INTRODUCTION

The cardiovascular system is the first functional organ system to develop in the vertebrate embryo. It starts out with differentiation of mesodermal cells into hemangioblasts, bipotential stem cells capable of development into hematopoietic and endothelial lineages. Angioblasts form a primitive vascular plexus de novo in a process known as vasculogenesis. New blood vessels form by angiogenesis, sprouting or splitting from the pre-existing primary blood vessels, extending this network throughout the embryo. The emerging vascular plexus undergoes rapid pruning and remodeling to form a mature, tree-like network with larger vessels feeding into many smaller capillaries (reviewed in Folkman and D’Amore, 1996; Risau, 1997).

Multiple signaling molecules, including fibroblast growth factor-2 (Flamme and Risau, 1992), vascular endothelial growth factor (Carmeliet et al., 1996; Ferrara et al., 1996), angiopoietin-1 (Suri et al., 1996), platelet-derived growth factor (Leveen et al., 1994), Ephrin-B2 (Wang et al., 1998) and transforming growth factor-β1 (TGF-β1) (Dickson et al., 1995), as well as their receptors (Dumont et al., 1994; Fong et al., 1995; Oshima et al., 1996; Sato et al., 1995; Shalaby et al., 1995; Soriano, 1994), are implicated in vasculogenesis and angiogenesis. The TGF-β superfamily consists of over 40 secreted signaling molecules that are known to have important roles in controlling cell fate by regulating proliferation, differentiation and apoptosis (reviewed in Kingsley, 1994; Roberts and Sporn, 1990). TGF-β homologs, particularly TGF-β1, have been shown to regulate new blood vessel formation, both in vivo and in vitro (reviewed in Beck and D’Amore, 1997; Pepper, 1997).

Recently, SMAD proteins have been identified as important components of TGF-β signaling pathways. They function downstream of TGF-β receptors and directly transduce signals from the cell membrane into the nucleus. SMAD proteins are highly conserved across species from invertebrates to man. There are nine vertebrate SMADs, including the pathway-specific SMADs, SMADs 1-3, 5 and 8, MADH6, the common mediator SMAD4, and the inhibitory SMADs, SMAD6 and 7 (Heldin et al., 1997; Kawabata et al., 1998; Watanabe et al., 1997; Massague, 1998). The pathway-specific SMADs are phosphorylated by ligand-activated type I receptors on a conserved Ser-Ser-X-Ser motif in the most C-terminal region, called the MH2 domain (Abdollah et al., 1997; Souchelnytskyi et al., 1997). This releases the MH2 domain from inhibition by the N-terminal MH1 domain, and allows the pathway-specific SMADs to form hetero-oligomers with SMAD4, and translocate into the nucleus, triggering transcriptional responses (Lagna et al., 1996). In vitro studies suggest that SMAD2 and SMAD3 act downstream of the TGF-β and activin receptors (Baker and Harland, 1996; Graff et al., 1996; Zhang et al., 1996), while SMAD1 and SMAD5 respond to BMP signals (Kretzschmar et al., 1997; Lagna et al., 1996; Kawabata

SUMMARY

The transforming growth factor-β (TGF-β) signals are mediated by a family of at least nine SMAD proteins, of which SMAD5 is thought to relay signals of the bone morphogenetic protein (BMP) pathway. To investigate the role of SMAD5 during vertebrate development and tumorigenesis, we disrupted the Smad5 gene by homologous recombination. We showed that Smad5 was expressed predominantly in mesenchyme and somites during embryogenesis, and in many tissues of the adult. Mice homozygous for the mutation died between days 10.5 and 11.5 of gestation due to defects in angiogenesis. The mutant yolk sacs lacked normal vasculature and had irregularly distributed blood cells, although they contained hematopoietic precursors capable of erythroid differentiation. Smad5 mutant embryos had enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells, suffered massive apoptosis of mesenchymal cells, and were unable to direct angiogenesis in vitro. These data suggest that SMAD5 may regulate endothelium-mesenchyme interactions during angiogenesis and that it is essential for mesenchymal survival.

Key words: SMAD5, Gene targeting, Yolk sac, Vasculogenesis, Angiogenesis, Mouse, Apoptosis

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The cardiovascular system is the first functional organ system to develop in the vertebrate embryo. It starts out with differentiation of mesodermal cells into hemangioblasts, bipotential stem cells capable of development into hematopoietic and endothelial lineages. Angioblasts form a primitive vascular plexus de novo in a process known as vasculogenesis. New blood vessels form by angiogenesis, sprouting or splitting from the pre-existing primary blood vessels, extending this network throughout the embryo. The emerging vascular plexus undergoes rapid pruning and remodeling to form a mature, tree-like network with larger vessels feeding into many smaller capillaries (reviewed in Folkman and D’Amore, 1996; Risau, 1997).

Multiple signaling molecules, including fibroblast growth factor-2 (Flamme and Risau, 1992), vascular endothelial growth factor (Carmeliet et al., 1996; Ferrara et al., 1996), angiopoietin-1 (Suri et al., 1996), platelet-derived growth factor (Leveen et al., 1994), Ephrin-B2 (Wang et al., 1998) and transforming growth factor-β1 (TGF-β1) (Dickson et al., 1995), as well as their receptors (Dumont et al., 1994; Fong et al., 1995; Oshima et al., 1996; Sato et al., 1995; Shalaby et al., 1995; Soriano, 1994), are implicated in vasculogenesis and angiogenesis. The TGF-β superfamily consists of over 40 secreted signaling molecules that are known to have important roles in controlling cell fate by regulating proliferation, differentiation and apoptosis (reviewed in Kingsley, 1994; Roberts and Sporn, 1990). TGF-β homologs, particularly TGF-β1, have been shown to regulate new blood vessel formation, both in vivo and in vitro (reviewed in Beck and D’Amore, 1997; Pepper, 1997).

Recently, SMAD proteins have been identified as important components of TGF-β signaling pathways. They function downstream of TGF-β receptors and directly transduce signals from the cell membrane into the nucleus. SMAD proteins are highly conserved across species from invertebrates to man. There are nine vertebrate SMADs, including the pathway-specific SMADs, SMADs 1-3, 5 and 8, MADH6, the common mediator SMAD4, and the inhibitory SMADs, SMAD6 and 7 (Heldin et al., 1997; Kawabata et al., 1998; Watanabe et al., 1997; Massague, 1998). The pathway-specific SMADs are phosphorylated by ligand-activated type I receptors on a conserved Ser-Ser-X-Ser motif in the most C-terminal region, called the MH2 domain (Abdollah et al., 1997; Souchelnytskyi et al., 1997). This releases the MH2 domain from inhibition by the N-terminal MH1 domain, and allows the pathway-specific SMADs to form hetero-oligomers with SMAD4, and translocate into the nucleus, triggering transcriptional responses (Lagna et al., 1996). In vitro studies suggest that SMAD2 and SMAD3 act downstream of the TGF-β and activin receptors (Baker and Harland, 1996; Graff et al., 1996; Zhang et al., 1996), while SMAD1 and SMAD5 respond to BMP signals (Kretzschmar et al., 1997; Lagna et al., 1996; Kawabata
et al., 1998; Suzuki et al., 1997). However, the full range of inputs governing the activation of individual SMAD proteins is still not clear.

The functions of SMAD proteins have been studied in mouse using gene targeting. It has been shown that SMAD4 is essential for epiblast proliferation, egg cylinder formation and mesoderm induction (Yang et al., 1998; Sirard et al., 1998). Mutations made in different domains of SMAD2 revealed a role of this gene in multiple developmental processes, including mesoderm induction, and formation of the left-right and anterior-posterior axes (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). Disruption of SMAD3 indicated that this gene is required for mediating regulatory effects of TGF-β in T cells, and loss of SMAD3, consequently, results in susceptibility to severe mucosal infection and immune dysregulation (Yang et al., 1999), in addition to a high susceptibility to colon cancer (Zhu et al., 1998).

SMAD5 is also known as dawrin C, Jv5-1 and MAD5, and shares 95% amino acid sequence homology with SMAD1 (Riggins et al., 1996; Yingling et al., 1996). Both belong to the pathway-specific SMAD proteins. However, the exact pathways in which SMAD5 functions are still less than clear. Studies in Xenopus laevis showed that SMAD5 could transduce BMP signals and induce ventral mesoderm formation (Suzuki et al., 1997). However, antisense oligonucleotides to the Smad5 gene reversed the inhibitory effects of TGF-β on hematopoietic cell proliferation, suggesting an involvement of SMAD5 in TGF-β signal transduction (Bruno et al., 1998). SMAD5 has been mapped to human chromosome 5q31, deletion of which is one of the most frequent cytogenetic abnormalities in human leukemia and myelodysplastic syndrome. Therefore Smad5 is thought to be a candidate tumor suppressor gene in this region (Hejlik et al., 1997; Zavadil et al., 1997).

To investigate the role of SMAD5 during vertebrate development and tumorigenesis, we disrupted the Smad5 gene by homologous recombination in embryonic stem (ES) cells. Mouse heterozygous for the targeted mutation are phenotypically normal. However, homozygous mutant mice died between embryonic days 10.5 and 11.5 (E10.5-E11.5) as described (Suri et al., 1996). Yolk sacs from Smad5 heterozygous matings were harvested at E9.5 (MEC13.3, rat anti-mouse monoclonal, Pharmingen) were performed as described (Suri et al., 1996).

In vitro yolk sac hematopoietic progenitor assay

Yolk sacs from Smad5 heterozygous matings were harvested at E9.5 and disaggregated to single cell suspensions by incubation in 0.1% collagenase/20% FBS for 3 hours at 37°C. Cells were then washed twice in 4 ml α-MEM/2% FBS medium, and counted with a hemocytometer. A total of 10^6 cells from each yolk sac were plated in duplicate with methylcellulose matrix (MethoCult 3430; StemCell Technologies, Inc. Vancouver, Canada), 3 units/ml erythropoietin (Pharmingen; San Diego, CA), 100 ng/ml IL-3 (Gibco-BRL), 100 ng/ml IL-6 (Gibco-BRL) and 1500 units/ml SCF (Pharmingen; San Diego, CA). Cultures were then incubated at 37°C in the presence of 5% CO₂. Colony-forming units were scored under the microscope at day 7. The

MATERIALS AND METHODS

Targeting vector

Several BAC clones containing genomic DNA of the Smad5 locus were isolated from a 129 mouse library (Genome Systems, St Louis) by using a portion (478-1359 bp) of a mouse Smad5 cDNA (Yingling et al., 1996) as a probe. A 19 kb genomic DNA was further subcloned into a Bluescript SK vector. To construct the targeting vector for the Smad5 gene, a 2 kb HindIII fragment, which is 3’ to exon 6 of the Smad5 gene, was subcloned into Hpal sites of a vector containing the pLoxneo (Yang et al., 1998). The resulting construct was cleaved with ClaI, filled in, and then digested with BamHI, followed by insertion of a 10 kb HindIII-BamHI fragment (the BamHI site is from polylinker of the SK vector), which is 5’ to exon 6 of the Smad5 gene. The finished construct, pSmad5neo, is shown in Fig. 2A.

Homologous recombination in ES cells and generation of germine chimeras

TC1 ES cell line (Deng et al., 1996) was transfected with NotI-digested pSmad5neo, and selected with G418 and FIAU. The culture, electroporation and selection of ES cells was carried out as described (Deng et al., 1994). ES cell colonies that were resistant to both G418 and FIAU were picked and analyzed by Southern blotting for homologous recombination events within the Smad5 locus. Genomic DNAs from these clones and the parental TC1 cell line were digested with Asp718 and BamHI followed by Southern transfer analysis using a 1.8 kb Xhol-BamHI fragment 3’ to the targeting vector (Fig. 2A). ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts to obtain germine transmission. The injected blastocysts were implanted into the uterus of pseudopregnant Swiss Webster (Taconic) foster mothers and allowed to develop to term. Male chimeras (identified by the presence of agouti coat color) were mated with C57BL/6 and NIH Black Swiss females (Taconic). Germine transmission was confirmed by agouti coat color in the F1 animals, and all agouti offspring were tested for the presence of the mutated Smad5 allele by Southern analysis using the same conditions for the detection of the homologous recombination event in the ES cells.

Genotype analysis

Genotypes were determined by Southern blotting or PCR. For PCR analysis, the wild-type Smad5 allele was detected using a 5’-oligonucleotide (primer 1, 5′-AATGAATTGATCTGCCCAGTA-3′) and a 3’-oligonucleotide (primer 2, 5′-TAAACAATCGTTGGGGAAGC-3′). This primer pair flanks the pLoxneo insertion site and amplifies a 231 bp fragment from the wild-type Smad5 gene. DNA was also amplified using primer 3 (5′-CCCTTATTATCACAATGGCC-3′) and primer 4 in the pLoxneo gene (5′-CCGACTGCCTGGGAAAGC-3′) to detect the mutant Smad5 allele. In this case, a 449 bp fragment was detected in mice heterozygous or homzygous for the mutant Smad5 allele, while no signal was detected in wild-type mice.

Histological analysis, in situ hybridization and immunohistochemistry

Histology and in situ hybridization were carried out using standard procedures. The Smad5 probe is the same one we used for BAC genomic DNA library screening. Slides were dipped in emulsion (Kodak NTB-2) and exposed for 7-15 days before developing. Whole-mount embryo immunostainings using an anti-pECAM antibody (MEC13.3, rat anti-mouse monoclonal, Pharmingen) were performed as described (Suri et al., 1996).
scoring of colony-forming units was performed as previously described (Evans et al., 1995).

Detection of apoptotic cells
For whole-mount apoptosis staining, embryos were dissected out at E9.5, and incubated in Nile Blue A sulfate salt (Sigma) solution for 30 minutes at 37°C, then destained in PBS until the background clarified. Stained embryos were observed under a dissection microscope and were photographed immediately. TUNEL staining was performed using ApopTag in situ apoptosis detection kit (Oncor).

In vitro angiogenesis assay
Bovine capillary endothelial cells (ATCC) were cultured in MEM, supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FCS. For in vitro angiogenesis assay, 4x10^5 cells in 1 ml of medium were mixed with 1 ml of collagen gel. 400 μl of the mixture was sampled into 1 well of a 4-well dish. Pieces of embryos, containing the dorsal aorta, were put into the collagen gel plates before the gel solidified at 37°C. Samples were cultured in a 5% CO2 incubator at 37°C for 1-2 weeks before photography.

RESULTS

Embryonic expression of the Smad5 gene
The expression of Smad5 in embryonic and adult tissues was examined by northern blots (Fig. 1A). Smad5 transcripts were detected throughout embryogenesis, and in all of the adult tissues examined. Section in situ hybridization revealed that Smad5 was expressed in the mesenchyme of E8.5 embryos (Fig. 1B). At E10.5 Smad5 message was enriched in the somites and mesenchyme (Fig. 1D). Transcripts of Smad5 were also detected in the endothelium (Fig. 1C), while nucleated blood cells exhibited signal at a lower level (not shown).

Targeted disruption of the Smad5 gene
To study the function of SMAD5, a targeting construct, pSmad5neo (Fig. 2A), was used to disrupt the Smad5 gene in embryonic stem (ES) cells. This construct contains a 12-kb Smad5 genomic sequence interrupted in exon 6 with a pLoxpneo cassette (Yang et al., 1998). The mutation truncated 179 amino acids from the C-terminal end of SMAD5, including the receptor binding domain, the L3 loop (Lo et al., 1998) and the phosphorylation site (Kretzschmar et al., 1997; Macias-Silva et al., 1996). These domains are essential for the function of pathway-specific SMADs as shown previously (reviewed in Heldin et al., 1997). The targeted mutation, therefore, should create a candidate null allele at the Smad5 locus. Of 82 G418/FIAU doubly resistant ES clones screened by Southern blots, 16 were found to be correctly targeted (Fig. 2B). Two targeted ES clones were injected into C57BL/6 blastocysts, from which germline transmission of the mutant Smad5 allele was obtained. Smad5 chimeras were backcrossed with C57BL/6 or intercrossed with Black Swiss. Comparable phenotypes were found from mice in both genetic backgrounds. Northern analysis of RNA isolated from mutant embryos failed to detect any Smad5 transcript (Fig. 2C). The probe used was an 882-bp fragment containing nucleotides 478-1359 of mouse Smad5 cDNA (Yingling et al., 1996). The lack of hybridization in the mutant embryos indicates that there was little or no Smad5 message in our mutant mice. This was further substantiated by RT-PCR using primers that flank the exon 6 where the neo gene was inserted (Fig. 2D). Again no Smad5 transcript was seen in the mutant embryos, suggesting that the targeting resulted in a candidate null mutation. The analysis also revealed that there was no compensatory increase in Smad1 message (Fig. 2C), which might have been expected because Smad1 and Smad5 share a high degree of homology.

Loss of SMAD5 results in embryonic lethality at E10.5-E11.5
Mice heterozygous for the Smad5 mutant allele (Smad5<sup>ex6/+</sup>) appeared phenotypically normal. However, genotyping of 176 offspring from heterozygous intercrosses detected no homozygous mutant mice (Smad5<sup>ex6/ex6</sup>), indicating that the Smad5 mutation was a recessive embryonic lethal (Table 1). To
Bam Asp 718/ exhibited a restriction fragment shift from 10 to 12 kb upon blot analysis of DNA isolated from parental ES cells (lanes 1, 2 and mutant (lane 2) embryos derived from intercrosses of Bam flanking probe. Restriction sites are as follows: As, by arrows. The short line below the targeted allele represents the 3 neo and after targeting. Transcriptional directions of exon 6 of the pLoxPneo targeting construct contains a 12-kb genomic sequence with a  

At E8.5, both the mutants and their littermates had expanded 

Disrupted angiogenesis in Smad5 ex6/ex6 yolk sacs 

At E8.5, both the mutants and their littermates had expanded yolk sacs. Blood islands were found in mutant yolk sacs (not shown). Blood islands are formed de novo from the condensation of mesoderm, and their presence suggested that the initiation of vasculogenesis was largely unaffected despite the absence of SMAD5. As vasculogenesis proceeds, the peripheral cells in the blood islands flatten, differentiate into endothelial cells, and assemble into a primitive capillary plexus. This is followed by angiogenesis, in which new vessels are formed from pre-existing ones. At E9.5, control yolk sacs developed a well-formed vascular network full of blood cells (Fig. 3A). Capillary-like vessels were seen between the mesothelial and endodermal layers of the yolk sac (Fig. 3C). In contrast, the mutant yolk sacs were generally pale, with blood cells scattered in some regions. No distinct blood vessels were evident (Fig. 3B). Histological sections revealed large cavities lined by endothelial cells (Fig. 3D). These observations suggest that loss of SMAD5 blocked angiogenesis, leading to the death of Smad5ex6/ex6 embryos.

We next visualized the vascular endothelium in the developing yolk sacs using an antibody to platelet endothelial cell adhesion molecule (pECAM), a marker for endothelial cells. A well-organized network of blood vessels was seen in wild-type yolk sacs (Fig. 3E), which was not apparent in the mutants (Fig. 3F). Labeled yolk sacs were sectioned for histological analysis. Endothelial cells were found between the endodermal and mesothelial cells in both control (Fig. 3G) and mutant (Fig. 3H) yolk sacs, suggesting that the failure to form blood vessels in mutant yolk sacs was not due to a lack of endothelial cells.

**Smad5ex6/ex6** yolk sacs contain hematopoietic progenitor cells capable of erythroid differentiation

The discovery of Smad5ex6/ex6 embryos with pale yolk sacs prompted us to examine the function of SMAD5 during embryonic hematopoiesis. This is also of interest because SMAD5 was identified as a potential tumor suppressor in human myeloid leukemia (Hejlik et al., 1997; Zavadil et al., 1997). We analyzed the hematopoietic progenitors by an in vitro yolk sac hematopoietic progenitor colony assay. Equal numbers of resuspended E9.5 yolk sac cells from mutants and controls were cultured in methylcellulose supplemented with various growth factors (see Materials and methods) to assay for hematopoietic differentiation. Cells from representative colonies of each genotype were prepared by cytocentrifugation and analyzed under the microscope. There were no morphological differences detected in Smad5ex6/ex6-derived cells compared to those from controls (data not shown). However, twice as many myeloid colony-forming units (CFU-GM) were found from Smad5ex6/ex6 homozygous mutant yolk sacs compared to those from wild-type yolk sacs (Fig. 4), suggesting that Smad5 may affect the differentiation of yolk sac progenitors to the myeloid lineage. In contrast, no change was seen in the number of erythroid precursors (CFU-Ery; Fig. 4). This suggests that the loss of Smad5 did not affect the ability of hematopoietic precursors to differentiate into the erythroid lineage. It is not yet clear whether this change in the myeloid precursors reflects an increased ability of more primitive progenitors to differentiate into the myeloid lineage, or whether the myeloid cells have an increased ability to proliferate.
Developmental defects in Smad5<sup>ex6/ex6</sup> embryos

From E8-E8.25, there is no obvious difference between mutants and their littermates (not shown). At E8.5, however, Smad5<sup>ex6/ex6</sup> embryos were smaller than their littermate controls (wild type and Smad5<sup>ex6/+</sup>). Some of the homozygotes showed delayed fusion between the allantois and chorion (not shown). At E9.5 normal embryos had finished turning, while about 75% of the mutant embryos had not done so (Fig. 5A,B). Mutant embryos were retarded in development, and many exhibited a lateral spreading of the ventral mesoderm (Fig. 5A). The remaining mutants were able to turn normally (Fig. 5B). At E10.5, 75% of the mutants were much smaller than their littermate controls, were arrested midway in the turning process, and exhibited multiple abnormalities (Fig. 5C). 15% of the mutant embryos were completely resorbed, while the remainder (<10%) had relatively normal body structures (Fig. 5D). All the mutant embryos failed to develop a forebrain, and many exhibited abnormal accumulation of blood cells in various areas (arrowheads in Fig. 5B-D).

Smad5<sup>ex6/ex6</sup> embryos were further characterized by whole-mount in situ hybridization with several lineage markers. Staining for Otx2, which is located in forebrain and midbrain, confirmed the lack of forebrain in Smad5<sup>ex6/ex6</sup> embryos (arrowheads, Fig. 5E), although the midbrain appears unaffected. Staining for Fgf8, a marker for the isthmus, showed that the boundary between the mid- and hindbrain was also intact (Fig. 5F). Severely affected mutant embryos exhibited reduced expression of HoxD3 in the presumptive spinal cord (Fig. 5G, H), whereas mutant embryos more advanced in development did

### Table 1. Phenotype and genotype analysis of offspring from Smad5 heterozygous intercross

<table>
<thead>
<tr>
<th>Age</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Resorption</th>
<th>Genotype</th>
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<tr>
<td>E8.5</td>
<td>35</td>
<td>2</td>
<td>1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+/+</td>
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<tr>
<td>E9.5</td>
<td>79</td>
<td>22</td>
<td>2&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>E10.5</td>
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<td>13</td>
<td>5</td>
<td>+/-</td>
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<tr>
<td>E11.5</td>
<td>40</td>
<td>2</td>
<td>5</td>
<td>+/-</td>
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<tr>
<td>Postnatal</td>
<td>176</td>
<td>0</td>
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<sup>*</sup>These were not genotyped due to the difficulties of separating the embryos from the maternal tissues.

#### Fig. 3. Smad5<sup>ex6/ex6</sup> embryos exhibit defects in yolk sac angiogenesis. E9.5 yolk sacs isolated from sibling controls (left) or Smad5<sup>ex6/ex6</sup> embryos (right). (A,B) Whole-mount view of E9.5 yolk sacs. Large blood vessels can be seen in the control yolk sac (arrowhead) but are missing in the mutant. (C,D) Histological sections of yolk sacs showing a lack of normal vasculature in the Smad5<sup>ex6/ex6</sup> mutant. Endothelial cells (arrowhead in D) can be seen in both control and mutant yolk sacs. (E,F) Whole-mount staining with pECAM antibody. Note the lack of an organized vascular network in the mutant. (G,H) Histological sections of yolk sacs stained with an anti-pECAM antibody showing the presence of endothelial cells in the mutant (arrow). Bar, 253 μm (A,B); 43 μm (C,D); 78 μm (E,F); 41 μm (G,H).

#### Fig. 4. Increased number of Smad5<sup>ex6/ex6</sup> mutant hematopoetic precursors in vitro. Smad5<sup>ex6</sup> animals were intercrossed and progeny from nine litters analyzed, which included 15 mutant embryos. Hematopoetic precursor cells were isolated from mutant and sibling yolk sacs by collagenase digestion. Equal numbers of these were plated in methyleneblue and colonies were counted 7 days later. CFU-GM, myeloid colony-forming units; CFU-mix, mixed erythroid-myeloid colonies; CFU-ery, erythroid colony-forming units.
is shown in (D). (E-I) E9.5 embryos stained with left. Bar, 191 μm (C-F); 92 μm (G,H). Forebrain, which is missing in the mutants. In I the mutant is on the right, while mutants are shown on the right in (A-F). The arrowheads show abnormal pools of blood cells in (B-D). (A) E9.0 embryos. Note the lack of turning and the lateral spreading of the mesoderm in the most severely affected embryos (arrow). (B) E9.5 embryos. The arrow points out the lack of forebrain. (C,D) E10.5 embryos. The Smad5 ex6/ex6 embryo shown in (C) exhibits extreme distortion in the posterior region of the embryo (arrow), and resembles some Bmp-4 mutants. A less affected Smad5 ex6/ex6 embryo is shown in (D). (E-I) E9.5 embryos stained with Otx2 (E), Fgf8 (F), HoxD3 (G,H) and Shh (I). The arrowheads in E point to the forebrain, which is missing in the mutants. In I the mutant is on the left. Bar, 191 μm (A,B); 239 μm (C-F); 92 μm (G,H); 200 μm (I).

Abnormal vascularization observed in Smad5 ex6/ex6 embryos

In addition to the vascular defects seen in the yolk sacs, the Smad5 ex6/ex6 mutant embryos also exhibited pronounced malformation of blood vessels. Throughout the embryos, enlarged vessel-like structures could be seen in place of the normal vasculature (Fig. 6A,B,G,H). In addition, there was an almost complete lack of mesenchyme in the most affected embryos (Fig. 6B,D,H). The dorsal aorta, which could be seen adjacent to the somites, was misshapen in the Smad5 ex6/ex6 embryos, and could actually be seen invading into the ventral region of the somites themselves (Fig. 6C,D). Layers of endothelial cells were often found to be dissociated from mesenchymal cells in the Smad5 ex6/ex6 embryos, whereas the endothelial cells in control embryos tightly adhered to their surrounding mesenchymal cells (Fig. 6E,F). A misshapen dorsal aorta (Fig. 6I) and enlarged blood vessels (Fig. 6K,L) could also be seen in a less severely affected mutant that had retained much of its mesenchyme (Fig. 6I). In addition, these embryos often exhibited accumulation of blood cells both inside and outside blood vessels (Fig. 6K,L).

We examined the embryonic vascular network with whole-mount staining for the pECAM antibody, referred to earlier. The primitive vascular plexus had permeated the developing brain of E8.5 control and mutant embryos, which were not distinguishable at this stage (not shown). At E9.5 the vascular network had undergone angiogenesis and remodeling to form a branched and intricate tree-like network in control embryos (Fig. 7A). In contrast, Smad5 ex6/ex6 mutants exhibited a simplified and abnormal vascular network (Fig. 7B). These embryos were analyzed further by serial sectioning. Enlarged and misshapen blood vessels could be seen throughout mutant embryos (Fig. 7C,D). All of these blood vessels were lined with a layer of endothelial cells. These observations were confirmed by staining with an anti-CD34 antibody, another marker for the differentiated endothelial cells (not shown), demonstrating that SMAD5 is not required for the differentiation of endothelial cells.

To ascertain whether or not there was a defect in embryonic angiogenesis, we subjected mutant embryos to an in vitro angiogenesis assay (Vu et al., 1998). Wild-type endothelial cells were plated together with pieces of mutant or sibling embryos containing dorsal aorta in a collagen gel. Sibling control embryos were able to direct the organization of the endothelial cells into a network of tubes radiating away from the embryo (Fig. 7E). However, the Smad5 ex6/ex6 mutant embryos were unable to support this organization of endothelial cells, which grew in a disorganized fashion (Fig. 7F), indicating an angiogenic defect in these embryos.

We further analyzed the supporting cells of the dorsal aorta in both mutant and control embryos using an antibody against smooth muscle α-actin, a marker for vascular smooth muscle cells. At E10.5, the endothelial cells of the wild-type dorsal aorta were surrounded by a well-organized layer of smooth muscle cells (Fig. 7G). In contrast, a thinner disorganized smooth muscle layer could be seen in the Smad5 ex6/ex6 mutants (Fig. 7H). This observation was extended to E11.5 mutants as well (not shown).

Reduced mesenchyme in Smad5 ex6/ex6 embryos is accompanied by massive apoptosis

Histological analysis showed that most Smad5 ex6/ex6 embryos had necrotic cells restricted to particular regions and lacked mesenchyme generally. We therefore visualized the pattern of cell death in mutant embryos in whole-mount images and tissue sections. The Smad5 ex6/ex6 mutants showed massive apoptosis in head mesenchyme by whole-mount staining (Fig. 8A,B). We have also seen apoptosis in the somites, which was not (not shown). Both HoxD3 and Fgf8 are expressed normally in the limb buds, which were present in even the most severely affected embryos (Fig. 5G,H). Shh was also expressed normally in the Smad5 ex6/ex6 mutants (Fig. 5I). The expression of several other genes, including Bmp4, Lim1, T, HoxB9, Pax3, Mox1 and others (not shown) was also unaffected.

Fig. 5. Smad5 ex6/ex6 mice lack a forebrain and exhibit defects in morphogenesis of the posterior region of the embryos. Controls are on the left, while mutants are shown on the right in (A-F). The arrowheads show abnormal pools of blood cells in (B-D). (A) E9.0 embryos. Note the lack of turning and the lateral spreading of the mesoderm in the most severely affected embryos (arrow). (B) E9.5 embryos. The arrow points out the lack of forebrain. (C,D) E10.5 embryos. The Smad5 ex6/ex6 embryo shown in (C) exhibits extreme distortion in the posterior region of the embryo (arrow), and resembles some Bmp-4 mutants. A less affected Smad5 ex6/ex6 embryo is shown in (D). (E-I) E9.5 embryos stained with Otx2 (E), Fgf8 (F), HoxD3 (G,H) and Shh (I). The arrowheads in E point to the forebrain, which is missing in the mutants. In I the mutant is on the left. Bar, 191 μm (A,B); 239 μm (C-F); 92 μm (G,H); 200 μm (I).
appears to be most prevalent in the scleratome (Fig. 8C-E). To confirm the loss of scleratal cells, we examined the somites of Smad5<sup>ex6/ex6</sup> embryos with several markers of somitic lineages. Pax-3 stains the dermamyotome, as well as the dorsal neural tube, and is expressed in the Smad5<sup>ex6/ex6</sup> mutants (Fig. 8G,J). On the other hand, transcripts of Pax-1, which is a scleratoma-specific marker, were markedly reduced in the Smad5<sup>ex6/ex6</sup> embryos (Fig. 8H,K), confirming the dramatically reduced scleratome lineages. These results were verified by whole-mount in situ hybridization (not shown). We have shown earlier that Smad5<sup>ex6/ex6</sup> embryos lacked mesenchyme (Fig. 6B,D,H). It is conceivable that the apoptosis found in the mesenchymal cells accounts for this defect. This result is all the more noteworthy because the pattern of apoptotic cell death is coincident with the expression pattern of Smad5 shown earlier (Fig. 1D).

**DISCUSSION**

We have disrupted the Smad5 gene and found that the Smad5<sup>ex6/ex6</sup> embryos died at E10.5-11.5, primarily due to yolk sac defects. Analysis of the mutants revealed a lack of yolk sac vasculature, and in vitro analysis of mutant yolk sac hematopoiesis revealed a higher number of myeloid hematopoietic precursors. Further examination showed that the dilated embryonic blood vessels were surrounded by reduced smooth muscle cells. The embryos also showed apoptosis of the mesenchyme, often leading to a nearly complete loss of this cell type in the most severely affected individuals. These observations suggest that SMAD5 plays an essential role during vascular development.

**Defective angiogenesis in Smad5<sup>ex6/ex6</sup> embryos**

Our analysis of the Smad5<sup>ex6/ex6</sup> embryos revealed that both the yolk sac and embryo can initiate vasculogenesis, as the yolk sac can form blood islands and the embryo can form a vascular plexus. However, no blood vessels are seen in the yolk sac, suggesting a failure of yolk sac angiogenesis. Mutant embryos were challenged in an in vitro angiogenesis assay in which they were unable to direct the organization of endothelial cells, indicating that the abnormalities in the vasculature of embryos were also due to an angiogenic defect.

Angiogenesis requires extensive interactions of endothelial cells with themselves, and extracellular matrix, pericytes or smooth muscle cells (reviewed in Folkman and D’Amore,

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**Fig. 6.** Histological analysis of Smad5<sup>ex6/ex6</sup> mutants. Embryos shown are either sibling controls (left) or Smad5<sup>ex6/ex6</sup> embryos (right). (A,B) Parasagittal section of E9.5 embryos. Note the lack of mesenchyme in the mutant. (C,D) Enlargement of the boxed areas in A and B showing the abnormal dorsal aorta in the mutant. Note the intrusion of the dorsal aorta into the somites (arrowhead in D). (E,F) Enlargement showing the dissociation of endothelium (arrowhead) away from the mesenchyme (arrow) in mutant. (G,H) Cross sections of E9.5 embryos, showing enlarged blood vessels (arrowheads) and lack of mesenchyme in the mutant. (I,J) Dorsal aorta (arrows) in E10.5 control (I) and a less severely affected mutant (J). The mutant dorsal aorta is misshaped. (K,L) Enlargement of the boxed areas in I and J showing abnormal accumulation of blood cells in the Smad5<sup>ex6/ex6</sup> mutant permeating the tissues (arrowheads). Bar: 368 μm (A,B); 210 μm (C,D); 34 μm (E,F); 222 μm (G,H); 228 μm (I,J); 99 μm (K,L).
We found that the abnormal blood vessels observed in the Smad5\textsuperscript{ex6/ex6} mutants have an increase in their lumen, such that they are many times the size of normal blood vessels. Histological analysis revealed that the layer of endothelial cells was dissociated from mesenchymal cells in mutant embryos, suggesting that the interaction between the endothelial and mesenchymal cells may be affected. Many Smad5\textsuperscript{ex6/ex6} embryos suffered a complete loss of mesenchymal cells; however, we were able to recover a sizeable number of less severely affected embryos and examine the supporting cells surrounding the dorsal aorta. This type of Smad5\textsuperscript{ex6/ex6} embryo exhibited a less pronounced loss of mesenchyme, formed a normal antero-posterior axis, and proceeded further in development. Although the blood vessels are considerably reduced in size compared with the more severely affected embryos, they are still enlarged and display a decrease in the thickness of the smooth muscle cell layer surrounding them, indicating that the differentiation of mesenchymal cells into smooth muscle cells was impaired in Smad5\textsuperscript{ex6/ex6} mutants. The weakened vessel walls may have hemorrhaged, leading to the accumulation of blood cells which were seen in many Smad5\textsuperscript{ex6/ex6} embryos.

Notably, we found that the Smad5\textsuperscript{ex6/ex6} embryos exhibited phenotypes similar to those of TGF-\(\beta\)1 and TGF-\(\beta\) type II receptor knockouts (Dickson et al., 1995; Oshima et al., 1996). All these mutants died at same time of gestation (E10.5) due to very similar defects in the yolk sacs. TGF-\(\beta\)1 mutant embryos also suffer increased cell death in the head mesenchyme and have dilated blood vessels in the embryo.

Fig. 7. The Smad5\textsuperscript{ex6/ex6} mutants exhibit defects in angiogenesis. Sibling controls are shown on the left, while Smad5\textsuperscript{ex6/ex6} mutants are shown on the right. (A,B) At E9.5, the wild-type vascular plexus undergoes angiogenesis and remodeling to form a tree-like blood vessel network (A, arrowhead), while the mutant shows disorganized blood vessels in the brain region (B, arrowhead). (C,D) Histological sections through the embryos in A and B showing abnormal blood vessels in the limb bud. Arrowheads point to endothelial cells. (E,F) In vitro angiogenesis assay. Sibling control (E) or mutant (F) embryos were plated together with endothelial cells in a collagen gel. The control embryos were able to direct the endothelial cells to form a network (arrowheads), whereas the mutant embryos were unable to do so. (G,H) Staining for smooth muscle \(\alpha\)-actin reveals an attenuated layer of smooth muscle (arrowhead) surrounding the mutant dorsal aorta (da). Bar, 127 \(\mu\)m (A,B); 131 \(\mu\)m (C,D); 299 \(\mu\)m (E,F); 198 \(\mu\)m (G,H).

Fig. 8. Smad5\textsuperscript{ex6/ex6} mutants exhibit increased apoptosis of mesenchymal cells. (A-C) Whole-mount staining of apoptotic cells with Nile Blue Sulfate. Siblings exhibit little staining in the head (A) or somites (not shown), while the mutants have a large number of apoptotic cells (arrowheads) in the head (B) and somites (C). (D,E) TUNEL staining of histological sections. Controls show few apoptotic cells in the somites (D), while apoptosis is prevalent in the sclerotome of the mutants (E). (F-K) Section in situ analysis of somitic lineages in E9.5 sibling control embryos (F-H) and mutants (I-K). F and I are Hematoxylin-stained sections, while G and J are adjacent sections labeled with Pax3. H and K are labeled with Pax-1. Arrowheads in H and K point out the sclerotomal region, which is missing in the mutant (K). Bar, 91 \(\mu\)m (A-C); 117 \(\mu\)m (D,E); 148 \(\mu\)m (F-K).
proper (Dickson et al., 1995; Fig. 2), which have also been shown in Smad5ex6/ex6 embryos. TGF-β1 is expressed in the endothelial and mesenchymal cells (Akhurst et al., 1990), and is believed to mediate interactions between these two types of cells during angiogenesis (reviewed in Folkman and D’Amore, 1996; Pepper, 1997). In this study, we show that the Smad5 expression is highly coincident with the expression of TGF-β1 and TGF-β type II receptor (Lawler et al., 1994) in both cell populations. However, similarities between these mutants need to be interpreted cautiously because the majority of previous investigations placed Smad5 in the BMP, and not in the TGF-β signaling pathway (Kawabata et al., 1998; Suzuki et al., 1997). Thus, although our experiment raises a possibility that Smad5 may mediate TGF-β signals during angiogenesis, further experiments are needed to clarify this.

**Smad5ex6/ex6 embryos exhibit increased levels of mesenchymal apoptosis**

The more severely affected mutants consisted of animals in which mesenchymal apoptosis was severe, resulting in a nearly complete loss of mesenchymal cells. These embryos exhibited extremely enlarged and abnormal blood vessels, which filled the embryonic interior. We feel that the severity of the angiogenesis defect in these very severely affected embryos was a secondary consequence of the mesenchymal apoptosis. If the mesenchymal cells were absent they could not communicate any angiogenic signals to the endothelium, differentiate into vascular support cells, or physically support the blood vessels.

The mesenchymal apoptosis itself is likely a direct consequence of the loss of Smad5, and not secondary to the yolk sac defects. The regions of cell death correlate very well with the areas in which Smad5 itself is found. The apoptosis was most concentrated in the mesenchyme of the head and somites (Fig. 8A-E), where we saw the highest expression of Smad5 (Fig. 1D). In addition, a number of murine mutants have been examined that lack normal vasculature in the yolk sac or embryo, but do not exhibit a similar pattern of mesenchymal apoptosis. The more severely affected mutants consisted of animals in which Smad5 ex6/ex6 embryos exhibit increased levels of mesenchymal apoptosis.

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**Role of SMAD5 during hematopoiesis**

Smad5ex6/ex6 yolk sacs appeared pale because the erythroid cells were evenly distributed throughout the yolk sac, instead of being concentrated in blood vessels. There is also a possibility that these precursors were unable to differentiate in the Smad5ex6/ex6 yolk sacs into the erythroid lineage. However, if this is the case, our analysis would suggest that this failure to differentiate was not due to an intrinsic defect in the mutant precursors, but rather to a flaw in their environment due to the angiogenic defect in the mutant yolk sacs, as we were able to isolate normal numbers of erythroid progenitor cells from Smad5ex6/ex6 yolk sacs (Fig. 4). However, the mutant yolk sacs yielded an increased number of myeloid progenitors. This is of interest, given that Smad5 has been suggested as a candidate tumor suppressor gene for myeloid leukemia (Hejlik et al., 1997; Zavadil et al., 1997). Further study will be needed to address the functions of Smad5 in the differentiation and proliferation of hematopoietic cells, and to determine if Smad5 is indeed a tumor suppressor gene.

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