

Thymosin β 4 Is Cardioprotective after Myocardial Infarction

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ABSTRACT: Heart disease is a leading cause of death in newborns and in adults. Efforts to promote cardiac repair by introduction or recruitment of exogenous stem cells hold promise but typically involve isolation and introduction of autologous or donor progenitor cells. We have found that the G-actin-sequestering peptide thymosin β 4 promotes myocardial and endothelial cell migration in the embryonic heart and retains this property in postnatal cardiomyocytes. Survival of embryonic and postnatal cardiomyocytes in culture was also enhanced by thymosin β 4. We found that thymosin β 4 formed a functional complex with PINCH and integrin-linked kinase (ILK), resulting in activation of the survival kinase Akt/PKB, which was necessary for thymosin β 4's effects on cardiomyocytes. After coronary artery ligation in mice, thymosin β 4 treatment resulted in upregulation of ILK and Akt activity in the heart, enhanced early myocyte survival, and improved cardiac function. These findings suggest that thymosin β 4 promotes cardiomyocyte and endothelial migration, survival, and repair and may be a novel therapeutic target in the setting of acute myocardial damage.

KEYWORDS: thymosin β 4; cardiac repair; integrin-linked kinase

INTRODUCTION

Coronary artery disease results in acute occlusion of cardiac vessels leading to the loss of dependent myocardium. Such events are one of the leading causes 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 10 million cases in the United

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States alone.¹ While 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.³⁻⁵ While 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 stem cells may improve myocardial function,³⁻⁵ but this approach has been controversial,^{6,7} and requires isolation of autologous stem cells or use of donor stem cells along with immunosuppression. Efforts to efficiently coax pluripotent embryonic stem cells into a cardiomyocyte lineage remain unsuccessful,⁸ although discovery of pathways involved in early cardiac commitment may reveal methods to encourage cardiac differentiation.⁹ Technical hurdles of stem cell delivery and differentiation have thus far prevented broad clinical application of cardiac regenerative therapies.

Regulatory pathways involved in cardiac development may have utility in reprogramming cardiomyocytes to aid in cardiac repair.¹⁰ In our studies of genes expressed during cardiac morphogenesis, we found that the 43-amino acid peptide thymosin β 4 was expressed in the developing heart. Thymosin β 4 has numerous functions, with the most prominent involving sequestration of G-actin monomers and subsequent effects on actin-cytoskeletal organization necessary for cell motility, organogenesis, and other cell biological events.¹¹⁻¹³ Domain analyses indicate that β -thymosins can affect actin assembly based on their carboxy-terminal affinity for actin.¹⁴ Although thymosin β 4 promotes skin and corneal wound healing through its effects on cell migration, angiogenesis, inhibition of scar formation, and possibly cell survival,¹⁵⁻¹⁷ the precise molecular mechanism through which it functions and its potential role in solid organ wound healing remains unknown. We have found that thymosin β 4 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.¹⁸ These results are described below.

Secreted Thymosin β 4 Stimulates Cardiac Cell Migration and Survival

Although thymosin β 4 is found in the cytosol and nucleus and functions intracellularly,¹² we found that conditioned medium of Cos1 cells transfected with myc-tagged thymosin β 4 contained thymosin β 4 detectable by Western blot, consistent with previous reports of thymosin β 4 secretion and presence in wound fluid.^{17,19,20} Upon expression of thymosin β 4 on the surface of phage particles added extracellularly to embryonic cardiac explants, we found that an antiphage antibody coated the cell surface and was ultimately detected

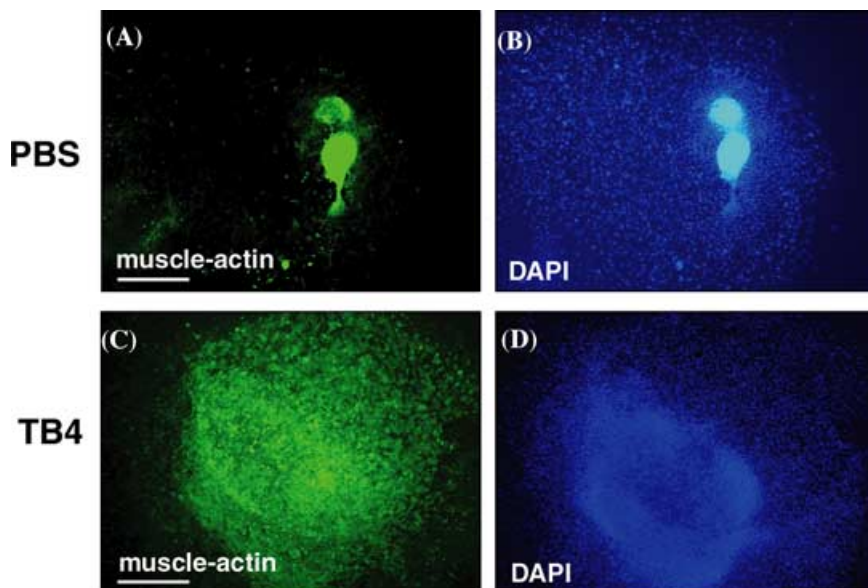


FIGURE 1. Thymosin $\beta 4$ promotes cardiac cell migration and survival. (A–D) Mouse E11.5 cardiac outflow tract explants stained with antimuscle actin antibody (*green*) after PBS (A) or thymosin $\beta 4$ (C) treatment. (B) and (D) are DAPI stains of (A) and (C), respectively. Bars represent 500 μm . (In color in Annals online.)

intracellularly in the cytosol and nucleus while control phage was not detectable.¹⁸ These data indicated that secreted thymosin $\beta 4$ may be internalized into cells, as previously suggested, although the mechanism of cellular entry remains to be determined.

To test the effects of secreted thymosin $\beta 4$ on cardiac cell migration, we took advantage of an embryonic heart explant system designed to assay cell migration and transformation events on a three-dimensional collagen gel.²¹ In this assay, explants of adjacent embryonic myocardium and endocardium from valve-forming regions are placed on a collagen gel with the endocardium adjacent to the collagen. Signals from cardiomyocytes induce endocardial cell migration, but myocardial cells do not normally migrate onto the collagen in significant numbers (FIG. 1A, B). In contrast, upon addition of thymosin $\beta 4$ to the primary explants, we observed a large number of spontaneously beating, cardiac muscle actin-positive cells that had migrated away from the explant (FIG. 1C, D; $P < 0.0001$).

Similar to embryonic cardiomyocytes, we found that the migrational distance of thymosin $\beta 4$ -treated neonatal cardiomyocytes was significantly increased compared to control. In addition to thymosin $\beta 4$'s effects on myocardial cell migration, we observed a similar effect on endothelial migration in the embryonic heart explant assay.

Primary culture of neonatal cardiomyocytes typically survived for approximately 1 to 2 weeks with some cells beating up to 2 weeks when grown on laminin-coated slides in our laboratory. Surprisingly, neonatal cardiomyocytes survived significantly longer upon exposure to thymosin β 4 with rhythmically contracting myocytes visible for up to 28 days.¹⁸

Thymosin β 4 Activates ILK and Akt/Protein Kinase B

To investigate the potential mechanisms by which thymosin β 4 might be influencing cell migration and survival events, we searched for thymosin β 4-interacting proteins. Using phage display, we found that PINCH, a LIM domain protein, was most consistently isolated in an interaction screen with thymosin β 4 and this was confirmed by coimmunoprecipitation. PINCH and integrin-linked kinase (ILK) interact directly with one another and indirectly with the actin cytoskeleton 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^{22,23} 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.^{22–25} All three proteins could be isolated as a complex and we have demonstrated that thymosin β 4 induces activation of signaling events downstream of ILK, particularly, phosphorylation and activation of Akt.¹⁸

Because recruitment of ILK to the focal adhesion complex is important for its activation, we assayed the effects of thymosin β 4 on ILK localization and expression. ILK detection by immunocytochemistry was markedly enhanced around the cell edges after treatment of embryonic heart explants or C2C12 myoblasts with synthetic thymosin β 4 protein or thymosin β 4-expressing plasmid (FIG. 2A). Western analysis indicated a modest increase in ILK protein levels in C2C12 cells, suggesting that the enhanced immunofluorescence may be in part due to altered localization by thymosin β 4 (FIG. 2B). We found that upon thymosin β 4 treatment of C2C12 cells, ILK was functionally activated, evidenced by increased phosphorylation of its known substrate Akt,²⁴ using a phosphospecific antibody to serine 473 of Akt (FIG. 2B), while total Akt protein was unchanged. The effects of thymosin β 4 *in vitro* could be inhibited using an ILK inhibitor suggesting that the survival effects on cardiomyocytes was likely via Akt activation.

Thymosin β 4 Promotes Cell Survival after Myocardial Infarction and Improves Cardiac Function

Because of thymosin β 4's effects on survival and migration of cardiomyocytes cultured *in vitro* and phosphorylation of Akt, we tested whether thymosin β 4 might aid in cardiac repair *in vivo* after myocardial damage. We

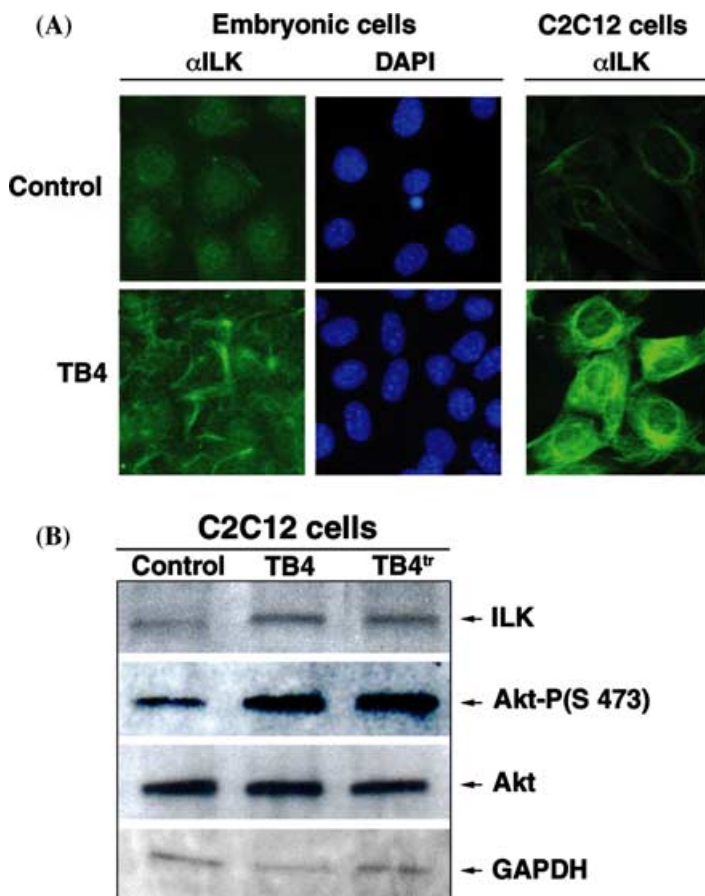


FIGURE 2. Thymosin β 4 forms a functional complex with PINCH and ILK resulting in phosphorylation of Akt. (A) Immunocytochemistry with anti-ILK antibody (*green*) and DAPI (*blue*) marking nuclei after thymosin β 4 treatment of embryonic cardiac explants or C2C12 myoblasts. (B) Western blot of C2C12 cells treated with thymosin β 4 protein or transfected with thymosin β 4-expressing plasmid (TB4^{tr}) using anti-ILK antibody, phosphospecific antibody to Akt-S⁴⁷³ or antibody to Akt. Loading controls with GAPDH levels are shown. (In color in Annals online.)

created myocardial infarctions in 58 adult mice by coronary artery ligation and treated half with systemic, intracardiac, or systemic plus intracardiac thymosin β 4 immediately after ligation and the other half with phosphate-buffered saline (PBS) (FIG. 3). Intracardiac injections were done with collagen (control) or collagen mixed with thymosin β 4. All 45 mice that survived 2 weeks later were interrogated for cardiac function by random-blind ultrasonography at 2 and 4 weeks after infarction by multiple measurements of cardiac contraction.

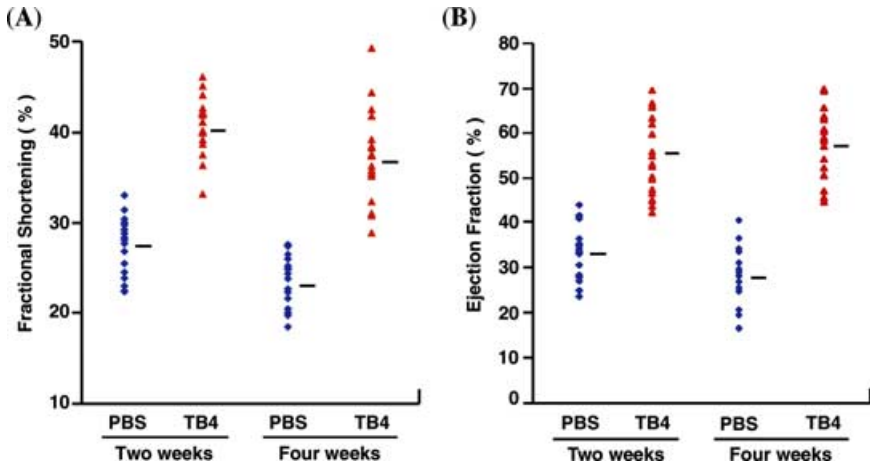


FIGURE 3. Thymosin $\beta 4$ treatment after coronary ligation improves myocardial function *in vivo*. (**A**, **B**) Distribution of left ventricular fractional shortening (**A**) or ejection fraction (**B**) at 2 and 4 weeks after coronary ligation with ($n = 23$) or without ($n = 22$) thymosin $\beta 4$ treatment. Values are averages of multiple echocardiographic measurements of each mouse with mean of each cohort indicated by a bar.

Four weeks after infarction, left ventricles of control mice had a mean fractional shortening of $23.2 \pm 1.2\%$ ($n = 22$, 95% confidence interval); in contrast, mice treated with thymosin $\beta 4$ had a mean fractional shortening of $37.2 \pm 1.8\%$ ($n = 23$, 95% confidence intervals; $P < 0.0001$) (FIG. 3A). As a second measure of ventricular function, two-dimensional echocardiographic measurements revealed that the mean fraction of blood ejected from the left ventricle (ejection fraction) in thymosin $\beta 4$ -treated mice was $57.7 \pm 3.2\%$ ($n = 23$, 95% confidence interval; $P < 0.0001$) compared to a mean of $28.2 \pm 2.5\%$ ($n = 22$, 95% confidence interval) in control mice after coronary ligation (FIG. 3B). The greater than 60% or 100% improvement in cardiac fractional shortening or ejection fraction, respectively, suggested a significant improvement with exposure to thymosin $\beta 4$, although cardiac function remained depressed compared to sham-operated animals ($\sim 60\%$ fractional shortening; $\sim 75\%$ ejection fraction). Finally, the end diastolic dimensions and end systolic dimensions were significantly higher in the control group, indicating that thymosin $\beta 4$ treatment resulted in decreased cardiac dilation after infarction, consistent with improved function. Remarkably, the degree of improvement when thymosin $\beta 4$ was administered systemically through intraperitoneal injections or only locally within the cardiac infarct was not statistically different, suggesting that the beneficial effects of thymosin $\beta 4$ likely occurred through a direct effect on cardiac cells rather than through an extracardiac source.

To determine the manner in which thymosin $\beta 4$ improved cardiac function, we examined multiple serial histologic sections of hearts treated with or without

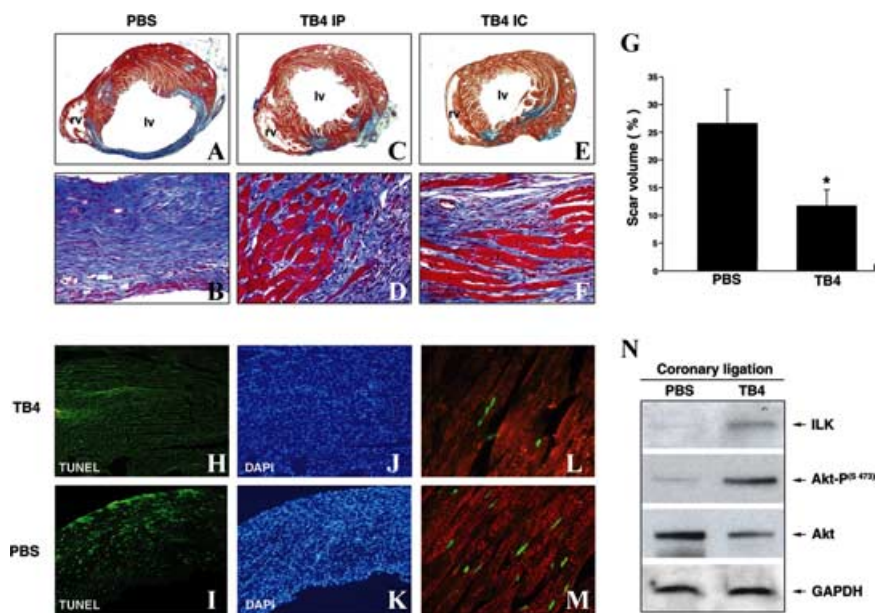


FIGURE 4. Thymosin β 4 promotes survival and alters scar formation after coronary artery ligation in mice. (**A–F**) Representative trichrome stain of transverse heart sections at comparable levels 14 days after coronary ligation and PBS (**A, B**) treatment or thymosin β 4 treatment delivered intraperitoneally (IP) (**C, D**) or intracardiac (IC) (**E, F**). (**B, D**), and (**F**) are higher magnifications of (**A**), (**C**), and (**E**), respectively. Collagen in scar is indicated in *blue* and myocytes in *red*. Images are typical of 20 separate animals examined histologically. (**G**) Estimated scar volume of hearts after coronary ligation and PBS or TB4 treatment calculated from six levels of section for six mice in a blinded fashion. Bars indicate standard deviation at 95% confidence limits. * $P < 0.02$. (**H, I**) TUNEL positive cells (*bright green*) 24 h after TB4 or PBS treatment demonstrating rare positive cells in TB4 sections. (**J, K**) DAPI stain of nuclei of (**H, I**). (**L, M**) Higher magnification of TUNEL positive nuclei (*bright green*) double-labeled with antimyosin antibody (*red*) to mark cardiomyocytes. TUNEL positive cells costained with muscle actin were rare in the TB4-treated hearts but abundant in the control hearts. (**N**) Western blot using anti-ILK antibody, phosphospecific antibody to Akt-S⁴⁷³ or antibody to Akt on heart lysates after coronary ligation and treatment with PBS or TB4. Loading controls with GAPDH levels are shown. (In color in Annals online.)

thymosin β 4. Trichrome stain at three levels of section revealed that the size of scar was reduced in all mice treated with thymosin β 4 but was not different between systemic or local delivery of thymosin β 4 (FIG. 4A–F), consistent with the echocardiographic data above. Quantification of scar volume using six levels of sections through the left ventricle of a subset of mice demonstrated significant reduction of scar volume in thymosin β 4-treated mice (FIG. 4G, $P < 0.05$). We did not detect significant cardiomyocyte proliferation or death

at 3, 6, 11, or 14 days after coronary ligation in PBS- or thymosin β 4-treated hearts. However, 24 h after ligation we found a striking decrease in cell death by TUNEL assay in thymosin β 4-treated cardiomyocytes (FIG. 4). TUNEL positive cells that were also myocytes were rare in the thymosin β 4 group but abundant in the control hearts. Consistent with this observation, we found that the left ventricle fractional shortening 3 days after infarction was $39.2 \pm 2.34\%$ ($n = 4$, 95% confidence interval) with intracardiac thymosin β 4 treatment compared to $28.8 \pm 2.26\%$ ($n = 4$, 95% confidence interval) in controls ($P < 0.02$); ejection fraction was $64.2 \pm 6.69\%$ or $44.7 \pm 8.4\%$, respectively ($P < 0.02$), suggesting early protection by thymosin β 4. The decreased scar volume and preserved function of thymosin β 4-treated mice were likely due to early preservation of myocardium after infarction through thymosin β 4's effects on survival of cardiomyocytes.

By Western blot we found that the level of ILK protein was increased in heart lysates of mice treated with thymosin β 4 after coronary ligation compared with PBS-treated mice (FIG. 4). Correspondingly, phosphospecific antibodies to Akt-S⁴⁷³ revealed an elevation in the amount of phosphorylated Akt-S⁴⁷³ in mice treated with thymosin β 4 (FIG. 4), consistent with the effects of thymosin β 4 on ILK described earlier. These observations *in vivo* were consistent with the effects of thymosin β 4 on cell migration and survival demonstrated *in vitro* and suggest that activation of ILK and subsequent stimulation of Akt may in part explain the enhanced cardiomyocyte survival induced by thymosin β 4, although it is unlikely that a single mechanism is responsible for the full repertoire of thymosin β 4's cellular effects. Indeed, we have found that thymosin β 4 is also a potent stimulator of neoangiogenesis in the hypoxic myocardium following myocardial infarction (I. Bock-Marquette *et al.*, unpublished observations). The combination of cytoprotection followed by angiogenesis likely underlies its potent effects. The recent discovery that thymosin β 4 can regulate coronary angiogenesis is consistent with this notion.²⁶ In addition, thymosin β 4 is highly upregulated in the heart of adult zebrafish during damage-induced regeneration, suggesting that this protein may be playing a role in a regenerative process after injury.²⁷

The early effect of thymosin β 4 in protecting the heart from cell death was reminiscent of myocytes that are able to survive hypoxic insult by "hibernating."²⁸ While the mechanisms underlying hibernating myocardium are unclear, alterations in metabolism and energy usage appear to promote survival of cells.²⁸ Future studies will determine if thymosin β 4 alters cellular properties in a manner similar to hibernating myocardium, possibly allowing time for endothelial cell migration and new blood vessel formation. Indeed, recent studies have shown that thymosin β 4 is highly secreted by marrow-derived progenitor cells that appear to have beneficial paracrine effects on the heart after coronary occlusion in rodents.²⁹ It will be interesting to determine if thymosin β 4 is one of the critical paracrine factors from such cells that serve to improve cardiac function postmyocardial infarction. Given the findings here,

the utility of thymosin β 4 for healing after cardiac injury holds promise and warrants further preclinical investigation.

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