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The epicardium as a candidate for heart regeneration

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Abstract

The mammalian heart loses its regenerative capacity during early postnatal stages; consequently, individuals surviving myocardial infarction (MI) are at risk of heart failure due to excessive fibrosis and maladaptive remodelling. There is an urgent need, therefore, to develop novel therapies for myocardial and coronary vascular regeneration. The epicardium-derived cells (EPDCs) present a tractable resident progenitor source with the potential to stimulate neovasclogenesis and contribute *de novo* cardiomyocytes. The ability to revive ordinarily dormant EPDCs lies in the identification of key stimulatory factors, such as T β 4, and elucidation of the molecular cues used in the embryo to orchestrate cardiovascular development. MI injury signalling reactivates the adult epicardium; understanding the timing and magnitude of these signals will enlighten strategies for myocardial repair.

Keywords

Epicardium; EPDCs; neovascularisation; myocardial regeneration; cardiac development; epicardial signalling; injury signalling; Thymosin β 4

Introduction

While cardiovascular diseases (CVD) remain the major worldwide cause of mortality and morbidity, there is an urgent need to tackle the clinical and economic burden of heart failure. In the United Kingdom alone, CVD kills over 191,000 individuals each year, with coronary heart disease (CHD) causing the majority of deaths; this costs the UK economy over £14 billion *per annum* (British Heart Foundation website⁽¹⁰¹⁾).

Since the mammalian heart is unable to adequately regenerate beyond early postnatal stages⁽¹⁾, individuals surviving acute myocardial infarction (AMI) are at risk of heart failure and dysrhythmia due to the replacement of lost muscle with a non-contractile scar, fibrosis and ensuing maladaptive remodelling. Despite improved management of acute coronary syndromes using drugs such as statins, beta blockers and ACE inhibitors⁽²⁾, there is currently no treatment to incite cardiac regeneration. Thus, the only possible cure for patients with end-stage heart failure is transplantation, which is fraught with issues around limited donor hearts and host immune rejection. Therefore, as a fundamental mechanistic strategy to treat heart failure, new approaches are sought towards restoring damaged heart muscle tissue, through repair and regeneration.

The prospects for utilising gene therapy towards improving angiogenesis, inhibiting fibrosis, limiting cell death, modulating cardiomyocyte contractility as well as reactivating the cardiac cell cycle are promising and consequently widely pursued, with particular attention towards overcoming issues of viral vector immunogenicity and risk of insertional mutagenesis⁽³⁾. However, since a typical human infarct results in the loss of approximately one billion cardiomyocytes⁽⁴⁾, the seemingly impossible challenge of restoring an equivalent

complement has focussed efforts on the identification of new cellular sources for cardiomyocyte replacement (reviewed in⁽⁵⁾). A number of embryonic and adult cell types have been explored in both preclinical and patient trials but, due to modest outcome, impaired graft survival and limited trans-differentiation, attention has turned to the identification of tractable progenitor populations that reside within the adult heart. A contemporary paradigm in regenerative medicine is that tissue repair in the adult is frequently underpinned by a re-activation of the embryonic programme that created the tissue in the first instance. As such there is much to gain from understanding the embryonic mechanisms of vasculogenesis and cardiogenesis. The success of this approach lies in the identification of a tractable progenitor cell population and the development of appropriate strategies for their redeployment in the adult, based on defined embryonic roles. Due to their fundamental role in heart development, the epicardium-derived cells (EPDCs) have emerged as a population that fulfil this remit and have come under intense scrutiny as a new source for myocardial regeneration. A prerequisite for utilising EPDCs in this regard is the identification of factors to reactivate this ordinarily dormant reservoir of cells in order to exploit their restorative power. Reactivation of the adult epicardium was first revealed using the actin-monomer binding protein Thymosin β 4 (T β 4) and subsequently shown by the venom-related protein family member prokineticin-2. However, until large-scale small molecule screens yield further insight, we are limited in our ability to pharmacologically stimulate the adult epicardium. Herein we review the potential of T β 4-activated EPDCs to contribute towards repair of the adult heart, with regeneration of the coronary vasculature as well as the myocardium. Since myocardial injury is itself sufficient to promote epicardial activation and neovascularisation and T β 4 appears to act synergistically to enhance the extent of repair⁽⁶⁾, we discuss the potential for injury-associated signals to reactivate the epicardium. While the intrinsic injury response is inadequate to fully repair the adult myocardium, an understanding of the signalling pathways that underlie epicardial activation should inform strategies to therapeutically realize a full-scale response, recapitulating development, towards myocardial regeneration via the targeting of adult EPDCs.

The Epicardium in Development and Disease

The developing heart receives cellular contributions from three main sources⁽⁷⁾. The cardiogenic mesoderm establishes the primitive heart tube, an avascular structure with myocardial and endocardial layers. As the myocardium increases in size and the heart transitions to a more complex multilayered organ, diffusion becomes unfeasible and the heart requires a dedicated vascular system⁽⁸⁾. Following looping of the heart tube, cardiac neural crest cells migrate from the dorsal neural tube to contribute vascular smooth muscle cells (VSMCs) to the great arteries and, at the same time, proepicardial precursors contact the surface, to form the epicardium^(9,10).

The epicardium gives rise to EPDCs, multipotent cardiac progenitors that were proposed by Wessels and Perez-Pomares in 2004 to be “true cardiac stem cells”⁽¹¹⁾, due to their potential to differentiate into endothelial cells (ECs)⁽¹²⁾, coronary VSMCs⁽¹⁰⁾, cardiac fibroblasts⁽¹³⁾, possibly AV cushion mesenchyme^(13,14),⁽¹²⁾ and cardiomyocytes⁽¹¹⁾. In addition to direct contribution of cardiac cells, the epicardium plays a critical role, via paracrine secretion of key signalling factors, in myocardial compaction, Purkinje fibre development and inhibition of endocardial EMT (epithelial-mesenchymal transition; reviewed in⁽¹⁵⁾).

That EPDCs give rise to VSMCs, pericytes and fibroblasts is widely accepted⁽¹⁶⁾, however, contribution to the cardiomyocyte and endothelial lineages is more divisive. While recent studies proposed an epicardial origin for a proportion of cardiomyocytes^(17,18), both in development and disease, has been challenged⁽¹⁹⁾⁽²⁰⁾. A number of factors have confounded a consensus, including the heterogeneity of the epicardium and the paucity of

suitable epicardium-specific markers and EPDC cre-expressing strains for conventional lineage tracing. Generic caveats of cre-based lineage tracing notwithstanding^(21,22), those reported for epicardial fate mapping have to date have utilised cre knocked in to either the *Tbx18*⁽¹⁸⁾ or *Wt1*⁽¹⁷⁾ locus or a *Gata5*^{cre} transgenic strain⁽²³⁾. The latter strain is potentially compromised for use in lineage tracing due to the possibility of ectopic activation within the myocardium. Of the knock-in epicardial lines, the *Tbx18*^{cre} is compromised by the fact that *Tbx18* is expressed in cardiomyocytes between E10.5 and E16.5⁽¹⁹⁾ ⁽²⁴⁾. In a direct comparison with the *Wt1*^{cre}, Zeng and colleagues excluded *Wt1* expression in cardiomyocytes⁽²⁴⁾, establishing the basis for its use in a number of studies to date^(17,25,26,27,28). The embryonic study utilising this trace demonstrated that *Wt1*+ progenitors differentiated into fully functional cardiomyocytes that comprised up to 10% of ventricular and 18% of atrial cardiomyocytes in the developing heart ⁽¹⁷⁾. Contrary to this, lineage tracing in zebrafish failed to demonstrate a contribution of epicardial (*tcf21*⁺) cells to the myocardium of either the embryonic or injured adult heart⁽²⁹⁾. Whether this disparity reflects species variance including the unique ability of more primitive zebrafish cardiomyocytes to dedifferentiate and proliferate following injury, or the differential contribution of heterogeneous sub-populations of epicardial cells, identified by distinct markers, remains to be resolved.

Additional problems arise when lineage tracing in the adult since expression of most known epicardial genes is restricted to embryonic stages and silenced in the adult. Thymosin β 4, combined with myocardial injury, was exploited to enable adult epicardial reactivation, *Wt1* re-expression and the use of constitutive (*Wt1*^{GFPCre/+} knock-in) and conditional (*Wt1*^{CreERT2/+} knock-in) epicardial cell tracking in the adult⁽²⁸⁾. With this approach, a modest contribution of cardiomyocytes from the adult epicardium was observed⁽²⁸⁾, as discussed in full below.

Equally unresolved is the origin of the coronary endothelium ; formerly thought to originate from the epicardium^(30,12), it is increasingly argued that very few, if any, are (pro-)epicardium-derived ^(31,32,33). For a detailed recent discussion, please refer to ⁽²²⁾. At least in mouse, coronary vessels have been shown to arise from angiogenic sprouts of the sinus venosus, with a secondary contribution from the endocardium ⁽³²⁾. As they traverse a stereotyped path through the myocardium, sinus venosus cells dedifferentiate with a switch from venous (*EphB4*+) to arterial (*ephrinb2*+) fate; vessels that invade the myocardium develop into mature arteries while those that remain superficial re-express venous markers and give rise to veins. Regardless of origin, endothelial precursors within the subepicardial space migrate over the heart in the same direction as epicardial growth (in an anterior and ventral progression). Following migration, endothelial precursors coalesce to form a primitive vascular plexus which undergoes extensive branching to form the mature coronary tree, following connection with the aorta ⁽³⁴⁾. Upon perfusion, capillaries are remodelled into larger vessels via vascular wall matrix enrichment and recruitment of VSMCs and pericytes from the epicardium (arteriogenesis)⁽³⁴⁾.

Reciprocal paracrine signalling between the myocardium and epicardium is important throughout development⁽³⁵⁾; epicardial-derived signals direct proliferation of the underlying cardiomyocytes and prepare the epicardium for EMT (e.g. ⁽³⁶⁾) while signals from the myocardium serve to induce epicardial EMT (e.g. ⁽³⁷⁾), directing the differentiation of the subepicardial mesenchyme into cardiomyocytes and components of the coronary vasculature. The epicardium may continue to nurture the myocardium beyond foetal life with evidence for myocardial homeostasis in the zebrafish⁽³⁸⁾ and the demonstration that the differentiated phenotype of adult rat ventricular myocytes, including appropriate sarcomeric organisation and contractile function, is preserved upon coculture with epicardial cells;

interestingly, this study reveals an epicardial role beyond paracrine stimulation since long term maintenance required direct epicardial-myocardial contact⁽³⁹⁾.

While epicardial-myocardial signalling has not been extensively studied following myocardial injury, a number of preliminary observations suggest that the epicardium responds by transmitting and receiving signals, extending its embryonic role, and may influence the outcome of MI. Valuable insight into the epicardial response may be derived from studies in zebrafish. Following resection of the adult fish heart, the epicardium exhibits a rapid and robust response to injury, which includes the re-expression of embryonic epicardial markers, *Tbx18* and *Raldh2* and proliferation of EPDCs within 1-2 days of resection⁽⁴⁰⁾. The activated epicardium envelopes the cardiac chambers, including the injured apex, and a subpopulation of cells invades the sub-epicardial space and myocardium to contribute endothelial and VSMCs to form new coronary vessels; this is an exact recapitulation of the processes involving the mammalian epicardium during development of the coronary vasculature. Consistent with this notion, microarray analysis of regenerating fish hearts revealed up-regulation of genes encoding several key secretory factors implicated in coronary development, including *vegfc*, *pdgf-a*, *pdgf-b* and *Tβ4*⁽⁴¹⁾, along with the receptor for PDGF-B, *Pdgfrβ*⁽⁴²⁾. Inhibition of PDGF signalling in this context impaired epicardial cell and cardiomyocyte proliferation and inhibited EMT and coronary vessel formation^(42,41). The epicardium, as well as the endocardium in the regenerating zebrafish, is a dynamic injury-responsive source of potent myocardial mitogens, such as retinoic acid which stimulates myocardial regeneration via the dedifferentiation and proliferation of cardiomyocytes^(43,44). While comparatively meagre in their ability to regenerate, recent studies point to a retention of the epicardial activation mechanism, albeit diminished, in mammals^(28,26). Epicardial thickening in response to MI has recently been shown to act as a source of trophic paracrine factors which condition the underlying myocardium for repair in an analogous manner to nurturing myocardial growth during development⁽²⁶⁾. Indeed, it has been speculated that epicardial activation and thickening may underlie the human phenomenon observed in Dressler syndrome, a complication of MI and cardiac surgery, in which inflammation of the epicardium and pericardium occur in response to injury, associated with chest pain and fever⁽⁴⁵⁾. Far from simply being an immune-mediated response, it may reflect an evolutionarily-conserved mesothelial activation with the potential to contribute to repair. Although difficult to define the impact of epicardial reactivation on the outcome of MI injury, it may be significant that the subepicardium is the main region of myocardial salvage post- MI and infarct repair initiates earlier than in the mid-myocardium⁽⁴⁶⁾. While this may be attributable to its higher residual blood flow, compared with the mid-myocardium and sub-endocardium, it is possible that faster repair and larger myocyte mass may result from the delivery of both pro-survival and trophic paracrine factors to the myocardium as well as the contribution of myocardial and vascular precursors from the epicardium. In support of this, transplantation of human adult EPDCs into the ischaemic mouse myocardium improved cardiac function and attenuated adverse remodelling⁽⁴⁷⁾.

Application of Thymosin β4 to Harness the Regenerative Potential of the Epicardium

Lacking the intrinsic regenerative capacity of fish, the mammalian epicardium requires a helping hand to achieve even a modest improvement in myocardial regeneration. The potential for so inducing the adult epicardium was first revealed in rodent cardiac explant studies when Tβ4 was shown to stimulate EPDC migration, restoring the quiescent adult epicardium to its pluripotent embryonic state to enable the derivation of vascular progenitors⁽⁴⁸⁾. As well as inducing epicardial markers in the adult, Tβ4 treatment was

associated with the induction of FGF17, FGFR-2, FGFR4, VEGF, Flk-1, TGF β and β -catenin, factors that promote EMT, in the epicardium⁽⁴⁹⁾. These studies represent a significant milestone towards translation of the developmental role for T β 4 to that of myocardial regenerative therapy.

Epicardial Potential for Neovascularisation

To date, attempts to develop therapies to promote neovascularization in the adult heart have met with limited success. The potential of EPDCs for repair was extrapolated to determine the ability of T β 4 to promote neovascularization in a murine MI model⁽⁶⁾. T β 4-treated, infarcted hearts revealed dramatic EPDC proliferation and large numbers of ECs and VSMCs in the expanded sub-epicardial space which assembled to form a capillary network. Compared with a modest degree of VSMC recruitment in vehicle-treated hearts, extensive VSMC migration and differentiation and a significant increase in the number of perfused, functional VSMC-lined arterioles was observed following T β 4 treatment. Arteriogenesis may therefore explain the beneficial effects of T β 4 treatment post-MI, beyond the relatively unstable and grossly inadequate endogenous capillary response.

T β 4-activated EPDCs as a source of *de novo* cardiomyocytes

Just as the myocardium and coronary vasculature develop simultaneously in a coordinated manner, it is highly desirable, therapeutically, to reinstate all damaged components concurrently within the diseased adult heart. Indeed, attempts to replenish muscle in an ischaemic environment are futile. The epicardium, by contributing to the myocardium and coronary vasculature in the embryo offers a unique target for co-ordinately stimulating myocardial and coronary vascular repair. From the demonstration of a significant epicardial contribution to the cardiomyocyte lineage came a basis for translating myocardial potential into the adult heart⁽¹⁷⁾. The ability of T β 4 to reactivate the adult epicardium was exploited in order to “prime” adult Wt1^{GFP^{Cre}/+} and Wt1^{CreERT2/+} knock-in mice and reactivate *Wt1* expression (a key epicardial marker) to provide both constitutive (GFP+) and pulse- (YFP+) labelling of EPDCs, respectively. Thus, reactivated Wt1-driven reporter expression enabled the tracking of the spatiotemporal distribution of epicardium-derived cardiomyocyte precursors through to mature myocytes⁽²⁸⁾. This approach revealed YFP+ EPDCs, including cardiac progenitors, migrating from the epicardium (days 2-7) and, by day 14 post-MI, larger YFP+ cells, co-expressing sarcomeric markers and morphologically resembling mature cardiomyocytes, within the myocardium. Importantly, the *de novo* cardiomyocytes were appropriately integrated with resident myocardium via both adherens’ and gap junctions. Moreover, functional integration of YFP+ cardiomyocytes with existing myocardium was demonstrated upon measuring [Ca²⁺]_i transients with neighbouring YFP-cardiomyocytes. MRI analyses over a 28 day time course after MI revealed T β 4 treatment resulted in significant improvement in ejection fraction and end diastolic/systolic volumes alongside beneficial changes in infarct/scar volume with increased left ventricular mass over time consistent with replenishment of myocardium. Despite the beneficial outcome, a limitation of this approach is the requirement to pre-treat (“prime”) with T β 4 prior to injury which, from a translational perspective, would require the identification of patients considered at risk of MI. No cardiomyocyte replenishment from epicardial precursors was achieved without pre-treatment^(26,28), or when T β 4 was administered only after MI⁽²⁵⁾. Whether epicardial activation prior to injury is required to alter the subsequent fate of epicardial cells or whether it simply enhances the magnitude of response sufficiently, over and above the activation that occurs by injury alone, or in combination with T β 4 post-treatment, remains to be determined. Critically, however, the demonstration of myocardial regeneration via the epicardium paves the way for the discovery of novel compounds that more efficiently promote regeneration, whether given before or after injury. Replenishment of destroyed myocardium by activated EPDCs is a significant advance towards resident cell-

based therapy for acute MI in human patients. Thus, in addition to its pro-survival effects⁽⁵⁰⁾, T β 4 can activate adult epicardium to not only induce neovascularisation⁽⁶⁾ but promote myocardial regeneration⁽²⁸⁾ to restore a functional vasculature, maintain cardiomyocyte survival and replace lost muscle in the injured heart.

Myocardial Injury Signalling: Possible Interaction with the Epicardium

The myocardium responds to stresses by activating a multitude of adaptive mechanisms to limit cellular injury and to regenerate, as much as possible, the damaged tissue; however, the extent to which these processes can be induced intrinsically varies considerably between species. As described above, the zebrafish possesses an unparalleled capacity for regeneration, the like of which is lost in mammals beyond the earliest post-natal stages⁽⁵¹⁾. In ventricular resection models, regeneration of both fish⁽⁴⁰⁾ and neonatal murine hearts⁽⁵¹⁾ is underpinned by reactivation of the epicardium. While regeneration in these models can be achieved by a prevailing dedifferentiation and proliferation of existing “primitive” (mononuclear) cardiomyocytes, regeneration of the adult mammalian heart depends upon activating resident precursors and injury alone affords minimal regenerative impetus. However, injury signalling activates the epicardium in the adult mouse, to initiate a neovascular response which correlates with the extent of injury⁽⁶⁾. The molecular identity of injury signals and the mechanisms by which they activate the epicardium are currently unknown but a number of candidate pathways have emerged (Figure 1). Understanding the timing and magnitude of myocardial-epicardial signals, based upon defined embryonic roles and recapitulation in the injury setting, will enlighten strategies to direct epicardial EMT and proliferation and to influence EPDC fate. Collectively, enhancing positive signals and inhibiting repressive stimuli to achieve maximal activation alongside balancing differentiation factors to shift EPDC fate from predominantly fibroblast towards an adequate quota of cardiomyocytes and vascular precursors may enable the benefits of epicardium-based therapy to be realised.

After onset of MI, the left ventricle undergoes a succession of molecular, cellular, and extracellular changes that culminate in wall thinning, dilation, and fibrosis. Although a degree of fibrotic healing is necessary to prevent cardiac rupture, maladaptive remodelling hinders regeneration, promotes excessive dilation and dysfunction and ultimately leads to heart failure. Broadly speaking, the cardiac healing process consists of four overlapping phases: (1) cardiomyocyte death; (2) infiltration of monocytes and lymphocytes into the necrotic myocardium for the removal of dead cardiomyocytes; (3) formation of granulation tissue, characterized by the presence of fibroblasts, macrophages, myofibroblasts, new blood vessels, and extracellular matrix (ECM) components; and (4) collagen scar formation. Endothelial activation and injury, in response to ischaemia, increase vascular permeability and invasion by inflammatory cells, notably neutrophils, and platelets. Moreover, up-regulation of soluble factors such as SDF-1 stimulates the mobilization of bone marrow progenitor cells and their homing to the myocardium⁽⁵²⁾. Paracrine trophic factors, secreted from injured and border zone myocardium, as well as the vasculature, the infiltrating cells and transformed ECM components (Table 1) recapitulate much of the developmental myocardial signalling that regulates EPDC EMT, migration and differentiation and likely underlie intrinsic epicardial reactivation that occurs following injury.

Fibroblast Growth Factors

Reciprocal FGF signalling between the epicardium and myocardium is critical for coronary vessel and myocardial development. FGF receptor 1 (FGFR1) is expressed in the epicardium and is up-regulated in response to myocardial FGF. FGF-FGFR1 signalling appears sufficient but not necessary to promote epicardial EMT; it is, however, required for EPDC invasion of the myocardium. Moreover, FGF signalling has been shown to influence the

differentiation and remodelling of the subepicardial mesenchyme with FGF-2 shown to induce endothelial cells in avian heart⁽⁵³⁾, and promote angiogenesis. In return, FGFs constitute one of the epicardial signals that induce myocyte proliferation. A number of FGF family members are expressed in the epicardium including FGF-1, -2, -4, -9, -16, and -20 (reviewed in ⁽⁵⁴⁾) and expression of FGF receptors (FGFR1 and FGFR2c) renders embryonic cardiomyocytes competent to receive proliferative FGF signals.

Following MI, FGF-1 becomes up-regulated, particularly in inflammatory cells within the border zone of infarcted rat myocardium; elevated protein levels peak at 7 days and subside by 14 days⁽⁵⁵⁾; FGF-2 gene expression is significantly elevated for the full 14 days post-MI and is primarily produced by endothelial cells. It is relevant, therefore, that FGF receptor expression increased at day 3 and remained elevated in the infarcted myocardium and overlying epicardium⁽⁵⁵⁾. Myocardial FGF17b and epicardial FGFR2 and FGFR4 have been proposed to regulate epicardial EMT during regeneration post ventricular resection in adult zebrafish⁽⁴⁰⁾. Although FGF17, the murine homologue, is not expressed during development⁽⁵⁶⁾, its expression post-MI in the sub-epicardium is enhanced by T β 4 treatment⁽⁴⁹⁾. Myocardially secreted FGF-1⁽⁵⁷⁾ and FGF-2 levels⁽⁵⁸⁾ in the pericardial fluid of MI patients were found to correlate with the severity of ischaemia; moreover, the finding of higher FGF-2 in patients with unstable angina than in patients with non-ischaemic heart disease infers an important role in mediating collateral growth in humans. While a role for the epicardium in this process is highly speculative, the changes post-MI are reminiscent of development and would facilitate reactivation.

Vascular Endothelial Growth Factors

The VEGF family, comprising five ligands (VEGF-A, -B, -C, -D, and placental growth factor) with multiple splice isoforms and three receptors (VEGFR1/Flt-1, VEGFR2/Flk-1/KDR, and VEGFR3/Flt-4), plays multiple roles in the development of the coronary vasculature⁽⁵⁹⁾. VEGF-A, VEGF-B, and VEGF-D are expressed in cardiomyocytes, while VEGF-C is expressed in pericytes⁽⁶⁰⁾ and their receptors are expressed in the epicardium and subepicardium. Myocardial to epicardial VEGF signalling appears to induce epicardial EMT and subsequently induces coronary EC proliferation, migration and tube formation in conjunction with FGF-2⁽⁵³⁾.

Mediated by HIF-1 α , myocardial hypoxia leads to the up-regulation of the same cohort of pro-angiogenic growth factors that are implicated in developmental coronary vasculogenesis, including VEGF and its receptors, flk-1 and flt-1 ⁽⁶¹⁾. Following permanent LAD ligation in the rat heart, Vegf-a increases in the endocardium and cardiomyocytes of the border zone within 2 hours and remains elevated for two days⁽⁶²⁾. Moreover, VEGF isoforms are abundantly secreted from EPCs⁽⁶²⁾ and BMSCs⁽⁶³⁾. As well as potentially inducing coronary angiogenesis by stimulating ECs, VEGF signalling post-MI may contribute epicardium-mediated vasculogenesis to improve myocardial perfusion. The promising preclinical results obtained in animal studies have yet to be translated into man. The largest phase II VEGF trial (VIVA), involving 178 patients with coronary artery disease (CAD), recorded no significant effect in the primary end point of treadmill exercise capacity at four months ⁽⁶⁴⁾. Lack of efficacy in trials of therapeutic angiogenesis may be explained by the lack of concomitant arteriogenesis; capillaries that form by vasculogenesis, without smooth muscle (SM) support are unstable and regress over time.

Angiopoietin Signalling

Angiopoietins represent a family of endothelial growth factors expressed in the myocardium which act on the receptor, Tie-2, to direct coronary vessel development ⁽⁶⁵⁾. During development, Ang1 is highly expressed in the myocardium and signals to Tie2 in both the

epicardium and endocardium. Ang1 levels are critically regulated during development for appropriate coronary vasculogenesis and angiogenesis⁽⁶⁵⁾, with an excess or deficiency equally detrimental. The other major ligand for the Tie2 receptor, Ang2, also influences coronary development, with evidence that it behaves as a weak Tie2 agonist acting synergistically with VEGF to increase capillary density⁽⁶⁶⁾. Cardiac Ang2 and Tie2 are up-regulated within the first 48 hours of reperfusion following MI in the rat⁽⁶⁷⁾ and Ang2 levels were found to be elevated in the serum of AMI patients. Recapitulating vascular development, elevated Ang2 and VEGF may act in synergy via the epicardium and existing coronary vasculature to facilitate revascularisation of the ischaemic myocardium.

Transforming Growth Factor β

TGF signalling inputs at multiple stages in coronary vasculature formation. TGF β 2 is the predominant isoform expressed in the embryo during early epicardial and coronary vascular formation. TGF β 2 transduces its signals by interacting with heteromeric complexes of a type I and a type II TGF β receptor (TBRI and TBRII, respectively), and occasionally TBRIII. The TGF β superfamily ubiquitously regulates EMT in multiple tissues. TGF β induces loss of epithelial cell character in epicardial cells via TBRI, p38 MAP kinase and p160 rho kinase to modulate angiogenesis⁽⁶⁸⁾ and promotes VSMC differentiation. Downstream of this pathway, transcription of hyaluronan synthase 2 produces the ECM component hyaluronic acid (HA) in the epicardium to induce EPDC invasion and differentiation, via the hyaluronate receptor (CD44)⁽⁶⁹⁾. TGF β -mediated regulation of epicardial EMT is additionally imposed via myocardial TGF β -stimulated phosphorylation of the BMP receptor, ALK2, and smad-mediated transcriptional changes⁽⁷⁰⁾. Culturing human adult epicardial cells in the presence of TGF β induced EMT and SM differentiation via the ALK5 receptor and coincided with down-regulation of WT1, a marker of undifferentiated EPDCs, and vascular cell adhesion molecule (VCAM-1)⁽⁷¹⁾. VCAM-1 and WT1, therefore, present additional targets for therapeutic induction of epicardial cell-based regeneration. Reverse TGF β signalling from the epicardium also participates since TGF β ligands are expressed in the epicardium and the compact myocardium throughout coronary development, as is TBRIII and, while signalling via this receptor may negatively regulate epicardial EMT, it appears to promote invasion into the subepicardial and myocardial compartments⁽⁷²⁾.

Following MI, TGF β isoforms are up-regulated and, via their potent effects on the epicardium, are capable of mediating critical roles in cardiac repair. However, TGF β is additionally pro-fibrotic and pro-inflammatory in this setting⁽⁷³⁾; TGF- β 1 expression post-MI increases fibroblasts and collagen fibrils⁽⁷⁴⁾ and elevated TGF- β expression is associated with adverse remodelling during cardiac hypertrophy and heart failure. The epicardium, particularly in the ischaemic setting, is a rich source of fibroblasts; understanding the modifiers of TGF β signalling is therefore critical to therapeutically shift the balance of EPDC contribution towards myocardial and coronary vascular progenitors and away from fibroblasts, which, by and large, appears to be the default fate⁽²⁶⁾. High molecular weight HA stimulates epicardial cell differentiation *in vitro*⁽⁶⁹⁾. Due primarily to their pro-angiogenic and arteriogenic properties, various HA-based injectable hydrogels have been tested for their ability to regenerate the ischaemic heart⁽⁷⁵⁾. Application of HA proximal to the epicardium may engage EPDC activation to contribute towards repair and may present a means of diverting TGF β -induced EPDC fate away from the pro-fibrotic fibroblast phenotype.

Platelet-Derived Growth Factors

Platelet-derived growth factors (PDGFs) comprise four monomeric peptides, PDGF-A, -B, -C, and -D, that act as dimers in one of five forms: AA, AB, BB, CC, and DD, by binding transmembrane tyrosine kinase receptors. PDGF receptor (PDGFR) α binds all PDGF dimers

except PDGF-DD, whereas PDGFR β binds only -BB and -DD homodimers; both receptors are expressed in the epicardium^(76,77). PDGF signalling crucially supports epicardial EMT and possibly myocardial invasion, via PDGFR α -mediated WT1 repression⁽⁷⁸⁾, as well as PDGFR β -mediated Rho kinase and phosphatidylinositol-3 kinase pathway activation⁽⁷⁹⁾. PDGF signalling may additionally govern cell fate since activation of PDGFR α has been implicated in myocardial differentiation and fibroblast production whereas PDGF-BB/PDGFR β potently induces SM fate, in keeping with its requirement for VSMC and pericyte recruitment and differentiation in the coronary vascular bed.

Expression of PDGF-B, PDGFR α and PDGFR β was induced in reperfused mouse infarcts, with activation of PDGFR β signalling in perivascular cells within the infarct⁽⁸⁰⁾. The importance of this pathway in maturation of infarct vasculature was demonstrated by PDGFR β blockade, which resulted in enhanced capillary density; however, vessels were dilated, lacked mural cell support and displayed increased permeability to red blood cells and macrophages. Acquisition of a mural coat and maturation of the vasculature promotes resolution of inflammation and stabilization of the scar⁽⁸⁰⁾. PDGF-A and -D and PDGF receptors were significantly increased in rat myocardium, primarily in ECs, macrophages and myofibroblasts, to coincide with angiogenic, inflammatory and fibrogenic responses⁽⁸¹⁾. Inhibition of either PDGFR α or PDGFR β significantly decreased collagen deposition in the infarct⁽⁸⁰⁾. Improved myocardial repair may be achieved by therapeutic manipulation of receptor activation, particularly if due consideration is given to the epicardial component of PDGF signalling.

Increased plasma PDGF levels in AMI patients⁽⁸²⁾ may be contributed by increased activation of platelets or monocytes, two major sources of PDGF. Secretion of such factors has been utilised therapeutically with the development of autologous platelet gel, derived from concentrated platelets activated and induced to secrete stimulatory cytokines, growth factors, and chemokines. Gels provide a temporary and local hyperphysiological concentration of platelet secretory factors that may initiate adaptive myocardial healing⁽⁸³⁾. Local delivery of a combination of potent epicardial mitogens, by application of platelet gel to areas of cardiac injury may contribute towards the stimulation of myocyte regeneration, angiogenesis, and restoration of ECM composition.

Wnt Signalling

Wnt signalling, particularly via the canonical (β -catenin-stabilizing) pathway, is critical for heart development with expression of β -catenin in the epicardium required for myocardial proliferation⁽²³⁾ and epicardial EMT. Mice with epicardial-deleted β -catenin appropriately form a primitive capillary plexus in the subepicardial space and the coronary venous system remodels appropriately, however, the coronary arteries fail to recruit VSMCs suggesting a primary requirement in SM differentiation of EPDCs. Epicardial Wnt expression appears to be downstream of the RA pathway⁽²³⁾. A model has been proposed in which epicardial RA signalling activates FGF-2 expression, possibly indirectly via the myocardium, to induce epicardial Wnt9b expression and β -catenin activation to promote EPDC differentiation into coronary VSMC lineage for contribution to the coronary arterial tree⁽⁵⁴⁾. Disabled-2 (Dab2) is a TGF β receptor adaptor protein which interacts with two components of the β -catenin destruction complex, Dvl-3 and axin, and stabilizes axin to inhibition Wnt/ β -catenin-mediated signalling⁽⁸⁴⁾. miR-145 is down-regulated in the myocardium following acute MI and is associated with increased Dab2 expression to down-regulate Wnt/ β -catenin signalling in border zone cardiomyocytes⁽⁸⁵⁾. It would be informative to determine whether epicardial β -catenin is similarly repressed and whether this can be pharmacologically reversed in order for the epicardium to respond to myocardial FGF-2, as it does during development, to contribute towards arteriogenesis and repair. Indeed, T β 4 treatment enabled a modest

elevation of epicardial β -catenin expression over 24-72 hours post-MI⁽⁴⁹⁾, supporting the value of targeting epicardial Wnt signalling to facilitate repair.

Retinoic Acid

Retinoic acid signalling is critical for normal cardiogenesis, requiring tight control over its levels for appropriate embryonic development. RALDH2, the enzyme responsible for RA synthesis and retinoic acid receptors (RXRs and RARs) on the epicardium are required to non-cell autonomously promote cardiomyocyte proliferation and differentiation, ventricular maturation and coronary angiogenesis⁽⁸⁶⁾. Appropriate coronary vascular development requires the formation of a complex, hierarchical tree of coronary vessels within a provasculogenic environment before vessel structure is stabilized with recruitment of ECM-producing VSMCs. While elevated VEGF promotes endothelial differentiation and angiogenesis, a physiological delay in VSMC maturation is achieved by high levels of RA to continually repress SM fate, in the face of high myocardial serum response factor, TGF β and PDGF, strong promoters of VSMC differentiation⁽⁸⁷⁾. Following development of an extensive endothelial network, RA and VEGF levels decline, releasing the brake to allow EPDC differentiation into VSMCs. In recapitulating these embryonic mechanisms in the disease setting, it will be essential to reproduce the spatiotemporal and graded release of growth factors, rather than deliver a continuous sustained level of competing factors.

Following MI, RALDH2 is reactivated in mouse epicardium^(88,28) and, in fish, its organ-wide re-expression in both the epicardium and endocardium is indispensable for cardiomyocyte proliferation and regeneration⁽⁸⁹⁾. The lack of endocardial activation and poor intrinsic epicardial activation may, in part, account for the inadequate regeneration in mammalian hearts. Indeed, attempts to boost epicardial activation, for example using T β 4, increased *Raldh2* levels and facilitated myocardial regeneration⁽²⁸⁾.

Erythropoietin

The cytokine erythropoietin (Epo) similarly signals via its epicardially expressed receptor (EpoR) in the embryo, to both regulate vasculogenesis and angiogenesis⁽⁹⁰⁾ and to induce cardiomyocyte proliferation⁽⁹¹⁾. Here Epo functions cooperatively with RA to activate epicardial IGF2 which, in turn, stimulates compact zone growth⁽⁹²⁾. Epo was shown to protect the heart after MI by inducing angiogenesis and preventing cardiomyocyte apoptosis through sonic hedgehog signalling⁽⁹³⁾. In rats with heart failure, EPO increased VEGF expression predominantly in cardiomyocytes to induce a 37% increase in capillary density and improved cardiac performance⁽⁹⁴⁾ but it is unknown whether epicardial activation either directly, or via IGF2 or VEGF, mediates these effects.

Notch signalling

Notch signalling is implicated in establishing arterio-venous identity and in regulating endothelial-mural cell interactions⁽⁹⁵⁾. Activation of EC Notch induces arterial marker expression (ephrin B2, CD44 and neuropilin 1) and suppresses venous markers (Eph B4). Within the epicardium, Notch pathways regulate key cardiovascular cell fate decisions. Epicardial notch regulates arterial endothelium commitment and vessel wall maturation, being a crucial regulator of EPDC-SM differentiation, as well as myocardial growth^(96,33). Notch induces *Pdgfrb* expression in EPDCs and cooperates with TGF β signalling to induce SM genes.

The Notch pathway regulates heart regeneration in zebrafish⁽⁹⁷⁾ and is implicated, by virtue of its up-regulation, in repair of the mammalian heart⁽⁹⁸⁾. Using a powerful tool for identifying “Notch-activated” progenitors (CBF1-RE(x)₄-EGFP), Russell and colleagues revealed an abundance of Notch-activated epicardial-derived progenitor cells (NECs) within

the epicardium⁽⁹⁹⁾, a population which expanded following MI or thoracic aortic banding (a model of pressure overload hypertrophy and heart failure), consistent with reactivation of Notch signalling following MI and the up-regulation of Notch3 and Notch4 signalling in human heart failure⁽¹⁰⁰⁾. NECs were shown to secrete potent mitogenic and cardiogenic growth factors including Tβ4, PDGF-A, TGF-β3 and -2, BMP-1 and -4 and FGF-7, which can further potentiate epicardial activation. They contributed fibroblasts to the remodelling heart but, under appropriate conditions (cardiomyocyte coculture and engraftment into non-ischaemic heart), revealed a modest epicardial-cardiomyocyte differentiation potential⁽⁹⁹⁾. Thus, a dynamic Notch injury response activates adult epicardium, producing a multipotent cell population that contributes to repair. Fibrosis appears to be default programme, but the modest cardiomyocyte differentiation potential may be therapeutically exploited and preferentially enhanced over fibroblast differentiation. A current obstacle is the lack of understanding of the heterogeneity of epicardial cells; distinct populations have been identified based upon their activation stimuli e.g. Notch⁽⁹⁹⁾, Tβ4⁽²⁸⁾ or on expression of epicardial and progenitor marker e.g. WT-1⁽²⁸⁾ or c-kit⁽⁸⁸⁾ but the extent of overlap in marker profile, stimulatory factors and differentiation potential remains to be characterised and should inform efforts towards exploiting their regenerative potential.

Prokineticin

Prokineticins (PKs) are small, secreted peptides that signal through G protein-coupled receptors, Prokineticin receptor (PKR)-1 and -2⁽¹⁰¹⁾. In the heart, prokineticin-2 acts via PKR-1 to support coronary vessel development and protect cardiomyocytes after ischaemic injury⁽¹⁰¹⁾. Overexpression of PKR-1 in the mouse heart increases coronary capillary density, apparently increasing epicardial cell proliferation and potentially influencing their differentiation towards a vascular fate. Interestingly, PK2 and its receptors are elevated for 7 days following murine MI⁽¹⁰²⁾. PKR1 in mouse heart up-regulates its own ligand, PK2, which acts as a paracrine signal to promote vasculogenic differentiation of EPDCs⁽¹⁰¹⁾ and thus PK2 has emerged as a potential therapeutic agent in epicardial-based cardiac repair.

Extracellular Matrix Components

The ECM is a diverse and adaptable network of fibrillar and non-fibrillar proteins with critical signalling functions, regulating proliferation, adhesion and migration and provides mechanical support to enable coordinated cardiomyocyte contraction⁽¹⁰³⁾. ECM components include the basic structural proteins collagen and elastin, specialized proteins such as fibronectin, proteoglycans and matricellular proteins including osteopontin, thrombospondin-1/2, tenascin and periostin. While these proteins play a critical role during development, they are maintained at low level expression in normal adult tissue, but are markedly up-regulated during wound healing and tissue remodelling, including MI. The ECM impacts significantly on the epicardium, both during development and disease.

The subepicardial space, situated between the epicardium and myocardium, is rich in fibronectin, collagens (I, IV, V, VI) proteoglycans, laminin, vitronectin, fibrillin, elastin, tenascin-X and flectin⁽¹⁰⁴⁾ and their interaction with epicardial cells provide signals that induce EMT⁽¹⁰⁵⁾. α4 integrin represses EMT, invasion and migration, and additionally influences cell fate, being required for VSMC differentiation rather than the 'default' interstitial fibroblast phenotype in its absence⁽¹⁰⁵⁾. Similarly, the transmembrane glycoprotein podoplanin is required for coronary vasculogenesis and is proposed to stimulate EMT by negatively regulating E-cadherin. Subepicardial matrix components change as coronary vasculogenesis proceeds revealing its influence on vascular fate⁽¹⁰⁶⁾. The matrix is initially rich in vitronectin, then fibronectin is deposited to facilitate migration of EPDCs and endothelial precursors⁽¹⁰⁷⁾. Laminin deposition follows, which coincides temporally with endothelial tube formation, and subsequently collagens (type IV first,

followed by types I and III) are deposited⁽¹⁰⁸⁾. Fibulin is deposited by the epicardium as it migrates over the heart and is produced by ECs of budding coronary arteries⁽¹⁰⁹⁾.

These changes in matrix composition that occur during development are recapitulated in the adult heart in the extracellular matrix around the regions of active coronary collateral remodelling post-MI⁽¹¹⁰⁾. The response to MI generates distinct regions within the myocardium, with altered cellular environments⁽¹¹¹⁾, following deposition of plasma-derived proteins such as fibronectin and collagen in and around the site of infarction. Collagen, produced primarily by fibroblasts and myofibroblasts, is required to preserve ventricular integrity and prevent cardiac rupture. Osteopontin (OPN), thrombospondin-1/2 (TSP-1/2), tenascin-C/X (TNC/TNX) and periostin are among the matricellular proteins up-regulated following MI⁽¹¹²⁾. The deposition of these proteins proximal to the subepicardial space may contribute towards epicardial reactivation post-MI. Some EPDCs, at least those that, during development, contribute to the annulus fibrosus, were highly enriched for mRNAs encoding periostin, procollagen I, fibronectin I, vimentin and tenascin C⁽²⁷⁾. It remains to be determined whether this is a property shared by all EPDCs or only those that are required to synthesize structural components of the annulus fibrosus. The possibility that these synthetic properties may be (re)acquired would confer upon EPDCs the ability to remodel their immediate environment, to facilitate their inward migration and influence their differentiation.

TNC is normally only expressed in the chordae tendinae of the adult heart but becomes transiently up-regulated in the infarct border region during MI healing⁽¹¹³⁾ where it is suggested to accelerate adverse ventricular remodelling and fibrosis. Periostin, normally restricted to the valves of the heart, is strongly expressed in cardiac fibroblasts after MI. It binds to $\alpha v \beta 3$, $\alpha v \beta 5$ and $\alpha 4 \beta 6$ integrins, and transduces signals to facilitate EMT and migration⁽¹¹⁴⁾ and is critically required for myofibroblast recruitment and collagen fibre formation. Modulating $\alpha 4$ integrin signalling has been shown to alter EPDC migration and cell fate⁽¹⁰⁵⁾. Whereas periostin deficiency leads to increased incidence of cardiac rupture⁽¹¹⁵⁾, surviving periostin-deficient mice display reduced fibrosis and significantly improved cardiac performance⁽¹¹⁵⁾. Interaction of the epicardium with key matrix components such as periostin and TNC may, therefore, play a pivotal role in balancing cardiac remodelling with regeneration consequently the ECM may be targeted therapeutically to manipulate EPDC fate to achieve an optimal contribution of myofibroblasts, cardiomyocytes and vascular progenitors.

Pericardial Fluid

Several bioactive molecules released from the pericardium, epicardium and myocardium become concentrated within the pericardial fluid (PF) to affect epicardial cells, both physiologically and pathophysiologically. Many molecules have been identified in the PF of normal and diseased hearts, with their levels correlating with inflammation, collateral growth and cardiac remodelling⁽¹¹⁶⁾. Administration of PF into the pericardial cavity of non-infarcted mouse was sufficient to induce epicardial proliferation and embryonic gene expression⁽⁸⁸⁾. IGF1, HGF and HMGB1 were significantly elevated in PF from MI patients, factors that induce resident cardiac c-kit⁺ cells including, but not necessarily limited to, those progenitors within the epicardium that express c-kit. Hypoxia enhances pericardial mesothelial VEGF expression which, in addition to a demonstrated autocrine growth-regulatory role in pericardial cells, will likely signal to activate the adjacent epicardium⁽¹¹⁷⁾. Thus, PF contains trophic factors which might be harnessed to invoke epicardial activation and repair.

Conclusion and Future Perspective

The epicardial–myocardial signalling that directs coronary vasculogenesis and myocardial growth is complex, with multiple signalling pathways underlying each step and several pathways serving multiple roles. Additional complexity derives from the crosstalk, cooperation and feedback regulation implicit in such systems. The first critical step in coronary development involves the establishment of the epicardium and autocrine signalling (Epo and RA) for induction of cardiomyocyte proliferation and epicardial FGF secretion. FGFs not only stimulate cardiomyocyte proliferation but also induce other growth factors that signal back to the epicardium. Myocardial-epicardial signalling then directs epicardial EMT to generate EPDCs fated to become fibroblasts, cardiomyocytes, VSMCs and perhaps endothelial cells. The Shh-VEGF-Ang2 pathway may influence the generation of ECs, whereas PDGF, Wnt/ β -catenin, and TGF β promote differentiation of coronary VSMCs. Since the epicardium is effectively dormant in adulthood, the first critical step for engaging epicardial involvement in regeneration is its reactivation and restoration of pluripotency. To date, this has been achieved with T β 4, prokineticin and pericardial fluid. While the search is on-going for more potent compounds with which to reactivate adult epicardium, attention should also be focussed on understanding the intrinsic mechanisms that unfold during the injury response. The potential exists to not only exploit and enhance native signalling pathways to a threshold sufficient to induce meaningful repair but to disentangle detrimentally competing pathways. The ability to relieve the inhibition on pro-regenerative signalling may be sufficient to release the heart's own healing potential, which is clearly retained, to effect self-repair. A number of injury-induced factors promote EPDC EMT; their failure to do so to a sufficient extent may simply reflect the inability to attain the signal threshold for EMT induction or, equally likely, the presence of a competing signal that maintains epithelial state. Moreover, what potential exists within the adult mammalian epicardium requires therapeutic exploitation to derive maximal regenerative benefits. Beyond EMT the apparent heterogeneity of the adult epicardial lineage needs to be considered, which in turn likely affords alternate cardiovascular cell fates on sub-populations once activated. Epicardial activation in response to injury alone produces principally fibroblast derivatives which may contribute towards the prevailing fibrotic remodelling but fail to restore the remaining myocardial components. T β 4 increases vascular contribution⁽⁶⁾ to the ischaemic heart and even upholds the contribution of epicardium-derived cardiomyocytes⁽²⁸⁾; nevertheless the response is insufficient to realise significant myocardial regeneration. The potential for a localised response of the mammalian epicardium, as opposed to organ-wide (as in zebrafish), is also a key factor in terms of not only determining which progenitor cells become what, but where, and in response to which key signals along the proximal-distal axis relative to the site of injury. Understanding how injury signals or, as yet unidentified, novel compounds, modulate EPDC fate may enable the development of therapies to supplement a substantial proportion of vascular progenitors and cardiomyocytes to orchestrate a more complete myocardial restoration.

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Executive Summary

Clinical Problem

- Cardiovascular diseases are the major worldwide cause of mortality and morbidity; consequently there is an urgent need to tackle the clinical and economic burden of heart failure.
 - Coronary heart disease, leading to acute myocardial infarction (MI), is the primary cause of death.

The Need for Regenerative Therapies

- The mammalian heart is unable to adequately regenerate beyond early postnatal stages.
 - Myocardial healing post-MI consists of replacement of lost muscle with a non-contractile scar, fibrosis and (maladaptive) hypertrophic remodelling.
 - Transplantation is the only known cure, confounded by a lack of donor hearts and host immune rejection.
 - A number of embryonic and adult cell types have been explored for replacement of lost cardiomyocytes while angiogenic therapies have attempted to re-vascularise the ischaemic myocardium. Overall, such approaches have delivered meagre benefits.
 - Recent attention has focussed on the identification of a tractable resident progenitor population within the adult heart.
 - Understanding the embryonic mechanisms of vasculogenesis and cardiogenesis, as well as the mechanisms retained for regeneration in species such as the zebrafish, will inform on strategies for human myocardial repair.

The Epicardium as a Candidate for Regeneration

- Due to their fundamental role in heart development, the epicardium-derived cells (EPDCs) have emerged as a population with the potential to restore myocardium and coronary vasculature.
 - During development, EPDCs give rise to coronary vascular smooth muscle cells, pericytes, fibroblasts, up to 10% of the heart's cardiomyocytes and, potentially, a proportion of coronary endothelial cells.
 - A prerequisite for utilising EPDCs for regeneration is the identification of factors to reactivate this ordinarily dormant reservoir of progenitors.
 - Reactivation of the adult epicardium was first revealed using Thymosin β 4 (T β 4) and subsequently prokineticin-2; however, until large-scale small molecule screens yield further insight, we are limited in our ability to pharmacologically stimulate the adult epicardium.

Application of Thymosin β 4 to Harness the Regenerative Potential of the Epicardium

- T β 4 restores the quiescent adult epicardium to its pluripotent embryonic state
 - The ability of T β 4 to reactivate the adult epicardium was first revealed in cardiac explant cultures; T β 4 stimulates EPDC migration and subsequent differentiation into vascular and myocardial progenitors.
 - T β 4-treated, infarcted hearts revealed dramatic EPDC proliferation and a network of perfused, functional VSMC-lined new vessels.
 - The ability of T β 4 to reactivate the adult epicardium was exploited in order to restore *Wt1* expression (a key embryonic epicardial marker) in the adult lineage and demonstrate the contribution of epicardium-derived myocardial precursors and, subsequently, mature cardiomyocytes following MI. Importantly, the *de novo* cardiomyocytes were structurally and functionally coupled and may contribute towards the functional recovery of T β 4-treated hearts post-MI.

Myocardial Injury Signalling: Possible Interaction with the Epicardium

- The mammalian myocardium responds to stresses by activating adaptive mechanisms to limit cellular injury and to regenerate, as much as possible, the damaged tissue.
 - Myocardial injury is itself sufficient to promote epicardial activation and a degree of neovascularisation
 - While the intrinsic injury response is inadequate to fully repair the adult myocardium, understanding the timing and magnitude of myocardial-epicardial signals, based upon defined embryonic roles, will inform strategies to direct epicardial EMT and proliferation and to influence EPDC fate.
 - Enhancing positive signals and inhibiting repressive stimuli may lead to maximal activation alongside a shift in EPDC fate from predominantly fibroblast towards an adequate quota of cardiomyocytes and vascular precursors.
 - A number of candidate signalling pathways have emerged, which include:
 - Fibroblast Growth Factors
 - Vascular Endothelial Growth Factor
 - Angiopoietin Signalling
 - The transforming Growth Factor β family
 - Platelet-Derived Growth Factors
 - Canonical Wnt Signalling
 - Retinoic Acid
 - Erythropoietin
 - Notch signalling
 - Prokineticins

○ Extracellular Matrix Components

- Signalling components of these pathways are up-regulated and secreted from injured myocardium, or the reactivated epicardium. Additional factors that may potentiate activation of the epicardium may be derived from
 - Infiltrating inflammatory cells
 - Platelets
 - Damaged and activated vascular cells
 - Infiltrating fibroblasts
 - Progenitor cells mobilized from the bone marrow
 - Changes to the extracellular matrix

Therapeutic Perspective

- Being essentially dormant in adult, the first critical step for engaging the regenerative potential of the epicardium is reactivation and restoration of pluripotency.
 - The search is on-going for potent compounds with which to reactivate adult epicardium; starting with candidate signals which function to ensure epicardium development and contribution of cardiovascular cell types in the embryonic heart.
 - Understanding the intrinsic signalling mechanisms that activate the epicardium post-MI will inform the development of novel therapies.

Recognizing how injury signals or, as yet unidentified, novel compounds modulate EPDC fate should enable the therapeutic activation of myocardial regeneration over maladaptive remodelling.

Figure 1. Recapitulating Embryonic Signalling in the Ischaemic Heart

Following myocardial infarction, many of the reciprocal myocardial-epicardial signalling pathways that regulate coronary and myocardial development in the embryo are re-activated. The response to MI also generates distinct changes to components of the extracellular matrix (ECM), to recapitulate its role during development. Additional factors known to act on the embryonic epicardium are secreted from fibroblasts, activated vascular cells, infiltrating inflammatory cells and platelets, mobilised bone marrow-derived cells, including bone marrow mesenchymal stem cells (BMSCs) and endothelial progenitor cells (EPCs). The factors underlined indicate those in which embryonic expression is faithfully recapitulated (i.e. in the same cell type) following injury. Other listed factors are those which impact on the developing epicardium and are expressed following MI, but a signalling role from that cell type to affect the adult epicardium has not, to-date, been shown. Black arrows indicate signalling between cell types; blue arrows indicates differentiation of EPDCs into derivative smooth muscle cells, fibroblasts, cardiomyocytes and, possibly, endothelial cells.

Table 1
Epicardial Signalling in Development and Disease

A summary of the signalling pathway components involved in coronary and myocardial development which may contribute towards reactivation of the adult epicardium following myocardial infarction. BMSC: bone marrow mesenchymal stem cell; CM: cardiomyocyte; EC: endothelial cell; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; EPC: endothelial progenitor cell; EPDC: epicardium-derived cell; MI: myocardial infarction; PF: pericardial fluid; VSMC: vascular smooth muscle cell.

SIGNALLING FACTOR	DEVELOPMENTAL ROLE	RESPONSE FOLLOWING INJURY	REFERENCES
Fibroblast Growth Factors (FGFs)	FGF -1, -2 and -7; FGFRs -1 and -2 expressed in myocardium; FGF -1, -2, -4, -9, -16 and -20 and FGFR1 expressed in epicardium. Roles in EMT and invasion, as well as EC differentiation and myocardial proliferation.	FGF-1 ↑ in inflammatory cells and PF; FGF-2 ↑ in myocardium and PF; FGF-17 ↑ in myocardium and epicardium. FGF receptors -1 and -2 ↑ in epicardium.	[49]; [54]; [55]; [58]; [88];
Vascular Endothelial Growth Factor (VEGF)	VEGF-A, -B, and -D in cardiomyocytes, VEGF-C in pericytes; VEGF receptors expressed in epicardium and sub-epicardium. VEGF signaling induces epicardial EMT and EC proliferation, differentiation and angiogenesis.	↑ in myocardium, endocardium, PF, serum, BMSCs, EPCs and epicardium; Flk-1 ↑ in epicardium.	[49]; [59]; [62]; [63];
Angiopoietins	Myocardial Ang1 and Ang2 signal via epicardial Tie2 for coronary vasculogenesis and angiogenesis.	Ang2 ↑ in myocardium and serum; Tie2 ↑ in myocardium.	[65]
Transforming Growth Factor β (TGFβ)	TGFβ expressed in epicardium and compact myocardium; TGFβ receptors expressed in epicardium and myocardium; regulate EMT, invasion and VSMC differentiation.	↑ in myocardium, epicardium and platelets.	[49]; [54]; [83]
Platelet-Derived Growth Factors (PDGFs)	Signalling via epicardial PDGF-α and -β receptors regulates EMT and EPDC differentiation.	↑ in platelets, ECs, myofibroblasts, epicardium, mural cells, BMSCs.	[78]; [80]; [81]; [82]; [83]
Wnt/β catenin Pathway	Epicardial β-catenin critical for myocardial proliferation. Promotes EPDC differentiation to VSMCs.	β-catenin ↓ in myocardium but ↑ in epicardium following Tβ4 treatment.	[23]; [49]
Retinoic Acid	Synthesis in epicardium by RALDH2 critical for myocardial proliferation and coronary angiogenesis. Represses SM differentiation to allow Ec network branching.	↑ in epicardium; required for CM proliferation and regeneration in zebrafish.	[28]; [86]; [88]; [89]
Erythropoietin	Signals via epicardial receptor to induce vasculogenesis, angiogenesis and CM proliferation	Increases myocardial VEGF and capillary density; effect on epicardium unknown following injury.	[92]; [93]
Notch Signalling	Expressed in EPDCs to establish arterial versus venous identity. Regulates EPDC-VSMC differentiation	↑ in CMs and in some EPDCs (Notch-activated epicardial-derived progenitor cells).	[33]; [98]; [99]
Prokineticin (PK)	Myocardial PK2 acts via its epicardial receptor PKR-1 to support coronary vessel development.	PK2 and its receptors, PKR-1 and -2, ↑ following MI to induce vasculogenic EPDC differentiation and protect CMs.	[101]; [102]
Thymosin β4	Tβ4 myocardial-epicardial signaling critical for EPDC migration.	Tβ4 ↑ in CMs, EPDCs, EPCs, BMSCs. Reactivates adult epicardium; stimulates EPDC migration; promotes neovascularization and de novo CM production.	[6]; [28]; [48]
ECM Components	Interaction with ECM regulates integrin-mediated signaling, critical for EPDC EMT.	↑ collagen and ↑ periostin production by fibroblasts and epicardial cells; fibronectin, vimentin and tenascin C ↑ in epicardium; ↑ tenascin C in myocardium.	[18]; [104]; [112]