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Endoglin, an Ancillary TGFβ Receptor, Is Required for Extraembryonic Angiogenesis and Plays a Key Role in Heart Development

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Endoglin (CD105) is expressed on the surface of endothelial and haematopoietic cells in mammals and binds TGFβ isoforms 1 and 3 in combination with the signaling complex of TGFβ receptors types I and II. Endoglin expression increases during angiogenesis, wound healing, and inflammation, all of which are associated with TGFβ signaling and alterations in vascular structure. The importance of endoglin for normal vascular architecture is further indicated by the association of mutations in the endoglin gene with the inherited disorder Hereditary Haemorrhagic Telangiectasia Type 1 (HHT1), a disease characterised by bleeding from vascular malformations. In order to study the role of endoglin *in vivo* in more detail and to work toward developing an animal model of HHT1, we have derived mice that carry a targeted nonsense mutation in the endoglin gene. Studies on these mice have revealed that endoglin is essential for early development. Embryos homozygous for the endoglin mutation fail to progress beyond 10.5 days *postcoitum* and fail to form mature blood vessels in the yolk sac. This phenotype is remarkably similar to that of the TGFβ1 and the TGFβ receptor II knockout mice, indicating that endoglin is needed *in vivo* for TGFβ1 signaling during extraembryonic vascular development. In addition, we have observed cardiac defects in homozygous endoglin-deficient embryos, suggesting endoglin also plays a role in cardiogenesis. We anticipate that heterozygous mice will ultimately serve as a useful disease model for HHT1, as some individuals have dilated and fragile blood vessels similar to vascular malformations seen in HHT patients.

Key Words: **endoglin; HHT; TGFβ; angioigenesis; cardiogenesis; haematopoiesis.**

INTRODUCTION

Endoglin (CD105) is a transmembrane glycoprotein expressed by mammalian vascular endothelial cells, where it is found associated with TGFβ signaling receptors (Cheifetz *et al.*, 1992). The minimum TGFβ receptor complex required for signaling contains only the type I and II transmembrane receptors. These control the expression of TGFβ-responsive genes by ligand-activated phosphorylation of SMAD proteins, which then move into the nucleus (Heldin *et al.*, 1997; Massague, 1998). However, coexpression of ancillary nonsignaling receptors, such as endoglin, influence which TGFβ isoforms are bound and which signaling responses are made. In this regard, endoglin preferentially binds TGFβ isoforms 1 and 3, when associated with TbRII, the TGFβ type II receptor (Barbara *et al.*, 1999), and overexpression of endoglin in cell lines attenuates some responses to TGFβ1 (Lastres *et al.*, 1996; Letamendía *et al.*, 1998). The degree to which this ancillary role of endoglin is a requirement for TGFβ signaling *in vivo* has not previously been studied. Recently, endoglin has also been shown to bind, *in vitro*, at least three other members of the TGFβ superfamily (Activin A, BMP-2 and BMP-7) and to interact

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with the activin type II receptors (ActRII and ActRIIB), and so may be involved in a number of different signaling pathways (Barbara *et al.*, 1999).

TGFβ1 signaling is important for normal vascular development. It is involved in the differentiation of recruited smooth muscle cells by the endothelial cells in the early stages of vasculogenesis and also plays a critical role in angiogenesis (Folkman and D'Amore, 1996; Pepper, 1997). Mice homozygous for targeted mutations of the TGFβ1 gene exhibit severe deficiencies in haematopoiesis and vasculogenesis in the yolk sac and die around 10.5 days *postcoitum* (dpc) (Dickson *et al.*, 1995). A similar phenotype is exhibited by mice lacking TbRII (Oshima *et al.*, 1996). In contrast, deficiencies of either TGFβ2 or TGFβ3 are compatible with development to birth, although both result in perinatal death (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995; Sanford *et al.*, 1997). These phenotypes suggest that binding of TGFβ1 to a receptor complex containing the TbRII subunit leads to critical signaling events required for haematopoiesis and vasculogenesis in the yolk sac.

Humans, heterozygous for deleterious mutations in the endoglin gene, develop the disease Hereditary Haemorrhagic Telangiectasia Type 1 (HHT1) (McAllister *et al.*, 1994), an autosomal dominant disease characterised by small, localised vascular malformations called telangiectases, which develop in the nasal septum, oral mucosa, and gastrointestinal tract, and are prone to prolonged bleeding episodes (Guttmacher *et al.*, 1995; Shovlin *et al.*, 1997). Larger arterio-venous malformations may also develop at pulmonary, hepatic, and cerebral sites with potentially fatal consequences. HHT1 patients have an overall 50% reduction in the basal level of endoglin expression on vascular endothelium, demonstrating that normal levels of endoglin protein are important in maintaining vascular structure, but the specific molecular events which cause the vascular malformations are not yet understood (Pece *et al.*, 1997).

Interestingly, defects in Alk1, a type I receptor of the TGFβ receptor family, which appears to signal through SMAD1, result in a clinically similar form of HHT (Johnson *et al.*, 1996), suggesting that endoglin and Alk1 act in the same signaling pathway. The similar expression pattern of ALK1 and endoglin in the vascular endothelium is consistent with this suggestion (Panchenko *et al.*, 1996; Roelen *et al.*, 1997).

In order to study the role of endoglin *in vivo* in more detail and to work toward developing an animal model of HHT1, we have introduced a targeted stop codon, similar to some mutations found in HHT1 patients (McAllister *et al.*, 1995), into the murine endoglin gene (Fig. 1). We report here our preliminary analysis of these mice.

MATERIALS AND METHODS

Construction of the targeting vector and generation of chimeric mice. Genomic clones containing the endoglin gene were isolated from a mouse strain 129/Ola lambda library by probing with the 0.43-kb *Bam*H1/*Bsp*H1 fragment spanning exons 6 and 7 of murine endoglin from clone pCDNA1-7/18 (Ge and Butcher, 1994). Linearised targeting construct DNA (Fig. 1A) was added to 1.2×10^8 E14Tg2a embryonic stem (ES) cells (from mouse strain 129/Ola) and exposed to a 0.8 kV, 3.0 μF pulse in a BioRad gene pulser. Cells were plated out at 5×10^6 cells/10-cm petri dish. Recombinant clones were selected after 1 day by the addition of G418 (at a final concentration of 150 μg/ml) and gancyclovir (at a final concentration of 0.1%) for a total of 8 days. Clones containing the targeted deletion of the central portion of the endoglin gene were identified by Southern blotting, using two probes external to the targeting fragment. Eight positive clones out of 60 recombinant ES clones tested were then confirmed by Southern blotting with a third probe derived from the neomycin gene to ensure that only single integration events had occurred (Fig. 1B). Chimeric males were derived from injection of three independent ES clones into blastocysts of 3.5 dpc C57Bl/6J mice implanted into pseudopregnant foster females 2.5 dpc. Five out of seven male chimeras gave 100% germ-line transmission of ES-derived 129/Ola genes, detected by the agouti coat colour of the progeny. The endoglin genotype of the mice was assessed either by Southern blotting (Fig. 1C) or by PCR using template DNA prepared from tail biopsies.

Timed matings and characterisation of offspring. All the analyses reported here have been performed on mice from a mixed 129/Ola and C57Bl/6J genetic background. Embryos were collected at various intervals from females following timed matings between heterozygous ($eng^{+/-}$) mice, where noon of the day of the vaginal plug was taken as 0.5 day. Embryos and yolk sacs were dissected from the decidua and examined under the microscope. For genotyping, DNA was isolated from yolk sacs of embryos or from tail biopsies of 3-week-old anaesthetised mice by digesting with 0.2 mg/ml proteinase K in tail lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) overnight at 55°C (Laird *et al.*, 1991). DNA was precipitated with isopropanol, washed with 70% ethanol, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

For routine genotyping, PCR contained two primer pairs simultaneously. For the *neo* gene, primers were d(ACGTACTCGGATGGAAGCC) and d(CAAGCTCTTCAGCAATATCACG); to detect the wild type endoglin gene, primers from the region deleted in the mutant allele were used, d(ACCATCTTGTCCTGAGTAGCG) and d(TGAGCCTGACGGGAAACTG). Annealing temperature was 58°C and 30 cycles were used for tail DNA while 35 cycles were used for the less concentrated yolk sac DNA. PCR products were resolved on 1.5% agarose gels.

Total protein was isolated from embryos and separated by SDS-PAGE. Endoglin was detected by Western blotting using anti-endoglin antibody (Pharmingen). Total RNA for rtPCR was prepared using Trizol (Life Technologies).

X-gal staining, histology, and immunohistochemistry. Embryos or small pieces of adult tissue were lightly fixed in 2.5% paraformaldehyde in PBS, washed three times in PBS, and stained for β-galactosidase activity by incubating overnight at 30°C in 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 10 mM sodium phosphate buffer, pH 7.3, 15 mM sodium chloride, 1 mM magnesium chloride, and 2 mg/ml X-gal. X-gal-stained and unstained tissues were fixed in 4% formaldehyde/PBS at room temperature, washed in PBS, dehydrated through ethanol, cleared in histoclear, and embedded in paraffin fibrowax. Sections (4 μm) were lightly counterstained with eosin or stained with haematoxylin and eosin or elastin van Gieson according to standard procedures. For immunohistochemistry, antigen retrieval was carried out by incubation with 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.6) at

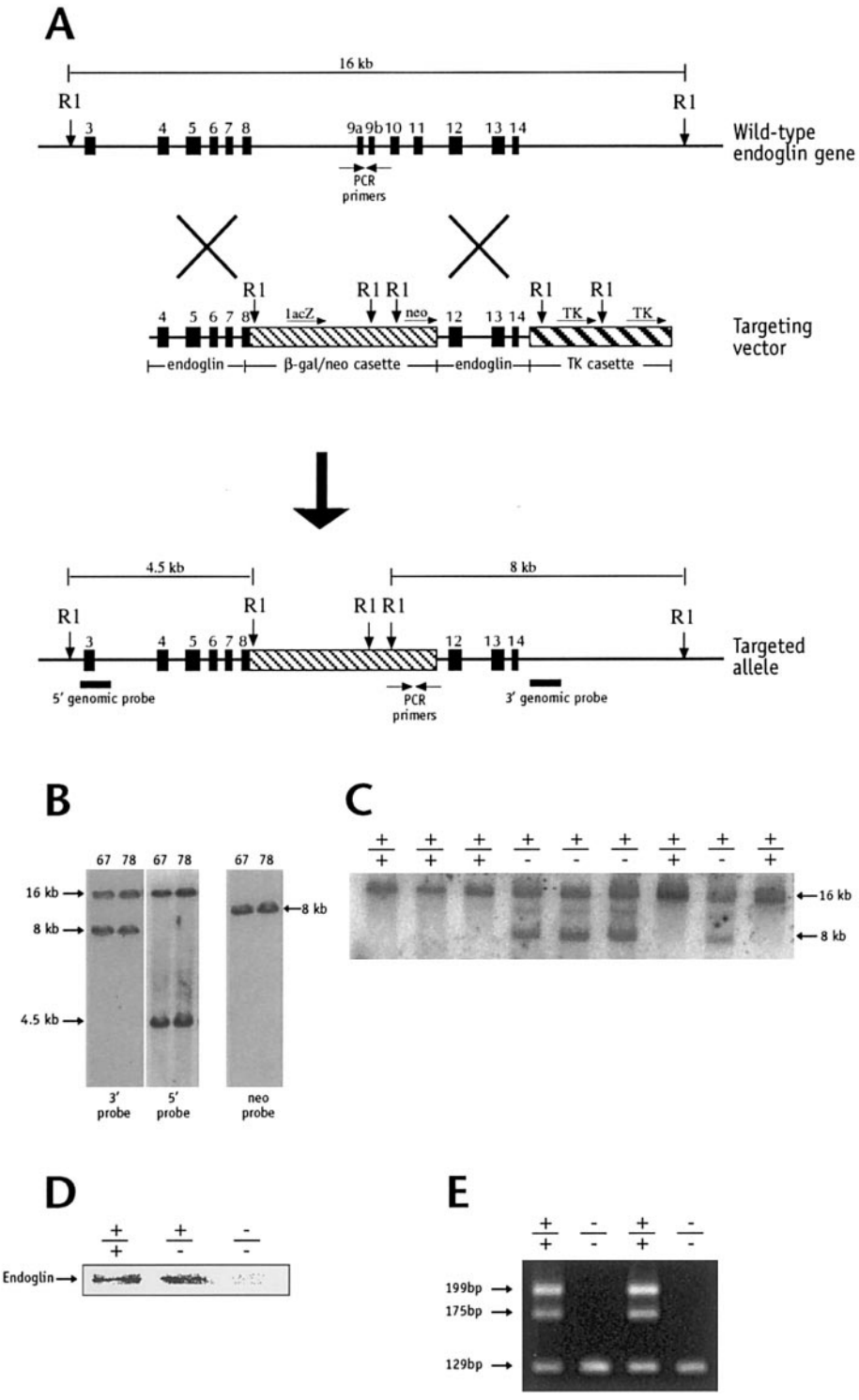


FIG. 1. Targeted disruption of the endoglin gene in ES cells and mice. (A) Wild-type endoglin gene, targeting construct and targeted allele. Endoglin exons (boxed regions) and introns are drawn approximately to scale. In the targeting construct a deletion of approximately 5-kb endoglin DNA, including exons 9a, 9b, 10, and 11, means that PCR primers from exons 9a and 9b can be used to detect the wild-type allele. The β-gal/*neo* cassette has a promoterless *lacZ* gene immediately downstream of an IRES site, allowing transcription driven by the endoglin promoter to be measured using β-galactosidase. The *MC1neo* gene driven by the Tk promoter and F9 enhancer enabled selection for integration of the construct. Two copies of the *Tk* gene from HSV were used for selection against nonhomologous recombination events.

TABLE 1
Analysis of Progeny from Heterozygote ($eng^{+/-}$) Intercrosses

Age	Genotype +/+	Genotype +/−	Genotype −/−	Total	χ^2	Probability
3 weeks postpartum	**42** (25)	**58** (50)	**0** (25)	**100**	37.84	⩽0.001 (2 *df*)
Embryo (9.5 dpc)	**25** (26.75)	**56** (53.5)	**26** (26.75)	**107**	0.252	Not significant (almost perfect fit)

Note. Observed numbers of progeny of each genotype are in bold type and expected numbers, based on the Mendelian ratio 1:2:1, are in parentheses.

37°C for 18 min. Endogenous peroxidase was quenched in hydrogen peroxide and immunohistochemical staining was carried out using the Histomouse-SP kit (Zymed Laboratories) using antibodies raised against human fibronectin, smooth muscle actin, or Von Willebrand factor (all from DAKO).

RESULTS

Targeted Disruption of the Endoglin Gene Causes Embryonic Lethality

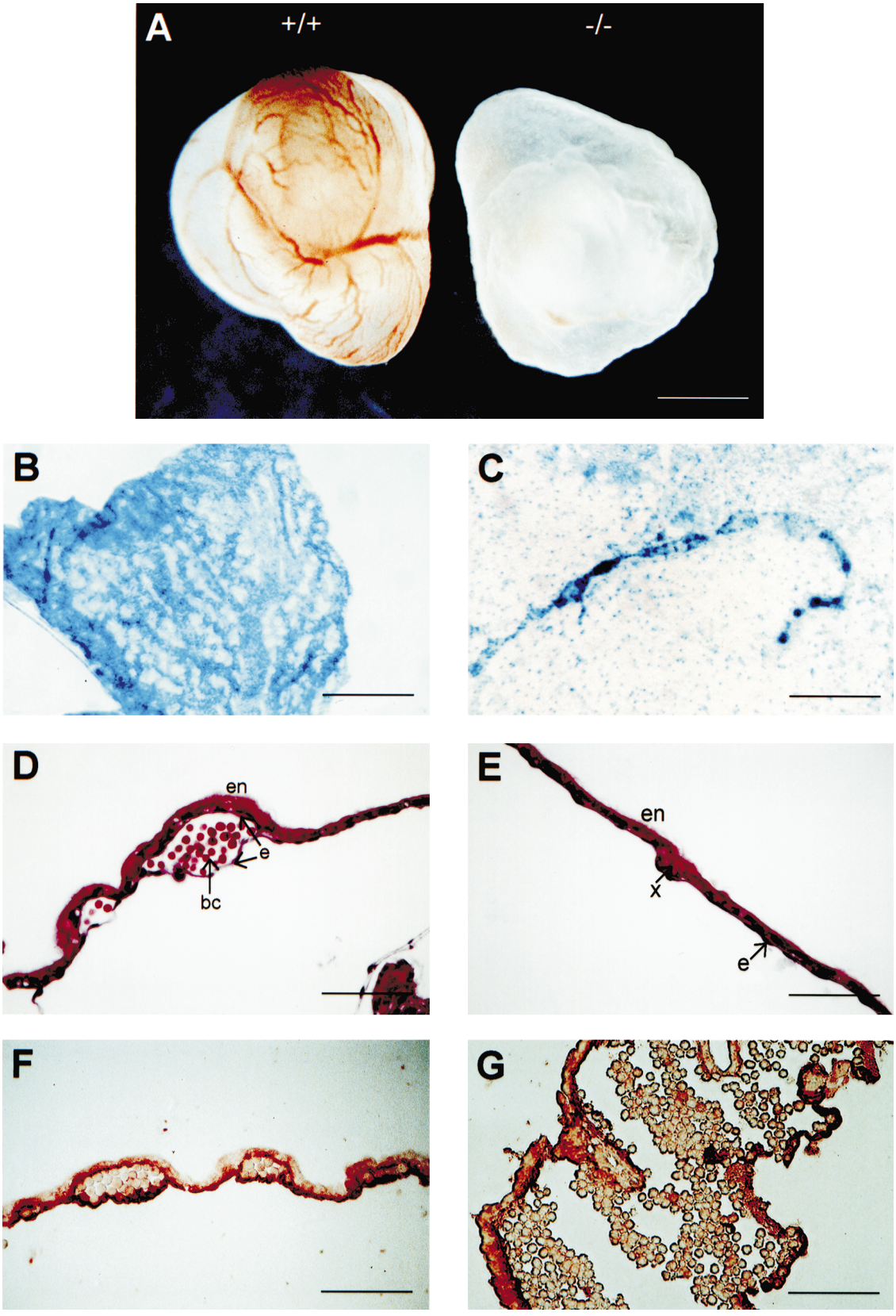
The targeting construct used to disrupt the endoglin gene had the following features: (1) a premature stop codon was introduced into the *Bsp*H1 site in exon 8 of the endoglin gene with the expected effect of producing a truncated endoglin protein; (2) this stop codon was immediately preceded by an influenza haemagglutinin (HA) epitope to facilitate immunological detection of the truncated product; (3) the *lacZ* coding region of *Escherichia coli* with an internal ribosome entry site was positioned immediately downstream of the endoglin mutation, so that expression from the endoglin promoter could also be monitored using β-galactosidase; and (4) a deletion of exons 9, 10, and 11 was introduced to minimise the chances of producing a functional endoglin molecule (Fig. 1A). Full-length endoglin protein was not present in $eng^{-/-}$ embryos showing that the endoglin gene had been successfully disrupted during targeted mutagenesis (Fig. 1D). However, we were unable to detect the expected truncated protein of 37 kDa, indicating it may be unstable, as has also been reported for HHT1 patients (Gallione *et al.*, 1998; Pece *et al.*, 1997). As expected, exons 5 (not shown), 6, and 7 were transcribed in endoglin-deficient ($eng^{-/-}$) progeny whereas exons 10 and 11 were not transcribed, as shown by rtPCR (Fig. 1E).

Crosses between animals which were heterozygous for this mutant allele of endoglin ($eng^{+/-}$) yielded very low litter sizes (typically 4 or 5 pups) and no homozygous $eng^{-/-}$ pups have been born among the 100 progeny tested to date. This clearly indicates that the endoglin mutation is lethal in the homozygous state (see Table 1). To determine at which stage of development the $eng^{-/-}$ embryos died, heterozygous ($eng^{+/-}$) females from timed matings with $eng^{+/-}$ males were euthanised at various stages of pregnancy. Yolk sacs were then examined prior to use for DNA preparation and retrospective genotyping. It became clear that when embryo progeny are examined at 9.5 dpc, the observed number of homozygous mutants is at the expected Mendelian frequency (Table 1), but by 10.5 dpc all $eng^{-/-}$ embryos had died and were in the process of being reabsorbed.

$eng^{-/-}$ Embryos Show Severe Defects in Haematopoiesis and Angiogenesis of the Yolk Sac

At 9.5 dpc, the most striking features of the $eng^{-/-}$ embryos were the abnormal vasculature and anaemia of the yolk sac (Fig. 2A). Endoglin appeared to be essential for the formation of mature vessels in the extraembryonic vasculature. When endoglin expression was assessed using β-galactosidase transcribed from the endoglin promoter in the mutant allele, it was seen in the endothelium of the

(B) Southern blot analysis of two clones (67 and 78) of double-resistant ES cells. Introduction of the β-gal/*neo* cassette results in three additional *Eco*R1 (R1) sites, which are used to differentiate between the targeted and the wild-type alleles. The left panel shows genomic DNA digested with *Eco*R1 and probed with 3′ and 5′ genomic probes, which reveal novel 8- and 4.5-kb fragments, respectively, in addition to the wild-type 16-kb fragment. The right panel shows the same digests probed with DNA derived from the *neo* gene. (C) Southern blot analysis of agouti progeny of a germ-line chimera probed with the 3′ probe. As before, the 8-kb *Eco*R1 fragment is associated with the mutant endoglin allele. (D) Western blot of reduced endoglin protein from $eng^{+/+}$, $eng^{+/-}$, and $eng^{-/-}$ embryos shows that $eng^{-/-}$ embryos lack full length endoglin protein. (E) rtPCR of total RNA from $eng^{+/+}$ and $eng^{-/-}$ embryos. Primers designed to amplify cDNA across intron boundaries show that a 129-bp PCR product corresponding to exons 6 and 7 was amplified from the cDNA prepared from embryos of both genotypes, while the 199- and 175-bp PCR products corresponding to exons 10 and 11 were only amplified from cDNA prepared from $eng^{+/+}$ embryos.



newly formed yolk sac vessels of $eng^{+/-}$ embryos (Fig. 2B), but only in isolated cells in many of the $eng^{-/-}$ yolk sacs (Fig. 2C). Upon closer examination, there were fewer and less well-developed vascular channels and dramatically reduced numbers of red blood cells in the yolk sac of many $eng^{-/-}$ embryos (Fig. 2E) compared with wild-type or $eng^{+/-}$ embryos (Fig. 2D), indicating defects in yolk sac haematopoiesis. However, this was not always the case, as distended vascular channels which contained large numbers of blood cells also occurred in $eng^{-/-}$ yolk sacs (Fig. 2G). Histological sections of yolk sac were immunostained for fibronectin, an extracellular matrix protein whose expression is regulated by TGFβ1 signaling and modulated by endoglin (Lastres *et al.*, 1996). Fibronectin expression was not quantified, but did not appear to be significantly altered in $eng^{-/-}$ compared with $eng^{+/-}$ or wild-type embryos and was clearly seen in the subendothelium of the yolk sac of all three genotypes (Figs. 2F and 2G).

$eng^{-/-}$ Embryos Exhibit Defects in Cardiogenesis

Overall the $eng^{-/-}$ embryos were much more fragile and smaller than their wild-type and heterozygous littermates (Fig. 3). Many exhibited developmental delay of about 0.5 day, which may have been due to yolk sac insufficiency and this may have been the reason for the delayed fusion of the dorsal aorta seen in $eng^{-/-}$ embryos. However, in contrast to the severe vascular defects seen the yolk sac, the appearance of the vasculature of the $eng^{-/-}$ embryos was largely normal, including development of the capillary network in most cases (Fig. 3). Exceptions included occasional embryos with a reduced cephalic capillary bed (Fig. 3B). There was severe anaemia and reduced red cell count in nearly all cases, presumably due to the reduced levels of haematopoiesis in the yolk sac, the main source of embryonic red blood cells at this stage in development (Dzierzak and Medvinsky, 1995). In addition, many of the $eng^{-/-}$ embryos had evidence of abnormal cardiac development. Almost all had enlarged ventricles (Fig. 4B) and very dilated outflow tracts (Figs. 4D and 4F). The endothelial surface of the truncal cushions failed to organise in about half the $eng^{-/-}$ embryos (Fig. 4D). No difference in fibronectin expression in endocardial cushions was seen between $eng^{+/+}$ and $eng^{-/-}$ embryos, indicating that endoglin is not required for fibronectin expression in the cardiac jelly (Figs. 4E and 4F).

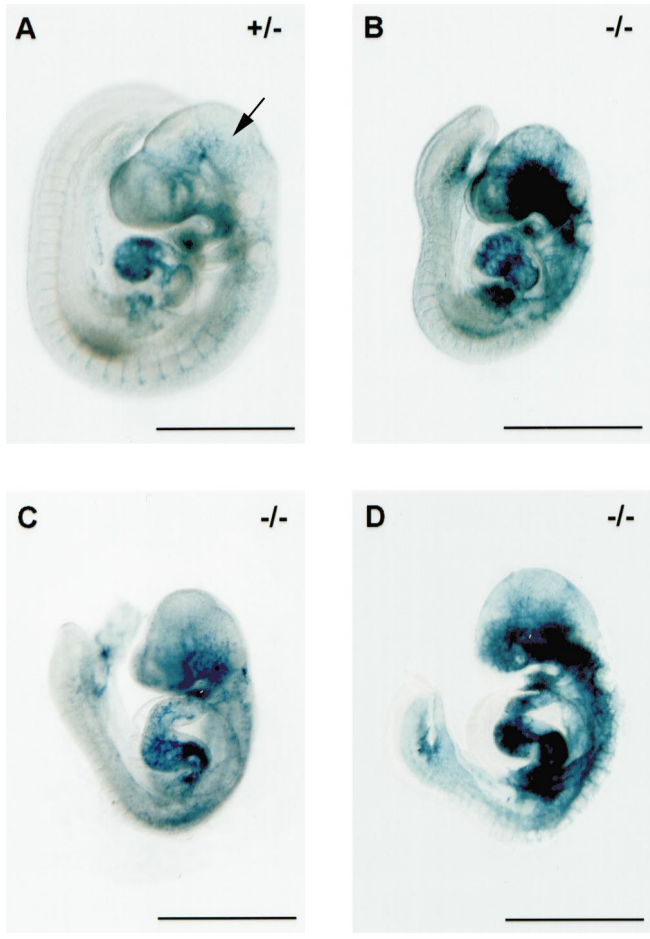


FIG. 3. Whole mount 9.5 dpc embryos stained with X-gal showing endoglin expression associated with the developing vasculature. The genotype of each embryo is indicated on the figure and an arrow points to the cephalic capillary bed in the $eng^{+/-}$ embryo. Scale bar: (A, B, C, D) 1 mm.

However, endoglin was expressed by individual migrating mesenchymal cells of the endocardial cushion (Fig. 4G), but these cells were rarely seen in $eng^{-/-}$ embryos. In addition, many of the $eng^{-/-}$ embryos had abnormal cardiac looping and exhibited pericardial effusion (Figs. 3C and 3D).

FIG. 2. Analysis of the yolk sac of 9.5 dpc embryos. (A)Whole mount view of yolk sacs showing an extensive vasculature full of red blood cells in the $eng^{+/+}$ yolk sac (which is also seen in $eng^{+/-}$ yolk sacs), but no vessels and very few red blood cells in the $eng^{-/-}$ yolk sac. Detection of endoglin expression using X-gal staining shows endoglin associated with the newly formed vessels in the $eng^{+/-}$ yolk sac (B), whereas it is associated with isolated cells in the $eng^{-/-}$ yolk sac (C) and the umbilical vessel (folded flat beneath the yolk sac in this view) was the only normal vessel seen in $eng^{-/-}$ yolk sacs. Histological analysis of yolk sacs from $eng^{+/-}$ (D and F) and $eng^{-/-}$ (E and G) embryos. Haematoxylin and eosin-stained sections of yolk sac showed normal development of vascular channels containing red blood cells in the $eng^{+/-}$ yolk sac (D), but poor differentiation of vessels typical of large regions of the $eng^{-/-}$ yolk sac (E). Sections immunostained for fibronectin show this protein associated with both endothelial layers of the yolk sacs of $eng^{+/-}$ (F) and $eng^{-/-}$ embryos (G). bc, blood cells; e, endothelial cell; en, endoderm; x, undifferentiated vessel. Scale bars; (A) 1 mm; (B, C) 400 μm; (D, E, F, G) 50 μm.

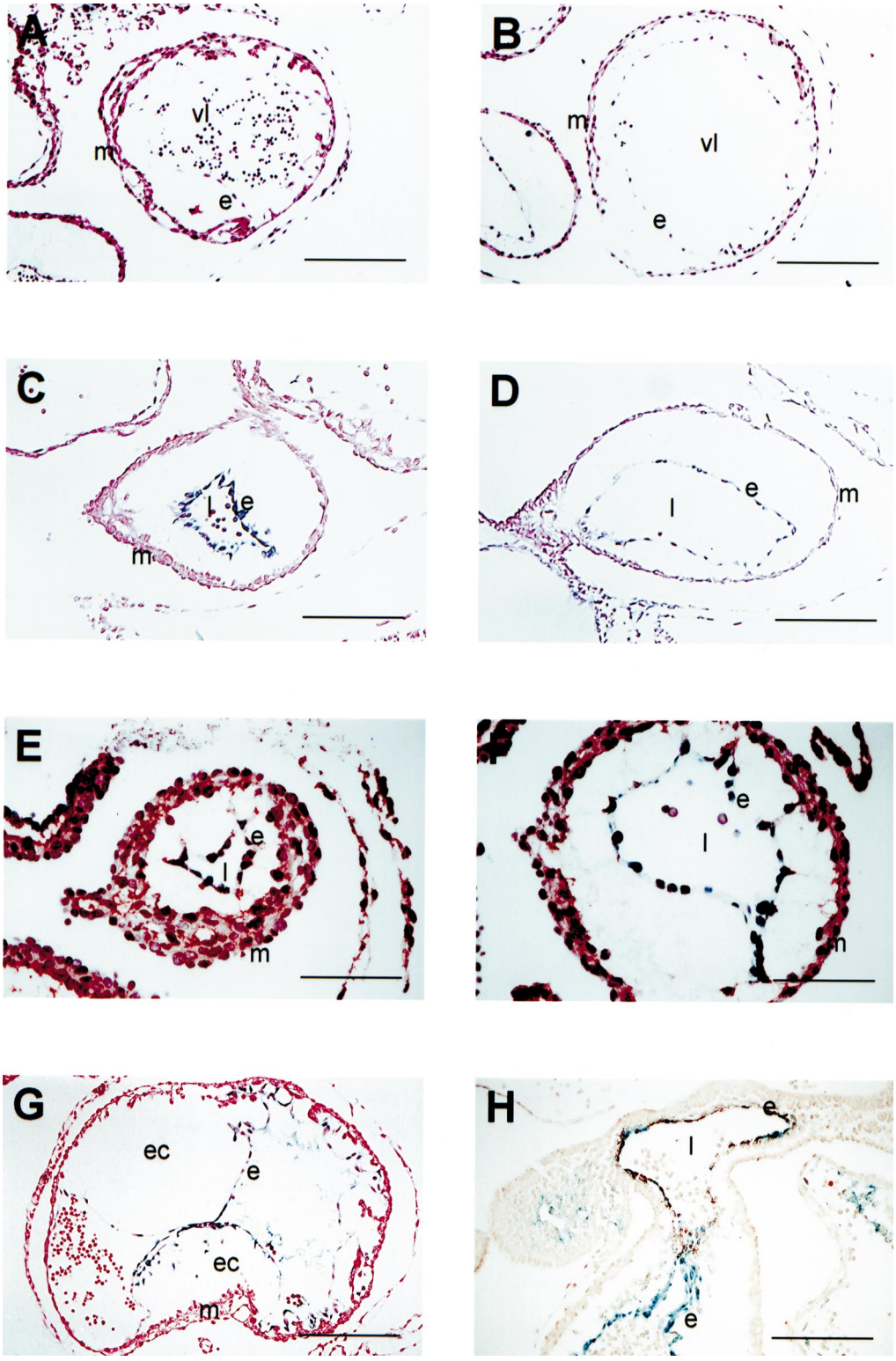


FIG. 4. Examination of heart development in 9.5 dpc embryos illustrated by sagittal sections of *eng*$^{+/-}$ (A, C, E, G, and H) and *eng*$^{-/-}$ (B, D, and F) embryos. Haematoxylin and eosin-stained sections through primitive ventricle (A and B) showing no red blood cells in the ventricular lumen, dilated ventricle, and thinner myocardium in the *eng*$^{-/-}$ embryo (B). X-gal-stained sections through outflow tract (C and D) show strong endoglin expression in the organising heart tissue of the *eng*$^{+/-}$ embryo (C), while the *eng*$^{-/-}$ embryo shows ill-formed

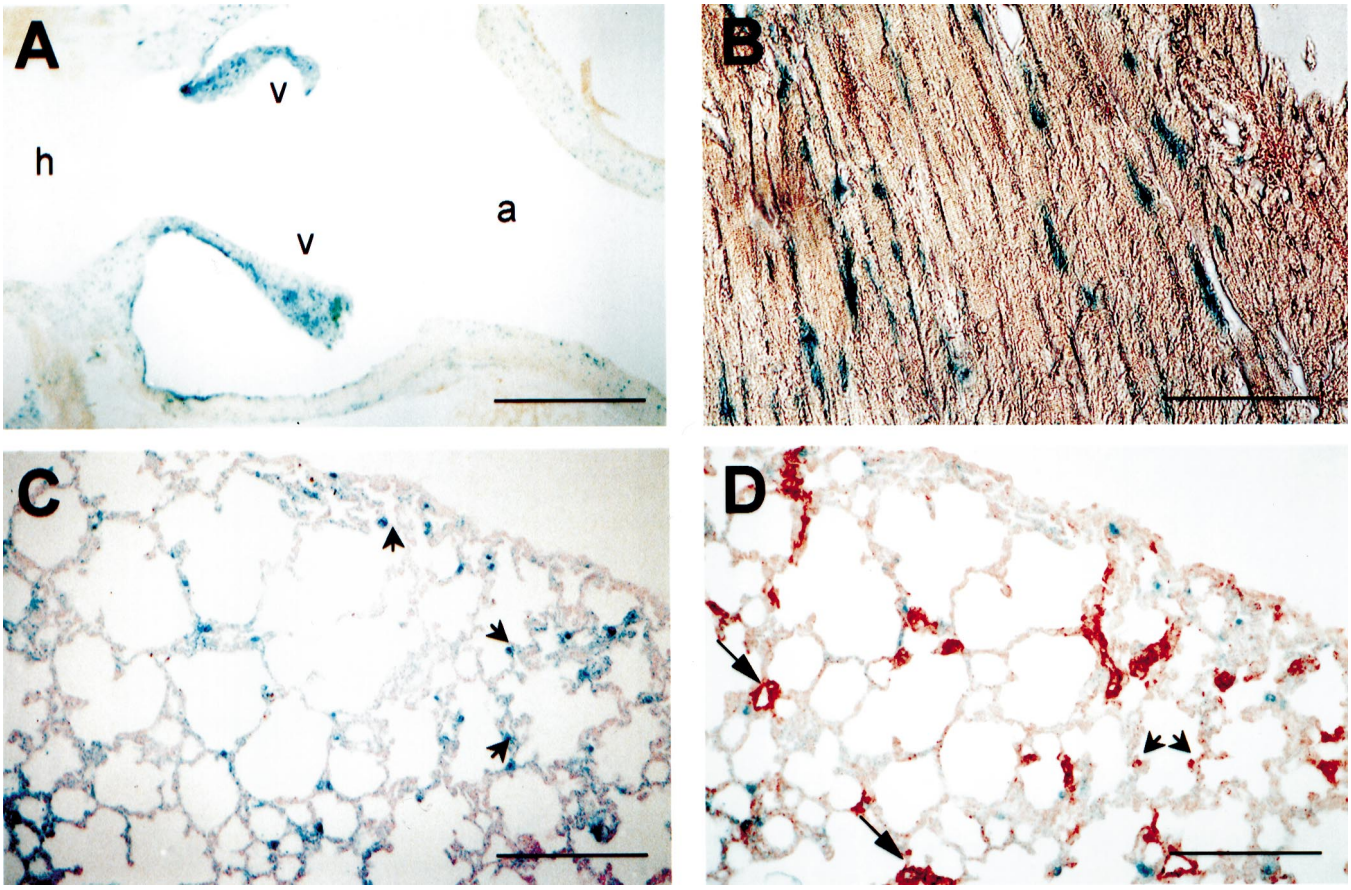


FIG. 5. Examination of endoglin expression in adult heart and lung tissue. Pulmonary valve, stained with X-gal (A), shows high endoglin expression particularly on the pulmonary artery side of the valve. Myocardium, stained with X-gal (B), shows endoglin expression associated with the capillary network supplying the cardiac muscle. (C and D) Adjacent sections of lung, stained with X-gal (blue) and eosin and immunostained brown for von Willebrand factor. (C) Strong endoglin expression is seen in alveolar capillary endothelial cells (arrows), while weaker expression is associated with stromal cells within the pulmonary interstitium. (D) Some colocalisation of von Willebrand factor and endoglin is seen in the capillary endothelium (short arrows), but von Willebrand factor is mainly associated with the larger vessels particularly veins (long arrows). Scale bar: 100 μm. h, heart; v, valve; a, pulmonary artery.

Endoglin Expression in Vascular Endothelium Is Heterogeneous

In 9.5 dpc $eng^{+/-}$ embryos, endoglin expression can be seen in the endothelial cells throughout the developing vascular system and is strongest in the endocardium (Fig. 3A). We compared endoglin expression with that of another endothelial marker, von Willebrand factor, and found that larger embryonic vessels express both endoglin and von Willebrand factor (e.g., dorsal aorta and branchial arch arteries), but endocardial cells expressed endoglin alone and we located a junction of expression of von Willebrand factor in the aortic sac region of the outflow tract (Fig. 4H). This observation is in agreement with previous data showing

endocardial cells (D). Sections through outflow tract, immunostained (brown) for fibronectin, show expression in truncal cushions of both $eng^{+/-}$ (E) and $eng^{-/-}$ (F) embryos. X-gal and eosin-stained section through a central portion of the developing heart (G) shows endoglin expression (blue) associated with mesenchymal cells within the endocardial cushion. X-gal and von Willebrand factor-stained section through the heart (H) showing endoglin expression throughout the endocardium while von Willebrand factor (brown) is associated only with the endothelium of the aortic sac region of the outflow tract. Scale bar: (A,B,G) 100 μm; (C,D,E,F,H) 50 μm. e, endothelial cells; ec, endocardial cushion; l, lumen of outflow tract; m, myocardial cells; vl, lumen of primitive ventricle.

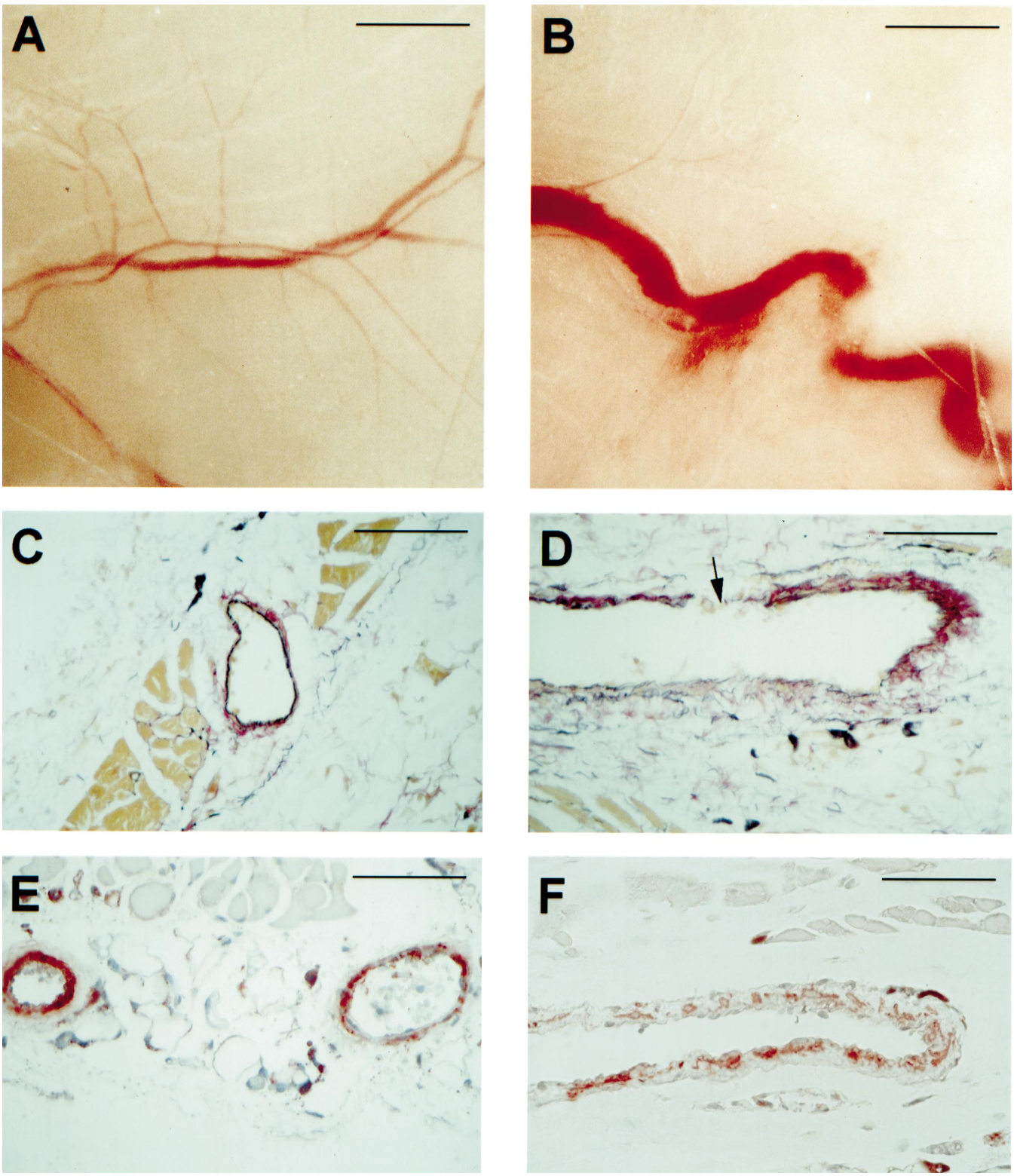


FIG. 6. Comparison of blood vessels in age-matched $eng^{+/-}$ and $eng^{+/+}$ mice. Convoluted and dilated blood vessel seen in the abdominal skin of an $eng^{+/-}$ mouse (B) compared with normal vasculature from a similar region of skin from an $eng^{+/+}$ mouse (A). Transverse sections of these vessels stained with elastin van Gieson show the sparse and uneven distribution of elastin fibres (stained black) in the abnormal dilated vessel from the $eng^{+/-}$ mouse (D) compared with a normal venule in C. The arrow in D points to a region of vessel rupture which probably occurred when the skin was removed from the animal and serves to illustrate the fragility of this vessel. Immunohistochemical staining for smooth muscle actin (stained brown) shows disorganised smooth muscle cells surrounding the abnormal vessel in the $eng^{+/-}$ mouse (F) compared with the normal vessels in E. Scale bars: (A, B) 500 μm, (C, D, E, F) 25 μm.

that expression of von Willebrand factor is limited to the larger vessels at this stage in development (Coffin *et al.*, 1991) and illustrates differences between endothelial cells from different regions of the vasculature.

In adult mice, endoglin expression is much more extensive. It is associated with both endothelial and stromal tissue (St-Jacques *et al.*, 1994) and to a lesser extent with smooth muscle cells (Adam *et al.*, 1998). We have focused our analysis on expression in heart and lung tissue. In heart, the highest endoglin expression was seen in the aortopulmonary and atrioventricular valves and was associated with the valve mesenchymal cells (Fig. 5A). Expression is also seen in the endothelium of small blood vessels in the compact layers of the ventricular walls but not in the outer layer of the myocardium (Fig. 5B). In the lung, endoglin was expressed in some stromal cells within the interstitium and in most endothelial cells of the vasculature, but was particularly high in the pulmonary microcapillaries (Fig. 5C). Von Willebrand factor was expressed in a complementary fashion in the pulmonary vasculature, strongly staining large vessel endothelium (Fig. 5D), but weakly staining the microvasculature, in agreement with previous reports (Yamamoto *et al.*, 1998).

Mice, Heterozygous for the Endoglin Mutation, Sometimes Have Abnormal and Dilated Blood Vessels

Occasional mice, of strain 129/Ola, carrying a single copy of the endoglin mutation exhibited extensive dilated and weak-walled vessels typical of HHT. One such example is shown in Fig. 6. A large vessel, which appeared to be venous, was extremely dilated and somewhat convoluted (Fig. 6B). Detailed examination showed that the elastin support fibres (Fig. 6D) and smooth muscle cells (Fig. 6F) surrounding this vessel were both disorganised and sparsely distributed, properties which may contribute to the fragility of these vessels.

DISCUSSION

This work clearly shows that expression of endoglin is essential during angiogenesis in the yolk sac during early development. The phenotype of the endoglin knockout mouse is very similar to the TbRII knockout mice (Oshima *et al.*, 1996) and to the fraction of TGFβ1 knockout mice, which die *in utero* (Dickson *et al.*, 1995). This contrasts with the less severe phenotype of mice without TGFβ2 or TGFβ3 (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995; Sanford *et al.*, 1997). As endoglin is known to bind TGFβ1 in association with TbRII (Barbara *et al.*, 1999), the evidence suggests that endoglin is required for TGFβ1 signaling during extraembryonic angiogenesis. Many, although not all, of the $eng^{-/-}$ embryos showed defects in haematopoiesis. Some of the variation seen in haematopoietic defects may be due to the random segregation of alleles at multiple loci in the embryos, a common and often overlooked problem affecting the examination of knockout mice in a mixed genetic background. Endoglin is temporarily expressed in differentiating pro-erythrocytes in foetal and adult bone marrow (Rokhlin *et al.*, 1995) and this may correspond to a parallel stage at which haematopoiesis may stall in the yolk sac of endoglin-deficient 9.5 dpc embryos.

The gross defects described in the $eng^{-/-}$ 9.5 dpc embryos are in the extraembryonic vasculature of the yolk sac, while the embryonic vasculature, with the exception of the heart, appears to form almost normally, indicating that there are differences in the signaling pathways involved in the formation of extraembryonic and embryonic vascular systems. In contrast, the VEGF receptor flk-1 is required for both pathways and its absence causes severe disorganisation of vessels in both extraembryonic and embryonic tissue (Shalaby *et al.*, 1995).

Endoglin is expressed in endothelial cells throughout the developing vascular system (Fig. 3A) in a strikingly similar pattern to that of avian TbRII expression at a similar stage of development (Brown *et al.*, 1996). Expression of endoglin in mesenchymal cells of the endocardial cushion (Fig. 4G) is also seen in human tissue (Qu *et al.*, 1998). As there is abnormal truncal cushion formation in the $eng^{-/-}$ embryos (Figs. 3, and 4D), endoglin may also play a role as an ancillary TGFβ receptor during endocardial cushion formation. It is likely that TGFβ1, as a ligand of both endoglin and TbRII, is involved, as it too is expressed in the developing heart valve (Heine *et al.*, 1987) and disorganised valves have been reported for TGFβ1 null mice developing in the complete absence of circulating maternal TGFβ1 (Letterio *et al.*, 1994). The role of endoglin may relate to the essential role recently shown for betaglycan in avian endocardial cell transformation (Brown *et al.*, 1999). Betaglycan is a type III TGFβ receptor, which shares a region of high sequence homology with endoglin in the cytoplasmic domain (Lopez-Casillas *et al.*, 1991), but unlike endoglin, augments TGFβ2 signaling (Lopez-Casillas *et al.*, 1993). However, there are also some differences between endoglin expression in developing heart tissue of chick and mouse (Vincent *et al.*, 1998), and further studies are required to determine its role in heart development of both animals.

Endoglin expression remains high in adult mouse heart valves, in agreement with previous reports (St-Jacques *et al.*, 1994), suggesting a continued role in this tissue. In addition, the abnormal cardiac looping, enlarged cardiac ventricles and pericardial sac and the particularly high expression of endoglin in the endocardium in the 9.5 dpc embryos provide evidence that endoglin has a more general role to play during cardiogenesis. Transgenic expression of a constitutively activated mutation of Alk5 in myocytes severely disrupts cardiac looping (Charng *et al.*, 1998) and it is possible that loss of endoglin upsets the balance of signaling between different receptor complexes of the TGFβ family expressed in endocardial cells leading to a similar but less severe effect. However, as we observed significant variation in the cardiac defects, which may have been due

to random segregation of modifier alleles in the progeny, we are now breeding the endoglin mutation into a number of pure lines to reduce this problem and ultimately allow the identification of modifier genes.

In the presence of a modifier allele on chromosome 5 of the NIH strain, the defects in haematopoiesis and vasculogenesis of theTGFβ1 knockout (KO) mice are suppressed and they can progress to birth (Bonyadi *et al.*, 1997). However, they die at about 3 weeks from severe inflammatory disease (Kulkarni *et al.*, 1993; Shull *et al.*, 1992). We have now bred the endoglin mutation into the NIH background and have seen no rescue to birth of the lethal phenotype of animals homozygous for the endoglin mutation, suggesting that endoglin acts downstream of this modifier.

There appear to be no deleterious effects of a single copy of the endoglin mutation on longevity (the oldest $eng^{+/-}$ mice are now aged 18 months) or fertility in the mixed (129 plus C57BL/6) genetic background. However, the fragile, dilated, and convoluted vessels seen in occasional heterozygous adults have to date only been observed in the 129/Ola genetic background and it may be significant that this strain has low levels of circulating TGFβ1 (Rosemary Akhurst, personal communication). It is not clear why, in an isogenic background, only some individuals develop these abnormal vessels, but we intend to identify the triggers that induce the vascular defects and thereby develop a reliable model of HHT1.

In conclusion, we have shown that endoglin is indispensible in early development and we propose that it is an essential component of the TGFβ1 signaling pathway required for the formation of blood vessels in the yolk sac and for normal cardiogenesis. This proposal does not concur with data from *in vitro* experiments showing that endoglin is not required for TGFβ1 signaling and can even block some cellular responses to TGFβ1, such as growth-arrest (Lastres *et al.*, 1996; Letamendía *et al.*, 1998). A possible explanation of these apparently contradictory data is that endoglin is required for activation and/or release of TGFβ1 from the extracellular matrix *in vivo*, in order to make it available to the signaling receptors and this role is bypassed in *in vitro* experiments when free, active TGFβ1 is used. Clearly, much remains to be learned about the role of endoglin in vascular development and maintenance and we are confident that the endoglin KO mouse will be an invaluable tool for further studies.

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