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Thymosin β4 induces invasion and migration of human colorectal cancer cells through the ILK/AKT/β-catenin signaling pathway

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ABSTRACT

Thymosin β4 (Tβ4) is a 43-amino-acid peptide involved in many biological processes. However, the precise molecular signaling mechanism(s) of Tβ4 in cell invasion and migration remain unclear. In this study, we show that Tβ4 was significantly overexpressed in colorectal cancer tissues compared to adjacent normal tissues and high levels of Tβ4 were correlated with stage of colorectal cancer, and that Tβ4 expression was associated with morphogenesis and EMT. Tβ4-upregulated cancer cells showed increased adhesion, invasion and migration activity, whereas Tβ4-downregulated cells showed decreased activities. We also demonstrated that Tβ4 interacts with ILK, which promoted the phosphorylation and activation of AKT, the phosphorylation and inactivation of GSK3β, the expression and nuclear localization of β-catenin, and integrin receptor activation. These results suggest that Tβ4 is an important regulator of the ILK/AKT/β-catenin/Integrin signaling cascade to induce cell invasion and migration in colorectal cancer cells, and is a potential target for cancer treatment.

1. Introduction

Colorectal cancer is the third most common cancer globally. Early detection and improved treatment techniques have decreased colorectal cancer death rates over the past few decades [1]. However, the high mortality rate of late-stage and/or metastasized colorectal cancer reflects our lack of understanding of the etiological factors and pathogenesis. Colorectal carcinogenesis results from a complex interplay between genetic and environmental factors that deregulate potentially oncogenic signaling pathways. A comprehensive understanding of the oncogenic molecular mechanism associated with colorectal carcinogenesis is critical for the development of innovative therapeutic strategies for this disease.

Late-stage colorectal cancer has a tendency to metastasize to the liver, and metastatic colorectal cancer has been correlated with a poor prognosis. β-Catenin is a proto-oncogene that plays a key role in migration, invasion and metastasis of cancer cells. β-Catenin protein levels are regulated by the serine-threonine kinase GSK3β. GSK3β phosphorylates multiple regulatory residues within the N-terminus of β-catenin, which leads to its degradation via the ubiquitin–proteasome signaling pathway. Overexpression and mutations in the regulatory region of β-catenin prevent GSK3β-dependent phosphorylation, which in turn leads to stabilization of β-catenin and an interaction between β-catenin and T-cell factor (TCF)/lymphocyte enhancer factor (LEF) [2]. The β-catenin-TCF/LEF complex is then translocated into the nucleus and regulates the transcription of target genes such as c-Myc, Cyclin D1, MMP7, fibronectin, Fra-1 and c-Jun, which are associated with cancer proliferation, apoptosis, migration, and invasion [3–7]. Several studies have suggested that the nuclear accumulation of β-catenin facilitates the epithelial-to-mesenchymal transition (EMT) [8].

Tβ4 was first detected in the calf thymus and has subsequently been identified in a variety of tissues and cells [9]. Tβ4 is thought to regulate actin polymerization and has been reported to be involved in many critical biological processes, including angiogenesis, wound healing, the inflammatory response and cell migration [10–13]. Tβ4 overexpression is known to be associated with increased invasion and distant metastasis of human colorectal cancer cells [14]. However, the precise molecular signaling mechanism(s) of Tβ4 in cell invasion and migration remain unclear.

In this study, we report that Tβ4 is overexpressed in human colorectal cancer cells and closely associated with stage of colorectal cancer. Furthermore, Tβ4 promotes cell invasion and migration

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through the ILK/AKT/β-catenin signaling pathway. We also show that Tβ4 interacts with ILK, which promotes activation of AKT, β-catenin and integrin. These results suggest that Tβ4 is a positive regulator of the ILK/AKT/β-catenin/Integrin signaling cascade.

2. Materials and methods

2.1. Tissue samples and cell culture

The tissue samples for this study were provided by the Chonnam National University Hwasun Hospital National Biobank of Korea, a member of the National Biobank of Korea, which is supported by the Ministry of Health Welfare and Family Affairs. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols. Human colorectal cell line Caco-2, SW-480 and DKO-1 were cultured in DMEM (Lonza, Walkersville, MD) supplemented with 10% FBS (Gibco-BRL, Rockville, MD) and 1% penicillin/streptomycin (Gibco-BRL).

2.2. Up- and downregulation of Tβ4 in colorectal cancer cell lines

To generate Tβ4 overexpressing plasmid, about 135-bp PCR fragment containing full length of Tβ4 were amplified from Caco-2 cDNA by using the following primers; 5′-GGATCCACATGTCTGACAAACCCGATATGG-3′ and 5′-CTCGAGCGATTCGCCTGCTTGCTTC-3′. Restriction sites of *Bam*HI and *Xho*I are underlined. A pcDNA6-Tβ4 plasmid was constructed by cloning the PCR fragment into the pcDNA6. Transfection was performed using Lipofectamin 2000 (Invitrogen, Carlsbad, CA). At 48 h post-transfection, 5 μg/ml Blasticidin (Sigma, St. Louis, MO) was added and lived cells were selected as stably transfected cells. To downregulate Tβ4 expression, the Tβ4 siRNA (No. 1173234, UUCCCAUCUGUCUAUCUAU, from 384 to 402 of NM_021109) and negative control siRNA (SN-1003) were purchased from Bioneer (Daejeon, Korea). Transfection of siRNAs was performed using RNAiMAX (Invitrogen). Up- and downregulation of Tβ4 was assessed RT-PCR and Western blotting.

2.3. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using Trizol (Invitrogen). Reverse transcription was carried out on 1 μg of total RNA for 1 h at 42 °C. PCR was performed with Taq DNA polymerase using the following primers: 5′-GGATCCACATGTCTGACAAACCCGATATGG-3′ and 5′-CTCGAGCGATTCGCCTGCTTGCTTC-3′ for Tβ4; 5′-CAGGG AGTAGTGTTTGTATTTC-3′ and 5′-AGGAGAAGTTGAGAGCGATG-3′ for integrin α5; 5′-TTTGCTGTGTGTTTGCTC-3′ and 5′-CACAAATG AGCCAAATCC-3′ for integrin β1; 5′-GTGAAGGTCGGAGTCAAC-3′ and 5′-GTTGAGGTCAATGAAGGG-3′ for GAPDH; 5′-CGGCGACGACC-CATTCGAAC-3′ and 5′-GAATCGAACCCTGATTCCCCGTC-3′ for 18S rRNA.

2.4. Adhesion, invasion and migration assay

Cells were seeded on cell culture cover slip coated with 5 μg/ml fibronectin (Calbiochem, La Jolla, CA). After 30 min incubation, adherent cells were fixed with 70% ethanol, and stained with Diff Quik solution (Sysmex, Kobe, Japan), and counted the stained cells in five selected fields. Cell invasion assay was carried out using the transwell filters chambers that were coated with 1% gelatin in culture media for overnight and dried out it at room temperature. Cells were seeded at 2×10^5 cells in 150 μl medium with 0.2% bovine serum albumin (BSA) on upper chamber. Then, 500 μl medium with 0.2% BSA and 10 μg/ml fibronectin (Calbiochem) was loaded into lower chamber. After 24 h incubation, cells that invaded to the bottom surface of the transwell were fixed with 70% ethanol, stained with Diff Quik solution (Sysmex), and counted in five selected fields. Cell migration was measured by using the Culture-Inserts (Ibidi, Regensburg, Germany). The Culture-Inserts were transferred into 6-well culture plate, and cells were seeded at a density of 5×10^4 cell/100 μl in each well of Culture-Inserts. After 24 h incubation, the Culture-Inserts were removed, and cell-free gap were created. Images of the closed gap were captured at the indicated incubation time.

2.5. Western blotting analysis

Whole-cell lysate were isolated with RIPA buffer. The nuclear and cytoplasmic fractions were isolated using Nuclear Extraction Kit (Panomics, Redwood, CA). The protein concentrations were measured using BCA (Pierce, Rockfold, IL). The following antibodies were used: anti-ILK, anti-AKT1, anti-p-AKT-S473, anti-p-GSK3β, anti-β-catenin, anti-Cyclin D1, anti-Integrin β1, anti-Integrin α5 from Cell Signaling Technology (Danvers, MA); anti-GAPDH, anti-Lamin-A, anti-β-tubulin, anti-E-cadherin, anti-N-cadherin, anti-Vimentin, anti-c-Myc, anti-Fibronectin from Santa Cruz Biotechnology; anti-MMP-2 from Chemicon (Temecula, CA).

2.6. Statistical analysis

Statistical analysis was performed using the SPSS 12.0 (SPSS Inc., Chicago, IL). Statistical significance was assessed by student *t*-test or Mann–Whitney U test, as appropriate. *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Tβ4 is overexpressed in colorectal cancer cells

To determine whether Tβ4 is overexpressed in cancer tissues, Tβ4 staining was performed in 102 samples of colorectal cancer tissues and adjacent noncancerous tissues by immunohistochemistry (Fig. 1A). Tβ4 was highly expressed and was mostly localized in the cytosol of colorectal cancer cells. Tβ4 staining in colorectal cancer tissues was weak (1+) in 3.9% (4/102), moderate (2+) in 22.5% (23/102) and strong (3+) in 73.5% (75/102) cases. Tβ4 staining in normal tissues exhibited intensity scores of 0 to 2: score 0, 20 patients (19.6%); score 1+, 65 patients (63.7%); and score 2, 17 patients (16.7%). Therefore, Tβ4 staining in cancer tissues with intensity score of 3 was characterized as high expression. The association of Tβ4 expression and clinicopathological features was analyzed and summarized in Table S1. High expression of Tβ4 in the colorectal cancer tissues closely correlated with LN metastasis and TNM stage of colorectal cancer. We also examined Tβ4 mRNA expression in colorectal cancer tissues and seven colorectal cancer cell lines. The Tβ4 mRNA expression was higher in colorectal cancer tissues than in noncancerous tissues and was detected in all cancer cell lines tested (Figs. 1B and S1A). Tβ4 was expressed weakly in the Caco-2 cell line compared with other cell lines. In addition, Tβ4 was highly expressed in the KM12-SM, SW-480 and SW-620 cell lines and was intermediately expressed in DLD1, DKO-1 and HCT116 cell lines. To investigate the effects of Tβ4 expression on tumor cell behavior, Caco-2 cells were used to upregulate Tβ4 expression since Caco-2 cells exhibited a lower level of endogenous Tβ4 expression than other cell lines. The Caco-2 (low Tβ4 expression), DKO-1 (moderate Tβ4 expression) and SW-480 (high Tβ4 expression) cell lines were used to downregulate Tβ4 expression. Tβ4 was stably upregulated using a pcDNA6 vector encoding

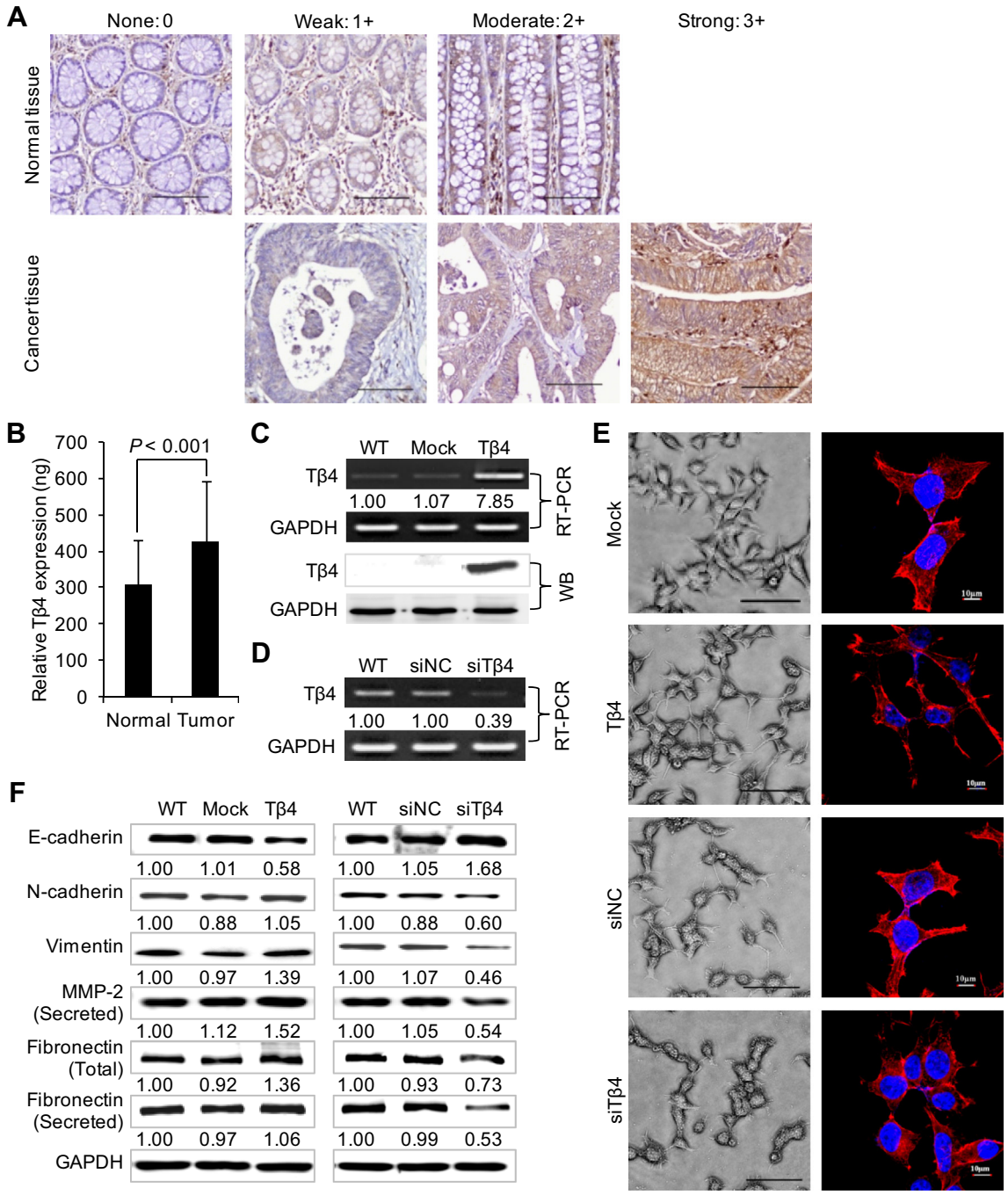

Fig. 1. Tβ4 is overexpressed in colorectal cancer cells. (A) Representative images of colorectal cancer and normal tissues were immunostained with Tβ4 antibody. Bar = 200 μm. (B) Tβ4 mRNA levels in colorectal cancer and noncancerous tissues was measured using RT-PCR and normalized against the expression level of 18S rRNA. Values represent means ± SD. (C) Tβ4 was upregulated by a stable Tβ4-expressing plasmid in Caco-2 cells. Tβ4 expression levels were measured using RT-PCR and Western blotting. (D) The expression of Tβ4 mRNA was transiently downregulated by a Tβ4-specific siRNA in Caco-2 cells and was measured using RT-PCR. (E) Pseudopodia formation was observed in monolayer culture of Caco-2 cells. Photographs were obtained under phase contrast microscopy (left). Bar = 200 μm. Elongated actin-base tails were observed by phalloidin staining. Images were obtained by confocal laser scanning microscopy (right). Bar = 10 μm. (F) The expression levels of EMT markers in Tβ4 up- and downregulated cells were measured using Western blotting.

human Tβ4 cDNA. Tβ4 upregulation was confirmed using RT-PCR and Western blotting with an anti-Myc-Tag antibody (Fig. 1C). Tβ4 expression in the stably upregulated Caco-2 cells was increased six fold compared to parental and pcDNA6 vector alone (Mock) cells. Application of gene-specific siRNA significantly decreased Tβ4 mRNA expression compared with parental and control siRNA-treated cells at 48 h after transfection (Figs. 1D and S1B).

3.2. Tβ4 induces morphological changes and the EMT

Tβ4 stably upregulated cells showed pseudopodia-like extensions in monolayer culture, whereas vector control cells showed short and fewer pseudopodia-like extensions (Fig. 1E). The pseudopodia extension in Tβ4-upregulated cells showed a filopodia-like morphology. Elongated actin-base tails were observed in Tβ4-upregulated cells by phalloidin staining. Consistent with these

observations, cells in which Tβ4 was downregulated by a specific siRNA showed decreased pseudopodia-like extensions and shortened actin-base tails compared with control-siRNA-treated cells. These morphological changes suggested that the Tβ4-upregulated cells experienced trans-differentiation from epithelial to mesenchymal (EMT) cells. During EMT, the expression of mesenchymal markers, such as vimentin, fibronectin, and N-cadherin, is upregulated, while that of epithelial markers, such as E-cadherin, and claudins, is downregulated [15]. Consistent with these reports, upregulation of Tβ4 resulted in upregulation of N-cadherin, vimentin, MMP2 and fibronectin expression, and downregulation of E-cadherin expression (Fig. 1F). Conversely, the downregulation of endogenous Tβ4 significantly increased E-cadherin expression and decreased N-cadherin, vimentin, MMP-2 and fibronectin expression compared with parental and control-siRNA-treated cells. Moreover, upregulation and activation of MMP2 induced the cleavage and secretion of a greater quantity of fibronectin. The secretion of fibronectin, which is an activated form and ligand of the Intergrin α5/β1 receptor complex, was significantly decreased in Tβ4 siRNA-treated cells.

3.3. Tβ4 promotes cell adhesion, invasion and migration

To explore the biological function of Tβ4 in colorectal cancer cells, a proliferation assay was performed using the MTT assay. The growth rate of stable Tβ4-upregulated cells was similar between parental and vector control cells (data not shown). This indicated that Tβ4 is not associated with the growth of colorectal cancer cells. Cell adhesion assays were used to investigate the effect of Tβ4 on cell–matrix interactions that may influence invasion. Tβ4-upregulated Caco-2 cells exhibited increased adhesion to fibronectin (by 2.3-fold) compared with vector control cells, and Tβ4 specific siRNA-treated cells showed reduced adhesion compared to control siRNA-treated cells (Figs. 2A and S2). These results indicated that Tβ4 regulates fibronectin expression and activation, and modulates cell–matrix interactions via regulation of cell–matrix adhesion receptors such as integrin(s). To investigate the involvement of integrin in Tβ4-mediated adhesion, integrin α5 and β1 expression was assessed by Western blotting. Integrin β1 expression was increased by upregulation of Tβ4 and significantly decreased by downregulation of Tβ4 (Fig. S3A);

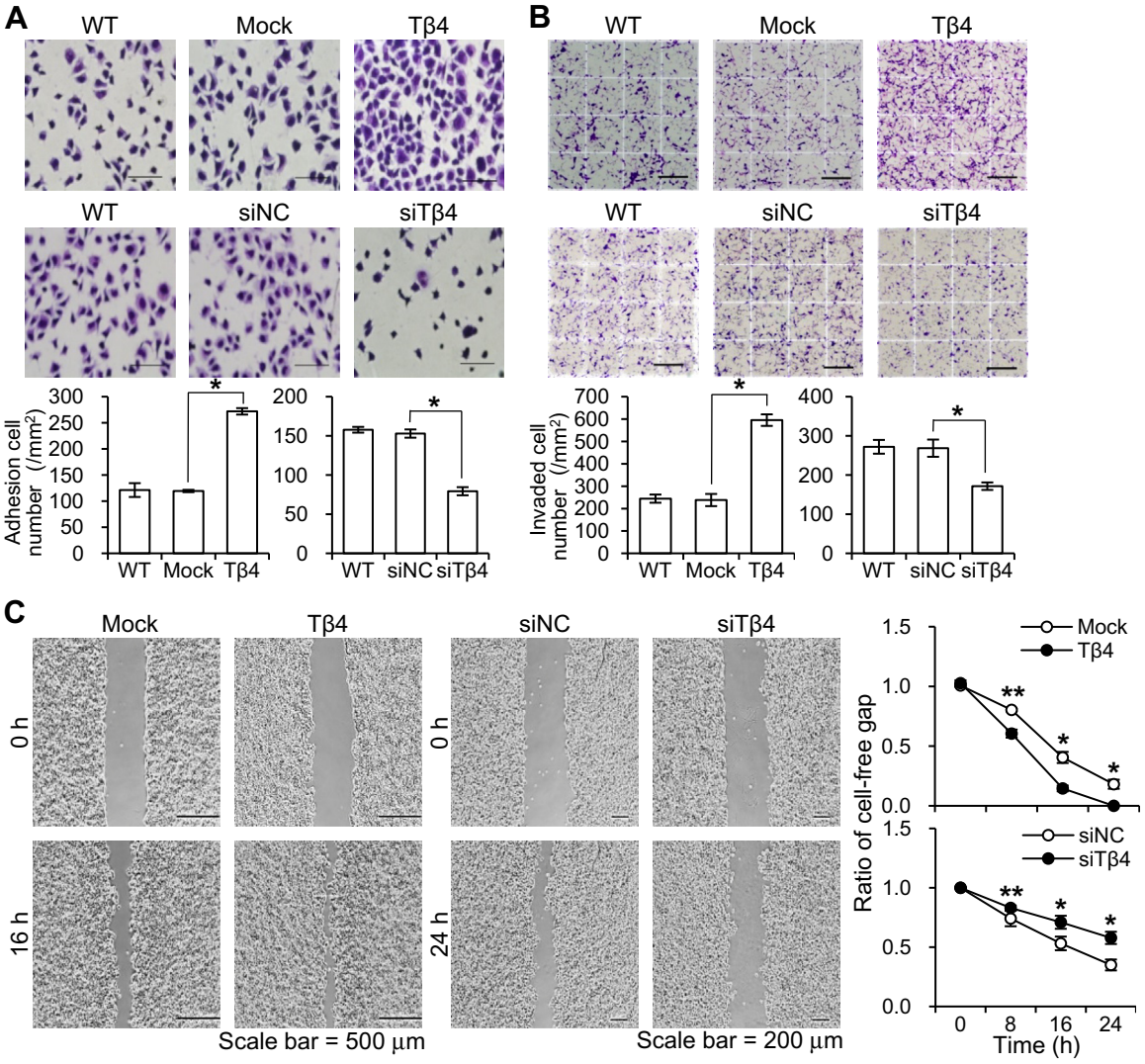

Fig. 2. Tβ4 promotes cell adhesion, invasion and migration. (A) Cell adhesion to fibronectin of Caco-2 cells was assessed. The number of adherent cells was counted in five representative photographs. Bar = 100 μm. (B) Cell invasion ability was determined using an invasion assay. The number of invaded cells was counted in five representative photographs. Bar = 200 μm. (C) The migration ability was determined using a wound-healing assay. The width of the wound was measured at five sites for the indicated periods of time and normalized to that at 0 h. Values represent means ± SD. $^{*}P < 0.001$; $^{**}P < 0.05$.

however, integrin α5 expression levels were similar in Tβ4 up- and downregulated cells compared with control cells. These results indicated that Tβ4 positively regulates the expression of integrin β1. To determine whether Tβ4 regulates the expression of integrin β1 through transcriptional or translational regulation, RT-PCR and cycloheximide (a translation inhibitor) treatments were performed. Integrin α5 and β1 mRNA levels were unaffected by regulation of Tβ4 in RT-PCR (Fig. S3A). After cycloheximide treatment, the integrin α5 and β1 protein levels decreased, and the degradation ratios in Tβ4-overexpressing plasmid and control vector-treated cells were identical (Fig. S3B). This indicated that protein stability of integrin β1 was not associated with Tβ4 expression, and integrin β1 expression was associated with the increased protein synthesis induced by Tβ4 expression.

Cell migration and invasion are basic characteristics of tumor metastasis. To examine the effect of Tβ4 on migration and invasion in colorectal cancer cells, invasion and wound-healing migration assay were performed using Tβ4 up- and downregulated cells. Tβ4-upregulated cells showed significantly increased motility toward human plasma fibronectin compared with control cells in invasion assay (Fig. 2B). Similar results were observed in wound-healing migration assay. The wound-healing ability of Tβ4-upregulated cells was significantly increased compared with control cells (Fig. 2C). Therefore, the wound-healing and invasion ability of Tβ4-downregulated cells decreased significantly compared with control

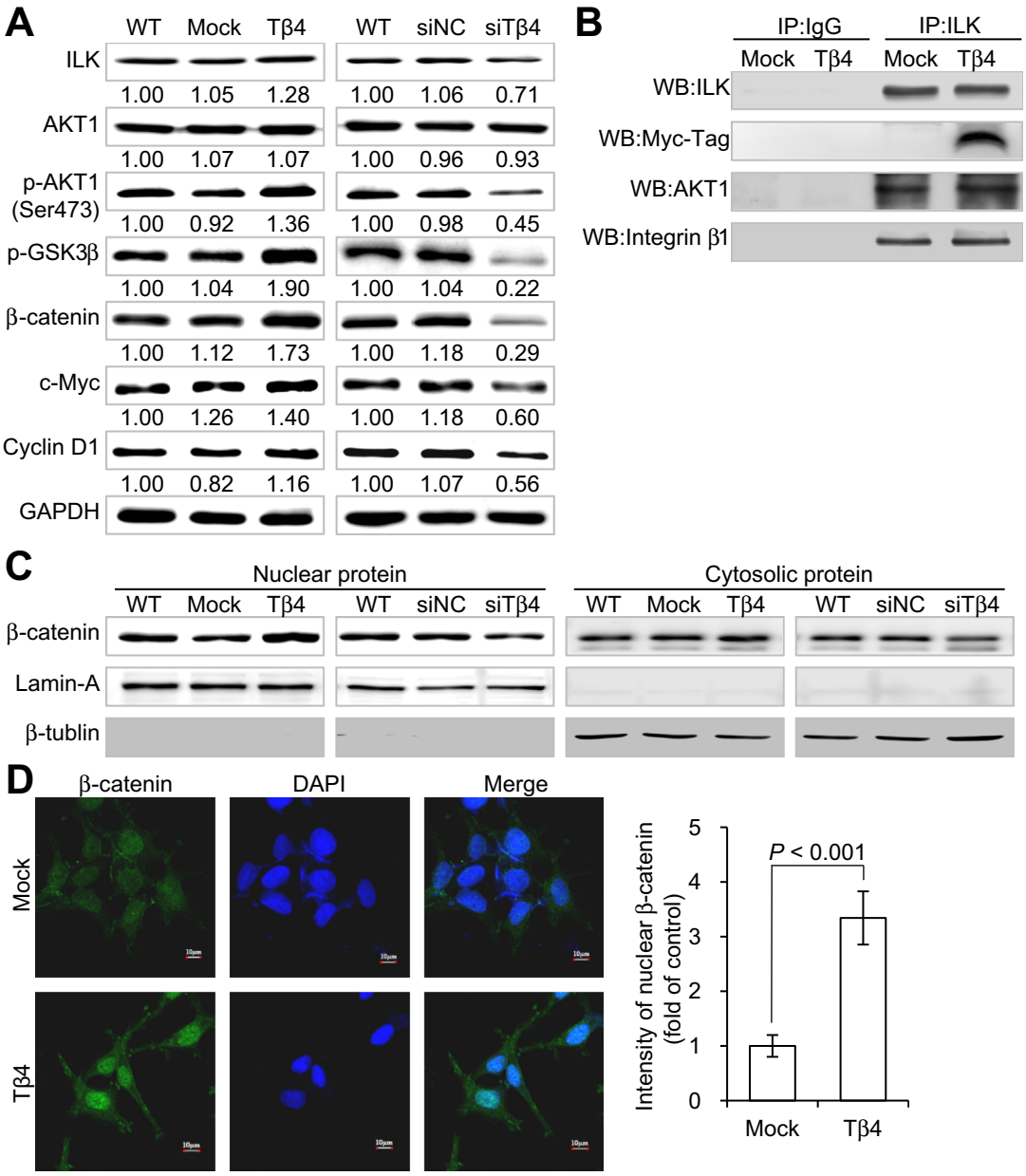

Fig. 3. Tβ4 activates the ILK/AKT/β-catenin signaling cascade. (A) Levels of proteins related to ILK/AKT/β-catenin signaling cascade were measured using Western blotting. (B) Molecules that interact with Tβ4 were detected by immunoprecipitation using an anti-ILK antibody. Immunoprecipitation by IgG was used as the control. (C) Intercellular localization of β-catenin was determined using Western blotting. Nuclear and cytoplasmic fractions were isolated and equal amounts of each were immunoblotted using an anti-β-catenin antibody. Lamin-A and β-tubulin were used as nuclear and cytoplasmic controls. (D) Nuclear localization of β-catenin in Tβ4-upregulated Caco-2 cells was detected and quantified by immunofluorescence staining. The histogram shows relative intensities compared to empty vector controls. Values represent the relative means ± SD. Bar = 10 μm.

cells (Figs. 2B, C and S4). Thus Tβ4 likely plays a role in the migration and invasion of colorectal cancer cells.

3.4. Tβ4 activates the ILK/AKT/β-catenin signaling pathway

Integrin-linked kinase (ILK) is upregulated in various cancers and is implicated in cellular processes such as EMT, invasion, migration and regulation of the actin cytoskeleton [16]. ILK expression has previously been shown to be stimulated by Tβ4.[13,17,18] To determine whether ILK is involved in Tβ4-mediated EMT and invasion in Caco-2 cells, we evaluated ILK expression in Tβ4 up- and downregulated Caco-2 cells using Western blotting. ILK expression was positively regulated by Tβ4 in these cells (Fig. 3A). Immunoprecipitation was performed to determine whether Tβ4 and ILK form a dimeric complex in Caco-2 cells. ILK could bind to Tβ4, AKT and integrin β1 (Fig. 3B). Tβ4-ILK complex induced AKT activation, which promoted cell migration and survival, as described previously [13,19]. Consistent with these reports, Ser473 phosphorylation of AKT1, an active form of AKT1, was induced by upregulation of Tβ4 and reduced by downregulation of Tβ4 (Fig. 3A). In parallel, activated AKT1 inhibited GSK3β through phosphorylation, after which β-catenin accumulated in the cytoplasm and translocated into the nucleus to play a role as an oncogenic transcriptional factor (Fig. 3C). Corroborating the Western blotting results, Tβ4-upregulated Caco-2 cells showed 3.3-fold higher nuclear β-catenin expression than vector control cells, based on immunofluorescence staining (Fig. 3D). Nuclear β-catenin positively regulated the expression of c-Myc, Cyclin D1, fibronectin and MMP2, which resulted in cell migration and invasion (Figs. 1F and 3A). Consistent with the Western blotting results in Caco-2 cells, Tβ4-downregulated DKO-1 and SW-480 cells showed downregulated expression of Ser473-phosphorylated AKT1, phosphorylated GSK3β, β-catenin, c-Myc and Cyclin D1 (Fig. S5).

To characterize the role of Tβ4 and the ILK/AKT/β-catenin signaling pathway in cell migration and invasion, GSK3β ability to phosphorylate β-catenin was inhibited in Tβ4-downregulated cells by Lithium chloride (LiCl) treatment. Since Ser9 phosphorylation of GSK3β inactivates its kinase activity, the phosphorylation level of GSK3β was examined in Caco-2 cells in the presence of 10 mM LiCl. Treatment with LiCl increased the Ser9 phosphorylation of GSK3β and the expression of β-catenin by preventing GSK3β-dependent phosphorylation and degradation (Fig. 4A). Inhibition of GSK3β activity significantly increased cell invasion in parental and control siRNA-treated cells, and was restored in Tβ4 downregulated cells (Fig. 4B).

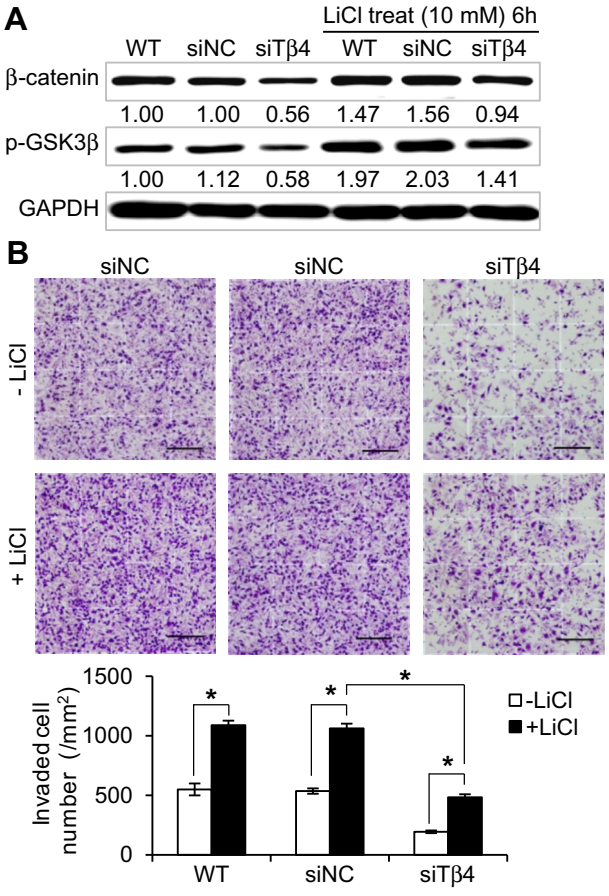

Fig. 4. Invasion ability of Tβ4-downregulated Caco-2 cells is restored by a GSK3β inhibitor. (A) The expression levels of β-catenin and phosphorylated GSK3β in the presence or absence of 10 mM LiCl were measured using Western blotting and normalized to GAPDH expression. (B) Cell invasion ability in the presence or absence of 10 mM LiCl was determined by an invasion assay. The number of invaded cells was counted in five representative photographs. Bar = 200 μm. Values represent means ± SD. *$P < 0.001$.

4. Discussion

Here, we show that Tβ4 is overexpressed in colorectal cancer and plays a role in adhesion, invasion and migration, and as well as in the EMT. In addition, high levels of Tβ4 were closely correlated with stage of colorectal cancer. We also found that Tβ4 modulates the formation of actin-based pseudopodia. The extension of pseudopodia is a basic element for movement of eukaryotic cells, and the direction of the pseudopodia determines the direction of cell migration [20]. Upregulation of Tβ4 led to the extension of pseudopodia, whereas downregulation led to shorter and less numerous pseudopodia. These morphological changes suggest that Tβ4 expression is associated with cell migration and the EMT.

The EMT is an essential process during the developmental period and is known to occur during wound healing and invasion, which are critical for initiation of cancer metastasis. EMT is a process by which epithelial cells undergo significant morphological changes and lose their cell polarity and cell–cell adhesion to become mesenchymal cells. Epithelial cells are closely connected through tight junctions, whereas mesenchymal cells are connected only through focal points and have a spindle-shaped morphology. EMT could be characterized by downregulation of E-cadherin, upregulation and translocation of β-catenin from the cell membrane to the nucleus, and upregulation of mesenchymal molecular markers such as vimentin, fibronectin and N-cadherin [15]. Upregulation of N-cadherin in addition to loss of E-cadherin is positively associated with tumor cell invasion and metastasis [21]. Here, we showed that Tβ4 is positively correlated with EMT by demonstrating its effects on the expression of EMT markers, such as E-cadherin, N-cadherin, vimentin and fibronectin, and promotes cell adhesion, invasion and migration. Furthermore, Tβ4 upregulated the expression of integrin β1 and activated its function via upregulation of the expression and secretion of fibronectin. This finding is consistent with previous reports that functional integrin α5β1 is implicated in induction of the EMT [22].

We showed that Tβ4 interacts with ILK, AKT1 and integrin β1. ILK is a ubiquitously expressed multifunctional protein kinase that interacts with the cytoplasmic domain of integrin β1 and acts as a proximal receptor kinase regulating integrin-mediated signal transduction [16]. In addition, ILK interacts with AKT and stimulates phosphorylation of AKT, leading to its activation. ILK also stimulates phosphorylation of GSK3 in a direct and/or indirect manner, leading to its inhibition and relieving several negatively regulated signaling pathways. Here, we demonstrated that Tβ4 promoted cell invasion and migration through the ILK/AKT/β-catenin signaling pathway. However, Tβ4 could not directly interact

with AKT and GSK3 and could not directly regulate their phosphorylation [19]. An interaction between Tβ4 and ILK may be required to promote cell invasion and migration. This result indicates that Tβ4 is a key initiator of the ILK-activated signaling pathway during movement of colorectal cancer cells.

β-catenin is the central mediator of the Wnt signaling pathway and plays a central role in cell adhesion, invasion, and migration. AKT also regulates the Wnt signaling pathway through the Ser9 phosphorylation of GSK3β, leading to inactivation of GSK3β and augmentation of β-catenin stabilization and its nuclear translocation. Here, we showed that downregulation of Tβ4 by siRNA decreased the phosphorylation of GSK3β, and decreased the expression and nuclear localization of β-catenin. This indicates that GSK3β is a negative mediator of Tβ4-induced cell invasion and migration. Inhibition of GSK3β activity by LiCl treatment significantly increased invasion in parental and control siRNA-treated cells, and restored invasion in Tβ4-downregulated cells. However, LiCl treatment did not completely restore invasion in Tβ4-downregulated cells to the levels of parental and control siRNA-treated cells. It has been reported that ILK transcriptionally regulates E-cadherin expression through PARP-1, Snail and Zeb1 [23,24], and that E-cadherin interacts with β-catenin near the plasma membrane, reducing β-catenin/TCF/LEF transcriptional activity [25]. The partial restoration of cell invasion in Tβ4-downregulated cells by LiCl treatment might have been due to reexpression of E-cadherin. This indicates that Tβ4-mediated cell invasion and migration are closely associated with β-catenin activation and E-cadherin downregulation.

Previous studies have reported that overexpression of Tβ4 elevated cell invasion and migration potential *in vitro* and tumorigenicity and metastatic potential *in vivo* [14,17,18]. Moreover, the stimulated cell migration in Tβ4-overexpressing SW-480 cells is caused by upregulation of ILK, AKT and β-catenin activities. Here, we confirmed that Tβ4 promoted cell invasion, migration and EMT via enhancing ILK/AKT/β-catenin signaling pathway in three colorectal cancer cell lines, Caco-2, DKO-1 and SW-480. We also newly showed that the expression and nuclear localization of β-catenin is induced in Tβ4-overexpressing Caco-2 cells. Furthermore, we demonstrated that Tβ4 positively upregulates the expression of integrin β1 and thus regulates its signaling transduction activity.

In summary, we showed that downregulation of Tβ4 significantly decreased ILK/AKT/β-catenin signaling cascade activity and upregulated E-cadherin expression in colorectal cancer cells, which were correlated with significant decreases in migration and invasion ability. These results demonstrate that Tβ4 is an important component of ILK-mediated colorectal cancer progression, and suggests its possible application as a molecular target for therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.012.

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