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Original Article

Targeting TGF-β1 suppresses survival of and invasion by anaplastic thyroid carcinoma cells

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Abstract: Background and aims: Overexpression of transforming growth factor (TGF)-β1 has been implicated in promoting cell survival, migration and invasion in many cancers, including anaplastic thyroid cancer (ATC). In the present study, we studied the effect of suppressing TGF-β1 by RNA silencing on the survival, invasion and metastasis of ATC cells. Methods: Small interfering RNA (siRNA) constructs targeting TGF-β1 were validated and used to develop clonal derivatives of the ATC cell line, 8505C. The cells were used in several *in vitro* assays, including migration, invasion, survival rate, colony formation and apoptosis. A wound healing assay was used to determine the migration of cells in culture and a Boyden chamber transwell assay was used for invasion. Further, clones were used in an *in vivo* mouse model to study the kinetics of tumor growth and metastatic growth in lungs. Results: Targeting TGF-β1 expression in 8505C cells caused a 70% decrease in migration and a 78% decrease in invasion, as well as a 68% decrease in proliferation and a 19% increase in apoptosis *in vitro*. The growth of primary tumors *in vivo* was also inhibited when compared with parental 8505C cells; however, the number of mice bearing lung metastases was not significantly decreased. Conclusions: Targeting TGF-β1 may be effective in inhibiting primary tumor formation, but not metastasis, by ATC cells. TGF-β1 inhibition in combination with other tumor-targeted therapies may be more effective in inhibiting ATC.

Keywords: Anaplastic thyroid cancer, metastasis, TGF-β1

Introduction

Anaplastic thyroid cancer (ATC) is one of the most lethal human malignancies. It accounts for less than 2% of all thyroid cancers, but results in nearly one-third of thyroid cancer-related deaths [1]. The median survival rate of patients with ATC is less than six months, with 90% of patients presenting with unresectable ATC at the time of diagnosis and most patients developing recurrent ATC even after complete tumor resection [2, 3]. Currently, there is no standard or effective therapy for ATC, and patient survival rates have not improved in over six decades [2, 4]. In spite of this, targeted molecular therapy is a promising approach in the treatment of ATC patients.

TGF-β1 is a ubiquitous cytokine that can induce cancer cells to proliferate and form an invasive phenotype as characterized by epithelial to mesenchymal transition (EMT) [5]. Cells undergoing EMT demonstrate cellular morphological changes and increased expression of mesenchymal markers such as vimentin and decreased expression of E-cadherin [6, 7], which aid in invasion and metastasis. TGF-β1 additionally promotes metastasis by regulating the composition of the extracellular matrix, as well as proteolysis and inflammatory responses [8, 9]. Proteomic studies have shown that different proteins are involved in different stages of metastasis [10, 11]. All this evidence points to the interatomic nature of the TGF-β1 induced metastatic process.

TGF-β1 is present as three different isoforms: TGF-β1, TGF-β2, and TGF-β3. In normal thyroid-tissue, expression of the three TGF-β isoforms is barely detectable. However, in carcinomas, almost all epithelial cells displayed immunoreactivity for the three TGF-β isoforms [12]. TGF-β1 is overexpressed in human papillary thyroid carcinomas (PTCs) compared to the normal thy-

roid tissue after analysis by oligonucleotide microarray of microscopically dissected intratumoral samples taken from central and invasive regions [13]. These data, together with reduced levels of mRNAs encoding proteins involved in cell-cell adhesion and communication, and an overexpression of vimentin, strongly support the hypothesis that TGF-β1, responsible for EMT induction, increases tumor invasiveness in PTCs [13]. In addition, strong activation of TGF-β1 signaling was found in ATCs, suggesting the inhibition of TGF-β1 signaling represents a new and promising approach for the treatment of ATCs [14].

The present study determined the effects of TGF-β1 inhibition on proliferation, apoptosis, migration, and invasion in the human ATC cell line, 8505C, by small RNA interference (siRNA). We found that siRNA-mediated silencing of TGF-β1 decreased migration, invasion, and proliferation, and increased apoptosis *in vitro*. Furthermore, short hairpin RNA (shRNA)-mediated silencing of TGF-β1 inhibited primary tumor growth, but did not inhibit lung metastasis *in vivo*.

Materials and methods

Ethics

All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital Qingdao University.

Cell lines

The ATC cell line, 8505C, was purchased from American Type Culture Collection (ATCC, Shanghai, China). High endogenous levels of TGF-β1 are apparent in 8505C. The cells were maintained in DMEM with 10% fetal calf serum (FCS), at 37°C in 5% carbon dioxide and 95% air.

siRNA transfection

Cells in 6-well or 96-well plates were grown to 50% confluence and transfected with double-stranded siRNA against a TGF-β1 target sequence (Sense 5'TGF-β1 GATGCCTACACAGGTGTGTAT 3', Antisense 3'GCAGACTAGACTACGGTTCAA 5') or with a siRNA nonspecific control in serum-free medium without antibiotic supplements using HiperfectTransfectin Reagent (Qiagen, Inc., Hilden, Germany). Cells were incubated under these conditions for 72 h and silencing was then confirmed by reporter assays, as well as western blotting.

The construction of TGF-β1 shRNA plasmid and transfection

Constructs of pcDNA3.1 containing two short hairpin RNA (shRNA) sequences were generated by the RNAi Consortium: GATGCCTACACAGGTGTGTAT and GCAGACTAGACTACGGTTCAA. Two strands of oligonucleotides underwent annealing, double enzymecutting by the restriction endonucleases Hind and BamH1, ligation, and transformation, as well as the polymerase chain reaction (PCR) process to identify positive clones. The pcDNA3.1-non-shRNA vector was used as a negative control, which did not interfere with the studied intrinsic gene. The constructed pcDNA3.1/TGF-β1 shRNA expression plasmid was sequenced by Shanghai Invitrogen Biotechnology Company.

Suspensions of 8505C cells were seeded into 6-well plates at 3×10^5 cells per well. After the cells adhered to the bottom, they were transfected with Lipofectamine 2000 following the instructions of the test kit. To acquire stably transfected colonies, cells were transiently transfected with pcDNA3.1/TGF-β1 shRNA or pcDNA3.1 for 48 h, then selected with G418 (400 μg/mL, Sigma-Aldrich) for 10-12 days. TGF-β1 knockdown was confirmed by western blot. 8505C cells were transfected with pcDNA3.1 as the negative control. Experiments were performed at least three times in triplicate to ensure the reproducibility of results.

Western blot

Equal amounts of protein (40 μg) from cell lysates were mixed with 2× SDS loading buffer and separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with the primary antibodies, anti-TGF-β1 and anti-β-actin (Santa Cruz, Shanghai, China). An enhanced chemiluminescence system was used for the detection of immunoreactive proteins, with horseradish peroxidase-conjugated immunoglobulin G (IgG) used as secondary antibodies.

Reverse transcription polymerase chain reaction

Total RNA was isolated from transfected cells using the Qiagen RNeasy kit (Qiagen, Inc., Shanghai, China) according to the manufacturer's protocol. A One Stepreverse transcriptase polymerase chain reaction (RT-PCR) kit (Qiagen) was used for detecting TGF-β1 mRNA expression. First-strand cDNA was prepared using Omniscript and Sensiscript reverse transcriptases at 50°C for 30 min. Polymerase chain reaction (PCR) amplification was then carried out under the following conditions: 95°C for 15 min, followed by 35 cycles at 95°C for 1 min, and 47°C for the amplification of TGF-β1 and β-actin (as an internal control) for 1 min. The final extension was completed at 72°C for 10 min. The primers used for TGF-β1 were 5'-ACCATGCAGGCCATCAAGTGTGTGG-3' and 5'-TTACAACAGCAGGCATTTTCTCTTC-3'; for β-actin they were 5'-GGAGAAACGTACGGTAAGGATATAACC-3' and 5'-GGCAATCGGCTTGTCTTTGCCC-3'.

Transwell migration and matrigel invasion assays

Transwell migration and matrigel invasion assays were performed using a transwell membrane (8-μm pore size, 6.5-mm diameter; Corning Incorporated, Corning, NY, USA) in a 24-well plate according to the manufacturer's instructions. A matrigel matrix (1:5 dilution, 50 μL/well, BD Biosciences, San Jose, CA, USA) was coated onto the transwell membrane (8-μm pore size, 6.5-mm diameter; Corning Incorporated) and used for the cell invasion assay. The lower chambers of the transwell plates were filled with 600 μL IMDM medium containing 10% FBS. After detachment of pcDNA3.1/TGF-β1 shRNA/8505C or pcDNA3.1/8505C cells from tissue culture plates, cells were resuspended in IMDM medium containing 1% FBS and then loaded onto the upper side of the chamber (200 μL/well). For migration and invasion assays, 8505C cells were used (1×10^4 and 2×10^4 cells/well, respectively). Cells were placed in incubators at 37°C for 24 h. The filter inserts were then removed from the wells. Cells on the upper surface of filters were removed using cotton swabs. Those on the lower surface were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), stained with 0.1% crystal violet and counted. Cells that migrated or invaded were counted in five random fields of each filter under a microscope at 200× magnification.

Wound healing assays

Wound healing assays were performed to confirm invasion and migration data. To start, pcDNA3.1/TGF-β1 shRNA/8505C cells or pcDNA3.1/8505C cells were grown in McCoy's 5A with 10% FBS in six-well cell culture plates until they reached 80-100% confluence. A small linear scratch was introduced in the middle of the confluent cells using a 10 μL pipette tip and a photomicrograph was taken for the zero time point. All scratches were carefully performed to ensure a scratch of equal width was made. All photomicrographs after time 0 were taken at the same point of the scratch. Cells were then returned to the incubator for continued growth. Cells were only removed from the incubator at the designated time points (24 h), when photomicrographs were taken to monitor the progression of cell migration.

Determination of the proportion of apoptotic cells

Control and transfected cells were harvested after 72 h of transfection with siRNA for TGF-β1, or control siRNA. To evaluate proportions of apoptotic cells, an Annexin V staining kit (Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's instructions. The stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BDBiosciences). Fluorescence emission was measured at 530 nm and 575 nm.

Cell viability assay

Cell viability was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay according to the manufacturer's protocol (Cell Proliferation Kit, Roche). After 24 h of transfection with TGF-β1 or control siRNA (100 nM), 8505C cells were trypsinized and placed in a 96-well plate at 5,000 cells per well for an additional 24-96 h. Absorbances at 570 nm were measured using a microtiter plate reader (Multiskan MK3; Thermo Electron Corporation, USA). Data were obtained from three separate experiments. The percentage decrease of viability in the TGF-β1

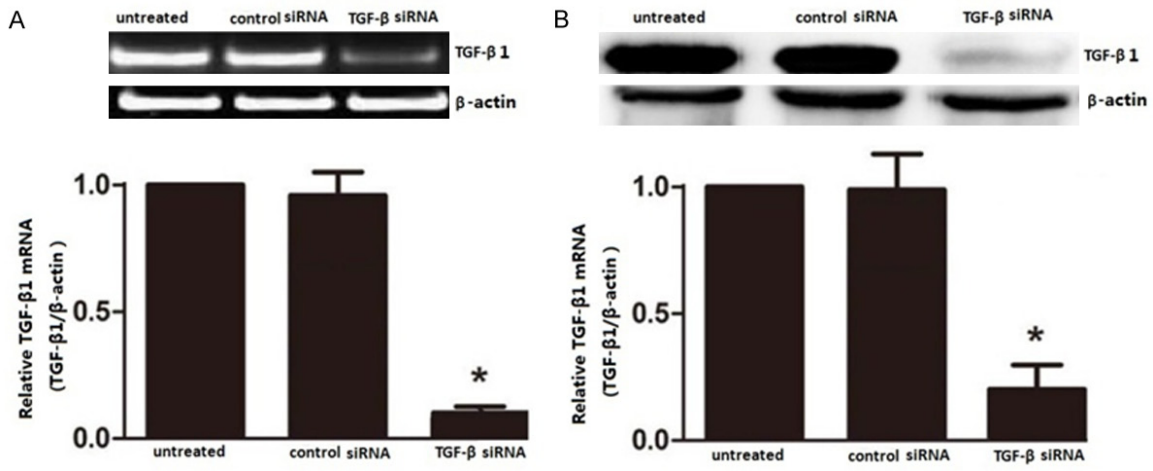

Figure 1. Effect of siRNA on TGF-β1 expression in 8505C cells. The vectors, pcDNA3.1/TGF-β1 siRNA orpcDNA3.1, were transfected into 8505C cells for 48 h. TGF-β1 mRNA and protein expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR) (A) and western blot assays, respectively (B). Results from western blots and RT-PCR are representative of three independent preparations. $^{*}P<0.05$ vs. control.

siRNA groups was determined by comparing absorbance values with those of the siRNA controls. Each experiment was repeated at least three times in quadruplicate wells.

Colony formation assays

Colony formation by 8505C cells was examined by anchorage-independent soft agar assay. Briefly, 1.5 mL FBS-supplemented medium containing 0.5% agarose was poured into 35-mm cell culture dishes and allowed to solidify (base agar). Next, pcDNA3.1/TGF-β1 shRNA/8505C or pcDNA3.1/8505C cells (5000 cells/well) were mixed with 1.5 mL FBS-supplemented medium containing 0.35% agarose and added to the top of the base agar. The cells were then cultured for 12 days at 37°C under 5% carbon dioxide. The dishes were stained with 0.005% crystal violet, and the colonies were examined with a microscope and digital camera.

Xenograft experiments using nude mice

Male BALB/c nude mice (4-5 weeks old) were purchased from the Medical Experimental Animal Center of Shandong Province (Jinan, China). All protocols were approved by the Animal Care and Use Ethics Committee. To set up a xenograft or metastatic model of anaplastic thyroid carcinoma in nude mice, pcDNA3.1/TGF-β1 shRNA/8505C or pcDNA3.1/8505C cells (1×10^7) were subcutaneously injected in the dorsal flank or intravenously injected through the tail vein, respectively, of mice. For tumor xenograft assays, 42 days after inoculation, the developing tumors were measured in two dimensions with a digital caliper and tumor volume was calculated as follows: volume $=X^2\times Y/2$, where X and Y are the short and long tumor dimensions, respectively. For lung metastasis assays, mice were sacrificed, the lungs were fixed, paraffinembedded, cut, and stained with hematoxylin and eosin staining after intravenous injection through the tail vein of mice for 3 weeks.

Statistical analysis

The significance of results was determined by Student's *t* test (two-tailed). Values are expressed as mean ± standard deviation (SD) from at least three separate experiments and differences were considered significant at $P<0.05$.

Results

Effect of siRNA on TGF-β1 expression in 8505C cells

As shown in **Figure 1**, high levels of endogenous TGF-β1 mRNA and protein expression were found in 8505C cells and these were therefore chosen for siRNA experiments. As determined by RT-PCR analysis, cells transfected with TGF-β1 siRNA for 48 h displayed a significant reduction in the expression level of TGF-β1 Mrna (**Figure 1A**; $P<0.05$). Nonsilencing siRNA did

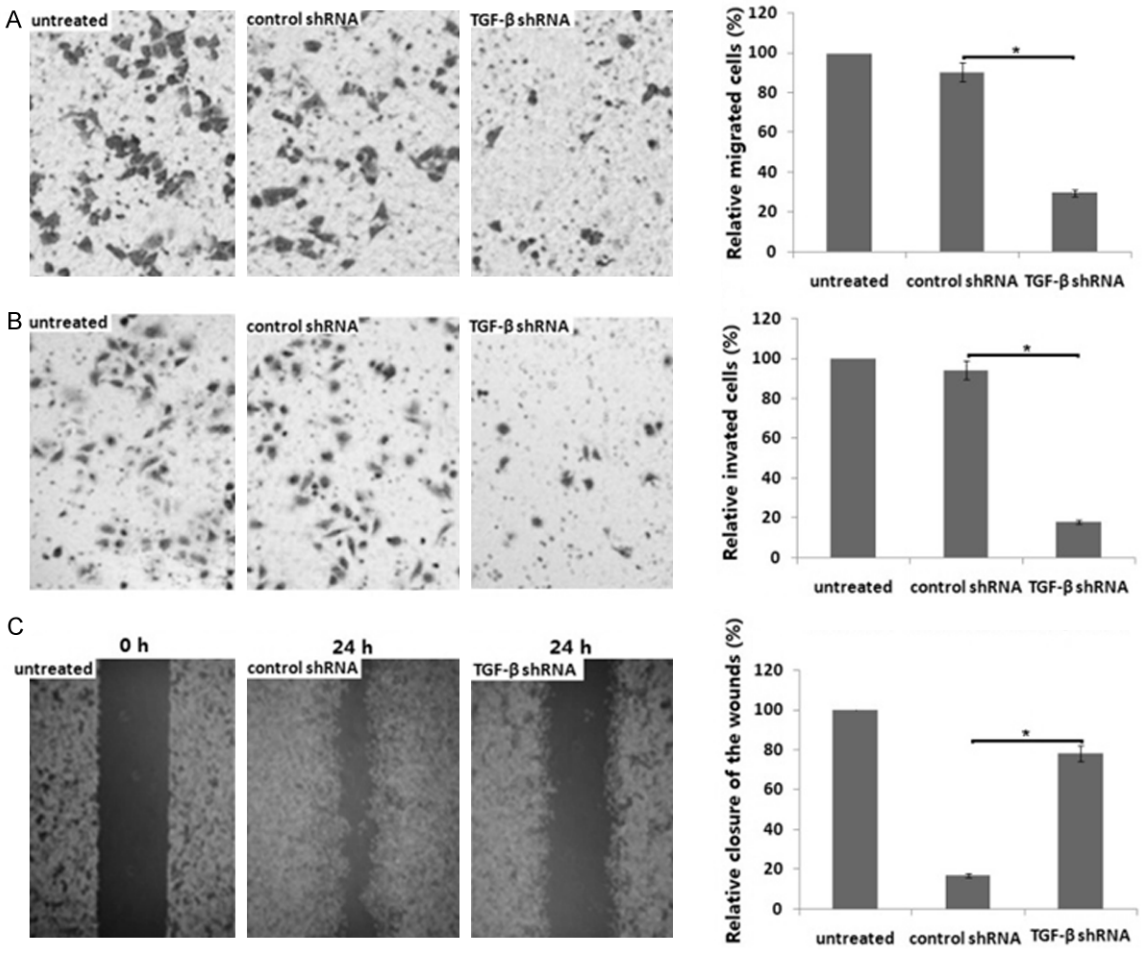

Figure 2. Knockdown of TGF-β1 inhibits migration and invasion by 8505C cells*in vitro*. (A) Cell migration, (B) cell invasion and (C) cell mobility assays were performed in 8505C cells, with or without transfection with TGF-β1 shRNA. Magnification, ×200. Data is presented as the mean ± SD; *P* values were calculated using Student's *t*-test. $^{*}P<0.05$ vs. control.

not exhibit any effect on mRNA levels of TGF-β1 (**Figure 1A**). Further, the significant suppression of TGF-β1 by siRNA in 8505C cells was confirmed by western blot analysis (**Figure 1B**; $P<0.05$). These data confirmed the suppressive effect of siRNA on TGF-β1 at the mRNA and protein levels and established the efficiency of siRNA transfection.

Effect of TGF-β1 gene knockdown on invasive capability of 8505C cells in vitro

Stably transfected (pcDNA3.1/TGF-β1 shRNA or pcDNA3.1) 8505C cells were seeded in Matrigel-coated invasion chambers. After 24 h, cells that migrated through the Matrigel barrier were stained and counted. TGF-β1 shRNA exhibited a significant reduction in cell migration and invasion (**Figure 2A**, B; $P<0.05$ for both). To investigate this, a wound healing assay was performed with stable TGF-β1 shRNA transfected 8505C cells. Results showed that the closure of wounds was significantly faster in pcDNA3.1 transfected cells ($P<0.05$), whereas wounds remained open in TGF-β1 shRNA transfected cells (**Figure 2C**; $P<0.05$). Thus, the results of these experiments suggest that TGF-β1 is a critical mediator of 8505C cell migration and invasion.

Effect of TGF-β1 gene knockdown on cell proliferation, colony formation, and apoptosis

To study the effect of TGF-β1 silencing on cell proliferation in 8505C cells, we used an MTT assay. We found that the proliferation of siRNA (TGF-β1) transfected cells was significantly attenuated in a time-dependent manner, as

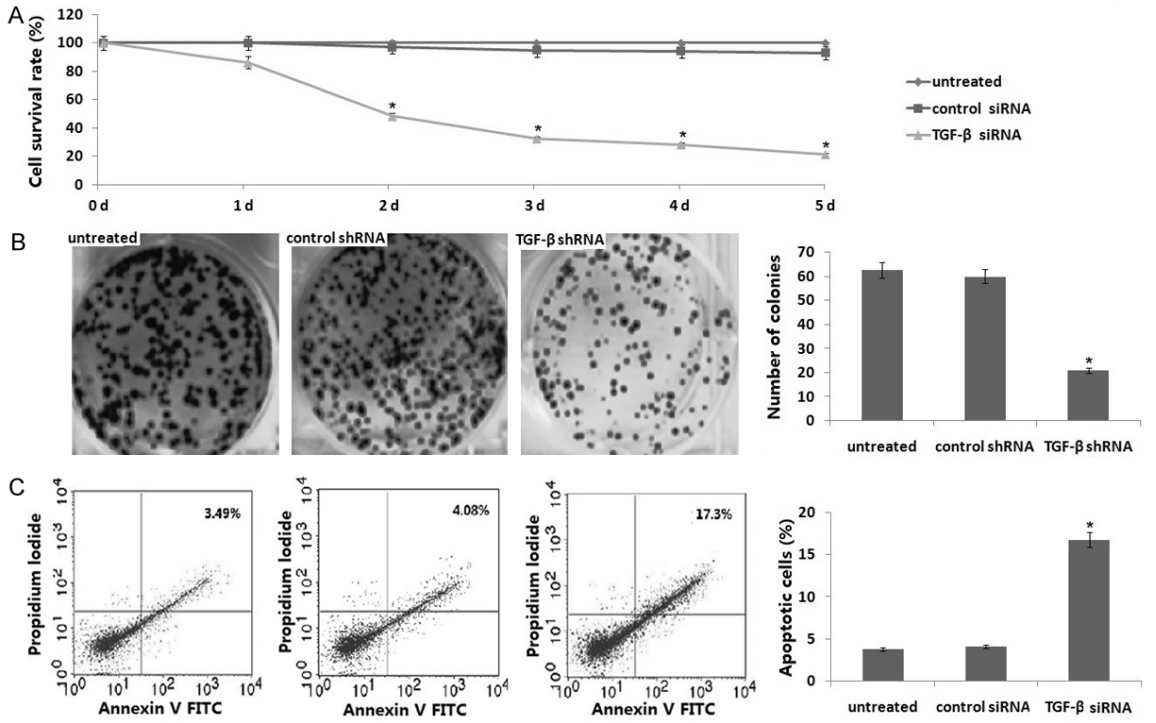

Figure 3. Knockdown of TGF-β1 inhibits proliferation and induces apoptosis in 8505C cells *in vitro*. A. Cell viability detected by MTT assay. B. Cell anchorage-dependent proliferation detected by colony formation assay. C. Cell apoptosis was detected by flow cytometry analysis. *P* values were calculated with Student's *t*-test. **P*<0.05 vs. control.

compared with control siRNA (-) transfected cells (**Figure 3A**; *P*<0.05). Additionally, colony forming assays showed that after 12 days' incubation, shRNA (-) had no effect on the growth of 8505C cells, whereas we observed significantly fewer aggregates in shRNA (TGF-β1) transfected cells (**Figure 3B**; *P*<0.05). The colony forming assay revealed that the efficiency of shRNA (TGF-β1) transfected cells was more than 50% slower than that of siRNA (-) cells. Therefore, we show that knockdown of TGF-β1 by siRNA can attenuate the growth of 8505C cells.

Apoptosis was quantified using an Annexin V staining kit and flow cytometry to evaluate the correlation between TGF-β1 siRNA and apoptosis in 8505C cells (**Figure 3C**). The apoptotic index was 17.3±3.12% in siRNA TGF-β1 cells, which is significantly higher than siRNA (-) cells (4.08±0.82%; *P*<0.05). The data indicates that TGF-β1 siRNA induces apoptosis in 8505C cells.

Knockdown of TGF-β1 inhibits xenograft tumor growth but not lung metastasis in vivo

Given that suppression of TGF-β1 inhibited the proliferation and invasion of 8505C cells *in vitro*, we tested whether knockdown of TGF-β1 could also affect tumorigenesis and metastasis *in vivo*. As shown in **Figure 4A**, tumors grew at a significantly slower rate and displayed smaller volumes in the pcDNA3.1/TGF-β1 shRNA/8505C group compared with the pcDNA3.1 group (*P*<0.05). Next, we determined whether TGF-β1 suppression could decrease cancer metastasis *in vivo*. We injected pcDNA3.1/TGF-β1 shRNA/8505C or pcDNA3.1/8505C cells (1×10^7) through the tail veins of mice. After 3 weeks, we detected no significant decrease in lung metastasis of mice with TGF-β1 shRNA (**Figure 4B**; *t*-test, *P*>0.05). This result provides strong *in vivo* evidence that knockdown of TGF-β1 did not inhibit metastasis by 8505C cells.

Discussion

TGF-β1 has emerged as a promising new target for the treatment of cancer metastasis. In breast cancer, blockade of TGF-β1 signaling may reduce tumor cell viability and migratory potential [15]. McEarchern et al. [16] found that exposure to TGF-β1 caused 4T1 cells to undergo morphological changes associated with the metastatic phenotype, and to invade more

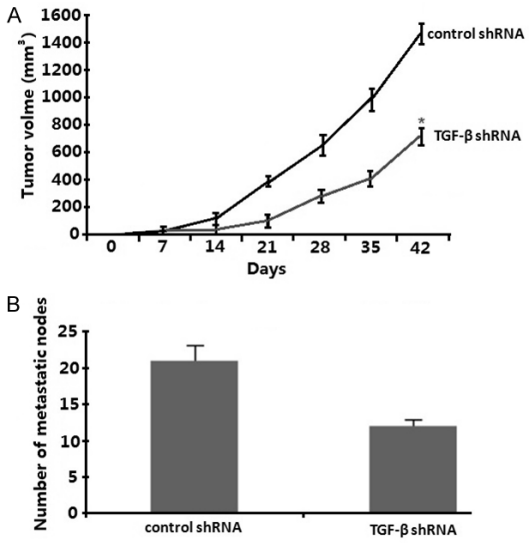

Figure 4. Effect of TGF-β1 gene knockdown on xenograft tumor growth and lung metastasis *in vivo*. A. Representative growth curves of tumor volumes. B. The number of lung metastatic nodes in each group. Tumors were counted with the naked eye. The data represent the mean ± SD (n=6). *P* values were calculated using Student's *t*-test. *$P<0.05$ vs. control.

readily through collagen-coated matrices. Furthermore, expression of a dominant negative truncated type II receptor diminished TGF-β1 signaling and significantly restricted the ability of 4T1 cells to establish distant metastases, suggesting TGF-β1 signaling is required for tumor invasion and metastases formation.

The present study indicates that suppression of TGF-β1 altered the metastatic characteristics of highly invasive 8505C cells. Suppression of TGF-β1 with TGF-β1 siRNA significantly decreased cell migration and invasion, and reduced the closure of wounds *in vitro*. However, lung metastatic nodes were not significantly decreased compared to controls, suggesting that TGF-β1 silencing did not significantly inhibit organ metastasis.

TGF-β is a physiological regulator of thyroid cell differentiation and proliferation. TGF-β is normally expressed and secreted by thyrocytes, acting as a potent inhibitor of thyroid cell growth [17]. TGF-β1 appears to show a dual effect in tumorigenesis. It can act as a tumor suppressor at the pretumor stage, and as a tumor promoter during the late stages of tumorigenesis. It is likely that during tumorigenesis, as a result of genetic and/or epigenetic changes, the balance between these opposing functions of TGF-β1 changes resulting in a switch to tumor promotion [18].

In granulosa cell tumors of the ovary, TGF-β1 increased viability by inhibiting apoptosis, and decreased viability by inducing apoptosis when TGF-β1 was inhibited [19]. In breast cancer cells, TGF-β inhibited apoptosis by suppressing the expression of the BH3-only protein, Bim, which promotes programmed death signaling via the release of cytochrome c from mitochondria [20].

In the present study, we found that the clonal derivatives of 8505C cells constitutively expressing TGF-β1 shRNA showed that cell proliferation and colony formation were decreased significantly, and the cell apoptotic rate was increased significantly. This decrease in proliferation and colony formation in response to TGF-β1 silencing seems to be due to an increase in cell apoptosis. *In vivo*, we also found that tumors grew at a slower rate and had smaller volumes in the TGF-β1 shRNA group than the non-targeting shRNA group, suggesting that TGF-β1 silencing inhibited *in vivo* tumor growth.

In conclusion, the present study suggests that therapies targeting TGF-β1 in tumor cells may be effective in inhibiting growth, and in inducing apoptosis, but not metastasis, in ATC cells. This suggests that TGF-β1 inhibition in combination with other metastasis-targeted therapies may be more effective in treating anaplastic thyroid carcinoma.

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Disclosure of conflict of interest

None.

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