The epicardium: development, differentiation and its role during heart regeneration

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Abstract | In the past few decades, the mortality rate after myocardial infarction has been significantly reduced through the use of thrombolytic therapy, primary percutaneous coronary intervention and bypass surgery to restore coronary circulation. However, reconstitution of the damaged coronary vessels is usually not achieved, leading to myocardial damage. Lost myocardium is replaced with scar tissue instead of healthy cells, impairing cardiac function. The human heart has classically been considered a postmitotic organ, incapable of self-repair. However, new evidence demonstrates that, to a limited extent, cardiomyocytes are renewed during adulthood and after myocardial damage.[1-3] The mechanisms controlling cardiomyocyte proliferation and coronary vessel regeneration and the development of therapeutic methodologies to enhance these processes are the focus of current research.

The coronary vasculature is partly derived from the epicardium, an epithelial layer enclosing the myocardium. Investigation of the epicardial structure has become a higher priority in the past decade, because it was shown to be a source of progenitor cells and to promote cardiomyocyte proliferation during heart development and regeneration.[4-6] This review summarises the most recent advances in epicardial development and its role during heart regeneration.

Origin of the epicardium

Coelomic mesothelial cells lining the pericardial cavity form an aggregate at the base of the inflow tract, which is named the proepicardium (for review see ref. 4-6) (Fig. 1a). In chicks, the proepicardium has a characteristic cauliflower-like structure, while in other species, such as dogfish and teleost fish, it is less morphologically distinct. Most of the epicardium covering the heart is derived from the proepicardium; however a small portion enveloping the distal outflow tract originates from the cephalic pericardium.[7] Recent lineage tracing experiments have shown that the proepicardium is derived from a common cardiac precursor pool that generates most of the cell types that form the heart. The early cardiac marker gene encoding the transcription factor *GATA4* is expressed in the proepicardium and its loss of function disrupts proepicardium formation.[8] Early cardiac precursor cells are also defined by the expression of the transcription factor genes *Nkx2.5* and *Isl1*. *Nkx2.5* and *Isl1* are not expressed in proepicardial (PE) cells. However, PE cells are descendents of *Nkx2.5*$^+$ *Isl1*$^+$ cells.[9,10] The proepicardium does not form correctly in *Nkx2.5* null mice; in contrast, *Isl1* is not necessary for proepicardial development.[10]

Some of the earliest genes expressed in the proepicardium are those encoding Retinaldehyde-dehydrogenase 2 *(Raldh2)*,[11,12] the T box transcription factor 18 *(Tbx18)*,[13,14] Wilm´s tumour protein *(Wt1)*[15,16] and the extracellular matrix protein podoplanin[17,18] (Fig.1a' and data not shown). None of these genes is exclusively expressed in the proepicardium and epicardium. *Raldh2* is expressed in posterior heart precursors at early stages of heart tube formation and is later expressed in the epicardium, the pericardium and the endothelial cells of the atrioventricular cushions. Loss of *Raldh2* function, which is involved in retinoic acid (RA) synthesis, leads to severe heart malformations. Epicardium-specific ablation of *Raldh2* has not been reported. However, inactivation of the RA receptor, *RXRα*, using a *GATA5*-enhancer–driven Cre construct, which is predominantly active in the epicardium, disrupts epicardial layer formation.[19] *Tbx18* is expressed in the coelomic wall of the pericardium and the myocardium of the sinus horns and is required for the development of the sinus horns but not for epicardium development.[13,14,20] Sites of *Podoplanin* expression include the coelomic wall of the pericardium, the cardiac conduction system and sinus venosus,[18] and *Podoplanin* null mutants display defective epicardium development as well as alterations in the sinus venosus and sinoatrial myocardium.[17] Similar to *Tbx18* and *Podoplanin*, *Wt1* expression is not confined to PE cells and the epicardium, but can also be detected in the mesothelial cells lining the pericardial cavity.[15,16] *Wt1* mutants also display a defective formation of the epicardium and its derivatives.[21,22]

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The formation of the proepicardium at the venous pole of the heart seems to be controlled by opposing actions of Fibroblast Growth Factor 2 (FGF2) and Bone Morphogenetic Protein 2 (BMP2). [23] In the chick, *FGF2* is expressed in PE cells, whereas *BMP2* is expressed in myocardial cells at the heart inflow tract. Inhibition of FGF2 signalling blocks PE formation. Instead, precursor cells become incorporated into the inflow tract (Fig. 1a").

PE cells proliferate and adhere to the myocardial surface, forming an enveloping epithelial layer. Two mechanisms for PE cell translocation to the myocardium have been proposed (Fig. 1b). In avian animal models, attachment of the proepicardium to the myocardial surface at the level of the atrioventricular boundary allows the transfer of PE cells to the myocardial surface.[24-26] Increased extracellular matrix accumulation at the contact site favours adhesion; and treatment with heparinase impedes the attachment of the proepicardium to the ventricular surface.[25] In dogfish and mouse, PE cell aggregates adhere progressively to the myocardium.[27,28] This phenomenon has been proposed to be driven by the heartbeat: the beating heart is suggested to promote alternating attachment and detachment between proepicardium and myocardium, leading to the formation of PE cell aggregates that remain adhered to the myocardial wall or temporarily free-float in the pericardial cavity until they reattach.[29] The possibility that both mechanisms could coexist in some species cannot be excluded. Furthermore, alternative mechanisms might be involved, since in the axolotl mutant *lethal*, which lacks a heartbeat, PE cells still attach to the myocardium although their spreading seems to be impaired.[30] Little is known on the molecular basis of PE cell migration and adhesion to the myocardium. The vascular cell adhesion molecule, VCAM-1, and its receptor, α4β1 integrin, mediate adhesion of α4β1-integrin-positive epicardial cells to VCAM-1 expressing cells on the myocardial surface.[31,32] The T-box transcription factor, *Tbx5*, has been suggested to control PE cell migration, since *Tbx5* gain or loss of function impairs PE cell migration and survival.[33]

An extracellular-matrix rich space, the subepicardial space, is established between the epicardium and myocardium. Some epicardial cells undergo an epithelial-mesenchymal transition (EMT) and delaminate from the epicardium to invade this space (Fig. 1c). Some of these epicardial-derived cells (EPDCs) remain as mesenchymal cells or form blood islands; others migrate further into the myocardium and endocardial cushions, where they differentiate into a subset of cell types described below (reviewed in ref. 4-6). Epicardial cells express the ATP-dependent SWI/SNF chromatin-remodelling component, *BAF180*, and analysis of *BAF180* null mutant mice identified a regulatory role for this gene in epicardial cell EMT.[34] Proper establishment of gap junctions is essential for correct EMT and epicardial cell migration, which was revealed by gain and loss of function analysis of *Connexin43* mutants.[35] EMT and EPDC migration are mainly controlled by paracrine signals from the myocardium, which are generated through interaction of the transcription factors GATA4 and FOG-2, among others.[36,37] Several studies have shown that Transforming Growth Factor β (TGFβ), via its receptor, Alk5, promotes epicardial cell EMT.[38-40] FGF Receptor 1 (FGFR1) signalling is important for myocardial invasion by EPDCs, since FGFR1 knockdown in PE cells results in a reduced number of coronary vessels in the myocardium and their accumulation in the subepicardial space.[41] A similar phenotype was observed in mice deficient for the actin-binding peptide Thymosin β4 (Tβ4),[42] which is normally expressed in the myocardium and promotes EPDC migration.

Epicardial derived cell differentiation

Much knowledge about the formation of the epicardium and its derivatives comes from studies in the chick. Analysis of quail-chick chimeras and retroviral labelling experiments suggest a primitive epicardium origin for the mesenchymal cells of the subepicardial layer, the endothelium and smooth muscle cells (SMCs) of the coronary vasculature, the perivascular and intermyocardial fibroblasts and some cells in the atrioventricular valves (reviewed in ref. 4-6) (Fig. 1e).

More recent studies in mouse using the Cre-lox technique examined the fate of cells expressing the epicardial marker genes *Wt1* and *Tbx18* [9,43] (Fig. 1e'). These studies showed that *Wt1*/*Tbx18* descendants contribute to the coronary vessel SMCs and the fibroblasts of the myocardial interstitium. Unlike reports in the chick, these studies found that in mice only a small population of coronary vascular endothelial cells is derived from the epicardium. Interestingly, a *Tbx18*-negative population expressing the endothelial precursor cell marker *Flk1* was found in the proepicardium, suggesting that different mesothelial-derived cell types coexist in this structure.[43] Alternatively, the *Flk1*-positive cell population might be derived from a different source. In chicks, the proepicardium has been reported to contain a mixture of hepatic endothelial precursors and mesothelial cells.[44] The authors suggest that the hepatic derived endothelial cell precursors give rise to the endothelial cells of the coronary vasculature, whereas the mesothelial component of the proepicardium contains the SMC progenitors. Contrasting with the observations in chicks, the mouse epicardium is suggested to make a considerable contribution to the myocardium.[9,43] However, this suggestion is questionable, since the supposed epicardium-derived cardiomyocytes might instead be a *Tbx18*-positive population residing within the myocardium.[45]

EPDC differentiation is controlled by epicardial and myocardial signals. A wave of epicardial *Sonic hedgehog* (*Shh*) expression precedes the formation of the coronary vasculature (for review see ref. 46). Epicardial Shh activates the production of proangiogenic factors, such

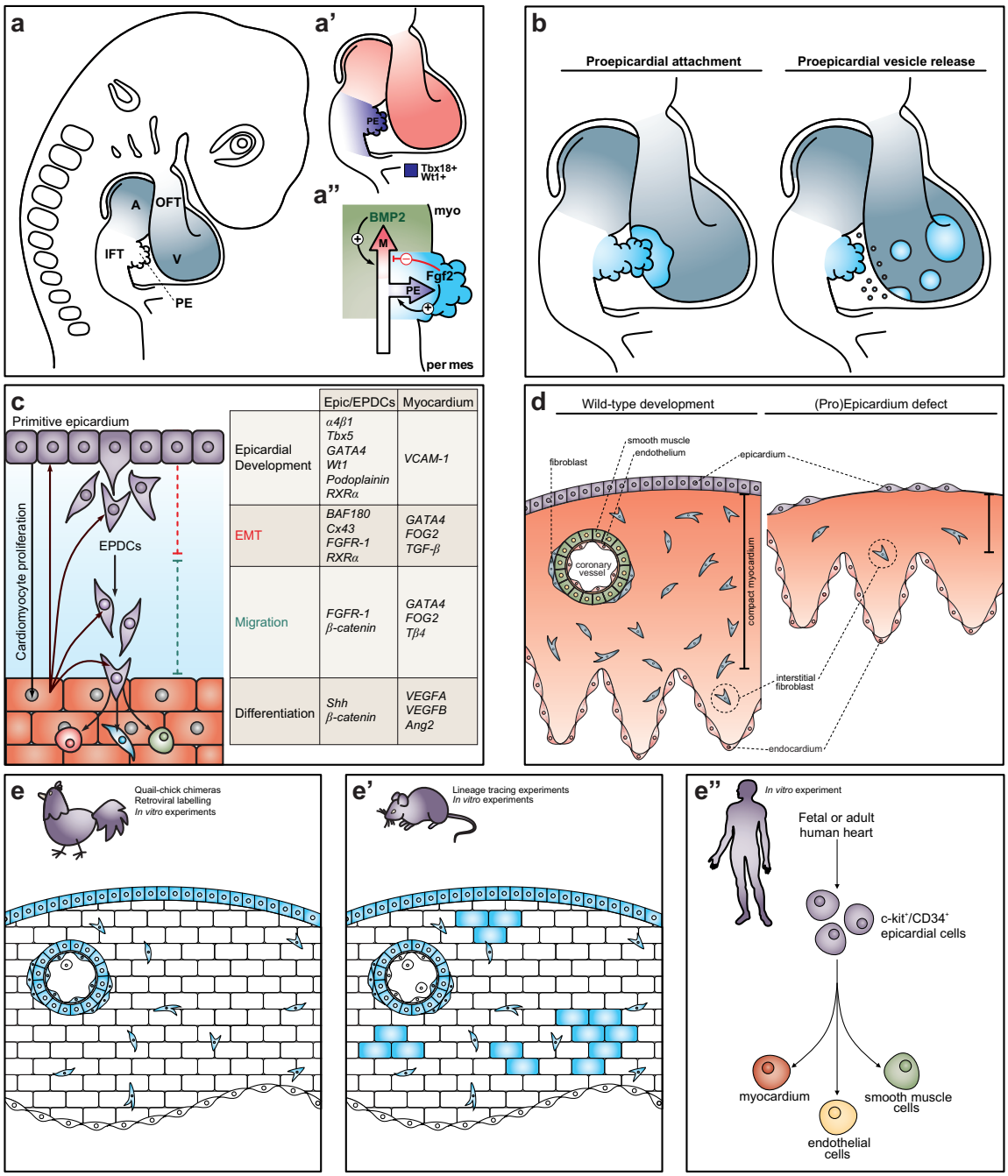

Figure 1 | Schematic representation of epicardial development and Epicardial Derived Cell (EPDC) differentiation. **a** | Lateral view of the anterior portion of a chicken embryo showing the proepicardium at the inflow tract of the heart. The heart tube is depicted in grey. **a'** | *Tbx18* and *Wt1* expression can be detected in the proepicardium (violet). The heart tube is depicted in red. **a"** | FGF signalling (blue) is required to specify proepicardial (PE) cell lineage (violet) and counteracts BMP signalling (green), which drives differentiation of the pericardial mesoderm into cardiomyocytes of the inflow tract (red). **b** | In the chick and quail, attachment of the proepicardium (blue) to the myocardial surface (grey) leads to transfer of proepicardial cells to the myocardium. In mouse and dogfish, PE cell aggregates have been suggested to progressively attach to the myocardial wall. **c** | Epicardial cells undergo an epithelial-mesenchymal transition (EMT) and accumulate in the subepicardial space. Some cells migrate into the myocardium where they differentiate into a subset of cell types. These processes are controlled in an autocrine and paracrine manner (brown arrows) by genes expressed in the epicardium or EPDCs and in the myocardium, respectively. The epicardium also influences cardiomyocyte proliferation (black arrow). **d** | Lack of correct epicardium formation- due to physical ablation or in null mutant mice of epicardial-development regulating genes- leads to alterations, including gaps within the epicardial layer, impaired formation of the coronary vasculature and thin compact layer of the myocardium. **e-e"** | Cell fate of EPDCs in chicks (e), mice (e') and humans (e"). A, atrium; EMT, epithelial-mesenchymal transition; Epic, epicardium; EPDCs, epicardial derived cells; IFT, inflow tract; myo, myocardium; OFT, outflow tract; PE, proepicardium; per mes, pericardial mesoderm; V, ventricle. Panel a has been adapted from ref. 5.

as Vascular Endothelial Growth Factor A (VEGFA), VEFGB and Angiopoietin 2 in the myocardium and VEGFC in the perivascular mesenchyme. Platelet Derived Growth Factor b (PDGFb) is involved in smooth muscle cell differentiation and *PDGFb* null mice have defective coronary arteriogenesis, with reduced numbers of pericytes and SMCs surrounding endothelial capillaries.[47,48] Correct angiogenesis of the coronary vasculature also involves Wnt signalling, since epicardial-specific suppression of *β-catenin* impairs myocardial invasion by EPDCs and their differentiation into SMCs.[49]

The epicardium as a signalling source

Experiments in the chick, where the proepicardium was removed or PE-cell adhesion to the myocardium was prevented, demonstrated that correct development of the myocardium requires the epicardium. In the absence of a healthy epicardial layer, the myocardium develops as a thin compact layer, and the development of the atrioventricular (AV) valves and intraventricular septum is impaired[50-52] (Fig. 1d). Defective epicardium also leads to a bulging of the ventricular myocardium, suggesting that the epicardium controls the passive mechanical properties of the myocardial wall.[52] The effects of epicardium ablation in chicks are similar to the phenotype of *Wt1* null mutant mice, which have a thin myocardial wall, impaired AV valve development and incomplete intraventricular septation.[21,22]

There is evidence that epicardium-derived secreted molecules signal to the underlying myocardium. *FGF9*, *16* and *20* are expressed in the epicardium and myocardium-specific inactivation of FGF receptors 1 and 2 leads to defects in cardiomyocyte development.[53] Epicardial-derived RA triggers a signalling cascade that upregulates myocardial FGF2, thereby leading to cardiomyocyte proliferation.[19] EPDCs are also a source of paracrine signals for cardiomyocytes. Intermyocardial fibroblasts, which are at least partly epicardially derived, promote cardiomyocyte proliferation during development.[54]

Role of the epicardium during heart regeneration

The role of the epicardium during myocardial regeneration has attracted growing interest within the scientific community in recent years. In a zebrafish heart regeneration model, resection of the ventricular apex leads to complete regrowth of the amputated region, including the coronary vasculature, myocardium and endocardial tissues.[55] Up to 20% of the heart can be regenerated in this species. The epicardium has been suggested to play a role during heart regeneration in zebrafish.[56] Amputation of heart tissue induces a strong expression of epicardial marker genes and vascular endothelial precursor cells accumulate at the amputation site. To date, it is unclear whether the epicardium contributes in a paracrine manner, stimulating myocardial cell proliferation, or as a cell source, providing EPDCs that populate the regrowing myocardial wall. Studies of the epicardium in the zebrafish suggest that EPDCs are incorporated into the heart not only during regeneration, but also during continuous growth of the adult heart, since DiI-labelled epicardial cells are incorporated into the compact layer of the myocardium.[57] The supportive action of the epicardium during regeneration is not exclusive to teleosts; it has also been reported in mammals. Cells positive for the stem cell markers c-kit and CD34 have been detected in the subepicardial space of foetal and adult human hearts.[58] In culture, these cells can acquire myocardial marker gene expression and an endothelial phenotype (Fig. 1e"). In a mouse myocardial infarction model, c-kit-positive and CD34-positive cells in the subepicardial space proliferate and differentiate into myocardial, endothelial and smooth muscle cell lineages;[59] further, human epicardial cells also can differentiate *in vitro* into smooth muscle cells.[59] Moreover, myocardial infarction in mice reactivates *Wt1* expression in the epicardium and coronary vessels;[60] suggesting that tissue damage in adult animals reactivates the developmental gene regulatory network. In a recent study, transplantation of human EPDCs into a mouse heart after coronary artery occlusion resulted in improved cardiac function, demonstrating that there is a genuine therapeutic potential for EPDCs to restore heart function after myocardial infarction.[61] However, while hEPDCs were clearly incorporated into the heart, it is uncertain as to which exact cell type they gave rise to.

The potential for ectopic factors to promote EPDC migration, or proliferation, has also been investigated. Administration of *Tβ4* enhances epicardial marker gene expression and slightly improves capillary formation at the infarction border zone in adult mice following coronary artery ligation.[62] The effect of FGF and VEGF administration on angiogenesis after myocardial ischemia-reperfusion injury are also being tested (reviewed in ref. 63). In addition, activation of Hedgehog signalling in mice or *Shh* gene therapy promotes neovascularisation and protects against ischemic injury in the adult heart.[64,65]

Future Perspectives

Given that regeneration mechanisms in a mature organ generally recapitulate the developmental processes of its formation, understanding the developmental mechanisms that underlie the formation of the epicardial layer, trigger EMT by EPDCs and drive their differentiation into diverse cell types will be crucial for the development of strategies to manipulate these events in adults. Functional genetic studies and lineage tracing experiments in animals have been hampered by the lack of a suitable epicardial-specific enhancer. To date, most studies have relied on reporter lines that are not exclusive to the epicardium, thus making interpretation of the results challenging. Identification of a more

suitable enhancer will therefore assist greatly. In light of the differences between chick and mouse EPDC lineages, it will be interesting to analyse the contribution of EPDCs to the growing heart in other species, such as teleost fish. This will also clarify whether the extraordinary regenerative capacity of the teleost heart is reflected in the differences in the composition, or responsiveness to injury, of their epicardium or EPDCs. In suitable models, such as zebrafish, in vivo imaging of the processes through which PE cells attach and spread over myocardial tissue will provide novel insights and help to confirm in vitro findings. Small molecule screens to find new factors involved in epicardial development might lead to the discovery of pharmacological compounds with therapeutic applications for the ischemic heart.

It is important to continue efforts to define ways to promote neovascularisation by mobilising resident cells or by administering cells or factors ectopically. However, off-target effects of administered factors need to be carefully examined to exclude deleterious consequences. It will also be important to promote the replacement of damaged tissue by newly formed myocardium that is functionally coupled to the rest of the myocardium. In the context of the immense efforts being made to identify resident stem cells in the adult heart,[66] it seems appropriate to consider the epicardium as another potential source of pluripotent cells.

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