Enhancing epicardial EMT to repair the heart

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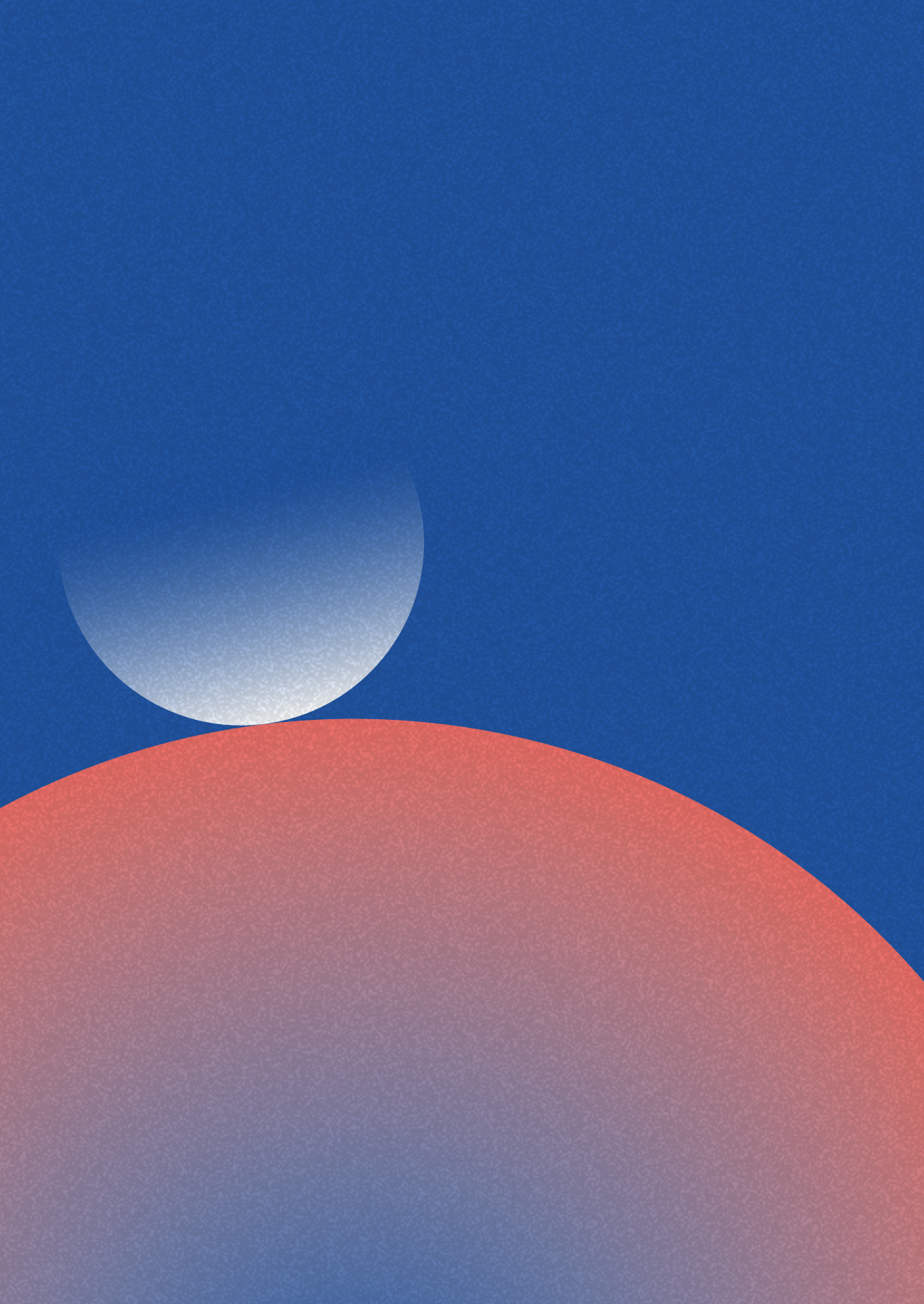

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The epicardium as a source of multipotent adult cardiac progenitor cells: Their origin, role and fate

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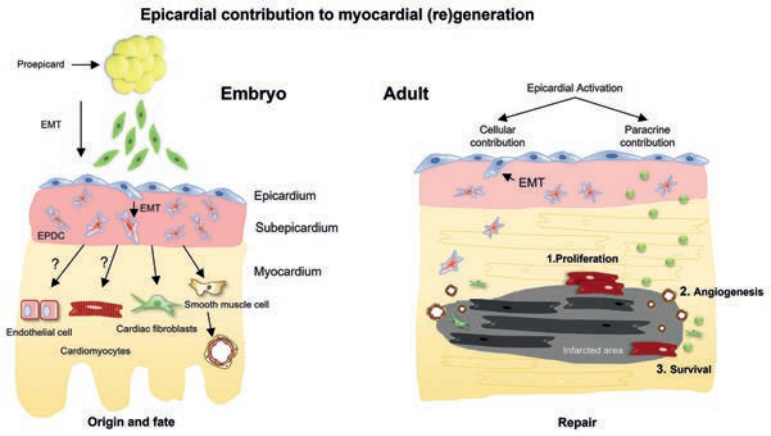
ABSTRACT

Since the regenerative capacity of the adult mammalian heart is limited, cardiac injury leads to the formation of scar tissue and thereby increases the risk of developing compensatory heart failure. Stem cell therapy is a promising therapeutic approach but is facing problems with engraftment and clinical feasibility. Targeting an endogenous stem cell population could circumvent these limitations. The epicardium, a membranous layer covering the outside of the myocardium, is an accessible cell population which plays a key role in the developing heart. Epicardial cells undergo epithelial to mesenchymal transition (EMT), thus providing epicardial derived cells (EPDCs) that migrate into the myocardium and cooperate in myocardial vascularisation and compaction. In the adult heart, injury activates the epicardium, and an embryonic-like response is observed which includes EMT and differentiation of the EPDCs into cardiac cell types. Furthermore, paracrine communication between the epicardium and myocardium improves the regenerative response. The significant role of the epicardium has been shown in both the developing and the regenerating heart. Interestingly, the epicardial contribution to cardiac repair can be improved in several ways. In this review, an overview of the epicardial origin and fate will be given and potential therapeutic approaches will be discussed.

Graphical abstract



Keywords

Cardiac progenitor cell, Epicardium, Myocardial infarction, Cardiac development, Cardiac repair

1. INTRODUCTION

Myocardial infarction (MI) is one of the most frequently occurring consequences of coronary heart disease. It has been the leading cause of death in the western world for many decades, and it is expected to remain so for years to come [1]. The obstruction of blood flow in a coronary artery and the ensuing sudden cessation of oxygen supply to regions of the muscle results in massive cell death, followed by an influx of inflammatory cells and collagen producing myofibroblasts [2]. Although adult cardiomyocytes may still possess some residual proliferative ability [3], it is evident that their contribution is insufficient to repair the heart. As a result, injured tissue is replaced by a rigid non-contractile scar. This scar tissue provides tensile strength that potentially prevents the rupture of the damaged myocardial wall. Importantly, scar tissue also severely impairs cardiac contraction which will result in cardiac dysfunction because of pathological compensatory remodelling. Eventually, this combination of factors will progress into heart failure.

Current therapies for MI mainly aim at reopening the culprit blood vessel, thereby reinstating perfusion of the damaged area. These approaches are very effective, and have greatly reduced the number of patients that acutely die after MI. Conversely, it has resulted in an increase in the number of people prone to developing heart failure, for which a heart transplant is the only option for treatment. To repair the heart, it is necessary to find other ways to increase the number of cardiac myocytes after damage.

Over the last two decades, extensive research has been invested in identifying cells with the ability to generate new cardiac tissue (reviewed in [4], [5]). Several of these cell sources, which include the bone marrow, blood, and adipose tissue, have been tested in clinical trials but the influence on cardiac function has been lower than was anticipated [6]. However, one of the most important observations in this era of cell-based therapy is the fact that the adult heart itself harbours cardiac progenitor cells. These cells bear stem cell-like features and have the ability to differentiate into new cardiac cells *in vitro* and *in vivo* [7], [8], [9], [10], [11]. The direct transplantation of cultured human cardiac progenitor cells into the ischemic mouse heart positively influenced cardiac function [12], but only resulted in a marginal increase in newly formed cardiac tissue [7]. Nevertheless it has opened new avenues to explore for treating cardiac damage. The current cell-based therapy approach would require the isolation of progenitor cells from biopsies of cardiac tissue, and a subsequent lengthy culture protocol to increase the number of these rare cells to obtain sufficient

material to transplant. If it is possible to locally stimulate progenitor cells, this could be a valuable addition to current therapies. As such, the epicardium is an intriguing cell population to study since it is an easily accessible source of cells as it is located on the outside of the heart. In this review we will highlight the origin and fate of the epicardium, and why it can potentially be used in therapeutic approaches to cure the damaged heart.

2. THE EPICARDIUM

Anatomically the epicardium is part of the pericardium, which is a double layered membranous sac covering the heart and the root of the large vessels. The outer, most superficial layer of the pericardium is known as the fibrous pericardium and consists of connective tissue. It anchors the heart to the diaphragm, the pleura, and the sternum. The inner fibrous pericardium is lined with a serous membrane that folds and covers the heart, thereby forming the pericardial sac. The serous layer lining the fibrous pericardium is called the parietal pericardium, while the layer lining the heart is known as the visceral pericardium or epicardium.

2.1. The proepicardium: developmental origin of the epicardium

Early in development, the embryonic heart consists of only two layers: the myocardium and the endocardium. The epicardium, which is not yet present at this tubular heart stage, derives from an extra-cardiac mesothelial cell cluster known as the proepicardium (PE, Fig. 1).

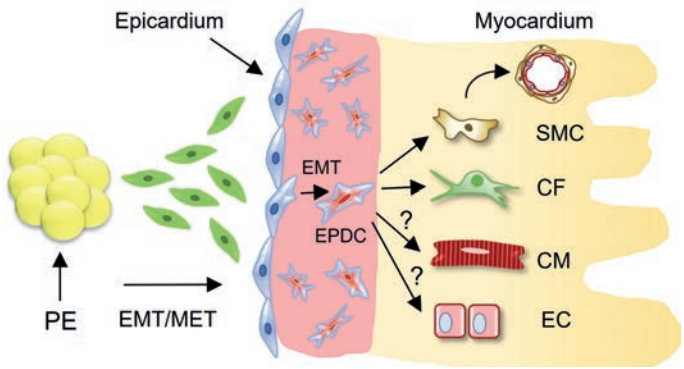


Fig. 1 | Development of the epicardium and epicardial lineages. The epicardium originates from the proepicardium (PE). Proepicardial cells go through epithelial to mesenchymal transition (EMT) and translocate to the myocardial surface of the looping heart where they adhere, migrate,

proliferate and undergo mesenchymal to epithelial transition (MET) to form a squamous epithelial layer; the epicardium. While the epicardium remains an intact layer, some of the epithelial cells will undergo a second round of EMT and migrate into the matrix rich sub-epicardial layer (pink). Fate mapping and genetic lineage tracing has demonstrated the multi-lineage potential of these epicardium derived cells (EPDCs). EPDCs differentiate into smooth muscle cells (SMCs), contributing to the coronary vasculature and cardiac fibroblasts (CF) of the mature heart. The contribution of EPDCs to cardiac endothelial cells (ECs) and cardiomyocytes (CMs) has been described but is matter of an ongoing debate.

This structure arises from the pericardial coelomic mesothelium in close proximity of the heart and the liver (Reviewed in [13]). The liver provides additional inductive signals that aid the development of the PE [14]. In mouse, the PE becomes visible from embryonic day 8.5 (E8.5) as a transient cauliflower-like cluster of cells located at the base of the venous inflow tract of the developing heart. The cells of the PE are located adjacent to the primitive heart tube, but do not directly interact with the cardiogenic mesoderm that will form the future myocardium [15], [16].

The PE is composed of different cell populations, with an outer layer of cuboidal epithelial cells expressing Wilms' tumour 1 (WT1) that overlies an inner core of extracellular matrix harbouring several mesenchymal cell types, as well as endothelial cells [17]. The WT1 positive cells within the PE originate from the early cardiac progenitor fields that express Nkx2.5 and Isl-1 [18]. Together with the T-box transcription factor Tbx18 and the basic helix-loop-helix transcription factor Tcf21, WT1 is used to identify PE and (later in development) epicardial (-derived) cells [19], [20]. A distinct subset of PE cells express Scleraxis (Scx) and Semaphorin 3D (Sema3D) and both proteins only partially overlap with WT1 and Tbx18 [21], thereby emphasising the heterogeneity of the cell cluster.

Once the embryonic heart has looped, the bare heart tube will be covered with an epicardial layer of cells derived from the PE that translocate to the heart via different mechanisms. For instance in chick, villous protrusions of the PE extend and directly attach to the myocardial surface, generating a "bridge" for cells to cross [22], while in mice islands of PE cells are formed as they are either pulled off from the villi [23] or are released as free-floating vesicles [24]. Recently, another mechanism was reported where PE cells reach the ventricle by migrating along the surface of the inflow tract [25]. Next, the adhering clusters of PE cells start to proliferate and spread to cover the developing myocardium with a squamous epithelial layer. Epicardial coverage occurs in the mouse between E9.5 and E10.5 in a dorsal to ventral pattern. In

addition, the left ventricle is covered first, and with a more dense layer as compared to the right ventricle [26].

To unravel the role of the PE in the formation of the epicardium and myocardial wall, studies were conducted where the PE was prevented from outgrowth. Both microsurgical and genetic inhibition of the PE cells' migration and adhesion reduced the proliferation of cardiomyocytes, resulting in a thin-walled compact myocardium [20], [27], [28]. Moreover, genetic mouse models causing disturbed epicardial development are associated with defects in endocardial valve development and heart looping [29], cardiomyocyte proliferation and alignment [30], development of the coronary vasculature [27], [31], [32], and the cardiac conduction system [33]. Given the crucial role of the (pro)epicardium in the formation of a fully functioning heart, it is important to understand its contribution in development and disease.

2.2. The heterogeneous composition of the epicardium

When the epicardium completely covers the heart around E11.5 in mouse [26], and week 5 during human cardiac development [34], it has established a multi-cellular epithelium lining the ventricles [35]. The detailed composition of the epicardium is not fully known, but several epicardial cell specific proteins have been suggested, although not necessarily expressed in the same cell. These include WT1 [36], Tbx18 [37], Tcf21 [38], Gata5 [39] and cytokeratin [40]. Additionally, Semad3D, and Scx are expressed in a subset of cells [21]. Furthermore, within the epicardium, clusters with bone marrow-derived CD45+ cells are present, demonstrating that the epicardium is not solely composed of PE-derived cells [41]. Whether the cell populations within the PE are related, i.e. if they represent distinct stages of a continuum of differentiation, or if they are distinct populations with specific abilities, remains to be investigated.

2.3. The epicardium in the formation of the myocardium

2.3.1. Epithelial to mesenchymal transition and migration of epicardial (-derived) cells

Once the epicardium is established, epicardial cells will directly start to participate in the formation of the cellular composition of the myocardium. A subset of the epicardial cells will undergo a process known as epithelial-to-mesenchymal transition (EMT) (reviewed in [42]; Fig. 1). EMT starts with epicardial cells losing epithelial characteristics like their apical-basal polarity, and their cell–cell contacts, by reducing the expression of the transmembrane adhesion proteins E-cadherin and zonula occludens-1 (ZO-1). Subsequently the epicardial cells acquire mesenchymal cell characteristics; they gain a spindle shape morphology and upregulate the expression of fibronectin,

N-cadherin and matrix metalloproteases (MMPs). This endows the EPDCs with the ability to migrate and populate the subepicardial space: an amorphous matrix-rich layer which is present between the epicardium and the myocardium (Fig. 1). From the subepicardium, EPDCs migrate into the myocardial interstitium where they can differentiate into different cell types and contribute to the development and maturation of the myocardium [43] (Fig. 1).

Epicardial EMT and migration are controlled via various myocardial- and epicardial-derived growth factors like TGFβ [44], PDGF [45], and FGF [46] (reviewed in [42]). EMT is regulated by transcription factors such as WT1, TCF21, as well as the snail family members Snail1 and Snail2 [42]. For instance, WT1 stimulates EMT via downregulation of E-cadherin expression [47], by increasing the expression of Snail, by enhancing Wnt signalling, and via upregulation of Raldh2 resulting in enhanced retinoic acid signalling [48]. Furthermore, loss of neurofibromin (encoded by *Nf1*) resulted in increased EMT and EPDC proliferation [49]. Interestingly, when TCF21 is specifically deleted from the epicardium, migration of epicardial cells into the myocardium is hampered due to a defect in EMT [50].

Migration of epicardial cells into the myocardium is important for proper myocardial development and it is controlled by a set of transcription factors like Nfatc1 and MRTFs. Nfatc1 is expressed in a subset of epicardial cells and when deleted it resulted in reduced levels of matrix degrading enzymes and a subsequent impaired migration of EPDCs into the myocardium [51]. The myocardin related transcription factors (MRTF)-A and −B are induced when EPDCs are treated with TGFβ *in vitro*, resulting in enhanced migration. In vivo, when MRTF-A/B are knocked out specifically in the epicardium, migration of EPDCs into the sub epicardial layer is impaired [52].

The involvement of all these factors in a temporal and spatial controlled manner to regulate epicardial EMT and migration of EPDCs into the myocardium is crucial for proper development of the myocardial wall.

2.3.2. Differentiation of EPDCs into cardiac cell types

Once EPDCs have invaded the myocardium, they will start to differentiate into several cardiac cell types (Fig. 1). The predominant cell types are interstitial fibroblasts that produce the cardiac extracellular matrix, smooth muscle cells (SMCs), and adventitial fibroblasts that sustain the coronary vasculature [36], [53], [54]. In contrast, contribution to endothelial cells and cardiomyocytes is subject to debate [55], [56] (Fig. 1).

To explore the differentiation capacity of EPDCs, *in vitro* models have been developed to culture EPDCs from mouse embryonic heart explants. These culture systems have confirmed differentiation of EPDCs into fibroblasts and SMCs after addition of TGFβ [44], [57], [58]. Furthermore, addition of thymosin β4, combined with VEGF/FGF7, resulted in a highly significant increase in Tie2-positive endothelial cells [59]. Additionally, cardiomyocyte differentiation *in vitro* was observed using embryonic $WT1^{+}$ cells [36]. While these *in vitro* models may predict the ability of epicardial cells to differentiate into many cell types that compose the heart, it is important to realise that these conditions are artificial and may not represent the actual *in vivo* situation where interaction with other cells and signals occurs.

In vivo, the first studies investigating the differentiation potential of epicardium were performed using retroviral labelling in quail-chick chimera experiments. These experiments indicated an ability of EPDCs to contribute to fibroblast, SMC and coronary endothelial lineages [60], [61], [62]. Subsequent genetic fate-mapping experiments in mice where the expression of Cre-recombinase (Cre) is driven by epicardial-cell specific promoters (e.g. WT1, Tbx18, Tcf21, Gata5, Sema3d and Scx) generally confirmed the epicardial origin of fibroblasts and SMCs (see Table 1, and Tian et al. [63])

Concerning the origin of endothelial cells, lineage tracing studies have demonstrated different results depending on the promoter that was used to follow the cells. Tcf21 and Tbx18 lineage tracing studies were unable to establish epicardial derived endothelial cells [37], [50], [64], while WT1, GATA4, SEMA3D and Scx fate-mapping studies have shown co-localization of genetically labelled cells with EC proteins (Table 1) [21], [32], [36], [65]. Controversy between these studies could be explained by the fact that the PE and epicardium contain heterogeneous cell populations that could reflect distinct populations with a diverse differentiation potential (see Section 2.2). This was further demonstrated by Katz et al., showing that a TBX18/WT1 negative and SEMA3D/Scleraxis positive cell population in the PE gives rise to endothelial cells [21]. Furthermore, some results should be interpreted cautiously. Chick-quail chimeras can be contaminated with cells of the sinus venosus; a structure known to contribute to the coronary vasculature [66].

Table 1 | Overview of *in vivo* epicardial lineage tracing in mouse embryos. Only studies aiming to use the mouse model for epicardial lineage tracing were included. *Did not investigate other cell types, $ Sporadic, # investigated CMs only in right ventricle

Mouse model	Reporter gene	EPDC differentiation	Identified markers	Reference
SEMA3D$^{GFPCre/+}$	R26R^{LacZ}	EC SMC CM$^{\$}$ Fibro	cTnT, SM22α, Vimentin, Flk-1, PECAM	[21]
Scx^{GFPCre}	R26R^{LacZ}	EC SMC$^{\$}$ CM Fibro Endocardial$^{\$}$	cTnT, Cx43, SMM, FSP, Flk-1, PECAM, Nfatc1, NRP-1, Ephrin B2	[21]
G2-Gata4^{Cre}	Rosa26^{YFP}	EC SMC *	PECAM, IB4, αSMA, NOTCH1	[32]
Wt1$^{CreYFP+}$	Rosa26^{YFP}	EC SMC *	PECAM, αSMA	[32]
Wt1^{CreERT2}	Rosa26^{YFP}	EC *	PECAM	[32]
Wt1^{GFPCre}	Rosa26^{fsLz} and Z/Red	SMC EC CM	PDGFRβ, SM22, SM-MHC, Flk-1, PECAM, Tnnt2, Actn1, GATA4, Nkx2.5, Cx43, functional coupling	[36]
Wt1^{CreERT2}	Rosa26^{fsLz} and Z/Red	SMC EC CM	Actn1, PECAM, PDGFR, Tnnt	[36]

Table 1 *(Continued)*

Mouse model	Reporter gene	EPDC differentiation	Identified markers	Reference
Tbx18^{Cre}	R26R^{LacZ}	CM SMC Fibro	Tnnt, cTnI, MF20, GATA4, Nkx2.5, SM-MHC, NRP-1, PDGFrb, Col1a2	[37]
Tcf21$^{iCre/+}$	R26R^{YFP} and R26R^{tdT}	Fibro	PDGFRα, Col1	[50]
Wt1^{Cre}	R26R^{mTmG}	Fibro CM*	FlnA, Vimentin, Col1a1, MF20	[54]
Tbx18$^{cre/+}$	Rosa26^{mTmG}	Fibro SMC #	Nos3, Emcn, SMaActin, Notch3, Postn, Tnni3, Myhc	[64]
Wt1^{CreER}	Rosa26^{RFP}	SMC Pericyte EC *	SM22, SM-MHC, SMA, PECAM	[65]
Tbx18^{Cre}	Rosa26^{RFP}	SMC *	SMA	[65]
Wt1^{CreERT2}	Rosa^{mTmG}	Pericyte *	CSPG4, PDGFRβ	[115]

Abbreviations: Actn1: Alpha-actinin-1, CM: Cardiomyocyte, Col: Collagen, CSPG4: Chondroitin Sulfate Proteoglycan 4, cTnI: cardiac Troponin I, cTnT: cardiac muscle troponin T, Cx43: Connexin 43, EC: Endothelial Cell, Emcn: Endomucin, Fibro: Fibroblast, Flk-1: Fetal liver kinase 1, FlnA: FilaminA, FSP: Fibroblast-specific Protein, IB4: Isolectin B4, MF20: Myosin Heavy Chain, Myhc: Myosin heavy chain cardiac muscle, Nfatc1: Nuclear factor of activated T-cells, cytoplasmic 1, Nkx2.5: NK2 Homeobox 5, Nos3: Nitric oxide synthase 3, Notch: Neurogenic locus notch homolog protein, NRP-1: Neuropilin-1, Pdgfr: Platelet-derived growth factor receptor, PECAM: Platelet Endothelial Cell Adhesion Molecule, Postn: Periostin, SM22: Smooth Muscle Protein 22, SMaActin: Actin α2 Smooth Muscle aorta, SMC: Smooth Muscle Cell, SMM: Smooth muscle myosin, SM-MHC: Smooth Muscle-Myosin Heavy Chain, Tnni3: Troponin I cardiac 3, Tnnt: Troponin T, αSMA: α Smooth Muscle Actin

Additionally, WT1 was shown to be expressed in cardiac endothelial cells [34], [56], [67], which may obscure the results from lineage tracing experiments. Therefore, although some studies conclude that endothelial cells can derive from the epicardium, it remains an ongoing debate to what extend the PE/epicardium contributes to the coronary vasculature in the developing heart. Overall the direct contribution of epicardial cells, if any, appears to be low and both the sinus venosus and endocardium [21], [68] are considered the major contributors (reviewed in [63]).

With respect to epicardial-derived cardiomyocytes, lineage tracing studies using Scleraxis, WT1 and TBX18 to trace cells have demonstrated epicardial-derived functionally active cardiomyocytes (Table 1). However it must be noted that these findings are controversial, since some of the genes driving the expression of Cre recombinase in these studies, e.g. *Tbx18* [37], and *WT1* [36], have been shown to be present within cardiomyocytes at certain stages during development [55], [67].

Although the full differentiation potential *in vivo* is yet to be confirmed, it is however clear that embryonic EPDCs have an essential impact on cardiac development via direct contribution of cells to the myocardium (Fig. 1).

2.3.3. Contribution of epicardial paracrine signalling to the formation of the myocardium

Another process that occurs once the epicardium has covered the developing heart is that epicardial cells will start to produce paracrine factors that support myocardial growth [69], [70], [71]. These factors provide an essential exchange of signals between the myocardium and the epicardium that are crucially important for the development of the coronary vessels (reviewed in [72]), as well as the growth and differentiation of the heart muscle [73]. For instance, Kolander et al. showed that disrupting GATA4 and GATA6 signalling specifically in the epicardium results in defective coronary vascular development by regulation of the number of subepicardial endothelial cells via secreted factors [74]. Another example of epicardial-myocardial signalling is retinoic acid (RA), a known contributor to cardiomyocyte proliferation. Retinoid X receptor α (RXRα) knock out mice are lethal as a result of hypoplastic myocardium, and demonstrate a poorly attached epicardium [75]. Interestingly, this phenotype was demonstrated to be epicardial-related since deletion of RXRα in GATA5 expressing cells also demonstrated impaired cardiac compaction, as a consequence of a decreased cycling of cardiomyocytes [76]. Furthermore, multiple Fibroblast Growth Factors (FGFs) are expressed in the epicardium and are shown to be essential for proper myocardial formation [46], [77], [78]. For example, depletion of the epicardially expressed Fibroblast

Growth Factor 9 (FGF9), or depletion of the concomitant FGF receptors present on premature cardiac myoblasts, results in embryonic lethality and reduced cardiomyocyte proliferation in the developing heart [77]. Also other produced factors, such as Hedgehog [78], [79] and CXCL12 [80] were shown to be involved in myocardial morphogenesis and coronary development. This emphasises the importance of the epicardium in cardiac development, by contributing cells as well as essential cytokines and growth factors to induce the myocardial development.

3. THE EPICARDIUM IN THE ADULT MAMMALIAN HEART

3.1. The quiescent epicardium in the adult intact heart

Contrary to development, the postnatal mammalian epicardium is a dormant single-cell layer. This was shown via the analysis of genes involved in epicardial activation such as *WT1*, *Tbx18* and *Raldh2*. These genes are abundantly present in the embryonic heart, but are rapidly downregulated postnatally, only to be barely detectable at 3 months of age in the mouse [81]. The same conclusion on the dormancy of the epicardium was drawn by using a mouse model where the Green Fluorescent Protein (GFP) gene was knocked-in into the WT1 locus ($WT1^{GFPCre}$). When investigating the WT1-driven expression of GFP in different stages of heart development, Zhou et al. observed that in the embryo, its expression was restricted to the epicardium and labelled approximately 90% of the epicardial cells [82]. In contrast, in the adult heart, GFP expression was observed in fewer than 25% of the cells within the epicardium; thereby revealing that WT1 is present at stages where the epicardium actively plays a role in heart development. This lack of activation in the adult heart was further underscored by using a tamoxifen inducible $WT1^{CreERT2}$ mice crossed onto a Cre reporter line ($Rosa^{mTmG}$). In this model, the activation of WT1 only results in translocation of Cre recombinase to the nucleus when tamoxifen is administered. By crossing these mice onto the $Rosa^{mTmG}$ reporter line, nuclear translocation of Cre results in the irreversible exchange of expression of red fluorescent Tomato into the indefinite expression of green fluorescent GFP. This model, which is extensively used in the field of epicardial research, thereby provides the opportunity to label $WT1^{+}$ cells at any given time point, and to track their fate based on the continuous expression of GFP. Investigating uninjured adult hearts using this model up to eight weeks after tamoxifen injection revealed no migration of WT1/GFP expressing cells into the myocardium [82]. Together, these data show that in the adult uninjured heart, the epicardium is a quiescent cell layer, as shown by the downregulation of epicardial specific genes and the lack of migration of cells after the formation of the heart is completed.

3.2. The activated epicardium in the infarcted adult heart

3.2.1. Activation of the embryonic gene programme in the epicardium post-MI

In the adult heart, the epicardium can be awakened from its dormant state. Several studies have shown that MI or ischemia/reperfusion can result in reactivation of the epicardial layer, including its proliferation and expansion, EMT, and migration of epicardial derived cells (reviewed in [42], [83]).

Within the first days after MI, the epicardial layer displays a partial recapitulation of the embryonic gene programme, which is evident by the upregulation of the epicardial genes Wt1, Raldh2 and Tbx18 [82], [84], [85]. At five days post-MI, the expression of these genes peak, and they remain present in approximately 75% of the epicardial cells at 14 days after injury, after which the expression decreases gradually [82]. Interestingly, the upregulation of genes is observed not only at the site of injury, but throughout the epicardium. One theory is that this occurs via factors secreted into the pericardial fluid. Studies inducing MI with an intact pericardium have shown an attenuated cardiac function post-MI compared to MI where the pericardium was opened prior to ligation [84], [86].

How the activation of the epicardium is regulated on a transcriptional level is not fully understood, however it appears to be partially controlled via the C/EBP transcription factor family [85]. Interestingly, in contrast to MI, mice undergoing transverse aortic constriction (TAC) to induce hypertrophy and fibrosis display no activation of $WT1^{+}$ cells within the epicardium [87], suggesting that cardiac damage through ischemia is likely a potent activator of this layer.

3.2.2. Proliferation and EMT of epicardial cells post-MI

Following MI, the epicardium covering the injured area is initially completely lost and is rebuilt from the surviving epicardium within three days post-injury [88]. The tamoxifen inducible $WT1^{CreERT2}Rosa26^{mTmG}$ lineage tracing model was able to confirm that this is due to proliferation of pre-existing epicardial cells. At two days post-MI, phospho-histone H3 and BrdU staining revealed many proliferating $WT1^{+}/GFP^{+}$ epicardial cells [82]. This resulted in a transition from a single cell epithelium into a multi-layered sheet, in an organ wide fashion, but most pronounced near the injured area [82], [88]. This process is specific to acute ischemic injuries like MI and ischemia reperfusion, as thickening of the epicardium is absent in models for hypertrophy like TAC [89].

Further recapitulation of the embryonic activation process in the ischemic heart is shown by the upregulation of several EMT-related transcription factors including *Smad1*, *Snail*, *Slug* and *Twist* in EPDCs [82], [88]. Interestingly, BrdU incorporation experiments revealed that the epicardial cells positive for EMT-markers are responsible for the newly generated subepicardial mesenchyme [88].

The activation and expansion of the epicardial layer is crucial for the post-injury response, as was illustrated by Duan and colleagues [90].The specific disruption of Wnt signalling within epicardial cells in a mouse model of cardiac ischemia reperfusion injury revealed less epicardial EMT, and a reduced subepicardial collagen deposition. As a result, ventricular dilatation and a decreased fractional shortening were observed [90]. Equally, in a study were the epicardium was primed by systemic injection of thymosin β4 prior to MI, a thicker layer of activated epicardium, and more migration was observed and resulted in a subsequent positive effect on cardiac function [81]. These studies emphasize the importance of understanding and optimising the epicardial activation and EMT to enhance the post-injury response [42]. Our recent *in vitro* data show that both human adult and foetal epicardial cells undergo activation and EMT [91]. Interestingly, foetal cells were activated more efficiently, and underwent EMT spontaneously. This potentially reflects the differences in efficiency of activation *in vivo* during development and in the adult heart. As such the foetal processes may serve as a blueprint for an optimal adult post-injury response.

3.2.3. Migration of EPDCs into the injured adult heart

Whether adult EPDCs retain the ability to migrate into the damaged area is subject to debate. Two studies using the $WT1^{CreERT2}Rosa26^{mTmG}$ injected with tamoxifen to trace the epicardium with GFP indeed confirmed the expansion of this layer after MI, but labelled $WT1^{+}$ cells appeared to be retained subepicardially showing no indication of migrating into the damaged myocardium [82], [92]. However, in a later study using the same mouse model, the injection of modified-RNA encoding for VEGF-A protein (modRNA VEGF-A) after injury did reveal migration of cells into the heart [93].

Several alternative methods to mark cells within the epicardial layer have been applied. The first one is to inject fluorescent protein-producing lentiviruses into the pericardial fluid, which is in direct contact with the epicardial layer, allowing infection and labelling of the epicardium [53], [94]. A second approach is to apply a biocompatible gel containing a modified RNA for Cre on the epicardium of the $Rosa^{mTmG}$ reporter mouse [93]. The patch was applied two weeks prior to MI to prevent labelling of non-epicardial cells in the complicated injured environment. Both methods revealed

that epicardial-derived cells were found within the infarcted myocardium at 7 and 21 days post-MI [93], [94]. Although these studies rely on epicardial labelling based on location rather than using epicardial specific markers, they coincide with the results of a genetic labelling study where in the Bacterial Artificial Chromosome (BAC)-WT-1^{Cre}; R26R mice, beta-galactosidase expressing cells were observed in the infarcted area after 1 month [88].

It is challenging to correlate these different findings in mouse lineage trace models. In the non-inducible BAC model, background labelling can occur if cells within the myocardial wall start to express WT1 [56], which is less likely to happen in the inducible lineage trace model since the labelling occurs during a short period of time. However, a downside of the tamoxifen labelling is that the short duration of labelling may result in missing of many cells and therefore an underestimation of the epicardial contribution.

Although these different conclusions regarding migration may be the result of technical issues, it appears that, at least partially, migration is a component of the adult reactivating response to injury.

3.3. Differentiation of adult epicardial cells into cardiac cell types in vivo

3.3.1. Fibroblasts

As mentioned above, the most prominent contribution of epicardial cells to the formation of the embryonic myocardium is via differentiation into coronary SMCs, and interstitial and adventitial fibroblasts. In the adult injured heart, a similar differentiation profile is observed (Table 2). Lineage tracing models using WT1 as a Cre driver, showed that a majority of traced cells transition into fibroblasts [82], [88], [92]. Additionally, after ischemic injury, the epicardium thickness increases up to 6-fold, and the deposition of collagen in the subepicardial space is also dramatically increased [89] indicating a rise in fibroblast activity.

Table 2 | Overview of *in vivo* epicardial lineage tracing following myocardial infarction in mice. Only studies aiming to use the mouse model for epicardial lineage tracing were included. * Other cell types not reported, $ Sporadic, ^ Displayed no mature characteristics, remained rounded.

Mouse model	Reporter gene	EPDC differentiation	Identified markers	Treatment	Reference
WT1$^{CreERT2/+}$	R26R^{EYFP}	CM *	cTnT, sαActin, Cx43, N-Cad, $Ca2^{+}$transients, Functional coupling	TB4	[81]
WT1$^{CreERT2/+}$	R26R^{mTmG}	SMC Fibro EC$^{\$}$	SM-MHC, αSMA, SM22α, FN1, ColIII, FSP1, ProCol1, PECAM	-	[82]
Ad:Msln-Cre	R26R^{mTmG}	Fibro	FSP1	-	[82]
(BAC)WT1^{EGFPCre}	R26R	SMC EC CM$^{\$}$ Fibro	αSMA, PECAM + location in vessel wall, cTnI, SERCA2, via exclusion of other markers	-	[88]
WT1$^{CreERT2/+}$	R26R^{mTmG}	SMC Fibro	αSMA, DDR2, ProCol1, desmin, FSP1, ColIII	TB4	[92]
WT1^{CreERT2}	R26R^{mTmG}	EC CM *	PECAM1, Kdr, Tnnt2, TNNI3	VEGF-A modRNA	[93]

Table 2 *(Continued)*

Mouse model	Reporter gene	EPDC differentiation	Identified markers	Treatment	Reference
Cre modRNA gel	$R26R^{mTmG}$	SMC EC CM	SM-MHC, PECAM1, TNNI3	VEGF-A modRNA	[93]
LV-CMVGFP	-	CM *	sαActin, morphology	-	[94]
Gata5-Cre	$R26R^{EYFP}$	SMC^ EC^ Fibro	αSMA, PECAM, ProCol1	TB4	[97]

Abbreviations: Ad: Adenovirus, CM: Cardiomyocyte, ColIII: Collagen type III, cTnI: cardiac TroponinI, cTnT: cardiac muscle TroponinT, Cx43: Connexin 43, DDR2: Discoidin Domain Receptor2, EC: Endothelial Cell, Fibro: Fibroblast, FN1: Fibronectin 1, FSP1: Fibroblast-specific protein 1, Kdr: Kinase insert domain receptor, Msln: Mesothelin, N-Cad: N-Cadherin, PECAM: Platelet Endothelial Cell Adhesion Molecule, proCol1: procollagen1, SERCA2: Sarcoplasmic Reticulum Ca2+ ATPase 2, SM22: Smooth Muscle Protein 22, SMC: Smooth Muscle Cell, SM-MHC: Smooth Muscle-Myosin Heavy Chain, sαActin: sarcomeric α Actin, Tnni3: Troponin I cardiac 3, Tnnt: Troponin T, Tβ4: Thymosinβ4, αSMA: α Smooth Muscle Actin

Russell et al. [95] used a Notch reporter mouse line which revealed significant activation of the reporter in the epicardium. Notch reporter positive epicardial cells were isolated and subjected to microarray analysis which showed that these cells have a fibroblast signature with high expression of collagen-I, elastin and fibronectin. Therefore, fibroblasts likely represent the default programme in the activated epicardial layer [95]. Although too much activity of cardiac fibroblasts could result in excessive scar formation, they represent a vital cellular component in cardiac homeostasis and wound healing [96]. Reducing Wnt/βcatenin specifically in cardiac fibroblasts resulted in reduced collagen deposition and a worse cardiac function post-injury revealing a beneficial role of cardiac fibroblasts in cardiac repair [90].

3.3.2. Endothelial and smooth muscle cells

With respect to neovascularisation post-injury, many studies have identified lineage-traced EPDCs that differentiate into smooth muscle cells post-MI [82], [88], [92], [93], [97], [98] (Table 2), which was anticipated based on the described embryonic fate of EPDCs. In contrast, most analyses failed to identify EPDCs that differentiated into endothelial cells [81], [82], [92] (Table 2). However, a study using the BAC $WT1^{Cre}$;R26R mouse line to determine the fate of epicardial cells based on β-galactosidase (β-gal) expression revealed $\beta\text{-gal}^{+}$ cells co-expressing PECAM were found to line vessels within the infarcted area [88]. Although intriguing, this is likely due to the fact that WT1 can be re-expressed in endothelial cells post-injury [56] and could therefore represent an artefact, making it unlikely that there is a direct contribution to endothelial cells in the normal response of the heart to MI.

Interestingly, injection of modRNA VEGF-A into the mouse myocardium after MI resulted in an increased capillary density and a smaller infarct size. This coincided with an enhanced activation of epicardial cells, which showed an upregulation of the VEGF receptor KDR in WT1-GFP expressing cells in the $WT1^{GFPCre}$ mouse line. After isolation and sorting of epicardial cells post-MI, cultured WT1+ cells responded to VEGF-A by increasing their proliferation. Moreover, VEGF-A was able to shift the differentiation of epicardial progenitor cells towards the endothelial lineage as shown by an increase of VE-Cadherin, KDR and PECAM1 expression. This was further corroborated by *in vitro* clonal assays and lineage trace experiments using either the $WT\text{-}1^{CreRT2}$x $ROSA^{mTmG}$ and or a modRNA Cre expressing patch on the epicardium [93]. This study emphasises the plasticity of epicardial cells, provided the right cues are delivered at the right time (Table 2).

3.3.3. Cardiomyocytes

The differentiation of EPDCs into cardiomyocytes in the embryo is controversial, and the same holds true in the adult (Table 2). In a study by Limana et al., the epicardium was labelled via injection of a GFP producing lentivirus 3 days before experimentally induced MI, which revealed that some cells that migrated into the infarcted myocardium expressed α-sarcomeric actin, albeit in very low numbers [94]. The observation that EPDCs have the ability to differentiate into cardiomyocytes was confirmed in the BAC-WT1^{Cre};R26R mouse line. Epicardial derived β-gal^{+} cells co-expressing troponin I were found within the myocardium but only after one and three months post-MI. These EPDC-derived cardiomyocytes maintained an immature phenotype, being small round cells lacking sarcomeric organisation, and numbers were still very low [88].

An enhanced post-injury activation of the epicardium was observed when animals were treated with thymosin β4 prior to myocardial infarction. Interestingly, in this study using the WT1^{CreERT2};R26R^{EYFP} line, differentiation of epicardial derived cells into troponin T and α-sarcomeric actin expressing YFP^{+} cells that coupled to the surrounding myocardial tissue was observed. To counter that these results are due to extra-epicardial expression of WT1, YFP^{+} epicardial derived cells were isolated from thymosin β4 treated mice 4 days after myocardial infarction and transplanted into the infarcted myocardium of a recipient wildtype mouse; a small number of the YFP^{+} donor cells differentiated into α-sarcomeric actin expressing cells. A similar effect was observed when modRNA encoding VEGF-A was injected into the *peri*-infarct zone to enhance the epicardial response; besides endothelial cells, a slight increase in cardiomyocytes was found based on TNN3 expression in lineage traced epicardial cells [93]. Although the occurrence of cardiomyocyte differentiation is still extremely low in these two studies [81], [93], it provides a proof-of–concept that cardiomyocyte formation from EPDCs could be a possibility.

In contrast, there are several studies using a similar lineage tracing approach (WT-1^{CreERT2}; R26^{mTmG}) that did not observe differentiation into cardiomyocytes [82], [98]. Of note is that these studies also did not observe migration of EPDCs from the subepicardial to the myocardium after injury, whether this is due to technical issues needs to be addressed.

The data argue that in the adult, the epicardial potential for cardiomyocyte formation is comparable to the embryonic heart: its ability to deliver cardiomyocytes directly is low to non-existent but can be enhanced by priming the epicardium using specific factors.

3.4. Paracrine contribution of epicardial cells to cardiac repair

Besides contributing directly to the formation of cardiac cell types, epicardial cells can participate in repair of the heart via paracrine processes.

Zhou et al. observed many blood vessels within the thickened subepicardium after MI. These newly formed vessels were often located in close proximity to EPDCs. *In vitro* experiments with the GFP^+ lineage-traced EPDCs confirmed that these cells indeed have proangiogenic properties [82]. Interestingly, the injection of conditioned medium from cultured EPDCs into the infarcted heart resulted in an increase in vessel density and reduced the adverse remodelling of the heart post-MI in short and long-term follow-up [82]. A similar finding was observed when injecting human EPDCs into the infarcted heart of NOD-SCID mice. Although human cell survival was minimal at 6 weeks post-MI, a significant effect on vascularisation was apparent, again emphasising an angiogenic effect of EPDCs [99] (and our own unpublished observations). Interestingly, EPDCs may also influence cardiomyocytes within the myocardium via paracrine mechanisms. Co-culture experiments of EPDCs and cardiomyocytes resulted in increased myocyte proliferation and enhanced levels of cardiomyocyte differentiation [30], [100].

Besides using conditioned medium or cells, a single factor excreted by epicardial cells was identified that significantly aids in cardiac repair. Follistatin-like 1 was identified as a highly enriched factor produced by epicardial cells with the ability to induce cardiomyocyte proliferation [101] and to increase cardiomyocyte survival [102], [103]. By delivering this factor directly to the infarcted myocardium via an epicardially applied patch Wei et al. showed an increase in proliferation of local cardiomyocytes resulting in an increased survival, a reduction of fibrosis and prevented deterioration of cardiac function [101]. Follistatin-like 1 did not have an effect on the epicardium itself.

Conversely, epicardial cells can be influenced to partake in cardiac repair by addition of a single factor. Foglio et al. identified clusterin as highly enriched protein within the pericardial fluid of MI patients [86]. *In vitro*, clusterin was shown to induce proliferation and EMT of epicardial cells. *In vivo*, the injection of this single protein into the pericardial fluid of the infarcted mouse heart was sufficient to enhance epicardial EMT, and induce cell survival, arteriole density, and resulted in an ameliorated cardiac function [86].

These studies stress that epicardial cells do not only participate in cardiac repair by providing cells, but have a major contribution to the repair mechanisms within the

heart via production of essential proteins. Moreover, since epicardial cells themselves are sensitive to induction via cytokines and growth factors, one can imagine a feedforward loop were single proteins enhance the number of epicardial cells that produce paracrine factors, thereby further stimulating cardiac repair after injury.

4. THE EPICARDIUM AS A SOURCE OF MULTIPOTENT PROGENITOR CELLS?

From the data described above, the question arises whether EPDCs should be considered cardiac progenitor cells. It becomes clear that the adult epicardium in the infarcted heart is activated, undergoes EMT and contributes to several cardiac cell types. Epicardial EMT has been postulated to be involved in the formation of resident cardiac progenitor cells [104]. The adult epicardium may therefore function as a reservoir of mesenchymal progenitor cells [94].

Classically, stem or progenitor cells can be defined as a self-maintaining population of relatively undifferentiated, proliferative cells that can produce a variety of differentiated progeny with the ability to regenerate tissue of parts thereof [105]. Based on the expression of an embryonic gene programme, their ability to display proliferation upon damage, and their differentiation into several lineages, EPDCs partially fulfil these criteria. However, if they fulfil all criteria to be true *cardiac* progenitor cells remains to be established. To this end it is important to define which progenitors are necessary to deliver the cells of the developing heart [106].

Within the adult heart, cells have been discovered expressing stem cell markers like the tyrosine kinase receptor c-Kit [11], and stem cell antigen (Sca)-1 [10] on their cell surface. These c-Kit and Sca-1 expressing cells are considered to be stem- or progenitor cells based on their ability to form colonies, to display telomerase activity and to show long-term label retention. These cellular abilities are generally linked to stem-cell features. Moreover, these cells have been shown to differentiate into cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts, thereby indicating their ability to differentiate into all cell types required to generate cardiac tissue and participate in cardiac regeneration (please refer to the reviews included in this special edition of Pharmacological Research, Volume 127).

The human foetal and adult (sub)epicardium is highly heterogeneous [107] and was shown to harbour a minor population of c-Kit and CD34 expressing cells [84], [94].

In vitro, these c-Kit expressing epicardial cells differentiated into the endothelial and smooth muscle cell lineage [94]. A similar population was observed in the adult mouse heart. Interestingly, the percentage of proliferative c-Kit^{+} cells increased after induction of MI and these cells appeared to participate in the formation of subepicardial blood vessels [94]. Bollini and colleagues [108] performed a direct comparison between the expression profiles of mouse embryonic EPDCs, and adult EPDCs isolated after thymosin β4 stimulation and MI [108]. They observed a low expression of Sca-1 in the WT1^{+} embryonic cells, while in the adult, Sca-1 was present on approximately 60% of the WT1^{+} EPDCs. Moreover, the mesenchymal markers endoglin (CD105), the hyaluronan receptor CD44, major T-cell antigen 1 (Thy-1 or CD90) and Platelet Derived Growth Factor Receptor (PDGFR)β were observed in the activated adult EPDCs. They continued to show that within the adult epicardium post-MI, the WT1^{+}, Sca-1^{+}, CD90^{hi}, CD44^{hi} populations retain cardiovascular multipotency, as based on the expression of cardiac progenitor markers in this population [108]. These data indicate that thymosin β4 in combination with MI can stimulate a cardiac progenitor cell type within the epicardium.

Cells with mesenchymal-like properties have been isolated and cultured from the adult mouse [109] and adult human heart [110], [111]. These cells, termed cardiac colony-forming units fibroblast (cCFU-F), originated from the epicardium, and were defined as mesenchymal stem cells because they showed clonogenic propagation, long-term growth for over 40 passages without senescence, and multi-lineage differentiation. Furthermore, these mesenchymal progenitors express mesenchymal cell markers including CD105, CD44, CD90, and the stem cell markers Oct4 and c-Myc. Additionally, they are MSC-like in their transcriptome profile [112]. Although cCFU-Fs express Sca-1 and PDGFRα, lineage tracing using the Nkx2.5^{Cre} fate mapping mouse line suggested that they do not arise from the Nkx2.5 cardiac progenitor lineage [109].

A comparable cell culture population of mesenchymal-like EPDCs was isolated from the human epicardium. These spindle EPDCs (sEPDCs) are abundantly decorated with the mesenchymal stem cell markers CD44, CD90 and CD105, express the early cardiac transcription factor GATA-4 and show multi-lineage differentiation. When stimulated *in vitro* with TGF-β, sEPDCs express proteins of the smooth muscle cell lineage and calcify when cultured in osteogenic medium [58]. Interestingly, while sEPDCs only express GATA-4, freshly isolated cCFU-Fs are also reported to express the early cardiac marker Nkx2.5. Furthermore, sEPDCs differentiate into the mesenchymal lineage, but cCFU-Fs have the additional ability to acquire an endothelial phenotype, which has not been convincingly established for EPDCs. sEPDCs and cCFU-Fs are therefore

very alike, but whether they are similar is not clear to date. Russell et al. isolated a population of notch-reporter positive cells, that were related to the epicardium. These cells were not only able to differentiate into fibroblasts, but under the right culture conditions formed cardiomyocytes [95]. Additionally, in vitro studies using VEGF-A on WT-1 positive isolated cells showed that these cell could be instructed to proliferate and become cardiovascular cell types [93].

Overall, epicardial cells and EPDCs appear to behave like multipotent progenitor cells in the embryo proper. However in the adult, it appears that this potential is not fully exploited yet, but can be enhanced by providing the right cues.

5. FUTURE PERSPECTIVES

In the embryo, the contribution of the epicardium to the formation of the heart is essential *via* the contribution of cardiac cell types as well as the production of growth factors and cytokines that influence myocardial growth [113] (Fig. 1). As we have described in this review, many of these abilities are retained in the adult injured heart but appear to occur less efficient compared to the embryo. Therefore, the question arises whether the epicardial response can be optimised to more efficiently partake in the repair of the injured heart.

Possible approaches include inducing proliferation and subsequent EMT of the epicardium. This would provide a larger pool of cells that has the ability to migrate into the heart (Fig. 2, left). This method appears to be feasible based on the finding that thymosin β4 treatment prior to MI increases the number of activated epicardial cells, and enhances cardiac function and differentiation into cardiac cell types [81]. Unfortunately, this treatment is only successful when applied prior to injury [92], making it difficult to imagine thymosin β4 treatment as a clinical approach. Promising results came from applying modRNA for VEGF-A which resulted in an increase in the proliferation and migration of epicardial cells and direct their differentiation capacity into endothelial as well as cardiomyocytes. Importantly, this approach increased cardiac function post-injury [93]. Identifying the most potent activators of the epicardium will therefore be an important goal to pursue.

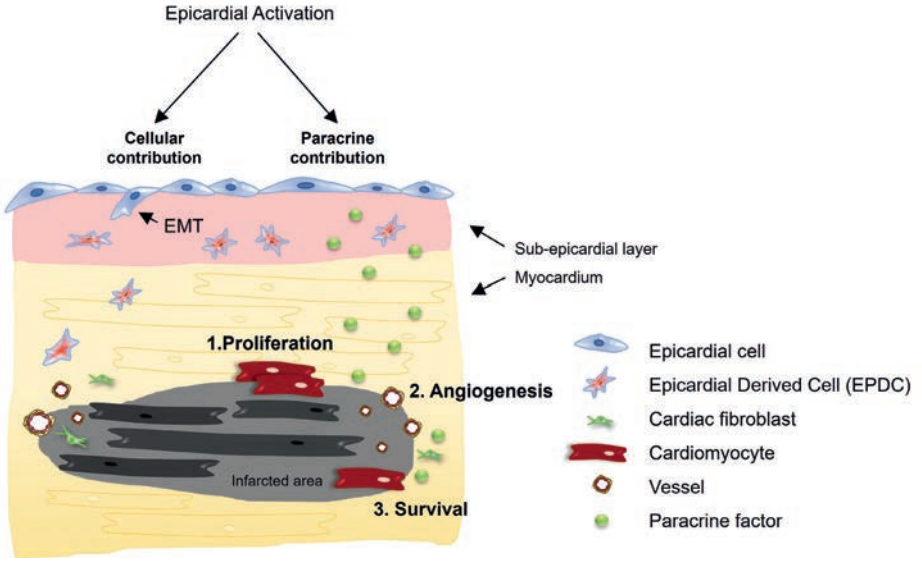


Fig. 2 | Potential mechanisms of epicardial-derived contribution to cardiac repair. Activated epicardial cells can have a direct cellular contribution to cardiac regeneration as epicardial cells undergo EMT, migrate into the myocardium and differentiate towards cardiac cells, such as cardiac fibroblasts, smooth muscle cells and potentially other cell types. A second epicardial contribution to cardiac repair acts via paracrine signalling, inducing (1) proliferation of cardiomyocytes, (2) angiogenesis and (3) survival of cardiomyocytes in the infarcted area.

Since the contribution of the epicardium is optimal during cardiac development, a potentially interesting method is to explore the embryonic or foetal epicardium as a paradigm to optimise the adult post-injury response. We have recently developed a cell culture system to efficiently isolate human foetal as well as adult epicardial cells, and culture them in their epithelial-like state [91]. This culture model allows direct comparison between these two cell sources. We have observed that EMT occurs spontaneously in foetal EPDCs, while adult cells require stimulation by TGFβ [91]. Identifying the differences in signalling pathways and receptor levels between foetal and adult EPDCs may help us understand how to unlock the full potential of the adult epicardial post-injury response.

Furthermore, it is important to explore the paracrine properties of the epicardial cells in more detail (Fig. 2, right). Zhou et al. showed that the epicardium can produce pro-angiogenic factors which positively influence the functional outcome post-MI [82]. A similar effect was observed by Winter et al. [99] where the transplantation of human EPDCs into the infarcted mouse heart resulted in increased vascularisation without

direct differentiation of the donor cells [99]. A more specific approach was used by Wei and colleagues [101], where the epicardial derived cytokine follistatin-like 1 was identified as a potent stimulator of cardiomyocyte proliferation [101]. A patch containing cells or growth factors can also be used to stimulate the epicardial layer directly. When loading a PCL/gelatine patch with MSCs, a beneficial effect was observed on the myocardium, but also induced proliferation and migration of the endogenous epicardial cells, thereby achieving a double effect on cardiac repair [114]. We envision that the foetal epicardium can again serve as a template to understand the optimal cocktail of growth factors and cytokines to achieve a maximal effect on the adult injured heart.

To summarize, several strategies are available to unlock the full potential of the epicardium as an endogenous cardiac progenitor cell source (Fig. 2). To which extend this will impact cardiac function after injury is part of future investigations.

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