Thymosin β4 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 β4 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 β4. We found that thymosin β4 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 β4's effects on cardiomyocytes. After coronary artery ligation in mice, thymosin β4 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 β4 promotes cardiomyocyte and endothelial migration, survival, and repair and may be a novel therapeutic target in the setting of acute myocardial damage.

KEYWORDS: thymosin β4; 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 cardiomycytes to aid in cardiac repair.[10] In our studies of genes expressed during cardiac morphogenesis, we found that the 43-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, 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 β4 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 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.[18] These results are described below.

Secreted Thymosin β4 Stimulates Cardiac Cell Migration and Survival

Although thymosin β4 is found in the cytosol and nucleus and functions intracellularly,[12] we found that conditioned medium of Cos1 cells transfected with myc-tagged thymosin β4 contained thymosin β4 detectable by Western blot, consistent with previous reports of thymosin β4 secretion and presence in wound fluid.[17,19,20] Upon expression of thymosin β4 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

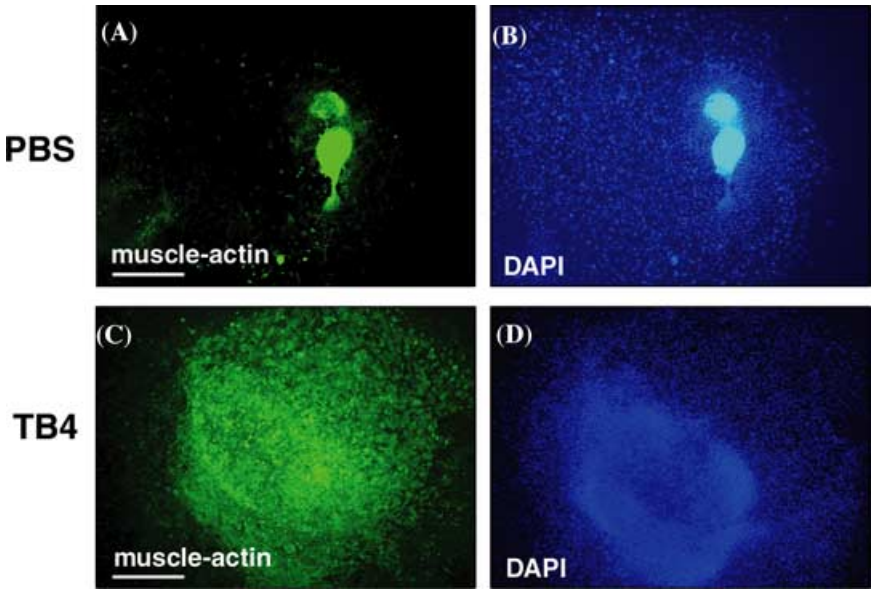

FIGURE 1. Thymosin β4 promotes cardiac cell migration and survival. (**A**–**D**) Mouse E11.5 cardiac outflow tract explants stained with antimuscle actin antibody (*green*) after PBS (**A**) or thymosin β4 (**C**) treatment. (**B**) and (**D**) are DAPI stains of (**A**) and (**C**), respectively. Bars 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 β4 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 β4 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 β4 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 β4-treated neonatal cardiomyocytes was significantly increased compared to control. In addition to thymosin β4'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 β4 with rhythmically contracting myocytes visible for up to 28 days.[18]

Thymosin β4 Activates ILK and Akt/Protein Kinase B

To investigate the potential mechanisms by which thymosin β4 might be influencing cell migration and survival events, we searched for thymosin β4-interacting proteins. Using phage display, we found that PINCH, a LIM domain protein, was most consistently isolated in an interaction screen with thymosin β4 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 β4 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 β4 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 β4 protein or thymosin β4-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 β4 (FIG. 2B). We found that upon thymosin β4 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 β4 *in vitro* could be inhibited using an ILK inhibitor suggesting that the survival effects on cardiomyocytes was likely via Akt activation.

Thymosin β4 Promotes Cell Survival after Myocardial Infarction and Improves Cardiac Function

Because of thymosin β4's effects on survival and migration of cardiomyocytes cultured *in vitro* and phosphorylation of Akt, we tested whether thymosin β4 might aid in cardiac repair *in vivo* after myocardial damage. We

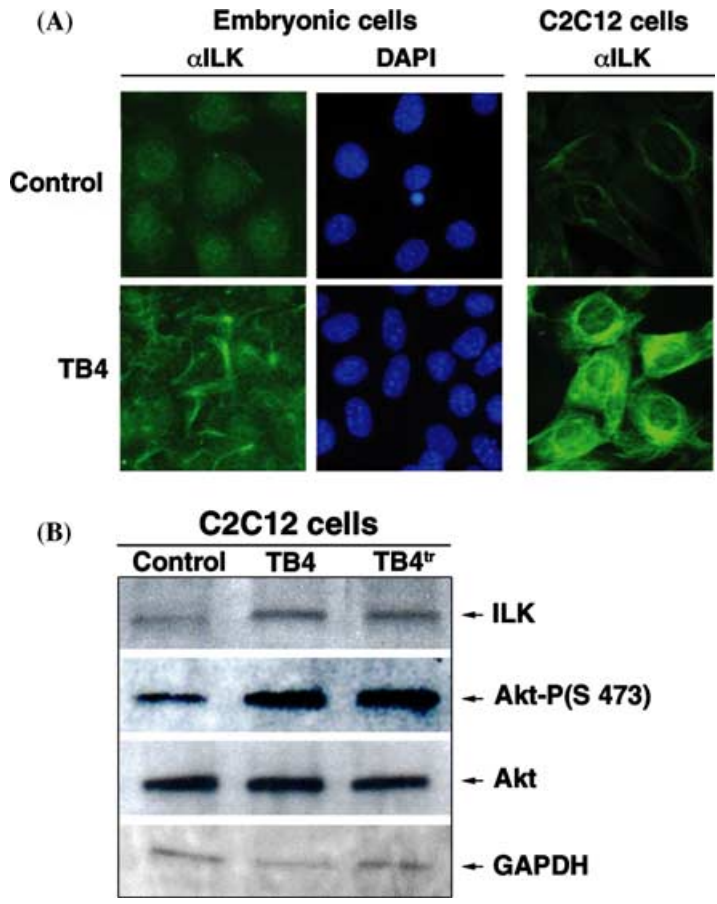

FIGURE 2. Thymosin β4 forms a functional complex with PINCH and ILK resulting in phosphorylation of Akt. (**A**) Immunocytochemistry with anti-ILK antibody (*green*) and DAPI (*blue*) marking nuclei after thymosin β4 treatment of embryonic cardiac explants or C2C12 myoblasts. (**B**) Western blot of C2C12 cells treated with thymosin β4 protein or transfected with thymosin β4-expressing plasmid ($T\beta4^{tr}$) using anti-ILK antibody, phosphospecific antibody to Akt-S^{473} 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 β4 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 β4. All 45 mice that survived 2 weeks later were interrogated for cardiac function by random-blind ultrasonagraphy at 2 and 4 weeks after infarction by multiple measurements of cardiac contraction.

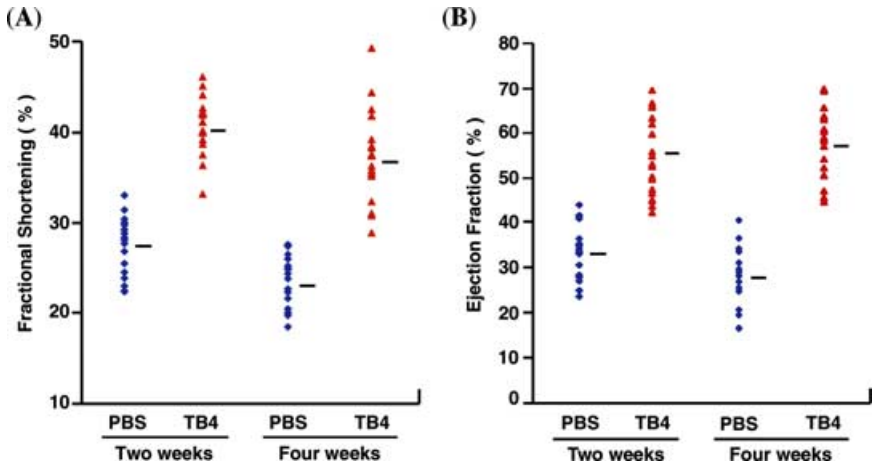

FIGURE 3. Thymosin β4 treatment after coronary ligation improves myocardial function *in vivo*. (**A**, **B**) Distribution of left ventricular fractional shortening (**A**) or ejection fraction (**B**) at 2 and 4 weeks after coronary ligation with ($n = 23$) or without ($n = 22$) thymosin β4 treatment. 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 ± 1.2% ($n = 22$, 95% confidence interval); in contrast, mice treated with thymosin β4 had a mean fractional shortening of 37.2 ± 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 β4-treated mice was 57.7 ± 3.2% ($n = 23$, 95% confidence interval; $P < 0.0001$) compared to a mean of 28.2 ± 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 β4, although cardiac function remained depressed compared to sham-operated animals (~60% fractional shortening; ~75% ejection fraction). Finally, the end diastolic dimensions and end systolic dimensions were significantly higher in the control group, indicating that thymosin β4 treatment resulted in decreased cardiac dilation after infarction, consistent with improved function. Remarkably, the degree of improvement when thymosin β4 was administered systemically through intraperitoneal injections or only locally within the cardiac infarct was not statistically different, suggesting that the beneficial effects of thymosin β4 likely occurred through a direct effect on cardiac cells rather than through an extracardiac source.

To determine the manner in which thymosin β4 improved cardiac function, we examined multiple serial histologic sections of hearts treated with or without

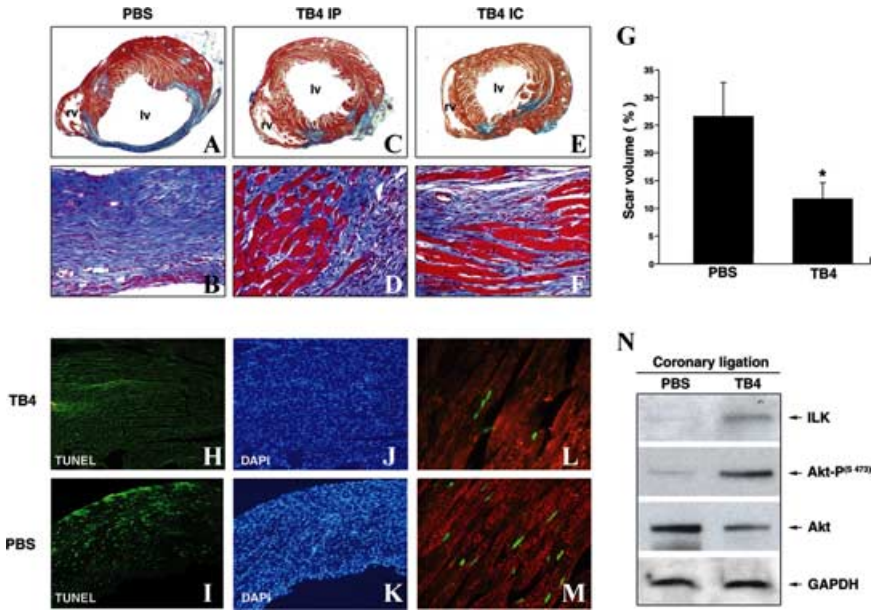

FIGURE 4. Thymosin β4 promotes survival and alters scar formation after coronary artery ligation in mice. **(A–F)** Representative trichrome stain of transverse heart sections at comparable levels 14 days after coronary ligation and PBS **(A, B)** treatment or thymosin β4 treatment delivered intraperitoneally (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 Tβ4 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 Tβ4 or PBS treatment demonstrating rare positive cells in Tβ4 sections. **(J, K)** DAPI stain of nuclei of **(H, I)**. (**L**, **M**) Higher magnification of TUNEL positive nuclei (*bright green*) double-labeled with antimuscle actinin antibody (*red*) to mark cardiomyoctyes. TUNEL positive cells costained with muscle actin were rare in the Tβ4-treated hearts but abundant in the control hearts. (**N**) Western blot using anti-ILK antibody, phosphospecific antibody to Akt-S^{473} or antibody to Akt on heart lysates after coronary ligation and treatment with PBS or Tβ4. Loading controls with GAPDH levels are shown. (In color in Annals online.)

thymosin β4. Trichrome stain at three levels of section revealed that the size of scar was reduced in all mice treated with thymosin β4 but was not different between systemic or local delivery of thymosin β4 (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 β4-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 β4-treated hearts. However, 24 h after ligation we found a striking decrease in cell death by TUNEL assay in thymosin β4-treated cardiomyocytes (FIG. 4). TUNEL positive cells that were also myocytes were rare in the thymosin β4 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 β4 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 β4. The decreased scar volume and preserved function of thymosin β4-treated mice were likely due to early preservation of myocardium after infarction through thymosin β4'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 β4 after coronary ligation compared with PBS-treated mice (FIG. 4). Correspondingly, phosphospecific antibodies to Akt-S^{473} revealed an elevation in the amount of phosphorylated Akt-S^{473} in mice treated with thymosin β4 (FIG. 4), consistent with the effects of thymosin β4 on ILK described earlier. These observations *in vivo* were consistent with the effects of thymosin β4 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 β4, although it is unlikely that a single mechanism is responsible for the full repertoire of thymosin β4's cellular effects. Indeed, we have found that thymosin β4 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 β4 can regulate coronary angiogenesis is consistent with this notion.[26] In addition, thymosin β4 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 β4 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 β4 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 β4 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 β4 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 β4 for healing after cardiac injury holds promise and warrants further preclinical investigation.

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REFERENCES

1. AHA. 2004. Heart Disease and Stroke Statistics—2004 update. 11–14.
2. Hoffman, J.I. & S. Kaplan. 2002. The incidence of congenital heart disease. J. Am. Coll. Cardiol. **39:** 1890–1900.
3. Orlic, D. *et al*. 2001. Bone marrow cells regenerate infarcted myocardium. Nature **410:** 701–705.
4. Beltrami, A.P. *et al*. 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell **114:** 763–776.
5. Anversa, P. & B. Nadal-Ginard. 2002. Myocyte renewal and ventricular remodelling. Nature **415:** 240–243.
6. Balsam, L.B. *et al*. 2004. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature **428:** 668–673.
7. Murry, C.E. *et al*. 2004. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature **428:** 664–668.
8. Srivastava, D. & K.N. Ivey. 2006. Potential of stem cell-based therapies for heart disease. Nature **441:** 1097–1099.
9. Srivastava, D. 2006. Making or breaking the heart: from lineage determination to morphogenesis. Cell **126:** 1037–1048.
10. Srivastava, D. & E.N. Olson. 2000. A genetic blueprint for cardiac development. Nature **407:** 221–226.
11. Safer, D. *et al*. 1991. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J. Biol. Chem. **266:** 4029–4032.
12. Huff, T. *et al*. 2001. Beta-Thymosins, small acidic peptides with multiple functions. Int. J. Biochem. Cell Biol. **33:** 205–220.
13. Sun, H. *et al*. 1996. Beta-Thymosins are not simple actin monomer buffering proteins. Insights from overexpression studies. J. Biol. Chem. **271:** 9223–9230.
14. Hertzog, M. *et al*. 2004. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. Cell **117:** 611–623.
15. Malinda, K. *et al*. 1999. Thymosin beta4 accelerates wound healing. J. Invest. Dermatol. **113:** 364–368.
16. Sosne, G. *et al*. 2002. Thymosin beta 4 promotes corneal wound healing and decreases inflammation *in vivo* following alkali injury. Exp. Eye. Res. **74:** 293–299.
17. Grant, D.S. *et al*. 1999. Thymosin beta 4 enhances endothelial cell differentiation and angiogenesis. Angiogenesis **3:** 125–135.

18. BOCK-MARQUETTE, I. *et al*. 2004. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature **432**: 466–472.
19. FROHM, M. *et al*. 1996. Biochemical and antibacterial analysis of human wound and blister fluid. Eur. J. Biochem. **237:** 86–92.
20. HUANG, W.Q. & Q.R. WANG. 2001. Bone marrow endothelial cells secrete thymosin beta4 and AcSDKP. Exp. Hematol. **29:** 12–18.
21. RUNYAN, R. & R. MARKWALD. 1983. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev. Biol. **95:** 108–114.
22. FUKUDA, T. *et al*. 2003. PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. J. Biol. Chem. **278:** 51324–51333.
23. ZHANG, Y. *et al*. 2002. Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites. J. Cell. Sci. **115:** 4777–4786.
24. TROUSSARD, A. *et al*. 2003. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J. Biol. Chem. **278:** 22374–22378.
25. BRAZIL, D.P. *et al*. 2002. PKB binding proteins. Getting in on the Akt. Cell **111:** 293–303.
26. SMART, N. *et al*. 2007. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature **445:** 177–182.
27. LIEN, C.L. *et al*. 2006. Gene expression analysis of zebrafish heart regeneration. PLoS Biol. **4:** e260.
28. DEPRE, C. *et al*. 2004. Program of cell survival underlying human and experimental hibernating myocardium. Circ. Res. **95:** 433–440.
29. GNECCHI, M. *et al*. 2006. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. **20:** 661–669.