Published in final edited form as:

Expert Opin Biol Ther. 2018 July ; 18(SUP1): 177–184. doi:10.1080/14712598.2018.1478961.

Thymosin β 4 inhibits PDGF-BB induced Activation, Proliferation, and Migration of human Hepatic Stellate Cells via its Actin-binding Domain

Ruchi Shah^{1,2,*}, Karina Reyes-Gordillo^{1,2,*}, Marcos Rojkind^{2,§}

¹Lipid Research Laboratory, VA Medical Center, 50 Irving Street NW, Washington, DC, USA

²Department of Biochemistry and Molecular Medicine, The George Washington University Medical Center, 2300 I Street NW, Washington, DC, USA.

Abstract

Objectives: Hepatic stellate cells (HSC) trans-differentiation is central to the development of liver fibrosis, marked by the expression of pro-fibrogenic genes and the proliferation and migration of activated HSC. Therefore, preventing and/or reverting the activation, proliferation, and migration of HSC may lead to new therapies for treating fibrosis/cirrhosis. Thymosin β 4 (T β 4) inhibits PDGF-BB-induced fibrogenesis, proliferation and migration of HSC by blocking Akt phosphorylation. Here, we utilized T β 4-derived peptides: amino-terminal-Ac-SDKPDMAEIEKFDKS (1–15aa) and actin-binding-LKKTETQ (17–23aa) to investigate the molecular mechanisms in the anti-fibrogenic actions of T β 4.

Methods: We used RT-PCR, Western blot, and proliferation and migration assays in early passages of human HSC cultures treated with PDGF-BB and/or T β 4 peptides.

Results: We showed that 17–23aa but not 1–15aa inhibited PDGF-BB-dependent up-regulation of PDGF β receptor, α -SMA, and collagen 1. It also blunted the phosphorylation of Akt at T 308 and S473, resulting in the inhibition of phosphorylation of PRAS40, and HSC proliferation and migration. Interestingly, 1–15aa blocked Akt phosphorylation at S473, but not T308 by inhibiting mTOR phosphorylation, thus, it did not have any effect on HSC proliferation and migration.

Conclusion: These findings suggest that while 1–15aa has a minor effect on Akt phosphorylation, the anti-fibrogenic actions of T β 4 are exerted *via* 17–23aa.

Keywords

hepatic fibrosis; hepatic stellate cells; Akt pathway; thymosin beta 4

Correspondence should be addressed to: Karina Reyes-Gordillo, PhD, Assistant Research Professor, The George Washington University, DC VA Medical Center, 50 Irving Street, NW, Washington, DC 20422., Ph. (202) 745 8000 Ext. 57692, Fax. (202) 462 2006, karrygor@gwu.edu.

[§]MR is deceased.

*RS and KRG contributed equally to this work.

Author contributions

MR led and conceptualized the project; RS designed and performed experiments and wrote the manuscript; KRG designed and performed the experiments and revised the manuscript.

1. Introduction

Liver fibrosis is a pathological wound healing response of the liver arising from chronic liver injury¹. Although, it occurs in the advanced stages of the disease, it is a reversible event, and hence, receptive to treatment². Hepatic stellate cells (HSC) are the major cell type involved in the fibrotic process. In response to injury, these cells undergo a phenotypic transformation and trans-differentiate from quiescent HSC to activated myofibroblasts. The activated HSC proliferate and synthesize extracellular matrix proteins to produce a fibrous scar. Several cytokines and growth factors are central to the fibrotic process, including transforming growth factor beta 1 (TGF- β 1) and platelet-derived growth factor (PDGF), which is the most potent proliferative cytokine for HSC. Activation of HSC is also accompanied by the expression of PDGF- β receptor and α -smooth muscle actin (α SMA), the markers of activated myofibroblasts, that further lead to the deposition of type 1 collagen (Col1), type 3 collagen (Col3), and fibronectin and result in fibrosis^{1, 3, 4}.

Thymosin β 4 (T β 4) is a 43 amino acid polypeptide with a molecular weight of 4.9 kDa that belongs to a large family of highly conserved, small biologically active molecules. It is present in many concentrations ranging from 10 nM - 600 μ M in different tissues and cell types⁵. T β 4 is involved in a diverse range of biological activities such as promoting wound healing, tissue repair and regeneration, and angiogenesis as well as preventing inflammation, apoptosis, and fibrosis in several different tissues⁵⁻⁷. T β 4 has three main functional domains. Its N-terminal sequence, made up of 1-4 amino acid residues (ac-SDKP), is known to have anti-inflammatory and anti-fibrotic properties. Another N-terminal sequence that includes the N-terminal Ac-SDKP consists of 1-15 amino acid residues (ac-SDKPDMAEIEKFDKS), and promotes cell survival and blocks apoptosis. The actin-binding domain (LKKTTETQ) is a short sequence consisting of 17-23 amino acid residues and promotes angiogenesis, wound healing, and cell migration. Amongst these 3 biologically active sites, T β 4 exerts a variety of physiological functions in the body⁸.

Previous work from our laboratory demonstrated that T β 4 prevented carbon tetrachloride induced acute liver injury in rat⁹. Moreover, we have also shown that T β 4 decreases the expression of PDGF- β receptor in human HSC and prevents PDGF-BB induced HSC proliferation and migration by inhibiting the phosphorylation of Akt and binding to actin¹⁰. To understand the underlying mechanism by which T β 4 exerts its anti-fibrogenic effects, in this study, we investigated which of the T β 4-derived peptides, if any, is responsible for the T β 4-mediated events. Our data shows that the actin-binding peptide (17-23aa) prevents PDGF-BB induced up-regulation of HSC activation markers, PDGF- β receptor and α SMA, as well as Col1 gene expression. It also blocks the phosphorylation of protein kinase B (Akt) at S473 and T308, thereby inhibiting cell proliferation and migration. In contrast, the amino-terminal peptide (1-15aa) only inhibits the phosphorylation of mammalian target of Rapamycin (mTOR), thus, blocking the phosphorylation of protein S6 kinase (P70S6K) and Akt at S473, but has no effect on HSC activation, proliferation, and migration.

2. Materials and Methods

2.1 Cell Culture

Human HSC were isolated as described by collagenase and protease digestion and fractionation on an Optiprep (Sigma, Saint Louis, MO) gradient from human liver biopsies of patients with morbid obesity, subject to bypass surgery by an approved protocol (IRB 070701). Early passages of culture activated HSC (passage 4–6) were grown and used as described previously¹⁰. Cells were treated with 50 ng/mL of PDGF-BB, 10 ng/mL of Tβ4 (1–15) and 10 ng/mL of Tβ4 (17–23) alone or in combination with PDGF-BB, and maintained at 37°C and in a 5% CO₂ incubator. Tβ4 peptides: Tβ4 peptide (1–15) and Tβ4 peptide (17–23) were a kind gift courtesy of RegeneRx (Rockville, MD) and produced by Bachem (Torrance, CA).

2.2 RNA Extraction and Quantitative RT-PCR

These experiments were performed as previously described¹⁰. All reagents were purchased from Applied Biosystems (Foster City, CA). Relative gene expression was calculated as 2^{-C_t} ($C_t = C_t$ of GAPDH).

2.3 Western Blotting

Cells were lysed for protein extraction, quantification and Western blotting as described previously¹⁰. A 1:1000 dilution of polyclonal rabbit anti-human antibodies against Akt, p-Akt (T308), phosphatase and tensin homolog (PTEN), p-PTEN, PDK1, p-PDK1, proline-rich Akt substrate (PRAS40), p-PRAS40, mTOR, p-mTOR and a 1:400 dilution of polyclonal rabbit phosphoinositide 3-kinase (PI3K) p85 was used. All the polyclonal rabbit antibodies were obtained from Cell Signaling Technology, Inc., Danvers, MA. A 1:1000 dilution of monoclonal mouse anti-human PDGFβR and p-Akt (S473) (Cell Signaling Technology, Inc., Danvers, MA) and P70S6K and p-P70S6K (Santacruz Biotechnology, Inc., Santa Cruz, CA) were used. Antigen antibody complexes were detected by chemiluminescence detection system (NEN Life Sciences Products, Boston, MA).

2.4 Growth Proliferation Assay

Cell proliferation was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were plated in 96-well tissue culture plates at a concentration of 3000 cells/well. The cells were cultured for 24 hours and 48 hours with media containing 0.1% fetal bovine serum. At the end of the treatment, 20 μL MTT solution (5 mg/mL in PBS) was added to each well and incubated for an additional 2 hours at 37°C. The colored formazan product was then dissolved in 150 μL of MTT solvent (4 mmol/L HCl and 0.1% Nonidet P-40 in isopropanol). The mitochondrial activity was evaluated by measuring the optical density at 570.

2.5 Cell Migration Assay

The migration of cells was measured in a scratch-wound assay as described previously¹⁰. The distances between the edges of the HSC migrating from both sides were measured.

2.6 Statistical Analysis

All of the experiments were performed (at least) in triplicate, and data are expressed as mean $\pm$ SE. Statistical differences between experimental groups were analyzed by Student's *t*-test and $P < 0.05$ was considered to be significantly different (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA).

3. Results

3.1 The Actin Binding Domain of T β 4 Prevents PDGF-BB induced HSC Activation and Fibrogenesis

To elucidate the molecular mechanism by which T β 4 exerts its anti-fibrotic effects, we examined the effect of the two T β 4-derived peptides on PDGF-BB induced activation of HSC. To this end, we incubated human HSC cultures with 50 ng/mL of PDGF-BB with and without 10 ng/mL of the T β 4 peptide (1–15aa) or 10 ng/mL of the T β 4 peptide (17–23aa). As illustrated in Figure 1, we observe that PDGF-BB up-regulated the mRNA expression PDGF- β receptor and α -SMA by 2.5 fold ($p < 0.05$) (Figure 1A–1B) as well as α 1(I) collagen by 1.9 fold ($p < 0.05$) (Figure 1C). While T β 4 peptide (1–15aa) had no effect on the PDGF-BB induced up-regulation of these genes, treatment with T β 4 peptide (17–23aa) significantly down-regulated the PDGF-BB induced mRNA expression of PDGF- β receptor, α -SMA, and α 1(I) collagen by 65% ($p < 0.05$), 53% ($p < 0.05$) and 55% ($p < 0.05$), respectively as compared to PDGF-BB treated group. Thus, the actin binding peptide and not the amino terminal peptide of T β 4 inhibits PDGF-BB induced HSC activation and fibrogenesis.

3.2 The Actin binding Domain of T β 4 prevents the Reappearance of PDGF- β Receptor after its degradation by PDGF-BB

Our previous work on full-length T β 4 showed that it prevents the PDGF-BB induced reappearance of PDGF- β receptor that occurs 48–72 hours after the PDGF-BB induced degradation of the receptor. Therefore, we investigated the time dependent changes in the expression of the receptor after treatment with the two T β 4 peptides. As observed in Figure 2A, PDGF-BB decreased the expression of the receptor by 68% ($p < 0.05$) by 30 minutes, and was almost undetectable by 3 hours with a 95% decrease in receptor expression (Figure 2C) and reappeared at 72 hours with receptor expression comparable to control level (Figure 2D). Although T β 4 peptide (17–23aa) had no initial effect on the PDGF-BB induced degradation of the receptor, it decreased with time and followed a similar pattern as the cells were treated only with PDGF-BB. However, while the receptor expression in PDGF-BB treated cells reappeared, T β 4 peptide (17–23aa) prevented the PDGF-BB induced reappearance of the receptor protein once it is already degraded. On the other hand, T β 4 peptide (1–15aa) had no effect on the PDGF-BB induced expression on the receptor and followed a similar expression pattern as PDGF-BB.

3.3 The Actin Binding Domain of T β 4 Prevents PDGF-BB induced HSC Proliferation and Migration

Because PDGF- β receptor is the main receptor involved in HSC proliferation and migration, and its pharmacological inhibition results in amelioration of liver fibrosis, we investigated

whether any of the T β 4 derived peptides have any effect on HSC proliferation and migration. As expected, PDGF-BB increased HSC proliferation by 2 fold ($p<0.05$) after 24 hours of treatment, and by 3 fold ($p<0.05$) by 48 hours (Figure 3A). While T β 4 peptide (1–15 aa) had no effect on HSC proliferation by itself, it did not prevent proliferation induced by PDGF-BB. Contrastingly, T β 4 peptide (17–23 aa) prevented PDGF-BB dependent proliferation by 35% ($p<0.05$) by 24 hours and 56% ($p<0.05$) by 48 hours (Figure 3A). Similarly, PDGF-BB also promoted HSC migration and only 15% ($p<0.05$) of the wound remained open after 24 hours as compared to the untreated control group (Figure 3B). T β 4 peptide (1–15 aa) did not prevent the PDGF-BB induced migration of HSC, and the cells migrated towards each other and only 18% ($p<0.05$) of the wound remained open as compared to the control (Figure 3B). On the other hand, T β 4 peptide (17–23 aa) prevented the HSC migration and the wound remained open by 93% ($p<0.05$) after 24 hours as compared to PDGF-BB treated cells (Figure 3B). Thus, only the actin binding peptide and not the amino terminal peptide of T β 4 inhibits PDGF-BB induced HSC proliferation and migration.

3.4 T β 4-derived Peptides have Different Effects on the Phosphorylation of Akt

We have previously shown that T β 4 inhibits the phosphorylation of Akt and both S473 and T308 sites¹⁰. Because the actin binding peptide inhibits HSC proliferation and migration and Akt phosphorylation is necessary for the proliferation and migration of these cells, we considered it important to evaluate the effects of the two peptides on the phosphorylation of Akt. Although there was no significant change in the expression of total Akt between the various groups (Figure 4A), PDGF-BB increased the phosphorylation of Akt at both phosphorylation sites, S473 by 4.7 fold ($p<0.05$) (Figure 4B) and T308 by 33 fold ($p<0.05$) (Figure 4C). Surprisingly, while only T β 4 peptide (17–23 aa) blocked the PDGF-BB induced phosphorylation of Akt at T308 (Figure 4C), both the peptides prevented the PDGF-BB induced phosphorylation of Akt at S473 (Figure 4B). Thus, the two bioactive peptides act on different phosphorylation sites of Akt, thereby exerting diverse effects.

3.5 T β 4 Peptides Have a Divergent Effect on the Kinases involved in the Akt signaling pathway

To further investigate if the diverse effects of the two T β 4 peptides on the phosphorylation of Akt are a consequence of their effect on the kinases upstream of Akt, we studied the protein expression of the various kinases involved in the Akt signaling pathway. As shown in Figure 5A–5E, the total protein expression as well as the phosphorylation status of the kinases upstream of Akt, including PI3K, PTEN, and PDK1 mostly remained unchanged between the various groups of cells, with or without PDGF-BB and T β 4 peptides treatments. However, although the expression of total mTOR did not show a significant difference between the various groups (Figure 5F), PDGF-BB increased the phosphorylation of mTOR, by 2.7 fold ($p<0.05$) as compared to the untreated control (Figure 5G). Interestingly, only T β 4 peptide (1–15aa) prevented the PDGF-BB induced phosphorylation of mTOR by 90% as compared to the PDGF-BB treated cells, while the actin binding peptide: T β 4 peptide (17–23 aa) was unable to block the PDGF-BB induced phosphorylation of mTOR. Since mTOR phosphorylates Akt at S473, and T β 4 peptide (1–15 aa) also blocked the phosphorylation of Akt only at S473, and not at T308 (Figure 4B–4C), T β 4 seems to inhibit the activation of

Akt through both the bioactive peptides, however, the effect of the amino terminal peptide appears to be minor. The different effect of the T β 4 peptides on the phosphorylation of Akt and mTOR were further confirmed by measuring the expression of downstream kinases in the mTOR/Akt-signaling pathway. While no significant changes in the protein expression of PRAS40, a substrate of Akt (Figure 6A) was noted, its phosphorylation increased by 5.5 fold ($p<0.05$) in PDGF-BB treated cells (Figure 6B). Treatment with only T β 4 peptide (17–23 aa), and not T β 4 peptide (1–15 aa) inhibited the increase in phosphorylation of PRAS40 by 96% as compared to the PDGF-BB treated cells (Figure 6B). On the other hand, the total protein expression of P70S6K mostly remained unaltered between the various groups (Figure 6C). However, even though PDGF-BB did not significantly increase the phosphorylation of P70S6K, as expected, T β 4 peptide (1–15 aa) inhibited P70S6K phosphorylation by 54% ($p<0.05$) as compared to the PDGF-BB treated cells (Figure 6D).

4. Discussion

There has been immense growth in understanding the molecular mechanisms involved in the development of hepatic fibrosis, yet, there continues to be an urgent need for specific anti-fibrotic therapies, due to the rising incident of cirrhosis¹¹. Currently, apart from liver transplantation, there are no fibrosis specific liver disease therapies that have been shown to be effective or approved for clinical use in chronic human liver disease¹². Nevertheless, because of its reversibility¹³, targeting and inhibiting the fibrogenic changes during HSC activation and proliferation is critical for the prevention and treatment of the disease.

Although T β 4 is expressed in the liver, the specific cell types that express T β 4 are not well established. One report has revealed that T β 4 is expressed in hepatocytes from healthy human liver¹⁴, while another study showed that T β 4 was expressed in Kupffer cells in the damaged liver¹⁵. Others have determined that T β 4 is expressed by HSC in chronically damaged liver¹⁶. In addition, depletion of T β 4 has shown to promote the proliferation, migration, and activation of HSC¹⁷. Furthermore, T β 4 treatment has shown to up-regulate HGF mRNA and down-regulate the expression of PDGF- β receptor in cultured HSC¹⁸.

PDGF-BB and its receptor play a key role in the trans-differentiation of HSC from a quiescent to a myofibroblastic phenotype^{4, 19–21}, which play a vital role in the formation of scar tissue in the liver during fibrosis and cirrhosis²¹. Generally, myofibroblasts are said to be derived from bone marrow cells or epithelial-mesenchymal transition, but mounting evidence suggest that most of the hepatic myofibroblasts that contribute to fibrosis development are derived from HSC trans-differentiation³. PDGF- β receptor, a hallmark of HSC activation, has been a target for many anti-fibrotic therapies^{1, 3}. It has been shown that a dominant negative form of the receptor prevents liver fibrosis²¹. Previously, we have shown that there is no internal protein pool of PDGF- β receptor in culture-activated HSC and it takes as much as 24 to 48 hours for the receptor to reappear after PDGF-BB induced degradation in mouse HSC and as much as 72 hours in human HSC^{10, 22}. This process of reappearance of the receptor after treatment with PDGF-BB is slow and dependent on *de novo* protein biosynthesis²². We have also shown that PDGF-BB treatment resulted in the complete disappearance of the receptor by 3 hours, and only reappeared at 72 hours, which was prevented by full-length T β 4¹⁰. In this study, we show that the actin binding peptide

alone is able to replicate the effects of full-length T β 4 on the reappearance of the receptor. PDGF-BB elicits the expression of PDGF- β receptor resulting in the activation of the PI3K/Akt signaling pathway, that causes the activated HSC to proliferate and migrate to the site of injury^{4, 22}. Akt, also known as serine/threonine protein kinase B, is an important kinase that is involved in the regulation of several cellular processes, such as cell growth, migration, and apoptosis^{23, 24}. For this reason, it is considered to be a perfect target to inhibit the activation of HSC, fibrogenesis, and scar formation after liver injury. The kinases involved in the Akt signaling pathway, PDK1 and mTOR phosphorylate and activate Akt at T308 and S473, respectively^{3, 10}. In this study, PDGF-BB induced the phosphorylation of Akt at both the sites, T308 and S473. While the amino terminal T β 4 peptide (1–15) blocked the phosphorylation of Akt at S473 but not T308, the actin-binding T β 4 peptide (17–23) completely blocked this PDGF-BB induced Akt phosphorylation at both T308 as well as S473. Due to this, only the actin-binding peptide was able to prevent the PDGF-BB induced proliferation and migration of HSC. We further confirmed our findings on the diverse effects of the two peptides on Akt phosphorylation by examining the expression of the kinases of the Akt signaling pathway. The amino terminal peptide blunted the phosphorylation of mTOR that regulates the phosphorylation of P70S6K as well as Akt phosphorylation at S473, both of which were also affected by the amino terminal peptide. In contrast, the phosphorylation of the kinase downstream of Akt, PRAS40 was inhibited by the actin-binding peptide alone. These results are in accordance with our previous study, which shows that full-length T β 4 blocks the binding of Akt to actin, thus preventing the phosphorylation of Akt, thereby inhibiting HSC proliferation and migration¹⁰. Indeed, in the present study, the actin-binding domain of T β 4 exerts its beneficial effects on HSC activation, proliferation and migration.

In summary, this is the first study to show that the actin binding T β 4 peptide (17–23 aa) inhibits PDGF-BB induced HSC activation and fibrogenesis, as well as HSC proliferation and migration. In contrast, the amino terminal T β 4 peptide (1–15 aa) has no effect of HSC activation and fibrogenesis or HSC proliferation and migration. The molecular mechanism of action of the peptides revealed that the actin-binding peptide prevented the proliferation and migration of HSC by blocking the phosphorylation of AKT at both T308 and S473 residues. Although the amino-terminal peptide had no effect on the phosphorylation of T308, it blunted the PDGF-BB induced phosphorylation of S473. This effect of the amino-terminal peptide appeared to be associated with the inhibition of mTOR phosphorylation, the kinase responsible for the phosphorylation of Akt at S473. Thus, the amino terminal peptide has only a minor effect and the actin-binding peptide exerts most of the anti-fibrotic effects of T β 4 on PDGF-BB induced fibrogenesis as well as proliferation and migration of HSC. In our previous work on full-length T β 4, we showed that T β 4 prevented the binding of Akt to actin. Because Akt is recruited to the plasma membrane for its phosphorylation, and T β 4 binds to actin, it suggests the possibility that actin is required for the recruitment of Akt to the plasma membrane. Thus, T β 4 exerts its action on proliferation and migration of HSC *via* its actin-binding region, which was confirmed in the current study by using the individual bioactive peptides of T β 4. Because actin is found on the surface of activated HSC/myofibroblasts¹⁰, it may function as a cell surface receptor, although this has not been

shown directly. Altogether, these findings provide an insight on the molecular mechanisms by which T β 4 exerts its anti-fibrotic effects on activated HSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was presented in part at the 5th International Symposium on Thymosins in Health and Disease (2017) in Washington, DC. T β 4 peptides used for this work were a kind gift from RegeneRx. We thank Dr. Allan L. Goldstein for his encouragement and advice.

Funding

This work is supported by the NIH grants RO1 10541 (to M. Rojkind) and 1K01 AA025140-01 (to K. Reyes-Gordillo). This paper has been published as part of a supplement issue covering the proceedings of the Fifth International Symposium on Thymosins in Health and Disease and is funded by SciClone Pharmaceuticals.

Bibliography

- [1]. Bataller R, Brenner DA: Liver fibrosis. *The Journal of clinical investigation* 2005, 115:209–18. [PubMed: 15690074]
- [2]. Nieto NaR M: Pathophysiology of Alcoholic Liver Disease. *The Liver: Biology and Pathobiology* Edited by Arias IM. Fifth Edition ed. Oxford, UK: Wiley-Blackwell, 2009.
- [3]. Rojkind MaR-G K: Hepatic Stellate Cells. *The Liver: Biology and Pathobiology* Edited by Arias IM. Fifth Edition ed. Oxford, UK: Wiley-Blackwell, 2009.
- [4]. Shah R, Reyes-Gordillo K, Arellanes-Robledo J, Lechuga CG, Hernandez-Nazara Z, Cotty A, Rojkind M, Lakshman MR: TGF-beta1 up-regulates the expression of PDGF-beta receptor mRNA and induces a delayed PI3K-, AKT-, and p70(S6K) -dependent proliferative response in activated hepatic stellate cells. *Alcoholism, clinical and experimental research* 2013, 37:1838–48.
- [5]. Goldstein AL, Hannappel E, Kleinman HK: Thymosin beta4: actin-sequestering protein moonlights to repair injured tissues. *Trends Mol Med* 2005, 11:421–9. [PubMed: 16099219]
- [6]. Crockford D, Turjman N, Allan C, Angel J: Thymosin beta4: structure, function, and biological properties supporting current and future clinical applications. *Annals of the New York Academy of Sciences* 2010, 1194:179–89. [PubMed: 20536467]
- [7]. Sosne G, Kleinman HK: Primary Mechanisms of Thymosin beta4 Repair Activity in Dry Eye Disorders and Other Tissue Injuries. *Invest Ophthalmol Vis Sci* 2015, 56:5110–7. [PubMed: 26241398]
- [8]. Sosne G, Qiu P, Goldstein AL, Wheeler M: Biological activities of thymosin beta4 defined by active sites in short peptide sequences. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2010, 24:2144–51. [PubMed: 20179146]
- [9]. Reyes-Gordillo K, Shah R, Arellanes-Robledo J, Rojkind M, Lakshman MR: Protective effects of thymosin beta4 on carbon tetrachloride-induced acute hepatotoxicity in rats. *Annals of the New York Academy of Sciences* 2012, 1269:61–8. [PubMed: 23045971]
- [10]. Reyes-Gordillo K, Shah R, Popratiloff A, Fu S, Hindle A, Brody F, Rojkind M: Thymosin-beta4 (Tbeta4) blunts PDGF-dependent phosphorylation and binding of AKT to actin in hepatic stellate cells. *The American journal of pathology* 2011, 178:2100–8. [PubMed: 21514425]
- [11]. Scaglione S, Kliethermes S, Cao G, Shoham D, Durazo R, Luke A, Volk ML: The Epidemiology of Cirrhosis in the United States: A Population-based Study. *Journal of clinical gastroenterology* 2015, 49:690–6. [PubMed: 25291348]
- [12]. Rudnick DA: Antifibrotic therapies in liver disease: Ready for primetime? *Clinical Liver Disease* 2017, 9:138–40. [PubMed: 30992978]

- [13]. Huang Y, Deng X, Liang J: Modulation of hepatic stellate cells and reversibility of hepatic fibrosis. *Experimental cell research* 2017, 352:420–6. [PubMed: 28238836]
- [14]. Nemolato S, Van Eyken P, Cabras T, Cau F, Fanari MU, Locci A, Fanni D, Gerosa C, Messina I, Castagnola M, Faa G: Expression pattern of thymosin beta 4 in the adult human liver. *European journal of histochemistry : EJH* 2011, 55:e25. [PubMed: 22073372]
- [15]. Paulussen M, Landuyt B, Schoofs L, Luyten W, Arckens L: Thymosin beta 4 mRNA and peptide expression in phagocytic cells of different mouse tissues. *Peptides* 2009, 30:1822–32. [PubMed: 19631707]
- [16]. Kim J, Wang S, Hyun J, Choi SS, Cha H, Ock M, Jung Y: Hepatic stellate cells express thymosin Beta 4 in chronically damaged liver. *PloS one* 2015, 10:e0122758.
- [17]. Xiao Y, Qu C, Ge W, Wang B, Wu J, Xu L, Chen Y: Depletion of thymosin beta4 promotes the proliferation, migration, and activation of human hepatic stellate cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2014, 34:356–67.
- [18]. Barnaeva E, Nadezhda A, Hannappel E, Sjogren MH, Rojkind M: Thymosin beta4 upregulates the expression of hepatocyte growth factor and downregulates the expression of PDGF-beta receptor in human hepatic stellate cells. *Annals of the New York Academy of Sciences* 2007, 1112:154–60. [PubMed: 17584975]
- [19]. Kastanis GJ, Hernandez-Nazara Z, Nieto N, Rincon-Sanchez AR, Popratiloff A, Dominguez-Rosales JA, Lechuga CG, Rojkind M: The role of dystroglycan in PDGF-BB-dependent migration of activated hepatic stellate cells/myofibroblasts. *American journal of physiology Gastrointestinal and liver physiology* 2011, 301:G464–74. [PubMed: 21659621]
- [20]. Lechuga CG, Hernandez-Nazara ZH, Hernandez E, Bustamante M, Desierto G, Cotty A, Dharker N, Choe M, Rojkind M: PI3K is involved in PDGF-beta receptor upregulation post-PDGF-BB treatment in mouse HSC. *American journal of physiology Gastrointestinal and liver physiology* 2006, 291:G1051–61. [PubMed: 16990448]
- [21]. Borkham-Kamphorst E, Herrmann J, Stoll D, Treptau J, Gressner AM, Weiskirchen R: Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. *Laboratory investigation; a journal of technical methods and pathology* 2004, 84:766–77. [PubMed: 15077122]
- [22]. Lechuga CG, Hernandez-Nazara ZH, Dominguez Rosales JA, Morris ER, Rincon AR, Rivas-Estilla AM, Esteban-Gamboa A, Rojkind M: TGF-beta1 modulates matrix metalloproteinase-13 expression in hepatic stellate cells by complex mechanisms involving p38MAPK, PI3-kinase, AKT, and p70S6k. *American journal of physiology Gastrointestinal and liver physiology* 2004, 287:G974–87. [PubMed: 15246963]
- [23]. Fan H, Ma L, Fan B, Wu J, Yang Z, Wang L: Role of PDGFR-beta/PI3K/AKT signaling pathway in PDGF-BB induced myocardial fibrosis in rats. *American journal of translational research* 2014, 6:714–23. [PubMed: 25628782]
- [24]. Son G, Hines IN, Lindquist J, Schrum LW, Rippe RA: Inhibition of phosphatidylinositol 3-kinase signaling in hepatic stellate cells blocks the progression of hepatic fibrosis. *Hepatology* 2009, 50:1512–23. [PubMed: 19790269]

Figure 1. Effect of Tβ4 peptides on PDGF-BB induced HSC activation and fibrogenesis in HSC culture.

Total RNA was extracted from human HSC treated for 24 hours with PDGF-BB (50 ng/mL), in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) and quantitative RT-PCR was performed to analyze the mRNA expression of (A) PDGF-β receptor, (B) α-SMA, and (C) Collagen1α1. All values are means of triplicate experiments ± SE after correcting for the expression of GAPDH mRNA. * p<0.05 v control; ** p<0.05 v PDGF-BB.

Figure 2. Effect of Tβ4 peptides on PDGF-β receptor expression after PDGF-BB-induced Degradation of the Protein.

Time-course analysis of the expression of PDGF-β receptor in total protein extracted from untreated HSC cultures or HSC treated with PDGF-BB in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) for (A) 30 minutes, (B) 1 hour, (C) 3 hours, (D) 72 hours was measured by Western Blot analysis. Values are means of triplicate experiments $\pm$ SE and were corrected for difference in loading after reprobing with an antibody to β -actin. * $p < 0.05$ v control; ** $p < 0.05$ v PDGF-BB.

Figure 3. Effect of Tβ4 peptides on PDGF-BB induced HSC proliferation and migration. Untreated HSC cultures or cells treated with 50 ng/mL of PDGF-BB in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) were used to measure (A) cell proliferation by MTT assay after 24 hours and 48 hours and (B) cell migration by scratch wound assay after 24 hours. (C) Representative images of the scratch wound migration assay taken at 0 hour and 24 hours. * $p < 0.05$ v control; ** $p < 0.05$ v PDGF-BB.

Figure 4. Effect of Tβ4 peptides on PDGF-BB induced activation of Akt.

Total protein was extracted from human HSC treated for 30 min with PDGF-BB (50 ng/mL), in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) and the expression of (A) Akt, (B) pAkt (S473), and (C) pAkt (T308) was assessed by Western Blot analysis. Values are means of triplicate experiments $\pm$ SE and were corrected for difference in loading after reprobing with an antibody to β -actin. * $p < 0.05$ v control; ** $p < 0.05$ v PDGF-BB.

Figure 5. Effect of Tβ4 peptides on kinases upstream of Akt.

Total protein was extracted from human HSC for 30 min with PDGF-BB (50 ng/mL), in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) and the expression of (A) PI3K, (B) PTEN, (C) pPTEN, (D) PDK1, (E) pPDK1, (F) mTOR, and (G) pmTOR was assessed by Western Blot analysis. Values are means of triplicate experiments $\pm$ SE and were corrected for difference in loading after reprobing with an antibody to β -actin. * $p < 0.05$ v control; ** $p < 0.05$ v PDGF-BB.

Figure 6. Effect of Tβ4 peptides on kinases downstream of Akt.

Total protein was extracted from HSC treated for 30 min with PDGF-BB (50 ng/mL), in the presence or absence of Tβ4 peptides: 1–15 aa or 17–23 aa (10 ng/mL) and the expression of (A) PRAS40, (B) pPRAS40, (C) P70S6K, and (D) pP70S6K was assessed by Western Blot. Values are means of triplicate experiments $\pm$ SE and were corrected for difference in loading after reprobing with an antibody to β -actin. * $p < 0.05$ v control; ** $p < 0.05$ v PDGF-BB.