

Knockdown of Cul4A increases chemosensitivity to gemcitabine through upregulation of TGFBI in lung cancer cells

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Abstract. Cullin 4A (Cul4A) promotes oncogenesis through overexpression and then ubiquitination-mediated proteolysis of tumor suppressors in various types of cancers. Transforming growth factor β -induced (TGFBI) has been implicated as a tumor suppressor, which enhances gemcitabine chemosensitivity in lung cancer cells. The present study aimed to investigate the association of TGFBI and Cul4A and the mechanism by which Cul4A regulates TGFBI. In addition, we also evaluated the therapeutic value of Cul4A RNAi using adenoviral transfection of Cul4A RNAi in nude mouse xenograft models. We observed that knockdown of Cul4A was associated with increased sensitivity to gemcitabine through upregulation of TGFBI in lung cancer cells. Cul4A regulated TGFBI through direct interaction and then ubiquitin-mediated protein degradation. In the nude mouse xenograft models, adenoviral transfection of Cul4A RNAi in combination with gemcitabine chemotherapy inhibited lung cancer tumor growth. As the result, combination of Cul4A RNAi with chemotherapy may provide a new approach to lung cancer treatment.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. The majority of non-small cell lung cancer (NSCLC) patients are in the advanced stage of the disease at the time of diagnosis. Systemic platinum-based chemotherapy is usually the treatment of choice for advanced lung cancer (1). Unfortunately, conventional chemotherapy regimens are hampered by limited efficacy, significant toxicity and a high relapse rate, indicating an urgent need for developing alternative therapeutic approaches such as targeted therapy. The development of tyrosine kinase inhibitors targeting mutant epidermal growth factor receptor (EGFR) is a good example of lung cancer targeted therapy (2). However, the identification of novel targets is still required in the future development of lung cancer therapy.

Cullin 4A (Cul4A) is a member of the evolutionarily conserved cullin protein family, which is related to the ubiquitin proteasome pathway. Overexpression of Cul4A has been observed in breast (3,4), mesothelioma (5) and liver cancers (6). Cul4A has also been implicated in the ubiquitination and proteolysis of tumor suppressors, including p53 (7), NF2 (8), RASSF1A (9) and p21 (10,11), to promote oncogenesis. Our previous study demonstrated that the expression of Cul4A causes ubiquitination and destabilization of p21 and p27. Consequently, downregulation of Cul4A expression resulted in G0/G1 arrest; and overexpression, increased growth of mesothelioma cells (5). Cul4A is also a critical gene for the survival of hematopoietic cells and development and growth of cancer cells (12). In a transgenic mouse study, conditional expression of Cul4A resulted in the formation of lung tumors (11,13).

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Since Cul4A is overexpressed in cancers and is associated with oncogenesis, Cul4A can be a candidate for targeted therapy.

Transforming growth factor β -induced, 68-kDa protein (TGFBI) is known as keratopithelin or β Ig-h3, and contains four conserved fasciclin-1 (FAS1) domains and a carboxyl-terminal Arg-Gly-Asp (RGD) integrin-binding sequence. TGFBI mediates integrin binding to extracellular matrix proteins such as collagen, laminin and fibronectin (14). Loss of TGFBI expression has been reported in several types of cancers including lung cancer (15), and it has been suggested to act as a tumor-suppressor gene (16). Additionally, overexpression of TGFBI in lung cancer cells increased the sensitivity to gemcitabine (17), and overexpression of TGFBI has also been reported to be associated with a better response to gemcitabine chemotherapy in lung cancer cells and patients. Although the Cul4A-DDB1 E3 ligase complex has been reported to ubiquitinate and degrade many tumor-suppressor proteins, it is unclear whether Cul4A causes ubiquitination of TGFBI to decrease its stability.

The present study aimed to investigate the association of TGFBI and Cul4A and the mechanism by which Cul4A regulates TGFBI. In addition, we also evaluated the therapeutic value of Cul4A RNAi using adenoviral transfection of Cul4A RNAi in a nude mouse xenograft model.

Materials and methods

Cell culture. The NSCLC cell lines A549 (ATCC CCL-185) and H460 (ATCC HTB-177) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both A549 and H460 cell lines were grown in complete RPMI-1640 growth medium supplemented with 10% fetal calf serum, 10 U/ml penicillin and 10 μ g/ml streptomycin at 37°C and in 5% CO₂.

Retroviral production and transduction. The pBabe-puro retroviral vector (Addgene, Cambridge, MA, USA) was used to transduce the Cul4A gene, and the pSUPER-retro-puro vector (Oligoengine, Seattle, WA, USA) was used to express Cul4A shRNA. The resultant pBabe Cul4A vector expressing the Cul4A-myc-His fusion protein and the pSuper Cul4A vector expressing Cul4A shRNA were constructed as described previously (5). Retroviral vectors were transfected into the HEK 293 Phoenix amphi packaging cells (ATCC) using Fu-GENE6 transfection reagent (Roche, Lewes, UK). After transfection for 48 h, the supernatant was filtered using a 0.45-mm syringe filter. Retroviral infection was performed by adding the filtered supernatant to lung cancer cell lines cultured on 10-cm dishes with 50% confluency in the presence of 8 μ g/ml of Polybrene (Sigma-Aldrich, St. Louis, MO, USA). Six hours after infection, the culture medium was replaced with fresh medium. The infected cells were allowed to recover for 48 h. Infected cells were selected by adding 1 mg/ml puromycin (Sigma-Aldrich) for 48 h and then maintained in complete medium with 0.5 mg/ml puromycin. Empty retroviral-infected stable cell lines were also produced following the above protocols (5).

Western blot analysis. Whole protein was extracted using Mammalian Protein Extraction Reagent (M-PER) from the

cell lines added with Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA, USA) and Complete Protease Inhibitor Cocktails (Roche) according to the manufacturer's protocols. The proteins were separated on 4-15% gradient SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The following primary antibodies were used: anti-Cul4A (Abcam, Cambridge, MA, USA), anti- β -actin (Sigma Chemical, St. Louis, MO, USA), anti-TGFBI (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-HA (Cell Signaling, Danvers, MA, USA). After being incubated with the appropriate secondary antibodies, the antigen-antibody complexes were detected using an ECL blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometry of western blot analysis was determined by ImageJ (v1.44m for Windows; National Institutes of Health).

Semi-quantitative reverse transcription-PCR (RT-PCR) analysis. RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) from cell pellets according to the manufacturer's instructions. Total RNA was then transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Munich, Germany). Reverse-transcribed cDNA (2 μ l) was subjected to with a total volume of 20 μ l and the Bio-Rad CFX96™ quantitative PCR system (Bio-Rad Laboratories). The following primers were used for PCR: Cul4A, 5'-GCACTGGAGCGAGTACATCA-3' (sense) and 5'-CACATGCTTTGCGATCAGTT-3' (antisense); TGFBI, 5'-AGCCCTGCCACCAAGAGAA-3' (sense) and 5'-CTCCGCTAACCAGGATTTATC-3' (antisense); GAPDH, 5'-CATCCATGACAACCTTGTATCGT-3' (sense) and 5'-CAGTCTTCTGGGTGGCAGTGA-3' (antisense). Amplification conditions were as follows: 1 cycle of 95°C for 5 min and 30 cycles of 95°C for 20 sec, 58°C for 30 sec, 72°C for 30 sec and 1 cycle of 72°C for 5 min and then maintained at 4°C.

Immunofluorescence. For immunofluorescence microscopy, the cells were grown on coverslips, fixed in cold methanol for 10 min at -20°C, and blocked with 2% bovine serum albumin for 30 min. The cells were incubated with the primary anti-TGFBI (Santa Cruz Biotechnology, Inc.) antibody in 2% bovine serum albumin for 1 h at room temperature. The cells were washed with PBS and subsequently incubated with FITC-conjugated secondary antibodies in 2% bovine serum albumin for 1 h at room temperature. After washing with PBS, the cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a TCS SP5 confocal microscope (Leica, Wetzlar, Germany). The intensity of TGFBI was quantified by MetaMorph® Microscopy Automation & Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA).

Cell viability assay. H460 and A549 stable cells (1x10⁴/ml) were cultured in 6-well plates for 48 h and then treated with the indicated concentration of gemcitabine for 72 or 96 h as indicated. Then, cells were trypsinized. The number of viable cells was counted by trypan blue dye exclusion using a hemocytometer. The result was expressed as a percentage, relative to

the empty virus-transfected control groups. The half maximal inhibitory concentration (IC₅₀) value was determined using GraphPad Prism® log (inhibitor) vs. response (variable slope) software (version 6; La Jolla, CA, USA).

Transfection of small interfering RNA (siRNA) and vectors. Pre-designed and validated TGFBI and universal negative control siRNA were purchased from Santa Cruz Biotechnology, Inc. Transfection was performed using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's manual. The cells were plated in 6-well plates in antibiotic-free media, and transfection was performed with cells at 80% confluency with a final concentration of 50 nM for each siRNA. At 96 h after transfection, the cells were treated with the indicated concentration of gemcitabine for 72 h and then the number of viable cells was counted.

The pCMV6-TGFBI-GFP (OriGene, Rockville, MD, USA) and empty pCDNA3 (Invitrogen Life Technologies) vectors were transfected with OmniFect™ transfection reagent (TransOMIC, Huntsville, AL, USA), according to the manufacturer's manual. The cells were plated in 6-well plates in antibiotic-free media, and transfection was performed with cells at 80% confluency with a final concentration of 0.5 µg for each vector. At 96 h after transfection, the cells were treated with the indicated concentration of gemcitabine for 72 h and then the number of viable cells was counted.

Protein degradation assay. A protein degradation assay was used to evaluate the effects of Cul4A on the decay of TGFBI in lung cancer cells. Lung cancer cells that were retrovirally transfected with Cul4A shRNA and Cul4A were plated on 6-cm culture dishes. At 80% confluency, the cells were exposed to 20 mg/ml of cycloheximide. Then, the cells were harvested at the indicated time points. Total cellular proteins were extracted and analyzed by western blot analysis using β-actin as a loading control.

Co-immunoprecipitation assay. 293T cells were transiently co-transfected with the pBabe-Cul4A-myc-his and pCMV6-TGFBI-GFP (OriGene) vectors with Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies). Twenty-four hours after transfection, the cells were treated with 10 mM of MG132 (Sigma-Aldrich) and then harvested in NP-40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP40], protease inhibitor and phosphatase inhibitor cocktail (Roche). Immunoprecipitation was performed by the Catch and Release v2.0 reversible immunoprecipitation system (Millipore) according to the manufacturer's protocols. Anti-GFP (OriGene) and anti-Myc tag (Cell Signaling, Danvers, MA, USA) antibodies were used for immunoprecipitation, respectively.

In vivo ubiquitination assay. 293T cells were cotransfected in combination with pBabe-Cul4A-myc-his and pCMV6-TGFBI-GFP (OriGene) with or without pRK5-HA-Ubiquitin-WT (Addgene). All cells were treated with 10 µM of MG132 for 24 h prior to being lysed. Anti-GFP antibody was used for immunoprecipitation. Anti-HA antibody was used for western blot analysis.

Adenovirus expressing Cul4A RNAi. An adenovirus expressing Cul4A RNAi was generated with BLOCK-iT™ adenoviral RNAi expression system (Invitrogen, Life Technologies) according to the instructions from the manufacturer's manual. Briefly, double-stranded oligonucleotides that encode Cul4A siRNA were cloned into the pENTR™/U6 vector. The following Cul4A siRNA sequences were used for cloning: 5'-GGUUAUCCACGGUAAAGA-3' (5) and 5'-GCAGAA CUGAUCGCAAAGCAU-3' (18). The resulting clones were verified by DNA sequencing. This study used the pAd/BLOCK-iT™-DEST vector and a pENTR™/U6 entry clone in an LR recombination reaction to generate an expression clone containing the U6 RNAi cassette of Cul4A. After digesting the DNA with *PacI*, the pAd/BLOCK-iT™-DEST expression construct was used to transfect the 293A cell line to produce an adenoviral stock. An empty virus was also generated using the same method. The adenoviral stock was amplified and titered using the Adeno-X™ Rapid Titer kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Knockdown of Cul4A was confirmed by immunoblotting using the empty virus-transfected group as the control. The adenovirus was used for the xenograft study.

Nude mouse xenograft models. After approval from the Institutional Animal Care and Use Committee at Chang Gung Memorial Hospital, Chiayi, Taiwan, female Balb/c athymic nude mice of 5-6 weeks of age were housed under specific pathogen-free conditions. H460 or A549 lung cancer cells (1x10⁶) in 100 µl of serum-free RPMI-1640 medium and 20% Matrigel (BD Biosciences, San Jose, CA, USA) were injected subcutaneously into the flank areas of the mice. When tumors reached the mean size of ~50-100 mm³, the mice were divided randomly into groups of five and injected with either AdCul4A or AdEV viruses with or without gemcitabine, at 120 mg/kg intraperitoneally (i.p.) weekly for three weeks. An intratumoral injection of 2x10⁹ plaque forming units (PFU) of the AdCul4A virus or AdEV control virus was performed at days 0, 7 and 14 from the day of injection. Tumors were measured with a caliper twice a week for 6 weeks, and the tumor volume was calculated by the formula (L x W²/2), where L represents the largest tumor diameter and W represents the smallest tumor diameter. Then, the mice were sacrificed, and tumors were excised for further studies at the indicated times.

Statistical analysis. The Student's t-test was used to compare variables in the different groups studied. Statistical analysis was carried out using SPSS (version 10.0; SPSS, Inc., Chicago, IL, USA). Significance was defined as P<0.05 with two-sided analysis.

Results

Overexpression of Cul4A downregulates TGFBI expression in lung cancer cells. To study the correlation between Cul4A expression and TGFBI in lung cancer cells, we established stable cell lines expressing Cul4A using retroviral transfection with the pBabe-puro vector in H460 and A549 (H460 pBabeCul4A and A549 pBabeCul4A) lung cancer cells. In the stable cell lines overexpressing Cul4A, downregulation of TGFBI was observed when compared to the expression level

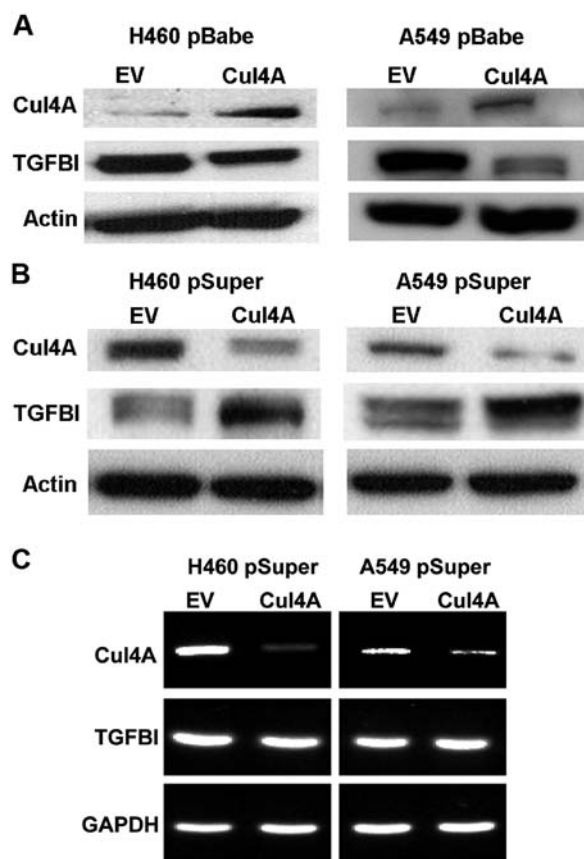


Figure 1. Western blot analysis for TGFBI in H460 and A549 lung cancer cells. (A) H460 and A549 lung cancer cells were stably transfected with pBabe Cul4A (Cul4A) for overexpression of Cul4A. The empty pBabe vector (EV)-transfected cells were used as the control group. (B) H460 and A549 lung cancer cells were stably transfected with pSuper Cul4A (Cul4A) for downregulation of Cul4A. The empty pSuper vector (EV)-transfected cells were used as the control group. β -actin was used as the internal control. (C) RT-PCR analysis for Cul4A and TGFBI in the H460 and A549 lung cancer cells stably transfected with pSuper Cul4A (Cul4A). The empty pSuper vector (EV)-transfected cells were used as the control group. GAPDH was used as the internal control.

in the empty vector-transfected stable cells (H460 pBabeEV and A549 pBabeEV) (Fig. 1A).

Downregulation of Cul4A upregulates TGFBI expression in lung cancer cells. To further study the association of Cul4A and TGFBI expression in lung cancer cells, knock-down of Cul4A expression in the H460 and A549 lung cancer cells was performed using retroviral transfection of Cul4A-specific shRNA with the pSuper-puro vector. In the Cul4A-knockdown H460 and A549 (H460 pSuperCul4A and A549 pSuperCul4A) lung cancer cells, upregulation of TGFBI was observed compared to the expression level in the empty vector-transfected stable cells (H460 pSuperEV and A549 pSuperEV) (Fig. 1B). To further evaluate whether upregulation of TGFBI is associated with increased transcription of TGFBI mRNA, RT-PCR was performed to evaluate the expression of Cul4A and TGFBI in the Cul4A-knockdown H460 and A549 lung cancer cells. The expression of TGFBI mRNA was not changed in both the Cul4A-knockdown lung cancer cell lines (Fig. 1C). Thus, Cul4A may not regulate TGFBI through transcriptional regulation of mRNA. Immunofluorescent

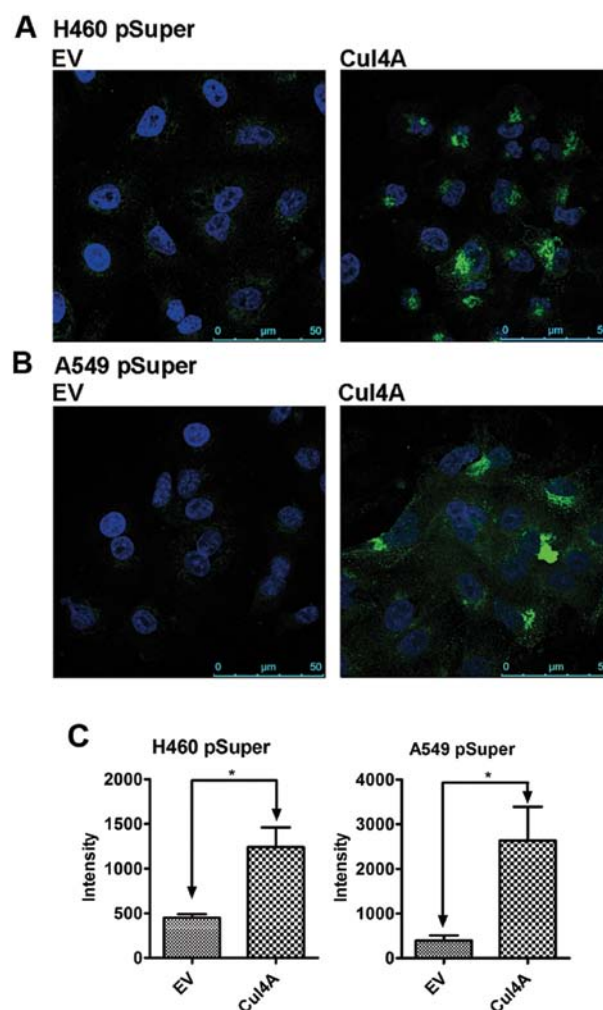


Figure 2. Immunofluorescent analysis of (A) H460 and (B) A549 lung cancer cells transfected with pSuper Cul4A (Cul4A) for downregulation of Cul4A. The empty pSuper vector (EV)-transfected cells were used as the control group. TGFBI was stained green and nuclei were stained blue. Scale bar, 50 μ m. Original magnification, x200. (C) The intensity of TGFBI in the each cells was quantified and shown as a mean \pm standard deviation in triple experiments. * $P < 0.05$.

analysis was also performed to evaluate the expression of TGFBI in the Cul4A-knockdown H460 (Fig. 2A) and A549 (Fig. 2B) (H460 pSuperCul4A and A549 pSuperCul4A) lung cancer cells. Upregulation of TGFBI was also observed in these cells when compared to the expression level in the empty vector-transfected stable cells (H460 pSuperEV and A549 pSuperEV), and TGFBI was mainly expressed in the cytoplasm and on the surface of the lung cancer cells studied.

Downregulation of Cul4A increases chemosensitivity to gemcitabine in lung cancer cells. We next studied the chemosensitivity to gemcitabine in the Cul4A-knockdown H460 and A549 lung cancer cells. We treated Cul4A-knockdown (H460 pSuperCul4A and A549 pSuperCul4A) and empty vector-transfected (H460 pSuperEV and A549 pSuperEV) lung cancer cells with the indicated concentrations of gemcitabine for 96 h. Viable cells were counted and normalized to the cell number in the empty vector-transfected groups without treatment. IC_{50} values were determined in the H460

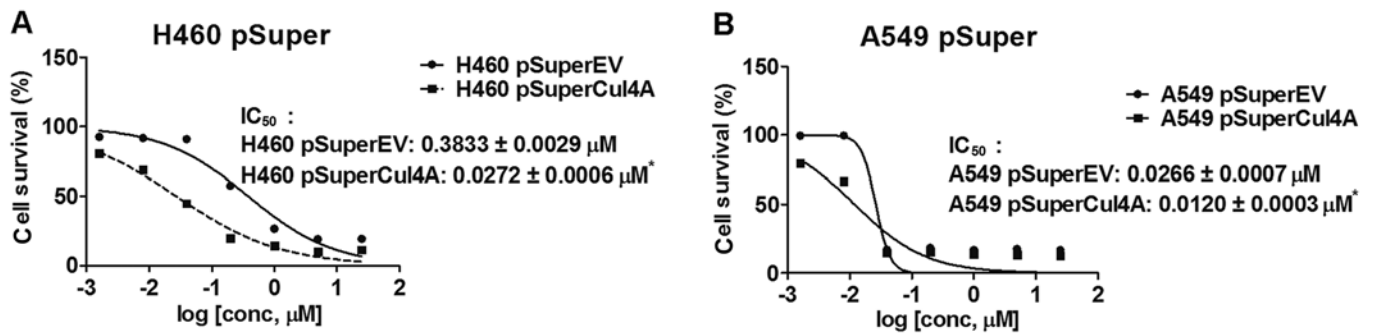


Figure 3. Sensitivity to gemcitabine of (A) H460 and (B) A549 lung cancer cells transfected with pSuper Cul4A (Cul4A) for downregulation of Cul4A. All groups of lung cancer cells were treated with the indicated concentrations of gemcitabine for 96 h. The percentage of surviving cells was normalized to the empty vector-transfected groups without gemcitabine treatment and shown as mean \pm standard deviation in triple experiments. * $P < 0.05$.

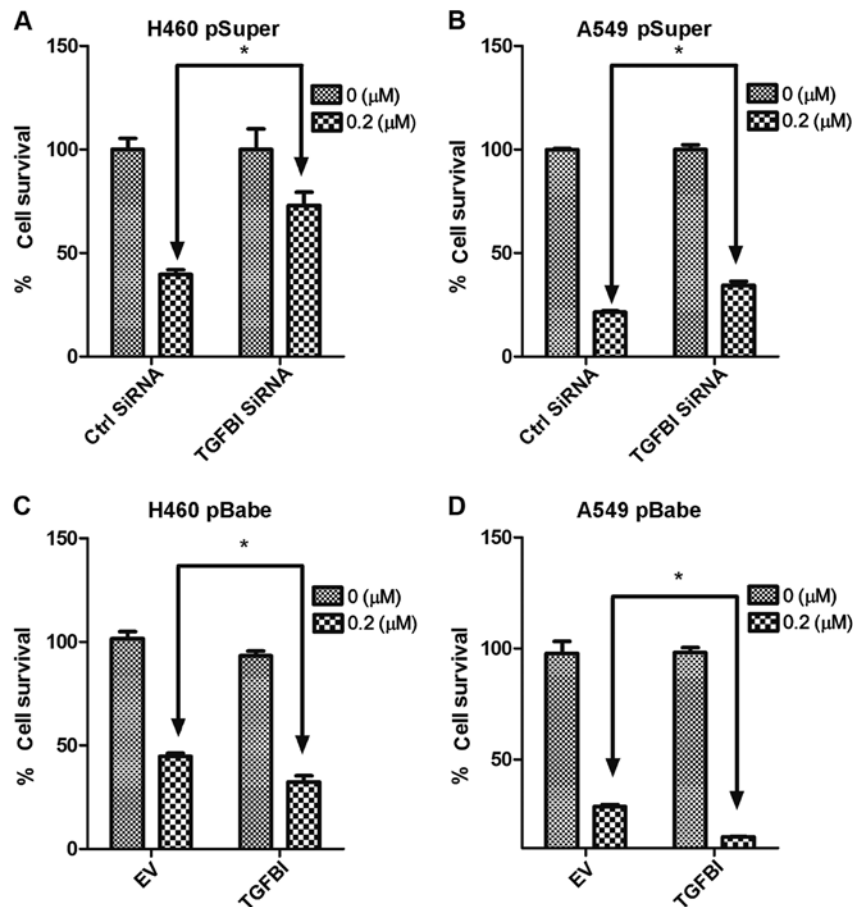


Figure 4. (A and B) Sensitivity to gemcitabine of the H460 and A549 lung cancer cells transfected with pSuper Cul4A for downregulation of Cul4A and TGFB1 siRNA. Both H460 pSuper Cul4A and A549 pSuper Cul4A lung cancer cells were transfected with control (Ctrl siRNA) or TGFB1 siRNA (TGFB1 siRNA) for 96 h and then treated with the indicated concentrations of gemcitabine for 72 h. (C and D) Sensitivity to gemcitabine of the H460 and A549 lung cancer cells transfected with pBabe Cul4A for overexpression of Cul4A and TGFB1. Both H460 pBabe Cul4A and A549 pBabe Cul4A lung cancer cells were transfected with empty (EV) or TGFB1 expression (TGFB1) vectors for 96 h and then treated with the indicated concentrations of gemcitabine for 72 h. The percentage of surviving cells was normalized to groups without gemcitabine treatment and shown as mean \pm standard deviation in triple experiments. * $P < 0.05$.

pSuperCul4A ($0.0272 \pm 0.0006 \mu\text{M}$), A549 pSuperCul4A ($0.0120 \pm 0.0003 \mu\text{M}$), H460 pSuperEV ($0.3833 \pm 0.0029 \mu\text{M}$) and A549 pSuperEV ($0.0266 \pm 0.0007 \mu\text{M}$) lung cancer cells. Significantly lower IC_{50} values were observed in the Cul4A-knockdown (H460 pSuperCul4A and A549 pSuperCul4A) cells when compared to the corresponding empty vector-transfected (H460 pSuperEV and A549 pSuperEV) lung cancer cells (Fig. 3A and B).

Downregulation of TGFB1 decreases chemosensitivity to gemcitabine in Cul4A-knockdown lung cancer cells. To further evaluation the effects of TGFB1 on chemosensitivity to gemcitabine, we downregulated TGFB1 expression in the Cul4A-knockdown H460 pSuperCul4A and A549 pSuperCul4A lung cancer cells. All groups of cells were treated with $0.2 \mu\text{M}$ of gemcitabine for 72 h. Compared to the control siRNA-treated groups, significantly decreased chemosensitivity to

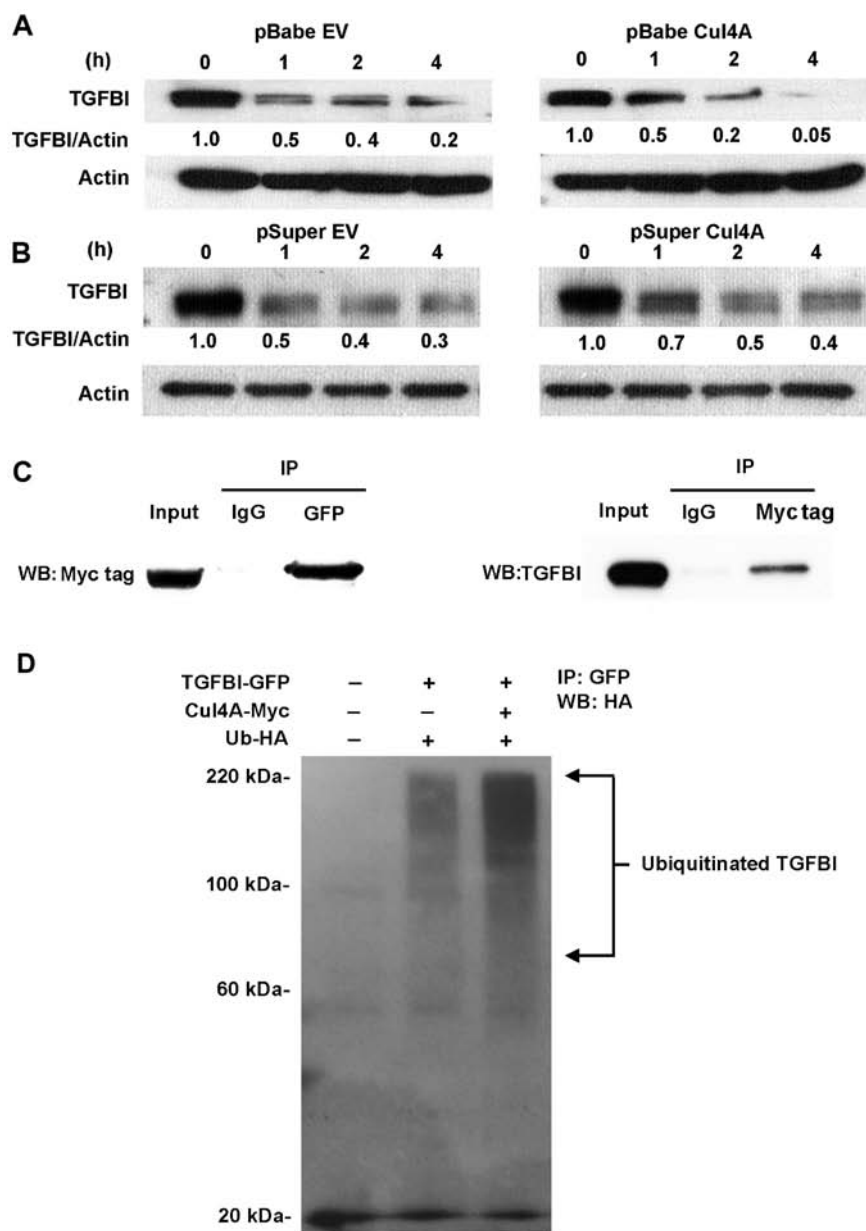


Figure 5. (A) Western blot analysis of TGFBI in the H460 lung cancer cell line. H460 lung cancer cells were stably transfected with the pBabe Cul4A overexpression vector or the empty pBabe EV vector. H460 cells were incubated with 20 μ g/ml cycloheximide for the indicated time periods. (B) Western blot analysis for TGFBI in H460 lung cancer cell line. H460 lung cancer cells were stably transfected with the pSuper Cul4A to downregulate Cul4A or the empty pSuper EV vector. H460 cells were incubated with 20 μ g/ml cycloheximide for the indicated time periods. (C) Reciprocal immunoprecipitation of Cul4A and TGFBI. The pBabe-Cul4A-myc-his and pCMV6-TGFBI-GFP vectors were cotransfected into 293T cells. Anti-GFP and anti-Myc tag antibodies were used for immunoprecipitation, respectively. (D) *In vivo* ubiquitination assay demonstrated that TGFBI is ubiquitinated by Cul4A in the cells.

gemcitabine was observed in the TGFBI siRNA-treated H460 pSuperCul4A (Fig. 4A) and A549 pSuperCul4A (Fig. 4B) lung cancer cells.

Overexpression of TGFBI increases chemosensitivity to gemcitabine in Cul4A-overexpressing lung cancer cells. The effects of TGFBI overexpression on chemosensitivity to gemcitabine were also studied. We overexpressed TGFBI in the H460 pBabeCul4A and A549 pBabeCul4A lung cancer cells. All groups of cells were treated with 0.2 μ M of gemcitabine for 72 h. Compared to the empty vector-transfected groups, significantly increased chemosensitivity to gemcitabine was observed in the TGFBI-overexpressing H460

pBabeCul4A (Fig. 4C) and A549 pBabeCul4A (Fig. 4D) lung cancer cells.

Cul4A regulates TGFBI stability through protein degradation and ubiquitination. Since Cul4A-DDB1 E3 ligase complex has been reported to ubiquitinate and degrade many tumor-suppressor proteins, we thus studied whether Cul4A regulates TGFBI stability. A protein degradation assay was performed using the Cul4A-overexpressing H460 pBabeCul4A lung cancer cells. Compared to the empty vector-transfected H460 pBabeEV lung cancer cells, increased degradation of TGFBI was noted in the H460 pBabeCul4A lung cancer cells (Fig. 5A), which indicated that overexpression of Cul4A

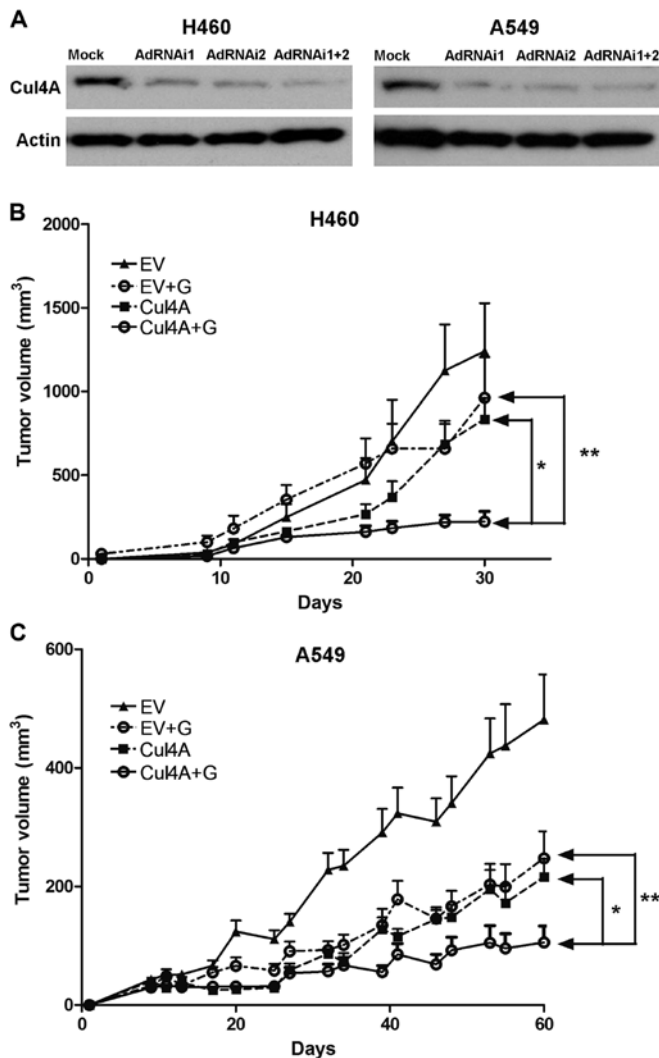


Figure 6. (A) Adenoviruses expressing Cul4A RNAi (AdRNAi1, AdRNAi2 or combination of both) were transfected into H460 and A549 lung cancer cells. Totally 200 MOI of each adenovirus was transfected. Mock, empty virus. Ninety-six hours post transfection, the cells were harvested and cell lysates were subjected to western blot analysis. (B) H460 lung cancer cell nude mouse xenograft model. Data points represent the average of tumor volume \pm standard deviation (n=6 in each group). * $P=0.0014<0.05$, ** $P=0.0345<0.05$. (C) A549 lung cancer cell nude mouse xenograft model. Data points represent the average of normalized tumor volume \pm standard deviation (n=6 in each group). * $P=0.0241<0.05$, ** $P=0.0241<0.05$; EV, empty virus; Cul4A, Cul4A RNAi virus (100 MOI of each AdRNAi1 and AdRNAi2); G, gemcitabine.

is related to increased degradation of TGFBI. A protein degradation assay was also performed using Cul4A-knockdown H460 pSuperCul4A lung cancer cells. Compared to the empty vector-transfected H460 pSuperEV lung cancer cells, decreased degradation of TGFBI was noted in the H460 pSuperCul4A lung cancer cells (Fig. 5B), which indicated that downregulation of Cul4A is related to decreased degradation of TGFBI.

The interaction between Cul4A and TGFBI was also evaluated in our study. Reciprocal immunoprecipitation of Myc-tagged Cul4A and GFP-tagged TGFBI proteins was performed. Direct and preferential binding between the Cul4A and TGFBI proteins was noted (Fig. 5C). Since Cul4A exerts its functions through forming the multifunctional

ubiquitin-protein ligase E3 complex by interacting with ROC1 and DDB1, we further performed *in vivo* ubiquitination assays to determine whether Cul4A is a TGFBI E3 ligase. 293T cells were cotransfected with HA-tagged ubiquitin and Myc-tagged Cul4A in combination with GFP-tagged TGFBI. Our results showed that TGFBI was ubiquitinated by Cul4A (Fig. 5D).

Adenovirus-mediated transfection of Cul4A RNAi inhibits tumor growth in lung cancer xenograft models. Since the susceptibility of lung cancer cells to gemcitabine was elevated after the knockdown of Cul4A in the stable lung cancer cell lines (Fig. 3A and B), we further studied the therapeutic effects of Cul4A RNAi and the synergistic effects of Cul4A RNAi with gemcitabine in nude mouse xenograft models. Transient transfection of cells with Cul4A RNAi was performed in this model. Adenoviruses expressing Cul4A RNAi (AdRNAi1 and AdRNAi2) targeting different regions of the Cul4A gene were generated. Compared to the empty virus-transfected cells, the combination of both AdRNAi1 and AdRNAi2 viruses resulted in downregulation of Cul4A in the H460 and A549 lung cancer cells (Fig. 6A). Nude mouse xenografts derived from the H460 and A549 lung cancer cells were then established. Compared to the empty virus combined with gemcitabine and the Cul4A RNAi-transfected groups, significantly decreased tumor growth was observed in the groups which combined Cul4A RNAi and gemcitabine in the H460 (Fig. 6B) and A549 (Fig. 6C) lung cancer xenograft models.

Discussion

In the present study, we observed that knockdown of Cul4A enhanced chemosensitivity to gemcitabine in lung cancer cells. Upregulation of TGFBI was noted in the Cul4A-knockdown lung cancer cells and downregulation of TGFBI was noted in the Cul4A-overexpressing lung cancer cells. Further knockdown of TGFBI expression in the Cul4A-knockdown lung cancer cells decreased chemosensitivity. In contrast, overexpression of TGFBI in the Cul4A-overexpressing lung cancer cells increased chemosensitivity. Thus, the mechanisms by which Cul4A affects chemosensitivity to gemcitabine in lung cancer cells may be through regulation of TGFBI protein.

In addition, Cul4A knockdown significantly upregulated TGFBI protein levels and decreased its degradation, whereas overexpression of Cul4A markedly enhanced TGFBI protein ubiquitination and increased degradation of TGFBI protein. We also observed that Cul4A regulated TGFBI protein stability through direct interaction and ubiquitin mediated proteolysis. Thus, TGFBI is a target of the Cul4A E3 ligase complex. Cul4A has been reported to play a role in the ubiquitination and proteolysis of some well-defined tumor suppressors, including p53 (7), NF2 (8), RASSF1A (9), p27 (19), DDB2 (20) and p21 (10,11). To the best of our knowledge, the association of Cul4A and TGFBI has not been previously reported.

Knockdown of Cul4A has been reported to increase chemosensitivity to cisplatin in lung cancer cells (13). Overexpression of Cul4A was also reported to participate in multiple drug resistance (MDR) in breast cancer cells through upregulation of MDR1/P-gp expression at both the transcription and protein

levels, and knockdown of Cul4A increased chemosensitivity to P-gp substrate drugs, paclitaxel, vincristine and adriamycin in breast cancer cells (21). In the present study, we observed that knockdown of Cul4A is related to chemosensitivity to gemcitabine through upregulation of TGFBI in lung cancer cells for the first time. TGFBI-mediated chemosensitivity to gemcitabine has been reported to be related to proteolytic fragments derived from TGFBI-induced cell death through binding to the $\alpha\beta3$ integrin on the surface of NSCLC cells and the subsequent activation of caspase-8 and caspase-3/7 signaling and resultant apoptosis of NSCLC cells (17). Gemcitabine is one of the most widely used drugs for the treatment of NSCLC. Cisplatin plus gemcitabine regimen has become a commonly used combination for advanced NSCLC (22) and maintenance therapy (23). When used as a first-line monotherapy, the objective response rate to gemcitabine is 16-22% (24). Thus, developing novel adjuvant chemotherapy in combination with gemcitabine chemotherapy is urgent. Our study may provide the rationale to improve the efficacy of gemcitabine chemotherapy as well as other chemotherapy drugs in NSCLC in combination with anti-Cul4A targeted therapy in the future.

Moreover, we also established an animal model to demonstrate the therapeutic value of Cul4A RNAi. We observed that tumor size was significantly decreased in the group of mice treated with Cul4A RNAi and gemcitabine. To date, there is no Cul4A-specific small compound reported. RNAi is a potential new class of pharmaceutical drugs. RNAi-based therapeutics can offer a powerful method for rapidly identifying specific and potent inhibitors of disease targets from all molecular classes. The broad potential application of RNAi therapeutics has been demonstrated by numerous proof-of-concept studies in animal models of human diseases (25). The use of adenoviral vectors is considered to be a very powerful tool in cancer gene therapy as they have been shown to transduce genes efficiently into many types of cancer cells (26). In the present study, we designed recombinant adenoviral vectors encoding siRNAs against Cul4A and investigated their efficacy in regard to the suppression of Cul4A expression in cancer cells and consequent antitumor potential. Through *in vivo* studies, our results support the feasibility of adenoviral-mediated transfer of Cul4A RNAi for treatment of NSCLC and other cancers in the future.

In summary, the present study showed that knockdown of Cul4A is associated with increased sensitivity to gemcitabine through upregulation of TGFBI in NSCLC cells. Cul4A regulates TGFBI through direct interaction and then ubiquitin-mediated protein degradation. In nude mouse xenograft models, adenoviral-mediated transfer of Cul4A RNAi in combination with gemcitabine chemotherapy inhibited NSCLC tumor growth. Therefore, combination of Cul4A RNAi with chemotherapy may provide a new approach to lung cancer treatment.

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