Thymosin β4 induces epicardium-derived neovascularization in the adult heart

Paul R. Riley1 and Nicola Smart
UCL-Institute of Child Health, 30 Guilford Street, London WC1N 1EH, U.K.

Abstract
The inability of the human heart to effectively repair itself after acute ischaemic injury has driven the search for efficacious means of promoting cardiac regenerative growth. Central to this has been the emergence of cell-based strategies to stimulate and augment both myocardial regeneration and neovascularization. Autologous cell transplantation of a variety of adult progenitor cells has been taken forward in clinical trials and, in parallel, investigators have begun to focus on the activation of resident cardiac cell populations as a means to stimulate endogenous repair. The latter approach depends on characterizing native progenitors with self-renewal, clonality, multipotency and arguably an analogous embryological counterpart. Recently, we have focused on adult EPDCs (epicardium-derived progenitor cells), which, when induced by the actin monomer-binding protein 1/4 (thymosin β4), are able to revert to their embryonic phenotype and give rise to endothelial cells and vascular smooth muscle cells ex vivo. Studies are ongoing to determine whether activated adult EPDCs can contribute to bona fide neovascularization in the injured adult mammalian heart proper, as a therapeutic means to support surviving cardiac muscle cells and sustain regenerating myocardium.

Introduction
Ischaemic heart disease resulting in MI (myocardial infarction) causes irreversible cell loss and scarring, which in turn predisposes to a second infarction event or maladaptive remodelling, myopathy and heart failure. As such MI is the major source of morbidity and mortality in humans. The adult human heart, as is the case in adult mammals in general, is unable to effectively repair itself after ischaemic injury and, as a result, there has been a recent, widespread focus on mechanisms to offer cardioprotection and regeneration post-MI. Approaches to mitigate the adverse consequences of MI include manipulation of cell death pathways to increase survival of remaining resident cardiac cells, influencing cell cycle re-entry to induce cell proliferation and re-differentiation and provision of both new cardiac muscle (de novo cardiomyocytes) and blood vessel cells (endothelial and smooth muscle cells) either via cell transplantation strategies (e.g. with bone marrow mononuclear cells, endothelial precursor cells or skeletal myoblasts) or via the stimulation of resident cardiac stem cells and/or progenitors (reviewed in [1]). In this regard, the generation of new coronary vessels, termed neovascularization, is an extremely important area of study since not only are de novo coronary vessels required to support the increased load on surviving muscle, thus protecting against further MI, but also an expanded vasculature is essential to replace lost vessels in the region of injury and to establish blood supply for regenerating muscle in tandem with strategies to introduce replacement myocardium.

Embryonic insight into neovascularization
The key signalling molecules and cell types that could play critical roles in inducing adult neovascularization are likely to be the same factors that contribute to coronary vessel development. That studies on the embryonic heart, including the identification of multipotent cardiovascular progenitors [2–4], might be instructive towards understanding how to manipulate an adult reparative response is a rapidly emerging paradigm in cardiovascular regenerative medicine. During embryogenesis the coronary vessels are derived from cells of the epicardium, an outer mesothelial layer of the developing heart, which is, itself, derived from a temporary structure of the heart tube has commenced to give rise to the epicardium [6]. These cells are referred to as EPDCs (epicardium-derived cells) and have been shown to give rise to a restricted proportion of coronary endothelial cells, VSMCs (vascular smooth muscle cells), perivascular and cardiac interstitial fibroblasts as well as, as more recently described, cardiomyocytes of the myocardial lineage [7–10].

Key words: cardiovascular regenerative medicine, epicardium, ischaemia, neovascularization, regeneration, thymosin β4.
Abbreviations used: EMT, epithelial–mesenchyme transition; EPDC, epicardium-derived progenitor cell; hEPDC, human EPDC; MI, myocardial infarction; Tβ4, thymosin β4; VSMC, vascular smooth muscle cell.

1To whom correspondence should be addressed (email p.riley@ich.ucl.ac.uk).
capillary plexus, subsequent angiogenesis, collateral growth and arteriogenesis on connection to the developing aorta (reviewed in [11]). The end product is an intact coronary vasculature in communication with the systemic circulation.

**Tβ4 (thymosin β4) is essential for coronary vessel development**

We recently revealed that Tβ4, a 43-amino-acid actin- monomer-binding protein known to regulate actin cytoskeletal dynamics, lamellipodia formation and directed cell movement, is essential for the migration of EPDCs from the epicardium into the underlying myocardium [12]. In a cardiac-specific, transgenic mouse shRNA (small-hairpin RNA) knock-down model for Tβ4, EPDCs were retained in the epicardium where they differentiated in situ and failed to give rise to coronary vessels. Coincident with failed vasculogenesis, we observed cardiomyocyte cell death and embryonic lethality at E14.5 (embryonic day 14.5). Since Tβ4 was evidently essential for epicardium-derived coronary vessel development, we explored whether it might be sufficient to activate the ordinarily quiescent adult epicardium, a lineage confined to the overriding notion of the adult heart as a terminally differentiated post-mitotic organ that lacks homoeostatic replenishment of existing muscle or vascular cells.

**Adapted adult epicardium-derived progenitors**

We extrapolated the developmental role for Tβ4 into the adult heart (Figure 1) by treating adult epicardial explants with synthetic Tβ4. Compared with a lack of response with vehicle alone, Tβ4 invoked a significant outgrowth of epicardin-positive EPDCs, which differentiated in culture into Flk1+ endothelial cells, aSMA+ VSMCs and procollagen I-positive fibroblasts. Thus Tβ4 (re-)activated the dormant adult epicardium, restoring embryonic pluripotency, such that EPDCs were able to undergo EMT, migrate away from the epicardium and differentiate into vascular precursors with the potential to contribute towards neovascularization of the adult heart.

**Adult EPDCs: a tractable lineage?**

That the adult epicardium may be even more tractable, and respond to multiple signalling pathways, has been demonstrated in subsequent studies both in rodents and humans. In the mouse, it was revealed that prokineticsins, related to venom-like proteins, implicated in sensory function, circadian rhythms and survival and differentiation of inflammatory cell lineages, can induce pluripotency of EPDCs and promote new vessel growth in the postnatal heart [13]. In humans, adult epicardial cells were shown to undergo EMT and obtain characteristics of smooth muscle cells in vitro in response to TGFβ1 (transforming growth factor β1) or BMP2 (bone morphogenetic protein 2), thus recapitulating, at least in part, the differentiation potential of their embryonic counterparts [14]. Furthermore, transplantation of hEPDCs (human EPDCs), by the same group, into infarcted mouse hearts preserved left ventricular function and attenuated pathological remodelling [15]. The mechanism of action following the injection of hEPDCs was not determined, but among candidate pathways suggested were protection against DNA damage and putative paracrine effects on resident cells, notwithstanding a neovascular response given the propensity of the transplanted cells to adopt an endothelial and smooth muscle cell fate in vivo [15]. The vascular commitment of hEPDCs was subsequently confirmed in a separate investigation on human fetal and adult epicardium from autopsy samples [16]. This study revealed the presence of distinct populations of c-kit+ and CD34+ cells, thus highlighting the heterogeneous nature of the adult epicardium; however, both subpopulations expressed early and late cardiac transcription factors and acquired an endothelial phenotype in vitro [16].

**Activation of the epicardium underpins zebrafish heart regeneration**

Compelling evidence of an evolutionary role for the epicardium in cardiac regeneration, and in particular neovascularization, has emerged from a significant body of work in the adult zebrafish (reviewed in [17]). The zebrafish possesses a unique capacity for cardiac regeneration, such that 2 months after resection of the ventricular apex the adult heart is fully regenerated [18]. The mechanism underpinning

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**Figure 1 | The adult epicardium and coronary vessels**

Histological section through the adult mouse heart. Shown in red are the epicardium, the outer cell layer and coronary vessels residing in the underlying myocardium (which is shown in blue). The vessels are expanded in size 2-fold for illustration purposes. Scale bar, 500 μm.
this regeneration is a dynamic organ-wide response of the epicardium to injury. Chamber epicardium induces developmental markers (Tbx18/Raldh2) and expands to create a new epithelial cover for the exposed myocardium; subsequently, a subpopulation of epicardial cells undergoes EMT, invades the wound and provides new vasculature to regenerating muscle in an FGF (fibroblast growth factor)-dependent manner [19]. These studies emphasize the importance of the epicardium in response to injury in a non-mammalian vertebrate that has maintained an inherent ability to regenerate its heart. Moreover, it has been proposed that the ability to mobilize epicardial cells and cultivate a cardiogenic environment is the primary reason why zebrafish, as opposed to mammals, effectively regenerate. This is further supported by the fact that mammalian hearts typically show insufficient neovascularization after MI. Experimental attempts, therefore, to promote neovascularization in a paracrine manner, by directly utilizing epicardial cells or EPDCs, could prove favourable for encouraging mammalian (including human) cardiac regeneration.

Tβ4 facilitates neovascularization in vivo
Studies are now ongoing within our group to investigate whether Tβ4-activated EPDCs contribute new vasculature in both the intact and injured (mouse MI model) adult rodent heart and to determine the mechanism by which Tβ4 exerts these effects on the target EPDC population. Thus we seek to elevate the status of the adult mammalian epicardium to that of a definitive source of pluripotent cardiac progenitors and Tβ4 to a facilitator of the vascular component of cardiac regeneration. Therefore collectively our studies are of general interest to the fields of stem/progenitor cell biology and cardiovascular regenerative medicine and, more specifically, of significant clinical relevance with respect to therapy for ischaemic heart disease and acute MI.

Funding
This work was generously supported by grants from the British Heart Foundation and Medical Research Council.

References

Received 1 May 2009
doi:10.1042/BST0371218