Published in final edited form as:

Ann N Y Acad Sci. 2012 October ; 1269: 92–101. doi:10.1111/j.1749-6632.2012.06708.x.

Myocardial Regeneration: Expanding the Repertoire of Thymosin β 4 in the Ischaemic Heart

Nicola Smart^a, Sveva Bollini^a, Karina N. Dubé^a, Joaquim M. Vieira^a, Bin Zhou^{b,c}, Johannes Riegler^{d,e}, Anthony N. Price^f, Mark F. Lythgoe^d, Sean Davidson^g, Derek Yellon^g, William T. Pu^{b,c}, and Paul R. Riley^{a,#}

^aDepartment of Physiology, Anatomy and Genetics, Sherrington Building, University of Oxford, South Parks Road, Oxford OX1 3PT

^bHarvard Stem Cell Institute and Department of Cardiology, Children's Hospital Boston, 300 Longwood Avenue, Boston, Massachusetts 02115, USA

^cDepartment of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA

^dCentre for Advanced Biomedical Imaging (CABI), Department of Medicine and Institute of Child Health, University College London (UCL), London, UK

^eCentre for Mathematics and Physics in the Life Sciences and Experimental Biology (CoMPLEX), UCL, London, UK

^fMRC Clinical Sciences Centre, Faculty of Medicine, Imperial College London, London, W12 0NN, UK

^gThe Hatter Cardiovascular Institute, University College London, London, WC1E 6HX

Abstract

Efficient cardiac regeneration post-infarction (MI) requires the replacement of lost cardiomyocytes, formation of new coronary vessels and appropriate modulation of the inflammatory response. However, insight into how to stimulate repair of the human heart is currently limited. Utilising the embryonic paradigm of regeneration, we demonstrated that the actin-binding peptide Thymosin β 4 (T β 4), required for epicardium-derived coronary vasculogenesis, can recapitulate its embryonic role and activate quiescent adult epicardial cells (EPDCs). Once stimulated, EPDCs facilitate neovascularisation of the ischaemic adult heart and, moreover, contribute *bona fide* cardiomyocytes. EPDC-derived cardiomyocytes structurally and functionally integrate with resident muscle, to regenerate functional myocardium, limiting pathological remodelling and effecting an improvement in cardiac function. Alongside pro-survival and anti-inflammatory properties, these regenerative roles, via EPDCs, markedly expand the range of therapeutic benefits of T β 4 to sustain and repair the myocardium after ischaemic damage.

[#]To whom correspondence should be addressed: Paul R. Riley, paul.riley@dpag.ox.ac.uk.

Keywords

Thymosin β 4; EPDCs; epicardium; *Wt1*; myocardial regeneration; *de novo* cardiomyocytes

The Quest for Effective Cardiac Regenerative Strategies

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality worldwide, with myocardial infarction (MI) the most common cause of cardiac injury. The consequence of losing over a billion functional cardiomyocytes in mammals is replacement by a non-contractile scar, pathological remodelling and progression to heart failure (HF).¹ Since the mammalian heart is unable to adequately regenerate beyond early postnatal stages,² transplantation is the only possible cure, although confounded by host immune rejection and a limited supply of donor hearts. Conventional palliative medication seeks to preserve the already compromised cardiac function, rather than regenerating lost myocardium.

Consequently, there has been an intensive effort to develop stem-cell based strategies for cardiac repair to both regenerate heart muscle and promote coronary vasculogenesis after MI, culminating in a number of clinical trials. The majority of such trials have relied on the use of autologous cell types, including bone marrow stem cells, however, there is no consensus that these cells can differentiate to contribute new muscle efficiently. Perhaps for this reason, the clinical improvement has been generally disappointing.³ Hence, there continues to be an urgent need to identify the most promising cardiovascular stem cells; either for transplantation and engraftment or activation of a resident population for induction towards a cardiomyocyte fate (reviewed in⁴).

Redeploying Embryonic Mechanisms in the Adult Heart for Repair

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. The epicardium gives rise to EPDCs, multipotent cardiac progenitors that were proposed to be true cardiac stem cells,⁵ due to their potential to differentiate into endothelial cells (ECs)⁶, coronary VSMCs,⁷ cardiac fibroblasts⁸ and cardiomyocytes.⁵ 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).⁹

Thymosin β 4 Reactivates the Quiescent Adult Epicardium

A prerequisite for utilising EPDCs for repair in the adult heart is the identification of factors to reactivate this ordinarily dormant reservoir of cells in order to exploit their restorative

power. Valuable insight into the epicardial response to injury 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.¹⁰ Lacking the intrinsic regenerative capacity of fish, the mammalian epicardium requires a boost to achieve even a modest improvement in myocardial regeneration. Reactivation of the mammalian adult epicardium was first revealed using the actin-monomer binding protein Thymosin β 4 (T β 4).¹¹ 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. Myocardial injury is itself sufficient to promote epicardial activation and neovascularisation and T β 4 appears to act synergistically to augment the extent of repair.¹² 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.

Thymosin β 4 and EPDCs: a Joint Force for Myocardial Regeneration

Just as the myocardium and coronary vasculature develop simultaneously in a coordinated manner in the embryo, 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 during development offers a unique target for coordinately stimulating myocardial and coronary vascular repair. Moreover, 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.¹³

From the demonstration of a significant epicardial contribution to the cardiomyocyte lineage emerged a basis for translating myocardial potential into the adult heart.¹⁴ However, epicardial lineage tracing is confounded in the adult since expression of most known epicardial genes is restricted to embryonic stages and silenced in the adult. From a peak of expression at E11.5, coincident with epicardial formation and EPDC contribution, *Wt1*, alongside other epicardial genes, is down-regulated by early post-natal stages to leave the adult epicardium effectively dormant (Fig. 1a). We exploited the ability of T β 4 to reactivate the adult epicardium in order to restore *Wt1* expression (Fig. 1b). “Priming” of adult *Wt1*^{GFP^{Cre}+} and *Wt1*^{CreERT2}; *R26R*^{YFP/+} knock-in mice with T β 4 in this manner enabled both constitutive (GFP+) and pulse- (YFP+) labelling of EPDCs, respectively, to facilitate the tracking of epicardium-derived precursors through to mature cardiomyocytes.¹⁵ Proliferative YFP+ cells were shown to expand in number over time (d2-d14) within the epicardium, extending into the myocardium, towards the region of injury (Fig. 1c-h). Two-photon molecular excitation laser scanning microscopy revealed live YFP+ cells in the epicardium and sub-epicardial region at day 7 post-MI and a large number of cells which had migrated into the underlying myocardium to a depth of <100 μ m (Fig 1i).

In epicardial explants¹⁶ from T β 4-primed Wt1^{GFP^{Cre/+}} adult hearts, a proportion of outgrowing GFP+ cells were positive for Isl-1 and Nkx2.5, indicative of cardiomyocyte precursors (Figure 2a-h).¹⁷ After 14 days of culture, cells adopted a more differentiated cardiac muscle phenotype, with expression of sarcomeric structural components, cardiac troponin T (cTnT), sarcomeric α -actinin (S α A) and cardiac myosin binding protein C (cMyBPC).

In an *in vivo* model of MI (ligation of the left anterior coronary artery), YFP+ cells positive for Isl-1 or Nkx2.5 cells were found to reside in the epicardium and adjacent subepicardial region of T β 4-treated Wt1^{CreERT2}; R26R^{EYFP/+} hearts within two days post-MI (Fig. 2i-q). This was accompanied by elevated *Isl-1* expression from 2 days post-MI onwards (Fig. 2r). By day 14 post-MI, larger YFP+ cells, co-expressing sarcomeric markers and morphologically resembling mature cardiomyocytes, were detected within the left ventricular wall, specifically in the border zone and peri-infarct region (Fig. 3a-h). The total mean % of YFP+ EPDCs that became cardiomyocytes was 0.59 $\pm$ 0.18% (serial sections through n=7 hearts $\pm$ SEM). Approximately 82% of YFP+ cardiomyocytes were located proximal to the site of injury, residing either in the border zone or within the immediate surrounding healthy myocardium. Consistent with tracking labelled cardiomyocytes from EPDCs, BrdU injections of Wt1-ERT2YFP mice revealed proliferating BrdU+ EPDCs at day 4 traced to BrdU+/YFP+ cardiomyocytes at day 14 (data not shown).¹⁵ Importantly, the de novo cardiomyocytes were appropriately integrated with resident myocardium and with each other, via both adherens' (Fig. 3d-e) and gap junction formation (Fig. 3g). Moreover, functional integration of YFP+ cardiomyocytes with existing myocardium was demonstrated upon measuring [Ca²⁺]_i transients with neighbouring YFP- cardiomyocytes. We recorded evoked cellular calcium transients [Ca²⁺]_i between YFP+ and YFP- cells in Langendorff-perfused, T β 4-primed, Wt1-ERT2YFP hearts loaded with the fluorescent indicator rhod-2/AM (Fig. 3i-n). We observed small, immature YFP+ rod-shaped cells within the border zone which failed to elicit [Ca²⁺]_i transients, as well as mature YFP+ cardiomyocytes, in which calcium transients were synchronous with kinetics indistinguishable from those of neighbouring YFP-cardiomyocytes (Fig. 3o). Minor differences in resolution between resident YFP- and the de novo YFP+ cardiomyocyte transients (compare Fig. 6m with 6n) likely reflect the newly acquired function of the YFP+ population. These results strongly suggest that T β 4-primed EPDC-derivatives couple with surviving cardiomyocytes following injury to form a functional syncytium with resident myocardium.

Excluding the potential for inadvertent tracing of resident cardiomyocytes due to ectopic activation of the fluorophore from the *Wt1* knock-in alleles, required the transplantation of FACS-isolated donor GFP+ cells into non-transgenic host hearts (data not shown).¹⁵ Extensive analyses on the prospective donor cells, isolated at day 4, characterised them as EPDCs and ruled out any co-isolation of non-epicardial cells which may have up-regulated *Wt1*. Immunostaining confirmed restricted localisation of Wt1+ cells to the epicardium and expanded sub-epicardial region. GFP+ cells were excluded from the entire myocardium and no cardiomyocyte marker expression was detectable from the FACS-isolated donor cells at day 4. These data collectively confirmed that donor Wt1+ cells were exclusively epicardial

and were not myocytes which had simply up-regulated GFP via the targeted *Wt1* allele, consistent with the fact that GFP⁺ or YFP⁺ cardiomyocytes were never observed at day 4 post-MI. FACS-isolated donor cells from Tβ4/injury-primed male or female *Wt1*-GFP animals (between 3-6 × 10⁴ cells/host) were injected into the subepicardial space of Tβ4 primed non-transgenic gender mis-matched hosts, following LAD ligation. At 24 hours following transplantation, small round GFP⁺ cells were restricted to the site of injection within the host heart. By day 7 GFP⁺ cells, residing within the sub-epicardium were Nkx2.5⁺, indicative of myocardial progenitor commitment, whilst other GFP⁺ cells, in the underlying myocardium, although morphologically immature, were found to co-express cTnT. More definitive donor GFP⁺ cardiomyocytes with myofibrillar structure, co-expressing cTnT were observed within the host myocardium by day 14. Collectively the detection of these staged donor derivatives suggested progressive differentiation towards a mature cardiomyocyte fate. Fluorescence in situ hybridisation on GFP⁺ cardiomyocytes confirmed a single XX or XY karyotype in female or male-derived donor cells, respectively, implying transdifferentiation of EPDCs in the absence of cell fusion¹⁵.

MRI analyses over a 28 day time course after MI revealed that Tβ4 treatment resulted in significant improvement in ejection fraction and end diastolic/systolic volumes over time (Fig. 4a-e). Improvements in LV mass, relative to scar volume (Fig. 4f-g), suggested a degree of muscle regeneration attributable, in part, to the primed EPDC contribution of functional cardiomyocytes.

Therapeutic Application of Thymosin β4 for the Ischaemic Heart

Despite the beneficial outcome, a limitation of this approach is the requirement to pre-treat with Tβ4 prior to injury which, from a translational perspective, would require the prior identification of patients considered at risk of MI. No cardiomyocyte replenishment from epicardial precursors was achieved without pre-treatment^{13,15} or when Tβ4 was administered only after MI.¹⁸ Whether epicardial activation prior to injury is required to alter the subsequent fate of epicardial cells or whether it simply enhances the magnitude of epicardial reactivation sufficiently, over and above the activation that occurs by post-treatment with Tβ4 remains to be determined. In addition, the incidence of Tβ4-induced cardiomyocytes from activated EPDCs was very low and certainly insufficient to replenish losses in the region of 10⁹ cardiomyocytes post-MI, however, the potential myocardial regeneration via the epicardium paves the way for the discovery of novel compounds that more efficiently promote regeneration without a requirement to pre-treat. 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,¹⁹ Tβ4 can activate adult epicardium to not only induce neovascularisation¹² but promote myocardial regeneration¹⁵ to restore a functional vasculature, maintain cardiomyocyte survival and replace lost muscle in the injured heart.

Pleiotropic Roles of Thymosin β4 in Cardiac Repair

The direct reactivation of adult EPDCs by Tβ4, to underpin the initiation and migration of adult epicardium-derived cardiovascular progenitors towards a cardiomyocyte fate,

constitutes one of several pleiotropic benefits of T β 4 therapy for acute myocardial infarction (Fig. 4h). Previously, T β 4 was shown to activate Akt signaling to enhance cardiomyocyte survival post-ischaemic injury.¹⁹ In addition, we and others have revealed that T β 4 can induce the adult epicardium, in the same setting, to contribute coronary endothelial and smooth muscle cells and initiate vascular repair.^{12,20}

Alongside neovascularization and myocardial regeneration, efficient therapies to combat cardiac dysfunction also require appropriate modulation of inflammatory responses to prevent maladaptive remodeling and fibrosis.²¹ Following injury, local production of cytokines instructs recruitment of phagocytes for the removal of necrotic debris and macrophages to promote the formation of granulation tissue.²² Uncontrolled inflammation, as manifests post-MI, inhibits wound healing and manipulation of the early response presents a therapeutic target to salvage viable myocardium and improve repair. T β 4 is markedly up-regulated around the sites of inflammation,²³ and exhibits anti-inflammatory properties in many pathological settings.²⁴⁻²⁸ Pro-inflammatory cytokines, including IL-1 β and TNF α , produced during MI, converge to stimulate activation and nuclear transport of the transcription factor NF κ B. Chronic activation of NF κ B in the ischaemic heart, exacerbates cardiac remodelling by imparting pro-inflammatory, pro-fibrotic and pro-apoptotic effects²⁹. In models of MI and heart failure, T β 4 was shown to suppress NF κ B activation and collagen expression, hallmarks of cardiac fibrosis.³⁰ While the mechanism of T β 4-induced NF κ B suppression has not been defined, T β 4 modulates the PINCH-ILK complex, the activation of which is known to sensitize NF κ B signaling after TNF α stimulation in acute inflammatory conditions,³¹ suggesting a possible involvement of this pathway. Further anti-fibrotic properties of T β 4 may be mediated via the biological activity of its tetrapeptide cleavage product, AcSDKP, which inhibits collagen deposition by cardiac fibroblasts in the failing heart.³²

Prospects for further extending the limits of T β 4's therapeutic utility are emerging with the recent demonstration that T β 4 enhances *in vivo* reprogramming of cardiac fibroblasts into induced cardiomyocytes (iCMs) upon viral transduction with the transcription factors Gata4, Mef2c and Tbx5.³³ By activating EPDCs, thereby increasing the number and proliferation of cardiac fibroblasts, T β 4 treatment enhanced the yield of iCMs upon transduction.

The multiple facets of myocardial repair addressed by delivery of a single peptide distinguish T β 4 as an attractive therapeutic agent, particularly given its intrinsic capacity to generate potent anti-inflammatory and anti-fibrotic derivatives such as AcSDKP. Further contributions via the epicardium to regenerate lost myocardium and restore blood flow to the ischaemic heart expand its therapeutic repertoire immeasurably. The multiple beneficial facets of T β 4 are delineated with time post-MI. The pro-survival and anti-inflammatory activities of T β 4 are early injury responses, to maintain the quotient of surviving myocardium, whilst neovascularisation and *de novo* cardiogenesis are longer term regenerative functions acting through the common target of adult EPDCs.

Acknowledgments

This work was funded by the British Heart Foundation.

References

1. Reinecke H, Minami E, Zhu WZ, Laflamme MA. Cardiogenic differentiation and transdifferentiation of progenitor cells. *Circ Res*. 2008; 103:1058–1071. [PubMed: 18988903]
2. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. *Science*. 2011; 331:1078–1080. [PubMed: 21350179]
3. Bartunek J, Dimmeler S, Drexler H, Fernandez-Aviles F, Galinanes M, Janssens S, Martin J, Mathur A, Menasche P, Priori S, Strauer B, Tendera M, Wijns W, Zeiher A. The consensus of the task force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for repair of the heart. *Eur Heart J*. 2006; 27:1338–1340. [PubMed: 16543252]
4. Laflamme MA, Murry CE. Heart regeneration. *Nature*. 2011; 473:326–335. [PubMed: 21593865]
5. Wessels A, Perez-Pomares JM. The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat Rec A Discov Mol Cell Evol Biol*. 2004; 276:43–57. [PubMed: 14699633]
6. Perez-Pomares JM, Carmona R, Gonzalez-Iriarte M, Atencia G, Wessels A, Munoz-Chapuli R. Origin of coronary endothelial cells from epicardial mesothelium in avian embryos. *Int J Dev Biol*. 2002; 46:1005–1013. [PubMed: 12533024]
7. Mikawa T, Gourdie RG. Pericardial Mesoderm Generates a Population of Coronary Smooth Muscle Cells Migrating into the Heart along with Ingrowth of the Epicardial Organ. *Developmental Biology*. 1996; 174:221–232. [PubMed: 8631495]
8. Gittenberger-De Groot AC, Vrancken Peeters MP, Mentink MMT, Gourdie RG, Poelmann RE. Epicardium-Derived Cells Contribute a Novel Population to the Myocardial Wall and the Atrioventricular Cushions. *Circulation Research*. 1998; 82:1043–1052. [PubMed: 9622157]
9. Winter EM, Gittenberger-De Groot AC. Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell Mol Life Sci*. 2007; 64:692–703. [PubMed: 17380310]
10. Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns C, Poss KD. A Dynamic Epicardial Injury Response Supports Progenitor Cell Activity during Zebrafish Heart Regeneration. *Cell*. 2006; 127:607–619. [PubMed: 17081981]
11. Smart N, Risebro CA, Melville AAD, Moses K, Schwartz RJ, Chien KR, Riley PR. Thymosin β 4 induces adult epicardial progenitor mobilization and neovascularization. *Nature*. 2007; 445:177–182. [PubMed: 17108969]
12. Smart N, Risebro CA, Clark JE, Ehler E, Miquelol L, Rossdeutsch A, Marber MS, Riley PR. Thymosin β 4 facilitates epicardial neovascularization of the injured adult heart. *Ann N Y Acad Sci*. 2010; 1194:97–104. [PubMed: 20536455]
13. Zhou B, Honor LB, He H, Ma Q, Oh JH, Butterfield C, LIN RZ, Melero-Martin JM, Dolmatova E, Duffy HS, Gise A, Zhou P, Hu YW, Wang G, Zhang B, Wang L, Hall JL, Moses MA, McGowan FX, Pu WT. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest*. 2011; 121:1894–1904. [PubMed: 21505261]
14. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, Von GA, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008; 454:109–113. [PubMed: 18568026]
15. Smart N, Bollini S, Dube KN, Vieira JM, Zhou B, Davidson S, Yellon D, Riegler J, Price AN, Lythgoe MF, Pu WT, Riley PR. De novo cardiomyocytes from within the activated adult heart after injury. *Nature*. 2011; 474:640–644. [PubMed: 21654746]
16. Smart N, Riley PR. Derivation of epicardium-derived progenitor cells (EPDCs) from adult epicardium. *Curr Protoc Stem Cell Biol*. 2009; Chapter 2 Unit2C.
17. Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, Qyang Y, Bu L, Sasaki M, Martin-Puig S, Sun Y, Evans SM, Laugwitz KL, Chien KR. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell*. 2006; 127:1151–1165. [PubMed: 17123592]
18. Zhou B, Honor LB, Ma Q, Oh JH, Lin RZ, Melero-Martin JM, Von GA, Zhou P, Hu T, He L, Wu KH, Zhang H, Zhang Y, Pu WT. Thymosin β 4 treatment after myocardial infarction does not reprogram epicardial cells into cardiomyocytes. *J Mol Cell Cardiol*. 2011

19. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin β 4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature*. 2004; 432:466–472. [PubMed: 15565145]
20. Bock-Marquette I, Shrivastava S, Pipes GC, Thatcher JE, Blystone A, Shelton JM, Galindo CL, Melegh B, Srivastava D, Olson EN, Dimaio JM. Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J Mol Cell Cardiol*. 2009; 46:728–738. [PubMed: 19358334]
21. Sutton MG, Sharpe N. *Circulation*. 2000; 101:2981–2988. [PubMed: 10869273]
22. Frangogiannis NG. Targeting the inflammatory response in healing myocardial infarcts. *Curr Med Chem*. 2006; 13:1877–1893. [PubMed: 16842199]
23. Frohm M, Gunne H, Bergman AC, Agerberth B, Bergman T, Boman A, Liden S, Jornvall H, Boman HG. Biochemical and antibacterial analysis of human wound and blister fluid. *Eur J Biochem*. 1996; 237:86–92. [PubMed: 8620898]
24. Sosne G, Chan C, Thai K, Kennedy M, Szliter E, Hazlett L, Kleinman H. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. *Exp Eye Res*. 2001; 72:605–608. [PubMed: 11311052]
25. Girardi M, Sherling MA, Filler RB, Shires J, Theodoridis E, Hayday AC, Tigelaar RE. Anti-inflammatory effects in the skin of thymosin-beta4 splice-variants. *Immunology*. 2003; 109:1–7. [PubMed: 12709011]
26. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, Hazlett LD. Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp Eye Res*. 2002; 74:293–299. [PubMed: 11950239]
27. Badamchian M, Fagarasan MO, Danner RL, Suffredini AF, Damavandy H, Goldstein AL. Thymosin beta(4) reduces lethality and down-regulates inflammatory mediators in endotoxin-induced septic shock. *Int Immunopharmacol*. 2003; 3:1225–1233. [PubMed: 12860178]
28. Zhang J, Zhang ZG, Morris D, Li Y, Roberts C, Elias SB, Chopp M. Neurological functional recovery after thymosin beta4 treatment in mice with experimental auto encephalomyelitis. *Neuroscience*. 2009; 164:1887–1893. [PubMed: 19782721]
29. Sosne G, Qiu P, Christopherson PL, Wheeler MK. Thymosin beta 4 suppression of corneal NFkappaB: a potential anti-inflammatory pathway. *Exp Eye Res*. 2007; 84:663–669. [PubMed: 17254567]
30. Sopko N, Qin Y, Finan A, Dadabayev A, Chigurupati S, Qin J, Penn MS, Gupta S. Significance of thymosin beta4 and implication of PINCH-1-ILK-alpha-parvin (PIP) complex in human dilated cardiomyopathy. *PLoS One*. 2011; 6:e20184. [PubMed: 21625516]
31. Qiu P, Wheeler MK, Qiu Y, Sosne G. Thymosin beta4 inhibits TNF- alpha-induced NF-kappaB activation, IL-8 expression, and the sensitizing effects by its partners PINCH-1 and ILK. *FASEB J*. 2011; 25:1815–1826. [PubMed: 21343177]
32. Peng H, Carretero OA, Raj L, Yang F, Kapke A, Rhaleb NE. Antifibrotic effects of N-acetyl-seryl-aspartyl-Lysyl-proline on the heart and kidney in aldosterone-salt hypertensive rats. *Hypertension*. 2001; 37:794–800. [PubMed: 11230375]
33. Qian L, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu JD, Srivastava D. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature*. 2012

Figure 1. Myocardial injury and Tβ4 reactivate embryonic epicardial gene expression in the adult heart

Real time qRT-PCR revealed a significant down-regulation ($p^* 0.05$; $p^{**} 0.01$) of the epicardial genes *Wt1*, *Tbx18*, *Raldh2* and *EndoB* in intact hearts from embryonic day E11.5 through to post-natal day 7 (P7) and 4 months of age (**a**). Tβ4 priming resulted in a precocious up-regulation in *Wt1* ($p^{***} 0.001$) and *Tbx18* ($p^* 0.05$) as early as day 2 post-MI, the extent of which correlated with severity of injury. MI group 1 = mild injury; MI group 2 = severe injury (**b**). An increase in the number of EPDCs was observed following injury from days 2, 4, 7 ($p^{**} 0.01$) to day 14 ($p^{***} 0.00$; **c-g**), with evidence of migration into the underlying myocardium (**h-i**). Multi-photon-acquired 3D reconstructions revealed the extent of EPDC migration from the epicardial surface to the underlying myocardium in response to injury and Tβ4 (**i**). Scale bar in **h** represents 50μm, applies to **d-h**. ep, epicardium, my, myocardium; sc, scar region. All statistics: Student's t-test.

Figure 2. Tβ4-primed EPDCs give rise to cardiac progenitors

Primed GFP+ EPDCs (white arrowheads) migrated from epicardial explants (a-d). GFP+ EPDCs co-stained for Isl-1 (e, f; white arrowhead indicates an Isl-1 negative EPDC surrounded by an Isl-1+ cluster) or Nkx2.5 (g, h; white arrowheads indicate Nkx2.5+ EPDCs) providing evidence of cardiac progenitor formation. *In vivo*, YFP+ EPDCs co-expressing Isl-1 (i-l) or Nkx2.5 (m-p) resided in the epicardium, indicative of an early progenitor response 2 days post-MI. Significant increases in Isl-1+/YFP+ cells and Nkx2.5+/YFP+ cells were observed within 7 days post-MI, alongside increased phospho-histone H3+ (P-HH3+) proliferating YFP+ progenitors at day 7 compared to sham operated controls (q). Real time qRT-PCR revealed a significant increase in *Isl-1* expression in primed hearts, following MI, correlating with the extent of injury (r; $p=0.05$; $p^{**}=0.01$; $p^{***}<0.001$; MI group 1 = mild injury; group 2 = severe injury). All p-values calculated by paired ANOVA (q) and Student's t-test (r). Scale bars in a, 100μm; b, 500μm; c-e, g, l, m, applies to c-p, 50μm. ep, epicardium; my, myocardium; sc, scar region.

Figure 3. Activated adult Wt1+ EPDCs differentiate into structurally coupled, functional cardiomyocytes

YFP+ cells (detected by α -GFP), co-expressing cTnT, residing within the border zone and peri-infarct region (a; white arrowheads; dashed white line demarcates extent of peri-infarct region). The YFP+ cells had evident sarcomeric structure, which were S α A+ (b, c, f, g) or cTnT+ (d, e, h), with sarcomeric banding (white arrowheads) and evidence of structural coupling to resident YFP- cardiomyocytes through N-Cad+ adherens junction (d, e) and Cx43+ gap junction formation (f, g). Multiphoton imaging on Langendorff perfused T β 4-primed Wt1-ERT2YFP hearts loaded with rhod-2/AM at day 14 post-MI. Rhod-2 loading (i) of YFP+ cardiomyocytes within the peri-infarct region (j) was compared against distal YFP- cardiomyocytes (k). EYFP and rhod-2/AM excited simultaneously at 990 nm allowed measurement of calcium transients ([Ca²⁺]_i) line traces) across clustered YFP- and YFP+ cardiomyocytes (l) as evidence of functional coupling both between YFP- with YFP+ and YFP+ with YFP+ cardiomyocytes. Distal YFP- spontaneous calcium transients were visualized without electrical pacing (m) and compared against YFP+ transients (n). YFP- and YFP+ calcium transients displayed comparable amplitude and synchrony. Scale bars represent 150 μ m (a); 20 μ m (b-h); bz, border zone, sc, scar region.

Figure 4. EPDC-derived cardiomyocytes contribute towards improved cardiac output and myocardial regeneration post-MI

MRI analyses with representative short axis images (mid ventricular and apical slices) of infarcted hearts 28 days post-MI with late gadolinium enhancement (scar tissue bright) following treatment with either Tβ4 (**a**) or PBS (**b**). Scatter plots for end diastolic volume (EDV; **c**), end systolic volume (ESV; **d**) and infarct volume (**e**) indicate improved cardiac function following Tβ4 treatment. Horizontal bars indicate the means for each group. Trichrome stained transverse sections from PBS- (**f**) and Tβ4- (**g**) treated hearts 28 days post MI revealed reduced scarring and fibrosis with Tβ4 treatment (blue represents collagen deposition). Note the increased proportion of healthy myocardium highlighted by the black box in (**g**) relative to the comparative region in (**f**). * $p < 0.05$; all statistics by one way repeated measures ANOVA. Scale bar in **g** represents 1mm applies to **f**, **g**. Myocardial replenishment constitutes one of the beneficial effects of Tβ4 in the ischaemic heart, alongside promotion of cell survival and neovascularization and modulation of the inflammatory response (**h**).