Signaling During Epicardium and Coronary Vessel Development

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Abstract: The epicardium, the tissue layer covering the cardiac muscle (myocardium), develops from the proepicardium, a mass of coelomic progenitors located at the venous pole of the embryonic heart. Proepicardium cells attach to and spread over the myocardium to form the primitive epicardial epithelium. The epicardium subsequently undergoes an epithelial-to-mesenchymal transition to give rise to a population of epicardium-derived cells, which in turn invade the heart and progressively differentiate into various cell types, including cells of coronary blood vessels and cardiac interstitial cells. Epicardial cells and epicardium-derived cells signal to the adjacent cardiac muscle in a paracrine fashion, promoting its proliferation and expansion. Recently, high expectations have been raised about the epicardium as a candidate source of cells for the repair of the damaged heart. Because of its developmental importance and therapeutic potential, current research on this topic focuses on the complex signals that control epicardial biology. This review describes the signaling pathways involved in the different stages of epicardial development and discusses the potential of epicardial signals as targets for the development of therapies to repair the diseased heart. (Circ Res. 2011;109:1429-1442.)

Key Words: cardiac repair ■ coronary vessels ■ differentiation ■ epicardium ■ signaling

The epicardium is the outermost tissue layer of the vertebrate heart. Its importance during cardiac development frequently has been overlooked to the extent that it was traditionally regarded as part of the pericardium (the “visceral pericardium”),1 and it was thought to derive from the myocardium. It was not until the late 1960s that the myocardium was refuted as the origin of the epicardium and its derivatives.2 Numerous studies in a variety of vertebrate models followed this seminal work and demonstrated that the epicardium has an extracardiac origin, the proepicardium (PE) (Figure 1A–C, G).3–6 Cell-tagging analyses revealed that the epicardium is not the quiescent tissue it was first thought to be, but rather an active cell population that contributes to the development of diverse cardiac structures,7–11 leading to proposals that proepicardial progenitors should be classified as multipotent cells.5 The most recent studies on epicardial development have focused on tracing the origin and developmental fate of this tissue and its derivatives in avian and mouse embryos.10–13 Studies in chick embryos12,14 demonstrate that some PE cells can differentiate into cardiomyocytes (CM) (Figure 1D). However, in vitro and in vivo studies suggest that the default differentiation pathway for avian and mouse PE cells is toward smooth muscle, not cardiac muscle,15,16 (Figure 1E, H, I), and the epicardium is thus generally accepted to be the main source of coronary smooth muscle cells (CoSMC). The contribution of PE cells to the coronary endothelium (CoE) is less clear. Avian PE gives rise to endothelial cells both in vivo17–19 and in vitro.16,20,21 In these in vitro experiments, endothelial differentiation was dependent on the addition to the culture medium of the provascular growth factors vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2; Figure 1F); these factors, which are produced by the embryonic myocardium, are required for coronary vessel development in the embryo.22 Whereas endothelial differentiation from avian PE cells has been demonstrated, genetic tracing in mice has failed to confirm a significant contribution of the PE to CoE.10,11,23 Moreover, a recent report suggests that the mouse CoE arises from angiogenic sprouts of the sinus venosus24 (see the section on coronary development for discussion of this topic). PE/epicardial cells also contribute some of the non-muscle cells resident in the interstitial space between CM. Although these cells are often classed as fibroblasts, only some interstitial cells actively secrete collagen and can be considered true cardiac fibroblasts (CF); other epicardial-derived interstitial cells do not normally differentiate into mature fibroblasts (CF); other epicardial-derived interstitial cells do not normally differentiate into mature fibroblasts and continue to express low levels of genes characteristic of undifferentiated epicardial cells, such as Wt1 and Gata4.11,25

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Knowledge of epicardial cells and epicardial mesenchymal derivatives can provide the basis for the potential use of these cells in reparative and regenerative cardiovascular medicine. However, little is known about the signals and downstream effectors that regulate epicardial development. This review discusses recent findings related to signals involved in the coordination of epicardium-related developmental events and their potential therapeutic implications.

**Signals Involved in PE Specification**

The epicardium originates from the PE, a cauliflower-shaped mass of coelomic cells protruding toward the pericardial cavity. The PE forms at the posterior limit of cardiac inflow myocardium, covering the septum transversum in the mouse and analogous regions in nonmammalian vertebrates (Figure 1A–C, G).

Various signals determine the outgrowth and precise location of the PE. In the chick embryo, two PE primordia appear bilaterally, but only the right one develops. This asymmetry is proposed to be under the control of the right determining molecular system including Fgf8/Sna1.26 Fgfs are also suggested to support PE proliferation, thereby sustaining PE identity and preventing PE cells from differentiating into CM,14,28 Bmp has also been identified as an important signal in PE specification in zebrafish,29 because inhibition of Bmp signaling via expression of a dominant-negative form of Bmpr (or genetic ablation of the activin-like receptor type 1) leads to reduced expression of the PE markers Tbx18 and Tcf21 (transcription factor 21).29 Gain-of-function studies in which Bmp2 was expressed before PE specification under the control of a heat-shock promoter show ectopic expression of Tbx18 but not of Tcf21, indicating that Bmp2 alone is not enough to drive mesodermal commitment to PE fate.29 In addition, Bmp2 expression is crucial for attachment of PE cells to the heart.30 All these events require highly patterned expression of Bmp2 in the PE/septum transversum region.31 Bmps thus can be postulated to play a role in establishing the posterior-most myocardial limit (venous pole) of the developing heart. The signals active in this region are detailed in Figure 2.

**Formation of the Epicardium and Its Spreading Over the Myocardium**

Formation of the epicardial sheet requires that PE cells attach to the myocardial surface. In avian embryos, this is accomplished by formation of a PE tissue bridge over the myocardium. The results of ectopic Bmp2 expression in chicken myocardium suggest a model in which Bmps (presumably Bmp2 and Bmp4) expressed in the atrioventricular (AV) canal direct PE protrusion toward the heart tube.30 In mammals, the predominant mode of PE cell transfer to the heart seems to be the release of PE cell cysts to the pericardial cavity, with the PE connecting to the heart relatively late in epicardial development.34

Once PE cells adhere to the myocardial surface, they flatten and spread to form a characteristic cobblestone epithelial monolayer (Figure 3A). PE cell polarity is an important aspect of this process, because inactivation of the cell polarity protein PAR3 severely impairs epicardial development.32 In PAR3 mutants, baso-apical cell polarity is disrupted and the cells that bud from the PE do not form the cysts that would normally form the primitive epicardial epithelium.32

As development proceeds, the epicardium grows over the heart in a fixed spatio-temporal pattern, starting from the dorsal AV groove.33,34 Most research into the molecular mechanisms of epicardial ensheathing of the heart has been driven by the belief that such movements are likely to participate, via cytoskeleton-mediated signal transduction, in the activation of molecules important for epicardial development. Examination of the distributions of α4 integrin and the α4β1 integrin ligand vascular cell adhesion molecule-1 (VCAM1) indicates that VCAM1 is expressed in the developing myocardium and α4 integrin the epicardium. This complementary pattern suggests that α4/VCAM1 interaction stabilizes adhesion of epicardium to myocardium and enables superficial migration. Targeted inactivation of VCAM135 or α4 integrin36 in mice confirmed this hypothesis, with mutants dying at approximately E14.5 and displaying impaired formation of epicardium and coronary vessels. Interfering with α4 integrin in the chick increases the invasive behavior of epicardial mesenchymal derivatives, indicating a role for α4 integrin in modulating epicardial-derived cell migration.37 Further studies in the chick show that communication be-

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<th>Non-standard Abbreviations and Acronyms</th>
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<tr>
<td>AV</td>
<td>atrioventricular</td>
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<td>Bmp</td>
<td>bone morphogenetic protein</td>
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<td>CF</td>
<td>cardiac fibroblast</td>
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<td>CoE</td>
<td>coronary endothelium</td>
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<td>CoSMC</td>
<td>coronary smooth muscle cell</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
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<td>EPDC</td>
<td>epicardium-derived cell</td>
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<tr>
<td>Fgf</td>
<td>fibroblast growth factor</td>
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<td>Hh</td>
<td>hedgehog</td>
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<td>Igf2</td>
<td>insulin growth factor-2</td>
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<td>N1ICD</td>
<td>Notch1 intracellular domain</td>
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<td>PE</td>
<td>proepicardium</td>
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<tr>
<td>Pdgfr</td>
<td>platelet-derived growth factor receptor</td>
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<td>RA</td>
<td>retinoic acid</td>
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<td>Raldh2</td>
<td>retinaldehyde dehydrogenase 2</td>
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<tr>
<td>RBPJK</td>
<td>recombination signal binding protein for immunoglobulin kappa J region</td>
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<td>Rxra</td>
<td>retinoic X receptor alpha</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>Tβ4</td>
<td>thymosin-β4</td>
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<td>Tbx18</td>
<td>T-box transcription factor 18</td>
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<tr>
<td>Vegf</td>
<td>vascular endothelial growth factor</td>
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<td>WT1</td>
<td>Wilms tumor 1</td>
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between integrin receptors facilitates epicardial cell adhesion and subepicardial matrix organization. Other important genes involved in epicardial ensheathing of the myocardium are \( \text{Rxra} \), \( \text{Gata4} \), and \( \text{Fog2} \). Targeted inactivation of \( \text{Rxra} \) in mice is embryonic lethal between E13.5 and 15.5, and affected embryos have a thin compact myocardium layer. \( \text{Fog2} \), a cofactor for Gata transcription factors, is highly expressed in the heart. At E12.5, \( \text{Fog2} \) mutants show a normal epicardium that ensheathes the myocardium; however, this epicardium does not undergo epithelial-to-mesenchymal transition (EMT), and thus coronary vessel formation does not occur, resulting in embryonic death at approximately E14.5. Among Gata transcription factors, Gata4 is required for coronary vessel angiogenesis, a process it promotes via direct Vegf activation.

**Epicardial EMT**

As soon as PE cells form the primitive epicardium, some epicardial epithelial cells transform into a population of highly migratory invasive mesenchymal cells called epicardium-derived cells (EPDC) through a process known as epithelial to mesenchymal transition (EMT). EMT provides mesenchyme to developing embryonic structures at various locations throughout development. An interesting aspect of EMT is how epithelial cells are specified for transformation. Two scenarios are possible for epicardial

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**Figure 1. Proepicardium growth and developmental potential.**

A. HH17 chick embryo. The proepicardium (blue) is shown in relation to the early looped heart tube (red) and secondary heart field progenitors (green). B and C. SEM images. B. Right lateral view of a HH17 chick embryo showing the extracardiac mass of cells that forms the proepicardium (arrow). C. Frontal view of a HH19 chick embryo; the proepicardium is now attached to the myocardium (arrow). D to F. Differentiation of avian proepicardial cells. Proepicardial cells differentiate into cardiac striated muscle (D; MF20\(^{17} \), green), smooth muscle (E; \( \alpha \)-smooth muscle actin\(^{17} \), red), and angioblastic vascular progenitors (F; QH1\(^{17} \), green) in vitro. G. The anatomic position of the E9.5 mouse proepicardium is analogous to that in the chick embryo. The myogenic differentiation potential of the mouse proepicardium is revealed by chemical inhibition of Notch signaling. Compare (H) (control) to (I) (DAPT-treated).

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**Figure 2.** Diagram showing myocardial and epicardial differentiation from the pericardial mesoderm adjacent to the inflow tract of the heart based on in vitro data from chicken and in vivo data from zebrafish and mouse. Green delineates the myocardial differentiation zone, red shows the proepicardial differentiation zone, and yellow shows the transition zone. Modified from Kruithof BP, van Wijk B, Somi S, Kruithof-de Julio M, Perez Pomares JM, Weesie F, Wessels A, Moorman AF, van den Hoff MJ. BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. Dev Biol. 2006;295:507–522.
EMT. In one, a subset of epicardial cells would be specified early in development to acquire competence for EMT. In the second scenario, all epicardial cells would be equally able to transform, the activation of EMT depending on locally restricted signals. In either case, spindle orientation during cell division is an important determinant of the ability of epicardial cells to undergo EMT.47

Several signaling pathways activate EMT.46 These signals must function coordinately and target defined effectors for epithelial cells to downregulate intercellular adhesion, reorganize their cytoskeleton, lose baso-apical polarity (changing gene expression to acquire a mesenchymal invasive phenotype), and degrade the extracellular matrix (Figure 3B, C). Epicardial EMT involves the exchange of cytokeratin for vimentin cytoskeletal filaments.7,48 Vimentin influences cell migration and proliferation by scaffolding the activity of a variety of signals, but it is not clear how increased vimentin content influences EMT cell-shape changes.49 Many cells undergoing EMT also express α-smooth muscle actin, a molecule associated with the onset of smooth muscle differentiation. However, embryonic α-smooth muscle actin expression is also strongly related to activation of a migratory program in mesenchymal cells, a feature of cells undergoing EMT,50,51 and is considered a hallmark of the myofibroblastic cell type in adults.52

Various myocardial signals influence epicardial EMT. Transforming growth factor-β was originally described as a negative regulator,48 but more recent studies indicate this growth factor promotes EMT.53 The role of Fgfs in epicardial EMT is difficult to distinguish from the other functions of these potent mitogens,54 and Fgfs are also suggested to be required for EPDC migration into the myocardium.55 Other growth factors involved in epicardial biology, such as Vegf and platelet-derived growth factor (Pdgf)-BB, have not been demonstrated to directly affect epicardial EMT, and instead seem to control cell fate decisions taken by EPDC once EMT is completed.56

A key regulator of gene expression changes during epicardial EMT is Wilms tumor suppressor protein (Wt1). This transcription factor is expressed in many cells of the early PE and in epicardium and EPDC,57 and has been used to trace epicardial derivatives in the embryo. Epicardial-specific Wt1 mutant embryos show deficient EMT and impaired EPDC generation (Figure 3D–G). Wt1 binds and transactivates the Snail1 promoter, thus reducing E-cadherin levels. This function is also achieved via binding of Wt1 to the E-cadherin promoter to inhibit its transcription.58 Recent work with a novel Wt1 mutant allele suggests an alternative mechanism for Wt1-mediated regulation of epicardial EMT.59 No effect was detected on E-cadherin expression and there was no reduction in Snail1 or Snail2 expression, and impaired EMT in this model was thus attributed to the small number of EPDC in Wt1 mutants secondary to impaired epicardial EMT. In contrast, the authors found a marked reduction in Wnt/β-catenin signaling.59 β-catenin is required for epicardial EMT,60 so these results suggest that Wt1 functions upstream of Wnt/β-catenin to activate epicardial EMT.59 Signals implicated in EMT are summarized in Figure 5.

Non-cell Autonomous Roles of Epicardium in Heart Development

The importance of epicardial signaling during embryonic myocardium development is evidenced by the severe myocardial defects displayed by animal models with defective epicardial development.56,61 This non-cell autonomous role of the epicardium is mediated by a characteristic epicardial secretome, including growth factors and other soluble molecules. Retinoic acid (RA) (mostly via Rxra) plays a key role in the production of part of this secretome, although RA itself does not promote growth of compact myocardium.62 Mice deficient for Wt1,57,63 Rxra,62,64 or the erythropoietin receptor65 have epicardial and myocardial defects. In a recent study, the involvement of RA in the control of ventricular myocyte proliferation was shown to be dependent on erythropoietin production by the liver. This hepatic erythropoietin activates production of Igf2 in the epicardium,66 which in turn regulates compact ventricular myocardium proliferation via ERK signaling.67 Other growth factors secreted by the epicardium, mainly Fgfs, are reported to regulate cardiac myocyte growth. Among them, Fgf9 and Fgf16 are likely to be crucial signals regulating this process.68
The prominent actions of epicardial cells and EPDC on ventricular myocyte development have overshadowed the equivalent role of these cells at the venous pole of the heart. Most epicardial-related functions at the cardiac inflow seem to depend on the PE itself and the underlying mesenchyme that populates the septum transversum area. Some mesodermal cells at the cardiac inflow seem to be lineage-related, expressing markers like Wt1 and Tbx18.13 Wt1-deficient mice show reduced levels of the RA-synthesizing enzyme retinaldehyde dehydrogenase-2 (Raldh2)63,69 (Figure 3F–G), recently shown to be a direct Wt1 target.64 Wt1 mutants show anomalies in the formation of sinus venous horns, a defect interpreted as a result of deficient RA signaling.69 Targeted inactivation of Tbx18 specifically affects sinus horns but not pulmonary venous return development, implying that different regulatory mechanisms are required for the morphogenesis of these structures.70

Thus, the alteration of signals relevant for noncell autonomous functions of the epicardium in heart development could partially explain some congenital defects, particularly those affecting the growth of the ventricular myocardium (Figure 5). In this context, RA deficiency is known to produce severe cardiovascular malformations.71 Because RA depletion causes early embryonic death, RA-related cardiac defects seen in newborns are more likely to be caused after the formation of the heart primordium, in parallel with the proliferation of chamber myocardium. The epicardial synthesis of RA, which correlates in space and time with ventricular myocardial growth, could explain this phenomenon.

**EPDC Differentiation**

The differentiation of epicardial mesenchymal derivatives depends not only on signals provided by the epicardial epithelium and EPDCs themselves but also on instructions from the adjacent myocardium. Moreover, as discussed, the myocardium depends on epicardial signals for its development. This complex network of reciprocal epicardial–myocardial cross-talk produces a progressive and coordinated maturation of myocardium and epicardial-derived tissues. Unfortunately, the mechanistic relation between these signals is largely unknown.

It is clear that the contact established between the newly formed epicardium and the myocardium strongly influences the fate of epicardial derivatives. The proximity of epicardial cells and cardiac muscle fully activates the vascular potential of epicardial cells and promotes massive differentiation of vascular progenitors in the subepicardium, the extracellular matrix located between the epicardial epithelium and the myocardium.20 This is probably attributable to the increased availability of myocardially secreted growth factors like Vegf and Fgfs.22 Myocardial Fgf signaling also triggers a wave of hedgehog (Hh) activation that is essential for the expression of Vegf-A, Vegf-B, Vegf-C, and angiopoietin-2.72 Thus, Hh does not promote myocardial proliferation directly,73 but its role in supporting coronary morphogenesis might be essential for coronary-induced maturation of the ventricular myocardium. This accords with the proposed role of β-catenin in the growth of coronary vessels, which has been shown to impact ventricular myocyte proliferation.60 The β-catenin activity suggests the involvement of canonical Wnt signaling in epicardial–coronary development, as confirmed by altered expression of Wnt9b in Ruxo-deficient epicardial cells.64 Defining the role of Wnt signaling during cardiac development is nonetheless a formidable task, given the complexity of this pathway.74

An epicardial origin for most coronary mural cells is widely accepted. Epicardial cells and EPDC have been shown to differentiate into CoSMC under standard culture conditions, almost spontaneously.20,75,76 Pdgf-BB promotes serum response factor-dependent epicardial expression of smooth muscle cell (SMC) markers, establishing a role for serum response factor in CoSMC differentiation.76,77 Nevertheless, the mechanisms determining the late muscularization of coronary vessels have remained a mystery for decades. A recent report proposed RA as a key regulator of the late differentiation of CoSMCs with respect to CoE.16 This is compatible with the robust expression of the RA-synthesizing enzyme Raldh2 in epicardial epithelium and the earliest EPDC,18,78 the transcription of Raldh2 being partially controlled by Wt1.59,63 Azambuja et al suggest that late differentiation of CoSMC could permit extended remodeling of the primary coronary endothelial plexus in the absence of the stabilizing signals provided by vascular smooth muscle/mural cells.16 The role of RA in CoSMC differentiation might be linked to the essential function of Pdgfr in the same process,56 because Pdgfr expression responds to RA.79 Signals involved in EPDC differentiation are summarized in Figure 5.

As noted, newly formed primitive epicardium is unable to differentiate into CM in vitro.14 This is surprising because other studies report a contribution of epicardial cells to the CM population during mouse heart development in vivo. These findings, based on the use of Tbx18- and Wt1-Cre drivers,10,11 have been challenged by further research providing evidence for the expression of the epicardial marker Tbx18 in a population of chamber myocardial cells before EPDC invade the cardiac walls.13 While research continues, it now seems that the number of epicardial cells that might spontaneously transdifferentiate into myocytes in the developing heart is small; because the so-called epicardial markers Tbx18, Gata5, and Wt1 are also expressed by nondifferentiated mesodermal cells at the venous pole of the heart before the PE forms,80 CM genetically tagged as epicardial-derived might not, in fact, directly derive from the embryonic epicardium.

**The PE-Epicardium-Coronary Transition**

The idea that coronary vessel morphogenesis is tightly associated with the development of the epicardium is relatively modern. The first experimental evidence supporting central involvement of the epicardium in coronary vessel formation came from pioneering research in chick embryos.15,17 This study was followed by others evaluating the ability of the primitive epicardial epithelium to undergo EMT,48 and the developmental fate of EPDC.9,18,19 Many of these studies cultured isolated epicardial progenitor cells (PE) in vitro or simply transplanted them into a host embryo *in vivo*. As a result of both procedures, the PE, after making contact with the myocardial surface, recapitulates its normal
transformation into a monolayered epicardial epithelium. Because the epicardium determines the formation of the subepicardial space (where the earliest coronary blood vessels form) and directly contributes to the cellular and extracellular subepicardial milieu, epicardial development cannot be separated from coronary development. Therefore, we view the PE-epicardium-coronary transition as a developmental continuum and believe that the process of epicardial development should be envisioned as a highly interactive cell system in which the progressive appearance of new cell types increases the level of complexity of signaling networks.

A key issue regarding the contribution of (pro)epicardium to coronary vessel formation is the origin of the mesenchymal cells in the subepicardial space. Although the cells populating the embryonic subepicardium were originally named subepicardial mesenchymal cells, it was the term “epicardially derived cells” that became established in the field. Unfortunately, EPDC has often been used for all mesenchymal cells in the subepicardium. This is problematic because it is not possible to rule out a contribution of nonepicardial sources to the subepicardial cell population (eg, cells already present at the PE core or cells from the dorsal mesocardium).

A second frequent assumption is that any embryonic mesenchymal cell is, by definition, a multipotent progenitor. The use of various molecular markers to genetically trace mouse epicardial cells and their derivatives using Cre-Lox technology has provided valuable data, but also contributed some confusion to our understanding of epicardial development. It is evident that the “one molecule, one cell lineage” approach needs qualification. First, few molecules are expressed only by a single cell type; Wt1, Tbx18, and Gata5 are not found only in PE or epicardial cells. Because the subepicardium is an open space for the incorporation of extracardiac cells in the developing heart, it is possible that it contains non-epicardial derived cells expressing these markers. Second, the expression level of the gene driving Cre activity varies depending not only on the identity of the gene but also on the sensitivity of the reporter driving Cre activity;82 a corollary to this is that it is difficult to accurately determine the moment when recombination takes place. This situation could be circumvented by using inducible systems, but the efficiency of recombination is variable and the number of cells expressing the reporter is low compared with standard Cre-mediated recombination.

Thus, disruption of the mechanisms regulating PE-epicardial-coronary transition is likely to impact coronary morphogenesis. In this context, anomalous coronary vessel patterning is critically dependent on epicardial embryonic development.83

Epicardium and Coronary Endothelium

There is a general agreement on the contribution of epicardial derivatives to CoSMC and coronary adventitial fibroblasts, but it remains controversial whether the epicardium gives rise to CoE, with avian and mammalian models yielding different results; experiments in chicks indicate that PE cells differentiate into CoE,16,17,18,19,21 whereas genetic tracing in mice indicates a minor contribution, if any, of epicardial cells to the endothelial component of coronary vessels.15,23,60
epicardial derivatives to mammalian CoE could reflect intrinsic interspecies differences, but it is also possible that the use of a single tracer molecule as a general marker of (pro)epicardial cells might exclude epicardial subpopulations. An alternative possibility is that the scaffolding and coordination of coronary vessel formation and patterning might be controlled by a small population of epicardial-derived CoE cells with special signaling properties.

The previous discussion can be conveniently summarized by considering a recent article by Red-Horse et al claiming that CoE is derived entirely from the endothelium of the sinus venosus.24 This report is based on the finding that the endocardium of the embryonic sinus venosus (cardiac inflow) produces a characteristic budding of small vessels that expand toward the subepicardial space. This finding, however, is not novel, being first described in the human embryo8 and later in avian embryos.85 Because the anatomic connection of coronary veins to the systemic blood flow takes place at the cardiac inflow, these vessels are normally regarded as prospective coronary veins. However, Red-Horse et al24 claim that this endothelium is “reprogrammed” into arterial endoderm, giving rise to the arterial component of the coronary vascular tree. This view is based on information obtained from Ephrin- and Eph-LacZ mouse lines, which show dynamic expression of molecules associated with the arterial (EphrinB2) or the venous (EphB4) endothelia. Additional data from a mouse line carrying apelin-LacZ, a marker unable to distinguish between arterial and venous endothelia, is unhelpful in this context. Moreover, given the absence of an extensive and specific permanent tag for sinus venosus endocardial cells, the results of this study do not support a definitive conclusion: the vascular endothelial (VE)-CreER inducible driver used might be activated in any vascular cell, vascular progenitor (angioblast), or even hematopoietic cell, with special signaling properties.

Further research is needed to determine whether the specification of arterial and venous lineages during the vascular plexus stage responds to a general mechanism governing vascular development.

A third type of endothelium is found in the lymphatic circulation. Cardiac lymphatics form side-to-side with the coronary vessels but do not originate from the PE,93 as suggested by experiments in avian embryos.9,19,75,94 Labeling studies in chick and mouse suggest that cardiac lymphatic endothelial cells originate from systemic embryonic veins, with precursor cells entering at the venous pole of the heart.93,95,96 A study by Srinivasan et al,96 using a Proxl-Cre driver to label the earliest lymphatic progenitors, provided clear evidence that, as previously proposed,97 lymphatic endothelial cells sprout from venous-derived lymph sacs, proliferate, and migrate to give rise to the entire lymphatic vascular tree.96

### Epicardial Origin of Cardiac Fibroblasts

During cardiac development, diverse nonmyocardial cells colonize the extracellular space surrounding CM. These cells produce extracellular matrix (ECM) components, including collagens and fibronectin, and the cells and their milieu constitute the cardiac interstitium.98 Interstitial ECM strongly influences cell shape and function and is crucial for transmitting information from the extracellular environment during development and in the onset of disease.98,99

Although most cardiac interstitial cells are thought to be CF, multiple studies have shown that these cells form a heterogeneous population (we nonetheless use the term “CF” here for simplicity). The functions of CF are to maintain ECM homeostasis through the production of growth factors and other signaling molecules, sustain homeostasis of cardiac vessels, and even contribute to correct cardiac electrophysiological activity.100 Morphology is not a sufficiently rigorous criterion for identifying fibroblastic cells, because the shape of a fibroblast changes depending on its position and physiological status. The term “activated fibroblast” seeks to define a fibroblast functionally by its active synthesis of collagen, but the use of “fibroblast” should perhaps be more restrictive and applied only to cells that actually participate in building the ECM. The well-known diversity of “fibroblast markers,” including vimentin, FSP1, Thy.1,CD90, DDR-2 and α-smooth muscle actin,101 suggests that we are trying to create a single category from a number of distinct cell types. The problems with these definitions are crystallized by the use of α-smooth muscle actin as a marker for CF, which are hence called “myofibroblasts.”102 This is confusing because a cell expressing proteins of the contractile apparatus should not be immediately assumed to be muscle, given that many of these proteins are critical to the migration of nonmuscle cell types.

The EPDC produced by epicardial EMT are considered a major source of CF,103 and several studies in avian9,15,17,19 and mouse embryos10,11,80 indicate that the embryonic epicardium is the first and main contributor of embryonic CF (Figure 4B, C). Other sources of CF are the EMT that occurs in the embryo during the formation of cardiac cushions104 or post-
naturally via the recruitment of circulating bone marrow cells. The latter process also has been suggested to occur during disease.

The interaction of CF with CM in the embryonic and adult heart is an important issue. A recent report demonstrates that the emergence of embryonic CF coincides with the expansion of the compact layer of the ventricular myocardium and stimulates the proliferation of CM progenitors. Culturing embryonic CM and CF together or CM alone, these authors observed a strong stimulatory effect of fibroblasts on CM proliferation. Gene expression profiling revealed that CFs express high levels of growth factors, cytokines, and ECM components. Examination of the effect of different ECM molecules on CM proliferation revealed that fibronectin and collagen potentiate stimulate embryonic CM proliferation. These molecules signal through integrins, and embryonic β1-integrin expressed on CM has been reported to mediate this proliferative effect via activation of the ERK1/2 and PI3K/Akt pathways. Nkx2.5-Cre–mediated myocardial deletion of β1-integrin corroborated this finding in vivo, resulting in weak myocardial proliferation, a thin compact layer at E14.5, and perinatal death.

The biomedical relevance of CF is enormous, as is demonstrated by the massive fibrosis that follows myocardial infarction. The classic cardiac scar that appears in response to myocardial death is a growing tissue that progressively limits the biomechanical properties of the ventricles, ultimately causing heart failure. CF are likely to interact with other interstitial cells, an important consideration given that the cardiac interstitium contains a great variety of resident cardiac stem cells. Cardiac stem cells support cardiac muscle homeostasis by steadily providing new CM but seem unable to support extensive myocardial repair after massive heart damage. These cells nonetheless provide hope for future treatment of the infarcted heart, and the likely stromal function of CF thus warrants detailed attention. Surprisingly, most approaches to repairing the infarcted heart are exclusively aimed at regeneration of the lost tissue, without considering the need to simultaneously control cardiac fibrosis. The unique features of CF make them an appealing target for reducing the pathological remodeling occurring as a consequence of myocardial infarction or hypertension, and it is therefore essential to define the target subpopulations for such therapeutic approaches, characterizing the cells that sustain fibrotic progression and the signals to which they respond. An important first step will be the identification of cell-specific markers to define CF populations and allow their isolation and further characterization.

**Signaling Pathways in Epicardium and Coronary Vessel Development**

**Retinoic Acid**

RA actions in the embryo are regulated by tight control of dosage. Targeted inactivation of Raldh2 causes embryonic death, with embryos showing interrupted heart development. RA was later identified as an epicardial factor required for CM proliferation. Inhibition of RA signaling in cultured heart slices or removal of the epicardium from these slices reduces CM proliferation. RA does not activate CM proliferation directly, but rather induces the production of a proliferative signal in epicardial cells. Deletion of the nuclear Rxra receptor gene in mice results in myocardial hypoplasia and death, and conditional Rxra inactivation in epicardial-derived cells using the Gata5-Cre driver causes a partially penetrant lethal phenotype characterized by a detached epicardium and a thin subepicardial mesenchyme layer secondary to reduced Fgf2 production in the epicardium. Formation of the coronary arteries is impaired in this model, implying defective angiogenesis, and ventricular cells maintain expression of early cardiac differentiation markers. This suggests that the epicardium not only supports CM proliferation but also instructs myocardial cell differentiation. RA signaling via Rxra thus sustains heart development by stimulating the epicardium to produce signals that promote CM proliferation (Figure 5).

**Fibroblast Growth Factor**

Various Fgf family members are expressed in the epicardium, including Fgf-1, Fgf-2, Fgf-4, Fgf-9, Fgf-16, and Fgf-20. Fgf-1, Fgf-2, Fgf-4, Fgf-9, Fgf-16, and Fgf-20 are expressed in epicardium in partially overlapping patterns starting at E10. Fgf-9 mutant mice die at birth and show a hypoplastic left ventricle associated with weak CM proliferation. Interestingly, Fgf-9 is induced by RA in primary epicardial cell cultures, linking epicardial RA signaling and myocardial proliferation. Embryonic CM express Fgfr1 and Fgfr2, suggesting that they are able to process Fgf signals. Deletion of Fgfr1 and Fgfr2 in CM impairs myocardial proliferation and results in delayed coronary vascular plexus formation, similar to the loss of Fgf-9. These data suggest that Fgfs form part of the epicardium-to-myocardial signaling that regulates myocardial proliferation and, indirectly, coronary vasculogenesis. A recent report showed that Fgfr10/Fgfr2b signaling from myocardium to epicardium is crucial for EPDC migration into compact myocardium and for myocardial growth, indicating that the myocardium also influences the epicardium. In this regard, coordination of myocardial and epicardial activities by Fgf signaling has been proposed to vascularize new CM during heart regeneration in zebrafish.

**Hedgehog**

Sonic hedgehog (Shh) is an Fgf target during coronary vasculogenesis. At E12.5, Shh is expressed in epicardial cells of the AV groove and the base of the heart, later spreading to the rest of the epicardium. Its receptor, Patched (Ptc), is expressed in the AV groove and the CM at the base of the heart, suggesting that the myocardium responds to epicardial-derived Shh signals. Epicardial Shh expression is delayed in conditional knockouts of Fgf9 and Fgfr1/2 (Mlc2v-Cre), and the associated delay in coronary plexus formation is rescued ex vivo by exogenous Shh. Furthermore, inhibition of Hh signaling in wild-type hearts blocks coronary plexus formation, and conditional inactivation of the Hh downstream effector Smoothened also leads to defective coronary plexus formation at E13.5. Together, these data suggest that epicardial Fgf activate myocardial Fgfrs, which by a still unknown mechanism activate Shh expression in the...
epicardium. Interestingly, vessel identity within the coronary plexus appears to depend on Hh signaling.63 Abrogation of Hh signaling in embryonic CM results in the loss of subepicardial mesenchyme and a lack of EphB4-positive venous vessels, but intramyocardial EphrinB2-positive arterial vessels still form. Loss of Hh signaling in cardiac perivascular mesenchymal cells impairs the development of intramyocardial EphrinB2-positive vessels. Lineage analysis of Ephrin- and Eph-expressing cells indicates that coronary vessel identity is already established at E12.5 before vascular remodeling has occurred.73 Hh signaling to CM and perivascular mesenchymal cells induces expression of various angiogenic molecules, including Vegfs and angiopoietin-2.23,80 These and other growth factors instruct developing vascular endothelial cells to regulate coronary vasculogenesis.

Wnt
Various Wnt/wingless genes and their receptors are expressed during cardiac development.117 Canonical Wnt signaling occurs via β-catenin stabilization,118 and epicardial-specific deletion of β-catenin causes death between late gestation and birth.60 Mutant mice have a thin compact zone myocardium, presumably a result of weak CM proliferation. The subepicardial mesenchyme is also thin, suggesting impaired epicardial EMT. In these mice, coronary arteries do not develop, despite the formation of a primitive capillary plexus in the subepicardial space and an intact coronary venous system. This defect appears to be caused by the lack of SMC recruitment in vivo and in vitro, because the explanted epicardium does not express SMC markers on treatment with Tgfβ1.60 Together, these data suggest that epicardial cells from β-catenin mutant embryos are unable to differentiate into SMC.

Notch
Two recent reports demonstrated that the Notch pathway is central to the development of mouse epicardium and coronary vessels.23,80 Various conditional mutants combined with epicardial-specific drivers have been used to ascertain the function of Notch in the epicardium and its derivatives. In the study by Grieskamp et al, a Tbx18-Cre line was bred with a conditional allele of the RBPJk gene,119 Tbx18-Cre; RBPJk flox/flox mice survive to adulthood but show abnormal coronary vessel development and enlarged lumens of the coronary vessels. E18.5 mutant embryos also present dilated intramyocardial and superficial vessels.23 Although the large coronary arteries and veins are correctly specified, a reduction in the numbers of intramyocardial capillaries suggests that arteries and veins are expanded at the expense of capillaries.23 The authors conclude that the differentiation potential of mutant EPDC is compromised and that RBPJk-dependent Notch signaling is required for differentiation of perivascular cells into smooth muscle cells.23 Interestingly, when N1ICD is constitutively expressed under the Tbx18-Cre driver, PE explants from E9.5 embryos appear mesenchymal and show reduced Tbx18 and Wt1 expression and increased expression of CoSMC markers, suggesting that ectopic N1ICD expression is sufficient to induce EMT and CoSMC differentiation.23 Pdgfr-β and Tgf-β are required for CoSMC differentiation during murine coronary arteriogenesis,120,121 and their expression is severely impaired in the hearts of E18.5 Tbx18-Cre and RBPJk flox/flox mutants, and is increased in epicardial cells of E14.5 Tbx18-Cre/+ and Rosa26N1ICD/+ mice, indicating that Notch acts upstream of PDGF receptor-β and Tgf-β signaling in EPDC.23

Figure 5. Epicardial-myocardial signaling during coronary vessel development. Scheme showing the crosstalk between signals (black arrows) derived from epicardium (blue) and cardiomyocyte (green) during the early stages of coronary development. Blue arrows indicate processes in which several molecules have been shown to participate. (Illustration credit: Cosmocyte/Ben Smith).
4G, H). CoSMC marker expression is reduced (a finding confirmed by treatment of wild-type proepicardia with the Notch inhibitor DAPT), as is the expression of the EphrinB2 arterial marker, confirming the arterial nature of the affected vessels (Figure 4I, J). Furthermore, a 30% reduction in compact myocardium thickness in Wt1Cre:Notch1fl/fl;RBPJkfl/fl mutants is associated with a marked reduction in CM proliferation and Raldh2 expression in the epicardium.

Consistent with the canonical function of Notch, two reports appear to indicate that Notch inhibits CM differentiation in the PE, sustaining primitive epicardial features. The phenotypic consequences of Notch manipulation using the two driver lines are similar, and the minor differences found can be attributed to the different genetic backgrounds of the conditional Notch1 and RBPJk alleles. Abnormal coronary artery development in Wt1-Cre:Notch1fl/fl mice might be caused by disrupted specification of coronary artery progenitors, defective migration of these progenitors from the subepicardial space into the compact myocardium, or an altered microenvironment around these progenitors. In Tbx18-Cre:RBPJkfl/fl mice, specification of coronary arterial endothelial fate is unaffected, but CoSMC differentiation is impaired. Notch has been shown to regulate SMC differentiation in the systemic vasculature, suggesting that this might be a general role for Notch. Notch regulates SMC differentiation through cooperation with TGF-β.23 Notch has proved. Data from GFP tagging of epicardial cells supports a contribution of EPDC to myocardium, although it is possible populations, excluding a role for stem or progenitor cells from this process. The role of the epicardium in zebrafish heart regeneration is an area of intense study. It is now generally accepted that EPDC do not differentiate into CM in the fish heart, but it is also established that the epicardium is necessary for cardiac regeneration to occur, as shown by the requirement for epicardial Raldh2-RA epicardial signaling in CM proliferation during this regeneration. Several studies have analyzed the capacity for cardiac regeneration in mice. A recent report links the cardiac response after mechanical or ischemic injury to Notch activity. These authors used a CBF1Creesr;EGFP reporter line to identify all Notch-dependent signals in the damaged heart, including in native progenitor-like cell populations. This study was based on an earlier publication showing that Notch protects the heart after myocardial injury by triggering a CM survival response mediated by activation of c-Met and Akt. Using the CBF1Creesr;EGFP line, these authors identify an EGFP+/CD45−/CD31− cell population with a gene expression signature of multipotent stromal cells called Notch-activated epicardial-derived cells. These cells express muscle-related and epicardial genes and localize in the epicardium. After ischemic injury, Notch-activated epicardial-derived cells expand, downregulate muscle genes, and upregulate repair pathways involving fibrosis repair, suggesting that they are involved in fibrosis, thedefault repair response of the adult mammalian heart. Initial experiments indicate that the cardiogenic potential of these cells is quite modest. Data from in vitro analysis also suggest that Notch-activated EPDC might act as modulators of tissue repair, eliciting a trophic response in the damaged heart.

Adult Heart Repair: The Embryo Revisited
Epicardial derivatives are a potential source of cells for the development of therapies to repair the damaged heart. The heart was classically regarded as a postmitotic organ, although it was clear that the average lifespan of CM necessitates continual replacement of cells to support adult human life. This paradox triggered the efforts that led to the discovery of cardiac stem cells. Despite controversies about the origin, diversity, abundance, and differentiation potential of cardiac stem cells, their existence endorses the hypothesis that the vertebrate heart has some intrinsic regenerative capacity. Among vertebrates, fish and amphibians are able to regenerate some body parts, including the heart. This cardiac regeneration capacity is not seen in mammals, although a recent report showed that the neonatal mouse heart might transiently display some regenerative capacity. Because the zebrafish heart was shown to regenerate after surgical resection (ablating 20% to 25% of the ventricle), much has been published on this topic. A crucial insight into zebrafish heart regeneration was provided by genetic fate-mapping experiments, which indicated that surviving CM are the source of new muscle after cardiac damage. These studies show that after damage, CM in the subepicardial ventricular layer re-express the embryonic cardiac gene Gata4 and dedifferentiate. Electric conduction is ultimately re-established between existing and regenerated CM a few weeks after injury. These reports demonstrate that functional cardiac muscle regenerates after resection injury primarily through the activation and expansion of CM populations, excluding a role for stem or progenitor cells from this process. The role of the epicardium in zebrafish heart regeneration is an area of intense study. It is now generally accepted that EPDC do not differentiate into CM in the fish heart, but it is also established that the epicardium is necessary for cardiac regeneration to occur, as shown by the requirement for epicardial Raldh2-RA epicardial signaling in CM proliferation during this regeneration.

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Although in zebrafish the epicardium has been discarded as the origin of new CM in the damaged heart, whether mammalian epicardial cells can differentiate into CM has yet to be studied in depth. In a recent report, Tβ4 “priming” of the adult mouse heart was shown to reactivate Wt1 expression and induce CM differentiation by Wt1+ GFP-tagged cells. After Tβ4 priming, Wt1 and Tbx18 expression in epicardial cells was precociously increased 2 days after myocardial infarction (compared with 7 days without Tβ4), and a subpopulation of epicardial cells was found to be positive for the early cardiac progenitor markers Isl1 and Nkx2.5. These cells progressively increased in number and by day 14 had migrated and located within the border zone and peri-infarct region. Analysis by MRI indicates that these de novo generated CM functionally integrate with the resident myocardium, whose function is significantly improved. Data from GFP tagging of epicardial cells supports a contribution of EPDC to myocardium, although it is possible that Wt1+ progenitors arise from a nonepicardial source. As for the mechanism, Tβ4 was previously shown to upregulate integrin-linked kinase and protein kinase C activity, favoring CM survival and migration after ischemic damage; however, it remains unclear how these data can be reconciled with the ability of Tβ4 to promote adult epicardial cell mobilization and differentiation into CoE and CoSMC. Further work is needed to discriminate the relative contribution of each proposed Tβ4 function and their cellular targets in the damaged heart.
**Conclusions**

The embryonic development of the epicardium is a complex process that has recently emerged as a central aspect of cardiac development with wide biomedical implications. Three intrinsic features of epicardial research create difficulties for understanding the role of the epicardium during cardiac development. First, the process is highly dynamic, initially involving a small number of cells (PE cells) that migrate toward different cardiac locations and differentiate into various cell types. Second, the epicardium is subject to a great diversity of cell and molecular interactions (including liver, mesodermal progenitors at the venous pole of the heart, myocardium, and endocardium). Third, the use of a variety of animal models and technical approaches provides data sets that have to be carefully compared to produce an integrated understanding of epicardial biology. This last consideration is particularly important when trying to distinguish between the true fate of a given embryonic cell (what the cell actually does in the embryo) and the full developmental potential of such a cell (what the cell is capable of doing under certain experimental conditions). Research aimed at the use of epicardial cells or EPDC for cardiac repair or regeneration must first confirm the robustness of each identified differentiation potential, the efficiency of this differentiation, and its physiological significance.

Altered epicardial development is implicated in the origin of various manifestations of congenital heart diseases. These congenital heart diseases include those affecting cardiac structures that incorporate cells of epicardial origin (mainly coronary vessels), but probably also other cardiac conditions affecting tissues such as the myocardium and heart valves. This might reflect the ability of epicardium to signal to adjacent tissues in a paracrine manner and it highlights the potential implication of epicardial cells in the progression of acquired disease during the adult life, including atherosclerosis and cardiac valve disease. Unfortunately, no report has shown a direct relationship between defective epicardial development and congenital heart diseases, and further research efforts are therefore needed to reveal the impact of epicardial embryonic development on the postnatal and adult heart.

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**Disclosures**

None.


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