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

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Abstract

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

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Introduction

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

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

The expansive erythropoiesis of the fetal liver is thought to be mechanistically similar to stress erythropoiesis in adult mice. In response to acute anemia, erythropoiesis is up-regulated in the spleen and robustly produces new erythrocytes (Lenox et al., 2005). Fetal liver erythropoiesis and adult stress erythropoiesis are in stark contrast to adult bone marrow erythropoiesis, which is primarily homeostatic. This relationship between fetal liver and adult stress erythropoiesis is evident in mice with a mutation at the flexed-tail (f) locus. ff embryos exhibit a severe microcytic, hypochromic fetal anemia (Bateman and Cole, 1972; Gruneberg, 1942a,b). The livers of these embryos are smaller and have only about half the normal number of CFU-E (Cole and Regan, 1976). Despite this severe defect in the erythroid lineage, the number of CFU-S in the fetal liver is not different from the control embryos suggesting this defect is specific to the erythroid lineage (Bateman and Cole, 1972; Gruneberg, 1942a,b; Thompson et al., 1966). The anemia is most severe early in fetal development and gradually improves such that the anemia resolves about 2 weeks after birth. This time corresponds to the development of the bone marrow as the primary erythropoietic organ. As adults, ff mice have normal blood values but are unable to respond rapidly to acute erythropoietic stress. When challenged with an acute anemia, the control mice respond by inducing the rapid expansion of erythroid progenitors in the spleen but in the ff mice, this response is delayed (Coleman et al., 1969; Lenox et al., 2005). These data demonstrate that ff mice have a defect in expansive erythropoiesis both during fetal life and during response to acute anemia in the adult.

We have previously shown that ff mice have a mutation in the Smad5 gene, which results in a defect in their ability to respond to acute anemia (Hegde et al., 2007; Lenox et al., 2005). Smad5 functions as a receptor activated Smad downstream of the receptors for BMP2, 4 and 7 (Huber et al., 1998; Massague, 2000; Massague and Chen, 2000). Previous work has implicated BMP’s, in particular BMP4, in the development of mesodermal cells that will give rise to hematopoietic cells early in development (Huber et al., 1998). We have observed that the BMP4 is rapidly induced during expansive erythropoiesis in the spleen and is involved in the mobilization of a distinct population of stress erythroid progenitors, which we term stress BFU-E, during the recovery from acute anemia (Lenox et al., 2005). Similar to human fetal liver BFU-E, stress BFU-E exhibit faster doubling times and are capable of forming BFU-E colonies in the presence of Epo alone. These observations suggest that fetal liver erythropoiesis may be mechanistically similar to splenic stress erythropoiesis and utilize the BMP4/Smad5 signaling pathway to expand progenitors.

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

Methods

Preparation of fetal liver

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

Immunofluorescence studies

Paraffin embedded fetal liver sections were deparaffinized and rehydrated through an ethanol series as described previously (Lenox et al., 2005). Sections were incubated for 1 h at each temperature with the primary antibody and then washed with PBS. Anti-BMP4 (Novocastra Laboratories/Vector Laboratories, Burlingame, CA) and Smad5 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used at the manufacturer’s recommended dilutions (Chagraoui et al., 2003; Lenox et al., 2005). Alexa Fluor (Molecular Probes, Eugene, OR) fluorescent secondary antibodies were then added. For negative controls, appropriate isotype controls were used. Sections were mounted in Slowfade (Molecular Probes, Eugene, OR) and analyzed by digital microscopy (Olympus BX-60 Epi-Fluorescence Digital digital microscope).

Erythroid progenitor assays

5 × 10⁵ fetal liver cells were plated in methylcellulose media containing 3 U/ml Epo (M3334 Stem Cell Technologies, Vancouver, BC). 15 ng/ml BMP4 (R&D Systems, Minneapolis, MN) and 2.5 ng/ml IL-3 (Sigma, St. Louis, MO) were added wherever indicated. Replicates were plated and at least three independent experiments were performed for each time point. BFU-E was scored by acid Benzidine staining after incubation for 5 days (Finkelstein et al., 2002).

TUNEL assay for apoptosis

E14.5 fetal liver sections were prepared and permeabilized as described earlier. Apoptosis in tissue sections was detected using an in situ cell death
detection fluorescence of activated cells sorting analysis

E14.5 fetal liver single cell suspensions were stained with biotin-conjugated Mouse lineage panel (BD Pharmingen, San Diego, CA). Biotinylated Sca-1 (BD Pharmingen, San Diego, CA) was added to the cocktail and the cells were negatively selected for using EasySep Biotin Selection Kit for Mouse Cells (StemCell Technologies, Vancouver, BC, Canada). Cells were stained with fluorescein isothiocyanate-conjugated anti-Kit antibody (BD Pharmingen, San Diego, CA) and phycoerythrin-conjugated anti-CD31 (Mec 13.3) (BD Pharmingen, San Diego, CA) and sorted using a Coulter Elite ESP flow cytometer (Coulter Electronics, Hialeah, FL) for Lin−Sca1−cKit+ CD31+CD45− erythroid cell populations (Baumann et al., 2004). Fetal liver MEPs were sorted as previously described (Lenox et al., 2005; Traver et al., 2001). 25,000 cells each were plated in triplicates in methylcellulose and scored as described above.

Differentiation of fetal erythroid progenitors

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

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

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

Western blot analysis

Whole fetal livers were dissected from mutant and control mice on the indicated days. The livers were lysed in RIPA buffer and whole cell lysates were generated. Equal amounts of protein were loaded on SDS-PAGE gels and blotted to nitrocellulose membrane. The membranes were blocked and then incubated with anti-Smad5, anti-Phospho Smad1, 5, 8 (Cell Signaling, Danver, MA) or anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. In separate experiments, western blots of fetal liver lysates were probed with anti-BMP4 antibodies (Novocastra Laboratories/Vector Laboratories, Burlingame, CA) and then probed with anti-actin antibodies as a loading control. The membranes were washed and incubated with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase. Protein antibody complexes were visualized using ECL plus western blotting detection system (GE Healthcare, Buckinghamshire, UK).

Results

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

In our previous work we showed that during the recovery from acute anemia, a specialized population of stress erythroid progenitors, which we termed stress BFU-E, are rapidly expanded in the adult spleen. The properties of these BFU-E are distinct from bone marrow steady state BFU-E. Stress BFU-E are resident in the spleen and rapidly form large BFU-E colonies when cultured in the presence of Epo alone (Lenox et al., 2005). Similar to the spleen, some fetal liver erythroid progenitors have characteristics distinct from their adult bone marrow counterparts. While bone marrow BFU-E require the presence of a BPA along with Epo to form BFU-E, studies in human fetal liver have shown that some fetal BFU-E can respond to Epo alone without addition of any BPA (Emerson et al., 1989; Valtieri et al., 1989). In many ways, these fetal liver erythroid progenitors are more similar to the stress BFU-E, which require BMP4, SCF and hypoxia for their expansion (Lenox et al., 2005; Perry et al., 2007). Iff mice exhibit a delayed expansion of stress BFU-E because of impaired BMP4 dependent signaling. Because iff mutant embryos also have a defect in the fetal liver erythropoiesis, we looked for the presence of stress BFU-E in the fetal liver and asked whether these cells were defective in the iff mice. We observed that there is a significant expansion in the relative number of stress BFU-E at E15.5 in control mice, which correlates with the maximal erythropoietic activity in the organ and this expansion is sustained until E16.5 (Fig 1A). At E18.5, when most of the erythropoietic activity in the fetal liver is finished, the number of stress BFU-E had decreased to the low levels observed at E12.5. In contrast, the expansion in the relative numbers of stress BFU-E in iff embryos is not observed until E16.5. At this time iff and control embryos exhibit similar numbers of stress BFU-E, which are not significantly different from high levels of stress BFU-E observed in control embryos at E15.5. Similar to control embryos, the relative numbers of stress BFU-E in iff embryos decreases between E16.5 and E18.5 such that by E18.5 the number of stress BFU-E is similar to the low levels observed at E12.5. These data demonstrate that iff embryos exhibit not only a delay in the increase in the relative numbers of stress BFU-E but also a diminished response when compared to control embryos.

It has been shown that iff mice have smaller fetal livers and contain decreased number of mature erythroid progenitors and identifiable erythroblasts (Cole and Regan, 1976). We determined the number of cells in the fetal liver at various time points and plotted the number of stress BFU-E colonies per fetal liver (Fig 1B). The difference between stress BFU-E in iff and control fetal livers is even more striking when the smaller fetal livers of the iff embryos are taken into account. At E15.5, there is approximately a 7 fold decrease in the number of stress BFU-E in iff fetal livers. The maximal increase in stress BFU-E in iff embryos was observed at E16.5. Similar to what we observed when relative numbers of stress BFU-E were examined, the total number of stress BFU-E at E16.5 iff embryos was not significantly different than control E16.5 embryos and this number, although less, was not significantly fewer than the total number of stress BFU-E observed in E15.5 control embryos. These data mirror the data for the relative numbers of stress BFU-E and show that, even though the iff fetal liver was capable of expanding stress BFU-E at E16.5 similar to controls, the impaired expansion at E15.5 makes their
response significantly weaker. The anemia of the $ff$ mutant embryos is most severe early in fetal development (Gruneberg, 1942a,b; Kamenoff, 1935). Our data suggests that the delayed and diminished expansion of stress BFU-E in $ff$ embryos results in the fetal anemia.

Unlike the spleen, which contains primarily stress BFU-E, the fetal liver also contains BFU-E, which are similar to bone marrow steady state BFU-E and require both a BPA and Epo to form a BFU-E colony. We assayed for steady state BFU-E at various times during embryogenesis by culturing fetal liver cells in media containing Epo alone or Epo and IL-3, scoring total BFU-E colonies (Epo+IL-3) and then subtracting out the number of Epo-only BFU-E. We observed that at no time point is there a significant difference in the relative numbers of steady-state BFU-E in the control mice compared to the $ff$ mice (Fig. 2A). Although the relative numbers of steady state BFU-E are similar between $ff$ and control mice, we took into account the smaller fetal livers present in the $ff$ embryos and calculated the total number of “steady state” BFU-E per fetal liver. In control embryos the peak expansion of steady state BFU-E is observed at E15.5 and E16.5. After this time the number of steady state BFU-E decreases such that at E18.5 low levels are observed. In contrast, $ff$ embryos exhibit a small, but significant deficit at E14.5 and E15.5 (Fig. 2B). By E16.5, however, the total number of steady state BFU-E in $ff$ embryos is not significantly different than control E16.5 embryos or E15.5 embryos. These data show that the delayed and diminished expansion of steady state BFU-E in the mutant embryos is similar to what we observed for stress BFU-E. However, the defect in steady state BFU-E is much less severe in that the difference at E14.5 or E15.5 is approximately 2 fold, which parallels the difference in fetal liver cellularity.

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

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

Fig. 2. Expansion of steady state BFU-E in the fetal liver of $ff$ and control mice. Fetal liver cells were isolated on the indicated days during fetal development and plated in methylcellulose media containing Epo + IL-3. BFU-E were scored after 5 days in culture. The number of Epo+BPA BFU-E was calculated by subtracting the number of Epo-only BFU-E from the number of Epo+IL-3 BFU-E. (A) Relative BFU-E per $5 \times 10^5$ fetal liver cells. (B) Total number of steady state BFU-E per fetal liver. The significance of the differences between $ff$ and control discussed in the text are indicated on the figure. If no other $p$ value is given then the difference was not significant.
BFU-E suggests that the decrease in stress BFU-E would have a significant impact on fetal red cell production (Lenox et al., 2005; Perry et al., 2007).

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

We investigated the expression of BMP4 between E12.5 and E18.5 when erythropoiesis in the fetal liver is active. We observed very little expression of BMP4 at E12.5, when the fetal liver is still being seeded by hematopoietic cells from yolk sac and AGM regions. BMP4 expression starts at approximately E13.5 and reaches maximal levels by E14.5, which coincides with the maximal erythropoietic activity in the organ (Cole and Regan, 1976) (Fig. 3A). Thereafter, the BMP4 protein level decreases, reaching basal levels by E18.5. In contrast, f/f embryos exhibit very little expression of BMP4 protein up to E14.5. However at E15.5, BMP4 expression is observed and reaches maximal levels by E16.5; which coincides with the maximal erythropoietic activity in f/f mice (Cole and Regan, 1976) (Fig. 3A). This delay is similar to the delay in the expression of BMP4 seen in the spleens of adult f/f mice following induction of acute anemia (Lenox et al., 2005). We further compared the expression of f/f fetal liver BMP4 expression with control fetal liver expression by western blot analysis. We examined expression at E14.5, 15.5 and 16.5; timepoints when the differences in stress BFU-E expansion were observed. Fig. 3B shows that at E14.5 control fetal livers show significantly more BMP4 expression by western blot. Over the next 2 days of development, the expression of BMP4 decreases in control embryos while it increases in f/f fetal livers. These data support the idea that f/f embryos exhibit a defect in BMP4 expression in the fetal liver.

BMP4 expression does not co-localize with CD45+ or Kit+ cells, which shows that hematopoietic progenitor cells in the fetal liver are not the source of BMP4. This situation is similar to what we have observed in the adult spleen during the recovery from acute anemia (P. Porayette and RF Paulson unpublished observations). However, when we stain fetal liver sections with antibodies to hepatocyte specific markers, we observed that a subset of BMP4 expressing cells were hepatocytes. In addition, staining for stromal cell markers, cytokeratin 8 (CK8) and α-smooth muscle actin (ASMA) demonstrated that BMP4 is also expressed by stromal cells in the fetal liver (Supplementary Fig. 1).

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**Fig. 3. Expression of BMP4 in the fetal liver of f/f and control mice.** (A) (top) Fetal livers from control and f/f embryos isolated at the indicated days were sectioned and stained with anti-BMP4 antibodies. (Bottom) Negative control staining with isotype control antibodies of fetal liver sections from C57BL/6-f/f and C57BL/6 control embryos. (B) Western blots of whole fetal liver lysates from the indicated days of development probed with anti-BMP4 antibodies and anti-actin antibodies as a loading control.
**BMP4 increases the number of stress BFU-E in vitro**

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

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

Our previous work showed that BMP4 had no effect on bone marrow steady state BFU-E (Lenox et al., 2005). However, our observation that the total number of steady state BFU-E in the f/f fetal liver is decreased approximately 2 fold suggests that BMP4 signaling may play a role the expansion of steady state BFU-E. We tested this possibility by treating E12.5 fetal liver cells from f/f and control embryos with BMP4 and determined whether the number of steady state BFU-E increased. Fig. 4B shows that stress BFU-E are significantly increased when BMP4 is added to the culture, but this treatment had no effect on the number of steady state BFU-E. As shown in Fig. 4A, f/f fetal

![Fig. 4. Effect of BMP4 on Epo only and Epo+IL-3 BFU-E formation. (A) (left) Fetal liver cells were isolated on the indicated days during fetal development and plated in methylcellulose media containing Epo alone or Epo+BMP4. The fold increase in the number of BFU-E (Epo+BMP4/Epo-only) was calculated at each time point. Significant increases in the number of Epo-only BFU-E following BMP4 treatment are indicated on the figure. (right) Western blot of cell lysates from E14.5 f/f and control fetal liver cells probed with anti-Phospho-Smad1, 5, 8, anti-Smad5 and β-actin as a loading control. (B) Fetal liver cells were isolated on E12.5 and plated in the indicated media±BMP4 to test the effect of BMP4 on the formation of Epo-only or Epo+IL-3 BFU-E. Significant differences are indicated on the figure. If no other p value is given then the difference was not significant.](image-url)
liver cells did not respond to BMP4 treatment by increasing the number of stress BFU-E. Furthermore, steady state BFU-E from \( f/f \) exhibited responses indistinguishable from control embryos following treatment with BMP4. These observations are consistent with our model that BMP4 does not affect the number of steady state BFU-E and that the \( f/f \) mutation primarily affects the stress BFU-E in the fetal liver.

The \( f/f \) mutation does not cause increased apoptosis or cause defects in erythroid differentiation

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

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

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

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

Stress BFU-E are present in the yolk sac and are reduced in \( ff \) embryos

Recent work in zebrafish has shown that Smad5 and Smad1 are required for definitive hematopoiesis (McReynolds et al.,

Fig. 5. \( ff \) embryos do not exhibit excessive apoptosis or defects in erythroid terminal differentiation. (A) Fetal liver sections from E14.5 C57BL/6-ff and C57BL/6 control embryos were stained for apoptotic cells by TUNEL assay. Bright field views are shown to the left. (B) Fetal liver cells from E14.5 C57BL/6-ff and C57BL/6 control embryos were incubated in erythroid differentiation media for 48 h. The cells prior to culture (top) and after culture (bottom) were stained with Wright–Giemsa and o-Dianisidine stain to identify hemoglobinized cells.
Murine Smad5−/− embryos die prior to the onset of definitive erythropoiesis and no studies using conditional alleles of Smad5 have examined the role of Smad5 in the development of definitive erythroid progenitors (Yang et al., 1999). Erythroid progenitors do not develop de novo in the fetal liver and it has been proposed that stem cells and progenitors from the yolk sac and AGM region of the embryo seed the fetal liver (McGrath and Palis, 2005; Palis et al., 1999). Definitive erythroid progenitors, BFU-E, are first observed in the yolk sac at E8.25. These BFU-E rapidly expand during the next 2 days of development and are thought to seed the fetal liver with an initial wave of definitive erythroid progenitors. In order to determine whether stress BFU-E are present in the yolk sac, we directly plated yolk sac cells from E10.5, E11.5 and E12.5 embryos in methylcellulose media containing Epo or Epo + IL-3 (Fig. 7A). In control E10.5 embryos, yolk sac cells generated significantly more total BFU-E than mutant E10.5 yolk sac cells; while at E11.5 and E12.5 the total number of BFU-E generated in control versus mutant yolk sacs was not significantly different (Fig. 7B). When we analyzed stress BFU-E, we observed...
Fig. 7. Yolk sac contains stress BFU-E and progenitor cells that develop into stress BFU-E when Yolk sac cells are plated on fetal liver stromal cells. (A) Schematic of the direct plating of yolk sac cells and co-culture with AFT024 cells prior to plating. (B) Total number of BFU-E (stress BFU-E+ Steady State BFU-E) present in the yolk sac of C57BL/6-ff and C57BL/6 control mice on the indicated days of embryonic development. *p<0.05 when E10.5 control yolk sacs were compared with E10.5 mutant and E11.5 mutant or control yolk sacs. (C) Left panel: Yolk sac cells from E10.5, E11.5 and E12.5 control and ff embryos were directly plated in media containing Epo alone or Epo+IL-3. BFU-E were scored 5 days later. Right panel: Yolk sac cells from E10.5 and E11.5 control and ff embryos were co-cultured on AFT024 fetal liver stromal cells for 48 h and then plated in methylcellulose media containing Epo alone or Epo+IL-3. BFU-E were scored 5 days later. Epo+IL-3 BFU-E were calculated by subtracting the number of Epo-only BFU-E from the total number of BFU-E formed in Epo+IL-3. The significance of the differences between different time points in ff and control yolk sacs as discussed in the text are indicated on the figure. If no other p value is given then the difference was not significant. (D) The fold increase in stress BFU-E observed when E10.5 and E11.5 yolk sac cells were co-cultured AFT024 cells.
that at E10.5, both control and mutant yolk sacs contained similar relative numbers of stress BFU-E. However, at E11.5, the number of stress BFU-E had significantly increased in the control yolk sac when compared to control E10.5 yolk sacs (p<0.05). In contrast, the relative number of stress BFU-E in E11.5 ff yolk sacs was unchanged from the number observed at E10.5 and significantly less than the number observed in control E11.5 yolk sacs (p<0.05) (Fig. 7C). At E12.5, both mutant and control yolk sacs exhibited similar, low relative numbers of stress BFU-E. This analysis also demonstrates that the percentage of total BFU-E that are stress BFU-E dramatically increases in control yolk sacs from E10.5 to E11.5 (6.2% stress BFU-E at E10.5 to 45% stress BFU-E at E11.5). This increase is not observed in ff yolk sacs where the percentage of total BFU-E that are stress BFU-E remains constant (13.2% stress BFU-E at E10.5 and 11% stress BFU-E at E11.5). This increase in the percentage of stress BFU-E occurs despite the fact that there is no significant difference in the relative numbers of total BFU-E between control and mutant yolk sacs. These data show that the expansion of stress BFU-E in the ff mutant yolk sacs is defective.

The observation that ff yolk sacs exhibit a defect in the expansion of stress BFU-E at E11.5 is not consistent with our observation that at E12.5 ff and control fetal livers contain similar numbers of stress BFU-E. One possibility is that stress BFU-E could develop in the AGM region and then seed the fetal liver. We tested whether AGM region cells could generate stress BFU-E when plated in methylcellulose media, however no BFU-E were observed in these cultures even when IL-3 was included in the media (Data not shown). These results are consistent with earlier reports that the AGM region is not a site of erythropoiesis in the mouse embryo (Godin et al., 1999). A second possibility is that progenitors from the yolk sac differentiate into stress BFU-E when they seed the fetal liver. These progenitors are not stress BFU-E, but would differentiate into stress BFU-E in response to a signal or signals in the fetal liver microenvironment. Recently we have demonstrated that bone marrow cells can differentiate into stress BFU-E when they come in contact with the spleen microenvironment (J. Perry and RF Paulson unpublished observations). Using these data as a guide we tested whether co-culturing yolk sac cells on fetal liver stromal cells could induce the development of stress BFU-E (Fig. 7A). We used AFT024 fetal liver stromal cells, which were derived from E14.5 fetal liver (Moore et al., 1997). These cells have been shown previously to support the expansion of stem and progenitor cells in culture (Chagraoui et al., 2003; Moore et al., 1997). Plating control E10.5 yolk sac cells on AFT024 cells led to a 10 fold increase (p=0.004) in the number of stress BFU-E when compared to direct plating (Fig. 7D). In contrast ff yolk sac cells exhibited a small (~2 fold) increase in stress BFU-E when compared to direct plating that was not statistically significant (p=0.078). However the opposite was observed when E11.5 yolk sac cells were co-cultured with AFT024 cells. Co-culturing control yolk sac cells resulted in a small increase (~2 fold, p=0.08), while ff yolk sac cells showed a 6 fold increase in stress BFU-E (p<0.001) when compared to direct plating. Co-culturing either mutant or control E12.5 yolk sac cells with AFT024 cells did not result in an increase in stress BFU-E. In addition to the increase in stress BFU-E, co-culturing on AFT024 cells lead to an increase in the percentage of total BFU-E that are stress BFU-E such that stress BFU-E become the predominate form of BFU-E in the cultures (Fig. 7C). This increase in the percentage of stress BFU-E was not observed when E10.5 mutant yolk sac cells were co-cultured with AFT024 cells. These data show that E10.5 control yolk sacs contain more progenitors with the potential to become stress BFU-E when co-cultured with fetal liver stromal cells than E10.5 ff yolk sacs. However, the situation is reversed at E11.5 where ff yolk sacs exhibit a greater potential to generate stress BFU-E following co-culture with fetal liver stromal cells (Fig. 7D). These observations suggest a model where definitive hematopoietic progenitors in the yolk sac interact with fetal liver stromal cells and develop into stress BFU-E. Our data suggests that despite the deficit in stress BFU-E in E11.5 yolk sacs of ff embryos, they contain progenitors that can develop into stress BFU-E when they interact with a signal or signals in the fetal liver microenvironment. It appears that the maximum development of these progenitors is delayed in ff yolk sacs from E10.5 to E11.5, but despite this delay, the ff E12.5 fetal livers contain similar numbers of stress BFU-E as control fetal livers.

Discussion

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

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

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

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

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

The prevailing model in the field is that definitive progenitors in the yolk sac will seed the fetal liver. Paradoxically, we observe that despite the defect in stress BFU-E in the f/f yolk sac, E12.5 f/f mutant fetal livers have numbers of stress BFU-E that are not significantly different from controls. Recent work from our lab may explain this discrepancy. In the adult spleen, all stress BFU-E differentiate during the recovery from acute anemia, so they must be replaced. We observe that bone marrow progenitors can replenish the BMP4 responsive stress BFU-E if they migrate to the spleen. These data suggest that signals in the spleen microenvironment cause the differentiation of bone marrow progenitors into stress BFU-E (J. Perry and RF Paulson unpublished data). Our data shows that co-culturing yolk sac cells on fetal liver stromal cells can also promote the development of stress BFU-E. This observation suggests that the yolk sac produces progenitor cells that respond to signals in the fetal liver microenvironment, which promote their differentiation into stress BFU-E. In control embryos we observe a larger increase in stress BFU-E when E10.5 yolk sac cells are co-cultured than when E11.5 cells are used. The opposite was true when f/f yolk sac cells were used. In this case, co-culturing f/f E11.5 yolk sac cells with AFT024 resulted in significantly more stress BFU-E and a preferential increase in stress BFU-E. From these observations we conclude that f/f mutant yolk sacs exhibit a delay in the generation of progenitor cells in the yolk sac that can respond to the fetal liver stromal signals, however, they are able to overcome this delay such that by E12.5 the number of stress BFU-E and CD31+Kit+Sca1−Lin− cells (Data not shown) in the fetal liver are approximately equal to control mice. Earlier work supports this model where the yolk sac progenitors respond to signals in the fetal liver and differentiate into definitive erythroid progenitors. These experiments showed that co-culture of yolk sac with the fetal liver rudiment induced the expansion of definitive erythropoietic progenitors (Cudélec et al., 1981). This expansion was promoted by a signal secreted by the liver. The identity of the signal in the fetal liver is unclear. Our experiments used AFT024 cells, which were made from E14.5 fetal liver (Moore et al., 1997) and express BMP4 (P. Porayette and RF Paulson unpublished observations). However, treatment of yolk sac cells with BMP4 did not affect the number of stress BFU-E or the ratio of stress BFU-E to steady state BFU-E (P. Porayette and RF Paulson unpublished observations). Future work will be needed to identify the signals involved in this process.

The most penetrant phenotype of f/f embryos is the severe fetal anemia, which is in contrast to the lethality of the Smad5−/− embryos. Our previous work showed that f mutant mice have a defect in splicing of the Smad5 gene, where the majority of the Smad5 mRNA is one of two mis-spliced mRNAs. One of these aberrant splice forms when overexpressed in an osteoblast cell line can inhibit BMP4 dependent differentiation. Furthermore, f/fSmad5−/− mice exhibit a less severe phenotype than f/f mice, which suggests that f/f represents a gain of function allele. So these data lead to the question of why f/f mutant embryos survive, but Smad5−/− embryos die. Recently, we have demonstrated that the splicing defect in f/f mice is caused by a mutation in an intronic splicing regulatory element.
In addition our analysis has shown that the splicing defect is tissue specific in its severity, in that some tissues exhibit low levels of mis-spliced Smad5 mRNA. Furthermore the splice forms vary between tissues (Hegde et al., 2007). These data suggest that f represents an allele of Smad5 that specifically affects stress erythropoiesis and in some strains, where tail flexures and white belly spots are penetrant, chondrogenesis and the development of melanocytes are also affected. However, other tissues that express Smad5 are unaffected.

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.047.

References