THE BMP4-SMAD5 SIGNALING PATHWAY IS REQUIRED FOR THE RAPID EXPANSION OF ERYTHROID PROGENITORS IN THE EMBRYO

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by
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During development, the embryo grows at a very rapid pace, unlike seen anytime during adult life. This rapid growth places a severe demand on the embryo to deliver oxygen to the increasing tissue mass. Hence, one of the first systems to be established in the embryo is the circulatory system consisting of a network of vessels capable of carrying blood cells. This process of blood formation is predominantly erythroid initially, which lends the embryo the capacity to deliver oxygen efficiently to the growing tissues. This expansive erythropoiesis seen in the fetus is mechanistically very different from the erythropoiesis seen in the adult which is predominantly homeostatic. The only situation in the adult where there is expansive erythropoiesis as seen in the embryo is under conditions of acute anemia or rapid blood loss, which leads to a rapid expansion of erythroid progenitors in the spleen; a condition termed stress erythropoiesis. This relationship between fetal liver and adult stress erythropoiesis is evident in mice with a mutation at the flexed-tail (f) locus. \textit{ffe} embryos exhibit a severe microcytic, hypochromic fetal anemia. The anemia is most severe early in fetal development and gradually improves such that the anemia resolves about two weeks after birth. As adults, \textit{ffe} mice have normal blood values but are unable to respond rapidly to acute erythropoietic stress. When challenged with an acute anemia, the control mice respond by inducing the rapid expansion of erythroid progenitors in the spleen but in the \textit{ffe} mice, this response is delayed.
We have identified the mutation in the \textit{f/f} mice to be in a gene called \textit{Smad5}. Smad5 belongs to the Smad family of transcription factors and functions downstream of BMPs, specifically BMP2, 4, and 7. We have shown that the BMP4-Smad5 signaling pathway is responsible for initiating the stress erythropoietic response in the adult spleen in response to acute anemia. Work described in this thesis shows that the same BMP4-Smad5 signaling pathway is responsible for the anemia seen in the \textit{f/f} fetal livers during development. The observations described in the thesis underscore the similarities and the differences observed between fetal erythropoiesis and stress erythropoiesis seen in the adult using \textit{f/f} mice as a model for studying expansive erythropoiesis.
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Chapter 1

LITERATURE REVIEW

Hematopoiesis

Hematopoiesis can be defined as the cellular processes by which the various lineages of blood cells are generated and mature into functionally viable cells. These terminally differentiated blood cells cannot replicate themselves and hence have to be derived from a more primitive blood cell called the hematopoietic stem cell (HSC). HSCs by definition have the capacity to both self renew and differentiate to form all the different blood lineages (Potten and Loeffler 1990) Figure 1-1. The pool of HSC are maintained by the process of an asymmetric division in which one HSC divides to form another HSC and a more differentiated multipotent progenitor (MPP). The MPP, like the HSC has the capacity to form multiple lineages of blood, but is more committed than a HSC and also has a reduced capacity to self renew. A MPP can differentiate further, depending on the signals it receives into one of the many hematopoietic lineages. It is generally accepted that the process of differentiation of these stem/progenitor cells into mature cells is an irreversible process and is associated with loss of their capacity for self renewal.

Among the first evidence for a pleuripotent HSC came from an in vivo clonal assay called colony forming unit spleen (CFU-S) which laid the foundations for the
investigations into stem cell biology and the paradigm of hematopoietic hierarchy as we know it today (Till and Mc 1961). In this assay when bone marrow cells were injected into lethally irradiated mice, after 8 to 12 days these donor cells gave rise to macroscopic splenic colonies that contained differentiated cells of various blood types derived from the donor cells. It is now appreciated that these colonies contained two different populations of cells. Cells that arose within 8 days called CFU-S\textsubscript{8} were mainly uni- or oligo-lineage committed progenitors and the cells that arose around 12 days called the CFU-S\textsubscript{12} colonies consisted of cells of various lineages suggesting that at least a
portion of these colonies were derived from a pleuripotent HSC (Magli, Iscove et al. 1982; Ploemacher and Brons 1988).

A more stringent assay for measuring HSC activity is the long term repopulation assay (LTRA) as it satisfies the two important definitions for a HSC: self renewal and multilineage repopulation (Li and Johnson 1992; Uchida and Weissman 1992). In this assay, either unfractionated or purified HSCs are transplanted into lethally irradiated recipient mice and screened after 8 to 10 weeks to look for reconstitution of the hematopoietic system by the donor cells. Nowadays, a modified version of LTRA is used to quantify the number of HSCs transplanted (Szilvassy, Humphries et al. 1990). Here along with a limited number of donor HSCs, bone marrow cells isolated from the host are also transplanted to provide protection against pancytopenia during the initial phase when the hematopoietic reconstitution by the transplanted HSCs have not occurred. As the limited number of donor HSCs have to compete with the host HSCs (either from the co-transplanted HSCs from the bone marrow or from the HSCs which escaped irradiation), these assays are called competitive repopulation or CRU assays.

Central to the identification of various stem/progenitor cells is their functional capacity to form mature blood cells when isolated and plated in vitro in semi-solid methylcellulose media called colony assays. These colony assays allow identification of clonal population of cells derived from single progenitor/stem cells. The morphology of these colonies can be studied for their composition to identify the terminally differentiated blood cells formed by these stem/progenitor cells. A MPP when plated in these colony assays can form colonies containing multiple cell types; whereas a more
differentiated bipotent progenitor can form only two of many blood types. A much more committed erythroid progenitor can form only erythroid colonies in these colony assays.

Over the years, a great deal of data point to the role of cytokines and growth factors in the survival and proliferation of HSC and their more differentiated progenitors. The role of these signals in hematopoietic development may be two fold. The instructive model for the role of cytokines suggests that a cell will commit to one or the other lineage depending on the extrinsic signal it receives (Socolovsky, Lodish et al. 1998). A stochastic model suggests that growth factor signal is required for the survival and proliferation of committed cells but plays no role in the commitment decisions per se. A clear support for the instructive hypothesis comes from the role cytokines play in lineage commitment of bipotential granulocyte-macrophage colony-forming cells (GM-CFC). In the presence of SCF or G-CSF, the GM-CFC form granulocytes but when exposed to M-CSF, they form macrophages. The stochastic model was formed when the first colony assay studies like CFU-S was shown to form variable number of cells per spleen and best fitted the model where the formation of differentiated cells compared to the self renewal of the progenitors was under probabilistic control (Till and Mc 1961). But it is important to realize that these models were formed during times when the various cytokines and their roles were not yet characterized and hence, could not rule out their role in lineage commitment (Nakahata, Gross et al. 1982; Ogawa, Porter et al. 1983). The strongest evidence for the stochastic model comes from studies done on IL-7 knockout mice. IL-7 has been shown to be required for the development of thymocytes in which it primarily acts as a survival factor by repressing Bax activation and inducing $bcl-2$ synthesis.
Hybrid hypotheses incorporating both the models have also been proposed.

The growth factors along with many transcription factors are extremely important considering some recent work showing that many of these factors which play a role in hematopoiesis are also involved in development of various leukemias. For example, stem cell leukemia (SCL) is normally expressed in cells of erythroid and megakaryocytic lineages, but when the SCL gets expressed in T cells due to a chromosomal translocation, they develop a form of leukemia like T Cell Acute Lymphocytic Leukemia (T-ALL) (Engel and Murre 1999). The *Ikaros* gene is an essential regulator of lymphoid differentiation and is required for B cell commitment. Mice lacking Ikaros do not form mature B cells (Wang, Nichogiannopoulou et al. 1996) suggesting the importance of these growth and transcription factors in both normal hematopoiesis and development of hematological malignancies.

Clonal cultures where cells from resuspended colonies were recultured have been helpful in understanding how multipotential cells generate cells committed to a single blood lineage. Analysis of daughter cells has shown that cells are generated with almost any combination of single or multilineage potentiality (Suda, Suda et al. 1984). Despite the overall stochastic pattern by which lineage commitment occurs, analysis has shown that certain bipotential progenitor cells are more common than others. Megakaryocyte Erythroid Progenitor (MEP) which can give rise to both megakaryocytes and erythroid cells when grown in culture is a common finding both in the adult bone marrow as well as in the embryonic stage (Akashi, Traver et al. 2000). On the other hand, the combination of bipotentiality to form eosinophils and erythroid cells together is never
seen. This would argue against hematopoietic lineage commitment being a completely random process and indicate that there is some level of structure in how lineage commitment occurs.

Irrespective of how the terminally differentiated cells are formed, the cells have been shown to have specialized functions. The myeloid cells (granulocytes and macrophages) are involved in responding to inflammation and in allergic responses. The lymphocytes are critical for adaptive immune functions. The erythrocytes are involved in oxygen delivery to tissues and the platelets are involved in blood coagulation.

**Erythropoiesis**

Erythropoiesis is the process of formation of red blood cells (RBC). RBCs are specialized cells whose role is to carry oxygen obtained from the respiratory system into all the tissues through the systemic circulation. Adult humans have about $3 \times 10^{13}$ RBCs at any given time. They have a life span of about 120 days and senescent cells are removed from circulation when they are unable to squeeze through the small sinusoids in the spleen. The mature RBC is a biconcave disc shaped cell about 8 microns in diameter. It lacks organelles and so cannot synthesize new proteins. It also depends exclusively on anaerobic glycolysis for its energy needs as they lack mitochondria.

Traditionally, erythroid progenitors have been identified and described using functional *in vitro* colony assays. One of the first progenitors known to form erythroid colonies are the CFU-GEMM. They can form not only erythrocytes, but also granulocytes, macrophages and megakaryocytes, showing that the erythroid lineage is derived from the myeloid lineage. Recent advances using cells surface markers have identified a common precursor for these lineages that has been named Common Myeloid
Progenitor (CMP) (Akashi, Traver et al. 2000). The CMP can give rise to various types of myeloid colonies including CFU-Mix, CFU-MegE, BFU-E, CFU-Meg, CFU-GM, CFU-G, and CFU-M when plated in vitro in methylcellulose media containing SCF, FL, IL-3, IL-6, IL-11, EPO, and TPO. This CMP has been shown to exhibit specific surface markers, Lin- Kit+ Sca1- IL7Rα- FcγRlo CD34+. This population can give rise to either a bipotent Megakaryocyte Erythroid Progenitor (MEP) with a cell surface characteristic of Lin- Kit+ Sca1- IL7Rα- FcγRlo CD34- or a Granulocyte Macrophage Progenitor (GMP) with a cell surface characteristic of Lin- Kit+ Sca1- IL7Rα- FcγRhí CD34+ (Traver, Miyamoto et al. 2001). The MEPs gave rise exclusively to CFU-Meg, CFU-E, or CFU-MegE colonies that contained only megakaryocytes or erythrocytes in response to the above growth factors, but they did not form colonies in the absence of TPO and EPO suggesting the requirement for these factors in the terminal differentiation of erythrocytes and megakaryocytes.(Figure 1-2)

Figure 1-2: Maturation of HSC into various lineages through the production of different multi-potent progenitors (Akashi, Traver et al. 2000).
The first committed erythroid progenitor to be identified has been called the Burst forming unit-erythroid (BFU-E). The BFU-E when plated in methylcellulose media, require high concentrations of Erythropoietin (Epo) of about 3U/ml and a burst promoting factor (BPF) like Stem cell factor (SCF) but in vitro SCF can be replaced by Interleukin-3 (IL-3). They form large colonies which are predominantly erythroid in nature which can be identified using stains that bind to hemoglobin. The incubation times required to form these colonies vary between 3 days to about 10 days (Gregory and Eaves 1977; Gregory and Eaves 1978). It has been suggested in both human and mouse adult bone marrow that there are two different BFU-Es: mature and primitive BFU-E. The mature BFU-E are formed earlier, more sensitive to Epo and less dependent on BPF, and have more proliferative capacity compared to the primitive BFU-E (Eaves and Eaves 1978; Gregory and Eaves 1978; Peschle, Migliaccio et al. 1981). This implies that the populations forming these colonies are heterogeneous and that there are both immature and more mature cells which can form these colonies. BFU-E characteristics also vary in their characteristics depending on the site of their isolation. Human fetal BFU-E has a much higher proliferative capacity compared to their adult counterparts. Human fetal BFU-E does not require the presence of a BPF for their activity unlike the bone marrow, where very few erythroid colonies form with the absence of a BPF. The mature fetal BFU-E have also been shown to be much more sensitive to added Epo compared to their adult counterparts (Peschle, Migliaccio et al. 1981).

The next committed erythroid progenitor described is colony forming unit-erythroid (CFU-E). The colonies formed are much smaller and more tightly held together when compared to a BFU-E. They are formed in about 2 days of incubation with Epo and
do not require the addition of other cytokines. The CFU-E then differentiates further to undergo series of histologically identified stages of development which can be characterized by staining with Wright Giemsa stain (Figure 1-3). During this process, these cells lose their nuclei and organelles and accumulate hemoglobin. Late in this process after they have extruded their nucleus they become reticulocytes, which have only residual strands of RNA in them along with hemoglobin. The reticulocytes then mature into an erythrocyte which is then released into the circulation.

Figure 1-3: Terminal Differentiation of Erythroid Precursors into an Erythrocyte. Courtesy Wikimedia Commons.
Erythropoietin (Epo) is the master regulator of erythropoiesis. The essential role of Epo in the control of erythroid development is well established (Ratcliffe 1993; Sawyer and Penta 1994; Semenza 1994). Epo binds to its receptor on erythroid progenitor cells and stimulates their proliferation, differentiation, and resistance to apoptosis (Kelley, Koury et al. 1993; Liboi, Carroll et al. 1993; Watowich, Hilton et al. 1994). It works in concert with other factors like SCF, and Insulin-like growth factor (IGF-1) for optimal erythroid proliferation and differentiation. Both Epo and EpoR homozygous mice die of severe anemia between E13 and E15. In spite of this severe phenotype, there was still formation of BFU-E and CFU-E in the fetal livers of these mice suggesting that the commitment towards of erythroid lineage did not require Epo (Wu, Liu et al. 1995; Lin, Lim et al. 1996). The period of Epo dependence has been shown to be from just before the CFU-E stage until about the basophilic erythroblast stage (Koury, Kelley et al. 1994). The proliferation, differentiation, and survival of the later progenitors have been shown to be dependent on Epo (Kieran, Perkins et al. 1996; Lin, Lim et al. 1996). Erythrocytes and reticulocytes do not express the receptor for Epo (EpoR).

Epo is primarily expressed by the interstitial cells in the kidney and by the liver during fetal and neonatal stages of development. Hypoxia is one of the key regulators of Epo. In response to hypoxia, there is an increase in the number of cells that produce Epo rather than increased production of Epo by the cells producing them (Koury, Koury et al. 1989). Epo is regulated by hypoxia mostly at the transcriptional level (Schuster, Badiavas et al. 1989). The enhancer region of the Epo gene has a response element where Hypoxia Inducible Factor-1 (HIF-1) binds to regulate Epo transcription (Ebert and Bunn 1999).
Binding of Epo to its receptor (EpoR) on erythroid progenitors causes a conformational change in the EpoR leading to oligomerization of the receptors. This allows phosphorylation and activation of two receptor associated Janus family receptor tyrosine kinases (JAK2) which further phosphorylates the tyrosine residues in the intracellular domain of EpoR. These tyrosine residues now act as docking sites for other proteins to bind through their SH2 domains. The signaling molecules involved downstream include the signal transducers and activator of transcription-5 (STAT5), phospholipid modifying enzymes like PI-3 kinase and PLC-γ, suppressors of cytokine signaling like SOCS3, and Src family kinases (Sasaki, Yasukawa et al. 2000; Cheung and Miller 2001). The signal transduction pathways involving Epo have been well described and include interaction with other transcription factors like GATA-1, NF-E2, SCL, EKLF, and NF-kB.

**Origins of Hematopoiesis**

Before I describe erythropoiesis during development, I will describe how the hematopoietic cells are first derived from non-hematopoietic mesodermal tissue. These hematopoietic cells once derived, will contribute to hematopoiesis throughout the remainder of the life of the organism. Cell fate determination during development is controlled by signals exchanged between various cell populations. This process is called embryonic induction. Gastrulation is a process where cells of the primitive ectoderm also called the epiblast move into the interior through the primitive streak, resulting in the formation of the three germ layers in the embryo: ectoderm, mesoderm, and endoderm (Tam and Behringer 1997) (Figure 1-4). It is one of the first examples of
inductive process occurring in the embryo. In nearly all vertebrates, time of gastrulation coincides with the onset of hematopoiesis and results from the induction of extraembryonic mesoderm.

The yolk sac is the first site of hematopoiesis in the embryo. It is a bilaminar tissue in which a layer of mesoderm is laminated on a layer of primitive visceral endoderm. It functions like a placenta before the establishment of circulation in mammals (Jollie 1990). The process of formation of yolk sac begins around gastrulation at about E6.5, when the mesodermal cells from the early posterior primitive streak migrate into the proximal region of yolk sac (Lawson, Meneses et al. 1991). A thin layer of mesodermal cells along with the overlying visceral endoderm forms the visceral yolk sac.

Figure 1-4: Mesodermal Patterning during Gastrulation (Baron 2005).
Microscopy of embryos in the early neural plate stage of development (E7.5-E7.75) revealed proliferation of mesodermal sheets in the extraembryonic mesoderm which were named mesodermal masses (Haar and Ackerman 1971; Silver and Palis 1997). By E8.0, these masses had formed angioblastic cords with two types of cells in them which were suggested to be endothelial and hematopoietic progenitors (Haar and Ackerman 1971). By E8.25, the ‘blood islands’ of yolk sac form clusters of erythroid cells which are surrounded by a layer of vascular endothelial cells. These endothelial cells then join to form vessels for the vascular network. By E8.5, the yolk sac vessels join the embryonic vessels and the circulation gets established once the heart starts beating (Flamme, Frolich et al. 1997) (Figure 1-5).

Figure 1-5: Yolk Sac Blood Islands. Yolk sac blood islands form between the single cell layer of endoderm cells (E) and mesothelial cells (open arrow). Blood islands develop from undifferentiated mesoderm cells (E7.5, closed arrow) that give rise to inner blood cells surrounded by an outer endothelial lining (E9.5, closed arrow). (Palis, Chan et al. 2001)

Recent studies have given more insight into the sequence of events concerning the emergence of hematopoietic and endothelial cells in the yolk sac. In many ways, these
data challenge the traditional knowledge about how the blood islands are formed and contribute to yolk sac erythropoiesis. Colony assays done to detect primitive erythroid progenitors called the EryP-CFC found that these hematopoietic cells are present by E7.25 (Palis, Robertson et al. 1999) suggesting that the first wave of hematopoiesis begins much before the formation of blood islands which are identified first at around E8.25. In addition to the temporal evidence, it has been shown that the EryP-CFC activity was present near the primitive streak of the embryo proper along with in the region of prospective blood islands suggesting that hematopoietic development can occur independent of endothelial development as seen in the case of primitive streak (Kinder, Tsang et al. 1999; Palis, Robertson et al. 1999).

A recent study using CD41 as a marker for erythroid progenitors showed that by E7.75, a band of blood cells circumferentially surround the whole embryo and forms a single large blood island. This band of blood cells is separated from the distal embryo proper by a honeycomb like series of vascular plexus (Ferkowicz, Starr et al. 2003). Furthermore, the relationship between hematopoietic cells and endothelial cells suggest that primitive erythropoiesis may be occurring extravascularly in contrast to the traditional belief that blood island formation occurs surrounded by endothelial sheath. It has been suggested that the blood cells are only partially covered by endothelial cells and they only subdivide the already formed blood cell clusters into smaller channels to eventually connect it to the vascular plexus (Ferkowicz, Starr et al. 2003; Li, Ferkowicz et al. 2005). (Figure 1-6).

Flk-1 is a receptor tyrosine kinase belonging to the Vascular Endothelial Growth Factor Receptor family (VEGFR). It is also known as VEGFR-2 or KDR. It was observed
that inactivation of *Flk-1* resulted in embryos lacking both endothelial and hematopoietic cells, indicating that *Flk-1* is required in the formation of both vasculogenesis and hematopoiesis (Shalaby, Rossant et al. 1995). It was later shown that *Flk-1* was involved in the migration of cells from the posterior primitive streak to the appropriate extra-embryonic and embryonic mesodermal compartments, where the hematopoietic and endothelial cells arise (Shalaby, Ho et al. 1997). Studies in *Xenopus* support this finding as it has been shown that VEGFR-2 expressing angioblasts migrate to the site of dorsal aorta formation in response to VEGF signaling which is the ligand for the flk-1 receptor (Cleaver and Krieg 1998). Hence the migration of mesodermal cells from the primitive streak to the ‘blood islands’ of yolk sac is a signal driven tightly regulated process.

Tissue recombination studies done in chick embryos had shown that for the yolk sac mesoderm to form hematopoietic cells, they have to interact with the extraembryonic endoderm (Miura and Wilt 1969). Only recently was it shown in mouse models that visceral endoderm is required for the induction of hematopoiesis. To examine this, pre-streak embryos were isolated and ectoderm separated from other tissues and then either incubated with or without visceral endoderm and then examined for hematopoietic activity (Belaoussoff, Farrington et al. 1998). It was further shown, that a diffusible signal and not cell-cell contact from the visceral endoderm was responsible for inducing hematopoiesis in the ectodermal tissue of the embryo. One of these diffusible signals has been shown to be Indian Hedgehog (Dyer, Farrington et al. 2001; Byrd, Becker et al. 2002). It is believed that BMP4 might be the downstream target of hedgehog signaling in this system even though it has not been characterized. BMP4 knockout embryos die in
early post-implantation stage because of mesodermal formation defects and they lack yolk sac blood islands (Winnier, Blessing et al. 1995). ES cell derived embryoid bodies can form mesodermal and hematopoietic cells in the presence of exogenous BMP4 (Johansson and Wiles 1995).

Figure 1-6: Novel view of yolk sac blood island. (A) Late neural plate (E7.75) embryo with maturing Flk-1+ vascular plexus (green) and distinct CD41+ blood band in the proximal yolk sac (red). Note the paucity of Flk-1+ (green) cells in the blood band region. (B) Higher magnification cross-section of the blood band. The Flk-1+ endothelial cells (green cells highlighted with asterisks) in the blood band region are found between the extravascular blood band cells and the outer visceral endoderm of the yolk sac (blue cells depicted as endo). (C) Blood island image depicting an angioblastic cord (a) from the same stage embryo as in panels (A) and (B). Note the similarity to the cross-section of the blood band in panel (B). Figure from (Ferkowicz and Yoder 2005)
As mentioned earlier, the mesodermal cells leave the primitive streak and migrate proximally to appose the visceral endoderm, embryonic ectoderm, and extra embryonic ectoderm. The association of mesoderm with endoderm is termed splanchnopleure. Both the mesodermal and endodermal cells of the bilaminar yolk sac have distinct functions. The visceral endoderm metabolizes maternally derived nutrients and makes serum proteins while the mesodermal cells form the first hematopoietic cells of the embryo in the blood islands of yolk sac.

**Hemangioblast**

The term hemangioblast was first coined in the 1930s from keen observations that there was a close spatial and temporal association between the appearances of blood cells and endothelial cells in the yolk sac blood islands leading to the hypothesis that a common precursor, the hemangioblast, forms both these lineages. Subsequently, several pieces of work have pointed to the existence of this cell. In addition, there are a large number of genes that are co expressed in both the endothelial and hematopoietic lineages. These include *Scl, Lmo-2, EpoR, Tie2, CD31, and Gata2*, which suggest the concept of a common precursor (Anagnostou, Liu et al. 1994; Kallianpur, Jordan et al. 1994; Watt, Gschmeissner et al. 1995; Hashiyama, Iwama et al. 1996). Disruption of many of these genes, like *Flk-1* (Shalaby, Rossant et al. 1995; Schuh, Faloon et al. 1999), *Scl* (Robb, Lyons et al. 1995; Shivdasani, Mayer et al. 1995), *Lmo-2* (Warren, Colledge et al. 1994; Yamada, Pannell et al. 2000) had defects in both hematopoietic and vascular cells in the mouse embryo. The most direct evidence for the hemangioblast comes from ES cell
experiments where a unique precursor cell, Blast-CFC has been identified which gives rise to both endothelial and hematopoietic cells. These cells express Flk-1 and were shown to respond to its ligand vascular endothelial growth factor (VEGF) (Choi, Kennedy et al. 1998; Faloon, Arentson et al. 2000). Even though the embryos lacking Flk-1 did not form any hematopoietic cells, Flk1-/- ES cells cultured in vitro showed formation of some erythroid cells despite the fact that they did not form any endothelial cells. These data suggested that at least some hematopoietic cells were formed independent of Flk1 (Shalaby, Ho et al. 1997). It was soon shown that Scl was required for the formation of colonies from the Blast-CFC (Robertson, Kennedy et al. 2000). The Flk-1 positive cells from ES cell culture were later on shown to be able to form not only endothelial and blood cells but also smooth muscle cells (Nishikawa, Nishikawa et al. 1998; Yamashita, Itoh et al. 2000). The Blast-CFC was recently identified in the mouse embryo in the posterior primitive streak and was shown to give rise to hematopoietic, endothelial, and smooth muscle cells (Huber, Kouskoff et al. 2004).

**Primitive erythropoiesis**

The first blood cells formed in the yolk sac between E7 and E8 are called the primitive erythroblasts. These cells are the only lineage of blood which is transient in nature. They are large nucleated cells, about 465 to 530 μm in size, which make embryonic hemoglobins and enter the circulation at around E8.5 (Chui and Russell 1974). These cells keep dividing in the circulation till about E13 (Sangiorgi, Woods et al. 1990) and are seen in the peripheral blood until E18 even though the nucleated ones are present only till E16. The primitive erythroblasts differentiate and enucleate in the bloodstream,
and as they differentiate, they accumulate more and more hemoglobin (Sasaki and Matsumura 1986) such that they have about 80 pg/cell compared to a mature adult erythrocyte which has about 22 pg/cell (Steiner and Vogel 1973). The primitive erythroblasts initially synthesize embryonic globins, but switch to adult globin synthesis later (Brotherton, Chui et al. 1979). Table 1-1 Charts the differences between definitive and primitive erythroblasts.

The growth factor requirements for primitive erythroblasts are different from those of adult definitive counterparts. The Dominant white-spotted (W) and Steel (Sl) mutant mice which have defective Kit and SCF function respectively, develop anemia at around midgestation (Chui and Russell 1974; Ogawa, Nishikawa et al. 1993). This suggests that Kit/SCF signaling is required for definitive erythropoiesis but is dispensable for primitive erythropoiesis. The role of Epo in the initial differentiation of yolk sac erythroblasts seems controversial. Culture of E7.5 yolk sac explants with exogenous Epo results in increased numbers of erythroid cells (Palis, McGrath et al. 1995). The primitive erythroid progenitors have been shown to be more sensitive to Epo than definitive erythroid progenitors both in the embryo and in the adult bone marrow (Zimmermann and Rich 1996). On the other hand, mouse mutants involving Epo signaling molecules like the EpoR and Jak2 kinase mutants, have shown very little defect in primitive erythropoiesis compared to the severe defects seen in definitive erythropoiesis (Wu, Liu et al. 1995; Lin, Lim et al. 1996; Neubauer, Cumano et al. 1998). So the role of Epo signaling in primitive erythropoiesis has not been clearly elucidated.

The lack of phenotype for primitive erythropoiesis in the knockouts of various growth factors brings out a crucial question- Are primitive erythroblasts independent of
growth factor requirement? Further studies might be required to understand the growth requirement of these erythroblasts.

Table 1-1: Differences between Primitive and Definitive Erythropoiesis. Adapted from Hematopoiesis: A Developmental Approach. Zon, LI.

<table>
<thead>
<tr>
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<th>Primitive</th>
<th>Definitive</th>
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<tr>
<td><strong>Site of Synthesis</strong></td>
<td>Yolk Sac</td>
<td>Liver</td>
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<tr>
<td><strong>Site of Differentiation</strong></td>
<td>Bloodstream</td>
<td>Liver</td>
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<tr>
<td><strong>Temporal appearance</strong></td>
<td>E7.5</td>
<td>E9.5</td>
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<tr>
<td><strong>Morphology</strong></td>
<td>Nucleated/Non-nucleated</td>
<td>Non-nucleated</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>465um</td>
<td>145um</td>
</tr>
<tr>
<td><strong>Hb content/cell</strong></td>
<td>80pg</td>
<td>27pg</td>
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<tr>
<td><strong>Globin chain synthesis</strong></td>
<td>Embryonic-&gt;adult</td>
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Unlike the growth factor mutants, various transcription factor mutants have defects in primitive erythropoiesis. Targeted deletion of the Stem cell leukemia (SCL) gene causes death of the embryos at around E10.5 due to severe anemia, which is caused by reduced blood formation in the yolk sac (Shivdasani, Mayer et al. 1995). Lmo2 belongs to the LIM domain family of transcription factors and mediates protein-protein
interactions. Lmo2/- embryos lack yolk sac erythroid cells, leading to embryonic death by E10.5 (Warren, Colledge et al. 1994). GATA-1 belongs to GATA family of zinc finger proteins. The members of this family have a conserved DNA binding domain which is capable of binding to a conserved sequence which has been shown to be present in the promoter regions of many erythroid specific genes (Ko and Engel 1993). Deletion of GATA-1 led to severe defects in primitive erythropoiesis, causing embryonic lethality by E11 (Fujiwara, Browne et al. 1996). Considering the similar phenotypes of the targeted mutations, it is not surprising that SCL, Lmo2, and GATA proteins have been shown to form a transcriptional complex (Wadman, Osada et al. 1997). Many other transcription factors like Myb (Mucenski, McLain et al. 1991), AML-1 (Okuda, van Deursen et al. 1996), EKLF (Nuez, Michalovich et al. 1995; Perkins, Sharpe et al. 1995) have been shown to affect definitive erythropoiesis without affecting yolk sac primitive erythropoiesis.

**Yolk Sac Hematopoiesis**

Hematopoietic development studies in the mouse embryo showed that there are two waves of erythroid development occurring in the yolk sac. The first wave of primitive erythroid progenitors, called EryP-CFC, arise on E7 during the late primitive streak stage, in the region of embryo which is going to form the yolk sac (Palis, Robertson et al. 1999). These progenitors expand over the next 36 hours but by E9, these cells have disappeared from the yolk sac. Along with the erythroid progenitors, the yolk sac also has macrophage progenitors (Mac-CFC) and megakaryocyte progenitors (Meg-CFC) (Xu, Matsuoka et al. 2001; Xie, Chan et al. 2003). The second wave of
erythropoiesis is definitive erythropoiesis that occurs in the yolk sac at E8.25 before circulation is established (Palis, Robertson et al. 1999). These cells expand in the yolk sac and are released into the circulation at E8.5. These progenitors are thought to migrate out and seed the fetal liver. This wave of definitive erythropoiesis is associated with Mac-CFC, GM-CFC and Mast-CFC formation.

High proliferative potential colony forming cells (HPP-CFC) is a term coined for multipotential progenitors first found in the bone marrow, which have the capacity to form macroscopic colonies with more than 50,000 myeloid cells (Bertoncello 1992; Palis, Chan et al. 2001). These are the earliest cells in the hematopoietic hierarchy which can be cultured in vitro without any stromal support. At E8.5, these cells are found exclusively in the yolk sac and coincide with the formation of definitive BFU-E in the yolk sac. These cells expand in the yolk sac until about E11 when the fetal liver becomes the primary site of definitive erythropoiesis, which continues for the rest of embryonic life.

**Definitive Hematopoiesis in the Embryo**

In the adult mice, hematopoiesis occurs almost completely in the bone marrow and in the spleen under specific conditions. Unlike in the adult, the site of hematopoiesis changes multiple times during development in the mouse embryo. Hematopoietic stem cell expansion occurs in the yolk sac until about E12.5, when the fetal liver becomes the main site of hematopoiesis (Johnson and Moore 1975; Kumaravelu, Hook et al. 2002). Other sites which contribute to fetal liver hematopoiesis include the placenta and the aorta gonad mesonephros (AGM) region. It is believed in the field that for these
hematopoietic cells to be formed there has to be a presence of a multipotent HSC in these various tissues (Zon 1995).

In one of the first studies done, yolk sac cells from E8 embryos were transplanted into the yolk sacs of congenic nonablated mice in utero. This study showed that these E8 yolk sacs had HSC activity. In a later study, E9 yolk sac cells were transplanted into Kit deficient fetal mice in utero via a trans-placental route. The authors of this study showed erythroid reconstitution in these transplanted mice about 6 months post transplantation (Toles, Chui et al. 1989). But attempts to repeat this experiment using yolk sac younger than E11 and transplanting them into lethally irradiated adult mice or adult c-kit deficient mice have been unsuccessful (Harrison, Astle et al. 1979; Yoder, Hiatt et al. 1997; Medvinsky and Dzierzak 1999; Cumano, Ferraz et al. 2001). So clearly, the yolk sac HSCs are incapable of reconstituting an adult irradiated mouse, even though these cells can reconstitute embryonic recipients in utero. To check if this inability of yolk sac to engraft adult host was due to their failure to home and engraft in the marrow compartment of adult mice, an experiment was done where these yolk sac cells were transplanted into newborn mice. This model was based on the assumption that the fetal liver was still a hematopoietic organ in the newborn, the yolk sac HSC may engraft in a conditioned newborn mouse (Yoder and Hiatt 1997; Yoder, Hiatt et al. 1997). Both E9 and E11 yolk sac HSCs were able to reconstitute the sublethally myeloablated newborn mice with formation of all the blood lineages and successful transplant of secondary recipients(Yoder, Hiatt et al. 1997). To check if the failure of yolk sac HSCs to engraft adult recipients was because of limited number of cells, Cumano and group developed a system to expand the HSC population isolated from the yolk sac in vitro and checked if
now the yolk sac could rescue an adult irradiated mouse and found that even after 4 days in the expansion culture, there was no long term engraftment (Cumano, Ferraz et al. 2001). Another reason believed for the failure of yolk sac to engraft myeloablated adult mice was that the NK cells in the recipient may be destroying the yolk sac cells which express low levels of Class I MHC. The same group tested this hypothesis by transplanting yolk sac cells into immunodeficient mice lacking NK cells. However, the transplanted yolk sac cells again failed to show evidence for long term reconstitution activity (Cumano, Dieterlen-Lievre et al. 1996; Cumano, Ferraz et al. 2001).

There have been few instances when the pre circulation yolk sac has been able to reconstitute an adult recipient. When yolk sac cells from E8 embryos were isolated and co cultured on a layer of AGM region derived endothelial cell line (AGM-S3), the yolk sac cells could now engraft adult myeloablated mice (Matsuoka, Tsuji et al. 2001). Also, yolk sac cells isolated and transduced with HoxB4, a homeotic transcription factor, also developed the capacity to engraft myeloablated adult mice (Kyba, Perlingeiro et al. 2002). Hence, the pre circulation yolk sac had the ability to engraft an adult myeloablated mouse, if it was exposed to specific conditions which are yet to be understood.

**Intra-Embryonic Hematopoiesis**

In the embryo proper, the site which gives rise to hematopoietic stem cells is called the aorta-gonad-mesonephros (AGM) region. This region surrounds the developing aorta and is in close proximity to the developing gonads, mesonephros, and the mesentery in the splanchnopleura. This region is called the para-aortic splanchopeluric (P-Sp) region before E10 and AGM region after E10 (Godin, Garcia-Porrero et al. 1993). The first time
the P-Sp and AGM region can form long term reconstituting HSC activity when transplanted into adult myeloablated mice was at E10. This is before the same activity can be seen in the yolk sac at E11 (Muller, Medvinsky et al. 1994). E8 P-Sp cells can be isolated and successfully reconstitute an adult myeloablated mouse, if these cells are cultured in vitro (Cumano, Ferraz et al. 2001). Similar to the yolk sac cells, E9 P-Sp cells can be successfully used to engraft a new born mouse (Yoder, Hiatt et al. 1997). Hence, like the yolk sac, cells from the P-Sp and AGM region can successfully engraft an adult myeloablated mouse, once they have been conditioned in vitro but unlike the yolk sac, the maturation process occurs independently by exposure to its own AGM region. Hence, none of the information available at present can conclusively state the site of formation of the first HSC being either the yolk sac or the presumptive AGM region.

Figure 1-7: Dual Stem Cell Origin Model. Adapted from Hematopoiesis: A developmental approach; Zon LI.
One of the most commonly accepted models is the Dual Stem Cell Origin Model where it is agreed upon that on the basis of the information available, the HSCs might be originating from both extra- and intra-embryonic sites and seeding the fetal liver (Godin, Dieterlen-Lievre et al. 1995)

One of the biggest differences between intra- and extra-embryonic hematopoiesis is that unlike in the yolk sac, where the mesoderm gives rise to hematopoietic and endothelial cells simultaneously; in the intra-embryonic compartment, vascular development occurs without concomitant hematopoietic development. In the AGM region, two structures were associated with hematopoietic cell generation. The first one was called the hematopoietic intra-aortic clusters (HIACs). These clusters were found in the ventral aspect of the dorsal aorta in many species including mouse and human (Tavian, Coulombel et al. 1996; Wood, May et al. 1997). These hematopoietic cells were shown to have long term reconstituting capacity suggesting that they were HSCs. These cells are seen only during the peak of precursor generation in this region around E10.5 to E11 (Godin, Garcia-Porrero et al. 1999) and not during the initial formation of hematopoietic precursors. They have been found to be physically inserted between the endothelial cells, which disrupts the endothelial layer (Tavian, Coulombel et al. 1996).

The other structures associated with hematopoietic cell generation in the embryo proper are called the sub-aortic patches (SAPs). These are present below the aorta preferentially below the HIACs but are present throughout the hematopoietic generation phase of the AGM region(Godin, Garcia-Porrero et al. 1999). These structures, unlike HIACs, have not yet been identified in all the species. Also, unlike the HIACs, they are not present in the other developing vessels like the umbilical or omphalo-mesenteric arteries.
Figure 1-8: Different models of HSC generation in the AGM region.
The only direct evidence showing that the HIACs and SAPs can form HSCs comes from lineage tracing experiments done in *Xenopus* embryos where it was shown that blastomeres which give rise to DLP, which is equivalent to the AGM region in the mice, expressed hematopoietic markers (Ciau-Uitz, Walmsley et al. 2000).

**Fetal liver hematopoiesis**

Hepatic development begins around E9 when the endodermal cells from the foregut infiltrates the septum transversum (Rifkind, Chui et al. 1969). Immediately after the liver starts to develop, the hematopoietic cells start to seed the hepatic tissue around E9.5 (Rifkind, Chui et al. 1969; Moore and Metcalf 1970). By E12.5, hematopoiesis is well established in the fetal liver and definitive erythrocytes can be seen in circulation. Once hematopoiesis has been established in the fetal liver, it remains the main site of hematopoiesis until birth and about 2 weeks post-natally (Harrison and Astle 1997; Kurata, Mancini et al. 1998). It is believed that two waves of hematopoiesis colonize the liver. At E9, primitive erythroblasts from the yolk sac form the first wave and later definitive hematopoietic progenitors and HSCs form the second wave at E11 (Dzierzak and Medvinsky 1995). The fetal liver functions to expand the hematopoietic cells during the initial stages of hematopoiesis between E10 and E14. Since the contribution of HSCs from the AGM region is only about 500 to 1000 in number, most of the initial activity seen in the fetal liver is from the expansion of yolk sac hematopoietic cells (Morrison, Hemmati et al. 1995; Godin, Garcia-Porrero et al. 1999). Within 24 hours between E10 and E11, the percentage of primitive erythrocytes drops from 80% to about 10% in the
peripheral circulation suggesting that in the fetal liver, the primitive erythroblast undergo
terminal differentiation, probably in response to the Epo expressed in the fetal
liver (Cumano and Godin 2007). This drop may also represent the fact that there is a rapid
expansion of definitive erythroid progenitors in the fetal liver starting at E10, leading to a
lesser contribution of primitive erythrocytes to the circulating erythrocytes in the
circulation. The HSCs in the fetal liver expand their pool until about E15 (Morrison,
Hemmati et al. 1995). The fetal liver also supports the expansion and differentiation of
hematopoietic progenitors along with the HSC.

**Fetal liver erythropoiesis**

The fetal liver is the first source of abundant definitive erythrocytes in the
developing embryo. Unlike the adult liver, fetal liver is almost completely hematopoietic
and unlike bone marrow, which forms blood types of all lineages, the fetal liver is
primarily erythropoietic. During development, due to the requirements of a rapidly
growing embryo, the fetal erythroid progenitors undergo an exponential expansion of
erythroid progenitors. When compared to the adult progenitors, they exhibit maximal
cycling activity and have a shorter doubling time. In colony assay cultures, fetal liver
BFU-E mature faster than their bone marrow counterparts, but still express globin genes
characteristic of fetal erythrocytes. Studies done on human fetal liver cells show that the
BFU-E exhibit altered growth factor requirements (Peschle, Migliaccio et al. 1981). As
mentioned above, they can give rise to bursts in the presence of Epo alone in the absence
of Burst Promoting Factors like SCF or IL-3, which is required by the bone marrow
progenitors (Valtieri, Gabbianelli et al. 1989). The definitive erythroid progenitors from
the yolk sac start seeding the fetal liver beginning E10 and by E11.5, most of the
erythroid activity in the embryo has moved into the fetal liver. The fetal liver remains the
predominant site of erythropoiesis until after birth, when the bone marrow becomes the
main site of hematopoiesis.

The erythroid progenitors in the fetal liver proliferate, expand and enucleate in
specialized niches termed erythroblastic islands. These islands are characterized by a
central macrophage surrounded by developing erythroblasts (Naito, Hasegawa et al.
1997). Most of the genes shown to play a role in fetal liver erythropoiesis have been
identified because of the roles they play in the formation of these erythroblastic islands.
One of the first molecules shown to play a role in the formation of erythroblastic islands
in vitro was the Erythroblast macrophage protein (Emp) (Hanspal and Hanspal 1994). It
was expressed in both the erythroblasts and the macrophages. Recent work done by the
same group using Emp null mice showed that Emp null embryos die perinatally due to
complete lack of erythroblastic islands in the fetal liver (Soni, Bala et al. 2006). The other
molecules shown to facilitate interaction between the macrophages and erythroblasts in
the fetal liver are vascular cell adhesion molecule-1 (VCAM-1) which is expressed on the
erythroblasts and very late antigen-4 (VLA-4), the counter receptor for VCAM-1, which
is expressed on macrophages. Using blocking antibodies against both molecules, it was
shown that interaction between them was necessary for the formation of erythroblastic
islands (Sadahira, Yoshino et al. 1995). The DNase II gene was shown to be essential for
fetal liver erythropoiesis and it was shown that DNase II released from macrophages was
responsible for the digestion of the nuclei material extruded from the
erythroblasts (Kawane, Fukuyama et al. 2001). The importance of the physical interaction
between macrophages and erythroblasts is exemplified in the retinoblastoma (Rb) mutation mice. Rb null embryos have defects in the macrophages such that the physical interaction between the macrophages and erythroblasts is disrupted leading to prevention of enucleation and terminal differentiation of the erythroblasts leading to severe anemia and mortality (Iavarone, King et al. 2004). However, more recent work has shown that the defect in enucleation seen in the Rb null erythroblasts is independent of macrophage function. The defect in the Rb null embryos to form erythroblastic islands seems to be due to hypoxic stress either in the form of a defective placental function or due to induced hemolytic anemia by Phenylhydrazine injection (Spike, Dibling et al. 2007).

**Fetal liver microenvironment**

The unique microenvironment seen in the fetal liver which allows for expansion of HSCs and progenitors is unlike that of bone marrow and very little work has been done to understand the uniqueness of the fetal liver environment in supporting and expanding hematopoiesis. One study showed that the hematopoiesis in the fetal liver occurred extravascularly in the cords of presumptive hepatocytes (Medlock and Haar 1983). Studies done on rat fetal livers suggested that the hematopoietic cells were in close association with the endothelial cells during their development in the fetal liver (Bankston and Pino 1980). The role of stromal cells in the fetal liver erythroid development was suggested when desmin positive cells were shown to be in close association with the hematopoietic cells in the fetal liver during midgestation (Kiassov, Van Eyken et al. 1995). When the activity of CFU-F was studied, which measures number of fibroblasts formed (which ultimately form the stromal cells in the fetal liver), and correlated with the
hematopoietic activity, there was a clear temporal correlation between the two suggesting that hematopoietic cells need fetal liver stromal cells for their lodgment and expansion (Wolf, Bertoncello et al. 1995).

More recently, it was shown that the fetal liver had a specialized population of cells called the epithelial to mesenchymal transition (EMT) cells (Chagraoui, Lepage-Noll et al. 2003). These cells expressed both epithelial and mesenchymal markers on them and they were present in the fetal liver throughout the hematopoietic phase of fetal liver development and go down as the fetal liver matures into an adult hepatic organ. They went on to show that these cells were present in the stromal cells derived from E14 fetal liver which could support hematopoietic growth. They also showed that OncostatinM reduced the hematopoietic supporting capacity of fetal liver at least in part by differentiating these EMT cells into either epithelial or mesenchymal cells which no longer could support hematopoietic activity.

Mouse stromal cell lines have been established from E13 fetal livers and these have been shown to support proliferation and differentiation of erythroid progenitors in colony assays (Ohneda, Yanai et al. 1990). When erythroid progenitors from either fetal liver or bone marrow were layered on these stromal cells, large Benzidine positive cells were formed within 4 days. These cells were more mature than a BFU-E and closer to a CFU-E but with properties different from CFU-E. These cells, when in contact with the fetal liver stromal layer, could divide rapidly and form large colonies (Ohneda, Yanai et al. 1990; Obinata and Yanai 1999). These results show that the stromal environment in the fetal liver might be playing a pivotal role in the rapid expansion of hematopoietic progenitors seen in the fetal liver during midgestation.
Seeding of the adult hematopoietic organs

The hematopoietic stem/progenitor cells following expansion in the fetal liver start seeding the fetal spleen and bone marrow at around E15 and E17.5 respectively. The process of seeding occurs gradually over 2 to 3 days and is not due to a sudden influx of HSCs into the organ (Christensen, Wright et al. 2004). Once the fetus is born, the fetal liver still functions as a site of hematopoiesis, but gradually is taken over by the spleen and bone marrow; which become fully functional sites of hematopoiesis about 2 weeks following birth.

Homeostatic and Expansive Erythropoiesis

The role of homeostatic or steady state erythropoiesis is to replace the erythrocytes that are worn out due to ageing and removed by the spleen. Expansive or stress erythropoiesis on the other hand, is induced when there is a need for a rapid expansion of the erythroid precursors to compensate for a depleted erythroid pool. The expansive erythropoiesis can occur in response to an acute induction of acute anemia like sudden massive blood loss, or can be in response to inefficient erythropoiesis as in hemoglobinopathies.

The mechanisms regulating expansive erythropoiesis are distinct from the ones regulating steady state erythropoiesis. This regulation can occur at the level of microenvironment where the erythroid pool can expand rapidly, it can involve a new set of signals which may not play a pivotal role in steady state erythropoiesis, and it can
involve set of erythroid progenitors which are poised to respond only under conditions of rapid erythroid depletion.

Analysis of murine erythropoiesis has shown that after phlebotomy, the contribution of spleen to erythropoiesis increased from 10% to 40% (Sadahira, Yasuda et al. 2000). A study done showed the increase in splenic activity to migration of progenitors from the bone marrow to spleen (Hara and Ogawa 1977). Following a 3 day regimen of Phenylhydrazine (PHZ), which induces a rapid hemolytic anemia, they saw an increase in CFU-E activity in the bone marrow, but declining numbers of BFU-E. But in the spleen, the numbers of both BFU-E and CFU-E increased until day 4 before going down to normal numbers. They also observed BFU-E in the blood, which was maximal at day 2. This suggested that there was migration of BFU-E from the bone marrow to the spleen through the bloodstream. Work in our laboratory suggested a new model. Similar to the earlier findings, we see that there is no elevation in the numbers of BFU-E in the bone marrow following PHZ treatment and in fact reduced over time. But, we never see any BFU-E activity in the peripheral blood at any time points following PHZ treatment. More importantly, there was an expansion of BFU-E in the spleen that peaked at just 36 hours post anemia induction. These results suggest a model where erythroid progenitors, which are resident in the spleen, are poised to respond immediately to an induction of acute anemia (Lenox, Perry et al. 2005).

Spleen has been shown to be a site of hematopoiesis where erythropoiesis prevails. CFU-S assays look for the formation of macroscopic colonies formed in the spleen or irradiated recipients about 9-12 days after injection of cells. This assay detects for the capacity of multipotential progenitors to differentiate into the erythroid lineage. In
a CFU-S assay, erythroid colonies predominate in the spleen, with the red pulp retaining most of the erythroid activity (Obinata and Yanai 1999). Many cell lines have been established from new born spleens which can support the formation of erythroid colonies with either fetal liver or adult bone marrow cells. The erythroid colonies supported by these spleen stromal cell lines are distinct from those formed by co-culture on bone marrow stromal cell lines like PA6. The colonies are composed of up to 1000 benzidine staining erythroid cells which are formed in 4 to 6 days and were much larger than a typical CFU-E. They were distinct from a typical BFU-E in that they were less dependent on Epo, colony size, benzidine staining, and the time of appearance (Yanai, Matsuya et al. 1989). Conditioned medium from these cell lines did not support the erythroid colony formation suggesting that cell-cell contact was required was required for their supportive function.

Hypoxemia (reduced oxygen delivery to the tissues) has been known to stimulate expansive erythropoiesis. Hypoxemia can be induced by hypoxia (reduced oxygenation in the lung), anemia (reduced RBC or hemoglobin in the blood), or by conditions which prevent proper association or disassociation of oxygen from hemoglobin (like carbon monoxide or cyanide poisoning). Epo production is induced in the kidney in response to hypoxia leading to increased circulating Epo titers. Epo can also be induced experimentally in the laboratory by phlebotomy, hemolysis using drugs like PHZ, or cobalt administration (Bozzini, Alippi et al. 1994). The increase in serum Epo concentration that occurs in response to acute anemia is due to formation of a complex involving Hypoxia inducible factor-1α (HIF-1α), leading to increased transcription of Epo (Ebert and Bunn 1999).
Apart from Epo, many other signals are involved in the recovery from acute anemia. Stem cell factor (SCF) plays an important role in the development and expansion of erythroid progenitors (Russell 1979; Bernstein, Forrester et al. 1991; Ashman 1999). It is encoded by the murine Steel (Sl) locus and its receptor Kit is encoded by the murine Dominant white spotting (W) locus (Geissler, Ryan et al. 1988; Copeland, Gilbert et al. 1990; Huang, Nocka et al. 1990; Zsebo, Williams et al. 1990). Both of these mutations cause severe anemia, due to a defect in the development of erythroid progenitors CFU-E (Nocka, Majumder et al. 1989). In addition to this, they also exhibit a delayed recovery from PHZ induce acute anemia (Harrison and Russell 1972; Perry, Harandi et al. 2007) due to a delayed expansion of progenitors in the spleen suggesting they play a role in expansive erythropoiesis too.

More recently, we have shown that SCF and hypoxia work in concert with BMP4 to drive the expansion of stress erythroid progenitors in the spleen during the recovery from acute anemia. We also show that hypoxia alters the response of erythroid progenitors to BMP4 and SCF. Spleen erythroid progenitors cultured in vitro in the presence of BMP4 and SCF in hypoxic conditions results in the expansion of a population of erythroid progenitors that express kit, CD71 and TER119 together on their cell surface (Perry, Harandi et al. 2007). The combination of early hematopoietic (Kit) and late erythroid markers (TER119) together is not observed on bone marrow erythroid progenitors. Moreover, the differentiation pattern of these stress erythroid progenitors is different from that described for bone marrow progenitors(Perry, Harandi et al. 2007).

Glucocorticoids are lipophilic hormones, which are involved in various physiological processes. They have been shown to increase colony size and enhance
proliferation of erythroid precursors in vivo (Golde, Bersch et al. 1976; Udupa, Crabtree et al. 1986). The glucocorticoid receptor was shown to induce the expansion of a particular population of erythroid progenitors following stress situations like hemolysis and hypoxia (Bauer, Tronche et al. 1999). They went on to show that an erythroid progenitor in the spleen with cell surface characteristics of both early hematopoietic (CD34+kit+) and late erythroid markers (TER119) required the glucocorticoid pathway for its expansion under condition of acute anemia. This is similar to the stress erythroid progenitors described earlier expressing kit, CD71 and TER119 together on their surface. Our analysis of the Kit+ CD71+ TER119+ cells has shown that they are CD34+ suggesting the role for glucocorticoids along with BMP4, SCF, and hypoxia in stress erythropoiesis.

Bone morphogenetic proteins (BMPs) particularly BMP4 has been implicated in the development of mesodermal cells which give rise to hematopoietic cells early during development (Huber, Zhou et al. 1998). We have shown that in response to acute anemia, BMP4 is induced in the spleen. It acts on an immature progenitor called the BMP4R cell causing it to differentiate into an Epo responsive erythroid progenitor which we call a stress BFU-E. The BMP4R cell is contained within the spleen megakaryocyte erythroid progenitor (MEP) but is not seen in the bone marrow. The stress erythroid progenitor can form BFU-E in the presence of Epo only and do not require the addition of a burst promoting factor like SCF or IL-3. The colonies formed by the stress BFU-E are larger than those formed by bone marrow BFU-E and take only about 5 days to mature (Lenox, Perry et al. 2005). This is similar to the human fetal liver erythroid progenitors, which
also required only Epo to form BFU-E and had much faster cycling than the bone marrow BFU-Es (Peschle, Migliaccio et al. 1981).

**flexed tail Mutant Mice**

The *flexed tail* (*f*) mutant mice were first identified in 1928 (Hunt, Mixter et al. 1933). It was a spontaneous autosomal recessive mutation first characterized by a tail flexure. The phenotype which was of interest to our lab is the fetal-neonatal anemia which was present during fetal development and resolves about 2 weeks after birth (Mixter and Hunt 1933). The *f/f* mice then exhibit normal steady state blood parameters as adults, but are defective in responding to acute erythroid stress (Coleman, Russell et al. 1969). This early work suggested that the *f* locus was involved in the regulation of erythropoiesis that is observed only under conditions of rapid proliferation (Thompson, McCulloch et al. 1966). This observation that fetal liver erythropoiesis and response to acute anemia is defective in the *f/f* mice suggested a common genetic link between fetal erythropoiesis and expansive erythropoiesis seen in adult mice.

Adult *f/f* mice appear normal under homeostatic conditions. They have normal numbers of erythrocytes and erythroid progenitors like BFU-E and CFU-E both in the bone marrow and spleen (Gregory, McCulloch et al. 1975). The sensitivity to Epo for erythroid progenitors were similar between *f/f* and control mice (Gregory, McCulloch et al. 1975; Cole and Regan 1976). When CFU-S assay was done with bone marrow cells, even though the numbers between *f/f* and control were similar, the CFU-S were devoid of erythroid cells in the *f/f* mice which normally form majority of the CFU-S colony (Thompson, McCulloch et al. 1966). Also, the *f/f* mice were incapable of forming
transient endogenous spleen colonies called TE-CFU. These are committed erythroid progenitors which rapidly form colonies in the spleen in response to lethal doses of irradiation in vivo and hence makes this a much better assay compared to BFU-E and CFU-E which are analyzed in vitro (Gregory, McCulloch et al. 1975). These cells have the capacity to produce a large number of progeny (~10^5) in about 5 days on exposure to Epo and radiation (Gregory, McCulloch et al. 1975). Hence, the characteristics of TE-CFU make it a candidate for a specialized progenitor which responds to conditions of acute erythroid stress. When an experiment was done comparing the time course of recovery of f/f and control mice from irradiation, the maximal TE-CFU activity was seen at 6 days in the control mice whereas in the f/f mice there was minimal formation of TE-CFU. Hence the f locus plays a critical role in the formation of TE-CFU. f/f mice also exhibit a delay in recovery from PHZ induced acute anemia, as shown by a delay in the expansion of erythroid progenitors in the spleen along with a delay in the appearance of reticulocytes in the peripheral blood (Coleman, Russell et al. 1969). Taken together, these data demonstrate that the f locus plays a key role in the expansion of erythroid progenitors in the adult mice following acute anemia.

The f/f embryos have a transient microcytic, hypochromic anemia which resolves about 2 weeks after birth. The red cell count is approximately 80% of the normal embryos in the f/f embryos. The f/f fetal reticulocytes contain only about 70% of the normal hemoglobin present within a red cell, thus the f/f newborn mice have only about half the hemoglobin of a normal newborn mice (Cole, Garlick et al. 1974). Along with the anemia, the reticulocytes of f/f mice have non-heme iron granules which are called siderocytes. These siderocytes are localized within the mitochondria of the reticulocytes
(Chui, Sweeney et al. 1977). There are two kinds of defects in the $f/f$ embryos. One which affects the proliferation of erythroid progenitors leading to anemia and the other where the terminal differentiation and maturation of erythroid precursors is defective leading to defective hemoglobinization and formation of siderocytes in the fetal reticulocytes (Bateman and Cole 1972; Cole, Garlick et al. 1974).

The anemia in the $f/f$ embryos is especially pronounced between E13 and E16 suggesting that the defect may be primarily in definitive erythropoiesis occurring in the fetal liver (Russel, Thompson et al. 1968). The $f/f$ fetal livers are smaller in size and contain decreased numbers of erythroid progenitors and identifiable erythroblasts (Coleman, Russell et al. 1969; Bateman and Cole 1972; Cole and Regan 1976). As seen in the spleen of mice receiving bone marrow cells from $f/f$ mice, the fetal liver cells when injected into irradiated recipients formed CFU-S devoid of the erythroid cells suggesting that the erythroid differentiation in CFU-S was severely compromised (Bateman and Cole 1972). The same group also looked at the differentiation capacity of erythroid progenitors by measuring radiolabeled iron incorporation into the blood of recipients transplanted with fetal liver cells from $f/f$ and control mice. They showed that there were reduced numbers of erythroblasts in circulation. They also showed that the peak of CFU-E numbers reached much slower in the $f/f$ transplanted mice and the peak values of CFU-E in $f/f$ fetal liver transplanted reached only half in numbers compared to the control mice (Bateman and Cole 1972).

Hemoglobin synthesis is a highly regulated process where the synthesis of $\alpha$ and $\beta$ globin chains are tightly regulated with the production of heme. $f/f$ fetal reticulocytes have a 50% reduction in $\beta$ globin chain synthesis which leads to an imbalance in the $\alpha:\beta$
chain ratio (Chui, Sweeney et al. 1977). There has been some evidence of reduced activity of enzymes involved in the heme biosynthetic pathway, but no direct evidence has been shown for them to be the cause of the accumulation of the iron granules in the fetal reticulocytes (Coleman, Russell et al. 1969; Cole, Regan et al. 1972; Cole, Garlick et al. 1974; Chui, Sweeney et al. 1977). Regardless of the cause, it’s clear that on a whole, the siderocytic granules and reduced hemoglobin seen in the \textit{f/f} embryos arises from the overall disruption in the coordinate disruption of hemoglobin components.

The \textit{f} mutation was initially reported using linkage mapping to be due to a frameshift mutation in a mitochondrial transmembrane protein called \textit{Sideroflexin1} (\textit{Sfxn1}) (Fleming, Campagna et al. 2001). But work in our laboratory has clearly shown that the mutation in the \textit{f/f} mice is in a gene called \textit{Smad5}, a transcription factor functioning downstream of the bone morphogenetic protein (BMP) pathway (Lenox, Perry et al. 2005). Since there is only a single allele of \textit{f} mutation, all \textit{f/f} mice should carry the same mutation. Work in our lab suggests that the \textit{f} mutation has separated from the \textit{Sfxn1} mutation by recombination and hence, mutation of \textit{Sfxn1} cannot be responsible for the phenotype seen in \textit{f/f} mice. Further analysis of the mutation has shown that the mutation causes altered mRNA splicing of \textit{Smad5}. The predominant mRNA in the \textit{f/f} spleens were mis-spliced with very little normal full length transcripts being formed. The consistent features of these mis-spliced mRNAs have been the deletion of exon 2, which contains the initiator AUG codon for \textit{Smad5}. Further work has been done to better identify the mutation and has been included in Appendix B of this thesis. (Figure 1-9)
The TGFβ (transforming growth factor) superfamily of cytokines has more than 30 signaling molecules which include the BMP, activin, nodal, TGFβ and other related proteins (ten Dijke and Hill 2004). BMPs were originally identified for their role in bone formation and since then have been implicated in a variety of functions (Hogan 1996). BMP is the vertebrate homolog of drosophila Decapentaplegic (Dpp) protein (Padgett, Wozney et al. 1993). Most of the members, including BMP4, upon binding induce the formation of a heterodimeric complex between Type I and Type II receptors which are serine threonine kinases (Derynck and Zhang 2003). Within this tetrameric complex, the Type II receptors can then transphosphorylate the Type I receptors. The activated Type I

BMP4 Signaling in Hematopoiesis

The TGFβ (transforming growth factor) superfamily of cytokines has more than 30 signaling molecules which include the BMP, activin, nodal, TGFβ and other related proteins (ten Dijke and Hill 2004). BMPs were originally identified for their role in bone formation and since then have been implicated in a variety of functions (Hogan 1996). BMP is the vertebrate homolog of drosophila Decapentaplegic (Dpp) protein (Padgett, Wozney et al. 1993). Most of the members, including BMP4, upon binding induce the formation of a heterodimeric complex between Type I and Type II receptors which are serine threonine kinases (Derynck and Zhang 2003). Within this tetrameric complex, the Type II receptors can then transphosphorylate the Type I receptors. The activated Type I
receptors can now phosphorylate downstream intracellular molecules like Smads following their recruitment to the complex (Massague 2000). The various combinations of Type I and Type II receptors allow for differential signaling following binding with the same ligand (Derynck and Feng 1997; Moustakas, Souchelnytskyi et al. 2001) (Figure 1-10).

The name Smad was coined from its sequence similarity to the Mad (mothers against decapentaplegic) gene in Drosophila and the related Sma genes in Caenorhabditis elegans both of which have been implicated in signal transduction by factors of the TGFβ family (Derynck, Gelbart et al. 1996). Smads are subdivided based on their functions into Receptor regulated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, and Smad8), the common mediator Smad (Co-Smad or Smad4), and the inhibitory Smads (I-Smads: Smad6 and Smad7). Among the R-Smads, Smad2 and Smad3 carry out TGFβ signaling whereas Smad1, Smad5, and Smad8 transduce BMP signaling. The N-terminal end of Smad, also called the Mad-homology-1 domain or MH-1 domain contains the DNA binding domain and is required for nuclear localization whereas the MH-2 domain is required for the receptor Smad interaction. Both domains have been shown to be needed for protein-protein interactions (Xiao, Watson et al. 2001). The activated Smad binds to Smad4 and then translocates into the nucleus where gene specific activation occurs.

A new level of complexity was added to the TGFβ Smad signaling pathway when transcriptional intermediary factor 1 (TIF1γ) was identified. It was shown that TIF1γ can also bind to the R-Smads in place of Smad4 and in hematopoietic stem/progenitor cells.
R-Smads had different effects depending on whether they were bound to TIF1γ or Smad4 (He, Dorn et al. 2006). Association of TIF1γ with Smad2/3 stimulated erythroid differentiation in hematopoietic stem/progenitor cells whereas association of Smad4 with Smad2/3 led to the inhibition of proliferation in these cells in response to TGFβ. The

Figure 1-10: TGFβ Smad signaling. (Moustakas, Souchelnytskyi et al. 2001)
interaction between TIF1γ and Smad2/3 was shown to be specific in that Smad1 could not interact with TIF1γ. The closest homologue of TIF1γ in zebrafish is encoded by moonshine (mon), an essential gene in blood formation (Ransom, Bahary et al. 2004). Interestingly, TIF1γ is expressed in high levels in the blood islands of yolk sac (He, Dorn et al. 2006) eventhough no role has been yet shown for TIF1γ in yolk sac hematopoiesis.

Work in our laboratory has shown that in response to acute anemia, BMP4 is rapidly induced in the spleen and is turned off after the initial expansion of erythroid cells (Lenox, Perry et al. 2005). BMP4 acts on an immature progenitor called the BMP4R cell causing it to differentiate into an Epo responsive erythroid progenitor which we call stress BFU-E. The BMP4R cell is contained within the spleen megakaryocyte erythroid progenitor (MEP) but is not seen in the bone marrow. The stress BFU-E can form BFU-E in the presence of Epo only and do not require the addition of a burst promoting factor like SCF or IL-3. The colonies formed by the stress erythroid progenitors are larger than those formed by bone marrow progenitors and take only about 5 days to mature (Lenox, Perry et al. 2005).

In the f/f mice, BMP4 expression is completely deregulated following PHZ treatment. This deregulation suggests that a Smad5-dependent signal may be needed for the expression of BMP4 in the spleen of untreated mice. f/f spleens do not increase the formation of stress BFU-E in response to BMP4 unlike the control mice and they are delayed in responding to the acute anemia and the expansion of stress BFU-E in the spleen is delayed until 4 days following PHZ treatment unlike the control mice which have a peak of stress BFU-E formation within 36 hours (Lenox, Perry et al. 2005). Hence, the work in our laboratory shows that the f/f mice have defect in expanding the erythroid
pool rapidly at time of acute erythropoietic stress due a defective BMP4-Smad5 signaling.

**Hedgehog signaling in Hematopoiesis**

The Hedgehog (Hh) family of proteins comprises Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh) in mammals. Shh is the most well studied. Once secreted, Shh exerts most of its action through a dual receptor system comprised of Patched1 (Ptch1) and Smoothened (Smo) (Riobo and Manning 2007). Ptch1 is a twelve-pass transmembrane protein that normally represses the activity of a seven-pass transmembrane protein called Smoothened (Smo). When Shh binds to Ptch1, it causes the inhibition of the repressive action of Ptch1 on Smo. Smo can now act on Gli family of transcription factors to transduce the hedgehog signaling pathway. Members of the Gli family of zinc finger transcription factors can then function either as activators or repressors to carry out Hh signaling. Figure 1-11 represents a simplified version of mammalian hedgehog signaling.

**Role of Hedgehog signaling in yolk sac hematopoiesis**

It was known for quite some time that Ihh is expressed specifically in the VE on the gastrulating embryo and in the mature yolk sac (Echelard, Epstein et al. 1993; Farrington, Belaoussoff et al. 1997) it was only shown recently that Ihh alone is sufficient to induce formation of hematopoietic and endothelial cells in the embryo (Dyer, Farrington et al. 2001). Actually, VE can be separated from the epiblast and still Ihh
alone can induce blood formation in the epiblast tissue (Belaoussoff, Farrington et al. 1998) and can convert the neuroectodermal fate of anterior ectoderm into a hematopoietic fate which is normally the fate of posterior ectoderm (Belaoussoff, Farrington et al. 1998). All the receptors for Hh including Ptch, Smo, and Gli1 have been shown to be expressed in the target epiblast tissue which is adjacent to the VE and forms the blood cells suggesting that the epiblast is capable of responding to Hh signaling from the VE (Dyer, Farrington et al. 2001). When anterior epiblast cells after exposure to Hh was plated in secondary cultures, both primitive (Dyer, Farrington et al. 2001) and definitive

Figure 1-11: Overview of Hedgehog signaling (Riobo and Manning 2007).
(Baron 2003) hematopoietic colonies were formed suggesting that Hh signaling can produce functional hematopoietic stem/progenitor cells. A recent study has suggested that SCL might be a direct target of Hh signaling during hemangioblast specification (Hochman, Kinston et al. 2006). Despite the seemingly important role for Hedgehog signaling in yolk sac hematopoiesis, analysis of both Ihh-/- and Smo-/- yolk sac showed a severe defect in angiogenesis with comparatively normal hematopoietic formation (Byrd, Becker et al. 2002). (Figure 1-12)

![Presumptive Model for role of Hh and BMP4 signaling in Yolk Sac Blood Formation](image)

**Figure 1-12**: Presumptive Model for role of Hh and BMP4 signaling in Yolk Sac Blood Formation. (Byrd, Becker et al. 2002)
Work on \( ptc-1^{+/+} \) mouse, which has increased hedgehog expression, showed that there was increased cycling and expansion of hematopoietic stem cells and progenitors leading to hematopoietic regeneration. However, deregulated expression of Hh leads to HSC exhaustion leading to reduced ability for hematopoietic regeneration on secondary transplantation (Trowbridge, Scott et al. 2006). The hematopoietic lineages are formed in the same percentages suggesting that the expansion of hematopoietic cells was not skewed towards any particular lineage. The same group had shown earlier that this proliferative capacity of Hh was specifically through its downstream target BMP4 as they could inhibit the Hh activity by addition of Noggin which inhibits BMP4 signaling (Bhardwaj, Murdoch et al. 2001). A recent article has shown that Hh receptor Ptch controls the lineage commitment of a multipotent progenitor into lymphoid progenitors without affecting the myeloid lineage by blocking the formation of common lymphoid progenitor (CLP) (Uhmann, Dittmann et al. 2007). Hence, clearly Hh signaling plays a key role in the formation and establishment of hematopoietic cells, possibly through BMP4 dependent and independent mechanisms, but its role in erythroid development is not understood. Some of the work in our lab studying the role of Hh signaling in erythroid commitment and development will be covered in Appendix D in this thesis.
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Chapter 2

BMP4/Smad5 dependent stress erythropoiesis is required for the expansion of erythroid progenitors during fetal development

Foreword

The following chapter is adapted from the manuscript prepared for submission to the journal *Developmental Biology*. The manuscript was written with Dr. Robert F Paulson. All the work in the manuscript is done by the author along with Dr. Robert F Paulson.

Abstract

The rapid growth of the embryo places severe demands on the ability of the cardiovascular system to deliver oxygen to cells. To meet this need, erythroid progenitors rapidly expand in the fetal liver microenvironment such that by E14.5, erythropoiesis predominates in the fetal liver. In this report we show that the BMP4/Smad5 dependent stress erythropoiesis pathway plays a key role in the expansion of erythroid progenitors in the fetal liver. These data show that the fetal liver contains two populations of erythroid progenitors. One population resembles the steady state erythroid progenitors found in the adult bone marrow. While the second population exhibits the properties of stress erythroid progenitors found in adult spleen. Here we demonstrate that defects in BMP4/Smad5 signaling preferentially affect the expansion of the stress erythroid progenitors in the fetal liver leading to fetal anemia. These data suggest that steady state erythropoiesis is unable to generate sufficient erythrocytes to maintain the rapid growth of the embryo leading to the induction of the BMP4 dependent stress erythropoiesis
pathway. These observations underscore the similarities between fetal erythropoiesis and stress erythropoiesis.

Key words: Fetal liver, Erythropoiesis, BMP4

Introduction

Hematopoiesis during embryogenesis occurs in distinct anatomical locations of the mammalian embryo. In the mouse, the first blood cells arise in yolk sac blood islands at embryonic day 7.5 (E7.5)(Palis et al., 1999). These ‘primitive’ erythroblasts are large, initially nucleated, and synthesize embryonic globins (Kingsley et al., 2004). In contrast, ‘definitive’ erythrocytes are smaller, enucleated, and express adult globins. From E9.5 to E11.5, definitive progenitors formed in the yolk sac and the Aorta-Gonad-Mesonephros (AGM) region are thought to seed the fetal liver, which remains the predominant site of definitive erythropoiesis in the embryo until birth (McGrath and Palis, 2005).

Unlike the bone marrow, where myelopoiesis predominates, erythropoiesis is the primary function of the fetal liver from E12.5 until about E18.5, after which the bone marrow becomes the primary erythropoietic organ in the adult (Johnson and Moore, 1975; Palis et al., 1999). Fetal erythroid progenitors exhibit maximal cycling activity and have shorter doubling times than adult bone marrow erythroid progenitors resulting in a rapid expansion of these progenitors during development. The most immature lineage committed erythroid progenitor is the Burst Forming Unit Erythroid (BFU-E), which form large burst colonies after 7 days in culture. Analysis of bone marrow BFU-E showed that they required erythropoietin (Epo) and a second signal referred to as a Burst promoting activity or BPA to form colonies in vitro. Unlike bone marrow progenitors,
human fetal liver progenitors can give rise to bursts in the presence of Epo alone without any added BPA, which further underscores the differences between adult bone marrow erythropoiesis and fetal liver erythropoiesis (Emerson et al., 1989; Valtieri et al., 1989). In addition to the progenitors, the fetal liver microenvironment also contributes to the maintenance of erythropoiesis in the embryo. In vitro experiments have shown that fetal liver stromal cell lines have the unique ability to promote erythropoiesis when compared to bone marrow stromal cell lines (Ohneda et al., 1990; Slaper-Cortenbach et al., 1987).

The expansive erythropoiesis of the fetal liver is thought to be mechanistically similar to stress erythropoiesis in adult mice. In response to acute anemia, erythropoiesis is up-regulated in the spleen and robustly produces new erythrocytes (Lenox et al., 2005). Fetal liver erythropoiesis and adult stress erythropoiesis are in stark contrast to adult bone marrow erythropoiesis, which is primarily homeostatic. This relationship between fetal liver and adult stress erythropoiesis is evident in mice with a mutation at the flexed-tail (f) locus. f/f embryos exhibit a severe microcytic, hypochromic fetal anemia (Bateman and Cole, 1972; Gruneberg, 1942a; Gruneberg, 1942b). The livers of these embryos are smaller and have only about half the normal number of CFU-E (Cole and Regan, 1976). Despite this severe defect in the erythroid lineage, the number of CFU-S in the fetal liver is not different from the control embryos suggesting this defect is specific to the erythroid lineage (Bateman and Cole, 1972; Gruneberg, 1942a; Gruneberg, 1942b; Thompson et al., 1966). The anemia is most severe early in fetal development and gradually improves such that the anemia resolves about two weeks after birth. This time corresponds to the development of the bone marrow as the primary erythropoietic organ. As adults, f/f mice have normal blood values but are unable to respond rapidly to acute erythropoietic stress.
When challenged with an acute anemia, the control mice respond by inducing the rapid expansion of erythroid progenitors in the spleen but in the *f/f* mice, this response is delayed (Coleman et al., 1969; Lenox et al., 2005). These data demonstrate that *f/f* mice have a defect in expansive erythropoiesis both during fetal life and during response to acute anemia in the adult.

We have previously shown that *f/f* mice have a mutation in the *Madh5* or *Smad5* gene, which results in a defect in their ability to respond to acute anemia (Lenox et al., 2005). Smad5 functions as a receptor activated Smad downstream of the receptors for BMP2, 4 and 7 (Huber et al., 1998; Massague, 2000; Massague and Chen, 2000). Previous work has implicated BMPs, in particular BMP4, in the development of mesodermal cells that will give rise to hematopoietic cells early in development (Huber et al., 1998). We have observed that BMP4 is rapidly induced during expansive erythropoiesis in the spleen and is involved in the mobilization of a distinct population of stress erythroid progenitors, which we term stress BFU-E, during the recovery from acute anemia (Lenox et al., 2005). Similar to human fetal liver BFU-E, stress BFU-E exhibit faster doubling times and are capable of forming BFU-E colonies in the presence of Epo alone. These observations suggest that fetal liver erythropoiesis may be mechanistically similar to splenic stress erythropoiesis and utilize the BMP4/Smad5 signaling pathway to expand progenitors.

In this report we show that the fetal liver contains two populations of erythroid progenitors. One population behaves like steady state bone marrow BFU-E in that they require both Epo and a BPA to form colonies. The second population exhibits the properties of stress BFU-E in that they are able to form BFU-E in the presence of Epo
alone and respond to BMP4. \(f/f\) mutant mice exhibit a delay in the expansion of these fetal stress BFU-E, which leads to the fetal anemia in these mutant embryos. The expansion of the stress BFU-E in the fetal liver is correlated with the induction of BMP4 expression by stromal cells in the fetal liver. The delay in stress BFU-E expansion in \(f/f\) embryos is not caused by excessive apoptosis or defects in terminal erythroid differentiation, but rather a delay in the expansion of progenitor cells. In addition we demonstrate that stress BFU-E are present in the CD31+Kit+Sca1-Lin- (CD31+) fraction of fetal liver cells. This population is reduced by 50% in the \(f/f\) mice and is defective in forming stress BFU-E when compared to the CD31+ fetal liver cells from wild type controls. Furthermore, we show that the yolk sac contains stress BFU-E and \(f/f\) yolk sacs contain fewer stress BFU-E than controls, which may also contribute to the increased severity of their anemia early in development. Taken together these data support a model where the BMP4 dependent stress erythropoiesis pathway plays a key role in the expansion of erythroid progenitors at critical junctures during development in the yolk sac and fetal liver.

**Methods**

*Preparation of fetal liver*

C57BL/6 and C57BL/6-\(ff\) mice were bred in our colony. All mice were approximately 2 months old, controls were age matched. Timed pregnancies were set up in the late afternoon and the appearance of vaginal plug in the morning was designated as 0.5 days (E0.5). Pregnant mice were sacrificed at different time points between E12.5 and E18.5. Fetal livers were dissected from embryos and single cell suspension was made by
passing liver cell suspensions through 18G and 21G needles and through a nylon cell strainer. Absolute numbers of fetal liver cells were determined by standard cell counting. For immunofluorescence, the isolated fetal livers were fixed in 4% Paraformaldehyde, embedded in paraffin and sectioned for analysis. All procedures were approved by the IACUC at the Pennsylvania State University.

Immunofluorescence studies

Paraffin embedded fetal liver sections were deparaffinized and rehydrated through an ethanol series as described previously (Lenox et al., 2005). Sections were incubated for 1 hour each at room temperature with the primary antibody and then washed with PBS. Anti-BMP4 (Novocstra Laboratories/Vector Laboratories, Burlingame, CA) and Smad5 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used at the manufacturer’s recommended dilutions (Lenox et al., 2005). Alexa Fluor (Molecular Probes, Eugene, OR) fluorescent secondary antibodies were then added. For negative controls, appropriate isotype controls were used. Sections were mounted in Slowfade (Molecular Probes, Eugene, OR) and analyzed by digital microscopy (Olympus BX-60 Epi-Fluorescence Digital digital microscope).

Erythroid progenitor assays

5X10^5 Fetal liver cells were plated in methylcellulose media containing 3U/ml Epo (M3334 Stem Cell Technologies, Vancouver, BC). 15ng/ml BMP4 (R&D Systems, Minneapolis, MN) and 2.5ng/ml IL-3 (Sigma, St. Louis, MO) were added wherever indicated. Replicates were plated and at least three independent experiments were
performed for each time point. BFU-E was scored by acid Benzidine staining after incubation for 5 days (Finkelstein et al., 2002).

**TUNEL assay for apoptosis**

E14.5 fetal liver sections were prepared and permeabilized as described earlier. Apoptosis in tissue sections was detected using an in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer’s recommendations.

**Fluorescence activated cell sorting analysis**

E14.5 fetal liver single cell suspensions were stained with biotin-conjugated Mouse lineage Panel (BD Pharmingen, San Diego, CA). Biotinylated Sca-1 (BD Pharmingen, San Diego, CA) was added to the cocktail and the cells were negatively selected for using EasySep Biotin Selection Kit for Mouse Cells (StemCell Technologies, Vancouver, BC, Canada). Cells were stained with fluorescein isothiocyanate-conjugated anti-cKit antibody (BD Pharmingen, San Diego, CA) and phycoerythrin-conjugated anti-CD31 (Mec 13.3) (BD Pharmingen, San Diego, CA) and sorted using a Coulter Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL) for Lin- Sca1- cKit+ CD31+/- cell populations (Baumann et al., 2004). Fetal liver MEPs were sorted as previously described (Lenox et al., 2005; Traver et al., 2001). 25,000 cells each were plated in triplicates in methylcellulose and scored as described above.
Differentiation of fetal erythroid progenitors

E14.5 fetal liver cells were isolated and sorted for Lin-Kit+ cells as described above. Sorted cells were either analyzed directly or plated in an erythroid differentiation media (Isocove’s Modified Dulbecco’s Medium was supplemented with 5% Fetal Bovine Serum, 3U/ml Epo and 10µg/ml Insulin) for 2 days and then analyzed (Panzenbock et al., 1998). The cells were then cytospun on to slides and then stained with o-Dianisidine (Sigma, St. Louis, MO) and counterstained with Wright-Giemsa cytology stain.

Yolk sac cell isolation and co-culture with fetal liver stromal cells.

Yolk sacs were dissected out of embryos at particular time points as described earlier. Single cell suspensions of tissues were obtained by treating with 0.25% Trypsin/EDTA (Cellgro) for 3-4 minutes at 37 degrees with vigorous pipetting as described earlier (Palis et al., 2001). Cell count and viability was quantitated after staining with Trypan Blue. 0.67 embryo equivalents were plated in each well in triplicates either directly or onto AFT024 cells. Co-culture with AFT024 cells was done as follows. AFT024 cells were grown into confluency at 33C and then were moved to 37C to stop their proliferation. A single cell suspension of yolk sac cells was layered onto the AFT024 monolayers. The cells were co-cultured for 48 hours. The cells were trypsinized and plated in methylcellulose media containing Epo or Epo + IL-3 as described above. BFU-E were scored following acid benzidine staining after 5 days of methylcellulose culture.
Western blot analysis

Whole fetal livers were dissected from mutant and control mice on the indicated days. The livers were lysed in RIPA buffer and whole cell lysates were generated. Equal amounts of protein were loaded on SDS-PAGE gels and blotted to nitrocellulose membrane. The membranes were blocked and then incubated with anti-BMP4 (Abcam Inc. Cambridge, MA), anti-Smad5, anti-Phospho Smad1, 5, 8 (Cell Signaling, Danver, MA) or anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The membranes were washed and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase. Protein antibody complexes were visualized using ECL plus western blotting detection system (GE Healthcare, Buckinghamshire, UK). The expression of BMP4 was normalized to ß-actin expression and quantified using ImageQuant TL (Amersham Biosciences, Piscataway, NJ).

Results

Fetal liver contains two BFU-E populations, which exhibit properties of either stress BFU-E or steady State BFU-E

In our previous work we showed that during the recovery from acute anemia, a specialized population of stress erythroid progenitors, which we termed stress BFU-E, are rapidly expanded in the adult spleen. The properties of these BFU-E are distinct from bone marrow steady state BFU-E. Stress BFU-E are resident in the spleen and rapidly form large BFU-E colonies when cultured in the presence of Epo alone (Lenox et al., 2005). Similar to the spleen, some fetal liver erythroid progenitors have characteristics
distinct from their adult bone marrow counterparts. While bone marrow BFU-E require the presence of a BPA along with Epo to form BFU-E, studies in human fetal liver have shown that some fetal BFU-E can respond to Epo alone without addition of any BPA (Emerson et al., 1989; Valtieri et al., 1989). In many ways, these fetal liver erythroid progenitors are more similar to the stress BFU-E, which require BMP4, SCF and hypoxia for their expansion (Lenox et al., 2005; Perry et al., 2007). f/f mice exhibit a delayed expansion of stress BFU-E because of impaired BMP4 dependent signaling. Because f/f mutant embryos also have a defect in the fetal liver erythropoiesis, we looked for the presence of stress BFU-E in the fetal liver and asked whether these cells were defective in the f/f mice. We observed that there is a significant expansion in the relative number of stress BFU-E at E15.5 in control mice, which correlates with the maximal erythropoietic activity in the organ and this expansion is sustained until E16.5 (Fig 1A). At E18.5, when most of the erythropoietic activity in the fetal liver is finished, the stress BFU-E number had decreased to low levels. In contrast, the f/f mice exhibit a delayed and less sustained increase in stress BFU-E at E16.5, a day after the expansion observed in the WT mice. By E18.5, the number of stress BFU-E had returned to values similar to the control mice.

It has been shown that f/f mice have smaller fetal livers and contain decreased number of mature erythroid progenitors and identifiable erythroblasts (Cole and Regan, 1976). We determined the number of cells in the fetal liver at various time points and plotted the number of stress BFU-E colonies per fetal liver (Fig 1B). The difference between stress BFU-E in f/f and control fetal livers is even more striking when the smaller fetal livers of the f/f embryos is taken into account. At E15.5, there is approximately a 7 fold decrease in the number of stress BFU-E in f/f fetal livers. The
maximal increase in stress BFU-E in \textit{f/f} embryos was observed at E16.5, however this number of stress BFU-E was less than that observed in control fetal livers at E15.5 and not different from that observed in control fetal livers at the same time point. These data show that, even though the \textit{f/f} fetal liver was capable of expanding stress BFU-E at E16.5, the overall reduction in fetal liver cellularity makes their response significantly weaker.

The anemia of the \textit{f/f} mutant embryos is most severe early in fetal development (Gruneberg, 1942a; Gruneberg, 1942b; Kamenoff, 1935). Our data suggests that the delayed and diminished expansion of stress BFU-E at E15.5 in \textit{f/f} embryos results in the fetal anemia.

Unlike the spleen, which contains primarily stress BFU-E, the fetal liver also contains BFU-E, which are similar to bone marrow steady state BFU-E and require both a BPA and Epo to form a BFU-E colony. We assayed for steady state BFU-E at various times during embryogenesis by culturing fetal liver cells in media containing Epo alone or Epo and IL-3, scoring BFU-E colonies and then subtracting out the number of Epo-only BFU-E. We observed that at no time point is there a significant difference in the relative numbers of steady-state BFU-E in the control mice compared to the \textit{f/f} mice (Fig 2A). Although the relative numbers of steady state BFU-E are similar between \textit{f/f} and control mice, when we took into account the smaller fetal livers present in the \textit{f/f} embryos and calculated the total number of “steady state” BFU-E per fetal liver, \textit{f/f} embryos exhibit a small, but significant deficit at E14.5 and E15.5 (Fig 2B). However the difference at E15.5 is only 2 fold, which parallels the difference in fetal liver cellularity.

Taken together the analysis of BFU-E demonstrates that two populations of BFU-E exist in the fetal liver. One population responds to Epo alone and exhibits properties of
stress BFU-E (Lenox et al., 2005). These progenitors are strongly affected in \( f/f \) mutant embryos. While the second population, which exhibits properties of steady state BFU-E in that they require a BPA, is less affected in \( f/f \) mice. This observation is similar to adult \( f/f \) mice, which do not exhibit a defect in the number of bone marrow steady state BFU-E, but exhibit a delayed expansion of stress BFU-E in the spleen during the recovery from acute anemia (Gregory et al., 1975; Lenox et al., 2005). The decrease in stress BFU-E could potentially make a larger contribution to the severity of the anemia beyond the simple decrease in BFU-E numbers. Our earlier observations that stress BFU-E generate significantly larger bursts than steady state BFU-E suggests that the decrease in stress BFU-E would have a significant impact on fetal red cell production (Lenox et al., 2005; Perry et al., 2007).

**BMP4 expression correlates with the level of erythropoietic activity in the fetal liver**

We investigated the expression of BMP4 between E12.5 and E18.5 when erythropoiesis in the fetal liver is active. We observed very little expression of BMP4 at E12.5, when the fetal liver is still being seeded by hematopoietic cells from yolk sac and AGM regions. BMP4 expression starts at approximately E13.5 and reaches maximal levels by E14.5, which coincides with the maximal erythropoietic activity in the organ (Cole and Regan, 1976) (Fig. 3A). Thereafter, the BMP4 protein level decreases, reaching basal levels by E18.5. In contrast, \( f/f \) embryos exhibit very little expression of BMP4 protein up to E14.5. However at E15.5, BMP4 expression is observed and reaches maximal levels by E16.5; which coincides with the maximal erythropoietic activity in \( f/f \) mice(Cole and Regan, 1976) (Fig. 3A). This delay is similar to the delay in the
expression of BMP4 seen in the spleens of adult f/f mice following induction of acute anemia (Lenox et al., 2005). Quantitation of our immunofluorescence studies suggested that the maximal level of BMP4 expression in f/f embryos may be less than that observed in control embryos. We extended these studies by performing western blot analysis of BMP4 expression from E14.5 control fetal livers and E16.5 f/f fetal livers, which represent the time points with the greatest expression of BMP4. We observed that the level of BMP4 expression when normalized for β-actin expression was significantly less at E16.5 in f/f embryos when compared to E14.5 control embryos (Fig. 3B) suggesting that f/f embryos exhibit decreased and delayed expression of BMP4 in the fetal liver.

**Hepatic parenchymal and stromal cells secrete BMP4**

The E14.5 fetal liver is comprised of many subpopulations of cells including hepatic, biliary parenchymal and stromal cells as well as the hematopoietic cells colonizing the organ. In order to determine which cell population is responsible for BMP4 secretion, we performed double immunostaining experiments with BMP4 and other markers. Kit is expressed on most of the hematopoietic progenitors. We used Kit as a hematopoietic marker and did co-localization studies with BMP4. BMP4 expression does not co-localize with CD45+ (data not shown) or Kit+ cells (Supplementary figure.1A), which shows that hematopoietic progenitor cells in the fetal liver are not the source of BMP4. This situation is similar to what we have observed in the adult spleen during the recovery from acute anemia (P. Porayette and RF Paulson unpublished observations). However, when we stain fetal liver sections with antibodies to hepatocyte specific markers like hepatocyte specific antigen (HSA), we observed that a subset of
BMP4 expressing cells were hepatocytes (Supplementary Figure.1B). However, there were many BMP4-positive cells which were not hepatocytes suggesting that multiple cell types might be expressing BMP4. Consistent with this idea, selective ablation of the BMP4 gene in fetal hepatocytes by crossing a conditional allele of BMP4 (Kulessa and Hogan, 2002) with a hepatocyte specific Cre mouse (Parviz et al., 2002) did not affect the expression of BMP4 in the fetal liver (Data not shown). Fetal liver stroma consists of cells in epithelial to mesenchymal transition (EMT) and these cells have been shown to be capable of supporting the expansion of hematopoietic progenitor cells \textit{in vitro} (Chagraoui et al., 2003). In order to investigate the potential ability of EMT cells to secrete BMP4, we stained fetal liver sections from control mice and did double immunostaining with BMP4 and markers co-expressed on EMT cells, \(\alpha\)-smooth muscle actin, (ASMA) and cytokeratin-8, (CK-8). As shown by others, we were able to stain for EMT cells using ASMA and CK-8 as markers (Supplementary Figure 1C Top). Also, we could show that cells expressing ASMA and CK-8 also expressed BMP4 (Supplementary Figure 1C Middle and Bottom). Again we found that many BMP4 secreting cells were actually EMT cells, but still these cells did not wholly account for all the BMP4-positive cells, suggesting that both hepatic parenchymal and stromal cells may be responsible for secreting BMP4.

\textit{BMP4 increases the number of stress BFU-E in vitro}

Our analysis of stress BFU-E in adults showed that culturing spleen cells from non-anemic mice with BMP4 resulted in an increase in stress BFU-E (Lenox et al., 2005; Perry et al., 2007). We tested whether culturing fetal liver cells from control and \textit{f/f}
embryos in media containing BMP4 and Epo resulted in increased BFU-E when compared with cultures containing Epo only. At E12.5 and E13.5 we observed a small but significant increase in BFU-E when cells were plated in media containing BMP4. However the effect of BMP4 was gradually lost such that by E15.5 no effect of BMP4 was observed (Fig 4A). In contrast, in \( f/f \) fetal liver cells, BMP4 is unable to increase the number of BFU-E at any of the time points due to the defect in \( Smad5 \) gene. The lack of a response to BMP4 in the control mice at E15.5 and later time points might be because these cells have already been exposed to BMP4, which is induced in vivo at E14.5 (Fig 3A) and thus are refractory to the effects of any BMP4 added in vitro. We have made a similar observation in the spleen during the recovery from acute anemia. Once BMP4 expression starts 24 hours after anemia induction, the response of spleen cells to BMP4 is lost (J. Perry and RF Paulson unpublished).

We examined the expression of Smad5 and the extent of BMP4 induced phosphorylation of Smad5 in E14.5 fetal liver cells (Figure 4A). We observed that in control fetal liver cells, Smad5 is highly expressed and phosphorylated whereas in \( f/f \) embryos little Smad5 is expressed. However, despite the low levels of Smad5, it appears to be phosphorylated. Therefore the defect in BMP4 dependent expansion of stress BFU-E appears to be caused by low levels of full length Smad5.

Our previous work showed that BMP4 had no effect on bone marrow steady state BFU-E (Lenox et al., 2005). However, our observation that the total number of steady state BFU-E in the \( f/f \) fetal liver is decreased approximately 2 fold suggests that BMP4 signaling may play a role the expansion of steady state BFU-E. We tested this possibility by treating E12.5 fetal liver cells from \( f/f \) and control embryos with BMP4 and
determined whether the number of steady state BFU-E increased. Figure 4B shows that stress BFU-E are significantly increased when BMP4 is added to the culture, but this treatment had no effect on the number of steady state BFU-E. As shown in Fig 4A, f/f fetal liver cells did not respond to BMP4 treatment by increasing the number of stress BFU-E. Furthermore, steady state BFU-E from f/f exhibited responses indistinguishable from control embryos following treatment with BMP4. These observations are consistent with our model that BMP4 does not affect the number of steady state BFU-E and that the f/f mutation primarily affects the stress BFU-E in the fetal liver.

The f/f mutation does not cause increased apoptosis or cause defects in erythroid differentiation.

Recent work has suggested that the regulation of Fas mediated apoptosis of erythroid progenitors plays a role in the expansion of erythroblasts in response to erythropoietic stress (Liu et al., 2006). The defect in the expansion of stress BFU-E in f/f mutant embryos could result from an increase in apoptosis. We investigated this possibility by performing TUNEL staining on sections of E14.5 fetal livers from f/f and control embryos. Figure 5A shows that there is no difference in the number of TUNEL+ cells in f/f fetal livers when compared with control, suggests that apoptosis is not the cause of the delayed expansion of stress BFU-E.

The scoring of the BFU-E colony assay relies on our ability to stain terminally differentiated erythroid cells with benzidine. Therefore a defect in terminal erythroid differentiation would affect our ability to identify BFU-E. We tested whether fetal liver cells from f/f mice could terminally differentiate when plated in media containing Epo
and insulin which promotes the terminal differentiation of erythroid cells in vitro (Panzenbock et al., 1998). We observed no difference in the ability of \( f/f \) fetal liver cells to differentiate when compared with control cells (Fig 5B). Taken together these data support a model where the anemia of \( f/f \) embryos is caused by defect in the expansion of progenitor cells.

The majority of stress BFU-E are present in the CD31+Kit+Sca1-Lin- population

Our analysis of erythroid progenitors in the spleen has shown that stress BFU-E capable of forming Epo-only BFU-E share the same surface phenotype as the Megakaryocyte-Erythroid Progenitor (MEP) (Akashi et al., 2000; Lenox et al., 2005). Analysis of fetal liver progenitors has shown that MEPs and their precursors, Common Myeloid Progenitors (CMP) are present in the fetal liver (Traver et al., 2001). We sorted MEPs from E14.5 fetal liver and analyzed their ability to form stress BFU-E. Our analysis of MEPs showed that they have only minimal ability to form stress BFU-E colonies under our culture conditions (Fig 6A). Recently, another population of erythroid progenitors was identified that are distinct from the MEPs and are characterized by the expression of CD31 or PECAM (Baumann et al., 2004). This population (CD31+) exhibits a Kit+ CD31+ Sca1- Lin- surface phenotype. Analysis of the CD31+ population in adult bone marrow showed that these cells could provide effective short-term radioprotection by transiently contributing to the erythroid lineage. We sorted these cells from the fetal liver and showed that this population was capable of forming stress and steady state BFU-E (Fig 6A). In contrast, the CD31- population was able to form very few steady state BFU-E and no stress BFU-E, suggesting that the majority of the stress
BFU-E forming activity was within the CD31+ population. We extended our analysis of the CD31+ population by analyzing these cells in the fetal liver of E14.5 f/f embryos, which showed that their numbers are reduced 2 fold in these embryos compared to the control (Fig 6B). Functional analysis of BFU-E colony forming ability of the CD31+ population from f/f mice showed that these cells form only half the number of stress BFU-E compared to the control mice despite the observation that the total number of BFU-E (stress + steady state) were not different (Fig 6C). These data show that the CD31+ erythroid progenitor population contains the majority of the stress BFU-E and CD31+ cells from f/f fetal liver exhibit defects in stress BFU-E formation.

*Stress BFU-E are present in the yolk sac and are reduced in f/f embryos.*

Recent work in zebrafish has shown that Smad5 and Smad1 are required for definitive hematopoiesis (McReynolds et al., 2007). Murine Smad5-/- embryos die prior to the onset of definitive erythropoiesis and no studies using conditional alleles of Smad5 have examined the role of Smad5 in the development of definitive erythroid progenitors (Yang et al., 1999). Erythroid progenitors do not develop de novo in the fetal liver and it has been proposed that stem cells and progenitors from the yolk sac and AGM region of the embryo seed the fetal liver (McGrath and Palis, 2005; Palis et al., 1999). Definitive erythroid progenitors, BFU-E, are first observed in the yolk sac at E8.25. These BFU-E rapidly expand during the next 2 days of development and are thought to seed the fetal liver with an initial wave of definitive erythroid progenitors. In order to determine whether stress BFU-E are present in the yolk sac, we directly plated yolk sac cells from E10.5, E11.5 and E12.5 embryos in methylcellulose media containing Epo alone or Epo +
IL-3 (Figure 7A). At E10.5, only a small fraction of the total BFU-E are stress BFU-E, however by E11.5 approximately 45% of the BFU-E respond to Epo alone (Fig 7B). By E12.5, a time when the fetal liver is an active site of erythropoiesis, the total number of BFU-E drops to low levels. The situation is different in f/f yolk sacs, where the percentage of stress BFU-E remains low even at E11.5 (Fig 7B). These data suggest that the stress BFU-E are first present in the yolk sac and f/f mutant yolk sacs contain fewer stress BFU-E than controls.

The observation that f/f yolk sacs contain fewer stress BFU-E than controls at E10.5 and E11.5 is not consistent with our observation that at E12.5 f/f and control fetal livers contain similar numbers of stress BFU-E. One possibility is that stress BFU-E could develop in the AGM region and then seed the fetal liver. We tested whether AGM region cells could generate stress BFU-E when plated in methylcellulose media; however no BFU-E were observed in these cultures even when IL-3 was included in the media (Data not shown). These results are consistent with earlier reports that the AGM region is not a site of erythropoiesis in the mouse embryo (Godin et al., 1999). A second possibility is that progenitors from the yolk sac differentiate into stress BFU-E when they seed the fetal liver. These progenitors are not stress BFU-E, but would respond to a signal or signals in the fetal liver microenvironment. Recently we have demonstrated that bone marrow cells can differentiate into stress BFU-E when they come in contact with the spleen microenvironment (J. Perry and RF Paulson unpublished observations). Using these data as a guide we tested whether co-culturing yolk sac cells on fetal liver stromal cells could induce the development of stress BFU-E (Fig 7A). We used AFT024 fetal liver stromal cells, which were derived from E14.5 fetal liver (Moore et al., 1997). These
cells have been shown previously to support the expansion of stem and progenitor cells in culture (Chagraoui et al., 2003; Moore et al., 1997). Plating control E10.5 yolk sac cells on AFT024 cells lead to a 10 fold increase in the number of stress BFU-E when compared to direct plating (Figure 7C). In addition, stress BFU-E were now the predominate type of BFU-E in these cultures. E11.5 yolk sac cells co-cultured with AFT024 also showed an increase in stress BFU-E, but the fold increase in stress BFU-E when compared with direct plating was less (~2 fold) than that observed with E10.5 cells. At E12.5 little effect of co-culture was observed. In contrast, E10.5 f/f yolk sac cells exhibited only a modest increase in stress BFU-E (~2 fold) and the majority of the BFU-E were steady state BFU-E. However, when E11.5 f/f yolk sac cells were co-cultured with AFT024 cells, we observed a 6 fold increase in stress BFU-E when compared with direct plating and now stress BFU-E were the predominate erythroid progenitor. In addition, f/f E12.5 yolk sac cells showed a small but significant increase in stress BFU-E when co-cultured with AFT024 cells. These observations suggest a model where definitive hematopoietic progenitors in the yolk sac interact with fetal liver stromal cells and develop into stress BFU-E. Our data suggests that despite the deficit in stress BFU-E in the yolk sacs of f/f embryos, they contain progenitors that can develop into stress BFU-E when they interact with a signal or signals in the fetal liver microenvironment. It appears that the maximum development of these progenitors is delayed in f/f yolk sacs from E10.5 to E11.5, but despite this delay, the f/f E12.5 fetal livers contain similar numbers of stress BFU-E as control fetal livers.
Discussion

The need for oxygen necessitates that the hematopoietic and cardiovascular systems develop early during embryogenesis. To meet the demands of the fetus, fetal liver hematopoiesis is predominately erythropoietic. Using our previous work on the erythroid response to acute anemia as a paradigm, these data suggest that the oxygen needs of the rapidly expanding embryo activates the BMP4 dependent stress erythropoiesis pathway in the fetal liver, which drives the expansion of stress erythroid progenitors. Our observations suggest that mid gestation (E13.5-E15.5) is a critical juncture in development where the rapid up-regulation of erythropoiesis is required and steady state erythroid progenitors are unable to produce sufficient erythrocytes. The BMP4 dependent expansion of stress BFU-E fills this need because stress BFU-E differentiate faster and have a larger capacity to generate mature erythrocytes than steady state BFU-E (Lenox et al., 2005; Perry et al., 2007). Taken together these data suggest a new model for fetal erythropoiesis where the activation of the BMP4 dependent stress erythropoiesis pathway is required to generate sufficient erythrocytes for the developing embryo.

BMP4 regulates the expansion of stress BFU-E in the spleen, which exhibit properties distinct from bone marrow steady state BFU-E (Lenox et al., 2005). Analysis of erythroid progenitors in control embryos showed that the progenitors that expand at E14.5 exhibit properties of stress BFU-E. Like stress BFU-E, these fetal liver BFU-E are able to form colonies in media containing Epo alone in 5 days of culture. This population of BFU-E initially make up approximately 30% of BFU-E at E14.5, but by E15.5 when the maximum number of BFU-E are present in the fetal liver, stress BFU-
E make up the majority of the fetal liver BFU-E. Later during development (E18.5) the percentage of Epo-only BFU-E returns to approximately 30%. Previous work has shown that human fetal liver also contains a population of BFU-E that respond to Epo alone. Interestingly this population is present primarily during early gestation (9-10 weeks), while during later fetal development (>17 weeks) BFU-E require both Epo and a BPA (Emerson et al., 1989; Valtieri et al., 1989). The role of BMP4 signaling in the expansion of these human fetal liver BFU-E however, is not known.

Our previous analysis of adult stress erythropoiesis showed that essentially all of the BFU-E in the spleen were stress BFU-E, in contrast, the bone marrow contained primarily steady state BFU-E. Here we show that the fetal liver contains both steady state and stress BFU-E. Our data show that \textit{f/f} exhibit a significant decrease in both the relative number and total number of stress BFU-E in the fetal liver at E15.5. These observations are consistent with a model where the fetal anemia of \textit{f/f} mutant mice primarily results from their impaired ability to expand stress BFU-E in response to BMP4 signaling. The delayed expansion of stress BFU-E observed in \textit{f/f} fetal livers also explains the severe anemia exhibited early in fetal development, which progressively improves during development.

In addition to the decrease in stress BFU-E, we observed a small, approximately two fold, decrease in the number of steady state BFU-E in \textit{f/f} fetal livers. This decrease suggests the possibility that BMP4/Smad5 dependent signaling could also regulate the expansion of steady state BFU-E. However, the addition of BMP4 to methylcellulose cultures of fetal liver cells does not affect the number of steady state BFU-E in control or \textit{f/f} fetal liver cells suggesting that \textit{f/f} steady state BFU-E do not
exhibit a cell intrinsic defect in responding to BMP4. We feel that the most likely explanation for this observation is that the two fold decrease in steady state BFU-E is a consequence of the smaller fetal livers present in f/f embryos. Previous work demonstrated that f/f fetal livers were approximately two fold smaller than control fetal livers (Bateman and Cole, 1972; Cole and Regan, 1976). The fetal liver may contain only a certain number of niches for steady state BFU-E, which would be decreased in number in the smaller f/f fetal livers. This situation would lead to fewer steady state BFU-E. This model would also predict that the decrease would parallel the difference in fetal liver size and the relative numbers of steady state BFU-E should not be significantly different between f/f and control, which is what we observe.

Our analysis also showed that f/f embryos express BMP4 at reduced levels and its expression is delayed when compared to control fetal livers. Our previous work on stress erythropoiesis in adults also showed that the expression of BMP4 was delayed in the spleens of f/f mutant mice (Lenox et al., 2005). These data further support our assertion that a Smad5 dependent signal regulates the expression of BMP4 (Lenox et al., 2005). We have examined the expression of Smad5 and BMP4 in the fetal liver at E14.5 in control embryos (Supplementary figure 2) and have observed that Smad5 is co-expressed in a subset of BMP4 expressing cells. These observations are consistent with a model where initial BMP4 expression is expanded to other cells in a Smad5 dependent manner. This situation would be similar to that observed in Drosophila where Decapentaplegic (Dpp), the fly ortholog of BMP4, auto-regulates its expression in a Mad dependent fashion (Chanut and Heberlein, 1997; Hepker et al., 1999; Hursh et al., 1993; Yu et al., 1996). Our previous analysis of adult expression of BMP4 showed that hypoxia
can induce BMP4 expression in a spleen stromal cell line (Lenox et al., 2005), which suggests the possibility that hypoxia in the fetal liver may induce the initial expression of BMP4, while the increase in BMP4 expressing cells relies on the BMP4/Smad5 dependent signaling to induce BMP4 expression. Further analysis will be needed to investigate this possibility.

The initial erythropoiesis in the yolk sac is primitive erythropoiesis. Early work on f/f mutant embryos showed that primitive erythropoiesis was unaffected in these mice (Russell, 1979). Similarly, Smad5/- mice do not exhibit a defect in primitive yolk sac erythropoiesis (Yang et al., 1999). Recent work from zebrafish has shown that Smad1 and Smad5 are required for definitive erythropoiesis (Gupta et al., 2006; McReynolds et al., 2007). The initial definitive erythropoiesis in the embryo is first observed in the yolk sac. Our data shows that stress BFU-E develop in the yolk sac at E10.5 and E11.5 and that f/f embryos exhibit a defect in this process, which may contribute to the early anemia in these embryos.

The prevailing model in the field is that definitive progenitors in the yolk sac will seed the fetal liver. Paradoxically, we observe that despite the defect in stress BFU-E in the f/f yolk sac, E12.5 f/f mutant fetal livers have numbers of stress BFU-E that are not significantly different from controls. Recent work from our lab may explain this discrepancy. In the adult spleen all stress BFU-E differentiate during the recovery from acute anemia, so they must be replaced. We observe that bone marrow progenitors can replenish the BMP4 responsive stress BFU-E if they migrate to the spleen. These data suggest that signals in the spleen microenvironment cause the differentiation of bone marrow progenitors into stress BFU-E (J. Perry and RF Paulson unpublished data). Our
data shows that co-culturing yolk sac cells on fetal liver stromal cells can also promote
the development of stress BFU-E. This observation suggests that the yolk sac produces
progenitor cells that respond to signals in the fetal liver microenvironment, which
promote their differentiation into stress BFU-E. In control embryos we observe a larger
increase in stress BFU-E when E10.5 yolk sac cells are co-cultured than when E11.5 cells
are used. The opposite was true when /f/f yolk sac cells were used. In this case, co-
culturing /f/f E11.5 yolk sac cells with AFT024 resulted in significantly more stress BFU-
E and a preferential increase in stress BFU-E. From these observations we conclude that
/f/f mutant yolk sacs exhibit a delay in the generation of progenitor cells in the yolk sac
that can respond to the fetal liver stromal signals, however, they are able to overcome this
delay such that by E12.5 the number of stress BFU-E and CD31+Kit+Sca1-Lin- cells in
the fetal liver are approximately equal to control mice (Data not shown). Earlier work
supports this model where the yolk sac progenitors respond to signals in the fetal liver
and differentiate into definitive erythroid progenitors. These experiments showed that co-
culture of yolk sac with the fetal liver rudiment induced the expansion of definitive
erthropoietic progenitors (Cudennec et al., 1981). This expansion was promoted by a
signal secreted by the liver. The identity of the signal in the fetal liver is unclear. Our
experiments used AFT024 cells, which were made from E14.5 fetal liver (Moore et al.,
1997) and express BMP4 (P. Porayette and RF Paulson unpublished observations).
However, treatment of yolk sac cells with BMP4 did not affect the number of stress BFU-
E or the ratio of stress BFU-E to steady state BFU-E (P. Porayette and RF Paulson
unpublished observations). Future work will be needed to identify the signals involved in
this process.
The most penetrant phenotype of \( f/f \) embryos is the severe fetal anemia, which is in contrast to the lethality of the Smad5-/- embryos. Our previous work showed that \( f \) mutant mice have a defect in splicing of the \( Smad5 \) gene, where the majority of the \( Smad5 \) mRNA is one of two mis-spliced mRNAs. One of these aberrant splice forms when over expressed in an osteoblast cell line can inhibit BMP4 dependent differentiation. Furthermore, \( f/Smad5 \)- mice exhibit a less severe phenotype than \( f/f \) mice, which suggests that \( f/f \) represents a gain of function allele. So these data lead to the question of why \( f/f \) mutant embryos survive, but Smad5-/- embryos die. Recently, we have demonstrated that the splicing defect in \( f/f \) mice is caused by a mutation in an intronic splicing regulatory element. In addition our analysis has shown that the splicing defect is tissue specific in its severity, in that some tissues exhibit low levels of mis-spliced Smad5 mRNA. Furthermore the splice forms vary between tissues (S Hegde et al. submitted). These data suggest that \( f \) represents an allele of Smad5 that specifically affects stress erythropoiesis and in some strains chondrogenesis and the development of melanocytes where tail flexures and white belly spots are penetrant whereas other tissues that express Smad5 are unaffected.

In summary, we have demonstrated that the BMP4 dependent stress erythropoiesis pathway plays a key role in the expansion of erythroid progenitors in the fetal liver that exhibit the properties of stress BFU-E found in the adult spleen. These data suggest a model where the acute oxygen needs of the growing fetus induce stress erythropoiesis to rapidly generate large numbers of erythrocytes at a critical time in fetal development.
Acknowledgements:

We would like to thank Shailaja Hegde and Michele Yon for excellent technical assistance and the members of the Paulson lab for their comments and support.

References:


Gruneberg, H., 1942a. The anaemia of the flexed-tail mice (Mus musculus L.) II.


Figure Legends

Figure 1. Expansion of Stress BFU-E in the fetal liver of $f/f$ and control mice. Fetal liver cells were isolated on the indicated days during fetal development and plated in methylcellulose media containing only Epo. BFU-E were scored after five days in culture. (A) Relative number of BFU-E per $5 \times 10^5$ fetal liver cells. (B) Total number of stress BFU-E per fetal liver. Significant differences between $f/f$ and control are indicated on the figure. If no p value is given then the difference was not significant.

Figure 2. Expansion of steady state BFU-E in the fetal liver of $f/f$ and control mice. Fetal liver cells were isolated on the indicated days during fetal development and plated in methylcellulose media containing Epo + IL-3. BFU-E were scored after five days in culture. The number of Epo+BPA BFU-E was calculated by subtracting the number of Epo-only BFU-E from the number of Epo+IL-3 BFU-E. (A) Relative BFU-E per $5 \times 10^5$ fetal liver cells. (B) Total number of steady state BFU-E per fetal liver. Significant differences between $f/f$ and control are indicated on the figure. If no p value is given then the difference was not significant.

Figure 3. Expression of BMP4 in the fetal liver of $f/f$ and control mice. (A) (top) Fetal livers from control and $f/f$ embryos isolated at the indicated days were sectioned and stained with anti-BMP4 antibodies. (Middle) Negative control staining with isotype control antibodies of fetal liver sections from C57BL/6-$f/f$ and C57BL/6 control embryos. (Bottom) The fluorescent intensity of the staining was quantified using
ImageQuant software. The relative intensity of the green pixels is indicated. (B). Western blot analysis of BMP4 and β-actin expression in E14.5 control and E16.5 f/f fetal liver (left). Expression of BMP4 normalized to β-actin expression is quantified on the right.

**Figure 4. Effect of BMP4 on Epo only and Epo + IL-3 BFU-E formation.** (A) (left) Fetal liver cells were isolated on the indicated days during fetal development and plated in methylcellulose media containing Epo alone or Epo + BMP4. The fold increase in the number of BFU-E (Epo+BMP4/Epo-only) was calculated at each time point. Significant increases in the number of Epo-only BFU-E following BMP4 treatment are indicated on the figure. (right) Western blot of cell lysates from E14.5 f/f and control fetal liver cells probed with anti-Phospho-Smad1, 5, 8, anti-Smad5 and β-actin as a loading control. (B) Fetal liver cells were isolated on E12.5 and plated in the indicated media + BMP4 to test the effect of BMP4 on the formation of Epo-only or Epo + IL-3 BFU-E. Significant differences are indicated on the figure. If no p value is given then the difference was not significant.

**Figure 5. f/f embryos do not exhibit excessive apoptosis or defects in erythroid terminal differentiation.** (A) Fetal liver sections from E14.5 C57BL/6- f/f and C57BL/6 control embryos were stained for apoptotic cells by TUNEL assay. Bright field views are shown to the left. (B) Fetal liver cells from E14.5 C57BL/6- f/f and C57BL/6 control embryos were incubated in erythroid differentiation media for 48 hours. The cells prior to culture (top) and after culture (bottom) were stained with Wright-Giemsa and o-Dianisidine stain to identify hemoglobinized cells.
Figure 6. CD31+Kit+Sca1-Lin- population contains both Epo-only and Epo+IL-3 BFUE. (A) Fetal liver cells were isolated from E14.5 control embryos and sorted for CD31+Kit+Sca1-Lin-, CD31-Kit+Sca1-Lin- or MEP cells. 25,000 cells were plated in methylcellulose media containing Epo or Epo+IL3. BFU-E were scored after five days in culture. (B) Representative flow diagrams from E14.5 control and f/f fetal liver cells gated on Sca1-Lin- cells. (right) Graph showing the average percentage of fetal liver cells that are CD31+Kit+Sca1-Lin- in from E14.5 control and f/f mice. (C) Stress BFU-E and total BFU-E present in the CD31+Kit+Sca1-Lin- population of cells from the fetal livers of control or f/f mice were determined. Significant differences are indicated on the figure. If no p value is given then the difference was not significant.

Figure 7. Yolk sac contains stress BFU-E and progenitor cells that develop into stress BFU-E when Yolk sac cells are plated on fetal liver stromal cells. (A) Schematic of the direct plating of yolk sac cells and co-culture with AFT024 cells prior to plating. (B) Yolk sac cells from E10.5, E11.5 and E12.5 control and f/f embryos were directly plated in media containing Epo alone or Epo+IL-3. BFU-E were scored 5 days later. Epo + IL-3 BFU-E were calculated by subtracting the number of Epo-only BFU-E from the total number of BFU-E formed in Epo+IL-3. (C) Yolk sac cells from E10.5 and E11.5 control and f/f embryos were co-cultured on AFT024 fetal liver stromal cells for 48 hours and then plated in methylcellulose media containing Epo alone or Epo+IL-3. BFU-E were scored 5 days later.
Figure 1

A.

![Graph A showing BFU-E per 5x10^5 cells across Embryonic Day 12.5 to 18.5.]

B.

![Graph B showing BFU-E per fetal liver across Embryonic Day 12.5 to 18.5.]

- p<0.05
- p>0.08
- p>0.7
Figure 2

A.

B.
A.

<table>
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</table>

B.

- **BMP4**
  - C57BL/6 E14.5
  - C57BL/6-t/f E16.5

- **ACTIN**
  - C57BL/6
  - C57BL/6-t/f
Figure 4

A.

![Graph showing fold increase over embryonic day](image)

B.

![Bar chart showing BFU-E counts](image)
Figure 5

A. C57BL/6-/- vs C57BL/6

B. T = 0 vs T = 48 hours
Figure 6.

A. 

![Graph showing BFU-E per 25,000 gated cells for CD31+ and CD31- Kit+Sca1-Lin- cells. The graph compares Epo alone and Epo+IL3 conditions.]

B. 

![Flow cytometry plots showing the percentage of CD31+ Kit+ Sca1-Lin- cells in control and E14.5 f/f conditions. The right graph compares the percent of CD31+ Kit+ Sca1-Lin- cells between control and E14.5 f/f conditions with a p-value of 0.005.] 

C. 

![Bar graph showing BFU-E per 25,000 CD31+ Kit+ Sca1-Lin- cells. The graph compares E14.5 WT and E14.5 f/f conditions, with p-values of <0.01 and <0.01, respectively.]
Figure 7.

A. Yolk sac cells

- Direct plating of Yolk sac cells
  - Epo
  - Epo + IL-3
  - 5 days

- Plating after incubation with fetal liver stromal cells
  - 48 Hour co-culture
  - AFT024 Stromal cells
  - Epo
  - Epo + IL-3
  - 5 days

B. Stress BFU-E

- Steady state BFU-E

C. p<0.05

- Epo only
- Epo+IL3

p>0.4

- Epo only
- Epo+IL3

C57BL/6 control C57BL/6-/-
Supplementary Figure 1A. BMP4 expressing cells do not express Kit receptor. E14.5 fetal liver sections from control embryos were stained with anti-BMP4, anti-Kit antibodies and DAPI to show nuclei. Overlaying the BMP4 and Kit signals show that there is no overlap in expression. Arrows point out examples of Kit+ cells that are BMP4-. The circle shows BMP4 expression where in Kit-cells. We observe similar results when sections were stained with anti-BMP4 and either anti-CD45 or anti-Ter119 (Data not shown). These data support the conclusion that hematopoietic cells do not express BMP4.

Negative control staining. Isotype controls for the anti-BMP4 and anti-Kit antibodies were used to stain fetal liver sections.
Supplementary Figure 1B. A subset of the BMP4 expressing cells are hepatocytes. Fetal liver sections from E14.5 control embryos were stained with anti-Hepatocyte specific antigen (HSA), anti-BMP4 antibodies and DAPI to visualize nuclei. Overlaying BMP4 expression and HSA expression shows that a subset of BMP4 expressing cells are hepatocytes (small arrows). Larger arrows show BMP4 expressing cells that are not HSA+. Similar data was observed when sections were stained with anti-HNF4α antibodies (Data not shown).

Negative control staining. Isotype controls for the anti-BMP4 and anti-HSA antibodies were used to stain fetal liver sections.
Supplementary Figure 1C. BMP4 staining overlaps with markers for epithelial (CK8) and mesenchyme (α smooth muscle actin, ASMA). (Top panels) E14.5 fetal liver sections were stained with anti-CK8 and anti-ASMA. The overlap of the two signals is shown in the center panel in yellow. (Middle panels) E14.5 fetal liver sections were stained with anti-BMP4 and anti-ASMA. Overlap in signals is shown in yellow in the center panel. (Bottom panels) E14.5 fetal liver sections stained with anti-BMP4 and anti-CK8 antibodies. Overlap in signal is shown as yellow in the center panel. Please note these images are 40X magnification and therefore BMP4 expression appears different than that observed in Supplementary figures 1A, 1B and 2, which are 60X.
Supplementary Figure 1C continued. Negative control staining. Isotype control antibodies for the indicated antibodies were used to stain E14.5 fetal liver sections.
Supplementary Figure 2. E14.5 fetal liver section from C57BL/6 control mice were stained anti-BMP4 (green) and anti-Smad5 (red) antibodies. The signal from each antibody as well as the overlap in signal are shown. Overlapping signal is indicated by yellow color. (Lower right) DAPI staining of nuclei to show the presence of cells in the section is included as a control. These data show that a subset of BMP4+ cells also express Smad5. These images were taken at 60X magnification.

Negative control staining. Isotype controls for the anti-BMP4 and anti-Smad5 antibodies were used to stain fetal liver sections.
We have shown that the fetal liver has two populations of erythroid progenitors. One population exhibits properties similar to the stress erythroid progenitors found in the adult spleen. The other population is similar to the steady state erythroid progenitors found in the bone marrow. The stress erythroid progenitors can form BFU-E in the presence of Epo alone whereas the steady state progenitors need both Epo and a BPF like IL-3 to form BFU-E. We have enriched the stress BFU-E population using cell surface markers to Lin-cKit+CD31+ (from now on referred to as CD31+) cells. The remainder of hematopoietic cells in the fetal liver including Lin-cKit+CD31-, Lin-cKit-CD31+ and Lin-cKit-CD31- do not form stress BFU-E. But the CD31+ population of cells also forms steady state BFU-E. Hence the CD31+ population of cells is not pure for stress BFU-E.

We have tried to purify the stress BFU-E population further. The platelet glycoporphin IIb (CD41) is highly expressed on platelets and has been known for some time to be important for platelet homeostatic function (Phillips, Charo et al. 1988). Recently, it has been shown to be the earliest marker for primitive erythroid progenitor cells at E7.0 in the yolk sac and its high expression marks most of the definitive hematopoietic progenitors in the E8.25 yolk sac (Ferkowicz, Starr et al. 2003). In the fetal liver, only a minority of cells were CD41+, even though they were much more enriched in hematopoietic progenitors compared to the CD41- population. We asked if adding CD41
to the cocktail of Lin-cKit+CD31+ cells would help in separating the stress BFU-E from the steady state BFU-E. Hence, CD41 did not separate the two populations of erythroid progenitors in the fetal liver (Data not shown).

The integrin family of molecules is established mediators of cell migration in the developing embryo and in the adults, including the hematopoietic tissues. \( \alpha_4 \) integrins have been suggested to play roles in attachment, transmigration, proliferation, and differentiation of hematopoietic progenitors \textit{in vitro} (Coulombel, Auffray et al. 1997). \( \alpha_4 \) integrins are not required for the migration of hematopoietic progenitors between sites of hematopoiesis (Arroyo, Yang et al. 1999). They are shown to play an important role in the regulation of proliferation of hematopoietic progenitors (Moritz, Patel et al. 1994; Levesque, Haylock et al. 1996). \( \alpha_4 \) integrins are expressed on early erythroid progenitors (BFU-E and CFU-E) and down regulated later, which conforms to its role in proliferation. Antibodies against \( \alpha_4 \) integrins inhibit erythroid development (Yanai, Sekine et al. 1994) and when injected \textit{in utero} led to perinatal anemia in pups (Hamamura, Matsuda et al. 1996). Interestingly, yolk sac primitive erythropoiesis is not affected in the yolk sac in \( \alpha_4 \) integrin null mice, but \( \alpha_4 \) integrin null fetal livers are smaller and have defective proliferation compared to control fetal livers (Arroyo, Yang et al. 1999). \( \alpha_4 \) integrins function by heterodimerizing with \( \beta \) integrins (called VLA-4) and binding with its counter receptors namely VCAM-1 and fibronectin (FN) (Oostendorp, Reisbach et al. 1995). The interaction between erythroblasts and macrophages in the erythroblastic islands in the fetal liver may be mediated by VLA-4 binding to VCAM-1 (Sadahira, Yoshino et al. 1995). This might explain the specific defect in definitive erythropoiesis seen in the fetal liver in the \( \alpha_4 \) null embryos. We asked if differentiating
the CD31+ population of cells on their expression of α4 would help us separate the stress BFU-E from the steady state BFU-E. Most of the CD31+ population was positive for α4 and did not differentiate stress BFU-E from steady state BFU-E. The α4 negative CD31+ population did not form BFU-E efficiently suggesting that most of the CD31+ erythroid progenitors were α4+ (Data not shown).

Further studies will be required to separate the functionally different stress BFU-E from steady state BFU-E using cell surface markers in the fetal liver.

**Signals regulating expansion of Stress BFU-E**

We have shown that stress BFU-E are present in the fetal liver and expand in response to BMP4 signals induced in the fetal liver at E14.5. These stress BFU-E are also resident in the yolk sac at both E10.5 and E11.5, but they do not expand within the yolk sac and have to migrate to the fetal liver before they undergo expansion. Interestingly, yolk sac stress BFU-E do not respond to BMP4 signals unlike the fetal liver stress BFU-E (Porayette and Paulson, unpublished results); suggesting that another signal present in the fetal liver must be priming these cells to respond to BMP4 in the fetal liver. Work in our laboratory, which is described in more detail in the Appendix section of this thesis, has suggested that Desert hedgehog (Dhh) may be the signal in the fetal liver which may be helping the fetal liver primed to respond to BMP4. On induction at E14.5, BMP4 can then expand the Stress BFU-E and rapidly form erythrocytes for the rapidly developing embryo. Further work will be required to confirm this hypothesis.
Regulation of BMP4 Signaling

Our analysis of BMP4 expression in \textit{f/f} and control fetal livers showed that BMP4 is delayed in the \textit{f/f} mice. This is similar to the situation in the spleens of adult mice induced with acute anemia where the expression of BMP4 was delayed in response to acute anemia (Lenox, Perry et al. 2005). These observations raise an important question of why would there be a delay in BMP4 expression because of a mutation in the downstream target gene \textit{Smad5}. As mentioned in Chapter 2, we believe that BMP4 auto regulates its own expression through a Smad dependent pathway. We have shown that hypoxia induces the expression of BMP4 in the spleen (Lenox, Perry et al. 2005). Once BMP4 is turned on, BMP4 may induce its own expression. There is precedence for such an occurrence in Drosophila where \textit{Decapentaplegic (Dpp)}, the fly orthologue of BMP4, auto regulates its expression in a Smad dependent manner (Hursh, Padgett et al. 1993; Chanut and Heberlein 1997). If the problem in \textit{f/f} mice is in auto-regulating its BMP4 expression, then the initial induction of BMP4 in response to hypoxia should be normal. Further studies are needed to study this process.

Expansive erythropoiesis in mice is distinct from the erythropoiesis occurring under homeostatic conditions. Expansive erythropoiesis occurring in the fetal liver is mechanistically similar to stress erythropoiesis in the adult mice. A unique set of erythroid progenitors in the fetal liver is poised to respond to specific signals and rapidly form erythrocytes in large numbers. This is similar to the spleen, where in response to acute anemia, there is a rapid expansion of stress erythroid progenitors, rapidly replacing the acute loss of erythrocytes.
The expansive or stress erythropoiesis utilizes the BMP4-Smad5 signaling pathway to replenish the erythroid loss with this pathway being used only under these conditions and not under homeostatic conditions. The $f/f$ mice which are defective in the BMP4-Smad5 signaling are not able to launch a rapid response to acute blood loss. They are also anemic during fetal development as they are not able to rapidly expand their erythroid pool in the fetal liver. Both the phenotypes can be explained by their lack of stress erythroid progenitors both in the spleen in the adult mice and in the liver of the embryo. Therefore, the $f/f$ mouse provides a powerful example of the link between the stress erythropoiesis in the adult and the expansive erythropoiesis in the fetal liver.

The importance of microenvironment is demonstrated by the fact that even though the stress erythroid progenitors are resident in the yolk sac, they have to be exposed to the fetal liver environment for them to be able to expand proficiently. It compliments our findings in the adult mice, where the spleen and not bone marrow is poised to respond under conditions of acute erythroid stress.

The findings described in the thesis characterize the similarities and the differences in the fetal and adult erythroid microenvironments. They share a unique signaling pathway utilized specifically for stress erythropoiesis and not during homeostatic erythropoiesis. They both utilize the stress erythroid progenitors to rapidly restore their depleted erythroid pool. But unlike the spleen or the bone marrow which host the stress or steady state erythroid progenitors respectively, the fetal liver has both these progenitors resident in them. Also, unlike the spleen, which is almost exclusively an erythroid organ, the fetal liver even though predominantly erythroid, has the capacity to not only expand HSCs, but also other lineages of blood simultaneously.
Reference:


Appendix A

Friend virus utilizes the BMP4 dependent stress erythropoiesis pathway to induce erythroleukemia

Foreword

This chapter has been adapted from the manuscript submitted and accepted in Journal of Virology titled ‘Friend virus utilizes the BMP4 dependent stress erythropoiesis pathway to induce erythroleukemia’ and the author list includes Aparna Subramanian, Shailaja Hegde, Prashanth Porayette, Michele Yon, Pamela Hankey and Robert F. Paulson. The author contributed to work shown in figures 1B, 5D, 7D and work described in the Results section in the first five lines of Page 20. The author also contributed to the preparation of the manuscript.

Abstract

Over 50 years of genetic analysis has identified a number of host genes that are required for the expansion of infected cells during the progression of Friend virus induced erythroleukemia. In this report we show that Friend virus induces the Bone morphogenetic protein 4 (BMP4) dependent stress erythropoiesis pathway in the spleen, which rapidly amplifies target cells, propagating their infection and resulting in acute splenomegaly. This mechanism mimics the response to acute anemia, where BMP4 expressed in the spleen
drives the expansion of a specialized population of stress erythroid progenitors. Previously we demonstrated that these progenitors termed “stress BFU-E” are targets for Friend virus in the spleen. Here we extend those findings by showing that Friend virus infects two distinct populations of bone marrow cells. One population, when infected differentiates into mature erythrocytes in an Epo-independent manner. While a second population migrates to the spleen following infection where it induces BMP4 expression and acts as a reservoir of virus. The activation of the stress erythropoiesis pathway in the spleen by Friend virus results in the rapid expansion of stress BFU-E providing abundant target cells for viral infection. These observations suggest a novel mechanism where a virus induces a stress response pathway which amplifies target cells for the virus leading to acute expansion of infected cells.
Introduction

Friend erythroleukemia virus (FV) is a complex of two retroviruses, the replication defective Spleen Focus Forming Virus (SFFV), which is the pathogenic component and the replication competent Friend Murine Leukemia virus (F-MuLV). Friend virus induces an acute erythroleukemia that proceeds through a characteristic two stage progression(4, 35). The initial stage is characterized by the polyclonal expansion of infected cells in the bone marrow and spleen of susceptible mice. In the late stage of disease, a clone of infected cells acquires new mutations, specifically mutation of p53(34) and proviral insertional activation of $Spi1/Pu.1$(33), which leads to the emergence of a leukemic clone and eventually erythroleukemia.

The tropism of the virus for the erythroid lineage and the characteristic two stage progression has allowed for the identification of a number of host loci involved in the pathogenesis of Friend erythroleukemia(22, 42). Two genes $Fv1$(7) and $Fv4$(25) directly affect the ability of the virus to infect target cells by interfering with the retroviral life cycle. Four other genes regulate the expansion of infected cells during the initial stage of Friend disease, the $Friend virus susceptibility gene 2 (Fv2)$(28), $Dominant white spotting (W)$(40), $Steel (Sl)$(5) and $flexed-tail (f)$(2). Fv2 encodes a naturally occurring truncated form of the Macrophage stimulating 1 receptor (Mst1r), which is referred to as Short-form Stk or Sf-Stk(38). Sf-Stk interacts with the viral envelop glycoprotein from SFFV, gp55, and the Erythropoietin receptor, EpoR(16, 36). This complex drives the expansion of infected cells and the Epo-independent differentiation of
cells infected with the polycythemia inducing variant of Friend virus, FVP. Recent work has shown that Sf-Stk can drive the expansion of infected cells in the absence of EpoR in vivo suggesting that Sf-Stk signaling is responsible for the polyclonal expansion of infected cells during the initial stage of Friend disease(48). Sf-Stk is produced from an alternative promoter located in intron 10 of the gene. Fv2 resistant strains (Fv2<sup>r/r</sup>) fail to express Sf-Stk due to a mutation in the Sf-Stk promoter and consequently fail to expand infected cells in the spleen(38).

<sup>W</sup> and <sup>Sl</sup> encode the Kit receptor tyrosine kinase and its ligand Stem cell factor (SCF) respectively(9, 14, 17, 49). Mutation of either locus results in severe macrocytic anemia. The original work demonstrating that <sup>W</sup> and <sup>Sl</sup> mice were resistant to Friend virus suggested that the defect in erythropoiesis that leads to the severe anemia was responsible for the resistance(5, 40). However, recent work from our lab has demonstrated that pathogenic targets for Friend virus require the Kit/SCF signaling pathway in the spleen, but not the bone marrow(45). These studies identified the target cells for Friend virus in the spleen and showed that they were present in the spleen megakaryocyte erythroid progenitor (MEP) population originally described by Akashi et al(1). <sup>W/W</sup><sup>v</sup> mutant mice exhibit a 10 fold reduction in spleen MEPs, but have normal numbers of bone marrow target cells. These data supported the early work from Mirand and colleagues that showed that a spleen was necessary for the pathogenesis of Friend erythroleukemia, thus the defect in spleen target cells in <sup>W/W</sup><sup>v</sup> mice accounts for their resistance(29, 31).
f/f mutant mice exhibit a fetal-neonatal anemia that resolves two weeks after birth (19, 20, 23). As adults the mice exhibit normal steady state blood parameters and have normal numbers of erythroid progenitors in the bone marrow. However when challenged with an acute anemia, f/f mice are slow to recover, which suggests that they have a defect in expansive erythropoiesis that occurs at times of acute erythropoietic stress (13, 18). We cloned the f locus and showed that it encoded Madh5 or Smad5, a receptor activated Smad that acts downstream of the receptors for BMPs 2, 4 and 7 (27). Analysis of the f/f mutant phenotype showed that in response to acute anemia, BMP4 is induced in the spleen and drives the expansion of a specialized population of stress erythroid progenitors which we term stress BFU-E. BMP4 signaling is only required transiently and induces the differentiation of an earlier cell, the BMP4 responsive (BMP4R) cell into the Epo responsive stress BFU-E. These progenitors exhibit properties distinct from bone marrow steady state BFU-E in that they require only Epo to rapidly generate large colonies. Analysis of spleen cell populations showed that the BMP4 responsive stress BFU-E are present in the spleen MEP fraction, which are also the targets for Friend virus in the spleen suggesting that the BMP4 dependent stress erythropoiesis pathway is involved in the pathogenesis of Friend erythroleukemia (45). This link between Friend virus and stress erythropoiesis is also supported by the observation that f/f mice are resistant to Friend virus induced erythroleukemia (2).

In this report we show that Friend virus utilizes the BMP4 dependent stress erythropoiesis pathway during the initial stage of Friend erythroleukemia.
f/f mutant mice lack target cells in the bone marrow and spleen which results in resistance to Friend virus. Conditions that lead to the expansion of stress BFU-E such as induction of acute anemia in vivo or treatment of spleen cells with BMP4 in vitro significantly expand the number of target cells in the spleen. Infection with Friend virus leads to BMP4 expression in the spleens of wildtype, but not f/f mutant mice suggesting that in addition to the target cell defect, f/f mice have a defect in the spleen microenvironment. Our earlier analysis of W/Wv mice identified MEPs as the target cells in the spleen(45). Here we identify two distinct populations of target cells in the bone marrow, one that expresses Sf-Stk and forms Epo-independent (Epo\textsuperscript{ind}) BFU-E following Friend virus infection and a second population that migrates to the spleen following infection and induces BMP4 expression. These latter cells act as infectious center (IC) cells propagating infection of the stress BFU-E, which are rapidly expanding in response to BMP4. The acute expansion of infected cells in the spleen and the rapid progression to erythroleukemia are a direct result of the ability of Friend virus to activate the BMP4 dependent stress erythropoiesis pathway. These observations suggest a novel mechanism where a virus induces a physiological response that results in the amplification of target cells allowing for the acute propagation of the infection.

### Materials and Methods

**Mice.** BALB/c and BALB/c-f/f mice were bred and maintained in our colony. The f mutation was bred 4-5 generations onto the BALB/c background. Induction of
acute anemia by the injection of Phenylhydrazine (PHZ) was done as previously described(27). All research involving the use of mice was performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

**BFU-E Colony Assays**

For *in vitro* BFU-E formation, total bone marrow and spleen cells were harvested from mice. All assays for Epo<sup>ind</sup> BFU-E formation used FVP derived from a single batch of supernatant from FP63 cells, which produce the polycythemia-inducing form of Friend virus (FVP). FP63 cells were a kind gift from Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada. The amount of FP63 supernatant used was determined to produce the maximal number of Epo<sup>ind</sup> BFU-E when tested by infecting control bone marrow cells. FP63 supernatant or an equivalent volume of Dulbecco’s Modified Eagle Medium (DMEM) (mock infection) were incubated with bone marrow (2.5x10<sup>6</sup> cells total) on ice for 1 hour or spleen (6.5x10<sup>6</sup> cells total) for 1.5 hours. For bone marrow assays, Friend virus infected cells were plated in Methocult media (Stem Cell Technologies, Vancouver, BC) containing IL-3 (2.5 ng/ml). Control cells were plated in Methocult media containing IL-3 (2.5 ng/ml) + Epo (3 U/ml). The cells were then plated in 6-well plates in triplicates at a final concentration of 5x10<sup>5</sup> cells per well. For spleen assays, FV infected cells were plated in Methocult containing IL-3 (2.5 ng/ml) + SCF (100 ng/ml) (Peprotech Inc, Rocky Hill, NJ). Control cells were plated in IL-3 (2.5 ng/ml) + SCF (100 ng/ml) + Epo (3 U/ml). Final concentrations
of cells were 1.5x10^6 cells per well in triplicates in 6-well plates. The cultures were scored for BFU-E using acid benzidine staining as previously described(16). For assays examining the ability of BMP4 to increase spleen target cells, spleen cells were pre-incubated with BMP4 15 ng/ml BMP4 (R&D Systems, Minneapolis, MN) in DMEM prior to infection. The cells ± BMP4 were infected and plated as described above.

**Infectious center cell transplantation assays**

Bone marrow cells were isolated from BALB/cJ and BALB/c-f. Red blood cells were lysed using 0.16M NH4Cl. For unfractionated bone marrow cells, the cells were infected on ice with supernatant from FP63 cells as described above for Epo^ind colony assays. The infected cells were washed three times with PBS to remove surface bound virus. 4x10^6 infected cells were injected into tail veins of respective recipient mice. 2 weeks later, mice were sacrificed, spleens isolated, weighed and fixed in Bouin's fixative (Sigma, St Louis, MO). IC cell transplantation assays with sorted bone marrow cells were done as described above expect fewer cells were infected and later transplanted. For CD31+Kit+Sca1-Lin- cells 1 X 10^5 infected cells were transplanted per recipient and for CD31+CD41+Kit+Sca1-Lin- 5 X 10^4 infected cells were transplanted.

**Analysis of Sf-Stk expression.**

Total RNA was isolated from sorted populations of bone marrow and spleen cells. The different sorted populations were lysed in Trizol (Invitrogen, Carlsbad,
CA) and reverse transcribed into cDNA. Primer sequences for Sf-STK were as follows: Sf-stk–specific PCR primers were 5´–TCTGGCTGATCCTTCTGTCTG–3´ and 5´–GCAGCAGTGGGACACTTGTCC–3´ (456-bp product)(38). HPRT or β-actin were used as internal controls.

**Analysis of BMP4 expression in spleen sections**

Spleens were harvested on the indicated days post Friend virus infection, fixed in 4% paraformaldehyde and paraffin embedded tissue sections were cut. Sections were deparaffinized in Histo-clear II (National Diagnostics, Atlanta, GA), rehydrated through an ethanol series to 50% ethanol, blocked in Protein blocking agent (Immunotech, Westbrook, ME) for 1hr to overnight and rinsed in phosphate-buffered saline (PBS). Sections were incubated with primary anti-BMP4 antibody (Novocastra Labs, Vector Labs, Burlingame, CA) conjugated to Alexa 667 using the labeling kit from Molecular Probes (Eugene, OR). Some sections were also labeled with mAb34(12) (provided by Dr. K Hasenkrug, Rocky Mountain Labs, NIAID) conjugated to Alexa 488 labeling dye. Labeling antibodies were diluted at 1:20 concentration. Slides were incubated 2-4 hours in a dark humid chamber. Sections were washed in PBS and mounted in Slow Fade (Molecular Probes). Slides were analyzed by confocal microscopy using an **Olympus Fluoview 300 Confocal Laser Scanning Microscope**.

**FACS analysis of bone marrow and spleen target populations.**
Bone marrow and spleen cells were stained for MEP’s as described earlier(1, 27). Bone marrow and spleen cells were sorted for the CD31+ subsets using biotinylated lineage markers (Molecular Probes) and biotin–conjugated Sca-1 (Pharmingen, San Diego, CA). Sca+Lin+ cells were removed using streptavidin conjugated magnetic beads and the Easy-SEP magnet (Stemcell Technologies, Vancouver, BC). The remaining cells were stained with FITC-conjugated anti-Kit and PE-conjugated anti-CD31 (Pharmingen, San Diego, CA). CD41+ subsets were obtained by removing Sca+Lin+ subsets and staining the remaining cells with FITC-conjugated c-Kit, APC-conjugated CD31 and PE-conjugated CD41 (Pharmingen). Cells were washed and sorted using a Coulter Elite ESP flow cytometer. For sorting peripheral blood cells, peripheral blood was collected into tubes containing heparin. Peripheral blood mononuclear cells were isolated by layering the blood onto a Ficoll gradient (Histopaque 1077, Sigma St. Louis, MO) and collecting the cells at the interface following centrifugation. Analysis of CD31+Kit+CD41+Sca1-Lin- cells was done as described above.

**Results**

*f/f* mutant mice exhibit decreased numbers of Friend virus target cells in the bone marrow and spleen.

*f/f* mice are maintained on the C57BL/6 background, which is $Fv2^r/r$ and is resistant to Friend virus. In order to circumvent this problem we crossed the *f* mutant allele 4-5 generations onto the BALB/c background (BALB/c-*f/f*), which is
Fv2<sup>s/s</sup> and sensitive to Friend virus infection. *In vitro* infection of bone marrow and spleen cells from sensitive mice with the polycythemia inducing strain of Friend virus (FVP) results in the formation of erythropoietin-independent (Epo<sup>ind</sup>) BFU-E colonies. These colonies have also been referred to as viral BFU-E or vBFU-E, however, in this paper we will refer to them as Epo<sup>ind</sup> BFU-E(26). Previous work from our lab has shown that this assay accurately reflects the *in vivo* sensitivity of different mouse strains to Friend virus induced erythroleukemia and is a measure of the number of pathogenic targets for Friend virus(16, 26, 45). We tested whether bone marrow and spleen cells from BALB/c-*f/f* and BALB/c-*f/+* mice could generate Epo<sup>ind</sup> BFU-E following Friend virus infection *in vitro*. Figure 1A shows that BALB/c-*f/f* bone marrow and spleen cells generated significantly fewer Epo<sup>ind</sup> BFU-E following FVP infection than the number of BFU-E generated when cells are cultured with Epo. In contrast, bone marrow and spleen cells from BALB/c-*f/+* mice exhibited no significant difference between the number of Epo<sup>ind</sup> BFU-E generated following FVP infection and the number of BFU-E generated when Epo was included in the media. Furthermore, BALB/c-*f/+* mice are sensitive to infection *in vivo*, exhibiting splenomegaly 14 days after infection that is indistinguishable from BALB/c control mice (data not shown). These data demonstrate that *f/f* mice have decreased number of target cells in their bone marrow and spleen. This observed decrease in the numbers of target cells could be due to an actual reduction in the number of target cell numbers or an inability of these target cells to respond to Friend virus infection. Previously we showed that the targets for Friend virus in the spleen are contained in the MEP
population(45). We sorted MEPs from BALB/c and BALB/c-f/f and observed no difference in the number of spleen MEPs (data not shown). These observations suggest that f/f MEPs must be defective in their ability to respond to Friend virus. Sf-Stk is required for Friend virus to induce Epo\textsuperscript{ind} BFU-E formation. Analysis of Sf-Stk expression in the spleens of f/f and control mice demonstrated that f/f spleen MEPs fail to express Sf-Stk. These data suggest that Smad5 dependent signals are required for the proper expression Sf-Stk in these cells (Figure 1B). However, despite repeated attempts, treatment of spleen cells with BMP4 \textit{in vitro} does not induce the expression of Sf-Stk (data not shown), which suggests that the regulation of Sf-Stk by Smad5 is indirect.

\textbf{Acute anemia induces Friend virus target cells in the spleen, but not the bone marrow.}

Phenylhydrazine (PHZ) injection induces an acute hemolytic anemia from which control mice take 6-7 days to recover. In contrast, f/f mice exhibit a delayed recovery, which takes approximately 8-9 days. Our analysis of the recovery from acute anemia has demonstrated that a specialized population of stress erythroid progenitors, which we term stress BFU-E, rapidly expand in the spleen during the recovery period. The peak expansion of stress BFU-E is observed at 36 hours after PHZ treatment. f/f mutant mice exhibit a delayed expansion of stress BFU-E, which occurs at 4 days after treatment and results in the delayed recovery(27). The number of bone marrow BFU-E does not change significantly during recovery except in the later stages where their numbers start
to decrease(27). Stress BFU-E are contained in the spleen MEP population, which also contains the targets for Friend virus(27, 45). Based on these data, we would predict that treatment of control mice with PHZ would cause an expansion of Friend virus target cells at 36 hours after treatment, however in f/f mice the expansion would be delayed until 4 days after treatment. Figure 2A shows that in BALB/c mice the number of FVP induced Epo\textsuperscript{ind} BFU-E is significantly increased 36 hours after PHZ treatment. In contrast, BALB/c-f/f mice show no change in the number of FVP induced Epo\textsuperscript{ind} BFU-E 36 hours after treatment with PHZ. However, at 4 days after treatment, BALB/c-f/f mice exhibited a significant increase in Epo\textsuperscript{ind} BFU-E following FVP infection, which correlates with the delayed expansion of stress BFU-E, we previously observed in f/f mice(27). Control mice exhibit decreased levels of Epo\textsuperscript{ind} BFU-E 4 days after PHZ treatment, which is also consistent with our observations that the numbers of stress BFU-E decline 4 days after PHZ treatment of control mice. This expansion of target cells in the spleen of BALB/c-f/f mice following acute anemia induction also translates into sensitivity to FVP induced disease. BALB/c-f/f mice were treated with PHZ and 4 days after treatment they were infected with FVP. Two weeks later, these mice developed extensive splenomegaly that was indistinguishable from that observed when control mice were infected with FVP (average spleen weight: 3.15g FVP infected BALB/c-f/f PHZ treated v. 1.2g uninfected BALB/c-f/f PHZ treated v. 0.2g untreated mouse, n=2. For comparison FVP infected BALB/c average spleen weight 2.5 g). This experiment also shows that Friend virus can replicate in the mutant mice. So defects in viral replication
are not the root cause of the resistance in f/f mice. In contrast, the number of bone marrow Epo\(^{\text{ind}}\) BFU-E was unaffected by PHZ treatment (Figure 2B). These data suggest that the resistance in the f/f mice is caused by a lack of target cells in the spleen. If you induce the expansion of target cells by activating the BMP4 dependent stress erythropoiesis pathway with PHZ, f/f mice are sensitive to Friend virus.

**Treatment of spleen cells with BMP4 in vitro increases the number of target cells.**

Acute anemia induces the expression of BMP4 in the spleen, where it acts on an immature cell, the BMP4 responsive (BMP4\(^{R}\)) cell causing it to become an Epo responsive stress BFU-E. Treatment of spleen cells in vitro with BMP4 results in a significant expansion of stress BFU-E(27). Based on our observation that acute anemia increases the number of target cells in the spleen, we next tested whether culturing spleen cells with BMP4 could increase the number of Friend virus target cells in vitro. Spleen cells from BALB/c mice were cultured in media containing BMP4 (15 ng/ml) for 24 hours and then tested for the ability of FVP to induce Epo\(^{\text{ind}}\) BFU-E. As shown in Figure 3A, culturing cells in BMP4 significantly increased the number of Epo\(^{\text{ind}}\) BFU-E following infection with FVP in BALB/c mice. Thus, acute anemia expands Friend virus target cells in vivo and treatment with BMP4 in vitro increases Friend virus targets, which further implicates the BMP4 dependent stress erythropoiesis pathway in the pathogenesis of Friend erythroleukemia.
**Friend virus infection induces the expression of BMP4 in the spleen.**

The expansion of stress BFU-E in the spleen during the recovery from acute anemia is driven by the expression of BMP4. We would predict that Friend virus infection would lead to a similar induction of BMP4 in the spleen, if Friend virus utilizes this pathway. We tested whether BMP4 is expressed in the spleen following infection with FVP by staining spleen sections with anti-BMP4 antibodies at different times post infection. No expression of BMP4 is seen at 1 day post infection, but by days 8 and 12 after infection, BMP4 is expressed in small patches in the red pulp of spleen. By day 15, BMP4 expression is observed throughout the red pulp of the spleen (Figure 3B). The kinetics of BMP4 expression correlates with our previous data showing that the expansion of Friend virus infected Epo$^{ind}$ BFU-E in the spleen does not occur until after day 6(45). These data demonstrate that Friend virus infection can induce the expression of BMP4 in the spleen, which suggests that the activation of the BMP4 dependent stress erythropoiesis pathway plays a role in the pathogenesis of Friend virus.

**FVP infected bone marrow cells can not induce BMP4 expression or splenomegaly when transplanted into f/f mice.**

Early work showed that bone marrow cells infected in vitro with Friend virus could be transplanted into susceptible mice leading to the propagation of infection in the spleen. Surprisingly these early experiments showed that the
transplanted cells did not expand in the spleen, but rather acted as a reservoir of virus that infected recipient spleen cells resulting in splenomegaly (41, 44). These cells were referred to as infectious center (IC) cells. In previous figures we showed that signals induced by acute anemia and in particular, BMP4, increased target cells in the spleen and that Friend virus infection of control mice induced the expression of BMP4 in the spleen. These observations suggest that Friend virus induces the BMP4 dependent stress pathway to amplify target cells in the spleen. Based on these observations we would predict that control bone marrow IC cells infected in vitro with Friend virus could not propagate an infection when transplanted into f/f mice because the recipient spleen cells are unable to respond to BMP4. We tested this hypothesis by transplanting FVP infected control bone marrow cells into f/f and control mice and assaying whether IC cells could propagate an infection leading to splenomegaly. BALB/c bone marrow cells were infected in vitro with FVP and transplanted in BALB/c control or BALB/c-f/f mice. Spleen weight was measured at day 4 and day 15 post transplant. FVP infected bone marrow cells very efficiently induced splenomegaly by day 15 when transplanted into control recipients, however when infected bone marrow cells were transplanted into f/f recipients no splenomegaly was observed (Figure 4A). The homing of bone marrow cells to f/f spleens is similar to the homing of bone marrow cells to control spleens, so a defect in homing to the spleen cannot explain these observations (O. Harandi and RF Paulson unpublished data).

We next examined whether this defect in the ability of IC cells to transplant infection was due to a defect in the progenitor cells or due to a defect in the
expression of BMP4. During the recovery from acute anemia the expression of
BMP4 is very tightly regulated in the spleen. However previous work from our lab
has shown that f/f mice exhibit a delayed expression of BMP4, which suggests
that Smad5 dependent signals regulate BMP4 expression in the spleen
stroma(27). We tested the expression of BMP4 in the spleens of control and f/f
mice transplanted with control bone marrow cells infected in vitro with FVP.
Figure 4B shows that the spleens of control mice transplanted with control FVP
infected cells express BMP4 at high levels at days 8 and 15 post transplantation,
but f/f mice transplanted with FVP infected bone marrow cells expressed little to
no BMP4 at any of the time points. Based on these observations we propose that
impaired BMP4 stress erythropoiesis pathway in the spleen results in the
resistance of f/f mice to Friend virus infection. This defect is caused by a
combination of the inability of f/f progenitor cells to respond to BMP4 and the
inability of IC cells to induce BMP4 expression. Together, these defects block the
rapid expansion of target cells leading to resistance.

The induction of BMP4 expression in the spleen by Friend virus infection
is puzzling. Our previous analysis showed that hypoxia was capable of inducing
BMP4 expression in spleen stromal cells. However because of the increased red
cell production caused by Friend virus, it is unlikely that hypoxia induces BMP4
expression following Friend virus infection. Furthermore the observation that FVP
infected control bone marrow cells were unable to induce the expression of
BMP4 when transplanted into f/f mice suggested that the mutant mice also have
a defect in the cells expressing BMP4. We examined spleen sections to
determine which cells were expressing BMP4 in the infected spleens. At day 12 and 15 after infection, we stained spleen sections with anti-BMP4 antibodies and mAB34, a monoclonal antibody that recognizes Friend virus infected cells(12, 45). At day 12, there is clear overlap between infected cells and BMP4 expressing cells, however by day 15 BMP4 expressing cells are distinct from Friend virus infected cells (Figure 4C). These observations suggest that Friend virus infection could itself induce BMP4 expression. However, when we examined the expression of BMP4 in bone marrow cells infected in vitro with Friend virus, we could not detect any expression (data not shown). Taken together these data suggest a model where FVP infected bone marrow cells migrate into the spleen where they encounter a signal in the spleen microenvironment, which induces BMP4 expression by the infected cells. The subsequent expression of BMP4 by the surrounding stromal cells is Smad5 dependent and is defective in f/f mice.

**Bone marrow CD31+Kit+Sca1-Lin- cells form Epo\textsuperscript{ind} BFU-E following FVP infection in vitro.**

Previous work from our lab showed that the spleen MEP population contained the targets for Friend virus, while the bone marrow MEP population did not contain targets for Friend virus as they failed to form Epo\textsuperscript{ind} BFU-E following infection in vitro(45). Recent work from Bauman et al. identified a new population of progenitor cells that exhibit erythroid potential(3). These cells are characterized by their expression of the endothelial cell marker CD31 or PECAM.
This work showed that CD31+Kit+Sca1-Lin- cells sorted from bone marrow were able to form BFU-E and provide short-term erythroid radioprotection when transplanted into irradiated recipients. They also showed that these cells were distinct from the MEP population in the bone marrow. We tested whether these progenitors were targets for Friend virus infection by sorting CD31+Kit+Sca1-Lin- cells from the bone marrow of BALB/c mice. Approximately, 38% of Lin-Sca1-cells were CD31+Kit+ in the bone marrow. These cells were infected with FVP and scored for Epo_{ind} BFU-E in methylcellulose assays. Figure 5A shows that this population contains Friend virus target cells, in fact the CD31- fraction failed to form Epo_{ind} BFU-E (data not shown) suggesting that this population contains most if not all the target cells for Friend virus in the bone marrow. We also sorted CD31+Kit+Sca1-Lin- cells from spleen and found that these cells do not form Epo_{ind} BFU-E following FVP infection (Figure 5B). These data show that the target cells for Friend virus in the bone marrow are distinct from the target cells in the spleen.

f/f bone marrow contains few functional Friend virus target cells.

We tested whether the mutation in Smad5 resulted in a decrease in the number of CD31+Kit+Sca1-Lin- cells in the bone marrow or an inability of these cells to respond to the virus. Flow cytometry analysis of f/f bone marrow showed that these mice had slightly fewer CD31+Kit+Sca1-Lin- cells, approximately 33% of the Sca1-Lin- cells were CD31+Kit+. However, when this population was sorted and infected in vitro, f/f CD31+Kit+Sca1-Lin- cells generated significantly fewer Epo_{ind} BFU-E (Figure 5C). When we analyzed the expression of Sf-Stk in
CD31+Kit+Sca1-Lin- cells from f/f and control mice, we observed that the expression in the mutant cells was indistinguishable from control (Figure 5D). We next tested whether CD31+Kit+Sca1-Lin- cells from BALB/c-f/f mice were infectable by Friend virus. Analysis of f/f bone marrow cells infected in vitro by Friend virus showed that CD31+Kit+Sca1-Lin- cells were infected as measured by flow cytometry with mAB34(12, 45) (data not shown). These data show that Smad5 dependent signaling is not required in the bone marrow for the development of these target cells or for Sf-Stk expression but is required for FVP induced BFU-E formation. At the present time we do not understand why f/f CD31+Kit+Sca1-Lin- cells that express Sf-Stk, fail to form Epo\textsuperscript{ind} BFU-E following FVP infection. Further work will be needed to explain the role of Smad5 dependent signals in these cells.

**CD31+Kit+CD41+Sca1-Lin- cells act as IC cells.**

Friend virus target cells can be subdivided into two populations, target cells capable of forming Epo\textsuperscript{ind} BFU-E following FVP infection and target cells that are capable of acting as IC cells, propagating the infection in the spleen. Early work that separated cells in the bone marrow and spleen by velocity sedimentation analysis showed that these two target cell populations were distinct(43). We fractionated bone marrow and spleen cells into MEPs (Kit+CD34-FcgR\textsuperscript{lo}Sca1-IL-7R\alpha-lin-) or CD31+Kit+Sca1-Lin- cells and tested whether these fractions when infected \textit{in vitro} with FVP could act as IC cells by inducing splenomegaly when transplanted into susceptible control mice. Despite
the fact that earlier work showed that IC cells were distinct from Epo\textsuperscript{ind} BFU-E forming cells, we observed that bone marrow CD31+Kit+Sca1-Lin- cells functioned very efficiently as IC cells (Figure 6A), while bone marrow or spleen MEPs failed to induce splenomegaly. Furthermore, bone marrow CD31+Kit+Sca1-Lin- cells induced BMP4 expression 14 days post transplant, while spleen MEPs, the targets of FVP in the spleen failed to induce BMP4 expression (Figure 6B).

Given that earlier work showed that IC cells were distinct from Epo\textsuperscript{ind} colony forming cells, we further subdivided the CD31+Kit+Sca1-Lin- cells. We chose CD41 as an additional marker. CD41, which encodes platelet \(\alpha\) integrin IIb, was originally shown to play a role in platelet function. Recent work, however demonstrated that CD41 is also expressed on progenitor cells in the yolk sac and fetal liver in a pattern similar to what was observed with CD31\textsuperscript{(15, 30, 32)}. Approximately 5% of the CD31+Kit+Sca1-Lin- cells were CD41+ (Figure 7A). Sorted CD31+Kit+CD41+Sca1-Lin- (CD41+) and CD31+Kit+CD41-Sca1-Lin- (CD41-) cells were infected \textit{in vitro} with FVP and plated in methylcellulose media to test for Epo\textsuperscript{ind} BFU-E formation or transplanted into susceptible control mice to assay for IC cell activity. When cells were plated for Epo\textsuperscript{ind} BFU-E formation, the CD41- cells readily formed Epo\textsuperscript{ind} BFU-E, while the CD41+ fraction did not (Figure 7B). However the opposite was true in the IC cell assay, where the CD41+ population induced splenomegaly, while the CD41- population had no effect (Figure 7C). The spleen also contains IC cells\textsuperscript{(44)}, so we tested whether the CD31+Kit+CD41+Sca1-Lin- cells from the spleen could
also propagate an infection when infected *in vitro* and transplanted into susceptible recipients. The number of CD41+ cells in the spleen was significantly less than what was observed in the bone marrow. Despite the decreased numbers, spleen CD41+ cells infected in vitro with FVP were able to propagate the infection and induce splenomegaly when transplanted into BALB/c mice (data not shown). Sf-Stk is required for Friend virus to induce Epo\textsuperscript{ind} BFU-E formation.

We tested Sf-Stk expression by RT-PCR in bone marrow CD31+Kit+Sca1-Lin- cells fractionated for CD41 expression. Figure 7D shows that the CD41- cells express Sf-Stk and form Epo\textsuperscript{ind} BFU-E, while the CD41+ cells fail to express Sf-Stk and their role is limited to IC cell. The lack of Sf-Stk expression by the CD41+ IC cells is consistent with earlier work that showed that IC cells from Fv2\textsuperscript{rr} congenic mice could propagate infection when transplanted into Fv2\textsuperscript{ss} recipients(10).

BALB/c-f/f mice have a defect in target cells in the bone marrow and control cells infected *in vitro* are unable to propagate an infection in a f/f spleen. We next investigated whether f/f mice also have a defect in IC cells. CD31+Kit+CD41+Sca1-Lin- cells were sorted from bone marrow and approximately 7.6% of CD31+Kit+Sca1-Lin- cells were CD41+. However, given the fact that BALB/c-f/f mice have slightly fewer CD31+Kit+Sca1-Lin- cells, the CD41+ populations in the two strains, as measured by the percentage of Sca1-Lin- cells, is approximately equal (1.9% in BALB/c v. 2.4% in BALB/c-f/f) (Figure 8A). We further tested whether the f/f mutation could affect the ability of f/f bone marrow cells infected *in vitro* with FVP to propagate infection in the spleen when
transplanted into control recipient mice. After 15 days post transplant, FVP infected f/f bone marrow could induce splenomegaly in recipient mice. However the extent of splenomegaly was slightly, but significantly less. In addition the kinetics of splenomegaly was significantly slower in the mice transplanted with f/f infected bone marrow cells when compared with control bone marrow cells (Figure 8B). The expansion of infected cells in the f/f transplants is delayed and the majority of the expansion occurs in the final three days, while in the control transplants, the expansion begins at day 8 and continues through day 15. These observations are consistent with the possibility that f/f mutant IC cells have a defect in their ability to propagate infection in the spleen. Two alternative explanations could explain these data. First, f/f mutant IC cells could have a defect in homing to the spleen. We have examined this possibility in the context of other experiments and have observed no difference in the homing of f/f mutant bone marrow cells to the spleen (O. Harandi and RF Paulson unpublished). Second, as discussed earlier f/f mice can replicate Friend virus in the spleen, however, decreased viral titers in the mutant IC cells could also lead to a delay in the expansion of infected cells in the spleen. This possibility can not be ruled out by our experiments.

**CD31+Kit+CD41+Sca1-Lin- cells increase in the peripheral blood during**

**Friend virus infection.**

The pathogenesis of Friend virus infection requires the expansion of infected target cells in the spleen(29, 31, 45) and our data demonstrates that the
activation of the BMP4 dependent stress erythropoiesis pathway is required for
this expansion. The mechanism by which the infection spreads to the spleen is
not clear. IC cells from the bone marrow when infected in vitro and transplanted
into susceptible mice propagate the infection in the spleen. However during the
natural course of infection do infected IC cells migrate to the spleen to initiate
infection? We tested whether the number of CD31+Kit+CD41+ cells in the
peripheral blood increased following FVP infection in vivo. Figure 9 shows that
there is 6 fold increase in the number of CD31+Kit+CD41+ cells in the peripheral
blood of BALB/c mice infected with FVP when compared to uninfected controls.
This observation supports a model where IC cells infected in the bone marrow
migrate to the spleen, induce BMP4 expression and infect stress BFU-E.
However, we have also observed IC cells in the spleen, which could be directly
infected by Friend virus and contribute to the expansion of target cells. Our
earlier data showing that the expansion of infected Epo\textsuperscript{ind} BFU-E in the spleen
does not occur until after day 6 post infection suggests that immediate direct
infection of spleen IC cells does not make a significant contribution to the initial
expansion of target cells(45).

Discussion

Friend virus is capable of inducing an acute splenomegaly caused by the
rapid expansion of infected cells, which progresses to erythroleukemia. Our data
shows that Friend virus activates the BMP4 dependent stress erythropoiesis
pathway and takes advantage of the ability of this pathway to rapidly expand a
specialized population of stress BFU-E, which are the targets for Friend virus in the spleen. Based on these observations we propose a new model for the pathogenesis of Friend virus, where the bone marrow contains two populations of target cells. One population, CD31+Kit+CD41-Sca1-Lin- cells, express Sf-Stk and form Epo\textsuperscript{ind} BFU-E upon infection with FVP. The second population, CD31+Kit+CD41+Sca1-Lin-, does not express Sf-Stk. These cells act as IC cells during infection, which migrate to the spleen following infection. Once in the spleen, IC cells respond to signals in the spleen microenvironment and begin expressing BMP4. We speculate that the expression of BMP4 in the spleen stroma is initiated and maintained by a BMP4/Smad5 dependent mechanism. BMP4 causes the expansion of stress BFU-E, which are the targets for Friend virus in the spleen(27, 45). The BMP4 stress erythropoiesis pathway in the spleen has enormous proliferative potential as demonstrated by our observation that stress BFU-E expand 48 fold 36 hours after anemia induction. The ability of Friend virus to activate this pathway and utilize stress BFU-E as targets results in the acute splenomegaly, polycythemia and rapid progression to erythroleukemia associated with FVP infection. This new model proposes that Friend virus manipulates a physiological stress response pathway to amplify target cells resulting in an acute expansion of infected cells.

The close relationship between Friend virus and stress erythropoiesis is further strengthened when previous work is re-examined in the light of this new model. The four host genes that are required for the expansion of Friend virus infected cells all appear to play a role in the BMP4 dependent
stress erythropoiesis pathway. Our earlier work on the resistance of $W$ and $Sl$ mice showed that the defect in these mice was caused by a lack of target cells in the spleen (45). Similarly, we have demonstrated that $W$ mutant mice have a severe defect in the expansion of stress BFU-E in response to acute anemia and in vitro, SCF plays a key role in the expansion stress BFU-E in culture (37). Sf-Stk is absolutely required for the expansion of infected cells (38) and preliminary data suggests that Sf-Stk is up-regulated in the spleen during the recovery from acute anemia, where it appears to play a role in the differentiation of stress BFU-E (O. Harandi, L. Shi and RF Paulson unpublished). Based on these observations, our identification of two target cell populations in the bone marrow suggests that these cells may also be involved in the BMP4 dependent stress erythropoiesis pathway. Although both of these populations exhibit erythroid potential in vitro their exact role in stress or steady state erythropoiesis will await further investigation.

Our model proposes that IC cells must migrate to the spleen to induce BMP4 expression and act as reservoirs of infectious virus. Our observation that infection of bone marrow cells with FVP does not induce BMP4 expression suggests that the induction of BMP4 expression in the spleen is indirect. We propose that it is the interaction between infected IC cells and the spleen microenvironment that induces the expression of BMP4. This situation is very similar to some recent observations we have made about the maintenance of the BMP4 dependent stress erythropoiesis pathway. Our data show that transplanted bone marrow cells can home to the spleen and replenish the BMP4 responsive
stress BFU-E. Donor bone marrow cells express BMP4 when they migrate to the spleen, which is very similar to what we observed 12 days after FVP infection of BALB/c control mice. However by 15 days after infection, we observed that FVP infected cells no longer express BMP4. Together these data support a model where initial BMP4 expression by infected cells is induced by interactions with the spleen microenvironment and the subsequent expression of BMP4 by the spleen stroma requires BMP4/Smad5 dependent signals. The ability of BMP4 to induce its own expression in neighboring cells and expand the area of BMP4 expression has been observed with Dpp, the Drosophila ortholog of BMP4 and this auto-regulation is responsible for the spread and maintenance of Dpp expression (11, 21, 24).

An essential component of our model proposes that bone marrow IC cells infected with virus migrate to the spleen where they induce BMP4 expression and act as reservoirs of virus for the infection of the expanding stress BFU-E. Earlier work in the field investigated the ability of helper free SFFV to cause disease in mice(6, 8, 39, 46, 47). In some instances, infection with helper free SFFV was able to induce disease (splenomegaly, polycythemia and erythroleukemia)(39, 46, 47). On the surface these results would appear to be inconsistent with our model. If direct infection of spleen targets could result in disease then IC cells are not necessary to acts as reservoirs of virus or induce the BMP4 dependent expansion of target cells in the spleen. However, on closer inspection of the data, helper free SFFV causes disease in mice only when they have been treated with PHZ prior to infection. PHZ treatment activates the BMP4
dependent stress erythropoiesis pathway leading to the expansion of stress BFU-E, which can be directly infected by helper free virus.

In summary, we have demonstrated that Friend virus utilizes the BMP4 dependent stress erythropoiesis pathway during the early stages of Friend erythroleukemia. We have identified two distinct target cells for Friend virus in the bone marrow, one which forms Epo\textsuperscript{ind} BFU-E and a second that functions as an IC cell propagating infection in the spleen. These data led us to propose a new model for Friend virus pathogenesis where infection of cells in the bone marrow leads to either Epo independent erythropoiesis or migration of infected IC cells to the spleen. Once in the spleen, these cells induce BMP4 expression, which leads to the expansion of stress BFU-E, the target cells for Friend virus. This model suggests a novel mechanism where Friend virus activates the stress erythropoiesis pathway resulting the rapid amplification of target cells leading to acute splenomegaly and polycythemia.
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We want to dedicate this paper to Dr. Alan Bernstein on the occasion of his retirement as Director of the Canadian Institutes of Health Research in honor of his service to Canadian Science and his contributions to Friend virus research. We want to thank Andy Henderson for comments on the manuscript. This work was funded by Public Health Service Grant HL070720 from the National Heart Lung and Blood Institute (RFP). This project is funded, in part, under a grant with the Pennsylvania Department of Health using Tobacco Settlement Funds. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.
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Figure legends

Figure 1. *f/f* mice have decreased numbers of target cells in the spleen and bone marrow. (A) BALB/c-*f/f* and BALB/c-*f/+* bone marrow (left) and spleen (right) cells were mock infected or infected with FVP and plated in the indicated cytokines. BFU-E were scored. (B) RT-PCR analysis of Sf-Stk expression in MEPs that were sorted from BALB/c-*f/f* and BALB/c spleen. Bars represent the average ± standard deviation of one representative experiment of 4 independent experiments.

Figure 2. Phenylhydrazine induced acute anemia expands Friend virus target cells in the spleen but not the bone marrow. (A) BALB/c and BALB/c-*f/f* mice were treated with PHZ to induce acute anemia. 36 hours and 4 days after treatment spleen cells were harvested and either mock infected or infected with FVP. The cells were then plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) and BFU-E scored. (B) BALB/c mice were treated with PHZ to induce anemia and bone marrow cells were harvested 36 hours after treatment. The cells were either mock infected or infected with FVP and plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) and BFU-E scored. Significant differences as measured by t-test are indicated. The bars represent average ± standard deviation of one of 4 independent experiments.
Figure 3. BMP4 treatment increases the number of target cells in the spleen and its expression is induced in the spleen by FVP infection. (A) BALB/c spleen cells were either mock infected or infected with FVP and plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) with or without added BMP4 (15ng/ml). BFU-E were scored. Significant differences as measured by t-test are indicated. Bars indicate average ± standard deviation of one representative experiment of 3 independent experiments. (B) Spleen sections of BALB/c mice isolated on the indicated days post infection with FVP. The sections are stained with anti-BMP4 antibodies. Bright field pictures are below.

Figure 4. FVP infected bone marrow cells when transplanted into f/f recipients cannot propagate the infection in the spleen. BALB/c control bone marrow cells were infected in vitro with FVP and transplanted into BALB/c-/-f/f or BALB/c controls recipients. (A) On the indicated days spleens were removed and weighed to determine splenomegaly. Significant differences as measured by t-test are indicated. (B) Spleen sections were stained with anti-BMP4 antibodies to determine BMP4 expression. (C). Sections from spleens isolated from BALB/c mice infected with FVP on day 12 (Top) or day 15 (Bottom) after infection were stained with anti-BMP4 and mAB34 which recognizes Friend virus infected cells. BMP4 staining is shown in red, mAB34 staining in green and the overlap is shown in yellow. A bright field image is included in the lower left panel for each day.
Figure 5. CD31+Kit+Sca1-Lin- cells are Friend virus target cells in the bone marrow. (A) (Left) Flow cytometry analysis of BALB/c Lin-Sca1- cells analyzed for expression of CD31 and Kit. The box indicated CD31+Kit+ Sca1-Lin- population. (Right) CD31+Kit+Sca1-Lin- cells sorted from BALB/c bone marrow were either mock infected or infected with FVP and plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) and BFU-E scored. (B) Spleen CD31+Kit+Sca1-Lin- cells were either mock infected or infected with FVP and plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) and BFU-E scored. (C) (Left) Flow cytometry analysis of BALB/c-/-f/f Lin-Sca1- cells analyzed for expression of CD31 and Kit. The box indicated CD31+Kit+ Sca1-Lin- population. (Right) CD31+Kit+Sca1-Lin- cells sorted from BALB/c-/-f/f bone marrow were either mock infected or infected with FVP and plated in methylcellulose media containing Epo + SCF + IL-3 (Mock infected) or SCF + IL-3 (FVP infected) and BFU-E scored. (D) RT-PCR analysis of Sf-Stk expression in CD31+Kit+Sca1-Lin- cells isolated from BALB/c-/-f/f and BALB/c control mice. Significant differences as measured by t-test are indicated. The bars represent average ± standard deviation of one representative experiment of 3 independent experiments.

Figure 6. CD31+Kit+Sca1-Lin- cells act as IC cells. (A) Bone marrow cells from BALB/c control mice were sorted into CD31+Kit+Sca1-Lin- (CD31+) or MEP (Kit+CD34-FcγRIIα-IL-7Rα-Sca1-Lin-) populations and spleen MEPs were isolated.
The sorted cells were infected \textit{in vitro} with FVP and transplanted into BALB/c control mice. 14 days later the spleens were removed and weighed to test for splenomegaly. Significant differences are indicated by t-test. The data represent the average + standard deviation of 3 independent experiments. (B) Spleen sections from mice transplanted with FVP infected bone marrow CD31+ and Spleen MEP cells were stained with anti-BMP4 antibodies. Bright filed images are presented below.

\textbf{Figure 7. CD41 expression marks the IC cells present in the bone marrow CD31+Kit+Sca1-Lin- population.} (A) Flow cytometry analysis of CD41 expression in the bone marrow CD31+Kit+Sca1-Lin- population of cells. (B) Bone marrow CD31+Kit+Sca1-Lin- cells were sorted into CD41+ and CD41- populations. Each population was tested for the ability to form Epo\textsuperscript{ind} BFU-E following infection \textit{in vitro} with FVP. Cells were either mock infected or infected with FVP and plated in methylcellulose media containing the indicated cytokines. BFU-E were scored. (C) Bone marrow CD31+Kit+Sca1-Lin- cells were sorted into CD41+ and CD41- populations. Each population was tested for the ability to propagate infection in the spleen when infected with FVP \textit{in vitro} and transplanted into BALB/c control mice. Spleens were removed and weighed 14 days post transplant (right). Pictures of representative spleens are shown (right). (D) RT-PCR analysis of Sf-Stk expression in CD31+Kit+Sca1-Lin- cells sorted into CD41+ or CD41- populations. Expression is analyzed in each population from both BALB/c and BALB/c-\textit{f/f} mice. Significant differences as measured by t-
test are indicated. The bars represent the average ± standard deviation of one representative experiment of 2 independent experiments.

Figure 8. BALB/c-<i>f/f</i> mice exhibit a defect in IC cells in the bone marrow. (A) Flow cytometry analysis of CD41+ cells in the CD31+Kit+Sca1-Lin- cells in the bone marrow of BALB/c-<i>f/f</i> mice. (B) BALB/c-<i>f/f</i> and BALB/c control bone marrow cells were infected <i>in vitro</i> with FVP and transplanted into BALB/c control mice. On the indicated days, spleens were isolated and weighed. Significant differences as measured by t-test are indicated. The bars in (B) represent average ± standard deviation of one representative experiment of two independent experiments.

Figure 9. CD31+Kit+CD41+Sca1-Lin- cells are mobilized into the peripheral blood during infection with FVP <i>in vivo</i>. Peripheral blood mononuclear cells from uninfected (top) and infected (bottom) BALB/c control mice were analyzed by flow cytometry for CD31+Kit+CD41+Sca1-Lin- cells.
Subramanian et al. Figure 1

A. Bone marrow

- Epo + IL-3
- FVP + IL-3

B. Spleen

- Epo + IL-3 + SCF
- FVP + IL-3 + SCF

B. BALB/c MEPs BALB/c-f/f MEPs

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Subramanian et al. Figure 2

A.

![Bar graph showing BFU-E levels over time and cell treatments.](image)

- Untreated
- 36 hours
- 4 Days
- Time post PHZ treatment

**Comparisons:**
- Epo + SCF + IL-3 vs. FVP + SCF + IL-3
  - p < 0.01
- Epo + IL-3 vs. FVP + IL-3
  - p > 0.10

B.

![Bar graph showing BFU-E levels with and without PHZ treatment.](image)

- Bone marrow
- Bone marrow + PHZ
- Epo + IL-3
- FVP + IL-3

**Comparisons:**
- p < 0.01
Subramanian et al. Figure 3

A.

![Bar graph showing BFU-E counts with error bars for Epo + IL-3 + SCF and FVP + IL-3 + SCF conditions. The graph includes a control group and a group treated with BMP4. The p-value for the comparison is p<0.05.](image)

B.

![Images showing cellular proliferation at different days post Friend virus infection.](image)

Days post Friend virus infection:
- Day 1
- Day 8
- Day 12
- Day 15
Subramanian et al. Figure 4

A. Spleen Weight (g)

![Graph showing spleen weight comparison between BALB/c and BALB/c-ff recipients.](image)

Days post transplant

- Day 4
- Day 15

p < 0.001

B. Days post transplant

- Day 4
- Day 8
- Day 15

FVP infected BALB/c bone marrow transplanted into BALB/c mice.

FVP infected BALB/c-ff bone marrow transplanted into BALB/c-ff mice.
Subramanian et al. Figure 4 continued.

C.

Day 12

Day 15

Days post Friend virus infection
Subramanian et al. Figure 5

A.

B.

C.
Subramanian et al. Figure 5 continued.
Subramanian et al. Figure 6

A.  

B.  

FVP infected Bone marrow CD31+Kit+Sca1-Lin-  

FVP infected Spleen MEPs
Subramanian et al. Figure 7

A. BALB/c Lin-Sca1-

B. Bar graph showing BFU-E counts for Epo + IL-3 and FVP + IL-3 conditions.

C. Graph showing spleen weight comparison.
Subramanian et al. Figure 7 continued

D.

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Subramanian et al. Figure 8

A. 

B. 

CD31

Kit

CD41

Spleen weight (g)

Days post transplant

FVP infected BALB/c bone marrow
FVP infected BALB/c-/- bone marrow

p<0.01

p<0.01

p<0.01

p<0.01
Subramanian et al. Figure 9
Appendix B

An intronic sequence mutated in flexed-tail mice regulates splicing of Smad5

Foreword

This chapter is adapted from the manuscript submitted to Mammalian Genome. The author list of the manuscript includes Shailaja Hegde, Laurie E. Lenox, Andrew Lariviere, Prashanth Porayette, John M. Perry, Michele Yon and Robert F. Paulson. The author contributed to work shown in Figure 1 and Figure 4A.

Abstract

Recent work has identified a growing body of evidence that subtle changes in non-coding sequences can result in significant pathology. These mutations, which would have been called silent polymorphisms in the past, affect gene transcription and mRNA splicing and lead to drastic changes in gene expression. Previous work from our lab has characterized the murine flexed-tail (f) mutation, which encodes Smad5, a transcription factor that functions downstream of the receptors for Bone morphogenetic proteins (BMPs). f/f mice are unable to rapidly respond to acute anemia. Our analysis of these mice lead to the development of a new model for stress erythropoiesis, where BMP4 expression in the spleen leads to the Smad5 dependent expansion of a specialized population of stress erythroid progenitors during the recovery from
acute anemia. f/f mutant mice exhibit a defect in Smad5 mRNA splicing in the spleen such that the majority of Smad5 transcripts are two mis-spliced mRNAs. One of these mRNAs encodes a truncated form of Smad5 that inhibits BMP4 signaling when over expressed. Here we show that a mutation in a poly(T) element in intron 4 causes the splicing defect in f/f mutant mice. This subtle mutation (loss of 1 or 2 Ts in a 16T element) results in defects in splicing throughout the Smad5 gene. Furthermore, we show that this mutation results in tissue specific splicing defects, which may explain why f/f mice are viable when Smad5/- mice are embryonic lethal.
Introduction

Tissue hypoxia induces a physiological response designed to increase oxygen delivery to the tissues. One component of this response is increased erythropoiesis. At times of acute need, large numbers of new erythrocytes must be rapidly generated (Socolovsky 2007). This process utilizes progenitors and signals that are distinct from those used by steady state erythropoiesis (Lenox et al. 2005; Perry et al. 2007). Previous work from our lab has shown that BMP4 acts in concert with Stem cell Factor (SCF) and hypoxia to induce the expansion of differentiation of a specialized population of stress erythroid progenitors in the spleen (Perry et al. 2007). These progenitors exhibit a greater capacity to generate erythrocytes and differentiate faster than steady state erythroid progenitors.

The key role for BMP4 in this process was first demonstrated by the analysis of the murine flexed-tail (f) mutation. f/f mice are unable to rapidly generate erythrocytes at times of acute need (Russell 1979). During embryogenesis f/f embryos exhibit a fetal neonatal anemia, which resolves two weeks after birth, which coincides with the time that the bone marrow becomes the primary site of steady state erythropoiesis (Gruneberg 1942a, b; Mixter and Hunt 1933; Palis and Segel 1998). As adults, f/f mice exhibit normal steady state blood parameters (Gregory et al. 1975). However, when challenged with an acute anemia, f/f mice are significantly slower to recover (Coleman et al. 1969; Lenox et al. 2005). We cloned the f locus and showed that it encoded Smad5, a receptor activated Smad that functions downstream of the receptors for BMP4. f/f
mice have a defect in splicing such that the majority of the Smad5 mRNA in the f/f mutant spleen is mis-spliced (Lenox et al. 2005). Two mis-spliced messages are observed. The common feature of these messages is the deletion of exon two which contains the initiator ATG. Over-expression of one of these mRNAs in a BMP4 responsive osteoblast cell line showed that it was capable of inhibiting BMP4 signaling. These data and experiments using f/Smad5- trans-heterozygotes demonstrated that the severity of the f mutant phenotype depended on the dose of mis-spliced Smad5 mRNA (Lenox et al. 2005). In our initial report, we were unable to identify the molecular lesion responsible for the splicing defect. In this report, we show that f/f mice have a mutation in a poly(T) element in intron 4. Wildtype mice have 16 (T)’s while f/f mice have 14 or 15. This subtle change when introduced into a BAC clone containing the Smad5 gene is sufficient to cause a splicing defect. In addition, the splicing defect caused by the mutation appears to be tissue specific, in that the severity of aberrant splicing is variable depending on the tissue and different tissues exhibit different mis-spliced Smad5 mRNAs. These data explain why f/f mice are viable, while Smad5/- mice are embryonic lethal (Chang et al. 1999; Yang et al. 1999).

Methods and materials

Sequencing the f locus

Genomic DNA was isolated from C57BL/6 and C57BL/6-f/f mice using standard methods. The 10 fragments spanning the Smad5 locus were generated by PCR using the following primers. The PCR fragments were cloned into PCR
XL-Topo vector according to the manufacturer’s instructions. Multiple independent clones were sequenced for each fragment.

Primers used for amplifying the segments of the Smad5 locus.

Fragment 1
Forward 5’ AAGTCAAAAGGATTGTGGAGG 3’
Reverse 5’ TCAGCAGAATTCCTCTCTACCTTGCTA 3’

Fragment 2
Forward 5’ GGGTAGATGATGTGTGTGTTGCCTTT 3’
Reverse 5’ CTCCAAGAACGTATATCATCAGGGC 3’

Fragment 3
Forward 5’ GTTAGCCAGGTACAGTAACGCATGCTTTC 3’
Reverse 5’ GCTGACCAGTTATCACTGTCACAGAGTGATC 3’

Fragment 4
Forward 5’ GTGGTGAGCTTTATATCAGCTTTGTT 3’
Reverse 5’ ACAAGCATGAGCCAGCACATCTTTAT 3’

Fragment 5
Forward 5’ TTCAGGAGAAGAGAGCAAGAAGCAA 3’
Reverse 5’ ACAAGCATGAGCCAGCACATCTTTAT 3’
Gap repair cloning of intron 4 from Smad5
A plasmid containing fragment 8 of the Smad5 locus was digested with NheI according to manufacturer’s instructions. The digested plasmid was purified by agarose gel electrophoresis. This digest generates a linear plasmid containing the 5’ 263 bp and 3’ 1779 bp of Fragment 8, which was used as a cloning vector. Genomic DNA was isolated from C57BL/6 control and C57BL/6-f/f mutant mice. In addition, we used genomic DNA isolated from C58, BALB/c and 129SvJ to rule out the possibility that the poly(T) mutation was a sequence polymorphism. The genomic DNA was sheared by sonication to generate smaller fragments of DNA. The λ phage recombineering strain DY380 was grown at 30C until O.D. 0.4 and then the culture was heat shocked at 42C for 15 minutes to induce the expression of the λ phage recombination proteins as previously described (Hegde and Paulson 2004; Yu et al. 2000; Yu et al. 2003). The induced DY380 cells were made electroporation competent by washing in ice cold sterile water. The cloning vector (5ug) and genomic DNA (24 ug) were electroporated into induced DY380 bacteria. Recombinant colonies were selected by Kanamycin resistance (Kan^R). Full length recombinants were identified by PCR of plasmid DNA using Fragment 8 primers. For each mouse, two independent recombination reactions were done and multiple full length recombinant clones were sequenced.

**Generation of mutations in the poly (T) sequence of the Smad5 BAC**

A Smad5 containing BAC clone (RP24 267-I21) from a C57BL/6 BAC library was obtained from the BACPAC resources center. The generation of ΔT
and ΔT:poly(T)15 mutants was done by recombineering techniques. We initially constructed the ΔT mutant using overlapping oligonucleotides (Yu et al. 2003). We utilized the galK co-selection technique to identify recombination events, which screens for the targeting of Kan^R to the galK locus on the E.coli chromosome (Hegde and Paulson 2004). 30 ng of ΔT forward and ΔT reverse oligonucleotides and 80 ng of a PCR fragment containing the Kan^R gene flanked by homology to galK were electroporated into Smad5 BAC DY380 bacteria that had been heat shocked at 42°C to induce the λ phage recombination system as described above. Kan^R colonies that formed white galK- colonies when grown of MacConkey galactose indicator agar were selected and the ΔT mutation was confirmed by sequencing. The ΔT mutation BAC was retransformed into DY380 and the ΔT:poly(T)15 mutation was induced using the same procedure except that different oligonucleotides were used.

ΔT Mutation primers:

ΔT Forward 5' GGTTTTTTGTTTGCTTGGGAACCTAT 3'
ΔT Reverse 5' ATAGCCAAAAAGAACAAATAGGTT 3'

ΔT:poly(T)15 primers:

ΔT:poly(T)15 Forward 5' TTGTTTGCTTGTTTGTTTTTTTTTTTTTTTTT 3'
ΔT:poly(T)15 Reverse 5' AAATAGCCAAAAAGAACAAATAGGTT 3'

Introduction of mutated and control Smad5 BACs into MSS31 cells by infection
The initial step in these experiments was to generate BAC clones that contained the selectable markers neomycin/kanamycin resistance \((\text{neo}^R)\) and EGFP integrated into BAC vector sequences. Oligos that contained homology to the Chloramphenicol resistance \((\text{Cm}^R)\) of the BAC vector (Frengen et al. 2000) at their 5’ ends and homology to neo\(^R\)/EGFP cassette from pEGFPN3 (Clontech) were used to PCR amplify a fragment that contained the neo\(^R\)/EGFP cassette flanked by 50 bp of BAC vector homology. This fragment was electroporated into DY380 bacteria which had been heat shocked to induce the expression of \(\lambda\) phage recombination proteins as described above. BAC DNA was isolated from Kan\(^R\), Cm\(^{\text{sensitive}}\) colonies and assayed for the correct insertion into the BAC vector by PCR.

Primers for neo\(^R\)/EGFP recombineering

Forward

5' TTCCGGTCACACCACATACGTTCCGCCATTCCTATGCGATGC
ACATGCTGTATGCCGGTATAACCGTATTCACGCCATGC 3'

Reverse

5'AGACTTCCGTGAACTGATGGACTTATGTCCCATCAGGCTTTGCA
GAACCTTTCAGCGTAACGACCCAACACCGTGCGTT 3'

We next inserted the *invasin* (inv) gene from *Yersinia pseudotuberculosis* into the *Aspartate \(\beta\)-semialdehyde* (asd) locus of each Smad5 BAC containing DY380 strain (Narayanan and Warburton 2003). The invasin plasmid, pRI203,
was a kind gift of Dr. Ralph Isberg (Isberg et al. 1987). The invasin gene was PCR amplified from the plasmid using primers that contained homology to the \textit{asd} locus at their 5’ ends. The fragment generated was purified on an agarose gel. A second fragment containing the \textit{Cm\textsuperscript{R}} gene flanked by 50 bp of homology to the \textit{galK} gene was generated so that co-selection for \textit{Cm\textsuperscript{R}} and \textit{galK-} could be used as previously described. DY380 strains containing Smad5 BAC clones that had been fitted with \textit{neo\textsuperscript{R}}/EGFP cassettes were heat shocked at 42C to induce the \textit{\lambda} phage recombination system as described above. 600ng of the \textit{inv} fragment and 100 ng of the \textit{galK-Cm\textsuperscript{R}} fragment were electroporated into the induced bacteria. The bacteria were allowed to recover for 90 minutes at 37C in 1ml of LB medium containing 0.5M diaminopimelic acid (DAP) and bacteria were plated on LB agar contain 0.5M DAP and Kanamycin and Chormphenicol. Kan\textsuperscript{R}, Cm\textsuperscript{R} colonies were tested on MacConkey galactose indicator agar and those that formed white \textit{galK-} colonies were selected. The insertion of \textit{inv} into \textit{asd} was then confirmed by PCR.

\begin{verbatim}
Primer used to generate \textit{inv} fragment with homology to \textit{asd} at each end.
5’GAGACCGGCACATTATACAGCACACATCTTTCAGGAAAAAACGCTATGGTTTCCAGCCAATCGAGTTT 3’

5’CTCCTGTATTACGCACTAACAAGGCGGCATCGCGCCCCAGATTATATTTATTTGACAGCGACAGCGAAGACG 3’
\end{verbatim}

Primers used to verify the insertion of \textit{inv} into \textit{asd}.
5' TGGTGAAGATGTGCCAAGA 3'
5' TCCCGGTAAATCATGAAACA 3'

The infection of MSS31 spleen stromal cells with *inv+asd* Smad5 BAC containing DY380 bacteria was done as previously described (Narayanan and Warburton 2003). In brief, overnight cultures (O.D. 1.2) were grown in brain-heart infusion broth supplemented with 0.5M DAP. The bacteria were spun down and resuspended in 1 ml of RPMI 1640 media. The bacteria were overlaid onto monolayer cultures of MSS31 cells (1 x 10^5 cells/well) at an MOI of 4500. The plates were centrifuged at 1000 rpm for 10 minutes. After 2 hours at 37°C, the cells were washed with media and then incubated in media containing G418 (0.5 mg/ml). G418R colonies were pooled after 2 weeks of selection and analyzed for Smad5 splicing defects.

*RT-PCR analysis of Smad5 expression*

Total RNA was isolated from MSS31 cells and the BAC containing MSS31 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. cDNA was generated and Smad5 cDNA was amplified using 5'-GGGGCCGAGCTGCTAAT-3' and 5'-CTATGAAACAGAAGAAATGGGG-3' primers (Lenox et al. 2005). For the analysis of Smad5 splicing in other tissues, these tissues were isolated from C57BL/6-\textit{f/f} and C57BL/6 control mice. The tissues were homogenized in TRIzol reagent according to the manufacturer’s instructions and total RNA was isolated. cDNA was generated and Smad5 expression analyzed by RT-PCR as described for MSS31 cells. For both MSS31...
cells and primary tissues, RT-PCR reactions were separated on agarose gels and the major bands were cloned into the PCR XL-TOPO vector. Multiple independent clones were sequenced to determine the alterations in splicing.

Results

Our initial analysis of the splice donor and acceptor sites in the Smad5 gene failed to identify any mutations that could explain the aberrant mRNA splicing observed in f/f mice (Lenox et al. 2005). However these sequences are only a small part of the cis acting sequences that can regulate mRNA splicing. A growing body of work has identified a large number of both intronic and exonic sequences that can affect splicing (Baralle and Baralle 2005; Buratti et al. 2006; Faustino and Cooper 2003). In order to determine whether f mice had a mutation in such a sequence, we sequenced the entire Smad5 locus from f/f mice. As shown in Figure 1A, the locus was subdivided into 10 fragments of 3-5 kb in length, which were amplified by PCR and cloned. The fragments were sequenced and compared to the C57BL/6 reference sequence. Over the entire 36.4 kb region, we identified only one consistent change in sequence. In intron 4 we identified a homopolymer run of 16 T residues that was reduced to 14 in f/f mice (Figure 1). PCR amplification of homopolymer sequences can be problematic due to polymerase slippage. In order to circumvent this problem, we cloned this region by gap repair cloning using the λ phage recombineering system (Copeland et al. 2001; Lee et al. 2001). Starting with a plasmid that contained fragment number 8, we generated a cloning vector by digesting the
plasmid with NheI to generate a linear plasmid that contained the 5’ 263 bp and the 3’ 1779 bp. Genomic DNA was isolated from C57BL/6 control and C57BL/6-\(f/f\) mice. The DNA was sheared to generate smaller DNA fragments. The genomic DNA and the linear fragment 8 cloning vector were electroporated into the \(\lambda\) phage recombineering strain DY380 (Yu et al. 2000). Kan\(^R\) recombinants were identified and the isolated plasmids sequenced. The analysis of 3 independent \(f/f\) mice showed that they contained either 14 or 15 T residues, while the analysis of control C57BL/6 mice showed that they all contained 16 T residues (Figure 1B). A difference of 1-2 T residues is a minor change and could represent a polymorphism in the C57BL/6-\(f/f\) strain. We analyzed this sequence in other strains to determine whether this change represented a polymorphic sequence or a potential mutation. Using gap-repair cloning we isolated this region of intron 4 from the C57BL/6 related strain C58 and two unrelated strains, BALB/c and 129SvJ. Analysis of the sequence of this region in these strains showed that all of the strains had 16 T’s like the wildtype C57BL/6 mice. This observation suggests that 16T’s is the normal sequence and the change observed in C57BL/6-\(f/f\) mice may represent a mutation. We also compared this sequence in the mouse with the sequences of intron 4 in rat and human to determine whether this element is conserved in other species. We observed no similar sequences any where in human or rat \textit{Smad5}, which suggests that this element is mouse specific (data not shown). The loss of a single T residue would seem unlikely to result in a mutant phenotype as severe as the \(f/f\) phenotype; however, previous work on several genes including the \textit{CFTR} (Niksic et al. 1999;
Nissim-Rafinia et al. 2000; Zuccato et al. 2004) and MRE11 (Giannini et al. 2004) has shown that changes in poly T sequences can have a profound effect on splicing.

In order to demonstrate that this change in the poly T sequence was the cause of the splicing defect, we mutated this sequence in a Bacterial Artificial chromosome (BAC) clone that contained the entire Smad5 locus and tested whether these mutants affected Smad5 mRNA splicing. Our rationale for using BAC clones was that the defect that we observed in f/f mice did not occur in the splicing of the exons flanking the mutation, but rather affected upstream and downstream sites. Thus, we needed to study the mutation in the context of the entire locus. We utilized λ phage recombineering to first delete the entire poly T sequence to generate the ΔT allele. We then used this allele to reintroduce the poly (T)₁₅ mutant allele into the BAC, which generated ΔT:poly(T)₁₅ allele (Figure 2A). Transfection of BAC DNA into mammalian cells can be difficult and poor transfection efficiencies are common. An alternative strategy to introduce BACs into tissue culture cells utilizes bacterial infection to deliver the BAC DNA. Ectopic expression of the invasin (inv) gene from Yersinia pseudotuberculosis makes bacteria competent to invade mammalian cells (Narayanan and Warburton 2003). Inv binds β-integrin on the target cell, which then facilitates the uptake of bacteria (Isberg and Leong 1990). Coupling inv expression with mutation of the Aspartate β-semialdehyde (asd) locus, which causes a cell wall defect due to auxotrophy for diaminopimelic acid, leads to efficient uptake of
bacteria into cells where they lyse upon division and deliver the BAC DNA into the cells (Narayanan and Warburton 2003) (Figure 2B).

A selectable marker, neomycin resistance/EGFP (neo\(^R\)/EGFP) was inserted into the BAC vector sequences of the Control, \(\Delta T\) and \(\Delta T:\text{poly(T)}15\) Smad5 BAC clones by recombineering. Inv was inserted into the \(\text{asd}\) locus by recombineering in \(E.coli\) DY380 to generate DY380 \(\text{inv+asd-}\) strains that contained control or mutant BAC clones. The three BAC strains were incubated with the spleen stromal cell line, MSS31. These cells express endogenous Smad5. The cells were washed with PBS to remove non-adherent bacteria and then cultured in new media containing G418 to select for cells that have integrated the BAC clone. Pools of neo\(^R\)/EGFP+ colonies for each BAC clone were expanded and the expression of \(\text{Smad5}\) was determined by RT-PCR.

Figure 3 shows that uninfected MSS31 and those infected with bacteria containing the control BAC clone express wildtype \(\text{Smad5}\) mRNA. However, the cells that received the \(\Delta T\) exhibited a new \(\text{Smad5}\) mRNA. Sequencing this RT-PCR product revealed that it encoded a mis-spliced transcript which lacked exon 4 and included an insertion of 15 nucleotides between exons 6 and 7. This insertion was observed previously in one of the mis-spliced mRNAs we observed in the spleen of C57BL/6-\(f/f\) mice (Lenox et al. 2005). However that aberrant mRNA, had a deletion of exon 2 in addition to the deletion of exon 4. In addition, we observed several small RT-PCR products, which contained portions of exon 4 and intron 4. Reintroduction of the mutated poly(T) sequence in the \(\Delta T:\text{poly(T)}15\) Smad5 BAC clone resulted in the appearance of the same larger mis-spliced
mRNA as observed with the ∆T BAC. However, the smaller RT-PCR products were not observed. Based on these observations, we conclude that the poly(T) element in intron 4 of the Smad5 gene plays a key role in the proper regulation of mRNA splicing.

The analysis of MSS31 cells containing different Smad5 BAC clones showed that mutation of the poly (T) sequence caused defects in splicing similar but not identical to what we observed in the spleen erythroid progenitors. These data suggest that in vivo, the poly(T)14/15 mutation may have different effects in different tissues. We tested this possibility by isolating mRNA from spleen, bone marrow, kidney and liver of C57BL/6-f/f and control mice. The different Smad5 mRNA isoforms were analyzed by RT-PCR and sequence analysis. Figure 4 shows that altered splicing is observed in all of the tissues tested. However, the severity of the splicing defect was variable. Spleen showed an almost complete lack of full length wildtype Smad5 mRNA as we previously reported (Lenox et al. 2005). In contrast, bone marrow, kidney and liver all exhibited substantial amounts of wildtype Smad5 mRNA and lower levels of aberrantly spliced Smad5 mRNAs. Similar to our observations in vitro with MSS31 cells expressing mutant BAC clones, the sequence of the aberrantly spliced mRNA in bone marrow, kidney and liver was distinct from the mis-spliced mRNAs we observed in the spleen and also distinct from those observed in MSS31 cells. This mRNA showed some similarity to those cloned from f/f spleen in that exon 1 is spliced into the same cryptic splice acceptor in the middle of exon 3. In addition, this mRNA also contains the 15bp insert, which corresponds
to sequences in intron 6. Taken together, these data support a model where the mutation of the poly(T) sequence in \textit{f/f} mice leads to defects in Smad5 mRNA splicing, which vary from tissue to tissue.

**Discussion**

Our analysis of the Smad5 locus in \textit{f/f} mice has identified a poly(T) element that regulates mRNA splicing. Mutation of this element leads to aberrant use of splice sites and activation of cryptic splice sites, which leads to the skipping of whole or parts of exons and the inclusion of intronic sequences in the mature Smad5 mRNA. The regulation of splicing is complex. Only in recent years has work expanded on the relatively minimal sequence motifs initially used to characterize splice sites and identified intronic and exonic enhancers and suppressors of splicing (For a review see Baralle and Baralle 2005; Buratti et al. 2006; Faustino and Cooper 2003). The defects associated with the poly(T) mutation in \textit{f/f} mice suggest that this element may function as a negative regulator of splicing that prevents the inappropriate use of cryptic splice sites. Furthermore these data underscore the idea that mutations or polymorphisms, previously thought to be silent because they did not affect coding sequences, can have profound effect on splicing and result in significant pathology.

These data also address an important question concerning the relationship between the \textit{f} allele of Smad5 and the role of Smad5 during development. The phenotype of the Smad5-/- mice is embryonic lethal between embryonic day 10.5-11.5 (E10.5-11.5)(Chang et al. 1999; Yang et al. 1999).
cause of the lethality in these embryos is complex. Smad5-/- embryos exhibit defects in angiogenesis and have defective yolk sac vasculature (Chang et al. 1999; Yang et al. 1999). In addition, one group noted that the mutant embryos exhibited a high level of mesenchymal apoptosis and suggested that the interactions between endothelial and mesenchymal cells were disrupted in the mutants (Yang et al. 1999). f/f mutant mice do not exhibit embryonic lethality. The primary developmental phenotype is severe fetal anemia, which resolves by two weeks after birth (Gruneberg 1942a, b; Mixter and Hunt 1933; Russell 1979). The effects of the Smad5 null mutation on fetal erythropoiesis are unknown because the embryos die prior to the onset of fetal liver erythropoiesis. How might these differences in the phenotypes be reconciled? Our data suggest that the variable splicing defects associated with the poly(T) sequence mutation lead to multiple Smad5 mRNA isoforms. Our previous data showed that in the spleen, only one of the aberrant Smad5 mRNAs can inhibit BMP4 signaling when over expressed in a BMP4 responsive cell line (Lenox et al. 2005). This mRNA contained a deletion of exon 2 and an insertion of the same 15 nucleotides between exons 6 and 7. This insertion was also observed in the MSS31 cells that contained the mutated Smad5 BAC clones. The other predominant mis-spliced mRNA in the spleen of f/f mice had no effect on BMP4 signaling. Its sequence is more similar to the aberrant mRNAs observed in other tissues in f/f mice and MSS31:Smad5 ∆T BAC cells (Figure 4B). It is important to remember that f is a recessive mutation. Heterozygotes do not exhibit a phenotype (Lenox et al. 2005). However, our previous genetic analysis showed that f is not a Smad5 loss of function allele
in that f/Smad5- mice do not exhibit a more severe phenotype than f/f (Lenox et al. 2005). The increased level of mutant protein in f/f mice coupled with the low levels of wildtype Smad5 mRNA in the spleen results in a defect in BMP4 signaling in spleen progenitor cells. Based on these data we propose that the specific phenotypes in f/f mice are caused by an almost complete absence of wildtype Smad5 mRNA and the expression of an inhibitory Smad5 protein in BMP4 dependent components of the stress erythroid lineage which leads to fetal anemia and defects in the response to acute anemia in adult mice. f/f embryos survive because the splicing defect in f/f embryos is not severe and does not lead to the expression of inhibitory proteins in critical tissues during embryogenesis.

f/f mice also exhibit two other phenotypes, namely tail flexures from which the mutant receives its name and white belly spots (Hunt and Premar 1928; Kamenoff 1935; Mixter and Hunt 1933). Both of these phenotypes can be traced to BMP4 dependent pathways(Christiansen et al. 2000; Murtaugh et al. 1999; Sela-Donenfeld and Kalcheim 1999). In C57BL/6-f/f mice tail flexures are not observed. Similarly, the white belly spots are incompletely penetrant. These observations suggest that the splicing defect in f/f can be affected by differences in strain background and indicates that splicing control elements may be regulated by allelic differences in trans acting factors that segregate in inbred strains of mice.

The feature that distinguishes the mutation in the poly(T) element of Smad5 from other examples of poly(T) sequences affecting splicing is that the poly(T) mutation in Smad5 does not exclusively affect the splicing of the two
flanking exons. Other examples such as \textit{CFTR} and \textit{Mre11} exhibit skipping of the exon downstream of the homopolymer sequence. These elements are part of the polypyrimidine stretch that marks the branch point splice acceptor region of the intron (Giannini et al. 2004; Niksic et al. 1999; Nissim-Rafinia et al. 2000). Analysis of these mutations has suggested that the fewer T residues the greater the probability that the downstream exon will be skipped (Niksic et al. 1999; Nissim-Rafinia et al. 2000; Zuccato et al. 2004). In contrast, the \textit{f} polyT element appears to regulate the use of a number of cryptic and normal splice sites.

In summary, we have identified a mutation in a poly(T) element in intron 4 of the \textit{Smad5} gene in \textit{f/f}. This mutation leads to a tissue specific splicing defect, which varies in its severity and the aberrant mRNAs produced. We propose that the variability in the tissue distribution of the splicing defect is responsible for the differences in the phenotype of \textit{f/f} and Smad5/-/- mice.
Acknowledgements:

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Figure Legends

**Figure 1. Sequencing of the Smad5 locus identifies a mutation in a poly(T) element in intron 4.** (A). Schematic of the Smad5 locus showing the position of the ten PCR fragments used to clone the mutation in \( f \) mice. Black exons denote 5’ and 3’ UTRs and grey exons coding sequences. Below fragment 8 is the sequence of the poly (T) element. (B). Sequence of the poly (T) element from three C57BL/6 control, three C57BL/6-\( f/f \), C58, BALB/c and 129SvJ mice derived from gap-repair cloning. The three control C57BL/6 mice all showed identical sequence.

**Figure 2. Generation of mutations in the poly(T) element in Smad5 BAC clones and the introduction of mutated BACs into MSS31 cells by infection.** (A). Schematic of the mutations introduced into the Smad5 BAC clone by recombineering to generate the \( \Delta T \) and \( \Delta T:poly(T)15 \) mutants. (B). A schematic of the strategy used to introduce BAC clones into MSS31 cells by infection.

**Figure 3. Mutated Smad5 BAC clones generate mis-spliced mRNAs.** Smad5 mRNA was analyzed by RT-PCR using RNA isolated from MSS31 control cells and MSS31 cells which contained mutant and control Smad5 BAC clones. Arrows indicate major Smad5 mRNAs and their exon structure. Arrowheads indicate small aberrantly spliced mRNAs that contained variable portions of exon4 and intron 4. All other bands were non-specific in that they
were not consistently observed and did not contain sequences from the Smad5 locus.

**Figure 4. Analysis of Smad5 mRNA splicing in other adult tissues.**

(A). RNA was isolated from bone marrow, kidney and liver (top) and spleen (bottom) of C57BL/6 and C57BL/6-ff mice. Smad5 mRNA expression was measured by RT-PCR. The major Smad5 mRNAs are indicated by the arrows and their exon structure is given. Bands indicated by * are non-specific in that they are not consistently observed and do not contain sequences from the Smad5 locus. (B). Schematic of the aberrant spliced forms of Smad5 observed in the spleen (Lenox et al. 2005) or in this study. * indicates pathogenic Smad5 mRNA isoform.
Hegde et al. Figure 1

A.

36.37 kb

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

C57BL/6 control

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

C57BL/6-f/f #1

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

C57BL/6-f/f #2

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

C57BL/6-f/f #3

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

C58

CCTTGTAGAGGTTTTTGTTTGCTTGGTTTTTTTTTTTTTTTTAACCTATTTGTTCTTTT

129SvJ

B.
Hegde et al. Figure 2

A.

AGGTTTTGTTGCTTGGTTTTTTTTTTTTTTTTAACCTATAGTTCT

First recombineering step

AGGTTTTGTTGCTTGGACCTATAGTTCT \( \Delta T \)

Second recombineering step

AGGTTTTGTTGCTTGG-TTTTTTTTTTTTTTTTTAACCTATAGTTCT \( \Delta T:poly(T)_{15} \)

B.

Smad5 BAC control
\( \Delta T \) or \( \Delta T:poly(T)_{15} \) alleles
Neo\(^R\)/EGFP inserted into the BAC vector

Inv+asd- Bacteria used to infect Mss31 cells. BAC+ clones are selected for G418\(^R\).
Hegde et al. Figure 4

A.

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Liver</th>
<th>Kidney</th>
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<tbody>
<tr>
<td>M</td>
<td></td>
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C57BL/6 control

C57BL/6-f/f

Spleen

C57BL/6-f/f

C57BL/6

B.

1 2 3 4 5 6 7

* 1 3 5 6 7

CTGTTCATTTCCC

1 3 4 5 6 7

Observed in MSS31 cells expressing BAC polyT(15T) mutant and other tissues

Wildtype

1 2 3 4 5 6 7

Observed in f mutant spleen

1 3 4 5 6 7

CTGTTCATTTCCC

1 3 4 5 6 7

Observed in MSS31 cells expressing BAC polyT(15T) mutant and other tissues
Appendix C

Role of PERK and ATF4 in Stress Erythropoiesis

Secretory proteins are transported into endoplasmic reticulum (ER), where they are folded and processed before being transported to the Golgi apparatus. When the secretory demand exceeds the folding capacity of the ER, it leads to ER stress launching the unfolded protein response (UPR) (Wek and Cavener 2007). One of the most important sensors of ER stress is PERK. PERK is a member of a family of protein kinases that phosphorylate the \( \alpha \) subunit of the eukaryotic initiation factor eIF2. Phosphorylation of eIF2\( \alpha \) leads to a global repression of new protein synthesis (Harding, Novoa et al. 2000). A central regulator of PERK stress response is the transcriptional activator, ATF4 (CREB2). ATF4 is upregulated in response to ER stress and most of this upregulation has been shown to be translationally controlled. ATF4 regulates gene transcription by forming homo- or hetero-dimers with other transcription factors like FOS, JUN, and NRF2. Gene microarray studies showed that ATF4 regulated a wide array of genes including those involved in amino acid metabolism, oxidative stress, apoptosis regulation, and mitochondrial function (Harding, Novoa et al. 2000).

Homozygous inactivation of ATF4 gene in mice led to severe fetal anemia. The E15.5 embryos were pale and the fetal liver was small. The fetal liver contained only half the number of hematopoietic progenitors and there was a 6 fold reduction in the size of hematopoietic colonies (Masuoka and Townes 2002). In spite of the severe fetal anemia, the steady state erythropoiesis in the adults was relatively normal, with only a mild
anemia. This phenotype parallels the f/f mouse model we have used in our laboratory. Interestingly, the ATF4-/- mice have defects in recovering efficiently from Phenylhydrazine induced acute anemia (Masuoka and Townes 2002). Hypoxia has been shown to upregulate ATF4 expression (Blais, Filipenko et al. 2004). These data suggest a model, where hypoxia, induced during acute anemia, upregulates ATF4 expression through phosphorylation of eIF2α, which helps in rapid expansion of erythroid progenitors using the stress erythropoietic mechanism.

Work in our laboratory has corroborated the findings that in the ATF4-/- mice, there is a delay in the recovery from acute hemolytic anemia induced by Phenylhydrazine (Lariviere and Paulson, unpublished results). We have shown earlier that SCF acts in concert with hypoxia to expand the stress BFU-E (Perry, Harandi et al. 2007) (Figure 3-4). Preliminary studies have shown that the ATF4-/- mice are not able to expand their stress BFU-E in response to hypoxia suggesting that ATF4, induced by hypoxia, may be expanding the stress BFU-E in response to acute anemia. Also, erythroid progenitors from ATF4-/- mice are not able to increase the number of stress BFU-E in response to addition of SCF into the in vivo culture conditions, suggesting that ATF4 might be a downstream target of SCF (Perry, Lariviere and Paulson, unpublished results).

ATF4-/- mice do not expand stress BFU-E in response to hypoxia. This suggested that ATF4 might be activated in response to hypoxia in the spleen. To check this, we isolated lineage negative hematopoietic cells from spleen and cultured them under hypoxia. We observed a 2.5 fold increase in ATF4 protein levels when spleen cells were cultured in hypoxia compared to the levels seen under normoxic conditions (Figure 3-1).
Since ATF4 knockout mice had a delayed recovery from acute anemia, we checked if ATF4 was induced in the spleen following recovery from acute anemia in the control mice. We see that ATF4 is expressed minimally at the protein level before the induction of anemia. But about 24 hours post PHZ treatment, there is a rapid induction of
ATF4 in the spleen which reaches maximal levels at around 36 hours post PHZ treatment. Following that, the levels of ATF4 start going down until reaches low levels by about 6 days post PHZ treatment. (Figure 3-2)

Therefore, ATF4 is induced in the spleen following both hypoxia and acute anemia and is required for the formation of stress BFU-E in the spleen following acute anemia. This explains the defective stress erythropoiesis seen in the spleen following induction of acute anemia in the ATF4 knockout mice.

Most of the PERK knockout mice die either pre-natally or within a few days of post-natal life due to multiple defects during development (Zhang, McGrath et al. 2002). We have obtained the ‘floxed’ PERK mice and crossed them to a IFN inducible Cre-recombinase expressing strain. We treated these mice with Poly I: C, which induces
IFN and hence induces the Cre-lox recombinase mechanism to delete the ‘floxed’ Perk gene. We isolated spleen from these mice and did erythroid colony assays on them to see if they form stress BFU-E. Earlier work in the lab has shown that hypoxia can act in concert with BMP4 and SCF to expand erythroid colony formation (Figure 3-4). We observed that the Perk knockout mice are not able to expand their Stress BFU-E in response to hypoxia in vitro similar to the ATF4 knockout mice (Figure 3-3) suggesting that ATF4 might be functioning downstream of PERK in the hematopoietic system similar to that observed in other systems. Further studies will be required to elucidate the similarities and differences between PERK and ATF4 in responding to acute anemia and understanding their roles in stress erythropoiesis.
Figure 3-3: Response of spleen cells from PERK-/− mice to various cytokines under normoxic and hypoxic conditions.
Figure 3-4: Response of spleen cells from control mice to various cytokines under normoxic and hypoxic conditions. (Perry, Harandi et al. 2007)

References:


Appendix D

Replenishing the Stress Erythroid Response

Expansive or stress erythropoiesis is induced when there is a need for a rapid expansion of erythroid progenitors to compensate for a depleted erythroid pool. As discussed earlier, the mechanisms involving expansive erythropoiesis are distinct from those regulating homeostatic erythropoiesis. We have shown that, in response to acute anemia, BMP4 is induced in the spleen. It acts on an immature progenitor called the BMP4<sup>R</sup> cell causing it to differentiate into an Epo responsive erythroid progenitor which we call a stress BFU-E. The BMP4<sup>R</sup> cell is contained within the spleen and is not seen in the bone marrow. The stress erythroid progenitor can form BFU-E in vitro in the presence of Epo only and do not require the addition of BPA. The colonies formed by the stress BFU-E are larger and mature faster than the bone marrow BFU-E forming large number of erythrocytes in response to the acute anemia (Lenox, Perry et al. 2005). We have shown that SCF, BMP4, and hypoxia act in concert to drive the expansion of stress erythroid progenitors in the spleen in response to acute anemia (Perry, Harandi et al. 2007).

Although we have identified a unique stress erythroid response resident in the spleen which is poised to respond to acute anemia, neither the BMP4<sup>R</sup> cell nor the stress BFU-E are present in the bone marrow making spleen the unique site for stress erythroid response. 36 hours post PHZ induced acute anemia, almost all the stress BFU-E as well as the BMP4<sup>R</sup> cells are mobilized to differentiate in the spleen which brings up
the important question of how does the body replenish these cells in the spleen? We have shown that once the stress BFU-E are mobilized at 36 hours, it takes about 3 weeks before the stress response can be initiated again in the spleen (Perry and Paulson, unpublished observations). These data suggest that these cells do not self renew and have to be replenished either from an immature cell present in the spleen or from an extra-splenic source, most probably bone marrow. Transplantation studies have demonstrated that bone marrow cells migrate to the spleen following recovery from acute anemia. Once in the spleen, they encounter a signal that causes them to differentiate into a BMP4^{R} cell (Perry and Paulson, unpublished observations). Once the BMP4^{R} cells are formed, the spleen is now poised to respond to an acute anemic insult by launching the stress erythroid response.

We have been curious to identify the signal which is responsible for differentiating an immature progenitor into the BMP4^{R} progenitor in the spleen. One such signal might be the Hedgehog proteins, namely Sonic, Indian, or Desert Hedgehog (Shh, Ihh, and Dhh respectively). Work on chondrocyte development has demonstrated that Shh is required for the somitic mesoderm to become responsive to BMP4 (Murtaugh, Chyung et al. 1999; Zeng, Kempf et al. 2002). It has been shown that Shh induces proliferation of human HSCs by inducing BMP4 (Bhardwaj, Murdoch et al. 2001). So we analyzed for the expression of the three hedgehog proteins in the spleen and we found that only Dhh mRNA is expressed in the spleen (Figure 4-1A). We confirmed the findings by looking at Dhh protein expression at various time points following PHZ treatment and found that Dhh is expressed constitutively in the spleen at all the time points (Figure 4-1B). Treatment of bone marrow cells with Shh induces the formation of
both BMP4\textsuperscript{R} cells and Stress BFU-E (Perry and Paulson, unpublished results). Shh
treatment also induced expression of BMP4 in the bone marrow cells. We also saw clear
co-localization between Dhh and BMP4 expressing cells in the spleen suggesting that
Dhh expressing cells overlap with BMP4 expressing cells in the spleen stroma (Figure 4-1C).

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Figure 4-1: Dhh expression in the spleen during recovery from acute anemia. (A) RT-PCR analysis of Dhh expression. (B) Western Blot analysis of lysates from whole spleen.
(C) Expression of BMP4 and Dhh protein in the same cells at 24 hours post PHZ injection.
Further studies are being done to elucidate the roles Dhh plays in recovering from acute anemia in mice using Smoothened mutant mice. This will give us a better understanding of the process of replenishing the stress erythroid response resident in the spleen.

References:


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