Hedgehog signaling to distinct cell types differentially regulates coronary artery and vein development

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Vascular development begins with formation of a primary capillary plexus that is later remodeled to give rise to the definitive vasculature. Although the mechanism by which arterial and venous fates are acquired is well understood, little is known about when during vascular development arterial and venous vessels emerge and how their growth is regulated. Previously, we have demonstrated that a hedgehog (HH)/vascular endothelial growth factor (VEGF) and angiopoietin 2 (ANG2) signaling pathway is essential for the development of the coronary vasculature. Here, we use conditional gene targeting to identify the cell types that receive HH signaling and mediate coronary vascular development. We show that HH signaling to the cardiomyoblast is required for the development of coronary veins, while HH signaling to the perivascular cell (PVC) is necessary for coronary arterial growth. Moreover, the cardiomyoblast and PVC appear to be the exclusive cell types that receive HH signals, as ablation of HH signaling in both cell types leads to an arrest in coronary development. Finally, we present evidence suggesting that coronary arteries and veins may be derived from distinct lineages.

KEY WORDS: Hedgehog (HH), Vascular endothelial growth factor (VEGF), Angiopoietin (ANG), Heart development, Coronary vascular development, Myocardium, Pericyte

INTRODUCTION

Vascular development is governed by two processes: vasculogenesis and angiogenesis. Vasculogenesis refers to the formation of blood vessels via de novo differentiation of either angioblast or hemangioblast precursors, while angiogenesis is defined as the growth or remodeling of established blood vessels. In general, vascular systems undergo a stereotyped pattern of development, beginning with the formation of a primary capillary plexus that is later remodeled, giving rise to the mature vasculature. It is thought that the primary capillary plexus forms by vasculogenesis and is remodeled via angiogenesis (Flamme et al., 1997; Risau, 1997).

A critical component of capillary plexus remodeling is the emergence of a vascular tree composed of larger proximal and smaller distal vessels. In addition, it is thought that arterial and venous vessels differentiate during this remodeling process. Thus, the remodeling process yields many of the components of the mature circulatory system, including larger arteries and veins, medium-sized arterioles and venules, and smaller capillaries (Risau, 1997). Interestingly, it has been reported that capillaries also acquire arterial and venous fates (Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998).

The molecular mechanism underlying acquisition of arterial versus venous cell fates has been recently described. These analyses were based on the observation that arterial and venous endothelial cells differentially expressed ephrin B2 (EFNB2) and its receptor, EPHB4, respectively (Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998). Intriguingly, although deletion of either Ephb2 or Ephb4 in mice severely affected vascular development, the differential expression of Ephb2 and Ephb4 was not affected, indicating that factors acting upstream of these genes control arterial versus venous identity (Lawson and Weinstein, 2002). Further work has identified these factors as components of the Notch signaling pathway. Notch1, Notch3, Notch4 and the notch ligand Dil4 are expressed in arterial endothelial cells, are required for vascular development and control Ephb2 expression (Domenga et al., 2004; Duarte et al., 2004; Fischer et al., 2004; Krebs et al., 2000). Activation of Notch signaling is sufficient to promote acquisition of the arterial cell fate, and in the absence of Notch signaling, blood vessels initially form but all express the venous markers Ephb4 and Flt4 (Lawson et al., 2001).

Further analysis in zebrafish has identified that a sonic hedgehog (SHH)/vascular endothelial growth factor (VEGF) pathway acts upstream of Notch, functioning to select which endothelial cells will receive Notch signaling and take on the arterial fate. During somitogenesis, notochord-derived SHH signals regulate expression of Vegfa in the somitic mesoderm. In turn, VEGFA activates the expression of Notch5 preferentially in dorsally situated endothelial precursors, thus restricting Notch signaling to cells that will give rise to the dorsal aorta (Lawson et al., 2002).

In addition to the role of hedgehog (HH) signaling in arterial specification in zebrafish, several studies have provided evidence that HH signaling functions more broadly in vascular development. Mouse embryos lacking smoothened (transducer of HH signaling) display defects in vasculogenesis; SHH promotes vascular plexus formation in cell culture, and activation of HH signaling in the adult mouse is sufficient to promote neovascularization in several different tissues (Kanda et al., 2003; Pola et al., 2001; Vokes et al., 2004). Moreover, we have previously reported that a HH/VEGF/angiopoietin 2 (ANG2) signaling pathway is essential for coronary vascular development (Lavine et al., 2006).

Similar to other vascular systems, coronary development begins with the formation of a vascular network that is later remodeled to give rise to the mature coronary tree (Kattan et al., 2004; Morabito et al., 2002). Interestingly, the initial coronary vascular plexus consists of two sets of blood vessels located in different positions:
the subepicardial mesenchyme and the myocardial wall. We have shown that HH signaling controls the growth of both sets of blood vessels via induction of Vegfa, Vegfb, Vegfc and Ang2 expression (Lavine et al., 2006). However, the mechanism by which HH signaling can coordinate the development of both vascular structures is unclear.

We have proposed that HH signaling to two different cell types, cardiomyoblasts and perivascular cells, controls the growth of blood vessels located within the subepicardial mesenchyme and within the myocardial wall, respectively. Additionally, we hypothesized that subepicardial and intramyocardial blood vessels represent distinct vessel types. In this work, we identify the recipients of HH signaling in the embryonic heart using conditional gene targeting. We show that the cardiomyoblast and the perivascular cell are the functionally relevant targets of HH signaling during coronary vascular development. We demonstrate that HH signaling to the cardiomyoblast controls subepicardial blood vessel development, while HH signaling to the perivascular cell controls intramyocardial development. Furthermore, we show that subepicardial vessels are veins and intramyocardial vessels are arteries.

MATERIALS AND METHODS

Mouse lines

Mlc2v-Cre (Chen et al., 1998a), Dermin1-Cre (Yu et al., 2003), Smo0/0 (Long et al., 2001), Ephb2-lacZ (Wang et al., 1998), Ephb4-lacZ (Gerety et al., 1999) and ROSAI6R (Soriano, 1999) mice were maintained on a C57/B6J background or bred at least three generations onto C57/B6J. Littermate controls for conditional knockout experiments included animals with a genotype of Smo0/0, Cre or Smo0/0 (and no Cre allele).

Whole-mount PECAM immunohistochemistry

PECAM staining was performed as described (Lavine et al., 2005). Briefly, tissues were fixed in 4% PFA and dehydrated in a methanol series, incubated in methanol/hydrogen peroxide, rehydrated and blocked in PBSST (5% goat serum/PBS 0.1% Triton X-100). The primary antibody was rat anti-mouse PECAM (Pharminogen, 1:200). Biotinylated goat anti-rat IgG (Vector, 1:200) was used followed by Vectastain ABC-peroxidase reagent and DAB visualization (Vector). All antibody and ABC reagent dilutions were carried out in PBSST. Antibody and ABC reagent incubations were carried out at 4°C overnight. Following each overnight incubation, tissues were washed five times (1 hour each at 4°C) with PBSST. Following photography, PECAM stained hearts were paraffin embedded and sectioned. Paraffin sections (4 μm) were then dewaxed, rehydrated, counterstained with Hematoxylin (Sigma) and mounted. Whole-mount specimens were photographed at 25× and histological sections at 400× magnification. lacZ staining was performed as described previously (Soriano, 1999).

Blood vessel density was quantified by counting the number of vessels per unit area (10,000 square pixels). At least six biological specimens were examined per genotype. For quantification of the number of vessels per 20× field, three representative fields were analyzed from three biological samples for each genotype. The number of subepicardial and intramyocardial blood vessels were quantified and plotted. The error bars represent one standard deviation from the mean.

Fluorescent immunohistochemistry

Cryosections (12 μm) were cut from E12.5-E13.5 hearts and stained with primary antibodies to PECAM (R&D) (1:200), human CD4 (R&D) (1:200), CD45 (R&D) (1:200), SCA1 (R&D) (1:50), cardiac actin (Sigma) (1:400), VEGFA (Santa Cruz) (1:200), VEGFB (Santa Cruz) (1:200), VEGFC (Santa Cruz) (1:200) and β-galactosidase (Abcam) (1:250). The following secondary antibodies were used at 1:200: anti-rat Alexa 555, anti-rabbit Alexa 488, anti-rabbit Alexa 647 (Molecular Probes) and anti-mouse IgM FITC (Vector Labs). Immunofluorescence was visualized on a Zeiss Apotome Microscopy system. All specimens were photographed at 400× magnification.

For 3D reconstruction of PECAM staining, 40 μm cryosections were cut from E12.5-E13.5 hearts and stained with a primary antibody to PECAM (R&D) (1:200) and anti-rat Alexa 555 secondary antibody (Molecular Probes) (1:200). Using the Zeiss Apotome system, 40 images, each spanning 1 μm, were acquired per section and processed with Zeiss Axiovision software to produce a compressed z-stack image. Representative images are at 400× magnification.

Quantification of VEGF protein expression

VEGFA, VEGFB and VEGFC expression was quantified by measuring the immunofluorescent signal in both the myocardium and perivascular cells from control, Smomlc2v and Smomlc2v E12.5 hearts. Regions containing myocardial and perivascular cells were identified by co-labeling with PECAM. Perivascular regions corresponded to cells within the immediate proximity of intramyocardial blood vessels, while myocardial areas corresponded to those not in the immediate vicinity of intramyocardial blood vessels. Average pixel intensity was measured in nine total tissue sections for each genotype (three sections each from three independent hearts). Pixel intensity was quantified using Adobe Photoshop software (and confirmed using other software packages including Metamorph and Canvas), and displayed as relative to control. Error bars represent one standard deviation from the mean. P-values were calculated using Student’s t-test.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Lavine et al., 2005). Tissues were photographed and then cryosectioned (16 μm), mounted on slides and re-photographed. In situ probes used were for VEGFA, VEGFB, VEGFC and PTC1 (Lavine et al., 2006). All comparisons shown are between littermates and all experiments were repeated at least three times. Cryosections are at 400× magnification.

RESULTS

Subepicardial and intramyocardial blood vessels represent distinct venous and arterial lineages

Subepicardial and intramyocardial blood vessels represent two vascular subsets that appear to be preferentially regulated by HH signaling to different cell types. Activation of HH signaling in the myocardium promotes subepicardial blood vessel growth but not intramyocardial blood vessel growth (Lavine et al., 2006). These data suggest that subepicardial and intramyocardial blood vessels may represent distinct vascular lineages. Consistent with this hypothesis, corrosion casting of late gestation rat hearts has demonstrated that coronary arteries are positioned within the myocardial wall, while veins are located closer to the epicardial surface (Ratajska et al., 2003), suggesting that subepicardial and intramyocardial blood vessels may represent venous and arterial blood vessels, respectively.

To identify arterial and venous blood vessels within the developing heart, we examined mice harboring lacZ inserted into either the Ephb2 or Ephb4 loci. Ephb2-lacZ and Ephb4-lacZ mice specifically express β-galactosidase in arteries and veins, respectively (Gerety et al., 1999; Wang et al., 1998). Immunofluorescent staining of E12.5 hearts with antibodies against the endothelial marker PECAM (CD31) and against β-galactosidase demonstrated that ephrin B2 is expressed within intramyocardial blood vessels and Ephb4 is expressed within subepicardial blood vessels during the vascular plexus stage (Fig. 1A-F). Similar to the embryonic heart, immunofluorescent staining of adult hearts demonstrated that, in general, larger arteries are located deeper within the myocardial wall, whereas larger veins are positioned closer to the epicardial surface. By contrast, smaller arterial and venous blood vessels are dispersed throughout the myocardium (Fig. 1G-L). These analyses indicate that subepicardial and intramyocardial vessels represent coronary veins and arteries, respectively. Moreover, coronary artery and vein identity is specified.
In addition to the cardiomyoblast, the cardiomyoblast and perivascular cell (PVC) are maintained in the adult heart. Larger coronary arteries and veins, established during vascular plexus development, are formed during or prior to the vascular plexus stage, and the relative positions of larger coronary arteries and veins, established during vascular plexus development, are maintained in the adult heart.

The cardiomyoblast and perivascular cell (PVC) are the exclusive targets of HH signaling

In addition to the cardiomyoblast, Ptc1 (previously known as Ptc1) is expressed in perivascular cells (PVCs), suggesting that PVCs are also targets of HH signaling. Consistent with this, Vegfa, Vegfb and Vegfc are also expressed in these cells (Lavine et al., 2006).

PVCs act as supporting cells for developing blood vessels and give rise to vascular smooth muscle and adventitial fibroblasts (Yoshida and Owens, 2005). Previously, we inserted the Cre-recombinase cDNA into the Dermo1 (Twist2) locus (Yu et al., 2003). Using the Rosa26-lacZ reporter mouse (Soriano, 1999), we have identified sites of Dermo1-Cre activity in the developing heart. Beginning at E11.5, segmental regions of the epicardium display Dermo1-Cre activity (data not shown). At E12.5, Dermo1-Cre activity is present at epicardial sites that appear to be undergoing an epithelial mesenchymal transformation (EMT) and in cells located adjacent to developing intramyocardial blood vessels (Fig. 2A,D-F). Further characterization revealed that cells of the Dermo1-Cre lineage express smooth muscle actin and do not express cardiac actin (Fig. 2G-L). Moreover, these cells express VEGFA, consistent with the notion that Dermo1-Cre marks the PVC lineage (Fig. 2M-O). Given that PVCs and the subepicardial mesenchyme are derived from epicardium (Dettman et al., 1998; Merki et al., 2005; Mikawa and Gourdie, 1996; Vrancken Peeters et al., 1999), these data suggest that Dermo1-Cre marks epicardial cells fated to undergo EMT that later take residence within either subepicardial mesenchymal or perivascular locations. This is consistent with proposed functions of Twist in EMT (Kang and Massague, 2004). lacZ staining of postnatal Dermo1-Cre/Rosa26-lacZ hearts demonstrated lacZ activity in cells located within the interstitial space, including both vascular smooth muscle cells and fibroblasts beyond the region surrounding the atrial-ventricular groove (Fig. 3A-B). Interestingly, myocardial cells adjacent to the atrial-ventricular groove are not targeted by Mlc2v-Cre (Fig. 3C), potentially explaining why blood vessels are still present in the proximity of this region.

Consistent with loss of HH signaling, cryosections of Smomlc2v; dermo1 CKO hearts stained with a Pich1 in situ probe showed loss of Pich1 expression in both cardiomyoblasts and perivascular cells (Fig. 3D,E). Moreover, immunofluorescent analysis for VEGFA, VEGFB and VEGFC revealed that, compared with controls, Smomlc2v; dermo1 CKO hearts expressed significantly lower levels of VEGFA protein (Fig. 3F-G) and undetectable levels of VEGFB and VEGFC protein (Fig. 3H-K). In addition, whole-mount in situ hybridization for Vegfa, Vegfb and Vegfc demonstrated that, compared with controls, Smomlc2v; dermo1 CKO hearts displayed reduced Vegfa, Vegfb and Vegfc expression (see Fig. S1 in the supplementary material). Together with the failure of vascular development in Smomlc2v; dermo1 CKO hearts, these data indicate that the cardiomyoblast and perivascular cells are the predominant cell types that receive HH signaling during coronary development.
HH signaling to the cardiomyoblast and perivascular cells are respectively necessary for coronary vein and artery development

Previously, we have shown that activation of HH signaling in the myocardium preferentially promotes subepicardial blood vessel growth with little effect on intramyocardial vessel growth, suggesting that subepicardial and intramyocardial blood vessel development is controlled by HH signaling to different cell types (Lavine et al., 2006). To test this hypothesis, we examined vascular development in hearts in which HH signaling was inactivated in either the myocardial or perivascular cell.

![Diagram of HH signaling](image)

**Fig. 2.** Dermo1-Cre is active in the perivascular cell lineage. (A-C) lacZ staining of Dermo1-Cre/Rosa26-lacZ hearts showing that at E12.5 (A) Dermo1-Cre marks cells localized in patches of the epicardium that are undergoing EMT (arrow) and around intramyocardial blood vessels (arrow). (B) lacZ staining of postnatal day 10 (P10) hearts demonstrating that Dermo1-Cre-expressing cells give rise to perivascular interstitial cells (arrow), but not the vascular endothelium (arrowhead). (C, inset) Dermo1-Cre-positive cells also give rise to the interstitium of the valvular leaflets (asterisk). (D-F) Immunofluorescent staining of cryosections for lacZ (D, red) and PECAM (E, green) confirming that Dermo1-Cre-expressing cells give rise to perivascular interstitial cells (arrow), but not the vascular endothelium (arrowhead). (C, inset) Dermo1-Cre-positive cells also give rise to the interstitium of the valvular leaflets (asterisk). (D-F) Immunofluorescent staining of cryosections for lacZ (D, red) and PECAM (E, green) confirming that Dermo1-Cre-expressing cells give rise to perivascular interstitial cells (arrow), but not the vascular endothelium (arrowhead). (C, inset) Dermo1-Cre-positive cells also give rise to the interstitium of the valvular leaflets (asterisk). (D-F) Immunofluorescent staining of cryosections for lacZ (D, red) and PECAM (E, green) confirming that Dermo1-Cre-expressing cells give rise to perivascular interstitial cells (arrow), but not the vascular endothelium (arrowhead). (C, inset) Dermo1-Cre-positive cells also give rise to the interstitium of the valvular leaflets (asterisk).

![Diagram of HH signaling](image)

**Fig. 3.** HH signaling to the cardiomyoblast and pericyte is essential for coronary development. (A, B) Whole-mount PECAM immunohistochemistry demonstrating that, compared with control (A), Smomlc2v; dermo1 CKO heart (B) displays an arrest in coronary development. Bracket indicates atrial ventricular groove. (C) β-Galactosidase staining of an E12.5 Mlc2v-Cre/Rosa26R heart, demonstrating that Mlc2v-Cre does not efficiently target cardiomyoblasts located in the atrial ventricular groove (AVG, bracket). By contrast, Mlv2v-Cre efficiently targets the ventricular myocardium (area below bracket). (D, E) Cryosections of hearts stained with in situ probes for Ptc1 revealing that, compared with controls (D), Smomlc2v; dermo1 CKO hearts (E) lack both myocardial (asterisk) and perivascular (arrow) sources of Ptc1 expression. (F-K) Immunofluorescent staining of cryosections for PECAM (red) and VEGF ligands (green) revealing that, compared with controls (F-H), Smomlc2v; dermo1 CKO hearts (G, K) display diminished expression of VEGF (F, G), VEGFB (H, I) and VEGFC (J, K). White arrowhead indicates PECAM-positive endocardial cells that appear unaffected in these conditional mutants. Red arrow in A, D denotes orientation of tissue sections in relation to whole-mount photographs (B, base; A, apex).
expression. However, no Ephb4-expressing vasculature was
detected, indicating that Smomlc2v CKO hearts contained arterial but
lacked venous vasculature (Fig. 4I-U).

In addition to containing only arterial blood vessels, Smomlc2v
CKO hearts lacked a subepicardial mesenchyme. Co-labeling with
PECAM and cardiac actin antibodies confirmed that Smomlc2v CKO
hearts contained only blood vessels located within the myocardial
wall and lacked blood vessels growing within the subepicardial space
(Fig. 5A-F). Immunohistochemistry for PECAM, cardiac actin, WT1
and DAPI demonstrated that Smomlc2v CKO hearts contained
fewer intramyocardial blood vessels per 20× field compared
with controls (71.7±3.6 and 45.0±5.9, P<0.001). Histological
analysis demonstrated that Smomlc2v CKO hearts displayed specific
defects in intramyocardial blood vessel development. Smomlc2v
CKO hearts contained 8.0±0.7 intramyocardial blood vessels/20×
field, whereas control hearts contained 16.2±1.3 intramyocardial
blood vessels/20× field (P<0.0001). Interestingly, subepicardial
blood vessel development remained intact in Smomlc2v CKO hearts
(Fig. 6E-I).

To confirm that decreased intramyocardial blood vessel number
in Smomlc2v CKO hearts represented a deficit in arterial blood vessel
development, we bred the Efnb2- and Ephb4-lacZ alleles into the
Smomlc2v CKO background. Immunohistochemistry for PECAM
and β-galactosidase revealed that Smomlc2v CKO hearts contained
fewer ephrin B2-expressing vessels but normal numbers of Ephb4-
expressing vessels, indicating that Smomlc2v CKO hearts display
defects specific to the arterial vasculature (Fig. 6J-V).
HH signaling to the cardiomyoblast and perivascular cell is required for the expression of specific VEGF ligands

We have previously shown that HH signaling promotes coronary vascular development by inducing Vegfa, Vegfb, Vegfc and Ang2 expression (Lavine et al., 2006). To determine whether HH signaling to the cardiomyoblast regulates a specific subset of these factors, E13.5 control and Smomlc2v CKO hearts were analyzed by in situ hybridization for Ptch1 and by immunohistochemistry for VEGFA, VEGFB and VEGFC.

Compared with controls, Smomlc2v CKO hearts displayed diminished levels of Ptch1 in the myocardium of Smomlc2v CKO hearts. However, perivascular Ptch1 expression was present in Smomlc2v CKO hearts (Fig. 7A,B). Similar to that of Ptch1, immunofluorescent analysis with antibodies against VEGFA and VEGFB confirmed the loss of myocardial expression and retention of perivascular expression of VEGFA and VEGFB protein in Smomlc2v CKO hearts (Fig. 7C-F). Immunolabeling for VEGFC demonstrated that both control and Smomlc2v CKO hearts displayed comparable perivascular VEGFC protein expression (Fig. 7G,H). Quantitative analysis of fluorescent intensity confirmed that, compared with controls, Smomlc2v CKO hearts had significant (P<0.001) reductions in myocardial VEGFA and VEGFB expression (Fig. 7Q). In situ hybridization for Vegfa, Vegfb and Vegfc confirmed reductions in myocardial expression of Vegfa and Vegfb in Smomlc2v CKO hearts (see Fig. S1 in the supplementary material).

These analyses indicate that HH signaling to the cardiomyoblast regulates Vegfa and Vegfb expression in a cell-autonomous manner. This is consistent with our previous finding that activation of HH signaling in the cardiomyoblast can upregulate Vegfa expression cell autonomously (Lavine et al., 2006). Together with the finding that HH signaling to the myocardium is essential for subepicardial vessel growth, these data support the conclusion that HH signaling to the myocardium controls subepicardial vessel development by regulating myocardial VEGFA and VEGFB expression.

Analysis of Smomlc2v CKO hearts revealed that although subepicardial vessel development was disrupted, intramyocardial vessel development was intact. Moreover VEGFA and VEGFB expression was specifically lost from the myocardium but retained in PVCs of Smomlc2v CKO hearts. These data suggest that perivascular expression of VEGF ligands regulates intramyocardial blood vessel development. Given that disruption of HH signaling in PVCs leads to specific defects in intramyocardial vessel development, we hypothesized that HH signaling to PVCs regulates expression of VEGF ligands in these cells. This hypothesis is supported by the loss of both cardiomyoblast and perivascular sources of VEGF ligand expression in Smomlc2v; dermo1 CKO hearts (Fig. 3). Consistent with loss of perivascular HH signaling, in situ hybridization for Ptch1 revealed decreased expression in perivascular cells of Smomlc2v CKO hearts compared with controls. Myocardial Ptch1 expression was unchanged in Smomlc2v CKO hearts (Fig. 7I,J). Similarly, immunofluorescent analysis of VEGFA, VEGFB and VEGFC protein expression confirmed specific loss of VEGFA and VEGFB expression in perivascular cells of Smomlc2v CKO hearts. Myocardial VEGFA and VEGFB protein expression was unaffected (Fig. 7K-N). VEGFC, which is specifically expressed in perivascular cells, could not be detected in the ventricles of Smomlc2v CKO hearts (Fig. 7O,P). Quantitative analysis of fluorescent intensity confirmed that, compared with controls, Smomlc2v CKO hearts had statistically significant
reductions in perivascular VEGFA, VEGFB and VEGFC expression (Fig. 7R). In situ hybridization for Vegfa, Vegfb and Vegfc confirmed reductions in perivascular expression of these factors in Smo\textsuperscript{dermo1} CKO hearts (see Fig. S1 in the supplementary material).

In contrast to Smo\textsuperscript{mhc2v} CKO hearts, which demonstrate an absence of subepicardial blood vessels and reduced myocardial VEGF ligand expression, Smo\textsuperscript{dermo1} CKO hearts showed a quantitative decrease in intramyocardial blood vessels despite diminished perivascular VEGF expression. Given that subepicardial and intramyocardial blood vessels express identical VEGFRs (Lavine et al., 2006), these data suggest that myocardial sources of VEGFA and VEGFB preferentially promote subepicardial vessel growth, but can also support intramyocardial vessel growth to a lesser degree. However, perivascular sources of VEGFA, VEGFB and VEGFC specifically control intramyocardial vessel growth, as no subepicardial vessels are present in the ventricles of Smomhc2v CKO hearts (Fig. 4).

**Coronary arteries and veins may be derived from distinct vascular lineages**

The finding that HH signaling to different tissues differentially controls coronary artery and vein development raises the possibility that coronary arteries and veins may develop via distinct mechanisms and potentially may be derived from different
endothelial cell precursors. Consistent with this hypothesis, examination of H&E stained histological sections of E12.5 hearts revealed that whereas coronary arteries contained only red blood cells, coronary veins contained both red blood cells and rosette-like clusters of undefined cells located within the vascular lumen (Fig. 8A,B). These clusters of cells were reminiscent of hemangioblast precursors present within vascular channels of the yolk sac (Ema and Rossant, 2003).

To determine whether hemangioblasts are present within coronary veins, we examined the expression of the hemangioblast markers, CD45, SCA1 (Bailey et al., 2004) and SCL/TAL1 (Chung et al., 2002) in E12.5 hearts. In contrast to PECAM, which marks both coronary veins and arteries, CD45 expression could only be detected in coronary veins (Fig. 8C,D). Consistent with control hearts (C,E), which express VEGFA and VEGFB in both cardiomyoblasts (asterisk) and perivascular cells (arrow), Smomlc2v CKO hearts (D,F) express VEGFA and VEGFB in only perivascular cells (arrow). VEGFC is expressed in perivascular cells of both control (G) and Smomlc2v CKO (H) hearts (arrows). (K) Cryosections of hearts stained with an in situ probe for Ptc1. Although control hearts (i) express Ptc1 in both cardiomyoblasts (asterisk) and perivascular cells (arrow), Smomlc2v CKO hearts (j) express these transcripts in only the cardiomyoblast (asterisk). (K-P) Immunofluorescent staining of cryosections for PECAM (red) and VEGF ligands (green). Compared with control hearts (K,M), which express VEGFA and VEGFB protein in both cardiomyoblasts (asterisk) and perivascular cells (arrow), Smomlc2v CKO hearts (L,N) express VEGFA and VEGFB in only cardiomyoblasts (asterisk). VEGFC is expressed in perivascular cells of control (O), but not Smomlc2v CKO (P) hearts. (Q,R) Quantitative analysis of VEGF expression demonstrating statistically significant alterations in VEGF ligand expression in Smomlc2v CKO (Q) and Smodermo1 CKO (R) hearts compared with control hearts. Black bars represent myocardial expression and grey bars represent perivascular expression. Asterisk indicates a statistically significant difference compared with controls (P<0.01).

**DISCUSSION**

**HH signaling coordinately regulates coronary artery and vein development**

Previously, we hypothesized that HH signaling to the cardiomyoblast and perivascular cell regulates coronary vascular development (Lavine et al., 2006). Through conditional gene targeting, we have now shown that the cardiomyoblast and perivascular cells are the relevant targets of HH signaling. Furthermore, we have demonstrated that myocardial and perivascular HH signaling controls the development of distinct vascular subtypes. HH signaling to the cardiomyoblast is required for coronary vein development and HH signaling to the perivascular cell specifically supports coronary artery development. Moreover, coronary veins probably arise from hemangioblasts, whereas coronary arteries are probably derived from another cell type, possibly from previously established vasculature (Fig. 8I-K).
HH signaling controls coronary artery and vein development
coronary artery and vein development possibly through vasculogenesis. This suggests that subepicardial vessels probably develop from hemangioblasts, whereas coronary arteries are probably derived from hematopoietic origin. Blue, PECAM; green, CD45; Red, SCA1. (F-H) Immunofluorescent staining of SCL/TAL1-CD4 knock-in E12.5 hearts, demonstrating that SCL/TAL1 is expressed in coronary veins (arrowhead) but not in coronary arteries (arrow). Red, PECAM; green, CD45 (SCL/TAL1). H, merge of fluorescent signals in F-G. (C-G) Immunofluorescent signals are superimposed on DIC images. (A,C-H) Taken at 400X magnification. (I) The developing coronary vascular plexus is composed of two distinct subsets of blood vessels: coronary arteries and veins. Coronary vessels are derived from hemangioblasts, whereas coronary arteries are probably derived from endothelial cells. (K) Model describing the signaling events that coordinately control coronary artery and vein development. Perivascular HH signaling controls coronary artery growth by regulating perivascular expression of VEGFA, VEGFB and VEGFC. Myocardial HH signaling controls myocardial VEGFA and VEGFB expression, which is required for coronary vein growth, and in combination with perivascular VEGF expression, positively regulates coronary artery growth.

RESEARCH ARTICLE

The ability of HH ligands to coordinately control the development of coronary arteries and veins by signaling to distinct cell types implies that coronary artery and vein growth can be uncoupled by differentially altering the ability of cardiomyoblasts and perivascular cells to receive HH signaling. Although there is no evidence that this occurs during the vascular plexus stage, it may be important during the remodeling stage, as in the adult, coronary arteries and veins are not always located in the same positions and do not always follow the same routes (Icardo and Colvee, 2001).

In addition, the ability of HH signaling to differentially promote the development of distinct vessel types by signaling to different tissues may be important beyond differentially promoting artery and vein growth. It is possible that differential HH signaling may promote distinct patterns of vascular growth. Perivascular HH signaling may promote angiogenic growth, such as vascular sprouting, via localized production of VEGF ligands. By contrast, HH signaling to the cardiomyoblast probably produces a more diffuse pattern of VEGF expression and thus may promote a vasculogenic or vascular plexus pattern of growth. Consistent with this, subepicardial vessels probably develop from hemangioblasts possibly through vasculogenesis.

**Coronary arterial and venous lineages are established during the vascular plexus stage**

Vascular development is thought to proceed through a stereotyped series of events beginning with the formation of a vascular plexus that is later remodeled, giving rise to the mature vasculature. Although much is known about the signaling mechanisms that confer arterial and venous cell fates, it remains unclear when this signaling occurs and when such fates are established.

Studies analyzing spatiotemporal patterns of Efnb2 and Ephb4 expression have produced contrasting results. In the yolk sac, arterial and venous cell fates appear to be established during the vascular plexus stage. However, in the embryonic head region, arterial and venous gene expression is not observed until the remodeling phase (Gerety et al., 1999; Wang et al., 1998).

In the embryonic heart, we have observed that coronary arterial and venous fates are established either during or prior to the vascular plexus stage. Thus, the coronary vascular plexus is not merely a network of capillaries, but rather consists of two distinct and superimposed sets of arterial and venous blood vessels. Interestingly, the spatial relationship between these two sets of blood vessels is conserved in the adult heart, indicating that processes that pattern the vascular plexus may influence the organization of the mature vasculature.

**Vascular plexus remodeling**

The molecular mechanisms that control vascular remodeling are largely unknown. However, a key observation that has been made is that classical axon guidance factors can influence vascular remodeling and patterning (Carmeliet, 2003). Netrin signaling through the UNC5B receptor acts as a repulsive cue during vascular morphogenesis. Loss of Unc5b leads to ectopic vascular sprouting.
and subsequent defects in vascular patterning (Lu et al., 2004). Similarly, plexin signaling through semaphorin receptors also serves as a repulsive cue, as mutations in plexin D1 (Ptxd1) result in ectopic vascular sprouting (Torres-Vazquez et al., 2004). Consistent with a role in vascular remodeling, Ptxd1–/– and semaphorin 3C–/– mice display profound defects in aortic arch remodeling (Gitler et al., 2004). Notably, neuropilins (NRPs) are co-receptors for both semaphorin and VEGF receptors, and Nrp1 and Nrp2 knockout mice display severe vascular growth and pattern phenotypes (Kawasaki et al., 1999; Takashima et al., 2002; Yuan et al., 2002).

In addition to netrin and semaphorin, ephrins have also been implicated in the control of vascular patterning. The ephrin B2 ligand is expressed on arterial endothelial cells and signals to its receptor, Ephb4, which is expressed on venous endothelial cells. In addition, similar to netrin and semaphorin signaling, ephrin signaling serves as a repulsive cue. Loss of either Efnb2 or Ephb4 leads to vascular patterning defects affecting both arterial and venous lineages, suggesting that interactions between arterial and venous blood vessels are crucial for proper vascular patterning (Gerety et al., 1999; Wang et al., 1998). Consistent with this, deletion of HH signaling in the myocardium (Simox2+/–cko) not only resulted in loss of coronary veins, but also led to mislocalization of coronary arteries.

Implications for therapeutic neovascularization

Previously, we and others have demonstrated that activation of HH signaling in the adult heart can promote the formation of new coronary vessels (Kusano et al., 2005; Lavine et al., 2006). Furthermore, HH induced neovascularization protected the heart from ischemic insult and preserved cardiac function following myocardial infarction, implicating the HH pathway as a potentially important therapeutic target for treating ischemic heart disease (Kusano et al., 2005). Similar to the mechanism by which HH signaling regulates coronary development, activation of HH signaling promoted blood vessel growth in the adult heart by inducing expression of VEGF and angiotropin (Kusano et al., 2005; Lavine et al., 2006).

Interestingly, forced expression of Shh in the adult heart led to the growth of multiple vascular types, including capillaries and larger blood vessels. Moreover, the HH receptor and target of signaling, Ptc1/h, was expressed in both cardiomyocytes and in cells surrounding the vasculature (Kusano et al., 2005). The ability of HH signaling to induce growth of multiple blood vessel types and expression of Ptc1/h in several different cell types suggests that, as during coronary development, HH signaling differentially promotes the growth of distinct vascular types by signaling to different tissues.

The potential to selectively trigger the growth of particular blood vessel types would have profound implications for therapeutic intervention. Therapies could be rationally tailored to specific diseases and/or individual patients based on the type of vasculature that would be most efficacious. Further understanding of whether and how HH signaling controls the growth of distinct blood vessel types in the adult heart may provide the first steps towards designing such strategies.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/18/3161/DC1

References


