The role of erythropoietin in regulating angiogenesis

Nathalie Kertesz[a], Jun Wu[a], Tim H.-P. Chen[b], Henry M. Sucov[b], Hong Wu[a],*

[a]Howard Hughes Medical Institute and Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA, 90095-1735, United States
[b]Institute for Genetic Medicine, University of Southern California Keck School of Medicine, Los Angeles, CA, 90033, United States

Abstract

Erythropoietin (EPO) is an essential growth factor that regulates erythrocyte production in mammals. In this study, we demonstrate a novel role of EPO in regulating angiogenesis in vivo. *Epo* and *Epo* receptor (*EpoR*) are expressed in the vasculature during embryogenesis. Deletion of *Epo* or *EpoR* leads to angiogenic defects starting at E10.5, 2 days before ventricular hypoplasia and 3 days before the onset of the embryonic lethal phenotype. Overall, angiogenesis was severely affected in the mutant embryos: vascular anomalies included decreased complexity of the vessel networks. However, de novo vasculogenesis remained intact, consistent with the differential expression of *Epo* and *EpoR* during the early stages of embryonic development. The aforementioned angiogenesis defect can be partially rescued by expressing human EPO during embryogenesis. Moreover, Ang-1 expression is regulated by EPO/EPOR under normoxic conditions. Taken together, our results suggest important roles of EPO and EPOR in angiogenesis.
© 2004 Elsevier Inc. All rights reserved.

Keywords: Erythropoietin; Angiogenesis; Gene targeting; VEGF; Angiopoietin

Introduction

Erythropoietin is a principal hematopoietic growth factor responsible for the proliferation, survival, and differentiation of erythroid progenitor cells. Through specific binding to its cognate receptor, EPOR (D'Andrea et al., 1989a,b), EPO triggers a chain of intracellular signaling events, including activation of the receptor-associated tyrosine kinase JAK2 and phosphorylation and nuclear translocation of STAT5, leading to progenitor cell proliferation and differentiation (Watowich et al., 1996). In addition to erythropoiesis, EPO has been found to be important for megakaryocyte proliferation and differentiation.

Recent studies suggest that the functions of EPO and EPOR are not strictly limited to erythroid or hematopoietic lineages. For instance, *EpoR* expression has been detected in umbilical cord and placental endothelial cell lines, and EPO was capable of stimulating endothelial cell prolifer-

ation in vitro (Anagnostou et al., 1990, 1994). In addition, EPO was known to induce a pro-angiogenic phenotype in cultured endothelial cells and stimulated neo-vascularization in the chick chorioallantoic membrane (Ribatti et al., 1999). EPO plays an important role in cardiac morphogenesis (Wu et al., 1999), in myoblast proliferation (Ogilvie et al., 2000), and possibly in neurogenesis (Yu et al., 2002). Despite these advances, a detailed analysis of *Epo* and *EpoR* expression patterns and their in vivo functions in these non-erythroid lineages are currently lacking.

To understand the possible roles of EPO in these nonhematopoietic lineages and during embryogenesis, we have performed a detailed in situ hybridization analysis comparing the expression profiles of *Epo* and *EpoR* from 6.5 to 11.5 dpc. High levels of *Epo* and *EpoR* expression can be detected in the vasculature and the heart. Consistent with their expression patterns, we found that *Epo* and *EpoR* null embryos are defective in angiogenesis while relatively normal in vasculogenesis. Thus, our results provide the first in vivo evidence for the functions of EPO and EPOR in vivo in angiogenesis.

---

* Corresponding author. Fax: +1 310 267 0242.
E-mail address: hwu@mednet.ucla.edu (H. Wu).

Materials and methods

Animal breeding and embryo harvesting

Mice with deletions in the *Epo* or *EpoR* genes have been described (Wu et al., 1995, 1999). The morning when the vaginal plug could be detected was defined as embryonic day 0.5 (E0.5). Somite and presomite embryos were staged according to criteria previously described (Downs and Davies, 1993).

In situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde (in PBS) at 4°C. Nonradioactive whole-mount mRNA in situ hybridization technique was performed as previously described (Belo et al., 1997). Digoxigenin-labeled ribo-probes were synthesized from a *Bam*H1/*Eco*R1 500-bp fragment of murine *Epo* cDNA corresponding to exons 4 and 5, and a *Xho*I/*Hin*dIII 949-bp fragment corresponding to 270–1219 of the *EpoR* cDNA (Lee et al., 2001). Embryos, post in situ hybridization, were embedded in liquid paraplast and sectioned at 7 μm. The sections were photographed.

Histology and immunohistochemistry

Whole-mount immunohistochemistry for visualization of vasculature was performed according to protocol described by Koblizek et al. (1998) using an antibody specific to mouse PECAM (anti-CD31, Pharmingen). HRP-conjugated secondary Antibody (Roche) was used followed by DAB staining.

Northern blot analysis

RNA was isolated with Qiagen RNA miniprep kit according to the manufacturer's protocol. Total RNA were separated by a 1% agarose gel and transferred onto a nylon membrane. The RNA was immobilized on the membrane by UV cross-linking and dried at 80°C for 2 h. The blot was prehybridized at 65°C in QuickHyb hybridization solution (Stratagene) for 30 min and then hybridized for 1 h with VEGF and Ang-1 cDNA probes labeled with [α-^{32}P] dCTP by using a random primer labeling kit (Stratagene). The blot was washed as recommended by Stratagene. Hybridization signals were visualized by radiography and quantified using Scion Image software (NIH, Bethesda, MD), using a Kodak gray color scale as a standard. Each membrane was stripped and reprobed with labeled actin probe for loading control.

HUVEC cell culture

Normal HUVEC were obtained from Cambrex (Bio-Whittaker) and maintained in EBM2 medium supplemented with 0.1 mg/ml endothelial cell growth supplement (crude extract from bovine brain), penicillin (50 U/ml), streptomy-cin (50 U/ml), 2 mmol/l glutamine, and 0.1 mg/ml sodium heparin. Aliquots of cells were preserved frozen between passages 1 and 3. For all experiments, HUVECs were used at passages 4 or below and collected from a confluent dish. Cells were treated with 10 Units of rHEPO (a gift from Amgen) and cells were collected at 2 and 5 h after stimulation.

Epo transgenic mice

The full-length human *Epo* cDNA (*Hin*dIII–*Apa*I) was cloned into the *Hpa*I site of the plasmid bMHC, which contains the beta myosin heavy chain promoter and 5′ untranslated sequence of the *hEpo* start and termination codons, as well as a human growth hormone 3′ UT and poly A sequence (Rindt et al., 1993). Transgenic mice were made by pronuclear injection and founder mice were identified by PCR analysis using primers of 5′-ACTCCTGAGTGCT-GAGCA-3′ and 5′-CCTAGTCAAACAAAATGATGCA-3′, which amplifies a fragment of 800 bp. Five founders survived to adulthood but three died 4–7 weeks after birth due to, at least in part, splenomegaly and extremely high hematocrit (97%). Two surviving lines, with hematocrit ranging 60–75%, were used for further studies.

FITC-lectin injection

Half milliliter of FITC-Lectin (Vector Laboratories, 40 μg/ml in PBS) was injected into each mouse via tail vein under anesthesia (40 μl/mouse of ketamine 100 mg/ml and xylazine 20 mg/ml mixed 4:1 solution). One minute later, the mice were perfused with 1% paraformaldehyde (in PBS) for 2 min and then perfused with PBS for 2 min by cardiac perfusion. Then, the peritoneal membranes were dissected out, mounted on slide, and observed under fluorescent microscope.

Real-time quantitative PCR

Assays were performed using an Applied Biosystems 7700 sequence detector. Briefly, 1 μg of total RNA was reverse transcribed with random hexamers using the Taqman reverse transcription reagent kit (Applied Bio-systems) according to the manufacturer's protocol. Each Taqman reaction (50 μl) contained 50 ng of cDNA, 900 nM forward primer, 900 nM reverse primer, 100 nM dual-labeled fluorogenic probe (IDT), and 25 μl of Universal PCR Master mix (Qiagen). PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. All samples were analyzed for 36B4 expression in parallel. The quantitative expression values were extrapolated from standard curves for VEGF or 36B4. Each sample was run in duplicate and was normalized to 36B4. The replicates were then averaged and fold induction was determined. Statistical analysis of

mRNA expression data was performed by using the two-tailed, homoscedastic *t* test. Probe and primer sequences are available upon request.

Results

Differential expression of Epo and EpoR during early vasculogenesis

The vascular system develops earlier during embryogenesis, both extraembryonically and intraembryonically, to satisfy the metabolic requirements for gastrulation and organogenesis. To study the possible roles of EPO and EPOR in early vasculogenesis, we performed detailed in situ hybridization analysis on E6.5–E9.0 embryos to investigate the timing and location of *Epo* and *EpoR* expression.

Whole-mount in situ hybridization analysis revealed that *EpoR* expression starts as early as E7.5 in the extraembryonic coelemic cavity and mesoderm derived allantois (all, arrows) (Figs. 1a,d). This expression pattern continues until late neural fold stage, at which time *EpoR* expression is clearly evident in the yolk sac blood islands (bi) (Fig. 1b). During early embryogenesis, vasculogenesis in the yolk sac blood islands is closely associated with primitive hematopoiesis. Blood islands are formed by aggregation of mesoderm cells: Cells at the periphery of these aggregates differentiate into endothelial precursors, while cells inside become primitive blood cells (Risau, 1991). Fusion of these blood islands eventually results in the formation of a continuous vascular channel system throughout the yolk sac. *EpoR* expression can be detected in both endothelial cells (green arrow) and primitive blood cells (red arrow, Fig. 1e). At E9.0, *EpoR* expression is strong in the extraembryonic vessel networks (yolk sac vasculature, ysv; Fig. 1c) as well as vitelline vessels (Fig. 1f), which develop embryonically and are responsible for connecting the embryonic vasculature with the yolk sac vasculature (Rugh and Somogyi, 1968).

In contrast to high levels of *EpoR* expression in extraembryonic tissues, *Epo*, which encodes the only known ligand for the EPOR, is undetectable, either extra- or intraembryonically during these stages of embryonic development (Figs. 1g–i). Thus, similar to initiation of primitive erythrocytes in the yolk sac (Lee et al., 2001; Lin et al., 1996; Wu et al., 1995), the initiation and formation of the extraembryonic vasculature is probably EPO-independent (see below).
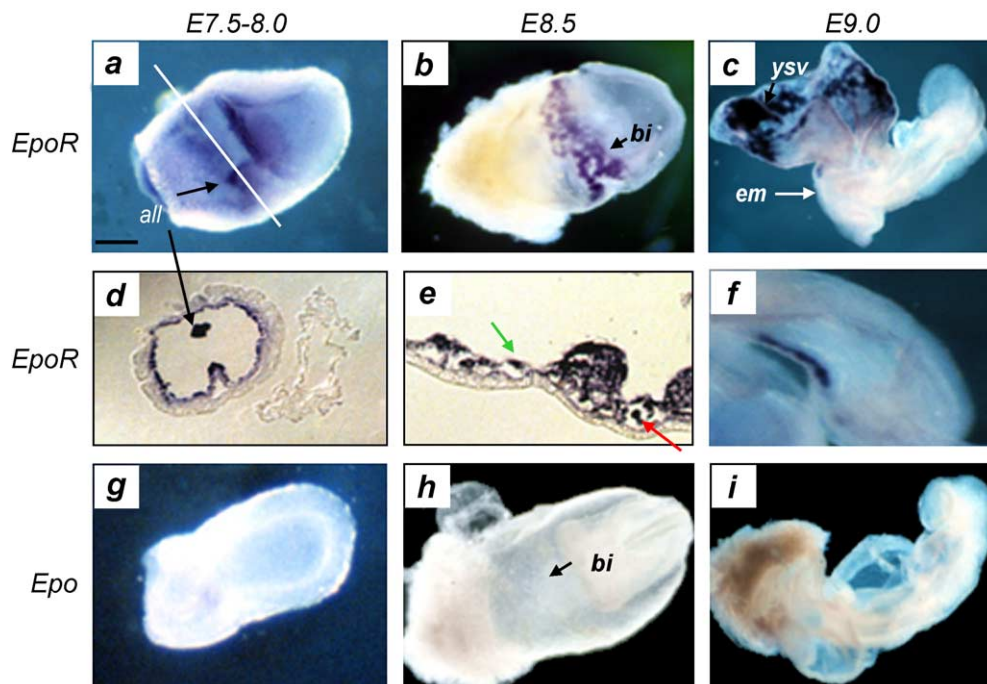

Fig. 1. Whole-mount in situ hybridization analysis of *EpoR* and *Epo* expressions during early embryogenesis. Embryos are viewed laterally. (a) Early head-fold stage embryo (E7.5) showing *EpoR* expression in the extraembryonic coelemic cavity, the chorion, and in the mesoderm derived allantois (all). (b) High level of *EpoR* expression in the blood islands of the yolk sac. (c) E8.75 embryo displaying *EpoR* expression in the extraembryonic vessel network, specifically in the yolk sac vasculature, and a lack of expression in the embryo proper (em). (d) Transverse section of (a) shows expression is confined to extraembryonic ectoderm and mesoderm-derived allantois. (e) Transverse section of (b) showing *EpoR* expression in both endothelial cells (green arrow) and primitive blood cell component of the blood islands (red arrow). (f) Lateral view of embryo in (c) with anterior to the left; *EpoR* expression is present in the vitelline vessels. (g) Late streak stage embryo (E8.0). No *Epo* expression could be detected either embryonically or extraembryonically. (h) Lack of *Epo* expression in head-fold stage (E8.0–8.5) embryo. (i) E9.0 embryo showed a lack of *Epo* expression in both the extraembryonic and embryonic regions. No expression could be detected when embryos were hybridized with sense probes in all stages studied (data not show). Scale bar = 250 μm in a–c and g–i; 1000 μm in d–f.

Overlapping expressions of Epo and EpoR during midgestation

Around E8.5–9.0, the embryonic vessels connect with the extra-embryonic vasculature through umbilical and vitelline vessels and fetal circulation begins. The yolk-sac-derived primitive nucleated red blood cells fill the embryonic vasculature. One day later, the liver rudiment is formed and soon receives hematopoietic stem cells and progenitors generated from the yolk sac or/and from the aorta-genital ridge-mesonephros (AGM) region. Figs. 2a and b show the overlapping expression of *Epo* and *EpoR* at E10.0 in the vasculature, which leads to the hepatic primordium (hpv). At E10.5, both *Epo* and *EpoR* are expressed in the dorsal aorta (da, Figs. 2c,d), a site enriched in definitive hematopoietic stem cells (Dieterlen-Lievre and Martin, 1981; Medvinsky and Dzierzak, 1996), suggesting that EPO may play an autocrine role in the expansion of the erythroid/megakaryocyte co-progenitors or erythroid progenitor cells in these regions. The overlapping expression of *Epo* and *EpoR* during this stage of embryonic development fits precisely the roles of EPO and EPOR in definitive erythropoiesis since targeted disruption of *EpoR* or *Epo* leads to a complete block in fetal liver erythropoiesis and causes embryonic lethality (Lin et al., 1996; Wu et al., 1995). Whole-mount in situ hybridization analysis also indicated that both *EpoR* (Fig. 2e) and *Epo* (Fig. 2f) are expressed in the intersomitic vessels, with *EpoR* expression extending more rostral and at higher levels as compared to *Epo* expression. The fact that Epo expression is mainly restricted to the caudal intersomitic vessels, which are less mature than the rostral ones, suggest the possible involvement of EPO signaling in initiation of angiogenesis.

Normal vasculogenesis in Epo$^{-/-}$ and EpoR$^{-/-}$ embryos

Functional significance of *Epo* and *EpoR* expression in the vessels outside of the heart was ascertained by further
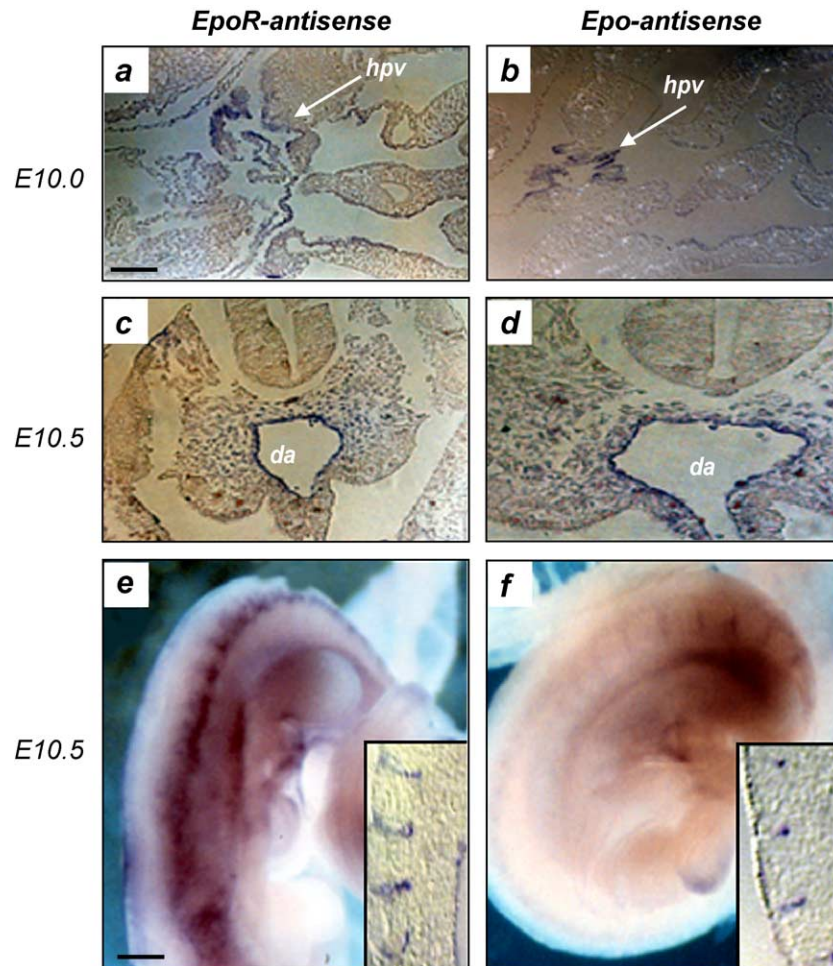

Fig. 2. Overlapping expressions of *Epo* and *EpoR* during mid-gestation stage. (a,b) Transverse section of E10 embryos through the AGM region. Note the overlapping expression of *Epo* and *EpoR* in the vasculature leading to the hepatic primordium (hpv). (c,d) *Epo* and *EpoR* are expressed in the dorsal aorta in E 10.5 embryo sections. Transverse section at the level of the anterior part of the dorsal aorta reveals specific expression in cells lining the lumen of the dorsal aorta (da). (e) *EpoR* expression in the intersomitic vessels extends rostrally. Parasagittal section showing vessels in between the somites are positive for *EpoR*. (f) Whole mount of E10.5 embryo demonstrating positive expression of *Epo* in the caudal intersomitic vessels. Scale bar = 540 μm in a and b; 250 μm in c; and 125 μm in d.

examination of $Epo^{-/-}$ and $EpoR^{-/-}$ embryos. Blood vessel development consists of two distinct phases, vasculogenesis and angiogenesis. Vasculogenesis is a process in which individual endothelial progenitors assemble vessels de novo. This contrasts with angiogenic assembly, in which new vessels arise from existing vessels through endothelial branching, sprouting, migration, proliferation, and anastomic interconnection with endothelial cells residing in existing vessels (Daniel and Abrahamson, 2000). Studies performed in quail-chick chimeras suggest that vasculogenesis mainly accounts for the formation of the heart, dorsal aorta, cardinal and vitelline vessels, as well as the extraembryonic vessels of the yolk sac (Coffin and Poole, 1988, 1991; Pardanaud et al., 1989). Angiogenesis is thought to be responsible for the formation of vessels such as the intersomitic arteries and the vessels in individual organs (Coffin and Poole, 1988). As shown in Fig. 3, no
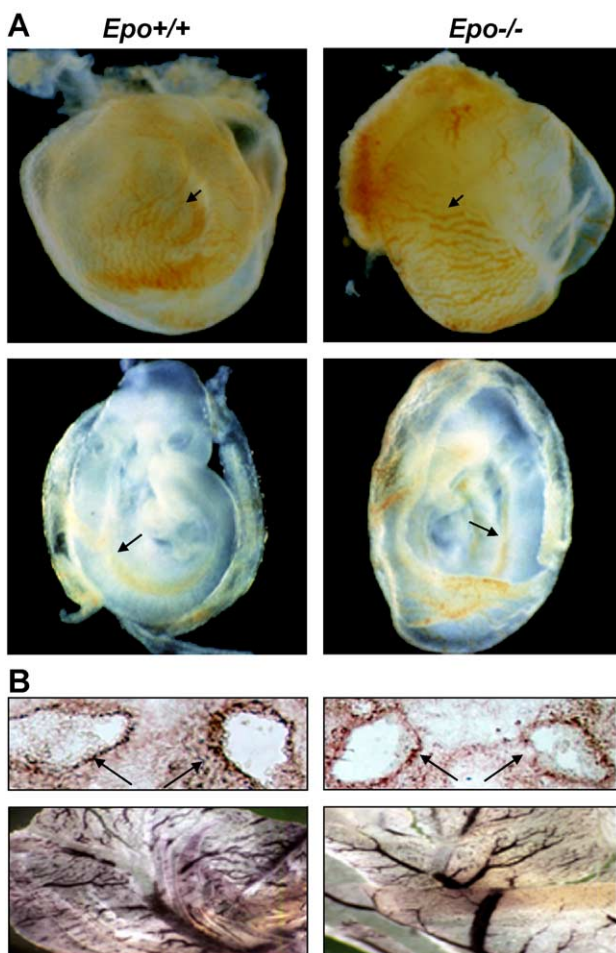

Fig. 3. Analysis of vasculogenesis in the knock-out embryos. (A) Embryonic stage 9.5. The blood vessels are clearly noticeable in the extra-embryonic yolk sac (arrows in upper panels) and dorsal aorta (arrows in lower panels) of both WT (left panels) and mutant (right panels) embryos at this stage. (B) PECAM-1 staining of embryo sections (upper) and yolk sacs. Arrows indicate dorsal aorta; notice the diameter of lumen in the mutant is comparable to WT (upper panel). Yolk sac whole mounts show comparable PECAM-1 staining in WT and mutant at this stage (lower panel). Scale bar = 600 μm in A and 250 μm in B.

significant differences in vascular patterning were detected in the null embryos, E9.5 or younger, as compared to the WT or heterozygous littermates. Both intra- (lower panels, arrows point to dorsal aorta) and extra-embryonic vasculatures (upper panels) are well developed in the mutant embryos (Fig. 3A). PECAM-1 staining, a specific marker for vascular endothelial cells, on the mutant yolk sac whole mounts and embryonic sections further revealed that the extraembryonic vessel network and the diameter of the mutant dorsal aorta and the intensities of PECAM-1 staining are comparable to that of the WT littermates (Fig. 3B). Because earlier endothelial cell differentiation and formation of blood islands, dorsal aorta, heart, and primordial vasculature are normal in $Epo^{-/-}$ and $EpoR^{-/-}$ embryos and yolk sacs, our results suggest that earlier vasculogenesis is EPO and EPOR independent, which is consistent with our expression studies (Figs. 1 and 2).

Angiogenesis defects in $Epo^{-/-}$ and $EpoR^{-/-}$ embryos

Starting from E10.5, 1.5 days after the commencement of blood circulation, which connects the extra-embryonic compartment with the embryo proper, both $Epo^{-/-}$ and $EpoR^{-/-}$ embryos appeared pale due to the absence of definitive erythropoiesis (Lee et al., 2001; Wu et al., 1995). Interestingly, both mutant embryos also showed defects in the second wave of blood vessel formation, especially in the head region where active angiogenesis takes place. To confirm that this observation is not simply due to the lack of red blood cells in the vessels, we performed whole-mount PECAM-1 staining. The WT head contains well-branched blood vessel networks, whereas the complexity of cranial vasculatures in $Epo^{-/-}$ and $EpoR^{-/-}$ embryos was decreased: with narrower vessel diameter and fewer branches (Fig. 4, upper panels). PECAM-1 staining is also weaker in both mutant intersomitic vessels (Fig. 4, middle panels), where high levels of Epo and EpoR expression are detected in the WT embryos by in situ hybridization analysis (Figs. 2e,f). This angiogenesis defect happens 2 days before the onset of observable cardiac defects (Wu et al., 1999) and is therefore likely to be a primary defect in angiogenesis.

At E12.5, angiogenesis defects became even more severe. We performed PECAM-1 staining on embryonic sections across the neural tube areas since (1) angiogenesis is very active during this stage of embryogenesis; and (2) high levels of both Epo and EpoR expressions can be detected in the neural tube region (Yu et al., 2002 and our unpublished observation). Lower panels in Fig. 4 clearly demonstrated angiogenesis defects in the neural tube regions: capillary sprouting is severely diminished in both mutants, as compared to the WT control where angiogenic sprouting is numerous in the neural tube and surrounding mesenchyme tissues. Taken together, these results suggest that EPO and EPOR are vital factors for angiogenesis but dispensable for vasculogenesis.
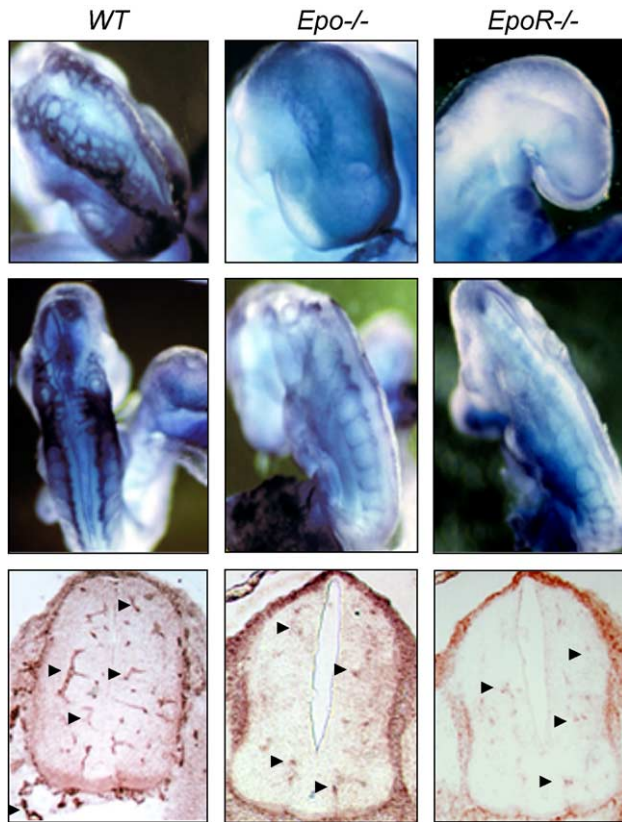
Fig. 4. Analysis of angiogenesis defects in the mutant embryos. Upper and middle panels, embryonic stage 10.5. Notice clear defects of vascular network in the head region (upper panels) or intersomitic region (middle panels) of both mutant embryos based on whole mounts for PECAM staining. Lower panels, embryonic stage 12.5. PECAM-stained sections show lack of extensive vascular network in neural tube regions in both types of mutant animals. Capillary sprouting is severely diminished in the neural tube in the mutant embryos, as compared to the WT control where angiogenic sprouting is numerous (arrowheads).

Overexpression of Epo leads to increased vascularization and partial rescue of the angiogenic defects in Epo$^{-/-}$ embryos

If the above defects were a direct result of lacking EPO or EPOR signaling, we would expect to rescue the vascular defects of *Epo$^{-/-}$* embryos with an *Epo* transgene. For this, we generated transgenic lines carrying human *Epo* cDNA (see Materials and methods), using bMHC promoter (Rindt et al., 1993). Because EPO is a secreted hormone, its effects will be seen systemically. Five founders survived to adulthood but three died 4–7 weeks after birth due to, at least in part, spleenomegaly and extremely high hematocrit (data not shown). Two surviving lines, with hematocrit ranging 60–75%, were used for further studies.

Based on RT-PCR analysis, *hEpo* transgene expression can be detected as early as E11 (Tran and Sucov, unpublished observation). As a result of overexpression of *hEpo*, a significant increase in the number of vessels, as visualized by FITC-labeled lectin (Fig. 5A, upper

right), was observed in adult mice of two independent transgenic lines. We also detected significant morphological changes in the vessel structures. Instead of straight small vessels branching out from the large vessels, the transgenic mice contained abundant tortuous vessels (Fig. 5A, lower right). When crossed to EPO-deficient mice, we found that the vascular defects in the null embryos were partially rescued by the *hEpo* transgene expression (Fig. 5B).

EPO and the expression of angiogenic factors

Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) are two important factors involved in the vascular formation during embryogenesis. VEGF is crucial for de novo vasculogenesis, the first wave of vasculogenesis, as well as for angiogenesis. Ang-1, on the other hand, has less effect on vasculogenesis but is indispensable for angiogenesis. To investigate the possible mechanism involved in EPO-controlled angiogenesis, we first compared the expression levels of *Vegf* and *Ang-1* in E12.5 *EpoR$^{+/+}$* and *EpoR$^{-/-}$* embryonic head region by Northern blot analysis. Interestingly, the expression of *Vegf* is up-regulated by at least 4-fold in the receptor null embryos (Fig. 6A, left panel), which is most likely due to the hypoxic condition caused by erythropoietic defects (Wu et al., 1995), since *Vegf* expression levels are comparable in the placenta tissue of WT and mutant embryos (Fig. 6A, right panel), a tissue that is not hypoxic due to maternal blood circulation. However, the expression of *Ang-1* is reduced by 2-fold, as compared with WT controls (Fig. 6A, left panel), suggesting that the EPO/EPOR signaling pathway may somehow modulate *Ang-1* expression.

To prove that the decrease in *Ang-1* levels is a direct result of the lack of EPO signaling, we employed HUVEC cells as well as primary murine aortic-derived endothelial cells (data not shown), which express the endogenous *EpoR* and can respond to EPO stimulation (Anagnostou et al., 1990, 1994; Carlini et al., 1995). Consistent with our in vivo studies, EPO stimulation under normoxic conditions resulted in a 2- to 4-fold increase in *Ang-1* expression in HUVEC cells by Northern blot analysis (Fig. 6B, left), or 2.5-fold based on Q-PCR (Fig. 6B, right). Interestingly, under the normoxic condition, *Vegf* levels are also induced upon EPO stimulation (Fig. 6B). This provides the first evidence that EPO/EPOR signaling pathway controls the expressions of *Ang-1* and *Vegf*, two crucial angiogenic factors, and suggests that decreased *Ang-1* expression may directly or indirectly contribute to the angiogenesis defects seen in the mutant embryos.

Discussion

In this study, we addressed the potential roles of EPO and EPOR in angiogenesis. Our in situ hybridization analysis
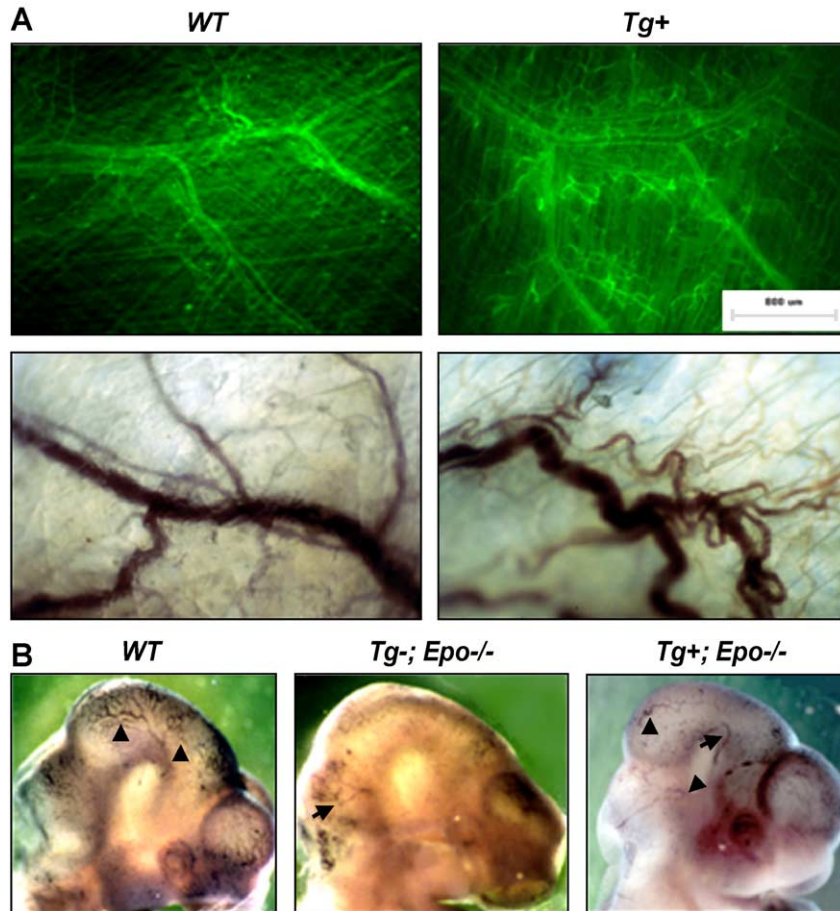
Fig. 5. Overexpression of h*Epo* induces adult angiogenesis and significantly rescues the vascular defects seen in *Epo* null embryos. (A) FITC-Lectin staining of the WT (left panels) and *Epo* transgenic (right panels) mice. Notice many more small tortuous vessels in the transgenic animal. Lower panels show the tortuous vessels in the transgenic ear. Scale bar = 800 μm in upper panels and 200 μm in lower panels. (B) Vascular network of the *WT* vs. *Epo*$^{-/-}$; *EpoTg* embryo: PECAM staining, showing partial rescue of *Epo*$^{-/-}$ embryo with significantly improved vessel structure and network (right) compared to *Epo* null embryo (middle).

clearly demonstrated that *Epo* and *EpoR* are expressed in situ in the vasculature. By analyzing *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ mice as well as mice overexpressing *Epo*, we also provided the first genetic evidence for the roles of EPO and EPOR in angiogenesis.

EPO and EPOR are crucial for angiogenesis

The formation of new blood vessels (angiogenesis) is essential for embryonic development and contributes to the pathogenesis of numerous disorders. In contrast, insufficient angiogenesis may lead to tissue ischemia and failure. The effect of EPO on in vivo vasculogenesis has not been previously elucidated. However, the potential roles of EPO and EPOR in vascular function have been indicated in both in vitro and semi-in vivo studies: rEPO has been shown to increase microvascular branch formation from rat aortic rings in a standard angiogenic assay. In addition, rEPO has been shown to up-regulate expression of several genes involved in vascular function, signal transduction, and energy transfer, in cultured endothelial cells (Banerjee et al., 2000; Carlini et al., 1995; Fodinger et al., 2000; Heidenreich et al., 1991; Wang and Vaziri, 1999). Recent studies also link EPO with malignant tumor growth (Yasuda et al., 2003), at least in part, due to its angiogenic effects (Ribatti et al., 2003). Our study demonstrates that EPO and EPOR regulate angiogenesis in vivo, a process involving the formation of new vessels on existing vessels, but not de novo vasculogenesis. Interestingly, vasculatures in both *Epo*$^{-/-}$ and *EpoR*$^{-/-}$ embryos are equally affected, suggesting EPOR is the signaling receptor for EPO in regulating angiogenesis.

The defects seen in the mutant vasculatures fit precisely with the patterns of *Epo* and *EpoR* expressions: *EpoR* is expressed in the yolk sac blood islands and vascular structures. However, *Epo* is absent during this early stage of embryogenesis, the period that vasculogenesis takes place. Thus, similar to initiation of primitive erythropoiesis in the yolk sac blood islands (Lee et al., 2001; Wu et al., 1995), the initiation of vascular structures is also EPO-independent. It is only by E9.0–E10.5 when EPO starts to
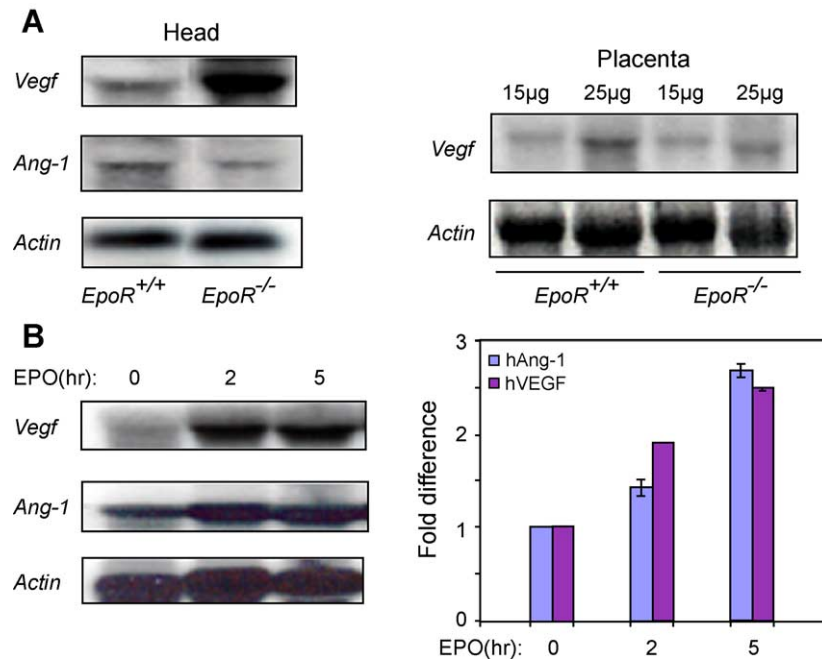
Fig. 6. EPO regulates *Ang-1* and *Vegf* expression. (A) Northern blot analysis of *Ang-1* and *Vegf* expressions in E12.5 *EpoR* null (lane 2) and WT embryo (lane 1) heads (left) and placenta (right). *Vegf* expression is significantly increased by 4-fold in the mutant embryos as compared to the WT (upper panel). The same blot was reprobed with *Ang-1* probe (middle). *Ang-1* expression is significantly lower (about 2 fold) in the null embryo compared to the WT. *Vegf* expression, on the other hand, is not changed in the placental tissues: 15 and 25 μg of total RNA were loaded, respectively. Actin is used as loading control. (B) The levels of *Ang-1* and *Vegf* were determined by Northern blot analysis (left) or real-time quantitative PCR (right). EPO (10 U) stimulation leads to increased *Vegf* and *Ang-1* expression at 2 and 5 h.

be produced intraembryonically that angiogenesis defects become apparent.

Recently, Suzuki et al. (2002) showed rescue of lethality of *EpoR* null mutant mice upon expression of *EpoR* under an erythroid specific promoter GATA-1 and concluded that nonhematopoietic expression of *EpoR* is dispensable to normal mouse development. This discrepancy could be due to the (1) different *EpoR*-deficient animals were used. The *EpoR* knockout mice Suzuki et al. used carries an exon-3-specific deletion (Kieran et al., 1996) that leads to erythroid defects less severe than our mutant line in which the promoter and exons 1–6 are completely deleted (Wu et al., 1995). Importantly, the erythroid colony formation defects of our $EpoR^{-/-}$ progenitors cannot be rescued by TPO (Wu, unpublished observation), different from what was reported (Kieran et al., 1996). (2) The angiogenesis defects seen in *Epo*- and *EpoR*-deficient embryos may happen transiently during a specific stage of embryonic development, similar to the erythroid defects seen in $RXR\alpha^{-/-}$ embryos at E10.25–11.25 (Makita et al., 2001) or Stat5a$^{-/-}$5b$^{-/-}$ mice during fetal stage (Socolovsky et al., 1999). (3) Because GATA-1 is expressed in the Flk1+ extra-embryonic mesoderm, as well as the hematopoietic cells (Fujimoto et al., 2001), the *EpoR* transgene, under the control of the GATA-1 promoter, may be expressed at low levels in the hemangioblasts or vessel endothelium, and although difficult to be detected, sufficient to rescue the vessel defects. Further investigation will help in distinguishing these possibilities.

Angiogenic defects and hypoxia

The lack of EPO or EPOR is clearly accompanied by alterations in the relative oxygen tensions in the mutant animals. However, the angiogenesis defects described in this study are unlikely due to general hypoxia. Hypoxia will induce the expression and activation of transcription factor HIF-1α, which in turn activates gene expression, such as *Vegf* and promotes vessel formation. Therefore, hypoxia conditions usually cause increased angiogenesis, rather than decreased angiogenesis, as we observed in this study. Furthermore, the onset and location of angiogenesis defects correlate well with endogenous *Epo* expression and EPO production, suggesting that EPO/EPOR plays an active role in promoting new vessel formation.

During vertebrate embryogenesis, blood cell formation and vasculogenesis are intertwined temporally and spatially to satisfy the metabolic requirements of the developing embryo. It is interesting that *EpoR* is expressed early in the murine yolk sac where the blood cell and vessel formation takes place, and EPO and EPOR are required for the second, definitive waves of erythropoiesis (Lee et al., 2001) as well as the second wave of vasculogenesis, as indicated in this study. Whether the low shear stress, i.e., decreased hematocrit due to anemia, in the vasculature of the mutant embryo could contribute to the angiogenesis defects observed in this study needs to be further investigated. However, studies on zebrafish mutants affecting either the

cardiovascular system (Stainier et al., 1996) or hematopoiesis (Paw and Zon, 2000; Weinstein et al., 1996) seem to suggest that the formation of the zebrafish circulation system are not severely influenced by the low shear stress caused by hematopoietic defects.

EPO and Vegf/Ang-1 expressions

Although the precise mechanism by which EPO regulates angiogenesis remains to be studied, it is most likely initiated by binding of EPO to EPOR on endothelial cells and activation of the downstream signaling cascades, since both *Epo*- and *EpoR*-deficient mice demonstrated identical phenotypes throughout our studies.

We have shown decreased *Ang-1* expression in the mutant embryos. Conversely, EPO stimulation leads to increased *Ang-1* levels, indicating that EPO may regulate angiogenesis through, at least in part, by modulating *Ang-1* expression. The mechanism whereby EPO affects *Ang-1* expression remains to be further elucidated. Recent studies have demonstrated that *Ang-1* induces endothelial cell sprouting, endothelial cell migration, and tubule-like structure formation in vitro (Fujikawa et al., 1999; Hayes et al., 1999; Koblizek et al., 1998). In addition, increased branching and vessel density is observed in vivo upon overexpression of *Ang-1*, providing strong evidence of an active role for *Ang-1* in both embryonic and adult angiogenesis (Suri et al., 1998). Interestingly, increased vessel density is also evident in *hEpo* transgenic animals.

Ang-1 regulates endothelial cells by binding to its cognate receptor, Tie2 (Yancopoulos et al., 2000). Recent studies suggest that EPO and Ang-1 may employ similar signaling pathways for their biological functions; for example, STAT3 and STAT5 were found to be potential targets of Tie2 activation. As STAT3 and/or STAT5 have been implicated in the regulation of EPO-mediated biological effects (for a review, see (Akira, 1999), some of the Tie2 functions in endothelial cells may be regulated by the cross-talk from the EPOR signaling pathway. Both *Epo* and *Vegf* are under the control of HIF-1α, a transcription factor whose activity is up-regulated under hypoxic conditions. It is interesting to observe up-regulation of *Vegf* expression upon EPO stimulation under normoxic conditions. The precise mechanism and biological significance of such regulation needs further investigation.

Multifunction of EPO and EPOR: a mode of action

Generation of animals lacking either *Epo* or *EpoR* genes established genetic systems to address the function of this ligand/receptor pair in erythropoiesis (Wu et al., 1995), in cardiac morphogenesis (Wu et al., 1999), in neurogenesis (Yu et al., 2002), and in angiogenesis (this study). The mutant phenotypes observed in the mutant mice fit precisely the temporal and spatial expression patterns of *Epo* and *EpoR* genes. First, *EpoR* is always expressed before that of *Epo* during embryonic development to "prime" or prepare cells to respond to EPO stimulation. Secondly, *Epo* and *EpoR* expression are spatially separated in the yolk sac: *EpoR* is expressed in the yolk sac blood island and vasculatures, but *Epo* expression is absent in the yolk sac. This spatially uncoupled expression pattern provides a cellular mechanism as to why the initiation of primitive erythropoiesis and primitive vasculogenesis in the yolk sac are independent of EPO and EPOR even though the cells are fully competent to respond to EPO stimulation. Only until later when both *Epo* and *EpoR* are co-expressed intra-embryonically can defects in definitive erythropoiesis in the fetal liver, hypoplasia in the myocardium, and defects in angiogenesis be observed.

References

Akira, S., 1999. Functional roles of STAT family proteins: lessons from knockout mice. Stem Cells 17, 138–146.

Anagnostou, A., Lee, E.S., Kessimian, N., Levinson, R., Steiner, M., 1990. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 87, 5978–5982.

Anagnostou, A., Liu, Z., Steiner, M., Chin, K., Lee, E.S., Kessimian, N., Noguchi, C.T., 1994. Erythropoietin receptor mRNA expression in human endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 91, 3974–3978.

Banerjee, D., Rodriguez, M., Nag, M., Adamson, J.W., 2000. Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity. Kidney Int. 57, 1895–1904.

Belo, J.A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M., De Robertis, E.M., 1997. Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. Mech. Dev. 68, 45–57.

Carlini, R.G., Reyes, A.A., Rothstein, M., 1995. Recombinant human erythropoietin stimulates angiogenesis in vitro. Kidney Int. 47, 740–745.

Coffin, J.D., Poole, T.J., 1988. Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. Development 102, 735–748.

Coffin, J.D., Poole, T.J., 1991. Endothelial cell origin and migration in embryonic heart and cranial blood vessel development. Anat. Rec. 231, 383–395.

D'Andrea, A.D., Fasman, G.D., Lodish, H.F., 1989a. Erythropoietin receptor and interleukin-2 receptor beta chain: a new receptor family. Cell 58, 1023–1024.

D'Andrea, A.D., Lodish, H.F., Wong, G.G., 1989b. Expression cloning of the murine erythropoietin receptor. Cell 57, 277–285.

Daniel, T.O., Abrahamson, D., 2000. Endothelial signal integration in vascular assembly. Annu. Rev. Physiol. 62, 649–671.

Dieterlen-Lievre, F., Martin, C., 1981. Diffuse intraembryonic hemopoiesis in normal and chimeric avian development. Dev. Biol. 88, 180–191.

Downs, K.M., Davies, T., 1993. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development 118, 1255–1266.

Fodinger, M., Fritsche-Polanz, R., Buchmayer, H., Skoupy, S., Sengoelge, G., Horl, W.H., Sunder-Plassmann, G., 2000. Erythropoietin-inducible immediate-early genes in human vascular endothelial cells. J. Investig. Med. 48, 137–149.

Fujikawa, K., de Aos Scherpenseel, I., Jain, S.K., Presman, E., Christensen, R.A., Varticovski, L., 1999. Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. Exp. Cell Res. 253, 663–672.

Fujimoto, T., Ogawa, M., Minegishi, N., Yoshida, H., Yokomizo, T., Yamamoto, M., Nishikawa, S., 2001. Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. Genes Cells 6, 1113–1127.

Hayes, A.J., Huang, W.Q., Mallah, J., Yang, D., Lippman, M.E., Li, L.Y., 1999. Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. Microvasc. Res. 58, 224–237.

Heidenreich, S., Rahn, K.H., Zidek, W., 1991. Direct vasopressor effect of recombinant human erythropoietin on renal resistance vessels. Kidney Int. 39, 259–265.

Kieran, M.W., Perkins, A.C., Orkin, S.H., Zon, L.I., 1996. Thrombopoietin rescues in vitro erythroid colony formation from mouse embryos lacking the erythropoietin receptor. Proc. Natl. Acad. Sci. U. S. A. 93, 9126–9131.

Koblizek, T.I., Weiss, C., Yancopoulos, G.D., Deutsch, U., Risau, W., 1998. Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr. Biol. 8, 529–532.

Lee, R., Kertesz, N., Joseph, S.B., Jegalian, A., Wu, H., 2001. Erythropoietin (Epo) and EpoR expression and 2 waves of erythropoiesis. Blood 98, 1408–1415.

Lin, C.S., Lim, S.K., D'Agati, V., Costantini, F., 1996. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. Genes Dev. 10, 154–164.

Makita, T., Hernandez-Hoyos, G., Chen, T.H., Wu, H., Rothenberg, E.V., Sucov, H.M., 2001. A developmental transition in definitive erythropoiesis: erythropoietin expression is sequentially regulated by retinoic acid receptors and HNF4. Genes Dev. 15, 889–901.

Medvinsky, A., Dzierzak, E., 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897–906.

Ogilvie, M., Yu, X., Nicolas-Metral, V., Pulido, S.M., Liu, C., Ruegg, U.T., Noguchi, C.T., 2000. Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts. J. Biol. Chem. 275, 39754–39761.

Pardanaud, L., Yassine, F., Dieterlen-Lievre, F., 1989. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development 105, 473–485.

Paw, B.H., Zon, L.I., 2000. Zebrafish: a genetic approach in studying hematopoiesis. Curr. Opin. Hematol. 7, 79–84.

Ribatti, D., Presta, M., Vacca, A., Ria, R., Giuliani, R., Dell'Era, P., Nico, B., Roncali, L., Dammacco, F., 1999. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood 93, 2627–2636.

Ribatti, D., Marzullo, A., Nico, B., Crivellato, E., Ria, R., Vacca, A., 2003. Erythropoietin as an angiogenic factor in gastric carcinoma. Histopathology 42, 246–250.

Rindt, H., Gulick, J., Knotts, S., Neumann, J., Robbins, J., 1993. In vivo analysis of the murine beta myosin heavy chain gene promoter. J. Biol. Chem. 268, 5332–5338.

Risau, W., 1991. Embryonic angiogenesis factors. Pharmacol. Ther. 51, 371–376.

Rugh, R., Somogyi, C., 1968. Pre and postnatal normal mouse blood cell counts. Proc. Soc. Exp. Biol. Med. 127, 1267–1271.

Socolovsky, M., Fallon, A.E., Wang, S., Brugnara, C., Lodish, H.F., 1999. Fetal anemia and apoptosis of red cell progenitors in Stat5a−/−5b−/− mice: a direct role for Stat5 in Bcl-X (L) induction. Cell 98, 181–191.

Stainier, D.Y., Fouquet, B., Chen, J.N., Warren, K.S., Weinstein, B.M., Meiler, S.E., Mohideen, M.A., Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., Zwartkruis, F., Stemple, D.L., Malicki, J., Driever, W., Fishman, M.C., 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. Development 123, 285–292.

Suri, C., McClain, J., Thurston, G., McDonald, D.M., Zhou, H., Oldmixon, E.H., Sato, T.N., Yancopoulos, G.D., 1998. Increased vascularization in mice overexpressing angiopoietin-1. Science 282, 468–471.

Suzuki, N., Ohneda, O., Takahashi, S., Higuchi, M., Mukai, H.Y., Nakahata, T., Imagawa, S., Yamamoto, M., 2002. Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. Blood 100, 2279–2288.

Wang, X.Q., Vaziri, N.D., 1999. Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. Hypertension 33, 894–899.

Watowich, S.S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S.N., Lodish, H.F., 1996. Cytokine receptor signal transduction and the control of hematopoietic cell development. Annu. Rev. Cell Dev. Biol. 12, 91–128.

Weinstein, B.M., Schier, A.F., Abdelilah, S., Malicki, J., Solnica-Krezel, L., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Driever, W., Fishman, M.C., 1996. Hematopoietic mutations in the zebrafish. Development 123, 303–309.

Wu, H., Liu, X., Jaenisch, R., Lodish, H.F., 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 83, 59–67.

Wu, H., Lee, S.H., Gao, J., Liu, X., Iruela-Arispe, M.L., 1999. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 126, 3597–3605.

Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J., Holash, J., 2000. Vascular-specific growth factors and blood vessel formation. Nature 407, 242–248.

Yasuda, Y., Fujita, Y., Matsuo, T., Koinuma, S., Hara, S., Tazaki, A., Onozaki, M., Hashimoto, M., Musha, T., Ogawa, K., Fujita, H., Nakamura, Y., Shiozaki, H., Utsumi, H., 2003. Erythropoietin regulates tumour growth of human malignancies. Carcinogenesis 24, 1021–1029.

Yu, X., Shacka, J.J., Eells, J.B., Suarez-Quian, C., Przygodzki, R.M., Beleslin-Cokic, B., Lin, C.S., Nikodem, V.M., Hempstead, B., Flanders, K.C., Costantini, F., Noguchi, C.T., 2002. Erythropoietin receptor signalling is required for normal brain development. Development 129, 505–516.