REGULATION OF STRESS ERYTHROPOIESIS: INTERACTION BETWEEN MICROENVIRONMENT AND STRESS ERYTHROID PROGENITORS

A Dissertation in
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by

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Abstract

Steady-state erythropoiesis occurs in the bone marrow and produces erythrocytes at a constant rate. Its role is to replace “worn out” red blood cells that are removed by the spleen. While in response to acute anemia and tissue hypoxia, another type of erythropoiesis, stress erythropoiesis, is induced to rapidly generate new erythrocytes to compensate the loss. Previously our lab showed that stress erythropoiesis utilizes various signals and stress erythroid progenitors that are distinct from steady state erythropoiesis. Initially, bone marrow CD34+KSL cells migrate into the spleen and interact with hedgehog ligands, which induce the expression of BMP4. Hedgehog and BMP4 together specify the stress erythroid fate. Further work showed that there are three distinct populations of stress erythroid progenitors that expand in the spleen during the recovery from bone marrow transplantation. They were identified as Population I (Kit + CD71 -med Ter119 lo/), Population II (Kit + high CD71 med Ter119 ) and Population III (Kit + CD71 -med Ter119 high). The appearance of three populations suggested the temporal order for their development: Population I gives rise to Population II and Population II gives rise to Population III. Although the number of Population I cells increased in the pre-recovery stage, we didn’t observe stress BFU-E until day 8, which indicated that these cells proliferated but couldn’t differentiate in the early time. Then they acquired the ability to differentiate into stress BFU-E. Finally, BMP4, SCF and hypoxia are required for the expansion and differentiation of stress BFU-E during the late stage of the recovery from acute anemia.
My work mainly focuses on the following questions: 1. What is the role of GDF15 in the regulation of stress erythropoiesis? 2. How to identify the self-renewing Population I cells and differentiating Population I cells? 3. How does Epo regulate the transition from expansion to differentiation of stress erythroid progenitors? These questions will be answered in Chapters 2, 3 and 4. In Chapter 2, my work showed that GDF15-/- mice are deficient in recovery from acute anemia and fail to provide short-term radioprotection. GDF15 plays a critical role in stress erythropoiesis by inducing and maintain hypoxia dependent BMP4 expression. My work in chapter 3 further described the development of distinct stress erythroid progenitors with increasing maturity based on their expression of cell surface markers CD34, CD133, Kit and Sca1. Here we developed an in vitro culture system and identified CD34+CD133+KS population as the early stress erythroid progenitors that have self-renewal ability and CD34-CD133-KS population as the later stress erythroid progenitors that have differentiation ability. The bone marrow transplantation data also showed CD34+CD133+KS cells expanded on day6 and CD34-CD133-KS cells were increased on day 12 post-transplantation. Then I further demonstrated that similar to murine bone marrow, human bone marrow contains cells can exhibit stress erythropoiesis ability and generate BMP4 dependent stress BFU-E.

Finally my work in Chapter 4 showed that hypoxia and Epo induce the transition from expansion to differentiation of stress erythroid progenitors. The interaction between microenvironment and stress erythroid progenitors contributes a key part in regulating stress erythropoiesis. Wnt signaling produced by macrophages involves in the expansion of early stress erythroid progenitors. Epo stimulates macrophages
through Stat5 signaling pathway to down regulate Wnt signaling and increase Prostaglandin E2 production to induce the differentiation of stress erythroid progenitors. The characterization of stress erythroid progenitors and the signals that regulate their development potentially provide new therapeutics to treat anemia and improve the recovery from bone marrow transplants.
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<table>
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<th>Description</th>
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<tr>
<td>AGM</td>
<td>Aorta Gonad Mesonephros</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
</tr>
<tr>
<td>BFU-E</td>
<td>Burst Forming Unit-Erythroid</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone Marrow Derived Macrophage</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>BMP4R</td>
<td>Bone Morphogenetic Protein 4 Receptor</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete Blood Cell Count</td>
</tr>
<tr>
<td>c-Kit</td>
<td>Kit receptor tyrosine kinase</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony Forming Unit-Erythroid</td>
</tr>
<tr>
<td>CFU-S</td>
<td>Colony Forming Unit-Spleen</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP Responsive Element</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>EI</td>
<td>Erythroblastic Island</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EpoR</td>
<td>Erythropoietin Receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow Associated Cell Sorter</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>GDF15</td>
<td>Growth and Differentiation Factor 15</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/Monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HH</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HRE</td>
<td>HIF2α Responsive Element</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin 3</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte Erythroid Progenitor</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotential progenitor</td>
</tr>
<tr>
<td>OHT</td>
<td>4-Hydroxytamoxifen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHZ</td>
<td>Phenylhydrazine</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td>SBE</td>
<td>Smad2/3 Binding Element</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Stress Erythropoiesis Medium</td>
</tr>
<tr>
<td>SEEM</td>
<td>Stress Erythroid Expansion Medium</td>
</tr>
<tr>
<td>SEDM</td>
<td>Stress Erythroid Differentiation Medium</td>
</tr>
<tr>
<td>Sf-Stk</td>
<td>Short-form Stk</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Stk</td>
<td>Stem-cell-derived tyrosine kinase</td>
</tr>
<tr>
<td>μl</td>
<td>Micro liter</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel Lindau</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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Chapter 1

Introduction

1.1 Hematopoiesis: A Brief Overview

Hematopoiesis is the highly regulated development of diverse blood cells that perform different functions. It is described as a hierarchal system and provides the regeneration and replacement of all the blood cell types. The process of hematopoiesis starts from a pool of self-renewing and multipotential cells that can give rise to all blood cell types, which are called Hematopoietic Stem Cells (HSC)[2-5].

The existence of HSCs was firstly proposed by Till and McCulloch in 1961. A series of experiments found that bone marrow contains proliferative cells that can give

Figure 1.1: Development of different blood cells during hematopoiesis[10].
rise to various types of colonies of myeloid, erythroid and lymphoid cells in the spleen of irradiated host[8]. Almost 0.05% of cells in the bone marrow are HSCs, which are responsible for the radioprotective and reconstitution effects after bone marrow transplantation. According to their self-renewal ability, HSC can be divided into short-term (ST) and long-term (LT) reconstituting HSC and they can be separated by phenotype. LT-HSCs have infinite replicating ability while ST-HSC can only self renew for a few weeks[4] (Figure 1.1).

HSCs can be isolated by fluorescence-activated cell sorting (FACS) with the specific cell surface markers Kit+Sca1+Lin- (K+S+L-). They express Kit receptor and Sca1 but no lineage specific surface markers (Lin-)[11-13]. LT-HSCs and ST-HSCs are identified separately as CD34⁻K⁺S⁺L⁻ and CD34⁺K⁺S⁺L⁻ cells, respectively. ST-HSCs can give rise to Multipotential Progenitors (MPP). MPPs will further differentiate into lineage-restricted progenitors: common lymphoid progenitor (CLP) and common myeloid progenitors (CMP)[11, 14]. CMPs subsequently give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte /erythrocyte progenitors (MEPs) with differentiate into erythrocytes and platelets[15]. CLPs will further give rise to B-cells, T-cells and NK-cells [14]. Both CLPs and CMPs can differentiate into dentritic-cells[16]. According to the cell surface markers, people have identified and purified various progenitors by using FACS[17, 18]. MPPs are CD150⁻CD48⁻CD244⁺; Lineage-restricted progenitor cells (LRPs) are CD150⁻CD48⁻CD244⁺; CMPs are Lin-Sca1⁻Kit⁺CD34⁺CD16/32mid; GMPs are Sca1⁻Kit⁺CD34⁺CD16/32hi and MEPs are Sca1⁻Kit⁺CD34⁻CD16/32low.
There are two types of hematopoiesis, primitive and definitive hematopoiesis. In mice, primitive hematopoiesis starts with the embryonic HSCs in the yolk sac between E7 and E8 during fetal development, which will make embryonic hemoglobins. Then cells migrate into embryo body. Fetal liver is the place for mid- and late-stage of primitive hematopoiesis in mammals[19-21]. Around 10 days post-conception, adult hematopoiesis begins to take the place of primitive hematopoiesis. Adult HSCs originate from aorta-gonad-mesonephros (AGM) at E10-11 in mouse. Then hematopoietic progenitors migrate into fetal liver. In the later fetal life, bone marrow is the main place for hematopoiesis[21-24]. Adult hematopoiesis follows the hierarchy of development.

1.2 Erythropoiesis

Erythropoiesis is characterized by the maturation of hematopoietic stem cells to erythroid lineage specific progenitors and it is a critical process that produces red blood cells (RBC) with oxygen delivery function. In this process, MEPs give rise to burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E), which immediately form proerythroblasts[25]. They then go through several stages of development (basophilic erythroblast, polychromatic erythroblast and normoblast) and finally lose their nucleus to form erythrocytes or red blood cells (RBC) (Figure1.2). CFU-Es are identified to express Epo-receptor, Kit (stem cell factor receptor), transferrin receptor (CD71+) and they are Ter119-negative. Epo can induce CFU-E to terminally differentiate within 2 or 3 days in vitro culture. In contrast, it takes longer time (about 8
days) for BFU-E to differentiate into RBCs[26]. Both BFU-E and CFU-E are defined by colony formation in methylcellulose medium with specified cytokines, Epo alone for CFU-E or Epo+SCF for BFU-E. Recently people used CD44, CD71 and Ter119 to identify stages of terminal differentiation[27].

Erythropoiesis is strictly regulated by multiple factors that are involved mainly in cell fate determination and maturation of RBCs. Erythropoietin (Epo)-EPOR signal pathway plays a critical role in erythropoiesis, which regulates both proliferation and differentiation in erythroid lineage. Systemic hypoxia can increase red cell production by inducing Epo synthesis[28]. The lineage commitment also involves the action of other transcription factors, including TAL1, LMO2, GATA1 and GATA2. Transcriptional factors like NF-E2 and KLF are important in differentiation of committed progenitor
cells[26]. Dysregulated expression of GATA-1 in primitive hematopoietic cells enlarges the pool of erythroid progenitor cells[29]. It has been shown that KLF2-/- mice have a significant defect in primitive erythropoiesis[30]. SCF/c-Kit is also a critical element in the development of early progenitor cells[31-33]. Later more and more factors were found regulating erythropoiesis. NF-kappaB are involved in normal erythropoiesis and Fas/Fas ligand system regulate the apoptosis during erythropoiesis[34, 35]. Lodish lab showed that Lnk-deficient mice have elevated numbers of erythroid progenitors and exhibit superior recovery after erythropoietic stress[36]. Runx1-/- mice also display abnormal morphology in primitive erythrocytes[37]. Recently another type of regulation- microRNA has been found contributing a significant impact on
erythropoiesis. MiR-221 and miR222 inhibit erythropoiesis by kit-receptor down-modulation[38]. Mir-24 also inhibits erythropoiesis by targeting activin type I receptor ALK4[39].

Primitive erythropoiesis differs from definitive erythropoiesis and it occurs in yolk sac. The primitive erythroid progenitors (EryP-CFC) were identified by capability of forming colonies of erythroid cells in vitro and they first merge within the yolk sac approximately on E7.25 [40]. Then EpoR transcripts were detected in blood islands of mouse embryos at E7.5. Then maturation of primitive erythroid precursors happens together with cell divisions for the amplification of erythroblasts [41]. It has been known that erythroblasts contain different hemoglobins compared with definitive erythropoiesis [42]. In the mouse, primitive red blood cells express high levels of embryonic globins βH1 and εy as well as low levels of adult globin β1 and β2 [41]. After birth, the switch to adult β globin gene expression is completed. People have shown that BCL11A knock out mice exhibit the disruption of embryonic/fetal to adult globin gene switch. BCL11A also involves long-range interactions and cooperation with SOX6 to induce the transcriptional silencing of β globin [43, 44].

1.3 Homeostatic Versus Stress Erythropoiesis

Erythrocytes are essential blood cells in that they deliver the oxygen to the body tissue through the circulatory system. Because they are enucleated and cannot proliferate, they must be produced constantly through erythropoiesis throughout one’s
lifetime. Normally, in human body, the RBCs have a lifespan of 100-120 days. When they reach the end of lifespan, they will be senescent and removed from circulation. Thus the body needs to produce fresh RBCs to replace the worn-out cells at a constant rate. A low level of RBCs causes anemia and insufficient absorption of oxygen by tissues. A high level of RBCs is referred to as polycythemia. It increases the viscosity of blood, which will cause clotting and hemorrhage. Homeostatic erythropoiesis or steady-state erythropoiesis constantly generates new RBCs to maintain the level of tissue oxygenation. It mainly occurs in the bone marrow during adult life and relies on local source of Epo. Most of what we know about erythroid progenitors such as MEP, BFU-E, CFU-E and later stages were well defined using steady state erythropoiesis as a model.

In addition to the homeostatic erythropoiesis, there is another type of erythropoiesis that occurs in response to anemic stress, like tissue hypoxia, massive blood loss, acute anemia and irradiation. It is called stress erythropoiesis and it must rapidly produce sufficient number of erythrocytes to compensate for the loss of red blood cells. Stress erythropoiesis differs from steady-state erythropoiesis in several aspects: (1) Steady-state erythropoiesis happens in bone marrow microenvironment while stress erythropoiesis is occurring extramedullary mainly in the fetal liver and adult spleen in mouse[45, 46]. It has been known that following bone marrow transplantation, the hematopoietic activity was enhanced and the donor progenitor cells were immediately found in recipient spleen[47]. (2) Steady-state erythropoiesis needs Epo and other factors described above. However, stress erythropoiesis relies on high Epo concentration and in addition it needs hypoxia, bone morphogenetic protein 4
(BMP4), stem cell factor (SCF), Hedgehog and GDF15[48, 49]. The role of these factors will be discussed later in this chapter. (3) Stress erythropoiesis utilizes unique progenitors that are distinct from steady state erythroid progenitors. In the chapter 3, these specific progenitors will be described.

In order to assay stress erythropoiesis, people commonly use short-term radioprotection after bone marrow transplantation or phynelhydrazine-induced acute anemia as models to study stress erythropoiesis. In recent years, in addition to Epo, BMP4, SCF, GDF15, Hedgehog and hypoxia, multiple other signals involved in regulation of stress erythropoiesis have been discovered. Glucocorticoids receptor is required for rapid expansion of erythroid progenitors[50]. Foxo3 is required for the regulation of oxidative stress in erythropoiesis. Foxo3 deficient mice exhibited decreased expression of ROS scavenging enzymes and consequent increase in oxidative damage[51].

1.4 Model of Stress Erythropoiesis In flexed-tail Mice: BMP4

Signaling Pathway In Stress Erythropoiesis.

The BMP4 signaling pathway is critical for stress erythropoiesis and this requirement was discovered by the analysis of flexed-tail (f/f) mice. Previous work from our lab also showed that BMP4 is induced during the recovery from PHZ induced tissue hypoxia. Flexed-tail is a recessive mutation in mouse that discovered by Hunt in the rodent colony of department of zoology at the Michigan State College in 1928. This type of mutation is characterized by spiral or angular bends in the tails and white spotting in
the belly. The *flexed-tail* mice are on the average deficient at birth both in hemoglobin and erythrocytes. The newborn flexed-tail mice are severe fetal-neonatal anemia. But this will disappear within two weeks after birth[52, 53]. The adult flexed-tail mice exhibit normal erythropoiesis and they have normal reticulocyte counts. But they are deficient in response to acute eythroid stress[48, 54]. During the maturation of erythrocytes in the spleen, the early multipotential colony forming unit-spleen (CFU-S) has been identified to differentiate into BFU-E and CFU-E. In 1975, it was found that there was an intermediate committed erythroid progenitor identified to form transient endogenous spleen colonies, which was called TE-CFU and it was between CFU-S and CFU-E. TE-CFUs can produce excessive number of erythroid progeny when mice were irradiated or injected continuously with Epo. Thus TE-CFUs are characterized as the candidate progenitor that can respond to erythropoietic stress conditions[55-57]. The f/f mice exhibit normal BFU-E and CFU-E colonies but they are defective in expanding TE-CFUs in vivo when exposed to high Epo concentration and irradiation. Due to this defect, f/f mice cannot respond rapidly to erythropoietic stress and exhibit a delay expansion of erythropoietic progenitors. They also have a delay in the recovery of hematocrits after phenylhydrazine (PHZ) injection that resembles the hypoxia state that occurs after irradiation[58]. However, except this defect, f/f mice do not have defect in other hematopoietic lineage. Taken together, the f/f mouse is an excellent model to study stress erythropoiesis.

Based on those findings, our laboratory has demonstrated that f/f mice have a delay in BFU-E expansion in spleen after PHZ treatment, which causes a delayed
recovery from PHZ induced acute anemia. The control mice exhibited an expansion of BFU-E in the spleen at 36 hours post PHZ injection while the expansion was observed at 4-6 days after injection in f/f mice[48]. In 2004, our lab determined that f/f locus contains a dominant negative mutation in the Smad5 gene that leads to the production of SMAD5. Smad5 is a downstream factor in the BMP4 signaling pathway. BMP4 is a member of transforming growth factor-beta (TGF-β) family. The binding of BMP4 to its receptors leads to phosphorylation of SMAD1/5/8. Then SMAD1/5/8 and the Co-SMAD (SMAD4) form the complex and translocate into nucleus to regulate gene transcription[59, 60]. It is a critical signaling molecule required for bone development and early differentiation of embryo. In response to acute anemia, BMP4 is rapidly induced in the spleen. BMP4 acts on the stress BMP4 responsive cells (BMP4R) and cause them to rapidly generate stress BFU-Es. SCF/Kit signaling pathway, hypoxia and hedgehog signaling also are required in stress erythropoiesis but they play different roles. BMP4 acts on several levels during stress erythropoiesis. It induces BMP4R cells to form stress BFU-E. SCF and hypoxia in combination with BMP4 promote the expansion of stress BFU-E. Stress erythroid progenitors in the spleen are derived from bone marrow stem cells. Bone marrow progenitors do not respond to BMP4, but Hedgehog in spleen microenvironment allows cells to adopt the stress erythroid fate. Hedgehog induces BMP4 expression. Hedgehog and BMP4 are required for the specification of the stress progenitor fate. In addition, Hedgehog appears to play a role in the expansion of stress erythroid progenitors in the spleen[61] (Figure 1.3).
Data published in 2010 from our lab showed that in response to acute anemia, short-term reconstitution HSCs (STR-HSC, CD34+Kit+Sca1+Lin-) migrate into spleen and give rise to stress BFU-Es[1]. Resident in the spleen we observed Kit+CD71+Ter119+ population, which differentiate in response to acute anemia. BMP4, SCF and hypoxia promote the expansion of this population. Dr. Harandi from our lab further identified three distinct populations in spleen during the recovery from bone marrow transplantation by flow cytometry analysis and called them Population I, II and III (Figure 1.4). He also demonstrated that during the recovery of acute anemia induced by bone marrow transplantation, the three population emerged in an order. Population I cells (Kit'CD71lo/medTer119lo/−) give rise to Population II cells (Kit'CD71hi/Ter119med) and Population II cells give rise to Population III cells (Kit'CD71lo/medTer119hi).
Population 1 cells have self-renewal ability and they can provide short-term radioprotection when serially transplanted into irradiated recipients[1]. In Chapter 3, I will further describe those populations by using new cell surface markers.

Figure 1.4: Functional and morphological analysis of the stress erythroid progenitors in spleen at day8 post-transplantation[1]. Flow diagram shows three populations in spleen at day 8 gated on Kit+ and analyzed for CD71 and Ter119 expression. These populations were sorted for colony assays to evaluate their erythroid potential in vitro.

1.5 Signals In Stress Erythropoiesis

Erythropoiesis is a dynamic developmental process regulated by intrinsic and extrinsic signals. Stress erythropoiesis has the same terminal erythroid differentiation and requirement of erythroid specific factors as the steady state erythropoiesis. However, stress erythropoiesis not only requires Epo and GATA2, but also needs BMP4, SCF/KIT, Hedgehog, GDF15 and hypoxia. In addition, this process requires special
microenvironment for the proliferation and differentiation of stress erythroid progenitors. In this section, I will introduce the major signals that have been demonstrated playing a critical role in stress erythropoiesis.

1.5.1 Role Of Hypoxia And Erythropoeitin (Epo) In Stress Erythropoiesis

Acute anemia will cause tissue hypoxia, which leads to a systematic response designed to increase oxygen delivery to tissues[62]. One of the response is stress erythropoiesis, so hypoxia is the main factor that stimulates this process. Once the body senses a hypoxic state, the erythropoiesis will happen faster and produce more erythrocytes in a short time to increase the oxygen concentration. Previously people showed that if bone marrow and fetal liver BFU-E and CFU-E were grown in hypoxic condition, an increased number of cells was observed when compared to those grown in normoxia condition (atmospheres oxygen). In addition, the cells grown in hypoxic condition have more sensitivity to Epo[63]. Hypoxia also regulates stress BFU-E differentiation[49].

Hypoxia alters cellular responses in many ways. Chief among these responses is the regulation of gene transcription by the hypoxia inducible transcription factor (HIF). In addition, HIF independent mechanism of altering gene transcription can occur, which rely on the changes in transcription factor translation regulated by the unfolded protein response. This pathway is regulated by the sensor kinase PERK, which leads to the expression of ATF4, a CREB family member. One of the genes that are regulated by
hypoxia is BMP4, a key factor in stress erythropoiesis. In response to acute anemia, HIF2α binds to the two HIF response element (HRE) sites in the BMP4 locus and promotes the transcription, which initiates the BMP4-dependent stress erythropoiesis[64]. Activating transcription factor 4 (ATF4) belongs to the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family that consists of basic-region leucine zipper transcription factors that bind to the consensus cAMP responsive element (CRE) binding site[65]. The expression of ATF4 can be induced by hypoxia, ER stress and oxidative stress[66]. ATF4--/-- mice display severe fetal anemia and a decrease in size of progenitor colonies in the fetal liver. Interestingly, adult ATF4 mice have only slightly impaired erythropoiesis[67].

Hypoxia induces a transcriptional response through the stabilization of the alpha subunit of HIF[68]. At normal oxygen concentration, HIF alpha subunit is hydroxylated on specific proline residues by prolyl-hydroxylases. These hydroxyl-prolines form a binding site for the VHL-E3 ubiquitin ligase complex that marks it for proteasomal degradation. One of the target genes regulated by HIF is Epo, a crucial factor that involved in erythropoiesis. The hypoxic state induces kidney to produce Epo, which leads to an increased circulating Epo titer. The regulation of Epo occurs by mRNA level. Under hypoxic condition, hypoxia inducible factor-1α (HIF-1α), hepatic nuclear factor 4 (HNF4) and the general transcriptional activator p300 form a trimetric complex. This complex binds to the regulatory domain of the Epo gene and induces Epo expression[69].
Epo signal is the key factor that regulates proliferation, differentiation and self-renew of hematopoietic cells[70]. Extensive studies of Epo helped the development of the recombinant Epo as the antianemia agents[71]. When Epo binds to the receptor, the tyrosine kinase Jak2 is activated and promotes the tyrosine phosphorylation of EpoR. Then the transcription factor Stat5 is recruited to EpoR and activated by phosphorylation. The activated Stat5 translocates into the nucleus and promotes the transcription of target genes involved in erythropoiesis (Figure 1.5). A role for Epo signaling in stress erythropoiesis was shown in the work from Mennon et al. They showed that Stat5-/- mice exhibit normal steady-state erythropoiesis but cannot respond to acute erythropoietic stress. They also showed that a defect in stress erythropoiesis occurs in mice expressing a truncated EpoR that lacks the ability to activate Stat5[72]. Other studies also showed that the Stat5a-/-5b-/- mice have ineffective erythropoiesis due to decrease survival of early erythroblasts[73]. In addition to its role in erythroid progenitors, Stat5 also functions in stem cells where it promotes the expansion of immature progenitor cells. The different roles of Stat5, depend on the expression of Gata1, which changes the Stat5 response from proliferation to differentiation[74]. Taken together, hypoxia and Epo represent two crucial signals that regulate stress erythropoiesis[75].
1.5.2 Role Of Hedgehog Signaling In Stress Erythropoiesis

Hedgehog (HH) was identified as one of several genes that involved in the development of the body segments in Drosophila. It also plays a critical role in the embryo development, erythropoiesis and disease by regulating the transcription of target genes. In embryos, the concentration of hedgehog signaling proteins in different body parts identified the developmental process. In adults, mutations in this pathway result in various phenotypes, including nevoid basal cell carcinoma syndrome and Pallister-Hall syndrome[77]. It also regulates the maintenance and proper behavior of stem cells and progenitors.
There are three Hedgehog homologues in mammals, Desert Hedgehog (DHH), Indian Hedgehog (IHH) and Sonic Hedgehog (SHH). In the absence of Hedgehog, the transmembrane protein called Patched (Ptc) binds to a positive acting co-receptor Smoothened (Smo) to inhibit its activity. When extracellular Hedgehog is present, it binds and inhibits Patched activity, which allows Smoothened to release and accumulate. The downstream factor of Smoothened is called Gli, which is a family of zing finger transcription factors. Smoothened will inhibit the proteolytic cleavage of Gli protein and Gli can translocate into nucleus and regulate the transcription of target genes[78]. Gli transcription factors have three members, Gli1, Gli2 and Gli3. Gli1 and Gli2 are the activators and Gli3 is the repressor[79].

The role of Hedgehog signal pathway in hematopoiesis has been studied for years. In 1995, it has been shown that lhh-/- and smo-/- yolk sac had a severe defect in angiogenesis development, which indicates that hedgehog is involved in hematopoiesis[80, 81]. Later, it was demonstrated that sonic hedgehog can induce the proliferation of primitive human hematopoietic cells. The BMP-4 inhibitor, Noggin, was capable of inhibiting Shh-induced proliferation and this suggested that Shh signaling works through BMP4[82]. Our lab also showed that hedgehog induces the expression of BMP4 in the spleen microenvironment during stress erythropoiesis. Hedgehog and BMP4 together regulate the maintenance of stress erythroid progenitors[61].
1.5.3 Role Of SCF/Kit Receptor Signaling In Stress Erythropoiesis

Many spontaneous chemical-induced and radiation-induced dominant white spotting (W) and steel (Sl) mutations have been identified in the mouse. Russell showed that W and Sl mutations have similar effects including deficiencies in blood cells, pigment cells and germ cells. Mice bearing mutant W alleles or mutant Sl alleles have severe macrocytic anemia[83, 84]. Mutation at this locus also effects the proliferation and migration of cells during early embryogenesis and a defect in hematopoietic stem cell hierarchy[85]. W locus encodes a putative tyrosine kinase receptor c-kit and Sl locus encodes a ligand for c-kit, stem cell factor (SCF)[84, 86-88]. In other developmental systems, SCF and c-kit play a key role in the determination of cell fate and the elaboration of developmental programs[32]. Broudy et al. showed that W mice and Sl mice have a defect on acute expansion of hematopoietic cells in the spleen after phenylhydrazine injection. The interaction of Kit and SCF is required for normal homing of hematopoietic progenitor cells in response to acute anemia[45]. It is also identified that recombinant human stem cell factor enhances the formation of colonies[89]. Recently our lab found that the resistance to friend-virus-induced erythroleukemia in $W^v/W^v$ mice is caused by a spleen-specific defect that results in a severe reduction in target cells and a lack of Sf-stk expression[90]. SCF, hypoxia and BMP4 cooperatively promote the expansion and differentiation of stress BFU-E during recovery from acute anemia[49].

In addition to those signals described above, we found some new signals that
involve in the regulation of stress erythropoiesis, which include GDF15, Wnt signaling and Prostaglandins. We also discovered an important interaction between macrophages and stress erythroid progenitors. In the following section, I will introduce these new signals and their function in erythropoiesis.

Figure 1.6: TGF-β signaling pathway. (A) TGFb signaling pathway. (B) BMP pathways with their corresponding Smad proteins and mechanisms of inhibition by I-Smads (Smad6/7). (C) Non-Smad signaling pathways downstream of the TGFb receptors[7].

1.6 GDF15 Signaling In Erythropoiesis

Growth and differentiation factor 15 (GDF15) is a protein in the transforming growth factor beta (TGF-β) superfamily that has a role in regulating inflammatory and apoptotic pathways during disease processes. When TGF-β superfamily ligands bind to a type II receptor, it recruits and phosphorylates a glycine-serine rich juxtamembrane domain in a type I receptor and form a heterotetrameric signaling complex. This
complex then phosphorylates receptor-regulated SMADs (R-SMADs) including Smad1, Smad2, Smad3, Smad5 and Smad8, which can bind the coSMAD (Smad4). RSMADs/coSMAD complexes accumulate in the nucleus where they act as transcription factor and regulate gene expression (Figure 1.6).

GDF15 can be induced in hepatocytes by surgical and chemical injury and heat shock. The induction of GDF15 was independent of protein synthesis, which is a hallmark of immediate early gene regulation[91]. It also has been found that circulation concentration of GDF15 is associated with chronic heart failure (CHF) and it is a new biomarker of the risk of death in patients with CHF[92, 93]. TNFα, TGF-β and IL-1 also can induce GDF15 rapidly in macrophages to limit macrophage activation and inflammation[94]. Kempf et al found that GDF15 is induced in cardiomyocytes on ischemia/reperfusion (I/R) and unregulated by other cardiovascular[95].

GDF15 is closely related to erythropoiesis and it was identified as a hepcidin-suppression factor that is expressed at high levels in patients with ineffective erythropoiesis like thalassemia and related anemias[96]. The suppression of hepcidin production in hepatocytes leads to iron overload in thalassemia[97, 98]. Although GDF15 plays a critical role in erythroid iron homeostasis, a direct role of this signal in regulating erythropoiesis has not been identified. GDF15-/- mice do not exhibit any steady state defects in erythropoiesis[99]. However the in vitro studies showed erythroid-specific production of GDF15 and dependence on erythropoietin (Epo). Downregulation of endogeneous GDF15 expression by siRNA in erythroblasts compromised erythroid differentiation[100]. Recently, our lab also showed that GDF15
is also involved in stress erythropoiesis. The GDF15/- mice have delayed recovery in
the short-term radioprotection. They also failed to recovery from the acute anemia
induced by high dose injection of PHZ. In Chapter 2, our recent discoveries about the
role of GDF15 in BMP-4 dependent stress erythropoiesis will be presented.

1.6 Wnt Signaling In Hematopoiesis

Roel Nusse and Harold Varmus first discovered the Wnt signaling in 1982. When
they tried to see the genes that cause breast tumors, they identified a new mouse proto-
oncogene int1 (integration). Later they discovered further genes related to int1 and the
int family was renamed Wnt family that stands for Wingless-related integration
site[101, 102]. Wnt signaling pathway contains a group of secreted proteins that pass
signals out side of a cell through cell surface receptor to the inside of the cell. There are
three Wnt signaling pathways activated by the binding of a Wnt-protein ligand to the
receptor Frizzled: the canonical Wnt pathway, the noncanonical planar cell polarity
pathway and the noncanonical Wnt/calcium pathway. Among 19 wnt ligands, Wnt2,
Wnt2b, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a and
Wnt10b are canonical Wnt ligands. Wnt1, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt11 and
Wnt16 are noncanonical wnt ligands.

For canonical wnt signaling pathway, without Wnt binding to the receptor, Axin,
GDK and APC will form a destruction complex to destroy β-Catenin by ubiquitination.
When Wnt protein binds the extracullular domain of a Frizzled (Fz) family receptor and
some co-receptors like lipoprotein receptor-related protein (LRP)-5/6, receptor tyrosine kinase (Ryk), the phosphoprotein Dishevelled (Dsh) is recruited and binds to Fz receptor. The destruction complex becomes disrupted and translocates to the membrane. GSK3 activity is inhibited by Dsh, which allows β-Catenin to accumulate and localize to the nucleus and then regulate the gene transcription together with transcription factors TCF/LEF[103, 104]. The other two noncanonical Wnt signaling pathways do not involve β-Catenin accumulation and they regulate the restructuring of the cytoskeleton and calcium release from the ER in order to control intracellular calcium levels[103, 105]. Noncanonical Wnt signaling pathway can inhibit canonical signaling in K562 cells[106].

Wnt signaling plays a critical role in the embryonic development of a variety of organisms, axis patterning, cell fate specification, cell proliferation and migration. It also involves in the development of malignant breast tumors[107-109]. Here I mainly focus on the role of Wnt signaling in hematopoiesis. Wnt signaling can induce blood formation from stem cells and promote hematopoietic stem cell self-renewal and progenitor development[110]. Wnt5a, Wnt2b and Wnt10b were identified and cloned from human fetal bone stromal cells. Wnt5a was expressed in CD34+Lin- hematopoietic progenitors. The exposure to stromal cell layers expressing Wnt genes will increase the number of hematopoietic progenitors. Wnt10b showed differential activity on BFU-E[111]. Activation of canonical Wnt pathway in the early phase during embryoid body formation enhances ES cell differentiation into cardiomyocytes while activation of Wnt signal pathway in the late phase during embryoid body formation enhances the
expression of hematopoietic/vascular marker genes[112, 113]. The study of mouse embryonic hematopoiesis showed that Wnt 3,5a, 8a, Fzd4 and LRP5 were upregulated during primitive hematopoiesis, followed by Wnt3a, 6, 7b, 10b and 16 during definitive hematopoiesis. Specifically, Wnt16 was detected when the earliest lymphoid progenitors are formed[114, 115]. Other people also found that ectodermal BMP4 expression is dependent on Wnt4 signals from the mesoderm[116]. Luis et al demonstrated that different, lineage-specific Wnt dosages regulate hematopoietic stem cells, myeloid precursors, and T lymphoid precursors during hematopoiesis[117, 118]. Although the role of Wnt signaling in hematopoiesis has been identified for years, it has not been known very well that how Wnt signaling involves in the stress erythropoiesis. In Chapter 4, recent findings from our lab regarding Wnt signaling pathway in stress erythropoiesis will be further presented.

1.7 Prostaglandins Function

Prostaglandins are a group of lipid compounds that were first isolated from seminal fluid in 1935. They are derived from enzymatically from fatty acids (Figure 1.7). Prostaglandins are autocrine or paracrine molecules that behave as acting messenger molecules. It has been known that prostaglandins have a broad function in the body. They can regulate inflammatory mediation and calcium movement, control cell growth and regulate the contraction and relaxation of smooth muscle tissue. Prostaglandins are synthesized following the sequential oxidation of AA or EPA by cyclooxygenases (COX1 and COX2). PGH2 then goes though terminal prostaglandin
synthases and finally produces different prostaglandins, such as PGE2, PGI2, PGD2 and PGJ2 [119, 120].

Prostaglandin E2 (PGE2) produces high stimulation of endogenous heme synthesis and promotes erythropoiesis[121, 122]. Data showed that PGE2 works together with Epo to promote the synthesis of fetal and adult hemoglobin in peripheral blood-derived erythroid burst colonies from normal adults and from patients with sickle cell anemia[123]. The genetic interaction of PGE2 and Wnt signaling also regulates developmental specification of stem cells and regeneration[124]. Work from Leonard Zon lab also showed that the enhancement of PGE2 synthesis increased HSC numbers and it regulates the HSC homeostasis and engraftment[125, 126]. Taken together, PGE2 serves as an important mediator in microenvironment of hematopoiesis. Our lab recently demonstrated a crucial role of PGE2 in stress erythropoiesis. Epo can stimulate the production of PGE2 to promote the differentiation of stress erythroid progenitors, which will be illustrated in Chapter 4.
1.8 Role Of Macrophages In Erythropoiesis

Macrophages are derived from precursor cells (CMP) in the bone marrow. They play many roles in the body. Macrophages can get rid of the worn-out cells as scavengers and they have a crucial role in initiating an immune response. As the secretory cells, macrophages and monocytes are important in the regulation of immune response and the development of inflammation. There are various types of macrophages identified by their location. Adipose tissue macrophages reside in adipose
tissue; monocytes stay in bone marrow and blood; red pulp macrophages are in the spleen; peritoneal macrophages locate in peritoneal cavity. Macrophages can be identified by flow cytometry due to the expression of a number of proteins including CD14, CD11b, F4/80, MAC-1/MAC-3, CD64 and CD68[127].

Resident macrophages in hematopoietic tissue can be distinguished from other stromal cells and monocytes by immunostaining with antibody F4/80 and anti-Frossman glycosphingolipid body. It plays a supportive role in erythropoiesis. In the presence of Epo, the erythroid colony-forming units adhere to a resident macrophage and differentiate to erythroblasts, resulting in the formation of an erythroblastic island (EI)[128]. EIs are the specialized niches for the proliferation, differentiation and enucleation of erythroid precursors[129]. The splenic macrophages are a source of inflammatory mediators accelerating the expansion of murine erythroleukemic cells[130]. Later people identified CD163 on macrophages as an adhesion receptor for erythroblasts in EIs[131]. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in mesenchymal stem cell niche[132]. F4/80+ splenic macrophages participate in fetal erythropoiesis and in the formation of the splenic architecture[133]. Without macrophages, an increasing apoptosis was observed in erythroid progenitor cells.

Rivella’s lab demonstrated that macrophages contribute decisively to recovery from induced anemia and the pathological progression of polycythemia vera and beta-thalassemia. Mice treated with clodronate showed markedly impaired recovery from
anemia. In the human cells culture, they also showed that macrophages stimulate proliferation and delay enucleation of erythroblasts[134]. Our lab recently found another role of macrophages in stress erythropoiesis. They response to Epo and change the signals in the microenvironment to regulate the expansion and differentiation of stress erythroid progenitors, which I will discuss further in Chapter 4.
1.10 References


Chapter 2

Growth And Differentiation Factor 15 (GDF15) Regulates Stress Erythropoiesis By Inducing Hypoxia Dependent BMP4 Expression

Forward

The following chapter is a manuscript that will be submitted as “Jie Xiang, Dai-Chen Wu, Shailaja Hegde, Jefery L. Miller and Robert F. Paulson, Growth and Differentiation Factor 15 (GDF15) regulates stress erythropoiesis by inducing hypoxia dependent BMP4 expression.” This manuscript was written by Robert F. Paulson and Jie Xiang. Figure 2.1 was done by Dai-Chen Wu. Figure 2.4A-B, Figure 2.7B-C were done by Shailaja Hegde. Figure 2.7A was adapted from a published paper appearing in “Prashanth Porayette, Robert F. Paulson, BMP4/Smad5 dependent stress erythropoiesis is required for the expansion of erythroid progenitors during fetal development. Developmental Biology. 2008 May 1;317(1):25-35.” The work in the remaining figures is from the author.

Abstract

Tissue hypoxia leads to a systemic response designed to increase oxygen delivery to tissues. One major component of this response is stress erythropoiesis. This response utilizes progenitor cells and signals that are distinct from steady state
erythropoiesis. Previously we showed that bone morphogenetic protein 4 (BMP4) dependent signaling is essential for the expansion and differentiation of stress erythroid progenitors where it functions in concert with stem cell factor (SCF) and hypoxia. The expression of BMP4 in the spleen is also regulated by hypoxia, which demonstrates the central role of hypoxia dependent signaling in stress erythropoiesis. Here we show that Growth and differentiation factor 15 (GDF15), a Transforming Growth Factor β (TGFβ) related growth factor plays a key role in stress erythropoiesis. GDF15-/- mice are unable to recover from anemic challenge because stress erythropoiesis is compromised. The defect in these mice is an inability to maintain BMP4 expression. Our analysis shows that GDF15 expressed by stress erythroid progenitors acts on the microenvironment and inhibits the expression of the von Hippel Lindau (VHL) gene, which maintains hypoxia dependent BMP4 expression.
**Introduction**

Stress erythropoiesis is a specialized response whose function is to rapidly generate new erythrocytes to relieve tissue hypoxia[1]. This process utilizes progenitor cells and signals that are distinct from steady state erythropoiesis and have a greater capacity to rapidly generate large numbers of new erythrocytes. Stress erythropoiesis is best characterized in the mouse, where it is extra medullary and occurs in the fetal liver during embryonic development and in the adult spleen and liver[2-4]. One of the key signals regulating stress erythropoiesis is BMP4. Mutations that affect BMP4 signaling cause defects in stress erythropoiesis, which cause a delay in the recovery from anemia. BMP4 acts in concert with SCF and hypoxia to drive the expansion and differentiation of stress erythroid progenitors termed stress BFU-E[5-7]. This observation coupled with the demonstration that BMP4 is regulated by hypoxia underscore the central role for the hypoxia response in promoting stress erythropoiesis and limiting the response to anemic stress[8].

GDF15 is a member of the TGFβ super family of cytokines[9-12]. It is expressed by erythroblasts. High levels of GDF15 are observed in the serum of patients with anemia associated with ineffective erythropoiesis[13-15]. Tanno et al. first showed that high levels of GDF15 could cause iron overload in these patients. GDF15 can inhibit hepcidin expression in hepatocytes, which leads to increased iron uptake[15, 16]. These data suggest that GDF15 may function during anemia to regulate iron uptake. Although GDF15 may play a role in erythroid iron homeostasis, a direct role for this signal in
regulating erythropoiesis has not been identified. GDF15-/- mice do not exhibit any steady state defects in erythropoiesis[10], but knock down of GDF15 by siRNA in human cord blood cells compromised erythroid differentiation in vitro[17]. In this study we demonstrate an essential role for GDF15 in regulating stress erythropoiesis. GDF15-/- mice are unable to recover from phenylhydrazine (PHZ) induced acute hemolytic anemia. Furthermore GDF15-/- donor bone marrow cells fail to provide erythroid short-term radioprotection following transplant. The defect in GDF15-/- mice is an inability to maintain BMP4 expression in the spleen. Our data show that initial Hif2α dependent expression of BMP4 is down-regulated in GDF15-/- mice because Hif2α also induces VHL expression which feeds back to degrade Hif2α and inhibit the hypoxia response[18]. In wildtype mice, GDF15 expressed by stress erythroid progenitors inhibits the expression of VHL, so Hif2α dependent activation of BMP4 is maintained.
Materials And Methods

Mice and cell culture

C57BL/6 and C57BL/6-Tg(UBC-GFP)30Scha/J (hereafter referred to as GFP mice) mice were purchased from The Jackson Laboratory. GDF15-/− mice are the kind gift from Dr. Se-jin Lee. All the mice were 8-12 weeks old. All procedures are approved by the IACUC of the Pennsylvania State University. For the culture of spleen cells in vitro, mouse spleen cells were isolated and cultured in stress erythropoiesis media (Gibco IMDM (Invitrogen) with 10% fetal bovine serum (Equiec-Bio, Kerrville, TX), 1% Penicillin-Streptomycin, 10μg/ml insulin, 200μg/ml transferrin, 2mM L-glutamine, 0.01g/ml Bovine Serum Albumin and 7ul/L 2-Mercaptoethanol) supplemented with Epo (3U/ml), BMP4 (15ng/ml), SCF (50ng/ml) and SHH (25ng/ml) (R&D systems). Cells were treated with GDF15 (30ng/ml) or not and cultured in the hypoxia tank which provides 2% O₂ for the indicated period of time. Then cells were harvested for the RNA analysis.

Bone marrow transplantation and phenylhydrazine treatment

For transplantation assay, 5x10⁵ bone marrow cells were isolated from C57BL/6 mice or GDF15-/− mice and transplanted into lethally irradiated C57BL/6 or GFP recipients. Before the transplantation, all the recipients were treated with acidified water (pH 2.5-3.0) and antibiotics for 1 week. On the indicated days, mice were sacrificed to isolate bone marrow cells and spleens. For the homing assay, spleen cells were isolated from the GFP recipients that transplanted with C57BL/6 or GDF15-/− bone marrow cells. Non-GFP donor cells were analyzed by using LSR-II Fortessa Flow
cytometer (BD Biosciences). For the acute anemia, it was induced by injection of phenylhydrazine (Sigma) at the concentration of 100mg/kg mouse in phosphate-buffered saline (PBS) buffer. After the injection, mice were sacrificed on indicated time for the analysis. For each time point, n=3.

**RT-PCR and gene expression assay**

Total RNA was isolated by TriZol reagent (Invitrogen) from cultured cells or homogenized spleen tissues. 2ug RNA was used for RT-PCR to prepare cDNA by using the SuperScript-II system (Invitrogen). qRT-PCR was done by using Taqman probe for BMP4 (Mm00432087_m1), VHL (Mm00494137_m1) and 18s (Hs99999901_s1, Applied Biosystems). The qRT-PCR analysis was done using an ABI7300 Real-time PCR system.

**Western Blotting**

Western blot analysis was performed using the following primary antibodies: anti-VHL (556347, BD biosciences) at 1:1000 dilution, anti-HIF2α (NB100-122, Novus Biologicals) at 1:500 dilution and anti-β actin (sc-130656, Santa Cruz Biotechnology) at 1:2000 dilution for overnight at 4°C. Secondary antibody goat anti-rabbit and goat anti-mouse were used at 1:10000 dilution for 1h at room temperature. The bands were visualized with Amersham ECL prime western blotting detection reagent.

**Chromatin Immunoprecipitation (CHIP) assay**

The CHIP assay was performed by using the EZ-Magna CHIP A kits (#17-408, Millipore). Samples were sheared for 3 rounds of 10 cycles of 30 sec ON/ 30 sec OFF with
Bioruptor UCD200 (Diagenode). The antibodies used for immunoprecipitation were anti-HIF2α (NB100-122, Novus Biologicals), anti- SMAD2/3 (5678S, Cell Signaling Technology). Quantitative reverse transcriptase PCR was performed using SYBR-green Master Mix (Applied Biosystems). The following primers were used for amplification of precipitated DNA. HRE4: Rev: 5’-CAATCTCGACCATCAAGGC-3’; Fw: 5’-GACCAGAGTCTGCTCTGA-3’. SBE4: Rev: 5’-CAATCTCGACCATCAAGGC-3’; Fw: 5’-GACCAGAGTCTGCTCTGA-3’.

Prediction of potential SBEs (Smad2/3 Binding Elements)

Mouse VHL gene sequence with 3000 extra bp on either side of the gene was obtained from UCSC Genome Browser. The sequence was analyzed for potential transcription factor binding sites by rVista 2.0 software. The regulatory potential (RP) scores for the potential binding sites were generated by UCSC Regulatory potential 7 species analysis. Then the sequences of the predicted SBEs were aligned.

Short hairpin RNA and transduction

Mouse shRNA constructs in lentiviral GFP vector that targeting VHL gene (TL502408) and shRNA lentiviral packaging kit (TR30022) were purchased from Origene Technologies (Rockville, MD). Spleen cells were isolated from GDF15-/- mice and transduced with shRNA lentivirus for 48 hours. Then Cells were cultured in stress erythropoiesis media supplemented with Epo (3U/ml), BMP4 (15ng/ml), SCF (50ng/m), SHH (25ng/ml) and cultured in the hypoxia chamber which provides 2% O₂
for the indicated period of time. On the indicated days, cells were harvested for RNA isolation and protein extraction.

**Statistical analysis**

The standard deviation indicates the variation within each experiment (vitro) and each mouse (vivo). P-values were calculated by Student t-test (2-tailed). Statistical significance was taken at values of * p<0.05, ** p< 0.01, *** p<0.001. Error bars indicate ±SEM. All assays in vivo were done with n>3 mice per time point. For in vitro experiments, each treatment was done in triplicate and the data is representative of two independent experiments.
Results

GDF15-/- mice exhibit severely impaired stress erythropoiesis

Successful recovery from bone marrow transplant relies not only on the engraftment of donor hematopoietic stem cells but also on the ability of short-term radioprotective cells to generate new erythrocytes, platelets and neutrophils in the immediate post-transplant period[19]. The BMP4 dependent stress erythropoiesis pathway is the mechanism by which new erythrocytes are generated in the immediate post-transplant period[20]. In order to test whether GDF15 plays a role in regulating stress erythropoiesis, we transplanted GDF15-/- or control donor bone marrow cells into lethally irradiated recipient mice. The data in Figure 2.1A show that recipient mice transplanted with GDF15-/- bone marrow do not survive longer than 20 days after transplant. Analysis of complete blood counts showed that donor mutant bone marrow failed to provide erythroid short-term radioprotection as the recipient mice developed a lethal anemia. In addition, the recovery of platelets and neutrophils were also significantly impaired, which means GDF15-/- bone marrow cells were also deficient in the development of other lineages during recovery (Figure 2.1B). However, this observation may be due to overall lack of viability in the GDF15-/- transplants. On day 8 and day 10 post-transplantation, whole spleen cells were isolated from the recipients and plated in methylcellulose medium with Epo for stress BFU-E colony assay. Recipients transplanted with GDF15-/- bone marrow cells displayed profoundly reduced BFU-E colonies compared to the control case, which is consistent with observed anemia and low hematocrits values (Figure 2.2A). The mechanism underlying
the deficiency in reconstitution capacity of GDF15-/- bone marrow cells could also include a defect in homing to the bone marrow and spleen niche. To rule out this possibility, 5x10^5 bone marrow cells isolated from C57BL/6 mice or GDF15-/- mice were transplanted into GFP mice. We analyzed the donor non-GFP cells in both bone marrow and spleen of the recipients from on days 1-3. Flow cytometry analysis showed that the homing of both the control and GDF15-/- donor cells to the bone marrow and spleen of recipient mice on days 1 and 2 was not significantly different, indicating that homing is not the reason for the observed difference in the recovery of hematocrits. Although the percentage of donor cells in the bone marrow on day 3 post-transplant did differ between control and GDF15-/- transplants, the percentage of donor control cells in the spleen was significantly greater than that observed in GDF15-/- transplants. These data suggest that GDF15-/- donor cells fail to expand in the spleen which is consistent with the defect in expansion of stress BFU-E observed at days 8 and 10 post-transplant (Figure 2.2B-C).

One of the key signals regulating the development of erythrocytes by stress erythropoiesis is BMP4. Previously we showed that BMP4 expression is induced in the immediate post-transplant period. Analysis of BMP4 mRNA expression in the spleen following transplant showed that recipient mice transplanted with wild type bone marrow exhibited a significant increase in expression on day 6 after transplant. In contrast the recipient mice transplanted with GDF15-/- bone marrow failed to up regulate BMP4 expression in the spleen (Figure 2.3A). Inability to up-regulate BMP4 expression would impair the expansion of stress BFU-E in the spleen.
Stress erythropoiesis also plays an essential role in the recovery from phenylhydrazine (PHZ) induced acute hemolytic anemia. 100% of control mice recovered from the anemia induced by treatment with a dose of PHZ of 100mg/Kg mouse. Despite the fact that they showed no defects in steady state erythropoiesis, GDF15-/- mice were unable to activate a stress erythropoietic response and could not survive this treatment (data not shown). At lower doses of 75mg/Kg mouse, approximately 25% of the mice survived. Analysis of BMP4 expression in these mice again showed that control mice up-regulated BMP4 expression on 36 hours after treatment, but GDF15-/- mice fail to increase BMP4 expression (Figure 2.3B). These data show that GDF15-/- mice have a defect in stress erythropoiesis caused by their inability to up-regulate BMP4 expression during the recovery from anemia.

**GDF15 and hypoxia act in concert to maintain BMP4 expression**

BMP4 expression is regulated in the spleen by hypoxia. Previously we showed that the BMP4 gene contained two hypoxia inducible elements that bind Hif2α during the recovery from anemia[8]. Our observations showing that GDF15-/- mice are unable to up-regulate BMP4 expression suggest that GDF15 dependent signaling may also regulate BMP4 expression. We tested this possibility using MSS31, a spleen stromal cell line[21]. MSS31 cells were stimulated for 30 minutes with different doses of GDF15. At higher doses of 10 and 30 ng/ml, we observed an increase in BMP4 expression (Figure 2.4A). GDF15 signaling leads to the activation of Smad2/3[22]. Although this
observation suggested that GDF15 dependent Smad2/3 could directly regulate BMP4 expression, we could not identify Smad2/3 binding sites that mediated GDF15 dependent regulation of BMP4 (Data not shown). During the recovery from anemia in vivo, GDF15 would act in a hypoxic environment. We next tested the response of MSS31 cells under conditions that more closely mimicked the anemic spleen. Using a 10ng/ml dose of GDF15, We tested the ability of GDF15 to induce BMP4 mRNA expression in the presence and absence of hypoxia. This analysis showed that at atmospheric O₂ levels, BMP4 expression was transiently up-regulated peaking at 15 minutes and returning to base line by 60 minutes. However if we repeated this assay under hypoxia conditions (2% O₂), we observed that GDF15 and hypoxia act in concert to robustly induce BMP4 expression that is maintained for the full 60 minutes tested (Figure 2.4B). These data suggested that GDF15 acts in concert with hypoxia to induce and maintain BMP4 expression.

**GDF15 represses VHL and maintains Hif2α to induce BMP4 expression**

Hypoxia induces a transcriptional response through the stabilization of the alpha subunit of hypoxia inducible factor[23, 24]. At normal oxygen concentrations, Hif2α is hydroxylated on specific proline residues. These hydroxyl-prolines form a binding site for the VHL-E3 ubiquitin ligase complex that marks it for proteasomal degradation[24]. Hif dependent transcription is a stress response designed to respond to changing O₂ conditions[23, 25]. In addition to activating genes required to respond to hypoxic stress, Hif2α also activates VHL as part of a negative feedback loop to inhibit the
hypoxia response[18]. We tested whether GDF15 regulated Hif2α dependent BMP4 mRNA expression by regulating VHL expression. Primary spleen cultures from GDF15-/− mice were cultured in media containing Shh, BMP4, SCF and Epo at 2% O2 (conditions designed to mimic the environment of the spleen during the recovery from anemia) supplemented with and without GDF15. BMP4 mRNA expression significantly increased between 48 and 72 hours regardless of whether GDF15 was added, but the increase was significantly greater in the cultures with added GDF15. The greater increase in BMP4 expression was inversely correlated with the decreased expression of VHL in the cultures supplemented with GDF15 (Figure 2.4C).

To further determine whether repression of VHL is necessary for the regulation of BMP4 by GDF15, shRNA knockdown of VHL expression was performed in GDF15-/− primary spleen cell cultures. Three shRNAs were tested. Infection of primary spleen cells with lentiviruses expressing two of the shRNAs, shRNA-1 and shRNA-3, reduced the VHL expression significantly. After infection with shRNA, GDF15-/− spleen cells were cultured in stress erythropoiesis medium without GDF15 treatment for 3 days. Cells were harvested on 72 hours for RNA analysis. Compared to scramble control, cells with reduced VHL expression exhibited increased expression of BMP4, which showed that either GDF15 treatment or decreasing VHL expression could upregulate BMP4 expression (Figure 2.4D). Taken together, these observations support the conclusion that GDF15 induces BMP4 expression by down regulating VHL.
We next examined the expression of VHL during the recovery from bone marrow transplant and PHZ induced acute anemia. In both cases we observed that VHL mRNA and protein expression was maintained at low levels. Similarly, in control transplanted mice and in control mice treated with PHZ, Hif2α protein was stabilized in recipients receiving control donor cells or in control mice recovering from PHZ induced anemia. In contrast, recipient mice receiving GDF15-/- donor cells or GDF15-/- mice recovering from PHZ induced anemia failed to inhibit VHL expression, which correlated with decreased Hif2α protein and a loss of BMP4 expression (Figure 2.5 A-B and Figure 2.3A-B).

Hif2α binds to two hypoxia responsive elements in the BMP4 gene[8]. We analyzed Hif2α binding to one of these sites HRE4 by Chromatin immunoprecipitation (ChIP) during the recovery from bone marrow transplant and PHZ induced anemia. Once again in control transplanted mice and control mice treated with PHZ, Hif2α binding to HRRE4 corresponded to increased BMP4 expression (Figure 2.5C and Figure 2.3 A-B) and a decrease in VHL expression (Figure 2.5C and Figure 2.5 A-B). Recipients of GDF15-/- transplant and GDF15-/- PHZ treated mice exhibited little binding of Hif2α, which connected with the decreased BMP4 expression and increased VHL.

**Smad2/3 complex binds to VHL gene and repress its transcription**

GDF15 signaling leads to the phosphorylation of Smad2/3. We examined the VHL gene for conserved Smad2/3 binding sites and analyzed the putative sites for
regulatory potential using the RP score algorithm[26]. Based on this analysis, we identified 4 potential sites. Among these four, one site (SBE4) showed high regulatory potential and contained an overlapping conserved Smad4 binding site, which suggested that this site would bind the Smad2/3-Smad4 heterodimer (Figure 2.6A). ChIP analysis for Smad2/3 binding to this site during the recovery from bone marrow transplant and PHZ induced anemia showed that this site was bound in control mice during recovery and binding correlated with decreased VHL expression. No binding was observed when GDF15 was mutated (Figure 2.6B). Taken together, these data suggest that GDF15-activated Smad2/3 complex binds to VHL gene locus and represses its expression, which in turn stabilizes Hif2α protein. Hif2α further induces BMP4 expression maintaining the expansion and differentiation of stress erythroid progenitors.

Discussion

GDF15 dependent regulation of the hypoxia response in stress erythropoiesis may also shed light on its function in other tissues. Previously, GDF15 signaling was shown to play a role in cardioprotection[27]. GDF15 expression is upregulated in response to ischemia and is an important biomarker for cardiac injury [28, 29]. Overexpression of GDF15 protects cardiomyocytes from ischemia reperfusion injury[22]. This response requires the activation of Smad2/3 dependent signaling, which is consistent with previous work showing that activation of HIF dependent transcription can protect against the potential damage caused by ischemia reperfusion
injury [30]. Therefore, GDF15 functions as a protective signal whose expression is increased at times of tissue injury when tissue regeneration is ongoing.

In thalassemia patients, GDF15 expression is increased and that increase leads to decreased expression of hepcidin and iron overload[16]. Although that suggests a role for GDF15 in regulating iron utilization during erythropoiesis, recent work has identified other signals that may play a greater role. It was found that erythroferrone is an erythroid regulator of iron metabolism and it leads to the suppression of hepcidin and the iron overload characteristic of thalassemia[31]. Our data would suggest that GDF15 plays an additional role in regulating stress erythropoiesis [32]. GDF15 signaling is part of the stress erythropoietic response designed to regenerate the erythroid mass. Increased iron uptake is part of this regenerative process, but defects in the erythropoietic differentiation in thalassemia result in constitutive expression of GDF15 and unintended pathology. Despite the fact that GDF15 appears to be a potential target for treating iron overload in thalassemia and other iron loading anemia’s, our data would predict that blocking GDF15 expression in thalassemia patients would have dire consequences to the stress erythroid response to the anemia. We tested this possibility directly by crossing GDF15⁻/⁻ mutation into the thalassemia intermedia model Hbb<sup>th3</sup>/+. No live born GDF15⁻/⁻;Hbb<sup>th3</sup>/+ mice pups were observed. Analysis of embryos showed that double mutant embryos did not survive beyond day E14.5 of severe anemia. BMP4 signaling plays a key role in fetal erythropoiesis and stress erythropoiesis [2]. GDF15⁻/⁻;Hbb<sup>th3</sup>/+ embryos failed to express BMP4 in the fetal liver (Figure 2.7). This observation
demonstrates that GDF15 signaling in the context of thalassemia is required for stress erythropoiesis to maintain survival of the embryo.

In summary, our data supports a model where GDF15 is a signal upregulated in response to tissue injury that promotes tissue regeneration in part through the regulation hypoxia dependent gene transcription, which leads to the expansion and differentiation of new cells that will repair the tissue.

Acknowledgements

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References


Figure 2.1: Analysis of the ability of GDF15−/− bone marrow cells to provide short-term radioprotection. (A) Bone marrow cells from control and GDF15−/− mice were transplanted into lethally irradiated recipients. Survival of recipient mice over the first 3 weeks post transplant is shown. \( P < 0.001 \) (Long rank test, Gehan-Breslow-Wilcoxon test) Representative of 4 individual transplants (n=9 for GDF15+/+ and n=16 for GDF15−/−). (B) Analysis of hematocrit, white blood cells (WBC), Platelet and neutrophil counts during the recovery from bone marrow transplant. * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \). Error bars indicate ±SEM. Each time point reflects >5 mice analyzed and the data is representative of two independent experiments.
Figure 2.2: GDF15-/- bone marrow cells exhibit a delayed production of BFU-E colonies but normal homing in recipients after bone marrow transplantation. 5x10^5 bone marrow cells isolated from GDF15-/- or C57BL/6 mice were transplanted into GFP recipients (for each type, n=6). (A) Spleen cells were isolated from recipients on day 8 and 10 post-transplantation and cultured in methylcellulose medium for BFU-E colony assay. (B) Both bone marrow cells and spleen cells were analyzed for non-GFP donor cells by flow from day 1 to day 3 post-transplantation. (C) Histogram for flow cytometry. For each time point, n=3. * p<0.05, ** p<0.01 and *** p<0.001. Error bars indicate ±SEM.
Figure 2.3: Analysis of BMP4 expression during the recovery from bone marrow transplant (BMT) and Phenylhydrazine (PHZ) induced acute hemolytic anemia. Analysis of relative BMP4 mRNA expression in the spleen by qRT-PCR at the indicated times during the recovery from bone marrow transplant (A) and PHZ treatment (B). *** p<0.001. Error bars indicate ±SEM. All assays were done in triplicate with n>3 mice per time point.
Figure 2.4: GDF15 dependent induction of BMP4 expression in MSS31 cells and primary spleen cultures. (A) MSS31 cells were treated with the indicated dose of GDF15 for 30 minutes. Expression of BMP4 and control β-actin mRNA was determined by RT-PCR. (B) MSS31 cells were treated with 10ng/ml GDF15 for the indicated time under atmospheric (20% O₂) or hypoxia (2% O₂) conditions. Expression of BMP4 and control β-actin mRNA was determined by RT-PCR. (C) Primary spleen cells cultures derived from GDF15-/- mice were treated ± GDF15 10ng/ml for the indicated time. BMP4 and VHL mRNA expression levels were determined by qRT-PCR and expressed relative to GAPDH or 18S rRNA respectively. (D) Primary spleen cells isolated from GDF15-/- mice were transduced with shRNA targeting VHL for 48 hours and then cultured in stress erythropoiesis medium for 72 hours. BMP4 and VHL expression were analyzed by qRT-PCR. Error bars indicate ± SEM. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
Figure 2.5. Analysis of GDF15 dependent regulation of BMP4 expression during the recovery from bone marrow transplant (BMT) and Phenylhydrazine (PHZ) induced acute hemolytic anemia. (A) Analysis of relative VHL mRNA expression in the spleen during the recovery from BMT (left) and PHZ treatment (right). (B) Analysis of VHL and Hif2α protein expression in the spleen. Whole cell lysates made from spleen cells on the indicated days during the recovery from BMT (left) and PHZ treatment (right) were western blotted and probed with anti-VHL, anti-Hif2α and control β-actin antibodies. Representative experiments are from two independent experiments. (C) Chromatin immunoprecipitation analysis of the Hif2α binding to HRE4 in the BMP4 gene in the spleen during the recovery from BMT (left) and PHZ treatment (right). Binding is expressed as fraction of input DNA. Error bars indicated ± SEM. All assays were done in triplicate with n>3 mice per time point. * p<0.05, ** p<0.01.
Figure 2.6. Smad2/3 complex binds to VHL gene to regulate its expression. (A) Schematic of the VHL locus and RP score of the binding site. The locations of the four potential putative Smad Binding Elements (SBE) are indicated. The conservation of the functional SBE4 site across multiple species is demonstrated by sequence alignment. RP scores of SBE4 site are generated by UCSC regulatory potential 7 species analysis. (B) Chromatin immunoprecipitation analysis of Smad2/3 binding to the SBE in the VHL locus during the recovery from BMT (left) and PHZ (right). Binding is expressed as fraction of input DNA. Error bars indicated ± SEM. All assays were done in triplicate with n>3 mice per time point. * p<0.05, ** p<0.01.
Figure 2.7. GDF15\(^{-/-}\);Hbb\(^{thal3/+}\) E14.5 embryos are severely anemic and deficient in BMP4 expression. BMP4 is expressed in the fetal liver and promotes the expansion of stress BFU-E. (A) (Top) Expression of BMP4 in control fetal liver sections stained with fluorescent anti BMP4 antibodies. (Bottom) Number of total stress BFU-E colonies in the fetal liver on the indicated days. (Stress BFU-Es are defined as BFU-E colonies formed in media containing only Epo.) (B) Representative E14.5 embryos. Note the lack of erythropoiesis in the fetal liver of the GDF15\(^{-/-}\);Hbb\(^{thal3/+}\) embryo. (C) Analysis of BMP4 expression in the fetal liver of E14.5 embryos. RT-PCR analysis of BMP4 expression in fetal liver RNA isolated from E14.5 embryos of the indicated genotypes. Beta actin is included as an RNA loading control. These results are representative of 3 independent experiments.
Chapter 3

In Vitro Expansion Of Stress Erythroid Progenitors Identifies Distinct Progenitor Populations And Analogous Human Stress Erythroid Progenitors

Forward

The following chapter is taken from the manuscript appearing in “Jie Xiang, Dai-Chen Wu and Robert F. Paulson, In vitro expansion of stress erythroid progenitors identifies distinct progenitor populations and analogous human stress erythroid progenitors. (under review for Blood 2014)” This manuscript was written by Robert F. Paulson and Jie Xiang. Jie Xiang and Dai-Chen Wu are co-first authors in this paper. The work presented in this chapter is the work of the author.

Abstract

Acute anemia induces tissue hypoxia caused by the lack of oxygen delivery to tissues. Increased erythropoiesis is necessary to compensate for the loss of erythrocytes and increase tissue oxygenation. Steady state erythropoiesis is primarily homeostatic producing new erythrocytes to replace old erythrocytes removed from circulation by the spleen. In response to anemia, the situation is different. New erythrocytes must be rapidly made to increase hemoglobin levels. At these times stress
erythropoiesis predominates. Stress erythropoiesis is best characterized in the mouse, where it is extra-medullary and utilizes progenitors and signals that are distinct from steady state erythropoiesis. In this report, we use an in vitro culture system that recapitulates the in vivo development of stress erythroid progenitors. We identified cell surface markers that delineate a series of stress erythroid progenitors with increasing maturity. We further confirmed this observation by the in vivo analysis of stress erythroid progenitors development after bone marrow transplantation. In addition, we used the in vitro culture system to expand human stress erythroid progenitors that express analogous cell surface markers. Consistent with previous suggestions that human stress erythropoiesis is similar to fetal erythropoiesis, we demonstrated that human stress erythroid progenitors express fetal hemoglobin (HbF) upon differentiation. These data showed that similar to murine bone marrow, human bone marrow contains cells that generate BMP4 dependent stress BFU-E when cultured under stress erythropoiesis conditions.
Introduction

Anemic stress leads to tissue hypoxia which induces a systemic response designed to increase oxygen delivery to the tissues. A key component of this response is stress erythropoiesis, which rapidly generates large numbers of new erythrocytes[1-3]. Stress erythropoiesis is best understood in mice where it is extramedullary occurring in the adult spleen, liver and in the fetal liver during development[4-6]. Stress erythropoiesis utilizes signals and progenitor cells that are distinct from steady state erythropoiesis[7-9]. In response to anemia, hypoxia induces the expression of BMP4 in the spleen[10]. BMP4 acts on an immature population of stress erythroid progenitors and promotes their differentiation into stress BFU-E[7, 11, 12]. The efficient expansion and differentiation of stress BFU-E also requires Epo, SCF and hypoxia in addition to BMP4[7, 11]. During the response to anemia most of the stress erythroid progenitors are mobilized into stress BFU-E. Following recovery, stress erythroid progenitors are replenished in the spleen by bone marrow progenitors that migrate into the spleen and adopt the stress erythroid fate[13]. Hedgehog signaling is required for this process and blocking hedgehog signaling impairs the ability of bone marrow progenitor cells to replenish spleen stress erythroid progenitors[14, 15].

Recently, we demonstrated that BMP4 dependent stress erythropoiesis mediates erythroid short-term radioprotection following bone marrow transplant. Mice with defects in stress erythropoiesis exhibit difficulties in generating new erythrocytes in the immediate post transplant period prior to HSC engraftment[16]. Using bone marrow transplant as an assay, we identified CD34+Kit+Sca1+Lin- (CD34KSL) cells, which were
previously shown to be short-term reconstituting hematopoietic stem cells, as the population of cells that migrates from the bone marrow to the spleen and replenishes the stress erythroid progenitor population. Following transplant, the development of donor derived stress erythroid progenitors proceeds in a regulated manner. Initially donor cells proliferate in the spleen, but during the first 8 days after transplant they are unable to differentiate. At this point a switch in development occurs and the stress progenitors acquire the ability to develop into stress BFU-E. Over the following 8 days stress BFU-E expand and differentiate. Following recovery new cells migrate into the spleen to replenish the progenitor cells. Stress erythroid progenitors express cell surface markers that are different than steady state erythroid progenitors. They express both immature cell markers like Kit and Sca1 and late erythroid markers like CD71 and TER119. Analysis of the stress progenitors in the spleen during the recovery from bone marrow transplant showed that stress erythroid progenitors could be sorted into three distinct populations. The most immature population (Population I) also expressed Sca1 and contained all the stress BFU-E activity. Purified Population I cells were capable of rescuing erythropoiesis in lethally irradiated mice, maintaining their survival until endogenous HSCs that had survived radiation could repopulate the animals. In addition, Population I progenitors could be serially transplanted, which demonstrated their ability to self renew in vivo.

Stress erythropoiesis plays an essential role in the response to tissue hypoxia and anemia, but unregulated stress erythropoiesis can lead to pathology – polycythemia and erythroleukemia[17-22]. Friend erythroleukemia virus causes an acute erythrocytosis
that progresses to erythroleukemia. Friend virus induces BMP4 expression in the spleen and activates the BMP4 dependent stress erythropoiesis pathway[17, 23-25]. Infection of late stage stress erythroid progenitors (Kit+Sca1-CD71loTer119lo) leads to the development of Epo-independent BFU-E and erythrocytosis, while infection of immature stress erythroid progenitors (Kit+Sca1+CD71loTER119lo) leads to the development of self renewing leukemia stem cells and erythroleukemia. Analysis of the leukemia stem cell population showed that these cells also expressed CD34 and CD133[25], which suggested that these markers might also be useful for delineating sub-populations of stress erythroid progenitors.

In this chapter we outline an in vitro culture system that recapitulates the development, expansion and differentiation of stress erythroid progenitors. Utilizing unfractionated bone marrow cells, we show that culturing cells in a combination of Sonic hedgehog (Shh), BMP4, GDF15, SCF, Epo and low oxygen leads to the development of stress BFU-E. Previously, we showed that the stress BFU-E were contained in Population I stress progenitors (Kit+Sca1+/CD71loTer119lo/), analysis of cells generated in vitro shows that this population can be separated into three distinct populations based on their expression of CD34 and CD133. Each of these populations has the ability to rescue erythropoiesis when transplanted into irradiated animals and is capable of self-renewal in vivo. The in vitro culture system allowed us to extend our findings to human stress progenitors. Culturing human bone marrow in media under the same conditions leads to the expansion of human stress erythroid progenitors that express a set of cell surface markers similar to those observed in murine stress
erythroid progenitors. The human stress erythroid progenitors form BFU-E colonies, which require BMP4, Hedgehog, GDF15, SCF, Epo and the hypoxia environment. In addition, analysis of human stress erythroid progenitors showed that they expressed gamma-globin and fetal hemoglobin (HbF), which is consistent with previous findings that human stress erythropoiesis is similar to fetal erythropoiesis.
Materials And Methods

Mice and cell culture

C57BL/6 mice and C57BL/6-Tg(UBC-GFP)30Scha/J (hereafter referred to as GFP mice) mice were purchased from The Jackson Laboratory. All the mice were 6-10 weeks old. All procedures are approved by the IACUC of the Pennsylvania State University. For the culture of bone marrow cells in vitro, mouse bone marrow cells were isolated and cultured in stress erythropoiesis expansion media (SEEM): (Gibco IMDM (Invitrogen) with 10% fetal bovine serum (Equiec-Bio, Kerrville, TX), 1% Penicillin-Streptomycin, 10ug/ml insulin, 200ug/ml transferrin, 2mM L-glutamine, 0.01g/ml Bovine Serum Albumin and 7ul/L 2-Mercaptoethanol) supplemented with GDF15 (30ng/ml), BMP4 (15ng/ml), SCF (50ng/ml) and SHH (25ng/ml) (Mybiosouce Company and R&D sysetms). For the differentiation, they were treated with Epo (3U/ml) and cultured in the hypoxia tank which provides 2% O₂ for the indicated period of time (stress erythropoiesis differentiation media, SEDM). Then cells are harvested for the RNA and flow analysis. Human bone marrow mononuclear cells were purchased from Reachbio [26-200] and cultured in the stress erythropoiesis medium described above. On the indicated days, cells were harvested for analysis.

Bone marrow transplantation and WBC isolation

For transplantation assay, bone marrow cells were isolated from CD45.2 mice and cultured in specific medium. Then stress erythroid progenitors were sorted and transplanted into lethally irradiated CD45.1 recipients (5000 sorted cells/mice). For
each type of donor cells, 5 mice were used. Before the transplantation, all the recipients were treated with acidified water (pH 2.5-3.0) for 1 week and antibiotics for 3 days. On the indicated days, peripheral blood was obtained by retro-orbital sinus through heparin-coated microhematocrit tubes and transferred to tubes coated with EDTA for complete blood cell (CBC) analysis. On day 16, White blood cells were isolated from peripheral blood by Histopaque-1077 (Sigma-Aldrich) for the flow assay. For the flow analysis of stress erythroid progenitors in vivo, 5x10^5 GFP bone marrow cells were transplanted into lethally irradiated C57BL/6 recipients. On the indicated days, spleen cells were isolated from recipients and stained with antibodies for flow assay.

**BFU-E colony assay**

Cells cultured in stress erythropoiesis medium were plated in methylcellulose media (StemCell Technologies) containing 3U/ml Epo alone at 20% O_2_ or in combination with 50ng/ml SCF and 15 ng/ml BMP4 at 2% O_2_ at the concentration of 2x10^5/ml. Stress BFU-Es were scored as described after 5 days incubation [7].

**RT-qPCR and gene expression assay**

Total RNA was isolated from cultured cells by TriZol reagent (Invitrogen). 2ug RNA was used for RT-PCR to get cDNA using the SuperScript-II system (Invitrogen). RT-qPCR was done using Taqman probes and primers in table 1. The RT-qPCR analysis was done using an ABI7300 Real-time PCR system.
**Western Blotting**

Western blot analysis was performed using the following primary antibodies for human cells: anti-BCL11A (sc-33093, Santa Cruz Biotechnology) at 1:500 dilution and anti-β actin (sc-130656, Santa Cruz Biotechnology) at 1:1000 dilution for overnight at 4°C. Secondary antibody goat anti-rabbit and donkey anti-goat were used at 1:10000 dilution for 1h at room temperature. The bands were visualized with Amersham ECL prime western blotting detection reagent.

**Flow Cytometry and Cell Sorting**

Cultured mouse bone marrow cells were labeled with different combinations of antibodies listed in table 2. After labeling, cells were washed with 2% fetal bovine serum in PBS. Flow cytometry analysis was done using LSR-II Fortessa Flow cytometer (BD Biosciences). The percentage of dead cells was measured by propidium iodide staining. All the data were analyzed with FlowJo software. Cell populations were sorted as CD34+CD133+KS and CD34-CD133-KS by using a Cytopia Influx V-GS Cytometry Workbench with Spigot software.

**Statistical analysis**

The standard deviation indicates the variation within each experiment (vitro) and each mouse (vivo). P-values were calculated by Student t-test (2-tailed). Statistical significance was taken at values of, * p<0.05, ** p< 0.01, *** p<0.001.
Results

Development of an in vitro culture system to generate stress erythroid progenitors.

Previous work demonstrated that several growth factors are required for expansion and development of murine stress BFU-E, which include BMP4, SCF, Hedgehog, Epo, Growth and Differentiation Factor 15 (GDF15) and hypoxia. Using these factors we set out to identify conditions that allow for the in vitro expansion of stress erythroid progenitors and stress BFU-E. Unfractionated bone marrow cells were cultured in stress erythropoiesis medium containing BMP4, SCF, Hedgehog, Epo and GDF15 at 2% O₂ for 5 days to mimic hypoxic tissue conditions (Hereafter referred to as stress erythropoiesis differentiation media, SEDM). Then cells were plated in methylcellulose medium containing Epo at 20% O₂ and BFU-E colonies were scored after 5 days. The combination of factors promoted the expansion of stress BFU-Es. The removal of any of the factors from the culture resulted in a significant decrease in stress BFU-E formation (Figure 3.1A), indicating that each of these factors contributed to the expansion and differentiation of stress erythroid progenitors. Previously we showed that CD34+Kit+Sca1+Lin- cells migrate into spleen and provide the short-term radioprotection after the bone marrow transplantation. Here we generate a culture system that mimic the microenvironment in the spleen and successfully produce stress BFU-E colonies in vitro.
In addition, our previous work showed that Population I cells could be transplanted into lethally irradiated mice where they provided erythroid rescue until endogenous stem cells that had survived myeloablation could re-populate the mouse[8]. Population I stress erythroid progenitors generated in vitro rescued lethally irradiated mice. In fact as few as 5000 purified population I stress erythroid progenitors could provide erythroid short-term radioprotection, which further demonstrates that this culture system generates functional stress erythroid progenitors (personal communication with Dai-Chen Wu and Robert F. Paulson).

Previous work suggested that stress erythropoiesis exhibits properties of fetal erythropoiesis[27-29]. To investigate whether stress erythroid progenitors produced by in vitro culture system expressed embryonic β-globin genes, beta H1 globin and εy globin expression, which are enriched during primitive erythropoiesis in mouse, mouse bone marrow cells were cultured in SEDM or further cultured in 2%+Bmp4+SCF+Epo for 3 additional days (SEDM+Diff.). For comparison, bone marrow cells were also cultured in steady-state differentiation medium with Epo and SCF only for 5 days as a control condition (Steady-state). Cells were collected on the indicated time for the mRNA analysis of βH1, εy and β major expression and the results were presented as the βH1/βMajor and εy/βMajor ratios. The culture in SEDM exhibited significantly higher βH1/βMajor and εy/βMajor ratios compared to the case in steady-state erythropoiesis. The extended culture (SEDM+Diff.) induced even much more βH1 and Ey globin production. The βH1/βMajor ratio reached to 1.5, which means that during the terminal differentiation of stress erythropoiesis, βH1 is the dominant β-globin (Figure 3.1B-C).
This data confirmed our hypothesis that stress erythropoiesis induces the expression of embryonic hemoglobin, which has stronger ability to bind and deliver oxygen. It has been shown that BCL11A represses fetal/embryonic hemoglobin expression[30-33]. We further tested the expression of BCL11A under the three culture conditions. As shown in Figure 3.1D, under extended stress erythropoiesis culture condition, a dramatic decrease of BCL11A mRNA expression was observed. This data further demonstrated that the repression of BCL11A increases the production of embryonic globin genes during stress erythropoiesis. Our data here provides an insight into the role of stress erythropoiesis in fetal hemoglobin production.

Epo promotes the transition from expansion to differentiation and hypoxia potentiates Epo-dependent response

Previously we showed that during the recovery from bone marrow transplant, donor cells rapidly proliferate in the spleen. However, prior to day 8 after transplant, donor derived cells isolated from the spleen by flow cytometry failed to form stress BFU-E colonies despite the expression of signals known to be required for stress BFU-E colonies formation. This observation suggested that a signal is required for the transition from proliferating stress progenitors to differentiating stress BFU-E. We suggested that Epo and or hypoxia could be the signal in that the expression of Epo mRNA in the kidney, which is also a surrogate marker for tissue hypoxia, peaked on day 8 after transplant – the time when stress BFU-E were first observed in the spleen[8].
To identify the roles of Epo and hypoxia playing in the stress erythropoiesis, we use an in-vitro culture system to generate stress progenitors. Unfractionated bone marrow cells isolated from C57BL/6 mice were cultured in SEDM or in SEDM lacking Epo at 20% O2 (hereafter referred as Stress erythropoiesis expansion media, SEEM) for 5 days. On each day, cells were plated in methylcellulose medium and stress BFU-E colonies were scored. The figure 3.2A shows that bone marrow cells cultured with Epo and hypoxia differentiated and produced a great number of BFU-E colonies, however the cells cultured without Epo and hypoxia failed to differentiate and few colonies were observed. To further delineate the requirements for this transition, we tested whether Epo alone could promote the differentiation. Unfractionated bone marrow cells were cultured in SEEM for 5 days and then treated with Epo or Epo+hypoxia for 1,3 and 5 days. BFU-E colony assay was performed on each time point. Figure 3.2B shows that Epo is sufficient to promote the development of stress BFU-E; however the response is delayed. Significant numbers of stress BFU-E were not observed until day 5 of culture and the magnitude of the response is less than that observed when Epo and hypoxia are used. This observation suggested that hypoxia potentiates the Epo dependent signal. The switch from expansion to differentiation of the stress erythroid progenitors is the key component of our model. By altering culture conditions, we can recapitulate the in-vivo switch from expansion to differentiation of stress erythroid progenitors, which ensures that enough progenitors are produced so that once differentiation starts, sufficient new erythrocytes are generated to prevent lethal anemia.
CD34 and CD133 expression mark a developmental progression of stress erythroid progenitors.

Using short term erythroid radio protection following bone marrow transplant as an assay, we identified three distinct populations of stress erythroid progenitors based on the expression of Kit, Ter119 and CD71: Population I cells (Kit⁺CD71lo/medTer119lo/–), Population II (Kit⁺CD71high Ter119med) and Population III (Kit⁺CD71lo/medTer119high) [8]. However, this population I cells include progenitors in both the expansion stage and the differentiation stage. Thus we need to find more markers to further delineate the expanding population I cells from Population I cells capable of differentiating. In our analysis of the progression of Friend virus induced erythroleukemia, we observed that Friend virus infection induced the BMP4 dependent stress erythropoiesis pathway and infection of Sca1+ Population I cells led to the development of self renewing Leukemia stem cells (LSCs). Analysis of Friend virus LSCs showed that they also expressed CD34 and CD133. The presence of CD34 is consistent with our previous data showing that CD34+KSL cells are the bone marrow sub-population that gives rise to stress erythroid progenitors in the spleen[25]. CD133 is a marker associated with tissue and cancer stem cells [34, 35], which is also expressed in the progenitors in cord blood[36, 37]. Based on these studies, we next tested whether CD34 and CD133 could further delineate the populations of stress erythroid progenitors.

Unfractionated bone marrow cells were isolated and cultured in SEEM for 7 days and then cultured in Stress Erythroid Differentiation Medium (SEDM,
Bmp4+SCF+Hedgehog+Gdf15+Epo+2%O2) for 5 days. On indicated time points, flow cytometry analysis was performed to check the expression of cell markers. Data in figure 3.3A showed that the Kit+Sca1+(KS) population of unfractionated bone marrow cells had low or negative expression of CD34 and CD133. After cultured in SEEM for 7 days, 77% of Kit+Sca1+ (KS) cells were CD34+CD133+. When we shifted the cells to SEEM+Epo for 5 days, 90% of the CD34+CD133+KS cells became either CD34-CD133+KS (45%) or CD34-CD133-KS cell (45%) with remaining cells maintained as CD34+CD133+KS. In contrast, if we shifted cells to SEDM, almost all (>97%) of the CD34+CD133+KS cells transitioned to CD34-CD133-KS cells, which suggests that hypoxia and Epo are more efficient in promoting the development of CD34-CD133-KS cells. Epo and hypoxia promote a change in the cell surface phenotype of Population I stress erythroid progenitors and the use of CD34 and CD133 as markers allows for the identification of three distinct progenitor populations. Based on this observation, we identified CD34+CD133+KS population as the early stress erythroid progenitors that have self-renewal ability and CD34-CD133-KS population as the later stress erythroid progenitors that have the differentiation ability.

To further confirm the progress of stress erythroid progenitors, CD34+CD133+KS, CD34-CD133+KS and CD34-CD133-KS populations were sorted from the GFP bone marrow culture. Then they were co-cultured with GFP- unfractionated bone marrow cells in SEEM for 7 days and switched into SEDM for 5 days. At each step, GFP+ populations were analyzed by flow cytometry. Figure 3.3B shows that when cultured in the SEEM, GFP+ cells were maintained as CD34+CD133+KS population. But once
cultured in the differentiation medium, about 10% of the CD34+CD133+KS population transitioned to CD34+CD133+KS cells and 85% of them were CD34+CD133+KS cells. Over 90% of the CD34+CD133+KS population became CD34+CD133+KS cells. These observations confirmed what we observed previously that the CD34+CD133+KS cells generate CD34+CD133+KS and CD34+CD133+KS cells. However, when we plated CD34+CD133+KS or CD34+CD133+KS cells in SEEM, they reverted to the more immature CD34+CD133+KS cells (Figure 3.3C). Similarly, we observed that donor derived CD34+CD133+KS cells were maintained in vivo even when purified CD34+CD133+KS cells were transplanted into irradiated mice (Data not shown). These data suggest that the developmental stage of the stress erythroid progenitors is driven by signals in the microenvironment and they are able to move back and forth along the developmental continuum. Collectively, our data demonstrated a developmental progression of the three populations of stress progenitors the developmental stage of these populations appears to be plastic both in vitro and in vivo.

We further performed stress BFU-E colony assay and bone marrow transplantation assay to functionally test these two populations. CD34+CD133+KS and CD34+CD133+KS cells were sorted and cultured in methylcellulose medium. After 5 days, BFU-E colonies were scored. Only CD34+CD133+KS cells have the ability to give rise to BFU-E colonies (Figure 3.3D). This data suggested that CD34+CD133+KS cells represent the most immature stress progenitors, while CD34+CD133+KS cells are the most mature population. This characterization was further strengthened by the analysis of gene expression in the different populations. CD34+CD133+KS cells expressed higher levels
of genes associated with stem cell self-renewal Bmi1 and Yap1[38-40]. In addition Pu.1[41], which is required for the self-renewal of Friend virus LSCs and fetal liver BFU-E and Scl[42], which is required for BFU-E formation are also preferentially expressed in the CD34+CD133+KS cell. In contrast, genes associated with erythroid differentiation, Gata2, Gata1 and p53 are expressed at higher levels in CD34-CD133-KS population[43, 44]. Similarly, Numb, whose expression is associated with progenitor cells produced by asymmetric divisions in stem cells, is also expressed at higher levels in the more mature cells[45] (Figure 3.3E). These data suggest the idea that CD34+CD133+KS cells are more immature stem cell like cells, while CD34-CD133-KS cells have upregulated the erythroid differentiation pathway.

Next we transplanted CD34+CD133+KS, CD34-CD133+KS and CD34-CD133-KS populations from CD45.2 mice into the lethally irradiated CD45.1 mice (5000 cells/mouse). The hematocrits were measured during the immediate post-transplant period. All the recipients recovered, which showed that these populations are all capable of providing short-term radioprotection. To further check whether they were erythroid-lineage specified, on day 16 after bone marrow transplantation when the white blood cells (WBCs) start to show up, WBCs were isolated from peripheral blood and analyzed by flow cytometry. The data in figure 3.3F showed that the white blood cell markers CD3ε, B220, Gr-1 and Mac1 were mainly expressed on CD45.1 cells, which confirmed the conclusion that the stress erythroid progenitors are erythroid-lineage specified.
The development of stress erythroid progenitors in vivo

Using in vitro culture system, we identified three distinct populations of stress erythroid progenitors and proved that they functionally and erythroid-specifically provide short-term radioprotection. To further investigate how stress erythroid progenitors progress in vivo, we use bone marrow transplantation as a model and analyze the donor progenitors by flow cytometry. Bone marrow cells were isolated from GFP mice and transplanted into lethally irradiated C57BL/6 recipients. GFP donor cells in the spleen of recipients were analyzed every the other day. Based on the data in figure 3.4A-B, the post-transplantation can be divided into 4 stages. In stage 1, the homing and migration of donor cells happened, which was from d0 to d4. Very few donor cells were observed in the spleen at this stage (0.4%) and they were mainly CD34+CD133+KS population. Stage 2 was the expansion stage, which occurred from d4 to d6. Donor cells expanded rapidly (46.6%) at day 6 and most of KS cells were CD34+CD133+, which is consistent with our previous findings that CD34+CD133+KS cells have self-renewal ability that are incapable of differentiation. These two stages together were defined as pre-recovery stage. Previously we defined pre-recovery stage according to the decreasing hematocrits value and the absence of BFU-E colony. Now by using more biomarkers, we modified the pre-recovery stage, which included the migration of donor cells and expansion of CD34+CD133+KS population. Stage 3 was the differentiation stage, from d6 to d12, CD34+CD133+KS population started to differentiate towards CD34-CD133-KS population. Tissue hypoxia and Epo that is produced in the kidney in response to acute anemia induce the differentiation of stress
erythroid progenitors. This stage is the recovery stage in which BFU-E colonies started to appear following the differentiation of stress erythroid progenitors. It was noticed that in this stage KS populations decreased and remained around 5% of donor cells. Because of the differentiation, the percentage of donor GFP cells in the spleen continued to increase. The last stage was from d12 to d18 that correlated to post-recovery stage in which hematocrits value continued to increase. CD34⁺CD133⁺KS population started to increase and CD34⁻CD133⁻KS population decreased, which indicated that when the differentiated cells were sufficient to provide the recovery from acute anemia and the CD34⁺CD133⁺KS population needed to be replenished to allow for the second wave of expansion. At this stage, the KS cells did not increase, but they were maintained as CD34⁺CD133⁺KS population. These progenitors may reside in the spleen until a subsequent anemic stress would induce a second wave of expansion and differentiation.

The in vivo data demonstrated the whole developmental process of stress erythroid progenitors in the spleen and it consisted with what we observed in our culture system. These data show that once the CD34⁺KSL cells were in the spleen, they became CD34⁺CD133⁺KS population. However, it is not known whether the cells migrating into the spleen express CD133 or not. Taken together, we proposed a model that delineates our findings. As shown in figure 3.5, when acute anemia is induced, CD34⁺KSL cells migrate into spleen and they become CD34⁺CD133⁺KS population, which have self-renewal ability and the ability to rapidly expand. This expansion needs signals such as BMP4, GDF15, Hedgehog and SCF. Around day 8 Epo and hypoxia promote differentiation. CD34⁺CD133⁺KS cells differentiate into CD34⁺CD133⁺KS
population and then CD34-CD133-KS population. Finally, the proliferation and differentiation of stress BFU-Es produce enough erythrocytes leading to the recovery from acute anemia.

**Identification of human stress erythroid progenitors that express γ-globin**

Stress erythropoiesis is best understood in the mouse because of our ability to induce anemia and analyze their recovery. The development of an in vitro culture system allows us to now interrogate human stress erythropoiesis. Unfractionated human bone marrow mononuclear cells were cultured in SEEM supplemented with human factors for 7 days and analyzed by flow cytometry on cell surface markers CD133, CD34 and Kit (Sca1 is not a marker for human progenitor). We observed that uncultured bone marrow cells had very few CD133+ and CD34+ populations, while the cells cultured in SEEM generated a marked increase in the proportion of CD34+CD133+ Kit+ cells, the early stage stress erythroid progenitors (Figure 3.6A). To further test the role of BMP4, GDF15, HH and SCF used in culture medium, human bone marrow cells were cultured in SEDM for 5 days and then cultured in methylcellulose media for stress BFU-Es. Figure 3.6B-C showed that the complete SEDM rapidly generated stress BFU-E colonies. Removal of any one factor led to a significant decrease in stress BFU-E development, especially in the absence of BMP4, the key factor that required in stress erythropoiesis. These data showed that like mouse bone marrow culture, BMP4, GDF15, HH and SCF are playing critical roles in human stress erythropoiesis. Collectively, our studies demonstrated that human bone marrow cells have the similar response to
generate stress erythroid progenitors when cultured in stress erythropoiesis medium.

Human stress erythropoiesis is also associated with fetal erythropoiesis and fetal hemoglobin (HbF) production. Fetal erythrocyte characteristics and antigens as well as the expression of fetal hemoglobin are observed during the recovery from erythropoietic stress[28, 46]. They are also observed following bone marrow transplant, in acute anemia syndromes and in some patients with thalassemia and sickle cell anemia[47-56]. Herein, we investigated whether stress erythropoiesis in human bone marrow cells produces gamma globin and fetal hemoglobin. Cells were cultured in steady-state erythropoiesis medium containing Epo and SCF, SEDM for 5 days or SEDM for 5 days and further culture in BMP4+SCF+EPO+2%O₂ for an extended 3 days (SEDM+Diff.) for the complete differentiation. Expression levels of gamma globin and beta globin were analyzed by RT-qPCR and the data was presented as a Y/β ratio. Figure 3.7A showed that the uncultured HBM cells exhibited pretty low gamma globin expression and the steady-state condition exhibited a baseline of gamma/beta ratio, which may due to the effect of SCF on inducing gamma globin expression[57]. Cells cultured in SEDM displayed an elevated gamma/beta ratio as high as 0.5. For the extended culture, the gamma/beta ratio reached to approximately 1.5, indicating that stress erythropoiesis in human bone marrow culture induces significant gamma globin expression. Flow cytometry analysis was used to further confirm the expression of feta hemoglobin in human stress erythropoiesis. Cells cultured in steady-state erythropoiesis condition had little fetal hemoglobin expression, which was evident in the flow data that most of CD235a+ cells were HbF negative. However, for the SEDM
culture, approximately 1/3 of CD235a+ cells expressed HbF and the HbF/HbB ratio was close to 0.5. Compared to steady-state erythropoiesis, stress erythropoiesis generates higher level of fetal hemoglobin, which is consistent with the previous findings that stress erythropoiesis exhibits properties of fetal erythropoiesis.

To further investigate the regulation of gamma globin, we analyzed the expression of BCL11A, a repressor that plays a key role in silencing the gamma globin gene in adult[30-33, 58]. BCL11A has four isoforms, XL, L, S and XS. When we used the probe to detect all the forms of BCL11A, there was no significant difference on the expression (data not shown). However, only the XL and L isoforms are associated with \( \gamma \) globin repression. We designed a taqman probe that only recognized XL and L forms. RT-qPCR data showed that HBM cells cultured in SEDM had lower expression on XL/L isoforms, while steady-state erythropoiesis of HBM exhibited higher level of BCL11A-XL/L. The removal of any factor in the media showed that the absence of Hedgehog also induced elevated expression of BCL11A. Western blotting data further confirmed our hypothesis that steady-state erythropoiesis produces more expression of BCL11A-XL/L isoforms, which further inhibits gamma globin expression. These data demonstrated that Hedgehog is required for the repression of BCL11A and it may or may not directly regulate BCL11A-XL/L expression. However, HBM cells cultured in stress erythropoiesis medium exhibits no obvious expression of BCL11A-XL/L, which is correlated with high expression of gamma globin. Taken together, all the data showed that stress erythropoiesis conditions in human bone marrow cell cultures inhibit BCL11A expression and thus generates high level of gamma globin expression.
Discussion

Unlike steady state erythropoiesis, stress erythropoiesis utilizes unique progenitors to rapidly produce new erythrocytes. In mice where it is best characterized, it mainly occurs in the spleen. This developmental process is tightly regulated. Previous studies from our lab discovered the important factors that are required for stress erythropoiesis[7, 8, 11-13]. Based on this, we developed an in vitro culture system that functionally produces stress BFU-Es. This culture system recapitulates the events that occur in vivo. Analysis of erythroid short-term radioprotection showed that CD34+KSL cells gave rise to Population I stress progenitors in the spleen following transplant. However, our analysis in vitro showed that this population is unable to generate stress BFU-Es. Only culture of unfractionated bone marrow cells, which allows for the development of a microenvironment, will lead to the development of stress erythroid progenitors. In fact, it is the macrophages that develop in these cultures that are critical for the generation of stress erythroid progenitors. This observation supports the recent work from Rivella and Frenette labs demonstrating a key role for macrophages in stress erythropoiesis in vivo[59, 60]. In addition to the role of macrophages, this culture also identified Epo and hypoxia as signals that promote the transition of stress erythroid progenitors from an expansion stage to a differentiation stage. Our previous work showed that despite the presence of all the factors necessary for stress BFU-E formation, we did not observe donor derived stress BFU-E until we could detect the expression of Epo in the kidney. Our data here show that culturing cells in the absence of Epo and hypoxia (SEEM) leads to an expansion of cells that are unable to
differentiate. However when cells are switched into media containing Epo and grown at 2% O\textsubscript{2}, they rapidly form stress BFU-E. These data support a model where immature stress erythroid progenitors expand in the spleen, but are unable to differentiate until tissue hypoxia has reached such levels that Epo is induced in the kidney. This mechanism ensures that a large number of progenitor cells are generated, so that once differentiation starts, sufficient erythrocytes are produced to alleviate the anemia.

Previously, our lab identified the Population I, II and III cells according to the expression of Kit, Ter119 and CD71[8]. However, this classification was not specific enough to separate the expansion stage and differentiation stage. Here we further divided the Population I cells into three subgroups by the combination of stem cell markers CD34, CD133, Kit and Sca1. Both the in vitro and in vivo data consistently showed the developmental process of stress erythroid progenitors from CD34\textsuperscript{+}CD133\textsuperscript{+}KS population to CD34\textsuperscript{-}CD133\textsuperscript{+}KS and CD34\textsuperscript{-}CD133\textsuperscript{-}KS populations. The CD34\textsuperscript{+}CD133\textsuperscript{+}KS cells have self-renewal ability and they are dominant in the expansion stage. Then they go through the intermediate stage CD34CD133\textsuperscript{+}KS cells and become CD34\textsuperscript{-}CD133\textsuperscript{-}KS population which acquires the ability to differentiate. Although we have strong data to delineate the progress of stress erythroid progenitors, it remains unknown about the migration stage from bone marrow to spleen. Previously we identified the ST-HSCs can provide the short-term radioprotection, but whether these cells that migrate into spleen carry CD133 or not need further studies. Here we showed that once in the spleen, the cells expressing CD133 are enriched, which raised the possibility that the CD34\textsuperscript{+}CD133\textsuperscript{+}KS cells migrate from bone marrow to spleen and then
proliferate to ensure enough erythrocytes can be made.

Stress erythropoiesis is closely associated with severe anemia. Although it has been difficult to study the erythropoiesis in human patients due to the complicated situation of indirect effects of disease pathology, several studies have identified some properties of human stress erythropoiesis. It is associated with fetal hemoglobin production. Work from the Malik lab showed that analysis of peripheral blood and bone marrow progenitors from sickle cell anemia patients identified a population of progenitors that express CD34, GlycophorinA, CD71 and Kit. These cells give rise to a higher proportion of HbF. Furthermore, hypoxia increased the production of HbF[61]. Other studies also showed that acute anemia induces the expansion of a fetal erythroid progenitor[62]. These results are highly consistent with our findings in this chapter. The in vitro culture of human bone marrow cells in stress erythropoiesis medium exhibits an increased gamma-globin and HbF proportion. Stuart Orkin’s lab previously identified that BCL11A works with SOX6 to suppress gamma-globin expression[33]. Our data here showed similar results and further found that the BCL11A-L/XL form regulates human gamma-globin expression. Similarly we observed that murine stress erythroid progenitors fail to express BCL11A and as a consequence express high levels of embryonic globin βh1 and εy. These data suggest that expression of elevated fetal/embryonic globins may be a characteristic of stress erythropoiesis. This property could be exploited to treat thalassemia and sickle cell anemia patients by selectively promoting the differentiation of progenitors predisposed to express HbF.
References


Figure 3.1. In vitro culture system of stress erythropoiesis generates stress BFU-Es and induces fetal hemoglobin. (A) Stress BFU-E colony assay. Unfractionated bone marrow cells were isolated from C56BL/6 mice and cultured in stress erythropoiesis differentiation media (SEDM) containing BMP4, SCF, Hedgehog, Epo and GDF15 under hypoxia condition (control media, C) or in absence of each of these factors for 5 days. Then cells were plated in methylcellulose medium for BFU-E colonies. (B-D) The expression of murine embryonic β-globin genes βH1 (B) and εy (C) and Bcl11A (D) was examined by qRT-PCR in murine bone marrow cells cultured in SEDM for 5 days (SEDM), SEDM for 5 days then switched to stress BFU-E media Epo+BMP4+SCF at 2% O₂ for 3 days (SEDM+Diff.) or cultured in steady state conditions (Epo + SCF at 20% O₂). Error bars indicate ± SEM. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
Figure 3.2. Epo and hypoxia promote the differentiation of stress erythroid progenitors. (A) BFU-E colony number per 1X10⁵ cells. Bone marrow cells isolated from C57BL/6 mice were cultured in SEDM or SEEM for 5 days. Then cells were plated in methylcellulose medium. BFU-E colonies were scored after 5 days. (B) Cells pre-cultured in medium absent of Epo and hypoxia were treated with Epo alone or Epo plus hypoxia. BFU-E colony assay was performed on the indicated days. Error bars indicate ± SEM. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
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A

CD34

Kit+Sca1+

CD133

SEEM

7 days

B

CD34

Kit+Sca1+

CD133

SEEM

7 days

Co-culture

5 Days

SEDM

Co-culture
CD34-CD133-KS
CD34+CD133+KS
CD34-CD133-KS
CD34+CD133+KS

BFU-E Colony Number/1X10^5 cells

p=0.001
p=0.021

C

D

2%+BMP4+Scf+Epo
Epo only
CD34+CD133+KS

Recipient

CD34+CD133+KS

Donor
Figure 3.3. Identification of stress erythroid progenitors and their development in vitro. (A) Representative flow cytometry analysis of unfractionated mouse bone marrow prior to culture (left), after 7 days in SEEM (center) and after 5 days in SEEM + Epo or SEDM (right). Cells were gated on Kit+Sca1+ and the expression of CD34 and CD133 is shown. (B) GFP+ CD34+CD133+KS cells were sorted by flow cytometry (left) and co-cultured with GFP- bone marrow cells in SEEM for 7 days (center) and then switched SEDM (right) for 5 days. At each step, GFP+ cells were gated on Kit+Sca1+ and analyzed for CD34 and CD133 expression. (C) GFP+ CD34-CD133+KS (left) and GFP+ CD34-CD133-KS (right) cells were sorted by flow cytometry (top) and co-cultured with GFP- bone marrow cells in SEEM for 7 days (center) and then switched into SEDM (bottom) for 5 days. At each step, GFP+ cells were gated on Kit+Sca1+ and analyzed for CD34 and CD133 expression. (D) BFU-E colony assay on distinct stress erythroid populations. (E) qRT-PCR analysis of gene expression in sorted stress erythroid progenitors. (F) Flow analysis on white blood cells in recipient mice on day 16 post-transplant. Sorted populations were transplanted into C57BL/6 mice (5000 cells /mouse). White blood cells were isolated on day 16 from the peripheral blood. Data in vitro is representative for three independent experiments. 3 mice were analyzed on each time point in each type of transplantation.
Figure 3.4. Analysis of stress erythroid progenitors in vivo. (A) Flow analysis for stress erythroid progenitors in the spleen of recipients after bone marrow transplantation. $5 \times 10^5$ GFP donor bone marrow cells were transplanted into each C57BL/6 recipient. Spleen cells were isolated and analyzed on every two days. 2 mice were analyzed for each time point. (B) The diagram of percentage and cell numbers for the flow analysis. Error bars indicate ± SEM.
Figure 3.5. Model for expansion and differentiation of stress erythroid progenitors. HH, Hedgehog; SCF, Stem cell factor.
Figure 3.6. Human stress erythroid progenitors can be cultured from human bone marrow. (A) Human bone marrow cells were cultured in stress expansion medium for 7 days. Cells were then analyzed by flow for CD34, CD133 and Kit. (B-C) Human bone marrow cells were cultured in stress erythropoiesis medium containing BMP4, SCF, Hedgehog, Epo and GDF15 under hypoxia condition (control media, C) or in absence of each of these factors for 5 days. Then cells were plated in methylcellulose medium with Epo alone for BFU-E colonies. Data is representative of 3 independent experiments using bone marrow from 3 different human donors.
Figure 3.7. Human stress BFU-E express embryonic globin genes. (A) qRT-PCR analysis of β and γ globin expression in human bone marrow cells prior to culture (control), cultured in SEDM for 5 days (SEDM), cultured in SEDM for 5 days then switched to stress BFU-E media Epo+BMP4+SCF at 2% O₂ for 3 days (SEDM+Diff) or cultured in steady state conditions (Epo + SCF at 20% O₂). Expression is expressed as a γ-globin to β-globin ratio. (B) Flow cytometry analysis of HbF in CD235a+ cells in human bone marrow cells cultured in SEDM (left) or cultured in steady state erythopoiesis media, Epo + SCF at 20% O₂ (right). (C) Expression of BCL11A-L/XL isoform in cultured human bone marrow cells. (D) Western blotting assay of BCL11A in cultured human bone marrow cells. Data is representative of 3 independent experiments using bone marrow from 3 different human donors.
Table 3.1 Probes used in qRT-PCR.

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Table 3.2 Antibodies used in flow cytometry.

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Chapter 4

Analysis Of Signals Regulating Stress Erythropoiesis: New Roles For
Epo, Wnt And PGE₂ Signaling In Stress Erythropoiesis

Forward

The following chapter is a manuscript that will be submitted as “Jie Xiang and Robert F. Paulson, Analysis of signals regulating stress erythropoiesis: New roles for Epo, Wnt and PGE₂ signaling in stress erythropoiesis.” This manuscript was written by Robert F. Paulson and Jie Xiang. The work presented in this chapter is the work of the author.

Abstract

Acute anemia results in tissue hypoxia. This condition activates a systemic response designed to increase oxygen delivery to the tissues. One component of this response is stress erythropoiesis, which relies on the rapid expansion and differentiation of stress erythroid progenitors in the spleen. This process utilizes a specialized population of progenitors that utilize signals distinct from steady state erythropoiesis. Recent work from our lab demonstrated that stress erythropoiesis proceeds through a two-stage process. Initially, stress progenitors that exhibit stem cell properties rapidly expand, but at this stage, the progenitor cells are unable to
differentiate. In response to microenvironmental signals, a transition occurs and the stress progenitors acquire the ability to differentiate. This change in signaling leads to the synchronous generation of new erythrocytes. Here we show that during the initial expansion stage, macrophages in the microenvironment make Wnt family factors (Wnt2b and 8a) that promote the expansion of the stem cell like stress erythroid progenitors. Tissue hypoxia promotes the transition from expansion to differentiation by increasing the concentration of systemic erythropoietin, Epo. Epo acts on macrophages in the spleen to down regulate Wnt expression and increase the production of Prostaglandin E2 (PGE₂), which induces the differentiation of stress erythroid progenitors. The loss of the expansion signal coupled with the increase PGE₂ results in the switch from expansion to differentiation.
Introduction

Acute anemia and tissue hypoxia result in stress erythropoiesis which is BMP4-dependent and it utilizes unique signals and progenitors that are different from steady state erythropoiesis. Previous studies in our lab have shown that in response to acute anemia, stress erythropoiesis leads to a rapid expansion and differentiation of the specialized progenitors that reside in the spleen[1-4]. This process requires multiple signals that tightly work at different stages of development[5-7]. It is known that BMP4, GDF15, Hedgehog and SCF are involved in the proliferation of early stress erythroid progenitors. While, hypoxia, SCF and BMP4 regulate the expansion and differentiation of stress BFU-Es. The increased numbers of stress BFU-E rapidly produce large numbers of new erythrocytes to promote the recovery from acute anemia and eliminate tissue hypoxia[6]. Using bone marrow transplantation as a model, our lab has shown donor stress BFU-E are not observed in the spleen of recipients until day 8 post-transplant. This observation indicated that the proliferating stress erythroid progenitors are unable to differentiate before day 8. Correspondingly we found that Epo expression was also up-regulated by tissue hypoxia in the kidney on day 8[4]. We developed an in vitro culture system to reconstitute the stress erythropoiesis and found that Epo and hypoxia were the signals that promote the transition from proliferation to differentiation. Previously published data shows that Stat5a/b-/- mice and mice with EpoR mutations that are deficient in their ability to activate Stat5a/b are unable to respond to anemic stress[8, 9]. These supported the hypothesis that Epo/EpoR/Stat5a/b signaling played a key role in stress erythroid progenitors. However, they did not examine the role of Epo-Stat5a/b signaling in
macrophages. Here we show that Epo activates Stat5a/b signaling pathway in macrophages to regulate the differentiation of stress erythroid progenitors.

Stress erythropoiesis requires the interaction of progenitors and microenvironment[10]. People have shown that macrophages play a critical role in erythropoiesis[11]. In the presence of Epo, erythroid colony forming unit (CFU-E) adhere to macrophages, resulting in the formation of erythroblastic island (EI)[12-14]. F4/80+ splenic macrophages participate in fetal erythropoiesis and in the formation of the splenic architecture[15]. Without macrophages, an increasing apoptosis is observed in erythroid progenitor cells. Spleen CD169+ macrophages promote erythropoiesis under homeostasis and stress and bone marrow CD169+ macrophages promote the retention of HSC and progenitor cells in the mesenchymal stem cell niche[16, 17]. Recent work showed that ablating macrophages in vivo with Clodronate liposomes or a toxin transgene impairs stress erythropoiesis. Work done in Rivella's lab demonstrated that macrophages contribute decisively to the recovery from induced anemia and pathological progression of polycythemia vera and beta-thalassemia[18]. Mice treated with clodronate showed markedly impaired recovery from anemia. In this chapter, we showed that macrophages are involved in the expansion and differentiation of stress erythroid progenitors. The rise in serum Epo induced by tissue hypoxia induces changes of the signals in microenvironment from signals that promote proliferation to ones that induce differentiation. We identified two important signals, Canonical Wnts and Prostaglandin E2 (PGE2) that can respond to Epo and regulate stress erythropoiesis. Based on previous work, it is known that
Wnt signaling can promote hematopoietic stem cell self-renewal and progenitor development[19-25]. PGE$_2$ belongs to the prostaglandin family of lipid signaling molecules. It can produce stimulation of endogeneous heme synthesis and promote erythropoiesis[26, 27]. Here, we showed that Epo works on macrophages to turn off Canonical Wnt signals and increase PGE$_2$ production to promote the differentiation of stress erythroid progenitors. This observation demonstrates the crucial role of macrophages in stress erythropoiesis. Furthermore we identified that in response to Epo treatment, phosphorylated Stat5a/b binds to the promoter of Canonical Wnts (Wnt 2b and 8a) and recruits the histone-lysine N-methyltransferase EZH2, a member of the Polycomb-group (PcG) family leading to an increase in H3K27Me3 in the promoter region, which represses Wnt gene transcription.
Materials And Methods

Mice and cell culture

C57BL/6 mice and C57BL/6-Tg(UBC-GFP)30Scha/J (hereafter referred to as GFP mice) mice were purchased from The Jackson Laboratory. Stat5a/b<sup>b<sub>k</sub>/<sup>k<sub>x</sub></sup> mice were obtained from Dr. Lothar Henighausen's lab and they were crossed with B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J transgenic mice (hereafter referred to as Cre mice) from The Jackson Laboratory[28, 29]. To delete Stat5a/b in vivo, Tamoxifen was injected into mice every day for 5 days (75mg/kg). To induce the deletion in the culture, cells were treated with 4-hydroxytamoxifen (OHT) (Sigma Aldrich) at a final concentration of 1uM for 48 hours. All procedures are approved by the IACUC of the Pennsylvania State University. For the culture of bone marrow cells in vitro, mouse bone marrow cells were isolated and cultured in stress erythropoiesis expansion media (SEEM): Gibco IMDM (Invitrogen) with 10% fetal bovine serum (Equiec-Bio, Kerrville, TX), 1% penicillin-streptomycin, 10ug/ml insulin, 200ug/ml transferrin, 2mM L-glutamine, 0.01g/ml Bovine Serum Albumin and 7ul/L 2-mercaptoethanol) supplemented with GDF15 (30ng/ml, Cloud-Clone Corporation), BMP4 (15ng/ml, R&D systems), SCF (50ng/ml, R&D systems) and SHH (25ng/ml, R&D systems). For the differentiation, cells were cultured in SEEM supplemented with Epo (3U/ml) at 2% O<sub>2</sub> for 5 days (SEDM). L929 cell line was cultured in DMEM with 10% fetal bovine serum (Equiec-Bio, Kerrville, TX) and 1% penicillin-streptomycin to collect L929 medium. For the bone marrow derived macrophages, primary bone marrow cells were cultured in DMEM with 10% fetal bovine, 1% Penicillin-Streptomycin and 10% L929 medium. For the treatment of PGE<sub>2</sub>,
Indomethacin, BIO and WIF1, the following final concentrations were used: PGE\textsubscript{2} (50nM), Indomethacin (10\textmu M), BIO (1\textmu M) and WIF1 (100ng/ml).

**Bone marrow transplantation and complete blood cells assay**

For transplantation assay, bone marrow cells were isolated from C57BL/6, Stat5a/b\textsubscript{fx/fx}:Cre or Stat5a/b\textsuperscript{−}/\textsuperscript{−} mice and cultured in stress erythroid expansion medium. Then sorted stress erythroid progenitors were transplanted into lethally irradiated C57BL/6, Stat5a/b\textsubscript{fx/fx}:Cre or Stat5a/b\textsuperscript{−}/\textsuperscript{−} recipients (1x10\textsuperscript{5} cells/mice). For each type of donor cells, 5 recipients were used. Before the transplantation, all the recipients were treated with acidified water (pH 2.5-3.0) for 1 week and antibiotics for 3 days. On the indicated days, peripheral blood was obtained by retro-orbital sinus through heparin-coated microhematocrit tubes and transferred to tubes coated with EDTA for complete blood cell (CBC) analysis.

**BFU-E colony assay**

Cells (2x10\textsuperscript{5}/ml) cultured in stress erythropoiesis medium were plated in methylcellulose media (StemCell Technologies) containing 3U/ml Epo alone at 20% O\textsubscript{2} or in combination with 50ng/ml SCF and 15 ng/ml BMP4 at 2% O\textsubscript{2}. Stress BFU-Es were scored after 5 days incubation as described [6].

**RT-qPCR and gene expression assay**

Total RNA was isolated by TriZol reagent (Invitrogen) /chloroform/isopropanol. 2ug RNA was used for RT-PCR to get cDNA by using the SuperScript-II system (Invitrogen).
RT-qPCR was done by using Taqman probes from Applied Biosystems (Table 1). The RT-qPCR analysis was done using an ABI7300 Real-time PCR system.

**Western Blotting**

Western blot analysis was performed using the following primary antibodies: anti-Stat5a/b (#9363S, Cell Signaling Technology) at 1:1000 dilution and anti-Ezh2 [30S, Cell Signaling Technology] at 1:1000 dilution for overnight at 4°C. Secondary antibody goat anti-rabbit was used at 1:10000 dilution for 1h at room temperature. The bands were visualized with Amersham ECL prime western blotting detection reagent.

**Immunocytochemistry**

Spleen macrophages and BMDMs were fixed with 4% formaldehyde (Sigma-Aldrich) for 20min at room temperature. Then cells were washed with PBST (PBS containing 0.1% Tween20) and permeabilized by 0.3% Triton X-100. After being washed with PBST for 3 times, samples were blocked with 1% BSA in PBST for 30min at RT and incubated with primary antibodies (F4/80 (#123102, Biolegend) and Phospho-Stat5a/b (#ab32364, Abcam)) at 4 °C overnight. Then samples were washed with PBST for 3 times and incubated with secondary antibodies (FITC-conjugated goat anti-rabbit IgG (sc-2012, Santa Cruz Biotechnology) and Alexa 647-conjugated goat anti-rabbit IgG (A21244, Life Technologies)) for 1h in dark. After 3 times wash with PBST, samples were mounted in Prolong Gold Antifade Mountant with DAPI (P-36931, Life Technologies). Then samples were observed on FV1000 confocal microscope (Olympus).
**Flow Cytometry and Cell Sorting**

Cells were labeled with different combinations of the following antibodies: CD133-PE, CD34-FITC and CD34-APC (eBiosciences), Kit-Alexa Fluor 647, F4/80-PE-CY7, and Sca1- Pacific Blue (BioLegend), Phospho-Stat5a/b-Alexa Fluor 647 (BD biosciences). After labeling, cells were washed with 2% fetal bovine serum in PBS. Flow cytometry analysis was done using LSR-II Fortessa Flow cytometer (BD Biosciences). The percentage of dead cells was measured by propidium iodide staining. All the data were analyzed with FlowJo software. F4/80+ macrophages or cell populations CD34-CD133-KS and CD34-CD133+KS were sorted by using Cytopia Influx V-GS Cytometry Workbench with Spigot software.

**PGE₂ extraction and LC-MS/MS-MRM analysis**

The splenic macrophages or BMDM cell culture supernatants were acidified with HCl (1 N) to pH 3.0 and extracted with ethyl acetate. The PGE₂ was quantitated using an LC-MS/MS-MRM system as described previously[31]. The HPLC system consisted of LC-20AD pumps with a SIL-20AC autosampler (Shimadzu Corporation, Columbia, MD). A Luna (Phenomenex, Torrance, CA) phenyl-hexyl analytical column (2 × 150 mm, 3 µm) with a 30 min isocratic elution with methanol/ water (70:30 v/v) containing 0.1% acetic acid at a flow rate of 150 µL/min was used for the quantitation of PGE₂. The injection volume was 50 µL. Negative ion electrospray tandem mass spectrometric analysis was carried out using AB Sciex (Foster city, CA) API 2000 triple quadrupole mass spectrometer at unit resolution with multiple reaction monitoring mode (MRM). The source temperature was at 450 °C,
electrospray voltage was -4500 V and the declustering potential was set at -16 V. Nitrogen was used as collision gas at -20 eV and the dwell time was 150 ms/ion. During MRM, PGE₂ was measured by recording the signal for the transition of the deprotonated molecule of m/z 351 to the most abundant fragment ion of m/z 271. Data were acquired and analyzed using Analyst software version 1.5 (AB Sciex, Foster city, CA).

**Epo and PGE₂ Elisa assay**

Serum Epo concentration was measured by Mouse Erythropoietin Quantikine ELISA Kit (MEP00B, R&D systems). PGE₂ concentrations in spleen and serum were measured by Prostaglandin E₂ Express EIA Kit (500141, Cayman Chemical Company) and Prostaglandin E₂ Metabolite EIA Kit (514531, Cayman Chemical Company), separately.

**Short hairpin RNA and transduction**

Mouse shRNA constructs in lentiviral GFP vector targeting Stat5a/b gene and shRNA lentiviral packaging kit were purchased from Origene Technologies (Rockville, MD). Spleen cells were isolated from C57/BL6 mice and transduced with shRNA lentivirus for 48 hours. Then Cells were cultured in stress erythropoiesis media supplemented with Epo (3U/ml), GDF15 (30ng/ml), BMP4 (15ng/ml), SCF (50ng/m), SHH (25ng/ml) and cultured in the hypoxia chamber which provides 2% O₂ for the indicated period of time. On the indicated days, cells were harvested for further analysis.
**Chromatin Immunoprecipitation (CHIP) assay**

The CHIP assay was performed by using the EZ-Magna CHIP A Kits [32-408, Millipore]. Samples were sheared for 3 rounds of 10 cycles of 30 sec ON/ 30 sec OFF with Bioruptor UCD200 (Diagenode). The antibodies used for immunoprecipitation were, anti- H3K27Me3 (5678S, Cell Signaling Technology) and IgG. Quantitative reverse transcriptase PCR was performed using SYBR-green Master Mix (Applied Biosystems). The primers and probes in table 2 were used for amplification of precipitated DNA.

**Statistical analysis**

The standard deviation indicates the variation within each experiment (in-vitro) and each mouse (in-vivo). P-values were calculated by Student t-test (2-tailed). Statistical significance was taken at values of, * p<0.05, ** p< 0.01, *** p<0.001. Sample sizes were described in the figure legends.
Results

Epo regulates the differentiation of stress erythroid progenitors through Stat5a/b pathway.

Previously we found that Epo alone can promote the transition from the expansion to differentiation and hypoxia potentiates this response. It has been known that Stat5a/b-/- mice have inefficient erythropoiesis and they are deficient in the recovery from acute anemia. We tested whether Stat5a/b is involved in the Epo-induced differentiation of stress erythroid progenitors. Short hairpin RNAs targeting Stat5a/b were used to knockdown the expression. Bone marrow cells were transduced with lentivirus for 48 hours. Figure 4.1A showed that the three different shRNAs had 50-60% knockdown efficiency. Then cells were cultured in stress erythropoiesis expansion medium as described in Chapter 3 for 5 days and treated with Epo for 3 or 5 days. At the indicated time points, cells were plated in methylcellulose medium for stress BFU-E colony assay. Both control and non-targeting condition exhibited induced BFU-E colonies in response to Epo treatment on day 5. However, when Stat5a/b was knocked down, there was a significant decrease in the BFU-E colony numbers compared to control (Figure 4.1B). These data show that Stat5a/b plays a critical role in Epo-dependent differentiation.

To further test whether Stat5a/b deficient bone marrow cells could provide short-term radioprotection, 5x10^5 whole bone marrow cells with or without
knockdown of Stat5a/b expression were transplanted into C57BL/6 recipients. Hematocrits were analyzed after bone marrow transplantation. Although all the recipients were recovered around day 18, mice transplanted with Stat5a/b-knockdown bone marrow cells displayed delayed recovery (Figure 4.1C). These data show that Stat5a/b is involved in the differentiation stage of stress erythropoiesis. However, from other studies, Stat5a/b-/- mice exhibit much more severe deficiency on stress erythropoiesis [8, 9]. The better recovery in our data is most likely due to the 50% knockdown efficiency, however taken together these data show that Epo promotes the differentiation of stress progenitors through Stat5a/b pathway.

**Epo works on macrophages rather than stress erythroid progenitors to initiate the differentiation.**

Our previous data shows stress erythroid progenitors can be divided into three sub-populations: CD34⁺CD133⁺KS, CD34⁺CD133⁺KS and CD34⁺CD133⁻KS cells. The CD34⁺CD133⁺KS population is the most immature population of progenitors. They can proliferate but cannot differentiate or form stress BFU-E colonies. After Epo treatment, they become CD34⁻CD133⁻KS population and acquire the ability to differentiate. One possible mechanism by which Epo induces differentiation is through the activation of EpoR on the surface of CD34⁺CD133⁺KS cells. We tested the EpoR expression on the stress erythroid progenitors. Surprisingly, CD34⁺CD133⁺KS cells have little to no detectable EpoR expression based on three independent sortings, but the later stage cells, CD34⁻CD133⁻KS population exhibit high EpoR expression (Figure
4.2A). This indicates that the CD34⁺CD133⁺KS cells are not the direct targets of Epo signal. After the Epo treatment, they progress into CD34⁻CD133⁻KS cells and EpoR expression is induced. When we isolated the early stress erythroid progenitors (CD34⁺CD133⁺KS population) and cultured them alone without supporting stromal cells in the differentiation medium with Epo treatment, almost all of the cells were dead on day 5 (data not shown). These data demonstrate that Epo does not act directly on CD34⁺CD133⁺KS cells and the microenvironment is important for their survival and transition to differentiation.

Because of the lack of EpoR expression in CD34⁺CD133⁺KS cells, we sorted CD34⁺CD133⁺KS population from Stat5a/b<sup>fx/fx</sup>:Cre mice or Stat5a/b<sup>fx/fx</sup> control mice. Cells were treated with 4-OHT to induce deletion of Stat5a/b<sup>a/b</sup> and transplanted them into C57BL/6 recipients. If Epo directly affects the early stress erythroid progenitors, this Stat5a/b-deficient donor population will fail to provide the radioprotection and the recipients will exhibit severe anemia and slow recovery. However, the hematocrit data showed that a significant difference was observed between control and Stat5a/b<sup>−/−</sup> groups only on days 18 and 21 in post recovery stage and there was no significant difference on later time points (Figure 4.2C). Furthermore most (80%) of the recipients transplanted with Stat5a/b<sup>−/−</sup> CD34⁺CD133⁺KS cells survived (Figure 4.2B). These data support the hypothesis that the deficiency of Epo-Stat5a/b pathway in stress progenitors is not enough to cause a severe defect in stress erythropoiesis. This difference in phenotype suggests that Epo might work on stromal cells in microenvironment to regulate differentiation. To further test whether
mutation of Stat5a/b in the microenvironment will cause the defect in stress erythropoiesis, the same numbers of CD34*CD133*Ks cells from Stat5a/b<sup>fx/fx</sup> mice were transplanted into Stat5a/b<sup>-/-</sup> recipients. Compared to the C57BL/6 recipients, the Stat5a/b<sup>-/-</sup> recipients also exhibited a defect of recovery after day 18 (Figure 4.2D). Furthermore if we transplanted Stat5a/b<sup>-/-</sup> stress progenitors into Stat5a/b<sup>-/-</sup> recipients, we observed the most severe anemic condition (Figure 4.2E). The hematocrits curves indicate that both Stat5a/b<sup>-/-</sup> donor CD34*CD133*Ks cells and Stat5a/b<sup>-/-</sup> microenvironment contribute to the slow recovery. Taken together, all these data show that Stat5a/b plays a critical role in both stress erythroid progenitors and microenvironment.

Published data from the Rivella and Frenette labs showed that the elimination of macrophages by clodronate or toxic ablation impairs the response to stress erythropoiesis in wildtype mice[18]. Recent work also showed that macrophages are a novel target cell for Epo. Epo contrastingly negatively regulates NFκB mediated response in macrophages[33, 34]. All of these data suggest that Epo may work on macrophages to regulate the differentiation of stress erythroid progenitors. To test this hypothesis, we analyzed the EpoR expression in sorted primary spleen F4/80+ macrophages as well as bone marrow derived macrophages generated using cultures in L929-conditioned medium. Consistent with other studies, both primary spleen macrophages and BMDM express detectable EpoR expression, which is much higher than sorted CD34*CD133*Ks cells (Figure 4.2F). To further confirm the activation of Stat5a/b pathway in macrophages in response to Epo, Spleen cells isolated from
Stat5a/b<sup>fx/fx</sup> mice and Stat5a/b<sup>-/-</sup> mice were cultured in stress erythroid expansion medium with Epo treatment for 1 hour and then phosphorylation of Stat5a/b was analyzed by flow cytometry. As shown in Figure 4.2G, Epo induced the Stat5a/b phosphorylation in F4/80+ macrophages from wildtype mice but not in Stat5a/b<sup>-/-</sup> macrophages. Taken together, these data show that Epo activates Stat5a/b dependent signaling in macrophages, which could mediate the microenvironment for the stress erythropoietic differentiation.

Epo regulates Canonical Wnt signaling and Prostalglandin E2 secretion in macrophages.

Among the signals involved in the regulation of stress erythropoiesis, Wnt and PGE<sub>2</sub> have been well studied. Wnts and their receptors are highly expressed in embryonic hematopoietic tissues indicating an essential role for Wnt signaling in hematopoiesis[22]. Recently studies also clarified the role of Canonical Wnt signaling and Non-Canonical signaling in hematopoietic ontogeny. Wnt signal regulates hematopoiesis in a dose-dependent manner. The moderate activation of beta-catenin pathway results in increased differentiation potential whereas high levels of activation results in a differentiation block and rapid proliferation[25]. Canonical Wnt signaling also promotes early hematopoietic progenitor formation and erythroid specification during embryonic stem cell differentiation[35]. Prostalglandin E<sub>2</sub> (PGE<sub>2</sub>) belongs to the prostalglandin family, a group of lipid mediators that are derived from Arachidonic acid (AA) [36, 37]. PGE2 is involved in stimulation of endogenous heme synthesis and
promotion of erythropoiesis[38, 39]. It has been known that PGE₂ works together with Epo to mediate the synthesis of fetal and adult hemoglobin in blood erythroid bursts[26].

Based on the previous conflicting views regarding the importance of β-catenin for normal HSC function[40-45], we tested both an inhibitor (WIF-1) and an activator (BIO) of Canonical Wnt signaling in stress erythropoiesis culture system. For PGE₂, we also used an inhibitor of COX-1 and COX-2, indomethacin [46] to block the total prostaglandin synthesis. To test whether Epo signaling interacts with Wnt and PGE₂, bone marrow cells isolated from wildtype mice were cultured in stress erythroid expansion medium with the treatment of WIF-1, BIO, PGE₂ and indomethacin in a combination of Epo or not. Cells then were plated in methylcellulose medium to generate BFU-E colonies. The data in figure 4.3A showed that cells cultured in the absence of Epo, but supplement with PGE₂ increased differentiation as measured by stress BFU-E colony formation. Similarly inhibition of Wnt signaling by WIF1 also induced differentiation, while the combination of WIF1+PGE₂ was not greater than either treatment alone. In contrast, activation of Wnt signaling by BIO blocked stress BFU-E development to untreated levels. Inhibition of PGE₂ synthesis with indomethacin blocked the ability of WIF1 to induced differentiation; while addition PGE₂ to BIO treated cells rescued the development of stress BFU-E. These data suggest that Wnt signaling may negatively regulate PGE₂ production. Finally addition of BIO and indomethacin leads to a complete loss of stress BFU-E development, suggesting that the background levels of stress BFU-E in untreated cultures were due to PGE₂
production that was independent of Wnt regulation. In cultures where Epo was added, we observed that PGE$_2$ and WIF1 increased the number of stress BFU-E colonies, while addition of BIO blocked stress BFU-E development back to ground levels. These data support the hypothesis that Epo signaling blocks Wnt dependent signaling that in turn relieves the expression of PGE$_2$ production. PGE$_2$ promotes the differentiation of stress erythroid progenitors.

Based on our previous analysis of stress erythroid progenitors (Chapter 3), we would predict that BIO would maintain the CD34+CD133+KS immature cell population. Cells were cultured in stress erythroid expansion medium with different combination of BIO and Epo for 7 days and then cells were collected for flow analysis on stress erythroid progenitors. Data in figure 3B shows that treatment with BIO increased the early stress erythroid progenitors, CD34+CD133+KS population. Notably, BIO treatment blocks the differentiation that induced by Epo. This data indicated that inhibition of Wnt signaling and up-regulation of PGE$_2$ production enhance differentiation whereas activation of Wnt signaling promotes the expansion of stress erythroid progenitors. To further test whether the expanded stress erythroid progenitors induced by BIO are functional, stress BFU-E colony assays were performed. Bone marrow cells were cultured in SEEM for 7 days supplemented with BIO. The cells were than switched to SEDM for 5 days, after which the cells were plated in methylcellulose medium with Epo alone or Epo plus BMP4, SCF at 2% O$_2$. Data in figure 4.3C show that BIO treatment in expansion stage resulted in increased number of BFU-
E colonies, which demonstrated that activation of Wnt signaling leads to the expansion of stress erythroid progenitors.

Using the in vitro culture model, we demonstrated a role for canonical Wnt signal in regulating the expansion of early stress erythroid progenitors. Next we used bone marrow transplantation to further check the Wnt expression, Epo levels and upregulation of PGE$_2$ production in the spleen during stress erythropoiesis. $5 \times 10^5$ wildtype bone marrow cells were transplanted into wildtype recipients. Spleen and kidney cells were isolated every two days for the mRNA expression analysis. Our previously studies show that the CD34$^+$/CD133$^+$KS population expand in the pre-recovery stage. In the recovery stage, Epo and hypoxia are induced and initiate the differentiation. The kidney data showed that Epo was induced on day 6 and the serum data also confirmed that the levels of serum Epo started to increase on day 6. Based on this observation, we proposed that the pre-recovery stage or expansion stage was before day 6. The spleen weight curve also showed an increase starting on day 6, which corresponded with the initial increase in serum Epo (Figure 4.3D). We next analyzed the expression of Wnt family members during the pre-recovery stage in the spleen. As shown in Figure 3E, an increased expression was observed in most of Canonical Wnts on day 4 and disappeared on day 6, the time when Epo was induced. However, for the non-Canonical Wnts, no obvious elevated expression was observed. Among Canonical Wnts, Wnt 2b, Wnt 7b, Wnt8a, Wnt9b had significant up-regulation. This data showed that during expansion stage, Canonical Wnt family members are induced and the increased Epo concentration leads to repression of Wnt expression, which initiates a differentiation. We chose Wnt 2b and Wnt 8a as the typical Canonical
Wnts to test their expression within 22 days post-transplantation. Using the same set of data in the bone marrow transplantation model, we checked the expression of Wnt 2b and Wnt 8a as well as the PGE\(_2\) concentration in the spleen until day 22. The peak of Epo concentration was related to the “turn-off” of Wnt 2b and Wnt 8a expression (Figure 4.3F). Moreover, we observed that PGE\(_2\), the synthases Ptges, Hpgds and COX2 were induced following high Epo expression (Figure 4.3G). Taken together, these data indicated that high Canonical Wnt activation regulates the expansion of stress erythroid progenitors. Epo initiates the differentiation by repressing Canonical Wnts expression and elevating PGE\(_2\) concentration.

The previous data show that Epo activates Stat5a/b signaling pathway in macrophages and changes the microenvironment to one that favors differentiation. Based on these data, we tested whether Epo stimulates macrophages to change the Wnt expression and PGE\(_2\) production. Primary F4/80+ macrophages in the spleen were sorted and cultured in SEEM for 24 hours. Cells then were treated with Epo. On indicated time points, cells were harvested for mRNA analysis. Culture medium was collected for extracting lipids. The PGE\(_2\) concentration was determined from mass spectrometry. Upon Epo treatment, both Wnt 2b and Wnt8a expression were decreased dramatically within 1 hour. In contrast the production of PGE\(_2\) was observed to increase within 3 hours post-treatment (Figure 4.3H). The production of PGE\(_2\) requires the action of PGE\(_2\) synthase (mPGES-1), when we checked the expression of mPGES-1, we found it was upregulated in response to Epo (Figure 4.3I). These data show that macrophages-derived Canonical Wnt signaling regulates the proliferation of
stress erythroid progenitors. In response to Epo, the expression of Canonical Wnt signals is repressed and the production of PGE$_2$ in the macrophages is increased. Together these two signaling events initiate the differentiation. Previously we showed that Epo signaling in macrophages during stress erythropoiesis was dependent on Stat5a/b. We used Stat5a/b$^{-/-}$ macrophages to test whether Stat5a/b mediated the repression of Canonical Wnts expression. The primary F4/80+ macrophages were sorted from control Stat5a/b$^{fx/fx}$ mice or Stat5a/b$^{-/-}$ mice and cultured in stress erythroid expansion medium for 24 hours with the following Epo treatment for 3 hours. As shown in figure 4.3J, a reduced expression of Wnt 2b and Wnt 8a as well as an elevated concentration of PGE$_2$ were observed in Stat5a/b$^{fx/fx}$ macrophages, but not in Stat5a/b$^{-/-}$ macrophages. This observation indicates that Stat5a/b$^{-/-}$ macrophages fail to change the macrophage microenvironment in response to Epo. Taken together, these data confirmed our hypothesis that Epo activates Stat5a/b pathway in macrophages to regulate Canonical Wnt and PGE$_2$ signals.

**L929 bone marrow derived macrophages response similarly to the Epo signal.**

Previous data demonstrate the role of macrophages in regulating differentiation of stress erythroid progenitors. To establish a microenvironment for stress erythropoiesis in vitro, we needed to develop an in vitro co-culture system including macrophages and CD34$^+$CD133$^+$KS population. However, the primary F4/80+ macrophages sorted from spleens cannot be re-cultured for a long time due to their inability to attach and grow. First we tested RAW 264.7, a murine macrophage cell line
for their response to Epo. Unfortunately, this cell line did not exhibit the same response as primary macrophages (Data not shown). Finally, we tried bone marrow derived macrophages (BMDM) generated using L929 conditioned media. Following generation of BMDMs, we cultured them in stress erythroid expansion medium for 24 hours. After Epo treatment for 3 hours, cells were harvested for analysis on Stat5a/b phosphorylation and Canonical Wnts expression. As shown in Figure 4.4, both immunostaining and flow cytometry confirmed the phosphorylation of Stat5a/b in response to Epo stimulation in BMDMs (Figure 4.4A-B). Analysis of Wnt expression by qRT-PCR showed that unlike the whole spleen analysis, some of wnt ligands were not expressed. However, the expression of Canonical Wnt 2b, Wnt 7b, Wnt 8a and Wnt 9b as well as the Non-Canonical Wnt4 were repressed in response to Epo, which indicated that the BMDM cells display similar properties to the sorted F4/80+ primary macrophages in spleen (Figure 4.4C). We next examined PGE$_2$ production by collecting the culture medium with longer treatment of Epo. The ELISA data showed that both wildtype and Stat5a/b$_{fx/fx}$ bone marrow derived macrophages exhibit an increase production of PGE$_2$ in response to Epo, whereas there was pretty low PGE$_2$ concentration in Stat5a/b$_{-/-}$ BMDM culture (Figure 4.4D). All of these data demonstrate that BMDMs can provide a strong and efficient microenvironment to study stress erythropoiesis.
Stat5a/b deficient macrophages/microenvironment fails to promote the differentiation.

To further test whether BMDMs could function as a microenvironment for stress erythropoiesis, bone marrow cells were isolated from GFP mice and cultured in SEEM for 7 days. GFP+CD34+CD133+KS population were sorted and co-cultured with Stat5a/b\textsuperscript{fx/fx} or Stat5a/b\textsuperscript{-/-} BMDM cells in SEEM with Epo treatment for 5 days. The cells were analyzed by flow cytometry or plated in methylcellulose medium for 5 days for stress BFU-E colony assay. Data in Figure 4.5 showed that the CD34+CD133+KS population could differentiate when cultured together with Stat5a/b\textsuperscript{fx/fx} macrophages but not in Stat5a/b\textsuperscript{-/-} microenvironment as demonstrated by the ability of cells grown on Stat5\textsuperscript{fx/fx} BMDM to form stress BFU-E colonies (Figure 4.5A-B). Flow cytometry analysis further showed that when co-cultured with Stat5a/b\textsuperscript{fx/fx} macrophages, CD34+CD133+KS population progress into CD34+CD133+KS cells in response to Epo treatment, which is reflected in their ability to differentiate. However, in the Stat5a/b\textsuperscript{-/-} microenvironment, about half of the CD34+CD133+KS cells fail to differentiate into CD34+CD133+KS cells and maintained in the culture (Figure 4.5C). This co-culture experiment confirmed our previous conclusions and demonstrated the role of Epo-dependent signaling in regulating the signals generated by macrophages during stress erythropoiesis.
**Epo represses Canonical Wnt expression by recruiting Ezh2.**

Although Stat5 is more often associated with activation of gene expression, Stat5 can act to repress gene expression. One prominent example is the B cell Igk locus. IL-7/Stat5 dependent signaling silences Igk expression in pre B cells. This process requires Stat5a/b-mediated recruitment of the histone methyltransferase Ezh2 to repress the Igk locus[47]. In our study, we found that Epo represses Canonical Wnts expression through Stat5a/b dependent pathway during stress erythropoiesis. Here we want to test whether Stat5a/b-mediated repression of Canonical Wnt genes involves in the recruitment of Ezh2. BMDMs were cultured in SEEM for 24 hours and then treated with Epo for 1 and 3 hours. Cells were crosslinked and harvested on the indicated time points for CHIP assay. The immunoprecipitation assay showed that Stat5a/b and Ezh2 interact in macrophages only with Epo treatment (Figure 6A). Ezh2 is part of the polycomb complex (PRC2) that leads to the tri-methylation of lysine 27 in histone H3, a repressive chromatin mark. Next we tested whether there was a corresponding H3K27Me3 in the promoter region of Canonical Wnt 2b and Wnt8a. Three pairs of primers were designed around -5kb, -1kb and +5kb in Wnt genes. The promoter region (-1kb) exhibited an increase of H3K27Me3 modification with Epo treatment, while the -5kb and +5kb sites did not show this response (Figure 6B). Taken together, all these data demonstrate that activated Stat5a/b recruits Ezh2 to repress the expression of Canonical Wnts in macrophages.
Discussion

Here we describe the mechanisms by which Epo can promote differentiation of stress erythroid progenitors and how Canonical Wnt and PGE$_2$ dependent signaling is involved in stress erythropoiesis. Most importantly, our studies identified the important interaction between macrophages and stress erythroid progenitors. Macrophages have been identified as scavengers that can phagocytose worn-out cells and play a role in immune responses. Previous work also found that macrophages are important in erythropoiesis by forming the erythroblastic island[12, 14, 48]. Distinct populations of macrophages interact at different times and places to regulate hematopoiesis. CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche of the bone marrow[16], while F4/80+ macrophages participate in fetal erythropoiesis in the fetal liver. Recently, other labs found that the elimination of macrophages by clodronate or genetic ablation impairs the recovery from anemia, suggesting that they play a critical role in stress erythropoiesis. However, the mechanism by which macrophages regulate stress erythropoiesis has not been identified. Epo is the key signal in erythropoiesis. Most of findings about Epo are related to erythroid progenitor cells. However recent work showed that macrophages are novel target cells for Epo[33]. Epo inhibits NF-kappaB-inducible immune pathways in macrophages, which dampens inflammation in the bacterial infection and experimental colitis models[34]. Our data provide strong and significant evidences to show that macrophages can respond to Epo to regulate the signal production in the microenvironment and mediate the expansion and
differentiation of stress erythroid progenitors, which uncovers a new connection between Epo and macrophages.

We demonstrate that the Canonical Wnt signal pathway promotes the expansion of early stress erythroid progenitors. Both Canonical Wnt and Non-canonical Wnt signals have been shown important in hematopoiesis[24, 49]. Recently Tarafar et al showed that Canonical Wnt signaling promotes early hematopoietic progenitor formation and erythroid specification during embryonic stem cell differentiation. The activation of β-catenin induces expression of genes involved in maintaining stem cell potential for sustaining the rapid proliferation during the establishment of the hematopoietic system[35]. These data are consistent with our findings that Canonical Wnts regulate the proliferation of early stress erythroid progenitors that have the self-renew potential. However, other lab found that macrophages-derived Canonical Wnt3a opposes Notch signaling to specify hepatic progenitor fate and drive hepatocyte differentiation in chronic liver disease. In their study, they mainly identified the role of Wnt3a in maintaining Numb expression to promote the specification to hepatocytes[50]. It has been discovered that Wnt3a is also involved in the differentiation of embryonic stem cells[51]. In our findings, Epo does not affect Wnt3a expression and not all the Canonical Wnts have the potential to regulate expansion. It is highly possible that Canonical Wnt ligands have various functions in different cell types. Our work identified the role of Canonical Wnt expression in macrophages.
We developed the co-culture system by using BMDMs to study the role of signals that produced from microenvironment in stress erythropoiesis. The Stat5a/b deficient macrophages fail to promote the differentiation of stress erythroid progenitors due to the incapability in changing Canonical Wnts and PGE$_2$ signals. This data supports our observation that Stat5-/- progenitors and microenvironment are needed for efficient expansion and differentiation of stress erythroid progenitors following bone marrow transplant. All of these results demonstrate that the interaction between microenvironment and stress erythroid progenitors is complex and essential. Epo is not only working on hematopoietic progenitor cells to specify the erythroid fate and promote terminal differentiation, but also targeting macrophages to modulate signals in microenvironment to initiate the differentiation during stress erythropoiesis.
References


Figure 4.1. Stat5a/b is required in Epo-dependent differentiation of stress erythroid progenitors.

(A) Relative Stat5a/b mRNA expression in cells transduced with lentiviral vectors. NT: Non-targeting. V2, V3847, V3849 are three shRNA constructs targeting Stat5a/b. (B) Stat5a/b-knockdown and control bone marrow cells were cultured in stress erythroid expansion medium for 5 days and then treated with Epo for 3 or 5 days. Then cells were cultured in methylcellulose medium supplemented with Epo only or with BMP4, SCF and Epo at 2% O₂ for 5 days. Bone marrow cells with Stat5a/b knockdown fails to increase the BFU-E colonies. Each treatment was done in triplicate and this experiment is representative of two independent experiments. (C) Each lethally irradiated C57/BL6 recipient was transplanted with 5x10⁵ Stat5a/b-knockdown bone marrow cells or control cells. Hematocrits were measured by CBC assay. For each time point, n=3.
**A**

Relative EpoR expression

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<th>CD34+CD133+KS</th>
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</table>

**B**

Percent survival

- Stat5a/b<sup>-/-</sup> to C57BL/6
- Stat5a/b<sup>fx/fx</sup> to C57BL/6

Days Post-transplantation: 0, 5, 10, 15, 20

**C**

% Hematocrit

- Stat5a/b<sup>-/-</sup> to C57BL/6
- Stat5a/b<sup>fx/fx</sup> to C57BL/6

Days Post-transplantation: 0, 3, 6, 9, 12, 15, 18, 21, 24
Figure 4.2. The activation of Stat5a/b pathway in macrophages in response to Epo involves in the regulation of stress erythropoiesis.

(A) Relative EpoR mRNA expression in sorted stress erythroid progenitors. CD34 CD133 KS cells exhibited significantly low EpoR expression. The data represented 3 independent sorting.

(B-C) Sorted CD34 CD133 KS cells from cultured Stat5a/b fx/fx or Stat5a/b −/− bone marrow cells were transplanted into lethally irradiated C57/BL6 recipients (5000/mice). The survival curve (B) and the hematocrit value (C) were measured. For each type of transplantation, n=5.

(D-E) Sorted CD34 CD133 KS cells from cultured Stat5a/b fx/fx or Stat5a/b −/− bone marrow cells were transplanted into lethally irradiated Stat5a/b fx/fx or Stat5a/b −/− recipient (5000/mice). Recovery of hematocrit value was analyzed by CBC assay. The deficiency of Stat5a/b in microenvironment also contributed to the delayed recovery (D). The defect of Stat5a/b in both the donor stress erythroid progenitors and the microenvironment in recipients displayed the slowest recovery (E). For each type of transplantation, n=5.

(F) Relative EpoR expression in stress erythroid progenitors, bone marrow derived macrophages and primary spleen macrophages. This data represented 3 independent sortings.

(G) Flow analysis of the phosphorylated Stat5a/b in spleen macrophages from Stat5a/b fx/fx and Stat5a/b −/− mice. The data represented 2 independent experiments.
D

Days Post-transplantation

Epo concentration (pg/ml)

Days Post-transplantation

Epo Relative expression

Days Post-transplantation

Spleen Weight (mg)

Days Post-transplantation
E

**Canonical Wnts Expression**

**Non-Canonical Wnts Expression**
PGE2 concentration (ng/ml) induced by EPO in Macrophages

Relative expression of Wnt2b and Wnt8a over time after EPO stimulation:

- M-E
- 1hr
- 3hr
- 6hr
- 12hr
- 24hr

PGES (relative expression):

- M-E
- M+E 1
- M+E 3
- M+E 6
- M+E 12
- M+E 24
Figure 4.3. Epo regulates Canonical Wnt signaling and PGE$_2$ concentration via macrophages.

(A) BFU-E colony assay. Wild type bone marrow cells were cultured in stress erythropoiesis medium with or without Epo, WIF1, BIO, PGE$_2$ and Indomethacin (INDO) for 1d or 5d. Then cells were cultured in methylcellulose medium for 5 days and BFU-E colonies were scored. Each treatment was done in triplicate and this experiment is representative of two independent experiments.

(B) Flow analysis of stress erythroid progenitors in cultures treated with or without Epo and BIO. This data represented 2 independent experiments.

(C) BFU-E colony assay on stress erythroid progenitors with or without BIO treatment. Each treatment was done in triplicate and this experiment is representative of two independent experiments.

(D-G) Bone marrow cells isolated from C57/BL6 mice were transplanted into lethally irradiated C57/BL6 recipient (5x10$^5$ cells/mice). Samples were collected every 2 days for analysis. (D) Epo concentration in serum (top), Epo mRNA expression in the kidney (middle), spleen weight (bottom) in 22 days post-transplantation. (E) Canonical and Non-Canonical Wnt ligands mRNA expression in the spleen from d0 to d10 post-transplantation. (F) Wnt2b and Wnt 8a mRNA expression from d0-d22 post-transplantation. (G) The mRNA expression level of PGE$_2$ (top left), PGE$_2$ concentration in spleen (top right), mRNA expression levels of COX2 (bottom left) and PGDS (bottom right) from d0-d22 post-transplantation. For each time point, n=2.

(H-I) Wnt 2b and Wnt 8a mRNA expression (left), PGE$_2$ concentration (right) and PGES mRNA expression (I) in sorted spleen macrophages treated with Epo. Each treatment was done in triplicate and this experiment is representative of two independent experiments.

(J) Wnt2b and Wnt 8a mRNA expression in cultured spleen macrophages from Stat5a/b$^{fx/fx}$ and Stat5a/b$^{-/-}$ mice (left). ELISA assay of PGE$_2$ concentration in the culture of the spleen macrophages from Stat5a/b$^{fx/fx}$ and Stat5a/b$^{-/-}$ mice (right). *p<0.05. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
Figure 4.4. L929 bone marrow derived macrophages respond to Epo signal.
(A) Immunocytochemistry analysis on co-localization of phosphorylated Stat5a/b, F4/80 and DAPI in L929 bone marrow derived macrophages (BMDM).
(B) Flow analysis on phosphorylated Stat5a/b in L929 BMDM.
(C) The mRNA expression of Canonical and Non-Canonical Wnt ligands in BMDM treated with or without Epo.
(D) ELISA assay of PGE\textsubscript{2} concentration in Stat5a/b\textsuperscript{fx/fx}, Stat5a/b\textsuperscript{-/-} and C57BL/6 (control) BMDM culture with Epo treatment. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
Figure 4.5. Stat5a/b deficient microenvironment fails to initiate the differentiation.
(A-B) BFU-E colony assay of stress erythroid progenitors co-cultured with BMDM from Stat5a/b<sup>fx/fx</sup> and Stat5a/b<sup>−/−</sup> mice. (C) Flow analysis on stress erythroid progenitors co-cultured with BMDM from Stat5a/b<sup>fx/fx</sup> and Stat5a/b<sup>−/−</sup> mice. The data represents two independent experiments.
Figure 4.6. Epo represses Canonical Wnt signaling by recruiting Ezh2. (A) Immunoprecipitation assay on Ezh2 and Stat5a/b in BMDM. (B) Schematic diagram of Wnt 2b and Wnt 8a genes including the position of designed probes. (C) Chromatin Immunoprecipitation assay of H3K27Me3 in Wnt2b and Wnt8a genes. Each treatment was done in triplicate and this experiment is representative of two independent experiments.
Figure 4.7. Mechanism of Epo/macrophages-dependent regulation in expansion and differentiation of stress erythroid progenitors. (Top) In the absence of Epo, macrophages produce high Canonical Wnt expression and low PGE$_2$ concentration to promote the self-renewal of the early stress erythroid progenitors identified as CD34$^+$ CD133$^+$ KS cells. (Bottom) Epo activates Stat5a/b pathway in macrophages to turn off Canonical Wnt signal and increase PGE$_2$ concentration, which together initiate the differentiation.
Table 1. Probes used in qRT-PCR

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Table 2. Primers and probes used in CHIP assay.

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Chapter 5

Future Studies And Conclusion

The characterization of stress erythroid progenitors and the discovery of Epo and macrophages-dependent differentiation provided us a better model for studying acute anemia. The findings in previous chapters explored new signals that are required in stress erythropoiesis as well as the interaction between progenitors and microenvironment. However, there are still some questions that we need to figure out based on these results. Studies mentioned below will help us better understand the mechanism in stress erythropoiesis.

Future Studies

*Identification of the role of hypoxia in the regulation of differentiation*

In Chapter 3, hypoxia was identified as potentiating the Epo-dependent differentiation of stress erythroid progenitors. Hypoxia alters cellular responses in many ways [1-3]. The chief one among these responses is the regulation of gene transcription by the Hypoxia Inducible Factor (HIF)[4-6]. In addition, there is also a HIF-independent mechanism of altering gene transcription that regulated by unfolded protein response. This pathway is regulated by the sensor kinase PERK, which leads to the expression of ATF4, a Activating Transcription Factor/Cyclic AMP Response
Element Binding (ATF/CREB) family member which consists of basic-region leucine zipper transcription factors that bind to the consensus cAMP responsive element (CRE) binding site[7]. ATF4 can be induced by anoxia/hypoxia, ER stress and oxidative stress. ATF4 is regulated at the translation level by the PERK pathway, and post-translationally by phosphorylation (Figure 5-1)[8]. ATF4−/− mice display severe fetal anemia and a decrease in size of progenitor colonies in the fetal liver. Interestingly, adult ATF4−/− mice have only slightly impaired erythropoiesis[9]. Previous data from our lab also showed that ATF4−/− mice have a defective stress erythropoiesis in exhibiting a delayed recovery after bone marrow transplantation. The complete blood cell count assay shows that the hematocrit value for ATF4−/− mice is significantly lower than wildtype (Figure 5-2, work done by Lauren A. Aldinger), but other lineages are not significantly affected (Figure 5-3, work done by Lauren A. Aldinger). In addition, during the recovery from acute anemia induced by PHZ, the phosphorylation of eIF2α and ATF4 protein expression coincide with the expression of BMP4. These data point out the role of PERK/eIF2α/ATF4 pathway in stress erythropoiesis (Figure 5-4 work done by Omid F. Harandi).

Based on those results, we need to identify how this pathway affects differentiation. First of all, we need to measure cellular hypoxia during recovery from bone marrow transplantation by injecting pimonidazole hydrochloride into mice on time points post-transplantation. This compound forms adducts in hypoxic cells and these adducts can be visualized by antibody staining. Then we need to identify whether the phosphorylation of eIF2α and ATF4 expression are induced in stress erythroid
progenitors under hypoxic condition. PERK inhibitors also can be used to test the differentiation of stress erythroid progenitors both in culture and in vivo by flow analysis and BFU-E colony assay. At last, we need to test this hypothesis in PERK−/− mice. These experiments will tell us the role of HIF-independent pathway in regulating differentiation in stress erythropoiesis.

Regulation of EpoR expression

Data in chapter 4 showed that early stress erythroid progenitors, CD34+CD133+Kit+Sca1+ population, are EpoRlow/− but later stage cells, CD34−CD133−Kit+Sca1+ population are EpoRhigh. It is obvious that the expression of EpoR is highly induced during the differentiation. However, the mechanism of how EpoR is regulated is still unknown. Previous data suggested that Wnt, GDF15, Hedgehog and BMP4 are involved in the expansion of stress erythroid progenitors. Hypoxia and Epo turn down the Canonical Wnt signal and turn up PGE2 to promote the differentiation. Based on this model, we propose that Wnt, HH, GDF15 and BMP4 may repress EpoR expression. Once the differentiation starts, these signals are turned off and EpoR is induced. To test this hypothesis, we can use CHIP-seq assay on CD34+CD133+Kit+Sca1+ and CD34−CD133−Kit+Sca1+ populations to figure out the transcriptional regulation of EpoR.
Regulation of BCL11A by Hedgehog

In Chapter 3, the human bone marrow data showed that stress erythropoiesis induces fetal hemoglobin expression by repressing BCL11A-L/XL form. When each factor was taken out from the culture, we found that hedgehog is the signal that regulates BCL11A expression. Data in Chapter 3 also indicated that hypoxia may regulate the translation of fetal hemoglobin protein. Thus we need to identify how hypoxia and hedgehog play a role here. Previous work showed that ATF4 and Nrf1/2 form complexes, which bind to globin LCR and the gamma promoter[9-11]. Nrf1 KO mice have fetal anemia but not adult anemia[12]. Based on this, we have a hypothesis that during stress erythropoiesis, PERK dependent signaling activates the integrate stress response, which leads to translation of ATF4 protein and phosphorylation of NRF1/2. These events lead to increase in gamma globin expression and expression of genes required for rapid production of hemoglobin. Stress erythroid progenitors are predisposed to express globin because Hedgehog signaling inhibits the expression of BCL11A.

Identification of the signals that induce Canonical Wnt expression

In Chapter 4 we found that Canonical Wnt expression is highly induced during the expansion stage and turned down by Epo in the differentiation stage in the spleen after bone marrow transplantation. However, signals that upregulate Canonical Wnt expression are still unknown. It is highly possible that BMP4 and GDF15 induce
Canonical Wnt pathway. To test this hypothesis, we can use BMP4−/− mice or GDF15−/− mice to conduct bone marrow transplantation assay and examine Canonical Wnt expression in the pre-recovery stage. Alternatively, we can test the effect of BMP4 and GDF15 in our L929 macrophages culture system. These studies will provide us a connection among signals that we identified in stress erythropoiesis.

Role of Dexamethasone in stress erythropoiesis

Dexamethasone is a potent synthetic member of the glucocorticoid class. It has been shown that the glucocorticoid receptor is required for stress erythropoiesis[13]. It cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro[14, 15]. However, the role of glucocorticoid in stress erythropoiesis has not been well studied. We can treat the bone marrow cells with dexamethasone in our stress erythropoiesis culture system and check the expansion and differentiation of distinct populations by flow and BFU-E colony assay. To identify when glucocorticoids are upregulated in vivo, we can use bone marrow transplantation assay to check time course for the concentration of glucocorticoids in the serum of recipients. These studies will tell us when and how glucocorticoids regulate stress erythropoiesis.


Conclusion

Stress erythropoiesis has been identified as a BMP-4 signaling-dependent process. The regulation of BMP4 is important for us to understand the mechanism in stress erythropoiesis. Previously our lab showed that hypoxia induces BMP4 expression through HIF2α. In chapter 2, we found that GDF15 also plays an important role in stress erythropoiesis and it can elevate BMP4 expression through HIF pathway by repressing VHL. The bone marrow transplantation data further showed that GDF15 is upregulated in both expansion and differentiation stages, which confirmed the function of GDF15 in stress erythropoiesis.

The discovery of stress erythroid progenitors in chapter 3 and the interaction between microenvironment and progenitors in chapter 4 provide us a new model for stress erythropoiesis (Figure 5-5). In stage 1, CD34+KSL population migrates into spleen in response to acute anemia, and then Hedgehog and BMP4 specified the stress erythroid fate. In stage 2, CD34+CD133+KS cells proliferate quickly in the spleen in response to signals in both progenitors and microenvironment, such as Hedgehog, GDF15, BMP4 and Wnt signaling. In stage 3, Epo is induced by tissue hypoxia and it represses Wnt signaling and induces PGE2 concentration through macrophages. At this time, a decrease of BMP4 and GDF15 expression is also observed. All of these together trigger the progress from CD34+CD133+KS to CD34-CD133-KS population and initiate the differentiation to stress BFU-Es. In last stage, hypoxia, SCF and BMP4 together promote the expansion and differentiation of BFU-Es. Finally, the large numbers of erythrocytes compensate the blood loss and rescue the acute anemia.
The research presented in this thesis provides a satisfying continuation of previous study. Hopefully, it will contribute new paths for further researchers to explore and refine.
References


Figures

Figure 5.1. Activation cascade of Aft4. (Ameri K, Harris AL. Activating transcription factor 4. Int J Biochem Cell Biol. 2008;40:14-21.)
Figure 5.2. *Aft4*<sup>−/−</sup> donor cells exhibit a defect in erythroid short-term radioprotection. (A) Survival curve for recipients transplanted with Aft4<sup>−/−</sup> or Wt donor cells. (B) Hematocrit values and Reticulocyte recovery of mice transplanted with 5x10<sup>5</sup> cells. *p<0.05, **p<0.01, ***p<0.005.
Figure 5.3. Atf4\textsuperscript{-/-} defect in short-term radioprotection is erythroid specific. 5x10\textsuperscript{5} Atf4\textsuperscript{-/-} donor cells were transplanted into lethally irradiated control recipients. On the indicated days, complete blood cell counts were done. Overall, the recovery of platelets (A), white blood cells (B) and neutrophils (C) is about the same. *p<0.05, **p<0.01, ***p<0.005
Figure 5.4. ATF4 protein expression is up-regulated during the recovery from acute anemia. (A) Analysis of eIF2a phosphorylation during the recovery from PHZ induced acute anemia. (B) Western blot analysis of ATF4 expression in the spleen during recovery from PHZ induced acute anemia.
Figure 5.5. Model of stress erythropoiesis in erythroid short-term radioprotection during the recovery from bone marrow transplantation. Details are in the text. HH, hedgehog; PGE2, prostaglandin E2; SCF, stem cell factor. RBC, red blood cell.
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PUBLICATIONS AND MANUSCRIPTS

1. **Jie Xiang**, Dai-chens Wu, Shailaja Hegde, Jeffrey L. Miller and Robert F. Paulson. Growth and Differentiation Factor 15 (GDF15) regulates stress erythropoiesis by inducing hypoxia dependent BMP4 expression. **(2014)** (manuscript in preparation);
2. **Jie Xiang**, Dai-chens Wu and Robert F.Paulson. In vitro expansion of stress erythroid progenitors identifies distinct progenitor populations and analogous human stress erythroid progenitors. (under revision for Blood Journal);
3. **Jie Xiang** and Robert Paulson. Analysis of signals regulating stress erythropoiesis: New roles for Epo, Wnt and PGE2 signaling in Stress erythropoiesis. (manuscript in preparation);
5. Ling-bing Sun, Yan Zhang, Qiang Wang, Hao Zhang, Wei Xu, **Jie Xiang**, Chen-yu Zhang et al. Serum palmitic acid-oleic acid ratio and the risk of coronary artery disease: a case-control study. **Journal of Nutritional Biochemistry. 2011. 311-317**;

CONFERENCES AND PRESENTATIONS

1. **2013, Graduate Exhibition at Penn State University.** Poster: **Jie Xiang** and Robert F. Paulson. New Roles for Epo, Wnt and PGE2 Signaling in Stress Erythropoiesis;
2. **2013, July 7-12** (Andover, NH), **Gordon Conference, Red Cell Meeting.** Poster: **Jie Xiang** and Robert F. Paulson. New Roles for Epo, Wnt and PGE2 Signaling in Stress Erythropoiesis;
3. **2011, Oct 17** (Rockville, MD), **Stress Erythropoiesis Workshop;**
4. **2011, July 24-29** (Andover, NH), **Gordon Conference, Red Cell Meeting.** Poster: **Jie Xiang** and Robert F. Paulson. GDF15 augments hypoxia dependent activation of the BMP4 expression during the recovery from acute anemia;