Thymosin beta-4 modulates post-hypoxic fibrotic remodeling of the adult mammalian heart

Doctoral (PhD) Thesis

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1. Introduction

1.1. Hypoxic heart disease

According to most recent data of Heart Disease and Stroke Statistics, more than 1.5 million Americans died from cardiovascular disease (CVD) and heart failure (HF) in 2025 [1]. Moreover, the American Heart Association estimates over 9% of the population of the United States will suffer from coronary heart disease (CHD) by the year of 2030 [2]. With the global population rapidly aging and by the limitations of our therapeutical tools towards preserving or improving the injured heart, brain or blood vessels, searching for novel approaches to limit life threatening consequences became essential.

Among all potential solutions, replacement of irreversibly lost cardiomyocytes stands as a primary goal. Although cardiac cells are capable of proliferation, the overall regenerative potential of the heart is extremely limited. Consequentially, fibrotic scar tissue is formed at the site of the injury causing mechanical complications, arrhythmias or contractility disorders eventually leading to heart failure [3].

1.2. Organ / tissue fibrosis

To maintain structural and functional integrity of the organs following injury, fibrotic response mechanisms are often initiated and scar tissue is formed at the damaged area. The process is characterized by excessive extracellular matrix (ECM) (mostly collagen) deposition as a result of imbalance in production and degradation of matrix proteins [4]. In the heart, acute necrosis due to an MI, or chronic diseases such as high blood pressure or diabetes, even aging itself may induce fibrotic alterations [5, 6]. In the healthy myocardium, fibroblasts constantly modify the extracellular environment by the production or degradation of the ECM in a well-balanced way [3, 5, 7]. During cardiac injury however, fibroblasts become activated and differentiate into a new form referred to as myofibroblasts [5]. The term ‘myofibroblast’ is utilized to describe a heterogenous population of cells, which participate in the healing processes of practically all organs in the body. The most cardinal functions of myofibroblasts include the ability of exerting contractile force through

actin-myosin bundles and to metabolically and functionally adapt to various stages of tissue repair over the course of wound healing and maturation [5, 8, 9].

Although vigorously studied, the precise mechanism and signals which trigger the differentiation process into myofibroblasts still remain unclear. According to recent results, once activated, myofibroblasts become responsive to many secreted molecules including various hormones, growth factors or pro-inflammatory cytokines resulting in increased proliferation, migratory behavior, secretion of ECM-degrading metalloproteinases (MMPs) and collagen turnover all critical for the formation of a pathologic scar [5, 8]. *TGF-β pathway* is one of the most studied and most fundamental pathways affecting fibrotic processes in the heart. This pathway induces ECM protein production primarily through activation of Mothers against decapentaplegic homolog-4 (SMAD) transcription factors [7]. Clinical utilization of TGFβ inhibitors however are currently not possible due to serious side effects [10]. In addition to TGFβ, activation of systematic neurohumoral pathways such as the *Renin-Angiotensin System (RAS)* is equally pivotal regarding maladaptive pathological fibrotic remodeling processes [11]. Activation of the RAS system leads to increased blood pressure, influences cardiac fibroblast proliferation, migration and promotes myofibroblast conversion through various ways [7, 12-15].

1.3. The impact of cellular microenvironment on fibroblast activation

The importance of cellular microenvironment in relation to cell transformation and behavior became increasingly apparent and supported by recent investigations. Fibroblasts engage with the extracellular environment through a cohesive structural complex which includes the extracellular matrix (ECM), integrin-associated focal adhesions and actin cytoskeleton, which are responsible to integrate environmental inputs into consequential transcriptional events [16, 17]. Clearly, the arrangements made in the actin cytoskeletal system are equally critical regarding cellular behavior, transformations and thus, myofibroblast differentiation [18].

RhoGTPases are recognized to reorganize actin cytoskeleton into stress fibers, which in a myofibroblast contain primarily smooth muscle isoforms [19]. The most investigated RhoGTPases, Rho, Rac and cell division control protein 42 homolog (CDC42) are activated by profibrotic receptor complexes and regulate effector proteins which modulate the polymerization equilibrium of glomerular-actin (G-actin) and filamentous-actin (F-actin) in the cytoplasm [19]. Such regulatory downstream targets are the *Rho*

associated kinases (ROCK1, also known as ROKß and ROCK2, also known as ROKα [20]) and the diaphanous family of formins (mDia), which are both required for polymerization and interconnection of stress fibers. ROCK isoforms share approximately 65% sequence homology and 92% kinase homology [21]. Based on the specific phosphorylated substrate molecules, ROCK proteins have extensive intracellular effects. The pathway is central in stress-fiber formation and contraction, cytoskeletal rearrangement, cell-substratum and cell-cell adhesion, apoptosis, cell migration and cytokinesis [22, 23]. In recent years, numerous studies examined the cardiovascular impact of the RhoA/ROCK pathway. Results indicate ROCKs have significant role in cardiovascular diseases such as cardiac hypertrophy, hypertension, atherosclerosis and fibrosis [24]. Notably, pharmacologic inhibition of ROCKs blocks cytoskeletal remodeling and matrix gene expression in TGFβ stimulated fibroblasts [25-27], while ischemic hearts from ROCK1 knockout mice failed to form myofibroblasts and revealed less fibrosis [27] all suggesting a critical role for the molecule during tissue remodeling and scar formation. With special regards to fibrosis, ROCKs enhance the process through the myocardin related transcription factor-A (MRTFA)/ Serum Response Factor (SRF) pathway [28]. In response to injury like MI, RhoA/ROCK pathway becomes activated, which leads to a shifted balance of F-actin and G-actin [29]. As the cytoplasmic G-actin levels are decreasing, G-actin dissociates from MRTF-A. Unbound MRTF-A translocates to the nucleus and contributes in the induction of collagen secretion by fibroblasts [26, 29]. Subsequent studies have revealed that inhibition of MRTF activity blocks dermal, colonic and lung fibrosis in vivo [30-34]. Together, these studies demonstrate the potential for targeting signal responsive transcription factors such as MRTF-A to regulate fibroblast response.

Targeting the TGFβ/Smad/Scx (scleraxis) [35-37] or Rho-ROCK/MRTF/SRF pathways [38, 39] has already proven efficacious in blocking the progression of fibrosis in animal models of disease. Naturally, in addition to the aforementioned players, actin assembly may be equally influenced by numerous additional small molecules and pathways within the cell, such as microRNAs or secreted peptides.

1.4. Small peptides / Thymosin β4 (TB4)

Presently, there is substantial interest in recognizing physiological changes or identifying molecules which can be affected by positive or negative physical or psychological stress. Utilization of such information holds great potential for identifying different states of the human body. Importantly, these small secreted molecules, which are commonly referred to

as biomarkers may equally pave the path for the development of novel therapeutical targets in everyday clinical practice [40].

Thymosins are a group of small secreted proteins, first isolated from calf thymus and initially thought to be a single thymic hormone [41]. Further research later confirmed that the purified thymic extract contains a mixture of biologically active compounds [42]. Based on isoelectric focusing pattern, the constituents were named based on their isoelectric points: alpha thymosins (pH below 5.0), beta thymosins (pH between 5.0-7.0), and gamma thymosins (pH above 7.0) [43].

Currently sixteen beta thymosins are known in various organisms, however only three are expressed in humans: TB4, thymosin β10 (TB10), and thymosin β15 (TB15) [44-47]. Among all, TB4 is the most abundant isoform representing 70-80% of all beta thymosin content in cells [48]. It is present in high concentrations (up to 0.4 mM) in various adult tissues, especially in the spleen, lungs, thymus, brain and heart [49], as well as in macrophages, tumor and human blood cells, while serum contains less than 1% of the TB4 amount present in the entire blood system. Apart from blood, TB4 can be detected in wound fluid or in additional body fluids, such as saliva or tears [44-47].

TB4 has been implicated in modulating the availability of actin monomers by sequestering G-actin, however, overexpression of TB4 indicated an alternative function for the molecule as well [50, 51]. Accordingly, in addition to its G-actin sequestering function, TB4 also possesses the power to control the steady state of actin assembly similarly to capping proteins and profilin, however, in contrast to profilin, it is capable of performing its action at both ends [51].

1.5. Role of TB4 in the heart

With respect to embryonic development TB4 is expressed in the heart and vessels in mice [52], chicks [53] as well as in humans [54], suggesting a pivotal role in healthy organogenesis. During mammalian heart development it has been reported to be highly expressed in the interventricular septum, ventricles, and the outer layer of the myocardium [52].

The significance of TB4 regarding cardiac regeneration and repair was first published by members of our research group affiliating a leading role for the peptide in aiding cardiac function following hypoxia [52]. We discovered that external administration of TB4 promotes myocardial cell migration and survival in embryonic tissue in vitro and retains this property following birth [52, 55]. Following coronary artery ligation in mice,

the peptide enhanced myocyte survival, reduced scaring and improved cardiac function suggesting TB4 may be a novel therapeutic target in the setting of acute myocardial damage from heart attacks and other myocardial diseases among children and adults [56-58]. Remarkably, the degree of improvement when TB4 was administered systemically through intraperitoneal injections or only locally within the infarction area 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 [52].

2. Objectives and Specific Aims

One of the major advantages regarding tissue repair is TB4's capability for reducing rigid malfunctioning scar tissue, which is achieved likely through reduction of the inflammatory processes [59-61], inhibition of cellular death by activating AKT [52] and general positive promotion of repair mechanisms [6, 62-64]. Most notably, TB4 is capable of reversing, or at least influencing fibroblast/myofibroblast transition in vitro, which attribute lends the molecule a broad applicational perspective as an anti-inflammatory agent [65, 66].

While the molecular mechanisms triggered by TB4 during repair processes in the heart are widely investigated [62, 65], there are numerous questions yet to be explained regarding its impact on post-ischemic heart repair. Accordingly, **the general aim of this present thesis is to reveal further targets and novel mechanisms through which TB4 mitigates pathological scarring in the heart.** Accordingly, we delved into the molecular alterations by which TB4 may influence scar formation in adult mammalian hearts by systemic administration following hypoxia.

Accordingly, the Specific Aims of this thesis are the following:

Aim 1.: To identify altered small molecules (miRNAs) related to scar formation following systemic TB4 administration in adult hypoxic mice hearts.

Aim 2.: To determine the molecular roleplayers and mechanisms governed by the altered expression of identified miRNA targets regarding scar formation.

Aim 3: To specify and compare the initiated alterations in various cell types of the adult mouse and human heart to predict potential clinical relevance.

3. Methods

3.1. Animal procedures

Myocardial infarctions were produced in C57BL/6J male mice at 16 weeks of age (25–30 g) by a ligation of the left anterior descending (LAD) coronary artery as previously described [52]. All animal protocols were reviewed and approved by the University of Texas Southwestern Medical School Institutional Animal Care Advisory Committee and were in full compliance with the rules governing animal use as published by the NIH.

3.2. miRNA microarray

To perform miRNA microarrays, we isolated RNA from the core (area of risk) and remote areas of five TB4 treated, and five PBS treated hearts utilizing Trizol Reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's protocol (Invitrogen). Microarray assay was performed by using a service provider (LC Sciences).

3.3. Real-time quantitative PCR

Total murine heart RNA was extracted utilizing Trizol Reagent (Invitrogen, Carlsbad, CA) in full accordance with the manufacturer's protocol (Invitrogen). Following RNA quantification, 50 ng of total RNA from core and remote areas was transcribed to cDNA via TaqMan Reverse Transcriptase Kit (Thermo Fisher Scentific). mRNA and miRNA specific probes (Applied Biosystems) were purchased and tested using cDNA templates from day one and day three TB4 and PBS treated hearts in accordance with the manufacturer's protocol, respectively.

3.4. Western blot

Protein expression was analyzed by Western blotting. Cells or tissues were lysed in the presence of protease inhibitors to obtain total protein. Equal amounts of protein were denatured and separated by SDS–PAGE. Proteins were then transferred onto a PVDF membrane. Following blocking with milk proteins, the membrane was incubated with target specific primary and HRP-conjugated secondary antibodies. Protein bands were detected using chemiluminescent methods. Band intensities were analyzed to evaluate relative protein expression.

3.6. Cell culturing and immunostaining– normoxic and hypoxic conditions

Adult human umbilical vein endothelial cells (HUVECs), adult human cardiac fibroblasts (HCFBs) and adult human cardiac myocytes (HCMCs) (PromoCell GmbH, Heidelberg, Germany) were cultured on collagen treated cover glasses (Rat tail collagen, Roche) and incubated in cell specific culturing medium (supplemented by the distributor; PromoCell GmbH, Heidelberg, Germany) until 30-60% confluency was achieved. TB4 in PBS (n=4) or PBS (n=4) alone was directly added to the culturing medium for 24 hours under regular tissue culture conditions (20% O_2, 5% CO_2, 75% N_2). To achieve hypoxic stress, an equal number of cells with similar treatment conditions were incubated under hypoxic conditions (5%CO_2, 1%O_2, 94% N_2). Following incubation, cell culture experiments were terminated utilizing 4% PFA and immunostaining was performed using target protein specific primary and fluorophore conjugated secondary antibodies. Protein expression was documented via Zeiss LSM-710 confocal microscopy.

3.7. Statistical analyses

For microRNA microarrays, statistical tests and clustering analyses were provided by LC Sciences as part of the miRNA microarray service. The signal values were derived by background subtraction and normalization. Detectable transcripts were subjected to data processing statistics and signal intensities were listed as average values of repeating spots. One-way ANOVA and *t*-test were utilized to determine significance. $p < 0.05$ was considered statistically significant.

RT-PCR results and quantification of western blots were processed utilizing Microsoft® Excel 16.96 or GraphPad® Prism 10.1.1 software. Quantitative results were expressed as means +/− SD. $p < 0.05$ was considered statistically significant, whereas $p < 0.01$ was highly significant.

4. Results

4.1. Specific Aim 1. To identify altered small molecules (miRNAs) related to scar formation following systemic TB4 administration in adult hypoxic mice hearts.

4.1.1. MicroRNA microarray of TB4 treated hypoxic mouse hearts

Our previous results suggested reduction of cellular death is one, but most likely not the only mechanism responsible for TB4's positive impacts following hypoxic events in the

heart [52]. To further elucidate the potential molecular mechanisms beyond our observations, we performed miRNA microarrays one and three days following infarction, with or without TB4 treatment utilizing samples from the core and remote areas of adult mouse hearts (n=4 core/remote one day/three days/each, respectively). In our screen we discovered 18% of the altered miRNAs may have potential impact on cardiac fibrosis and ECM remodeling (Figure 1.)

In addition to numerous altered potential miRNA targets, we discovered miR139-5p became significantly elevated (1.5-fold) one and three days following systemic treatment with TB4 when compared to PBS treated controls by microarray in the infarcted core. To confirm microarray results, we performed real-time PCR utilizing miR139-5p specific probe on the core and remote areas of the hearts. We did not observe significant alterations one day following infarction, however our results indicated a significant upregulation in miR139-5p expression on the third day in both the core and remote areas of the hearts when compared to PBS treated controls.

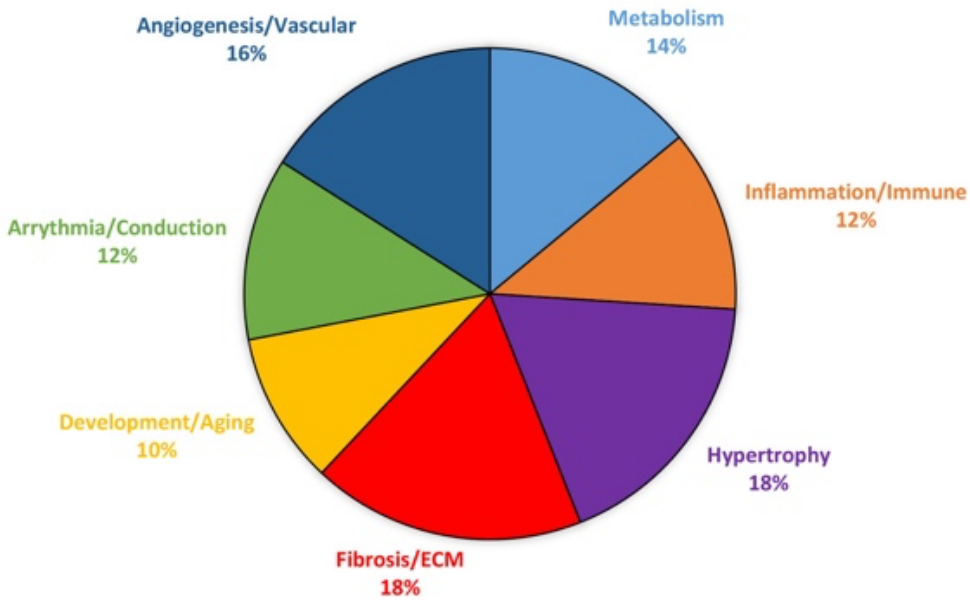

Figure 1. Representative chart summarizing potential functions of altered miRNAs following systemic TB4 injections in mouse hearts after infarction (Multiple categories were allowed for each miRNA target)[67].

4.2. Specific Aim 2.: To determine the molecular roleplayers and mechanisms governed by the altered expression of identified miRNA targets regarding scar formation.

Next, we searched for predicted potential targets of miR139-5p utilizing Targetscan (targetscan.org) software in parallel with the literature. Our attention was primarily focused on molecules which are expressed in the heart and described as critical during the processes of remodeling and fibrotic scar formation. One potential target of miR139-5p that meets these criteria and was previously identified by Zhao et al. is ROCK1 [68], a well-known regulator of actin assembly in various cell types [69].

To reveal whether TB4 may influence ROCK1 protein expression in general, we first performed western blot analyses utilizing protein samples from the core and remote uninjured areas of mouse hearts one and three days following infarction and simultaneous systemic TB4 or PBS treatments. The source for protein samples was the same tissue used for microarray analyses. Our results demonstrated, TB4 significantly decreases ROCK1 protein levels in the core and remote areas of the heart one and three days following cardiac infarction respectively (Figure 2.).

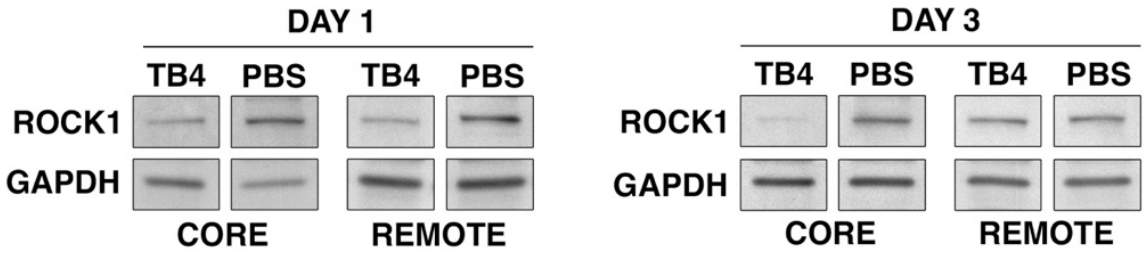

Figure 2. TB4 downregulates ROCK1 protein levels in vivo following cardiac infarction. Representative western blot images of the core and healthy remote areas utilizing ROCK1 primary antibody [67].

Given to the 92% sequence homology of the kinase domains of ROCK1 and ROCK2, we next asked, whether the impact of TB4 is ROCK1 specific. In contrary to the findings with ROCK1, western blots with ROCK2 specific primary antibody did not reveal significant alterations of the protein following systemic TB4 treatment in infarcted mouse hearts.

Finally, because our western blot results indicated a drop in ROCK1 protein levels while miR139-5p remained unaltered one day following infarction, we searched for additional potential mechanisms responsible for ROCK1 alterations. Accordingly, we investigated the levels of ROCK1 mRNA at these time points in the heart. We found

ROCK1 mRNA decreases visibly yet not significantly in all remote and healthy tissues of the infarcted mouse hearts during the first 24 hours following systemic TB4 treatment at the core and remote regions, respectively. Notably, the RQ values of TB4 of all regions and treatments were close or similar to those detected in the sham-operated hearts.

4.3. Specific Aim 3: To specify and compare the initiated alterations in various cell types of the adult mouse and human heart to predict potential clinical relevance.

4.3.1. TB4 decreases ROCK1 protein levels in the hypoxic adult mammalian mouse heart in vivo—Immunohistochemistry

To understand and to identify the localization and type of cells in which ROCK1 protein expression becomes altered, we performed immunohistochemistry on cryo-preserved post-hypoxic mouse hearts. Correspondingly to western blots, ROCK1 was equally decreased in the core, border and remote regions following TB4 treatment. In the core and border areas, we found cardiac myocytes with increased TB4 positivity reveal a decrease in ROCK1 protein in comparison to those with low TB4. In the remote, non-infarcted areas, ROCK1 was decreased in the TB4 treated hearts. Finally, we checked the alterations of ROCK1 in the mature cardiac vessels. We found the most significant differences to be detected in the outermost layer of the vessels (tunica adventitia), where the presence of ROCK1 was visibly increased in the hypoxic non-treated hearts. TB4 expression in the smooth muscle cell layer (sm) was increased, but ROCK1 remained low in both TB4 and PBS-treated mature heart vessels. Finally, although the endothelial layer (e) was visibly positive for ROCK1, we did not detect significant alterations in this cell type by histochemistry (Figure 3.).

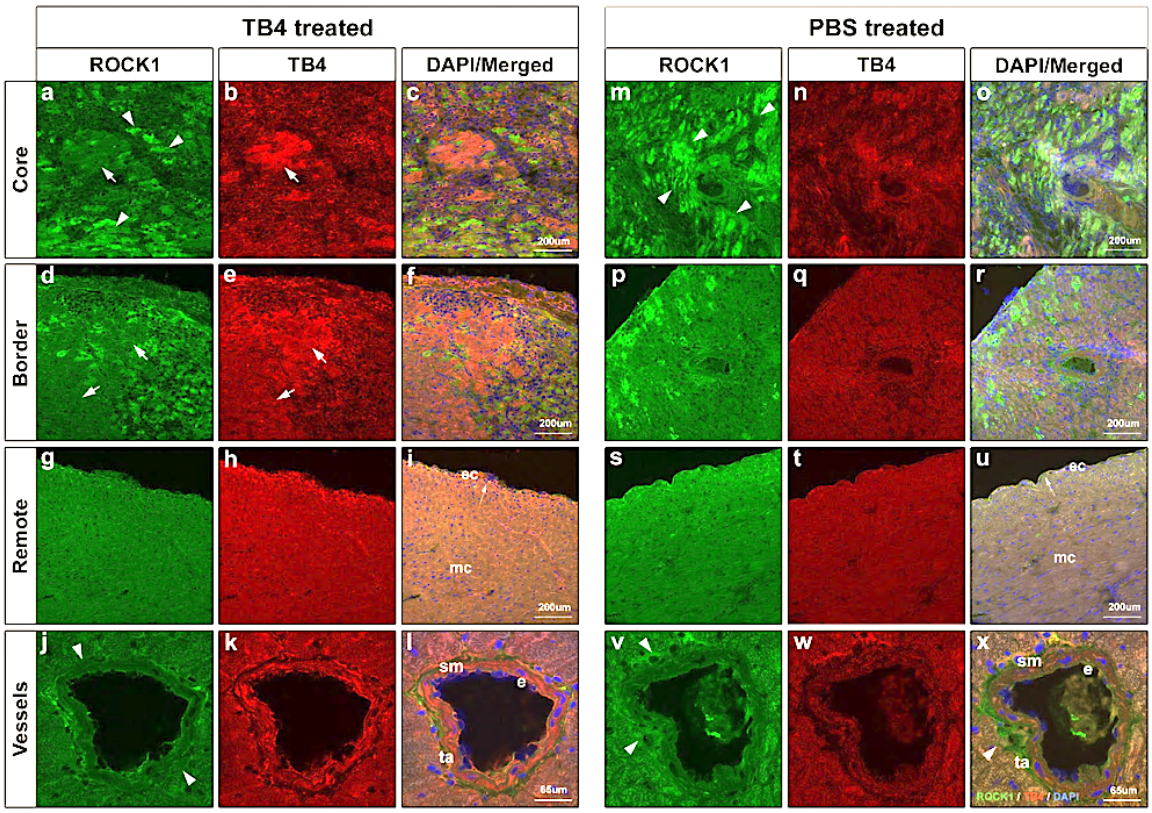

Figure 3. Immunohistochemical investigations of TB4 and PBS treated infarcted mouse hearts three days following MI. (e: vessel endothel; ta: tunica adventitia, sm: smooth muscle; mc: myocardium; ec: epicardium) (n = 3/each treatment)[67].

4.3.2. In vitro effect of TB4 on ROCK1, actin and MRTFA levels in adult human cardiac cells

Since our immunohistochemical findings indicate TB4 alters ROCK1 levels in endothelial, myocardial and fibroblast cells of hypoxic mouse hearts, we asked whether these effects can be equally detected in human cardiac cells in vitro. Moreover, to elucidate the downstream effects of TB4-initiated ROCK1 alterations, we investigated the expression of actin and MRTFA [70, 71] in all mentioned cell types, respectively.

4.3.2.1. Human umbilical vein endothelial cells (HUVECs)

First, we treated HUVECs with TB4 and PBS as control under normoxic and hypoxic conditions respectively. Although our results indicated no significant alterations in ROCK1 expression under normoxic conditions by TB4, we detected a slight shift towards actin filament assembly when TB4 was not present in the culturing medium. During hypoxia, our results revealed TB4 decreases ROCK1 levels in HUVECs. This impact was underscored by a visible shifting towards actin disassembly in the cells. TB4 was formerly described to impact translocation, and thus activation of MRTFA towards the nuclei of cells [72], a

transcription factor knowingly interacting with G-actin and critical in activating profibrotic genes with SRF [70, 71]. Therefore, we investigated the impact of TB4 on MRTFA expression and translocation in HUVECs. Our result revealed no alterations in MRTFA localization as it was exclusively present in the nuclei of the cells during both hypoxic and normoxic conditions.

4.3.2.2. Adult human cardiac myocytes (hCMs)

Next, we asked how TB4 influences the behavior of the enlisted molecules in hCMs. Much like our findings in adult mouse hearts, immunostaining of hCMs revealed a visible decrease in ROCK1 levels in both norm- or hypoxic conditions, which phenomenon was underscored by a decrease in actin filament structures in TB4 treated cells when compared to PBS controls.

4.3.2.3. Adult human cardiac fibroblasts (hCFBs)

Finally, we examined the impact of TB4 on adult hCFBs. In addition to the previously enlisted molecules, we investigated the alterations of alpha-smooth muscle Actin (sm a-Actin), a respected marker of fibroblast-myofibroblast transformation in the heart [73, 74]. Our results revealed a substantial decrease in ROCK1 protein levels following TB4 treatment, which was most significant under hypoxic conditions. Our observations regarding ROCK1 alterations were further supported by the proportional diminishing of filamentous actin structures in TB4-treated hCFBs, especially during hypoxia. By investigating sm a-Actin, we found the levels of the protein decreased under both hypoxic and normoxic conditions by TB4. Notably, TB4′s inhibition of fibroblast to myofibroblast transformation was most striking in hCFBs with hypoxia. Finally, in contrast to hCMs, we did not detect visible alterations in MRTFA localization by TB4 in this cell type, as MRTFA remained primarily restricted to the nucleus of hCFBs.

5. Discussion

The array of cardiovascular diseases and dysfunctions represent some of the most extensively studied health conditions and remain a leading cause of mortality worldwide. Stemming from a multifaceted background, the development of inflammation and its often consequential scarring contribute to malfunctions and decreased performance of the heart. Therefore, influencing distinct molecular mechanisms to reduce pathological scar formation through various agents holds significant clinical relevance and potential.

In our earlier studies we demonstrated systemically administered TB4, a 43 amino acid peptide, is capable of enhancing cardiac function in adult mice and pigs respectively [52, 56, 75]. In addition to inhibition of cell death and activation of vessel growth, functionality was restored and supported by a significant reduction in scar volume in both species [56, 75]. To understand how TB4 may achieve these alterations, in our research we performed screens such as miRNA microarrays focusing on targets potentially influencing proteins related to cardiac remodeling. Accordingly, in this study, we identified a novel mechanism by which TB4 may mitigate scar formation following hypoxia in the heart. We found the peptide may indirectly or directly modulate ROCK1 protein levels in the adult mouse heart following infarction in vivo. The inhibitory action was most evident in the core and border regions, where TB4 positive cells or cell islands exhibited visibly lower ROCK1 levels. Moreover, ROCK1 became downregulated in both infarcted core and remote regions, suggesting the effect is not exclusively hypoxia dependent. In mature vessels, we identified an involvement of endothelial cells and cells of the adventitial layer, which includes fibroblasts and stem/progenitors within a collagen-rich connective tissue matrix [76].

Results of miRNA microarray and real-time PCR suggested upregulation of miR139-5p may be one of the mechanisms by which TB4 influences ROCK1 levels in the heart [77]. The precise mechanisms of TB4′s direct impact on ROCK1 mRNA expression however remain still unclear and are currently under investigation. Moreover, because of the relatively small sample size of our study, future research with an increased sample number will be equally important to fully validate our findings to ensure reproducibility and generalizability in a clinical setting.

The significance and role of ROCK1 have been investigated in the context of cardiac remodeling and consequential scar formation by many investigators [24, 27, 38, 78]. The protein plays a crucial role in actin dynamics, which is essential for regulating various aspects of cellular physiology, including cell migration, proliferation, neurite extension and vesicle trafficking [79]. These processes are closely linked to actin polymerization and its association with stress fibers within cells [19]. These molecular assemblies are anchored to the plasma membrane through their association with focal adhesions and are responsible for generating contractile force via myosin-mediated anti-parallel motion of F-Actin. Reports show that inhibition of ROCK1 expression resulted in a decrease of stress fiber formation and focal adhesion assembly [80, 81], while overexpression of the protein enhanced the number of fibers, thus contraction [79, 80]. Consistent with knockout or inhibitory

observations, in our present investigation we detected a visible decrease in actin filaments, especially in human cardiac fibroblasts and myocytes.

Fibroblasts are tightly related to scar formation, not only as key sources for the extracellular matrix of the healthy but also in the ischemic heart, where the significant loss of cardiomyocytes becomes replaced by cardiac fibroblasts, which then can differentiate into myofibroblasts [27, 78, 82-84]. In these cells, the expression of contractile proteins such as sm a-Actin becomes up-regulated together with an increased expression and secretion of MMPs and collagen fibrils [85] which eventually leads to the development of cardiac fibrosis. To alter these pathological and functionally unbeneficial processes, influencing ROCK1 activity was widely suggested. In our present results, the external administration of TB4 to human cardiac fibroblasts revealed a significant decrease of sm a-Actin not only during hypoxia but also under normoxic conditions. This suggests a strong influence on the aforementioned critical transformation and thus on the consequential cellular alterations and pathologies in humans.

By activation of Rho/ROCK signaling, polymerization of globular actin becomes initiated and a consequential release of MRTFs from their actin-bound state leads to MRTF translocation to the nucleus. There, it induces the expression of various profibrotic genes through the activation of SRF, a MADS-box transcription factor which regulates numerous muscle-specific genes including ANF and α-skeletal actin through binding to CArG box elements in their promoter/enhancer sequences [86, 87]. Recently, numerous studies have demonstrated that inhibition of the ROCK/MRTF/SRF signaling prevents myofibroblast activation and also promotes myofibroblast apoptosis, thus limiting the development of fibrosis in various organs, including the heart or the lung [28, 38, 88, 89]. Moreover, a potential connection between TB4 and MRTF/SRF signaling has been equally reported, suggesting that an increase of TB4 in the cytosol initiates MRTF's nuclear translocation by competitively sequestering G-actin and thus, releasing MRTF-A from its G-actin binding [70, 71]. Our present findings however suggest TB4 initiated translocation of MRTF-A is strongly cell type specific. While external addition of the peptide to human adult cardiac myocytes accelerated MRTF-A translocation to the nucleus under both hypoxic and normoxic conditions, we did not detect a similar transition in HUVECs or in cardiac fibroblasts. On the contrary, in these cells, we found MRTF-A was primarily located in the nucleus independently of TB4 addition, while ROCK1 levels were still decreased in each cell type. This suggests the detected decrease of sm a-Actin in TB4-treated adult fibroblasts may occur through MRTF-A-independent mechanisms. By observing the discrepancies

between ROCK1 expression, sm a-Actin expression and MRTF-A's altered behavior in human fibroblast and endothelial cells in comparison to cardiac myocytes, further investigation of TB4′s effects on downstream signaling pathways may be critical and is a subject of our current research.

Finally, in our settings with TB4, the reduced activity of ROCK1 was likely due to lowered mRNA production in both remote and core regions of the heart one and three days following infarction, which was most likely further regulated by a significant increase of miR139-5p three days after infarction. Since externally administered TB4 is capable of reaching the nucleus of cells [90], elucidating the potential mechanisms of TB4′s impact on ROCK1 mRNA transcription warrants further analyses.

6. Conclusions

Heart regeneration is a complex process aimed at restoring cardiac function following injury. Sadly, the adult mammalian heart possesses limited regenerative capacities. Consequential myocardial fibrosis contributes to pathological remodeling and heart failure [3, 7, 91]. To counter these unprofitable transformations, identification of the molecular triggers and networks responsible for the missing functionality is undoubtedly critical. Therapeutic agents targeting key profibrotic pathways, such as ROCK signaling, have shown promise in supporting tissue repair [38]. However, further research is undoubtedly needed to refine these approaches and to minimize or eliminate side effects. Our current findings on TB4′s impact on ROCK1 expression and its ability to reduce profibrotic activity reveal a novel mechanism by which the peptide influences actin dynamics, making the molecule a promising candidate for future clinical applications.

7. Original findings of the thesis

I. Systemic administration of TB4 alters miRNA expression of the hypoxic mammalian heart.

II. Systemic administration of TB4 upregulates miR139-5p in the hypoxic mouse heart three days following administration.

III. TB4 decreases miR139-5p target ROCK1 levels of the hypoxic mammalian heart following systemic administration.

IV. TB4 decreases ROCK1 levels in cardiac endothelial cells, fibroblasts and myocardial cells in vivo following systemic injection *in mice*.

V. Systemic TB4 injection does not have significant impact on ROCK2 levels of the infarcted mouse hearts in vivo.

VI. Systemic administration of TB4 does not significantly alter ROCK1 mRNA levels of the hypoxic mouse hearts in vivo.

VII. TB4 decreases ROCK1 expression in hypoxic human cardiac endothelial, myocardial and fibroblast cells in vitro.

VIII. TB4 decreases sm a-Actin levels in human cardiac fibroblasts.

IX. TB4 decreases filamentous actin assembly in human cardiomyocytes, endothelial and fibroblast cells.

X. TB4 initiated translocation of cytosolic MRTF-A to the nucleus is cell type specific in the human heart cells.

8. Publications and conference attendances

8.1. Publications related to the topic of the thesis

Maar, Klaudia; Thatcher, Jeffrey E.; Karpov, Egor; Rendeki, Szilard; Gallyas, Ferenc; Bock-Marquette, Ildiko; Thymosin Beta-4 Modulates Cardiac Remodeling by Regulating ROCK1 Expression in Adult Mammals. ***INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*** 26: 9 Paper: 4131, 21 p. (2025) (Q1/D1) IF: 4.9

Maar, Klaudia; Hetenyi, Roland; Maar, Szabolcs; Faskerti, Gabor; Hanna, Daniel; Lippai, Balint; Takatsy, Aniko; Bock-Marquette, Ildiko; Utilizing Developmentally Essential Secreted Peptides Such as Thymosin Beta-4 to Remind the Adult Organs of Their Embryonic State-New Directions in Anti-Aging Regenerative Therapies. ***CELLS*** 10: 6 Review Paper: 1343, 15 p. (2021) (Q1) IF: 7.666

Bock-Marquette, Ildiko; **Maar, Klaudia**; Maar, Szabolcs; Lippai, Balint; Faskerti, Gabor; Gallyas, Ferenc Jr; Olson, Eric; Srivastava, Deepak; Thymosin beta-4 denotes new

directions towards developing prosperous anti-aging regenerative therapies ***INTERNATIONAL IMMUNOPHARMACOLOGY*** 116 Review Paper: 109741, 10 p. (2023) (Q1) IF: 4.8

Total IF of relevant publications: 17.366

8.2. Publications not related to the topic of the thesis

Toldi, Janos; Kelava, Leonardo; Marton, Sandor; Muhl, Diana; Kustan, Peter; Feher, Zsolt; **Maar, Klaudia**; Garai, Janos; Pakai, Eszter; Garami, Andras; Distinct patterns of serum and urine macrophage migration inhibitory factor kinetics predict death in sepsis: a prospective, observational clinical study. ***SCIENTIFIC REPORTS*** 13: 1 Paper: 588, 15 p. (2023) (Q1) IF: 3.8

Total IF of all publications: 21.166

8.3. Citable abstracts related to the topic of the thesis

Bock-Marquette, I.; **Maar, K.**; Maar, S.; Lippai, B.; Faskerti, G.; Hanna, D.; Thatcher, J.; Olson, E. N.; Gallyas, F.; Enhancing cardiac regenerative therapies by reminding the adult heart on its embryonic state. ***EUROPEAN JOURNAL OF HEART FAILURE*** 24 pp. 281-282., 2 p. (2022) (Q1/D1) IF: 18.2

Maar, K.; Thatcher, J.; Gallyas, F.; Bock-Marquette, I.; Thymosin beta-4 alters hypoxia induced ROCK1 expression in the adult mammalian heart. ***EUROPEAN JOURNAL OF HEART FAILURE*** 24 pp. 282-282., 1 p. (2022) (Q1/D1) IF: 18.2

Bock-Marquette, I.; Gallyas, F.; **Maar, K**.; Utilizing Secreted Peptides To Remind the Adult Organs on Their Embryonic State – New Directions in Anti-Aging Regenerative Therapies***. SCRIPTA MEDICA*** 52: Suppl. 1 Paper: S67 (2021)

Maar, K.; Thatcher, JE.; Olson, EN. Gallyas, F.; Bock-Marquette, I.; Thymosin beta-4 alters ROCK1 expression in the adult hypoxic mammalian heart. ***SCRIPTA MEDICA*** 52: Suppl. 1 Paper: S74 (2021)

Szabolcs, Maar; Lilla, Adrienn Czuni; Szilard, Rendeki; Aniko, Takatsy; **Klaudia, Maar**; Gabor, Faskerti; Ferenc, Gallyas; Ildiko, Bock-Marquette; Identification of potential physical and psychological stress-initiated peptides in support of tissue regeneration and repair in humans. ***SCRIPTA MEDICA*** 52: Suppl. 1 Paper: S73 (2021)

Bock-Marquette, I.; Hinkel, R.; Ball, H.; Thatcher, J.; Faskerti, G.; **Maar, K**.; Olson, E. N.; Kupatt, C.; The effect of thymosin beta-4 domains on post-hypoxic cardiac regeneration and repair. ***EUROPEAN JOURNAL OF HEART FAILURE*** 22 pp. 263-264., 2 p. (2020) (Q1/D1) IF: 15.534

Total IF of all citable abstracts: 51.93

Cumulative IF of all publications: 73.096

8.4. Additional conference Abstracts/Posters *(7)*

Bock-Marquette, Ildikó; **Maar, Klaudia**; Maar, Szabolcs; Fáskerti, Gábor; Lippai, Bálint Takatsy Anikó; Gallyas, Ferenc; Enhancing cardiac regenerative therapies by reminding the adult heart on its embryonic state. In: István, Baczkó; Norbert, Nagy; Péter, Bencsik (szerk.) *8th European Section Meeting of the International Academy of Cardiovascular Sciences: September 28 – October 1, 2022, Szeged, Hungary*: Programme and Abstract Book Szeged, Magyarország: Szegedi Tudományegyetem (2022) 126 p. pp. 58-58., 1 p. - poster presentation

Maar, Klaudia; Jeffrey, E. Thatcher; Eric, N. Olson; Ferenc, Gallyas; Bock-Marquette, Ildikó; Thymosin beta-4 alters miR-139-5p expression in the hypoxic mammalian heart. In: István, Baczkó; Norbert, Nagy; Péter, Bencsik (szerk.) *8th European Section Meeting of the International Academy of Cardiovascular Sciences: September 28 – October 1, 2022, Szeged, Hungary*: Programme and Abstract Book Szeged, Magyarország: Szegedi Tudományegyetem (2022) 126 p. pp. 100-100., 1 p.- poster presentation

Klaudia, Maar; Jeffrey, E. Thatcher; Santwana, Shrivastava; Michael, DiMaio; Eric, N. Olson; Ferenc, Gallyas; Ildiko, Bock-Marquette; Thymosin β 4 increases mmu-mir-1196 expression in hypoxic adult mammalian hearts. In: Csiszár, Beáta; Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia (szerk.) *Medical Conference for PhD Students and Experts*

of Clinical Sciences 2021: Book of Abstracts Pécs, Magyarország: University of Pécs, Doctoral Student Association (2021) 128 p. pp. 112-112., 1 p. - poster presentation

Maar, K., Thatcher, J., Olson, EN., Gallyas, F., Bock-Marquette, I. Thymosin Beta-4 Alters ROCK1 Expression in the Adult Hypoxic Mammalian Heart. *7th Meeting of European Section and 8th Meeting of North American Section of the International Academy of Cardiovascular Sciences (IACS)* (Bosznia és Hercegovina 2020) - poster presentation

Maar, K., Thatcher, J., Shivastava, S., DiMaio, JM., Olson, EN., Gallyas, F., Bock-Marquette, I. Thymosin Beta-4 influences miR1196 expression following cardiac infarction in adult mice. *6th Meeting of European Section and 7th Meeting of North American Section of the International Academy of Cardiovascular Sciences* (IACS) (Szerbia 2019) - poster presentation

Faskerti, G., Czuni, L., Takatsy, A., Ronai, D., Nagy, Z., Rendeki, S., Rendeki, M., Koltai, A., **Maar, K.,** Gallyas, F., Bock-Marquette, I. Identification of potential excercise initiated small molecules to support organ regeneration and repair in humans. *6th Meeting of European Section and 7th Meeting of North American Section of the International Academy of Cardiovascular Sciences (IACS)* (Szerbia 2019) - poster presentation

Bock-Marquette, I., Hinkel, R., Ball, H., Thatcher, J., Faskerti, G., **Maar, K.,** Olson, EN., Kupatt, C., Gallyas, F. The effect of Thymosin beta-4 domains on post-hypoxic cardiac regeneration and repair. *6th Meeting of European Section and 7th Meeting of North American Section of the International Academy of Cardiovascular Sciences (IACS)* (Szerbia 2019) - oral presentation

8.5. Awards

2021 **First price:** 2nd Poster section, Medical Conference for PhD Students and Experts of Clinical Sciences (MedPECS 2021) conference

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