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Issue: *Thymosins in Health and Disease***Thymosin β 4 and cardiac repair**Santwana Shrivastava,¹ Deepak Srivastava,² Eric N. Olson,³ J. Michael DiMaio,¹
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Hypoxic heart disease is a predominant cause of disability and death worldwide. As adult mammals are incapable of cardiac repair after infarction, the discovery of effective methods to achieve myocardial and vascular regeneration is crucial. Efforts to use stem cells to repopulate damaged tissue are currently limited by technical considerations and restricted cell potential. We discovered that the small, secreted peptide thymosin β 4 (T β 4) could be sufficiently used to inhibit myocardial cell death, stimulate vessel growth, and activate endogenous cardiac progenitors by reminding the adult heart on its embryonic program *in vivo*. The initiation of epicardial thickening accompanied by increase of myocardial and epicardial progenitors with or without infarction indicate that the reactivation process is independent of injury. Our results demonstrate T β 4 to be the first known molecule able to initiate simultaneous myocardial and vascular regeneration after systemic administration *in vivo*. Given our findings, the utility of T β 4 to heal cardiac injury may hold promise and warrant further investigation.

Keywords: cardiac regeneration; coronary development; PKC; progenitor cells; Thymosin β 4

Introduction

Heart failure is a consequence of an injured or diseased heart undergoing pathological remodeling to match cardiac output with the metabolic needs of the body. Eighty million people are confirmed with cardiovascular disease in the USA alone.¹ With few exceptions the prognostic benefits of current treatments are limited, resulting in high rates of morbidity and mortality. As the mammalian adult heart is incapable of sufficient regeneration, immense efforts have been devoted to promote cardiac repair.² Direct use of stem cells to repopulate damaged tissue is limited by maintaining transplanted cell survival, ensuring appropriate homing to the site of injury, and limited trans-differentiation.^{3,4} Accordingly, we hypothesize that the use of small, secreted molecules could be an alternative to support healing and increase cardiac function in adults.

Among the numerous causes that result in heart failure the defects in the coronary vascular system

have the most significant impact on heart function and disease. A variety of stimuli can initiate the formation of new blood vessels in the heart, presumably through common downstream signaling cascades that trigger quiescent endothelial or other progenitor cells to form nascent tubular structures.⁵ Although many of the cellular and molecular mechanisms of embryonic coronary development are well investigated, the molecular basis of angiogenesis in the embryo seems to differ from the pathological vessel regeneration in adults.⁶ Blood vessels in the embryo form primarily through vasculogenesis, a differentiation of precursor cells (angioblasts) to endothelial cells that assemble into a vascular network. New vessels in adults arise mainly through angiogenic sprouting, although vasculogenesis may also occur.⁶

Recent studies to reveal the molecular and cellular mechanisms that support cardiac regeneration in adult hearts showed that zebrafish epicardial cells invade the myocardium and create a vascular

network likely to encourage cardiac regeneration in adults.⁷ Thus, the injured adult zebrafish heart can recall signaling pathways essential during embryonic coronary development, and the ability to mobilize epicardial cells may be the primary reason they effectively regenerate myocardium. Since adult mammalian hearts typically show insufficient neovascularization after myocardial infarction, attempts to modify this deficiency by directly utilizing epicardial cells or their progenitors could prove favorable for cardiac regeneration.

We previously demonstrated that Thymosin β 4 (T β 4), a 43-amino-acid G-actin-sequestering peptide is expressed in the embryonic heart, stimulates cardiomyocyte migration *in vitro*, and increases cardiac function while promoting the survival of cardiomyocytes in adult mice *in vivo*.⁸ Our recent results indicate that T β 4 initiates capillary-like tube formation of adult coronary endothelial cells, induces endothelial cell migration and proliferation in embryonic cardiac explants *in vitro*, and supports revascularization *in vivo*. Importantly, it induces an organwide epicardial thickening and progenitor cell activation in adults similar to the changes in developing embryos and in regenerating adult zebrafish, while initiating the expression of numerous proangiogenic developmental genes. T β 4 initiated protein kinase C (PKC) activity revealed to be essential for the epicardial activation. Our findings indicate that T β 4 supports cardiac regeneration not only by inhibiting myocardial cell death after infarction, but also through induction of vessel growth, myocardial progenitor mobilization, and by reactivating the embryonic developmental program of the adult epicardium *in vivo*. These results are described below.

Results

T β 4 *in vitro*

We tested the effects of T β 4 on myocardial and cardiac endothelial cell migration by embryonic heart explant assays on rat tail collagen.⁹ Since T β 4 is expressed in myocardial and endothelial cells and is absent in cardiac mesenchyme, we used T β 4 primary antibody to identify endothelial cells. Exogenously administered T β 4 significantly increased embryonic endothelial and myocardial cell migration by facilitating the number of round actively moving cells *in vitro* (Fig. 1A–F). We did not de-

tect changes in cellular death. Phospho-histone H3 staining and immunocytochemistry after BrdU administration indicated that T β 4 significantly affects endothelial cell proliferation (Fig. 1G–I). An explanation for increased proliferation rate could be the activation or upregulation of β -Catenin expression by T β 4 *in vitro* and *in vivo* (see Fig. 3M,N).¹⁰

Human umbilical vein endothelial cells (HUVECs) form capillary structures when plated on matrigel.^{11,12} To analyze the effect of T β 4 on cardiac vessel formation we checked whether adult human coronary endothelial cells (HCECs) behave similarly to HUVECs and if T β 4 may alter the process *in vitro*. Our results indicated that HCECs are capable of capillary structure formation on matrigel and that T β 4 expedites this process (Fig. 1J–L).

T β 4 *in vivo*

Our *in vitro* observations on cardiac and vessel endothelial cells suggested that T β 4 might affect coronary vascular growth in adult mice. To test our assumption we created cardiac infarctions by ligating the left anterior descending coronary artery followed by immediate systemic T β 4 or PBS administration. Simultaneous staining with platelet endothelial cell adhesion molecule-1 (Pecam-1) and smooth muscle α -actin (sm α -actin) specific primary antibodies revealed significant increase in capillary density 3 days after T β 4 injection at the infarction border zone and noninfarcted remote areas of the hearts (see Fig. 2).

Coronary vessels are believed to originate from the epicardium during development. Recent work from Lepilina *et al.*⁷ demonstrated that an elaborate sequence of organwide and local responses by epicardial cells increases cardiac regeneration and revascularization in adult zebrafish. The changes of the epicardium in adult fish are similar to the alterations in developing embryos. Mammalian hearts, however, are incapable of such neovascularization or general regeneration after cardiac injury. Thus, we hypothesized that experimental attempts to activate the embryonic coronary developmental program in mammals could enhance cardiac regeneration. To determine if T β 4 may stimulate an epicardial response, we analyzed the changes in blood vessel/epicardial substance (Bves) expression after systemic T β 4 administration. Bves is widely expressed in the developing coronary vascular system^{13,14} and is used as one of the markers of

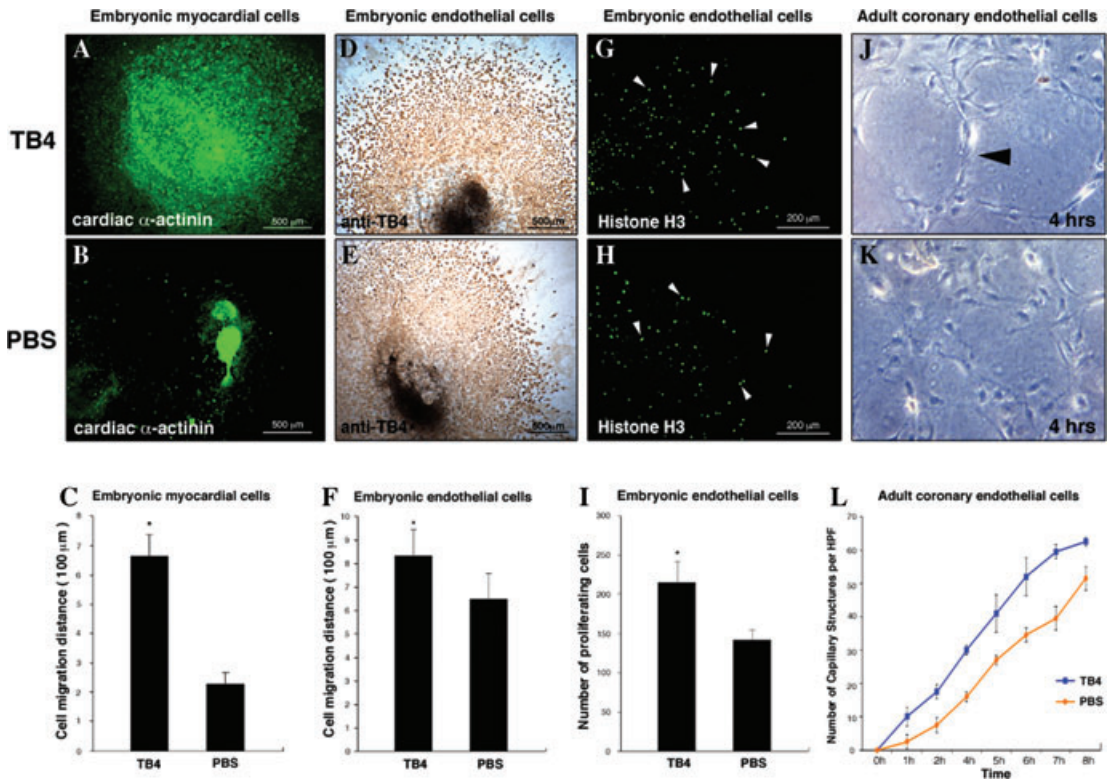


Figure 1. $T\beta$ 4 induces embryonic myocardial and endothelial cell migration, endothelial cell proliferation, and initiates capillary-like structure formation of HCECs *in vitro*. A, B, D, E: Mouse E11.5 cardiac outflow tract myocardial (A, B) and endothelial (D, E) cells after $T\beta$ 4 or PBS treatment. C, F: Distance of embryonic myocardial (C) and endothelial (F) cell migration in E11.5 cardiac outflow tract explants with or without $T\beta$ 4 treatment. G, H: Staining of mouse E11.5 cardiac outflow tract explants with phospho-histone H3 antibody shows increased endothelial cell proliferation (*white arrowheads*) after $T\beta$ 4 treatment compared to PBS. I, Number of proliferating embryonic endothelial cells with or without $T\beta$ 4 treatment. J, K: Adult HCECs form capillary structures (J, *black arrowhead*) in advance when treated with $T\beta$ 4 on matrigel. L: $T\beta$ 4 increases the number of HCEC capillary-like structures on matrigel. Bars indicate standard deviation at 95% confidence limits ($n = 6$), $*P < 0.05$.

epicardial cells or cells of epicardial origin in adult and embryonic tissues. We observed elevated Bves expression 24 h after $T\beta$ 4 treatment, and an increase in Bves positive cells with general organ-wide thickening of the adult epicardium 72 h after peptide administration in the noninfarcted remote regions of the hearts (data not shown). We found that most of the adult epicardial cells also express sm α -actin (Fig. 2C and D) and that the number of sm α -actin positive cells significantly increases proximal to the thickened epicardium after $T\beta$ 4 treatment, indicating direct connection between the epicardium and new capillary outgrowths.

$T\beta$ 4 initiates embryonic developmental gene expression in adult mouse epicardium

To support the hypothesis that $T\beta$ 4 might activate the adult epicardium and initiate vessel growth we investigated the expression of proteins essential during coronary development in embryos by Western blot and by immunohistochemistry 24 and 72 h after systemic $T\beta$ 4 injection. We found that $T\beta$ 4 affects developmental gene expression as early as 24 h after systemic injection, while alterations in epicardial morphology were first observed 3 days after the initial peptide treatment. We detected significant increase in VEGF, VEGF receptor-2 (Flk-1), and TGF- β expressions and moderate elevation

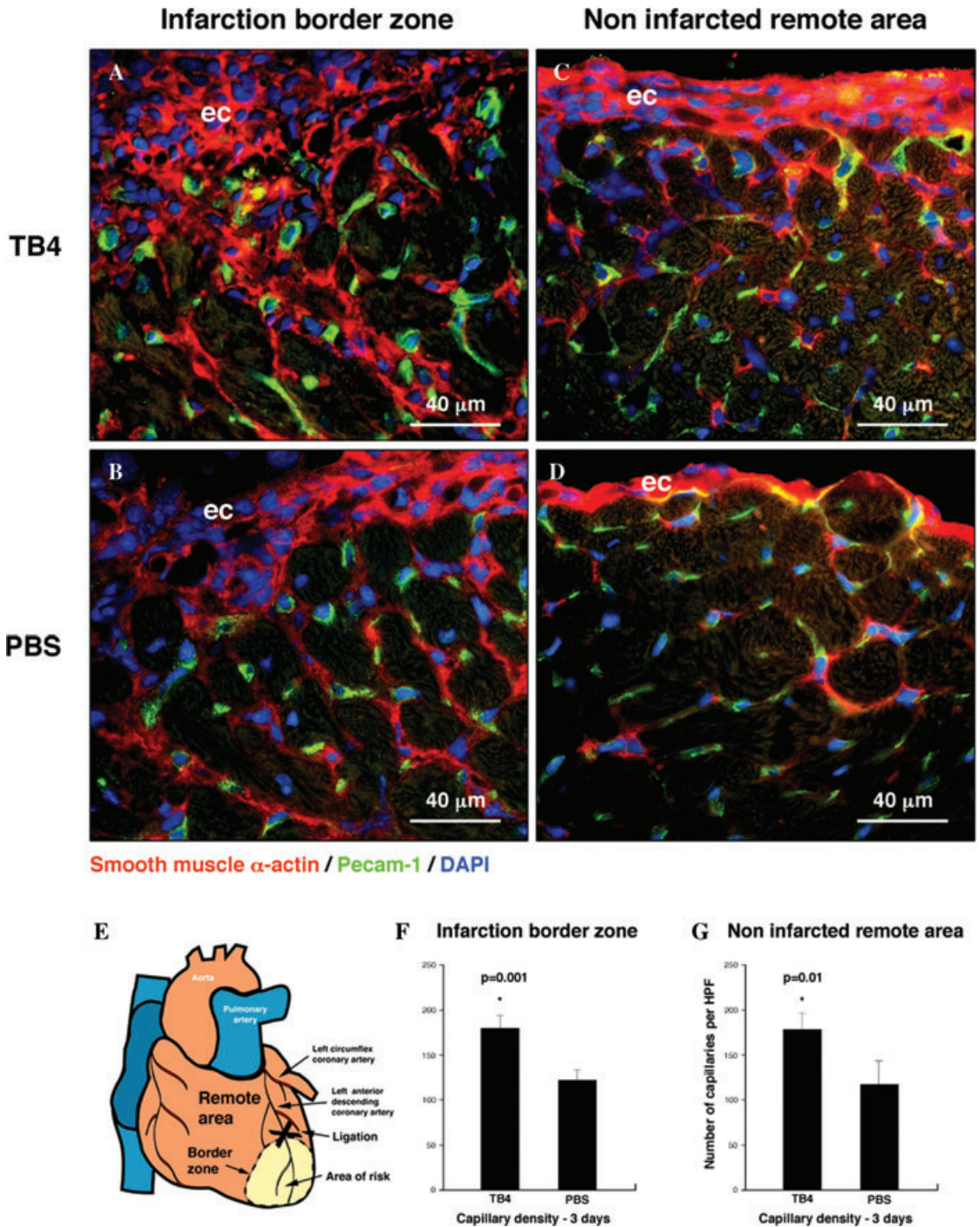


Figure 2. T β 4 initiates cardiac vessel formation *in vivo*. A–D: Immunohistochemistry using sm α -actin (red) and Pecam-1 (green) specific antibodies with DAPI staining (blue) revealed increase in capillary structures at the margin of the scar (A, B) and in the remote areas (C, D) 3 days after T β 4 treatment. E: Diagram of adult mouse heart ligation. F, G: Number of capillaries increases significantly after T β 4 treatment compared to PBS control. Bars indicate standard deviation at 95% confidence limits ($n = 6$), * $P < 0.05$.

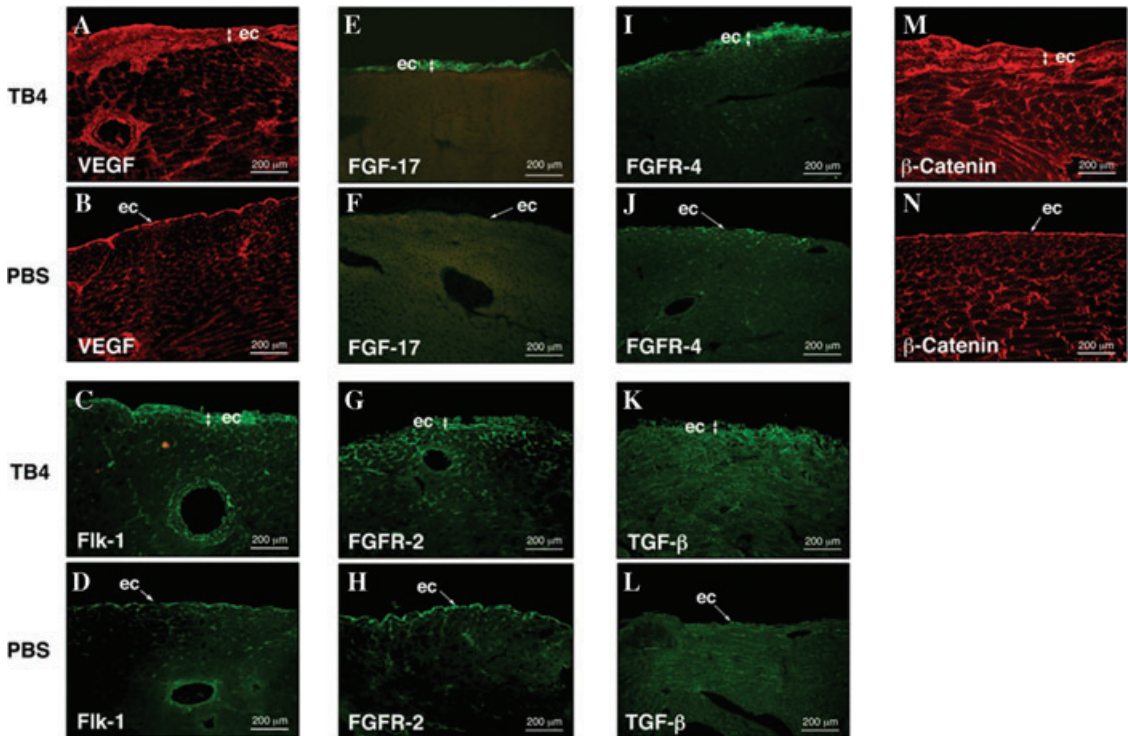


Figure 3. Systemic T β 4 injection beneficially alters the expression of proteins essential for embryonic coronary development in mammals and during cardiac regeneration in adult zebrafish. Immunohistochemical analysis shows increase in VEGF (A, B), Flk-1 (C, D), FGF-17 (E, F), FGFR-2 (G, H), FGFR-4 (I, J), TGF- β (K, L), and β -Catenin (M, N) expressions notably in the thickened epicardium at the intact areas of ligated adult mouse hearts 3 days after T β 4 treatment when compared to PBS. ec, epicardium

in FGF-17, FGF receptor-2 (FGFR-2), and FGFR-4 levels by Western blot after 24 h of treatment. Immunohistochemistry after 3 days of injection indicated that the changes are primarily manifested in the thickened epicardium (Fig. 3). The alterations in gene expression were consistent with the findings in regenerating adult zebrafish hearts.⁷

In our previous work, we showed that T β 4 significantly reduces scar volume by inhibiting myocardial cell death after infarction.⁸ Because of recent studies suggesting that the epicardium serves as a source of cardiomyocytes in the embryo^{15,16} we asked whether T β 4 could also initiate a long-term post ischemic muscle regeneration by myocardial progenitor activation in adult mouse hearts. We found that T β 4 raises the expression of Tbx-18 and Wt-1 in the noninfarcted remote areas after 24 h and significantly increases the number of Tbx-18 and Wt-1 positive cells after 3 days (see Fig. 5K and

L). Tbx-18 positive cells were distributed equally in the epicardium and myocardium, while Wt-1 positive cells were primarily located in the subepicardial space, suggesting that Wt-1 and Tbx-18 may mark different progenitor populations in the activated epicardium. Finally, the analysis of additional regenerative proteins revealed that T β 4 significantly increases Jun N-terminal kinase (JNK) expression, while p38 expression and p38 and JNK activation were significantly reduced. We detected minor alterations in extracellular signal regulated kinase1/2 (Erk1/2) activation, inducible isoform of nitric oxide synthase (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) levels in the noninfarcted cardiac tissue in the first 24 h of treatment. These observations strongly suggest an early molecular support for new vessel formation and myocardial regeneration by initiation of the embryonic epicardial developmental program and by activation of

myocardial progenitors in adult mouse hearts after T β 4 injection *in vivo*.

T β 4 activates PKC in the adult epicardium

To further identify molecules that respond to T β 4 and are expressed in the adult epicardium, we analyzed adult mouse hearts with Mouse Genome 430 2.0 Affymetrix cDNA microarrays. While focusing on genes significant in angiogenesis, myristoylated alanine-rich C-kinase substrate (Marcks) was up-regulated 2.8-fold after T β 4 administration (Fig. 4A, I). Marcks is a prominent intracellular substrate for PKC, a regulator of angiogenesis,¹⁷ and is distributed in numerous cell types, including vascular endothelial cells.¹⁸ It mediates PKC signaling through its phosphorylation,¹⁹ resulting in a release of Marcks from the cell membrane to the cytosol. These responses are commonly used to indicate PKC activity *in vitro*.²⁰

To determine if T β 4 affects PKC activation in cell culture, we investigated Marcks phosphorylation and localization in adult HCECs by Western blot and immunocytochemistry after T β 4 treatment. Our results indicate that external administration of T β 4 increases Marcks expression, phosphorylation, and translocation from the cell membrane to the cytosol, suggesting that T β 4 modulates PKC activity (Fig. 4B, C, H). PKC lies on the signal transduction pathways by which VEGF augments development and angiogenesis during initial and later stages of vessel development.⁵ Since VEGF expression was significantly increased after T β 4 treatment (Fig. 3) and PKC is known to activate Akt by Ser473 phosphorylation,^{5,21} we speculate that VEGF mediated PKC activation represents an ILK-independent means of Akt activation by T β 4 in cardiac cells.

To confirm our *in vitro* results, we examined the effect of T β 4 on Marcks expression and phosphorylation after cardiac ligation in adult mice. As shown by Western blot (Fig. 4J) and immunohistochemistry, the expression (Fig. 4D, E, J) and phosphorylation (Fig. 4F, G, J) of Marcks increased, especially in the thickened epicardium, indicating a role for PKC in epicardial activation and coronary re-growth in adults after T β 4 treatment *in vivo*. Additionally, analysis of Bves, T β 4, VEGF, and p-Marcks expression showed an increase and co-localization of Bves, T β 4, and VEGF in the upper layer of the thickened epicardium after T β 4 treatment, while p-Marcks-positive cells were mainly found in the

subepicardium. This is consistent with an indirect activation of PKC by T β 4, possibly through increasing VEGF expression in the primary epicardial cells.

Inhibition of PKC activity suppresses T β 4 initiated epicardial activation in adult mice

To define whether PKC activity might be regulatory for T β 4 induced epicardial progenitor activation, we tested the effect of Bisindolylmaleimide-I (PKC inhibitor) on HCECs and adult epicardial cells *in vitro*, and injected 10 μ g of PKC inhibitor intraperitoneally with or without T β 4 into infarcted adult mice. Our *in vitro* experiments indicated that PKC inhibition alters HCEC capillary structure formation and inhibits epicardial cell differentiation on matrigel. The newly formed structures contained irregular sm α -actin positive cells and showed disordered morphology. In live animals immunohistochemistry with Bves, VEGF (data not shown) or p-Marcks (Fig. 5E–H) specific primary antibodies showed reduction of epicardial thickening. We also observed a significant decrease in capillaries and sm α -actin positive cells at the noninfarcted remote areas (Fig. 5A–D, I, J) or borders of the infarction after Bisindolylmaleimide-I injection. This suggests reduction of coronary outgrowths from the epicardium in the T β 4 + PKC inhibitor treated infarcted hearts. In addition, Bisindolylmaleimide-I significantly suppressed the number of T β 4 activated Tbx-18 and Wt-1 positive progenitor cells in the noninfarcted remote areas of the hearts (Fig. 5K and L). Our results suggest that T β 4 mediated direct or indirect PKC activation is essential for epicardial cell transformation and migration in the adult mouse heart *in vivo* (Fig. 6).

Discussion

Our studies show that T β 4 minimizes cardiomyocyte loss after cardiac infarction, increases or activates proteins and epicardial progenitors important for myocardial regeneration or vascular growth, and initiates organwide activation of the adult epicardium reminiscent of embryonic coronary development by restimulation of signaling pathways essential during embryogenesis. It reveals a novel role for PKC in this process.

Despite the specificity of the signaling systems triggering angiogenic responses in endothelial cells, the signaling pathways or transcription factors

A

GenBank ID	Gene name	Spotfire	SAM	Gene Spring	ANOVA
BB332426	Myristoylated alanine rich protein kinase C substrate (Marcks)	2.8	2.8	2.8	0.002066892

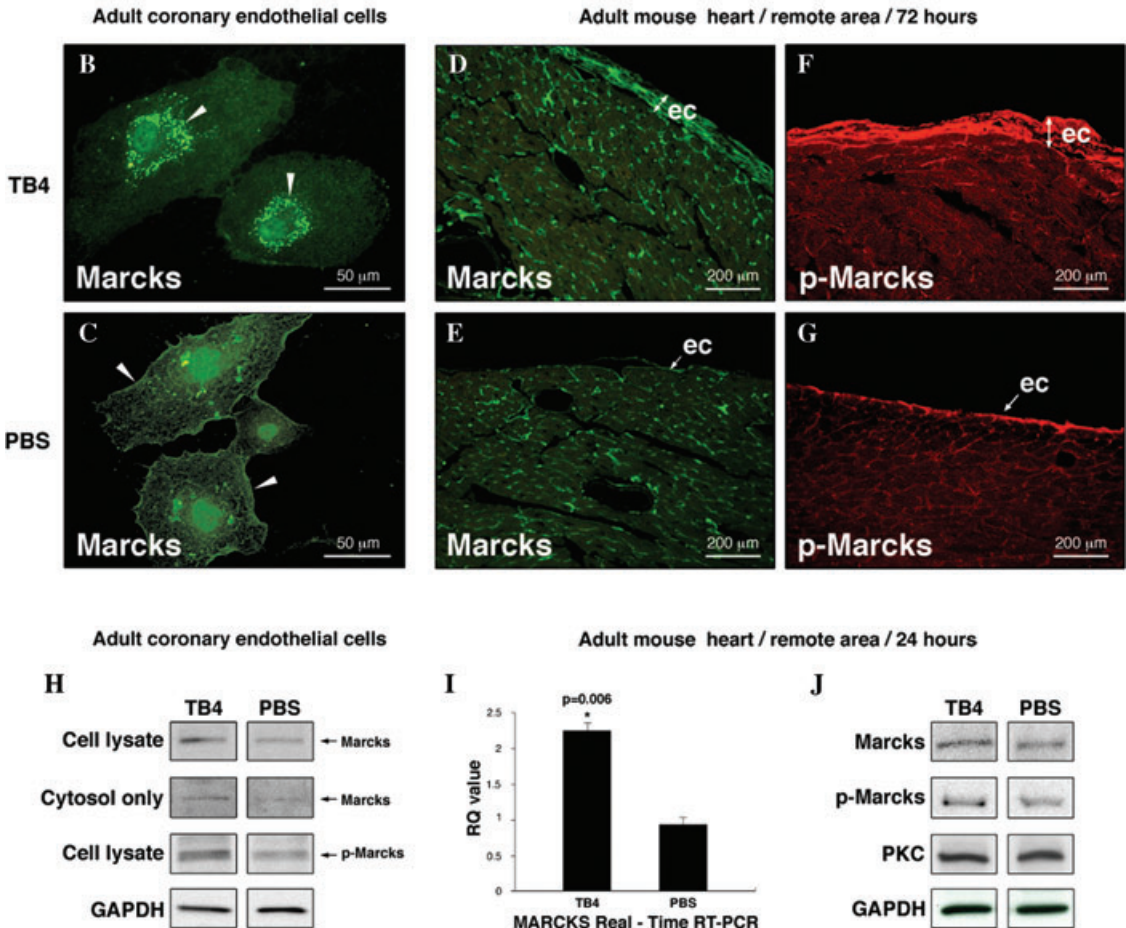


Figure 4. T β 4 alters Marcks expression and activates PKC in adult epicardium *in vivo*. **A:** cDNA microarray on adult mouse hearts reveals 2.8-fold increase in Marcks expression 24 h after T β 4 treatment. **I:** Real-time RT-PCR of mouse heart RNA confirms the *in vitro* cDNA microarray results. **B, C:** Immunocytochemical analysis of HCECs with Marcks-specific antibody (green) shows increased Marcks expression and translocation from the cell membrane (**C** white arrowheads) to the cytosol (**B** white arrowheads) after T β 4 treatment, suggesting a change in PKC activity by T β 4 *in vitro*. **H:** Western blot supports translocation of Marcks protein into the cytosol and indicates increase in Marcks activation by PKC after T β 4 treatment *in vitro*. **D-G:** Immunohistochemical analysis shows significant increase in Marcks and phospho-Marcks expressions in thickened epicardium at the intact areas of ligated adult mouse hearts 3 days after T β 4 treatment. **J,** Western blot analysis of adult cardiac tissue from the remote areas by Marcks, p-Marcks, PKC, and GAPDH antibodies 24 h after treatment show increase in Marcks expression and phosphorylation without significant change in PKC levels 24 h after T β 4 treatment *in vivo*. Bars indicate standard deviation at 95% confidence limits ($n = 6$), * $P < 0.05$.

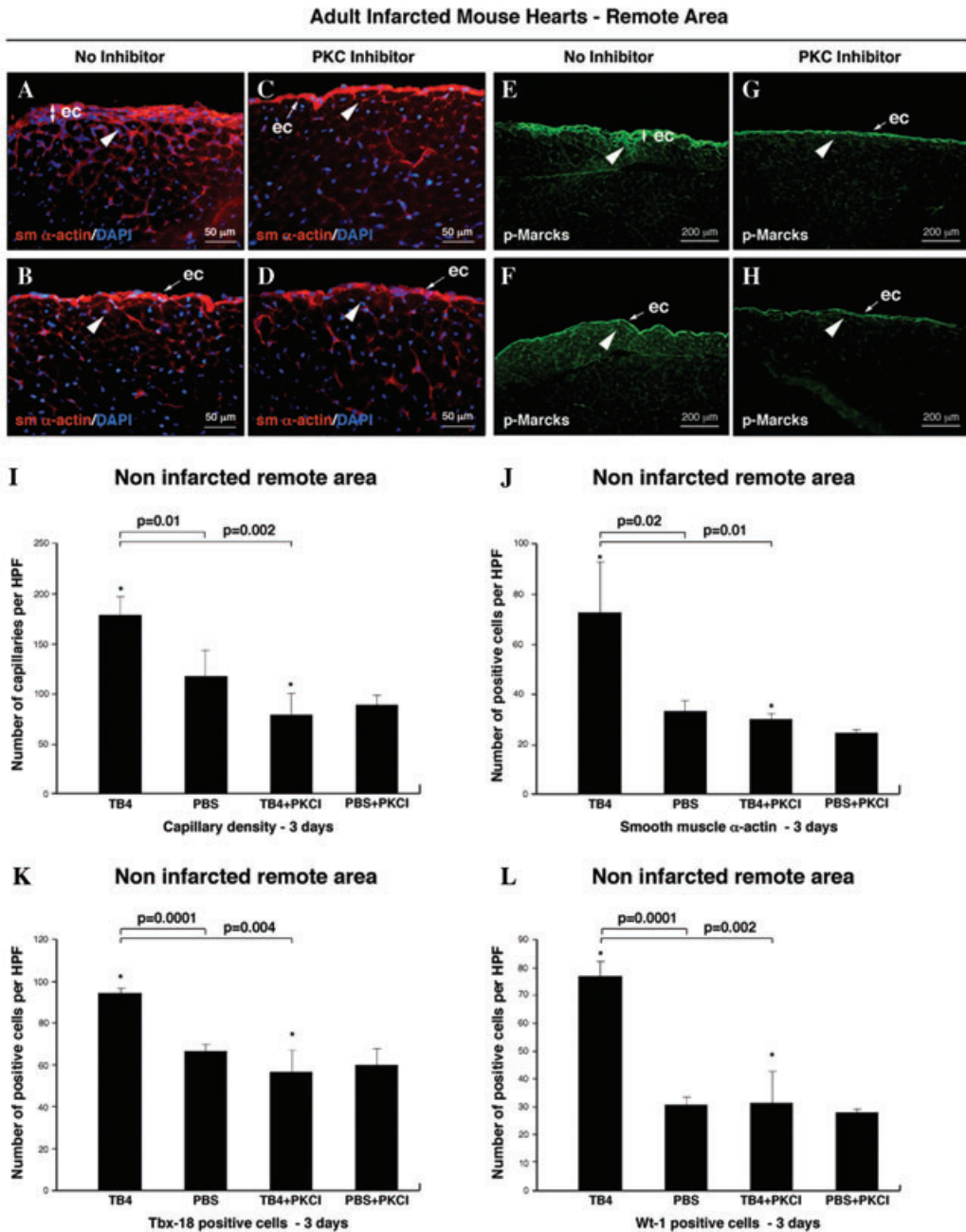


Figure 5. PKC activity is essential for T β 4 induced epicardial activation in adult mice *in vivo*. A-H: Immunohistochemical analyses with anti-sm α -actin (A-D) and anti-p-Marcks (E-H) antibodies show reduced epicardial thickening and inhibition of capillary outgrowths (C, G *white arrowheads*) in T β 4+PKC inhibitor treated adult hearts when compared to T β 4 and no inhibitor treated controls. Decrease in p-Marcks expression indicated sufficient reduction of PKC activity in the inhibited control hearts (H). I, J: Bisindolylmaleinimide-I significantly suppresses the number of capillaries (I) and sm α -actin positive cells (J) in the noninfarcted remote areas of T β 4 treated hearts. K, L: Inhibition of PKC activity significantly suppresses the number of T β 4-activated Tbx-18 and Wt-1 positive myocardial progenitor cells in the noninfarcted remote areas. Bars indicate standard deviation at 95% confidence limits ($n = 6$), * $P < 0.05$.

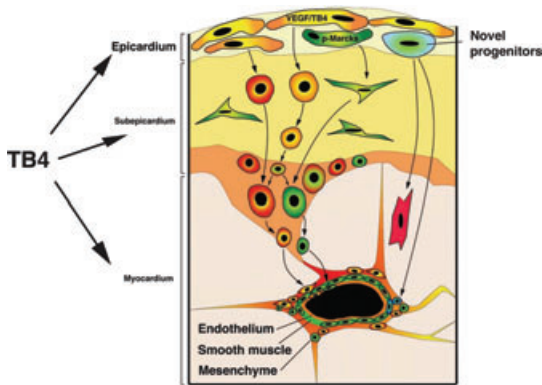


Figure 6. Effects of T β 4 on cardiac progenitor cell differentiation. The demonstrated effects of T β 4 on functional recovery and cardiac progenitor activation makes T β 4 an outstanding tool to understand and analyze the biological and molecular changes during cell reprogramming, and to identify and characterize novel progenitor cell populations of the adult mammalian heart.

known to regulate the entire process are diverse.⁵ Given the roles of Pecam-1, VEGF, MAP kinases, ILK, Akt, FGFs, NOSes, β -Catenin, and PKC in vessel formation, our findings suggest that T β 4 may initiate broad angiogenic events that promote cardiac regeneration after cardiac injury in adults. This is consistent with the recent observations that T β 4 regulates epicardial development in embryonic mice.²²

Our findings also suggest that T β 4 augments cardiac regeneration and increases cardiac function in the adult hypoxic heart through at least two steps. First, it inhibits myocardial cell death 24 h after cardiac ligation.⁸ Second, T β 4 can initiate signaling pathways responsible for late-phase regeneration, such as vascular regrowth or progenitor cell activation.¹⁰ This process may be initiated by organwide epicardial thickening and activation of endothelial–mesenchymal transformation of adult epicardial cells, is most likely regulated by PKC, and is first visible 3 days after T β 4 administration.

Coronary vessel growth is independent of cells outside the heart once the epicardium is formed.²³ Thus the potential for T β 4 to activate dormant cardiac stem cells that exist in the adult mammalian heart is critical for cardiac regeneration. Furthermore, as T β 4 also inhibits inflammation,^{24–26} this property may be also supportive during cardiac regeneration in adults.

Generating novel therapies to achieve coupled myocardial and vascular regeneration by recalling the embryonic program in adults is a rapidly expanding concept²⁷ and may be a solution to aid the failing heart. The work presented here describes a molecule with such capabilities. While T β 4 can augment an organism's ability to heal surface wounds,^{25,28} our results demonstrate T β 4's efficacy in repairing of a solid organ and reveal novel mechanisms through which T β 4 affects cellular functions.

As the discovery of innovative methods to enhance cardiac regeneration is important toward future therapies, the continued investigation of molecular signals initiated by T β 4 is highly essential. Given the findings here, the utility of T β 4 for healing after cardiac injury suggests promise and warrants further pre-clinical investigation.

Conflicts of interest

The authors declare no conflicts of interest.

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