

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Circ Res. 2012;110:1628-1645

doi: 10.1161/CIRCRESAHA.111.259960

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Endocardial and Epicardial Epithelial to Mesenchymal Transitions in Heart Development and Disease

Alexander von Gise, William T. Pu

Abstract: Epithelial to mesenchymal transition (EMT) converts epithelial cells to mobile and developmentally plastic mesenchymal cells. All cells in the heart arise from one or more EMTs. Endocardial and epicardial EMTs produce most of the noncardiomyocyte lineages of the mature heart. Endocardial EMT generates valve progenitor cells and is necessary for formation of the cardiac valves and for complete cardiac septation. Epicardial EMT is required for myocardial growth and coronary vessel formation, and it generates cardiac fibroblasts, vascular smooth muscle cells, a subset of coronary endothelial cells, and possibly a subset of cardiomyocytes. Emerging studies suggest that these developmental mechanisms are redeployed in adult heart valve disease, in cardiac fibrosis, and in myocardial responses to ischemic injury. Redirection and amplification of disease-related EMTs offer potential new therapeutic strategies and approaches for treatment of heart disease. Here, we review the role and molecular regulation of endocardial and epicardial EMT in fetal heart development, and we summarize key literature implicating reactivation of endocardial and epicardial EMT in adult heart disease. (*Circ Res.* 2012;110:1628-1645.)

Key Words: cardiac repair ■ differentiation ■ endocardium ■ epicardium ■ epithelial ■ mesenchymal ■ signaling ■ transition

A major step forward in the evolution of metazoa occurred approximately 600 million years ago, when epithelial cells developed the ability to form mesenchymal cells through a process known as epithelial to mesenchymal transition (EMT).^{1,2} Typical epithelia are organized as sheets of cells polarized along an apical–basal axis, perpendicular to the epithelial plane. An apical belt-like arrangement of tight and adherens junctions interconnects the cells within the epithelial sheet, providing mechanical stability and partitioning the cells into apical and basolateral membrane domains.^{1–3} Epithelial cells commonly rest on a basal lamina, and cell–cell and cell–basal lamina interactions stabilize and coordinate the epithelial architecture. Unlike epithelial cells, mesenchymal cells lack the belt of tight and adherens junctions and have an amoeboid or stellate morphology, features that facilitate their movement as individual cells through the extracellular matrix. Mesenchymal cells are also developmentally pliable, often capable of differentiating into a number of distinct cell types.¹ Thus, the evolution of EMT and mesenchymal cells enabled development of more complex body plans featuring new types of internal organs.^{2–4}

In higher metazoa, most adult tissues and organs arise from a series of EMTs, followed by formation of new epithelial structures by the reverse process of mesenchymal to epithelial transition.¹ The heart is no exception, and all of the cells of

the heart arise through one or more EMTs (Figure 1).^{1,3} In a primary EMT during gastrulation, epiblast cells are converted to mesenchymal cells, a portion of which form the lateral plate mesoderm.^{1,5} These cells then form a secondary epithelium, the splanchnopleure, which undergoes a secondary EMT to form heart cell precursors, which differentiate into cardiomyocyte and endocardial lineages.^{1,5,6} Nearly all of the nonmyocyte cells that populate the heart are generated by additional EMT events. In the heart itself, endocardial EMT in the endocardial cushions forms valve progenitor cells necessary to build the heart valves,^{7,8} and epicardial EMT generates vascular cells, fibroblasts, and possibly even a subset of cardiomyocytes.^{9–19} Errors of EMT likely contribute to congenital heart disease, and in the adult heart there is growing awareness that disease-mediated activation of EMT may contribute to cardiac fibrosis, degenerative valve disease, and the myocardial injury response. Here, we review EMT of the epicardium and endothelium in heart development and our current understanding of how these processes are redeployed in adult heart disease.

Overview of EMT Regulatory Pathways

Although EMT events occur in diverse settings, they typically share general cell biological features (Figure 2) and bear similarities in molecular regulation.^{3,5} To undergo EMT, an

Original received March 24, 2012; revision received April 24, 2012; accepted May 2, 2012. In April 2012, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 12.79 days.

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Circulation Research is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.111.259960

epithelial cell must disassemble the cell–cell junctions that connect it to neighboring cells. The cell must lose epithelial cell polarity and undergo cytoskeletal and gene expression changes to acquire a motile phenotype. Expression of extracellular matrix remodeling enzymes facilitates focal breakdown of the basement membrane, permitting the nascent mesenchymal cell to exit the epithelium and migrate into the subjacent tissue.

At a molecular level, a number of signaling pathways have been implicated in EMT. Two key events in the EMT process are the downregulation of intercellular adhesion junctions and the expression of EMT-inducing transcription factors.²⁰ The cell adhesion protein E-cadherin is the prototypical epithelial adhesion protein, and downregulation of E-cadherin is a key event in EMT in many different contexts. Aside from forming intercellular junctions that help to restrain cells to the epithelial layer, E-cadherin likely participates in intracellular signaling pathways that promote epithelial identity or repress mesenchymal identity. Thus, expression of the cytoplasmic portion of E-cadherin caused cells that have passed through an EMT to lose their mesenchymal phenotype.²¹

The Snail proteins (encoded by *Snail* and *Snai2*) are prototypical EMT-inducing transcription factors.²² Snail is activated by EMT-inducing stimuli such as transforming

Non-standard Abbreviations and Acronyms	
AV	atrioventricular
AVC	atrioventricular canal
EMT	epithelial to mesenchymal transition
EndMT	endothelial EMT
EPDC	epicardium-derived cell
HA	hyaluronic acid
MI	myocardial infarction
MSC	mesenchymal stem cell
NTICD	Notch1 intracellular domain
OFT	outflow tract
RA	retinoic acid
TB4	thymosin beta 4
VEC	valve endocardial cell

growth factor (TGF)- β and Notch. Snail binds to conserved E-box elements within the promoters of adhesion protein genes such as E-cadherin, directly repressing their transcription. Snail also upregulates RhoA and Vimentin, which stimulate cytoskeletal remodeling, and activates expression of

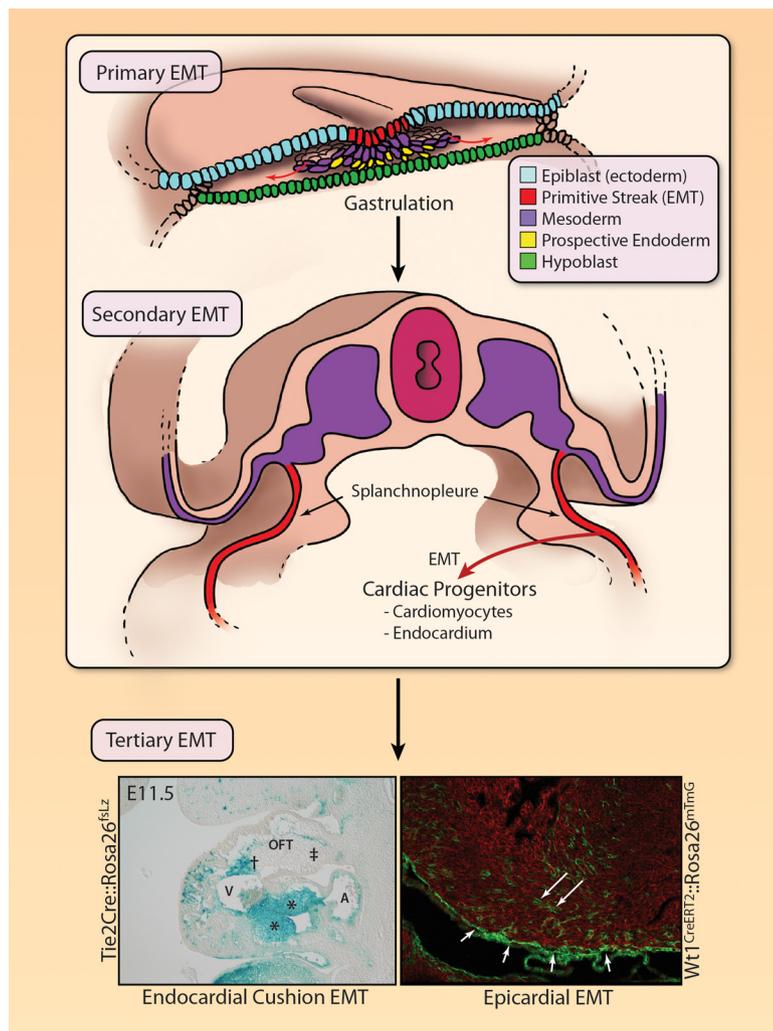


Figure 1. Formation of the heart through a series of epithelial to mesenchymal transitions (EMTs). Myocardium and endocardium arise from two EMTs. Additional lineages present in the adult heart are formed by additional EMT events: neural ectoderm undergoes a primary EMT to yield cardiac neural crest (not shown); endocardium overlying the endocardial cushions undergoes a tertiary EMT to yield mesenchyme of the atrioventricular (AV) canal (*) and proximal outflow tract (OFT†) cushions; and epicardium undergoes a tertiary EMT to yield cardiac fibroblasts, vascular smooth muscle, and potentially a subset of cardiomyocytes. Endothelium or epicardium-restricted Cre (Tie2Cre or Wt1^{CreERT2}, respectively) were used to activate Cre-dependent reporters Rosa26^{fsLz} or Rosa26^{mTmG}, labeling descendants with LacZ (blue) or GFP (green), respectively. Distal OFT cushion (§) was not labeled by Tie2Cre, reflecting mixed contributions from multiple precursor populations. **Short arrows**, epicardium, **Long arrows**, epicardium-derived cells. Illustration credit: Cosmocyte/Ben Smith.

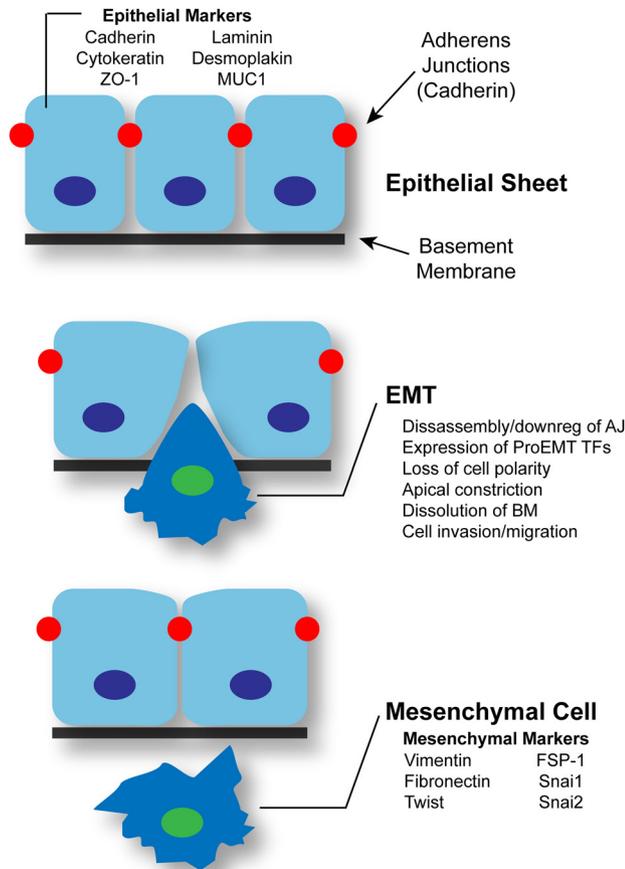


Figure 2. Cellular changes during epithelial to mesenchymal transition (EMT). A typical epithelial sheet contains polarized epithelial cells atop a basement membrane (BM). Cells are joined to one another by adherens junctions (AJs) containing cadherins. EMT requires disassembly and downregulation of adherens junction components, expression of pro-EMT transcription factors (TFs) like SNAI1 and SNAI2, and delamination of the cell through a combination of apical constriction and dissolution of the basement membrane. The resulting mesenchymal cell is mobile and developmentally plastic, and it expresses typical markers such as those indicated.

matrix metalloproteases, which degrade the basal membrane and facilitate cell migration. Thus, Snail and similar EMT-inducing transcription factors coordinate reprogramming of epithelial cells toward a mesenchymal phenotype.

Endocardial/Endothelial EMT in Heart Development and Disease

Endothelium is a specialized type of epithelium, and endothelial cells undergo EMT in developing and adult animals, designated by some as endothelial EMT (EndMT).²³ The best-studied endothelial EMT event occurs in the developing heart valves. In the adult animal, EndMT may contribute to valve homeostasis and disease. In addition, adult microvascular endothelial cells appear to engage in EndMT to yield fibroblasts and myofibroblasts, and this form of EndMT is likely an important mediator of fibrosis in diseased organs, including the heart.

Endocardial EMT and Valve Development

EMT of the endothelial lining of the heart, the endocardium, forms heart valve progenitor cells. The early developing heart

consists of an endocardial layer and a surrounding myocardial layer that are separated by extracellular matrix known as cardiac jelly. At embryonic day 9 of murine development, the endocardial cushions, focal expansions of the cardiac jelly at the atrioventricular (AV) and ventriculo-arterial junctions, become visible. These cushions will develop into the AV and outflow tract (OFT) valves, respectively. At approximately embryonic day 9.5, endocardial cells that line the AV cushions undergo EMT to populate the cushions with mesenchymal cells, precursors of the future valve interstitial cells. OFT cushion development similarly involves endocardial EMT, but occurs somewhat later in gestation and also involves important contributions from the cardiac neural crest. Subsequent steps of valve development involve proliferative expansion of the cushion mesenchyme, followed by elongation and remodeling of the valves to form mature, thin, pliable valve leaflets with a characteristic trilaminar structure. This review focuses on the initial formation of valve mesenchymal cells by endocardial EMT. For information on valve maturation and remodeling, readers are referred to excellent recent reviews.^{24,25}

Endocardial cushion EMT has been well-studied, in part because of the pioneering work of Markwald et al, who established an *in vitro* culture system that recapitulates many aspects of this process.^{26,27} Endocardial cushion cells of chick atrioventricular canal (AVC) explants, consisting of the endocardial cushions and the surrounding cuff of myocardium, undergo EMT when cultured on collagen gels. EMT requires both the AVC myocardium and the endocardial cushions, because ventricular myocardium or endocardium failed to substitute for their respective counterparts.^{27,28} Thus, signals exchanged between specialized AVC myocardium and cushion endocardium are required for endocardial EMT. The AVC explant assay has been extended to mouse²⁹ and used in combination with genetically modified mice to achieve additional insights. Considerable work over the past 20 years in both chick and mouse systems have defined a number of key signaling pathways that mediate specification of AVC myocardium and its induction of endocardial cushion EMT (Figure 3). Details such as isoform usage often differ between chick and mouse systems, and for the sake of brevity here we focus on studies performed in the mammalian system.

Transforming Growth Factor- β /Bone Morphogenic Protein

Signaling by TGF β superfamily members is essential for AVC EMT, (reviewed in detail in references 30 and 31). Two major TGF β superfamily members, the TGF β and bone morphogenic protein (BMP) families, have been implicated.

Among the BMP family, BMP2, BMP4, BMP5, BMP6, and BMP7 participate in AVC or OFT EMT. BMP5, BMP6, or BMP7 null hearts have normal development, but double-mutant embryos develop heart malformations including endocardial cushion defects, suggesting redundant function of these BMPs in cushion development, maturation, or remodeling.³¹ BMP2 mRNA³² and protein³³ are expressed in AVC myocardium at the onset of EMT, and expression then shifts to AV cushion mesenchyme.³³ BMP2 substituted for AVC myocardium in the AVC explant assay, suggesting that it is

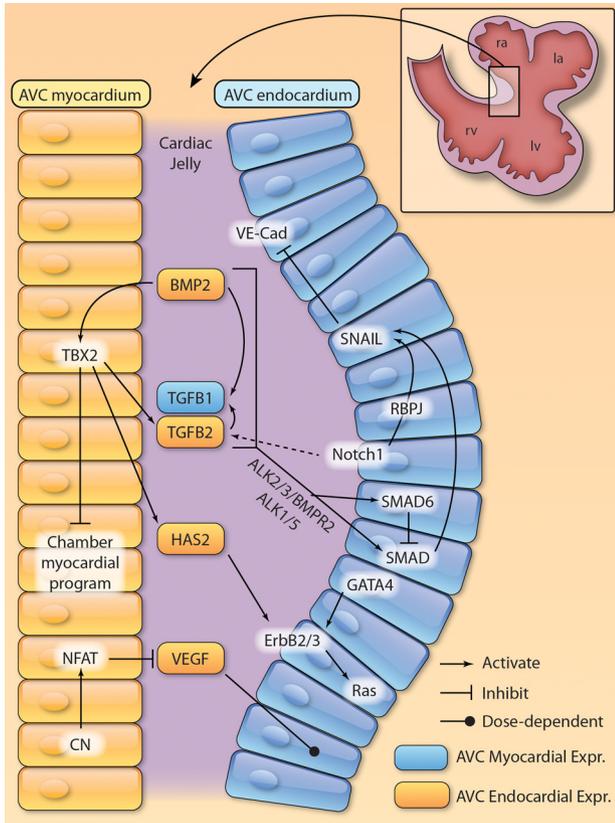


Figure 3. Molecular regulation of endocardial cushion epithelial to mesenchymal transition (EMT). Endocardial EMT to form valve mesenchyme is regulated by signaling between specialized atrioventricular canal (AVC) myocardium and overlying cushion endocardium. Cardiac jelly, the extracellular matrix of the cushions, is also required for endocardial EMT. (Illustration credit: Cosmocyte/Ben Smith).

the key myocardially expressed EMT-inducing factor.³³ Consistent with this, BMP2 inactivation by *Nkx2-5^{Cre}* reduced AV cushion formation and cellularity.^{34,35} BMP2 also was required for maintenance of AVC myocardial gene expression, because BMP2 mutants failed to express *Tbx2*, a key regulator of the AV myocardium gene expression program, and ectopically expressed the chamber myocardial marker *Nppa*.^{34, 35} Restriction of BMP2 to AVC myocardium is important for restraining endocardial EMT from occurring ectopically in ventricles, because BMP2 induced endocardial EMT in ventricular explants normally lacking this activity.³⁶ In large part, this may be attributable to expanded expression of *Tbx2*, because forced expression of *Tbx2* in ventricular myocardium activated key EMT-inducing genes *Tgfb2* and *Has2* and was sufficient to permit endocardial EMT in ventricular explants.³⁷

BMP4 is expressed robustly in OFT, but not AVC, myocardium at the onset of EMT.³⁸ Consistent with its expression pattern, BMP4 was dispensable for AVC EMT but was required for later stages of AV valve development and for septation of the AV canal.³⁸ BMP4 also was not required for OFT EMT, but it was essential for later OFT development and proper ventriculo-arterial alignment.³⁸

BMPs signal through a receptor complex containing type I and type II receptors. In the heart, these are encoded by

Alk2/3^{39,40} and *Bmpr2*,³⁴ respectively. Endothelial-restricted deletion of *Alk2*, *Alk3*, or *Bmpr2* severely impaired endocardial EMT. *Snai1* was markedly downregulated in endocardial *Alk2* knockouts, suggesting that BMP signaling is required for expression of this key pro-EMT transcription factor.³⁹ Thus, myocardial BMP2 signals to endocardial cells through ALK2/BMP2 and ALK3/BMP2 complexes to stimulate AVC endocardial EMT.

The TGFβ family signaling promotes endocardial EMT.⁴¹ In mice, *Tgfb2* is expressed at high levels in AVC myocardium and mesenchyme, where it is a downstream target of BMP2.³³ TGFβ1 is expressed in AVC endocardium before EMT and is upregulated in these cells as they undergo EMT. TGFβ3 is not expressed in the AVC until after the onset of EMT.

Genetic knockout of each TGFβ factor individually did not abolish EMT in vivo, suggesting genetic redundancy (or alternatively a nonessential role for TGFβ in AVC EMT in vivo). TGFβ1-null hearts developed normally; however, these fetuses were not fully deficient for TGFβ1 because of placental transfer of maternal TGFβ1.^{42,43} To exclude effects of maternal TGFβ1, rare TGFβ1 fetuses of TGFβ1-null mothers were studied and found to have multiple abnormalities including severe cardiac defects, suggesting an essential (but as yet poorly characterized) role in heart development.^{42,43} TGFβ2-neutralizing antibody inhibited EMT in murine explants²⁹ and TGFβ2-null hearts had cardiac defects including AV and OFT abnormalities, although these were likely attributable to defects in valve maturation and remodeling rather than mesenchyme formation via EMT.⁴⁴ In contrast, TGFβ3-neutralizing antibody did not affect explant EMT,²⁹ and TGFβ3-null hearts were phenotypically normal,⁴⁵ indicating that TGFβ3 does not have unique essential functions in heart development. Collectively, these studies suggest that myocardial signals (BMP2±TGFβ2) activate AV endocardium, upregulating TGFβ1 and further stimulating EMT in an autocrine fashion.³⁰

Like BMPs, TGFβs also signal through a complex between type I (ALK1/5) and type II (TGFBR2) receptors. ALK1 and ALK5 are expressed in murine AV endocardium. ALK1 knockout or endocardial-restricted ALK5 inactivation caused markedly hypocellular endocardial cushions, suggesting an in vivo requirement for both TGFβ type I receptors in cushion EMT.⁴⁶ Unexpectedly, *Tgfb2* was dispensable for in vivo endocardial EMT in the mouse.⁴⁷ In contrast, AVC canal explants from these mutant mice failed to undergo EMT in explant culture. One explanation for these results is that AVC EMT in explant culture does not exactly model AVC EMT in embryos, so that TGFβ signaling is essential for AVC EMT in explant culture but is dispensable for this process in embryos because of redundancy with other cytokine signaling pathways. Alternatively, this result might be attributable to redundancy with a different type II receptor, such as BMPRII, which may permit TGFβ signaling in embryos that lack the type II TGFβ receptor.³⁰ However, at present *Tgfb2* is the only solidly supported type II receptor for TGFβ signaling.

The TGFβ superfamily influences gene expression through SMAD transcription factors. The receptor SMADs (R-SMADs; SMAD2/3 for TGFβ ligands and SMAD1/5/8 for

BMP ligands) heterodimerize with a common SMAD, SMAD4, to activate transcription. SMAD transcriptional activity is required in both AVC myocardium and endocardium for endocardial EMT. In myocardium, SMAD activation by BMP2 directly stimulates a *Tbx2* transcriptional enhancer, thereby specifying the AVC myocardium.³⁷

Endocardium-restricted inactivation of *Smad4* abolished endocardial EMT,⁴⁸ reinforcing the essential role of TGF β superfamily signaling on endocardial cells in promoting EMT. Interestingly, SMAD4 physically interacted with GATA4,⁴⁸ a transcription factor strongly expressed in endocardium, where it is essential for endocardial EMT.⁴⁹ Heterozygous GATA4 mutation caused endocardial cushion defects in humans and mice,^{48,50} and GATA4 and SMAD4 interacted genetically to promote endocardial cushion development.⁴⁸ Human GATA4 mutations associated with endocardial cushion defects impaired GATA4–SMAD4 interaction,⁴⁸ suggesting that these mutations attenuate pro-EMT TGF β superfamily signaling through *Smad4*.

The inhibitory SMADs, *Smad6/7*, reduce TGF β superfamily signaling. *Smad6* is expressed primarily in the heart (including AV cushion endocardial cells) and vascular system. *Smad6* inactivation resulted in excessive valve mesenchymal cells and heart valve hyperplasia,⁵¹ and *Smad6* overexpression in AV explants was shown to directly inhibit AV cushion EMT.⁵² TGF β superfamily signaling stimulated *Smad6* expression, suggesting that it functions as a native feedback mechanism to limit TGF β superfamily signaling. These data indicate that *Smad6* is essential to limit endocardial cushion EMT.

Notch

The Notch signaling system regulates cell fate specification and differentiation, and its function in heart valve development and disease was recently reviewed.⁵³ The Notch receptor (encoded by *Notch1-4* in mammals) contains a single-pass transmembrane domain, a large extracellular domain, and a Notch intracellular domain. Notch interaction with a ligand (*Jagged 1/2* or *Delta 1/3/4*) expressed on the surface of an adjacent cell stimulates Notch proteolytic cleavage by γ -secretase activity and nuclear translocation of Notch intracellular domain. Nuclear Notch intracellular domain interacts with the DNA-binding transcription factor RBPJ, converting it from a repressor to an activator.

Notch signaling is required for several steps in valve development. The ligand DLL4 and nuclear-localized intracellular domain of Notch1 (NIICD) are present in AV and OFT endocardium at the time of EMT. *Notch1* or *Rbpj* deletion caused severe endocardial cushion hypoplasia.⁵⁴ In *Rbpj*-null mutants, *Snail* failed to be upregulated in AV endocardium. This blocked EMT by preventing downregulation of the major endothelial intercellular adhesion protein, vascular endothelial (VE)-cadherin. The pro-EMT growth factor TGF β 2 also was severely downregulated in AVC myocardium, suggesting a Notch1-dependent endocardial to myocardial signal required for local TGF β 2 expression.⁵⁴

Widespread endothelial and endocardial expression of NIICD activated expression of pro-EMT and mesenchymal genes, including *Snail*, *Snai2*, *Tgfb2*, *Twist2*, and *Alk3*.^{36, 55}

but it was not sufficient to induce invasive endocardium-derived mesenchyme in ventricular explants.³⁶ Further addition of *Bmp2* to these explants did lead to full endocardial EMT. Consistent with these results, nuclear NIICD was present in endocardium throughout the normal developing heart, albeit it was most strongly detected in endocardium overlying the cushions.⁵⁶ Thus, NIICD confers some but not all signals for endocardial EMT, and activation of Notch does not account for restriction of endocardial EMT to the endocardial cushions.

ErbB Receptor Signaling

The ErbB receptor tyrosine kinases (ErbB1–ErbB4) are critical for heart and valve development and for adult heart function. ErbB ligands include epidermal growth factor (EGF)-like ligands (eg, EGF and heparin-binding [HB]-EGF) and neuregulins (NRG1–4). ErbB receptor tyrosine kinase receptors signal through Ras, PI3K-Akt, and MAPK pathways. Here, we focus on regulation of EMT by neuregulin signaling through ErbB2–ErbB3, although ErbB1 (also known as EGFR) and ErbB4 are essential for other aspects of heart and valve development.⁵⁷

ErbB2 is an “orphan” receptor that does not by itself bind known ligands. It is a frequent dimerization partner of other ErbB family members that increases ligand binding affinity and augments tyrosine kinase activity. ErbB3 binds neuregulin family ligands but lacks its own tyrosine kinase activity. Neuregulin, ErbB2, and ErbB3 are expressed in the endocardium of the developing cushions at the time of EMT initiation.²⁹ Cushion mesenchymal cells were not formed in ErbB2^{-/-} or ErbB3^{-/-} hearts or in explant culture. Furthermore, EMT in wild-type explants was inhibited by inactivation of ErbB3 ligands. Interestingly, expression of ErbB3 in AV endocardium required endocardial expression of the transcription factor GATA4,⁴⁹ mutation of which causes human endocardial cushion defects.^{48,50}

Hyaluronic acid (HA), a glycosaminoglycan and a major component of the endocardial cushion extracellular matrix, is synthesized by HA synthetase 2 (*Has2*). *Has2*-null endocardium failed to undergo cushion EMT, a defect that was rescued in explant culture by addition of exogenous HA.⁵⁸ This effect was not attributable to general disruption of cushion structure, because impaired synthesis of versican, another major component of the cushion extracellular matrix, did not block endocardial cushion EMT in the in vitro assay.⁵⁹ Furthermore, HA was effective at low micromolar concentrations, suggesting a high-affinity interaction.

Interestingly, HA induces ErbB tyrosine kinase activity, which activates RAS. The requirement for HA in endocardial EMT was bypassed by activated RAS, whereas dominant-negative RAS blocked endocardial EMT of wild-type explants and *Has2*^{-/-} explants that had been rescued by HA treatment.⁵⁸ ErbB3 tyrosine phosphorylation in AVC explants required HA or stimulation with exogenous neuregulin.²⁹ Thus, HA augments ErbB2/B3 heterodimer activation, which promotes AVC EMT through a RAS-dependent pathway.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF)-A is a key regulator of endothelial cells. The myocardium is the major

site of VEGF-A production at the time of AVC EMT.^{60,61} The endocardium of the AVC and OFT may also produce VEGF-A.⁶¹ At EMT initiation (embryonic day 9.5), myocardial VEGF-A expression was uniform, but later in the process of cushion mesenchymalization (embryonic day 10.5), VEGF-A expression became concentrated in the AVC myocardium.⁶⁰ VEGF-A acts on endocardial cells, because endocardial cells, but not endocardially derived mesenchyme, expressed VEGFR1, a VEGF-A receptor.⁶⁰

Proper levels of VEGF-A exposure are essential for normal endocardial EMT. Transgenic myocardial VEGF-A expression inhibited endocardial EMT in embryos, and addition of exogenous VEGF-A blocked EMT in AV explant culture.^{60,62} However, treatment of AV explants with the VEGF-A antagonist sFlt-1 blocked endocardial EMT, indicating that too little VEGF-A is also incompatible with normal endocardial cushion mesenchymalization.⁶³ Thus, upregulation of VEGF-A in AV myocardium appears to be a mechanism to regulate the timing and extent of cushion mesenchymalization through endocardial EMT.

Calcineurin–NFAT signaling regulates myocardial expression of VEGF-A. Triple knockout of *NFATc2*, *NFATc3*, and *NFATc4* caused failure of endocardial EMT, as did pharmacological (cyclosporin A) inhibition of calcineurin, the calcium-calmodulin–regulated phosphatase that activates NFAT.⁶² Germline mutation of the calcineurin regulatory subunit CNB1 also caused reduced endocardial EMT in AVC explants,⁶² although this finding was not recapitulated in embryos with endocardium or myocardium-restricted *Cnb1* inactivation.^{62,64} Further work revealed that NFAT directly represses VEGF-A transcription.⁶² Thus, myocardial calcineurin–NFAT signaling prevents excessive VEGF-A expression and thereby permits endocardial EMT.

Abnormal regulation of VEGF-A expression has been implicated in congenital heart disease associated with environmental stresses or in Down syndrome. Hypoxia increased AV cushion expression of VEGF and inhibited cushion EMT. EMT could be rescued in hypoxic cushions by VEGF-A inhibition with sFlt-1.^{60,65} Hyperglycemia reduced cushion VEGF-A expression and also inhibited cushion EMT.⁶³ Hyperglycemia-mediated reduction of cushion EMT was rescued in explant culture by addition of VEGF-A. Trisomy of the Down syndrome critical region is associated with high risk of endocardial cushion defects. This region contains the gene *MCIP1*, also known as Down syndrome critical region 1, which regulates calcineurin–NFAT signaling.^{66,67} The hypothesis that excessive *MCIP1* copy number contributes to the risk of abnormal cushion development was tested by normalizing *MCIP1* copy number in the trisomy 16 mouse model of Down syndrome. *MCIP1* copy number did not influence the incidence of congenital heart disease in this mouse model,⁶⁸ suggesting that *MCIP1* copy number is not sufficient to account for endocardial cushion defects in this disorder.

Wnt/ β -Catenin Signaling

Canonical signaling by Wnt ligands releases β -catenin from cellular adhesion junctions and permits its nuclear entry, where it regulates gene transcription by interacting with

DNA-binding transcription factors, most notably members of the LEF/TCF family. This signaling pathway is important for both formation of endocardial cushion mesenchyme and for later valve remodeling. Endocardial ablation of β -catenin disrupted endocardial EMT in embryos and in explant culture.⁶⁹ However, the upstream Wnt ligands and the downstream targets of Wnt/ β -catenin signaling in AV EMT have not been well-studied. These signals may be extremely transient, because the BAT-gal transgenic reporter of canonical Wnt signaling is activated in very few cells of the cushion endocardium or cushion mesenchyme.⁶⁹

Studies in zebrafish have revealed that canonical Wnt signaling is required to properly specify AV myocardium. The adenomatous polyposis coli (*apc*) gene encodes an essential component of the β -catenin destruction complex, which limits canonical β -catenin signaling. In normal zebrafish, nuclear β -catenin localized to AV cushion endocardium and myocardium. However, in *apc* mutant zebrafish, all endocardial and myocardial cells exhibited nuclear β -catenin.⁷⁰ Mutant hearts did not complete looping, contracted poorly, and did not properly restrict myocardial *Bmp* expression (*bmp4* for zebrafish rather than murine *Bmp2*) to AV myocardium. This prevented normal differentiation of chamber myocardium, which instead expressed *has2* and *tbx2b*, normally restricted to AV myocardium.^{70,71}

Endothelial EMT in Disease

Although developmental EMT of cushion endocardium has been extensively studied, the role of endothelial EMT in adult heart and valve disease is an emerging area of research. Here, we review the growing literature suggesting that EndMT participates in adult heart and valve homeostasis and disease.

EndMT in Adult Valve Disease

Valve disease causes significant morbidity and mortality and poses a significant societal burden in health costs. The majority of valve disease cases involve a congenitally malformed valve. Thus, valve disease is attributable to the combined effects of predisposing genotypes, aberrant developmental mechanisms, and abnormal tissue maintenance.²⁴

The role of endocardial EMT in adult valve homeostasis and disease is only just beginning to be investigated. Diseased valves are characterized by valvular interstitial cell (VIC) expression of mesenchymal/myofibroblast markers, such as vimentin and smooth muscle actin (SMA).^{72,73} During development, these markers are also expressed by valve progenitor cells derived by endocardial EMT, suggesting a potential reactivation of endocardial EMT in valve disease. This possibility is also supported by the activation of these mesenchymal markers in a subset of diseased valve endocardial cells (VECs) in animal models and human patients.^{74–76}

Clonal analysis of VECs has yielded additional insights. Single juvenile ovine aortic VECs, plated in 96 well dishes, expanded to a confluent monolayer and subsequently continued to proliferate through repeated subculture, suggesting robust self-renewal.⁷⁴ Moreover, when the clones were stimulated with TGF β 1, a subset downregulated the endothelial marker CD31, upregulated the marker mesenchymal SMA, and adopted mesenchymal morphology, changes consistent

with EMT.⁷⁴ Other clones were refractory to TGF β 1 stimulation. These findings suggested the hypothesis that valve endothelium contains a subpopulation of progenitor-like cells that undergo EMT to replenish mesenchymal cells within the valves. This hypothesis was further supported in an experimental model of mitral regurgitation. In this model, surgical tethering of the papillary muscles induced mitral regurgitation and increased mitral valve leaflet area. VEC EMT (defined as VEC expression of SMA) was strongly increased in animals with regurgitation compared with sham-operated controls, suggesting that enhanced VEC EMT contributes to pathological changes in diseased leaflets.⁷⁵ As in embryonic cushion endocardium EMT, TGF β , VEGFA, and Notch were found to regulate VEC expression of this mesenchymal cell marker.⁷⁶

Might VEC EMT contribute to valve calcification, a hallmark of adult valve disease? A clonal analysis of ovine mitral valve VECs showed that a subset of VECs were readily induced to differentiate toward osteogenic or chondrogenic phenotypes, which was not observed in a number of other primary endothelial cell cultures.⁷⁷ Thus, a subset of VECs and their EMT-derived progeny may display unique developmental plasticity that contributes to adult valve disease. Missense mutations of Notch1 were found to cause familial premature calcific aortic valve disease.⁷⁸ The underlying mechanisms linking Notch1 mutation to aortic valve disease remain unclear but may involve impaired regulation of aortic VEC EMT.

The observation that in disease VECs express mesenchymal genes suggests the possibility that these cells are undergoing EMT. However, full EMT of adult VECs to vascular interstitial cells has not been directly demonstrated in valve homeostasis or disease. If it occurs, then the extent of VEC to vascular interstitial cell transition and the importance of this transition for adult onset valve disease remain to be determined. A key step forward will be the use of lineage tracing techniques to show and quantify VEC to VIC transformation in adult valve disease models. This will require development of lineage tracing approaches in larger animal models of valve disease or application of genetic lineage tracing approaches to adult VEC EMT in murine models of valve disease.

EndMT and Cardiac Fibrosis

Cardiac fibrosis is a major consequence of most forms of adult heart injury, such as myocardial infarction (MI). During cardiac fibrosis, the number of fibroblasts in the heart increases, but until recently little was known about the source of the newly formed fibroblasts. Potential sources included resident fibroblasts, circulating cells originating from the bone marrow, and differentiation of resident nonfibroblasts, such as endothelial cells.⁷⁹

Genetic lineage tracing shed new light on sources of cardiac fibrosis. In TielCre::Rosa26^{fsLacZ} mice, TielCre catalyzes recombination of the Cre-activated reporter, so that LacZ marks endothelial cells and their descendants. In the context of cardiac injury, Zeisberg et al²³ showed that the genetic LacZ label was expressed in a subset of cardiac fibroblasts and myofibroblasts, indicating their origin from

endothelial cells. A substantial fraction of cells was labeled, approximately 30% of cardiac fibroblasts and 75% of cardiac myofibroblasts. Formation of fibroblasts by EndMT likely contributes to the perivascular pattern of cardiac fibrosis. One limitation of this and most current EndMT studies in adult injury models is the possibility that injury induces ectopic expression of Cre activity, so that it is no longer restricted to endothelial cells. This possibility is difficult to control for using constitutively active forms of Cre. Similar genetic lineage tracing strategies have implicated EndMT in fibrosis of other organs, including the kidney⁸⁰ and lung,⁸¹ and EndMT may also participate in cancer pathobiology.⁸² EndMT implicated in organ fibrosis has been compared with developmental endocardial cushion EMT and principles may be similar. However, given that cushion EMT is strictly limited to the endocardial cushions, whereas fibrosis-related EndMT occurs in multiple different types of endothelial cells, the relationship between these processes is unclear.

TGF β 1 was implicated as a major signaling pathway that stimulates EndMT in cardiac injury. Recombinant BMP7, which antagonized the action of TGF β 1 on cultured endothelial cells, reduced EndMT and cardiac fibrosis in aortic banding models.²³ The plasminogen activator inhibitor-1 restrains TGF β signaling in the normal heart, and mutants lacking plasminogen activator inhibitor-1 developed cardiac fibrosis.⁸³ TGF β -induced EndMT was implicated in this process, because TGF β -stimulated expression of mesenchymal markers was reduced in plasminogen activator inhibitor-1-null endothelial cells. However, direct evidence through *in vivo* lineage tracing was not obtained. TGF β -induced EndMT may require “druggable” signaling molecules, such as c-Abl or PKC δ ,⁸⁴ suggesting a strategy for developing novel therapeutic approaches to cardiac fibrosis.

Canonical Wnt signaling also appears to be involved in EndMT-mediated cardiac fibrosis after MI. After experimental MI, canonical Wnt signaling increased in the evolving infarct scar, as assessed using the TOPGAL Wnt reporter mouse.⁸⁵ This activity was primarily confined to endothelial and SMA-expressing mesenchymal cells, approximately 40% of which was traced to endothelial precursors by Cre-loxP genetic lineage tracing. Similarly, activation of canonical Wnt signaling induced morphological and molecular changes toward a mesenchymal phenotype.⁸⁵ Activation of Wnt expression also has been reported in epicardium and in fibroblasts after ischemic myocardial injury⁸⁶ (see Paracrine Activity of Reactivated Epicardium), and the relative contribution of epicardium and endocardium to Wnt-related post-MI fibrosis requires further investigation.

Epicardial EMT in Heart Development

The epicardium is a specialized epithelium related to the mesothelium that lines most visceral organs. Ablation of the epicardium or genes expressed in the epicardium disrupts myocardial growth, coronary vessel development, and formation of cardiac interstitial cells. Thus, the epicardium plays critical roles in heart development because of extensive myocardial–epicardial signaling and direct contribution to multiple cardiac lineages through EMT.

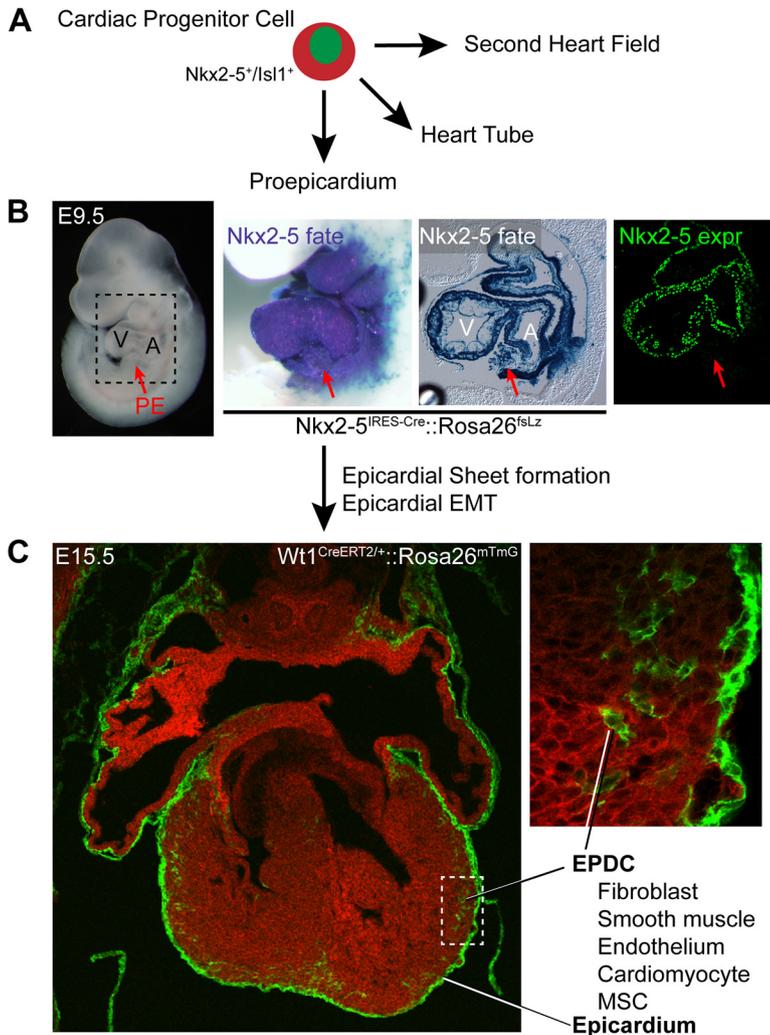


Figure 4. Developmental plasticity of epicardial progenitor cells. **A**, Progenitor cells expressing Isl1 and Nkx2-5 generate proepicardium (PE, red arrow) and the heart tube, containing the nascent atria (A) and ventricles (V). Thus, PE cells are labeled by Nkx2-5IRES-Cre (**B**) and Isl1Cre (not shown), but they do not actively express these markers. Whether precursors of proepicardium and heart tube are the same or merely express the same markers is presently unknown. **C**, Subsequently, PE cells migrate over the surface of the heart to form epicardium, which undergoes epithelial to mesenchymal transition (EMT) to form epicardium-derived cell (EPDCs). EPDCs differentiate into interstitial and annulus fibrosis fibroblasts, smooth muscle cells, endothelial cells, cardiomyocytes, and mesenchymal stem cells. Cre-labeled cells expressed GFP (green), and unlabeled cells expressed RFP (red). **B**, Modified from Zhou et al, BBRC, 2008, with permission.⁸⁷

The epicardium arises from the proepicardium, a vesicular outgrowth continuous with the coelomic mesothelium¹ (Figure 4). The relationship of the proepicardial lineage to other cardiac lineages is not well-understood. Cre recombinase driven by regulatory elements of *Isl1* or *Nkx2-5*, markers of cardiac progenitor cells, activated Cre-dependent reporters in proepicardium, but neither *ISL1* nor *NKX2-5* was actively expressed in proepicardium^{16,87} (Figure 4B). Regulatory elements of another key cardiac transcription factor gene, *Hand1*, similarly labeled proepicardium without active expression in this tissue.⁸⁸ Thus, proepicardium likely shares a lineage relationship to cardiac progenitor cells, although it remains uncertain whether proepicardium and heart tube progenitors overlap or express overlapping markers.

Cells from the proepicardium migrate onto and cover the myocardium as an epithelial sheet known as the epicardium. The intimate relationship of epicardium to myocardium and the nascent coronary vasculature uniquely position it to regulate myocardial growth and coronary vessel development. Epicardial signals are required for normal expansion of the myocardium, and cells and factors originating from the epicardium are required for normal growth of coronary vessels. These aspects of epicardial function were recently reviewed.^{89,90} Here, we focus on epicardial cell fate and regulation of epicardial EMT.

Epicardial Cell Fate

Initially, a thin layer of extracellular matrix separates epicardium from myocardium. Epicardial cells undergo EMT, forming mesenchymal cells known as epicardium-derived cells (EPDCs). EPDCs invade the subepicardial matrix and subsequently migrate into the myocardium, where they differentiate into several lineages of the heart. Initially, the fate of EPDCs was analyzed in chick embryos by proepicardial injection of limiting doses of replication-deficient LacZ-expressing retrovirus. The fate of infected proepicardial cells was determined by evaluating the distribution of the indelible LacZ label later in development. Epicardial dye labeling and quail chick chimeras are additional approaches that have been used to determine the fate of epicardial cells in avian systems. These pioneering studies showed that proepicardial cells differentiate into vascular smooth muscle, endothelial, and fibroblast lineages of the mature heart.⁹⁻¹² In addition, some labeled cardiomyocytes were noted, although it was unclear if these represented cardiomyocytes that were inadvertently labeled during the injection process.¹⁰ Importantly, these techniques provide information on the types of lineages that arise from proepicardial cells but do not reveal the extent to which EPDCs contribute to a specific lineage. For instance, from these studies it was not possible to ascertain what

fraction of coronary vascular endothelial cells arises from proepicardium.

In mammals, genetic Cre-loxP-based lineage tracing strategies have been used to examine the fate of epicardial cells (Figure 4C). Interpretation of these studies has been more difficult than that of comparable studies of AV endocardial EMT for several reasons. First, mesenchymal cells arising from AV cushion EMT migrate into a largely acellular space, whereas EPDCs migrate into the highly cellular myocardium. Second and more importantly, it has been challenging to generate Cre alleles with highly restricted epicardial expression, so that there has been controversy over what labeling reflects EPDC differentiation versus labeling attributable to endogenous Cre activity in a nonepicardial lineage. Several Cre alleles with primarily epicardial expression in the heart have been reported: cGATA5-Cre,¹⁴ based on a chicken GATA5 enhancer; Tbx18^{Cre15}; and Wt1-Cre,^{13,91} Wt1^{GFP^{Cre16}}, and Wt1^{CreERT216}. A Tcf21-MerCreMer line with epicardial expression also was recently described.⁹² Defining the specificity of Cre expression for these alleles has been problematic, leading to uncertainty over some lineage tracing results.⁹³

Vascular smooth muscle cells were consistently labeled using these Cre drivers. A Wt1-Cre BAC transgene labeled 92% of cardiac vascular smooth muscle, suggesting that the large majority of these cells arise from epicardium via EMT.¹³ Robust labeling of vascular smooth muscle also was reported for Tbx18Cre¹⁵ and cGATA5-Cre.¹⁴

The origin of coronary vascular endothelial cells is more controversial. cGATA5-Cre, Tbx18^{Cre}, Wt-based Cre, and Tcf21-MerCreMer labeled 0% to 14% of vascular endothelial cells,^{13–16} indicating that the subsets of epicardium that express these Cre drivers do not contribute to the majority of coronary vascular endothelium. Tabin et al recently reported novel Scx-Cre and Sema3D-Cre alleles that are restricted to the proepicardium and epicardium prior to E11.¹⁷ In the proepicardium, most Sema3D⁺ cells expressed neither TBX18 or WT1. Similarly, most Scx⁺ cells expressed neither WT1 nor Tbx18. Unlike Wt1-Cre-labeled cells, Scx-Cre-marked and Sema3D-Cre-marked cells contributed to coronary endothelial cells with similar or greater frequency than to the smooth muscle lineage. However, the fraction of coronary endothelial cells labeled by these Cre alleles remained low, so that the origin of the majority of coronary endothelial cells remains uncertain. Note that these results do not necessarily conflict with the lineage tracing data from avians, which shows epicardial contribution to the endothelial lineage but not the extent of contribution. Potential sources of the majority of coronary vascular endothelial cells remain controversial and include subsets of epicardium that do not express these Cre drivers, sinus venosus endothelial cells,⁹⁴ liver microvasculature,⁹⁵ and heart chamber endocardial cells.⁹⁴ Given likely multiple sources of coronary endothelial cells, quantitative approaches are required to define the predominant origins of these cells.

Cardiac fibroblasts also derive from epicardium through EMT. Much of the data linking epicardial derivatives with cardiac fibroblasts arose from studies in avians.^{9–12} In mammals, relatively less has been reported about EPDCs differ-

entiating into fibroblasts, in part because of the paucity of definitive markers of cardiac fibroblasts. cGATA5-Cre, Tbx18^{Cre}, and Wt1^{CreERT2} each labeled cardiac fibroblasts.^{14–16} Mutant hearts lacking epicardial EMT attributable to epicardium-restricted ablation of both platelet-derived growth factor receptor (PDGFR)- α and PDGFR β exhibited a striking lack of fibroblasts, indicating that the large majority of cardiac fibroblasts arise from epicardial EMT.⁹⁶ Epicardial derivatives are especially abundant around the AV groove, and in this region EPDCs differentiate into cells of the annulus fibrosis,^{12,18,19} the fibrous insulation that separates atrial and ventricular myocardium to permit sequential atrial and then ventricular contraction. Perturbation of epicardial migration over the heart in chick embryos impaired annulus fibrosis formation, resulting in myocardial bridges between atria and ventricles and causing ventricular pre-excitation similar to that seen in Wolff-Parkinson-White syndrome, a common human arrhythmia.¹⁸

EPDCs have been reported to differentiate into cardiomyocytes during normal heart development,^{15,16} although this remains controversial.⁹³ In vitro, proepicardial explants spontaneously differentiated into cardiomyocytes, and this was modulated by BMP, fibroblast growth factor (FGF), and Notch signaling.^{56,97} These experiments clearly demonstrated the cardiomyocyte differentiation potential of proepicardium and its derivatives. Subsequently, Tbx18-driven and Wt1-driven Cre alleles were shown to label a subset of cardiomyocytes in the normal developing heart.^{15,16} Most recently, adult Wt1-marked cardiac cells were shown to differentiate into cardiomyocytes in adult hearts primed with the peptide thymosin beta 4 (see section below titled “Targeting Epicardial Plasticity in Therapeutic Myocardial Regeneration”), consistent with the potential of EPDCs to differentiate into cardiomyocytes.⁹⁸

However, the question of whether EPDCs differentiate into cardiomyocytes in vivo continues to be debated. In zebrafish, an epicardium-restricted Tcf21-CreERT2 BAC transgene did not label fetal or adult cardiomyocytes,⁹⁹ suggesting that this epicardial subset does not differentiate into cardiomyocytes in zebrafish. However, this does not exclude a cardiomyocyte fate of other epicardial subsets or cardiogenic differentiation in response to therapeutic manipulations. This experiment also does not directly address epicardial differentiation and function in mammals. The function of epicardium may differ considerably between zebrafish and mammals, given the marked differences between zebrafish and mammalian heart structure, eg, in adult zebrafish the large majority of myocardial mass is in cardiac trabeculations remote from the epicardium, whereas the bulk of myocardial mass in adult mammals resides in compact myocardium immediately subadjacent to epicardium.

Wt1^{CreERT2} and Tbx18-Cre did label mammalian cardiomyocytes,^{15,16,98} but the interpretation has been muddied by limitations of the Cre-loxP approach. First, it is important to note that the Cre-loxP system has a binary readout (Cre reporter recombined or not), which necessarily imposes a threshold on the results. Second, the response of reporters is not linear or uniform, so that the same Cre can yield very different recombination maps with different reporters.¹⁰⁰

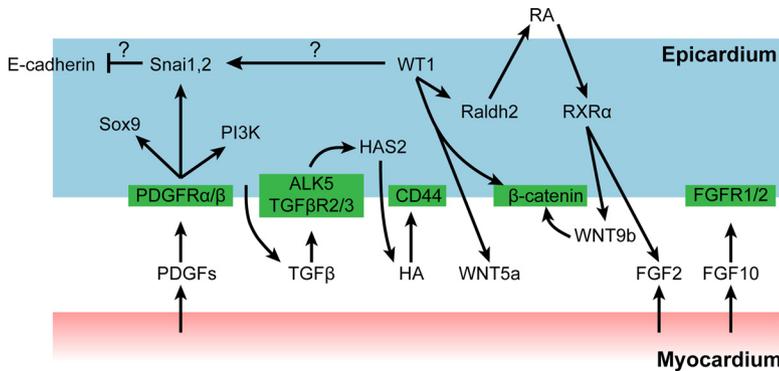


Figure 5. Molecular regulation of epicardial epithelial to mesenchymal transition (EMT). Pathways that regulate epicardium-derived cell (EPDC) fate, myocardial growth, and coronary vessel development without directly influencing epicardial EMT are not shown. Question marks indicate inconsistency in existing data.

Third, rigorous interpretation requires that the “epicardial” Cre not be expressed in cardiomyocytes, but it can be difficult to distinguish low-level or infrequent stochastic expression from background staining. The thresholding and variable reporter responses make it difficult to determine whether an epicardial Cre is or is not expressed below an acceptable “background” level in cardiomyocytes. *Tbx18* and *Tbx18^{Cre}* were expressed in a subset of cardiomyocytes, so that lineage tracing with *Tbx18^{Cre}* was not conclusive.⁹³ *Wtl* expression in cardiomyocytes has not been reported, although a zebrafish *wtlb*-based transgene was expressed in a subset of fetal cardiomyocytes⁹⁹ (the endogenous gene product was not assayed). Nevertheless, complementary approaches that do not critically hinge on the expression or lack of expression of Cre in cardiomyocytes are essential to resolve this controversy.

Additional concerns regarding the hypothesized differentiation of EPDCs to cardiomyocytes also have been raised. It also has been speculated that the *Wtl*-based lineage tracing results were attributable to presence of a neomycin resistance cassette in the targeted locus.⁹⁰ To clarify, in this case the lineage tracing results were the same with or without the neo cassette (and, in fact, most of the published data^{16,19,101–103} were obtained using the allele after neo cassette removal). Also, it has been noted that *Tbx18*, *GATA5*, and *Wtl* are expressed in nondifferentiated mesodermal cells at the venous pole of the heart, so that cells labeled by these Cre alleles might derive from sources other than epicardium.⁹⁰ These caveats were in part addressed by complementary approaches involving epicardial dye labeling in explant culture and fluorescence activated cell sorting (FACS) purification and in vitro differentiation of fetal epicardial cells into cardiomyocytes,¹⁶ but clearly additional studies are required.

Mesenchymal stem cells (MSCs) resident in the adult heart also appear to originate largely from the fetal epicardium.¹⁰⁴ Multipotent MSCs were shown to reside in the fetal and adult heart. The adult MSCs did not arise from adult bone marrow; rather, they were efficiently labeled by epicardial Cre drivers. Fetal activation of *Wtl^{CreERT2}* labeled adult heart MSCs, suggesting that these cells arise from fetal epicardium. Future work will elucidate the function of these cardiac MSCs in heart homeostasis, injury response, and regeneration.

The lineage hierarchy of EPDCs has yet to be established. It is likely that proepicardium and epicardium contain distinct subsets of progenitors that differ in their signaling interactions and fate.¹⁷ For instance, one epicardial subset may

proliferate to form more epicardium, another may differentiate into smooth muscle cells, and another may differentiate into fibroblasts. Currently available Cre drivers may only label a subset, so that not all epicardium-derived cells are labeled (eg, possibly coronary endothelial cells). Alternatively, an epicardial progenitor might be multipotent and capable of differentiating into multiple definitive cell types. Additional work using clonal labeling approaches will be required to establish the EPDC lineage hierarchy and to discover the signals that govern lineage choices.

Molecular Regulation of Epicardial EMT

Elaborate signaling between epicardium and myocardium regulates myocardial growth and coronary vessel formation, and is covered in excellent recent reviews.^{89,90} Here, we focus on molecular regulation of epicardial EMT. Relatively less is known about epicardial EMT than endocardial EMT, in part because tools to label, track, isolate, and genetically manipulate epicardium in vitro and in vivo were not available until recently. The widespread availability of epicardium-restricted Cre alleles will allow a combination of conditional mutagenesis and genetic lineage tracing strategies to probe the activity of signaling molecules in epicardial EMT. Interestingly, current studies suggest that distinct mechanisms regulate EMT within the epicardium versus the endocardium, nearby subcompartments of the same organ (Figure 5).

TGFβ

The TGFβ superfamily appears to promote both epicardial and endocardial EMT. The proepicardium expresses *Tgfb2*, whereas by embryonic day 12.5 the epicardium expresses all three TGFβ genes.¹⁰⁵ Myocardium underlying AV and OFT cushions also express *Tgfb2*, but the remaining myocardium is largely negative for TGFβ expression after embryonic day 11.5.¹⁰⁵ When cardiac explants are grown on collagen matrices, epicardial cells migrate onto the gel as an epithelial sheet. Experimental manipulations can then be scored for loss of epithelial markers (ZO1, cytokeratin), increased expression of mesenchymal markers (SMA, actin stress fibers), and invasion of the collagen gel. Using this assay, TGFβ1 and TGFβ2 were shown to stimulate EMT of epicardial explants via the type I TGFβ receptor ALK5.^{106,107} The requirements of ALK5 and, thus, TGFβ signaling in epicardium were confirmed in mouse embryos, where cGATA5-Cre-mediated *Alk5* inactivation abrogated EMT in vitro and caused abnormal epicardial attachment, impaired myocardial growth, and

coronary vessel abnormalities *in vivo*.¹⁰⁸ TGF β signaling through ALK5 similarly stimulated EMT of cultured human epicardial cells.¹⁰⁹ Interestingly, ALK2, a BMP receptor, rather than ALK5 was primarily responsible for activation of proepicardial explants.¹¹⁰ Thus, TGF β appears to regulate EMT of epicardial cells, whereas BMPs predominantly regulate this process in proepicardium.

Epicardial TGF β signaling also requires the type II and type III TGF β receptors, TGF β R2 and TGF β R3, respectively. *Tgfb2* inactivation in myocardium plus epicardium by SM22a-Cre caused myocardial hypoplasia and coronary vascular abnormalities,¹¹¹ whereas inactivation selectively in myocardium was compatible with normal heart development,⁴⁷ implicating epicardial *Tgfb2* in both myocardial growth and coronary vessel development. Mutation of the type III TGF β receptor gene *Tgfb3* also decreased EPDCs *in vivo*.¹¹² In explant assays, *Tgfb3*-null epicardial cells underwent EMT, but mutant EPDCs exhibited reduced motility. These results indicate that epicardial TGF β signals through epicardial ALK5, TGF β R2, and TGF β R3 to promote epicardial EMT and EPDC invasion of myocardium.

Interestingly, TGF β 2 also upregulated *Has2* in epicardial explants.¹¹³ As noted in the section ErbB Receptor Signaling, *Has2* synthesizes HA, an important component of the subepicardial extracellular matrix. In the epicardial explant assay, HA and its receptor CD44 were essential for induction of mesenchymal genes and for TGF β 2 enhancement of EPDC cell motility and invasion.¹¹³ Furthermore, HA-stimulated epicardial cell invasion required *Tgfb3*.¹¹² These results indicate that TGF β -stimulated HA secretion is important for epicardial EMT, and suggest a TGF β -HA feed-forward circuit that enhances epicardial EMT or migration.

Retinoic Acid

Retinoic acid (RA) signaling is crucial for formation of multiple organs, including the heart. RA binds to RA receptors and retinoid X receptors (RXRs), nuclear hormone receptors that are ligand-dependent transcription factors.¹¹⁴ RA receptors and RXRs act as dimers with RXRs, and there are three RA receptors and three RXR isoforms (α , β , and γ). RXRs also heterodimerize with non-RA-dependent nuclear hormone receptors, such as the vitamin D or thyroid hormone receptors. Functional redundancy has greatly complicated analysis of RA receptor and RXR function. Of the six single mutant knockouts, only RXR α leads to cardiac defects and embryonic lethality with reduced cardiomyocyte proliferation, myocardial hypoplasia, and VSDs.¹¹⁵ Subsequent tissue-restricted conditional knockout revealed that RXR α is only essential in the epicardium, where its inactivation by cGATA5-Cre recapitulated most features of the null phenotype and caused defects in coronary vessel development.¹⁴ RXR α stimulated expression of *Wnt9b* and *Fgf2*, which promoted epicardial EMT and EPDC differentiation toward vascular smooth muscle.¹⁴

Retinal dehydrogenase, encoded in the heart fields by *Raldh2* (MGI: *Aldh1a2*), catalyzes the rate-limiting step in the synthesis of RA. At the linear heart tube stage, *Raldh2* is expressed in the dorsal mesocardium, sinus venosus, and

proepicardium,¹¹⁶ and by embryonic day 12.5 cardiac *Raldh2* is highly localized to the epicardium. *Raldh2*-null hearts formed a single dilated cardiac chamber, and the grossly malformed embryos died at approximately embryonic day 9.5.¹¹⁷ Maternal RA supplementation bypassed this early lethality. By embryonic day 12.5, the rescued *Raldh2*-null hearts showed thinning of the compact myocardium and abnormal coronary vessel development. Unlike RXR α mutants, rescued *Raldh2*-null epicardium was competent for EMT, and *Wnt9b* and β -catenin activation were unaltered.¹¹⁸ This discrepancy might suggest that RXR α promotes Wnt/ β -catenin signaling as a heterodimer with a different non-RA-dependent nuclear hormone receptor.

Notch

Notch ligands and receptors are expressed within proepicardium and epicardium, where their functions have been reviewed recently in depth.⁹⁰ In proepicardium, Notch signaling maintains multipotent progenitor cells and prevents them from differentiating into cardiomyocytes when cultured *in vitro*.⁹¹ In epicardium, Notch signals direct EPDC differentiation into smooth muscle cells, regulate coronary vessel development, and promote *Raldh2* expression and compact myocardial growth.^{91,119} Although Notch signaling is essential for endocardial EMT, it appears dispensable for epicardial EMT, because epicardium-restricted inactivation of RPB1, the nuclear target of Notch signaling, did not alter the number of Tbx18^{Cre}-labeled EPDCs within right ventricular myocardium.¹¹⁹

Platelet-Derived Growth Factors

Epicardium expresses both platelet-derived growth factor receptors α and β . These receptors are coexpressed in epicardium, but their expression in EPDCs becomes mutually exclusive by embryonic day 16.5.⁹⁶ Epicardium-restricted inactivation of *Pdgfrb* caused loss of vascular smooth muscle around coronary vessels. The number of EPDCs was reduced in *Pdgfrb*-deficient hearts, suggesting impaired EMT. *Pdgfrb* inactivation also reduced EPDC migration, and this was linked to aberrant PI3K regulation of the actin cytoskeleton. *Pdgfra* likewise was required for appearance of EPDCs within myocardium, because the number of these cells was reduced by global or epicardium-restricted *Pdgfra* inactivation.^{120,121}

Epicardium-restricted inactivation of both *Pdgfra* and *Pdgfrb* was compatible with formation of a largely intact epicardium, but the epicardial cells were unable to undergo EMT and migrate into the myocardium.⁹⁶ *In vitro*, these double knockout cells were unresponsive to EMT-inducing growth factors TGF β 1 and FGF2. At a molecular level, this block of EMT was linked to downregulation of *Snail*, *Slug*, and *Sox9*, a transcription factor gene expressed in the epicardium and in developing valves. Forced *Sox9* expression restored growth factor-induced EMT, suggesting that it is an important regulator of epicardial EMT downstream of *Pdgfr-a/b*.

In addition to promoting EMT, each *Pdgfr* isoform appeared to regulate EPDC differentiation into distinct lineages.⁹⁶ Whereas *Pdgfrb* inactivation blocked EPDC differentiation into

vascular smooth muscle, *Pdgfra* loss of function impaired EPDC differentiation into cardiac fibroblasts.

FGFs

There are 22 mammalian FGFs that signal through tyrosine kinase receptors encoded by four distinct genes (*Fgfr1-4*).¹²² *Fgfr1* through *Fgfr3* are alternatively spliced with one of two mutually exclusive exons, yielding b and c isoforms with differing ligand-binding specificity. Epithelial cells tend to express the b isoforms, whereas mesenchymal tissues tend to express the c isoforms.¹²³ Epicardium expresses FGF2, FGF9, FGF16, and FGF20.^{14,124,125} FGF9, and potentially FGF16 and FGF20, signal to cardiomyocytes through FGFR1c and FGFR2c to control myocardial proliferation and to indirectly regulate coronary vessel formation.¹²⁵ FGF9 expression was stimulated by RA in epicardial cell culture, and may represent one pathway by which RA regulates myocardial growth and coronary vessel development.

FGF signals also regulate epicardial EMT and EPDC migration. FGF1, FGF2, and FGF7 stimulated EMT in cultured epicardial cells,¹²⁴ and inhibition of FGF signaling reduced EMT and myocardial invasion.¹²⁶ Recently, it was shown that myocardial FGF10 signals to FGFR1 and FGFR2b expressed on epicardial cells and EPDCs.¹²⁷ This signal stimulated epicardial EMT, enhanced EPDC motility, and stimulated their differentiation into cardiac fibroblasts. The FGF10 and FGFR2b mutant hearts also were smaller than those of controls, possibly because fetal cardiac fibroblasts support cardiomyocyte proliferation.¹²⁸ These data indicate that myocardial and epicardial FGF signals stimulate epicardial EMT via epicardial FGF receptors.

Wnt/ β -Catenin

The *Bat-gal* transgenic reporter of canonical Wnt signaling is activated in epicardial cells.¹⁰³ Epicardial inactivation of β -catenin by cGATA5-Cre or *Wt1*^{CreERT2} caused myocardial hypoplasia and decreased cardiomyocyte proliferation.^{103,129} As in endocardial EMT, β -catenin is required for epicardial EMT, as shown by the paucity of EPDCs in embryos with epicardium-restricted β -catenin inactivation. The major coronary arteries did not form after epicardial β -catenin knockout, and supporting vascular smooth muscle cells were markedly deficient.

Although requirement of canonical Wnt signaling is inferred from β -catenin loss-of-function studies, β -catenin is also an important component of intercellular adhesion complexes, and disruption of these complexes may contribute to the β -catenin loss of function phenotype. Tallquist et al¹³⁰ showed that β -catenin is required to establish epicardial cell polarity. Epicardial EMT depended on epicardial cell division, and cell divisions oriented with the mitotic spindle perpendicular to the epicardial surface preferentially underwent EMT. β -catenin mutation randomized spindle orientation and reduced epicardial EMT. These defects were rescued in whole heart culture by adenoviral expression of truncated β -catenin lacking the transactivation domain. This result suggests that epicardial EMT requires β -catenin to establish epicardial cell polarity and direct mitotic spindle orientation, rather than to drive transcription in canonical Wnt signaling.

However, this rescue experiment was limited to whole heart culture, and β -catenin may function in both capacities in vivo. The result also does not exclude later roles of canonical Wnt signaling in EPDC differentiation. In vivo experiments using β -catenin conditional alleles that distinguish its transcriptional and structural roles¹³¹ will yield further insights.

Transcriptional Regulation

Epicardial EMT is carefully regulated at the transcriptional level. The epicardial cell functions of *Sox9* and *Rxra* in epicardial EMT were described. The transcription factor WT1 is a third transcription factor known to be essential in epicardial cells for EMT.^{103,132,133} *Wt1* is expressed in the developing genitourinary system, and also in the mesothelia covering most visceral organs.¹³⁴ Global *Wt1* loss-of-function caused malformation of most visceral organs, likely by disrupting normal mesothelium–parenchyma interactions. In the heart, *Wt1* was essential for both epicardial paracrine activity and for epicardial EMT. Loss of *Wt1* caused pericardial edema, myocardial hypoplasia, and impaired coronary vessel development.^{103,132,133}

Epicardial EMT was dramatically impaired in *Wt1*-null hearts, likely attributable to transcriptional dysregulation of genes involved in EMT. Canonical Wnt/ β -catenin signaling was reduced in *Wt1* mutant heart, because of downregulation of multiple pathway component genes including *Ctnnb1* (encoding β -catenin) and its interacting transcription factor gene *Lef1*, and upregulation of *sFrp2*, encoding a secreted Wnt antagonist.¹⁰³ Levels of *Wnt5a*, a noncanonical Wnt ligand selectively expressed in epicardium, were also substantially reduced in *Wt1*-null heart.¹⁰³ *Wnt5a* is essential for compact myocardial growth¹⁰³ and may have a role in regulating epicardial cell polarity and EMT based on studies of its function in planar cell polarity pathways.¹³⁵ *Wt1* inactivation also markedly decreased expression of *Raldh2*,^{103,136} and this may have contributed to the EMT defect as maternal supplementation with RA ameliorated the EMT defect.¹⁰³ *Snai1* and *Snai2* were downregulated in *Wt1* mutant hearts,¹³³ although whether this was the cause or effect of loss of EPDCs is uncertain.¹⁰³ E-cadherin, in other contexts a target of *Snai1* and *Snai2* repression and often downregulated during EMT, was reported to be ectopically expressed in *Wt1*-null epicardium,¹³³ although we were unable to reproduce this result.¹⁰³

Epicardial EMT is also regulated by transcriptional programs in nonepicardial cells, presumably through yet-to-be-identified cell surface or secreted factors that act cell-nonautonomously. Mutations of the cardiac transcription factors GATA4 and FOG2 caused dramatic reduction of the coronary plexus of embryonic day 13.5 hearts.^{137–139} Both factors are expressed in epicardium, myocardium, and endocardium, but tissue-restricted transgenic rescue and loss of function approaches showed that both FOG2 and GATA4–FOG2 interaction are required in cardiomyocytes.^{137,139} Initially, when it was presumed that coronary endothelial cells arose from epicardium, the cardiomyocyte GATA4–FOG2 interaction was hypothesized to be required for epicardial EMT and, hence, coronary plexus formation.¹³⁷ However, as summarized in the section Epicardial Cell Fate, subsequent

studies suggested that most coronary endothelial cells do not arise from epicardium. Furthermore, direct genetic lineage tracing approaches showed that FOG2 is not required for epicardial EMT.¹³⁹ Thus, it is likely that GATA4–FOG2 regulates coronary vessel development by promoting secretion of angiogenic factors and recruitment of coronary endothelial cells.^{139,140}

The transcriptional coactivator p300 is also required for epicardial EMT.¹⁴¹ Knock-in of a dominant-negative p300 allele caused lethal heart malformations that included myocardial hypoplasia and deficient coronary vasculogenesis. The number of WT1⁺ epicardial cells was reduced, as was the formation of EPDCs in the subepicardial region. However, it is unclear if p300 activity was required in epicardial cells or nonepicardial cells.

Epicardial EMT in Disease

Because of its developmental plasticity and its critical role in heart development, there has been great interest in the role of epicardium in adult heart homeostasis and disease, and its potential applications to cardiac regeneration. Here, we review the current understanding of epicardium and epicardial EMT in the normal and diseased adult heart.

Reactivation of Epicardium in Disease

Epicardial function has been examined in adult zebrafish heart. Fetal epicardial genes such as *raldh2* and *tbx18* were downregulated in normal adult zebrafish epicardium.¹⁴² Amputation of the cardiac apex stimulated robust reactivation of these markers. *raldh2* and *Tbx18* were reactivated in the epicardium throughout the heart by 3 days postamputation and became localized to the injury site by 7 days postamputation.^{142,143} Interestingly, *raldh2* was also activated in endocardium, within distinct kinetics from epicardium.¹⁴³ Endocardial *raldh2* was broadly upregulated within hours of injury, and then became restricted to endocardium at the injury site by 24 hours. RA signaling was essential for zebrafish cardiac regeneration, suggesting an essential role of epicardium (and endocardium) in the myocardial repair process.¹⁴³ Apex amputation also upregulated expression of *Pdgfrb* in regenerating myocardium and epicardium, and *Pdgfrb* signaling was required for epicardial cell proliferation and for coronary vessel formation in regenerating myocardium.¹⁴⁴

In mammals, fetal epicardial markers *Raldh2*, *Tbx18*, and *Wt1* were likewise downregulated in normal adult epicardium.¹⁰¹ Adult epicardial cells showed little cell-cycle activity and did not actively undergo EMT to maintain cardiac homeostasis. MI stimulated a robust epicardial response, marked by re-expression of fetal epicardial markers *Raldh2*, *Tbx18*, and *Wt1*. Interestingly, myocardial injury did not stimulate endocardial upregulation of *Raldh2* as it did in zebrafish, suggesting that lack of robust immediate endocardial *Raldh2* upregulation may be an important difference between regenerative zebrafish myocardium and nonregenerative mammalian myocardium.¹⁴³ Post-MI epicardium and EPDCs were mitotically active, and their cell number increased substantially. Injury activated a partial EMT response: EPDCs expressed mesenchymal markers and disso-

ciated post-MI epicardial cells appeared mesenchymal rather than epithelial. However, the EPDCs did not invade the subjacent myocardium, but rather remained in a thickened epicardial layer on the surface of the heart. A somewhat different conclusion was reached by lentiviral labeling of epicardial cells, achieved by pericardial injection of virus expressing fluorescent protein from a ubiquitous promoter.¹⁴⁵ These experiments suggested that in the setting of MI, some EPDCs may migrate into the myocardium, although the extent of myocardial penetration was not quantitated and the potential for nonepicardial labeling was not excluded.

We analyzed the fate of cells derived from adult epicardium after MI. In the absence of exogenous stimulation, epicardial cells labeled by *Wt1*^{CreERT2} and Tam pulse before MI did not significantly differentiate into cardiomyocyte or endothelial cell lineages, unlike what we observed in fetal heart.¹⁰¹ EPDCs primarily differentiated into myofibroblast/fibroblast and smooth muscle lineages and mainly adopted a perivascular location in the thickened epicardium. These cells robustly stimulated blood vessel formation in angiogenesis assays. The limited range of cell types derived from post-MI EPDCs may have been attributable to “partial” EMT of epicardial precursors, or lack of EPDC mobilization and migration into a suitable environment within the myocardium. Using a lentiviral labeling approach, Limana et al¹⁴⁶ reported that cells resident in epicardium differentiate into cardiomyocytes after MI. The extent of cardiomyocyte labeling was not measured, and the possibility of infrequent direct cardiomyocyte labeling was not excluded. The difference in results between lentiviral and *Wt1*^{CreERT2} labeling might suggest a epicardial progenitor outside of the *Wt1*^{CreERT2} recombination domain with cardiomyogenic potential.

Schneider et al¹⁴⁷ used an independent approach to identify a progenitor cell population activated in the injured adult heart. Using a transgenic GFP reporter of Notch activation, they identified a population of cells primarily localized to the epicardium that underwent Notch activation after myocardial injury. FACS sorting of dissociated adult heart for transgenic GFP reporter of Notch activation led to the isolation of a Notch-activated population that was negative for blood and endothelial lineage markers. These cells, designated Notch-activated epicardium-derived cells, increased after myocardial injury and had a transcriptional signature of MSCs. Unlike other MSCs, these cells expressed epicardial markers. Interestingly, Notch-activated epicardium-derived cell coculture with neonatal rat cardiomyocytes promoted long-term cardiomyocyte survival, suggesting secretion of cardiotropic factors. Reciprocally, neonatal cardiomyocytes enhanced Notch-activated epicardium-derived cell cardiac differentiation, manifested by upregulation of cardiac sarcomere genes. This study suggests that injury stimulates expansion of a Notch-activated epicardial population active in the myocardial injury response. Notch-activated epicardium-derived cells potentially may be therapeutically recruited for myocardial regeneration.

Paracrine Activity of Reactivated Epicardium

Fetal epicardium is intimately related to the developing myocardium and coronary vasculature, and myocardium–

epicardium signaling promotes myocardial growth and vascularization.^{89,90} Recent studies have shown that adult epicardium reactivated by myocardial injury is likewise active in paracrine signaling that modifies the myocardial injury response.^{101,148,149} Injection of human EPDCs into murine myocardium at the time of MI improved ventricular function at 2 and 6 weeks.¹⁴⁸ Improvement of ventricular function was associated with greater vascularization in the EPDC-treated group. However, few EPDCs were present by 6 weeks after injection. Based on the time course of the response and on histological analyses showing improved host tissue properties rather than formation of EPDC-derived tissue, the functional improvement was inferred to be attributable to paracrine effects of the transplanted cells.

To further study the paracrine activity of adult EPDCs, we developed strategies to isolate highly pure populations of post-MI EPDCs and amplify them in culture.^{101,102} We showed that post-MI EPDCs upregulate a number of genes encoding potent secreted factors, including *Vegfa*, *Fgf2*, *Tgfb2*, *Sdf1*, and *Mcp1*. Post-MI EPDC-conditioned media stimulated angiogenesis. Moreover, injection of this conditioned media into the ischemic area border zone at the time of coronary artery ligation improved capillary density and ventricular function, and reduced adverse left ventricular remodeling. These results indicate that epicardial signals, amplified by MI-induced gene expression changes and dramatic epicardial expansion, influence the myocardial injury response and reduce MI size. Enhanced vessel density contributes to the beneficial effect, but recruitment of circulating cells through SDF1, MCP1, and other chemotactic factors and stimulation of cardiac progenitor cells, also may play a role. Further studies are needed to investigate these hypotheses.

Canonical Wnt/ β -catenin signaling is essential for fetal epicardial EMT, and this signaling pathway is redeployed in myocardial injury. Two studies showed upregulation of several Wnt ligands after myocardial ischemia.^{85,149} The studies did not agree on which Wnt ligands were upregulated or on whether the upregulation occurred primarily in epicardial, endocardial, or fibroblast tissue compartments, perhaps because one study used permanent LAD occlusion whereas the other used ischemia/reperfusion.^{85,149} Duan et al¹⁴⁹ reported rapid seven-fold upregulation of *Wnt1* after ischemia/reperfusion in the epicardium and subepicardial region and later expansion of *Wnt1* expression into fibroblasts in the injury area. Consistent with *Wnt1* upregulation, ischemic injury activated the TOPGAL reporter of canonical Wnt signaling in epicardium and later in the injury region. Canonical Wnt- β -catenin signaling promoted epicardial EMT, and epicardium-restricted ablation of β -catenin by *Wt1*-Cre reduced epicardial EMT and compromised cardiac function after ischemic injury. These data implicate epicardial activation of canonical Wnt/ β -catenin signaling in the myocardial injury response.

Targeting Epicardial Plasticity in Therapeutic Myocardial Regeneration

The epicardium is developmentally malleable and uniquely situated to influence both myocardial and coronary vessel function through cellular differentiation and paracrine mech-

anisms. The extensive myocardial–epicardial signaling observed in heart development is likely reiterated in adult heart disease, and the epicardial microenvironment is highly accessible through the pericardial space. These aspects of the epicardium provide an opportunity for therapeutic manipulation of epicardial cell activity and differentiation to improve outcomes in adult heart disease.

The G-actin monomer binding protein thymosin beta 4 (TB4) has been shown to reduce infarct size in experimental MI,¹⁵⁰ and it is currently in phase 2 clinical trials for treatment of MI. The mechanisms underlying the beneficial effect of TB4 in MI models likely involve direct cardiomyocyte protection from apoptosis¹⁵⁰ and modulation of epicardial activity.^{96,102,151,152} TB4 is expressed by myocardium and epicardium. In shRNA knockdown models, TB4 was necessary for coronary vessel development.¹⁵¹ However, this was not supported by subsequent studies using genetic ablation approaches.¹⁵³ Exogenous TB4 increased epicardial progenitor cell plasticity and mobilization. TB4 increased vascular cell outgrowths from fetal epicardial explants.¹⁵¹ Whereas unstimulated adult epicardial explants were nearly devoid of epicardial cell outgrowth, TB4 stimulated extensive outgrowth of cells that differentiated into fibroblast, smooth muscle, and endothelial cell lineages. Systemic administration of TB4 to mice substantially increased epicardial thickness at baseline and after MI.^{102,152} Capillary density was also increased in the infarction border zone and in myocardium remote from the infarct.

Based on the potential of fetal EPDCs to differentiate into cardiomyocytes and TB4 augmentation of adult epicardial cell mobilization and developmental plasticity, Riley et al⁹⁸ tested the hypothesis that pretreatment with TB4 would enable adult epicardial cells to differentiate into cardiomyocytes in response to myocardial injury. TB4 “priming” in the context of myocardial injury stimulated de novo formation of cardiomyocytes from *Wt1*-expressing epicardial precursors, suggesting that epicardial cells might represent a resident cardiac progenitor that could be recruited for therapeutic myocardial regeneration. Interestingly, the cardiogenic activity of TB4 required additional signals induced by myocardial injury. Furthermore, the TB4 effect also required “priming” (initiation of treatment before MI), because we did not observe cardiomyocytes derived from the *Wt1* lineage when TB4 was started concurrent with LAD ligation.¹⁰² In future work it will be important to determine the signaling mechanisms that underlie the synergistic effect of TB4 priming and myocardial injury, and the need for TB4 pretreatment.

Conclusion

EMT is a powerful mechanism deployed during development to diversify and augment cell lineages. Reactivation of these mechanisms likely contributes to disease pathogenesis, and redirection and amplification of disease-related EMT offer means to mitigate disease processes and to stimulate regeneration.

Studies of heart development have demonstrated the key role of EMT in fashioning the mature heart. Applying these lessons of developmental biology to adult heart disease has led to new insights on heart disease mechanisms, which, in

turn, suggest new therapeutic strategies. Continued research promises to lead to exciting advances in our understanding and treatment of cardiac valve disease, cardiac fibrosis, and cardiac muscle disease.

Sources of Funding

W.T.P. was supported by funding from National Institutes of Health (U01 HL100401 and R01 HL094683) and by charitable donations from Edward Marram, Karen Carpenter, and Gail Federici Smith.

Disclosures

None.

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