

# Thymosin $\beta$ 4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair

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**Heart disease is a leading cause of death in newborn children and in adults. Efforts to promote cardiac repair through the use of stem cells hold promise but typically involve isolation and introduction of progenitor cells. Here, we show that the G-actin sequestering peptide thymosin  $\beta$ 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  $\beta$ 4. We found that thymosin  $\beta$ 4 formed a functional complex with PINCH and integrin-linked kinase (ILK), resulting in activation of the survival kinase Akt (also known as protein kinase B). After coronary artery ligation in mice, thymosin  $\beta$ 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  $\beta$ 4 promotes cardiomyocyte migration, survival and repair and the pathway it regulates may be a new therapeutic target in the setting of acute myocardial damage.**

Coronary artery disease results in acute occlusion of cardiac vessels leading to loss of dependent myocardium. Over thirteen million individuals in the United States alone suffer from coronary artery disease and this condition is one of the leading causes of death in the Western world<sup>1</sup>. Because the heart is incapable of sufficient muscle regeneration, survivors of myocardial infarctions typically develop chronic heart failure. Although more commonly affecting adults, heart disease in children is the leading non-infectious cause of death in the first year of life and often involves abnormalities in cardiac cell specification, migration or survival<sup>2</sup>.

Recent evidence suggests that a population of extracardiac or intracardiac stem cells may contribute to maintenance of the cardiomyocyte population under normal circumstances<sup>3–5</sup>. 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 stem cells may improve myocardial function<sup>3–5</sup>, but this approach has been controversial<sup>6,7</sup> and requires isolation of autologous stem cells or the use of donor stem cells along with immunosuppression. Technical hurdles of stem cell delivery and differentiation have thus far prevented broad clinical application of cardiac regenerative therapies.

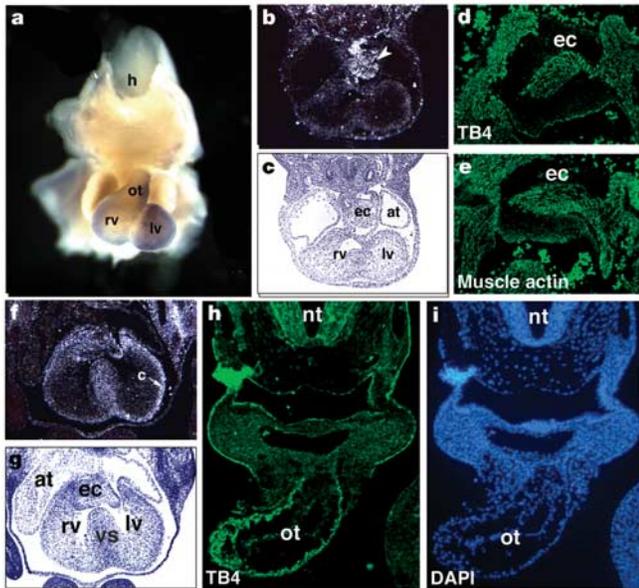
Regulatory pathways involved in cardiac development may have utility in reprogramming cardiomyocytes to aid in cardiac protection or repair<sup>8</sup>. In our studies of genes expressed during cardiac morphogenesis, we found that the 43-amino-acid peptide thymosin  $\beta$ 4 was expressed in the developing heart. Thymosin  $\beta$ 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<sup>9–11</sup>. Recent domain analyses indicate that  $\beta$ -thymosins can affect actin assembly based on their carboxy-terminal affinity for actin<sup>12</sup>. In addition to cell motility, thymosin  $\beta$ 4 may affect transcriptional events by influencing Rho-dependent gene expression or chromatin remodelling events regulated by nuclear actin<sup>13,14</sup>. Although thymosin  $\beta$ 4 promotes skin and corneal wound healing through its effects on cell migration,

angiogenesis and possibly cell survival<sup>15–17</sup>, the precise molecular mechanism through which it functions and its potential role in solid organ wound healing remain unknown.

Here, we show that thymosin  $\beta$ 4 can stimulate migration of cardiomyocytes and endothelial cells and promotes survival of cardiomyocytes. The LIM domain protein PINCH<sup>18</sup> and ILK<sup>19</sup>, both of which are necessary for cell migration and survival, formed a complex with thymosin  $\beta$ 4 that resulted in phosphorylation of the survival kinase Akt. Inhibition of Akt phosphorylation reversed the effects of thymosin  $\beta$ 4 on cardiac cells. Treatment of adult mice with thymosin  $\beta$ 4 after coronary ligation resulted in increased phosphorylation of Akt in the heart, enhanced early myocyte survival and improved cardiac function. These results indicate that an endogenous protein expressed during cardiogenesis may be re-deployed to protect myocardium in the setting of acute coronary events.

## Developmental expression of thymosin $\beta$ 4

Expression of thymosin  $\beta$ 4 in the developing brain was previously reported<sup>20</sup>, as was expression in the cardiovascular system<sup>21</sup>, although not in significant detail. Whole-mount RNA *in situ* hybridization of embryonic day (E)11.5 mouse embryos revealed thymosin  $\beta$ 4 expression in the left ventricle, outer curvature of the right ventricle and cardiac outflow tract (Fig. 1a). Radioactive *in situ* hybridization indicated that thymosin  $\beta$ 4 transcripts were enriched in the region of cardiac valve precursors known as endocardial cushions (Fig. 1b, c). Cells in this region are derived from endothelial cells that undergo mesenchymal transformation and invade a swelling of extracellular matrix separating the myocardium and endocardium. We found that thymosin- $\beta$ 4-expressing cells in the cushions (Fig. 1d) co-expressed muscle actin (Fig. 1e), suggesting that thymosin  $\beta$ 4 was present in migratory cardiomyocytes known to invade the endocardial cushion<sup>22</sup>. Thymosin  $\beta$ 4 transcripts and protein were also expressed at E9.5–E12.5 in the ventricular septum and the more proliferative region of the myocardium, known as the compact layer, which migrates into the trabecular region as the cells



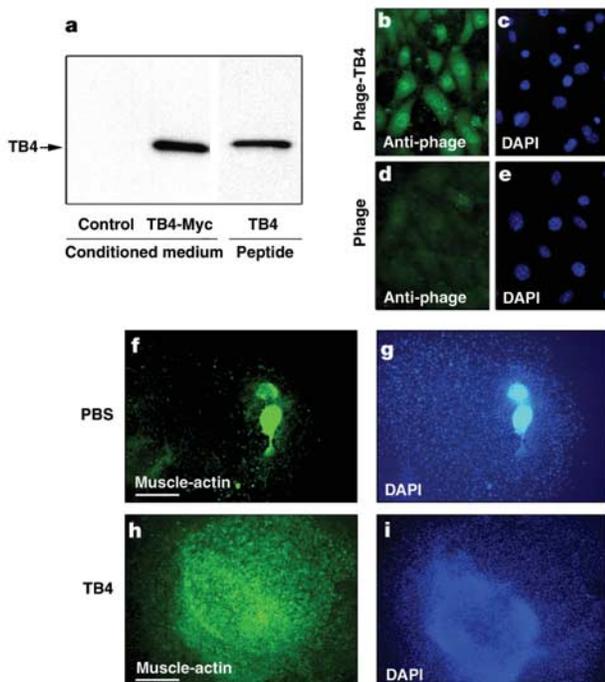
**Figure 1** Thymosin  $\beta$ 4 is expressed in specific cardiac cell types during development. **a**, Thymosin  $\beta$ 4 mRNA transcripts at E10.5 by whole-mount *in situ* hybridization in frontal view. h, head; lv, left ventricle; ot, outflow tract; rv, right ventricle. **b, c**, Radioactive section *in situ* hybridization at E11.5 in transverse section through heart. Arrowhead indicates endocardial cushion (ec). at, atria. **d, e**, Immunohistochemistry using thymosin  $\beta$ 4 (**d**) and muscle actin (**e**) antibodies focused on cushion cells at E11.5. TB4, thymosin  $\beta$ 4. **f, g**, Expression of thymosin  $\beta$ 4 mRNA at E12.5 in compact layer (c) of ventricles and ventricular septum (vs). Note absence in atria. **h, i**, Thymosin  $\beta$ 4 protein or 4,6-diamidino-2-phenylindole (DAPI) in outflow tract myocardium by immunohistochemistry of E9.5 transverse section. nt, neural tube.

mature (Fig. 1f, g). Finally, outflow tract myocardium that migrates from a secondary heart field also expressed high levels of thymosin  $\beta$ 4 protein<sup>23</sup> (Fig. 1h, i).

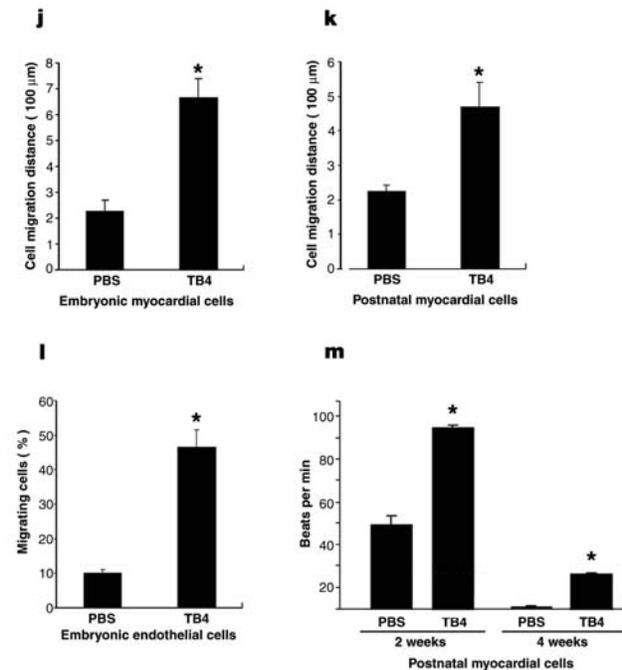
**Thymosin  $\beta$ 4 induces cardiac cell migration and survival**

Although thymosin  $\beta$ 4 is found in the cytosol and nucleus and functions intracellularly<sup>10</sup>, we found that conditioned medium of Cos1 cells transfected with Myc-tagged thymosin  $\beta$ 4 contained thymosin  $\beta$ 4 detectable by western blot (Fig. 2a), consistent with previous reports of thymosin  $\beta$ 4 secretion and presence in wound fluid<sup>17,24,25</sup>. Upon expression of thymosin  $\beta$ 4 on the surface of phage particles added extracellularly to embryonic cardiac explants, we found that an anti-phage antibody coated the cell surface and was ultimately detected intracellularly in the cytosol and nucleus, whereas control phage was not detectable (Fig. 2b–e). Similar observations were made using biotinylated thymosin  $\beta$ 4 (data not shown). These data indicated that secreted thymosin  $\beta$ 4 was 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 used an embryonic heart explant system designed to assay cell migration and transformation on a collagen gel<sup>26</sup>. Cardiomyocytes from valve-forming regions secrete signals that induce endocardial cell migration onto collagen, but myocardial cells do not normally migrate in significant numbers (Fig. 2f, g). In contrast, upon addition of thymosin  $\beta$ 4, we observed a large number of spontaneously beating, muscle actin-positive cells that migrated away from the explant (Fig. 2h–j,  $P < 0.0001$ ). No significant difference in cell death or proliferative rate based on TdT-mediated dUTP nick end labelling (TUNEL) assay or phospho-histone H3 immunostaining, respectively, was observed in these cells compared to control cells (data not shown).



**Figure 2** Thymosin  $\beta$ 4 is secreted and promotes cardiac cell migration and survival. **a**, Western blot of supernatant from thymosin  $\beta$ 4 (TB4) transfected Cos cells using thymosin  $\beta$ 4 antibodies. **b–e**, Immunocytochemistry using anti-phage antibody or DAPI after thymosin- $\beta$ 4-expressing T7 phage (**b, c**) or control phage (**d, e**) administration in the medium of embryonic cardiac explants. **f–i**, Mouse E11.5 cardiac outflow tract explants stained with anti-muscle actin antibody (green) or DAPI (blue) after PBS (**f, g**) or thymosin  $\beta$ 4 (**h, i**) treatment. Scale bars, 500  $\mu$ m. **j, k**, Distance of migrating myocardial cells in



E11.5 cardiac outflow tract explants (**j**,  $P < 0.0001$ ) or rat neonatal cardiomyocytes (**k**,  $P < 0.03$ ) with or without thymosin  $\beta$ 4 treatment. **l**, Per cent of embryonic endothelial cells migrating with or without thymosin  $\beta$ 4 ( $P < 0.01$ ). **m**, Beating frequency of rat neonatal cardiomyocytes with or without thymosin  $\beta$ 4 (see Supplementary Figs 1 and 2 for movies;  $P < 0.02$ ). Means and standard deviation bars with 95% confidence limits are shown. Asterisk,  $P < 0.05$ .

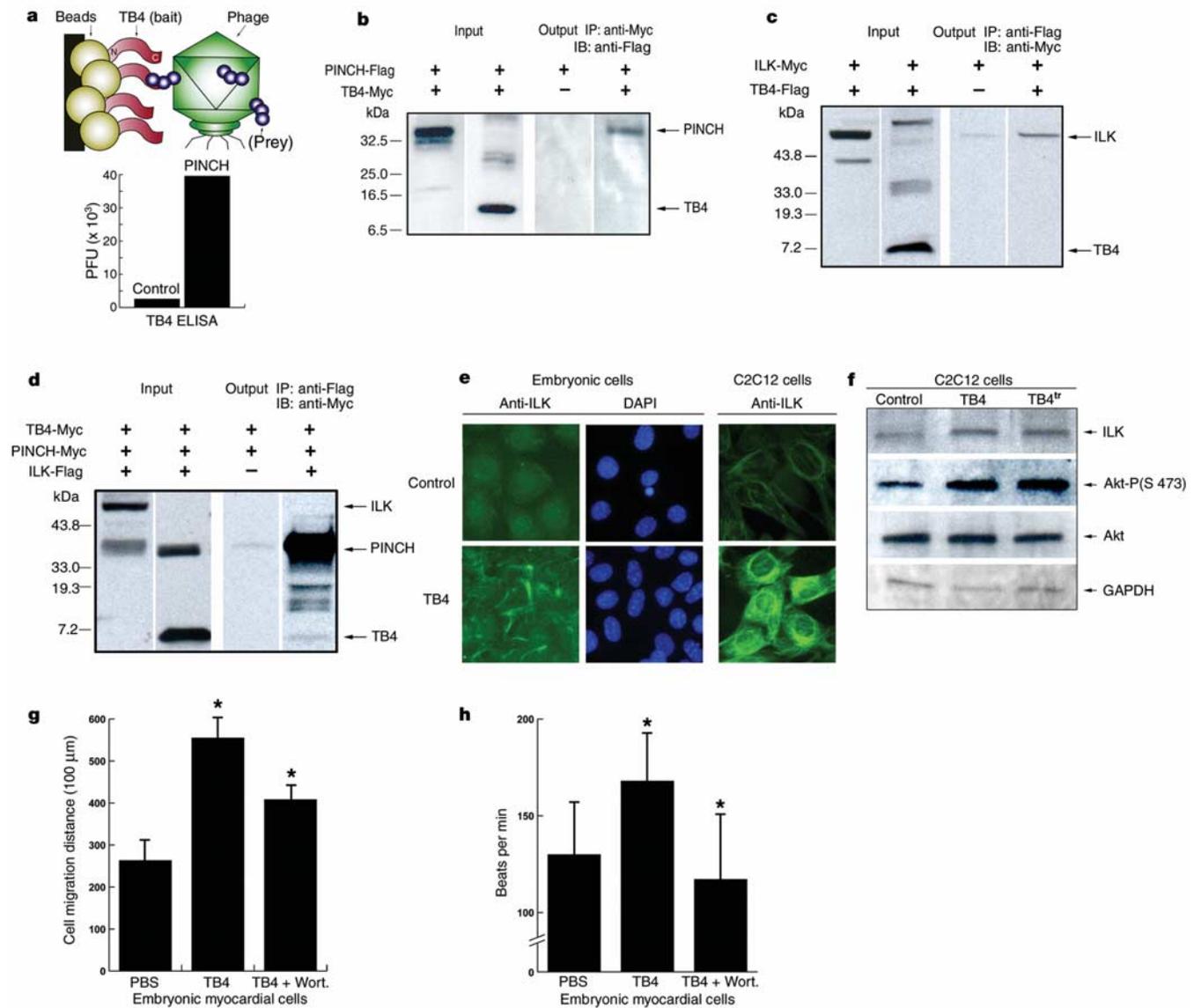
To test the response of postnatal cardiomyocytes, we cultured primary rat neonatal cardiomyocytes on laminin-coated glass and treated the cells with phosphate-buffered saline (PBS) or thymosin  $\beta$ 4. Similar to embryonic cardiomyocytes, the migrational distance of thymosin- $\beta$ 4-treated neonatal cardiomyocytes was significantly increased compared with control (Fig. 2k,  $P < 0.03$ ). In addition to the effects of thymosin  $\beta$ 4 on myocardial cell migration, we observed a similar effect on endothelial migration in the embryonic heart explant assay (Fig. 2l,  $P < 0.01$ ).

Primary culture of neonatal cardiomyocytes typically survives for approximately 1 to 2 weeks, with some cells beating for up to 2 weeks when grown on laminin-coated slides in our laboratory. Surprisingly, neonatal cardiomyocytes survived significantly longer upon exposure to thymosin  $\beta$ 4, with rhythmically contracting

myocytes visible for up to 28 days (Fig. 2m). In addition, the rate of beating was consistently faster in thymosin- $\beta$ 4-treated neonatal cardiomyocytes (95 versus 50 beats per minute,  $P < 0.02$ ), indicating either a change in cell–cell communication or cell metabolism (Fig. 2m; see also Supplementary Figs 1 and 2).

**Thymosin  $\beta$ 4 activates ILK and Akt**

To investigate the potential mechanisms through which thymosin  $\beta$ 4 might be influencing cell migration and survival events, we searched for thymosin  $\beta$ 4 interacting proteins. The amino terminus of thymosin  $\beta$ 4 was fused with Affi-gel beads resulting in exposure of the C terminus, which allowed identification of previously unknown interacting proteins but prohibited association with actin. We synthesized and screened an E9.5–E12.5 mouse heart T7



**Figure 3** Thymosin  $\beta$ 4 forms a functional complex with PINCH and ILK resulting in phosphorylation of Akt. **a**, Phage display strategy for isolating thymosin  $\beta$ 4 (TB4) interacting proteins, and ELISA confirmation of PINCH interaction. PFU, plaque-forming units. **b**, **c**, Immunoprecipitation (IP) for thymosin  $\beta$ 4 and immunoblot (IB) for PINCH (**b**) or ILK (**c**). **d**, Immunoprecipitation of ILK and immunoblot for PINCH and thymosin  $\beta$ 4. Cell lysate input for each protein is shown along with protein from the immunoprecipitation (output). **e**, Immunocytochemistry with anti-ILK antibody (green) and DAPI (blue) after

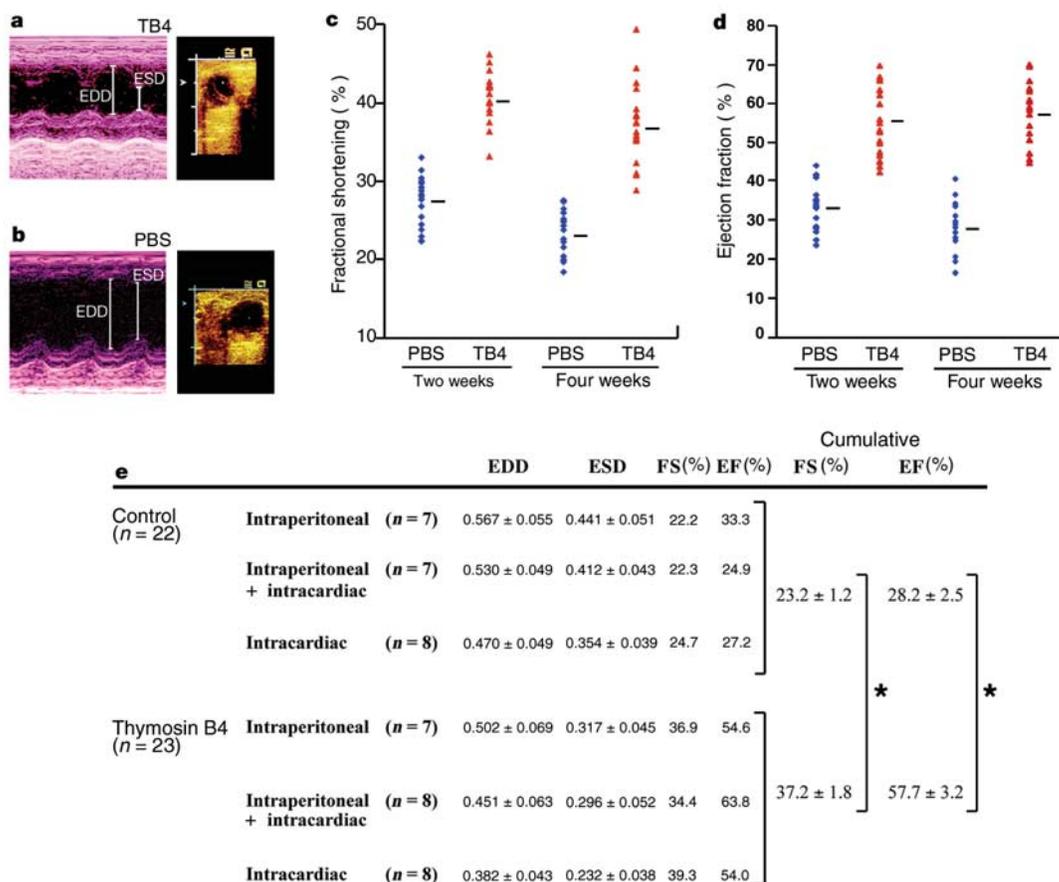
thymosin  $\beta$ 4 treatment of embryonic cardiac explants or C2C12 myoblasts. **f**, Western blot of C2C12 cells treated with thymosin  $\beta$ 4 protein or transfected with thymosin- $\beta$ 4-expressing plasmid (TB4<sup>tr</sup>) using antibodies for ILK, Akt, GAPDH or phospho-specific antibody to Akt-S473. **g**, **h**, Myocardial migration (**g**) or beating frequency (**h**) of E11.5 cardiac explants induced by thymosin  $\beta$ 4 in the presence or absence of wortmannin (Wort). Bars indicate standard deviations with 95% confidence interval. Asterisk,  $P < 0.05$ .

phage complementary DNA library by phage display, and thymosin-β4-interacting clones were enriched and confirmed by enzyme-linked immunosorbent assay (ELISA, Fig. 3a). PINCH, a LIM domain protein, was most consistently isolated in this screen and interacted with thymosin β4 in the absence of actin (Fig. 3a). PINCH and 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<sup>18,27</sup> and for cell survival, in part by promoting phosphorylation of the serine-threonine kinase Akt, a central kinase in survival and growth signalling pathways<sup>18,19,27,28</sup>. We transfected plasmids encoding thymosin β4 with or without PINCH or ILK in cultured cells and found that thymosin β4 co-precipitated with PINCH or ILK independently (Fig. 3b, c). Moreover, PINCH, ILK and thymosin β4 consistently immunoprecipitated in a common complex, although the interaction of ILK with thymosin β4 was weaker than with PINCH (Fig. 3d). The PINCH interaction with thymosin β4 mapped to the fourth and fifth LIM domains of PINCH, whereas the N-terminal ankyrin domain of ILK was sufficient for thymosin β4 interaction (data not shown).

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 (10 ng per 100 μl) or thymosin-β4-expressing plasmid (Fig. 3e). 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. 3f). We found that upon thymosin β4 treatment of C2C12 cells, ILK was functionally activated—evidenced by increased phosphorylation of its known substrate Akt<sup>19</sup> using a phospho-specific antibody to serine 473 of Akt (Fig. 3f)—whereas total Akt protein was unchanged. The similar effects of extracellularly administered thymosin β4 and transfected thymosin β4 were consistent with our previous observations of internalization of the peptide, and suggested an intracellular rather than an extracellular role in signalling for thymosin β4. Because thymosin β4 sequesters the pool of G-actin monomers, we asked whether the effects on ILK activation were dependent on the role of thymosin β4 in regulating the balance between polymerized F-actin and monomeric G-actin. We inhibited F-actin polymerization using C3 transferase and also promoted F-actin formation with an activated Rho<sup>29</sup>, but neither intervention affected the ILK levels detected by immunocytochemistry after treatment of COS1 or C2C12 cells with thymosin β4 (data not shown).

To determine whether activation of ILK was necessary for the observed effects of thymosin β4, we used a well-described ILK inhibitor, wortmannin, which inhibits ILK's upstream kinase, phosphatidylinositol-3-OH kinase (PI(3)K)<sup>30</sup>. Using myocardial cell migration and beating frequency as assays for thymosin β4



**Figure 4** Thymosin β4 treatment after coronary ligation improves myocardial function *in vivo*. **a, b**, Representative echocardiographic M-mode images of left ventricles after coronary ligation with **(a)** or without **(b)** thymosin β4 (TB4) treatment. Two-dimensional images are shown to the right. **c, d**, Distribution of left ventricular fractional shortening (FS) **(c)** or ejection fraction (EF) **(d)** at 2 and 4 weeks after coronary ligation with (n = 23)

or without (n = 22) thymosin β4 treatment. Bars indicate means. **e**, Echocardiographic measurements for intraperitoneal, intracardiac or intraperitoneal and intracardiac administration of thymosin β4 or PBS (Control) at 4 weeks. Means and 95% confidence limits are shown. Asterisk, P < 0.0001.

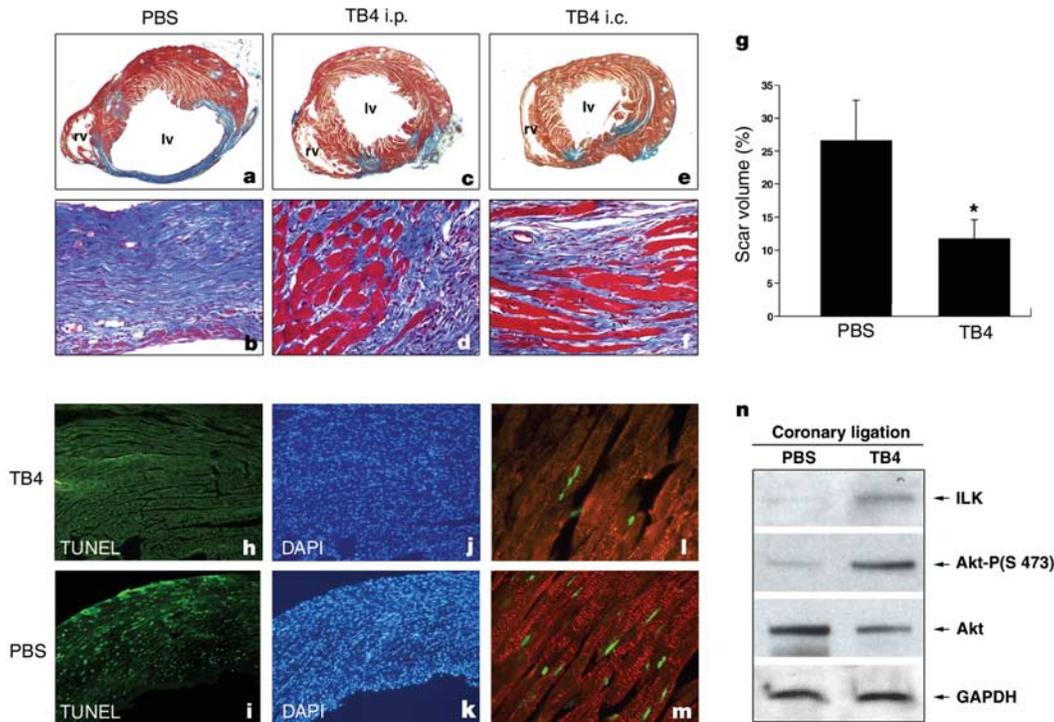
activity, we cultured embryonic heart explants as described above in the presence of thymosin  $\beta$ 4 with or without wortmannin. Although inhibiting PI(3)K affects many pathways, we observed a significant reduction in myocardial cell migration and beating frequency upon inhibition of ILK, consistent with ILK mediation of the effects of thymosin  $\beta$ 4 (Fig. 3g, h,  $P < 0.05$ ). Together, these results supported a physiologically significant interaction of thymosin  $\beta$ 4–PINCH–ILK within the cell and suggested that this complex may mediate some of the observed effects of thymosin  $\beta$ 4 relatively independently of actin polymerization.

**Thymosin  $\beta$ 4 protects cells after myocardial infarction**

Because of the effects of thymosin  $\beta$ 4 on cardiac cells *in vitro*, we tested whether thymosin  $\beta$ 4 might aid in cardiac repair *in vivo* after myocardial damage. We created myocardial infarctions in 58 adult mice by coronary artery ligation and treated half with systemic, intracardiac, or systemic plus intracardiac thymosin  $\beta$ 4 immediately after ligation and the other half with PBS (Fig. 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 (Fig. 4a–d). 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 interval;  $P < 0.0001$ ) (Fig. 4c, e). 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;  $P < 0.0001$ ) com-

pared with a mean of  $28.2 \pm 2.5\%$  ( $n = 22$ , 95% confidence interval) in control mice after coronary ligation (Fig. 4d, e). 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 with sham-operated animals ( $\sim 60\%$  fractional shortening;  $\sim 75\%$  ejection fraction). Finally, the end diastolic dimensions (EDDs) and end systolic dimensions (ESDs) were significantly higher in the control group, indicating that thymosin  $\beta$ 4 treatment resulted in decreased cardiac dilation after infarction, consistent with improved function (Fig. 4e). 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 probably occurred through a direct effect on cardiac cells rather than through an extracardiac source.

Trichrome stain at three levels of section revealed that the size of scar was reduced in all mice treated with thymosin  $\beta$ 4 but was not different between systemic or local delivery of thymosin  $\beta$ 4 (Fig. 5a–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- $\beta$ 4-treated mice (Fig. 5g,  $P < 0.02$ ). We did not detect significant cardiomyocyte proliferation or death at 3, 6, 11 or 14 days after coronary ligation in PBS or thymosin- $\beta$ 4-treated hearts (data not shown). However, 24 h after ligation we found a marked decrease in cell death by TUNEL assay (green) in thymosin- $\beta$ 4-treated cardiomyocytes (Fig. 5h–k), marked by double-labelling with muscle-actin antibody (red)



**Figure 5** Thymosin  $\beta$ 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**) or thymosin  $\beta$ 4 (TB4) treatment delivered intraperitoneally (i.p.) (**c, d**) or intracardiac (i.c.) (**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. lv, left ventricle; rv, right ventricle. **g**, Estimated scar volume of hearts after coronary ligation and PBS or thymosin

$\beta$ 4 treatment. Bars indicate standard deviation at 95% confidence limits. Asterisk,  $P < 0.02$ . **h, i**, TUNEL-positive cells (bright green) 24 h after coronary ligation and thymosin  $\beta$ 4 or PBS treatment. **j, k**, DAPI stain of **h, i**. **l, m**, Higher magnification of TUNEL-positive nuclei (green) double-labelled with anti-muscle actin antibody (red striations) to mark cardiomyocytes. **n**, Western blot on heart lysates after coronary ligation and treatment with PBS or thymosin  $\beta$ 4.

(Fig. 5l, m). TUNEL-positive cells that were also myocytes were rare in the thymosin  $\beta 4$  group but abundant in the control hearts. Consistent with this observation, we found that the left ventricle fractional shortening 3 days after infarction was  $39.2 \pm 2.3\%$  ( $n = 4$ , 95% confidence interval) with intracardiac thymosin  $\beta 4$  treatment compared with  $28.8 \pm 2.3\%$  ( $n = 4$ , 95% confidence interval) in controls ( $P < 0.02$ ); ejection fraction was  $64.2 \pm 6.7\%$  or  $44.7 \pm 8.4\%$ , respectively ( $P < 0.02$ ), suggesting early protection by thymosin  $\beta 4$ . Finally, we failed to detect any differences in the number of c-kit, Sca-1 or Abcg2 positive cardiomyocytes between treated and untreated hearts, and the cell volume of cardiomyocytes in thymosin- $\beta 4$ -treated animals was similar to mature myocytes, suggesting that the thymosin- $\beta 4$ -induced improvement was unlikely to be influenced by recruitment of known stem cells into the cardiac lineage (data not shown). Thus, the decreased scar volume and preserved function of thymosin- $\beta 4$ -treated mice were probably due to early preservation of myocardium after infarction through the effects of thymosin  $\beta 4$  on survival of cardiomyocytes.

Similar to cultured cells, the level of ILK protein was increased in heart lysates of mice treated with thymosin  $\beta 4$  after coronary ligation compared with PBS-treated mice (Fig. 5n). Correspondingly, phospho-specific antibodies to Akt-S473 revealed an elevation in the amount of phosphorylated Akt-S473 in mice treated with thymosin  $\beta 4$  (Fig. 5n), consistent with the effects of thymosin  $\beta 4$  on ILK described earlier (Fig. 3e, f). These observations *in vivo* were consistent with the effects of thymosin  $\beta 4$  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  $\beta 4$ , although it is unlikely that a single mechanism is responsible for the full repertoire of thymosin  $\beta 4$ 's cellular effects.

## Discussion

The evidence presented here suggests that thymosin  $\beta 4$ , a protein involved in cell migration and survival during cardiac morphogenesis, may be re-deployed to minimize cardiomyocyte loss after cardiac infarction. Given the known roles of PINCH, ILK and Akt, our data are consistent with this complex having a central role in the effects of thymosin  $\beta 4$  on cell motility, survival and cardiac repair. The ability of thymosin  $\beta 4$  to prevent cell death within 24 h after coronary ligation probably leads to the decreased scar volume and improved ventricular function observed in mice. Although thymosin  $\beta 4$  activation of ILK is likely to have many cellular effects, the activation of Akt may be the dominant mechanism through which thymosin  $\beta 4$  promotes cell survival. This is consistent with Akt's proposed effect on cardiac repair when overexpressed in mouse marrow-derived stem cells administered after cardiac injury<sup>31</sup>, although this probably occurs in a non-cell-autonomous fashion. Whereas thymosin  $\beta 4$  can augment an organism's ability to heal surface wound and stimulate angiogenesis<sup>16,17,32</sup>, the work presented here is the first demonstration of thymosin  $\beta 4$ 's efficacy in healing of a solid organ, and reveals a new mechanism through which thymosin  $\beta 4$  affects cellular functions. Whether thymosin  $\beta 4$  directly affects stabilization of ILK or transcription of ILK through actin-dependent regulation of transcription factors, and which cell types are affected by these or other pathways, remain to be determined.

The early effect of thymosin  $\beta 4$  in protecting the heart from cell death was reminiscent of myocytes that are able to survive hypoxic insult by "hibernating"<sup>33</sup>. Although the mechanisms underlying hibernating myocardium are unclear, alterations in metabolism and energy usage seem to promote survival of cells<sup>33</sup>. Future studies will determine whether thymosin  $\beta 4$  alters cellular properties in a manner similar to hibernating myocardium, possibly allowing time for endothelial cell migration and new blood vessel formation. Given the findings here, thymosin  $\beta 4$ , or the discovery of small

molecules that mimic its function, may prove useful for protecting patients from cardiac injury and therefore warrant further pre-clinical investigation. □

## Methods

### RNA *in situ* hybridization

Whole-mount or section RNA *in situ* hybridization of E9.5–E12.5 mouse embryos was performed with digoxigenin-labelled or <sup>35</sup>S-labelled antisense riboprobes synthesized from the 3' untranslated region of mouse thymosin  $\beta 4$  cDNA that did not share homology with the closely related transcript of thymosin  $\beta 10$ , as previously described<sup>34</sup>.

### Immunohistochemistry

Embryonic or adult cardiac tissue was embedded in paraffin and sections used for immunohistochemistry. Embryonic heart sections were incubated with anti-thymosin  $\beta 4$  (a gift of H. Yin) that does not recognize thymosin  $\beta 10$  (ref. 35). Adult hearts were sectioned at ten equivalent levels from the base of the heart to the apex. Serial sections were used for trichrome sections and reaction with muscle actin, c-kit, Sca-1, Abcg2 and BrdU antibodies and for TUNEL assay (Intergen Company S7111).

### Collagen gel migration assay

Outflow tract was dissected from E11.5 wild-type mouse embryos and placed on collagen matrices as previously described<sup>26</sup>. After 10 h of attachment explants were incubated in 30 ng per 300  $\mu$ l thymosin  $\beta 4$  in PBS, PBS alone or thymosin  $\beta 4$  and 100 nM wortmannin. Cultures were carried out for 3–9 days at 37 °C 5% CO<sub>2</sub> and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were counted for quantification of migration and distance using at least three separate explants under each condition for endothelial migration and eight separate explants for myocardial migration.

### Immunocytochemistry on collagen gel explants

Paraformaldehyde-fixed explants were permeabilized for 10 min at room temperature with Permeabilize solution (10 mM PIPES pH 6.8, 50 mM NaCl, 0.5% Triton X-100, 300 mM sucrose, 3 mM MgCl<sub>2</sub>) and rinsed with PBS twice for 5 min each at room temperature. After a series of blocking and rinsing steps, detection antibodies were used and explants rinsed and incubated with equilibration buffer (Anti-Fade kit) for 10 min at room temperature. Explants were scooped to a glass microscope slide, covered and examined by fluorescein microscopy. TUNEL assay was performed using ApopTag plus fluorescein *in situ* apoptosis detection kit (Intergen Company S7111) as recommended.

### Embryonic T7 phage display cDNA library and phage biopanning

Equal amounts of messenger RNA were isolated and purified from E9.5–E12.5 mouse embryonic hearts by using Straight A's mRNA Isolation System (Novagen). cDNA was synthesized by using T7Select10-3 OrientExpress cDNA Random Primer Cloning System (Novagen). The vector T7Select10-3 was used to display random-primed cDNA at the C terminus of 5–15 phage 10B coat protein molecules. 10<sup>9</sup> plaque-forming units of the T7 phage embryonic heart library (100 × of the complexity) in 500  $\mu$ l of PBST was applied to a column of Affi-gel bound to thymosin  $\beta 4$  to achieve low-stringency biopanning to identify thymosin  $\beta 4$  interacting partners. See Supplementary Methods for details of phage packaging, phage biopanning and ELISA confirmation.

### Co-immunoprecipitation

Cos1 and 10T1/2 cells were transfected with thymosin  $\beta 4$ , PINCH and/or ILK and lysates precipitated with antibodies to each as previously described<sup>36</sup>. Western blots were performed using anti-ILK polyclonal antibody (Santa Cruz), anti-thymosin  $\beta 4$  polyclonal antibody<sup>35</sup> (gift of H. Yin) and anti-Myc or anti-Flag antibody against tagged versions of PINCH.

### Animals and surgical procedures

Myocardial infarction was produced in 58 male C57BL/6J mice at 16 weeks of age (25–30 g) by ligation of the left anterior descending coronary artery as previously described<sup>37</sup>. All animal protocols were reviewed and approved by the University of Texas Southwestern Medical Center Institutional Animal Care Advisory Committee and were in compliance with the rules governing animal use as published by the NIH. Twenty-nine of the ligated mice received thymosin  $\beta 4$  treatment immediately after ligation and the remaining 29 received PBS injections. Treatment was given intracardiac with thymosin  $\beta 4$  (400 ng in 10  $\mu$ l collagen) or with 10  $\mu$ l of collagen; intraperitoneally with thymosin  $\beta 4$  (150  $\mu$ g in 300  $\mu$ l PBS) or with 300  $\mu$ l of PBS; or by both intracardiac and intraperitoneal injections. Intraperitoneal injections were given every 3 days until mice were killed. Doses were based on previous studies of thymosin  $\beta 4$  biodistribution<sup>38</sup>. Hearts were removed, weighed and fixed for histological sectioning. Additional mice were operated on in a similar fashion for studies 0.5, 1, 3, 6 and 11 days after ligation.

### Analysis of cardiac function by echocardiography

Echocardiograms to assess systolic function were performed using M-mode and two-dimensional measurements as described previously<sup>37</sup>. The measurements represented the average of six selected cardiac cycles from at least two separate scans performed in random-blind fashion with papillary muscles used as a point of reference for consistency in level of scan. End diastole was defined as the maximal left ventricle diastolic dimension and end systole was defined as the peak of posterior wall motion. Single outliers in each group were omitted for statistical analysis. Fractional shortening (FS), a surrogate of systolic function, was calculated from left ventricle dimensions as follows:

FS = ((EDD – ESD)/EDD) × 100%. Ejection fraction (EF) was calculated from two-dimensional images.

## Calculation of scar volume

Scar volume was calculated using six sections through the heart of each mouse using Openlab 3.03 software (Improvision) similar to that previously described<sup>6</sup>. Per cent area of collagen deposition was measured on each section in a blinded fashion and averaged for each mouse.

## Statistical analyses

Statistical calculations were performed using a standard *t*-test of variables with 95% confidence intervals.

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