Vascular defects in a mouse model of hypotrichosis-lymphedema-telangiectasia syndrome indicate a role for SOX18 in blood vessel maturation

Meredith Downes1, Mathias François1, Charles Ferguson1,2, Robert G. Parton1,2 and Peter Koopman1,*

1Institute for Molecular Bioscience and 2Centre for Microscopy and Microanalysis, The University of Queensland, Brisbane, QLD 4072, Australia

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Mutations in the transcription factor gene SOX18 cause vascular, lymphatic and hair follicle defects in humans with dominant and recessive forms of hypotrichosis-lymphedema-telangiectasia (HLT) syndrome. Here, we clarify the role of SOX18 in the vascular dysfunction in HLT by ultrastructural, immunofluorescence, molecular and functional analysis of vascular anomalies in embryos of the naturally occurring Sox18-mutant mouse strain ragged-opossum (RaOp). Early genesis and patterning of vasculature was unimpaired in RaOp embryos, but surface capillaries became enlarged from 12.5 dpc and embryos developed massive surface hemorrhage by 14.5 dpc. Large focal breaches in the endothelial barrier were observed, in addition to endothelial hyperplasia associated with impaired pericyte recruitment to the microvasculature. Expression of the genes encoding the endothelial factors MMP7, IL7R and N-cadherin was reduced in RaOp embryos, suggesting that these are downstream targets of SOX18. Together, our results indicate that vascular anomalies in HLT arise from defects in regulation of genes required for the acquisition of structural integrity during microvascular maturation.

INTRODUCTION

Disorders in blood vessel development and function are a feature of a large number of human genetic diseases, and can arise at any one of a complex series of steps. During vasculogenesis, angioblasts originate in the blood islands of the yolk sac and either differentiate in situ to form the yolk sac vasculature or migrate to sites in the embryo proper to form the primary vascular plexus: the dorsal aortae, cardinal veins and endocardial tubes (1). During angiogenesis, this plexus is remodeled and extended through endothelial proliferation, migration and association to form a vascular network throughout the embryo (2). Maturation of these vessels then involves the formation of a basal lamina and the recruitment of pericytes, and interaction among and between endothelial cells and pericytes to optimize vascular structure and function (3), but a comprehensive picture of the regulatory networks involved in the progression of vessels into and through the maturation phase is lacking.

A growing number of studies have indicated a role for the transcription factor SOX18 in vascular development. A member of the SRY-related HMG box (SOX) family of transcription factors, SOX18 is characterized by a HMG-type DNA-binding domain and a C-terminal transcriptional transactivation domain (4,5). Sox18 is transiently expressed in endothelial cells of all developing blood vessels, whether formed by vasculogenesis or angiogenesis, in embryos of mice, chickens and fish (6–11). Expression is reactivated during adult neovascularization associated with wound healing and tumorigenesis (12,13).

Mutations of SOX18 are responsible for the human syndrome hypotrichosis-lymphedema-telangiectasia (HLT), indicating an involvement of SOX18 in the development or function of blood vessels, the lymphatic system and hair.
rupture of peripheral vasculature (20). The most severe loss-of-function of SOX18 (9,12,13,15,16). All four HLT. These mutations result in dominant-negative region, mirroring the dominant mutation observed in RaOpations of the patients (14,17–23). mutants show a similar phenotypic spectrum to human HLT. However, because of a number of factors including inability to identify mutants by molecular genotyping, only limited phenotypic analyses were possible after the initial inability to identify mutants by molecular genotyping, only limited phenotypic analyses were possible after the initial discovery of ragged alleles in the 1950s and 1960s. Investigations of the Ra allele mutant revealed dilation and rupture of peripheral vasculature (20). The most severe allele, RaOp, was found to cause edema in some putative heterozygous embryos after 11 dpc (days post coitum) and death in putative homozygotes before 11 dpc (19). A significant number of heterozygous RaOp neonates were also reported to show edema, cyanosis, chyloous ascites and small regions of superficial epidermal hemorrhage (18). However, because the blood vessels of these ragged mutants were not characterized during this early research, the role of Sox18 in establishing a functional vascular system in mice has not been established.

In this study, we examine the development of blood vasculature in RaOp mouse embryos. We demonstrate abnormal microvascular enlargement in homozygous mutant embryos from 12.5 dpc, followed by focal rupture of the microvasculature with increasing severity, culminating in gross hemorrhage, edema and death around 14.5 dpc. Using histological, molecular and ultrastructural analysis of the RaOp endothelium, we show that these defects are owing to focal lesions in the endothelium and endothelial hyperplasia associated with impaired pericyte recruitment, and identify a number of potential target genes involved in blood vessel maturation. These findings indicate a critical role for SOX18 in the maturation rather than the early modeling of the blood vasculature, explaining the telangiectasia associated with HLT syndrome.

RESULTS

In these studies we compared the phenotypes of homozygous RaOp/RaOp mouse embryos (hereafter also referred to as ‘mutant’ or ‘RaOp mutant’ embryos) with those of wild-type littermates derived from RaOp/+ × RaOp/+ matings.

Early cardiovascular development is normal in RaOp mutant embryos

We first established the time of death of RaOp homozygous embryos to gain insights into the likely cardiovascular defects in these mice. RaOp homozygotes from litters of RaOp/+ × RaOp/+ matings were represented at close to the expected Mendelian ratio of 25% at 8.5, 9.5, 10.5, 12.5, 13.5 and 14.5 dpc (Fig. 1A). A reduced proportion of homozygotes (10%; n = 30) was observed at 11.5 dpc, but this reduction is unlikely to be significant because it was not sustained at later stages. No homozygous mutant embryos survived beyond 14.5 dpc. Clearly, mutant embryos survive the critical period for establishment of a functional cardiovascular system.

RaOp homozygous embryos at 8.5–10.5 dpc (n = 15) showed no gross phenotypic differences from wild-type siblings (Fig. 1B). This period represents a key window for the appearance of primary defects in cardiovascular development (24,25). In situ hybridization of RaOp homozygotes at 10.5 dpc, using a probe for the vascular endothelial marker Flk-1 (26), confirmed the presence of an extensive surface vascular network with overtly normal vessel density and branching (n = 3; Fig. 1B). Moreover, histological examination at 11.5 dpc showed normal development of the cardinal veins, dorsal aortae and cranial vessels (Fig. 1C). These observations suggest that differentiation, proliferation, migration and association of endothelial cells, and patterning and establishment of the primary vascular plexus are unaffected by Sox18 dysfunction.

Development of gross vascular anomalies in RaOp mutant embryos

Phenotypic differences between RaOp and wild-type embryos appeared from 12.5 dpc onward. At 12.5 dpc, surface microvasculature in the trunk, head, proximal limb and neck, in most cases, appeared enlarged when compared with wild-type embryos (67%, n = 9; Fig. 2). Many mutants (63%, n = 11) also showed severe subcutaneous edema in the trunk and neck regions (Fig. 2A)

Vascular defects became increasingly severe in mutant embryos with age. At 13.5 dpc, all mutant embryos showed enlarged surface microvasculature, multiple regions of vascular hemorrhage and generalized edema (n = 5; Fig. 2). By 14.5 dpc, large-scale vascular hemorrhage was seen in all mutant embryos, and hematoma was visible over large areas of the trunk, neck and head, together with persistent enlarged surface microvessels, particularly around the anterior cranial region (n = 8; Fig. 2). Enlarged capillaries also frequently appeared ruptured. By this stage, edema had mostly dissipated with the degradation of epidermal tissue, and blood circulation in the yolk sac vasculature of these embryos had ceased.

These observations indicate that Sox18 mutation results in severe vascular dysfunction during mouse embryo development, and that these defects arise later in development than previously suggested (18,19). In addition, most RaOp embryos develop edema, which may be owing to either cardiovascular or lymphatic defects, or both. We have analyzed the development of the lymphatic system in Sox18 mutant mice in
a separate study (27), and focus here on a detailed characterization of blood vascular development in \( Ra^{Op} \) homozygous mutants.

**Vascular enlargement and hemorrhage in \( Ra^{Op} \) embryos**

To gain a clearer understanding of the role of Sox18 in blood vessel development, we examined the histology of \( Ra^{Op} \) mutant embryos. At 12.5 and 13.5 dpc, subdermal microvasculature was enlarged in \( Ra^{Op} \) homozygotes (\( n = 7 \); Fig. 3A, black arrowheads). On an average, microvessel diameter was 21% higher in mutant embryos than in wild-type embryos at 12.5 dpc (\( P < 0.005 \)), and at 13.5 dpc, this difference was 35% (\( P < 0.005 \)). Analysis of the distribution of vessel diameters showed an increasing trend towards larger microvessel sizes in the mutant. At 12.5 dpc, there was an increased frequency of very large microvessels (>15 \( \mu \)m) in mutants and by 13.5 dpc, vascular enlargement caused an increase in the frequency of all microvessels greater than 10 \( \mu \)m (Fig. 3B).

Vascular enlargement in \( Ra^{Op} \) homozygotes appeared to be specific to microvasculature. Enlargement of jugular veins was observed between 12.5 and 13.5 dpc in some \( Ra^{Op} \) homozygotes but was associated with the dysgenesis of associated lymph sacs (Fig. 3A, black arrowheads). Minor variations in
the size of thoracic vessels were observed in all embryos between 12.5 and 13.5 dpc, but most RaOp homozygous vessels were comparable to wild-type (Fig. 3A). From 13.5 dpc, the RaOp subdermal microvasculature also showed evidence of vascular rupture and hemorrhage (n = 5; Fig. 3A, red arrowheads). By 14.5 dpc, hemorrhage was observed throughout the subdermal mesenchyme in all embryos examined (n = 3; Fig. 3A, red arrowheads). The extent of microvascular rupture in RaOp homozygotes at 14.5 dpc (see below) was so high that it precluded the accurate measurement of vessel diameter in the subdermal tissue at this stage.

In summary, qualitative and quantitative histological analysis showed microvascular enlargement correlating with hemorrhage, from 12.5 dpc, in RaOp mutant embryos. These findings point to primary defects in blood vessel maturation and integrity in these mutants.

Loss of vascular integrity is due to endothelial rupture

We next examined the development of cytostructural components of the maturing microvasculature required for vascular integrity. Due to the effects of edematous swelling on the tissue architecture of the trunk region, only subdermal tissue from the tail region could be examined at this level. The structure of blood microvessels from wild-type and mutant embryos was compared at 12.5 and 13.5 dpc by transmission electron microscopy. Small capillary walls showed comparable morphology of endothelial cells, endothelial tight junctions and basal laminae in mutants and wild-type tissue (Fig. 4A). However, ruptures of the endothelial barrier were observed in some larger microvessels in the mutant embryos. These ruptures were found across inter-endothelial junctions (Fig. 4B, upper, arrowheads) and also through the endothelial cell membranes (Fig. 4B, lower, arrowheads), indicating that rupture was not necessarily due to the failure of junctional components in mutant vessels.

We performed a series of dye injection experiments to functionally determine the pattern, frequency and severity of the vascular ruptures (Fig. 5). Examination of the surface microvasculature of India ink-perfused embryos at 12.5–13.0 dpc showed small focal regions of ink extravasation in one embryo (n = 3; Fig. 5B). By 13.5 dpc, larger focal regions of extravasation were visible in the epidermal microvasculature of the trunk and head of all mutant embryos examined (n = 4; Fig. 5A and B). In histological sections of some

**Figure 2.** RaOp/RaOp embryos show severe vascular defects from 12.5 to 14.5 dpc. (A) Edema (white arrowheads) in some embryos at 12.5 dpc, and in all mutant embryos by 13.5 dpc, accompanied by localized hemorrhage of surface vasculature (black arrows). At 14.5 dpc, hemorrhage and blebbing in surface vasculature was visible. (B) Enlarged vessels in some RaOp/RaOp homozygous embryos from 12.5 dpc (n = 6, black arrowheads). By 13.5 dpc greater enlargement of surface vessels (black arrowheads) and hemorrhagic rupture (blue arrowhead) were observed in RaOp/RaOp homozygotes. In 14.5 dpc mutant embryos, large regions of surface vasculature exhibited severe hemorrhage. Scale bars: (A) 1 mm; (B) 250 μm.
injected embryos, the pattern of ink extravasation correlated to evidence of hemorrhage, marking vascular breaches large enough to accommodate 6 μm erythrocytes. However, examples of ink extravasation in areas of no hemorrhage were also observed (Fig. 5C), indicating the presence of smaller vascular lesions between 50 nm and 5 μm in size.

The presence of these smaller vascular breaches indicated that loss of vascular integrity was more widespread in mutant embryos than was indicated by evidence of hemorrhage and thus that loss of vascular integrity may be more severe at earlier stages (12.5 dpc), when edema was observed in the presence of little or no hemorrhage in mutant embryos.

**Figure 3.** Enlargement and rupture of blood vasculature in RaOp/RaOp embryos. (A) Subdermal vessels at 12.5–14.5 dpc (first and second row) showed enlargement (black arrowheads) and rupture (red arrowheads) in RaOp/RaOp homozygotes [scale bar (first row), 50 μm and scale bar (second row), 25 μm]. Enlargement of cephalic major vessels (third row, black arrowheads, JV, scale bars, 100 μm) was also observed in some RaOp/RaOp embryos and was associated with the dysgenesis of associated lymph sacs. The thoracic major vessels (fourth row, TA, scale bars, 25 μm) of RaOp/RaOp embryos predominantly did not appear enlarged (12.5–13.5 dpc). (B) Quantitation of lumen diameter in subdermal vessels at 12.5 and 13.5 dpc. Note that the overall shift towards larger vessels, particularly at 13.5 dpc. n = 3–4 for each time point and genotype; **P < 0.005. CA, carotid artery; JV, jugular vein; IJV, internal jugular vein; EJV, external jugular vein; LS, lymph sac; TA, thoracic aorta; CV, cardinal vein.
Endothelial hyperplasia associated with defects in pericyte recruitment

In addition to vascular hemorrhage, \( Ra^{Op}/Ra^{Op} \) embryos showed evidence of microvascular enlargement at the gross level and in histological sections of the trunk (Fig. 3A). To investigate the cause of this enlargement, we counted the number of endothelial cells per vessel cross-section in 12.5 and 13.5 dpc subdermal vasculature after hematoxylin–eosin staining, and immunofluorescence staining of 13.5 dpc embryo sections using an antibody directed against the endothelial marker, PECAM-1 (Fig. 6A). Quantitation of endothelial cell nuclei in \( Ra^{Op} \) confirmed a higher number of endothelial cells (229/95 vessels) than in wild-type (180/95 vessels; Fig. 6A). Normalization of the number of endothelial cells by vessel diameter showed a significantly greater value at 12.5 dpc in \( Ra^{Op} \) animals and no difference at 13.5 dpc (data not shown). Therefore, vessel enlargement appeared to be owing to endothelial hyperplasia rather than distension of endothelial cells.

Study of other vessel maturation mutants has shown a correlation between endothelial hyperplasia in the microvasculature and inadequate recruitment of pericytes to the maturing blood microvessels (28). We therefore tested whether this was true of \( Ra^{Op} \) mutants by immunofluorescence analysis of subdermal microvessels for NG2 proteoglycan, a pericyte marker. Pericytic investment of microvasculature was quantified by counting the proportion of subdermal vessels in immunofluorescence sections which were associated with NG2-stained

Figure 4. Endothelial wall breaches in enlarged vessels in \( Ra^{Op}/Ra^{Op} \) embryos. Transmission electron microscopy. (A) Small capillaries presented normal interendothelial tight junctions (insert, higher power view, arrows). (B) Endothelial cells of larger microvessels in wild-type embryos (left) exhibited junctions with a normal morphology and an intact endothelium (left, inset and arrows), whereas homozygous embryos (right) displayed severe disruption of the endothelial layer (upper and lower right panels, arrowheads). L, lumen; n, nuclei. Scale bars: 1 \( \mu \)m.
cells with pericytic morphology (i.e. specifically associated with the wall of the endothelial vessel). NG2-positive pericytes were found to associate with the vascular wall of both RaOp and wild-type vessels (Fig. 6B; Supplementary Material, Fig. S1). However, quantitation of sectioned microvessels in the trunk region showed that significantly fewer microvessels (9.3%) were associated with NG2-positive pericytes in RaOp at 13.5 dpc compared with wild-type (17.8%; Fig. 6B). This result indicates an impairment of pericyte recruitment to subdermal blood microvessels in RaOp mutant embryos, likely contributing to both endothelial hyperplasia and reduced structural integrity of these vessels.

Molecular analysis of vascular defects in RaOp embryos

To explore the molecular basis of the endothelial defects in RaOp embryos, we assayed expression levels of genes encoding several key proteins required for vascular structure and function. These included occludin, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-1 (Tie1), zonula occludens-1 (ZO-1), platelet-derived growth factor-B (Pdgfb), matrix metalloprotease-7 (Mmp7), interleukin-7 receptor (Il7r) and N-cadherin.

Quantitative real-time RT-polymerase chain reaction analysis was performed using 10.5 dpc tissue from the trunk region
of RaOp homozygous and wild-type siblings. No significant difference in the transcript levels of occludin, Tie1, ZO-1 or Pdgfb was observed at this stage (Fig. 7). However, Il7r, Mmp7 and N-cadherin were downregulated in RaOp homozygotes by 2.2-, 4.1- and 12-fold, respectively (Fig. 7). Thus, Il7r, Mmp7 and N-cadherin were identified as candidate downstream targets of SOX18 regulation.

DISCUSSION

Mutations in SOX18 in patients with HLT indicate a role for SOX18 in the development or function of the blood vasculature. We show here that loss of SOX18 function in mice causes defects in blood vessel maturation of microvasculature resulting in vessel enlargement and hemorrhage from 12.5 dpc, rather than defects in the initial differentiation and organization of the primary vascular plexus. Our findings illustrate the etiology of the telangiectasia associated with HLT, and suggest that SOX18 acts as a transcriptional activator of genes required for vascular structural integrity and the recruitment of pericytes to developing blood vessels.

Early versus late roles for SOX18 during vascular development

Previous studies suggested a role for SOX18 in the early specification of endothelial cells during vasculogenesis and/or the migration and proliferation of endothelial cells during angiogenesis. Sox18 is expressed during endothelial cell differentiation in mouse, chicken and zebrafish (6,10,29). A role for SOX18 and SOX7 in arteriovenous specification is reported in zebrafish (7,29,30). Over-expression of SOX18 in HUVEC endothelial cells promoted tube formation in vitro, whereas expression of dominant-negative RaOp SOX18 resulted in short tubes that failed to associate into a network (13). Expression of RaOp SOX18 in cell culture also impaired cell migration by disruption of the actin cytoskeleton, and impeded proliferation through changes in cell cycle regulation (13). Finally, in vitro assays of tube formation using liver endothelial cells of Sox18<sup>-/-</sup>/Sox17<sup>-/-</sup> mice demonstrated a reduction in branch number and vessel length during the remodeling of the primary tubes formed (31).

Our present data indicate a role for SOX18 in establishing and maintaining the structural integrity of the developing blood vessels during vessel maturation. The endothelial hyper-
plasia, focal membrane rupture and reduced ability to recruit pericytes in \( Ra^{Op} \) embryos are likely to be related phenomena, since pericytes stabilize vessel structure and induce endothelial quiescence (28,32,33). The time of death of \( Ra^{Op} \) embryos, around 14.5 dpc, is similar to that of mutant mouse models of several factors involved in vessel maturation (34–38).

Available evidence therefore suggests that SOX18 acts at multiple points in the pathway of blood vessel development. If this is the case, any early phenotypes resulting from loss of SOX18 function must be masked in \( Ra^{Op} \) mice. This phenomenon may involve the related SOX factors, SOX7 and SOX17, given their role in early vascular development (11,13,16,31,39–41).

Vascular and lymphatic defects in hypotrichosis-lymphedema-telangiectasia syndrome

SOX18 has an additional primary role in lymphatic vascular development (27). Defects specific to lymphatic failure, such as chylous ascites, have been observed in both mouse and human mutants of SOX18 (14,18,20–23), so that vascular defects alone may not explain the edema seen in \( Ra^{Op} \) embryos and HLT patients. Conversely, the edema in \( Ra^{Op} \) mutants appeared at 12.5 dpc, prior to the formation of the first functional lymphatic vessels (13.5 dpc) and prior to the age of onset of lymphedema in mouse mutants of lymphatic differentiation factors, such as PROX1 and VEGFC (42,43). These observations suggest that edema in \( Ra^{Op} \) and HLT is caused by a combination of high levels of fluid extravasation and impaired lymphatic vascular resorption.

Targets of SOX18 in vascular development

SOX18 has previously been shown to regulate two vascular endothelial target genes. One of these, \( Vcam1 \) (44) has no apparent role in vascular maturation: \( Vcam1 \)-null embryos show no generalized edema or other features of \( Ra^{Op} \) embryos (45,46). In addition, regulation of \( claudin-5 \) expression by SOX18 has recently been demonstrated in cultured endothelial cells (47). However, null mouse analysis has indicated that \( claudin-5 \) is predominantly required for the integrity of the blood-brain barrier in late-stage embryonic development (48). Claudin-5 does not appear to play a role in vascular integrity or development in other contexts (47).

Our present studies identified three factors, \( Il7r \), \( Mmp7 \) and \( N-cadherin \), that are downregulated in \( Ra^{Op} \) homozygotes. While some null animal models for \( N-cadherin \) have indicated its early requirement in embryonic vascular development, several studies have shown a pivotal role for this cell adhesion molecule in the association of pericytes with the endothelium of developing vessels (49–54). In particular, studies of the angiogenic behavior of cultured and \textit{in vivo} endothelial cells null for \( N-cadherin \) expression have shown a decrease in the recruitment of pericytes to newly forming vessels but no impairment of the earlier stages of angiogenesis (53,55,56). Given our present evidence for reduced pericyte recruitment to microvessels in the \( Ra^{Op} \) homozygote and the presence of endothelial hyperplasia, a defect associated with impaired pericyte investment, the downregulation of \( N-cadherin \) may provide a molecular mechanism to explain this aspect of the SOX18 mutant phenotype (28).

Similarly, it is possible that decreased levels of Mmp7 contribute to the \( Ra^{Op} \) phenotype. Mmp7 is required for the regulation of adherens junction stability via degradation of VE-cadherin and regulation of \( VE-cadherin \) expression during vessel formation (57–60), although Mmp7 null mice do not show \( Ra^{Op} \)-like defects (61). However, any role for \( Il7r \) in regulating blood vessel maturation and integrity remains to be determined. Previous studies have indicated that endothelial \( Il7r \) is involved in the induction of lymphangiogenesis and upregulation of endothelial cell proliferation (62,63). Future studies will focus on characterizing the regulatory relationship of SOX18 to the putative target genes \( Il7r \), \( Mmp7 \) and \( N-cadherin \), and investigating the role these factors might play in the \( Ra^{Op} \) and HLT phenotypes.
MATERIALS AND METHODS

Animals and genotyping

RaOp mice were obtained from the Jackson Laboratory and maintained on a B6/DBA/2J genetic background. Homozygous RaOp embryos (n = 55) were obtained by breeding RaOp heterozygotes (35 matings). Genomic DNA was extracted from embryonic tissue for genotyping; the RaOp Sox18 allele was detected by PCR amplification and sequencing. Mouse work was performed in accordance with the regulations of University of Queensland Animal Ethics Committee.

Quantitative polymerase chain reaction analysis

cDNA was amplified in triplicate in a reaction volume of 25 μl using SYBR Green Gene Expression Assay (Applied Biosystems) and an ABI/Prism 7000 HT thermocycler using a pre-PCR step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Preparations of RNA template without reverse transcriptase were used as negative controls. C_1 values were normalized to 18S expression (64).

Wholemount in situ hybridization

Samples were fixed overnight in 4% paraformaldehyde, and dehydrated in methanol. Whole embryos (n = 4) were hybridized overnight with riboprobe for Flk-1 (26) labeled with digoxigenin (Roche, Castle Hill, Australia) according to the manufacturer’s instructions. Embryos were then incubated with anti-digoxigenin antibody coupled to alkaline phosphatase (1:2500; Roche) until color reaction was seen.

Histology and electron microscopy

Embryos (n = 26) for histological examination were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Blocks were then sectioned (6–10 μm) and stained with hematoxylin–eosin. For transmission electron microscopy, embryo tissue was rapidly fixed in 2.5% glutaraldehyde, embedded in epon and sectioned transversely.

Immunofluorescence

Cryosectioned embryos (10 μm, n = 2) were pre-incubated with blocking solution [100 mM maleic acid pH 7.4, 10% horse serum in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBSTx)] at room temperature for 1 h, then with the primary antibody overnight at 4°C. Secondary antibody was applied at room temperature for 1 h. Slides were mounted in PBS/60% glycerol. Antibodies were rat anti-PECAM-1 (BD-Pharmingen, Palo Alto, CA, USA) and mouse anti-NG2 proteoglycan (Chemicon, Boronia, Australia).

Intravascular dye injection

Embryos (n = 11) with yolk sac and placenta intact were dissected and placed immediately into PBS at 4°C for 5–10 min. Small volumes (1–5 μl) of 10% India ink were injected into the yolk sac vasculature and allowed to circulate via the umbilical vein. After 5 min at room temperature, injected embryos were returned to 4°C for at least 10 min before analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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