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Cardiac repair with thymosin β4 and cardiac reprogramming factors

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Heart disease is a leading cause of death in newborns and in adults. We previously reported that the G-actin–sequestering peptide thymosin β4 promotes myocardial survival in hypoxia and promotes neoangiogenesis, resulting in cardiac repair after injury. More recently, we showed that reprogramming of cardiac fibroblasts to cardiomyocyte-like cells *in vivo* after coronary artery ligation using three cardiac transcription factors (Gata4/Mef2c/Tbx5) offers an alternative approach to regenerate heart muscle. We have combined the delivery of thymosin β4 and the cardiac reprogramming factors to further enhance the degree of cardiac repair and improvement in cardiac function after myocardial infarction. These findings suggest that thymosin β4 and cardiac reprogramming technology may synergistically limit damage to the heart and promote cardiac regeneration through the stimulation of endogenous cells within the heart.

Keywords: thymosin β4; cardiac reprogramming; myocardial infarction; cardiac repair

Introduction

Heart disease is the leading cause of death in the Western world.[1] Because the heart is incapable of sufficient muscle regeneration, survivors of myocardial infarctions typically develop chronic heart failure, with over five million cases in the United States alone.[1] Although more commonly affecting adults, heart disease in children is the leading noninfectious cause of death in the first year of life and often involves abnormalities in cardiac cell specification, migration, or survival.[2]

Recent evidence suggests that a population of extracardiac or intracardiac stem cells may contribute to maintenance of the cardiomyocyte population under normal circumstances.[3] Although the stem cell population may maintain a delicate balance between cell death and cell renewal, it is insufficient for myocardial repair after acute coronary occlusion. Introduction of isolated adult stem cells may improve myocardial function,[4–6] but this approach has been controversial, and may not contribute substantially to new muscle.[7,8] Pluripotent embryonic stem cells and induced pluripotent stem cells can be efficiently differentiated into the cardiomyocyte lineage using timed delivery of growth factors, and as a result, large numbers of cardiomyocytes can now be made from pluripotent stem cells. However, these cells remain immature, at least *in vitro*. Although technical hurdles of stem cell purification, delivery, and integration have thus far prevented clinical application of pluripotent stem cell–derived cardiomyocytes, the use of embryonic stem cell or induced pluripotent stem cell technology holds great promise for the future.

Regulatory pathways involved in cardiac development may have utility in cardiac repair.[9] In our studies of genes expressed during cardiac morphogenesis, we found that the forty-three amino acid peptide thymosin β4 was expressed in the developing heart. Thymosin β4 has numerous functions, with the most prominent involving sequestration of G-actin monomers and subsequent effects on actin-cytoskeletal organization necessary for cell motility,

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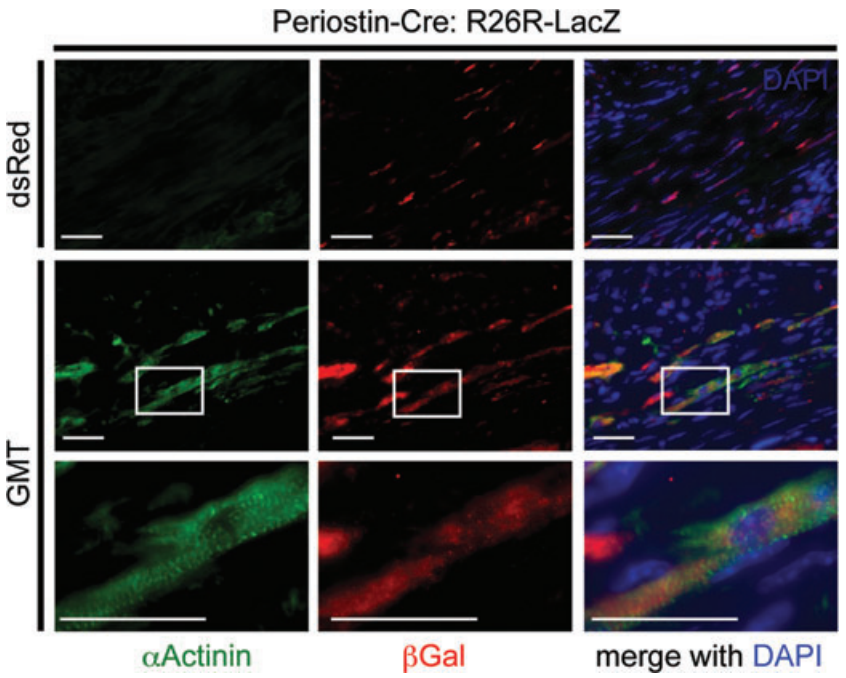

Figure 1. **Genetic lineage tracing demonstrates *in vivo* reprogramming of cardiac fibroblasts to cardiomyocyte-like cells. Immunofluorescent staining for αActinin, βGal, and DAPI on injured areas of dsRed- or GMT-injected Periostin-Cre:R26R-lacZ mouse hearts four weeks post-MI. Boxed areas indicate regions of magnification shown in lower panels. Scale bar, 50 μm. Data are from Ref. 22.**

organogenesis, and other cell biological events.[10–12] Domain analyses indicate that β-thymosins can affect actin assembly based on their carboxy-terminal affinity for actin.[13] Thymosin β4 can promote skin and corneal wound healing through its effects on cell migration, angiogenesis, and cell survival.[14–16] We previously reported that thymosin β4 can activate the survival kinase Akt and can play a potent role in protecting cardiac muscle from death after ischemic damage as occurs in the setting of a myocardial infarction.[17] Thymosin β4 can also promote angiogenesis in ischemic areas[18,19] and was reported to prime epicardial-derived progenitors to differentiate into cardiomyocytes.[20] Thus, there appear to be pleiotropic effects of thymosin β4 related to its role in promoting cardiac repair.

Most recently, we described the ability of three central cardiac developmental factors, Gata4, Tbx5, and Mef2c (GMT), to reprogram resident nonmyocytes in the heart into newly born cardiomyocyte-like cells.[21,22] We initially discovered this method *in vitro* and subsequently described its utility *in vivo* in the setting of myocardial infarction in a mouse model. Over half of all cells in the mammalian heart are cardiac fibroblasts that provide secreted signals to neighboring myocytes, and are activated after injury to form scar, but do not normally become muscle. Delivery of GMT into the nonmuscle population using retroviruses that can only infect dividing cells resulted in transdifferentiation of cardiac fibroblasts into cardiomyocyte-like cells that integrated with other myocytes *in vivo* and contributed to force generation. We also revealed that thymosin β4, in conjunction with delivery of GMT, could induce even greater cardiac repair than GMT alone in the setting of acute coronary occlusion in mice. We will describe the reprogramming process here and the effects of thymosin β4 in this new technology for cardiac repair.

Identification of cardiac reprogramming factors

To find a combination of reprogramming factors, we developed an assay to quantitatively analyze the reprogramming of fibroblasts toward the cardiomyocyte lineage by reporter-based fluorescence-activated cell sorting (FACS).[21] We generated

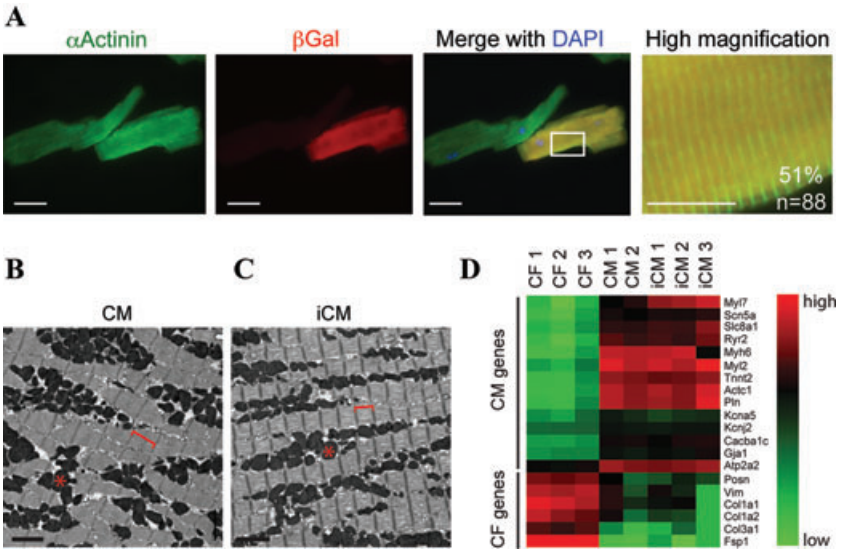

Figure 2. Single-cell analysis of the degree of cardiac reprogramming *in vivo*. (A) Immunofluorescent staining for αActinin, colabeled with βGal and DAPI, on isolated cardiomyocytes (CMs) from the infarct/border zone of periostin-Cre:R26R-lacZ hearts four weeks after GMT injection. Scale bar, 50 μm for the first three panels, 20 μm for the last panel. Red or yellow cells represent iCMs, green cells represent endogenous CMs. (B, C) Electron microscopy of endogenous CMs or iCMs. Asterisk indicates mitochondria and brackets indicate sarcomeric units. Scale bar, 2 μm. (D) Heat map of gene expression for a panel of CM- or fibroblast-enriched genes in isolated adult cardiac fibroblasts (CFs), CMs, or iCMs. Data are from Ref. 22.

αMHC promoter–driven transgenic mice (αMHC-green fluorescent protein), in which cardiomyocytes expressed GFP. Transduction of a mixture of retroviruses expressing 14 transcription factors involved in cardiac development into GFP-negative cardiac fibroblasts *in vitro* resulted in a small number of fibroblasts that became GFP$^+$ cells (1–2%), indicating activation of the cardiac-enriched αMHC gene. Serial deletion of each factor led to a combination of three transcription factors (Gata4, Mef2c, and Tbx5 (GMT)) that were necessary and sufficient to reprogram approximately 15% of postnatal cardiac fibroblasts into αMHC–GFP$^+$ cells that exhibited global reprogramming of gene expression.[21] The shift in cellular state was epigenetically stable and did not require ongoing ectopic gene expression of the reprogramming factors. Most cells assembled sarcomeres and displayed calcium transients, and ∼30% expressed high levels of cardiac Troponin T (cTnT). However, less than 1% of *in vitro* reprogrammed cells spontaneously contracted at five weeks and had action potentials similar to adult ventricular cardiomyocytes, suggesting most were only partially reprogrammed. We termed the reprogrammed cells *induced cardiomyocytes* (iCMs). Others have found that fibroblasts can be directly converted to neuron-like cells,[23] hematopoietic-like cells,[24] and hepatocyte-like cells[25,26] using various cocktails of transcription factors and microRNAs.

In vivo cardiac repair with reprogramming technology

More recently, we introduced GMT directly into the mouse heart by intramyocardial injection of viral vectors to assay *in vivo* reprogramming of fibroblasts.[22] Using a genetic lineage-tracing approach, we showed that nonmyocytes in the heart, mostly cardiac fibroblasts, converted into cardiomyocyte-like cells four weeks after coronary ligation and intramyocardial injection of GMT (Fig. 1). Approximately one-third of cells in the border zone of injury that expressed sarcomeric genes were positive for β-Galactosidase (β-Gal) activity or the yellow fluourescent protein (YFP), activated by the fibroblast enriched promoter of Periostin or Fsp1 driving cre-recombinase. This result suggested the β-Gal$^+$ cells that developed sarcomeres were descendants of periostin or Fsp1-expressing nonmuscle cells. Importantly, we did not find evidence for cell fusion after genetically labeling endogenous cardiomyocytes and marking cells that were infected with retrovirus.

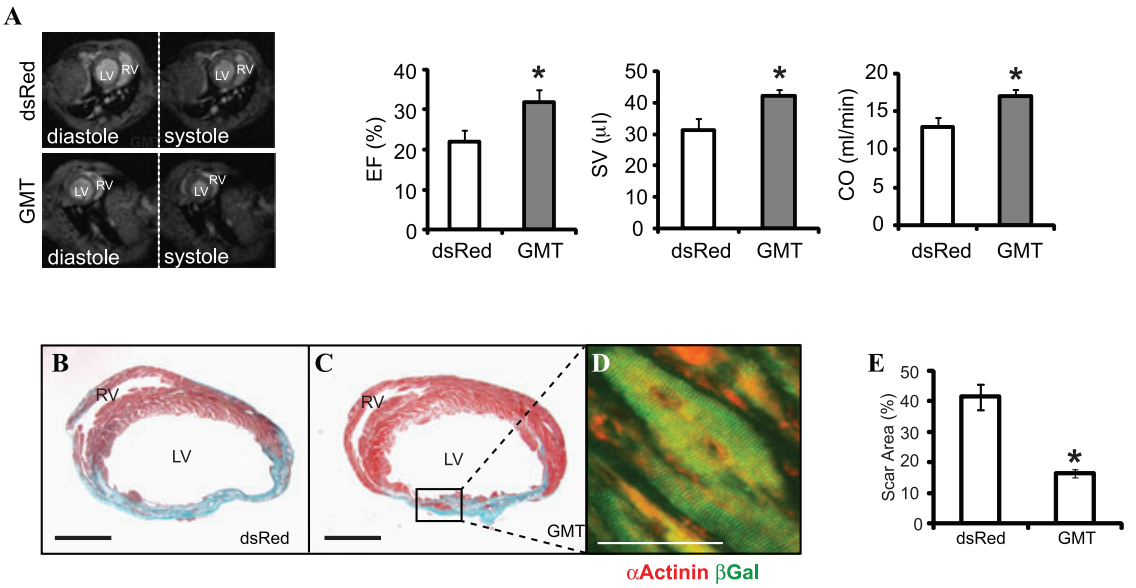

Figure 3. *In vivo* delivery of cardiac reprogramming factors improves cardiac function after myocardial infarction. (A) Ejection fraction (EF), stroke volume (SV), and cardiac output (CO) were quantified by MRI (left panels) 12 weeks after MI ($n = 9$/group, $^*P < 0.05$). Representative MRI thoracic images are shown in diastole and systole with dsRed or GMT injection. (B, C) Masson–Trichrome staining on heart sections of Periostin-Cre: R26RLacZ mice eight weeks post-MI injected with dsRed or GMT. Scale bars, 500 μm. (D) αActinin + βGal$^+$ cells in the infarct area. Scale bar, 50 μm. (E) Calculation of scar area (dsRed, $n = 8$; GMT, $n = 9$; $^*P < 0.05$). Scale bars: 500 μm. Error bars indicate standard error of the mean (S.E.M.). Data are from Ref. 22.

Morphologically, half of iCMs were large with a rod-shaped appearance and were binucleated, closely resembling endogenous cardiomyocytes from the same isolation. Further analyses revealed that, in addition to α-actinin, the β-galactosidase$^+$ cells expressed multiple sarcomeric markers, including tropomyosin, cardiac muscle heavy chain (αMHC), and cTnT. Examples of cells that showed nearly normal sarcomeric structures throughout the cell, representing ∼50% of cells, are shown in Figure 2A, while others had intermediate levels of sarcomeric organization in the cell. Similar results were obtained by electron microscopy, with a range of ultrastructural organization, although almost all had well-developed and abundant mitochondria (Fig. 2B and C). qPCR on a panel of 20 genes normally enriched in CMs or cardiac fibroblasts indicated the gene expression of iCMs was similar to that of CMs (Fig. 2D). Importantly, isolated cells also expressed and localized gap junction proteins, such as connexin 43 and N-cadherin. Small dyes could pass through the gap junction, and calcium waves could be propagated from an induced cardiomyocyte to an endogenous cardiomyocyte. Individual YFP$^+$ iCMs isolated from the heart had ventricular-like action potentials by patch-clamp studies and ∼50% of isolated YFP$^+$ cells beat similarly to endogenous cardiomyocytes upon stimulation, while others were incompletely depolarized, suggestive of only partial reprogramming.

Because *in vivo* reprogrammed iCMs had contractile potential and electrically coupled with viable endogenous cardiomyocytes, we determined if converting endogenous cardiac fibroblasts into myocytes translates into partial restoration of heart function after MI. Mice injected with GMT or dsRed alone into the border/infarct zone immediately after coronary ligation were evaluated after three months for cardiac function by magnetic resonance imaging (MRI) and high resolution 2-D echocardiography. MRI provides the most accurate three-dimensional assessment of the fraction of blood ejected with each ventricular contraction (ejection fraction), the volume of blood ejected (stroke volume), and the total cardiac output per minute (Fig. 3A). Each of these parameters was significantly improved with GMT infection, particularly the stroke volume and cardiac output, possibly due to cardiac enlargement. Serial echocardiography showed similar results, with improvement detectable by eight weeks after injection.

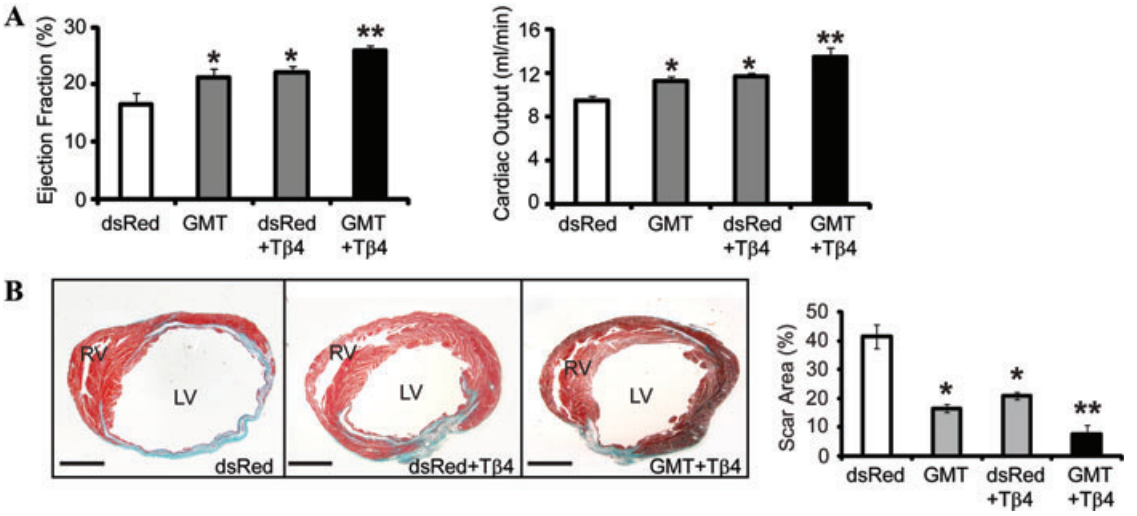

Figure 4. Thymosin β4 (Tβ4) enhances the benefits of *in vivo* cardiac reprogramming with GMT. (A) Ejection fraction (EF) and cardiac output (CO) of the left ventricle were determined using high-resolution echocardiography eight weeks postsurgery after injection of dsRed ($n = 9$); GMT ($n = 10$); dsRed + Tβ4 ($n = 10$); GMT + Tβ4 ($n = 8$); ($^{*}P < 0.05$, $^{**}P < 0.01$). (B) Scar area was calculated in a blinded fashion from multiple heart sections eight weeks post-MI after dsRed ($n = 8$), GMT ($n = 9$), dsRed + Tβ4 ($n = 7$), or GMT + Tβ4 ($n = 8$) injection. Representative Masson–Trichrome staining on heart sections is shown. Scale bars: 500 μm. Quantification of scar size was calculated by measuring the scar area in a blinded fashion. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars indicate standard error of the mean (S.E.M.). Data are from Ref. 17.

In agreement with the improvement of cardiac function, calculation of scar area with 16 sections at four levels of the heart revealed a significantly smaller scar size eight weeks after MI in the GMT-treated group, with presence of iCMs in the area of scar (Fig. 3B–E).

Enhanced cardiac repair with thymosin β4 and cardiac reprogramming factors

We and others had previously shown that thymosin β4 could promote cardiac cell migration, activate proliferation of cardiac fibroblasts and endothelial cells, and promote neoangiogenesis.[17–19] One mechanism by which thymosin β4 functions is through interaction with PINCH and integrin linked kinase (ILK) as part of a larger complex involved in cell-extracellular matrix interactions known as the focal adhesion complex. PINCH and ILK are required for cell motility[27,28] and for cell survival, in part, by promoting phosphorylation of the serine-threonine kinase Akt/protein kinase B, a central kinase in survival and growth signaling pathways.[27–30] All three proteins could be isolated as a complex, and we have demonstrated that thymosin β4 induces activation of signaling events downstream of ILK, particularly, phosphorylation and activation of Akt.[17]

In rodent and pig models of myocardial infarction, thymosin β4 had potent effects in limiting the amount of damage caused by coronary ligation.[17,31] Thymosin β4 administration appeared to promote cell survival and initiate neoangiogenesis in the area of hypoxia. More recently, thymosin β4 administration before injury appeared to prime a population of epicardium-derived progenitor cells to become new cardiomyocytes.[20]

Although GMT delivery significantly affected cardiac repair after MI, we hypothesized that concurrent administration of thymosin β4 might further enhance the degree of repair, in part by increasing the number of fibroblasts that became activated and proliferative, and through promoting angiogenesis. To test the effects of thymosin β4 on cardiac fibroblast migration, we used a cardiac explant migration assay.[17,21] The average time for fibroblasts to migrate from adult heart explants was approximately three weeks; however, with thymosin β4, equivalent fibroblast migration was observed after only two weeks and occurred within three days after MI. Similarly, the proliferation of Vimentin^{+} cells increased after MI and was even more increased following administration of thymosin β4, as marked by phosphohistone H3. Consistent with the activation of fibroblasts by thymosin β4, the percent

of Thy1$^+$ or vimentin$^+$ cells (markers of fibroblasts) infected by retrovirus in the setting of MI more than doubled upon intramyocardial injection of thymosin β4. The improved delivery of GMT-expressing retrovirus to more cells by addition of thymosin β4 resulted in an increase in the percentage of iCMs compared to total CMs in single-cell CM culture from the infarct/border zone of periostin-Cre:R26R-lacZ hearts (51% vs. 35%, $P < 0.05$).

Injection of thymosin β4 immediately after coronary ligation resulted in improvement of cardiac function, as previously reported.[17,31] Coinjection of thymosin β4 and GMT yielded further functional improvement in ejection fraction and cardiac output eight weeks after infarction (Fig. 4A). In agreement with this, coinjection of thymosin β4 and GMT caused a greater reduction in scar size than either thymosin β4 or GMT injection alone (Fig. 4B), despite the area at risk and initial infarct size being similar in both groups. The combined effects of GMT and thymosin β4 on cardiac function were particularly powerful and suggest that the two approaches may synergize with one another to limit damage to the heart and promote cardiac repair.

The full mechanism of cardiac repair with GMT and/or with thymosin β4 remains to be determined. Future studies examining the DNA-occupancy of the critical transcription factors will be informative, as will evaluation of the time course of the reprogramming process. It is possible that the small population of epicardium-derived cells that may give rise to new myocytes after pretreatment with thymosin β4 could be contributing to the effects observed in our study, although no effect was observed by Smart *et al.* when thymosin β4 was given at the time of injury.[20] Nevertheless, it is possible that transduction of GMT into these progenitors, or other rare progenitors yet to be identified, could promote their differentiation into cardiomyocytes. Because thymosin β4 is also a proangiogenic factor,[18,19] the full mechanism of cooperativity between GMT and thymosin β4 may be multifaceted and will be interesting to explore.

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Conflicts of interest

The authors declare no conflicts of interest.

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