The Pennsylvania State University
The Graduate School
Department of Biochemistry and Molecular Biology

FROM EXPLORING ROLES OF SIGNALS IN STRESS ERYTHROPOIESIS
TO IN VITRO EXPANDING STRESS ERYTHROID PROGENITORS
THAT ALLEVIATE ANEMIA

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by
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ABSTRACT

Bone marrow erythropoiesis is primarily homeostatic, producing new erythrocytes at a constant rate. However at times of acute anemia, new erythrocytes must be rapidly produced much faster than bone marrow steady state erythropoiesis. At these times stress erythropoiesis predominates. Stress erythropoiesis occurs in the fetal liver during embryogenesis and in the adult spleen and liver. In adult mice, stress erythropoiesis utilizes a specialized population of stress erythroid progenitors that are resident in the spleen. In response to acute anemia, these progenitors rapidly expand and differentiate.

Our lab previously identified a BMP4-dependent stress erythropoiesis pathway. The initiating event in the activation of this pathway is the up-regulation of BMP4 expression in the spleen. The work described in Chapter 2 shows that hypoxia plays a key role in initiating the BMP4 dependent stress erythropoiesis pathway by regulating BMP4 expression. I uncovered that hypoxia transcription factor HIFs (Hypoxia Inducible Factors) binds two cis regulatory sites in the BMP4 gene, which induces BMP4 expression in the spleen during the recovery from phenylhydrazine-induced acute anemia (published in *PLoS ONE*). Following activation, essentially all of the BMP4-dependent stress erythroid progenitors are mobilized and differentiate. Previous work from the lab showed that Hedgehog signaling is required for the maintenance and replenishment of stress progenitors in the spleen. The work described in Chapter 3 dissects the role of Hedgehog signaling during stress erythropoiesis, and shows that Hedgehog signaling is essential for stress erythropoiesis. Firstly by using short-term radioprotection as a stress model, I showed that Hedgehog signaling is required for stress
erythropoiesis by regulating differentiation and expansion of stress progenitors
Sca1+Kit+CD34+/ Sca1+Kit+CD34- cells, and stress erythroid progenitors
Sca1+Kit+CD71+ cells. In addition, Hedgehog signaling is required for the induction of BMP4 expression during the recovery. The data suggest that Hedgehog signaling is necessary to initiate the BMP4-dependent stress erythropoiesis pathway and may play a role in specifying the stress erythroid fate. Secondly by using the in vivo culture system that recapitulates in vivo stress erythropoiesis (described in Chapter 4), I analyzed why Hedgehog signaling is still required following the specification of stress erythroid fate. It maintains self-renewal, expansion of BMP4-responsive cells, and the ability of Sca1+Kit+CD34- cells to generate erythrocytes. This study also identified stress erythroid progenitor populations, “CD34+CD133+Sca1+Kit+ CD71+Ter119- cells” and “CD34-CD133-Sca1+Kit+ CD71+Ter119- cells”. In addition to hypoxia and Hedgehog signaling, role of another factor, GDF15, in stress erythropoiesis was also explored. In the first part of Chapter 4, my studies on role of GDF15 in stress erythropoiesis are summarized, which demonstrate that GDF15 is an essential factor for stress erythropoiesis. Moreover, I applied my findings from studying these signals and stress erythropoiesis as well as the knowledge from our lab’s publications to establishing an in vitro culture system that expands stress erythroid progenitors. The work in the second part of Chapter 4 demonstrates “proof of principle” experiments where stress erythroid progenitors provide erythroid-specific rescue in a murine model of β0 thalassemia which is similar to the most severe form of human Cooley’s anemia. This in vitro culture system successfully generates functional stress erythroid progenitors that alleviate lethal anemia and allow a normalization of erythropoiesis including iron utilization in murine β0
thalessemia. The fact that these thalassemia recipients are alive for more than 10 months (up to date) suggests that erythroid stem cells exist in these stress erythroid progenitors (Scal+Kit+CD71+Ter119- cells). Taken together, this study explored the interactions among signals, microenvironment and progenitor cells in stress erythropoiesis. The working model of stress erythropoiesis established in our lab was further proved by the in vitro generation of functional stress erythroid stem cells. The in vitro culture system established in this study provides therapeutic insight and a tool for studying stress erythropoiesis.
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Chapter 1

Literature review and introduction

Hematopoiesis

Hematopoiesis is a developmental process by which all lineages of mature blood cells are produced through a series of divisions that begin with stem cells. The classical view of hematopoietic hierarchy is as outlined in Figure 1-1 (Reya et al., 2001). More recently, this traditional model has been modified given new data over the past decade (Akashi, 2009; Kawamoto and Katsura, 2009; Kawamoto et al., 2010; Luc et al., 2008). All models include three main hierarchies: stem cells, committed progenitors, and precursor cells. Stem cells are defined as cells capable of both self renewal and the ability to give rise to multiple lineages of cells. Transplantation assays and purification of stem cell populations using cell surface markers using FACS (Fluorescence Activated Cell Sorting, or flow cytometry) were the primary means to characterize stem cells. The pool of stem cells is maintained by asymmetric division where one hematopoietic stem cell (HSC) divides into another HSC and a multipotent progenitor (MPP), which is more differentiated and has reduced capacity to self renew. In the murine system, HSCs can be enriched by selecting for the cell surface profile of Kit+, Sca1(Ly-6A/E)+ without expression of the lineage specific cell surface markers (Lin-, or Ter119-Mac1-B220-CD3-Gr1-) (Orkin, 2000; Spangrude et al., 1988). Committed progenitors, defined through in vitro functional assays, can produce differentiated progeny. Scientists have
been working on elucidating pathways which commit stem cells to multi-lineages or uni-lineage. Experimental evidence to date suggests that it is an irreversible process. Multipotent committed progenitors, give rise to colonies of multiple lineages *in vitro*, are more primitive than unipotential committed progenitor which only produce a single kind of progeny. The progeny of progenitors acquire more distinct properties of a specific lineage and move away from the shared characteristics as they differentiate. **Precursor cells** are defined by their morphologies, which are reflected by the acquired properties such as lineage-specific proteins and mature through a sequence of event that affects the structure of nucleus or organelle. As shown in Figure 1-1, at least eight lineages of mature blood cells are known to be produced throughout a vertebrate’s lifetime. The end-stage effector cells can be produced faster than normal in response to acute needs. Intrinsic regulators such as cytokines, growth factors, lineage-specific transcription factors and receptors, as well as microenvironment interactions with stromal cells or extracellular matrix, are crucial for hematopoiesis.
Figure 1. Development of haematopoietic stem cells. (Adapted and modified from Nature 414:105-111, 2001 (Reya et al., 2001)) HSCs can be subdivided into long-term self-renewing HSCs, short-term self-renewing HSCs and multipotent progenitors (red arrows indicate self-renewal). They give rise to common lymphoid progenitors (CLPs; the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells). Both CMPs/GMPs (granulocyte macrophage precursors) and CLPs can give rise to all known mouse dendritic cells. ErP, erythrocyte precursor; MEP, megakaryocyte erythrocyte precursor; MkP, megakaryocyte precursor; NK, natural killer.
Experimental methods for analyzing stem cells and progenitors

The first evidence of pluripotent HSCs came from an in vivo clonal assay, CFU-S or colony formation unit spleen in the early 1960’s (Till and McCulloch, 1961). Lethally irradiated mice were injected with bone marrow cells; it was noticed that macroscopic nodules were developed in their spleens at short post-engraftment times. Colonies that arise within 8 days are called CFU-S$_8$, which are mainly uni- or oligo-lineage committed progenitors. Colonies that arise at 12 days are called CFU-S$_{12}$, which consist of various lineages of cells (Magli et al., 1982; Ploemacher and Brons, 1988a, b, c). Extensive strategies using transplantation assay were developed after this first attempt to measure stem cells. Nowadays, a competitive repopulation assay is used to study HSCs function on a regular basis. This assay utilizes a tester population that is mixed with competitor population, usually bone marrow cells. Both populations are co-transplanted into lethally irradiated mice. Cell surface markers, which differentiate tester from competitor, are used to distinguish the sources of differentiated progeny. The stem cell functionality, or long-term repopulation ability, of the tester is measured relative to the competitor (Li and Johnson, 1992a, b; Szilvassy et al., 1990; Uchida and Weissman, 1992). The advantage of this assay is that the measurement of different sources of cells, such as spleen or fetal liver, can be all compared to the same standard.

Colony formation assay is used to identify the functional capacity of progenitor cells to form certain lineages. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The colonies formed can be enumerated and characterized according to their unique morphology. The hematopoietic progenitors that
can be assayed in clonal culture systems represent a continuum of differentiation, which includes multipotential progenitors and very late-committed progenitors with only limited cell-division capabilities. In this assay, cells are isolated and plated in semi-solid methylcellulose media with various cytokines. The further from a stem cell a population is, the more committed a single lineage it becomes and the fewer types of colonies it can form.

Later, the advances in flow cytometry together with above technologies further allow the identification and study of progenitor populations in hematopoietic tissues. Irving Weissman's group was the first to isolate mouse hematopoietic stem cells in 1988, and also the first to work out the cell surface markers to distinguish the mouse LT (long-term) -HSCs, ST (short-term) -HSCs, and MPPs. His group’s and others’ findings are reviewed and summarized in Figure 1-2 (Bryder et al., 2006; Weissman and Shizuru, 2008). This outline was established according to studies on homeostatic hematopoiesis in marrow, providing a start point for further work. As one can expect, development of populations in other microenvironments or under stress condition would be different (for example, see Figure 1-3). Indeed, works from our lab showed that stress erythropoiiesis is regulated by distinct signaling pathways, and also showed that acute erythroid stress changes the hematopoietic hierarchy. This topic will be discussed in a later section.
Figure 1-2. Schematic of hematopoietic development indicating intermediates in the hierarchy of hematopoietic differentiation. (Adapted from *Am J Pathol.* 169:338-346 (2006) (Bryder et al., 2006)) Surface markers used for isolation are indicated at left for human (bottom) and mouse (top) for each stem and progenitor cell. HSC indicates long-term reconstituting, self-renewing; MPP, multipotent progenitors with limited self-renewal leading to transient but multilineage reconstitution; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; BLP, B lymphocyte progenitor; ProT, T-cell progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythroid progenitor; MkP, megakaryocyte progenitor; EP, erythroid progenitor.
Growing knowledge and advance in technology have been facilitating the understanding of hematopoiesis model. Recently, a few modified versions of models were proposed (Akashi, 2009; Kawamoto and Katsura, 2009; Kawamoto et al., 2010; Luc et al., 2008). In the classical model, HSC first generates a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP). The CLP produces only T or B cells. This model is strongly supported by the prospective identification and purification of CMPs and CLPs, respectively, as well as subsequent megakaryocyte/erythroid (MEP) and granulocyte/monocyte (GMP) progenitors (Akashi et al., 2000; Kondo et al., 1997). However, this idea of “myeloid-lymphoid dichotomy” is now challenged by new findings (see reviews (Akashi, 2009; Kawamoto and Katsura, 2009; Kawamoto et al., 2010; Luc et al., 2008)). Studies in fetal hematopoiesis (Katsura, 2002; Kawamoto et al., 1997; Lacaud et al., 1998; Lu et al., 2002), and in part in adult bone marrow (Montecino-Rodriguez et al., 2001), had suggested that myelo-lymphoid precursors lacking erythroid potential exist in early hematopoiesis before cells are fully committed to the lymphoid lineage. This “CLP-independent”, or “Lymphoid Lineage-Primed MPP” model (Figure 1-3) suggests that the first lineage commitment step of HSCs does not result in a strict separation between common myeloid and lymphoid pathways. Its comparison with the traditional model is shown in Figure 1-4.
Figure 1-3. Comparative models for adult and fetal hematopoietic lineage commitment (Luc et al., 2008). (Adapted from Seminars in Immunology 20:213–220, 2008) Cell types with question mark inside, indicate commitment steps implicated but not unequivocally and prospectively identified. LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; GMTP, GM T cell progenitor; GMBP, GM B cell progenitor; MkEP, megakaryocyte/erythroid progenitor; MPP, multipotent progenitor; LMPP, lymphoid-primed MPP; CMLP, common myelo-lymphoid progenitor.
Figure 1-4. Models for cellular pathways in adult murine hematopoiesis. (Adapted from Hematopoietic Stem Cells VII: Ann. N.Y. Acad. Sci. 1176: 18–25, 2009) (A) The conventional model of myeloid versus lymphoid developmental pathways. In this model, HSCs give rise to MPPs, which choose to become CLPs or CMPs to differentiate into lymphoid or myeloid-erythroid cells, respectively. (B) The new developmental pathway model based on heterogeneity of the murine MPP population. The CD34+ MPP is composed of multiple progenitor populations. The LMPP is the MPP population expressing Flt3 and is largely committed to the myelo-lymphoid lineage. The upregulation of GATA-1 or PU.1 marks the lineage specification of the MPP into the myelo-erythroid or the myelo-lymphoid lineages, respectively. GATA-1+ MPPs are functional CMPs, whereas PU.1+ MPPs are functional GMLPs. RAG1+ ELPs are committed to the lymphoid lineage but retain a minor GM potential. The LMPP contains PU.1+ GMLPs and RAG1+ ELPs, and these progenitor populations might represent continuous developmental steps from the MPP to the lymphoid-committed CLP.
In addition, another "myeloid-based model" was also proposed (See reviews (Kawamoto and Katsura, 2009; Kawamoto et al., 2010)). In this model, HSC first generates a common myelo-erythroid progenitor (CMEP) and a common myelo-lymphoid progenitor (CMLP). CMLP generates T and B cell progenitors through a bipotential myeloid-T progenitor and a myeloid-B progenitor stage (Figure 1-5). This model postulates that the myeloid potential is still retained in all erythroid, T and B lineage cells.

Figure 1-5. Representative contemporary models of hematopoiesis. (Adapted from Trends in Immunology 30:193-200, 2009) (a) Classical dichotomy model. HSC diverge into CMEP and CLP. Note that CMEP are sometimes referred to as common myeloid progenitors (CMP). E, M, T, and B represent the progenitor potential for erythroid, myeloid, T, and B cells, respectively. (b) Myeloid-based model. In this model, the first branch point generates CMEP and CMLP, and myeloid potential persists in the T and B cell branches even after these lineages have diverged. Abbreviations: CLP, common lymphoid progenitor; CMEP, common myeloid-erythroid progenitor; CMLP, common myeloid-lymphoid progenitors; HSC, hematopoietic stem cell; MBP, myeloid-B cell progenitor; MTP, myeloid-T cell progenitor.
Notably, one problem with all these models is that each of them uses different markers. Common markers and assays could unify the models. Ongoing and future work will elucidate the model by which terminal differentiated cells are formed via interactions among progenitors, cytokines and microenvironments.

Irrespective of the models, each lineage is known to have specialized functions. The erythrocytes carry oxygen throughout the body. The platelets are in charge of blood coagulation. The lymphocytes, mainly composed of T cell and B cell, are important to adaptive immune system. Basophil, neutrophil, eosinophil, and monocytes that are developed from myeloblasts are involved in responding to allergy and inflammation. Our lab focuses on erythroid lineage and the special mechanism that regulates erythropoiesis under stress condition, such as acute anemia and tissue hypoxia. Erythropoiesis and its regulations related to my studies are reviewed in the following sections.
Erythropoiesis

Erythropoiesis is the process of red blood cell (RBC, or erythrocyte) development. Erythrocytes are specialized to transport oxygen from respiratory system to tissues through circulation. An erythrocyte’s cytoplasm is rich in hemoglobin, an iron-containing molecule, which binds oxygen and causes the red color of blood. The mature erythrocyte is a small biconcave disk, lacking a nucleus and mitochondria. It is therefore assumed that the mature erythrocyte has no protein biosynthesis. It depends almost exclusively on anaerobic glycolysis for its energy requirement. In human, a RBC is 6-8um in diameter, 2um thick, has a volume of 90fL with a surface of 136um². Adult humans have about 2-3 x 10¹³ RBCs, comprising around one quarter of total cell number. Human RBCs are developed from stem cells in approximately 7 days. They have a lifespan of 100-120 days, then become senescent and are removed from circulation. In mouse, the lifespan of RBCs is between 44-52 days (Goodman and Smith, 1961; Hoffmann-Fezer et al., 1993). It decreases under hypoxia conditions. Experiments showed that the average lifespan of mice are 46.9, 44, and 39.2 days at 1, 0.7 and 0.5 atm, respectively (Abbrecht and Littell, 1972).
Hematopoiesis in adult life begins in bone marrow at the level of pluripotent long-term HSCs, which then give rise to short-term HSCs and multipotent hematopoietic progenitors. These cell types then yield committed progenitors and finally mature blood cells. Among various hematopoietic cell-restricted pathways illustrated above, erythropoiesis represents a unidirectional pathway of maturation. The erythrocytic pathway of differentiation (highlighted) begins from megakaryocytic/erythroid pluripotent progenitor (MEP) that gives rise to BFU-E, CFU-E, intermediate forms of proerythroblasts (ProEB), and then to orthochromatic normoblasts (ON). Enucleation of ONs leads to birth of reticulocytes (RETs). The latter cells finally yield mature enucleated red blood cells (RBCs). BFU-E, burst forming unit-erythroid; CMP, common myeloid progenitor; CFU-E, colony forming unit-erythroid; CLP, common lymphoid progenitor; DCs, dendritic cells; EB, erythroblast; GMP, granulocyte-myeloid progenitor; MEP, megakaryocytic/erythroid progenitor; MPP, multipotent progenitors; NK, natural killer; ON, orthochromatic normoblast; PLTs, platelets; RBCs, red blood cells; RET, reticulocyte.
The known hierarchy of erythropoiesis is as outlined in the white box in Figure 1-6 (Tsiftsoglou et al., 2009). Traditionally, colony assays are used to identify erythroid cells. The first committed progenitor that was identified is the BFU-E, or burst forming unit- erythroid. When plated in methylcellulose media, BFU-E requires high concentration of Epo (3 units) and a burst promoting factor (BPF) to form. Stem cell factor (SCF) is probably the BPF in vivo, but can be replaced by Interluekin-3 (IL-3) or granulocyte monocyte- colony stimulating factor (GM-CSF) in vitro. BFU-E colonies are recognized by staining with benzidine dye that binds to hemoglobin. They are large colonies which usually form within 3 days to about 10 days (Gregory and Eaves, 1977, 1978), suggesting that BFU-E colonies actually represent a heterogeneous population at different stages of maturation. From day by day observation of their growth, it seems that some BFU-E colonies are derived from more immature progenitors than others because they require longer time than others to form. Some form larger colonies faster, presumably are derived from progenitors with higher proliferative capacity or shorter cycling time. In addition, their properties vary depends on their origin. For example, fetal BFU-E is more proliferative, and more sensitive to Epo concentration than its adult counterpart (Peschle et al., 1981). Therefore, further fine characterization and categorization of cells within this population is needed.

The next committed erythroid progenitor which was also identified by colony assay is the CFU-E, or colony formation unit- erythroid. They do not require BPF to form. These colonies are much smaller than BFU-Es, composed of 8-32 cells that tightly held together. CFU-Es require only Epo to form and form in 2 days. In culture CFU-E lyse after a few days. It is generally accepted that CFU-Es are derived from a more
differentiated progenitor whose proliferative capacity is less, presumably the most mature form of BFU-E. CFU-Es are composed of proerythroblasts, the first histologically recognizable type of erythroid cell. Proerythroblasts synthesize globins and differentiate to erythroblasts that synthesize hemoglobin. In mammals, the nucleus is expelled when the cell undergoes maturation, and the cell becomes a reticulocyte. The reticulocyte uses the remaining RNA to synthesize globin, and eventually becomes a mature erythrocyte which does not divide nor synthesize RNA and protein. These mature erythrocytes are then released into circulation (Stamatoyannopoulos, 2000).

Later the advances of flow cytometry allow further characterization of erythroid progenitors in vivo. Erythroid populations in the bone marrow can be categorized by using the markers CD71 and Ter119, as shown in Figure 1-7. CD71 is the transferrin receptor, which is essential for iron transport into proliferating cells. CD71 is expressed on marrow stromal cells from bone marrow (Pittenger et al., 1999), present on reticulocytes and erythroid progenitors in fetal liver, cord blood, and peripheral blood, yet it is lost as these cells differentiate to mature erythrocytes (Judd et al., 1980; Loken et al., 1987; Phillips et al., 1984). Ter-119 is used as terminal differentiation marker for erythroid cells. Kina et al. (Kina et al., 2000) reported that Ter119 antibody reacts with mature erythrocytes, 20-25% of bone marrow cells, 2-3% spleen cells, 30-40% of embryonic day10 yolk sac cells, 80-90% of embryonic day14 fetal liver cells and 40-50% of newborn liver cells. Ter119 antibody reacts with erythroid cells at late stage (proerythroblasts to mature erythrocytes). It does not react with BFU-E and CFU-E. Expression of Ter119 is not detectable in thymocytes or lymph node cells. Using CD71 and Ter119, four distinct stages of erythroid maturation can be observed. These
populations I-IV characterized by Socolovsky, M and Lodish, H.F. are now commonly used for analyzing erythropoid populations among literatures (Figure 1-7). Under conditioning acute erythroid stress, our lab recently identified erythroid populations that co-express CD71 and Ter119 with stem cell marker, Kit (Harandi et al., 2010; Perry et al., 2007b) (Appendix); these small populations which express Kit emerge under stress condition.
Figure 1-7. Definition of flow cytometric erythroblast subsets. (Adapted from *Blood* 108(1): 123-233, 2006 (Liu et al., 2006))

(A) Flow cytometric analysis of freshly isolated bone marrow cells labeled with antibodies against Ter119 and CD71. Dead cells were excluded with 7-AAD. The left panel shows all viable bone marrow cells. Ter119\textsuperscript{high} cells are further analyzed with respect to their forward scatter (FSC) in the right panel. (B) Cytospin preparations of cells sorted from the ProEs and Ery.A, B, and C subsets shown in panel A are stained for hemoglobin with diaminobenzidine and counterstained with Giemsa. Scale bar represents 5 μm.
**Homeostatic versus stress erythropoiesis**

As mentioned earlier, precursor cells in several hematopoietic lineages can be produced faster than normal sequence in response to acute needs. Under homeostatic condition, erythropoiesis constantly produces erythrocytes to maintain hematocrit (HCT) while the worn-out red cells are removed by spleen. Under certain circumstances such as acute anemia, a distinct mechanism of erythropoiesis is predominant. For example, during fetal development, erythropoiesis is critically important for the survival and growth of the embryo. During the third trimester, the fetus grows rapidly and produces red cells approximately 3 to 5 times of adult steady state levels. Birth brings dramatic changes in oxygenation and erythropoietin production which result in a tenfold drop in red cell production (Palis and Segel, 1998). This rapid production of new erythrocytes is called stress erythropoiesis. The regulation of stress erythropoiesis occurs at the following levels. First, the *microenvironment*, regulates the expansion of this erythroid pool. In addition to the fetal liver during embryogenesis, the adult spleen is where stress erythropoiesis occurs in mice. It was shown that the contribution of spleen to erythropoiesis increased to 40% from 10% after phlebotomy (Sadahira et al., 2000). The function of the microenvironment refers to adjacent cells such as fibroblasts, endothelial cells and stromal cells that support and maintain growth and differentiation of hematopoietic cells via paracrine signaling. It was suggested that the hematopoietic inductive microenvironments in different organs are different (Obinata and Yanai, 1999). This property is evident when colony formation following lethal irradiation is examined. In spleen the erythroid colonies are dominant, while in bone marrow granulocyte colonies are dominant (Curry et al., 1967). The second aspect of stress erythropoiesis is that the
properties of the progenitor cells active in the stress state are different from their counterparts in steady state. Thirdly, the signals that activate the sequence of robust erythrocyte production are specific to stress erythropoiesis. Our lab previously identified a BMP4-dependent stress erythropoiesis pathway. I will discuss the BMP4-dependent stress erythropoiesis pathway in more detail in a subsequent section.

Previous Models for Stress Erythropoiesis
Studies by Hara and Ogawa (Hara and Ogawa, 1977) suggested an early model for stress erythropoiesis. In the bone marrow, decreased BFU-E colonies were observed after the traditional method of Phenylhydrazine (PHZ) injection (day0, 1, and 3). Whereas in spleen, both number of BFU-E and CFU-E colonies increased until day4. In peripheral blood, number of BFU-E was maximal at day2. The temporal kinetics of BFU-E expansion suggested a model where erythroid progenitors migrate from the bone marrow to the spleen via the blood upon stress stimulation. Furthermore, their examination of DNA synthesis showed no change in the proliferation of progenitors under anemia condition, demonstrating that different number of BFU-E colonies was due to migration but not proliferation. The model established by Hara and Ogawa postulated two key conclusions. First, Epo is the key signal. Second, Erythroid progenitors that responding to stress are the same as the bone marrow progenitors. One caveat of this experiment, however, was that it did not rule out the possibility that the progenitors resident in spleen can also respond to anemia. Using higher dose of PHZ treatment and irradiation which both induced acute anemia much robustly, we were able to analyze the immediate response of cells. We have established a different model through our understanding of
BMP4-dependent stress erythropoiesis. In our model, stress progenitors poised to respond to acute needs are resident in spleen; these progenitors have distinct properties from their bone marrow counterparts and emerge only under stress condition. These findings on **BMP4-dependent stress erythropoiesis** are summarized in the following.

**BMP4-dependent stress erythropoiesis pathway**

BMP4 (bone morphogenic protein 4) belongs to the TGFβ (transforming growth factor) superfamily, which includes more than 30 molecules such as TGFβ, activin, nodal, BMPs and others. The TGFβ signaling pathway is outlined in Figure 1-8. The ligands bind to the type II receptor, which recruits and phosphorylates the type I receptor. The type I receptor phosphorylates receptor-regulated SMADs (R-SMADs), which allows the formation of R-SMAD/coSMAD(SMAD4) complex. In nucleus, the R-SMAD/coSMAD complex acts as a transcription factor and regulates the expression of target genes (Reviewed in (Massague, 2000)).
Figure 1-8. The basic SMAD pathway. (Adapted from *Nature Review Mol Cell Biol* 1:169-178, 2000 (Massague, 2000)) Receptor-regulated SMAD transcription factors (RSMADs) require transforming growth factor-β (TGF-β) induced phosphorylation to assemble transcription regulatory complexes with partner SMADs (co-SMADs). R-SMADs can move into the nucleus on their own but, to be accessible to membrane receptors, R-SMADs are tethered in the cytoplasm by proteins such as SARA (SMAD anchor for receptor activation). Receptor activation occurs when TGF-β induces the association of two type I and two type II receptors. Both receptor components have a serine/threonine protein kinase domain in the cytoplasmic region. In the basal state, the type I receptor is kept inactive by a wedge-shaped GS region, which presses against the kinase domain, dislocating its catalytic centre. In the ligand-induced complex, the type II receptor phosphorylates the GS domain and this activates the type I receptor, which catalyses R-SMAD phosphorylation. Phosphorylation decreases the affinity of R-SMADs for SARA and increases their affinity for co-SMADs. The resulting SMAD complex is free to move into the nucleus and competent to associate with transcriptional coactivators or corepressors. SMADs can contact DNA, but effective binding to particular gene regulatory sites is enabled by specific DNA-binding cofactors. R-SMADs that move into the nucleus may return to the cytoplasm, but their ubiquitylation- and proteasome-dependent degradation in the nucleus provides a way to terminate TGF-β responses.
Previously, our lab established a model of stress erythropoiesis based on our growing findings of BMP4/Smad5 pathway’s role in stress erythropoiesis. The story started with the analysis of the murine *flexed-tail* (*f*) mutation. Homeostatic erythropoiesis of *ff* mice is nearly normal. These mutant mice, however, exhibit fetal-neonatal anemia which resolves about two weeks after birth, and delayed recovery from PHZ- induced hemolytic anemia (Bateman and Cole, 1972; Cole and Regan, 1976b; Hunt et al., 1933; Law, 1952). These phenotypes suggested that *ff* mice would be a model for studying erythropoiesis specifically under stress condition. Using a modified protocol of PHZ treatment which induce anemia more acutely, our lab found the expansion of BFU-E is delayed in the spleen, but not bone marrow, of *ff* mice compared to the controls (Lenox et al., 2005b). This observation is consistent with the idea that erythroid progenitors respond to stress condition are distinct from those in homeostatic state. The finding suggested a new model for stress erythropoiesis where a population of specialized stress erythropoid progenitors is resident in spleen. Cloning of *ff* locus revealed the splicing mutation of Smad5 (Hegde et al., 2007; Lenox et al., 2005b). Smad5 is a transcription factor acting downstream of the receptor for BMP2, 4, and 7 (Massague et al., 2005). Subsequent analyses showed that BMP4 expression is first induced then declined upon PHZ treatment (Lenox et al., 2005b). The induction of BMP4 in spleen post PHZ treatment is regulated via the binding of HIF (Hypoxia- inducible factor) to the HRE (HIF- responsive element) sites in the BMP4 locus (described in Chapter 2) (Wu and Paulson, 2010). Using colony formation assay, our lab also demonstrated that BMP4 induced the expansion of BFU-E (Lenox et al., 2005b). Taken together, these findings showed that BMP4/Smad5- dependent stress erythropoiesis is required for expansion of
erythroid progenitors that are resident in adult spleen in response to acute anemia. In addition to adulthood, this pathway is also required for the expansion of erythroid progenitors during fetal development (Porayette and Paulson, 2008). Our lab’s previous analysis in murine system showed that in the absence of spleen, stress erythropoiesis occurs in the liver. This extramedullary erythropoiesis in liver also requires the BMP4/Smad5 dependent signaling (Lenox et al., 2009).

In addition to PHZ- induced anemia as a stress model, our lab later adopted another experimental system, bone marrow transplantation (BMT). In order to recover from lethal anemia post lethal irradiation, new erythrocytes must be made prior to the engraftment of the donor stem cells. By transplanting the cells of interest, we were able to examine the autonomous defect of the donor cells. The BMP4-dependent stress erythropoiesis were further studied by taking advantage of BMT system (described in detail in Appendix B). By transplanting f/f cells, our lab showed that the short-term radioprotection also required BMP4/Smad5 signaling (Appendix B) (Harandi et al., 2010). Kit+CD71+Ter119+ cells were found to emerge post BMT. These cells can be divided into three distinct populations which we termed as population I, II, and III (described in detail in Appendix). Among which, partial population I also express Sca1. The coexistence of hematopoietic stem cell markers, Sca1 and Kit, with erythroid surface marker suggested their potential to self renew. Indeed, transplanting population I (Kit+CD71loTer119- cells) from spleen of recipients at day 8 post BMT into lethally irradiated secondary recipients rescued the erythropoiesis, without contributing to other lineages. These data demonstrated that murine short-term radioprotection requires self-renewing stress erythroid progenitors that are regulated by BMP4-dependent pathway
The BMP4-dependent stress erythropoiesis model based on these findings is outlined in Figure A-6 (Appendix) (Harandi et al., 2010).

In summary, our lab’s findings of BMP4-dependent stress erythropoiesis pathway suggest that manipulation of this pathway and cultivation of the self renewing stress progenitor may alleviate human anemia. Further studies on regulation of this pathway and characterization of the analogous pathway in human will advance the therapeutic potential in human.

**Other Signals involved in Stress Erythropoiesis**

Researchers have been studying signals predominant in response to acute anemia. Among those, knowledge regarding *Erythropoietin (Epo)* probably is most abundant by far. Epo plays an essential role in both steady and stress states (see reviews (Socolovsky, 2007; Wojchowski et al., 2010)). It is the lineage-specific cytokine required for erythropoiesis (Stamatoyannopoulos, 2000). It was suggested that in the absence of Epo, the default pathway for the erythroid progenitors is apoptosis and Epo prevents this pathway (Fang et al., 2008; Koury and Bondurant, 1990a, b; Liu et al., 2006; Socolovsky, 2007; Socolovsky et al., 2007). Under anemia condition, the level of Epo in the serum is greatly increased and more progenitors survive which results in increased erythrocyte production. Epo is highly expressed in fetal liver and adult kidney (Zanjani et al., 1981). In response to acute anemia caused by lethal irradiation followed by transplantation, indeed we observed increased Epo mRNA in kidney during the recovery period (Figure A- S9) (Harandi et al., 2010). In addition to site and level of Epo expression, it is evident
that different downstream effectors are activated under homeostatic versus stress conditions at the level of Epo receptor (EpoR) signaling (Figure 1-9) (Longmore, 2006). Menon et al. showed that EpoR-activated Stat5 signaling pathway uniquely contribute to stress erythropoeisis (Menon et al., 2006). Two mutant strains of mice were generated by knocking in different alleles of the mutant EpoR into the EpoR gene locus, allowing mutant EpoR expression in the appropriate tissues and cells. One allele is EpoR-H containing the single Stat5-interacting tyrosine residue Y343, which serves to recruit and activate Stat5. The other allele was EpoR-HM mice that express a truncated EpoR that is mutated at tyrosine (Y343F). EpoR-HM mice, who had normal level of circulating erythrocytes, could not appropriately respond to anemia stress responding to anemic stress. Similar response as wildtype controls was seen in EpoR-H mice. This finding is consistent with the defects in adult Stat5a, b−/− mice, in that they are compromised in response to PHZ- induced acute anemia (Socolovsky et al., 2001).
Contributors to stress-induced erythropoiesis include EpoR-stat5 signaling, synergy between SCF and Epo, oncostatin-M, BMP4, and possibly Gas6. EpoR-Stat5 signals were found to induce the expression of oncostatin-M, and oncostatin-M cooperates with SCF/Epo signals to enhance erythroid survival. Thus EpoR-Stat5 signals set up a positive feedback loop whereby the signal induces secretion of oncostatin-M, which then contributes to regulation of erythropoiesis during times of acute anemic stress. c-Kit, SCF receptor; Gas6, growth arrest–specific 6; Osm, oncostatin-M; OsmR, oncostatin-M receptor; Pim-1, proviral integration site 1.
**Stem Cell Factor (SCF)** is also known to play important roles in both steady and stress states. Murine SCF is encoded by *Steel (Sl)* locus; its receptor, Kit, is encoded by *Dominant white spotting (W)* locus (Chabot et al., 1988; Geissler and Kobberling, 1988; Martin et al., 1990; Matsui et al., 1990; Williams et al., 1990; Zsebo et al., 1990). Mutations in both genes cause defective development of CFU-E which results in severe anemia (Nocka et al., 1989). It has been shown that SCF is required for development and expansion of erythroid progenitors (Adelman et al., 1999; Bernstein et al., 1991; Russell, 1979b). Upon treatment with PHZ, the mutant mice exhibited delayed recovery and expansion of progenitors in spleen (Harrison and Russell, 1972). Our lab have also previously demonstrated that SCF increase the progeny of BFU-E in *vivo*; SCF acts in concert with BMP4 and hypoxia, another two signals which play important roles in response to acute anemia (Lenox et al., 2005b; Perry et al., 2007b). Mutant mice harboring knocked-in KIT(Y567F/Y567F), KIT(Y569F/Y569F), KIT(Y719F,Y719F), and KIT(Y567F/Y567F:Y569F/Y569F) alleles were used to dissect regulation of SCF/Kit signaling pathway in stress condition. Homeostatic erythropoiesis was not affected whereas recovery from PHZ or 5-fluorouracil (5-FU)-induced anemia was impaired in KIT (Y567F/Y567F) mice. In KIT (Y567F/Y567F) mice, expansion of erythroid progenitors was deficient in both bone marrow and spleen (Agosti et al., 2009). *Ex vivo* culture of bone marrow cell from KIT(Y567F/Y567F) mice showed skewed development from Kit⁺CD71⁺ to Kit⁻CD71⁺ compartment. These defects were not seen in the case of KIT(Y719F/Y719F) (Agosti et al., 2009). MEK-1,2/ERK-1,2 signaling was unaffected while AKT, p70S6K, and especially JNK2/p54 pathways were selectively attenuated in Kit(Y567F/Y567F) erythroblasts (Agosti et al., 2009).
Early work on lipophilic hormones, **corticosteroids or glucocorticoids** were also shown to increase the proliferation of BFU-E colonies *in vivo* (Golde et al., 1976; Udupa et al., 1986). The glucocorticoid derivative, dexamethasone, was used together with the activated EpoR and SCF/Kit pathways to maintain erythroblast in long-term culture *in vitro* (Bauer et al., 1999). Mice with mutant glucocorticoid receptor (GR) have no defects in homeostatic erythropoiesis, but exhibit defective response to acute anemia (Bauer et al., 1999). In addition, GR is required for splenic erythroid progenitor to co-express terminal differentiated marker Ter119 with CD34 and Kit when acute anemia occurred (Bauer et al., 1999). CD34 is a cell surface glycoprophosphoprotein belongs to sialomucin family. It is expressed by bone marrow stromal cells, hematopoietic stem cells, progenitor cells, vascular endothelium and some fibroblasts (Simmons et al., 1992). It has been commonly used to identify hematopoietic stem cells. It is known that CD34 is involved in cell-cell adhesion (Drew et al., 2005; Sato et al., 1999) and facilitates cell migration (Blanchet et al., 2007). It is suggested that it mediates the attachment of stem cells to stromal cells or extracellular matrix in marrow (Satterthwaite et al., 1992; Simmons et al., 1992). In mouse, the RAM34 monoclonal antibody that reacts with mouse CD34 is used to detect expression of CD34. Simultaneous staining of mouse bone marrow cells with a cocktail of antibodies to lineage markers reveals a subset of cells that stain with the RAM34 antibody and express undetectable to low levels of the indicated lineage markers (antibody datasheet from eBioscience). Expression of these markers and the role of these progenitors appears to be restricted only to stress erythropoiesis. This observation is actually very intriguing to us. In my study on stress erythropoiesis regulated by Hedgehog signaling pathway (Chapter 3), increasing Sca1⁺Kit⁺CD34⁺
(S⁺K⁺34⁺) cells and S⁺K⁺CD71⁺ cells were found in mice spleen when Hedgehog signaling pathway was blocked. Co-expression of CD71 was found in S⁺K⁺34⁺ cells, but not S⁺K⁺34⁻ cells, that were sorted from culture which contained factors important for stress erythroid progenitors (Figure 3-19). Taken together, Bauer et al.’s finding reported in 1999 (Bauer et al., 1999) and our present studies in Chapter 3 demonstrated that the stress erythroid progenitors co-express Sca1, Kit and CD34 at certain stage during this differentiation, and these cells are regulated by Hedgehog and glucocorticoids pathways. The co-existence of Sca1, Kit and CD34 in erythroid populations that express CD71 has not yet be reported; this population should be included in the hierachy of stress hematopoiesis.

A second hormone that may play a role in erythropoiesis is T3, or thyroid hormone. TRalpha(-/-) mice have compromised fetal and adult erythropoiesis, and recovered very poorly from hemolytic anemia (Kendrick et al., 2008). Elevated glucocorticoid level was seen in TRalpha(-/-) mice (TR, Thyroid hormone receptor) (Kendrick et al., 2008). Despite having increased levels of glucocorticoids, the mice exhibite a defect suggesting that the increasing glucocorticoids in TRalpha(-/-) mice might be compensation for a T3-dependent process.

Another factor that related to CD34 and stress erythropoiesis is podocalyxin. Podocalyxin-like protein (PCLP) is a sialomucin-type membrane protein structurally related to CD34 and endoglycan. Miettinen’s and McNagny’s groups analyzed expression of podocalyxin in human and murine system, respectively. Kerosuo et al. demonstrated its expression in human CD34+ cells, in lineage committed erythroid, megakaryocyte and myeloid progenitors, in K562 leukaemia cells, and in peripheral
blood leukocytes (Kerosuo et al., 2004). Its expression is found higher in developing cells than in mature leucocytes (Kerosuo et al., 2004). In mouse, it was found highly expressed in the first primitive hematopoietic progenitors and nucleated red blood cells, as well as definitive multi-lineage hematopoietic progenitors and erythroid precursors in fetal liver (Doyonnas et al., 2005). Its expression declines gradually with maturation of embryo (Doyonnas et al., 2005). However its expression is boosted again at the timing when new hematopoietic progenitors in the spleen and bone marrow are seeded (Doyonnas et al., 2005). Later, the expression is restricted to Lin−S+K+ bone marrow cells (Doyonnas et al., 2005). It was shown that the podocalyxin-expressing cells are capable of serially reconstituting myeloid and lymphoid lineages (Doyonnas et al., 2005). Later McNagny’s group examined expression of podocalyxin in response to stress. Its expression on Ter119+ erythroblasts is upregulated following treatment of Epo or PHZ (Maltby et al., 2009). However, it is dispensable for efficient erythroblast expansion, erythroid differentiation, or reticulocyte release in response to Epo stimulation in vivo (Maltby et al., 2009).

In 2010, more new signals which contribute to stress erythropoiesis were reported. Regulation of erythropoiesis by microRNAs (miRNAs), the small noncoding RNAs that regulate gene expression and protein synthesis post-transcriptionally, was revealed very recently (reviewed in (Zhao et al., 2010)). It was reported that mice lacking miR-451 exhibited delayed recovery from PHZ-induced anemia (Patrick et al., 2010; Yu et al., 2010), in addition to their defects in homeostatic erythropoiesis (Dore et al., 2008; Masaki et al., 2007; Pase et al., 2009; Patrick et al., 2010; Rasmussen et al., 2010). It was shown that 14-3-3ζ is up-regulated in miR-451−/− erythroblasts, and inhibition of 14-3-3ζ...
rescues their differentiation defect (Patrick et al., 2010; Yu et al., 2010). In addition to revealing the role of miR-451 and 14-3-3ζ in stress and homeostatic erythropoiesis, these findings also highlighted the therapeutic potential of miR-451 inhibitors. Another significance of these findings lies in the ineffective erythropoiesis in response to oxidative stress when lacking miR-451. 14-3-3ζ inhibits nuclear accumulation of a transcription factor FoxO3, a positive regulator of erythroid anti-oxidant gene such as Cat and Gpx1. Indeed, overexpression of 14-3-3zeta in erythroid cells and fibroblasts inhibited nuclear localization and activity of FoxO3. The findings identified a miRNA-regulated pathway that protect erythropoiesis from oxidant stress and showed that miRNA can affect gene expression by altering the activity of transcription factor.

**Sox6**, which was actually one of our candidates downstream of Hedgehog signaling pathway in stress erythropoiesis, was recently reported necessary for efficient erythropoiesis in adult mice in response to acute anemia. Sox6 is a transcription factor with a Sry-related high-mobility DNA-binding domain and a highly conserved coiled-coil homodimerization domain, but without any known transactivation or transrepression domains (Lefebvre, 2010). Unlike some Sox proteins that are required for cell fate specification and differentiation in discrete lineages, Sox6 acts as a significant modulator rather than determinant of cell fate (Lefebvre, 2010). Homologous deletion of Sox6 causes death at birth or the third week of life with skeletal, glial, and cardiac developmental defects (Cohen-Barak et al., 2007; Dumitriu et al., 2006; Hagiwara et al., 2005; Han and Lefebvre, 2008; Lefebvre, 2010; Lefebvre et al., 1998; Smits et al., 2001; Stolt et al., 2006; Yi et al., 2006). Mutant mice exhibit nucleated definitive red blood
cells and are anemic, despite the increased serum Epo level and hepatomegaly (Cohen-Barak et al., 2007; Dumitriu et al., 2006; Yi et al., 2006). By using conditional knockout of Sox6, Dumitriu et al. examined role of Sox6 in adulthood erythropoiesis (Dumitriu et al., 2010). 25% of mutants died post PHZ treatment and the rest recovered slowly. These mutants increased Epo level promptly. Upon anemia induction, mutants amplified the erythroid population pool first and then exhibited erythroblast and reticulocyte defects. In fact, the defective phenotypes reported in this study are similar to what we saw when Hedgehog signaling was blocked during the response to acute anemia (as described in Chapter 3). In both systems, expansion of erythroid population pool and defective erythroblast populations were observed, except that the Lefebvre group only analyzed these relatively late-stage cells whereas we also examined immature progenitors and populations enriched in stress erythroid progenitors. Under anemia stress, inactivation of Sox6 resulted in downregulation of erythroid terminal marker Bcl2l1 and anti-apoptotic factor Bcl-xl.

The transcription factor GATA-1 is a key player in erythropoiesis. The role of GATA-1 was also examined under stress condition in adults recently. Previously, determining the role of the GATA-1 in adult erythropoiesis has precluded due to the lethality of Gata1-null embryos. Gutierrez et al. solved this by using conditional GATA1 knockout mice. Mx-Cre-mediated GATA1 recombination, although incomplete, caused maturation arrest of GATA1-null erythroid cells at the proerythroblast stage (Gutierrez et al., 2008). Tamoxifen-Cre-mediated Gata1 recombination depleted the erythroid compartment in bone marrow and spleen (Gutierrez et al., 2008). Plus, these mice failed
to activate a stress erythropoietic response upon treatment of PHZ, despite the rising erythropoietin levels (Gutierrez et al., 2008). This study showed that GATA1 is necessary for both steady-state and stress erythropoiesis in adult.

Regulation of stress erythropoiesis in the adult mice by members of the integrin, family of adhesion molecules, was also demonstrated. Ulyanova et al. examined specific members of integrins by using three genetic models (Ulyanova et al., 2010). The survival of β1-conditionally deficient mice after PHZ treatment is severely compromised. Different response was seen in the marrow versus spleen. In bone marrow, only defects in late differentiation existed. The expression of stress BFU-E is defective despite normal expression of BMP4 in the spleen. α4Δ/Δ mice showed only a kinetic delay in recovery with terminal maturation defects in both bone marrow and spleen. These defects were not present in VCAM-1Δ/Δ mice. This study suggested that the presence of α5β1 integrin in all hematopoietic progenitor cells that interact with spleen microenvironment is instrumental for their survival and accumulation during hemolytic stress; whereas presence of α4, or of both α5 and α4, is important for completion of terminal maturation steps (Ulyanova et al., 2010).

While loss of many factors results in impaired response to acute anemia, one gene was found enhanced recovery. Hfe- knockout mice was shown to overcome phlebotomy-induced anemia more rapidly than wildtype mice (Ramos et al., 2010). Mutations of HFE lead to iron overload through abnormally low levels of hepcidin in hereditary hemochromatosis. Ramos et al.’s study suggested that lack of Hfe is
advantageous in conditions of increased erythropoietic activity, due to augmented iron mobilization driven by deficient hepcidin response (Ramos et al., 2010).

**Role of hypoxia in stress erythropoiesis**

During an erythrocyte’s lifespan, they accumulate hemoglobin and transport oxygen. Therefore it is not surprising that concentration of oxygen and regulation of oxidative stress are critical for erythropoiesis. The most well-known example of hypoxia-promoted erythropoiesis is the induction of Epo regulated by HIF1α. At high altitude where oxygen is low, Epo is induced transcriptionally which is predominantly regulated by hypoxia-inducible factor (HIF) family and nuclear factor kappa B (NF-kB) (Jelkmann, 2004; Rogers et al., 2008; Smith et al., 2008). The studies on hypoxia in regulating Epo establish a role for hypoxia and HIFs in stress erythropoiesis. HIFs belong to the basic helix-loop-helix (bHLH) PAS (Period-ARNT Single minded) family. HIF is a heterodimer composed of an oxygen labile α-subunit and a constitutively expressed β-subunit. After the discoveries of its activation of Epo expression, HIF was subsequently found to control a wide range of responses to hypoxia. In addition to Epo, HIFs regulate vascular endothelial growth factor (VEGF), glucose uptake transporter-1, transferrin, transferrin receptor, plasminogen activator inhibitor-1, aldolase C, and endothelin-1 (reviewed in (Smith et al., 2008)). Like production of Epo, the DNA-binding activity of HIF-1 was found to be tightly regulated by cellular oxygen concentration. HIF-α subunit is synthesized continuously but is rapidly degraded in the presence of oxygen. In addition to O₂, iron is also required for the regulation. The stability of HIF is regulated by specific prolyl hydroxylase-domain (PHD) enzymes. Oxygen- and iron-dependent PHD enzymes
hydroxylate specific proline residues in HIF-α, which increases its affinity for the von Hippel-Lindau tumor suppressor protein (VHL). VHL binds to the HIF-α subunit and targets it for ubiquitin-mediated proteasomal degradation (Figure 1-10). Another level of regulation is by another oxygen- and iron-dependent enzyme, factor inhibiting HIF (FIH). FIH hydroxylates an asparagine residue in HIF-α, thereby reduces its ability to activate transcription by inhibiting binding of the transcriptional coactivator complex p300/CBP. Taken together, hydroxylation of HIF-α by PHDs and FIH is inhibited and proteasomal degradation is minimized under hypoxia condition. HIF-α dimerizes with HIF-β, leading to recruitment of coactivator p300/CBP followed by upregulation of hypoxia-responsive genes (Figure 1-10).
Figure 1-10. Schematic diagrams illustrating the major events occurring during erythropoiesis under normal oxygen consumption and hypoxia. (Adapter from IUBMB Life 61(8):800-30, 2009 (Tsiftsoglou et al., 2009)). (A) In normal oxygen (O$_2$) conditions (normoxia), the HIF-1α protein is subjected to hydroxylation by PDHs and FIH, both requiring 2-oxoglutarate and oxygen as cofactors. This allows recruitment of VHL, a component of ubiquitin ligase complex that ubiquitinitates (Ub) HIF-1α and thereby targets it for proteasome-mediated degradation. (B) During hypoxia, when PDHs are not active, HIF-1α is stabilized, dimerizes with HIF-1β-CBP, a fact that results in transcription at HREs of hypoxia-inducible genes involved in angiogenesis, apoptosis, glucose transport and metabolism, erythropoiesis, inflammation, cellular invasion, and stress response. As far as erythropoiesis is concerned, hypoxia stimulates EPO production mainly in the adult kidneys and cell–cell contacts of erythroblasts and endothelial cells in the bone marrow through hypoxia-induced accumulation of HIF-2α. In the bone marrow, HIF-2α drives the expression of VCAM-1 and VLA-1 (α1β1 integrin), molecules that facilitates cell–cell contacts between stromal and hematopoietic progenitors, thus modulating bone marrow microenvironment and finally inducing erythropoiesis. Epo, erythropoietin; FIH, factor inhibiting HIF-1; HREs, hypoxia-responsive elements (TACGTG); p300/CBP, CREB-binding protein (CREB, CCAAT enhancer-binding protein-ε); PHD, propyl hydroxylases; VCAM-1, vascular adhesion molecule-1; VHL: von Hippel-Lindau tumor-suppressor protein; VLA-1, very late antigen-1.
Mutations in these genes that participate in hypoxia-regulated erythropoiesis further show the importance of the pathway in erythropoiesis. In Chuvash polycythemia patients, homozygous 598C>T mutation in VHL causes change in amino acid from arginine to tryptophan. This mutation diminishes the binding affinity of VHL for hydroxylated HIFα (Ang et al., 2002). As a consequence, the rate of HIF1-α degradation is reduced and resulting in increased expression of downstream targets, such as Epo, transferrin and VEGF. In familial polycythemia (erythrocytosis) and unrelated patients of erythrocytosis, mutations in EGLN1 gene which encodes for PHD2 were found (Al-Sheikh et al., 2008a; Al-Sheikh et al., 2008b; Eltzschig et al., 2009; Ladroue et al., 2008; Percy et al., 2007; Percy et al., 2006).

Later HIF-2α was identified, and studies demonstrated its important role in regulating Epo synthesis in mice and in humans as well (Rankin et al., 2007; Scortegagna et al., 2005). HIF-2α knockdown mice exhibited normocytic anemia. While Epo level is unaffected, hypoxia-inducible VCAM expression is regulated by HIF-2α. In HIF-2α knockdown mice, the defective erythropoiesis can be rescued by restoration of HIF-2α expression in endothelial cells. VCAM-1 is known to support the interaction of hematopoietic and endothelial cells in the bone marrow microenvironment and is required for maturation of erythroid cells. These data provided another insight of hypoxia-regulated erythropoiesis - hypoxia increases erythropoiesis by facilitating hematopoietic cells interact with stromal cells (Fandrey, 2008; Yamashita et al., 2008).

At the time I joined our lab (2005), our lab had identified that BMP4/Smad5 pathway regulates stress erythropoiesis. Upregulation of BMP4 in response to PHZ-induced acute anemia leads to expansion of stress BFU-E in mice spleen, which was not
seen in f/f mice that exhibit mutant splicing of Smad5. It was important to address how expression of BMP4 is upregulated in stress erythropoiesis. Regulation of BMP4 via a mechanism similar to the upregulation of Epo by HIF1α was hypothesized. While HIF1α did not give consistent results, HIF2α was also tested at time that its role in erythropoiesis just reported (Scortegagna et al., 2005). Our study links two important molecular events in stress erythropoiesis, BMP4 induction and hypoxia. Our data demonstrate that BMP4 is primarily regulated by HIF2 in murine spleen under hypoxia, and establishes the mechanism whereby BMP4-dependent stress erythropoiesis is initiated. Later mutations in HIF2 gene associated with erythrocytosis were reported (Percy, 2008; Percy et al., 2008a; Percy et al., 2008b; van Wijk et al., 2010), further showing essential role of HIF2 and also validating our findings. The study on regulation of BMP4 by hypoxia is described in Chapter 2 (Wu and Paulson, 2010).

Hedgehog Signaling in Hematopoiesis

In vertebrates, the Hedgehog signaling pathway is regulated by three ligands, Sonic hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) (Figure 1-11). In the absence of ligand, the pathway is inactive; the Hedgehog receptor Patched (Ptch) inhibits the activity of Smoothened (Smo). In this case, the downstream transcription factor Gli is prevented from entering the nucleus through interactions with cytoplasmic proteins. As a result, target genes are not activated. Activation of the pathway is initiated through binding of any of the three mammalian ligands to Ptch, resulting in de-repression of Smo. In this case Gli translocates to nucleus, which leads to activation of target genes,
including Ptch and Gli itself, as well as Hip, a Hedgehog binding protein that attenuates ligand diffusion and increases Hedgehog signaling.

Figure 1-11. Hedgehog signaling pathway. (Adapted from Nature Reviews Cancer 3: 903-911, 2003 (Pasca di Magliano and Hebrok, 2003))
The dissection of the biological role of Hedgehog signaling *in vivo* was initially limited due to embryonic lethality resulted from targeted disruption of its receptor Ptch and Smo (Goodrich et al., 1997). The function of Hedgehog in hematopoietic tissues remained undefined, and the studies were limited to *in vitro* analysis using cell lines. Bhardwaj *et al.* was the first to show that Shh, a Hedgehog ligand, and the receptors Smo and Ptch were expressed in the purified human primitive blood population (CD38− CD34+Lin− cells). They showed that Shh induced the expansion of human HSCs (CD38− CD34+Lin− cells) *in vitro* (Bhardwaj *et al.*, 2001). After this initial report, Martin *et al.* used zebrafish as a tool to study function of Hedgehog signaling in hematopoiesis. They showed that zebrafish embryos mutant in the Hedgehog signaling display defects in the adult HSC formation (Gering and Patient, 2005). Subsequently, availability of conditional alleles of genes in the Hedgehog signaling pathway allowed the studies on the functions of Hedgehog signaling in development, cancer and stem cells. Regarding the functions of Hedgehog signaling in hematopoiesis specifically, emerging reports have been published since my study began at the end of 2007, yet the conclusions have been contradictory. The studies and findings focused on the role of Hedgehog signaling in hematopoiesis using mouse model are summarized and outlined in Table 1-1. To date, these reports all focused on homeostatic hematopoiesis in bone marrow; among which a few also tested the aberrant Hedgehog signaling in response to 5-FU- induced myeloablation. However these studies lacked extensive and systematic analysis of stress hematopoiesis.
Table 1-1. Studies on Hedgehog in regulating hematopoiesis.

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<tr>
<th>Experimental Model</th>
<th>Experimental Assays</th>
<th>Phenotypes and Findings</th>
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<tr>
<td>1. Genetic model (gain-of-function): Ptc-1+/- from Goodrich et al, 1997.</td>
<td>Repopulation ability examined by transplantation ($10^5$ Lin’ bone marrow) to sublethally irradiated (350rads) NOD/SCID mice; analyzed at 5 and 8 weeks.</td>
<td>Under homeostatic conditions, increased Hedgehog activity had no effect on mature hematopoietic cells, however led to larger LSK compartment, more cycling (S+G2M) LSK cells and functional increase in progenitor (CFU) capacity.</td>
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<td>2. Pharmacological model (inhibition of function): i.p. injection of Hedgehog pathway inhibitor cyclopamine (25mg/kg of body weight) or DMSO twice per week before sacrificing mice (5-8 weeks).</td>
<td>Secondary repopulation with 2.5X donor-derived bone marrow to sublethally irradiated NOD/SCID mice; analyzed at 6 weeks.</td>
<td>Under stress, increase of Hedgehog activity increased short-term (at 5weeks) repopulation capacity (% donor cells) and cycling (S+G2M) Sca1+ cells; yet eventually lost these abilities (at 8weeks), and incapable of regenerating hematopoiesis (CFU) after 6 weeks upon 2nd challenge.</td>
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<tr>
<td>3. Model to induce hematopoietic stress: (1) 150ug of 5-FU per gram of weight (single dose); (2) irradiation.</td>
<td>CFU assay with donor-derived bone marrow from recipients 8weeks post transplantation.</td>
<td>Administration of cyclopamine complemented the long-term (at 8weeks) functional defects in Ptc-1+/- mice.</td>
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<td>4. Cell cycle analysis on donor-derived Lin’ bone marrow from mice i.p. injected with BrdU (2mg) at 12 and 24h before sacrifice.</td>
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<td>5. Stress model (5-FU): leukocyte counts analyzed before, 24h and 9days after injection.</td>
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Hedgehog signaling is dispensable for adult murine hematopoetic stem cell function and hematopoiesis. (Cell Stem Cell, 2009) (Hofmann et al., 2009)

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<tr>
<th>Experimental Model</th>
<th>Experimental Assays</th>
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<tbody>
<tr>
<td>1. Smo² f/f (Long et al., 2001)</td>
<td>1. Analyzed from conditional knockout mice and controls at 1-18 months.</td>
<td>1. Loss of Smo has no effect on the followings: terminally differentiated hematopoietic cells, LSK cells, progenitor cells, bone marrow repopulation, thymocyte development.</td>
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<tr>
<td>2. Cre system for conditional Knockout: Interferon-inducible Mx1-Cre transgenic mice; Cre was induced with poly(I)poly(C) (start at ~10 weeks after birth, and every 2 days for totally 3 injections).</td>
<td>2. Repopulation ability examined by competitive transplantation to lethally irradiated mice (1:0, 3:1, 1:1, 1:3, and 0:1, 2x10⁶ in total); analyzed at 4, and 12 weeks.</td>
<td>2. Hedgehog signaling is dispensable under hematopoietic stress, and for MLL-AF9-mediated leukemogenesis.</td>
</tr>
<tr>
<td>3. Model to induce hematopoietic stress: 150µg of 5-FU per gram of weight (3 weekly i.p. injection).</td>
<td>3. Stress model (5-FU): survival and CBC analyzed at day 5, 10, and 15 post induction.</td>
<td>3. HhAntag or rshh has no effect on colony-forming potential and blood cells counts.</td>
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<td>4. In vitro study: colony assays after culture of bone marrow with inhibitor HhAntag or rshh agonist.</td>
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**Hedgehog signaling is dispensable for adult hematopoietic stem cell function.** (Cell Stem Cell, 2009) (Gao et al., 2009)

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<tr>
<th>Experimental Model</th>
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<tr>
<td>1. <em>Smo</em>^f/f^ (Long et al., 2001); R26SmoM2 where enhanced YFP was fused with the constitutively active <em>SmoW539L</em>.</td>
<td>1. Analyzed from conditional knockout mice and controls at 4-6 weeks.</td>
<td>1. Loss of Smo has no effect on the followings: LSK cells, progenitor cells, bone marrow repopulation, HSC self-renew, apoptosis, expression of HSC-specific genes in LSK and MPPs.</td>
</tr>
<tr>
<td>2. Cre system for conditional Knockout: Interferon-inducible Mx1-Cre transgenic mice; Cre was induced with poly(I)poly(C) (start at 14 days after birth and every 2 days for totally 3 injections).</td>
<td>2. Repopulation ability examined by competitive transplantation to lethally irradiated mice.</td>
<td>2. Hyperactivation of Hedgehog pathway does not expand HSC compartment.</td>
</tr>
<tr>
<td>3. Model to induce hematopoietic stress: 150ug of 5-FU per gram of weight (3 weekly i.p. injection).</td>
<td>3. (5x10^5 bone marrow or 500LSKs + 5x10^5 wildtype bone marrow); analyzed at 4, 8, and 12 weeks.</td>
<td>3. Hedgehog signaling is dispensable for maintenance or induction of T-cell acute lymphoblastic leukemia.</td>
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<td>4. Stress model (5-FU): survival analyzed every 5 days for 23 days post induction.</td>
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The Hedgehog receptor Patched controls lymphoid lineage commitment. (Blood, 2007) (Uhmann et al., 2007)

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<th>Experimental Model</th>
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| 1. *Ptch* 
  generated. | 1. Analyzed from 
  conditional knockout mice and 
  controls (vehicle-treated *Ptch* 
  ERT2+/− and tamoxifen-treated 
  *Ptch* 
  ERT2−/−) at 19 days post 
  injection. | 1. Lin− bone marrow 
  cells from *Ptch*−/− had no 
  defects in myeloerythroid 
  colony-forming (CFU-GEMM, 
  CFU-GM, CFU-G, CFU-M, and 
  BFU-E); Similar blood 
  parameters except 
  neutrophils and lymphocytes. |
| 2. Cre system for 
  conditional Knockout: 
  tamoxifen-inducible 
  Rosa26CreERT2+/− transgenic mice; Cre 
  was induced with 
  tamoxifen (i.p. 1mg for 
  5days). | | 2. Thymic atrophy and 
  defective T-cell development. |
| | | 3. Loss of splenic B cells 
  (CD24−CD21+), block of B-lineage 
  commitment prior to 
  pro-B cell stage in bone 
  marrow. |
| | | 4. Increase of HSC-containing Lin−Kit hi Sca1 hi population and myeloid 
  progenitor- containing Lin− Kit hi Sca1 -/lo population, 
  decrease of CLP-containing 
  Lin−Kit lo Sca1 -/lo population in 
  *Ptch*−/−. |
The Hedgehog receptor Patched1 regulates myeloid and lymphoid progenitors by distinct cell-extrinsic mechanisms. (Blood, 2009) (Siggins et al., 2009)

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<tr>
<td>1. Ptch&lt;sup&gt;ff&lt;/sup&gt; (Ellis et al., 2003)</td>
<td>1. Analyzed from conditional knockout mice and controls.</td>
<td>1. Apoptosis of B- and T-cell progenitors in MxPtch&lt;sup&gt;-/-&lt;/sup&gt; mice.</td>
</tr>
<tr>
<td>2. Cre system for conditional Knockout: Interferon-inducible Mx1-Cre transgenic mice; Cre was induced with poly(I)poly(C) (every 2 days for totally 3 injections); MxPtch&lt;sup&gt;-/-&lt;/sup&gt; mice were examined at 4-6 weeks post poly(I)poly(C) injection.</td>
<td>2. Repopulation ability examined by competitive transplantation to lethally irradiated mice (2x10&lt;sup&gt;6&lt;/sup&gt; bone marrow + 2x10&lt;sup&gt;6&lt;/sup&gt; wildtype bone marrow); analyzed at 4, 8, and 12 weeks.</td>
<td>2. Bone marrow cells from MxPtch&lt;sup&gt;-/-&lt;/sup&gt; had no defects in myelerythroid colony-forming (granulocyte, Macrophage, GM, megakaryocyte, GEMM, blast); similar blood parameters except neutrophils.</td>
</tr>
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<td>3. HSCs-specific deletion by tamoxifen-inducible Scl-CreER; Cre was induced with tamoxifen (i.p. 10mg for 3days).</td>
<td>3. increased of HSC-containing Lin&lt;sup&gt;hi&lt;/sup&gt;Kit&lt;sup&gt;hi&lt;/sup&gt;Sca1&lt;sup&gt;hi&lt;/sup&gt; population, and increased cycling of LKS cells.</td>
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<td>4. B cell-specific deletion by CD19Cre.</td>
<td>4. MxPtch&lt;sup&gt;-/-&lt;/sup&gt; had no defect in repopulating ability (4 &amp; 16 weeks) in both 1&lt;sup&gt;st&lt;/sup&gt; and 2&lt;sup&gt;nd&lt;/sup&gt; transplantation.</td>
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<td>5. T cell-specific deletion by LckCre.</td>
<td>5. Hematopoietic abnormalities in MxPtch&lt;sup&gt;-/-&lt;/sup&gt; are cell-extrinsic due to: (1) No defects in hematopoietic-specific knockout mice but similar or stronger defects to MxPtch&lt;sup&gt;-/-&lt;/sup&gt; in K14Ptch&lt;sup&gt;-/-&lt;/sup&gt;; and (2) MxPtch&lt;sup&gt;-/-&lt;/sup&gt; mice reconstituted with wildtype BONE MARROW display more LSK and reduced pre-B cell numbers compared to wilttype mice reconstituted with MxPtch&lt;sup&gt;-/-&lt;/sup&gt; bone marrow.</td>
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Gli regulates the proliferation and differentiation of hematopoietic stem cell and myeloid progenitors. (Blood, 2010) (Merchant et al., 2010)

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<tr>
<td>1. <em>Gli1</em>&lt;sup&gt;−/−&lt;/sup&gt; (<em>Gli&lt;sub&gt;LacZ/LacZ&lt;/sub&gt;</em>)</td>
<td>1. Analyzed from knockout mice and controls.</td>
<td>1. No difference in the blood parameters.</td>
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<tr>
<td>2. Model to induce hematopoietic stress: 150ug of 5-FU per gram of weight</td>
<td>2. Repopulation ability examined by competitive transplantation to lethally irradiated mice (500 KSL + 2.5x10&lt;sup&gt;5&lt;/sup&gt; wildtype bone marrow); analyzed at 4, 8, and 12 weeks.</td>
<td>2. Reduced number and size of CFU-Granulocytes from <em>Gli1&lt;sup&gt;−/−&lt;/sup&gt;</em> bone marrow.</td>
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<td>3. Increased FcR&lt;sub&gt;γ&lt;/sub&gt;&lt;sup&gt;lo&lt;/sup&gt;CD34&lt;sup&gt;−&lt;/sup&gt;Kit&lt;sup&gt;−&lt;/sup&gt;Sca1&lt;sup&gt;−&lt;/sup&gt;Lin&lt;sup&gt;−&lt;/sup&gt; (CMP) and decreased FcR&lt;sub&gt;γ&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;CD34&lt;sup&gt;−&lt;/sup&gt;Kit&lt;sup&gt;−&lt;/sup&gt;Sca1&lt;sup&gt;−&lt;/sup&gt;Lin&lt;sup&gt;−&lt;/sup&gt; (GMP), suggesting loss of <em>Gli1</em> impairs myeloid differentiation.</td>
<td>3. Increased FcR&lt;sub&gt;γ&lt;/sub&gt;&lt;sup&gt;lo&lt;/sup&gt;CD34&lt;sup&gt;−&lt;/sup&gt;Kit&lt;sup&gt;−&lt;/sup&gt;Sca1&lt;sup&gt;−&lt;/sup&gt;Lin&lt;sup&gt;−&lt;/sup&gt; and decreased FcR&lt;sub&gt;γ&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;CD34&lt;sup&gt;−&lt;/sup&gt;Kit&lt;sup&gt;−&lt;/sup&gt;Sca1&lt;sup&gt;−&lt;/sup&gt;Lin&lt;sup&gt;−&lt;/sup&gt;, suggesting loss of <em>Gli1</em> impairs myeloid differentiation.</td>
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<td>4. <em>Gli1&lt;sup&gt;−/−&lt;/sup&gt;</em> HSCs and progenitor cells are less proliferative (BrdU-positive).</td>
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<td>5. After 5-FU treatment, delayed recovery in neutrophil and platelet count (within 2weeks period).</td>
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<td>6. 4 months post transplantation, higher percentage of donor chimerism, increased LT-HSC in mutant mice.</td>
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**Hedgehog signaling is essential for maintenance of cancer stem cell in myeloid leukemia.** (Nature, 2009) (Zhao et al., 2009)

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<tr>
<td>1. Smo&lt;sup&gt;fl&lt;/sup&gt; (Long et al., 2001).</td>
<td>1. Analyzed from conditional knockout mice and controls at 8-10 weeks.</td>
<td>1. Loss of Smo does not alter the frequency of hematopoietic lineages and Lin&lt;sup&gt;-/lo&lt;/sup&gt; Kit&lt;sup&gt;-&lt;/sup&gt; Sca1&lt;sup&gt;-&lt;/sup&gt; Flk2&lt;sup&gt;+&lt;/sup&gt; cells and homing (6h post transplantation).</td>
</tr>
<tr>
<td>2. Cre system for conditional Knockout: Vav-Cre (Cre driven by Vav regulatory element) transgenic mice.</td>
<td>2. Repopulation ability examined by competitive transplantation to lethally irradiated mice.</td>
<td>2. Loss of Smo impaired long-term (24weeks) HSCs repopulation in 1&lt;sup&gt;st&lt;/sup&gt; and 2&lt;sup&gt;nd&lt;/sup&gt; transplantation.</td>
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<td>3. (2x10&lt;sup&gt;5&lt;/sup&gt; bone marrow + 2x10&lt;sup&gt;5&lt;/sup&gt; wildtype bone marrow; analyzed at 7 weeks; 500 Lin&lt;sup&gt;-/lo&lt;/sup&gt; Kit&lt;sup&gt;-&lt;/sup&gt; Sca1&lt;sup&gt;-&lt;/sup&gt; Flk2&lt;sup&gt;+&lt;/sup&gt; + 2x10&lt;sup&gt;5&lt;/sup&gt; wildtype; analyzed at 24weeks).</td>
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Expansion of Bcr-Abl-positive leukemic stem cell is dependent on Hedgehog pathway activation. (Cancer Cell, 2008) (Dierks et al., 2008)

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<th><strong>Phenotypes and Findings</strong></th>
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<tr>
<td>1. Mutants generated by transplanting fetal liver cells at embryonic day14.5 (<em>Ptch1</em>+/−, <em>Ptch1</em>−/−, <em>Smo</em>+/−, <em>Smo</em>−/−).</td>
<td>1. Analyzed from fetal livers of mutants and controls at embryonic day14.5.</td>
<td>1. No difference in the number of fetal liver HSCs and lineage populations between different genotypes.</td>
</tr>
<tr>
<td>3. Repopulation ability examined by transplantation (fetal liver cells at e.d14.5) to sublethally irradiated mice; analyzed at 3,6,10, and 13weeks.</td>
<td>3. Enhanced repopulation ability in <em>Ptch1</em>−/− fetal liver cells yet similar in <em>Smo</em>−/− compared to controls (no growth after replating in both cases).</td>
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</tr>
<tr>
<td>4. Stress model (5-FU): survival and CBC analyzed at day10 and day40 post induction.</td>
<td>4. Lineage distribution was affected in mutant (<em>Ptch1</em>−/−, <em>Smo</em>−/−) recipients at 10 weeks post transplantation.</td>
<td>4. Lineage distribution was affected in mutant (<em>Ptch1</em>−/−, <em>Smo</em>−/−) recipients at 10 weeks post transplantation.</td>
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</table>
| 5. Short-term (10 days post 5-FU inj.) but not long-term (40 days) regeneration capacity was reduced in *Smo*−/− transplants. Both short-term and long-term (more than 4 months) regeneration capacities were enhanced in *Ptch1*−/− transplants. | | }
Researchers have used different approaches to examine the effects of aberrant Hedgehog signaling. As listed in Table 1-1, these approaches included analysis of Ptch heterozygotes, conditional knockout of Smo and Ptch using Cre recombinases expressed from several different promoters, knockout of downstream factor Gli1, the constitutively active form of Smo, pharmacological inhibitors, or usage of fetal liver cells from mutants. These groups used various assays to dissect functions of HSCs in their mutant models. Some phenotypical studies included analysis of stem cell and/or progenitor populations in bone marrow, colony forming ability (CFU) of bone marrow cells, and peripheral blood counts directly from mutants to study role of Hedgehog signaling in homeostatic state. Some groups performed functional examinations, which included repopulation ability followed by bone marrow transplantation into irradiated recipients, serial transplantation or serial colony plating assays. A few groups also did cell cycle analysis. Some studies showed that Hedgehog signaling was essential to hematopoiesis such as regulating proliferation of hematopoietic stem cell (HSCs) and lineage commitment; whereas other studies showed that it is dispensable for normal adult hematopoiesis. The different findings among groups could be due to disruption of specific components of the pathway, which may not show complete phenotypes. There are complex interactions and feedback regulations among ligands, receptors and the downstream transcription factors in Hedgehog signaling pathway. In addition, crosstalk between signaling and redundancy could also contribute to the range of experimental results. Despite this fact, discordant phenotypes were still observed in the same mutant genotypes. One major cause of these contradictory observations could be the experimental strategies. The study methods and these possibilities are discussed below.
Ptch mutants. Ptch has been examined using Ptch<sup>fl/fl</sup> mice generated from tamoxifen-treated Ptch<sup>fl/fl</sup>CreERT2<sup>+/−</sup> (Uhmann et al., 2007), or from poly(I)poly(C)-treated Mx1CrePtch<sup>fl/fl</sup> (Interferon-inducible) (Siggins et al., 2009), Ptch<sup>+/−</sup> mouse (Trowbridge et al., 2006), and Ptch<sup>+/−</sup> fetal liver cells (embryonic day14.5) (Dierks et al., 2008). These experiments addressed the effects of hyperactivation of Hedgehog signaling. No defects in mature lineage populations were seen in Ptch<sup>+/−</sup> fetal liver cells and Ptch<sup>+/−</sup> mice (Dierks et al., 2008; Trowbridge et al., 2006), but increased neutrophil counts was observed in both Ptch<sup>−/−</sup> Mx1Cre and Ptch<sup>−/−</sup>CreERT2<sup>−/−</sup> models (Siggins et al., 2009; Uhmann et al., 2007). Increased LSK (Lin+Sca1<sup>+</sup>Kit<sup>+</sup>) cells and increased cycling of this population were observed in Ptch<sup>+/−</sup>- and Ptch<sup>−/−</sup>- mice (Siggins et al., 2009; Trowbridge et al., 2006; Uhmann et al., 2007), but not in Ptch<sup>+/−</sup>- fetal liver cells (Dierks et al., 2008), which may reflect the differences between fetal liver and adult HSCs. Interestingly, repopulation activity used to examine function of HSC was increased in Ptch<sup>+/−</sup>- fetal liver cells and Ptch<sup>+/−</sup>- mice compared to controls (Dierks et al., 2008; Trowbridge et al., 2006), but not the homozygous deleted Ptch<sup>−/−</sup>- mice (Siggins et al., 2009; Uhmann et al., 2007). In my analysis focus on role of Hedgehog signaling in stress erythropoiesis, as described in Chapter 3, we actually observed faster recovery and less severe anemia and better recovery in all blood lineages than controls in Ptch<sup>−/−</sup>- recipients during the one month recovery period post transplantation. Several groups including ours directly transplanted mutant cells into irradiated mice to examine their own repopulation activity; whereas Siggins et al. co-transplanted equal amount (2x10<sup>6</sup>) of wildtype bone marrow cells, which would show non-autonomous defects. In the Ptch<sup>+/−</sup> model, the increased repopulation activity observed at 5 weeks post transplantation ended up in exhaustion at 8 weeks.
(Trowbridge et al., 2006). However, this long-term exhaustion was not seen in the Ptch
fetal liver cell model during the course of observation (3-13 weeks) (Dierks et al., 2008). In terms of lineage specification, Ptch-CreERT2 model showed that Hedgehog signaling controls lymphoid lineage commitment (Uhmann et al., 2007), and the Ptch
fetal liver recipients also showed changed lineage distribution post transplantation to wildtype recipients (Dierks et al., 2008). However, other studies did not suggest function of Ptch in regulating lineage commitment.

**Smo mutants.** The phenotypes resulting from loss of Smo were even more contradictory among studies. Some reported that loss of Smo led to severe defects in HSC function, while some reported modest effects or none at all. Smo-generated from poly(I)poly(C)-treated Mx1CreSmo
(Interferon-inducible) (Gao et al., 2009; Hofmann et al., 2009), or from VavVreSmo
, and Smo-
fetal liver cells (embryonic day14.5) (Dierks et al., 2008) were used to address the effects of inhibiting Hedgehog signaling. Dierks et al. did not observe differences in HSC or lineages in Smo-
fetal liver cells compared to controls. This piece of data was consistent with the reports from Gilliland Lab and Aifantis Lab, where both groups showed no defects in terminally differentiated hematopoietic cells, LSK cells, and progenitor cells in Smo-
mice (Dierks et al., 2008; Gao et al., 2009; Hofmann et al., 2009). Indeed, we also found that inhibiting Hedgehog signaling with a chemical inhibitor had no effect on homeostatic hematopoiesis, as described in Chapter 3. However, effects of mutant Smo in other facets were inconclusive. 3,6,10 and 13 weeks post transplantation, Smo-
fetal liver cells had similar repopulation ability to controls,
however the distribution of lineages was altered in the peripheral blood (Dierks et al., 2008). This finding by Dierks et al. suggested that inhibiting Hedgehog signaling affected lineage specification. Using competitive repopulation strategy, Zhao et al. showed results opposite to both Gilliland Lab and Aifantis Lab’s data. While Zhao et al. found decreased long-term (24 weeks) repopulation activity in Smo−/− (Zhao et al., 2009), the other two groups did not see difference within 4-12 weeks (Gao et al., 2009; Hofmann et al., 2009). One difference among these groups was the Cre transgenic mice used for conditionally knocking out Smo. Zhao et al. used Vav-Cre system in which Vav expresses mostly in the hematopoietic system, including HSC-enriched fractions. Progeny from this cross with it would be expected to delete the floxed gene predominantly in hematopoietic system (Almarza et al., 2004). Although by using various Cre systems, Siggins et al. concluded that the hematopoietic abnormalities in MxPtch−/− are cell-extrinsic since no defects was seen in hematopoietic- specific knockout of Ptch mutants (Siggins et al., 2009). Whether different Cre models are comparable is unclear. In addition, one recent study showed that interferon-α (IFNα) activates dormant hematopoietic stem cells in vivo (Essers et al., 2009). This paper showed that in response to treatment of IFN-α, HSCs entered an active cell cycle, increased phosphorylation of STAT1 and PKB/Akt, expressed IFN target genes and up-regulated Sca1. While chronic activation of the IFN pathway in HSCs impairs their function, acute IFNα treatment promotes the proliferation of dormant HSCs in vivo. The Mx1-Cre is interferon-inducible; whether the interplay between interferon and activation of dormant HSCs could affect the analysis of Hedgehog signaling using interferon-inducible Cre has not yet been studied.
Gli1\textsuperscript{null}. Like most Ptch and Smo mutant models, there was no difference in blood counts in Gli1\textsuperscript{null} mice compared to wild type (Merchant et al., 2010). Consistent with some findings in Ptch mutants where LSK cells and colony forming ability are increased; bone marrow of Gli1\textsuperscript{null} mice had less proliferative HSCs and progenitors. In addition, Gli1\textsuperscript{null} bone marrow formed less and smaller CFU-Granulocytes, showing that Gli1 regulates myeloid lineage commitment under homeostatic condition. One caveat of addressing role of Hedgehog signaling by using Gli1\textsuperscript{null} mutants is that the downstream target, Gli1, is functionally redundant. Three Gli factors, Gli1, Gli2, as well as Gli3, function downstream of Smo (Riobo and Manning, 2007; Taipale and Beachy, 2001). The role of Gli factors in Hedgehog signaling is complex. In some cases, Gli1 is induced by Hedgehog, whereas in some case Gli2 is the main factor and Gli1 get induced later. Furthermore, in some cases like kidney development, Hedgehog signaling is required to eliminate Gli3 repression but not Gli1/2 activation.

5-FU hemoablation as a model for stress hematopoiesis. To date, all studies on role of Hedgehog signaling in stress condition were addressed by 5-Flourouracil (5-FU) hemoablation model. 5-FU model is often used to test the short-term regeneration capacity. Upon intraperitoneally injection of 5-FU into mice, the cycling cells are depleted and quiescent stem cells are forced to proliferate and repopulate the marrow. The two groups who concluded that Hedgehog signaling is dispensable for HSC function
did not see effects of defective Hedgehog signaling in 5-FU model either because they did not observe significantly difference between mutants and controls (Gao et al., 2009; Hofmann et al., 2009). Both groups utilized weekly injection of 5-FU, and actually neither controls nor mutants survived after 2 weeks (Hofmann et al., 2009) or 3.5 weeks (Gao et al., 2009). However, another three groups using different genetic models showed defects. Recipients of Smo⁻/⁻ fetal liver cells had less repopulation (% of donor-derived cell, by the group’s definition) at day10 post injection (however similar to controls afterwards); Gli1null mice exhibited slightly delayed recovery in neutrophil and platelet lineages (Dierks et al., 2008; Merchant et al., 2010). In contrast, Ptc+/- mice had more leukocytes and recipients of Ptc⁻/- fetal liver cells increased repopulation (% of donor-derived cell, by the group’s definition) upon stress induction (Dierks et al., 2008; Trowbridge et al., 2006). In my studies on role of Hedgehog in regulating stress hematopoiesis with focus on erythropoiesis, stress hematopoiesis was examined by analyzing short-term radioprotection. Opposite responses in Ptc⁻/- and Smo⁻/- transplants were observed. Similar to these three studies, our research demonstrated that Hedgehog plays essential roles in stress condition. The analyses of progenitor cells and the kinetics of their expansion and differentiation are described in Chapter 3.

In summary, several possibilities could explain the controversial role of Hedgehog signaling in hematopoiesis. The complex regulation of the pathway, difference in the properties of fetal and adult bone marrow cells and their studies in the context of adult environment, the use of different Cre recombinase systems, and the design of assays may lead to different conclusions. In my study which focused on stress erythropoiesis,
two genetic models and a pharmacological strategy were used. The data were consistent among these models, as described in detail in Chapter 3.

**Hedgehog signaling in stress erythropoiesis**

The above described studies focused on the role of Hedgehog signaling in the regulation of HSCs and steady state hematopoiesis. Work in our lab has demonstrated a role of Hedgehog in stress erythropoiesis (Perry et al., 2009a). We previously identified that BMP4 signaling promote differentiation and expansion of stress BFU-Es (Lenox et al., 2005b). However, acute anemia leads to an almost complete mobilization of these stress BFU-Es, suggesting that new stress progenitors are recruited to replenish stress erythroid progenitors in the spleen (Perry et al., 2009a). In search of the signals that are required for the maintaining BMP4-dependent stress erythropoiesis, our lab tested Hedgehog signaling. Overnight pre-incubation of wildtype bone marrow cells with Sonic Hedgehong (Shh) results in increase of stress BFU-E colonies. BMP4 mRNA expression is found induced after 2hrs of Shh treatment. Notably, bone marrow cells from Smo conditional knockout are defective in forming BMP4-responsive BFU-Es, showing that bone marrow cells fail to respond to BMP4 unless they encounter Hedgehog first. Smo conditional knockout mice treated with PHZ recovered normally, however none of the mice survived post 48 hours after the second challenge of PHZ. These data showed that Smo conditional knockout mice could not replenish stress erythroid progenitors. This previous study demonstrates that maintenance of BMP4- dependant stress erythropoiesis
requires Hedgehog signaling. Taken together with other previous findings from our lab, a model for the role of Hedgehog signaling in regulating BMP4-dependent stress erythropoiesis was proposed, as shown in Figure 1-12. My work which delineates how Hedgehog signaling regulates stress erythropoiesis is described in Chapter 3.

Figure 1-12. Model for the role of Hedgehog in the recovery of the BMP4-dependent stress erythropoiesis pathway in the spleen. (Adapted from Blood 113(4): 911-918, 2009)(Perry et al., 2009a)
References


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Chapter 2

Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia

Forward

The following chapter is adapted from the manuscript appearing in Wu, D.-C., and Paulson, R.F. Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia. PLoS One 5(6):e11303 (2010). This paper was written with Dr. Robert Paulson.

Abstract

Background: Bone marrow erythropoiesis is primarily homeostatic, producing new erythrocytes at a constant rate. However at times of acute anemia, new erythrocytes must be rapidly produced much faster than bone marrow steady state erythropoiesis. At these times stress erythropoiesis predominates. Stress erythropoiesis occurs in the fetal liver during embryogenesis and in the adult spleen and liver. In adult mice, stress erythropoiesis utilizes a specialized population of stress erythroid progenitors that are resident in the spleen. In response to acute anemia, these progenitors rapidly expand and differentiate in response to three signals, BMP4, SCF and hypoxia. In absence of acute anemic stress, two of these signals, BMP4 and hypoxia, are not present and the pathway...
is not active. The initiating event in the activation of this pathway is the up-regulation of BMP4 expression in the spleen.

**Methodology/Principal Findings:** In this paper we analyze the regulation of BMP4 expression in the spleen by hypoxia. Using stromal cell lines, we establish a role for hypoxia transcription factor HIFs (Hypoxia Inducible Factors) in the transcription of BMP4. We identified putative Hypoxia Responsive Elements (HREs) in the BMP4 gene using bioinformatics. Analysis of these elements showed that in vivo, Hif2α binds two cis regulatory sites in the BMP4 gene, which regulate BMP4 expression during the recovery from acute anemia.

**Conclusions and Significance:** These data show that hypoxia plays a key role in initiating the BMP4 dependent stress erythropoiesis pathway by regulating BMP4 expression.
Introduction

Acute blood loss leads to tissue hypoxia, which induces a systemic response designed to increase oxygen availability to the tissues. Increased erythropoiesis is part of this response. Under steady state conditions, the bone marrow produces new erythrocytes at a constant rate to maintain homeostasis. In response to acute anemia stress, new erythrocytes must be rapidly produced. At these times stress erythropoiesis is the predominant form of erythropoiesis (Socolovsky, 2007). Stress erythropoiesis relies on a specialized population of stress erythroid progenitors that are primarily resident in the spleen (Lenox et al., 2005a). These cells possess the ideal properties of stress response cells in that they are rapidly mobilized in response to acute anemia and are able to generate larger numbers of new erythrocytes much faster than bone marrow steady state erythroid progenitors (Lenox et al., 2005a; Perry et al., 2007a).

Three signals regulate the expansion of stress erythroid progenitors in the spleen, BMP4, SCF and hypoxia (Perry et al., 2007a). BMP4 acts on an immature cell, the BMP4 responsive cell (BMP4*R), which causes it to differentiate into stress BFU-E. BMP4 also acts in concert with SCF and hypoxia to promote the proliferation and differentiation of stress BFU-E. Hypoxia plays a key role in this process by altering the response of progenitor cells to the other signals, which maximizes the expansion and differentiation of stress erythroid progenitors (Perry et al., 2007a). Acute anemia results in the complete mobilization of stress progenitors in the spleen. Following recovery, these progenitors are replenished by bone marrow cells that migrate into the spleen. Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) in the spleen induce the bone marrow progenitor cells to adopt
the stress erythroid progenitor cell fate, which makes them competent to respond to BMP4 in response to acute anemia (Perry et al., 2008).

The BMP4 dependent stress erythropoiesis pathway has the potential to rapidly produce large numbers of new erythrocytes. Inappropriate activation of this pathway could result in polycythemia and lead to pathological consequences. However in the absence of anemic stress, this pathway is quiescent. Two levels of control maintain the pathway in the inactive state. Our previous work demonstrated that three signals are required for the expansion of stress progenitors, BMP4, SCF and hypoxia. Of these three signals only SCF is constitutively expressed in the spleen (Perry et al., 2007a). Tissue hypoxia is present only in response to anemia, and BMP4 expression is also limited to times of anemia. In our original analysis of this pathway, we proposed that BMP4 may be regulated by hypoxia (Lenox et al., 2005a). This hypothesis would support the idea that anemic stress leading to tissue hypoxia would regulate two of the three signals needed for the expansion and differentiation of stress erythroid progenitors.

Hypoxia regulates gene expression primarily through the action of a family of transcription factors referred to as Hypoxia Inducible Factors or HIFs (for review see (Nakayama, 2009; Semenza, 2009; Simon and Keith, 2008)). These transcription factors are made up of two subunits, an α subunit (Hif1α, Hif2α or Hif3α) which is stable under hypoxic conditions but rapidly degraded at normal O2 levels, and a β subunit (Hifβ or ARNT) that is unaffected by changes in O2 concentration. The HIF complex binds to a Hypoxia responsive element (HRE), where it recruits co-activators p300/CBP to promote gene transcription (Arany et al., 1996). At normal levels of O2, the α subunits are hydroxylated on a proline residue by a family of proline hydroxylases (PHDs) (Epstein et
al., 2001; Ivan et al., 2001; Kaelin, 2005; Maxwell et al., 1999). The hydroxylated proline is recognized by the product of the Von Hippel Lindau tumor suppressor gene, VHL, which targets the protein for ubiquitination and destruction. At low levels of $O_2$, the PHDs are inhibited and the $\alpha$ subunits are stable. The interaction of HIF with co-activators is also regulated by $O_2$ concentration. FIH-1 is an asparaginyl hydroxylase which functions at moderate to high $O_2$ levels (Hewitson et al., 2002; Lando et al., 2002; Mahon et al., 2001). Asparagine hydroxylated HIF is stable, but cannot bind co-activators, which allows for fine tuning of the hypoxia response. Hif1$\alpha$(Iyer et al., 1998; Yoon et al., 2006) and Hif2$\alpha$(Gruber et al., 2007; Scortegagna et al., 2005; Scortegagna et al., 2003) have been shown to be involved in the regulation of erythropoiesis. The analysis of targeted mutations of Hif1$\alpha$ or Hif2$\alpha$ or in genes that affect the stability of these molecules (VHL (Hickey et al., 2007) and PHD2 (Minamishima et al., 2008)) showed that these mutations cause defects in murine erythropoiesis. The central role of this pathway in regulating erythropoiesis was further underscored by the identification of patients with erythrocytosis that have mutations in PHD2 (Percy et al., 2006) and HIF2 $\alpha$(Percy et al., 2008a; Percy et al., 2008b; van Wijk et al., 2009) and the demonstration that Chuvash Polycythemia was caused by mutations in VHL (Ang et al., 2002; Gordeuk et al., 2004).

In this report, we have characterized the hypoxia dependent regulation of BMP4 expression during the recovery from acute anemia. We demonstrate that BMP4 expression is regulated at the transcriptional level by HIF. Using bioinformatics we identified five putative HREs and show that two of these potential regulatory sequences
are bound by HIF *in vitro* and *in vivo*. Our data also show that *in vivo* during the recovery from acute anemia, Hif2α is the primary regulator of BMP4 expression in spleen.
Study Design

Cell culture, mice and phenylhydrazine treatment

Murine spleen stromal cell line MSS31 (Yanai et al., 1989) (Japan Health Science Foundation, Health Science Research Resources Bank, Osaka Japan) was cultured in Gibco IMDM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), Penicillin-Streptomycin, 10ug of transferrin, 0.2g of BSA, 1mg of insulin, and 10ug of EGF. Murine bone marrow stromal cell line W2017 (Binnerts et al., 2004) (American Type Culture Collection, Manassas, VA) was cultured in Gibco DMEM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), Penicillin-Streptomycin, and 1.5g of Na$_2$CO$_3$. Murine fetal liver stromal cell line AFT024 (Moore et al., 1997) (American Type Culture Collection, Manassas, VA) was cultured in Gibco DMEM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), Penicillin-Streptomycin, 1.5g of Na$_2$CO$_3$, and 0.05mM beta-mercaptoethanol. HEK293T cells were cultured in Gibco DMEM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), and Penicillin-Streptomycin. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were approximately 6 to 8 weeks old, and controls were matched for gender and age. Acute anemia was induced by injection of phenylhydrazine (Sigma, St Louis, MO) at the concentration of 100 mg/kg mouse in phosphate-buffered saline (PBS) buffer. All procedures using mice were approved by the IACUC of the Pennsylvania State University (IACUC Protocol #30584).
Transfection of shRNA

shRNA plasmids target for Hif1α, Hif2α, GATA2 (served as control) (TCR3-54449, TCR3-54450, TCR3-82303, TCR3-82306, TRC5-85419, Open Biosystem, Huntsville, AL) were transfected into HEK293T cells with TransIT®-293 transfection reagent (Mirus, Madison, WI).

Prediction of potential HREs (HIF responsive elements)

Mouse BMP4 DNA sequence with 5000 extra bp upstream and 5000 extra bp downstream was gained from UCSC Genome Browser created by the Genome Bioinformatics Group of UC Santa Cruz, and analyzed with transcription factor analysis program MatInspector (Quandt et al., 1995). The acquired sequences data were as of Sep, 2005. The sequences of the predicted HREs were then aligned and compared the conservation among mammal species for further selection of candidates.

RT-PCR

Total RNA isolated from cell lines or spleen cells was homogenized in TriZol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA by using SuperScriptII system (Invitrogen, Carlsbad, CA). BMP4 gene expression was determined with primers 5’-CCTGGTAACCGAATGCTGAT-3’ and 5’-TGTGATGAGGTGTCCAGGAA-3’; β-actin gene expression was determined with primers 5’-AGCCATGTACGTAGCCATCC-3’ and 5’-TTTGATGTCACGCACGATTT-3’; 28s rRNA gene expression was determined with primers 5’-TTGAAAATCCGGGGGAGAG-3’ and 5’-
ACATTGTCCAACATGCCAG-3’. Relative quantification of BMP4 expression was also determined by TaqMan probe using 18s rRNA as the internal control (Applied Biosystems, Foster City, CA).

**Chromatin immunoprecipitation (ChIP) assay**

Spleens were diced into small pieces and resuspended in phosphate-buffered saline (PBS) containing freshly added Complete Protease Inhibitor Cocktail (Roche, Indianapolis, USA) and homogenized. Cross-linking of proteins to the DNA of cells (isolated from spleen or cell culture) was achieved by adding formaldehyde to a final concentration of 1% for 15 min at 37°C with occasional inversion. Glycine was then added to a final concentration of 0.25M, and the reaction was incubated at room temperature for 10 min with occasional inversion. Cells then were washed twice with ice-cold PBS containing freshly added protease inhibitors. Cell pellets were collected at 1000rpm at 4 °C. Cells were resuspended in SDS cell lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS, and protease inhibitor) for 20min on ice. For spleen cells, prior to further lysis by SDS cell lysis buffer, pellets were first resuspended in cell lysis buffer (5mM PIPES [PH8.0], 85mM KCl, 0.5% NP-40, and protease inhibitor) for 20min on ice. Cells were sonicated to give a DNA size range from 200 to 900 bp. Samples were centrifuged for 10 min at 13,000rpm at 4 °C to remove debris and the supernatants were transferred to new microcentrifuge tube. DNA concentration of each sample was measured to ensure an equal amount of DNA for further immunoprecipitation. 10ug of samples were pre-cleared with salmon sperm DNA/protein A or G agarose slurry (Upstate Biotechnology, Lake Placid, NY) for 1hr at 4 °C with agitation and then spun down at 4 °C at 2000rpm. The
supernatant fractions were collected and diluted 10-fold with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, and protease inhibitor). 5 or 10% of each sample was kept as input control for PCR. Samples were incubated with antibodies overnight at 4 °C. Immune complexes were collected using salmon sperm DNA/protein A or G agarose slurry for 1 hr at 4°C, and collected at 2000rpm for 1 min. The samples were washed sequentially at 4°C with buffers as followings, low salt wash buffer (20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), high salt wash buffer (20 mM Tris-HCl (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), LiCl wash buffer (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA), and Tris-EDTA buffer. Immuno complexes were extracted from the agarose slurry with freshly prepared elution buffer (1% SDS and 0.1 M NaHCO₃) by rotating 15 min at room temperature. Cross-linking of samples was reversed by adding NaCl at final concentration of 0.3M to the eluates and incubating at 65°C for 4 hr to 16hr. Afterwards the samples were digested with proteinase K, and the DNA extracted with phenol-chloroform-isoamyl alcohol, purified by ethanol precipitation and stored at -80°C. The antibodies used for immunoprecipitation were anti- HIF1α (NB100-105, Novus Biologicals, Littleton, CO), anti-HIF2α (NB100-122, Novus Biologicals, Littleton, CO), anti-p300 (N-15, Santa Cruz Biotechnology, Santa Cruz, CA), anti-IgG (SC-2025, SC-2027, Santa Cruz Biotechnology, Santa Cruz, CA), anti- GATA2 (H116, Santa Cruz Biotechnology, Santa Cruz, CA), anti- SCL (E-14, Santa Cruz Biotechnology, Santa Cruz, CA), anti- Smad4 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA).
PCR was performed within linear range and PCR products were separated on agarose gels, and quantified using ImageQuant. The following primers were used for amplification of precipitated DNA. HRE-1(forward):
TGTCGGCGCTGTAAAGAGAC; HRE-1(reverse): TTGTCCCCGCCTGCTCTGAG;
HRE-2(forward): TCCATCACAATGTGACACGG; HRE-2(reverse):
ACTACGTTTTGGCCCTTCTGC; HRE-3(forward):
CATTCAACCACCTACACATACCAC; HRE-3(reverse):
GTCAAAATATATGATCAATATGGTCAAAAC; HRE-4(forward):
GCAATACCAGCACCCTACTTG; HRE-4(reverse): GTTCCTGTTGCTCTGGCTTG.
PCR is also performed by using primers amplifying Glut-1 promoter region as previously described, which served as positive and specificity controls for CHIP by Hif1α(Hu et al., 2006).

Plasmid constructs and luciferase assay
Regions spanning Bmp4 HRE’s-2 and -4 were amplified by PCR with the following primers, digested with restriction enzymes and ligated into plasmid MCSgLuc(Wang et al., 2006), HRE2PCRinsert(F): GGAGTTCTCGAGGCAACCCAATTATG;
HRE2PCRinsert(R): ATGCGGCCGCTGAGGTAACGATC; HRE4PCRinsert(F):
ACGTCTCGAGAACTCAGGAAAGAAG; HRE4PCRinsert(R):
ATGCGGCCGCTGAGGTAACGATC. Plasmid clones containing HRE-2 or HRE-4 were further mutated by using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA ) according to manufacturer’s instruction with the following primers,
HRE2mut(F): GACCCACCAGGGCCAGTTTTTCAGATACTACGGCAATCG;
HRE2mut(R): CGATCGGCTGATTCTGAAAAACTGGCCCTGGTGTCGTC;
HRE4mutbp(F): GAAAGCTCACCATGAGGCCTATTTTGGCAAATAATCAGC;
HRE4mutbp (R): GCTGATTATTTGCCAAATAGGCCTCATGGTGAGCTTTT.

These Lusiferase constructs were co-transfected with Renilla plasmid which serves as transfection efficiency control into HEK293T cells. After culture, luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to manufacturer’s instruction.

**Statistical analysis**

P values were calculated by student’s t-Test with two-tailed distribution and two-sample equal variance.
Results

Hypoxia regulates the transcription of BMP4.

Our analysis of the recovery from acute hemolytic anemia showed that BMP4 expression is induced in the spleen and our initial analysis suggested that BMP4 expression was regulated by hypoxia (Lenox et al., 2005a). In addition to the spleen, BMP4 is also expressed in the fetal liver during development and the BMP4 dependent stress erythropoiesis pathway drives the expansion stress erythroid progenitors in the fetal liver (Porayette and Paulson, 2008). We tested whether hypoxia could induce BMP4 expression in spleen stromal cells (MSS31 (Yanai et al., 1989)), fetal liver stromal cells (AFT024 (Moore et al., 1997)) and a murine osteoblast cells line (W-20-17 (Binnerts et al., 2004)). In figure 1A, the data show that in all three cells lines, culturing cells under hypoxic conditions (1% O₂) leads to an induction of BMP4 mRNA (Figure 1A). The expression of BMP4 in AFT024 cells decreased at 24 hours of hypoxia, suggesting that other factors in these stromal cells may feed back to turn off BMP4 expression.

Hypoxia induced transcription is mediated by the HIF transcription factor complex. However other mechanisms such increased mRNA stability could also account for the increase in BMP4 expression. We hypothesized that this up-regulation of BMP4 expression was due to an increase in transcription caused by the HIF complex, which is known to activate transcription under low O₂ conditions. To test the hypothesis, we first examined whether increased BMP4 expression by hypoxia is regulated at the transcriptional level. MSS31 cells were cultured in media alone or media plus the
transcription inhibitor actinomycin D (ActD), and incubated at 20% O$_2$ for 30 minutes. Cells were then cultured either at 20%O$_2$ or shifted to 1% O$_2$. The cells were harvested and BMP4 expression was examined by RT-PCR. ActD abolished the ability of hypoxia to induce BMP4 expression, indicating hypoxia regulates BMP4 expression by increasing the transcription of the BMP4 gene (Figure 1B).

**Hif1$\alpha$ and Hif2$\alpha$ are required for BMP4 induction under hypoxia.**

HIF is a heterodimer composed of $\alpha$ subunit encoded by Hif$\alpha$ genes and $\beta$ subunit encoded by Arnt genes. The $\beta$ subunit is not affected by oxygen change, however $\alpha$ subunits are unstable in the presence of oxygen. The N-terminal activation domain (NTAD) and C-terminal activation domain (CTAD) of HIF $\alpha$ subunit can activate transcription when bound to DNA in complex with $\beta$ subunit (For review see(Kaelin, 2005; Semenza, 2009; Simon and Keith, 2008)). Under very low O$_2$, HIF activates transcription in concert with the co-activator p300/CBP(Arany et al., 1996). To date, many HIF targets have been identified, which contain HIF responsive elements (HREs)(Kaelin, 2005). Previous work showed that Arnt-/- embryonic stem cells were defective in the development of hematopoietic progenitors(Maltepe et al., 1997) and mutations in Hif2$\alpha$, Phd2 and VHL all cause defects in erythropoiesis(Ang et al., 2002; Hickey et al., 2007; Percy et al., 2008a; Percy et al., 2008b; Percy et al., 2006; Scortegagna et al., 2005; Scortegagna et al., 2003; Semenza, 2009; van Wijk et al., 2009), indicating that hypoxia or the HIF complex regulates hematopoiesis and in particular erythropoiesis. Here, we hypothesize that the HIF complex is required for regulating BMP4 transcription in the spleen during the recovery from acute anemia. To verify this
hypothesis, we knocked down HIF1α or HIF2α expression by using short hairpin RNA (shRNA). In Figure 2A, the data show that clone HIF1α-50 and clone HIF2α-06 were able to efficiently knock down expression of HIF1α and HIF2α, respectively. HEK293T cells were transfected with these shRNA expression vectors. 24 or 48 hours post transfection, the cells were incubated at 20% O₂ or shifted to 1% O₂ for 16 hours. The expression of HIF1α or HIF2α and BMP4 were determined by RT-PCR with the expression of 28S rRNA used as control. Under hypoxic (1% O₂) conditions, expression of HIF1α, HIF2α, and BMP4 were induced in HEK293T cells transfected with control construct (Figure 2B). When cells were transfected with shRNA constructs targeting either HIF1α or HIF2α, expression of HIF1α or HIF2α were no longer increased under 1% O₂, showing that the knock-down by shRNA was efficient. The knockdown of HIF1α led to a loss of BMP4 expression in hypoxic cultures, while a decrease in HIF2α led to decreased expression of BMP4 compared to control when cells were grown in hypoxic cultures (Figure 2B). These results demonstrate that HIF1α and HIF2α are required for inducing BMP4 expression under hypoxia.

The BMP4 locus contains 5 putative HIF responsive elements (HREs).

Our previous work identified a putative HRE in the 3’ UTR of the BMP4 gene (Lenox et al., 2005a) (HRE-3 in Figure 3A). This element was highly conserved in mouse, human and rat BMP4 genes. In addition to this element which is highly similar to the HRE found in the 3’end of the Erythropoietin gene, we also analyzed the sequence of the BMP4 locus for other putative HREs. We used MATInspector (Quandt et al., 1995) to analyze the BMP4 gene and 5000bp on either side of the gene. Four additional potential binding
sites for HIF-1 and/or bHLH/PAS protein family were identified by MATInspector (HRE’s-1, 2, 4, 5 in Figure 3A). We next analyzed whether these five sites were conserved among species (Figure 3B). Sequence conservation among species is one measure that can be used to identify bona fide regulatory sites. Statistical models have been developed that provide additional information. We analyzed each of the sites for their regulatory potential (RP) scores (Figure 3B). RP scores are computed from genome-wide alignments of human and other organisms which take into account the conservation, composition and short-pattern structure information (King et al., 2005; Kolbe et al., 2004). Regulatory potential analysis was used to generate ESPERR 7 species RP scores (Taylor et al., 2006). This analysis in addition to sequence conservation showed that putative HRE’s 1-4 were potential candidates for hypoxia regulatory elements, while HRE-5 lacked sequence conservation and was not further examined by experiments.

**Hif1α, Hif2α and the transcriptional coactivator p300 are associated with BMP4 HRE’s 2 and 4 in response to hypoxia *in vitro.*

In order to examine whether the predicted HREs in the BMP4 locus are associated with the HIF complex in a hypoxia dependent manner, we performed chromatin immunoprecipitation (ChIP) assays with antibodies against HIF1α, HIF2α and the co-activator p300. MSS31 cells were cultured at either 20% O₂ or 1% O₂ for 16 hours, and then harvested for ChIP assay. IgG and other isotype control antibodies were used as specificity controls for immunoprecipitation. PCR primer pairs amplifying around 200bp spanning these predicted HREs were used to assess the binding of Hif1α, Hif2α and p300. 5% or 10% of input DNA from both culture conditions were served as positive
control for PCR and quantification control. In addition, a well-known Hif1α target, Glut-1, was served as positive and specificity control for ChIP assay by Hif1α (Figure 4C) (Hu et al., 2006).

Among our four candidates, we found that two HREs were associated with HIF and p300 in a hypoxia dependent manner. HRE-2, which is located within exon 3 of the BMP4 gene, exhibited hypoxia specific binding of Hif2α and p300 (Figure 4A). HRE-4, which is located approximately 4kb 3’ of exon 3, exhibited binding of Hif1α, Hif2α and p300 under hypoxic conditions. The association with Hif2α was stronger than Hif1α at the HRE-4 site (Figure 4B). When we analyzed binding at the HRE-1 and HRE-3 sites, no PCR products were detectable until we increased the template amount to tenfold greater than that used with HRE-2 and 4. The amount of the PCR product generated at HRE-1 and 3 was similar regardless of whether the cells were cultured at 20% O₂ or 1% O₂ for all antibodies used for immunoprecipitation, suggesting that the interaction was not hypoxia inducible and represented background binding (data not shown). These data show that Hif1α, Hif2α and the coactivator p300 are associated with BMP4 under hypoxia in vitro; demonstrating a mechanism whereby BMP4 is regulated by HIF under hypoxia.

Hif2α and the co-activator p300 are associated with HRE’s 2 and 4 in the spleen following phenylhydrazine-induced acute hemolytic anemia.

BMP4 expression is up-regulated in the spleen 24 hours after treatment with Phenylhydrazine to induce acute anemia (Lenox et al., 2005a). We next tested whether these putative HREs are bound by Hif1α, Hif2α and p300 in vivo during the recovery
from acute anemia. Tissue hypoxia is one of the physiological characteristics of acute anemia; we hypothesized that BMP4 is induced in spleen by HIF after phenylhydrazine-induced anemia. Spleen cells were harvested from control mice or mice treated with phenylhydrazine for RNA extraction and ChIP assay. In figure 5A, the data show that BMP4 expression was induced in the spleen at 24 hours post phenylhydrazine injection. At HRE-2 and HRE-4, we observed an increase in Hif2α and p300 binding following anemia induction (Figure 5B-C). Overall, the intensity of PCR bands, as quantified by ImageQuant software and normalized with input controls, showed that the association of Hif2α with HRE’s 2 and 4 was stronger than Hif1α in phenylhydrazine-treated mice when compared to control mice (Figure 5B-C). There was no detectable binding to HRE-1 site. There was very weak binding of Hif2α and p300 to HRE-3 site (data not shown). These data demonstrate a mechanism whereby BMP4 is regulated in stress erythropoiesis. The Hif2α complex and the co-activator p300 are associated primarily with HRE’s 2 and 4 in the BMP4 gene in murine spleen at 24 hours post phenylhydrazine treatment. This time corresponds to the time when BMP4 is induced, which initiates the expansion of stress erythroid progenitors (Lenox et al., 2005a).

**HRE’s 2 and 4 can confer hypoxia dependent expression to a luciferase expression plasmid.**

Our ChIP analysis identified two HREs that were bound by the HIF complex and p300 both in MSS31 cells *in vitro* and in the spleen at 24 hours after treatment with phenylhydrazine. These data suggest that these sites mediate hypoxia dependent expression of BMP4; we next tested whether these sites could confer hypoxia dependent
expression on an exogenous luciferase gene. We cloned 100bp fragments spanning either HRE-2 or HRE-4 or altered versions where these HREs were mutated (Figure 6A) upstream of luciferase gene expressed from a minimal murine β-globin promoter (Wang et al., 2006). The HRE-2, HRE-2mut, HRE-4 or HRE-4mut plasmids were co-transfected with Renilla plasmid respectively into HEK293T cells. 24-48 hours after transfection with the plasmids, cells were cultured at 20% O₂ or shifted to 1% O₂ for overnight. Both HRE-2 luciferase and HRE-4 luciferase exhibit significant hypoxia dependent increases in expression when compared to luciferase alone at 20% O₂ (Figure 6B). The hypoxia dependent induction was approximately 2.7 fold greater in HRE-2 luciferase and approximately 1.8 fold greater in HRE-4 luciferase when compared to the hypoxia dependent induction of the MCSgLuc vector. In both cases mutation of the HRE abolished the hypoxia dependent induction of luciferase activity (Figure 6B), showing that these HRE’s are required for hypoxia dependent luciferase induction. These data demonstrate that HRE’s 2 and 4 are capable of conferring hypoxia dependent transcription to exogenous genes and that they regulate BMP4 expression during the recovery from acute anemia.
Discussion

Our data show that the expression BMP4 in the spleen during the recovery from acute anemia relies primarily on the recruitment of Hif2α and p300 to specific sites in the BMP4 locus. We identified two HREs that exhibit hypoxia inducible binding of Hif2α and p300 both in vitro in MSS31 cells and in vivo in the spleen during recovery from acute anemia; these HREs are capable of conferring hypoxia inducible transcription when placed upstream of a luciferase reporter gene. These data show that BMP4 expression is regulated directly by hypoxia in spleen stromal cells.

Hif2α mutant mice exhibit embryonic lethality in inbred strains of mice, which complicated analysis of the role of Hif2α in adult hematopoiesis (Gruber et al., 2007). However, crossing the null mutation onto a mixed F1 background allowed mutant mice to survive. Analysis of these mice showed that Hif2α-/- mice developed pancytopenia. Further analysis of this phenotype showed that transplanted Hif2α-/- bone marrow could effectively generate new progenitor cells leading to normal hematopoiesis in recipient mice. In contrast, transplant of control bone marrow into Hif2α-/- recipients recapitulated the pancytopenia phenotype, which demonstrates that Hif2α functions in the hematopoietic microenvironment (Scortegagna et al., 2003). The primary target of Hif2α appears to be erythropoietin, as treatment of mutant mice with exogenous erythropoietin can rescue the defect in erythropoiesis (Scortegagna et al., 2005). Similar results were observed using Hif2α conditional alleles (Gruber et al., 2007). In these experiments, treatment with exogenous Epo also rescued the erythropoietic defects associated with
deletion of Hif2α in adults. Our data suggests that BMP4 should be added to the list of
genes regulated by Hif2α in the spleen microenvironment.

The BMP4 dependent stress erythropoiesis pathway is not limited to the spleen. Analysis of splenectomized mice showed that the adult liver can also support stress erythropoiesis (Lenox et al., 2009). BMP4 is normally expressed by hepatocytes in the liver. The expression pattern within each liver acinus forms a gradient of expression that fits a known oxygen gradient (Jungermann and Kietzmann, 2000). Highest expression of BMP4 is associated with regions that contain the lowest levels of oxygen. Our present study suggests a model where, in response to acute anemia, expansion of the hypoxia zone in the liver acini leading to an expansion of Hif2α dependent expression of BMP4 (Lenox et al., 2009).

Our data show that HRE-2 and HRE-4 can directly regulate hypoxia dependent induction of BMP4 expression. However, several observations suggest that the regulation of BMP4 during the recovery from acute anemia may be more complex. Studies examining hypoxia dependent transcription of BMP4 in hepatocellular carcinoma cells (HCC) showed that HIF1α dependent regulation of BMP4 expression is indirect in these cells (Maegdefrau et al., 2009). Hypoxia induces the HIF1α dependent expression of ETS1, which in turn induces BMP4 expression. The authors showed that HIF1α was required for the BMP4 induction by transfecting a dominant negative form of HIF1α, which abolished BMP4 expression. However this effect could be overcome by over expression of ETS1. This paper examined only one putative HRE in the promoter of the human BMP4 gene, which bound HIF1α weakly in gel mobility shift assay and did not correspond to any of HREs we characterized in this study. Our analysis showed that
HRE-2 and HRE-4 can confer hypoxia inducible expression of luciferase gene. This induction was completely abolished when the HRE was mutated, which demonstrates that Ets1 does not regulate BMP4 expression through these sites.

In addition to HIF, the role of other transcription factors that may act independently or in concert with HIF to regulate BMP4 expression during recovery from acute anemia is also indicated by our previous work. Hedgehog signaling induces BMP4 expression by bone marrow progenitor cells when they enter the spleen microenvironment (Perry et al., 2008). This induction is independent of hypoxia. Furthermore, we observed that BMP4 expression is delayed in the spleens of flexed-tail (f) mutant mice during the recovery from acute anemia (Lenox et al., 2005a) and in the fetal liver of mutant embryos (Porayette and Paulson, 2008). fj/fj mice have a mutant Smad5, which acts as a dominant negative form with the ability to inhibit the function of Smads1, 5 and 8, which are activated by BMP4 (Hegde et al., 2007; Lenox et al., 2005a). This observation suggests that Smad 1, 5 and/or 8 may function with Hif2α to regulate transcription of BMP4 in the spleen during the recovery from acute anemia. Further analysis will be needed to address this question.
References

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Figures and Figure Legends

Figure 2-1. Hypoxia induces the transcription of BMP4. (A) RT-PCR analysis of BMP4, Hif1α, Hif1β, Hif2α or β-actin (control) expression in the MSS31 spleen stromal cell line (top); BMP4 and 28S rRNA (control) in the W2017 osteoblast cell line (middle) and BMP4 and 28S rRNA (control) in the AFT024 fetal liver stromal cell line (bottom) at 20% O₂ or 1% O₂. BMP4 expression relative to 18S rRNA in these cell lines was also examined by quantitative PCR. (B) MSS31 cells were treated with Actinomycin D (ActD) (1µg/ml) for 30 minutes prior to shifting the culture to 1% O₂. At the indicated times cells were harvested, RNA was isolated and the expression of BMP4 was determined by RT-PCR.
Figure 2-2. HIF1α and HIF2α are required for BMP4 induction under hypoxia.

(A) RT-PCR analysis of the expression of HIF1α or HIF2α in HEK293T cells transfected with the indicated shRNA constructs. (B) RT-PCR analysis of BMP4 expression at 20% or 1% O₂ in HEK293T cells where HIF1α (left) or HIF2α (right) were knocked down with shRNAs.
**Figure 2-3. Identification of putative HREs in the murine BMP4 gene.** (A) Schematic diagram of the location of the 5 putative HREs in BMP4 locus with extra 5000bp both upstream and downstream, where the transcription start site is located at 6102bp. The acquired sequence data were from September 2005. (B) The sequences of the 5 putative HREs are aligned among mammals. Sequences in grey box are conserved among 6 species. Below each alignment is the ESPERR 7 species RP score for each nucleotide.
Figure 2-4. HIF and co-activator p300 are associated with BMP4 under hypoxia in MSS31 cells. The binding of Hif1α, Hif2α, and p300 to these sites was investigated by ChIP assay in MSS31 cells cultured at 20% O₂ or 1% O₂ for 16 hours. IgG or other antibodies served as controls. PCR were performed with primers amplify HRE-2 (A) and HRE-4(B). Representative gel data are shown. The relative intensity of PCR products normalized to the input lanes is shown by combining data from three to five individual ChIP assays. (C) Positive and specificity controls of Hif1α were examined at Glut1 locus.
**Figure 2-5. Binding of HIF and p300 to the BMP4 locus is induced by anemia.** (A) RT-PCR analysis of BMP4 or β-actin (control) expression in primary spleen cells isolated from mice at 24 hours post induction of acute hemolytic anemia with phenylhydrazine (PHZ) and untreated mice. (B) Spleen cells were analyzed by ChIP assay using anti- Hif1α, Hif2α, p300 or IgG antibodies. PCR was performed with primers amplify HRE-2 (B) and HRE-4(C). Representative gel data are shown. The relative intensity of PCR products normalized to the input lanes is shown by combining data from three individual ChIP assays.
Figure 2-6. HRE-2 and HRE-4 can confer hypoxia inducible expression to a luciferase gene. (A) Sequence of wildtype and mutated versions of HRE-2 and HRE-4. (B) Luciferase activity in HEK293T cells transfected with the indicated plasmids at 20% or 1% O₂. The firefly luciferase activity is normalized with renilla luciferase expression. Fold increase in luciferase activity was normalized with the increase observed in the control plasmid set to 1.0. p value was calculated to compare the luciferase activity under 20% versus 1% O₂. Each bar represents the average of three to twelve individual transfections.
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Author Contributions

Conceived and designed the experiments: DCW, RP. Performed the experiments: DCW. Analyzed the data: DCW, RP. Wrote the paper: DCW, RP.
Chapter 3

Hedgehog signaling initiates the BMP4-dependent stress erythropoiesis pathway and promotes the expansion of self-renewing stress erythroid progenitors in the murine spleen

Abstract

During fetal development and in response to acute anemia in adults, stress erythropoiesis rapidly produces large numbers of new erythrocytes by a mechanism that is distinct from bone marrow homeostatic erythropoiesis. Our laboratory has demonstrated that BMP4, SCF, and hypoxia act in concert to regulate the expansion and differentiation of stress erythroid progenitors that are resident in spleen in response to acute anemia. In addition to these three signals, we have also previously identified that Hedgehog signaling is required for the maintenance of the BMP4-dependent stress erythropoiesis pathway. Following recovery from acute anemia, bone marrow cells migrate into the spleen. Once in the spleen, hedgehog and BMP4 dependent signaling cause the bone marrow cells to adopt the stress erythroid progenitor fate.

In this chapter, I studied how Hedgehog signaling regulates the generation of erythrocytes at times of acute need. I utilized short-term radioprotection as a stress erythropoiesis model to dissect the roles of Hedgehog signaling in generating erythrocytes during the recovery following bone marrow transplantation. Both pharmacological and genetic approaches were utilized in this short-term radioprotection...
model. The donor bone marrow cells that are inhibited or mutant in the Hedgehog signaling pathway are unable to provide erythroid the short-term radioprotection. The recovery of the erythroid lineage was significantly delayed in the surviving mice. Defects in regenerating platelets and WBC in the immediate post-transplant period were also observed when Hedgehog signaling was inhibited. Conversely, transplanting donor bone marrow cells in which Hedgehog signaling is activated resulted in enhanced recovery compared to controls. Hedgehog signaling plays a critical role in initiating stress erythroid response by potentially regulating the specification of stress progenitors and expansion of stress progenitors Sca1+Kit+CD34+/ Sca1+Kit+CD34- cells, and stress erythroid progenitors Sca1+Kit+CD71+ cells. Hedgehog signaling is still required following the specification of stress erythroid fate. It maintains self-renewal, expansion of BMP4-responsive (BMP4R) cells, and the ability of Sca1+Kit+CD34- cells to generate erythrocytes. In conclusion, this study demonstrates that Hedgehog signaling is essential for bone marrow cells to adopt the stress erythroid fate and plays important roles in the expansion of stress erythroid stem cells. This study also reveals a stress erythroid progenitor population, Sca1+Kit+CD34+CD71lo/+ Ter119- cells, whose specification during stress erythropoiesis requires Hedgehog signaling.
Introduction

In response to acute anemia, stress erythropoiesis predominates and rapidly produces large numbers of new erythrocytes by a mechanism that is distinct from bone marrow homeostatic erythropoiesis (Lenox et al., 2005b). The following model for stress erythropoiesis summarizes previous studies from the lab. The rapid expansion of stress erythroid progenitor population in the spleen is driven by BMP4+SCF+hypoxia, which leads to complete mobilization and differentiation of the population (Perry et al., 2007b). During recovery, this population is replenished by bone marrow cells migrating into the spleen. Once in spleen, these cells encounter Hedgehog signal which promotes the development of new stress progenitors. Hedgehog induces bone marrow cells to express BMP4, and together these two signals promote the development of new stress progenitor in the spleen (Perry et al., 2009a).

The Hedgehog signaling pathway is inactive in the absence of ligands. In mammals, Hedgehog signaling pathways are initiated by binding of any of the three ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) or Desert hedgehog (Dhh), to the cell surface receptor Ptch. Upon binding of ligand to Ptch, the inhibition of Smo by Ptch is released. Smo signals then activates the downstream cascade, which leads to the translocation of the active form of the transcription factor Gli to the nucleus. Nuclear Gli activates target gene expression, including Ptch, Gli and the receptor Hip that attenuates ligand diffusion (reviewed in (Pasca di Magliano and Hebrok, 2003)). Whether Hedgehog signaling is required for adult hematopoiesis is a controversial topic (reviewed and discussed in Chapter 1, outlined in Table 1-1). Recently, it has been shown by a few
studies that Hedgehog signaling-defective hematopoietic cells exhibited a delayed recovery from 5-fluorouracil (5-FU) myeloablation when compared to controls. However the defects were minor and no mechanism was determined (reviewed in Chapter 1).

To understand the process in which Hedgehog signaling regulates stress erythropoiesis, I first extended our previous work by analyzing the ability of donor bone marrow cells defective in Hedgehog signaling to provide erythroid short-term radioprotection. Following transplantation, the quiescent hematopoietic stem cells (HSCs) are induced to proliferate during regeneration and give rise to mature progeny while maintaining the ability to self renew. Because deletion of Smo or Ptch is embryonic lethal, conditional knockout mice and chemical inhibitors of Hedgehog pathway were used as the models. Biological evidence showed that Hedgehog signaling is required for short-term radioprotection. To further dissect what causes defective hematopoietic regeneration when Hedgehog signaling is inhibited, and to investigate roles of Hedgehog in stress erythropoiesis; I next examined differentiation of erythroid progenitors and hematopoietic stem cells during the recovery. The results demonstrated that Hedgehog signaling is essential for bone marrow cells to adopt the stress erythroid fate. In addition, a role for Hedgehog signaling after specifying erythroid fate was investigated by in vivo and in vitro models. Once the erythroid fate is specified, Hedgehog signaling is required for maintaining expansion and differentiation of stress erythroid stem cells.
Study Design

Mice

Mouse strains used in this study are as follows. C57BL/6 (CD45.2) and congenic B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice were bred in our colony or purchased from Jackson Laboratory (Bar Harbor, ME). Smo()Smoothened (Smo) conditional allele Smo<sup>tm2amc</sup>/J (Long et al., 2001) mice were purchased from Jackson Laboratory (Bar Harbor, ME). The conditional Patched allele (Ptc<sup>fx</sup>) mice were provided by Brandon Wainwright (Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland, Australia) (Ellis et al., 2003). Smo and Ptc mutant alleles were crossed onto the C57BL/6 background in our lab at least 5 generations. C57BL/6-Smo<sup>tm2amc</sup>/J mice were crossed with the interferon-inducible Cre recombinase transgenic mouse line, B6.Cg-Tg(Mx1-cre)1Cgn/J mice (Kuhn et al., 1995) to generate C57BL/6-Smo<sup>tm2amc</sup>;Mx1cre. C57BL/6-Ptc<sup>fx</sup> mice were crossed with the tamoxifen-inducible Cre recombinase transgenic line, C57BL/6-CAGGC<sup>Cre-ER</sup> (Hayashi and McMahon, 2002) to generate C57BL/6-Ptc<sup>flxed</sup>;CAGGC<sup>Cre-ER</sup> mice. Deletion of Smo using poly(I)poly(C) injection to induce MX1-cre expression and deletion of Ptc using 4-hydroxytamoxifen to activate CreER were done as previously described (Indra et al., 1999) (Mikkola et al., 2003). The efficiency of deletion was measured by polymerase chain reaction (PCR) analysis as described (Ellis et al., 2003; Long et al., 2001). All mice were approximately 6-16 weeks old male unless mentioned in context, and the controls were age and gender matched. All procedures
were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

**Cyclopamine treatment**

Cyclopamine were purchased from LC Laboratories (C-8700). 25mg (dissolved in 2-hydroxypropyl-β-cyclodextrin) /kg mouse was used to inject to mice, and 5μM or 10μM was used to incubate with cells. Controls were treated with vehicles. All procedures were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

**Phenylhydrazine treatment**

Acute anemia was induced by injection of phenylhydrazine (Sigma, St Louis, MO) at the concentration of 100 mg/kg mouse in phosphate-buffered saline (PBS) buffer. All procedures were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

**Complete Blood Analysis**

Peripheral blood was collected from retro-orbital sinus by using heparin-coated microhematocrit tubes and transferred to EDTA-coated tubes for complete blood cell (CBC) analysis. CBC analysis was determined by Advia 120 Multi-species whole blood analyzer (Bayer, Tarrytown, NY).

**Flow Cytometry and Cell Sorting**
Single cell suspensions of splenocytes, BM and peripheral blood were isolated and incubated with anti-FcγRII/III for non-specific binding flowed by various combinations of antibodies on ice. The antibodies used in this study included anti-CD71-FITC, CD71-PE, Ter119-PE, Ter119-Alexa647, cKit-Alexa647, cKit-PE, Sca1-Pacific Blue, Sca1-PE-Cy7, CD34-FITC, CD34-PE-Cy5, CD45.2-FITC, CD45.2-APC-eFlour780, CD45.1-FITC, AnnexinV-FITC, CD3e-biotin, Mac1-biotin, Ter119- biotin, B220- biotin, Grl-biotin and streptavidin-PE (BD-Pharmingen, San Diego, CA) (BioLegend, San Diego, CA), (Invitrogen, Carlsbad, CA). Dead cells were determined by propidium iodide (PI) staining. Cells were suspended in 5% fetal bovine serum (Equitech-Bio, Kerrville, TX)/PBS after labeling with antibodies. FC500 Benchtop Flow Cytometer (Beckman-Coulter, Miami Lakes, FL) with CXP software was used for flow cytometry analysis. Cytopeia inFlux V-GS Cytometry Workbench with Spigot software was used for sorting to enrich specific population. All data present were analyzed by FlowJo software (Tree Star, Ashland, OR).

**Transplantation Assays**

Prior to transplantation all recipients were treated with acidified water (pH 2.5-3.0) and antibiotics for one week. Before transplantation recipients were irradiated by lethal dose (9.5Gy) or otherwise mentioned. Transplantation was done by retro-orbital sinus injection of cell populations described in context.
**Colony Assays**

Cells or populations mentioned in context were plated in methylcellulose media containing 3U/ml Epo (#M3334, StemCell Technologies, Vancouver, BC) alone or with addition of various cytokines mentioned in context. The cell numbers were as indicated. The concentrations of cytokines were SCF (50ng/ml), and BMP4 (15ng/ml) (R&D Systems, Minneapolis, MN). Stress BFU-E were scored after 5 days incubation, and stress CFU-E were scored after 2 days incubation.

**Immunofluorescence staining**

The spleens were harvested at indicated time and processed for paraffin sections. The sections were stained with anti-BMP4 antibodies (Novocastra, Newcastle, United Kingdom; and Vector Laboratories, Burlingame, CA) using an Alexa Fluor 660 (Invitrogen, Carlsbad, CA) secondary antibody and with anti-CD45.2-FITC (BD Biosciences Phar-Mingen, San Diego, CA). Slides were analyzed by Olympus FV300 confocal microscope (Tokyo, Japan).

**Preparation of cytospin or blood smear slides and staining**

Preparation of cytospin was done immediately after cells were collected. Cells were spun onto slides using a Cytospin 3 centrifuge (Shandon, Pittsburg, PA) at 500 rpm for 10 min, fixed in methanol for 10 min followed by air dry. Preparation of blood smear was done immediately after blood collection from mice. Blood was dropped on a slide and spread by a spreader slide at approximately 30-45 degree angle with rapid and even motion.
Slides were then fixed by incubation with methanol for 10min and air dried. These slides were then subjected to the indicated staining.

**Analysis of hemoglobin with cellulose acetate electrophoresis**

The Helena electrophoresis system (Helena Laboratories, Beaumont, Texas) was used according to the manufacturer’s instructions.

**RT-PCR and Gene Expression Analysis**

Total RNA isolated from cell populations or tissues was homogenized in TriZol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA by using SuperScriptII system (Invitrogen, Carlsbad, CA). The qRT-PCR was done using Taqman assays from Applied Biosystems according to the manufacturer’s instructions. The qRT-PCR analysis was done using an ABI 7300 Real time PCR system.

**In vitro culture of bone marrow cells**

Bone marrow cells were cultured in Gibco IMDM (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), Penicillin-Streptomycin, 10ug of transferrin, 0.2g of BSA, 1mg of insulin, and 0.05 mM beta-mercaptoethanol.

Supplementary of additional cytokines are mentioned in the context with the following concentration: SCF (50ng/ml), BMP4 (15ng/ml), shh (100ng/ml), GDF15 (30ng/ml), cyclopamine (10uM) (R&D Systems, Minneapolis, MN).

**Statistical Analysis**
Standard deviation was used for error bars. P values were calculated by Student’s t-Test with two-tailed distribution and two-sample equal variance.
Results

_Hedgehog signaling is required for stress hematopoiesis._

The hemolytic reagent PHZ induces an acute anemia, which activates the BMP4-dependent stress erythropoiesis pathway. This activation leads to the mobilization of stress BFU-E resident in the spleen. Following recovery, new stress erythroid progenitors are recruited to the spleen. Our lab has previously shown that deletion of Smo prevented development of new stress BFU-E in spleen (Perry et al., 2009a). To examine how Hedgehog signaling regulating the development of stress erythroid progenitors in more detail, in this present study I utilized short-term radioprotection as a model of stress hematopoiesis. After lethal irradiation, in the immediate post transplant recovery period, short-term radioprotective cells must rapidly produce new erythrocytes, platelets and neutrophils to alleviate the acute anemia and potential for infection or hemorrhage. To examine Hedgehog’s role in short-term radioprotection, the mutant bone marrow cells were transplanted into recipients without co-transplantation of any radioprotective dose of wild type cells. I first used donor cells isolated from Smo conditional knockout mice (Figure 3-1), as the Smo receptor is a non-redundant element of the Hedgehog signaling pathway. When I transplanted 100,000 cells, all the Smo/- recipients were dead by day13, which prevented analysis of their recovery (Figure 3-2). At the dose of 500,000 donor cells, most Smo/- recipients could not survive through the recovery stage (day 8-16) and all of them were dead by day18 (Figure 3-3). Neither recovery in RBC and platelet lineages, or generation of new WBCs in these mice was observed (Figure 3-3). To overcome the lethality so that enough mice survive for further analysis, I increased the
number of donor cells. When I transplanted 700,000 and 1,500,000 cells, the survival rates were increased to 30-60% (700,000 donor cells) (Figure 3- 4A) and 100% (1,500,000 donor cells) In all cases, surviving Smo-/ recipients exhibited poor and delayed recovery (Figure 3- 4B, 4C, 4E, 4F, 5A, 5B, 5D, and 5E). Under the conditions where doses of donor cells were higher, we were able to compare the reticulocyte counts in Smo-/ recipient survivors to wild type recipients, which measures how fast the newly generated erythrocytes are made and released into the blood. In response to acute anemia, at the beginning Smo-/ transplants generated significantly fewer reticulocytes (at days 8 and 10). However, later (at days16 and 18) Smo-/ transplants generated more reticulocytes than controls (Figure 3- 4D, 5C). In addition, increased splenomegaly was observed in Smo-/ recipient survivors later in the recovery period (Figure 3- 4G). These data suggest that Smo-/ cells adapt a mechanism to overcome insufficient erythropoieisis, possibly by generating more progenitors or increasing total cell numbers. In summary, our analysis of a loss-of-function genetic model demonstrates that Smo-deficient mice exhibit severe defects in short-term radioprotection.

I observed that survival rate of Smo-/ transplantd mice increased when the dose of donor cells was increased. There are a few potential explanations for this observation. One possibility is that Smo-/ short term radioprotection is insufficient. At high doses of donor cells, enough erythrocytes were made so that Smo-/ transplants survived until engraftment of stem cells. The other possibility could be incomplete deletion of Smo; in which case, the existence of a small percentage of wild type cells could alleviate the defects. Careful analysis of Smo expression in several experiments performed, we did one time find low expression of Smo in the polyI-polyC-injected mice
(Figure 3-6, all data from that set of experiment was considered failed and excluded.). To circumvent the potential incomplete deletion, we then took advantage of cyclopamine, a Hedgehog pathway-specific inhibitor (Figure 3-1) to confirm the phenotype observed in genetic model. Bone marrow cells were first incubated with cyclopamine for 2 hours prior to transplant. 24-36 hours post transplantation, recipients were then given daily injections of cyclopamine for the next 5 days after transplantation to continuously block Hedgehog signaling. Stronger defects were seen in the cyclopamine-treated transplants than Smo-deficient recipients. Among several batches of transplantation performed, none of the cyclopamine-treated mice transplanted with 500,000 cells survived longer than 11 days, whereas all vehicle-treated controls survived (Figure 3-7A). Analysis of complete blood counts in the transplants showed that the surviving cyclopamine-treated recipients generated fewer RBCs, platelets, and WBCs at days 8 and 11 post transplantation when compared to controls (Figure 3-7B-G). The increased splenomegaly observed in Smo-/-recipient survivors was also seen in the cyclopamine-treated transplants (Figure 3-7H). Thus, using both genetic and pharmacological approaches, these data show that Hedgehog is required for stress hematopoiesis.

**Hyperactivation of Hedgehog signaling results in a faster recovery from acute anemia.**

In addition to examining the effect of inhibition of the hedgehog pathway, I also analyzed the response of hyperactivation of Hedgehog signaling in the short-term radioprotection model by using Ptch conditional knockout mice (Figure 3-1). We have previously observed that deletion of Ptch, the negative regulator of Hedgehog signaling pathway which inhibits activity of Smo, leads to expansion of stress BFU-E in bone marrow
(Perry et al., 2009a). Given this observation and the defective short-term radioprotection recovery when Hedgehog signaling is blocked, we would expect accelerated recovery in short-term radioprotection when Hedgehog signaling is hyperactivated. Indeed, 100% of Ptc-h-/- recipients survived from lethal irradiation when given 500,000 donor cells. Their recovery in erythroid lineage was faster than controls; they generated more newly-made red cells during recovery stage (Figure 3-8A, 8B). They also generated more platelets and WBCs (Figure 3-8C, 8D). Taken together, the opposite phenotypes in loss-of function and gain-of-function cells demonstrate that Hedgehog signaling tightly regulates short-term radioprotection cells.

Inhibiting Hedgehog signaling results in defective differentiation of spleen erythroid stress progenitor.

Next, I analyzed the stress erythroid progenitor populations in spleen during the recovery post transplantation. Our lab previously identified three stress erythroid progenitors termed “Population- I, -II, and -III” (Harandi et al., 2010). Population I cell was Kit+CD71lo Ter119lo/-, Population II cell was Kit+CD71hi Ter119med, and Population III cell was Kit+CD71lo/med Ter119hi. Population I was the primary population present at the pre-recovery stage (days 2-6), whereas population II exhibited a wave of proliferation during the recovery stage (days 6-12). Population I is self-renewing erythroid-specific progenitor, as demonstrated by serial transplantation experiments. Population II develops from Population I, given that culturing the purified Population I ex vivo for 3 days gave rise to Population II (Harandi et al., 2010).
Surprisingly, expression of stem cell marker Kit was higher in mutant transplants than controls as they recovering (Figure 3- 9A, 9B). Within the Kit+ population, the percentage of CD71+ cells were increased in mutant transplants (Figure 3- 9C, 9D), showing that the differentiation from stress erythroid progenitor population- I to –II (i.e Kit+CD71lo to Kit+CD71hi ) was affected. Based on our initial analyses from different transplantation experiments by using Smo conditional knockout model, differentiation of population- I to –II tended to be delayed, and increased population- II cells were seen later during the recovery prior to their deaths (Figure 3- 9C). Similarly in cyclopamine-treated transplants, expansion of population- II was always seen prior to their deaths (Figure 3- 9D). Whether the development of population II was delayed or blocked in cyclopamine-treated transplants was unable to be determined, since no more survivors available afterwards. (In all performed transplantation experiments, the survival rates were 0-20% at day 10). Overall, I observed the same increase of CD71+ population in mature cells and the stress progenitor population (Kit+ CD71+ Ter119-lo) in both Smo-/- and cyclopamine- treated recipients. To prevent the complications with potential incomplete deletion of Smo, all of our subsequent analysis utilized the pharmacological model.

Previous analysis from our lab showed that population- I and -II contained a population of Sca1+ cells (Harandi et al., 2010). Here, I found that donor-derived Sca1+ Kit+CD71+Ter119+ population was significantly increased during the course of recovery in cyclopamine-treated transplants compared to controls (Figure 3- 10A). This data demonstrate that, the impaired short-term radioprotection of Hedgehog signaling-blocked cells is not caused by decreased number of stress progenitors. Rather, there is a defect in
the differentiation of stress erythroid progenitors, which may result in incorrect specification of erythroid fate. Indeed, analysis of the late stage erythroblast populations (Socolovsky et al., 2001) showed that the differentiation was held up in cyclopamine-treated transplants. The terminal differentiated Ter119+ cells can be further differentiated using forward scatter as a measure of cell volume. These populations are termed as EryA, B and C, where A is being earliest and C latest stage during differentiation (Liu et al., 2006). The data in Figure 3-10B show an increased percentage of donor-derived EryA population in the spleen of cyclopamine- treated transplants. In conclusion, inhibiting Hedgehog signaling causes defects in differentiation of stress erythroid progenitor and results in inefficient terminal differentiation of erythroblasts.

**Inhibiting Hedgehog signaling causes expansion of Sca1+Kit+CD34+ cells in response to acute anemia**

Our analysis on erythroid population demonstrates that Hedgehog signaling plays a role in regulating differentiation of stress erythroid progenitors. Given the cyclopamine-inhibition of Hedgehog signaling leads to defects in generating short-term radioprotection (RBCs, WBCs, and platelets), one would suspect that this phenotype resulted from either decreased or dysfunctional hematopoietic stem cell populations (HSCs). Whether Hedgehog signaling is dispensable in homeostatic hematopoiesis has been inconclusive among studies in the literatures (reviewed in Chapter 1, outlined in Table 1-1). To test this hypothesis and to examine the role of Hedgehog signaling in the hierarchy of stress hematopoiesis, following transplantation I also analyzed the repopulation ability of donor cells and a population enriched for HSCs, Sca1+Kit+ cells.
The numbers of donor-derived cells in bone marrow were similar between cyclopamine-treated transplants and controls (Figure 3-11A). While in the spleen, the predominant site of murine stress erythropoiesis, I observed increased numbers of donor-derived cells in spleen of cyclopamine-treated transplants before or at the time when severe anemia occurs (Figure 3-11A). In spleen, there were also more donor-derived Sca1+Kit+ cells in cyclopamine-treated transplants than controls (Figure 3-11B). These data show that the defect of cyclopamine-treated cells in generating new erythrocytes or other lineages is not due to inability to home and engraft. Instead, inhibiting Hedgehog signaling leads to increased numbers of Sca1+Kit+ cells in spleen during recovery; which could represent compensation to insufficient stress erythropoiesis.

In addition to Kit and Sca1, the initial analysis on expression of stem cell markers revealed striking differential expression of CD34 (Figure 3-12). Sca1+Kit+ population can be further fractioned into Sca1+Kit+CD34+ and Sca1+Kit+CD34- populations. I sorted bone marrow Lin-Sca1+Kit+CD34+ (i.e. short-term reconstituting hematopoietic stem cells, STR-HSCs) and Lin-Sca1+Kit+CD34- (i.e. long-term reconstituting hematopoietic stem cells, LTR-HSCs) populations and transplanted them respectively into lethally irradiated mice. Spleen cells from Lin-Sca1+Kit+CD34+ recipients gave rise to stress BFU-E colonies (Figure A-3), demonstrating that bone marrow Lin-Sca1+Kit+CD34+ cells come into spleen during recovery from bone marrow transplantation and form stress BFU-Es in the spleen. As shown in Figure 3-11C (Vehicle), Sca1+Kit+CD34+ cells emerged on (and possibly also before) day 6 post transplantation and this population was greatly diminished at day 9. These data suggest that the stem cell population, Sca1+Kit+CD34+ cell, is a stress progenitor play roles
during the early phase of stress erythropoiesis. As stress erythropoiesis progresses, the stress progenitor loses expression of CD34. However this phenomenon was not seen when Hedgehog signaling is inhibited (Cyclopamine, Figure 3-11). In the surviving cyclopamine-treated transplants, the Sca1+Kit+CD34+ population was expanded at day 9 during the recovery from lethal anemia prior to their deaths (Figure 3-11C, 11D). In conclusion, these data show that inhibiting Hedgehog signaling affects differentiation of Sca1+Kit+CD34+ to Sca1+Kit+CD34- cells and results in expansion of Sca1+Kit+CD34+ cells. Taken together, the data demonstrated that Hedgehog signaling is essential for bone marrow cells to adopt correct stress erythroid fate. The data also suggests a model where Hedgehog signaling specifies stress erythroid fate by regulating the differentiation and expansion of Sca1+Kit+CD34+/ Sca1+Kit+CD34- stem cells, the precursor of the previously identified stress progenitor “population I” in the spleen.

**Hedgehog signaling is required for stress erythroid progenitors to form stress BFU-Es in the spleen.**

Despite expansion of stress erythroid progenitor populations (day 8 and day 10, Figure 3-10A, 9C, 9D), expansion of Sc1+Kit+CD34+ cells (Figure 3-11C, 11D), increased donor- derived cells (Figure 3-11A), and increased Sca1+Kit+ cells (Figure 3-11B); cyclopamine- treated and *Smo-* transplants exhibited a defect in terminal differentiation and erythroid recovery in response to acute anemia. To dissect what steps in stress erythropoiesis went awry in these mutant transplants, I tested whether the stress progenitor populations were functional by BFU-E formation assay. We have previously showed that BMP4 acts in concert with hypoxia and SCF on BMP4-responsive (BMP4R)
cells and make these cells form stress BFU-Es in spleen (Perry et al., 2007b). Here the spleen cells at each time point were collected, washed by PBS, and plated in methylcellulose medium with two conditions, Epo only and Epo+hypoxia+BMP4+SCF. Epo+hypoxia+BMP4+SCF are signals known to maximally expand stress BFU-Es. By comparing the numbers of colonies formed in these two conditions, we can also see how many BMP4R are in these cultures. I first plated the same amount of donor-derived spleen cells from controls, Smo-/ and cyclopamine-treated transplants. Within the same number of donor-derived cells, Smo-/ and cyclopamine-treated cells contained larger percentage of progenitors than controls (according to flow cytometry data as mentioned above). However the mutant cells formed fewer stress BFU-Es; their responses to stress signals were either none (case of cyclopamine-treated cells) or delayed (case of Smo-/ cells) compared to controls (Figure 3-13A, 13B). I next sorted and plated the same amount of Kit+CD71+Ter119- cells, which we have known to form stress BFU-Es and CFU-Es, from Smo-/ transplants and controls. The progenitors from Smo-/ transplants had delayed ability to form stress CFU-Es and stress BFU-Es (Figure 3-13C, 13D). These data show that Hedgehog signaling is required for the stress erythroid progenitor (Kit+CD71+Ter119- cells) to develop into stress BFU-Es and expansion of BMP4R cells that respond to stress signals. Compared to controls, delayed and reduced induction of BMP4 expression in spleen during the recovery post transplantation was seen in Smo-/ transplants (Figure 3-14). This data demonstrates that BMP4-dependent stress erythropoiesis pathway is impaired when Hedgehog signaling is inhibited, which can also explain the inability to form stress BFU-Es. The expansion of stress erythroid progenitor (Sca1+Kit+CD34+ cells, Sca1+Kit+CD71+ cells) in recipients of Hedgehog-inhibited
cells might be a compensation for insufficient erythropoiesis resulted from defective differentiation and specification.

**Inhibition of Hedgehog signaling results in increased apoptotic cells in response to acute anemia**

Our colony forming assay demonstrated that, blocking Hedgehog signaling leads to a defective response to stress signals (BMP4, SCF and hypoxia). I next investigated whether blocking Hedgehog signaling lead to increased apoptosis of erythroid progenitors. In surviving cycloamine-treated transplants, I observed more early-apoptotic cells at day8 and late-apoptotic or necrotic cells in the spleen at day10 post transplantation (Figure 3- 15A). The apoptotic cells are donor-derived (Figure 3- 15B). These data suggest defects in Hedgehog signaling lead to increased apoptosis, which also contributes to the defects in erythroid recovery.

**Hedgehog signaling is required to maintain stress erythropoiesis after specifying erythroid lineage**

Our previous work and this thesis study have established role of Hedgehog signaling in specifying stress erythroid progenitors. Whether Hedgehog signaling plays a role after erythrocytes have been specified was addressed in the next set of experiments. I took advantage of previous work in the lab, or the Kit<sup>lo/+</sup>CD71<sup>lo</sup>Ter119- population (i.e., population I). Previously when we sorted population I on day 8 post transplantation and transplanted into secondary irradiated recipient, these cells rescued erythropoiesis without contribution to other lineages (Harandi et al., 2010). Here, I sorted Kit<sup>lo/+</sup>CD71<sup>lo</sup>Ter119-
cells at day 8 after transplantation, and transplanted these cells into secondary recipients. The secondary recipients were given injections of cyclopamine or vehicle control (Figure 3-16A). Only 25% of mice survived until day 7 after transplantation (n=1 out of 4), at which time erythroid populations were analyzed in the remaining mouse which showed signs of morbidity (Figure 3-16B). Flow cytometry analysis showed that there was an almost complete loss of late stage erythroblast (CD71-Ter119+) in the cyclopamine-treated recipient, showing mature red cells were not developed (Figure 3-16C). The high death rate after administering cyclopamine demonstrated that Hedgehog signaling is required to maintain stress hematopoiesis even after the erythroid fate is specified.

*Inhibiting Hedgehog signaling after erythroid commitment causes defects in expanding BMP4<sup>+</sup> cells and differentiation of stress progenitors*

I next examined how inhibiting Hedgehog signaling after specifying the stress erythroid lineage resulted in a failure of stress erythropoiesis. The severe phenotype in the *in vivo* model made it difficult to investigate. To overcome it, I utilized an *in vitro* culture system which provides stress signals that are required *in vivo* (Figure S3-1, and described in Chapter 4). Our previous work showed that, bone marrow cells first migrate into spleen where they encounter Hedgehog. Hedgehog induced BMP4 expression and together these two signals promote development of stress erythroid progenitors (Perry et al., 2009a). To mimic the development of stress erythroid progenitors *in vitro*, I grew bone marrow cells with cocktail of stress signals which include Epo, BMP4, hypoxia, SCF, GDF15, and Shh. We have previously shown that BMP4 expression was induced after overnight incubation of bone marrow with Shh (Perry et al., 2009a); induction of BMP4 expression
initiates the BMP4-dependent stress erythropoieisis pathway (Lenox et al., 2005b). To access the role of Hedgehog signaling after specifying erythroid lineage, Hedgehog signaling was blocked by addition of cyclopamine after 24hrs of culture (Figure 3-17A). The development of erythroid population within 10 days of culture was analyzed by functional assay and flow cytometry.

Here I first tested the ability of these cultured cells to form stress BFU-Es. The cultured cells (Shh vs. Shh → Cy) at each time point were collected, washed by PBS, and plated in methylcellulose medium with two conditions, Epo only and Epo+hypoxia+BMP4+SCF. In general, fewer and also smaller BFU-Es can be formed in Epo-only condition than in Epo+hypoxia+BMP4+SCF condition after the same length of incubation. Stress BFU-Es that are formed in these two conditions are generated from different precursors. The data showed that cyclopamine-treated cultures generated more Epo-only stress BFU-Es than controls in the beginning (Figure 3-17C, upper left, day1+1 & 1+3); however these cyclopamine-treated cells formed fewer stress BFU-Es than controls in Epo+hypoxia+BMP4+SCF condition at all time points (Figure 3-17C, upper right). These data show that, inhibiting Hedgehog signaling following specifying erythroid fate causes defects in expanding BMP4R cells and gives rise to stress erythroid progenitors with different properties. Hedgehog signaling was required to expand progenitors that respond to hypoxia+BMP4+SCF to maximize their capability in generating stress BFU-Es. As far as colony size is concerned, cyclopamine-treated cells formed much bigger colonies earlier than controls in both conditions (Figure 3-17C). The sizes of colonies were similar between cyclopamine-treated cells and controls since
day1+5. This data again shows that inhibiting Hedgehog signaling after specification of the stress erythroid fate gives rise to stress erythroid progenitors with different properties.

As far as stress erythroid progenitors are concerned, flow cytometry data showed that there were higher percentage of Sca1+Kit+CD71+/hiTer119+ cells (labeled as II) but lower percentage of Sca1+Kit+CD71-/loTer119- cells (labeled as I) in cyclopamine-treated cells than controls (Figure 3-17D). Higher percentage of Sca1+Kit+CD34+ population at day1+3, higher percentage of Sca1+Kit+CD34hi population at day1+5, and again higher percentage of Sca1+Kit+CD34+ were observed at day1+9 in cyclopamine-treated culture (Figure 3-17G). In addition, the terminal differentiation of mature erythroid populations (CD71 vs. Ter119) was also affected in cyclopamine-treated cells (Figure 3-17E). Taken together, our colony assay and flow cytometry analysis demonstrate that Hedgehog signaling maintains erythroid development after specifying fate by regulating expansion and differentiation of BMP4R and stress progenitors (Sca1+Kit+CD34+ cells, Sca1+Kit+CD71loTer119- cells).

**Hedgehog signaling is required for maintaining expansion of Sca1+Kit+ cells after specifying erythroid fate**

In the *in vitro* culture model by which the role of Hedgehog signaling after specifying erythroid lineage was examined, total numbers of these stress progenitors were fewer in cyclopamine-treated culture (Figure 3-17B). The data show that Hedgehog signaling is required for expansion of these cells. This phenomenon also led us to speculate that cyclopaamine may also deplete self-renewal ability of stress progenitors besides causing defective differentiation once the erythroid fate is specified. I then analyzed Sca1+Kit+
cells, a self-renewing population enriched in stress erythroid progenitor (population I, and also Sca1+Kit+CD34+ cells) in the *in vitro* cultured cells. As shown in Figure 3-17F, the percentages of Sca1+ cells and Sca1+Kit+ cells were getting lower in cyclopamine-treated cells during culture. Taken together, inhibiting Hedgehog signaling after specification of erythroid fate results in skewed differentiation and decreased expansion of Sca1+Kit+ stem cells.

**Defects in cyclopamine-treated cells can be rescued by Sonic Hedgehog (Shh)**

One interesting question we also addressed was whether the defective stress erythropoiesis following specification caused by cyclopamine is reversible. The cells were cultured first as previously described (Shh, or Shh → Cy) for 7 days. Small portion of the culture was taken and analyzed by flow cytometry after 7 days (Figure 3-17C); Shh and other factors were then added to both of the remaining culture for another 5 days followed by analysis. Figure 3-18A showed the similar expression of CD71/Ter119, Kit, Sca1 and CD34 in both cultures, demonstrating that the defective differentiation caused by cyclopamine after specification of erythroid fate can be rescued.

To further examine whether the cyclopamine-treated cells can really function when they re-encounter Hedgehog signaling *in vivo*, I transplanted the *in vitro* cultured bone marrow cells (Shh, or Shh → Cy, for 7 days) into lethally irradiated wildtype mice without any blocking of Hedgehog signaling. All recipients of both cells survived post transplantation. Firstly these data demonstrate that, bone marrow cells not only can be expanded in our culture *ex vivo* for at least 7 days, but are also functional after culture (described in more detail in Chapter 4). Secondly these data show that cyclopamine-
treated cells can provide short-term radioprotection once they re-encounter Hedgehog signaling circulating in the body. The erythroid recovery in the recipients was similar in both cases (Figure 3-18B, n=4 each condition). Taken together, Hedgehog signaling replenishes stress progenitors following specification.

**Inhibiting Hedgehog signaling after specifying stress erythroid fate impairs ability of Sca1+Kit+CD34-cells to provide erythroid short-term radioprotection**

Our investigations have so far demonstrated that Hedgehog signaling plays a crucial role in stress erythropoiesis. It is required for specifying erythroid fate. Inhibiting Hedgehog signaling resulted in incorrect differentiation and expansion of stress progenitors (Sca1+Kit+CD71+ cells, Sca1+Kit+CD34+ cells), as well as inability of population I to form stress BFU-Es, leading to a defect in short-term radioprotection. It is also required following specification of erythroid fate. Inhibiting Hedgehog signaling causes death of transplants who received stress erythroid-specific progenitors (or, population I). Addition of cyclopamine after initial culture with Shh resulted in decreased proliferation, fewer Sca1+Kit+ cells, defective differentiation of stress erythroid progenitor (Sca1+Kit+CD71+ cells), different precursors to form stress BFU-Es, and defective expansion of BMP4<sup>®</sup> cells. In addition, increased percentage of Sca1+Kit+CD34+ cells and decreased percentage of Sca1+Kit+CD34- cells were seen in the cyclopamine-treated culture. These observations showed that Hedgehog plays roles in stress erythropoiesis by regulating differentiation and function of Sca1+Kit+CD34+ and Sca1+Kit+CD34- cells.

To characterize these two populations and to examine effect of inhibiting Hedgehog signaling following specifying erythroid fate on their regeneration, I sorted
Sca1+Kit+CD34+ and Sca1+Kit+CD34- populations after 6 days of in vitro culture as previously described (Figure 3- 17A). Flow cytometry analysis of the enriched samples post sort revealed existence of the previously characterized stress erythroid progenitors (Kit+CD71<sup>-/lo</sup>Ter119-, Kit+CD71+Ter119-) in these populations (Figure 3- 19A). This data reveals the existence of three stress erythroid progenitor population, CD34- Sca1+Kit+CD71<sup>-/lo</sup>, CD34+Sca1+Kit+CD71<sup>-/lo</sup>, and CD34+Sca1+Kit+CD71<sup>-/hi</sup> cells. Higher percentage of CD71<sup>-/hi</sup> cells existed in the Sca1+Kit+CD34+ cells sorted from cycloamine-treated culture compared to their control counterparts, which is consistent with our earlier observation (Figure 3- 17D). The data suggest that Hedgehog signaling is required for the development of CD34+Sca1+Kit+CD71+ to CD34+Sca1+Kit+CD71+ stress erythroid progenitor cells.

I next tested the potential of these enriched populations in generating different lineages by transplanting them to lethally irradiated mice. We have previous shown that population I (Kit+CD71+Ter119- cells sorted on day8 post transplanation) is a self-renewing erythropoiesis- specific stress progenitor (Harandi et al., 2010), therefore I expected the Sca1+Kit+CD34+ cells and Sca1+Kit+CD34- cells from our ex vivo culture to provide erythroid short-term radioprotection. As shown in Figure 3- 19B, both Sca1+Kit+CD34+ and Sca1+Kit+CD34- populations could give rise to all lineage cells. Among which, Sca1+Kit+CD34- cell from cycloamine-treated culture was defective in that its recipients had lower survival rate and lower nadir in RBC and WBC recoveries (Figure 3- 19B). One notion has to be realized in this experiment is that, Hedgehog signaling is replenished in the recipients since no further administration of cycloamine post transplantation. Complete block of Hedgehog signaling after transplantation is
known to be lethal as shown in our first and secondary transplants. I further analyzed whether the WBCs generated were from donor or not. Mononuclear cells were enriched from peripheral blood by gradient centrifugation and examined by lineage markers. CD3e, B220 and Mac-1 were only co-expressed with recipient cells but not donor-derived cells (Figure 3-19C), whereas erythrocytes were donor-derived based on the analysis of hemoglobin allele (Figure 3-19D). This data demonstrates that both CD34-Sca1+Kit+CD71$^{+/lo}$ cells and CD34+Sca1+Kit+CD71+ cells give rise to erythrocytes and provide short-term radioprotection. Importantly, it also demonstrates that our in vitro culture can recapitulate stress erythropoiesis in vivo and generated stress erythroid progenitors. In conclusion, Hedgehog signaling specifies erythroid restriction. Inhibiting Hedgehog signaling by cyclopamine impairs the expansion and development of stress erythroid “stem cell” population, as we saw the function of CD34-Sca1+Kit+CD71$^{+/lo}$ cells were defective.

**Inhibition of Hedgehog signaling does not affect homeostatic hematopoiesis.**

Role of Hedgehog in adult normal hematopoiesis has been one subject of intense study in recent years; however, the exact role of Hedgehog signaling is controversial (as discussed in Chapter 1). Using Hedgehog signaling inhibitor cyclopamine, I examine the effects of inhibiting Hedgehog in steady state hematopoiesis (Figure 3-20A). This assay served two purposes. First, to ensure the defects we observed in stress condition was not due to toxicity or other effects of cyclopamine. Second, to elucidate role of Hedgehog signaling in steady state hematopoiesis in parallel compared to our stress model. I analyzed complete blood counts, Sca1+Kit+ population and erythroid populations, and apoptosis in
spleen and bone marrow post injection. In contrast to the lethality and strong defects in erythroid short-term radioprotection, inhibition of Hedgehog by cyclopamine did not affect steady state hematopoiesis. Complete blood counts and flow cytometry analysis of progenitor populations showed no defects (Figure 3-20B-E). In conclusion, Hedgehog signaling regulates hematopoiesis at times of acute need, and is not required for homeostatic hematopoiesis.
Discussion

This present study demonstrates the roles of Hedgehog signaling in stress erythropoiesis, as summarized in Figure 3-21A and B.

The findings from previous work in the lab and the present study establish a model for stress erythropoiesis (Figure 3-21A). Short-term reconstituting hematopoietic stem cells (Lin-CD34+Sca1+Kit+) first migrate to spleen, where cells encounter Hedgehog signaling. Hedgehog signaling induces expression of BMP4; together these two signals act on stress progenitors to specify stress erythroid fate in spleen. The stress progenitor (Lin-CD34+Sca1+Kit+ population) is first amplified and specified to become the stress erythroid progenitor (CD34+Sca1+Kit+CD71+) by gaining the expression of erythroid lineage marker, CD71. Hedgehog signaling is required for this specification step. Once the erythroid fate is specified, this stress erythroid progenitor population goes on its expansion and further development. The progenitors then lose expression of CD34, as CD34+ cells were seen on day 6 but no longer seen in spleen on day 9 post transplantation (vehicle in Figure 3-11C). CD34 is a member of the sialomucin family and plays a role in adhesion. It was known to be expressed by capillary endothelial cells, bone marrow stroma, and small subpopulation of mouse bone marrow cells. The phenomenon that spleen stress erythroid progenitors express CD34 and then lose expression of CD34 as they differentiate is first discovered in this study, and the reason is unexplored yet. Hedgehog signaling plays an important role in expansion and development from CD34+ to CD34- cells. Later some of the CD34-Sca1+Kit+CD71+ cells lose expression of Sca1 and gain expression of the terminal differentiation marker
Ter119, according to previous study in the lab (Harandi et al., 2010). Our lab has demonstrated that the Kit+CD71loTer119- population (“population I”) is self-renewing and erythroid-restricted. Kit+CD71loTer119- cells then further develop into Kit+CD71hiTer119lo (“population II”) and Kit+CD71loTer119hi cells (“population III”). “Population I” was shown to form stress BFU-Es and “population II” was shown to form stress CFU-Es.

Previously by studying short-term radioprotection in f/f mice, our lab discovered a self-renewing stress erythroid progenitor “population I” (Kit+CD71+loTer119-), and showed that development of population I, II and III were delayed when BMP4/Smad5 pathway is mutant. This present study reveals the roles of Hedgehog signaling in the earlier event during stress erythropoiesis, as discussed in the following (data are summarized in Figure 3-21B). First, inhibition of Hedgehog signaling leads to a build-up of Sca1+Kit+CD71hi cells in transplants prior to their death, which suggests that the initial events of the BMP4-dependent stress erythropoiesis pathway requires Hedgehog signaling. In the spleens of cyclopamine-treated or Smo-/- transplants, numbers of donor-derived cells, Sca1+ cells, Kit+ cells, CD34 cells, Sca1+Kit+CD34+ cells were increased whereas numbers of Sca1+Kit+CD34- cells were decreased. These increased cells may be compensation to incorrect specification that results in inefficient stress erythropoiesis. Another possibility is that Hedgehog signaling also plays a role in the initial development of stem cells in the spleen. The donor-derived cells and “population I” in cyclopamine-treated or Smo-/- transplants have defects in responding to stress signals and forming stress BFU-Es. As a result, no new RBCs were generated and no survivors following transplantation. In addition, apoptosis and
delayed/low induction of BMP4 were also observed in these mutant transplants. Whether
the built-up donor-derived Sca1+Kit+CD71^{hi} Ter119^{lo} cells in mutant recipients also
expressed CD34 or other stem cell markers was unable to be determined simultaneously
at the times of study until recently. My recent analysis on the expression of CD133 in the
enriched CD34+Sca1+Kit+CD71+Ter119- and CD34-Sca1+Kit+CD71+Ter119- cells
revealed erythroid stress progenitor CD34+CD133+Sca1+Kit+CD71+Ter119- and
CD34-CD133-Sca1+Kit+CD71+Ter119- populations. In addition, my preliminary data
suggested that inhibiting Hedgehog signaling results in loss of CD133 expression in the
CD34+CD133+Sca1+Kit+CD71+Ter119- populations (Figure 3-S2). A bone marrow
transplantation experiment is currently set up to analyze the emergence and function of
these stress erythroid progenitors.

Second, Hedgehog signaling is also required after specification of stress
erythroid fate. Administration of cyclopamine to transplants received “population I”,
which were known to provide erythroid short-term radioprotection, resulted in death of
recipients. In vitro culture experiments were utilized to dissect the cause of death in vivo.
Cyclopamine- treated cells did not expend as well as control cells, showing that
Hedgehog signaling is required for expansion after specification. Although all cell
populations in cyclopamine- treated cultures were fewer than controls, the percentage of
Sca1+Kit+CD71^{hi}Ter119^{lo} and Sca1+Kit+CD34+ cells were higher whereas the
percentage of Sca1+Kit+CD71^{lo}Ter119- and Sca1+Kit+CD34- cells were lower. Given
the facility at the time of this study, not all markers (donor, Kit, Sca1, CD34, CD71,
Ter119 and/or vital dye) could be analyzed simultaneously. However, later analysis of
CD71/Ter119 on enriched/sorted Sca1+Kit+CD34+ and Sca1+Kit+CD34- populations
revealed the expression of CD71 in Sca1+Kit+CD34+ and Sca1+Kit+CD34- populations. The percentage of Sca1+Kit+CD34+CD71^{+hi} was higher in the cyclopamine- treated culture (Figure 3-20A). *In vivo* functional assay demonstrated by transplantation experiments showed that Sca1+Kit+CD34+CD71^{+hi} and Sca1+Kit+CD34-CD71^{-lo} populations both provide erythroid- specific short-term radioprotection. Although serial transplantation experiments were not done, one can speculate that the Sca1+Kit+CD34+CD71^{+hi} population is self-renewing and erythroid- restricted.

Inhibiting hedgehog signaling after specification impaired the ability of Sca1+Kit+CD34-CD71^{-lo} cells to short-term radioprotection. Taken together, Hedgehog signaling also regulates the development from Sca1+Kit+CD34+CD71^{hi} to Sca1+Kit+CD34-CD71^{lo}, in addition to expanding cells. The cyclopamine- treated cultures gave rise to stress BFU-Es with different properties than controls, and responded poorly to Epo+hypoxia+SCF+BMP4. The defects in expansion and development of stress erythroid progenitors after specification is reversible, however, demonstrated both *in vivo* and *in vitro* (Figure 3-18A, B). Taken together, following specification Hedgehog signaling replenishes and maintains expansion and development of stress erythroid progenitors.

In addition, Hedgehog signaling is also required for BMP4- dependent stress erythropoiesis. Low induction of BMP4 protein in *Smo/-* transplants during recovery suggested that BMP4- dependent stress erythropoiesis pathway is impaired when Hedgehog signaling is inhibited. This observation is consistent with the previous studies from our lab (Perry et al., 2009a). The defects in BMP4 expression suggests that the defects in stress progenitor development/expansion lead to lower/delayed BMP4
expression. One explanation for this data is that progenitors make a signal that induces BMP4 expression. Preliminary data in the lab suggests that this signal is GDF15.

Despite its essential role in regulating stress erythropoiesis, inhibiting Hedgehog signaling by cyclopamine has no effects on steady-state hematopoiesis.

Whether Hedgehog signaling is dispensable for hematopoiesis has been controversial, as discussed in Chapter 1. By using cyclopamine, our conclusion is consistent with two studies from other groups demonstrated by genetic models (Gao et al., 2009; Hofmann et al., 2009). This present study shows that Hedgehog signaling is required for stress, but not steady state, erythropoiesis. What genes that act downstream of Hedgehog signaling to regulate the specification of erythroid fate is currently under investigation.
References


Figures and Figure Legends

**Figure 3-1.** Experimental scheme of short-term radioprotection as the model of stress erythropoiesis.
Figure 3-2. Hedgehog signaling through Smo is required for short-term radioprotection (I). 100,000 of either Smo-/- or control bone marrow cells were transferred into lethally (950 rads) irradiated mice. The followings from recipients were analyzed during recovery. (A) Survival rate. (B)-(F) Complete blood counts (n=6 controls + 6 mutants, equal numbers of both genders). *p<0.1, **p<0.05, ***p<0.01.
Figure 3-3. Hedgehog signaling through Smo is required for short-term radioprotection (II). 500,000 of either Smo-/ or control bone marrow cells were transferred into lethally (950 rads) irradiated mice. The followings from recipients were analyzed during recovery. (A) Survival rate. (B)-(F) Complete blood counts (n=6 controls + 6 mutants, equal numbers of both genders). *p<0.1, **p<0.05, ***p<0.01.
**Figure 3-4. Smo-deficient mice exhibit defects in short-term radioprotection.** 700,000 of either Smo/- or control bone marrow cells were transferred into lethally (950 rads) irradiated mice. The followings from recipients were analyzed during recovery. (A) Survival rate (n=19 controls + 19 mutants, equal numbers of both genders). Data shown is representative of 3 individual transplantation experiments. (B)-(H) Complete blood counts (n=18 controls + 28 mutants, equal numbers of both genders). (G) Size and weight of spleens, and total live cell number (n=3-4 each). Data shown is representative of 2 individual transplantation experiments. *p<0.1, **p<0.05, ***p<0.01.
**Figure 3-5. Hedgehog signaling through Smo is required for short-term radioprotection (III).** 1,500,000 of either *Smo*−/− or control bone marrow cells were transferred into lethally (950 rads) irradiated mice. The followings from recipients were analyzed during recovery. (A) Survival rate. (B)-(F) Complete blood counts (n=4 controls + 6 mutants). *p<0.1, **p<0.05, ***p<0.01.
Figure 3-6. Residual expression of Smo in a conditional knockout mouse in one experiment.
Figure 3-7. Hedgehog signaling is required for short-term radioprotection. Bone marrow cells from wildtype mice were incubated with either cyclopamine or vehicle for 2 hours, and 500,000 cells were transferred into lethally (950 rads) irradiated mice. 24 hrs post transplantation, mice were given injections of either cyclopamine or vehicle for another 5 days. The followings from recipients were analyzed during recovery. (A)-(G) Survival rate and complete blood counts (n=20 vehicle + 20 cyclopamine-treated). (H) Size and weight of spleen (n=3-4 each). Data shown is representative of 3 individual transplantation experiments. **p<0.05, ***p<0.01.
Figure 3-8. Hyperactivation of Hedgehog signaling results in faster and better recovery from stress. 500,000 of either Ptch-/- or control bone marrow cells were transferred into lethally (950 rads) irradiated mice. Complete blood counts (A-D) were analyzed during recovery (n=6 controls + 10 mutants). *p<0.1, **p<0.05, ***p<0.01.
Figure 3-9. Flow cytometry analysis of spleen erythroid progenitor populations from Smo⁻/⁻ or cyclopamine-treated transplant survivors. Spleen cells were isolated and analyzed at the indicated times from control transplants or survivors transplanted with Smo⁻/⁻ or cyclopamine-treated bone marrow cells. These cells were then stained with antibodies for flow cytometry analysis. (A, B) Expression of cKit. (C, D) Flow cytometry analysis of stress erythroid progenitor populations.
Figure 3-10. Inhibition of Hedgehog signaling affects spleen erythroid stress progenitor differentiation and lineage specification in spleen.

(A) Flow cytometry analysis of donor-derived stress erythroid progenitors in spleen (upper panel), and bar graphs of subsets (lower panel). (B) Flow cytometry analysis of erythroid subsets in terminal-differentiated (Ter119+) cells. Quadrant gates were based on unstained and single-staining controls. **p<0.05, ***p<0.01.
A.

spleen

day8

day10

bone marrow

No Ab
Vehicle
Cyclopamine

CD45.2
D.

**Vehicle (n=3 each)**

- day6 bone marrow:
  - CD34+ 55.4%
  - CD34+ 49.9%
  - CD34+ 64.5%

- day9 bone marrow:
  - CD34+ 33.1%
  - CD34+ 30.1%
  - CD34+ 32.2%

**Cyclosporine (n=3 each)**

- day6 bone marrow:
  - CD34+ 55.4%
  - CD34+ 44.5%
  - CD34+ 55.3%

- day9 bone marrow:
  - CD34+ 52.1%
  - CD34+ 57.3%
  - CD34+ 52.1%

---

**Day 6 bone marrow**

- Cell number per 100,000 cells:
  - Vehicle: 250
  - CY: 275
  - p=0.077

**Day 9 bone marrow**

- Cell number per 100,000 cells:
  - Vehicle: 150
  - CY: 383
  - 3.83X (p=0.025)
**Figure 3-11. Inhibition of Hedgehog signaling causes increase of LSK and Sca1+Kit+CD34+ populations in response to acute needs.** Spleens or bone marrow cells from vehicle- or cyclopamine-treated transplant recipients were harvested at indicated times, and analyzed by multiparameter flow cytometry to identify various populations. (A) Analysis of donor-derived (CD45.2+) cells from spleens or bone marrow. (B) Analysis of donor-derived LSK cells in spleen. (C) Analysis of Sca1+Kit+CD34+/− in spleen. (D) Analysis of Sca1+Kit+CD34+ in bone marrow. Quadrate gates were based on unstained and single-staining controls.
Figure 3-12. Flow cytometry analysis of stem cell progenitor populations from cyclopamine-treated transplant survivors. (A) Spleens or (B) bone marrow cells from vehicle- or cyclopamine-treated transplant recipients were harvested at indicated times, and analyzed by multiparameter flow cytometry to identify various populations. Flow cytometry plots and bar graphs are shown. Quadrant gates were based on unstained and single-staining controls.
Figure 3-13. Hedgehog signaling is required for the formation of stress BFU-E in the spleen. 700,000 of either Smo-/- or control bone marrow cells were transferred into lethally irradiated mice (A, C, D). 500,000 of either cyclopamine- or vehicle- treated bone marrow cells were transferred into lethally irradiated mice, followed by another 5 injections (B). At day8 and 10 post transplantation, the spleen cells were harvested, the indicated populations were sorted and plated for BFU-E (A,B,C) or CFU-E (D) assays with various conditions. Data shown is the representative from 2 individual transplantation experiments for both genetic and pharmacological strategies. **p<0.05, ***p<0.01.
Figure 3-14. Expression of BMP4 protein in bone marrow transplants. Spleen sections obtained from transplants at the indicated days were stained with fluorescent antibodies. Protein expression (fluorescence) level was analyzed under confocal microscope. Expression of CD45.2 alone (donor-derived cells), BMP4 alone, bright field merged with stainings of both CD45.2 and BMP4, and merge of CD45.2 with BMP4 (co-localization of donor-derived cells with BMP4-expressing cells) are shown in the order from upper left, upper right, lower left to lower right.
Figure 3-15. Increased apoptotic cells and erythroblast EryA population in cyclopamine-treated transplant survivors. (A) Representative flow cytometry analysis of AnnexinV and PI-stained cells. (B) The apoptotic cells are from donor-derived cells. (C) Flow cytometry analysis of erythroblast subsets. Data shown is representative of 2 individual transplantations.
A.

Lethal Irradiation 950 cGy

Primary Transplantation

Sort spleen progenitor cells (Day 8)
(100,000 cKit<sup>lo</sup> CD71<sup>lo</sup> Ter119<sup>-</sup>cells)

Secondary Transplantation

Injection of Vehicle

Injection of Cyclopamine
Figure 3-16. HH signaling is required to maintain erythropoiesis after erythroid lineage specification. (A) Experimental scheme of secondary transplantation with the splenic erythroid lineage-specific stress progenitor. (B) Survival rate (n=2 vehicle- + 4 cyclopamine-treated). (C) Flow cytometry analysis on erythroid population in spleens from control recipients and the cyclopamine-treated transplant survivor.
A.

B. [Graph showing total cell number over time with different conditions: shh, shh 24hr + Cyclopamine]
D.

\[ \text{shh} \rightarrow \text{Cy} \]

\[ \text{gated from Sca1+Kit+} \]
E.

**whole bone marrow**

shh  \hspace{1cm} \text{Shh} \rightarrow \text{Cy}

day 1+1

day 1+3

day 1+5

day 1+7

day 1+9

Order of maturation

CD71 \hspace{1cm} \text{Ter}119

CD71 \hspace{1cm} \text{Ter}119
whole bone marrow

shh  Shh → Cy

F.

day1+1
day1+3
day1+5
day1+7
day1+9

cKit

Sca1
whole bone marrow

shh  Shh → Cy

day 1+1

day 1+3

day 1+5

day 1+7

day 1+9

gated from Sca1+  cKit
H.

Culture day 1+7

**Vehicle**

**Cyclopamine**

---

Culture day 1+7

**Shh**

**Shh → Cy**

---

gated from Kit+Scal+
Figure 3-17. *In vitro* culture of bone marrow cells and functional assay establish a system to demonstrate the roles of HH signaling in stress erythropoiesis after erythroid fate specified. Bone marrow cells were cultured in our *in vitro* culture system, followed by examinations on differentiation and proliferation during the course of culture. (A) Experimental scheme. (B) Total numbers of live cell post culture. (C) Total BFU-E number after culture for indicated time, and representatives of BFU-E colonies (20X) after culture. Cyclopamine-treated cells gave rise to bigger colonies after 2 days. No difference in size between Shh and Shh → Cy thereafter. (D) Flow cytometry analysis on stress erythroid progenitors. (E) Flow cytometry analysis on erythroid population. (F) Flow cytometry analysis on Sca1+ and cKit+ population. (G) Flow cytometry analysis on Sca1+Kit+CD34+/ populations. (H) Another set of representative data. Cell numbers and flow cytometry data shown are representatives of at least 4 individual culture experiments.
Figure 3-18. Defects in cycloamine- treated culture can be rescued by Shh \textit{in vitro} and \textit{in vivo}. 
D.

Figure 3-19. **Sca1+Kit+CD34+ and Sca1+Kit+CD34- cells provide short-term radioprotection.** Total bone marrow cells from wildtype mice were cultured with or without cyclopamine. Sca1+Kit+CD34+ and Sca1+Kit+CD34- cells were sorted after culture. Four types of samples in total were analyzed by flow cytometry and transplanted to lethally irradiated mice, respectively. (A) flow cytometry analysis of Sca1+Kit+CD34+/- populations grown in *ex vivo* culture post sort. (B) Survival curves of recipients transplanted with the sorted Sca1+Kit+CD34+ or Sca1+Kit+CD34- cells from *ex vivo* cultures. Regeneration curve of lineage cells post transplantation. (C) flow cytometry analysis of CD3e, Mac-1, B220 and Gr-1 at day55 post transplantation. (D) Hgb analysis by cellulose acetate gel electrophoresis.
A.

Stress Hematopoiesis (short-term radioprotation)

- Acidified Water (2 weeks)
- Antibiotic (3 days)

- CD45.1 Recipient

- Donor bone marrow cells:
  - Incubation with Cycloamine or Vehicle for 2 hrs

- Irradiation 950 cGy

- Transplantation

- Injection of Cycloamine or Vehicle

No irradiation & transplantation

B.

- RBC ($10^6$ cells/μL)
- % Hematocrit
- Hemoglobin (g/dL)
- Ret ($10^6$ cells/μL)
- Platelet ($10^6$ cells/μL)
- WBC ($10^6$ cells/μL)

Days post injection: 0, 14
C.

Day9 Bone Marrow without Transplantation

**Vehicle (n=3 each)**

**Cyclopamine (n=3 each)**

- **CD71**
- **Ter119**
- **gated from Ter119hi**
- **FSC**
- **Sca1**
D.

Day9 Spleen without Transplantation

**Vehicle** (n=3 each) ___________________________ **Cycloamine** (n=3 each) ___________________________

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Figure 3-20. Inhibition of HH signaling does not affect homeostatic erythropoeisis.

(A) Experimental scheme of the homeostatic control compared to stress erythropoeisis.

(B) Complete blood counts (n=4 controls + 4 cyclopamine-treated). (C, D) flow cytometry analysis on erythroid population and LSK cells in bone marrow cells or spleens. (E) Representative flow cytometry analysis of annexinV and PI-stained cells.

Data shown (C-E) is the representative of 2 individual experiments.
B.

Figure 3-21. Role of Hedgehog signaling in stress erythropoiesis. Model for role of Hedgehog signaling in stress erythropoiesis (B) Summery of in vivo and in vitro results observed in Hedgehog signaling- inhibited cells in the present study.
Figure S3-1. The *in vitro* culture model recapitulates the short-term radioprotection model. The *in vitro* culture model (described in Chapter 4) was served as a tool to circumvent the unavailability in the *in vivo* model due to the severe lethality. Bone marrow cells were cultured in complete liquid culture medium (Shh) or complete medium with cyclopmaine and without shh (Cyclopamine) to mimic the *in vivo* genetic and pharmacological models where Hedgehog signaling are mutant or blocked. The results are consistent with what were observed in spleens *in vivo* (Figure 3-9, 10, 11, and 12).
Figure S3-2. Expression of CD133 in the enriched CD34+Kit+Sca1+CD71+Ter119- and CD34-Kit+Sca1+CD71+Ter119- populations.
Acknowledgements

For the work described in this chapter, I would love to thank many people who once helped. Lab specialist Shailaja Hedge for retro-orbital injection of cells, and assistance when swamped; former member Dr. Omid Harandi who insisted to inject cells at the time I started this project. Susan Magargee, Dr. Ningchun Xu, and Nicole Zembower in flow cytometry and microscope facility for their help on machines; Susan Magargee and Dr. Ningchun Xu for sorting cells; Jeff Dodds for discussion with histology (data not shown); Ruth Haldeman in electron microscopy facility for preparing sections; and also staff members in Centralized Biological Lab.
Chapter 4.

From Exploring the Role of GDF15 in Stress Erythropoiesis to Expanding Stress Erythroid stem cells in vitro

Abstract

The following chapter includes two parts. The first part is the summary of my study on role of GDF15 in stress erythropoiesis. The second part is the summary of my study on establishment of a culture system which expands stress erythroid progenitor in vitro. The function of these in vitro expanded cells was examined by in vitro colony assays and in vivo bone marrow transplantation to wild type recipients and a murine thalassemia model. In a murine thalassemia model where no erythroid cells can be generated by the host, we successfully showed that the enriched stress erythroid progenitors expended in vitro can provide erythroid- specific rescue to prevent lethality, alleviate anemia and iron overload in the erythropoiesis- deficient mice. These thalassemia mice received 1,000 stress erythroid progenitors (Sca1+Kit+CD71+Ter119- cells) in September, 2010 are currently (July, 2011) alive with normal hematocrit, suggesting that these self- renewing stress erythroid progenitors are erythroid stem cells. This “proof of concept” study demonstrates the working model established in our lab.
Introduction

Through a complete understanding of the mechanisms that regulate stress erythropoiesis, including characteristics of stress erythroid progenitors and roles of signaling pathways, we may achieve one of our goals – recapitulating in vitro the in vivo expansion and differentiation of stress erythroid program, which could be applied to developing therapies beneficial to humans. Our lab has identified and characterized factors which are required for or play important roles in stress erythropoiesis. One focus of our work has been the signal(s) that regulate BMP4 expression in the BMP4-dependent stress erythropoiesis pathway. I have characterized that hypoxia induces BMP4 expression through binding of Hypoxia Inducible Factor (HIF) to HIF-responsive elements in the BMP4 locus during the recovery from PHZ-induced acute anemia (Wu and Paulson, 2010) (Chapter 2). In addition, the study done by former students (Perry et al., 2009a) and myself (described in Chapter 3) also demonstrated that Hedgehog signaling is required for maintaining the BMP4-dependent stress erythropoiesis pathway. Another candidate for regulating BMP4 expression is Growth Differentiation Factor 15 (GDF15), which is a member of the TGFβ (transforming growth factor-β) family. In an erythroblast transcriptome project, GDF15 expression was discovered during late erythroid differentiation. Jeff Miller’s group discovered that GDF15 was highly expressed in patients with ineffective erythropoiesis such as thalassemia syndromes, congenital dyserythropoiesis and some acquired sideroblastic anemias. In these patients, GDF15 suppresses hepcidin expression, which leads to iron overload. Hepcidin is a peptide formed in the liver that degrades ferroportin to negatively regulate iron uptake (Tanno et
High-level GDF15 expression is not a feature of normal erythropoiesis (reviewed in (Tanno et al., 2010)).

In collaboration with Dr. Miller’s group, we set out to examine the role of GDF15 in stress erythropoiesis. Preliminary experiments done by Shailja Hegde in our lab showed that mutation of GDF15 in a β thalassemia heterozygous background (GDF15−/−; Hbbthal3+/+) was embryonic lethal. This result was surprising given that GDF15−/− mice show no phenotype and Hbbthal3+/+ exhibit a mild anemia. Examination of embryos showed that double mutants were extremely anemic at E14.5. Fetal liver erythropoiesis requires BMP4-dependant stress erythropoiesis pathway (Porayette and Paulson, 2008). Double mutant embryos failed to express BMP4 in the fetal liver (data not shown).

The experiments done by me are summarized as follows. GDF15−/− spleen cells formed fewer and smaller stress BFU-Es than controls when plated with Epo+BMP4+SCF+hypoxia (Figure 4-1), showing that GDF15 is required for stress erythroid progenitors that are resident in spleen to respond to BMP4+SCF+hypoxia. In vitro culture with GDF15 overnight allowed bone marrow cells to adopt the stress progenitor fate leading to expansion of stress BFU-Es, both number-wise and size-wise (Figure 4-2). GDF15−/− and control bone marrow cells were cultured in vitro with various combinations of factors and followed by BFU-E assay and flow cytometry analysis. Lower percentage of Sca1+Kit+ population was observed in the GDF15−/− cells post culture (Figure 4-3B, 4-3D, and 4-4A). GDF15−/− bone marrow cells exhibited delayed expansion of stress BFU-Es post culture (Figure 4-4B). In general, GDF15−/− spleen and bone marrow cells formed smaller and fewer stress BFU-Es than controls among all
assays performed. *GDF15/-* cells appeared to have defects in response to acute anemia, demonstrated in both PHZ and short-term radioprotection models. Most of *GDF15/-* mice died early post treatment of PHZ (3 out of 4 died within 72 hours). The spleen cells were obtained from the remaining survivor (1 out 4) at 90 hours post treatment and assayed for BFU-E formation. *GDF15/-* spleen cells formed fewer and smaller stress BFU-Es (Figure 4-5). To compare the recovery of *GDF15/-* mice to controls, reduced dose of PHZ (75% of the original) was used in the later experiment. *GDF15/-* mice exhibited lower values of Hgb, WBC counts and platelet counts, and reduced expression of BMP4 post PHZ treatment (Figure 4-6). These data show that *GDF15/-* mice were defective in recovery from PHZ-induced acute anemia. GDF15/- cells also has defects in providing short- term radioprotection (Figure 4-7). Delayed and reduced induction of BMP4 (RNA and protein) was also observed during the recovery post transplantation (Figure 4-7E and F). In addition, preliminary data shows that stress erythroid progenitors in *GDF15/-* mice have defects in responding to Epo- promoted erythropoiesis (Figure 4-8). Analysis of stress erythroid progenitors in *GDF15/-* mice by flow cytometry suggested that, the progenitors resident in *GDF15/-* might be different from their wild type counterpart (Figure 4-9, 4-10). Increased apoptosis was also observed in the *GDF15/-* spleen erythroid populations (Figure 4-11). Lower expression of BMP4 was also observed in GDF15/- spleen cells compared to controls (Figure 4-12). I also tested mechanistic hypotheses whereby GDF15 upregulates BMP4 via hypoxia by *in vitro* assays, and designed another mechanistic study to examine GDF15 upregulates BMP4 expression via hypoxia by *in vivo* assays, which is currently tested by a lab member.
While more experiments are needed to be done for conclusion, these data showed that GDF15 plays a role in expanding stress erythroid progenitors at times of acute need.

My study regarding the establishment of an in vitro culture that expands stress erythroid progenitors is described as follows.
Results

*In vitro* incubation with GDF15 overnight promoted the expansion of stress BFU-Es capable of forming larger colonies (Figure 4-2). Following this observation, I tested whether addition of GDF15 to our known factors (BMP4, hypoxia, SCF, hedgehog, and Epo) promoted the expansion of cells *in vitro*. Our lab has previously demonstrated that BMP4 act in concert with SCF and hypoxia to promote expansion of stress BFU-Es (Perry et al., 2007b). Later we identified that Hedgehog signaling maintains the BMP4-dependent stress erythropoiesis pathway in the spleen (Perry et al., 2009a)(Chapter 3). Therefore, I cultured bone marrow cells using 4 conditions, (1) “Epo only” as the baseline, (2) “Epo+BMP4+SCF+hypoxia”, (3) “Epo+BMP4+SCF+hypoxia+Shh”, and (4) “Epo+BMP4+SCF+hypoxia+Shh+GDF15”. Both bone marrow and spleen cells were isolated from wild type, and were incubated with the various combinations of factors for 7 days. No further supplementary of fresh medium or cytokines was added after the initial culture in this pilot test; data were collected at 3 days and 7 days. As shown in Figure 4-3A, spleen cells and bone marrow cells responded very differently. The addition of factors had little effect on maintenance of spleen progenitor viability. In contrast, the addition of GDF15 increased the viability of bone marrow cells on both day3 and day7. In fact, the number of cells in the bone marrow culture actually increased when there were GDF15 with other factors (from day3 to day7, bone marrow in Figure 4-3A). The data showed that GDF15 promotes the expansion of bone marrow cell significantly. To examine whether GDF15 did indeed expand the population of stress erythroid progenitors, we measured Kit+Sca1+ cells, the population enriched in hematopoietic stem cells and self-renewing stress erythroid progenitor (Kit+Sca1+CD71+Ter119-). As
shown in Figure 4-3, addition of BMP4+SCF+hypoxia, BMP4+SCF+hypoxia+Shh, or BMP4+SCF+hypoxia+Shh+GDF15 all increased the percentage of Kit+Sca1+ cells in bone marrow cultures (Figure 4-3B). However, it is an opposite scenario in the case of spleen cells (Figure 4-3D). Erythroid populations were also analyzed. While subsets of CD71+Ter119- (CD71\textsuperscript{lo} and CD71\textsuperscript{hi}) could still be seen in bone marrow at 3 days and 7 days post cultures (Figure 4-3C), these erythroid populations in spleen were nearly gone after 3 days of \textit{in vitro} culture (Figure 4-3E). One hypothesis was that, these factors promote differentiation of erythroid progenitors in the spleen. Importantly, addition of GDF15 to Epo+BMP4+SCF+hypoxia+shh enhanced the \textit{in vitro} expansion of bone marrow cells. These factors together induced stress BFU-Es (Figure 4-13). Therefore this culture system provides a tool for \textit{in vitro} functional analysis, especially at times when \textit{in vivo} study is inaccessible (such as experiments described in Chapter 3).

Although GDF15 in combination with other factors promoted the expansion of stress BFU-Es, \textit{in vitro} colony formation experiments needed to be followed by \textit{in vivo} analysis of the ability of these progenitors to provide recovery from acute anemia. During the course of my study, roles of Hedgehog signaling and GDF15, as well as \textit{in vitro} liquid culture were investigated simultaneously. For the \textit{in vivo} study, I cultured bone marrow cells using a variety of growth factor combinations for 6.5 days. The cells were then tested by using the short-term radioprotection assay. The culture conditions included, (1) Epo+BMP4+SCF+hypoxia+shh+GDF15 (i.e., LC), (2) Epo+BMP4+SCF+hypoxia+shh (i.e., LC w/o GDF15), (3) Epo+BMP4+SCF+hypoxia+GDF15 (i.e., LC w/o shh), (4) Epo+BMP4+SCF+hypoxia (i.e, LC w/o shh&GDF15). Cells from these cultures (2x10\textsuperscript{5} cells, unfractioned) were
transplanted to lethally irradiated wild type mice. Same amount (2x10^5 cells) of fresh bone marrow cells from wild type served as a control and reference for transplantation (“fresh” in Figure 4-14A). *In vitro* culture of bone cells with all factors (“LC”) provided short-term radioprotection (Figure 4-14A). In fact transplantation of just 5,000 of cultured cells was sufficient to ensure 100% survival. Among the transplantation experiments done by the author and other members in lab, the survival rate of transplants with 1x10^5 of fresh wildtype bone marrow cells were usually less than 100%. Clearly the culture condition greatly amplify the number of stress progenitors such that lethally irradiated mice can be rescued by only 5,000 of cells grown in this culture system.

Analysis of survival of mice transplanted with various cultured conditions showed that, cells generated with “LC without shh” provided short-term radioprotection. 50% of “LC without GDF15” and 100% of “LC without shh & GDF15” transplants died in the immediate post transplantation period. The data showed both Shh and GDF15 are the necessary ingredients of this culture system, which is consistent with our studies on Hedgehog signaling and GDF15. Analysis of apoptosis revealed that, addition of GDF15 decreased apoptosis (Figure 4-14B). This data is consistent with two of my previous observation. First, more apoptotic cells existed in GDF15-/− spleens. Second, GDF15 increased the number of viable cells in bone marrow culture (Figure 4-3). In summary, cells grown from an *in vitro* liquid culture system containing BMP4+SCF+shh+GDF15+Epo+hypoxia can provide short-term radioprotection.

The next test was to examine the erythroid recovery of the recipients transplanted with these cultured cells. Heterozygous thalassemia mice were served as recipients in this set of experiment as a pilot test of stress erythroid progenitors’ effects.
on an anemia model. 2x10^5 cultured cells (unfractioned) were transplanted to lethally irradiated mice. 2x10^5 fresh bone marrow cells were served as a control. As shown in Figure 4-15, erythroid recovery of the recipients with cultured cells was similar to controls, demonstrating that these cultured cells provided erythroid short-term radioprotection. The recovery in other lineage (platelet and white blood cells) was delayed compared to controls, suggesting that the short-term protection provided by these cultured cells was erythroid- restricted, which is consistent with our previous work using population I (Kit+CD71^{lo}Ter119-) cells isolated from recovering mice post transplantation (Harandi et al., 2010).

These tests established our confidence in generating functional stress erythroid progenitors with this \textit{in vitro} culture system. To demonstrate the ability of these erythroid progenitors to generate new red blood cells in a clinical situation, we turned to a strategy that used recipients who were incapable of generating red blood cells on their own – a murine model of β^0 thalassemia. We utilized the Hbb^thal3 allele generated by Oliver Smithies (Yang et al., 1995), which deleted both the β major and β minor genes. Because the homozygote is embryonic lethal, this thalassemia model is generated by transplanting fetal liver cells from homozygous embryos (at embryonic day14) into lethally irradiated wildtype recipients (Levasseur et al., 2003). These transplants gradually develop a lethal anemia and die between 3-5 weeks post transplantation (Figure 4-16A). Cells grown \textit{in vitro} for 6 days were collected, washed, stained with antibodies followed by sorting. The enriched stress erythroid progenitor population “Sca1+Kit+CD71^{lo}Ter119- cells” (20,000 or 1,000 cells) were transplanted to the thalassemia model mice after they had developed anemia (3 weeks post generation). Two conditions of myeloablative conditioning were
tested, (1) 200 rads of irradiation given over 2 doses, or (2) no irradiation. 500,000 cultured cells without fraction served as a control. 100% of transplants survived post transplantation (experiments #1: Sep, 2010- present; #2: Feb, 2011- present). As shown in Figure 4-16, these stress erythroid progenitors generated in vitro not only completely prevented the lethality but gave rise to red blood cells (Figure 4-16B). The values for hematocrit and hemoglobin were significantly improved. In fact, these values at the end of analysis were similar to healthy normal mice. The recovery curves of other lineages were also analyzed (Figure 4-16C). Analysis of the hemoglobin alleles confirmed that the generation of erythroid lineage in these transplants was donor-derived (Figure 4-16, left). The origin of other lineages was examined by staining with antibodies against Mac-1, B220 and CD3ε; the data showed that these lineages were derived from recipients (Figure 4-16D, right). In conclusion, stress erythroid progenitors (Sca1+Kit+CD71loTer119-) generated from this culture system recapitulate the in vivo self-renewing stress erythroid progenitors (Kit+CD71loTer119- spleen cells sorted at day8 post transplantation) that provided erythroid-specific short-term radioprotection (Harandi et al., 2010). Moreover, these progenitors prevent lethality and alleviate anemia, when transplanted into a murine model of β0 thalassemia.

In addition to alleviating lethal anemia, it is also important to determine whether other pathological phenotypes in this model can be corrected by progenitors generated in our culture system. One of the pathological characteristics of thalassemia is iron overload. Sections of spleen and liver were stained for iron using Prussian blue reaction. In the spleens of β0 thalassemia mice, severe iron overload was observed at the time of examination (day 22 post generation) (Figure 4-17A); such iron overload was not
seen in liver. After just 40 days post treatment with the in vitro cultured cells, positive iron staining was significantly reduced, if not completely absent (Figure 4-17B). In addition, the size of spleens and hearts of these recipients became similar to wild type mice (Figure 4-18). The data demonstrate the therapeutic potential of these stress erythroid progenitors to alleviate chronic anemia and allow a normalization of iron utilization in murine β^0^ thalassemia.

In conclusion, this in vitro culture system with a cocktail of growth factors that includes BMP4, SCF, Shh and GDF15 successfully generated functional stress erythroid progenitors. In addition, I show “proof of principle” experiments that in vitro generates stress erythroid stem cells can rescue a murine model of β^0^ thalassemia. I am presently finishing our characterization of these mice by examining hepcidine expression and the level of serum ferritin, which is the primary intracellular iron- storage protein.
References


Figure 4-1. Stress BFU-Es formation in *GDF15/-* mice and controls.
Figure 4-2. GDF15 promotes bone marrow cells to form stress BFU-Es. Bone marrow cells were incubated with indicated factor(s) for 20 hours and then assayed for (A) stress BFU-E formation and (B) BMP4 expression (representative of two experiments).
A.

**BM_total live cell number**

- medium + Epo
- med. + 1%O2 + Epo + BMP4 + SCF
- med. + 1%O2 + Epo + BMP4 + SCF + shh
- med. + 1%O2 + Epo + BMP4 + SCF + shh + GDF15

**Spleen_total live cell number**

- medium + Epo
- med. + 1%O2 + Epo + BMP4 + SCF
- med. + 1%O2 + Epo + BMP4 + SCF + shh
- med. + 1%O2 + Epo + BMP4 + SCF + shh + GDF15
bone marrow

WT

day3

GDF15/-

day7

WT

GDF15/-
spleen

WT

day3

GDF15-/-

Medium+Epo +BMP4 +SCF +1%O2 +shh +shh+GDF15

Medium+Epo +BMP4 +SCF +1%O2 +shh +shh+GDF15

Medium+Epo +BMP4 +SCF +1%O2 +shh +shh+GDF15

Medium+Epo +BMP4 +SCF +1%O2 +shh +shh+GDF15

WT

day7

GDF15-/-
Figure 4-3. Analysis of cells post culture with various factors that play important roles in stress erythropoiesis (I). (A) Numbers of total live cells post culture. (B) Kit and Sca1 population in cultured bone marrow cells. (C) Erythroid population in cultured bone marrow cells. (D) Kit and Sca1 population in cultured spleen cells. (E) Erythroid population in cultured spleen cells.
A.

WT

GDF15-/-

day2
day6

Kit

Sca1
stress BFU-Es formed in Epo + 1%O₂ + SCF + BMP4

Day2 post LC
Day4 post LC
Day6 post LC

WT

GDF15-/-
Figure 4-4. Analysis of cells post culture with various factors that play important roles in stress erythropoiesis (II). (A) Flow cytometry analysis of Sca+Kit+ populations (Representative data of 3 individual liquid culture experiments). (B) Formation of stress BFU-Es post *in vitro* culture.
Figure 4-5. Stress BFU-Es formation post PHZ- treatment.
A.

RBC post-PHZ treatment

Hemoglobin post-PHZ treatment

Hematocrit post-PHZ treatment

Reticulocytes post-PHZ treatment

n = 6-7 each, * p<0.05, ** p<0.01, *** p<0.005, ****p<0.001

B.

Platelet post-PHZ treatment

WBC post-PHZ treatment

n = 6-7 each, * p<0.05, ** p<0.01, *** p<0.005, ****p<0.001
Figure 4-6. Response of GDF15-/- mice to PHZ treatment (75% of regular dose in the lab). (A) Erythroid recovery. (B) Recovery of platelets and white blood cells. (C) BMP4 expression by qRT-PCR (n=4 each). (D) Preliminary examination on genes by RT-PCR (n=4 each).
A.

Survival post Bone Marrow Transplantation

Survival Rate

Days post bone marrow transplantation

B.

Spleen Weight

Spleen Weight (mg)

days post BMT

WT

GDF15/-

P=0.013
C.

Hematocrit

Reticulocyte

Hemoglobin

D.

WBC

Platelets
E.

Relative Quantification by qPCR (internal = 18s rRNA)

BMP4 expression in transplants
Figure 4-7. Analysis on recipients post transplantation with GDF15-/- or wildtype bone marrow cells. (A) Survival curve. (B) Spleen weight. (C) Recovery of erythroid lineage. (D) Recovery of platelet and white blood cells. (C-D: * p<0.05, **p<0.01, ***p<0.005, ****p<0.001) (E) Expression of BMP4 in the spleen by qRT-PCR (n=2 each). (F) Expression of BMP4 in the spleen by immnostaining (representative data from each time point). (G) Preliminary examination of gene expression in the spleen by RT-PCR (n=2 mice each).
**Figure 4-8.** Response of GDF15/- mice to Epo. (A) Flow cytometry plots of stress erythroid progenitors. (B) Percentage and numbers of stress erythroid progenitors shown in (A).
B.

unstained control

GDF15-/- spleen (n=3)

Control spleen (n=3)
Figure 4-9. Flow cytometry plots of populations of the spleen cells in *GDF15/-* mice and wild types. (A) Erythroid population. (B) Kit, Sca1, and Kit+Sca1+ stress erythroid progenitors.
Figure 4-10. Flow cytometry plots of populations of the bone marrow cells in 

*GDF15/-* mice and wild types.
Figure 4-11. Analysis of apoptosis in *GDF15/-* mice and wild types. (A) Whole spleen cells. (B) Erythroblast subsets in spleen cells. (C) Erythroblast subsets in bone marrow cells. Flow cytometry plots shown (left) are representative of 3 mice for each genotype.
Figure 4-12. BMP4 expression in GDF15-/- mice and wild types.
Figure 4-13. Stress BFU-Es colonies and flow cytometry plot of stress erythroid progenitors post *in vitro* culture.
Survival post- Bone Marrow Transplantation
Figure 4-14. Survival rate (A) and apoptosis (B) post transplantation with in vitro cultured cells.
Figure 4-15. Recovery from anemia post transplantation with *in vitro* cultured cells in a thalassemia model (heterozygous).
A. Hematocrit of thalassemia model mice (n=6 mice)

B. Enriched population

\[ S+K^{lo/hi}CD71^{+/lo} Ter119^{-} \text{ (20K or 1K)} \]

Sub-lethally irradiated (100 rad x2) or non-irradiated th3 / th3
C.

**Platelet**

![Platelet Graph]

**WBC**

![WBC Graph]
D.

Figure 4-16. Recovery from anemia post transplantation with *in vitro* cultured cells in a thalassemia model (homozygous). (A) Development of anemia in the thalassemia model mice. (B) Erythroid recovery. (C) Recovery of platelets and white blood cells. (D) The origin of erythroids (left) and monocytes (right, day41 post transplantation with *in vitro* cultured cells.)
Figure 4-17. Staining of spleen sections by Prussian blue reaction. (A) 20X or 40 X images of thalassemia mice without treatment of cultured cells. (B) 20X images of thalassemia mice treated with cultured cells, from two individual experiments. (blue: iron; red: nuclei)
A.

- Day 41
- Day 63

Day 148 (of expl#2, upper left); 10 months (of expl#1, lower left); Thal/+ (upper right); WT (lower right)
B.

Figure 4-18. Size of organs of thalassemia mice post treatment of stress erythroid progenitors. (A) spleens (Yellow bar= 1 inch, red bar= 1cm) and (B) hearts (scale in inch).
Acknowledgements

For my work described in this chapter, I would love to first thank my former labmates for their published findings -- Dr. Laurie Lenox for cloning $f/f$ gene; Dr. John Perry for his studies on BMP4, SCF, hypoxia and Hedgehog signaling in stress erythropoiesis; Dr. Prashanth Porayette for his study on BMP4-dependent stress erythropoiesis pathway in fetal liver; Dr. Omid Harandi for his study on BMP4-dependent stress erythropoiesis pathway in short-term radioprotection and “population I”. In addition, lab specialist Shailja Hegde for retro-orbital injection of cells and setting up a repetition experiment while my plans of travel and experiments were pending due to visa application; John Cantolina in electron microscopy facility for preparing sections; Susan Magargee and Nicole Zembower in flow cytometry and microscope facility for their help on machines; Susan Magargee for sorting cells; and also staff members in Centralized Biological Lab.
Appendix

Murine Erythroid Short-term Radioprotection Requires a BMP4 Dependent, Self Renewing Population of Stress Erythroid Progenitors

Forward

The following chapter is adapted from the manuscript appearing in Omid F. Harandi, Shailaja Hedge, Dai-Chen Wu, Daniel McKeone and Robert F. Paulson. Murine erythroid short term radioprotection requires a BMP4 dependent, self renewing population of stress erythroid progenitors. Journal of Clinical Investigation. 120(12): 4507 (2010). I performed experiments and analyzed data, which included analysis of bone marrow erythroid population during the recovery (day6, 8, and 10) post transplantation by flow cytometry and colony assays (described in context), culture of spleen stress progenitor population-I and analysis of population-I, II and III after culture by flow cytometry (Figure S5), RT-PCR analysis of Epo expression in kidney during the recovery post transplantation (Figure S9); and also suggested on the manuscript. My discoveries from studying the roles of Hedgehog signaling in stress erythropoiesis led to experiments shown in Figure 3 and Figure S6 which identified the bone marrow population that give rise to spleen stress erythroid progenitor. The following manuscript was written by Dr. Robert Paulson.
Abstract

Acute anemic stress induces a systemic response designed to increase oxygen delivery to hypoxic tissues. Increased erythropoiesis is a key component of this response. Recovery from acute anemia relies on stress erythropoiesis, which is distinct from steady state erythropoiesis. In this study we show that the BMP4 dependent stress erythropoiesis pathway is required and specific for erythroid short-term radioprotection following bone marrow transplant (BMT). BMP4 signaling regulates the expression of Scl and Gata2 to promote the development of three populations of stress erythroid progenitors which expand in the spleen following BMT. These progenitors do not correspond to previously identified bone marrow steady state progenitors. The most immature population of stress progenitors is capable of self renewal, while maintaining erythropoiesis without contribution to other lineages when serially transplanted into irradiated secondary and tertiary recipients. These data show that during the immediate post transplant period, the microenvironment of the spleen is altered, which allows donor bone marrow cells to adopt a stress erythropoietic fate and promotes the rapid expansion and differentiation of stress erythroid progenitors. Overall, these studies show that acute stress can profoundly impact the development of progenitors and promote the rapid production of key cell lineages needed for survival.
Introduction

Steady state erythropoiesis occurs in the bone marrow and is primarily homeostatic. New erythrocytes are continuously generated at a constant rate. The situation is dramatically different during embryogenesis and in response to acute anemic stress in adults. During these times, stress erythropoiesis predominates, which rapidly generates new erythrocytes at a rate beyond the capability of steady state erythropoiesis (Longmore, 2006; Socolovsky, 2007). The mechanisms that regulate this response are distinct from those that regulate steady state erythropoiesis. In response to anemic stress, the microenvironments of the fetal liver and adult spleen and liver promote the expansion of a specialized population of stress erythroid progenitors (Lenox et al., 2005a; Lenox et al., 2009; Perry et al., 2007a; Perry et al., 2008; Porayette and Paulson, 2008). Previous work has identified a mechanistic link between fetal liver erythropoiesis and adult stress erythropoiesis through the analysis of the murine flexed-tail (f) mutation (Coleman et al., 1969; Gruneberg, 1942; Mixter and Hunt, 1933; Russell, 1979a). f/f mutant mice exhibit near normal steady state erythropoiesis, but are defective in stress erythropoiesis. Our analysis of these mice showed that they have a defect in the expansion of a specialized population stress erythroid progenitors in the spleen, which we term stress BFU-E (Lenox et al., 2005a). These progenitors have a greater ability to generate large numbers of new erythrocytes faster than bone marrow steady state progenitors (Perry et al., 2007a). We identified a mutation in Smad5 in f mutant mice (Hegde et al., 2007; Lenox et al., 2005a), which implicated BMP signaling in stress erythropoiesis. We showed that BMP4 drives the expansion of stress BFU-E in the spleen during the recovery from acute anemia. The f mutation causes a defect in Smad5 splicing, which leads to the tissue specific expression
of a dominant negative form of Smad5. This mutated version of Smad5 inhibits BMP4 signaling by Smads1, 5 and 8 (Hegde et al., 2007; Lenox et al., 2005a).

In addition to BMP4, the expansion of stress BFU-E relies on the action of two additional signals, SCF and hypoxia. These three signals act in concert and are necessary and sufficient to recapitulate in vitro, the 45 fold expansion of stress BFU-E observed in the spleen during the recovery from acute anemia in vivo (Perry et al., 2007a). In vitro culture of spleen cells showed that the action of these three factors results in the expansion of a distinct stress erythroid progenitor cell (Kit+CD71+Ter119+), which expresses immature progenitor (Kit) and erythroid lineage specific markers (Ter119).

Previously we demonstrated that the stress erythroid progenitors in the spleen were completely mobilized in response to acute anemia. Following initial recovery it takes an additional two weeks to replenish the stress progenitor population. Our analysis showed that bone marrow cells repopulate the spleen and Hedgehog and BMP4 dependent signals are required for the development of new erythroid stress progenitors (Perry et al., 2009b).

Based on these observations, we developed a model where acute anemia leads to tissue hypoxia, which induces BMP4 expression in the spleen. These two signals plus SCF drive the rapid expansion of stress progenitors, which differentiate into new erythrocytes. Following recovery, bone marrow cells migrate to the spleen and develop into new stress erythroid progenitors primed to respond to the next anemic challenge.

Our previous work utilized phenylhydrazine (PHZ) induced acute hemolytic anemia as a model system to study stress erythropoiesis. This model however has several limitations. First, PHZ treatment only affects the erythroid lineage so it was not possible to analyze the potential of stress erythroid progenitors to develop into other myeloid lineages.
Second, the analysis of stress erythropoiesis in the PHZ model is focused on late stage events, the expansion and differentiation of stress progenitors resident in the spleen. For this report, we utilized a robust experimental system that allows us to analyze all steps in the BMP4 dependent stress erythropoiesis pathway and study the development of stress erythroid progenitors in vitro and in vivo. This system takes advantage of the observation that new erythrocytes must be rapidly generated immediately following bone marrow transplant. Although a single stem cell is sufficient to reconstitute the hematopoietic system of an irradiated mouse, in practice, short-term radioprotective cells must also be transplanted along with stem cells to alleviate the lethal anemia and potential for infection or hemorrhage caused by the myeloablative conditioning prior to transplant (Jones et al., 1996; Jones et al., 1990). In this study, we demonstrate that defects in the BMP4 dependent stress erythropoiesis pathway lead to a specific delay in erythroid short-term radioprotection, while the generation of platelets and neutrophils was unaffected. We show that spleen stress erythroid progenitors are derived from short-term reconstituting hematopoietic stem cells (STR-HSCs) which are characterized as CD34+Kit+Sca1+Lin- cells. Once in the spleen they develop into three distinct stress erythroid progenitor populations, each with a different erythroid potential. Donor cells with defects in BMP4 signaling (f/f) when transplanted at low dose ($10^5$) fail to provide erythroid short-term radioprotection which results in a lethal anemia. At higher doses ($5 \times 10^5$) mutant donor cells exhibit a delayed erythroid recovery, which was associated with the delayed expression of Scl and Gata2. Over expression of either of these molecules in mutant donor cells rescued this defect. We further characterized the spleen stress erythroid progenitors and showed that the most immature population of these
progenitors, when transplanted into irradiated secondary and tertiary recipients, is capable of maintaining erythropoiesis with no contribution to other myeloid or lymphoid lineages until surviving recipient stem cells recovered. Despite the fact that recipient HSCs have repopulated these mice, donor stress erythroid progenitors rapidly responded to PHZ induced acute anemia. These data demonstrate that the BMP4 dependent stress erythropoiesis pathway generates a population of self renewing stress erythroid progenitor cells that can maintain erythropoiesis in the absence of HSCs and represent an anemic stress response compartment.
Material and Methods

Mice

C57BL/6-f, C57BL/6 (CD45.2) and congenic B6.SJL-Ptprc<sup>a</sup> Pep3<sup>b</sup>/BoyJ (CD45.1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were approximately 6-10 weeks old, controls were age matched. All procedures were approved by the IACUC of the Pennsylvania State University.

Complete Blood Analysis

Peripheral blood was obtained by retro-orbital sinus through heparin-coated microhematocrit tubes and transferred to tubes coated EDTA for complete blood cell (CBC) analysis. CBC analysis was determined using an Advia 120 Multi-species whole blood analyzer (Bayer, Tarrytown, NY).

Flow Cytometry and Cell Sorting

Single cell suspensions of splenocytes, BM and peripheral blood were isolated and labeled (Perry et al., 2007a) with different combination of antibodies: anti-FcγRII/III, CD71-FITC, Ter119-PE, CD45.1-FITC, CD45.2-FITC, CD61-PE and CD31-FITC (BD-Pharmergen, San Diego, CA), anti- Kit-Alexa647, and Sca1-Pacific Blue ( BioLegend, San Diego, CA), anti- Kit-PE-Alexa610 and Sca1-Alexa647 (Invitrogen, Carlsbad, CA). After labeling, cells were washed with 2% fetal calf serum in PBS. Flow cytometry analysis was done using a FC500 Benchtop Flow Cytometer (Beckman-Coulter, Miami
Lakes, FL) with CXP software. The percentage of dead cells was measured by propidium iodide staining. All the data were analyzed with FlowJo software (Tree Star, Ashland, OR). Cell populations were sorted as Kit$^+$CD71$^{\text{low}}$Ter119$^{\text{low}}$ (Population-I), Kit$^+$CD71$^{\text{high}}$Ter119$^{\text{med}}$ (Population-II) and Kit$^+$CD71$^{\text{med}}$Ter119$^{\text{high}}$ (Population-III) by using a Cytopenia inFlux V-GS Cytometry Workbench with Spigot software. Some cell populations were sorted and spun onto slides using a Cytospin 3 centrifuge (Shandon, Pittsburg, PA) at 500 rpm for 10 min, fixed in methanol for 10 min and subjected to Hematoxylin and eosin, neutral Benzidine or Giemsa staining according to the manufacturer’s recommendations.

**Transplantation Assays**

For primary transplantation, 1x10$^5$ and 5x10$^5$ BM mononuclear cells from C57BL/6 (CD45.2+) and C57BL/6-$f/f$ (CD45.2+) mice isolated and transplanted into the retro-orbital sinus of lethally irradiated (9.5Gy) B6.SJL-Ptprc$^a$ Pep3$^b$/BoyJ (CD45.1+) recipients. Prior to transplantation all recipients were treated with acidified water (pH 2.5-3.0) and antibiotics for one week. Reconstitution was monitored by FACS analysis of BM and spleen cells at post-transplantation time points. The primary transplantation experiments were repeated several times under the same condition for different analysis purposes. For secondary transplantation experiments, Population I and II (CD45.2+) cells were sorted from spleens of primary transplanted animals (CD45.1+) at day 8 and 4x10$^5$ sorted cells were transplanted into lethally irradiated CD45.1+ secondary recipients. A cohort of secondary recipients was used for further analysis. The rate of reconstitution was measured by FACS analysis.
**Colony Assays**

Donor derived splenocytes were isolated FACS on the indicated days from B6.SJL-\textit{Ptprc}^{a} \textit{Pep3}^{b}/BoyJ (CD45.1+) mice transplanted with C57BL/6 or C57BL/6-\textit{f/f} bone marrow. 2x10^{6}/ml nucleated splenocytes were plated in methylcellulose media (StemCell Technologies, Vancouver, BC) containing 3U/ml Epo (US Biological, Swampsott, MA). Stress BFU-E were scored as described after 5 days incubation (Perry et al., 2007a). For the analysis of BFU-E and CFU-E potential of Populations I, II and III, spleen cells were isolated from B6.SJL-\textit{Ptprc}^{a} \textit{Pep3}^{b}/BoyJ (CD45.1+) mice transplanted with C57BL/6 or C57BL/6-\textit{f/f} bone marrow by FACS on day 8 after transplant. The indicated number of cells was plated in methylcellulose media (StemCell Technologies, Vancouver, BC) containing 3U/ml Epo (US Biological, Swampsott, MA) \textpm 50ng/ml SCF \textpm 15ng/ml BMP4 \textpm Noggin (R&D Systems, Minneapolis, MN) as indicated in the figures. Stress BFU-E were scored as described after 5 days incubation (Perry et al., 2007a) and CFU-E were scored after 2 days. For assays done at 2% O\textsubscript{2}, plates were incubated in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) as previously described (Perry et al., 2007a).

**RT-PCR and Gene Expression Analysis**

RNA was isolated from the indicated cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. cDNA was prepared using the SuperScript-II system for RT-PCR (Invitrogen). Primers sequences are listed in the supplementary data. qRT-PCR was done using Taqman assays for Scl (Mm00441665-
A1), Gata2 (Mm00492302-g1) and Gapdh (Mm03302249-g1) from Applied Biosystems according to the manufacturer’s instructions. The qRT-PCR analysis was done using an ABI 7300 Real time PCR system.

**Statistical Analysis**

For all experiments with error bars, standard deviation was calculated to indicate the variation within each experiment. Mean differences between groups were examined by Student $t$ test (2-tailed). Statistical significance was taken at values of *$P$* less than 0.05, **$P$** less than 0.01, and ***$P$*** less than 0.005.
Results

*BMP4 dependent signaling is required and specific for erythroid short-term radioprotection following bone marrow transplant.*

In the immediate post transplant recovery period, short-term radioprotective cells must rapidly produce new erythrocytes, platelets and neutrophils. We tested whether the BMP4 dependent stress erythropoiesis pathway regulated the development of new erythrocytes following transplant and whether BMP4 dependent signaling regulated the development of other myeloid lineages, in particular megakaryocytes and neutrophils, during the recovery from transplant. We transplanted 1 x10^5 bone marrow cells isolated from C57BL/6 or C57BL/6-f/f mice, which carry the CD45.2 allele, into lethally irradiated B6.SJL-Ptprc<sup>α</sup>Pep3<sup>β</sup>/BoyJ recipients (CD45.1+) and followed their recovery over a 26 day period. 90% (9/10) of mice transplanted with control bone marrow survived, in contrast none (0/10) of the mice transplanted with mutant bone marrow lived longer than 20 days (Figure 1A). Analysis of hematocrit during the recovery period showed that control transplanted mice reached a nadir at 14 days post transplant after which their hematocrits steadily increased. The mutant transplants failed to increase their hematocrits at any time post transplant (Figure 1B). This defect was further demonstrated when we examined reticulocyte production, which is a direct measure of new erythropoiesis. Control transplanted mice exhibited a robust reticulocyte response which paralleled their hematocrit recovery, while f/f transplanted mice showed no reticulocyte development (figure 1C). These data demonstrate that at limiting numbers of transplanted cells, f/f mutant donor bone marrow cells are unable to provide erythroid short term radioprotection.
In order to overcome the lethality, we increased the number of donor bone marrow cells to $5 \times 10^5$. At this dose, we observed that all transplanted mice survived regardless of whether they received control or mutant bone marrow cells. Despite their survival, mice transplanted with $ff$ mutant bone marrow exhibited a delayed erythroid recovery (Figure 1D). When we examined different erythroid parameters, the $ff$ transplanted mice exhibited a significant delay in all parameters, which was characterized by significantly lower nadir values and delayed recovery (Figure S1A-C). In addition, the onset of the expansion of donor cells in the spleen was delayed and the overall expansion of cells in the spleen was diminished in mice transplanted with $ff$ bone marrow (Figure S1D-E).

In contrast to the defects in erythroid recovery, mice transplanted with $ff$ donor cells generated new platelets, neutrophils, lymphocytes and white blood cells equally as well as control transplanted mice (Figure S1F-I). Flow cytometry and histological analysis showed that the number of donor derived megakaryocytes (CD45.2+CD61+) in spleens of $ff$ and control transplanted mice were not significantly different (Figure S1J,K). The delayed recovery observed in $ff$ transplants is not due to a generalized defect in hematopoietic stem cells (HSCs). $ff$ bone marrow cells are as capable as control bone marrow cells in reconstituting hematopoiesis long term. Four months after transplant, $ff$ and control transplanted mice exhibited similar levels of reconstitution and hematological recovery (Figure S2). Our data are completely consistent with recent work showing that Smad1 and 5 are dispensable for long term reconstitution (Singbrant et al.; Singbrant et al., 2006). Furthermore, previous analysis of several multi-potential progenitors that have
been implicated in short-term radioprotection (Baumann et al., 2004; Na Nakorn et al., 2002) showed that these progenitors were not decreased in $f/f$ mutant mice (Subramanian et al., 2007).

**BMP4 is expressed in the spleen during the recovery from bone marrow transplant.**

We next examined the expression of BMP4 in the spleen during the recovery of mice transplanted with control or $f/f$ mutant bone marrow (figure S3). BMP4 expression was confined to the red pulp (Figure S3D). In control transplants, early BMP4 expression (24 hours) was associated with donor cells, but by 48 hours, recipient stromal cells in the spleen expressed BMP4. The level of BMP4 increased up to day 12 when the highest levels of BMP4 were observed. After that time the expression decreases (Figure S3A,B). In addition to the early expression by donor cells, we also observed donor cell expression at day 12 where the majority of BMP4 expression is donor derived. These observations are consistent with our model, showing that when bone marrow cells migrate into the spleen, they encounter a Hedgehog signal which induces them to express BMP4. Our data support a mechanism where on day 1 the initial wave of progenitors migrate into the spleen, encounter hedgehog and express BMP4, which leads to the development of BMP4 responsive stress erythroid progenitors. While donor derived BMP4 expression on day 12 represents a second wave of progenitors migrating to the spleen, which leads replenishment of stress progenitors post recovery (Figure S3C).

In contrast to control transplants, $f/f$ mutant transplants exhibited a delayed and diminished expression of BMP4, which is consistent with our previous work on the recovery from acute anemia (Lenox et al., 2005a; Porayette and Paulson, 2008). The
expression of BMP4 by donor derived cells was prolonged in mutant transplants. This observation suggests that defects in Smad5 dependent signaling leads to aberrant regulation of BMP4 expression by mutant donor derived cells, which would lead to prolonged expression of BMP4 (Figure S3).

**Stress BFU-E proliferate in the spleen during the recovery phase, but the expansion of these progenitors is delayed in mice transplanted with f/f mutant bone marrow.**

One of the hallmarks of the BMP4 dependent stress erythropoiesis pathway is the expansion of stress BFU-E in the spleen (Lenox et al., 2005a; Perry et al., 2007a; Perry et al., 2008). We examined donor derived stress BFU-E in the bone marrow and spleen by sorting CD45.2+ cells from the bone marrow and spleen and plating them in methylcellulose media containing only Epo, which specifically assays stress BFU-E (Lenox et al., 2005a; Perry et al., 2007a). At no time did we observe stress BFU-E in the bone marrow (data not shown). Prior to day 8 post-transplant, no donor derived stress BFU-E were observed in the spleen (Figure 1E). Stress BFU-E were first observed in control transplants on day 8 post transplant, but we did not detect stress BFU-E in the spleens of mice transplanted with mutant bone marrow at this time point. However, stress BFU-E were observed in the spleen on day 12 in mice transplanted with f/f mutant bone marrow. Although the frequency of stress BFU-E was slightly greater in f/f transplanted mice, the spleens of f/f transplanted mice were still significantly smaller than control transplants at this time (Figure S1D,E), which suggests that control transplanted mice had greater total numbers of stress BFU-E. In contrast at day 20, the frequency of stress BFU-E in the spleens of f/f transplanted mice is greater and the spleens of f/f are slightly larger
than control transplanted mice (Figure S1D,E), which suggests that f/f transplants exhibit delayed expansion of stress BFU-E in the spleen. Later in the post recovery stage, at day 28, f/f mice have similar numbers of stress BFU-E when compared to control mice.

Overall, careful examination of data concerning the control transplants shows that the erythroid recovery in the immediate post transplant period proceeds through three distinct phases. An initial stage, which we will refer to as “pre-recovery” (Days 0-8), is characterized by steadily decreasing RBC numbers, and absence of compensatory stress erythropoiesis as no reticulocytes are observed in the peripheral blood and spleen size is unchanged. A second “recovery” stage (Days 8-16) where erythrocyte counts reach their nadir and active stress erythropoiesis occurs leading to an expansion of stress BFU-E and an increase in RBC numbers, hemoglobin and reticulocytes in the peripheral blood as well as an increase in spleen weight. Finally, the third stage or post-recovery (Days 16-28) is characterized by normal hematocrit, RBC numbers and decreasing reticulocytes and spleen weight (Shown in Figure 1D).

Three distinct populations of stress erythroid progenitors expand in the spleen during the recovery from bone marrow transplant.

Previous analysis of bone marrow erythroid progenitors identified several distinct populations based on their expression of CD71 and Ter119. These cells represent late stage erythroid progenitors that do not express Kit and do not form BFU-E colonies (Wojchowski et al., 2006; Zhang et al., 2003). When we analyzed these populations in the bone marrow of mutant and control transplanted mice, we did not observe any differences in the frequencies of different progenitor populations on days 6
and 8 after transplant. However on day 10, f/f transplanted mice showed a decreased frequency of the most mature Ter119+CD71- cells (Data not shown). Our previous work showed that BMP4, SCF and hypoxia promote the expansion of a Kit+CD71+Ter119+ population of spleen stress erythroid progenitors (Perry et al., 2007a). Careful flow cytometry analysis of spleen cells following bone marrow transplant showed that the Kit+CD71+Ter119+ cells could be subdivided into three populations with distinct staining characteristics, morphology and erythroid potential. We term these populations I, II and III. Population-I cells are Kit+CD71^{lo/med}Ter119^{lo/-}, Population-II cells are Kit+CD71^{high}Ter119^{med}, and Population-III cells are Kit+CD71^{lo/med}Ter119^{high} (Figure 2A).

We calculated the total number of each of these three populations during the recovery time. Population-I is the primary population present at the pre-recovery stage (days 2 - 6) with a slight increase in the total number throughout the post-recovery time (day 16) (Figure 2A,B). Population-I is also present in non anemic spleen (day 0) which suggests it represents the population of stress erythroid progenitors resident in the spleen (data not shown). Population I cells exhibit morphology similar to primitive progenitor cells (Figure S4). In contrast, Population-II exhibited a wave of proliferation which starts at day 6 (early recovery time). We observe a 30 fold increase in these cells by day 12 (recovery time), which diminishes by day 16 (post-recovery) (Figure 2A,B). The expansion of these cells corresponds to a critical time during the recovery, which tightly correlates with the increase of spleen size at day 12 (Figure S1D) and high production of reticulocytes (Figure S1A). Morphological analysis of Population II showed that the cell resemble early basophilic erythroblasts (Figure S4). Population-III with high expression of Ter119 exhibited a smaller window of appearance with significant increase in the
number of cells at recovery time (day 12), which also correlates with the maximum of spleen size, number of reticulocytes and early hematocrit recovery (Figure S1A,D). Population III cells resemble late stage chromatophilic and orthochromatophilic erythroblasts (Figure S4). In addition approximately 50% of these cells stain positive for benzidine (Data not shown). Further analysis of these populations showed that Population-I also contained a population of Sca1+ cells (Figure 2D). The percentage of Sca1+ cells in untreated mice was approximately 65-70%. During recovery this percentage decreased to approximately 45% at day 8 and 12, but it increased to 65% by day 16. In addition to Population-I, we observed that a small subset of (~10%) of Population-II cells expressed Sca1. However, this expression was only observed during the recovery stage and corresponded to the expansion of Population-II cells. These findings suggest that Populations II cells lose their Sca1 expression as differentiation proceeds during recovery.

The kinetics of appearance of the three populations suggests a temporal order for their development where Population–I gives rise to Population-II and Population–II gives rise to Population-III. We tested this model by sorting Population-I cells from the spleens of mice transplanted with control bone marrow on day 8 of recovery. The Population-I cells were plated in media containing growth factors known to expressed in the spleen during the recovery period which included BMP4, SCF, Epo, Sonic hedgehog (Shh) and hypoxia and cultured cells were analyzed on days 3 and 6 by flow cytometry (Figure S5). This analysis shows that Population-I cells give rise to Population-II cells, which suggests that in vivo the differentiation of Population-I is the initial event in the erythroid
recovery from bone marrow transplant. We observed very few Population III cells in these cultures, which suggests that their differentiation may require other factors.

Based on flow cytometry and morphology, these three populations appear to be distinct cell populations. We next examined the erythroid potential of these cells. In control transplanted mice, we observed the greatest expansion of donor derived stress BFU-E on day 8 post transplant (Figure 1). So we sorted Populations I, II and III on day 8 after transplant and assayed their ability to form erythroid colonies in methylcellulose (Figure 2G-I). Stress BFU-E are capable of forming BFU-E colonies in cultures supplemented with Epo alone in the absence of other added factors (Lenox et al., 2005a). In addition, stress BFU-E maximally expand when grown in media containing BMP4, SCF and Epo at 2%O₂. When we examined the colony forming ability of Population-I, we observed that they formed stress BFU-E. In fact, Population I contained all the stress BFU-E present in the spleen. Furthermore, these progenitors responded to the different combinations of these factors in a manner that was identical to what we observed previously with stress BFU-E present in the spleen (Perry et al., 2007a) (Figure 2G).

BMP4, SCF and hypoxia promoted the greatest expansion of stress BFU-E. These data demonstrate that Population-I cells are BMP4 responsive stress BFU-E. These cells also formed CFU-E colonies, however unlike bone marrow CFU-E, which respond primarily to Epo, the production of CFU-E by Population-I cells was greatly enhanced when the cells were grown in media supplemented with BMP4, SCF and hypoxia (Figure 2H). In contrast to Population-I, Population-II cells only form CFU-E colonies. However, these cells exhibit properties more similar to bone marrow CFU-E, which respond primarily to
Epo and hypoxia (Figure 2I). Population-III failed to form any colonies, which is consistent with their morphology and temporal development as late stage erythroblasts.

**Mice transplanted with f/f mutant bone marrow exhibit a defect in the differentiation of stress erythroid progenitors.**

Unlike control transplanted mice, mice transplanted with f/f mutant BM did not exhibit an expansion of donor derived stress BFU-E at day 8 post transplant (Figure 1). We next examined whether the delayed expansion of stress BFU-E correlated with a defect in the development of Population-I stress progenitors in the spleen. Spleen cells isolated from mice transplanted with f/f mutant bone marrow on days 6, 8 and 12 after transplant (Figure 2E, F). Our analysis shows that early during the recovery phase (day 6) there was a significant reduction in the number of Population-I cells in the mice transplanted with f/f bone marrow. However, this defect was not present at day 8 or 12 post transplant. Despite the fact that f/f transplants have similar numbers of Population-I cells on day 8 after transplant, they do not exhibit any stress BFU-E. Indeed, when f/f mutant Population-I cells were plated for BFU-E, few BFU-E were observed even when cells were plated in media supplemented with BMP4, SCF and hypoxia (Figure 2G). Similar results were observed when f/f Population-I cells were plated for CFU-E (Figure 2H). Few CFU-E colonies were formed in any of the culture conditions, which demonstrates that Population I cells derived from f/f donors exhibit a delayed development of stress BFU-E and CFU-E. Similar to Population-I cells, the numbers of Population-II progenitor cells at day 8 after transplant were not significantly different than control cells, although at day 12 f/f transplanted mice appear to have more cells (Figure 2F). Analysis
of CFU-E from day 8 Population II cells showed that f/f Population-II cells were unable to form large numbers of CFU-E and did not respond to different growth factor conditions like control cells (Figure 2I). Unlike Population-I and II, Population-III was significantly reduced at day 8 in the f/f transplanted mice (Figure 2F), which correlates with the delayed production of reticulocytes in the mutant transplants (Figure S1).

**Bone marrow Short-term reconstituting HSCs (CD34+Kit+Sca1+Lin-) generate stress erythroid progenitors in the spleen.**

The expression of Kit and Sca1 on Population I progenitors suggested that these cells may be derived from a more primitive progenitor population. We tested whether bone marrow Kit+Sca1+Lineage- (KSL) cells could give rise to donor derived stress BFU-E in the spleen on day 8 after transplant. In figure 3A, the data show that transplanting 4x10^5 KSL cells leads to the development of stress BFU-E that respond to BMP4, SCF and hypoxia. Control experiments using Kit+Sca1-Lin- donor cells also lead to BFU-E in the spleen, but these progenitors failed to expand in response to BMP4, SCF and hypoxia.

The KSL fraction contains hematopoietic stem cells (HSCs). This population can be further fractionated into long term reconstituting (LTR-HSC), which are CD34-, and short term reconstituting (STR-HSC) stem cells, which are CD34+. We fractionated bone marrow KSL cells into CD34+ and CD34- populations and transplanted 2 x10^5 donor cells into lethally irradiated mice. On day 8 after transplant donor derived cells were sorted from the spleen and plated for stress BFU-E. The spleens of mice transplanted with CD34+KSL cells were significantly larger than the spleens of mice transplanted with CD34-KSL cells (Figure S6). This difference in spleen size also reflected a
difference in the number of stress BFU-E. CD34+KSL cells generated significant numbers of stress BFU-E which responded to BMP4, SCF and hypoxia like stress BFU-E (Figure 3B). In contrast the mice transplanted with the CD34-KSL donor cells generated few stress BFU-E. These data indicate that STR-HSCs generate erythroid stress progenitors during the immediate post transplant recovery. It is interesting to note that although STR-HSCs are CD34+, Population I and II cells isolated from the spleen of transplanted mice did not express CD34 (data not shown), which suggests that upon migration to the spleen they lose expression of CD34, but maintain expression of Kit and Sca1.

*Stress erythroid progenitors derived from f/f mutant donor bone marrow cells exhibit a delayed expression of Scl and Gata2.*

Several transcription factors have been shown to play key roles in regulating erythropoiesis. We hypothesized that the defect in BMP4 signaling in the f/f mutant donor cells would impair the expression of factors required for the development or differentiation of stress BFU-E. We focused on two transcription factors, Scl and Gata2, which are known to play a key role in the development of primitive progenitor cells. Scl is required for erythroid short-term radioprotection(Curtis et al., 2004; Hall et al., 2003; Hall et al., 2005). Mice transplanted with Scl−/− bone marrow fail to generate donor erythrocytes in competitive repopulation assays. Furthermore, CFU-S from these mice do not produce erythroid cells, which phenocopies the defect observed in f/f CFU-S(Cole and Regan, 1976a; Hall et al., 2005). These observations suggest that Scl expression may be a target of BMP4 signaling during the recovery from bone marrow transplant. Gata2
on the other hand is known to be a target of BMP4 signaling (Lohmann and Bieker, 2008; Lugus et al., 2007). Gata2+- mice exhibit defects in generating progenitor cells in competitive transplants and the expansion of KSL cells in the bone marrow following 5-FU treatment is significantly impaired (Ling et al., 2004). Given that Scl and Gata2 function in concert to regulate erythropoiesis, we analyzed the expression of Scl and Gata2 by qRT-PCR in Population I cells isolated from spleen of day 6 after transplant, which is just prior to the appearance of stress BFU-E and on day 8 after transplant when stress BFU-E are present. In control transplants, we observed high expression of both Scl and Gata2, however in mice transplanted with f/f mutant bone marrow, Population I cells expressed significantly lower levels of Scl and Gata2 (Figure 4A). This difference in expression was also apparent when we examined Scl protein expression in the spleens of mutant and control transplanted mice. We examined Scl protein expression by staining spleen sections with antibodies to Scl and CD45.2 to identify donor derived cells. Like BMP4, Scl expression is limited to the red pulp of the spleen (Figure S7). At day 4, Scl expression is first observed, however, only a low level of Scl is associated with donor derived cells. The expression changes dramatically at day 8 when donor derived cells have expanded in the spleen and nearly all donor derived cells express SCL. By day 12 the expression of Scl is less while expression further decreases by day 20. Similar to what we observed with BMP4 expression, donor derived f/f mutant cells exhibited a delayed expression of SCL. At day 8 very little SCL expression is present in donor derived cells (Figure S7). At day 12 most donor f/f cells express SCL but at a lower level than control donor cells. While at day 20, the levels of SCL expression in f/f donor cells were similar to control transplants. Overall, these studies demonstrate that SCL mRNA and protein
and Gata2 mRNA are delayed and diminished in mice transplanted with f/f mutant bone marrow, which correlates with the delayed erythroid recovery.

If Scl and Gata2 are required for the expansion of stress erythroid progenitors, then the signals that promote the expansion of these cells should increase the expression of these factors. We sorting donor derived cells from mice transplanted with control bone marrow on days 6, 8 and 10 after transplant. The cells were incubated for two hours in the presence or absence of all the signals that previously we showed were necessary for the development and expansion of stress BFU-E – namely SCF, BMP4, Shh and hypoxia (Perry et al., 2008). The treatment of donor derived cells with these factors resulted in the up-regulation of Scl and Gata2 mRNA expression (Figure 4B). These data show that Scl and Gata2 are rapidly induced by these signals. In order to test the role of Scl and Gata2 in the expansion of stress BFU-E in vivo, we infected f/f mutant bone marrow with MSCV virus expressing Scl, Gata2 or a MSCV vector control. 24 hours after infection, GFP+ cells were sorted and transplanted into irradiated mice. On day 8 after transplant, spleen cells were plated for stress BFU-E. In Figure 4C, the data show that donor cells infected with either the Scl or the Gata2 virus rescued the defect in the expansion of stress BFU-E in f/f donor cells. These data demonstrate that Scl and Gata2 are key targets of BMP4 signaling, which promote the expansion of stress BFU-E in the spleen following bone marrow transplant.

*Population-I cells can provide erythroid short-term radioprotection and self renew when serially transplanted into irradiated recipients.*
The co-expression of Kit and Sca1 by Population I and a subset of Population II cells is a surprising finding given that these cells are erythroid progenitors. Kit and Sca1 expression is associated with stem cells and multipotential progenitor cells (Holmes and Stanford, 2007; Spangrude et al., 1988). This observation suggested that these cells may be able to generate multiple cell lineages or potentially self renew if placed in the correct developmental context. We directly tested this hypothesis by isolating donor derived Population I and II cells from the spleens of recipient mice on day 8 after transplant and transplanting them into secondary recipients (Figure 5A). Donor CD45.2+;Hbb\textsuperscript{D} bone marrow cells were transplanted into irradiated CD45.1+;Hbb\textsuperscript{S}. On day 8 after transplant, donor derived Population I and II cells were isolated by FACS. 4 \times 10^5 Population I or II cells were transplanted into irradiated CD45.1+;Hbb\textsuperscript{S} recipients and the recovery followed over a 6 week period. Mice transplanted with Population II cells exhibited a transient rise in hematocrit (>50% at day 2) (Figure 5C). However, despite the initial rise in red cell number, the Population II transplanted mice rapidly developed a lethal anemia and none of the mice survived longer than 15 days (Figure 5B). Population II cells were unable to generate appreciable numbers of reticulocytes, which is consistent with these cells being CFU-E with little proliferative capacity (Figure 5D). The situation was quite different in the mice transplanted with Population I. These mice also exhibited a transient increase in hematocrit (>50%) at day 2 after transplant, which is consistent with our observation that Population I cells exhibit CFU-E activity (Figure 2H). However, unlike the Population II transplanted mice, more than 60% of the mice transplanted with Population I cells survived in our initial experiments and by changing the radiation dose from a single dose of 950 rads to two doses of 475 rads, we were able to increase the survival of Population
I transplanted mice to near 100% (Figure 5B). In comparison to mice transplanted with whole bone marrow, the mice transplanted with Population I cells exhibited a more severe anemia, which resolved slower (Figure 5C). Using cellulose acetate gel analysis, we observed that from day 16-42, 100% of the erythrocytes were donor derived, Hbb<sup>D</sup> (Data not shown). In Figure 2B, the data show that the spleens of transplanted mice contain approximately 4 x10⁶ Population-I cells on day 8 after transplant which is almost 10 fold more Population-I cells than we transplanted in this experiment. Despite the low number of transplanted progenitors, these cells were able to maintain erythropoiesis until surviving recipient stem cells could repopulate the hematopoietic system. Murine erythrocyte half life is approximately 17-23 days (Goodman and Smith, 1961; Hoffmann-Fezer et al., 1993). We waited until all the erythrocytes produced by the initial wave of donor derived stress progenitors would have turned over and then tested the expression of Hbb alleles. At this time, we observed that the erythrocytes in the transplanted mice had reverted to 100% recipient derived, Hbb<sup>S</sup>, which demonstrates that Population I progenitors do not contribute to steady state erythropoiesis (Data not shown).

The most surprising result, however, came when we analyzed the contribution of these cells to other lineages. Platelet recovery was significantly delayed (Figure 5G). We did not observe an increase in platelet number until 28 days after transplant. The recovery of WBC counts was extremely limited, which suggests that these cells have limited myeloid potential (Figure 5H). Flow cytometry analysis of peripheral blood leukocytes showed that all the WBCs were recipient derived (Figure 5I) including cells expressing the megakaryocyte markers CD41 and CD61. These observations suggest that the few
myeloid cells produced are products of recipient derived progenitors that survived irradiation.

In order to test whether Population I cells could self renew in vivo, we isolated donor derived spleen cells from secondary transplanted mice and transplanted into tertiary irradiated recipients (Figure 5A). Once again we observed that the mice survived the transplant and erythroid recovery was similar to that observed in the secondary transplants (Figure S8). Hemoglobin analysis showed erythroid recovery was 100% donor derived (Data not shown). In contrast, analysis of peripheral blood mononuclear cells by flow cytometry indicated that these cells were recipient derived (Figure S8). Similar to what we observed in the secondary transplants, surviving recipient stem cells repopulated the mice and erythrocytes reverted to recipient HbbS. These data show that Population I stress erythroid progenitors self renew in vivo and are capable of maintaining erythropoiesis in the absence of hematopoietic stem cells.

Our observation that Population I cells can be serially transplanted shows that they can self renew in vivo. We next tested whether donor derived Population I cells were maintained in the secondary recipients and could respond to acute anemia even after the mice had been repopulated by surviving HSCs and the erythrocytes had reverted back to recipient derived HbbS+. We treated secondary recipients with PHZ to induced acute anemia and then 7 days after treatment analyzed erythrocytes for Hbb alleles. In Figure 5J, the data show that donor derived HbbD is induced during the recovery from acute anemia. We extended this observation by allowing the mice to fully recover from the PHZ induced anemia and the HbbD donor derived erythrocytes had turned over (52 days). We treated these mice once again with PHZ and analyzed the contribution of donor
derived stress progenitors to the recovery from anemia. Analysis of Hbb alleles showed that PHZ induced anemia once again lead to the production of donor derived erythrocytes (Figure 5J). These data show that although recipient HSCs have repopulated the mouse and are driving steady state erythropoiesis, stress erythropoiesis utilized the donor derived progenitors, which suggests that the stress erythroid response is maintained by a stable stress progenitor cell compartment.

Transplanted Population I progenitor cells preferentially expand in the spleens of recipient mice. Flow cytometry analysis in secondary transplanted mice shows that on day 21 approximately 97% of spleen cells are donor derived, while only 4% are donor derived in the bone marrow (data not shown). These data were similar in tertiary transplants. Our in vivo data show that Population I progenitors are erythroid restricted. We next tested whether donor derived cells sorted from the spleen or the bone marrow of tertiary transplanted mice exhibited greater developmental potential when plated in methylcellulose media containing a complete set of cytokines (IL-3, IL-6, SCF and Epo). Donor derived spleen cells primarily generated only BFU-E when plated in this media, while donor derived bone marrow cells generated mainly BFU-E (~70%), but also other myeloid colonies (Figure 5K). These data show that in vivo and in vitro spleen population I progenitors are erythroid restricted, however, when these cell are in the bone marrow microenvironment, they can reacquire the potential to form other myeloid lineages.
Discussion

Our analysis of erythroid short-term radioprotection shows that the BMP4 dependent stress erythropoiesis pathway plays an essential role in the rapid generation of new erythrocytes in the immediate post-transplant period. Our work suggests a new model for stress erythropoiesis (Figure 6). In the initial stage donor derived CD34+KSL cells migrate to the spleen. The signals that induce the migration or regulate the homing of progenitor cells to the spleen are not known. In the second stage, these cells adopt the stress erythroid progenitor cell fate. CD34+KSL cells interact with Hedgehog (Dhh and Ihh) in the spleen, which induces them to express BMP4. Hedgehog and BMP4 act in concert to specify the stress erythroid progenitor fate. Once the stress erythroid fate is specified, the stress erythroid progenitors expand in the spleen without differentiating. Our data in Figure 2 show that number of population I cells in the spleen rapidly increases in the first 8 days after transplant. However despite the fact that BMP4, SCF and Hedgehog are present in the spleen, we do not observe stress BFU-E until day 8 after transplant. The development of stress BFU-E correlates with the nadir in the hematocrit and the induction of Epo expression by the kidney, which is a direct readout of systemic tissue hypoxia (Figure S9). This observation is consistent with our previous work showing that stress BFU-E require BMP4, SCF and hypoxia to maximally expand in vitro(Perry et al., 2007a). The transition from expanding stress progenitors to stress BFU-E capable of differentiating marks the last stage in our model. We propose that switch from proliferation to differentiation requires high level expression of Scl and Gata2 and is mediated by tissue hypoxia. Although Epo could play a role in this process, Epo-/- mice still develop BFU-E suggesting that Epo is not necessary for BFU-E development(Wu et
al., 1995). The presence of BMP4, SCF, hypoxia and a rise in serum Epo concentration drives the terminal differentiation of stress BFU-E and the eventual erythroid recovery of the transplanted mice. Following recovery at day 12 after transplant, we observe a second wave of progenitors migrating into the spleen as evidenced by the high levels of donor derived BMP4 expression. These cells would replenish the stress erythroid progenitors that have recently differentiated (Perry et al., 2008). However, the lack of a hypoxia signal prevents their differentiation so they remain dormant.

Our analysis points to a defect in the differentiation of stress progenitors as the cause of the delayed erythroid recovery in the mice transplanted with f/f mutant bone marrow. The f mutation causes a defect in splicing that results in the production of a dominant negative form, which can inhibit signaling by Smads 1, 5 and 8 (Hegde et al., 2007; Lenox et al., 2005a). On day 8 after transplant the numbers of Population I progenitors were equivalent in the mutant and control transplants, however, the mutant transplants lack stress BFU-E. We hypothesize that the progression from expanding stress erythroid progenitors to stress BFU-E capable of terminal differentiation relies on the expression of Scl and Gata2. Analysis of the expression of these genes in Population I cells showed that mutant progenitor cells exhibited significantly lower Scl and Gata2 expression. Treatment of control donor derived cells isolated from spleen with BMP4, Hedgehog, SCF and hypoxia induced the expression of Scl and Gata2 in two hours suggesting that these genes are direct or indirect targets of these signals. The importance of these genes in the development of stress BFU-E is underscored by the data in Figure 4, which shows that retroviral expression of Scl or Gata2 rescues the delay in stress BFU-E development. These data are consistent with previous work showing that Scl expression
can be regulated by Hedgehog signaling (Hochman et al., 2006) and Gata2 is a BMP4 inducible gene (Lohmann and Bieker, 2008; Lugus et al., 2007), further analysis will be needed to establish the relationship between Scl and Gata2 expression and BMP4 and Hedgehog signaling in the spleen during the recovery from bone marrow transplant.

Surprisingly, mice transplanted with f/f mutant bone marrow also exhibited a defect in BMP4 expression by the spleen stroma (Figure S3). Our recent work showed that BMP4 is regulated by hypoxia in the spleen and we have identified 2 hypoxia responsive elements in the BMP4 gene (Wu and Paulson, 2010). However, hypoxia is not the whole story. Mock transplanted mice exhibit only a transient increase in BMP4 expression despite the low hematocrits (data not shown). This observation suggests that the regulation of BMP4 expression is more complex and we propose that the donor cells make a signal that cooperates with hypoxia to maintain the expression of BMP4.

The most surprising result of our study is the observation that purified Population I cells can maintain erythropoiesis when transplanted into secondary and tertiary recipients without contribution to other lineages until surviving recipient derived HSCs expand and repopulate the mice. These serial transplant experiments clearly demonstrate that Population I cells self renew. Furthermore donor stress progenitor cells are maintained in the secondary and tertiary transplants even after the recipient HSCs repopulate the mice. The donor stress progenitor cells primarily populate the spleen, but a small percentage of donor derived cells are present in the bone marrow. Donor cells in the spleen are restricted to the erythroid lineage, consistently generating only BFU-E in colony assays. Although donor cells in the bone marrow preferentially develop into BFU-E they can also generate other lineages. This observation suggests that spleen
The donor derived stress progenitors are functional in secondary and tertiary recipients and are capable of generating new erythrocytes in response to PHZ induced acute anemia despite the fact that the surviving recipient HSCs have repopulated the mice. This donor derived stress response is maintained through multiple anemic challenges, which further underscores their self renewal capability. These observations suggest that Population I stress erythroid progenitors and potentially STR-HSCs may represent a stress response compartment that is maintained separately from LTR-HSCs.

The directed differentiation of HSCs or multipotential cells at times of physiological stress was previously observed when treatment of HSCs and MPPs with TLR ligands resulted in the development of macrophages, granulocytes and dendritic cells by a process that bypassed the normal differentiation pathways (Nagai et al., 2006). The authors of this work suggested that the response of early progenitors to these signals during infection may promote the rapid replenishment of innate immune cells. In our case, anemic stress alters the spleen microenvironment, which induces the differentiation of STR HSCs that rapidly develop into an erythroid lineage restricted stress progenitor, which appears to occur without proceeding through the intervening stages of development. These data suggest that at times of acute life threatening conditions the hematopoietic system can adapt to rapidly produce new mature cells to alleviate the stress condition.

Our analysis presented here focused on murine stress erythropoiesis, which leaves the question of whether this pathway functions in humans. The analysis of human stress erythropoiesis has been limited to studies of erythropoiesis in severely anemic patients,
which can be complicated by indirect effects of disease pathology. Despite these difficulties, some general aspects of human stress erythropoiesis have been reported. Similar to murine stress erythropoiesis, human stress erythropoiesis exhibits properties of fetal erythropoiesis (Alter, 1979; Stamatoyannopoulos et al., 1985). The expression of fetal antigens and fetal hemoglobin (HbF) has been observed following bone marrow transplant (Galanello et al., 1989; Meletis et al., 1994; Weinberg et al., 1986) and in patients with anemia (Bank, 2006; Link and Alter, 1981; Papayannopoulou et al., 1980). In addition, treatment of non-human primates with PHZ to induce anemia results in increased expression of HbF (DeSimone et al., 1982; DeSimone et al., 1978; DeSimone et al., 1979). Analysis of peripheral blood and bone marrow progenitors in sickle cell anemia patients identified a putative stress erythroid progenitor that expressed CD34, Kit, CD71 and glycophorin A (GpA) on their surface. These cells readily produced HbF+ erythrocytes in culture and the production of F cells was increased when the cells were incubated at low O2 (Luck et al., 2004). It is tempting to speculate that these cells are the human equivalent to the Kit+Sca1+CD71\textsuperscript{lo}Ter119\textsuperscript{lo} Population I stress erythroid progenitors we observe in mice. More work will be necessary to determine the similarities between these two systems.
References


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Figure and Figure Legends

A. % Survival

B. % Hematocrit

C. % Reticulocyte

D. % Hematocrit

E. Number of Epo-only BFU-E

ND 8 12 20 28

Days after transplantation

Pre-Recovery Recovery Post-Recovery
Figure A-1. Mice transplanted with f mutant bone marrow exhibit a defect in erythroid short-term radioprotection. C57BL/6 mice were irradiated and transplanted with C57BL/6-ff mutant or C57BL/6 control bone marrow. (A-C) Analysis of mice transplanted with 1x10^5 unfractionated bone marrow cells. (A) Survival of mice transplanted with mutant or control bone marrow, (B) Hematocrit recovery and (C) Reticulocyte production following transplant. (D) Analysis of hematocrit recovery in mice transplanted with 5x10^5 unfractionated bone marrow cells. For each figure, each time point represents 4-18 recipients from at least 3 independent experiments. (E) Donor derived (CD45.2+) spleen cells were isolated by FACS on the indicated days from CD45.1 mice that had been transplanted with 5x10^5 mutant or control bone marrow cells. 2x10^6 cells were plated in methylcellulose media containing Epo only. Stress BFU-E were scored 5 days later. No colonies were observed prior to day 8 post transplant. ND: Not detected. *p<0.05, **p<0.01, ***p<0.005.
Figure A-2. Analysis of stress erythroid progenitor populations by flow cytometry and colony assays. Spleen cells were isolated on the indicated days from CD45.1 mice transplanted with $5 \times 10^5$ mutant (C57BL/6-<i>f/f</i>) or control (C57BL/6) bone marrow cells. (A) Spleen cells from mice transplanted with control bone marrow cells were labeled with anti-Kit, CD71 and Ter119 antibodies. The cells were gated on Kit+ cells and the expression of CD71 and Ter119 analyzed by flow cytometry. Three populations were identified as indicated by the gates on the individual flow diagrams. (B) Analysis of the total number of each stress progenitor population in the spleen on the indicated day after transplant. The number represents the average of 3 mice at each time point. (C) Analysis of total cells in each population on days 2-6 is enlarged to show differences in the numbers of stress progenitors in each population during the early recovery period, which corresponds to the box in panel B. (D) Analysis of the total number of Sca1+ cells in Populations I, II and III in the spleen following transplant. (E) Comparison of stress erythroid progenitors Populations I, II and III in the spleens of mice transplanted with $5 \times 10^5$ mutant (C57BL/6-<i>f/f</i>) or control (C57BL/6) bone marrow cells on days 6, 8 and 10 after transplant. Cells were analyzed as described in A above. (F) Analysis of the total number of Populations I, II and III in mice transplanted with $5 \times 10^5$ mutant (C57BL/6-<i>f/f</i>) or control (C57BL/6) bone marrow cells. (G) Analysis of the BFU-E colony forming potential of Population I cells isolated by FACS from the spleens of mice transplanted with $5 \times 10^5$ mutant (C57BL/6-<i>f/f</i>) or control (C57BL/6) bone marrow cells on day 8 after transplant. Cells were plated in methylcellulose media containing the indicated growth factors and cultured at 20% O₂ or 2% O₂. For all conditions control cells produced significantly more BFU-E than mutant cells, <i>p</i>&lt;0.05. (H) Analysis of the CFU-E colony
forming potential of Population I cells isolated by FACS from the spleens of mice transplanted with $5 \times 10^5$ mutant (C57BL/6-ff) or control (C57BL/6) bone marrow cells on day 8 after transplant. Cells were plated in methylcellulose media containing the indicated growth factors and cultured at 20% O$_2$ or 2% O$_2$. For all conditions control cells produced significantly more BFU-E than mutant cells, p<0.05. (I) Analysis of the CFU-E colony forming potential of Population II cells isolated by FACS from the spleens of mice transplanted with $5 \times 10^5$ mutant (C57BL/6-ff) or control (C57BL/6) bone marrow cells on day 8 after transplant. Cells were plated in methylcellulose media containing the indicated growth factors and cultured at 20% O$_2$ or 2% O$_2$. For all conditions control cells produced significantly more CFU-E than mutant cells, p<0.05. *p<0.05, **p<0.01, ***p<0.005. For each time point at least three independent mice were analyzed.
Figure A- 3. Analysis of bone marrow progenitor populations that give rise to BMP4 dependent stress erythroid progenitors in the spleen following transplant. (A) Stress BFU-E generated by bone marrow Kit+Sca1+Lin- (KSL) cells on day 8 after transplant. (B) Stress BFU-E produced by bone marrow CD34+ KSL and CD34-KSL on day 8 after transplant.
Figure A- 4. Scl and Gata2 expression is regulated by BMP4 during the differentiation of stress erythroid progenitors. (A) qRT-PCR analysis of Scl (left) and Gata2 (right) expression in Population I progenitor cells sorted from mice transplanted with $f/f$ mutant or control bone marrow on days 6 and 8 after transplant. Expression is expressed relative to Gapdh. (B). Population I stress progenitors were sorted on the indicated days after transplant and incubated $\pm$ BMP4+Shh+SCF+Hypoxia for two hours. Scl (left) and Gata2 (right) expression was determined by qRT-PCR. Expression is expression relative to Gapdh. (C) Rescue of the $f/f$ defect in stress BFU-E development by retroviral expression of Scl or Gata2. Bone marrow cells infected with the indicated viruses were transplanted in to mice and on day 8 after transplant spleen cells were isolated and plated for stress BFU-E in methylcellulose media containing either Epo alone of Epo+BMP4+Hypoxia.
**Figure A- 5. Population-I cells can provide erythroid short-term radio protection when transplanted into secondary recipients.** (A) Population I and II cells isolated by FACS from the spleens of CD45.1 mice transplanted with $5 \times 10^5$ control bone marrow cells on day 8 after transplant were transplanted into lethally irradiated CD45.1 secondary recipients. The secondary transplants were analyzed for (B) survival, Erythroid recovery parameters – (C) Hematocrit recovery, (D) Reticulocyte generation, (E) RBC count, (F) Hemoglobin, Myeloid recovery – (G) Platelet count and (H) WBC count in Population I and II secondary transplants. The values for the recovery of mice transplanted with unfractionated bone marrow transplants and control untreated mice are indicated on the graphs. (I) Flow cytometry analysis of peripheral blood mononuclear cells. Cells were harvested 42 days after secondary transplant and (Top) stained with anti-CD45.2 and the indicated antibodies or (bottom) isotype controls. Data is representative of 3 independent mice. (J) Analysis of Hbb alleles on cellulose acetate gels. Secondary transplants (>48 days after transplant) were treated with PHZ to induce anemia. Hbb alleles were tested 7 days later (1st PHZ). The mice were allowed to recover 52 days then treated a second time with PHZ and Hbb alleles analyzed 7 days later (2nd PHZ). (K). Donor derived cells were sorted from the spleen or bone marrow of secondary recipients. The cells were plated in “Complete” methylcellulose media containing SCF+IL-3+IL-6+Epo. Colonies were counted and scored as BFU-E or “other” myeloid colonies.
Figure A-6. Model for erythroid short-term radioprotection during the recovery from bone marrow transplant. Details are in the text.
Supplemental methods

Immunofluorescence Microscopy

Paraffin embedded spleen sections were deparaffinized and rehydrated through an ethanol series as described previously (3, 7). Sections were incubated for 1 hour each at room temperature with the primary antibody and then washed with PBS for several times. BMP4 (Novocastra Laboratories/Vector Laboratories, Burlingame, CA) and SCL (Santa Cruz Biotech, CA) and CD45.2 (BD-Pharmingen, San Diego, CA) antibodies were used at the manufacturer's recommended dilutions. Alexa Fluor (Molecular Probes, Eugene, OR) fluorescent secondary antibodies were then added. For negative controls, appropriate isotype controls were used. Sections were mounted in Slowfade (Molecular Probes, Eugene, OR) and analyzed by digital microscopy (Olympus BX-60 Epi-Fluorescence Digital digital microscope). Fluorescence quantification was analyzed by ImagePro (Media Cybernetics, Bethesda, MD) and images were processed with Adobe Photoshop 8.0 software (Adobe Systems, San Jose, CA).
## Primer Sequences

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<th>Reverse</th>
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Supplementary Figure and Figure Legends
Figure A- S1. Mice transplanted with f mutant bone marrow exhibit a defect in erythroid short-term radioprotection. C57BL/6 mice were irradiated and transplanted with 5x10^5 cells of unfractionated bone marrow of C57BL/6-f/f mutant or C57BL/6 control bone marrow. (A) Absolute Reticulocyte Count, (B) Hemoglobin, (C) RBC, (D) Spleen Weight, (E) Representative photos of spleens isolated from mice transplanted with mutant or control donor cells on the indicated days after transplant. A ruler (mm) is shown to the left for comparison. (F) WBC count, (G) Lymphocyte, (H) Neutrophil, (I) Platelet count, (J) Flow cytometry analysis of donor derived megakaryocytes (CD45.2+CD61+) in the spleens on mutant and control transplanted mice on Day 8 and 12 after transplant. (K) On the indicated days spleens were isolated, fixed and paraffin sections generated. The spleen sections were stained with Hematoxylin and Eosin. The number of megakaryocytes were counted in each section. The total number of megakaryocytes was normalized to spleen weight. The values for day 0 indicate non-transplanted mice. For each figure, each time point represents 4-18 recipients from at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.005.
Figure S2, Harandi et al.

A. Percentage of Reticulocyte
B. 10^6 cells/μl of RBC
C. 16 g/dl of Hemoglobin
D. Percentage of Hematocrit
E. 10^3 cells/μl of WBC

F. 10^3 cells/μl of Neutrophil
G. 10^3 cells/μl of Basophil
H. 10^3 cells/μl of Monocyte
I. 10^3 cells/μl of Eosophil
J. 10^3 cells/μl of Lymphocyte

K. 10^9 cells/μl of Platelet
L. mg of Spleen Weight

M. CD45.2 in BM
N. CD45.2 in Spleen

Wt: Transplant, f/f: Control
Figure A- S2. *ff* mutant donor cells do not exhibit a defect in long term reconstitution. CD45.1+ recipient mice were transplanted with 5x10^5 *ff* or control bone marrow cells (CD45.2+). Analysis of reconstitution was done 128 days after transplant. (A) Reticulocytes, (B) RBC counts, (C) Hemoglobin concentration, (D) Hematocrit, (E) WBC counts; Differential count: (F) Neutrophils, (G) Basophils, (H) Monocytes, (I) Eosinophils, (J) Lymphocytes, and (K) Platelet count were done using the Advia 120 hematology autoanalyzer with Veterinary software. (L) Spleen weight. Contribution of donor derived (CD45.2+) cells was measure by flow cytometry analysis of (M) Bone marrow cells and (N) spleen cells. The dashed line is the unstained control cells. There was no significant differences between mice transplanted with *ff* or control wildtype bone marrow or control untransplanted wildtype and *ff* mice.
Figure A- S3. Expression of BMP4 is induced in the spleen during recovery from bone marrow transplant. Paraffin sections of spleens isolated on the indicated day after transplant with 5x10^5 cells of C57BL/6-/-f/f and C57BL/6 control donor cells into CD45.1 recipients. The sections were double immunostained with antibodies to BMP4 (red) and CD45.2 (green). (A) The sections were examined by confocal microscopy at 20X magnification. Scale bar is 100µm. (B) 40X magnification. (C) Co-localization of BMP4 and CD45.2 was determined by Pearson’s correlation. Scale bar is 20µm. (D) Expression BMP4 and donor derived cells on day 8 after transplant is limited to the red pulp (RP) and excluded from the white pulp (WP).
Pop-I: Kit+ CD71^med Ter119^low
Pop-II: Kit+ CD71^high Ter119^med
Pop-III: Kit+ CD71^med Ter119^high
Figure A- S4. Functional and morphological analysis of the stress erythroid populations in spleen at day 8 post-transplantation. (center) Flow diagram of Populations I, II and III in spleen at day 8 (early recovery stage) gated on Kit+ cells analyzed for CD71 and Ter119 expression. The three populations were sorted, subjected to colony assays to evaluate their erythroid potential in vitro and Spun onto slides and stained with Hematoxylin and eosin to evaluate their morphology. (left) Population I cells exhibited primitive "blast" morphology and formed BFU E and CFU E colonies. (top) Population-II cells exhibited basophilic erythroblast morphology and formed CFU-E only. (right) Population-III cells exhibited orthochromic erythroblast morphology and formed no colonies. Only Population-III stained positive for hemoglobin by benzidine staining, with approximately 50% benzidine positive.
Figure A- S5. Population-I progenitors develop into population II and III

progenitors in vitro. Donor derived Population-I cells purified by FACS from the spleens of mice on day 8 after transplant with control bone marrow. The cells were cultured in the presence of BMP4, SCF, Epo, SHH and hypoxia (2% O2) and analyzed on days 3 and 6 for the development to other Populations II and III by flow cytometry. Population-I gave rise to population II and III at day3 and Population-II cells were predominant at day6. This experiment is representative of two independent experiments done with cells isolated from independent transplants.
Figure A - S6.
Figure A- S7. SCL expression is defective in mice transplanted with C57BL/6-Δf/f mutant donor cells. (A) paraffin section of spleen isolated on day 12 after transplant with 5x10^5 of C57BL/6 donor cells were stained with antibodies to SCL (red) and CD45.2 (green). The section was examined by confocal microscopy. The donor derived CD45.2+ cells overlaid (yellow) with SCL expression and both localized to the red pulp areas in spleen. (B) Analysis of SCL protein expression during the post transplant recovery in spleen sections from mice transplanted with 5x10^5 mutant (C57BL/6-Δf/f) or control (C57BL/6) bone marrow cells. SCL expression is indicated in red and CD45.2 in green. Scale bar is 20µm. (C) Analysis of colocalization of CD45.2 and SCL in spleen sections by Pearson’s correlation (Rr).
Figure A- S8. Analysis of tertiary transplants. CD45.1;HbbS mice were irradiated with a split dose of radiation (two doses of 475rads) and transplanted with donor derived spleen cells sorted from secondary transplanted mice. (A) Survival of tertiary transplanted mice. (B) Hematocrit analysis, (C) RBC counts. (D) Analysis of peripheral blood mononuclear cells 50 Days after tertiary transplant.
Figure A-S9. Expression of Epo in the kidney of CD45.1 mice transplanted with $5 \times 10^5$ control (left) or mutant (right) bone marrow cells on the indicated days after transplant. 28S rRNA was used as RNA loading control.
Acknowledgements

We thank Susan Magargee, Elaine Kunze and Nicole Zembower in flow cytometry facility for their excellent help and advice on cell sorting, Jeff Dodds for his help with histology and in developing the bone marrow transplant protocol, and Ruth Haldeman in electron microscopy facility. Gerd Blobel provided the MSCV-Scl and MSCV-Gata2 virus. We also thank Paulson Lab members for their support. This work is dedicated to the memory of Gholamreza Sharifi, who devoted his life to inspire and support many young scientists (OFH). This work was funded by a grant from the National Blood Foundation (RFP), Seed funding (RFP), NIH grant DK080040 (RFP) and graduate student competitive research awards (OFH and DCW) from the College of Agricultural Sciences at Penn State University.
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Scientific Publication
- Eirini Trompouki*, Teresa V. Bouwmans*, Lee N. Lawton, Zi Peng Fan, Dai-Chen Wu, Anthony DiBiase, Corey S. Martin, Jennifer N. Cech, Anna K. Sessa, Jocelyn L. Leblanc, Pulin Li, Garrett Heffner, George Daley, Robert F. Paulson, Richard Young and Leonard I. Zon. BMP and Wnt pathway are currently in preparation for submission (chapter 3 and 4); another project as the major contributor is ongoing in the lab (Chapter 4, GDF15).

Scientific Presentation (selected)
- Role of Hedgehog signaling in stress erythropoiesis. May 2, 2011. Dissertation Seminar, Eberly College of Science, The Pennsylvania State University, University Park, PA, USA
- Wu DC and Paulson RF. Hedgehog signaling is required for erythroid short-term radioprotection during the recovery from bone marrow transplantation. Poster, Annual Graduate Exhibition, March 26-28, 2010, The Pennsylvania State University, University Park, PA, USA
- Wu DC and Paulson RF. Hedgehog signaling is required for erythroid short-term radioprotection during the recovery from bone marrow transplantation. Poster, Gordon Research Conference - Red Cell, June 28 - July 3, 2009, University of New England, ME, USA
- Regulation of stress erythropoiesis by Bone Morphogenetic Protein 4 and Hedgehog signaling. April 24, 2008. Research Seminar Series, Eberly College of Science, The Pennsylvania State University, University Park, PA, USA
- Regulation of Bone Morphogenetic Protein 4 by hypoxia. September 2006. Short talk, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA, USA

Award / Fellowship
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- Fellowship, Ministry of Education, Taiwan (2000-02)
- Excellent Undergraduate Research Creativity Award, National Science Council, Taiwan (2000)
- Excellent Undergraduate Research Project Grant, National Science Council, Taiwan (1999-00)
- Fellowship, National Science Council, Taiwan (1998-00)
- 1st place, creative writing – story, the 14th National Literature and Art Conference, Taiwan (1999)

Professional Society
- Member of International Society of Stem Cell Research (2010- present)