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VEGF-C and aortic cardiomyocytes guide coronary artery stem development


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Introduction
The vascular beds of each individual organ are shaped to optimize blood flow through the different tissues of the body. In the heart, 2 coronary artery (CA) stems are located at specific sites on the aorta, one at the right sinus and one at the left sinus of the aortic valve (Figure 1A). The stem sites are positioned to efficiently deliver oxygenated blood to the ventricular myocardium. Congenital anomalies causing stems to arise at abnormal sites can cause myocardial ischemia and sometimes lead to sudden death (1, 2). Most dramatic is when CAs stem from the pulmonary artery, which carries deoxygenated blood. Usually fatal if left untreated, this malformation (anomalous left CA from the pulmonary artery [ALCAPA]) requires surgery soon after birth and often entails lifelong complications (3, 4). Despite the clinical relevance of proper stem formation, the mechanisms that dictate this process during embryonic development are unknown.

Much of what we have learned about CA stem formation is from studies performed in chick embryos (5–8). For over a century, observations made using general histological stains suggested that CA stems arise by endothelial budding off the aorta (9, 10). A breakthrough in our understanding came from careful histological analyses and transplantation studies in chicks showing that, instead of outward budding, blood vessels that surround the cardiac outflow tract — termed peritruncal vessels — grow inward and fuse with the aorta (6, 7, 11). Recent lineage tracing has suggested a similar invasion process in the mammalian heart (12). These studies led to the current model in which peritruncal vessels first invade and anastomose with the aorta at multiple sites around its base, before remodeling into 2 larger-bore vessels at the right and left stem sites (Figure 1A and ref. 5). In chicks, this process requires PDGF, FGF, and VEGF-B, since inhibiting these molecules during coronary development decreases the number of stems formed (5, 13, 14). In mice, the remodeling of CA stems requires regulation of BMP signaling by BMP-binding endothelial regulator (BMPER) (15). However, the specific mechanisms patterning their spatial arrangement on the aorta are unknown.

In all species studied, including humans, correct positioning of CA stems depends on proper morphogenesis of the outflow tract. Inhibition of cardiac neural crest (16) and second heart field development (17) results in outflow tract septation and rotation defects that are associated with abnormally placed CA stems on the aorta. Interestingly, CAs do not connect with the pulmonary artery under these conditions, even in the presence of dramatic morphological defects. Similar phenotypes have been observed in Cx43-, Tbx1-, and perlecan-null mice (18–20) as well as in retinoic acid-treated embryos (21). Coronary stems are also abnormal in human hearts with rotation defects called...
Our study provides insight into the factors around the outflow tract and aorta that facilitate CA stem formation at the proper location. VEGF-C is highly expressed around the outflow tract and is required for normally patterned stems because it attracts transposition of the great arteries (TGA) (22). Thus, the mechanisms patterning CAs on the aorta are altered when outflow tract rotation is disturbed, but the factors restricting them from the pulmonary artery remain intact.
robust vessel growth near the presumptive stem sites on the aorta. Although necessary for the proper establishment of peritruncal vessels, VEGF-C expression throughout the aorta and pulmonary artery suggests that it does not specify the exact location of the stem sites. Instead, we found that cardiomyocytes, a highly angiogenic cell type (23, 24), are present specifically in the wall of the aorta, but not in the pulmonary artery, and that they surround developing stems in both mouse and human hearts. Heterozygosity for islet 1 (Isl1) decreases aortic cardiomyocytes and leads to delayed, abnormally targeted stems. Misplaced stems remain tightly associated with aortic cardiomyocytes on hearts with outflow tract rotation defects, and cardiomyocytes induce ectopic endothelial connections with the pulmonary artery in vitro. Our data suggest that VEGF-C first induces vascular expansion around the outflow tract, followed by cardiomyocyte-assisted vessel growth specifically at the CA stem sites. Inhibition of either mechanism results in mistargeted CA stems. Understanding this process will begin to shed light on the molecular defects that cause congenitally misplaced CA stems and could suggest methods of generating new CAs following injury or disease.

Results
CA stem formation in the mammalian heart. A stepwise, high-resolution characterization of CA stem development has not been performed in the mammalian heart. Therefore, we used whole-mount confocal microscopy to image blood vessels in mouse hearts isolated from E10.5 to birth. We found that peritruncal vessels surrounding the aortas and pulmonary arteries first appeared at E11.5 and arose from 2 different locations. First, coronary vessels migrated toward the base of the outflow tract as they grew over and into the heart (Figure 1B). Second, a previously undescribed plexus was detected on the aorta, which began as a single vessel that circled its lateral side before expanding at later developmental stages (Figure 1B and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI77483DS1). These vessels developed directly beneath the aortic epicardium (Figure 1C) and were therefore termed aortic subepicardial vessels (ASVs). Similar vessels were seen occasionally in the analogous region of the pulmonary artery but with much less frequency and at lower densities (data not shown). ASVs appeared to be blood vessels, since they contained erythrocytes (Supplemental Figure 2A) and connected to aortic endothelium (see below). However, a subset of early ASV endothelial cells was positive for the lymphatic marker PROX1 but negative for the mature lymphatic marker LYVE-1 (Supplemental Figure 2, B and C). LYVE-1+ vessels appeared on the aorta much later at E16.5 (Supplemental Figure 2D). Both coronary vessels and ASVs expanded as development progressed (Figure 1B), but only coronary vessels persisted while ASVs mostly disappeared by E17.5 (Supplemental Figure 1). Thus, peritruncal vessels surrounding the outflow tract are composed of developing coronary vessels and ASVs.

Prior to any anastomosis event between the aorta and peritruncal vessels, the aortic endothelium often extended sprouts (Figure 1, D and E, and Supplemental Figure 3A), which never appeared on the pulmonary artery (Figure 1D and Supplemental Figure 3A). Sprouts were frequently 1 cell extending into the medial layer surrounding the aorta (Figure 1, D and E), but those consisting of 2 to 5 cells were also observed (Figure 1F). Extending cells and adjacent aortic endothelial cells expressed apelin, a marker for sprouting endothelium (Figure 1E). These sprouts were independent of coronary vessel or ASV development, since they still formed when peritruncal vessels were inhibited by an early...
injection of the VEGFR antagonist axitinib (Supplemental Figure 3B). The presence of aortic sprouts is in contrast to that in avian experiments, which report only the ingrowth of peritruncal vessels, with no budding of the aorta. This could be due to species differences or our use of high-resolution confocal microscopy, which can detect single-cell processes deep within tissue. It does not appear that the sprouts robustly contributed to the stem, since they were very small (1–5 cells) and did not grow extensively in the absence of peritruncal vessels (Supplemental Figure 3B). Taken together, these observations suggest that the environment surrounding the aorta is angiogenic, while the analogous region on the pulmonary artery is not, but that ingrowth of coronary vessels is the primary mechanism of stem formation.

Connections between the aorta and peritruncal vessels emerged between E12.5 and E13.5 and formed at locations that include both future CA stem sites (Figure 1G) and various points higher on the artery (Supplemental Figure 3A and Supplemental Figure 4). Connections never formed on the pulmonary artery (Supplemental Figure 3A). Initial fusions consisted of lumenless, single-cell thick bridges (Figure 1G). If these connections were positioned at the future stem site adjacent to the valve, they expanded in the next developmental stage. Expansion involved the formation of multiple, clustered connections with increased diameters that began to receive blood from the aorta (Figure 1H and Supplemental Figure 5A). The stem site was not located at the exact same spot in each heart but occurred within a range at or above where the valve leaflets converge (Supplemental Figure 6, A and B). At E14.5, vascular smooth muscle development was initiated around the CA stems (Supplemental Figure 5B), and, by E16.5, the mature stem morphology was seen (Figure 1I). In summary, the process of CA stem formation in the mammalian heart includes anastomoses of peritruncal vessels with a sprouting aortic endothelium, followed by the remodeling and maturation of only those connections at the future stem site adjacent to the valve, they expanded in the next developmental stage. Expansion involved the formation of multiple, clustered connections with increased diameters that began to receive blood from the aorta (Figure 1H and Supplemental Figure 5A). The stem site was not located at the exact same spot in each heart but occurred within a range at or above where the valve leaflets converge (Supplemental Figure 6, A and B). At E14.5, vascular smooth muscle development was initiated around the CA stems (Supplemental Figure 5B), and, by E16.5, the mature stem morphology was seen (Figure 1I). In summary, the process of CA stem formation in the mammalian heart includes anastomoses of peritruncal vessels with a sprouting aortic endothelium, followed by the remodeling and maturation of only those connections at the stem sites (Figure 1 and Supplemental Figure 4).

Coronary vessels originate from at least 3 progenitor populations, the sinus venosus (25, 26), endocardium (25, 27), and pro-epicardium (28), the latter of which may first transition through the former cell types (28). We next performed Cre recombinase–based lineage tracing with Cre lines expressed in the sinus venosus and endocardium to assess their contribution to CA stems. Mice containing either the Rosam<sup>tm1cre</sup> or Rosam<sup>tdTom</sup>Cre reporter alleles were crossed with Apj-CreER or Nfatc1-CreER transgenic mice to lineage label the sinus venosus and endocardium, respectively. When analyzed at later embryonic stages, stems contained lineage-labeled cells from both Apj-CreER and Nfatc1-CreER traces, showing that both populations contributed to the CA stem endothelium (Figure 2). Lineage-labeled cells from both Cre lines were regularly found in both the right and left CA stems. Thus, both progenitor cell types contribute to right and left CA stem formation, a reasonable outcome given the close apposition of the stem region to growing sinus venosus–derived coronary vessels and the endocardium.

**VEGF-C is required for peritruncal vessel development.** We next sought to identify the molecules involved in CA stem formation. Previous studies identified a role for VEGF-B in avian stem development (13), although, in mice, it appears to have a more prominent role in regulating cardiac vascularization and metabolism during injury rather than in development (29–32). To investigate whether other VEGF family members could have a role during mammalian coronary development, embryos were treated with the pan-VEGFR inhibitor axitinib in utero. This treatment could completely inhibit coronary vessel growth, identifying the VEGF pathway as a potential player during stem genesis (data not shown). Investigating the expression of different VEGF family members using lacZ knockin alleles revealed that VEGF-C was expressed in a pattern highly suggestive of a role in stem development. Vegf-lacZ expression was detected within the walls of the outflow tract vessels and around their base (Figure 3, A–C). VEGF-C receptors were expressed in nearby cardiac endothelial cells. Peritruncal vessels (coronary vessels and ASVs) and the endocardium were positive for VEGFR2 and VEGFR3 (Figure 3D), while antibodies recognizing VEGFR2 also labeled aortic endothelium (Figure 3D). VEGF-A was also expressed in the heart, but it was largely expressed in cardiomyocytes throughout the ventricle (33) and not robustly expressed in the aortic wall or near its endothelium where stems normally form (Supplemental Figure 7). Thus, VEGF-C expression overlaps with the outflow tract and stem-forming regions (Figure 3C) and could signal to peritruncal and endocardial cells through VEGFR2 and VEGFR3.

Loss-of-function experiments were next performed to investigate the role of VEGF-C in CA stem formation. Depletion of VEGF-C had a dramatic effect. Although heart morphogenesis was grossly normal and other vessel beds were not affected (26), ASVs were completely missing and peritruncal coronary vessels were severely reduced in VEGF-C knockout animals (Figure 3E). Heterozygous animals exhibited an intermediate phenotype (Figure 3F). Many VEGF-C-deficient hearts did not have CA stems (Figure 3, G–I). Interestingly, in these knockout hearts, sprouts connected to the endocardium appeared to extend up toward the aorta (Figure 3G). When mutant animals did have stems, they were abnormally low on the aorta, connecting to the trough of the valve and pointing downward instead of to the side of the aorta above the aortic valvular sinus (Figure 3, H–J). This low positioning was closer to the deeper coronary vasculature that forms in knockout hearts. Data are shown for the right lateral side (right CA), but in most cases the left CA exhibited the same phenotype (Supplemental Figure 8A). These data indicate that VEGF-C is required for normal CA stem development due to its role in stimulating the first step of the process: the establishment of peritruncal vessels. In the absence of this growth factor, anomalous compensatory stems arise, possibly through interactions with endocardial sprouts.

**Cardiomyocytes specifically populate the aortic wall where they are associated with developing CA stems.** The knockout analysis and expression data described above show that VEGF-C is necessary for proper CA stem formation but that it does not pattern the precise connection site on the aorta. More specifically, VEGF-C is a powerful inducer of peritruncal vessel growth, but its expression extends into areas in which endothelial cells are not found, including the pulmonary artery and the vessel-free zone that surrounds the outflow vessels (Figure 1C and Figure 3, A–C). These observations suggest that other factors collaborate with VEGF-C–induced vessel growth to specify stem sites on the aorta. To identify candidate mechanisms, we looked for differences between the aorta and pulmonary artery by examining the spatial distribution of different cell types present at the relevant developmental stages. Our analysis included neural crest cells,
Whole-mount imaging of E13.5 hearts showed that cardiomyocytes were abundant within the wall of the aorta but not in the pulmonary artery, in which they formed a sharp border at the level of the valve (Figure 4A). When this parameter was measured smooth muscle cells, neurons, macrophages, epicardial cells, and cardiomyocytes. Of these, cardiomyocytes were the only cell type displaying an aorta-specific localization, suggesting a possible role in CA stem patterning.
in tissue sections, cardiomyocytes were found to be significantly more distal from the valve in the aorta than in the pulmonary artery (Figure 4B). Measuring cardiomyocytes around the circumference of the aorta showed that they are significantly more concentrated at the left and right coronary sinuses when compared with the noncoronary sinus (Figure 4, C–E). The presence of aortic cardiomyocytes preceded stem formation, with aortic cardiomyocytes first observed at E11.5 (Figure 4E). At this time,
they were in direct contact with luminal endothelial sprouts from the aorta (Figure 4F). In contrast, cardiomyocytes were mostly separated from the pulmonary artery endothelium where sprouting was never seen (Figure 4G). Sectioning through the entire outflow tract and using serial sections to quantify the total amount of contact between cardiomyocytes and luminal endothelium revealed that there are significantly more direct interactions with the aorta compared with the pulmonary artery (Figure 4H). Thus, cardiomyocytes are present at the correct location and appropriate developmental stage to pattern CA stem formation.

We next observed the relationship between aortic cardiomyocytes and developing CA stems. Both the right and left stems were always closely associated with aortic cardiomyocytes in embryonic hearts (Figure 4I and Supplemental Figure 9, A and B). However, these cardiomyocytes disappeared postnatally, and stems were surrounded instead by smooth muscle (Figure 4J). A similar association between aortic cardiomyocytes and CA stems was seen during human embryogenesis (Figure 4K). This was particularly notable, since human CAs, unlike rodent CAs, are located on top of ventricular myocardium. We observed that human CAs connect with the aorta through a layer of aortic cardiomyocytes before they localize to the heart surface (Figure 4K). Thus, cardiomyocytes could play an important role in guiding CA stem development in mouse and human hearts.

Understanding how aortic cardiomyocytes arise on the aorta could be useful in devising loss-of-function experiments that test their role in stem formation. There are at least 2 routes by which cardiomyocytes could localize to the aorta: by migrating up from the ventricle or by directly differentiating from progenitor cells present in the aortic wall. The latter would be analogous to the mechanism of early heart growth, in which there are cardiomyocyte progenitors at the outflow and inflow poles that continuously differentiate and add to the heart tube (34, 35). To investigate these possibilities, we performed time-lapse microscopy on isolated hearts with fluorescently labeled cardiomyocytes (Myh6-Cre, Rosa26tmG). While labeled cells could be seen proliferating, they never appeared to migrate (Figure 5). Instead, we frequently observed the initiation and gradual increase in cardiomyocyte-specific GFP expression on the aorta distal to the main ventricle, suggesting de novo differentiation (n = 7 hearts). This was in contrast to control experiments in which fluorescently labeled endothelial cells (VE-cadherin-CreER, Rosa26tmG) migrated rapidly while continuously extending and retracting filopodia (data not shown). These data suggest that aortic cardiomyocytes differentiate in situ and that inhibiting progenitor populations could decrease their presence.

Isl1 heterozygous hearts exhibit decreased aortic cardiomyocytes and abnormally low CA stems. Deletion of the transcription factor Isl1 causes embryonic lethality and cardiac developmental defects (36). Without Isl1, cardiomyocyte progenitors in the second heart field fail to expand and contribute to heart growth, resulting in the lack of later developing structures, such as the right ventricle and outflow tract. We crossed the Mef2c-AHF-Cre mouse (37) to a Rosa26tmG Cre reporter to lineage trace the second heart field and found that aortic cardiomyocytes descend from this cell population (Figure 6A). Since aortic cardiomyocytes arise from the second heart field lineage and appear to develop de novo from progenitor cells within the outflow tract, we tested whether deletion of one Isl1 allele would affect their cell numbers in the aorta. Observing cardiomyocytes using whole-mount confocal micrographs of wild-type and Isl1 heterozygous hearts revealed a reduction of this cell type in the aortic wall at both the right and left CA stem regions (Figure 6, B and C, and Supplemental Figure 8B). Measuring the presence of cardiomyocytes distal to the valves via optical sections showed a significant decrease in the cardiomyocyte distribution in mutant embryos (Figure 6D). We did not find any gross defects in heart morphogenesis, as outflow tract rotation was normal (Figure 6B) and myocardial thickness and total heart size were similar in wild-type littermates (Supplemental Figure 10). The amount of smooth muscle around the aorta near the stem site was not ser-
and F). Although we cannot rule out differences in cell types or functions not analyzed, Isl1 heterozygous mice appear to have a relatively well-isolated defect in aortic cardiomyocytes, making them useful in testing the role of these cells in CA stem formation.

Figure 6. ISL1 heterozygosity decreases aortic cardiomyocytes and disrupts normal stem development and positioning. (A) Second heart field lineage tracing (Mef2c-AHF-Cre, RosamTmG) labels aortic cardiomyocytes (CMs, arrows). (B) Confocal images of wild-type and Isl1 heterozygous embryos showing decreased numbers of cardiomyocytes within the aorta (dotted line). The pulmonary artery is also outlined (solid lines). Cardiomyocytes (cTnT+) are shown in red; peritruncal vessels (VE-cadherin+) are shown in blue. The bottom panel shows only the cTnT channel where red lines indicate the stem position. (C) Boxed regions in A showing only the cTnT channel. (D) Quantification of the length distal to the valve occupied by aortic cardiomyocytes shows a significant decrease in Isl1 mutant hearts. (E and F) Other developmental parameters, (E) smooth muscle cell development and (F) coronary vessel growth, are unchanged in mutant hearts. (D–F) Data represent mean ± SD. Each dot represents a value obtained from one sample. (G) Isl1 heterozygous hearts have abnormally low stem positioning with respect to the valves (lines) in comparison to wild-type hearts. (H) Distribution of stem phenotypes observed in Isl1 mutant and wild-type hearts. n values are shown on the right. (I) The height of CA stems does not significantly rise above the level of aortic cardiomyocytes in both wild-type and mutant hearts, as evidenced by the paucity of points in the pink area (y > x) of the graph. These data were measured at E14.5. A linear regression for the data set (dotted line, $y = 0.5999x - 36.39$) indicates a positive correlation. ****P < 0.0001; NS ≥ 0.05. Scale bars: 100 μm.
CA stems in $Isl1$ mice were dramatically different than those in wild-type mice. Stems attached much lower on the aorta, often localizing to the trough of the valve (Figure 6G and Supplemental Figure 6, A and B). In addition, initial connections between peritruncal vessels and the aorta were frequently delayed (Figure 6H). This phenotype was seen in both the right and the left CA stems (Figure 6 and Supplemental Figure 8C). Stem sites were quantified by drawing a line at the top of the valves where the leaflets converge and measuring the distance from this line to where the stem connects to the aorta (Figure 6G and Supplemental Figure 6, A and B). Positive values indicate the stem orifice was above (distal to) the valve. Negative values indicate the stem orifice was below the valve. A value of 0 was assigned to a stem that connects at the exact level of the valve. Plotting this location against the region occupied by cardiomyocytes revealed a positive correlation between the 2 parameters (Figure 6I). This analysis highlighted that stems did not attach above the region where cardiomyocytes were found (i.e., no data points were significantly above the line $y = x$), even in $Isl1$ mutants with dramatically lowered aortic cardiomyocytes (Figure 6I). These data support a
Analyzing Pax3 mutants that exhibit TGA (outflow tract septation without proper rotation) revealed that neither normal neural crest migration nor complete rotation was required for aorta-specific cardiomyocyte growth (Figure 7, C–H). Although the aorta and pulmonary artery were side by side in mutant hearts, cardiomyocytes were specifically present in large numbers on the aorta (Figure 7, D and F). Mutant hearts also had coronary vessels that exited the aorta ventrally (from the front) and dorsally (from the back) instead of laterally, from the right and left sides (Figure 7, A and B). However, the misplaced CA stems were always associated with a concentration of cardiomyocytes growing within the aortic wall (Figure 7, D, F, and H) \((n = 12\) hearts). These data provide further evidence of a developmental relationship between cardiomyocytes and CA stems.

Cardiomyocytes can induce ectopic connections with the pulmonary artery in vitro. We next performed gain-of-function experiments to investigate whether cardiomyocytes could induce vas-
cicular connections where they normally do not occur. Outflow tract explants were cultured adjacent to ventricular myocardium or control tissues. After 2 to 3 days, the explants were immunostained to determine whether ectopic vascular connections occurred specifically in the presence of cardiomyocytes. When cultured alone, explanted pulmonary arteries maintained their general shape, with a nonsprouting luminal endothelial layer surrounded by a vessel-free region (Figure 8A). Endothelial cells from lung explants did not invade the vessel-free region, even though they readily migrated into the myocardium at the base of the artery explant (Figure 8, B and D). In contrast, culturing myocardium adjacent to outflow tracts frequently resulted in connections between ventricular coronary vessels and pulmonary artery luminal endothelium (Figure 8, C and D). Thus, myocardium can mediate ectopic connections between the pulmonary artery and the coronary vessels in vitro, supporting the hypothesis that aortic cardiomyocytes facilitate this process during stem development.

Discussion

In this study, we describe 2 morphological steps that are required for CA stem patterning — (a) the establishment of the peritruncal vessels that surround the aorta and (b) anastomosis of these vessels specifically with the aorta — and provide insight into the molecular and cellular mechanisms guiding both processes. Our data show that VEGF-C mediates step one. It is highly expressed throughout the outflow tract, and VEGF-C–deficient mice have absent ASVs and hypoplastic peritruncal coronary vessels. Step one is required for CA stem patterning, as evidenced by the delayed, misplaced stems that form in VEGF-C mutant hearts when the peritruncal vessels are reduced or missing. VEGF-C is expressed throughout the outflow tract, including regions in which endothelial cells do not grow, suggesting that it alone is not sufficient for stimulating connections with the aorta at defined locations. We found that cardiomyocytes are localized in a pattern suggestive of a role in step two. They are concentrated along the wall of the aorta in which stems form but are mostly absent from the pulmonary artery. Isl1 heterozygosity decreases aortic cardiomyocytes and results in delayed, abnormally placed stems. Misplaced stems on hearts with outflow tract rotation defects formed within regionally shifted aortic cardiomyocytes, and cardiomyocytes induced ectopic connections with the pulmonary artery in culture. These data support a step-wise model in which peritruncal vessels are attracted to the outflow tract from nearby vessel beds (the sinus venosus, endocardium, and surface of the aorta) in response to VEGF-C, after which cardiomyocytes facilitate the positioning of their fusion sites on the aorta (Figure 9). Failure to complete either step results in abnormal CA stem patterning.

CA anomalies in humans can range from nonstandard stem origin on the aorta to defects in which the left coronary stem branches from the pulmonary artery (ALCAPA). Anomalous origins on the aorta have been estimated to occur in approximately 1% of the population (1). These are frequently asymptomatic but can cause myocardial ischemia and sudden cardiac death in a significant number of cases (2, 39). Anomalous CA stems can attach to the aorta in a number of different configurations, which may explain the spectrum of symptoms. The most clinically concerning anomalies for sudden cardiac death occur when the artery stem attaches to the opposite aortic sinus, which causes the vessel to travel between the aorta and pulmonary artery, e.g., the left CA arising from the right sinus. The direct cause of ventricular ischemia in these cases is not known but is thought to arise when the aorta and pulmonary artery apply pressure to the intervening CA, obstructing the vessel lumen. Understanding how to handle CA anomalies is particularly important for athletes and military recruits preparing to undergo intense training. CA anomalies are estimated to be responsible for 17% of sudden cardiac deaths in young competitive athletes (40). An American Armed Forces Institute of Pathology study found that 33% of cardiac-related deaths in new recruits undergoing training were associated with anomalous left CAs arising from the right coronary sinus (41). Specialists in the field are focused on developing imaging techniques for accurate screening and devising prophylactic strategies following diagnosis (42).

The condition in which coronary arteries connect to the pulmonary artery (ALCAPA) is a much more serious and life-threatening anomaly than instances of nonstandard stem placement on the aorta. ALCAPA has been calculated to occur in 1 in 300,000 births, and approximately 90% of patients with this condition do not survive past infancy without surgical repair (43, 44). Those who...
Our results identified cardiomyocytes as a cellular feature specific to the aorta. Cardiomyocytes are known to foster vascular growth by secreting angiogenic molecules (23, 24, 55), supporting the hypothesis that they facilitate the establishment of stems at the correct location. However, preliminary experiments deleting Vegfa using various cardiac Cre lines did not suggest a role for this growth factor. Future experiments will focus on identifying the specific cardiomyocyte-derived molecule mediating stem positioning. An additional possibility is that these cells are antagonizing an antiangiogenic factor present in the walls of the outflow tract. Indeed, large arteries are frequently surrounded by an avascular zone, as is the case with the developing aorta and pulmonary artery. In our experiments, endothelial cells did not migrate into the region surrounding outflow tract explants (unless cardiomyocytes were present), suggesting the existence of antiangiogenic factors. Precedent for such a mechanism is seen with the extracellular matrix protein chondromodulin-1, which restricts vessel growth in the cardiac valves during development and in the adult (56). Similarly, the vessel-free zone around the outflow arteries expresses numerous extracellular matrix molecules, which, in other systems, have been shown to liberate antiangiogenic peptides following proteolytic processing (57).

The mechanisms by which cardiomyocytes specifically populate the aortic wall are actively under investigation. Aortic cardiomyocyte development is disrupted by heterozygosity for the transcription factor Isl1. This transcription factor marks the second heart field, which contributes to heart growth, by adding cells onto the early heart tube to produce the outflow tract, right ventricle, and atria (58). Functionally, Isl1 supports cardiomyocyte precursor proliferation, causing Isl1-null animals to lack the majority of second heart field–derived cardiac structures (36). In fact, we found that aortic cardiomyocytes derive from the second heart field. These observations suggest that aortic cardiomyocytes may develop via mechanisms analogous to those of second heart field maturation but in an aorta-specific manner. There may be an aorta-specific delay in the differentiation of second heart field progenitors in response to molecules specifically expressed in this outflow vessel. There are genetic distinctions between the aorta and pulmonary artery. The base of the pulmonary artery specifically expresses a Mys5 promoter fragment (96-16 transgene) (59) and Sema3C (18), while the corresponding region on the aorta expresses an Fgf10 fragment (T55 transgene) (18). Ultimately, insight into the embryonic mechanisms that trigger CA stem development on the aorta could be commandeered to establish new vessels in the adult as treatments for congenital cardiac defects and coronary heart disease.

Methods

Animals

The following mouse strains were used: wild-type (CD1, Charles River Laboratories), Apj-CreER (see below), Vegfc-lacZ (provided by K. Alitalo, University of Helsinki; ref. 60), Vegfa-lacZ (provided by L. Miquerol, Aix Marseille Université, Developmental Biology Institute of Marseille, Marseille, France; A. Damert, Babes Bolyai University, Cluj-Napoca, Romania; and A. Nagy, Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario, Canada; ref. 33), Vegfc6 (provided by K.
Alitalo and P. Saharinen, University of Helsinki; see below), Isp1loxCreER (provided by S. Evans, University of California, San Diego, La Jolla, California, USA; ref. 61), Rosa26loxCre reporter (The Jackson Laboratory, Gt(Rosa26)4B+Zb(Tm.Tflm,ERFlp), Rosa26loxFlp Cre reporter (The Jackson Laboratory, B6.CgGr(Rosa26)4B+Zb(Tm.Tflm,ERFlp)), Pax3 (The Jackson Laboratory, Pax2tm17(Tm.Tflm,ERFlp)), B6.FVB-Tg(Myh6-cre)(2182Mds) Myh6-Cre, The Jackson Laboratory, Mf2c-AHF-Cre (provided by B. Black, University of California, San Francisco, San Francisco, California, USA; ref. 37), VE-cadherin-CreER (provided by L. Iruela-Arispe, University of California, Los Angeles, Los Angeles, California, USA; ref. 62), Nfatc1-CreER;Rosa26loxP/loxP (provided by B. Zhou, Shanghai Institutes for Biological Sciences; ref. 63), and Aplin-lacZ (provided by T. Quertermous, Stanford University; ref. 64). CD1 mice were used for wild-type characterization studies. In general, other mouse lines were maintained on a mixed background of C57BL/6J and FVB unless otherwise noted. Vegfc knockout and floxed mice were ICR. The floxed line was crossed to Rosa-CreER on a mixed C57BL/6J and 129Sv background (described below). In all cases, wild-type littermate controls were compared with mutant animals. Among the mouse lines used, we did not detect evidence of any strain-dependent differences in early stem targeting.

The majority of VEGF-C-deficient embryos were produced by crossing a ubiquitously expressed, tamoxifen-inducible Cre line to a Cre-dependent (conditional) deletion allele. Mice with conditionally targeted Vegfc alleles were created by inserting the mouse Vegfc cDNA and a Frt-flanked Neo cassette in the first exon of the mouse Vegfc genomic locus, thereby deleting the first exon and part of the first intron of the mouse Vegfc gene. Additionally, the inserted Vegfc cDNA was flanked by LoxP sites. Heterozygous mice were mated with the Fdp deleter mouse strain to remove the Neo cassette and were subsequently mated to homozygosity. The homozygous Vegfc−/− mice were viable, with no abnormal phenotype. Mating of the Vegfc−/− mice with the R26R-Cre mice resulted in efficient deletion of the gene and caused the expected embryonic lethality based on previous data from constitutive Vegfc knockout mice (60).

To create an Apj-CreER mouse line, recombineering (65, 66) was used to insert a CreERT2 gene at the Apj start site of a BAC. The Apj BAC was procured from the Children’s Hospital Oakland Research Institute and represents 153,553 base pairs of chromosomal DNA derived from a male C57BL/6J mouse (clone RP24-301A16, Children’s Hospital Oakland Research Institute). Modified Apj BACs were injected into oocyte pronuclei (Cyagen Biosciences) that were transferred into pseudopregnant females. Resulting pups were screened for incorporation of BAC DNA, and 2 founder lines were established and found to exhibit similar expression patterns.

To generate embryos expressing Cre plus an allele of interest, a male parent containing the Cre gene (and the floxed allele, if applicable) was mated with a female parent containing either the Cre-inducible reporter or the floxed gene. This breeding scheme was performed to avoid flossing out genes in the female parent during pregnancy, minimizing potentially confounding variables in the resulting embryo phenotypes. For all breeding schemes, mice were between 2 and 6 months of age.

In some cases, mice were treated with tamoxifen to induce CreERT2-based recombination. Apj-CreER and Nfatc1-CreER lineage-tracing experiments were carried out by injecting pregnant dams intrauterinely with 4 mg tamoxifen dissolved in corn oil. To obtain VEGF-C-deficient animals, the Vegfc−/− line was crossed to mice containing the R26R-CreER transgene, which expresses a tamoxifen-inducible Cre recombinase in most cells. To activate Cre expression, mice were given 100 μl 4-OH-tamoxifen (25 mg/ml) by oral gavage on E6.5 and E7.5 (prior to Vegfc expression).

For assessment of aortic sprouting in the absence of peritruncal vessels, coronary vessels and ASVs were completely inhibited with intraperitoneal injections of the VEGFR antagonist axitinib (Sigma-Aldrich) on E10.5 and E11.5 (25 mg/kg, i BID). Hearts were isolated at E12.5 and immunostained as described below.

**Immunohistochemistry and imaging**

To obtain staged embryos and hearts, timed pregnancies (morning plug designated E0.5) were dissected and fixed in 4% paraformaldehyde and stored in PBS. Fixed tissues were left intact or sectioned and then processed for either whole-mount or section immunofluorescence. Some tissues were subjected to an X-gal reaction. Immunofluorescence was performed in either 1.5-ml tubes (whole mount) or on microscope slides (tissue sections) using the same protocol, except the former was subjected to constant rotation. Primary antibodies were diluted in blocking solution (5% goat or donkey serum, 0.5% Triton X-100) and incubated with tissues overnight at 4°C. Tissues were then washed with PBT 3 times for 1 hour before another overnight incubation with secondary antibodies diluted in blocking solution or in PBT. Specimens were then washed again, placed in Vectashield (Vector Laboratories), and imaged using either an inverted Zeiss LSM-700 confocal microscope (whole-mount tissue) or Axioimager A2 epifluorescence microscope (tissue sections). Images were digitally captured and processed using ImageJ (NIH), Photoshop (Adobe Systems), Zen (Carl Zeiss), and AxioVision (Carl Zeiss) software packages. The following primary antibodies were used: VE-cadherin (BD Biosciences, 550548, 1:100); cTnT (Developmental Studies Hybridoma Bank, CT3-c, 1:500); SM-MHC (Biomedical Technologies, BT-562, 1:300); WT-1 (Abcam, ab15249, 1:100); CD31 (BD Biosciences, 550274, 1:100); PROX1 (Developmental Studies Hybridoma Bank, CT3-c, 1:500); SM-MHC (Biomedical Technologies, BT-562, 1:300); WT-1 (Abcam, ab15249, 1:100); CD31 (BD Biosciences, 550274, 1:100); PROX1 (R&D Systems, AF2727, 1:300), ERG-1/2/3 (Santa Cruz Biotechnology, C20, 1:100); LVEF-1 (eBiosciences, 14-0443-80, 1:100); VEGFR2 (R&D Systems, AF644, 1:100); and VEGFR3 (R&D Systems, AF743, 1:100). Secondary antibodies were Alexa Fluor conjugates 488, 555, 594, 633, 635, and 647 (Life Technologies) used at 1:250. DAPI (Sigma-Aldrich, 1:2000) was also used.

**Perfusion experiments**

The patency of CA stems was assessed by injecting FITC-conjugated tomato lectin (Vector Laboratories) into the left ventricle of dissected embryos. The lectin was distributed throughout the vasculature via cardiac contraction. Tissue sections were prepared and imaged as described above, and vessels containing the fluorescent lectin were considered patent.

**Organ cultures**

For cocultures, dissected outflow vessels, ventricles, and lungs from E12.5 or 13.5 wild-type embryos were placed on 8-μm Millicell cell culture inserts (EMD Millipore) at the air-liquid interface. Cultures were maintained in DMEM media with 10% fetal bovine serum and 100 U ml−1 penicillin and 100 μg ml−1 streptomycin at 37°C/5% CO2 for 48 to 72 hours and then fixed and subjected to whole-mount immunofluorescence as described above.
Time-lapse imaging of aortic cardiomyocytes and cardiac endothelial cells was performed using embryonic hearts from Myh6Cre, Rosa26tm1cre and VE-cadherin-CreER, Rosa26tm1ScaF mice, respectively. As described above, hearts (with the atria and sinus venosi removed) were cultured with aortas facing upward. Images documenting their development were captured every 15 minutes for 20 hours by an inverted Zeiss Observer.Z1 microscope configured with a XL-3 stage incubation chamber (with temperature and CO2 control) using AxioVision SE64 Rel. 4.9 software.

Quantification

Aortic cardiomyocyte and CA stem height. In wild-type hearts, the location of cardiomyocytes in the wall of the aorta and pulmonary artery was measured in sagittal tissue sections. For Isl1 heterozygous and wild-type littermates, optical sections from confocal z-stacks imaged from the right lateral side were used to quantify the position of aortic cardiomyocytes and stems. In all cases, values were obtained by measuring the distance between the valvular endothelium where the leaflets converge and the distal-most cardiomyocyte or stem. CA stems at or below the level of the valve leaflet meeting point were considered “low,” since this was rare for wild-type samples (Figure 6I and Supplemental Figure 6). AxioVision and Zen software packages from Zeiss were used to perform measurements in tissue and optical sections, respectively.

The area occupied by cardiomyocytes at the different aortic valve sinususes (left coronary, right coronary, and noncoronary) was measured by imaging entire aortas from the right and left lateral sides. Right lateral images were transferred into ImageJ, and the area above the troughs of the valves covered in cardiomyocytes was measured for the right coronary sinus and noncoronary sinus regions (see Figure 4D). A similar measurement was performed for the left lateral images. The noncoronary sinus values from the right and left views were summed while the right and left coronary sinus values were taken solely from the respective images. Values were reported as pixels (Figure 4C).

To quantify the total amount of contact between cardiomyocytes and outflow tract luminal endothelium, the length in which the 2 cell types were directly adjacent was measured in every serial section through the entire vessel. The values from every section were summed to determine the total contact for one individual aorta or pulmonary artery.

Myocardial and smooth muscle thickness. Myocardial and smooth muscle thickness were quantified using optical sections from confocal z-stacks of hearts immunolabeled for cTnT and SM-MHC, respectively. Myocardial measurements were taken from sagittal views through the dorsal wall of the right ventricle. Smooth muscle thickness measurements were taken from sagittal views through the ventral wall of the aorta. All measurements were performed using Zeiss Zen software.

Heart size and coronary vessel growth. Total heart size and coronary vessel growth was measured for Isl1 heterozygous and wild-type hearts at E12.5 using ImageJ software. The freeform selection tool was used to circumscribe the whole dorsal side of the heart (considered to be the total heart size) and/or the area covered by coronary vessels. The percentage of each individual heart covered by coronary vessels was used to assess the extent of coronary growth.

Tissue samples

Fetal heart tissue was procured from medical waste (StemExpress). Three samples were analyzed, which all displayed the same characteristics.

Statistics

All measurements were compiled, plotted, and analyzed using Prism 6 (GraphPad). Two-tailed, unpaired parametric t tests with Welch’s correction were used to determine the P value for comparisons between groups. A P value of less than 0.05 was considered significant. Linear regression analysis was performed to measure the strength of association between 2 variables.

Study approval

All animal experiments performed at Stanford University, at the University of Helsinki, and at the Shanghai Institute for Biological Sciences (SIBS) were conducted in accordance with guidelines of the Stanford University Institutional Animal Care and Use Committee, the Committee for Animal Experiments of the District of Southern Finland, and the SIBS Institutional Animal Care and Use Committee, respectively. All human tissues were obtained with informed consent and used in a manner approved by the Stanford University Institutional Review Board.

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