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Rasip1 is required for endothelial cell motility, angiogenesis and vessel formation

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Abstract

Ras proteins are small GTPases that regulate cellular growth and differentiation. Components of the Ras signaling pathway have been shown to be important during embryonic vasculogenesis and angiogenesis. Here, we report that *Rasip1*, which encodes a novel Ras-interacting protein, is strongly expressed in vascular endothelial cells throughout development, in both mouse and frog. Similar to the well-characterized vascular markers *VEGFR2* and *PECAM, Rasip1* is specifically expressed in angioblasts prior to vessel formation, in the initial embryonic vascular plexus, in the growing blood vessels during angiogenesis and in the endothelium of mature blood vessels into the postnatal period. *Rasip1* expression is undetectable in *VEGFR2* null embryos, which lack endothelial cells, suggesting that *Rasip1* is endothelial-specific. siRNA-mediated reduction of *Rasip1* severely impairs angiogenesis and motility in endothelial cell cultures, and morpholino knockdown experiments in frog embryos demonstrate that *Rasip1* is required for embryonic vessel formation in vivo. Together, these data identify *Rasip1* as a novel endothelial factor that plays an essential role in vascular development.

Keywords

vasculogenesis; angiogenesis; blood vessel; endothelium; migration; proliferation; Ras; Rasip1; VEGFR2; Flk-1

Introduction

The cardiovascular system, which includes the entire network of blood vessels and the heart, is the first functional organ system to form in the embryo. Defects in the structure and/or function of the cardiovascular system inevitably lead to early embryonic lethality (Cleaver and Krieg, 1999). Initially, the vasculature emerges from aggregation of *angioblasts*. Angioblasts are endothelial precursors that arise from mesodermal cells that differentiate either within blood islands, structures composed of hematopoietic cells (blood cell precursors) surrounded by a mantle of angioblasts, or within embryonic tissues as scattered cells. Vessels subsequently

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form via v*asculogenesis*, or the coalescence of individual angioblasts "in situ" to form primitive vascular 'cords', which then undergo tubulogenesis (Risau and Flamme, 1995). The first vessels consist of a relatively simple and homogeneous endothelial cell (EC) network of vessels, often termed a 'plexus'. Subsequently, the complexity of the vasculature increases dramatically as new vessels sprout and extend from pre-existing vessels, via a process called *angiogenesis* (Risau, 1997). Angiogenic remodeling of blood vessels then transforms the initially simple, net-like, primary plexus, into a complex hierarchical network of large and small vessels, which includes specialized ECs, such as arteries and veins. As these vessels mature and stabilize, they become ensheathed by smooth muscle cells and pericytes. However, they continue to grow coordinately with organs and tissues, providing the tissues they perfuse with the nutrients and oxygen required for viability.

Most of the molecular mechanisms responsible for blood vessel formation are not yet well understood. For decades, much attention was given to the role of vascular endothelial growth factor (VEGF) and its influence on EC migration and proliferation (Ferrara et al., 2003; Yancopoulos et al., 2000). Recently, however, discovery of a host of endothelial 'guidance' cues, which either attract or repel ECs and shape individual blood vessels, has broadened our understanding of how cell-cell signaling influences the morphogenesis of individual vessels and the vascular network as a whole. These signaling molecules include the Eph-ephrins (Kuijper et al., 2007), bone morphogenetic proteins (BMPs) (Lebrin et al., 2005; Moser and Patterson, 2005; Park et al., 2006), transforming growth factors (TGFβs) (Lebrin et al., 2005), Notch and Notch ligands (Roca and Adams, 2007) and many others. In addition, a number of cell-autonomous factors have also recently been shown to be critical for proper EC behavior and blood vessel formation. Many of these factors, such as small GTPases Ras, Rho, Rac, Cdc42, Pak and their many effectors/modulators (Fryer and Field, 2005; Garnaas et al., 2008; Gitler et al., 2003; Kranenburg et al., 2004; Merajver and Usmani, 2005; Tan et al., 2008) are already known to drive basic cell processes such as cell migration, cell proliferation and establishment of cell polarity. Despite recent advances, the molecular mechanisms underlying much of blood vessel formation in vivo remain unclear, and elucidation of both extracellular signaling events and cell autonomous regulatory signaling cascades will advance our understanding of vascular specification and patterning, in both normal and pathological conditions (Coultas et al., 2005).

Many Ras family members and their regulators have been implicated in vascular development (Gitler et al., 2003; Henkemeyer et al., 1995; Tan et al., 2008), including EC migration (Sosnowski et al., 1993; Tan et al., 2008), capillary tube assembly (Connolly et al., 2002), angiogenesis (Aitsebaomo et al., 2004; Fryer and Field, 2005; Kranenburg et al., 2004; Merajver and Usmani, 2005), blood vessel homeostasis (Komatsu and Ruoslahti, 2005) and vascular permeability (Serban et al., 2008). Ras molecules are small GTPases widely shown to function as molecular switches coordinating multiple cellular behaviors like growth, proliferation, migration and differentiation. Ras GTPases cycle between the GTP-bound (active) and GDP-bound (inactive) states, under the influence of GAPs (**G**TPase **A**ctivating **P**roteins), and GEFs (**G**TPase **E**xchange **F**actors). Ras family proteins have been shown to activate signaling cascades downstream of VEGF (Cross et al., 2003; Kranenburg et al., 2004; Roberts et al., 2004). VEGF stimulation of ECs increases the amount of activated Ras, while dominant negative Ras constructs inhibit VEGF-induced endothelial proliferation, migration and assembly (Meadows et al., 2001). However, although the Ras pathway proteins have been implicated in vascular development, their exact role is not well understood.

Recently, Mitin and colleagues reported the identification of a novel Ras-interacting protein, Rasip1/Rain, which displays the characteristics of an endomembrane Ras effector (Mitin et al., 2004). Their experiments showed that Rasip1 possesses a Ras-associating domain (RA), homologous to the RA domains of other Ras effectors, and that Rasip1 preferentially binds to

the GTP-loaded form of Ras, both in vitro and in vivo. In addition, they demonstrated that transfected Rasip1 localizes to a perinuclear, juxta-Golgi region in intact COS cells and is recruited to the Golgi by active Ras. Its enrichment in adult lung and high expression in transformed EC lines suggested the possibility that Rasip1 is expressed by ECs (Mitin et al., 2006).

In an effort to discover unknown regulators of blood vessel development, we performed a microarray screen that transcriptionally profiled embryonic aortal ECs (Xu and Cleaver, unpublished). Among numerous EC-enriched transcripts, we identified *Rasip1*. Here, we show that expression of *Rasip1* is strikingly restricted to the endothelium of the developing vasculature, in both frog and mouse, and we demonstrate that *Rasip1* is essential for proper endothelial cell angiogenic assembly and migration, both in vivo and in vitro. We propose that *Rasip1* plays important roles during vasculogenesis and angiogenesis, possibly regulating the function of Ras proteins in ECs.

Materials and Methods

Isolation of *Rasip1* sequence

A pYX plasmid containing mouse *Rasip1* cDNA piece (1047–3170bp, spanning exons 4 through 7) was obtained from OpenBiosystems (**BC072584**). For making longer in situ probes, the full-length coding region (2886bp) of *Rasip1* was amplified from E8.5 mouse cDNA by RT-PCR, using 5’ primer ATGCTATCTGGTGAACGAAAG and 3’ primer TCAAGGTGTCGAAGCCACCG. PCR fragments were inserted into pGEM-T-Easy Vector (Promega) by TA cloning. *Xenopus tropicalis Rasip1* partial coding region sequence (1151bp, exon2-exon7) was cloned by RT-PCR using primers: 5’ primer ATTAAGGGAAAGAGAAGAAAGCATCT and 3’ primer GCATACAGTGTCTTGGTCAGATAATATAC. The amplified fragment was subcloned into pGEM-T-Easy Vector (Promega) by TA cloning.

Embryos and Histology

CD1 embryos were collected from pregnant females (E7.5 through E15.5) after dissection in ice-cold PBS buffer and fixed in 4% paraformaldehyde in PBS solution overnight at 4°C with gentle rocking. The amnion was removed during dissection for better probe penetration. Embryos were washed three times in PBS for 5 min, and dehydrated using a series of ethanol washes. Embryos were then stored in 75% ethanol at −20°C. Postnatal tissue was collected and fixed in a similar manner.

For wax sectioning of embryos following in situ hybridization, the embryos were fixed and dehydrated as described above. Embryos were rinsed twice in 100% ethanol for 5 min, twice in xylene at room temperature (RT) for 10 min, then a mixture of 1:1 paraplast:xylene at 60° C for 10 min, then a series of 100% paraplast at 60°C (McCormick Scientific). The embryos were then embedded and sectioned with a Biocut 2030 microtome. For examination, the sections were placed on glass slides, deparaffinized in xylene twice for 5 min each and mounted on SuperfrostPlus glass slides (Fisher) using Permount (Fisher).

Digoxigenin-labeled RNA probes

Rasip1 (in pYX-plasmid) was linearized using EcoRI and an antisense Digoxigenin(Dig)UTP-labeled RNA probe was synthesized using T3 polymerase. VEGFR2 coding region was amplified from mouse E8.5 cDNA with primers 5’-GACGGAGAAGGAGTCTGTGC and 3’-GGGACAGGACCACTTCCAT and cloned into pGEM-T-Easy vector. This VEGFR2 clone was linearized using SpeI and an antisense Dig-labeled RNA probe was synthesized using T7 polymerase. A PECAM clone, containing 950bp of 3’UTR (and kindly provided by D. Melton),

was linearized using XhoI and an antisense Dig-labeled RNA probe was synthesized using T3 polymerase. Probe synthesis was carried out at 37°C for 2 hrs: 1μg linearized plasmid, 2.0μl DIG-RNA labeling mix (Roche), 2.0μl 10X transcription buffer (Roche), 1.5μl Placental ribonuclease inhibitor (Promega), 1.0μl T3/T7 RNA polymerase (Roche), RNase free water to a final volume of 20μl. DNA template was removed using 2μl RQ1 DNase I (Promega), at 37° C for 15 min. The probes were then purified with Micro Biospin columns (Bio-RAD). 10x hybridization stock solution was prepared at a concentration of 10μg/ml in 'prehyb' solution: 50% Formamide (Fisher), 5×SSC (pH 4.5), 50μg/ml Ribonucleic acid from Torula yeast, Type VI (Sigma), 1% SDS, 50μg/ml Heparin (Sigma). Stock solution is stored at −80°C.

Whole mount in situ hybridization

Whole mount in situ hybridization in mouse embryos was carried out using a protocol adapted from D. Wilkinson's Method (Wilkinson, 1999). Briefly, embryos stored in 75% ethanol at −20°C were rehydrated in stepwise fashion to PBST. Then, the embryos were treated with 10μg/ml proteinase K (time treated varied with age of tissue; 2min-30min), fixed in a 0.2% gluteraldehyde/4% paraformaldehyde (PFA) solution, and pre-hybridized at 65°C for 1 hour. The samples were transferred into hybridization mix, containing 1μg/ ml Dig-labeled probes described above. The in situ hybridization post-hybridization washes and antibody incubation were carried out using a Biolane HTI automated incubation liquid handler (Holle & Huttner). Color development was carried out using BM purple solution (Roche). Frog in situ hybridization was carried out using a similar standard in situ hybridization protocol (Costa et al., 2003).

In situ hybridization on sections

Paraffin sections (on glass slides) were washed 3×3min in PBS, followed by a 10min treatment with 15μg/ml proteinase K. Sections were then rinsed in PBS, fixed in 4% PFA for 5 min, and incubated for 10min in acetylation solution: mix of 2.66ml Triethanolamine, 350μg HCl, 750μg acetic anhydride and 200ml water. Prehybridization was carried out in plastic slide mailers (Fisher) containing hybridization buffer at RT for 1 hour. Slides were then transferred to a humidified chamber (humidified with 50% formamide/5×SSC) for probe hybridization (probe at 1μg/ml) with 100μl probe/slide (covered with glass coverslips) at 68°C overnight.

Slides were washed post-hybridization in 2×SSC at 72°C just long enough to allow coverslips to separate. Then slides were rinsed in 0.2×SSC at 72°C and RT for 1x1min, respectively, then MBST buffer at RT (100mM Maleic acid, 150mM NaCl, pH7.5, 0.1% Tween20). Slides were incubated in blocking solution (2% blocking reagent (Roche) and 5% heat-inactivated sheep serum in MBST) for 1 hour at RT. Anti-Dig alkaline phosphatase conjugated antibody was applied on slides in a chamber humidified with MBST (250μl of 1/4000 anti-Dig antibody (Roche)), covered with parafilm and incubated at 4°C overnight. Slides were washed for 3×30min in MBST after antibody incubation, and treated in NTMT (100mM NaCl, 100mM Tris, pH9.5, 50mM MgCl2, 0.1% Tween20) for 3×5min. Color reaction was carried out using BM purple as described above. For microscopic examination, slides were sealed and coverslipped using Permount (Fisher).

VEGFR2 null embryo generation and β-Galactosidase reaction

VEGFR2 null embryos were generated by mating Flk1(VEGFR2)-lacZ heterozygous males and females (kindly provided by Drs. Janet Rossant and Eli Keshet). Embryos were dissected manually in ice cold PBS. Embryos lacking blood vessels were identified visually, by the absence of yolk sac blood vessels, and genotypes (of either embryos or adults) were confirmed by PCR, using primers to lacZ; 5'primer GGTGGCGCTGGATGGTAAGC, 3'primer CGCCATTTGACCACTACC, which yield a 630bp PCR fragment. For the β-Galactosidase reaction Flk-1(VEGFR2)+/- and Flk-1(VEGFR2)-/- embryos (or isolated organs) were fixed

in 5mM EGTA (pH 8.0), 0.2% gluteraldehyde, 2mM $MgCl_2$ and PBS solution for 15 min on ice. After fixation, embryos were rinsed 3 times for 5 min in PBS. 50 mM Potassium Ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) and Potassium Ferricyanide ($K_3Fe(CN)_6$) solutions, stored at RT in dark, were used to make lacZ staining solution: 20 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 20 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, add water or 1×PBS to 500 μl. Staining solution was warmed to 37°C before adding X-Gal (Growcells) to avoid X-Gal precipitation. 4 μl of 100 mg/ml X-Gal stock (in dimethyl formamide) was then added to the lacZ staining solution. Embryos were placed in staining solution and color reaction was allowed to develop at 37°C overnight. When staining was evaluated to be optimal, embryos were washed with PBS 3 times for 5 min each, post-fixed in 4%PFA overnight, and transferred to 80% glycerol for viewing.

siRNA transfection and endothelial cell assays

siRNAs were ordered from IDT-DNA as the TriFECTa Kit. Sequences: si*HPRT*: 5'-AAUUUCAAAUCCAACAAAGUCUGGCUU. si*Rasip1*: 5' -CCAUCUCUAGCACUUUCUCCUGUACAA. The transfection was carried out in the 24-well plate format. For each well, 1.25μl of 20μM dicer substrate siRNA was diluted in 50μl of Opti-MEM I Reduced Serum Medium (Invitrogen). 1 μl of Lipofectamine 2000 (Invitrogen) transfection reagent was diluted in another 50μl of Opti-MEM I Reduced Serum Medium. After 5 minutes incubation at RT, the diluted siRNA and the transfection reagent were combined together, and incubated for 20 minutes at RT. MS1 cells (ATCC) were plated on a 24-well plate with a density of 5×10^4 cells/well in 400μl DMEM containing 10% FBS and without penicillin/streptomycin. The pre-mixed 100μl transfection complexes were then added drop-wise on top of the cells. After gentle mixing by rocking the plate back and forth, the cells were incubated at 37°C in a 5% CO2 incubator prior to following assays.

For analyzing transcription of the targeted genes in these assays, cells were trypsinized 72 hours post transfection, and the total RNAs were isolated using an Rneasy Mini Kit (Qiagen). First strand cDNAs were made using M-MLV Reverse Transcriptase (Promega) based on manufacturer standard protocols. *Rasip1* primers: 5' primer GGAGCAGCTTACGGACTGAC, 3' primer CCATCGTCTACCAACCCAAC. HPRT primers: idtDNA HPRT primer set. β-Actin primers: 5' primer GTTGGTTGGAGCAAACATCC, 3' primer AGGGAGACCAAAGCCTTCAT. The transcripts were amplified in a 30-cycle polymerase chain reaction.

'Tube-formation assays' were carried out in a 96-well plate. 50μl of Matrigel (BD Matrigel 354234) was thawed on ice and plated on the bottom of each well. ECs cultured in one well of a 24-well plate (90% confluency) were trypsinized, plated in one Matrigel coated well of a 96-well plate and cultured at 37°C. When using wild type cells, the angiogenic aggregation of ECs (or 'tubes) starts to occur within a few hours. For better viewing, cells were stained with 1μM fluorescent dye Calcein-AM (Cell Biolabs) before microscopic examination. Quantification of angiogenic branchpoints was accomplished by counting observable branchpoints within 8 representative areas within each plated well. 'Branchpoints' are defined as the intersection point of two linear, vessel-like vascular structures, as previously defined by others (Hellstrom et al., 2007).

'Wound-healing' assays were carried out 72 hours post-transfection. Briefly, the cell monolayer is scratched using a sterile P200 pipet tip to create a 'cell-free' area (the wound, width of ~600μm). The cells were then immediately washed once with DPBS to remove detached cells from the wound area. Cells on the scratched plate are then allowed to recover and migrate into the 'cell-free' area. Images were acquired immediately after scratching and rinsing, and also after an overnight incubation at 37°C for comparison of wound width. Distance migrated was calculated as half of the total change in width.

Cell proliferation was analyzed in cultured ECs by Ki67 staining. 72 hours post-transfection of *siRasip1*, cells were washed 3 times in PBS, and then fixed in 4% PFA for 10 min at RT. For better antibody penetration, the cells were incubated in PBSN (0.1% NP-40 in PBS) for 15 min with gentle rocking. Cells were then incubated in blocking solution (5% donkey serum (sigma), 1% BSA (Fisher) in PBSN) for 30 min at RT. Primary rabbit anti-Ki67 antibody (Vector laboratories) and secondary antibodies (Alexa488 conjugated anti-rabbit, Invitrogen) were applied at 1:500 dilution in blocking solution at RT for 1 hour. Cells were rinsed 3×5 min in PBSN following each antibody incubation. Cells were then mounted in Vectashield mounting media (Vector laboratories) and examined using a Zeiss Axiovert fluorescent microscope.

Morpholino (MO) knockdown of *Xenopus Rasip1*

Xenopus tropicalis embryos were injected with 16ng *Rasip1*-MO (Gene-tools) into 1 cell at the 2-cell stage for assessment of vascular defects using in situ hybridization, or into both cells for assessment of transcript knockdown by RT-PCR. Embryos were allowed to develop to stage 32, then fixed in preparation for in situ hybridization. Morpholino-injected embryos were fixed in MEMFA (0.1M MOPS pH7.4, 2mM EGTA, 1mM $MgCl_2$, 4% PFA), transferred to 100% ethanol and stored at -20°C. For evaluation of transcript knockdown efficiency, embryos were allowed to develop to either stage 25/26 or 29/30 and frozen directly on dry ice for RT-PCR.

Results

Identification of *Rasip1* expression in murine endothelial cells

To identify sequences enriched in the embryonic dorsal aortae, we carried out Affymetrix microarray screening of aortal ECs from E8.25 mouse embryos (Xu and Cleaver, unpublished). dChip (Li and Wong, 2001) and Genespring software analysis was used to compare array data (to non-vascular array sets) and extract endothelial-enriched sequences. We initially identified *Rasip1* as an EST (**AI853551**) showing 50-fold enrichment in ECs over other tissues. A longer clone was acquired commercially (OpenBiosystems), allowing production of Dig-labeled antisense probes encompassing the region from exon 4 through exon 12 (~2000bp) of the *Rasip1* transcript. The *Rasip1* genomic structure has been previously described (Mitin et al., 2004), however no developmental expression or function has been reported.

Rasip1 is expressed in vascular endothelium during vascular plexus formation (E7.5-E10.0)

Using in situ hybridization, we characterized embryonic expression of *Rasip1* in mouse embryos and found it to be principally expressed in vascular endothelium. At E7.0 *Rasip1* is initially detected in the parietal yolk sac, in a punctate ring of cells (data not shown). Soon thereafter, at E7.5, expression expands to scattered cells of the extraembryonic yolk sac blood islands (Fig.1A). At E8.0, individual cells expressing *Rasip1* within the extraembryonic mesoderm can be observed at increasingly ventrolateral locations, in regions previously described as containing angioblasts (Drake et al., 2000;Ferkowicz and Yoder, 2005). The punctate appearance of *Rasip1* expression in extraembryonic tissues at this stage suggests that these cells are angioblasts (Fig.1B), as it closely resembles that of vascular endothelial growth factor receptor 2, *VEGFR2* (or *Flk1/KDR*), and Tal1 both established markers for early angioblasts (Drake and Fleming, 2000). At E8.25-E8.5, *Rasip1* is strongly expressed throughout the embryonic and extraembryonic endothelium in a pattern recognizable as the primary vascular plexus, including the endocardium, the forming dorsal aortae and the primordia of the cardinal veins (Fig.1C,D). During these stages, vasculogenesis of the principal embryonic blood vessels is occurring and major vessels are taking shape (i.e. parallel dorsal aortae in Fig.1D) (Walls et al., 2008).

As embryogenesis continues, *Rasip1* expression continues to be expressed in developing blood vessels. After embryonic turning, at E8.75, expression is evident in all large and small blood vessels, including the sprouting intersomitic/intersegmental vessels (ISVs) (Fig.1E). Expression of *Rasip1* within ISVs of later embryos (Fig.1E,F,K) suggests a role not only during vasculogenesis, but also during extension of vascular sprouts, or angiogenesis. Transverse sections through E8.5 embryonic tissues reveal that expression is restricted to the endothelium in all tissues examined, including the dorsal aortae (Fig.1G) and yolk sac vessels (Fig.1H). In addition, *Rasip1* is expressed within the endothelium of the endocardium, but not in myocardium (Fig.1I).

We compared expression of *Rasip1* with that of other known vascular markers, such as *VEGFR2* and *PECAM* (Fig.1J-L and Suppl. Fig.1), and found that *Rasip1* outlined almost identical vascular structures in the embryo. For instance, expression of all three markers was observed in aortae, ISVs, endocardium and vessels of the lateral plate and head mesoderm. Of note, different vascular beds appeared to express these three vascular markers with varying intensity. For instance, the head plexus of E9.5 embryos expressed *VEGFR2* more robustly, while *Rasip1* was more strongly expressed than either *VEGFR2* or *PECAM* in the ISVs and endocardium (Fig.1J-L and Suppl. Fig.1). These differences reveal surprising endothelial heterogeneity at early stages of vascular development. Nonetheless, overall expression analysis suggests that *Rasip1* is primarily restricted to vascular endothelium.

Rasip1 during late embryogenesis (E10.5-birth)

Analysis of *Rasip1* transcripts later during development reveals their expression in established vessels. Expression could indeed be detected in the blood vessels of various organs throughout midgestion stages (Fig.2 and Suppl. Fig.2). Specifically, we found *Rasip1* strongly expressed in the vessels of all embryonic organs and tissues examined, including heart (Fig.2B,E, Suppl. Fig.2B,C,E,H), lung (Fig.2B',E', Suppl. Fig.2C,D,G), head (Fig.2B"), limb bud (Fig.2E"), pancreas, spleen and stomach (Suppl. Fig.2J-L). When compared to the expression of *VEGFR2* (Fig.2 columns A,D) and *PECAM* (Fig.2 columns C,F), we found that *Rasip1* generally marked identical vascular beds, with only slight variations in expression intensity (Fig.2 columns B,E). Expression of *Rasip1* in later embryonic vessels, after their formation via either vasculogenesis or angiogenesis, implies that it has a maintenance function in mature vessels. Indeed, *Rasip1* continued to be expressed in the endothelium of vessels into postnatal stages (Suppl. Fig.3) and was detected in adult organs, particularly in the highly vascularized lung (Mitin et al., 2004).

Rasip1 expression is absent in vascularless embryos

To definitively test whether *Rasip1* is restricted to vascular endothelium, we assessed its expression in *VEGFR2* mutant embryos that lack vascular endothelium (Shalaby et al., 1995). First, we compared *Rasip1* expression (Fig.3 column B) to that of *Flk1(VEGFR2)*-lacZ (Fig.3 column A) in *VEGFR2*+/- heterozygous mice, which display no detectable abnormalities, and found both outlined the developing vasculature as expected. However *VEGFR2*-/- homozygotes, which lack all blood vessels, exhibited no trace of *Rasip1* expression by in situ hybridization (Fig.3 column C). *Rasip1* and *VEGFR2* are expressed in similar vascular domains in *VEGFR2* heterozygotes, from E8.25 to E9.0, while in *VEGFR2* null embryos we observed no *Rasip1* expression in any embryonic region. These findings suggest that *Rasip1* is expressed exclusively in *VEGFR2*-dependent cell types. Given that *VEGFR2* is primarily expressed in and required for ECs (Shalaby et al., 1995; Yamaguchi et al., 1993), it is likely that *Rasip1* is also expressed exclusively in those cell types, but not in other non-vascular mesodermal or mesenchymal cell populations. We propose that *Rasip1* is a novel and largely specific marker of embryonic blood vessels throughout development.

Rasip1 is required for angiogenesis in cultured ECs

To identify the potential role of *Rasip1* in ECs, we used an in vitro siRNA approach to knockdown endogenous *Rasip1* expression in cultured mouse ECs (Fig.4A). To identify endothelial cell lines that expressed *Rasip1*, we screened a number of lines using RT-PCR. We found that MS1, bEnd.3 and SVEC endothelial cell lines (ATCC) all expressed *Rasip1* to varying degrees, while non-vascular lines such as HEK293, Balb3 and NIH3T3 did not (data not shown), supporting the notion that *Rasip1* is a marker of endothelium. MS1 cells expressed the highest levels of both *Rasip1* and *VEGFR2*, therefore we chose this line for subsequent assays. When siRNAs targeting *Rasip1* were transfected into MS1 cells, the cells displayed an abnormal 'elongated' morphology, compared to untreated (wildtype, WT) or positive (siHPRT) control cells within 5 days of transfection (Suppl. Fig.4). This suggested a specific effect, resulting from loss-of-function of *Rasip1* in MS1 cells.

To examine whether reduction of *Rasip1* levels might have a functional impact on MS1 cell behavior, we carried out both in vitro tube-formation and wound-healing assays. These assays allowed evaluation of the effect of *Rasip1* knockdown on endothelial angiogenesis and cell motility, respectively. Strikingly, si*Rasip1*-treated MS1 cells, in which *Rasip1* transcript levels were significantly reduced (Fig.4A), almost completely lost the ability to form plexus-like vascular structures when cultured on Matrigel (Fig.4B,D-F). This observation supports the notion that *Rasip1* is required for endothelial function. To evaluate the effect, we quantified the number of branch points created by the coalescence of ECs into cords/tubes, and found that these were reduced by over 85%.

In addition, ablation of *Rasip1* function in MS1 cells also dramatically decreased EC migration ability. Using an in vitro scratch assay, the 'healing rate' of a scratch 'wound', across a monolayer of ECs, was significantly reduced. While unmanipulated or siHPRT transfected cells were able to heal the wound following overnight incubation (i.e. fill in the ~600μm cell-free wound area), si*Rasip1* transfected cells migrated only about 50% the distance over that same timeframe (Fig.4D'-F',D"-F"). In sum, control cells could migrate approximately 300μm on each side to fill in the gap, while si*Rasip1* treated cells migrated less than 150μm. It is unlikely that this effect is indirect, as a result of decreased cell proliferation, since the timeframe of the healing study is too short to allow significant proliferation within the gap (wound healing assay is carried out overnight, and MS1 doubling time is approximately 24 hrs). In support, we detect no significant difference in endothelial proliferation by Ki67 staining in si*Rasip1*-treated cells, following a short siRNA treatment of 3 days (Suppl. Fig.5). (Of note, we do detect a mild effect on EC proliferation over a longer treatment period of 6 days). Together, these results indicate that *Rasip1* function is required in cultured ECs, for both angiogenic coalescence and cell motility, and is therefore likely to play important roles in blood vessel development.

Rasip1 is required for embryonic blood vessel formation

Bioinformatic comparison of *Rasip1* sequences revealed that it was highly conserved across many different species, from human to lower vertebrates, including frog. The DILute domain of Rasip1, for instance, displayed almost 85% identity at the amino acid level between mouse and *Xenopus tropicalis* (Suppl. Fig.6). This high level of similarity suggested *Rasip1* might also be expressed in the vessels of other species, such as frog, and might play a conserved role in vessel formation. Thus, to assay *Rasip1* function during embryonic vessel formation in vivo, we examined its expression and function in *Xenopus tropicalis* embryos. This tractable model system provided us with an avenue for in vivo assays.

RT-PCR was used to amplify a fragment containing approximately 1900bp of the *Rasip1* coding region from *Xenopus tropicalis* cDNA. Using this construct, we generated Dig-labeled

probe for in situ hybridization and compared *Rasip1* expression to known vascular markers (Fig.5A-H). We note that *erg* (Fig.5A,B), *VE-Cadherin* (Fig.5C), and *msr* (Fig.5D) expression patterns outline the early vasculature in *Xenopus tropicalis*, during both vasculogenesis and angiogenesis, as previously described in *Xenopus laevis* (Baltzinger et al., 1999;Devic et al., 1996). Similarly, we found that *Xenopus Rasip1* is expressed in the developing vasculature throughout development, including early expression in emerging angioblasts (stage 25) and during vessel coalescence (Fig.5E-G). In addition, *Rasip1* is expressed strongly during angiogenesis, in sprouting ISVs (Fig.5G), and remains expressed after initiation of heart beat (st.34) and blood circulation (Fig.5H).

To determine if *Rasip1* function is required for vessel development in vivo, we targeted the sequence in *Xenopus tropicalis* embryos with antisense *Rasip1* morpholino oligos (*Rasip1*-MO) designed to inhibit splicing of *Rasip1* transcripts (Suppl. Fig.7). RT-PCR analysis with primers spanning the exon-intron boundary confirmed that *Rasip1* splicing, and hence expression of mature transcripts, was effectively abolished in embryos radially injected with 30ng of *Rasip1*-MO (Fig.5J). To specifically examine the effect of *Rasip1* knockdown on embryonic blood vessel development, we injected the *Rasip1*-MO (16 ng), into one side of the embryos at the 2-cell stage and then assayed them by in situ hybridization at stage 32 with the endothelial marker *msr* (Fig.5K-O).

We found that blood vessel development was severely inhibited in *Rasip1*-MO injected embryos as detected by *msr* staining (compare Fig.5K to L). Most strikingly, we found that the posterior cardinal vein failed to form on the injected side in 77% of injections that were assayed at later stages of development (st.33-35) (Suppl.Fig.8). In addition, we noted a significant reduction in both the number of ECs, as marked by *msr*, along the flank of the embryo, and the organization of these cells into the vitelline plexus (reduction of average branch points from 23 to 4) (Fig.5L,P). The uninjected side, in contrast, remained unaffected and displayed normal vascular structures, including a normal posterior cardinal vein (Fig. 5K,M). We also observed that sprouting ISVs failed to appear, which was not surprising as they originate from the posterior cardinal vein (Fig.5N,P). Sectioning of injected embryos revealed the distinct absence of the posterior cardinal vein and ISVs on the injected side (Fig. 5O). Quantification of these observations showed that the cardinal vein and ISVs were lost over 90% of the time, while the plexus EC branching was reduced by over 80%. We detect these effects on the vasculature using the endothelial markers *msr*, *flk1*, *erg* or *ve-cadherin* (Suppl.Fig.8). Interestingly, assays at earlier stages of development (st.20-21), using *msr*, do not reveal reduction in the number of angioblasts at the location of the posterior cardinal vein, indicating that vasculogenesis, not specification, is affected in MO-injected embryos. These experiments provide evidence that *Rasip1* is required in vivo for proper vessel development.

Discussion

This report provides the first evidence that *Rasip1* is both expressed and required in the endothelium of the embryonic vasculature. We show that *Rasip1* is specifically expressed in the endothelium of the developing blood vessels of both mouse and frog embryos. Additionally, we demonstrate that this expression initiates early, in angioblasts prior to their aggregation into vessels, and continues in established vessels, which grow and remodel via angiogenesis. We also demonstrate that *Rasip1* is fundamentally required in ECs, both in vitro and in vivo. Knocking down *Rasip1* in cultured ECs inhibits their migration and coalescence into vessel-like structures, while knocking down *Rasip1* in amphibian embryos results in failure of vessel development. These findings establish *Rasip1* as a novel and robust marker of embryonic ECs, throughout their specification and differentiation, and as a likely important regulator of vascular development.

Rasip1 is a novel endothelial marker

Few vascular genes have proven useful as specific markers of the endothelium. VEGFR2, PECAM, Tie2 and VE-Cadherin are the most frequently used markers available to date, in that they are highly enriched in ECs and are often used as specific markers. However, they are not completely endothelial specific, as they are often transiently expressed in other tissues, at some point during development. For instance, VEGFR2 is also detected in hematopoietic cells (Yamaguchi et al., 1993), PECAM is also in macrophages (Lee, 1991), Tie2 is found in mesenchymal cells of heart outflow tracts (Kisanuki et al., 2001), VE-Cadherin is also expressed in liver hematopoietic stem cells (Kim et al., 2005) and both VEGFR2 and PECAM are also found in lymphatic vessels (Enholm et al., 2001). In addition, most other commonly used endothelial markers such as Dll4, Egl7, ephrin-B2, EphB4, Jagged1, Notch1 and many more, are widely expressed in a number of other organs and tissues (Conway et al., 2001; Eichmann et al., 2005; Torres-Vazquez et al., 2003).

This report adds *Rasip1* to the short list of useful endothelial-enriched sequences, which can be used to study the developing cardiovascular system. Like *VEGFR2*, *Rasip1* transcript levels are highly enriched in embryonic ECs. This is in contrast to *PECAM* and *Tie2* whose transcript levels are lower, and thus difficult to visualize by in situ hybridization. While we have assayed commercially available antibodies to RASIP1 protein, both in vitro and in vivo, none have proven useful for either immunofluoresence or immunohistochemistry. Until effective antibody reagents become available, assays for *Rasip1* transcripts will be useful for studies ranging from examination of early angioblast specification to vasculogenesis and angiogenic vessel formation. Given its expression conservation across species, in both frog and mouse, it will very likely be more widely applicable to vascular studies in other species as well.

Rasip1 is essential for endothelial cell function

Knockdown of *Rasip1* levels in cultured ECs reveals a critical role for Rasip1 in basic cellular functions, such as cell motility and angiogenesis. When Rasip1 function is reduced, both these basic endothelial behaviors are severely abrogated. si*Rasip1*-treated cells, but not si*HPRT*-treated cells, display a reduction in their propensity to aggregate and form cords or vessels, in a matrigel angiogenesis assay. In addition, whereas untreated or control siRNA-treated ECs will normally migrate actively across tissue culture-treated plastic in vitro, such as in a ‘wound healing’ assay, cells lacking Rasip1 function fail to migrate. We propose that this reflects a direct effect on EC motility, since we detect no decrease in the rate of endothelial proliferation in si*Rasip1*-treated cells when we use Ki67 to assay dividing cells.

Given that these basic cellular behaviors are likely to comprise the foundations of vessel formation, we predicted that endothelial cells in emerging blood vessels would also require Rasip1 function. Indeed, reduction of *Rasip1* in vivo leads to a dramatic failure of embryonic vessel formation. Using a MO-based approach, we knocked down endogenous expression of *Rasip1* in *Xenopus tropicalis* embryos, which led to a clear failure of the posterior cardinal vein and associated ISVs to form. These results demonstrate that *Rasip1* is required for the proper formation of vascular structures that develop via both vasculogenesis (posterior cardinal vein) and angiogenesis (ISVs). Interestingly, vessels of the flanking vitelline plexus, within the lateral plate mesoderm, while disorganized when Rasip1 function is reduced, were not completely abrogated. The basis for the difference in the response of these different vascular beds to the absence of *Rasip1* is unclear. However, it has been proposed that inherently different populations of hematopoietic, and perhaps endothelial, cells arise within these different embryonic regions (Kau and Turpen, 1983; Maeno et al., 1985). It is possible that they may represent ‘primitive’ (ventral/flank) versus ‘definitive’ (dorsal lateral plate/somite) ECs, and that these two populations have a differential requirement for *Rasip1* function.

Rasip1 is not required for angioblast specification

Significantly, despite disruption of vessels when Rasip1 function is knocked down in frog embryos, angioblasts still emerge within the mesoderm. This finding indicates that Rasip1 plays a role sometime after initial angioblast specification. This observation is supported by the timing of *Rasip1* expression initiation during vessel development in frogs, as angioblast specification is conveniently separated in time from the process of vessel formation via vasculogenesis. In frogs, angioblasts are specified within the mesoderm of late neurula stage embryos (st.18-22), many hours prior to vessel formation that occurs at the late tailbud stage (st.30-32). We find that *Rasip1* expression in frogs initiates later (st.22) than *VEGFR2* (st.18) (Cleaver et al., 1997), thus displaying a marked delay and appearing distinctly later than the earliest known angioblast markers. In addition, when Rasip1 function is knocked down using MOs, we observe that early angioblasts emerge relatively normally as assayed by *msr* expression at st.20-21. We therefore suggest that *Rasip1* is likely to function in angioblasts and ECs following their initial specification, possibly during their migration, cord formation or tubulogenesis.

In contrast, we observe that *Rasip1* expression in mouse appears to be initiated remarkably early, around the same time that *VEGFR2* and other vascular markers begin transcription within the earliest endothelial precursors of the yolk sac. This is not surprising since most murine endothelial markers initiate almost simultaneously during a relatively short timeframe, making it difficult to establish the order of vascular gene onset (Drake and Fleming, 2000). It will therefore be necessary to carry out more detailed expression analyses of vascular markers, or functional epistases experiments, during this initial phase of vasculogenesis to determine when Rasip1 may exert its function and to place it within potential regulatory genetic cascades.

Summary

Data presented here supports a requirement for Rasip1 function within developing ECs during blood vessel formation. Knockdown of *Rasip1* in frog embryos results in failure of the posterior cardinal vein and ISVs to develop and in disorganization of the vitelline plexus. However, at this point, the cellular and molecular mechanisms that prevent the proper formation of these vessels remain unclear. At the cellular level, it is likely that the observed vascular defects are a result of either a reduction of angioblast survival, or a failure of cord formation via vasculogenesis or vascular tube formation, with subsequent cell death or dedifferentiation. At the molecular level, it is not yet clear how, when and whether Rasip1 modulates Ras downstream of VEGF signaling, or whether it impacts alternative pathways. Studies are currently underway to clarify these molecular relationships during vascular development.

Together, our studies demonstrate the critical role of the Ras effector Rasip1 for proper vessel formation and normal endothelial cell behavior. In addition, we identify *Rasip1* as a novel tool for studies of embryonic vessel development. In vitro experiments demonstrate that *Rasip1* is required for EC migration and coalescence of angioblasts into vessels, while in vivo experiments show that it is required for formation of blood vessels in frog embryos. Due to its conservation, we predict that *Rasip1* will also be required for proper formation of the vasculature in other species, including mammals. Further studies of *Rasip1* will open novel and exciting questions regarding the role of intracellular signaling molecules in vascular development, including members of the Ras family, both areas which to date have been relatively unexplored. Given the central importance of blood vessels during many diseases, such as cancer and tumor angiogenesis, we propose that further understanding of the mechanism and impact of Ras and Rasip1 signaling in ECs will prove to be of great clinical relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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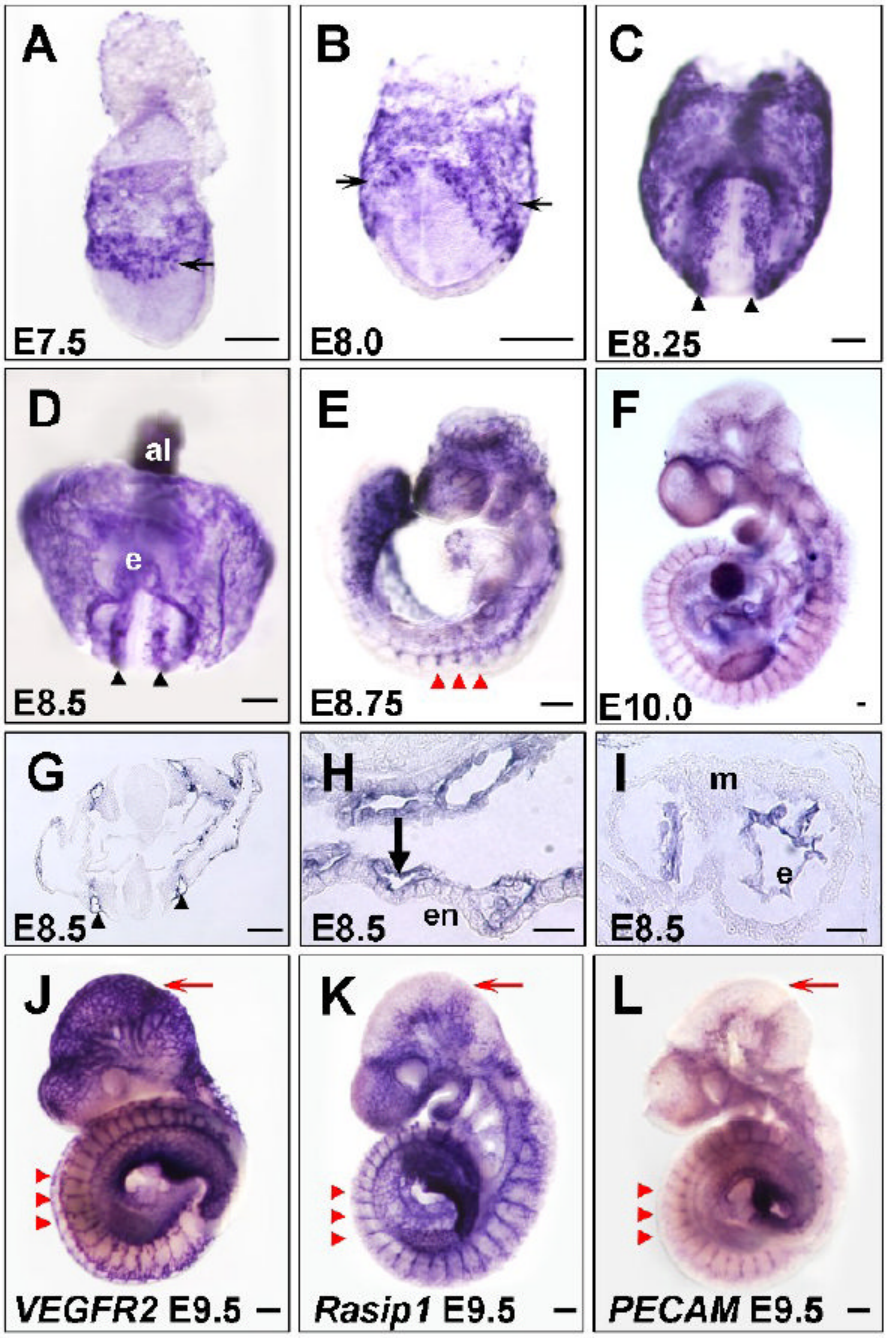

Figure 1. Expression of *Rasip1* in vascular endothelium during early embryogenesis

In situ hybridization showing expression of *Rasip1* in embryonic vessels at stages indicated (A-I, K). A-F) Whole mount in situ hybridization showing whole stained embryos. Note expression in both scattered angioblasts (thin arrows), forming dorsal aortae (black arrowheads), intersomitic vessels (ISVs) (red arrowheads) and yolk sac vessels (thick arrows). G-I) Transverse sections of in situ hybridizations showing endothelial-specific expression of *Rasip1* in G) dorsal aortae, H) yolk sac vessels, and I) heart endocardium. J-L) Comparison of *Rasip1* expression with that of the vascular markers *VEGFR2* and *PECAM*, in E9.5 embryos. Note overall similarity of expression, especially in the ISVs (red arrowheads) and trunk vessels. Note difference in intensity of vascular staining in distinct regions, such as the cephalic vessels

(red arrows). al, allantois; e, endocardium; en, endoderm; m, myocardium. The scale bars represent 200μm in all panels except J-L, where they represent 50μm.

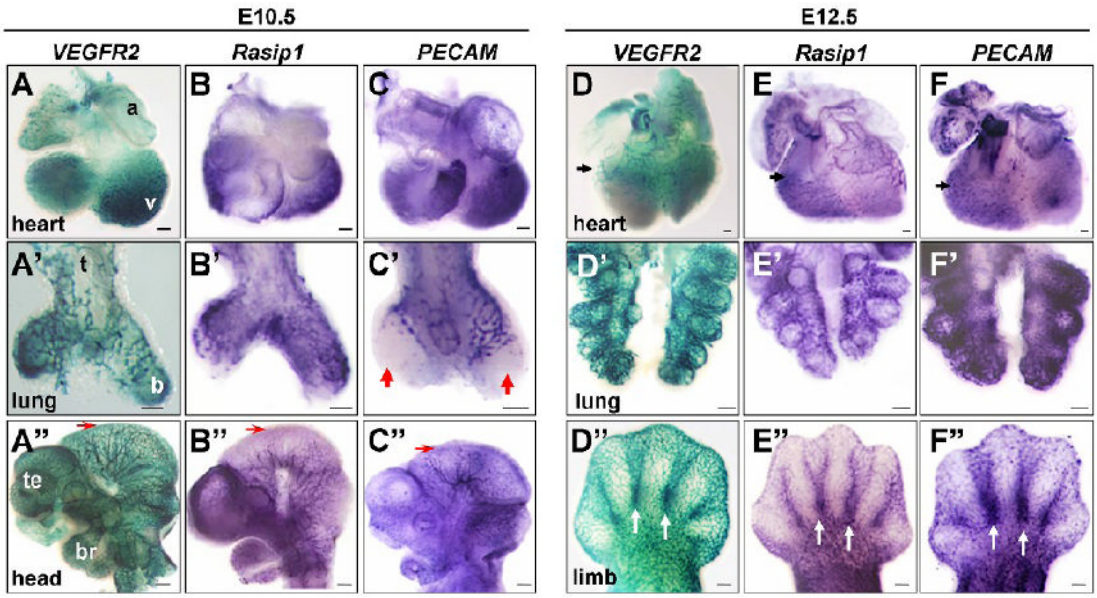

Figure 2. Vascular expression of *Rasip1* in embryonic organs and tissues

Flk1(*VEGFR2*)-lacZ whole mount β-galactosidase staining and whole mount in situ hybridization of vascular markers in isolated embryonic tissues, at stages indicated. A,D columns) Whole mount β-galactosidase staining using *Flk1*(*VEGFR2*)-lacZ embryos. Whole mount in situ hybridization of *Rasip1* (B,E columns) and *PECAM* (C,F columns). A-F) Hearts. A'-F') Lungs. A"-C") Heads. D"-F") Limb buds. Note similarity of expression of *Rasip1* in most vessels, as marked by *VEGFR2* and *PECAM* expression (black arrowheads). Expression of all three vascular markers can be observed in the endocardium of the ventricle trabeculae in the heart (A-C) and of the coronary vasculature (arrows, D-F). Expression of all three markers is evident in the proximal ECs of the early lung buds (A'-C'), although *PECAM* is not expressed in the ECs of the most distal tips of the buds at E10.5 (red arrowheads), while both *VEGFR2* and *Rasip1* are observed in this population. This heterogeneity is also observed in the cephalic vessels at E10.5, where *VEGFR2* is robustly expressed in the most mediolateral/distal vessels of the mesencephalon, while *Rasip1* and *PECAM* are expressed at lower levels (red arrows, A"-C"). Rasip1 is expressed in the vessels of the developing limb buds, including the interdigit vessels (white arrows, D"-F"). a, atria; b, bronchus; br, branchial arches; t, trachea; te, telencephalon; v, ventricle. The scale bars represent 100μm in A-F' and 250μm in A"-F".

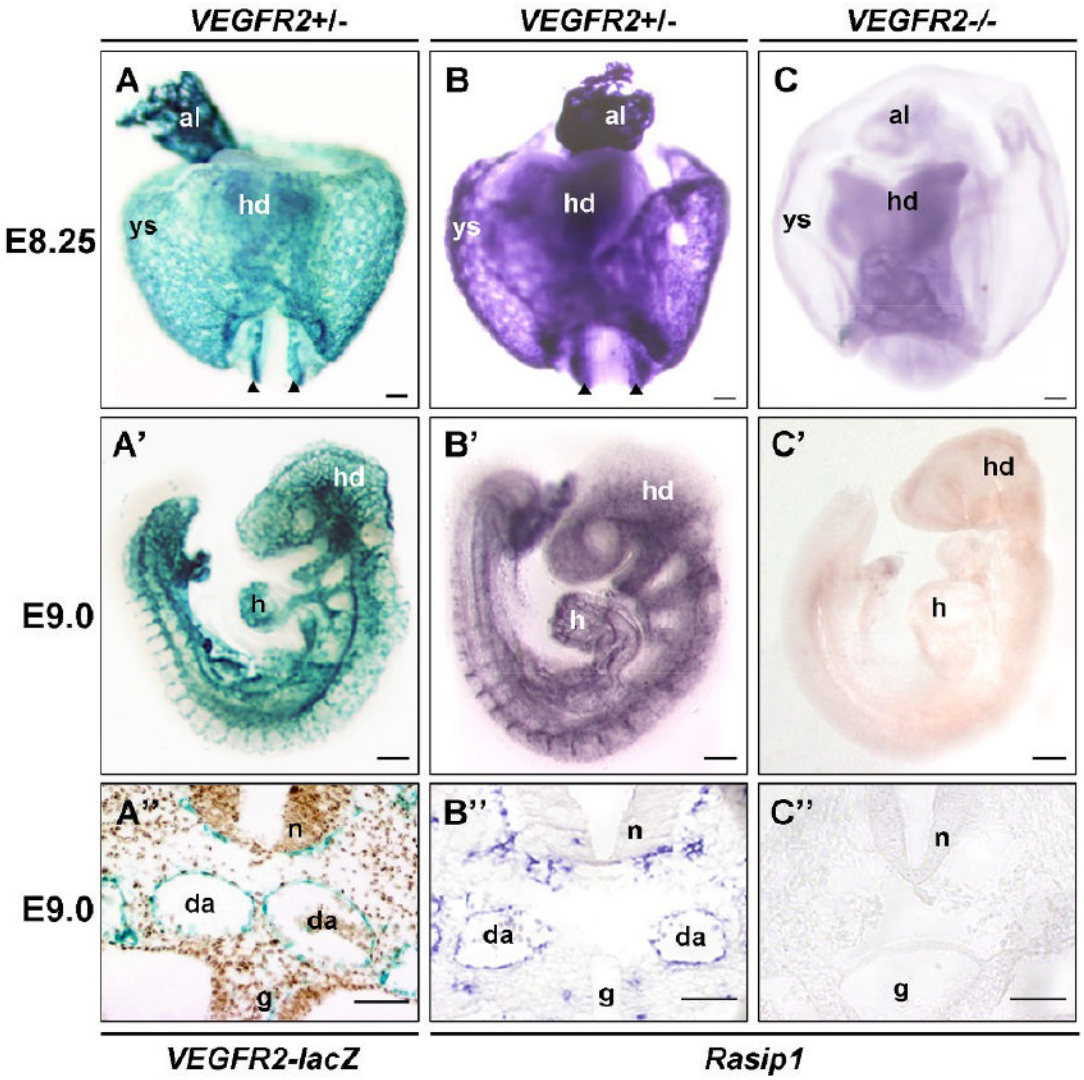

Figure 3. Expression of *Rasip1* is restricted to *VEGFR2*-dependent endothelium

Whole mount in situ hybridization and β-galactosidase staining to detect expression of *Rasip1* and *VEGFR2*. A,B,A',B') Comparison of *Flk1*(*VEGFR2*)-lacZ staining and *Rasip1* expression. Note overall similarity of expression. *Rasip1* expression closely resembles *Flk1* (*VEGFR2*)-lacZ expression at both E8.5 (A,B) and E9.0 (A',B'). C,C') VEGFR2-/- null embryos, lacking all endothelium. Embryos in C-C" have been stained by in situ hybridization for *Rasip1* expression and allowed to develop same length of time as wildtype embryos in B-B". Note complete lack of *Rasip1* expression in these mutants. A"-C") Sections through embryos in A'-C' showing presence of aortae and perineural vascular plexus in wildtype embryos, while these vascular structures are missing in VEGFR2-/- embryos. al, allantois; g, gut tube; h, heart; hd, head; da or black arrowheads, dorsal aortae; n, neural tube; ys, yolk sac. The scale bars represent 200μm (A'-C') and 50 μm (A-C, A"-C").

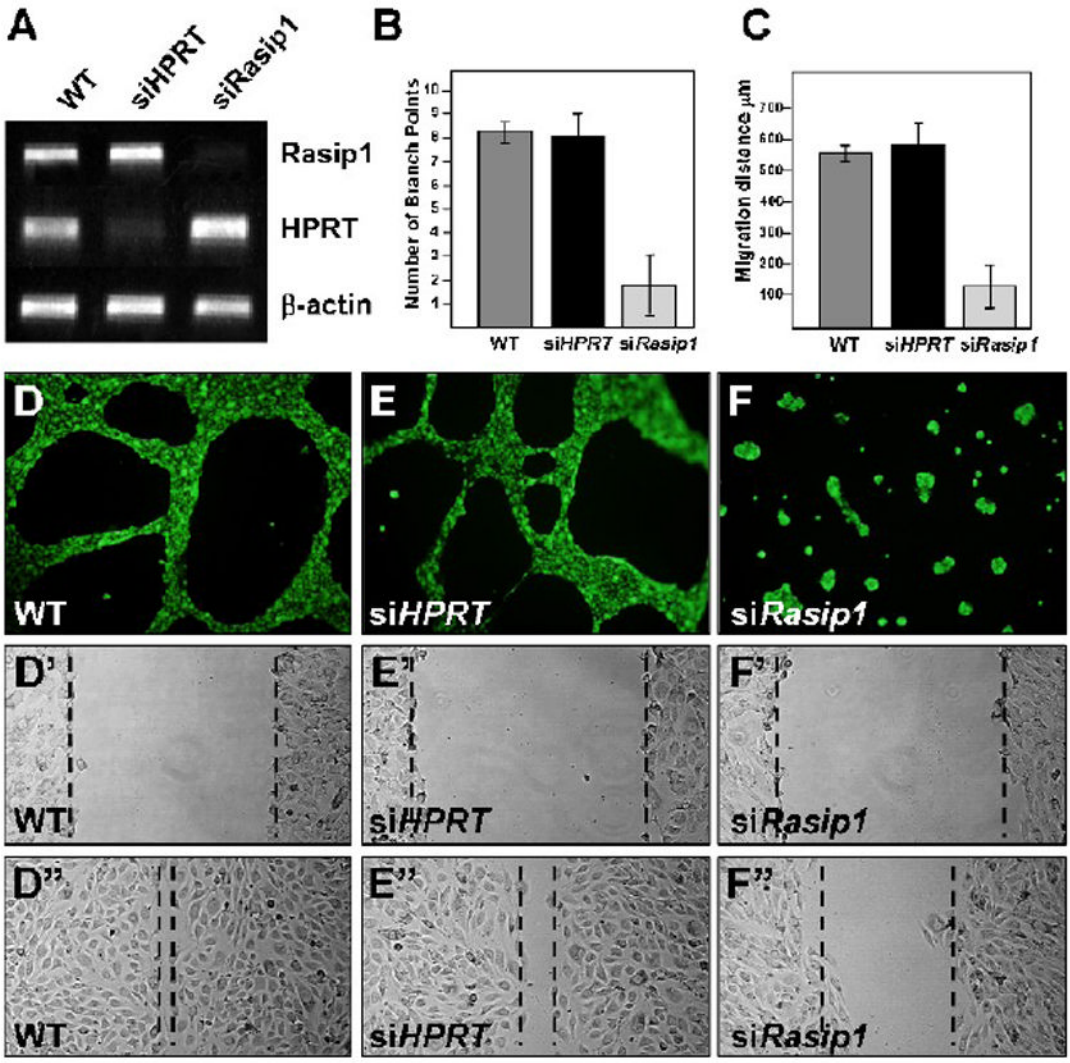

Figure 4. *Rasip1* ablation in MS1 cells by transient siRNA transfection hinders endothelial tube formation and migration ability

A) Semi-quantitative RT-PCR (30 cycles) shows the knockdown of *Rasip1* at the mRNA level. HPRT control knockdown is also shown. B,D-F) si*Rasip1* treated MS1 cells fail to form "tubes" when plated on Matrigel. B) Quantification shows that formation of linear structures (tubes or cords) as measured by counting branching points in the vascular plexus, is decreased by approximately 85%. C,D'-F', D"-F") Knockdown of *Rasip1* inhibits endothelial cell migration. si*Rasip1*-treated cells show slow healing rate in "scratch assay". While untreated cells migrate quickly into the cell free area (D',D"), si*Rasip1*-treated cells migrate less than half the distance during the same time period (F',F"). C) Quantification of endothelial migration in the scratch assay was determined by difference in relative diameter of scratch, before and after healing (D'-F', compared to D"-F"), measured in µm on y axis (as shown).

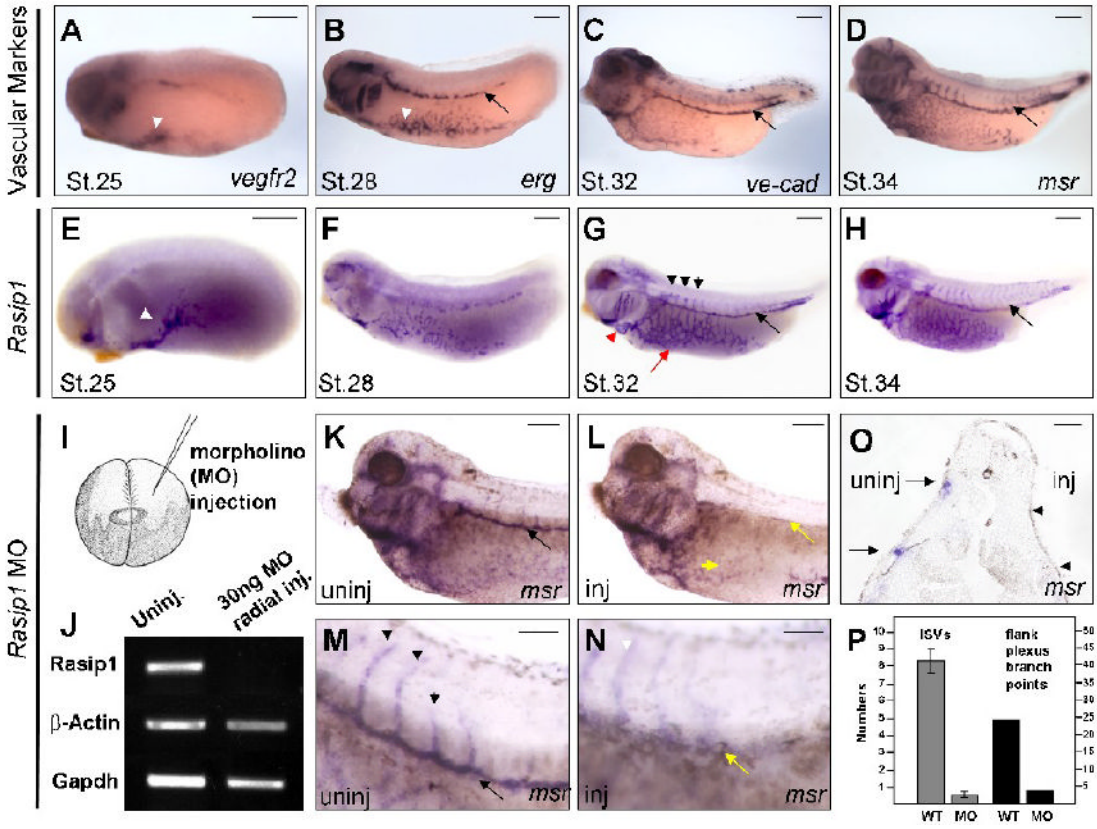

Figure 5. *Rasip1* knockdown in frog embryos results in failure of blood vessel formation

Expression of *Xenopus tropicalis Rasip1* transcripts in frog embryos by in situ hybridization marks the developing embryonic blood vessels (in all panels anterior is to the left). A-D) Vascular markers reveal the embryonic vasculature at stages indicated, including angioblasts (white arrowheads in A, B) and developing blood vessels, such as the posterior cardinal vein (black arrow, B-D). A) *vegfr2*; B) *erg*, vascular ETS factor; C) *ve-cad*, vascular endothelial cadherin; D) *msr*, vascular G-protein coupled receptor. E-H) *Rasip1* initially marks scattered angioblasts (E, st.25), but progressively marks aggregating flank vessels (F, st.28). G) As vessels form, *Rasip1* marks all embryonic frog vessels examined, including the flank plexus (red arrow), endocardium (red arrowhead), cardinal veins (black arrow) and ISVs (black arrowheads). H) Expression of *Rasip1* declines slightly in vessels as they mature (including data not shown). I) Schematic of microinjection of *Rasip1* morpholino injections into blastomeres of early cleavage stage embryo. J) Semi-quantitative RT-PCR shows the knockdown of *Rasip1* transcript expression in radially injected embryos (24 cycles). (K-N) *msr* in situ hybridization of injected embryo, outlining vasculature on either uninjected (K,M) or injected (L,N) sides. K,M) Uninjected side of embryo displays major blood vessels, including the prominent cardinal vein (black arrow) and ISVs. M) Higher magnification of embryo in panel K, showing ISVs sprouting from the cardinal vein on the uninjected side (black arrowheads). L,N) MO injected side of embryos shows a severe reduction in vascular structures, including absence of cardinal vein (yellow arrow) and reduced plexus vessels (yellow arrowhead). N) Higher magnification of embryo in panel L, showing complete absence of both ISVs (white arrowhead) and cardinal vein (yellow arrow). Note that faint outline of ISVs in panel N results from ISVs from uninjected side appearing through the dorsal fin tissue (white arrowhead). O) Transverse sections through injected embryos show that cardinal veins and ISVs are lost on the injected side (arrowheads) but not the uninjected side (arrows). P) Quantification of observations in K-O. Total number of ISVs and flank plexus branch points

were counted from the injected versus uninjected sides of MO-injected embryos. Y-axis for ISVs is to left; Y-axis for flank branch points is to right. The scale bars represent 250μm (A-H), 100μm (K, L), and 50μm (M, N, O), respectively.

Table 1

Rasip1 is required for proper blood vessel formation

Listed in table are the numbers of embryos with absent/severely reduced angioblasts (st.20-27) or posterior cardinal vein (st.33-37) observed on injected side of embryo (x), out of the total number assayed (y): x/y. Controls include uninjected embryos (top row) and uninjected side of injected embryos (data not tabulated).

	***msr*(st.20-21)**	***flk1*(st.25-27)**	***erg*(st.33-35)**	***ve-cadherin* (st.33-35)**	***msr* (st.35-37)**
uninjected	1/21	1/17	0/13	0/16	0/25
16ng Rasip1-MO	1/19	10/14	12/15	11/15	4/5