ONCOLOGY REPORTS 24: 1389-1394, 2010

# Cooperation of actin-sequestering protein, thymosin ß-4 and hypoxia inducible factor-1α in tumor cell migration

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Received May 28, 2010; Accepted July 16, 2010

DOI: 10.3892/or_00000997

**Abstract.** Cell migration plays an important role in many physiological and pathological processes, including tumor metastasis. Tumor cell migration is increased through the sequential induction of HIF-1α and VEGF under hypoxic conditions. Thymosin ß-4 (Tß4) is an actin-sequestering protein which controls cytoskeletal reorganization. Here, we investigated whether tumor cell migration could be cooperatively controlled by hypoxia and Tß4. Cell migration was measured by wound healing assay with scratching confluent monolayers of tumor cells. Cell migration was enhanced 18 h after scratching cells. In addition, we found that the expression of HIF-1α, VEGF-A isoform 164/120 and Tß4 was increased by scratching cells. Cell migration was decreased by the inhibition of Tß4 or HIF-1α expression with lentiviral shRNA of Tß4 or siRNA of HIF-1α, respectively. In contrast, cell migration was increased by the treatment with Tß4 proteins. The inhibitory effect of Tß4-shRNA or HIF-1α-siRNA was also attenuated by treatment with Tß4 proteins. Collectively, these findings suggest that Tß4 and HIF-1α cooperatively enhance tumor cell migration.

## Introduction

Thysmosin-ß-4 (TB4), a small, naturally occurring 43-amino acid peptide, was initially isolated in 1981 from the thymus. TB4 is the most abundant member of the ß-thymosins, a family of highly conserved polar 5-kDa peptides (1). TB4 is the major G actin-sequestering molecule in mammalian cells (2,3) and can be also cross-linked to some proteins including fibrin and collagen increased in the sites of tissue damage (1,4,5). TB4 led to paclitaxel-resistance (6,7), tumor growth and metastasis (8,9).

Cell migration plays a role in many physiological and pathological processes, including tumor metastasis (10). Cell migration requires the integration and temporal coordination of many different processes that occur in spatially distinct locations in the cell (11). Migration can be demonstrated as a multistep cycle including extension of a protrusion, formation of stable attachments near the leading edge of the protrusion, translocation of the cell body forward, release of adhesions and retraction at the cell rear (12,13). Spatially controlled polymerization of actin is at the origin of cell motility and actin networks continuously generated at the leading edge (14,15). The cellular factor that generates new filaments in a site-directed, signaling-controlled fashion is the Arp2/3 complex (16). Profilin, G-actin-binding protein is involved in motile processes mediated by actin polymerization. Actin-sequestering protein, TB4, do not modify filament dynamics or the rate of movement but it is thought to buffer the free ATP-G-actin concentration (17). Tß4 proteins induce HIF-1α stabilization, through Erk activation (18). In the meanwhile, hypoxic condition enhances tumor cell migration through the sequential induction of HIF-1α and VEGF (19). However, little is known about whether tumor cell migration could be cooperatively controlled by HIF-1α stabilization and Tß4 expression under normoxia.

Here, we investigated whether HIF-1α stabilization and Tß4 expression cooperatively regulate tumor cell migration. We made a wound by scratching a monolayer of B16F10 melanoma cells and observed a migration of cells into the space left by the scratch. We found that Tß4 and HIF-1α expression was increased by scratching cells. Our data also showed that tumor cell migration was inhibited by the reduction of Tß4 expression and HIF-1α stabilization. In addition, Tß4 protein increased tumor cell migration and attenuated the inhibitory effect of Tß4-shRNA or HIF-1α-siRNA on tumor cell migration. It suggests that Tß4 and HIF-1α cooperatively regulate tumor cell migration.

## Materials and methods

*Reagents*. Thymosin ß-4 (TB4) proteins were kindly provided by Dr Hynda Kleinman, Gorge Washington University, USA. Antibodies which are reactive with HIF-1α and VEGF came from Santa Cruz Biotechnology (Santa Cruz, CA). Small interference RNA of HIF-1α was obtained from Bioneer Corp.

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*Abbreviations*: Tß4, thymosin-ß-4; HIF-1α, hypoxia inducible transcription factor 1 α; VEGF, vascular endothelial growth factor; shRNA, small hairpin RNA; siRNA, small interference RNA

*Key words:* thymosin ß-4, hypoxia conditioning, HIF-1α, VEGF, tumor cell migration

(Taejeon, Korea). Except where indicated, all other materials are obtained from the Sigma Chemical Company (St. Louis, MO).

*Cell culture*. B16F10 mouse melanoma cells were obtained from the Korea Institute of Radiological and Medical Science (KIRMS) cell bank (Seoul, Korea). Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Kansas City, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

*Lentiviral shRNA of TB4*. The shRNA-expression lentiviral vectors for targeting TMSB4X were constructed by inserting synthetic double strand oligonucleotides (5'-CGGAATTCC GGCTTCCAAAGAAACGATTGAttcaagagaTCAATCG TTTCTTTGGAAGTTTTTGATATCTAGACA-3') into *Eco*RI-*Xba*I restriction enzyme sites of shLenti 3.4G lentiviral vector and verified by nucleotide sequencing. The shLenti 3.4G lentiviral vector was designed to produce shRNAs promoted from U6 promoter and to express GFP from hCMV promoter.

Recombinant lentivirus was produced as described earlier by Follensi *et al* (20) and Dull *et al* (21). Briefly, three plasmids, a transfer vector, an envelope glycoprotein expression vector and a packaging vector, were co-transfected into 293T cells at a 1:1:1 molar ratio by using lipofectamine Plus (Invitrogen, USA). The culture supernatant containing viral vector particles was harvested 48 h after transfection, clarified with a 0.45-$\mu$ membrane filter (Nalgene, USA), and stored at -70˚C immediately. Titers were determined by p24 ELISA or infection into HeLa cells. The RFP expression of transduced cells was observed and photographed under fluorescence microscope; in our routine preparation, the titer was ~106-107 transduction unit (TU) per $\mu$l without further concentration.

*Wound migration assay*. When B16F10 cell density was confluent in 35-mm culture dish (Corning, NY, USA), three wound lines in the form of a cross were made by scratching cellular layer with a plastic pipette tip. Then, floating cells were washed out and fresh medium was added. As the incubation time passed under normoxia condition, the width of scratch was narrowed and it was recorded by taking photographs under phase contrast microscope from 6 h after the scratch.

*RT-PCR*. RNA was isolated from B16F10 cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 $\mu$g of total RNA, using oligo-$dT_{18}$ primers and superscript reverse transcriptase in a final volume of 21 $\mu$l (Bioneer, Taejeon, Korea). For standard PCR, 1 $\mu$l of the first strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer). PCR amplification proceeded as follows: 30-33 thermocycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec, using oligonucleotides specific for mTß4 (forward, ata tgg ctg aga tcg aga aa; reverse, gct tgc ttc tct tgt tca at), mVEGF variant 164 (358 bp) and mVEGF variant 120 (226 bp) (forward, gtg ccc acg tca gag agc aa; reverse, cgg tct ttc cgg tga gag gt), and mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) (forward, tcc acc acc ctg ttg ctg ta; reverse, acc aca gtc cat gcc atc ac).

*Western blot analysis*. Cells were lysed in ice-cold lysis buffer, containing 0.5 % Nonidet P-40 (vol./vol.) in 20 mM Tris-HCl, at a pH of 8.3; 150 mM NaCl; protease inhibitors (2 $\mu$g/ml aprotinin, pepstatin, and chymostatin; 1 $\mu$g/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM $Na_4VO_3$. Lysates were incubated for 30 min on ice prior to centrifugation at 14,000 rpm for 5 min at 4˚C. Proteins in the supernatant were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5 % Tween-20), then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

*Statistical analyses*. Experimental differences were tested for statistical significance using ANOVA and Student's t-test. P-value of <0.05 was considered to be significant.

## Results

*Tumor cell wound enhanced HIF-1α stability and Tß4 gene expression*. In order to investigate the role of HIF-1α and Tß4 in tumor cell migration, we used wound migration assay by scratching B16F10 melanoma tumor cell monolayer. As shown in Fig. 1A, wound was narrowed 15 h after a monolayer of tumor cells was scratched. Given that tumor cell migration was enhanced through the induction of HIF-1α and VEGF in hypoxic condition (19), we examined HIF-1α stabilization and VEGF gene expression. We found that HIF-1α stabilization and the expression of VEGF-A isoform 165/121 were increased in a time-dependent manner after scratching a monolayer of B16F10 cells (Fig. 1B and C). In addition, given that HIF-1α was stabilized by Tß4 peptide (18), we also examined whether Tß4 gene expression could be induced during tumor cell migration. As shown in Fig. 1D, TB4 gene expression was increased by scratching B16F10 cell monolayer (Fig. 1D). It suggests that tumor cell migration could be mediated by HIF-1α stabilization and Tß4 gene expression.

*Tumor cell migration was inhibited by Tß4-shRNA and HIF-1α-siRNA*. To confirm the involvement of HIF-1α and TB4 gene in tumor cell migration, HIF-1α and TB4 gene expression was inhibited by the transfection of B16F10 cells with siRNA of HIF-1α and by the infection of B16F10 cells with lentiviral shRNA of Tß4, respectively. Wound migration of B16F10 cells was decreased by HIF-1α-siRNA (Fig. 2A). Inhibitory effect of HIF-1α-siRNA on HIF-1α expression was confirmed by the reduction of HIF-1α-mediated VEGF-A isoform 165/121 expression under normoxia and hypoxia

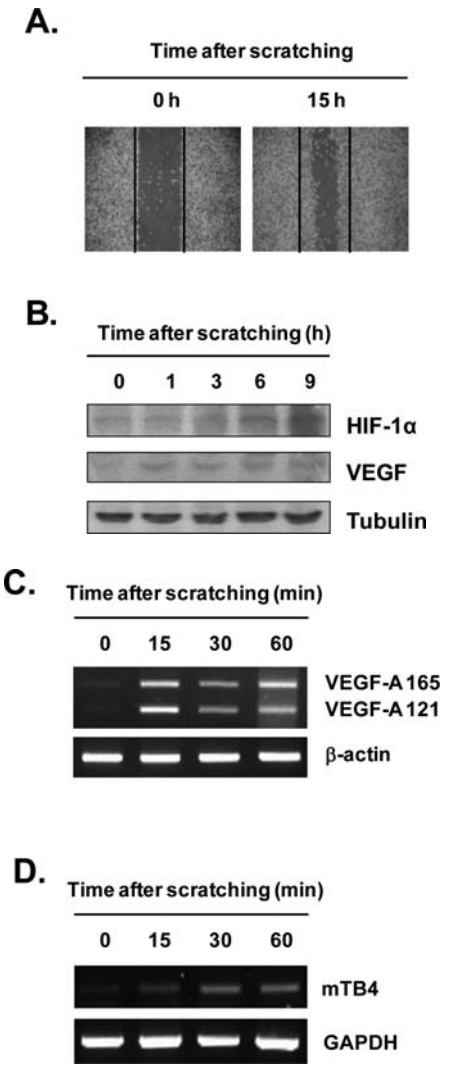


Figure 1. Cell migration was increased by scratching a monolayer of B16F10 melanoma cells. (A) A monolayer of B16F10 cells was scratched and incubated under normoxia condition for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. Space between vertical lines indicate an original wound area before incubation. Cells inside two vertical lines in each photograph indicate the cells which migrated into the space left by the scratch. (B) B16F10 cells were scratched and incubated for various appropriate time. Cell lysates were prepared and each molecule was separated by SDS-PAGE. HIF-1α and VEGF were detected by Western blot analysis. (C) and (D) B16F10 cells were scratched and incubated under normoxia condition for 15, 30, and 60 min. VEGF-A 165/121 and mTß4 transcript level was measured by RT-PCR.

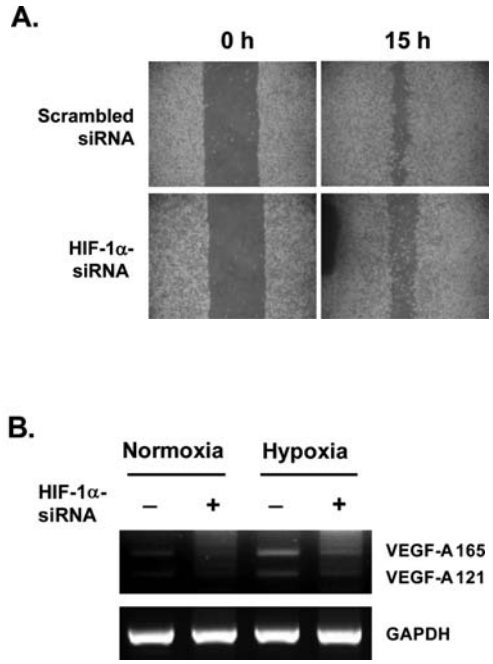


Figure 2. Cell migration was inhibited by the transfection with HIF-1α-siRNA. (A) B16F10 cells were transfected with control scrambled siRNA or HIF-1α-siRNA for 18 h. A monolayer of B16F10 cells was scratched and incubated under normoxia condition for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. (B) B16F10 cells were transfected with scrambled siRNA or HIF-1α-siRNA for 18 h. Then, cells were incubated under normoxia or hypoxia condition for 1 h. VEGF-A 165 and 121 transcript level was level was measured by RT-PCR.

condition (Fig. 2B). In addition, wound migration of B16F10 cells was inhibited by the infection with lentiviral shRNA of Tß4 (Fig. 3A). Tß4 expression was reduced by the infection with Tß4-shRNA (Fig. 3B). Data demonstrate that HIF-1α and Tß4 could be involved in tumor cell migration. It suggests that tumor cell migration could be induced by HIF-1α stabilization and TB4 gene expression.

*Tß4 protein attenuated an inhibitory effect of Tß4-shRNA and HIF-1α-siRNA on tumor cell migration.* To examine the cooperation of HIF-1α and Tß4 in tumor cell migration, we first determined the effect of Tß4 peptide on tumor cell migration. As shown in Fig. 4A, tumor cell migration was enhanced by the treatment with Tß4 peptide. Given that

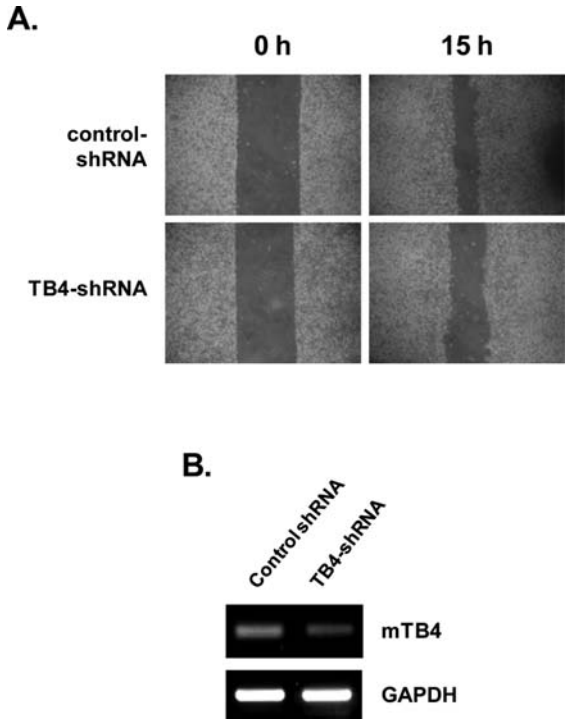


Figure 3. Cell migration was inhibited by the infection with lentiviral Tß4-shRNA. (A) B16F10 cells were infected with lentiviral control or lentiviral Tß4-shRNA for 18 h. A monolayer of B16F10 cells was scratched and incubated under normoxia condition for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. (B) B16F10 cells were infected with lentiviral control or Tß4-shRNA for 18 h. Then, mTß4 transcript level was measured by RT-PCR.

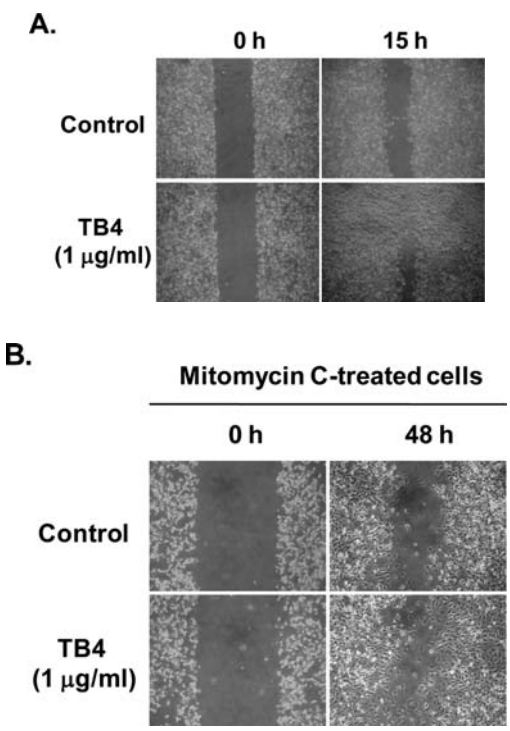


Figure 4. Tß4 peptide enhanced cell migration. (A) A monolayer of B16F10 cells was scratched and incubated in the presence or absence of 1 $\mu$g/ml Tß4 peptide for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. (B) B16F10 cells were treated with 25 $\mu$g/ml mitomycin C for 30 min. After washing cells with fresh medium twice, cells were plated and incubated for 18 h. A monolayer of B16F10 cells was scratched and incubated in the presence or absence of 1 $\mu$g/ml Tß4 peptide for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope.

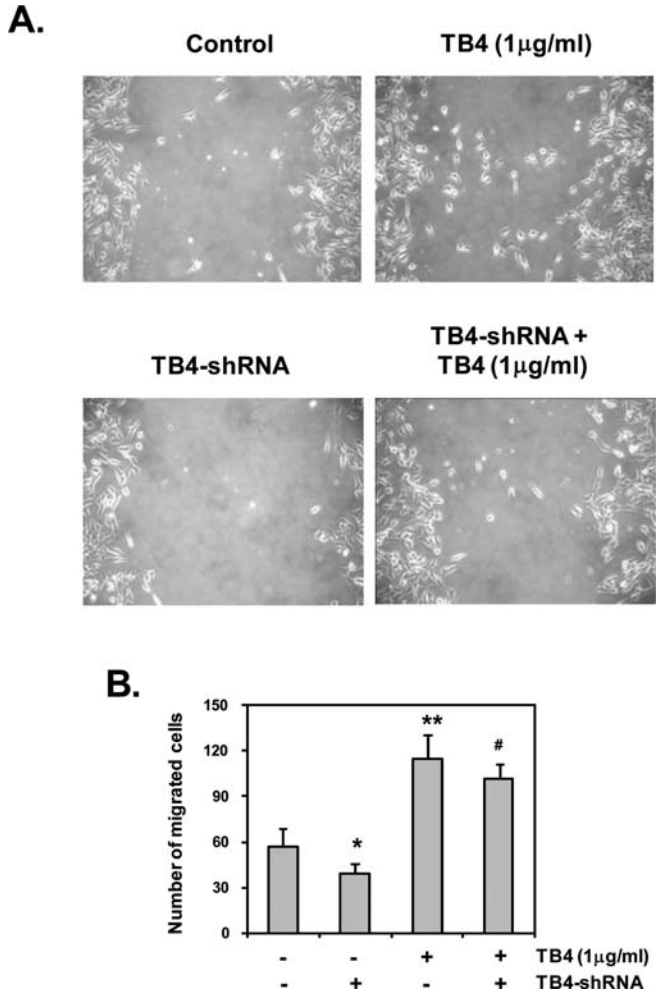


Figure 5. Tß4 protein attenuated an inhibitory effect of Tß4-shRNA on tumor cell migration. (A) B16F10 cells were infected with lentiviral control or Tß4-shRNA for 18 h. A monolayer of B16F10 cells was scratched and incubated in the presence or absence of 1 $\mu$g/ml Tß4 peptide under normoxia condition for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. (B) Number of cells that were migrated into the space left by the scratch was counted. Data in bar graph represent mean ± SED. $^{*}p<0.05$; $^{**}p<0.01$, significantly different from Tß4-shRNA-not infected control in normoxia condition; #$p<0.05$, significantly different from Tß4-shRNA non-infected control in hypoxia condition.

cell density was increased by the treatment with Tß4 peptide (18), we treated B16F10 cells with mitomycin C that inhibited cell proliferation. As shown in Fig. 4B, mitomycin C-treated tumor cell migration was also enhanced by the treatment with Tß4 peptide. Decreased wound migration in cells infected with lentiviral shRNA of Tß4 was recovered by the addition of Tß4 peptide (Fig. 5). Data indicate that tumor cell migration is dependent on Tß4 expression. The cooperation of HIF-1α and Tß4 in tumor cell migration was confirmed by the treatment of Tß4 peptide on B16F10 cells that was transfected with HIF-1α-siRNA. Tß4 protein attenuated an inhibitory effect of HIF-1α-siRNA on tumor cell migration (Fig. 6). It suggests that Tß4 gene expression and HIF-1α stabilization cooperatively enhanced tumor cell migration at least *in vitro*.

## Discussion

Cell migration plays a role in many physiological and pathological processes (10). Cell migration required actin polymerization continuously generated at the leading edge (14,15). Actin-sequestering protein, Tß4, is thought to buffer the free ATP-G-actin concentration (17). Tß4 proteins induce HIF-1α stabilization through Erk activation (18). Decreased HIF-1α expression inhibits prostate and breast cancer cell migration (22,23). Here, we investigated whether HIF-1α stabilization and Tß4 expression cooperatively regulate tumor cell migration. In our experimental condition, Tß4 and HIF-1α expression was increased by scratching cells. Tumor cell migration was inhibited by the infection with lentiviral Tß4-shRNA or by the transfection with HIF-1α-siRNA (Figs. 2 and 3). The inhibitory effect of Tß4-shRNA or HIF-1α-siRNA on tumor cell migration was reversed by the treatment with Tß4 protein (Figs. 4 and 5). It suggests that Tß4 and HIF-1α cooperatively enhanced tumor cell migration.

Spatially controlled polymerization of actin is at the origin of cell motility and actin networks continuously generated at the leading edge (14,15). The small G proteins of the Rho family are involved in reorganization of the actin cytoskeleton, cell migration and in the regulation of gene transcription (11,12). When cells were scratched in

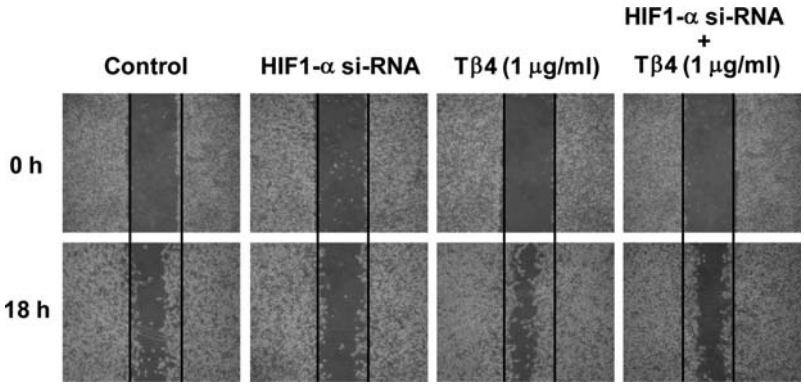


Figure 6. Tß4 protein attenuated an inhibitory effect of HIF-1α-siRNA on tumor cell migration. B16F10 cells were transfected with control scrambled siRNA or HIF-1α-siRNA for 18 h. A monolayer of B16F10 cells was scratched and incubated in the presence or absence of 1 μg/ml Tß4 peptide under normoxia condition for 15 h. Then, migration of cells into the space left by the scratch was photographed by phase contrast microscope. Space between vertical lines indicated an original wound area before incubation. Cells inside two vertical lines in each photograph indicated the cells which migrated into the space left by the scratch after incubation.

our experimental condition, cytoskeleton reorganization was stimulated by actin polymerization to induce cell migration. We found that Tß4 and HIF-1α expression was increased by scratching cells. It indicates that the small G proteins of the Rho family could regulate Tß4 and HIF-1α expression. It has to be defined by further study.

According to previous reports, MUC1 is directly regulated by HIF-1α and affects the invasive and migration properties of renal cancer cells (24). Chemokine receptor, CXCR6 and CXCR4 contributes significantly to cell migration during hypoxia (23,25). Therefore, Tß4 expression could be regulated by HIF-1α in a monolayer of scratched cells. Even though Tß4 expression was inhibited by Tß4-shRNA, cells were still migrated due to HIF-1α existence, which may control Tß4 expression (Figs. 3 and 4). It suggests that Tß4 protein produced by HIF-1α also conversely induce HIF-1α stabilization. However, it could not be ruled out that Tß4 expression was not completely inhibited by Tß4-shRNA.

Previous report also showed that Tß4 proteins induce HIF-1α stabilization, through Erk activation (18). Even though HIF-1α expression was inhibited by HIF-1α-siRNA, cells were still migrated due to Tß4 existence, which may control HIF-1α stabilization (Figs. 2 and 5). HIF-1α is phosphorylated in hypoxia by an ERK-dependent pathway (26). Erk activation was detected by scratching cells (data not shown), which may activate HIF-1α. However, it could not be also ruled out that HIF-1α expression was not completely inhibited by HIF-1α-siRNA, then control to produce Tß4. Therefore, our data demonstrate that Tß4 and HIF-1α cooperatively enhanced tumor cell migration.

In conclusion, even though we could not explain all of the mechanism on tumor cell migration, our data showed that tumor cell migration was inhibited in response to Tß4-shRNA or HIF-1α-siRNA. Data also showed that Tß4 protein attenuated their inhibitory effect on tumor cell migration. It suggests that Tß4 and HIF-1α cooperatively enhanced tumor cell migration. It also suggests that Tß4 could be a novel modulator to control tumor cell migration through the cooperation with HIF-1α Tß4 might be applied to develop anti-metastatic therapeutics.

## Acknowledgements

We thank Dr Hynda Kleinman, National Cancer Institute, USA for providing thymosin beta-4 protein (TB4P). This study was supported by grants (#0920270) from National Cancer Control Program, and (#A080076) of the Korea Healthcare technology R&D Project, Ministry of Health and Welfare, Korea.

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