

Extra-embryonic vasculature development is regulated by the transcription factor HAND1

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Summary

The basic helix-loop-helix (bHLH) transcription factor HAND1 (also called eHAND) is expressed in numerous tissues during development including the heart, limbs, neural crest derivatives and extra-embryonic membranes. To investigate the role of *Hand1* during development, we generated a *Hand1* knockout mouse. *Hand1*-null mice survived to the nine somite stage at which time they succumbed to numerous developmental defects. One striking defect in *Hand1*-null embryos was the accumulation of hematopoietic cells between the yolk sac and the amnion because of defects in the yolk sac vasculature. In *Hand1*-null yolk sacs, vasculogenesis occurs but vascular refinement was arrested. Analysis of angiogenic genes in extra-embryonic membranes showed that most are expressed at normal levels in *Hand1*-null embryos but several, including *Vegf*, *Ang1* and ephrin B2, and gene components of the Notch pathway are upregulated. In the absence of *Hand1* the expression of the

bHLH factor *Hand2* is also enhanced. Although HAND1 and HAND2 share many structural features, and *Hand2* is required for vasculature development in yolk sacs, enhanced expression of *Hand2* is insufficient to compensate for the loss of *Hand1*. The most striking aspect of the vascular defect in *Hand1* mutant yolk sacs is the abnormal distribution of smooth muscle cells. During normal angiogenesis, vascular smooth muscle precursors are recruited to the peri-endothelial tissue before differentiation, however, in *Hand1* null yolk sacs, smooth muscle cells are not recruited but differentiate in clusters distributed throughout the mesoderm. These data indicate that *Hand1* is required for angiogenesis and vascular smooth muscle recruitment in the yolk sac.

Key words: bHLH, HAND1, Angiogenesis, Smooth muscle, Yolk sac

Introduction

The two HAND transcription factors HAND1 (also called eHAND/Hxt/Thing1) and HAND2 (dHAND/Hed/Thing2) belong to the Twist family of basic helix loop helix (bHLH) transcription factors (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995). Hand genes are expressed in numerous tissues, including the heart, lateral mesoderm and neural crest derivatives. In addition, *Hand1* is expressed at high levels in extra-embryonic membranes, whereas *Hand2* is expressed at high levels in the deciduum and at lower levels in extra-embryonic membranes. Both Hand genes are expressed in similar embryonic tissues during development but often with complementary instead of overlapping expression within the tissues.

Previous studies have shown that the *Hand1* gene is essential for embryonic viability beyond 9.5 dpc (Firulli et al., 1998; Riley et al., 1998). *Hand1*-null mice exhibit numerous embryonic and extra-embryonic defects. Based on the expression pattern of HAND1, many abnormalities in *Hand1*-null mice are probably indirect and arise as a consequence of defects in extra-embryonic tissues. A requirement for *Hand1* during trophoderm development suggests that part of the

embryonic defects could be due to the failure to fully develop this lineage (Riley et al., 1998). However, experiments designed to rescue the trophoderm-induced defects did not significantly rescue the extra-embryonic or the embryonic defects (Riley et al., 2000). Another defect in extra-embryonic tissues that could account for the extensive developmental abnormalities found in *Hand1*-null embryos is defects in the yolk sac. A major defect in *Hand1*-null yolk sac is the lack of fully developed vasculature (Firulli et al., 1998). However, the regulation of extra-embryonic membrane development by HAND1 has not been investigated.

Embryonic vasculature development proceeds through a multi-step processes (Conway et al., 2001; Neufeld et al., 1999; Patan, 2000). Embryonic blood vessels initially form in the yolk sac early during development with angioblasts first appearing around 7.0-7.5 dpc in mice. These migrate, aggregate, proliferate and eventually differentiate to form a vascular plexus through the process called vasculogenesis. Endothelial cells form from the angioblasts within the mesoderm adjacent to the extra-embryonic endoderm in part through signaling by vascular endothelial growth factor (VEGF). VEGF binds two tyrosine kinase receptors implicated

in the VEGF-directed vasculogenesis and hematopoiesis. One receptor, FLK1 (KDR – Mouse Genome Informatics), is required for vasculogenesis and blood island formation (Shalaby et al., 1995). Another VEGF receptor FLT1 regulates blood island formation but is not required for endothelium differentiation (Fong et al., 1995).

Angiogenesis is the expansion and elongation of the primitive vascular network through sprouting and remodeling from pre-existing vessels. Angiogenic growth factors angiopoietin 1 and 2 (ANG1 and ANG2) play a key role in this process. ANG1 directs phosphorylation of the endothelial tyrosine kinase receptor TIE2 (TEK – Mouse Genome Informatics) and acts as a chemoattractant for endothelial cells (Gale and Yancopoulos, 1999; Suri et al., 1996). ANG2 destabilizes the smooth muscle cells that form around the endothelial cells and also induces endothelial sprouting by antagonizing ANG1 (Gale and Yancopoulos, 1999; Maisonpierre et al., 1997). Mice lacking *Tie2* show extensive vascular remodeling defects, while loss of *Tie1*, a gene closely related to *Tie2*, shows impaired blood vessel integrity (Sato et al., 1995). Vasculature refinement is thought to occur through a combination of activation and inhibition of the ANG/TIE signal transduction pathways.

In addition to their role in vasculogenesis, VEGF and its receptors FLK1 and FLT1 are required for angiogenesis by promoting the migration, proliferation and tube formation in endothelial cells (Patan, 2000). Loss of a single copy of *Vegf* results in embryonic lethality between 8.0 and 9.0 dpc resulting in vasculature defects, suggesting that the concentration of VEGF is crucial for angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). Endothelial cells respond to VEGF through the VEGF₁₆₅ specific receptors neuropilin 1 and 2 (NRP1 and NRP2) (Gitay-Goren et al., 1992). Mice lacking neuropilins have angiogenic defects similar to mice lacking VEGF (Takashima et al., 2002), suggesting that they are key receptors of VEGF mediated vascular formation.

After endothelial tubes form, vessel maturation requires recruitment of peri-endothelial cells, including vascular smooth muscle cells (SMCs) and pericytes. Platelet-derived growth factor (PDGF) BB and PDGFR β are recruitment factors for SMCs (Hellstrom et al., 1999). Transforming growth factor- β 1 (TGF β 1) also regulates SMC differentiation and recruitment although its exact roles are unknown (Orlandi et al., 1994). Mice lacking the TGF β 1 receptors TGF β RII (Hirschi et al., 1998) and endoglin (Li et al., 1999), and the TGF β signal transduction molecule Smad5 (Yang et al., 1999) all show a decrease in the number of SMCs surrounding endothelial tubes.

Several transcription factors have been identified as regulators of vasculogenesis, angiogenesis and SMC recruitment (Oettgen, 2001). We examined the role of the bHLH transcription factor *Hand1* during vascular development in yolk sacs, the tissue where blood vessel formation originates in the embryo. We have generated a *Hand1* knockout (KO) mouse line and examined the role of *Hand1* in vasculature development during yolk sac development. Our data shows that *Hand1* is not required for vasculogenesis, but is required for elaboration of the primitive endothelial plexus to refine into a functional vascular system. One function of *Hand1* during yolk sac development is the recruitment of SMCs to the endothelial network.

Materials and methods

Generation of a targeting construct

A genomic lambda library generated from 129/Sv mouse DNA was screened with *Hand1* cDNA and three overlapping *Hand1* genomic clones were isolated. These inserts were cloned into pBSKII (Stratagene) prior to restriction analysis. A construct was designed to replace part of the *Hand1*-coding region in the first exon with the gene encoding β -galactosidase (β -gal) (Fig. 1). First, β -gal from Hsp60- β -gal (a gift from Dr Janet Rossant, Samuel Lunenfeld Research Institute, Toronto) was subcloned into the *NcoI*-*Bam*HI site of Litmus 28 (New England BioLabs) to generate Lit28- β -gal. Then, the 5' region of *Hand1* was cloned as a PCR product using Vent polymerase (New England BioLabs). One primer (5'-actccatggactagtgttg-agaggctcctggcc-3') mutagenized the initiating methionine of HAND1 to generate an *NcoI* site and the other primer was located 6 kb downstream from the start of translation (5'-actccatggact-agtgagaggctcctggactgtgtgc-3'). The 6 kb PCR product was cut with *NcoI* to produce a 3.5 kb *NcoI* fragment that was then cloned into the *NcoI* site of the Lit28- β -gal. The 3' region of the KO construct was generated in pBSKII (Stratagene) by cloning the PGKNeo gene from PGKneoSXRO (a gift from Dr Richard Behringer, University of Texas, MD Anderson Cancer Center, Houston) as a *SalI*-*XhoI* fragment into the *SalI* site of pBSKII (pBSK-Neo). An *NruI*-*XhoI* fragment from the *Hand1* gene was cloned into the *HincII*-*XhoI* site of pBSK-Neo. This construct was digested with *Bam*HI and blunted with Klenow polymerase. The *Hand1*- β -gal fusion was cloned into a blunted *Bam*HI site as a blunted *Acc65I*-*XhoI* fragment. For negative selection, the MC1TK cassette was cloned into the *XhoI* site downstream of the *Hand1* gene. The *Hand1*/ β -gal construct was linearized using *NotI* prior to electroporation into ES cells.

Generation of *Hand1* targeted ES cells

The generation of targeted ES cells followed protocols outlined in Hogan et al. (Hogan et al., 1994). The *Hand1*/ β -gal knock-in construct was electroporated into CJ7 ES cells (Swiatek and Gridley, 1993) (a gift from Dr Tom Gridley, Jackson Laboratories, Bar Harbor) and ES clones containing stably integrated DNA were selected using G418 and FIAU. Clones were picked and replica plated in 96-well microtiter dishes. Targeted clones were identified by genomic Southern blot analysis using an *EcoRI*-*XhoI* fragment that flanks the KO construct as probe (Fig. 1A). Targeted clones were injected into blastocysts by the Herbert Irving Comprehensive Cancer Center Transgenic Mouse Facility at Columbia University. Three mouse lines were generated from two independently targeted ES clones.

β -Gal staining

Embryos were dissected and fixed briefly in 4% paraformaldehyde/PBS. Embryos were rinsed three times with PBS and stained in 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂/PBS containing 1 mg/ml X-gal.

Whole-mount PECAM staining

Yolk sacs were fixed in 4% paraformaldehyde/PBS for 30 minutes, blocked in immunoblock reagent (PBS, 5% goat serum, 1% DMSO) for 1 hour at room temperature and incubated with 1:200 anti-PECAM antibody (PharMingen) overnight at 4°C. Yolk sacs were washed five times with PBS and incubated with 1:500 anti-rat AP-conjugated antibody (Zymed) overnight at 4°C. After washing, a chromogen was generated using BCIP and NBT as substrates.

Section immunofluorescence and immunohistochemical analysis

Immunohistochemistry was performed on frozen sections. For detection of β -gal and PECAM, sections were incubated with rabbit anti- β -gal (5 prime-3 prime, Boulder, CO) and rat anti-PECAM antibodies, washed, and incubated with anti-rabbit rhodamine

conjugated and anti-rat FITC conjugated antibodies (Santa Cruz). To detect smooth muscle cells, rhodamine conjugated anti-mouse smooth muscle α -actin antibody (Sigma) was used.

RT-PCR analysis

Total RNA was extracted from extra-embryonic membranes using Trizole reagent (Gibco). RNA samples were treated with RNase free DNase I prior to reverse transcription. RNA was primed with oligo(dT) and the first strand was synthesized using SuperscriptII (Invitrogen). PCR amplification was with MasterPCR mix (Qiagen) with gene specific primer pairs. All samples were normalized to *Gapd*. Each PCR reaction was performed with dilution of cDNA samples to maintain the products in a linear range. Each primer pair was tested in three independent PCR amplifications for each sample. RT reactions were performed three times with different membranes. Primer sequences are available upon request.

Results

Hand1 is essential for extra-embryonic membrane formation and embryonic viability

To examine the roles *Hand1* plays during development, we targeted the *Hand1* gene in mice using a gene replacement strategy (Fig. 1A). The gene replacement targeting vector contained a β -galactosidase (β -gal) gene fused in frame to the initiation methionine of *HAND1*, ablating the first 174 amino acids of exon 1. The deleted region contained the bHLH, DNA binding and dimerization domains. The targeting strategy preserved all transcriptional regulatory elements to allow for a detailed examination of the expression pattern of *Hand1* throughout development and in adult mice by examining β -gal expression.

Hand1 ^{β -gal/ β -gal} embryos develop normally up to 7.5 dpc at which time they reabsorb when grown in a 129sv background. When the mice were outcrossed to C57/Bl6 or Swiss-Webster mice, *Hand1* ^{β -gal/ β -gal} embryos were found in the predicted Mendelian frequency up to 7.75 dpc after which the frequency of *Hand1* ^{β -gal/ β -gal} embryos decreased with a corresponding increase in the number of absorption sites. As reported previously (Firulli et al., 1998; Riley et al., 1998), *Hand1* ^{β -gal/ β -gal} embryos out-crossed appear grossly normal up to ~8.0 dpc after which time development is severely retarded and embryonic defects are more severe. However, we found a large number of embryos survive up to 9.5 dpc although they did not develop past the formation of nine somites.

Analysis of the expression of the inserted β -gal gene in *Hand1* ^{β -gal/+} embryos during development revealed that the expression pattern recapitulates the expression of the endogenous *HAND1* gene (Cserjesi et al., 1995) (Fig. 1B-E). *HAND1* is first observed in the extra-embryonic mesoderm at 7.5 dpc. High expression levels of β -gal expression are maintained in the yolk sac and the amnion in the mesodermal layer throughout development. By 7.75 dpc β -gal expression was seen throughout the lateral plate mesoderm and in the forming heart (Fig. 1B). Expression of *Hand1* is maintained in the SMCs of the gut, a lateral plate derivative, throughout development and in adult mice.

β -gal expression in the heart become progressively restricted and by 10.5 dpc β -gal activity was seen predominantly in the left ventricle and outflow tract. Expression was also seen in neural crest derived tissues of the first and second branchial arches and in the forming sympathetic nervous system (Fig.

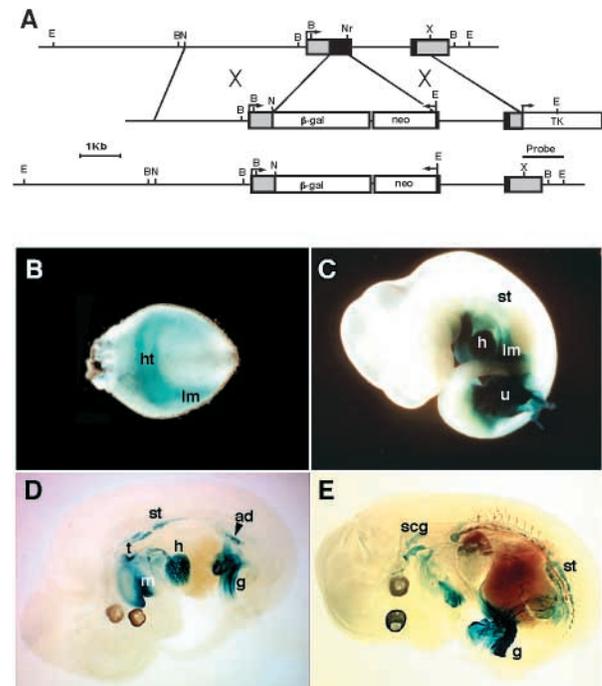


Fig. 1. Targeting strategy and expression pattern of *Hand1*. (A) A region of the first exon of the *Hand1* gene was replaced with the *lacZ* gene to monitor *Hand1* expression. The coding region is shown as black shading and the 5' and 3' untranslated regions shown as gray shading. The targeting vector is shown in the middle. The *lacZ* gene was inserted in frame with the initiating methionine of the *Hand1* gene and PGKneo gene was inserted in the opposite orientation to the transcription of *Hand1*. The MC1TK cassette was linked to the 3' untranslated region of *Hand1* to provide negative selection with FIAU. (B-E) *Hand1* expression is recapitulated by β -gal expression during *Hand1* ^{β -gal/+} mouse development at 7.75 dpc (B), 10.5 dpc (C), 12.5 dpc (D) and 14.5 dpc (E). Embryos in D and E were cleared using benzyl alcohol and benzyl benzoate (1:2 ratio). ad, adrenal gland; h, heart; ht, heart tube; g, gut; lm, lateral mesoderm; m, mandible and tongue; scg, superior cervical ganglia; st, sympathetic trunk; t, thyroid; u, umbilical cord and gut.

1C). Expression of β -gal is high in components of the umbilicus including the blood vessels and smooth muscle of the umbilical gut (Fig. 1C).

To better visualize the expression of the β -gal knock-in gene within developing embryos, we cleared later stages with benzyl benzoate and benzyl alcohol (Fig. 1D,E). By 12.5 dpc, expression of β -gal is restricted to the superior-lateral region of the left ventricle (Fig. 1D). Expression continues in the branchial arch-derived tissues, most prominently in the tongue and mandible and thymus. Extensive expression was seen throughout the developing sympathetic nervous system, including the trunk and splanchnic ganglia. Expression was also observed in the developing adrenal gland in cells of the adrenal medulla, the exocrine component of the sympathetic nervous system. Extensive expression of β -gal continues in the developing gut and by 14.5 dpc, expression of β -gal was seen throughout the gut distal to the duodenum (Fig. 1E). Within the heart, expression was found only in the apex of the left ventricle. Expression continues in the tongue and mandible at the midline and in the sympatho/adrenal lineage. After 14.5

dpc, expression decreases in all tissues, except the gut and a subset of cells of the sympatho/adrenal lineages.

Vascular abnormalities in *Hand1* mutant yolk sac

The yolk sac of *Hand1* ^{β -gal/ β -gal} embryos shows multiple abnormalities soon after formation (Firulli et al., 1998; Riley et al., 1998). Wild-type yolk sac has an extensive and highly organized vasculature filled with blood by 9.5 dpc (Fig. 2A,C) while the vasculature within the *Hand1* ^{β -gal/ β -gal} yolk sac was absent or poorly developed (Fig. 2B,D,E). In *Hand1* ^{β -gal/ β -gal} embryos, blood formation proceeds without an intact vasculature leading to extensive leakage of hematopoietic cells (Fig. 2B).

Histological analysis comparing *Hand1* ^{β -gal/+} and *Hand1* ^{β -gal/ β -gal} yolk sacs indicated that mature blood vessels were absent in yolk sacs lacking *Hand1* (Fig. 2F,G). In *Hand1* ^{β -gal/+} yolk sac, blood vessels were formed and contained hematopoietic cells (Fig. 2F). *Hand1*-null yolk sac also have other abnormalities including folded endoderm and gaps between the endodermal and mesodermal layers (Fig. 2G) (Firulli et al., 1998). The expression of β -gal in the mesodermal layer of both *Hand1* ^{β -gal/+} and *Hand1* ^{β -gal/ β -gal} yolk sacs suggests that *Hand1* expression is not regulated by an auto-regulatory mechanism.

Lack of blood vessel remodeling in *Hand1* mutant yolk sac

During normal vessel development, formation begins with

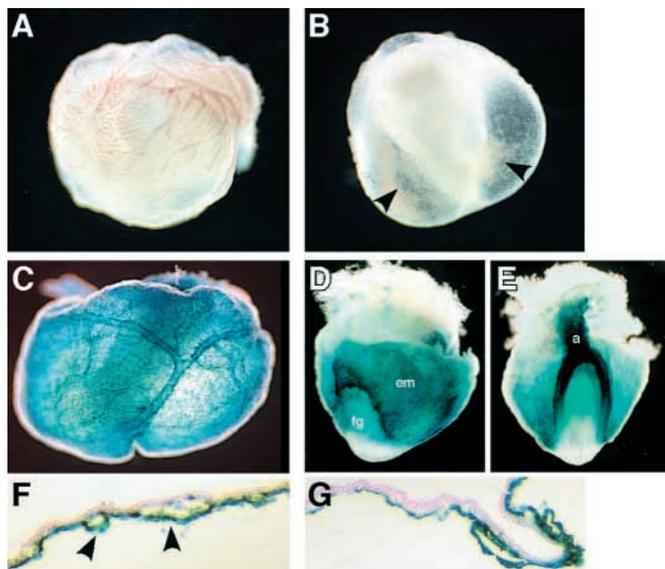


Fig. 2. *Hand1*-null embryos fail to develop an extra-embryonic vasculature. (A,C) A 9.5 dpc wild-type mouse embryo shows a well-developed vasculature within the yolk sac filled with red blood cells. (B,D,E) A 9.5 dpc *Hand1* ^{β -gal/ β -gal} embryo appears to be devoid of a vasculature containing red blood cells within the yolk sac. Arrowheads indicate red blood cells collecting within the yolk sac. (C-E) β -Gal staining. (F) Yolk sac section of 9.5 dpc wild-type mouse showing blood vessels between the endodermal and mesodermal layers (arrowheads). *HAND1* expression is restricted to the mesodermal cells in the yolk sac (blue staining). (G) Yolk sac section of 9.5 dpc *Hand1* ^{β -gal/ β -gal} yolk sac showing a disorganized mesodermal layer. a, allantois; em, extra-embryonic membrane; fg, foregut.

vasculogenesis, the aggregation of endothelial cells. In the yolk sac, vasculogenesis occurs in conjunction with hematopoiesis during the formation of blood islands. Blood islands are composed of aggregates of endothelial and hematopoietic precursor cells that form distinct lineages. We examined the organization of endothelial cells in *Hand1* mutant yolk sacs using the endothelial marker platelet endothelial cell adhesion molecule, PECAM. Immunofluorescence analysis of PECAM and β -gal expression was used to localize endothelial cells and *HAND1* expressing cells in yolk sacs. Endothelial cells were located between the extra-embryonic endoderm and mesoderm layers in both *Hand1* ^{β -gal/+} (Fig. 3C) and *Hand1* ^{β -gal/ β -gal} (Fig. 3D) embryos. *HAND1* is only expressed in extra-embryonic mesoderm and its expression pattern does not overlap with PECAM (Fig. 3E-H). The presence of endothelial cells

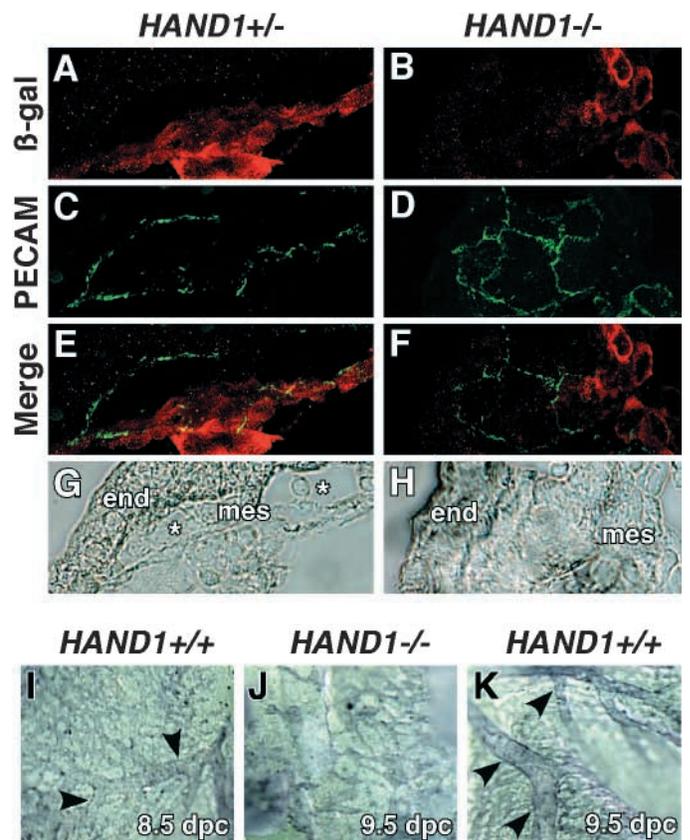


Fig. 3. Distribution of endothelial cells in *Hand1* mutant yolk sacs. *Hand1* is expressed in the mesodermal layer of the yolk sac but not in differentiated endothelial cells. (A-F) Co-localization of PECAM protein and *Hand1* expression. (G,H) Phase contrast images of A-F. Immunohistochemistry was performed with yolk sac sections of 8.5 dpc *Hand1* ^{β -gal/+} (A,C,E,G) and 9.5 dpc *Hand1* ^{β -gal/ β -gal} (B,D,F,H). Anti- β -gal antibody was used to visualize *HAND1*-expressing cells (A,B). Both yolk sacs expressed PECAM (C,D). end, extra-embryonic endoderm; mes, extra-embryonic mesoderm. Asterisks in G indicate blood vessels. (I,J) Visualization of tissues expressing PECAM in yolk sacs. Whole-mount staining of 8.5 dpc *Hand1* ^{β -gal/+} (I), 9.5 dpc *Hand1* ^{β -gal/ β -gal} (J) and 9.5 dpc *Hand1* ^{β -gal/+} littermate (K) yolk sacs with anti-PECAM antibody. Wild-type yolk sacs show integration into large vessels in both 8.5 dpc and 9.5 dpc (arrowheads). *Hand1* ^{β -gal/ β -gal} yolk sac lacks integrated vasculature.

suggests the early steps of vasculogenesis, the formation and clustering of endothelial cells is not dependent on *Hand1*.

Vasculogenesis is followed by a remodeling phase involving sprouting and branching of the endothelial cells and recruitment of support cells to form the functional vasculature. We examined the vasculature architecture in yolk sacs of *Hand1* ^{β -gal/ β -gal} embryos by whole-mount PECAM staining (Fig. 3I-K). Endothelial cells of *Hand1* ^{β -gal/ β -gal} yolk sacs were distributed in a honeycomb-like structure (Fig. 3J), characteristic of an immature vascular plexus. By contrast, the vasculature of wild-type littermates (Fig. 3K) and yolk sacs obtained from wild-type 8.5 dpc embryos (Fig. 3I) show extensive organization of both large vessels and capillary beds. Lack of refinement of endothelial cells in *Hand1* ^{β -gal/ β -gal} yolk sac suggests that vascular development in *Hand1*-null yolk sac is arrested at late vasculogenesis or during early angiogenesis.

Hand1 regulates the expression of angiogenic genes

Numerous signaling molecules, receptors and signaling transduction pathways regulate angiogenesis (Conway et al., 2001; Patan, 2000; Sullivan and Bicknell, 2003). To determine which genes were misregulated in *Hand1* mutant extra-embryonic membranes, we analyzed their expression using semi-quantitative RT-PCR (Fig. 4). All genes examined were expressed in *Hand1* mutant extra-embryonic membranes, indicating that *Hand1* is not required for their activation. However, several of the genes involved in vasculature development were misexpressed in *Hand1* mutant extra-embryonic membranes, suggesting that *Hand1* is required for their regulation.

The angiogenic growth factor genes, *Vegf* and *Ang1* were upregulated in *Hand1* mutant membranes. Both factors are co-expressed in extra-embryonic mesoderm (Patan, 2000) with *Hand1*, suggesting that they are potentially regulated by *Hand1* directly. However, hypoxia is also known to upregulate VEGF expression (Iyer et al., 1998), the regulation of VEGF we observe is unlikely to be due to a hypoxic condition of the

embryos. Embryos at the developmental stages that were used for these analysis are not dependent on a functional vasculature, instead all the requirements of the embryo and extra-embryonic tissues are met by passive diffusion. In addition, hypoxia represses *Ang1* expression (Enholm et al., 1997) and enhances the expression of the hypoxia-inducible factor *Hif1a*. In *Hand1* mutant membranes, *Ang1* expression is enhanced and *Hif1a* expression remains unchanged (data not shown). Another possible explanation for an apparent enhanced expression of *Vegf* and *Ang1* is a change in the ratio of the cell populations in wild-type versus mutant membranes. However, histological examination of mutant and normal membranes did not reveal gross differences in cell populations (Fig. 2F,G).

Angiogenesis is regulated through interactions between soluble angiogenic factors and their receptors. As VEGF and ANG1 can signal through a number of different receptors, we examined the expression of the VEGF receptors *Flk1*, *Flt1/4* and *Nrp1/2*, and the ANG1 receptor gene *Tie1/2*, all of which are expressed in endothelial cells. Of these receptors, the expression of *Flk1*, *Flt1*, *Nrp1* and *Tie1* were upregulated in *Hand1* mutant extra-embryonic membranes, while the expression of *Flt4*, *Nrp2* and *Tie2* was unchanged (Fig. 4).

The Eph/ephrin pathways play complex roles during vessel formation during development (Adams, 2002). Both ephrin B ligands and EphB receptors are expressed in yolk sac blood vessels and disruption of this pathway leads to extra-embryonic vascular defects morphologically similar to those of the *Hand1*-null mice (Adams et al., 1999). We therefore examined if Eph/ephrin signaling in extra-embryonic membranes were affected in *Hand1* ^{β -gal/ β -gal} mice. *Efnb2* was upregulated while *Efnb1* and the *Ephb* receptor expression were unaffected in *Hand1* mutant extra-embryonic membranes (Fig. 4).

Hedgehog (HH) signaling is also required during yolk sac angiogenesis, although the action of the HH family member Indian hedgehog (IHH) (Byrd et al., 2002) and the loss of IHH results in severe vascular defects. In the extra-embryonic membrane, IHH acts through binding to its receptor patched 1

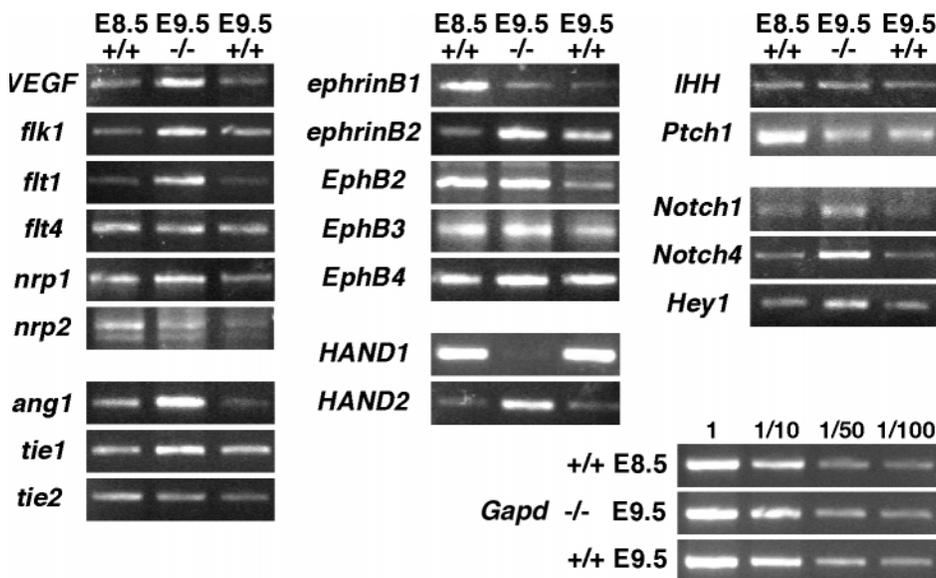


Fig. 4. RT-PCR analysis of angiogenic genes in *Hand1* ^{β -gal/ β -gal} and *Hand1*^{+/+} extra-embryonic membranes. cDNA was synthesized from RNA extracted from the individual extraembryonic membranes of 8.5 dpc *Hand1*^{+/+} embryos and 9.5 dpc *Hand1* ^{β -gal/ β -gal} and 9.5 dpc *Hand1*^{+/+} littermates. Semi-quantitative RT-PCR was performed with synthesized cDNA. Expression of growth factor *Vegf* and its receptors are shown on the left (top). Expression of angiogenic growth factor *Ang1* and its receptors are shown on the left (bottom). Expression of *Hand1* was monitored to confirm our genotype analysis. Expression of related bHLH factor *Hand2* was also examined. *Vegf*, *Flt1*, *Flk1*, *Nrp1*, *Ang1*, *Efnb2*, *Bmp4*, *Notch1*, *Notch4*, *Hey1* and *Hand2* were upregulated in *Hand1* ^{β -gal/ β -gal} extraembryonic membranes. *Gapd* was used to normalize each sample.

(*Ptch*) (Byrd et al., 2002). In *Hand1* mutant extra-embryonic membranes, neither *IHH* nor *Ptch1* transcript levels were affected (Fig. 4).

Another signaling pathway that plays numerous roles during yolk sac vasculature development is the Notch pathway (Iso et al., 2003). Loss of *Notch1* arrests angiogenesis in the yolk sac and embryo, while the loss of *Notch4* has minor effects on vascular development. The combined loss of both *Notch1* and *Notch4* leads to a more severe vascular phenotype (Krebs et al., 2000). When we examined the effect of the loss of *Hand1* on expression of *Notch1/4* (Fig. 4) we found that both *Notch1* and *Notch 4* were expressed at higher levels in *Hand1* mutant membranes. To determine if the enhanced expression of the Notch genes correlates with enhanced activity, we examined the expression of a target of notch signaling, *Hey1* (Maier and Gessler, 2000). We found that *Hey1* was also upregulated in *Hand1* mutant extra-embryonic membranes (Fig. 4), suggesting that the enhanced *Notch* expression corresponds to enhanced signaling.

Foxf1, a winged helix transcription factor, is involved in epithelial-mesenchymal interactions and loss of *Foxf1* leads to vasculature defects. The extra-embryonic membranes of *Foxf1*-null embryos morphologically resemble those of the *Hand1* ^{β -gal/ β -gal} mice. To determine if *Hand1* regulates vascular development through the regulation of *Foxf1*, we examined the level of *Foxf1* transcripts in *Hand1* ^{β -gal/ β -gal} membranes. *Foxf1* transcript levels were unaffected by the loss of the *Hand1* gene (data not shown), suggesting that the genes regulate vascular development through different mechanisms.

Regulation of *Hand2* expression by *Hand1*

The two HAND proteins share many structural features and function in a similar manner to regulate limb polarity (McFadden et al., 2002). However, during heart development, the Hand genes have complementary expression patterns (Cserjesi et al., 1995; Srivastava et al., 1995) suggesting that they may repress each other's expression. Both *Hand1* and *Hand2* are expressed in the yolk sac mesoderm and *Hand2* mutant embryos show defects in yolk sac angiogenesis and in neural crest-derived vascular smooth muscle development within the embryo. In the yolk sac, *Hand2* regulates the VEGF receptor *Npr1*, suggesting a direct role in yolk sac vascular development (Yamagishi et al., 2000). We examined the potential interaction between the two Hand genes during yolk sac formation. *Hand2* transcripts are upregulated in *Hand1* mutant embryos (Fig. 4). This is consistent with the proposed role of HAND1 as a negative regulator of *Hand2* (Bounpheng et al., 2000; Cross et al., 1995). As the upregulation of *Hand2* did not rescue the *Hand1* mutant phenotype, *Hand1* and *Hand2* appear to regulate vascular development through different pathways.

To determine if *Hand2* also regulates *Hand1*, we examined the expression of *Hand1* in *Hand2* mutant embryos (a gift from Dr Eric Olson, University of Texas Southwestern Medical Center, Dallas) by whole embryo β -gal staining. When we examined the expression of the β -gal knock-in gene in a *Hand2*^{-/-} background (Fig. 5). We found that both the level and pattern of *Hand1* expression were the same in wild type and *Hand2* mutant backgrounds. RT-PCR was used to quantify the levels of *Hand1* transcripts in *Hand2* mutant and wild-type extra-embryonic membranes. *Hand1* transcript levels were not

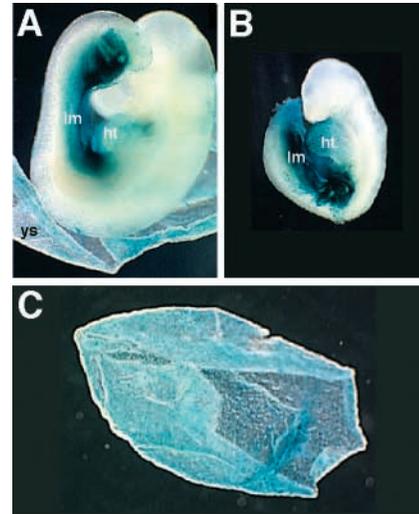


Fig. 5. *Hand1* expression in yolk sacs is not regulated by *Hand2*. The expression of *Hand1* was examined in yolk sacs of *Hand1* ^{β -gal/+}*Hand2*^{-/-} mice by staining for β -gal activity. Wild-type (A) and *Hand2*-null embryo (B) showed similar expression patterns of *Hand1*. *Hand1* was broadly expressed in the mesoderm of *Hand2* null yolk sacs (C). All embryos are to the same scale. ht, heart; lm, lateral mesoderm; ys, yolk sac.

affected by the genetic backgrounds (data not shown), indicating that *Hand2* does not regulate *Hand1* expression in extra-embryonic membranes.

Regulation of smooth muscle cell recruitment by *Hand1*

In blood vessel development, endothelial cells pattern the vasculature and are surrounded by smooth muscle cells and pericytes during maturation to give vessels the strength to carry blood under pressure. As endothelial cells develop in *Hand1* mutant mice, we asked if the leakage of blood was due to defects in tube maturation. Using the early SMC marker smooth muscle α -actin (SMA), we examined whether SMCs are recruited to the vascular plexus in *Hand1* mutant yolk sacs. Yolk sac sections were analyzed by double labeling of SMA for SMCs and β -gal to monitor *Hand1* expression. In *Hand1* ^{β -gal/+} yolk sacs, SMA is expressed around the endothelial cells and peri-endodermal cells (Fig. 6A,D,G), indicating normal migration and recruitment of SMCs. In *Hand1* ^{β -gal/ β -gal} yolk sacs, SMA-positive cells were found in extra-embryonic mesoderm but rarely seen surrounding blood islands adjacent to endothelial cells (Fig. 6B,E,H). The SMA-expressing cells were scattered in clusters within the extra-embryonic mesoderm (Fig. 6C,F,I).

As SMA is an early marker for SMCs and pericytes, an alternative explanation for the defect in *Hand1* mutant yolk sacs is an inability of SMCs to terminally differentiate. To address this further, we examined the expression of the SMC markers, SM22- α and calponin by RT-PCR. All SMC markers tested were upregulated in the *Hand1* mutant extra-embryonic membranes (Fig. 7A), indicating that the ability of SMCs to differentiate is unaffected in *Hand1* mutant yolk sac.

Yolk sac smooth muscle cells are thought to be of mesodermal origin (Gittenberger-de Groot et al., 1999).

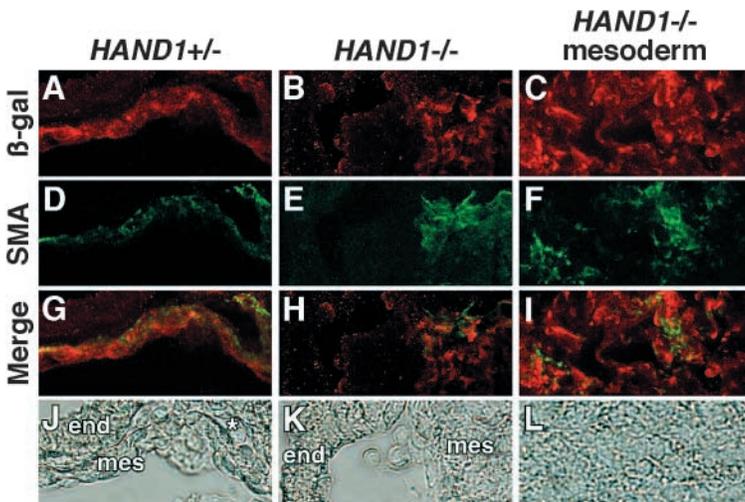


Fig. 6. Distribution of SMCs in yolk sacs. *Hand1* is required for the SMC in yolk sacs to migrate from the mesoderm and surround the endothelial tubes of the developing vasculature. Sections of yolk sacs from 8.5 dpc *Hand1*^{β-gal/+} (A,D,G,J) and 9.5 dpc *Hand1*^{β-gal/β-gal} mice (B,C,E,F,H,I,K,L) were stained with anti-β-gal antibody to visualize *Hand1* expression (A-C) and anti-SMA antibody to visualize SMCs (D-F). SMCs were located in the peri-endothelial and peri-endothelial cells in *Hand1*^{β-gal/+} yolk sac (D). In *Hand1*^{β-gal/β-gal} yolk sacs, SMCs were rarely found in regions surrounding the developing vasculature (E). (G-I) Merged images of A-F. (J-L) Phase contrast images of A-I. Instead, SMCs were scattered in clusters in extra-embryonic mesoderm of *Hand1*^{β-gal/β-gal} yolk sacs (F). end, extra-embryonic endoderm; mes, extra-embryonic mesoderm. Asterisk indicates blood vessel. C,F,I,L are images of *Hand1*^{β-gal/β-gal} extra-embryonic mesoderm only.

Endothelial cells signal to SMC precursors that then migrate to peri-endothelial tissue and differentiate into SMCs. As differentiated SMCs were found in *Hand1* mutant mesoderm, the molecular pathways that regulate SMC migration and recruitment may be defective. TGFβ1 and PDGFβ stimulate SMC migration by signaling through their receptors PDGFRβ, TGFβRII, endoglin and the signal transduction molecule SMAD5 (Conway et al., 2001). Mice lacking the genes for TGFβRII (Oshima et al., 1996), endoglin (Li et al., 1999) and SMAD5 (Yang et al., 1999) all show defects in yolk sac angiogenesis. Expression of the genes for TGFβ1, PDGFB and their receptors was analyzed in *Hand1* mutant extra-embryonic membranes by RT-PCR and shown to be unaffected (Fig. 7B).

As VEGF and ANG1 are known to regulate smooth muscle recruitment as well as vasculature remodeling, and the levels

of VEGF and ANG1 were upregulated in *Hand1* mutant yolk sacs (Fig. 4), we examined the localization of both proteins in yolk sac using anti-VEGF and anti-ANG1 antibodies. The distribution of VEGF and ANG1 was unaffected in *Hand1* mutant yolk sac (data not shown).

Discussion

Owing to the early embryonic lethality associated with loss of *Hand1* in mice, the role *Hand1* plays during development has not been examined in detail. We investigate the role of HAND1 in extra-embryonic membrane development by deletion of the *Hand1* gene through a gene replacement strategy. Loss of *Hand1* leads to severe defects in both extra-embryonic tissues and tissues within the developing embryo. Most defects observed within the embryo cannot be explained by a cell-autonomous role for *Hand1*. The abrupt arrest of growth and development in the embryo suggests *Hand1* regulates a key developmental checkpoint.

The extra-embryonic membrane is a region where *Hand1* is normally expressed that is severely affected by loss of *Hand1* function. Severe disruption of the vasculature occurs in the yolk sac of *Hand1*-null embryos. The data presented here suggest that *Hand1* is required for maturation of the vasculature after extensive vasculogenesis has occurred and that *Hand1* is required for both elaboration of the primitive vasculature and recruitment of SMCs. Interestingly, the lack of recruitment of SMC to the vasculature did not arrest SMC development.

Hand1 is expressed in the extra-embryonic mesoderm throughout development. The number of mesodermal cells in the *Hand1*-null membranes does not appear to be affected, suggesting that *Hand1* is not required for their formation and survival. However, the yolk sac mesodermal layer is disorganized with extensive detachments of the mesoderm from the endoderm. Another major defect in *Hand1*-null yolk sacs is the loss of blood from the vasculature because of vascular defects. In the yolk sac, hematopoiesis accompanies angiogenesis in discrete clusters of cells that form the blood islands. This represents the beginnings of vascular development and hematopoiesis in mammals.

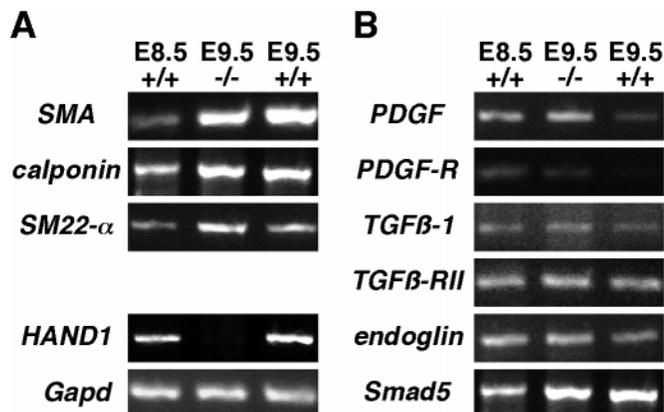


Fig. 7. The yolk sacs of *Hand1*^{β-gal/β-gal} contain differentiated SMC and express genes required for SMC recruitment. Semi-quantitative RT-PCR was performed with cDNA from extra-embryonic membranes of 8.5 dpc *Hand1*^{+/+} embryos and 9.5 dpc *Hand1*^{β-gal/β-gal} and 9.5 dpc *Hand1*^{+/+} littermates. (A) Analysis of SMC specific differentiation markers. Early and late markers of SMC differentiation were expressed in *Hand1*^{β-gal/β-gal} extra-embryonic membranes. (B) Analysis of SMC migration/recruitment signaling genes. Expression of these genes was not significantly different between wild type and mutant.

Regulation of angiogenesis by *Hand1*

Vascular development occurs in two stages: vasculogenesis, in which angioblasts differentiate into endothelial cells to form the vascular primordium; followed by angiogenesis, when the primitive vascular network is extended by budding and branching. While endothelial cells differentiate in *Hand1* mutant yolk sacs, remodeling of the endothelial cells is defective. The distribution of endothelial cells in a honeycomb-like plexus in *Hand1* mutant yolk sacs is similar to that seen in mice carrying mutations for the angiogenic receptor tyrosine kinases *Tie1/2* (Sato et al., 1995) and VEGF receptor neuropilin 1/2 (Takashima et al., 2002). Thus, in yolk sacs lacking *Hand1*, vasculogenesis occurs but angiogenesis is arrested at an early stage.

Angiogenesis is directed by a signaling system combining a number of soluble growth factors that signal through a set of receptors. In *Hand1* mutant yolk sac, angiogenic genes are expressed showing that *Hand1* is not required for their activation. However, several angiogenic genes are misexpressed in *Hand1* mutant extra-embryonic membranes. The angiogenic growth factor genes, *Vegf* and *Ang1* are upregulated in *Hand1* mutant membranes. Overexpression of VEGF (Larcher et al., 1998) or ANG1 (Suri et al., 1998) in transgenic mice produces increased vascularization during embryogenesis and in adults. It is unclear why increased expression of VEGF and ANG1 would lead to a defect in vascular development. It is possible that the misregulation of these factors in combination with other angiogenic genes leads to the vascular phenotype. It has been reported that ANG1 regulates angiogenesis in a paracrine manner (Suri et al., 1996) and that ANG1 acts as a chemoattractant; thus, the local concentration of the signal is likely to influence angiogenesis.

The expression of the VEGF receptors *Flk1*, *Flt1* and *Nrp1*, and ANG1 receptor *Tie1* is all upregulated in *Hand1*-null yolk sac. As these genes are expressed in the endothelial lineage that does not express *Hand1*, upregulation of these genes is not a cell autonomous effect. Because expression of *Flk1*, *Flt1*, *Nrp1* and *Tie1* is enhanced by VEGF (Barleon et al., 1997; Kremer et al., 1997; McCarthy et al., 1998; Oh et al., 2002), the increased expression of these genes may be a result of enhanced VEGF signaling in *Hand1* mutant mice. The levels of the VEGF receptor genes that are not regulated by VEGF signaling, *Flt4* and *Nrp2* (Oh et al., 2002), were not affected in *Hand1*-null yolk sacs, supporting an indirect role for HAND1 in regulating VEGF receptor genes.

VEGF is an upstream signal of the Notch pathway in arterial endothelial differentiation (Lawson et al., 2002). The enhanced activity of Notch in combination with other signaling pathways may lead to the vascular phenotype we observe in *Hand1*-null mice. The enhanced expression of the *Notch1/4* genes in *Hand1* mutant yolk sac, and the upregulation of the downstream gene *Hey1*, suggests enhanced Notch function. Enhanced Notch4 activity produces angiogenic defects (Leong et al., 2002; Uyttendaele et al., 2001) suggesting that HAND1 functions in part by regulating the Notch pathway during vascular development. *Hand1* may regulate the Notch pathway through enhanced expression of *Vegf* or by direct regulation of the Notch genes.

Another signaling pathway that is enhanced in the absence of *Hand1* is the Eph/ephrin family of receptors and ligand. In the absence of *Hand1*, *Efnb2* is up-regulated. Overexpression

of ephrin B2 in mouse embryos results in abnormal blood vessel formation (Oike et al., 2002), suggesting its overexpression in the yolk sac may contribute to the vascular defects.

Regulation of *Hand2* expression by *Hand1*

A wide and diverse array of transcription factors is required for development of the vasculature (Oettgen, 2001). For example, the Ets family members ELF1, FLI1 and TEL; the MADS box family member MEF2C; bHLH family members SCL/tal1 and EPAS and the nuclear receptor COUP-TFII (NR2F2 – Mouse Genome Informatics) are known to regulate yolk sac vascular development. A number of these factors have been shown to regulate angiogenesis through the direct regulation of angiogenic genes. Loss of the Ets family transcription factor members *Elf1* (Dube et al., 2001), *Fli1* (Hart et al., 2000) and *Nerf2* (Dube et al., 1999) leads to reduced expression of the ANG1 receptor *Tie2* while expression of *Ang1* is reduced in COUP-TFII knockout mice (Pereira et al., 1999). The lack of total loss of angiogenic gene expression by the loss of an individual transcription factor suggests that several different transcription factors simultaneously regulate angiogenesis. In contrast to other transcriptional regulators of angiogenic genes, where loss of function leads to decreased expression, the loss of *Hand1* results in enhanced expression of angiogenic genes. If HAND1 regulates angiogenic genes directly, it appears to suppress their expression. This is consistent with previous results that show that HAND1 can act as a negative or positive regulator of transcription (Bounpheng et al., 2000; Cross et al., 1995).

HAND1 also suppresses expression of the bHLH factor *Hand2*. *Hand2* is expressed in the yolk sac and is essential for vascular development, but these defects in *Hand2*-null mice have not been examined in detail (Yamagishi et al., 2000). As the two Hand genes are expressed in a complementary manner during heart development, this suggests that they may negatively regulate each other's expression during heart development. Our results argue that although *Hand1* regulates *Hand2* expression in the yolk sac, *Hand2* does not regulate *Hand1*. The inability of enhanced *Hand2* expression to compensate for the loss of *Hand1* suggests the Hand genes have unique functions during extra-embryonic vascular development. This is supported by the observation that the yolk sac vasculature defects in *Hand2*-null mice are not identical to those seen in the *Hand1* mutant mice.

Regulation of smooth muscle cell development by *Hand1*

During vasculature maturation, endothelial cells are surrounded by peri-endothelial cells and SMCs that give the vessels strength. In *Hand1* mutant yolk sacs, SMCs differentiate but do not surround the endothelial tubes. The loss of SMCs in the peri-endothelial region may account for the leakage of hematopoietic cells from the yolk sacs into the yolk sac-amniotic space. Mice lacking *Tie2* also exhibit hemorrhaging and the absence of peri-endothelial cells observed in *Hand1*-null yolk sacs (Sato et al., 1995). However, in *Hand1* null yolk sacs, *Tie2* expression does not appear to be affected, precluding *Tie2* misregulation as the *Hand1* target causing the abnormal distribution of the SMCs.

A number of other transcription factors are involved in

vascular SMC development. Mice lacking *Fli1* (Hart et al., 2000), *Mef2c* (Lin et al., 1998), *Smad5* (Yang et al., 1999), endoglin (Li et al., 1999) and *Hand2* (Yamagishi et al., 2000) show reduced smooth muscle development around the vessel. It is not known whether these genes are directly involved in SMC recruitment.

The abnormal distribution of SMCs in *Hand1* yolk sac mesoderm suggests SMC migration is defective. TGF β 1 and PDGF β B regulate vascular SMC migration through their receptors, TGF β RII, endoglin, PDGFR β and signal transduction factor SMAD5. We examined the expression of these genes in *Hand1* mutant yolk sac and found the levels of these transcripts are unaffected. This suggests that regulation of SMC migration by HAND1 is downstream of, or independent from, these pathways. *Vegf* and *Ang1* are also involved in smooth muscle recruitment, based on analysis of their loss of function, but it has not been determined whether enhanced expression of these two genes affects SMC recruitment. All signaling pathways that account for the SMC migration defect and whether *Hand1* acts solely through these pathways are unknown.

Upregulation of angiogenic genes in the *Hand1*-null mice can account for defective vasculature remodeling and smooth muscle migration. In *Hand1* mutant yolk sac, a number of angiogenic genes, especially the genes involving in signaling, are upregulated. It is unclear whether misregulation of these genes is the cause or the effect of the defective vasculature development.

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