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Requirement for p38α in Erythropoietin Expression: A Role for Stress Kinases in Erythropoiesis

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Summary

Activity of the p38α MAP kinase is stimulated by various stresses and hematopoietic growth factors. A role for p38α in mouse development and physiology was investigated by targeted disruption of the *p38*α locus. Whereas some *p38*$\alpha^{-/-}$ embryos die between embryonic days 11.5 and 12.5, those that develop past this stage have normal morphology but are anemic owing to failed definitive erythropoiesis, caused by diminished erythropoietin (Epo) gene expression. As p38α-deficient hematopoietic stem cells reconstitute lethally irradiated hosts, p38α function is not required downstream of Epo receptor. Inhibition of p38 activity also interferes with stabilization of *Epo* mRNA in human hepatoma cells undergoing hypoxic stress. The p38α MAP kinase plays a critical role linking developmental and stress-induced erythropoiesis through regulation of Epo expression.

Introduction

Exposure to environmental stress and strong deviations from normal conditions, including elevated temperature, increased or decreased osmolarity, nutrient deficiency, and decreased oxygen tension, results in activation of specialized signal transduction pathways and gene expression programs that allow the organism to adapt, survive, and maintain homeostasis. Stress-activated signaling pathways may also be involved in development. An important role in adaptation, homeostasis, and specialized stress responses is played by the stress-activated protein kinases (SAPKs). This evolutionary conserved subfamily of mitogen-activated protein (MAP) kinases (MAPKs) includes the Jun kinases (JNKs) and the p38s (Lee and Young, 1996; Su and Karin, 1996; Ip and Davis, 1998; New and Han, 1998). In budding yeast, the p38 homolog Hog1 mediates the adaptive response to increased osmolarity (Brewster et al., 1993). A similar function as well as responses to other stressors are mediated by the fission yeast homolog Spc1/Sty1/Phh1 (Kato et al., 1996; Shiozaki and Russell, 1996; Degols and Russell, 1997). In addition to stress signaling, Spc1/Sty1/Phh1 is also involved in development (Kato et al., 1996). The mammalian p38 SAPKs, which include p38α, p38β, p38γ, and p38δ, are similarly regulated (Waskiewicz and Cooper, 1995). In addition to stress stimuli, which include osmotic shock (Han et al., 1994) and hypoxia (Conrad et al., 1999), the p38 MAPKs are responsive to proinflammatory stimuli and hematopoietic growth factors (Lee et al., 1994; Crawley et al., 1997; Foltz et al., 1997; Nagata et al., 1997). The availability of highly specific p38 inhibitors (Lee et al., 1994) led to identification of a role for p38 SAPKs in regulation of inflammatory gene expression (Lee and Young, 1996; Dean et al., 1999). In addition, it was suggested that p38 SAPKs may be involved in induction of myoblast differentiation (Zetser et al., 1999; Puri et al., 2000; Wu et al., 2000). However, until now genetic analysis of p38 function in mammals has not been conducted. In particular, the role of p38 MAPKs in normal development and physiology has been little explored.

We have used a gene targeting approach to explore the function of the p38α isozyme. Here we describe the analysis of p38α-deficient mice that revealed an unexpected function for p38α in mammalian development and physiology. Whereas some *p38*$\alpha^{-/-}$ mouse embryos die between embryonic days (E) 11.5 and 12.5, probably due to a placental defect (Adams et al., 2000), those that survive beyond this point are morphologically normal but are anemic and display defective definitive erythropoiesis. This erythropoietic defect is caused by a dramatic decrease in erythropoietin (Epo) gene expression. As *p38*$\alpha^{-/-}$ hematopoietic stem cells (HSC) can fully reconstitute hematopoiesis in lethally irradiated hosts, p38α does not have an essential function in mediating downstream responses to Epo or other hematopoietic growth factors. A general role for p38 in regulation of *Epo* gene expression is suggested by inhibition of *Epo* mRNA accumulation in hypoxic human hepatoma cells upon treatment with a specific p38 inhibitor. In this system, p38 acts posttranscriptionally, most likely through stabilization of *Epo* mRNA. These results strongly suggest that p38α is an important regulator of stress erythropoiesis in addition to its role in definitive erythropoiesis during fetal development.

Results

Disruption of the Murine *p38*α Locus

To disrupt the mouse *p38*α locus, the targeting vector illustrated in Figure 1A was constructed. Homologous integration of this vector into the *p38*α locus inserts a neomycine resistance (*neo*) gene into the *p38*α open reading frame at codon 97. As the *neo* cassette contains termination codons in all three reading frames, it is expected to prevent translation of a p38α fusion protein. In addition, the N-terminal 97 amino acids of p38α are not sufficient for kinase activity. Whereas mice that were heterozygotes for the targeted *p38*α mutation (*p38*$\alpha^{+/-}$) were alive and fertile, no live *p38*α homozygote null mice were found among progeny of *p38*$\alpha^{+/-}$ intercrosses (Figure 1B; Table 1). However, analysis of timed pregnancies

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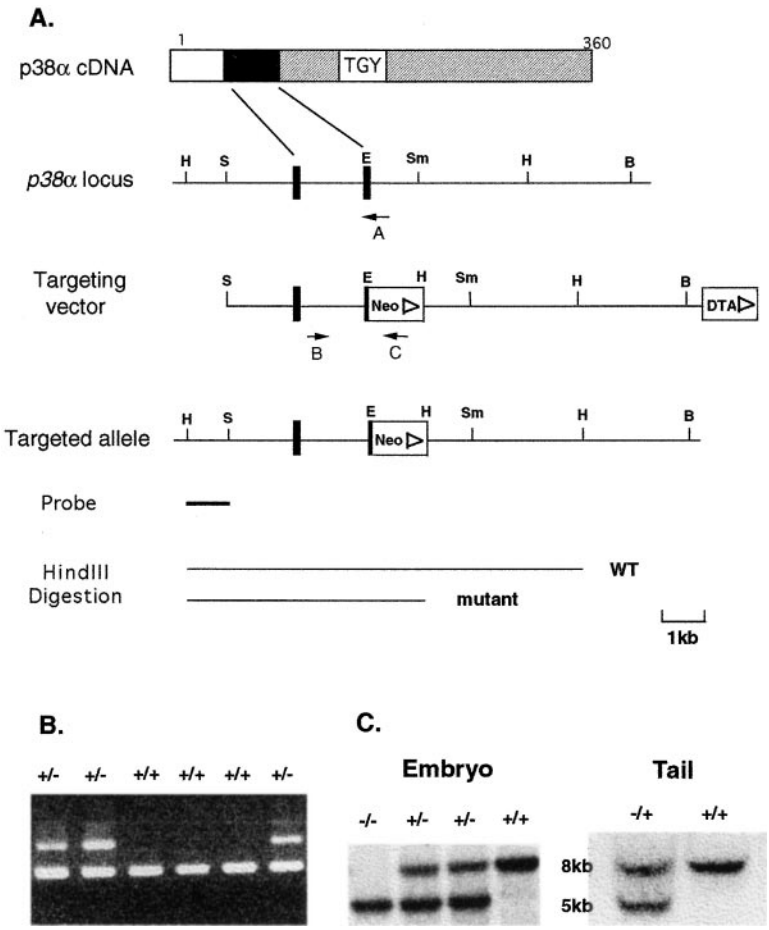

Figure 1. Targeted Disruption of the *p38*α Locus

(A) Schematic structures of *p38*α cDNA, *p38*α genomic clone, the targeting vector, and the targeted allele. TGY-activating phosphoacceptor sites. The black box within *p38*α cDNA corresponds to the two exons shown by black bars in the genomic clone of the *p38*α locus. The neomycine resistance positive selection marker (*neo*) was inserted within the EcoRI site in the second exon corresponding to codon 97 of the p38α open reading frame. The vector also includes a diphtheria toxin negative selection marker (*DTA*). Location of hybridization probe and PCR primers (A, B, and C) used for genotyping are indicated. Digestion of genomic DNA with HindIII should generate 8 kb and 5 kb fragments from wt and mutant alleles, respectively. Restriction sites are also indicated: H, HindIII; S, SacI; E, EcoRI; Sm, SmaI; B, BamHI.

(B) PCR analysis of genomic DNA obtained from live progeny of *p38*$\alpha^{+/-}$ intercrosses.

(C) Southern blot analysis of HindIII-digested genomic DNA isolated from E9.5 embryos and tails of adult mice generated by *p38*$\alpha^{+/-}$ intercrosses.

revealed that until E10.5 *p38*$\alpha^{-/-}$ embryos are present at a frequency consistent with Mendelian inheritance (Table 1; Figure 1C).

To biochemically determine whether disruption of the *p38*α locus abolished p38α expression or activity, murine embryonic fibroblasts (MEFs) were derived from E10.5 to E11.5 progeny of *p38*$\alpha^{+/-}$ intercrosses. The genotypes of the different MEF cultures were determined by Southern blotting, and p38α expression was examined by immunoblot analysis. No expression of full-length or truncated p38α polypeptides reactive with an antiserum raised against a C-terminal p38α peptide could be detected in *p38*$\alpha^{-/-}$ MEFs (Figure 2A). Similar results were obtained using two different antibodies raised against full-length p38α or an N-terminal peptide (data not shown). In addition, the level of p38α expression in *p38*$\alpha^{+/-}$ MEFs was reduced in comparison to *p38*$\alpha^{+/+}$ MEFs. No compensatory change in expression of the closely related p38β isozyme could be detected. Immunecomplex kinase assays revealed complete absence of p38α kinase activity in UV-irradiated *p38*$\alpha^{-/-}$ MEFs (Figure 2B). Thus, the mutant *p38*α allele generated by our targeting vector is a null allele.

Table 1. Genotypes of Offspring from *p38*$\alpha^{+/-}$ Intercrosses

Stage	Total (Litters)	Genotype +/+	Genotype +/−	Genotype −/−	Phenotype Live[a] −/−	Phenotype Dead −/−
Postnatal	44 (6)	13	31	0		
E19.5	11 (2)	4	7	0		
E18.5	9 (2)	4	5	0		
E16.5	31 (4)	10	15	6	6	
E15.5	37 (5)	13	21	3	3	
E14.5	40 (5)	14	20	6	6	
E14.0	8 (1)	3	4	1	1	
E13.5	52 (7)	19	29	4	3	1
E12.5	91 (10)	21	53	17	11	6
E11.5	92 (11)	33	44	15	10	5
E10.5	169 (16)	42	87	40	40	0
E9.5	81 (11)	23	36	22	22	0
E8.5	79 (10)	20	40	19	19	0

[a]Surviving embryos were defined as those with beating hearts at time of dissection.

p38$\alpha^{-/-}$ Embryos Die in Midgestation and Are Anemic

To identify at which developmental stage p38α-deficient embryos die, the phenotypes and genotypes of offspring from *p38*$\alpha^{+/-}$ intercrosses were examined between E8.5 and E19.5. Live *p38*$\alpha^{-/-}$ embryos were detected up to E16.5, but after E11.5 their frequency was considerably lower than what was expected based on Mendelian inheritance (Table 1). We did notice, however, that the actual time at which the *p38*$\alpha^{-/-}$ mice died varied from one animal facility to another: while animals housed in

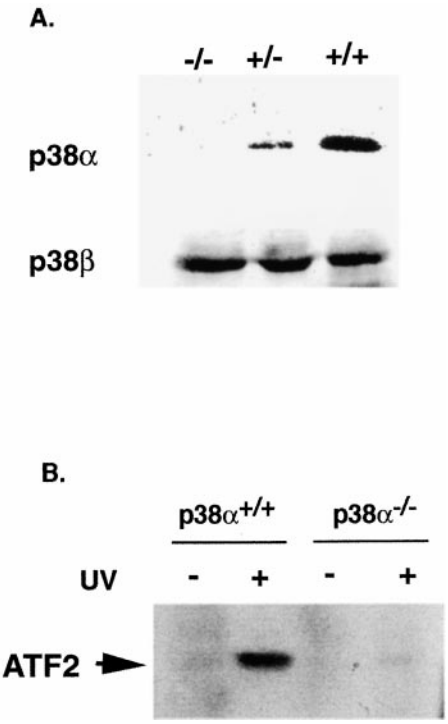

Figure 2. Analysis of p38α Expression and Function in WT and Mutant Embryonic Fibroblasts

(A) MEF cultures were established from E10.5–E11.5 embryos of the indicated genotypes. Expression of p38α and p38β was examined by immunoblotting with specific antibodies.

(B) p38α was immunoprecipitated from nonstimulated or UV-irradiated wt and *p38*α-null MEFs. Kinase activity was measured using a GST-ATF2 fusion protein as a substrate. Protein levels were normalized by coomassie blue staining.

San Diego did not generate *p38*$\alpha^{-/-}$ embryos that survived beyond E13.5, those that were kept in Saitama, Japan generated *p38*$\alpha^{-/-}$ embryos that developed to E16. The basis for this difference is not clear, but we found that the frequency of live *p38*$\alpha^{-/-}$ that develop beyond E11.5 had dramatically decreased upon backcrossing the *p38*$\alpha^{+/-}$ heterozygotes [which were of (129/Sv × 129/J) F1 × C57BL/6J background] into the C57BL/6J background. Further examination suggested the existence of two distinct defects caused by the p38α deficiency. Whereas a certain proportion *p38*$\alpha^{-/-}$ mice die between E11.5 and E12.5 and some of them appear growth retarded, those that survive beyond that point were morphologically similar to their wild-type (wt) counterparts except for a slight pallor. In those embryos that died at E11.5, histological examination revealed a marked paucity of embryo-derived blood vessels in the labyrinth layer of the placenta (H. Yoshida and K. T., unpublished data). The work described below was based on the analysis of only those *p38*$\alpha^{-/-}$ embryos that were morphologically normal and alive at E11.5 or later time points. The fetal livers (FLs) of the live *p38*$\alpha^{-/-}$ embryos were smaller than those of wt counterparts and their major blood vessels lacked color (Figure 3A). In addition, the yolk sacs that surrounded these embryos were of normal size but contained few circulating red blood cells (Figure 3B).

During normal murine development, hematopoiesis is initiated in the yolk sac, followed by the aorta-gonad-mesonephros (AGM) region. After establishment of blood circulation at E8.0, multipotent cells can be found in the blood vessels and these cells colonize the liver, spleen, thymus, and omentum (Godin et al., 1999). At midgestation, the FL becomes the major hematopoietic organ with hematopoietic activity starting around E11.0 to E12.5 until 1 week postnatally. Visual and histological examination revealed that *p38*$\alpha^{-/-}$ yolk sac blood vessels and the vasculature of the embryo proper appeared normal using PECAM antibody (data not shown). However, histological examination of wt and *p38*$\alpha^{-/-}$ FLs revealed a marked decrease in the number of circulating hemoglobinized red blood cells and erythropoietic foci in E11.5 to E13.5 *p38*$\alpha^{-/-}$ embryos (Figures 3C and 3D). While blood vessels of wt embryos contained both enucleated and nucleated erythrocytes, the small number of erythrocytes found in the blood vessels of *p38*$\alpha^{-/-}$ embryos were mostly nucleated and very few enucleated red blood cells were detected (Figure 3D). The total number of nucleated cells in E11.0 *p38*$\alpha^{-/-}$ FLs was 4-fold lower than in wt counterparts. Cytospin analysis of wt FL cells revealed cells in all stages of erythropoiesis, whereas similar preparations of p38α-deficient FLs contained mostly hepatocytes with few proerythroblasts and an even lower number of more mature erythroid cells (Figure 3E).

Defective Erythropoiesis in *p38*$\alpha^{-/-}$ Embryos

We examined FL hematopoiesis in E11.5 wt and *p38*$\alpha^{-/-}$ embryos by flow cytometry using markers that are characteristic of different hematopoietic cell types and developmental stages, including CD34 (detecting hematopoietic progenitors), CD44 (detecting hematopoietic cells), c-kit (hematopoietic stem cells), and Ter-119 (erythroid cells) (Ikuta et al., 1990; Kansas et al., 1990; Neubauer et al., 1998; Ghaffari et al., 1999). Analysis of single-cell suspensions with the CD34 and CD44 markers revealed a modest but significant increase in the relative fraction of $CD34^{low}CD44^{high}$ cells in *p38*$\alpha^{-/-}$ FLs, whereas the frequency of $CD34^{neg}CD44^{low}$ cells had decreased (Figure 4). A similar but more dramatic change in CD44 expression occurs in *EpoR*$^{-/-}$ and *Jak2*$^{-/-}$ FLs (Neubauer et al., 1998). Although CD44 is expressed by all types of mature blood cells, its expression level changes with cell differentiation, and $CD34^{low}CD44^{high}$ cells represent hematopoietic progenitors (Ghaffari et al., 1999). Moreover, expression of CD44 is downregulated during erythroid cell differentiation and maturation (Kansas et al., 1990). FLs of *p38*$\alpha^{-/-}$ embryos also exhibited a modest increase in the relative abundance of $CD34^{low}$c-kit^{pos} cells (Figure 4). Expression of c-kit marks HSCs capable of long term reconstitution (Ikuta and Weissman, 1992). On the other hand, expression of Ter-119, which marks committed erythropoietic precursors beyond the erythroid colony-forming unit (CFU-E) stage (Ikuta et al., 1990; Neubauer et al., 1998), was reduced in *p38*$\alpha^{-/-}$ FLs. The *p38*$\alpha^{-/-}$ FLs contained a higher relative level of Ter-$119^{neg}CD44^{high}$ hematopoietic progenitors and Ter-$119^{pos}CD44^{neg}$ primitive erythroid cells and a lower level of more mature Ter-119^{pos}c-kit^{neg} cells. These results strongly suggest that the absence of p38α results in a severe block to erythroid differentiation.

Further evidence for a defect in definitive erythropoiesis in *p38*$\alpha^{-/-}$ embryos was provided by reverse transcription (RT)–polymerase chain reaction (PCR) analysis

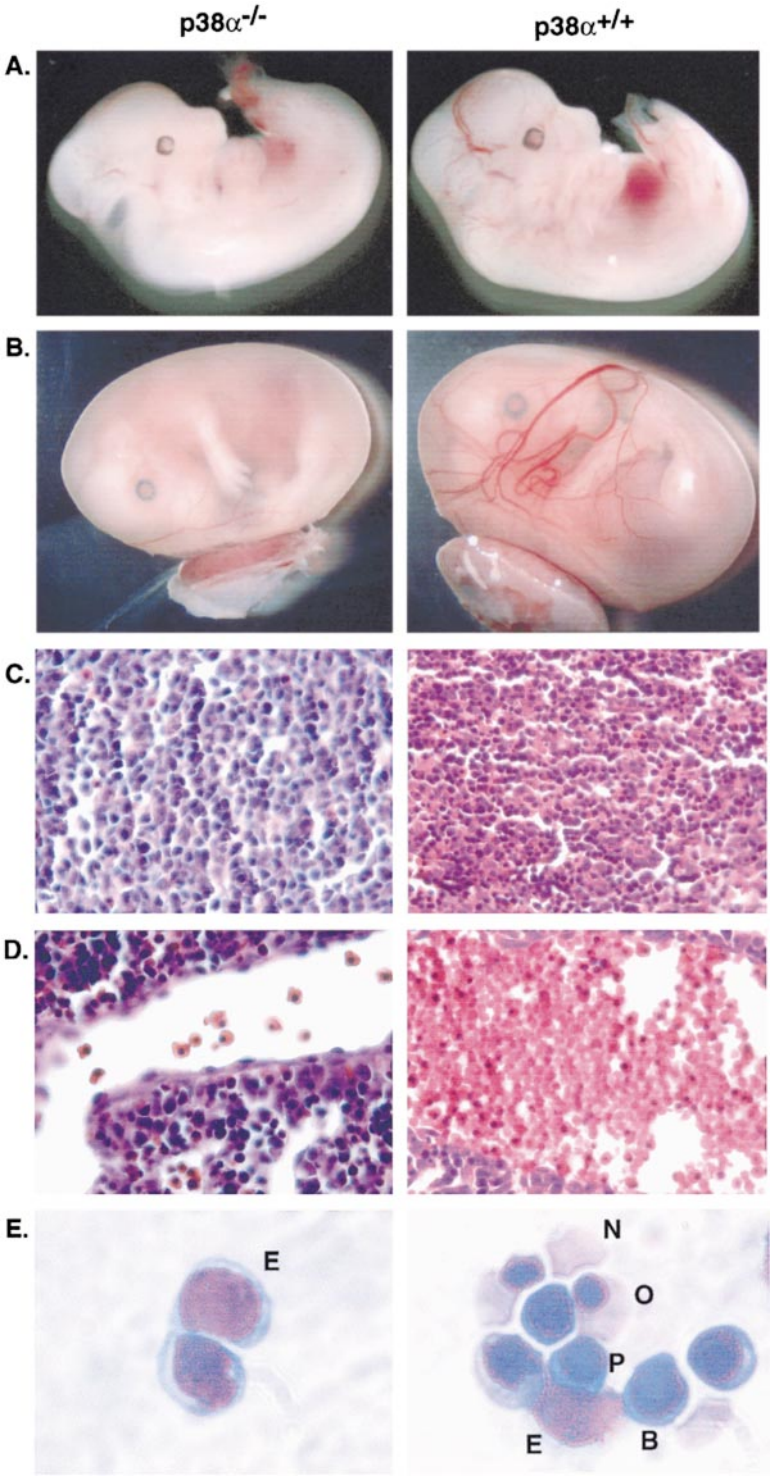

Figure 3. Morphological Phenotype of *p38*$\alpha^{-/-}$ Embryos

(A) Appearance of *p38*$\alpha^{+/+}$ and *p38*$\alpha^{-/-}$ E12.5 mouse embryos. Note the smaller liver, absence of color in major blood vessels, and general pallor of the *p38*$\alpha^{-/-}$ embryo.

(B) E16.5 *p38*$\alpha^{+/+}$ and *p38*$\alpha^{-/-}$ mouse embryos within their yolk sacs. Note the reduced amount of blood in the *p38*$\alpha^{-/-}$ embryo and yolk sac.

(C) Sections of FLs from *p38*$\alpha^{-/-}$ and *p38*$\alpha^{+/+}$ E11.5 embryos were stained with hematoxilin and eosin (H&E). The *p38*$\alpha^{+/+}$ FL contains numerous dark staining erythropoietic elements, while *p38*$\alpha^{-/-}$ FL contains primarily hepatocytes and only a small number of primitive nucleated erythrocytes.

(D) Sections through blood vessels in FLs of *p38*$\alpha^{-/-}$ and *p38*$\alpha^{+/+}$ E13.5 embryos stained with H&E. Note the almost complete absence of enucleated erythrocytes in the *p38*$\alpha^{-/-}$ sample.

(E) Liver cytospin preparations from E12.5 *p38*$\alpha^{+/+}$ and *p38*$\alpha^{-/-}$ embryos. Giemsa staining reveals numerous erythropoietic cells in all stages of differentiation, including proerythroblasts (E), basophilic erythroblasts (B), polychromatic erythroblasts (P), orthochromatic erythroblasts (O), and enucleated erythrocytes (N), within cells isolated from *p38*$\alpha^{+/+}$ FL. In contrast, FL cytospin preparations from a *p38*$\alpha^{-/-}$ embryo show proerythroblasts (E), but erythroid cells at later stages of differentiation are rare.

of *globin* gene expression. The levels of β*h1*-, ϵ*y2*-, and ζ-*globin* transcripts, which are expressed predominantly during embryonic (primitive) erythropoiesis (Whitelaw et al., 1990), were similar in wt and knockout FLs (Figure 5A). The levels of α-*globin* transcripts, which are produced during both embryonic and definitive erythropoiesis, were also not substantially different between wt and *p38*$\alpha^{-/-}$ FLs. However, expression of β^{maj}-*globin* transcripts, which first occurs in FLs during definitive erythropoiesis, was highly reduced in *p38*$\alpha^{-/-}$ embryos (Figure 5A). Collectively, these results indicate that embryonic (or primitive) erythropoiesis proceeds normally in *p38*$\alpha^{-/-}$ embryos but definitive erythropoiesis is severely defective.

To better define the block in definitive erythropoiesis, we measured the ability of cells derived from wt and *p38*$\alpha^{-/-}$ E10.5 FLs to form CFU-Es and the more immature erythroid burst-forming units (BFU-E). These colony types reflect the presence of committed erythroid pro-

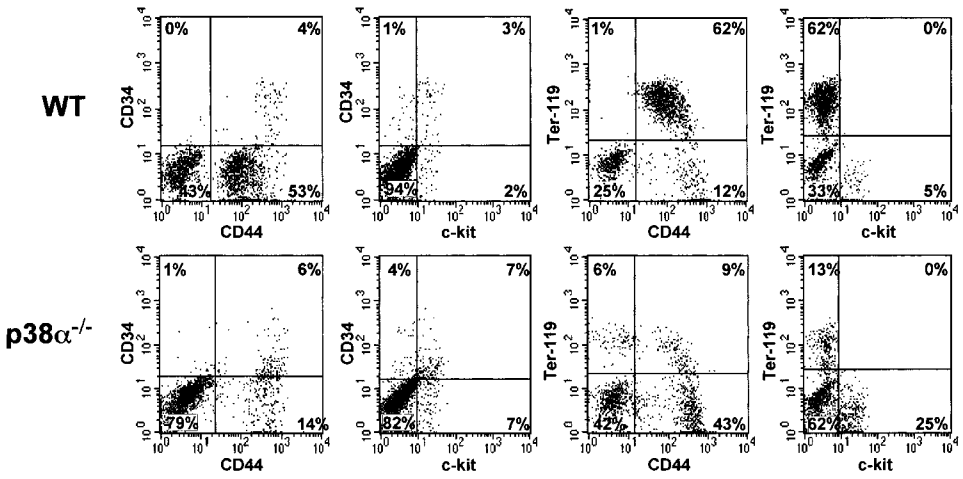

Figure 4. Flow Cytometry of FL Hematopoietic Cells from E11.5 WT and Mutant Embryos

Expression of different cell surface markers is depicted. Cell suspensions from E11.5 FLs were labeled with anti-c-kit, which marks hematopoietic stem cells, anti-CD44, which marks all hematopoietic cells, anti-Ter-119 to identify differentiated erythroid cells, or anti-CD34 to mark hematopoietic progenitors. A minimum of 5×10^5 live cells were analyzed for each FL.

genitors (Gregory and Eaves, 1978). While the total number of CFU-E and BFU-E in *p38*$\alpha^{-/-}$ FLs was considerably lower than in those of wt littermates, upon normalization to the total number of nucleated cells per FL, no decrease in the frequency of BFU-Es and CFU-Es between mutant and wt was detected. Most important, *p38*$\alpha^{-/-}$ erythroid progenitors were capable of complete terminal differentiation in vitro, as indicated by benzidine staining. Collectively, the phenotype of *p38*$\alpha^{-/-}$ embryos is comparable to that of *Epo*$^{-/-}$ embryos (Wu et al., 1995), suggesting that the major defect in p38α-deficient embryos is in the expansion and differentiation of CFU-E progenitors to more mature erythroid cells.

p38α Is Required for Optimal Epo Expression

In addition to stress stimuli, p38 catalytic activity is stimulated by several hematopoietic growth factors, including Epo, stem cell factor (SCF), and IL-3 (Crawley et al., 1997; Foltz et al., 1997; Nagata et al., 1997). Although such findings suggest that p38α may act downstream to Epo receptor (EpoR), the hematopoietic defect of *p38*$\alpha^{-/-}$ embryos described above was more similar to that of *Epo*$^{-/-}$ embryos than embryos lacking EpoR or a downstream component of its signaling pathway (Wu et al., 1995; Neubauer et al., 1998; Parganas et al., 1998). Furthermore, *p38*$\alpha^{-/-}$ erythropoietic progenitors undergo terminal differentiation in vitro in the presence of exogenous Epo. We therefore explored the possibility that p38α may be required for optimal expression of Epo and other hematopoietic growth factors. RNA was extracted from FLs of morphologically normal and viable E11–E11.5 wt and *p38*$\alpha^{-/-}$ fetuses. The relative amount of *Epo* transcripts was first examined by RT–PCR analysis. To ensure linearity, we examined the relative abundance of *Epo* cDNA as different cycles. The results indicated a dramatic reduction in the level of *Epo* mRNA expression in *p38*$\alpha^{-/-}$ FLs (Figure 6A). By comparison, only a modest decrease in expression of *SCF* mRNA was detected in *p38*$\alpha^{-/-}$ FLs (Figure 6B). To more accurately determine the differences in *Epo* and *SCF* mRNA levels between wt and *p38*$\alpha^{-/-}$ FLs, we analyzed samples of FL RNA by quantitative real-time PCR (Heid et al., 1996). When normalized to the levels of *GAPDH* mRNA, the relative amount of *Epo* mRNA in *p38*$\alpha^{-/-}$ FLs was nearly ten times lower than in wt, whereas the relative amount of *SCF* mRNA was 2-fold lower in *p38*$\alpha^{-/-}$ FLs than in wt FLs (Figure 6C). We also observed a small decrease in *Thrombopoietin* (*Tpo*) mRNA levels in *p38*$\alpha^{-/-}$ FLs, as measured by real-time PCR (data not shown). We also examined expression of mRNAs coding for GM-CSF, IL-3, IL-6, IL-9, and IL-13, but their levels were too low to be detected in E11.5 FL (K. T., unpublished data).

The *Epo* gene is expressed primarily in FL and adult kidney and is regulated in response to oxygen availability (Ebert and Bunn, 1999). Unfortunately, there is a limited number of tissue culture models for studying the regulation of *Epo* gene expression. Most useful for such studies are the human hepatoma cell lines Hep3B and HepG2 (Ebert and Bunn, 1999). Due to their dedifferentiated nature, hepatoma cell lines may resemble FL cells, which are the major site of *Epo* synthesis during development (Ebert and Bunn, 1999). To examine the general involvement of p38 MAPKs in the regulation of *Epo* gene expression, we used the specific p38 inhibitor SB203580, which mostly affects the p38α and p38β isozymes (Lee and Young, 1996). Treatment with SB203580 inhibited the accumulation of *Epo* mRNA in Hep3B cells incubated with the hypoxiamimetic inducer $CoCl_2$ (Figure 6D). To determine at which level p38 regulates *Epo* gene expression, we examined the effect of SB203580 on induction of *Epo* transcription using a run-on assay. Despite its major effect on *Epo* mRNA accumulation, treatment with SB203580 had only a marginal effect on the rate of *Epo* gene transcription in the absence or presence of $CoCl_2$ (Figure 6E). We attempted to measure the effect of the p38 inhibitor on *Epo* mRNA turnover, but as previously described (Goldberg et al., 1991), we found that inhibition of transcription with actinomycin D resulted in *Epo* mRNA stabilization (data not shown). As the only other way to prevent new *Epo* gene transcription entailed the removal of the inducer, $CoCl_2$, which also causes *Epo* mRNA stabilization (Goldberg et al., 1991), we resorted to a different approach. Cells were treated with $CoCl_2$ for 15 hr to induce accumulation of

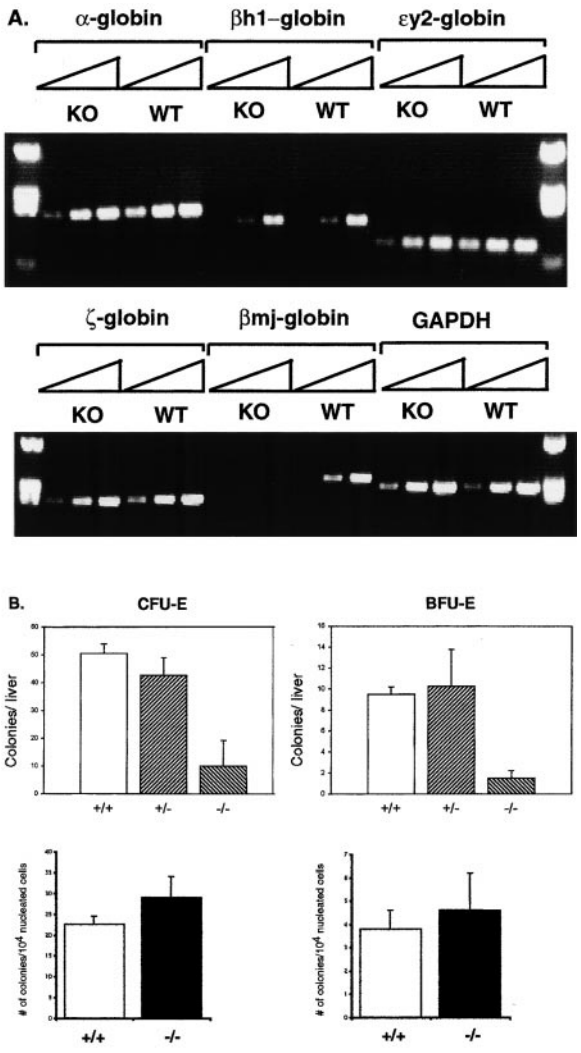

Figure 5. A Defect in Definitive Erythropoiesis in *p38*$\alpha^{-/-}$ Embryos

(A) Expression of *globin* genes in E11 FL of wt and *p38*$\alpha^{-/-}$ (KO) embryos. Samples were amplified for 20, 23, or 26 cycles, using gene specific primers. *GAPDH* mRNA levels were measured as a control.

(B) Analysis of CFU-E- and BFU-E-forming ability of FL cells from *p38*$\alpha^{+/+}$, *p38*$\alpha^{+/-}$, and *p38*$\alpha^{-/-}$ E11 embryos. Top panel, the total number of colony-forming units per FL was determined. Bottom panel, the frequency of progenitor cells per 10^4 nucleated FL cells. The depicted values are averages ± standard errors determined for three embryos of each genotype.

Epo transcripts. At that time, the cells were left with $CoCl_2$ and were incubated for 3 additional hr in the absence or presence of SB203580. Despite the small effect on *Epo* gene transcription (Figure 6E), the 3 hr treatment with SB203580 resulted in a 2-fold decrease in *Epo* mRNA level (Figure 6F). Given that the half-life of *Epo* mRNA under normoxic conditions is 3 hr (Goldberg et al., 1991), these results suggest that inhibition of p38 activity results in *Epo* mRNA destabilization. We therefore conclude that p38 acts posttranscriptionally, most likely by increasing the stability of *Epo* mRNA. As previously reported for PC12 cells (Conrad et al., 1999), treatment of Hep3B cells with $CoCl_2$ resulted in p38α activation (data not shown) and this activity was sensitive to SB203580 (Figure 6G). We also find that p38α is active in FL cells of E11.5 fetuses, but consistent with the reconstitution results shown below, its activity is not stimulated by Epo. (Figure 6H).

p38$\alpha^{-/-}$ Hematopoietic Stem Cells Reconstitute Lethally Irradiated Hosts

The results described above suggest that the major aberration in *p38*$\alpha^{-/-}$ embryos that leads to the defect in definitive erythropoiesis is Epo deficiency. Furthermore, in FL Epo itself does not seem to be involved in regulation of p38 activity. Yet, other experiments suggested that p38 activity is stimulated by a variety of hematopoietic growth factors, including Epo and SCF (Crawley et al., 1997; Foltz et al., 1997; Nagata et al., 1997). To rigorously examine the possibility that p38α function may also be required downstream to EpoR or other hematopoietic growth factor receptors, we tested the ability of *p38*$\alpha^{-/-}$ FL HSC to reconstitute hematopoiesis in lethally irradiated hosts.

Adult B6.SJL mice, which express the CD45.1 antigen, were lethally irradiated and injected with single-cell suspensions of E11.5 wt and *p38*$\alpha^{-/-}$ FLs, which express the CD45.2 antigen. While mock-injected lethally irradiated mice did not survive beyond 2 weeks (data not shown), mice reconstituted with either wt or *p38*$\alpha^{-/-}$ FL cells survived for at least 20 weeks. At 14 weeks postreconstitution, peripheral blood was analyzed for cell surface markers characteristic of donor-derived erythroid, myeloid, and lymphoid cells. The reconstitution capacity of *p38*$\alpha^{-/-}$ FL cells was very similar to that of wt cells (Figure 7). In both cases, complete reconstitution of erythroid, myeloid (granulocytes and monocytes), and lymphoid (B and T) cells was observed. Thus, the hematopoietic deficiency of *p38*$\alpha^{-/-}$ mice is not cell autonomous and can be corrected by host derived factor(s).

Discussion

Like other stress-activated protein kinases, members of the p38 group of MAPKs are rapidly activated in response to a wide variety of stress stimuli, including heat shock, osmotic shock, UV irradiation, and proinflammatory cytokines (Lee and Young, 1996; Su and Karin, 1996; Ip and Davis, 1998). It was therefore expected that the major, if not sole, function of the p38 MAPKs is in activation of stress responses and induction of proinflammatory cytokines, chemokines, and other proteins involved in inflammation. The development of specific p38 inhibitors that target both the p38α and p38β isozymes has verified the important role of p38 MAPKs in activation and regulation of inflammatory and innate immune responses, at least in cultured cells (Lee and Young, 1996; Crawley et al., 1997; Dean et al., 1999). Further evidence for the involvement of p38α in proinflammatory signaling was recently provided by the analysis of *p38*$\alpha^{-/-}$ ES cells (Allen et al., 2000). Based on such results, a considerable current effort is being dedicated to the development of p38 inhibitors as antiinflammatory drugs. Yet, the function of p38 MAPKs in development and normal physiology has not been extensively explored. We and others (Adams et al., 2000) addressed the function of the p38α isozyme in mouse development and physiology through targeted disruption of the *p38*α locus. As described above and elsewhere (Adams et al., 2000), the p38α deficiency results in two distinct developmental defects that appear to be influenced by the genetic background of the mice. Using 129XC57BL/6J hybrids,

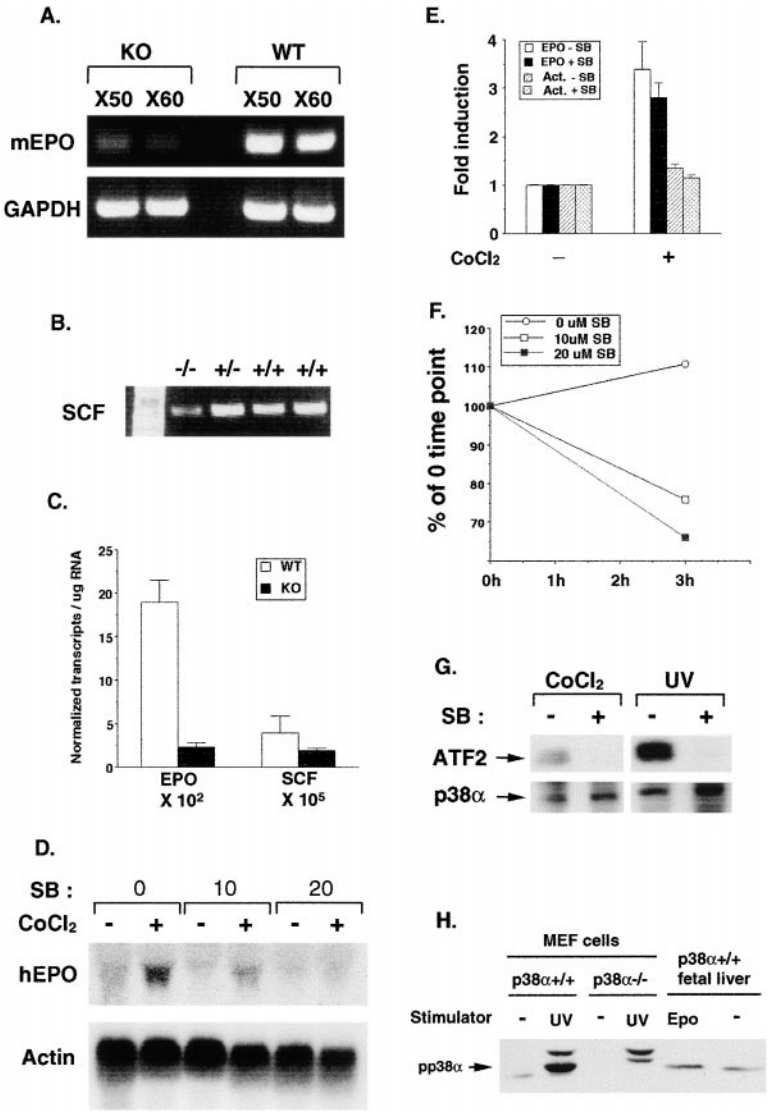

Figure 6. p38α Is Required for Epo Expression

(A) RT–PCR analysis of *Epo* mRNA expression in E11 FL of wt and *p38*$\alpha^{-/-}$ (KO) embryos. Samples were amplified for 50 or 60 cycles. *GAPDH* mRNA levels were measured as a control.

(B) RT–PCR analysis of *SCF* mRNA expression in E11 FL of wt and *p38*$\alpha^{-/-}$ (KO) embryos. Samples were amplified for 30 cycles.

(C) Quantitation of *Epo* and *SCF* mRNA levels by real-time PCR. The levels of *Epo*, *SCF*, and *GAPDH* transcripts in RNA samples of E11.5 wt and *p38*$\alpha^{-/-}$ FL were determined by real-time PCR. The concentrations of *Epo* and *SCF* transcripts were normalized relative to those of *GAPDH* transcripts. Note that the scales for *Epo* and *SCF* are different.

(D) Hep3B cells were cultured in medium containing 50 μM $CoCl_2$ for 9 hr. The cells were also treated with SB203580 (10 or 20 μM) or vehicle (DMSO), as indicated. Expression levels of human *Epo* mRNA were determined by Northern blot hybridization. *Actin* mRNA levels were measured as a control.

(E) p38 inhibitor does not interfere with induction of *Epo* gene transcription. The transcription rates of the *Epo* and β-*actin* genes were measured in nuclei isolated from Hep3B cells that were untreated or treated with 50 μM of $CoCl_2$ for 4–8 hr. Cells were incubated or not with 20 μM SB203580. The values represent relative fold induction and are averages of two experiments.

(F) p38 inhibitor destabilizes *Epo* mRNA. Hep3B cells were incubated in medium containing 50 μM $CoCl_2$ for 15 hr. One dish was left in 50 μM $CoCl_2$, one dish incubated with 50 μM $CoCl_2$ plus 10 μM SB203580, and the third dish in 50 μM $CoCl_2$ plus 20 μM SB203580. After 3 hr, RNA was isolated and analyzed by Northern blot hybridization using human *Epo* and β-*actin* probes. Expression of *Epo* mRNA was normalized to that of β-*actin* mRNA after quantitation by phosphoimaging.

(G) $CoCl_2$ induced SB20350-sensitive p38α kinase. Hep3B cells were incubated with 50 μM of $CoCl_2$ or exposed to UV-C radiation (40 J•m^{-2}). After 8 hr lysates were prepared and p38α kinase activity was determined by immunecomplex kinase assays in the presence (plus signs) or absence (minus signs) of 10 μM SB203580.

(H) Detection of activated p38α in fetal liver. MEFs from wt and *p38*$\alpha^{-/-}$ fetuses were exposed or not to UV-C radiation a potent p38 MAPK activator. Single-cell suspensions of FLs from E11.5 wt fetuses were incubated with or without 30 U/ml murine Epo for 5–10 min at 37° C. Cell lysates were separated by SDS–PAGE, transferred to Immobilon membrane, and immunoblotted with an antibody that specifically recognizes the activated form of p38 (phospho-p38). The migration position of phospho-p38α is indicated.

we find that some of the *p38*$\alpha^{-/-}$ embryos die between E11.5 and E12.5, while a considerable proportion of the p38α-deficient embryos develop beyond this point and survive up to E16.5 with normal morphology but highly anemic appearance. Whereas Adams et al. (2000) have focused their studies on the basis for the earlier lethality, which is the predominant phenotype after backcrossing to the C57BL/6 background, and suggest that it is due to a requirement for p38α in placental development, we have investigated the basis for the anemia that is observed in those p38α-deficient embryos that do not die due to placental insufficiency. The basis for the anemic phenotype has been traced to a deficiency in *Epo* gene expression.

Not only do those *p38*$\alpha^{-/-}$ embryos that survive beyond E12.5 exhibit a dramatic reduction in accumulation of *Epo* mRNA, but the characteristics of their hematopoietic defect are almost identical to those of *Epo*$^{-/-}$ mice (Wu et al., 1995). In both cases, the homozygous null mice die during midgestation and prior to their death exhibit severe anemia, decreased FL cellularity, and impaired definitive erythropoiesis. However, unlike mouse bryos that are deficient in essential downstream components of the EpoR signaling pathway, for instance *Jak2*$^{-/-}$ embryos (Neubauer et al., 1998; Parganas et al., 1998), the FLs of *p38*$\alpha^{-/-}$ and *Epo*$^{-/-}$ (Wu et al., 1995) embryos contain BFU-Es and CFU-Es that are formed with normal frequency and can reconstitute erythropoiesis in lethally irradiated hosts or undergo terminal differentiation in vitro in the presence of exogenous Epo. It is therefore clear that despite the ability of several hematopoietic growth factors, including Epo itself, to activate p38

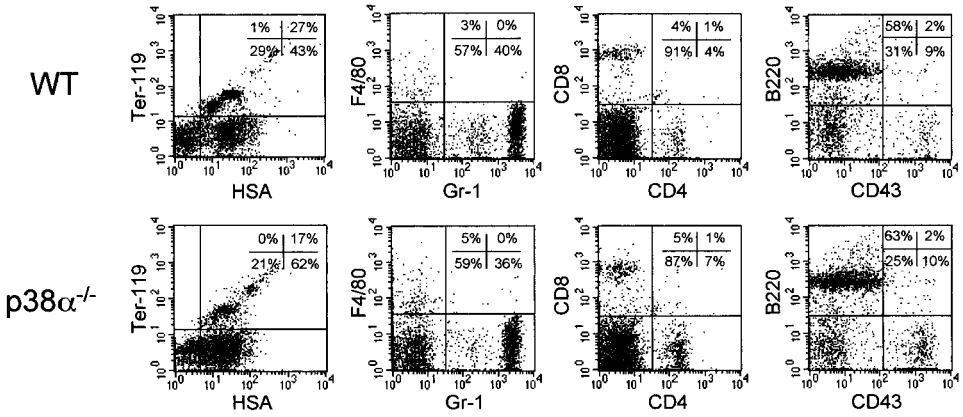

Figure 7. Reconstitution of Hematopoiesis by Adoptive Transfer of WT and *p38*$\alpha^{-/-}$ FL Cells

Peripheral blood cells were withdrawn 14 weeks after adoptive transfer of FL cells from wt or *p38*$\alpha^{-/-}$ E11.5 embryos into lethally irradiated hosts. Cell surface expression of different lineage markers is shown. The dot plots represent the log of fluorescence intensities of cells falling into the CD45.2 gate. Monoclonal antibodies used were against Ter-119 (erythroid cells), HSA (heat stable antigen, lymphoid precursors), Gr-1 (granulocytes), F4/80 (monocytes), CD4 and CD8 (T lymphocytes), B220 (B lymphocytes), and CD43 (leukocytes). The percentages of cell populations in the different quadrants are indicated.

MAPKs in cultured cells (Crawley et al., 1997; Foltz et al., 1997; Nagata et al., 1997), at the least the p38α isozyme is not required for expansion and differentiation of hematopoietic progenitors in response to these factors. The most likely molecular defect in the anemic *p38*$\alpha^{-/-}$ embryos that accounts for their hematopoietic deficiency is the inability to accumulate sufficient amounts of *Epo* mRNA. Formally, however, it is also possible that p38α may not be directly involved in the regulation of *Epo* mRNA metabolism and instead is required for formation or maintenance of the cells responsible for Epo production. Although it is essentially impossible to rule out this possibility, as the only marker for Epo-producing cells is Epo itself, the experiments conducted with Epo-producing human hepatoma cells strongly suggest that p38α is directly involved in regulation of *Epo* mRNA metabolism.

In addition to p38α-deficient mouse embryos, inhibition of *Epo* mRNA accumulation was also observed in human Hep3B hepatoma cells subjected to hypoxia and treated with the specific p38 inhibitor SB203580. Although in adults the major site of Epo production is the kidney, hepatoma cells are similar in their differentiation state to FL cells, which are the major producers of *Epo* during fetal life (Ebert and Bunn, 1999). As SB203580 targets both the p38α and p38β isozymes (Lee and Young, 1996), it is not clear whether the observed effect is solely due to inhibition of p38α. Nevertheless, we found that treatment of Hep3B cells with the hypoxiamimetic agent $CoCl_2$ resulted in p38α activation (K. T., unpublished data), and previous studies have shown that only p38α and p38γ (which is insensitive to SB203580) are responsive to hypoxia (Conrad et al., 1999). The hypoxic induction of *Epo* gene expression is mediated both at the transcriptional and posttranscriptional levels, the latter through mRNA stabilization (Ebert and Bunn, 1999). Our results suggest that p38α affects *Epo* gene expression at the posttranscriptional level, most likely through mRNA stabilization. The major transcription factor responsible for the hypoxic induction of the *Epo* gene is HIF-1 (Ebert and Bunn, 1999), but so far it appears that HIF-1 is not a direct target for SB203580-sensitive p38 MAPKs, and the induction of HIF-1α phosphorylation in *p38*$\alpha^{-/-}$ MEFs is unaltered (A. M. D. and R. J., unpublished data; Richard et al., 1999). Just like the JNKs, the p38 MAPKs were first found to phosphorylate and regulate the activity of transcription factors, but recent results indicate that both JNKs (Chen et al., 1998) and p38 MAPKs (Dean et al., 1999; Winzen et al., 1999) are also involved in the regulation of mRNA turnover.

Due to the absence of appropriate culture models, the developmental cues that regulate *Epo* gene expression during embryonic development remain unknown (Ebert and Bunn, 1999). Also, because of the rather early embryonic lethality of *Hif1*$\alpha^{-/-}$ embryos (Iyer et al., 1998; Ryan et al., 1998), it has been impossible so far to evaluate the role of HIF-1, the major hypoxia-induced transcription factor, in the developmental control of *Epo* gene expression. Our results, however, which attribute a critical role for p38α in regulation of *Epo* mRNA accumulation both in mouse embryos and in human hepatoma cells subjected to hypoxia, suggest that p38α is important for regulation of fetal *Epo* gene expression. However, it remains to be examined whether p38 is involved in the hypoxic induction of *Epo* mRNA in the kidney during adult life. Nevertheless, given the activation of p38α and p38γ by hypoxia (Conrad et al., 1999; K. T., unpublished data), it is reasonable to postulate that hypoxia may provide an important signal that triggers *Epo* gene expression during embryogenesis. In support of this hypothesis, it was observed that a hypoxia-responsive reporter is activated during mouse embryogenesis, suggesting that the embryo is subject to mild hypoxia during certain periods of development (Ryan et al., 1998). Furthermore, Epo serum concentrations are increased in anemic or hypoxic human fetuses (Fahnenstich et al., 1996). Hence, it is likely that fetal erythropoiesis may be regulated to maintain oxygen homeostasis in the embryo, in much the same way it is regulated in response to environmental cues in the adult.

In addition to revealing the unexpected role of p38α in regulation of Epo production and erythropoiesis, as well as placental development (Adams et al., 2000), the

phenotypes associated with *p38α* null mutations suggest that the function of p38α, at least during mouse embryogenesis, is nonredundant. This is quite different from what has been revealed by mutant mice with disruptions of only one of the *Jnk* loci or the *Erk1* locus (Yang et al., 1997; Dong et al., 1998; Kuan et al., 1999; Pagès et al., 1999; Sabapathy et al., 1999a, 1999b). None of these mutants has revealed a critical role for any of the affected MAPKs in development, and therefore, it has been assumed that these protein kinases may have overlapping functions, at least during development, as revealed by the severe developmental defect of *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ double mutants (Sabapathy et al., 1999b).

In summary, our results identify a clear function for the p38α MAPK in normal development through its ability to regulate the accumulation of *Epo* mRNA. As p38α activity is also regulated by hypoxia, the major environmental signal that controls Epo production and erythropoiesis (Ebert and Bunn, 1999), p38α provides an important yet previously unexpected link between stress-induced erythropoiesis and developmental erythropoiesis.

Experimental Procedures

Generation of *p38α* Knockout Mice

Conditions for blastocyst injection of correctly targeted and karyotypically normal ES clones and breeding to generate mice homozygous for the mutated *p38α* gene were as described (Hu et al., 1999; Sabapathy et al., 1999a). Mouse genomic DNA library was screened to isolate a genomic DNA clone covering the N-terminal portion of the *p38α* coding region. This clone was used to construct the targeting vector described in Figure 1, using standard procedures (Hu et al., 1999). Details of the construction are available upon request. The targeting vector was transfected into R1 ES cells derived from the 129 strain (Hu et al., 1999). Two independent *p38α*$^{+/-}$ ES clones were injected into blastocysts of C57BL/6J mice. Male mice with a high degree of chimerism were crossed to C57BL/6J females to generate *p38α*$^{+/-}$ mice. Mouse tail DNA was prepared from ~1 mm tail snips of 1- to 2-week-old progeny. Genotyping was performed by Southern blot analysis or by PCR analysis of tail- and embryo-derived DNA. Multiplex PCR with three primers per reaction was used. The primers were as follows: A, 5′-CCCTATACTCCCTCTCTG TGTAACTTTTG-3′; B, 5′-CCCAAACCCCAGAAAGAAATGATG-3′; and C, 5′-TTCTGTGACAACGTCGAGCACAGCTG-3′. Using these primers at 1 cycle at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, with an extension step of 10 min at 72°C at the end of the last cycle, produced 800 bp and 450 bp fragments from the mutant and wt alleles, respectively.

Analysis of p38α Activity and Expression

Embryonic fibroblasts from *p38α*$^{+/+}$, *p38α*$^{+/-}$, and *p38α*$^{-/-}$ embryos were cultured in DMEM supplemented with 10% fetal bovine serum. Cell lysates were analyzed for expression of *p38α* and *p38β* as described (Sudo and Karin, 2000) using anti-p38α (c-20, Santa Cruz) and anti-p38β (c-16, Santa Cruz) polyclonal antibodies. In addition, we used anti-p38α antibodies raised against the intact protein (Sudo and Karin, 2000) or an N-terminal peptide (N20G, Santa Cruz). To measure p38α kinase activity, cells were stimulated or not, as indicated, lysed, and p38α was immunoprecipitated with anti-p38α antibody (c-20). Immune complex kinase assays with GST-ATF2(1-99) as a substrate were performed as described (Sudo and Karin, 2000).

Reconstitution of Hematopoiesis in Lethally Irradiated Mice

FL cells from wt or *p38α*$^{-/-}$ E11−E13 embryos were used to reconstitute lethally irradiated B6.SJL (CD45.1) female mice, exposed to 950 rad of γ irradiation. A total number of 1×10^6 FL cells were injected into the tail vein of the host. Reconstitution of hematopoiesis was examined by flow cytometric analysis performed 14 weeks later.

RNA and Transcription Rate Analysis

Total RNA was isolated from livers of wt and p38α-deficient E11–E11.5 embryos using the Trizol purification method as recommended by the manufacturer. For cDNA synthesis, 1 μg of total RNA in a final volume of 12 μl of DEPC-treated water was heated to 70°C for 5 min and chilled on ice for 2 min. One microliter of oligo (dT) primer (500 ng/μl) was added to the reaction mix, which included 5 μl of 10× first-strand buffer, 1 μl of 10 mM dNTPs, and 1 μl of MMLV-RT (50 U/μl). The reactions were incubated at 42°C for 50 min. The primers and the conditions for PCR were previously described (Weiss et al., 1994; Eferl et al., 1999). Nuclear run-on experiments were conducted as described (Greenberg, 1987) using nuclei isolated from Hep3B cells untreated or treated with 50 μM $CoCl_2$ for different lengths of time. After in vitro transcription in the presence of [α-^{32}P]UTP, radiolabeled RNA was isolated using the Trizol method. Equal amounts of [^{32}P]RNA were hybridized to nitrocellulose filters to which single-stranded human *Epo* and β-*actin* cDNAs were spotted. Results were quantitated using a phosphoimager.

Real-Time PCR

Quantitative real-time PCR based measurement of RNA abundance (Heid et al., 1996) was carried out using gene-specific double fluorescently labeled probes and the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Norwalk, CT). 6-carboxy fluorescein (FAM) was the 5′ fluorescent reporter, and tetramethylrhodamine (TAMRA) was added to the 3′ end as a quencher. The following primer and probe sequences were used: mGAPDH-247 forward primer (F), 5′-CAACGGGAAGCCCATCAC-3′; mGAPDH-311 reverse primer (R), 5′-CGGCCTCACCCCATTTG-3′; mGAPDH-171 probe, 5′-TGGCACCGTCAAGGCTGAGAACG-3′; mEPO-174F, 5′-CAGGCC CTGCTAGCCAATT-3′; mEPO-251R, 5′ACGTAGACCACTGATGGCTT TGT-3′; mEPO-196 probe, 5′-CCAAGCCACCAGAGACCCTTCAGC TTC-3′; mSCF-6F, 5′GATCTGCGGGAATCCTGTGA-3′; mSCF-103R, 5′-CGGCGACATAGTTGAGGGTTA-3′; mSCF-39 probe, 5′-AGACAT TACAAAACTGGTGGCTCTTCCAAAT-3′; mTPO-254F, 5′-CCTGTGA CCCCAGACTCCTAAT-3′; mTPO-323R, 5′-CACTGACTCAGTCGGC TGTGA-3′; mTPO-278 probe, 5′-AACTGCTGCGTGACTCCCACC TCC-3′. The specificity of PCR primers was tested under normal PCR conditions before quantitation. Real-time PCR was performed with 25 ng of reverse transcription products in a total volume of 50 μl in triplicates. PCR was performed at 50° C for 2 min, at 95°C for 10 min and then for 45 cycles at 95° C for 15 s, and at 60° C for 1 min on the ABI PRISM 7700 Detection System. Standard curves were generated using serial dilutions of a plasmid containing the cDNA of interest.

Colony Formation Assays

Cells were prepared from livers of E11–E12 embryos in α-MEM medium (GIBCO–BRL) and counted in the presence of 3% acetic acid to lyse erythrocytes. Cell suspensions and recombinant cytokines specific for each assay were mixed with MethoCult M3230 (StemCell Technologies) as described (Neubauer et al., 1998). Cells were plated in 35 mm dishes and cultured at 37°C, 5% CO_2. For the CFU-E assay, cells were cultured in 0.2 U/ml recombinant murine (rm) erythropoietin (R&D Systems), and benzidine-positive CFU-E colonies were scored at day 3. For the BFU-E assay, cells were cultured in 3 U/ml rmEpo and 10 ng/ml rmIL-3 (R&D Systems), and benzidine-positive BFU-E colonies were scored at day 8.

Flow Cytometry

Single-cell suspensions of wt and mutant FLs were obtained at E11.5. For analysis of reconstituted mice, peripheral blood was collected from retroorbital veins, and ammonium chloride lysis of erythrocytes was performed. FL cells or peripheral blood cells (5×10^5) were stained with monoclonal antibodies for 30 min at 4°C in 75 μl phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. Cell suspensions were then incubated on ice with rat anti-mouse CD16/CD32 (PharMingen) to block nonspecific binding to Fc receptors. Subsequently, cells were incubated with rat anti-mouse biotin-conjugated CD34 (RAM34)/streptavidin-phycoerythrin (PE), Cy-Chrome-conjugated anti-CD44 (IM7), fluorescein isothiocyanate (FITC)–conjugated anti-c-kit, anti-Ter-119

(PE), anti-CD24 (HSA) (FITC, M1/69), anti-CD43 (PE, 1B11), and anti-Gr-1 (PE, RB6-8C5) (all from PharMingen). Cells were also stained with anti-CD4 (PE, CT-CD4), Tri-Color-conjugated anti-CD8α (TC, CT-CD8a), anti-B220 (TC, RA3-6B2), and anti-F4/80 (TC) (all from Caltag). Appropriate isotype control antibodies were used. Cell surface expression of the different markers was analyzed in a Becton Dickinson FACScan using CellQuest software.

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