TGF-β1 promotes the migration and invasion of bladder carcinoma cells by increasing fascin1 expression

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Received January 28, 2016; Accepted March 5, 2016

DOI: 10.3892/or.2016.4889

Abstract. Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that is reported to regulate cellular motility and invasive capability during tumor progression. Fascin1, an actin-bundling protein, increases cell motility, migration and adhesion. To investigate the function of TGF-β1 and test whether fascin1 is an important mediator of the tumor response to TGF-β1 in bladder carcinoma cells, real-time RT-PCR and western blot analysis were used to test changes in fascin1 expression after TGF-β1 (10 ng/ml) treatment in T24 and BIU87 cells. Small interfering RNA (siRNA) technique was performed to silence fascin1. Cell viability and biological behavior changes were evaluated by cell growth (MTT), wound-healing and Matrigel invasion assays. In the present study, we found that the mRNA and protein levels of fascin1 in the T24 and BIU87 cells were significantly increased after 10 ng/ml TGF-β1 treatment ($p<0.05$). The proliferation of T24 cells ($p=0.005$) was also significantly increased, while no significant change was observed in BIU87 cells ($p=0.318$). In addition, the migratory and invasive potential of the two cell lines were promoted. Furthermore, we successfully silenced fascin1, and observed that fascin1 siRNA significantly attenuated the migration and invasiveness induced by TGF-β1. The findings suggested that TGF-β1 can promote invasion and migration of T24 and BIU87 bladder carcinoma cells, and the increase in fascin1 expression may be the key point of this impact of TGF-β1.

Introduction

As is well known, transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that regulates cell proliferation, growth, differentiation as well as cell movement (1,2). In addition, TGF-β1 has been shown to play a critical role during tumor progression potentially by regulating cellular motility and invasive capability. However, the underlying mechanism remains largely unknown.

Fascin is a 55-kDa globular protein that belongs to a unique family of actin-bundling proteins (3). There are three isoforms of fascin that are found in vertebrate cells. Fascin1 is widely expressed in mesenchymal tissues as well as in the nervous system, while fascin2 is expressed by retinal photoreceptor cells, and fascin3 is a typical testis-specific protein (4). Fascin (also known as fascin1) is found to be located in the microspikes, membrane ruffles and stress fibers, and is reported to induce membrane protrusions. Its principal function is to form the parallel actin bundles that support lamellipodial and filopodial cell protrusions, which are the key cellular structures involved in environmental guidance and cell motility, migration and adhesion (5-7).

Recent studies show that the fascin1 protein level is significantly increased in transformed epithelial cells and various types of carcinomas (8-11). Overexpression of fascin1 has been reported in bladder carcinoma (12,13). Previously, we reported that fascin1 plays an important role in migration and invasion of urothelial carcinoma of the bladder (14,15).

Given the fact that the cytoskeleton proteins are involved in TGF-β1-induced tumor progression and metastasis (16), and that the expression of fascin1 was upregulated by TGF-β1 in various types of cancers such as lung and gastric cancer (17,18), we hypothesized that fascin1 could be a potential mediator of TGF-β1-induced cell invasion and tumor metastasis in bladder cancer. We thus undertook the present study to investigate the effect of TGF-β1 on the expression of fascin1 as well as the role of fascin1 in the TGF-β1-induced cellular biological changes in human bladder urothelial carcinoma cell lines T24 and BIU87.

Materials and methods

Cell culture. The human urothelial carcinoma cell lines T24 and BIU87 were purchased from the China Type Culture Center (Wuhan, China) and maintained according to the manufacturer's instructions. Cells were cultured in a suitable incubator with sufficiently moisturized conditions

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Key words: bladder carcinoma, TGF-β1, fascin1, invasion, migration

(monolayer cells grow in a 37˚C environment with an atmospheric composition of 5% CO_2 and 95% air) in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (EuroClone, West York, UK), 100 U/ml penicillin and 100 mg/ml streptomycin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). When cells grew to 70% confluency, the routine medium was removed and replaced with FBS-free RPMI-1640 medium without or with 10 ng/ml TGF-β1 (RayBiotech, Atlanta, GA, USA) for 24 h.

Small interfering RNA (siRNA) preparation and transfection. T24 or BIU87 cells were seeded to grow for 48 h to ~80% confluency in RPMI-1640 medium containing 10% FBS. One OD fascin1 siRNA (GenePharma, Shanghai, China) was diluted in 125 μl diethylpyrocarbonate (DEPC) solution. Then, 8 μl siRNA solution and 8 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added to 242 μl of fresh RPMI-1640 medium, incubated for 5 min at room temperature, and mixed for another 20 min at room temperature. The transfection complex mixture was added to 1,500 μl RPMI-1640 medium without FBS to the cells. Scrambled siRNA with Lipofectamine 2000 alone was used as control. After 6 h, the medium was replaced with RPMI-1640 containing 10% FBS, and the cells were cultured for 24 or 48 h with or without 10 ng/ml TGF-β1 until ready for further assay.

Real-time RT-PCR. Total RNA was isolated from cultured cells with ice-cold TRIzol reagent (Invitrogen), according to the manufacturer's instructions. The concentration of RNA was determined by Thermo Scientific NanoDrop ND-100 (Wilmington, DE, USA). Total RNA was used for reverse transcription using SYBR® PrimeScript® RT-PCR kit (Perfect Real-Time) (Takara, Kyoto, Japan). Real-time PCR was performed using Thermal Cycler Dice™ Real-Time system TP800 (Takara). The reaction system was maintained at 60˚C for 2 min and heated to 95˚C for 10 min followed by 45 cycles, denaturation of the mixture at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The sequence of primers designed for fascin1 and GAPDH are listed in Table I. The $2^{-\Delta\Delta Ct}$ method was used to calculate mRNA expression of the target gene.

Western blotting. Western blot analysis was carried out to investigate the expression of fascin1 before and after TGF-β1 treatment in T24 and BIU87 cells. Cells were lysed in lysis buffer with a cocktail of protease inhibitors (both from Sigma, St. Louis, MO, USA). Total proteins were measured using the BCA protein assay kit (Sigma) according to the manufacturer's protocol. A sample consisting of 30 μg of total protein was analyzed by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Sigma). The membranes were subsequently blocked with 5% skim milk for 2 h at room temperature and incubated overnight at 4˚C with a specific primary antibody against fascin1 (1:5,000; Abcam, Hong Kong, China) or β-actin (1:2,000; Santa Cruz Biotechnology). β-actin was used as an internal control. Next, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,500; Santa Cruz Biotechnology), and signals were developed using Western Blotting Luminol Reagent (Gene Company Ltd., Hong Kong, China).

Table I. Sequences of primer pairs for real-time RT-PCR analysis.

Gene		Nucleotide sequence (5'-3')	Length (bp)
Fascin1	F	GGCAAGTTTGTGACCTCCAAGAA	136
	R	AGCCGATGAAGCCATGCTC	
β-actin	F	CTCCATCCTGGCCTCGCTGT	268
	R	GCTGTCACCTTCACCGTTCC	

F, forward; R, reverse.

Cell proliferation assay. The MTT assay was used for measuring cell proliferation with and without TGF-β1 treatment. T24 and BIU87 cells were seeded at a density of $1x10^4$ cells/well into 96-well plates. After 24 h, the culture medium was removed and fresh medium contained 0 or 10 ng/ml TGF-β1 was added. After a 48-h treatment, MTT working solution (Sangon Biological Company, China) (20 μl, 5 mg/ml) was added to each well, and the cells were incubated at 37˚C for 4 h. Then, the medium in each well was completely removed, 150 μl of dimethyl sulfoxide (DMSO) solution (Sigma) was added into each well and the plate was shaken for 10 min. The 490 nm absorbance of the dissolved chemical crystals was measured by a plate reader (model 680; Bio-Rad, Hertfordshire, UK). All assays were performed three times in sets of six replicate wells.

Cell migration assay. The migration activities of the T24 and BIU87 cells were evaluated by wound-healing assay. Cells were plated in a 24-well plate and cultured to reach a confluency before the assay. The wound was made by scraping using a conventional pipette tip across the monolayer. The monolayer was softly washed with phosphate-buffered saline (PBS), and then the cells were continued to be cultured in the medium supplemented with 0 or 10 ng/ml TGF-β1. The initial gap length (0 h) and the residual gap length 24 and 48 h after wounding was measured from photomicrographs.

Cell invasion assay. Cell invasion was determined by an invasion chamber assay. Cells ($1x10^4$) in RPMI-1640 medium containing 5% FBS were seeded onto the top chamber of a 24-well Matrigel-coated micropore membrane filter with 8-μm pores (Corning, Corning, NY, USA), and the bottom chamber was filled with RPMI-1640 medium containing 10% FBS as a chemoattractant. After 36 h of incubation in a 5% CO_2 humidified incubator at 37˚C, the cells on the upper surface were carefully removed with a cotton swab, and the membranes were fixed with 4% paraformaldehyde and stained with crystal violet staining solution. Invasion was quantified by counting all of the cells that had migrated through the membrane in five random fields under a light microscope (magnification, x200). The mean value was calculated from data obtained from three separate chambers.

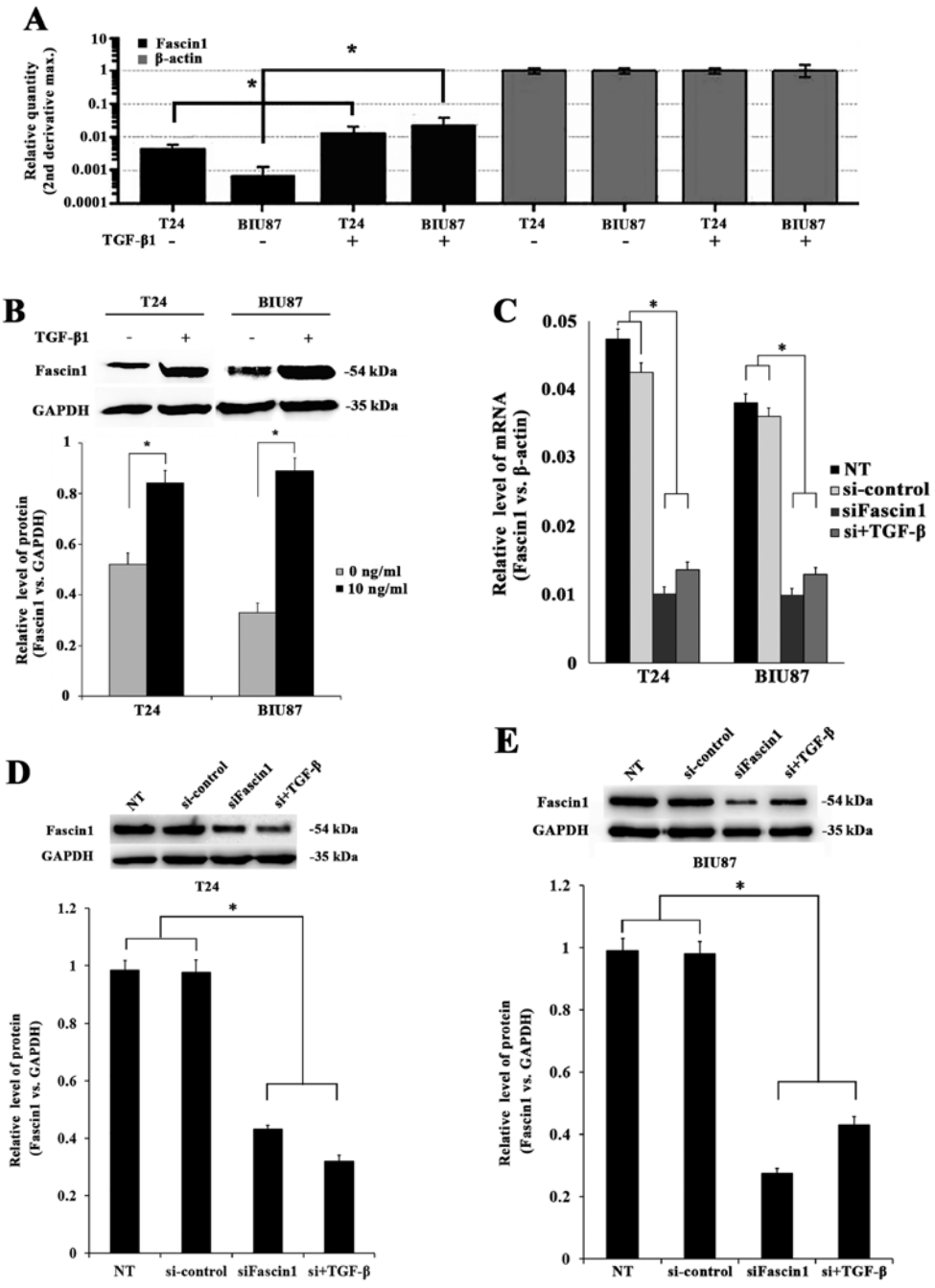

Figure 1. Effect of TGF-β1 (10 ng/ml) and fascin1 siRNA on fascin1 expression in T24 and BIU87 cells. (A and B) Fascin1 expression in T24 and BIU87 cells pre-treated without or with TGF-β1. (C-E) The mRNA and protein levels of fascin1 were effectively suppressed by fascin1 siRNA in the T24 and BIU87 cells. β-actin and GAPDH were used as control for the western blot and real-time RT-PCR analyses, respectively. Data are expressed as mean ± SD; *p<0.05. NT, untransfected.

Statistical analysis. All values are expressed as means ± standard deviation (SD), and were analyzed using SPSS for windows, version 16.0 (SPSS, Inc., Chicago, IL, USA). The independent and paired t-tests were used and the values were considered to be significant at $p<0.05$.

Results

Effects of TGF-β1 and fascin1 siRNA on fascin1 expression in the T24 and BIU87 cells

TGF-β1 increases the expression of fascin1. Expression of fascin1 in the T24 and BIU87 cells after treatment with TGF-β1 is shown in Fig. 1A and B. As shown in Fig. 1A, compared with the corresponding control, the mRNA levels of fascin1 in the T24 and BIU87 cells were significantly increased after a 24-h treatment with TGF-β1 ($p<0.05$). The protein levels of fascin1 in the T24 and BIU87 cells were significantly increased after a 48-h treatment with TGF-β1 ($p<0.05$) (Fig. 1B).

Fascin1 siRNA effectively suppresses fascin1 expression. The inhibitory efficiency of fascin1 siRNA on fascin1 expression was assessed at both the mRNA and protein levels (Fig. 1C-E). As shown, compared with the si-control cells, fascin1 mRNA levels were significantly reduced by 78.7±2.3 and 73.8±2.1%

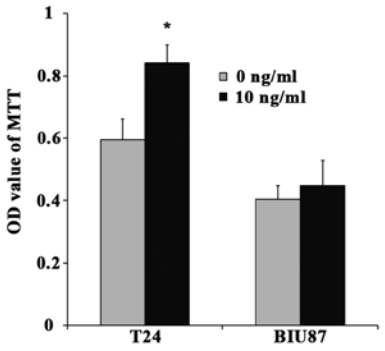

Figure 2. Effect of TGF-β1 on the proliferation of T24 and BIU87 cells. MTT assays were used to measure the proliferation of T24 and BIU87 cells with or without TGF-β1 (10 ng/ml) treatment. Columns represent the average of 490 nm absorbance values from six replicates in three independent experiments. Data are expressed as mean ± SD; $^{*}p<0.05$.

following transfection with fascin1 siRNA in the T24 and BIU87 cells, respectively (Fig. 1C), while fascin1 protein level was significantly reduced by 56.3±1.7% in the T24 and 72.4±2.2% in the BIU87 cells, respectively ($p<0.05$). Moreover, after treatment with 10 ng/ml TGF-β1, the fascin1 protein levels did not increase in the fascin1 siRNA-transfected cells compared with the non-transfected (NT) cells ($p>0.05$).

Effect of TGF-β1 on the proliferation of the T24 and BIU87 cells. Changes in the proliferation of T24 and BIU87 cells after TGF-β1 treatment are summarized in Fig. 2. As shown in this figure, the proliferation of T24 cells was significantly increased after treatment with 10 ng/ml TGF-β1 ($p=0.005$). However, the proliferation of the BIU87 cells treated with 10 ng/ml TGF-β1 failed to show significant difference compared with the TGF-β1-untreated cells ($p=0.318$).

Effects of TGF-β1 and fascin1 siRNA on the migratory abilities of the T24 and BIU87 cells

TGF-β1 promotes the migration activities of T24 and BIU87 cells. Changes in the migration activities of T24 and BIU87 cells after TGF-β1 treatments are shown in Fig. 3A and B. As shown in Fig. 3A, after the wound was made at 24 h, the initial width of the wound was closed by 40% in the TGF-β1-untreated T24 cells, while it was closed by 60% in the TGF-β1-treated T24 cells, which was a significant increase compared with the TGF-β1-untreated cells. After the wound was made at 48 h, there still existed a significant 'unhealed' wound in the TGF-β1-untreated cells; however, the TGF-β1-treated cells almost 'healed' the wound by 90%, which also increased significantly compared with the TGF-β1-untreated cells. The similar response of 'wound healing' to TGF-β1 was also found in another bladder cancer cell line BIU87 (Fig. 3B).

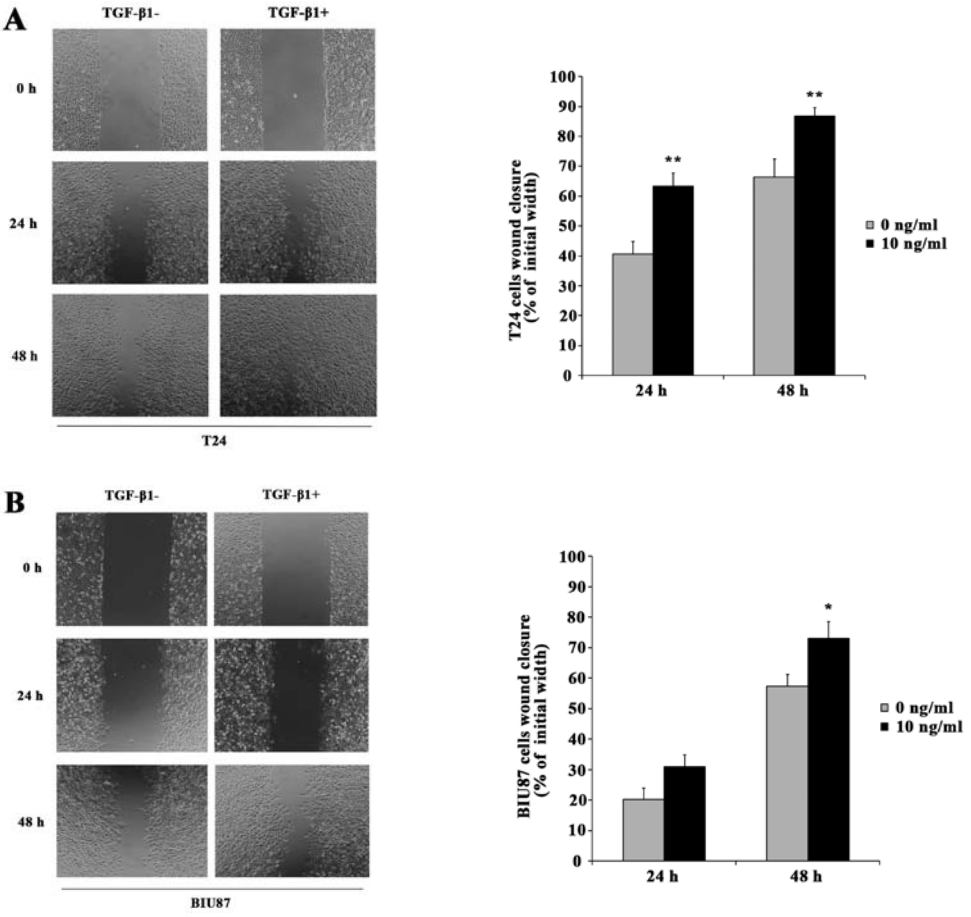

Figure 3. Effects of TGF-β1 (10 ng/ml) and fascin1 siRNA on the migratory abilities of the T24 and BIU87 cells. The cell migration abilities of non-transfected (NT) (A) T24 and (B) BIU87 cells were assessed by wound-healing assay; $^{*}p<0.05$; $^{**}p<0.01$.

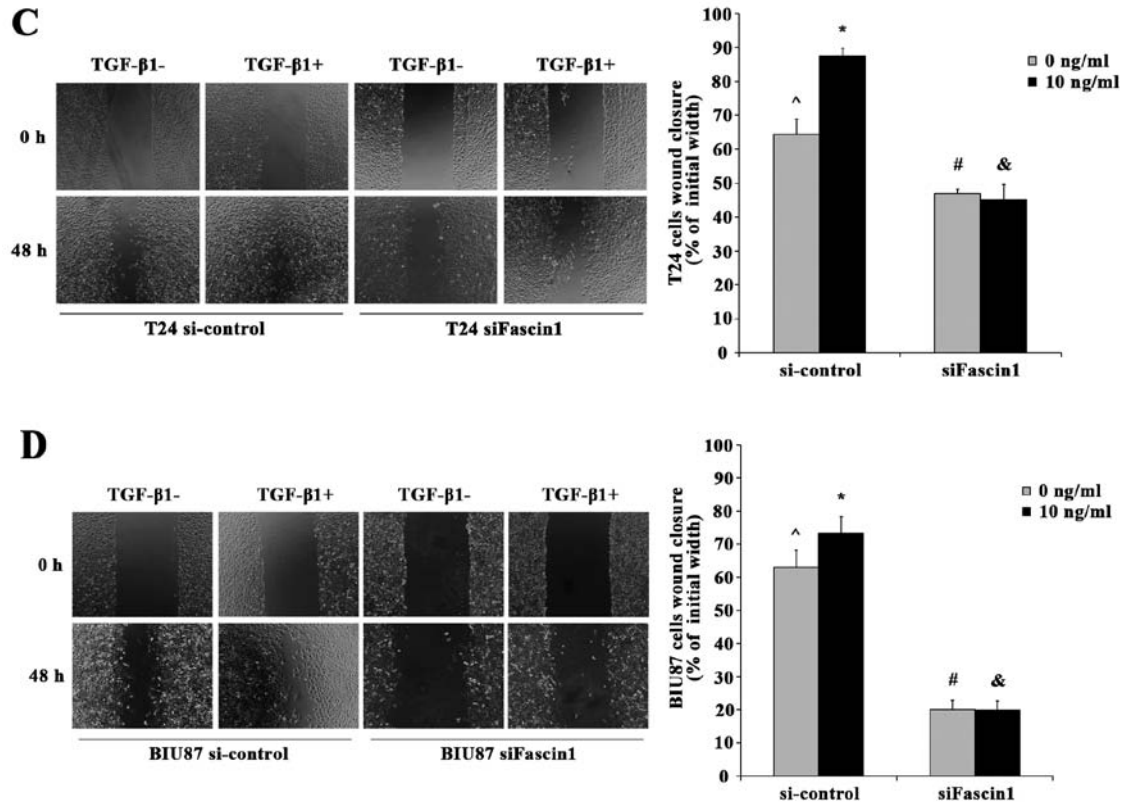

Figure 3. Continued. Transfected (C) T24 and (D) BIU87 cells were assessed by wound-healing assay. Images are the representative results of three separate experiments for each group. Columns represent the average quantitation of cell wound closure proportion for each group. Data are expressed as mean ± SD; *p<0.05 vs. ^; #p<0.005 vs. ^; &p<0.005 vs. *; &p>0.05 vs. #.

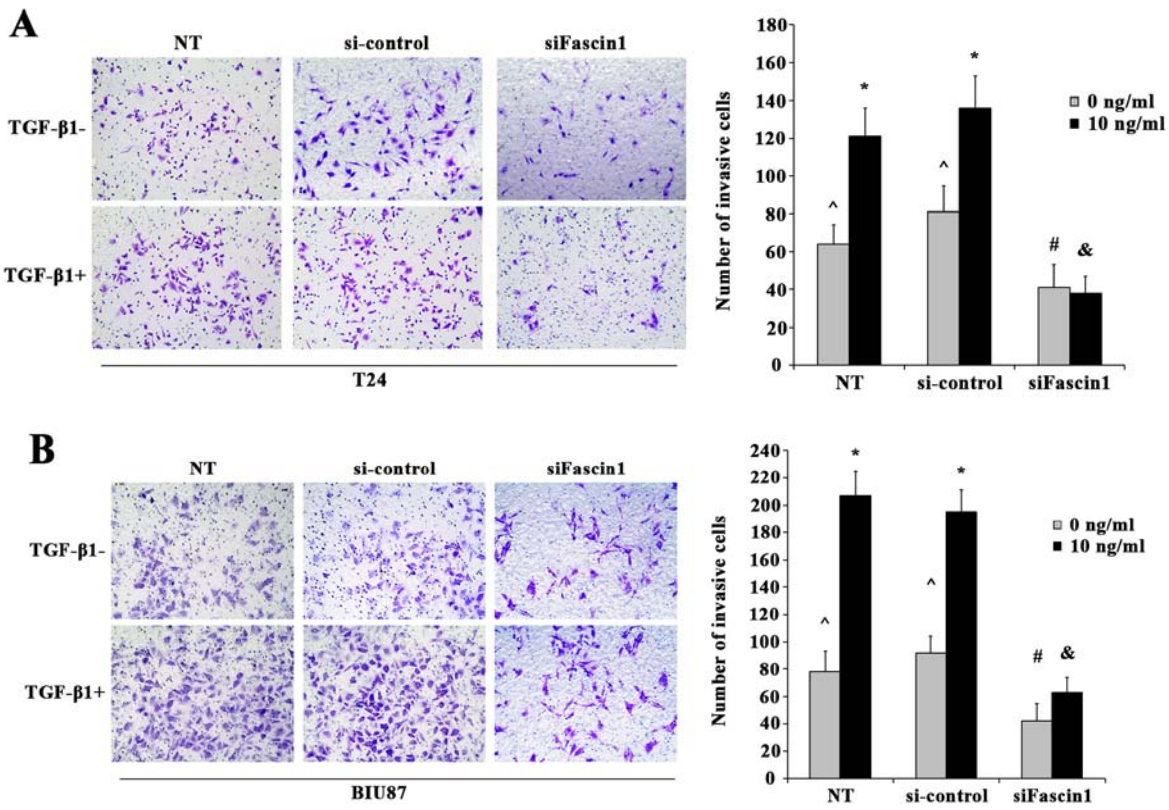

Figure 4. Effects of TGF-β1 (10 ng/ml) and fascin1 siRNA on the invasive abilities of T24 and BIU87 cells. The invasiveness of (A) T24 and (B) BIU87 cells was examined by Matrigel-coated Transwell chambers. Images are the representative results of three separate experiments for each group. Columns represent the average invasive cell numbers in each group. Cells were counted in 10 random fields under a light microscope (magnification, x200). Data are expressed as mean ± SD; *p<0.05 vs. ^; #p<0.05 vs. ^; &p>0.05 vs. #. NT, untransfected.

Fascin1 siRNA inhibits the cell migratory abilities induced by TGF-β1. Changes in the migration activities of T24 and BIU87 cells after fascin1 siRNA and TGF-β1 treatment are summarized in Fig. 3C and D. As shown in this figure, compared with the si-control cells, the fascin1 siRNA treatment decreased the cell migration in both the TGF-β1-untreated and TGF-β1-treated cells in the T24 and BIU87 cell lines, respectively. Importantly, the treatment of TGF-β1 significantly promoted the migration of both cell lines, and this effect was largely attenuated by lowering the expression of fascin1 using fascin1 siRNA in these cells, as the migration of cells in the fascin1 siRNA treatment group was significantly lower than that in the si-control group.

Effects of TGF-β1 and fascin1 siRNA on the invasive abilities of the T24 and BIU87 cells

TGF-β1 promotes the invasive abilities of the T24 and BIU87 cells. Changes in the invasive abilities of the T24 and BIU87 cells after TGF-β1 treatments are illustrated in Fig. 4A and B. As shown in this figure, the numbers of invasive cells penetrating through the membrane in the 10 ng/ml TGF-β1 treatment cells were significantly increased in the T24 (p=0.005) and BIU87 cells (p=0.001), compared with the NT T24 and BIU87 cells.

Fascin1 siRNA attenuates the invasiveness induced by TGF-β1. Changes in the invasive abilities of the T24 and BIU87 cells after fascin1 siRNA and TGF-β1 treatment are summarized in Fig. 4A and B. Compared with si-control cells in TGF-β1-untreated group, less cells revealed invasiveness in the sifascin1 group (T24, p=0.014; BIU87, p=0.003). When compared with the TGF-β1-untreated cells, treatment with 10 ng/ml TGF-β1 significantly promoted the invasiveness of the si-control cells (T24, p=0.000; BIU87, p=0.000), however, this failed to have significant effects on the invasiveness of the fascin1 siRNA cells (T24, p=0.746; BIU87, p=0.100).

Discussion

TGF-β1 is a multifunctional cytokine that is known to induce G1 arrest during the cell cycle in order to end proliferation, induce differentiation, or promote apoptosis in normal cells, thus being a natural tumor-suppressive agent (19). In the early stages of cancer development, cells respond to the antimitotic effect of TGF-β1 (20). However, at the entry of tumor cells into the phase of uncontrollable growth, most of them lose sensitivity to the inhibitory effect of TGF-β1. It is surprising that this occurs despite the presence on the tumor cell surface of the receptors for TGF-β1. Moreover, these cancer cells begin to secrete TGF-β1 themselves. The TGF-β1-dependent immunosuppressive activity increases the affinity of cancer cells to cell adhesion molecules (21), creates a microenvironment favorable to tumor growth and metastasis, and increases invasiveness of cancer cells (20). Sun *et al* (22) found that TGF-β1 induced invasion and filopodia formation in spindle-shaped tumor cells, and Fu *et al* (18) found that TGF-β1 promoted invasion and metastasis of gastric carcinoma cells. In agreement with our previous data, increased migration and invasiveness potential in TGF-β1-treated bladder cancer cells were confirmed in the present study.

Fascin1 has been found to play an important role in migration and invasion of various tumor cells (11,12,15), and fascin1 overexpression has been previously reported in many cancer tissues (13,23,24). Thus, fascin1 was recently used as an identifier of metastatic carcinoma to differentiate carcinoma metastasis, particularly in urothelial carcinoma (25,26).

Recent studies have indicated that upregulation of fascin1 may be the key point in the process of TGF-β1-promoted invasion and migration of tumor cells (18,22), and we obtained a similar conclusion in bladder cancer cells according to the data in the present study. After exposure to TGF-β1 (10 ng/ml), fascin1 expression was increased in the T24 and BIU87 bladder cancer cells. Meanwhile, the migration and invasiveness of these cells were enhanced. However, we did not observe the same impact of TGF-β1 when the expression of fascin1 was decreased by specific siRNA. We could speculate that fascin1 is an important mediator of the response of bladder cancer cells to TGF-β1.

However, in our previous study, we observed that fascin1 did not influence cell proliferation of 5637 or BIU87 cells (15). In the present study, we observed that the cell proliferation of T24 was promoted after TGF-β1, and there was no significant increase on cellular growth in the BIU87 cells. These results suggested that the effect of TGF-β1 on cell proliferation was cell line-dependent. Lee *et al* (27) recently reported that six cell lines showed growth inhibition after TGF-β1 treatment and proved that TGF-β1 did not stimulate cellular proliferation but was a growth inhibitory factor in bladder cancer cells. Thus, the effects of TGF-β1 on promoting cell proliferation still need further research for confirmation.

Moreover, the signaling pathways through which TGF-β1 upregulates fascin1 expression and increases migration and invasion potential in cancer cells have not been fully defined. One study speculated that fascin is regulated by TGF-β through the canonical TβRI-Smad pathway (22). In contrast to this conclusion, another study showed that TGF-β1 increased fascin1 expression via the ERK and JNK signaling pathways in gastric cancer cells (18). In summary, to fully understand the effect of TGF-β1 on the proliferation, motility and invasive potential of bladder carcinoma cells, further studies are needed.

In conclusion, the present study demonstrated for the first time that TGF-β1 can promote the invasion and migration abilities of T24 and BIU87 bladder carcinoma cells and an increase in fascin1 expression may be a key factor for this impact of TGF-β1.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (81372722), and the Natural Science Foundation of Liaoning Province (2013225021).

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