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Endoderm is required for vascular endothelial tube formation, but not for angioblast specification

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SUMMARY

Angioblasts, the precursor cells that comprise the endothelial layer of blood vessels, arise from a purely mesodermal population. Individual angioblasts coalesce to form the primary vascular plexus through a process called vasculogenesis. A number of reports in the literature suggest that signals from the adjacent endoderm are necessary to induce angioblast specification within the mesoderm. We present evidence, using both embryological and molecular techniques, indicating that endoderm is not necessary for the induction of angioblasts. *Xenopus* embryos that had endoderm physically removed at the onset of gastrulation still express vascular markers. Furthermore, animal caps stimulated with bFGF form angioblasts in the absence of any detectable endodermal markers. These results show that endoderm is not required for the initial formation of angioblasts. While *Xenopus* embryos lacking endoderm contain aggregates of angioblasts, these angioblasts fail to assemble into endothelial tubes. Endothelial tube formation can be rescued, however, by implantation of endodermal tissue from sibling embryos. Based on these studies in *Xenopus*, and corroborating experiments using the quail embryo, we conclude that endoderm is not required for angioblast specification, but does play an essential role in the formation of vascular tubes.

Key words: Vasculogenesis, Tubulogenesis, Endoderm, Induction, *Xenopus laevis*

INTRODUCTION

The primary network of blood vessels in the embryo is formed by the process of vasculogenesis, which is defined as the de novo formation of blood vessels by the aggregation of individual angioblasts. At a fundamental level, the first step in vasculogenesis involves the specification of endothelial cell precursors (angioblasts) from mesoderm. Subsequently, the angioblasts proliferate and coalesce into cords that then form continuous strands of endothelial cells. These cells then form tubular vascular structures. The process of tube formation is initiated when a 'slit-like' space opens up between two angioblasts. These spaces enlarge, combining with other such spaces to form a hollow endothelial tube (Houser et al., 1961). At least in some cases, lumen formation precedes the formation of a continuous endothelial network (Drake and Jacobson, 1988); reviewed elsewhere (Risau and Flamme, 1995; Wilting and Christ, 1996; Roman and Weinstein, 2000; Vokes and Krieg, 2002). Subsequent elaboration of the vascular network occurs via angiogenesis, which is the growth and extension of vessels from the pre-existing vascular network [for a recent review, see Carmeliet (Carmeliet, 2000)].

A number of signaling pathways are known to play regulatory roles during embryonic vasculogenesis. At the earliest stages of vascular development, the VEGF signaling pathway is essential for blood vessel formation (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). The VEGF ligand is bound by two high-affinity receptors, VEGFR2 (Flk-1/KDR) and VEGFR1 (Flt-1), both of which belong to the tyrosine kinase receptor family. Flk-1 is expressed exclusively in vascular endothelial cells, and represents the earliest known specific marker of endothelial cells. In addition to its role as a mitogen, VEGF also acts as a chemoattractant for endothelial cells (Waltenberger et al., 1994; Cleaver and Krieg, 1998; Ash and Overbeek, 2000), and is also involved in the correct assembly of endothelial cells into lumenated vessels (Drake et al., 2000). Ablation of VEGF expression results in an almost complete block to vascular development (Carmeliet et al., 1996; Ferrara et al., 1996). On the other hand, expression of excess VEGF ligand in the embryo results in both hypervascularization and formation of abnormally large vascular lumens (Drake and Little, 1995; Flamme et al., 1995; Cleaver et al., 1997). Following the formation of the original vascular network, numerous other growth factor signaling pathways are involved in the subsequent remodeling and maturation of the vascular system (reviewed by Yancopoulos et al., 2000).

In amniotes, the formation of primary vascular networks occurs in two distinct regions. Extraembryonic vasculogenesis is observed in the yolk sac blood islands, while intraembryonic vasculogenesis occurs within the developing embryo itself. Classical embryological experiments have demonstrated that

formation of the two vascular systems is not developmentally linked, since assembly of the intraembryonic vascular network is completely independent of extraembryonic vasculogenesis (Hahn, 1909; Miller and McWhorter, 1914; Reagan, 1915). On the other hand, in organisms such as teleosts (bony fishes) and amphibians, all vasculogenesis occurs intraembryonically (Stockard, 1915). A major difference between extraembryonic angioblasts and intraembryonic angioblasts lies in their organization. Extraembryonic angioblasts originate in blood islands, containing an outer layer of endothelial cells and an inner layer of red blood cells. In contrast, intraembryonic endothelial precursors are almost always first observed as solitary angioblasts (Risau, 1995) and these can arise in any mesodermal tissue in the embryo with the exception of the prechordal mesoderm (Noden, 1989; Wilms et al., 1991; Wilting et al., 1995). Only in certain specific, rare, instances are these intraembryonic angioblasts closely associated with blood cells (Cormier and Dieterlen-Lièvre, 1988; Olah et al., 1988; Jaffredo et al., 1998; Ciau-Uitz et al., 2000). Based on the remarkable ability of diverse mesodermal tissues to form angioblasts, it appears that the tissue environment in and around a specific region of mesoderm is responsible for regulating vascular endothelial cell specification and commitment (Noden, 1989; Pardanaud et al., 1989; Pardanaud and Dieterlen-Lièvre, 1999; Cox and Poole, 2000). Although both intraembryonic and extraembryonic angioblasts are of mesodermal origin, the different environments in which they arise and the differences in the fate of associated cells raises the possibility that the two populations may be specified by different mechanisms.

At present, the precise origin of the embryonic angioblast lineage is uncertain. Numerous anatomical studies have shown that angioblasts in the extraembryonic blood islands, and also in the earliest intraembryonic blood vessels, arise in close proximity to endoderm (Mato et al., 1964; Gonzalez-Crussi, 1971; Mobbs and McMillan, 1979; Meier, 1980; Kessel and Fabian, 1985; Pardanaud et al., 1989). Based on these observations, it was proposed (Wilt, 1965) that direct interactions between the endoderm and mesoderm might be required for angioblast induction, and this possibility has been investigated in a number of different studies carried out using the avian embryo (Wilt, 1965; Miura and Wilt, 1969; Pardanaud et al., 1989; Pardanaud and Dieterlen-Lièvre, 1993). In chick tissue culture experiments, when specific portions of the area vasculosa that form the extraembryonic blood islands were separated into the mesectodermal and endodermal components, the mesectodermal component failed to generate detectable endothelial cell enclosed blood islands (Wilt, 1965). Endothelial cell differentiation could be restored if the mesectoderm was recombined with endoderm. This suggests that an endodermally derived inductive signal is necessary for extraembryonic endothelial cell formation, at least in the context of blood island formation. This result was corroborated in a subsequent study (Miura and Wilt, 1969). While these studies implied that endoderm is required for blood island formation, in the absence of molecular markers it was not possible to identify individual angioblasts prior to blood vessel formation, and so the results are not necessarily conclusive.

In studies of intraembryonic vasculogenesis, it was also proposed (Pardanaud et al., 1989) that interactions between mesodermal and endodermal tissues are necessary for vasculogenesis. Once again, this proposal was based on the fact that vasculogenic mesoderm is always observed in the immediate vicinity of endoderm. This hypothesis was extended in a subsequent study showing that, when grafted onto chick limb buds, quail splanchnopleuric mesoderm (which is in contact with endoderm) generated greatly more endothelial cells than somatopleuric mesoderm (not in contact with endoderm). On the basis of this result, it was concluded that an endodermal factor is necessary to promote the emergence of endothelial cells (Pardanaud and Dieterlen-Lièvre, 1993). More recently, it has been argued that an indian hedgehog signal from the visceral endoderm is necessary for specifying endothelial cell fate in mouse embryos (Belaoussoff et al., 1998; Dyer et al., 2001). Overall, these studies imply that interactions between endoderm and mesoderm are required for vascular endothelial cell specification. Notwithstanding a large number of assumptions and the relative paucity of experimental support, this relationship is routinely stated in the literature and has largely assumed the status of dogma (Wilt, 1965; Miura and Wilt, 1969; Gonzalez-Crussi, 1971; Augustine, 1981; Kessel and Fabian, 1985; Pardanaud et al., 1989; Pardanaud and Dieterlen-Lièvre, 1993; Risau and Flamme, 1995; Sugi and Markwald, 1996; Belaoussoff et al., 1998; Waldo and Kirby, 1998; Cleaver and Krieg, 1999; Roman and Weinstein, 2000; Dyer et al., 2001; Poole et al., 2001).

Despite the widespread acceptance of a role for endoderm in angioblast specification, the results of a number of experiments using several different organisms have called this conclusion into question (see Discussion). It is important to acknowledge, however, that none of these studies had been designed to specifically address the requirement of endoderm for angioblast formation, and so none of them were fully controlled. To formally address this question, we have used a combination of molecular and classical embryology techniques to examine the role of endodermal tissues during vasculogenesis. We find that large numbers of angioblasts are formed in frog embryos that contain no detectable endoderm. However, angioblasts in these endoderm-depleted embryos fail to assemble into endothelial tubes. This observation was confirmed in complementary experiments using avian embryos. In summary, our studies indicate that endoderm is indeed important for vascular development, not for angioblast specification, but for the formation of tubular blood vessels.

MATERIALS AND METHODS

Embryology

Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Animal caps were dissected from stage-8 embryos and cultured in 50% NAM (Normal amphibian medium) containing 0.1% BSA and penicillin-streptomycin until sibling embryos were at stage 30. When applicable, caps were cultured in medium containing 100 ng/ml *Xenopus* bFGF (a gift from David Kimelman) at 13°C overnight (until sibling embryos were at stage 12.5), and then transferred into 50% NAM to generate a population of mesoderm completely devoid of endoderm. While activin treatment is a more routine method for generating mesodermal populations in animal caps and is effective in the induction of endothelial cell markers, it also induces the expression of endodermal genes (data not shown), making these experiments uninterpretable. Embryological manipulations were performed using electrolytically sharpened tungsten needles and hair

loops in 75% NAM. Embryos were subsequently incubated in 50% NAM until the appropriate stage. Presumptive endoderm was removed from stage-10 embryos as described (Nascone and Mercola, 1995). In the rescued endodermless embryos, endoderm was removed as above, and a small core of vegetal mass from a sibling embryo was inserted into the embryo. Embryos were then allowed to heal under glass bridges overnight.

Japanese quail (*Coturnix coturnix japanica*) embryos were incubated until stage 5 (Hamburger and Hamilton, 1951) and whole embryo New culture explants were mounted on glass rings on albumen agar dishes (New, 1955). Endoderm was then removed from one side of the embryo, and the adjacent extraembryonic domain, using electrolytically sharpened tungsten needles, while the other side was left intact to serve as an internal control. These embryos were then incubated to the 6-somite stage (stage 9–), at which time vascular structure was examined.

VegT antisense-treated embryos

cDNA from VegT antisense oligonucleotide-treated embryos was generously provided by Matt Kofron and Janet Heasman. The samples, obtained following the host-transfer technique, are identical to those used by Kofron et al. (Kofron et al., 1999), and represent oocytes injected with 5-8 ng of phosphorothioate antisense VegT oligonucleotides and subsequently implanted into host females prior to fertilization. Embryos were harvested at stage 34 for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

RT-PCR

Approximately eight animal caps were harvested for each sample and total RNA was prepared using a standard SDS-Proteinase K method. cDNA samples were prepared from one-half of the total RNA (with the other have serving as a –RT control) and radioactive RT-PCRs were performed using $1/25^{th}$ of the cDNA reaction as template and 0.3 μCi of [γ-^{32}P]dATP in a 50 μl reaction. The number of cycles for each primer was empirically determined so that they would be in the linear range of amplification. PCR samples were run on non-denaturing 5% acrylamide gels.

Primers

Cardiac α-actin (Niehrs et al., 1994) (T_m=63°C); cardiac Troponin I: forward: 5′TCGGTCCTATGCCACAGAACCAC3′, reverse: 5′TTTTGAACTTGCCACGGAGG3′ (T_m=63°C); Endodermin: forward: 5′GAGACTTGGCTTTGGGACCTTGTTG3′, reverse: 5′CCATTTCCTGCGAGCACAGTAACC3′ (T_m=62°C); Erg (detects both isoforms): forward: 5′CCTCAACAAGACTGGCTCTCACAG3′, reverse: 5′TGCTCCACAAAGTAGGGTCAGC3′ (T_m=66°C); Flk-1: forward: 5′AAGAGGGAACAAGAATGAGGGC3′, reverse: 5′TGCTGCTGCTGTGAAGAAACC3′ (T_m=64°C); IFABP: (Henry et al., 1996) (T_m=60°C); Insulin (Henry et al., 1996) (T_m=63°C); Mixer: forward: 5′GCTTTGTTCAGAATCCACCTACGC3′, reverse: 5′AGTGATGGTCTTGTTGGGAGGG3′ (T_m=61°C); Ornithine decarboxylase (ODC) (Bouwmeester et al., 1996) (T_m=64°C); SCL/tal-1: forward: 5′CCCAAATGAAAGGCAAACGG3′, reverse: 5′CAGTTCTGTGGCTGGTGTCAAAG3′ (T_m=64°C); Xbra: forward: 5′GGAGTAATGAGTGCGACCGAGAGC3′, reverse: 5′GCCACAAAGTCCAGCAGAACCG3′ (T_m=60°C); Xlhbox8: forward: 5′AAGGACAGTGGACAGATG3′, reverse: 5′GGATGAAGTTGGCAGAGG3′ (T_m=65°C); Xsox17-α: forward: 5′TGCCAATAATGATGACTGGACTCG3′, reverse: 5′TCTTCACCTGTTTCCTCCTGCG3′ (T_m=61°C).

In situ hybridization and histology

Digoxigenin-labeled RNA probe was transcribed using MEGAscript (Ambion). Embryos were assayed by in situ hybridization with the endothelial marker *X-msr* as previously described (Gerber et al., 1999), and developed in either BM-Purple (Roche) or NBT-BCIP (Roche). *X-msr* (Devic et al., 1996; Cleaver et al., 1997) is the *Xenopus* orthologue of the mammalian APJ receptor (Devic et al., 1999), which is the receptor for the apelin peptide (Tatemoto et al., 1998). While the precise physiological role of this ligand-receptor system is unclear, it is thought that it plays a related role to the structurally related angiotensin II signaling system (Lee et al., 2000). Paraffin sections on embryos assayed by in situ hybridization were carried out by dehydrating the embryos in a graded ethanol series, washing twice for 10 minutes each in xylene, and then three times in Paraplast at 60°C for a total of 2 hours. Embryos were then embedded in Paraplast and sectioned at a thickness of 12 μm. Slides were dewaxed in xylene and viewed by DIC optics. For plastic sections, embryos were fixed in 1/2 strength Karnovsky's solution in 0.1 M cacodylate buffer, embedded in Spurr resin, post-fixed in 2% OsO_4, sectioned at a thickness of 1 μm and stained with Toluidine Blue (semi-thin histological sections) or 3 μm (in situ hybridized sections). For electron microscopy imaging, thin sections (approximately 0.06 μm) were stained with uranyl acetate and lead citrate and imaged on a Philips CM12 transmission electron microscope.

Immunohistochemistry

Quail endothelial cells were detected with the QH1 monoclonal antibody (Pardanaud et al., 1987) (Developmental Studies Hybridoma Bank). The procedure was performed as described by Sugi and Markwald (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 1:500 dilution.

RESULTS

Angioblast formation after manual removal of endoderm

Using the frog embryo, we performed a series of experiments to test whether interactions between endodermal and mesodermal tissues are required for the formation of angioblasts. Our initial experiments used standard embryological techniques to physically remove the vast majority of endoderm from the gastrula stage *Xenopus* embryo. This dissection is closely modeled on methods previously described (Cooke, 1989; Nascone and Mercola, 1995). Both of these studies showed that endoderm acts as a permissive signal that is essential for cardiac development. We used tungsten needles and hair loops to carefully remove all detectable endoderm from stage-10 embryos (Fig. 1A) and then allowed the manipulated embryos to develop until control embryos showed the presence of a beating heart (about stage 34). As expected, none of the endoderm-depleted embryos (0/24) showed the presence of beating cardiac tissue (data not shown), thereby indicating successful removal of endoderm (Cooke, 1989; Nascone and Mercola, 1995). Apart from the loss of a large proportion of the total tissue mass, endodermless embryos exhibited a generally normal overall body pattern, including segmented somites and morphologically intact notochords and neural tubes. As described by Cooke (Cooke, 1989), the ventral region of the embryos consisted primarily of 'lateroventral mesoderm', although the precise nature of this tissue is uncertain. Endoderm-depleted embryos, at the equivalent of stage 34, were assayed for the presence of angioblast cells by in situ hybridization using several distinct angioblast marker probes, including *X-msr*, *flk-1* and *erg*. Using the in situ method, expression of these markers is first detected in developing vascular tissues at the late neurula stage

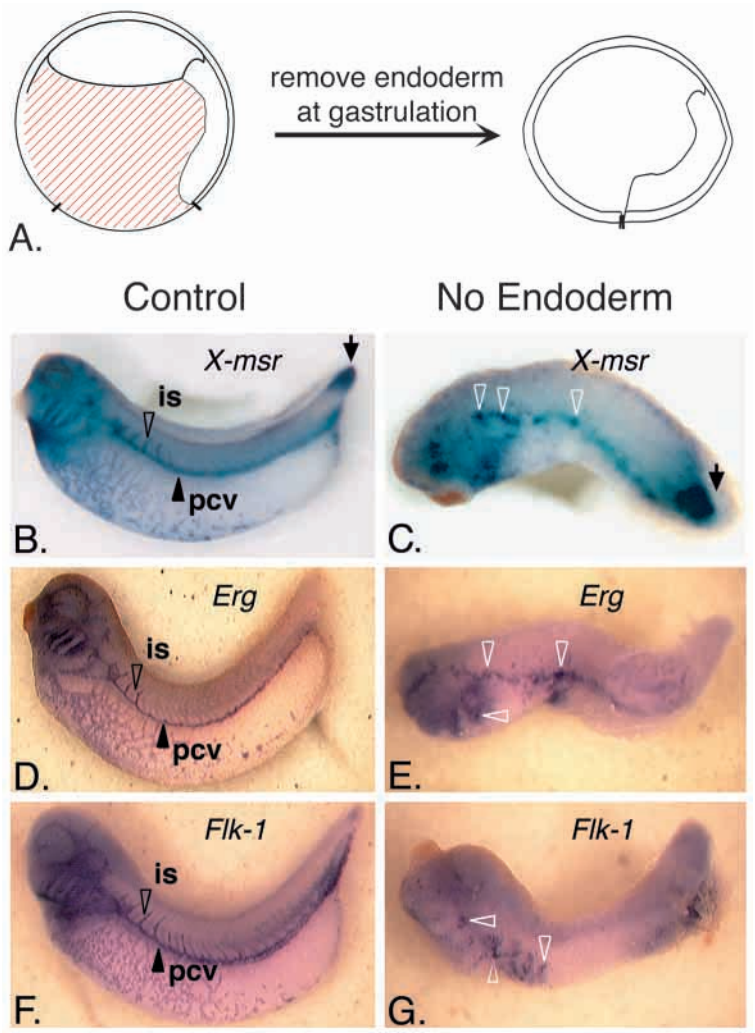

Fig. 1. Endoderm is not necessary for in vivo angioblast specification. (A) Diagram of the dissection used to remove endoderm. The vegetal core (red), comprising future endoderm, was removed from embryos at the onset of gastrulation, and the resulting endoderm-depleted embryos were incubated until stage 34. (B,D,F) Control embryos assayed with *X-msr*, *erg* and *flk-1* probes, respectively. These show elaborate vascularization, including posterior cardinal veins (pcv; closed arrowheads), intersomitic vessels (is; open arrowheads) and a ventrolateral vascular plexus. (C,E,G) Endoderm-depleted embryos, assayed with *X-msr*, *erg* and *flk-1* probes, respectively, contain angioblasts (white open arrowheads), but these are not organized into patent blood vessels.

(approx. stage 18) (Cleaver et al., 1997; Baltzinger et al., 1999). Surprisingly, all endoderm-depleted embryos examined showed the presence of significant numbers of angioblasts. This was particularly evident in lateral regions of the embryos, which showed strong expression of *X-msr* (14/14 embryos; Fig. 1C), *erg* (5/5 embryos; Fig. 1E) and *flk-1* (5/5 embryos; Fig. 1G). In order to confirm, and control, these in situ hybridization observations, endodermless embryos were assayed for vascular markers and a number of endodermal markers by RT-PCR analysis (Fig. 2). In this analysis, the presence of angioblasts was assessed using *erg* and *flk-1*. We did not use *X-msr* in the RT-PCR assays because this gene is expressed in an additional, apparently non-endothelial, domain at the tip of the tail (Fig. 1B,C) that might confuse interpretation of the results. As shown in Fig. 2, RT-PCR analysis indicates that angioblast markers *flk-1* and *erg*, and the angioblast/hematopoietic cell marker, *SCL/tal-1* (Mead et al., 1998) are expressed at significant levels in endoderm-depleted embryos. To determine the efficiency with which endodermal cells were eliminated by physical dissection, RT-PCR analysis was carried out on RNA samples from the same manipulated embryos, using a number of different markers of endodermal tissue. This assay reveals that expression of the definitive endodermal markers *insulin*, *IFABP* and *xlhbox8*, is almost completely eliminated in manipulated embryos, indicating that removal of endoderm, although not complete, has been very effective. Another commonly used endodermal marker, *endodermin*, is detected at quite high levels in endoderm-depleted embryos, but this is presumably because of its additional expression domain in the paraxial mesoderm, especially the notochord (Sasai et al., 1996) (data not shown). Note also that the general muscle marker, *cardiac α-actin* is expressed at normal levels in the manipulated embryos while, as expected, expression of the heart-specific marker *cardiac troponin I* is undetectable (Fig. 2). We believe that the slightly reduced levels of angioblast markers in endoderm-depleted embryos may be due to reduced angioblast proliferation, because in normal embryos the endoderm expresses substantial amounts of VEGF (Cleaver et al., 1997), which is a potent mitogen for angioblasts (Keyt et al., 1996). Overall, these dissection experiments indicate that angioblasts are specified at significant levels in embryos from which endoderm has been greatly depleted or eliminated.

Angioblast formation in embryos with reduced-VegT function

VegT function is essential for endoderm formation in the frog embryo and has recently been shown to be the crucial initiating molecule underlying all endoderm specification (Xanthos et al., 2001). Treatment of embryos with VegT antisense oligonucleotides results in abolition of all detectable endodermal tissue (Zhang et al., 1998) and, at higher doses, can cause elimination of as much as 90% of mesodermal tissue (Kofron et al., 1999). To complement our studies where endoderm was physically removed, we assayed for angioblast formation in embryos treated with VegT antisense phosphorothioate oligonucleotides at levels sufficient to eliminate endodermal tissue (5-8 ng per embryo). Since the antisense oligonucleotide acts prior to fertilization, optimally treated embryos should not contain endoderm at any stage of development. Analysis of treated embryos by RT-PCR shows severe reduction or elimination of expression of all endodermal markers tested, including endodermin, *IFABP*, *Xlhbox8* and *insulin* (Fig. 3). In this experiment, we believe that *endodermin* expression is completely absent because VegT depletion also results in downregulation of many mesodermal genes (Kofron et al., 1999). Importantly, however, the experimental embryos continue to show expression of the vascular-specific markers

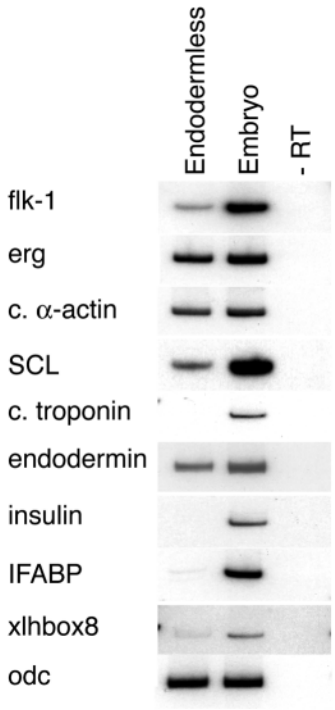

Fig. 2. Endodermless embryos show a marked reduction in expression of endodermal markers but still express endothelial markers. RT-PCR was performed on total RNA from a stage-34 endodermless embryo. Expression levels of the endodermal markers *insulin*, *IFABP* and *xlhbox8* are either severely reduced or eliminated relative to unmanipulated controls, while the vascular markers *flk-1* and *erg* and the angioblast/hematopoietic cell marker *SCL/tal-1*, are still present. –RT, RT not performed.

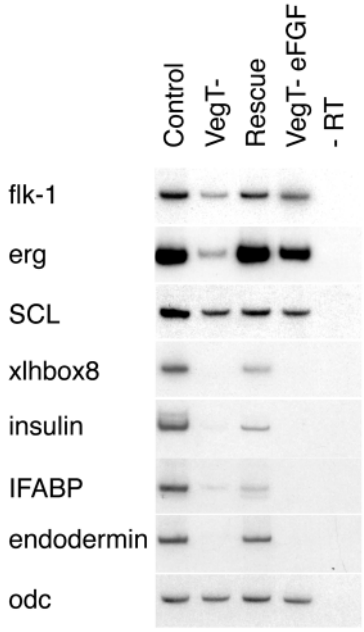

Fig. 3. Embryos depleted of endoderm by treatment with VegT antisense oligonucleotides continue to express vascular markers. RT-PCR analysis of RNA from stage-34 embryos shows a lack of endodermal markers in VegT-treated embryos (labeled VegT–), while vascular markers are still present. Rescue by coinjection of VegT mRNA (labeled Rescue) restores both endodermal and mesodermal markers to control levels, whereas partial rescue with eFGF (labeled VegT-eFGF) restores mesodermal marker expression but has no effect on endodermal markers (Kofron et al., 1999). –RT, RT not performed.

flk-1 and erg, and the angioblast/hematopoietic cell marker *SCL/tal-1*, although at somewhat reduced levels compared to wild-type embryos. Complete rescue of embryos, by injection of *VegT* mRNA, restores *flk* and *erg* expression to normal levels. When antisense VegT-treated embryos are partially rescued by microinjection with *eFGF* mRNA, which restores ventrolateral mesodermal levels to those of wild type while specifically excluding endoderm (Kofron et al., 1999), expression of the vascular markers *flk-1* and *erg* is restored to wild-type levels (Fig. 3). No expression of endodermal markers is detected in the *eFGF*-rescued embryos. Taken together, these experiments using VegT-depleted embryos strongly imply that formation of embryonic angioblasts is not dependent on the presence of endodermal tissue.

Angioblasts form in FGF-treated animal caps that contain no endoderm

The experiments described above do not formally preclude the possibility that very small amounts of endoderm are sufficient for the induction of angioblasts or, in the case of the embryonic dissection experiments, that a transient interaction of mesoderm and endoderm, prior to stage 10.5, is adequate to specify the lineage. To address these two possibilities, we employed animal cap techniques to generate mesodermal tissue that has never come into contact with endoderm. Specifically, we treated animal caps with basic fibroblast growth factor (bFGF) under conditions that generate mesoderm completely free of endoderm (see Materials and Methods) (Cornell et al., 1995; Gamer and Wright, 1995). At the equivalent of stage 30, the caps were assayed using RT-PCR for expression of a range of endothelial, mesodermal and endodermal tissue markers. Stage 30 was chosen because all endothelial markers are expressed at significant levels in wild-type embryos at this time. As shown in Fig. 4A, animal caps treated with 100 ng/ml of bFGF express the endothelial markers *flk-1* and *erg*, and the angioblast/hematopoietic cell marker *SCL/tal-1*, as well as the general mesodermal marker *cardiac α-actin*. There is, however, no detectable expression of the endodermal markers *endodermin*, *Xsox17-α*, *insulin*, *IFABP* and *xlhbox8*. While endodermin is detected in manually dissected endodermless embryos, probably due to its expression in paraxial mesoderm, it is not present in bFGF-induced animal caps (Sasai et al., 1996). This is most likely because bFGF does not induce the expression of genes representing more dorsal mesodermal tissues such as notochord (Green et al., 1990). We also note that the early endodermal marker *mixer* is not present in stage-30 control embryos, in agreement with its published expression pattern (Henry and Melton, 1998). To ensure that endodermal tissue was not transiently present soon after bFGF treatment, animal caps were also assayed for marker expression at the late gastrula stage (stage 12.5). Whereas treated caps express *Xbra*, indicating the presence of early mesodermal tissue, they do not express the early endodermal markers *endodermin*, *Xsox17-α* and *mixer*. The vascular markers *flk-1* and *erg* are also absent

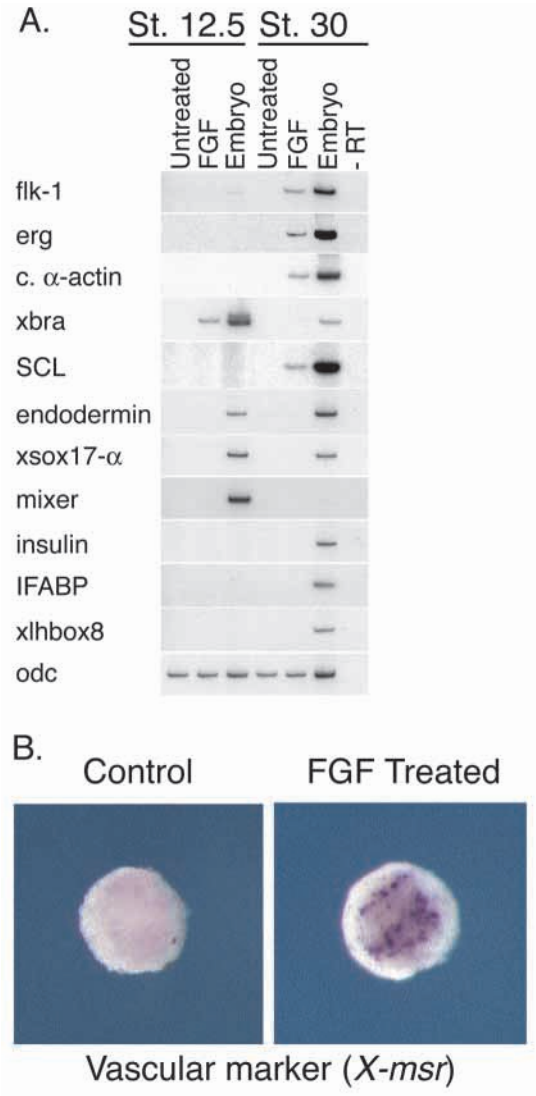

Fig. 4. Animal caps treated with bFGF form mesoderm containing endothelial markers in the absence of detectable endoderm. (A) Animal caps were incubated in bFGF and cultured until the appropriate stage (either 12.5 or 30). Caps were then assayed for early or late markers of endoderm and for endothelial markers using RT-PCR. While the animal caps show expression of both endothelial and mesodermal markers, there is no detectable expression of endodermal markers at either stage. Note that significant expression of endothelial markers is not expected in the stage-12.5 samples. (B) Stage-30 animal caps treated with bFGF express the vascular marker *X-msr* in discrete patches when assayed by in situ hybridization.

at this stage, in agreement with their known embryonic expression profiles (Cleaver et al., 1997; Baltzinger et al., 1999). In order to determine the distribution of endothelial cells in FGF-treated caps, we examined the stage-30 animal caps for the presence of vascular markers by in situ hybridization. As shown in Fig. 4B, the vascular marker *X-msr* reveals the presence of individual angioblasts in treated caps, but not in untreated control caps.

Endoderm is required for endothelial tubule assembly

We performed three independent sets of experiments, physical dissection, VegT ablation and induction of mesoderm in animal caps, all of which suggest that angioblast specification is independent of interactions with endoderm. Does this imply that endoderm plays no role at all in the development of the embryonic vascular system? In fact, the results presented below strongly support a role for endoderm during assembly of angioblasts into patent vascular tubes.

As described above, embryos from which endoderm had been physically dissected at stage 10.5 showed the presence of an abundance of aggregated cords of angioblasts during later development (stage 34) (Fig. 1C,E,G). In no case, however, did we observe angioblasts assembling into the patent blood vessels visible in the control embryos. In order to ensure that this was not merely the consequence of a developmentally delayed phenotype, endoderm-depleted embryos were incubated until stage 37. At this stage, all embryos contained dark eye pigment and melanocytes, clear indications that they had developed past the stage when blood vessel tube formation would normally occur (about stage 34) (Cleaver et al., 1997). When these endoderm-depleted embryos were assayed by in situ hybridization for the vascular marker *X-msr*, angioblasts, but no endothelial tubes, were visible in whole-mount embryos (Fig. 5B,C). In sectioned embryos, thick assemblages of angioblasts were visible in lateral regions of the embryo (Fig. 5F). However, despite the presence of large numbers of angioblasts, none of the endoderm-depleted embryos (0/21) contained any detectable vascular tubes. On the other hand, patent vessels were readily visible in all control embryos (15/15 examined; Fig. 5E).

To ensure that the absence of tube formation by angioblasts in endoderm-depleted embryos was indeed due to the absence of endoderm rather than a dissection artifact, we carried out a rescue experiment. In this experiment, stage-10 embryos from which endoderm had been removed were implanted with a small amount of vegetal core tissue from a sibling embryo. We estimate that approximately 20% of the normal amount of endodermal tissue was restored to the embryo. In all cases (11/11), the rescued embryos exhibited much improved overall morphology and also a substantial degree of vascular assembly and tube formation. Phenotypes ranged from formation of a vascular plexus, generally restricted to the ventral region of the embryo, up to an almost complete vascular network that contained paired posterior cardinal veins and intersomitic vessels (Fig. 5D). The presence of patent blood vessel morphology was examined more closely in serial, plastic, semi-thin sections from additional endoderm-depleted and rescued embryos. These embryos were not assayed by in situ hybridization because we find that the in situ procedure makes the embryos brittle and compromises histological quality, especially for delicate structures like blood vessels. In this experiment, only 2/11 endoderm-depleted embryos showed any discernible endothelial tubes in any section along the length of the embryo (Fig. 6B) for a total of 55 sections examined. In contrast, 6/8 rescued embryos showed the clear presence of vascular tubes (Fig. 6C). These results are statistically significant ($P<0.05$). Representative transverse sections through posterior cardinal veins from wild-type and rescued embryos were also examined by electron microscopy (Fig. 6D,E, respectively). Based on examination of numerous sections, endothelial tube structures in the rescued embryos were morphologically indistinguishable from those in wild-type embryos.

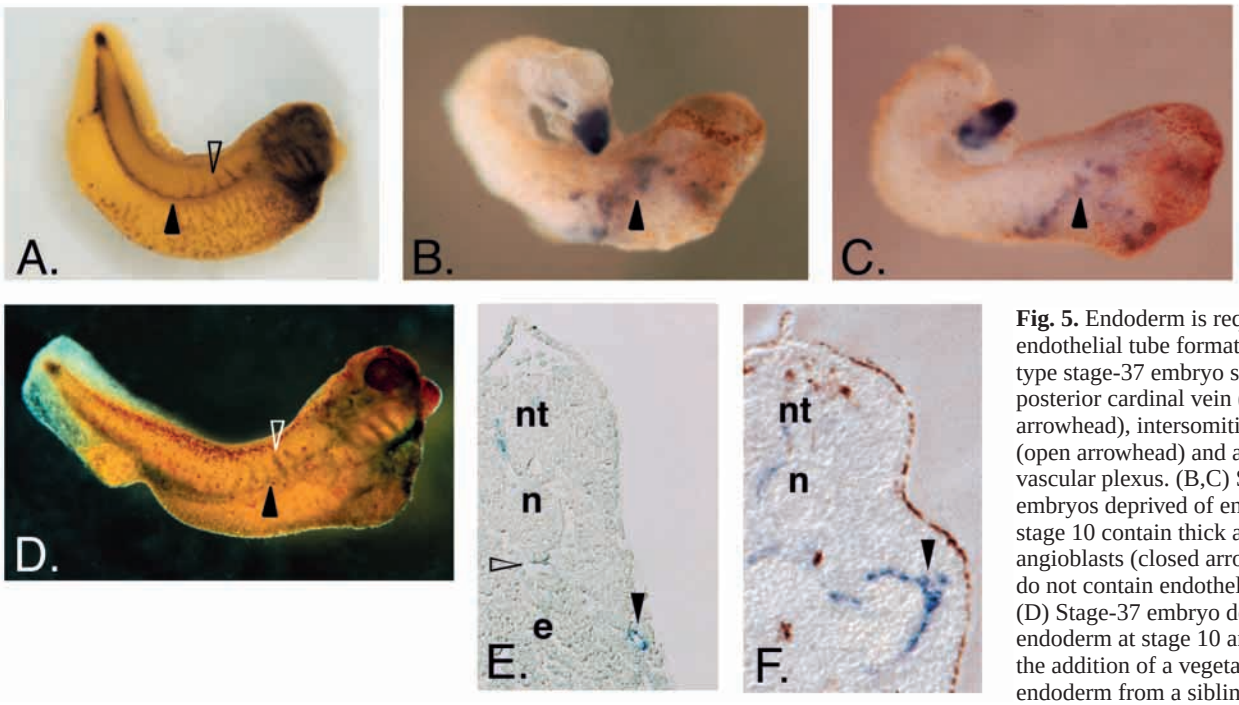

Fig. 5. Endoderm is required for endothelial tube formation. (A) Wild-type stage-37 embryo showing posterior cardinal vein (closed arrowhead), intersomitic vessels (open arrowhead) and a prominent vascular plexus. (B,C) Stage-37 embryos deprived of endoderm at stage 10 contain thick assemblages of angioblasts (closed arrowheads), but do not contain endothelial tubes. (D) Stage-37 embryo deprived of endoderm at stage 10 and rescued by the addition of a vegetal plug of endoderm from a sibling embryo. Note the presence of posterior cardinal veins (closed arrowhead) and intersomitic vessels (open arrowhead). (E) Cross-section through a wild-type stage-37 embryo showing posterior cardinal vein (closed arrowhead) and dorsal aorta (open arrowhead). (F) Cross-section through a stage-37 endodermless embryo showing presence of angioblasts (closed arrowhead) but no assembly into endothelial tubes. All embryos were assayed by in situ hybridization with the vascular marker *X-msr*. e, endoderm; n, notochord; nt, neural tube.

The original experiments of Wilt and Miura suggesting a role for endoderm in angioblast specification were carried out using avian embryos (Wilt, 1965; Miura and Wilt, 1969). These experiments clearly indicated an absence of endothelium-enclosed blood islands in endoderm-depleted embryos but, without the aid of molecular markers, it was not possible to determine whether angioblasts were indeed present. To address this question, we have examined vascular development in endoderm-depleted quail embryos using the angioblast marker, QH1 (Pardanaud et al., 1987). For these experiments, both intra- and extraembryonic endoderm was removed from the left side of stage-5 embryos, with the unmanipulated right side serving as an internal control. Embryos were cultured for approximately 12 hours until they had approximately 6 somites (stage 9–). We assayed embryos at this stage, rather than later in development, to ensure that the vasculature was formed exclusively by vasculogenic mechanisms. Using the quail endothelial cell-specific antibody QH1, 8/8 embryos examined contained no discernible endothelial tubes on the side lacking endoderm, although all embryos had robust vascular development on the control side (Fig. 7A,B). This result is statistically significant ($P<0.01$). Despite the absence of blood vessels, all embryos showed the presence of abundant QH1-positive cells on the endodermless side, indicating that angioblasts were still specified in the absence of endoderm. Basically these experiments in the quail embryo support the original observations of Wilt (Wilt, 1965) that endoderm is required for formation of organized endothelial structures. The underlying reason, however, is not the absence of angioblasts, but the failure of these cells to assemble into patent blood vessels.

DISCUSSION

Angioblast specification does not require endoderm

Based on three distinct experimental approaches using two different model systems, our results indicate that embryonic specification of angioblasts is independent of the presence of endoderm. Using the *Xenopus* embryo, consistent results are obtained when endoderm is removed by embryonic dissection, when endoderm is ablated using antisense VegT oligonucleotides, and when mesodermal tissue is induced in animal caps in the complete absence of detectable endoderm. In the avian embryo, angioblasts still form when endoderm is physically removed. Overall, these results directly challenge the broadly cited proposition that interactions between mesoderm and endoderm are necessary to specify endothelial cells (Wilt, 1965; Miura and Wilt, 1969; Gonzalez-Crussi, 1971; Kessel and Fabian, 1985; Pardanaud et al., 1989; Pardanaud and Dieterlen-Lièvre, 1993; Sugi and Markwald, 1996; Belaoussoff et al., 1998; Dyer et al., 2001), and stated in numerous reviews (Augustine, 1981; Risau and Flamme, 1995; Cleaver and Krieg, 1999; Roman and Weinstein, 2000; Poole et al., 2001).

Although this study is amongst the first to use molecular markers to directly address the role of endoderm in angioblast specification, it is important to acknowledge that a number of previous studies, using different experimental systems, have hinted that endodermal-mesodermal interactions are not essential for the formation of angioblasts. For example, it has been shown that mouse embryoid bodies lacking activity of the transcription factor GATA-4, fail to form extraembryonic endoderm. In the absence of endoderm, these embryoid bodies

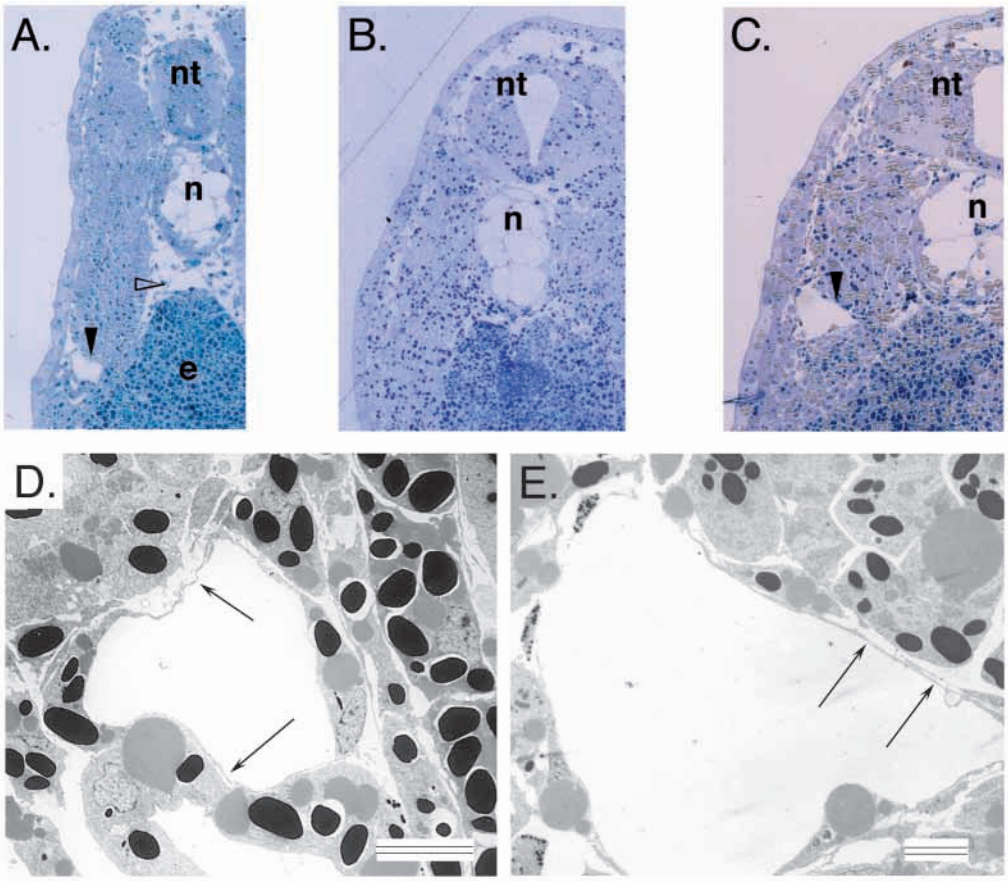

Fig. 6. Embryos without endoderm lack patent blood vessels. (A-C) 1 μm plastic sections stained with Toluidene Blue. (A) Cross-section through a wild-type stage-37 embryo showing endothelial tubes, including a posterior cardinal vein (closed arrowhead) and dorsal aorta (open arrowhead). (B) Endothelial tubes are not present in stage-37 endodermless embryo but are present in stage-37 embryos that have been rescued by the addition of endoderm (C, closed arrowhead). (D,E) Transmission electron microscopy showing transverse sections through the posterior cardinal veins of a wild-type embryo (D) and an endodermless embryo rescued by the addition of endoderm from a sibling donor embryo (E). Arrows indicate the characteristic thin-walled endothelial cell morphology in each section. Scale bars, 1 μm. Black objects in sections are lipid droplets generated during histological preparation. e, endoderm; n, notochord; nt, neural tube.

are unable to form endothelial cell enclosed blood islands. This observation is in apparent agreement with the endoderm induction model. However, use of specific markers indicated that vascular endothelial cells were still present in these embryoid body cultures (Bielinska et al., 1996). Similar results were obtained embryologically (Palis et al., 1995); they showed that murine yolk sac explants that contained extraembryonic mesoderm, but were separated from endoderm, still developed endothelial cells, but lacked organized blood vessels. In this experiment, however, dissections were performed at E7.5. Since extraembryonic angioblasts are initially detected at E6.5 (Drake and Fleming, 2000), it is possible that angioblasts had already been specified prior to the separation of mesoderm from endoderm.

Further evidence that angioblasts form in the absence of endoderm is provided by a series of experiments using quail-chick heterochronic chimeras. In these experiments, quail blastoderm treated with cytochalasin B to block gastrulation was grafted to host limb buds. The presence of endothelial cells was then assessed using the antibody QH-1. Because limb buds do not contain endoderm, the presence of quail endothelial cells in these chimeras implied that the endodermal germ layer is not necessary for vascular cell specification (Christ et al., 1991; von Kirschhofer et al., 1994; Wilting and Christ, 1996). However,

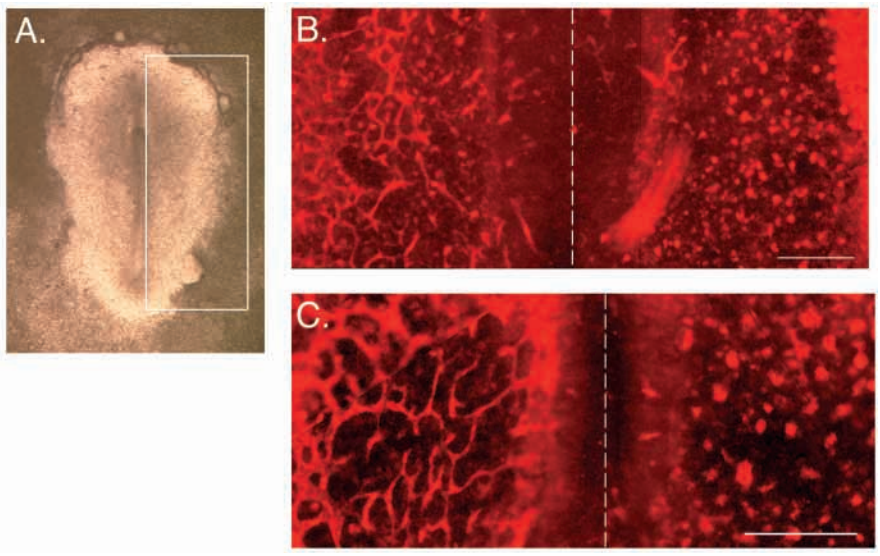

Fig. 7. Removal of endoderm in quail embryos does not prevent angioblast formation. (A) Ventral view of quail embryo showing the approximate region from which endoderm was removed. (B,C) Ventral images of fluorescent staining of the endothelial cell marker, QH1 at approximately the 4- and 6-somite stages, respectively. The endoderm-depleted side is on the right. Note the presence of endothelial cells within the manipulated region, but the absence of vascular assembly. The approximate midline of the embryo is indicated by the dashed line. Scale bars, 10 μm.

interpretation of the limb bud experiments in the context of endothelial cell specification is difficult since they utilized an older, already specified population of mesoderm that contained a complex and specific set of growth factors involved in limb bud patterning.

Studies of zebrafish mutants that are deficient in endoderm formation also support our suggestion that endoderm is not necessary for vascular specification. For example, *one-eyed pinhead* (*oep*) mutants lack almost all endoderm (Schier et al., 1997), but still contain abundant angioblasts (Brown et al., 2000). In these mutants, however, at least some endodermal tissue is still present and so the absolute requirement for endoderm in angioblast formation is difficult to ascertain.

Some recent molecular studies using mouse tissues would appear to directly contradict our conclusions. In particular, Belaoussof et al. (Belaoussof et al., 1998) have suggested that an early signal from the visceral endoderm can respecify neurectoderm to a posterior mesodermal cell fate containing both endothelial and blood markers. It was concluded that a secreted signal from the visceral endoderm is needed to induce endothelial cell fate. Subsequent work has suggested that indian hedgehog (Ihh) is the secreted signaling factor (Dyer et al., 2001). This result is challenged by gene ablation studies in mice, which show that embryos lacking function of either *Ihh* or *Smoothened* (the receptor for all hedgehog proteins) still contain at least rudimentary endothelial tubes in the yolk sac (Byrd et al., 2002). This result conclusively demonstrates that hedgehog signaling is not necessary for angioblast specification, at least in an in vivo context. The tissue recombination work (Belaoussof et al., 1998) implying that visceral endoderm is required to induce endothelial cells, is a more complicated issue. However, we propose that the function of visceral endoderm in these experiments is in fact the induction of mesodermal tissue, since this is not present in the original explants. Once mesoderm is present, it is then capable of forming angioblasts, precisely as observed in our experiments. Alternatively, it is possible that the mechanism leading to specification of angioblasts in frog and avian embryos differs from that operating in the mammalian embryo.

Endoderm is required for endothelial tube formation

Our experiments show that angioblasts are indeed present in embryos containing no endoderm. However, these angioblasts fail to assemble into patent vascular tubes. Serial sectioning through endoderm-depleted embryos shows that formation of tubular blood vessels is absent or severely reduced (Fig. 6B), although in situ hybridization indicates that angioblasts have assembled into dense, cord-like aggregations throughout the trunk of the embryo (Fig. 5B,C). These observations suggest that vasculogenesis in endoderm-depleted embryos is interrupted at a step prior to tube formation. This view is supported by the rescue experiments in which endoderm from a donor embryo is implanted into the endoderm-depleted embryo. Despite the trauma caused by this rather crude manipulation, the majority of rescued embryos show vascular tube formation. In the most effective cases, the rescued embryos showed clear organization of the posterior cardinal veins and intersomitic vessels. Variation in the amount of vascular structure observed in different rescued embryos is presumably due to differential healing, but we cannot exclude the possibility that pre-patterning of the endoderm has already occurred and therefore the degree of vascular rescue may be related to the orientation of the implanted endodermal tissue. In agreement with our results using *Xenopus*, we note that zebrafish *oep* mutants, which lack most endoderm, contain angioblasts but exhibit dramatic defects in axial vascular formation, and lack a functional circulatory system (Brown et al., 2000), suggesting that endoderm is indeed required for vascular assembly. Likewise, murine extraembryonic mesoderm, when isolated from endoderm, forms endothelial cells that fail to assemble into vascular tubes (Palis et al., 1995; Bielinska et al., 1996).

The results of our experiments raise two fundamental questions relating to the mechanisms underlying vascular development. First, what is the molecular nature of the endodermal signal necessary for vascular tubulogenesis? At present, the composition of this signal is completely unknown. One could imagine it being a secreted molecule influencing expression of a subset of endothelial genes, or perhaps a secreted structural protein or extracellular matrix component that is necessary for vascular fusion. Given the close juxtaposition of endoderm with vasculogenic mesoderm, a cell surface signal is also plausible. It also remains to be determined whether the signal arising from endoderm is expressed exclusively in areas adjacent to mesodermal tissues or if it is distributed throughout the endoderm. The second question is related to the observation that endoderm is not involved in angioblast specification. This implies that any signal for angioblast specification arises within the mesoderm itself. The ectodermal germ layer, the only other theoretically possible source of inductive signals, is not likely to contribute to vasculogenesis because it has been shown to profoundly inhibit vasculogenesis (Feinberg et al., 1983; Wilson et al., 1989; Pardanaud and Dieterlen-Lièvre, 1993; Pardanaud and Dieterlen-Lièvre, 1999). While our results suggest that the origin of the angioblast specification signal is likely to be exclusively mesodermal, the molecular nature of the signal is completely unknown. Because almost all mesoderm has the potential to express angioblasts (Noden, 1989), it is possible that angioblast specification occurs by an inherent patterning mechanism, perhaps analogous to the Delta/Notch signaling pathway responsible for neuroblast specification in *Drosophila*. Inhibitory signals from ectodermal tissues may subsequently help to determine the boundaries of the vasculogenic network.

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