Chapter 3

A Novel Endothelial Specific Gene, *D1.1*, is a Marker of Adult Neovasculature

ABSTRACT

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, is essential for tumor growth and wound healing in adults, as well as for proper embryonic development. Here we describe a novel endothelial-specific gene, *D1.1*, which encodes a predicted transmembrane protein that is not homologous to any other genes in the mammalian genome. We have examined the expression and function of *D1.1* using a *tau-lacZ* knock-in to the endogenous chromosomal locus. We find that *D1.1* is strongly expressed by most or all endothelial cells during embryonic development, and is subsequently down-regulated in the majority of adult microvessels. *D1.1* is highly up-regulated and expressed by the majority of endothelial cells during tumor angiogenesis, wound healing, and in the corneal micropocket assay. *D1.1* homozygous null mutant mice appear phenotypically normal. However, a soluble D1.1-Fc fusion protein impairs endothelial cell migration and blood vessel formation in several different acute assays. These data identify *D1.1* as a novel marker of neovasculature, and suggest it may play a functional role in angiogenesis that is compensated in vivo by other, structurally distinct proteins.

INTRODUCTION

Neovascularization, the formation of new blood vessels in adult tissues, is essential for tumor growth, wound healing, and a variety of additional physiological and pathological process. For example, inhibition of new blood vessel formation impairs tumor growth, and causes necrosis or apoptosis of tumor cells (Hanahan and Folkman, 1996), while inhibition of neovascularization in the skin significantly delays wound healing (Streit et al., 2000).

At present, there are only a handful of molecular markers of neovascularization. Such markers are important, for several reasons. First, they are essential for monitoring the process of neovascularization, and its response to different experimental manipulations, including drug treatments, in vivo. Second, they could be used to acutely isolate endothelial cells involved in neo-angiogenesis, to compare their properties to those of endothelial cells in stable vessels. Third, such markers could be useful, in humans, for early diagnosis of diseases involving new blood vessel formation. Finally, some markers may themselves provide novel targets for pharmacologic inhibition of neovascularization.

Despite the potential utility of such markers, relatively few genes have been described that mark endothelial cells involved in most or all settings of neo-angiogenesis, i.e., pan-neovessel markers. Numerous tumor-restricted endothelial genes have been identified (Madden et al., 2004; Oh et al., 2004; St Croix et al., 2000); however, it is not clear whether any of them serves as a definitive marker of adult neovascularization. For example, some tumor-restricted endothelial markers, such as Flk1, are substantially expressed in the endothelial cells of normal adult tissues (Madden et al., 2004). Others are expressed in a subset of endothelial cells of tumor tissues (St Croix et al., 2000), or

expressed in certain tumors but not in all tumors (Zheng et al., 2005). In general, it has been difficult to evaluate from the published literature whether any of these markers are expressed in all settings of neo-angiogenesis.

Here we characterize a novel endothelial-specific gene, *D1.1*, which encodes a predicted transmembrane protein. Using *D1.1-LacZ* knockin mice, we show that *D1.1* is highly expressed in most endothelial cells involved in neo-vascularization, using tumor angiogenesis, wound healing and corneal micropocket assays. By contrast, *D1.1* is barely detectable in the microvasculature of most normal adult tissues, although it is expressed in large-diameter vessels. Homozygous *D1.1* mutant mice do not show any obvious cardiovascular phenotypic defects. However, a soluble D1.1-Fc fusion protein has anti-angiogenic activity in several different assays, suggesting that D1.1 function may be compensated in vivo by other, structurally unrelated proteins.

RESULTS

Isolation of a novel endothelial-specific gene, *D1.1*

D1.1 was isolated from a screen originally designed to identify novel arterial- or venous-specific genes, using single cells from E12 yolk sac as previously described (Dulac and Axel, 1995). cDNAs from these cells were amplified using PCR, and analyzed by Southern blotting using four different genes as probes: a house keeping gene, *Tubulin*; two pan-endothelial markers, *Flk1* and *Flt1*; and an arterial marker, *ephrin-B2* (data not shown). cDNAs exhibiting comparable expression of these standard genes were selected for library construction and screening. While *D1.1* was originally isolated as an arterial-specific gene, in situ hybridization to E10.5 embryo sections revealed pan-endothelial

expression (Supplemental Fig. 1A, C; arrows and arrowheads). These data also revealed that *D1.1* expression appeared to be restricted to endothelial and endocardial cells (Supplemental Fig. 1B, arrows).

Reconstruction of a full-length *D1.1* cDNA from overlapping ESTs revealed an open-reading frame that encodes a predicted type I transmembrane protein (Fig. 1B). The N-terminal region of D1.1 contains a signal peptide, consisting of the first 18 amino acids, and many putative consensus serine- and threonine-glycosylation sites (Fig. 1A, blue and red circles), while the C-terminal region contains six putative consensus serine-phosphorylation sites (Fig. 1A, green circles). However, D1.1 does not contain any conserved structural motifs.

Sequences of rat, human, bovine, porcine, and chicken *D1.1* orthologs have been deposited in the public databases, but no apparent orthologs in zebrafish, fugu, or frog have been reported. In addition, there were no other *D1.1*-related genes in the mouse or human genomes. The transmembrane and cytoplasmic regions are highly conserved among the different species examined, whereas the extracellular domain is highly divergent (Fig. 1A). Three out of six putative serine-phosphorylation sites in the cytoplasmic region are conserved among the different species, suggesting that D1.1 may be involved in an intracellular signaling pathway.

Targeted mutagenesis of *D1.1*

To assess the function of D1.1 in vivo, and to characterize its expression in more detail, we replaced the first exon (containing the ATG start codon and part of the signal peptide) and part of the downstream intron with a *tau-LacZ* reporter, using homologous

recombination in embryonic stem (ES) cells (Fig. 1C). This construct is designed to create a functional null, by preventing membrane insertion of D1.1; a similar targeting strategy was previously used to inactivate *ephrin-B2* and *EphB4* (Gerety et al., 1999; Wang et al., 1998). Germline chimeric mice were then generated from these ES cells by blastocyst injection (Fig. 1D). We examined whether homozygous mutant mice transcribe *D1.1* mRNA by performing RT-PCR using two different 5' primers and three different 3' primers (Fig. 1F)**.** Green and red triangles in Fig. 1C and F indicate the relative positions of these 5' primers in the *D1.1* locus and *D1.1* cDNA; one primer is in the upstream of the deleted exon, and the other is downstream of the exon. The absence of any *D1.1* transcripts in a homozygous mutant embryo and the fact that any splicing to produce a truncated form of D1.1 can not make a functional protein due to the absence of the signal peptide, clearly indicate *D1.1* homozygous mutant is a null mutant (Fig. 1F).

Pan-endothelial expression of *D1.1* in embryonic vasculature

To examine *D1.1* expression in detail during embryonic development, we used the *tau-LacZ* reporter to perform X-gal staining in E7.5-E9.5 heterozygous embryos. At E7.5, *D1.1* was expressed in endocardial cells and the dorsal aorta (Fig. 2A, B; arrows and arrowheads); furthermore, it was expressed in small vessels in the head (Fig. 2A, B; open arrowheads). At E8.5 and E9.5, *D1.1* was expressed in endocardial but not in myocardial cells (Fig. 2C-F, arrows), and in intersomitic vessels as well as in the dorsal aorta (Fig. 2C, E; open and black arrowheads). *D1.1* was also expressed in endothelial cells around the neural tube at E8.5, and in endothelial cells penetrating the neural tube at E9.5 (Fig. 2D, F; open arrowheads). *D1.1* is expressed in both arterial and venous endothelial cells

at E9.5 (Fig. 1F, arrowhead vs. open arrow). By contrast, whole-mount X-gal staining revealed, surprisingly, that *D1.1* was preferentially expressed in arterial yolk sac endothelial cells (Fig. 2G vs. H). This arterial-enriched expression of *D1.1* in the yolk sac may explain why *D1.1* was originally identified as an arterial-specific gene by the differential screen.

The fact that *D1.1* appeared to be expressed in most or all embryonic endothelial cells prompted us to directly compare its expression to that of PECAM-1, a pan-endothelial marker, using double-immunofluorescent labeling with antibodies to ß-galactosidase and PECAM-1. *D1.1* expression was almost identical to PECAM-1 expression in most endothelial cells of the brain, the neck region, the liver, the lung, and the heart at E13.5 (Fig. 3A-I and data not shown). Double-labeling with antibodies to ß-galactosidase and podoplanin, a lymphatic endothelial marker, also revealed that *D1.1* was expressed in lymphatic endothelial cells (Fig. 3J-L, arrows). Taken together, these data suggest that *D1.1* is a pan-endothelial (blood endothelial and lymphatic endothelial) marker in the embryonic vasculature.

D1.1 expression is down-regulated in most adult microvessels

We next examined whether the endothelial expression of *D1.1* is maintained in adult vasculature, by performing double-labeling with antibodies to ß-galactosidase and PECAM-1. Strikingly, *D1.1* was strongly down-regulated in the microvessels of most organs and tissues examined. For example, such down-regulation was clearly detected in brain, liver, kidney, stomach, pancreas and heart (Fig. 4A-R, arrowheads). This down-regulation was particularly evident in brain, where *D1.1* was virtually absent (Fig. 4A-C).

However, a subset of vessels detectably expressed *D1.1* in some other tissues (Fig. 4D-R). For example, *D1.1* was relatively highly expressed in a subset of large-diameter vessels in kidney, stomach, pancreas, and heart (Fig. 4G-R, arrows). In the pancreas, the level of *D1.1* expression in some microvessels is similar to that in the large-diameter vessels (Fig. 4M-O, open arrows vs arrows). Expression of *D1.1* in the heart was particularly complex, with microvessels in different regions (atrium, interventricular septum) expressing the gene to different extents (Supplemental Fig. 2). Despite these exceptions, these data suggest that, in general, *D1.1* is down-regulated in most microvessels, but maintained in a subset of large-diameter vessels.

D1.1 expression is maintained in postnatal tissues undergoing active neo-angiogenesis

Interestingly, *D1.1* was relatively highly expressed in most vessels of the ovary and the uterus (Fig. 5A-F). The fact that physiological angiogenesis occurs in the ovary where *D1.1* was highly expressed prompted us to examine other physiologically angiogenic tissues such as the placenta and the retina. *D1.1* was highly, homogeneously expressed in all the vessels of the placenta taken from a pregnant heterozygous female bearing E9.5 embryos, whereas it was barely expressed in the vessels of the placenta from a female bearing E16.5 embryos (Fig. 5G-L). Furthermore, *D1.1* was strongly expressed in the vessels of P5 retina, whereas it was down-regulated in the vessels of adult retina (Fig. 5M-R). Whole-mount X-gal staining of P5 and adult retina clearly revealed the down-regulation of *D1.1* in adult retinal vessels (Fig. 5S, T). Taken together, these data

suggested that *D1.1* may be expressed when angiogenesis occurs, then down-regulated when angiogenesis ceases.

Strong up-regulation of *D1.1* in most vessels during tumor angiogenesis, wound healing, and in the corneal micropocket assay

To investigate further the idea that *D1.1* broadly marks endothelial cells involved in neovascularization, we examined *D1.1* expression in a variety of settings of adult neovascularization: tumors, wound healing and the corneal micropocket assay. *D1.1* was highly expressed in most endothelial cells of Lewis lung carcinoma (LLC) tumors and B16F10 melanoma tumors grown subcutaneously (Fig. 6A-C and data not shown). *D1.1* was also abundantly expressed in most vessels during wound healing, while in normal skin it was expressed in only a subset of vessels (Fig. 6G-I, arrows). In addition, *D1.1* was highly expressed in the corneal neovasculature induced by bFGF (Fig. 6P-R). Although *D1.1* was still expressed in most limbic endothelial cells of the untreated eye (Fig. 6M-O, arrows), its expression in the neovessels was a little higher than that in the limbic vessels of the untreated eye. Whole-mount X-gal staining also revealed that *D1.1* was strongly expressed in the corneal neovessels growing toward bFGF-containing pellets (Fig. 6T, U), but moderately expressed in the stable vessels (Fig. 6S).

Although *D1.1* was more strongly expressed in neovessels than in the stable vessels of surrounding tissues, it is not clear whether *D1.1* expression in neovessels is caused by the induction of *D1.1* in neovessels or by the recruitment of $D1.1^{+}$ endothelial cell from surrounding tissues. To clarify this issue, B16F10 melanoma cells were implanted into the brain, where *D1.1* was barely expressed, and the tumors taken from the brain were

processed to examine *D1.1* expression by double-labeling with antibodies to ß-galactosidase and PECAM-1. *D1.1* was expressed in the endothelial cells of the tumor vessels, but not of surrounding brain vessels (Fig. 6J-L, arrows vs. arrowheads), suggesting that *D1.1* expression in the endothelial cells of tumor vessels is caused by the induction of *D1.1*, not by the recruitment of $D1.1^{+}$ endothelial cells from surrounding tissues. Taken together, these data suggest that *D1.1* provides a marker of neovasculature.

_D1.1_ is not essential for vessel development or maintenance

The pan-endothelial expression of *D1.1* during development prompted us to examine the cardiovascular phenotype of *D1.1* homozygous mutant embryos. Whole-mount staining with anti-PECAM-1 antibodies revealed no obvious differences in vessel development or patterning of E9.5 embryos (Fig. 7A, B), E9.5 yolk sacs (Fig. 7C, D), or E12.5 trunk neural tubes between wild-type and homozygous mutant mice. Whole-mount X-gal staining also revealed no obvious differences in vessel development or patterning of the retina, uterus, intestine, bladder, stomach, or the testis between P5 or P9 heterozygous and homozygous mutant mice (Fig. 7G-L and data not shown). The increased X-gal intensity in homozygous mutant mice likely reflects the additional copy of the *D1.1-tauLacZ* gene. *D1.1* homozygous mutant mice develop normally without any apparent physiological or developmental defects, and are fertile in both a 129/c57Bl6 mixed background and a pure 129 background.

D1.1 was highly expressed in endothelial cells during tumor angiogenesis and wound healing. We therefore asked whether pathological angiogenesis in tumors and during wound healing was affected in homozygous mutant mice, by implanting Lewis lung

carcinoma or B16F10 melanoma cells subcutaneously or by making skin wounds in the back of the mutant mice. The tumor size and extent of vascularization, and the rate and extent of wound healing and skin vascularization in homozygous mutant mice, were similar to those in heterozygous and wild-type mice (data not shown). We also performed corneal micropocket assays by inserting bFGF-containing pellets into the corneas of the mutant mice (data not shown), but no obvious differences were apparent.

D1.1-Fc proteins impair angiogenesis in several angiogenesis assay

The lack of an obvious phenotype in *D1.1* homozygous mutant mice suggested that its function might be compensated during development by other genes. To circumvent this problem, we sought a means of acutely interfering with the function of D1.1 and/or interacting proteins. The functions of many single-pass transmembrane proteins have been analyzed using soluble proteins, consisting of their extracellular domain fused to the Fc region of human immunoglobulin (Davis et al., 1994; Suchting et al., 2005). We therefore generated a D1.1-Fc fusion protein using the baculovirus expression system (see Methods). These D1.1-Fc proteins were used to analyze the functions of D1.1 in various in vitro and in vivo angiogenesis assays.

The chick chorioallantoic membrane (CAM) assay measures natural vessel growth (Oreilly et al., 1994). This assay revealed that vessels did not grow around filter discs containing a D1.1-Fc protein, but did grow normally around filter discs containing a control Fc protein (Fig. 8A, B). Quantification of vessel density around the discs confirmed the robust inhibition of vessel growth by D1.1-Fc (Fig. 8C). Preliminary experiments using another CAM assay, which measures bFGF-induced vessel growth

(Brooks et al., 1994), indicated that D1.1-Fc significantly reduced bFGF-induced vessel branching in this assay as well (data not shown).

The allantois assay was performed to check whether the murine D1.1-Fc protein can impair angiogenesis occurring in mouse tissues, as well as in chick. This assay tests for anti-angiogenic activity using cultured E8.5 mouse embryonic allantoises incubated with test reagents for 18-22 hours (Argraves et al., 2002; Drake and Fleming, 2000). Rather than blocking vessel growth completely (Crosby et al., 2005), preclustered D1.1-Fc perturbed vascular patterning, resulting in a significant enlargement of average vessel diameter (Fig. 8D-F). This effect occurred mainly for vessels at or near the surface of the cultured allantoises, likely reflecting a lack of efficient penetration of the D1.1-Fc fusion deeper into the explant.

In addition, D1.1-Fc significantly inhibited spontaneous and VEGF-induced trans-well migration of human umbilical vein endothelial cells (HUVEC) (Fig. 8G). However, the effect of D1.1-Fc to impair cell migration in vitro can be overridden by factors that strongly promote the activity, such as bFGF (Fig. 8G). Taken together, these data suggest that a D1.1-Fc fusion protein may have anti-angiogenic activity in certain in vitro and in vivo assays.

DISCUSSION

In this study, we identified a novel endothelial-specific gene, *D1.1*, as a molecular marker of adult neovasculature as well as embryonic vasculature based on several features of *D1.1* expression, by generating *D1.1-LacZ* knockin mice and by taking advantage of the *LacZ* reporter for the detailed expression analysis of *D1.1*. First, *D1.1* is homogeneously

expressed in most endothelial cells of embryonic vasculature, whereas it is barely or weakly expressed in microvessels of most adult tissues. Second, *D1.1* is highly expressed in the vessels of physiologically angiogenic tissues such as the ovary, the placenta, and the retina, whereas it is down-regulated in the placenta and the retina when angiogenesis ceases. Third, *D1.1* is highly up-regulated in most vessels during tumor angiogenesis and wound healing and in the corneal micropocket assay. In addition to the characteristic of *D1.1* as a neovessel marker, *D1.1* expression in normal adult tissues reveals heterogeneity of endothelial cells among different tissues and even in the same tissue. Furthermore, a soluble D1.1-Fc protein impaired angiogenesis in the chick CAM and mouse allantois assays, and inhibited the migration of HUVEC cells, suggesting that D1.1 may play a role in angiogenesis.

D1.1 is a marker of adult neovasculature

The fact that *D1.1* is strongly, homogeneously expressed in most endothelial cells of various neo-angiogenic tissues, whereas it is barely or weakly expressed in microvessels of most normal tissues suggests *D1.1* as a marker of adult neovasculature. Many tumor-restricted endothelial genes have been reported (Madden et al., 2004; Oh et al., 2004; St Croix et al., 2000), but few of them have been used as a definitive marker of neovascularization.

In addition to these tumor-restricted genes, several genes expressed in endothelial cells during development were recently reported to be induced in tumor vasculature and down-regulated in normal adult tissues, suggesting that they may be a possible marker of adult neovasculature. EGF-like domain 7 (Egfl7), a secreted factor from endothelial cells, is

expressed at high levels in the vasculature associated with tissue proliferation, and is down-regulated in most mature vessels of normal adult tissues; however, it is expressed in a subset of the neovasculature, but not in most endothelial cells of the neovessels (Parker et al., 2004). In addition, Egfl7 is expressed in a subset of vessels in some normal organs such as the lung, the kidney, and the heart (Parker et al., 2004; Soncin et al., 2003). Delta-like 4 (Dll4), a membrane-associated Notch ligand, is induced in a subset of vessels in tumors, but not in most tumor vessels; is also expressed in smaller arteries and microvessels in normal adult tissues (Gale et al., 2004). Ras guanine-releasing protein 3 (RasGRP3), a guanine exchange factor, is up-regulated in maternal vessels of the decidua and in tumor vessels, and is expressed in the endothelial cells of the kidney glomerulus, but not of most adult tissues; however, it is not clear from the limited published data whether RasGRP3 is expressed in most, or just a subset, of the neovessels (Roberts et al., 2004).

In contrast to these genes, *D1.1* is strongly, homogeneously expressed in most vessels in tumors, during wound healing, and in bFGF-treated corneas, and barely or weakly expressed in the microvessels of most adult tissues although it is expressed in some of large-diameter vessels. Therefore, *D1.1-LacZ* mice will be a useful resource to monitor neovascularization in mouse models of angiogenesis-dependent diseases such as tumors.

The distinct expression of *D1.1* between large-diameter vessels and microvessels

Microvessels are the main sites for angiogenesis in adults, and many genes associated with angiogenesis are specifically expressed in microvascular endothelial cells but not in the endothelial cells of large vessels in adults (Chi et al., 2003), suggesting that adult

microvessels are more similar to embryonic vessels than to adult large vessels because angiogenesis occurs during development. For example, Flk1 is expressed in most embryonic endothelial cells, is maintained in microvessels but not in large vessels of adult tissues, and induced in adult neovasculature (data not shown).

In contrast to Flk1, *D1.1* is relatively highly expressed in large vessels but not in microvessesls of adult tissues (Fig. 4G-R, arrows vs. arrowheads), although it is similarly expressed in most endothelial cells of embryonic vessels and adult neovasculature. Therefore, *D1.1* expression is not consistent with the general concept that microvessels are more similar to embryonic vessels than to large vessels in adults. The robust expression of *D1.1* in adult neovessels suggests *D1.1* expression may be regulated by angiogenesis-related signals, which may not exist around stable large-diameter vessels; thus, *D1.1* expression in stable large-diameter vessels might be regulated independently of angiogenesis-related signals. It would be of interest to examine which enhancer elements in the *D1.1* locus and which factors control this distinct expression of *D1.1* between large vessels and microvessels in adults.

Possible Functions of D1.1 in angiogenesis based on D1.1-Fc activities

Despite its endothelial-specific expression and the absence of any other *D1.1*-related genes in the mouse genome, *D1.1* homozygous mutant mice did not show any detectable phenotypic defects during development or in adulthood, preventing us from deducing any possible functions of D1.1 from homozygous mutant mice.

Although D1.1 ECD sequences are divergent among different species and chicken D1.1 ECD sequence was not reported due to the incomplete chicken genome sequencing

(Fig. 1A), mouse D1.1-Fc protein impaired angiogenesis in the chick CAM assay and in vitro assay using HUVEC cells as well as in mouse allantois assay, suggesting that D1.1 ECD might be structurally conserved among different species and D1.1 may play roles in angiogenesis.

D1.1-Fc inhibited spontaneous vessel growth and bFGF-induced angiogenesis in the chick CAM assays, impaired proper vascular remodeling in mouse allantois assay, and inhibited the migration of HUVEC cells, indicating that D1.1-Fc appears to be an inhibitor of angiogenesis. However, it is not clear from the present studies whether D1.1-Fc acts as an agonist or antagonist. If D1.1-Fc acted as an agonist, D1.1 might have anti-angiogenic activities, of which blocking might increase angiogenesis, and the up-regulation of D1.1 in neovasculature might be a part of the negative feedback mechanism to control excessive vessel growth. If D1.1-Fc acted as an antagonist, D1.1 might have pro-angiogenic activities, of which blocking might inhibit angiogenesis, and the up-regulation of *D1.1* in neovasculature might be part of the positive feedback mechanism to promote angiogenesis.

EXPERIMENTAL PROCEDURES

Preparation and screening of Single-Cell cDNA libraries

The middle regions of E12 yolk sacs were dissected in HBSS, and dissociated into single cells by digestion with type 1 collagenase (Worthington). Under a microscope, single cells were transferred using a mouth pipette into tubes containing lysis buffer, and processed for single-cell based PCR amplification, essentially as described (Dulac and Axel, 1995). Each 5 μg of the amplified cDNAs were loaded on 1.5% agarose gels for

electrophoresis to check the quality of the cDNAs, and transferred into a Hybond-N^+ membrane (Amersham) followed by Southern blotting using 3'-biased cDNA probes against a house keeping gene, *Tubulin*; two pan-endothelial markers, *Flk1* and *Flt1*; and an arterial endothelial marker, *ephrin-B2*. The single-cell cDNAs positive for *Tubulin, Flk1, Flt1*, and *ephrin-B2* were considered arterial endothelial cDNAs, while ones, which are positive for *Tubulin, Flk1, and Flt1* and negative for *ephrin-B2,* were considered as venous endothelial cDNAs. One pair of cDNAs was selected for further steps of the differential screen.

Generation of *D1.1* null mutant allele

The arms of the *D1.1* targeting construct (left arm, 2.7 kb; right arm, 5.2kb) were subcloned from a 129/SvJ genomic DNA BAC clone (Incyte). A tau-LacZ reporter and a self-excised ACN selection cassette was inserted into the start codon of *D1.1*, by replacing the first exon and the part of the first intron of *D1.1* (total 51 bp). Homologous recombination was performed in mouse CJ7 embryonic stem (ES) cells following standard procedures. Correctly targeted ES cell clones were identified by Southern blot hybridization using 5' and 3' external probes. Chimeric mice were produced by blastocyst injection and were mated to C57Bl/6 mice to establish lines. The *D1.1* allele was deposited as **MGI:3526088**. The ACN selection cassette was self-excised in the chimeric sperm, whose progenies do not contain the ACN cassette in their genome (Bunting et al., 1999). To clarify whether this mutant is null or hypomorphic, two 5' primers (a, 5'-AGTACT CCCTCTCTCTTCTCTACT-3'; b, 5'-GAGAAGCATCTCTGGATGCTACTC-3') and three 3' primers (c, 5'-

GTTCACGTTGATGTTCCTCATGGA-3'; d, 5'-TTAAAGAATCTTCTCTGCTGACATGCTG-3'; e, 5'-CTAGTAGAATGGACAATCTACCTC-3') were used in all possible combinations for RT-PCR using E10.5 embryonic cDNAs from a heterozygote and a homozygote as a template (Fig. 1F).

Genotyping

The genotyping of *D1.1* mutant mice was performed by PCR using two primer sets detecting the wild-type and the mutant allele: WT1 (5'-CATCTCACCCCAGTACTCCCTC-3') and WT2 (5'-CCTTGGAGCAGGAGTAAACCGAGA-3') primers, 142 bp PCR products; LacZ1 (5'-CGCCCGTTGCACCACAGATG-3') and LacZ2 (5'-CCAGCTGGCGTAATAGCGAAG-3') primers, 370 bp PCR products.

Immunohistochemistry

Embryos and adult organs were fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, sunk in 30% sucrose/PBS overnight at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat. Staining was performed using anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C), anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C), Cy3-conjugated anti-α-SMA antibody (Sigma, 1:500, 40 minutes at room temperature), and anti-podoplanin antibody (Developmental Studies Hybridoma Bank, 8.1.1, 1:100, overnight at 4°C), For immunofluorescent detection; FITC-, Cy3-, Cy5-, Alexa-488-, or Alexa-568-conjugated

secondary antibodies (Jackson, 1:300; Molecular Probes, 1:250, 40 minutes at room temperature) were used. All confocal microscopy was carried out on a Leica SP confocal (Leica).

For whole-mount staining, embryos and organs were first fixed for 1-2 hours in 4% paraformaldehyde/PBS at 4°C, washed with PBS, and dehydrated in 100% methanol at -20°C. Staining was performed with anti-PECAM1 antibody (Pharmingen, 1:200 overnight at 4°C) and anti-ß-galactosidase antibody (5-prime 3-prime, 1:1000, overnight at 4°C); either HRP-conjugated secondary antibodies (Jackson, 1:300, overnight at 4°C) or secondary antibodies conjugated to Cy5- or Alexa-488 (Jackson, 1:300, and Molecular Probes 1:250, 1 hour at room temperature) were used.

LacZ staining

Embryos and organs were dissected, fixed in 0.25% glutaraldehyde/PBS for 5-15 minutes, rinsed twice with PBS, and stained at 37°C in X-Gal buffer [1.3 mg/ml potassium ferrocyanide, 1 mg/ml potassium ferricyanide, 0.2% Triton X-100, 1 mM $MgCl_2$ and 1 mg/ml X-Gal in phosphate-buffered saline (PBS, pH 7.2)]. The stained embryos were post-fixed in 4% paraformaldehyde/PBS at 4°C, washed with PBS, were embedded in 15% sucrose and 7.5% gelatin in PBS, and 20 µm sections were collected on a cryostat. All bright-field images were captured using an Axiocam CCD camera (Zeiss).

In situ hybridization

In situ hybridization was carried out essentially as described (Wang et al., 1998). Embryos were cryosectioned at 20 μm, and hybridized with a cRNA probe against *D1.1* (750 bp).

Pathological studies: tumors, cutaneous wounding, and corneal micropocket assay

All procedures were carried out under protocols reviewed and approved by the IACUC (Institute Animal Care and Use Committee). Mice were implanted subcutaneously with 200 μl of 2 x 10^6 Lewis lung carcinoma cells (LLC) or B16F10 melanoma cells, and sacrificed between 13 to 15 days post-implantation when the diameter of the tumors reached 1.5 cm. The tumors were dissected, weighed, fixed for 6-8 hours in 4% paraformaldehyde/PBS at 4°C, cryoprotected overnight in 30% sucrose/PBS at 4°C, frozen in OCT medium, and 20 μm sections were collected on a cryostat.

For brain tumors, 2 μl of 1 x 10^5 B16F10 melanoma cells were implanted into the brain, as described (Papadopoulos et al., 2004). The mice were sacrificed in 6 days post-implantation, and the brain was removed and processed for immunofluorescence staining like subcutaneous tumors.

Full-thickness cutaneous wounds were made on the back skin of mice using a sterile, disposable 6-mm dermal biopsy punch (Miltex). Seven days later, animals were sacrificed and the tissue was examined.

The mouse corneal micropocket assay was performed as described previously (Kenyon et al., 1996) using hydron-coated sucralfate pellets containing 100 ng of bFGF (PeproTech). For LacZ staining, the entire eye was first stained, and the cornea was

dissected out and flat-mounted on a slide whose coverslip was elevated by a bridge of two coverslips on each side to avoid crushing the cornea. For immunofluorescent staining, the entire eyes were fixed and sectioned on a cryostat.

Production of Fc and D1.1-Fc fusion proteins

D1.1 extracellular region including signal peptide sequences and human IgG1 Fc region were amplified by PCR, and inserted together into the baculovirus expression vector pBacPAK8 (CLONTECH) to make D1.1-Fc fusion construct. For Fc construct, human IgG1 Fc region was amplified by PCR with a primer containing D1.1 signal peptide sequences, and cloned into pBacPAK8 vector; thus, both D1.1-Fc and Fc constructs contain the same signal peptide sequences, which make the proteins secreted into the culture media. The baculovirus/insect cell expression system was used, and the secreted proteins were purified over protein-A columns (Pierce).

Cell culture

Human umbilical vein endothelial cells (HUVEC-2) were purchased from BD Bioscience and cultured in Medium 200 (Cascade Biologics) containing Low Serum Growth Supplement (Cascade Biologics), as recommended in the product sheet. Cells were not used beyond the sixth passage.

Endothelial cell migration assay

Analysis of HUVEC migration was performed using the angiogenesis endothelial cell migration kit (BD Bioscience); 5×10^4 HUVEC per well were seeded into the upper

chamber of the migration plate insert in migration media (Medium 200 + 0.4% FCS). The bottom chambers were loaded with or without VEGF (10 ng/ml) or bFGF (10 ng/ml) in 750 µl total volume of migration media in the presence of D1.1-Fc or Fc. After 22 h of incubation at 37°C, the insert membranes were stained with 4 µg/ml Calcein AM (Molecular Probes) in Hanks' balanced salt solution for 90 min. Fluorescence on the under side of the membrane was measured at excitation/emission wavelengths of 485/530 nm using a fluorescence microplate reader.

Chick CAM assay

The CAM assay to measure spontaneous vessel growth was performed, as described (Oreilly et al., 1994). Briefly, 3-d-old fertilized white Leghorn eggs (Spafas, Inc., Norwich, CT) were cracked, and chick embryos with intact yolks were placed in 100 X 20 mm plastic Petri-dishes. After 3 d of incubation in 3% CO_2 at 37°C, a disk of methylcellulose containing D1.1-Fc or Fc was implanted on the CAM of individual embryos. After 48 h of incubation, embryos and CAMs were analyzed for the formation of avascular zones by a stereomicroscope.

The CAM assay, which measures bFGF-induced angiogenesis, was performed as described (Brooks et al., 1994). Filter discs saturated with D1.1-Fc or Fc proteins along with 100 ng/ml bFGF were placed on the CAMs of 10-d-old chick embryos. After 72 h, filter discs and associated CAM tissues were harvested and quantified. Angiogenesis was assessed as the number of visible blood vessel branching points within the defined area of the filter discs.

Allantois explant culture

Allantoises were dissected from E8.5 mouse embryos and cultured for 18 hours (37°C, 5% CO_2) in the presence of pre-clustered D1.1-Fc or Fc proteins, as described (Argraves et al., 2002; Drake and Fleming, 2000). D1.1-Fc or Fc proteins were pre-clustered, by incubating with anti-human Fc antibodies (Jackson) for 30 minutes at room temperature. Allantoises were fixed and processed for immunofluorescence staining with anti-PECAM-1 antibody (Pharmingen, 1:300 overnight at 4°C).

ACKNOWLEDGMENTS

We thank Peter Snow and Inderjit Nangiana for the protein purification; Yoh-suke Mukouyama for helpful discussions; Shirley Pease and Jade Wang for performing blastocyst injections; Bruce Kennedy and the staff of the Transgenic Animal Facility at Caltech for assistance with mouse breeding and care; Janet Baer and Claire Lindsell for revision of animal protocols; Gwen Williams for surgical assistance; Shelley Diamond and Stephanie Adams for FACS assistance; David Mathog for sequence analysis; Gaby Mosconi for laboratory management; Jung Sook Chang and Monica Martinez for technical support; Gina Mancuso for administrative assistance; and other Anderson lab members for technical help and discussion. The anti-podoplanin antibody (8.1.1) developed by Andrew Farr was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. D.J.A. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Argraves, W. S., Larue, A. C., Fleming, P. A., and Drake, C. J. (2002). VEGF signaling is required for the assembly but not the maintenance of embryonic blood vessels. *Developmental Dynamics* **225,** 298-304.

Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T. H., Klier, G., and Cheresh, D. A. (1994). Integrin Alpha(V)Beta(3) Antagonists Promote Tumor-Regression by Inducing Apoptosis of Angiogenic Blood-Vessels. *Cell* **79,** 1157-1164.

Bunting, M., Bernstein, K. E., Greer, J. M., Capecchi, M. R., and Thomas, K. R. (1999). Targeting genes for self-excision in the germ line. *Genes & Development* **13,** 1524-1528.

Chi, J. T., Chang, H. Y., Haraldsen, G., Jahnsen, F. L., Troyanskaya, O. G., Chang, D. S., Wang, Z., Rockson, S. G., Van de Rijn, M., Botstein, D., and Brown, P. O. (2003). Endothelial cell diversity revealed by global expression profiling. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 10623-10628.

Crosby, C. V., Fleming, P. A., Argraves, W. S., Corada, M., Zanetta, L., Dejana, E., and Drake, C. J. (2005). VE-cadherin is not required for the fort-nation of nascent blood vessels but acts to prevent their disassembly. *Blood* **105,** 2771-2776.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M., and Yancopoulos, G. D. (1994). Ligands for Eph-Related Receptor Tyrosine Kinases That Require Membrane Attachment or Clustering for Activity. *Science* **266,** 816-819.

Drake, C. J., and Fleming, P. A. (2000). Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* **95,** 1671-1679.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83,** 195-206.

Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., Murphy, A. J., Adams, N. C., Lin, H. C., Holash, J., Thurston, G., and Yancopoulos, D. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 15949-15954.

Gerety, S. S., Wang, H. U., Chen, Z. F., and Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Molecular Cell* **4,** 403-414.

Gothert, J. R., Gustin, S. E., van Eekelen, J. A. M., Schmidt, U., Hall, M. A., Jane, S. M., Green, A. R., Gottgens, B., Izon, D. J., and Begley, C. G. (2004). Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* **104,** 1769-1777.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353-364.

Kenyon, B. M., Voest, E. E., Chen, C. C., Flynn, E., Folkman, J., and DAmato, R. J. (1996). A model of angiogenesis in the mouse cornea. *Investigative Ophthalmology & Visual Science* **37,** 1625-1632.

Madden, S. L., Cook, B. P., Nacht, M., Weber, W. D., Callahan, M. R., Jiang, Y. D., Dufault, M. R., Zhang, X. M., Zhang, W., Walter-Yohrling, J., Rouleau, C., Akmaev, V. R., Wang, C. J., Cao, X. H., Martin, T. B. S., Roberts, B. L., Teicher, B. A., Klinger, K. W., Stan, R. V., Lucey, B., Carson-Walter, E. B., Laterra, J., and Walter, K. A. (2004). Vascular gene expression in nonneoplastic and malignant brain. *American Journal of Pathology* **165,** 601-608.

Oh, P., Li, Y., Yu, J. Y., Durr, E., Krasinska, K. M., Carver, L. A., Testa, J. E., and Schnitzer, J. E. (2004). Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* **429,** 629-635.

Oreilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y. H., Sage, E. H., and Folkman, J. (1994). Angiostatin - a Novel Angiogenesis Inhibitor That Mediates the Suppression of Metastases by a Lewis Lung-Carcinoma. *Cell* **79,** 315-328.

Papadopoulos, M. C., Manley, G. T., Krishna, S., and Verkman, A. S. (2004). Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. *Faseb Journal* **18,** 1291-1293.

Parker, L. H., Schmidt, M., Jin, S. W., Gray, A. M., Beis, D., Pham, T., Frantz, G., Palmieri, S., Hillan, K., Stainier, D. Y. R., de Sauvage, F. J., and Ye, W. L. (2004). The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. *Nature* **428,** 754-758.

Roberts, D. M., Anderson, A. L., Hidaka, M., Swetenburg, R. L., Patterson, C., Stanford, W. L., and Bautch, V. L. (2004). A vascular gene trap screen defines RasGRP3 as

an angiogenesis-regulated gene required for the endothelial response to phorbol esters. *Molecular and Cellular Biology* **24,** 10515-10528.

Soncin, F., Mattot, V., Lionneton, F., Spruyt, N., Lepretre, F., Begue, A., and Stehelin, D. (2003). VE-statin, an endothelial repressor of smooth muscle cell migration. *Embo Journal* **22,** 5700-5711.

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. (2000). Genes expressed in human tumor endothelium. *Science* **289,** 1197-1202.

Streit, M., Velasco, P., Riccardi, L., Spencer, L., Brown, L. F., Janes, L., Lange-Asschenfeldt, B., Yano, K., Hawighorst, T., Iruela-Arispe, L., and Detmar, M. (2000). Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *Embo Journal* **19,** 3272-3282.

Suchting, S., Heal, P., Tahtis, K., Stewart, L. M., and Bicknell, R. (2005). Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *Faseb Journal* **19,** 121-123.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93,** 741-753.

Zheng, P. P., van der Weiden, M., and Kros, J. M. (2005). Differential expression of Hela-type caldesmon in tumour neovascularization: A new marker of angiogenic endothelial cells. *Journal of Pathology* **205,** 408-414.

FIGURE LEGENDS

Fig. 1. Alignment of *D1.1* orthologs and targeting of the *D1.1* locus. (A) Amino acid sequences of mouse, rat, human, bovine, porcine, and chicken *D1.1* orthologs are aligned. Signal peptide and transmembrane sequences (black bars) are marked over the mouse sequence; putative serine-phosphorylation (green circles), and threonine- (red circles) and serine-glycosylation (blue circles) sites are marked over the sequence. (B) Imaginary diagram of D1.1 topology to show the relative position of its N- and C-terminal. (C) Schematic representation of the wild-type (WT) *D1.1* locus, the targeting vector, and the targeted locus in ES cells and F1 progenies. The targeting vector contains a tau-LacZ reporter and a self-excised selection ACN cassette, by replacing the first exon and the part of the first intron of *D1.1* (total 51 bp). (D) Confirmation of homologous recombination of the targeting vector by Southern blotting. Genomic DNAs of the ES cells were digested with NcoI and RcaI, and hybridized with 5'external probe A and 3' external probe B, respectively. WT, wild-type locus (14 kb for probe A, 12 kb for probe B); TG, targeted locus (9.5 kb for probe A, 9 kb for probe B) (E) Genotyping of E10.5 progeny of a *D1.1* heterozygote intercross. LacZ primers reveal 370 bp TG band; *D1.1* wild-type primers, a 142 bp WT band. (F) Confirmation of *D1.1* null mutation by RT-PCR. Two different 5' primers (a, green triangle; b, red triangle) and three different 3' primers (c-e) were used to amplify cDNAs from heterozygous and homozygous mutants. Their relative position in *D1.1* cDNA was marked with start (ATG) and stop (TAA) codons; white box represents the deleted region in the targeting construct.

Fig. 2. *D1.1* expression during early development. E7.5 (A, B), E8.5 (C, D), and E9.5 (E, F) *D1.1*$^{LacZ/+}$ embryos were processed for whole mount X-gal staining, and sectioned (B, D, F); E9.5 arterial (G) and venous (H) part of *D1.1*$^{LacZ/+}$ yolk sac was processed for whole mount X-gal staining. Arrows indicate endocardial cells and arrowheads indicate dorsal aortic endothelial cells. Open arrowhead in A, endothelial cells in the head; ones in B, microvessels; ones in C and E, intersomitic vessels; one in D, endothelial cells around the neural tube; one in F, endothelial cells inside the neural tube; open arrow in F, venous endothelial cells. Dotted lines in A, C, and E represent the position of sections shown in B, D, and F, respectively. Scale bars, 300 μm.

Fig. 3. Pan-endothelial expression of *D1.1* in E13.5 embryos. E13.5 *D1.1*$^{LacZ/+}$ embryos were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red) (A-I), or with antibodies to β-galactosidase (Gothert et al.) and podoplanin, a lymphatic endothelial marker (red) (J-L). *D1.1* expression is very similar to PECAM-1 expression in the brain (A-C), the neck region (D-F), and the liver (G-I); *D1.1* is expressed in lymphatic endothelial cells (J-L, arrows) as well as arterial and venous endothelial cells (J-L, open and closed arrowheads). Scale bars, 100 μm.

Fig. 4. *D1.1* is barely or weakly expressed in the microvessels of most adult tissues. Adult *D1.1*$^{LacZ/+}$ mice were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). (A-C) *D1.1* is barely expressed in vessels of the brain. (D-E) *D1.1* is weakly expressed in a

small subset of the microvessels of the liver. (G-H) In the kidney, *D1.1* is mainly expressed in large-diameter vessels (arrows), but not in the microvessels (arrowheads). (J-L) *D1.1* is strongly expressed in the large vessels of the stomach (arrows), but barely expressed in the microvessels (arrowheads). (M-O) In the pancreas, *D1.1* is strongly expressed in the large vessels (arrows), but weakly expressed in a subset of the microvessels (arrowheads). (P-R) *D1.1* is expressed in a subset of the large vessels and the microvessels (arrows and arrowheads) of the heart. Scale bars, 100 μm.

Fig. 5. *D1.1* is highly expressed in adult tissues where physiological angiogenesis occurs. (A-L) Adult $D1.1^{LacZ/+}$ mice were sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is highly expressed in the ovary (A-C), the uterus (D-F), and the placenta of E9.5 bearing females (G-I), but barely expressed in the placenta of E16.5 bearing females (J-L). (M-R) P5 and adult $D1.1^{LacZ/LacZ}$ retinas were processed for whole-mount double-labeling with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is strongly expressed in the arterial and venous vessels of P5 retinas as well as the microvessels (M-O, arrows and arrowheads), but faintly expressed in a subset of the vessels of adult retinas (P-R). (S-T) P5 and adult $D1.1^{LacZ/+}$ retinas were processed for whole-mount X-gal staining, revealing the strong expression of *D1.1* in P5 retinas (S) and the down-regulation of *D1.1* in adult retinas (T). Scale bars, 100 μm.

Fig. 6. *D1.1* is highly up-regulated in the neovasculature in tumors, during wound healing, and in the corneal micropocket assay. Adult $D1.1^{LacZ/+}$ mice were used for *D1.1*

expression analysis. Immunofluorescence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red) was performed on the sectioned tissues of Lewis lung carcinomas grown subcutaneously (A-C), wounded skin (D-F), normal skin (G-I), B16F10 melanomas grown in the brain (J-L), normal sclerocorneal junction (M-O), and bFGF-treated corneas (P-R). *D1.1* is highly expressed in most neovessels, whereas it is expressed in a subset of the vessels of normal skin, so there are a number of *D1.1*$^{-}$ vessels in the skin (G-I, arrows and arrowheads). *D1.1* is induced in the vessels of B16F10 melanomas grown in the brain, in which *D1.1* is barely expressed (J-L, arrows vs arrowheads). The tumor boundary is marked by dashed lines. *D1.1* is expressed in the limbic vessels at the sclerocorneal junction and in the vessels of the iris (M-O, arrows and arrowheads). (S-U) An untreated cornea and bFGF-treated corneas for 3 days and 6 days were processed for whole-mount X-gal staining, and were flat-mounted for photograph. Arrows indicate bFGF-containing pellets. Scale bars, 100 μm.

Fig. 7. *D1.1* is not essential for normal vessel development. Whole-mount anti-PECAM-1 immunoperoxidase staining reveals no obvious differences between wild type and *D1.1* homozygous mutant mice in the vasculature of E9.5 embryo (A, B), yolk sac (C, D), and E12.5 neural tube (E, F). Whole-mount X-gal staining also reveals no obvious differences between P5 *D1.1*$^{+/-}$ and *D1.1*$^{-/-}$ littermates in the vasculature of the retina (G, H), the uterus (I, J), and the intestine (K, L). Scale bars, 300 μm.

Fig. 8. Soluble D1.1-Fc protein impairs angiogenesis. (A-C) The chick chorioallantoic membrane (CAM) assay measuring spontaneous vessel growth reveals that D1.1-Fc (4

μg) inhibits spontaneous vessel growth. The arrows denote the position of the methylcellulose pellets containing D1-1 Fc or Fc proteins. The vascular density around a disk from each 10 CAMs was measured; results are means and s.e.m. (D-F) Mouse allantois explant culture reveals that pre-clustered D1.1-Fc (250 μg/ml) impairs the proper vascular pattering. The diameter of vessels was randomly measured from the surface regions of three different allantoises, and results are means and s.e.m. Scale bars, 100 μm. (G) D1.1-Fc (100 μg/μl) inhibits spontaneous and VEGF-induced migration of HUVEC cells, but does not significantly inhibit bFGF-induced migration. Results are means and standard deviations from each triplicate.

Supplemental Fig. 1. In situ hybridization of *D1.1* at E10.5. The staining of the trunk (A, B) and the tail (C) regions reveals that *D1.1* is expressed in arterial (A,C; arrows) and venous (A,C; arrowheads) endothelial cells, and endocardial cells (arrows in B). Scale bars, 100 μm.

Supplemental Fig. 2. Heterogeneous expression of *D1.1* in adult heart. Adult $D1.1^{LacZ/+}$ heart was sectioned and processed for double-label immunoflouresence staining with antibodies to β-galactosidase (Gothert et al.) and PECAM-1 (red). *D1.1* is highly heterogeneously expressed in the different regions of the heart: the right ventricle (A-C), the interventricular septum (D-L), the left ventricle (M-O), and the left atrium (P-R). Scale bars, 100 μm.

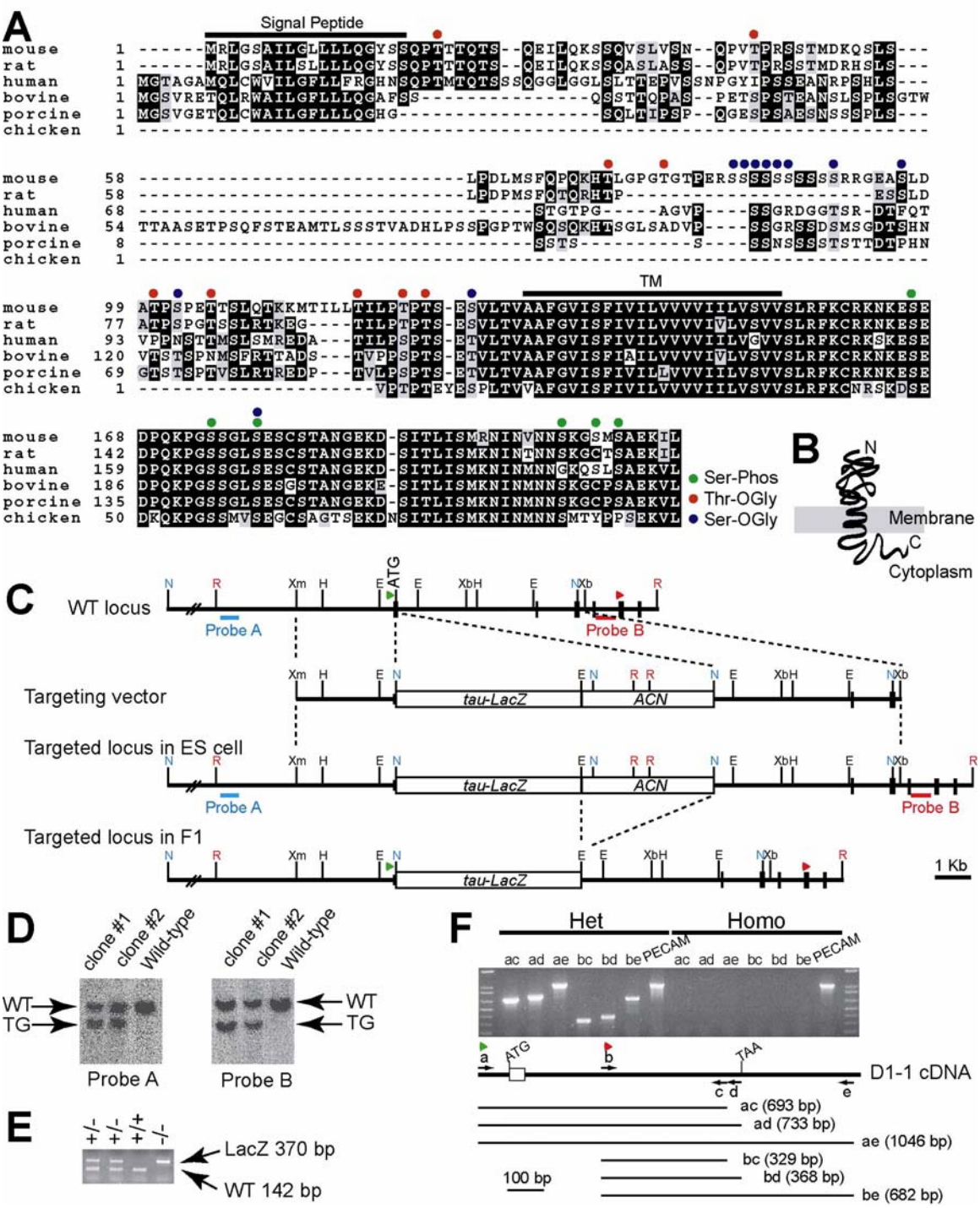


Fig. 1. Alignment of *D1.1* orthologs and targeting of the *D1.1* locus

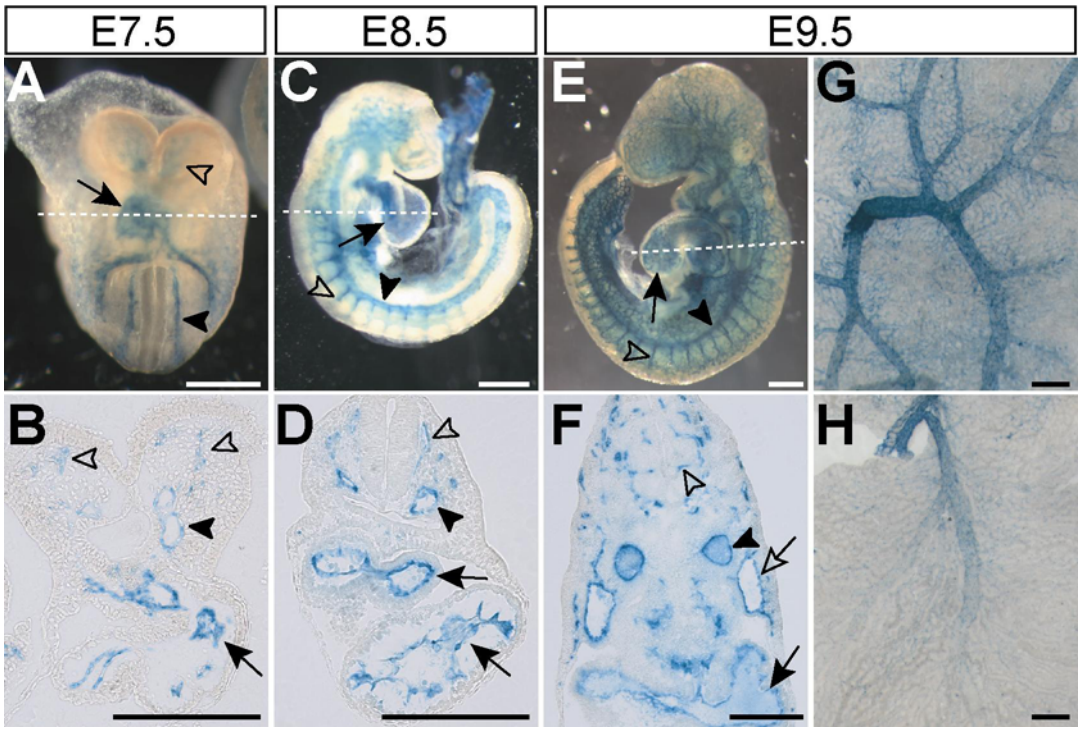


Fig. 2. *D1.1* expression during early development

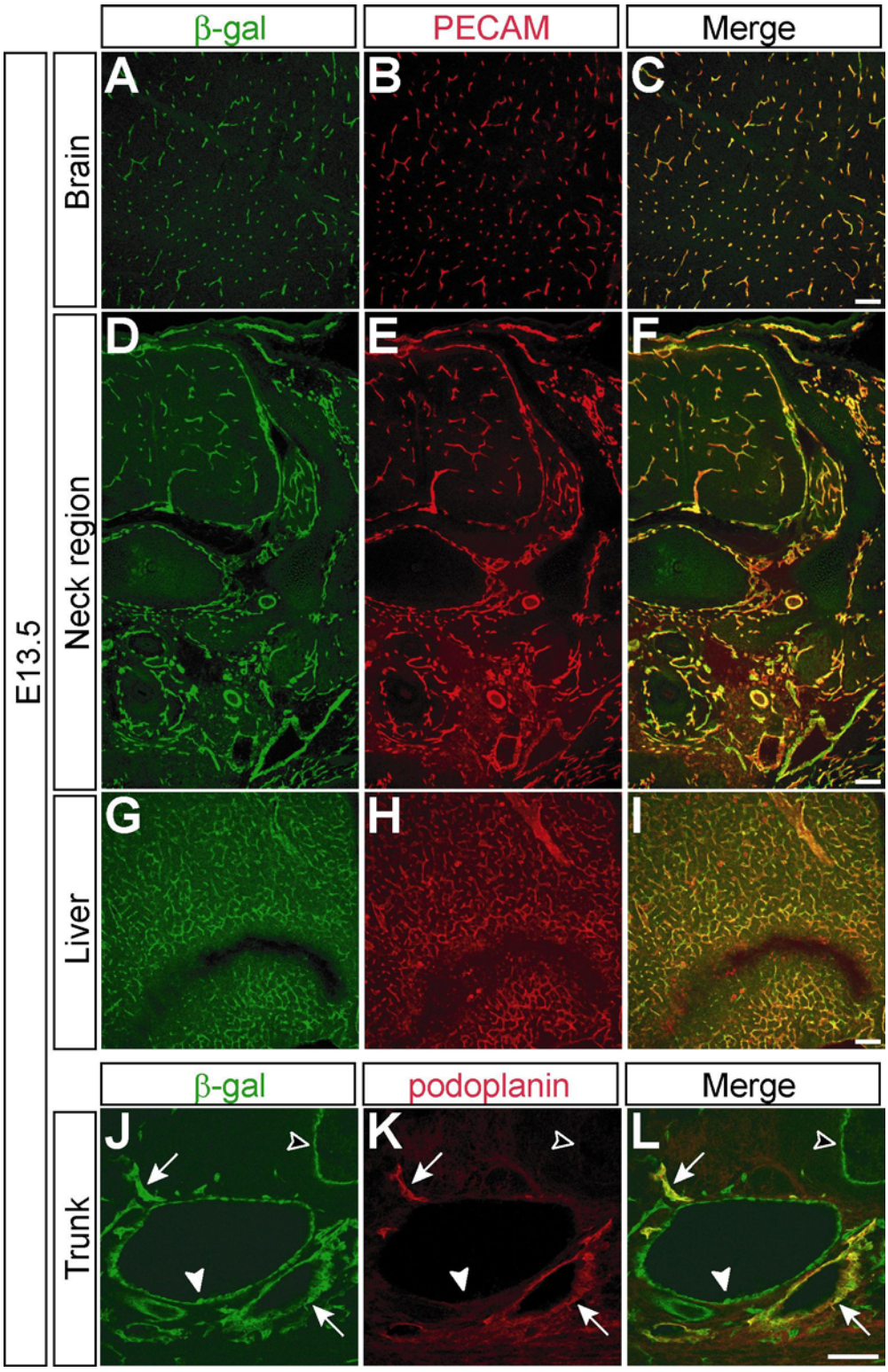


Fig. 3. Pan-endothelial expression of *D1.1* in E13.5 embryos

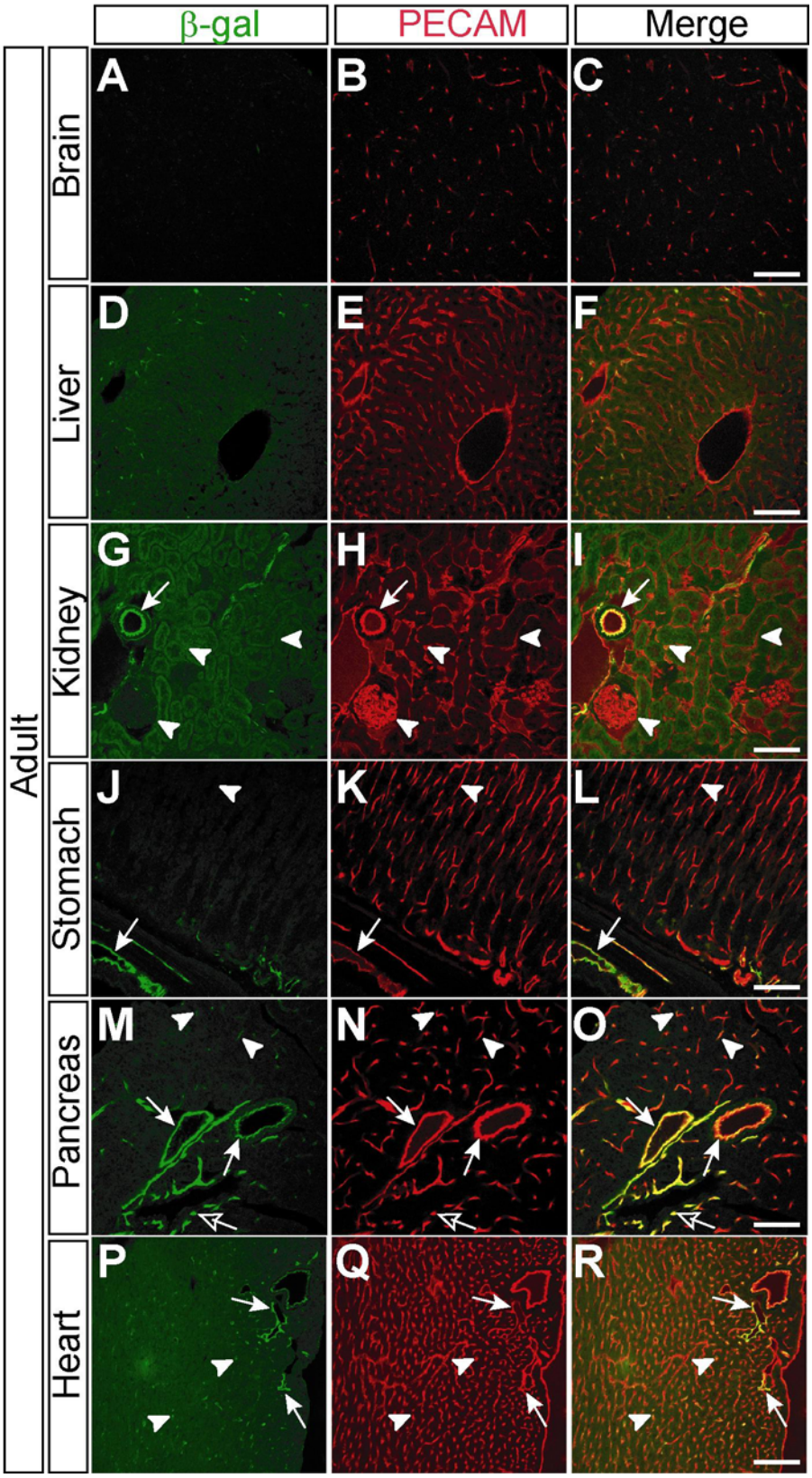


Fig. 4. *D1.1* is barely or weakly expressed in the microvessels of most adult tissues

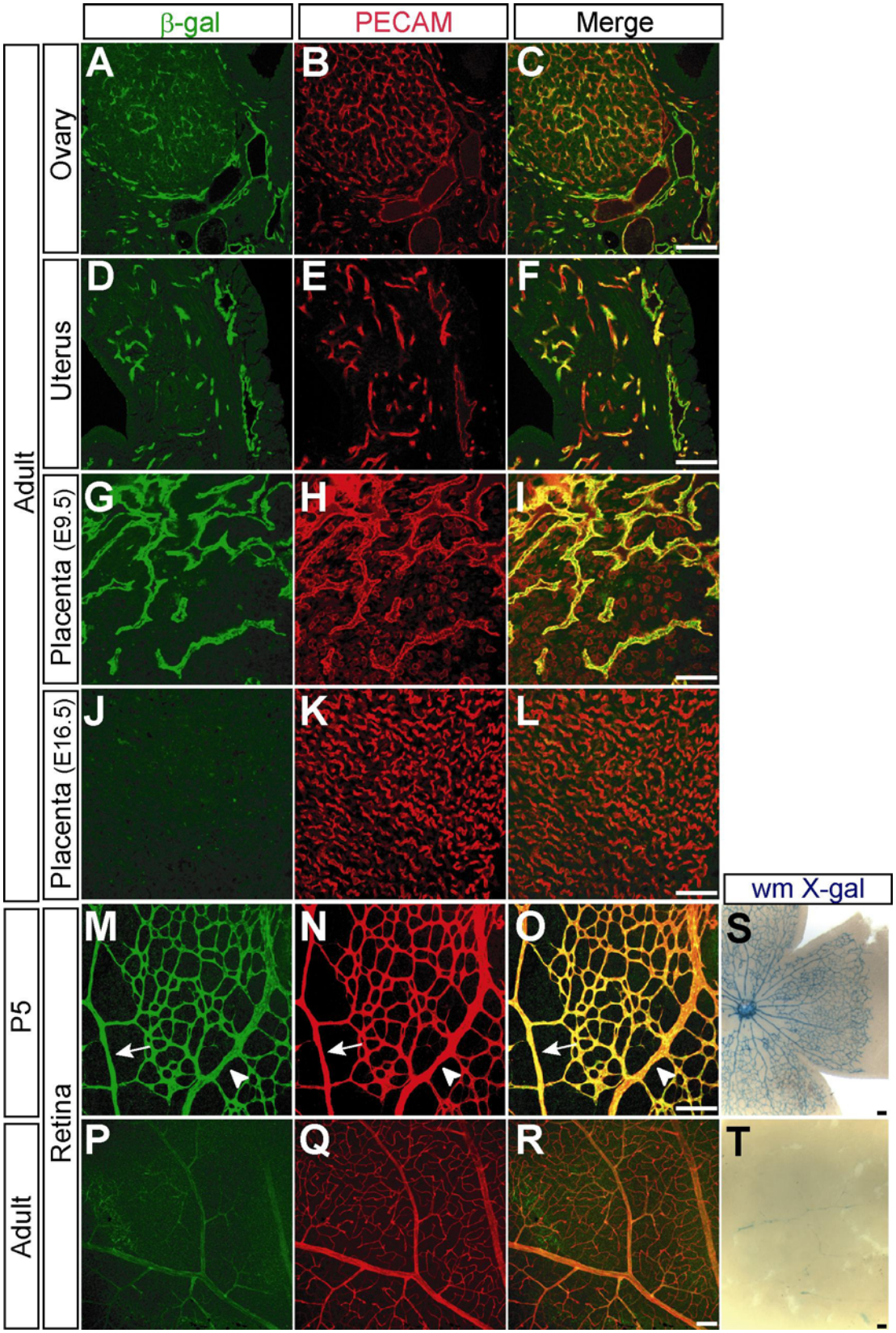


Fig. 5. *D1.1* is highly expressed in adult tissues where physiological angiogenesis occurs

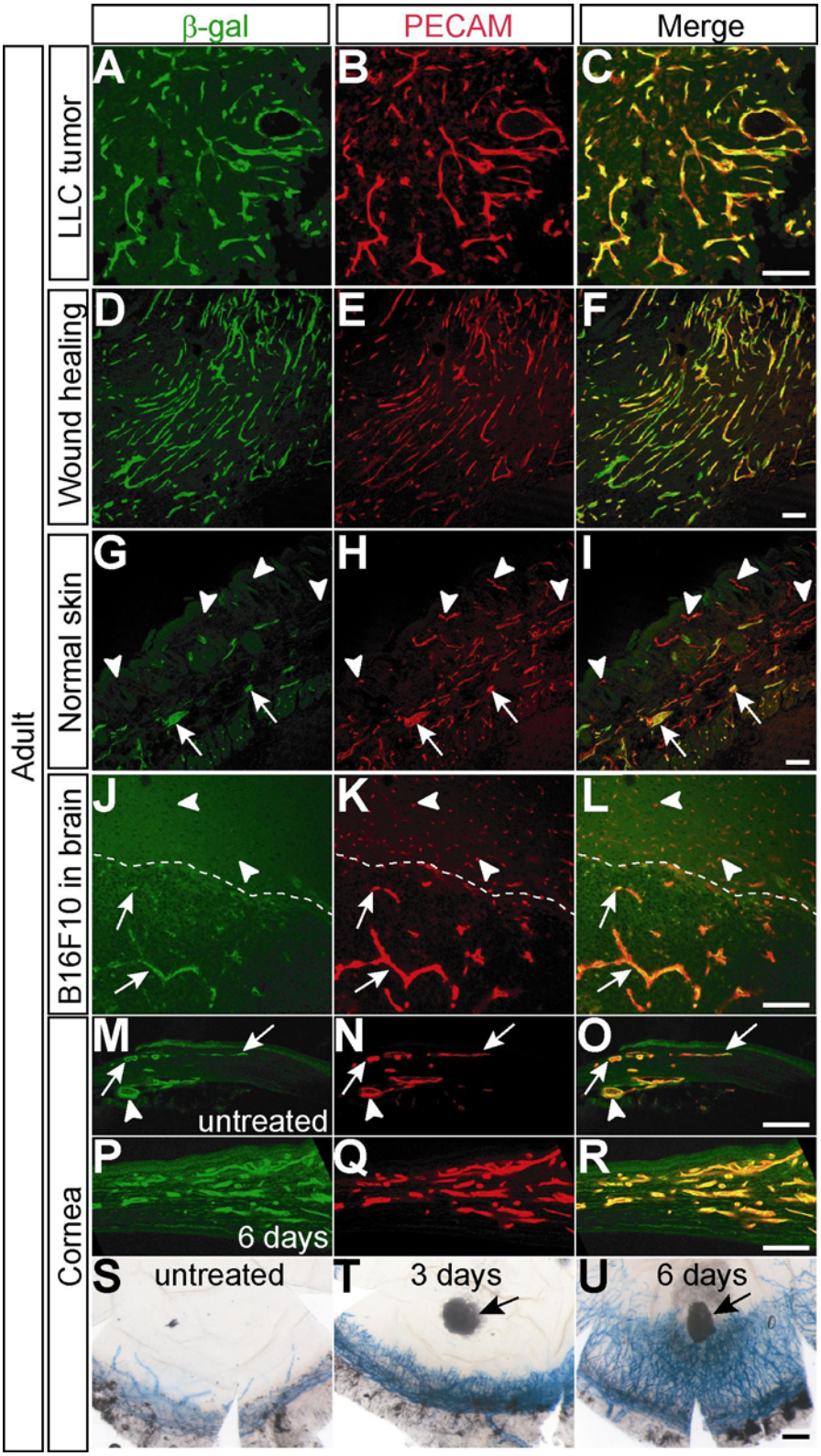


Fig. 6. *D1.1* is highly up-regulated in the neovasculature in tumors, during wound healing, and in the corneal micropocket assay

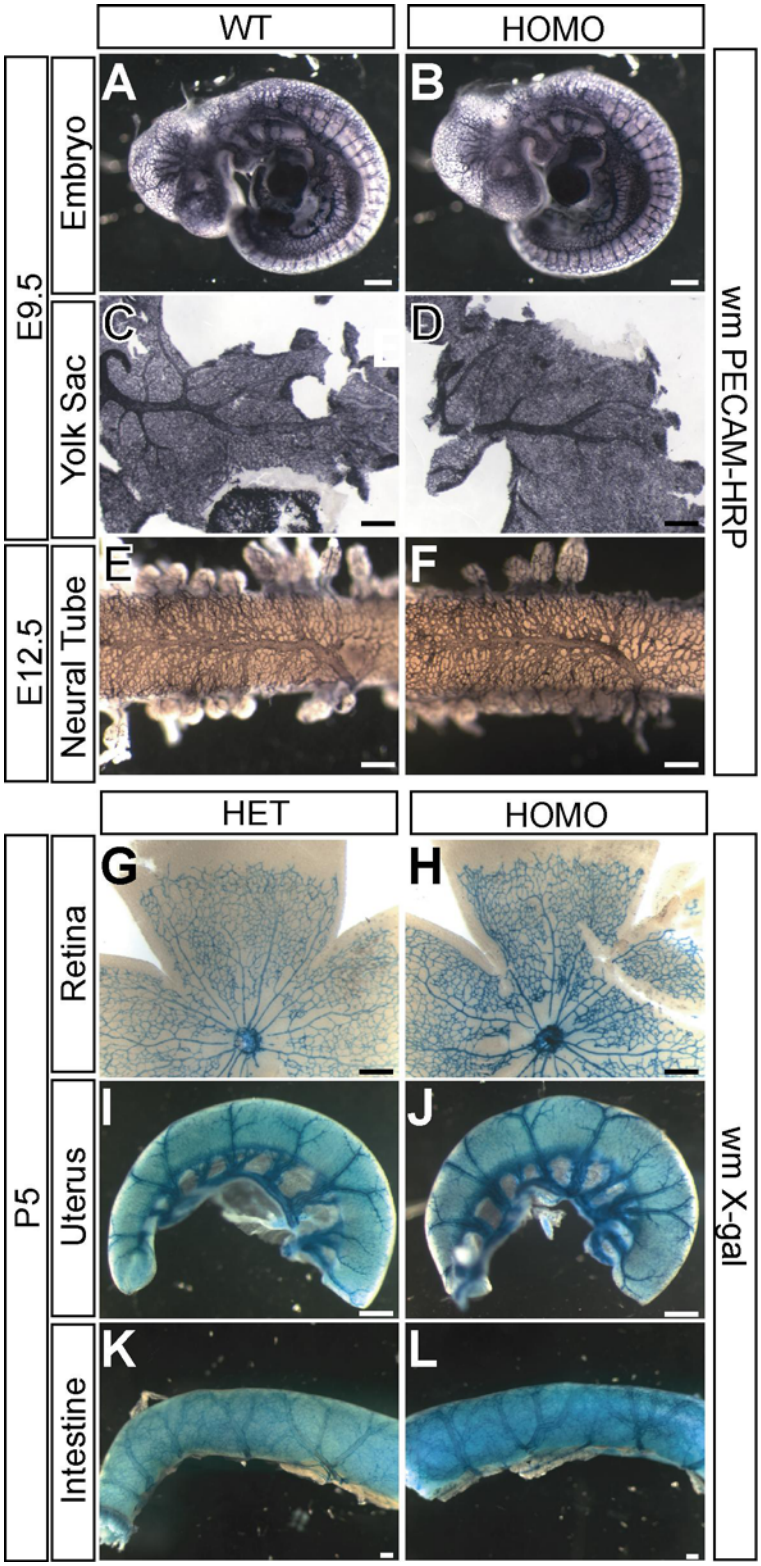


Fig. 7. *D1.1* is not essential for normal vessel development

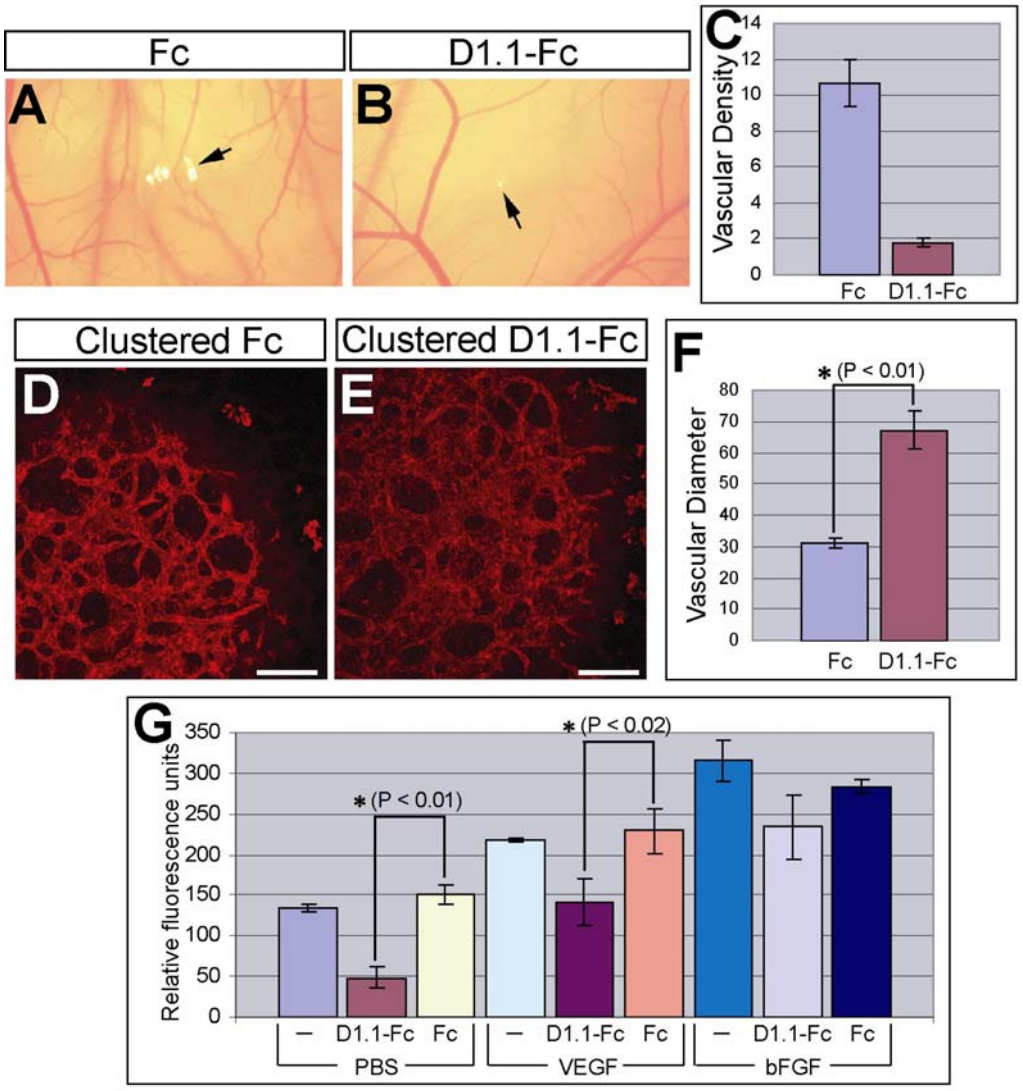
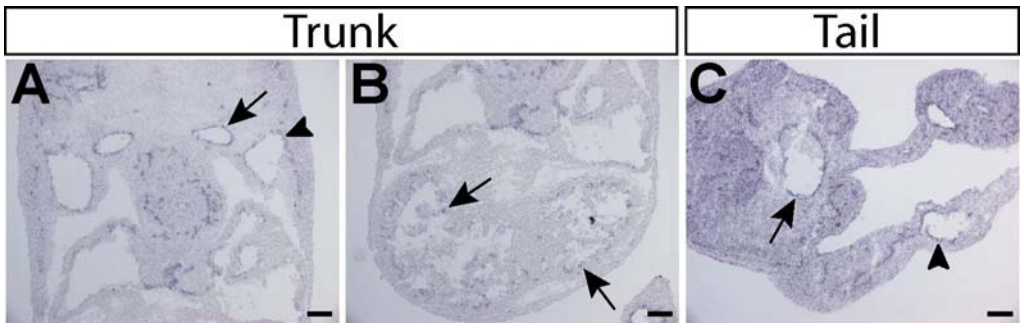
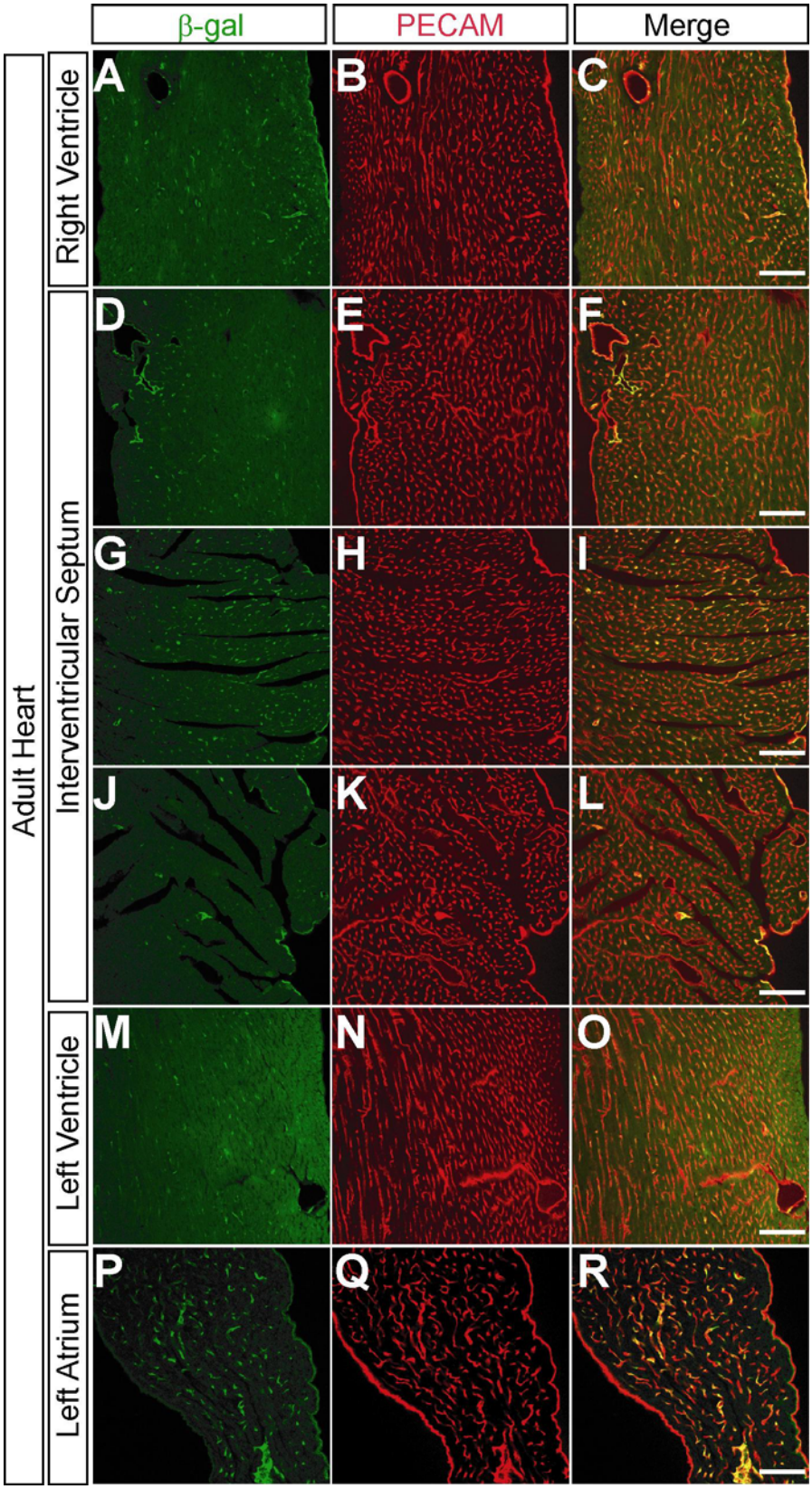


Fig. 8. Soluble D1.1-Fc protein impairs angiogenesis



Supplemental Fig. 1. In situ hybridization of *D1.1* at E10.5



Supplemental Fig. 2. Heterogeneous expression of *D1.1* in adult heart