DISTINCT ROLES OF CELLULAR COPPER AND ERYTHROPOIETIN RECEPTOR ON HEMATOPOIETIC ENGRAFTMENT

by

Xiaosong Huang

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SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

Xiaosong Huang

This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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Gerald SpaKgrude (J

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Vicente Planelles

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John Phillips

Sanchez Alvarado

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FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the dissertation of [redacted] its fmal form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

3AVo9 [redacted]

Date

Gerald SpangrudK (J
Chair: Supervisory Committee

Approved for the Major Department

[redacted]

Peter E. Jensen
Chair/Dean

Approved for the Graduate Council

[redacted]

David S. Chapman
Dean of The Graduate School

ABSTRACT

Cell differentiation is influenced by a combination of cues from both extracellular and intracellular sources. Recent advances in the regulation of hematopoiesis have focused on the roles of the growth factors, cytokines, transcription factors and microRNAs; little attention has been given to the role of cellular metabolism. In the first part of this dissertation, I explored the idea that an essential cellular nutrient, copper (Cu), is an active component of cell fate regulators that influence hematopoietic progenitor cell differentiation. I showed that reducing cellular Cu content by exposing the cells to the Cu chelator tetraethylenepentamine (TEPA) enhanced the generation or maintenance of erythroid progenitor cells from multipotent progenitor cells in culture. I also provided evidence suggesting that this effect is likely due to the modulation of cellular energy metabolism by cell Cu level. In the second part of this dissertation, I investigated the effects of modulating erythropoietin receptor (EPOR) signaling intensity on mouse tri-lineage hematopoietic engraftment and proliferation of hematopoietic stem/progenitor cells (HSC/HPCs). I showed that neither increasing nor reducing EpoR signaling intensity influence long-term leukocyte engraftment, an indication of HSC function. I confirmed the essential role of EpoR on erythropoiesis. I also provide evidence suggesting a potential role for EpoR in megakaryopoiesis. I also demonstrated that multipotent progenitor cells with naturally-expressed hyperactive EpoR can proliferate and differentiate in response to Epo stimulation alone.

This thesis is dedicated to my parents

Haicheng and Guolan

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CHAPTER 1

INTRODUCTION

Hematopoiesis

Hematopoiesis, the generation of blood cells, is a continuous developmental process happening in both embryonic and adult stages. Blood cells can be categorized into three major classes: erythrocytes (red blood cells), platelets, and leukocytes (granulocytes, monocytes and lymphocytes). Blood cells can also be categorized into myeloid and lymphoid lineages. Myeloid lineages include erythrocytes, megakaryocytes (the precursor of platelets), granulocytes and monocytes/macrophages, while lymphoid lineages include B, T and NK cells. Dendrtic cells can be derived from both myeloid and lymphoid cells. Erythrocytes carry and supply oxygen (O2) to the tissues. Granulocytes (neutrophils, eosinphils, basophils and mast cells) and macrophages engulf and clear invading pathogens and damaged tissue cells. T and B lymphocytes function in adaptive immune responses. Megakaryocytes fragment into platelets that control blood clotting. Thus, blood cells have important functions in supporting normal metabolism by providing an oxygen supply and a defense system against foreign pathogens and injuries. Blood is a tissue with a high turnover rate, and it is estimated that about 10" new blood cells are produced daily in a healthy adult.

All blood cells are produced from hematopoietic stem cells (HSCs), normally found within the bone marrow (BM) and in some species, the spleen. HSCs are defined as clonal precursors of additional HSCs (through a process termed self-renewal), as well as of all types of differentiated blood cells (multipotency). HSCs were first described more than 40 years ago (Becker et al., 1963). In an effort to identify cells capable of protecting humans from the hematopoietic failure caused by the lowest lethal dose of radiation, scientists noticed that the lead shielding of spleen prevented death of animals from otherwise lethal dose of radiation (Jacobson LO, 1949). It was later found that intravenous injection of syngeneic bone marrow cells or spleen cells can rescue animals from death caused by radiation (Jacobson et al., 1951; Lorenz et al., 1951), and it was shown that cells derived from injected bone marrow cells or spleen cells repopulated the hematopoietic system of the host animals (Ford et al., 1956; Makinodan, 1956; Nowell et al., 1956). The existence of HSCs in bone marrow was predicted by a series of seminal experiments showing that the donor bone marrow contains cells capable of giving rise to individual colonies of myeloid, erythroid, and megakaryocyte cells within the spleens of irradiated hosts (Till and Mcculloch, 1961; Wu et al., 1967). The same cell type can also give rise to lymphocytes (Wu et al., 1968). Since it was not initially known whether splenic colonies were of clonal origin or rather required a number of cells to initiate, the cells responsible for forming these colonies were termed spleen colony-forming units (CFU-S). Each spleen CFU-S could produce more CFU-S upon isolation and secondary transplantation (Siminovitch et al., 1963), thus proving the self-renewal capability of these cells. Based on these experiments, it was proposed that a single HSC capable of multilineage differentiation as well as self-renewal must exist within the bone marrow

(Siminovitch et al., 1963). It was later found that the majority of CFU-S actually represents a more mature multipotent progenitor cell (MPP) type rather than HSCs. The definitive proof of the existence of HSC came from the transplantation of clonally marked bone marrow cells (using chromosome breaks or integrated retrovirus as a clonal marker) and tracking the patterns of markers in differentiated blood cells from transplanted bone marrow cells (Abramson et al., 1977; Jordan and Lemischka, 1990). These experiments prove that a single HSC gives rise to all the blood cell types as well as more HSCs. HSCs are the only stem cells that are clinically utilized in the treatment of diseases such as breast cancer, leukemias and congenital immunodeficiencies. However, a shortage of HLA-matched donors and the inability to expand and genetically modify HSCs or HPCs in vitro have limited more widespread therapeutic applications. Thus, determination of the factors that favor HSC/HPC expansion (self-renewal) instead of differentiation or apoptosis is of great scientific and therapeutic interest, as these factors potentially represent a means for maintaining expansion of HSC/HPCs in vitro. Realizing this goal would have a significant impact on the clinical transplantation of HSC/HPCs.

HSCs can be subdivided into long-term (LT) HSCs and short-term (ST) HSCs. Long-term HSCs have extensive self-renewal capability and are defined by their capability of repopulating a myeloablated animal for more than 4 months. Short-term (ST) HSCs have limited self-renewal capability and can repopulate a myeloablated animal for only ~ 6 weeks (Christensen and Weissman, 2001). Although HSCs are ultimately responsible for the homeostasis of the entire blood system and act as a reserve for its regeneration in response to hematological stresses such as blood loss, infection, or exposure to cytotoxic agents, HSCs usually remain quiescent and the daily blood cell

generation demand is largely fulfilled by the transient amplifying progenitor cells. Hematopoietic progenitor cells (HPCs) are cells that may be multipotent, oligopotent, or unipotent, and they lack significant self-renewal capacity but are highly active in cell division. LT-HSCs give rise to ST-HSCs. Multipotent progenitors (MPPs) are immediate progenies of ST-HSCs and retain the differentiation potential to differentiate into all the blood lineages but have lost self-renewing capability. It has been shown that the cell cycle status of phenotypically defined HSC is mostly at the Go/Gi phase (Fleming et al., 1993). In vivo BrdU incorporation was used to examine cell cycle kinetics in HSCs and MPPs (Passegue et al., 2005). BrdU is an analogue of thymidine and can be incorporated into newly synthesized DNA (during the S phase of the cell cycle), thus BrdU incorporation indicates cells that were active in cell division. LT-HSCs showed the slowest kinetics of cell cycle (approximately 24% of cells 24 hrs post-BrdU injection) whereas ST-HSCs and MPPs displayed faster kinetics of cell cycle (>50% cells 24 hrs post-BrdU injection). In addition, the majority of LT-HSCs were found to be in the quiescent G_0 cell cycle state (75%) compared to ST-HSCs and MPPs (60% and 50%, respectively) (Cheshier et al., 1999). A more recent study has demonstrated that BrdU labeling actually damages HSC and induces entry into the cell cycle, suggesting that older work using this labeling approach overestimated the cell cycle entry of dormant HSC (Wilson et al., 2008).

The identities of downstream progenitors following the MPPs are very controversial. The classical model predicts that MPPs give rise to two types of oligopotent progenitor cells: the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) that are restricted to differentiation potential in myeloid

lineages or lymphoid lineages, respectively (Akashi et al., 2000; Kondo et al., 1997). Following this lineage bifurcation, the CMP further differentiates into bipotent progenitors: megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/monocyte progenitors (GMP) (Akashi et al., 2000), while the CLP gives rise to B, T or NK cell unipotent progenitors (Kondo et al., 1997). Recent data has challenged this classical model and suggests that the bifurcation of MPP into either myeloid or lymphoid lineages may not hold true for the in vivo situations (Adolfsson et al., 2005). MPP may give rise to progenitors with megakaryocyte and erythroid potential (MEP) and also progenitors with granulocyte, monocyte and lymphoid potential (LMPP) (Figure 1.1).

Assays for HSC and HPC

Because of the morphological similarities between HSC and various types of HPCs and the lack of easily-identifiable markers, the definitive identifications of HSC and HPC relies on retrospective examination of their progenies after proliferation and differentiation in vivo or in vitro.

Histological assay

The oldest records of the study of hematopoiesis trace to histological studies originating in the 1800s. This technique involved the staining of tissue or blood cells with dyes, such as Giemsa solution, and then evaluating cells based on their staining morphology. The staining solutions contain basic dyes and acid dyes. The basic dyes stain nuclei, granules of basophil granulocytes and RNA molecules of the cytoplasm of white blood cells as a blue color. The acidic dye eosin stains red blood cells and granules

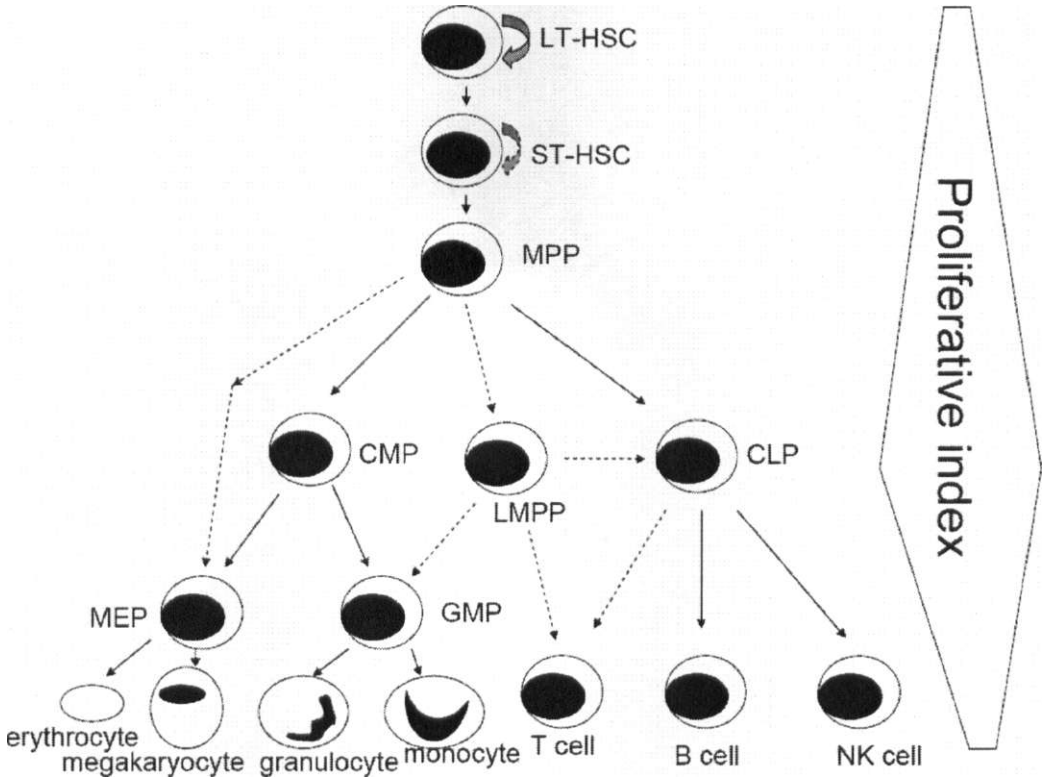

Figure 1.1. The current models of hematopoietic hierarchy. The classical model (solid arrow) predicts that the first lineage commitment is bifurcation of myeloid and lymphoid lineage from MPP into CMP and CLP. Recent alternative models (dashed arrow) suggest that MPP may give rise to three or four different progenitors that include MEP, CMP, LMPP and CLP without abrupt separation of myeloid and lymphoid potential. See text for abbreviations.

of eosinophil granulocytes as a red color. Although this was originally a good assay to look at the presence of various mature blood cell types, it cannot distinguish HSC from HPC. Any conclusions regarding the differentiation of blood cells based on morphology remained speculative.

FACS assay

Many of the cell surface proteins have been shown to have specific patterns of expression on different types of hematopoietic cells. Using fluorescence-activated cell sorting (FACS)-based methods, one can identify and quantify the cells with specific expression patterns of cell surface markers. This assay is relatively easy and fast. Problems associated with this method include the variation in the expression of some markers by different mouse strains, and the developmental stages, cell cycle stages, drug treatment, culture conditions, transplantation effects, and spontaneous or induced mutations in mice (Matsuoka et al., 2001; Morrison et al., 1996; Purton et al., 2006; Spangrude and Brooks, 1993; Spangrude et al., 1995; Walkley et al., 2005a; Walkley et al., 2005b; Walkley et al., 2007). Thus, the expression of one or several cell surface markers cannot be used to definitively identify stem/progenitor cell types and functions.

Colony-forming cell assays

The colony-forming cell (CFC) assays measure progenitor cell number and lineage potential using well-defined methylcellulose or agar-based semisolid culture media supplemented with various cytokines (Bradley and Metcalf, 1966). Because of the semisolid nature of these cultures, dividing cells form colonies derived from a single

progenitor cell. The majority of CFCs consist of lineage-restricted colonies: erythroid restricted burst-forming units-erythroid (BFU-E), colony-forming units erythroid (CFU-E); megakaryocyte-restricted CFU-Mk; colony-forming units-granulocytes (CFU-G), colony-forming units-macrophages (CFU-M); and colony forming units-granulocytes/macrophages (CFU-GM). The most immature CFC colony contains granulocytes, erythrocytes, macrophages, and often megakaryocytes (CFU-GEMM). While informative about the HPC content of a population of interest, the CFCs do not distinguish HSCs and HPCs.

In vivo repopulation assays

In vitro assays provide fast and easy methods to study hematopoietic cells; however, it is still the in vivo repopulation assays that provide the most convincing data on the function and quantity of HSC and HPCs. In vivo repopulation assays can be categorized as either short-term or long-term.

Short-term repopulation assay is usually used to assess the differentiation and proliferation potential of HPCs. The recipient is usually sublethally irradiated before transplantation to promote engraftment while assuring the survival of the recipient. The recipient can also be lethally irradiated prior to transplantation of HPCs if a low dose of normal "competitor" bone marrow cells are also transplanted to assure long-term survival. In either case, the transplanted HPC must be distinguishable from the recipient mouse and the competitor bone marrow in order to trace the fate of the progeny of the HPC. This is accomplished using genetic markers that distinguish the donor and recipient mouse strains.

Long-term repopulation assay is usually used to assess the quality and quantity of HSCs. Two criteria must be met to demonstrate the function of HSCs. First, transplanted cells must be able to generate both lymphoid and myeloid cells. Second, these cells must be able to reconstitute the hematopoietic system indefinitely posttransplant. The recipient is usually lethally irradiated before transplantation to eliminate their endogenous hematopoietic system. This assay is usually done in a competitive repopulation setting for quantitative analysis. This assay measures the functional potential of the unknown source of hematopoietic cells (donor cell population) against a fixed number of competitors HSC/HPC (usually whole bone marrow cells from congenic wild-type mice of the same genotype as the recipient). The ratio of donor to competitor peripheral blood cells is then monitored over time. The number of repopulating units (RU) in the donor cell population can be determined by the following formula: donor RU = % donor cells x C/(100-% donor cells), where C = the number of competing RU and 1 x 10^5 whole bone marrow cells is defined as 1 competing RU (Harrison et al., 1993; Yuan et al., 2005). This study can provide semiquantitative information about the HSCs within a given population, but it cannot distinguish between the number of HSCs and their quality (progeny produced per HSC). To accomplish this distinction, a semiclonal transplant model is used in which progeny of individual HSC are followed with time. The disadvantage of this approach is that many recipient mice must be transplanted with limiting numbers of putative HSC in order to detect rare clonal contribution of HSC in a subset of the transplant recipients.

The success of in vivo repopulation assays relies on the markers that distinguish the progeny blood cells derived from the transplanted cells from those derived from the competitor or recipient cells. Investigators have used radiation-induced chromosomal

breaks (Harrison et al., 1978) and retroviral insertions (Leary et al., 1984) for this purpose. More recently, alleles of the cell surface antigen Ly5 (CD45) have been widely used as a marker. However, Ly5 is only expressed on leukocytes thus can not track the repopulation in erythrocyte and platelet lineages. Erythrocyte repopulation is usually marked by naturally occurring hemoglobin (3 chain (Hbb) variants. These genetic variants have been bred onto common genetic backgrounds. The Hbb^s variant differs from the Hbb^d variant by a cysteine (Cys) to glycine (Gly) substitution at amino acid position 13. The distinction and quantitation of these two variants in erythrocytes can be achieved by reduction of thiol groups with cystamine which introduces a differential charge into the Hbb variants. The variants can then be detected by agarose electrophoresis under alkaline conditions (Whitney, 1978). However, the gel electrophoresis system is cumbersome for large number of samples and can be problematic in interpretation and quantitation due to gel irregularity and sample loading errors. Our lab developed an automated high-performance liquid chromatography (HPLC)-based method for mouse Hbb variant analysis. This technique can detect as little as 1% variant Hbb isoform in an easy and fast manner (Spangrude et al., 2006). Platelet chimerism is usually assayed by the intracellular glucose phosphate isomerase (Gpi) isoenzymes, $Gpi\text{-}1^a$ and $Gpi\text{-}1^b$. The Gpi isoenzymes are easily separated by agarose gel electrophoresis because of their net surface charge differences (Harrison et al., 1988a; Harrison et al., 1988b; Harrison et al., 1989). Platelets are first purified from peripheral blood by centrifugation before electrophoresis. To observe the protein bands, the gel is treated with an enzymatic stain specific for Gpi (Slayton et al., 2002). This method is also very time consuming and error prone.

Purification of mouse HSCs and HPCs

Mouse HSC/HPCs have been purified using different techhniques, including cell-surface marker selection (Spangrude et al., 1988b), elutriation (Jones et al., 1990), pharmacologic manipulation (Szilvassy and Cory, 1993) and supravital dye staining (Goodell et al., 1997) as well as intracellular enzyme content (Jones et al., 1995). The most powerful method for the purification of mouse HSCs or HPCs is by FACS. Fluorescent dye-labeled monoclonal antibodies specific for cell surface markers can be used to isolate HSC/HPCs by FACS (Table 1.1). Most HSC purification strategies utilize a negative selection against markers of mature hematopoietic cells (typically B220, CD2, CD3, CD4, CD8, CD 19, Gr-1, Mac-1, and Ter-119) combined with positive selection for the markers c-Kit (CD117, a tyrosine kinase receptor) and Sca-1 (a membrane glycoprotein). Although this Lin"Sca-l^{+}c-Kit^{+} (LSK) phenotype greatly enriches for hematopoietic reconstituting activity, it contains HPCs in addition to HSCs. In fact only 10% of KLS cells are bona fide HSCs, and as such the KLS compartment should be regarded as merely enriched for HSCs (Okada et al., 1992). The inclusion of Thy1.l expression in KLS purification can further purify HSCs (Spangrude et al., 1988a). Recently, the expression of CD34 and Flt3 (CD135) has been used to further purify long-term HSCs (KLS+CD34"Flt3") from short-term HSCs (KLS+CD34^{+}Flt3") and MPPs (KLS+CD34^{+}Flt3^{+}) (Adolfsson et al., 2001; Yang et a1, 2005). The unique property of effluxing or retaining some vital dyes by HSCs has been exploited as a method for HSC/HPC purification. Rhodaminel23 (Rhol23) is a fluorescence dye that accumulates in active mitochondrial membranes. Several studies have demonstrated that enriched progenitor fractions can be further subdivided with respect to Rhol23 uptake and that the

Table 1.1. The expression of cell surface markers by hematopoietic cells.

Cell surface marker	Major lineages of expression	Expression on HSCs	Expression on MPPs
Thy-1	T cells	Low	Negative
Sca-1	T cells, stem cells	High	High
c-Kit	Progenitor and stem cells, mast cells	High	High
Ly5	Leukocytes	Positive	Positive
Mac-1[1]	Granulocytes, monocytes	Negative	Low
Gr-1	Granulocytes, macrophages	Negative	Negative
CD4	T cells	Negative	Low
CD8	T cells	Negative	Negative
CD5	T cells, B cells	Negative	Negative
B220	B cells, progenitor cells	Negative	Negative
Terl 19	Erythroid progenitors, erythrocytes	Negative	Negative
CD34	Progenitors	Negative	Positive
Fit-3	Progenitors	Negative	Positive

1. Mac-1 is expressed by HSC found in fetal liver

Rho123^{low} fraction is more enriched for long-term HSCs than is the Rh123$^{med/hi}$ fraction (Li and Johnson, 1992; Spangrude and Johnson, 1990). Hoechst 33342 is another vital dye used extensively for the purification of HSCs. Hoechst 33342 is a DNA-binding dye that has at least two binding modes resulting in different spectral properties. The side population (SP) cells are visualized by detecting Hoechst 33342 emissions at two wavelengths simultaneously, resulting in a distinct "tail" profile (Goodell et al., 1996). The side-population phenotype of HSCs is due to their expression of Bcrpl, an ATP binding cassette transporter that is responsible for dye efflux. The dye efflux property of HSCs does not seem directly linked with their self-renewal and differentiation capacities (Zhou et al., 2002). Hoechst 33342 can not be used to purify MPPs.

Molecular mechanisms regulating HSC self-renewal

Throughout the adult life of a mammalian organism, HSCs must replicate themselves to maintain the constant HSC pool in the marrow. The process of HSC replication through mitosis is called self-renewal. The fate choice of HSCs to either self-renew or differentiate is controlled by a complex interplay between intrinsic mechanisms and extrinsic signals from the surrounding environment, or stem cell niches (Moore and Lemischka, 2006). Understanding the mechanisms of self-renewal of HSCs will facilitate the expansion of HSCs in culture while maintaining the stem cell identity for cell and gene therapies.

Currently, several transcription factors are implicated in the regulation of HSC self-renewal through various gain- and loss-of-function studies. One of them is the transcription factor Tel (translocation Ets leukemia), which has been demonstrated to be

required for HSC self-renewal (Hock et al., 2004b). Inactivation of Tel leads to the depletion of HSCs in BM without influencing their committed progenitors. Another interesting factor is HoxB4, a member of the homeobox gene family that encodes a set of transcription factors regulating embryonic body patterning and organogenesis. Overexpression of HoxB4 in HSCs has been shown to enhance the self-renewal of HSCs in both in vitro and in vivo models (Sauvageau et al., 2004). Interestingly, HoxB4 knock-out mice exhibit normal hematopoiesis, with only a mild defect in the proliferative potential of HSC (Brun et al., 2004). This discrepancy is likely due to the redundant function of other Hox family members. Gfil (Growth factor independence 1) is a zinc finger-containing transcriptional repressor. Two studies independently identified Gfil as a positive regulator of HSC self-renewal that functions by restraining HSC proliferation (Hock et al., 2004a; Zeng et al., 2004). Gfil$^{7"}$ HSCs exhibit increased proliferation and decreased capability for repopulation of irradiated recipients. It has been suggested that Gfil might function through upregulating the cell cycle inhibitor p21, since p21 expression is greatly decreased in Gfil$^{7"}$ HSCs. Consistent with this hypothesis is the observation that p21 deficient HSCs also show impaired repopulation capability compared to wildtype HSCs (Cheng et al., 2000). Another cell cycle inhibitor, pi 8, has an opposite effect on HSC self-renewal compared to p21 such that pl8-deficient HSCs have improved repopulation capability compared to wildtype HSCs (Yu et al., 2006; Yuan et al., 2004). These results suggest that a delicate balance of cell prolix feration and cell cycle repression is essential for HSC self-renewal. Stat3 and Stat5 are transcription factors involved in the Janus family kinase-signal transducer and activator of transcription (JAK-STAT) pathway that are implicated in the induction of leukemia,

lymphoma and some solid tumors. Gain and loss of function studies have shown that both Stat5 and Stat3 are positive regulators of HSC self-renewal (Chung et al., 2006; Kato et al., 2005).

In addition to transcription factors, other proteins involved in the modulation of gene expression have been found to regulate self-renewal of HSCs. Bmi-1 is a polycomb group (PcG) protein that forms the PRC1 (Polycomb repression complex 1) protein complex with other members of the PcG family, such as Mel-18, Mphl/Rae28, M33, Scmhl and Ringl A/B. This group of proteins represses the activity of the SWI-SNF chromatin remodeling complex, leading to the repression of transcriptional activity. The Bmi-1 protein complex also binds to methylated histone H3 and contributes to themaintenance of epigenetic memory (Iwama et al., 2004). Mphl/Rae28-deficient FL cells include fewer HSCs compared to wildtype mice (Ohta et al., 2002). Bmi-1-deficient BM HSCs have profound defects in long-term repopulating activity (Park et al., 2003), while forced expression of Bmi-1 in HSCs enhances the ex vivo expansion of HSCs and their in vivo repopulating activity (Iwama et al., 2004). The mechanisms whereby Bmi-1 and its protein complex maintain HSC self-renewal are unclear, but it may be attributed to the repression of pi 6 and pi 9, and/or repression of differentiation-related gene expression programs.

Extrinsic environmental signals are connected to HSC self-renewal-specific gene expression programs by numerous signal transduction pathways. Notch, Wnt, BMP and Sonic hedgehog (Shh) signaling pathways have all been implicated in the regulation of HSC self-renewal. Notch proteins are highly conserved cell surface receptors that include four members in mammals. The ligation of Notch ligands (five members in mammals)

with Notch receptors results in cleavage and release of the intracellular domain of Notch receptors, which enters the nucleus and bind with the transcriptional repressor CSL, converting it into a transcriptional activator and inducing target gene expression. Notch signaling has important functions in cell fate determination in many contexts of embryogenesis as well as in T cell versus B cell lymphoid lineage commitment. Forced activation of Notchl in hematopoietic cells increases the self-renewal capability of HSCs and favors lymphoid lineage commitment (Stier et al., 2002). Notch signaling is active in HSCs and is downregulated as HSCs differentiate (Duncan et al., 2005). One of the Notch ligands, Jagged 1, is expressed at high level in osteoblasts, an essential component of HSC niches, suggesting that Notch signaling is important in controlling HSC self-renewal (Calvi et al., 2003). Using a 'pan' Notch signaling inhibitor to inhibit Notch signaling in HSCs results in the accelerated differentiation and depletion of HSCs (Duncan et al., 2005). In contrast to these findings, a recent study showed that the inactivation of Notchl and/or its ligand Jagged 1 in BM hematopoietic cells and stromal cells has no effect on HSC self-renewal. Notchl deficient HSCs repopulated irradiated mice with Jagged 1 deficient BM stroma normally, even under competition with wild-type HSCs (Mancini et al., 2005). A possible explanation for this discrepancy is the functional redundancy of other members of the Notch family of receptors and/or ligands.

Wnt signaling has also been implicated in the regulation of HSC self-renewal. Like Notch signaling, Wnt signaling is highly conserved and acts during embryogenesis as well as in adult tissue homeostasis. The Wnt family comprises secreted proteins and includes many members. The receptors for Wnt proteins are the Frizzled family of seven-pass transmembrane proteins and LDL-receptor related proteins (Duncan et al., 2005).

Wnt proteins can trigger at least three intracellular signaling pathways: the canonical P-catenin pathway, the noncanonical calcium pathway, and the c-Jun N-terminal kinase pathway (Scheller et al., 2006). P-catenin is the central player of canonical Wnt pathway. In the absence of Wnt signaling, P-catenin is associated with a large protein complex called the destruction complex, which is targeted for degradation by ubiquitilation. Wnt protein binding to its receptor inhibits the degradation of P-catenin, resulting in the accumulation of P-catenin in the cytosol. P-catenin then translocates into nucleus and binds with members of the LEF-TCF family of transcription factors, resulting in the expression of their target genes. Purified Wnt3A protein has been shown to promote self-renewal of HSCs (Willert et al., 2003). It has also been demonstrated that Wnt signaling is active in HSCs, and that the overexpression of an active form of P-catenin in HSCs leads to the expansion of immature cells that can reconstitute irradiated recipients (Reya et al., 2003). In contrast, HSCs transduced with inhibitors of Wnt signaling lost their in vivo repopulation capability. However, these experiments were performed using HSCs that expressed the anti-apoptotic gene bcl-2, making the interpretation of the sole role of Wnt signaling in HSC self-renewal unclear. In contrast, another group showed that mice with conditionally deleted P-catenin had no hematopoietic defects, and that P-catenin deficient HSCs had normal self-renewal capability (Cobas et al., 2004). More recently, two independent studies addressed the role of canonical Wnt signaling in hematopoiesis by engineering mice that conditionally express dominantly active forms of P-catenin in hematopoietic cells. These mice showed widespread hematopoietic abnormalities and died within 2-3 weeks. The HSCs from these mice had lost the capacity to repopulate irradiated recipients, even though the number of cells expressing the HSC surface

antigens increased (Kirstetter et al., 2006; Scheller et al., 2006). Taken together, the existing studies suggest that canonical Wnt signaling may not be strictly required for HSC function, but that canonical Wnt signaling may affect self-renewal and differentiation of HSCs depending on the extent of canonical Wnt signaling and on the context of expression of additional genes. Noncanonical Wnt signaling and/or other signaling pathways may also compensate for the absence of canonical Wnt signaling in maintaining the self-renewal of HSCs.

ex vivo HSC/HPC expansion

Since HSC undergo self-renewing cell divisions in order to maintain and expand their numbers in vivo, many studies have been devoted to the identification of cell culture conditions that support HSC/HPC expansion. Ex vivo expansion of HSC/HPCs is of great significance in the quest for hematopoietic reconstitution of chemotherapy or radiotherapy-treated patients and development of gene therapy for inherited hematopoietic malfunctions. Several strategies have been investigated, including the coculture of HSC/HPC with stromal cells derived from in vivo hematopoietic sites, the identification of cytokines, growth factors and other culture conditions that favor HSC/HPC expansion, and the genetic modification of HSC/HPCs. Historically, cytokines and growth factors have been the main targets of study for HSC/HPC expansion in vitro. The proper cocktail of growth factors and cytokines used in ex vivo culture conditions for HSC expansion has not yet been determined. A variety of combinations have now been described that permit enormous expansion of progenitor cells. However, the ex vivo expansion of rigorously defined HSC has largely proven unsuccessful. Most ex vivo

conditions involve stem cell factor (SCF), Flt3 ligand (FL), IL-6 and thrombopoietin (TPO). SCF is a cytokine that binds with its corresponding cell surface receptor c-kit, which is crucial for proliferation and differentiation of HSCs and HPCs, mainly in myeloerythroid lineages (Broudy, 1997). The cytokine TPO is key in regulating megakaryocyte and platelet production, and an important role for TPO in stimulating self-renewal of HSCs has also been revealed (Fox et al., 2002; Kaushansky, 2003; Ku et al., 1996). Flt3 is a tyrosine kinase receptor expressing on MPPs and committed lymphoid progenitors (Christensen and Weissman, 2001; Lai et al., 2005). The ligand for this receptor (FL) was shown to be an active proliferative stimulus for stem and progenitor cells, particularly when acting in synergy with other cytokines. IL-6 also stimulates the proliferation of MPPs in concert with other cytokines (Ikebuchi et al., 1987). The careful studies in mouse and human hematopoietic stem/progenitor cell transplantation models have identified SCF, FL and TPO as the most important cytokines for expansion of HSC and MPPs (Kaushansky, 1999; Luens et al., 1998). Notably, culture in low oxygen conditions seems to promote the expansion of HSCs (Danet et al., 2003), an observation that is likely related to the fact that these cells naturally reside in a hypoxic environment (bone marrow) (Parmar et al., 2007). The most significant ex vivo HSC expansion has been achieved by ectopically expressing transcription factors in cultured HSCs. Mouse bone marrow cells transduced with Hoxb4, a homeodomain-containing transcription factor, were cultured in the presence of serum. The HSC number at various time points in the culture was determined by competitive repopulation assay. The results showed a net 5 to 7 fold increase of HSC number at day 6 of culture and a 40-fold increase by day 12 of culture (Antonchuk et al., 2002).

Copper biology: acquisition and transportation

Copper (Cu) is the 29th element in the periodic table. It is an essential trace element for most aerobic organisms. An average adult male contains about 110 mg of Cu, distributed between the skeleton (46 mg), skeletal muscle (26 mg), liver (10 mg), brain (7 mg) and blood (6 mg) (Kim et al., 2008; Macreadie, 2008). Typically, the amount of Cu consumed is about equal to the amount excreted each day. Cu is an important catalytic and structural cofactor for proteins that function in many important biochemical processes that are essential for life. Cu is able to accept and donate single electrons as it switches oxidation states between Cu (cuprous ion) and Cu (cupric ion). This property makes copper an excellent biological cofactor in biochemical reactions that require electron transfer (Leary and Winge, 2007). By binding to proteins through sulfur, oxygen or nitrogen, Cu can also influence protein structures and protein-protein interactions, thereby controlling protein functions in various biochemical and regulatory processes. (Kim et al., 2008) Bioinformatics approaches estimate the Cu containing protein coding genes to be about 1% of the total genes in bacterial, archaeal and eukaryotic genomes (Andreini et al., 2008) (Table 1.2). However, the same property also allows free Cu to catalyze the formation of reactive oxygen species (ROS). Free Cu^{+} within the cells can react readily with hydrogen peroxide (H_2O_2) to yield the detrimental hydroxyl radical (OH). Free Cu ions within the cells can also stick to structural proteins and therefore alter protein structures and impair their functions. Because of this potential for damage, intracellular Cu homeostasis is regulated very tightly, and the concentration of unbound Cu ions is extremely low in cells (Prohaska and Gybina, 2004). Cellular Cu content is safeguarded by various mechanisms that regulate import, distribution, sequestration,

Table 1.2. Cu binding and regulating proteins

Protein	Function
Amyloid precursor protein (APP)	Neuronal development and potentially Cu metabolism protein
Atoxl	Metallochaperone
ATP7A	Cu^{+}-transporting P-type ATPase
ATP7B	Cu'-transporting P-type ATPase
Ceruloplasmin	Serum ferroxidase
CCS	Metallochaperone
Cox 17	Metallochaperone
Ctrl	High-affinity Cu^{+} importer
Cu/Zn SOD (SOD1)	Cu-containing antioxidant enzyme
Cytochrome c oxidase	Cu-containing enzyme
Hephaestin	Transmembrane multi-Cu ferroxidase
Lysyl oxidase	Cu-containing enzyme
Peptidylglycine-a-amidating mono-oxygenase (PAM)	Cu-containing enzyme
Prion protein (PrP)	Protein binds Cu via the N-terminal octapeptide repeats
Tyrosinase	Cu-containing enzyme

storage and export of copper both at systematic and cellular levels. These include Cu importers, metallochaperones that bind free Cu and deliver it to subcellular regions for storage or the synthesis of Cu-proteins, and Cu exporters (Leary and Winge, 2007). Cu is absorbed from the diet in the proximal gastrointestinal tract by intestinal epithelial cells (IECs). IECs transport Cu into peripheral blood in conjugation with unknown ligands where it is taken up by the liver through the portal circulation. Liver is the most important organ responsible for Cu storage and homeostasis. Liver mobilizes Cu into the peripheral circulation for the use by other organs. Liver also secretes Cu into the bile for excretion in the case of excessive copper. Biliary excretion is the only physiological way of excess Cu elimination (Gitlin, 2003). In the liver cell Cu is incorporated into mainly ceruloplasmin, which is a multicopper-containing ferroxidase that catalyzes the oxidation of Fe^{2+} to Fe^{3+} in serum. Ceruloplasmin plays an important role on facilitating Fe^{3+} loading onto transferrin for iron transportation (Hellman and Gitlin, 2002). Although more than 95% of the serum Cu is bound by ceruloplasmin, mice lacking ceruloplasmin have no apparent defect in Cu absorption or distribution suggesting ceruloplasmin does not play an essential role on Cu transportation (Harris et al., 1999).

The best known Cu importer for intestinal epithelial cells and other tissue cells is Ctrl, a transmembrane protein that is structurally and functionally conserved from yeast to mammals (Puig and Thiele, 2002). Ctrl is a heavily glycosylated protein, and is serine (Ser) - and methionine (Met)-rich in amino acid composition. Ctrl has a structural motif M-X-X-M repeated 11 times. Ctrl likely forms a homo-trimer across the cell membrane. This complex likely contains a pore which is highly specific for Cu^{1+} but not Cu^{2+} traverse (Balamurugan and Schaffner, 2006). Because Ctrl can only import Cu^{+} and Cu

exists as Cu^{2} at the extracellular environment, it requires metalloreductases reducing Cu^{2+} to Cu for the reduction of Cu" to Cu before it is imported into the cells (Kim et al., 2008). The mechanisms that drive Cu^{4} movement across the cell membrane are still unclear. Cu transportation across Ctrl does not require ATP. Cu transport by Ctrl is stimulated by extracellular K^{+} and probably facilitated by the much lower concentration of free Cu^{+} within the cells compared to that outside the cells (Lee et al., 2002a; Rae et al., 1999). There is evidence suggesting that Ctrl-independent Cu uptake mechanisms might exist in mammalian cells (Lee et al., 2002b).

Once Cu^{+} is imported into the cells, it is rapidly sequestered by Cu cheperones, a class of intracellular proteins that bind and deliver Cu to subcellular sites for incorporation into cuproproteins or for Cu storage (Balamurugan and Schaffner, 2006). Unbound free Cu ions do not appreciably exist within cells because they are rapidly sequestered by cellular Cu chaperones or vesicles (Rae et al., 1999). Antioxidant-1 (Atox 1) is a Cu chaperone that delivers Cu to cuproproteins that are secreted out of cells. During this process, Atoxl first delivers Cu' to the trans-Golgi network through membrane-associated Cu transporter P-type ATPases ATP7A and ATP7B by directly interacting with them. ATP7A is expressed in most tissues other than liver while ATP7B is expressed specifically in liver cells. ATP7A and ATP7B are associated with the trans-Golgi network membrane and deliver Cu into the trans-Golgi network where Cu is incorporated into secreting Cu-dependent proteins, such as tyrosinase, dopamine P-monooxgenase, peptidylglcine a-amidating monooxygenase, lysyl oxidase and ceruloplasmin, depending on cell types (Prohaska and Gybina, 2004). If the intracellular Cu levels elevate above a threshold, ATP 7A and ATP7B can be redistributed from the

trans-Golgi network membrane to the plasma membrane and deliver excess Cu out of the cell. ATP7A and ATP7B return to the trans-Golgi network as soon as the intracellular copper levels return to normal level (Balamurugan and Schaffner, 2006). These two ATPases may arise from gene duplication of common ancestor gene. ATP7B can respond to excess cellular Cu in liver cells by trafficking to the cell membrane and excreting excess Cu into the bile duct where Cu is disposed into the digestive tract. ATP7B thus functions in systematic Cu elimination (Suzuki and Gitlin, 1999; Wijmenga and Klomp, 2004).

The Cu chaperone for Cu, Zn-superoxide dismutase (SOD) is CCS protein (Schmidt et al., 2000). CCS is a 70-kDA protein that is structurally homologous to Cu/Zn SOD and is required for the delivery of Cu to SOD. CCS delivers Cu to SOD by direct pairing and ligand exchange reactions (Furukawa et al., 2004; Lamb et al., 2001). Three Cu chaperons, Coxl 7, Coxl 1 and Scol, function in the delivery of Cu to mitochondrial cuproprotein cytochrome c oxidase (CCO) (Horng et al., 2004). Cox17 functions in delivering Cu ion to the inner mitochondrial membrane proteins Coxl 1 and Scol, which in turn may transfer Cu to CCO (Horng et al., 2004; Lode et al., 2000).

It is estimated that less than one free Cu ion exists in a single eukaryotic cell (Rae et al., 1999). Besides Cu chaprones, other cellular molecules, for example glutathione (GSH) and metallothionein, may also function to sequester free Cu. GSH has no target specificity in Cu transportation and may function in Cu storage or as a protective molecule against Cu toxicity. Metallothioneins are used for Cu storage rather than uptake (Harris, 2000).

Copper-associated diseases

Cu deficiency or overload can cause diseases in humans. Menkes disease and Wilson disease are the known genetic diseases of Cu metabolism in humans. Menkes disease is an X-linked recessive disorder caused by the loss of function mutations of ATP7A gene. Menkes disease affects about 1 in 200,000 births and is typically lethal by 3-4 years of age (Daniel et al., 2004). Normal ATP7A is expressed in interstinal epithelia cells and functions as a Cu transporter for transporting Cu out of IECs and into peripheral blood. The Menkes mutations result in a profound Cu deficiency due to a failure to transport Cu out of intestinal epithelia cells (IECs) after its absorption from the gut, thus leads to systemic Cu deficiency. This systemic Cu deficiency causes impaired activity of various cuproenzymes that are essential for specific organ functions. Central nervous system and connective tissue are most profoundly affected by Cu deficiency (Danks, 1988). The symptoms of Menkes disease include: mental retardation and neurodegeneration, altered bone and connective tissue structure, pigment and keratin abnormalities (Madsen and Gitlin, 2007). Another genetic disease of Cu metabolism is Wilson disease caused by loss-of-function mutations in the ATP7B gene. Wilson disease is an autosomal recessive disease which causes a deficiency in biliary copper secretion affects about 1 in 40,000 people. Because of the deficiency in biliary Cu secretion, Cu accumulates in hepatocytes and causes liver damage. Eventually, excess Cu ions in liver cells leak into the plasma and cause systematic Cu overload (Gitlin, 2003; Tao and Gitlin, 2003). Besides liver cirrhosis, Wilson disease can also present with neurological symptoms such as behavioral disturbances and movement disorders due to Cu overload in the brain (Brewer and Askari, 2005; Madsen and Gitlin, 2007). Wilson disease is

typically treatable through Cu chelation and zinc loading therapies if detected before permanent damage takes place (Daniel et al., 2004).

Interestingly, Cu has been linked to cancer and atherosclerosis (Itoh et al., 2008; Volker et al., 1997). Hyperproliferative cancer cells and atherosclerosis tissue cells contain higher Cu content than normal tissue cells suggesting Cu might play a role on the pathology of these diseases (Arnold and Sasse, 1961; Daniel et al., 2004; Fuchs and de Lustig, 1989; Gupte and Mumper, 2008). Cu chelation treatment inhibits tumor growth by inhibiting angiogenesis (Brewer, 2005; Mandinov et al., 2003; Pan et al., 2002). Cu has been proposed to be required for angiogenesis, the formation of new blood vessels that are important for tumor growth (Finney et al., 2008). Cu stimulates proliferation and migration of human endothelial cells (Hu, 1998). Furthermore, Phase I and II clinical trials for the treatment of solid tumors by copper chelation showed its efficacy in disease stabilization (Brewer, 2005; Harris, 2004).

Cu has also been linked to neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and prion disease. In Parkinson's Disease, Cu can bind to a-synuclein and promote the aggregation of this protein (Bharathi et al., 2007; Paik et al., 1999) and dopamine may be affected by Cu to become toxic (Paris et al., 2001; Snyder and Friedman, 1998). Prion proteins (PrP) have also been found to have high-affinity N-terminal Cu binding sites that may alter the structure of PrP upon Cu binding to form prion disease proteins (Brown et al., 1997; Hornshaw et al., 1995; Jones et al., 2004). The amyloid precursor protein (APP) and the Ap peptide derived from APP involved in Alzheimer's disease are strongly implicated in Cu metabolism (Atwood et al., 1998; Bayer et al., 2003; Hesse et al., 1994; Macreadie, 2008; Maynard et al., 2002; Phinney et

al., 2003). APP is processed by the proteases to produce the AP peptide which exhibits neurotoxicity, and is a major component of the extracellular AP plaques associated with Alzhermer's disease. These plaques are a reservoir for a number of metals, including Cu, and it is has been considered that the Cu in these plaques may facilitate the production of reactive oxygen species (ROS) which could cause neuronal loss and brain damage.

Copper and hematopoiesis

Cu is essential for normal hematopoiesis as demonstrated by the hematological manifestations in Cu deficient humans and animals. Acquired Cu deficiency usually presents with hematological symptoms like anemia, neutropenia and rarely thrombocytopenia in adults (Kumar et al., 2005; Wu et al., 2006). Acquired Cu deficiency can be caused by parenteral or enteral feeding with Cu-deficient formulation, partial gastrectomy, prolonged zinc consumption and with the use of Cu chelating agents (Fiske et al., 1994; Gregg et al., 2002; Hayton et al., 1995; Hirase et al., 1992; Karpel and Peden, 1972; Nagano et al., 2005; Perry et al., 1996; Schleper and Stuerenburg, 2001; Tamura et al., 1994; Wu et al., 2006). Oral or intravenous Cu supplement usually results in prompt and complete resolution of the hematologic symptoms suggesting hematopoietic stem cells are not affected by Cu deficiency.

Morphological examination of the bone marrow of Cu-deficient patients has shown the characteristics typical of myelodysplastic syndrome (MDS), such as hypocellular marrow, vaculolated erythroid or granulocytic precursors, trilineage dysplasia, erythropoiesis blocked at proerythroblast stage, increased storage iron and occasional ring sideroblasts (nucleated erythrocytes with granules of iron in their cytoplasm)

(Dunlap et al., 1974; Gregg et al., 2002; Summerfield et al., 1992; Tamura et al., 1994; Zidar et al., 1977). The in vitro colony-forming assay of bone marrow cells showed that myeloid progenitor cells are present in patient bone marrow in normal numbers but the differentiation of these progenitors to mature blood cells is arrested due to the lack of Cu thus causing anemia, neutropenia and thrombocytopenia (Zidar et al., 1977).

The cause of anemia and other cytopenias in Cu deficient patients remains largely unknown. It may due to the reduction of activity of copper-containing enzymes, such as ceruloplasmin ferroxidase, cytochrome c oxidase and superoxide dismutase. Ceruloplasmin is a serum ferrioxidase that is essential for the mobilization of iron from macrophages to serum and maturing erythrocytes (Osaki and Johnson, 1969; Osaki et al., 1966). Cu deficiency leads to impaired ceruloplasmin production and ferrioxidase activity. Cu-deficiency causes reduced activity of cytochrome c oxidase in mitochondria which may lead to inhibition of heme synthesis from ferric iron and protoporphyrin, thus causing the iron accumulation seen in mitochondria of Cu-deficient animals (Gregg et al., 2002). Superoxide dismutase activity is decreased in Cu deficient erythrocytes which may have a reduced life span compared to normal erythrocytes (Hirase et al., 1992). Suppression of heme production by heme synthetase (Williams, 1983) was reported in copper deficient erythrocytes. These data together suggest that Cu through Cu-containing enzymes might play an important role in hematopoiesis, especially erythropoiesis. The influence of Cu deficiency on iron utilization by erythrocytes may also be one reason for anemia caused by Cu deficiency (Fox, 2003).

In the 19th and early 20th centuries, physicians noticed that dietary Cu supplement can increase hemoglobin content and red blood cell number in chlorosis (a form of

anemia) patients and used diet Cu supplement as a treatment for chlorosis and other anemias (Fox, 2003). These observations suggest that Cu has an important function in hemoglobin formation. Dr. Cartwright and Dr. Wintrobe and colleagues used Cu deficient swine models to study the function of Cu in hematopoiesis. They noticed a marked increase of normoblasts in bone marrow of Cu deficient swine (Lahey et al., 1952). The normoblast is the immediate precursor of a normal erythrocyte. They found that dietary Cu deficient swine developed anemia (microcytic and hypochromic), mild leucopenia and neutropenia but no thrombocytopenia (Lahey et al., 1952). They further studied iron metabolism in these Cu deficient swine and found that iron absorption is decreased, and iron mobilization and utilization is impaired (Gubler et al., 1952). This finding was explained by the discovery of ceruloplasmin and hephaestin, two Cu-containing ferroxidases that are essential for iron transportation (Fox, 2003).

Recently, Cu has been shown to regulate hematopoietic stem/progenitor cell proliferation and differentiation in culture. This discovery has led to the development of in vitro-expanded cord blood grafts as a potential medicinal product for the treatment of cancer and hematological malignancies. The expansion technology claims to enable preferential expansion of hematopoietic stem and early progenitor cells and is based on the finding that copper chelators can regulate the balance between self-renewal and differentiation of stem cells (de Lima et al., 2008; Peled et al., 2005).

Erythropoiesis

Erythropoiesis is the process of HSCs differentiating into red blood cells (erythrocytes). The mean lifespan of a normal human mature erythrocyte is about 120

days. In a normal adult, approximately 200 billion erythrocytes (about 1% of the total erythrocytes in the body) are replaced every day by an equal number of newly differentiated erythrocytes. Erythropoiesis is marked by the sequential generation of a series of progenitor cell types that are increasingly committed to erythroid lineage from HSC. These progenitor cells sequentially include multipotent progenitors (MPP), common myeloid progenitor (CMP), megakaryocyte/erythrocyte progenitor (MEP), burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E), proerythroblast, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic (normoblast) erythroblasts and reticulocytes prior to mature erythrocytes. Cell division is active before polychromatic erythroblast stage. After the polychromatic erythroblast stage, erythroid cells do not divide, but they undergo highly specialized maturation and transformation. The earliest erythroid lineage committed progenitor is BFU-E (Gregory and Eaves, 1978). BFU-E cells divide and further differentiate into CFU-E. CFU-E divide three to five times over 2 to 3 days as they differentiate and undergo many significant changes including a decrease in cell volume, chromatin condensation and hemoglobin synthesis leading up to expulsion of nucleus and other organelles. These morphological changes are used to define the sequential stages of late erythroid differentiation: proerythroblasts, basophilic and polychromatophilic erythroblasts, orthochromatic erythroblasts and reticulocytes.

Erythropoietin and its receptor

Erythropoietin (EPO) is a single chain glycoprotein of 166 amino acids with a molecular weight of 39KD that is synthesized mainly by kidney. Epo has long been

known as the major growth factor for enhancing erythropoiesis (Richmond et al., 2005); EPO functions through binding to the EPO receptor (EPOR) on the cell surface of erythroid progenitor cells and promotes cell survival, proliferation and erythroid differentiation. Epo recptor (EPOR) expression is upregulated at the BFU-E stage and maximal at the CFU-E and proerythroblast stages of differentiation (Koury et al., 2002; Wognum et al., 1990). EPOR expression is downregulated as erythroid progenitor cells undergo terminal differentiation and hemoglobin synthesis (Koury and Ponka, 2004; Zhang et al., 2003). The EPO receptor belongs to the type I cytokine receptor family, which includes the thrombopoietin (TPO) receptor (Mpl) and the G-CSF receptor. EPOR is a single transmembrane protein. Two EpoR molecules form a homodimer and bind to one Epo protein. This binding causes conformational changes in EPOR leading to transphosphorylation and activation of Janus family tyrosine protein kinase 2 (JAK2) associated with the membrane proximal region of the EPOR cytoplasmic domain (Witthuhn et al., 1993). Activated JAK2 phosphorylates all eight tyrosine residues on the EpoR intracellular domain. Phosphorylated tyrosines provide docking sites for Src homology 2 (SH2) domain-containing signaling proteins that are thereafter phosphorylated by JAK2 and become activated. One target is signal transducer and activator of transcription 5 (STAT5). Phosphorylated STAT5 translocates to the nucleus and induces target gene expression. Several other signaling pathways are activated by EPOR, including the phosphatidylinositol 3-kinase/protein kinase B (PI 3-kinase/Akt) and mitogen-associated protein kinase/extracellular signal-related kinase (MAPK) pathways. The C-terminal region of EPOR also contains a negative-regulatory domain

that contain docking site for SHP1 protein tyrosine phosphatase which can downregulate EPOR signaling by dephosphorylating JAK2.

The expression and function of EPOR is not restricted to erythroid progenitor cells. Endothelial, neural, muscle, cardiovascular and renal tissues express functional EPOR (Noguchi et al., 2008). Even within hematopoietic cells, the EPOR is also expressed by some multipotential hemopoietic progenitors and stem cell lines (Heberlein et al., 1992; Migliaccio et al., 1991; Schmitt et al., 1991), and whether it plays a role in vivo at an early stage of hematopoiesis is currently unclear.

This dissertation comprises two studies on mouse hematopoiesis. In Chapter 2,1 investigate the effect of modulating cellular copper content on mouse hematopoietic stem/progenitor cell self-renewal and differentiation using an ex vivo culture model. In contrast to previous human cell studies, I find no enhancing effect of reducing cellular copper content on the self-renewal of HSCs. I find that erythroid lineage committed progenitor cell generation or expansion is enhanced by reducing cellular copper content in the culture of multipotent progenitor cells. In Chapter 3,1 investigate the physiological effect of hyper- or hypo- active EPOR signaling on hematopoietic stem cell and progenitor cell function. I find that EPOR signaling does not play a role on HSC self-renewal naturally. EPOR signaling is essential for erythropoiesis and may play a minor role in platelet differentiation. EPOR signaling may also play a role on multipotent progenitor cell proliferation or differentiation. In Chapter 4,1 summarize my thesis work and provide some directions for further investigation.

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CHAPTER 2

COPPER MODULATES THE PROLIFERATION AND DIFFERENTIATION OF MOUSE HEMATOPOIETIC PROGENITOR CELLS IN CULTURE

Abstract

Copper chelation has been shown to favor the expansion of human hematopoietic stem/progenitor cells in vitro. To further understand the effects of copper modulation on defined subsets of stem cells versus progenitor cells, I extended the studies in a mouse system. I isolated mouse hematopoietic stem cells (HSCs) or hematopoietic progenitor cells (HPCs) and cultured them with or without the copper chelator tetraethylenepentamine (TEPA) or CuC^. Cytokine-stimulated HPC cultures treated with TEPA for 7 days generated about two to three times more total and erythroid colony-forming cells (CFCs) compared to control cultures. In contrast, C11CI2 treatment decreased the CFC numbers. Similar results were seen with HSC after 14, but not 7, days of culture. Transplant studies showed that HPCs cultured for 7 days in TEPA had about 2 fold higher short-term erythroid repopulation potential compared to control cultures, while CuCli decreased the erythroid potential of cultured HPCs compared to control cultures. There are no significant differences in leukocyte and platelet engraftment among

culture cells under different conditions. HSCs cultured with TEPA for 7 days did not exhibit significantly higher repopulation potential in either leukocyte, erythrocyte and megakaryocyte/platelet lineages compared to control cultures in short-term or long-term assays. Based on JC-1 staining, the mitochondrial membrane potential of HPCs cultured with TEPA was lower relative to control cultures. Our data suggest that decreasing the cellular copper content with TEPA results in preferential expansion or maintenance of HPC that are biased for erythroid differentiation in vivo, but does not enhance the maintenance of HSC activity in culture.

Introduction

Metal elements, such as iron (Fe), calcium (Ca), magnesium (Mg), zinc (Zn) and copper (Cu), are fundamental and essential for life. Cu is a cofactor for many cellular enzymes, such as cytochrome c oxidase, superoxide dismutase, tyrosinase, peptidylglycine alpha-amidating mono-oxygenase, lysyl oxidase (Harris, 2000; Pena et al., 1999; Uauy et al., 1998). Cu has also been shown to regulate gene expression, cellular protein functions and cell differentiation (Barbara Birkaya, 2005; Dahl et al., 2005; Hainaut et al., 1995; Iseki et al., 2000; Kang et al., 2004; Kudrin, 2000; Ostrakhovitch et al., 2002; Vanacore et al., 2000). However, excess intracellular Cu is toxic due to its role in catalyzing the generation of reactive oxygen species (ROS) through Fenton chemistry (Leary and Winge, 2007). Specifically, Cu is essential for normal hematopoiesis in which mature blood cells are constantly replenished from hematopoietic stem cells in the bone marrow. In adult mammals, hematopoietic stem cells (HSCs) gradually lose their self-renewal capability and multipotency and restrict to

specific lineages. HSC must replicate through self-renewing cell divisions to maintain the pool of HSCs. This process is tightly regulated by the interplay of extrinsic and intrinsic molecular mechanisms in order to both sustain blood production and maintain the HSC compartment (Kondo et al., 2003). In vitro studies showed that addition of Cu salts augmented the retinoic acid-induced differentiation of the human myeloid leukemia cell line, HL-60 (Bae and Percival, 1993). Lowering of the cellular Cu level by the Cu chelator tetraethylenepentamine (TEPA) blocked 1,25-dihydroxyvitamin D3 or phorbol 12-myristate 13-acetate-induced U937 cell differentiation and this blocking was reversed by adding back Cu (Huang et al., 2001). The importance of Cu in regulating hematopoiesis is also inferred from inherited or acquired Cu deficiency in human and mouse. Cu deficiency due to genetic mutations or malnourishment in humans causes anemia, neutropenia, and thrombocytopenia due to the arrested differentiation at the hematopoietic progenitor cell level (Cordano et al., 1966; Fuhrman et al., 2000; Goyens et al., 1985; Halfdanarson et al., 2008; Hirase et al., 1992; Naveh et al., 1981; Porea et al., 2000; Simon et al., 1988; Zidar et al., 1977). Interestingly, recent studies showed that the high-affinity copper chelator TEPA, together with early-acting cytokines (TPO, SCF, IL-6, FL), can promote the expansion of both hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs) from human cord-blood derived $CD34^+$ cells in culture (Peled et al., 2005; Peled et al., 2002). The expansion of HPCs was shown by in vitro colony-forming assay and FACS analysis of the expression of cell surface markers. The expansion of the HSCs was shown by the repopulation of the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with cultured cells (Peled et al., 2004a; Peled et al., 2004c). This finding has justified a clinical trial using TEPA together with

cytokines for the ex vivo expansion of human HSC/HPCs prior to transplantation into patients (de Lima et al., 2008; Peled et al., 2004b). Although the expansion of HPCs is clearly established by both the in vitro and in vivo assays, the expansion of HSCs in these cultures is less convincing. There are still many controversies about the faithfulness of the NOD/SCID mouse model in detection and quantification of human HSCs (Coulombel, 2004). Because the differences of the physiological environment of mouse and human and the differences of the life span and proliferation capacities of mouse and human hematopoietic cells, the detection of human cells in NOD/SCID mouse bone marrow 8 weeks after the transplantation does not prove the contribution from the human HSCs (Horn and Kiem, 2006). As a result, it is unclear whether TEPA significantly promotes the self-renewal and expansion of HSCs in vitro. The objective of present study was to clarify these issues by extending the study in the mouse system, where isolation and unequivocal assays for HSC and HPC are better defined. To separate mouse HSCs from HPCs for analysis of the effect of Cu modulation on each of these two populations, I used cell surface markers in combination with the mitochondrial dye rhodamine-123 to fractionate mouse bone marrow cells. The most primitive HSC and HPC reside in a population of bone marrow cells that do not express mature blood cell surface markers (lineage negative or Lin") and express Sca-1 and c-Kit cell surface markers. These bone marrow cells are commonly referred to as the LSK population (Spangrude et al., 1988). I further divide the LSK population by rhodamine-123 staining. Rhodamine-123 (Rho) is a mitochondrial dye that stains cells based on their state of activation, in which the more metabolically active cells fluoresce brightly while more quiescent cells fluoresce dimly (Bertoncello et al., 1988). Segregation of the LSK population using Rho has

demonstrated that the LSKRho^{low} population contains long-term HSC with a frequency of about 25%, while the LSKRho^{hi} population lacks HSC but is highly enriched for multipotent and lineage-restricted progenitor cells (Li and Johnson, 1992; Spangrude and Johnson, 1990). I cultured these two populations separately with early-acting cytokines (SCF, TPO, IL-6, FL) and used TEPA or $CuCl_2$ to modulate the Cu content within the cultured cells. I used in vitro colony-forming assays and in vivo mouse competitive repopulation assays to characterize and quantify the progenitor and stem cells within the cultures. Our results show that TEPA promotes the expansion or maintenance of progenitor cells that are mainly erythroid lineage-biased within the HPC culture. In contrast, TEPA did not significantly promote the expansion or maintenance of HSC in culture. The effects of TEPA on HPC correlate with its effect of reducing the mitochondrial membrane potential of cultured HPC.

Materials and methods

Mice

Mice carrying the Thy-1.1 and Ly-5.1 alleles on a C57BL background were generated in our breeding colony by mating the BKa.AK-Thyl^a/Ka and B6.SJL-Ptprc^a Pep3^b/BoyJ strains and selecting for cosegregation of Thy-1.1 (CD90^a) and Ly-5.1 (CD45^a). Breeding pairs of B6.Cg-Gpil^a Hbb^d Hl^b/DehJ mice (Harrison et al., 1988) were kindly provided by David Harrison (Jackson Laboratory, Bar Harbor, ME). I refer to these mice as B6-Hbb^d. GFP transgenic mice were provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan). This transgenic strain was generated by pronuclear injection of fertilized eggs obtained from C57BL/6 matings as previously described

(Nakanishi et al., 2002) and are here referred to as B6-GFP. Mice carrying both GFP transgene and Hbb^d allele were generated in our breeding colony by mating B6-Hbb^d mice with B6-GFP mice and selected for cosegregation of GFP and Hbb^d. I refer to these mice as B6-GFP/Hbb^d. All animals were maintained in the animal resources center at the University of Utah under protocols approved by the institutional animal care and use committee.

Antibodies

Monoclonal antibodies against CD2 (Rm2.2), CD3 (KT3-1.1), CD5 (53-7.3), CD8 (53-6.7), CD1 lb (Ml/70), Ly-6G (RB6-8C5), TER119, CD45R (B220; RA3-6B2) and CD 19 (1D3) were purified from media of cultured hybridoma cell lines. PE-conjugated monoclonal antibodies to Sca-1 and CD 19 were purchased from PharMingen (San Diego, CA, USA). Anti-c-kit mAb 3C11 and anti-Ly5.1 mAb A20.1 were purified and conjugated to Alexa Fluor 647 in our laboratory. PE-conjugated monoclonal antibodies to CD4, CD8, B220, Mac-1 and Gr-1 were purchased from eBioscience (San Diego, CA, USA) or purified and conjugated in our laboratory.

Cytokines

Recombinant mouse SCF was expressed in bacteria and purified from lysates as previously described (Yee et al., 1993). Recombinant mouse Flt3L, IL-3, GM-CSF and IL-6 were purchased from Peprotech (Rocky Hill, NJ, USA). The peptide mimetic of thrombopoietin (TPO) was synthesized as described (Cwirla et al., 1997). Recombinant

human erythropoietin (EPO) was purchased from Ortho Pharm. Corp. (Raritan, NJ, USA).

Isolation of bone marrow hematopoietic stem
and progenitor cells (HSC/HPCs)

Mouse bone marrow cells were incubated in a cocktail of antibodies to CD2, CD3, CD5, CD8, CD1 lb, Ly-6G, TER119, CD45R, and CD19. Lineage depletion was performed by two successive incubations in sheep anti-rat Ig-coupled magnetic beads (Dynal, Oslo, Norway). Lineage-negative (Lin^{1168}) cells were incubated with 0.2 rhodomine-123 (Molecular Probes, Eugene, OR) in Hank's balanced salt solution containing 5% fetal calf serum (HBSS) at 37°C for 20 min. After washing once, the cells were resuspended in fresh HBSS and incubated at 37°C for another 20 min to allow for efflux of the dye. After washing, the cells were stained with phycoerythrin (PE)-conjugated anti-Sca-1 and AlexaFluor 647-conjugated anti-c-kit antibodies. Sorting was done using FACS Vantage or FACS Aria instruments (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). I sorted $Lin^{neg}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) bone marrow cells as $LSKRho^{low}$ and $LSKRho^{l}$" populations using Rho-stained bone marrow cells to determine the placement of the Rho gates. This usually resulted in $LSKRho^{l}$™ cells comprising the lower 20% of the Rho distribution and $LSKRho^{hl}$ cells comprising the upper 50% of the Rho distribution. An aliquot of each sorted cell population was taken for reanalysis to evaluate purity and cell count.

In vitro liquid culture of HSCs and HPCs

Purified LSKRho^{low} and LSKRho^{hi} cells were cultured in 24-well plates at 1000 cells/ml in alpha-MEM medium supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mM 1-glutamine, 100 |iM 2-mercaptoethanol, 1% each sodium pyruvate and pen/strep stock solutions (Invitrogen Corp, Carlsbad, CA) and the following mouse recombinant cytokines: stem cell factor (SCF) at 100 ng/ml, thrombopoietin mimetic (TPO) at 5 nM, interleukin 6 (IL-6) at 10 ng/ml and FLT-3 ligand (FL) at 10 ng/ml. Cellular Cu content was modulated by supplementing the culture with either TEPA and/or CuCb (both obtained from Sigma-Aldrich, St. Louis, MO) as indicated. At weekly intervals, cell cultures were demi-depopulated and supplemented with fresh medium, serum, cytokines and other components as indicated. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. At various time points, cells were harvested and counted by hemocytometer following staining with trypan blue. The intracellular Cu content of cultured cells was determined using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Perkin Elmer Life Sciences) as described (Leary et al., 2007).

Colony-forming cell (CFC) assay

Cells were plated in alpha-MEM-based methylcellulose containing 10% fetal bovine serum (FBS), 10% deionized bovine serum albumen (BSA), antibiotics, 1-glutamine, and 2-mercaptoethanol. Colony growth and differentiation were stimulated with recombinant cytokines in combination as indicated, including stem cell factor (100 ng/ml), G-CSF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (20 ng/ml), thrombopoietin mimetic (5

nM), and erythropoietin (4 U/ml). After 7 days of culture, quadruplicate plates were stained using benzidine hydrochloride and scored for total colonies and for colonies containing hemoglobinized erythroid lineage cells colonies based on benzidine staining.

Mouse transplantation assay

Radiation was delivered to B6-Hbb^{d} or B6-GFP/Hbb^{d} recipient mice in a split dose (2x6 Gy) with a 3 hr interval between doses, using a Shepherd Mark 1 ^{137}Cs source (JL Shepherd and Associates, Glendale, CA) at a dose rate of 0.8 Gy/min. Cultured cells mixed with competitor BM cells were transplanted by the retro-orbital route under isoflurane anesthesia (IsoSol; Vedco Inc., St. Joseph, MO). Peripheral blood samples were collected from the retroorbital sinus under isoflurane anesthesia delivered using the E-Z Anesthesia system (Euthanex Corp., Palmer, PA). Blood was collected in heparinized capillary tubes and mixed with acid citrate dextrose at a 10:1 ratio prior to determination of the complete blood count using a Serono System 9010^{+} CP hematology counter (Serono Diagnostics, Allentown, PA). Samples were then mixed with an equal volume of 2% Dextran T500 (Amersham Biosciences, Piscataway, NJ) in phosphate-buffered saline (PBS) and incubated at 37°C for 30 min. The upper layer, containing leukocytes, platelets, and residual erythrocytes, was collected for flow cytometry analysis, whereas the sedimented erythrocytes were used for Hbb analysis.

Determination of Hbb variants by HPLC

A cation exchange protocol was developed to separate and quantitate Hbb variant alleles by HPLC (Spangrude et al., 2006). A stock solution of 100 mM 5,5'-dithiobis-(2-

nitrobenzoic acid) (DTNB) (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 10 mg of DTNB in 250 |il of dimethyl sulfoxide and stored at -20°C. Erythrocytes obtained from heparinized blood were washed three times in dextrose-gelatin-veronal buffer and stored as packed pellets at 4°C for up to 2 weeks prior to analysis. Samples were derivatized by adding 10 of packed erythrocytes to 200 [il of 40 mM NaCl, 2 mM DTNB and incubating at room temperature for 30 min. Following centrifugation (12,000g for 2 minutes), samples were analyzed using a VARIANT hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

All results are expressed as the mean +/- the standard error of the mean. Statistical significance was determined using the paired Student t-test with significance at a p value <0.05.

Results

Effects of TEPA and Cu supplementation on intracellular Cu content of cultured progenitor cells

I did a titration experiment to determine the optimal concentration of TEPA for the growth of HSC/HPCs in the culture and found that TEPA at 40 jiM is optimal for the progenitor cell proliferation in HSC/HPC cultures. I determined the effect of supplementing TEPA and CuCb on intracellular Cu content of HPC cultures. I did two independent measurements using inductively coupled plasma-optical emission spectrometry (ICP-OES) (Leary et al., 2007). LSKRho^{hi} HPCs were cultured in serum

and cytokine-containing medium for 7 days. Under these conditions, ICP-OES measurements indicated that cultured cells contained 7.6 - 12.7 ng Cu per 10^7 cells. When the Cu chelator TEPA was added to the culture at 40 pM for 7 days, the cellular Cu content decreased to 2.4 - 2.8 ng per 10^7 cells. TEPA-treated cultures showed only modest changes in other metals (Fe 30% decrease, Zn 8% decrease) suggesting that the TEPA effect is copper-specific. Addition of 10 pM $CuCl_2$ to the culture medium increased the cellular Cu content to 686.3 - 3088.5 ng per 10^7 cells (Table 2.1). These results indicate that the cellular Cu content of HPC can be modulated by the addition of TEPA or $CuCl_2$ to the culture medium.

Effects of TEPA and Cu supplementation in HSC/HPC cultures

I analyzed of the progeny of HSC or HPC after 7 and 14 days of liquid culture by counting total cell numbers, and used the in vitro methylcellulose-based hematopoietic colony-forming assay to measure CFC activity in the cultures (Figure 2.1 and 2.2). LSKRho^{hi} HPC cultured with 40 or 80 pM TEPA plus FBS and cytokines for 7 days produced two to three times more total CFCs than control cultures maintained with FBS and cytokines alone (Figure 2.1 A). Interestingly, in the presence of 80 pM TEPA the expansion of cell numbers was reduced relative to controls while the CFC numbers increased significantly (Figure 2.1 A). In contrast, LSKRho^{hl} HPC cultured with 10 pM C1lCl2 plus FBS and cytokines for 7 days produced about half of the CFCs of control culture with a similar expansion of cell numbers. TEPA at 80 pM also increased the erythroid CFC number compared to control while 40 pM had no effect on erythroid

Table 2.1. Intracellular Cu content of cultured hematopoietic progenitor cells is modulated by Cu chelator and Cu salt. $LSKRho^{hl}$ HPCs were cultured with cytokines +/- TEPA or $CuCl_2$ for 7 days. The copper content of the cultured cells was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The ng of Cu per 10 million cells in each culture condition is presented.

	40|j.M TEPA	IOjiM $CuCl_2$	Control
Cu (ng) /10^7 cells (experiment 1)	2.4	686.3	7.6
Cu (ng)/10^7 cells (experiment 2)	2.8	3088.5	12.7

CFCs (Figure 2.1 A). After 14 days of culture, LSKRh^{hl} HPCs had decreased number of total cell numbers and CFC numbers compared to day 7 cultures, but cultures supplemented with 40 or 80 |iM TEPA had much more total CFC and erythroid CFC number than cultures supplemented with CuCh or control cultures (Figure 2.IB). In parallel cultures initiated with LSKRho^{low} HSC, I observed no difference in total CFC number between cell cultures with TEPA, CuCh and control in one week of culture (Figure 2.2A). Relative to control cultures, I observed an increase in erythroid CFC in cultures containing 40 jaM TEPA and a decrease in erythroid CFC in cultures containing supplementary Cu (Figure 2.2A). After 2 weeks of culture, LSKRho^{low} cells cultured with 40 (iM TEPA generated about 30% more total CFCs compared to control cultures containing cytokines only, while treatment with 10 |iM CuCl2 decreased the total CFCs in culture compared to control (Figure 2.2B).

I examined the morphology of the cells in cultures established from LSKRho^{hl} HPCs by May-Grunwald-Giemsa staining of cytospins. Cultures supplemented with 40 joM TEPA contained cells with a uniform and blast-like morphology, while control cultures contained more cells with morphological characteristics of differentiation (Figure 2.3 A, 2.3B). Supplementation with 10 (iM CuCh increased the portion of cells with differentiated cell morphology, while adding TEPA to CuC^-supplemented culture reversed the effect of Cu (Figure 2.3C, 2.3D).

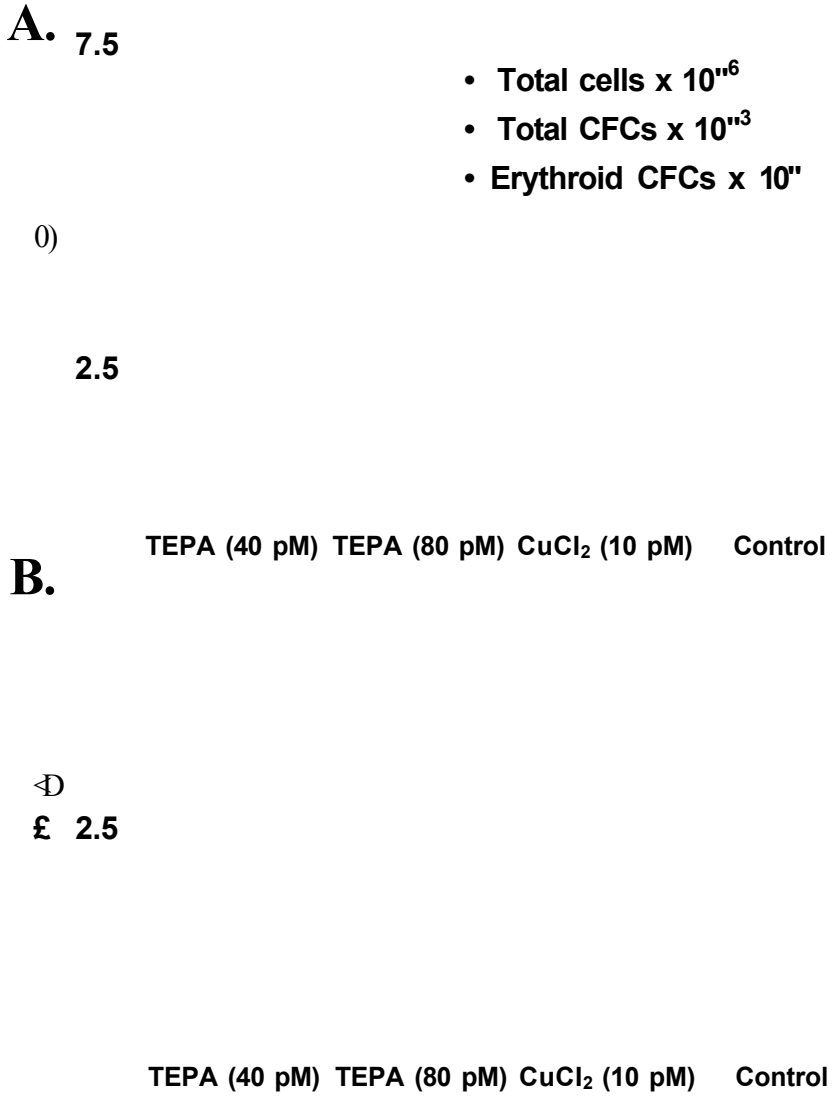


Figure 2.1. The effects of TEPA or Cu supplementation on HPC cultures. LSKRho^{111} HPCs (1000 cells) were cultured with cytokines +/- TEPA/Cu at indicated concentrations for 7 days (A) or 14 days (B). The total cell number and total CFC and erythroid CFC number were determined at the end of culture.

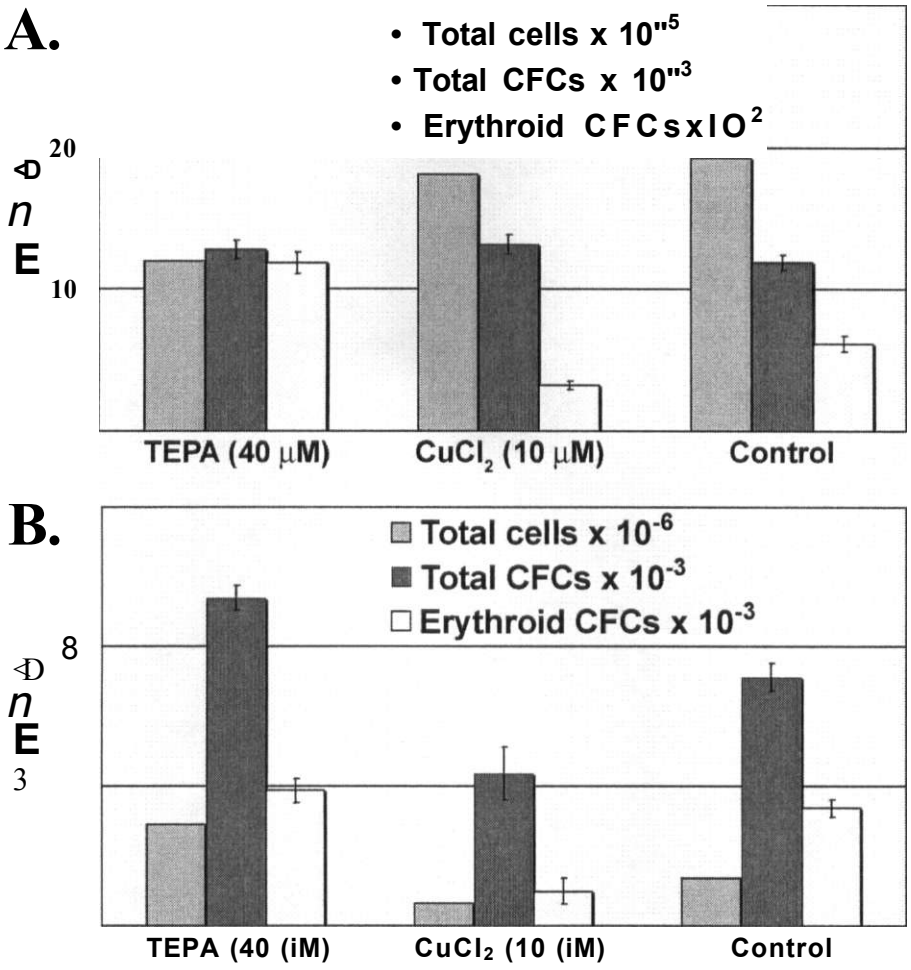


Figure 2.2. The effects of TEPA or Cu supplementation on HSC cultures. LSKRho^{low} HSCs (1000 cells) were cultured with cytokines +/- TEPA/Cu at indicated concentrations for 7 days (A) or 14 days (B). The total cell number and total CFC and erythroid CFC number were determined at the end of culture.

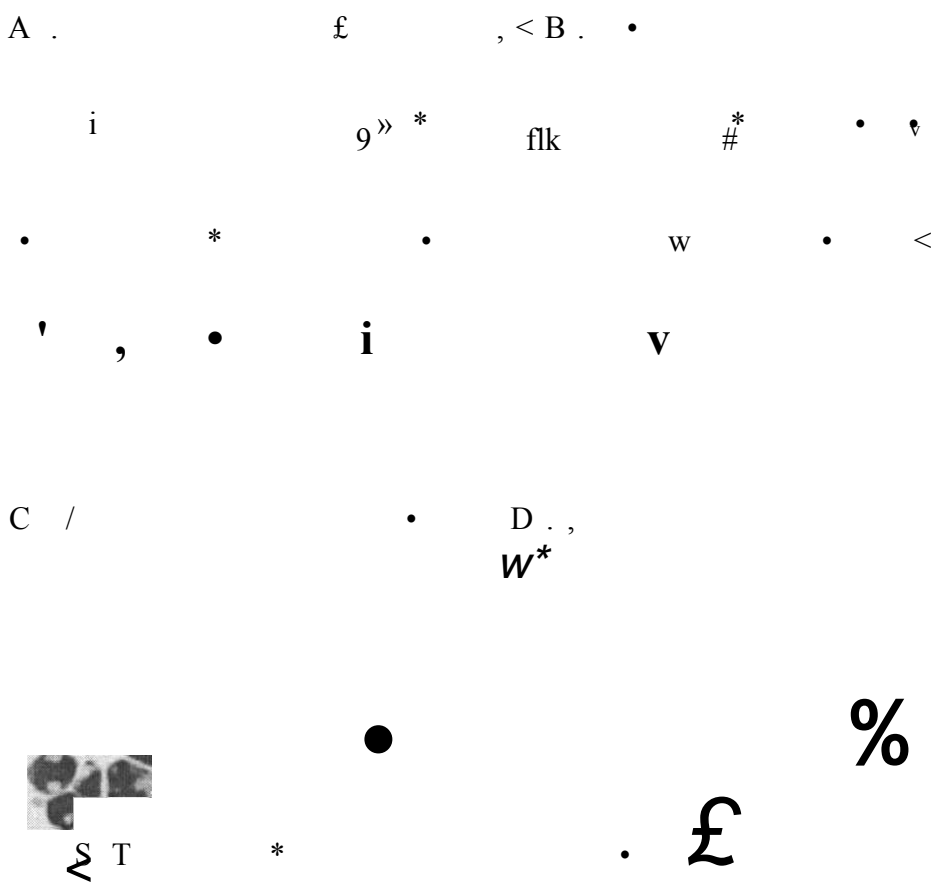
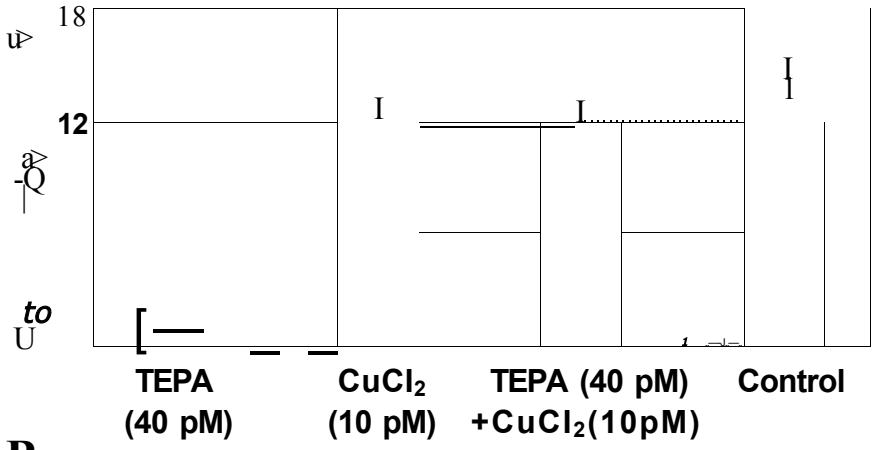


Figure 2.3. The effects of TEPA or Cu supplementation on the morphology of HPC cultures. LSKRho^{hi} HPCs were cultured with cytokines +/- TEPA/Cu at the indicated concentrations for 7 days. Cultured cells were cytospun into slides and stained with May-Grunwald-Giemsa. The culture conditions for cells shown in each panel were: A. control; B. 40 pM TEPA; C. 10 pM $CuCl_2$; D. 40 pM TEPA + 10 pM $CuCl_2$. Scale bar: 10 pm.

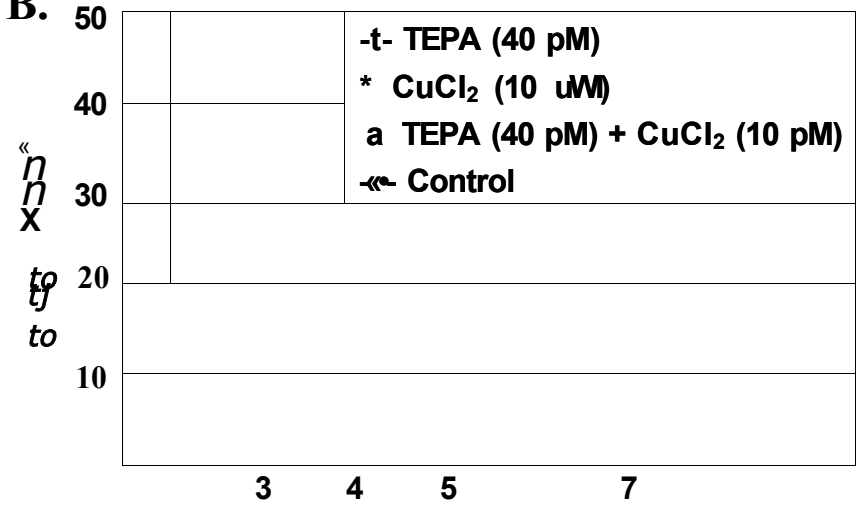
Effects of TEPA and Cu supplementation on in vivo short-term tri-lineage repopulating activity of cultured HPCs

To evaluate the in vivo repopulation activity of cultured cells in the three major blood lineages, I harvested 1-week cultures of LSKRho^{1}" HPCs and transplanted these cells together with a fixed number of competitor bone marrow cells into lethally irradiated congenic recipient mice. Cultured cells were tracked based on expression of the Ly5.1 allele on the cell surface of white blood cells and the hemoglobin-P single (Hbb^{s}) variant in red blood cells and lack of GFP expression on platelets (Spangrude et al., 2006). Competitor bone marrow and recipient mice cells expressed the Ly5.2 allele, the hemoglobin-P diffuse (Hbb^{d}) variant, and GFP. This transplant model allows us to track the in vivo contribution of cultured cells into the leukocyte, platelet and erythrocyte lineages. I transplanted cultured cells obtained from a 7-day culture of 1000 LSKRho^{hl} cells, together with 10^{5} freshly-obtained competitor bone marrow cells into lethally irradiated mice and followed the relative contributions of cultured cells and competitor BM cells into the leukocyte, platelet, and erythrocyte lineages in the peripheral blood of recipient mice over time. LSKRho^{hl} cells cultured with 40 pM TEPA generated about 2 fold higher percentage of reconstitution of the erythroid lineage repopulation compared to LSKRho^{1}" cells cultured with cytokines alone, in spite of a lower degree of cellularity in the cultures expanded under this condition (Figure 2.4A and 2.4B). The red blood cell count of the recipient mice was similar among the groups (data not shown). This enhancement of erythroid engraftment persisted over the time course of progenitor cell activity in the recipients, and was limited to the erythroid lineage as these cultures had

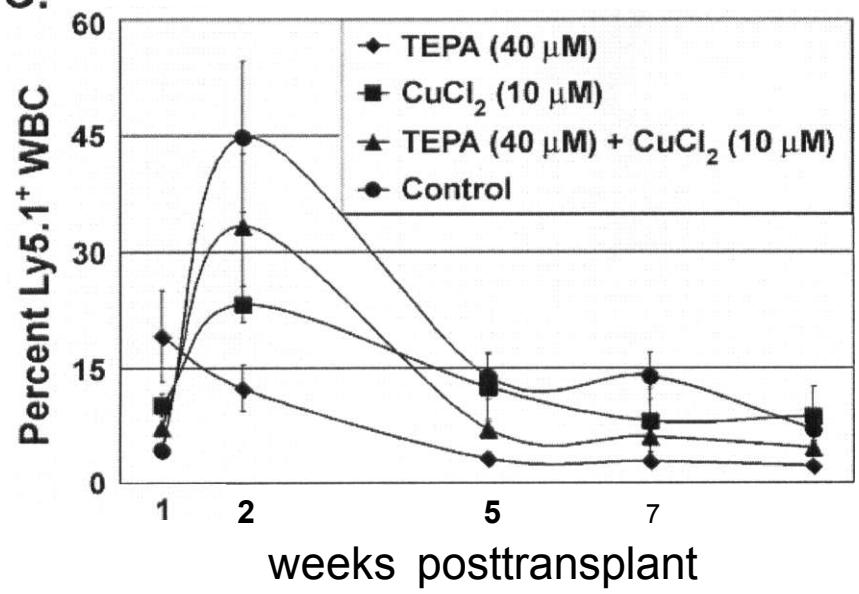
Figure 2.4. The effects of TEPA or Cu supplementation on the short-term erythrocyte and leukocyte repopulation potential of HPC cultures. LSKRho^{hi} HPCs (1000 cells) were cultured with cytokines +/- TEPA/Cu at the indicated concentrations for 7 days. The total cell numbers were determined at the end of culture (A). The total cells were mixed with 10^5 whole BM cells and injected intravenously into lethally irradiated mice. The percentage contribution of cultured cells to peripheral blood erythrocytes (B) and leukocytes (C) were determined at the various times post-transplantation.


18
12
TEPA (40 pM)
CuCl2 (10 pM)
TEPA (40 pM) +CuCl2(10pM)
Control

B.


50
40
30
20
10
-t- TEPA (40 pM)
* CuCl2 (10 uM)
a TEPA (40 pM) + CuCl2 (10 pM)
-«- Control
3
4
5
7
weeks posttransplant

C.


60
45
30
15
0
Percent Ly5.1+ WBC
TEPA (40 μM)
CuCl2 (10 μM)
TEPA (40 μM) + CuCl2 (10 μM)
Control
1
2
5
7
weeks posttransplant

about 30% of the leukocyte lineage repopulation activity of LSKRho^{hl} cells cultured with cytokines alone (Figure 2.4C). The effect of TEPA was reversed by addition of CuCh into the culture medium. TEPA and Cu had no obvious effects on platelet lineage repopulation activity of cultured LSKRho^{h}" cells (data not shown). The contribution of cultured cells to the peripheral blood of recipient mice was limited to 9 weeks posttransplantation, consistent with the absence of stem cells in the LSKRho^{l}" population and its cultured progeny. Collectively, our results suggest that TEPA preferentially promotes the maintenance or expansion of erythroid-biased progenitor cells in cultures established from primitive progenitor cells.

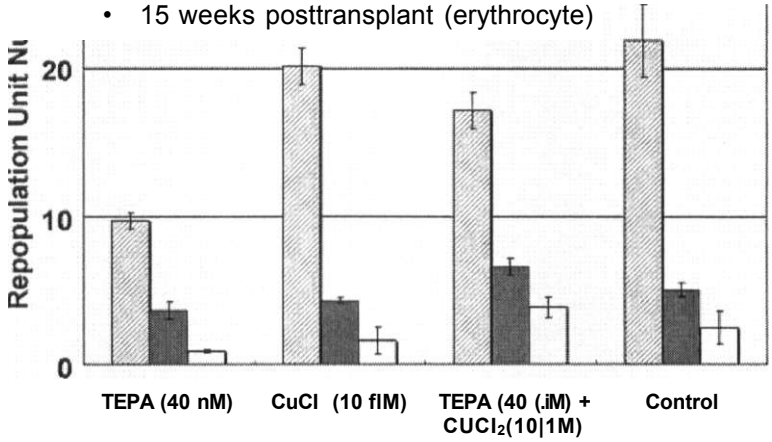
Effects of TEPA and CuCb supplementation on the short-term
and long-term repopulation capacity of cultured HSCs

To evaluate the effects of TEPA on expansion of HSC number and function, I tested the activity of cultured LSKRho^{low} HSC in the long-term competitive repopulation assay. The 7-day cultured cells derived from 10^3 LSKRho'^{ow} BM cells were cotransplanted with 10^6 fresh competitor BM cells into lethally irradiated recipient mice. The relative contribution of cultured cells and competitor cells into peripheral blood leukocyte and erythrocyte lineages were analyzed using Ly5 and Hbb variants at 4 weeks, 8 weeks, 15 weeks and 20 weeks posttransplantation. At 4 weeks, LSKRh^{low} HSCs cultured with 40 jiM TEPA had significantly less repopulation capacity in the leukocyte lineage than control cultures or cultures supplemented with 10 (iM CuCl2 (Figure 2.5B). At 15 weeks posttransplantation, there was no significant difference of leukocyte and erythrocyte repopulation potential of cultured cells under different conditions (Figure

Figure 2.5. The effect of TEPA or Cu supplementation on the short-term and long-term repopulation potential of HSC cell cultures. LSKRho^{low} HSC (1000 cells, equals approximately 100 RU) were cultured under the indicated conditions for 7 days and then transplanted together with 10^6 competitor BM cells into lethally irradiated recipients. The peripheral blood was sampled at various times and the contribution of cultured cells to leukocyte and erythrocyte pools was determined by flow cytometry and HPLC. Repopulation unit (RU) number was calculated based on 1 RU=10^5 competitor BM cells. Results from 3 independent experiments are shown in panels A, B, and C.

A.

- 4 weeks posttransplant (leukocyte)
- 15 weeks posttransplant (leukocyte)
- 15 weeks posttransplant (erythrocyte)

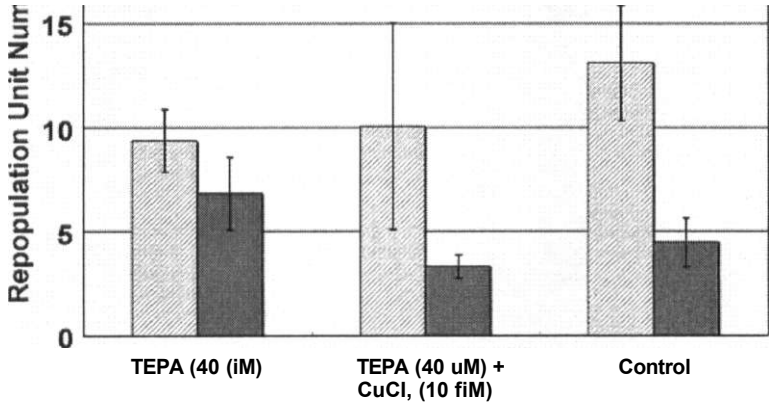


B.

20

m Two months posttransplant (leukocyte)

• Five months posttransplant (leukocyte)



c .

8

B Five months posttransplant (leukocyte)

• Five months posttransplant (erythrocyte)

6

4

2

mm

TEPA (40 yM)

TEPA (40 pM) + $CuCl_2$ (10)iW!)

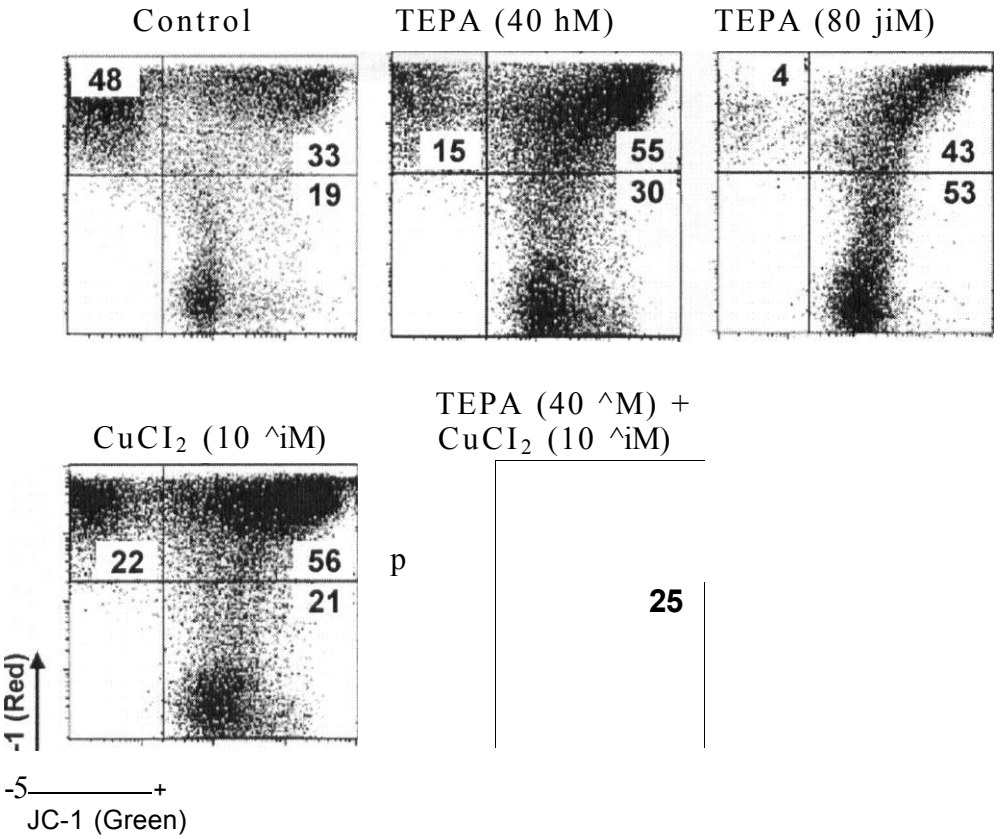
Control

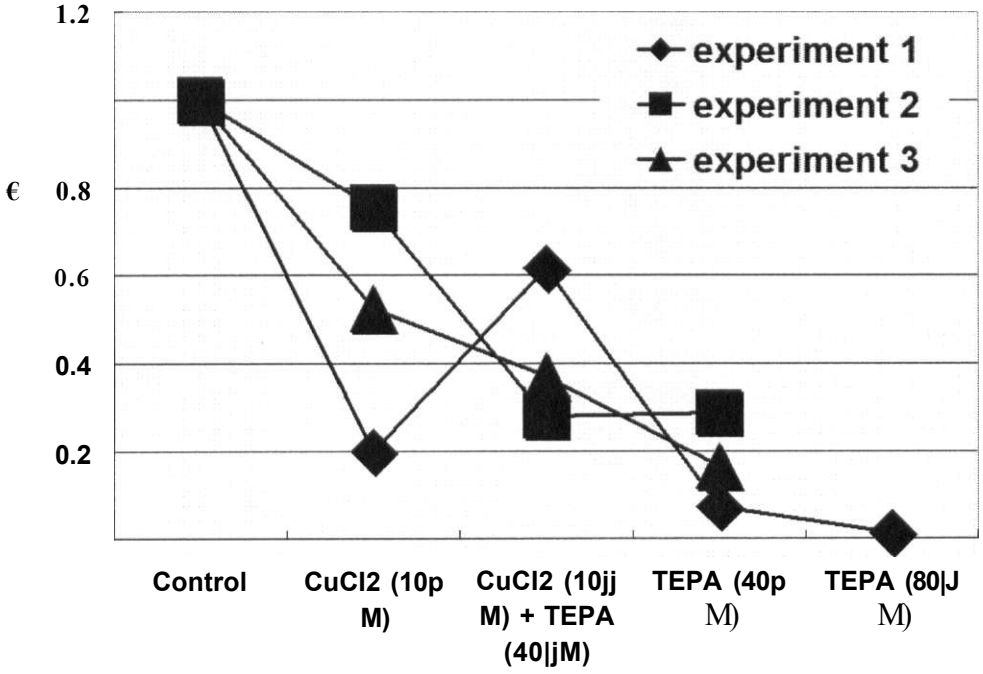
2.5B). At 5 months posttransplantation, HSCs cultured with 40 pM TEPA showed a slight increase in the repopulation in leukocyte and erythrocyte lineages, but this increase was not statistically significant (Figure 2.5A, 2.5C). Based on these data, I conclude that TEPA does not significantly increase the maintenance or expansion of short-term or long-term HSCs in culture.

Effects of TEPA and Cu supplementation on the mitochondrial membrane potential (A^m) of cultured progenitor cells

To gain some insights into the mechanisms of TEPA's effect on LSKRho^{111} progenitor cells and their progenies in culture, I measured the mitochondrial membrane potential (A4V) of day-7 cultured cells from LSKRho^{1}" cells. I used the cationic dye JC-1 that indicates increased mitochondrial polarization by shifting its fluorescence emission from green to red. JC-1 staining of LSKRho'^{11} bone marrow cells cultured for 7 days showed that 40 pM TEPA increased the number of cells that have relatively low AT_m and decreased the number of cells that have relatively high ATm compared to control cultures containing only cytokines (Figure 2.6A). I did three independent cultures and AT_m measurement. The results are summarized in Figure 2.6B. The ratio of JC-1 red to green fluorescence is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape and density that may influence single-component fluorescence signals. To compare the results from different experiments, the ratio is normalized to 1 in control culture and the ration of other culture conditions are divided by the ration of control culture in the same experiment. The effect of TEPA on mitochondrial membrane potential of cultured cells was reversed by adding a low amount

Figure 2.6. The effect of TEPA or Cu supplementation on the mitochondrial membrane potential of cultured HPC. LSKRho^{hi} cells were cultured under the indicated conditions for 7 days, stained with JC-1 and analyzed by flow cytometry. The relative fluorescence emission in FL1 (green=low mitochondrial membrane potential) and FL2 (red=high mitochondrial membrane potential) channels indicates the relative mitochondrial potential of the cultured cells. The percentages of cells in each quadrant are shown in A. The normalized JC-1 red to green fluorescence ratios from three independent experiments are shown in B.


Control
TEPA (40 hM)
TEPA (80 jiM)
48
33
19
15
55
30
4
43
53
CuCI2 (10 ^iM)
TEPA (40 ^M) +
CuCI2 (10 ^iM)
22
56
21
p
25
-1 (Red)
-5
+
JC-1 (Green)


1.2
0.8
0.6
0.4
0.2
experiment 1
experiment 2
experiment 3
Control
CuCl2 (10p M)
CuCl2 (10jj M) + TEPA (40|jM)
TEPA (40p M)
TEPA (80|J M)

of exogenous Cu into TEPA-supplemented cultures, suggesting reduction of Cu concentration within the cell is responsible for the effect of TEPA (Figure 2.6).

Effects of TEPA and Cu supplementation on the reactive oxygen species (ROS) content of cultured progenitor cells

Free Cu ions within the cells are very potent catalyst for the generation of ROS. I measured the relative ROS content in my cultured cells using fluorescent dye dichlorodihydrofluorescein diacetate (DCF-DA) (Kohen and Nyska, 2002). The results from two independent experiments are shown in Figure 2.7. The mean fluorescence intensities (MFI) of culture HPCs stained with DCF-DA under different conditions are normalized to the value of control culture in each experiment (Figure 2.7). From the result, it is clear that TEPA treatment does not decrease the ROS content in HPCs compared to the control culture with cytokines only, actually TEPA treatment slightly increased ROS content within the culture HPCs.

Discussion

Copper has long been known to be essential for normal hematopoiesis in mammals from both in vitro and in vivo studies. However, the effects of fine-tuning of intracellular copper content on the regulation of HSC/HPCs self-renewal and differentiation are relatively unknown. Recently, some interesting findings suggested that reducing the intracellular Cu content by Cu chelation in human HSC/HPCs is beneficial for their expansion or maintenance in culture. While this finding is very intriguing and promising,

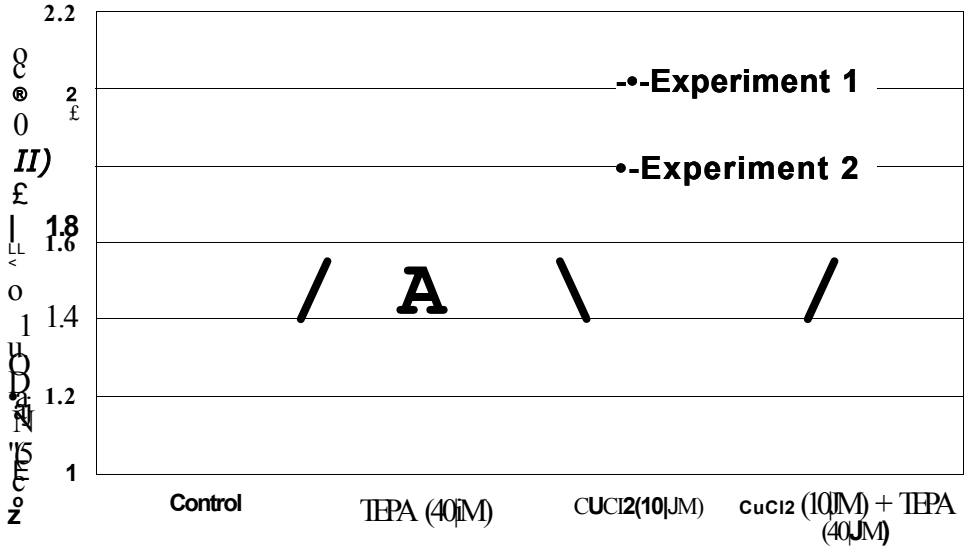


Figure 2.7. DCF-DA fluorescence intensity of culture LSKRh[1]" cells after 7 days in culture and stained with DCF-DA. The mean fluorescence intensity of culture cells under different conditions is normalized to value of control culture cells within the same experiment.

it is unclear whether copper chelation expands or maintains the activities HSC, HPC or both, due to the difficulty of faithfully separating and measuring human stem and progenitor cell activities. HSC are strikingly different from HPC in that HSC can repopulate the hematopoietic system for a life-long period, while HPC can only function for a limited time. The in vivo repopulation potential of human HSC/HPCs can be estimated using xenotransplantation models, usually using the NOD/SCID mouse as a transplant recipient. The NOD/SCID mouse is a widely used model for assessing human HSC/HPCs, however it is still uncertain how well it is able to faithfully measure and quantify human HSC/HPCs, and the utility of the model in predicting human engraftment is controversial. To better address this issue and to gain some insights into the mechanisms of Cu regulation on HSC/HPC fate decisions, I performed experiments using the more defined mouse model system. Using cell surface markers and mitochondrial dye Rho, I isolated HSCs and HPCs as separate populations from mouse bone marrow (Kim et al., 1998; Spangrude and Johnson, 1990). When these two populations were cultured separately in medium containing FBS and cytokines, they responded differently to Cu modulation. I performed in vitro CFC assays to quantify the progenitor cell content in the day 7 and day 14 HPC and HSC cultures. TEPA supplementation at 40 pM and 80 pM increased the total and erythroid CFC number in the HPC culture by two to three fold compared to control cultures after 7 days of culture, and the relative increase was even more striking after 14 days of culture although the overall CFC number was decreased from day 7. In contrast, the HSC cultures showed no difference in total CFC number due to TEPA or Cu supplementation after 7 days of culture. There were slight increases in total CFC numbers in HSC cultures supplemented with TEPA compared to controls and

cultures supplemented with Cu for 14 days. These in vitro CFC assay data suggest that Cu chelation has a positive effect on progenitor cell expansion or maintenance. I also assessed the in vivo repopulation potential of the cultured HPCs or HSCs by competitive repopulation assays. When the cultured cells competed with a fixed number of normal competitor BM cells, the HPCs cultured with 40 pM TEPA showed a 2 fold higher short-term erythroid repopulation potential compared to control culture cells. In contrast, the leukocyte and platelet lineage repopulation from cultured cells was decreased or unchanged by TEPA treatment. Cu addition had the opposite effect, and Cu reversed most effects of TEPA, indicating that intracellular Cu reduction is largely responsible for TEPA's activities in our experiments. I transplanted cultures established from HSCs into lethally irradiated hosts in a competitive repopulation setting, and followed the contribution of cultured cells to the peripheral blood of hosts periodically over a time frame up to 5 months posttransplantation. At 4 weeks posttransplantation, HSC cultures treated with TEPA showed a decreased leukocyte repopulation potential compared control cultures. At 3 to 5 months post-transplantation, HSC cultures treated with TEPA showed a slightly increased repopulation potential in both leukocyte and erythroid lineages, but this increase was not statistically significant. These results suggest that TEPA does not promote the expansion or maintenance of HSCs in cytokine-supplemented cultures. Interestingly, I did not see a similar effect of TEPA on short-term erythroid-biased progenitors in $LSKRh^{low}$ HSC culture as in $LSKRh^{hi}$ HPC culture. This suggests that TEPA acts on a very specific population of progenitor cells that are contained in the $LSKRh^{hl}$ cell population or their progenies but not contained

significantly in LSKRh^{low} cells and their cultured progenies. Further studies are needed to identify and characterize this cell population.

Cu is a co-factor of a key enzyme in the mitochondrial electron transport chain, cytochrome c oxidase (CCO). Reducing Cu content in cells has been shown to result in decreased activity of CCO and mitochondrial membrane potential in myogenic cells (Chen et al., 2005). To investigate whether Cu chelation decreases the mitochondrial membrane potential of cultured HPCs, I stained day-7 HPC cultures with JC-1, a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green to red due to concentration-dependent formation of red fluorescent J-aggregates (Smiley et al., 1991). HPC cultured with 40 and 80 jaM TEPA contained a higher number of cells with low mitochondrial potential compared to control cultures. These cells may represent erythroid-biased progenitor cells. Currently, I do not know whether reducing the mitochondrial membrane potential of these cells is responsible for the enhanced generation or maintenance of erythroid-biased progenitor cells in culture. Further studies are required to answer this question.

Reducing cellular Cu content by TEPA may attenuate CCO activity, and this may in turn lead to a switch in metabolic flux towards glycolysis. Metabolic influences may be one mechanism by which HPCs regulate the choice between proliferation and differentiation (Chen et al., 2008). Mammalian cultures with impaired CCO maturation do show a shift toward glycolysis. TEPA treatment can also reduce the reactive oxygen species (ROS) in some cell types, including HPCs (Prus and Fibach, 2007). Reducing ROS level has been shown to be essential for the maintenance of HSCs in bone marrow (Ito et al., 2004; Ito et al., 2006; Yalcin et al., 2008), and control of ROS level is also

essential for normal erythropoiesis (Marinkovic et al., 2007). My measurement of ROS level in culture cells did not show that TEPA treatment decreases ROS level. So I do not think that TEPA modulates HPC proliferation by modulating intracellular ROS level.

There are several mechanisms that can possibly explain the effect of TEPA in hematopoietic cultures. One possibility is the increased survival of progenitor cells, especially erythroid progenitor cells in TEPA-supplemented culture. Secondly, the inhibition of differentiation of progenitor cells into mature blood cells in TEPA-supplemented cultures may explain our results. TEPA has been shown to have anti-differentiation effects on some established hematopoietic progenitor cell lines, and whether it has similar effects on primary hematopoietic progenitor cells should be investigated. Third, increased proliferation of erythroid progenitor cells in the TEPA-supplemented culture is possibly involved in the selective increases in this lineage in both in vitro and in vivo settings. I are currently investigating whether one or a combination of these mechanisms accounts for TEPA's effects on hematopoietic progenitor cells.

Although I did not see a positive effect of TEPA on HSC expansion or maintenance in our culture system, I can not rule out the possibility that TEPA may have positive effects on stem cell maintenance and expansion in the context of other culture environments or with human cells. It will be very interesting to investigate the effect of TEPA on HSCs together with supportive stromal cells (Huang et al., 2007; Monga et al., 2001) and some recently identified signaling molecules that have been shown to promote the self-renewal of HSCs in culture, such as caspase inhibitors (Wiesmann et al., 2002), Wnt proteins, fibroblast growth factor-1 (FGF-1) (de Haan et al., 2003), insulin like

growth factor-2 (IGF-2) (Zhang and Lodish, 2004) and angiopoietin-like proteins (Zhang et al., 2006). The specific effect of TEPA on erythroid lineage progenitor cells in cultures of HPCs is very intriguing; this effect may be exploited in clinical situations where rapid erythroid repopulation is desired. Furthermore, it will be interesting to study whether Cu modulation has similar effects on the progenitor cells from other tissues.

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CHAPTER 3

THE ROLE OF ERYTHROPOIETIN RECEPTOR SIGNALING ON HEMATOPOIETIC STEM/PROGENITOR CELL FUNCTION

Abstract

The function of erythropoietin (Epo) and its receptor (EpoR) on erythropoiesis is well established. However, the role(s) played by the EpoR in hematopoietic stem cells (HSC), multipotent progenitor cells (MPP), and other hematopoietic lineage-restricted progenitors are largely unknown. It has been previously shown that a transgenic gain-of-function truncated human EPOR gene leads to expansion of the HSC pool in response to exogenous human EPO. MPPs that were enforced to express EPOR gene can also proliferate and differentiate into multiple lineages in response to EPO stimulation (Dubart et al., 1994). But it is unclear whether EpoR plays any roles on HSC and MPP proliferation and differentiation under natural gene expression situation. I have re-investigated this issue using a gene knock-in mouse model, wherein the mouse EpoR gene was replaced in its proper genetic locus by a single copy of either a wild type human or a hyperactive truncated human EPOR gene. Using the competitive repopulation assay, I demonstrate that augmented Epo signaling increases erythroid repopulation as expected, but has no effect on leukocyte repopulation both short-term and long-term.

Interestingly, bone marrow cells obtained from the wild type human EPOR knock-in mouse exhibited diminished erythroid engraftment, suggesting human EPOR is not fully competent in mouse cells, and provided decreased platelet repopulation compared to bone marrow cells from the mutant human EPOR knock-in mouse or normal B6 animals, and this decrease in platelet repopulation was persistent after secondary transplantation. This result suggested a role for EpoR signaling in megakaryopoiesis in vivo. In vitro colony-forming assay demonstrated that MPPs but not HSCs from mthEpoR knock-in mouse can proliferate and differentiate in response to Epo stimulation alone. Normal B6 and wthEpoR knock-in mice HSCs and MPPs can not grow in response to Epo stimulation. This result suggests that MPPs do express EPOR on cell surface naturally and EPOR on MPPs may transduce a proliferation stimulating signals into MPPs. The synergistic responses of HSCs and MPPs to multiple cytokines including EPO are very similar among normal mice and two knock-in mouse models, suggesting EPOR only palys a minor role on MPP proliferation.

Introduction

Erythropoietin (Epo) is the major regulator of red blood cell production. It inhibits the apoptosis of late erythroid progenitors and stimulates their proliferation, ultimately expanding red cell mass.(Koury, 2005) In recent years, Epo has shown expanding roles outside of the erythroid lineage.(Sasaki, 2003) Roles for Epo signaling in hematopoietic stem cell (HSC) and multipotent progenitor cell (MPP) proliferation have recently been suggested by some studies. Murine HSC carrying a gain-of-function truncated human erythropoietin receptor (EPOR) transgene were reported to have a competitive advantage

over wild-type mouse HSC when exogenous human Epo was provided following bone marrow transplantation.(Kirby et al., 2000; Kirby et al., 1996; Urbinati et al., 2005) Murine MPPs that are enforced to express wildtype EPOR gene can proliferate and differentiate into multiple lineages in response to Epo stimulation alone.(Dubart et al., 1994) These observations suggest that murine HSCs and MPPs contain all the necessary intracellular signaling molecules for signal transduction induced by Epo binding to EpoR. These observations may have relevance to the rare subset of polycythemia patients that go on to develop hematologic malignancies. However, due to the nature of the transgenic model system, in which multiple copies of the transgene under the transcriptional regulation of the constitutively active promoter are incorporated into the genome outside of their usual regulatory context, EPOR transgenes are likely to be abnormally expressed in HSCs or early progenitor cells. This misregulated expression could result in functional consequences that would not normally occur under proper regulatory control of the genetic context of endogenous mouse EPOR gene. It is unclear whether under physiological regulation of gene expression, EPOR plays any roles on HSC and MPP proliferation and differentiation.

I have re-examined the effect of hyperactive or hypoactive EpoR signaling on HSC, MPP and lineage-specific progenitor function, utilizing mouse models harboring wild type human EPOR (wthEPOR) or mutant human hyperactive EPOR (mthEPOR) genes knocked into the mouse EPOR locus. (Divoky et al., 2001) This animal model has functional EpoR signaling in all tissues that normally express EpoR and recapitulate normal EpoR expression pattern. Phenotypically, wthEPOR homozygous mice are anemic with decreased Epo-mediated signal transduction, likely due to decreased affinity

of endogenous mouse Epo for the wthEpoR, or decreased signaling downstream of the receptor. In contrast, mthEPOR homozygous mice are polycythemic with increased JAK2 and STAT5 phosphorylation. To evaluate HSC and hematopoietic progenitor cell function in the context of these two knock-in models, I compared the engraftment efficiency of mthEPOR or wthEPOR knock-in mice bone marrow cells relative to normal C57BL/6 mouse bone marrow cells in a competitive bone marrow transplantation assay. I tracked the relative contribution of donor bone marrow cells to peripheral blood leukocytes, erythrocytes and platelets over time using flow cytometry and HPLC. In contrast to previous studies in transgenic mice where EpoR is forced to express on all somatic cells including HSCs, I show that naturally-expressed hyper-responsive human EpoR had no effect on HSC function in spite of a pronounced enhancement of erythrocyte differentiation. Interestingly, wthEPOR knock-in mouse bone marrow cells showed impaired platelet engraftment in concert with the previously reported defect in erythropoiesis compared to either normal mouse bone marrow or mthEPOR mouse bone marrow cells, suggesting a role for Epo signaling in early megakaryocyte differentiation. In vitro colony-forming assay suggested that EpoR hyperactive HSCs do not proliferate in response to Epo stimulation alone and when stimulated with multiple cytokines containing Epo, EpoR hyperactive HSCs give rise to enhanced generation of both erythroid and non-erythroid progenitor cells. EpoR hyperactive MPPs unexpectedly proliferate and form mostly erythroid cell-containg colonies in response to Epo stimulation alone. Normal B6 or wthEPOR mice MPPs do not respond to Epo stimulation alone. These experiments suggest that EPOR influences the proliferation and differentiation of hematopoietic progenitor cells besides erythroid lineage.

Material and methods

Mice

Mice harboring wthEPOR or mthEPOR knocked into the endogenous mouse EpoR locus were generated by gene targeting (Divoky et al., 2001) and then and backcrossed to the C57BL/6 background. C57BL/6 mice carrying the hemoglobin-(3 diffuse variant allele (B6-Hbb^d) were kindly provided by Dr. David Harrison (Harrison, 1980) and were bred in our animal facility. GFP transgenic mice (Nakanishi et al., 2002) were provided by Dr. Masaru Okabe (Osaka University, Osaka, Japan) and are referred to as B6-GFP. Mice carrying both the GFP transgene and the Hbb^d allele were generated in our breeding colony by mating B6-Hbb^d mice with B6-GFP mice and selecting for cosegregation of GFP and Hbb^d. I refer to these mice as B6-GFP/Hbb^d. All animals were maintained in the animal resources center at the University of Utah under protocols approved by the institutional animal care and use committee.

Bone marrow transplantation

Donor bone marrow cells were obtained from either wthEPOR or mthEPOR knock-in mice or normal C57BL/6 mice. The competitor bone marrow cells were obtained from B6-GFP/Hbb^d mice. Equal numbers of donor and competitor bone marrow cells were mixed and transplanted intravenously into lethally irradiated (Cs, 13Gy delivered in a split dose) recipient mice (B6-GFP/Hbb^d) through the retro-orbital route. For secondary transplantation, bone marrow cells were harvested from femora and tibia of the primary transplant recipients and the cells from the recipient mice within each

group were pooled. These pools were transplanted at a dose of 10^7 cells into each lethally irradiated recipient through the retro-orbital route.

Peripheral blood analysis

Peripheral blood samples were collected from the retro-orbital sinus under isoflurane anesthesia delivered using the E-Z Anesthesia system (Euthanex Corp., Palmer, PA). Blood was collected in heparinized capillary tubes and mixed with acid citrate dextrose at a 10:1 ratio prior to determination of the complete blood count using a Serono System 9010^+ CP hematology counter (Serono Diagnostics, Allentown, PA). Samples were then mixed with an equal volume of 2% Dextran T500 (Amersham Biosciences, Piscataway, NJ) in phosphate-buffered saline (PBS) and incubated at 37°C for 30 min. The upper layer, containing leukocytes, platelets, and residual erythrocytes, was collected for flow cytometry analysis, in which platelets and leukocytes derived from test and competitor bone marrow grafts were distinguished by GFP expression. The sedimented erythrocytes were collected and evaluated for relative Hbb^s and Hbb^d ratios using HPLC (see below).

Determination of Hbb variants by HPLC

A cation exchange protocol was developed to separate and quantitate Hbb variant alleles by HPLC.(Spangrude et al., 2006) A stock solution of 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 10 mg of DTNB in 250 |al of dimethyl sulfoxide and stored at -20°C. Erythrocytes obtained from heparinized blood were washed three times in dextrose-gelatin-veronal

buffer and stored as packed pellets at 4°C for up to 2 weeks prior to analysis. Samples were derivatized by adding 10 pi of packed erythrocytes to 200 pi of 40 mM NaCl, 2 mM DTNB and incubating at room temperature for 30 min. Following centrifugation (12,000g for 2 minutes), samples were analyzed using a VARIANT hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA) with a modified p-thalassemia short program (details provided on request).

Phenotypic analysis of HSC populations

Bone marrow cells from wthEPOR and mthEPOR knock-in mouse strains were incubated in a cocktail of antibodies to CD2, CD3, CD5, CD8, Mac-1, Gr-1, TER119, CD45R, and CD 19. Lineage depletion was carried out by 2 successive incubations in sheep anti-rat Ig-coupled magnetic beads (Dynal, Oslo, Norway). Lineage-negative (Lin^{neg}) cells were incubated with 0.2 pM rhodomine-123 in Hank's balanced salt solution (HBSS) containing 5% newborn calf serum (HBSS) at 37°C for 20 min. After washing once, the cells were resuspended in fresh HBSS and incubated at 37°C for another 20 min to allow for efflux of the dye. After washing, the cells were stained with phycoerythrin (PE) conjugated anti-Sca-1 and allophycocyanin (APC) conjugated anti-c-kit antibodies. The cells were then washed and resuspended in HBSS containing propidium iodide (PI) for exclusion of dead cells and analyzed using a modified two-laser FACScan flow cytometer.

In vitro CFC assay

Lin"®[8] cells prepared as described above were stained with PE-conjugated anti-Sca-1, APC-conjugated anti-c-kit, and biotinylated anti-Flt3. The biotinylated antibody was detected using streptavidin-FITC, and dead cells were excluded based on PI staining as described above. The Lin^{neg} c-kit^{+} $Sca\text{-}1^{+}$ $Rhodamine^{low}$ ($LSKRho^{low}$) or Lin^{neg} c-kit^{+} $Sca\text{-}1^{+}$ $Rhodamine^{high}$ ($LSKRho^{hi}$) cell populations were isolated by aseptic cell sorting using a FACSVantage or FACSAria instrument and plated in alpha-modified Eagle's medium (alpha-MEM)-based methylcellulose containing 10% fetal calf serum, 10% deionized bovine serum albumen, antibiotics, glutamine, and 2-mercaptoethanol. Colony growth and differentiation were stimulated with recombinant cytokines in combination as indicated, including stem cell factor (SCF; 100 ng/ml), G-CSF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (20 ng/ml), a peptide thrombopoietin (Tpo) mimetic (5 nM), and erythropoietin (4 U/ml) (cytokines obtained from PeproTech, Rocky Hill, NJ; the Tpo mimetic was synthesized by the University of Utah core peptide synthesis facility as described (Cwirla et al., 1997)). After 7 days of culture, quadruplicate plates were stained using benzidine hydrochloride and scored for total colonies and for colonies containing hemoglobinized erythroid lineage cells. Results are reported as mean number of colonies per 400 cells plated, and error bars represent standard error values. Statistical analysis utilized a one-tailed, unpaired T-test with equal variance.

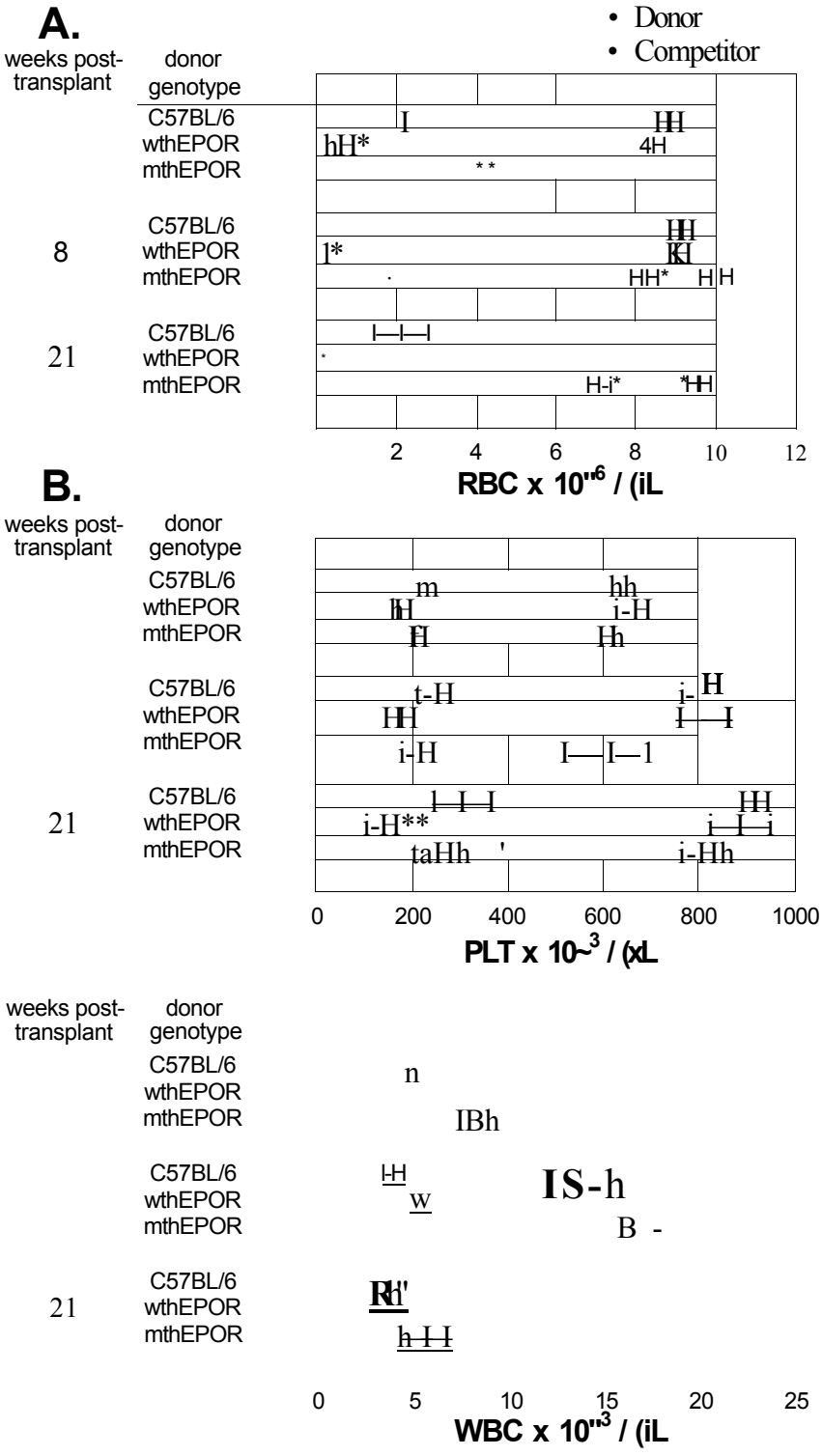
Results

Tri-lineage engraftment of wthEPOR and mthEPOR knock-in bone marrow cells in recipient peripheral blood

To evaluate the effects of augmented or decreased Epo signaling on the engraftment potential of HSC/HPCs, I performed competitive repopulation assays by co-transplanting 2 x 10^6 either wthEPOR or wthEPOR knock-in mice bone marrow cells (donor) together with 2 x 10^6 B6-GFP/Hbb^d bone marrow cells (competitor) into lethally irradiated B6-GFP/Hbb^d recipient mice. As a control, I co-transplanted 2 x 10^6 normal C57BL/6 mouse bone marrow cells together with 2 x 10^6 B6-GFP/Hbb^d mouse bone marrow cells into lethally irradiated B6-GFP/Hbb^d recipient mice. Using GFP and hemoglobin-P variants as markers (Spangrude et al., 2006), I tracked the relative contributions of donor and competitor bone marrow cells into leukocyte, platelet and erythrocyte pools in the peripheral blood of recipient mice up to five months post-transplantation. Secondary transplantations were also performed to evaluate the long-term HSC function in the primary transplant recipients.

The data in Figure 3.1 show the relative contributions of the donor marrow (shaded bars) and competitor marrow samples (open bars) relative to the total cell counts for each lineage over time. Bone marrow cells obtained from mthEPOR knock-in donors provided a much higher contribution to engraftment of the erythroid lineage compared to competitor bone marrow cells over the time course of the experiment (Figure 3.1 A), and recipients of mthEPOR cells exhibited a mild polycythemic phenotype. For example, the total RBC count from recipients of wthEPOR knock-in donors at 21 weeks post-transplantation was 8.60 + 0.46 x 10^6 cells/pL (mean + SD) compared to a count of 9.63

Figure 3.1. Competitive repopulation of wthEPOR or mthEPOR knock-in mouse bone marrow with normal mouse bone marrow. The erythrocyte, platelet and leukocyte cell numbers from each genotype of mouse bone marrow at various times posttransplantation are shown here. A. Erythrocyte lineage engraftment. B. Platelet lineage engraftment. C. Leukocyte lineage engraftment. Error bars represent standard errors (n=5). *, significantly different from corresponding value for C57BL/6 ($p < 0.01$); **, significantly different from corresponding value for C57BL/6 ($p = 0.02$) and mthEPOR (p - 0.003).


A.
• Donor
• Competitor
weeks post-transplant
donor genotype
C57BL/6
wthEPOR
mthEPOR
8
21
2
4
6
8
10
12
RBC x 10"6 / (iL
B.
PLT x 10~3 / (xL
0
200
400
600
800
1000
WBC x 10"3 / (iL
0
5
10
15
20
25

+ 0.42 x 10^6 cells/pL in recipients of mthEPOR bone marrow ($p = 0.003$). Consistent with the anemic phenotype of wthEPOR mice, bone marrow cells from these animals competed poorly with competitor bone marrow cells in the context of erythroid reconstitution, to the extent that erythroid reconstitution from wthEPOR cells was not detectable 21 weeks after transplantation (Figure 3.1 A). These observations are consistent with the observation that wthEPOR is not optimally stimulated by mouse Epo in vivo, as previously described (Divoky et al., 2001).

Analysis of platelet repopulation in this experiment revealed that wthEPOR knock-in bone marrow showed a progressively reduced contribution to the platelet pool compared to the competitor graft, while normal C57BL/6 and mthEPOR knock-in bone marrow maintained a consistent level of engraftment relative to the competitor bone marrow cells (Figure 3. IB). At 21 weeks posttransplant, recipients of wthEPOR knock-in bone marrow had about one half the number of donor-derived platelets relative to C57BL/6 recipients ($p = 0.02$) or mthEPOR recipients ($p = 0.003$). The decrease in relative contribution of wthEPOR knock-in mice bone marrow cells to the platelet pool was seen throughout the time course of our study; however, the contribution of competitor marrow to platelet production compensated for the decrease in donor-derived platelet counts. These observations indicate that the defect in platelet production was intrinsic to the wthEPOR knock-in mice bone marrow cells, and implicates a role for Epo signaling in the regulation of platelet production, consistent with previous in vitro studies (Broudy et al., 1995; Papayannopoulou et al., 1996).

In contrast to the alterations in erythroid and platelet chimerism noted above, leukocyte chimerism in transplant recipients is similar in all three transplant groups

(Figure 3.1C). No significant differences were noted in the degree of competition between the three genotypes of donor cells relative to the competitor cells. I conclude that leukocyte engraftment and chimerism was not affected by the EpoR signaling.

Selective alterations in erythroid and platelet chimerism without a specific effect on white cell chimerism indicates that expression of mthEPOR or wthEPOR impacts progenitor cells for these lineages but does not influence the HSC compartment. To further test this hypothesis, I evaluated myeloid and lymphoid cell distribution within the donor-derived leukocyte populations. This analysis showed that donor leukocyte populations in all three donor transplant groups included T and B lymphocytes as well as myeloid lineage cells (macrophage and granulocyte) in similar proportions (Figure 3.2). This observation strengthens the interpretation that the selective loss of erythroid lineage reconstitution and the decrease in platelet reconstitution in wthEPOR mice occurred at the level of lineage specific progenitor cells selective for those lineages rather than at the HSC level.

To confirm the interpretation that mthEPOR or wthEPOR expression does not impact the HSC compartment, I performed secondary transplantation studies. I harvested the bone marrow cells from the mice shown in Figure 3.1 and transplanted cells pooled from each group of primary recipients into lethally-irradiated secondary recipients. Following secondary transplantation, I observed results similar to those seen in primary transplantation in erythrocyte, leukocyte and platelet lineages (Figure 3.3). As in the primary transplants, I observed enhanced competition of mthEpoR-expressing cells and essentially no competition of wthEpoR-expressing cells in the erythroid lineage (Figure 3.3 A), reduced competition of wthEpoR-expressing cells in the platelet lineage (Figure

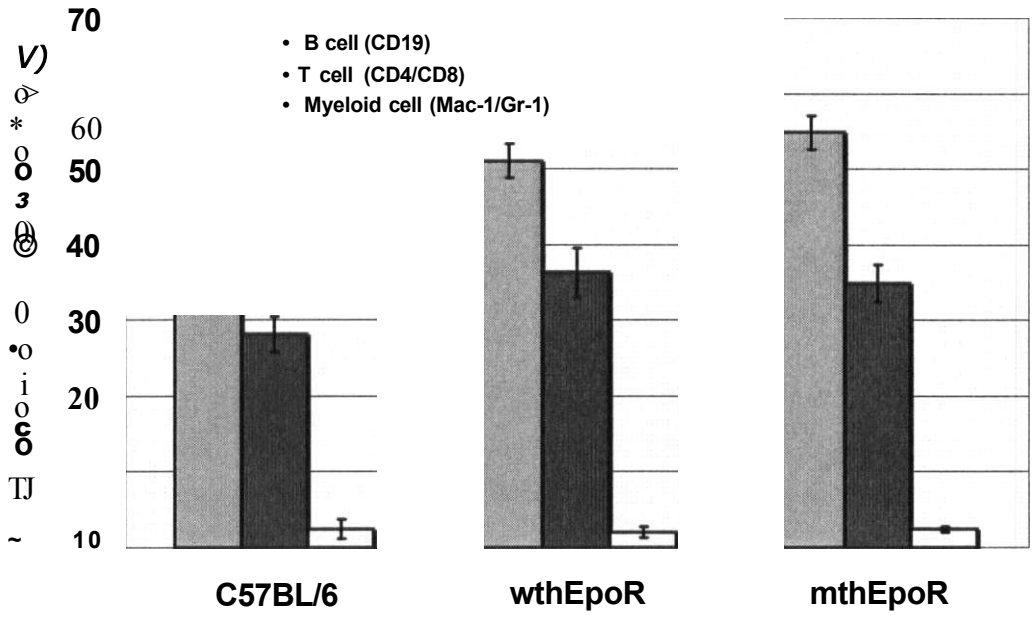
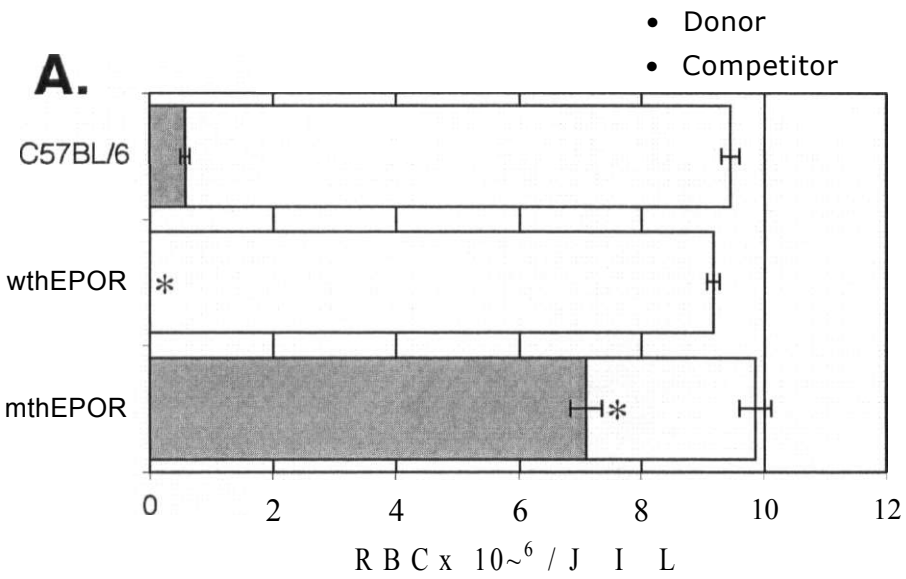


Figure 3.2. Myeloid and lymphoid cell distribution within the donor-derived leukocyte populations 3 months posttransplantation. Peripheral blood leukocytes collected from recipient animals were stained with fluourochrome-conjugated monoclonal antibodies to B cell, T cell and myeloid cell markers and analyzed by flow cytometry.

Figure 3.3. Secondary transplantation of bone marrow obtained from the transplant recipients shown in Figure 3.1. The erythrocyte, platelet and leukocyte cell numbers from each genotype of mouse bone marrow at 16 weeks posttransplantation are shown here. A. Erythrocyte lineage engraftment. B. Platelet lineage engraftment. C. Leukocyte lineage engraftment. Error bars represent standard errors (n=6). *, significantly different from corresponding value for C57BL/6 group ($p < 0.001$).



B.

C57BL/6

H * H

wthEPOR

h

mthEPOR

200 400 600 800 1000

PLT x 10"3 / \iL

C.

I-H

C57BL/6

wthEPOR

HH

mthEPOR

5 10 15 20

WBC X 10"3 / JIL

3.3B), and equivalent competition of all three genotypes in the leukocyte lineage (Figure 3.3C). Analysis of the lineages of leukocytes confirmed the presence of T and B lymphocyte as well as myeloid lineage cells in all donor-derived populations with similar proportions. This result demonstrates that the EpoR do not influence the function of the HSC compartment, but functions to modulate both productive erythropoiesis and megakaryopoiesis.

wthEPOR knock-in mice have a lower proportion of phenotypically- defined HSC compared to mthEPOR knock-in or wild-type mice

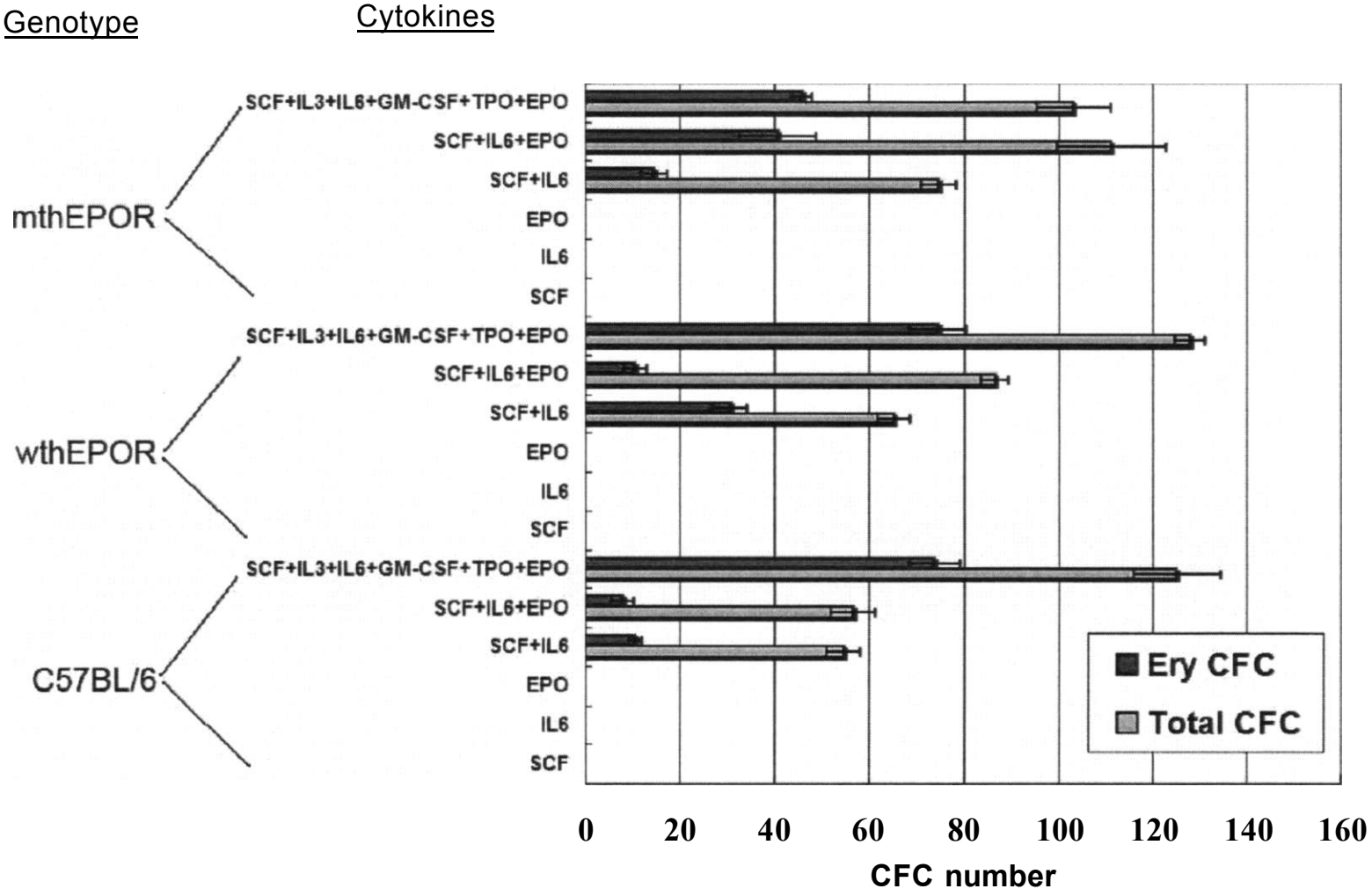
To investigate whether there are effects of Epo signaling on the frequency of phenotypically-defined HSC, I determined the proportion of these cells in bone marrow cells from wthEPOR and mthEPOR knock-in mice. In wthEPOR knock-in mice, the $Rh123^{low}$ subset of $Lin^{neg}c\text{-}kit^{+}Scal^{+}$ (KLS-R) cells represented 0.011±0.003% of total bone marrow cells while in mthEPOR knock-in mice these cells represented 0.023±0.006% of total bone marrow cells (mean ± SEM, n= 8, p=0.025). In normal C57BL/6 mice, these cells represented 0.031±0.003% of total bone marrow cells (mean ± SEM, n=4), which is not significantly different from mthEPOR knock-in mice. I conclude from the combined results of phenotypic and functional analysis that the replacement of the endogenous mouse EpoR gene with the polycythemic mthEPOR gene does not alter the characteristics of the HSC compartment, in contrast to results reported for the transgenic models of polycythemia. The reduction of the frequency of KLS-R cells in the marrow of wthEPOR knock-in mice may reflect a reduced frequency of early

multipotent progenitor cells that are not resolved from primitive HSC by our methods, since transplantation studies did not reveal a significant decrease in leukocyte reconstitution.

In vitro proliferation and differentiation of HSCs and MPPs from wthEPOR, mthEPOR and C57BL/6 mice in response to different cytokines

One hallmark characteristic of primitive HSCs and MPPs is their proliferation responsiveness to cytokine stimulation and form colonies containing differentiated cells in vitro, particularly with cytokine combinations that include stem cell factor (SCF). Synergy between SCF and EPO is particular relevant to erythropoiesis (Sui et al., 1998), and the impact of hyperactive EPOR signaling in the context of cytokine stimulation and synergy of cytokines has not been investigated. To evaluate the effect of augmented or reduced EPOR signaling on cytokine responsiveness and differentiation potential of HSCs and MPPs obtained from the two knock-in mouse strains compared to normal C57BL/6 mouse, I isolated LSKRho^{low} (HSCs) and LSKRho^{hi} (MPPs) bone marrow cells from each strain and cultured these cells in the presence of various combinations of cytokines in methylcellose-based semisolid medium. Colonies were evaluated after 7 days of culture, and colonies that included hemoglobinized erythroid cells were distinguished based on benzidine staining. As shown in Figure 3.4A, HSCs from all three genotypes requires multiple cytokines for proliferation and differentiation. In the presence of Epo together with SCF and IL-6, mthEPOR mouse HSCs generate more

Figure 3.4. Cytokine responsiveness of HSCs and MPPs isolated from normal C57BL/6, wthEPOR or mthEPOR knock-in mouse bone marrow. The HSC (A) and MPP (B) population were isolated from the bone marrow of mice as described in Methods. Cell cultures were established in methylcellulose medium containing the indicated cytokines. The plates were stained with benzidine hydrochloride after 7 days of culture and scored for total colonies and for colonies containing hemoglobinized cells 400 cells plated. Data reflects the means of triplicate or quadruplicate plates, and error bars represent standard errors.


Genotype
Cytokines
mthEPOR
wthEPOR
C57BL/6
SCF+IL3+IL6+GM-CSF+TPO+EPO
SCF+IL6+EPO
SCF+IL6
EPO
IL6
SCF
SCF+IL3+IL6+GM-CSF+TPO+EPO
SCF+IL6+EPO
SCF+IL6
EPO
IL6
SCF
SCF+IL3+IL6+GM-CSF+TPO+EPO
SCF+IL6+EPO
SCF+IL6
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SCF
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Total CFC
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20
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120
140
160
CFC number

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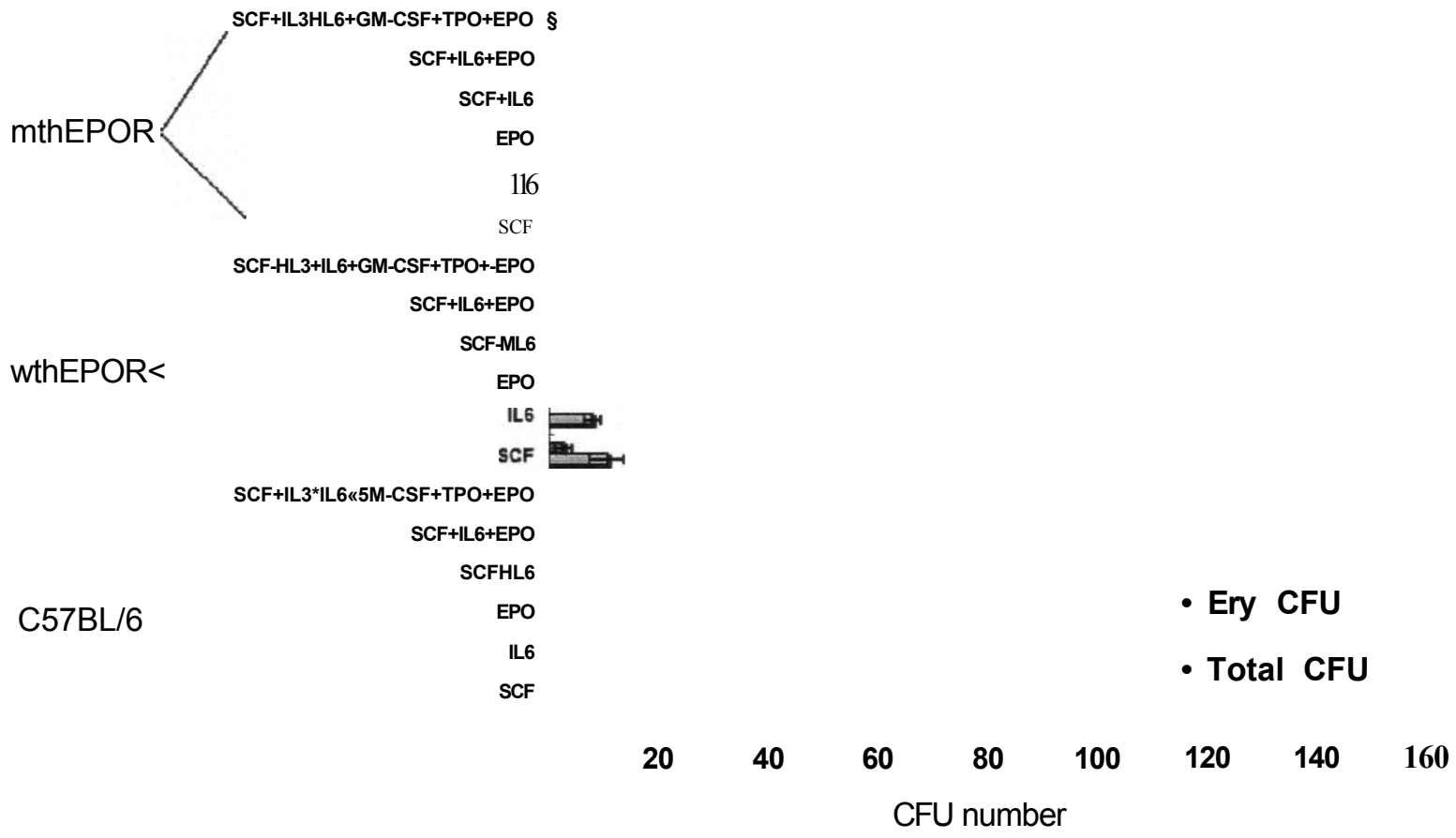
Genotype
Cytokines
mthEPOR
SCF+IL3HL6+GM-CSF+TPO+EPO §
SCF+IL6+EPO
SCF+IL6
EPO
116
SCF
wthEPOR<
SCF-HL3+IL6+GM-CSF+TPO+-EPO
SCF+IL6+EPO
SCF-ML6
EPO
IL6
SCF
C57BL/6
SCF+IL3*IL6«5M-CSF+TPO+EPO
SCF+IL6+EPO
SCFHL6
EPO
IL6
SCF
• Ery CFU
• Total CFU
20 40 60 80 100 120 140 160
CFU number

o

erythroid colonies compared to C57BL/6 (p=0.018) or wthEPOR (p=0.025) mouse, consistent with the in vivo result. As shown in Figure 3.4B, both the number of total colonies as well as the number of colonies containing erythroid cells were similar between wthEPOR, mthEPOR and C57BL/6 mouse MPPs stimulated with SCF as a single cytokine. This result demonstrates that the hyperactive EPOR by itself is not sufficient to synergize with SCF. Stimulation with Epo alone resulted in colonies formation from mthEPOR MPPs but not from wthEPOR or C57BL/6 MPPs. Synergy between SCF, IL-6 and EPO was similar among the three genotypes. The combination of SCF, IL-3, IL-6, G-CSF and Tpo, with or without human Epo, resulted in about 30% cloning efficiency, demonstrating that MPP cells in both knock-in strains require multiple cytokines to achieve optimal proliferation. Under these conditions, neither maximal colony formation nor the frequency of colonies containing hemoglobinized cells differed between the two knock-in strains or wild type C57BL/6 mice in replicate experiments. I conclude that when present in the mouse genome under appropriate regulatory control, mthEPOR itself does not contribute additional growth or differentiation signals in HSCs compared to wthEPOR or the cells with endogenous EpoR gene, mthEPOR may increase the sensitivity of erythroid progenitors to EPO stimulation. MPP cells may express functional EPOR on cell surface and hypoactive mthEPOR can stimulate the growth and differentiation of MPPs in response to EPO stimulation alone while MPP cells expressing wildtype human or mouse EPOR can not respond to EPO stimulation alone.

Discussion

Epo stimulates growth, prevents apoptosis, and promotes differentiation of erythroid progenitors. However, expression of Epo is not restricted to erythrocytes and erythroid progenitor cells. The functions of EpoR in hematopoietic stem cells and multipotent progenitor cells are controversial. Transgenic mice expressing gain-of-function EPOR under the control of the (3-actin promoter showed an enhanced HSC self-renewal capability when exogenous human Epo was provided.(Kirby et al., 2000) MPPs enforced to express EPOR have enhanced proliferation potential when they are stimulated with Epo.(Dubart et al., 1994) In the present study, I evaluated HSC and progenitor cell function in a mouse model in which a hyperactive form of the human EPOR (mthEPOR) or the wild type human EPOR (wthEPOR) were placed under the control of the endogenous mouse EpoR genetic regulatory elements. The mthEPOR knock-in mouse model showed increased Epo signaling and increased red blood cell mass and erythroid progenitor cell number, while the wthEPOR knock-in mouse model showed decreased Epo signaling and an anemic phenotype (Divoky et al., 2001). Using these mouse models, I performed a competitive repopulation assay to assess the effect of enhanced or reduced EpoR signaling on the functions of HSC and progenitor cells. Consistent with the phenotypes of the knock-in mouse models, mthEPOR mouse bone marrow in competition with normal mouse bone marrow showed dominant repopulation of erythrocytes in recipient mice, while the wthEPOR mouse bone marrow had a severe defect in erythrocyte repopulation. These results coincide with the polycythemia and anemia phenotypes of these two mouse models, respectively. I observed no significant difference of leukocyte repopulation between mthEPOR, wthEPOR, and control mouse

bone marrow cells up to 5 months posttransplantation in the primary transplantation and 16 weeks after secondary transplantation. Furthermore, mthEPOR knock-in mice have an equivalent number of phenotypically-defmed HSC relative to wild-type mice, and this together with the transplant data suggests that hyperactive EpoR signaling does not influence the number and function of HSC.

Unexpectedly, I observed impaired production of platelets in recipients of wthEPOR mouse bone marrow compared to recipients of mthEPOR or normal mouse bone marrow. This decreased platelet production was sustained over time (Figure 3.IB) and was also present after secondary transplantation (Figure 3.2B). Total platelet numbers (the sum of donor plus competitor contributions) were normal in both primary and secondary transplant recipients, suggesting that the defect in megakaryopoiesis was intrinsic to the wthEPOR bone marrow cells. The decrease in the percentage of platelets derived from wthEPOR bone marrow cells was significant, as donor-derived platelet numbers were decreased by 50% or greater relative to control or mthEPOR cells at 21 weeks postprimary transplant and at 16 weeks postsecondary transplant. This observation is consistent with previous studies suggesting that Epo/EpoR signaling might play a role in megakaryopoiesis.(Beguin, 1999) Tpo, the major cytokine influencing platelet production, is clearly the primary regulator of megakaryopoiesis (Kaushansky, 1995). However, targeted deletion of Tpo or of its receptor c-mpl fails to completely ablate thrombopoiesis (Carver-Moore et al., 1996), suggesting that additional cytokines function to regulate platelet production in vivo. A number of cytokines, including IL-3, IL-6, IL11, and leukemia inhibitory factor, have been shown to promote megakaryocyte growth, but compound knockout studies have failed to provide evidence supporting the

role of these cytokines in the residual production of platelets in the absence of c-mpl signaling (Chen et al., 1998; Gainsford et al., 2000). Due to the embryonic lethal phenotype of epo or epor knockouts, similar experiments combining either of these mutations with targeted deletion of tpo or mpl would require embryo aggregation in an experimental setting that would allow distinction of platelet reconstitution, as I have utilized in our studies. To our knowledge, such studies have not been reported.

The extensive homology between c-mpl and EpoR (Vigon et al., 1992). as well as the common downstream signaling pathways activated by these receptors (Nagata et al., 1997), make EpoR an attractive candidate as a regulator of platelet function in vivo. Epo will act with Tpo in a synergistic manner to promote megakaryocyte colony growth in vitro (Broudy et al., 1995; Papayannopoulou et al., 1996) and with IL-6 to promote proplatelet process formation (Tange and Miyazaki, 1996). In this context, Epo/EpoR signaling could act on a common progenitor for the erythrocyte and megakaryocyte lineages, or on committed progenitors of the megakaryocyte lineage in order to exert its role in megakaryopoiesis. However, in light of the lack of a positive effect of the mthEPOR on platelet reconstitution in our experiments (Figures 3.1 and 3.2), our data are consistent with the conclusion that Tpo provides the dominant regulatory effect on platelet numbers in vivo.

I also studied the cytokine responsiveness of HSCs and MPPs from these knock-in mouse models. It was shown that erythroid committed progenitor cells from mthEPOR knock-in mouse are hypersensitive to Epo stimulation.(Divoky et al., 2001) It was unknown whether HSCs and MPPs express functional EPOR on their cell surface or not. I provided in vitro colony-forming assay data indicating that MPPs and downstream

erythroid committed progenitor cells do express functional EPOR and can respond to EPO stimulation in hypesensitive situation. Hypersentitive EPOR signaling does not influence the synergistic response of HSC/MPP to optimal cytokines stimulation. Overall, these results suggest EPOR signaling function is largely restricted to erythroid lineage while there are additional minor influences of EPOR signaling on platelet differentiation and multipotent progenitor cell proliferation.

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CHAPTER 4

DISCUSSION

Hematopoiesis is a hierarchically structured cell differentiation process with a series of progenitors or intermediates that are controlled by the interplays of extracellular and intracellular cues. The roles of extracellular signaling molecules (growth factors, cytokines, extracellular matrix, etc.) and intracellular gene expression regulators (transcription factors, chromatin modifier, microRNAs, etc.) on hematopoiesis has been investigated extensively (Huang et al., 2007). But the roles of metabolism on hematopoiesis, especially at the levels of HSC/HPCs, are largely unexplored. In Chapter 2 of this dissertation, I studied the roles of cellular copper (Cu) content on HSC/HPCs proliferation and differentiation using an ex vivo cell culture model and a novel mouse bone marrow transplantation assay. I demonstrated a novel function of Cu metabolism on the erythroid progenitor cell generation or expansion. I also provided strong evidence suggesting that the effect of Cu content on erythroid progenitor cell is related to the cellular energy metabolism pathways but not cellular reactive oxygen species (ROS) modulation. Previous studies have implicated that EPOR signaling may function in promoting HSC self-renewal. In Chapter 3 of this dissertation, I studied the roles of erythropoietin receptor (EPOR) signaling on mouse bone marrow HSC/HPC function using knock-in transgenic mouse models, mouse bone marrow transplantation assay and

in vitro colony-forming assay. I provided direct evidence confirming essential function of EPOR signaling on erythroid differentiation. I also found a minor function of EPOR signaling on platelet production, but no function on HSC self-renewal. I also demonstrated that EpoR signaling may have physiological functions in promoting the proliferation and differentiation of multipotent progenitor cells. Though the two parts of my thesis are quite different from one another, each addressed issues important for understanding the regulators of HSC/HPC expansion and differentiation and shed insights into the design of ex vivo culture system for HSC/HPC expansion for clinical transplantation.

Cellular Cu content regulates hematopoietic progenitor cell differentiation in culture

HSC/HPCs hold enormous potential in regenerative medicine and gene therapy. But the lack of ex vivo culture system for expanding these cells greatly hampered their application. Ex vivo culture of HSC/HPC usually results in rapid differentiation and expansion of mature myeloid blood cells instead of maintaining or expanding immature HSC/HPCs in culture, suggesting that most current culture conditions lack the proper extra cellular environment that is crucial for HSC/HPC maintenance or expansion. A lot of research effects have been invested to optimize ex vivo culture conditions for promoting the maintenance and expansion of HSC/HPCs while inhibiting their differentiation, but with little success. Most of these efforts have been focusing on the roles of cytokines, growth factors and transcription factors. Little attention has been paid to the roles of cellular metabolism on HSC/HPC maintenance. One notable exception is

the studies of the role of oxygen tension on HSC/HPCs maintenance and expansion. Ex vivo culture of HSC/HPC in low oxygen tension condition together with early-acting cytokines has been shown to favor the maintenance and expansion of HSC/HPC in culture modestly (Kovacevic-Filipovic et al., 2007). The mechanisms by which hypoxia favors HSC/HPC proliferation while inhibits their differentiation is currently unclear. Reduction of intracellular reactive oxygen species (ROS) was proposed as an explanation (Fan et al., 2008). Copper (Cu) is an essential nutrient for all types of cells and a redox active element like oxygen. A similar role of cellular Cu level on HSC/HPC maintenance has recently been suggested. Peled and collegues showed that TEPA, a high affinity and specificity Cu^{2+} chelator, when added together with cytokines into the ex vivo culture of human HSC/HPCs (human cord blood-derived $CD34^{+}$ cells), decreased the intracellular Cu level of culture cells and enhanced the expansion of immature progenitor cells and possibly stem cells while inhibiting their differentiation (Peled et al., 2005; Peled et al., 2002). These studies used cell surface marker expression, in vitro colony-forming cell (CFC) assay and severe-combined immunodeficiency (SCID) mice repopulation assay to assess the content of human HPC and HSC after the culture with TEPA. While these are good assays for human myeloid HPC analysis, they can not distinguish different lineage-specific HPCs faithfully and can not quantify bona-fide HSCs. Our lab developed an analysis system to simultaneously analyze the tri-lieage engrftment (leukocyte, erythrocyte and megakaryocyte/platelet) of transplanted cells in the hosts both short-term and long-term in a competitive repopulation setting thus able to analyze and quantify different lineage specific HPCs and short-term and long-term HSCs. I re-examined this issue using mouse bone marrow-derived HSC/HPCs for culture and rigorous in vitro and

in vivo assays, including FACS assay, colony-forming cell (CFC) assay, short-term and long-term competitive repopulation assays. The experiments presented in Chapter 2 demonstrated that HSC maintenance in culture is neither enhanced nor impaired by modestly modulating cellular Cu content in culture. Interestingly, erythroid lineage specific progenitor cell expansion or generation is enhanced by reducing cellular Cu content using Cu chelation demonstrated by an increased number of erythroid CFC number and increased short-term erythroid lineage repopulation in recipient mice compared to control culture, while other myeloid/lymphoid and megakaryocyte progenitor number were not influenced by modulating cellular Cu content in culture.

I investigated the possible mechanisms behind the effect of Cu regulating the erythroid progenitor cells in culture. One possibility is that Cu modulates cellular energy metabolism pathways. There are two major pathways in eukaryotic cells to generate ATP, one is glycolysis (or anaerobic respiration) which does not require oxygen and happens in cytoplasm, and the other is oxidative phosphorylation (or aerobic respiration) which requires oxygen and happens in mitochondria. Glycolysis converts glucose into pyruvate and release the free energy as ATP and NADH. Pyruvate is converted to lactate under anaerobic respiration or enter mitochondria for oxidative phosphorylation. Oxidative phosphorylation produces ATP by coupling electron transfer to oxygen with the phosphorylation of ADP by ATP synthase at the inner membrane of mitochondria. During this process, protons are pumped into the intermembrane space of the mitochondria, producing a membrane potential. Oxidative phosphorylation produces much more ATP molecules from glucose than glycolysis. Local bone marrow hypoxia environment may favor glycolysis over oxidative phosphorylation for energy generation

in HSC/HPCs. It is interesting to notice that many types of cancer cells predominantly dependent on glycolysis for cellular energy metabolism even in the presence of enough oxygen, this phenomena is called Warburg effect (DeBerardinis, 2008; Kim and Dang, 2006). In some sense, stem cells and progenitor cells are similar to cancer cells (Reya et al., 2001). In addition, the enzymes involved in glycolysis are relatively enriched in HSC/HPCs (Unwin et al., 2006). Cu is an essential cofactor for cytochrome c oxidase (CCO) which is an essential component of electron transport chain for oxidative phosphorylation. Reducing cellular Cu content may reduce CCO activity leading to reduced oxidative phosphorylation and enhanced glycolysis. Indeed, some studies showed that Cu deficiency promotes glycolysis in erythrocytes (Brooks et al., 2003). The switch from oxidative phosphorylation to glycolysis is usually accompanied by a decrease of mitochondrial membrane potential (A^m) because A^Vi is a result of proton pumping by the electron transport chain (van Raam et al., 2008). I measured the relative AT™ of cultured HSC/HPCs using fluorescent dye JC-1 by flow cytometry. The data showed that TEPA decreased the relative A^m of cultured HPCs compared to control (Figure 2.6). This result supports the idea that TEPA inhibits oxidative phosphorylation and promotes glycolysis in cultured HPCs. This may lead to enhanced generation or maintenance of erythroid progenitor cells.

One possible link between cellular Cu content and glycolysis pathway is transcription factor hypoxia inducible factor (HIF). HIF is a heterodimer containing a constitutive beta unit and an alpha unit whose protein stability is upregulated by hypoxia. HIF activate the transcription of genes involved in angiogenesis, erythropoiesis and glycolysis (Seagroves et al., 2001). Cell surface glucose transporter GLUT1 and nearly

all the core enzymes of glycolysis are transcriptionally induced by HIF-1 (Semenza et al., 1994). HIF-1 can also directly inhibit oxidative phosphorylation. In aerobic respiration, pyruvate goes into the mitochondria and is converted to acetyl-CoA by the pyruvate dehydrogenase (PDH), allowing it to enter the citric acid cycle and oxidative phosphorylation. PDH activity is negatively regulated by pyruvate dehydrogenase kinase (PDK) which phosphorylates and inhibits PDH. PDK can be induced by active HIF-1 and reduce the activity of PDH thus reduces pyruvate from entering into the citric acid cycle in mitochondria for oxidative phosphorylation (Kim et al., 2006; Papandreou et al., 2006). In addition to hypoxia, HIF can also be activated through other signaling pathways even under normoxic conditions (Shaw, 2006). It is possible that HIF protein stability or activity is regulated by cellular Cu content. Indeed, Cu chelator has been shown to stabilize active HIF-1 protein by blocking its ubiquitination (Choi et al., 2006). It would be interesting to see if TEPA increases the stability of HIF-1 in the cultured HSC/HPCs in our culture model.

Another possible mechanism for TEPA's effect on erythroid progenitors is related to the intracellular reactive oxygen species (ROS) generation. Recent follow-up studies suggested that the observed effect of TEPA on human HSC/HPC expansion is due to reduction of cellular ROS level (Prus and Fibach, 2007). Excess free Cu ion in the cell is involved in the generation of ROS (Leary and Winge, 2007). It has been shown that HSCs contain relatively low level of ROS (Jang and Sharkis, 2007). Antioxidant supplement in the culture of HSC/HPCs enhanced the maintenance and expansion of HSC/HPCs (Gupta et al., 2006). Cu chelator may reduce the cellular ROS level in the HSC/HPCs culture thus enhance the maintenance or expansion of erythroid progenitor

cells in the culture. To test this possibility, 1 measured the relative ROS content in my cultured cells using fluorescent dye dichlorodihydrofluorescein diacetate (DCF-DA) (Figure 2.7). The ROS level in cultured cells treated with TEPA is slightly higher than control culture. These results do not support reduced ROS products as an possible mechanism for TEPA's effect on HPCs. Overall, my experiments suggest that alternation of metabolic pathways from oxidative phosphorylation to glycolysis but not reducing ROS might be the mechanism for Cu chelator TEPA to enhance the generation or maintenance of erythroid progenitor cells in culture.

To directly test this hypothesis, I need to measure the activity of CCO in cultured HPCs under different Cu modulating conditions. I need to purify the culture cells with low A^m to test if these cells are enriched in erythroid progenitor cell activity. I need to measure the oxygen and glucose consumption, lactate production of HPCs in different culture conditions to see if TEPA switches HPC metabolism from oxidative phosphorylation to glycolysis. I need to measure the cell surface expression of glucose transporter GLUT1 to see if it is up regulated by TEPA because enhanced glycolysis will require more glucose consumption for the cellular energy metabolism. To test if HIF-1 is involved in Cu modulating erythroid progenitor cells, I need to use HIF-1 deficient HPCs for the culture and test if the effect of Cu reduction on erythroid progenitor cell diminished.

The roles of EPOR on hematopoietic stem and progenitor cells

Since the discovery of erythropoietin (EPO) in 1977, the essential role of EPO for red blood cell generation has been well established. The molecular cloning of the EPO

gene and mass manufacturing of recombinant human EPO and its derivatives led to the widespread use of EPO in treating anemia. However, the biological role of EPO is not restricted to red blood cell production. EPO receptor (EPOR) expression is also found in endothelial, brain, cardiovascular and other tissues (Maiese et al., 2008). Even within hematopoietic tissue, EPOR expression is beyong erythroid lineage. EPOR message RNA is also expressed by multipotent hemopoietic progenitor cells and possibly stem cells (Heberlein et al., 1992; Migliaccio et al., 1991; Schmitt et al., 1991), and it may play a role on HSC and MPP self-renewal or proliferation. It is unknown whether EPOR expression in HSC/MPP is functional. Normal HSCs or MPPs do not proliferate or differentiate in response to EPO stimulation alone. This can be due either to an insufficient amount of EPOR expression on the surface of these cells or to the lack of necessary intracellular signaling molecules. Previous studies using transgenic mouse models showed that enforced expression of a gain-of-function truncated human EPOR gene or normal EPOR gene in mouse HSCs leads to expansion of HSC pool or EPO-stimulated proliferation of MPPs (Kirby et al., 2000; Kirby et al., 1996; Urbinati et al., 2005). These studies suggest that HSC and MPP do include the necessary intracellular signaling molecules downstream of EPOR for stimulation of cell proliferation. It was unclear whether naturally-expressed hyperactive EPOR influences HSC or MPP proliferation. I have examined this question using knock-in mouse models wherein the mouse EPOR gene reading-frame was replaced in its natural genetic locus by a single copy of either a wild type human (wthEPOR) or a polycythemia-causing hyperactive truncated human EPOR gene (mthEPOR) reading-frame. WthEPOR mice have hypoactive EPOR signaling, which leads to anemia. This may be due to the incompability

between mouse and human EPOR signaling, however this has not been proven. Using a mouse bone marrow transplantation model, I demonstrated that augmented EPOR signaling increases erythroid repopulation potential of bone marrow cells as expected, but has no effect on short-term or long-term leukocyte repopulation from bone marrow cells. Bone marrow cells obtained from the hypoactive wthEPOR mouse provided decreased platelet repopulation compared to bone marrow cells from normal B6 mice, while bone marrow cells obtained from the hyperactive mthEPOR mouse provided same level of platelet engraftment compared to bone marrow cells from normal B6 mice. These results suggested a possible role for Epo signaling in megakaryopoiesis in vivo, but the exact effects need to be determined by more experiments. For the future studies, the platelet number in peripheral blood, megakaryocyte number in bone marrow and bone marrow megakaryocyte progenitor cell content from both knock-in mice and normal B6 mice need to be compared in same conditions. More bone marrow transplantation experiments need to be done. To minimize the experimental variations, donor bone marrow cells should be taken from a large number of age and sex matched mice and recipient mice should be age and sex matched too.

I studied the proliferating potential of HSC and MPP from these knock-in mice using in vitro colony-forming assay. I purified HSCs or MPPs from wthEPOR, mthEPOR mice or normal B6 mice and cultured them clonally in methylcellulose-based medium supplemented with single or combination of various hematopoietic cytokines. HSCs need multiple cytokines combination to stimulate their proliferation. Single cytokines do not stimulate proliferation of HSCs. Synergistic response of HSCs from three kinds of mice are similar except HSCs from mthEPOR are more responsive to EPO stimulation together

with SCF and IL-6. SCF and IL-6 alone can stimulate the proliferation of MPPs. MPPs from hyperactive mthEPOR knock-in mice can proliferate in response to EPO stimulation alone. These results suggest that EPOR is expressed on MPPs cell surface naturally and can transduce EPO stimulation downstream to activate a cell proliferation program. The discrepancy between the previous studies and our study on the effect of augmented EPOR signaling on HSC proliferation may be explained by different transgene expression system used. Previous studies used ubiquitously active promoter to drive the transgene expression which may result in unnaturally high-level of expression of EPOR on HSCs. In contrast, our system used endogenous EPOR promoter and regulatory locus to drive expression of hyperactive EPOR, thus faithfully representing the in vivo physiological expression pattern. The combination of results from previous studies and our study suggest: 1) EPOR signaling provide a signal for HSC self-renewal when EPOR is engineered to be expressed at high levels on HSCs. 2) In normal conditions, HSCs don't express functional EPOR on cell surface while MPPs express functional EPOR on the cell surface. 3) EPOR signaling has a minor positive effect on megakaryocyte/platelet differentiation.

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