Cellular Origin and Developmental Program of Coronary Angiogenesis

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Abstract

Coronary artery disease causes acute myocardial infarction and heart failure. Identifying coronary vascular progenitors and their developmental program could inspire novel regenerative treatments for cardiac diseases. The developmental origins of the coronary vessels have been shrouded in mystery and debated for several decades. Recent identification of progenitors for coronary vessels within the endocardium, epicardium and sinus venosus provides new insights into this question. In addition, significant progress has been achieved in elucidating the cellular and molecular programs that orchestrate coronary artery development. Establishing adequate vascular supply will be an essential component of cardiac regenerative strategies, and these findings raise exciting new strategies for therapeutic cardiac revascularization.

Keywords

Coronary origin; lineage tracing; coronary vascular endothelial cells; epicardium; endocardium; sinus venosus

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To satiate its voracious appetite for oxygen and nutrients, the heart has a dedicated circulatory system, the coronary vasculature. Coronary artery disease causes myocardial infarction (MI) and heart failure, making it the leading cause of death worldwide.[1–3] In MI, coronary artery occlusion causes death of as many as one billion cardiomyocytes.[4] Lack of adequate numbers of functional cardiomyocytes coupled with chronic overload and subsequent dysfunction of remaining cardiomyocytes eventually results in heart failure and death.[5, 6] Currently, angioplasty and coronary artery bypass surgery are the mainstays for coronary artery disease treatment.[7] Although pro-angiogenic approaches have shown

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promise in animal models,[8, 9] these have not yet been efficacious in clinical trials. Clearly, improved approaches for coronary revascularization and cardiomyocyte regeneration after myocardial infarction are urgently needed.[9, 10] A better understanding of how coronary vessels are built during development will provide important insights that might be therapeutically deployed for cardiac regeneration.

Benjamin Franklin stated, "Originality is the art of concealing your sources". Nature has done a good job of concealing the sources of the coronary arteries. Simply speaking, the coronary arteries consist of two layers of cells: the inner endothelial cell layer and outer smooth muscle cells. Overwhelming evidence indicates that most coronary smooth muscle cells originate from epicardium.[11–14] However, the origin of coronary endothelium has ignited controversies over the past two decades and remains an enigma. This review focuses on the origin of coronary vascular endothelial cells (VECs) and on the developmental programs that regulate coronary angiogenesis in development and disease.

Genetic lineage tracing and its caveats

Most recent studies of coronary vessel development were performed in mouse, and state-of-the-art techniques have been used to trace the origin of coronary VECs. It is critical to first introduce genetic lineage tracing techniques and to discuss its caveats so that experiments can be appropriately analyzed and interpreted. Lineage tracing in mammalian systems largely relies on Cre-loxP recombination.[15, 16] This technology is dependent upon two components: a Cre allele that selectively expresses in the progenitor cell of interest, and a Cre-activated reporter allele (Figure 1A). Selective expression of Cre recombinase in a progenitor cell of interest is achieved most commonly by identifying an endogenous marker gene selectively expressed in the progenitor cell and then placing Cre under control of the marker gene's transcriptional regulatory sequences, e.g. by replacement of the marker gene's open reading frame with Cre in the endogenous locus (knock-in). Typically, Cre-activated reporters are located in a widely permissive locus (e.g. Rosa26) and use a broadly expressed promoter (e.g. CAG) to drive expression of a reporter gene. In the absence of Cre, a loxP-flanked transcriptional stop cassette blocks reporter gene expression. Cre expression in the progenitor cell leads to excision of the floxed stop cassette and allows subsequent expression of the reporter gene (Figure 1A). Because this genetic label is heritable and permanent, the cell and all of its descendants are indelibly marked by reporter gene expression, regardless of whether or not their descendants actively express Cre.

The powerful Cre-loxP system provides a history of a cell and its descendants by showing where they migrate and also the cell types into which they differentiate. For example, progenitor cell *A* expresses typical marker *a* strongly and specifically. Cre recombinase under promoter *a* would lead to lineage labeling of *A* cells. Cell *B* expresses neither marker *a* nor Cre recombinase from promoter *a* (Figure 1B). As lineage tracing is irreversible and inheritable, if *B* cells are labeled by *a*-Cre, then the straightforward interpretation is that *A* cells differentiate into *B* cells (Figure 1B). However, this interpretation should be made cautiously. If *B* cells express low level of *a* and therefore Cre, then one would not be able to interpret *B* cell marking by Cre as indicative of an *A* to *B* fate transition (Figure 1C). The interpretation of a Cre-loxP experiment therefore hinges on the "negative" expression of Cre

in *B* cells, or indeed in any non-*A* cell type in the organism's developmental history that could differentiate into *B* cells.

This type of expansive negative data can be difficult to acquire. Direct measurement of Cre expression is more desirable than measurement of the endogenous gene (because the Cre allele's expression characteristics may differ from the endogenous gene). Since the expression of a gene is dynamic with time and strength, negative expression at one time point does not exclude its possible expression at another. This is an important limitation to the rigorous interpretation of lineage tracing data obtained with constitutively active Cre alleles. Thus, it is desirable to perform these experiments using inducible Cre alleles that are active only in the presence of an inducing agent, since then one can restrict the temporal window in which one needs detailed Cre expression data. On the other hand, inducible Cre alleles can be difficult to fully activate. As a result, labeling may be incomplete and so underestimate the extent of contribution of lineage *A* to lineage *B*. Complementary approaches that do not critically hinge on the expression or lack of expression of Cre in target cells (*B* cells) are essential to provide definitive evidence of *A* to *B* trans-differentiation.

A final factor that must be considered to interpret Cre-loxP data, and to compare between studies, is that Cre reporter genes differ significantly in their susceptibility to recombination. [17, 18] This is true even when comparing between Cre reporters positioned in the same locus (e.g. Rosa26). While expression differences are graded, Cre recombination is all-or-none. As a result, the susceptibility of a Cre reporter to recombination imposes a "threshold" for Cre labeling, and slightly different thresholds can lead to very different Cre labeling maps (discussed in detail in ref. [18]). A recombination-sensitive reporter would reveal a broader fate map that includes progenitors with lower level or transient Cre expression, while an insensitive reporter would reveal a more narrow fate map that corresponds with higher level or longer duration of Cre expression.[19] Apparent descrepancies between the lineage tracing results reported by different labs can be linked to the use of Cre reporters with different sensitivities.[18–20]

We will incorporate these caveats in our interpretation of the different candidate origins for coronary vascular endothelium being most heavily investigated: (pro)epicardium, sinus venosus, and ventricular endocardium.

Coronary VECs from (Pro)epicardium

Proepicardium is a transient cauliflower-like protrusion originating from transversum septum and wedged below the atrioventricular junction at E9.5 (Figure 2A). Proepicardial cells migrate onto and spread over the surface of the heart, eventually covering the heart surface with an epithelial sheet, the epicardium.[21–24] The epicardium plays an essential role in heart development and disease through two general mechanisms. First, it undergoes epithelial-to-mesenchymal transition (EMT) to form mesenchymal epicardium-derived cells (EPDCs), which subsequently differentiate into most of the non-cardiomyocyte cell types of the myocardial wall.[22] Second, the myocardial-epicardial interface forms a critical signaling

center that is necessary for both myocardial growth and coronary vessel development.[22, 25, 26]

For two decades, the (pro)epicardium was proposed to give rise to the coronary arteries based on three types of lineage tracing experiments in chick embryos: retroviral labeling, dye labeling, and quail-to-chick cell transplantation.[11, 27, 28] In pioneering work, Mikawa and colleagues injected replication-deficient, lacZ expressing retrovirus into proepicardium. Later in heart development, lacZ-expressing cells were found in the endothelial, smooth muscle, and fibroblast lineages.[27] These key experiments established the paradigm that coronary VECs and smooth muscle cells arise from proepicardium. This view was reinforced by transplantation of microdissected quail proepicardium into chicken embryos. Quail cells, detected by the quail-specific antibody QCPN, formed coronary VECs and smooth muscle cells.[28]

These seminal experiments had two key limitations. First, both faced potential contamination issues. For example, sinus venosus (SV) was co-transplanted with the proepicardium in the chimera assay,[28] which may have been important in retrospect since the SV was recently found to be an important source of VECs in mouse embryos (see below).[29] Second, neither study could quantitatively determine the fraction of coronary VECs that arise from pro-epicardium, an important issue if there are multiple different VEC sources. In addition, transplantation assays, although informative about the trans-differentiation potential of proepicardium into coronary endothelium, may not inform us on the actual differentiation of epicardial cells under physiological conditions. For example, mammary myoepithelial cells can adopt a multipotent fate and are able to regenerate a complete mammary gland upon transplantation. However, it still remains a controversy whether these myoepithelial cells really behave in this manner in vivo under physiological conditions.[30, 31]

The retroviral and transplantation assays described above suggest that at least some coronary VECs arise from proepicardium. The proepicardium is a transient structure that contains several morphologically distinct cell types. In addition to superficial mesothelial cells, the avian proepicardium also contains angioblast, endothelial and hematopoietic cells.[32, 33] Poelmann et al. showed that endothelial cells from the SV expand into the proepicardium to reach the dorsal side of the atrioventricular sulcus, where most coronary vessels are first detected.[34] Distinct populations of endothelial cells – those associated with SV or liver bud, and those not associated with either SV or liver bud – were also found in murine proepicardium.[35] These results lead to the question of whether those coronary VECs found to arise from proepicardium develop from endothelial cells already present in proepicardium, or if they develop by transdifferentiation of the proepicardium's non-endothelial cell types. Addressing this question requires use of cell-type specific labeling reagents, rather than spatial labeling that is the basis of retroviral or transplantation assay.

The origin of coronary VECs has been studied in the murine system using the Cre-loxP system, which achieves cell-type specific labeling. Cre alleles driven by regulatory elements of epicardial markers *Wt1*,[14, 36–39] *Gata5*,[40] *Tbx18*,[41] *Tcf21*,[42] *Sema3d*, or *Scx*,[43] were used to study the fate of epicardium in the developing mammalian heart. These studies

showed that at most a small fraction of coronary VECs arise from (pro)epicardium in mouse (Table 1), a surprising finding given the long-standing view, established by the avian studies, that coronary VECs develop from (pro)epicardium. Why were such divergent conclusions reached in avians compared to mice? In retrospect, the avian studies did not provide quantitative information on the fraction of coronary VECs that originate from (pro)epicardium. Second, the key cell population that generated coronary VECs in the regional labeling in the avian systems may not have the mesothelial populations labeled by these Cre alleles. For instance, proepicardial vascular cells may have yielded coronary VECs in the avian experiments. Third, Katz et al. demonstrated that proepicardial cells are heterogeneous and suggested that different proepicardial progenitors may contribute differently to specific lineages.[43] However, the relevance of this hypothesis to coronary VECs awaits additional experimental data. Interestingly, in this case, the proepicardium itself may contribute to sinus venosus, which was also lineage labeled (Figure 2A, B).

Another caveat in lineage tracing of epicardial cells into coronary VECs is that the Cre allele used should not be expressed in coronary VECs themselves (Figure 1C). The most widely used epicardial Cre lines are based on *Wt1* regulatory elements, using different strategies including BAC and YAC transgenics and endogenous locus knockins.[14, 36–39, 44] Although *Wt1* is highly expressed in epicardium, it was also reported that *Wt1* could be expressed in coronary vasculature in adult heart after myocardial infarction.[45] Epicardial cells labeled by tamoxifen treatment of Wt1[CreERT2] embryos at E10.5 contribute to few coronary endothelial cells at E18.5. However, tamoxifen treatment in late embryonic or early neonatal stages (e.g. E14.5-P4) labeled a substantial number of coronary endothelial cells at E18.5 and P7 respectively.[19] Labeling of endothelial cells by E14.5 tamoxifen treatment likely reflected expression of Cre in situ in these coronary endothelial cells rather than their origin from epicardial cells.[19] During development, epicardial cells mainly contribute to coronary smooth muscle cells and fibroblasts (Figure 3). Likewise, the inducible lineage tracing in adult heart after myocardial injury captures a small population of coronary VECs.[46] However, these labeled coronary VECs were quite isolated in myocardium and far from reactivated epicardial layers in injured heart.[46] As there was no spatial relationship between these labeled VECs and the epicardium even shortly after injury, labeling of these coronary VECs are likely due to *Wt1*-driven CreERT2 expression in some coronary VECs rather than EPDCs that had migrated into the myocardium.[45, 46] These epicardial cells adopt smooth muscle and fibroblast fates in injured heart (Figure 3). Other studies based on a different *Wt1*-IRES-GFPCre line showed that a small population of coronary endothelial cells were labeled and they were interpreted to form de novo after myocardium infarction from re-activated epicardial cells.[47] This interpretation should be made cautiously given the aforementioned caveats of Cre lineage tracing and the potential for Wt1 expression in occassional coronary endothelial cells.

Taken together, epicardium likely contributes to coronary vessels in the developing mouse heart, but quantitatively, its contribution is small (Table 1). The same is likely true in the avian system, although definitively establishing this would require use of assays in which the cell-type specific contribution of mesothelial cells to coronary VECs could be quantitatively estimated.

Crosstalk Between Epicardium and Myocardium

Although epicardial cells provide few coronary VECs, the epicardium is essential for coronary vessel formation during heart development. Epicardial cells secrete essential growth factors and cytokines that regulate cells of the nascent coronaries directly and that impact the underlying myocardium, which in return provides angiogenic factors that promote coronary vasculogenesis and angiogenesis.[25, 48–50] Gene knockdown or knockout approaches have identified a number of epicardial or myocardial genes that are essential for coronary angiogenesis (Table 2). Vascular cell adhesion molecule 1 (*Vcam1*), erythropoietin receptor, connexin 43, and α4 integrin are required for formation, migration, or integrity of the epicardium.[51–54] The transcriptional regulators *Wt1* and *Rxra* are required within epicardial cells, where they influence the integrity of the epicardium as well as the formation of vascular cells by EMT.[40, 55, 56] *Wt1* and *Rxra* both regulate epicardial EMT through Wnt signaling pathways.[40, 56] *Wt1* also regulates expression of *Ntrk2*, a cytokine receptor necessary for normal coronary vessel development,[57] and *Raldh2*, a key regulator of retinoic acid signaling confined to the epicardium was also regulated by *Wt1*.[56] *Tbx18*, another epicardial transcription factor, is also required for proper development of coronary arteries.[58]

Within the myocardium, several major signaling pathways have been implicated in coronary vessel development. Here we will highlight three of the most studied pathways. First, normal formation of the coronary vascular plexus requires fibroblast growth factor (FGF) signaling. FGF9, secreted by the epicardium, stimulates FGFR1 and FGFR2c receptors on cardiomyocytes. The expression of FGF9 is regulated by retinoic acid signals from epicardium.[59] Disruption of this FGF signal results in impaired myocardial secretion of angiopoietin-2 (Ang-2), vascular endothelial growth factor-A (VEGF-A), and VEGF-B[60] VEGF was shown to be highest in the compact myocardium nearest the epicardium and subsequently become more evenly distributed transmurally, which coincides with sites of coronary vessel formation.[61, 62] Additional fibroblast growth factors such as FGF1, 2, 7 secreted by either myocardium or epicardium, also positively regulate epicardial EMT.[63] Sonic hedgehog is a critical mediator that links FGF signaling to VEGF-A expression and coronary vessel formation during development as well as maintenance in disease.[60, 64] In addition, hedgehog signaling to distinct cell types differentially regulates coronary artery and vein development.[65] Cardiomyoblast hedgehog signaling regulates the development of coronary veins, while perivascular cell hedgehog signaling is required for coronary arterial growth.[66]

Second, the secreted myocardial factor thymosin β4 is necessary for migration of EPDCs into the myocardium to form the coronary vascular plexus.[67–69] In thymosin β4 knockdown hearts, the epicardium was detached and contained nodules of cells expressing endothelial and smooth muscle markers. These cells were deficient within the myocardium. In both embryonic and postnatal heart, thymosin β4 promoted endothelial cell migration and proliferation[70] and initiated the embryonic coronary developmental program and epicardial progenitor cell activation.[71–73] These studies suggest that thymosin β4 is a facilitator of coronary neovascularization and highlight epicardial cells as resident progenitors which, when instructed by thymosin β4, have the capacity to sustain the myocardium after ischemic damage.[72, 74] However, two recent studies showed that thymosin β4 inactivation is

dispensable for embryonic viability and coronary vessel development.[75, 76] Further studies are required to further delineate the role of thymosin β4 in coronary vessel development and postnatal angiogenesis.

Third, normal formation of the coronary vascular plexus requires interaction between GATA4, a transcription factor that is critical for cardiac development and function[77–80], and its cofactor FOG2. FOG2 binds to GATA4 to both positively and negatively regulate transcriptional activity in a context-specific manner.[81, 82] Germline mutation of *Fog2* resulted in severe impairment of coronary vessel development and hypoplasia of the compact myocardium, as well as structural heart defects.[82] Introduction of a point mutation into *Gata4* that blocks interaction with *Fog2* resulted in a similar phenotype.[83] Cardiomyocyte-specific deletion of *Fog2* or disruption of Fog2-Gata4 interaction recapitulated the defected coronary vessel formation during embryonic development.[84] Fog2-Gata4 interaction promoted expression of proangiogenesis factors and suppressed expression of angiogenesis inhibitors.[84, 85]

Besides these major players, there are additional molecular signals that control epicardial integrity, coronary angiogenesis, and compact myocardium formation. It is becoming increasingly clear that coronary angiogenesis in both development and regeneration depends on an orchestrated crosstalk between the epicardium, coronary VECs and the myocardium in part through secreted growth factors. This complex epicardium-myocardium communication and its impact on the coronary angiogenesis has been described in detail in other previous reviewers.[22, 48, 86]

Coronary VECs from SV: vein-to-artery reprogramming

If epicardial cells contribute to at most a small fraction of coronary VECs in mice, then what is the ontogeny of the majority of coronary VECs? For the initial coronary vessels emerging at atrioventricular groove, the closest connecting structures are the epicardium and the sinus venosus (Figure 2A). The SV is a transient stucture in cardiovascular development that receives venous blood from the vitelline vein, umbilical vein and common cardinal vein and returns it to the atrium.[87] As the heart matures, the SV becomes integrated into the right atrium as the coronary sinus. Over a century ago, the coronary vascular network was reported to develop in the human embryo as a circulatory system with connections to the SV. [88] Later, Bennett studied the development of coronary vessels in swine embryos and found that coronary vessels arose from "the descending pocket of the SV".[89] His visionary analysis showed that venous outpockets of the SV penetrated the connective tissue between atrium and SV, and these venous sprouts formed capillary networks in the subepicardial space that shared a continuous endothelial lining with the SV.[89] Poelmann and colleagues investigated the origin of coronary VECs by detailed description of quail coronary development, and through the study of chimeras formed by quail tissue transplantation into chick embryos. [87, 90] These studies showed that pure proepicardial transplants did not contribute to coronary VECs formation, whereas transplants containing or exclusively composed of liver sinusoids did. More importantly, these studies showed that the first coronary VECs communicated with the heart at the SV. The data were interpreted to suggest ingrowth of coronary VECs

into the SV.[87, 90] However, this early work on the contributions of SV to coronary vessel development remained peripheral while most attention focused on the role of proepicardium.

Recent seminal work from Red-Horse, Krasnow, and colleagues has brought to the fore the importance of vascular sprouting from the SV in coronary vessel development.[29] Single cell labeling and clonal analysis were used to show a lineage relationship between SV endothelium and coronary VECs, including those of intramyocardial coronary arteries. Since SV endothelium is initially venous, the study proposed a novel process in which sprouting angiogenesis is accompanied by endothelial de-differentiation into progenitors capable of forming an entirely new vascular bed containing arteries, capillaries, and veins (Figure 4). Understanding the mechanisms that underlie vein-to-artery reprogramming might unlock numerous therapeutic opportunities by improving the durability of revascularization performed using venous grafts and by informing regenerative approaches to revascularization.[91]

The model of venous cell "reprogramming" into arteries received different views afterwards. [92] Because there are no tools currently available to selectively label sinus venosus endothelial cells, the Red-Horse et al. study used a vascular endothelial cell specific Cre (VE-Cadherin-CreERT2)[93] in combination with clonal analysis to establish the lineage relationship between sinus venosus ECs and coronary VECs.[29] However, this Cre labels many different cell types, including endocardial cells, angioblasts, and hematopoietic cells.[92] In addition, SV endothelial cell dedifferentiation and subsequent redifferentiation into coronary artery VECs was challenged by the notion that a subset of SV endothelial cells already express the arterial marker Notch1, underlining the plasticity of the embryonic microvascular endothelial cells rather than a dedifferentiation/redifferentiation program.[94, 95] SV endothelial cell heterogeneity was reinforced by a recent study showing that SV contains both apelin recepetor (APJ)-positive and -negative ECs.[96] The immature APJ-negative subset constituted the population that emigrated from the sinus venosus, and under the influence of angiopoietin-1 differentiated into coronary vein endothelium. Thus this study proposed selective emigration and differentiation of an immature, APJ-negative SV EC population, rather than dedifferentiation/redifferentiation of mature venous endothelium.[96]

We developed a novel reagent, Apln-CreER, that selectively labels subepicardial endothelial precursors but not endocardial cells. Using this reagent, we demonstrated that subepicardial endothelial precursors form most coronary VECs, including most of the intramyocardial coronary arteries.[97] This conclusion was functionally reinforced by a recent study that showed that GATA factors (Gata4 and 6) regulate coronary plexus development by recruiting endothelial cells to the sub-epicardium, and that loss of subepicardial vessels in hearts lacking epicardial GATA4 and GATA6 disrupts intramyocardial endothelial cell formation.[39] A subset of subepicardial vessels remained in the subepicardial space and became coronary veins.

What is the lineage relationship between subepicardial endothelial cell precursors labeled by Apln-CreER and sinus venosus endothelial cells? We used in vitro explant assays to develop supportive data that subepicardial endothelial precursors derive from sinus venosus endothelial cells by vascular sprouting,[97] as suggested by Red-Horse, Krasnow, and

colleagues. However, as pointed out previously, direct, quantitative in vivo data linking subepicardial endothelial cells labeled by Apln-CreER to sinus venosus is currently lacking. [98] A suitable SV-specific promoter driven Cre line is needed to more firmly establish this lineage relationship.

An unexpected result of the Tian et al. study was that Apln-CreER did not efficiently label coronary VECs within the ventricular septum, when it did efficiently label those in the ventricular free walls.[97] This result indicates that coronary VECs in ventricular septum are not descendants from subepicardial endothelial cell precursors. Clonal analysis and direct lineage tracing of endocardial cells indicated that endocardial cells contribute to these septum vessels (Figure 4B,D,F).[29, 99, 100] Blood islands budding from endocardium are found most abundantly at E12.5-E13.5 on the ventral surface of the developing heart, overlying the ventricular septum. This location coincides with a point where subepicardial vessels extending from the dorsal surface of the heart are least likely to reach by angiogenic sprouting. These blood islands have been suggested to be sites of endocardium-to-vessel formation,[29, 97] and potentially this process may happen more frequently within the ventricular septum and lead to the formation of most coronary VECs within the ventricular septum. Thus the embryonic coronary vessels seem to be patterned in two distinctive compartments that irrigate the ventricular free wall and ventricular septum, respectively (Figure 4).

Taken together, recent mouse embryonic lineage tracing studies suggest that, instead of epicardium, SV is the origin for the majority of coronary vessels, including the intramyocardial coronary arteries. However, this conclusion is inferred from a combination of clonal lineage tracing and in vitro transplantation studies, and direct evidence or a quantitative estimate of the fraction of coronary VECs dervied by this mechanism, is currently lacking. It should be kept in mind that the SV is a transient structure named by its anatomic location, and it receives endothelial cells from other organs or tissues, including proepicardium[43] and liver sinusoid VECs.[90] SV is also partly covered by myocardium and the endothelium of sinsus venosus closely connected with and in continuity with atrium endocardium, so it may be technically challenging to dissect clearly the boundary between migrating endocardial cells of SV and atrium endocardium (discussed in the following section). Future studies are needed to find a more specific marker that could be used to label SV endocardium specifically and perform direct lineage tracing in vivo.

Currently, the molecular regulation of the coronary vessel formation by SV sprouting, dedifferentiation, and redifferentiation are incompletely understood. Recent studies identified VEGFC and angiopoietin-1 (Ang1) as key paracrine signals that promote SV sprouting. VEGFC, expressed from epicardium, regulates the formation and growth of coronary sprouts from sinus venosus.[101] Similarly, cardiomyocyte-secreted Ang1 promoted the sprouting of APJ-negative ECs from SV.[96] Interestingly, Ang1 stimulated the differentiation of immature endothelial sprouts into coronary vein endothelia, and cardiomyocyte-specific Ang1 deletion selectively impaired formation of subepicardial veins but not intramyocardial coronary arteries. These findings demonstrate that distinct signaling mechnisms promote formation of coronary arteries and coronary veins. Improved

understanding of the process of SV sprouting and coronary artery and vein formation could significantly advance efforts in myocardial regeneration and revascularization.

Coronary VECs from endocardium

Endocardium is the specialized endothelium that lines the inner surfaces of the heart. During early heart development, endocardial cells overlying the cardiac cushions delaminate and form mesenchymal cells through epithelial to mesenchymal transition (EMT). These mesenchymal cells develop into the interstitial cells of the heart valves.[22] This endocardial EMT is critical to cushion formation, and disruption of key molecular signaling impairs EMT and results in cardiac valve malformation.[102–106] Since endocardium represents a large reservoir of endothelium distributed along the luminal surface of the ventricular myocardium, it would be reasonable to hypothesize that a similar delamination and migration process could yield coronary VECs.

Recently, Cre alleles based on *Nfatc1* regulatory elements have permitted lineage tracing of endocardial cells. Wu et al. used constitutive Nfatc1-Cre to assess endocardial contributions to the coronary vasculature.[99] This study was interpreted to show that endocardial cells overlying the ventricular trabeculae contribute to the majority of the intramyocardial coronary arteries of the embryonic ventricular wall. Sub-epicardial vessels, primarily coronary veins, were infrequently labeled by Nfatc1-Cre, which was interpreted to indicate that these arise from a different developmental mechanism (e.g., from SV endothelial cells). The Nfatc1-Cre lineage tracing data was supported by functional data obtained through manipulation of VEGFA signaling. Myocardium-specific VEGFA knockout or Nfatc1-Cre-driven endocardial VEGFR2 knockout showed significant reduction in intramyocardial coronary arteries but had less effect on subepicardial coronary veins.[99] Based on these data, this study proposed a new model of coronary vascular development in which a subset of ventricular endocardial cells invade the myocardial wall from the luminal (endocardial) surface, migrate towards the outer (epicardial) surface, and differentiate into intramyocardial coronary artery endothelial cells (Figure 5 left panel). One important concept advanced by this model is that endocardial cells are not only an endothelial sheet that covers the ventricular myocardium; in addition, endocardium also contains progenitor cells that migrate into compact myocardium and trans-differentiate into coronary VECs.[99] A second important possibility raised by the study is that coronary vessels may be developmentally heterogeneous, with intramyocardial coronary arteries formed primarily from endocardial sources and subepicardial veins originating from alternative precursor populations (Figure 5 left panel).[99]

The proposed model conflicts with results from other labs using different experimental approaches. First, retroviral analysis of chicken embryonic heart development showed that VECs have a different clonal origin than endocardial cells.[27] Second, analysis of single cell labeling by VE-cadherin-CreERT2 showed that many VEC clones were related to SV endothelial cells, whereas only rare VEC clones were related to endocardial cells.[29, 97] There is indeed a big gap between these studies, as Wu et al. found that the majority of coronary arteries (as high as 81%) originated from ventricular endocardium, but Red-Horse et al. found that very few coronary VECs were likely to arise by this mechanism.[29] We

showed that Apln-CreER initially labels sub-epicardial endothelial precursors, and that these give rise to over 90% of coronary VECs, including the intramyocardial subset.[97, 100] These differences can perhaps be reconciled by reviewing the caveats of Cre-loxP lineage tracing, namely that interpretation hinges upon reliably excluding Cre activity in the target cell type or its other potential precursors. Wu et al. used a constitutive Nfatc1-Cre, which makes careful exclusion of Cre expression in all potential VEC sources other than endocardial cells difficult. Of particular interest is the SV, which is adjacent to atrial endocardium. Wu et al. present expression data suggesting that Nfatc1-Cre would not label SV, but this is difficult to exclude particularly using a constitutive Cre allele, and it remains possible that a portion of SV was labeled by Nfatc1-Cre (Figure 2A, B). Additionally, *Nfatc1* was reported to be expressed in coronary VECs,[107] which would prevent accurate assessment of ventricle endocardial contribution to coronary VECs.

Recently, Chen et al. re-investigated this controversial issue by comparing lineage tracing results from Nfatc1-Cre, Sema3d-Cre, and Apj-CreER, the latter a bacterial artificial chromosome transgenic line in which regulatory elements of *Apj* drive CreERT2.[108] *Apj* is selectively expressed in sinus venosus but not epicardium nor endocardium. Nfatc1-Cre labeled most coronary vessels in ventricular septum (>80%) and a significant portion of coronary vessels in ventricle wall (eg. 65% in left ventricle), consistent with the results of Wu et al.[99] Sema3d-Cre, expressed in epicardium, labeled a minority of coronary vessels (9–11%), as previously reported.[43] Apj-CreER significantly labeled SV and contributed to a substantial fraction (40%) of coronary vessels in left and right ventricles, but very few (<5%) of the vessels within the ventricular septum (Figure 5 middle panel).[108] Unfortunately, this study was unable to assess overlapping labeling between the Cre alleles and so was unable to resolve the relative contributions of endocardium versus SV to coronary VECs (Figure 5 middle panel). Nfatc1-Cre labeling of 65–81% of VECs[108,99] may overestimate the endocardial contribution as a result of partial labeling of SV using constitutive Nfatc1-Cre or direct Cre activation in coronary VECs.[107] The 40% coronary VECs labeled by Apj-CreER in the ventricle wall[96] may under-estimate the contribution of SV to coronary vessels, due to incomplete Cre activation by tamoxifen or failure of the transgenic line to fully recapitulate endogenous APJ expression in SV. High labeling (>90%) by Apln-CreER in the ventricle wall may over-estimate the contribution of SV to coronary vessels[97] (Figure 5 right panel), as Apln-CreER might label a subset of endocardium-derived VECs in addition to subepicardial VECs. Taken together, endocardium appears to be the main source of coronary VECs in the ventricular septum, and endocardium and SV are likely to be the main sources for coronary VECs in the embryonic ventricle wall. Current tools and analyses have been unable to definitively determine the relative contribution of each source to ventricular wall VECs, and resolution of this important question awaits development of improved lineage tracing tools and potentially the use of dual labeling strategies. At least some developing coronary vessels arise from endocardial progenitors, raising the tantalizing possibility that endocardium could be a source for coronary VECs during postnatal and adult heart growth, and could be recruited for therapeutic cardiac regeneration.

Postnatal coronary vessels: endocardium revisited

Until recently, most studies on the origins of the coronary vessels focused on the initial stages of embryonic coronary vascular development, based on the assumption that later angiogenic expansion of the coronary vascular tree occurred by outgrowth of these initial coronary vessels. Technical limitations, namely the lack of tools to selectively label either endocardial or coronary vascular endothelial cells at a chosen developmental stage, precluded rigorous testing of this assumption. We took advantage of the genetic regulatory elements of genes selectively expressed in endocardium but not VECs (*Nfatc1*) or in VECs but not endocardium (*Apln*), to achieve selective genetic labeling using the inducible Cre/loxP strategy.[100] To achieve temporally regulated pulse labeling, tamoxifen-activated CreERT2 fusion protein[109] was used instead of constitutive Cre. Combining these technologies yielded new genetic reagents to track the fate of endocardial or vascular endothelial cells labeled at a specific developmental stage.

Apln-CreER, induced by a single dose of tamoxifen at the earliest stages of coronary VEC formation (E10.5), labeled nearly all coronary VECs at late gestation (E15.5) in the ventricular walls but did not label endocardium.[97] Building on this observation, we tested the assumption that the adult coronary vascular tree forms by outgrowth of embryonic coronary vessels.[100] In postnatal heart, most of the coronary VECs of the outer myocardial wall expressed the lineage mark made by Apln-CreER, indicating that these VECs originate from the fetal coronary tree (red staining, Figure 6, left panel). Surprisingly, a large portion of the coronary VECs in the inner myocardial wall of the postnatal heart did not express this lineage mark (green pseudo-color, Figure 6, right panel), suggesting that a substantial fraction of postnatal coronary VECs arise de novo, rather than by angiogenic expansion of pre-existing fetal coronary vessels. This result was independently verified by a different inducible Cre line (Fabp4-CreER), which like Apln-CreER selectively labeled fetal coronary vessels but not endocardium.[110] To test the hypothesis that these de novo postnatal coronary VECs of the inner myocardial wall originated from endocardial progenitors, we used Nfatc1-CreERT2 (activated at E8.5) to track the fate of fetal endocardial cells in the postnatal heart. We found that endocardium formed coronary VECs de novo in the postnatal heart.[100] Overall, these experiments show that coronary VECs continue to be formed de novo in the postnatal heart. Furthermore, the postnatal endocardium is not a static lining of the myocardium, but rather a dynamic source of these de novo formed postnatal coronary VECs.

How could a single layer of endocardium form such a large amount of vascular plexus at the core of a heart? Previous work in the embryonic heart suggested that endocardial cells invade the myocardium in response to high VEGFA expression in the ventricle wall, proliferate, and undergo angiogenic expansion to form the intramyocardial vascular plexus (Figure 5 left panel).[99] Our recent study[100] supported an alternative vessel formation model in the neonatal heart ventricular free walls. To accommodate the acute increase in wall stress that occurs due to the switch from fetal to postnatal circulations, trabecular myocardium rapidly coalesces with the inner portion of the compact myocardium, resulting in rapid expansion of compact myocardium. During this process of trabecular "compaction", the endocardial cells on the surface of trabeculae become trapped within the compacting

myocardium. In response to hypoxia and their new intramyocardial environment, the trapped endocardial progenitors rapidly form the vessels that supply the newly established compact myocardium of the inner myocardial wall.[100] Similar trabecular compaction also occurs earlier in development during formation of the ventricular septum. Like the inner myocardial wall, the coronary VECs of the ventricular septum are also largely derived from endocardium (Figure 4B,D,F, VS). We describe the VECs that originate from the endocardium as the 2nd coronary vascular population, which supplies blood to the core of the heart (the inner myocardial wall and the ventricular septum). In this model, trabeculae are reservoirs of myocardium that are rapidly deployed to increase compact myocardial mass. Intrinsic to these myocardial reservoirs are endocardial progenitors which will expand to form the vasculature that will supply the newly formed myocardium. Defining the molecular mechanisms that regulate trabecular compaction and the conversion of endocardial cells to coronary VECs will be important areas for future study.[111]

This trapping and trans-differentiation model not only represents a new mechanism for vessel formation, but also provide critical information for the pathogenesis of congenital heart diseases such as non-compaction cardiomyopathy. The model may also provide insights on the development of coronary artery anomalies in pulmonary atresia with intact septum, where fistulae between the ventricular chamber and the coronary circulation are frequently observed. There is evidence of Thebesian vessels in developing heart: the vessels directly connect between coronary arteries and chambers of the heart.[112] Interestingly, in the event of gradual closure of the orifices of the coronary arteries, the Thebesian vessels can supply the heart muscle with sufficient blood to enable it to maintain an efficient circulation.[113] It will be important to understand the genetic, epigenetic, and signaling mechanisms that links coronary vessel formation with trabecular coalescence in normal development and in congenital heart disease. Unveiling the endogenous mechanisms that permit rapid development of a functional vascular supply from endocardial progenitors likewise has direct implications for both congenital heart diseases and therapeutic cardiac regenerative approaches to coronary artery disease.

Coronary orifice and truncal/stem origin

In some congenital heart diseases, the coronary stems may arise at abnormal sites, which could cause serious consequences from myocardial ischemia to sudden death.[114, 115] In an extreme form, anomalous origin of the left coronary artery from the pulmonary artery (ALCAPA), infants present with myocardial ischemia, left ventricular dysfunction, mitral regurgitation,[116] and the mortality rate is high if left untreated.[117] More clear understanding of the developmental mechanisms that govern how coronary artery stems form and anastomose to the nascent coronary vascular plexus is a fascinating question in developmental biology with significant ramifications for congenital heart disease. Moreover, knowledge of the molecular pathways that guide coronary stem connection would also have implications for regenerative approaches to myocardial revascularization in the setting of coronary artery disease.

The nascent coronary plexus formed during early heart development initially develops in the absence of effective perfusion. At about E13.5-E14.5 of murine gestation, the nascent plexus

connects to the aorta and subsequently provides nutrients and oxygen to the heart. It was once believed that coronary arteries grow out of the aorta by sprouting angiogenesis.[89, 118] Elegant studies on avian embryos questioned this long-held belief and provided a reverse model in which the coronary stem and orifice forms by coalescence and remodeling of a pre-existing peritruncal coronary plexus. By staining of endothelial cells in quail embryos or ink injection of chick embryos, several groups reached the novel conclusion that the proximal coronary arteries did not grow outward from the aorta as commonly believed. Instead peritruncal capillaries encircle the outflow tract and grow into the aorta.[119–121] This impression was reinforced by quail-to-chicken tissue transplantation assays, in which quail rather than chick endothelial cells formed the endothelial cells of the coronary stems.[90] Mouse genetic lineage tracing studies verified this in-growth model.[122] In spite of these advances, the origin remain largely undefined. Recent lineage tracing studies using Apj-CreER and Nfatc1-CreER identified at least two different sources of these peritruncal coronary VECs: SV and endocardium.[123] The possibility of angiogenic sprouting from the aorta has also been re-visited,[123] although direct evidence by specific lineage tracing of aortic endothelium is needed to provide more definitive evidence.

The signals that guide peritruncal capillaries to penetrate the aorta and form stable coronary stems are beginning to be revealed. At least three different cell types have been implicated. Since EPDCs act as a source of secreted mitogens and exhibit proangiogenic properties,[46, 49, 124] EPDCs were among the first cell lineage investigated. Studies in chicken embryos showed that invasion of peritruncal vessels into the aorta and the formation of coronary orifices and coronary artery stems were spatially and temporally closely associated with apoptosis in the surrounding myocardium.[125] When proepicardial outgrowth was delayed, apoptosis in the outflow tract diminished. Further studies revealed that EPDCs produce Fas ligand, which induces apoptosis at sites of coronary ingrowth. These EPDCs not only influence myocardial development and vascularization of compact myocardium, but also induce apoptosis in the peritruncal region during the time window when peritruncal coronary vessels connect to the aorta.[126, 127] Indeed, delay of proepicardial outgrowth malfunction caused defective myocardial vascularization and absent coronary orifices. Although EPDCs contribute to few coronary VECs, the majority of coronary smooth muscle cells arise from EPDCs.[14, 69, 128] The presence of EPDCs in the orifice region may provide possible cues that guide ingrowth and subsequent persistence of the proximal coronary artery.

Preotic neural crest cells are a second lineage that influences coronary stem and orifice formation. These cells preferentially distribute in the conotruncal region and differentiate into coronary smooth muscle cells. Ablation of preotic neural crest or disruption of endothelial signalling caused abnormal coronary orifice formation.[129]

A third cell lineage that regulates coronary stem and orifice formation was recently discovered.[123] With the aorta, patches of cardiomyocytes were identified at stem sites. These intra-aortic cardiomyocytes were critical for normal formation of the coronary stems and orifices. Interestingly, in heart malformations involving outflow tract rotation defects, abnormal coronary stem implantation correlated with misplacement of these intra-aortic cardiomyocytes.

Understanding the regional signaling that governs the establishment, penetration, maintenance and growth of these two coronary artery stems would shed new light on the molecular defects that cause congenitally misplaced coronary artery stems, and may also aid in guiding or generating coronary arteries in therapies for coronary artery diseases.

Remaining challenges and future directions

Recent work has significantly advanced our understanding of the cellular origins and developmental program that regulates development of the coronary vessels. Nevertheless, major questions remain that need to be answered. What is the relative contribution of SV and ventricular endocardium to the free wall coronary vasculature, particularly the intramyocardial coronary arteries? What signals regulate SV endothelial cell outgrowth and lineage conversion to arterial VECs? What signals stimulate endocardial cells to transdifferentiate into VECs in the fetal interventricular septum or in the postnatal inner myocardial wall? What is the role of endocardium-myocardium signaling in coronary vessel formation? Does formation of vessels during coalescence of trabecular myocardium itself initiate or facilitate the compaction process? Does endothelial or endocardial plasticity exhibited in fetal life extent postnatally, during physiological growth or pathophysiological conditions? Can they be re-awakened by therapeutic stimulation? Answering these questions require new genetic tools as well as alternative methods and experiments that do not hinge on current Cre-loxP systems. Ultimately, a more complete understanding of the developmental program of coronary arteries will provide new opportunities for therapeutic cardiac revascularization and regeneration.

Acknowledgments

Non-standard Abbreviations and Acronyms

CA	coronary artery
EC	endothelial cell
VEC	vascular endothelial cell
SV	sinus venosus
PE	proepicardium
EMT	epithelial to mesenchymal transition
EPDC	epicardium-derived cell
MI	myocardial infarction

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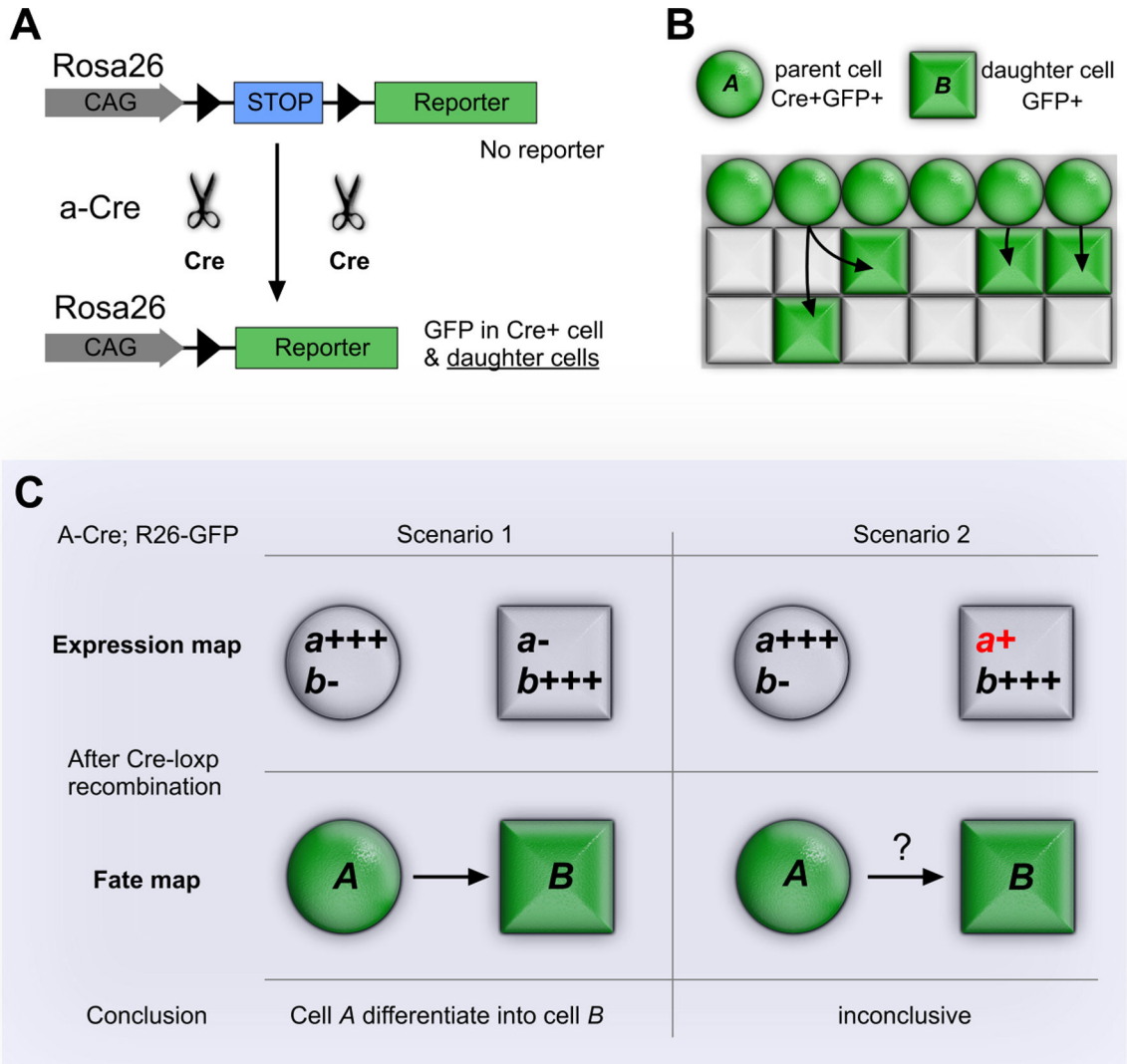
Figure 1. Cre-loxP mediated genetic lineage tracing system.

A, Cre-loxP mediated recombination for lineage tracing is heritable and irreversible. In Cre expressing cells (eg. cell *A*), the loxP-flanked transcriptional stop cassette is removed, permitting reporter (e.g. GFP) expression in cell *A* and its descendants. Cre is driven by promoter a, which is strong in cell *A* but absent in cell *B*. Rosa26-loxp-stop-loxp-GFP (R26-GFP) is the Cre reporter line. **B,** Schematic figure showing cell *A* (Cre+) migrate and differentiate into cell *B* (Cre-). *A* and *B* cells both express lineage tracing marker GFP. **C,** Correct interpretation of lineage tracing data hinges on the data supporting negative Cre expression in Cell *B* or its non-*A* ancestors.
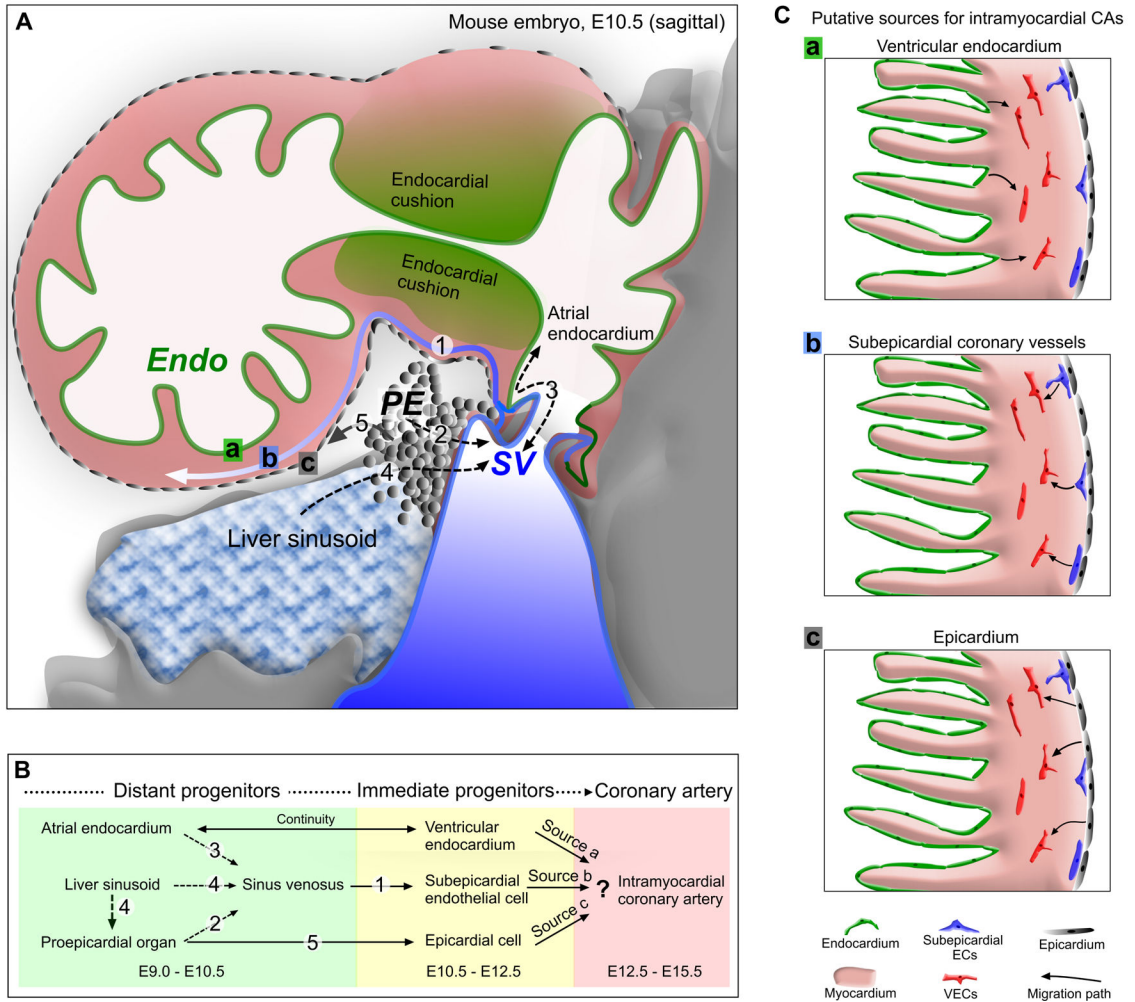
Figure 2. Formation of the nascent coronary vessel plexus in the developing heart.
A and B, The three major sources of coronary vessels, the proepicardium (PE), sinus venosus (SV) and endocardium (Endo) are intimately associated with each other during heart development. The PE is a transient structure (grey) that wedged into the atrioventricular groove between liver sinusoids and SV, and eventually gives rise to the epicardium covering the heart. The SV (blue) is the venous inflow tract. Venous cells from SV sprout onto the heart and produce subepicardial coronary vessels. The endocardium (green) lines the heart lumen. Black dashed arrows denote movement from one compartment to another, potentially complicating lineage-tracing experiments. Numbers _in B correspond to those in A showing location of migration events. **C,** Three putative sources for intramyocardial coronary arteries (CAs) in the developing heart. Arrows indicate corresponding migration path.
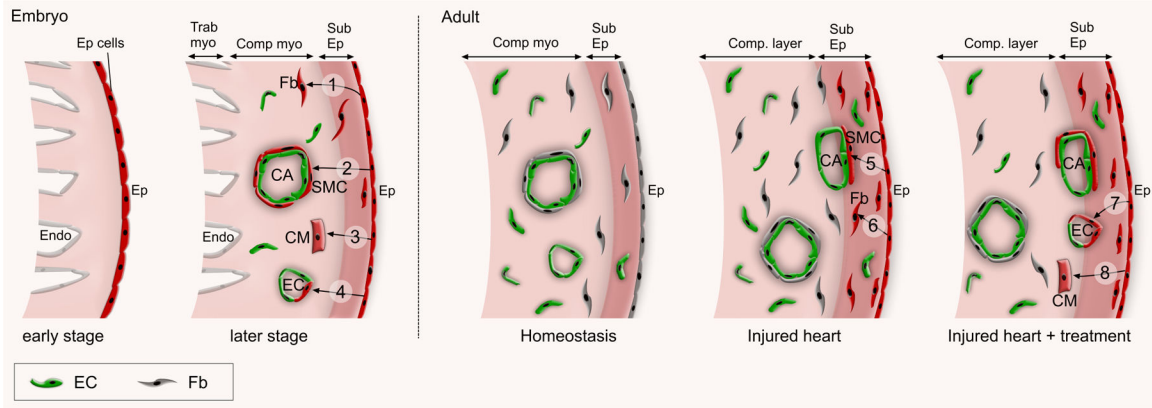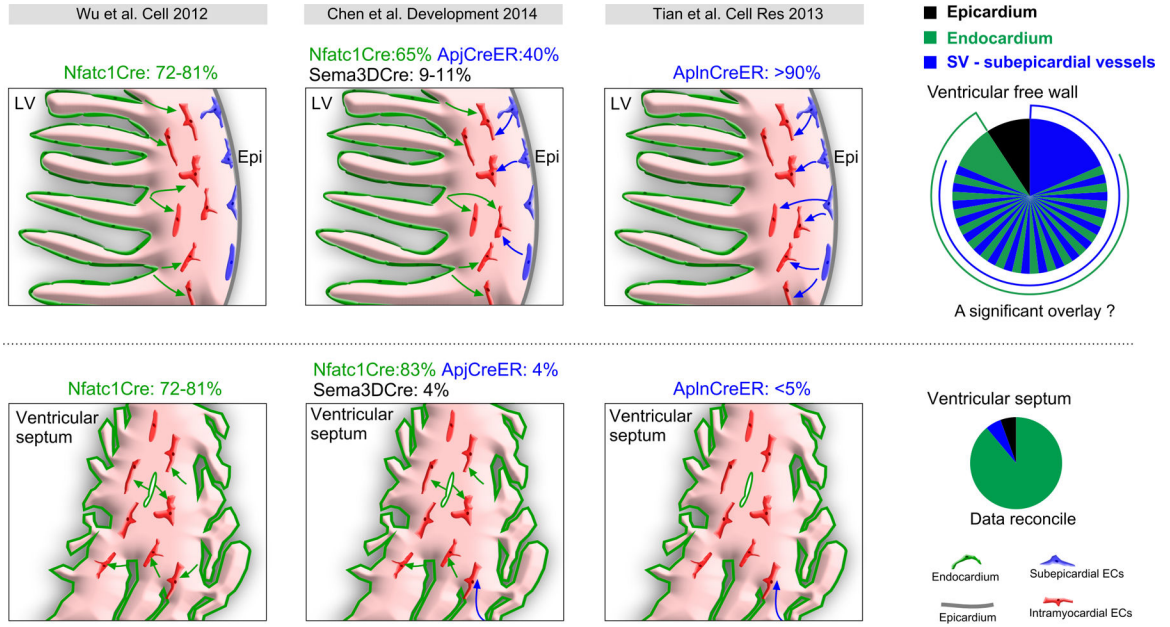
Figure 3. Epicardial contribution to developing and adult heart.

In early embryonic stage, epicardial cells form an epithelial sheet that covers the heart. At later embryonic stages, epicardial cells (Ep cells) form mesenchymal epicardium-derived cells (EPDCs) by EMT. EPDCs appear in the subepicardial layer (Sub Ep) and migrate into compact myocardium (Comp myo), where they differentiated into fibroblasts (Fb, 1), smooth muscle cells (SMC, 2), cardiomyocytes (CM, 3) and endothelial cells (EC, 4). In adult heart under normal homeostatic conditions, most epicardial cells remain quiescent (grey color). MI activates the embryonic program (red) and these epicardial cells differentiate into SMC (5) and Fb (6), but rarely if at all to CMs or ECs. Paracrine factors such as modRNA encoding *Vegfa* or thymosin β4 stimulates a subset of EPDCs to differentiate into EC (7) or CM (8) lineages, respectively in the post MI hearts.
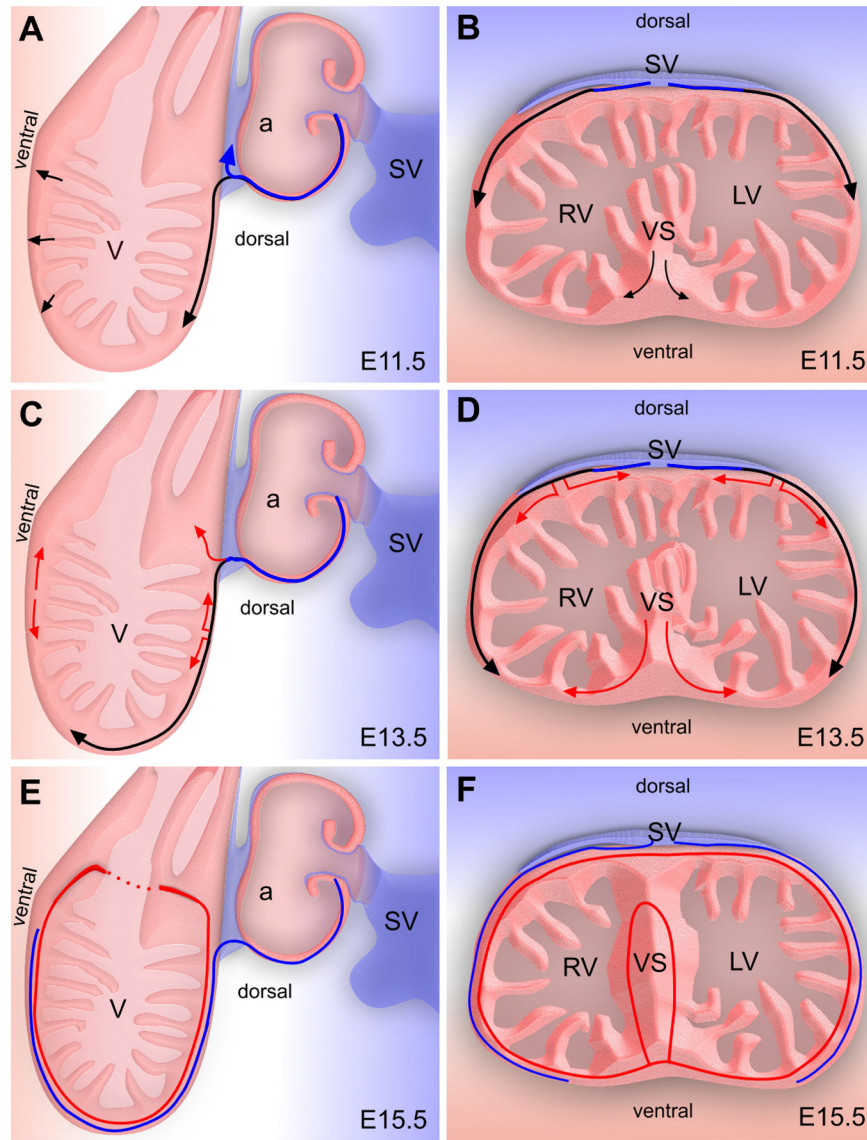
Figure 4. Coronary vessel formation in the ventricle wall and ventricular septum.
A, C, E, Sagittal view of developing heart; **B, D, F,** Cross-sectional view of the developing heart. Venous cells sprout from SV and dedifferentiate into undifferentiated subepicardial ECs (black) in the dorsal side of heart. As the heart continues to develop, these subepicardial ECs penetrate the myocardial wall and differentiate into arterial ECs (red), while the remaining subepicardial ECs redifferentiate into coronary veins (blue). On the ventral side of heart and in the ventricular septum, coronary ECs arise from endocardium during trabecular compaction (black) and sprout to form a coronary plexus that connects to the coronary vessels in the ventricle wall. V, ventricle; a, atrium; SV, sinus venosus; VS, ventricular septum.

Figure 5. Contribution of SV, endocardium and epicardium to coronary vessels in the developing hearts.

Comparison of quantitative measurements from the indicated references of the contribution of different sources to coronary VECs. Upper panels show the ventricular free walls, and the lower panels show the ventricular septum. Endocardium and SV-subepicardial endothelial progenitors were the two major sources, with endocardium making the predominant contribution to VECs in the ventricular septum. The relative contribution of SV/subepicardial endothelial progenitors and endocardium to coronary vessels in the ventricular free walls is currently a matter of debate. Resolution of this uncertainty requires new genetic tools that will distinguish endcoardium from SV/subepicardidal vessels.
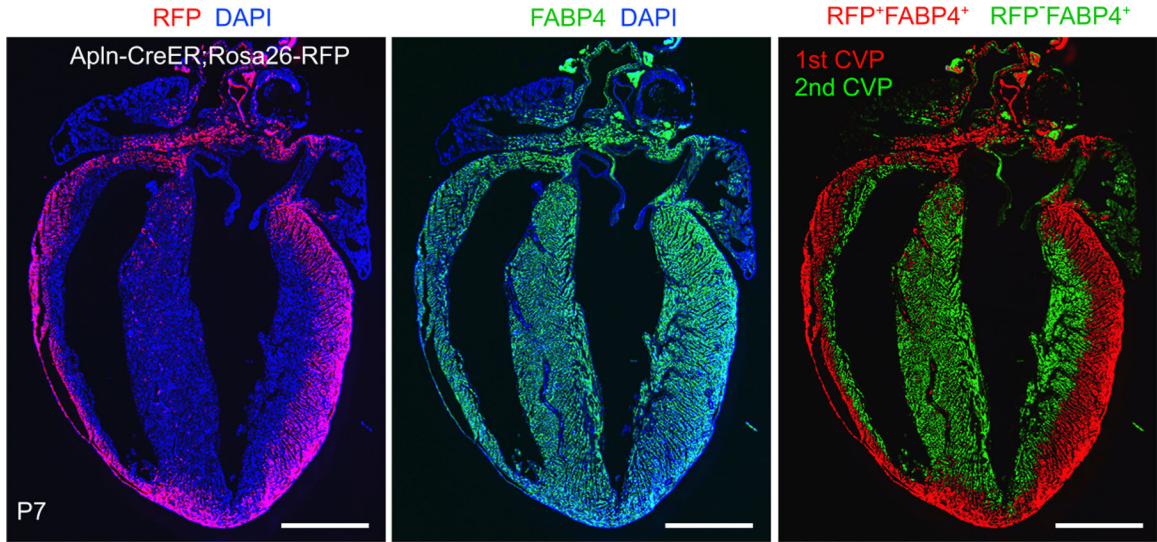
Figure 6. 1st and 2nd coronary vascular population in neonatal heart.

Apln-CreER, induced at E10.5, labeled fetal coronary VECs with Cre-activated RFP expression. In the P7 postnatal heart, VECs were present throughout the myocardial wall, as demonstrated by immunostaining for the VEC-selective marker FABP4 (middle panel), but the RFP lineage tracer of fetal VECs was only observed in the outer myocardial wall (left panel). The FABP4^{+}RFP[+] VECs, descended from fetal VECs, are designated the 1st coronary vascular population (CVP; red pseudocolor, right panel) while the remaining FABP4^{+}RFP[−] VECs, formed de novo in the postnatal heart, are designated the 2nd CVP (green pseudocolor, right panel). Bar = 1 mm.

Table 1.

Lineage tracing for intramyocardial coronary VECs in embryonic ventricle wall

Mouse line	Targeting strategy	Constitutive / inducible	Origins / sources	% VECs labeled	Note	References
WT280Cre, YAC	Transgenic	Constitutive	Proepicardium	14%	Labels most epicardium	(Wilm et al. 2005)
Wt1/IRES/GFP-Cre, BAC	Transgenic (RP23-266M16)	Constitutive	Proepicardium	Few	Labels most epicardium	(Wessels et al. 2012)
Wt1(RP23–8C14)-Cre	Transgenic (RP23–8C14)	Constitutive	Proepicardium	Few	Labels most epicardium	(Kolander et al. 2014)
Wt1-GFPCre	Knockin (ATG)	Constitutive	Proepicardium	Few	Labels most epicardium; caution regarding non-epicardial labeling under some conditions.	(Zhou et al. 2008, 2012; Rudat and Kispert 2012).
Wt1-CreERT2	Knockin (ATG)	Inducible	Proepicardium	Few	Labels most epicardium; caution regarding non-epicardial labeling under some conditions.	(Zhou et al. 2008, 2012)
Tbx18-Cre	Knockin (5' UTR)	Constitutive	Proepicardium	None	Labels most epicardium	(Cai et al. 2009)
Tcf21 MerCreMer	Knockin (ATG)	Inducible	Epicardium	None	Labels most epicardium	(Asha et al. 2011)
Tbx18-CreERT2	Knockin (ATG)	Inducible	Epicardium	None	Labels most epicardium	(Moore-Morris et al. 2014)
VE-Cad CreERT2	Transgenic	Inducible	Sinus venosus	Most	Clonal analysis	(Red-Horse et al. 2010)
Scx-GFPCre, BAC	Transgenic	Constitutive	Proepicardium	ND[*](1)	Labels part of SV	(Katz et al. 2012)
Sema3D-Cre	Knockin (ATG)	Constitutive	Proepicardium	ND[*](2)	Labels part of SV	(Katz et al. 2012)
Nfatc1-IRES-Cre	Knockin (3' UTR)	Constitutive	Endocardium	81%	Labels part of SV	(Wu et al. 2012)
Nfatc1-nrtTA, BAC	Transgenic	Inducible	Endocardium	Most	Clonal analysis	(Wu et al. 2012)
Nfatc1-CreERT2	Knockin (ATG)	Inducible	Endocardium	Few	Labels most 2nd CVP	(Tian et al. 2014)
Apj-CreERT2	Transgenic	Inducible	Sinus venosus	40% LV	Labels part of SV	(Chen et al. 2014)
Apln-CreERT2	Knockin (ATG)	Inducible	Subepicardial VECs	>90%	Labels subepicardial vessels	(Tian et al. 2013)

Few means the minority, as there is no quantitative data available from published papers.

Most means the majority of clones show their relationship by clonal analysis

ATG means the Cre or CreERT2 cDNA cassette was knocked into the endogenous start codon.

3'UTR means the Cre or CreERT2 cDNA cassette is inserted within the 3' untranslated region as IRES following endogenous gene.

ND, not determined; LV, left ventricle.

[*] (1) 24.3% or (2) 6.9% of Cre-labeled cells were coronary VECs.

Table 2.

Genetically Engineered Mice With Coronary or Epicardial Phenotypes

Gene	Expression			Model	Coronary plexus	Comp. Myo.	Additional Co/Epi Phenotypes	References
	Ep	**M**	**En**					
RXRa	X	X		MLC2vcre	Nl	Nl	no phenotype	(Chen et al., 1998)
				Gata5Cre	abnormal CA branching	thin	abnormal Wnt/b-catenin signaling	(Merki et al., 2005)
WT1	X			KO	absent	thin	decreased SEMC, epicardium fails to envelope heart	(Moore et al., 1999)
Ntrk2	X			KO	impaired	Nl	normal epicardium	(Wagner et al., 2005)
BDNF				KO	normal, but EC apoptosis	Nl	hemmorhage around subepicardial vessels	(Donovan et al., 2000)
EpoR	X		X	KO	impaired	thin	detached epicardium	(Wu et al., 1999) a4
integrin	X			KO	impaired		no epicardium; pericardial hemmorhage	(Yang et al., 1995)
VCAM1		X		KO	premature death	thin	no epicardium; pericardial hemmorhage	(Kwee et al., 1995)
Tbx1	X			KO	disorganized	NI	disordefective vascular plexus remodeling	(Wu et al. 2013)
PDGFrb	X			Gata5Cre	disrupted	thin	disrupted epicardial cell migration	(Mellgren et al. 2008)
Podoplanin	X			KO	abnormal	thin	impaired epicardial adhesion and spreading	(Mahtab et al. 2008)
ALK5	X	X	X	Gata5Cre	defective	thin	aberrant capillary vessel formation	(Sridurongrit et al. 2008)
FGF9	X		X	KO	impaired	thin	abnormal Hedgehog signaling	(Lavine et al., 2006)
FGFR1+2		X		MLC2vCre	impaired	thin	abnormal Hedgehog signaling	(Lavine et al., 2006)
Hedgehog signaling	X	X		Smo, Mlc2v-Cre or Dermo1-Cre	Reduced	Nl	Rudeced coronary veins or intramyocardial arterial vessels	(Lavine et al., 2008)
Thymosin b4		X		MLC2vCre-activated siRNA	Impaired	Nl	left ventricle noncompaction	(Smart et al., 2006)
Gata4	X	X	X	TR-KO	premature death	thin	no proepicardium	(Watt et al., 2004)
				Fog2-Gata4 loss	impaired	thin	epicardium intact, and decreased SEMC	(Crispino et al., 2001)
Fog2	X	X	X	KO	impaired	thin	epicardium intact, decreased SEMC	(Tevosian et al., 2000)
Vegfa		X		Tnnt2-Cre	imapired CAs	thin	epicardium intact, decreased intramyocardial CAs, no defect in coronary veins	(Wu et al., 2012)
VegfR2			X	Nfatc1-Cre	impaired CAs	thin	88% decrease in intramyocardial CAs, 37% decrease in coronary veins	(Wu et al., 2012)
Ang1		X		aMHC-Cre	impaired coronary veins	thin	74% decrease in intramyocardial CAs, 24% decrease in coronary veins	(Arita et al., 2014)
BAF180	X	X	X	KO	defective	NI	impaired EMT and epicardial maturation	(Huang et al., 2008)

Gene	Expression			Model	Coronary plexus	Comp. Myo.	Additional Co/Epi Phenotypes	References
BAF200	X	X	X	KO	defective	thin	reduced intramyocardial coronary vessels	(He et al. 2014)

TR-KO, tetraploid rescued knock out; Fog2-Gata4 loss, loss of interaction between Fog2 and Gata4; SEMC, subepicardial mesenchyme, Ep, epicardium; M, myocardium; En, endocardium; Comp. Myo., compact myocardium; Co/Epi, coronary/epicardium; NI, not indicated.