Epicardium in Heart Development

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The epicardium, the outermost tissue layer that envelops all vertebrate hearts, plays a crucial role in cardiac development and regeneration and has been implicated in potential strategies for cardiac repair. The heterogenous cell population that composes the epicardium originates primarily from a transient embryonic cell cluster known as the proepicardial organ (PE). Characterized by its high cellular plasticity, the epicardium contributes to both heart development and regeneration in two critical ways: as a source of progenitor cells and as a critical signaling hub. Despite this knowledge, there are many unanswered questions in the field of epicardial biology, the resolution of which will advance the understanding of cardiac development and repair. We review current knowledge in cross-species epicardial involvement, specifically in relation to lineage specification and differentiation during cardiac development.

The epicardium is the outermost mesothelial layer of all vertebrate hearts. First described more than a century ago, the presence of the epicardium was later confirmed by electron microscopy studies in the 1960s (Kurkiewicz 1909; Manasek 1968, 1969). The epicardium was previously considered to passively cover the myocardium, and only over the past few decades has its importance in heart development and diseases been revealed (Riley 2012). Empirical evidence suggests that the epicardium is a critical player in heart development and regeneration by acting as a source of progenitor cells and essential signals (Limana et al. 2011; Masters and Riley 2014). The epicardium and its derived cells have been reported to secrete factors for cardiomyocyte (CM) growth and maturation during development. In addition, these cells contribute to cardiac fibroblasts, vascular smooth muscle cells (vSMCs), and pericytes, with controversial contributions to endothelial cells and CMs (Mikawa and Gourdie 1996; Dettman et al. 1998; Gittenberger-de Groot et al. 1998; Manner 1999; Merki et al. 2005; Limana et al. 2007; Cai et al. 2008; Zhou et al. 2008a; Christoffels et al. 2009; Rudat and Kispert 2012). After cardiac injury, the epicardium is believed to reactivate developmental processes in both the highly regenerative teleost fish heart and the less regenerative mammalian heart by providing perivascular components and trophic signals for CM survival or division (Lepilina et al. 2006; Kikuchi et al. 2011; Smart et al. 2011; Zhou et al. 2011; Huang et al. 2012; Wang et al. 2013; Gemberling et al. 2015; Wei et al. 2015). Remarkably, the presence of an intact epicardium is essential for successful myocardial regeneration in zebrafish (Wang et al. 2015). All of these findings

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indicate that the epicardium is a putative therapeutic candidate for novel heart repair strategies. Precise manipulation of the epicardium to enhance secretion of proregenerative factors and differentiation to coronary cell types would augment the regenerative response of the epicardium for therapeutic purposes. Despite these studies, there are still substantial knowledge gaps in our understanding of epicardial formation and differentiation during development and reactivation of the developmental program after cardiac injuries. Epicardial functions during heart regeneration have recently been reviewed elsewhere (Cao and Poss 2018; Simões and Riley 2018). By focusing on heart development, here we summarize current knowledge and highlight the controversies and hurdles in the field of epicardial biology.

THE ORIGINS OF THE EPICARDIUM

Epicardial cells are primarily derived from a transient, mesoderm-derived cell cluster in the embryo called the proepicardial organ (PE). First described in the chick embryo as pericardial villi, the PE is a cell protrusion from the venous pole of the embryonic heart tube, between the sinus venous and the liver bud (Manner 1992, 1993). The PE is a highly conserved structure among vertebrates including zebrafish, *Xenopus*, axolotls, mice, rats, and even humans (Komiyama et al. 1987; Fransen and Lemanski 1990; Hirakow 1992; Nesbitt et al. 2006; Jahr et al. 2008; Serluca 2008). Several conserved transcription factors, including *Wilms tumor 1* (*Wt1*), *T-box factor 18* (*Tbx18*), and *Transcription factor 21* (*Tcf21*, also known as *epicardin*, *pod1*, or *capsuling* in mammals), are highly expressed in the PE and serve as a molecular signature of this structure.

Although the majority of PE induction studies were performed in the chick embryo because of the easy accessibility of the in ovo model, the exact mechanism of PE induction is still unclear. In chick embryos, gene expression analyses and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeling experiments suggested that the PE and inflow myocardium are both derived from the same precursor pool of the splanchnic mesoderm (van Wijk et al. 2009). Weak bone-morphogenetic protein (BMP) signaling is essential for PE specification, whereas strong BMP signals promote myocardial differentiation (Fig. 1; Schlueter et al. 2006; van Wijk et al. 2009). Also, fibroblast growth factor (FGF) signaling is reported to promote PE formation by suppressing BMP signaling in the PE region through phosphorylated extracellular signal-regulated kinase 1/2 (p-Erk1/2) (van Wijk et al. 2009). However, another study using chick embryos indicated that although FGF ligands are not involved in the induction or maintained expression of PE-specific genes such as *Tbx18*, they can act as either autocrine or paracrine growth factors to promote outgrowth of the PE (Fig. 1; Torlopp et al. 2010). Distinct from the

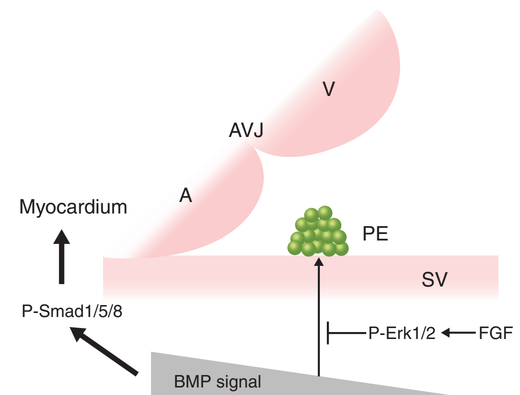

Figure 1. Bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signals are involved in the induction of the proepicardial organ (PE). In chick embryos, strong BMP signaling promotes myocardial differentiation of the precursor pool of the splanchnic mesoderm through p-Smad1/5/8, whereas weak BMP signaling stimulates PE formation. FGF signaling negatively regulates BMP signaling in the PE region via p-Erk1/2 to promote PE formation, although whether FGF signaling is required for the induction or maintained expression of PE-specific genes, such as *Tbx18*, is controversial (van Wijk et al. 2009; Torlopp et al. 2010). In contrast, blocking BMP signaling in zebrafish embryos reduces *tbx18* and *tcf21* expression and PE specification, whereas elevated *bmp2b* expression before PE formation induces ectopic expression of *tbx18* (Liu and Stainier 2010). A, Atrium; V, ventricle; AVJ, atrioventricular junction; SV, sinus venosus.

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findings in the chick embryo, blocking BMP signaling in zebrafish (through the overexpression of a dominant-negative form of a Bmp receptor) led to a significant reduction in *tbx18* and *tcf21* expression, whereas overexpression of *bmp2b* before PE specification led to the ectopic expression of *tbx18* (Liu and Stainier 2010). This suggests that the mechanisms for PE specification are organism-specific (Andrés-Delgado et al. 2019).

After PE formation, PE cells translocate to the myocardial surface to form the epicardium through direct contact and/or by releasing free-floating cell clusters (or cysts) into the pericardial cavity, depending on the model that was investigated (Fig. 2). In the chick, *Xenopus*, and axolotl models, the PE produces protrusions to form a tissue bridge between PE and the dorsal wall of the ventricle to allow the PE cells to migrate onto the myocardium (Fransen and Lemanski 1990; Nahirney et al. 2003; Jahr et al. 2008). BMPs expressed in the atrioventricular canal are believed to direct PE protrusion toward the heart tube in the chick embryo (Ishii et al. 2010). In mice, it was first reported that PE cell clusters detach from the proepicardial surface to form cysts, which then float freely across the pericardial cavity to attach to the myocardium around embryonic day 9 (E9.0) (Komiyama et al. 1987). Newer studies in mice suggest that both direct contact between the PE and myocardium and adhesion of free-floating PE cell cysts to the myocardium, underlie this translocation process (Rodgers et al. 2008; Li et al. 2017). Mice bearing a mutation of *Par3*, a polarity gene, display disrupted basoapical polarity and impaired cyst formation, suggesting that cell polarity is critical for PE cyst formation (Hirose et al. 2006). Similarly, an epicardium-specific deletion of *Cdc42* also disrupts cell polarity and blocks the formation of PE protrusions and floating epicardial clusters (Li et al. 2017). Recent live imaging of PE development and translocation in zebrafish have also found that PE cell release and adhesion occur through both direct contact with the myocardium and with the floating clusters, which are driven by the heartbeat-induced flow of pericardial fluid (Peralta et al. 2013; Plavicki et al. 2014). Apart from these mechanistic models, Li et al. discovered an approach in mice in which PE cells grow from the sinus venosus toward the heart along the surface of the inflow tract (Fig. 2; Li et al. 2017). Therefore, multiple concurrent mechanisms underlie the process of PE cell translocation.

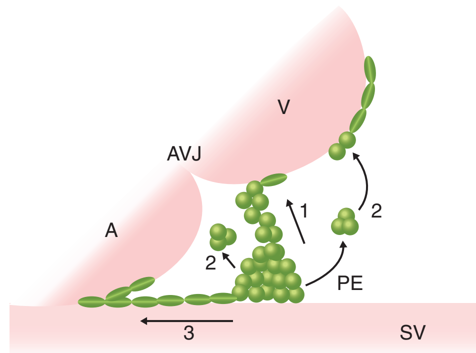

Figure 2. An overview of proepicardial organ (PE) translocation to the myocardium. Schematic of PE translocation to the myocardium showing three models across species. (1) Direct contact: In the chick, *Xenopus*, axolotl, zebrafish, and mouse, the PE produces protrusions to form a tissue bridge between the PE and the dorsal wall of the ventricle (or the PE contacts the ventricle directly) to allow the PE cells to migrate on to the myocardium. (2) Floating cysts: In zebrafish and mice, PE cell clusters detach from the proepicardial surface to form cysts, which float across the pericardial cavity to attach to the ventricular wall. (3) Migration: In mice, PE cells migrate from the SV toward the heart along the surface of the inflow tract. A, Atrium; V, ventricle; AVJ, atrioventricular junction; SV, sinus venosus.

In addition to cells coming from the PE, Balmer et al. identified an additional subset of adult mouse epicardial cells located proximal to coronary vessels that are derived from $CD45^+$ hematopoietic cells, thus adding further heterogeneity to epicardial cells (detailed below) (Balmer et al. 2014).

EPICARDIUM FORMATION AND EPITHELIAL-TO-MESENCHYMAL TRANSITION

After translocating to the myocardial surface, PE cells flatten and spread over the surface of the

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ventricle, atrium, and the outflow tract (bulbus arteriosus) to form a contiguous cell sheet. Cell-adhesion molecules between epicardial cells and the myocardium mediate epicardial cell migration. For example, migrating epicardial cells express α4-integrin, which interacts with the α4β1-integrin ligand vascular cell-adhesion molecule-1 (VCAM1) expressed by the developing myocardium. Null mutant mice for either *α4-integrin* or *Vcam1* displayed impaired epicardial cell attachment and migration and disrupted epicardium formation (Kwee et al. 1995; Yang et al. 1995; Sengbusch et al. 2002). In the chick embryo, epicardial migration is regulated by the epicardial expression of EphrinB ligands (the cell surface molecules for Eph tyrosine kinase receptors) and myocardial expression of the Eph receptor (Wengerhoff et al. 2010). In addition, the nuclear lamina protein lamin-B1 was reported to regulate epicardial cell migration by influencing the expression of cell-adhesion genes in mice (Tran et al. 2016). Despite these findings, it is still unclear what signals drive the migration process and whether there are heart chamber–specific differences in epicardium formation. Of note, the human ventricular epicardium has multiple cell layers, whereas the atrial epicardium is a monolayer (Risebro et al. 2015). Epicardial cells in both chambers also display differences in morphology and differentiation potential (Risebro et al. 2015). Consequently, it would be interesting to address how these differences are generated and how they might affect epicardial function in heart development and regeneration. Recent success in generating human embryonic stem cell–derived epicardial cells will facilitate further studies on epicardial cell differentiation and lineage commitment (Witty et al. 2014; Iyer et al. 2015).

As soon as a primitive epicardium forms, a group of epicardial cells delaminate from the epicardium and undergo epithelial-to-mesenchymal transition (EMT), enter the subepicardial domain, and give rise to epicardium-derived cells (EPDCs) (Lie-Venema et al. 2007). A study by Wu and colleagues found that epicardial cell proliferation is required for EMT and that directed spindle orientation of epicardial cells controls cell entry into the myocardium (Wu et al. 2010). The authors examined the spindle orientation of epicardial cells in E12.5 and E13.5 mouse embryos; they found that epicardial cells divide either parallel or perpendicular to the basement membrane. Parallel division keeps both daughter cells in the epicardium, whereas perpendicular division causes one daughter cell to remain in the epicardium while the second daughter cell enters the myocardium. Deletion of β-catenin or Numb, which are both colocalized to adherens junctions, disrupts the adherens junctions and leads to randomized mitotic spindle orientation and impaired EMT of epicardial cells (Wu et al. 2010). However, it is unclear whether the capacity for EMT is randomly distributed across the epicardium or specified early to a subset of cells.

Several signals are reported to influence epicardial cell EMT. These signals include, but are not restricted to, transforming growth factor β (TGF-β), FGF, Sonic hedgehog (Shh), retinoic acid (RA), Hippo/Yap, platelet-derived growth factor (PDGF), thymosin β4 (Tβ4), and extracellular matrix (ECM) components (e.g., hyaluronic acid [HA]; integrin) (Morabito et al. 2001; Dettman et al. 2003; Molin et al. 2003; Wada et al. 2003; Merki et al. 2005; Compton et al. 2006; Lavine et al. 2006; Olivey et al. 2006; Smart et al. 2007; Zamora et al. 2007; Austin et al. 2008; Sridurongrit et al. 2008; Craig et al. 2010; Vega-Hernandez et al. 2011; Missinato et al. 2015; Singh et al. 2016). For instance, the addition of the TGF-β ligands to epicardial explant cultures from chicken and mouse promotes epicardial EMT and SMC differentiation, whereas epicardial-specific deletion of the receptor *Alk5* in mice abolishes EMT and impairs the formation of the SMC layer and capillary vessels in vivo (Molin et al. 2003; Compton et al. 2006; Olivey et al. 2006; Austin et al. 2008; Sridurongrit et al. 2008). TGF-β also stimulates HA secretion, which is required for epicardial migration and EMT (Craig et al. 2010; Missinato et al. 2015). Similarly, the addition of PDGF ligands or FGF1/2/7 to epicardial explant cultures induces epicardial EMT and SMC differentiation (Morabito et al. 2001; Wada et al. 2003). In mice, myocardial-derived FGF10 interacts with its receptors FGFR1 and FGFR2b in

the epicardium to induce epicardial invasion into the myocardium and fibroblast differentiation (Vega-Hernandez et al. 2011). RA signaling regulates epicardial EMT through binding to the epicardial-expressed retinoid X receptors (RXRs) (Merki et al. 2005). Notably, RA induces the expression of FGF2 and FGF9 in the epicardial cells, which activates myocardial FGF signaling through FGFR1/2. The myocardial FGF signaling further triggers Shh activation in the epicardium, which enhances epicardial cell EMT and coronary vascular development, possibly by stimulating myocardial expression of vascular endothelial growth factors (VEGFs) and angiopoietin-2 (Ang-2) (Lavine et al. 2006, 2008). In addition, inhibition of Hippo signaling mediators Yap and Taz leads to impaired epicardial EMT and coronary vasculature formation (Singh et al. 2016). These findings suggest that there is intensive signaling cross talk between the epicardium and myocardium that underlie epicardial EMT. Notably, these signals not only influence epicardial EMT but also regulate epicardial cell differentiation. However, whether these two processes are sequential or occur concurrently remains unclear. Aside from these signals, transcription factors such as *Wt1*, *tcf21*, *Snail1*, *myocardin-related transcription factors* (*MRTFs*), and *Nuclear Factor of Activated T Cells 1* (*Nfatc1*) are also involved in the regulation of epicardial cell EMT (Martínez-Estrada et al. 2010; Combs et al. 2011; von Gise et al. 2011; Acharya et al. 2012; Trembley et al. 2015).

EPICARDIUM AS A SOURCE OF PROGENITOR CELLS

As outlined above, epicardial cells and EPDCs are highly plastic, giving rise to multiple cardiac cell types during development (Fig. 3). However, relevant regulatory sequences for specific pan-epicardial markers have yet to be identified; thus, the complete lineages of epicardial cells remain uncertain. This ambiguity results from an inability to perform genetic fate-mapping with specific regulatory sequences that induce a permanent label within the epicardial cell population and its progeny in transgenic animals. Below, we highlight our current understanding of the contribution of epicardial cells to various lineages within the heart.

Contribution to Coronary Cells and Fibroblasts

Studies in different animal models have reached a consensus that epicardial cells and EPDCs are

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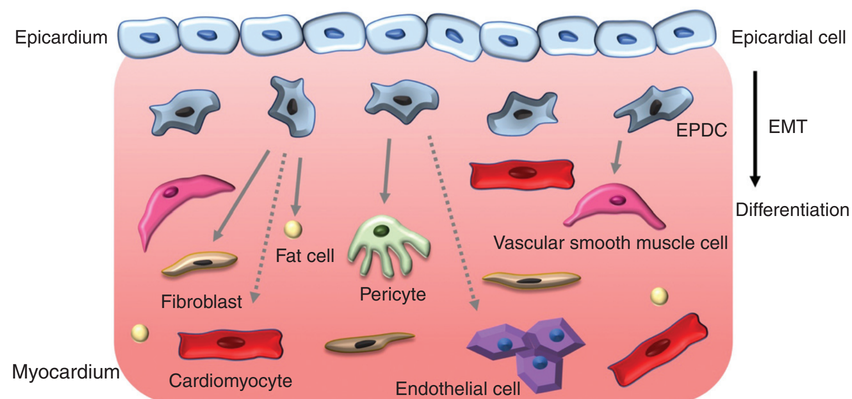

Figure 3. Cellular contributions of epicardium during heart development. Epicardial cells enter the myocardium through epithelial-to-mesenchymal transition (EMT) to form epicardium-derived cells (EPDCs), which differentiate into fibroblasts, vascular smooth muscle cells (vSMCs), pericytes, fat cells, and, possibly, endothelial cells and cardiomyocytes. Solid arrows denote consensus cell fates, whereas dashed arrows indicate controversial contributions.

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the major source of cardiac fibroblasts, while also contributing to perivascular SMCs and pericytes during development. Early studies in avian models suggested controversial epicardial contributions to coronary endothelial cells and endocardial cells, in addition to the consensus cell fates (Mikawa and Fischman 1992; Mikawa and Gourdie 1996; Manner 1999; Pérez-Pomares et al. 2002b; Guadix et al. 2006). These lineage-tracing studies used dye labeling, cell transplantation, and retroviral vector tagging to trace epicardial cell fates in chick embryos, which may not be as efficient and specific as a genetic fate-mapping approach (Table 1). In mammals and zebrafish, genetic fate-mapping has used regulatory elements to drive a tamoxifen-inducible Cre recombinase (Cre-ER) in conjunction with a floxed fluorescent reporter to trace the fate of *Cre*$^{+}$ cells. Regulatory sequences used thus far to trace epicardial cells have been derived from genes *Tcf21*, *Wt1*, *Tbx18*, *Scleraxia* (*Scx*), and *Semaphorin 3D* (*Sema3D*) (Cai et al. 2008; Zhou et al. 2008a,b; Christoffels et al. 2009; Katz et al. 2012; Rudat and Kispert 2012; Braitsch et al. 2013). These markers have overlapping expression domains, but also label distinct additional cell types. Also, some of the markers only label embryonic or injury-activated epicardial populations. Even so, studies in mammals using these regulatory sequences agree that epicardial cells are a primary source of cardiac fibroblasts during heart development (Acharya et al. 2012; Braitsch et al. 2012; Ali et al. 2014; Moore-Morris et al. 2014, 2016). Epicardial cells also contribute to the vSMCs and pericytes that compose the walls of coronary vessels (Cai et al. 2008; Zhou et al. 2008a; Grieskamp et al. 2011). The epicardial contribution to coronary endothelium was reported when tracing the PE or epicardial cells expressing *Scx* and *Sema3D* (Katz et al. 2012). However, the endothelial cell fate was not supported in other studies assessing *Tbx18*$^{+}$ or *Wt1*$^{+}$ epicardial cells (Cai et al. 2008; Zhou et al. 2008a; Grieskamp et al. 2011). Of note, *Scx* or *Sema3D* mark distinct PE subpopulations from *Tbx18*$^{+}$ and *Wt1*$^{+}$ cells. Generally, the consensus is that the epicardial contribution to endothelial cells is very minor in comparison to the more substantial sinus venous and endocardium contribution (for a review, see Leung et al. 2016). Nevertheless, the epicardium is an important signaling node for coronary vessel formation, as discussed in the section on the epicardium as an essential signaling node.

In zebrafish, *tcf21* is used as a pan-epicardial marker that has the widest and most specific expression (compared with *wt1* and *tbx18*) in epicardial cells and EPDCs in both quiescent and active (developing or heart-injured) cells (Lepilina et al. 2006; Perner et al. 2007; González-Rosa et al. 2011; Kikuchi et al. 2011; Schnabel et al. 2011). Genetic fate-mapping using the *tcf21:CreER* line showed that epicardial cells contribute to perivascular cells (likely SMCs), but not to CMs in developing and regenerating hearts (Kikuchi et al. 2011). Another study using the *wt1b:GFP* line and transplantation method similarly showed that there are epicardial contributions to perivascular SMCs and fibroblasts, but not to CMs (González-Rosa et al. 2012). In particular, zebrafish heart fibroblasts have not been well-characterized owing to a lack of specificity in the markers currently used. For example, in addition to fibroblasts, α-smooth muscle actin (α-SMA) labels SMCs and pericytes, vimentin labels endocardium, myosin light chain kinase (MLCK) labels SMCs and thrombocytes, and col1a2 and periostin label epicardial cells (Lane et al. 1983; Zeisberg et al. 2007; Tournoij et al. 2010; Zhou et al. 2010; Ito et al. 2014; Moore-Morris et al. 2014, 2015). Despite this specificity issue, it is generally accepted that the major epicardial lineage in zebrafish consists of perivascular cells and not endothelial cells or CMs, agreeing with the consensus in mammals. Of note, it is unclear whether there are *tcf21*$^{-}$ epicardial cells in zebrafish.

Contribution to Cardiomyocytes

The primary goal of heart regeneration is to generate new CMs. A critical question about the epicardium is whether it can contribute to CMs during development and heart regeneration in mammals and, if so, to what extent. Two reports in 2008 using transgenic mouse tools (albeit with the aforementioned specificity is-

Table 1. Epicardial cell fates during heart development

Cell fate	Species	Methods and models	Reference(s)
Smooth muscle cell	Chick	Dye labeling, retroviral labeling, and cell transplantation	Mikawa and Gourdie 1996; Perez-Pomares et al. 2002a
		Dye labeling, retroviral labeling, and cell transplantation (quail–chick chimeras)	Dettman et al. 1998
		Cell transplantation (quail–chick chimeras)	Gittenberger-de Groot et al. 1998; Manner 1999; Pérez-Pomares et al. 2002b; Guadix et al. 2006
	Mouse	$Wt1^{Cre}$;$Rosa26^{fsLz}$ or Z/Red $Wt1^{CreERT2}$;$Rosa26^{fsLz}$ or Z/Red	Zhou et al. 2008a
		$Tbx18^{Cre}$;$R26R^{lacZ}$	Cai et al. 2008
		$Tbx18^{Cre}$;$Rosa26^{mT/mG}$	Grieskamp et al. 2011
		Scx^{GFPCre};$R26R^{lacZ}$ $Sema3D^{GFPCre}$;$R26R^{lacZ}$	Katz et al. 2012
		$G2\text{-}Gata4^{Cre}$;$Rosa26^{YFP}$ $Wt1^{CreYFP+}$;$Rosa26^{YFP}$	Cano et al. 2016
		$Wt1^{CreER}$;$Rosa26^{RFP}$ $Tbx18^{Cre}$;$Rosa26^{RFP}$	Liu et al. 2016
Fibroblast	Chick	Dye labeling, retroviral labeling, and cell transplantation	Mikawa and Gourdie 1996; Perez-Pomares et al. 2002a
		Dye labeling, retroviral labeling, and cell transplantation (quail–chick chimeras)	Dettman et al. 1998
		Cell transplantation (quail–chick chimeras)	Gittenberger-de Groot et al. 1998; Manner 1999; Pérez-Pomares et al. 2002b; Guadix et al. 2006
	Mouse	$Tbx18^{Cre}$;$R26R^{lacZ}$	Cai et al. 2008
		$Tbx18^{Cre}$;$Rosa26^{mT/mG}$	Grieskamp et al. 2011
		$Wt1^{GFPCre}$;$R26R^{mT/mG}$	Wessels et al. 2012
		$Tcf21^{iCre}$;$R26R^{YFP}$ or $R26R^{tdT}$	Acharya et al. 2012
		Scx^{GFPCre};$R26R^{lacZ}$ $Sema3D^{GFPCre}$;$R26R^{lacZ}$	Katz et al. 2012
		$Tbx18^{Cre}$;$R26R^{mT/mG}$	Ali et al. 2014
Endothelial cell	Chick	Dye labeling, retroviral labeling, and cell transplantation	Mikawa and Gourdie 1996; Perez-Pomares et al. 2002a
		Cell transplantation (quail–chick chimeras)	Gittenberger-de Groot et al. 1998; Manner 1999; Pérez-Pomares et al. 2002b; Guadix et al. 2006
	Mouse	$Wt1^{Cre}$;$Rosa26^{fsLz}$ or Z/Red $Wt1^{CreERT2}$;$Rosa26^{fsLz}$ or Z/Red	Zhou et al. 2008a
		Scx^{GFPCre};$R26R^{lacZ}$ $Sema3D^{GFPCre}$;$R26R^{lacZ}$	Katz et al. 2012
		$G2\text{-}Gata4^{Cre}$;$Rosa26^{YFP}$	Cano et al. 2016
		$Wt1^{CreYFP+}$;$Rosa26^{YFP}$	Cano et al. 2016
		$Wt1^{CreERT2}$;$Rosa26^{YFP}$	Cano et al. 2016
		$Wt1^{CreER}$;$Rosa26^{RFP}$	Liu et al. 2016
Endocardial cell	Chick	Dye labeling, retroviral labeling, and cell transplantation (quail–chick chimeras)	Dettman et al. 1998
		Cell transplantation (quail–chick chimeras)	Gittenberger-de Groot et al. 1998; Manner 1999
	Mouse	Scx^{GFPCre};$R26R^{lacZ}$	Katz et al. 2012
Cardiomyocyte	Mouse	$Wt1^{Cre}$;$Rosa26^{fsLz}$ or Z/Red $Wt1^{CreERT2}$;$Rosa26^{fsLz}$ or Z/Red	Zhou et al. 2008a

Continued

Table 1. *Continued*

Cell fate	Species	Methods and models	Reference(s)
		Tbx18^{Cre};*R26R*^{lacZ}	Cai et al. 2008
		Scx^{GFPCre};*R26R*^{lacZ} *Sema3D*^{GFPCre};*R26R*^{lacZ}	Katz et al. 2012
Pericytes	Mouse	*Tbx18*^{Cre};*R26R*^{lacZ}	Cai et al. 2008
		Wt1^{CreER};*Rosa26*^{RFP}	Liu et al. 2016
		Wt1^{CreERT2};*Rosa*^{mTmG}	Trembley et al. 2015
Adipocyte	Mouse	*Tbx18*^{Cre};*R26R*^{YFP}	Yamaguchi et al. 2015
Perivascular cells	Zebrafish	*tcf21:CreER;gata5:RnG*	Kikuchi et al. 2011

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sues) indicated that epicardial cells contribute to CMs during development in mice (Cai et al. 2008; Zhou et al. 2008a). In a first report, Cai et al. generated a nuclear LacZ (*Tbx18*$^{nLacZ/+}$) and a Cre (*Tbx18*$^{Cre/+}$) knock-in into the endogenous *Tbx18* locus to label and trace the epicardial lineages during development (Cai et al. 2008). They found that the *Tbx18*$^+$ PE cells contribute to vSMCs and pericytes (marked by *Pdgfrb*), fibroblasts (marked by *Col1a2*), and cells that show a CM identity (positive for cardiac troponin T, troponin I, MF20, *Gata4*, and *Nkx2.5*). However, the CM contribution was later challenged by a finding that *Tbx18* itself is expressed in the myocardium (Christoffels et al. 2009). In a second report, a GFPCre cassette was knocked into the endogenous *Wt1* start codon (*Wt1*$^{GFPCre/+}$) to label and trace the *Wt1*$^+$ cells (Zhou et al. 2008a). Zhou et al. found that a subset of these cells differentiated into functional CMs (*Tnnt2*$^+$, *Actn1*$^+$, and electrically coupled with non-Wt1-derived CMs) that comprised 7%–10% of CMs in the ventricle and 18% in the atrium during normal heart development. In addition, lineage tracing using a CreERT2 knock-in line (*Wt1*^{CreERT2}) confirmed the contribution to CMs when the mice were treated with tamoxifen at E10.5 and E11.5 and assessed at E16.5 (Zhou et al. 2008a). The *Wt1*$^{GFPCre/+}$ and *Wt1*^{CreERT2} lines have been widely used to label the epicardial lineages ever since. A following study, however, challenged the cellular specificity of *Wt1* during development by showing endothelial cell expression in addition to PE and epicardium expression (Rudat and Kispert 2012). Moreover, the *Wt1*$^{GFPCre/+}$ line showed ectopic recombination in CMs before the formation of the PE (Rudat and Kispert 2012). Although there may be CMs derived from *tbx18*$^-$/*wt1*$^-$ epicardial cells in mice, the consensus in the field is that the epicardial contribution to CMs is minimal, if it occurs at all. Of note, the epicardial contribution to CMs has not been reported in the avian models, which needs further investigation. Even if epicardial cells do not naturally contribute to CMs, Tβ4 was identified as a regeneration factor that can prime epicardial cells to differentiate into myocytes in rare events when applied before myocardial infarction in mice, suggesting that the epicardium has a potential for reprogramming into CMs (Smart et al. 2007, 2011).

Contributing to Other Cell Types

Lineage-tracing studies in mice have also found that the *Tbx18*$^+$ or *Wt1*$^+$ epicardial cells are the origin of cardiac adipose tissue during development and after heart injury (Liu et al. 2014; Yamaguchi et al. 2015; Zangi et al. 2017). Of note, the cardiac adipose tissue is not only important for cardiac physiology but also a risk marker for cardiac diseases such as atrial fibrillation (Antonopoulos and Antoniades 2017), further supporting the important role of the epicardium in heart development. In addition, Chong et al. claimed that mouse PE cells (defined by *PDGFRα* expression) give rise to adult cardiac-resident mesenchymal stem cells, which have multilineage differentiation potential, including functioning in an adventitial location in the cardiac perivascular system (Chong et al. 2011). Lineage tracing indicated that these PE-derived cells autonomously emerge from the

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coronary vascular bed and are still present after fetal development. Nevertheless, extensive lineage mapping of these cardiac-resident mesenchymal stem cells is required to further illuminate epicardial differentiation potential.

Cellular Heterogeneity

An unanswered question regarding epicardial lineages is whether cell fates are committed within the epicardium during development. This notion is favored by several lines of evidence that show the cellular heterogeneity of PE and epicardial cells. The widely used markers *Tcf21*, *Tbx18*, and *Wt1* display mosaic expression patterns by labeling subpopulations of the developing epicardium in both the chick and mouse heart (Braitsch et al. 2012; Katz et al. 2012), with most of the *Scx*$^+$ and *Sema3D*$^+$ PE cells being *Tbx18*$^-$ and *Wt1*$^-$ in mice (Katz et al. 2012). Further, the *CD45*$^+$ hematopoietic cells were reported to give rise to a subset of the developing epicardium (Balmer et al. 2014). Moreover, the anatomical difference of human epicardial cells between heart chambers also supports the cellular heterogeneity with the epicardium (Risebro et al. 2015). In zebrafish, *tbx18* or *wt1* only mark subsets of *tcf21*$^+$ epicardial cells (Kikuchi et al. 2011). An experimental test of epicardial and EPDC heterogeneity has been performed in adult zebrafish through single-cell transcriptome analysis of the *tcf21*$^+$ cells (Cao et al. 2016). In this study, the investigators profiled dozens of GFP$^+$ cells purified from the uninjured hearts of zebrafish carrying a *tcf21:nucEGFP* reporter. Analysis suggested the presence of at least three cell subsets, defined by expression signatures. Although the origin and cellular plasticity of these subtypes were not addressed, this study identified potential candidate genes for labeling epicardial and EPDC subtypes, providing a resource to identify subset-specific markers. Further validation of those markers will derive new reporters for additional lineage-tracing experiments. In another study, Bollini et al. revealed the heterogeneity of Tβ4-primed EPDCs, which showed diverse cardiovascular potential among subsets (Bollini et al. 2014). Nevertheless, the above information regarding transcriptome heterogeneity is not enough to address when cell fates are committed owing to the fact that each subset labeled by a single marker gave rise to multiple lineages. Further fate-mapping analysis at the single-cell resolution with specific markers combined with human ES-derived epicardial cells (Witty et al. 2014; Iyer et al. 2015) will be necessary.

EPICARDIUM AS AN ESSENTIAL SIGNALING NODE

As mentioned above, multiple signaling pathways mediate epicardium–myocardium interactions to regulate epicardial EMT, which impact epicardial cell differentiation to perivascular cell types and fibroblasts as well as additional effects on heart development. Apart from these cell-autonomous signals, the epicardium and its derivatives serve as a signaling center for CM proliferation and coronary vessel development. The epicardium also provides ECM components to support cardiac tissue structure during development and regeneration. Identification of epicardial-derived mitogens for CMs and angiogenic factors is crucial for developing therapeutic strategies. This topic was reviewed elsewhere in the context of heart regeneration (Cao and Poss 2018; Simões and Riley 2018). Here, we summarize epicardial signals and ECM components (apart from EMT signals) for the development of the myocardium and coronary vessels.

Retinoic Acid

One of the most prominent regulators of heart morphogenesis is RA signaling, for which a tightly controlled dosage is critical (Niederreither et al. 1999; D'Aniello et al. 2013). *Retinaldehyde dehydrogenase 2* (*Raldh2*), which encodes an enzyme that catalyzes the synthesis of RA, is expressed by the developing epicardium. Deletion of *Raldh2* leads to embryonic death with interrupted heart development (Niederreither et al. 1999). Deletion of *retinoid X receptor α* (*RXRα*), which is also expressed by the epicardium, disrupted CM proliferation and compact myocardium formation in mice (Sucov et al. 1994). Remarkably, a myocardial defi-

ciency of *RXRα* led to normal heart formation, whereas epicardial deletion of *RXRα* recapitulated the phenotypes of global deletion, suggesting that epicardial RA signaling is necessary for heart formation (Chen et al. 1998, 2002; Merki et al. 2005). In chicken heart slice cultures, blockade of RA signaling from the epicardium inhibits CM proliferation and survival (Stuckmann et al. 2003). RA has been proposed to stimulate CM proliferation by inducing the secretion of trophic factors in the epicardium (Chen et al. 2002; Stuckmann et al. 2003; Merki et al. 2005); however, the identity of these trophic factors warrants further investigation.

Fibroblast Growth Factor

FGF is another well-known mitogen for CMs. During mouse heart development, *Fgf9*, *Fgf16*, and *Fgf20* are expressed in both the endocardium and epicardium, whereas the receptors *Fgfr1* and *Fgfr2* are expressed in the myocardium (Lavine et al. 2005, 2006). As mentioned above, *Fgfr1* and *Fgfr2* are also expressed in the epicardium, suggesting interactional regulations of FGF signaling between epicardium and myocardium (Vega-Hernandez et al. 2011). *Fgf9* mutant mice die at birth with a hypoplastic ventricle associated with reduced CM proliferation. Similarly, conditional knockouts of *Fgfr1* and *Fgfr2* in the myocardium dramatically reduced CM proliferation and led to heart hypoplasia (Lavine et al. 2005). A follow-up study found that epicardial–myocardial FGF signaling promoted coronary growth indirectly through the activation of Hh signaling, which further induced VEGF expression (Lavine et al. 2006, 2008). Moreover, RA can stimulate epicardial expression of *Fgf9*, which shows cross talk between these pathways (Lavine et al. 2005). In addition, myocardial *Fgf10* and epicardial *Fgfr1*, *Fgfr2b* signals also activate fibroblast differentiation of epicardial cells (Vega-Hernandez et al. 2011). In avian embryos, epicardial cells express *Fgf1* and *Fgf2* (Pennisi and Mikawa 2005). On surgical removal of the epicardium by blocking PE transition, there was reduction of myocardial proliferation and compact myocardium thickness. Further analyses suggested that decreased *Fgf2* and *Fgfr1* messenger RNA (mRNA) levels underlie this phenotype (Pennisi et al. 2003). On the other hand, in zebrafish, myocardial *fgf17b* and epicardial *fgfr2* and *fgfr4* are thought to regulate the homeostatic proliferation of CMs (Wills et al. 2008). Moreover, Fgf inhibition through overexpression of a dominant-negative fgfr1 blocked epicardial EMT, disrupted coronary neovascularization, and impaired heart regeneration (Lepilina et al. 2006). Evidently, FGF signaling is incorporated in epicardial-induced proliferative effects involving cross talk with other signaling pathways, including RA and Hh signaling.

Shh

In mice, epicardially expressed Shh interacts with myocardial Patched to regulate coronary vascular development (Lavine et al. 2006, 2008). Shh is one of the downstream targets of FGF signaling during coronary vessel development, and exogenous Shh can rescue the coronary plexus defect in the *Fgf9* and *Fgfr1/2* knockouts (Lavine et al. 2006). In chick embryos, blocking Shh signaling results in decreased second heart field proliferation, pulmonary atresia, and arterial pole defects (Dyer and Kirby 2009). Similarly, in zebrafish, inhibiting Shh diminishes processes relating to cell proliferation. For example, epicardial regeneration is stunted when outflow tract Shh signaling is hindered (Wang et al. 2015). Analogously, CMs display increased proliferation upon enhanced Shh signaling after heart injury (Choi et al. 2013). Although it is evident that Shh is implicated, and possibly evolutionarily conserved, in cardiac development and regeneration, the precise sources and cellular effects of Shh in the zebrafish model represents a vein of cardiac research that can be probed further.

Erythropoietin

During mouse heart development, erythropoietin (Epo) is expressed in the epicardium, and its receptor, EpoR, is expressed in the epicardium and other non-CM cells (Wu et al. 1999). Embryos deficient of either *Epo* or *EpoR* died at

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~E13.5, displaying ventricular hypoplasia and impaired coronary vascular plexus formation. Further, chimeric mice experiments indicated that the epicardial-derived Epo influences CM proliferation in a non-cell-autonomous manner (Wu et al. 1999). Another study using heart slice primary cultures from chickens showed that blocking Epo signaling from the epicardium inhibits CM proliferation and survival (Stuckmann et al. 2003). Interestingly, this proliferation blockade can be rescued by the addition of RA. Conversely, the blocked CM proliferation following RA inhibition can be rescued by exogenous Epo, suggesting that RA and Epo signaling act in parallel to regulate CM proliferation (Stuckmann et al. 2003). Further analysis proposed that Epo or RA signaling induced soluble mitogens from the epicardium to allow for CM proliferation, although the exact nature of these mitogens is still unknown (Stuckmann et al. 2003).

Vascular Endothelial Growth Factor

VEGF is an essential regulator of endothelial cell differentiation and vessel development (Kapuria et al. 2018). In mice, myocardial-derived VEGFA interacts with the receptor VEGFR2 in endocardial cells to regulate coronary plexus formation (Wu et al. 2012). Chen et al. found that mouse embryonic epicardial cells expressed VEGFC, which interacts with VEGFR2 and VEGFR3 in the cardiac endothelial cells. Additionally, deletion of *Vegfc* dramatically inhibited dorsal and lateral coronary artery growth without affecting vessels on the ventral side (Chen et al. 2014). In contrast, zebrafish *vegfaa*, the homolog of mammalian *Vegfa*, is expressed in the epicardium, and *vegfaa* overexpression enhances coronary angiogenesis and epicardial expansion and results in CM hyperplasia (Karra et al. 2018). The controlled regulation of *Vegfaa* may warrant further analysis as a potential therapeutic target for cardiac regeneration.

Insulin-Like Growth Factor

Mouse insulin-like growth factor 2 (IGF2) is reported to be secreted by epicardial cells to promote CM proliferation during heart development (Li et al. 2011). Global knockout of *Igf2* or CM-specific knockout of the receptors *Igf1r* and *Insr* both reduced CM proliferation and impaired ventricular development (Li et al. 2011). In zebrafish, the pharmacological inhibition of Igf signaling or the overexpression of a dominant-negative version of the receptor *Igf1ra* inhibited CM proliferation and reduced embryonic CM quantity (Huang et al. 2013). Although the source of Igf signals during zebrafish heart development is unknown, *igf2b* was detected in both the endocardium and epicardium of the postinjury wound region at a time that coincides with CM proliferation (Huang et al. 2013).

Notch

Notch signaling has been reported to regulate SMC differentiation of epicardial progeny (del Monte et al. 2011; Grieskamp et al. 2011). Using a *Tbx18-Cre* line, Grieskamp et al. found that epicardial-specific deletion of the intracellular mediator of Notch, Rbpj, impaired the differentiation of vSMCs in mice; whereas overexpressing the Notch1 intracellular domain (NICD) in the epicardium induced premature SMC differentiation and prevented coronary angiogenesis. Moreover, the authors further showed that Notch acts upstream of TGF-β and PDGF signaling in SMC differentiation (Grieskamp et al. 2011). In another study, epicardial-specific Notch1 ablation using a *Wt1-Cre* line both disrupted coronary artery differentiation and reduced CM proliferation (del Monte et al. 2011). Meanwhile, ectopic Notch1 activation in the epicardial lineage caused thinning of epicardium and ventricular myocardium, although SMC differentiation was not assessed in this setting (del Monte et al. 2011).

Wnt

Epicardial-specific deletion of β-catenin in mice caused death between E15.5 and birth (Zamora et al. 2007). The mutant heart displayed signs of reduced CM proliferation, impaired epicardial EMT, and blunted coronary artery formation. Because junctional β-catenin regulates epicardi-

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al EMT (Wu et al. 2010), it is unclear how Wnt/β-catenin signaling functions in this setting. Also, the source of Wnt ligands during coronary development is unclear.

Extracellular Matrix Components

ECM components are extracellular molecules secreted by cells that provide structural support and biochemical cues to the surrounding cells (Frantz et al. 2010). The ECM component fibronectin (Fn) was reported to be critical for heart morphogenesis in both the mouse and zebrafish (George et al. 1997; Trinh and Stainier 2004). Epicardial cells and their derived fibroblasts are the primary sources of ECM during heart development. Ieda et al. (2009) found that embryonic cardiac fibroblasts induced CM proliferation in a coculture system through the secretion of Fn and collagen, in addition to heparin-binding EGF-like growth factor (HBEGF). This proliferative effect was exerted through myocardial β1-integrin (Sengbusch et al. 2002), which when deleted in CMs has been shown to impair myocardial proliferation and ventricular compaction (Ieda et al. 2009). Also, as mentioned above, deletion of *α4-integrin* in mice disrupts epicardium formation during development by impairing epicardial cell attachment and migration (Sengbusch et al. 2002). During heart regeneration, there is intensive remodeling of ECM components, including Fn, collagen XII, tenascin-C, and HA (Mercer et al. 2013; Wang et al. 2013; Marro et al. 2016). Overall, epicardial ECM deposition may be manipulated to facilitate heart regeneration.

CONCLUDING REMARKS

The epicardium and its progeny constitute an essential part of heart development by simultaneously serving as a source of progenitor cells and as a signaling center. In terms of heart regeneration, the epicardium responds to injury through the reactivation of developmental signals across species. It is noteworthy that there could be regeneration-specific pathways in the injured adult heart that are distinct from the signaling in the embryonic heart, which warrant further investigation. Despite decades of study, there are still outstanding questions to be addressed in the field of epicardial biology, the answers to which could inspire new therapeutic strategies for heart diseases. The complete lineages of epicardial cells, mechanisms of lineage commitment, and strategies to activate the epicardium after cardiac injury are among the highest research priorities. Altogether, more refined lineage-tracing tools including next-generation sequencing–based, single-cell fate-mapping (Briggs et al. 2018; Farrell et al. 2018; Wagner et al. 2018) and dual-recombinase genetic systems (He et al. 2017), together with an explant culture approach and human stem cell–derived epicardial cells will ultimately move the field of epicardial research forward.

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