Development 126, 3597-3605 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV4186

Inactivation of erythropoietin leads to defects in cardiac morphogenesis

Hong Wu[1,2], Sang Hoon Lee[3,*], Jing Gao[1], Xin Liu[4] and M. Luisa Iruela-Arispe[3,‡]

[1]Howard Hughes Medical Institute and [2]Department of Molecular and Medical Pharmacology, University of California at Los Angeles, Los Angeles, CA 90095-1735, USA
[3]Department of Molecular, Cell and Developmental Biology, University of California at Los Angeles, Los Angeles, CA 90095-1735, USA
[4]Department of Pathology and Laboratory Medicine, University of California at Los Angeles, Los Angeles, CA 90095-1735, USA
*Present address: Stanford University School of Medicine, Department of Medicine, 300 Pasteur Drive, S-128, Stanford, CA 94305-5109, USA
‡Author for correspondence (e-mail: arispe@mbi.ucla.edu)

Accepted 31 May; published on WWW 19 July 1999

SUMMARY

Erythropoietin is an essential growth factor that promotes survival, proliferation, and differentiation of mammalian erythroid progenitor cells. Erythropoietin$^{-/-}$ and erythropoietin receptor$^{-/-}$ mouse embryos die around embryonic day 13.5 due, in part, to failure of erythropoiesis in the fetal liver. In this study, we demonstrated a novel role of erythropoietin and erythropoietin receptor in cardiac development in vivo. We found that erythropoietin receptor is expressed in the developing murine heart in a temporal and cell type-specific manner: it is initially detected by embryonic day 10.5 and persists until day 14.5. Both erythropoietin$^{-/-}$ and erythropoietin receptor$^{-/-}$ embryos suffered from ventricular hypoplasia at day 12-13 of gestation. This defect appears to be independent from the general state of hypoxia and is likely due to a reduction in the number of proliferating cardiac myocytes in the ventricular myocardium. Cell proliferation assays revealed that erythropoietin acts as a mitogen in cells isolated from erythropoietin$^{-/-}$ mice, while it has no effect in hearts from erythropoietin receptor$^{-/-}$ animals. Erythropoietin$^{-/-}$ and erythropoietin receptor$^{-/-}$ embryos also suffered from epicardium detachment and abnormalities in the vascular network. Finally, through a series of chimeric analysis, we provided evidence that erythropoietin acts in a manner which is non-cell-autonomous. Our results elucidate a novel role of erythropoietin in cardiac morphogenesis and suggest a combination of anemia and cardiac failure as the cause of embryonic lethality in the erythropoietin$^{-/-}$ and erythropoietin receptor$^{-/-}$ animals.

Key words: Endothelial cells, Endocardium, Heart, Myocardium, Mouse

INTRODUCTION

Erythropoietin (EPO) is best known as an essential growth factor that regulates erythrocyte production in mammals (Krantz, 1991). Through specific binding to its cognate receptor, EPOR (D'Andrea et al., 1989), EPO triggers a chain of intracellular signaling events which depend on activation of Jak2 tyrosine kinase (Witthuhn et al., 1993). In addition to erythropoiesis, EPO has been found to be important for megakaryocyte proliferation and/or differentiation (Ishibashi et al., 1987; Broudy et al., 1995) (Papayannopoulou et al., 1996). Recent studies have also provided evidence for non-hematopoietic effects of EPO. For instance, EPOR expression was detected in umbilical cord and placental endothelial cells (Anagnostou et al., 1990, 1994) and neurons (Liu et al., 1994; Digicaylioglu et al., 1995; Morishita et al., 1996; Liu et al. 1997). EPO has been shown to promote endothelial cell proliferation in vitro (Anagnostou et al., 1990, 1994), and to assist neuron recovery from injury (Digicaylioglu et al., 1995).

We and others have investigated the physiological role of EPO and EPOR on erythropoiesis and animal development by generating mice lacking EPO (Wu et al., 1995) or EPOR (Wu et al., 1995; Lin et al., 1996; Kieran et al., 1996). We showed that EPO and EPOR play a vital role in controlling processes such as proliferation, survival, and irreversible terminal differentiation of erythroid progenitors (Wu et al., 1995; Lin et al., 1996; Kieran et al., 1996). In this study, we demonstrated a novel function of EPO and EPOR in embryonic heart development. We showed that both EPO$^{-/-}$ and EPOR$^{-/-}$ mice suffer from ventricular hypoplasia which is coupled to defects in the intraventricular septum. The cardiac abnormalities are likely due to a reduction in cell proliferation. Reduction in cell division appears specific to the heart, since proliferation in other organs, including brain in EPOR null animals is indistinguishable from that in wild-type mice. Interestingly, no EPOR expression could be detected in myocardium. Instead, a high level of EPOR expression was detected in the endocardium, epicardium, and pericardium. By injecting marked EPOR$^{-/-}$ ES cells into wild-type blastocysts and analyzing ES cell contribution in the chimeric animals, we showed that EPOR$^{-/-}$ ES cells could contribute widely to the normal structure and development of cardiomyocytes, indicating that EPO triggers cardiomyocyte proliferation probably in a non-cell-autonomous manner.

MATERIALS AND METHODS

Animals

Mice with deletions in the EPO or EPOR genes have been described (Wu et al., 1995). Staged embryos were obtained from mating between heterozygous animals. By convention, the morning when the vaginal plug was detected was defined as embryonic day 0.5 (E0.5). Embryos were dissected from pregnant females at days 10.5, 11.5, 12.5, 13.5 and 14.5. Homozygous embryos were clearly recognizable due to their pale appearance (Wu et al., 1995). Nevertheless, genotype was confirmed by Southern blot (Wu et al., 1995) or PCR analysis. Primers used for PCR analysis are: primer I GCACTGAGTGTGTTCTG; primer II GCCTCACACTCTTCACC; and primer III GCTGCTAAAGCGCATGC. The primers will amplify 789 bp and 417 bp fragments for wild-type and mutant allele, respectively. Embryos were then either fixed or dissected for photography, as described below.

Immunocytochemistry and in situ hybridization

For immunocytochemistry, whole embryos or dissected hearts were fixed in 4% paraformaldehyde or metha-Carnoy's (60% methanol, 30% chloroform, 10% acetic acid) by direct immersion for 1-2 hours at 4°C. Tissues were embedded in paraffin and 5 μm sections were obtained for both immunocytochemistry and in situ hybridization.

Anti-actin antibody (Sigma, St. Louis, MO) was used at 5 μg/ml, followed by biotinylated anti-mouse IgG and avidin-FITC.

Whole-mount immunocytochemistry for visualization of epicardial vessels was performed using an antibody specific to mouse PECAM at 5 μg/ml (Vector labs) followed by biotinylated secondary antibody and avidin-peroxidase (Vector Labs).

Preparation of digoxigenin-labelled RNA probes and in situ hybridization was performed as previously described (Lee et al., 1997). The following probes were used. (1) EPOR, a 949 bp *Xho*I-*Hin*dIII fragment from the mouse EPOR cDNA; (2) natriuretic factor, a 352 bp fragment of the mouse atrial natriuretic factor (ANF) amplified by reverse transcriptase (RT)-PCR and subcloned into pGEMT and (3) histone 3, a 1.3 kb fragment from the histone 3 coding region. Each experiment was repeated at least five times at any given developmental stage, and sense probe was used as a control on a consecutive serial section.

Tissue culture and cell proliferation assays

E11.5 embryonic hearts were dissected and rinsed three times in DME medium. Individual hearts were then placed on top of a filter (VWR 28157-814) with the support of wire grids (Wire Mesh Corp FSS607548M) in an organ culture dish (Falcon 3037). Cultures were carried out in DME medium supplemented with 10% heat-inactivated serum with or without EPO (1.0 units/ml) at 37°C with 5% CO_2. Twelve hours later, hearts were removed from the organ culture dishes and fixed as described above.

For primary cultures, hearts were dissected individually and digested with 3 mg/ml collagenase (Sigma, St. Louis, MO) in DMEM at 37°C for 30 minutes under agitation. Samples were spun, washed several times with DMEM supplemented with 10% fetal calf serum, and plated onto 35mm^2 dishes pre-coated with 50 μg/ml vitrogen (Collagen Biomaterials, Palo Alto, CA). Sixteen hours later, cultures were rinsed and pre-incubated with serum-free DMEM for 30 minutes prior to treatment. Cells were treated either with no growth factor, or with rhEPO (kindly provided by Amgen, 10 units/ml). Cell growth was determined by a single 6-hour pulse with 1 μCi/ml [^{3}H]thymidine at the end of 18 hours treatment. Measurement of incorporated [^{3}H]thymidine was performed by trichloroacetic acid precipitation and scintillation counting as previously described (Iruela-Arispe and Sage, 1993).

Assessment of proliferating cells in the heart and brain sections was performed by determining the histone 3-positive cells per 1,000 total nuclei. Unstained nuclei (non-proliferating) were visualized by phase contrast microscopy. Because the distribution of histone 3-positive nuclei (proliferating cells) is uneven through the heart compartments, counts were performed in five areas: (1) ventricular septum; (2) lower right ventricular wall; (3) lower left ventricular wall; (4) upper right ventricular wall; (5) upper left ventricular wall. Both, compact layer and trabeculei, were included when counting the ventricular walls. All counts were then averaged. A total of three hearts were counted in this manner per experimental group. Finally the mean of all averages ±s.e.m. was obtained and represented in a histogram. For the brain sections, the same procedure was followed, however, the areas considered for counting were: (1) temporal cortex; (2) dorsal thalamus; and (3) preoptic area. Sections through the brain were coronal and at eye level in all experimental groups.

Generation of 'blue' ES cells and chimeric analysis

EPOR$^{+/-}$ Rosa$^{+/+}$ animals were obtained by crossing EPOR$^{+/-}$ mice with Rosa$^{+/+}$ mice, Rosa 11 (Friedrich and Soriano, 1991). ES clones were generated from early embryos derived from EPOR$^{+/-}$Rosa$^{+/+}$ × EPOR$^{+/-}$Rosa$^{+/+}$ mating using a standard procedure (Robertson, 1987). Individual 'blue' ES lines were genotyped by Southern blot or PCR analysis as described in this study. EPOR$^{+/+}$ or EPOR$^{-/-}$ 'blue' ES cells were injected into wild-type blastocysts to generate chimeric animals (Bradley, 1987).

X-gal staining

Hearts were dissected from E14 embryos or adult chimeric animals and rinsed three times in PBS. 10 μm frozen sections were fixed (0.2% glutaraldehyde, 2% formaldehyde, and 2 mM $MgCl_2$ in PBS) for 15 minutes at room temperature, rinsed twice with PBS, then stained with X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide) for 48-72 hours. Stained sections were rinsed and counter-stained with nuclear fast red (Vector Labs).

RESULTS

Abnormal cardiac development in EPO$^{-/-}$ and EPOR$^{-/-}$ embryos

As we reported previously, both EPO$^{-/-}$ and EPOR$^{-/-}$ animals are embryonic lethal and die in uteri around E13.5-14.5 of gestation. In addition to significant reduction in circulating erythrocytes and in the cell mass of the fetal liver, a secondary phenotype consistently observed was the abnormal development of the heart. At day 13.0-13.5 of gestation, homozygous embryos had enlarged chest bulge with visible pericardial edema (Fig. 1A). We could also detect generalized peripheral edema, an additional indicator of suppressed cardiac function. The phenotype was identical in EPO$^{-/-}$ and EPOR$^{-/-}$ embryos and independent of their genetic backgrounds. Thus, only results derived from EPOR$^{-/-}$ animals are shown; nonetheless EPO$^{-/-}$ embryos were also studied in most of the experiments.

To further define the stage at which cardiac growth was hampered, we examined hearts from wild type, EPO$^{-/-}$, or EPOR$^{-/-}$ embryos. No significant abnormalities could be detected at E11.5 (Fig. 1B, left panel). One day later, however, morphological differences between wild-type and mutant embryos were evident. The ventricular wall in the mutant hearts was often enlarged and rounded with little external evidence of interventricular sulcus (arrows) (Fig. 1B, middle panel). These differences became more obvious by E13.5 (Fig. 1B, right panel). The appearance of the interventricular sulcus in wild-type embryos is a consequence of interventricular septum formation. The lack of such anatomical marker is an

indication of abnormal morphogenesis of the septum in the EPOR$^{-/-}$ embryos.

During murine heart development, formation of ventricular trabeculae (T, Fig. 1C,a,c) starts normally around E9.5, which then forms a contractile lattice inside the ventricular chambers. At E10.5, four heart chambers can be easily distinguished (Rugh, 1990). Subsequently, the outer myocardial layer thickens by E11.5-12.5, giving rise to the compact myocardial zone (Fig. 1C, between arrows in b and d). This layer proliferates to generate the outer wall of the ventricular chambers and contributes to the formation of muscular ventricular septum (S, Fig. 1Cb,d). No significant differences could be detected between the mutant and the wild-type hearts at E11.5 (data not shown), consistent with our observation in Fig. 1B. At E12.5-13.5, the compact layer in EPO$^{-/-}$ or EPOR$^{-/-}$ hearts (arrows) was substantially thinner than in wild-type embryos, consisting of a single cell layer in some regions. The ventricular septum (Fig. 1C, S), which forms by proliferation of cardiomyocytes in the compact zone, was also markedly reduced, while the trabecular layer appeared to be less affected (Fig. 1C, T). Extensive histological analysis did not reveal significant increase in picnotic nuclei in the affected area, suggesting that the ventricular hypoplasia is not due to massive cell death.

The pericardial layer was also affected in the EPOR$^{-/-}$ embryos (Fig. 2). Examination of mutant hearts under a dissecting microscope revealed substantial detachment of the pericardium. This was confirmed by histological examination (Fig. 2F). Early during cardiac morphogenesis, a population of epicardial cells is responsible for contributing vascular endothelial cells to the coronary arteries of the heart. These endothelial cells first aggregate in the subepicardial space and subsequently migrates into the myocardial wall to form the coronary arteries. To evaluate the heart vasculature, we performed whole-mount PECAM staining on hearts from wild-type and EPOR$^{-/-}$ embryos (Fig. 2). PECAM, also known as CD-31, is a transmembrane molecule expressed on endothelial cells, and therefore a good marker for identification of capillary vessels (Albelda et al., 1991). In contrast to the wild-type heart (Fig. 2A,B), the vasculature in the EPOR$^{-/-}$ heart is severely affected (Fig. 2C,D). Instead of inter-connected, fine vascular networks, the EPOR$^{-/-}$ heart showed dilated and independent vascular clumps (arrows). Sectioning of this region revealed detachment of the epicardial wall and lack of defined capillary structures in the EPOR$^{-/-}$ heart (Fig. 2F), as compared to the tight association of epicardium to myocardium in the wild-type heart and discrete capillaries (Fig. 2E, arrows).

The cardiac abnormalities did not completely abolish all organized cardiac contractions. However, cardiac function in the null embryos was likely compromised by the hypoplasia as has been directly measured in another mouse model of compact zone hypoplasia (Dyson et al., 1995).

EPOR gene expression in the developing heart

EPO is a well known cytokine essential for the production of circulating red blood cells. EPO functions by binding to its cell surface receptor, EPOR. To further understand the mechanism that lead to the abnormal heart development in mutant animals, we studied EPOR expression in the developing heart at several gestational stages.

Whole-mount in situ hybridization revealed that EPOR is transiently expressed in the developing heart: beginning at E10.5 (Fig. 3A), high at E11-12 (Fig. 3C), and significantly diminished at day 13.5 (data not shown). By day 14.5, EPOR expression became undetectable (data not shown). No signal was seen when sense probe was used (Fig. 3A), or when the anti-sense probe was used on EPOR$^{-/-}$ hearts (Fig. 3B).

EPOR expression in the developing heart was rather cell type-specific. As show in Fig. 3C, it was positive in the endocardium (arrows), epicardium (arrowhead), pericardium (small arrowhead), as well as cells of the cardiac cushions (asterisk). However, no signal could be detected in the myocardium itself, where the hypoplasia defect was found. The results indicate that (1) EPOR is expressed in a subset of cardiac tissues, but not in the myocardium; and (2) its expression precedes the onset of ventricular hypoplasia observed in the mutant embryos.

Normal cell differentiation in EPO$^{-/-}$ and EPOR$^{-/-}$ hearts

Cardiac morphogenesis is accomplished by a combination of differential growth/folding of the initial cardiac tube and simultaneous differentiation of cardiomyocytes. To evaluate the state of cardiomyocyte differentiation we examined the expression of two early markers in wild-type and EPOR$^{-/-}$ hearts. ANF is expressed as early as E8.0 in the mouse embryo (Zeller et al., 1987). By E14, high levels of expression can be detected in both atria and the trabeculated regions of the left ventricle. Interestingly, expression in the right ventricle is significantly lower. This has been previously reported and it might relate to the differential pressure between the right and left ventricles, which appears to affect ANF expression levels (Zeller et al., 1987). No significant differences in the level of ANF expression were observed in EPOR$^{-/-}$ and wild-type controls (Fig. 4A,B).

Vascular smooth muscle α-actin expression is another milestone that characterizes early cardiomyocyte differentiation (Ruzicka and Schwartz, 1988). As shown in Fig. 4C,D, the levels of α-actin protein in the trabeculae, as well as in the compact layer were very similar between the wild-type and mutant hearts. Therefore, lack of EPO or EPOR does not appear to affect the initial commitment of cells to the cardiomyocyte fate. Expression of both α-actin and ANF indicate that undifferentiated mesenchymal cells have successfully transversed the developmental decision made around embryonic day 8 to become cardiomyocytes, and lack of EPOR signaling does not interfere with early differentiation events. In addition, the fact that the hearts are beating in EPOR$^{-/-}$ animals at embryonic day 13 indicates that acquisition of contractile proteins also does not appear to be affected.

EPO regulates cardiomyocyte proliferation

To test the role of EPO and EPOR signaling in myocardial cell proliferation, in situ hybridization analyses were performed on heart sections or whole embryo sections using a histone 3 riboprobe. histone 3 mRNA is expressed during S phase and has a short half-life. This makes it a good marker for detecting cycling cells (Gown et al., 1996; Lee et al., 1997). As shown in Fig. 5A, the relative number of proliferating cells was markedly reduced in hearts of null animals when compared to their wild-type littermates, especially in the compact layer

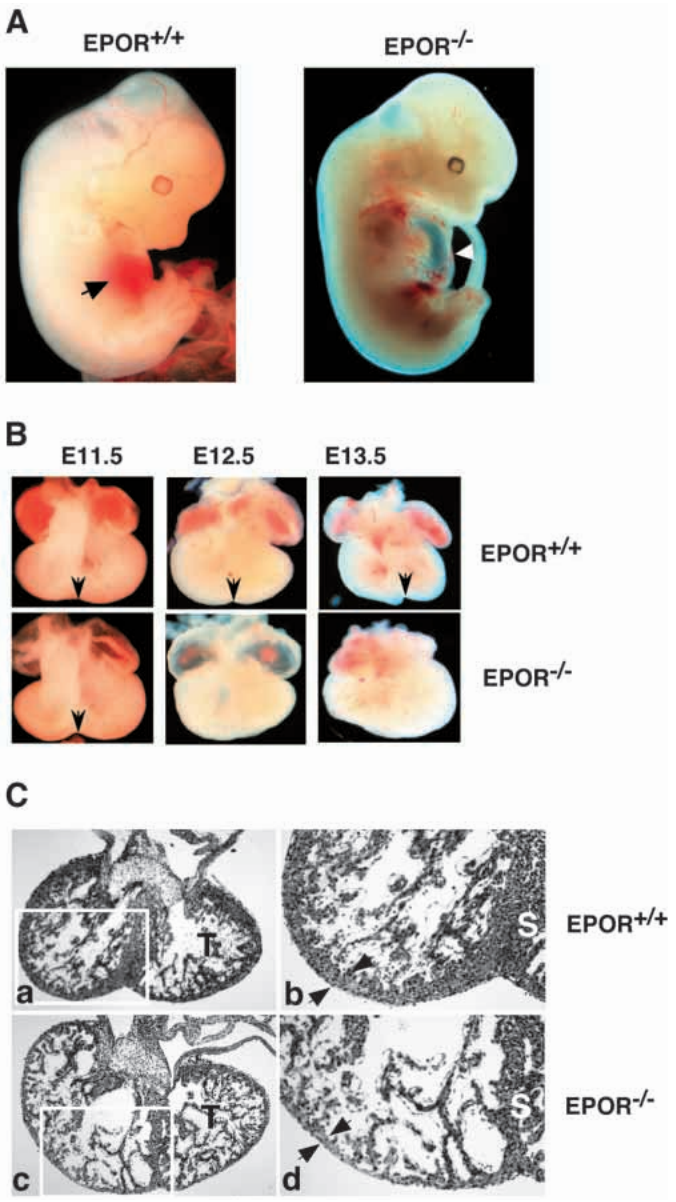

Fig. 1. Lack of EPO or EPOR affects heart development. (A) External appearance of the wild-type (+/+) and the EPOR null mutant (–/–) embryos at E14.0. Homozygous mutant embryo appears pale with no visible liver (position indicated by arrow in wild type), and edema in the pericardial space (arrowhead). (B) Heart development at different gestation stages. Hearts from wild-type and mutant embryos were dissected and photographed at E11.5 (left panel), E12.5 (middle panel) and E13.5 (right panel), respectively. Morphological differences between the wild-type and mutant hearts are evident at E12.5 and become obvious at E13.5. The wild-type hearts show clear interventricular sulcus (arrows) which is almost absent in mutant hearts (E12.5-13.5). (C) Histological analysis of wild-type (+/+) and EPOR null mutant (–/–) embryos at E12.5. T, trabeculae; arrows, compact layer; and S, ventricular septum.

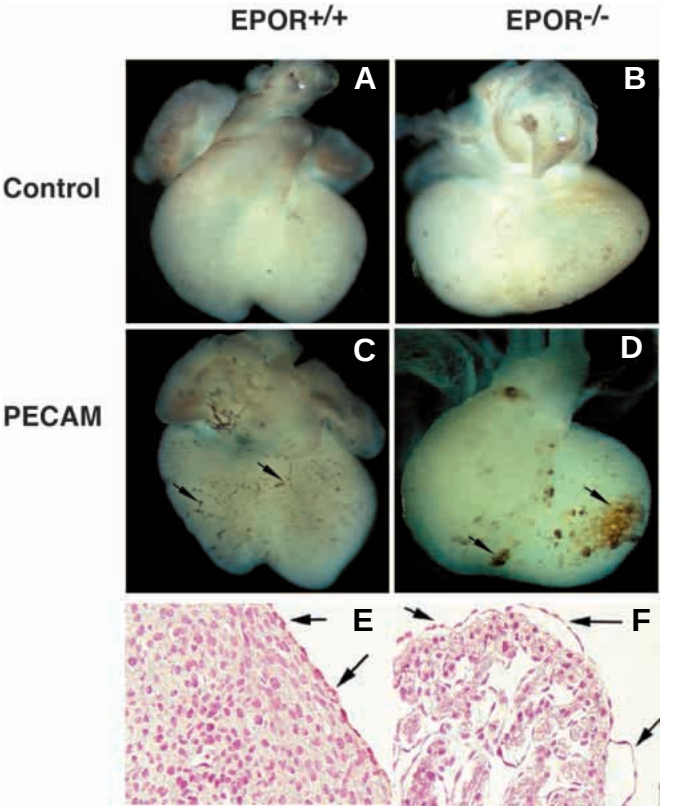

Fig. 2. Heart vasculature and epicardial defects in EPOR$^{-/-}$ heart. Heart vasculature was examined by staining E13.5 hearts with anti-PECAM antibodies. Note thin and abundant capillary network present in the wild-type heart (C, arrows), while the mutant heart shows enlarged vascular units that do not appear interconnected (D, arrows). Control for staining included normal rabbit IgG instead of anti-PECAM in both wild-type (A) and EpoR$^{-/-}$ hearts (B). Histological evaluation of mutant heart shows detachment of epicardial wall and lack of defined capillary structures (F) in contrast to wild-type hearts with tightly associated epicardium and discrete capillaries (arrows, E).

(arrows) and ventricular septum (arrowheads). The number of histone 3-positive cells per 1000 cells was determined (see Materials and Methods) and presented in Fig. 5B. Homozygous hearts had 39% fewer cycling cells than that of control samples at E13.5 (P<0.001). The difference in cell proliferation was also statistically significant at E12.5 (P<0.005), and E11.5 (P<0.007) (Fig. 5B, left panel). These results indicate that hypoplasia in the compact layer and ventricular septum is due to reduction in cell proliferation.

To determine whether decrease of cell proliferation in the mutant hearts was associated with or a result of the general level of hypoxia, we also examined the number of proliferating cells in the brain, since neurons are known to be sensitive to hypoxia. As shown in Fig. 5B, right panel, numbers of histone 3-positive cells in the wild type were similar to that in the mutant brain sections. This indicates that the reduction in cell proliferation is rather tissue specific, therefore, is probably not due to generalized hypoxia.

Based on the relative levels of cell proliferation in wild-type and EPO or EPOR mutant hearts, it is reasonable to anticipate that, at a certain time during heart development, EPO/EPOR may provide mitogenic signals to cardiomyocytes. To ascertain the potential role of EPO as a mitogen, we evaluated the responsiveness of hearts to exogenous EPO by culturing them in toto in organ culture dishes or as primary cultures. E11.5 hearts from EPO$^{+/+}$ and

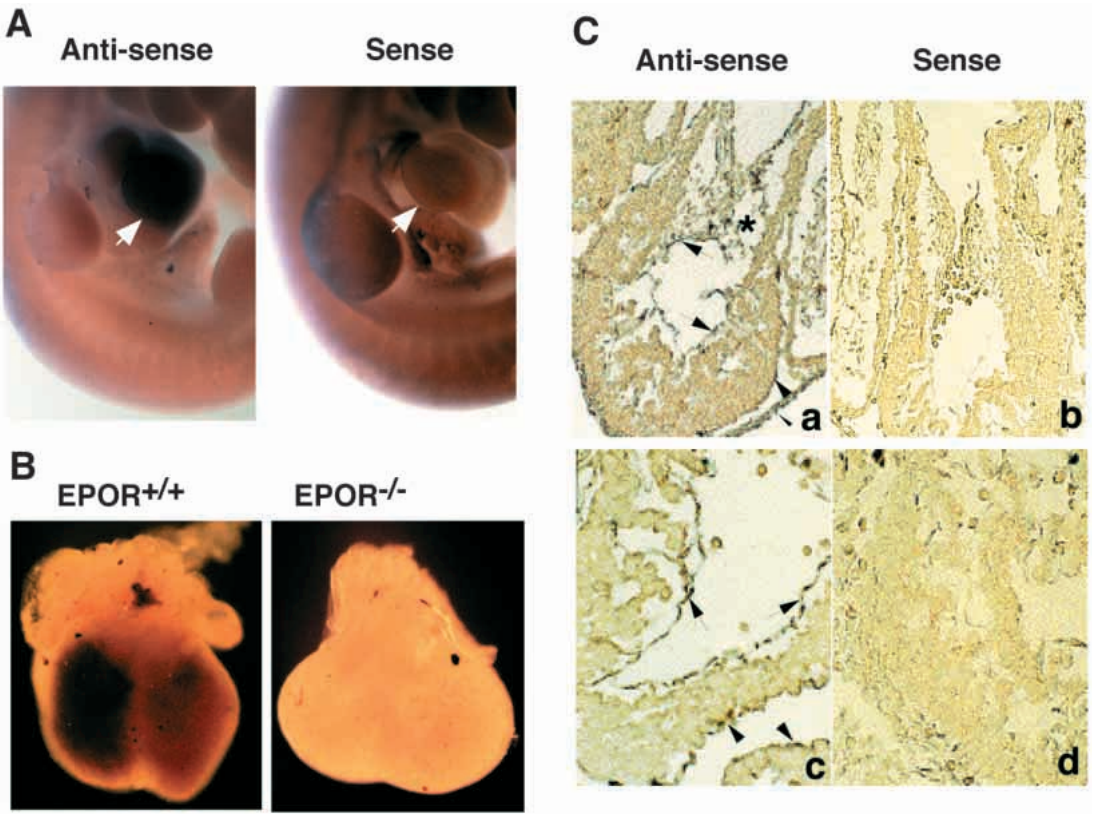

Fig. 3. Tissue and cell type-specific expression of the EPOR gene. (A) Whole-mount in situ hybridization for EPOR in E10.5. Left, anti-sense EPOR riboprobe. Arrow points to the ventricle, which shows more intense hybridization than the upper atrium. Right, sense EPO riboprobe. The reaction with the EPOR riboprobe was left for a longer time than in the anti-sense experiment to verify the specificity of the anti-sense reaction, therefore the embryo appears darker. (B) Whole-mount in situ hybridization using E12.5 heart dissected from wild-type and EPOR$^{-/-}$ embryos. In the wild-type heart, EPOR expression could be detected in both ventricles, but was more abundant in the right ventricle at this time. No signal could be detected in the EPOR$^{-/-}$ heart. (C) Cell type-specific expression of the EPOR. Antisense (a and c) and sense (b and d) riboprobes to EPOR were labeled with digoxigenin and hybridized to heart sections from wild-type embryos. Positive signal was detected in the endothelium (arrows), epicardium (arrowheads), pericardium (smaller arrowhead), and atrium-ventricular cushion (asterisk) when antisense probe was used.

EPO$^{-/-}$ embryos were harvested and cultured individually in organ culture dishes with or without EPO (1 unit/ml). Twenty-four hours later, the hearts were fixed and sectioned. Histone 3-positive cells were counted. As a positive control for EPO activity, fetal livers from the above animals were co-cultured in the same dishes. Erythroid progenitors in the fetal liver were highly responsive to EPO stimulation as evidenced by massive erythroid differentiation and red blood cell formation (data not shown). EPO also act as a mild mitogen for cardiomyocytes. Fig. 6A shows 33% increase in the histone 3 positive cells when WT hearts were cultured with EPO (P<0.05). EPO$^{-/-}$ hearts, which lack the endogenous EPO gene but are capable of responding to exogenous EPO stimulation (Wu et al., 1995), were even more responsive to EPO stimulation, with almost two-fold increase in proliferation compared to cultures without EPO (P<0.05). This different response is likely due to the endogenous EPO production by the wild-type heart (Kertesz and Wu, unpublished observation), resulting in higher basal level of histone 3-positive cells. The mitogenic activity could also be measured by [^{3}H]thymidine incorporation using primary cultures. Fig. 6B shows 33 and 46% increase (P<0.05) in [^{3}H]thymidine incorporation when primary cultures from wild-type and EPO$^{-/-}$ hearts were stimulated by EPO, respectively. However, no detectable increase was seen when

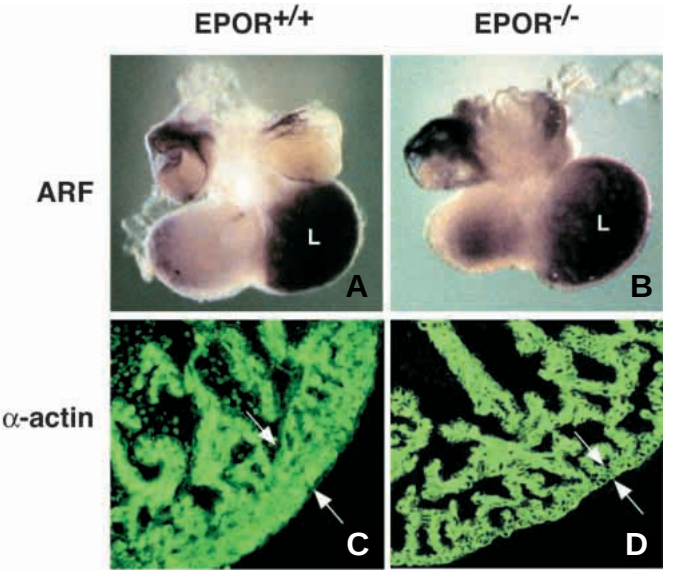

Fig. 4. EPO and EPOR have no affect on major cardial differentiation events. Expression of natriuretic factor (ARF) and α-actin were evaluated in wild-type (A and C) and EPOR null (B and D) animals. No significant differences could be detected in the expression of ANF by whole-mount in situ hybridization of E12.5 hearts (A and B). Intense signal for ARF was seen in the left (L) ventricle in both animals. Expression of α-actin protein was evaluated by immunocytochemistry (C and D). Although the overall thickness of the compact layer (arrows) in the ventricle was clearly different in the wild-type and mutant hearts, the intensity and distribution of α-actin were very similar.

EPOR$^{-/-}$ hearts were used, suggesting that this activity is EPOR signaling-dependent.

EPO affects cardiomyocyte proliferation in a cell non-autonomous manner

The major cardiac defect in EPO$^{-/-}$ or EPOR$^{-/-}$ embryos is located in the myocardium. Interestingly, EPOR expression was mostly detected in the endocardium (endothelial cells) and epicardium (mesothelial cells), but not in the cardiomyocytes themselves. This expression pattern may suggest that EPO affects myocardial cell proliferation indirectly. For instance, EPO may activate EPOR signaling pathways in endocardium and/or epicardium that could lead to the secretion of mitogenic factor(s) which then bind to its cognate receptor on the surface of myocardium. If this is the case, the myocardium defect caused by lack of EPO or EPOR should be compensated by the presence of wild-type endocardium or epicardium.

To test this hypothesis, we generated chimeric mice with 'blue' ES cells that were either EPOR$^{+/+}$ or EPOR$^{-/-}$ (see Material and Methods). Since any lineage derived from injected ES cells should express β-galactosidase, the fates of the ES cells can be directly assessed by X-gal staining of tissues from the chimeric mice. As shown in Fig. 7, the EPOR$^{-/-}$ 'blue' ES cells were able to contribute to all lineages in the chimeric hearts, including the myocardium (Fig. 7D), suggesting that EPO and EPOR influence cardiac morphogenesis in a non-cell-autonomous manner.

DISCUSSION

Homologous recombination in embryonic stem cells has become a powerful tool for the functional study of genes in vivo. Mice carrying various null mutations have been generated, and valuable information has been obtained by analysis of animals carrying these mutations. In some cases, the phenotype developed fits precisely with the predicted function of the gene. However, in other cases, unexpected roles may be revealed. In this study, we demonstrated a novel role of EPO in cardiac morphogenesis that likely contributes to the embryonic lethality of the knock-out mouse.

EPO function versus general hypoxia

Deletion of EPO or EPOR is clearly accompanied by alterations in the relative oxygen levels in the mutant animals. Therefore an obvious conclusion could be that the ventricular hypoplasia was a result of generalized hypoxia. However, the cardiac defects described in this study are unlikely to be due to general hypoxia. First, we have evaluated the proliferation index in two different organs known to be sensitive to the level of oxygen, brain and heart. We found that EPO$^{-/-}$ and EPOR$^{-/-}$ embryos, when compared to the wild-type controls, had similar numbers of proliferating cells in the brain, but significantly reduced numbers in the heart. Thus, reduction in cell proliferation is rather heart-specific in EPO$^{-/-}$ or EPOR$^{-/-}$ mice. Second, in c-kit and c-myb deficient mice, embryos suffer from anemia and die in mid-gestation stage (Besmer, 1991; Mucenski et al., 1991). However, no defects in heart development have been reported. In particular, we studied the W^{x}/W^{x} mutant, which lacks kinase activity and dies at mid gestation from anemia. We could not detect obvious cardiac morphogenesis defects in W^{x}/W^{x} mutant embryos (Wu et al., unpublished observations).

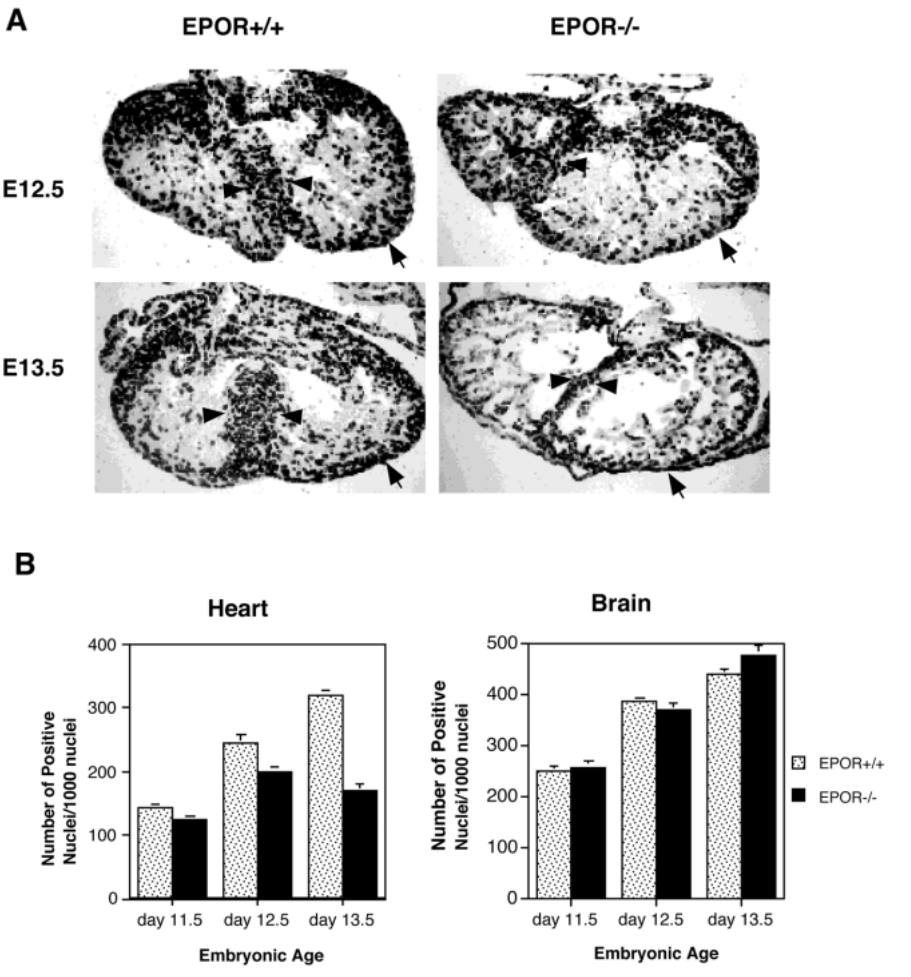

Fig. 5. EPO and EPOR affect cardiomyocyte proliferation. (A) Cell proliferation was evaluated in wild-type (left panels) and EPOR null embryos (right panels). Embryos and hearts at E12.5 (upper panels) and E13.5 (lower panels) were fixed, sectioned and hybridized to histone 3 anti-sense riboprobe. Proliferating cells were predominant in the ventricular septum (arrowhead) and compact layers (arrow). Interestingly, lower number of proliferating cells was seen in the EPOR null animals, especially at E13.5. (B) Numbers of histone 3-positive cells per 1,000 total cells in the brain and heart were counted from the same sections by two investigators independently. Quantification and statistic analysis were performed as described in the Materials and Methods. Left, number of histone 3-positive cells in the heart of wild-type and EPOR null animals; right, number of positive cells in the brain of wild-type and EPOR null animals. Each experiment was repeated 3 times and presented as mean ± s.d.

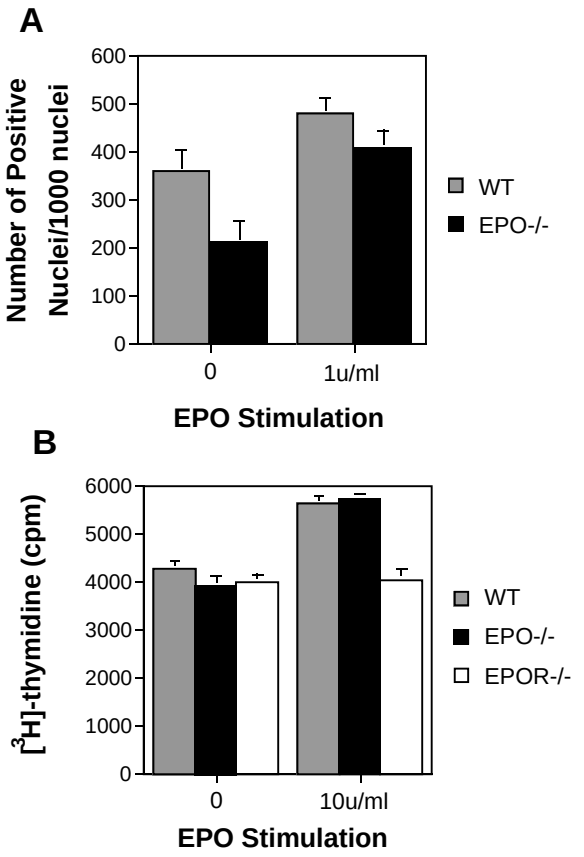

Fig. 6. EPO induces cardiac cell proliferation. (A) Whole hearts were isolated from wild-type and EPO null embryos (11.5 days) and placed in culture in the absence or presence of EPO (1 units/ml) for 16 hours. Hearts were fixed and processed for evaluation of proliferating cells using histone 3. The number of positive nuclei was scored by two investigators independently. A total of 3 to 4 hearts were used per experimental time-point. (B) Cells isolated from wild-type, EPO$^{-/-}$ and EPOR$^{-/-}$ hearts were subjected to proliferation assays in the absence or presence of EPO (10 units/ml). Equal numbers of cells were placed on 24-well COSTAR plates, then treated with EPO. [^{3}H]thymidine was added to the culture during the last 6 hours of treatment. Incorporated thymidine was measured by liquid scintillation counting.

Additional evidence that dissociates hypoxia from EpoR$^{-/-}$ cardiac defects relates to experiments on the hypoxia inducible factor (HIF-1α) null animal (Iyer et al. 1998). HIF-1α is a master regulator for EPO production and O_2 homeostasis. Massive cell death was observed in the neuronal lineage of HIF-1α$^{-/-}$ animals (Iyer et al. 1998). In contrast, higher levels of cell proliferation (hyperplasia) were observed in the cardiac tissues, suggesting that cardiac tissues are more resistant to hypoxia during embryonic development (Iyer et al. 1998). Taken together, these results suggest that general hypoxia is probably not the main cause of ventricular defects observed in EPO and EPOR mutant embryos.

Proliferation versus differentiation

We tested whether EPO influences heart development by promoting cardiomyocyte proliferation or by triggering differentiation. We showed that the initial cardiomyocyte differentiation events were not blocked in the mutant hearts. In contrast, cell proliferation, especially in the compact layer and ventricular septum, was severely hampered in the affected embryos. However, we cannot rule out the possibility that lack of EPO or EPOR causes earlier differentiation and results in reduction of the proliferation capacity of the ventricular cardiomyocytes, as observed in animals with vitamin A deficiency and mutations in RXRα, RXRβ, and RARα genes (Kastner et al. 1997).

We also determined whether, under culture conditions, EPO could enhance cardiomyocyte proliferation. Our results indicated that EPO significantly stimulated cell proliferation of the wild-type or EPO$^{-/-}$ hearts, while it had no effects on EPOR$^{-/-}$ hearts. Interestingly, mice lacking one of the major components in the EPOR signaling pathways, the Janus kinase 2, had a similar cardiac hypoplasia as we report in this study (Neubauer et al. 1998), suggesting that this mitogenic effect is EPOR signaling-specific and -dependent. Interestingly, cardiac-specific deletion of gp130 causes cardiac defects similar to those observed in EPO$^{-/-}$ EpoR$^{-/-}$ mice (Hirota et al., 1999). It is therefore tempting to speculate that EpoR may share similar mechanism or signaling pathways with gp130 during cardiac morphogenesis.

How does EPO function in cardiac morphogenesis?

At this point, it is difficult to elucidate the precise mechanism by which EPO influences cardiac morphogenesis. Expression of EPOR appears to be endocardium- and epicardium-specific, yet it is the myocardium that shows a consistent and statistically significant reduction in cell number. Chimeric analysis clearly showed that EPO stimulated cell proliferation in a cell non-autonomous manner, similar to recent studies on the RXRα gene (Tran and Sucov, 1998; Chen et al., 1998). It appears, therefore, that activation of EPOR in the endocardium or epicardium is required, perhaps as an initiator, for a cascade of events leading to the secretion of a mitogenic factor from the endocardium or epicardium. This factor(s) then promotes cell proliferation by binding to its receptor(s) on cardiomyocytes.

It has been postulated that endocardial-myocardial interactions are essential for normal cardiac morphogenesis and for the complete development of the ventricle. Nevertheless, of several potential endocardial-derived signals, only neuregulins and endothelins have a proven role in heart development. Neuregulins are highly expressed by the endocardium during early cardiac morphogenesis. Neuregulin receptors, on the other hand, are expressed on the myocardium. Targeted ablation of neuregulin (Meyer and Birchmeier, 1995), or its receptors erbB2 or erbB4 (Gassmann et al., 1995) all suppress development of trabeculae in the ventricles. However, neuregulin is unlikely to be the factor regulated by EPO/EPOR signaling pathway in endocardium. Neuregulin$^{-/-}$ mice die much earlier than that of EPO$^{-/-}$ or EPOR$^{-/-}$ embryos (9.5 vs. 13.5 dpc). Furthermore, in EPO$^{-/-}$ or EPOR$^{-/-}$ hearts, more severe defects were observed in the compact layer and interventricular septum rather than in the trabeculae.

Several lines of evidence also suggested that endothelin (ET), one of the most potent vessel contractors, might be a potential target for EPO/EPOR signaling pathway. As shown by both in vitro and in vivo experiments, EPO could stimulate ET-1 release from vascular endothelial cells, which might explain one of the

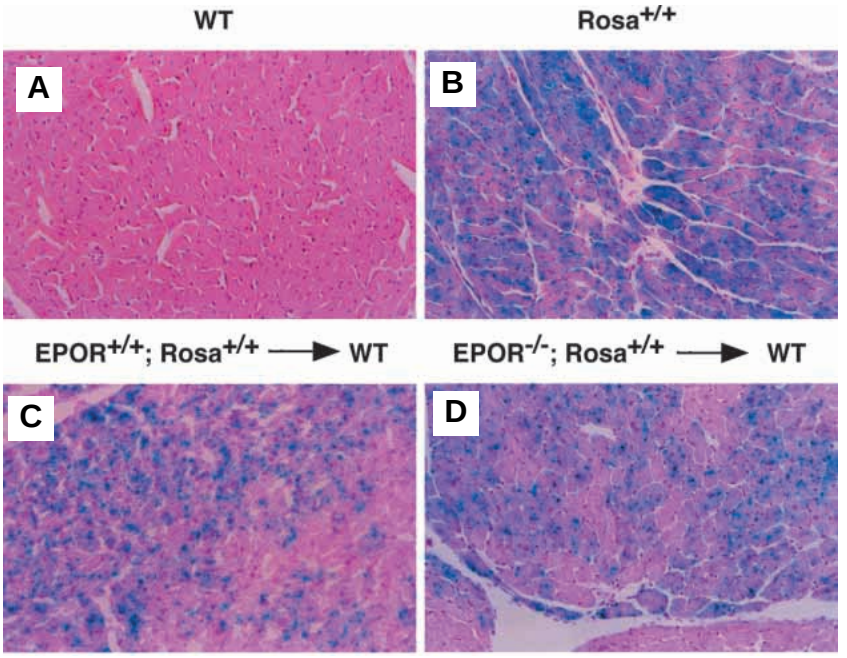

Fig. 7. EPO regulates cardiac morphogenesis in non-cell-autonomous manner. Chimeric mice were generated by injecting $EPOR^{+/+}Rosa^{+/+}$ or $EPOR^{-/-}Rosa^{+/+}$ ES cells into mouse blastocysts. Adult hearts were sectioned through the ventricles. Frozen sections were fixed and stained with X-gal. (A) Wild-type heart: a negative control for X-gal staining (red, counterstained with nuclear fast red); (B) $Rosa^{+/+}$ animal heart: a positive control for X-gal staining (blue). (C,D) Cells derived from $EPOR^{+/+}Rosa^{+/+}$ or $EPOR^{-/-}Rosa^{+/+}$ ES cells can contribute to myocardium as indicated by strong positive X-gal staining. ES-derived cells can also contribute to endocardium and epithelial cells in the blood vessels (data not shown).

major side effects of EPO administration, hypertension (Tojo et al., 1996). ET-1 can be produced by endocardium as well. By binding to its receptor on myocardium (ETA), ET-1 may exert its function in cardiac development (Clouthier et al., 1998). However, cardiac abnormalities found in ET-1 and ETA mutant mice are in the cardiac outflow tissues (Clouthier et al., 1998) and at later developmental stages than those observed in the $EPOR^{-/-}$ mice.

The epicardium is the outer layer of the heart. During development, it gives rise to populations of cells that form the vascular and connective tissues within the heart. Epicardium may contribute to myocardial development by direct cell-cell interaction or by secretion of local mitogenic factors. It is interesting to note that the highest rate of cell proliferation in the compact layer is located on the outer edge of the myocardium, immediately adjacent to the epicardium, as shown in our study and other metabolic labeling experiments. On the other hand, epicardium may indirectly contribute to the development of the myocardium by controlling coronary artery formation, which is crucial for nutrient and waste exchange or for providing blood-borne mitogenic factors. Cardiac defects have been reported in several mutant mice in which epicardium development or function was affected. α4 integrin is a subunit of a cell surface receptor that mediates cell-extracellular matrix and cell-cell adhesion by interacting with fibronectin and vascular cell adhesion molecule 1 (VCAM-1). α4 integrin is produced in the epicardium, whereas VCAM-1 is secreted by myocardium (Yang et al., 1995; Kwee et al., 1995). Interestingly, both α4 integrin and VCAM-1 knock-out mice show defects in epicardium and coronary vessels leading to cardiac hemorrhage (Yang et al., 1995; Kwee et al., 1995). These defects are most likely due to abnormalities in epicardium and coronary vessels, suggesting that myocardium-epicardium interaction may be crucial for normal heart morphogenesis and function. We have carefully examined the epicardium in $EPO^{-/-}$ or $EPOR^{-/-}$ mice. Although the epicardium was present in the mutant hearts, its morphology appeared abnormal. Firstly, the cell number was reduced and the remaining cells became extremely stretched (data not show). Secondly the epicardium layer was often detached from myocardium. Furthermore, the vascular network in the mutant heart was severely affected with a disorganized structure. In contrast, the epicardium in wild-type hearts was tightly associated with the myocardium. The defects in the epicardium could be detected at 11.5 dpc, prior to visualization of the defects in the compact layer and interventricular septum. Further experiments must be conducted to determine the molecular mechanism of this defect and to assess whether the myocardial and epicardial abnormalities are linked.

An alternative explanation for the coronary defects is the direct effect of Epo signaling on endothelial cells. In fact, Epo has been shown to act as a mitogen factor for cultured endothelial cells and to enhance angiogenesis in the chorioallantoic membrane (Ribatti et al., 1999). The defects in the heart displayed by the $EpoR^{-/-}$ mice are consistent with these findings.

We thank Dr Harvey Lodish for his support and encouragement at initial stage of this study. We thank Dr Sucov and members of our laboratories for helpful suggestions and discussions. H. W. is an assistant investigator of the Howard Hughes Medical Institute and a V Foundation Scholar. This work was supported by the American Heart Association Grant-in-Aid 96-1218 (L. I. A.) and the V Foundation (H. W.).

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