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THE DISCOVERY AND CHARACTERIZATION OF THE BMP4-MEDIATED STRESS ERYTHROID RESPONSE TO ACUTE ANEMIA

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Abstract

Acute anemia results in the rapid mobilization and differentiation of erythroid progenitors in the adult spleen. *flexed-tail (f)* mice exhibit normal steady state erythropoiesis but are unable to rapidly respond to acute erythropoietic stress. Analysis of the *f* locus shows that the mechanisms that regulate expansive erythropoiesis are distinct from the mechanisms that regulate steady state erythropoiesis. We show that *f/f* mice have a mutation in *Madh5*, which impairs signaling downstream of the BMP4 receptor. Further analysis of mutant and control mice demonstrates that during the recovery from acute anemia, BMP4 expression is induced in the spleen, which drives the differentiation of a BMP4 responsive progenitor into an Epo responsive stress BFU-E. Stress BFU-E rapidly form large colonies in the presence of Epo alone, which distinguishes them from steady state bone marrow BFU-E. These findings suggest a new model where stress BFU-E in the spleen are poised to respond to changes in the microenvironment induced by acute anemia.

Analysis of the stress erythroid response has also demonstrated the importance of SCF/Kit signaling, which we show to be necessary for the normal stress BFU-E response. BMP4 responsive cells express Kit on their surface and previous work in the field has shown that mice mutant for the Kit receptor (W/W^V) are slow to recover from acute anemia. Although W/W^V mice are chronically anemic, they do not constitutively express BMP4; however, they rapidly up-regulate BMP4 expression in response to phenylhydrazine treatment. Despite rapid induction of BMP4, the expansion of stress BFU-E is significantly delayed. Unlike control mice, W/W^V mice do not maintain a pool

of BMP4 responsive cells in the spleen. *In vitro* analysis reveals that BMP4 and SCF play distinct roles in the response to acute anemia. BMP4 treatment increased the number of stress BFU-E, while SCF affected the size of the stress BFU-E-derived colonies. These results demonstrate that, while BMP4 regulates the differentiation of stress BFU-E, SCF/Kit signaling regulates the expansion of stress BFU-E progeny. Furthermore, we found that hypoxia increases both colony number and size, and hypoxia synergizes with SCF in order to further increase colony number. *In vitro* treatment of spleen cells with hypoxia, SCF and BMP4 recapitulate the stress BFU-E expansion observed *in vivo* in response to acute anemia.

Further characterization of the stress erythroid response demonstrates that BMP4 responsive cells and stress BFU-E do not self-renew in the spleen but must be replenished by bone marrow progenitors. Bone marrow-derived progenitors are found to give rise to BMP4 responsive cells and stress BFU-E upon exposure to the splenic microenvironment. Desert hedgehog is highly expressed in the spleen and is a potential candidate signal for converting bone marrow progenitors into BMP4 responsive cells. We show that exposure of bone marrow cells to Sonic hedgehog protein induces BMP4 expression as well as the expansion of stress BFU-E. Also, unlike controls, Smoothened mutant bone marrow cells, which are unable to transmit hedgehog signals, are incapable of giving rise to BMP4 responsive cells in the spleen. Together, our data demonstrate that certain bone marrow progenitors differentiate into BMP4 responsive cells upon exposure to hedgehog signals in the spleen and serve to replenish BMP4 responsive cells which have been exhausted during the recovery from acute anemia.

The discovery and characterization of stress BFU-E provides a new model for the response to acute anemia. Induction of BMP4 in the spleen following acute anemia results in the differentiation of BMP4 responsive cells into stress BFU-E. Hypoxia and SCF serve to increase the number and size of stress BFU-E and together with BMP4 are responsible for the stress BFU-E response to acute anemia. This stress BFU-E response is then replenished by bone marrow progenitors which become BMP4 responsive cells upon exposure to hedgehog signals in the spleen.

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Chapter 1

Literature Review

Hematopoiesis

Because all mature blood cells have a finite life span within an organism, they must be continually replenished. This process is called hematopoiesis (Figure 1-1). Terminally differentiated blood cells are typically incapable of replication. Consequently, blood cells must ultimately arise from pluripotent hematopoietic stem cells (PHSC). PHSCs are capable of giving rise to all mature blood cells. As defining characteristics, they exhibit long-term repopulation capacity of all hematopoietic lineages in the adult and the ability to self-renew (Bagby 1993). Their pool is maintained throughout life by the process of asymmetric division where mitosis results in the production of both a somewhat more committed multipotent progenitor as well as another PHSC (Potten and Loeffler 1990). Multipotent progenitors include the common myeloid progenitor (CMP) as well as the common lymphoid progenitor (CLP). These multipotent progenitors subsequently undergo their own asymmetric divisions producing more multipotent progenitors along with even more committed progenitors. These include the granulocyte-monocyte progenitor (GMP) and the megakaryocyte-erythrocyte progenitor (MEP) (Akashi, Traver et al. 2000) (Figure 1-2). These progenitors in turn give rise to more committed progenitors and eventually, after many terminal differentiation steps, all

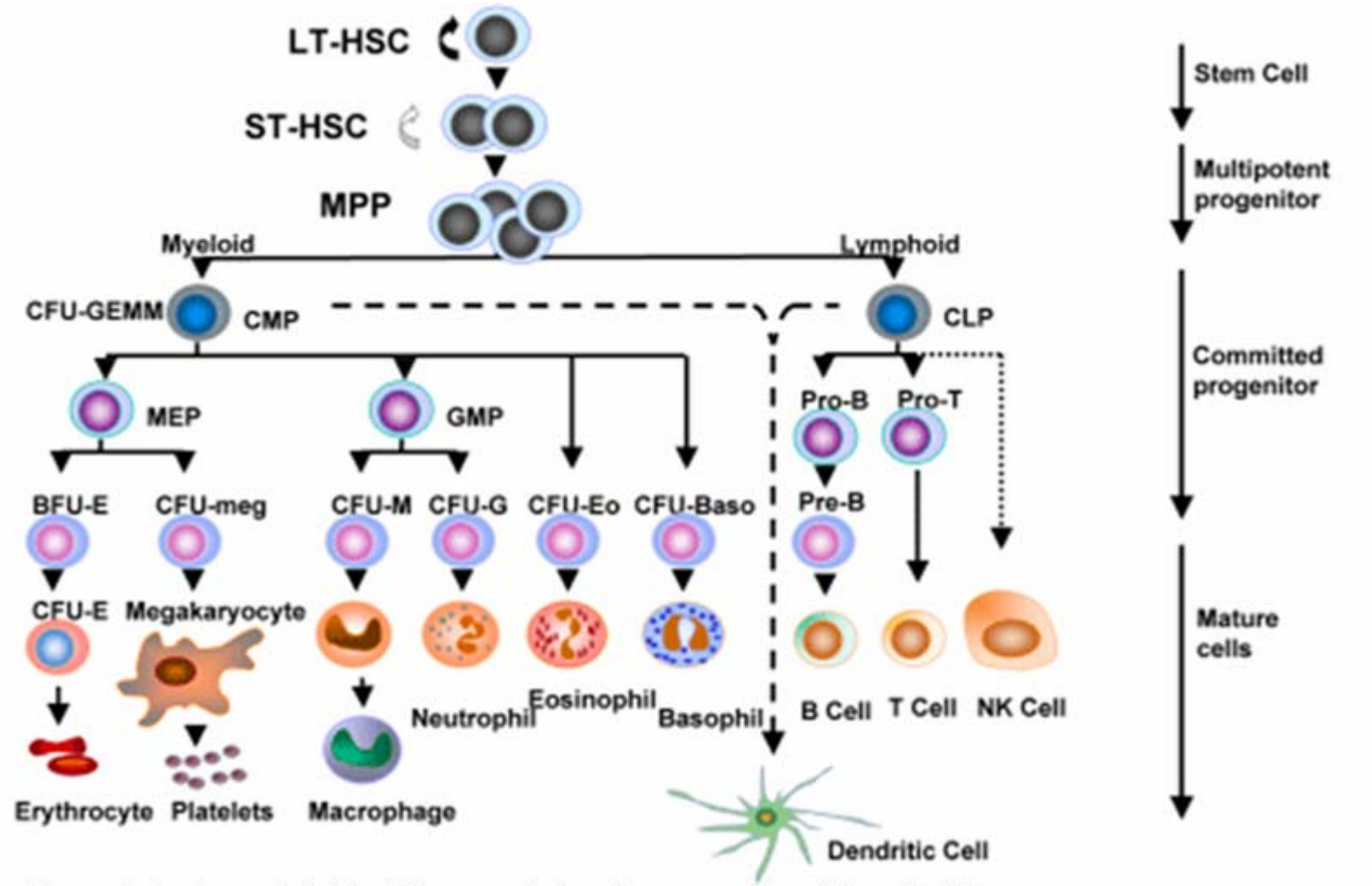

Figure 1-1. Current Model of Hematopoiesis. Courtesy of Lund Stem Cell Center

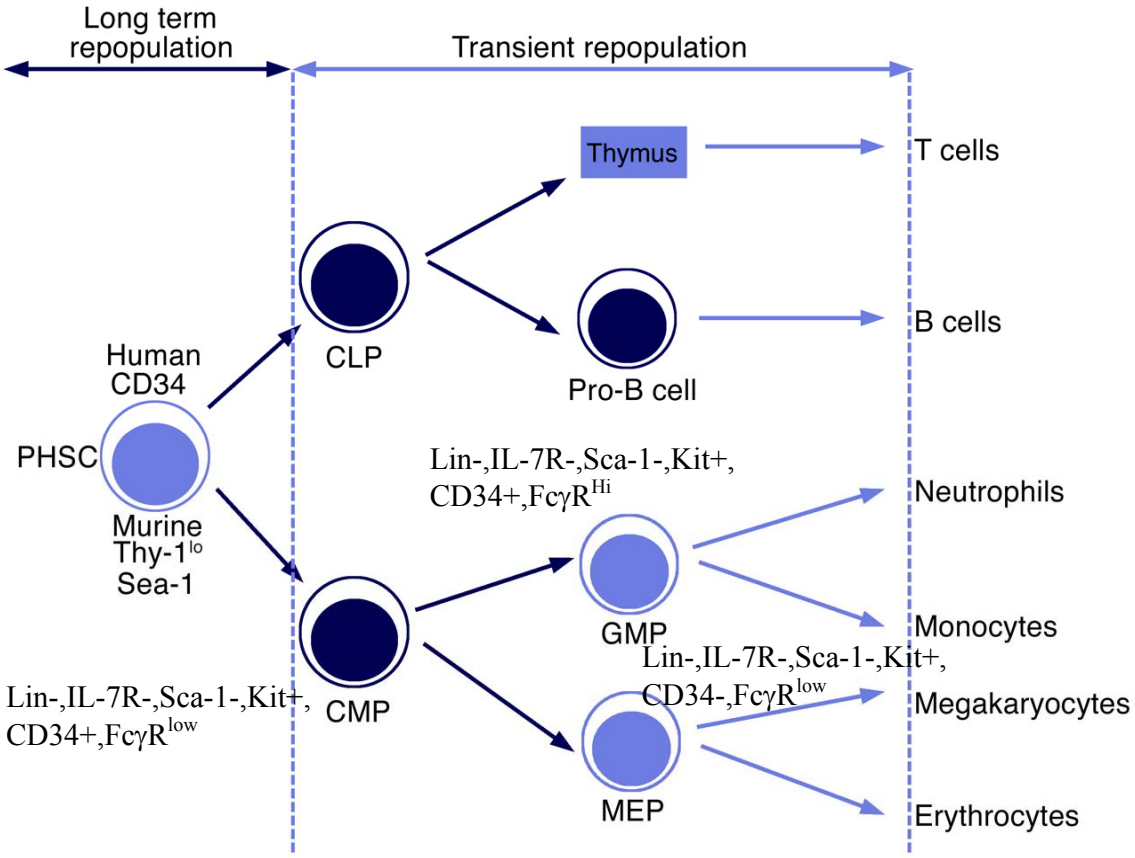

Figure 1-2. Characterization of CMPs, GMPs and MEPs in the Hematopoietic System. From Paquette and Dorshkind, 2002.

the mature blood cells including B and T cells, natural killer cells, basophils, eosinophils, neutrophils, monocytes, platelets and red blood cells (Bagby 1993). Of particular interest to this thesis is the MEP, which gives rise to only megakaryocyte and erythroid progenitors. Specifically, this thesis will focus on early committed erythroid progenitors, which eventually give rise to all the red blood cells or erythrocytes of an organism.

The hierarchal nature of hematopoiesis allows for the regulation of blood cell production at multiple levels. For instance, expanding the PHSC pool allows for a greater production of all hematopoietic cells. This is particularly important for

reconstituting the hematopoietic system after, for instance, total body irradiation. On the other hand when only a subset of hematopoietic cells is needed, such as lymphocytes, the pool of CLPs can be selectively expanded. Thus, the hematopoietic system must be capable of selective expansion of one particular or several particular lineages as needed. This selective expansion is controlled by various cytokines or growth factors. The fate of any hematopoietic cell precursor is specifically determined by the particular repertoire of cytokines to which it is exposed, which is in turn dependent upon the particular microenvironment. Thus, the hematopoietic system is well adapted to meet the dynamic needs of blood cell production throughout an organism's life span (Bagby 1993).

The hematopoietic system was largely delineated by functional assays, namely the colony assay. Colony assays are performed by obtaining single-cell suspensions of cells from hematopoietic organs and plating in semi-solid methylcellulose media containing specific growth factors necessary for the development of the colonies of interest (Stamatoyannopoulos and Grosveld 2001). Multipotent progenitors give rise to colonies containing multiple cell types. For example, MEPs give rise to colonies of both megakaryoblasts and erythroblasts. However, a committed erythroid progenitor gives rise to a colony containing only erythroblasts (Akashi, Traver et al. 2000). In general, larger colonies requiring longer periods of incubation are derived from progenitors more immature than colonies forming after shorter incubations and being relatively small in size (Stamatoyannopoulos and Grosveld 2001). Of particular interest here, colonies that form relatively large colonies after a relatively brief incubation in response to a stress condition such as tissue hypoxia may be termed a "stress progenitor". Such cells are

activated only after stress and represent progenitors with higher-than-normal proliferative capacity requiring shorter-than-normal cycling times.

In addition to the colony assay, various transplantation assays have been useful for demarcating the hematopoietic hierarchy. For instance, the PHSC was, in part, identified by injecting bone marrow cells into mice with a deficiency in hematopoiesis. Later, blood cells of all lineages in the recipient could be traced to these donor cells (Abramson, Miller et al. 1977). Another progenitor identified by transplantation experiments is the CFU-S or *c*olony-*f*orming *u*nit-*s*pleen. CFU-S form macroscopic colonies on the spleens of lethally irradiated mice transplanted with bone marrow cells (Till and McCulloch 1961; Till 1981). Microscopic analysis of CFU-S shows them to be composed of granulocytes, erythrocytes, macrophages, and megakaryocytes, so they have also been termed CFU-GEMM. Similar to stem cells, the CFU-S has self-renewal capacity. This was demonstrated by the ability of single-resuspended CFU-S to form multiple CFU-S colonies on the spleens of recipient mice (Jursskova and Tkadlcek 1965). This ability is progressively lost as a progenitor cell becomes more and more committed to a particular fate. For instance, early committed erythroid progenitors, termed BFU-E for *b*urst *f*orming *u*nit-*e*rythroid, can not undergo such serial transplantations. In similar fashion by colony assay and/or transplantation assays, the whole hematopoietic system has been characterized. This includes the more restricted lineages such as the committed erythroid progenitors to be discussed in the section on erythropoiesis below.

Complementing colony and transplantation assays has been the study of the effect of various cytokines on hematopoietic cells as well as the characterization of hematopoietic cells based upon cell-surface marker phenotype. Indeed, the particular

lineage found in any colony assay is fundamentally dependent on the particular repertoire of cytokines added to the culture media. Committed progenitor cells such as the BFU-E require the presence of certain growth factors in order to survive and form colonies *in vitro*. These growth factors act both directly and indirectly on progenitor cells. That is, they can directly stimulate the growth or differentiation of a particular progenitor cell and they can induce the expression of additional growth factors, which also have the ability to act indirectly (Stamatoyannopoulos and Grosveld 2001). No doubt this regulative complexity is necessary for proper hematopoiesis; however, this complexity makes it exceedingly difficult for researchers to delineate a specific set of discreet biological functions for any cytokine. Nonetheless, four general categories of biological functions are typically assigned to cytokines. These include inductive, synergistic, multilineage, and lineage-specific activities (Stamatoyannopoulos and Grosveld 2001). Thus, cytokines can have these activities in various combinations in certain contexts, and they can act differently in combination with other cytokines.

Embryonic origins of hematopoiesis

In the common mouse, *Mus musculus*, hematopoiesis is first detectable at 7.5 days postcoitum (d.p.c.) in the extraembryonic yolk sac (Baron 2003). This embryonic or 'primitive' hematopoiesis is transient and quickly superseded by definitive hematopoiesis, which occurs in the embryo proper by 9.5 d.p.c. Many factors contribute to the specification and development of this ventral mesoderm into hematopoietic tissue. Of particular prominence among them is bone morphogenetic protein 4 (BMP4) (Kessler and Melton 1994). Indeed, the underlying mesenchyme of budding HSCs in the AGM

secretes BMP4 (Marshall, Kinnon et al. 2000). BMP4 directly and indirectly acts to instruct the development of hematopoiesis through transcription factors such as the Smads, GATA-1 and 2, SCL, LMO-2, and EKLF (Schmerer and Evans 2003). These definitive hematopoietic stem cells do not mature at their site of origin but migrate to the fetal liver where they expand and mature. Thus, there is a difference between the particular microenvironment supporting production of HSCs and that supporting differentiation and expansion. As we will see, this theme recurs in the adult under various physiological conditions. The fetal liver serves as the main hematopoietic organ until around the time of birth when the bone marrow takes over this role. The fetal liver is uniquely equipped for expansive red blood cell development (Ohneda, Yanai et al. 1990). Here, progenitors have greater proliferative potential than their steady-state adult counterparts (Rich and Kubanek 1980; Lansdorp, Dragowska et al. 1993). Also, fetal liver progenitors respond differently to cytokines in a manner that is similar, as will be discussed later, to expansive erythropoiesis of the adult spleen in response to acute anemia (Peschle, Migliaccio et al. 1981). From the fetal liver, hematopoietic progenitors migrate to the spleen (embryonic day 12 or E12) and then the bone marrow (E15-16) (Godin and Cumano 2002). Thus, hematopoiesis is a dynamic process involving cell migrations and unique microenvironments at different stages of development and for different effects and purposes.

Hematopoiesis along with its dynamic nature does not end after birth. Rather the hematopoietic system must maintain a specific level of all the various blood cells throughout the life of the organism. It must maintain levels appropriate to the situation at hand. This includes, for instance, during pathogenic assault when the appropriate

immune cells must be marshaled for the defense of the organism as well as during tissue hypoxia. In this case, red blood cells or erythrocytes must be produced in order to alleviate the hypoxic condition.

Erythropoiesis

Erythropoiesis is the branch of hematopoiesis which gives rise specifically to erythrocytes. Colony assays are used to identify erythroid progenitors. The BFU-E is the most immature erythroid lineage-restricted progenitor and is presumably derived directly from the MEP. The BFU-E can only form one cell type and is named because of its ability to form burst-like colonies in methylcellulose media. BFU-E require both Epo as well as a burst-promoting activator (BPA). Stem cell factor (SCF) serves as the *in vivo* BPA; however, interleukin-3 (IL-3) or granulocyte monocyte-colony stimulating factor (GM-CSF) can substitute *in vitro*. Interestingly, except at suboptimal Epo concentrations, BPAs do not increase the number of embryonic or fetal BFU-E. This is dramatically different from the bone marrow where only a very small fraction of BFU-E respond to Epo alone (Peschle, Migliaccio et al. 1981). BFU-E are typically identified after a seven day incubation; however, it is important to note that BFU-E can be identified after much longer and even shorter incubation times. It is, therefore, likely that BFU-E actually represent a heterogeneous population at different stages of maturity rather than a specific cell type. For instance, BFU-E forming after 15 days may simply be derived from 7-day colonies which have been given 15 days to grow, yielding a larger BFU-E. However, not all colonies are necessarily larger after longer incubations, and some colonies are reabsorbed after longer incubations. It is also true that BFU-E can be

identified after only 3 days incubation (Gregory and Eaves 1978). It would seem that some BFU-E are derived from more immature lineage-committed progenitors than others. Those requiring long incubation times are presumably more immature than those requiring only brief incubation times, and those forming larger colonies in shorter times are derived from progenitors with higher proliferative capacity and perhaps shorter than normal cycling times. Thus, it is important to keep in mind that the BFU-E is not actually a single-specific cell type.

The next erythroid colony type identified is termed the CFU-E (*c*olony-*f*orming *u*nit-*e*rythroid). This colony is relatively small in size (from about 8-32 cells) and forms in only two days. It is important to note that CFU-E are not simply small BFU-E which have not yet had enough time to form. Instead, CFU-E form after two days and are not seen subsequently. Consequently, they are derived from a more differentiated erythroid progenitor with low proliferative capacity, presumably from the most mature form of BFU-E. CFU-E require Epo but do not require a BPA. CFU-E are composed of proerythroblasts, the first recognizable differentiated member of the erythroid lineage. Proerythroblasts synthesize globins and subsequently differentiate into erythroblasts, which synthesize large quantities of hemoglobin, the main constituent of the mature erythrocyte. Erythroblasts then undergo a series of maturation steps which are readily identified by histologists and hematologists by their distinctive staining properties and morphology. Eventually, in mammals, the nucleus is expelled and the progenitor is termed a reticulocyte. Reticulocytes continue to utilize remaining mRNA to synthesize globin before forming the mature erythrocyte, which does not divide, synthesize mRNA, or even protein (Bagby 1993).

The lineage-specific cytokine required for erythropoiesis is Epo. Epo is the primary regulator of erythropoiesis (Stamatoyannopoulos and Grosveld 2001). Using serum-free cultures and purified recombinant Epo, Epo's method of action has been described. It seems that the default program of erythroid progenitor cells is apoptosis and Epo's role is to prevent this otherwise obligatory pathway (Koury and Bondurant 1990). Of particular interest to this thesis is the fact that erythroid progenitors can be shown to have a range of sensitivities to Epo. It has been suggested that such heterogeneity of Epo-sensitivity supports a model where, in times of erythroid cell excess, less Epo is available and only progenitors with extreme sensitivity to Epo survive, which results in low erythroid production. Conversely, under anemic conditions, Epo expression is boosted and all progenitors including those only sensitive to unusually high Epo concentrations survive, which results in increased erythroid production. In short, because progenitors vary in their sensitivity to Epo, increased Epo production allows more progenitors to survive thus boosting erythroid output (Stamatoyannopoulos and Grosveld 2001). This is an attractive model in that it is simple and intuitively makes sense. For the purposes of this thesis, it is important to remember that erythroid progenitors vary in their sensitivity to Epo and not only for its explanatory power in the above model. Specifically, although this conclusion seems to have escaped most researchers in the field, this heterogeneity of sensitivity suggests to this author a possible heterogeneity of erythroid progenitors. Admittedly, the extent of this heterogeneity is unknown, although later chapters will show that there are indeed different types of erythroid progenitors responding to different physiologic conditions.

Epo is most highly expressed in the fetal liver during embryogenesis and in the adult kidney (Zanjani, Ascensao et al. 1981). The requirement of high fetal liver expression is obvious in that the fetal liver supports high erythropoietic activity necessary for the rapidly developing fetus. Adult kidney expression, especially in response to acute anemic stress, is less apparent but readily explicable. First, it is important to realize that, because the main role of the kidney is blood filtration, renal oxygen consumption is directly linked to renal blood flow. Therefore, unlike other tissues, lower blood flow in the kidney will result in less need for oxygen. If hematocrit is too high, blood viscosity increases, which decreases flow thereby reducing oxygen tension. In any other organ, this would lead to a positive-feedback loop where too many erythrocytes would lead to production of yet more erythrocytes, further exacerbating the problem. So, the kidney with its unique utilization of oxygen dependent on blood flow makes it the only organ where Epo levels can be appropriately regulated at a systemic level (Ebert and Bunn 1999).

Homeostatic versus stress erythropoiesis

Throughout the lifespan of an organism, a constant supply of erythrocytes must be maintained. This continual production is tightly regulated so that the hematocrit (HCT), or packed red cell volume, is neither too low nor too high. If too low, proper oxygen saturation cannot be maintained, and if too high, blood viscosity increases and may lead to clotting, increased blood pressure and other pathologies. Of course, this maintenance is usually relatively fixed since erythrocytes wear out at a fairly fixed-rate. The situation is dramatically different in the case of stress erythropoiesis. Here, the erythropoietic

system must exhibit its dynamic abilities and erythroid progenitors must expand at an uncharacteristically high rate. The molecular and cellular mechanisms governing these two fundamentally similar yet strikingly different phenomena is a subject of great importance to this thesis.

Perhaps most notable among the differences between homeostatic and stress erythropoiesis is the difference in their primary sites of occurrence. While adult homeostatic erythropoiesis occurs predominantly in the bone marrow, stress erythropoiesis occurs predominantly in the spleen (Ou 1980; Broudy, Lin et al. 1996). Presumably, this has much to do with the fact that murine bone marrow is relatively dense and 'trapped' in the confines of bone. Thus, marrow can not physically expand. In contrast, the spleen can become many times larger than its homeostatic size without rupture.

It seems that, among the vertebrates there has been an evolutionary shift in the main homeostatic erythropoietic organ from the spleen to the liver and then the bone marrow, although as with all such generalizations exceptions abound. For example, in fish the spleen is the primary erythropoietic organ (Jordan 1938). Apparently, any organ with a fairly stagnant sinusoidal vascular system can be ideal for erythropoiesis (Robb-Smith 1961). So, the sinusoidal cavities of the bone marrow in larger mammals such as adult humans have simply been co-opted for this function. Thus, there may be little reason to concern one's self with the question of why erythropoiesis occurs in a particular organ. The fact is that different sinusoidal organs are utilized in different organisms and at different developmental stages within the same organism. Indeed, extramedullary erythropoiesis in the spleen and liver is easily reactivated if necessary (Erslev 1967).

There has been much discussion about the apparent lack of need for a spleen, particularly for humans. Indeed, it seems that the spleen merely shares most of its homeostatic functions such as the formation, storage, and destruction of blood cells and the production of antibodies with many other organs. However, the spleen does have a unique function in the filtration of blood and removal of debris, although this function is apparently dispensable in humans. In fact, the medical profession thinks little of splenectomizing a patient and, with the exception of an increased risk for sepsis, there is little in the way of noticeable side effects as a result (Erslev 2001). Thus, if the spleen is important for stress erythropoiesis, as this thesis concludes, why can it be thrown away with so little apparent consequence? There are several answers to this question, but perhaps the most obvious is that humans, in developed countries where such things are monitored, rarely experience acute anemia. Massive blood loss or acute drug-induced hemolysis is alleviated with transfused blood. On the other hand, chronic anemia is one of the most common human afflictions worldwide, but there has been little data regarding splenectomized versus non-splenectomized patients with chronic anemia. At any rate, as mentioned earlier, splenic function is redundant with other organs, and any sinusoidal organ can be commandeered for erythropoiesis. In fact, while mice utilize both the bone marrow and spleen for homeostatic erythropoiesis, humans apparently do not (Bozzini 1970). Still, it is not known why the human spleen can be discarded with little consequence. Nonetheless, whether ordinarily useful or not, the potential for the spleen to be tapped for therapeutic purposes remains an attractive proposition. Since humans retain a spleen just as mice do, perhaps humans also retain the potentially therapeutically

useful BMP4-responsive cells which respond to acute anemia exclusively in the spleens of mice as discussed in chapter 2.

At any rate, the liver has been shown to serve a similar erythropoietic role as the spleen in splenectomized mice (L. Lenox and R.F. Paulson, unpublished observations). First, the kinetics of recovery from PHZ-induced acute anemia is delayed in splenectomized mice versus controls. Further, the bone marrow does not compensate for this delay by increasing erythropoiesis. This underscores the importance of the spleen in stress erythropoiesis and further demonstrates that the bone marrow does not (and presumably can not) simply increase erythropoietic output on a relatively massive scale. Interestingly, although not typically resident in the liver, BFU-E do expand in the liver in response to acute anemia and this expansion is more than 3 times greater in splenectomized mice versus controls. In addition, this liver erythropoietic expansion exhibits some of the unique properties of splenic expansion in response to acute anemia as described in chapter 2.

More locally, another significant difference between homeostatic and stress erythropoiesis is the particular microenvironment which supports these processes. It would be difficult to overemphasize the importance of microenvironment in the formation of all the various hematopoietic cells. Microenvironment refers to the particular constituency of immediately adjacent stromal cells which support the growth, maintenance and/or differentiation of hematopoietic cells. The microenvironment is composed of fibroblasts and endothelial or stromal cells and influences target cells via juxtacrine and paracrine factors. Indeed, PHSC's self-renewal and commitment is regulated by what has been dubbed the hematopoietic inductive microenvironment

(HIM). It has been suggested that the HIM of different organs are also different, accounting for the difference in hematopoietic activity and devotion to particular blood cell lineages in the various hematopoietic organs (Obinata and Yania 1999). For instance, the fetal liver and the spleen preferentially support erythroid development, thus these organs may have what can be described as an erythropoietic inductive microenvironment (EIM). In contrast, the adult bone marrow preferentially supports granulocytes and thus may be termed a GIM. This categorization may not be entirely warranted; however, the general concept of having different microenvironments in different organs that preferentially support different hematopoietic lineages is most certainly a valid one. Indeed, one can develop stromal cell cultures from the various hematopoietic organs and these can be used to preferentially support different hematopoietic lineages. For example, mouse spleen stromal (MSS) cell lines have been established which preferentially support large BFU-E formation (Yanai, Satoh et al. 1991). Also fetal liver stromal (FLS) cell lines have been established which preferentially support erythroid progenitors (Ohneda, Yanai et al. 1990). This effect is not simply due to selection bias during the establishment of the lines. Several MSS cell lines have been established which support erythropoiesis. Furthermore, lethally irradiated mice given bone marrow cells intravenously form discrete microscopic colonies in the bone marrow and spleen, but erythroid colonies dominate the spleen whereas the bone marrow is dominated by granulocytic colonies (Curry, Trentin et al. 1967). The microenvironmental differences between organs become particularly significant when considering stress and homeostatic erythropoiesis, because each occurs in different organs and thus in different microenvironments.

Different microenvironmental effects are also not merely a variation between the particular cytokines which are secreted by the stromal cells in each particular microenvironment (although this is certainly important and will be discussed subsequently). That differential cytokine expression is not the complete story has been demonstrated by experiments using stromal cell conditioned media. These experiments show that conditioned media can not substitute for actual stromal cell contact, and transwell filters between stromal and hematopoietic cells also prevents large BFU-E formation (Yanai, Matsuya et al. 1989; Ohneda and Bautch 1997). Thus, direct cell-cell contact is required for the EIM to operate properly. Further, the differences between the various inductive microenvironments can not be compensated for by simply upregulating the expression of the necessary cytokines from one generic stromal microenvironment. Rather, the differences between these microenvironments are more fundamental and occur not only at the level of gene regulation but also at the cellular and extracellular matrix level.

In addition to being found in different organs as well as different microenvironments within those organs, homeostatic and stress erythropoiesis also differ in the particular signals to which they respond. The signals which are important for stress erythropoiesis are most notably Epo, SCF, and BMP4. For this thesis, these signals are important enough to justify their own sections and will be presented separately. In addition, there are several other signals which require special mention. Particularly, glucocorticoids have been implicated in stress erythropoiesis (Bauer, Tronche et al. 1999). Glucocorticoids regulate a wide variety of developmental and physiological responses via the glucocorticoid receptor (GR). Notably, in the adult, these responses are

often a reaction to stress conditions and corticosteroids are released during stress. In regard to erythropoiesis, glucocorticoids are known to support erythroid colony formation and increase the proliferation of erythroid cells (Golde 1976; Udupa 1986). Also, humans suffering from certain anemias can be treated with glucocorticoids in order to restore normal erythropoiesis (Liang 1994). Notably, *in vitro* cultures of erythroblasts can be maintained in long-term culture with the cooperation of activated Epo receptor (EpoR), Kit/SCF, and glucocorticoids (Bauer, Tronche et al. 1999). Importantly, the GR is apparently dispensable for normal erythropoiesis because GR mutant mice do not exhibit a deficiency in homeostatic erythropoiesis. However, Bauer et al. have shown that the GR is required for stress erythropoiesis. Specifically, GR mutant mice are defective in their response to acute anemia. Bauer et al. have identified a specific population of erythroid progenitors which are responsible for the GR-mediated response to acute anemia. These progenitors are relatively late-stage progenitors at the CFU-E stage of development and are not the same as the earlier stress erythroid progenitor identified by Lenox, Perry and Paulson (2005) but may represent the progeny of stress erythroid progenitors.

Of particular interest is the fact that the glucocorticoid derivative dexamethasone (Dex) has been shown to act synergistically with SCF in order to increase erythroid proliferation (Gabbianelli, Testa et al. 2003). In addition, this increase is coupled with an increase in fetal hemoglobin (HbF) production. In humans, HbF reactivation occurs during stress erythropoiesis. In adult life, HbF represents only a small fraction of hemoglobin production; however, HbF can be reactivated to dramatically increase during times of erythropoietic stress (Alter, Rappeport et al. 1976; Stamatoyannospoulos, Veith

et al. 1985). Importantly, HbF reactivation has been shown to alleviate the affects of sickle cell disease as well as β-thalassemia (Stamatoyannopoulos and Grosveld 2001). Thus, glucocorticoids are further implicated in stress erythropoiesis, and it has been hypothesized that Dex may be used to treat various β-hemoglobinopathies.

Perhaps the most widely known model for the source of erythroid progenitors which respond to acute anemia states that, in response to erythropoietic stress, erythroid progenitors are released from the bone marrow and travel via the peripheral blood to the spleen where they expand and mature (Hara and Ogawa 1977). Hara and Ogawa utilized a standard three-injection protocol of the anemia-inducing drug phenylhydrazine (PHZ) (days 0, 1 and 3) and assayed for erythroid progenitors in the bone marrow, peripheral blood and spleen at various time points following these injections. The model is based on their observations of the temporal kinetics of the expansion of erythroid progenitors found at these three sites in response to anemia. First, bone marrow BFU-E were observed to decline, at two days post-injection BFU-E were found to be at highly elevated levels in the peripheral blood and at four days post-injection, BFU-E began to expand in the spleen. Additionally, Hara and Ogawa observed the proliferative state of the progenitors by observing the proportion of cells in DNA synthesis phase under both anemic and hypertransfused-polycythemic conditions. Neither anemia nor polycythemia changed the proliferative state of the progenitors which suggests that the change in BFU-E number in the respective organs were due to migration of progenitors rather than proliferation in each respective organ. The migration model explains Hara and Ogawa's data; however, their particular experiment does not reveal everything behind the expansion of erythroid progenitors in the spleen. In particular, my own data shows that

the migration model does not represent the initial response to acute anemia. While there is no reason to doubt that progenitors do migrate between the respective organs as Hara and Ogawa report, it seems that this migration may come secondarily to an initial, endogenous splenic response (see Lenox, Perry and Paulson (2005) or chapter 2). The differences between the previous model and ours can be explained by differences in the experimental method. Mainly, while Hara and Ogawa utilized a three injection protocol, we observed that this protocol only gradually and moderately reduced hematocrits. Consequently, we modified this protocol by using a single, higher-dose PHZ injection, which induced a rapid and severe anemia. This allowed us to observe the more immediate response to a truly acute anemia. Furthermore, we observed only BFU-E which matured in a relatively brief period (5 days) while Hara and Ogawa scored BFU-E which were more immature (9-10 days). Thus, our modification likely reveals the initial (or at least more proximate) response while the previous model of Hara and Ogawa reveals aspects which are secondary to this primary response.

Nonetheless, as discussed in chapter 4, migration is at least secondarily important in our revised model of the response to acute anemia. Recently, a protein important for cell migration was identified. Podocalyxin is a CD34-related cell surface protein, which has been likened to “cellular Teflon”. Podocalyxin allows cells to lose their adhesion to the extracellular matrix and stromal cells. Cells expressing podocalyxin round up and migrate to other organs as necessary. Podocalyxin is expressed highest during early development, and this expression attenuates as development proceeds. In particular, erythroid precursors in the fetal liver express podocalyxin. There is an early post-natal burst of expression allowing for hematopoietic cells to seed the bone marrow and spleen.

Podocalyxin expression declines and is eventually restricted to a population of Sca-1+ Kit+ Lin- cells in the bone marrow. Podocalyxin expressing cells apparently have HSC capability since podocalyxin positive cells can serially reconstitute the lymphoid and myeloid lineages of irradiated recipients (Doyonnas, Nielsen et al. 2005). Since podocalyxin is associated with the normal hematopoietic cell migrations of ontogeny, it is possible that it may also be involved in the mobilization of hematopoietic cells during the response to acute anemia. If cells do migrate from the bone marrow to the spleen during the response to acute anemia, podocalyxin may be important for their dislodgement from the bone marrow microenvironment. It would be interesting to examine podocalyxin expression during the response to acute anemia in order to help determine this.

Short-term radioprotective cells

Perhaps related to the stress erythroid progenitors reported here in chapter 2 and in Lenox, Perry and Paulson (2005) are the recently discovered short-term radioprotective cells. Bone marrow transplantation following myeloablative radiation and/or chemotherapy is plagued (at least in allogenic transplants) by T cell-mediated graft-versus-host disease (GvHD). That is, the donor immune system contained within the transplanted bone marrow attacks the recipient, often resulting in death. One way to eliminate GvHD is to transplant only enriched PHSCs. These cells can reconstitute the hematopoietic system entirely but will not contain the more mature lymphocyte precursors that cause GvHD. The problem is that, while PHSC transplants do eliminate GvHD, mice die 2-3 weeks after transplantation due to neutropenia, thrombocytopenia, and anemia. This is likely due to the additional time required for purified PHSCs to

proliferate and differentiate. In order to circumvent this problem, PHSCs are supplemented with a small portion of unfractionated bone marrow. Apparently, these cells provide enough progenitors to alleviate the radiation/chemotherapy-induced thrombocytopenia/anemia in the interim before the PHSCs stably reconstitute the hematopoietic system (Paquette and Dorshkind 2002). Recently, the identity of the radioprotective fraction of the supplemental bone marrow was discovered. CMPs, GMPs and MEPs were sorted out of bone marrow and each population was transplanted into lethally-irradiated recipients. While CMPs and MEPs conferred a radioprotective effect, GMPs did not (Na Nakorn, Traver et al. 2002). Since CMPs contain both GMPs and MEPs, it seems that the radioprotective cells are MEPs or at least contained within the MEP population. Thus, erythropoiesis and thrombopoiesis are most critical in the initial rescue from the lethal effects of high-dose radiation/chemotherapy. In fact, Na Nakorn et al. showed that mice transiently rescued with radioprotective cells from otherwise lethal irradiation showed only host-derived hematopoiesis after 30 days and survived at least 6 months post-transplant. Apparently, rare PHSCs survive myeloablation and, if given the chance, can reconstitute the hematopoietic system. This suggests the extraordinary possibility that PHSC transplantation may become unnecessary following myeloablative therapy. Interestingly, the BMP4-responsive cells which respond to acute anemia and are presented in Lenox, Perry and Paulson (2005) have been shown to be within the MEP fraction, however, only spleen MEPs but not bone marrow-derived MEPs contain BMP4 responsive cells. Since patients and mice receiving myeloablative therapy exhibit splenomegaly and the spleen is the site of expansive thrombo/erythropoiesis, this

observation suggests that BMP4 responsive cells may represent a major arm of the radioprotective cell population or may in fact be the radioprotective cells.

More recently, Baumann et al. (2004) have identified a different population capable of conferring radioprotection to lethally irradiated mice. Cells expressing PECAM-1 (or CD31), lineage marker negative (Lin-), Kit+ and Sca-1- were found to be short-term radioprotective cells. Additionally, transplantation of these cells produces low numbers of CFU-S in recipients (Baumann, Bailey et al. 2004). Interestingly, far fewer CD31+ Lin- Kit+ Sca-1- cells than MEPs are needed for the radioprotective effect. Thus, this population more specifically represents the radioprotective cells whereas MEPs may be the precursors of CD31+ Lin- Kit+ Sca-1- cells. Alternatively, MEPs may simply be a different, albeit less potent source of radioprotective cells.

Flexed-tail **as a model of stress erythropoiesis**

Flexed-tail (f) mice were first described in 1928 (Hunt and Premar 1928). Their name was originally derived from their most conspicuous feature which is a tail flexure. Despite their name, there has been little interest in the tail flexure itself. In fact, later stains show that the tail flexure is incompletely penetrant as are other features associated with this mutant such as a white belly spot. The focus of most scientific investigation regarding *f* has been its peculiar anemia. *f* mice exhibit a fetal-neonatal anemia, which resolves within two weeks after birth (Gruneberg 1942). They then exhibit normal steady-state blood parameters, but are defective in their response to acute erythroid stress (Coleman, Russell et al. 1969). Because *f* is inherited as a recessive allele in typical Mendelian fashion, the defect reveals an association between expansive fetal-liver

erythropoiesis and expansive adult splenic erythropoiesis. Furthermore, identification of the specific genetic defect in *f/f* mice reveals the genetic regulator of these two forms of expansive erythrocyte production.

The *flexed-tail* anemia begins early in development at embryonic day 12.5 (E12.5), which is the time when the fetal liver first becomes erythropoietic. The expansion of hematopoietic (mainly erythropoietic) cells is defective as revealed by their relatively small livers (Bateman and Cole 1972). In addition, *f/f* fetal livers contain less than half the normal number of CFU-E and reduced numbers of erythroblasts showing that fetal erythroid development is severely compromised (Bateman and Cole 1972; Cole and Regan 1976). The *f* mutation also affects erythroid progenitor differentiation. This property is shown by mutant embryo's severely compromised incorporation of iron into heme (Bateman and Cole 1972). Improper heme biosynthesis is also suggested by the presence of siderocytes containing non-heme iron granules at unusually high levels in the fetal blood. It is possible that this decrease in heme biosynthesis may be caused as a secondary effect of an imbalance in α and β globin chain synthesis (Chui, Sweeney et al. 1977). Consequently, *f/f* embryos have a defect occurring early in the expansion of erythroid progenitors as well as a later defect in maturation of red blood cells. It is important to note that this later defect is not present in the adult in response to acute erythropoietic stress. In fact, siderocytes are not observed at elevated levels even in response to acute anemia in the adult *f/f* mice (Gruneberg 1942). Therefore, the defect in globin chain synthesis and/or iron incorporation into heme is a separate defect from the delayed expansion of erythroid progenitors and occurs only in fetal expansive erythropoiesis, not adult expansive erythropoiesis in response to acute anemia.

Adult *f/f* mice have normal steady-state blood parameters and even progenitors such as CFU-E and BFU-E numbers are similar to control (Gruneberg 1942; Gruneberg 1942; Gregory, McCulloch et al. 1975). Their response to Epo and colony stimulating factors is also normal (Gregory, McCulloch et al. 1975; Cole and Regan 1976). Of particular interest is the fact that CFU-S numbers from the bone marrow in *f/f* mice are similar to control; however, *flexed-tail*-derived CFU-S are dramatically different from normal CFU-S in that they are devoid of erythroid cells which are ordinarily a major part of CFU-S. Similarly, *f/f* mice are almost completely incapable of generating transient endogenous spleen colonies (TE-CFU) (Gregory, McCulloch et al. 1975). These colonies are committed erythroid progenitors, which rapidly form colonies on the spleen in response to lethal doses of radiation treatment. Notably, the TE-CFU must be a stress response cell since the only assay known to produce this cell is severe stress. Thus it would seem that *f* mice specifically have a defect in a stress response cell as will be discussed in later chapters. Additionally, *f/f* mutants exhibit a severe delay in recovery from PHZ-induced hemolytic anemia, which is revealed by a delay in the expansion of erythroid progenitors in the spleen with a resultant delay in the appearance of reticulocytes in the peripheral blood (Coleman, Russell et al. 1969). Therefore, the *f* gene product plays a key role in the regulation of erythroid progenitor expansion in the adult spleen in response to acute anemia.

The *flexed-tail* mutation was reported to be due to a frameshift mutation in a putative mitochondrial transmembrane protein designated Sideroflexin 1 (Sfxn1) (Fleming, Campagna et al. 2001). More recent work by Lenox, Perry and Paulson (2005) shows that the *f* mutation is actually due to a splicing defect in Madh5 or Smad5, a

transcription factor that acts downstream of the bone morphogenetic protein (BMP) signaling pathway. The discrepancy is described in more detail in the following chapter, where Lenox, Perry and Paulson clearly show that *f* encodes the Smad5 locus. In addition to the functional data presented to support this claim, we have demonstrated that the Sfxn1 mutation can be segregated away in mice which still exhibit the *f* phenotype. Thus, Sfxn1 cannot be responsible for the *f* phenotype.

Role of SCF/Kit receptor signaling in stress erythropoiesis

SCF and its receptor, Kit, have important roles in a diverse range of functions, especially during embryogenesis. SCF/Kit signaling has roles in spermatogenesis, melanogenesis, as well as growth of primitive hematopoietic stem cells. The SCF/Kit pathway is involved in the migration of HSCs from the fetal liver to the bone marrow, the migration of germ cells, as well as the migration of melanocytes (Matsui, Zsebo et al. 1990). This last function is dramatically revealed by *Dominant White Spotting (W)* mutant mice, which have a mutation in Kit. Heterozygous *W* mice exhibit a white belly spot due to improper melanocyte migration, while homozygous mutants are completely white. Interestingly, melanocytes are derived from the neural crest and *f* mice also occasionally exhibit a white belly spot. Importantly, BMP4 is a known regulator of neural crest cell migration (Sela-Donenfeld and Kalcheim 1999; Christiansen, Coles et al. 2000). This connection will be made clear in chapter 2.

Notably, SCF appears to be the more important erythropoietic colony stimulating factor *in vivo*. This is illustrated by the fact that, unlike *W* mice, GM-CSF or IL-3

deficient mice do not exhibit erythropoietic defects although these factors can be substituted for SCF in *in vitro* colony assays (Wu 1997).

SCF is produced in two forms, generated by alternatively spliced mRNAs. One form is secreted, while the other is membrane-bound. The membrane-bound form appears to be the most important for hematopoiesis, as mice lacking the membrane-bound form but not the secreted form exhibit the severe defects. This implies that more direct cell-cell contact is required between the SCF-secreting stroma of hematopoietic microenvironments and the Kit expressing hematopoietic cells (Flanagan, Chan et al. 1991; Morrison-Graham and Takahashi 1993). In fact, it has been demonstrated that the membrane-bound form is critical for erythropoiesis whereas the secreted form is important for myeloid development (Kapur, Majumdar et al. 1998).

It has been suggested that interaction of SCF with Kit is required for normal homing behavior of hematopoietic progenitor cells during the response to acute anemia. Broudy et al. (1996) confirmed previous data showing that the response to acute anemia occurs mainly in the spleen and then sought to clarify the role of Kit/SCF in this response. Their studies mainly involved the use of neutralizing quantities of an antibody to Kit named ACK2. Using ACK2 on mice treated with PHZ essentially ablated splenic hematopoiesis but merely, although significantly, reduced it in the bone marrow. Because splenic erythropoiesis was preferentially blocked, this suggested that perhaps the homing of progenitors to the spleen had been blocked by ACK2 treatment. In order to test this, lethally irradiated mice were injected with bone marrow cells treated with and without ACK2, which were taken from anemic mice. The fraction of BFU-E retrieved from both the bone marrow and spleen was markedly reduced in mice receiving

progenitors exposed to ACK2 versus controls. This result suggests that an interaction between Kit and SCF is required for proper homing behavior of progenitors in the adult (Broudy, Lin et al. 1996).

There are important similarities as well as differences between *W* and *f* mice, but it is clear that both loci encode genes which are important for stress erythropoiesis. Similar to *f* mice, *W* mice have an impaired response to PHZ and other treatments that induce anemia, and both exhibit a severe fetal anemia (Harrison and Russell 1972; Broudy, Lin et al. 1996). However, both *f* and *W* clearly work at different stages of differentiation given that *f* exhibits a microcytic, hypochromic anemia whereas *W* exhibits a macrocytic anemia. Other differences include the fact that *W's* anemia is present throughout the lifespan whereas *f's* anemia is transitory and siderocytes are present at birth at greatly elevated levels in *f* mice but not *W* mice (Russell, Thompson et al. 1968). Notably, while *f* appears to be important only at times of expansive erythropoiesis (i.e. fetal liver and adult spleen in response to anemia), *W* appears to be important to erythropoiesis in general, whether homeostatic or expansive.

Role of Hypoxia in Stress Erythropoiesis

A second regulator of stress erythropoiesis is ultimately hypoxia. The number of erythroid progenitors is altered in response to hypoxia. For instance, bone marrow and fetal liver BFU-E and CFU-E grown in hypoxic conditions exhibit increased numbers as well as sensitivity to Epo compared to those grown in normoxic conditions (Rich and Kubanek 1982). Additionally, splenic erythropoiesis increases from only 10% of the total erythropoietic output to over 40% in phlebotomized mice (Sadahira 2000). Of

course, hypoxic stress results in the upregulation of many genes. In terms of erythropoiesis and the response to anemia, the most important of these is Epo. Decreased oxygen tension alone results in dramatic cellular, vascular and erythropoietic effects. A common example is that people living at high altitudes and therefore low oxygen tensions develop polycythemia, the severity of which is determined by the magnitude of the altitude. Of course, low oxygen tension can also be caused by loss of erythrocytes, increased oxygen affinity for hemoglobin and myriad other hemoglobinopathies. Severe hypoxia can increase the production of Epo up to 1,000 fold. Regulation of Epo by hypoxia occurs at the mRNA level, which reaches maximum production 4 to 8 hours after induction with a magnitude proportional to the degree of the anemia (Ebert and Bunn 1999). This induction of the *Epo* gene above basal levels is carried out predominantly by the transcription factor hypoxia-inducible factor 1α or HIF-1α and its cofactors, hepatic nuclear factor 4 (HNF4) and the general transcriptional activator p300. This trimeric complex's regulatory binding domain at the 3' end of the *Epo* gene mediates the transcriptional response to hypoxia. The induction of Epo is the main action of hypoxia in the response to acute anemia, and its action regarding Epo occurs at many levels. For instance, Epo mRNA may be stabilized by the binding of a hypoxia-induced protein to the 3' untranslated region (UTR) (McGary, Rondon et al. 1997).

On the other hand, Epo may not be quite as important during hypoxic stress as is generally assumed. For example, Brannan et al. examined plasma Epo titers in mice subjected to hypobaric hypoxia over the course of 18 days. Plasma Epo titers rise only briefly and fall after only 1 or 2 days, reaching baseline values after only 3 or 4 days. While the bone marrow proliferative response was sustained but relatively minor, the

spleen exhibited a large burst of erythropoietic activity from days 6-10 (Brannan 1997). Thus, the massive erythropoietic output of the spleen under hypoxic stress appears to be independent of plasma Epo levels.

BMP4 Signaling in Hematopoiesis

The chapter "BMP4 and Madh5 regulate the erythroid response to acute anemia" will reveal our discovery of the role of BMP4 signaling in stress erythropoiesis and our discovery that *f/f* mice have a splicing mutation in Madh5 or Smad5, a downstream transcription factor of the BMP 2, 4, and 7 signaling pathways. Here, I will review BMP4 signaling and its role in hematopoiesis and stem cell differentiation.

BMP factors are part of the TGF-β (transforming growth factor) superfamily of cytokines. These cytokines control cell proliferation, differentiation and apoptosis depending on the specific environment of the target cells (Massague 2000). Indeed, along with the Wnt (wingless), FGF (fibroblast growth factor), N (notch), and HH (hedgehog) families, TGF-β family members are largely responsible for guiding the process of development. In addition to their indispensable roles in development, TGF-β family members also have important roles in the adult organism. For instance, TGF-β family members are important in tumorgenesis, and they are involved in processes of regeneration and healing in response to injury or disease (Grimaud E 2002; Johnson AN 2002).

The two main families within the 30+ member superfamily are the TGF-β factors themselves and the BMP family. Originally named for their role in bone formation, BMPs have since been implicated in numerous other functions such as the regulation of

apoptosis, cell division, migration, and differentiation (Hogan 1996). BMP4 is the vertebrate homolog of the well-known *Drosophila Dpp (Decapentaplegic)* protein, and, in an example of conservation among the metazoa, human BMP4 has been shown to rescue flies deficient in Dpp (Padgett, Wozney et al. 1993). This illustrates the importance of BMP4 in all animals.

The basic structure of the TGF-β signaling pathway is as follows (Figure 1-3). There are two serine/threonine kinase receptors which bind TGF-β cytokines. Ligand binding occurs initially at the type II receptor chain which results in the binding of the type I receptor into the complex. At this point, the constitutively active type II receptor can now phosphorylate the type I receptor, which activates its kinase activity. Receptor activated transcription factors (Smads in this case) are recruited to the complex as a result of type I receptor activation and are themselves activated via phosphorylation. The activated Smad then binds the common Smad and is translocated to the nucleus where gene activation or repression occurs (Liu, Hata et al. 1996; Massague 2000).

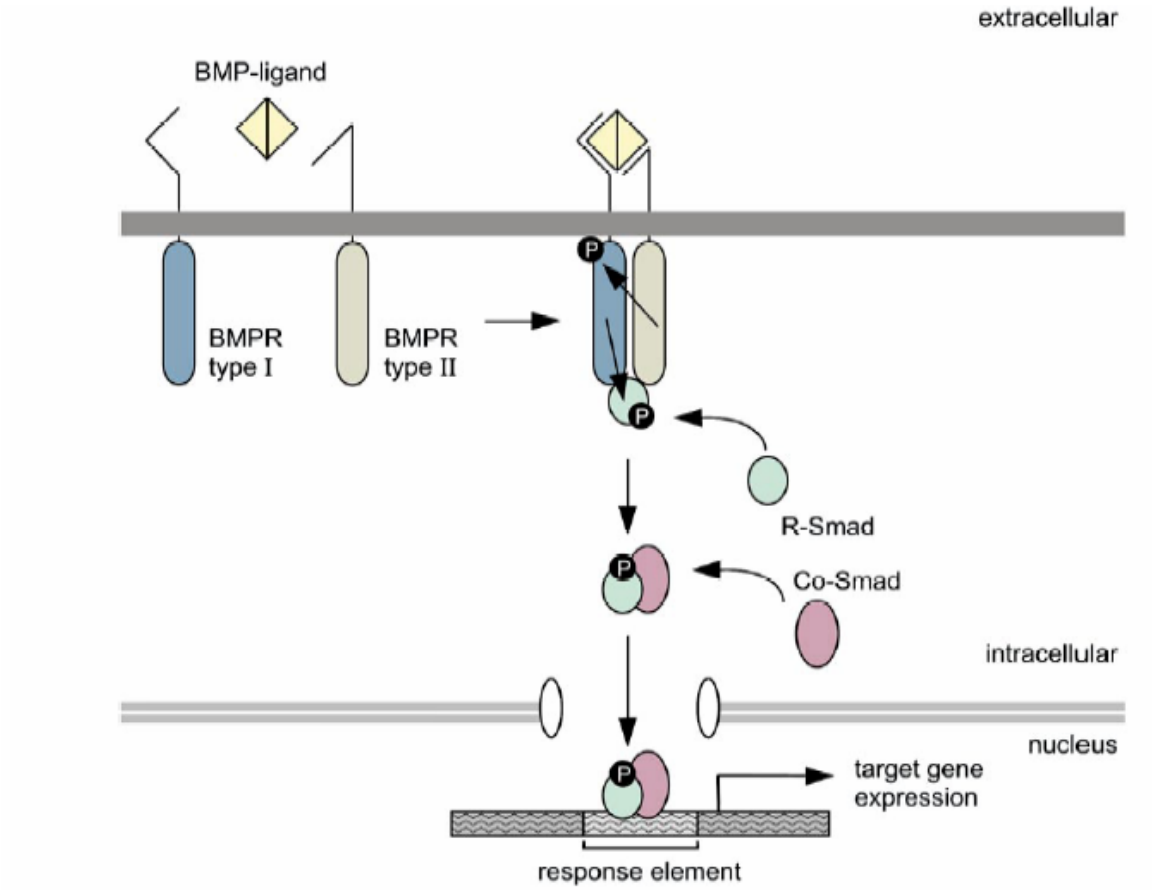

Figure 1-3. Cascade of BMP signaling. BMP dimers bind to serine/threonine kinase receptors type I and II. Upon ligand binding, type II receptors transphosphorylate type I receptors. The latter phosphorylate members of the Smad family of transcription factors. These Smads are subsequently translocated to the nucleus, where they activate transcription of target genes. From (Balemans and Van Hul 2002)

Paradoxically, despite having so many members, TGF-β superfamily signals are fed through a relatively simple set of common transcription factors termed Smads. The name 'Smad' is derived from the *Drosophila* Mad (Mothers Against Decapentaplegic) and *C. elegans* Sma (Small body size). While TGF-β members signal through Smads 2 and 3, BMP and other family members signal through Smads 1, 5, and 8. Together, these

Smads are termed R-Smads (receptor-phosphorylated Smads). Upon activation via phosphorylation, R-Smads undergo a conformational change which reveals their active domain called the MH2 (Mad homology) domain and along with heterodimerization with the common co-Smad (Smad4) and other transcriptional co-factors, this complex moves into the nucleus in order to activate the transcription of target genes (Liu, Hata et al. 1996). These signals are terminated by ubiquitylation- and proteasome-dependent degradation in the cytoplasm (Massague 2000).

The fact that a much larger number of signals converge on this relatively limited common set of Smad transcription factors begs the question: why do so many signals converge on so few known transcription factors? Furthermore, the ultimate output is anything but simple; TGF-β signals result in incredibly diverse physiological and developmental outcomes. One resolution to this apparent paradox is the recruitment of numerous cofactors such as p300 and CBP forming complexes with Smads. Since the Smad binding consensus sequence is only four nucleotides long, these cofactor complexes are largely responsible for the specific binding to particular gene promoters. No doubt the specific repertoire of cofactors depends on the cell's developmental history, physiologic and genetic state, interactions with other cells and other context-specific conditions. Indeed, it is becoming clear that key signaling pathways such as BMP are not so much pathways as they are networks (Massague and Wotton 2000; Bubnoff and Cho 2001). A network model is one resolution to the surprising paucity of genes found in complex animals such as mice and humans. No doubt organisms benefit from network signaling since networks tend to be more robust than simple pathways and signals can be transmitted even if part of the network is compromised.

TGF/BMP signaling is regulated by inhibitory Smads, namely Smads 6 and 7. Both of these lack the MH2 domain and thus their transcriptional activation abilities. Both are induced by TGF-β signals and apparently attenuate signal transduction by competitively binding to type I receptors thereby interfering with the binding of the other receptor-activated Smads (Imamura, Takase et al. 1997; Nakao, Afrakhte et al. 1997).

BMP4 has been shown to have a key role in the initial development of the hematopoietic system. The induction of hematopoiesis in the ventral mesoderm of *Xenopus* embryos has been shown to be accomplished by BMP4 (M Maeno 1965-1972 (1996)). BMP4's importance is such that, merely adding BMP4 to tissue normally fated to become skin or neuronal tissue changes its fate such that it will become blood if added at the appropriate developmental time. In fact, adding different amounts results in different fates thus leading to BMP4's designation as a mesodermic morphogen. Morphogens have a property such that, depending on the amount to which an undifferentiated tissue is exposed, the morphogen can simply "dial-up" a particular tissue fate (Zon 2001). In the case of BMP4, a diffusion gradient is set up in the early embryo with highest concentrations at its ventral source and continuously lower concentrations towards the dorsal aspect. Blood is formed at the highest concentrations of BMP4, mesenchyme or kidney at slightly lower concentrations as well as muscle and finally notochord at the lowest concentrations. Interestingly, primitive blood formation is mainly erythropoietic and BMP4 stimulates erythroid differentiation (Zon 2001). Thus, BMP4 has been implicated in hematopoiesis and particularly erythropoiesis.

More recently BMP4 along with one of its partner signaling pathways, hedgehog which will be discussed in the following section, have been implicated in regulating the

proliferation and differentiation of hematopoietic stem cells (Bhatia, Bonnet et al. 1999; Bhardwaj, Murdoch et al. 2001; Zon 2001). A role for BMP4 in adult HSCs was first shown by Bhatia et al. where BMP4 was shown to maintain human HSCs in long term cultures. More specifically, human CD34+CD38-Lin- stem cells have been shown to express the BMP type I receptors ALK-3 and ALK-6 as well as Smads 1, 4, and 5. Treatment of these cells with low concentrations of BMP4 induce proliferation and differentiation whereas higher concentrations allowed repopulation capacity to be maintained for a greater period of time in *ex vivo* culture suggesting that high BMP4 concentrations increase cell survival. Thus BMP4 has been shown to regulate blood development even after tissue specification in the adult. The following section will further discuss the role of BMP4 working in concert with hedgehog in human HSC self-renewal, proliferation and differentiation.

Smad5 is of particular interest to this thesis, but it is perhaps the least well characterized Smad. Smad5 knockout mice have been generated and characterized (Yang, Castilla et al. 1999). These mice die between E10.5 and 11.5 apparently due to defective angiogenesis. Yolk sacs of these mice have improper distribution of blood cells and abnormal vasculature. Despite these defects, differentiated erythroid cells were found in these knockouts. In addition *Smad5*-/- mice exhibit massive apoptosis of mesenchymal cells. Yang et al. postulate that these mutants show that Smad5 may regulate endothial-mesenchymal interactions which are essential for mesenchymal cell survival. It is possible that Smad5 may normally have an even greater range of roles which are not apparent in the knock out. This is because loss of a particular Smad may

be compensated for by other Smads either partially or completely. Also, the embryonic lethal effect prevents the inference of Smad5 function at later time points.

The DNA binding properties of Smad5 have also been characterized (Li, Chen et al. 2001). In studies of Smad5's ability to bind the Smad Binding Element (SBE) compared with other Smads, Smad5, unlike Smads 1 and 8, is able to bind the SBE with similar affinity as Smads 3 and 4; however, mutations in the SBE affect Smad5's binding affinity differently than Smad 3 or 4. Consequently, it may be that, while Smad5 falls in the same category as Smads 1 and 8 in transducing BMP signals, Smad5 may also transduce TGF-β signals to some degree in certain contexts. As discussed previously, this may not be surprising if TGF-β superfamily signals work in a network fashion as appears to be the case. In light of Smad5's potential dual capabilities, it may be of interest for future studies to determine potential TGF-β contributions to stress erythropoiesis as we have determined a role for BMP4/Smad5 signaling in stress erythropoiesis.

Hedgehog Signaling and its Interactions with the BMP4 Signaling Pathway

Hedgehog represents a strong candidate factor for a potential involvement in the BMP4-mediated response to acute anemia reported in chapter 2. The indicative data regarding this will be presented in chapter 4; here I will review hedgehog signaling and its interactions with the BMP4 signaling pathway.

One of the most dramatic teratogenic effects has been traced to aberrant hedgehog signaling. Ingestion of a plant-derived steroidal alkaloid, later termed cyclopamine, by grazing sheep resulted in cyclopic lambs. This striking phenotype was later found to be

the result of cyclopamine's blockage of hedgehog signaling (Keeler and Binns 1968; Chiang, Litingtung et al. 1996).

In mammals, there are three paralogs of hedgehog, Sonic, Indian and Desert hedgehog (Shh, Ihh, and Dhh, respectively). The hedgehog signaling pathway contains an interesting twist which is different from most signaling pathways. The receptor for hedgehog is called Patched (Ptc) and its co-receptor is Smoothened (Smo). In the absence of hedgehog signaling, Ptc binds to Smo and represses the activity of Smo. Upon hedgehog binding, Ptc releases Smo which allows Smo to activate targets, the most important of which are the Gli transcription factors. It is important to keep this in mind since Ptc knockouts actually allow for constitutive activation of the Hedgehog pathway rather than the opposite as one might reflexively conclude (Ingham and McMahon 2001).

After it was determined by Bhatia et al. that BMP4 regulates the development of adult human HSCs, Bhardwaj et al. showed that Shh induces proliferation of human HSCs via BMP regulation. First, Shh and its receptors are expressed in human HSCs, and Shh regulates the decision to self-renew or differentiate in a dose-dependent fashion. In culture, Shh increases the number of cells with repopulation capacity when transplanted into NOD-SCID recipients; however, treatment with antibodies to Shh inhibits cytokine-induced differentiation. These effects appear to be the result of controlling BMP4 homeostasis in the bone marrow because Shh induces both BMP4 and its antagonist, Noggin, which, as its name suggests, is expressed in the future head region of the embryo and prevents BMP4 from turning the head into blood. Specifically, while antibodies to Shh can not block BMP4's proliferative effect, Noggin is able to inhibit the effects of Shh, which suggests that Shh's influence is regulated via BMP4 (see Zon,

2001). Also, Shh's action does not appear to occur strictly via BMP4 as Shh, but not BMP4, was shown to induce the expansion of cells with repopulation ability (Bhardwaj, Murdoch et al. 2001).

Of the three known mammalian HH paralogs, Shh is the best characterized. It is clear that the three paralogs have distinct biological characteristics which go beyond spatial and temporal regulation. For instance, Pathi et al. looked at the activities of all three hedgehogs in a variety of cell-based and tissue explant assays. They determined that while equivalent in some contexts, the three paralogs had dramatically different activities in most of their tests (Pathi, Pagan-Westphal et al. 2001). Specifically Ihh has been shown to activate hematopoiesis and vasculogenesis in the mouse and also to have the ability to respecify prospective neural ectoderm into hematopoietic and endothelial lineages (Dyer, Farrington et al. 2001). Even less is known about Dhh, although the studies presented above regarding human HSCs also utilized Dhh in parallel with Shh in many of the experiments, and Dhh was shown to have an equivalent response in most of these experiments (Bhardwaj, Murdoch et al. 2001). Later, this thesis will show that Dhh is expressed in the adult spleen and is downregulated during the BMP4-mediated response to acute anemia thus establishing a role for HH/BMP signaling in adult expansive erythropoiesis. Further discussion of this is presented in chapter 4. Dhh has also been implicated in germ-cell proliferation and the developmental maturation of germ cells in the later stages of spermatogenesis (Bitgood and McMahon 1995). Thus, Dhh is further associated with stem cell proliferation and differentiation.

Several lines of evidence show that the BMP and HH signaling pathways interact. For instance, the downstream transcription factors of the HH pathway, Gli2 and Gli3 are

expressed at sites adjacent to BMP expression (Mason, Konrad et al. 1994). Also, various genetic analyses have indicated interactions between *Gli3* and *BMP4*. For instance, human syndromes resulting from mutations in *Gli3* have pleiotropic phenotypes that include similarities with mutations affecting BMP signaling (Liu, Massague et al. 1998). Also, Liu, Massague et al. report that Gli3 associates with the Smad proteins. Thus, in addition to their often complimentary developmental and hematopoietic stem cell roles, the HH and BMP pathways have also been shown to interact more directly.

ATF4 Mutant Mice and their Parallels with *f* Mutants

ATF4 mutant mice exhibit a very suggestive phenotype in that they exhibit parallels with the *f/f* mutants. Namely, ATF4-/- mice exhibit a severe fetal anemia and an impaired but certainly not severe adult anemia. The fetal liver of ATF4 null mice have fewer hematopoietic progenitors and also a dramatic decrease in the size of progenitor colonies (Masuoka and Townes 2002). Thus ATF4 is critical for normal expansive fetal-liver hematopoiesis. Unpublished experiments also suggest that adult ATF4-/- mice are more sensitive to PHZ treatment; therefore, ATF4 appears to be a critical regulator of expansive erythropoiesis in general (Lariviere and Perry, unpublished observations; Masuoka and Townes 2002). This phenotype is strikingly similar to *f/f*; however, it is clear that ATF4 plays a far more fundamental role in cell proliferation given that, unlike *f/f*, adult ATF4-/- mutants also exhibit slightly impaired homeostatic erythropoiesis as well as defects in other hematopoietic lineages such as granulocyte-monocyte progenitors. Additionally, unlike *f/f* mice, ATF4-/- mice exhibit severe growth retardation and severe microphthalmia. It seems that ATF4, along with hypoxia,

BMP4/Smad5 and SCF/Kit signaling plays key roles in expansive erythropoiesis but, while there is apparently some overlap, these signals work at distinct levels and in distinct ways.

Indeed, cellular stress pathways seem to converge on the transcription factor ATF4. Recently, it has become clear that although there are a variety of cellular stresses which can occur, many of these are funneled through a common stress response pathway. This pathway converges at the phosphorylation of the translational initiation factor eIF2α which subsequently activates translation of ATF4 (Kim and Sinha 2003). Notably, hypoxia has been shown to regulate ATF4 (Blais, Filipenko et al. 2004). Chapter 3 will present data concerning hypoxia's role in stress erythropoiesis while chapter 5 links ATF4 and hypoxia to my studies concerning stress erythropoiesis.

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Chapter 2

BMP4 and Madh5 regulate the erythroid response to acute anemia

Forward

The following chapter is taken from the manuscript appearing in Lenox, L. E., J. M. Perry and R. F. Paulson, *BMP4 and Madh5 regulate the erythroid response to acute anemia.* Blood, 2005. **105**(7): 2741-8. This manuscript was written with Robert F. Paulson and Laurie E. Lenox. Figures 2-2 and 2-6 as well as all supplementary figures present work done by Laurie E. Lenox. The work presented in the remaining figures is the work of the author.

Abstract

Acute anemia initiates a systemic response that results in the rapid mobilization and differentiation of erythroid progenitors in the adult spleen. *flexed-tail (f)* mutant mice exhibit normal steady state erythropoiesis, but are unable to rapidly respond to acute erythropoietic stress. Here we show that *f/f* mutant mice have a mutation in *Madh5*. Our analysis shows that BMP4/Madh5 dependent signaling, regulated by hypoxia, initiates the differentiation and expansion of erythroid progenitors in the spleen. These findings suggest a new model where stress erythroid progenitors, resident in the spleen are poised to respond to changes in the microenvironment induced by acute anemia.

Introduction

Erythropoiesis in the bone marrow is primarily homeostatic; however, the situation is dramatically different in the adult spleen in response to acute erythropoietic stress, where rapid, expansive erythropoiesis occurs. Previous work suggested a model where acute anemia leads to tissue hypoxia, which induces erythropoietin (Epo) expression in the kidney. Increased levels of serum Epo mobilize cells from the bone marrow, which migrate to the spleen where they expand and differentiate (Hara and Ogawa 1976; Broudy, Lin et al. 1996). The spleen contains a unique microenvironment that can support expansive erythropoiesis (Obinata and Yania 1999). However the signals that regulate the increase in splenic erythropoiesis in response to acute anemia are not clear. The expansive erythropoiesis observed in the adult spleen is similar to fetal liver erythropoiesis during development (Palis and Segel 1998). In both cases rapid erythroid development occurs.

Similar to the spleen, fetal liver stromal cells are capable of supporting the expansion of erythroid progenitors (Ohneda, Yanai et al. 1990). Because of these common features it has been suggested that splenic and fetal liver erythropoiesis may be mechanistically similar. This link between the fetal liver and spleen is apparent in mice with a mutation at the *flexed-tail (f)* locus. During fetal development, *f/f* mutant embryos exhibit a severe microcytic, hypochromic anemia (Mixter and Hunt 1933; Gruneberg 1942; Gruneberg 1942). *f/f* fetal livers contain about 50% the normal number of erythroid progenitors (Bateman and Cole 1972; Cole and Regan 1976) and have a maturation defect, which results in the production of large numbers of siderocytes or erythrocytes that contain non-heme iron granules (Gruneberg 1942; Chui, Sweeney et al.

1977). Despite these defects, the anemia of *f/f* mice resolves about two weeks after birth. Adult *f/f* mice exhibit normal numbers of steady state erythroid progenitors (Gregory, McCulloch et al. 1975). However, they are unable to respond rapidly to acute erythropoietic stress. This defect is manifested by a delay in the expansion of erythroid progenitors in the spleen and a delay in the appearance of reticulocytes following Phenylhydrazine (PHZ) induced acute anemia (Coleman, Russell et al. 1969). Despite the delayed response, adult *f/f* mice do not exhibit the maturation defect present during fetal liver erythropoiesis because no siderocytes are observed during the recovery from acute anemia (Cole, Regan et al. 1972). These observations demonstrate that the *f* gene product plays a key role in regulating the expansion and maturation of erythroid progenitors at times of great erythropoietic need.

Previous work has suggested that *f/f* mice have a mutation in *sideroflexin 1 (sfxn1)*, a putative mitochondrial transporter, which is proposed to play a role in the transport of molecules required for heme biosynthesis (Fleming, Campagna et al. 2001). In this report, however, we show that *f/f* mice have a mutation in the *Madh5* gene, which directly affects the ability of *f/f* mice to respond to acute anemia. Madh5 functions as a receptor activated Smad downstream of the BMP2, 4 and 7 receptors (Massague 2000; Massague and Wotton 2000). Previous work has implicated BMP's and in particular BMP4 in the development of mesodermal cells that will give rise to hematopoietic cells early in development (Huber, Zhou et al. 1998). Our work shows that in response to acute anemia, BMP4 is rapidly induced in the spleen. BMP4 acts on an immature progenitor cell causing it to differentiate into an Epo responsive stress erythroid progenitor. Cell sorting experiments showed that BMP4 responsive cells exhibit the

same cell surface phenotype as the bone marrow derived Megakaryocyte-Erythroid progenitors (MEPs) (Akashi, Traver et al. 2000), however, only spleen MEPs respond to BMP4. These results demonstrate that these spleen progenitors exhibit properties that are distinct from bone marrow erythroid progenitors suggesting that they represent a population of "stress erythroid progenitors" resident in the spleen whose function is to rapidly generate erythrocytes at times of great erythropoietic need.

Methods

Mice

C57BL/6 and C57BL/6-*f* mice were obtained from Jackson Laboratory. *Madh5+/-* mice were obtained from Dr. C. Deng (Yang, Castilla et al. 1999). All mice were approximately 6-8 weeks old, controls were age matched. Acute anemia was induced by injection of Phenylhydrazine (Sigma, St. Louis, MO) at a concentration of 100 mg/Kg mouse in PBS.

Colony Assays for BFU-E

Splenocytes, bone marrow, and peripheral blood cells were isolated from C57BL/6 control *f/+* and *f/f* mice. $1x10^5$/ml nucleated bone marrow and peripheral blood cells and $2x10^6$/ml nucleated splenocytes were plated in methylcellulose media (StemCell Technologies, Vancouver, BC) containing 3U/ml Epo + either 10 ng/ml IL3 (Sigma, St. Louis, MO) or 0. 15-15 ng/ml BMP4 (R&D Systems, Minneapolis, MN) where indicated. BFU-E were scored as described (Finkelstein, Ney et al. 2002). For the BMP4 pre-incubation experiment, splenocytes and bone marrow cells from C57BL/6 and *f/f* mice

were incubated for 24 hours in IMDM + 5% FCS + 15 ng/ml BMP4. Colony assays were then performed as indicated above + 15 ng/ml BMP4 for each.

Characterization of the Epo sensitivity of the stress BFU-E

Colony assays were performed as above on bone marrow and spleen cells in the presence of 0.1, 0.3, 1, 3, and 10 U/ml Epo as indicated. Additionally, bone marrow cells were supplemented with 50 ng/ml SCF while splenocytes were supplemented with 15 ng/ml BMP4. Colonies were scored as above.

Cloning of Madh5 mRNAs from f/f, f/+ and control mice

Total RNA was isolated from single cell suspensions of spleen cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was generated and *Madh5* cDNA was amplified using 5'-GGGGCCGAGCTGCTAAT-3' and 5'-CTATGAAACAGAAGAAATGGGG-3' primers.

Analysis of BMP4 expression

Total RNA isolated from bone marrow or spleen cells homogenized in TRIzol (Invitrogen, Carlsbad, CA) was reverse transcribed into cDNA. PCR was performed using primers 5'-TGTGAGGAGTTTCCATCACG-3' and 5'-TTATTCTTCTTCCTGGACCG-3'.

Staining of Spleen sections with anti-BMP4 antibodies

Spleens were harvested on the indicated days post PHZ induced anemia, fixed in Zinc fixative and paraffin embedded tissues sections were cut. The expression of BMP4 was analyzed as described (Marshall, Kinnon et al. 2000) using anti-BMP4 antibody (Novocastra Laboratories/Vector Laboratories, Burlingame, CA). Slides were analyzed by confocal microscopy.

Cell staining and sorting

Bone marrow and spleen MEPs were sorted as previously described (Akashi, Traver et al. 2000) with the exception that FITC-conjugated anti-c-kit was used (and APC-conjugated anti-c-kit and FITC-conjugated anti-CD34 eliminated) for spleen sorts after determining that FITCconjugated anti-CD34 did not stain spleen cells. Cells were washed twice and sorted using a Coulter Elite ESP flow cytometer. Cells were plated in methylcellulose and scored as described in Colony assays for BFU-E above.

Analysis of BMP4 signaling in W-20-17 osteoblast cells

The cDNAs coding the *f/f* truncated transcripts, as well as full length *Madh5* were cloned into the MSCVneo retroviral construct. Recombinant virus was generated as previously described (Melkun, Pilione et al. 2002) and used to infect W-20-17 cells (ATCC, Manassas, VA). Pools of neo^R colonies were plated and induction of alkaline phosphatase by BMP4 was measured as described (McNeice, Lanley et al. 1991).

Results

flexed-tail mutant mice exhibit a delayed expansion of erythroid progenitors in the spleen in response to acute anemia

In order to identify the origin of the defect, we characterized the response of *f/f*, *f/+* and control mice to acute anemia using a modified PHZ induced hemolytic anemia protocol that rapidly induced severe anemia (hematocrit 30% treated v. 50% untreated) in 12 hours. Similar to previous findings, we observed that early erythroid progenitors (BFU-E) in the bone marrow were not elevated in response to anemia and in fact gradually declined over time (Figure 2-1A). The greatest difference was observed in the spleen and

peripheral blood. In the spleen, control mice exhibited an expansion of BFU-E that peaked at 36 hours post anemia induction. Similarly, *f/+* mice showed the greatest expansion at 36 hours but unlike control mice we also observed a significant expansion later at 4 and 6 days post anemia. In contrast, the expansion was significantly delayed in the *f/f* mice where it peaked at 4 days post anemia induction (Figure 2-1B). These results correlate with previous data showing that the *f/f* mice were delayed in the expansion of erythroblasts in the spleen following anemia induction (Coleman, Russell et al. 1969). In the peripheral blood, however, we did not identify any BFU-E potentially migrating from the bone marrow to the spleen at any of the time points in the *f/f, f/+* or control mice (data not shown). These results suggest a new model where progenitor cells resident in the spleen mediate the response to acute anemia.

Splenic erythroid progenitors that expand in response to acute anemia exhibit distinct properties

Bone marrow BFU-E colonies have a distinct morphology, develop in 7 days in culture and require two signals to develop. The first signal being Epo and the second is a Burst-Promoting Activity (BPA), which *in vivo* is SCF, but *in vitro* IL-3 or GM-CSF can substitute. We observed that the BFU-E colonies from spleen 36 hours post anemia induction exhibited altered morphology. The colonies were larger, had more small satellite colonies associated with the BFU-E. They also grew faster such that spleen colonies grown for 5 days resembled bone marrow colonies that had grown for 7 days and spleen BFU-E were routinely grown for 5 days out of convenience. In many ways these erythroid progenitors resembled fetal liver erythroid progenitors, which are known

to exhibit a faster cell cycle than bone marrow BFU-E. Fetal liver BFU-E can also develop in media containing only Epo, without an added BPA (Peschle, Migliaccio et al. 1981). Given that *f/f* mice also have a defect in fetal liver, we repeated the analysis of bone marrow, peripheral blood and spleen BFU-E following induction of acute hemolytic anemia, however this time cells were cultured in media containing only Epo. The bone marrow contained very few cells that could form BFU-E colonies in Epo only media (Figure 2-1C). In the control and *f/+* spleens however, the expansion of BFU-E at 36 hours was completely recapitulated when the cells were plated in Epo only media (Figure 2-1D). If fact more splenic BFU-E colonies developed in this media. Similar to the initial observations, the *f/f* mice exhibited a delay in the expansion of BFU-E with the maximum number of colonies observed at 4 days post anemia induction. The number of spleen BFU-E observed under the Epo only culture conditions responded in a linear manner when increasing numbers of cells were plated, which suggests that the spleen cells are not producing a BPA (Data not shown). Once again we did not identify any BFU-E in the peripheral blood at any of the time points in the *f/f, f/+* or control mice indicating that these BFU-E are resident in the spleen.

In addition to shorter cell cycling times, fetal liver erythroid progenitors also exhibit an increased sensitivity to Epo (Peschle, Migliaccio et al. 1981). We tested the Epo-sensitivity of splenic erythroid progenitors. We observed that these progenitor cells were actually less sensitive to Epo than bone marrow cells (Figure 2-1E). Decreased Epo sensitivity is an ideal property of a stress progenitor, because differentiation of these progenitors would be dependent on the high serum Epo concentrations only present during the response to acute anemia. Taken together, these results suggest that the spleen

contains a distinct population of erythroid progenitors that are poised to respond to acute erythroid stress. These progenitors, which we will refer to as "stress BFU-E", form large burst colonies in 5 days, require only Epo at relatively high levels to develop and are resident in the spleen.

f/f mice have mutation in the Madh5 gene

The *flexed-tail* locus is located on mouse chromosome 13 (Lyon, Rastan et al. 1996). We generated a panel of 408 F2 progeny using a F1(C57BL/6-*f/f* X BALB/c) intercross. F2 progeny were scored at birth for anemia by hematocrit and for the presence of siderocyes by staining blood smears for iron deposits. We constructed a high resolution genetic linkage map of the *f* locus and initially localized the gene 0.6 cM distal to the microsatellite marker D13MIT13. Further analysis of markers showed that the *f* locus co-segregated with the marker D13Mit208 (Figure 2-2A). Our linkage mapping results differ from the recent work from Fleming et al. (Fleming, Campagna et al. 2001). They mapped the *f* locus to a more proximal position on chromosome 13 and identified a mutation in the *sideroflexin 1 (sfxn1)* gene, which they proposed caused the *f/f* mutant phenotype. Since there is only a single allele of the *f* mutation, all *f/f* must carry the same mutation (Hunt and Premar 1928). Like Fleming et al., our colony of C57BL/6J-*f* mice was derived from C57BL/6J-*f* mice obtained from the Jackson Laboratory. However, when we scored our *f/f* progeny for the presence of the mutation in exon 2 of *Sfxn1*, we identified *f/f* progeny that exhibited severe anemia and siderocytes at birth, but were heterozygous for the insertion mutation (Supplementary Figure 1). These results suggest that in our colony, the *f* mutation has been separated from the mutation in *Sfxn1* by

recombination and thus, mutation of *Sfxn1* cannot be the cause of the *f/f* mutant phenotype.

In order to identify candidate genes for the *f* locus, we initially took advantage of the fact that one of our flanking markers, D13Mit13, is located in the *IL-9* gene (Bult, Blake et al. 2004). This region of mouse chromosome 13 is homologous to human chromosome 5q31. Comparison of the human and mouse gene maps in region immediately surrounding *IL-9* revealed *MADH5* was located in this region. The possibility that *Madh5* was encoded by the *f* locus was supported by the recent mouse genome sequence release that showed that D13MIT208 is located in the *Madh5* gene. Previous work in Xenopus and mice has demonstrated that BMP4/Madh5 dependent signals play a key role in the development of erythroid cells (Huber, Zhou et al. 1998; Nakayama, Lee et al. 2000). *Madh5* is highly expressed in the fetal liver during development (Flanders, Kim et al. 2001) and we have observed *Madh5* expression in the spleen of mice recovering from PHZ induced acute anemia (Data not shown).

To determine whether *Madh5* is mutated in *f/f* mice, we cloned the entire coding region of *Madh5* by RT-PCR from spleen RNA isolated from C57BL/6J-+/+, C57BL/6J-*f*/+ and C57BL/6J-*f/f* mice. Only the expected product was observed in wildtype mice, however in both *f*/+ and *f/f* mice an additional band was observed (Figure 2-2B). The majority of the mRNA in *f*/+ mice is the wildtype fragment, while in *f/f* mice the majority of the mRNA is a truncated mRNA. The level of wildtype message in *f/f* mutant mice varies, but most express <30% wildtype mRNA. Analysis of the sequence of the truncated mRNA showed that it is a mixture of two mis-spliced mRNAs. The consistent feature of these mutant mRNAs was the deletion of exon 2, which contains the AUG

initiator codon. In one of the mis-spliced mRNAs we also observed deletion of exon 4 and insertion of a 15 nucleotide sequence at the splice junction between exons 6 and 7. In the other mis-spliced mRNA, we observed an aberrant splice into the middle of exon 3 (Figure 2-3). There were no other alterations in the coding sequence of *Madh5* observed in *f/f* mice. Southern blot analysis of the *Madh5* genomic region did not identify any deletions or rearrangements in the *Madh5* locus suggesting that the alterations in the *Madh5* mRNA in *f/f* mice are due to defects in mRNA splicing (Data not shown). We have sequenced the entire *Madh5* transcription unit from *f/f* and control mice and have not identified a consistent mutation, which suggests that the defect may lie in the promoter or 3' to the *Madh5*gene. We are currently investigating this possibility.

BMP4 expression is induced in the spleen just prior to the expansion of "stress BFU-E"

The identification of aberrantly spliced *Madh5* mRNA in *f/f* mice suggests a role for the BMP2, 4 and 7 signaling pathways in the response to acute anemia (Hogan 1996; Massague 2000). We investigated the expression of *BMP2, 4* and *7* in the spleen during the response to acute anemia by RT-PCR. *BMP2* is not expressed in the spleen at any time point during the recovery from acute anemia, while *BMP7* is expressed at low levels at all times tested (Data not shown). *BMP4* is not expressed in the spleen of untreated mice, however expression is initiated at 12 hours, peaks at 24 hours with lower levels at 36 and 48 hours post anemia induction. We also observe low levels at 6 and 8 days post anemia induction. The highest expression observed at 24 hours post anemia induction is just prior to the expansion of "Stress BFU-E" in the spleen (Figure 2-3A). Staining of spleen sections with anti-BMP4 antibodies showed that BMP4 protein was not present in

untreated mice, however high-levels of BMP4 was observed throughout the red pulp of the spleen at 24 and 36 hours post anemia induction, but expression is severely decreased by 48 hours and essentially off by 96 hours post anemia induction (Figure 2-3B). BMP4 was excluded from the white pulp.

The expression of BMP4 is tightly regulated during the response to acute anemia. During the analysis of the BMP4 expression by RT-PCR, we also tested the expression in *f/f* mice. Surprisingly, BMP4 expression is expanded in the mutant mice. Untreated mice and all the time points during the response to acute anemia exhibit BMP4 expression (Figure 2-3A). The expression of BMP4 in untreated *f/f* correlates with the observation of stress BFU-E in these mice (See Figure 2-1D). Despite the constitutive mRNA expression, BMP4 protein expression was not observed at all time points suggesting that BMP4 expression is regulated post-transcriptionally. These observed differences in BMP4 expression in *f/f* mice suggest that the regulation of BMP4 may require a Madh5-dependent signal to inhibit the expression of BMP4 in the spleens of untreated mice.

The increase in serum Epo concentration that occurs during the response to acute anemia is regulated at the transcriptional level by the hypoxia inducible transcription factor complex, HIF 1 (Ebert and Bunn 1999). Given that Epo expression is regulated by hypoxia we tested whether BMP4 expression is also hypoxia inducible. MSS31 spleen stromal cells (Yanai, Satoh et al. 1991), which support erythroid progenitor cell expansion *in vitro*, were grown at normoxic (20% O2) and hypoxic (6% O2) conditions. At low oxygen concentration, the expression of BMP4 was significantly increased (Figure 3C). Analysis of the BMP4 gene revealed the presence of a putative HIF element in the 3'UTR, which is conserved in the human, rat and mouse *BMP4* genes.

BMP4 causes the differentiation of an immature progenitor cell into an Epo responsive stress BFU-E

Given that BMP4 was induced in the spleen just prior to the expansion of stress BFU-E, we next tested whether culturing spleen cells from untreated mice in Epo and BMP4 could induce the expansion of stress BFU-E. Spleen cells from untreated *f/f* and control mice were plated in methylcellulose media containing 3U/ml Epo and various concentrations of BMP4. Control spleen cells responded in a dose dependent manner to BMP4 with a 6.1 fold increase in the number of stress BFU-E seen at a 15ng/ml BMP4 dose (Figure 2-4A). *f/f* spleen cells failed to respond to BMP4 except at the highest concentration, which is consistent with their defect in *Madh5*. BMP4 had very little effect on the number of BFU-E in the bone marrow suggesting that there are very few BMP4 responsive erythroid progenitor cells in the bone marrow (Figure 2-4A). We did not detect any BMP4 responsive cells in the peripheral blood in untreated or in mice treated with PHZ to induce anemia (Data not shown).

BMP4 can induce the expansion of stress BFU-E, but the mechanism by which BMP4 affects these cells is not clear. One might imagine two possible roles for BMP4. First BMP4 could synergize with Epo to promote the differentiation of stress BFU-E, much like SCF synergizes with Epo to increase the number and size of bone marrow BFU-E (McNeice, Lanley et al. 1991). Alternatively, BMP4 may act on an earlier cell inducing it to differentiate into an Epo responsive stress BFU-E. This possibility is similar to the situation in Xenopus embryos where BMP4 can induce mesodermal cells to become erythroid progenitors (Huber, Zhou et al. 1998). To test these possibilities, we

pre-incubated spleen cells from untreated mice with and without BMP4 for 24 hours, washed the cells and then plated them in methylcellulose media containing either Epo alone or Epo and BMP4. The cells pre-incubated with BMP4 and plated in Epo alone gave rise to as many stress BFU-E colonies as the cells plated in Epo and BMP4 (Figure 2-4B). These results suggest that a short exposure to BMP4 promotes the differentiation of an immature BMP4 responsive ($BMP4^R$) cell into an Epo responsive stress BFU-E.

f is a gain of function allele of Madh5

Our analysis shows that the *f* mutation maps to the *Madh5* locus, *f/f* mice express misspliced mRNAs, and spleen cells from *f/f* fail to respond to BMP4. Although all of these data are consistent with *f* being a mutation in *Madh5*, we crossed *f* mice with *Madh5*+/- mice to generate *f/Madh5*- mice to test whether *f* was allelic to *Madh5*. Figure 2-5A shows the expansion of Stress BFU-E in *f/Madh5*- and +/*Madh5*- during the recovery from acute anemia. Both genotypes exhibit an altered recovery when compared to control (Compare Figure 2-5A with Figure 2-1D). The peak expansion of stress BFU-E in *f/Madh5*- is delayed until 48 hours, while +/*Madh5*- exhibit an increase in stress BFU-Es at 36 hours, they continue to expand at 48 hours. Analysis of BMP4 mRNA expression in these mice showed that both *f/Madh5*- and +/*Madh5*- expressed BMP4 at all time points during the recovery (Figure 2-5B). These data are similar to what is observed in *f/f* and *f*/+ mice suggesting that a Madh5 dependent signal is required for the regulation of BMP4 in the spleen. These results show that the phenotype of *f/Madh5*- is more severe than *f*/+ and +/*Madh5*-, which demonstrates that *f* is allelic to a targeted mutation in *Madh5*. Classical genetic analysis, however, would predict that if *f* was a

hypomorphic or loss of function mutation in *Madh5* then *f/Madh5*- should have a more severe phenotype than *f/f*. We observed the opposite that the *f/f* phenotype was the most severe. These data suggest that the *f* mutation in *Madh5* is a neomorphic or gain of function mutation in *Madh5*. To test this possibility, we expressed each of the two mis-spliced *Madh5* mRNAs identified in *f/f* mice in a cell line that responds to BMP4 and analyzed whether these mRNAs could affect BMP4 dependent responses. W-20-17 is a mouse osteoblast cell line that differentiates in response to BMP4 (Binnerts, Wen et al. 2004). The mutant transcripts as well as controls were cloned in to the MSCV-neo retroviral vector and W-20-17 cells were infected with recombinant retroviruses. Although the mis-spliced messages lack the initiator ATG of wildtype *Madh5*, both messages contain in-frame ATGs that could be used to initiate the translation of truncated forms of the *Madh5* protein (Figure 6A). Treatment of W-20-17 cells with BMP4 induces the osteoblast differentiation program as measured by an increase in Alkaline Phosphatase (AP) activity. W-20-17 cells that express either *f* mutant message have a profound defect in BMP4 dependent induction of AP activity with mutant message #1 exhibiting the most severe defect (Figure 2-6B). These results show that the mis-spliced *Madh5* mRNAs present in *f/f* mice dominantly suppress BMP4 dependent signals. Furthermore, W-20-17 cells do not express endogenous *Madh5* (Data not shown), but rather rely of *Madh1* and *Madh8* to transmit BMP4 signals, which suggests that the mis-spliced mRNAs also inhibit signaling through Madh1 and Madh8. These results explain why *f/Madh5*- mice have a less severe phenotype than *f/f* because the *f/Madh5*- mice express lower levels of the mis-spliced mRNAs.

Spleen Megakaryocyte-Erythroid progenitors (MEPs) are the BMP4 responsive cells

In order to further characterize the $BMP4^R$ cell, we fractionated spleen cells and assayed for response to BMP4. Initially we sorted cells based on their expression of lineage restricted markers. $BMP4^R$ cells were observed in the Lin- population (Figure 2-7A). Previous work had demonstrated that mice with a mutation in the Kit receptor, *Dominant white spotting (W)* mice, failed to respond rapidly to acute anemia, which suggested that the $BMP4^R$ cell may also be Kit+ (Harrison and Russell 1972). Analysis of Lin- Kit+ spleen cells confirmed this hypothesis as $BMP4^R$ cells were not detected in Lin-Kit- spleen cells (Figure 2-7B). In the bone marrow, erythroid progenitors are derived from the Common Myeloid Progenitor (CMP) and the Megakaryocyte-Erythroid Progenitor (MEP) (Akashi, Traver et al. 2000). CMPs and MEPs are Kit+, but differ in their expression of CD34. We analyzed the expression of CD34 in Lin- Kit+ spleen cells and observed that they were CD34-, suggesting that the spleen does not contain CMPs (Data not shown). Isolation of MEPs from spleen showed that they responded to BMP4 (Figure 2-7C). Interestingly, MEPs from isolated from bone marrow failed to respond to BMP4. These results suggest that the unique microenvironment of the spleen alters the properties of MEPs rendering them responsive to BMP4 and further underscores our assertion that distinct erythroid progenitors are present in the spleen poised to respond to acute erythroid stress.

Discussion

Acute anemia induces a robust erythroid response that occurs in the spleen. Our data demonstrate that in response to PHZ induced anemia, dedicated stress progenitors

resident in the spleen respond to a BMP4 dependent signal, which leads to the rapid expansion of erythroid progenitors. These data differ from the previous model which suggested that high serum Epo levels induced by tissue hypoxia mobilized erythroid progenitors from the bone marrow to spleen where they expanded and differentiated (Hara and Ogawa 1976; Broudy, Lin et al. 1996). This early model was based on the observation that BFU-E were present in the peripheral blood during the recovery from acute anemia. However, we did not observe migration of erythroid progenitors in our studies and all of our data suggests that stress BFU-E are resident in the spleen. One reason for the differences in our data could be that earlier experiments cultured BFU-E for 10 days rather than the 5-7 days used in our studies. The longer culture conditions may have allowed more immature cells to develop. In addition, we used a modified protocol to induce anemia, which utilized a single injection of a high dose of PHZ. This protocol results in a synchronous and reproducible expansion of erythroid progenitors. The earlier experiments used multiple injections of a low dose of PHZ. We have found that multiple low doses of PHZ did not allow us to look at early events during the recovery and did not produce reliable results (L. Lenox and R.F. Paulson unpublished observations 1999).

The earlier model suggested that bone marrow progenitors develop in the spleen during the recovery. Our data however demonstrates that specialized $BMP4^R$ cells are resident in the spleen. BMP4 induces these cells to differentiate leading to the expansion of stress BFU-E in the spleen but not in the bone marrow. Stress BFU-E exhibit properties that are distinct from bone marrow BFU-E in that they form large burst colonies in 5 days rather than 7 days and they require only Epo without any added BPA

for BFU-E formation. Fractionation of spleen cells revealed that $BMP4^R$ cells are contained within the MEP population. However, only spleen MEPs are able to respond to BMP4. This observation suggests that the spleen microenvironment may provide a specialized signal that enables spleen MEPs to respond to BMP4 (Obinata and Yania 1999). This situation would be similar to chondrogenesis, where pre-somitic mesoderm cells require BMP signals to differentiate into chondrocytes, but are unable to respond to BMP unless they first encounter a Sonic hedgehog signal (Murtaugh, Chyung et al. 1999).

The notion that *f/f* mice have a defect in a spleen progenitor was first suggested almost 30 years ago. Gregory et al. showed that *f/f* mice had normal numbers of BFU-E and CFU-E, but were defective in transient endogenous colony forming units (TE-CFU). This population of cells was defined by an *in vivo* assay that identifies progenitor cells that form endogenous spleen colonies following sublethal irradiation and stimulation of erythropoiesis by Epo injection or bleeding (Gregory, McCulloch et al. 1975). $BMP4^R$ cells exhibit many of the properties expected in a putative TE-CFU. They are resident in the spleen, rapidly expand at times of great erythropoietic need and require high levels of Epo for differentiation. In addition to TE-CFU, previous work has identified other stress erythroid progenitors in the spleen. Mice with mutations in the glucocorticoid receptor are slow to respond to acute anemia and it has been suggested that a CD34+ Kit+ TER119+ population of cells fails to expand in these mutant mice (Bauer, Tronche et al. 1999). Another group identified a 4A5+ TER119+ bipotential megakaryocyte-erythroid progenitor that expands in the spleen following PHZ induced anemia (Vannucchi, Paoletti et al. 2000). However, both of these progenitors express the lineage-restricted

marker, TER119, which is not present on $BMP4^R$ cells or BFU-E in general (Kina, Ikuta et al. 2000). The relationship between these progenitors and the BMP4R cells and the stress BFU-E is not understood, but the most likely possibility is that they are derived from $BMP4^R$ cells.

Our analysis of *f/f* mice has identified a mutation in *Madh5*, which causes aberrant splicing. The mis-spliced Madh5 mRNAs exert a dominant negative effect on BMP4 signaling which suggests that *f* represents a gain of function allele of *Madh5*. These mis-spliced mRNAs inhibit BMP4 dependent signals in W-20-17 osteoblast cells, which do not express endogenous Madh5, suggesting that the *f* mutant mRNAs inhibit BMP4 signaling mediated by Madh1 and or Madh8. This possibility is consistent with our observation that *f/f* exhibits a more severe phenotype than *f/Madh5*- mice. In this case, *f/f* mice express higher levels of mis-spliced mRNAs and thus would have a more significant impairment of BMP4 dependent signaling than *f/Madh5*- mice.

Previous work from Fleming et al. suggested that *f* was a mutation in the putative mitochondrial transporter *Sfxn*. We show that the mutation in *Sfxn1*has been separated from the *f* locus by recombination in *f/f* mice in our colony. All of our observations characterizing the BMP4 dependent expansion of stress BFU-E in the spleen during the recovery from acute anemia and the defective response in *f/f* mice support the idea that the *f* locus encodes *Madh5*. In addition, other phenotypes associated with *f/f* mice, tail flexures and white belly spots, can easily be explained by defects in the BMP4 signaling pathway. The tail flexures are caused by defects in chondrogenesis that result in vertebrae fusion (Kamenoff 1935). BMP4 plays a key role in regulating chondrocyte development (Murtaugh, Chyung et al. 1999). Furthermore, white belly spots are caused

by defects in the migration of neural crest derived melanocytes (Christiansen, Coles et al. 2000). Inhibition of BMP4 signaling in chick embryos impairs the ability of neural crest cells to migrate (Sela-Donenfeld and Kalcheim 1999). Thus the combination of the observed defects in BMP4 dependent signals in *f/f* mice with the genetic interactions of *f* and *Madh5*- alleles demonstrates that the *f* locus encodes *Madh5*.

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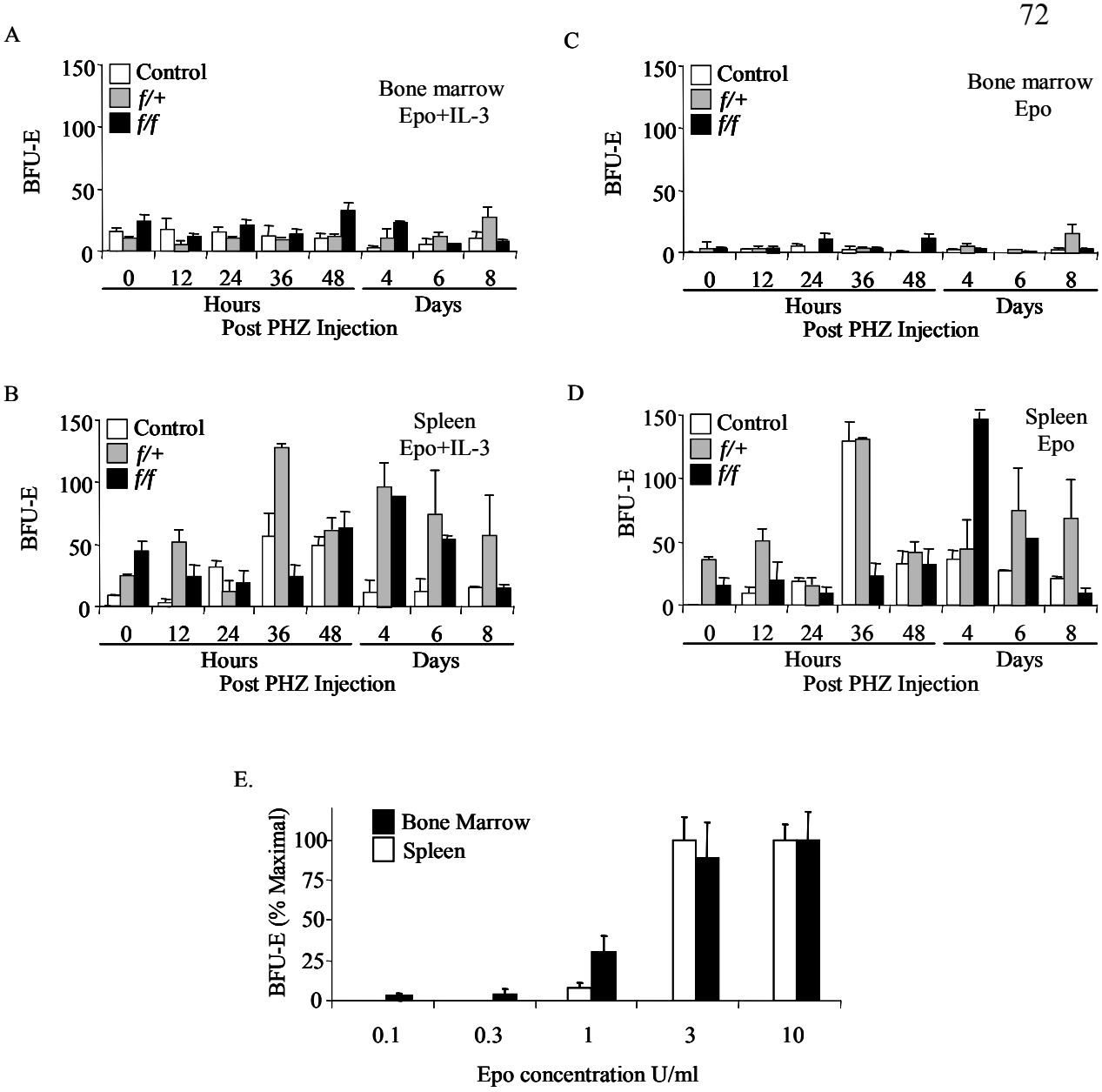


Figure 2-1. Analysis of BFU-E expansion during the recovery from PHZ induced acute hemolytic anemia. (A) Bone marrow and (B) spleen BFU-E from C57BL/6-*f/f*, C57BL/6-*f*/+ and C57BL/6-+/+ control mice during the recovery from PHZ induced acute anemia. Cells were plated in methylcellulose media containing Epo (3U/ml) and IL-3 (10 ng/ml). (C) Bone marrow and (D) spleen BFU-E from C57BL/6-*f/f*, C57BL/6-*f*/+ and C57BL/6-+/+ control mice during the recovery from PHZ induced acute anemia. Cells were plated in methylcellulose media containing only Epo (3U/ml). (E)Sensitivity of bone marrow and spleen BFU-E to Epo. $5x10^5$ Bone marrow or spleen

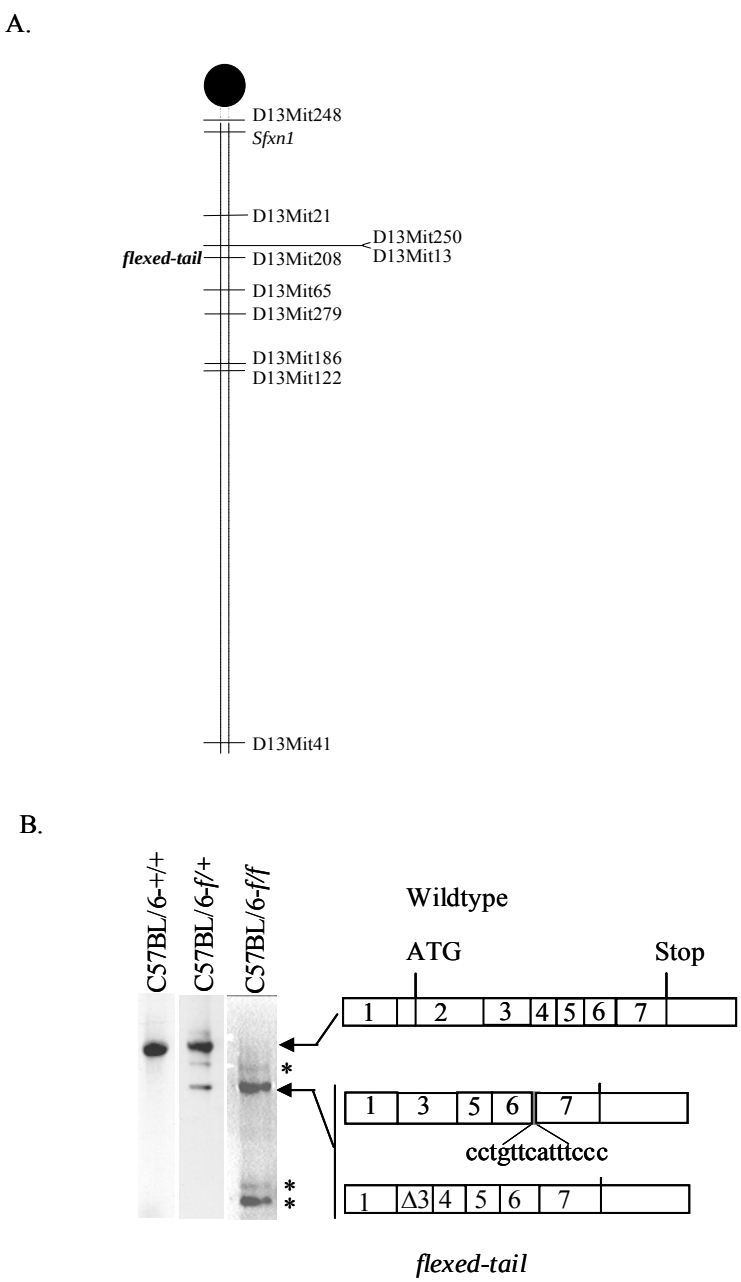


Figure 2-2. Genetic linkage map of the *f* locus and molecular analysis of *Madh5* transcripts in *f/f, f/+* and control mice. (A) Linkage map of the *f* locus on mouse chromosome 13. The position of the markers on chromosome 13 according to NCBI m33 mouse genome assembly as indicated. The number of recombinants in each interval among the scored intercross F2 progeny is indicted at the right. The position of *Madh5* and *Sfxn1* are shown. (B) The coding region of the *Madh5* was cloned by RT-PCR of spleen RNA from the indicated mice. The arrowheads indicate the position of the wildtype and mutant mRNAs. The * indicates a non-specific background band. The exon structure of the wildtype and *f/f* mRNAs is indicated at the right. The *f/f* mouse shown here is an example of a mutant mouse that expresses very little wildtype *Madh5* mRNA.

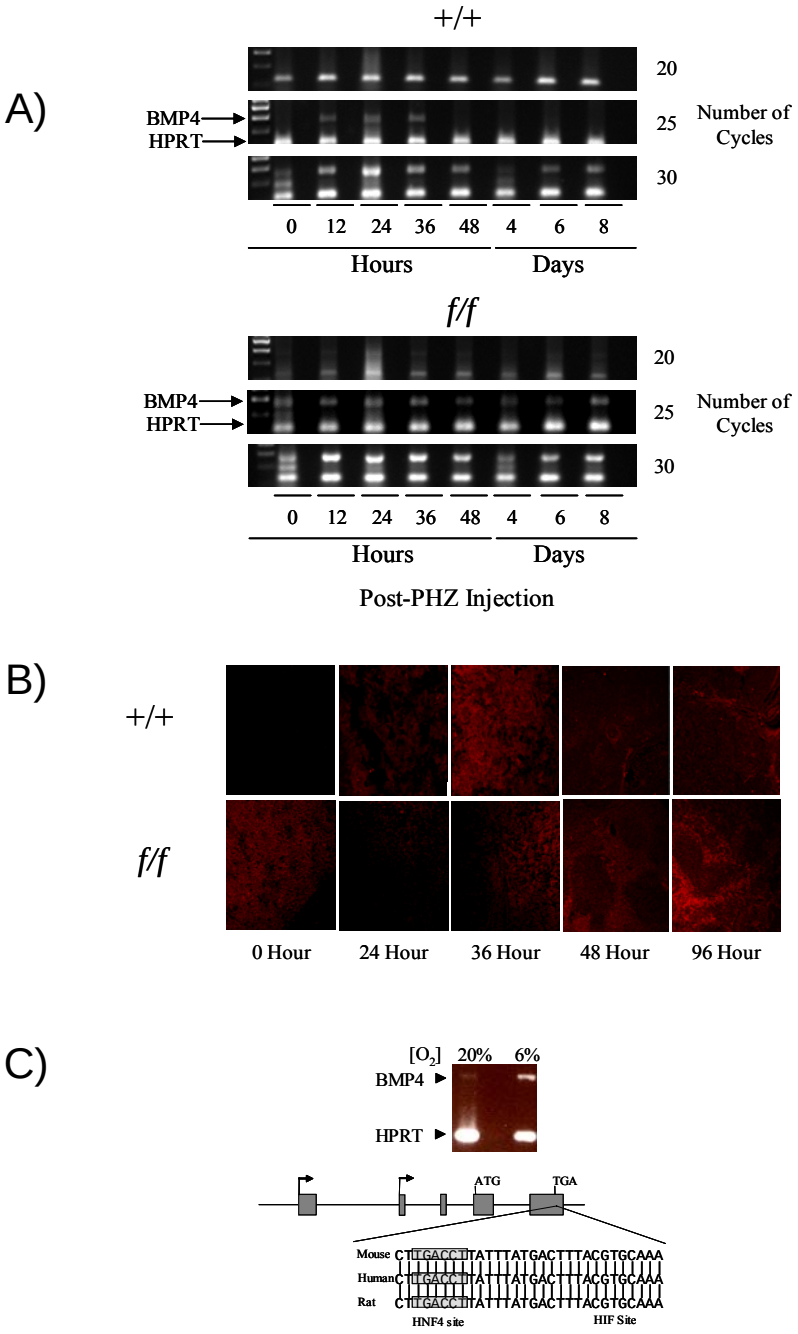


Figure 2-3. Analysis of BMP4 expression during the recovery from acute anemia. (A)RT-PCR analysis of *BMP4* expression in C57BL/6-+/+ control (Top) and C57BL/6-*f/f* (bottom) mice. Arrows indicate the positions of the BMP4 specific band and the HPRT control band and the number of PCR cycles is indicated at the right. (B) Spleen sections from C57BL/6-+/+ (Top) and C57BL/6-*f/f* (bottom) mice stained with anti-BMP4 antibodies at the indicated times following PHZ induced acute anemia. (C) RT-PCR analysis of BMP4 expression in MSS31 spleen stromal cells grown at normoxic (20%) and hypoxic (6%) conditions (Top). The position of the putative hypoxia inducible element in the BMP4 gene is indicated and an alignment of this sequence from mouse, human and rat is presented (Bottom).

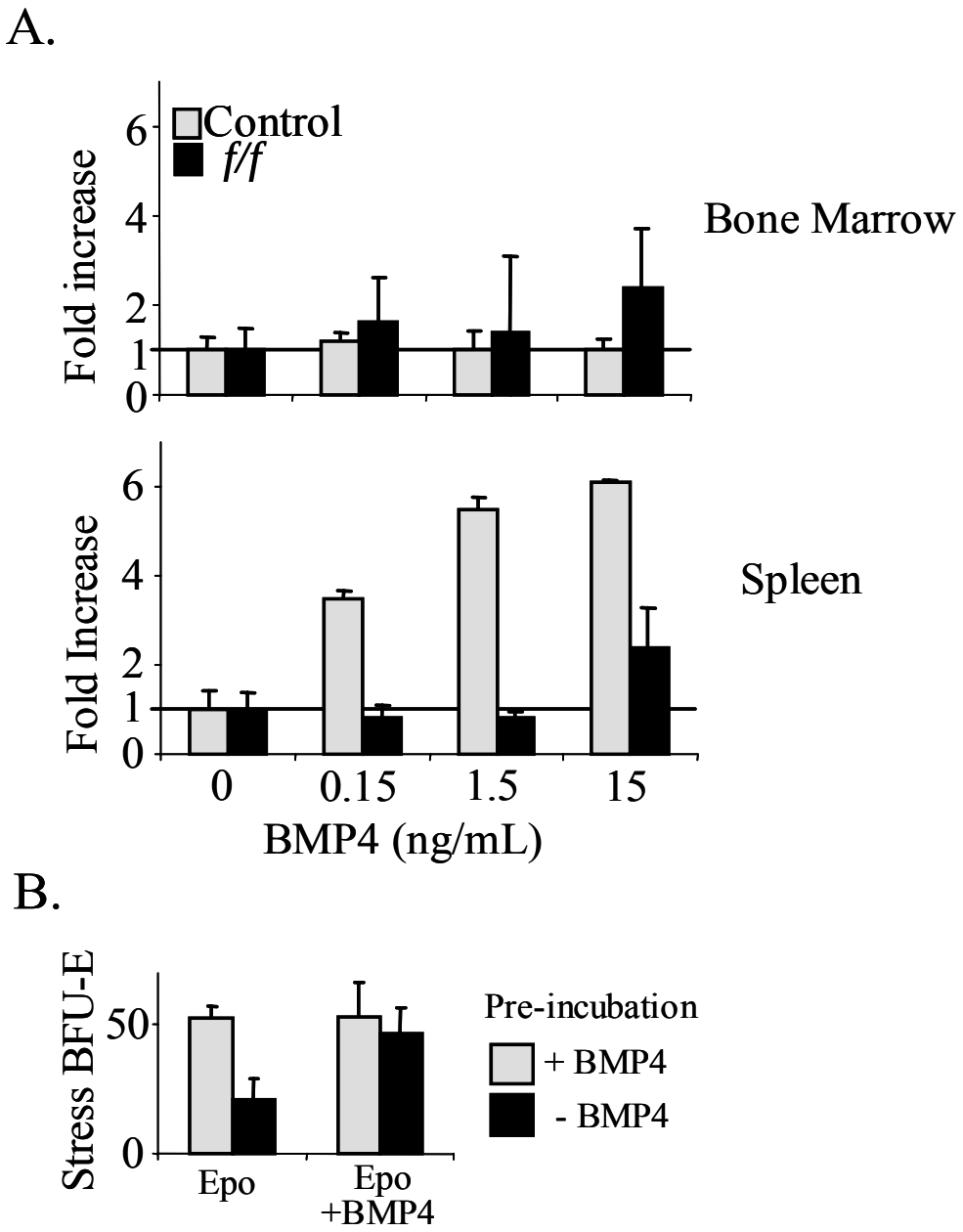


Figure 2-4. Analysis of the ability of BMP4 to induce the formation of stress BFU-E in spleen cells from untreated mice. (A) Bone marrow (Top) and spleen (Bottom) cells from untreated *f/f* and control mice were plated in methylcellulose media containing Epo (3U/ml) and the indicated concentration of BMP4. (B) Spleen cells fromC57BL/6-+/+ mice were preincubated + BMP4 (15ng/ml) for 24 hours, washed and then plated in the methylcellulose media containing either Epo (3U/ml) alone or Epo + BMP4 (15ng/ml).

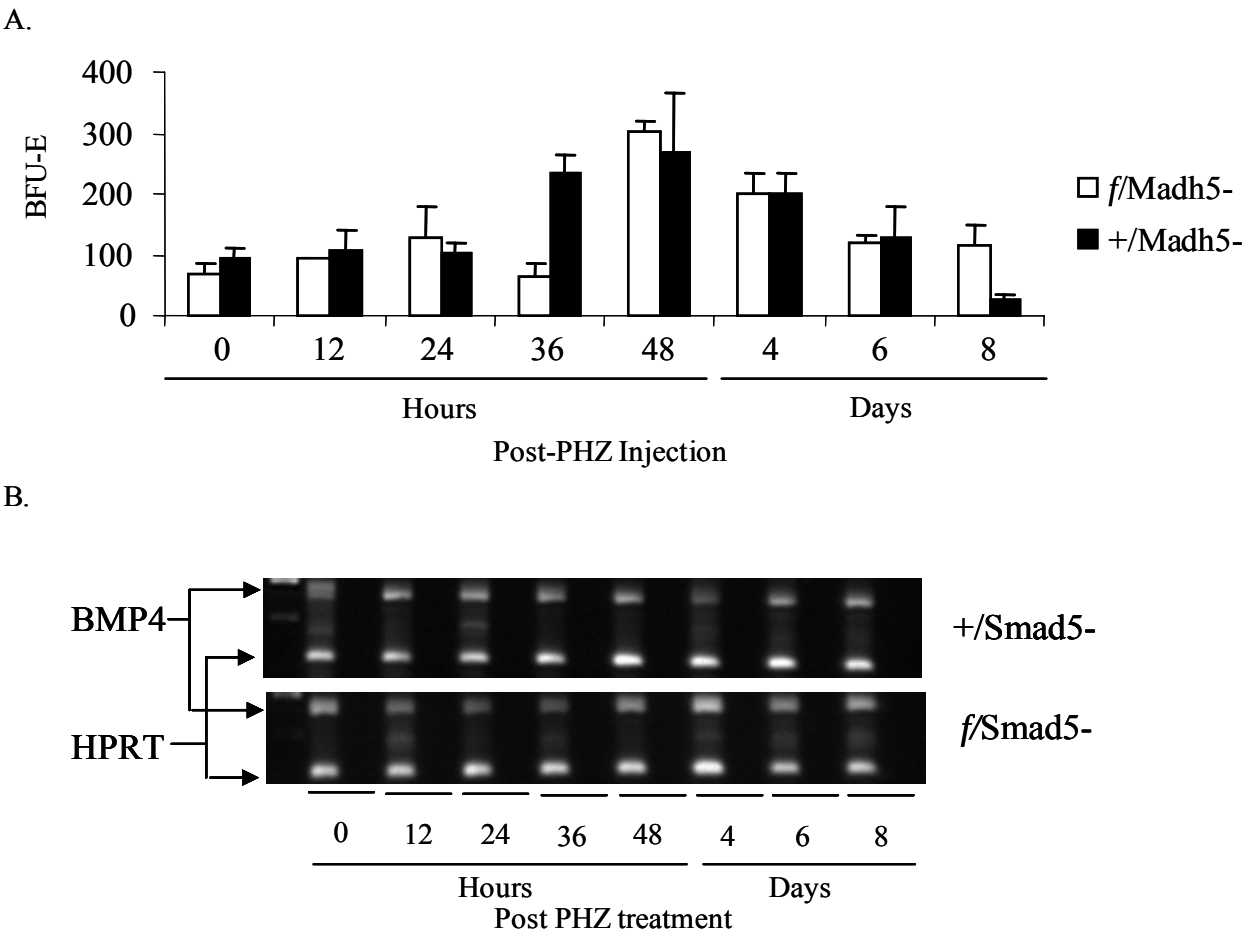


Figure 2-5. Analysis of the recovery from acute anemia in *f/Madh5*- and *+/Madh5*-mice. (A) Analysis of the expansion of stress BFU-E in the spleen of C57BL/6-*f/Madh5*- and C57BL/6-*+/Madh5*- mice. Spleen cells were plated in methylcellulose media containing 3U/ml Epo. (B) Expression of BMP4 in the spleen of C57BL/6-*f/Madh5*- and C57BL/6-*+/Madh5*- mice. The BMP4 and HPRT control bands are indicated by the arrows. These results are from 25 cycles of PCR.

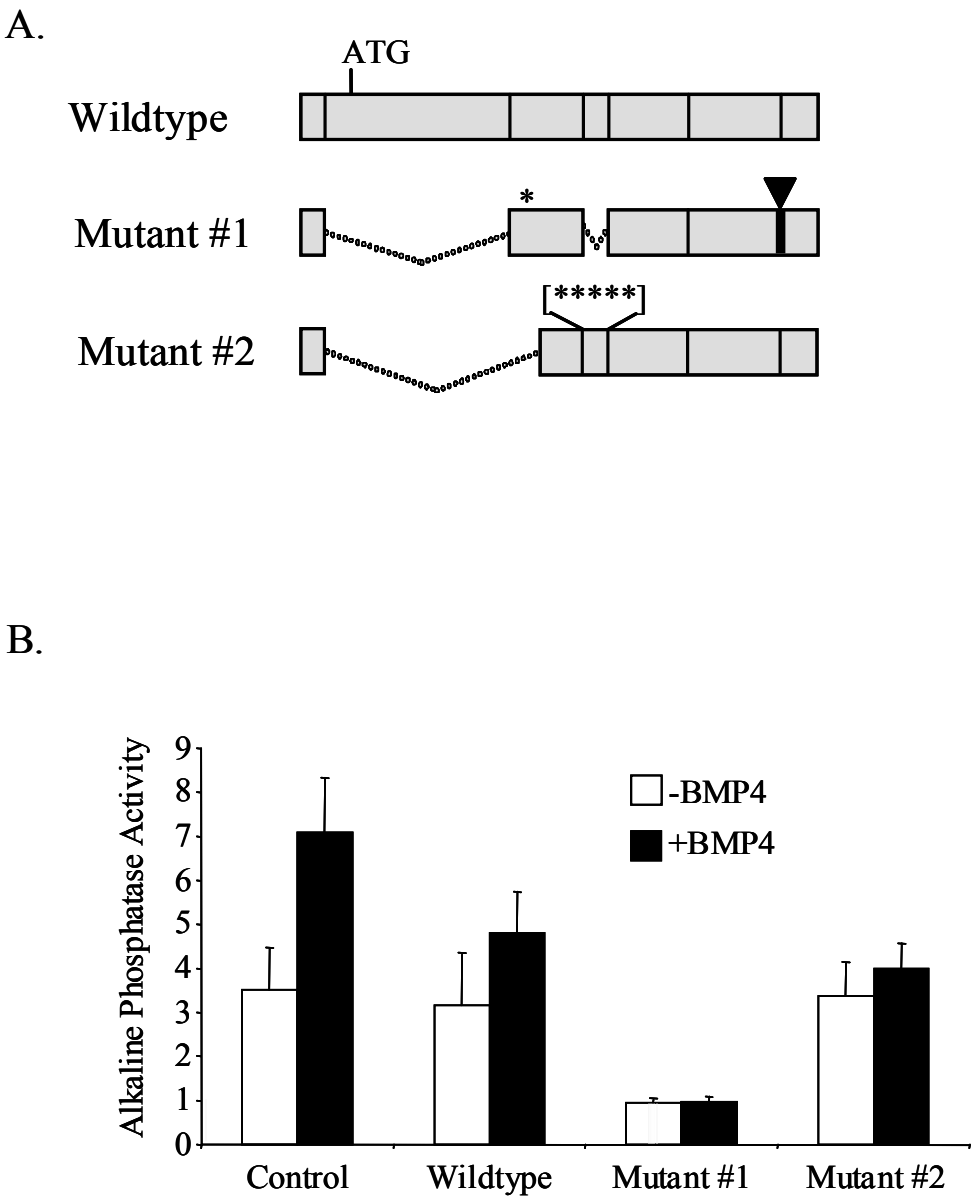


Figure 2-6. Analysis of the effect of over-expression of the *f* mis-spliced *Madh5* mRNAs on BMP4 signaling in W-20-17 osteoblast cells. (A) Schematic representation of the *f* mis-spliced *Madh5* mRNAs and control *Madh5* mRNA. The position of the endogenous ATG is indicated in the wildtype mRNA. * indicate the positions of putative in-frame ATGs in the mis-spliced mRNAs. (B) Induction of alkaline phosphatase activity by BMP4 in control W-20-17 cells and W-20-17 cells expressing wildtype or mis-splice *Madh5* mRNAs. Alkaline phosphatase activity was normalized to protein concentration and expressed in relative units.

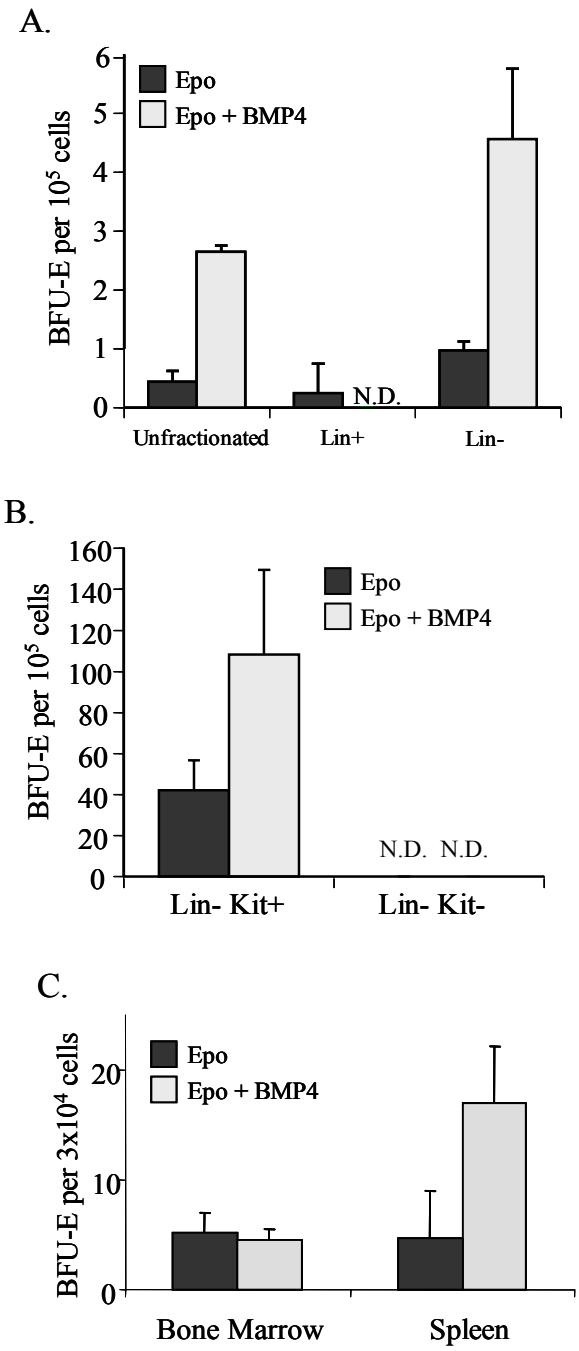
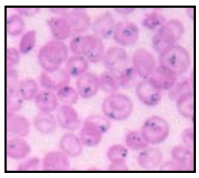


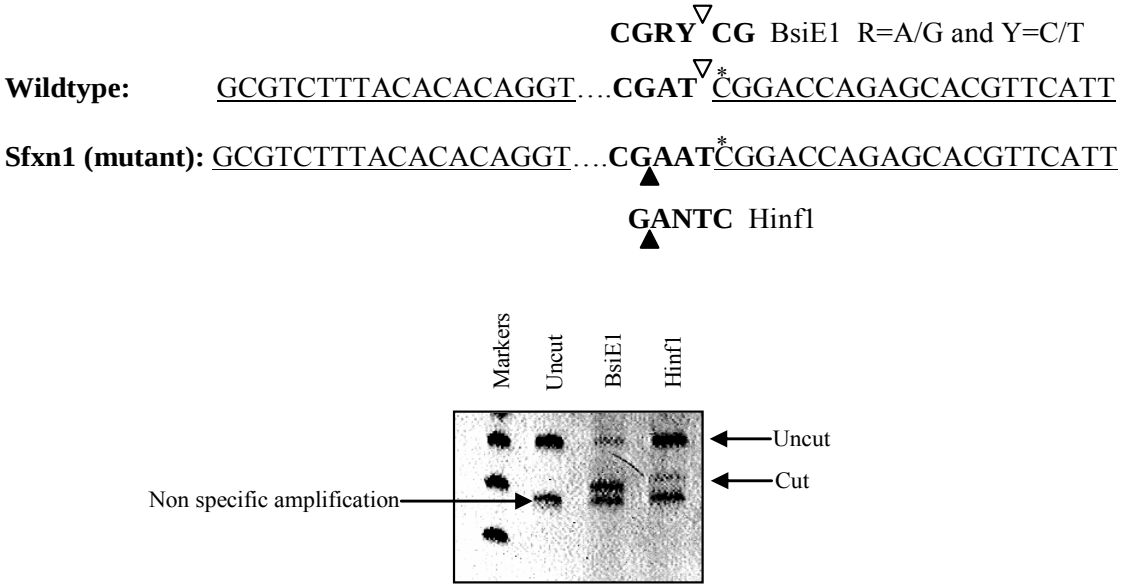
Figure 2-7. Identification of the sub-population of progenitor cells from untreated spleen that responds to BMP4. (A) Unfractionated, Lin+ and Lin – cells from untreated wildtype spleen were plated in 3 U/ML Epo + 15ng/ml BMP4 and the induction of stress BFU-E was analyzed. (B) Kit+ Lin- and Kit- Lin- cells from untreated wildtype spleen were plated in 3 U/ML Epo + 15ng/ml BMP4 and the induction of stress BFU-E was analyzed. (C) MEPs (Lin- Sca1- IL-7Rα- Kit+ CD34- FcγRlow) isolated from bone marrow and spleen of wildtype mice were plated in 3U/ML Epo + 15ng/ml BMP4 and the induction of stress BFU-E was analyzed. N.D.None Detected.

A.



Staining of a blood smear from a newborn *f/f* mouse with Prussian Blue stain to identify iron granule in the fetal erythrocytes. Blue dots in the erythrocytes indicate the presence of siderocytes.

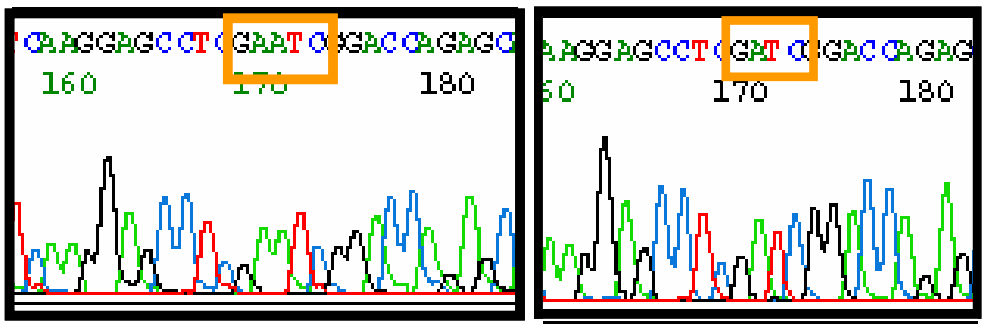
B. Genotyping the Sideroflexin mutation in *f/f* mice from our colony.



Supplementary Figure 1. Genotyping for the *sideroflexin* (*sfxn*) mutation

(Top) Schematic of PCR primers and the diagnostic cut sites used to genotype the *Sfxn1* mutation in *f/f* mice from our colony. The bold sequence is region around the A insertion mutant reported by Fleming et al.. Above and below this region are the sequences of the restriction sites used to differentiate between the alleles. The triangles mark the actual cut site. The * above the C base represents an inserted C nucleotide that generates the diagnostic restriction sites.

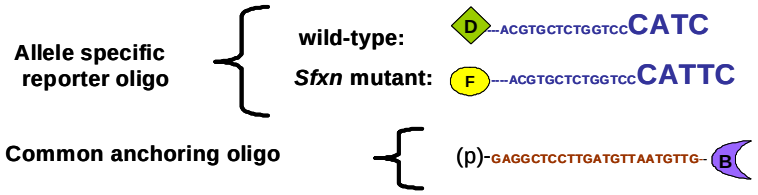
(Bottom) PCR analysis of DNA isolated from the mouse scored as a *f/f* in A. Both HinF1 and BsiE1 cut the PCR product indicating that this animal is heterozygous for the A insertion in *Sfxn1*. The lower band indicated by the arrow is a non-specific amplification.



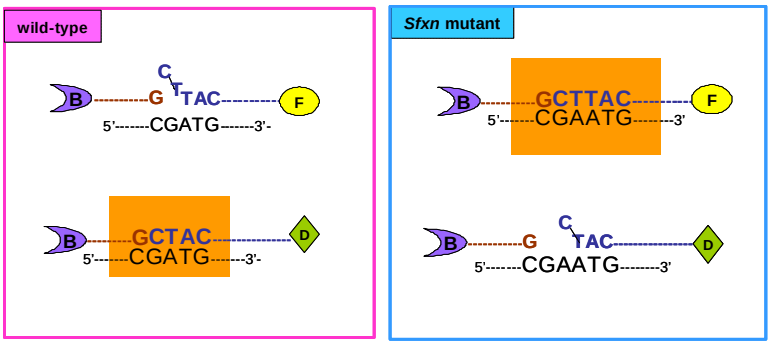
Direct sequencing of the *Sfxn1* exon 2 in *f/f* mice. *Sfxn1* exon 2 from the mouse in B-1(A) was PCR amplified and cloned in pTOPO-10 (Invitrogen). Multiple independent plasmids were sequenced. Two sequences were obtained as marked in the boxes, mutant-GAATC and wildtype-GATC.

Analysis of *Sfxn* mutations in *f/f* mice by Oligonucleotide ligation assay.

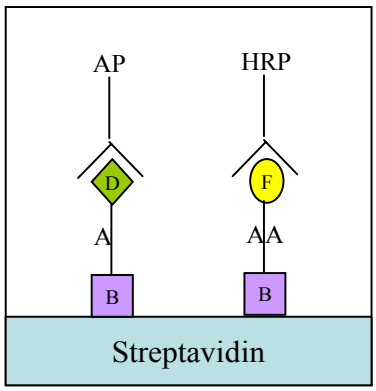
i.



ii.



iii.



Oligonucleotide ligation assay (OLA) was used to confirm all PCR genotypes. (i)The technique utilizes a common anchoring oligo that is phosphorylated at its 5' end and biotinylated at its 3' end. Allele specific oligos that contain either a digoxigenin modification (wildtype) or a fluorescein (mutant) at its 5' end. (ii) Ligation of the anchoring oligo to the allele specific oligo can only occur when the allele specific oligo binds the correct sequence. (iii) Anti-Dig conjugated to alkaline phosphatase and anti-Fitc conjugated to HRP are used to detect the ligated products by ELISA.

We tested all *f/f* mice in our colony by the PCR assay and OLA. Both assays gave identical results. We conclude that in our colony, the mutation in *Sfxn1* reported by Fleming et al. has been separated by recombination from the *f/f* mutant phenotype. We have identified numerous mice that exhibit the *f/f* phenotype, but are heterozygous for the *Sfxn1* mutation. The *f* locus therefore cannot encode *Sfxn1*.

OLA was done as described in Single-well genotyping of diallelic sequence variations by a two-color ELISA-based oligonucleotide ligation assay. VO Tobe, SL Taylor and DA Nickerson. Nucleic Acids research 24:3728-2732, 1996.

Chapter 3

BMP4, SCF, and hypoxia cooperatively regulate stress erythropoiesis

Abstract

Mice defective in the genes encoding stem cell factor (SCF) or its receptor, Kit, exhibit both a chronic anemia and an impaired response to acute anemia. Previously, we reported the discovery of a novel stress BFU-E, which undergoes a BMP4-mediated expansion in the murine spleen in response to acute anemia. Here, we extend our characterization of stress BFU-E by analyzing the role of the SCF/Kit signaling pathway. Although Kit mutant (W/W^V) mice are chronically anemic, they do not constitutively express BMP4; however, they rapidly upregulate BMP4 expression in response to phenylhydrazine (PHZ) induced anemia. Despite rapid induction of BMP4, the expansion of stress BFU-E is significantly delayed. Unlike control mice, W/W^V mice do not maintain a pool of BMP4 responsive cells in the spleen. *In vitro* analysis show that BMP4 and SCF play distinct roles in the response to acute anemia. BMP4 treatment increased the number of stress BFU-E, while SCF affected the size of the stress BFU-E-derived colonies. These results demonstrate that, while BMP4 regulates the differentiation of stress BFU-E, SCF/Kit signaling regulates the expansion of stress BFU-E progeny. Furthermore, we found that hypoxia increases both colony number and size, and hypoxia synergizes with SCF in order to further increase colony number. *In vitro*

treatment of spleen cells with hypoxia, SCF and BMP4 recapitulate the stress BFU-E expansion observed *in vivo* in response to acute anemia.

Introduction

Homeostatic erythropoiesis is dramatically different from the expansive erythropoiesis that occurs in response to acute anemia. While homeostatic erythropoiesis occurs predominantly in the bone marrow, stress erythropoiesis occurs predominantly in the adult spleen (Obinata and Yania 1999; Lenox, Perry et al. 2005). Previous data suggested that BFU-E present in the bone marrow migrated through the peripheral blood and subsequently seeded the spleen, where they expanded in order to respond to anemic stress (Hara and Ogawa 1976; Broudy, Lin et al. 1996). Recently, we demonstrated a new model for the erythroid response to acute anemia, where BMP4-responsive cells, endogenous to the spleen, differentiated in response to hypoxia-induced BMP4 expression. The resultant stress BFU-E represents a novel stress erythroid progenitor, which rapidly forms large colonies in the presence of erythropoietin (Epo) alone and without a requirement for an added burst promoting activator (BPA) such as SCF or IL-3 (Lenox, Perry et al. 2005).

Previously, we showed that *flexed-tail (f)* mutant mice have a mutation in *Madh5* (Lenox, Perry et al. 2005). Madh5 is a downstream transcription factor in the BMP4 signaling pathway. This mutation impairs BMP4 dependent signaling, which results in defective stress erythropoiesis in *f/f* mice. We sought to further our analysis of stress erythropoiesis by examining the stress BFU-E response in *Dominant White Spotting (W)* mutants. *W* mice have a mutation in Kit, the receptor for stem cell factor (SCF). The

allele combination W/W^V represents the most severe combination that survives to adulthood. W/W^V mice are anemic and have a dramatically reduced number of CFU-E but almost normal numbers of BFU-E. W/W^V mice also fail to respond rapidly to acute anemia, suggesting that their defect may be the result of an impaired stress BFU-E response.

We have previously shown that BMP4 responsive cells express Kit on their surface (Lenox, Perry et al. 2005) and now extend our characterization of the response to acute anemia by analyzing the role of SCF/Kit signaling. We show that, similar to *f/f* mice, W/W^V mice exhibit a significant delay in stress BFU-E expansion in response to acute anemic stress implicating Kit and its ligand, SCF, in the BMP4-mediated stress BFU-E expansion. Interestingly, although chronically anemic, W/W^V mice exhibit a normal induction of BMP4 expression in response to acute anemia and do not exhibit constitutive BMP4 expression. Furthermore, we show that SCF increases the size of stress BFU-E, while the presence of BMP4 increases the number of stress BFU-E. These data demonstrate that SCF serves to increase the expansion of stress BFU-E progeny. In addition, we further implicate SCF in expansive erythropoiesis by showing that SCF causes hypersensitivity of BFU-E to erythropoietin (Epo).

The *in vivo* response to acute anemia results in a 45-fold expansion of stress BFU-E number in the spleen; however, addition of BMP4 or BMP4 and SCF result in only a 6-fold expansion in *in vitro* colony assays. These data suggest that additional signals are required to recapitulate the *in vivo* expansion. One potential signal, hypoxia is already implicated in stress BFU-E production. Previously, we showed that hypoxia induces BMP4 expression in a spleen stromal cell line capable of supporting large BFU-E

formation (Lenox, Perry et al. 2005), but hypoxia's role goes beyond BMP4 induction. We found that hypoxia has a dramatic effect on both colony number and size. *In vitro*, addition of BMP4, SCF, and hypoxia together result in an expansion of BFU-E number similar to that observed *in vivo* in response to acute anemia. These data show that BMP4 and SCF play differential roles in the stress BFU-E response to acute anemia where BMP4 and hypoxia serve to expand the number of stress BFU-E in the spleen while SCF and hypoxia serve to increase both the number of stress BFU-E and the size of the stress BFU-E colonies.

Study Design

Mice

WBB6F1/J and WBB6F1/J-W/W^V as well as C57BL/6 and C57BL/6-*f* mice were obtained from Jackson Laboratory. All mice were approximately 6-8 weeks old, controls were age matched. Acute anemia was induced by injection of Phenylhydrazine (Sigma, St. Louis, MO) at a concentration of 120 mg/Kg mouse in PBS. All experiments were approved by the IACUC at the Pennsylvania State University.

Colony Assays for BFU-E

Splenocytes and bone marrow cells were isolated from control, *W/+*, and W/W^V mice. $1x10^5$/ml nucleated bone marrow and $2x10^6$/ml nucleated splenocytes were plated in methylcellulose media (StemCell Technologies, Vancouver, BC) containing 3U/ml Epo. BFU-E were scored as described (Finkelstein, Ney et al. 2002) after 5 days incubation. For SCF and BMP4 preincubation experiments, splenocytes were harvested from

C57BL/6 mice and incubated in IMDM + 5% FCS with and without 50 ng/ml SCF (US Biological) and/or 15 ng/ml BMP4 (R&D Systems) for 24 hours. Cells were then washed twice, resuspended in IMDM + 5% FCS, and plated in Methocult (M3334, Stem Cell Technologies) with and without 50 ng/ml SCF and/or 15 ng/ml BMP4. BFU-E were scored as above.

Hypoxia Experiments

Splenocytes were cultured in IMDM + 5% FCS with the indicated factors overnight in normoxia and hypoxia (2% oxygen) in a modular incubator chamber (Billups-Rothenberg). Cells were washed twice and colony assays were performed as described above. Colony diameters were determined with an ocular micrometer with the greatest width taken as the diameter. Only relatively uniform colonies were measured for size.

Analysis of BMP4 Expression

Total RNA was isolated from splenocytes, homogenized in TRIzol (Invitrogen, Carlsbad, CA), and reverse transcribed into cDNA. PCR was performed using the primers 5'-CCTGGTAACCGAATGCTGAT-3' and 5'-TTTATACGGTGGAAGCCCTG-3'.

Results

W/W^V mice exhibit delayed stress BFU-E expansion and lack BMP4 responsive cells

To determine the role of the SCF/Kit signaling pathway in the stress BFU-E expansion in response to acute anemia, colony assays were performed on bone marrow and splenocytes isolated from control, $W^V/+$ and W/W^V mice at various times during the recovery from PHZ-induce acute anemia. As shown in figure 3-1A, while control mice exhibit an expansion of stress BFU-E at 36 hours post-PHZ injection as previously

reported (Lenox, Perry et al. 2005), *W/W^V* mice do not expand stress BFU-E until day 6 post-PHZ injection. This delay in *W/W^V* mice is more severe than that observed in *f/f* mice (6 days vs. 4 days post-PHZ injection for *W/W^V* and *f/f*, respectively). *W^V*/+ mice exhibit a modest expansion beginning at 24 hours that continues throughout the recovery time. In contrast to the spleen, the bone marrow does not exhibit any significant expansion in BFU-E in response to PHZ. Because BMP4 treatment causes stress BFU-E number to increase in uninjected control mice but not *f/f* mice (Lenox, Perry et al. 2005), we next determined the effect of BMP4 on BFU-E from *W/W^V* and *W^V*/+ mice. As shown in figure 3-1B, BMP4 induces the expansion of stress BFU-E from control spleen cells; however, *W/W^V* spleens do not respond to BMP4 suggesting that spleens lack BMP4 responsive cells. Similar to the expansion *in vivo*, the response of *W^V*/+ cells is reduced about 4 fold relative to control mice.

While BMP4 is not expressed under homeostatic conditions, it is dramatically upregulated beginning at 12 hours post-PHZ injection with peak expression occurring at 24 hours. We determined whether a delay in BMP4 expression in response to acute anemia could explain the delayed expansion of stress BFU-E in *W/W^V* and control mice. As shown in figure 3-1C, *W/W^V* mice exhibit an induction of BMP4 expression which is similar to wild-type mice. Taken together, these data show that despite the fact that BMP4 is efficiently expressed in *W/W^V* mice, stress BFU-E exhibit a delayed expansion.

SCF increases the progeny of stress BFU-E

Because BMP4 and SCF/Kit signaling both play a role in stress BFU-E expansion, we sought to clarify the specific role of each. Previously, we showed that

BMP4 is transiently required to induce stress BFU-E differentiation. We now extend this analysis to determine requirements and roles of SCF/Kit signaling in stress BFU-E. We performed colony assays after a preincubation step where splenocyte cell suspensions were incubated in liquid media alone or in media containing SCF, BMP4 or SCF + BMP4. Preincubated cells were thoroughly washed and then plated in methylcellulose containing Epo, Epo + SCF, Epo + BMP4, or Epo + SCF + BMP4 as shown in figure 3-2A. Similar to our previous data, preincubation and/or growth in the presence of BMP4 result in an increase in the number of stress BFU-E. Thus, BMP4 appears to act early by differentiating BMP4 responsive cells into stress BFU-E. In contrast, preincubation and/or growth in SCF without BMP4 did not significantly increase the number of stress BFU-E. However, it was observed that stress BFU-E grown in SCF were larger than those grown without SCF. Consequently, stress BFU-E diameters were measured for all conditions. Interestingly, stress BFU-E are significantly larger only when grown in the presence of SCF and this is independent of BMP4 (figure 3-2B). Notably, preincubation in SCF alone is not sufficient to increase colony size likewise suggesting that SCF acts later in stress BFU-E development. Thus, while BMP4 acts early by differentiating BMP4 responsive cells into stress BFU-E, SCF acts later in stress BFU-E development by expanding stress BFU-E progeny.

SCF increases stress BFU-E sensitivity to Epo

In our previous work, we showed that stress BFU-E are less sensitive to Epo than 'classical' BFU-E. However, SCF is known to synergize with Epo in BFU-E assays. We next tested whether stress BFU-E sensitivity to Epo is affected by SCF/Kit signaling. We

plated splenocytes from wild-type mice in various low to high concentrations of Epo. As shown in figure 3-3, stress BFU-E do not form under low Epo concentrations in the absence of SCF. However, in the presence of SCF, stress BFU-E form even at low Epo concentrations. Thus, in addition to its role in expanding stress BFU-E progeny, SCF treatment increases the sensitivity of stress BFU-E to Epo.

Hypoxia increases number of BMP4 responsive cells and stress BFU-E

While BMP4 significantly expands stress BFU-E number, our experiments with BMP4 and SCF only result in a fraction of the expansion of stress BFU-E observed *in vivo* in response to acute anemia (figure 3-5). Consequently, we sought additional signals which might further serve to expand stress BFU-E *in vitro*. Acute anemia induces tissue hypoxia, so hypoxia represents a potential candidate signal. We tested this possibility by plating spleen cells in methylcellulose containing Epo with different combinations of SCF and BMP4 that were grown at normal (20%) or hypoxic (2%) oxygen concentrations. As shown in figure 3-4A, hypoxia results in a dramatic increase in stress BFU-E formation. At 20% O_2, BMP4 + SCF result in only a 6 fold expansion of stress BFU-E; however, at 2% O_2, a 41 fold expansion is observed. Thus, these three signals, hypoxia, BMP4 and SCF are necessary and sufficient for stress BFU-E expansion. However, addition of BMP4 in hypoxia does not induce any further BFU-E formation. While BMP4 increases the number of stress BFU-E at 20% O_2, hypoxia + SCF exhibits the same expansion as all three signals combined. This puzzling result can be explained by our previous results showing that the spleen stromal cell line, MSS31, which supports large BFU-E formation, expresses BMP4 in response to hypoxia (Yanai, Matsuya et al.

1989; Yanai, Satoh et al. 1991; Lenox, Perry et al. 2005). Consequently, the ability of BMP4 to increase the number of stress BFU-E in hypoxia may be due to an endogenous source of BMP4. In order to test this, we incubated primary splenocytes in both normoxia and hypoxia overnight and then observed BMP4 expression in each. As shown in figure 3-4C, hypoxia induces BMP4 expression in primary splenocytes demonstrating that BMP4's inaction in hypoxia is likely due to an endogenous source of BMP4. Notably, hypoxia's effect of inducing stress BFU-E formation is greater than that of BMP4 alone (6 vs. 22 fold). Thus, hypoxia's effect goes beyond BMP4 induction, although this accounts for a significant portion of hypoxia's effect. Furthermore, in hypoxic conditions, SCF is shown to further increase BFU-E number (figure 3-4A), which suggests that hypoxia changes the response of cells to SCF. Ultimately, as shown in figure 3-5, the maximal stress BFU-E response to acute anemia can be recapitulated *in vitro* with the combined effects of BMP4, SCF, and hypoxia.

We also tested hypoxia's effect on colony size. As shown in figure 3-4B, hypoxia resulted in a significant increase in colony size under all conditions tested ($p<0.01$). The magnitude of this increase was approximately 2.5 fold for all conditions. Thus, in addition to increasing BFU-E number, hypoxia also increases the size of BFU-E.

While the spleens of untreated control mice normally lack BMP4 expression and stress BFU-E but have BMP4 responsive cells, untreated *f/f* mice exhibit constitutive BMP4 expression, elevated numbers of stress BFU-E but lack BMP4 responsive cells (Lenox, Perry et al. 2005). Although it is not known how Madh5 regulates BMP4, we previously hypothesized that a Madh5-dependent signal may inhibit expression of BMP4 in the spleens of untreated control mice. In *f/f* mice, Madh5 signaling is deficient leading

to constitutive BMP4 expression which results in the lack of BMP4 responsive cells and the constitutive increase in stress BFU-E. In order to determine the effect of deficient Madh5 signaling on stress BFU-E, we cultured splenocytes from *f/f* mutants under both hypoxic and normoxic conditions with Epo and different combinations of SCF and BMP4. *f/f* mutants normally exhibit elevated stress BFU-E numbers, and hypoxia did not stimulate any further expansion of stress BFU-E (figure 3-4D). In contrast, stress BFU-E size is increased by hypoxia as well as SCF to the same degree as wild-type (figure 3-4E). Thus, the increase in size of stress BFU-E by hypoxia and SCF are unaffected by defective BMP4-dependent signaling. These data demonstrate that the effects of hypoxia and SCF/Kit signaling are separable from the effects of BMP4 on stress BFU-E development.

Discussion

We have shown that W/W^V mice exhibit a delay in stress BFU-E expansion in response to acute anemia. This delay occurs despite a normal induction of BMP4. Unlike control mice, W/W^V mice do not maintain a pool of BMP4 responsive cells in their spleens. These results are corroborated by analysis of $W^V/+$ spleens, which maintain a decreased number of BMP4 responsive cells relative to controls (figure 3-1). The lack of BMP4 responsive cells in W/W^V mice may be a consequence of W/W^V's chronic anemia. That is, BMP4 responsive cells may be continually depleted by conversion into stress BFU-E in an attempt to alleviate W/W^V's chronic anemia. Alternatively, BMP4 responsive cells may fail to expand. Such a failure of expansion may be similar to observations concerning CFU-S in W/W^V mice. CFU-S form macroscopic colonies on

the spleens of lethally irradiated mice transplanted with low concentrations of bone marrow cells (Till and McCulloch 1961); however, CFU-S derived from W/W^V mice are much smaller than controls and require microscopic analysis, which suggests that SCF/Kit signaling is required for the expansion of CFU-S progeny cells (Lewis, O'Grady et al. 1967; Barker and Starr 1991). The delay in stress BFU-E expansion in response to acute anemia may be explained by a deficiency in SCF/Kit signaling considering our data demonstrating that SCF acts synergistically with hypoxia to expand stress BFU-E. Regardless of the specific cause, we have demonstrated that SCF/Kit signaling plays a key role in stress BFU-E development where SCF acts later in stress BFU-E development by increasing the size of stress BFU-E (figure 3-2).

This role for SCF in increasing stress BFU-E colony size implicates SCF in stress BFU-E development but did not answer the question of what additional signals serve to expand the number of stress BFU-E in the spleen. In response to acute anemia, a maximal 45 fold expansion of stress BFU-E is observed *in vivo* whereas only about a 6 fold maximal increase is gained by treatment of splenocytes from uninjected mice with BMP4. By growing stress BFU-E under hypoxic conditions, we observed a much greater expansion compared with those grown in normoxia even with BMP4. Furthermore, while our normoxic experiments involving SCF showed only a small effect on stress BFU-E number, SCF and hypoxia together yield a synergistic effect. Thus, when the hypoxic signal is present, SCF also acts to increase stress BFU-E number (figure 3-4). In addition, we have shown that SCF increases the sensitivity of stress BFU-E to Epo (figure 3-3). Together, we have revealed three functions of SCF in stress BFU-E

development. SCF increases stress BFU-E number in the presence of hypoxia, the size of stress BFU-E, and the sensitivity of stress BFU-E to Epo.

Of the three signals studied, hypoxia, BMP4 and SCF, hypoxia is the most potent. No doubt part of hypoxia's potency lays in the regulation of additional signals affecting stress BFU-E development. Indeed, we show this to be the case for BMP4 given that hypoxia induces BMP4 expression in spleen cells and addition of BMP4 has no effect in hypoxia due to the apparent saturating effect of endogenous BMP4. Thus, part of hypoxia's influence lies in BMP4 induction; however, its influence runs deeper as shown by the fact that hypoxia + BMP4 yields a 22 fold increase in stress BFU-E compared to BMP4's 6 fold increase. Future studies will seek to determine the mechanism of hypoxia's additional effects. One possibility is that both hypoxia and SCF signals converge on the transcription factor ATF4, which serves as a key regulator of cellular stress responses (Kim and Sinha 2003). This possibility is explored in chapter 5.

While W/W^V mice exhibit defective homeostatic erythropoiesis, their defect is particularly acute during expansive erythropoiesis (Iscove 1978). It has been proposed that SCF/Kit interaction is necessary for the homing of erythroid progenitors which migrate from the bone marrow to the spleen in response to acute anemia (Broudy, Lin et al. 1996). In this model, W/W^V mice are delayed in their recovery due to insufficient homing ability. However, stress BFU-E expand prior to any migratory events (Lenox, Perry et al. 2005). Thus, W/W^V's deficient response to acute anemia is not due to insufficient homing of progenitors to the spleen but to the delay in stress BFU-E expansion (figure 3-1). There are many possible explanations for this delay; however,

considering the several functions of SCF in stress BFU-E development described here, it is likely that a convergence of these factors contribute to the delay.

The fact that SCF makes stress BFU-E hypersensitive to Epo may be particularly important to stress BFU-E development given that serum Epo concentrations are only briefly increased following anemic stress (Brannan 1997). This increase was thought to mobilize erythroid progenitors from the bone marrow to migrate to the spleen (Hara and Ogawa 1976; Broudy, Lin et al. 1996); however, we have shown that stress BFU-E resident in the spleen form the initial response to acute anemia. Thus, by increasing sensitivity to Epo, SCF allows for the development of stress BFU-E even at relatively low levels of Epo. Our stress BFU-E assays typically use a saturating concentration of Epo; however, *in vivo*, SCF likely plays a key role when Epo concentrations are otherwise limiting.

We have demonstrated that three signals, hypoxia, BMP4 and SCF, regulate stress BFU-E development. While BMP4 increases the number of stress BFU-E, hypoxia increases both the number and size of stress BFU-E derived colonies. Hypoxia acts by inducing BMP4, but its action goes beyond this. Future studies will seek to specifically determine these additional actions. In particular, we have identified three roles for SCF in stress BFU-E development. By expanding stress BFU-E in the presence of hypoxia, increasing the sensitivity of stress BFU-E to Epo, and increasing the size of stress BFU-E derived colonies, SCF/Kit signaling plays a key role in stress erythropoiesis.

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A

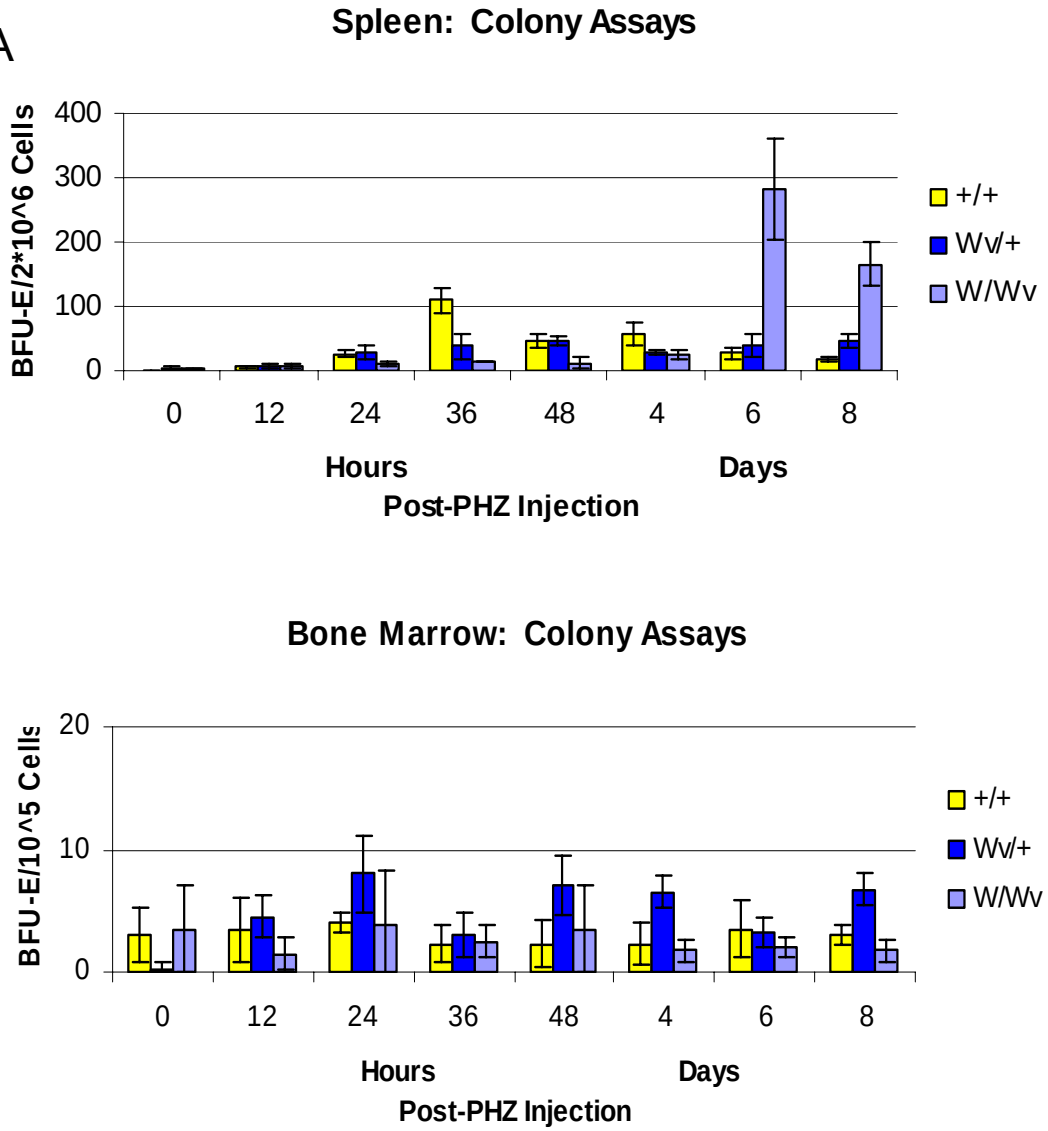


Figure 3-1A. Analysis of stress BFU-E expansion in response to PHZ-induced acute anemia A) Colony assays were performed on spleens and bone marrow from control, WV/+ and W/WV mice during the response to PHZ-induced acute anemia at the indicated time points. The indicated number of splenocytes and bone marrow cells were plated in methylcellulose media containing 3U/ml Epo and BFU-Es were scored after 5 days incubation by positive benezidine staining.

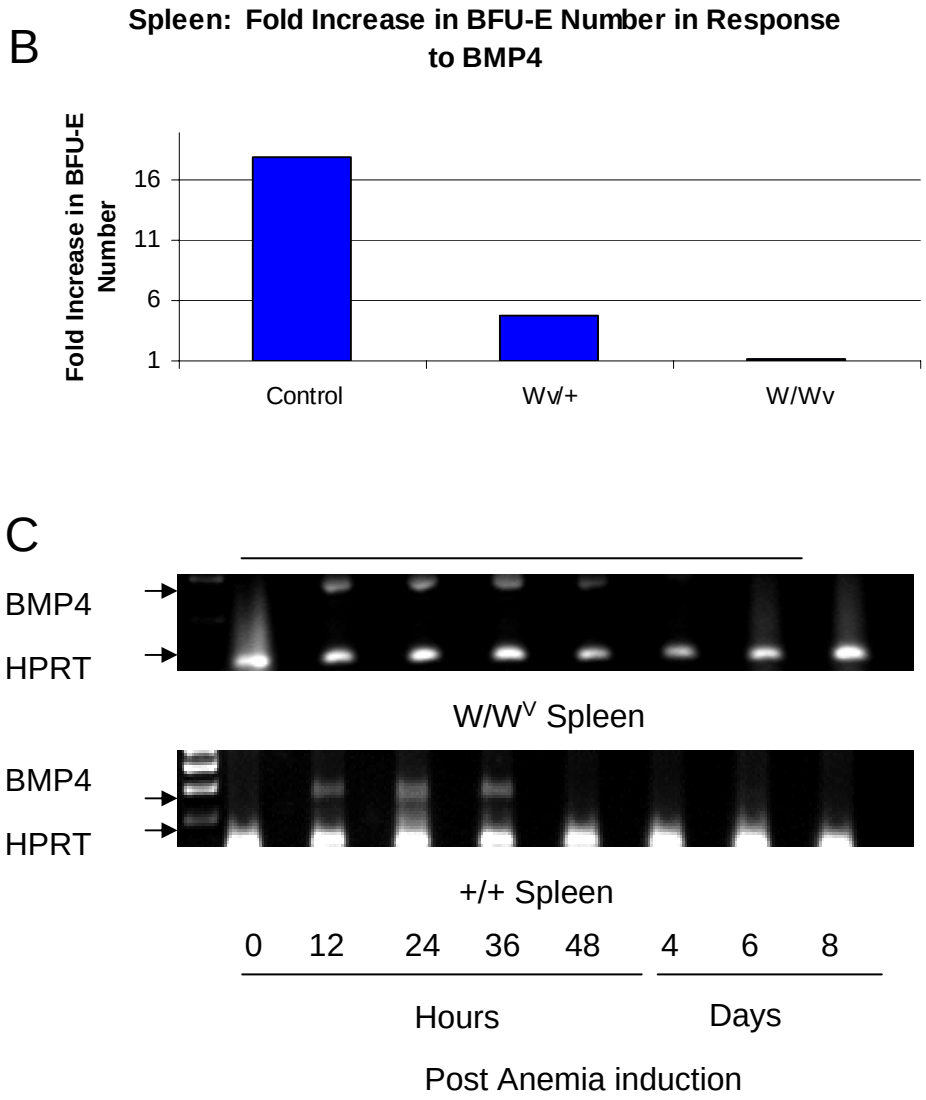


Figure 3-1B and C. Assay for BMP4 responsive cells and analysis of BMP4 expression during response to acute anemia. B) Colony assays were performed as described in figure 3-1A. Splenocytes from uninjected control, Wv/+ and W/Wv mice were plated in both Epo as well as Epo + 15 ng/ml BMP4. C) RT-PCR analysis of *BMP4* expression in W/Wv (Top) and control (bottom) spleens post-anemia induction as indicated. Arrows indicate the positions of the BMP4 specific band and the HPRT control band.

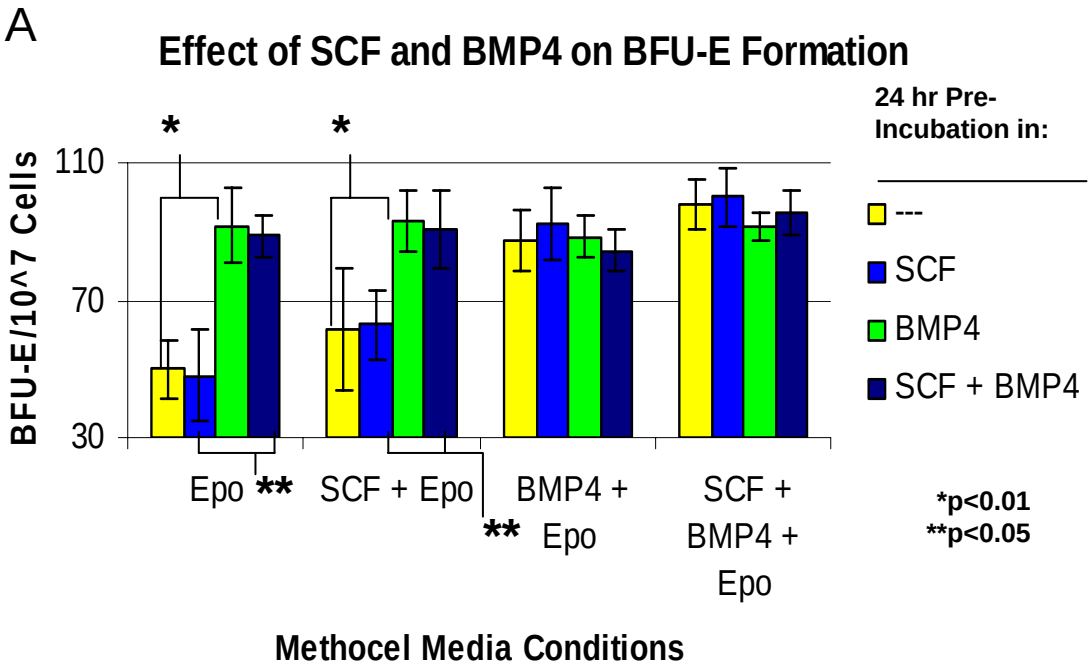


Figure 3-2A. Analysis of the effect of initial exposure to versus growth in SCF and/or BMP4 on stress BFU-E formation. Splenocytes from C57Bl/6 control mice were preincubated for 24 hours in media alone or media supplemented with 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated (legend) and then plated in methylcellulose containing 3U/ml Epo as well as Epo + 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated (X-axis). Colonies were scored as in Figure 1.

B

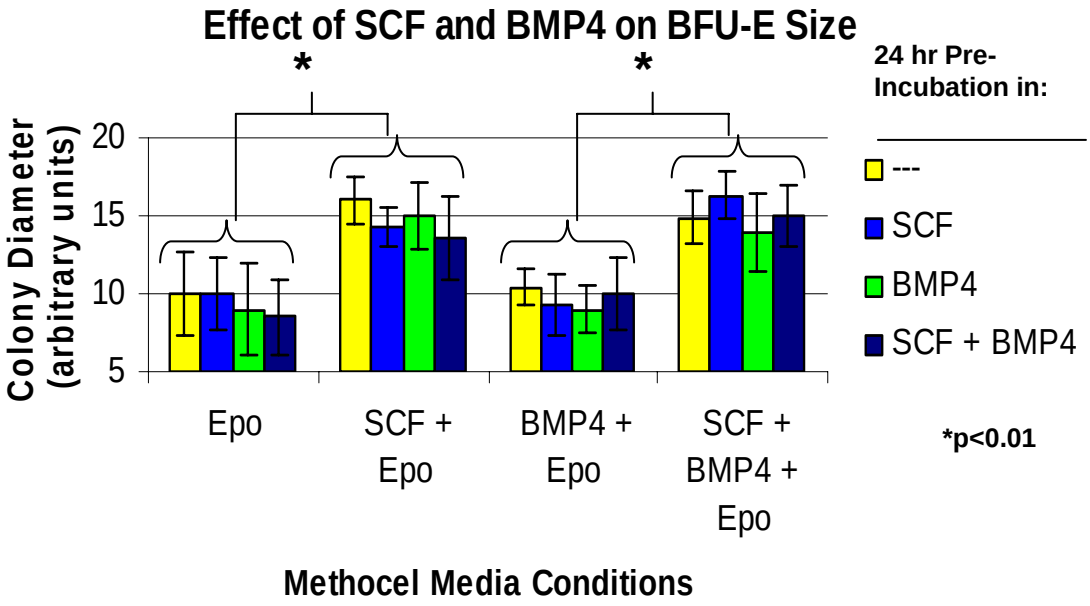
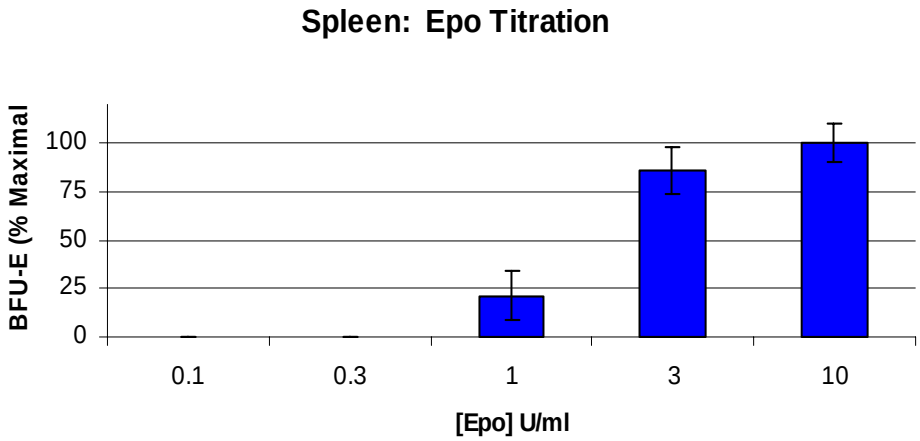


Figure 3-2B. Analysis of the effect of initial exposure to versus growth in SCF and/or BMP4 on stress BFU-E size. Splenocytes from C57Bl/6 control mice were preincubated for 24 hours in media alone or media supplemented with 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated (legend) and then plated in methylcellulose containing 3U/ml Epo as well as Epo + 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated (X-axis). Colony size was determine by measurement with an ocular micrometer.

A



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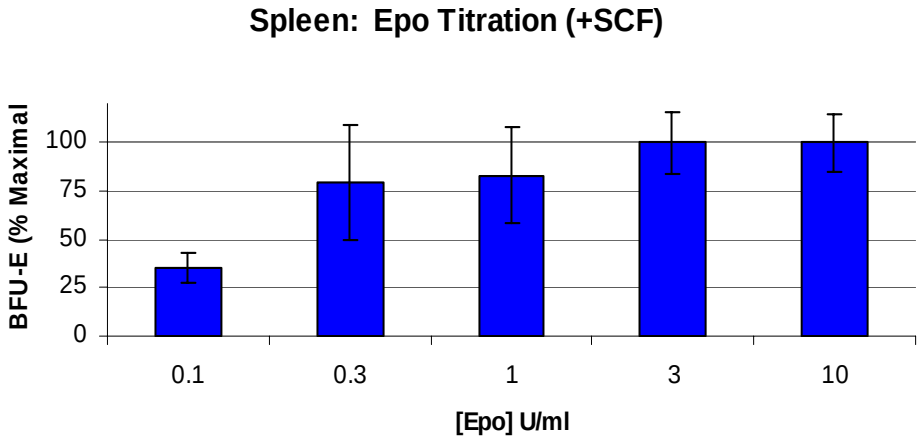
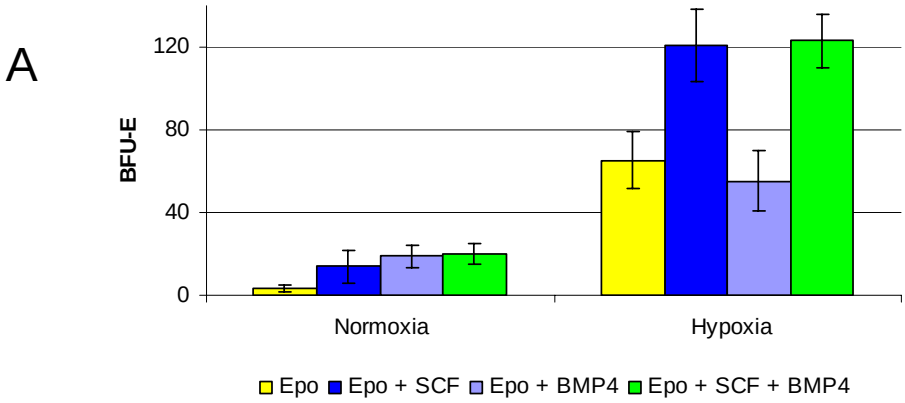
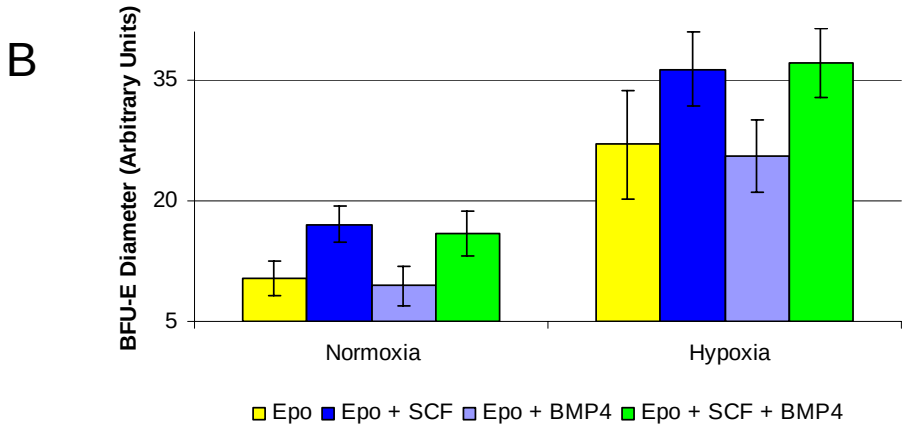


Figure 3-3. Analysis of the effect of SCF on stress BFU-E formation under various concentrations of Epo. Colony assays were performed as in figure 3-1 on splenocytes from C57Bl/6 control mice. Stress BFU-E formation was assayed for under the indicated concentrations of Epo (A) as well as Epo + 50 ng/ml SCF (B).





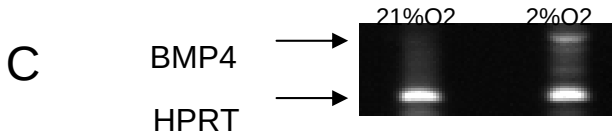
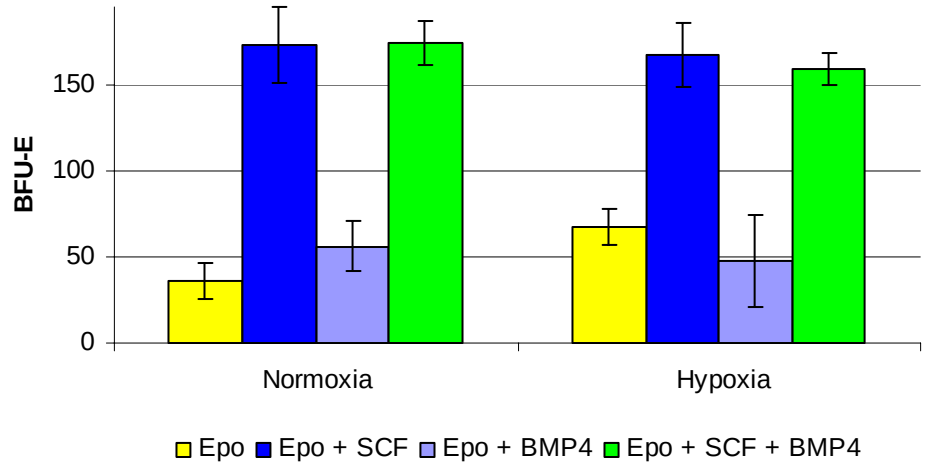


Figure 3-4A-C. Analysis of hypoxia's effect on stress BFU-E formation. Colony assays on splenocytes obtained from C57BL/6 control mice were performed as described in figure 3-1 in both normoxia (20% O2) and hypoxia (2% O2). Cultures contained 3U/ml Epo as well as Epo + 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated. Colony number was determined (A) as in figure 3-1 and colony size was determined (B) as in figure 3-2B. C) RT-PCR for *BMP4* expression was performed on primary splenocytes from C57BL/6 control mice cultured overnight in IMDM + 5% FCS in both normoxia and hypoxia. Arrows indicate the BMP4 and control HPRT specific bands.

D



E

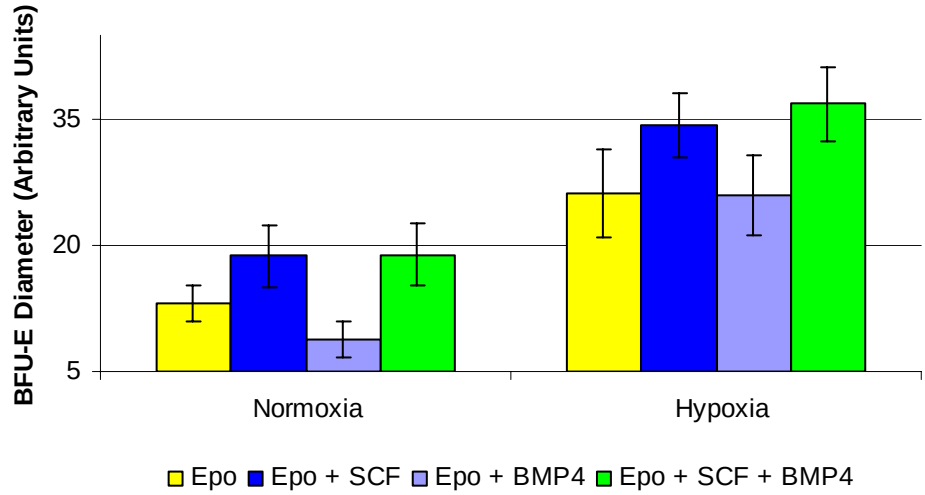


Figure 3-4D and E. Analysis of hypoxia's effect on stress BFU-E formation in *f/f* mice. Colony assays on splenocytes obtained from C57BL/6-*f/f* mice were performed as described in figure 3-1 in both normoxia (20% O2) and hypoxia (2% O2). Cultures contained 3U/ml Epo as well as Epo + 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated. Colony number was determined (D) as in figure 3-1 and colony size was determined (E) as in figure 3-2B.

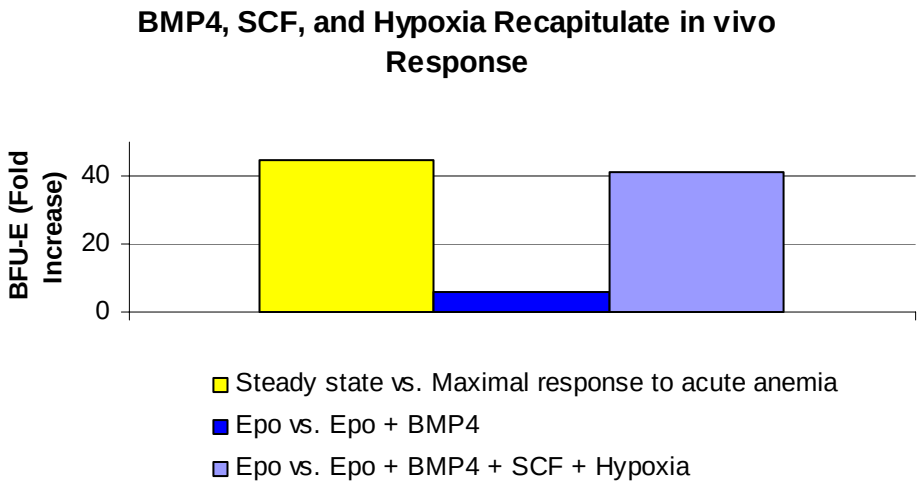


Figure 3-5. Comparison of *in vivo* vs. *in vitro* expansion of stress BFU-E. Number of stress BFU-E in control mice at 36 hours post-PHZ induced anemia divided by the number obtained under steady state (uninjected) conditions (yellow). Number of stress BFU-E obtained when splenocytes are plated in Epo + BMP4 (dark blue) or Epo + SCF + BMP4 + hypoxia (light blue) divided by number obtained when plated in Epo alone.

Chapter 4

Hedgehog differentiates bone marrow progenitors into BMP4 responsive cells in the spleen to replenish the stress BFU-E response to acute anemia

Abstract

Recently, our lab identified and characterized a stress erythroid progenitor (stress BFU-E) which forms the initial response to acute anemia. Stress BFU-E respond to Epo alone, are endogenous to the spleen and form large bursts after only brief incubation periods. Stress BFU-E are derived from BMP4 responsive cells, also endogenous to the spleen, which differentiate into stress BFU-E upon exposure to hypoxia-induced BMP4 expression. We find that BMP4 responsive cells and stress BFU-E do not self-renew in the spleen but must be replenished by bone marrow progenitors. Bone marrow-derived progenitors are found to give rise to BMP4 responsive cells and stress BFU-E upon exposure to the splenic microenvironment. Because bone marrow cells do not respond to BMP4 and do not contain stress BFU-E, we sought to discover what signal serves to convert bone marrow progenitors into splenic BMP4 responsive cells. Based on previous work in chondrocyte development, where Shh signals are required to make somitic mesoderm cells competent to respond to BMP4, we hypothesized that the hedgehog family of growth factors are likely candidates. Desert hedgehog (Dhh) is highly expressed in the spleen and exposure of bone marrow cells to Sonic hedgehog (Shh)

protein induces BMP4 expression as well as the expansion of stress BFU-E. Further, we show that, unlike controls, Smoothened mutant bone marrow cells, which are unable to transmit hedgehog signals, are incapable of giving rise to BMP4 responsive cells in the spleen. Together, our data demonstrate that certain bone marrow progenitors differentiate into BMP4 responsive cells upon exposure to hedgehog signals in the spleen and serve to replenish BMP4 responsive cells which have been exhausted during the recovery from acute anemia.

Introduction

The initial response to acute anemia relies on the expansion of a stress erythroid progenitor endogenous to the spleen. Termed stress BFU-E, these progenitors rapidly form large colonies in the presence of Epo alone without an added burst promoting activator (BPA) such as SCF or IL-3. Stress BFU-E expansion is triggered by BMP4, which is upregulated in the spleen in response to acute anemia. Our initial work showed that an immature cell, the BMP4 responsive cell, in the spleen differentiates upon BMP4 exposure into stress BFU-E (Lenox, Perry et al. 2005).

Several signals regulating various aspects of stress BFU-E development have been identified. In addition to the need for high levels of Epo and the role of BMP4, our lab has implicated the Kit/SCF signaling pathway as well as hypoxia as important to stress BFU-E development (chapter 3). We have demonstrated that BMP4, SCF, and hypoxia, in the presence of Epo, can serve to recapitulate the stress BFU-E expansion observed *in vivo* in response to acute anemia.

While we have identified the signals involved in stress BFU-E differentiation and expansion, many questions regarding the development of stress BFU-E remain. For instance, we have shown that the spleen, and not the bone marrow, contain BMP4 responsive cells, and stress BFU-E are largely absent from the bone marrow. However, up to this point the origin of BMP4 responsive cells is unclear. Given that fetal liver erythropoiesis is mechanistically similar to splenic stress erythropoiesis, BMP4 responsive cells could have populated the fetal spleen during development. Furthermore, it is not clear whether BMP4 responsive cells self-renew in the spleen or must be replenished from an extra-splenic source following recovery from acute anemia.

The identification of the BMP4-mediated stress BFU-E response has provided a new model for the response to acute anemia where BMP4 responsive cells differentiate into stress BFU-E in response to hypoxia-induced BMP4 expression in the spleen. However, the previous model suggested that the response involved a migration of BFU-E from the bone marrow to the spleen, where these BFU-E matured in the splenic microenvironment (Hara and Ogawa 1976; Hara and Ogawa 1977). We demonstrated that our stress BFU-E response formed the initial response to acute anemia and did not observe any migration event of BFU-E. Nonetheless, an earlier population of BMP4 responsive cell progenitors may migrate from the bone marrow in order to replenish the stress BFU-E response after it has been depleted following acute anemia. Here, we investigate this possibility.

We show that BMP4 responsive cells do not self-renew; however, the stress BFU-E response does recover within 3 weeks post-treatment. Thus BMP4 responsive cells must be replenished from an earlier population, probably from an extra-splenic source. If

bone marrow serves as a source of BMP4 responsive cells there must be a signal in the spleen which converts these bone marrow progenitors into BMP4 responsive cells. This signal cannot simply be BMP4 because BMP4 responsive cells and stress BFU-E are not found in the bone marrow. One possibility for this signal is one of the hedgehog (HH) factors, namely Sonic, Indian, or Desert hedgehog (Shh, Ihh, Dhh, respectively). The HH and BMP4 signaling pathways collaborate in many contexts, and there is precedent for a role for HH in making progenitors BMP4 responsive. For instance, work regarding chondrocyte development has demonstrated that Shh makes somitic mesoderm cells competent to respond to BMPs (Murtaugh, Chyung et al. 1999; Zeng, Kempf et al. 2002). HH and BMP4 signaling also have a precedent role in hematopoietic stem cell (HSC) regulation. For instance, Shh induces proliferation of human HSCs by regulating BMP4 (Bhardwaj, Murdoch et al. 2001). Here, we show that donor-derived BMP4 responsive cells develop in mice with transplanted bone marrow cells, which suggests that the bone marrow serves to replenish BMP4 responsive cells *in vivo*. We show that bone marrow-derived progenitors respond to HH signaling *in vitro* by differentiating into BMP4 responsive cells, and it is likely that Dhh provides this signal in the spleen.

Study Design

Mice

C57BL/6, B6.SJL, Smo^{tm2Amc}/J, WBB6F1/J and WBB6F1/J-*W* mice were obtained from Jackson Laboratory. All mice were approximately 6-8 weeks old, controls were age matched. Acute anemia was induced by injection of Phenylhydrazine (Sigma, St. Louis, MO) at a concentration of 120 mg/Kg mouse in PBS. The P-Lox flanked Smo allele

from Smo^{tm2Amc}/J mice was knocked out by poly-I, poly-C injection and scored as previously described (Long, Zhang et al. 2001). All experiments were approved by the IACUC at the Pennsylvania State University.

Transplantation Experiments

$2x10^6$ bone marrow or spleen cells from control mice or Smo^{tm2Amc}/J mice were resuspended in PBS and injected into W/W^V mice via the tail vain. W/W^V spleen and bone marrow were harvested 1, 2, and 3 weeks after transplantation as indicated and colony assays were performed as described below.

Colony Assays for BFU-E

Splenocytes and bone marrow cells were isolated from control, *W/+*, and W/W^V mice. $1x10^5$/ml nucleated bone marrow and $2x10^6$/ml nucleated splenocytes were plated in methylcellulose media (StemCell Technologies, Vancouver, BC) containing 3U/ml Epo and 10 ng/ml IL-3 (Sigma, St. Louis, MO) or 15 ng/ml BMP4 (R&D Systems, Minneapolis, MN) where indicated. BFU-E were scored as described (Finkelstein, Ney et al. 2002) after 5 days incubation. For preincubation experiments, bone marrow cells from C57BL/6 mice were cultured overnight in IMDM + 5%FCS with and without 200 ng/ml Shh protein (R&D Systems, Minneapolis, MN). Colony assays were then performed as described above.

Analysis of BMP4 and Dhh Expression

Total RNA isolated from bone marrow or spleen cells homogenized in TRIzol (Invitrogen, Carlsbad, CA) was reverse transcribed into cDNA. PCR was performed

using primers: forward 5'-CCTGGTAACCGAATGCTGAT-3' and reverse 5'-TTTATACGGTGGAAGCCCTG-3' for BMP4 and forward 5'-CATCCACGTA TCGGTCAAAG-3' and reverse 5'-GAACACCGGTGCAAAGTCAC-3' for Dhh.

Immunofluorescent staining of spleen sections

$2x10^6$ Bone marrow cells from B6.SJL (CD45.1+) mice were resuspended in PBS and transplanted into W/W^V (CD45.2+) mice as well as controls. Spleens were harvested one week after transplantation, fixed in Zinc fixative and paraffin embedded tissues sections were cut. The sections were deparafinized in Histo-clear II (National Diagnostics, Atlanta, GA), rehydrated through a methanol series to 50% methanol, blocked with PBS + 1% BSA for 45 min., and rinsed in PBS. Primary BMP4 antibody (Novocastra Laboratories/Vector Laboratories, Burlingame, CA) was diluted 1:100 and incubated overnight at 4°C in a humid chamber. Sections were washed in PBS and then incubated with 1:200 diluted Alexa Fluor 660 secondary antibody (Molecular Probes, Eugene, OR) and 1:100 diluted FITC conjugated anti-CD45.1 (BD Pharmingen) for 30 min. at room temperature. Sections were washed and mounted in Slowfade (Molecular Probes, Eugene, OR). Slides were analyzed by confocal microscopy.

Results

BMP4 responsive cells do not self-renew

Previously, we showed that stress BFU-E expand exclusively in the spleen in response to phenylhydrazine (PHZ) induced anemia. These stress BFU-E are derived from BMP4 responsive cells, which are present in unstimulated spleen and can be induced to form stress BFU-E by treatment with BMP4. We wanted to determine whether BMP4

responsive cells are present during and after the recovery from acute anemia or if all BMP4 responsive cells differentiate during the response. If BMP4 responsive cells possess the ability to self-renew, a constant number of BMP4 responsive cells should be maintained in the spleen. We assayed for BMP4 responsive cells at different times during the recovery from acute anemia. As shown in figure 4-1A, BMP4 responsive cells are present only in untreated spleen and at twelve hours post-PHZ injection. By 24 hours, no BMP4 responsive cells are observed, and they do not recover even by 8 days post-injection. Although the lack of BMP4 induction at earlier time points may be due to endogenous production of BMP4 mainly at 24 hours post-PHZ injection, we previously showed that BMP4 protein was generally absent by 96 hours (Lenox, Perry et al. 2005). Thus, all BMP4 responsive cells differentiate during the response to acute anemia.

Figure 4-1A reveals that, even by 8 days post-PHZ injection, BMP4 responsive cells have not replenished. Consequently, we sought to determine when and if the stress BFU-E response is reset. We treated mice with PHZ to induce acute anemia and exhaust their pool of BMP4 responsive cells; we then induced acute anemia with a second PHZ injection 7, 14, and 21 days after the first injection. Previously, we showed that the main stress BFU-E expansion occurs at 36 hours post-PHZ treatment, so we assayed for stress BFU-E at 7 days + 36 hours, 14 days + 36 hours, and 21 days + 36 hours. As shown in figure 4-1B, mice do not form the normal stress BFU-E response when only one week is given between injections (compare with Lenox, Perry et al. 2005). After 2 weeks the response is intermediate, but with 3 weeks between injections the normal 36 hour stress BFU-E expansion is observed (figure 4-1B). Thus the stress BFU-E response to acute anemia is regenerated, but three weeks are required for complete regeneration after it has

been depleted. Taken together, these data show that BMP4 responsive cells do not self-renew in the spleen and most likely must be replenished from an extra-splenic source.

BMP4 responsive cells are derived from bone marrow

The fact that BMP4 responsive cells are depleted and the stress BFU-E response to acute anemia is not regenerated for more than 2 weeks suggests that BMP4 responsive cells do not self-renew in the spleen. Therefore, it is likely that precursors of BMP4 responsive cells must replenish the spleen and may do so by migrating from a different hematopoietic organ, the most likely candidate being the bone marrow. Previously, we showed that W/W^V mice, which have a mutation in Kit, the receptor for SCF, lack BMP4 responsive cells in their spleens (figure 4-2 and chapter 3). We took advantage of this property of W/W^V mice as well as the well known property that W/W^V mice can be reconstituted without lethal irradiation. We utilized W/W^V mice to determine if BMP4 responsive cells could be transplanted from control mice and whether or not BMP4 responsive cells could be derived from the bone marrow. Both bone marrow and spleen cells from control mice were transplanted into W/W^V mice, and colony assays for BFU-E were performed with bone marrow and spleen cells isolated from the recipients 1, 2, and 3 weeks post-transplant. Analysis of W/W^V spleen following transplantation with control bone marrow reveals the presence of BMP4 responsive cells at 1 and 2 weeks post-transplant as well as progressively increased numbers of stress BFU-E at all post-transplant time points (figure 4-2A). Analysis of BFU-E from W/W^V bone marrow following transplantation of control bone marrow showed only the expected result of an increase in BFU-E requiring an added BPA but no increase in stress BFU-E or BMP4

responsive cells (figure 4-2B). *W/W^V* spleen transplanted with control spleen reveal an increase in stress BFU-E and, at 2 weeks post-transplant, BMP4 responsive cells (figure 4-2C). Thus, both BMP4 responsive cells and stress BFU-E can be transplanted from normal spleen to recipient spleen as expected. In contrast, after transplantation with control spleen, *W/W^V* bone marrow exhibit BMP4 responsive cells 3 weeks post-transplant but no increase in stress BFU-E (figure 4-2D). Thus, BMP4 responsive cells can be supported in the bone marrow but only when derived from the spleen. Despite the presence of BMP4 responsive cells in the bone marrow, stress BFU-E are not supported in the bone marrow microenvironment. Taken together these data demonstrate that BMP4 responsive cells can be derived from the bone marrow but require exposure to the microenvironment of the spleen and expansion of stress BFU-E also requires exposure to the spleen microenvironment. Furthermore, BMP4 responsive cells that home to the bone marrow maintain their ability to respond to BMP4 regardless of the bone marrow microenvironment.

Donor bone marrow cells seed the red pulp of the spleen and express BMP4

Having shown that BMP4 responsive cells are derived from bone marrow, we next tested whether transplanted bone marrow cells affect BMP4 expression in the spleen. As shown in figure 4-3A, while BMP4 is not normally expressed in *W/W^V* spleen, transplantation with bone marrow cells results in an increase in BMP4 mRNA expression in the recipient spleen. However, this analysis did not determine whether donor bone marrow express BMP4 or recipient splenocytes are induced by donor bone marrow to express BMP4. In order to determine which cells express BMP4, bone marrow cells from CD45.1 control

mice were transplanted into W/W^V mice, which express only the CD45.2 allele, and spleen sections from these recipients were stained with anti-BMP4 and anti-CD45.1 antibodies in order to mark donor-derived cells. Spleen sections from untransplanted control W/W^V mice did not exhibit any BMP4 or CD45.1 staining (data not shown). However, as shown in figure 4-3B, in transplanted W/W^V mice, donor-derived CD45.1+ cells can be seen throughout the peripheral red pulp area of the spleen. Donor-derived cells are particularly concentrated around the splenic artery and can be seen infiltrating the splenic cords throughout the red pulp. Interestingly, donor-derived cells are absent from the more interior white pulp, revealing that bone marrow cells tend to populate the erythropoietic red pulp but do not contribute to the lymphopoietic white pulp. BMP4 staining is more restricted with only a portion of donor-derived cells also staining with BMP4. Nonetheless, donor bone marrow cells do express BMP4 in the spleen. BMP4 staining is also seen in recipient spleen cells adjacent to donor-derived cells. It is unclear whether this represents host BMP4 expression or diffusion of BMP4 from donor cells; however, it is likely that the later is the case given that BMP4 alone staining is observed only immediately adjacent to where the concentration of donor-derived BMP4 expressing cells is the greatest (figure 4-3B). Alternatively, donor cells may also transiently induce BMP4 expression from adjacent recipient cells. Notably, this donor cell expression of BMP4 is restricted to only a section of the spleen, most donor-derived cells do not express BMP4. This may indicate that only certain donor cells are induced or that the induction is only transient upon entering the spleen. This later possibility may be the most likely given that it appears that only cells near the splenic artery show BMP4 expression. In either case, only a portion of the spleen expresses BMP4, which explains

why BMP4 responsive cells are present in our assays despite the induction of BMP4. Additionally, this may explain why stress BFU-E are induced in the spleen as early as 1 week following transplantation of bone marrow cells. That is, perhaps locally induced BMP4 differentiates newly formed BMP4 responsive cells into stress BFU-E.

Hedgehog treatment induces stress BFU-E in bone marrow cells

Since BMP4 responsive cells and stress BFU-E can be derived from the bone marrow but only when exposed to the splenic microenvironment, we sought to determine what signals convert bone marrow cells into BMP4 responsive cells in the spleen. Given that in chondrocyte development Shh makes cells competent to respond to BMP4, the HH signaling factors represented strong candidates for such a signal. We therefore determined the expression of Shh, Ihh, and Dhh following acute anemia induction by PHZ treatment. Shh and Ihh expression was not observed (data not shown). However, Dhh was highly expressed at all time points but was downregulated at 36 hours post-PHZ injection (figure 4-4). The timing of this downregulation corresponds to the main 36 hour expansion of stress BFU-E, thus Dhh's expression profile suggests it may be involved in the stress BFU-E response to acute anemia.

Because Dhh is highly expressed in the spleen even under homeostatic conditions, we wondered if Dhh may be the signal which converts bone marrow cells into BMP4 responsive cells upon reaching the spleen. Shh has been shown to have similar biological activities to Dhh, especially with regard to hematopoietic stem cell differentiation and expansion, and is, unlike Dhh, commercially available (Bhardwaj, Murdoch et al. 2001; Pathi, Pagan-Westphal et al. 2001). We treated bone marrow cells with and without Shh

overnight and then assayed for BMP4 responsive cells and stress BFU-E. As shown in figure 4-5, preincubation with Shh results in a 2.5 fold increase in the number of stress BFU-E. Thus, HH dependent signals are sufficient to induce bone marrow cells to become stress BFU-E.

One would predict that bone marrow cells would first differentiate into BMP4 responsive cells; however, no increase in BMP4 responsive cells was observed. It is possible that Shh simply induces BMP4 expression in bone marrow progenitors resulting in no observed increase in BMP4 responsive cells due to the possibility that endogenous BMP4 differentiates BMP4 responsive cells into stress BFU-E prior to our addition of BMP4. In order to test if BMP4 is induced by Shh treatment, we preformed RT-PCR on bone marrow cells incubated with and without Shh. As shown in figure 4-5B, Shh treatment induces BMP4 expression in bone marrow cells. Thus, the increase in stress BFU-E but not BMP4 responsive cells is likely due to endogenous BMP4 induction by Shh, and bone marrow cells likely pass through a BMP4 responsive cell intermediate before becoming stress BFU-E. Also, since donor bone marrow cells exhibit localized expression of BMP4 in the spleen (figure 4-3), this suggests that HH may induce the expression of BMP4 in bone marrow cells homing to the spleen.

Smoothened knockout bone marrow cells fail to generate BMP4 responsive cells in the spleen

Our data show that BMP4 responsive cells do not self-renew in the spleen, can be replenished by the bone marrow, and that HH signaling can lead to the differentiation of bone marrow progenitors into stress BFU-E. We sought to further confirm HH

signaling's role as well as further implicate BMP4 responsive cells in the pathway from bone marrow progenitors to stress BFU-E. To this end, we tested whether bone marrow cells unable to transmit HH signals could form BMP4 responsive cells when transplanted into W/W^V mice.

Control bone marrow cells transplanted into W/W^V mice result in BMP4 responsive cells in the recipient's spleen 1 and 2 weeks post-transplantation (figure 4-2D). HH signals are transmitted through their receptors, Ptc and Smo. Ptc normally inhibits Smo but, upon hedgehog binding, Ptc releases Smo and the signal is transmitted. Thus, inactivating Smo results in the inability to transmit HH signals. $Smo^{-/-}$ mice are embryonic lethal, so we obtained mice carrying a conditional allele of Smo that contains LoxP sites flanking a portion of the Smo coding region (Long, Zhang et al. 2001; Zhang, Ramalho-Santos et al. 2001). This floxed allele of Smo was crossed to mice carrying an interferon inducible Cre recombinase transgene (Mx1-Cre). Injection with poly-I, poly-C induces Cre and leads to the deletion of Smo in all tissues expressing Cre.

As shown in figure 4-6B, we were able to eliminate the majority of Smo transcripts in $Smo^{fx/fx}$ mice versus $Smo^{fx/+}$ controls. Bone marrow cells transplanted from $Smo^{fx/+}$ controls are expected to result in BMP4 responsive cells in the spleens of W/W^V recipients just as control bone marrow does. However, if HH is responsible for differentiating bone marrow progenitors into BMP4 responsive cells, one would expect that a lack of or severe reduction in Smo would compromise the ability of transplanted bone marrow cells to form BMP4 responsive cells in recipient spleen. After treatment with poly-I, poly-C, we transplanted bone marrow cells from $Smo^{fx/fx}$ and $Smo^{fx/+}$ controls into W/W^V recipients and assayed for BMP4 responsive cells 1 week post-transplant. As

shown in figure 4-6A, transplantation of $Smo^{fx/+}$ control bone marrow results in BMP4 responsive cells in the spleens of W/W^V recipients as expected; however, transplantation of $Smo^{fx/fx}$ bone marrow cells fails to result in recipient BMP4 responsive cells. Thus, transplanted bone marrow cells unable to transmit hedgehog signals are incapable of yielding BMP4 responsive cells. This result directly implicates hedgehog signals as being responsible for the differentiation of bone marrow progenitors into BMP4 responsive cells in the spleen.

Discussion

Our previous studies identified the BMP4-mediated stress erythroid response pathway to acute anemia. Later, we showed that SCF and hypoxia are required for this response. Here, we have shown that BMP4 responsive cells, although only found in the spleen, do not self-renew and are ultimately derived from an earlier bone marrow progenitor. These bone marrow progenitors only become BMP4 responsive cells when exposed to the spleen's microenvironment. Our studies demonstrate further that HH signaling, potentially Dhh, serves to convert these bone marrow-derived progenitors into BMP4 responsive cells.

Because the bone marrow gives rise to a diverse repertoire of cells, particularly hematopoietic cells, it was initially surprising that BMP4 responsive cells and stress BFU-E were only found in the spleen. However, the importance of the splenic microenvironment is clearly demonstrated by our transplantation experiments. Only in cases where transplanted cells are exposed to the spleen are BMP4 responsive cells found. Furthermore, once a cell has become a BMP4 responsive cell, it does not change

regardless of the microenvironment. BMP4 responsive cells are found within the bone marrow of recipients, but only when splenocytes were used as donor cells. The same is apparently not the case regarding stress BFU-E. An increased number of stress BFU-E are observed in the spleens of transplanted recipients, regardless of whether donor cells were bone marrow or spleen-derived. However, the bone marrow exhibited no increase in stress BFU-E in either case even when spleen-derived BMP4 responsive cells were transplanted (figure 4-2). Thus, unlike BMP4 responsive cells, stress BFU-E require the support of the splenic microenvironment. This result is consistent with previous evidence of an erythropoietic inductive microenvironment (EIM) in the spleen (Obinata and Yania 1999). In mice, the spleen is a hematopoietic organ where progenitor cells are predominantly committed to erythroid development (Curry, Trentin et al. 1967). In addition, the spleen specifically supports colonies derived only under stress conditions, namely the CFU-S (colony forming unit-spleen) and TE-CFU (transient-endogenous colonly forming unit), both of which form in response to myeloablative therapy (Till and McCulloch 1961; Till 1981). Notably, CFU-S and TE-CFU are largely composed of erythroid cells. Thus, the spleen has long been implicated in the support of erythroid stress-response progenitors.

We have also demonstrated that donor bone marrow cells quickly contribute to a substantial portion of the red pulp of the spleen (figure 4-3B). In addition, donor cells are shown to induce BMP4 expression in recipient spleen (figure 4-3A). Initially, it was surprising that BMP4 responsive cells were still found in recipient spleen since endogenous BMP4 expression typically prevents the *in vitro* addition of BMP4 from having any effect; however, BMP4 expression is shown to be restricted (figure 4-3B).

Thus, BMP4 responsive cells are still present in our assays despite endogenous BMP4 expression. One caveat to these results is that we do not know whether the anemia of the W/W^V mice affects these observations.

Previously, we showed that BMP4 is induced by hypoxia in the spleen stromal cell line, MSS31 (Yanai, Satoh et al. 1991; Lenox, Perry et al. 2005). Consequently, we suggested that stromal cells in the spleen provided the BMP4 for differentiating BMP4 responsive cells into stress BFU-E. Here, we show that HH induces BMP4 in bone marrow primary cultures (figure 4-3A) and that BMP4 is induced in donor bone marrow entering the spleen (figure 4-3B). HH induction of BMP4 in primary bone marrow cultures from control mice argues against the notion that BMP4 is only induced due to the W/W^V recipient's anemia and suggests that it is HH which induces the *in vivo* expression of BMP4 in donor cells. The fact that increased stress BFU-E are observed as early as 1 week post-transplant (figure 4-2D) suggests that this spatially restricted BMP4 induction leads to the differentiation of a portion of incoming BMP4 responsive cells into stress BFU-E. This observation raises the possibility that bone marrow cells may migrate to the spleen in response to acute anemia and, upon entering the spleen, Dhh may induce BMP4 expression thus differentiating BMP4 responsive cells into stress BFU-E.

Although we have not yet identified the bone marrow progenitor population which gives rise to BMP4 responsive cells, it is likely that this cell is, or is immediately derived from, either a megakaryocyte-erythrocyte progenitor (MEP) or a progenitor in the recently identified PECAM-1+ population (lineage marker (Lin)-, Kit+, Sca-1-, PECAM-1+) (Na Nakorn, Traver et al. 2002; Baumann, Bailey et al. 2004). Both populations have been shown to confer radioprotection on recipients. That is, these populations transiently

rescue mice from radiation/chemotherapy-induced thrombocytopenia/anemia in the interim before HSCs stably reconstitute the hematopoietic system. It is likely that BMP4 responsive cells/stress BFU-E provide the erythropoietic arm of the radioprotective effect conferred by these populations. Our previous data demonstrated that only spleen-derived MEPs give rise to BMP4 responsive cells (Lenox, Perry et al. 2005). However, recently our lab identified a potential bone marrow progenitor that could give rise to BMP4 responsive cells. Studies involving Friend virus have shown that Friend virus infects cells of the BMP4-dependent stress erythroid response pathway. Kit+, Sca-1-, Lin-, CD31+, CD41+ cells from the bone marrow infected with Friend virus induce BMP4 expression in the spleens of recipients (Aparna Subramanian and Robert F. Paulson, unpublished observations). Thus, this population is a strong candidate for the bone marrow progenitor giving rise to BMP4 responsive cells in the spleen.

We have also demonstrated an additional role for BMP4/HH cooperative signaling in the regulation of the differentiation of hematopoietic progenitors. Bhardwaj et al. previously showed that Shh induces proliferation of human HSCs by regulating BMP4. Although this effect was demonstrated in much earlier hematopoietic progenitors than our lineage-restricted ones, it is not surprising that these two signaling pathways are also utilized for the regulation of more specific differentiation events. Our results have demonstrated that Dhh is highly expressed in the spleen, HH treatment of bone marrow cells result in the expansion of stress BFU-E and bone marrow cells which cannot respond to HH, unlike controls, are unable to form BMP4 responsive cells and stress BFU-E. Thus, BMP4 responsive cells appear to be ultimately derived from bone marrow

progenitors and, upon exposure to Dhh in the spleen, differentiate into BMP4 responsive cells, which are poised to respond to acute anemia.

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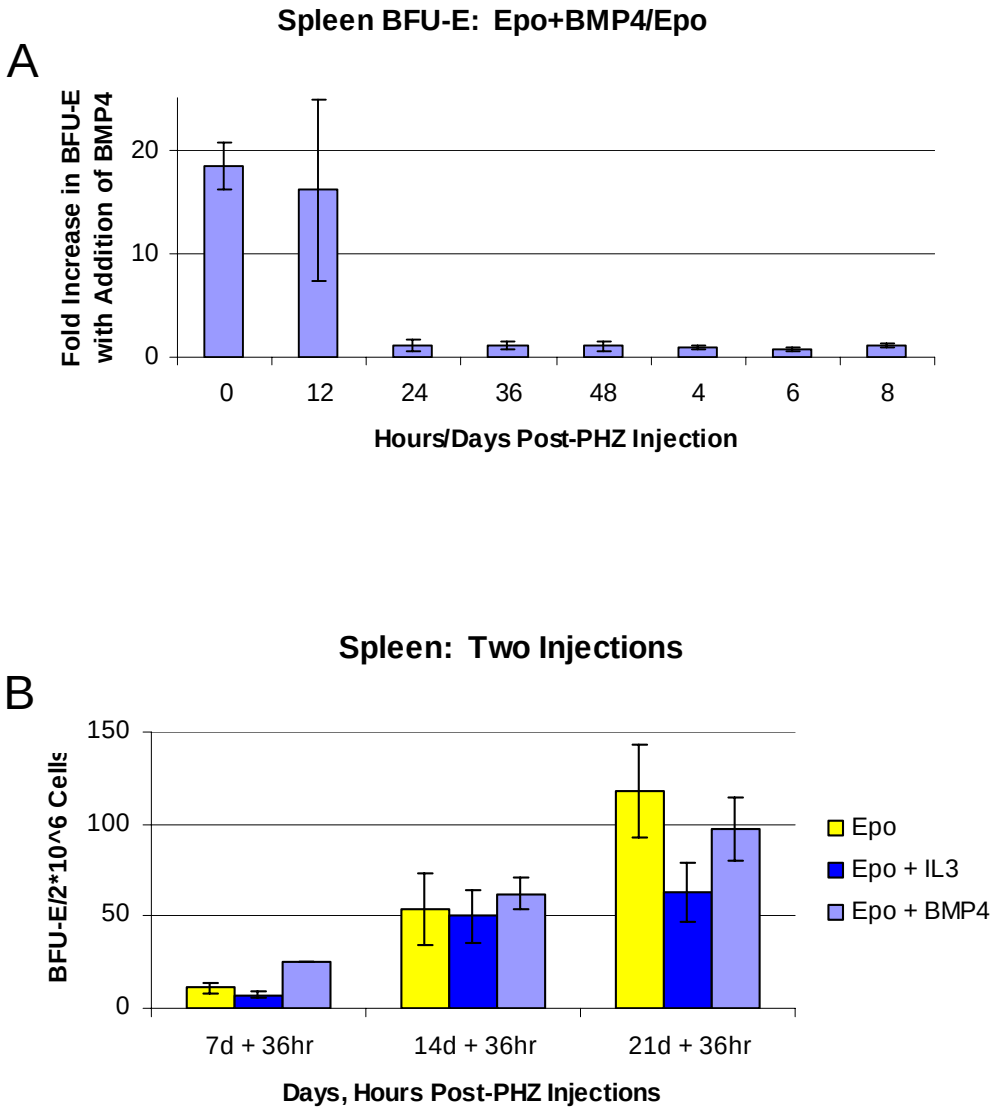
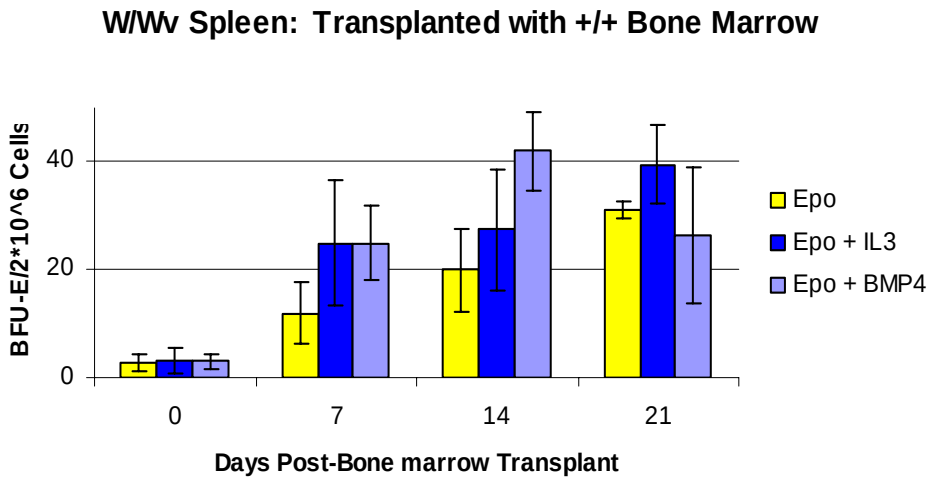


Figure 4-1. Analysis of self-renewal capability of BMP4 responsive cells and stress BFU-E during the response to acute anemia. Colony assays were performed on C57BL/6 control spleen cells during the response to acute anemia (A) and at 36 hours post-anemia induction 7, 14 and 21 days after an initial anemia induction (B). 2x10^6 spleen cells were plated in methylcellulose media containing 3U/ml Epo supplemented with 10 ng/ml IL-3 or 15 ng/ml BMP4 where indicated. BFU-Es were scored after 5 days incubation by positive benezidine staining.

A



B

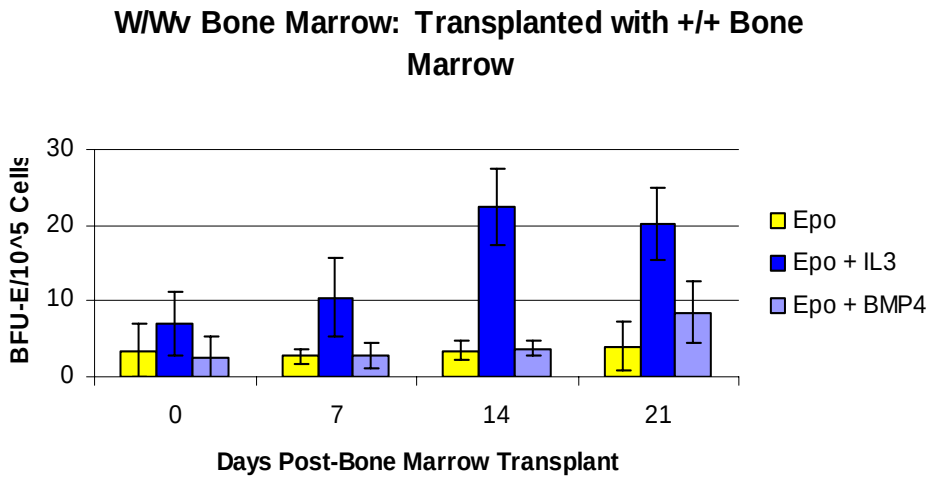
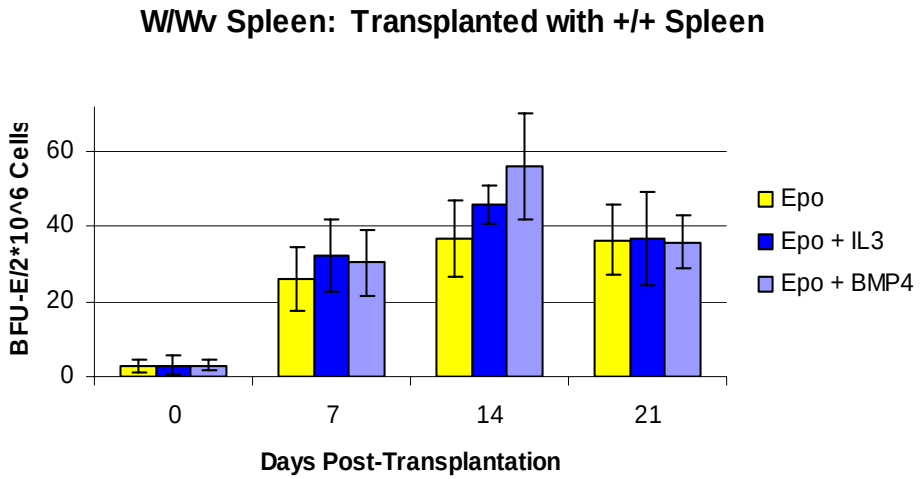


Figure 4-2 A and B. Determination of control bone marrow cell's ability to transplant BMP4 responsive cells and stress BFU-E to W/Wv spleen (A) and bone marrow (B). *W/Wv* mice were transplanted with 2x10^6 bone marrow from control mice. Spleen (A) and bone marrow (B) from recipients was assayed for BFU-E as described in figure 4-1 0, 7, 14, and 21 days post-transplant as indicated.

C



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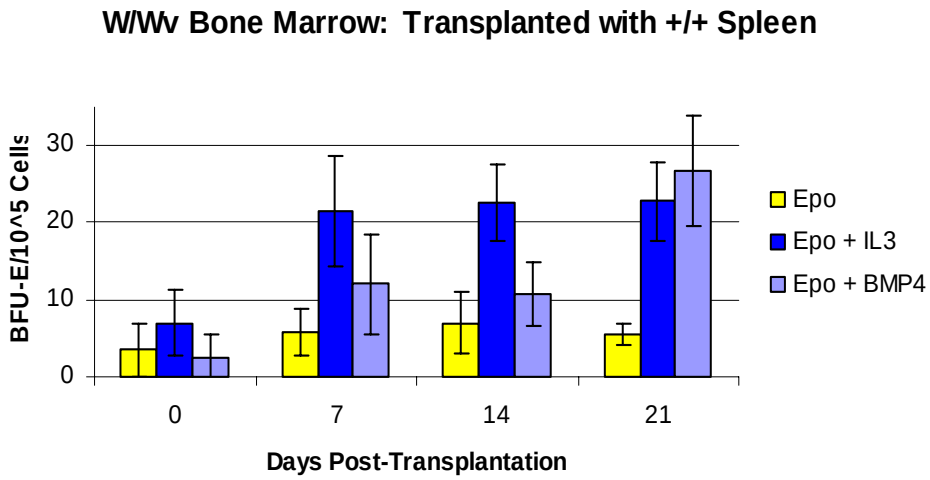


Figure 4-2 C and D. Determination of control spleen cell's ability to transplant BMP4 responsive cells and stress BFU-E to W/Wv spleen (C) and bone marrow (D). *W/Wv* mice were transplanted with 2x10^6 spleen cells from control mice. Spleen (C) and bone marrow (D) cells from recipients were assayed for BFU-E as described in figure 4-1 0, 7, 14, and 21 days post-transplant as indicated.

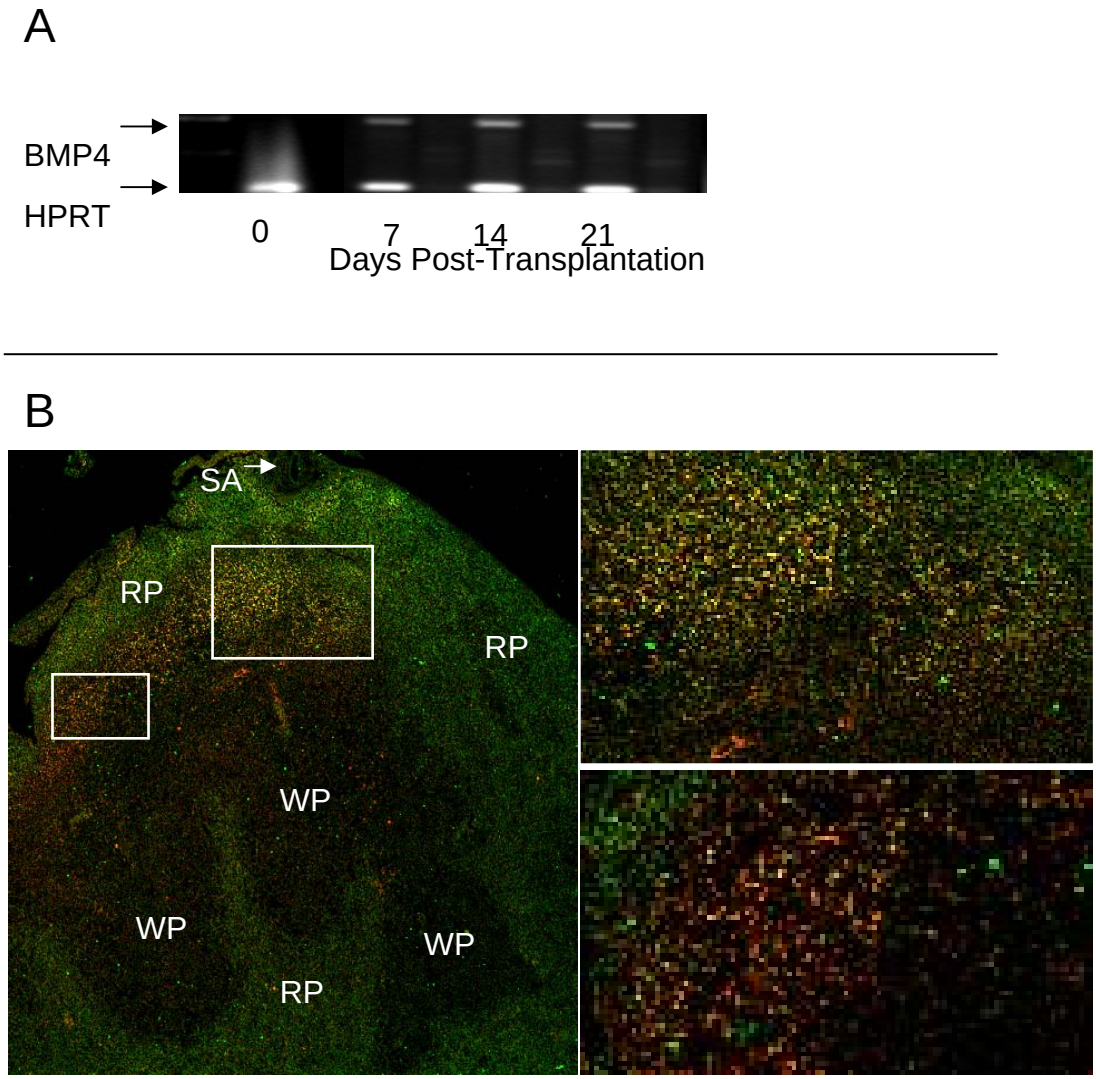


Figure 4-3. Analysis of BMP4 expression in donor spleen post-bone marrow cell transplantation. A) RT-PCR analysis of BMP4 expression in the spleens of W/Wv mice 0, 7, 14 and 21 days post transplantation with control bone marrow cells. Arrows indicate BMP4 and control HPRT specific product. B) Section of W/Wv spleen 7 days post-bone marrow cell transplant from CD45.1+ donor mice stained with anti-BMP4 (red) and anti-CD45.1 (green). Splenic artery (SA) is indicated by arrow. Red pulp (RP) and white pulp (WP) regions are labeled. Boxed areas are magnified at the right.

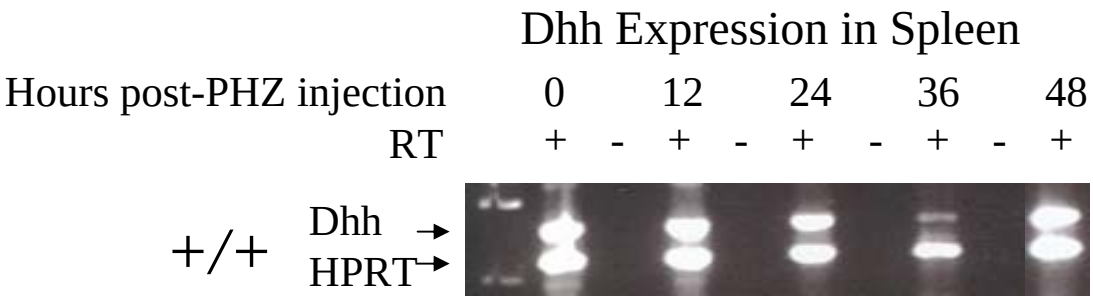
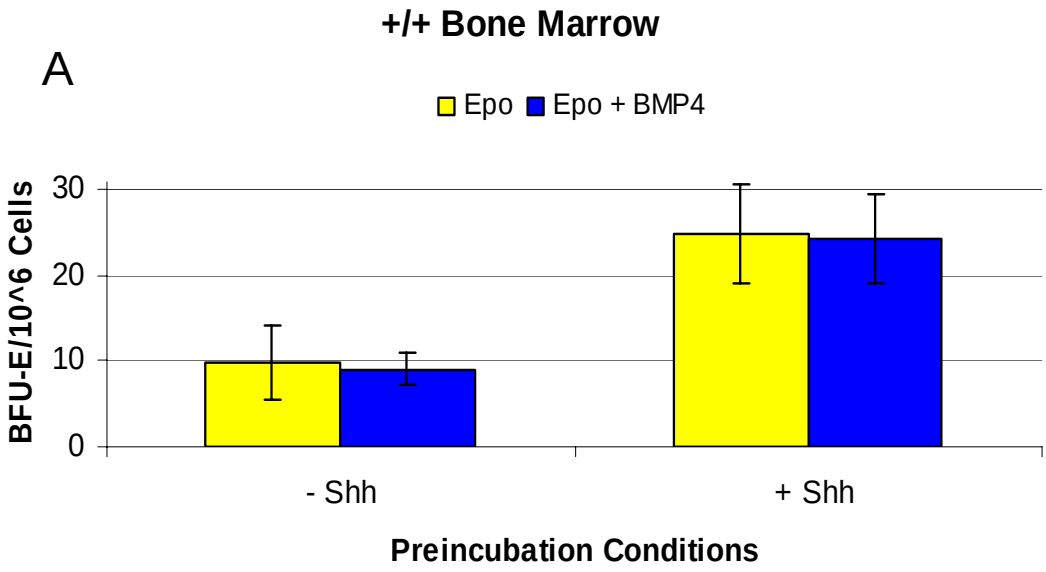


Figure 4-4. Analysis of *Dhh* Expression during the response to acute anemia. RT-PCR analysis of *Dhh* expression during the response to acute anemia in C57BL/6 control mice. Arrows indicate the position of the Dhh and control HPRT specific band.



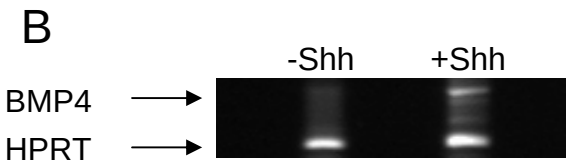
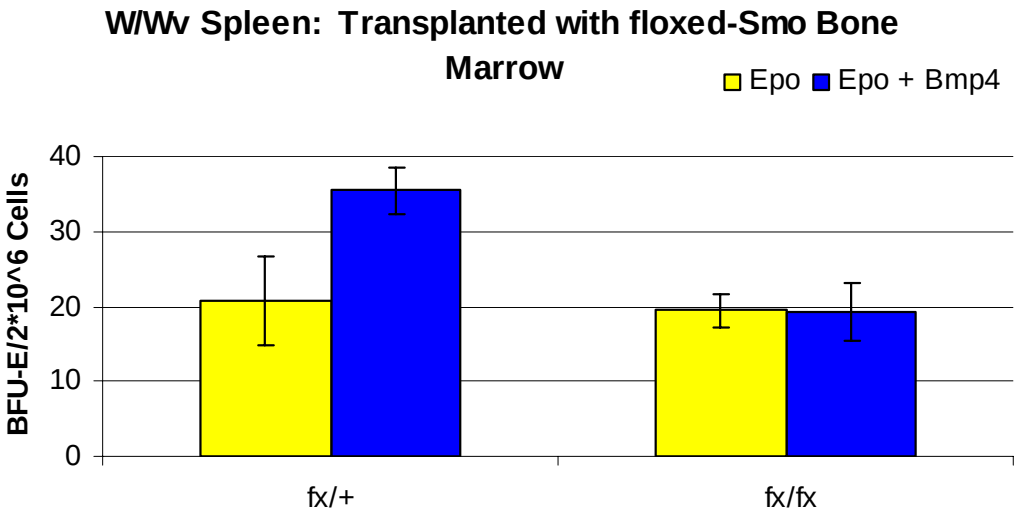


Figure 4-5. Effect of HH treatment of bone marrow cells. A) 1x10^7 primary bone marrow cells from C57BL/6 control mice were incubated overnight in IMDM + 5% FCS with and without 200 ng/ml Shh. Cells were then washed and colony assays were performed as described in figure 4-1. B) RT-PCR analysis of primary bone marrow cells incubated overnight in IMDM + 5% FCS with and without 200 ng/ml Shh. Arrows indicate the BMP4 and control HPRT specific bands.

A



B

Figure 4-6. Assay for BMP4 responsive cells in W/Wv spleen transplanted with Smo-/- bone marrow. A) Colony assays were performed as described in figure 4-1 on W/Wv spleen 7 days post-bone marrow cell transplant. Donor cells were derived from mice carrying floxed (fx) Smo alleles as well as Mx-Cre after treatment with poly-I, poly-C. fx/+ mice are used as control. B) RT-PCR analysis of *Smo* expression from bone marrow cells derived from fx/+ and fx/fx-Smo mice after treatment with a regimen of poly-I, poly-C. Arrows indicate the positions of the control HPRT and Smo specific products.

Chapter 5

Future Studies and Conclusion

The discovery and characterization of stress BFU-E as presented in this thesis has provided a new model for the response to acute anemia. The studies presented here originally began with an attempt to understand the cellular basis of the *flexed-tail (f)* defect. The *f* phenotype was particularly useful given that these mice are specifically defective at times of expansive erythropoiesis in the fetal liver and the spleen in response to acute anemia. Given that the fetal liver contains a unique class of BFU-E which respond to Epo alone, this led to the analysis of the spleen for BFU-E responding to Epo alone during the response to acute anemia. Finding a major expansion of such BFU-E and a delayed expansion in *f/f* mice led to the discovery of stress BFU-E. With Laurie Lenox's finding that the *f* locus encodes Madh5, this led to the examination of BMP expression and, eventually the discovery of BMP4 responsive cells. The characterization these progenitors forms the bulk of this thesis and much future work remains regarding this characterization.

Future Studies

Identification of bone marrow progenitors which replenish BMP4 responsive cells

Chapter 4 presents data showing that BMP4 responsive cells can be transplanted from the bone marrow to the spleen, and it is the bone marrow which may serve to

replenish BMP4 responsive cells that are depleted during the response to acute anemia. However, this chapter presents no data regarding the identity of these bone marrow-derived cells. Their identification is key to a full description of the ontogeny of stress BFU-E. Also, identification and purification would allow for further characterization of these cells. For instance, are these cells related to the short-term radioprotective cells discussed in chapter 1 and are they multipotential or restricted to the erythroid lineage?

Short-term radioprotective cells were shown to be within the MEP population of bone marrow progenitors (Na Nakorn, Traver et al. 2002). Indeed, we showed that BMP4 responsive cells are found within the MEP population in the spleen (chapter 2). However, only a small fraction of MEPs were shown to give rise to BMP4 responsive cells or radioprotective cells. Clearly, there is a need to refine this population. Initial experiments on BMP4's effect on megakarycyte progenitors (CFU-Meg) have shown that BMP4 does not affect their numbers (John M. Perry, unpublished observations). Thus, BMP4 responsive cells are likely restricted to the erythroid lineage. This suggests that BMP4 responsive cells are more differentiated than MEPs or short-term radioprotective cells. Still, the bone marrow progenitors giving rise to BMP4 responsive cells could be less differentiated and possibly multipotential, perhaps within the short-term radioprotective cell population.

Perhaps a more promising population giving rise to BMP4 responsive cells are the PECAM (CD31)+, Lin-, Kit+, Sca-1- population also discussed in chapter 1. Like MEPs, these progenitors were determined to confer short-term radioprotection, but far fewer of these cells were required for the protective effect (Baumann, Bailey et al. 2004). Thus, this population may more clearly define a potential source of BMP4 responsive cells.

Initial experiments on this population's ability to form BMP4 responsive cells were inconclusive (John M. Perry, unpublished observations); however, other results from the Paulson lab show that this population does contain BMP4 responsive cells (Aparna Subramanian and Robert F. Paulson, unpublished observations). As discussed in chapter 4, the potential population giving rise to BMP4 responsive cells is possibly within the PECAM+, Lin-, Kit+, Sca-1- CD41+ population. Nevertheless, our lab has yet to purify BMP4 responsive cells and stress BFU-E; however, focusing on the identification of the bone marrow progenitor giving rise to BMP4 responsive cells leads to the identification of its immediate progeny since they must be closely related. Thus, clear progress has been made in identifying the lineage of BMP4 responsive cells although much work remains.

Regulation of the migration event

One approach to identifying progenitors giving rise to BMP4 responsive cells is to determine which population migrates from the bone marrow following anemic stress. A population of progenitors which is present at abnormally high levels in the peripheral blood following anemic stress should contain the progenitors of interest. Indeed, the migratory event itself presents an important aspect of stress BFU-E development. There must be a signal in the bone marrow which initiates this migration, and one particularly attractive candidate for such a signal is the recently identified HSC and erythroid progenitor cell marker, Podocalyxin.

Podocalyxin is highly expressed on primitive hematopoietic progenitors in the embryo and fetus as well as erythroid precursors in the fetal liver (Doyonnas, Nielsen et

al. 2005). Its expression decreases dramatically in the adult; however, there is a burst of post-natal expression which correlates with the seeding of hematopoietic progenitors into the bone marrow and spleen. This makes sense in light of Podocalyxin's role, which has been described as cellular "Teflon" (see chapter 1). That is, Podocalyxin expression allows cells to lose their adhesive connections to the extracellular matrix and thus migrate to a different hematopoietic niche. Most interesting is the fact that Podocalyxin expression is reactivated in adult erythroid progenitors in response to acute anemia. Moreover, a population of Kit+, Podocalyxin+, Ter-119- progenitors expands following anemic induction, and our own analysis of BMP4-responsive cells shows them to be Kit+ and Ter-119- (chapter 2). Particularly notable is Doyonnas et al.'s note that, while Podocalyxin expression is generally restricted to the bone marrow, a small number of Podocalyxin+ cells are found in the spleens of anemic mice. Thus, Podocalyxin represents a prime candidate for allowing the emigration of bone marrow progenitors to the spleen in order to replenish BMP4 responsive cells as well as a marker which could significantly aid in the purification of such progenitors.

Additional regulators of the stress BFU-E response

We have examined numerous candidate factors which may serve to regulate stress BFU-E development. For instance, we examined the expression of Activin, SCF, IL-3, VEGF and others during recovery from acute anemia. Our initial analysis did not reveal any change in the expression of these factors in response to acute anemia. However, two candidates in particular exhibited a suggestive expression profile during the recovery from acute anemia. Smad6 is an inhibitory Smad, which lacks its MH2 domain and thus

it's transcriptional activity (Imamura, Takase et al. 1997). Smad6 is typically induced by TGF-β signals and attenuates signal transduction by competitively binding with type I receptors and thus interfering with the binding of other receptor-activated Smads (see chapter 1). As shown in figure 5-1, Smad6 is highly expressed in the spleen; however, Smad6 expression is dramatically downregulated at 36 hours post-PHZ injection. This corresponds to the expansion of stress BFU-E in response to acute anemia. Apparently, TGF-β signal transduction is actively inhibited in the spleen; however, this inhibition is removed during the main expansion of BMP4-induced stress BFU-E. It is unclear how Smad6 is regulated, but it is likely that this downregulation is necessary for the normal stress BFU-E response. Additional studies are needed to determine this.

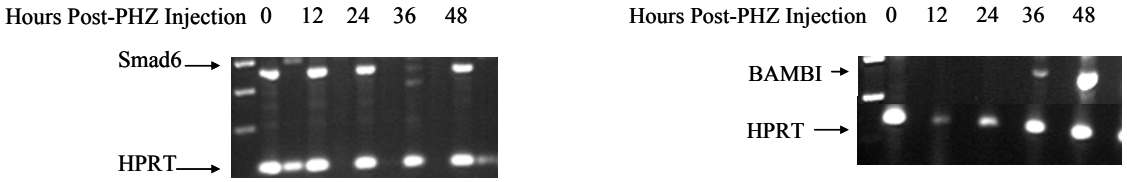


Figure 5-1. Expression in Smad6 and BAMBI during the response to acute anemia. RT-PCR analysis of Smad6 and BAMBI expression in the spleens of C57BL/6 mice during the recovery from acute anemia. Arrows indicate the positions of the Smad6, BAMBI and control HPRT specific products.

Another regulator of BMP signaling showing a suggestive expression profile in response to acute anemia is BAMBI. BAMBI is a TGF-β pseudoreceptor (Onichtchouk, Chen et al. 1999). It is related to type I receptors but lacks the intracellular kinase domain. BAMBI acts by competitively binding TGF-β signaling molecules such as BMP4. We examined BAMBI expression during the response to acute anemia and found that BAMBI is not expressed initially but is upregulated at 36 hours post-PHZ

injection and is highly expressed by 48 hours. Thus, BAMBI may be acting to negatively regulate BMP4 signaling following the main expansion of stress BFU-E.

Additional studies are needed to determine and confirm any potential role for Smad6 and BAMBI in the regulation of stress BFU-E development. Nonetheless, both represent strong candidates for such regulation. No doubt, many additional regulators await discovery.

Role of hypoxia

In chapter 3, hypoxia was identified as having a dramatic effect on stress erythropoiesis by increasing both the size and number of stress BFU-E. Indeed, hypoxia has the most dramatic effect of all the signals we have studied. It is clear that hypoxia's effect goes beyond its upregulation of BMP4 expression. As discussed in chapter 3, while BMP4 addition results in a 6 fold induction of stress BFU-E, hypoxia alone results in a 22 fold induction. So, hypoxia is obviously important, but it remains to be determined which genes, other than BMP4, hypoxia upregulates which specifically have an effect on stress BFU-E expansion. Many genes are induced by hypoxia, so identifying which ones are specifically important to stress BFU-E development may be difficult. Nonetheless, there are notable candidates with which we can initiate this undertaking.

ATF4 has been shown to be an important regulator of stress erythropoiesis. As discussed in chapter 1, ATF4-/- mice exhibit a similar phenotype with *f/f* mice, namely they have a severe fetal anemia and an impaired response to acute anemia (Lariviere and Perry, unpublished observations; (Masuoka and Townes 2002). However, ATF4 has a more ubiquitous role in stress response pathways. In fact, ATF4 is a key regulator of

multiple stress pathways (Kim and Sinha 2003). In terms of acute anemia, ATF4 has been shown to be activated by hypoxia (Blais, Filipenko et al. 2004). Given the phenotype of ATF4-/- mice as well as ATF4's central role in regulating stress responses, we examined the ability of ATF4-/- mice to form stress BFU-E and BMP4 responsive cells under both hypoxic and normoxic conditions. As shown in figure 5-2, ATF4-/- mice do not exhibit the normal increase in stress BFU-E under hypoxic conditions (compare with figure 3-4). Thus, it appears that ATF4, activated by hypoxia, regulates stress BFU-E expansion.

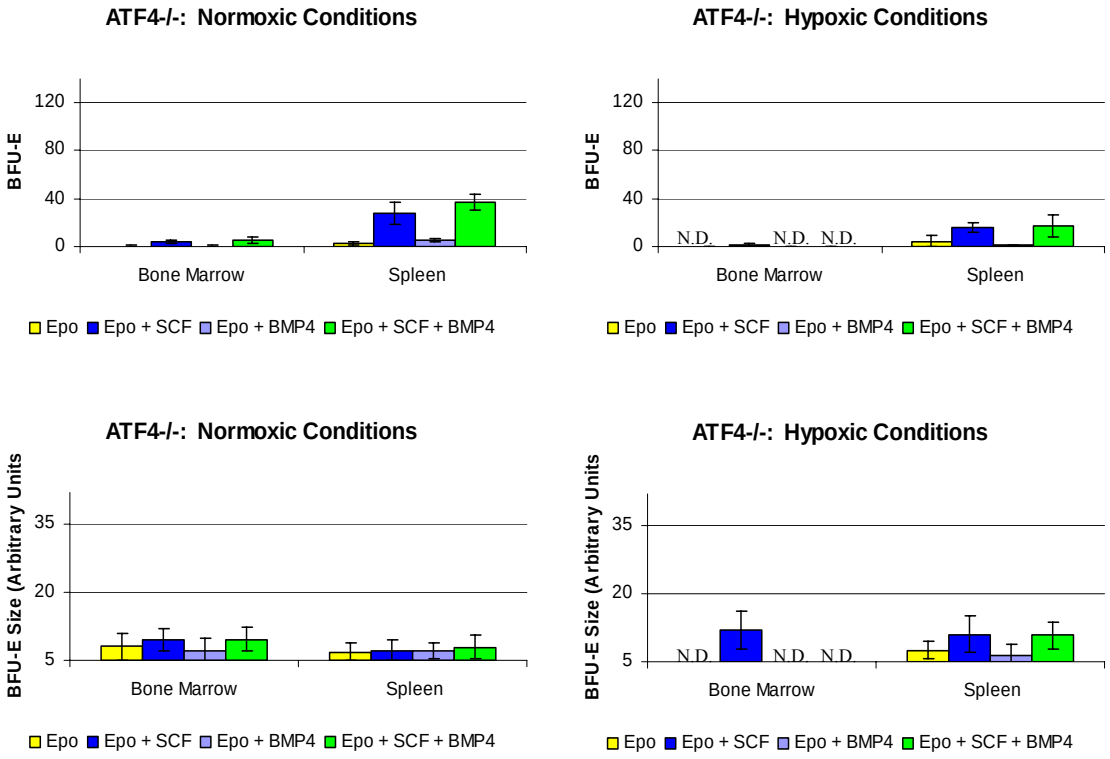


Figure 5-2. Hypoxic vs. normoxic analysis of BFU-E from ATF4 mutant mice. Colony assays on splenocytes obtained from ATF4-/- mice were performed as described in figure 3-1 in both normoxia (20% O2) and hypoxia (2% O2). Cultures contained 3U/ml Epo as well as Epo + 50 ng/ml SCF and/or 15 ng/ml BMP4 as indicated. Colony number was determined as in figure 3-1 and colony size was determined as in figure 3-2B.

We also examined the effect of SCF on stress BFU-E size and number. In chapter 3, we showed that hypoxia and SCF synergize to increase stress BFU-E number (figure 3-4); however, this expansion does not occur in ATF4-/- mice (figure 5-2). Chapter 3 also showed that both hypoxia and SCF increases stress BFU-E size (figure 3-2 and 3-4); however, ATF4-/- mice do not exhibit this increase in stress BFU-E size (figure 5-2). These data further show that hypoxia acts through ATF4 in regulating stress BFU-E development. It also reveals that SCF likely acts through ATF4. Indeed, it has been demonstrated that RSK, a downstream transcription factor of the Kit/SCF signaling pathway, activates ATF4. Figure 5-3 shows our working model where BMP4, hypoxia, and SCF cooperatively regulate stress erythropoiesis with hypoxia and SCF signaling converging on the activation of ATF4. Future studies will seek to determine which downstream factors are activated by Smad5 and ATF4.

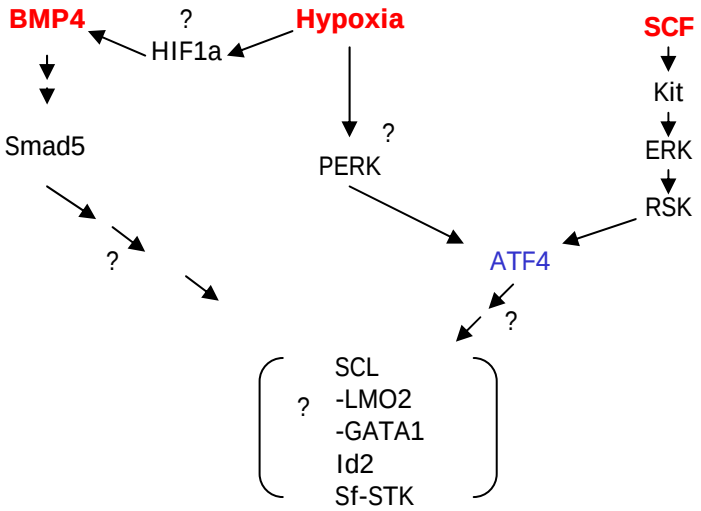


Figure 5-3. Working model for the signaling network regulating stress BFU-E expansion.

Stress BFU-E relevance to chronic anemia

As discussed in chapter 1, acute anemia is rarely a problem in the developed world. Severe blood loss, for instance, is treated with transfusion. Nonetheless, understanding how expansive erythropoiesis occurs in response to acute anemia could lead to the treatment for various anemic disorders by manipulating the stress BFU-E response toward therapeutic ends. Assuming there is a human equivalent of stress BFU-E, which remains to be determined by future studies, stress BFU-E could be exploited for the purposes of treating chronic anemia, which afflicts millions world-wide.

Initial studies on mice with β-thalassemia (Th-3/Th-3) have revealed that Th-3/Th-3 mice lack BMP4 responsive cells but maintain elevated numbers of stress BFU-E in the spleen (figure 5-4, compare with figure 1-1).

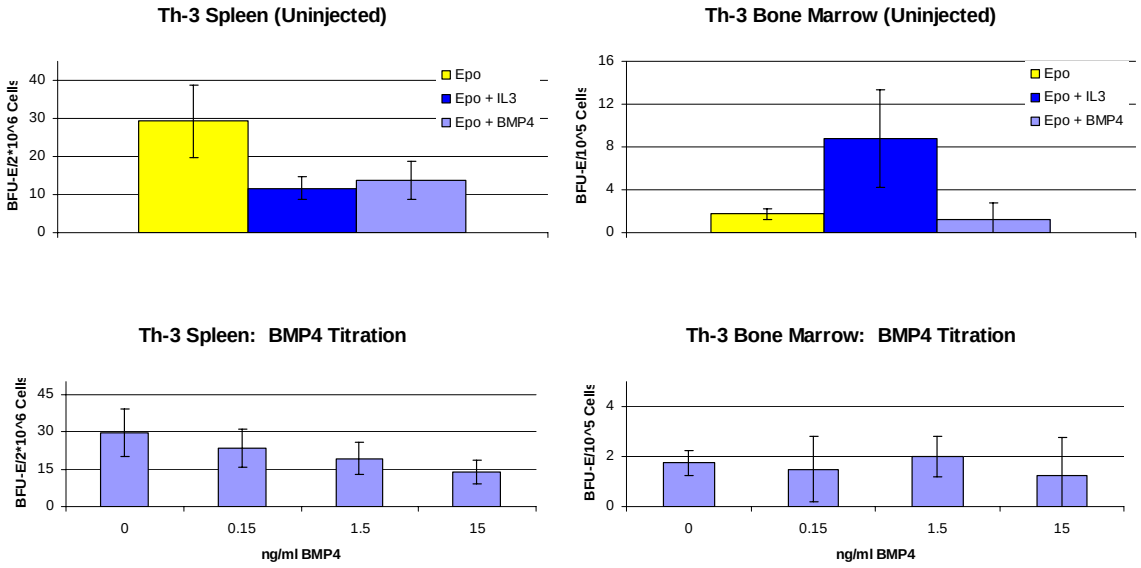


Figure 5-4. Analysis of stress BFU-E and BMP4 responsive cells in Th-3 mice. Colony assays were performed on untreated spleens from Th-3/Th-3 mutant mice as described in figure 3-1. 3U/ml Epo was added as well as 10 ng/ml IL-3 or 0.15-15 ng/ml BMP4 as indicated.

The severe chronic anemia of these mice may result in a constitutive activation of the stress BFU-E response. In order to test this hypothesis, we also examined *Bmp4* expression in these mutants. As shown in figure 5-5, Th-3/Th-3 mutants exhibit constitutive expression of BMP4.

Figure 5-5. BMP4 expression in uninjected Th-3 mouse spleen.
RT-PCR analysis of BMP4 expression in spleens derived from untreated Th-3/Th-3 mice. Arrows indicate the positions of the BMP4 and control HPRT specific products.

These data indicate that chronic anemia results in constitutive activation of the stress BFU-E response in an apparent attempt to cure the anemia. It is unclear to what extent this activation of the stress BFU-E response is successful at alleviating the anemic condition in these mutants; however, it is likely that stress BFU-E do or at least could alleviate chronic anemic conditions. Thus, knowledge of stress BFU-E regulation and development could lead to potential treatment for anemic conditions in general.

Concluding Remarks

The discovery of stress BFU-E has provided a new model for the response to acute anemia (figure 5-6). We have demonstrated that a unique class of erythropoietic progenitors responds to BMP4 by differentiating into stress BFU-E. We have characterized these progenitors as being related to short-term radioprotective cells and have demonstrated the importance of both SCF and hypoxia in their development. We

have also demonstrated the importance of HH signaling in stress BFU-E development. The discovery and characterization of stress BFU-E has also provided insight into the cellular basis of the *f* phenotype, which has intrigued hematologists for decades. Mainly, the research presented in this thesis provides the humble yet immensely satisfying continuation of previous studies and hopefully endows future researchers with new avenues to explore, refine, and build upon.

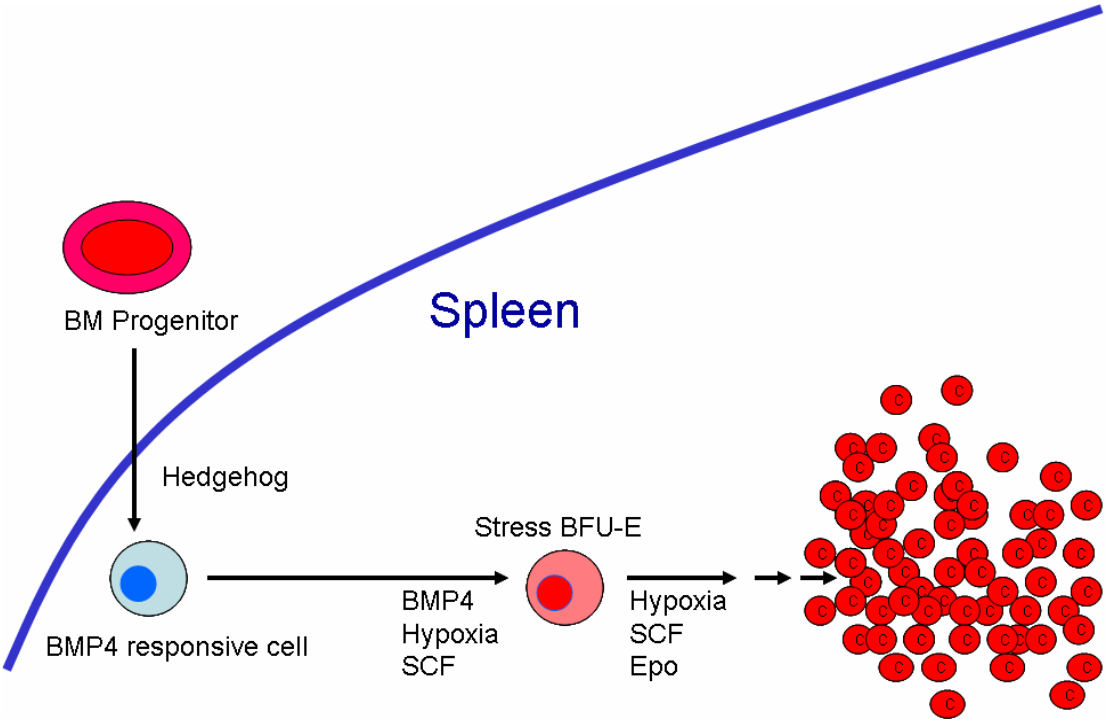


Figure 5-6. Current model for the response to acute anemia.

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