THE ROLE OF SF-STK IN STRESS ERYTHROPOIESIS

A Dissertation in
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by
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ABSTRACT

Erythropoiesis refers to the differentiation from LT-HSC through CMP, BFU-E and CFU-E to mature erythrocytes. Homeostatic erythropoiesis occurs in bone marrow in a slow and steady rate to replace old red blood cells. Stress erythropoiesis, on the other hand, occurs in adult liver and spleen to produce large quantity of red blood cell in a short period of time in response to acute anemia. It relies on high Epo concentration and utilizes unique erythroid progenitors other than homeostatic erythropoiesis. Except for Epo, BMP4, SCF and Shh are also required in stress erythropoiesis. The results in our lab in the past decades suggest that erythroid progenitors in bone marrow migrate to spleen where they respond to several factors including GDF15, Shh and SCF and differentiate into BMP4 responsive cells. BMP4⁺ give rise to stress BFU-E when BMP4 is present. Stress BFU-Es respond to high concentration of Epo and give rise to large quantity of erythrocytes in a short period of time.

Friend virus is another good model to study stress erythropoiesis. Friend virus induces erythroleukemia in Friend virus sensitive mice through a two stage progression. During the first step, Friend virus induces polyclonal expansion of infected erythroid progenitors in the absence of Epo. In the second stage, a proviral insertion activates Pu.1 gene which keeps infected erythroid progenitors in undifferentiating stage and leads to erythroleukemia. Only one viral protein – gp55, is required to induce this Epo independent polyclonal expansion. Recent research suggested that gp55 interact with a truncate form of stem-cell tyrosine kinase (Sf-Stk) on the cell surface to promote the proliferation of infected erythroid progenitors. Since the similarity between Friend virus induced polyclonal expansion of erythroid progenitors and stress erythropoiesis, it has
been proposed that Friend virus utilize the stress erythropoiesis pathway to infect target cells. Further experiments from our lab demonstrate that hypoxia and BMP4 can increase Friend virus target cells in spleen which are also stress erythroid progenitors. f/f mutant mice which have a delay recovery from acute anemia are defective in Friend virus targets. Since the important role Sf-Stk in Friend virus induced polyclonal expansion, we hypothesize that Sf-Stk may play an intrinsic role in stress erythropoiesis.

In the second chapter, we demonstrated that stress erythropoiesis is induced in adult liver during tissue hypoxia in the absence of spleen. Splenectomized f/f mice exhibit similar defects in stress erythropoiesis as f/f mice. BMP4 is expressed in liver corresponding to the expansion of stress BFU-E. Furthermore the splenectomized f/f mice are defective in liver erythropoiesis as well which correspond to the defect in BMP4 expression which indicates a role of BMP4 pathway in liver stress erythropoiesis as well. We concluded that BMP4 signal pathway plays a role in extramedullary erythropoiesis, which primarily occurs in spleen and fetal liver or adult liver when spleen is absent.

In the third chapter, we tested the expansion and differentiation of erythroid progenitors in the Sf-Stk deficient mice. The data reveal that Sf-Stk-/- mice have fewer stress BFU-Es as well as CFU-Es in spleen. The mutant stress BFU-Es have less potential to expand into erythrocytes. We also observed greater mortality rate in Sf-Stk-/- mice during the recovery from bone marrow transplantation which indicate that the expansion of erythroid progenitors in bone marrow is also affected by Sf-Stk deficiency. Further studies on bone marrow cells reveal that the Sf-Stk deficient bone marrow cells have an early expansion of stress BFU-Es during in vitro culturing than control mice which corresponds with the observation that mutant spleen cells have early terminal
differentiation during *in vitro* culturing. A study of the RBC life span indicates that there is an early expansion of RBCs in Sf-Stk-/- mice during the recovery from bone marrow transplantation. Those early emerged RBCs are defective and removed early during the recovery. This disruption of new RBC in Sf-Stk-/- mice during the recovery corresponds with the death of Sf-Stk-/- mice after bone marrow transplantation. Combining all those observations we propose that Sf-Stk may participate in promoting the proliferating and/or suppressing the differentiating of erythroid progenitors. When Sf-Stk expression is abrogated, stress erythroid progenitors fail to amplify and differentiate early which exhaust the pool of erythroid progenitors. The early expansion of erythroid progenitors generate defective new RBCs which were removed early leading to higher mortality rate during the recovery from bone marrow transplantation induced acute anemia.
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BFU-E</td>
<td>Burst Forming Unit - Erythroid</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone Morphogenetic Protein 4</td>
</tr>
<tr>
<td>BMP4R</td>
<td>BMP4 responsive cells</td>
</tr>
<tr>
<td>Kit</td>
<td>Kit receptor tyrosine kinase</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony Forming Unit - Erythroid</td>
</tr>
<tr>
<td>CHO cell</td>
<td>Chinese hamster ovary cell</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove's Modified Dulbecco's Medium</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EpoR</td>
<td>Erythropoietin Receptor</td>
</tr>
<tr>
<td>f/f</td>
<td>Flexed-tail</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>Friend Murine leukemia Virus</td>
</tr>
<tr>
<td>FV</td>
<td>Friend Virus</td>
</tr>
<tr>
<td>FVP</td>
<td>Polycythemia – inducing Fried Virus</td>
</tr>
<tr>
<td>FVA</td>
<td>Anemia – inducing Friend Virus</td>
</tr>
<tr>
<td>GATA-1</td>
<td>GATA-binding factor 1</td>
</tr>
<tr>
<td>GDF15</td>
<td>Growth differentiation factor 15</td>
</tr>
<tr>
<td>gp55</td>
<td>FVP envelop protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IL3</td>
<td>Interleukin 3</td>
</tr>
<tr>
<td>MEP</td>
<td>Megakaryocyte Erythroid Progenitor</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotential progenitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHZ</td>
<td>Phenylhydrazine</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
</tr>
<tr>
<td>SFFV</td>
<td>Spleen Focus Forming Virus</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>Spi-1</td>
<td>SFFV proviral integration 1</td>
</tr>
<tr>
<td>Sf-Stk</td>
<td>Short-form Stk</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>Stk</td>
<td>Stem cell-derived tyrosine kinase</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short term repopulating HSC</td>
</tr>
<tr>
<td>μl</td>
<td>Micro liter</td>
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</table>
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Chapter 1 Introduction

1.1 Hematopoiesis
Hematopoiesis is the process of blood cell development. The peripheral blood contains a wide variety of cell types which carry out different functions such as transporting oxygen, and immunological response. The process of hematopoiesis maintains the different types of blood cells by generating new cells at a steady rate on a daily basis or in response to stress conditions such as acute anemia.

All blood cells are continuously derived from hematopoietic stem cells (HSC’s) which are a pool of self renewing, multipotential progenitors that reside in bone marrow [1-4]. The division of HSCs gives rise to an identical daughter HSCs which will maintain HSC population, a process referred to as self renewal or multipotential progenitors which will further differentiate into multiple mature blood cells but lack self renewal ability. Based on the self renewing ability, HSCs can be classified as long term repopulating HSC (LT-HSC) which can replicate indefinitely, and short term repopulating HSC (ST-HSC) which can only replicate for 8 weeks [3].

ST-HSCs give rise to multipotential progenitors (MPP) which will differentiate into lineage restricted progenitors – common lymphoid progenitor (CLP) and common myeloid progenitors (CMP) through a series of functionally irreversible events [5-6]. CLP and CMP both have a determined fate during differentiation. CLPs will give rise to T lymphocytes, B lymphocytes and Natural killer cells (NK cells)[7]. While CMPs give rise to myeloid progenitors including granulocyte monocyte progenitor (GMP) which will give rise to macrophages and neutrophils, and megakaryocyte erythroid progenitors (MEP) which will give rise to megakaryocytes and erythrocytes[8]. Both CMP and CLP
can give rise to dendritic cells\[9\]. All those early stage progenitors – CMP, CLP, MEP and GMP were first identified by cell surface marker followed by \textit{in vitro} colony assays\[10\] (Fig 1.1). LT-HSCs differentiate into CMP which will give rise to MEP. Both CMP and MEP are identified by cell surface marker followed by functional assay. The CMP is characterized as Lin\(^-\)Sca1\(^-\)Kit\(^+\)IL-7Ra\(^-\)CD34\(^+\)FcgR\(^{low}\), while MEP is characterized as Lin\(^-\)Sca1\(^-\)Kit\(^+\)IL-7Ra\(^-\)CD34\(^+\)FcgR\(^{low}\) (Fig 1.2).

\section*{1.2 Erythropoiesis}

Erythropoiesis is a multistep differentiation process from MEP to enucleated red blood cells. It is part of hematopoiesis with unidirectional pathway of maturation. Burst forming units – erythroid (BFU-E) and colony forming unit – erythroid (CFU-E) are intermediate forms of proerythroblasts. They are identified by \textit{in vitro} functional assay called BFU-E assay and CFU-E assay. In culturing media with two growth factors – Epo and the burst promoting factor, it takes 8 days for BFU-E to differentiate into mature RBCs\[11\]. BFU-Es produce “burst” form of colonies in methylcellulose media. They have low proliferate activities\[12\]. The BFU-Es further differentiate into CFU-E which can differentiate into mature RBCs during \textit{in vitro} culturing in 2 days. The development from CFU-E to RBC can be measured by \textit{in vitro} functional assay called CFU-E assay. CFU-Es will produce colonies of 8-32 cells in methylcellulose media with Epo alone \[11\]. Past CFU-E stage, cells will go through a series of morphological change, where cells lose their nucleus and become a mature erythrocyte (Fig 1.2).

Multiple factors are involved in determining lineage commitments and maturation of erythrocyte. Most of the genes affecting cell fate determination during erythropoiesis are transcription factors such as SCL \[13-15\], Lmo2 \[16-17\], GATA 1 and GATA2 \[18\].
Some other factors are found to play a role in either affecting differentiation or proliferation of erythroid progenitors. Erythropoietin (Epo) and its receptor (EpoR) is the core signal pathway for erythropoiesis. It participates in both proliferation and differentiation during erythropoiesis [19-20]. Stem cell factor (SCF) and its receptor Kit receptor tyrosine kinase (Kit) are also found important in erythropoiesis[21]. Mice deficient in SCF signal pathway have anemia because of reduced erythropoiesis activity, indicating that SCF may involved in promoting the proliferation of erythroid progenitors[22]. Other factors involved in erythropoiesis are Interleukin 3 (IL-3), Granulocyte/Monocyte-colony stimulating factor (GSM-CSF) and Insulin-like growth factor 1 (IGF-1). In vitro, Epo with IL-3 are sufficient to promote the formation of erythroid colonies.

1.3 Stress erythropoiesis: flexed-tail mouse model and BMP4 signal pathway
There are two kinds of erythropoiesis that exist in organisms – steady state or homeostatic erythropoiesis and stress erythropoiesis. Steady state erythropoiesis maintains the pool of circulating RBCs. It produces mature red blood cells at a constant rate to replaces old erythrocytes that are removed from circulation. It relies on local source of Epo and occurs in the adult bone marrow. On the other hand, stress erythropoiesis produces large numbers of red blood cells in a short period time during some stress conditions such as blood loss, hemolysis, tissue hypoxia etc. It relies on high Epo concentration and mainly occurs in fetal liver, adult spleen and occasionally adult liver.

Tissue hypoxia which induces stress erythropoiesis can be caused by many situations such as high altitude, massive blood loss or increase oxygen demand. In the laboratory
environment, tissue hypoxia can be induced \textit{in vivo} by administration of Phenylhydrazine (PHZ) or bone marrow transplantation or \textit{in vitro} by simply culturing cells in 2\% oxygen. Stress erythropoiesis which mainly occurs in adult spleen requires high Epo concentration which is produced by kidney. Previously it was proposed that during stress erythropoiesis the BFU-E are migrated from bone marrow and reside in spleen to give rise to terminal differentiated erythroid cells[23]. The works in our lab demonstrate that stress erythropoiesis utilizes erythroid progenitors that differentiate through a different pathway comparing to normal steady state erythropoiesis. In addition the stress erythropoiesis progenitors respond to unique growth factors produced during the recovery from acute anemia. Previous work in our lab showed that bone morphogenetic protein 4 (BMP4) is induced during the recovery from PHZ induced tissue hypoxia. This factor acts on an intermediate erythroid progenitor called BMP4 responsive (BMP4R) cell to turn it into stress BFU-E [24]. Stress BFU-E can response to high level of Epo and differentiate into mature RBCs faster than stead state BFU-E. Epo is still required in stress erythropoiesis. Other factors such as SCF and hypoxia also play an important role in stress erythropoiesis [25-27].

The role of BMP4 pathway in stress erythropoiesis was discovered by the analysis of \textit{flexed-tail (ff)} mouse. \textit{flexed-tail} is a recessive mutation found in 1928[28]. Fetal \textit{ff} mutant mice are anemic and severely compromised in erythropoiesis. They exhibit a transitory, hypochromic, microcytic anemia [29-30], which resolves two weeks after birth. Adult \textit{ff} mice have normal erythropoiesis and are able to produce normal BFU-E and CFU-E colonies \textit{in vitro} [28]. The reticulocyte counts, hemoglobin content are also normal in adult flexed tail homozygous mouse. The abnormal phenotypes only occur
during fetal development in response to an increased demand for excess red blood cell
production [31]. It is already known that an early multipotential colony forming unit –
spleen (CFU-S) gives rise to BFU-E which will differentiate into CFU-E in the spleen
during stress erythropoiesis. In 1975, Gregory et al found an intermediate erythroid
progenitor named TE-CFU between CFU-S and CFU-E. This progenitor has the potential
to develop into a excessive number of erythroid progeny in about 5 days when
continuously exposed to Epo in vivo[32]. Although adult f/f mice have normal BFU-E
and CFU-E, they are severely defective in TE-CFU. Adult f/f mice can’t rapidly increase
TE-CFU in vivo under tissue hypoxia and exposed to high level of Epo. Because of this
defect f/f mutant mice can’t respond rapidly to tissue hypoxia and exhibit a delay
expansion of erythroid progenitor and a delay in the appearance of reticulocyte after PHZ
induced acute anemia [33]. Despite this defect f/f mice don’t have defect in other
hematopoietic lineages [34], which makes it an excellent model to study stress
erythropoiesis. And the characters of TE-CFU are good indications that stress
erythropoiesis may go through a different differentiation pathway than normal
erythropoiesis.

By using this model system, our laboratory has demonstrated that f/f mice have a delay in
expansion of BFU-E in spleen after PHZ treatment (4 days post PHZ injection in an f/f
mouse versus 36 hours post PHZ injection in a C57BL/6 control mouse) which causes the
delayed recovery from PHZ induced acute anemia. We also determined that f/f locus
contains a dominant negative Smad5 gene, which is a downstream factor of BMP4 signal
pathway. BMP4 is induced in the spleen by tissue hypoxia and act on an earlier cells
referred to as BMP4 responsive cell (BMP4R). SCF/Kit signaling pathway and hypoxia
are also required in stress erythropoiesis. Previous experiments in our laboratory demonstrate that during tissue hypoxia BMP4 and SCF act on the same cells in spleen, but play distinct roles in response to acute anemia. BMP4 activates erythroid progenitors to respond to Epo, while SCF promote proliferation of erythroid progenitor. BMP4\(^R\) gives rise to stress BFU-E in response to BMP4 signal. Hedgehog signaling is required in the spleen to promote the expansion of BMP4\(^R\) [35]. It also induces the expression of BMP4 in the erythroid progenitors that migrate from bone marrow to spleen. Our work has shown that stress BFU-E exhibit properties distinct from bone marrow steady state BU-E in that they are capable of expanding into red blood cells in a shorter period of time and require only Epo for differentiation [24]. Data from our laboratory suggest that short term reconstitution HSCs (STR-HSC) (CD34\(^+\)Kit\(^+\)Sca1\(^+\)Lin\(^-\)) give rise to stress BFU-Es [36]. We observed a Kit\(^+\)CD71\(^+\)Ter119\(^+\) population in spleen in acute anemia. BMP4, SCF and hypoxia promote the expansion of this population. A careful flow cytometry analysis reveals three distinctive populations with distinct staining properties. We termed them population I, population II and population II cells. Population I cells were Kit\(^+\)CD71\(^{lo/med}\)Ter119\(^{lo/-}\), Population II cells were Kit\(^+\)CD71\(^{hi}\)Ter119\(^{med}\), and Population III cells were Kit\(^+\)CD71\(^{lo/med}\)Ter119\(^{hi}\). Based on their emergence during the recovery of bone marrow transplantation induced acute anemia, population I cells give rise to population II cells, population II cells differentiate into population III. Population I cells are self-renew and provide short-term radioprotection. When serially transplanted to irradiated recipient, population I cells expanded for eight days then differentiated. Further functional assay indicates that population I cells are a mixture of BFU-Es and CFU-Es; population II cells contain CFU-Es [36].
1.4 Friend Virus infection: a model to study erythropoietic signaling pathway

In 1957, a virus inducing acute hyperplasia specific in erythroid lineage was first described by Charlotte Friend[37]. During the first 4-6 weeks of viral infection, erythroid progenitors have rapid expansion but can’t be transplanted, suggesting those progenitors are not tumor cell yet, but erythropoiesis expansion has been turned on. Later on, a small portion of those progenitors can induce leukemia in the irradiated secondary recipients[38] suggesting those erythroid progenitors became leukemia cells[39-40]. Two Friend virus strains have been isolated so far based on different phenotypes – Friend virus anemia (FVA) and Friend virus polycythemia (FVP). FVA induces anemia with splenomegaly[37]. However FVP induces polycythemia due to the terminal differentiation of infected erythroid progenitors[41]. Both strains induce Epo independent expansion of BFU-E and CFU-E. FVA infected progenitors required Epo for terminal differentiation [42-44]. While Epo is negligible in the terminal differentiation of FVP infected erythroid progenitors [45-48].

Friend virus is a complex of 2 viruses, a replication competent Friend helper virus (F-MuLV) and a replication defective spleen focus forming virus (SFFV) [49-51]. SFFV encodes a 55kD glycoprotein named gp55 which is the envelop protein of the virus [52-53]. This protein was found to play an important role in viral infection and Friend virus induced expansion of erythroid progenitors. The most important differences in genome sequence between FVP and FVA are located at 3’ of the envelop protein gene. Several amino acid changes have been identified at the C-terminal of envelop protein comparing between SFFV-P and SFFV-A. In SFFV-P, this envelop gene is modified after translation to gp65 which didn’t occur in SFFV-A. SFFV itself with multiple injections of virus stock can induce the expansion of erythroid progenitor as well as leukemia in the later
stage at a lower efficiency [54-55]. The changes in gp55 sequence determine its ability to interact with EpoR which is the key for the phenotype of Friend virus infection. Thus those modifications are probably the reason why FVP induces polycythemia while FVA induces anemia.

### 1.4.1 Pathogenesis for Friend virus induced leukemia

Friend virus infection induces erythroleukemia in Friend virus sensitive mice through a characteristic two stage progression. At the first stage, Friend virus infection causes rapid proliferation of erythroid progenitors in bone marrow. One population of proliferating progenitors undergoes erythroid differentiation. Depending on the strain of virus, FVA or FVP, the viral infection will cause anemia for FVA or polycythemia for FVP. However the anemia caused by FVA infection is not a true anemia but rather, it is a failed terminal differentiation due to the requirement for high Epo concentration which is negligible in FVP infection. Data from our laboratory suggest that a second population of infected erythroblasts migrate to spleen and form infectious centers, which propagate the infection in the spleen, resulting in splenomegaly [56]. During this early stage the expanded erythroblasts are not cancer cells because transferring these cells to another mouse doesn’t result in leukemia. In the second stage, it has been proposed that new mutations are acquired by proviral integration into preferred sites in the genome such as Spi-I[57] and Fli-I[58] and activation these genes. In addition, a mutation of \( p53 \) is observed leading to the loss of \( p53 \) function and development of leukemia [59-61]. Infection of mice with the polycythemia inducing strain of Friend virus, FVP, leads to a rapid rise in red cell counts caused by the Epo independent terminal differentiation of infected cells. Studies by different groups have shown that FVP envelop protein gp55 cooperates with host proteins controlling the induction of erythroleukemia.
1.4.2 Host loci for Friend virus infection
Several host loci have been identified to affect the susceptibility of cells to Friend virus infection. Based on their proposed mechanisms during Friend virus infection, those genes can be categorized into several groups (Table 1).

Group I are genes involved in the retroviral life cycle including *Friend virus susceptibility gene 1* (*Fv1*) and *Fv4*. *Fv1* acts on retrovirus after the entry into target cells but before the integration and formation of provirus [62-63]. *Fv4* encodes a protein that is associated with the envelop protein of F-MuLV which blocks the retroviral receptors in the target cells [64]. Group 2 are genes that affect the immune response to Friend virus such as *Fv3*, *Rfv1*, *Rfv2* and *Rfv3*. *Fv3* affects susceptibility of mice to Friend virus-induced immunosuppression [65]. Group 3 contains genes that regulate the proliferation and differentiation of infected cells. For example *Fv5* does not affect the early stage of erythropoiesis however it regulates terminal differentiation of Friend virus infected cells. Different *Fv5* alleles cause polycythemia response or anemic response to FVP infection [66]. *Dominate white spotting* (*W*) locus and *Steel* (*Sl*) locus encodes Kit receptor tyrosine kinase (Kit) and its ligand Stem cell receptor (SCF) respectively. Previous work demonstrated that Kit and SCF are required for steady erythropoiesis [67]. Recent work in our laboratory demonstrates that Kit mutant mice (*W/W* mice) lack Friend virus target cells in the spleen [68]. This work also demonstrated that the Friend virus target cells in spleen are the BMP4R cells. This observation may indicate a connection between Friend virus and stress erythropoiesis.
<table>
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<tr>
<th>Group 1: interference with retroviral infection</th>
<th>Gene</th>
<th>Function</th>
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<tbody>
<tr>
<td>Fv4</td>
<td>Blocks retroviral cell surface receptors</td>
<td></td>
</tr>
<tr>
<td>Fv1</td>
<td>Interferes with retroviral life cycle</td>
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<th>Group 2: altered immune response</th>
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<tr>
<td>Fv3</td>
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<tr>
<td>Rfv1, Rfv2</td>
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<td>Rfv3</td>
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<th>Group 3: regulators of erythroid cell proliferation and differentiation</th>
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<tr>
<td>Fv5</td>
</tr>
<tr>
<td>Kit, SCF</td>
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<tr>
<td>Fv2</td>
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<td>Flexed-tail</td>
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\textit{f} locus encodes \textit{Madh5 (Smad5)} which is a receptor activated Smad that acts downstream of BMP2, BMP4 and BMP7 [70]. Our work has shown that BMP4 induces BMP4\textsuperscript{R} cells in spleen to differentiate to stress BFU-E during the recovery from acute anemia. Work done by Aparna Subramanian in our laboratory also demonstrated that \textit{f/f} mice are resistant to Friend virus infection because Friend virus activates the BMP4 dependent stress erythropoiesis pathway to amplify target cells in the spleen. The defect in \textit{f/f} mice
in the BMP4 response blocks this expansion of target cells leading to resistance to the development of leukemia (unpublished data).

The Friend virus susceptibility gene 2, \textit{Fv2}, plays a key role in regulating the proliferation of Friend virus infected cells. \textit{Fv2} doesn’t affect the retroviral entry or the viral life cycle [71]. Instead \textit{Fv2} affects the proliferation of SFFV-infected erythroid progenitors [72] in a cell autonomous way [73]. \textit{Fv2} encodes a naturally occurring truncated form of the Met-related Macrophage stimulating 1-Receptor (Mst1r), also known as Stem cell-derived tyrosine kinase (Stk) referred to hereafter as short-form Stk (Sf-Stk). \textit{Fv2} locus is dominant for the susceptibility of Friend virus. Mice sensitive to Friend virus infection (\textit{Fv2}\textsuperscript{ss} and \textit{Fv2}\textsuperscript{sr}) express Sf-Stk. However mice homozygous for \textit{Fv2}\textsuperscript{rr}, also known as Friend virus resistant mouse such as C57BL/6, lack the expression of Sf-Stk [74]. Those mice fail to exhibit massive expansion of erythroid progenitor and progression to leukemia upon Friend virus infection. The Sf-Stk promoter is located in 10\textsuperscript{th} intron of Stk gene. The start codon of Sf-Stk gene is located at 11\textsuperscript{th} exon. The \textit{Fv2} resistant allele is caused by mutation in the Sf-Stk promoter. \textit{Fv2}\textsuperscript{rr} mice have a three nucleotide deletion in the promoter region which reduces Sf-Stk expression to a very low level [74]. Homologues of Stk as well as Sf-Stk have been found in human and chicken.

In human, the Stk and Sf-Stk homologues are called Ron and Sf-Ron respectively.

1.4.3 Interaction between EpoR, gp55 and Sf-Stk drive expansion and differentiation of infected cells

FVP envelop protein gp55 is the only viral protein required for inducing erythroleukemia. Gp55 induces BFU-E colony formation \textit{in vitro} in an Epo-independent manner [75].

Further studies reveal that gp55 activates EpoR and causes Epo-independent formation of red blood cells [76-77] by binding to and dimerizing EpoR through its transmembrane
domain [78-79] on the cell surface. A cell line study by Ruscetti 	extit{et al} in 1990 also showed that SFFV induces Epo independent growth in Epo-dependent cell lines [80]. In 1991, Casadevall and his colleagues demonstrated that the binding site for gp55 and Epo in EpoR are non-overlapping [81].

Mice that lack Sf-Stk expression are resistant to Friend virus infection and fail to develop erythroleukemia. However exogenous expression of Sf-Stk confers Friend virus sensitivity to erythroid progenitors from Friend virus resistant mice [82]. Gp55 covalently as well as noncovalently interacts with Sf-Stk \textit{in vitro}. This interaction causes constitutive tyrosine phosphorylation of Sf-Stk [83]. A constitutively active form of Sf-Stk induces Epo-independent colony formation in the absence of gp55. Coexpressing gp55 and Sf-Stk will prolong the half life of Sf-Stk in the cell line [84]. Recent experiment by our colleagues demonstrated that coexpressing gp55 and Sf-Stk in CHO cells localizes more Sf-Stk to the cell membrane [85]. This experiment suggests that gp55 can relocate Sf-Stk from cytoplasm to cell membrane where it can constitutively signal.

Based on all these experiments a possible model for gp55 induced Epo-independent formation of red blood cells is described below. Gp55 upregulates or stabilizes Sf-Stk and relocates it to the plasma membrane. On the cell membrane Sf-Stk interact with gp55 and EpoR to drive the proliferation and differentiation of infected cells.

1.4.4 Connection between Sf-Stk and stress erythropoiesis
The interaction among Sf-Stk, gp55 and EpoR are crucial for Friend virus induced Epo-independent erythroid progenitor expansion. Those expanded erythroid progenitors can terminal differentiate into red blood cells but they are not leukemia cells in the first stage of Friend virus infection. This phenotype is similar to stress erythropoiesis in the way that
erythroid progenitors rapidly expand and terminally differentiate leading to the production of large numbers of red blood cells in a short period of time. Our data indicate that Friend virus utilizes the BMP4-dependent stress erythropoiesis pathway to induce erythroleukemia. Given the important role of Sf-Stk in the pathogenesis of Friend virus induced leukemia, it is reasonable to hypothesize that Sf-Stk may play a key role in stress erythropoiesis. This thesis investigates the role of Sf-Stk in stress erythropoiesis.

It is already known that FVP induces Epo-independent colony formation in vitro. Using this as an assay, our former colleague Aparna Subramanian has determined that hypoxia increases Friend virus target cells in spleen which correlates with the upregulation of stress BFU-E colonies. BMP4 is upregulated during Friend virus infection and increases Friend virus target cells in spleen in vitro. Further experiments demonstrate that spleen stress progenitors are Friend virus target cells. Sf-Stk expression is found in these cells. BMP4^R cells differentiate into Epo responsive cells by BMP4 signal and rapidly expand into red blood cells in response to acute anemia stress. We hypothesize that Friend virus may mimic the stress erythropoiesis pathway to induce Epo-independent colony formation. Sf-Stk may be involved in stress erythropoiesis by facilitating the proliferation and differentiation of erythroid progenitors.
Figure 1-1: Development of different blood cells during hematopoiesis[86].

Courtesy of Lund stem cell center
Figure 1-2: Red blood cell development during erythropoiesis

CMPs differentiate into MEPs which will give rise to BFU-E and CFU-Es.

Progenitors defined by cell sorting followed by colony assays using multiple cytokines.

CMP: Lin-, Sca1-, Kit+, IL-7Rα-, CD34+, FcγRlow.

MEP: Lin-, Sca1-, Kit+, IL-7Rα-, CD34-, FcγRlow.

Progenitors defined by colony assay using small numbers of cytokines.

In bone marrow:
BFU-E: Epo + BPA
7 day culture period

CFU-E: Epo
2 day culture period

Proerythroblasts are defined by morphology following staining. They differentiate into mature erythrocytes through a series of morphologically distinct stages. This process requires Epo.

Reticulocytes are immature erythrocytes that still contain RNA and polyribosomes.
Figure 1-3: Sequence of the putative Sf-Stk promoter

The arrow marks the first nucleotide of the published Sf-Stk cDNA (ref. 34). Consensus transcription factor binding sites are shown by boxes. The black box shows 3 nt that are deleted from the DNA of C57BL/6 and related strains. Intron 10 is underlined. The first two amino acids of the predicted Sf-Stk protein are shown (M, T). (Persons DA, et al. 1999)
1.5 References


Chapter 2 Stress erythropoiesis in adult liver requires BMP4 signal pathway

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2.1 Abstract
In mice, homeostatic erythropoiesis happens in bone marrow. However stress erythropoiesis happens in spleen and fetal liver under the regulation of BMP4 pathway. In human erythropoiesis happens in bone marrow. However under certain pathological conditions erythropoiesis were observed in multiple organs including spleen and liver. Thus during stress conditions extramedullary erythropoiesis may be induced to help resolving tissue hypoxia in living organism. BMP4 signal pathway is important in regulating extramedullary erythropoiesis.

In this section we used a splenectomized wild type and splenectomized flexed-tail mice, which are defect in BMP4 signal pathway, to compare their recovery from tissue hypoxia induced by Phenylhydrazine treatment and the expansion of stress BFU-Es. Our experiment result indicates that stress erythropoiesis is induced in adult liver during tissue hypoxia in the absence of spleen. Splenectomized *f/f* mice exhibit similar defect in stress erythropoiesis as *f/f* mice. BMP4 is expressed in liver corresponding to the expansion of stress BFU-E. Furthermore the splenectomized *f/f* mice are defective in liver erythropoiesis as well which correspond to the defect in BMP4 expression which indicate a role of BMP4 pathway in liver stress erythropoiesis as well.
Thus we conclude that BMP4 signal pathway plays a role in extramedullary erythropoiesis, which primarily occurs in spleen and fetal liver or adult liver when spleen is absent.

### 2.2 Introduction

In adult mice, steady state erythropoiesis occurs in bone marrow. However during embryonic developmental or acute anemia, erythropoiesis will shift from bone marrow to fetal liver or adult spleen where stress erythropoiesis is induced to produce large amount of red blood cell in a short period of time. It has been long proposed that stress erythropoiesis in fetal liver and spleen may share similar pathway. In this section, our work in splenectomized $f/f$ mice support that hypothesis and BMP4 pathway regulates stress erythropoiesis occurred in both liver and spleen.

$f/f$ mutant mice exhibit a severe fetal-neonatal anemia which resolves three weeks after birth. Adult $f/f$ mice have normal erythropoiesis with normal erythrocyte counts [1-4]. In adult $f/f$ mice, their BFU-E and CFU-E counts as well as blood values are all comparable to wild type mice[5]. The situation is a lot different during stress conditions. In contrast to normal erythropoiesis, $f/f$ mice are severely defective in stress erythropoiesis which exhibit as a delayed recovery from both PHZ and bone marrow transplantation induced acute anemia [5-7]. This delayed recovery is caused by a delayed expansion of certain erythroid progenitor in spleen which we termed stress BFU-E [6]. Further studies show that the $f$ locus expresses a dominant negative Smad5 in erythroid progenitors, which is a transcription factor that functions downstream of receptors for Bone morphogenetic protein 2, 4 and 7 (BMP2, BMP4 and BMP7) [8-9]. BMP4 expression is upregulated in spleen prior the expansion of stress BFU-E. Except for BMP4, SCF and hypoxia are also
found to function in concert with Epo to regulate expansion of stress BFU-E in stress erythropoiesis [10]. All those data indicate that a unique signal pathway is activated to generate several unique intermediary erythroid progenitors during stress erythropoiesis. A tentative model for stress erythropoiesis in spleen during tissue hypoxia would be that a certain erythroid progenitor which we termed BMP4 responsive cell (BMP4$^R$) response to BMP4 in spleen to differentiate into stress BFU-E. Stress BFU-E can response to high level of Epo which work in concert with SCF, hypoxia and BMP4 to promote the rapid expansion of red blood cell. The work by Prashanth Porayette in our laboratory showed that the anemia observed during $f/f$ mice fetal development is caused by the defect in stress erythropoiesis in fetal liver [1]. BMP4 expression is observed at critical time point during embryonic development to produce red blood cells (Embryonic day 13.5-14.5) in accordance to expansion of stress BFU-E in fetal liver. The expansion of stress BFU-E in $f/f$ embryo is delayed which correspond to the delayed expression of BMP4 in fetal liver. Thus we could conclude that adult stress erythropoiesis and fetal liver erythropoiesis are regulated by similar signal pathways and share similar intermediary erythroid progenitors.

However, one big problem for that hypothesis is the lack of need for a spleen. Spleen is dispensable especially in human. There are little side effects for splenectomizing a patient except for an increased risk for sepsis [11]. Thus a question we would ask is that if spleen is the primary organ for stress erythropoiesis, why it is dispensable with so little side effects? One possible answer to this question is that extramedullary erythropoiesis can shift among multiple organs with suitable environment. Actually any organ with a stagnant sinusoidal vascular system could be an ideal place for erythropoiesis [12]. Different organisms may utilize different sinusoidal organs at different developmental
stages for erythropoiesis. Larger organisms such as human may use bone marrow as the primary location for erythropoiesis simply because the sinusoidal cavities of bone marrow. Some organism with small cavity within the bone such as fish, however, use spleen as the primary erythropoietic organ [13]. But since bone marrows are trapped in confines of bone, it has little space to expand which limit its role in stress erythropoiesis. Thus spleen, which can expand many times without rupture become a suitable place for stress erythropoiesis. Liver is another suitable place. Actually during embryonic development, mouse fetal liver is the primary place for erythropoiesis which exhibit characters much like adult stress erythropoiesis. Even though adult liver doesn’t carry much erythropoiesis activities, it can be easily reactivated if necessary [14]. In humans under certain pathological conditions, extramedullary erythropoiesis was observed in spleen, liver and other organs [15-23]. When mice were repeatedly treated with PHZ to induce tissue hypoxia, stress erythropoiesis was observed in liver as well [24-25].

In this chapter we investigated the BMP4 dependent stress erythropoiesis in splenectomized wild type mice and splenectomized f/f mice. We showed that stress erythropoiesis occurred in adult liver when spleen is absent. Splenectomized f/f mice exhibit a similar delay in recovery from PHZ treatment induced acute anemia as non-splenectomized f/f mice, which indicate that the stress erythropoiesis occurred in liver are also BMP4 dependent. Extramedullary erythropoiesis occurred in spleen and liver share some similar characters.
2.3 Material and method

2.3.1 Mice
C57BL/6 and C57BL/6 splenectomized mice were provided by Jackson Laboratory (Bar Harbor, ME). f/f mice were in C57BL/6 genomic background and bred in our mouse room located in the basement of Henning building. All the mice used are 6-10 weeks old. Wild type mice and f/f mice age are matched. Splenectomized f/f mice were generated by removing the spleen by surgery performed in our laboratory. C57BL/6-f/f mice were first anesthetized with Ketamine/Xylazine (80 mg/kg; 10mg/kg) by intraperitoneal (IP) injection. Then an incision was made in the skin and body muscles to expose spleen. After removing the spleen, the spleen vessel was immediately tied and the body wall incision was sutured and skin incision was closed with wound clips. The splenectomized f/f mice were allowed to recover for three weeks. Wound clops were removed one week after the surgery. Acute anemia was induced by PHZ (Sigma, St Louis, MO) treatment at a concentration of 100 mg/kg mouse in phosphate-buffered saline (PBS). All procedures were approved by the IACUC of the Pennsylvania State University.

2.3.2 Analysis of stress BFU-E
Liver were harvested at different time points post PHZ treatment. Single liver cells suspension was generated by smashing liver tissue and treat them with Type I collagenase (440ug/ml) (Worthington, Lakewood, NJ). Erythrocytes were lysed by cold 0.16M ammonium chloride. Mononuclear cells were separated from single cell suspension by using Nycoprep 1.077 step gradient (AXIS-SHIELD PoC AS, Oslo, Norway). Then those mononuclear cells are plated in methylcellulose media (StemCell Technologies, Vancouver, BC) in a 24 well plate containing either 3U/ml Epo + 10ng/ml IL3 or 3U/ml Epo itself (Sigma, St. Louis, MO). Cells were cultured at 37C for 5 days and then BFU-E
colonies were scored by acid benzindine staining [26]. For each mouse the BFU-E assay was done in triplicate.

2.3.3 Analysis of BMP4 expression in liver
Livers were harvested at different time points post PHZ treatment and fixed in 4% paraformaldehyde. Then the tissue was embedded in paraffin and cut into 0.5μ sections. When used for BMP4 staining, the sections were first warmed at 60°C for 20 minutes and deparaffinized in Histo-clear II (National Diagnostics, Atlanta, GA), rehydrated in an ethanol series (100% 2 times with 2 minutes each, 95%, 95%, 75% and H2O only with 1 minute each) and block endogenous peroxidase activity by incubating sections in 3% H2O2 solution in methanol for 10 minutes. Next the section was blocked with protein blocking reagent (Immunotech, Westbrook, ME) at room temperature for 20 minutes. Discarding the blocking reagent without washing, the sections were then incubated with primary anti-BMP4 antibody in a moist box for 1 hour. Finally after washing off the first antibody, the biotinylate conjugated secondary antibody was applied to the sections for 30 minutes in dark humid chamber. Streptavidin-HRP was then incubated with section for 30 minutes at room temperature and DAB substrate solution was added and incubated for 5 minutes after washing off Streptavidin-HRP. Sections were then washed in PBS and mounted in Slow Fade (Molecular Probes). HRP activity was detected by microscope.

2.4 Results

2.4.1 Splenectomized control mice recover from PHZ induced anemia with different kinetics
PHZ induces acute anemia by lysing red blood cells. The previous work in our lab showed that during the recovery from PHZ induced tissue hypoxia, special erythroid
progenitors, which we termed stress BFU-E, response to Epo, BMP4 and SCF and expand in spleen to generate massive red blood cells so that the hematocrit count will gradually become normal. f/f mouse is defect in the expansion of stress BFU-E, which causes a delayed recovery of hematocrit count. In this chapter we first compared the hematocrit at different time points after PHZ treatment for C57BL/6, f/f mice and their correspondent splenectomized mice. As shown in Fig2-1A, both C57BL/6 and splenectomized C57BL/6 mice are recovered on day 8 post PHZ treatment. However the kinetics is different. The hematocrit of wild type mice reaches a nadir 48 hours post initial PHZ injection and gradually increase to normal level until day 8. However the splenectomized C57BL/6 mice reach a nadir around day 4 post initial PHZ injections and rapidly catch up the recovery speed as control mice, indicating that in the absence of spleen extramedullary erythropoiesis switch to somewhere else and this process takes some time.

2.4.2 The bone marrow does not expand progenitors to compensate for the loss of spleen
Since both control mice and splenectomized mice are recovered from PHZ treatment at almost the same time, there are must some sorts of erythropoiesis in splenectomized mice to compensate for the loss of erythropoiesis in spleen. Except for liver, bone marrow may also increase the level of erythropoiesis to compensate the loss of spleen. To exclude this possibility, we tested the number of BFU-E in bone marrow during the recovery from PHZ treatment. Although we observed an increase in BFU-E at 12 hours post PHZ treatment, no other time points shows a significant increase of BFU-E numbers in splenectomized mice (Fig2-2). Based on those observations we concluded that bone
marrow erythropoiesis didn’t compensate for the loss of spleen erythropoiesis in splenectomized mice during the recovery from PHZ induced acute anemia.

2.4.3 Stress BFU-E expand in the liver of the splenectomized mice
Extramedullary erythropoiesis has been observed in some organs except for spleen, predominantly in the liver [27]. Recent work in our lab demonstrated that the BMP4 pathway also regulates the erythropoiesis occurred in fetal liver, which indicates that the liver has the potential to support stress erythropoiesis. First we tested whether BFU-Es were present in the liver during PHZ induced acute anemia. As shown in Fig2-3A, BFU-Es are present in the liver during the recovery period no matter whether spleen is present or not. The total number of BFU-E is increasing during the recovery. However there are significantly more BFU-Es in the liver of splenectomized mice 3 days and 5 days post PHZ treatment. The control mice, on the other hand, have less increase of BFU-E on those time points. This significantly bigger expansion of BFU-Es in the liver in splenectomized mice correlates with the rapid increase of hematocrit count on day 6, day 7 and day 8 during the recovery from PHZ treatment.

Previously we have shown that the spleen erythropoiesis is stress erythropoiesis and regulated by BMP4 pathway. A special erythroid progenitor, which we term stress BFU-E, expands in the spleen and differentiate into red blood cells in a short period of time in response to Epo only. We tested the stress BFU-E in the liver during PHZ induced acute anemia by plating the liver mononuclear cells in methylcellulose media with Epo only. As shown in Fig2-3B, we observed the same expansion pattern for stress BFU-E as total BFU-E indicating most of the BFU-Es in the liver during the recovery from PHZ treatment are stress BFU-Es. The stress BFU-E expansion in the liver in control mice is
greatly limited 3 days and 5 days post PHZ treatment. Splenectomized mice exhibit significantly more stress BFU-E in the liver than control mice.

To further demonstrate the erythropoiesis in the liver, we performed histological analysis on liver section. H&E staining of liver section for untreated mice shows little evidence of erythropoiesis which supports the hypothesis that liver erythropoiesis is induced in acute anemia. Starting on day 4 after treatment, numerous erythropoietic foci show up in the liver and peaked on day 6 after treatment. There are significantly more and bigger erythropoietic foci in the liver of splenectomized mice than control mice on day 6 and day 8 after PHZ treatments (Fig 2-4). Actually control mice exhibit no visible erythropoietic foci in the liver section on day 8 but splenectomized mice does. Those data indicate that erythropoiesis occurs in liver during acute anemia. When spleen is present, erythropoiesis in liver will be reduced to a low level during the recovery.

Those data suggested that during acute anemia, most of the erythroid progenitors move to spleen and give rise to stress BFU-E in response to tissue hypoxia. However, in the absence of spleen, the liver is reactivated for extramedullary erythropoiesis. Erythroid progenitors migrate to liver and give rise to stress BFU-E.

2.4.4 BMP4 expression is expanded in the liver of splenectomized mice and correlates with erythropoiesis

Our previous work demonstrates that the stress erythropoiesis occurred in spleen is regulated by BMP4 signal pathway. The expansion of stress BFU-E is preceded by the expression BMP4 in the spleen. Next we tested the expression of BMP4 in the liver during the recovery from acute anemia. Immunohistochemistry staining shows that the BMP4 expression is always on in the liver in both splenectomized mice and control mice
during the recovery. However, closer examination of the staining reveals that the area expressing BMP4 in the liver is different between splenectomized mice and control mice. Liver acinus, the functional unit of liver, consists of adjacent classic lobules partially separated by distributing vessels from the portal triad (consisting of the portal vein, hepatic artery and bile duct(s)) and central veins at each pole (Figure 2-5B) [28]. The oxygen concentration is different within the functional unit. As shown in Figure 2-5B, zone 3 has the lowest oxygen concentration and zone 1 has the highest [29-30]. BMP4 is expressed at zone 3 in the control mice which is consistent with our previous result showing that hypoxia upregulate BMP4 expression. The BMP4 expression temporally expand to zone 1 and zone 2 on day 1 post PHZ treatment but move back to zone 3 on day 4. However, in splenectomized mice BMP4 expression expand to all 3 zones during the whole observational period (6 days post PHZ treatment). Furthermore erythropoietic foci are present in the area where BMP4 is expressed. Those data suggested that the erythropoiesis occurred in liver during acute anemia corresponds with the expression of BMP4 in the same area where erythropoiesis take place.

2.4.5 Splenectomized f/f mice exhibit a more severe defect in the recovery from acute anemia
So far our data supports the hypothesis that erythropoiesis occurs in liver to compensate the loss of spleen erythropoiesis during acute anemia. BMP4 expression corresponds with the erythropoiesis in the liver. To further demonstrate whether the erythropoiesis in the liver is regulated by BMP4 or not, we analyzed the erythropoiesis in the liver of splenectomized f/f mice. Compared to C57BL/6 mice, f/f mice exhibit a delay in recovery from acute anemia. This defect is further exacerbated in splenectomized f/f mice. The hematocrit reaches a lower nadir during the recovery and doesn’t return to the initial
value before PHZ treatment on day 11 when the control mice fully recovered. 30% of the splenectomized f/f mice didn’t survive from the PHZ treatment which is likely due to the severe anemia in the splenectomized f/f mice (Fig2-6A and Fig2-6B).

The analysis of stress BFU-E in the liver also shows that the expansion of stress BFU-E in the liver of splenectomized mice is delayed as well (Fig 2-6C and Fig 2-6D). In the splenectomized C57BL/6 mice, the stress BFU-E in the liver was peaked on day 3 post PHZ treatment but the control splenectomized mice peaked on day 4. In addition the f/f splenectomized mice exhibit less stress BFU-E in the liver compare to control splenectomized mice on day 2. This delayed expansion of stress BFU-E in the liver corresponds with the delayed expansion of erythropoietic foci in the liver of splenectomized f/f mice (Fig 2-7A). However the BMP4 expression is expanded to all three zones in both splenectomized f/f mice and f/f mice. No obvious difference was observed between splenectomized mice and control mice (Fig 2-7B). But more detailed observation reveals that the BMP4 expression is on and expanded in untreated mice, which is consistent with our observation in the spleen of f/f mice during the recovery from acute anemia where the BMP4 is constitutively expressed.

Those data suggested that stress erythropoiesis could occur in both adult spleen and liver. BMP4 signal pathway plays a key role in the extramedullary erythropoiesis.

2.5 Discussion
Under acute anemia, stress erythropoiesis will be induced to produce massive red blood cells in a short period of time to resolve the tissue hypoxia. Spleen is the primary location for extramedullary stress erythropoiesis in adults. However extramedullary erythropoiesis was also observed in adult liver during acute anemia. Our data further extend this
observation to show that the extramedullary erythropoiesis in the liver is regulated by BMP4 signal pathway which, based on our previous findings, is one of the key pathway in regulating stress erythropoiesis in the spleen. The BMP4 expression in the liver is increased and temporarily expanded to all 3 zones of the liver functional units in control mice. The expanded expression is extended in splenectomized mice. The splenectomized f/f mice exhibit a defect in liver erythropoiesis indicating that BMP4 pathway also regulates the hepatic stress erythropoiesis.

Previous studies observed hepatic erythropoiesis in non-splenectomized mice in response to PHZ induced acute anemia [24-25]. However those studies gave mice multiple dose of PHZ injection, instead of one dose as we did which results severe tissue hypoxia than our experiment. In our experiment, we do observed erythropoietic foci as well as stress BFU-E in the liver in control mice. But the expansion of stress BFU-E is limited and the development of erythropoietic foci quickly disappeared during the recovery. This is probably due to the faster resolved tissue hypoxia in the present of spleen or competition for erythroid progenitor from spleen. Our data show that the control mice recover faster than splenectomized mice clearly due to the present of spleen. This will alleviate the tissue hypoxia and limit the expansion of BMP4 expression as well as the stress BFU-E in the liver. Furthermore, we observed that normally BMP4 is expressed in the zone 3, which has the lowest oxygen concentration, but expands to zone 1 and zone 2, which have higher oxygen concentration, under acute anemia. This expansion is corresponding to the expansion of erythropoietic foci in the liver. Thus we can conclude that the localization of erythropoiesis and BMP4 expression in the liver is regulated by tissue hypoxia.
The spleen maintains a resident population of BMP4 responsive stress progenitors [6]. The livers of untreated splenectomized and control mice contained very few stress BFU-E. Furthermore, treatment of liver mononuclear cells from untreated mice with BMP4 did not increase the number of stress BFU-E (data not shown), which suggests that the liver does not maintain a resident population of BMP4 responsive progenitors. These observations suggest that progenitors migrate into the liver during the recovery period, which may explain the delayed kinetics of recovery in splenectomized mice.

Extramedullary erythropoiesis is also observed in human under certain pathological conditions. In general, any pathology such as myelofibrosis, that limits the bone marrow erythropoiesis, will induce the extramedullary erythropoiesis. However the molecular mechanism that regulates this process is still unknown. Although the stress erythropoiesis pathway in human has not been identified yet, our work charactering some detailed role of BMP4 pathway for extramedullary stress erythropoiesis in mouse system cast a light on this research.

Overall, our data suggested that BMP4 dependent extramedullary erythropoiesis can be induced in the liver and spleen in acute anemia. BMP4 pathway is one of the key pathways in regulating stress erythropoiesis in mice.
Figure 2-1 Recovery from PHZ induced acute anemia (data by Laurie Lenox)

Splenectomized C57BL/6 and C57BL/6 control mice were treated with PHZ to induce anemia. Hematocrit measurements were done at the indicated times. The data shown are the average ±standard deviation of at least 3 mice per time point. *p<0.05, **p<0.01.
Analysis of BFU-E in bone marrow during the recovery from acute anemia. Bone marrow cells were plated in methylcellulose media supplemented with Epo (3U/ml) and IL-3 (50ng/ml). The data are the number of BFU-E per 1×10^5 bone marrow cells.
**Figure 2-3 Analysis of BFU-E in liver during the recovery from PHZ treatment (data by Laurie Lenox)**

Liver mononuclear cells (1×10⁴ per well) were plated in methylcellulose media supplemented with Epo + IL-3 (left) or Epo alone (right). Statistically significant differences are indicated on the figure. The figure shows a representative experiment of 2 independent experiments. The data shown are the average ± standard deviation of at least 3 mice per time point.
Figure 2-4 Hematoxylin and eosin stained liver section during the recovery from PHZ treatment (data by Laurie Lenox)

Hematoxylin and eosin stained liver sections from C57BL/6-splenectomized and C57BL/6 control at indicated time points following a PHZ induced acute anemia. Examples of the darker staining erythropoietic clusters are shown in gray boxed area marked in the day 6 in C57BL/6 splenectomized and control mice. Magnified views of the gray boxed regions are shown to the right of the panels. At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown.
Figure 2-5 Analysis of BMP4 expression in the liver during acute anemia (data by Laurie Lenox)

(A). Liver sections from C57BL/6-splenectomized and C57BL/6 control mice, recovering from an acute anemia stained with anti-BMP4 antibodies and HRP-conjugated secondary antibodies (BMP4) or secondary alone (control). At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown. (*) denotes portal vein of triad composed of portal vein, hepatic artery and bile duct. Arrow points to a central vein. BMP4 staining is brown, with blue hematoxylin counter-stain. Note an example of how BMP4 expression remains expanded into zones 1 and 2 (bracket) into Day 6 post-PHZ in splenectomized mice, while it returns to exclusively zone 3 (3) in wild type mice by 4 Days post anemia. (B). Schematic of liver acinus (functional interpretation of liver organization) formed from classical liver lobules. Blood enters the liver from portal triads composed of portal vein, hepatic artery and bile duct flows towards the central vein. This creates a gradient of oxygen concentration ranging highest in zone 1, intermediate in zone 2, to lowest in zone 3.
Figure 2-6 Analysis of recovery from PHZ treatment for splenectomized f/f mice

(A). Splenectomized C57BL/6-ff and C57BL/6 control mice were treated with PHZ to induce acute anemia. Hematocrit was measured on the indicated days. *p<0.05, **p<0.01, ***p<0.001. (B) Survival curve of splenectomized C57BL/6-ff and C57BL/6 control mice were treated with PHZ. Recovery from PHZ induced anemia was done in 2 independent experiments. 4–6 mice were used per time point. (C) Analysis of BFU-E in the liver of splenectomized C57BL/6-ff and C57BL/6 control mice were treated with PHZ. Liver mononuclear cells (1×10^4 per well) were plated in methylcellulose media supplemented with Epo + IL-3 (left) or Epo alone (right). Statistically significant differences are indicated on the figure. The figure shows average ± standard deviation of four mice per time point.
Figure 2-7 Analysis of BMP4 expression and erythropoiesis in the liver in splenectomized $f/f$ mice

(A). Hematoxylin and eosin stained liver sections from C57BL/6-$f/f$ splenectomized and C57BL/6-$f/f$ control mice at indicated time points following a PHZ induced acute anemia. Examples of the darker staining erythropoietic clusters are shown in gray boxed area marked on day 8 in the C57BL/6-$f/f$ splenectomized and control mice. Magnified views of the gray boxed regions are shown to the right of the panels. At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown. (B). C57BL/6-$f/f$ splenectomized and C57BL/6-$f/f$ control mice recovering from an acute anemia stained with anti-BMP4 antibodies and HRP-conjugated secondary antibodies (BMP4) or secondary alone (control). At least two independent livers were analyzed at each time point. Each liver was fixed, cut into several pieces and sectioned so that each section had fields from multiple areas of the liver. 2–3 sections per time point were analyzed and representative fields shown. (*) denotes portal vein of triad composed of portal vein, hepatic artery and bile duct. Arrow points to a central vein. BMP4 staining is brown, with blue hematoxylin counter-stain.
2.6 References


Chapter 3 The role of Sf-Stk in stress erythropoiesis

3.1 Abstract
Friend virus infection induces erythroleukemia in susceptible mice through a 2 step progression: (i) polyclonal proliferation and differentiation of infected erythroblasts (ii) acquisition of new mutations in erythroblast leading to leukemia. A naturally occurring truncated form of Stem cell derived tyrosine kinase (Stk), termed Sf-Stk has been identified to play a role in Friend virus induced Erythropoietin (Epo)-independent expansion of erythroid progenitors. Recent work in our laboratory has demonstrated a connection between Friend virus induced erythroleukemia and Bone morphogenetic protein 4 (BMP4) dependent stress erythropoiesis pathway. However the intrinsic function of Sf-Stk remains unknown. In this section, we tested the expansion and differentiation of erythroid progenitors in the Sf-Stk deficient mice. The data reveal that Sf-Stk-/- mice have fewer stress BFU-Es as well as CFU-Es in the spleen. The mutant stress BFU-Es have less potential to expand into red blood cells. We also observed greater mortality rate in Sf-Stk-/- mice during the recovery from bone marrow transplantation which indicate that the expansion of erythroid progenitors in bone marrow is also affected by Sf-Stk deficiency. Further studies on bone marrow cells reveal that the Sf-Stk deficient bone marrow cells have an early expansion of stress BFU-E during in vitro culturing than control mice which corresponds with the observation that mutant spleen cells have early terminal differentiation during in vitro culturing. A study of the RBC life span indicates that there is an early expansion of RBCs in Sf-Stk-/- mice during the recovery from bone marrow transplantation. Those early emerged RBCs are defective and removed early during the recovery. This disruption of new RBC in Sf-Stk-/- mice
during the recovery corresponds with the death of Sf-Stk-/- mice after bone marrow transplantation. Combining all those observations we propose that Sf-Stk may participate in promoting the proliferating and/or suppressing the differentiating of erythroid progenitors. When Sf-Stk expression is abrogated, stress erythroid progenitors fail to amplify and differentiate early which exhaust the pool of erythroid progenitors. The early expansion of erythroid progenitors generate defective new RBCs which were removed early leading to higher mortality rate during the recovery from bone marrow transplantation induced acute anemia.

3.2 Introduction
Steady state or homeostatic erythropoiesis in the bone marrow maintains the pool of circulating erythrocytes. Epo and stem cell factor (SCF) are produced locally at a low level to maintain a steady production of new red blood cells. The situation is different in stress erythropoiesis, which occurs at the time of acute erythroid stress. Stress erythropoiesis uses different mechanisms to promote the rapid expansion of red blood cells, which occurs in the adult spleen during the recovery from acute anemia and in fetal liver during embryogenesis [1].

Our knowledge of BMP4 dependent stress erythropoiesis first came from the research on f/f mutant mice. f/f mutant mice exhibit a fetal-neonatal anemia which resolves two weeks after birth. Adult f/f mice have normal erythropoiesis but are unable to rapidly respond to acute anemia [2]. f/f mutant mice exhibit a delayed expansion of erythroid progenitor and a delay in the appearance of reticulocytes after PHZ induced acute anemia [3]. This defect makes f/f mutant mice a good model to study stress erythropoiesis. By using this model system, our laboratory has demonstrated that a specialized population of early
erythroid progenitors, which we termed stress burst forming units-erythroid (BFU-E), is rapidly expanded in the spleen 36 hours post PHZ treatment. f/f mice have a delay in expansion of stress BFU-E after PHZ treatment (4 days post PHZ injection in a f/f mouse versus 36 hours post PHZ injection in a C57BL/6 control mouse). We also determined that f/f mice have a mutation in Smad5 gene, which is a transcription factor that acts in BMP4 signaling pathway [4]. BMP4 is induced in the spleen by tissue hypoxia and acts on an earlier cells referred to as BMP4 responsive cell (BMP4R) which differentiates into stress BFU-E. Our work has demonstrated that stress BFU-E exhibit properties distinct from bone marrow steady state BFU-E in that they are capable of expanding into red blood cells in a shorter period of time and require only Epo for differentiation [5].

These data lead us to propose a new model for stress erythropoiesis, where tissue hypoxia induces BMP4 expression in the spleen leading to the rapid expansion of specialized population of stress BFU-E. These progenitors rapidly differentiate to generate new erythrocytes.

SCF/Kit signaling pathway has also been found to be involved in stress erythropoiesis. W/W' mice which are defective in stem cell factor receptor gene (Kit), exhibit a chronic anemia [6] and are slow in recovery from acute anemia [7]. Recent experiments in our laboratory demonstrated that during the recovery from acute anemia BMP4, SCF and hypoxia work in concert to promote the expansion and differentiation of stress BFU-E.

The knowledge of Friend Virus pathogenesis suggests that it is a good model to study erythropoiesis. Friend virus is a complex of 2 viruses, a replication competent Friend murine leukemia virus (F-MuLV) and a replication defective spleen focus forming virus
(SFFV). Friend virus infection induces erythroleukemia in Friend virus sensitive mice through a characteristic two stage progression. (i) First stage: Friend virus infection causes a massive and polyclonal expansion of infected erythroid progenitors which is driven by Friend virus envelop protein gp55. (ii) Second stage: some of those erythroblasts acquire new mutations, loss of p53 function and proviral insertional activation of Spi1/Pu.1, which leads to the development a clone of leukemia [8]. The activation of Pu.1 plays a crucial role in the second stage of pathogenesis by keeping the erythroid progenitors in undifferentiated state. Pu.1 blocks differentiation by inhibiting GATA-1 function. RNA interference of Pu.1 promotes differentiation [9], while over expression of GATA-1 in the erythroleukemia cells triggers terminal differentiation [10]. Studies by different groups have shown that polycythemia inducing strain of Friend virus (FVP) envelop protein gp55 cooperating with host proteins plays an important role in inducing erythroleukemia.

Because of its characteristic progression and its tropism for the erythroid lineage, several host loci have been identified to affect the susceptibility of cells to Friend virus infection. Some of these loci encode regulators of erythroid cell proliferation and differentiation.

*Dominate white spotting (W)* locus and *Steel (Sl)* locus encode Kit receptor tyrosine kinase (kit) and its ligand SCF respectively. Previous work demonstrated that Kit and SCF are required for steady state erythropoiesis [11]. Recent work in our laboratory demonstrates that Kit mutant mice (W/Wv mice) lack Friend virus target cells in the spleen [12]. This work also demonstrated that the Friend virus target cells in the spleen are part of stress progenitors, which also contains the BMP4R cells. This observation suggests a connection between Friend virus infection and stress erythropoiesis.
f/f mice are also resistant to Friend virus. f locus encodes Madh5 (Smad5) which acts downstream of the receptor for BMP4 and promotes the expansion of stress BFU-E during the recovery from acute anemia. Our work has shown that BMP4 induces BMP4R cells in spleen to differentiate to stress BFU-E during the recovery from acute anemia. Recent work by Aparna Subramanian in our laboratory demonstrated that f/f mice are resistant to Friend virus infection because Friend virus activates the BMP4 dependent stress erythropoiesis pathway to amplify target cells in the spleen. The defect in f/f mice in the BMP4 response blocks this expansion of target cells leading to resistance [12].

Friend virus susceptibility gene 2 (Fv2) encodes a naturally occurring truncate form of Met-related Macrophage stimulating 1-Receptor (Mst1r), also known as short form Stem cell-derived tyrosine kinase (Sf-Stk). Fv2 regulates the polyclonal expansion of infected erythroid cells in the spleen [13-14]. Fv2 locus is dominant for the susceptibility of Friend virus. Mice sensitive to Friend virus (Fv2ss and Fv2sr) express Sf-Stk. However mice homozygous for Fv2rr lack the expression of Sf-Stk [15]. The Sf-Stk promoter is located at 10th intron of Stk gene. The start codon of Sf-Stk gene is located at 11th exon. Fv2rr mice have a three nucleotides deletion in promoter region which reduces Sf-Stk expression to a very low level [15].

FVP envelop protein gp55 is the only viral protein required for inducing erythroleukemia. Gp55 induces BFU-E colony formation in vitro in an Epo-independent manner [16]. Further studies reveal that gp55 activates EpoR and causes Epo-independent formation of red blood cells [17-18] by binding to and activating EpoR through its transmembrane domain [19-20]. A cell line study by SK Ruscetti et al in 1990 also showed that SFFV
induces Epo independent growth in Epo-dependent cell lines [21]. The binding site for gp55 and Epo in EpoR are non-overlapping [22].

Mice that lack Sf-Stk expression are resistant to Friend virus and fail to develop erythroleukemia. However exogenous expression of Sf-Stk confers Friend virus sensitivity to erythroid progenitors from Friend virus resistant mice [23]. Gp55 covalently as well as noncovalently interacts with Sf-Stk in vitro. This interaction causes constitutive tyrosine phosphorylation of Sf-Stk [24]. A constitutively active form of Sf-Stk induces Epo-independent colony formation in the absence of gp55. Coexpressing gp55 and Sf-Stk will prolong the half life of Sf-Stk in the cell line [25]. Recent experiments by our colleagues demonstrated that coexpressing gp55 and Sf-Stk in CHO cells increases the localization of Sf-Stk to the cell membrane [26]. This experiment suggests that gp55 will relocate Sf-Stk from cytoplasm to cell membrane which will expose the Sf-Stk to where it can activate signaling pathway. The interaction of gp55 and Sf-Stk will activate Stat3 in the early stage of pathogenesis which upregulate Pu.1 resulting in enhanced proliferation [27].

Based on all these observations a possible model for gp55 induced Epo-independent formation of erythrocytes is that gp55 upregulates or stabilizes Sf-Stk and relocates it to the cell membrane. Once on the cell membrane Sf-Stk forms a complex with gp55 and activates downstream pathways regulating proliferation. When gp55 interacts with EpoR, the signaling pathway regulating differentiation will be activated.

It’s already known that FVP induces Epo-independent colony formation in vitro. Using this property as an assay, our laboratory has determined that hypoxia increases Friend
virus targets in spleen which correlates with the upregulation of stress BFU-E colonies. BMP4 is upregulated during Friend virus infection and increases Friend virus target cells in spleen \textit{in vitro}. Further experiments demonstrate that stress progenitors were Friend virus target cells [28]. Sf-Stk expression is found in spleen MEPs [28]. These observations coupled with our previous work where we demonstrated that BMP4\textsuperscript{R} cells share the same cell surface marker with spleen MEPs suggest that Sf-Stk may be involved in stress erythropoiesis. We hypothesize that Friend virus may mimic the stress erythropoiesis pathway to induce Epo-independent colony formation. Sf-Stk may be involved in stress erythropoiesis by facilitating expansion of stress progenitors during recovery. We propose that Sf-Stk regulates the proliferation of Friend Virus infected cells in the spleen. Friend virus utilizes stress erythropoiesis pathway to induce erythroleukemia. Thus Sf-Stk may have an intrinsic role in regulating proliferation of stress erythropoiesis.

3.3 Materials and methods

3.3.1 Mice
FVB2 mice were purchased from Taconic\textsuperscript{®}. Sf-Stk knockout mice were provided by Susan Waltz at Cincinnati Children’s hospital. The Sf-Stk abrogation was generated by replacing the genome region containing the promoter of Sf-Stk with the corresponding Sf-Stk cDNA (Fig 3-1). As shown in Fig 3-1, a cDNA sequence for the exon 9, exon 10, exon 11 and exon 12 of Stk gene was inserted into genome to replace the original genome sequence which contains the promoter and start codon for the Sf-Stk gene.

Acute anemia was induced by PHZ (Sigma, St Louis, MO) treatment at a concentration of 100 mg/kg mouse in phosphate-buffered saline (PBS) by IP injection. All procedures were approved by the IACUC of the Pennsylvania State University.
3.3.2 Reverse transcriptase PCR
Spleen cells from wild type and Sf-Stk/- mice were collected at different time points post PHZ treatment. Then cells were lysed by TRIzol® reagent (invitrogen) for RNA isolation. cDNA were made from total mRNA by reverse transcriptase. PCR was performed using cDNA as template and primers for Sf-Stk which listed below [15]. β-actin was used as control.

Sf-Stk primer:

Forward: 5'-TCT GGC TGA TCC TTC TGT CTG-3'
Reverse: 5'-GCA GCA GTG GGA CAC TTG TCC-3'

3.3.3 Analysis of stress BFU-E
For the PHZ treatment experiment, spleen cells were collected at designated time points. Erythrocytes were lysed by treatment with cold 0.16M ammonium chloride. The single cell suspensions were then plated in methylcellulose media with Epo (3U/ml) at a concentration of 2X10^6 cells/ml and cultured in 20% O₂ for 5 days in triplicates.

For the BFU-E assay testing the effect of different growth factors, spleen cells were collected from untreated mice. Erythrocytes were lysed by cold 0.16M ammonium chloride as well. The single cell suspensions were plated in methylcellulose media at 2X10^6 cell/ml concentration with designated growth factors (Epo, Epo+BMP4, Epo+SCF, Epo+BMP4+SCF) at 2% O₂ in triplicates.

For the BFU-E assay testing the stress BFU-E in the bone marrow or spleen, spleen or bone marrow cells were collected from untreated mice. Erythrocytes in the spleen were lysed by cold 0.16M ammonium chloride. The bone marrow cells were cultured in the
liquid media for a certain period of time. Then the single cell suspensions were plated in methylcellulose media at designated conditions (20% O₂+3U/ml Epo, 2% O₂+3U/ml Epo+15ng/ml BMP4+50ng/ml SCF) for 5 days. The concentrations of cell for BFU-E assay are 2X10⁶ cell/ml for spleen cells and 1X10⁵ cell/ml for bone marrow cells.

After 5 days culturing, the BFU-E colonies were stained by benzindine (3% in a 90% Glacial Acetic Acid solution). The blue colonies were counted under microscope.

3.3.4 Analysis of CFU-E
Spleen cells were collected from untreated mice. Erythrocytes were lysed by 0.16M ammonium chloride. The single cell suspensions (1X10⁶ cell/ml) were plated in methylcellulose media with Epo (3U/ml) at 20% O₂ or 2% O₂. After 2 days culturing, the CFU-E colonies containing 8-32 cells, were stained by benzindine (3% in 90% Glacial Acetic Acid solution) and counted under microscope.

3.3.5 Bone Marrow transplantation
All bone marrow transplantation experiments were done according to the standard transplant protocol approved by IACUC of Pennsylvania State University. The recipient mice were fed with acidified water 7 days before irradiation. Then they were irradiated with 950 rads. Bone marrow cells were isolated from donor mice. A total of 0.5X10⁶ or 1X10⁶ bone marrow cells were transplanted to each recipient by retro-orbital injection.

_in vivo_ biotinylation of circulating blood cells was done for analysis of RBC life span in transplanted mice. The mice were given 1 IV injection of 1 mg of biotin-X-NHS dissolved in 20 µl dimethylformamide and 250 µl PBS one day before the bone marrow transplantation. Following treatment, most of the circulating blood cells are labeled by biotin. The recipient mice then were irradiated and transplanted as described above.
3.3.6  *In vitro* cell culture with spleen and bone marrow cells

Spleen and bone marrow cells were isolated from FVB2 and Sf-Stk-/ mice. The spleen cells were cultured in a differentiation media (IMDM (Gbico catalog number 12440)+15% Fetal Bovine Serum+10ng/ml insulin+200ng/ml transferin+2mM L-glutamine+0.1g/ml Bovine Serum Albumin+7ul/L 2-Mercaptoethanol) supplemented with Epo (3U/ml), BMP4(15ng/ml) and SCF(50ng/ml) for designated period of time.

For analysis of expansion of stress BFU-E in bone marrow cells during *in vitro* culturing, bone marrow cells were cultured in the same stress erythropoiesis expansion media with Epo (3U/ml) + BMP4 (15ng/ml) + SCF (15ng/ml) or Epo (3U/ml) + BMP4 (15ng/ml) + SCF (15ng/ml) + GDF15 (30ng/ml) +Shh (25ng/ml) for designated period of time.

3.3.7  Flow cytometry

Cells were centrifuged and resuspended in 100ul PBS. Before staining, cells were incubated with 5ul anti-CD16/32 antibody (Fc-γ block) for 5 minutes. Fic conjugated rat anti-mouse CD71, PE conjugated rat anti-mouse Ter119 and PE-Cy7 conjugated rat anti-mouse CD117 (c-Kit) were added to the cell suspension and incubated for 30 minutes at 4°C at concentrations recommended by manufacture. The cells were then washed and analyzed by a flow cytometer (FC-500 Beckman coulter). Computer software is used for acquisition (MXP V2.2 Beckman coulter) and analysis (Flowjo 7.61). A minimum of 30,000 events were analyzed for each sample.

For *in vivo* biotinylation of circulating blood cells experiment, the newly made red blood cells can be identified as biotin\(^{-}\)Ter119\(^{+}\)Draq5\(^{low/-}\). The percentages of biotin\(^{-}\)Ter119\(^{+}\)Draq5\(^{low/-}\), biotin\(^{low/-}\)Ter119\(^{+}\)Draq5\(^{low/-}\) and biotin\(^{high}\)Ter119\(^{+}\)Draq5\(^{low/-}\) populations are recorded at designated time points post bone marrow transplantation. The
percentages of these three populations are used as response variables for statistical analysis.

### 3.3.8 Statistical analysis

Student T test is traditionally used to compare the response variables between two groups. However that test is valid when there is only one explanatory variable with only two levels (e.g. FVB and Sf-Stk-/- mice). The T statistics is calculated based on the standard error to determine whether the null hypothesis should be rejected or not with certain type I error. Type I error is the probability that null hypothesis is right when we reject it. In other words, it is the probability that we are going to make a mistake when we reject null hypothesis. Typically alpha=0.05 is used as threshold in many areas. Any p-values less than 0.05 are considered significant. However if multiple tests are performed in one experiment, the type I error for the whole experiment will increase. Thus a correction for alpha or another method of analysis is required to control type I error for the whole experiment. For example, if there are two explanatory variables with 2 levels each (FVB and Sf-Stk-/- mice, 20% O2 and 2% O2), we may want to compare FVB and Sf-Stk-/- mice, normoxia and hypoxia. We also want to compare FVB in normoxia, and FVB in hypoxia. In this case if we insist using Student T test, we may need to control the type I error by dividing the original alpha value (0.05) with the number of comparisons (It is called Bonferroni correction). This correction could easily go out of control when there are multiple explanatory variables with several levels in the experiment. A more widely used method is analysis of variance (ANOVA). ANOVA partition variances into different explanatory variables so that an experimental-wise error excluding variance due to explanatory variables can be calculated. It provides a way to compare 3 or more means in the same experiment with the controlled type I error.
Conceptually there are 3 classes of explanatory variables in ANOVA: fixed effect, random effect and covariate. Fixed effect can be thought as “treatment” levels. We are only interested in how those certain levels of effect will affect the response variable. On the other hand, if the levels of effect are drawn from a larger population and our interested on this effect is not limited to those levels, we will treat this effect as random effect. Finally the covariate is the variable that may have a linear relationship the response variable.

In table 1, I listed all the response variables, explanatory variables and corresponding SAS codes to analyze the data for all the BFU-E assay and CFU-E assay in this section.

Table 1. List of response variables and explanatory variables

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Explanatory variable</th>
<th>SAS code</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-E assay for spleen cell</td>
<td>CFU-E colony number</td>
<td>oxygen (fixed) gene (fixed) trial (random)</td>
</tr>
<tr>
<td>BFU-E assay for spleen cell</td>
<td>BFU-E colony number</td>
<td>gene (fixed) condition(fixed) trial (random)</td>
</tr>
<tr>
<td>PHZ treatment BFU-E assay</td>
<td>BFU-E colony number</td>
<td>gene (fixed) time (fixed) trial (random)</td>
</tr>
<tr>
<td>BFU-E assay with different growth factors</td>
<td>BFU-E colony number</td>
<td>gene (fixed) factors (fixed) trial (random)</td>
</tr>
<tr>
<td>BFU-E assay for BM cell after in vitro culturing</td>
<td>BFU-E colony number</td>
<td>gene(fixed) oxygen(fixed) time (fixed) trial (random)</td>
</tr>
<tr>
<td><em>in vivo</em> biotinylation of circulating blood cells</td>
<td>Percentage of biotin Ter119'Draq5low/; Percentage of biotin Ter119'Draq5low/; Percentage of biotin Ter119'Draq5low/; Percentage of biotin Ter119'Draq5low/</td>
<td>gene(fixed) time(random, repeated)</td>
</tr>
</tbody>
</table>
Data analysis was done by SAS® 9.1. The BFU-E/CFU-E colonies at each well in the culture dish were counted 3 times. The mean of colony number were calculated and used as response variable for data analysis.

The explanatory variables oxygen/condition/factors represent different culture conditions (normoxia, hypoxia and different combination of growth factors). The fixed effect, gene, represents the two genotypes (FVB2 and Sf-Stk-/-). The fixed effect, time, represents different time points in the experiment. The glimmixed procedure was used to fit a general linear mixed model so that all the main effects, interaction effects and repeated measurement are properly estimated. The “lsmeans” statement with “slicediff=” option was used to compare FVB2 and Sf-Stk-/- mice at different culture conditions. In the BFU-E assay for bone marrow cells after in vitro culturing, bone marrow cells from the same mouse were used for BFU-E assay at different time points. This is actually a repeated measurement on the same experiment unit. Data collected from a repeated measurement violates the assumption of independence of ANOVA test, which assumes that data are independent from each other. The following statement was used in glimmixed procedure “random _residual_/subject=mice type=cs” to properly analyze the repeated measurement.

3.4 Results

3.4.1 Sf-Stk expression is increased during PHZ treatment
Sf-Stk is only expressed in Friend virus sensitive mice. Previous work by a former graduate student, Omid Harandi in our lab demonstrated that Sf-Stk could also be induced in Friend virus insensitive mice which express low levels of Sf-Stk in normal condition in response to acute anemia (data not shown). FVB2 mouse, which is the
control mouse, is sensitive to Friend virus infection. Acute anemia was induced in FVB2 mice by PHZ treatment. The Sf-Stk express at transcription level was tested by RT-PCR. Our data showed that Sf-Stk expression was increased during the recovery from acute anemia and peaked on day 2 and day 3 post PHZ treatments (Fig 3-2). This result indicates that Sf-Stk may involve in response to acute anemia.

3.4.2 Sf-Stk-/- mice have fewer and smaller stress BFU-E colonies
Sf-Stk is essential for Friend virus induced expansion of erythroid progenitors. Friend virus infected spleen cells can form Epo independent BFU-E colony in BFU-E colony assay. It has been proposed that Sf-Stk works in concert with Epo receptor (EpoR) and FVP envelop protein (gp55) to promote this Epo independent expansion of erythroid progenitors. Sf-Stk interacts with gp55 to promote proliferation while EpoR interact with gp55 to drive differentiation [29]. Thus except for Epo signal pathway, Sf-Stk signal pathway may play a role in regulating the expansion of erythroid progenitors. So we collected spleen cells from both Sf-Stk-/- mice and the control mice for BFU-E colony assay. 20% O₂+Epo is the basic condition for stress BFU-E colony formation in vitro. Only stress BFU-E will form colony in this condition. However, 2% O₂+Epo+BMP4+SCF is the optimal condition for stress BFU-E colony formation in vitro. The results indicate that in both culture conditions (20% O₂+Epo and 2% O₂+Epo+SCF+BMP4), there are significantly fewer BFU-E colonies in Sf-Stk-/- mice (P=0.0085 for normoxia and p=0.006 for hypoxia) (Fig 3-3 A C). The size of BFU-E colonies is also significantly smaller than control mice (Fig 3-3 B). These data indicate that there are fewer BFU-Es in the spleen of Sf-Stk deficient mice. The small BFU-E colonies indicate that those BFU-Es are defective in their ability to generate mature erythrocytes.
We cultured spleen cells from Sf-Stk-/- mice and control mice with different combinations of growth factors for BFU-E colony assay. Our data demonstrate that control mice have significantly more BFU-E in spleen compared to Sf-Stk deficient mice (p=0.002 for Epo+BMP4+SCF and p<0.0001 for the other three conditions) regardless what combinations of growth factors are used. BMP4 and SCF can also increase the number of BFU-E colonies in Sf-Stk-/- mice (Fig 3-4).

We hypothesize that Sf-Stk may play a role in regulating the expansion of stress erythroid progenitors such as stress BFU-E. So to test the number of stress BFU-E in spleen during acute anemia, we treated mice with PHZ and collected spleen cells during the recovery period for BFU-E assay. Sf-Stk deficient mouse shows an early expansion of stress BFU-E in spleen which reaches maximal at 12-24 hours after the initial PHZ treatment. In contrast the number of stress BFU-E in the spleen of control mice decrease significantly in the first 12 hours, after which the number of stress BFU-E gradually increased back to the level of untreated mice 48 hours post initial PHZ treatment. These data indicate that Sf-Stk deficient mice exhibit an early expansion of stress BFU-E in the spleen which is peaked 12-24 hours post PHZ treatment. The situation in Sf-Stk-/- mice, low numbers of BFU-E in the spleen of untreated mice, which expands to maximal level after PHZ treatment, is very similar to what is observed in C57BL.6 mice which express low levels of Sf-Stk (Fig 3-5).

3.4.3 Sf-Stk-/- mice have fewer CFU-E colonies in normoxia but more CFU-E colonies in hypoxia in spleen
During the erythropoiesis, BFU-E gives rise to CFU-E which will expand and differentiate into reticulocytes. The result of BFU-E colony assay indicates that in response to acute anemia, Sf-Stk deficient mice exhibit an early expansion of BFU-E as
well as a reduced number of BFU-E in the absence of tissue hypoxia in the spleen. Then the next question we asked is whether the expansion of CFU-E is also affected. Spleen cells from Sf-Stk-/- mice and control mice were plated in methylcellulose media with Epo+20% O₂ or Epo+2% O₂. Sf-Stk abrogation causes significantly reduced CFU-E colonies in normoxia (p=0.0319) but increased CFU-E colonies in hypoxia (p=0.0101) comparing to control mice (Fig 3-6). Those data suggested that late erythroid progenitors in Sf-Stk deficient mice readily differentiate under hypoxia conditions.

3.4.4 Sf-Stk-/- mouse can’t maintain stress progenitors during in vitro culturing in both spleen and bone marrow

Previous work by the graduate student Omid Harandi in our laboratory identified two cell populations by 3 cell surface marker that are enriched with BFU-E and CFU-E respectively. We termed them population I cells (Kit⁺CD71low/negTer119neg/low) which is enriched with BFU-E and CFU-E, and population II (Kit⁺CD71+/highTer1119+/high) which is enriched with CFU-E. Kit is the cell surface marker for stem cells. CD71 is the receptor for transferrin and Ter119 is cell surface marker for terminal differentiated erythroid cells. In steady state erythropoiesis, stem cell expressing Kit (Kit⁺) on their membrane gives rise to erythroid progenitors which start to make hemoglobin by expressing transferrin receptor (CD71) on their membrane. Cells start to become Kit⁻CD71⁺. Then erythroid progenitor goes on terminal differentiation which marked by Ter119 expressing on cell surface (Kit⁺CD71⁺Ter119⁻ >>> Kit⁺CD71⁺Ter119⁺ >>> Kit⁻CD71⁻Ter119⁻). In contrast, expansion of spleen stress erythroid progenitors showed that a triple positive population (Kit⁺CD71⁺Ter1119⁺). This population expands in spleen when stress erythropoiesis is induced but it is not found in steady state erythropoiesis. A
detailed analysis of data by flow cytometry demonstrated that population I and population II cells can be further categorized by these three cell surface marker and BFU-E and CFU-E are enriched in these two populations respectively which is confirmed by BFU-E and CFU-E functional assay [30].

To test how Sf-Stk mutation will affect population I and population II cells, I first cultured spleen cells in the differentiation media containing Epo, BMP4 and SCF. Cells were harvested at the indicated time and stained for Kit, CD71 and Ter119 with fluorescence conjugated antibodies. The data indicate that only FVB2 mice can maintain population II cells in hypoxia during 5 days in vitro culturing (Fig 3-7). Those data indicate that Sf-Stk knockout mice may be defective in stress erythroid progenitor proliferation which results in less BFU-E in spleen.

3.4.5 Sf-Stk-/- spleen cells exhibit early terminal erythroid differentiation
To further investigate the terminal differentiation of erythroid progenitors in Sf-Stk-/- mice, repeated the cultures of spleen cells in differentiation media were stained with fluorescence conjugated antibody for Ter119 and Draq5. Ter119 is the terminal differentiation marker which is expressed on reticulocyte and mature red blood cells. Draq5 stains cells with DNA. Thus a Ter119\(^+\)Draq5\(^{\text{low/}}\) population can be used to designate reticulocytes.

Over the five days of in vitro culture, Sf-Stk deficient spleen cells gave rise to a Ter119\(^+\)Draq5\(^{\text{low/}}\) population as early as day 2. This production of reticulocytes lasted until day 4 in both normoxia and hypoxia conditions (Fig 3-8). In contrast this reticulocyte population was not found in cultures of spleen cells from control mice grown
in normoxia or in hypoxia. These data indicate that Sf-Stk deficient erythroid progenitors differentiate earlier when cultured in conditions that mimic stress erythropoiesis.

3.4.6 Sf-Stk-/- mouse bone marrow cells exhibit altered stress BFU-E differentiation kinetic during in vitro culturing

Previous data suggested that Sf-Stk deficient mice have less BFU-E in spleen. These BFU-Es give rise to fewer descendants than control mice. Previous work in our lab indicates that stem cells in bone marrow migrate to spleen where they differentiate into BMP4 responsive cells. I next examined how mutation of Sf-Stk will affect the development and expansion of stress BFU-E in vivo and in vitro.

To investigate this question, I first did bone marrow transplantation with FVB2 mice as the recipients and both FVB2 mice and Sf-Stk-/- mice as the bone marrow donor. Surprisingly almost no mice transplanted with Sf-Stk-/- bone marrow can fully recover from bone marrow transplantation induced anemia. Most of them died between day 8 to day 12 post bone marrow transplantation (Fig 3-9) even with 2.5X10⁶ bone marrow cells transplanted (data not shown). These data suggested that Sf-Stk deficient bone marrows are defective in maintaining stem cells and progenitors.

Next to demonstrate the ability of bone marrow cells expanding into spleen stress progenitors, I cultured the bone marrow cells in differentiation media with GDF15, SHH, Epo, BMP4 and SCF. Cells were plated at the indicated time points in methylcellulose media for BFU-E assay. The data were analyzed by ANOVA with repeated measurement. The response variable is the number of BFU-E colony. As shown in Figure 3-10, Sf-Stk deficient mice have an early expansion of BFU-Es colonies in normoxia with maximal expansion on day 4. This is also the time point in normoxia where the biggest difference
between Sf-Stk deficient mice and control mice is ($p=0.0142$). While in hypoxia, the BFU-E colonies of Sf-Stk$^--/-$ mice became less 10 days post bone marrow transplantation ($p=0.002$). These data suggest that not only in the spleen but also in the bone marrow, the Sf-Stk deficient mice exhibit an early expansion of BFU-E in response to anemia but reduced number of BFU-E in the later time point of recovery.

3.4.7 Sf-Stk$^--/-$ mice have an decrease of newly made red blood cells during the recovery from bone marrow transplantation induced acute anemia

Based on previous data Sf-Stk$^--/-$ mice have an early expansion of erythroid progenitors in spleen and bone marrow during acute anemia and reduced BFU-E in the later time point. However, bone marrow transplantation experiment shows that Sf-Stk deficient mice can’t survive bone marrow transplantation induced acute anemia. Because of their death around day 8 post bone marrow transplantation, we are unable to determine whether stress BFU-E expansion is affected in those mice. Thus it is possible that Sf-Stk$^--/-$ died because of shortage of new erythrocytes which is caused by the defect in BFU-E. Or the early expansion of new erythrocytes in Sf-Stk$^--/-$ mice triggers an early turnover of old red blood cells which causes the death of mutant mice. To monitor the expansion of red blood cells and confirm the reason for Sf-Stk$^--/-$ mice’s death, I labeled the circulating cells in the recipient mice with biotin. The newly made erythrocytes can be identified as biotin$^{-/}$Ter119$^{+}/$Draq5$^{low/-}$. The percentages of biotin$^{-/}$Ter119$^{+}/$Draq5$^{low/-}$, biotin$^{low/-}$Ter119$^{+}/$Draq5$^{low/-}$ and biotin$^{high/-}$Ter119$^{+}/$Draq5$^{low/-}$ populations are response variables. Since there are three response variables, a Bonferroni correction was applied to control the overall type I error for the whole experiment. So $\alpha=0.05/3=0.017$ was used to determine the significant level.
Sf-Stk deficient mice have more new red blood cells than control mice on day 2 (p=0.0201) and day 8 (p=0.0233) (marginally significant), but significantly less new red blood cells on day 10 (p<0.0001), day 11 (p<0.0001) and day 12 (p<0.0001) (Fig 3-11). These data suggested that Sf-Stk deficiency causes an early increase in new erythrocytes. But those new red blood cells are removed from circulation in Sf-Stk-/- mice on day 8 and day 9. This loss of new erythrocytes is at critical time points when I observed the greatest mortality in mice transplanted with Sf-Stk-/- bone marrow cells. During the course of this experiment, approximately 50% Sf-Stk-/- bone marrow transplanted mice died prior to day 9 post bone marrow transplantation. Thus the early differentiation of erythrocytes in the knockout mice may be defective in their morphology which will trigger the rapid turnover. This major loss of new erythrocytes during the recovery cannot be compensated and causes mortality in Sf-Stk-/- bone marrow transplanted mice.

Consistent with this idea, I observed that control mice have significant more biotin+ erythrocytes on day 2 (p<0.0001), day 3 (p=0.0005), day 6 (p=0.0022) and day 7 (p=0.0005) than Sf-Stk deficient mice. The decrease of old red blood cells in the knockout mice correspond with the increase of new red blood cells in the early time points. These observations suggested that the emergence of new red blood cells may trigger the body to remove the old ones. The early expansion of erythroid cells in the Sf-Stk-/- mice may cause the early reduction of old red blood cells during the recovery. This reduction combined with the removal of defective new red blood cells in Sf-Stk deficient mice may be responsible for the high mortality rate for knockout mice during the recovery from bone marrow transplantation induced acute anemia.
3.5 Discussion
During the Friend virus infection and the Friend virus induced Epo-independent expansion of erythroid progenitors, Sf-Stk, gp55 and EpoR interact with each other on cell membrane, which will activate Sf-Stk by phosphorylating the kinase domain. The formation of complex and phosphorylation of Sf-Stk are crucial for Friend virus induced Epo-independent expansion of erythroid progenitors. Further experiments in our laboratory shed some light on the connection between Friend virus infection and stress erythropoiesis. The data indicate that the treatment of BMP4 and tissue hypoxia which induced by PHZ treatment can increase the Friend virus target cells in the spleen. $f/f$ mutant mice have almost no Friend virus target cells and defective in producing Epo-independent colony in vitro. $W/W'$ mutant mouse on the other hand, which is deficient in SCF/Kit pathway, has ten times fewer Friend virus target cells. Based on all those observations, we can reasonably assume that Friend virus may use the stress erythropoiesis signal pathway to infect Friend virus sensitive mice, which may cause the uncontrolled activation of this pathway and the Epo-independent expansion of erythroid progenitors in the first stage of pathogenesis.

Furthermore, we have evidence indicating that Friend virus induces Epo independent expansion of erythroid progenitors by activating BMP4 dependent stress erythropoiesis pathway. Sf-Stk is required for Friend virus induced leukemia. Therefore we hypothesized that Sf-Stk play a role in stress erythropoiesis.

My work in the Sf-Stk knockout mouse supports this possibility with some evidence. Data from the experiment on Sf-Stk-/- mice indicate that the mutant mice have less BFU-Es in spleen and bone marrow in normal condition. In acute anemia, Sf-Stk-/- mice
expand BFU-E as well as CFU-E population in both spleen and bone marrow earlier than control mice. Those erythroid progenitors gave rise to terminal differentiated erythroid cells earlier than control mice during the recovery from acute anemia. However, those early expanded erythroid progenitors have less potential to produce red blood cells. The terminal differentiated red blood cells, which are removed from body soon after they emerge seem defective and can’t function well.

Based on all those data, we proposed a simplified model for the role of Sf-Stk in stress erythropoiesis (Figure 3-12). Overall, Sf-Stk promotes the proliferation and/or suppresses the differentiation of erythroid progenitors. Erythroid progenitors in the bone marrow migrate to spleen where they are exposed to the special environment of spleen as well as some important growth factors such as GDF15, Shh and SCF, and give rise to stress erythropoiesis progenitors residing in spleen which we termed as BMP4 responsive cells (BMP4R). The BMP4R will respond to BMP4 and give rise to stress BFU-Es in the spleen. Stress BFU-Es will response to high concentration of Epo and give rise to large quantity of red blood cells in a short period of time. Sf-Stk may plays a role in suppressing differentiation or promote proliferation of erythroid progenitors at each steps. The presence of Sf-Stk may keep the erythroid progenitors staying in proliferating stage so that more committed erythroid cells can be made and progenies can be synchronized in differentiation stage. It may also ensure the quality of the terminal differentiated erythroid cells. When Sf-Stk is absent in the system, erythroid progenitors reduce the proliferation and differentiate early. The early terminal differentiated erythroid cells will be removed from system soon during the recovery possibly due to some defects which could be confirmed by some biophysics tests.
3.6 Future work
So far our data showed that at cellular level, lack of Sf-Stk promotes the differentiation of erythroid cells. The terminal differentiated erythroid cells from this early differentiation are defective and will be removed from system in a short period of time. However, current data are all at the cellular level. From the work in Friend virus induced expansion of erythroid progenitors, we know that Friend viruses envelop protein gp55 can extend the half life of Sf-Stk protein and relocate it to the cell surface. The stabilization and relocation of Sf-Stk protein may promote the interaction between Sf-Stk and gp55 which will activate both Sf-Stk pathway and Epo pathway. We hypothesized that Friend virus may mimic the hypoxia condition and use the stress erythropoiesis pathway to infect the sensitive mice. Thus could hypoxia stabilize and relocate Sf-Stk to activate the stress erythropoiesis pathway?

Next, it is known that during the first stage of Friend virus induced erythroleukemia Sf-Stk and gp55 activate Stat3 which upregulate the expression of Pu.1 leading to reduced differentiation which leads to the expansion of erythroid progenitors in the early stage. My data suggest that lack of Sf-Stk promotes differentiation and/or suppress proliferation in stress erythropoiesis. Thus Sf-Stk may act on Stat3 in the early stage of recovery from acute anemia to promote the expression of Pu.1 leading to reduced differentiation and/or enhanced proliferation. We could check the expression of Stat3 and Pu.1 in Sf-Stk-/- erythroid progenitors to find the relationship between Sf-Stk pathway and Stat3 pathway. Furthermore, we have found increased Sf-Stk expression in the spleen during the PHZ induced acute anemia. Previous work by Omid Harandi in our laboratory indicates that Sf-Stk expression can be induced by hypoxia in spleen in Friend virus resistant mice.
Thus how does hypoxia control the expression of Sf-Stk? What factors that are induced by hypoxia promote the expression of Sf-Stk? How the expression is shut down after the certain time point during the recovery from acute anemia? A possible candidate in associate with controlling the expression of Sf-Stk is GDF15. The work in our lab by Shailaja Hedge demonstrated that GDF15+BMP4+Shh can increase the expression of Sf-Stk in spleen cells. These data suggested that GDF15 could be a regulator of Sf-Stk (unpublished data).

Last Sf-Stk-/- mice have early expansion of red blood cells during the recovery from acute anemia. Those early terminal differentiated cells are removed from the body one day after they emerge possibly due to the defects in the cells themselves. To further confirm this, we could sort out the newly emerged red blood cells and check the expression of hemoglobin and morphological change in the Sf-Stk-/- mice. This experiment could reveal the direct reason why most of Sf-Stk deficient mice could not survive bone marrow transplantation.
Figure 3-1 Knockout Sf-Stk gene

The original genome sequence of exon 9-19 in the endogenous Ron (Stk) gene was replaced with the corresponding cDNA. This replacement removes all the introns between exon 9-19 which includes the start codon of Sf-Stk. Thus the transgenic mice express full length of Stk but lack the expression of Sf-Stk. (Wetzel, C. C. et al 2007)
Figure 3-2 Analysis of Sf-Stk expression in PHZ induced acute anemia

Spleen cells are collected from BALB/c mice at different time points post PHZ treatment. RT-PCR is performed to identify Sf-Stk.
**Figure 3-3 Analysis of stress BFU-E in spleen**

Spleen cells were plated in methylcellulose media at 2X10⁶/ml and cultured in designated condition for five days. The BFU-E colonies were stained by benzidine and counted. A. The number of BFU-E colonies in 20% O₂+Epo (Nor+Epo) or 2% O₂+Epo+BMP4+SCF (Hyp+Epo+BMP4+SCF). B. The pictures of BFU-E colonies for Sf-Stk⁻/⁻ mice and control mice. C. Statistical analysis for the spleen BFU-E colony assay.
Figure 3-4 Analysis of BFU-E in spleen with different factors

Spleen cells were plated in methylcellulose media at 2X10^6/ml and cultured in designated condition for five days. The BFU-E colonies were stained by benzidine and counted. A. Graph for the number of BFU-E colonies at different conditions. B. Statistical analysis for the experiment showing multiple comparisons between Sf-Stk/- mice and control mice at different conditions after adjusted alpha value.
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Figure 3-5 Analysis of the stress BFU-E in spleen post PHZ treatment

Spleen cells were plated in methylcellulose media at $2 \times 10^6$ /ml and cultured in designated condition for five days. The BFU-E colonies were stained by benzindine and counted. A. Graph for the number of BFU-E colonies at different time. B. Statistical analysis for the experiment showing multiple comparisons between Sf-Stk/-/- mice and control mice at different times after adjusted alpha value.
Figure 3-6 Analysis of CFU-E in the spleen

Spleen cells were plated in methylcellulose media at 1X10^5/ml and cultured in designated condition for two days. The CFU-E colonies were stained by benzidine and counted. A. Graph for the number of CFU-E colonies. B. Statistical analysis for the experiment showing multiple comparisons between Sf-Stk-/- mice and control mice.
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Figure 3-7 Analysis of triple positive cells in spleen during in vitro culture

Spleen cells were cultured in differentiation media as described with designated growth factors for 4 days. Cells were taken out at each time point and stained for cKit, Ter119 and CD71. The graph shows the populations that are cKit+. The x axis is Ter119 and y axis is CD71.
Figure 3-8 Sf-Stk-/- mice exhibit an early differentiation of reticulocyte in spleen

Spleen cells were cultured in differentiation media as described with designated growth factors for 5 days. Cells were taken out at each time point and stained for Ter119 and Draq5. The x axis is Ter119 and y axis is Draq5.
**Figure 3-9 Survival curve after Bone Marrow transplantation**

The recipient FVB2 mice were irradiated and the designated amount of bone marrow cells from the donor are injected to each mouse. A. The survive curve for the recipient mice that are transplanted with $1 \times 10^6$ bone marrow cells per mouse. B. The survive curve for the recipient mice that are transplanted with $0.5 \times 10^6$ bone marrow cells per mouse.

**A**

![Graph showing survival curve for 1M/mouse](image)

**B**

![Graph showing survival curve for 0.5M/mouse](image)
Figure 3-10 Analysis of BFU-E in bone marrow cells during in vitro culture

Bone marrow cells were cultured in differentiation media as described with designated growth factors for 10 days. Cells were taken out at each time point and plated in methylcellulose media as described. The BFU-E colonies were stained by benzidine and counted under microscope after five days culturing. A. Graph for the number of BFU-E colonies at different time point and condition for different mice. B. Statistical analysis of this experiment with the multiple comparisons between Sf-Stk-/- mice and control mice at different time points.
Type III Tests of Fixed Effects

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Figure 3-11 Analysis of red blood cell half life after bone marrow transplantation

The circulating blood cells of recipient mice were labeled with biotin prior the irradiation treatment as described and injected with 1X10^6 bone marrow cells from the donor. Approximately 1 ul blood were taken from the recipient mice at every designated time point and stained for Ter119, Draq5 and biotin. A. The graph for flow cytometry data from day 0 to day 4. The population showed is Ter119+ cells. X axis is Draq5 and Y axis is Biotin. B. The graph for flow cytometry data from day 5 to day 9. The population showed is Ter119+ cells. X axis is Draq5 and Y axis is Biotin. C. The graph for flow cytometry data from day 10 to day 12. The population showed is Ter119+ cells. X axis is Draq5 and Y axis is Biotin. D. Percentages for biotin Ter119+Draq5low/- cells (ie. the new red blood cells). E. Percentages for biotin Ter119+Draq5low/- cells. F. Percentages for biotin Ter119+Draq5low/- cells (ie. the old red blood cells). G. Statistical analysis for the percentage of biotin Ter119+Draq5low/- cells. H. Statistical analysis for the percentage of biotin Ter119+Draq5low/- cells. I. Statistical analysis for the percentage of biotin Ter119+Draq5low/- cells.
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**Simple Effect Comparisons of gene*time Least Squares Means By time**

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### Simple Effect Comparisons of gene*time Least Squares Means By time

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## Simple Effect Comparisons of gene*time Least Squares Means By time

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Figure 3-12 proposed model for the role of Sf-Stk in stress erythropoiesis

During acute anemia, bone marrow cells migrate to spleen where they are exposed to certain growth factors such as GDF15, SHH and SCF and the special environment in the spleen. Those stem cells give rise to the stress erythropoiesis specific progenitors which we termed as BMP4 responsive cells (BMP4R). The BMP4R cells can response to BMP4 and give rise to stress BFU-Es which will rapidly expand to erythrocytes with high concentration of Epo. Sf-Stk may play an important role in promoting proliferation and/or suppressing differentiation of erythroid progenitors. This function may be important for stress erythropoiesis by preventing the emergence defective red blood cells after an early differentiation.
Bone Marrow erythroid progenitor → Spleen erythroid progenitor → BMP4R → Stress BFU-E → erythrocytes

GDF15, Shh, SCF

BMP4

High Epo

Sf-Stk promotes proliferation and/or suppresses differentiation:
- Bone marrow exhibit altered BFU-E differentiation kinetic

Sf-Stk promotes proliferation and/or suppresses differentiation:
- Fewer and smaller BFU-E colony
- Reduced population II cells during *in vitro* culturing
- Bone marrow exhibit altered BFU-E differentiation kinetic

Sf-Stk promotes proliferation and/or suppresses differentiation:
- Reduced new erythrocytes after bone marrow transplantation
- Small BFU-E colonies
- Fewer CFU-E colonies in normoxia
- More CFU-E in hypoxia
- Early emergence of Δαq*low*-Ter119*+* population
3.7 References


3.8 Appendices

SAS code for spleen BFU-E analysis (Figure 3-4)

```sas
data working;
  infile 'H:\lab things\data\SAS data\FVB_SfStk_spleen_BFUE.csv' firstobs=2 DLM='';
  input mice gene $ condition $ trial BFUE;
  ModBFUE=log(BFUE);
run;

ODS html;
ODS graphics on;
proc glimmix data=working plot=studentpanel;
  class gene condition;
  model ModBFUE=gene condition trial gene*trial condition*trial gene*condition
    gene*condition*trial;
  random _residual_/subject=mice type=AR(1);
  lsmeans gene*condition/slice=condition slicediff=condition;
run;

ODS graphics off;
ODS html close;
```
SAS code for BFU-E analysis with different growth factors (Figure 3-5)

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infile 'H:\lab things\data\SAS data\FVB_SfStk_factors_BFUE.csv' firstobs=2 DLM=',';
input mice gene $ factors :$20 trial  BFUE;
modBFUE=log(BFUE);
run;

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model modBFUE=gene factors trial gene*trial factors*trial factors*gene
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SAS code for BFU-E analysis after PHZ treatment (Figure 3-6)

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infile 'H:\lab things\data\SAS data\FVB_SiStk_PHZ_BFUE.csv' firstobs=2 DLM=';';
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run;

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SAS code for spleen CFU-E analysis (Figure 3-7)

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ods graphics on;
proc glimmix data=working plot=studentpanel;
class oxygen gene trial;
model CFUE=oxygen|gene|trial;
random _residual_/subject=mice type=AR(1);
lsmeans oxygen*gene/slice=oxygen slicediff=oxygen;
run;
ods graphics off;
ods html close;
```
SAS code for BFU-E assay of liquid cultured bone marrow cells (Fig3-12)

data Fun;
infile 'h:\lab things\data\sas data\FVB_sfStk_liquid_BFUE.csv' DLM=',' firstobs=2;
input mice gene $ oxygen $ time trial BFUE cellnum;
AllBFUE=BFUE*cellnum/1.2;
total=sqrt(AllBFUE);
run;

ODS html;
ODS graphics on;

proc glimmix data=fun plot=studentpanel;
  class gene oxygen time trial;
  model total=gene oxygen time gene*oxygen gene*time oxygen*time gene*oxygen*time;
  random time/subject=mice type=cs residual;
  random trial/subject=mice type=cs residual;
  lsmeans gene*oxygen*time/slicediff=oxygen*time;
run;

ODS graphics off;
ODS html close;
SAS code for biotin labeled circulating cells (Fig3-13)

data bioneg;
  infile 'H:\lab things\data\SAS data\FVB-Sfstk_biotin RBC.csv' DLM=',' firstobs=2;
  input mice gene $:20. time type $ percent;
  if type='BioNeg' ;
  ModPercent=100*(sqrt(percent));

data biolow;
  infile 'H:\lab things\data\SAS data\FVB-Sfstk_biotin RBC.csv' DLM=',' firstobs=2;
  input mice gene $:20. time type $ percent;
  if type='Biolow' ;
  modpercent=100*sqrt(percent);

data biohigh;
  infile 'H:\lab things\data\SAS data\FVB-Sfstk_biotin RBC.csv' DLM=',' firstobs=2;
  input mice gene $:20. time type $ percent;
  if type='BioHigh' ;
  run;

ods html;
ods graphics on;
proc glimmix data=bioneg plot=studentpanel;
  class gene time;
  model Modpercent=gene time gene*time;
  random _residual_/subject=mice type=cs;
  lsmeans gene*time/slicediff=time;
  run;
ods graphics off;
ods html close;

ods html;
ods graphics on;
proc glimmix data=biolow plot=studentpanel;
  class gene time;
  model modpercent=gene time gene*time;
  random _residual_/subject=mice type=cs;
  lsmeans gene*time/slicediff=time;
  run;
ods graphics off;
ods html close;

ods html;
ods graphics on;
proc glimmix data=biohigh plot=studentpanel;
  class gene time;
model percent=gene time gene*time;
random _residual_/subject=mice type=cs;
lsmeans gene*time/slicediff=time;
run;
ods graphics off;
ods html close;
Lei Shi

Curriculum Vitae

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Academic Qualifications
2004-2011 Doctor of Philosophy (Ph.D) in Integrative Bioscience, The Pennsylvania State University, University Park, PA 16802
2001-2004 Master of Science (M.Sc) in physiology, Peking University, Beijing, China
1997-2001 Bachelor of Science (B.Sc) in Biotechnology, Peking University, Beijing, China

Research Experience
2004-2011 Ph.D in Integrative Bioscience, the Pennsylvania State University, University Park, PA 16802
• Research Advisor: Dr. Robert F. Paulson
• Dissertation Title: The role of Sf-Stk in stress erythropoiesis
2001-2004 M.S. in physiology, Peking University, Beijing, China
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• Dissertation Title: BioPD – the Web-based information center and its application in the analysis of signal peptide.
2000-2001 B.S. in biotechnology, Peking University, Beijing, China
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Awards and Honors
2007-2009 Predoctoral Fellowship by American Heart Association

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