

**Coronary vessel formation in development and disease: mechanisms and insight
towards therapy**

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Abstract

Formation of new blood vessels after myocardial infarction (MI) is essential for survival of existing and regenerated cardiac tissue. However, the extent of endogenous revascularisation is insufficient and needs to be enhanced. Angiogenic therapy has, to date, been ineffective in clinical trials, betraying a poor understanding of the processes required to form a functional coronary vasculature. In this review, we describe recent advances in our knowledge of coronary vessel formation, with mechanistic insight gleaned from developmental studies. Coronary vessels originate from multiple cellular sources during development and form through a number of distinct and carefully orchestrated processes. Since the ectopic reactivation of developmental programmes has been proposed as a paradigm for regenerative medicine, a complete understanding of these processes is crucial. Further, it is essential that we determine how these processes differ in adulthood and how they might be more closely recapitulated following injury. Such understanding may enable the identification of tractable molecular targets to therapeutically enhance neovascularisation and regeneration of the infarcted heart.

The growing burden of morbidity and mortality resulting from coronary artery disease (CAD) and myocardial infarction (MI) demands the urgent development of novel revascularisation therapies. Coronary artery occlusion inflicts ischaemic damage upon the downstream myocardium, after which blood flow becomes entirely contingent upon the existence of a collateral circulation. However, the extent of collateral growth varies between individuals and largely depends on prior adaptation to accruing stenosis. Emergency reperfusion therapy, by primary percutaneous coronary intervention and pharmacological thrombolysis, can effectively restore coronary blood flow to major epicardial vessels, but ischaemic endothelial damage and the dislodging of thrombotic debris from the primary lesion frequently leads to obstruction of downstream microvasculature. This so-called no-reflow phenomenon affects 30% of MI patients and is associated with poor healing, ventricular remodelling, progression to heart failure and premature death^{1,2}. Therefore, the development of new strategies to revascularise the infarcted heart is necessary.

Despite initially promising results in pre-clinical and phase I clinical studies, even the most potent angiogenic growth factors have proven ineffective, in terms of restoring coronary perfusion and cardiac function, during phase II and III clinical trials^{3,4}, as summarised in **Table 1**. These growth factors, including vascular endothelial growth factor (VEGF)A (primarily VEGFA₁₆₅ isoform)⁵ and fibroblast growth factor (FGF)-2⁶, have well-established roles in

angiogenesis, a term referring to the formation of new blood vessels from existing ones. However, even if effective, expansion of the microvasculature by angiogenesis without accompanying arterial differentiation to re-establish the hierarchical vascular network is unlikely to provide sufficient perfusion. Endogenously stimulated by haemodynamic forces, chiefly shear stress⁷, arteriogenesis has proven a challenging therapeutic target. The various attempts to regenerate the coronary vasculature via endothelial stem/progenitor cell therapy have been similarly unsuccessful⁸. Such disappointing outcomes likely reflect our currently incomplete understanding of which vascular sources and endogenous responses can most productively be pharmacologically leveraged post-MI. Until recently, most studies lacked the tools to distinguish newly-formed from pre-existing vessels, assumed that acute neovascularisation occurred solely via capillary sprouting, and were unable to determine cellular sources and mechanisms of any vessel formation^{9,10}.

In this review, we will discuss recent insights into the cellular sources and molecular mechanisms regulating neovascularisation in the heart, during both development and the response to ischaemic injury. We will specifically focus on the endogenous processes understood to occur in the heart in response to injury, rather than on the plethora of exogenous factors tested for their ability to induce coronary vessel growth. While non-coding RNAs¹¹⁻¹³, various progenitor cell types^{14,15} and extracellular vesicles¹⁶, either unmodified or tailored to deliver angiogenic factors, have shown promise in preclinical studies, they are either untested or have shown limited success in the clinic; moreover, the precise mechanisms underlying any enhanced neovascularisation have not been systematically investigated and are largely undefined. In contrast, emerging insights, for example from genetic lineage tracing and single cell transcriptomic studies are beginning to help us to understand the heart's intrinsic attempts to repair the coronary network and this may reveal novel targets for therapeutically enhancing such sub-optimal responses. Since the developing embryo provides a blueprint for establishing an optimally functioning coronary vasculature, we will first outline the current consensus on the contributory sources, cellular mechanisms and regulatory pathways underlying initial coronary vessel formation and differentiation. However, while injury responses recapitulate certain aspects of development, there are fundamental differences^{17,18}, which must be reconciled for an optimal therapeutic approach. As we gain increasing insights from animal models, such as zebrafish, with intrinsic capacity to regenerate coronary vasculature and myocardium, we will examine these, alongside the non-regenerative mammalian heart, seeking to identify promising approaches for clinical translation.

Establishing the coronary vasculature

The acquisition of coronary vasculature is a relatively new evolutionary trait, found in vertebrates that depend on pulmonary respiration: mammals, birds and reptiles¹⁹. The hierarchical coronary network consists of arteries, arterioles, capillaries, venules and veins²⁰, and a parallel network of lymphatic vessels^{21,22}. Arteries and veins are composed of an inner endothelial lining, a medial layer primarily made up of contractile tissue, and an outermost adventitial layer comprised of connective tissue. Arteries have a thicker medial layer, with more smooth muscle and elastin layers²³. Most cardiomyocytes are surrounded by coronary capillaries^{19,24} and this has facilitated the evolution of a denser myocardium that could not be sufficiently perfused via the chamber lumina. Amphibians, such as newts and salamanders, lack coronary vessels and utilise cutaneous respiration, while coronary vasculature in fish is variable; fast-swimming predatory fish and those in low oxygen environments have an extensive coronary network, while zebrafish possess fewer coronary vessels²⁵. Probably due to its recent evolution, the formation of the coronary vasculature is a rather complicated and unintuitive process, involving multiple sources.

Developmental sources of coronary endothelial cells

Historically, the coronary network was thought to form via angiogenic outgrowth from the aorta²⁶; however, quail-chick transplantation²⁷ and murine genetic lineage tracing²⁸ studies showed that coronary vessels connect into the aorta once a primitive vascular plexus has already formed. This called into question the cellular origin of coronary endothelial cells (CECs) until avian transplantation and retroviral labelling experiments²⁹ led to the theory that the proepicardial organ (PEO), the protrusion of coelomic mesothelium near the septum transversum (ST) and inflow tract, which gives rise to the epicardium, was the main source. This was further corroborated by studies in which PEO ablation/obstruction correlated with failed coronary formation^{30,31}. However, while the extent of PEO CEC contribution continues to be debated today, more precise genetic labelling in mouse suggests that it is extremely limited. Instead, seminal work in 2010 identified the sinus venosus (SV), the venous inflow tract in the embryonic heart, as a major source of CECs³². Further Cre-LoxP lineage tracing studies recognised substantial contributions of the ventricular endocardium, both during the embryonic^{33,34} and perinatal³⁵ periods, to CECs of the coronary vasculature (summarised in **Table 2**).

The sinus venosus

Both fate mapping and clonal analyses have demonstrated that, during embryonic development, endothelial cells from the SV sprout over the dorsal side of the heart and penetrate the ventricles from the outside-in. Although venous in origin, these cells give rise to both coronary arterial and venous ECs³². The switch from a venous EPHB4⁺ to arterial EFNB2⁺ CEC molecular signature in these emerging coronary sprouts was recently elaborated at a

molecular level by single-cell RNA-sequencing (scRNA-seq)³⁶. Gene set enrichment analysis demonstrated that venous-arterial CEC differentiation is a gradual process dependent upon a transcriptional threshold to specify pre-artery fate³⁶, an observation later corroborated in arterial transcriptional enhancer:reporter mouse models³⁷. Unexpectedly, scRNA-seq also demonstrated that pre-artery cells displayed diminished cell cycling: the vein-specifying transcription factor COUP-TFII was shown to inhibit pre-artery fate by inducing cell cycle genes, rather than by directly suppressing arterial or promoting venous gene expression³⁶. *In vitro* experiments confirmed that inhibition of cell proliferation is sufficient to promote pre-artery fate and these findings are consistent with the demonstration of arterial specification by G₁ cell cycle arrest in endothelial cells of the retinal vasculature³⁸. Further research is required to identify the stimulus required to repress COUP-TFII in selected SV cells in order to initiate cell cycle-controlled fate specification. In the retinal vasculature, cell cycle inhibition via a NOTCH1/connexin 37/CDKN1B axis was shown to be activated by fluid shear stress³⁸. However, in the SV, cell cycle control of arteriovenous fate curiously preceded the onset of coronary blood flow and, accordingly, E12.5 arterial cells were confirmed to lack *Notch1* expression; in fact, constitutive activation of Notch1 signalling in these cells could not overcome the influence of *Couptf2* in preventing pre-artery specification³⁶.

The Endocardium

Alongside the SV, clonal analyses by Red-Horse and colleagues³² indicated a modest CEC contribution from the ventricular and atrial endocardium by budding and blood island formation³². Subsequent *Nfatc1*-driven fate mapping suggested that the ventricular endocardium makes a more substantial contribution to the coronary endothelium via angiogenic sprouting in response to myocardial hypoxia-induced VEGFA signalling³³. However, constitutive *Nfatc1-Cre* lines were found to label some ECs within the SV, in addition to the ventricular endocardium³⁴, making definitive origin analysis impossible using these models. Later fate mapping using inducible *Nfatc1-Cre/ERT2*³⁵ and Natriuretic peptide receptor C *Npr3-Cre/ER*³⁴ models instead suggest that only interventricular septum (IVS) CECs are derived from endocardium during embryonic stages. These fate-mapping models were also used to investigate the coronary vasculature in the first three weeks after birth in mouse, when it undergoes a 3- to 4-fold expansion³⁹. These studies concluded that endocardial contribution to the coronary vascular increases from ~24% to ~60% during the perinatal period, to surpass the ~40% derived from the SV³⁴. Interestingly, coronary vessels after birth were inferred to form, not by sprouting of the existing coronary plexus, but by entrapment of the trabeculated endocardial surface upon myocardial compaction³⁵. The rapid coalescence of endocardial ECs into vessels and transition towards a CEC phenotype

efficiently increases vascularisation of the inner muscle wall and the IVS. In the zebrafish, coronary vessels form by angiogenic sprouting of arterial cells derived from the endocardium, specifically at the atrioventricular canal, which then migrate rostrally onto the bulbus arteriosus to form a vascular connection to the gills⁴⁰. Of note, while the site of sprouting corresponds to the position of the future coronary sinus in other species, the sprouts directly connect to the underlying endocardial layer and are arterial, rather than venous; thus it appears that the endocardium exclusively vascularises the zebrafish heart, with no evidence for a SV contribution⁴⁰. Interestingly, the coronary vasculature forms entirely post-embryonically 1-2 months post hatching in zebrafish, to coincide, as in other species, with the emergence of compact myocardium. This, and the absence of coronary vessels in the thinner-walled zebrafish atrium, support a conserved mechanism by which the endocardium adapts to supply the vascular demand of the myocardium. How the endocardium responds to developed hypoxia is further discussed below.

The proepicardium

What role, if any, does the (pro)epicardium play? Although originally implicated, genetic fate mapping with canonical proepicardial Cre models, specifically the inducible *Wt1-Cre/ERT2*⁴¹ and *Tcf21-MerCreMer*⁴² and constitutive *Tbx18-Cre*⁴³ lines, indicated minimal contribution of CECs (**Table 2**). This contrasts markedly with lineage tracing results from constitutive *Wt1-Cre* and *Tg(G2-Gata4-Cre)* models, which indicated ~25% and ~ 50% PEO origin of CECs, respectively⁴⁴. While these latter studies support a potential CEC progenitor population in the ST/PEO region, there are some caveats: *Tg(G2-Gata4-Cre)* traced cells were found in the ventricular endocardium, whilst *Wt1* expression has been documented in a subset of ST ECs⁴⁵ and widely in CECs, regardless of origin, after E13.5^{41,46}, making the route via which these progenitors transition unclear.

In 2012, Katz et al. postulated the existence of discrete cell populations within the murine PEO, roughly defined as either *Wt1/Tbx18+* or *Sema3d/Scx+*⁴⁷, however, the translation of this heterogeneity to the epicardium proper was not assessed. Lineage tracing using *Sema3d-Cre* labelled 6.9% of CECs at E16.5, whilst *Scx-Cre* labelled 24.3% of postnatal CECs, suggesting that this specific sub-population of PEO cells may contribute to CECs⁴⁷. However, this study may be confounded by *Sema3d-Cre* and *Scx-Cre* traced cells in the SV and endocardium⁴⁷, respectively, and also by possible expression of Cre in CECs themselves at later stages. Further, inducible Cre studies with the pan-epicardial marker *Tcf21-MerCreMer* robustly labelled the entire epicardium at E10.5 but demonstrated no contribution to CECs^{41,42,48}. Therefore, it is unlikely that PEO/ST cells contribute to CECs via the epicardial

layer; rather, a direct contribution from cells expressing the *Sema3d/Scx Cre* reporters in the PEO/ST to the primary angiogenic sources, the SV and endocardium, is more likely. Indeed, a recent avian-focused paper also demonstrated that SV and ventricular endocardium, not the epicardium as previously thought, are the main sources of CECs in chick, suggesting that this model is widely shared across vertebrates⁴⁹. The striking and consistent coronary vessel phenotypes observed in epicardial loss-of-function studies⁵⁰ may more likely be explained, not by impaired CEC contribution, but by loss of supportive mural cells, or by disruption of critical instructive cues, such as paracrine or extracellular matrix (ECM) signals.

A functional coronary vasculature from multiple sources?

It can be challenging to marry the somewhat disparate conclusions from these different fate-mapping analyses into a complete picture of the origins of coronary vessels (**FIG. 1**). A direct whole-mount comparison of embryonic hearts marked differentially by *Apj-CreERT2* (active in SV), *Nfatc1-Cre* (active in ventricular endocardium) and *Sema3d-Cre* (active in proepicardium) demonstrated that CECs originating from SV and endocardium segregate by anatomical region, rather than with arteriovenous fate⁵¹. CECs of SV origin appeared to be the main outside-in CEC contributor, primarily populating the dorsal and lateral walls⁵¹, whilst a complementary and largely non-overlapping inside-out contribution was made by the endocardium to the IVS and the ventral wall. *Sema3d*-lineage derived CECs were modest in number and more widely dispersed throughout the ventricular wall, calling further into question what role, if any, there might be for a PEO contribution: their skewed distribution with >4-fold more in dorsal regions than IVS may reflect the acknowledged labelling of the SV with *Sema3d-Cre*⁴⁷, in addition to the PEO. Indeed, with some reported expression of *Nfatc1* in SV³⁴ and *Apj* in endocardium³⁶, it is clear that more selective tools are required to unequivocally define the extent to which the three lineages contribute. On balance, however, the current evidence supports SV and endocardium as the dominant sources of CECs in the developing embryo.

How vessels from the SV and endocardium integrate to form a complete perfusion system is not yet understood, but endocardium-derived blood islands penetrating the myocardium and joining the SV-derived plexus have been visualised³². It is unclear why the mammalian coronary vasculature requires multiple progenitor sources. Certainly, the simultaneous outside-in and inside-out contributions from the SV and endocardium, respectively, facilitate a more efficient vascularisation to accommodate the rapidly increasing cardiac density of the growing heart. Whether by necessity or design, the system also allows for compensation if growth from one source is compromised, and a degree of redundancy is now recognised. In the epicardial VCAM-1 mutant, SV sprouting through the subepicardium is impaired, but results in an increased number of endocardial-derived blood islands ventrally⁵². Similarly, in *Elabela* and *Ccbe1* mutants⁵³, which also showed SV sprouting defects, compensatory

endocardial-derived vessel contribution was induced by the more hypoxic myocardium. Whether the SV can compensate for an endocardial deficit remains unexplored, although this is perhaps less likely, since hypoxia provides the stimulus to drive VEGFA-induced endocardial sprouting, while the SV requires non-hypoxic, developmentally-regulated cues from the epicardium. Moreover, the endocardium is the primary origin of *de novo* vessels in late embryonic/postnatal hearts³⁵; SV-derived vessels may not expand rapidly enough to compensate whilst maintaining function. Any functional distinctions between vessels of diverse origins require investigation, especially following the observation that endocardium- and SV-derived vessels are morphologically dissimilar⁵³ and scRNA-seq analyses may provide a useful starting point towards revealing key differences.

Molecular mechanisms regulating coronary plexus formation

The primitive coronary plexus initially develops in the absence of mechanical forces such as blood flow, indicating that other factors govern its formation. Extensive research has identified a complex epicardial-myocardial signalling axis as a major driver of coronary plexus formation from the SV (reviewed previously)^{54,55}. In this model, the apelin receptor (APJ)-expressing population of cells within the SV is stimulated by the epicardium-derived APJ agonists, Apelin (APN) and Elabela (ELA)⁵³, as well as VEGFC⁵¹, whilst the migration of APJ-negative SV cells is reportedly driven by myocardial Angiopoietin1 (ANG1)⁵⁶ (**FIG. 2**).

Although sprouting of the coronary plexus from the SV can occur in the absence of VEGFA⁵¹, angiogenic sprouting from the endocardium is lost after ablation of either myocardial VEGFA or endocardial *Vegfr2*³³. This supports a model in which endocardial-derived CECs form downstream of myocardial VEGFA-VEGFR2 signalling in response to the hypoxia created by rapid cardiac growth³³. Recent analysis of embryonic hearts from MEF2-dependent vascular enhancer-*LacZ* mouse models, in which *LacZ* is specifically expressed during angiogenesis directly downstream of VEGFA-VEGFR2 signaling, confirmed that VEGFA-driven angiogenesis was preferentially restricted to endocardial-derived coronary vessels during development³⁷. VEGFA-driven angiogenic-specific enhancers were also transiently active in the SV-derived vascular plexus soon after sprouting, suggesting an additional role for VEGFA-driven angiogenesis in the early SV-derived population prior to arteriovenous differentiation. Angiogenic VEGFA-MEF2 driven enhancer transgenes also remain active within the coronary vasculature throughout adulthood, implying a potential homeostatic role for VEGFA signaling in coronary vessel maintenance.

Maturation of the coronary vasculature

After the primitive coronary plexus has been established, the vessels that surround the aortic trunk grow into, and fuse with, the aorta between E12.5-14.5²⁷. Arterial stem growth requires the C-X-C motif chemokine, CXCL12⁵⁷ and VEGF-C⁵⁸ and the site of stem anastomosis is guided by aortic cardiomyocytes⁵⁸. Simultaneously, the veins form connections with the coronary sinus, driven by Semaphorin-3D signalling to an Erb-B2/Neuropilin1 heterodimer, to complete the circuit and initiate blood flow through the coronary circulation⁵⁹. Connection of the plexus to the aorta marks the onset of coronary remodelling to form the hierarchical network of vessels with differing diameters. Interestingly, whereas veins grow by proliferation, pre-artery cells are initially interspersed throughout the coronary plexus, then coalesce to form arteries in response to flow^{36,60}. The transcription factor DACH1 is key to their recruitment, promoting endothelial cell migration against the direction of flow through the CXCL12/CXCR4 signalling pathway⁶⁰. Analysis of the transcriptional regulation of early coronary arterial and venous differentiation has also indicated that these differentiation processes are influenced by similar transcriptional pathways as their systemic counterparts³⁷. Enhancer-*LacZ* transgenes downstream of the known arterial NOTCH/SOXF regulatory pathway become active as CECs transition to pre-arterial status. Conversely, vein-specific, BMP-SMAD1/5-driven enhancer-*LacZ* transgenes are rapidly switched off in the SV-derived plexus as it undergoes venous-arterial transition, before reactivating in mature coronary veins. Since the arterial-associated NOTCH/SOXF transcriptional pathway active in SV-derived arteries is downstream of VEGFA, this observation agrees with the reported loss of mature coronary arteries after VEGFA ablation³³, further suggesting that some aspects of SV-derived coronary vessel development are downstream of VEGFA signalling. Intriguingly, arterial cells are able to exit their differentiated state and migrate, even in the adult heart in the context of collateral artery formation (discussed later in this review)⁶¹.

Mural cell recruitment

Coronary vessels critically require perivascular support, with arteries surrounded by multiple layers of smooth muscle, while capillaries are in direct contact with single pericytes. The epicardium is the main source of mural cells in the mammalian heart, as evidenced by lineage tracing studies using an extensive list of epicardial Cre drivers (**Table 2; FIG. 1**). During early embryonic development, epicardium-derived cells (EPDCs) form from the epicardium via epithelial-to-mesenchymal transition (EMT). Regulated by platelet-derived growth factor (PDGF)-BB⁶² and retinoic acid signalling⁶³, EPDCs give rise to pericytes that subsequently differentiate to VSMCs in response to Notch3 signalling after interaction with coronary endothelium⁶⁴ (**FIG. 2**). This EMT takes place before E14.5 in the mouse heart, since tamoxifen induction in *Wt1-Cre/ERT2* and *Tcf21-MerCreMer* models beyond this stage labelled very few VSMCs⁶⁵⁻⁶⁷. Even the VSMCs of the postnatal endocardium-derived

vasculature originate predominantly from embryonically-specified EPDCs, which reside in the outer myocardial wall and subsequently migrate and differentiate to support new vessels as they arise in the inner myocardial wall⁶⁶. The neural crest (NC) and endocardium also make modest, but important, contributions to mural cell formation (**FIG. 1**); the NC provides VSMCs that line the proximal arteries and the septal branches in response to Edn1 signalling⁶⁸, whereas the endocardium provides VSMCs to septal vessels in response to canonical Wnt-Frizzled4- β -catenin signalling⁶⁴.

As is the case for the coronary endothelium, different VSMC sources can compensate for each other. For example, loss of epicardial PDGFR β resulted in absence of epicardium-derived VSMCs, but NC-derived VSMCs near the aortic root expanded to compensate⁶⁹. Similarly, *Rbpj*-depleted epicardium led to failed VSMC contribution and compensation from a non-targeted source. However, later in life, these alternative-origin VSMCs were prone to leakage and susceptible to disease, suggesting an important role for functional and physiological heterogeneity in VSMC origin⁷⁰.

Neovascularisation of the injured heart

While embryonic coronary vessel formation and differentiation is now understood in considerable detail, our comprehension of the extent, sources and mechanisms of new vessel growth in the adult heart following injury lags behind. Previous attempts to pharmacologically enhance neovascularisation in the clinic have failed to realise their goal (**Table 1**). This has led current research to focus on better understanding the endogenous vascular responses deployed by the heart in response to injury. In most cases, the model of choice has been permanent ligation of the left anterior descending (LAD) coronary artery in the rodent heart to mimic human myocardial infarction (MI). Understanding the limitations of these responses, when contrasted with the effective mechanisms used in the embryo and in regenerative species such as zebrafish⁷¹ and neonatal mice^{37,61}, may provide the vision to develop an amenable therapeutic strategy for neovascularisation of the human heart.

Cellular origins of neovascular growth in the injured heart

Since the coronary vasculature is fully established prior to adulthood, any expansion of the microcirculation that occurs endogenously in response to ischaemic injury was initially assumed to proceed via angiogenesis (which, by definition, indicates the formation of new blood vessels from existing ones). However, a number of other mechanisms have been proposed to contribute to neovascular growth in the heart, including EC differentiation from bone marrow-derived cells, vascular progenitor populations and cardiac fibroblasts (**FIG. 3**).

Vascular progenitor cells

Human endothelial progenitor cells (EPCs), phenotypically similar to embryonic hemangioblasts, have been shown to enhance new vessel growth and functional recovery in mouse⁶⁸ and rat⁷² LAD ligation models. These studies prompted various attempts to mobilize EPCs to the heart using a variety of cytokines (reviewed in⁷³), but any derived benefits have since been attributed primarily to paracrine angiogenic effects, rather than direct incorporation into the vasculature. Although EPCs were originally proposed to be bone marrow-derived, a recent study on male patients who received allogeneic bone marrow transplant from female donors determined that circulating EPCs did not originate from the bone marrow⁷⁴. Other potential CEC progenitor cell populations are thought to reside within postnatal blood vessels, specifically in the niche-like medial and adventitial layers⁷⁵⁻⁸¹. These have been investigated only after *ex vivo* expansion, are thought to be extremely rare and any endogenous role in neovascularisation is ill-defined⁸². Pericytes, that reside within the vessel wall, stand apart from these rarer putative progenitor populations, in being highly abundant with well-defined mural support roles in normal vessel function^{83,84}. Yet, in addition, pericytes are known multipotent progenitor cells, share phenotypic properties with mesenchymal stem cells⁸² and can, in response to injury, contribute to neovascularisation⁸⁵⁻⁸⁷, although this is also considered to be primarily via paracrine action⁸⁶.

Cardiac fibroblasts

Cre-based lineage tracing suggested that interstitial cardiac fibroblasts may transdifferentiate into CECs and contribute to neovascularisation via mesenchymal-endothelial transition (MEndT)⁸⁸. These studies used the Collagen1a2 enhancer-driven Cre line *Col1a2-Cre/ER*, considered to be fibroblast-specific, to demonstrate a significant contribution to neovascular growth three days post-MI (30-40% of Cre-labelled cells within the infarct border zone expressed EC markers). However, these findings were subsequently refuted: He and colleagues comprehensively traced cardiac fibroblasts, using not only the same *Col1a2-Cre/ER*⁸⁸ but also an independent *Col1a2-Cre/ERT* knock-in line and multiple fibroblast/mesenchymal lines, *Pdgfra-Dre/ER*, *Tcf21-MerCreMer*, *Sox9-Cre/ER* and *Postn-MerCreMer*¹⁰. This analysis concluded that fewer than 0.05% of lineage-labelled cells gave rise to PECAM1+ CECs.

Coronary endothelial cells via angiogenesis

It has long been established that a cohort of pro-angiogenic growth factors, including VEGF isoforms and their receptors, Flk1/VEGFR2 and Flt1/VEGFR1^{89,90}, FGF1 and -2 and Transforming Growth Factor (TGF)- β ^{91,92} are up-regulated in response to cardiac ischaemia,

mediated by hypoxia inducible factor (HIF)-1 α . Further, many descriptive accounts in rodent and canine hearts have been consistent with the mechanisms of sprouting angiogenesis and arterialisatation, inferring from static images that new vessel growth in the damaged heart is initiated by rapid capillary expansion into the infarcted territory, accompanied by CEC proliferation. However, the first direct evidence of an angiogenic mechanism of neovessel growth in the infarcted heart was provided only recently by Cre-driven lineage tracing. In this analysis, the activity of three independent, pan-endothelial Cre lines (*Cdh5-Cre/ER*⁹³, *Ap1n-Cre/ER*⁵², and *Fabp4-Cre/ER*⁹⁴) was induced two weeks prior to LAD ligation, timed to exclude the labelling of any cells that acquired CEC identity *de novo* post-injury. In these lineage dilution experiments, the proportion of labelled CECs was entirely unaltered post-MI, leading to the conclusion that all new vessels derive from pre-existing CECs, therefore by definition through some form of angiogenesis¹⁰. Lineage labelling studies have similarly confirmed that new vessels in the regenerating zebrafish also derive from pre-existing CEC or endocardial cells⁹⁵.

Which coronary endothelial cells contribute to neovascular growth?

These studies firmly support the notion that the pre-existing coronary endothelium should be the target of novel therapies to enhance the magnitude and rapidity of intrinsic neovascularisation. However, it is unclear whether there is a specific subpopulation of CECs that primarily contributes to neovascularization in the injured heart. Endothelial heterogeneity within the systemic and coronary vasculature underlies formation of the hierarchical network of arteries, veins, capillaries and lymphatic vessels and allows for organotypic function⁹⁶. This functional heterogeneity involves differential regulation by numerous signalling and transcriptional pathways in independent endothelial sub-populations^{37,97,98}. Given the multiple embryonic sources of CECs, further heterogeneity may be conferred by epigenetic memory of distinct origins, just as VSMC origin has been related to disease susceptibility^{65,99}.

Venous origin?

Systematic assessment of murine hearts over a two week time-course post-MI determined a greater propensity for proliferation by venous, rather than arterial or capillary, CECs¹⁸. In particular, the coronary sinus was identified as a site of overt vessel remodelling and apparent sprouting (**FIG. 3**). This finding may be relevant, given that the coronary sinus represents the adult derivative of the embryonic SV, the major venous source of CECs during development. Definitive proof of a coronary sinus origin of neovessel CECs post-MI is lacking and requires the identification of specific markers for selective fate mapping; while restricted expression of *Ap1nr/Apj* in the embryonic heart permits lineage tracing of SV-derived vasculature with *Tg(Ap1nr-cre/ERT2)*⁵¹, a more widespread expression in coronary capillary and venous ECs

of the adult heart at baseline precludes its use for assessing coronary sinus contribution post-MI⁶¹.

Endocardial origin?

A lineage dilution experiment using *Pdgfb-Cre/ERT2*¹⁰⁰, efficiently active in capillary, venous and arterial CECs, determined that a significant proportion (~26%) of border zone CECs were not lineage labelled. Since *Pdgfb*-driven Cre recombinase was selectively absent from the endocardium¹⁸, a putative contribution from this population was explored. The potential for neovascularisation from this source agrees with previous reports of endocardial plasticity in the adult heart^{101,102}. Miquerol and colleagues described vascular structures resembling 'flowers' within the endocardium overlying the infarct zone, and distinguishable from surrounding endocardium by marker profile. These 'flowers' were proposed to form by an angiogenic VEGFA-VEGFR2-mediated mechanism and to connect via stalk-like structures to the underlying coronary vasculature¹⁰¹. A further study by Kobayashi *et al.* proposed that a hypoxia-driven, VEGFR-dependent mechanism drives formation of a sub-endocardial vascular plexus to facilitate cardiomyocyte survival after LAD ligation in mice¹⁰² (**FIG. 3**). These angiogenic contributions appear, ostensibly, to recapitulate the hypoxia/VEGFA-driven endocardial sprouting that gives rise to coronary vessels during the embryonic period³³. In contrast, the large calibre *Pdgfb* lineage-negative subendocardial conduit vessels were proposed to form via MI-induced remodelling of the endocardium, a process resembling embryonic trabeculation and subsequent compaction¹⁸, more akin to the perinatal mechanism for rapid coronary expansion³⁵ (**FIG. 3**). Whilst there is precedence for endocardial remodelling in humans, with hypertrabeculation reported in MI and heart failure patients^{103,104}, it has yet to be demonstrated that these mechanisms can support neovascularisation. Moreover, when the *Npr3-Cre/ER* model was used to selectively label the endocardium, but not coronary ECs, in the adult heart, minimal contribution to the infarct/border zone neovascularisation was detected¹⁰⁵. In contrast, forced "trapping" of endocardial cells within the myocardium, by transplantation or a purse-string suture-like model, resulted in endocardium-derived new vessel formation, demonstrating that, if appropriately stimulated, endocardial cells have the potential to transdifferentiate and adopt a coronary phenotype¹⁰⁵. Thus, the key outstanding question is whether any degree of endocardial remodelling occurs endogenously following MI, as reported¹⁸, or whether it may be induced therapeutically to enhance neovascularisation.

Clonal expansion?

A step change in insight was recently gained by multispectral lineage tracing, in conjunction with transcriptomic analyses^{106,107}. Manavski and colleagues derived EC-restricted multicolour reporter (Confetti) mice, driven by *Cdh5*^{CreERT2}, and reported regionalised clonal expansion of CECs in response to MI. They concluded that large numbers of progeny could

be derived from a select few CECs with progenitor-like properties¹⁰⁷ (**FIG. 3**). Furthermore, by laser capture microdissection of EC clones and bulk RNA-seq, they inferred that a partial induction of endothelial-mesenchymal transition (EndMT) occurred within active, clonal CECs. EndMT was previously proposed to underlie endocardium-derived neovascularisation post-MI¹⁸ and, while the Manavski study did not map the origin of clones undergoing EndMT, support for an endocardial origin may be deduced by comparison with a subsequent clonal analysis. Li *et al.* utilised a similar confetti model, driven by *Pdgfb*^{CreERT2}, efficiently labelling arterial, venous and capillary ECs but excluded labelling of the endocardium¹⁰⁶. Clonally expanding CECs were subjected to single cell RNA-seq, demonstrating up-regulated cell cycle activity, ECM remodelling and stalk cell proliferation, but no evidence of EndMT. Thus, if EndMT provides a mechanism for CEC expansion, it may be restricted to the endocardial compartment.

The ability to prospectively identify, and target, CEC subsets with proliferative capacity depends upon identifying key transcriptional or metabolic differences to distinguish them from their neighbours. Progress in this regard was achieved by single cell RNA-seq of clonal populations¹⁰⁶. Ten transcriptionally discrete CEC states were identified, enabling the delineation of pathways through which selected CECs may enhance neovascularisation and repair. One such population was characterised by induction of *Plasmalemma vesicle-associated protein* (*Plvap*), an endothelial-specific regulator of permeability, leukocyte migration and angiogenesis¹⁰⁸⁻¹¹⁰, in proliferative border zone ECs in response to MI¹⁰⁶. *Plvap* was shown *in vitro* to be required for human umbilical vein EC (HUVEC) proliferation¹⁰⁶ and should be further explored, in terms of its therapeutic relevance, for revascularisation and cardiac repair.

Molecular mechanisms of neovascularisation

Since murine adult neovascularisation has been shown to primarily involve growth from existing vasculature, it has been hypothesised to occur by the redeployment of the same sprouting angiogenic mechanisms utilized in the developing embryonic heart¹⁸. However, a recent study revealed some divergence at the transcriptional level, suggesting that adult MI-induced neovascularization may differ from developmental angiogenesis. In this study, developmentally-active enhancer-*LacZ* transgenes downstream of VEGFA, highly active in angiogenic compartments of both embryonic and healthy adult hearts, were unexpectedly repressed in the infarct border zone of the adult heart post-MI³⁷, as were the arterial NOTCH/SOXF and venous BMP-SMAD1/5-mediated pathways (**FIG. 4**). These findings were recently supported by a genetic lineage tracing study showing that VEGFA overexpression in the (uninjured) myocardium was sensed by CECs, as demonstrated by apelin expression, however, in contrast to ECs of skeletal muscle, CECs failed to induce downstream

angiogenesis¹¹¹. Similarly, the angiogenic enhancer:*LacZ* constructs could be ectopically activated by VEGFA injection in other adult tissues, and indeed were still active in remote regions of the MI heart. Therefore, this suggests a specific repression (or failure of activation) of normal developmental angiogenic regulatory pathways in ischaemic myocardium. This repression was not seen when the experiment was repeated in neonatal (P1 or P7) mouse hearts, suggesting the response is specific to the adult heart. It is however unclear what process replaces this, as vascular proliferation still occurs in the ischemic adult heart, and neovascular growth is conclusively shown to derive from existing endothelium by some form of angiogenesis in these settings¹⁰. Further, there is considerable up-regulation of VEGFA post-MI in the epicardium¹¹², endocardium¹¹³ and border zone myocardium¹¹³, as well as its secretion, at high levels, from mobilized progenitor populations^{114,115}. Moreover, the clonally expanding CECs seen after MI express VEGFR2^{106,107} and the hypothesised endocardial contribution of vessels via both endocardial ‘flowers’¹⁰¹ and endocardial sprouting¹⁰² are thought to utilize VEGFA-VEGFR2 angiogenic signalling, for neovascular growth after ischemic injury. This is further supported by research in species with inherent regenerative capability: in zebrafish, angiogenic sprouting into the injured area occurred as early as 15 hours post-cryoinjury and was blocked in dominant negative *Vegfaa* mutants, resulting in impaired myocardial regeneration⁷¹. Since the VEGFA-induced, MEF2-driven angiogenic pathway has been the primary target of clinical trials for CAD/MI patients¹¹⁶, it should be determined whether human coronary ECs are capable of responding to exogenous VEGFA post-MI. Or, paradoxically, whether excessive VEGFA levels, from combined endogenous and exogenous sources, might in fact limit the extent of angiogenesis achieved, given that excess VEGFA has been shown to prevent EC proliferation, via p21-mediated cell cycle arrest¹¹⁷. The timing and mode of delivery of VEGFA treatment post-MI should also be considered. In clinical trials, sustained administration of VEGFA had little effect, whereas a rapid and transient burst of hVEGFA modified RNA at the time of LAD ligation improved neovascularisation, heart function and survival in a mouse model¹¹⁸. By uncoupling endothelial cell-cell junctions, VEGF-induced angiogenesis disrupts vascular barrier function, causing vascular permeability, oedema, and leukocyte invasion, which would exacerbate myocardial injury¹¹⁹. Thus, repression of VEGFA-VEGFR2 may represent an endogenous ‘damage limitation’ response. Alternatively, VEGFA may signal via different transcriptional pathways during adult coronary angiogenesis, or angiogenesis into the infarct/border zone may, instead, occur via non-VEGFA signalling pathways.

Recently, focus has turned to alternative VEGF isoforms, VEGFB₁₈₆ and VEGFD, which are more soluble than VEGFA due to lack of heparan sulfate proteoglycan binding¹²⁰. Adenoviral gene transfer of VEGFB₁₈₆ and a truncated VEGFD (VEGF-D^{ΔNAC}) into porcine myocardium

induced angiogenesis and enhanced coronary perfusion¹²¹. VEGF-D^{ΔNΔC} gene therapy is currently undergoing phase II trial in patients with refractory angina¹²²; being able to activate both VEGFR-2 and -3, VEGFD stimulates lymphangiogenesis, as well as angiogenesis, and may additionally provide benefit by promoting resolution of inflammation post-MI¹²³.

Studies in tumours after VEGF signalling blockade have demonstrated that angiogenesis can also proceed via a number of non-VEGF pathways, including those downstream of PDGFC¹²⁴, Ang2¹²⁵ and FGF2¹²⁶. Given the similarities between hypoxic tumours and the ischaemic myocardium, and the dilated, pericyte-poor vessels that form within these environments¹²⁷⁻¹²⁹, closer examination of these alternative pathways in the adult heart may be worthwhile. Indeed, deletion of FGF receptors 1 and 2 from murine CECs impaired cardiac functional recovery post-MI, with a failure of vascular remodelling, implicating a beneficial role for this pathway¹³⁰. Furthermore, studies of tumour^{131,132} and hindlimb¹³³ neovascularisation strongly support the value of further investigating the potential of combination treatments in the ischaemic heart. In these contexts, combined application of FGF2 and PDGF-BB synergistically enhanced angiogenesis and vascular stability¹³³. In tumours, the mechanism of synergy was demonstrated to occur via FGF2-induced PDGF receptor expression in ECs¹³¹ and in pericytes¹³², enhancing their sensitivity to PDGF-BB; whereas PDGF-BB-induced FGFR1 up-regulation in VSMCs reciprocally enhanced the sensitivity of these cells to FGF2¹³¹.

Collateral artery growth

Even if the microvasculature in the infarct/border zone can be sufficiently expanded, the increased flow resistance in proximal arteries will require concomitant replacement of damaged arteries. Collateral artery formation provides a functional bypass by bridging two existing arteries, and occurs endogenously as a chronic, adaptive response to arterial stenosis, to mitigate the effects of coronary artery disease (CAD)¹³⁴. A third of CAD patients possess sufficient collaterals to preserve up to 25% of the normal flow and prevent MI altogether²⁰. Understanding of the mechanisms that control arterial growth in the human heart may uncover strategies for its stimulation. Although the propensity for collateral growth varies between species, recent mechanistic insight has been garnered from murine studies. Using an inducible *ApIn^{CreER}* line to label capillary, but not arterial, CECs, collateral vessels were shown to form predominantly by the enlargement of pre-existing arteries (arteriogenesis), as opposed to capillary sprouting into the ischaemic region and recruitment of VSMCs (arterialization)¹³⁵. Yet, others have questioned whether collaterals in the infarcted mouse heart arise *de novo* or by remodelling of pre-existing arteries¹³⁶. A recent study offered clarity on this issue, demonstrating with whole mount imaging that collaterals form where no pre-existing arteries cross the watershed, the name given to a myocardial region that receives blood from distal branches of two large arteries. Therefore, it was concluded that the process

through which collateral arteries form is not strictly arteriogenesis⁶¹; rather, by a process dubbed “artery reassembly”, single arterial ECs migrate along existing capillaries in the watershed area, thereafter proliferating and coalescing to form collateral arteries. In the injured P1 neonatal heart, regeneration was accompanied by early collateral artery formation, an endogenous mechanism which entailed induction of CXCL12, in CECs, to stimulate their migration. Intrinsic collateral growth was restricted to the regenerative window, being lost by P7. Remarkably, however, injection of CXCL12 into the watershed area successfully reactivated collateral growth in the adult heart⁶¹.

Although their role is scarcely understood, myeloid cells are required for collateral growth¹³⁶. Monocyte chemoattractant protein-1, released following MI, serves to induce homing of CCR2+ monocytes from the bone marrow and their recruitment to the heart is, in some way, necessary for collateral growth. Macrophages, mast cells, dendritic cells and subsets of T cells, contribute to vessel growth in numerous ways, including by secreting angiogenic growth factors and coordinating cell-cell interactions^{137,138}

Induction of collateral vessel formation in large mammals was achieved in hibernating pig myocardium by adenoviral transduction with myocardin-related transcription factor-A, which activates serum response factor (SRF) and genes which promote capillary proliferation and pericyte recruitment¹³⁹. Since nuclear translocation (activation) of SRF occurs by dissociation from G-actin, collateral growth could similarly be enhanced by transgenic overexpression of the actin monomer-sequestering peptide Thymosin β 4 in pigs¹³⁹. Whether the same mechanisms of collateral growth characterised in rodents and large mammals can be triggered in CAD patients, with associated comorbidities, is difficult to determine but should be investigated in the clinic. Since a significant proportion of these patients cannot be treated by percutaneous coronary intervention or artery bypass grafting, the therapeutic induction of collateral growth may be a key treatment strategy in those individuals.

Prospects for future therapies

The reasons for the lack of a successful outcome to date in clinical trials for coronary neovascularisation therapies may be manifold. Generically, poor reproducibility of preclinical studies, reliance on severe “no option” patients as subjects and the strong placebo effect in expectant patients are among the frequently cited reasons, as are the altered vascular signalling and oxidative stress associated with hypercholesterolemia, diabetes and hypertension in CAD patients. A more explicit rationalisation, with regard to coronary neovascularisation, may be that putative therapies were predicated on a number of assumptions that are now partly invalid. Firstly, that angiogenesis of pre-existing capillaries

should be the principal target and that all CECs have an equal propensity to respond to stimulation. Secondly, that the classical angiogenic growth factors, such as VEGFA or FGF2, would elicit the maximal benefit from these cells. While recent studies reinforce the focus on angiogenesis, therapeutic approaches should now be refined based on understanding the extensive heterogeneity within the coronary vasculature. While some discrete sub-populations may respond positively to mitogenic stimulation, via appropriately defined pathways, recent studies caution against prodigiously boosting levels of mitogenic growth factors. In some contexts, excessive stimulation by VEGFA is now recognised to arrest endothelial cell proliferation¹¹⁷. If this holds true in the ischaemic heart, hypoxia-induced up-regulation of VEGFA may, in fact, be deleterious; if VEGFA levels do not endogenously reach the threshold to switch from pro- to anti-proliferative effects, then therapeutically enhancing VEGFA risks tipping the balance and inhibiting, rather than promoting, repair. If an optimal angiogenic response can initially be invoked, then, subsequently, an anti-proliferative effect may conversely be beneficial, since arterial specification appears to depend upon cell cycle inhibition^{36,38}. A key challenge remains to identify the most angiogenic subsets of endothelial cells, their distinguishing hallmarks and the stimulatory cues required to direct their activation. Single cell transcriptomics and genetic lineage tracing currently offer opportunities to identify and evaluate the CECs with clonogenic potential, whether they be broadly dispersed throughout the coronary network or resident within defined regions, such as the coronary sinus or endocardium. Expression profiling may not only disclose targets to allow their selective amplification for neovascularisation, but also a biomarker signature that can be used to evaluate the equivalent populations in human patient biopsies, in terms of their incidence, regulatory mechanisms and injury responses, and potential for therapeutic targeting.

Conclusions

That the heart mounts an intrinsic neovascularisation response to ischaemic injury, an attempt at self-repair, is well-recognized. However, the rapidity and magnitude of the response is inadequate to prevent the extensive myocardial damage that occurs within days after coronary artery occlusion. Clinical treatments to enhance coronary revascularisation do not currently exist, despite recent and ongoing trials. Whilst we might gain important clues for therapies to more efficiently reconstruct the coronary network after injury, based on understanding its formation during development, further research is needed to reconcile if, and how, these processes may be recapitulated in the adult heart. Regenerating species, such as zebrafish, appear not only to recall embryonic mechanisms for neovascularisation^{40,140,141} but efficiently utilise newly regenerated vessels to drive repair of the myocardium¹⁴². While replenishment of cardiomyocytes in the adult mammalian heart remains an overwhelming challenge, these

studies underscore the importance of neovascularisation within a holistic cardiac regenerative strategy.

Glossary

Angiogenesis: the process by which new blood vessels arise from pre-existing ones, typically by sprouting and/or splitting.

Arterialization: the sprouting of capillaries into the ischaemic region and transformation into arteries by recruitment of VSMCs.

Arteriogenesis: the enlargement of pre-existing arteries.

Collateral Arteries: arterial segments that bridge two original arteries, forming a natural bypass to ensure blood flow downstream of an obstruction.

Cre-LoxP based lineage tracing: a bacteriophage recombination system that is used (predominantly in mouse and fish studies) to genetically label a cell and trace its progeny.

Endocardium: the innermost layer of tissue that lines the chambers of the heart, comprising a specialised type of endothelial cell.

Endothelial-to-mesenchymal transition (EndoMT): The transformation of endothelial cells into mesenchymal cells, which may subsequently differentiate into fibroblasts or mural cells.

Enhancer: A cis-regulatory DNA sequence, to which proteins, such as transcription factors bind, to impose specific spatiotemporal control of gene expression.

Epicardium: a mesothelial layer of cells on the outer surface of the heart, which contributes progenitor cells to the embryonic heart and stimulates sprouting of vessels from the sinus venosus.

Epithelial-to-mesenchymal transition (EMT): A process by which epithelial cells, such as those of the epicardium, lose polarity and cell-cell contacts and transition into more migratory mesenchymal cells, which may subsequently differentiate (into fibroblasts or mural cells, in the case of epicardium-derived cells).

Myocardial Infarction: Also known as a heart attack, the sudden blockage of blood supply to the heart, usually caused by thrombus formation following atherosclerotic plaque rupture.

Neural Crest: a transient embryonic lineage that gives rise to the peripheral nervous system, as well as non-neural cell types, including vascular smooth muscle cells, pigment cells, and craniofacial bones, cartilage and connective tissue.

Proepicardium/proepicardial organ (PEO): a transient developmental outgrowth of mesothelial cells arising in the region of the septum transversum (~E8.5-E9.5 in mouse). Cells of the proepicardium delaminate over the outer surface of the heart to give rise to the epicardium.

Sinus venosus (SV): the venous inflow tract of the embryonic heart, continuous with the atria, which contributes a large proportion of coronary endothelial cells during embryonic stages. Later in development, the SV is incorporated into right atrium and coronary sinus veins.

Stenosis: the narrowing of a major artery, as a result of coronary artery disease, that impedes sufficient myocardial perfusion.

Watershed: a region of the heart that receives blood from distal branches of two large coronary arteries e.g. the capillary network at the anterior midline of the heart

Key Points

- The coronary vasculature is established during embryonic and neonatal development, with endothelial cells derived predominantly from the sinus venosus and endocardium and mural cells mainly from the epicardium.
- Neovascularisation post-myocardial infarction is essential to restore blood flow to injured myocardium, however the endogenous mechanisms by which new vessels grow is poorly understood.
- From clinical trials to date, no effective treatment for coronary revascularisation has been identified.
- The regulatory pathways that control angiogenesis during development are activated in the injured myocardium of the more regenerative neonatal mouse heart but are either repressed, or fail to activate, in the infarcted region of the adult mouse heart.
- Developmental comparisons, genetic lineage tracing and single cell transcriptomic analyses may provide insights into mechanisms that may be targeted to enhance neovascularisation and regeneration of the injured myocardium.

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Contributions

IEL and NS developed the outline plan of the Review, researched the literature and wrote the first draft. All authors reviewed and edited the manuscript before submission.

Figure Legends

Figure 1. Development of the embryonic coronary vasculature

Formation of the coronary plexus is initiated at E11.5, when sinus venosus-derived endothelial cells begin to migrate caudally over the dorsal side of the heart. Endocardial cells begin to sprout throughout the interventricular septum, onto the ventral surface and into the lateral walls towards the apex. Starting from E15.5, pericytes and vascular smooth muscle cells are recruited to the forming vessels. Most mural cells derive from the epicardium, with a small contribution near the base from the neural crest and to the interventricular septum from the endocardium. LA - left atrium, RA - right atrium, LV – left ventricle, RV - right ventricle, SV - sinus venosus, PEO – proepicardial organ, ENDO - endocardium, IVS - interventricular septum, EPI - epicardium, CEC - coronary endothelial cell, NC - neural crest.

Figure 2. Molecular mechanisms that control formation of the coronary vasculature

During development, the epicardium secretes mitogenic factors, including vascular endothelial growth factor (VEGF)-C and Elabela, which stimulate the sprouting of endothelial cells from the sinus venosus. As myocardial density increases, hypoxia-induced VEGFA and angiopoietin1 (ANG1) stimulate angiogenesis from the endocardium and sinus venosus, respectively. Endothelial-derived platelet-derived growth factor (PDGF)-B promotes EPDC formation from the epicardium, which differentiate into vascular smooth muscle cells in response to Notch3 signalling. epi - epicardium, epdc - epicardium-derived cell, myo - myocardium, vsmc - vascular smooth muscle cell, ec - endothelial cell.

Figure 3. Proposed mechanisms of coronary neovascularisation post-myocardial infarction

Multiple different mechanisms have been postulated to underlie endogenous coronary neovascularisation post-MI. In terms of sprouting angiogenesis, veins of the coronary sinus (**a**) and the endocardium (**c**) were noted for their overt sprouting capacity, while multispectral lineage tracing revealed the existence of discrete endothelial populations with a greater propensity for proliferation and clonal expansion (**d**). Various populations of resident vascular

progenitor cells, including pericytes, have also been reported to contribute or promote new vessel growth (**b**). Remodelling of the endocardium occurs in response to MI and is proposed to lead to new vessel growth. The extent to which these mechanisms contribute to improve coronary blood flow post-MI remains to be determined.

Figure 4. Regulatory pathways governing coronary vessel development are differentially regulated in the neonatal and adult heart following myocardial infarction

Three distinct regulatory pathways control coronary vessel formation in the embryo: a VEGFA-MEF2 pathway is principally active in angiogenic vessels that derive from the endocardium, while sinus venosus-derived arteries and veins show SOXF/RBPJ and BMP/SMAD pathway activity, respectively. Although diminished compared with embryonic stages, all three pathways remain active in the uninjured neonatal heart and, following MI, all are induced throughout the infarct border zone. However, these pathways either fail to activate, or are actively repressed, in the infarcted adult heart. This is particularly unexpected for the VEGFA-MEF2 pathway, as the classic hypoxia-induced pathway, which remains active throughout the remote myocardium after MI and the entire inner myocardial wall of uninjured/sham adult hearts.

TABLE 1: Clinical trials of angiogenic growth factors for coronary artery disease

Study	Growth factor	Therapeutic agent	Disease (n, total)	Primary endpoints	Adverse effects?	Main conclusion	Ref
Phase I							
	VEGF ₁₆₅	peptide	Refractory angina (7)	Safety, myocardial perfusion	Safe, well tolerated	Improved collateral density score	143
	VEGF ₁₆₅	plasmid	Refractory angina (5)	Safety, Myocardial perfusion	Safe	+5% LVEF in 2/5 patients	144
	VEGF-2 (VEGFC)	plasmid	Chronic MI (6)	Feasibility, safety, potential efficacy	Safe	Potential efficacy (trend towards reduced angina - preliminary)	145
	VEGF ₁₂₁	adenovirus	Severe CAD (with/without CABG)	Safety, Exercise tolerance ET, myocardial function	Safe, well tolerated	Reported improvement in angina class (underpowered)	146
	VEGF ₁₂₁	adenovirus	Severe CAD	Safety	Single case of moderate fever	Feasible, potentially safe	147
	VEGF ₁₆₅	plasmid	Severe CAD (35)	Appraise use of PET to evaluate angiogenic therapies.	Not reported	Reduced myocardial ischaemia	148
KAT 301	VEGFD _{ΔNAC}	adenovirus	Refractory angina (30)	Safety and feasibility	Safe, well tolerated	Improved myocardial perfusion	122
AGENT	FGF-4	adenovirus	Chronic stable angina (79)	Safety and potential efficacy	Fever <1 day in 3 patients who received high dose	Trend towards improvement in ET	149
	FGF-1	peptide	CAD (40)	Proof of concept	Not reported	Enhanced sprouting near site of injection	150
	FGF-2	peptide	Refractory CAD (8)	Safety and feasibility	1 perioperative MI but concluded safe and feasible	Improved LVEF in 7/8 patients	151
	FGF-2	peptide	CABG patients (24)	Safety and feasibility	No treatment-related adverse events	Safe and feasible	152
	FGF-2	peptide	Refractory CAD (52)	safety, preliminary efficacy	Safe, well tolerated	Improvement in angina and ET, reduction in ischaemic area	153
	FGF-2	peptide	CAD, stable angina (25)	Safety and feasibility	Moderate hypotension and bradycardia in some patients.	Generally well-tolerated	154
Phase II							
VIVA	VEGF ₁₆₅	peptide	Refractory angina (178)	ET, angina class, quality of life	Safe, well tolerated	High dose improved angina by 120 days, trend	5

						towards improved ET and angina frequency.	
Phase I/II	VEGF ₂ (VEGFC)	plasmid	Refractory angina (19)	Angina class, ET	Safe, well tolerated	Reduction in angina class, trend in improved ET	155
KAT II	VEGF ₁₆₅	adenovirus vs plasmid liposome	CAD (103)	Safety and feasibility	Feasible, well tolerated (Adv and plasmid)	Myocardial perfusion improved in VEGF-Adv group, no difference in mortality after 8 years	156,157
Euroinject One	VEGF ₁₆₅	plasmid	Severe IHD (80)	Myocardial perfusion, function, clinical symptoms	Safe, no adverse effects	No improvement in perfusion, improved regional wall movement	158
NOVA	VEGF ₁₂₁	adenovirus	Severe CAD (17)	Myocardial perfusion	Safe, minor adverse effects	No improvement in perfusion or ET. Terminated prematurely (company product portfolio decision)	159
	VEGF ₁₆₅ G-CSF	plasmid (VEGF ₁₆₅); peptide (G-CSF)	Severe chronic IHD (48)	Myocardial perfusion, clinical symptoms	Safe, no adverse effects	No improvement in perfusion or clinical symptoms	160
NORTHERN	VEGF ₁₆₅	plasmid	Severe CAD (93)	Myocardial perfusion	Musculoskeletal pains, other minor adverse effects not attributed to VEGF	No improvement in perfusion due to VEGF	4
FIRST	FGF-2	peptide	Refractory CAD (337)	Safety and feasibility	Adverse effects not attributed to FGF-2	No difference in perfusion or ET; reduced angina (trend towards improved symptoms)	6
VIF-CAD	VEGF ₁₆₅ /FGF-2	plasmid	Refractory CAD (52)	Safety, myocardial perfusion, function, clinical symptoms, ET	Safe after 1 year follow-up	No improvement in perfusion, marginal improvements in ET and angina class	161
Phase III							
AGENT-3/AGENT-4	FGF-4	adenovirus	Refractory angina (532)	ET, myocardial perfusion	Safe, no adverse effects	No difference in end points (albeit significant in subgroup of >55 year old women)	162

MI: myocardial infarction; LVEF: left ventricular ejection fraction; ET: exercise tolerance; CAD: coronary artery disease; CABG: coronary artery bypass grafting; IHD: ischaemic heart disease; G-CSF: granulocyte-colony stimulating factor; PET: positron emission tomography.

TABLE 2: Embryonic origins of coronary endothelial and mural cells

Endothelial cell origin						
Cre/reporter	Specificity (heart)	Induction	Stage analysed	Technique	Traced	Ref
<i>Apln</i> ^{tm1.1(cre/ERT2)Bzsh} ; <i>R26</i> ^{RFP}	All CECs	E10.5 E13.5 P4/6	P28	quantification of muscle volume occupied	OMW (40% of heart irrigated) IVS (60% of heart irrigated) IMW	35
<i>Nfatc1</i> ^{em1(cre/ERT2)Bzsh} ; <i>R26</i> ^{RFP}	Endo	E8.5	P0 P7		CECs in the IVS 76.7% of CECs in the IMW	
<i>Npr3</i> ^{tm1.1(cre/ERT2)Bzsh} ; <i>R26</i> ^{tdTomato}	Endo, Epi	E8.5	E15.5		88% of CECs in the IVS	34
<i>Nfatc1</i> ^{tm1.1(cre)Bz} ; <i>R26</i> ^{mTmG}	Endo, some SV	constitutive	E16.5	quantification of CD31 stained sections	80% of CECs in the IVS, ventral and Left lateral labelling	51
<i>Apj</i> ^{CreERT2} ; <i>R26</i> ^{mTmG}	SV, few Endo	E10.5	E16.5		80% of CECs on dorsal side and R lateral labelling	
<i>Wt1</i> ^{tm2(cre/ERT2)Wtp} ; <i>R26</i> ^{mTmG}	Epi Epi, EPDCs, CECs Epi, CECs	E10.5 E14.5 P4	E18.5 E18.5 P7		rare labelling of CECs substantial labelling of CECs 28% of CECs	41
<i>Wt1</i> ^{tm1(EGFP/cre)Wtp} ; <i>R26</i> ^{YFP}	PEO/ST/Epi/EPDCs/CECs	constitutive	E18.5	dissociated ventricles, CD31 Flow cytometry	11.3% of all CD31+ cells	44
<i>Tg</i> ^{(Gata4*G2-cre)#Roja} ; <i>R26</i> ^{YFP}	PEO/ST/Epi, vEndo	constitutive	E17.5, neonate		22.7% of CD31+ cells at E17.5, 35.7% in the neonate, some endocardial labelling	
<i>Tg</i> ^{(GATA5-cre)1Krc} ; <i>R26</i> ^{lacZ}	PEO/ST, Epi, some CMs	constitutive	P3	CD31 stained sections	none observed	163
<i>Tbx18</i> ^{tm2.1(cre)Sev} ; <i>R26</i> ^{lacZ}	PEO/ST, Epi, EPDCs, CMs, vSMCs	constitutive	adult	X-gal staining of affinity-purified endothelial cells	none observed	43
<i>Tcf21</i> ^{tm3.1(cre/Esr1*)Eno} ; <i>R26</i> ^{YFP}	Epi, AVC	E10.5	E18.5	CD31 stained sections	none observed	42
<i>Tg</i> ^{(Scx-GFP/cre)1Stzr} ; <i>R26</i> ^{tdTomato}	PEO, AVC, Epi	constitutive	P4-P7	Intact hearts, CD31+ NFATC1-Flow cytometry	24.3% of CECs, some endocardial labelling	47
<i>Sema3d</i> ^{tm2.1(GFP/cre)Joe} ; <i>R26</i> ^{lacZ}	PEO, IVS, AVC, Epi	constitutive	E16.5	quantification of CD31 stained sections	6.9% of CECs, some SV labelling	47,51
Mural cell origin						
<i>Tg</i> ^(Wnt1Cre) ; <i>R26</i> ^{lacZ}	NCC	constitutive	E17.5	αSMA stained sections	vSMCs of the proximal coronary arteries and septal branches	68
<i>Wt1</i> ^{tm2(cre/ERT2)Wtp} ; <i>R26</i> ^{RFP}	Epi Epi, EPDCs, CECs Epi, CECs	E10.5 E15.5 P1	P8w-P16w	quantification of αSMA stained sections	>80% of vSMCs <5% vSMCs v. few vSMCs labelled	66
<i>Tbx18</i> ^{tm2.1(cre)Sev} ; <i>R26</i> ^{RFP}	EPI, CMs, vSMCs	constitutive	P14w		vSMCs labelled, not quantified	43
<i>Tcf21</i> ^{tm3.1(cre/Esr1*)Eno} ; <i>R26</i> ^{YFP}	Epi, AVC EPDCs, CFs	E10.5 E14.5	E18.5	PDGFRβ, SM22α stained sections	none observed	42,67
<i>Tg</i> ^{(GATA5-cre)1Krc} ; <i>R26</i> ^{lacZ}	PEO/ST, Epi, some CMs	constitutive	E18.5, 4 and 8 months	αSMA stained sections	>80% of vSMCs	65
<i>Tg</i> ^{(Cdh5-cre/ERT2)1Rha} ; <i>R26</i> ^{mTmG}	ECs (CECs and Endo)	E8.5	E16.5	PDGFRβ, NG2 stained sections	some pericytes and vSMCs, not quantified	64
<i>Tg</i> ^{(Tek-cre)1Ywa} ; <i>R26</i> ^{mTmG}	ECs (CECs and Endo)	constitutive	E16.5	αSMA stained sections, PDGFRβ flow cytometry (cut below valves)	10.75% αSMA+ labelled 20.9% of PDGFRβ+ labelled	
<i>Apln</i> ^{tm1.1(cre/ERT2)Bzsh} ; <i>R26</i> ^{mTmG}	SV-derived CECs	E10.5	E16.5		none observed	
<i>Nfatc1</i> ^{em1(cre/ERT2)Bzsh} ; <i>R26</i> ^{RFP}	Endo	E8.5	E16.5	PDGFRβ, NG2 stained sections	some mural cells labelled, not quantified	

CEC: coronary endothelial cell; Endo: endocardium; Epi: epicardium; SV: sinus venosus; EPDC: epicardium-derived cell; PEO: proepicardial organ; ST: septum transversum; CM: cardiomyocyte; AVCu: atrioventricular cushion; OMW: outer muscle wall; IVS: interventricular septum; IMW: inner muscle wall; NC: neural crest; vSMC: vascular smooth muscle cell; CF: cardiac fibroblast.

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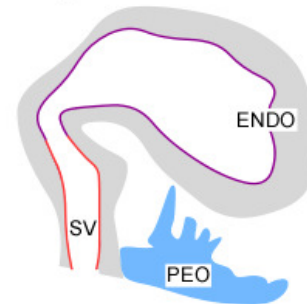
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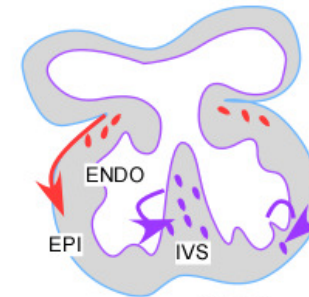
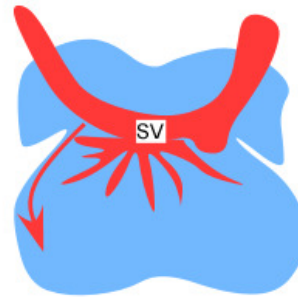
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E9.5-E11.5: Epicardium formation



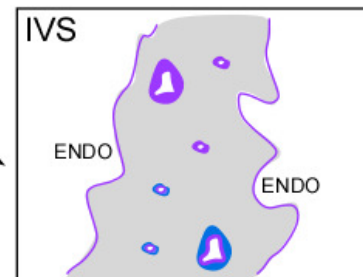
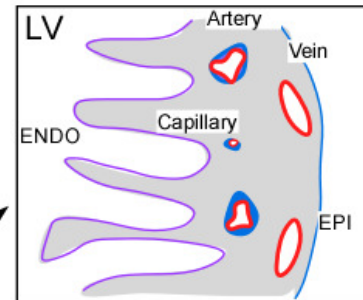
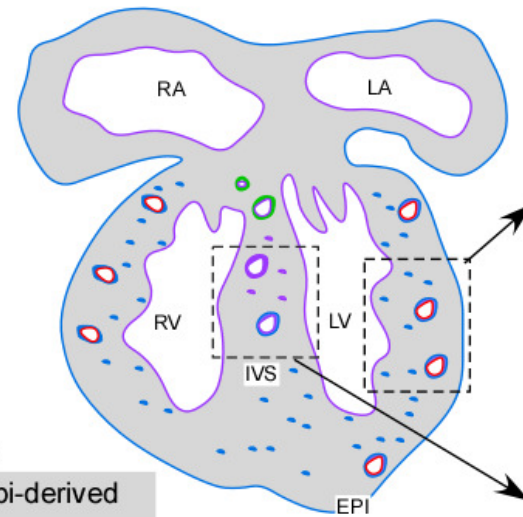
E11.5-E13.5: Coronary plexus formation



CEC

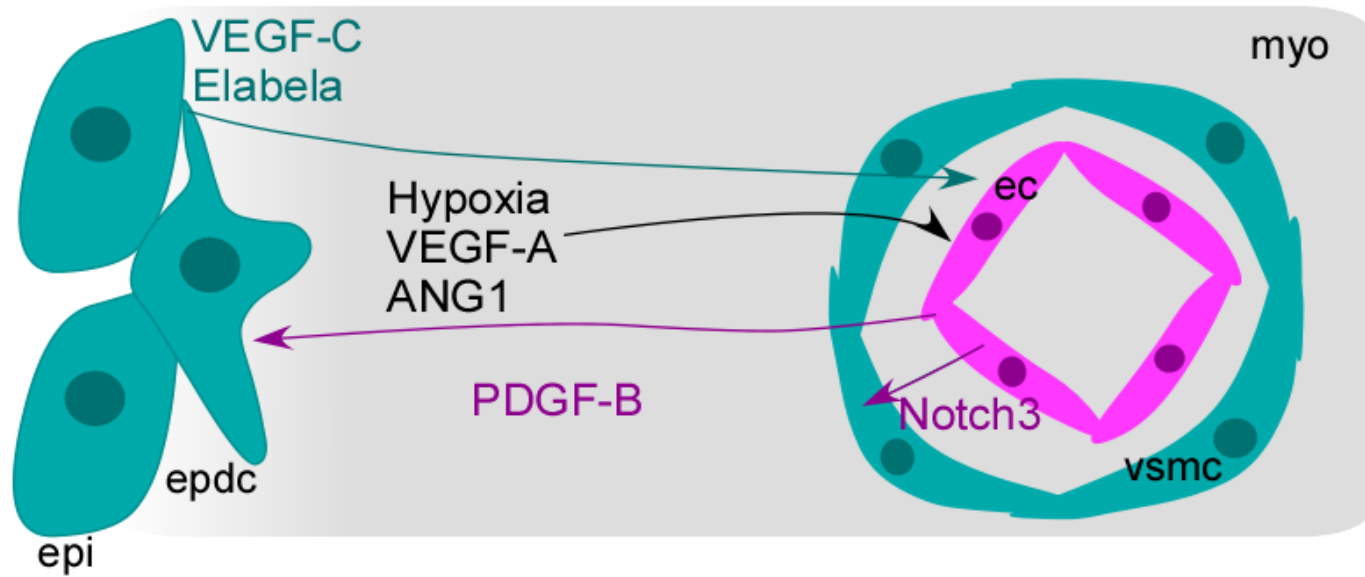
SV-derived
Endo-derived

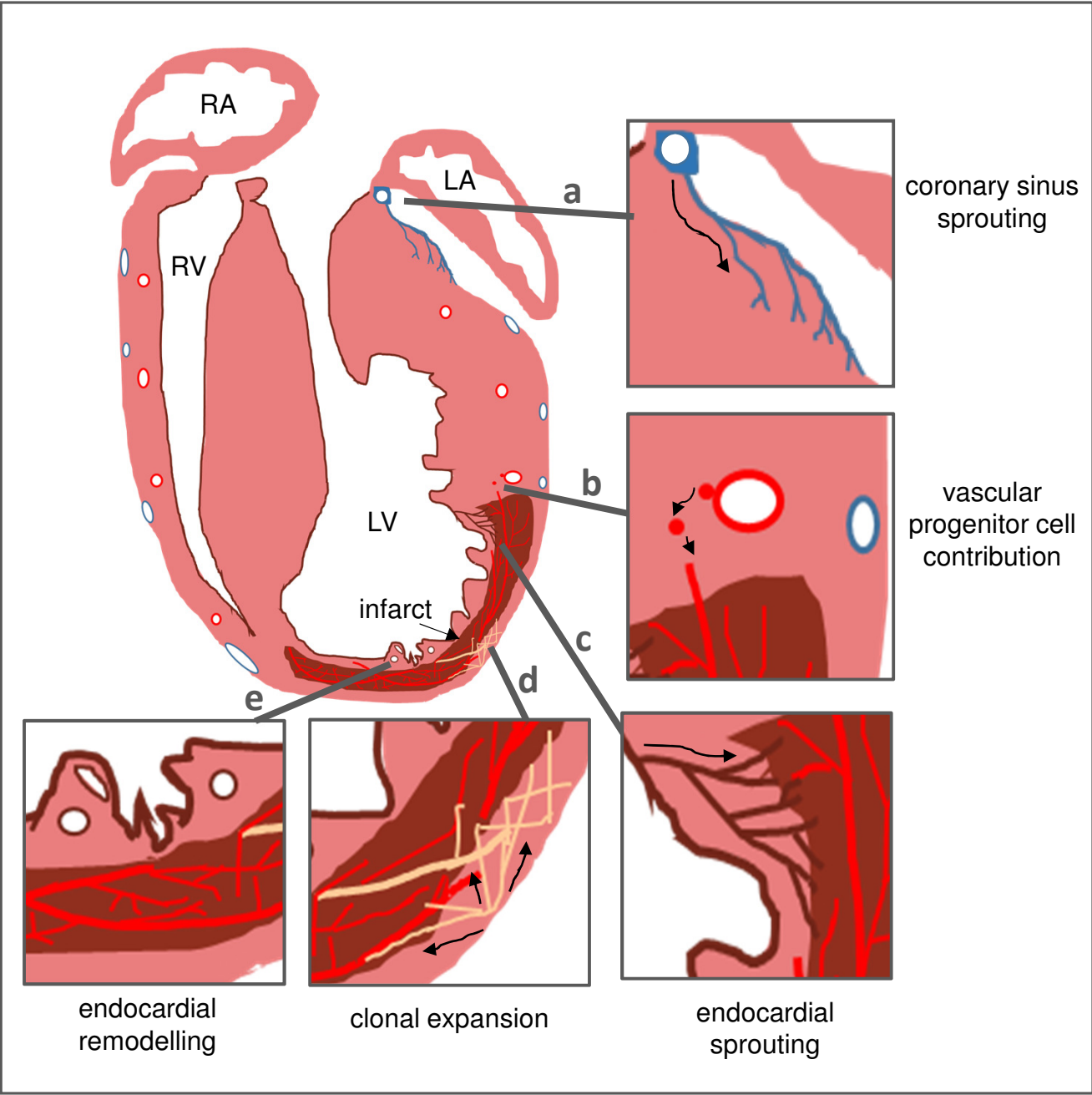
E13.5-P28: Coronary plexus remodelling

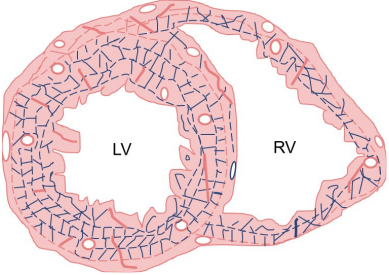
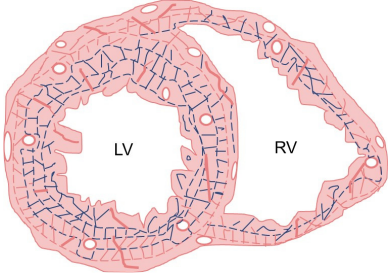
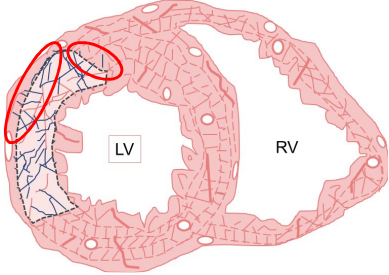
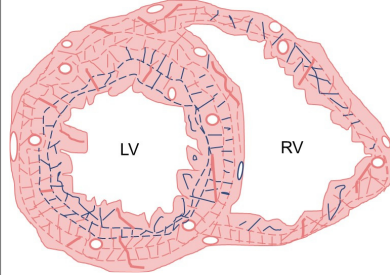
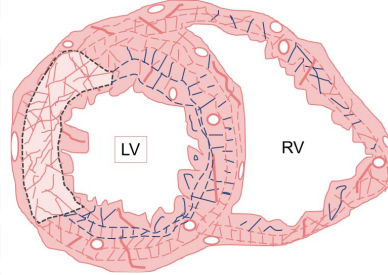
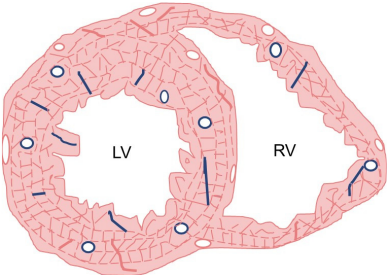
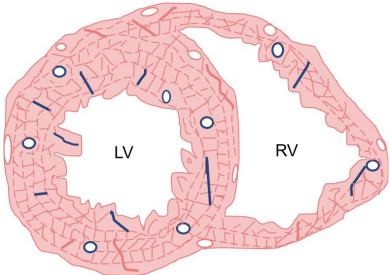
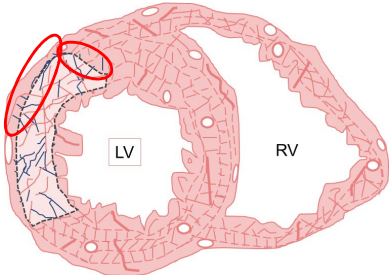
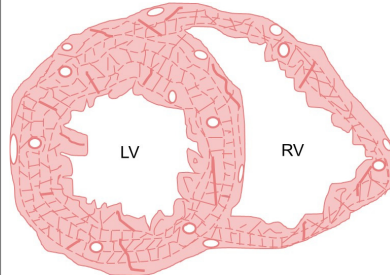
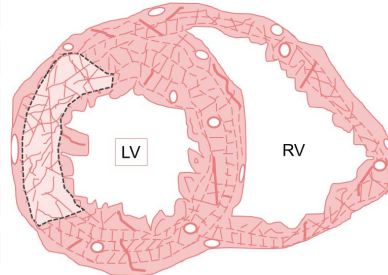


vSMC

Epi-derived
NC-derived
Endo-derived





	Embryo	Neonate		Adult	
		uninjured	MI	uninjured	MI
Angiogenic VEGFA- MEF2					
Arterial SOXF/ RBPJ					
Venous BMP/ SMAD	