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The Role of the Epicardium During Heart Development and Repair

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Abstract

The heart is lined by a single layer of mesothelial cells called the epicardium that provides important cellular contributions for embryonic heart formation. The epicardium harbors a population of progenitor cells that undergo epithelial-to-mesenchymal transition (EMT) displaying characteristic conversion of planar epithelial cells into multipolar and invasive mesenchymal cells prior to differentiating into non-myocyte cardiac lineages such as vascular smooth muscle cells, pericytes, and fibroblasts. The epicardium is also a source of paracrine cues that are essential for fetal cardiac growth, coronary vessel patterning, and regenerative heart repair. Although the epicardium becomes dormant after birth, cardiac injury reactivates developmental gene programs that stimulate EMT; however, it is not clear how the epicardium contributes to disease progression or repair in the adult. In this review, we will summarize the molecular mechanisms that control epicardium-derived progenitor cell migration, and the functional contributions of the epicardium to heart formation and cardiomyopathy. Future perspectives will be presented to highlight emerging therapeutic strategies aimed at harnessing the regenerative potential of the fetal epicardium for cardiac repair.

Subject Terms

Cell Biology/Structural Biology; Cell Signaling/Signal Transduction; Developmental Biology; Growth Factors/Cytokines; Myocardial Regeneration

Keywords

Epicardium; Cardiac Development; Fibrosis; Paracrine Signaling; Cardiac Repair and Regeneration

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Disclosures

None.

Introduction

Cardiovascular disease caused by ischemic injury remains the leading cause of death worldwide[1]. Millions of cardiomyocytes (CMs) are lost to myocardial infarction (MI), which rapidly triggers an innate immune response to clear dead or dying cells and activate collagen-producing myofibroblasts to remodel the extracellular environment[2]. Cardiac muscle damage is irreversible as adult mammalian CMs do not re-enter the cell cycle to replace ischemic tissue[3]. Over the last decade, several strategies have been employed to promote cardiac repair including stimulation of CM cell cycle re-entry, delivery of stem cells or stem cell-derived CMs, modulation of tissue repair using cell-free therapeutics, and applying viral vectors and bioengineered patches to deliver pro-regenerative factors[3–5]. However, it is becoming apparent that cardiac repair will require active contributions from numerous cardiac cell types.

The epicardium is an evolutionarily conserved layer of mesothelium covering the outermost cell layer of the vertebrate heart. During fetal development, the epicardium serves as a progenitor source, contributing multipotent cells that gives rise to cardiac mesenchyme. However, the role of the epicardium is not limited to *de novo* formation of non-myocyte lineages. This mesothelial layer also fosters a paracrine milieu critical for myocardial growth and coronary vessel patterning. As the heart matures, the epicardium undergoes a period of dormancy, functioning as a simple barrier between the myocardium and the pericardial cavity. However, recent studies in zebrafish and mice have elegantly shown that cardiac injury reanimates the epicardium and its derivatives to modulate tissue repair[6]. This review will summarize the dynamic roles of the epicardial lineage during heart development with a focus on developmental programs that may be harnessed to facilitate cardiac repair.

The Epicardium: From origin to a progenitor source

Formation of a functioning heart requires the assembly and integration of multiple progenitor cell populations that arise from embryonic structures such as the cardiogenic mesoderm, the cardiac neural crest, and the proepicardium (PE)[7]. The PE is an evolutionarily conserved and transient cluster of progenitor cells located at the venous pole of the fetal heart which has been described in many vertebrate species ranging from lampreys to humans[8–13]. PE-derived cells exhibit a migratory event at embryonic day (E)9.5 in mice and Hamburger-Hamilton stage 17–18 in chick, translocating from the PE and towards the atrioventricular canal (in chick) of the nascent heart. Here, PE-derived cells form a single cell layer of mesothelium, called the epicardium, that lines the outermost layer of the heart[11, 12, 14]. Here, PE-derived cells form a single cell layer of mesothelium, called the epicardium, that lines the outermost layer of the heart[11, 14]. Initially, the epicardium exhibits epithelial-like properties, serving as a barrier between the primitive myocardium and the pericardial space. However, a subset of epicardial cells delaminate from the epicardial sheet and invade the myocardium generating a majority of vascular smooth muscle cells (SMCs) and cardiac fibroblasts in the heart[15]. Thus, the epicardium is critical for providing support cells that contribute to myocardial integrity.

Epicardial Epithelial-to-Mesenchymal Transition

Epicardial-derived progenitor cells (EPDCs) arise from the epicardial layer through an epithelial-to-mesenchymal transition (EMT) event that initiates after Hamburger-Hamilton stage 18 in chick and approximately E12.5 in mice[16, 17]. In human, expression of EMT markers have been observed in the expanded epicardium surrounding the ventricles at fetal stage 3 (equivalent to E17.5–18.5 in mouse)[8]. EMT is an evolutionarily conserved process by which epithelial cells lose their apical-basal polarity and cell-cell adhesions, and acquire migratory and invasive characteristics akin to multipotent mesenchymal stem cells[18]. Progenitor cells that emerge from EMT often give rise to new cell lineages during organogenesis. Early studies in chick embryos employed dyes or retroviral vectors to label epicardial cells, which demonstrated the incorporation of EPDCs in cardiac mesenchyme and coronary vasculature[19–21]. Epicardial EMT and epicardial-derived cell fates (Figure 1) were subsequently described in mice using genetic fate-mapping approaches that indelibly label all progeny of Cre recombinase-expressing cells with beta-galactosidase (LacZ) or a fluorescent lineage reporter. This technology utilizes specific regulatory sequences to drive the expression of Cre in the epicardium of transgenic mice. Importantly, while it appears that no single gene exclusively marks the epicardium, several gene regulatory sequences have been used to trace epicardial cells and mesothelium-derived cells found in other tissues, including *Scx, Sema3d, Gata5, Tbx18, Tcf21* and *Wt1*. The use of tamoxifen-inducible Cre alleles (Cre recombinase fused to a mutant estrogen ligand-binding domain or CreERT2) has also provided temporal control to study the timing of epicardial EMT and to fate map descendants of the epicardium[22]. Tables 1 and 2 summarize the current genetic animal models available and the cells and tissues labeled by each marker.

Molecular Regulation of Epicardial EMT

Transcription factors (TFs) mediate transdifferentiation of epithelial cells into a mesenchymal phenotype through the repression of genes encoding epithelial adhesion molecules (E-cadherin, claudins, and occludens) and activation of mesenchymal genes (N-cadherin, collagens and fibronectin) necessary for ECM production and migration[18]. Here, we will highlight known transcriptional regulators, as well as upstream growth factor responsive pathways, implicated in epicardial EMT (Figure 2).

Wilms Tumor-1 (Wt1) is a zinc finger TF that is highly expressed in the epicardium during heart development and downregulated concomitant with epicardial EMT[23, 24]. Global or conditional deletion of *Wt1* in mice results in embryonic lethality during mid to late gestation and hearts display defective epicardial EMT and EPDC invasion, which leads to myocardial hypoplasia and abnormal coronary vascular plexus coverage[25, 26]. These defects may be attributed to altered transcription as WT1 regulates numerous targets during EMT[26–29], including activation of *Snai1* which represses the expression of epithelial *Cdh1* (E-cadherin)[25]. Wt1 has also been shown to act upstream of canonical Wnt/β-catenin and retinoic acid (RA) signaling, both critical for epicardial biology and EMT[17]. Consequently, loss of β-catenin in the epicardium disrupts adherens junctions and cell polarity, ultimately impairing EPDC migration and coronary artery formation[17, 30].

Tcf21 (Pod1/Epicardin/Capsulin) is a class II basic helix-loop-helix TF highly expressed in the PE and epicardium[31]. *Tcf21*-depleted *Xenopus* embryos fail to form a cohesive sheet of mesothelial cells lining the heart, suggesting that Tcf21 is critical for PE migration and proper formation of the epicardium[13]. In mice, Tcf21 regulates epicardial EMT as evidenced by downregulation of *Snai1, Sox9*, and *Zeb1* in *Tcf21* null epicardial explants[32]. The exact mechanisms linking *Tcf21*-dependent transcription to epicardial homeostasis and EMT remain unclear, although interactions with transcriptional co-repressors and direct binding to E-box consensus sequences (CAnnTG) may contribute to these processes[13, 33].

Regulation of EMT and motility gene programs by Hippo signaling and myocardin-related transcription factors (MRTFs)/Serum Response Factor (SRF) are required for the invasion of EPDCs into the myocardium. Yap (*Yap1*) and Taz (*Wwtr1*) are evolutionary conserved co-factors negatively targeted by the Hippo pathway to restrain organ size[34]. In addition to regulating contact inhibition, cell proliferation and stem cell self-renewal, Yap and Taz also mediate EMT in various contexts[34]. Conditional deletion of *Yap* and *Taz* using the $Wt1^{CreERT2}$ allele diminishes the expression of multiple EMT associated TFs including *Snai1, Snai2, Twist1, Wt1* and *Tbx18*[35]. Interestingly, the Hippo pathway must be tightly controlled in the epicardium as *Yap/Taz*-deficient (active Hippo) or *Lats1/2*-deficient (inactive Hippo) murine EDPCs exhibit defects in cell migration and fibroblast differentiation[35, 36].

SRF is a TF that is essential for the formation of the PE and epicardial EMT[37, 38]. SRF binds to a DNA motif called a CArG box [$CC(A/T)_6GG$], activating gene programs related to actin cytoskeletal rearrangements, cell adhesion and migration, and vascular cell commitment[37–40]. Nuclear SRF in epicardial cells corresponds with increased levels of smooth muscle genes, whereas expression of dominant negative SRF inhibits smooth muscle formation[38]. SRF transcriptional activity is greatly enhanced by interactions with the MRTF family of signal responsive co-factors[41]. MRTFs are tethered in the cytoplasm via interactions with monomeric actin, which conceals a nuclear localization signal[42]. Mechanical stretch or pathways that activate Rho-kinase and actin polymerization lead to nuclear accumulation of MRTFs and activation of SRF-dependent gene programs[42]. MRTF deletion using the $Wt^{CreERT2}$ allele results in decreased migration of EPDCs during developmental EMT coupled with delayed maturation of the coronary plexus[43]. These findings highlight the importance of signal responsive transcriptional regulation in epicardial EMT and EPDC differentiation. Generally, EPDCs display heterogeneous expression of epicardial TFs in both the mouse and chick epicardium[44]; thus, the diverse epicardium-derived lineages described in this review may arise in part due to intrinsic differences in EPDCs that modulate the response to paracrine cues.

Growth Factor-Dependent Regulation of Epicardial EMT

Transforming growth factor-β (TGFβ) is among the most well-studied upstream regulators of EMT and ECM production[45]. TGFβ1–3 are all expressed in the mouse epicardium by E12.5[46] and targeted deletion of type I TGFβ receptor *Alk5* using the chicken *Gata5* enhancer *(cGata5)-Cre* perturbs epicardial EMT, leading to myocardial hypoplasia, and attrition of SMCs and microvascular structures[47]. Likewise, global deletion of type III TGFβ

receptor (*Tgfbr3*$^{-/-}$ mouse model) abrogates the coronary plexus, which is attributed to dysregulated nuclear factor kappa-light-chain enhancer of activated B cells signaling in the epicardium and defects in bone morphogenetic protein 2-mediated EPDC motility[48, 49]. Mouse and human epicardial explants stimulated with TGFβ1 and/or TGFβ2 lose epithelial markers (Tight junction protein Zonula occludens-1 and Cytokeratin) and upregulate SMC markers (α-smooth muscle actin, Transgelin or SM22, Calponin, and Caldesmon) indicative of EMT[50, 51]. TGFβ2 stimulation also amplifies epicardial motility by indirectly stimulating expression of *hyaluronan synthase 2* (*Has2*) and the production of the ECM component hyaluronan (HA)[52].

Platelet-derived growth factor (PDGF) signaling is ubiquitous during mouse cardiogenesis and regulates critical processes such as cardiac neural crest cell recruitment to the outflow tract, coronary vessel maturation and myocardium development[53–55]. PDGF receptor (PDGFR)α and PDGFRβ are receptor tyrosine kinases that play critical roles in regulating epicardial EMT and EPDC specification[56]. Epicardial cells null for *Pdgfra/b* are unresponsive to pro-EMT stimuli (TGFβ, fibroblast growth factor-2, and PDGF-BB) and express low levels of EMT TFs (*Snai1* and *Sox9*) and mesenchymal genes[56]. Defects in EMT can be rescued by forced overexpression *Sox9* in PDGFR mutant cells, suggesting that Sox9 is downstream of PDGFRα/β signaling[56]. Conditional or global deletion of each receptor separately or together, using *cGata5-Cre* or *WT1*^{CreERT2} alleles, limits EPDC motility by disrupting phosphoinositide 3-kinase-mediated regulation of actin polymerization[56–58].

Epicardium-Derived Interstitial Lineages

Various cardiac interstitial cell lineages, including fibroblasts, vascular mural cells, and to a limited extent, endothelial cells (ECs), arise from the epicardium (Figure 1).

Cardiac fibroblasts

Lineage tracing and reporter mouse lines such as *Tcf21*$^{MerCreMer/+}$;*Rosa26*^{EGFP}[59], *Collagen1a1(Col1a1)-GFP*[60, 61] and *PDGFRα*$^{GFP/+}$[62] facilitate the identification of resident cardiac fibroblasts originating from EPDCs[63], which are especially populous in regions near the sinoatrial node, atrioventricular cushions and valves, and annulus fibrosis[64–67]. Notably, Tcf21 controls the specification of EPDCs to SMC or fibroblast lineages during murine embryonic development[68]. *Tcf21* null hearts have fewer Col1a1-GFP^{+} fibroblasts while the number of SMCs remain unaltered[32]. In contrast, activation of *Tcf21* through exogenous RA to embryonic chick hearts reduces the number of SMCs[44]. In addition to Tcf21, upstream cues such as PDGF signaling can influence cardiac fibroblast cell fate. PDGFR isoforms display overlapping expression patterns in the early stages of epicardial formation but become distinct by E13.5 in murine models[56]. *Pdgfra* loss-of-function impairs EPDC differentiation into cardiac fibroblasts while conditional deletion of *Pdgfrb* impacts the vascular SMC lineage[56]. These findings suggest that PDGFRα and PDGFRβ regulate distinct epicardium-derived developmental trajectories.

Vascular mural cells

Vascular SMCs lining the coronary arteries were first demonstrated to arise from PE-derived cells in avian models[19]. These findings were later substantiated in mice using Cre-dependent tracing of cells originating from *Tcf21*[56], *Wt1*[15] and *Tbx18*[69]-expressing progenitors. Tbx18 belongs to a large family of T-box TFs that regulate a variety of developmental processes in vertebrates and invertebrates and is enriched in the PE, epicardium, subepicardial mesenchyme, septum transversum, and a subset of the myocardium[70–72]. Tbx18 is dispensable for epicardial development and EMT; however, loss of *Tbx18* results coronary vessel maturation defects[70, 73]. Forced expression of *Tbx18* prevents SRF-dependent activation of smooth muscle reporters[73]. Conversely, conditional misexpression of *Tbx18VP16* (fusion of the *Herpes simplex virus* encoded protein VP16 to the C-terminus of Tbx18) results in hypoplastic hearts and ectopic smooth muscle differentiation mediated by Notch3 and TGFβ signaling[74, 75]. Mature SMCs (Smooth muscle myosin heavy chain, also known as MYH11^{+}) are reported to arise via the differentiation of *Tbx18* lineage-derived pericytes[76], which are stellate-like cells that encapsulate the microvasculature and preserve vascular integrity. Epicardium-derived cardiac pericytes, as defined by expression of PDGFRβ and chondroitin sulfate proteoglycan 4 (also known neuron-glial antigen 2), are required for the stability and growth of the embryonic coronary plexus[43, 77]. Indeed, EMT deficiency associated with genetic deletion of MRTFs in the epicardium depletes the heart of cardiac pericytes, leading to reduced vascular integrity and sub-epicardial hemorrhage[43].

Vascular endothelial cells

Coronary ECs are predominately derived from non-epicardial sources[78]. Lineage tracing studies in mice have excluded epicardial-derived ECs from *Tbx18*, *c-Gata5*, or *Tcf21* lineage-derived EPDCs[32, 69, 79]. However, *Wt1*-derived epicardial cells have been shown to give rise to ECs in the developing chick embryo[21] as well as EPDCs fate mapped from the *Wt1^{CreERT2}* lineage after administration of tamoxifen at E10.5 or early postnatal time points in murine models[15, 80]. However, the contribution of the epicardium towards the EC fate has been challenged by reports of endogenous *Wt1* expression in mature endothelium beginning at E12.5 and persisting throughout postnatal mouse development[81]. Scleraxis (*Scx*) and Sema3D positive epicardial cells form rare populations of ECs validated by *in vivo* fate mapping and *in vitro* cultures[82]. While the *Sema3D^{GFPCre}*, *Scx^{Cre}*, *Wt1^{CreERT2}*, and *G2-Gata4^{Cre}* epicardial Cre lines[82, 83] may sporadically label the endothelium, the majority of cardiac ECs are derived from the sinus venosus and endocardium[77].

The Epicardium-Derived Secretome

The previous sections highlight the complexity of epicardial EMT and epicardium-derived cell fate determination, which are controlled by both cell autonomous and cell non-autonomous mechanisms. The following sections will discuss some of the intercellular signaling cascades underlying paracrine regulation of CM and vascular development and homeostasis (See Figure 2).

Epicardium-Cardiomyocyte Crosstalk

In addition to directly differentiating into various cell lineages, the epicardium also supports CM proliferation and embryonic heart growth by providing a cocktail of secreted mitogens. Fibroblast growth factors (FGFs) FGF1, 2, 4, 6, 9, and 16 are all expressed by the fetal epicardium[84, 85]. In particular, both endocardial and epicardial FGF9 signals regulate CM proliferation via activation of fibroblast growth factor receptors (FGFR) FGFR1c and FGFR2c[84]. In contrast, FGF10 secreted from the myocardium promotes EPDC invasion and EPDC-to-fibroblast conversion by signaling through FGFR2b expressed on epicardial cells[86]. The mitogenic activity of CMs can be stimulated by the secretion of insulin growth factor-2 (IGF2) from epicardial cells *in vitro*, a phenomenon that is recapitulated through the pro-proliferative signals emanating from the developing epicardium *in vivo*[87, 88]. In fact, global deletion of *Igf2*, or conditional deletion of its receptor *Igfr1* in *Nkx2.5* expressing CMs, results in severe cardiac defects in the form of ventricular non-compaction[88, 89].

RA signaling is critical for proper cardiac morphogenesis[90, 91] and can induce the expression of epicardial-restricted TFs such as *Wt1* and *Tcf21*[44]. RA regulates transcription by binding to nuclear retinoic acid receptors which form heterodimers with retinoid X receptor (RXR)[92]. $RXR\alpha^{-/-}$ mice display normal cardiac mouse development up to E11.5; however, thereafter fail to undergo proper myocardial compaction and become severely hypoplastic[90, 91]. RA signaling stimulates epicardial secretion of trophic signals such as WNT9A, FGF2, FGF9 and IGF2 to promote myocardial growth and proper coronary vessel maturation[79, 84, 87, 88]. RA is synthesized by the oxidation of retinaldehyde by retinaldehyde dehydrogenases (Raldh 1, 2 and 3)[92], which are highly expressed in the fetal mouse epicardium at E12.5[93]. Global deletion of *Raldh2* results in embryonic lethality around E9.5 with a single dilated cardiac chamber[94]. Remarkably, this phenotype can be partially rescued by maternal supplementation of exogenous RA[94].

Epicardium-Endothelial Cell Crosstalk

The epicardium is also an important source of chemokines that regulate coronary vessel patterning. Prevention of pro-epicardial outgrowth, and genetic ablation of epicardial cells with diphtheria toxin results in disorganized coronary vasculature and reduced recruitment of sub-epicardial cardiac macrophages[95, 96]. *Wt1* knockout cells also express lower levels of vascular growth factors such as angiopoietin-1 and vascular endothelial growth factor A (VEGF-A)[26], and *Wt1* is needed to suppress the production of pro-inflammatory cytokines chemokine ligand 5 and C-X-C motif chemokine ligand (CXCL) 10 in epicardial cells[28]. Thus, the epicardium serves as an essential secretory hub to maintain vascular and inflammatory cell stability in the fetal heart. ECs also communicate with the developing epicardium by facilitating the induction of EMT and differentiation towards mural cell lineages via activation of PDGFRβ[97]. Pericyte recruitment to the microvasculature is coordinated in part by the expression of the Notch ligand Jagged-1 in ECs activating Notch3 receptors on epicardial-derived pericytes[76]. CXC chemokines and receptors (CXCR), such as the CXCL12-CXCR4 axis has been shown to play an essential role in coronary artery maturation[98]. *Cxcr4* in zebrafish is expressed in angiogenic ECs and mature coronary arteries lined by mural cells, whereas *cxcr4* deletion results in disorganized angiogenic sprouts[98]. In contrast, *Cxcl12* is expressed in the epicardium and epicardial-derived mural

cells in both zebrafish and mouse models[98, 99]. Although localized expression of CXCL12 is sufficient to increase capillary density through the recruitment of CXCR4$^+$ ECs, global myocardial overexpression disrupts vascular formation[98]. These findings indicate that discrete gradients of chemotactic cues are required during the intricate process of coronary vessel formation.

Overall, these data support a critical cell non-autonomous role for the epicardium in the fetal heart. Thus, the role of epicardial EMT appears to be two-fold: 1) Contribution of multipotent progenitors and 2) Secretion of growth factors necessary for embryonic heart formation (Figure 1 and 2). Harnessing fetal epicardium programs that build the developing heart may have therapeutic implications for the stimulation of scarless cardiac repair through regenerative medicine, which is discussed below.

Overview of Cardiac Repair and Regeneration

Advances in emergency medicine and cardiology have led to an abundance of patients surviving a MI. However, these successes have revealed deficiencies in supporting cardiac repair after an initial injury, which results in a growing population living with chronic heart disease. Adult mammalian CMs are postmitotic with a limited capacity for self-renewal following acute insult or over periods of chronic disease[100]. Therefore, progressive loss of CMs via apoptotic or necrotic cell death leads to the replacement of damaged contractile tissue with a fibrotic scar, irreversible cardiac remodeling and organ failure[2]. Recent studies have highlighted that CM renewal can be enhanced through molecular signaling pathways converging on the induction of critical cell cycle regulators (e.g. Cyclin A2), growth factors (Neuregulin1, IGF1/2), and intrinsic signaling pathways (e.g. Hippo)[3]. Therefore, understanding endogenous cardiac repair mechanisms is an emerging topic of intense investigation that may lead to new regenerative medicine strategies.

Reactivation of the Adult Epicardium

Fetal epicardial gene activation after myocardial injury is thought to represent a conserved cardiac repair mechanism[101]. *Wt1*, *Tbx18*, *Raldh1* and *Raldh2* transcripts are all transiently upregulated between 1 to 5 days after MI in mice, particularly surrounding the valves and atria, and wane in expression by 2 to 4 weeks after injury[102, 103]. Fetal epicardial gene induction correlates with increased proliferation and pronounced epicardial thickening, as documented in both adult mice and canine models[102, 103]. Unlike the fetal epicardium, which undergoes EMT and generates cardiac fibroblasts and vascular mural cells, lineage tracing experiments reveal minimal contribution of the epicardium to new adult fibroblasts or to collateral formation (Figure 3). In contrast, the adult epicardium is thought to serve as a source of paracrine signals with limited regenerative potential[103]. Thus, an emerging area of investigation includes developing methods to harness epicardial-derived developmental programs to promote cardiac repair and regeneration in adult hearts. The remainder of this review will highlight what is currently known regarding the role of the epicardium in cardiac regeneration and disease, and how we may take advantage of EPDC plasticity and signaling to promote efficient cardiac repair and remodeling and advance regenerative based medicine for the treatment of human cardiac disease.

Pre-existing Fibroblasts Contribute to Cardiac Fibrosis

The developmental origins of a majority of cardiac fibroblasts in the adult heart can be traced back to the fetal epicardium[32, 56]. These fibroblasts make up ~20% of the total composition of cells in the mature mammalian heart[104], however, mechanical and/or ischemic stress leading to the loss of CMs and vasculature can increase the total numbers and volume of fibroblasts following cardiac injury. Resident cardiac fibroblasts labeled with *Col1a1-GFP, PDGFRα* and *Tcf21* transgenic alleles display the ability to proliferate after injury and give rise to a majority of cells in the fibrotic scar[32, 105]. Myofibroblasts, labeled by Periostin (Postn), emerge from Tcf21 lineage-traced epicardium-derived fibroblasts when mice are subjected to MI or left ventricle pressure overload and are a major source of ECM[105, 106]. Alterations in fibronectin (FN), mitogen-activated protein kinase, or Wnt/β-catenin signaling in the Tcf21 lineage significantly alters fibroblast to myofibroblast conversion in response to cardiac stress[107–109]. Interestingly, it was proposed that subpopulations of myofibroblasts re-express Tcf21 following cardiac injury[105], which may indicate that a subset of myofibroblasts revert towards quiescence and/or senescence in chronic stages of disease. Although pre-existing resident cardiac fibroblasts originating from the epicardium during development are the primary source of cardiac scar tissue during ischemic and non-ischemic remodeling, nascent EPDCs and resident or infiltrating inflammatory cells have also been observed within the scar tissue, albeit to a lesser extent[60, 110] (Figure 3). *Tcf21*, *Tbx18* and *Wt1* serve as markers of both developmental and injury-induced epicardial-derived fibroblasts in zebrafish and mammalian adult hearts[60, 105, 110–112]. In both the human and adult mouse heart, fibroblasts expressing Wt1, Tbx18 and Tcf21 proteins are observed within interstitial fibrosis following ischemic injury[110]. In contrast, perivascular fibrosis was primarily composed of $Tcf21^+$ fibroblasts, but not $Wt1^+$ or $Tbx18^+$ cells, potentially indicating heterogeneity within epicardial derived fibroblast lineages. While this study depended on the endogenous protein labeling of epicardial markers, it is interesting to speculate that spatial diversity of epicardial-derived lineages may contribute to functional differences in cardiac repair and remodeling.

In addition to the *Tcf21* and *Postn* lineages, robust investment of *Wt1* lineage fibroblasts have been observed in the scar after cardiac injury. Tamoxifen-inducible tracing via *Wt1*-directed CreERT2 in the adult mouse heart reveals that EPDCs have enriched expression of *Snai1, Snai2* and *Smad1* and display a propensity towards secretion of pro-angiogenic factors as compared to non-EPDCs following MI[102, 103]. Re-activated *Wt1*-derived cells acquire a phenotype consistent with fibroblast and myofibroblast lineages as evidenced by the expression of FN, fibroblast-specific protein 1, collagen type III alpha 1, and smooth muscle markers, but are generally devoid of endothelial or cardiomyogenic cell markers[103]. Although the contribution of new $Wt1^+$ cells towards various intramyocardial fates is limited in the adult, the *Wt1* lineage originating during development is the major source of mesenchymal cells within the heart during stress (Figure 3). The constitutive *Wt1-Cre* line labels >90% of the fibroblasts present in the left ventricular free wall at baseline and following trans-aortic constriction surgery (TAC) or MI[60, 113]. Additionally, 96% of the *Wt1-Cre* lineage cells expressed the ECM gene *Col1a1*[60]. Inhibition of embryonic *Wt1-Cre* lineage mobilization via deletion of *Srf* or *Mrtfs* led to a significant reduction in the number of epicardium-derived fibroblasts colonizing the post-MI infarct[114]. Interestingly, the WT1

lineage depleted scar was populated with PDGFRα$^+$ cells, apparently from an alternative source[114].

In complementary lineage tracing experiments, resident fibroblast composition within the uninjured heart is ~75% Tbx18$^+$ and 90% Wt1$^+$ [60, 112, 113]. Tbx18$^+$ / Thy1 cell surface antigen$^+$ fibroblasts are dispersed throughout the heart and express markers of fibroblasts, SMCs and cardiac pericytes[112], whereas *Wt1*-lineage cells are primarily localized to the ventricular septum and left ventricle free wall[60, 113]. Although injury typically increases the total number of fibroblasts in the heart due to proliferation, the percentage of either *Tbx18* or *Wt1*-derived fibroblasts were unaltered following TAC[60, 112]. This data may be interpreted to indicate resident fibroblasts may arise from the heterogeneous compilation of various developmental epicardial lineages.

Mesenchymal cell derivatives such as cardiac pericytes have also been implicated in fibrotic diseases. However, *Tbx18*-lineage derived cardiac pericytes do not exhibit mesenchymal cell characteristics *in vivo* after TAC or prolonged aging in mice[115]. Due to these inconsistencies, further investigation is required to rule out a potential role of pericytes in adult cardiac repair processes, particularly in vascular remodeling during ischemic events. Taken together, these studies also conclude that epicardial activation does not contribute to the formation of *de novo* fibroblasts during adult pressure overload or following MI. Overall, understanding the cellular origin and heterogeneity of cardiac mesenchymal cells may offer critical insights for ameliorating the proliferation and/or differentiation of pathological myofibroblasts during the progression of heart disease.

The Epicardium Modulates Inflammation

Acute inflammation is a compensatory response that clears tissue of dead cells; however, chronic inflammation can increase cell death, formation of fibrosis and cardiac dysfunction[116]. Recent reports suggest that cellular and paracrine inflammatory processes are in part coordinated by the re-activated epicardium[95, 117]. CD45$^+$ hematopoietic cells emerge during fetal development alongside Wt1$^+$ EPDCs, and both become activated and migrate towards the subepicardium upon cardiac injury[118]. Resident cardiac macrophages with reparative characteristics (M2 macrophages) are observed in the healthy epicardium, but are significantly depleted in models of aging related cardiac dysfunction[119]. *Wt1b* is an early epicardial response gene that overlaps with expression of pro-regenerative macrophages in zebrafish[120] implicating the requirement of the epicardium in the recruitment of cardiac macrophages as observed during development[95]. Indeed, epicardial deficiency in YAP/TAZ signaling exacerbates cardiac inflammation after MI due to the limited recruitment of immunosuppressive regulatory T cells and depressed secretion of inflammatory cytokine interferon-γ from the adult mouse epicardium[121]. Attempts to modulate epicardial activation have harnessed the activity of CCAAT/enhancer binding proteins, a group of TFs that bind to enhancer elements controlling epicardial gene expression[117]. Adenoviral delivery of CCAAT/enhancer binding protein following ischemia/reperfusion (IR) injury in mice enhances activation of *Wt1* and *Raldh2*, blunting cardiac damage and the initiation of fibrosis, presumably through decreased acute neutrophil infiltration[117]. These studies emphasize the importance of epicardium reactivation in

inflammatory signaling and the promise of harnessing epicardial cell biology for cardiac repair.

Epicardium Contribution to Subepicardial Adipose Tissue

The human heart is surrounded by a layer fat called epicardial adipose tissue (EAT) that lies beneath the epicardium. The extent of EAT has prognostic value and correlates with various pathological conditions such as coronary artery disease and ischemic remodeling[122]. While a better understanding of the developmental source and molecular mechanisms that control EAT accumulation may have therapeutic implications, the study of EAT biology has been hampered by its paucity in experimental model organisms. Advances in genetic lineage tracing in mice have recently revealed that EAT originates from the epicardium in a process mediated by the metabolic regulator peroxisome proliferator-activated receptor-γ[123]. Furthermore, delivery of IGF-1 to mice augments epicardial cell conversion into adipocytes in experimental MI[124].

Importantly, disorders such as arrhythmogenic right ventricular cardiomyopathy are characterized by fibro-fatty tissue deposition that replaces functional heart muscle and markedly disturbs cardiac conduction in human patients[125–127]. Recent evidence suggests that mutations in the Plakophilin-2 gene are causative in arrhythmogenic right ventricular cardiomyopathy through disruption of the CM desmosome[128]. In addition, Plakophilin-2 may modulate epicardial cell migration and initiate an adipogenic program in epicardial cells, potentially relevant due to the epicardium-to-endocardium gradient of fibrofatty tissue deposition[129]. Defining the cellular and molecular avenues leading to fibrosis and the formation of EAT may thus lead to new clinical treatment options to prevent progression of arrhythmogenic cardiomyopathies. Collectively, reactivation of the adult heart due to cardiac disease encourages cell contributions and paracrine signals from the epicardium to take part in fibrotic, adipogenic, and vasculature remodeling, CM growth and cell death, and inflammatory signaling (Figure 4). Harnessing the regenerative power of the fetal epicardium at the expense of insult-responsive pathological programs holds promise for the advancement of new cardiac restoration strategies, as described in regenerative models below.

Mechanisms of Cardiac Regeneration

Many adult amphibians and fish possess an incredible capacity to completely regenerate damaged tissue, including the heart[101]. Tissue repair in axolotl and zebrafish is initiated by the migration of multipotent mesenchymal cells to the wound and the formation a regenerative blastema[6]. Particularly in zebrafish hearts, surgical removal of ~10–15% of the cardiac apex results in the formation of a reversible fibrin clot followed by the remarkable restoration of cardiac muscle via the proliferation of pre-existing CMs[130]. Zebrafish heart regeneration occurs efficiently due to four essential processes: 1) efficient CM proliferation, 2) endocardium activation, 3) re-activation of epicardial fetal gene expression, and 4) efficient resolution of fibrosis[130, 131]. In contrast, the adult mammalian heart is unable to regenerate following injury; however, neonatal mammals retain the capacity to regenerate cardiac tissue following apical resection, cryoinjury, or MI when the injury is performed

prior to 7 days after birth, which is within the window when pre-existing CM proliferation can be stimulated[132, 133]. Non-myocytes, including epicardial cells, are reported to play critical roles in cardiac regeneration by regulating inflammation, stimulating CM proliferation and modulating ECM turnover[134].

The ECM Landscape in Regeneration

ECM modulates the fate and function of EPDCs in the embryo or during regenerative cardiac repair; and likewise, epicardial cells provide a unique extracellular milieu that mediate cardiac healing. In the healthy adult mouse heart, clusters of 2 or more $Wt1^+$ cells are encased within ECM components such as FN, Collagen IV and HA, which help to define an epicardial-specific niche[118]. In zebrafish, the epicardium supports CM recruitment to sites of injury via the secretion of *fn1* and *fn1b*, which presumably bind the FN receptor (*integrinb3*) on CMs and promote motility required for regeneration[135]. In addition to FN, HA can also promote regeneration. HA signaling through the hyaluronan-mediated motility receptor (Hmmr) is induced 3 days post resection of the zebrafish heart; blocking Hmmr or inhibiting HA synthesis suppresses CM proliferation and exacerbates scar formation[136]. Importantly, inhibition of the HA-Hmmr axis perturbs cell-to-cell anchoring via focal adhesion kinase, which limits the expression of EMT genes *snail1a, snail1b, snail2, and twist1*[136]. Collectively, these data demonstrate that epicardial ECM production and EMT are essential in processes of adult cardiac regeneration.

Paracrine Regulation of Zebrafish Cardiac Regeneration

Intercellular signaling between the myocardium, epicardium, fibroblasts and endocardium coordinate regenerative processes in the adult zebrafish heart. PDGF, FGF, IGF, and VEGF ligands influence CM growth and proliferation in development and are proposed to promote CM dedifferentiation and the upregulation of cell cycle regulators during regeneration[137–140]. Disruption of the epicardium can lead to a marked reduction in regenerative programs consistent with the requirement of cell non-autonomous and epicardial-secreted factors in CM proliferation. Based on initial microarray analysis of the early zebrafish cardiac wound, *pdgfa* and *pdgfb* ligands are both highly expressed in regenerating tissue[141]. Administration of PDGF-BB is sufficient to induce CM proliferation, whereas PDGF inhibition prevents CM DNA synthesis in culture[141]. Zebrafish hearts exhibit an upregulation of classical EMT regulators (*snai2* and *twist1b)* that correlate with induction of the mural cell marker gene, Pdgfrβ, while Pdgfrα, a fibroblast marker, was unchanged[140]. Inhibition of PDGFR following injury limits epicardial proliferation *in vivo* and further suppresses the expression of EMT (*snai2*) and mural cell (*acta2*) markers[141]. Notably, PDGFR inhibition leads to impaired coronary vessel formation signifying the requirement of PDGF signaling in the process of revascularization and regeneration in the zebrafish heart[140].

FGF signaling functions downstream of RA signaling and is necessary for fin repair and cardiac regeneration in zebrafish[138, 142]. Global deletion of *fgfr1* results in a reduction of $Tbx18^+$ EPDCs in the ventricle and correlates with increased incidence of CM hypoplasia[142]. Following acute injury, *fgfr2* and *fgfr4* are increased in EPDCs surrounding the atria whereas *fgfr4* is expressed adjacent to the wound and up to 30-days post

resection[138]. Suppression of FGFR signaling in the epicardium disrupts EMT and neovascularization of damaged tissue[138].

IGF was discovered as a potent mitogen during cardiac development[87]. Activated *igf2b* is observed in the endocardium and epicardial surface and surrounding apical wounds of the adult injured zebrafish heart[117, 139]. Limiting *igf2* or *igf1r* suppresses DNA synthesis in CMs following resection[139]. Further examination of CM proliferation using the *gata4:EGFP* reporter confirms that IGF signaling is required for the proliferation of subepicardial CMs during wound repair[139]. Notch receptor (*notch1a, notch2, notch3*) expression is upregulated in both the epicardium and endocardium; Notch activity is required for regenerative replacement of CMs, but is dispensable for the formation of primitive endothelial tubes in the wound area[143]. The epicardium is also a source of soluble factors that control CM and vasculature regeneration through the secretion of *vegfaa*[137]. Interestingly, PDGF, FGF and VEGF ligands interact with Neuropilin transmembrane receptors, which are activated in response to cryoinjury in both endocardial and epicardial cells[144]. Loss-of-function of *neuropilin1a* significantly impairs *Wt1*-induced epicardial reactivation and EMT following cryoinjury[144].

Of note, the epicardium itself has a regenerative capacity. Deletion of the epicardium in the adult zebrafish resection model significantly reduces CM proliferation and regeneration[134]. Hedgehog signaling, expressed primarily in the bulbus arteriosus (analogous to the mouse outflow tract), is required for epicardial activation and regrowth[134]. Indeed, removal of the bulbous arteriosus impairs epicardial activation and migration towards the ventricle, as well as cardiac regeneration after injury, an outcome that can be reversed after implantation of Sonic hedgehog soaked beads in the ventricle[134]. Overall, studies directed at identifying the instructive cues to support the replenishment and recruitment of epicardial cells may lead to novel therapeutic strategies to enhance epicardium-mediated cardiac repair.

Epicardial-Derived Lineages in Zebrafish Regeneration

In zebrafish, *tcf21* is highly expressed in epicardial precursors that give rise to perivascular cells during development and regeneration[111]. Genetic ablation of *tcf21*$^+$ EPDCs results in prolonged fibrin clot deposition and collagen expression negatively impacting CM turnover and revascularization after injury[134]. However, even in cases where 80% of Tcf21$^+$ cells are depleted from the epicardium, Tcf21$^+$ cells re-populate the surface of the heart within 60 days post amputation indicating the endogenous reparative capacity of the epicardium[134]. Changes in mechanical tension have been recently shown to facilitate regeneration of the epicardium following ablation[145]. Utilizing a strategy to deplete *tcf21* in zebrafish[134], the migratory edge of the regenerating epicardium was populated by hypertrophic and multinucleated leader cells, which subsequently underwent apoptosis allowing for the persistence of mononucleated epicardial cells in regenerative tissue[145]. This study also indicated that mechanical signaling can regulate epicardial regeneration such as through modification of cellular behavior including cell death, nuclear polarity and migration; all factors that may significantly alter cellular processes of differentiation and/or secretion during cardiac repair.

Pan-epicardial reporters *wt1a* and *wt1b* have further revealed that the epicardium contributes to fibroblast plasticity in zebrafish heart regeneration[131]. The *wt1b* lineage reporter labels cells present at the epicardial border that contribute to fibroblastic *collagen1a2*$^+$ and *postn*$^+$ cells in response to cryoinjury[131]. Additionally, *wt1b*$^+$;*postn*$^+$ cells revert towards a quiescent state as evidenced by the noticeable downregulation of ECM genes, a process that appears to be conducive to CM proliferation[131]. This transient fibrosis mediated by cardiac fibroblast plasticity should be further examined in order to identify mechanisms that might lead to resolution of fibrosis during cardiac remodeling in adult mammalian models. In summary, the combination of *in vivo* and *in vitro* studies presented here contribute to the understanding of EPDC responsiveness and secretion of growth factors that impact potential downstream signaling programs associated with enhanced cardiac regeneration (Figure 5).

Mechanisms of Postnatal Heart Regeneration

Neonatal mammalian CMs demonstrate preserved cell cycle activity underlying the endogenous regenerative capacity of the mouse heart in the first week of life[132]. Apical resection (~15% of the ventricle) or permanent ligation induced-MI at postnatal day 1–2 results in complete regeneration by three weeks post injury due to increased CM proliferation[132]. Like zebrafish, acute upregulation of *Wt1* and EMT marker genes are detected following apical resection and cryo-injury, as well as permanent coronary artery ligation in the neonatal mouse heart[132, 146]. More recently, Tbx18$^+$ cell accumulation was observed in the neonatal heart following apical resection; however, *Tbx18* lineage-traced cells display minimal differentiation potential[147]. Despite the limited regenerative capacity of the adult EPDCs, recent studies have been aimed at discovering molecular and cellular programs that enhance and/or extend regeneration beyond the early postnatal window by preventing CM cell cycle arrest and limiting fibroblast activation. Previous reports in development show that FGF10 is critical for CM maturation[86], and although FGF10 treatment promoted the expansion of epicardial cells in the neonatal heart, FGF treatment did not significantly enhance cardiac regeneration[148]. However, pre-treatment of neonatal cardiac tissue with Thymosinβ4, which has been previously shown to enhance revascularization and cardiomyogenesis in the adult[149, 150], stimulated regeneration following resection at 7 days post birth, mediated in part by the enhanced migration and differentiation of Wt1$^+$ cells in the wound[151]. In addition to defining factors that have effective and combinatorial influence on CM proliferation and the prevention of fibrosis, stimulation of neoangiogenesis during neonatal cardiac regeneration has been largely overlooked. We previously described the importance of the positional cues stemming from the epicardium that can regulate the formation of the coronary vasculature[98, 99]. Similar to studies observed during cardiac development, efficient neonatal regeneration is prevented in models exhibiting *Cxcl12* deletion[152]. Exogenous CXCL12 administration facilitated arterial cell re-organization and collateral artery formation 14 days post-MI in the adult mouse heart, although adult cardiac physiology was not evaluated in this study[152].

Recruitment of inflammatory cells may also play a critical role in stimulation of angiogenesis and scar resolution in neonatal heart regeneration. Clodronate liposome-mediated depletion of monocytes and macrophages during the postnatal regenerative window augments fibrotic remodeling and impairs neovascularization in both peri-infarct

and distal regions[153]. Transcriptional analysis of macrophages between postnatal day 1 and day 21 suggests that loss of regenerative potential with time is associated with macrophage polarization and expression of anti-inflammatory factors[153]. However, these studies suggest that in addition to immune cells, a combination of cardiac cells, including ECs and epicardial cells, likely contribute secretory factors required for cardiac repair. Here, we presented some of the factors that may be harnessed to prolong the postnatal regenerative window, which may be adapted to induce cycling of adult CMs and/or stimulation of angiogenic processes that are needed to promote repair in human diseased hearts.

Future Perspectives

In this review, we have discussed the gene programs that regulate cardiac growth and tissue repair through both cell autonomous and cell non-autonomous functions of the epicardium. Our broad analysis describes the study of epicardial-secreted factors such as PDGFs, IGFs or Thymosin-β4, and the modulation of inflammatory factors that might enhance epicardial mediated cardiac repair[56, 88, 150]. Additionally, the development of therapeutic strategies that re-direct EPDCs towards pro-regenerative cells types at the expense of fibroblast and adipocyte differentiation could complement current regenerative medicine approaches that are largely based on stem and induced pluripotent cell delivery.

Enhancement of endogenous cardiac repair via the isolation, expansion and transplantation of progenitor cells derived from bone marrow and cardiac niches have seen limited success. Methods to derive primary EPDCs from zebrafish, mouse and human hearts have been validated and may lead to the creation of novel progenitor cell sources for therapies in the future[51, 154, 155]. Adult human EPDCs recapitulate EMT-like processes of fetal human EPDCs, including the ability to migrate and differentiate into mural cell lineages[154]. However, the promise of cell transplantation for regenerative purposes can be hampered by immune rejection if the donor is not autologous to the recipient. Recent advances in human induced pluripotent stem cell technologies have demonstrated the feasibility of differentiating pluripotent cells into epicardial cells and their derivatives through the modulation of bone morphogenic factor, Wnt, FGF and RA signaling pathways[156]. Co-culture of human embryonic stem cell derived epicardial cells (hESC-EPDCs)with hESC-derived CMs (hESC-CMs) was shown to promote cardiac contractility and CM maturation in engineered heart tissues[157]. Furthermore, co-transplantation of hESC-EPDCs and hESC-CMs into infarcted rat hearts enhances engraftment, improves systolic function and augments revascularization compared to hESC-CMs alone[157], making promising strides towards therapeutic practice.

Recent developments in evaluating epicardial activation and lineage commitment in various cardiac disease models indicate that cardiac regeneration is enhanced by facilitating cross talk between the epicardium and ECs or CMs. Replicating endogenous cross talk was accomplished via epicardial cell conditioned media delivery to the surface of the heart in a three-dimensional collagen patch, which promotes CM maturation and improves cardiac function after MI[158]. Furthermore, this study identified Follistatin-like 1 as the primary and potent epicardial cardiogenic factor to enhance cardiac function and repair after patch implementation[158]. Follistatin-like 1 treatment results in reduced fibrosis, improved re-

vascularization and increased CM proliferation in both adult mouse and porcine pre-clinical models indicating an applicability of epicardial-derived paracrine factors in future clinical practices[158]. Cell-free therapeutics, such as synthetic modified RNA and small molecules can promote the expression of pro-regenerative genes in multiple cell types including the epicardium[124, 149]. Delivery of a modified RNA expressing VEGF-A activates EPDCs and promotes their migration and differentiation towards EC fate after cardiac injury[159]. Therefore, as mentioned throughout this review, knowledge of upstream growth factor signaling can be utilized to enhance epicardial cell expansion *in vitro* or the generation of novel therapeutic strategies for clinical practice.

Finally, important questions remain regarding epicardial cell heterogeneity, which may complicate strategies to harness the epicardium for cardiac repair or may lead to identification of rare or unique EPDCs that are particularly amenable to pro-regenerative approaches. Transcriptional profiling of the epicardium using bulk and single cell RNA-sequencing platforms (RNA-seq or scRNA-seq, respectively) can prove useful in identifying genes and pathways that respond to cardiac injury. RNA-seq performed on laser captured epicardial tissue identified a number of markers that distinguish non-injured and injured epicardial cells[160]. In zebrafish, the Tcf21 lineage appears to be comprised of a number of distinct epicardium-derived cell populations[161]. This study reveals the complex heterogeneity within the Tcf21-lineage and encourages the use of single cell transcriptomics to study progenitor cell biology effectively in various model organisms and patient samples. A more comprehensive analysis of epicardial cell contributions to normal development, congenital cardiomyopathy, and heart failure, and may reveal unanticipated mechanisms that can be harnessed to improve cardiac regeneration and repair.

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Non-Standard Abbreviations

αSMA	alpha smooth muscle actin
CCL	CC chemokine ligand
CM	cardiomyocyte
Col1a1	collagen1a1
CXCL	chemokine receptor ligand
EAT	epicardial adipose tissue
EC	endothelial cell
ECM	extracellular matrix

EMT	epithelial-to-mesenchymal transition
EPDC	epicardium-derived progenitor cell
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
HA	hyaluronan or hyaluronic acid
IGF	insulin growth factor
IGFR	insulin growth factor receptor
MI	myocardial infarction
MRTF	myocardin-related transcription factor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PE	proepicardium
Postn	periostin
RA	retinoic acid
Raldh	aldehyde dehydrogenase
RXR	retinoid x receptor
Scx	scleraxis
SMC	smooth muscle cell
SRF	serum-response factor
TAM	tamoxifen
Tbx18	T-box protein 18
TF	transcription factor
TGFβ	transforming growth factor-β
VEGF	vascular endothelial growth factor
VIC	valve interstitial cell
WT1	wilms tumor-1
YAP	yes associated protein 1

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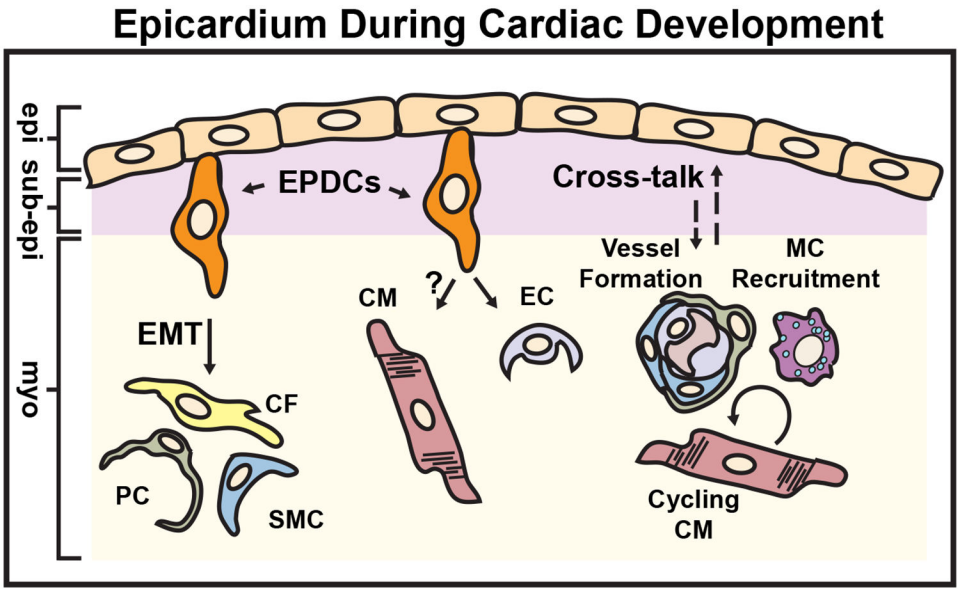

Figure 1. Epicardium During Cardiac Development.

Epicardium-derived progenitor cells (EPDCs) emerge from the fetal epicardium through the process of epithelial-to-mesenchymal transition (EMT) and contribute to various cardiac lineages such as cardiac fibroblasts (CFs), smooth muscle cells (SMCs) and pericytes (PCs). The ability of EPDCs to differentiate into endothelial cells (ECs) *in vivo* is limited, and CMs are not generally thought to derive from EPDCs. In addition to cellular contributions, the epicardium participates in reciprocal paracrine signaling (dashed arrows) to stimulate CM proliferation, macrophage (MC) recruitment and coronary vessel growth and maturation. epi = epicardium, sub-epi = sub-epicardium, myo = myocardium. (Illustration credit: Ben Smith)

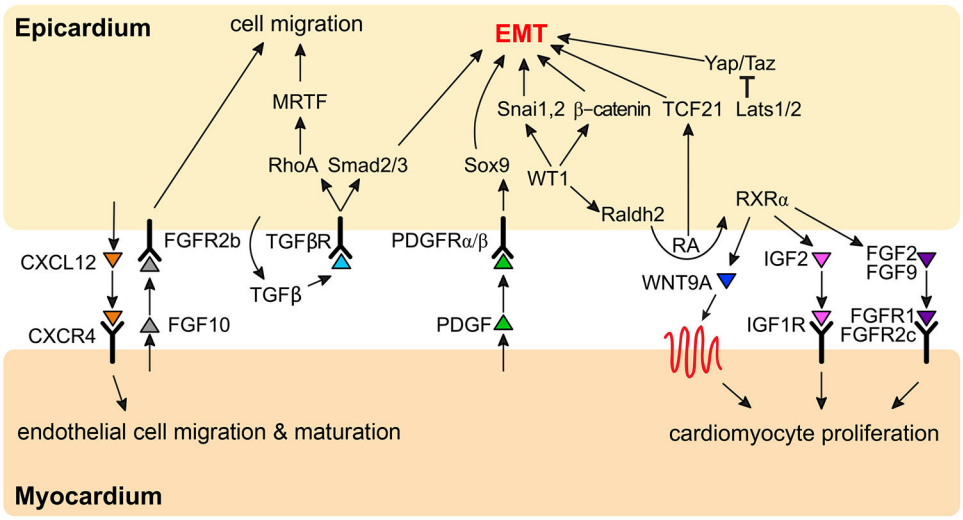

Figure 2. Molecular Control of Epicardial EMT.

Intrinsic and extrinsic molecular programs regulate epicardial epithelial-to-mesenchymal transition in mice, which include the regulation of transcription factors and molecular signaling. Cytokine mediated signaling between the epicardium and cardiomyocytes is reported to stimulate heart growth, and epicardium-derived signals support the growing coronary vasculature.

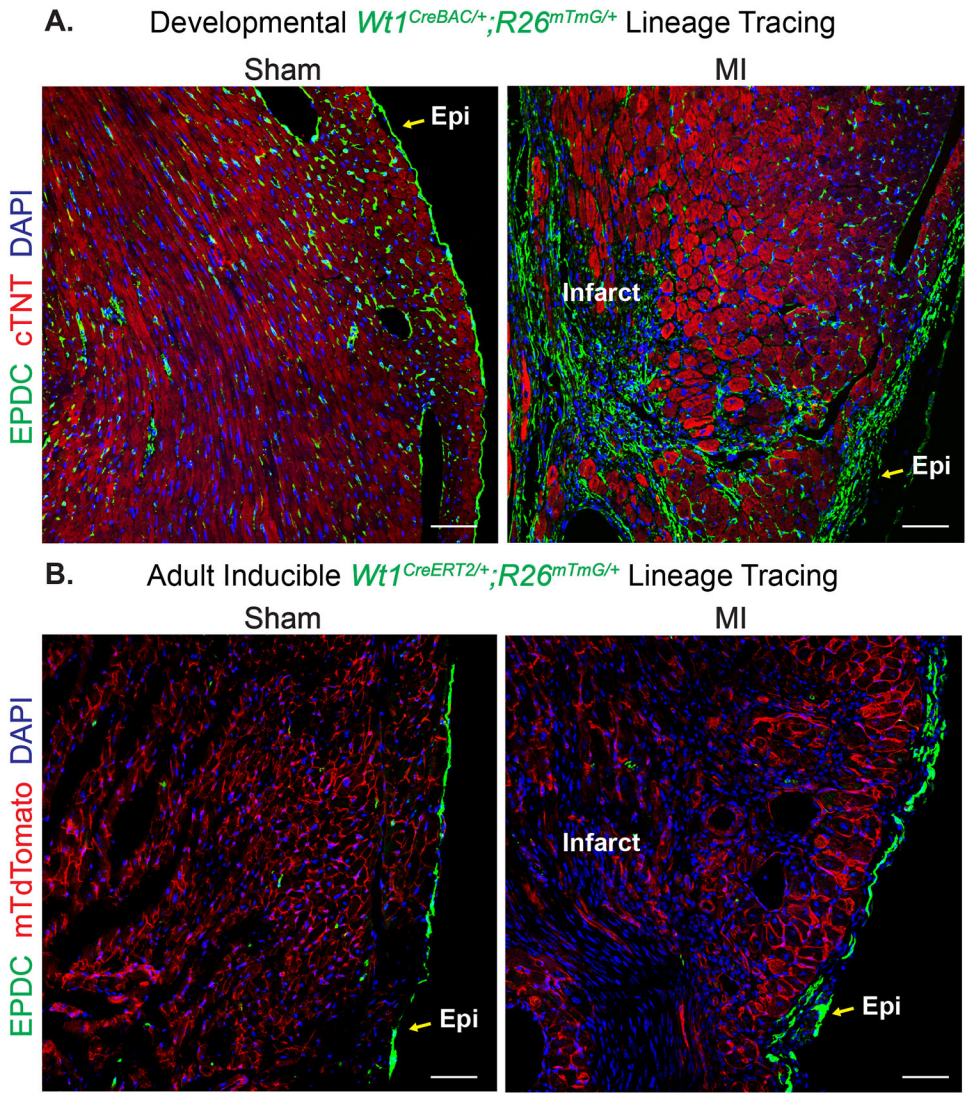

Figure 3. Resident Epicardium-Derived Fibroblasts Contribute to Fibrosis Following Myocardial Infarction.

Evaluation of epicardium-derived cell distribution in left ventricles of mice subjected to sham or myocardial infarction (MI). Epicardial cells are visualized (GFP^+=green) by crossing constitutive (*Wt1*$^{CreBAC/+}$) or tamoxifen-inducible (*Wt1*$^{CreERT2/+}$) Cre lines to the *Rosa26*^{mTmG} Cre-dependent fluorescent lineage reporter mouse line. **(A)** Epicardial cells of developmental origin (*Wt1*$^{CreBAC/+}$; *Rosa26*$^{mTmG/+}$) were observed on the epicardial surface (Epi), and in both perivascular and interstitial areas of the left ventricle of sham-operated hearts. Upon cardiac injury, pre-existing GFP^+ cells contribute to epicardial thickening and the formation of a cellularized scar. Green= GFP^+ EPDCs, Red = cardiac troponin T or cTNT, Blue = DAPI to visualize nuclei. **(B)** When tamoxifen was administered to 8-week old *Wt1*$^{CreERT2/+}$; *Rosa26*$^{mTmG/+}$ mice, GFP^+ cells were observed primarily on the epicardial surface of the left ventricle in sham operated animals. After MI, adult *Wt1*-lineage derived cells increase in number at the epicardial border surrounding the borderzone region of the left ventricle, but they do not directly contribute to scar formation. Green= GFP^+

EPDCs, Red = membrane tethered tdTomato (non-recombined cells), Blue = DAPI to visualize nuclei. Scale bars for (A) and (B) = 50μm.

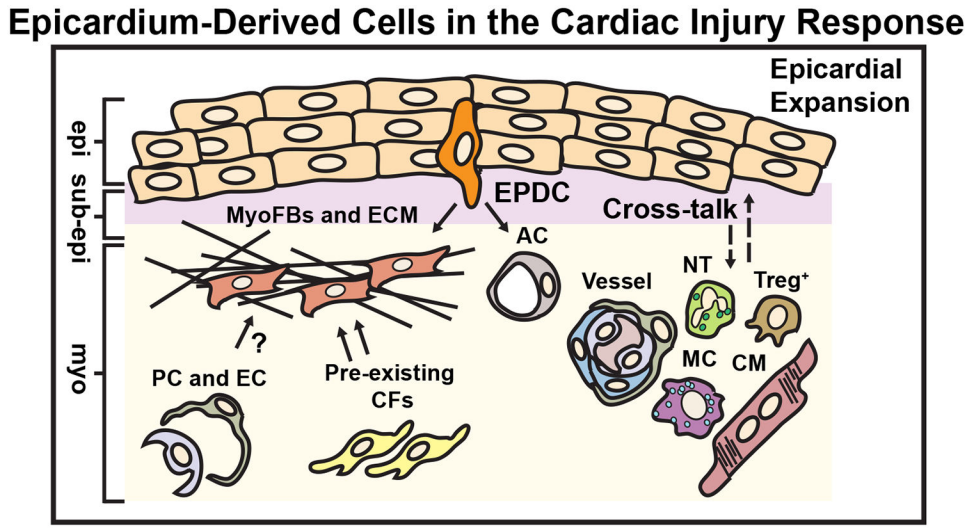

Figure 4. Epicardium-Derived Cells in the Cardiac Injury Response.

Pre-existing epicardium-derived resident cardiac fibroblasts are the primary source of MyoFbs that generate scar tissue during pressure overload or ischemic remodeling through the secretion ECM. Epicardium-derived pericytes (PC) or endothelial cells (EC) have also been reported to exhibit mesenchymal cell characteristics and produce ECM in response to injury. The adult epicardium is reactivated and expands in response to cardiac injury. A small proportion of epicardium-derived progenitor cells (EPDCs) undergo epithelial-to-mesenchymal transition (EMT) to become myofibroblasts (MyoFbs) that secrete extracellular matrix (ECM). In limited cases, EPDCs can become adipocytes (AC). The activated epicardium also promotes the recruitment of inflammatory cells (Neutrophils or NT, T-regulatory cells or $Treg^{+}$, and Macrophages or MC) and stabilizes coronary vasculature through the secretion of chemokines (dashed arrows). The epicardium may also promote cardiomyocyte (CM) survival and/or cell growth. epi = epicardium, sub-epi = sub-epicardium, myo = myocardium. (Illustration credit: Ben Smith)

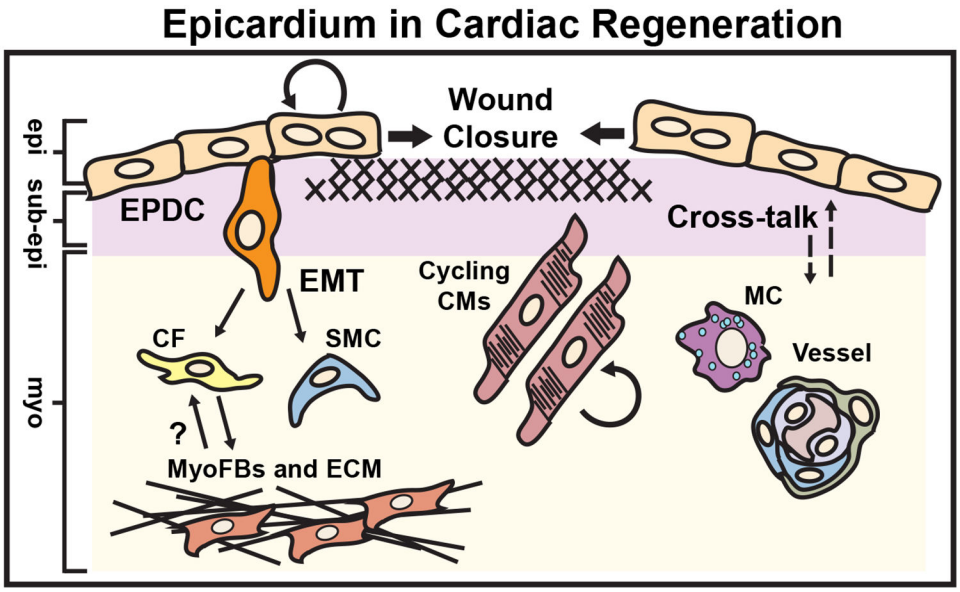

Figure 5. Epicardium in Cardiac Regeneration.

Cardiac regeneration occurs in response to a number of stimuli including apical resection, neonatal myocardial infarction, cryoablation or targeted ablation of the epicardium in both zebrafish and the early postnatal mammalian heart. The epicardium actively regenerates through the migration of “leader” binucleated epicardial cells initiating wound closure, and contributes to the formation of perivascular cells (smooth muscle cells or SMCs and fibroblasts or Fbs). Epicardium-derived fibroblasts have been shown to acquire myofibroblast phenotypes (MyoFb and ECM), which may be reversible during the resolution phase of the scar. The epicardium promotes macrophage (MC) recruitment, neovascularization as well as cardiomyocyte (CM) cell cycle re-entry via secretion of mitogens (dashed arrows). epi = epicardium, sub-epi = sub-epicardium, myo = myocardium. (Illustration credit: Ben Smith)

Table 1.

Epicardial Lineage Tracing Strategies.

Genetic Cre-loxP-based lineage-tracing strategies have been employed to fate map epicardial-derived cells in mice. Although an epicardium-specific reporter does not exist, a number of epicardium-enriched Cre lines have been described: *Wt1*[23], *Tcf21*[31, 163, 164, 171], *Tbx18*[70, 71], *cGata5* (chicken *Gata5* enhancer)[79], Mesothelin (*Msln*)[173], Raldh2 (*Aldh1a2*)[27, 93], Podoplanin (*Pdpn*)[174], *Gpm6a*[160], Scleraxis *(Scx*)[82], Sema3d (*Sema3d*)[82], *G2-Gata4* (*Gata4* enhancer)[83] and cytokeratin[175]. Among these markers, the most common transgenic mouse lines have been engineered to express Cre recombinase under the control of the *Wt1*[15, 65], *Tcf21*[59], *Tbx18*[69], *cGata5*[79], *Sema3d*[82], or *Scx*[82] promoters. To indelibly label epicardium-derived progenitor cells, a given Cre line is often bred into a reporter mouse strain (i.e. *Rosa*$^{\text{Lacz}}$, *Rosa*$^{\text{eGFP}}$). Fate mapping has demonstrated co-localization of labeled epicardial cells with markers of smooth muscle cells[15, 32, 69], cardiac fibroblasts[32, 64, 65, 69, 82], valvular interstitial cells[64, 65], pericytes[43], endothelial cells[15, 82, 83] and cardiomyocytes[15, 65, 69, 82]. In general, the epicardium predominately gives rise to cardiac fibroblasts and vascular mural cells, and to a lesser extent, endothelial cells and cardiomyocytes[70, 81].

Transgenic model	Organism	Description	Cardiac cell fates mapped
G2-Gata4^{Cre}; G2-Gata4^{LacZ} [83]	mouse	Constitutive, transgenic line expressing the conserved CR2 enhancer of Gata4	EP, EC, SMC[83]
cGata5^{Cre} [79]	mouse	Constitutive, transgenic line expressing chicken *Gata5* promoter fused to Cre	EP, SMC, CF[79]
Wt1^{CreERT2} [15]	mouse	Inducible, CreERT2 knock-in into the *Wt1* locus	EP, SMC, EC, CM, CF, VIC and PC (TAM E9.5 – E14.5)[15, 43, 64, 80] AC **(Re)**[124, 162], Mes **(Re)**[103]
Wt1^{Cre} [65]	mouse	Constitutive, BAC transgenic – IRES/EGFP-Cre inserted downstream of the *Wt1* translation stop site	EP, CF, VIC and CM[65] CFs **(Re)**[60, 113]
Wt1^{GFPCre} [15]	mouse	Constitutive, GFP-Cre fusion knock-in into the *Wt1* locus	EP, CM[15]
Tbx18^{CreERT2} [115]	mouse	Inducible, CreERT2 knock-in into the *Tbx18* locus	PC and SMC[115]
Tbx18^{Cre} [69]	mouse	Constitutive, Cre knock-in into the endogenous *Tbx18* locus	EP, SMC, PC, CF, and CM[69, 76] CFs **(Re)**[112]
Tcf21^{iCre} [59]	mouse	Inducible, MerCreMer knock-in into the *Tcf21* locus	EP, CF, SMC (TAM E10.5, E14.5)[32] and CF **(Re)**[105]
Tcf21^{Lacz} [163, 164]	mouse	Constitutive knock-in into the *Tcf21* locus	EP and CF[32]
tcf21:CreER [111]	zebrafish	Inducible, BAC transgenic – *CreERT2* knock-in into the *tcf21* locus	EP and SMC; EP and PV (**Re**)[111]
tcf21:dsRed [111]	zebrafish	Constitutive	EP, PV, and SMC; Mes and PV **(Re)**[111]
Scx^{GFPCre} [82, 165]	mouse	Constitutive	EP, EC, SMC, and CM[82] VIC[165]
Sema3D^{GFPCre} [82]	mouse	Constitutive	EP, EC, SMC, and CM[82]

EP = epicardial cells, SMC = smooth muscle cells, EC = endothelial cells, CM = cardiomyocytes, CF = cardiac fibroblasts, PC = Pericytes, PV = perivascular, AC = adipocytes, Mes = mesenchymal cells, VIC = valve interstitial cells, TAM = tamoxifen, **(Re)** = reactivation/regeneration

Table 2.

Labeling of Cre lines in non-cardiac tissue.

In addition to expression of *Wt1*, *Tbx18*, *Gata5*, *Tcf21*, *Scx*, and *Sema3d* in the pro-epicardium and epicardium, these genes are localized to various non-cardiac tissues.

Gene	Non-cardiac tissue labeling
WT1	adrenal gland & kidney[23], gut[166], liver[167], lung[168], diaphragm[169], adipose tissue[170]
TBX18	skeletal muscle, brain, retina, lymph nodes, skin, sino-atrial node, adipose tissue, bone marrow[115]
GATA5	septum transversum, liver, kidney[79]
TCF21	lung, kidney, gonads, spleen, gut, adrenal gland, skeletal muscle[59, 163, 171]
SCX	tendons and ligaments[172]
SEMA3D	pharyngeal arch endoderm[82]