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Effects of TGF-β signaling blockade on human A549 lung adenocarcinoma cell lines

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Abstract. Transforming growth factor β (TGF-β) is over-expressed in a wide variety of cancer types including lung adenocarcinoma (LAC), and the TGF-β signaling pathway plays an important role in tumor development. To determine whether blockade of the TGF-β signaling pathway can inhibit the malignant biological behavior of LAC, RNA interference (RNAi) technology was used to silence the expression of TGF-β receptor, type II (TGFβRII) in the LAC cell line, A549, and its effects on cell proliferation, invasion and metastasis were examined. Three specific small interfering RNAs (siRNAs) designed for targeting human TGFβRII were transfected into A549 cells. The expression of TGFβRII was detected by Western blot analysis. Cell proliferation was measured by MTT and clonogenic assays. Cell apoptosis was assessed by flow cytometry. The invasion and metastasis of A549 cells were investigated using the wound healing and Matrigel invasion assays. The expression of PI3K, phosphorylated Smad2, Smad4, Akt, Erk1/2, P38 and MMPs was detected by Western blot analysis. The TGFβRII siRNA significantly reduced the expression of TGFβRII in A549 cells. The knockdown of TGFβRII in A549 cells resulted in the suppression of cell proliferation, invasion and metastasis and induced cell apoptosis. In addition to the Smad-dependent pathway, independent pathways including the Erk MAPK, PI3K/Akt and p38 MAPK pathways, as well as the expression of MMPs and VEGF, were inhibited. In conclusion, TGF-β signaling is required for LAC progression. Therefore, the blockade of this signaling pathway by the down-regulation of TGFβRII using SiRNA may provide a potential gene therapy for LAC.

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Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1-2). Lung adenocarcinoma (LAC), which accounts for approximately 40% of all lung cancers, is currently one of the most common histological types and its incidence has gradually increased in recent years in many countries (3). Although advances in combined treatment of non-small cell lung cancer (NSCLC) have improved survival and quality of life, the majority of patients succumb to the disease within 2 years and prognosis is relatively poor compared with other common cancers (15% vs. 62-97% 5-year overall survival rate) (1,3-5). Human LAC cells are characterized by marked growth, invasion and metastasis and contribute to the poor patient outcomes.

The transforming growth factor-β (TGF-β) signaling pathway has been shown to play a key role in carcinogenesis and cancer progression (6-8). NSCLC specimens overexpress TGF-β, which has been detected by multivariate analysis to be an independent risk factor for pulmonary metastasis and correlates with decreased survival (9-10). TGF-β binds to TGF-β receptor, type II (TGFβRII), which then phosphorylates and activates the type I receptor in the complex, and activates SMAD-dependent and -independent pathways. The non-SMAD signaling pathways are likely to mediate the positive effects on pro-carcinogenesis (6-8). Several investigators have reported that inactivation of the TGF-β signaling pathway reduces the ability of human cancer cells to progress (11-14). Therefore, the TGF-β signaling pathway, and in paticular the critical role played by TGFβRII, may be a potential target for gene therapy in LAC.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene-silencing process, which is initiated by double-stranded RNA (e.g., chemically synthetic small interfering RNAs). Consequently, the RNA-induced silencing complex (RISC) degrades targeted mRNA and inhibits protein expression (15). RNAi technologies are currently widely used as knockdown genes in functional genomics due to the effective, stable gene suppression provided by siRNAs (16).

In this study, we used the RNAi strategy to silence TGFβRII expression and significantly inhibited the TGF-β signaling pathway in the LAC cell line, A549. In the experiments described herein, we demonstrate that the capability for proliferation, invasion and metastasis is reduced and apoptosis

is induced in TGFβRII-deficient A549 cells. In addition, the molecular mechanisms of the anti-tumor effects of the TGFβRII knockdown are initially revealed, which could lay the foundation for genetic therapy for LAC.

Materials and methods

Materials. The human LAC cell line, A549, was purchased from the Shanghai Institute of Biochemistry and Cell Biology, China Academy of Science. Cell culture medium, fetal bovine serum (FBS) and TRIzol reagent were from Life Technologies (Eggenstein, Germany). siRNAs (Genepharma, Shanghai, China) and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) were prepared for transfection. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and TGFβ1 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RevertAid™ first strand cDNA synthesis kit was obtained from Fermentas Europe. Matrigel (BD Biosciences, Bedford, MA, USA) and Transwell plates (Costar Corning, Cambridge, MA, USA) were prepared for the Matrigel invasion assay. The Annexin V-FITC apoptosis dectection kit was purchased from KeyGen Biotech Co. (Nanjin, China). Antibodies against TGFβRII, PI3K, p-Akt, p-Erk1/2, p-P38, p-Smad2, Smad4, VEGF, MMP-2, MMP-9 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against P38 and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from standard commercial suppliers unless otherwise specified.

Cell line and cell culture. The human LAC cell line, A549, was cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Throughout the experiment, cells were used in the logarithmic phase of growth.

Design and transfection of synthetic siRNAs. Three specific siRNA oligonucleotides targeting TGFβRII, which were based on the human TGFβRII gene (NM_003242.5), one scrambled non-targeting siRNA (mock) and one fluorescent siRNA were designed and synthesized. The target (5' to 3') and the siRNA sequences, designated as TGFβRII-3866(SiT1), TGFβRII-3047(SiT2) and TGFβRII-475(SiT3) are shown in Table I. Prior to transfection, A549 cells were cultured in the well plates for 24 h. After they had reached an 80% density/well, cells were transfected with 100 nM siRNA using Lipofectamine 2000 reagent, following the manufacturer's protocol. The cells were harvested 24, 48, or 72 h after transfection for analyses. Additionally, A549 cells that had been either untreated or treated only with Lipofectamine 2000 reagent were used as controls.

Western blot analysis. Cells were washed in phosphate buffered saline (PBS), and lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholic acid, 1 mM sodium orthovanadate, 1 mM PMSF and protease inhibitor cocktail). Protein concentrations were measured by the Bradford method using BCA protein assay reagent (17). Protein samples (20 μg/well) were separated by 10% SDS-PAGE, electrophoretically transferred to PVDF membranes, and the membranes were blocked for 2 h at room temperature with 5% non-fat dried milk in TBS-T (10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween-20). The membranes were then incubated with primary antibodies (1:2000) overnight at 4˚C, followed by secondary antibodies against rabbit or mouse IgG conjugated to horseradish peroxidase (1:3000) for 2 h at room temperature. Finally, they were developed with ECL detection reagents. Protein expression was quantified by densitometry and normalized to β-actin expression.

Purification of total cellular mRNA and reverse transcription-PCR. Total RNA was extracted from cells using the TRIzol reagent according to the manufacturer's protocol, cDNA was generated by reverse transcription of total cellular RNA with the First Strand cDNA Synthesis Kit. PCR amplification was performed in a 25 μl reaction mixture containing 12.5 μl RT-PCR Master Mix, 10 μl double distilled water, 1.5 μl cDNA, 0.5 μl forward primer and 0.5 μl reverse primer for 30 cycles. Primers used for amplification of cDNA were as follows: TGFβRII, forward, 5'-GAGGAGCGGAAGACGG AGTT-3' and reverse, 5'-TGTCATTTCCCAGAGCACCAG-3'; GAPDH,forward,5'-CCACCCATGGCAAATTCCATGGCA-3' and reverse, 5'-TCTAGACGGCAGGTCAGGTCCACC-3. The following conditions were used for PCR: 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 40 sec, 30 cycles and 72˚C for 5 min for final extension. The PCR products were separated on a 1.5% agarose gel, visualized under UV light and photographed.

Cell proliferation assay. Cell proliferation was detected by MTT assay. A549 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. After 24 h, the cells were transfected with siRNA and incubated with 2 ng/ml TGF-β1 for 0-72 h. Cell proliferation was determined by adding MTT (5 mg/ml) and further incubating the cells for 4 h, then the precipitate was solubilized by the addition of 150 μl/well DMSO (Sigma) and shaken for 10 min. Absorbance at a wavelength of 490 nm in each well was measured with a microplate reader (Bio-Tek ELX800, USA). All experiments were performed in triplicate and the experiment was repeated on at least three separate occasions.

Clonogenic assay. Cells transfected with siRNA after 48 h were seeded in 6-well plates at densities of 300 cells/well and incubated for 14 days at 37˚C in a humidified atmosphere of 5% CO_2. The colonies were fixed in 4% paraformaldehyde at room temperature for 30 min, stained with 0.1% crystal violet for 10 min and, finally, positive colony formation (>50 cells/colony) was counted and colony formation rate was calculated. These experiments were repeated three times.

Wound healing assay. A549 cells were transfected with siRNAs in 6-well plate. After 48 h, the cells were at 90% confluency/well, and scratched with a 200 μl pipette tip. Plates were washed twice with PBS to remove detached cells and incubated with the complete growth medium without FBS. Cells migrated into the wounded area, and photographs were taken immediately (0 h) and at 24 h (magnification, x40). Results were expressed as a migration index: the distance migrated by siRNA-treated cells (mock or targeted) relative

Table I. Sequences of human TGFβRII siRNA and negative control.

siRNA	Target sequences (5' to 3')	siRNA duplex sequences (5' to 3')
TGFβRII-3866 (SiT1)	(3866)GAAACTTGTTAATCAACAA	GAAACUUGUUAAUCAACAATT
	-	UUGUUGAUUAACAAGUUUCTT
TGFβRII-3047 (SiT2)	(3047)GGAGCAAATTCTCACTCTA	GGAGCAAAUUCUCACUCUATT
	-	UAGAGUGAGAAUUUGCUCCTT
TGFβRII-475 (SiT3)	(475)GGTTAATAACGACATGATA	GGUUAAUAACGACAUGAUATT
	-	UAUCAUGUCGUUAUUAACCTT
Negative control	-	UUCUCCGAACGUGUCACGUTT
	-	ACGUGACACGUUCGGAGAATT

to the distance migrated by untreated cells. Experiments were performed in triplicate and repeated at least three times.

Matrigel invasion assay. Matrigel invasion assay was performed using Transwell chambers. Briefly, the 8-μm pore size filters were coated with 100 μl of 1 mg/ml Matrigel 500 μl RPMI-1640 medium containing 10% FBS was added to the lower chambers. After transfection with siRNA for 48 h, cells were harvested and homogeneous single cell suspensions ($2x10^5$ cells/well) were added to the upper chambers. The invasion lasted for 24 h at 37˚C in a CO_2 incubator. Subsequently, noninvasive cells on the upper surface of the filters were carefully scraped off with a cotton swab, and cells migrated through the filters were fixed and stained with 0.1% crystal violet for 10 min at room temperature, examined and photographed by microscopy (magnification, x200). Quantification of migrated cells was performed.

Flow cytometric analysis of apoptosis. Following transfection for 48 h, cells were exposed to TGFβ1 (2 ng/ml) for 24 h. Cells in 6-well plates were harvested in 500 μl of binding buffer, stained with 5 μl Annexin V-FITC and 5 μl prodium iodide for 10 min, and subjected to flow cytometric analysis by a CycleTEST™ PLUS (Becton-Dickinson, San Jose, CA, USA) within 1 h. The results were quantified using CellQuest and ModFit analysis software.

Statistical analysis. All data were shown as the means ± standard deviation. Statistical significance was determined by analysis of variance using the SPSS 12.0 software package. The level for statistical differences was set at a value of $P<0.05$.

Results

Expression of TGFβRII protein and mRNA in NSCLC cell line A549. To determine whether TGFβRII was overexpressed in the NSCLC cell line, A549, its TGFβRII expression was examined using the Western blot, RT-PCR and immunochemical analyses, and HFL-1 (human fetal lung fibroblasts) and HepG2 (human hepatoma cells), which express TGFβRII, were used as the positive controls. The results of the Western blot analysis revealed that TGFβRII protein was significantly over-expressed in A549 cells in comparison with HFL-1 ($P<0.01$) and HepG2 cells ($P<0.05$) (Fig. 1A), results that were consistent with RT-PCR (Fig. 1B; $P<0.05$).

Transfection efficiency examination. To ensure the transfection efficiency of TGFβRII siRNA, 100 nM fluorescent siRNA mixed with Lipofectamine 2000 were transfected into A549 cells cultured in a 6-well plate. The transfection efficiency was examined by flow cytometry 6 h post-transfection and by fluorescence microscopy 6, 12, 24, and 48 h post-transfection. Flow cytometry revealed that approximately 86% of the cells were transduced (Fig. 2A). Fluorescence could be observed by fluorescence microscopy 6, 12, 24, and 48 h after transfection, and became gradually more intense (Fig. 2B). The results revealed that transfection was successful. Considering the toxicity of Lipofectamine 2000 and the transfection efficiency, 5 μl/well in 6-well plates was used in the following experiments.

Knockdown of TGFβRII by siRNA. Three TGFβRII -siRNA targeting different encoding regions of human TGFβRII mRNA, named siT1, siT2 and siT3 and scrambled siRNA (mock) at 100 nM were transfected into A549 cells for 48 h to identify the siRNA sequence that most markedly suppressed TGFβRII. The siRNA sequences for these siRNA duplexes are shown in Table I. We examined the protein expression of TGFβRII by Western blot analysis. As shown in Fig. 3, siT2 and siT3 efficiently inhibited the expression of TGFβRII (both $P<0.05$) compared with the scrambled siRNA (mock), and siT2 resulted in a greater inhibition than siT3, whereas there was little difference between the control and mock cells. Overall, the siT2 was identified as the most efficient siRNA to knock down TGFβRII expression and the subsequent experiments focused on the siT2.

Inhibition of A549 cell proliferation by TGFβRII siRNA. To investigate whether TGFβRII down-regulation affected the proliferation of A549 cells, cells were incubated with 2 ng/ml TGF-β1 for 0-72 h following transfection with TGFβRII siRNA and growth rates were determined by the MTT assay. As shown in Fig. 4, SiRNA-TGFβRII A549 cell growth was markedly inhibited at 24 h ($P<0.05$), 48 h ($P<0.01$) and 72 h ($P<0.01$), when compared to the control and mock cells. However, no difference was observed between control and mock cells. Thus, the results indicate that the knockdown of TGFβRII may have a negative effect on the proliferation of the LAC cell line, A549.

Inhibition of colony formation by TGFβRII siRNA transfection. To further examine the effects of TGFβRII knockdown on the growth properties of A549 cells, positive colony forma-

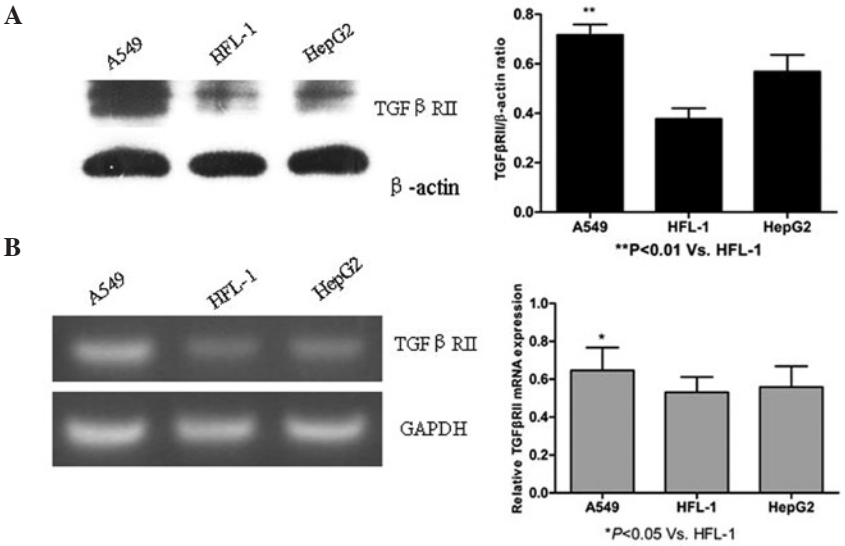

Figure 1. Expression of TGFβRII protein and mRNA in various cell lines. (A) TGFβRII protein levels. The cellular proteins were extracted with lysis buffer and TGFβRII protein levels were detected by Western blot analysis. The β-actin protein levels were examined as the control by Western blot analysis. (B) TGFβRII mRNA levels. The total RNAs were isolated from the cell lines, A549, HFL-1 and HepG2. The TGFβRIImRNA was analyzed by semiquantitative RT-PCR as described in Materials and methods. The detection of GAPDH levels was included as the control for RT-PCR analysis. *P<0.05, **P<0.01 vs. HFL-1.

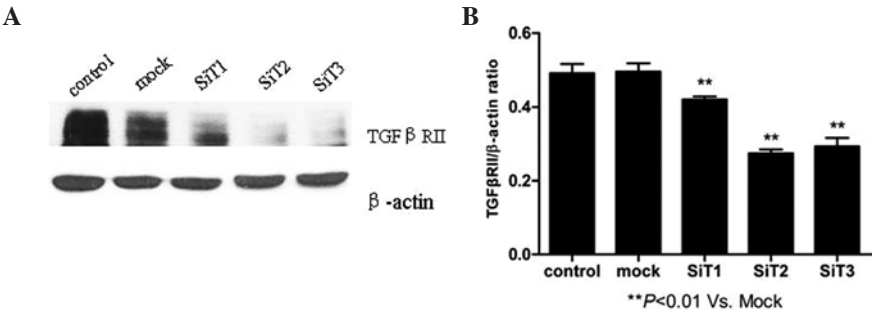

Figure 2. Three siRNAs suppressed the TGFβRII protein expression in the NSCLC cell line, A549. A549 cells were seeded into 6-well plates and transfected with three TGFβRII-specific siRNAs (siT1, siT2 and siT3) and scrambled non-targeting siRNA (mock). The untreated cells were used as the control. The protein expression of each siRNA was measured by Western blot analysis 48 h after transfection as described in Materials and methods. The siT2 was identified as the most efficient siRNA to knockdown TGFβRII expression. **P<0.01 vs. mock.

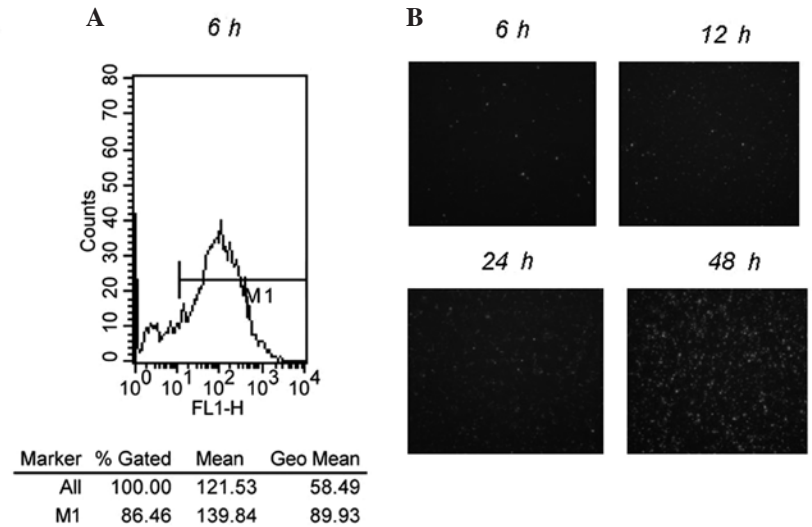

Figure 3. Efficient transfection of TGFβRII siRNA into A549 cells. (A) Detection of transfection efficiency by flow cytometry. A549 cells were transfected with 100 nM fluorescent siRNA for 6 h then examined by flow cytometry. Transfection efficiency was maintained at over 85%. (B) Detection of transfection efficiency by fluorescence microscopy. Efficiently transfected A549 cells were clearly identified at 6, 12, 24, and 48 h post-transfection by fluorescence microscopy.

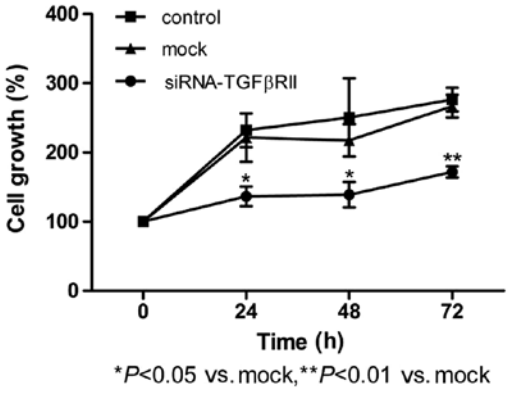

Figure 4. Blockade of the TGF-β signaling pathway with TGFβRII siRNA inhibited A549 cell proliferation. A549 cells were seeded into 96-well plates and transfected with TGFβRII and scrambled siRNAs (mock) at 100 nM. The untreated cells were used as the control. Three groups were incubated with 2 ng/ml TGF-β1 for 0-72h and measured at 0, 24, 48, and 72 h by MTT assay. SiRNA-TGFβRIIA549 cell growth was significantly attenuated at 24 h (P<0.05), 48 h (P<0.01) and 72 h (P<0.01) compared to the mock cells. *P<0.05, **P<0.01 vs. mock.

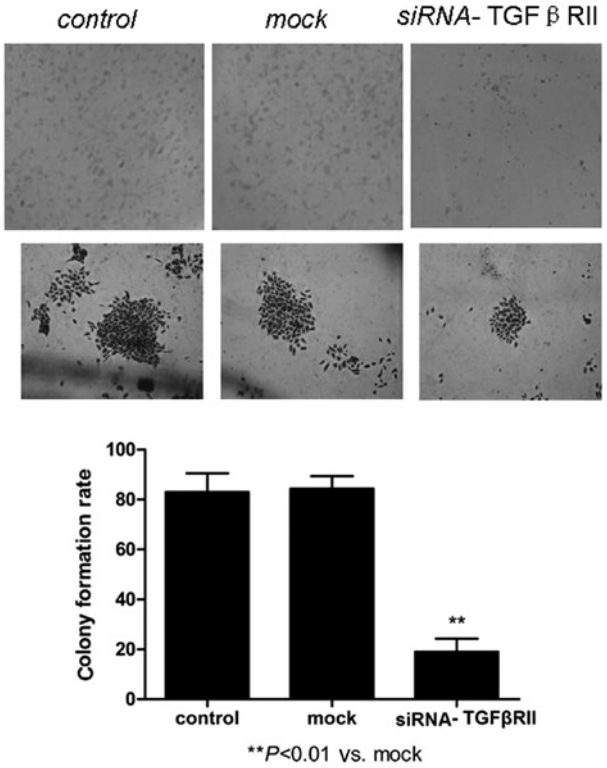

Figure 5. TGFβRII knockdown inhibits the colony formation of A549 cells. Cells transfected with TGFβRIIsiRNA after 48 h were seeded in 6-well plates at densities of 300 cells/well and incubated for 14 days. The cells were stained by 0.1% crystal violet and the positive colony formations (>50 cells/colony) were counted. Representative microscopy images are shown in addition to a bar graph representing the mean colony formation rate. The results demonstrated that knockdown of TGFβRII significantly attenuated the colony formation rate of A549 cells. **P<0.01 vs. mock.

tion (>50 cells/colony) was counted after the control, mock and TGFβRII siRNA cells were seeded at a density of 300 cells/ml in a 6-well plate and incubated for 14 days. As shown in Fig. 5, SiRNA-TGFβRII cells exhibited a significantly lower colony formation rate than the control and mock cells (P<0.01). Therefore, TGFβRII down-regulation resulted in a reduction in the ability of A549 cells to form colonies.

Targeting TGFβRII siRNA weakens the migratory/invasive ability of A549 cells. TGF-β signaling is associated with the biological behavior of tumor cells (7,14), and two critical steps of metastasis are migration and invasion (18). To investigate the effects of the TGF-β signaling blockade using TGFβRII siRNA on the metastasis of LAC cells, we examined the motility and invasiveness of targeted TGFβRII A549 using the wound healing migration and transwell Matrigel invasion assays. In the wound healing assay, TGFβRII siRNA-treated cells exhibited significantly decreased migration and were unable to close the wound when compared with the scrambled siRNA (mock) cells (P<0.01). The control and mock cells almost achieved complete wound closure (P>0.05). The results are represented as the migration index (Fig. 6). Furthermore, compared to the mock cells, transwell assays indicated that the number of invasive cells among those treated with TGFβRII siRNA was significantly reduced. It was also indicated that the invasive capacity was suppressed, but there was no difference between the control and mock cells (Fig. 7). These results indicate that the targeting of TGFβRII siRNA weakens the migratory and invasive abilities of LAC A549 cells *in vivo*.

Promotion of apoptosis in TGFβRII knockdown NSCLC A549 cells. To further evaluate whether the TGFβRII knockdown induced apoptosis in A549 cells, the cells, which were treated and then incubated with TGFβ1 (2 ng/ml) for 24 h, were analyzed by flow cytometry. As shown in Fig. 8, the apoptotic rate in TGFβRII siRNA cells was higher than that in the mock and control cells. The results indicated that the

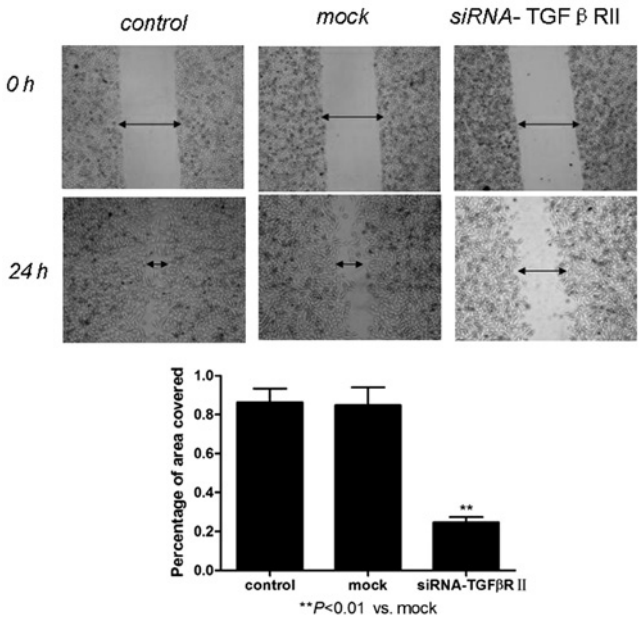

Figure 6. TGFβRII siRNA blocked A549 cell migration. A549 cells were transfected in 6-well plates for 48 h and scratched with a 200 μl pipette tips at approximately 90% confluency. Cells, which were then incubated with the complete growth medium without FBS, migrated into the wounded area and were photographed. Data and representative images are shown (magnification, x40) in addition to a bar graph showing the mean percentage of the wound area covered by migrating A549 cells. A549 cells treated with TGFβRII siRNA showed a marked decreased in cell motility. **P<0.01 vs. mock.

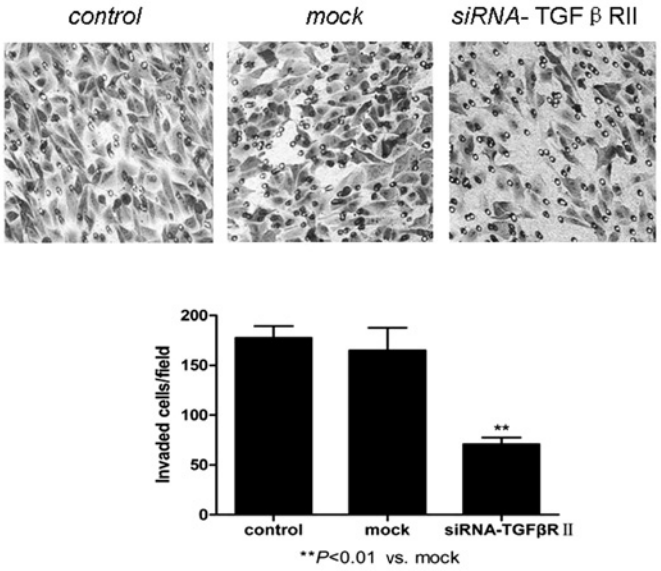

Figure 7. TGFβRII knockdown attenuated invasion of A549 cells. After transfection with siRNA for 48 h, Cells were harvested and 100 μl homogeneous single cell suspensions ($2x10^5$ cells/well) were added to the Matrigel-coated Transwell upper chambers. Cells penetrating the membrane were stained with 0.1% crystal violet and photographed. Representative microscopy images (magnification, x200) are shown. Bar chart represents the mean number of the cells per field. Knockdown of TGFβRII by siRNA in A549 cells inhibited cell invasion. **P<0.01 vs. mock.

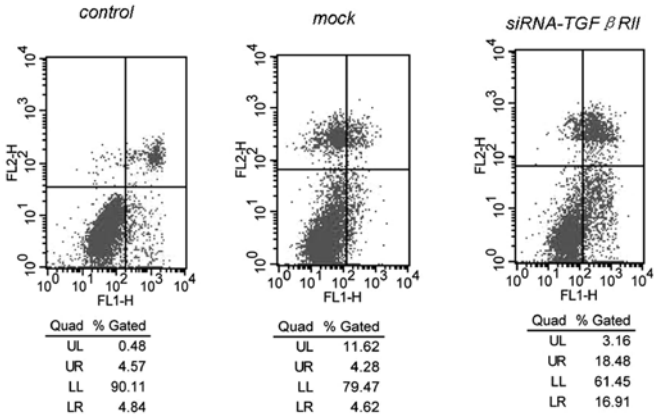

Figure 8. Blockade of the TGF-β signaling pathway with TGFβRII siRNA induced apoptosis of A549 cells. A549 cells were transfected in 6-well plates and exposed to TGFβ1 (2 ng/ml) for 48 h. The cells labeled with Annexin V-FITC and propidium iodide were subjected to flow cytometric analysis. TGFβRII siRNA increased the apoptotic rate in A549 cells, whereas scramble siRNA did not. Two parameter histogram Dot Plot displayed FL1-FITC on the x axis and FL2-PI on the y axis.

inhibition of TGFβRII expression promoted apoptosis in the TGFβRII-knockdown LAC A549 cells.

Molecular mechanisms of the anti-tumor effects by TGFβRII knockdown. The protein from cells in the three groups was extracted to examine the effects of the TGFβRII knockdown on the expression and phosphorylation status of specific cytokines and signaling molecules. We observed that the protein expression levels of PI3K, phosphorylated Akt, phosphorylated Erk1/2, phosphorylated P38, phosphorylated Smad2 and Smad4 were decreased in the TGFβRII siRNA cells, while those in the control and mock cells exhibited no difference (Fig. 9A and B). These results indicate that the TGFβRII knockdown significantly inhibits intracellular growth factor signaling molecules in LAC cells. In addition, TGFβRII knockdown decreased the protein expression of MMP-9 and VEGF, which have been demonstrated to directly promote carcinogenesis and cancer progression (19-21). However, MMP-2 expression was not changed (Fig. 9C). Together, these data demonstrate that TGFβRII knockdown in LAC cells plays a role in anti-tumor activity via the inhibition of downstream proliferation pathways.

Discussion

Despite advances in medical and surgical treatments, lung cancer is the leading cause of cancer deaths (approximately 1.3 million deaths/year) (2). Prognosis in main histological types of lung cancer is poor, due to intrinsic properties of LAC, whose cells exhibit a marked ability to rapidly progress (22-23). Tumor progression includes tumor cell proliferation, invasion (loss of cell-to-cell adhesion, increased cell motility and basement membrane degradation) and vascular intravasation and extravasation, establishment of a metastatic niche, and angiogenesis (24-25). Therefore, effective inhibition of the proliferative and metastatic biological behavior of LAC cells is crucial for the improvement ofpatient outcomes.

TGFβRII is a transmembrane receptor and mediates the TGF-β signaling pathway during TGF-β binding. In the present study, we revealed that TGFβRII overexpression can be detected in the LAC cell line, A549, whereas its expression in human normal HFL-1 and human hepatic carcinoma HepG2 cell lines is relatively low. In normal tissue/cells, the TGF-β signaling pathway sustains homeostasis and prevents tumorigenesis by inhibiting cellular proliferation, differentiation, and survival (6,26-27). Destruction of TGF-β signaling (especially TGFβRII) clearly induces tumorigenicity (28). However, tumors frequently lose their negative growth response to TGF-β and, in contrast, TGF-β signaling may promote tumor cell invasion and metastasis due to its ability to stimulate angiogenesis, alter the stromal environment, and significantly, cause local and systemic immunosuppression (8,28-31). Since it is still unclear as to whether TGF-β signaling plays a biological role in LAC progression, we focused on TGFβRII knockdown in LAC cells A549 to investigate the anti-tumor function of TGFβRII *in vitro* using RNA interference.

In this study, we designed and chemically synthesized three siRNAs to specifically target TGFβRII (named as siT1, siT2 and siT3) in human LAC cells A549. Transfection efficiency was above 85% and the efficacy of the siRNAs persisted for at least 48 h without significant degradation. In addition, Western blot analysis revealed that, of the three siRNAs tested, siT2 was the most efficacious in the two protein levels. The establishment of siRNA-mediated gene silencing was reliable due to the use of siRNAs designed against different regions of the TGFβRII gene (32).

When tissue and tumor cells overexpress TGF-β and lose the growth-inhibitory response to TGF-β, this is associated with malignant conversion and progression (26-28,30-31).

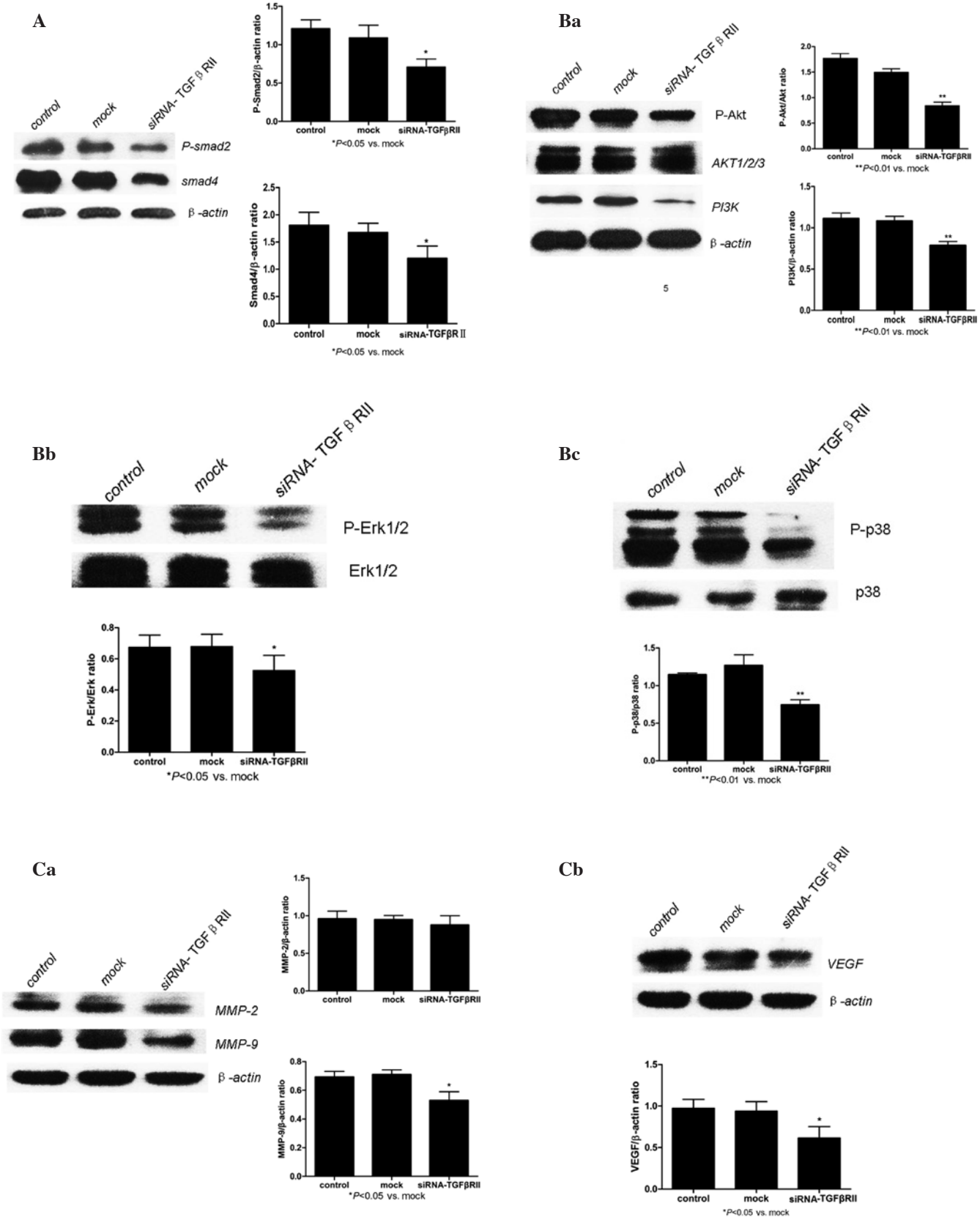

Figure 9. Western blot analysis of the anti-tumor effects by blockade of TGF-β signaling pathway with TGFβRII siRNA. The A549 cells treated with TGFβRII siRNA or scrambled siRNA for 48 h and control cells were incubated with TGF-βI (2 ng/ml) for 2 h, and then total cell protein was extracted. The levels of PI3K, phosphorylated Akt, phosphorylated Erk1/2, phosphorylated P38, phosphorylated Smad2 and phosphorylated Smad4 were examined by Western blot analysis. (A) Effects of TGFβRII knockdown on Smad protein-dependent pathway. Activation of Smad2 and Smad4 in A549 cells treated with TGFβRII siRNA were relatively diminished after TGF-β stimulation. (Ba-c) Effects of TGFβRII knockdown on the Smad protein independent pathway. Similar to the Smad protein dependent pathway, the activation of PI3K/Akt, Erk1/2 MAPK and P38 MAPK in cells transfected with TGFβRII siRNA was inhibited. (Ca-b) Effects of TGFβRII knockdown on MMPs and VEGF. The protein expression levels of MMP-9 and VEGF in TGFβRII-knockdown A549 cells were relatively diminished, whereas the MMP-2 was unchanged. *P<0.05, **P<0.01 vs. mock.

Numerous tumor types, including NSCLC, express a high level of TGF-β (9-10), but the relationship between LAC cells growth and TGF-β signaling has yet to be established. In the present study, we observed that the down-regulation of TGFβRII expression inhibited TGF-β signaling and led to suppression of the proliferation of A549 cells: these effects were time-dependent. Additionally, the tumor colony-forming assay revealed that the clonogenic potential of TGFβRII knockdown A549 cells was greatly reduced. Therefore, impairment of TGF-β signaling through the knockdown of the TGFβRII gene attenuated the ability of A549 cells to proliferate and form colonies. Furthermore, we observed that apoptosis was moderately induced by siRNA targeting TGFβRII for 48 h. The signaling pathways mediating LAC cell growth, including the Smad protein-dependent (e.g. Smad2, Smad4) and -independent pathways (e.g. Erk MAPK, PI3K/Akt, p38 MAPK), are complex (6-8). We observed the consequences of the down-regulation of TGFβRII expression on TGF-β signaling pathways, which were that the protein expression levels of phosphorylated Smad2, Smad4, PI3K, phosphorylated Akt, phosphorylated Erk1/2 and phosphorylated P38 decreased, whereas there were no differences between the control and mock cells. TGF-β signaling blockade through siRNA targeting TGFβRII may therefore inhibit the uncontrolled growth of LAC cells and lead to apoptosis via downstream signaling molecules.

In addition to the involvement in the growth of A549 cells, we observed that the capabilities for invasion and metastasis *in vitro* were attenuated following siRNA-mediated TGFβRII knockdown, which is consistent with previous studies showing that TGF-β signaling is required for carcinoma cell invasiveness and metastasis (e.g., in pancreatic cancer, breast carcinoma, glioblastoma and colon carcinoma cells) (11-14). The matrix metalloproteinases (MMPs) are a family of enzymes that degrade proteins in tissue extracellular matrices. These proteinases are clearly involved in cancer progression, including tumor cell degradation of basement membranes and stroma, blood vessel penetration, and angiogenesis (24). Although it has been established that the overexpression of MMP-2 and MMP-9 in lung cancer cells plays a role in invasion and metastasis (33-34), the manner in which TGF-β signaling regulates the expression of MMP-2 and/or MMP-9 in LAC remains unclear. The present study demonstrates for the first time that TGF-β signaling blockade suppresses the expression of MMP-9 but that MMP-2 expression was unaltered by the knockdown of TGFβRII using siRNA. In addition, LAC progression and metastasis depend upon angiogenesis, in which the vascular endothelial growth factor (VEGF) plays a core role (21). In the present study, we also observed the suppression of VEGF expression in the knockdown of TGFβRII A549 cells. Thus, we hypothesize that the TGF-β signaling pathway regulates MMP-9 and VEGF expression in LAC A549 cells to promote tumor progression. However, the mechanisms of this process require further investigation.

In conclusion, these results reveal that TGF-β signaling blockade using TGFβRII-targeting SiRNA can inhibit LAC cell proliferation, invasion and metastasis and induce cell apoptosisis. Therefore, we have identified that TGF-β signaling plays an important role in LAC progression and MMPs and VEGF may be closely associated with TGF-β signaling pathway. Efficient silencing of TGFβRII using RNA interference may be a potential modality for the gene therapy of LAC. Although the results were relatively favorable *in vitro*, further research *in vivo* is warranted to confirm the effectiveness and safety of anti-cancer therapy with TGFβRII-targeting SiRNA.

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