK4a}}$, $p15^{\text{INK4b}}$, $p18^{\text{INK4c}}$ and $p19^{\text{INK4d/Arf}}$ that inhibit CDK4/6; the other is the CIP/KIP family consisting of $p21^{\text{Cip1/Waf1}}$, $p27^{\text{Kip1}}$ and $p57^{\text{Kip2}}$ that inhibit CDK2 (Sherr and Roberts 1999). The first evidence that CDKIs play important roles in reinforcing a quiescent state came from the study of $p21^{-/-}$ mice, where the authors showed that the mutant HSCs exhibited accelerated cell division and increased HSCs pool size; however, upon serial transplantation, they were more rapidly exhausted (Cheng, Rodrigues et al. 2000). Loss of $p27$ somehow showed a milder phenotype, with only increased pool of progenitors, both under steady-state and stressed conditions (Cheng, Rodrigues et al. 2000). In contrast to $p21$ and $p27$, $p18^{\text{INK4c}}$ knockout mice showed increase in both the number and self-renewal ability of HSCs, even under stressed situation of serial transplantation (Yuan, Shen et al. 2004). Self-renewal involves two intimately linked processes, cell division and decision making. These somehow conflicting results of CDKI knockouts suggest that different CDKIs are influencing different aspects of these processes. Indeed, a model proposed by Scadden and colleagues posits that early G_1 phase is critical in HSC decision-making because this is where pro-differentiation signals exert their effects; $p18^{\text{INK4c}}$ inhibits cyclin D-CDK4/6 during this phase, therefore $p18^{-/-}$ HSCs transit faster through early G_1 phase, and are thus less susceptible to differentiation signals; whereas $p21$ acts during late G_1 phase after the restriction (R) point and therefore knockout mice are still susceptible to differentiating signals (Orford and Scadden 2008). Although elegantly

simple, further studies are certainly needed to support this model, especially when additional checkpoints during G₁ phase have been discovered (Foster, Yellen et al. 2011).

1.3.2 Signal transducers

Signal transducers provide links between extrinsic stimuli and intrinsic regulators. Gain/loss of functions of these transducers is interpreted by the cells as presence of specific stimuli. Moreover, many transducers are situated at the crossroad of different pathways; therefore their dysregulation can sometimes cause dramatic effects.

Lnk is a negative regulator of both c-kit signaling (Takaki, Morita et al. 2002) and TPO-MPL/Jak2 complex signaling (Bersenev, Wu et al. 2008). Loss of Lnk in mouse expanded long-term HSCs and improved their functional qualities (Ema, Sudo et al. 2005), thus providing one of the molecular explanations for TPO regulated quiescence. Interestingly, HSCs in Lnk^{-/-} mice started to increase exponentially at around 4 weeks of age, a time coincident with the diminishing expression of the deterministic transcription factor SOX17 in neonatal HSCs (Kim, Saunders et al. 2007), as well as the cease of active cell division (Bowie, McKnight et al. 2006; Bowie, Kent et al. 2007), suggesting a potential link between TPO signaling and fetal-to-adult HSC transition.

Interestingly, another negative regulator of the TPO/MPL signaling pathway, cellular-casitas B-cell lymphoma (c-Cbl), when deleted, can also cause hyper-proliferation of HSCs without compromising the *in vivo* reconstituting abilities in the long term (Rathinam, Thien et al. 2008). C-Cbl is an E3 ubiquitin ligase (Pickart 2001) and is expressed in all primitive bone marrow cells, with the highest expression in LT-HSCs. Its deletion is thought to cause hypersensitivity of cells to TPO stimulation via signal transducer and

activator of transcription 5 (STAT5) hyper-phosphorylation. Another member of the E3 ligase family Itch has also been demonstrated as a positive regulator of HSC quiescence. Itch shares with c-Cbl similar expression pattern and knockout phenotypes. However, the effects of Itch deletion were mediated, at least in part, via reduced Notch degradation (Rathinam, Matesic et al. 2011).

Members of STAT family, as their names suggest, transform exogenous cytokine stimuli into transcriptional changes. This is particularly relevant for HSCs, given that they are exposed to a multitude of cytokines in their microenvironment. However, due to the difference in upstream activators, dysregulation of different STATs can give different phenotypes. For example, constitutively active STAT5 overexpression in LT-HSC, but not ST-HSC, promotes uncontrolled self-renewal of HSCs *in vivo*, eventually leading to fatal myeloproliferative disease (Kato, Iwama et al. 2005). STAT3, in contrast, only enhances regeneration of HSCs under the stress condition of transplantation, in a well-controlled way (Chung, Park et al. 2006).

1.3.3 Metabolic sensors

Another important but largely neglected aspect of HSC maintenance is the regulation of HSC metabolism. To remain quiescent, HSCs have to maintain a delicate balance between cellular energetics and cell growth. Disruption of this fine balance can be detrimental to their functional integrity and even cause cell death. This is best illustrated in three recent papers (Gan, Hu et al. 2010; Gurumurthy, Xie et al. 2010; Nakada, Saunders et al. 2010), where the authors knocked out *Lkb1* and found that HSCs exited quiescence, followed by a metabolic catastrophe characterized by mitochondrial DNA degradation, mitochondrial

membrane potential and mass loss, and decrease of ATP levels, leading to quick apoptosis. The brief phase of enhanced cell division specifically in HSCs suggest that Lkb1 may represent another cell cycle checkpoint at the G1 phase that coordinates cell division and metabolism, ensuring that cells divide only when they are ready to do so. The reasons for the specific effect of Lkb1 on HSCs are unknown, but it is possible that HSCs are more sensitive to metabolic disturbance, given that they spend a large portion of their energy expenditure to keep themselves quiescent. Mechanically, the effects of Lkb1 are independent of its known downstream targets AMPK and mammalian target of rapamycin (mTOR), a central player in metabolic sensing.

mTOR is intricately linked to another interesting molecule that has gained more and more attention in both stem cell biology and cancer biology -- the tumor suppressor gene phosphatase and tensin homologue (PTEN). PTEN functions as a lipid phosphatase that dephosphorylates PIP3 to PIP2, thus inhibiting the PI3K, which is the major activating input of the mTOR pathway, a pathway that is frequently dysregulated in malignancies (Cully, You et al. 2006). Conditional knockout of PTEN in adult mice recruited HSCs into cell cycle, eventually leading to exhaustion of HSCs, both under steady-state condition and in a transplantation model. Moreover, these mice went on to develop transplantable leukemia within 4-6 weeks (Yilmaz, Valdez et al. 2006), probably via a mechanism distinct from its roles in HSC self-renewal. Remarkably, treatment of knockout mice with rapamycin, inhibitor of the mTOR pathway, can alleviate the exhaustion of HSCs and cure the acute leukemia. This differential ability makes PTEN an ideal target for intervention in leukemia (Rossi and Weissman 2006).

1.3.4 Transcription factors

A cell's identity is ultimately determined by a specific pool of transcription factors that establish a stable gene regulatory network, which in turn dictates the behaviors of the cell. Many transcription factors have been identified that regulate HSC fates under different conditions and these include "general" transcription factors that also function outside hematopoietic system, as well as more "restricted" transcription factors that are mainly acting within the hematopoietic system. One gene that is important for adult stem cell quiescence regulation is a member of the polycomb group genes Bmi-1. Bmi-1 is part of the polycomb repressive complex 1 that are important epigenetic regulators (Kerppola 2009). Overexpression of Bmi-1 enhanced self-renewal of HSCs (Iwama, Oguro et al. 2004; Rizo, Dontje et al. 2008), whereas deletion of Bmi-1 caused defects in HSC self-renewal (Park, Qian et al. 2003), possibly via up-regulation of p16^{INK4a} that leads to senescence and p19^{ARF} that leads to apoptosis (Park, Qian et al. 2003; Park, Morrison et al. 2004). Through a screening for Bmi-1-deficiency phenotype suppressors, Morrison and colleagues identified the PR-domain-containing 16 (PRDM16)--a fusion partner of RUNX1 in certain acute myeloid leukemia (AML) and is highly expressed in primitive bone marrow cells (HSC and MPP)--as a gene required for HSC and neural stem cell maintenance. Loss of PRDM16 led to neonatal death of mice and 20-fold decrease in ED14.5 fetal liver stem cells. In contrast to neural stem cells, defects in HSCs was independent of PRDM16's role in reactive oxygen species regulation, demonstrating a tissue-specific role of PRDM16 in adult stem cell regulation (Chuikov, Levi et al. 2010).

Interestingly, a recent paper also suggested a haploinsufficient effect of PRDM16 (Aguilo, Avagyan et al. 2011).

The “guardian of the genome” p53 has important roles in maintaining the quiescent state of HSCs. Knockout of p53 increased the frequency of actively cycling HSCs by two fold; although these HSCs eventually caused lymphoma when transplanted alone, they slightly improved chimerism in a competitive setting. P53 is also critical for the maintenance of HSC quiescence in MEF^{-/-} mice (Liu, Elf et al. 2009). MEF is a member of the ETS family of transcription factors and is one of the few negative regulators of HSC quiescence. Its absence increased the frequency as well as the number of quiescent HSCs by 3-4 fold (Lacorazza, Yamada et al. 2006). Effects of p53 seem to be mediated by its downstream targets p21 and Gfi-1. Gfi-1 is a zinc-finger transcription repressor that was originally identified as a collaborating gene of *c-Myc* and *Pim-1* induced lymphoma and found to be in the same complementation group as *Bmi-1* in lymphomagenesis (Scheijen, Jonkers et al. 1997). Although when overexpressed Gfi-1 can act as a dominant oncogene in lymphoma, in HSCs it restricts proliferation and maintains the functional integrity of HSCs, partly through up-regulation of the CDKI p21^{Cip1/Waf1} (Hock, Hamblen et al. 2004; Zeng, Yucel et al. 2004). P21 seems to be a common target for genes that regulates HSC quiescence. For instance, the oncoprotein c-Myc regulates HSC quiescence through p21; overexpression of c-Myc caused loss of self-renewal activity, whereas knockout of c-Myc increases HSCs quiescence and subsequent block in differentiation, eventually leading to the loss of competitiveness in transplantation (Wilson, Murphy et al. 2004; Baena, Ortiz et al. 2007).

Another subset of transcription factors are important for the specification of mesoderm into hematopoietic fate during embryonic development. Examples of these include Runx1, Scl/Tal1, PU.1, Gata-2 and Etv6. In adult HSCs, these genes are still expressed and may play important roles in defining their identities as well as determining cell fate choices. RUNX1 (or its partner CBF- β) null embryos, for instance, died at ED12.5 due to lack of definitive hematopoiesis in fetal liver; however, yolk sac primitive hematopoiesis is not affected (Okuda, van Deursen et al. 1996); moreover, the effect of RUNX1 is haploinsufficient (Cai, de Bruijn et al. 2000). In adult, RUNX1 seems to be dispensable for HSCs survival, as *RUNX1* mutant showed a 3-fold increase in LSK population (Gowney, Shigematsu et al. 2005), possibly due to up-regulation of Bmi-1 (Motoda, Osato et al. 2007). Paradoxical to its role in embryonic hematopoiesis, these mice develop mild myeloproliferation, a phenotype that can be exacerbated to leukemia with secondary mutations (Motoda, Osato et al. 2007; Jacob, Osato et al. 2010). In contrast, stem cell leukemia (SCL), which is critical for embryonic blood cell specification (Robb, Lyons et al. 1995), is dispensable for adult long-term HSCs (Mikkola, Klintman et al. 2003; Curtis, Hall et al. 2004); dramatic cell death of progenitors occurs only when its paralogue Lyl-1 is also deleted (Souroullas, Salmon et al. 2009).

1.3.5 HSC expansion by manipulating intrinsic factors

In principle, positive regulators of quiescence can be knocked down and negative regulators of quiescence can be overexpressed, to enhance HSC self-renewal. However, self-renewal behavior is a complicated process that involves the coordination of extrinsic

signals, cell cycle progression, cellular energetics and stem cell maintenance programs; many intrinsic factors are directly or indirectly involved in all these processes. Furthermore, there are almost no intrinsic factors that are uniquely expressed in HSCs and not in more differentiated progenitors or fully differentiated cells, making the effects of intrinsic factor manipulations very often unpredictable. Thus, knock-down of p18 and overexpression of Bmi-1 caused expansion of HSCs, whereas overexpression of c-Myc is inhibitory to self-renewal, although c-Myc has been involved as downstream targets of many genes capable of expanding HSCs. Furthermore, in none of the above-mentioned successful example of HSCs expansion, has rigorous *in vitro* HSC expansion been demonstrated.

Exceptions to this are transcription factors of the homeodomain protein family that hold great potential for *in vitro* HSC expansion.

1.4 Homeodomain-containing proteins

With minor exceptions, homeodomains are 60 amino acids long DNA-binding helix-loop-helix domains encoded by a 180 base-pair long DNA sequence called homeobox; they are present in all members of the homeodomain-containing protein superfamily (Gehring, Affolter et al. 1994; Banerjee-Basu and Baxevanis 2001). Homeodomains are so conserved that they allow a consensus sequence to be derived from a few hundreds of sequences; indeed, the homeodomains are the only unifying feature within this superfamily, which can be broadly categorized into two superclasses: dispersed (non-

clustered) and complex (clustered) superclasses. Outside the homeodomain, there are also features that are shared among different subclasses, as depicted in Figure 2.

Figure 2 Structural features of homeodomain protein subclasses

With the exception of Abd-B-like HOX genes, members of the complex (clustered), *ems*, *cad*, *TCL* share a hexapeptide IYPWMK located N-terminal to homeodomains. Engrailed class contains 4 highly conserved protein segments (EH) outside the homeodomains. Paired class contains a second DNA-binding domain of 128 amino acids called paired domain. POU class have a second 80 amino acid POU-specific DNA binding domain. Cut class is characterized by 3 copies of 80 amino acid cut-repeats upstream of homeodomain. LIM class contains a repeated motif of 60 amino acids called LIM motif that contains conserved cysteine and histidine residues. ZF class contains variable numbers of zinc fingers and homeodomains. NK-2 class contains a conserved peptide (CP) downstream of homeodomain. Adapted from (Burglin 2005).

1.4.1 Non-clustered homeodomain proteins

Non-clustered homeodomain proteins are a large collection encoded by genes dispersed across the genome. Representative subclasses and members are summarized in Table 3.

Table 3 Subclasses of Non-clustered homeodomain proteins

Sub-classes	Examples	Sub-classes	Examples
Caudal (Cad)	CDX1,CDX2, CDX4	Zinc-finger (zf)	ZFHX2-4,
Distaless (Dll)	DLX1-6	Paired (prd)	PAX5, OTX1/2, PITX1/2/3
NK-like	DBX1, DBX2, NANOG, LBX, HLX	POU	OCT1-4
Engrailed (En)	EN1, EN2	Cut	SATB1/2
TALE	MEIS1-3, PBX1-4, PREP1/2	LIM	LHX1-9

Many members from this superclass are intimately involved in the regulation of hematopoietic system homeostasis. For example, PAX5 is a critical transcription factor that specify B cell fates; RAE28 is part of the polycomb group repressing complex 1 and is important for sustaining the activity of HSCs (Ohta, Sawada et al. 2002; Kim, Sawada et al. 2004); CDX2 is overexpressed in most cases of AMLs (Scholl, Bansal et al. 2007); overexpression of CDX4 can cause leukemia in cooperation with MEIS1A (Bansal, Scholl et al. 2006); MEIS1 and PBX, in turn, act as co-factors for members of the HOX subclass, as discussed below.

1.4.2 Clustered homeodomain proteins (*HOX*)

The clustered homeodomain proteins, also called *HOX*, were first identified in *Drosophila* mutants where one body part was homeotically transformed into another body part. Genes responsible for these transformations in *Drosophila* form two clusters *ANTENAPEDIA* and *BITHORAX* that are present on one chromosome. It was later found that similar clusters of *HOX* genes are also present in mammals (Table 3), except that there are four clusters located on four different chromosomes. These four clusters are numbered from A to D. *HOX* genes within each cluster are somehow related, with interior genes (paralog group 6 and 7) deviate least from the consensus sequence, and dissimilarity increases gradually from interior genes to anterior and posterior genes. This allows the partition of 39 *HOX* genes present in mammals into 13 paralogous groups numbered from 1-13. Each cluster contains 9-11 paralogous genes; genes within each paralogous group are more similar to one another than to other paralogous groups, suggesting the different clusters are generated via series of duplication events during evolution (Burglin 2005).

HOX genes are first expressed in mesoderm during early gastrulation and are master regulators of anterior-posterior patterning of animals. Specific combination of active *HOX* genes in a particular body segment gives that segment a positional value so that cells within that segment can acquire a fate related to its position (Lewis Wolpert 2002). During development, *HOX* genes are expressed in a temporal and anterior-posterior spatial order that reflects their locations from 3' to 5' along the chromosomes, two properties referred to as temporal and spatial co-linearity, respectively.

Table 4 *Drosophila HOX* genes and their mammalian homologs

Drosophila	Labial	Proboscipedia		Deformed	Sex combs	Antennapedia		Abdominal-B
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	(Lab)	(pb)		(dfd)	reduced(scr)			(Abd-B)
Mammals	HOX1	HOX2	HOX3	HOX4	HOX5	HOX6, HOX7	HOX8	HOX9, 10, 11, 12, 13

In adulthood, *HOX* genes have distinct expression patterns in the hematopoietic system. In more primitive bone marrow cells, only *HOXA*, *B* and *C* cluster members are expressed, with *HOXA* members overrepresented compared to *HOXB* or *HOXC* members; moreover, 3' *HOX* genes are expressed in more primitive cells and down-regulated upon differentiation, whereas 5' *HOX* genes are more equally expressed in different hematopoietic subsets, roughly reflecting their co-linearity during development (Moretti, Simmons et al. 1994; Sauvageau, Lansdorp et al. 1994; Taghon, Thys et al. 2003; Abramovich, Pineault et al. 2005). Interestingly, consistent with *HOX* genes' roles in defining positional values, the expression patterns of *HOX* genes also change during migration and differentiation of hematopoietic cells (van Oostveen, Bijl et al. 1999; Taghon, Thys et al. 2003).

HOX genes have been known to cross-regulate one another and the combination of *HOX* genes in a particular cell type establishes a complicated and robust network. It is so robust that knockout of certain component *HOX* genes often fails to reveal any significant phenotypes, or at most mild phenotypes, in hematopoiesis. For example, knockout of *HOXB4* alone or *HOXB4* plus *HOXB3*, which are expressed in primitive BM cells but down-regulated upon differentiation, showed a slight defect (<2 folds) in HSCs only after serial transplantation, although HSC from both knockout mice displayed a proliferation defect (Björnsson, Larsson et al. 2003; Brun, Björnsson et al. 2004). Strikingly, when up

to 30% of *HOX* genes expressed in fetal liver HSCs are knocked out (*HOXB1* to *HOXB9*), the HSCs still retain competitive reconstitution ability and are largely normal (Bijl, Thompson et al. 2006). Moreover, in that particular study, expression of *HOXA* and *HOXC* genes was deregulated, suggesting that there is an extremely complicated interaction network with a high degree of redundancy among different *HOX* members. More pronounced phenotype has so far been reported only for *HOXA9*, which showed defects in lymphoid, myeloid and erythroid lineages, although the effects on HSCs have not yet been carefully examined (Lawrence, Helgason et al. 1997; Izon, Rozenfeld et al. 1998).

Whereas loss-of-function studies are often obscured by the redundancy of different *HOX* genes, overexpression studies can usually exaggerate the role of particular *HOX* genes in the putative *HOX* network and display overt phenotypes. The first *HOX* gene that was shown to cause leukemia when overexpressed is *HOXB8* (Perkins, Kongsuwan et al. 1990; Perkins and Cory 1993); co-expression of growth factor IL-3 drastically shortened the latency period from 12 month to 19 days, although some of the effects may be associated with IL-3 alone, which caused overproduction of granulocytes (Chang, Metcalf et al. 1989). Overexpression of other *HOX* genes, like *HOXB3*, *HOXB7*, *HOXB6*, *HOXA9* and *HOXA10*, all cause uncontrolled expansion of primitive cells that eventually leads to myeloid leukemia, albeit with long latencies as summarized in Table 5. The long latencies of leukemia caused by *HOX* genes overexpression suggest the requirement of secondary co-operating events. This is illustrated in the leukemia caused by *HOXB6* and *HOXA9*, where a nonrandom deletion of bands 2D-2E of chromosome 2 was recurrently observed (Zimonjic, Pollock et al. 2000; Fischbach, Rozenfeld et al. 2005). Inspection of this

chromosome region reveals genes that are important for hematopoietic system development, e.g., PU.1, among many others. Moreover, co-overexpression of the three amino acid loop extension (TALE) family of homeodomain proteins MEIS1A almost always shortens the latencies for leukemia development, whereas expression of MEIS1A alone does not result in any hematopoietic anomaly (Kroon, Kros et al. 1998; Thorsteinsdottir, Kroon et al. 2001; Fischbach, Rozenfeld et al. 2005), suggesting MEIS1A is, at least, one of the “second hit” components.

The TALE homeodomain proteins consist of *MEIS1A* (myeloid ecotropic viral integration site 1 homolog), *PBX1* (pre-B-cell leukemia transcription factor 1) and *PREP1* (PBX-regulating protein-1) (Chang, Shen et al. 1995; Shen, Rozenfeld et al. 1997). MEIS proteins interact with N-termini of 5' HOX proteins HOX9-HOX13, whereas PBX proteins interact with 3' HOX proteins HOX1 to HOX10 at a position N-terminal to the homeodomains. Therefore, only HOXA9 and HOXA10 are able to interact directly with both MEIS1 and PBX. However, over-expression studies have shown that 3' HOX proteins HOXB3, HOXB4, HOXB6, although lacking the MEIS1 interacting domain, are still able to cooperate with MEIS1 to induce aggressive leukemia, possibly via indirect interaction mediated by PBX, together forming a trimeric complex (Shen, Rozenfeld et al. 1999). Nonetheless, this could be one but definitely not the only mechanisms for MEIS1 cooperation, since as discussed in section 1.4.3, fusions of nucleoporin 98 (NUP98) to homeodomains, which lacked both PBX and MEIS1 interacting domains, are also able to cooperate with MEIS1. It is worth noting that although MEIS1 generally accelerate HOX-induced leukemogenesis, the latencies to leukemia development still vary, determined possibly by the intrinsic properties of individual HOX proteins.

Table 5 Phenotypes of HOX genes over-expression

Genes	Starting cells	Phenotypes	References
HOXB3	5-FU bone marrow, 4 days in culture	1.Transplantable progressive dominance of myeloid lineages, defective $\alpha\beta$ T cell development in thymus while $\gamma\delta$ T cell expanded, blocked B cell development, CRU not changed (reduced) 2.AML with a latency of 276 ± 64 days; cooperation with meis1 105 ± 14 days	(Sauvageau, Thorsteinsdottir et al. 1997; Thorsteinsdottir, Kroon et al. 2001)
HOXA5	Human CD34 ⁺ or CD34 ⁺ CD38 ⁻ CB and BM cells	<i>In vitro</i> experiment showing shift from erythropoiesis to myelopoiesis	(Crooks, Fuller et al. 1999)
HOXB6	5-FU BM cells	AML with latency of 223 ± 19 days; with MEIS1 within 5 weeks	(Fischbach, Rozenfeld et al. 2005)
HOXC4	Human peripheral blood CD34 ⁺ cells	Enhanced BFU-E and CFU-GM, as well as LTC-IC (13.1 fold)	(Daga, Podesta et al. 2000)
HOXA9	5-FU BM cells	Develop AML with long latency (185 ± 17 days); with MEIS1 54 ± 9 days	(Kroon, Krosi et al. 1998; Thorsteinsdottir, Kroon et al. 2001)
HOXB7	Human PBL CD34 ⁺ cells	Enhance myeloid progenitor growth, increase LTC-IC frequency	(Care, Valtieri et al. 1999)
HOXA10	5-FU BM cells	Increased megakaryocytic and blastic colonies <i>in vitro</i> ; block macrophage and B-cell development; caused AML from 19 week to 50 week	(Thorsteinsdottir, Sauvageau et al. 1997)
HOXB8	5-FU BM cells	4 out of 18 mice developed leukemia (both myeloid and erythroid) in 12 months;	(Perkins, Kongsuwan et al. 1990; Perkins and

		when IL-3 is expressed, aggressive leukemia developed with mean survival time of 19 days	Cory 1993)
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One exception to the “norm” of HOX-leukemogenesis is HOXB4 (Table 6). In a series of pioneering studies, Humphries and colleagues showed that overexpression of HOXB4 caused dramatic expansion of murine CRU in both primary and secondary recipients without exceeding the physiological limits (Sauvageau, Thorsteinsdottir et al. 1995; Thorsteinsdottir, Sauvageau et al. 1999). After two weeks in culture, HOXB4 was able to expand murine HSCs 40-fold before extensive *in vivo* expansion (Antonchuk, Sauvageau et al. 2002); interestingly, no leukemia was detected even after long period of observation. The success of HOXB4 led to a series of follow-up studies and surprisingly, HOXB4 could promote the maturation of yolk sac and embryonic stem cell (ESC)-derived hematopoietic cells and confer them the ability of long-term multi-lineage reconstitution (Kyba, Perlingeiro et al. 2002). However, the effects of HOXB4 on human HSCs seem to be more modest, reaching around 3-4 fold (Buske, Feuring-Buske et al. 2002; Amsellem, Pflumio et al. 2003; Kros, Austin et al. 2003). Moreover, in transplantation models using large animals, HOXB4 was also causally linked to leukemogenesis (Zhang, Beard et al. 2008; Thakar, Zhang et al. 2009).

Table 6 Summary of HOXB4 over-expression studies

Starting Cells	Delivery methods	Phenotypes	References
Murine 5-FU BM cells	MSCV retrovirus	900-fold expansion of CRU in both primary (20weeks) and secondary (16weeks) recipients	(Sauvageau, Thorsteinsdottir et al. 1995)
Murine 5-FU BM cells	MSCV retrovirus	HOXB4 transduced HSCs expanded to but not exceeding normal level over one year, suggesting HOXB4 is the limiting factor in normal serial bone marrow transplantation	(Thorsteinsdottir, Sauvageau et al. 1999)
Murine CCE and EFC-1 embryonic stem cells (ESC)	MSCV retrovirus	HOXB4 transduced ESC increases definitive erythroid (CFC-E) and myeloid progenitors (CFC-GEMM) output when induced to differentiate into hematopoietic lineages.	(Helgason, Sauvageau et al. 1996)
Murine 5-FU BM cells	MSCV retrovirus	HOXB4 transduced BMC were competitively advantageous and led to faster and higher recovery of HSC compartment (2 weeks reached 25%, normal after 12 weeks)	(Antonchuk, Sauvageau et al. 2001)
Human Lin ⁻ CB cells	MSCV retrovirus	HOXB4 increase SRC frequency by 3-4 fold	(Buske, Feuring-Buske et al. 2002)
Murine 5-FU BM cells	MSCV retrovirus	Expand input CRU 41 fold over 14 days, as compared to 29 fold decrease in untransduced control and 58 fold decrease in EGFP-transduced control , before extensive (5000 fold) <i>in vivo</i> amplification	(Antonchuk, Sauvageau et al. 2002)

ED8.25 yolk sac, ESC-derived hematopoietic progenitors	MSCV retrovirus and gene targeting	HOXB4 promote the maturation of yolk sac and ESC-derived hematopoietic progenitor, generating long-term repopulating definitive HSCs	(Kyba, Perlingeiro et al. 2002)
Murine 5-FU BM cells	MSCV retrovirus	Elimination of PBX1 in HOXB4 expressing cells increase repopulation ability over 20 fold	(Krosl, Beslu et al. 2003)
Murine 5-FU BM cells and Lin ⁻ Sca ⁺ cells;	TAT-HOXB4	Culture of BMC with TAT-HOXB4 for 4 days expand HSC to a level comparable to previous retrovirus transduction methods	(Krosl, Austin et al. 2003)
Human CD34 ⁺ CD38 ^{lo}	SP-HOXB4 secreted by MS-5 mouse stromal cell line	Co-culture with SP-HOXB4 secreting MS-5 over 2-5 weeks increases SRC content by 2.1-2.55 fold	(Amsellem, Pflumio et al. 2003)
Human CD34 ⁺ CB cells	Different retroviral constructs with 2A peptide	High level of HOXB4 can inhibit both lymphoid and myeloid development <i>in vitro</i> and <i>in vivo</i>	(Schiedlmeier, Klump et al. 2003)
Murine 5-FU BM cells	MSCV retrovirus	HOXB4-induced HSC expansion is DNA-binding dependent, PBX-interaction independent and HOXB4 expression level dependent	(Beslu, Krosl et al. 2004)
CD34 ⁺ cells from human, baboon and dog; Sca-1 ⁺ from mouse bone marrow	MSCV retrovirus	Overexpression of HOXB4 can immortalize dog and mouse cells but not human and baboon, possibly due to different expression level of HOXB4. Immortalized mouse cells showed bias towards myeloid <i>in vivo</i>	(Zhang, Schwartz et al. 2007)

		reconstitution.	
CD34 ⁺ cells from macaque and dog	MSCV retrovirus	Transplantable leukemia developed after 2 years. HOXB4 was thought to accelerate the leukemogenesis.	(Zhang, Beard et al. 2008; Thakar, Zhang et al. 2009)

The expression pattern and genetic studies discussed above clearly demonstrated the involvement of HOX genes in hematopoietic system homeostasis. As a third evidence, HOX genes are directly involved in the pathogenesis of leukemia, the majority of which are in the form of NUP98 fusions.

1.4.3 NUP98-HOX fusion proteins

HOX genes of the Abd-B-like cluster, namely, *HOXA9*, *HOXA11*, *HOXC11*, *HOXD11* and *HOXD13*, are recurrently found to be translocated to the *NUP98* locus in certain types of myeloid leukemia (Table 7). This translocation creates fusion proteins between the N-terminal FG/GLFG repeats of NUP98 and the DNA-binding homeodomain of HOX transcription factors (Borrow, Shearman et al. 1996; Nakamura, Largaespada et al. 1996; Raza-Egilmez, Jani-Sait et al. 1998; Fujino, Suzuki et al. 2002; Taketani, Taki et al. 2002; Taketani, Taki et al. 2002). NUP98 is a nuclear pore complex component that functions in normal nuclear transport (Radu, Moore et al. 1995), mitotic nuclear membrane integrity (Laurell, Beck et al. 2011) and possibly cell cycle control (Capelson, Liang et al. 2010; Kalverda, Pickersgill et al. 2010). Its function in the fusion context is unclear, although it has been shown that it can recruit transactivators CBP/p300 in 3T3 fibroblast (Kasper, Brindle et al. 1999). The recent finding that NUP98-HOXA9 interacts and mis-localizes endogenous NUP98 protein during interphase and interacts with mitotic chromosomes

may provide some new hints on the mechanisms of NUP98 fusion-mediated leukemogenesis (Xu and Powers 2010). Although the HOX fusion partners of NUP98 found in human leukemia all belong to the Abd-B-like paralogous groups, 3' HOX proteins when fused to NUP98 also become leukemogenic, especially when in collaboration with MEIS1 (Pineault, Abramovich et al. 2004). Although multiple NUP98-HOX fusions can cause leukemia, due to the intrinsic properties of individual *HOX* genes that are still retained in the fusion proteins, the latencies to onset of leukemia differ considerably. NUP98-HOXB4 for example, has not been associated with leukemogenesis in mouse transplantation models (Pineault, Abramovich et al. 2004; Ohta, Sekulovic et al. 2007; Ruedl, Khameneh et al. 2008), whereas NUP98-HOXA10 caused leukemia with substantially long latency. Moreover, both NUP98-HOXB4 and NUP98-HOXA10 were superior in sustaining HSC activity *in vitro* compared to intact HOX (Ohta, Sekulovic et al. 2007). Interestingly, this self-renewal promoting effect requires only an intact homeodomain from the HOX portion.

Table 7 NUP98-HOX fusion proteins and leukemia

Partner Genes	Phenotypes and translocation	References
<i>HOXA9</i>	AML FAB M2 and M4 t(7;11)(p15;p15)	(Borrow, Shearman et al. 1996; Nakamura, Largaespada et al. 1996)
<i>HOXA13</i>	AML with t(7;11)(p15;p15)	(Taketani, Taki et al. 2002)
<i>HOXD11</i>	AML with t(2;11)(q31;p15)	(Taketani, Taki et al. 2002)
<i>HOXC11</i>	De novo AML; t(11;12)(p15;q13)	(Taketani, Taki et al. 2002)
<i>HOXA11</i> and <i>HOXA13</i>	HOXA11 in CML and HOXA13 in MDS; t(7;11)(p15;p15) Interestingly, NUP98-HOXA9 transcript was also detected, as a result of long-distance splicing, instead of genomic rearrangement.	(Fujino, Suzuki et al. 2002)
<i>HOXA9</i>	NUP98-HOXA9 induced leukemia with long latency (AML onset 230±67 days), preceded by an MPD period; MEIS1 accelerate this process (142±52 days) , but not as dramatic as with HOXA9 (128±15days versus 57±6 days)	(Kroon, Thorsteinsdottir et al. 2001)
<i>HOXA9</i>	NUP98-HOXA9 upregulates HOXA9, HOXA7 and MEIS1. Interestingly, NUP98-HOXA9 is 3 times more stable than HOXA9	(Calvo, Sykes et al. 2002)
<i>HOXA9</i>	Identify some targets unique to NUP98 portion, independent of DNA binding	(Yassin, Sarma et al. 2009)
<i>HOXC13</i>	Adult AML, t(11;12)(p15;q13)	(La Starza, Trubia et al. 2003)

<i>HOXD13</i>	Leukemogenicity depends on intact homeodomain and NUP98; NUP98-HOXD13 (ND13) alone induced leukemia with very long latency (more than 13 months); however, cooperation with MEIS1 cause leukemia with median latency of 75 days. ND13 also increases HOXA7 and HOXA9 expression.	(Pineault, Buske et al. 2003)
<i>HOXD13</i>	ND13 transgenic mice develop MDS within 4-7 months and a wide spectrum of leukemia, including myeloid and lymphoid leukemia, within 14 months	(Lin, Slape et al. 2005)
<i>HOXD13</i>	Spontaneous mutations in N-ras, K-ras and Cbl occur in leukemia developed in ND13 transgenic mice, possibly as “second hit” genes.	(Slape, Liu et al. 2008)
<i>HOXB3</i> , <i>HOXB4</i> , <i>HOXD13</i> , <i>HOXA10</i>	Leukemogenicity of NUP98-HOX fusions is determined by the intrinsic properties of HOX partners; however, when MEIS1 is present, all become leukemogenic; moreover, homeodomain is enough for the HOX partner.	(Pineault, Abramovich et al. 2004)

1.5 Aims of the study

Significant advances have been made to expand HSCs *ex vivo* (Antonchuk, Sauvageau et al. 2002; Zhang, Kaba et al. 2008), both benefiting from and expanding our knowledge about HSC biology. However, the extents of expansion attainable by current methods are still far from the physiological limit; moreover, the “core” HSC self-renewal program remains elusive. Members of homeodomain protein family, especially when fused to NUP98, hold great potential for HSCs expansion, given the involvement of multiple HOX genes in leukemogenesis as well as homeostatic HSC regulation. Therefore, the aim of this study is to screen different branches of the homeodomain protein family for transcription factors that can robustly expand HSCs *in vitro* when fused to NUP98, using NUP98-HOXB4 as the “gold standard” for comparison. Identification of candidate genes would provide us a better platform for dissecting the self-renewal mechanism of HSC, as well as leukemogenesis.

Here we report that after surveying many branches of the homeodomain protein superfamily (Banerjee-Basu and Baxevanis 2001), we found CDX1, member of the extended HOX branch, had superior ability to expand HSC activity *in vitro* when expressed as NUP98 fusion in BM cells. Remarkably, NUP98-CDX1 can reprogram committed myeloid progenitors into cells with high HSC activity. Interestingly, another member of this branch, CDX4, can induce acute myeloid leukemia (AML) with the shortest latency among all NUP98-HOX fusions without the help of MEIS1 transcription factor. In addition by using an inducible system, we showed that the HSC expansion as well as leukemia strictly depends on the continual expression of NUP98 fusions.

As a first step towards understanding the mechanism of HSC self-renewal, we transcriptionally profiled NUP98-HOXA10 transduced bone marrow cells and found the aryl hydrocarbon receptor pathway that when inhibited can expand HSCs by at least 30 folds.

Chapter 2 Materials & methods

2.1 Chemicals and reagents

List of chemicals and reagents used in this study are presented in Table 8.

Table 8 List of chemicals and reagents used in this study

Chemical Names (Cat #)	Companies	Chemical Name	Companies
Primatone (P8388)	Sigma (St. Louis, MO, USA)	Neomycin Trisulfate (N1876)	Sigma (St. Louis, MO, USA)
Sodium citrate (1804)	Sigma (St. Louis, MO, USA)	Iscove's modified Dulbecco's medium (12200-069)	Invitrogen (Carlsbad, CA, USA)
Doxycycline (D9891)	Sigma (St. Louis, MO, USA)	Fetal Bovine Serum (optimized, S181P500)	Biowest (Nuaillé, France)
5-Fluorouracil (F6627)	Sigma (St. Louis, MO, USA)	Trizol (15596-018)	Invitrogen (Carlsbad, CA, USA)
Trypan Blue (93950)	Sigma (St. Louis, MO, USA)	CaCl ₂ dihydrate (517090)	JT Baker Chemicals B.V. (Deventer, Holland)
Sucrose (194018)	MP Biochemicals (Solon, Ohio, USA)	HEPES (H4034)	Sigma (St. Louis, MO, USA)
Ammonium Chloride (A0171)	Sigma (St. Louis, MO, USA)	D-Glucose (K27818937)	Merck (Damstadt, Germany)
Chloroquine (C-6628)	Sigma (St. Louis, MO, USA)	KCl (K41042236026)	Merck (Damstadt, Germany)
NaCl (106400)	Merck (Damstadt, Germany)	Na ₂ HPO ₄ ·2H ₂ O	Merck (Damstadt, Germany)
Polybrene (107689)	Sigma (St. Louis, MO, USA)	CH223191	Merck (Damstadt, Germany)
TotalPrep RNA Amplification Kit (IL1791)	Illumina (San Diego, CA, USA)	RNeasy minikit (74106)	Qiagen (Hilden, Germany)

2.2 Medium

Retrovirus packaging lines were maintained in Iscove's Modified Dulbecco's Medium (IMDM) with 10% Fetal Bovine Serum (FBS). Bone marrow cells were maintained in IMDM containing optimal concentrations of SCF and interleukin-6 (IL-6), 0.03% (w/v)

Primatone (Sigma) and 1.5-2.0% FBS (hereafter referred to as S6). For B cell reprogramming, IL-7 was added and the medium was referred to as S67.

2.3 Mice

All mice were maintained in specific pathogen free conditions in the animal facility of Nanyang Technological University, Singapore. All animal experiments were carried out according to institutional guidelines. Ly5.1 mice were bred at Nanyang Technological University. B6.Cg-*Gt(ROSA)26Sor^{tm1(rtTA^{M2})Jae}/J* mice (Jackson laboratory) that express rtTA-M2 reverse transactivator from the *Rosa26* locus (Hochedlinger, Yamada et al. 2005) were maintained in the same facility. Ly5.2 mice were purchased from the Comparative Medicine Centre in National University of Singapore.

2.4 DNA Constructs

Homeodomains (180bp) from HOXA1, HOXA2, HOXA3, HOXA4, HOXB4, HOXC4, HOXD4, HOXA5, HOXA6, HOXA7, HOXD8, HOXA9, HOXA10, HOXA11, HOXD12 and HOXA13 were amplified by PCR from bacterial artificial chromosomes (BAC/PAC resources) and cloned into the bicistronic vector pMYc-NUP98-HOXB4-IRES-EGFP (Ruedl, Khameneh et al. 2008) by replacing the HOXB4 fragment using the restriction enzymes EcoRI/XhoI or MfeI/Xho. The pMYc-NUP98-2A-EGFP vector was generated by replacing the internal ribosome entry site (IRES) of pMYc-NUP98-HOXB4-IRES-EGFP with a sequence encoding 2A peptide (de Felipe, Luke et al. 2006). Homeodomains from CDX1, CDX2, CDX4 and IPF-1 were amplified from murine intestinal cDNA and cloned into pMYc-NUP98-IRES-EGFP or pMYc-NUP98-2A-EGFP using EcoRI/XhoI. OCT4, NANOG and DBX2 homeodomains were amplified from plasmid templates used in the lab and the cloning was done by Miss Qi Lihua. No linker sequence except the

restriction site EcoRI was inserted between the NUP98 portion and the homeodomain portion. Therefore all resulting vectors have a homeodomain fused directly to NUP98; fusion point of NUP98 is the same as in (Pineault, Abramovich et al. 2004). Expression of the fusion proteins was driven by promoter and enhancer elements present in the retroviral long terminal repeat (LTR) derived from Moloney murine leukemia virus (MoMLV).

The inducible vector pSR-LP-TREtight-EGFP (Resnitzky, Gossen et al. 1994; Clontech 2003) was modified to contain a multiple cloning site that contains BamHI, HindIII, NotI and MfeI, followed by a 2A sequence in front of EGFP. NUP98-CDX1 and NUP98-CDX4 were then cloned between the MfeI and BamHI sites. Transactivator tTA plasmid for Tet-Off controlled expression was modified from rtTA-M2 (Urlinger, Baron et al. 2000) by site-directed mutagenesis.

2.5 Cell sorting

Single cell suspensions from bone marrow and spleen were stained with B220-PE antibody; DN1, DN2, DN3 and DN4 were stained with CD4/8-PE-Cy7, CD25-PE and CD44-FITC. Cells were double sorted using FACS Aria. Sorted B cells were stimulated with lipopolysaccharide (LPS) (1µg/ml) before transduction.

2.6 Bone marrow cultures and gene transfer

Fresh BM cells from tibiae, femurs and spines of 5-FU (160mg/kg) conditioned Ly5.1 mice (at the starting concentration of 5×10^5 cells/ml) were stimulated for 2 days in S6 medium before spinoculation.

Retrovirus-containing supernatants were generated by ecotropic phoenix or Plat/E packaging cells separately transfected with retroviral vectors containing different fusion

proteins. Polybrene (5µg/ml) was added to the supernatants to increase transduction efficiency. Spinoculation was done with a protocol modified from (Ruedl, Khameneh et al. 2008). Briefly, retrovirus-containing supernatants (4-5ml) were loaded twice (30 min each) onto fibronectin-coated 60mm dishes at 4°C, 4000rpm, with a final spin with cells at 37°C, 750g for 45min. This protocol was later modified such that only virus was spun at 4°C for 90min at 4000rpm before BM cells were placed, without further spinning, and incubated at 37°C to establish the cultures. This process was repeated if desired. Transduction frequency was routinely more than 50% based on EGFP expression. Transduced lines were maintained in S6 medium on bacteriological Petri dishes at average cell density of 1×10^6 cells/ml with regular counting and replating every 2 or 3 days. For Tet-On inducible cultures, Doxycyclin (DOX, 1µg/ml) were added and medium was changed every 2 days.

Tet-Off lines were made by co-transfecting tTA transactivator and the inducible vector pSR-LP-TREtight-NUP98-CDX4-2A-EGFP.

For inhibitor experiment, 5-FU BM cells were maintained in S6 medium containing 0.5µg/ml, 0.75µg/ml and 1µg/ml of aryl hydrocarbon receptor (AhR) inhibitor CH223191, as well as control DMSO (0.1%). Cells were counted and split every 2 days and stained every week. Day 14 and day 33 old cultures were injected into mice to test *in vivo* reconstituting abilities.

2.7 Bone marrow transplantation

The Ly5.1/Ly5.2 congenic system was used for the competitive repopulation assay (Szilvassy, Humphries et al. 1990). Briefly, for initial screening, 2×10^4 (low dose) or 2×10^5 (high dose) Ly5.1-expressing cultured cells were mixed with 2×10^5 Ly5.2-

expressing competitor cells and injected through retro-orbital route into Ly5.2 recipient mice lethally irradiated with 11Gray of gamma irradiation (separated into 2 equal doses 5.5 Gray, with a 4 hour interval). The functions of the competitor cells are three-fold. First, Since 2×10^5 cells were the minimal amount of cells required to rescue 100% of lethally irradiated mice, they ensure the survival of the irradiated mice by providing the progenitor cells and differentiated cells in cases where the testing cells do not produce functioning short-term progenitor cells or differentiated cells; second, it directly competes with the test cells so that we can identify test cells that out-compete the wildtype cells, either because the test cells have a repopulating advantage or because there are more HSC present in the culture of test cells. Third, when combined with limiting dilutions, this assay allows us to quantitate the amount of HSC present in the test cultures (Szilvassy, Humphries et al. 1990), that is, the reciprocal of the cell dose that reconstitute 63% of the mice is the frequency of HSCs in the culture.

For initial screening, each dosage group consists of 3 mice; HOXA4, HOXB4, HOXC4, HOXD4, HOXA9, HOXA10, HOXA11, HOXA13, CDX1, CDX2 and CDX4 cultures were injected with two doses whereas the other cultures were only injected with the low dose.

To compare the reconstituting activity of NUP98-HOXA10, NUP98-CDX1 and NUP98-HOXB4, 200 to 2×10^5 Ly5.1-expressing cultured cells expanded for 4 weeks *in vitro* were mixed with 2×10^5 Ly5.2-expressing competitor cells and injected into lethally irradiated Ly5.2 recipient.

Inhibitor cultures were injected at day 14 and day 33. Day 14 AhR inhibitor culture were injected into lethally irradiated mice at a 1:1 ratio with competitor cells (2×10^5 cells each) as compared to 5:1 ratio for day 14 DMSO control culture. Day 33 AhR inhibitor and DMSO cultures were both injected at 15:1 ratio with competitor cells (3×10^6 cultured cells: 2×10^5 competitor cells).

Peripheral blood from reconstituted mice was monitored monthly by flow cytometry analysis. After 4 months, all mice were sacrificed and both central and peripheral lymphoid organs were analyzed by multi-parameter immunostaining.

For bone marrow chimera reconstituted with Tet-Off-NUP98-CDX4 cells, mice were fed with DOX-containing water (200 μ g/ml) when overt signs of leukemia were detected.

2.8 Estimation of HSC frequency

The calculation is based on the principle of limiting dilution analysis with the equation $N = -\ln F_0$, where N is the number of injected bone marrow cells and F_0 is the fraction of mice that are negative for the donor cells, i.e., not reconstituted. Based on this principle, the reciprocal of cell number that gives rise to 37% negative readout would be the frequency of HSC present in the injected cells. Our experiment showed that injection of 200 NUP98-CDX1 cells was sufficient to reconstitute 100% of all mice without fluctuating positive and negative readouts. Therefore, on a semi-log paper, 100 cells would give rise to less than 37% negative readout. Hence, a frequency of 1% is a conservative estimation.

2.9 Flow cytometry analysis

Single cell suspension from blood, spleens, peripheral lymph nodes, thymi and bone marrows of chimeric mice were blocked with anti-CD16/CD32 antibodies in ice-cold PBS containing 2% FCS before staining with lineage markers. PE-, APC-, PE-Cy7-conjugated monoclonal antibodies were all purchased from eBiosciences (San Diego, USA) or Biolegend (San Diego, USA). Donor cells were recognized by both EGFP expression and Ly5.1 staining. T cells, B cells, and myeloid cells were defined as Thy1.2⁺, B220⁺ or CD19⁺ and Mac-1/Gr-1⁺, respectively. Stained cells were analyzed by FACSCalibur (Becton Dickinson, NJ, USA) and the data were processed by FlowJo software (Treestar, USA).

2.10 Blood cell count

Peripheral blood cells were manually counted according to protocols in (Michael P. McGarry 2010). For red blood cell count, 25µl of peripheral blood with anti-coagulant sodium citrate was diluted into 10ml of PBS, and number of red blood cells was counted at 400× magnification. For white blood cell count, 50µl peripheral blood with sodium citrate (Sigma, MO, USA) was diluted into 1ml PBS with 0.1% HCl and live cell in trypan blue were counted at 200× magnification.

2.11 RNA isolation and hybridization

RNA isolation and hybridization were done by Miss Ng Jia Hui from Dr Ng Huck Hui's lab in Genome Institute of Singapore (GIS). Briefly, total RNA was extracted using Trizol (Invitrogen) (Isopropanol precipitation method), DNase treated and purified with RNeasy minikit (Qiagen). Biotinylated cRNA were prepared using TotalPrep RNA Amplification Kit (Illumina) from 500ng of total RNA according to the standard protocol in the instruction manual. 1.5µg of cRNA was hybridized to Illumina Expression BeadChip for

18 hours at 58°C on Illumina Hybridization Oven. BeadChip was washed and stained with streptavidin-Cy3 before scanning using the Illumina Bead Reader.

2.12 Microarray data analysis

Microarray data analysis was done by Dr Chen Jinmiao from Singapore Immunology Network (SIgN). Image analysis, normalization, filtering, clustering and selection of differentially expressed genes (DEGs) were done using AMDA software developed by Genopolis (A-Star, Singapore). DEGs were mapped into pathways using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc. CA, USA)

Chapter 3 Screening

3.1 Construction of different retroviral vectors

As an initial search for genetic factors that sustain mouse HSC self-renewal, i.e. their expansion in culture, we selected representative members from different paralogous groups of the clustered HOX subclass, as well as the non-clustered homeodomain protein class. Homeodomains encoded by these genes were fused to NUP98 in a way that mimics the leukemogenic fusions generated by chromosomal translocations in certain, rather rare, AMLs, whereby NUP98 is fused to selected HOX family of transcription factors (Borrow, Shearman et al. 1996). The fusion constructs were cloned into the bicistronic retroviral vector that co-expresses EGFP under the control of IRES (Figure 3a). Expression of the fusion proteins was driven by promoter and enhancer elements present in LTR.

Figure 3 Experimental schemes.

a. Retroviral constructs used in the initial screening. Homeodomains from one representative member of the 13 HOX paralogous groups and CDX1, CDX2, CDX4 and IPF-1 were directly fused to the NUP98 protein. The fusion proteins were then cloned into the bicistronic pMYc-IRES-EGFP vector. Transcription was driven by the promoter present in the viral LTR.

b. Derivation of NUP98-HOX over-expressing bone marrow cell lines. All vectors were used to transfect the phoenix or Plat/E packaging lines. Virus supernatant from the packaging line was then used to infect BM cells isolated from B6 mice injected with 5-FU 4 days earlier as shown in **c**.

c. Experimental scheme for *in vivo* reconstitution. Ly5.1⁺ transduced cells were cultured for 4 weeks; then 2x10⁴ or 2x10⁵ cultured cells were injected together with 2x10⁵ Ly5.2⁺ competitor cells into lethally irradiated congenic Ly5.2⁺ mice.

3.2 Derivation of cell lines

Retroviral constructs were separately transfected into Phoenix or Plat/E packaging lines to generate virus supernatants, which was then used to spinoculate BM cells isolated from B6 mice injected with 5-FU four days earlier to enrich primitive cell populations (Harrison and Lerner 1991). The schematic protocol is shown in Figure 3b and 3c.

3.3 *In vitro* characterization of cultured cells

Growth of all cultures was monitored by cell counting and EGFP analysis every 2-3 days. Cell counting started from between 2-3 weeks after bone marrow isolation and continued for 2-4 weeks. A typical growth curve is shown in Figure 4a. The growth curves were used to calculate the average doubling time of the cultures (Figure 4b). Since by 2-3 weeks, the transduced population in almost all the cultures had already dominated, the growth of the total population reflected the growth of the transduced population; hence the EGFP⁺ population had a similar doubling time to the total population. Exceptions were NUP98-HOXA6 and NUP98-HOXA13 cultures, where gradual turning-off of EGFP expression in the transduced cells was observed (Figure 4c). Among all the cultures, NUP98-HOXA9, NUP98-HOXA10, NUP98-CDX1 and NUP98-CDX4 were fast growers with a doubling time between 21-25 hours, whereas the rest of the cultures grew more slowly, especially NUP98-HOXD4, which has a doubling time of around 40 hours. Data shown later suggested that there was a slight correlation between the growth phenotype and competitive capabilities.

Figure 4 *In vitro* characterizations of cultured cells.

a. Growth curve of NUP98-HOXA10 transfected culture. Cells were monitored by flow cytometry for EGFP expression every 2-3 days.

b. EGFP dynamics of NUP98-HOXA6 and NUP98-HOXA13 transduced cultures observed over 2 months.

c. Doubling time of different cultures as calculated from the growth curves.

d. Typical staining profile of NUP98-HOX cultures at the 3rd week. The profile is stable over months and is typical for most of the NUP98-HOX cultures.

Cultured cells were also regularly stained for different stem cell markers and lineage markers. Figure 4d shows the staining patterns for stem cell marker cocktails c-kit/Sca-1 and CD48/CD150 and lineage marker cocktails c-kit/FcεRI for mast cells and Mac-1/Gr-1 for myeloid cells. All cultures had a small population (<1%) of CD48⁺CD150⁺ cells and the majority (>90%) are c-kit positive, which is consistent with *in vivo* HSC phenotypes.

Sca-1 expression was not stable and can change according to the culture conditions. For example, cells cultured with recombinant SCF or overexpressing another transcription factor Sox17 showed a dramatic increase in Sca-1⁺ population. However, studies from our lab have shown that there is no difference in stem cell activity between Sca-1⁺ and Sca-1⁻ populations in these cultures (data not shown). Therefore, Sca-1 is not a reliable *in vitro* marker for expanded HSC. As expected, the percentage of monocytes/macrophage (Mac-1⁺) and mast cells (FcεRI⁺) were very low. Phenotypes were stable over at least two months.

3.4 *In vivo* reconstitution

We then went on to test the ability of these different cultures to competitively reconstitute lethally irradiated mice *in vivo* (Figure 3c). After one month of continual culture (c.a. at least 10¹⁸ fold exponential expansion), when only transduced cells were present, 2×10⁴ (low dose) or 2×10⁵ (high dose) Ly5.1-expressing cultured cells were injected into lethally irradiated mice with 2×10⁵ Ly5.2-expressing competitor cells. Peripheral blood from reconstituted mice was analyzed monthly by 4 color immunostaining with different lineage antibody cocktails together with antibody against the congenic antigen Ly5.1 to identify the cultured cells. After 4 months, all mice were sacrificed and different hematopoietic organs were analyzed. Figure 5b shows blood chimerism of different groups after four months of reconstitution. Among all cultures, NUP98-HOXA3, NUP98-HOXA9, NUP98-HOXA10, NUP98-HOXA11 and NUP98-CDX1, NUP98-CDX2, NUP98-CDX4, had the highest chimerism in all hematopoietic organs examined. However, all dosage groups from mice reconstituted with NUP98-HOXA3, NUP98-HOXA11, NUP98-CDX2 and NUP98-CDX4 cultures had at least one morphologically

sick mouse, which had splenomegaly, pale bones, small thymus and enlarged lymph nodes. We also noticed that the lower dose groups are more subject to stochastic effects, as suggested by the large standard error.

Figure 5 Chimerism in different hematopoietic compartments

a. Gating strategy used in this study for analysis of overall chimerism and lineage distributions. Nucleated cells and anucleated cells were identified by their characteristic positions in the FSC-SSC plot. Donor cells and Competitor cells in the nucleated gate were distinguished by the congenic marker Ly5. Differentiation into different lineages was then analyzed within the gated Ly5.1⁺ or Ly5.2⁺ populations. Anucleated cells contain mainly RBC and platelets; contribution of donor cells into these lineages was identified by EGFP expression.

b. Overall blood chimerism in recipient mice transplanted 4 months earlier with competitor cells (Ly5.2⁺, 2×10⁵) together with either 2×10⁴ (low dose) or 2×10⁵ (high dose) cultured cells (Ly5.1⁺). (means ± SEM, n=3)

c-f. Chimerism of RBC (**c**), myeloid cell (**d**), T cell (**e**) and B cell (**f**) in peripheral blood of bone marrow chimera described in **b**; both 1st month and 4th month were shown for RBC, myeloid cell and B cell; only 4th month results were shown for T cell because T cells were still in the process of development in the 1st month. Results shown are from the high dose group.

We then looked at the chimerism in different hematopoietic compartments to see whether the transduced cells were able to repopulate all lineages. Only results from the peripheral blood are shown, since chimerism in other hematopoietic organs basically followed the same trend, unless otherwise indicated. Red blood cells (RBC) and platelets were identified by their characteristic position in the FSC-SSC dot plot (Figure 5a). Because RBC and platelets do not express the Ly5 marker, chimerism analysis can only rely on the expression of EGFP. Considering the respective levels of average chimerism, NUP98-HOXA4, NUP98-HOXB4, NUP98-HOXC4, NUP98-HOXA11, NUP98-CDX1, NUP98-CDX2 and NUP98-CDX4 all had a high level of RBC/platelet chimerism (Figure 5c). In contrast, although NUP98-HOXA9 and NUP98-HOXA10 had a high level of chimerism in nucleated cells, they seemed to have a deficiency in reconstituting the megakaryocyte/erythrocyte lineage. B cell and T cell had similar level of chimerism as the average chimerism, except that genes that caused illness in the host seemed to have a

higher chimerism level in B cell compartment (Figure 5e and 5f). Significantly, almost all cultures, including the “gold standard” HOXB4, had high chimerism in myeloid lineage (Figure 5d).

Based on the screening results, NUP98-HOXA3, NUP98-HOXA9, NUP98-HOXA10, NUP98-HOXA11 and NUP98-CDX1/2/4 all have much higher chimerism as compared to NUP98-HOXB4. However, NUP98-HOXA9 caused a block in RBC/platelet development and was not as robust in reconstitution as compared to NUP98-HOXA10; NUP98-HOXA3 and NUP98-HOXA11 caused only incomplete penetrance of myeloproliferative disease (MPD) as compared to NUP98-CDX4. The best candidate that came out from this screening therefore is NUP98-CDX1, which gave high level of chimerism, as well as normal blood lineage development (shown in Chapter 4). NUP98-CDX2 behaved quite similarly to NUP98-CDX1 except that it also caused an early onset of MPD. Thus, we decided to further analyze NUP98-CDX1 and NUP98-HOXA10 for their HSC expansion activity; NUP98-HOXA10 was included because it has been reported by Humphries to cause maximal expansion of HSCs, although different lineages, especially the RBC lineage, were not carefully examined (Ohta, Sekulovic et al. 2007). We also selected NUP98-CDX4 for further studies because we believed the fast progression of bone marrow chimera towards MPD and leukemia can make it a useful model for leukemia study.

Chapter 4 NUP98-CDX

4.1 NUP98-CDX1 supports extensive self-renewal of HSC

As mentioned above, both NUP98-CDX1 and NUP98-CDX4 constructs established robust, growth factor dependent, lines in liquid cultures that could be maintained for months with regular doubling time of around 24 hours. From the initial screening batch of mice, we examined carefully the high dose (2×10^5) group for chimerism in different tissue compartments and lineage proportions within donor cells (cultured cells) by flow cytometry to score, as robustly as possible, the long-term HSC activity.

Despite long *in vitro* culture and extensive expansion, both lines outcompeted the normal cells in all tested tissue compartments: blood (nucleated and anucleated), BM, spleen and thymus (Figure 6b-6d). Typical staining profile was shown in Figure 6a. Surprisingly, NUP98-CDX1 culture sustained robustly HSC activity (Figure 6c) with normal myeloid to lymphoid ratios. Our analysis also showed active ongoing B cell and thymocyte production as revealed by the presence of normal numbers of B220⁺ BM cells and different thymocyte subpopulations, especially the presence of short-lived CD4/8 double positive (DP) cells (Figure 6d).

Figure 6 NUP98-CDX1 sustains robust HSC activities while NUP98-CDX4 caused overt myeloid proliferation.

Lethally irradiated mice transplanted with NUP98-CDX1 or NUP98-CDX4 as described in Figure 5 were further analyzed here. Results from the high dose group were shown here (means $\pm$ SEM, n=3).

a. Typical staining profiles from different organs of NUP98-CDX1 and NUP98-CDX4 bone marrow chimera 4 months after reconstitution. Cells are gated on Ly5.1⁺ donor cells.

b. *Left*, chimerism of NUP98-CDX1 and NUP98-CDX4 donor cells in different hematopoietic organs 4 months after reconstitution; *right*, chimerism of donor cells in different hematopoietic compartments in the blood.

c. Lineage distributions of donor cells as compared to age matched wildtype (WT) mice.

d. Distribution of different thymocyte subsets 4 months after reconstitution.

The recipients, however, that received NUP98-CDX4 cells showed clear signs of myeloproliferation and abnormal thymocyte development represented by the hugely expanded double negative (DN, CD4-/CD8-) cells (Figure 6d) that actually are myeloid cells (described in section 4.6).

Of note, NUP98-CDX1 cells after 94 days of continual culture (massive 10^{44} expansion) were still able to reconstitute all lineages and competed robustly with wild type normal BM cells in the standard long-term reconstitution assay. However, signs of myeloid preference could be observed (Figure 7).

Figure 7 NUP98-CDX1 culture still retains HSC activities after 3 months in culture.

20000 Ly5.1⁺ NUP98-CDX1 expressing cells after 3 months of culture were injected into lethally irradiated mice with 20 000 Ly5.2⁺ competitor cells (means $\pm$ SEM, n=3).

- a.** Overall chimerism in different hematopoietic organs after 4 months of reconstitution.
- b.** Percentage of donor cells (Ly5.1⁺) in different hematopoietic lineages in the blood.
- c.** Distribution of different hematopoietic lineages in donor cells in different organs.

4.2 Comparative analysis of sustenance of HSC activity by NUP98-CDX1, -HOXA10 and -HOXB4 transduced cells

New set of cultures were then set up in order to measure the HSC activity (content) more closely and in the same time compare NUP98-CDX1 cultures with those initiated with NUP98-HOXB4 and -HOXA10 that are known to support HSC activity remarkably well in expanding cultures (Ohta, Sekulovic et al. 2007) (Figure 8). All three BM lines established with these constructs grew indefinitely in growth factor dependent way with NUP98-HOXA10 having the fastest growth rate (not shown). In this case we cultured all the lines in parallel for 34 days and then quantified their HSC activity in long-term competitive repopulation assays.

Mixtures of 2×10^5 normal BM cells and 200 or 2000 cultured NUP98-CDX1 or NUP98-HOXA10 cells (1/1000 and 1/100) and 2000 or 20 000 NUP98-HOXB4 (1/100 and 1/10) cells were injected as above into lethally irradiated recipients that were then analyzed four months later for donor cell contribution in different cellular compartments (Figure 8a and 8b). Remarkably, even 200 NUP98-CDX1 or NUP98-HOXA10 cells gave robust multi-lineage reconstitution without gross myeloid bias in any compartments studied (Figure 9). Major difference between NUP98-CDX1 and NUP98-HOXA10 reconstitution was the cellularity of thymus and developmental progression of thymocytes. NUP98-CDX1 thymi were largely normal while NUP98-HOXA10 thymi were small and showed severe degrees of developmental block (abundance of DN cells) of T cell development (Figure 8c). Moreover, NUP98-HOXA10 seems to be inefficient in reconstituting the RBC/platelet lineage compared to NUP98-CDX1, as shown in Figure 6.

Figure 8 Comparative analysis of NUP98-CDX1, -HOXA10 and -HOXB4 transduced cells in sustaining HSC activity

a. Overall chimerism of donor cells (Ly5.1⁺) in different hematopoietic organs of recipient mice 4 months after transplantation. All donor cells were cultured for 34 days *in vitro* before transplantation. Competitors (Ly5.2⁺, 2×10⁵) were injected together with 200 (1:1000) or 2000 (1:100) NUP98-CDX1 or NUP98-HOXA10 transduced cells. In the case of NUP98-HOXB4 transduced cells, either 2000 (1:100) or 20000 (1:10) cells were used (means ± SEM, n=4).

b. Lineage distribution of donor cells (Ly5.1⁺) in the blood of recipient mice described in **a**. Other organs, except thymus, gave similar results (means ± SEM, n=4).

c. Representative analysis of thymopoiesis of two recipient mice from **a** transplanted with 1000 NUP98-CDX1 or NUP98-HOXA10 transduced cells profiled with indicated antibodies.

Figure 9 Lineage distributions of donor cells in different hematopoietic organs from recipient mice injected with 200 NUP98-CDX1 transduced cells and 200000 WT competitor cells 4 months earlier.

Since the recipients did not yet “fluctuate” even with 200 cells between non- and repopulated hosts (although the levels of chimerism vary), we can only estimate (conservatively) that transplanted cells from NUP98-CDX1 cultures contained at least 1% of cells that read out as HSC. NUP98-HOXB4 cultures maintained HSC activity c.a. tenfold less well but those cells reliably follow the normal developmental paths (Figure 8).

4.3 Continual expression of NUP98-CDX1 allows normal myeloid, erythroid/megakaryocytic and lymphoid development

In order to test if the expression of the fusion gene “stays on” during the differentiation of different hematopoietic lineages, we replaced the IRES sequence with that of 2A in our constructs. 2A or 2A-like peptides are viral peptides (originally found in picornaviruses like poliovirus and foot-and-mouth disease virus to mediate the cleavage of the viral proteins 2A and 2B) with the conserved motif DxExNPGP. During translation, through a ribosomal skipping mechanism (Donnelly, Luke et al. 2001), 2A peptide impairs the

peptide bond formation between G and P, leading to the separation of the upstream and downstream protein during translation. Unlike the IRES sequence, 2A peptide physically fuses EGFP with the upstream transcription factors; therefore, EGFP expression from this construct reflects more reliably the NUP98-CDX1 protein expression. 2A constructs “induced” continually growing lines similar to those of IRES constructs and cells from “2A” cultures repopulated all the hematopoietic lineages in typical *in vivo* reconstitution assays (Figure 10a)

Figure 10 Continual expression of NUP98-CDX1 is compatible with development of all hematopoietic lineages

a: Overall chimerism of different hematopoietic organs in recipients of NUP98-CDX1-2A-EGFP transduced cells. BM cells transduced with NUP98-CDX1-2A-EGFP vector were cultured for two weeks and then injected without competitors into lethally irradiated recipients. Four months later overall chimerism in different hematopoietic organs were analyzed by flow cytometry.

b. Analysis of different lineages in recipient mice described in **a**. Great majority of cells in all tested lineages expressed NUP98-CDX1. All the blood myeloid (Mac-1⁺) and most anucleated cells (analyzed as in Figure 6a) were expressing EGFP. Similarly, most developing BM B lymphocytes (B220⁺) and developing T lymphocytes (thymocytes) in thymus were EGFP⁺.

c. Flow cytometry of peripheral mature B (gated for B220⁺) and T (gated for Thy1.2⁺) lymphocytes isolated from lymph nodes from chimeras described in **a** show strong tendency of “shut-down” of NUP98-CDX1 expression--about half of these cells are EGFP⁻.

d. Comparison of EGFP expression in multipotent NUP98-CDX1 expressing cells from culture and *in vivo* blood nucleated cells from recipients in **a**. Overlay of EGFP expression histograms is shown.

e. Expression of EGFP in peripheral blood T and B cells from mice reconstituted with NUP98-CDX1-IRES-EGFP construct.

As shown in Figure 10b, NUP98-CDX1 is continually expressed in myeloid cells as well as during erythroid/megakaryocytic differentiation and that T and B cell development in thymus and BM, respectively, “tolerated” the level of NUP98-CDX1 expression comparable to that in the original transduced BM cells (Figure 10d). However, we could observe some selection against continual expression in long-lived T and B lymphocytes (four months after reconstitution, Figure 10c) that could not be explained by simple tissue-specific shut-off of the LTR promoter since in the case of IRES vectors EGFP was continually present in many mature lymphocytes (Figure 10e).

4.4 “Immortalized” HSCs are addicted to continual expression of NUP98-CDX1

We next analyzed the consequences of switching off the NUP98-CDX1 expression in established and continually growing lines in order to see if “immortalized” cells could be reverted to normal HSC. To do this we exploited Tet-On system whereby BM cells from mice expressing reverse transactivator (rtTA-M2) from Rosa26 locus (Hochedlinger, Yamada et al. 2005) were transduced with retroviral vectors containing doxycycline (DOX) inducible promoter (TRE) driving NUP98-CDX1-2A-EGFP expression.

In the presence of DOX, typical robust, continually growing lines could be established with ease as before. When DOX was removed after three weeks of culture, cells lost gradually the EGFP signal (Figure 11a), dropped out from cell cycle, became adherent and started to express myeloid marker Gr-1 (Figure 11b). All the phenotypic changes occurred as a synchronized cohort.

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Figure 11 Continual expression of NUP98-CDX1 is required to sustain HSC activity

a. BM cells were transduced with DOX inducible NUP98-CDX1-2A-EGFP vector and grown *in vitro* in the presence of DOX for two weeks, at which time all cells were EGFP⁺ due to selective advantage of transduced cells. Removal of DOX at day 14 led to baseline level of EGFP expression in 72 hours as followed by flow cytometry.

b. Morphology of cells in culture and immunophenotypes before and after DOX removal (5 days). Most of the cells differentiated to myeloid cells in the absence of DOX as shown by the appearance of Mac-1/Gr-1 markers. This was accompanied by their adherence to plastic.

c. Donor (in this case Ly5.2⁺) contribution in lethally irradiated recipients (Ly5.1⁺) 2 months after transplantation of 3×10^6 cells from two-week old cultures injected without competitor cells at the moment of DOX removal. Injected cells rescued the recipients from lethal irradiation but no signs of active hematopoiesis could be observed two months after transplantation—no donor Mac-1⁺ cells in the blood although some long-lived donor B (B220⁺) and T (Thy1.2⁺) lymphocytes were observed. Hematopoiesis was dominated by the host cells that were most likely generated from rescued rare host HSC.

To test the presence of HSC activity in the cultures withdrawn from DOX after 4 weeks of culture in its presence, we transplanted 3×10^6 cells without competitors at the day of DOX removal into lethally irradiated recipients. However, no signs of long-term HSC activity could be observed (Figure 11c). Instead, only short-term HSC-like cells are present. In half of the cases the transplants did not rescue the recipients from lethal irradiation and in those recipients that survived, the host stem cells took over the active hematopoiesis as indicated by the myelopoiesis (short-lived cells) that was completely of host origin. The presence of some long-lived donor lymphocytes most likely reflects a transient small wave of T cell and B cell production from rare non-stem cell progenitors.

4.5 NUP98-CDX1 can “reprogram” late myeloid progenitors into HSC

Most of our *in vivo* HSC assays above have used transduced cells from cultures established from fresh BM cells after 5-FU treatment (4 days before) that represents a heterogeneous mixture of HSC, multipotent and committed progenitors, as well as more differentiated cells, hence leaving the identity of potential “initiating” cells undetermined. To shed more light in the type of cells that can be turned into multipotent HSC-like cells, we transduced BM cells that had been cultivated in our standard conditions for 21 days. Cells from these cultures had all differentiated into myeloid precursors (Figure 12a) and at this stage of the culture, cells started losing their vigorous growth and turned increasingly into non-dividing adherent cells. Surprisingly, it was straightforward to derive robust lines from these cultures with our typical transduction frequencies (c.a. 50%, NUP98-CDX1-IRES-EGFP vector). We then analyzed the HSC content in the cultures after further 4 weeks of culture (total *in vitro* culture period 49 days) with competitive repopulation

assays. Like before (Figure 8), we transplanted 200, 2000 or 20 000 cultured cells with 2×10^5 competitors and analyzed the recipients 4 months later for overall chimerism and lineage distribution in different hematopoietic organs (Figure 12b and 12c). Remarkably, even 200 donor cells competed robustly with 2×10^5 normal BM cells and showed high chimerism in all tested organs without any major distortions in lineage distribution.

Figure 12 NUP98-CDX1 can “reprogram” differentiating myeloid progenitors

a. Flow cytometry for myeloid markers of normal BM cells grown for three weeks in culture. At this point of time adherent cells were dominant. The staining profile of non-adherent population that were also used for NUP98-CDX1 transduction is shown.

b. Chimerism in different hematopoietic organs of recipient mice that received 4 months earlier mixtures of competitor cells (Ly5.2⁺, 2×10^5) with 200 (1:1000), 2 000 (1:100) or 20000 (1:10) experimental cells that were transduced with NUP98-CDX1 at day 21 of *in vitro* culture and then further cultured for 4 weeks, i.e., total of 7 weeks of culture (means $\pm$ SEM, n=4).

c. Lineage distribution of cultured cells in different hematopoietic organs in mice described in **b**.

4.6 NUP98-CDX4 induces leukemia with short latency

Unlike NUP98-CDX1 transduced cells, “NUP98-CDX4” cells developed into leukemia with the shortest latency ever reported for NUP98-HOX fusion products when no collaborating MEIS1 was provided (Pineault, Abramovich et al. 2004). Half of the recipients of NUP98-CDX4 transduced cells succumbed in about 100 days into typical AML characterized by splenomegaly, lymph node enlargement and “anemic” bone marrow due to massive invasion of myeloid blasts (Figure 13a-13d). Examination of thymocytes also showed infiltration of myeloid cells (Figure 13e).

Figure 13 NUP98-CDX4 induced aggressive leukemia

a. Survival curves of sublethally irradiated mice transplanted either with 1×10^6 NUP98-CDX4 transduced BM cells ($n=6$, primary recipients) or with 1×10^6 BM cells from leukemic primary recipients ($n=3$). Median survival times are 112 days for primary and 34 days for secondary transplants.

b. Gross morphological comparisons of hematopoietic organs between leukemic and normal mice.

c. Red (*top*) and white blood cell (*bottom*) counts of normal and moribund mice of primary and secondary transplants (mean $\pm$ SEM, $n=3$).

d. Lineage distribution of nucleated cells in different hematopoietic organs of moribund hosts of primary transplants (mean $\pm$ SEM, $n=3$).

e. Flow cytometry analysis of thymopoiesis of moribund mice from **c**. Thymi had been invaded by leukemic cells (Mac-1/Gr-1) and no signs of normal T cell differentiation (gross aberrant CD4/CD8 staining profiles) was present.

Interestingly, full-blown leukemia could be “cured” by switching off the ectopic expression of NUP98-CDX4 in leukemic cells (Figure 14a). This was done by inducing first leukemia with Tet-Off NUP98-CDX4 constructs whose expression could be then turned off by DOX in the drinking water. This showed, perhaps not surprisingly due to short latency, that the leukemia was fully addicted to their “oncogene”. One of the leukemic mice was continuously treated with DOX water for another four months after the eradication of leukemia. Amazingly, four months after the “cure” and with DOX water, the majority of myeloid cells were still derived from the donor cells, indicating the presence of authentic HSCs in this mouse after the switch-off of NUP98-CDX4 expression.

Figure 14 Leukemia induced by NUP98-CDX4 can be cured by transgene turn-off

a. Lethally irradiated recipients (Ly5.2⁺) were transplanted with 1106 BM cells (Ly5.1⁺) that had been doubly transduced with NUP98-CDX4-2A-EGFP and transactivator (tTA) coding vectors (Tet-Off system) and then cultured in the absence of DOX (construct is “on”) for 20 days. Due to selective growth advantage of transduced cells, most cells after 20 days, at the time of transplantation, are EGFP⁺. After about 70 days when the clear signs of leukemogenesis could be observed in peripheral blood (*Top*), the mice were transferred to DOX-containing drinking water. Flow cytometric analysis for peripheral blood myeloid (Mac-1/Gr-1) and lymphoid (CD19/Thy1.2) cells at the time of transfer to DOX water (*bottom*). Typical example is shown. Analysis shown is gated on Ly5.1⁺ cells, although at the time of analysis great majority of all peripheral leukocytes are donor derived. Mostly long-lived lymphocytes, presumably generated before leukemogenesis, can be observed after the “cure” that eradicates the Mac-1/Gr-1 positive leukemic population.

b. One of the leukemic mice was treated with DOX continuously for another four months after the “cure”. After four months, peripheral blood was analyzed for donor derived (Ly5.1⁺) myelopoiesis (Mac-1⁺).

5.1 NUP98-HOXA10 can re-program B220⁺ cells *in vitro*

NUP98-HOXA10 has been shown by Humphries to be able to induce expansion of HSCs *in vitro* (Ohta, Sekulovic et al. 2007). Our initial screening results also identified it as a candidate gene for *in vitro* maintenance and expansion of HSC with relatively balanced lineage outputs, although with an obvious defect in RBC/platelet and thymocytes in around 50% of mice (Figure 5 and 8). To gain more insights into the effects of NUP98-HOXA10, we attempted to reprogram differentiated mature lymphocytes or lymphoid progenitors using NUP98-HOXA10. We first sorted the double negative population of thymocytes and the B220⁺ population of bone marrow cells (Figure 15a).

Figure 15. Establishment of cell lines from lymphocyte progenitors.

- a. Sorting of different lymphocyte progenitors. First dot plots in each panel show the sorting gates; the plots that follow show the purity for each sorted subset after sorting.
- b. Phenotypes of established lines transduced by NUP98-HOXA10 after 3 weeks of culture. Sorted B220⁺ cells were stimulated with LPS (1 μ g/ml) for 24 hours before transduction with NUP98-HOXA10. Transduced cells were cultured in S6 medium with IL-7 (B220+S67). Phenotyping was done at week 3, when the line was fully established. Removal of IL-7 after the line was established (week 2) did not change the phenotype. B220⁻ cells were sorted and transduced with NUP98-HOXA10; this is essentially similar to the usual 5-FU treated BM cells transduced with NUP98-HOXA10.
- c. PCR for DJ rearrangement in the established line. Spleenocyte DNA was used as a positive control; ES cell genomic DNA was used as a negative control.

Sorted B220⁺ cells were then stimulated with LPS (1 μ g/ml) immediately after sorting; both B cell and T cell were maintained in S6 (SCF and IL-6) medium or S67 (SCF, IL-6 and IL-7) medium. One day later when signs of cell division were obvious, cells were transduced with NUP98-HOXA10 virus supernatant. All the sorted T cell progenitors either didn't grow or grew for a few days before dying off. B220⁺ cells maintained in S6 medium eventually also died off; whereas those maintained in S67 formed dividing clusters on fibronectin-coated plates starting from two weeks in culture. At this time, the B220⁺ culture were separated into two halves, one still maintained in S67 (hereafter referred to as B220+S67), the other grown in S6 (hereafter referred to as B220+S6). By 3 weeks, rigorously growing lines were established from B220+S6 and B220+S67, with B220⁻ cells sorted at the same time and transduced with NUP98-HOXA10 as a positive control. By the time of injection, the transduced cells dominate all cultures. As shown in Figure 15b, the established lines were virtually indistinguishable from the B220⁻ line in terms of *in vitro* staining pattern. To confirm that the established lines were not derived from the <1% contamination of B220⁻ cells (Figure 15a), we checked the B cell receptor

rearrangement of the established lines. Remarkably, both lines showed DJ rearrangement (Figure 15c), indicating that they were derived from B cell progenitors.

The established lines were then injected at 1:10 ratio (2×10^4 cultured cells: 2×10^5 competitor cells) into lethally irradiated mice. Four months later all hematopoietic organs were analyzed. Our analysis showed that the B220⁺ lines behaved similarly to B220⁻ line *in vivo* (Figure 16a), although the B220⁻ line generally had higher overall chimerism in all hematopoietic compartments except the T cell lineage. Moreover, these B220⁺ reprogrammed lines had the ability to differentiate into all lineages of the hematopoietic system (Figure 16b). Surprisingly, a closer examination of the transgene expression showed that within lymphoid lineages, the percentages of EGFP⁺ cells within the donor cell population varied according to the lineages (Figure 16c). Specifically, in blood, spleen and bone marrow, expression of NUP98-HOXA10 (as indicated by EGFP) in B cells is shut off in B220+S6 and S67 lines, but not as much in B220⁻ lines. In T cell compartment, B220+S6 and S67 also shut off NUP98-HOXA10 expression in blood and spleen, less so in bone marrow, but showed high expression in thymus, suggesting differential molecular cues present in different hematopoietic organs.

Figure16. Cell lines established from B220⁺ cells by NUP98-HOXA10 overexpression behaved like HSC.

Sorted B220⁺ or B220⁻ cells were transduced with NUP98-HOXA10 after stimulation with LPS. Transduced B220⁺ cells were cultured in S67 medium and at around day 14 were split into two halves to be cultured in S6 (B220+S6) or S67 (B220+S67) medium.

Transduced B220⁺ cells were continuously cultured in S6 medium. After 25 days of culture, 20000 cultured cells (Ly5.1⁺) were co-injected with 200000 freshly isolated competitor cells (Ly5.2⁺) into lethally irradiated mice (Ly5.2⁺) (means $\pm$ SEM, n=4).

a. Overall chimerism in different hematopoietic organs of recipient mice.

b. Lineage distribution of donor cells (Ly5.1⁺) in different hematopoietic organs of recipient mice.

c. Percentage of EGFP⁺ cells in donor cells (Ly5.1⁺) in different hematopoietic lineages.

5.2 NUP98-HOXA10 microarray

To understand the mechanisms of NUP98-HOXA10 at the molecular level, we performed transcriptional profiling of NUP98-HOXA10 lines. By the time a stable line is established, it is very likely that both primary and secondary effects of NUP98-HOXA10 are responsible for the observed phenotypes. Therefore, to distinguish between these two different effects, we used the Tet-On inducible system as for NUP98-CDX1 before (Figure 17a). Tet-On system is ideally suited for this purpose because expression of the transcription factor can be precisely controlled temporally. The only drawback is that cells have to be expanded for 8 days, instead of the usual 2 days, to obtain enough cells for sorting before RNA isolation. Nevertheless, since NUP98-HOXA10 performed equally well as NUP98-CDX1 in the rescue experiments shown in Figure 12 to established lines that are essentially indistinguishable to those transduced at day 2 (data not shown), the effects of this time lapse, if any, are minimal.

5-FU BM cells were transduced with the inducible NUP98-HOXA10 (hereafter referred to as iNA10) and expanded without induction for 8 days. On day 8, transduced cells were induced by 1 μ g/ml DOX for 16 hours. EGFP⁺ and EGFP⁻ cells were sorted on day 9. Half of the sorted EGFP⁺ cells were immediately lysed in Trizol for RNA isolation as the early time point; the other half were grown in culture with continuous DOX induction until day

16. On day 16, RNA was isolated from the expanded EGFP⁺ cells; this was taken as the late time point (Figure 17a). The experiment was repeated for three independent iNA10 lines.

Figure 17 Microarray experimental design and results.

a. Experimental design of microarray experiment using the Tet-On inducible system. BM cells from 5-FU treated mice were isolated and stimulated for 2 days before transduction with iNA10 construct. The bulk culture was then expanded for 8 days. At day 8, DOX was added to the culture to induce NUP98-HOXA10 expression. 16 hours (day 9) after induction, EGFP⁻ and EGFP⁺ cells were sorted twice to ensure close to 100% purity. Sorted EGFP⁻ cells and half of the sorted EGFP⁺ cells were immediately lysed in Trizol for RNA isolation; the other half of EGFP⁺ cells were continuously cultured in DOX-containing medium for one more week before RNA isolation.

b. Number of DEGs at early (*left*) and late time points (*right*) at 1.5-fold threshold.

One-channel microarray was then performed using Illumina Expression BeadChip and the data were normalized and filtered using AMDA software. Two principle comparisons were performed to identify differentially expressed genes (DEG). The first is the comparison between the EGFP⁺ and EGFP⁻ populations at the early time point to see the direct targets of NUP98-HOXA10. The second comparison is between the late time point and the early time point EGFP⁺ cells to study the established transcriptome.

As shown in Figure 17b, overexpression of NUP98-HOXA10 immediately upregulates 57 genes. Among these 57 genes are cytokines, transmembrane receptors, enzymes and transcription factors (Table 9). Notably, the majority of transcription factors upregulated are *HOX* genes, *HOXA5*, *HOXA7*, *HOXA9* and *HOXB5*, consistent with extensive cross-regulation among *HOX* genes.

Table 9 DEGs at early time point

Log Ratio	Symbol	Type(s)
1.865	CXCL3	cytokine
1.203	IL1B	cytokine
1.015	CXCL3	cytokine
0.807	CXCL16	cytokine
2.436	GPNMB	enzyme
1.264	GPNMB	enzyme
0.945	IL4I1	enzyme
0.834	PLA2G7	enzyme
0.836	EMR1	G-protein coupled receptor
0.625	EMR1	G-protein coupled receptor
0.607	SGK1	kinase
1.552	C1QB	other
1.404	MS4A7	other
0.985	GM11428	other
0.907	EMP1	other
0.9	C1QC	other
0.878	IRG1	other
0.84	DAB2	other
0.778	OLFM1	other
0.741	MYL10	other
0.685	SLPI	other
0.674	CLEC4B1	other

Log Ratio	Symbol	Type(s)
0.65	LPXN	other
0.645	MANSC1	other
0.641	CD33	other
0.633	MS4A7	other
0.605	OLFM1	other
0.588	H2AFY	other
1.071	MMP14	peptidase
0.885	LGMN	peptidase
0.587	CTSS	peptidase
0.948	EYA1	phosphatase
1.402	HOXA5	transcription regulator
1.062	HOXA7	transcription regulator
0.951	HOXB5	transcription regulator
0.946	HOXA9	transcription regulator
0.806	HOXA9	transcription regulator
0.693	MEF2C	transcription regulator
0.606	MEF2C	transcription regulator
1.184	CD74	transmembrane receptor
1.107	CD74	transmembrane receptor
0.794	CD14	transmembrane receptor
0.652	CD74	transmembrane receptor
0.884	SAA1	transporter

Comparison of the late time point with early time point revealed 345 upregulated genes and surprisingly 467 downregulated genes at 1.5-fold threshold (Figure 17b). However, this number decreased to 47 up-regulated genes and 200 down-regulated genes when the threshold was changed to 2-fold. This suggests that HSC self-renewal is an active process that requires the suppression of large number of genes.

To make sense of the data from a larger perspective, instead of looking at single genes, we mapped all down-regulated genes onto pathways using Ingenuity Pathway Analysis (IPA) software. Interestingly, our analysis shows that the aryl hydrocarbon receptor (AhR) pathway was the most significantly repressed pathway (Table 10). AhR is a ligand-activated transcription factor that mediate the effects of the xenobiotic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), in the meantime activating the transcription of metabolizing enzymes like cytochrome P450 (Mimura and Fujii-Kuriyama 2003). Previous studies have suggested the involvement of this pathway in HSC biology; specifically, treatment of TCDD caused an expansion of phenotypical HSCs, but abolished their long-term reconstituting activities (Sakai, Kajiume et al. 2003; Singh, Casado et al. 2009; Singh, Wyman et al. 2009; Casado, Singh et al. 2010). Inhibitors of this pathway are commercially available. Therefore, we decided to pursue this pathway further.

Table 10 Top down-regulated pathways

Ingenuity Canonical Pathways	Molecules
Aryl Hydrocarbon Receptor Signaling	TGM2,FOS,MGST1,JUN,MGST2,GSTM5,IL1B,ALDH3B1
LPS/IL-1 Mediated Inhibition of RXR Function	MGST1,JUN,MGST2,GSTM5,CD14,ALAS1,IL1B,ALDH3B1,PAPSS2
Acute Phase Response Signaling	FOS,HP,JUN,C3,AMBP,SERPINF1,IL1B,SAA1
MIF Regulation of Innate Immunity	FOS,JUN,CD14,CD74
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	TLR2,OAS1,C3,IL1B,C1QB
Role of NFAT in Regulation of the Immune Response	FOS,JUN,HLA-DQA1,MS4A2,FCER1A,HLA-DRB1,FCGR3A
Toll-like Receptor Signaling	TLR2,FOS,JUN,CD14
Communication between Innate and Adaptive Immune Cells	TLR2,CCL4,CCL3L3,HLA-DRB1,IL1B
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	TLR2,FOS,HP,JUN,OSM,IL1B,FCGR3A,CEBPE,PRKCB
IL-10 Signaling	FOS,JUN,CD14,IL1B

Chapter 6 AhR inhibitor

6.1 AhR inhibitor culture

To study the effects of AhR inhibition on primitive BM cells, we treated 5-FU conditioned bone marrow cells with different concentrations of the AhR inhibitor CH223191 in S6 medium and monitored their growths *in vitro* extensively by cell counting and immunostaining. It is noteworthy that there is an initial lag period of around one week where AhR inhibitor does not have any obvious effects on growth. After 8 days, as shown in Figure 18a, AhR inhibitor dramatically enhanced the growth of primitive cells; combined with another two independent experiments, we found that the optimal concentration for AhR is 1 μ M. Higher concentration of AhR inhibitor (4 μ M) sensitized the cells to apoptosis. Remarkably, immunostaining of cells of the AhR inhibitor cultures showed that they were capable of maintaining a CD150⁺CD48⁻ and CD34⁻Flt3⁻ primitive population at much higher frequencies as compared to the DMSO control (Figure 18b). AhR inhibitor also repressed Sca-1 expression. However, as mentioned earlier, Sca-1 is not a reliable marker for *in vitro* expanded bone marrow cells.

Figure 18. *In vitro* characterization of AhR inhibitor culture.

BM cells from 5-FU treated mice were immediately culture in S6 medium containing DMSO (0.1% v/v) or 0.5 μ M, 0.75 μ M, or 1 μ M of AhR inhibitor CH223191.

a. Growth curves of DMSO and AhR inhibitor cultures. Cells were split and counted every 2-3 days and followed for 2 months.

b. Phenotypes of DMSO and AhR inhibitor cultures after 3 weeks of culture with the indicated stem cell markers.

6.2 AhR inhibitor culture reconstitution

Bone marrow cell culture at day 14 still contains a significant amount of HSC under our culture conditions, although dramatically decreased compared to the initiating cells. To study how well the AhR inhibitor expanded culture maintains HSC activity at this time point, we transplanted equal amount of day 14 AhR inhibitor expanded cells and freshly isolated cells (1:1 ratio, 2×10^5 each). Since cells in the DMSO culture did not grow as well as those in the AhR culture and did not show a preferable stem cell phenotype *in vitro*, we injected 5 times more cells, i.e., 1×10^6 cells. Using the growth curves in Figure 18a, we estimated that these correspond to 253 starting cells in AhR inhibitor culture and 8299 starting cells in DMSO culture. Figure 19 shows that injection of 253 starting cells from

AhR inhibitor culture generated comparable chimerism to 8299 starting cells from DMSO culture. This effect was even more dramatic at late time point, since as few as 1 cell equivalent of starting cells cultured with AhR inhibitor (3×10^6 cells at the time of injection) performed similarly to 158 cell equivalent of starting cells cultured in DMSO (3×10^6 cells at the time of injection). This shows that AhR inhibitor can expand HSCs *in vitro* for at least 30-100 fold compared to controls.

Figure 19 Overall chimerism of recipients that received day 14 and day 33 DMSO and AhR inhibitor cultured cells 4 months earlier.

Freshly isolated competitor cells ($\text{Ly5.2}^+, 2 \times 10^5$) were co-injected with cells cultured for either 14 days (2×10^5 for AhR inhibitor cultured cells, 1×10^6 for DMSO cultured cells) or 33 days (3×10^6 each for AhR inhibitor culture and DMSO culture) into lethally irradiated mice. Shown here was the contribution of donor cells (Ly5.1^+) in either total nucleated cells or different hematopoietic lineages in the peripheral blood of recipient mice. Thy1.2 for T cell; B220 for B cell; Mac-1/Gr-1 for myeloid cells.

Chapter 7 Discussion and Future Work

7.1 NUP98-CDX1

Chromosomal translocations are often initiating events in leukemia (Mitelman, Johansson et al. 2007). However, these first genetic hits can lead to long pre-leukemic phases where affected cells continue to follow normal physiological rules sometimes *ad infinitum* without progression to leukemia (Greaves and Wiemels 2003). Previous studies about the HSC expansion or leukemogenic properties of NUP98-homeodomain fusions were largely confined to the clustered *HOX* genes; and the *Abd-B*-like group were believed to have superior HSC expansion function compared to other paralogous groups (Abramovich, Pineault et al. 2005). These studies also suggest that there are significant overlaps between the *HOX* target genes and the HSC self-renewal circuitry, making them great candidates as tools for studying self-renewal of HSC, as well as leukemia stem cell (LSC). Here we have studied if different NUP98-*HOX* fusions that mimic oncogenic translocations in AMLs can induce physiologically “normal” pre-leukemic stem cells. Remarkably, we found that NUP98-CDX1 fusion could turn BM progenitors into cells with attributes of normal HSC. While the frequency of HSC is about 1/2000 (Szilvassy, Humphries et al. 1990) in the BM cells from 5-FU treated mice, we found that more than 1% of the NUP98-CDX1 transduced cells read out as HSC in long-term *in vivo* competitive repopulation assays even after massive *in vitro* expansion (c.a. 10^{18} fold). NUP98-CDX1 compared favorably to “gold standards”, NUP98-HOXB4 and -HOXA10 (Ohta, Sekulovic et al. 2007), sustaining most balanced HSC activity as reflected in physiological distribution of reconstituted lineages in all tested hematopoietic compartments. Hence we

think that potential translocations producing NUP98-CDX1 fusion proteins in human hematopoietic progenitors could be followed by long pre-leukemic phase. We have not observed leukemia in mice receiving NUP98-CDX1 transduced cells after 10 months, but undoubtedly, longer observation periods could reveal their leukemogenic potential, similarly like in the case of HOXB4 transduced cells in larger animal models (Zhang, Beard et al. 2008; Thakar, Zhang et al. 2009).

Interestingly, NUP98-CDX1 can rescue BM cells kept in culture for three weeks and about to cease their vigorous growth (normal fate of BM cells *in vitro* in our conditions) and confer these cells with high HSC activity comparable to that derived from fresh BM cells. However, we have not yet been able to measure the actual reprogramming frequency. Although the initial transduction frequency of these cultured cells is in usual range (c.a. 50%), we estimated the frequency of reprogramming to be somewhere between 1-10% based on the delays of the initiation of vigorous growth. It is certainly worthy to find out the cell types within the hematopoietic system that can be reprogrammed by NUP98-CDX1. However, better experimental approaches such as transgenic, retrovirus independent, inducible systems have to be developed to robustly explore these cellular limits.

Curiously, continual expression of NUP98-CDX1, at the original level, allowed normal differentiation of transduced cells in all tested lymphoid and myeloid compartments. This observation was based on BM cells transduced with 2A construct that reports the co-expression of NUP98-CDX1/EGFP proteins more reliably than conventional IRES containing vectors (de Felipe, Luke et al. 2006). However, there were clear signs for selection against the NUP98-CDX1 expression in mature lymphocytes. It will be

interesting to determine how the transcriptional circuitry of lymphoid development “tolerates” the presence of stem cell friendly NUP98-CDX1 that is able to reprogram committed cells to certain extent.

It is also clear that continual expression of NUP98-CDX1 is required to sustain the high HSC activity in the culture and *in vivo*. Switching off of the expression of NUP98-CDX1 in cultured cells led to almost immediate differentiation to adherent cells and the cease of growth factor dependent expansion. *In vivo* only a weak and transient wave of hematopoiesis could be observed following the switch-off. Clearly, NUP98-CDX1 establishes a modified transcriptional circuitry that can support HSC self-renewal without leaving permanent epigenetic marks that confer these activities.

Surprisingly, the ectopic expression of NUP98-CDX4 in mouse BM cells, unlike the related NUP98-CDX1/CDX2, induced AML with short latency. NUP98-CDX4 seems to be the most aggressive inducer of leukemia when compared to other overexpressed HOX or NUP98-HOX factors in mouse models in the absence of co-expressed MEIS1A co-factor (Pineault, Abramovich et al. 2004; Argiropoulos and Humphries 2007). Notably, aberrant expression of CDX2 is very common in human leukemia (Scholl, Bansal et al. 2007; Thoene, Rawat et al. 2009) and this is thought to be due to its ability to boost the expression of “leukemic” HOX transcription factors that alone, in translocations or otherwise, are only rarely primary drivers. Indeed, ectopic expression of intact CDX2, but not NUP98-CDX2 (unpublished observations), in mouse BM cells induces leukemia with kinetics approaching to that of NUP98-CDX4 (Rawat, Cusan et al. 2004).

However, CDX4 is only rarely dysregulated as compared to CDX2 in human AMLs (Bansal, Scholl et al. 2006); and perhaps consistently, overexpression of CDX4 in mouse BM cells induced leukemia with partial penetrance and long latency in the absence of co-expressed MEIS1A co-factor (Bansal, Scholl et al. 2006).

It is puzzling why NUP98-CDX4 fusions have not been reported in human AMLs while many other NUP98-HOX fusions can be recurrently found, albeit rather rarely (Slape and Apland 2004). It is possible that “*NUP98*” translocations involving *CDX4* occur only extremely rarely, likely due to the location of *CDX4* locus close to the centromeric region of X chromosome. Indeed, recent evidence suggests that translocations are not necessarily random (with subsequent selection) but biased due to the chromosomal locations of the fusion partners (Mani and Chinnaiyan 2010; Zhang, Gostissa et al. 2010). Similar phenomenon was observed in the case of NUP98-HOXA10, whose upstream (HOXA11 and HOXA13) and downstream (HOXA9) paralogs have been found fused to NUP98 in a single leukemia, whereas HOXA10 itself is somehow spared (Fujino, Suzuki et al. 2002). However, it is also possible that NUP98-CDX4 fusions are not leukemogenic in humans and interestingly, human and mouse CDX4 homeodomains differ in a single position (Q/R substitution) in the middle of homeodomain, at the beginning of helix II (Figure 20a). Future experiments should clarify this possibility.

What is puzzling also is that CDX1 and CDX4 caused such dramatic difference in reconstitution phenotypes, although their homeodomains are highly similar (Figure 20b), except in the loop-helix II-turn region. Our domain-swapping experiment indicates that this region is a critical determinant of the effects of NUP98-HOX/CDX that fine-tunes the functional properties of the fusion proteins (data not shown). This is similar to the

paralogous group 4 HOX genes, where the difference is even more diminutive (Figure 21c), but NUP98-HOXB4 behaved very differently as compared to NUP98-HOXC4 (Figure 5).

Our transcriptional profiling of NUP98-CDX1 and NUP98-CDX4 shows that genes regulated by NUP98-CDX1 are largely a subset of NUP98-CDX4 (data not shown). Amazingly, switching off of NUP98-CDX4 in leukemic cells seemed to return these cells into normal, physiological HSC (Figure 14b), in contrast to NUP98-CDX1. This is exciting because one can envision the scenario where HSCs are given pulses of NUP98-CDX4 stimulation to achieve maximal expansion while still retaining their HSC functional properties when expression of transgene is shut-off. However, the use of only one mouse and the inability to exclude the possibility that low level expression of NUP98-CDX4 (undetectable by FACS) is still present in this mouse prevent us from reaching any solid conclusion. Ongoing experiment in the lab is confirming this phenomenon with larger group of mice and eventually serial transplantation will be conducted to confirm the presence of “real” HSCs in these mice.

Figure 20. Sequence alignment of different homeodomain proteins.

- Protein sequence alignment of human and mouse CDX4 homeodomains. Secondary structures are shown at the top of the alignments.
- Protein sequence alignment of CDX1 and CDX4 homeodomains.
- Protein sequence alignment of HOXB4 and HOXC4 homeodomains.

It is noteworthy that the biological role of NUP98 part in the fusion products is not clear. There are some evidences that it could act as a transactivator (Kasper, Brindle et al. 1999) and interestingly very recent observations in *Drosophila* showed that NUP98 overexpression as such had general growth promoting transcriptional effects (Capelson, Liang et al. 2010; Kalverda, Pickersgill et al. 2010). Our own attempts to find “NUP98 alone” growth promoting effects in mouse BM cells have so far failed. In addition in our hands VP16, classical transactivator, cannot replace NUP98 in our constructs. BM cell transduced with VP16-CDX1 are “immortalized” *in vitro* and grew robustly in growth factor dependent way but show no *in vivo* HSC activity.

HSC and LSC share considerably similar molecular circuitry, especially in terms of self-renewal (Moore 2005; Rizo, Vellenga et al. 2006). Therefore, the NUP98-CDX1/4 pair

described here will give us now opportunity to dissect the molecular pathways of stem cell self-renewal and to look more closely at the genetic regulation of different stages of leukemogenesis.

7.2 NUP98-HOXA10

Lineage commitment in the hematopoietic system is a gradual process whereby progenitor cells gradually acquire lineage specific gene expression while repressing expression of genes specific to other lineages. It follows that cells during the process of differentiation must retain different degrees of flexibility with respect to lineage choices and the underlying gene expression. One extreme example is HSC, which maintains a promiscuous expression of lineage specific genes or keeps them in an epigenetically favorable state for expression, a phenomenon called lineage priming (Miyamoto, Iwasaki et al. 2002; Månsson, Hultquist et al. 2007). However, the best example of lineage conversion is illustrated by B cell lineage. Loss of PAX5 from either B cell progenitor or mature B cells can generate more primitive cells that have the potential to differentiate into T cell lineage *in vivo* and myeloid lineage *in vitro* (Nutt, Heavey et al. 1999; Rolink, Nutt et al. 1999; Cobaleda, Jochum et al. 2007). Moreover, overexpression of C/EBP α or C/EBP β can re-program mature B cells into macrophages (Xie, Ye et al. 2004) and constitutively active β -catenin can confer lymphoid and myeloid progenitors with multipotentiality (Baba, Garrett et al. 2005). However, in none of these studies can multipotentiality be demonstrated robustly *in vivo*. Our studies with NUP98-HOXA10 overexpression in B cell progenitors showed for the first time that reprogrammed B cell progenitors can differentiate into all hematopoietic lineages after 4 months. Importantly, the reprogrammed cells had the ability to differentiate into the megakaryocytic/erythroid

lineage, which branches from the MPP even earlier than the lymphoid/myeloid lineage (Iwasaki and Akashi 2007), further suggesting the existence of HSC in the reprogrammed donor cells.

The *in vitro* growth kinetics of the reprogrammed culture suggested that conversion of differentiated cells into multipotent cells is a slow and probably inefficient process because it occurs over 2-week period with only slow cell proliferation. Due to heterogeneity of the B220⁺ cells, we were unable to determine the population targeted by NUP98-HOXA10, nor could we determine the frequency of reprogramming. However, the IL-7-dependent nature of the reprogramming process and the D-J, but not V-D-J, rearrangement detected in established lines, strongly suggest that pro-B cells or earlier stages (Balciunaite, Ceredig et al. 2005) were targeted. It is interesting to note that the EGFP (hence the transgene) expression profiles between the B220⁺S6/S67 and B220⁻ donor cells were dramatically different (Figure 16c), further emphasizing their different origins. Although very unlikely, given the above-mentioned growth and reconstitution profiles, it is still formally possible that a small contamination of B220⁻ cells present in the sorted cells were also transduced and the established lines were a composite of B220⁺- and B220⁻-derived cells. Further studies (ongoing) using transgenic animals with inducible expression of NUP98-HOXA10 should be able to address these questions more adequately.

7.3 Small molecule inhibitors

Although transcription factor overexpression enables great expansion of HSC *in vitro*, ideally any *in vitro* manipulations should leave the genome intact (although in many cases the epigenome is more difficult to predict), due to concerns about retrovirus integration-induced leukemia, especially when it comes to clinical applications (Baum, Düllmann et al.

2003; Baum, von Kalle et al. 2004). Thus, the ultimate goal in pursuing HSC expansion *in vitro* using different protocols is to devise conditions that are capable of inducing extensive HSC self-renewal comparable to that induced by transcription factor overexpression. Small molecule chemicals are useful addition towards this goal. One early example is the inhibitor of GSK-3 β , which acts downstream of key HSC maintenance pathways Wnt, Hedgehog and Notch. Inhibition of this kinase has been shown to increase the output of HSC and enhance engraftment of *ex vivo* expanded HSCs (Trowbridge, Xenocostas et al. 2006; Ko, Holmes et al. 2011). However, in these studies, although engraftment enhancement was seen, there was little evidence of HSC expansion. Here we used a chemical inhibitor of AhR and showed that the inhibitor not only caused an expansion of phenotypically immature cells *in vitro*, but also enhanced their *in vivo* multi-lineage reconstitution abilities. This was recently confirmed by a study reporting that inhibition of AhR pathway can expand human cord blood HSCs as many as 17 fold over 3 weeks (Boitano, Wang et al. 2010). Our results show that the expansion in murine cells is likely to be more dramatic and ongoing experiments are quantifying the extent of expansion, especially in association with other cytokine components, with the hope that a “magic” cocktail can be obtained that enables maximal expansion of HSCs *in vitro*.

Conclusion

Our current knowledge about HSC self-renewal is not yet sufficient to allow us to expand HSC in a safe and efficient way. By the same token, lack of knowledge about LSC self-renewal also hampers the development of effective therapies. Expansion of knowledge can only be achieved with appropriate tools. In this study, we opted to use an overexpression system for these purposes. The advantage of using such genetic manipulations to expand HSC is that with the overexpression of transcription factors, the whole transcriptome could be swept towards a self-renewal dominated signature--the effects can be so dramatic that the manipulated HSC/progenitors are practically “immortalized”, as shown here and in other studies (Zhang, Schwartz et al. 2007). Although different NUP98-HOX fusions like NUP98-HOXB4, NUP98-HOXA10, NUP98-CDX1 and NUP98-CDX4 have overlapping functions in terms of HSC expansion and leukemogenesis, it is clear that they function in different ways, since they have different *in vitro* growth profiles, different latencies of leukemogenesis (Pineault, Abramovich et al. 2004) and enable different extents of HSC expansion. Therefore, comparison of transcriptomes commanded by these different fusions will set the stage for us to identify the common “core” program that underlies extensive HSC and/or LSC self-renewal, a step that is essential for understanding the basic biology of HSC and LSC, which is in turn a prerequisite for efficient and safe clinical expansion of HSC, as well as development of new treatment regimens for leukemia. With the mechanisms of HSC self-renewal better understood and the niche signals dissected, together with the advent of new systems and technologies that allow high-throughput screening of biologically relevant molecules, it is conceivable that

more effective (perhaps more complex) cocktails, which may include chemicals like the AhR inhibitor, can be devised that allows for safe expansion of HSCs *in vitro* for clinical applications, as well as applications beyond that.

Publications

Zhi-Hao Wu, Shuzk-Cheng Loh, Christiane Ruedl, Klaus Karjalainen. **First genetic hits inducing “pre-leukemic” stem cells can support extensive HSC-like self-renewal** (Submitted)

Zhi-Hao Wu, Jin-miao Chen, Shuzk-Cheng Loh, Christiane Ruedl, Klaus Karjalainen. **Identification of the “core” self-renewal signature of hematopoietic stem cells** (In preparation)

References

- Abramovich, C., N. Pineault, et al. (2005). "Hox Genes: From Leukemia to Hematopoietic Stem Cell Expansion." Annals of the New York Academy of Sciences **1044**(1): 109-116.
- Adams, G. B., K. T. Chabner, et al. (2006). "Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor." Nature **439**(7076): 599-603.
- Adolfsson, J., O. J. Borge, et al. (2001). "Upregulation of Flt3 Expression within the Bone Marrow Lin Sca1+c-kit+ Stem Cell Compartment Is Accompanied by Loss of Self-Renewal Capacity." Immunity **15**(4): 659-669.
- Aguilo, F., S. Avagyan, et al. (2011). "Prdm16 is a physiological regulator of hematopoietic stem cells." Blood.
- Akashi, K., D. Traver, et al. (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**(6774): 193-197.
- Amsellem, S., F. Pflumio, et al. (2003). "Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein." Nat Med **9**(11): 1423-1427.
- Antonchuk, J., G. Sauvageau, et al. (2001). "HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation." Experimental hematology **29**(9): 1125-1134.
- Antonchuk, J., G. Sauvageau, et al. (2002). "HOXB4-Induced Expansion of Adult Hematopoietic Stem Cells Ex Vivo." Cell **109**(1): 39-45.
- Arai, F., A. Hirao, et al. (2004). "Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche." Cell **118**(2): 149-161.
- Araki, H., N. Mahmud, et al. (2006). "Expansion of human umbilical cord blood SCID-repopulating cells using chromatin-modifying agents." Experimental hematology **34**(2): 140-149.
- Argiropoulos, B. and R. K. Humphries (2007). "Hox genes in hematopoiesis and leukemogenesis." Oncogene **26**(47): 6766-6776.
- Attema, J. L., P. Papathanasiou, et al. (2007). "Epigenetic characterization of hematopoietic stem cell differentiation using miniChIP and bisulfite sequencing analysis." Proceedings of the National Academy of Sciences **104**(30): 12371-12376.
- Audet, J., C. L. Miller, et al. (2001). "Distinct Role of gp130 Activation in Promoting Self-Renewal Divisions by Mitogenically Stimulated Murine Hematopoietic Stem Cells." Proceedings of the National Academy of Sciences of the United States of America **98**(4): 1757-1762.
- Baba, Y., K. P. Garrett, et al. (2005). "Constitutively Active β -Catenin Confers Multilineage Differentiation Potential on Lymphoid and Myeloid Progenitors." Immunity **23**(6): 599-609.
- Baena, E., M. Ortiz, et al. (2007). "c-Myc is essential for hematopoietic stem cell differentiation and regulates Lin-Sca-1+c-Kit- cell generation through p21." Experimental hematology **35**(9): 1333-1343.
- Balazs, A. B., A. J. Fabian, et al. (2006). "Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow." Blood **107**(6): 2317-2321.
- Balciunaite, G., R. Ceredig, et al. (2005). "A B220+ CD117+ CD19 $\pm$ hematopoietic progenitor with potent lymphoid and myeloid developmental potential." European Journal of Immunology **35**(7): 2019-2030.
- Banerjee-Basu, S. and A. D. Baxevanis (2001). "Molecular evolution of the homeodomain family of transcription factors." Nucleic Acids Research **29**(15): 3258-3269.

- Bansal, D., C. Scholl, et al. (2006). "Cdx4 dysregulates Hox gene expression and generates acute myeloid leukemia alone and in cooperation with Meis1a in a murine model." Proceedings of the National Academy of Sciences **103**(45): 16924-16929.
- Barker, J. E. (1994). "Sl/Sl(d) hematopoietic progenitors are deficient in situ." Experimental hematology **22**(2): 174-177.
- Baum, C., J. Düllmann, et al. (2003). "Side effects of retroviral gene transfer into hematopoietic stem cells." Blood **101**(6): 2099-2113.
- Baum, C., C. von Kalle, et al. (2004). "Chance or necessity? Insertional Mutagenesis in Gene Therapy and Its Consequences." Mol Ther **9**(1): 5-13.
- Benveniste, P., C. Cantin, et al. (2003). "Hematopoietic stem cells engraft in mice with absolute efficiency." Nat Immunol **4**(7): 708-713.
- Bersenev, A., C. Wu, et al. (2008). "Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2." The Journal of Clinical Investigation **118**(8): 2832-2844.
- Beslu, N., J. Kros, et al. (2004). "Molecular interactions involved in HOXB4-induced activation of HSC self-renewal." Blood **104**(8): 2307-2314.
- Bhardwaj, G., B. Murdoch, et al. (2001). "Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation." Nat Immunol **2**(2): 172-180.
- Bhatia, M., D. Bonnet, et al. (1997). "Quantitative Analysis Reveals Expansion of Human Hematopoietic Repopulating Cells After Short-term Ex Vivo Culture." The Journal of Experimental Medicine **186**(4): 619-624.
- Bhatia, M., D. Bonnet, et al. (1999). "Bone Morphogenetic Proteins Regulate the Developmental Program of Human Hematopoietic Stem Cells." The Journal of Experimental Medicine **189**(7): 1139-1148.
- Bhattacharya, D., A. Czechowicz, et al. (2009). "Niche recycling through division-independent egress of hematopoietic stem cells." The Journal of Experimental Medicine **206**(12): 2837-2850.
- Bijl, J., A. Thompson, et al. (2006). "Analysis of HSC activity and compensatory Hox gene expression profile in Hoxb cluster mutant fetal liver cells." Blood **108**(1): 116-122.
- Björnsson, J. M., N. Larsson, et al. (2003). "Reduced Proliferative Capacity of Hematopoietic Stem Cells Deficient in Hoxb3 and Hoxb4." Mol. Cell. Biol. **23**(11): 3872-3883.
- Boitano, A. E., J. Wang, et al. (2010). "Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells." Science **329**(5997): 1345-1348.
- Borrow, J., A. M. Shearman, et al. (1996). "The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP96 and class I homeoprotein HOXA9." Nat Genet **12**(2): 159-167.
- Bowie, M. B., D. G. Kent, et al. (2007). "Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties." Proceedings of the National Academy of Sciences **104**(14): 5878-5882.
- Bowie, M. B., K. D. McKnight, et al. (2006). "Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect." The Journal of Clinical Investigation **116**(10): 2808-2816.
- Bradford, S. B., T. A. Graubert, et al. (2005). "Roles of Sca-1 in hematopoietic stem/progenitor cell function." Experimental hematology **33**(7): 836-843.
- Brun, A. C. M., J. M. Björnsson, et al. (2004). "Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells." Blood **103**(11): 4126-4133.

- Burglin, T. (2005). "Homeodomain proteins." Encyclopedia of Molecular Cell Biology and Molecular Medicine **6**: 179 - 222.
- Buske, C., M. Feuring-Buske, et al. (2002). "Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells." Blood **100**(3): 862-868.
- Butler, J. M., D. J. Nolan, et al. (2010). "Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells." Cell stem cell **6**(3): 251-264.
- Cai, Z., M. de Bruijn, et al. (2000). "Haploinsufficiency of AML1 Affects the Temporal and Spatial Generation of Hematopoietic Stem Cells in the Mouse Embryo." Immunity **13**(4): 423-431.
- Calvo, K. R., D. B. Sykes, et al. (2002). "Nup98-HoxA9 immortalizes myeloid progenitors, enforces expression of Hoxa9, Hoxa7 and Meis1, and alters cytokine-specific responses in a manner similar to that induced by retroviral co-expression of Hoxa9 and Meis1." Oncogene **21**(27): 4247-4256.
- Capelson, M., Y. Liang, et al. (2010). "Chromatin-Bound Nuclear Pore Components Regulate Gene Expression in Higher Eukaryotes." Cell **140**(3): 372-383.
- Care, A., M. Valtieri, et al. (1999). "Enforced expression of HOXB7 promotes hematopoietic stem cell proliferation and myeloid-restricted progenitor differentiation." Oncogene **18**(11): 1993-2001.
- Casado, F. L., K. P. Singh, et al. (2010). "The aryl hydrocarbon receptor: Regulation of hematopoiesis and involvement in the progression of blood diseases." Blood Cells, Molecules, and Diseases **44**(4): 199-206.
- Challen, G. A., N. C. Boles, et al. (2010). "Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF- β 1." Cell stem cell **6**(3): 265-278.
- Chan, C. K. F., C.-C. Chen, et al. (2009). "Endochondral ossification is required for haematopoietic stem-cell niche formation." Nature **457**(7228): 490-494.
- Chang, C. P., W. F. Shen, et al. (1995). "Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins." Genes & Development **9**(6): 663-674.
- Chang, J., D. Metcalf, et al. (1989). "Nonneoplastic hematopoietic myeloproliferative syndrome induced by dysregulated multi-CSF (IL-3) expression." Blood **73**(6): 1487-1497.
- Cheng, T., N. Rodrigues, et al. (2000). "Stem cell repopulation efficiency but not pool size is governed by p27kip1." Nat Med **6**(11): 1235-1240.
- Cheng, T., N. Rodrigues, et al. (2000). "Hematopoietic Stem Cell Quiescence Maintained by p21cip1/waf1." Science **287**(5459): 1804-1808.
- Cheshier, S. H., S. J. Morrison, et al. (1999). "In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells." Proceedings of the National Academy of Sciences **96**(6): 3120-3125.
- Chow, A., D. Lucas, et al. (2011). "Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche." The Journal of Experimental Medicine **208**(2): 261-271.
- Christensen, J. L. and I. L. Weissman (2001). "Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells." Proceedings of the National Academy of Sciences **98**(25): 14541-14546.
- Chuikov, S., B. P. Levi, et al. (2010). "Prdm16 promotes stem cell maintenance in multiple tissues, partly by regulating oxidative stress." Nat Cell Biol **12**(10): 999-1006.
- Chung, Y.-J., B.-B. Park, et al. (2006). "Unique effects of Stat3 on the early phase of hematopoietic stem cell regeneration." Blood **108**(4): 1208-1215.

- Chung, Y. S., H. J. Kim, et al. (2009). "Undifferentiated hematopoietic cells are characterized by a genome-wide undermethylation dip around the transcription start site and a hierarchical epigenetic plasticity." *Blood* **114**(24): 4968-4978.
- Clontech (2003). "pTRE-Tight Vectors." *Clontechniques* **18**(3): 13-14.
- Cobaleda, C., W. Jochum, et al. (2007). "Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors." *Nature* **449**(7161): 473-477.
- Conneally, E., J. Cashman, et al. (1997). "Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice." *Proceedings of the National Academy of Sciences* **94**(18): 9836-9841.
- Crooks, G. M., J. Fuller, et al. (1999). "Constitutive HOXA5 Expression Inhibits Erythropoiesis and Increases Myelopoiesis From Human Hematopoietic Progenitors." *Blood* **94**(2): 519-528.
- Cully, M., H. You, et al. (2006). "Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis." *Nat Rev Cancer* **6**(3): 184-192.
- Cumano, A. and I. Godin (2007). "Ontogeny of the hematopoietic system." *Annual Review of Immunology* **25**: 745-785.
- Curtis, D. J., M. A. Hall, et al. (2004). "SCL is required for normal function of short-term repopulating hematopoietic stem cells." *Blood* **103**(9): 3342-3348.
- Daga, A., M. Podesta, et al. (2000). "The retroviral transduction of HOXC4 into human CD34+ cells induces an in vitro expansion of clonogenic and early progenitors." *Experimental hematology* **28**(5): 569-574.
- de Felipe, P., G. A. Luke, et al. (2006). "E unum pluribus: multiple proteins from a self-processing polyprotein." *Trends in Biotechnology* **24**(2): 68-75.
- de Haan, G., E. Weersing, et al. (2003). "In Vitro Generation of Long-Term Repopulating Hematopoietic Stem Cells by Fibroblast Growth Factor-1." *Developmental cell* **4**(2): 241-251.
- Dexter, T. M., T. D. Allen, et al. (1977). "Conditions controlling the proliferation of haemopoietic stem cells in vitro." *Journal of Cellular Physiology* **91**(3): 335-344.
- Donnelly, M. L. L., G. Luke, et al. (2001). "Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'." *Journal of General Virology* **82**(5): 1013-1025.
- Duncan, A. W., F. M. Rattis, et al. (2005). "Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance." *Nat Immunol* **6**(3): 314-322.
- Dzierzak, E. and N. A. Speck (2008). "Of lineage and legacy: the development of mammalian hematopoietic stem cells." *Nat Immunol* **9**(2): 129-136.
- Ema, H., K. Sudo, et al. (2005). "Quantification of Self-Renewal Capacity in Single Hematopoietic Stem Cells from Normal and Lnk-Deficient Mice." *Developmental cell* **8**(6): 907-914.
- Fischbach, N. A., S. Rozenfeld, et al. (2005). "HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo." *Blood* **105**(4): 1456-1466.
- Fleming, H. E., V. Janzen, et al. (2008). "Wnt Signaling in the Niche Enforces Hematopoietic Stem Cell Quiescence and Is Necessary to Preserve Self-Renewal In Vivo." *Cell stem cell* **2**(3): 274-283.
- Forsberg, E. C., S. S. Prohaska, et al. (2005). "Differential Expression of Novel Potential Regulators in Hematopoietic Stem Cells." *PLoS Genet* **1**(3): e28.
- Foster, D. A., P. Yellen, et al. (2011). "Regulation of G1 Cell Cycle Progression: Distinguishing the Restriction Point from a Nutrient-Sensing Cell Growth Checkpoint(s)." *Genes & Cancer*.

- Foudi, A., K. Hochedlinger, et al. (2009). "Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells." Nat Biotech **27**(1): 84-90.
- Fraser, C., C. Eaves, et al. (1990). "Expansion in vitro of retrovirally marked totipotent hematopoietic stem cells." Blood **76**(6): 1071-1076.
- Fraser, C. C., S. J. Szilvassy, et al. (1992). "Proliferation of totipotent hematopoietic stem cells in vitro with retention of long-term competitive in vivo reconstituting ability." Proceedings of the National Academy of Sciences **89**(5): 1968-1972.
- Fujino, T., A. Suzuki, et al. (2002). "Single-translocation and double-chimeric transcripts: detection of NUP98-HOXA9 in myeloid leukemias with HOXA11 or HOXA13 breaks of the chromosomal translocation t(7;11)(p15;p15)." Blood **99**(4): 1428-1433.
- Gan, B., J. Hu, et al. (2010). "Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells." Nature **468**(7324): 701-704.
- Gan, O. I., B. Murdoch, et al. (1997). "Differential Maintenance of Primitive Human SCID-Repopulating Cells, Clonogenic Progenitors, and Long-Term Culture-Initiating Cells After Incubation on Human Bone Marrow Stromal Cells." Blood **90**(2): 641-650.
- Gehring, W. J., M. Affolter, et al. (1994). "HOMEODOMAIN PROTEINS." Annual Review of Biochemistry **63**: 487-526.
- Godin, I. and A. Cumano (2002). "The hare and the tortoise: an embryonic haematopoietic race." Nat Rev Immunol **2**(8): 593-604.
- Gordon, M. Y., J. L. Lewis, et al. (2002). "Of mice and men ... and elephants." Blood **100**(13): 4679-4689.
- Greaves, M. F. and J. Wiemels (2003). "Origins of chromosome translocations in childhood leukaemia." Nat Rev Cancer **3**(9): 639-649.
- Growney, J. D., H. Shigematsu, et al. (2005). "Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype." Blood **106**(2): 494-504.
- Gurumurthy, S., S. Z. Xie, et al. (2010). "The Lkb1 metabolic sensor maintains haematopoietic stem cell survival." Nature **468**(7324): 659-663.
- Harrison, D. E. and C. P. Lerner (1991). "Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil." Blood **78**(5): 1237-1240.
- Hatzfeld, J., M. L. Li, et al. (1991). "Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides." The Journal of Experimental Medicine **174**(4): 925-929.
- Helgason, C., G. Sauvageau, et al. (1996). "Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated in vitro." Blood **87**(7): 2740-2749.
- Himburg, H. A., G. G. Muramoto, et al. (2010). "Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells." Nat Med **16**(4): 475-482.
- Hochedlinger, K., Y. Yamada, et al. (2005). "Ectopic Expression of Oct-4 Blocks Progenitor-Cell Differentiation and Causes Dysplasia in Epithelial Tissues." Cell **121**(3): 465-477.
- Hock, H., M. J. Hamblen, et al. (2004). "Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells." Nature **431**(7011): 1002-1007.
- Huber, T. L., V. Kouskoff, et al. (2004). "Haemangioblast commitment is initiated in the primitive streak of the mouse embryo." Nature **432**(7017): 625-630.
- Iscoe, N. N. and K. Nawa (1997). "Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion." Current biology : CB **7**(10): 805-808.
- Ivanova, N. B., J. T. Dimos, et al. (2002). "A Stem Cell Molecular Signature." Science **298**(5593): 601-604.
- Iwama, A., H. Oguro, et al. (2004). "Enhanced Self-Renewal of Hematopoietic Stem Cells Mediated by the Polycomb Gene Product Bmi-1." Immunity **21**(6): 843-851.

- Iwasaki, H. and K. Akashi (2007). "Myeloid Lineage Commitment from the Hematopoietic Stem Cell." *Immunity* **26**(6): 726-740.
- Izon, D. J., S. Rozenfeld, et al. (1998). "Loss of Function of the Homeobox Gene Hoxa-9 Perturbs Early T-Cell Development and Induces Apoptosis in Primitive Thymocytes." *Blood* **92**(2): 383-393.
- Jacob, B., M. Osato, et al. (2010). "Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis." *Blood* **115**(8): 1610-1620.
- Kalverda, B., H. Pickersgill, et al. (2010). "Nucleoporins Directly Stimulate Expression of Developmental and Cell-Cycle Genes Inside the Nucleoplasm." *Cell* **140**(3): 360-371.
- Kasper, L. H., P. K. Brindle, et al. (1999). "CREB Binding Protein Interacts with Nucleoporin-Specific FG Repeats That Activate Transcription and Mediate NUP98-HOXA9 Oncogenicity." *Mol. Cell. Biol.* **19**(1): 764-776.
- Kato, Y., A. Iwama, et al. (2005). "Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis." *The Journal of Experimental Medicine* **202**(1): 169-179.
- Keller, J. R., I. K. McNiece, et al. (1990). "Transforming growth factor β directly regulates primitive murine hematopoietic cell proliferation." *Blood* **75**(3): 596-602.
- Kent, D. G., M. R. Copley, et al. (2009). "Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential." *Blood* **113**(25): 6342-6350.
- Kerppola, T. K. (2009). "Polycomb group complexes - many combinations, many functions." *Trends in Cell Biology* **19**(12): 692-704.
- Kiel, M. J., Ö. H. Yilmaz, et al. (2005). "SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells." *Cell* **121**(7): 1109-1121.
- Kim, I., T. L. Saunders, et al. (2007). "Sox17 Dependence Distinguishes the Transcriptional Regulation of Fetal from Adult Hematopoietic Stem Cells." *Cell* **130**(3): 470-483.
- Kim, J. Y., A. Sawada, et al. (2004). "Defective long-term repopulating ability in hematopoietic stem cells lacking the Polycomb-group gene *rae28*." *European Journal of Haematology* **73**(2): 75-84.
- Ko, K.-H., T. Holmes, et al. (2011). "GSK-3 β Inhibition Promotes Engraftment of Ex Vivo-Expanded Hematopoietic Stem Cells and Modulates Gene Expression." *STEM CELLS* **29**(1): 108-118.
- Kollet, O., A. Dar, et al. (2006). "Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells." *Nat Med* **12**(6): 657-664.
- Kondo, M., A. J. Wagers, et al. (2003). "Biology of hematopoietic stem cells and progenitors: Implications for clinical application." *Annual Review of Immunology* **21**: 759-806.
- Kondo, M., I. L. Weissman, et al. (1997). "Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow." *Cell* **91**(5): 661-672.
- Kroon, E., J. Krosl, et al. (1998). "Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b." *EMBO J* **17**(13): 3714-3725.
- Kroon, E., U. Thorsteinsdottir, et al. (2001). "NUP98-HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice." *EMBO J* **20**(3): 350-361.
- Krosl, J., P. Austin, et al. (2003). "In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein." *Nat Med* **9**(11): 1428-1432.
- Krosl, J., N. Beslu, et al. (2003). "The Competitive Nature of HOXB4-Transduced HSC Is Limited by PBX1: The Generation of Ultra-Competitive Stem Cells Retaining Full Differentiation Potential." *Immunity* **18**(4): 561-571.
- Kunisato, A., S. Chiba, et al. (2003). "HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo." *Blood* **101**(5): 1777-1783.

- Kyba, M., R. C. R. Perlingeiro, et al. (2002). "HoxB4 Confers Definitive Lymphoid-Myeloid Engraftment Potential on Embryonic Stem Cell and Yolk Sac Hematopoietic Progenitors." Cell **109**(1): 29-37.
- La Starza, R., M. Trubia, et al. (2003). "Human homeobox gene HOXC13 is the partner of NUP98 in adult acute myeloid leukemia with t(11;12)(p15;q13)." Genes, Chromosomes and Cancer **36**(4): 420-423.
- Lacorazza, H. D., T. Yamada, et al. (2006). "The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells." Cancer cell **9**(3): 175-187.
- Lancrin, C., P. Sroczynska, et al. (2009). "The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage." Nature **457**(7231): 892-895.
- Larsson, J., U. Blank, et al. (2005). "Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-[beta] signaling in vivo." Experimental hematology **33**(5): 592-596.
- Laurell, E., K. Beck, et al. (2011). "Phosphorylation of Nup98 by Multiple Kinases Is Crucial for NPC Disassembly during Mitotic Entry." Cell **144**(4): 539-550.
- Lawrence, H. J., C. D. Helgason, et al. (1997). "Mice Bearing a Targeted Interruption of the Homeobox Gene HOXA9 Have Defects in Myeloid, Erythroid, and Lymphoid Hematopoiesis." Blood **89**(6): 1922-1930.
- Lewis Wolpert, R. B., Thomas Jessell, Peter Lawrence, Elliot Meyerowitz, Jim Smith (2002). Principles of development, Oxford University Press.
- Lin, Y.-W., C. Sape, et al. (2005). "NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia." Blood **106**(1): 287-295.
- Liu, Y., S. E. Elf, et al. (2009). "p53 Regulates Hematopoietic Stem Cell Quiescence." Cell stem cell **4**(1): 37-48.
- Luis, T. C. and F. J. T. Staal (2009). "WNT Proteins: Environmental Factors Regulating HSC Fate in the Niche." Annals of the New York Academy of Sciences **1176**(1): 70-76.
- Malhotra, S. and P. W. Kincade (2009). "Wnt-Related Molecules and Signaling Pathway Equilibrium in Hematopoiesis." Cell stem cell **4**(1): 27-36.
- Mani, R.-S. and A. M. Chinnaiyan (2010). "Triggers for genomic rearrangements: insights into genomic, cellular and environmental influences." Nat Rev Genet **11**(12): 819-829.
- Månsson, R., A. Hultquist, et al. (2007). "Molecular Evidence for Hierarchical Transcriptional Lineage Priming in Fetal and Adult Stem Cells and Multipotent Progenitors." Immunity **26**(4): 407-419.
- Marshall, C. J., C. Kinnon, et al. (2000). "Polarized expression of bone morphogenetic protein-4 in the human aorta-gonad-mesonephros region." Blood **96**(4): 1591-1593.
- Massberg, S., P. Schaerli, et al. (2007). "Immunosurveillance by Hematopoietic Progenitor Cells Trafficking through Blood, Lymph, and Peripheral Tissues." Cell **131**(5): 994-1008.
- Mendez-Ferrer, S., T. V. Michurina, et al. (2010). "Mesenchymal and haematopoietic stem cells form a unique bone marrow niche." Nature **466**(7308): 829-834.
- Michael P. McGarry, C. A. P. J. J. L. (2010). Mouse Hematology: a laboratory manual. New York, USA, Cold Spring Harbor Laboratory Press.
- Mikkola, H. K. A., J. Klintman, et al. (2003). "Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene." Nature **421**(6922): 547-551.
- Mikkola, H. K. A. and S. H. Orkin (2006). "The journey of developing hematopoietic stem cells." Development **133**(19): 3733-3744.

- Miller, C. L. and C. J. Eaves (1997). "Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability." Proceedings of the National Academy of Sciences **94**(25): 13648-13653.
- Mimura, J. and Y. Fujii-Kuriyama (2003). "Functional role of AhR in the expression of toxic effects by TCDD." Biochimica et Biophysica Acta (BBA) - General Subjects **1619**(3): 263-268.
- Mitelman, F., B. Johansson, et al. (2007). "The impact of translocations and gene fusions on cancer causation." Nat Rev Cancer **7**(4): 233-245.
- Miyamoto, T., H. Iwasaki, et al. (2002). "Myeloid or Lymphoid Promiscuity as a Critical Step in Hematopoietic Lineage Commitment." Developmental cell **3**(1): 137-147.
- Moore, K. A., H. Ema, et al. (1997). "In Vitro Maintenance of Highly Purified, Transplantable Hematopoietic Stem Cells." Blood **89**(12): 4337-4347.
- Moore, M. A. S. (2005). "Converging pathways in leukemogenesis and stem cell self-renewal." Experimental hematology **33**(7): 719-737.
- Moretti, P., P. Simmons, et al. (1994). "Identification of homeobox genes expressed in human haemopoietic progenitor cells." Gene **144**(2): 213-219.
- Morrison, S. J. and I. L. Weissman (1994). "The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype." Immunity **1**(8): 661-673.
- Motoda, L., M. Osato, et al. (2007). "Runx1 Protects Hematopoietic Stem/Progenitor Cells from Oncogenic Insult." STEM CELLS **25**(12): 2976-2986.
- Nagai, Y., K. P. Garrett, et al. (2006). "Toll-like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment." Immunity **24**(6): 801-812.
- Nakada, D., T. L. Saunders, et al. (2010). "Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells." Nature **468**(7324): 653-658.
- Nakamura, T., D. A. Largaespada, et al. (1996). "Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia." Nat Genet **12**(2): 154-158.
- Nemeth, M. J., K. K. Mak, et al. (2009). "β-Catenin Expression in the Bone Marrow Microenvironment Is Required for Long-Term Maintenance of Primitive Hematopoietic Cells." STEM CELLS **27**(5): 1109-1119.
- Nicolini, F. E., J. D. Cashman, et al. (2003). "NOD//SCID mice engineered to express human IL-3, GM-CSF and Steel factor constitutively mobilize engrafted human progenitors and compromise human stem cell regeneration." Leukemia **18**(2): 341-347.
- Nutt, S. L., B. Heavey, et al. (1999). "Commitment to the B-lymphoid lineage depends on the transcription factor Pax5." Nature **401**(6753): 556-562.
- Ohta, H., A. Sawada, et al. (2002). "Polycomb Group Gene rae28 Is Required for Sustaining Activity of Hematopoietic Stem Cells." The Journal of Experimental Medicine **195**(6): 759-770.
- Ohta, H., S. Sekulovic, et al. (2007). "Near-maximal expansions of hematopoietic stem cells in culture using NUP98-HOX fusions." Experimental hematology **35**(5): 817-830.
- Okuda, T., J. van Deursen, et al. (1996). "AML1, the Target of Multiple Chromosomal Translocations in Human Leukemia, Is Essential for Normal Fetal Liver Hematopoiesis." Cell **84**(2): 321-330.
- Omatsu, Y., T. Sugiyama, et al. (2010). "The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche." Immunity **33**(3): 387-399.
- Orford, K. W. and D. T. Scadden (2008). "Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation." Nat Rev Genet **9**(2): 115-128.
- Osawa, M., K.-i. Hanada, et al. (1996). "Long-Term Lymphohematopoietic Reconstitution by a Single CD34-Low/Negative Hematopoietic Stem Cell." Science **273**(5272): 242-245.

- Park, I.-K., S. J. Morrison, et al. (2004). "Bmi1, stem cells, and senescence regulation." The Journal of Clinical Investigation **113**(2): 175-179.
- Park, I.-k., D. Qian, et al. (2003). "Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells." Nature **423**(6937): 302-305.
- Pawliuk, R., C. Eaves, et al. (1996). "Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo." Blood **88**(8): 2852-2858.
- Perkins, A., K. Kongsuwan, et al. (1990). "Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia." Proceedings of the National Academy of Sciences **87**(21): 8398-8402.
- Perkins, A. C. and S. Cory (1993). "CONDITIONAL IMMORTALIZATION OF MOUSE MYELOMONOCYTIC, MEGAKARYOCYTIC AND MAST-CELL PROGENITORS BY THE HOX-2.4 HOMEBOX GENE." Embo Journal **12**(10): 3835-3846.
- Pickart, C. M. (2001). "MECHANISMS UNDERLYING UBIQUITINATION." Annual Review of Biochemistry **70**(1): 503.
- Pineault, N., C. Abramovich, et al. (2004). "Differential and Common Leukemogenic Potentials of Multiple NUP98-Hox Fusion Proteins Alone or with Meis1." Mol. Cell. Biol. **24**(5): 1907-1917.
- Pineault, N., C. Buske, et al. (2003). "Induction of acute myeloid leukemia in mice by the human leukemia-specific fusion gene NUP98-HOXD13 in concert with Meis1." Blood **101**(11): 4529-4538.
- Radu, A., M. S. Moore, et al. (1995). "The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex." Cell **81**(2): 215-222.
- Rathinam, C., L. E. Matesic, et al. (2011). "The E3 ligase Itch is a negative regulator of the homeostasis and function of hematopoietic stem cells." Nat Immunol **12**(5): 399-407.
- Rathinam, C., C. B. F. Thien, et al. (2008). "The E3 ubiquitin ligase c-Cbl restricts development and functions of hematopoietic stem cells." Genes & Development **22**(8): 992-997.
- Rawat, V. P. S., M. Cusan, et al. (2004). "Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia." Proceedings of the National Academy of Sciences of the United States of America **101**(3): 817-822.
- Raza-Egilmez, S. Z., S. N. Jani-Sait, et al. (1998). "NUP98-HOXD13 Gene Fusion in Therapy-related Acute Myelogenous Leukemia." Cancer Research **58**(19): 4269-4273.
- Resnitzky, D., M. Gossen, et al. (1994). "Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system." Mol. Cell. Biol. **14**(3): 1669-1679.
- Reya, T., A. W. Duncan, et al. (2003). "A role for Wnt signalling in self-renewal of haematopoietic stem cells." Nature **423**(6938): 409-414.
- Rizo, A., B. Dontje, et al. (2008). "Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1." Blood **111**(5): 2621-2630.
- Rizo, A., E. Vellenga, et al. (2006). "Signaling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche?" Human Molecular Genetics **15**(suppl 2): R210-R219.
- Robb, L., I. Lyons, et al. (1995). "Absence of Yolk sac Hematopoiesis from Mice with a Targeted Disruption of the scl Gene." Proceedings of the National Academy of Sciences of the United States of America **92**(15): 7075-7079.
- Rolink, A. G., S. L. Nutt, et al. (1999). "Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors." Nature **401**(6753): 603-606.
- Ross, J. and L. Li (2009). "HSC mobilization: new incites and insights." Blood **114**(7): 1283-1284.

- Rossi, D. J. and I. L. Weissman (2006). "Pten, Tumorigenesis, and Stem Cell Self-Renewal." Cell **125**(2): 229-231.
- Ruedl, C., H. J. Khameneh, et al. (2008). "Manipulation of immune system via immortal bone marrow stem cells." International Immunology **20**(9): 1211-1218.
- Sakai, R., T. Kajiume, et al. (2003). "TCDD Treatment Eliminates the Long-Term Reconstitution Activity of Hematopoietic Stem Cells." Toxicological Sciences **72**(1): 84-91.
- Sato, T., N. Onai, et al. (2009). "Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion." Nat Med **15**(6): 696-700.
- Sauvageau, G. and R. K. Humphries (2010). "The Blood Stem Cell Holy Grail?" Science **329**(5997): 1291-1292.
- Sauvageau, G., P. M. Lansdorp, et al. (1994). "Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells." Proceedings of the National Academy of Sciences **91**(25): 12223-12227.
- Sauvageau, G., U. Thorsteinsdottir, et al. (1995). "Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo." Genes & Development **9**(14): 1753-1765.
- Sauvageau, G., U. Thorsteinsdottir, et al. (1997). "Overexpression of HOXB3 in Hematopoietic Cells Causes Defective Lymphoid Development and Progressive Myeloproliferation." Immunity **6**(1): 13-22.
- Scheijen, B., J. Jonkers, et al. (1997). "Characterization of pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and Pim-1 transgenic mice." J. Virol. **71**(1): 9-16.
- Schiedlmeier, B., H. Klump, et al. (2003). "High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation." Blood **101**(5): 1759-1768.
- Scholl, C., D. Bansal, et al. (2007). "The homeobox gene CDX2 is aberrantly expressed in most cases of acute myeloid leukemia and promotes leukemogenesis." The Journal of Clinical Investigation **117**(4): 1037-1048.
- Shen, W.-F., S. Rozenfeld, et al. (1999). "HOXA9 Forms Triple Complexes with PBX2 and MEIS1 in Myeloid Cells." Mol. Cell. Biol. **19**(4): 3051-3061.
- Shen, W.-F., S. Rozenfeld, et al. (1997). "The Abd-B-like Hox Homeodomain Proteins Can Be Subdivided by the Ability to Form Complexes with Pbx1a on a Novel DNA Target." Journal of Biological Chemistry **272**(13): 8198-8206.
- Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes & Development **13**(12): 1501-1512.
- Singh, K. P., F. L. Casado, et al. (2009). "The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations." Biochemical Pharmacology **77**(4): 577-587.
- Singh, K. P., A. Wyman, et al. (2009). "Treatment of mice with the Ah receptor agonist and human carcinogen dioxin results in altered numbers and function of hematopoietic stem cells." Carcinogenesis **30**(1): 11-19.
- Sive, H. L. (1993). "The frog prince-ss: a molecular formula for dorsoventral patterning in *Xenopus*." Genes & Development **7**(1): 1-12.
- Slape, C. and P. D. Aplan (2004). "The role of NUP98 gene fusions in hematologic malignancy." Leukemia & Lymphoma **45**(7): 1341-1350.
- Slape, C., L. Y. Liu, et al. (2008). "Leukemic transformation in mice expressing a NUP98-HOXD13 transgene is accompanied by spontaneous mutations in *Nras*, *Kras*, and *Cbl*." Blood **112**(5): 2017-2019.

- Söderberg, S. S., G. Karlsson, et al. (2009). "Complex and Context Dependent Regulation of Hematopoiesis by TGF- β Superfamily Signaling." Annals of the New York Academy of Sciences **1176**(1): 55-69.
- Soma, T., J. M. Yu, et al. (1996). "Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor- β but not macrophage inflammatory protein-1 α activities." Blood **87**(11): 4561-4567.
- Souroullas, G. P., J. M. Salmon, et al. (2009). "Adult Hematopoietic Stem and Progenitor Cells Require Either Lyl1 or Scl for Survival." Cell stem cell **4**(2): 180-186.
- Stier, S., T. Cheng, et al. (2002). "Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome." Blood **99**(7): 2369-2378.
- Sugiyama, T., H. Kohara, et al. (2006). "Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches." Immunity **25**(6): 977-988.
- Suzuki, T., Y. Yokoyama, et al. (2006). "Highly Efficient Ex Vivo Expansion of Human Hematopoietic Stem Cells Using Delta1-Fc Chimeric Protein." STEM CELLS **24**(11): 2456-2465.
- Szilvassy, S., K. Weller, et al. (1996). "Leukemia inhibitory factor upregulates cytokine expression by a murine stromal cell line enabling the maintenance of highly enriched competitive repopulating stem cells." Blood **87**(11): 4618-4628.
- Szilvassy, S. J., R. K. Humphries, et al. (1990). "Quantitative Assay for Totipotent Reconstituting Hematopoietic Stem-Cells by a Competitive Repopulation Strategy." Proceedings of the National Academy of Sciences of the United States of America **87**(22): 8736-8740.
- Szilvassy, S. J., R. K. Humphries, et al. (1990). "Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy." Proceedings of the National Academy of Sciences **87**(22): 8736-8740.
- Taghon, T., K. Thys, et al. (2003). "Homeobox gene expression profile in human hematopoietic multipotent stem cells and T-cell progenitors: implications for human T-cell development." Leukemia **17**(6): 1157-1163.
- Takaki, S., H. Morita, et al. (2002). "Enhanced Hematopoiesis by Hematopoietic Progenitor Cells Lacking Intracellular Adaptor Protein, Lnk." The Journal of Experimental Medicine **195**(2): 151-160.
- Taketani, T., T. Taki, et al. (2002). "The chromosome translocation t(7;11)(p15;p15) in acute myeloid leukemia results in fusion of the NUP98 gene with a HOXA cluster gene, HOXA13, but not HOXA9." Genes Chromosomes & Cancer **34**(4): 437-443.
- Taketani, T., T. Taki, et al. (2002). "The HOXD11 Gene Is Fused to the NUP98 Gene in Acute Myeloid Leukemia with t(2;11)(q31;p15)." Cancer Research **62**(1): 33-37.
- Taketani, T., T. Taki, et al. (2002). "Novel NUP98-HOXC11 Fusion Gene Resulted from a Chromosomal Break within Exon 1 of HOXC11 in Acute Myeloid Leukemia with t(11;12)(p15;q13)." Cancer Research **62**(16): 4571-4574.
- Takizawa, H., R. R. Regoes, et al. (2011). "Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation." The Journal of Experimental Medicine **208**(2): 273-284.
- Thakar, M. S., X.-B. Zhang, et al. (2009). "Transmission and expansion of HOXB4-induced leukemia in two immunosuppressed dogs: Implications for a new canine leukemia model." Experimental hematology **37**(10): 1157-1166.
- Thoene, S., V. P. S. Rawat, et al. (2009). "The homeobox gene CDX2 is aberrantly expressed and associated with an inferior prognosis in patients with acute lymphoblastic leukemia." Leukemia **23**(4): 649-655.

- Thorsteinsdottir, U., E. Kroon, et al. (2001). "Defining Roles for HOX and MEIS1 Genes in Induction of Acute Myeloid Leukemia." Mol. Cell. Biol. **21**(1): 224-234.
- Thorsteinsdottir, U., G. Sauvageau, et al. (1997). "Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia." Mol. Cell. Biol. **17**(1): 495-505.
- Thorsteinsdottir, U., G. Sauvageau, et al. (1999). "Enhanced In Vivo Regenerative Potential of HOXB4-Transduced Hematopoietic Stem Cells With Regulation of Their Pool Size." Blood **94**(8): 2605-2612.
- Trowbridge, J. J., A. Xenocostas, et al. (2006). "Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation." Nat Med **12**(1): 89-98.
- Ueda, T., K. Tsuji, et al. (2000). "Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor." The Journal of Clinical Investigation **105**(7): 1013-1021.
- Urlinger, S., U. Baron, et al. (2000). "Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity." Proceedings of the National Academy of Sciences of the United States of America **97**(14): 7963-7968.
- van Oostveen, J. W., J. J. Bijl, et al. (1999). "The role of Homeobox genes in normal hematopoiesis and hematological malignancies." Leukemia **13**(11): 1675-1690.
- Varnum-Finney, B., L. Xu, et al. (2000). "Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling." Nat Med **6**(11): 1278-1281.
- Wagner, J. E., J. N. Barker, et al. (2002). "Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival." Blood **100**(5): 1611-1618.
- Wilson, A., M. J. Murphy, et al. (2004). "c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation." Genes & Development **18**(22): 2747-2763.
- Wineman, J., K. Moore, et al. (1996). "Functional heterogeneity of the hematopoietic microenvironment: rare stromal elements maintain long-term repopulating stem cells." Blood **87**(10): 4082-4090.
- Winkler, I. G., N. A. Sims, et al. (2010). "Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs." Blood **116**(23): 4815-4828.
- Winnier, G., M. Blessing, et al. (1995). "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse." Genes & Development **9**(17): 2105-2116.
- Wright, D. E., A. J. Wagers, et al. (2001). "Physiological Migration of Hematopoietic Stem and Progenitor Cells." Science **294**(5548): 1933-1936.
- Xie, H., M. Ye, et al. (2004). "Stepwise Reprogramming of B Cells into Macrophages." Cell **117**(5): 663-676.
- Xu, S. and M. A. Powers (2010). "Nup98-Homeodomain Fusions Interact with Endogenous Nup98 during Interphase and Localize to Kinetochores and Chromosome Arms during Mitosis." Mol. Biol. Cell **21**(9): 1585-1596.
- Yamazaki, S., A. Iwama, et al. (2009). "TGF- β as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation." Blood **113**(6): 1250-1256.
- Yang, L., D. Bryder, et al. (2005). "Identification of Lin⁻Sca1+kit+CD34+Flt3⁻ short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients." Blood **105**(7): 2717-2723.
- Yassin, E. R., N. J. Sarma, et al. (2009). "Dissection of the Transformation of Primary Human Hematopoietic Cells by the Oncogene NUP98-HOXA9." PLoS ONE **4**(8): e6719.

- Yilmaz, Ö. H., R. Valdez, et al. (2006). "Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells." Nature **441**(7092): 475-482.
- Yoshihara, H., F. Arai, et al. (2007). "Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche." Cell stem cell **1**(6): 685-697.
- Yuan, Y., H. Shen, et al. (2004). "In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C." Nat Cell Biol **6**(5): 436-442.
- Zeng, H., R. Yucel, et al. (2004). "Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells." EMBO J **23**(20): 4116-4125.
- Zhang, C. C., M. Kaba, et al. (2006). "Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells." Nat Med **12**(2): 240-245.
- Zhang, C. C., M. Kaba, et al. (2008). "Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation." Blood **111**(7): 3415-3423.
- Zhang, C. C. and H. F. Lodish (2004). "Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells." Blood **103**(7): 2513-2521.
- Zhang, X.-B., B. C. Beard, et al. (2008). "High incidence of leukemia in large animals after stem cell gene therapy with a HOXB4-expressing retroviral vector." The Journal of Clinical Investigation **118**(4): 1502-1510.
- Zhang, X.-B., J. L. Schwartz, et al. (2007). "Effects of HOXB4 Overexpression on Ex Vivo Expansion and Immortalization of Hematopoietic Cells from Different Species." STEM CELLS **25**(8): 2074-2081.
- Zhang, Y., M. Gostissa, et al. (2010). The Role of Mechanistic Factors in Promoting Chromosomal Translocations Found in Lymphoid and Other Cancers. Advances in Immunology. W. A. Frederick, Academic Press. **Volume 106**: 93-133.
- Zimonjic, D. B., J. L. Pollock, et al. (2000). "Acquired, nonrandom chromosomal abnormalities associated with the development of acute promyelocytic leukemia in transgenic mice." Proceedings of the National Academy of Sciences **97**(24): 13306-13311.