

Hedgehog signaling is essential for endothelial tube formation during vasculogenesis

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Summary

During embryonic development, the first blood vessels are formed through the aggregation and subsequent assembly of angioblasts (endothelial precursors) into a network of endothelial tubes, a process known as vasculogenesis. These first vessels generally form in mesoderm that is adjacent to endodermal tissue. Although specification of the angioblast lineage is independent of endoderm interactions, a signal from the endoderm is necessary for angioblasts to assemble into a vascular network and to undergo vascular tube formation. In this study, we show that endodermally derived sonic hedgehog is both necessary and sufficient for

vascular tube formation in avian embryos. We also show that Hedgehog signaling is required for vascular tube formation in mouse embryos, and for vascular cord formation in cultured mouse endothelial cells. These results demonstrate a previously uncharacterized role for Hedgehog signaling in vascular development, and identify Hedgehog signaling as an important component of the molecular pathway leading to vascular tube formation.

Key words: Vasculogenesis, Tubulogenesis, Endoderm, VEGF, Mouse, Chick

Introduction

The formation of a functional vascular system is a prerequisite for embryonic survival and organogenesis. The initial step in this process, termed vasculogenesis, involves (1) the specification of angioblasts within mesodermal tissues, (2) the proliferation and coalescence of these angioblasts into cords, and (3) the formation of endothelial tubes that fuse to form a continuous network. With few exceptions, subsequent growth and elaboration of the embryonic vascular system occurs via angiogenesis, a process that entails the branching and reorganization of existing vessels (Wilting and Christ, 1996). Angiogenesis is the exclusive source of new blood vessels in the adult organism. In avian embryos, discrete angioblasts are initially detected shortly after the onset of somite formation. Over a period of ~6 hours, these angioblasts generate a polygonal network of angioblast cords at the precise locations of the future endothelial tubes (Coffin and Poole, 1988). In a process that is still poorly characterized, the cords of angioblasts then form lumens to generate the complex tubular network that corresponds to the original vascular plexus. The process of vascular tube formation, which usually involves two or more endothelial cells, initiates with the appearance of 'clear, slit-like spaces' between angioblasts (Houser et al., 1961). The expansion of these slits into a lumen appears to involve the formation of intracellular vacuoles that coalesce with other vacuoles and fuse with the cell membrane to enlarge

the enclosed space between the cells (Clark and Clark, 1939; Folkman and Haudenschild, 1980; Davis and Camarillo, 1996; Meyer et al., 1997; Lubarsky and Krasnow, 2003). In the avian embryo, the process of vascular tubulogenesis occurs over a period of ~4 hours (Hirakow and Hiruma, 1981). Thus, the formation of the entire primary vascular system in the avian embryo takes ~10 hours, from the first appearance of angioblasts to the creation of a complex tubular network. Although these morphological processes are not as completely characterized during murine development, they appear largely similar to those described in the avian embryo (Drake and Fleming, 2000).

Vasculogenesis occurs at two distinct embryonic locations during development. In extra-embryonic tissues, angioblasts initially appear in blood islands in the splanchnic mesoderm adjoining the extra-embryonic endoderm in the posterior half of the embryo. In contrast to extra-embryonic angioblasts, which form structures that are closely associated with blood cells, intra-embryonic angioblasts are only rarely associated with blood cells (Cormier and Dieterlen-Lièvre, 1988; Olah et al., 1988; Jaffredo et al., 1998; Ciau-Uitz et al., 2000). In avian embryos, the first intra-embryonic angioblasts, which form slightly later than the extra-embryonic angioblasts, are visible as discrete cells at bilateral sites near the headfolds that correspond to the future endocardium (at two somites), and slightly later at the lateral edges of the anterior intestinal

portal (Coffin and Poole, 1988; Sugi and Markwald, 1996). Angioblasts subsequently become visible throughout broad regions of the embryo proper. Although the extra-embryonic and intra-embryonic vessels will ultimately form a continuous vascular network, each area develops independently of the other (Hahn, 1909; Miller and McWhorter, 1914; Reagan, 1915). At present, it is not known if extra and intra-embryonic angioblasts are specified by the same mechanism, or whether different genetic pathways regulate their formation.

Although the morphological events underlying vascular cord formation and endothelial tubulogenesis have been described in some detail, less is known about the signaling pathways involved in vascular development. One of the most important signaling molecules involved in early blood vessel development is vascular endothelial growth factor A (hereafter VEGF), which acts through its high-affinity receptor VEGFR2 (FLK1/KDR). VEGF activity is essential for the formation of blood vessels, and embryos lacking either VEGF or VEGFR2 develop few (or no) angioblasts and die early in development (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). In various contexts, VEGF has been shown to act as a potent mitogen (Keyt et al., 1996; Wilting et al., 1996; Park et al., 1993), chemoattractant (Waltenberger et al., 1994; Cleaver and Krieg, 1998; Ash and Overbeek, 2000) and survival factor (Gerber et al., 1998a; Gerber et al., 1998b). Additionally, the proper regulation of VEGF is crucial for the formation of normal endothelial channels (Fong et al., 1995; Fong et al., 1999; Drake et al., 2000). Fibroblast growth factors (FGFs) have also been implicated as proliferative agents during vascular development; however, the large number of FGF ligands, and the early lethality of knockout embryos have impeded research into the developmental roles of individual family members (Javerzat et al., 2002). During subsequent vascular development, additional molecules are important for promoting the maturation of specified endothelial cells into a patent vascular system, for conferring arterial or venous identity, and for recruitment of the vascular smooth muscle layer of blood vessels (for reviews, see Lawson and Weinstein, 2002; Vokes and Krieg, 2002b). However, although known signaling pathways are essentially linked to endothelial cell specification and proliferation, the specific signaling pathways required for vascular tubulogenesis have not been identified (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003).

Within the embryo, the initial specification of angioblasts in the mesoderm is independent of tissue interactions with other germ layers (Vokes and Krieg, 2002a). However, the first blood vessels within the embryo always form in mesoderm that is in close proximity to endoderm (Mato et al., 1964; Wilt, 1965; Gonzalez-Crussi, 1971; Mobbs and McMillan, 1979; Meier, 1980; Kessel and Fabian, 1985; Pardanaud et al., 1989). Furthermore, we have recently demonstrated that a signal originating from the endoderm is essential for the assembly of angioblasts into tubes (Vokes and Krieg, 2002a). We present evidence that sonic hedgehog (SHH) signaling by the vasculogenic endoderm plays a central role in organizing specified angioblasts into vascular tubes. SHH is the first growth factor identified that specifically regulates vascular tube formation.

Materials and methods

In situ hybridization and immunocytochemistry

Whole-mount in situ hybridization with digoxigenin-labeled antisense probes were carried out using standard protocol. In situ hybridization to sections was performed as described (Grapin-Botton et al., 2001). Quail endothelial cells were detected with the QH1 monoclonal antibody (Developmental Studies Hybridoma Bank). Embryos were stained using previously described methods (Sugi and Markwald, 1996), except that embryos were blocked in 5% normal donkey serum and a donkey anti-mouse Texas Red-conjugated IgG secondary antibody (Jackson ImmunoResearch) was used at a concentration of 3 µg/ml. Immunostained sections were generated by embedding previously stained embryos in 30% gelatin and fixing overnight in 5% paraformaldehyde. Specimens were then vibratome sectioned at 40 µm and imaged using deconvolution microscopy. To detect co-localized QH1 and *ptc1* expression, in situ hybridization was performed on 10 µm paraffin sections, and the embryos were post-fixed in 4% paraformaldehyde for 20 minutes prior to QH1 antibody staining using standard conditions. Whole-mount mouse embryos were fixed with 4% PFA/PBS and assayed with antibodies to VEGFR2 (PharMingen Avas 12α1) at 0.3 µg/ml and PECAM (PharMingen Mec 13.3) at 0.5 µg/ml. Embryos were then incubated with biotinylated anti-rat secondary at 0.25 µg/ml (Vector Laboratories) and ABC reagent (Vector Laboratories). Staining was visualized with diaminobenzidine (DAB) substrate. After viewing and imaging stained embryos as wholemounts, they were embedded in paraffin wax and sectioned at 10 µm.

Embryology

Avian embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Stage 5 (late gastrula) quail embryos (unless otherwise specified) were placed on plastic rings and endoderm was removed from one half of the embryo using tungsten needles. No enzymatic treatment was used with stage 5 embryos, but older embryos were dissected in media containing 0.01% trypsin, which was subsequently inactivated with 0.02% trypsin inhibitor. Embryos were then incubated as New Cultures (New, 1955) at 37°C until the appropriate stage (usually 7-8 somites) (see Fig. 1A,B). When necessary, heparin acrylic beads (Sigma) were implanted immediately after endoderm removal. In these experiments, heparin beads were rinsed in PBS and soaked for 1 hour or more in the appropriate concentration of growth factor, on ice. The beads were then briefly rinsed in PBS before being implanted in embryos. For Hedgehog inhibition experiments, embryos at 1-2 somites were incubated as New cultures immersed in DMEM containing 0.5% ethanol and 100 µm cyclopamine (Toronto Research Chemicals) or DMEM containing 0.5% ethanol for controls and incubated at 37°C in 95% oxygen until approximately the eight-somite stage. Mouse embryos were harvested between E8 and E8.5, and were a combination of 129/SvJ outbred with Swiss Webster.

Cell culture

The mouse endothelial cell lines bEnd3 (Montesano et al., 1990) and EOMA (Obeso et al., 1990) were obtained from ATCC and cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS (Hyclone), penicillin and streptomycin. For treatment studies, bEnd3 cells were plated at 3×10^4 cell/cm². When the cells had reached confluence the media was switched to serum-free DMEM and rm-SHH-N (mouse recombinant N-terminal fragment, R&D Systems) or rhuman VEGF (R&D Systems) added at the indicated concentrations and incubated for 72 hours. To determine proliferation, cells were pulsed with 10 µm BrdU for 2 hours, fixed in methanol and stained with FITC-anti BrdU (Roche). Cells were then counterstained with propidium iodide. Random fields and more than a 1000 total nuclei for each condition were counted.

RT-PCR

Chick embryos at the five-somite stage were placed on New Culture rings and submerged in 0.01% trypsin. To obtain endodermal tissue, the intra-embryonic endoderm was removed from both halves of the embryo using tungsten needles. Mesodermal tissue samples were obtained by then stripping the exposed somites and lateral plate mesoderm from the ectoderm. The notochord was excluded from this sample. Total RNA was extracted from these tissues, and from whole chick embryos using TRIzol® (Invitrogen). For cultured cells, total RNA was isolated using guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). Precipitated RNA was treated with DNase to remove residual genomic DNA and phenol-chloroform extracted prior to the generating cDNA. The number of cycles for each primer pair was empirically determined to be in the linear range of amplification.

Primers

Chick embryos

GAPDH, (forward) 5'CAGGTGCTGAGTATGTTGTGGAGTC3' and (reverse) 5'TCTTCTGTGTGGCTGTGATGGC3' (Tm=62°C); SHH, (forward) 5'ATCTCGGTGATGAACCAGTGGC3' and (reverse) 5'TTTGACGGAGCAGTGGATGTGC3' (Tm=58°C); VEGFA (core sequence common to all isoforms), (forward) 5'CAAATCCTGGAAGTCTACGAACG3' and (reverse) 5'AATTCTTGCATCTCATCTGTG3' (Tm=62°C).

Mouse cell lines

GAPDH, (forward) 5'CAGTATGACTCCACTCACGG3' and (reverse) 5'GTGAAGACACCAGTAGACTCC3' (166 bp); patched 1, (forward) 5'CTGCTGCTATCCATCAGCGT3' and (reverse) 5'AA-GAAGATAAGAGGACAGG3'; smoothened, (forward) 5'GTGATGATGAGCCCAAGAGA3' and (reverse) 5'AGGGGCAGAGTGGTGAAGC3' (422 bp); VEGFR2, (forward) 5'GCCCTGAGTCTCAGGAC3' and (reverse) 5'GGGTCTCCACGCAGAACC3' (344 bp).

VEGFA (three PCR product are produced by these primers [766bp (VEGF₁₈₈), 644bp (VEGF₁₆₅) and 512bp (VEGF₁₂₀)], (forward) 5'GCGGGCTGCCTCGCAGTC3' and (reverse) 5'TCACCGCCTTG-GCTTGTAC3' (Marti and Risau, 1998).

Results

An endodermal signal is required immediately prior to, or during, endothelial tube formation

In previous studies, we have demonstrated that a signal arising from the embryonic endoderm is essential for vascular tubulogenesis (Vokes and Krieg, 2002a). As a preliminary step towards molecular characterization of this signal, we carried out experiments to determine the time at which endoderm signaling is required for vascular assembly. Quail embryos were used for these experiments, because removal of the single-cell thick endodermal layer is readily achieved and because the QH1 antibody can be used to detect vascular precursor cells (Pardanaud et al., 1987). Endoderm was removed from half of the three-somite embryo (Fig. 1A), at which stage numerous angioblasts have differentiated, but have not yet begun to assemble into vascular cords (Coffin and Poole, 1988; Sugi and Markwald, 1996). When manipulated embryos were assayed for vascular tube formation ~8 hours later, at the eight-somite stage (Fig. 1B), no vascular tubes were observed on the side lacking endoderm, although abundant unassembled angioblasts were present (12/12 embryos; Fig. 1D). By contrast, the unmanipulated control side showed the presence of an extensive network of tubular vessels (Fig. 1C). We conclude from this

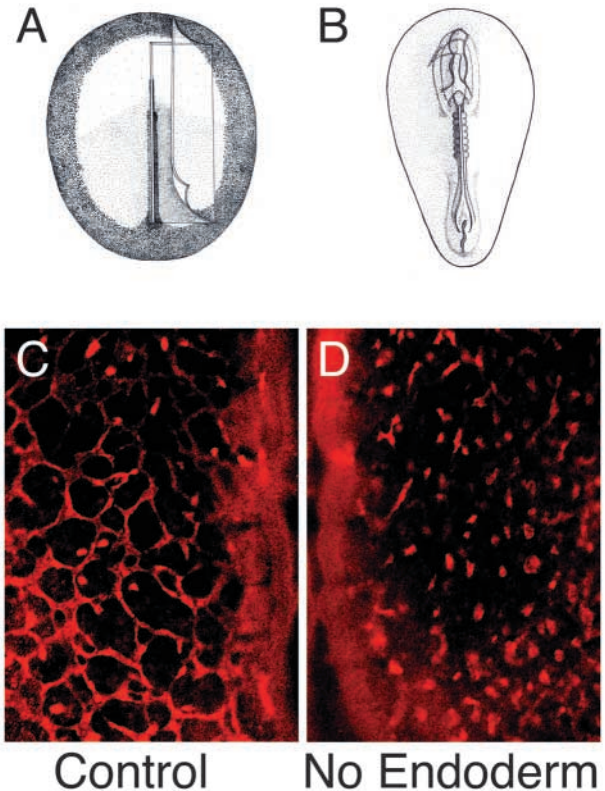


Fig. 1. (A) Endoderm was removed from one half of stage 5 quail embryos with the unmanipulated half serving as an internal control. (B) The embryos were then assayed at the seven- to eight-somite stage, after tube formation had occurred, using whole-mount staining with QH1 antibody and subsequent sectioning and analysis with deconvolution microscopy. (C) Endoderm was removed from one half of the embryo (typically at stage 5, but at three somites in this example) with the other half serving as a control. (D) As shown by QH1 staining of vascular cells in the eight-somite embryo, removal of endoderm results in failure of vascular assembly and tube formation.

experiment that the endodermal signal is acting immediately prior to, or during, vascular tube formation.

Shh is expressed in endoderm at the time of tube formation

The signaling factors that may be required for blood vessel tubulogenesis are currently unknown. Therefore, we used an RT-PCR approach to examine the expression of candidate growth factors in both the mesodermal and endodermal layers of chick embryos at the time when tube formation commences (five somites). Tissue samples were limited to the intra-embryonic region of the embryo, and did not include axial tissues, which express high levels of many different growth factors. Of the growth factor sequences examined, only *Shh* was present in the endoderm and absent from the mesoderm (Fig. 2A; data not shown). Sequences corresponding to Indian hedgehog (IHH) were not detected at significant levels in either the mesodermal or endodermal tissue samples at this stage (data not shown), and the chick ortholog of Desert hedgehog has not yet been reported. Transcripts encoding the essential vasculogenic growth factor, VEGF were also detected in the

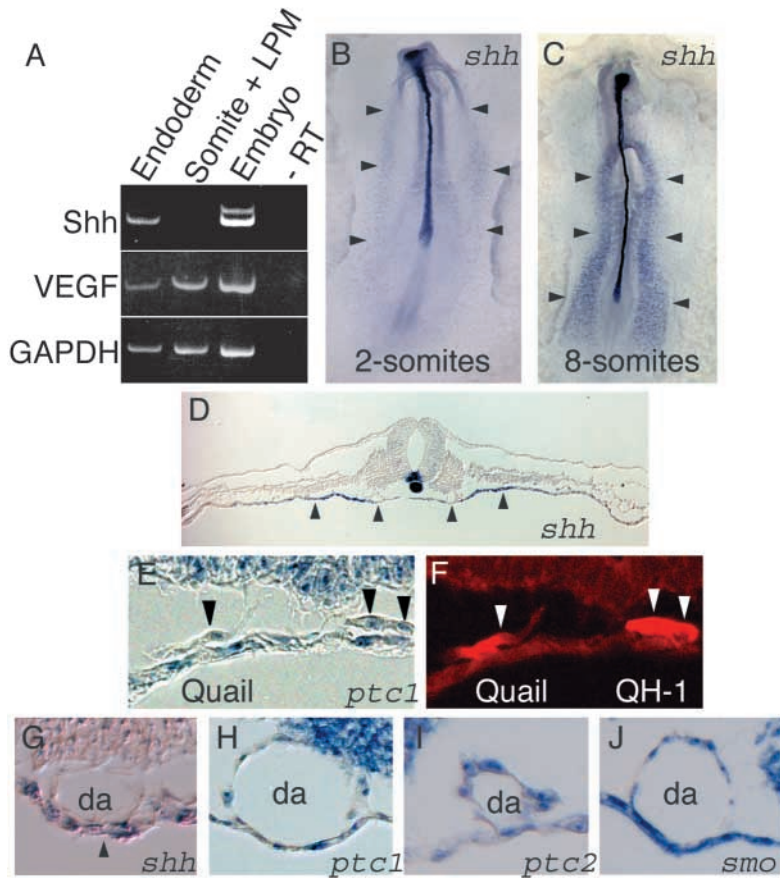


Fig. 2. Expression of Hedgehog signaling components correlates with vascular assembly. (A) RT-PCR analysis of five-somite chick tissues, showing presence of *Shh* in the endoderm and absence in non-axial mesoderm. VEGF is present in both the mesoderm and endoderm. (B,C) *Shh* in situ hybridization on a two-somite chick embryo showing low levels of endodermal expression (indicated by arrowheads) and an eight-somite chick embryo showing greatly increased endodermal staining (arrowheads). (D) Transverse section through an eight-somite chick embryo showing *Shh* expression in the endoderm (arrowheads). (E,F) In situ hybridization analysis of a six-somite quail embryo with *Ptch1* (bright field) and QH-1 (fluorescence) showing that *Ptch1* is present in angioblasts that have not yet formed tubes (arrowheads). (G-J) Transverse sections through seven-somite chick embryos analyzed by in situ hybridization. The expression of *Shh* in the endoderm (G, arrowhead), but not in the dorsal aorta (da). (H-J) The Hedgehog receptors *Ptch1* and *Ptch2*, and the transducer *Smo* are present in endothelial cells of seven-somite embryos.

endoderm, but were present in the mesoderm in higher amounts (Fig. 2A).

Endodermal expression of SHH has been described at later stages (ten somites) (Roberts et al., 1995), but not at this earlier time. In situ hybridization revealed that in addition to the well-characterized domain of expression in the notochord and floor plate of the neural tube, *Shh* is also detected at low levels in the lateral endoderm of two- and three-somite embryos (Fig. 2B). At the four-somite stage, expression intensifies and expands to include almost all embryonic endoderm, a pattern that persists throughout early development (Fig. 2C,D). Hedgehog ligands signal by binding to the patched (PTCH1) receptor, thereby relieving repression on the Hedgehog transducer smoothened (SMO) (reviewed by Ingham and McMahon, 2001). In addition, *Ptch1* and a related gene, *Ptch2*, are transcriptional targets of SHH signaling. We have carried out additional in situ assays in the chick embryo which show that the Hedgehog signaling components *Ptch1*, *Ptch2* and *Smo* are expressed in mesodermal tissues, including angioblasts (Fig. 2E-J). These data suggest that angioblasts not only express essential components of the signaling pathway, but are actively responding to a Hedgehog signaling input.

Inhibition of hedgehog signaling leads to disruption of vascular assembly

To determine whether Hedgehog signaling may play a role in regulating embryonic vasculogenesis, quail embryos were treated with cyclopamine, a highly specific inhibitor of SMO (Incardona et al., 1998; Taipale et al., 2000; Chen et al., 2002),

from the two-somite until the eight-somite stage. This is the period during which most vascular tubulogenesis occurs in the untreated embryo (Hirakow and Hiruma, 1983). Analysis of blood vessel formation by QH1 immunofluorescence showed that all embryos treated with 100 μ M cyclopamine exhibited vascular abnormalities (13/13). These alterations ranged from the presence of small, interrupted tubes, with a corresponding increase in unassembled clusters of angioblasts, to instances where virtually no discernible tubular structures were detected (Fig. 3B). In the latter case, angioblasts remained abundant and were located in the regions where blood vessel formation would normally occur. By contrast, vascular development was mostly normal in control embryos treated with carrier solution alone (Fig. 3A). Defects were observed in a small proportion of carrier treated embryos (2/9), but the extent of abnormalities

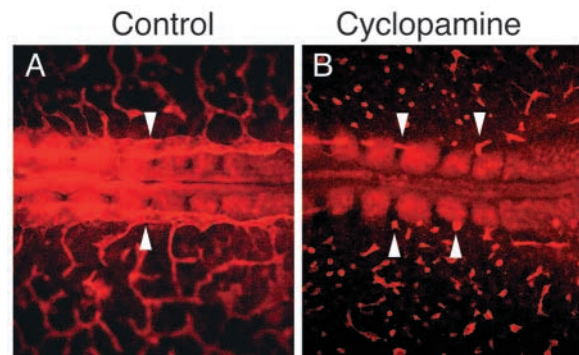


Fig. 3. Hedgehog signaling is essential for vascular assembly. Quail embryos treated with 100 μ M cyclopamine from the two-somite until the eight-somite stage (B) show severe deficiencies in vascular assembly when assayed with the endothelial cell specific antibody, QH-1. Dorsal aortae formation (arrowheads) is not present and there is almost complete lack of vascular assembly when compared with embryos treated with control media (A).

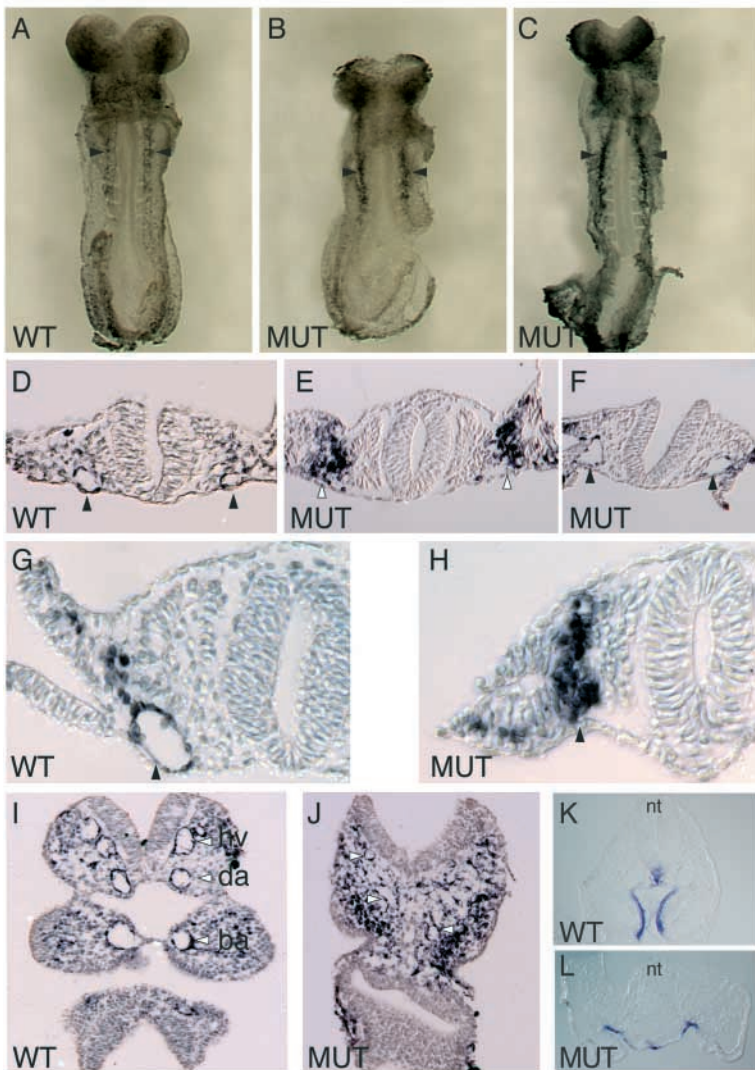


Fig. 4. Mouse embryos lacking SMO have severe defects in vascular tube formation. Vascular cells are visualized using an antibody that recognizes VEGFR2. *Smo* mutant embryos fail to form anterior dorsal aortae, although abundant VEGFR2-positive angioblasts are still present. (A,D) Wild-type embryo at four-somites contains patent dorsal aortae (arrowheads). (B,C,E) Mutant embryos (four to eight somites) show no apparent anterior dorsal aortae, either in wholemount embryos (arrowheads in B,C) or section (E, arrowheads show numerous angioblasts, but no dorsal aortae). (F) Posterior section through embryo depicted in E shows that dorsal aortae in mutant embryos (indicated by black arrowheads) appear histologically normal at the caudal end of the embryo. (G) Transverse section through additional wild-type and (H) mutant embryos showing complete lack of a patent dorsal aorta (arrowheads) in the mutant embryo. Transverse sections through branchial arch of wild-type (I) and mutant (J) sibling embryos at 11 somites showing severe vascular defects. The mutant embryo contains only a few, very poorly formed, endothelial tubes (white arrowheads), while an equivalent section through a wild-type embryo shows well-formed blood vessels (white arrowheads), including the primary head vein (hv), dorsal aorta (da) and first branchial arch artery (ba). Both sections are approximately 90 μ m anterior to the start of the heart. (K,L) In situ hybridization for *Shh* expression on wild-type (K) or mutant (L) embryos at approximately eight somites showing equivalent expression of *Shh* in endoderm.

observed, and the proportion of embryos showing defects, was similar to that observed in unmanipulated embryos maintained under New Culture conditions (data not shown). These experiments demonstrate that inhibiting hedgehog signaling prevents angioblasts from undergoing normal vascular assembly and tube formation.

Mouse *Smo* mutants exhibit severe defects in vascular tube formation

Smoothed (SMO) is an essential component of the Hedgehog signaling pathway. To determine whether SMO is required for the formation of the primary vascular network, we examined *Smo* mutant embryos between the four- and 12-somite stage [prior to embryonic arrest (Zhang et al., 2001)], focusing on the paired dorsal aortae, which are the first prominent vessels to form within the mouse embryo. All mutant embryos (12/12) contained defects in dorsal aorta tube formation, while wild-type stage-matched littermates exhibited normal vasculature and patent tubes (9/9 embryos examined). In the most severe *Smo*^{-/-} mutants (7/12), the anterior half of the dorsal aorta was completely absent, although abundant angioblasts were still present (compare Fig. 4A,D with 4B,C, E and Fig. 4G with 4H).

In less extreme cases (5/12), very small tubes formed on one or both sides of the embryo. Interestingly, all later stage *Smo* mutants (six somites and later) contain severely defective or absent dorsal aortae in the anterior two-thirds of the embryo (Fig. 4E), but exhibit detectable tubular dorsal aortae in the posterior third of the embryo (Fig. 4F shows a caudal section through same embryo shown in 4E). As the formation of the dorsal aortae occurs bi-directionally, from both the rostral and caudal ends in the mouse embryo (Drake and Fleming, 2000), our observations suggest that

Hedgehog signaling is required for tubulogenesis of a distinct anterior domain of the dorsal aorta. To confirm that the defects in vascular tube formation were not caused by a deficiency of angioblasts, we quantitated the VEGFR2-positive cells in cross-sections of *Smo*^{-/-} embryos lacking dorsal aortae. Stage-matched wild-type and mutant embryos contained 14.3 or 26.5 angioblasts, respectively, on one side of the embryo (average of three adjacent sections from three different embryos). This indicates that failure of vascular tube formation is not caused by a reduction in angioblast number. Although we focused our analysis on the dorsal aortae, vascular tube formation in many other areas in the anterior embryo, including the plexus in the cephalic mesenchyme and the aortic arches, also exhibited missing or defective tube formation (compare Fig. 4I with 4J; data not shown). Although *Smo*^{-/-} embryos are incapable of responding to hedgehog signaling, previous studies have indicated that endodermal development is morphologically normal in these animals (Zhang et al., 2001). In addition, we show that the endoderm of the mutant embryos still exhibits normal sonic hedgehog expression (compare Fig. 4K with 4L). It seems likely, therefore, that the failure of vascular tube formation in mutant embryos results from lack of hedgehog

signaling to angioblasts, rather than being an indirect consequence of altered endoderm development.

SHH rescues vascular patterning in endodermless embryos

The cyclopamine experiments in avian embryos strongly suggest that Hedgehog signaling is required in the pathway leading to vascular assembly and tubulogenesis. To learn more about the action of SHH, we carried out bead implantation experiments to determine whether addition of SHH protein is sufficient to rescue vascular assembly and tubulogenesis in embryos lacking endoderm. As an initial control, quail embryos from which endoderm had been removed were implanted with beads carrying buffer alone. Small clusters of angioblasts were observed in the vicinity of the control beads, but vascular assembly and tubulogenesis did not occur (1/12), and the aggregates of angioblast cells lacked any discernible lumen as assayed by deconvolution microscopy (compare Fig. 5A,B with 5C,D,E). When beads soaked in SHH (3 $\mu\text{g}/\mu\text{l}$) were added to quail embryos from which the endoderm had been removed, well-formed blood vessels were produced in the majority of cases (11/15). These were typically linear vessels that formed in close proximity to the surface of the bead (Fig. 5F). Examination of transverse sections through these vessels indicated that they did indeed contain lumens (Fig. 5G). The effect of SHH on vascular assembly is not due to a general, non-specific effect of growth factor stimulation, as no appreciable vascular tube formation was observed when beads carrying BMP4, FGF-2 or TGF β 1 were added to endodermless embryos (data not shown). As shown in Fig. 2A, VEGF transcripts are present at significant levels in the embryonic endoderm. It is possible that removal of this tissue is sufficient to drop VEGF levels below a threshold required for vascular tube formation. We therefore tested whether addition of this potent vascular mitogen was sufficient to bring about vascular tubulogenesis in endodermless embryos. As expected, when beads soaked in VEGF at varying concentrations (1 ng/ μl , 10 ng/ μl and 100 ng/ μl) were added to embryos, a dramatic increase in angioblast number was observed and these cells formed into broad sheets of QH1-positive tissue surrounding the bead. The primary difference between the different VEGF doses was the radius over which angioblast proliferation was observed. In no case did VEGF treatment lead to formation of vascular tubes or to organization of a vascular network (0/32 embryos; Fig. 5H,I). Thus, VEGF does not substitute for endodermal tissues in the induction of tubulogenesis. However, when VEGF and SHH are combined (beads soaked in 100 ng/ μl and 3 mg/ μl respectively), endodermless embryos formed a robust vascular plexus (6/7 embryos) that more closely resembled the wild-type vascular network than SHH treatment alone (compare Fig. 5J,K with Fig. 5A,B).

Addition of SHH to endothelial cells in culture promotes vascular assembly

The preceding experiments have addressed the role of Hedgehog in promoting vascular tube formation from nascent angioblasts. To determine whether Hedgehog signaling could function similarly in mature endothelial cells, we first asked if cultured endothelial cells express Hedgehog signaling components. Both *Ptch1* and the *Smo* were detected by RT-PCR in two separate mouse endothelial cell lines, bEnd3 and

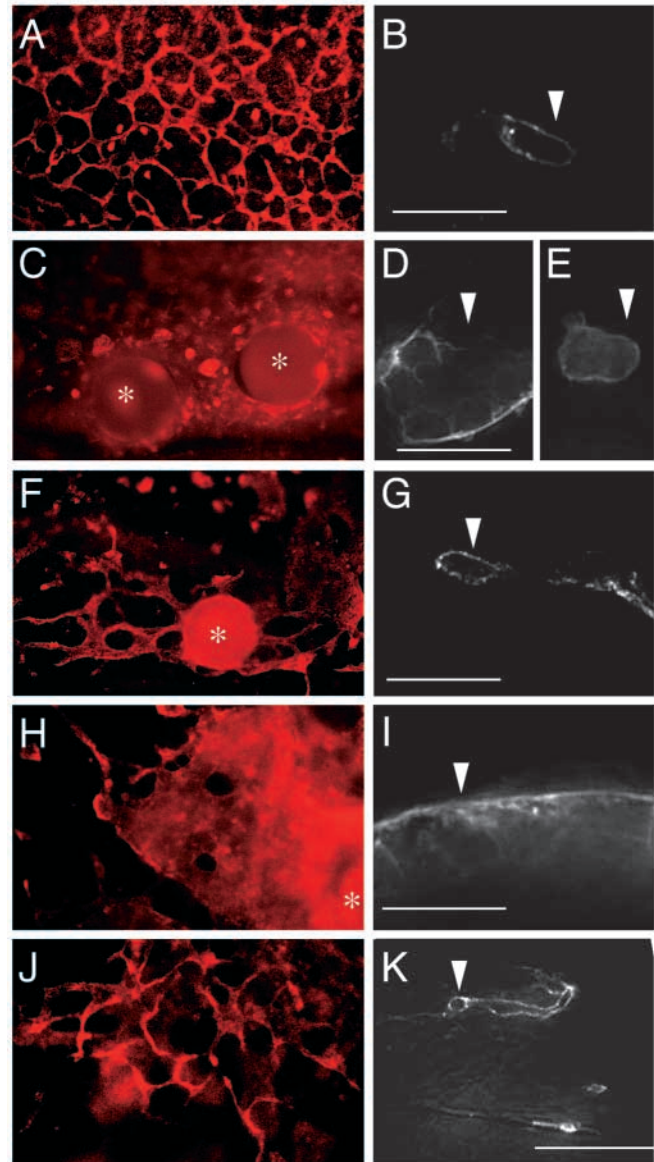
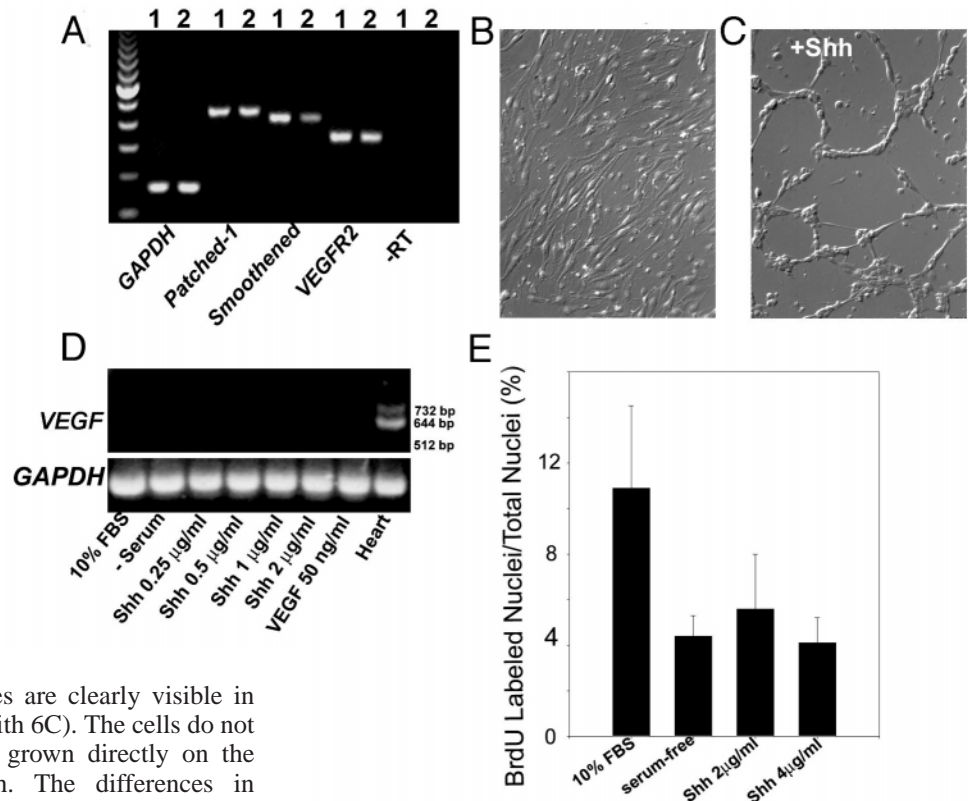


Fig. 5. Sonic hedgehog (SHH) signaling is sufficient to rescue vascular assembly in the absence of endoderm. The panels on the left show a whole-mount view of QH-1 stained quail embryos, while those on the right show deconvolution microscope sections through the same or similar embryos. (A,B) The control side of embryos contains a robust vascular plexus with patent tubes (arrowhead in B). (C-E) By contrast, the side lacking endoderm contains unassembled clusters of angioblasts that do not contain a lumen (arrowheads in D,E; asterisks indicate control beads). (F,G) Beads containing SHH rescue vascular assembly and tube formation (arrowhead in G). (H,I) VEGF causes massive endothelial cell proliferation, but is not able to rescue vascular assembly or tubulogenesis. QH-1-positive cells form apparent sheets of tissue (arrowhead in I). (J,K) When both SHH and VEGF beads are added (not in field of vision), a robust tubular vascular plexus is formed. The arrowhead in K indicates a longitudinal section through a connecting blood vessel. Scale bar: 15 μm .

Eoma (Fig. 6A). To determine whether SHH can regulate endothelial cell morphology in culture, 2 $\mu\text{g}/\text{ml}$ of SHH was added to confluent bEnd3 cells in serum-free media. After 72

Fig. 6. Vascular assembly in cell culture in response to exogenous SHH. (A) RT-PCR analysis detects *Ptch1*, *Smo* and *Vegfr2* transcripts in bEnd3 cells (lane 1) and Eoma cells (lane 2). (B) Confluent bEnd3 cells were switched to serum-free DMEM or (C) DMEM plus 2 $\mu\text{g}/\text{ml}$ of SHH. SHH induces assembly of vascular endothelial cells into tube-like structures. (D) RT-PCR analysis of *Vegf* transcripts. The cDNA was synthesized from untreated and treated bEND3 cultures 48 hours after the addition of SHH or VEGF. cDNA synthesized from adult mouse heart total RNA was used as a positive control. (E) bEnd3 cells were assayed for proliferation by BrdU labeling, following addition of SHH at concentrations indicated. Data were averaged from three separate experiments. Addition of SHH does not increase endothelial cell proliferation.



hours, vascular network-like structures are clearly visible in SHH-treated cells (compare Fig. 6B with 6C). The cells do not form patent tubes, because they are grown directly on the plastic surface of the culture dish. The differences in morphology cannot be attributed to an upregulation of VEGF, as there is no detectable VEGF expression in these cells (Fig. 6D). Furthermore, the assembly of a vascular network after SHH treatment is not caused by an increase in cell number because there is no stimulation of cellular proliferation when SHH is added to the culture media (Fig. 6E). Thus, SHH exerts a direct influence on the morphological properties of endothelial cells, independently of cell proliferation.

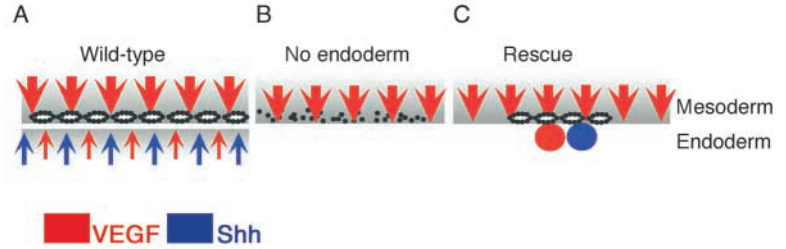
Discussion

Although tubulogenesis has been studied in some detail, particularly in the formation of tracheal tubes in *Drosophila* and during mammalian kidney tube development, very little is known of the mechanisms that control the process of vascular tube formation (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). In this study, we present multiple lines of evidence, indicating that SHH is an important factor regulating blood vessel assembly and tubulogenesis. First, SHH is expressed in endodermal tissues, immediately adjacent to the developing vascular network, but not in the mesodermal tissue itself. This is consistent with previous studies indicating that a signal originating in the endoderm is required for vascular tube formation (Vokes and Krieg, 2002a). Second, angioblasts, nascent endothelial tubes and cultured endothelial cells express Hedgehog transducing molecules, and are therefore capable of responding to Hedgehog signaling. Third, the loss or inhibition of Hedgehog signaling results in a dramatic reduction in vascular assembly in the mouse, and completely eliminates vascular tube formation in avian embryos. Finally, the implantation of beads containing SHH into endodermless avian embryos is sufficient to rescue vascular tube formation, and addition of SHH to cultured endothelial cells causes the formation of vascular network-like structures.

Collectively, our findings provide compelling evidence in birds and mammals that SHH, which is produced by the endoderm, is an important regulator of vascular tube formation from specified angioblasts. This conclusion is strengthened by several previous reports that also implicate SHH signaling in vascular tube formation. Murine embryoid bodies derived from ES cells lacking *Smo* initially express endothelial cell markers but fail to form endothelial enclosed blood islands (Byrd et al., 2002), and an initial examination of *Smo* mutant embryos reported that extra-embryonic yolk sac vessels were poorly formed and greatly reduced in number (Byrd et al., 2002). Zebrafish *Shh* mutant embryos contain angioblasts but do not form vascular tubes in the trunk region of the embryo (Brown et al., 2000), and overexpression of SHH by injection of *Shh* mRNA, causes the formation of lumenized ectopic vessels (Lawson et al., 2002). Transgenic mouse embryos ectopically expressing *Shh* in the neural tube display hypervascularization (Rowitch et al., 1999), and treatment of mouse embryonic neurectoderm explants with IHH is reported to respecify explants to form tissues containing blood vessels (Dyer et al., 2001). Finally, in culture, the addition of SHH causes endothelial cells to assemble into capillary networks (Kanda et al., 2003). Our demonstration of a specific requirement for Hedgehog signaling in intra-embryonic vascular assembly and tubulogenesis significantly extends our understanding of Hedgehog action in the developing vascular system.

Our analysis of the *Smo*^{-/-} phenotype indicates that the intra-embryonic vascular phenotype is more severe than that previously described for the yolk sac (Byrd et al., 2002), suggesting that development of intra-embryonic blood vessels is in some respects distinct from that of the extra-embryonic vasculature. This is not

Fig. 7. Model for vascular assembly. (A) In the unmanipulated embryo, both the mesodermal and endodermal layers contribute VEGF, although the mesodermal contribution is greater (as indicated by the larger red arrows). The presence of VEGF leads to the proliferation of angioblasts (black dots) in the mesodermal layer. In combination with SHH signaling from the endoderm (blue arrows), angioblasts assemble into a vascular network with patent tubes. (B) When endoderm is experimentally removed from embryos, angioblasts are



specified, but are unable to organize into vascular tubes. (C) The addition of SHH beads (blue circles) to endodermless embryos is sufficient to rescue vascular tubulogenesis. When VEGF beads (red circles) are added in combination with SHH beads, a more complete vascular plexus is formed, suggesting that VEGF from the endoderm (small red arrows) is also important for vascular development.

altogether unexpected, as the first extra-embryonic blood vessels are derived from endothelial encased blood islands while intra-embryonic vessels are derived from aggregations of individual angioblasts. However, based on the severe intra-embryonic vascular phenotype of the *Smo*^{-/-} mutants, it is surprising that mice lacking SHH signaling do not exhibit obvious vascular defects (Chiang et al., 1996). Our results suggest that this is probably the result of functional redundancy of SHH with IHH, which also signals through the SMO pathway and which appears to be expressed in an overlapping pattern in the intra-embryonic endoderm of the mouse embryo (Zhang et al., 2001). Finally, we note that unlike avian embryos treated with cyclopamine, mouse embryos that lack Hedgehog signaling still form a limited number of vascular tubes. This implies that an alternative, Hedgehog-independent pathway is sufficient for vascular tube formation in certain regions of the mouse embryo. The nature of this alternative pathway is currently unknown.

SHH has also been implicated as an indirect angiogenic factor in the postnatal mouse. In assays examining corneal angiogenesis and surgically induced hindlimb ischemia, addition of SHH resulted in a significant increase in angiogenesis. SHH is believed to act indirectly in these experiments by upregulating VEGF and angiopoietins 1 and 2 (Pola et al., 2001). Likewise, the microinjection mRNA encoding *Shh* into zebrafish embryos caused an upregulation of VEGF in the somites (Lawson et al., 2002). Our studies strongly suggest that a simple epistatic relationship between SHH and VEGF is unlikely to account for vascular tube formation. First, cyclopamine inhibition of SHH signaling completely eliminates vascular tubulogenesis in the avian embryo (Fig. 3), even in the presence of abundant VEGF in the endodermal and mesodermal layers (Fig. 2A; data not shown). Second, the addition of SHH alone is sufficient to mediate efficient tubulogenesis in endodermless embryos. This is in contrast to administration of VEGF alone, which results in the proliferation and adhesion of angioblasts, but never causes detectable vascular tube formation. Third, our data show that Hedgehog receptor components are expressed in angioblasts (Fig. 2E,H,I,J) and in cultured endothelial cells (Fig. 6A), consistent with a mechanism in which SHH acts directly upon endothelial cells to initiate the tubulogenesis pathway. Fourth, we observe that addition of both VEGF and SHH facilitates the formation of a more extensive plexus that closely resembles a wild-type network (compare Fig. 5A,F,J), again suggesting that SHH is not merely required to upregulate VEGF levels. Finally, we find that cultured endothelial cells respond to Hedgehog signaling by aggregating into vascular cords (Fig. 6C). During this process, there is no detectable VEGF expression in the

cultured cells (Fig. 6D), and no increase in cellular proliferation (Fig. 6E). Overall, these results strongly suggest that SHH signaling acts independently of VEGF to mediate tube formation; however, we do not exclude the possibility that SHH plays an additional role in upregulating VEGF expression under certain circumstances. In Fig. 7, we present a model in which SHH operates in concert with VEGF to promote normal vasculogenesis. In this model Hedgehog signaling is the crucial factor for initiating the tubulogenesis pathway, as no vascular tubes are formed in the absence of SHH activity. The model also indicates that VEGF, which originates in both mesodermal and endodermal tissues, is necessary for the proliferation of normal numbers of angioblasts. The idea that VEGF levels must be maintained within a narrow range for normal vascular development is illustrated by a recent study of yolk-sac vasculogenesis in mouse embryos carrying a targeted mutation of *Vegf* expression in the visceral endoderm, but not the adjacent mesodermal tissue. These embryos showed defects in yolk sac blood vessel formation, demonstrating that VEGF originating from the mesodermal layer alone is not sufficient for normal yolk sac vasculogenesis (Damert et al., 2002). Our data suggests that VEGF may also function to promote cell-cell interactions. This latter point is evidenced by the formation of the extended sheets of angioblasts following treatment with VEGF alone (Fig. 5H,I).

Current models for tubulogenesis indicate that three general cellular processes are involved. The first is increased contact between the individual cells that will constitute vascular cords. The second is the establishment of apical basal polarity, which appears to be an absolute prerequisite for the formation of cellular tubes (Lubarsky and Krasnow, 2003). Finally, interactions between angioblasts and the surrounding extracellular matrix are believed to play important roles in tube formation (Vernon et al., 1995; Drake et al., 1998). Several cell adhesion molecules have been described in the developing vascular system, including PECAM (CD31), cadherin 5 (VE-cadherin) and N-cadherin. While the *Pecam* knockout is viable with no discernible vascular phenotype (Duncan et al., 1999), cadherin 5 mutants show a severe vascular phenotype that is embryonic lethal. However, these embryos still form a significant number of endothelial tubes, indicating that other molecules, such as N-cadherin may also be involved in cell adhesion (Gory-Fauré et al., 1999; Carmeliet et al., 1999). The establishment of apicobasal cell polarity is also thought to play an integral role in the formation of cellular tubes (Lubarsky and Krasnow, 2003). For example, during the epithelial tubulogenesis of MDCK cell in response to hepatocyte growth factor/scatter factor, cells transiently lose cell polarity, but this is restored prior to lumen formation (Pollack et

al., 1998). To the best of our knowledge, there are no markers of apicobasal polarity in embryonic blood vessels, and so the role of cell polarity during vascular tubulogenesis remains completely uncharacterized. Interactions between angioblasts and the extracellular matrix (ECM) are also thought to play a crucial role in vascular tube formation. An increasing body of evidence implicates interactions between integrins, primarily $\alpha_5\beta_1$ and fibronectin as being crucial for this process. This evidence is based, in part, on the observation that some murine embryos lacking fibronectin contain angioblasts, but fail to form endothelial tubes (George et al., 1997; Francis et al., 2002). In addition, the injection of blocking antibodies to the β_1 integrin causes a failure of tube formation in chick embryos (Drake et al., 1992).

Given the sparse knowledge of the events leading to tubulogenesis, the precise role played by SHH remains speculative. One possibility is that SHH functions in promoting a specific type of cell adhesion. This is consistent with models in *Drosophila* proposing a Hedgehog-mediated cell adhesion pathway that controls cell segregation in the wing imaginal disc (Dahmann and Basler, 2000). Alternatively, given the evidence that SHH signaling within mouse dental epithelial cells is required for their polarization (Gritli-Linde et al., 2002), SHH signaling may be required to establish apicobasal polarity in angioblasts prior to lumen formation. Clearly, the identification of cell polarity markers in angioblasts would greatly facilitate studies to further elucidate the cellular mechanisms underlying vascular tube formation.

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