Introduction

The two HAND transcription factors HAND1 (also called eHAND/Hx1/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 trophectoderm 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 trophectoderm-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 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 though 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 ANG2 from pre-existing vessels. Angiogenic growth factors ANG1 and ANG2 (Gitay-Goren et al., 1992). Mice lacking Maligne et al., 1997). Mice lacking by antagonizing ANG1 (Gale and Yancopoulos, 1999; Suri et al., 1996). ANG2 that was cloned as a PCR product using Vent polymerase (New England BioLabs). One primer (5′-acctcgactagtagttg-agaggctctggcc-3′) mutagenized the initiating methionine of HAND1 to generate an Ncol site and the other primer was located 6 kb downstream from the start of translation (5′-acctcgactagtagttg-agaggctctggcc-3′). The 6 kb PCR product was cut with Ncol 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 Sall-Xhol fragment into the Sall site of pBSKII (pBSK-Neo). An Nrul-Xhol fragment from the HAND1 gene was cloned into the HincII-Xhol site of pBSK-Neo. This construct was digested with BamHI and blunted with Klenow polymerase. The HAND1-β-gal fusion was cloned into a blunted BamHI site as a blunted Acc65I-Xhol fragment. For negative selection, the MC1TK cassette was cloned into the Xhol site downstream of the HAND1 gene. The HAND1/β-gal construct was linearized using NotI prior to electroporation into ES cells.

**Materials and methods**

**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 C57 ES cells (Swiatek and Gridley, 1993) (a gift from Dr Tom Gridley, Jackson Laboratories, Bar Harbor) and ES cells 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 PECA1 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-PECA1 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 PECA1, sections were incubated with rabbit anti-β-gal (5 prime-3 prime, Boulder, CO) and rat anti-PECA1 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 Gapdh. 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-/-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-/-gal/-gal embryos were found in the predicted Mendelian frequency up to 7.75 dpc after which the frequency of Hand1-/-gal/-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-/-gal/+-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-/-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. 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 (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.

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 sympathetic/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\(b\)-gal/\(b\)-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\(b\)-gal/\(b\)-gal yolk sac was absent or poorly developed (Fig. 2B,D,E). In Hand1\(b\)-gal/\(b\)-gal embryos, blood formation proceeds without an intact vasculature leading to extensive leakage of hematopoietic cells (Fig. 2B).

Histological analysis comparing Hand1\(b\)/+ and Hand1\(b\)/\(b\)/- yolk sacs indicated that mature blood vessels were absent in yolk sacs lacking Hand1 (Fig. 2F,G). In Hand1\(b\)/+ 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 \(b\)-gal in the mesodermal layer of both Hand1\(b\)/+ and Hand1\(b\)/\(b\)/- 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 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 \(b\)-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\(b\)/+ (Fig. 3C) and Hand1\(b\)/\(b\)/- (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. 2](image-url) **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\(b\)/\(b\)/- 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) \(b\)-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\(b\)/\(b\)/- yolk sac showing a disorganized mesodermal layer. a, allantois; em, extra-embryonic membrane; fg, foregut.

![Fig. 3](image-url) **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\(b\)/+ (A,C,E,G) and 9.5 dpc Hand1\(b\)/\(b\)/- (B,D,F,H). Anti-\(b\)-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\(b\)/+ (I), 9.5 dpc Hand1\(b\)/\(b\)/- (J) and 9.5 dpc Hand1\(b\)/+ 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\(b\)/\(b\)/- 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. 3I), 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. *Efnb1* and *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
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 Notch4 were expressed at higher levels in *Hand1* mutant embryos. 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.

**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*-null embryos (Fig. 4). This is consistent with the proposed role of HAND1 as a negative regulator of HAND2 (Boumpheng 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*-null 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 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 SMCs and β-gal to monitor *Hand1* expression. In *Hand1* mutant yolk sacs, SMA-positive cells were found in the extra-embryonic mesoderm, but only sporadically at the interface between endothelial cells and peri-endothelial cells (Fig. 6A,E,H). The SMA-positive 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).
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 PDGFβB 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, PDGFβ 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.
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, Ephb2 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-TFI1 (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-TFI1 knockout mice (Pereira et al., 1999). The lack of total loss of angiogenetic 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 Flt1 (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. TGFβ1 and PDGFBB regulate vascular SMC migration through their receptors, TGFBRII, endoglin, PDGFRβ 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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