Thymosin Beta 4 Induces Hair Growth via Stem Cell Migration and Differentiation

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ABSTRACT: Thymosin beta 4 is a small 43-amino-acid molecule that has multiple biological activities, including promotion of cell migration angiogenesis, cell survival, protease production, and wound healing. We have found that thymosin beta 4 promotes hair growth in various rat and mice models including a transgenic thymosin beta 4 overexpressing mouse. We have also determined the mechanism by which thymosin beta 4 acts to promote hair growth by examining its effects on follicle stem cell growth, migration, differentiation, and protease production.

KEYWORDS: cell migration; hair follicle growth; angiogenesis; wound healing; stem cells; cell survival; inflammation; gene expression; laminin-5; proteases; zyxin; endothelial cells; thymosin beta 4; keratinocytes; MMPs; TIMPs

THYMOSIN BETA 4 HAS MULTIPLE BIOLOGICAL ACTIVITIES

Thymosin beta 4 is a small, 43-amino-acid, actin-binding, intracellular protein that is present in all cells.[1] It was originally identified in endothelial cells as a gene that was increased during early endothelial cell capillary formation.[2] It was later shown to actually promote capillary formation *in vitro* and angiogenesis *in vivo* in a number of model systems.[3,4] Since then, an unexpectedly large number of important biological activities of thymosin beta 4 have been identified (TABLE 1). Because it is high in platelets, its role in wound healing was investigated, and it was found to promote dermal healing in normal rats and mice as well as in impaired models of dermal wound healing, including aged mice, diabetic mice, and steroid-treated rats.[5,6] Thymosin beta 4 was also shown to have an important role in corneal wound healing and in cardiac

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TABLE 1. Biological activities of thymosin beta 4

Increased migration: endothelial cells, epithelial cells, stem cells, keratinocytes
Increased cell survival
Increased differentiation, endothelial cells, stem cells
Increased proteases: monocytes, fibroblasts, endothelial cells, stem cells, corneal cells
Decreased inflammation
Increased angiogenesis: *in vitro*, *ex vivo*, *in vivo*
Increased dermal healing: rats, mice, aged mice, diabetic mice, steroid-treated rats
Increased corneal healing: rats, mice
Increased hair growth
Increased tumor growth
Increased gene expression: laminin-5, zyxin, TFG beta, TIMPs (1–3)

repair.[7,8] Part of its role in healing may be due to its ability to reduce inflammation.[9] Surprisingly, it has been shown to induce proteases by a number of cell types, and this activity is important in cell migration and in wound repair.[10,11] We have also found that TIMPs, which are protease inhibitors, are also increased by thymosin beta 4 (TABLE 1).

Thymosin beta 4 has been found by a number of labs to promote tumor growth.[12–14] This activity is due to its ability to promote cell migration and angiogenesis.[12] It also increases vascular endothelial growth factor (VEGF) expression, which can promote cell migration and angiogenesis. It does not appear to be due to oncogenic activity, but rather has a passive role in promoting tumor growth. When animals are treated topically every day with thymosin beta 4, no tumors have been observed. Also, mice overexpressing high levels of this gene in their skin do not form an increased number of spontaneous tumors with age.

While we are beginning to understand a lot about the biological functions of thymosin beta 4 and its potential uses in the clinic, a number of unanswered questions remain on how it regulates it biological functions. We have begun to identify regions on the molecule important for some of the activities, such as the central actin-binding domain that promotes wound healing and angiogenesis.[15] The amino terminal amino acids are important in regulating inflammation.[16] Such progress has defined important activities, but a receptor has not been identified. Also, it is not clear how the signal is transduced in the cells.

THYMOSIN BETA 4 PROMOTES HAIR GROWTH

While studying full-thickness dermal wound healing in rat skin, we unexpectedly observed visually and at the histological level increased hair growth at the wound margins after topical treatment with thymosin beta 4. The hairs appeared very long, thicker than the hairs in the nonshaved areas and also a dark yellow in color. This unexpected hair growth was then observed in subsequent studies using nonwounded rats where we have shaved the skin of healthy rats

TABLE 2. Topical thymosin beta 4 and rat hair growth

Treatment	% Active hair follicles
Control	56.0 ± 6.8
2% Rogaine	56.9 ± 5.3
100 μg thymosin beta 4	68.3 ± 4.2
2% Rogaine + thymosin beta 4	62.4 ± 12.4

TABLE 3. Models of increased hair growth observed with thymosin beta 4

Normal and wounded rats
Normal mice
Nude mice
Aged mice
Cyclophosphamide-treated mice
Transgenic thymosin beta 4 overexpressing mice

and applied thymosin beta 4 topically on one side of the shaved area and the control vehicle on the opposing lateral side of the same animal. After 7 days of treatment, we observed an increased number of anagen-phase hair follicles in the skin areas treated with thymosin beta 4. The number of anagen follicles was approximately twofold greater than in rats treated with vehicle alone. The number of hairs in anagen phase was retained with continued treatment every other day for over 30 days. If thymosin beta 4 was withdrawn, the number of active hair follicles decreased to control levels by 2 weeks. We compared the effects of rogaine, a commercial preparation used to promote hair growth, with thymosin beta 4 in rats (TABLE 2). We found that rogaine alone was not affective in the 2-week time frame. Normally in humans, rogaine is used for a longer period of time to obtain hair growth.

We next tested whether thymosin beta 4 could promote hair growth in mice. The 8-week-old mice used in this experiment have all of their hair follicles in the telogen stage. The mice were shaved and thymosin beta 4 was applied topically on the shaved area. Separate control animals were treated with vehicle alone. The thymosin beta 4-treated (but not control) animals displayed quick hair regrowth. Histological examination confirmed the thymosin beta 4-induced activation of the hair follicles. Hair was also grossly visible in the treated mice and appeared darker than the hairs in the nonshaved areas.

The effect of thymosin beta 4 on hair growth in impaired models was then tested (TABLE 3). We found that thymosin beta 4 increased hair growth in aged animals. These animals were over 26 weeks of age and had sparse hair and thin skin. The actin-binding central peptide also was able to promote hair growth in these mice. We next tested athymic nude (nu nu) mice, which have very sparse hairs, for their response to thymosin beta 4. After topical treatment with thymosin beta 4, the hair grew rapidly on these mice and was thicker than the normal hairs on these mice. In many cases, the hair was curly with a corkscrew type appearance. Finally, we tested cyclophosphamide-treated

mice to determine if thymosin beta 4 would cause hair to regrow more quickly. Within 24 h of cyclphosphamide treatment, the hair on the mice fell out with gentle rubbing. Thymosin beta 4 was then applied topically and hair regrowth was observed within a few days while the control vehicle treated mice had a much delayed hair growth. Cyclophosphamide-treated mice are a model for chemotherapy-induced hair loss. These findings suggest that cancer patients on chemotherapy may benefit from thymosin beta 4 treatment.

A transgenic mouse overexpressing thymosin beta 4 on the keratin 5 promoter was created. At 8 weeks of age when the mouse hair was in the telogen phase, hair regrowth was tested after removal. The overexpressing mice more quickly grew back their hair than the littermate controls. These data confirm that thymosin beta 4 has a role in activation of hair follicle growth.

THYMOSIN BETA 4 LOCALIZES IN THE HAIR FOLLICLES TO THE STEM CELLS IN THE BULGE REGION AND TO THE MIGRATING STEM CELLS

Since thymosin beta 4 was promoting the activation of hair follicle growth we determined which cells in the follicle were making this protein. The location and timing of endogenous thymosin beta 4 in hair follicles during depilation-induced, synchronized adult hair cycling in mice was determined.[17] Low levels of thymosin beta 4 protein were observed in follicles at the telogen (resting) phase, prior to depilation. Thymosin beta 4 protein was found only in a small number of cells residing in the bulge region. The bulge region is know to contain the stem cells important in hair growth and in dermal healing.[18,19] Hair follicle transition to early anagen (day 4 after depilation) was associated with an increased number of thymosin beta 4-expressing cells in the bulge region. Moreover, some thymosin beta 4-positive-stained cells were detected in the migrating cells in the lower part of the follicle. By late anagen (day 9 after depilation), many of the cells located in the lower follicle (matrix-surrounding part of the outer root sheath) expressed thymosin beta 4. Thus, with the progression of the hair growth cycle, thymosin beta 4-positive cells, initially detected only in the bulge, were observed at the bulb area, suggesting that they are migrating from the bulge region. These data show that thymosin beta 4 is elevated in the stem cells in the bulge region and in the matrix cells that subsequently generate the hair shaft.

RAT VIBRISSAE *EX VIVO* CULTURES SHOW INCREASED HAIR GROWTH WITH THYMOSIN BETA 4

Intact vibrissae were isolated from rats and cultured for 5 days in the presence and absence of thymosin beta 4 (TABLE 4) to determine if hair could be observed

TABLE 4. Growth of isolated and cultured rat vibrissae (whiskers) exposed to thymosin beta 4

	0	10	100 μg thymosin beta 4
growing vibrissae $n = 6$	0/6	2/6	3/6

growing *ex vivo*. We found that thymosin beta 4 increased the length of the hair growing out of the vibrissae in a dose-dependent manner. It was surprising that a dose as low as 10 ng was active in this assay.

THYMOSIN BETA 4 IS EXPRESSED BY ISOLATED CLONOGENIC KERATINOCYTES FROM RAT VIBRISSAE

Rat vibrissae follicle keratinocytes from the bulge region were isolated and cultured to determine if isolated stem cells express thymosin beta 4. Previously, hair follicle stem cells were identified as bulge-residing keratinocytes with a high *in vitro* clonogenic potential.[20] Hair follicle stem cells preferentially express keratin 15 (K15). We isolated clonogenic keratinocytes (stem cells) from the rat vibrissa bulge region and found that the isolated cells were highly clonogenic and were positive for the stem cell marker keratin 15 as well as for keratins 5, 6, and 14, also known to be expressed by bulge stem cells.[21] These cells lacked keratin 10, a known early marker of terminal keratinocyte differentiation. These cells expressed thymosin beta 4 after 7–10 days *in vitro*. Treatment of the cells with exogenous thymosin beta 4 decreased the expression of the multi-potent undifferentiated stem cell marker K15, which is associated with stem cell differentiation. Thymosin beta 4 had no effect on stem cell proliferation as found with other cell types. These data indicate that thymosin beta 4 is important for early hair follicle stem cell differentiation. Such data on stem cell differentiation induced by thymosin beta 4 have also be confirmed in the heart.[22]

THYMOSIN BETA 4 PROMOTES HAIR FOLLICLE STEM CELL MIGRATION

Thymosin beta 4 promotes the migration of various cells including endothelial cells, keratinocytes, and corneal epithelial cells.[4] We found that cultured clonogenic keratinocytes (stem cells) migrate to thymosin beta 4 after 4.5 h in Boyden chamber assays (TABLE 5). In the presence of thymosin beta 4, cell migration was increased almost twofold at 1 ng over migration in the presence of medium containing vehicle alone (negative control). The effect of thymosin beta 4 on cell migration was greatest at 1 ng/mL and at 100 and 1000 ng/mL

TABLE 5. Thymosin beta 4 increases migration of isolated hair follicle stem cells in a 4.5-h Boyden chamber assay

Amount added	No. of migrated cells/field
0	69
1 μg thymosin beta 4	113
100 μg thymosin beta 4	85

migration was decreased. Previously, we found that 1 ng/mL was very potent for endothelial and keratinocyte migration.[3]

THYMOSIN BETA 4 INCREASES HAIR FOLLICLE STEM CELL PRODUCTION OF MATRIX METALLOPROTEINASE-2 (MMP-2)

Enzymatic degradation and remodeling of extracellular matrix and particularly, the basement membrane that separates the epithelial and stromal compartments of the follicle, is a necessary step in normal hair differentiation and growth.[23] We have examined the effect of thymosin beta 4 on the enzymatic activity of matrix metalloproteinases (MMPs). Increases in MMP proteases have been reported in the skin and in fibroblasts, endothelial cells, monocytes, and corneal epithelial cells after treatment with thymosin beta 4.[10,11] Treatment of the hair follicle stem cells with exogenous thymosin beta 4 caused almost a twofold increase in MMP-2. The increase in the levels of secreted and cell-derived enzyme MMP-2 was dose-dependent and specific since the levels of MMP-9 did not change. MMP-2 degrades collagen type IV and laminin, key proteins of the basement membrane. The degradation of laminin by MMP2 is important in cell migration.[24] Since MMP-2 plays a role in both hair growth associated extracellular matrix remodeling and cell migration, our data suggest that this enzyme may be one of the downstream effectors through which thymosin beta 4 exerts its effect on hair growth.

CONCLUSIONS

Thymosin beta 4 is a small 43-amino-acid peptide that has many biological activities.[4] It is upregulated during endothelial cell differentiation, and when added exogenously, it promotes endothelial cell differentiation and migration. *In vivo*, it promotes wound repair and is a potent anti-inflammatory agent. Thymosin beta 4 is overexpressed in metastatic tumors and its up-regulation results in increased tumor cell motility, angiogenesis, and metastasis. A related family member, thymosin beta 15, is also important in the metastasis of breast and prostate cancers.[25] Thymosin beta 4 exerts its effects on cell locomotion through specific interactions with actin that regulate cytoskeletal organization.[26] Thymosin beta 4 promotes hair growth in normal rats and

mice.[17] When examining the distribution of endogenous thymosin beta 4 during hair growth, we found that in the resting follicle it is expressed in the small number of cells originating in the bulge region. As the follicles enter active growth phase, the subset of thymosin beta 4-expressing cells in the outer root sheath extends toward the base of the follicle. At the peak of hair growth, many thymosin beta 4-expressing cells localize in the bulb area. Furthermore, hair follicle stem cells produce thymosin beta 4 when cultured for at least a week. In addition, the presence of exogenous thymosin beta 4 caused a dose-dependent decrease in the expression of the stem cell marker K15, suggesting that thymosin beta 4 may promote early stem cell differentiation. Treatment of the bulge-derived hair follicle stem cells with exogenous thymosin beta 4 also increased their migration and production of MMP-2.[17]

In addition to its role in cell locomotion mediated by interaction with actin, the effect of thymosin beta 4 on MMP-2 expression plays an important role in cell migration.[24] MMP-2 was previously shown to contribute to cellular migration, both by means of degrading extracellular matrix barriers for cell movement and, through direct effects of the degraded matrix. Fragments of laminin-5 are important in cell migration activity. MMP-2 is involved in hair-cycle-associated remodeling of the basement membrane and this remodeling is necessary for signaling between epithelial and stromal elements of the growing follicle and for elongation and invasion of the lower follicle into subcutaneous tissue during initiation of hair follicle growth.[23]

Thymosin beta 4 promotes hair growth in part due to its effects on stem cells, and due to its angiogenic activity. VEGF promotes hair follicle development presumably, due to its angiogenic activity.[27] Recently, another angiogenic molecule, hepatocyte growth factor, has been identified in hair follicles and found to promote hair growth.[28,29] Hepatocyte growth factor, also an angiogenic factor, upregulates thymosin beta 4 expression and may be acting by increasing thymosin beta 4 and/or synergizing with it.[30] Furthermore, steroids have been used successfully to treat certain types of hair loss. Thymosin beta 4 is the anti-inflammatory molecule that is increased in steroid-treated monocytes.[9] Thus, treatment of hair loss with steroids may also involve the activity of thymosin beta 4.

In summary, our results suggest that thymosin beta 4 exerts a profound hair-promoting effect through a combined action on several critical events in hair follicle growth, such as stem cell migration, extracellular matrix-degrading enzyme production, and differentiation.

REFERENCES

1. Low, T.L.K., S.K. Hu & A.L. Goldstein. 1981. Complete amino acid sequence of bovine thymosin: a thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations. Proc. Natl. Acad. Sci. USA **78:** 1162–1166.

2. GRANT, D.S., J.L. KINSELLA, M.C. KIBBEY, *et al*. 1995. A novel role for thymosin beta 4: a Matrigel-induced gene involved in endothelial cell differentiation and angiogenesis. J. Cell Sci. **108:** 3685–3694.
3. MALINDA, K.M., A.L. GOLDSTEIN & H.K. KLEINMAN. 1997. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. FASEB J. **11:** 474–481.
4. GOLDSTEIN, A.L., E. HANNAPPEL & H.K. KLEINMAN. 2005. Thymosin beta 4: actin-sequestering protein moonlights to repair injured tissues. Trends Mol. Med. **11**:421–429.
5. MALINDA, K.M., G.S. SIDHU, H. MANI, *et al*. 1999. Thymosin beta 4 accelerates wound healing. J. Invest. Dermatol. **113:** 364–368.
6. PHILP, D., M. BADAMCHIAN, B. SCHEREMETA, *et al*. 2003. Thymosin beta 4 and a synthetic peptide containing its actin-binding domain promote dermal wound repair in db/db diabetic mice and in aged mice. Wound Rep. Reg. **11:** 19–24.
7. SOSNE, G., E.A. SZLITER, R. BARRETT, *et al*. 2002. Thymosin beta 4 promotes corneal wound healing and decreases inflammation *in vivo* following alkali injury. Exp. Eye Res. **74:** 293–299.
8. BOCK-MARQUETTE, I., A. SAXENA, M.D. WHITE, *et al*. 2004. Thymosin beta 4 activates integrin-linked kinase and promotes cardiac cell migration, survival, and cardiac repair. Nature **432**:466–472.
9. YOUNG, J.D., A.J. LAWRENCE, A.G. MAC LEAN, *et al*. 1999. Thymosin beta 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. Nat. Med. **5:** 1424–1427.
10. QIU, P., M. KURPAKUS-WHEATER & G. SOSNE. 2007. Matrix metalloproteinase activity is necessary for thymosin beta 4 promotion of epithelial cell migration. J. Cellul. Physiol. In press.
11. PHILLP, D., B. SCHEREMETA, K. SIBLISS, *et al*. 2006. Thymosin beta 4 promotes matrix metalloproteinase expression during wound repair. J. Cellul. Physiol. **208:** 195–200.
12. CHA, H.J., M.J. JEONG & H.K. KLEINMAN. 2003. Role of thymosin beta 4 in tumor metastasis and angiogenesis. J. Nat. Cancer Inst. **95**:1674–1680.
13. KOBAYASHI, T, F. OKADA, N. FUJII, *et al*. 2002. Thymosin beta 4 regulates motility and metastasis of malignant mouse fibrosarcoma cells. Am. J. Pathol. **160:** 869–882.
14. CLARK, E.A., T.R. GOLUB, E.S. LANDER & R.O. HYNES. 2000. Genomic analysis of metastasis reveals an essential role for RhoC. Nature **406:** 532–535.
15. PHILP, D., T. HUFF, Y.S. GHO, E. HANNAPPEL & H.K. KLEINMAN. 2003. The actin binding site on thymosin beta 4 promotes angiogenesis. FASEB J. **17**:2103–21105.
16. CAVASIN, M.A. 2006. Therapeutic potential of thymosin beta 4 and its derivative N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after infarction. Am. J. Cardiovasc. Drugs **6:** 305–311.
17. PHILP, D., M. NGUYGEN, B. SCHEREMETA, *et al*. 2004. Thymosin beta 4 increases hair growth by activation of hair follicle stem cells. FASEB J. **18:** 385–387.
18. OSHIMA, H., A. ROCHAT, C. KEDZIA, *et al*. 2001. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell **104:** 233–245.
19. TAYLOR, G., M.S. LEHRER, P.J. JENSEN, *et al*. 2000. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell **102:** 453–461.
20. ROCHAT, S., K. KOBAYASHI & Y. BARRANDON. 1994. Location of stem cells of human hair follicles by clonal analysis. Cell **76:** 1063–1073.

21. FUCHS, E. 1995. Keratins and the skin. Annu. Rev. Cell. Dev. Biol. **11:** 123–153.
22. SMART, N., C.A. RISEBORO, A.A. MELVILLE, *et al*. 2007. Thymosin beta 4 induces adult cardiac progenitor mobilization and neovascularization. Nature **445**:177–182.
23. KARELINA, T.V., G.A. BANNIKOV & A.Z. EISEN. 2000. Basement membrane zone remodeling during appendageal development in human fetal skin. The absence of type VII collagen is associated with gelatinase-A (MMP2) activity. J. Invest. Dermatol. **114:** 371–375.
24. GIANELLI, G., J. FALK-MARZILLIER, O. SCHIRALDI, *et al*. 1997. Induction of cell migration by matrix metalloproteinase-2-cleavage of laminin-5. Science **277**:225–228.
25. BAO, L., M. LODA & B.R. ZETTER. 1998. Thymosin beta 15 expression in tumor cell lines with varying metastatic potential. Clin. Exp. Metastasis **16:** 227–233.
26. ROY, P., Z. RAIFUR, D. JONES, *et al*. 2001. Local photorelease of caged thymosin beta 4 in locomoting keratocytes causes cell turning. J. Cell. Biol. **153:** 1035–1048.
27. YANO, K., L.F. BROWN & M. DETMAR. 2001. Control of hair growth and follicle size by VEGF-mediated angiogenesis. J. Clin. Invest. **107:** 409–417.
28. YAMAZAKI, M., R. TSUBOI, Y.R. LEE, *et al*. 1999. Hair cycle-dependent expression of hepatocyte growth factor (HGF) activator, other proteinases, and proteinase inhibitors correlates with the expression of HGF in rat hair follicles. J. Invest. Dermatol. Symp. Proc. **4:** 312–315.
29. LINDNER, G., A. MENRAD, E. GHERARDI, *et al*. 2000. Involvement of hepatocyte growth factor/scatter factor and met receptor signaling in hair follicle morphogenesis and cycling. FASEB J. **14:** 319–332.
30. OH, I.S., S.S. SO, K.Y. JAHNG & H.G. KIM. 2002. Hepatocyte growth factor upregulates thymosin beta 4 in human umbilical vein endothelial cells. Biochem. Biophys, Res. Commun. **296:** 410–405.