Coronary Artery Development: Progenitor Cells and Differentiation Pathways

Bikram Sharma,[1] Andrew Chang,[1,2] and Kristy Red-Horse[1]

[1]Department of Biology, Stanford University, Stanford, California 94305; email: kredhors@stanford.edu

[2]Department of Developmental Biology, Stanford University, Stanford, California 94305

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Abstract

Coronary artery disease (CAD) is the number one cause of death worldwide and involves the accumulation of plaques within the artery wall that can occlude blood flow to the heart and cause myocardial infarction. The high mortality associated with CAD makes the development of medical interventions that repair and replace diseased arteries a high priority for the cardiovascular research community. Advancements in arterial regenerative medicine could benefit from a detailed understanding of coronary artery development during embryogenesis and of how these pathways might be reignited during disease. Recent research has advanced our knowledge on how the coronary vasculature is built and revealed unexpected features of progenitor cell deployment that may have implications for organogenesis in general. Here, we highlight these recent findings and discuss how they set the stage to interrogate developmental pathways during injury and disease.

INTRODUCTION

Coronary artery disease (CAD) is the number one cause of mortality worldwide and results from pathological dysfunction of the coronary arteries, the blood vessels that supply oxygen and nutrients to heart muscle (1). CAD occurs when changes in the artery wall lead to the accumulation of atherosclerotic plaques, thus narrowing the vessel lumen and restricting blood flow. Complete blockage of blood flow, frequently via a thrombotic event, induces a myocardial infarction that starves cardiac muscle of oxygen (2). Developing improved methods of repairing and regenerating coronary arteries is a major, yet unrealized, goal in cardiovascular medicine (3). Knowledge of the cellular and molecular interactions that drive coronary artery development during embryogenesis could provide new, more effective strategies (4, 5).

Recent years have seen much progress in our understanding of coronary artery development. One of the most important findings is that the coronary endothelium and smooth muscle layer are assembled from cells deriving from multiple progenitor sources, which combine to produce the mature vasculature. It is also becoming clear that distinct regulatory mechanisms control coronary growth from the different progenitor sources. The presence of multiple pathways leading to a coronary artery presents the exciting possibility that there may be more than one option for repair. Another area of recent focus is the precise mechanisms of coronary arteriogenesis, where the immature coronary endothelial plexus, covered in pericytes, undergoes a morphogenic process that transforms small vessels into mature, large-diameter arteries coated in vascular smooth muscle. This process requires the establishment of blood flow, where specific molecules direct attachments between the developing coronary plexus and aorta. The cell types involved in coronary arteriogenesis are present in the adult, leaving open the possibility that they could be reactivated to stimulate new arteries in the adult. In this review, we focus on the recent findings in coronary vessel development, which have built upon past work comprehensively reviewed elsewhere (6–9). The overarching future goal of research in this field is to transform information on coronary artery development into meaningful clinical treatments that can lower the incidence of CAD-induced mortality and morbidity.

OVERVIEW OF CORONARY VESSEL DEVELOPMENT

Coronary vessels develop through a stepwise angiogenic program, first involving the formation of an immature vessel plexus and then its subsequent remodeling into a mature vascular bed (**Figure 1*a***). Early in embryogenesis prior to coronary development, the heart consists of a thin myocardial muscle layer that is easily oxygenated by blood flowing through its lumen and therefore does not initially require its own vascular bed. Later, when heart size increases, coronary blood vessels start to emerge on the ventricles as an immature vascular plexus (10, 11). A vascular plexus is a highly branched network of small, similarly sized vessels. This plexus undergoes branching morphogenesis and massive expansion to cover and infiltrate the entire myocardium. Although it is initially devoid of blood flow, the plexus eventually anastomoses with the aorta, which provides a source of arterial blood flow (12). Subsequent to aortic attachment and establishment of blood flow is a vascular remodeling stage in which the plexus is transformed into the hierarchal arrangement of arteries, capillaries, and veins (12, 13). The result of plexus growth and remodeling is a coronary vasculature arranged in a pattern that efficiently supports the oxygenation of myocardial tissue (**Figure 1*a***).

The mature coronary vascular system is composed of multiple important cell types that should be understood when designing heart regeneration strategies (**Figure 1*b***). Coronary cells represent more than 60% of the nonmyocyte populations in the heart (14) and are critical in providing blood flow as well as regulating myocardial metabolism (15). All coronary blood vessels are lined

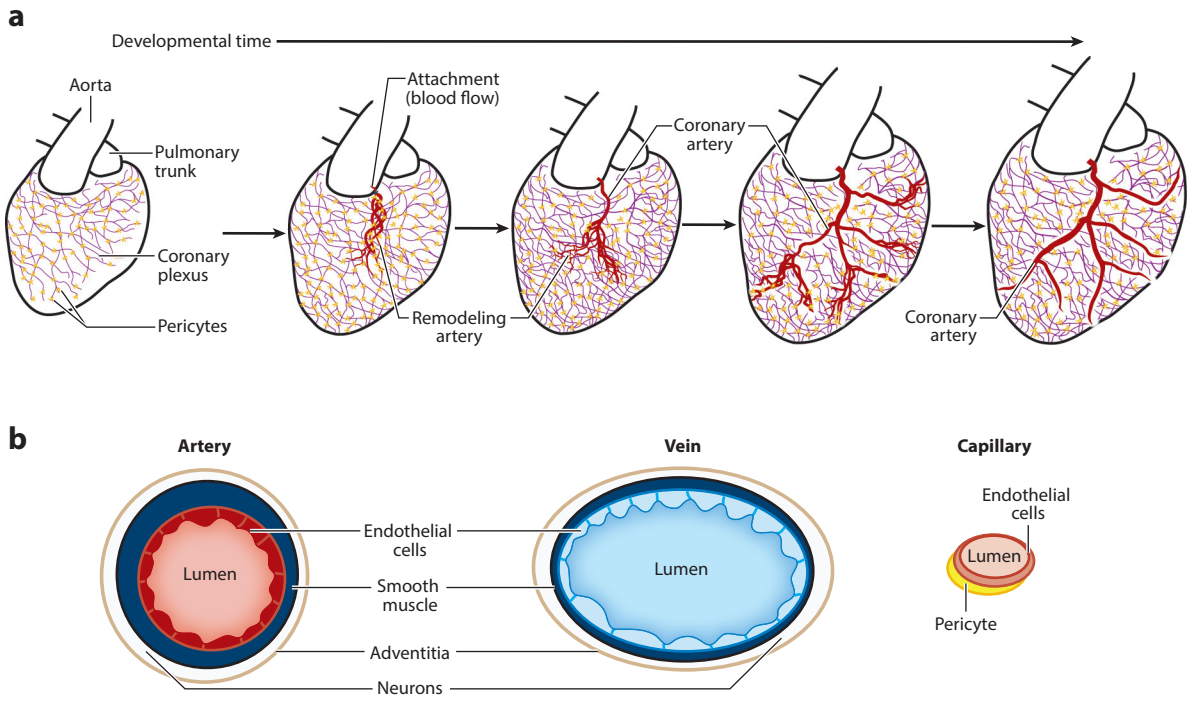


Figure 1

Structure and cellular components of the coronary vasculature. (*a*) Schematic of the developmental events leading to mature coronary arteries. First, a coronary plexus (*purple*) covered in pericytes (*yellow*) migrates over the surface of the heart and into the myocardium. Then, plexus vessels attach to the aorta to initiate blood flow, triggering arterial remodeling (*red*) that ultimately leads to mature arteries. Panel adapted from Reference 68. (*b*) Illustration of the prominent cell types comprising the coronary vasculature.

luminally by a single-cell layered, polarized endothelium, which comprises endothelial cells that have specialized functions, such as being antithrombotic and sensing/responding to mechanical shear signals from blood flow (16). Surrounding the endothelial layer are murals cells, including smooth muscle cells, covering arteries and pericytes around capillaries (17). Veins also contain smooth muscle but at a lower density than arteries. Furthermore, in the outermost layer of larger coronary artery segments is the adventitia, which contains fibroblasts and other cell types (18, 19). Finally, select coronary vessels, particularly arteries and veins, are innervated (20, 21) and associate with lymphatic vessels (22). Interestingly, in humans, larger arteries and veins are located in a subepicardial position, whereas in rodents, arteries are deep within the myocardium (intramyocardial), and veins are on the surface (subepicardial). In both species, the majority of capillaries are within the myocardium. Understanding the mechanisms underlying the differentiation and integration of all these cell types is important to recapitulate their development during regeneration and/or repair, particularly because despite some structural differences, many of the events observed in model systems also appear to occur in human hearts (23).

CORONARY VESSEL PROGENITOR CELLS AND THEIR DIFFERENTIATION PATHWAYS

The origins of coronary vessels have been a topic of great interest for over a century and one that has evolved as new techniques and experimental models have emerged. The first reports on this

subject came from early histological studies performed almost a century ago, when researchers observed connections between nascent coronary arteries and aortic endothelium, leading to the conclusion that arteries likely bud from the aorta (24, 25). Connections between coronary veins and the sinus venosus, the vein that empties into the embryonic heart, were also noted, and it was suggested that veins might arise from this structure (13, 24, 25). A formal testing of the progenitor cells for coronary vessels came from transplantation studies performed on avian species, which allows for progenitor cell lineage tracing using species-specific antigen immunolocalization. Studies from quail-chick chimeras and clonal analysis using replication-incompetent viruses showed that coronary progenitors invested the heart from a region outside the initial primitive heart tube (26–30). These studies identified this extracardiac source as being the proepicardium, which is a cluster of cells overlaying the septum transversum and sinus venosus during early heart development. It attaches to and migrates onto the heart, forming its outer layer called the epicardium. Quail-chick chimeras transplanting the proepicardium gave rise to donor-derived epicardium, coronary vessel endothelial cells, coronary artery smooth muscle, and cardiac fibroblasts. These seminal studies advanced the field's understanding of heart development and its stromal components. Thus, a model in which all the cells of coronary vasculature arise from the proepicardium became accepted over the subsequent decades and was presumed to hold true for other vertebrate species, including humans.

Origins and Development of the Primary Coronary Plexus Endothelium

The proepicardial origin model for the coronary endothelial layer was later challenged in the murine heart with the advent of Cre recombinase–mediated progenitor cell lineage tracing. This method involves creating transgenic mice that express Cre recombinase under a specific enhancer/promoter restricting Cre expression to particular cell lineages. These mouse lines can be used for lineage tracing when coupled with a Cre reporter allele, so that Cre excises a loxP-flanked stop cassette and relieves transcriptional inhibition of a marker protein, such as β-galactosidase or green fluorescent protein (31). Because the excision is a permanent lesion in the DNA, all cells expressing Cre and their progeny are labeled. When initial mouse lines were created to express Cre in epicardial cells and used for lineage tracing (*Wt1-Cre*, *Gata5-Cre*, and *Tb18-Cre*), epicardium, coronary smooth muscle, and cardiac fibroblasts were traced, but few or no coronary endothelial cells arose from the labeled epicardial cells (32–34). These data suggested that coronary endothelial cells arose from a different, unidentified source that collaborated with epicardial-derived smooth muscle to produce the coronary vasculature.

Following the apparent lack of epicardial contribution to coronary endothelium in the mouse heart, several studies emerged describing alternative coronary endothelial progenitors. These studies identified at least three different progenitor populations: the sinus venosus, endocardium, and a newly identified subset of proepicardial cells. First, by combining whole heart histology, clonal analysis, and ex vivo tissue recombination studies, it was shown that the endothelium of coronary arteries, capillaries, and veins arises from progenitor cells in the sinus venosus and endocardium (10). Histological and molecular marker analysis indicated that the sinus venosus undergoes sprouting angiogenesis, sending vessels onto the surface of the heart. These vessels subsequently migrate perpendicularly into the underlying myocardium, an observation later supported by lineage-tracing sinus venosus sprouts (35, 36). Clonal and histological observations indicated that the endocardium buds from the lumen of the heart onto the surface in a process that produces structures termed blood islands. These initially circular endothelial-lined structures filled with blood cells first arise at the surface of the dorsal and ventral midline, where they then appear to sprout into the myocardium (10, 36). Evidence suggests that the blood cells within these structures arise because the

endocardium is hemogenic and able to differentiate into hematopoietic cells (37, 38). Blood island formation and their hemogenic activity is negatively regulated by sVegfr1, TgfbrIII, and the RasGap activity of Nf1 (39–41). These studies showed the sinus venosus and endocardium to be sources for coronary endothelial cells and proposed a migratory pathway by which they populate the heart.

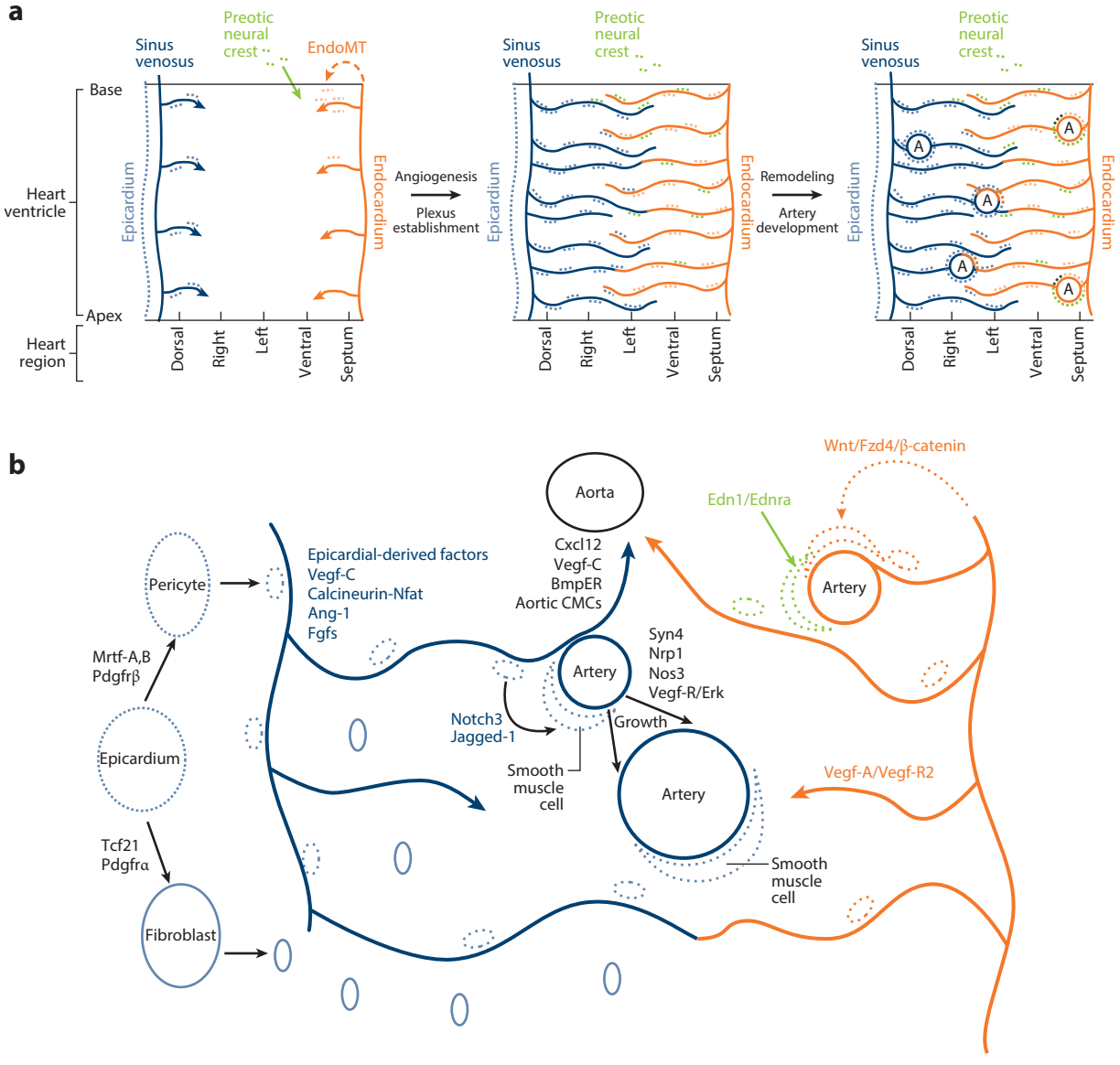
Parallel experiments taking a different approach provided evidence that the endocardium was a prominent source for coronary endothelium. Using the *Nfatc1-Cre* mouse line in lineage tracing, clonal analysis, and quail-chick chimera experiments showed that a large number of coronary arteries and capillaries arise from the endocardium, but few veins derived from this source (42). This activity was attributed to myocardial-derived Vegf-A, which signals to Vegfr2 in endocardial cells. Knockout of either molecule diminished the number of coronary vessels in the compact myocardium, although some of this effect may be impacted indirectly by the accompanying thin myocardium, which would provide less physical space for intramyocardial vessels to develop. However, supporting evidence for a role of Vegf-A in the endocardial-to-coronary pathway comes from *Nfatc1-Cre*-driven deletion of Vegfr1. The *Vegfr1* gene produces a soluble receptor that is secreted and acts as a trap for Vegf-A. Disrupting this mechanism resulted in overexuberant endocadial budding (39). Thus, the endocardium is not terminally differentiated and possesses angiogenic potential, which responds to signals from the myocardium via Vegf-A/Vegfr2 to grow into intramyocardial coronary vessels.

The third source of coronary endothelium in the mouse heart was revealed with Cre-based lineage-tracing studies on a subset of epicardial cells not labeled with the initial epicardial Cre lines (43). These results discovered a previously unappreciated heterogeneity within the epicardial cell layer of the heart. Specifically, a population of Scx^+ or $Sema3D^+$ cells was found to be located within the proepicardial organ, which only partially overlapped with $Tbx18^+$ and $Wt1^+$ cells. For example, approximately one-third of $Sema3D^+$ proepicardial cells coexpress either Tbx18 or Wt1, and only half of $Tbx18^+$ epicardial cells had seen Scx expression during their cell history. Lineage tracing using *Scx-Cre* or *Sema3D-Cre* lines revealed that both can give rise to some coronary endothelial cells. *Sema3D-Cre* also lineage labeled endothelial cells of the sinus venosus, whereas *Scx-Cre* gave rise to endocardium, suggesting that at least some proepicardial-derived endothelial cells could transit first through these cell types. Recent studies have begun to question the mechanisms underlying epicardial-to-endothelial cell differentiation. $Sema3d^+$ epicardial cells express the Hippo pathway transcription factors Yap/Taz. These proteins are required for proper proliferation, epithelial–mesenchymal transformation (EMT), and differentiation into coronary endothelial cells (44). Thus, the proepicardium is a heterogeneous progenitor population with only certain subsets possessing the ability to differentiate into coronary endothelial cells.

The above studies provided evidence that the coronary endothelium derived from three different sources, but some confusion remained regarding the level of contribution from each progenitor. A subsequent report aimed to address this issue through quantifying lineage-tracing data from all three sources in a single study of many different regions of the heart. The results revealed a compartmentalization of the areas populated by different progenitors (**Figure 2*a***) (35). Counting *Apj-CreER*-traced (sinus venosus derived), *Nfatc1-Cre*-traced (endocardial derived), and *Sema3D-Cre*-traced (proepicardial derived) endothelial cells in five different cardiac regions coupled with visualization by whole mount imaging observed the following distribution: Sinus venosus tracing gave rise to the majority of coronary arteries, capillaries, and veins on the dorsal and right lateral sides and almost half of those vessels on the left lateral side, with minimal contribution to the mid-ventral aspect and in the ventricular septum. Endocardium lineage tracing gave rise to vessels in a complementary pattern; i.e., the areas with most sinus venosus contribution had the least contribution from endocardium and vice versa. Contributions from *Sema3D-Cre*-expressing

proepicardial cells were notably fewer when compared to the sinus venosus and endocardium (<20%) and were distributed evenly among the outer circumference of the heart with the least in the septum. This distribution would be expected from cells originating directly from the epicardial covering. Although the simultaneous use of sinus venosus and endocardial lineage-tracing reagents clearly showed complementary contributions between these two progenitors, there is a small percentage of cross labeling with the Cre lines, particularly the *Nfatc1*-Cre recombination in the sinus venosus and potential sporadic Nfatc1 expression in the coronary endothelium itself.

Zhang et al. (45) have further refined these experiments by using intersectional lineage tracing and a more specific endocardial Cre line (*Nrp3-CreER*) to fully exclude sinus venosus labeling in endocardial lineage traces. This strategy observed minimal contribution from the endocardium



in the lateral free walls of the left and right ventricle but detected lineage labeling in most vessels within the septum and in the ventral wall adjacent to the septum, consistent with results from Chen et al. (35). Because coronary vessels in the septum and ventral wall are also as essential for cardiac function (the latter being where the left anterior descending artery resides), it can be concluded that both the sinus venosus and endocardium are important sources that combine to produce the mature coronary vasculature.

A study of the postnatal heart demonstrated further compartmentalization of endothelial cell sources during coronary development. The production of new coronary vasculature after birth in the inner myocardial wall was shown to arise from postnatal differentiation of the endocardium, whereas that found in the outer myocardial wall arose from the expansion of coronary vessels that had developed during embryogenesis (46). The postnatal contribution to the inner myocardial wall was proposed to occur by a process of trabecular compaction. In this model, endocardial cells are trapped in fusing trabeculae and transform into coronary endothelial cells. The existence of such trabecular coalescence still needs to be validated but if confirmed would provide an interesting mode of vascular expansion within the compact myocardium. Together, the lineage-tracing studies reported to date provide support for a model in which multiple sources serve as progenitors to the coronary endothelium by infiltrating the heart from different locations and populating their own respective subregions of the myocardium (**Figure 2*a***).

Following the identification of multiple coronary endothelial cell progenitors, it is important to ascertain whether similar or distinct molecular pathways induce the differentiation and morphogenesis of the different sources. Accumulating evidence supports the possibility that the sinus venosus and endocardium respond to different molecular pathways to form coronary vessels (**Figure 2*b***). For instance, the sinus venosus and endocardium differ in their expression of Apj, a G protein–coupled receptor that stimulates vascular growth and angiogenesis (35). In addition, sinus venosus vessel sprouting requires Vegf-C (expressed in the epicardium), yet *Vegf-C*-deficient hearts have normal vascular growth in endocardial-derived areas (35). Another pathway that affects sinus venosus sprouting is calcineurin-Nfat signaling. Deletion of *Cnb1* in endothelial cells delayed vascular growth in the region derived from the sinus venosus (11). Pharmacological

Figure 2

Multiple progenitor cells contribute to the mature coronary vasculature in a region-specific manner. (*a*) Schematic of the sinus venosus and endocardium progenitor contributions to vessel components in different regions of the heart ventricle. Endothelial cells are indicated by continuous lines, and mural cells (pericytes and smooth muscle) and their progenitors are indicated by dashed lines. Progenitor sources are color-coded and arteries are denoted with an "A." The heart ventricle is schematized as a box and the different heart regions are indicated below. (*b*) Schematic of the molecular pathways regulating different aspects of progenitor cell differentiation and morphogenesis during coronary development. The majority of the vascular endothelium arises through sprouting angiogenesis from the sinus venosus (*solid blue lines*) and endocardium (*solid orange lines*), guided by the molecules listed in blue or orange, respectively. The resulting immature vascular plexus migrates into the aorta forming a coronary artery stem, or ostia, under the guidance of the indicated factors. Following aortic attachment, a subset of the vascular plexus remodels into arteries, whose proper size is regulated by the growth factors and signaling pathways listed. During this process, the coronary endothelium is covered with pericytes that differentiate from both the epicardium (*blue dashed circles*) and cushion endocardium (*orange dashed arrow*). Both of these sources can produce coronary artery smooth muscle, and the epicardium does so through a pericyte intermediate (*black curved arrow*). Preotic neural crest also contributes to coronary artery smooth muscle (*green arrow*), primarily in the ventricular septum. The epicardium also differentiates into fibroblasts (*blue circles*) in a pathway distinct from that producing coronary mural cells (pericytes and smooth muscle), and this cell fate decision is made at the heart surface. Note that for simplicity the schematics in this figure do not include epicardial-derived endothelial cells, which represent a minor and less well-studied population. Abbreviations: BmpER, BMP binding endothelial regulator; CMC, cardiomyocyte; EndoMT, endocardium to mesenchymal transformation; Fgf, fibroblast growth factor; Mrtf, myocardin-related transcription factor; Pdgfrα, platelet-derived growth factor receptor alpha; Pdgfrβ, platelet-derived growth factor receptor beta; Vegf, vascular endothelial growth factor.

inhibition of calcineurin during different developmental windows established that its activity was only required during the initial stages of sprouting. Sinus venosus sprouting in ex vivo culture requires myocardial Ang-1, and cardiomyocyte-specific deletion results in a specific defect in surface veins leaving intramyocardial vessels intact (47). This study also presented histological evidence that Apj-negative cells within the sinus venosus form coronary sprouts. However, this is at odds with lineage tracing of Apj-positive cells within the sinus venosus using *Apj-CreER*, which results in labeling of a large number of coronary arteries, capillaries, and veins (35). It will be interesting to further discriminate the dependence of different coronary progenitors on individual angiogenic factors where differences in regulators could arise from the variation in cell types located along each source's migratory pathways.

The epicardium is a vital source for angiogenic growth factors that stimulate coronary plexus development. Its location on the surface of the heart where sinus venosus sprouts initially spread suggests that it may be particularly important for stimulating early coronary growth. Mouse knockouts that lack proper epicardial behavior are usually accompanied by defects in some aspect of coronary vessel development (reviewed in 7, 8). For example, *Wt1*- and *VCAM*-deficient mice have defective epicardial coverage of the heart, lack epicardial EMT, and exhibit a thin myocardium (48, 49). They also lack a coronary plexus and sinus venosus sprouting (48) (K. Red-Horse, unpublished observations). Tbx18, an epicardially expressed transcription factor, is important for subepicardial coronary plexus development, potentially thorough its induction of epicardial-derived angiogenic molecules (50). Epicardial-derived fibroblast growth factors (fgfs) also impact coronary plexus development, but indirectly. Fgfs support coronary growth through signaling the myocardium, which activates the expression of angiogenic molecules such as Vegf-A, -B, -C, and Ang2 (51). Given its location, the epicardium is likely a primary component during sinus venosus sprouting.

To date, few studies have focused on the molecular regulation of endocardial angiogenesis. Because the endocardium is in direct contact with the myocardium, these cells may be a particularly important source of stimulatory molecules. Myocardial Vegf-A binds endocardial Vegfr1 and Vegfr2 to inhibit and activate endocardial sprouting, respectively (39, 42). This pathway was more specific to intramyocardial capillaries and arteries rather than surface veins, suggesting a possible specificity for endocardial sprouting. Myocardial-expressed transcription factors, such as Fog2, were also critical for coronary angiogenesis in general, possibly through induction of proangiogenic genes and suppression of antiangiogenic factors (52). Whether these factors are specific to endocardial angiogenesis is unknown. There is much to be learned about the specific pathways that drive angiogenesis of the primary coronary plexus from different sources. With the identification of distinct progenitor populations and the availability of experimental tools to differentially manipulate each, the goal moving forward is to continue discovering additional angiogenic regulators, determining whether they have distinct or overlapping effects on the different coronary progenitors, and unravelling the intersections between the different signaling pathways involved.

Coronary Artery Smooth Muscle

Another important cell type of coronary arteries is the smooth muscle layer, which is also established during embryonic development during the plexus remodeling stage. Smooth muscle forms one or multiple cell layers directly surrounding the endothelium to give arteries structure and regulate lumen size. In mice, coronary artery segments proximal to the aorta have multiple layers, whereas those more distal have a single layer (53, 54). In contrast, human coronary arteries have multiple layers far distally. This difference could arise from the observation that rodent coronary

arteries course intramyocardially, whereas those of humans are in a subepicardial location. As to their origins, original observations using quail-chick chimeras and clonal analysis concluded that the proepicardium gives rise to smooth muscle, and this was largely corroborated in murine epicardial lineage-tracing studies. To date, *Tbx18-Cre*, *Wt1-Cre*, *Gata5-Cre*, *Sema3D-Cre*, *Scx-Cre*, and *Tcf 21-CreER* all trace into the smooth muscle layer (32–34, 43, 55). Lineage tracing with these tools has detected large contributions of epicardial cells to coronary smooth muscle. Thus, it is well documented in multiple species using multiple tools that epicardial cells are a predominant progenitor source for coronary artery smooth muscle under normal developmental conditions (**Figure 2*a***).

Despite a large contribution from the epicardium, the smooth muscle layer has additional progenitor cell types, much like the endothelial layer (**Figure 2*a***). Murine lineage-labeling experiments marking neural crest cells using *Wnt1-Cre* showed that this cell type gives rise to proximal coronary artery segments closest to the aorta (56). Neural crest contributions to chick coronary arteries were not observed in transplantation experiments using donor postotic cardiac neural crest (57, 58). However, when preotic neural crest, whose derivatives migrate prior to postotic cardiac neural crest, is transplanted, coronary artery smooth muscle is lineage labeled. Similar to coronary endothelium, compartmentalization was also seen in these experiments, as preotic neural crest produced smooth muscle that was mostly located in arterial branches within the ventricular septum. In mice and humans, differentiation of preotic neural crest into coronary artery smooth muscle requires Edn1/Ednra signaling. Expression patterns suggest that Edn1 expressed by coronary endothelium binds to the Ednra receptor on migrating neural crest cells to induce smooth muscle differentiation. The absence of Edn1/Ednra or postotic neural crest results in the specific dilation of septal coronary arteries and ectopic connections with the aorta, showing that neural crest is an important component of building the coronary vascular tree.

Recently, a third source for coronary smooth muscle was discovered. Endocardial-derived cardiac cushion mesenchyme differentiates into coronary mural cells (i.e., smooth muscle and pericytes). Cushion mesenchyme arises when endocardial cells at the atrioventricular junction and outflow tract undergo EMT and invade the underlying space. These cells express Pdgfrα and Pdgfrβ and ultimately sculpt into the valves that separate the heart chambers. They are also progenitors that, in response to Wnt-Fzd4-β-catenin signaling, migrate into the ventricular myocardium and differentiate into Pdgfrβ$^+$Pdgfrα mural cells (59). Cushion mesenchyme gave rise to coronary mural cells at a much greater rate in the ventricular septum when compared to left and right lateral walls. These data indicate that the developing embryo has multiple cellular pathways available for the production of coronary artery smooth muscle.

There is evidence that the alternative sources of smooth muscle can compensate for each other on mutant backgrounds. An undefined nonepicardial source for coronary smooth muscle was observed in the context of defective epicardial differentiation, for example, with epicardial-specific deletion of *Pdgfrβ* or *Rbpj* (60, 61). These mutations trigger a severely delayed investment of coronary artery smooth muscle, but eventually the cells appear. However, this delayed compensatory response does not occur in full *Pdgfrβ* knockouts, suggesting that the Pdgfrβ$^+$ cushion mesenchyme could be involved. Interestingly, upon epicardial *Rbpj* deficiency, the compensatory pathway rendered coronary arteries more susceptible to disease at adult stages (60). The mechanistic basis of this phenomenon has yet to be explored. The emerging theme in coronary development that multiple progenitor sources exist may allow for the activation of compensatory pathways ensuring the establishment of this critical vascular bed.

Although the epicardium is a progenitor source for smooth muscle, important regulatory steps guide fate decisions and migratory paths between the two cell types (**Figure 2*b***). This is most

appreciated in the rodent heart where developing coronary arteries are located deep within the myocardium many cell layers from the epicardium. To form cardiac stroma (cardiac fibroblasts or coronary artery smooth muscle), epicardial cells must undergo an EMT that allows them to leave the surface and migrate into deeper layers of the myocardium. Evidence indicates that the cell fate decision of becoming either smooth muscle or fibroblast occurs on the surface of the heart, where it may be coupled with the EMT process. Deletion of either *Pdgfrα* or *Pdgfrβ* specifically interrupts the ability of epicardial-derived cells to produce either the fibroblast or smooth muscle lineage, respectively (61, 62). This interruption is accompanied by a specific defect in the relevant progenitors (fibroblast or smooth muscle) to undergo EMT and enter the myocardium. In addition, the transcription factor Tcf21 becomes restricted to the fibroblast lineage around the time of EMT and before much myocardial invasion of epicardial-derived cells has occurred (55). Thus, a cell fate decision distinguishing the fibroblast from smooth muscle lineage has occurred prior to epicardial migration into the myocardium. Furthermore, one of Tcf21's functions is to suppress the smooth muscle gene *Smα* in the fibroblast lineage, and the ectopic expression of *Smα* in *Tcf21* mutants is seen in epicardial cells at the surface (63). A lineage decision within the epicardium is supported by inducible lineage labeling using *Wt1-CreER*, which shows that most smooth muscle cells are derived from the early epicardium (60, 64), which is consistent with the epicardium itself being a heterogeneous population (43, 65). Further experiments are required to confirm whether the initiation of EMT is part of the differentiation program. Interestingly, in other systems, core EMT transcription factors facilitate cell reprogramming (66) and mediate acquisition of stem cell fate (67). Thus, evidence to date indicates that the epicardial cells differentiate down either the smooth muscle or fibroblast lineage early in development at the heart surface prior to EMT and migration into the myocardium.

Although initiation of the smooth muscle differentiation pathway appears to occur at the heart surface, epicardial cells in this location do not express mature smooth muscle markers. Cells with these markers are only initially present directly adjacent to differentiating arteries and later maturing veins. This observation then raises the question of the identity and migratory path of the epicardial-derived cell type transiting through the myocardium fated to become coronary artery smooth muscle in deep arteries. Clonal analysis labeling a single epicardial cell and all its progeny and direct lineage tracing using *Ng2-Cre* and *Notch3-Cre* lines have identified cardiac pericytes as intermediate progenitors for coronary artery smooth muscle (68). Fitting with the differentiation of the pericyte/smooth muscle lineage at the surface, coronary plexus endothelial cells migrating beneath the epicardium during early development acquire epicardial-derived pericytes. This process requires Pdgfrβ (68) and myocardin-related transcription factors A and B, the latter of which induces the expression of factors involved in motility and pericyte differentiation (69). The coronary vascular plexus, which is covered with pericytes, migrates into and fills the ventricular myocardium. Then, the pericytes that have migrated onto plexus vessels that receive arterialization signals differentiate into smooth muscle. Notch signaling in epicardial-derived cells positively regulates smooth muscle differentiation (70). Specifically, Notch3 stimulates the induction of contractile proteins (Smα, Sm-Mhc) in pericytes surrounding developing coronary arteries, which is in response to *Jagged-1* that is expressed by the endothelium of remodeling artery (68, 71). Ultimately, *Jagged-1* deficiency results in improper arterial branching (72). In addition to its role during embryonic development, Jagged-1 must be maintained in adult coronary arteries through endothelial Akt/mTOR signaling to prevent smooth muscle degradation (73). Clonal studies showed that pericytes related to coronary smooth muscle persist in adult hearts, suggesting a potential for artery regeneration (68). Future studies should illuminate whether adult pericytes can be a source for new coronary artery smooth muscle following injury and whether the pericyte-to-smooth muscle transition occurs in vascular beds other than the heart.

ESTABLISHMENT OF CORONARY BLOOD FLOW

Coronary artery development is unique from many other organ-specific vascular beds in that its immature vascular plexus develops initially without significant amounts of blood flow (74). The immature coronary plexus, derived mostly from the sinus venosus and endocardium, migrates through the heart from the outside in and inside out, respectively. The resulting unperfused plexus also extends branches toward the aorta and connects with aortic endothelium at stereotyped locations to initiate arterial blood flow. Plexus vessels directly downstream of the connection point, which presumably experience the highest levels of blood flow, begin to arterialize ultimately forming the left and right coronary arteries. Mutants with delayed aortic connections have delayed artery remodeling, indicating that blood flow is critical for triggering the arterialization of undifferentiated plexus vessels (68, 74, 75). Thus, the coronary vasculature is a good model for investigating the role of blood flow in arterial differentiation during development.

There is great interest in understanding the connection event between the developing coronary plexus and aorta (reviewed in 9). This event is interesting from a developmental biology standpoint because the vessel growth here is highly targeted, requiring anastomosis with the aorta, but at the same time, requiring avoidance of the adjacent pulmonary artery, which will ultimately carry deoxygenated blood. The underlying mechanisms should provide a greater understanding of how cell types properly navigate each other to form mature organ structures. Coronary–aorta connections, called ostia or stems, are normally seen on the left and right valvular sinuses of the aorta nearest to the pulmonary artery and not the noncoronary sinus that is 180° from the aorta-pulmonary artery interface. Congenital coronary anomalies arise when aortic targeting is not carried out properly, which can range from coronary artery misplacements on the aorta to faulty connections with the pulmonary artery (76, 77). Such anomalies can be asymptomatic but are often associated with arythmia or sudden cardiac events. Ectopic pulmonary attachments are usually incompatible with life and in humans are referred to as anomalous left coronary artery from the pulmonary artery, or ALCAPA (78, 79). These usually require surgical repair in affected infants (80). Understanding the mechanisms targeting coronary arteries to the aorta should reveal interesting cell biology, and recapitulating these processes could lead to methods of forming new arterial blood supply in diseased hearts.

Although there are still many unknowns, recent progress has illuminated some of the factors involved in coronary artery ostia/stem formation. Morphologically, plexus vessels migrate toward the aorta and invade through the outer layers of the aorta (12, 74, 81, 82). The aortic wall at this immature state is composed of undifferentiated second heart field cells and an incomplete one- or two-cell-thick neural crest–derived smooth muscle layer (74) (K. Red-Horse, unpublished observations). The invading plexus vessels initially form multiple single-celled connections with the aortic endothelium, which at subsequent stages normally remodel into two larger (left and right) coronary artery stems.

Molecular regulation of coronary artery stem formation involves secreted molecules and their regulators locally expressed around the outflow tract (**Figure 2*b***). In mice, Vegf-C and the chemokine Cxcl12 are highly expressed in the walls of the outflow tract vessels during the time of stem formation (74, 75). Coronary plexus vessels also express Vegfr2 and -3, which bind Vegf-C and the Cxcl12 receptor, Cxcr4. Deletion of Vegf-C causes a delay in coronary artery stem formation by one or more days, and those that do form are located in an abnormal location low on the aorta (74). Stem development delays in the absence of Vegf-C are secondary to defective recruitment of coronary plexus vessels to the outflow tract (at this location coronary vessels are called peritruncal vessels) because mutants are completely devoid of vasculature at this site during the normal window of stem development. Endothelial-specific deletion of Cxcr4 also results in

missing stems and small coronary arteries. Deletion of Cxcl12 in the second heart field territory, which includes the outflow tract vessel walls, produces a similar phenotype (75). Interestingly, Cxcl12 deletion predominately affects the left coronary artery and results in one heart exhibiting ALCAPA. Bone morphogenetic protein (BMP) signaling is also involved in stem formation. BMP binding endothelial regulator (BmpER) is a secreted modulator of BMP signaling (83) that is proangiogenic (84). It is expressed in coronary endothelial cells and the cushion mesenchyme of the developing outflow tract valves (85). Deletion of *BmpER* results in defective formation of the coronary artery stem with a significant portion of mutants lacking connections with the aorta and others having mislocalizations such as high take off. In contrast to Vegf-C, Cxcl12/Cxcr4 signaling and BmpER were not required for vessels to approach the outflow tract but had a more localized function of facilitating peritruncal vessel migration across the aortic wall to connect with aortic endothelium (75, 85, 86). Thus, angiogenic ligands expressed around the outflow tract attract coronary plexus vessels and facilitate their connections with the aorta.

One conundrum regarding the role of Vegf-C and Cxcl12 in stem development is that Vegf-C and Cxcl12 are expressed at equal levels within the aorta and pulmonary trunk, whereas the coronary plexus only invades the aorta (74, 75). To date, no individual gene was identified as specifically expressed in the aorta. However, a cellular difference was discovered between the two outflow vessels. Cardiomyocytes, the muscle cells of the heart, develop specifically in the wall of the aorta but not the pulmonary artery (74). Within the aortic wall, cardiomyocytes are correlated with stem location being specific to the left and right coronary sinus and rarely in the noncoronary sinus. These cardiomyocytes develop in an *Isl1*-dependent manner, and coronary artery stems are delayed and mislocalized in *Isl1* mutants. This suggests a role for aortic cardiomyocytes in guiding anastomoses between the coronary plexus and aorta and that their absence in the pulmonary artery wall may help exclude coronary invasion. Further studies should identify cardiomyocyte-specific factors that may collaborate with Vegf-C and Cxcl12 to provide aortic specificity to coronary artery stem development.

ARTERIAL REMODELING AND MATURATION

Coronary arteries develop through transformation of the plexus vessels following stem formation in a process called developmental arterial remodeling. Developmental arterial remodeling refers to the process whereby immature plexus vessels reconfigure to produce a larger-bore artery covered in smooth muscle (**Figure 1*a***). This is distinct from adult arterial remodeling, which refers to the process in which arteries increase their lumen by expanding outward. Both processes are stimulated by increases in blood flow that are sensed as shear stress by endothelial cells (87, 88). Developmental arterial remodeling is the method by which coronary arteries initially form during embryogenesis, but adult arterial remodeling occurs in the adult when blood flow rates shift due to injury or disease associated with CAD. Relatively little is known about either process because it occurs in the heart, but some information has been gained and is described below.

Developmental arterial remodeling in the heart occurs once the plexus attaches to the aorta to receive blood flow in vessels directly downstream of the attachment site. The absence of attachment is why arterial remodeling is absent and/or delayed in *Vegf-C*- and *Cxcl12/Cxcr4*-deficient hearts (74, 75). Later knockouts induced postattachment could reveal roles during the remodeling processes itself, a possibility that is supported by observations in the regenerating fin where Cxcr4 facilitates artery growth by stimulating endothelial cell migration into growing arteries (89). Lineage-tracing (35, 36) and clonal analysis (36) experiments have definitively shown that plexus vessels from the sinus venosus and endocardium are those that are remodeled into arteries. Upon attachment to the aorta, downstream vessels upregulate arterial markers, including Jagged-1, in a

flow-dependent manner (68). Jagged-1 is functionally involved in arteriogenesis, as its knockout in endothelial cells results in abnormal artery patterning (72). Studies in the retina show that endothelial Notch signaling is required during remodeling. Deletion of Delta-like ligand 4 (*Dll4*) or *Rbpj* in periarterial capillaries, i.e., the arterial remodeling zones, led to shortened arteries, whereas expression of a constitutively active Notch intracellular domain increased artery length (90). In this system, Notch function was specific to periarterial capillaries and was no longer required once endothelial cells became incorporated in the mature artery. Given the timing of aortic attachment during coronary development, this system would be a good model to study how blood flow regulates Notch signaling during artery formation (91).

It is difficult to study the cell dynamics of arterial remodeling in the heart, but live imaging of other, more accessible vascular beds has revealed cell biology that may be applicable to coronary arteries. Remodeling in the mammalian and avian yolk sac revealed that arterial remodeling involves plexus vessel fusion. In addition, in these systems and the regenerating zebrafish fin, endothelial cells migrate against the direction of blood flow toward the growing artery (89, 92, 93). This migration involves Cxcr4a in the regenerating zebrafish fin (89). Both of these cellular behaviors likely contribute to the expansion of the arterial lumen during remodeling, particularly because endothelial cells of the remodeling and mature arteries dramatically decreased their proliferation (90). Future research illuminating the molecular control of these cell behaviors will increase our understanding of how arterial size is established and identify methods of manipulating this process to enhance regeneration.

Vegfr signaling is a prominent driver of arteriogenesis in many organs, including the heart (94). In multiple tissues and organs, it induced arterial identity by increasing arterial-specific genes (*Notch*, *ephrinB2*, and others). Deletion of *Vegf-A* in the myocardium impairs establishment of the coronary plexus from the endocardium, precluding specific interrogation of arteriogenesis (42). In contrast, signaling pathways downstream of Vegfrs were shown to specifically affect arteries. Deletion of synectin-4 results in small coronary (and other) arteries with decreased branching but normal veins and plexus angiogenesis (95, 96). This is due, at least in part, to synectin-4 expression in endothelial cells (97). A similar phenotype is obtained with the deletion of the intracellular domain of Nrp1 (98). Synectin-4 and Nrp1 form a complex with Vegfr2 and Myosin V1 to facilitate endocytosis of the Vegf-bound receptor. Endocytosis is required for Vegfr signaling because movement into $EEA1^{+}$ endosomes protects it from PTP1b dephosphorylation, allowing signaling to be maintained (94). Following these events, phosphorylated Vegfr2 increases arterial growth by ultimately stimulating Erk signaling, and overactive Erk results in an increase in arterial diameter. These studies systematically show that Vegfr/Erk signaling is required for coronary artery branching and lumen size to reach levels seen in wild-type animals. However, in all loss of function mutants, arterial vessels are only decreased in size and number and not completely absent, indicating that additional pathways are either normally involved in arterial development or are induced to compensate for defective arteriogenesis.

One mechanism by which Vegfr/Erk (and other signals) could modulate arterial development is through stimulating the production of nitric oxide (NO). Vegf induces the expression of endothelial nitric oxide synthase (NOS3), and *Nos3*-deficient mice have hypoplastic coronary arteries (99). The phenotype of small coronary arteries arises during development at the time of arterial remodeling and is associated with lethal postnatal myocardial infarctions in the first week of life. Nos3 is expressed in coronary endothelial cells and the endocardium, but the cell source and localization of the ligands binding Vegf receptors to activate Erk signaling specifically during arterial remodeling are still under investigation. This information is likely outstanding because deletion of Vegf members often affects plexus formation, precluding a direct analysis of arterial maturation (35, 39, 42). Timed and tissue-specific deletions of Vegfs and their receptors after the plexus is firmly

established could delineate a specific effect on arterial growth and remodeling. Vegf-A is expressed in the heart by cardiomyocytes, although it is not restricted to areas surrounding arterializing vessels, but instead throughout the myocardium. Vegf-B is also expressed throughout the myocardium from midgestation onward (100, 101). Although no significant developmental defects in knockout coronary arteries were reported (100, 102), Vegf-B plays an important role in stimulating vascular growth during the injury response (103). Vegf-B knockout impairs recovery from injury and ectopically applied Vegf-B stimulates coronary vascular growth, including increasing the number and size of arterial vessels, thus leading to better outcomes following cardiac injury (15). One cell type that could be a source of Vegfs and other growth factors that impact coronary artery development are macrophages. These cells are the source of angiogenic and arteriogenic factors, and in other systems they increase the development of arterial collaterals (94, 104, 105). The heart possesses a population of yolk sac–derived macrophages distinct from those descended from the blood monocyte lineage (106) that are required for remodeling of the coronary plexus (107). Mice deficient in these tissue macrophages have dilated cardiac veins and increased arterial branching, indicating a defect in plexus maturation. These macrophages expressed Vegf and insulin-like growth factor, the latter of which activated endothelial cells during in vitro angiogenesis assays.

CONCLUSION AND FUTURE PERSPECTIVES

Understanding normal developmental processes contributes to developing methods to repair organ dysfunction caused by congenital defects or disease. It also helps reveal reprogramming pathways that can be utilized for tissue engineering and regenerative medicine. For example, identification of developmental transcriptional networks, such as for pancreatic beta cells or cardiomyocytes, has led to the generation of replacement cells in disease models through in vivo reprogramming (108–111). Similarly, understanding the forces regulating the different aspects of coronary artery development should identify pathways that stimulate arteriogenesis in the malformed or damaged heart. One example is ALCAPA, where the left coronary artery is connected to the pulmonary trunk instead of the aorta (78, 79). Although this is a rare cardiac anomaly, it can be fatal if left untreated and usually requires surgical intervention. As described above, we now know the cellular and molecular guidance cues that connect murine coronary vessels to the aorta (Cxcl12, Vegf-C, and aortic cardiomyocytes), and that aspects of this process (aortic cardiomyocytes) can be observed in humans. It may be possible to target the expression of these molecules and the differentiation of these cells in the aorta to induce the growth of a new artery, both in patients suffering from the ALCAPA congenital malformation and in adults needing a new artery as a result of CAD.

New information on the cell biology of coronary arteriogenesis specifically (the process of transforming the immature vascular plexus into a mature artery) is critical for creating interventions that stimulate arterial growth during regeneration. One of the current limitations of proangiogenic treatments, such as Fgfs or Vegfs, is that they effectively generate new microvasculature but fail to generate the arterial growth required to sufficiently increase blood flow (5, 94). Coronary arteriogenesis studies have shown that blood flow is an important regulator of this process and have identified molecular pathways regulating artery size. Activating the mechanisms downstream of blood flow in situations when it is interrupted could provide a means of increasing artery size to make current proangiogeneic therapies more effective. Similarly, using the signaling cues that drive remodeling of small vessels could create new collateral arteries, the number of which are an important survival factor following myocardial infarction.

There have been significant advancements in our current understanding of coronary artery development, made possible through technological advancements in mouse genetics (particularly lineage tracing) and imaging possibilities. A predominant and unexpected theme is the existence of

multiple progenitor sources for the same cell types within the coronary system. Several questions remain: Why do multiple progenitor cells exist and what is the interplay between them in wild-type and mutant contexts? How similar or different at the epigenetic level are the same cell types when they arise from distinct progenitors? Do vessels derived from specific progenitors preferentially participate in the injury response? These and related questions will be interesting to address in future research, the results of which will hopefully shed further light on how to coax the regrowth of coronary vasculature during disease.

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