Role of Thymosin β4 During Cardiac Development and Myocardial Cell Survival

Doctoral (PhD) Thesis

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2009

1. Introduction

Coronary artery disease results in acute occlusion of cardiac vessels leading to loss of dependent myocardium. Such events are one of the major causes of death in the Western world. Because the heart is incapable of sufficient muscle regeneration, survivors of myocardial infarctions typically develop chronic heart failure with over ten million cases in the United States alone. While 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.

Recent evidence suggests that a population of extracardiac or intracardiac stem cells may contribute to maintenance of the cardiomyocyte population under normal circumstances. 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, but this approach has been controversial and requires isolation of autologous stem cells or 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 cardiomycytes to aid in cardiac repair. As an alternative to stem cell therapy ***we hypothesized the use of small, secreted molecules may be a potential way to stimulate cardiac regeneration*** averting technical hurdles associated with progenitor cell applications. Moreover, a systematic approach to understanding the signaling mechanisms actuated by such proteins may be beneficial in designing novel therapeutic strategies following an onset of cardiac dysfunction due to hypoxic conditions in children and adults.

In our studies of genes expressed during cardiac morphogenesis, we found the forty-three amino acid secreted peptide ***thymosin β4*** (TB4), which has been shown to be present in high concentrations in various adult tissues especially in the spleen, lungs, thymus, brain as well as in the developing heart.

TB4 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. Recent domain analyses indicate β-thymosins can affect actin assembly based on their carboxy-terminal affinity for actin. In addition to cell motility, TB4 may affect transcriptional events by influencing Rho-dependent

gene expression or chromatin remodeling events regulated by nuclear actin. Although TB4 promotes skin and corneal wound healing through its effects on cell migration, angiogenesis and inflammation, the precise molecular mechanism through which TB4 functions and its potential role in adult solid organ wound healing currently remain unknown.

2. Objectives / Specific Aims

Specific Aim 1. *Investigation of TB4's expression during embryonic heart development and its effect on cardiac cell migration and proliferation in vitro*. The goal of this aim is to provide detailed description of the expression of TB4 in the embryonic heart and to develop a reliable *in vitro* assay to investigate the cellular and molecular alterations initiated by small, secreted molecules in cardiac cells.

Specific Aim 2. *Molecular and functional investigations to characterize TB4 initiated alterations in the adult hypoxic myocardium in vivo*. The fundamental problem in myocardial infarction and congestive heart failure is an insufficient number of properly functioning cardiomyocytes. Repairing defective and/or regenerating cardiomyocytes is an important experimental means to improve heart function. To establish fully functioning cardiac muscle cells, a detailed comprehension of myocyte differentiation is required on cellular level. Thus, a systematic approach to understanding the signaling mechanisms actuated by proteins effective in cardiac protection and regeneration may be beneficial as it will be instrumental in designing novel therapeutic strategies.

Specific Aim 3. *Analysis of the effect of TB4 on vascular re-growth after hypoxic events in the adult mammalian heart in vivo*. Defects in the coronary vascular system have significant impact on heart function and disease. A proper angiogenic response after infarction is critical for healing and repair. This aim describes whether TB4 affects long term cardiac regeneration by inducing coronary re-growth in adult mammalian heart after hypoxia *in vivo*.

Specific Aim 4. *Investigation of the effect of TB4 on endogenous cardiac progenitor cells of the adult mammalian heart in vivo*. Many studies have attempted to identify progenitor cells capable of cardiac repair in adult hearts. In zebrafish, epicardial cells invade the myocardium and create a vascular network likely to encourage cardiac regeneration in adults. Thus, the injured adult zebrafish heart can recall signaling pathways essential during embryonic coronary development and the ability to mobilize epicardial

progenitor cells may be the primary reason they effectively regenerate myocardium. Since adult mammalian hearts typically show insufficient regeneration after myocardial infarction, experimental attempts to modify this deficiency by directly utilizing epicardial progenitor cells might prove favorable for cardiac repair.

3. Methods

3.1. RNA in situ hybridization

Whole-mount or section RNA in situ hybridization of E 9.5-12.5 mouse embryos was performed with digoxigenin-labeled or ^{35}S-labelled antisense riboprobes synthesized from the 3' UTR region of mouse TB4 cDNA that did not share homology with the closely related transcript of thymosin β10 (TB10).

3.2. Collagen gel migration assay

Outflow tract was dissected from E11.5 wild type mouse embryos and placed on collagen matrices. After 10 hours of attachment explants were incubated in 30ng of TB4, TB4 and 100nM wortmannin or PBS only in 300μl OPTIMEM medium (Invitrogen/Gibco). Cultures were carried out for 3-9 days and fixed in 4% paraformaldehyde. Cells were counted for quantification of migration and distance.

3.3. Animals and surgical procedures

Myocardial infarction was produced in male C57BL/6J mice at 16 weeks of age (25-30 g) by ligation of the left anterior descending (LAD) coronary artery. Immediately after ligation, half of the mice were injected intracardially with TB4 (200ng in 10ul collagen) or with 10 ul of collagen, intraperitoneally with 150μg of TB4 in 300 μl PBS and half with 300 μl PBS or by both intracardiac and intraperitoneal injections. Intraperitoneal injections were given every three days until mice were sacrificed. Doses were based on published studies of TB4 biodistribution. For *in vivo* inhibition of PKC activity mice were treated systemically with 10μg of Bisindolylmaleinimide-I hydrochloride in 100 μl PBS and with 150μg of TB4 in 300 μl PBS or 300 μl PBS in two boluses after myocardial infarction. Hearts were removed and processed for further investigations.

3.4. Analysis of cardiac function by echocardiography

Echocardiograms to assess systolic function were performed using M-mode and 2-dimensional measurements. 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 (LV) 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 LV dimensions as follows: FS = EDD - ESD / EDD x 100%. Ejection fraction (EF) was calculated from two-dimensional images. EDD, end diastolic dimension; ESD, end systolic dimension.

3.5. cDNA Microarray

RNA from the core and remote areas of four TB4 treated, and four PBS treated hearts were isolated 24 h after treatment using Trizol (Invitrogen, Carlsbad, CA) reagent following the manufacturer's protocol. The quality and quantity of RNA were determined and processed with Mouse Affymetrix Genome 430 2.0 arrays at the Microarray Core Facility at UT Southwestern Medical Center. Four independent experimental and four control arrays were analyzed with GeneChip operating software (GCOS, Affymetrix), GeneSpring 7.0 (Silicon Genetics), significance analysis of microarrays (SAM, Stanford University, Palo Alto, California, USA), and Spotfire Decision Site 8.2 (Spotfire). Computational hierarchical cluster analysis was performed with Spotfire and CLUSFAVOR 6.0 (Baylor College of Medicine, Houston, Texas, USA). Analysis of variance was performed with Spotfire. The data were normalized by mean values (for Spotfire pair-wise comparisons and SAM two-class comparisons) or percentile values (for GeneSpring analyses). Gene expression changes were considered significant if the p value was less than 0.05, the fold change at least 1.5, and gene expression was altered in all replicate comparisons. Genes expressed at different levels in untreated controls were excluded from analysis as they most likely represent experimental variation between samples.

3.6. Real-time RT-PCR

Real-time RT-PCR reactions were performed using 50 ng of mouse heart RNA from non-infarcted areas of three adult mouse hearts after systemic TB4 or PBS treatment respectively. TaqMan One-Step RT-PCR Master Mix Reagent for Marcks and 18s control were designed

by Applied Biosystems (Foster City, CA), and reactions were done in triplicate following the manufacturer's protocol on ABI 7000 cycler.

3.7. Epicardial progenitor cell isolation

Adult mice hearts were carefully perfused with PBS and removed to avoid epicardial damage. After digestion in 0.5% collagenase II (Worthington, Lakewood, NJ) cells were collected. Followed by centrifugation small epicardial progenitors were resuspended in plating medium, counted and an equal number was directly plated on gelatin coated tissue culture dishes, on matrigel (BD Biosciences, San Jose, CA), or incubated with p-Marcks primary / FITC anti-rabbit secondary antibody cocktail for p-Marcks positive cell separation. Cells were counted and sorted on MoFlo Laser sorter equipped with 488nm laser (Beckman-Coulter, Ft Collins, CO). Sorted cells were cultured on gelatin coated culture dishes and analyzed by immunocytochemistry.

4. Results

4.1. Specific Aim 1. Investigation of TB4's expression during embryonic heart development and its effect on cardiac cell migration and proliferation in vitro

4.1.1. TB4 is expressed in the developing heart

Expression of TB4 in the developing brain was previously reported, as was expression in the cardiovascular system, although not in significant detail. Whole mount RNA in situ hybridization of embryonic day (E) 10.5 mouse embryos revealed TB4 expression in the left ventricle, outer curvature of the right ventricle and cardiac outflow tract. Radioactive in situ hybridization indicated that TB4 transcripts were enriched in the region of cardiac valve precursors known as endocardial cushions. 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 TB4-expressing cells in the cushions co-expressed cardiac muscle actin, suggesting TB4 was present in migratory cardiomyocytes known to invade the endocardial cushion. TB4 transcripts and protein were also expressed at E 9.5 - E 11.5 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 mature. Finally, outflow tract myocardium that migrates from a secondary heart field also expressed high levels of TB4 protein.

4.1.2. Secreted TB4 stimulates cardiomyocyte migration and survival *in vitro*

Although TB4 is found in the cytosol and nucleus and functions intracellularly, we found that conditioned medium of Cos1 cells transfected with myc-tagged TB4 contained TB4 detectable by Western blot, consistent with previous reports of TB4 secretion and presence in wound fluid. Upon expression of TB4 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 while control phage was not detectable. Similar observations were made using biotinylated TB4. This data indicated that secreted TB4 was internalized into cells, as previously suggested, although the mechanism of cellular entry remains to be determined.

To test the effects of secreted TB4 on cardiac cell migration, we employed an embryonic heart explant system designed to assay cell migration and transformation on a collagen gel. Cardiomyocytes from valve-forming regions secrete signals that induce endocardial cell migration onto collagen but myocardial cells do not normally migrate in significant numbers. In contrast, upon addition of TB4, we observed a large number of spontaneously beating, cardiac muscle actin-positive, cells that migrated away from the explant. No significant difference in cell death or proliferative rate based on TUNEL assay or phosho-histone H3 immunostaining, respectively, was observed in these cells compared to control cells. To test the response of post-natal cardiomyocytes, we cultured primary rat neonatal cardiomyoctyes on laminin-coated glass and treated the cells with phosphate buffered saline (PBS) or TB4. Similar to embryonic cardiomyocytes, the migrational distance of TB4 treated neonatal cardiomyocytes was significantly increased when compared to control. Primary culture of neonatal cardiomyocytes typically survive for approximately one to two weeks with some cells beating up to two weeks when grown on laminin-coated slides in our laboratory. Surprisingly, neonatal cardiomyocytes survived significantly longer upon exposure to TB4 with rhythmically contracting myocytes visible for up to 28 days.

4.1.3. TB4 induces embryonic endothelial cell migration, proliferation and initiates capillary structure formation of adult coronary endothelial cells *in vitro*

In addition to cardiomyocyte migration, we also tested the effects of TB4 on cardiac endothelial cell migration by embryonic heart explant assays. Exogenously administered TB4

significantly increased embryonic endothelial cell migration by facilitating the number of round actively moving cells *in vitro*. We did not detect increase in cellular death. Phospho-histone H3 staining and immunocytochemistry after BrdU administration however indicated that TB4 significantly affects endothelial cell proliferation.

Human umbilical vein endothelial cells (HUVEC) form capillary structures when plated on matrigel. To analyze the effect of TB4 on cardiac vessel formation we checked whether adult human coronary endothelial cells (HCEC) behave similarly to HUVECs and if TB4 may alter the process *in vitro*. Our results indicated that HCECs are capable of capillary structure formation when cultured on matrigel and that TB4 expedites this process when compared to PBS treated controls.

4.2. Specific Aim 2. Molecular and functional investigations to characterize TB4 initiated alterations in the adult hypoxic myocardium in vivo

4.2.1. TB4 promotes cell survival after myocardial infarction and improves cardiac function

Because of TB4's effect on cardiac cells *in vitro*, we tested whether TB4 might aid cardiac repair *in vivo* after myocardial damage. We created myocardial infarctions in adult mice by coronary artery ligation and treated half with systemic, intracardiac, or systemic plus intracardiac TB4 immediately after ligation and the other half with PBS. All forty-five mice that survived two weeks later were interrogated for cardiac function by random-blind ultrasonagraphy at 2 and 4 weeks after infarction by multiple measurements of cardiac contraction. 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 TB4 had a mean fractional shortening of 37.2 +/- 1.8 % (n=23, 95% confidence intervals; $p<.0001$). 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 TB4 treated mice was 57.7 +/- 3.2 % (n=23, 95% confidence interval; $p<.0001$) compared to a mean of 28.2 +/- 2.5 % (n=22, 95% confidence interval) in control mice after coronary ligation. The greater than 60% or 100% improvement in cardiac fractional shortening or ejection fraction, respectively, suggested a significant improvement with exposure to TB4, although cardiac function remained depressed compared to sham operated animals (~60% fractional shortening; ~75% ejection fraction). Finally, the end

diastolic dimensions (EDD) and end systolic dimensions (ESD) were significantly higher in the control group, indicating that TB4 treatment resulted in decreased cardiac dilation after infarction, consistent with improved function. Remarkably, the degree of improvement when TB4 was administered systemically through intraperitoneal injections or only locally within the cardiac infarct was not statistically different, suggesting the beneficial effects of TB4 likely occurred through a direct effect on cardiac cells rather than through an extracardiac source. Trichrome stain at three levels of section revealed the size of scar was reduced in all mice treated with TB4 but was not different between systemic or local delivery of TB4, consistent with the echocardiographic data. We did not detect significant cardiomyocyte proliferation or death at three, six, eleven or fourteen days after coronary ligation in PBS or TB4 treated hearts. However, twenty-four hours after ligation we found a striking decrease in cell death by TUNEL assay in TB4 treated cardiomyocytes.

4.2.2 TB4 activates ILK and Akt / Protein Kinase B

To investigate the potential mechanisms through which TB4 might be influencing myocardial cell migration and survival events, we searched for TB4 interacting proteins. We synthesized and screened an E 9.5 - E 12.5 mouse heart T7 phage cDNA library by phage display and TB4-interacting clones were enriched and confirmed by ELISA. PINCH, a LIM domain protein, was consistently isolated in this specific screen and interacted with TB4 in the absence of actin. 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 and for cell survival, in part by promoting phosphorylation of the serine-threonine kinase Akt/PKB, a central kinase in survival and growth signaling pathways. We confirmed that TB4 co-precipitated with PINCH or ILK independently and in a common complex, although the interaction of ILK with TB4 was weaker than with PINCH.

Because recruitment of ILK to the focal adhesion complex is important for its activation, we assayed the effects of TB4 on ILK localization and expression. ILK detection by immunocytochemistry was markedly enhanced around cell edges after treatment of embryonic heart explants or C2C12 myoblasts with synthetic TB4 protein or TB4-expressing plasmid. Western analysis indicated a modest increase in ILK protein levels in C2C12 cells, suggesting the enhanced immunofluoresence may be in part due to altered localization by TB4. ILK was functionally activated, evidenced by increased phosphorylation of its known

substrate Akt using a phospho-specific antibody to serine 473 of Akt, while total Akt protein was unchanged. Because TB4 sequesters the pool of G-actin monomers, we asked if the effects on ILK activation were dependent on TB4's role 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, but neither intervention affected the ILK activation observed.

To determine if activation of ILK was necessary for the observed effects of TB4, we employed a well-described ILK inhibitor, wortmannin, which inhibits ILK's upstream kinase, phosphatidylinositol 3-kinase (PI3-kinase). Using myocardial cell migration and beating frequency as assays for TB4 activity, we cultured embryonic heart explants as described above in the presence of TB4 with or without wortmannin. Although inhibiting PI3-kinase affects many pathways, we observed a significant reduction in myocardial cell migration and beating frequency upon inhibition of ILK, consistent with ILK mediation of TB4's effects. Together, these results support a physiologically significant interaction of TB4-PINCH-ILK within the cell and suggested this complex may mediate some of the observed effects of TB4 relatively independent of actin polymerization.

Similar to cultured cells, the level of ILK protein was increased in heart lysates of mice treated with TB4 after coronary ligation compared with PBS treated mice. Correspondingly, phospho-specific antibodies to Akt-S^{473} revealed an elevation in the amount of phosphorylated Akt-S^{473} in mice treated with TB4. The observations *in vivo* were consistent with the effects of TB4 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 TB4, although it is unlikely that a single mechanism is responsible for the full repertoire of TB4's cellular effects.

4.3 Specific Aim 3. Analysis of the effect of TB4 on vascular re-growth after hypoxic events of the adult mammalian heart *in vivo*

4.3.1. TB4 stimulates vascular growth and initiates organ-wide thickening of the adult epicardium *in vivo*

Because of our *in vitro* observations on cardiac and vessel endothelial cells, we assessed the effect of TB4 on coronary vascular growth in adult mice after hypoxia. We created cardiac infarctions by ligating the LAD coronary artery in adult mice followed by immediate

systemic TB4 or PBS administration. Simultaneous staining with platelet endothelial cell adhesion molecule-1 (Pecam-1) and smooth muscle α-actin (sm α-actin) specific primary antibodies revealed significant increase in capillary density three days after TB4 injection at the infarction border zone and non-infarcted remote areas of the hearts.

Coronary vessels are believed to originate from the epicardium during development. Recent work from Lepilina et al. demonstrated an elaborate sequence of organ-wide and local responses by epicardial cells increases cardiac regeneration and revascularization in adult zebrafish. The changes of the epicardium in adult fish are similar to the alterations in developing embryos. Mammalian hearts however, are incapable of such neovascularization or general regeneration after cardiac injury. Thus, experimental attempts to activate the embryonic coronary developmental program in mammals could enhance cardiac regeneration. To determine if TB4 may stimulate an epicardial response, we analyzed the changes in blood vessel/epicardial substance (Bves) expression 24 and 72 hours after systemic TB4 administration. Bves is widely expressed in the developing coronary vascular system and is also used as one of the markers of epicardial cells or cells of epicardial origin in adult and embryonic tissues. In our experiments, we observed elevated Bves expression 24 hours after TB4 treatment, and an increase in Bves positive cells with general organ-wide thickening of the adult epicardium 72 hours after peptide administration in the non-infarcted remote regions of the hearts similar to the changes in adult zebrafish.

4.3.2. TB4 initiates the expression of embryonic developmental genes in adult mouse epicardium

Our *in vivo* results suggested TB4 may activate the adult epicardium and initiate vessel growth. To support our hypothesis we investigated the expression of proteins essential during coronary development in embryos by Western blot and by immunohistochemistry 24 and 72 hours after systemic TB4 injection. Our data indicate TB4 affects developmental gene expression as early as 24 hours after systemic injection while alterations in epicardial morphology were first observed three days after the initial peptide treatment. We detected significant increase in VEGF, VEGF receptor-2 (Flk-1) and TGF-β expressions and moderate elevation in FGF-17, FGF receptor-2 (FGFR-2) and FGFR-4 levels by Western blot after 24 hours of treatment. Immunohistochemistry after 3 days of TB4 injection indicated these changes are primarily manifested in the thickened epicardium. The alterations in gene expression were consistent with the findings in regenerating adult zebrafish hearts. Since

FGF and WNT signaling pathways can function jointly to sustain mesenchymal growth or to coordinate epithelial morphogenesis during development, and epicardium-derived progenitor cells also require β-Catenin for coronary artery formation, we asked whether TB4 might also alter β-Catenin expression *in vivo*. Our findings revealed an increase in β-Catenin expression in the thickened epicardium and in developing vasculature of the adult mouse hearts, which potentially indicate a role for β-Catenin and a regulatory convergence for FGF and WNT signaling in the epicardial initiation process. Finally, the analysis of additional known regenerative proteins revealed TB4 significantly increases Jun N-terminal kinase (JNK) expression while p38 expression and p38 and JNK activation were significantly reduced. We detected minor alterations in extracellular signal regulated kinase1/2 (Erk1/2) activation, inducible isoform of nitric oxide synthase (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) levels in the non-infarcted cardiac tissue in the first 24 hours of treatment. These observations strongly suggest an early molecular support for new vessel formation and myocardial regeneration by initiation of the embryonic epicardial developmental program and by activation of myocardial progenitors in adult mouse hearts after TB4 injection *in vivo*.

4.3.3. TB4 increases the number of Marcks positive cells and activates PKC in the adult epicardium

To further identify molecules responding to TB4 and are expressed in the adult epicardium, we analyzed mouse hearts with Mouse Genome 430 2.0 Affymetrix cDNA microarrays 24 h after cardiac infarction. While focusing on genes significant in angiogenesis, myristoylated alanine-rich C-kinase substrate (Marcks) was up-regulated 2.8-fold after TB4 administration. Marcks is a prominent intracellular substrate for PKC, a regulator of angiogenesis, and is distributed in numerous cell types, including vascular endothelial cells. It mediates PKC signaling through its phosphorylation, resulting in a release of Marcks from the cell membrane to the cytosol. These responses are commonly used to indicate PKC activity *in vitro*.

To determine if TB4 affects PKC activation in cell culture, we investigated Marcks phosphorylation and localization in adult HCECs by Western blot and immunocytochemistry after TB4 treatment. Our results indicate that external administration of TB4 increases Marcks expression, phosphorylation and translocation from the cell membrane to the cytosol suggesting that TB4 modulates PKC activity. PKC lies on the signal transduction pathways by which VEGF augments development and angiogenesis during initial and later stages of

vessel development. To confirm our *in vitro* results, we examined the effect of TB4 on Marcks expression and phosphorylation after cardiac ligation in adult mice. We detected increased Marcks expression and phosphorylation especially in the thickened epicardium, indicating a role for PKC in epicardial activation and coronary re-growth in adults after TB4 treatment *in vivo*.

4.3.4. Inhibition of PKC activity suppresses TB4 initiated epicardial activation in adult mice

To define whether PKC activity may be regulatory for TB4 induced epicardial activation, we tested the effect of Bisindolylmaleimide-I (PKC inhibitor) on HCECs and adult epicardial cells *in vitro*, and injected 10μg of PKC inhibitor intraperitoneally with or without TB4 into infarcted adult mice. Our *in vitro* experiments indicated that PKC inhibition alters HCEC capillary structure formation and inhibits epicardial cell differentiation on matrigel. The newly formed structures contained irregular sm α-actin positive cells and revealed disordered morphology. In live animals immunohistochemistry with Bves, VEGF or p-Marcks specific primary antibodies showed reduction of epicardial thickening. We also observed a significant decrease in capillaries and sm α-actin positive cells at the non-infarcted remote areas or borders of the infarction after Bisindolylmaleimide-I injection. This suggests reduction of coronary outgrowths from the epicardium in the TB4+PKC inhibitor treated infarcted hearts. Our results suggest that TB4 mediated direct or indirect PKC activation is essential for epicardial cell transformation and migration in the adult mouse heart *in vivo*.

4.4. Specific Aim 4. Investigation of the effect of TB4 on endogenous cardiac progenitor cells of the adult mammalian heart *in vivo*

4.4.1. p-Marcks positive adult epicardial cells show endothelial and smooth muscle fate

Recent results suggest that the epicardium is highly heterogenous and serves as a source of various progenitors in the embryonic heart. Consistent with these observations, immunohistochemistry on adult hearts showed several p-Marcks positive cells lacking VEGF while expressing Bves in wild type or in TB4 activated adult epicardium. Because of these findings, we asked whether p-Marcks positive cells might represent a distinct epicardial cell population of the adult heart. First, we cultured non-separated or FACS sorted p-Marcks

positive and p-Marcks negative adult mouse epicardial cells on matrigel or gelatin coated dishes. Our data revealed that purified p-Marcks positive epicardial cells are small, have narrow cytoplasm and express both smooth muscle and/or endothelial markers in cell culture, while p-Marcks negative epicardial cells differentiate into large sm α-actin positive and Cytokeratin negative lineages. Consistent with our *in vitro* results, co-immunostaining with p-Marcks, sm α-actin, Cytokeratin and Pecam-1 antibodies indicated an overlap of smooth muscle and early and late endothelial markers in the newly developing capillaries and in the endothelial and smooth muscle layers of the mature vessel wall.

Similar to p-Marcks and VEGF expression, the adult epicardium contained a mixed population of p-Marcks, sm α-actin and Cytokeratin positive cells but did not stain for the late endothelial marker Pecam-1. External administration of TB4 to non-separated adult epicardial cultures resulted in a large number of p-Marcks and Cytokeratin specific endothelial colonies when compared to PBS treated controls. Thus, our *in vitro* and *in vivo* results suggest that p-Marcks positive epicardial cells are multipotent and may be a source for smooth muscle and endothelial cells of the newly developing vessels in the injured adult heart.

4.4.2. TB4 activates myocardial progenitor cells of the adult epicardium *in vivo*

Recent studies suggest the epicardium is not only a resource for coronary vessel progenitor cells but also may provide stem cells for future cardiomyocytes in the embryo. Specifically, Tbx-18 and Wt-1 revealed to label myocardial progenitors in the adult epicardium. Because of these findings we asked whether TB4 could initiate long-term post ischemic muscle regeneration by myocardial progenitor activation in the adult hearts. Our results revealed TB4 raises the expression of Tbx-18 and Wt-1 proteins in the non-infarcted remote areas as early as 24 hours after injection and significantly increases the number of Tbx-18 and Wt-1 positive cells after three days. Tbx-18 positive cells were distributed equally in the epicardium and myocardium, while Wt-1 positive cells were primarily located in the subepicardial space, suggesting Wt-1 and Tbx-18 may mark different progenitor populations in the activated epicardium. In addition, Bisindolylmaleimide-I significantly suppressed the number of TB4 activated Tbx-18 and Wt-1 positive progenitor cells in the non-infarcted remote areas of the hearts. Our results indicate systemic TB4 administration increases the number of endogenous myocardial progenitor cells of the adult mammalian heart, and TB4

mediated direct or indirect PKC activation is essential for precursor migration and transformation from the adult epicardium *in vivo*.

5. Discussion

Acquired evidence presented in this work suggest that TB4, a small, secreted protein involved in cell migration and survival during cardiac morphogenesis may be re-deployed to minimize cardiomyocyte loss after cardiac infarction. We demonstrate that TB4 increases or activates proteins and epicardial progenitors important for myocardial regeneration or vascular growth and initiates organ-wide activation of the adult epicardium reminiscent of embryonic coronary development by re-stimulation of signaling pathways essential during embryogenesis. Our work also reveals a novel role for PKC in the epicardial activation process and suggests that p-Marcks positive epicardial cells may serve as novel endothelial and smooth muscle progenitors for future capillaries in the adult mammalian heart. Given the known roles of PINCH, ILK and Akt, the data are consistent with this complex playing a central role in TB4's effects on cell motility, survival and cardiac repair. TB4's ability to prevent cell death within twenty four hours after coronary ligation is probably one of the dominant factors leading to decreased scar volume and improved ventricular function observed in mice. Although TB4 up-regulation of ILK is likely to have many cellular effects, the activation of Akt may be the most dominant mechanism through which TB4 promotes cell survival. This is consistent with Akt's proposed effect on cardiac repair when over-expressed in mouse marrow-derived stem cells administered after cardiac injury, although this likely occurs in a non-cell autonomous fashion. Whether TB4 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.

In addition to direct inhibition of myocardial cell death we discovered that TB4 significantly increases coronary re-growth after cardiac infarction. Given the roles of Pecam-1, VEGF, MAP kinases, ILK, Akt, FGFs, NOSes, β-Catenin, and PKC in vessel formation, our research suggests TB4 may also initiate broad angiogenic events that promote regeneration after cardiac injury in adults.

Our results suggest TB4 augments cardiac regeneration and increases cardiac function in the adult hypoxic heart through at least two steps. First, it inhibits myocardial cell death 24

h after ligation. Second, TB4 can initiate signaling pathways responsible for late-phase or chronic regeneration, such as vascular re-growth or progenitor cell activation. This later process may be initiated by organ-wide epicardial thickening and activation of endothelial-mesenchymal transformation of adult epicardial cells, is most likely regulated by PKC, and is first visible 3 days after TB4 administration.

Coronary vessel growth is independent of cells outside the heart once the epicardium is formed. Thus, the potential for TB4 to activate dormant cardiac stem cells that exist in the adult mammalian heart is critical for cardiac regeneration. Furthermore, the anti-inflammatory effect of TB4 may also support adult cardiac repair.

Generating novel therapies to achieve coupled myocardial and vascular regeneration by recalling the embryonic program in adults is a rapidly expanding concept and may be a solution to aid the failing heart. The work presented here first describes a molecule with such capabilities. While TB4 can augment an organism's ability to heal surface wounds, our results demonstrate TB4's efficacy in repairing of a solid organ and reveal novel mechanisms through which TB4 affects cellular functions.

Since the discovery of innovative methods to enhance cardiac regeneration is important towards future therapies, the continued investigation of molecular signals initiated by TB4 is highly essential. Given the findings here, the utility of TB4 for healing after cardiac injury suggests promise and warrants further pre-clinical investigation.

6. Summary of Original Observations

I. Our results support that Thymosin β4 (TB4), a small, secreted peptide is expressed in the mammalian heart during embryonic development and in adults.

II. We showed TB4 is capable of increasing embryonic myocardial cell migration and endothelial cell proliferation, and initiates capillary tube formation of adult coronary endothelial cells *in vitro*.

III. We demonstrated local or systemic injection of TB4 inhibits myocardial cell death and initiates coronary re-growth after cardiac infarction in adult mammals.

IV. We presented TB4 to be the first known molecule capable of inducing the embryonic coronary developmental program in adults by activating the epicardium after systemic injection *in vivo*.

V. We demonstrated systemic administration of TB4 activates endogenous vessel and myocardial progenitor cells of the adult heart *in vivo*.

VI. We presented TB4 significantly increases cardiac function irrespectively of local or systemic administration in the infarcted adult mammalian heart.

VII. Analyzing the potential molecular mechanisms responsible for TB4's effects we found TB4 activates Akt / Protein Kinase B to inhibit cardiac cell death *in vitro* and *in vivo*.

VIII. We discovered TB4 initiated Protein Kinase C activation is essential for coronary regeneration and epicardial progenitor activation in the adult mammalian heart.

7. Relevant Publications

1. *Bock-Marquette I.*, Saxena A., White M.D., Dimaio J.M., Srivastava D..Thymosin beta-4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. ***Nature.*** 432(7016):466-72. (2004). Impact factor: 32.182

2. Srivastava D, Saxena A, Dimaio JM, *Bock-Marquette I.* Thymosin beta4 is Cardioprotective After Myocardial Infarction. ***Ann. N. Y. Acad. Sci.***. 1112:161-70. (2007). Impact factor: 1.731

3. Hinkel, R., Olson, T., Horstkotte, J., El Aouni, C., Mueller, S., Mayer, S., *Bock-Marquette, I.*, DiMaio, M., Hatzopoulos, A., Boekstegers, P., Kupatt, C. Cardioprotective Potential of Thymosin ß4 after Ischemia/Reperfusion in a Preclinical Pig Model. ***Circulation,*** 116, (16) II 130 (2007) Impact factor: 12.755

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Cumulative impact factors: 107.175

10. Acknowledgements

I wish to thank to my present and former mentors, Drs. Béla Melegh, Eric Olson, J. Michael DiMaio, Deepak Srivastava and Jon Wolff for their encouraging support and teachings. My former and present colleagues and my friends, especially István Dankó, Santwana Shrivastava, Michael White, Ankur Saxena, Jesse Williams, Stephen Johnson and members of the Srivastava, Olson, Wolff, Melegh and Sümegi laboratories for technical support and friendship. I am extremely grateful to my collaborator, Dr. Christian Kupatt for scientific help.

I express my warmest appreciation to my family, Jon and Antonio Marquette and my parents, Katalin Weber and Antal Bock for their love, patience and encouragement in the preparation of this research.