microRNA-182 inhibits the proliferation and invasion of human lung adenocarcinoma cells through its effect on human cortical actin-associated protein

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Abstract. Lung cancer is one of the main causes of cancer death worldwide. The cortactin gene, *CTTN*, may play a pivotal role in the proliferation and invasion of tumors. A microRNA (miR-182) was cloned and used to study the expression of *CTTN* and its regulatory effects on the proliferation and invasion of the lung cancer cell line, A549. Cortactin protein and *CTTN* mRNA expression decreased in A549 cells that were transfected with the miR-182 expression plasmid. A cell proliferation assay indicated that miR-182 expression affected cell cycle regulation and suppressed proliferation of lung cancer cells *in vitro*. In addition, xenograft experiments confirmed the suppression of tumor growth *in vivo*, which was due to the promotion of apoptosis. In conclusion, endogenous mature miR-182 expression may have an important role in the pathogenesis of lung cancer through its interference with the target gene *CTTN* by epigenetic modification.

Introduction

Lung cancer is the leading cause of cancer death worldwide. In 2008, it claimed the lives of approximately 1.4 million people around the world (1,2) and its 5-year survival rate is ~15% (3). In the past 20 years, lung cancer mortality has increased in China, especially in urban areas and among older people; treatment outcomes remain unsatisfactory (4,5) and the mortality is high. Hence, there is great interest in studying this disease in order to better understand its biology and to translate research findings into improved therapies (6).

Since their first description in 1993 (7), microRNAs (miRNAs) have been shown to play key roles in developmental regulation, and in the maintenance of undifferentiated, or incompletely differentiated, cell types, such as stem cells (8,9). The human genome is estimated to contain nearly 900 unique miRNAs, which control, in part, the expression of more than one third of the human genes (7). In human cancer, several dysregulated miRNAs have been shown to have oncogenic or tumor suppressive activity (4). For example, miRNA-155 (miR-155) is overexpressed in a variety of human cancers and immune diseases (10). Expression of BIC and miR-155 in three latency type III, EBV-positive Burkitt lymphoma (BL) cell lines, and in all cases of primary post-transplant lymphoproliferative disorder (PTLD) suggests a possible role for EBV latency type III-specific proteins in the induction of BIC expression (11,12). In addition, miR-128 inhibits glioma cell proliferation by targeting the transcription factor, E2F3a (13), and miR-200c and miR-141 (members of the miR-200 family) are important regulators of the epithelial to mesenchymal transition in normal and cancer cells (7,14). Furthermore, fragments of miRNA can be found and measured in the serum of patients with cancer, and could potentially act as a novel class of biomarkers for diagnosis or cancer staging; for instance, tumor-suppressive activities in human hepatocellular carcinoma cells are decreased by up-regulation of miR-23a~27a~24 (15). However, the complexity of the structures and cloning processes for miRNAs has prevented the analysis of large numbers of clinical samples.

Cortical actin-associated protein (cortactin), an 80 and 85 kDa protein, is a widely expressed actin-binding protein, which contains 6.537-amino acid repeats, of which the fourth is required for filamentous actin-binding activity (16-18). It was first identified as one of the major substrates for Src kinase (19) and has four major domains of interest, the N-terminal acidic and tandem repeat domains, and the C-terminal proline-rich and SH3 domains (19). The *CTTN* gene, also called *EMS1*, which codes the cortactin protein, is frequently overexpressed in breast, and head and neck cancers due to its presence in

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the 11q13 amplicon (19). This amplification has been associated with higher pathological stage, lymph node and distant metastases, and decreased survival. In addition, cortactin can stimulate the actin-nucleation activity of the Arp2/3 complex, alone or in combination with N-WASP (19-21). It also inhibits debranching and disassembly of dendritic actin networks.

Cellular roles for cortactin include the bridging of the endocytic machinery with components and regulators of the actin cytoskeleton, and the binding of the mechanochemical GTPase dynamin 2, which regulates the fission of endocytic vesicles. The function of cortactin has been examined in tumors grown in mice (19). Since cortactin overexpression increases the motility of fibroblasts and endothelial cells and increases bone metastasis in MDA-MB-231 breast cancer cells in a nude mouse model (20), one possible explanation for *CTTN* amplification in human cancers is that cortactin overexpression promotes tumor cell invasion and metastasis. The regulation of the endocytosis of integrins and growth factor receptors, or the secretion of molecules, such as proteases or extracellular matrix (ECM), by cortactin might have major effects on cell motility that would be cell context dependent (19). *In vivo* evidence supports a role for cortactin in metastasis, which is consistent with its well-characterized cell biological roles in cell motility and ECM degradation. Although known to be involved in cell motility and tumor occurrence, the mechanisms and characteristics of its regulation have not been addressed. Therefore, we sought to determine whether a miRNA existed that could specifically target cortactin to regulate its expression in different cells over time.

In this study, the miR-182 expression vector was transfected into the human lung adenocarcinoma cell line A549 to determine whether it could specifically regulate *CTTN* and inhibit cell proliferation and invasion. If miR-182 expression were involved in this cell line, it could potentially be an important therapeutic target in human lung adenocarcinoma.

Materials and methods

Cell lines, animals. The human lung adenocarcinoma cell line A549 was grown in DMEM (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Lab. Inc., Queensland, Australian), penicillin (100 U/ml), streptomycin (100 U/ml), and 2 mM L-glutamine (all from Hyclone). A549 cells were maintained at 37˚C in a humidified atmosphere of air containing 5% CO_2.

BALB/c nude mice were of 6-7 weeks of age and 20-22 g of weight. All animal procedures were carried out at Tongji University with the approval of the Institutional Animal Care and Use Committee and in accordance with institutional guidelines.

Vector construction. For expression experiments, plasmid pRNAT-CMV32-cGFP-mir182 (pre-miRNA of miRNA-182 expression element), oligonucleotide pairs for pre-miRNA of miRNA-182 and linker sequences with *Bam*HI and *Xho*I sites were chemically synthesized (10). The sequences of the oligonucleotides were: top strand, 5'-GTggatccCTG**TTTGGCAATGGTAGAACTCACACT**TTTTGCCTCCAACTGACTCCTACATATTAGCATTAACAGctcgagCC-3', and bottom strand, 5'-GGctcgagCTGTTAATGCTAATATGTAGGAGTCAGTTGGAGGCAAAAAAG**TGTGAGTTCTACCATTGCCAAA**CAGggatccAC-3' (sequences corresponding to miRNA-182 seed sequences are capitalized, underlined and bold; restriction enzyme sites are in lower case and underlined). To build the expression plasmid the pairs of oligos were annealed and inserted into the multiple cloning sites between the *Bam*HI and *Xho*I sites in the pRNAT-CMV32-cGFP/Neo vector (GenScript, New Jersey, USA). The negative control plasmid pRNAT-CMV32-cGFP-mir182-Mut was similarly built, except that 17 nucleotides in sequences corresponding to the miRNA-182 seed sequences were mutated (change from **TTTGGCAATGGTAGAAC** to **TaTGcgAtTGGaAcAtg**, mutations shown in lower-case). Co-transfection of the A549 cell line was conducted to transfer 0.3 μg miRNA-182 expressed vector or miRNA-182 mutant vector, respectively, with Lipofectamine 2000 reagent according to the manufacturer's protocol.

RNA extraction and analysis by quantitative real-time PCR (qRT-PCR). Total-RNA from each group of cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA samples were treated with DNase I (Sigma-Aldrich, St. Louis, MO, USA), quantified, and reverse-transcribed into cDNA using the ReverTra Ace-α first strand cDNA synthesis kit (Toyobo). qRT-PCR was conducted using a RealPlex4 real-time PCR detection system from Eppendorf Co. Ltd. (Germany), with SYBR-Green real-time PCR Master mix (Toyobo) used as the detection dye. qRT-PCR amplification was performed over 40 cycles with denaturation at 95˚C for 15 sec and annealing at 58˚C for 45 sec. Target cDNA was quantified using the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator) and steady-state mRNA levels are reported as an n-fold difference relative to the calibrator. For each sample, the maker genes Ct values were normalized using the formula ΔCt = Ct_genes–Ct_18sRNA. To determine relative expression levels, the following formula was used $\Delta\Delta Ct$ = ΔCt_all_groups–ΔCt_blankcontrol_group. The values used to plot relative expressions of markers were calculated using the expression $2^{-\Delta\Delta Ct}$. The mRNA levels were calibrated based on levels of 18S rRNA. The cDNA of each gene was amplified as previously described using the following primers: CTTN-forward: 5'-TAGAGCCTGGTGCCTGGG-3', CTTN-reverse: 5'-TAATCCAATGAGGAATTTCCAG-3'.

Methyl thiazolyl tetrazolium (MTT) assay for cell proliferation. Each group of A549 cell lines was seeded at $2x10^3$/well in 96-well plates and cultured in DMEM supplemented with 10% FBS at 37˚C with 5% CO_2, until 85% confluent. MTT (Sigma-Aldrich) reagent (5 mg/ml) was added to the maintenance cell medium at different time points, and incubated at 37˚C for an additional 4 h. The reaction was terminated with 150 μl dimethylsulfoxide (DMSO, Sigma-Aldrich) per well. The cells were lysed for 15 min and the plates were gently shaken for 5 min. Absorbance values were determined by using the enzyme-linked immunosorbent assay (ELISA) reader (Model 680, Bio-Rad) at 490 nm.

Flow cytometric (FCM) analysis of cell cycle by propidium iodide (PI) staining. Each group of A549 cells was seeded at

$3x10^5$/well in 6-well plates and cultured until 85% confluent. Each group of cells was washed with PBS three times, and the cells were then collected by centrifugation (Allegra X-22R, Beckman Coulter) at 1000 x g for 5 min. The cell pellets were resuspended in 1 ml of PBS, fixed in 70% ice-cold ethanol, and kept in a freezer for more than 48 h. Before flow cytometric analysis, The fixed cells were centrifuged, washed twice with PBS, and resuspended in PI staining solution (Sigma-Aldrich) containing 50 μl/ml PI and 250 μg/ml RNase A (Sigma-Aldrich). The cell suspension, which was hidden from light, was incubated for 30 min at 4˚C and analyzed using FACS (FCM-500, Beckman Coulter). A total of 20,000 events were acquired for the analysis using the CellQuest software.

Luciferase reporter assay. All steps of the luciferase reporter assay were performed as previously described (15,22). NIH-3T3 cells were seeded at $3x10^4$ cells/well in 48-well plates and co-transfected with 400 ng of pRNAT-CMV32-cGFP-mir182 or pRNAT-CMV32-cGFP or pRNAT-CMV32-cGFP-mir182-Mut, 20 ng of pGL3cm-CTTN-3UTR-WT or pGL3cm-CTTN-3UTR-Mut, and pRL-TK (Promega, Madison, USA) using the Lipofectamine 2000 reagent according to the manufacturer's protocol. After 48 h of transfection, the luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Wound-healing assay. All steps of the assay were conducted according to a previously described protocol (17). A549 cells were grown to confluence and then wounded using a 200 μl pipette tip. Three wounds were made for each sample, and all were photographed at the zero time point and at subsequent time points. Assays were repeated thrice in each clone.

Northern blotting. All steps of Northern blotting were performed as previously described (23). For all groups, 20 μg of good quality total RNA were analyzed on a 7.5 M urea, 12% PAA denaturing gel and transferred to a Hybond-N^+ nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using UV light for 30 sec at 1200 mJ/cm^2. Hybridization was performed with the miRNA-182 antisense StarFire probe, 5'-TTTGGCAATGGTAGAACTCACACT-3' (IDT, Coralville, IA), to detect the 22-nt miRNA-182 fragments according to the instruction of the manufacturer. After washing, membranes were exposed for 20-40 h to Kodak XAR-5 films (Sigma-Aldrich). As a positive control, all membranes were hybridized with a human U6 snRNA probe, 5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3'. Exposure times for the U6 control probe varied from 15-30 min.

Immunofluorescence staining analysis for relative protein expression. The cultured cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min. After blocking, the cells were incubated with a rabbit anti-human CTTN polyclonal antibody (1:200; Santa Cruz Biotechnology, Sana Cruz, CA) overnight at 4˚C, and then with a Cy3-conjugated goat anti-rabbit IgG antibody (1:200; Abcam, Cambridge, UK) and 5 mg/ml DAPI (Sigma-Aldrich) at room temperature for 30 min. The cells were then thoroughly washed with TBST and viewed under a fluorescence microscope (DMI3000; Leica, Allendale, NJ, USA).

Western blotting. Total protein extracts of each group of cells were resolved by 12% SDS-PAGE and transferred on PVDF (Millipore) membranes. After blocking, the PVDF membranes were washed 4 times for 15 min with TBST at room temperature and incubated with the primary antibody (rabbit anti-human CTTN polyclonal antibody (1:500). Following extensive washing, membranes were incubated with the secondary peroxidase-linked goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology) for 1 h. After washing 4 times for 15 min with TBST at room temperature once more, the immunoreactivity was visualized using the enhanced chemiluminescence ECL kit (Pierce Biotechnology), and the membranes were exposed to Kodak XAR-5 film.

In vivo xenograft experiments. About $1x10^6$ logarithmically growing A549 cells were inoculated in BALB/c nude/nude mice. Each experimental group consisted of four mice. After 4 weeks of observation, the mice were sacrificed and tumors were stripped (24). Tumor weight was measured and tumor volume was calculated according to the formula: Tumor volume (mm^3) = length (mm) x width (mm) x height (mm).

Statistical analysis. Each experiment was performed at least three times, and the data are shown as the mean ± SE where applicable. Differences were evaluated using Student's t-tests. The probability of <0.05 was considered to be statistically significant.

Results

Bioinformatic analysis of miR-182 and the 3'-untranslated region of CTTN mRNA. The precursor miRNA (pre-miRNA) sequences, mature miRNA sequences, chromosomal locations, and length of miR-182 and the target gene *CTTN* were analyzed in multiple species using the online research tool, the miRBase Target database (http://www.mirbase.org) (23,25). Seven putative miRNA target sites were identified in the 3'-untranslated region (3'-UTR) of CTTN mRNA, depending on the species. For this study, we focused on the human miR-182, which may target the human CTTN 3'-UTR, because these sites are conserved, to varying degrees, across species (Fig. 1).

Identification of miR-182 binding sites in the CTTN 3'-UTR region. Plasmid DNA of each CTTN mRNA 3'-UTR site [wild-type (wt) CTTN, empty plasmid, and mutant CTTN] was co-transfected with the miR-182 expression vector (wt miR-182, empty vector, and mutant miR-182) into the mouse embryonic fibroblast cell line, NIH-3T3, to examine whether *CTTN* gene expression was regulated by the mature miR-182. Luciferase activity of the CTTN 3'-UTR sites was significantly inhibited by wt miR-182 (Fig. 1), while luciferase activity of the mutated CTTN 3'-UTR sites was not inhibited, which indicate that miR-182 targets *CTTN*.

miR-182 specifically influences the expression of cortactin protein in the lung cancer cell line. Northern blot analysis showed that the hybridized signal of mutant miR-182 in the lung cancer cell line, A549, was weaker than in one of the miR-182 transfected. Immunofluorescence (IF) staining,

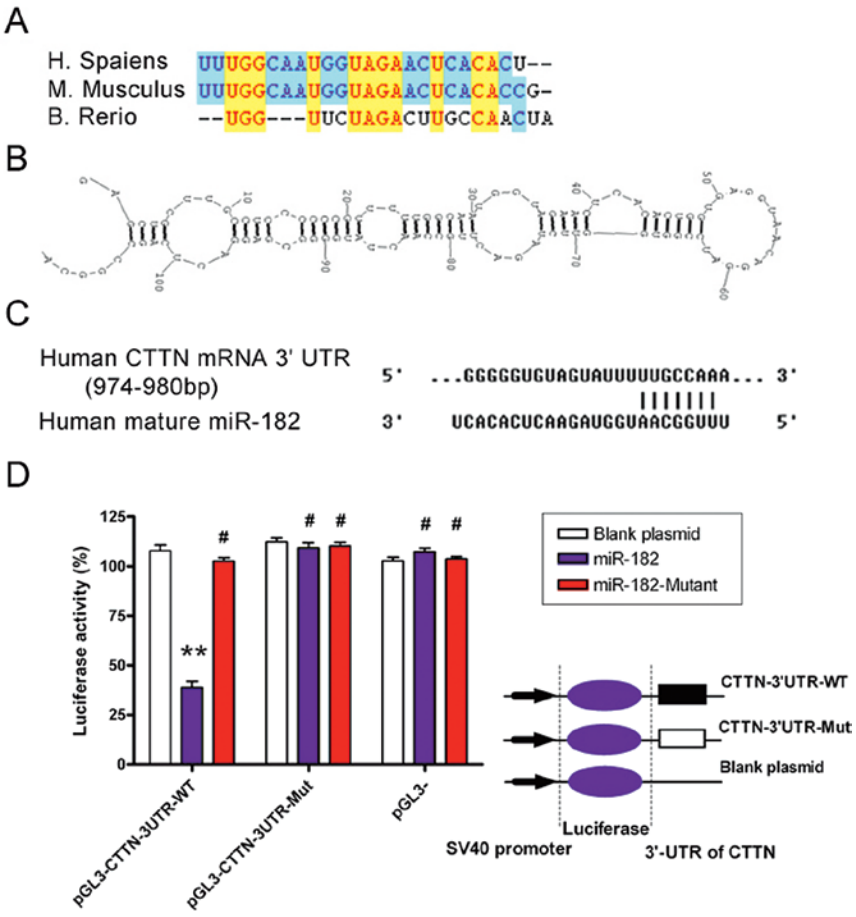

Figure 1. The human CTTN microRNA (miRNA) 3'-untranslated region (3'-UTR) contains microRNA-182 (miR-182) binding sites. The expression of miR-182 and its interference with the target gene *CTTN* was assessed by a luciferase assay. (A) The mature miR-182 sequences of multiple species analyzed and contrasted by bioinformatic tools. (B) The typical secondary structure of the precursor miRNAs (pre-miRNAs) with miR-182. There are stem-loop and hairpin structures in the pre-miRNA, and the common binding site is located in an unstable region with a multi-branching loop-like RNA structure. The mature miRNAs are bound in the 3'-UTR of the target gene. (C) Complementarity between miR-182 and the putative human CTTN 3'-UTR site target (974-980 bp downstream) showing that the conserved bases of the putative miR-182 target sequence are present in the human CTTN 3'-UTR. (D) Wild-type (wt) reporter or mutated control luciferase plasmids were transfected into NIH-3T3 cells with miR-182 expression plasmids or mutant-miR-182 expression plasmids. Luciferase activity within the CTTN 3'-UTR sites is inhibited by miR-182 ($^{**}P<0.01$ and $^{\#}P>0.05$ vs. the blank plasmid; n=3).

quantitative real-time PCR (qRT-PCR) and Western blotting were used to determine whether exogenous and endogenous miR-182 could influence the expression of cortactin. The IF assay revealed that cortactin protein expression decreased in A549 cells that were transfected with the miR-182 expression plasmid compared to miR-182 mutant transfected and to untransfected cells (Figs. 2 and 3). Therefore, miRNA-182 expression may influence endogenous cortactin expression.

The qRT-PCR results confirmed the IF results: when the miR-182 expression vector was transfected into the A549 cells, the CTTN mRNA expression decreased compared to that of untransfected and mutant-miR-182 transfected cells. The mRNA expression was normalized to that of the 18S ribosomal RNA (rRNA), which served as an internal control (Fig. 2). Interestingly, Western blotting revealed that cortactin levels in untransfected cells or mutant-miR-182 transfected cells were 0.892±0.107 and 0.795±0.137 of the GAPDH levels, respectively (Fig. 2). These values were significantly higher than those for the miR-182 transfected group (0.289±0.179 of GAPDH), which indicated that exogenous miR-182 down-regulated cortactin expression.

Cell cycle changes induced by miR-182. To detect changes in cell cycle progression, miR-182 transfected, mutant-miR-182 transfected, and untransfected A549 cells were stained with PI and analyzed by flow cytometry. As shown in Fig. 3, most of the miR-182 transfected cells were arrested in the G_0/G_1 phase of the cell cycle and the percentage of cells in the S and G_2/M phases were markedly decreased (Table I). In contrast, there were no significant differences in the cell cycle distribution of mutant-miR-182 transfected and untransfected cells. These cell cycle results suggest that miR-182 expression affects cell cycle regulation in lung cancer cells *in vitro*.

Proliferation of A549 cells is inhibited by miR-182. The results of the proliferation assays performed are shown in Fig. 2. Using an MTT assay, the survival rate of miR-182 transfected cells at both 72 h and 96 h post-transfection was shown to be markedly lower than untransfected cells and mutant-miR-182 transfected cells. In contrast, the viability of untransfected cells, mutant-miR-182 transfected cells, and miR-182 transfected cells showed no differences at 24 h. Survival rates remained unchanged for untransfected and

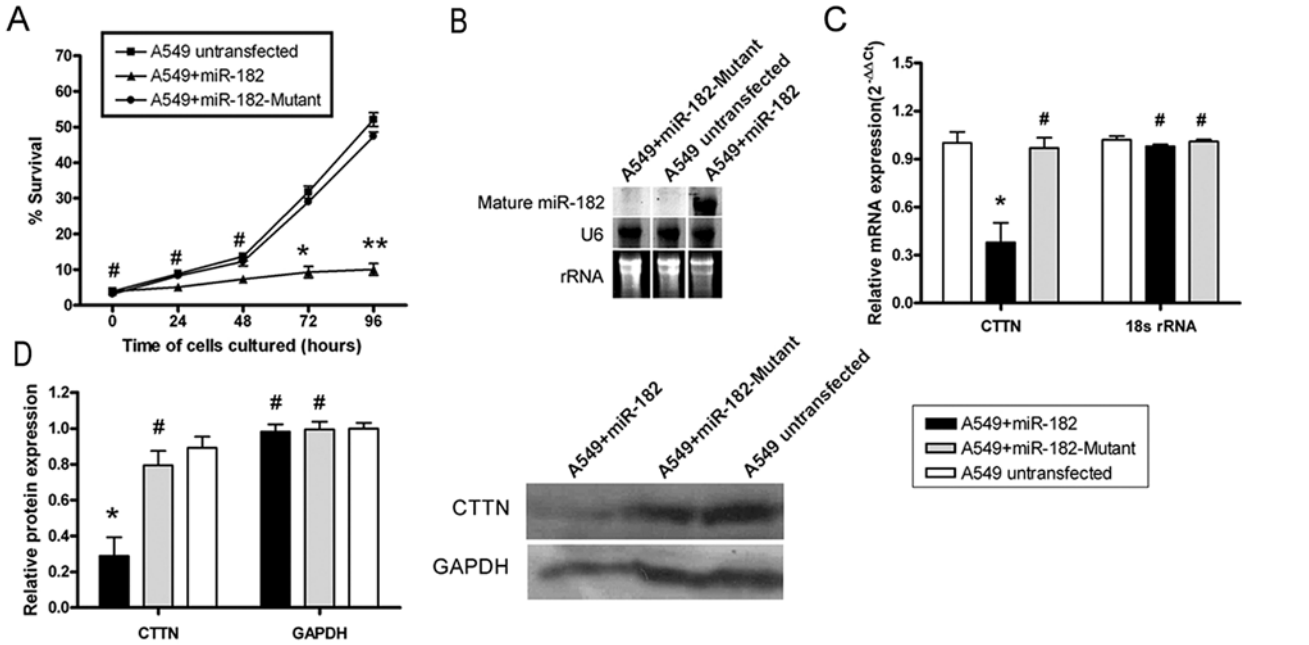

Figure 2. miR-182 and *CTTN* expression in different groups and the effect of miR-182 on cell proliferation. (A) MTT assay showing that the survival rate of miR-182 transfected cells is markedly lower than untransfected and mutant-miR-182 transfected cells at both 72 h and 96 h post-transfection (*P<0.05, **P<0.01 and $^{\#}$P>0.05 vs. the blank plasmid; n=3). (B) Northern blot analysis showing that the hybridized signal of mutant miR-182 in the A549 cell line is weaker than that of miR-182 transfected. The human U6 probe was used as a loading control. (C) qRT-PCR indicating that the CTTN mRNA expressed in the miR-182 transfected group is lower than in the untransfected or mutant-miR-182 transfected group. Relative mRNA expression is shown after normalization to 18S rRNA, which served as an internal control. (D) Western blot analysis showing the expression of CTTN. CTTN levels in untransfected cells or mutant-miR-182 transfected cells are significantly higher than those of the miR-182 transfected group. The data indicate that exogenous miR-182 down-regulates *CTTN* expression (*P<0.05 and $^{\#}$P>0.05 vs. the untransfected group; n=3).

Table I. Cell cycle changes of A549 cells induced by miR-182.

	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
miR-182	84.61±1.23	7.83±1.09	7.56±1.44
Untransfected	64.31±1.16	15.30±1.27	20.39±1.31
Mutant-miR-182	60.19±2.01	17.58±0.94	22.23±1.76

mutant-miR-182 transfected cells for the remainder of the time course, which indicated that induced exogenous miR-182 expression inhibited the growth of lung cancer A549 cells *in vitro*.

Wound-healing assays also showed that the miR-182 transfected cells migrated more slowly than the mutant-miR-182 transfected cells and untransfected cells. Both the mutant-miR-182 transfected and the untransfected cells had become confluent at 24 h post wounding, but the miR-182 transfected cells had migrated only a 2- to 4-cell distance (Fig. 3).

Expressed miR-182 in A549 cells inhibits subcutaneous tumor growth in nude mice. The effect of miR-182 expression on tumor growth was investigated *in vivo* by subcutaneous inoculation of the miR-182 transfected A549 cell line or the untransfected cell line into two groups of nude mice. All mice in the untransfected group developed tumors approximately 28 days after injection, whereas, only 1 in 4 mice from the miR-182 transfected group developed tumor by this time. Although both groups developed tumors, the tumors formed by miR-182 transfected cells grew more slowly than those in the untransfected group (Fig. 4). In addition, when the mice were sacrificed 56 days after injection, the tumor weights of the untransfected group were significantly heavier than those of the miR-182 transfected group. Furthermore, miR-182 expression in the A549 cell lines led to a significant decrease in the tumor volume (Fig. 4).

Ki-67 (a cell proliferation-related protein) was analyzed on the tumor sections by immunohistochemistry because tumor growth is determined by the balance of cell proliferation and programmed cell death. The tumors formed by untransfected A549 cells showed positive or strongly positive staining compared to the controls, but the tumors formed by miR-182 transfected cells exhibited only weak immunoreactions (Fig. 4). Kaplan-Meier survival curves for the mice inoculated with miR-182 transfected cells and untransfected cells were significantly different. In contrast, more apoptotic cells were detected in tumors from the miR-182 transfected cells. These results indicate that miR-182 expression in lung cancer cells suppresses *in vivo* tumor growth by promoting apoptosis.

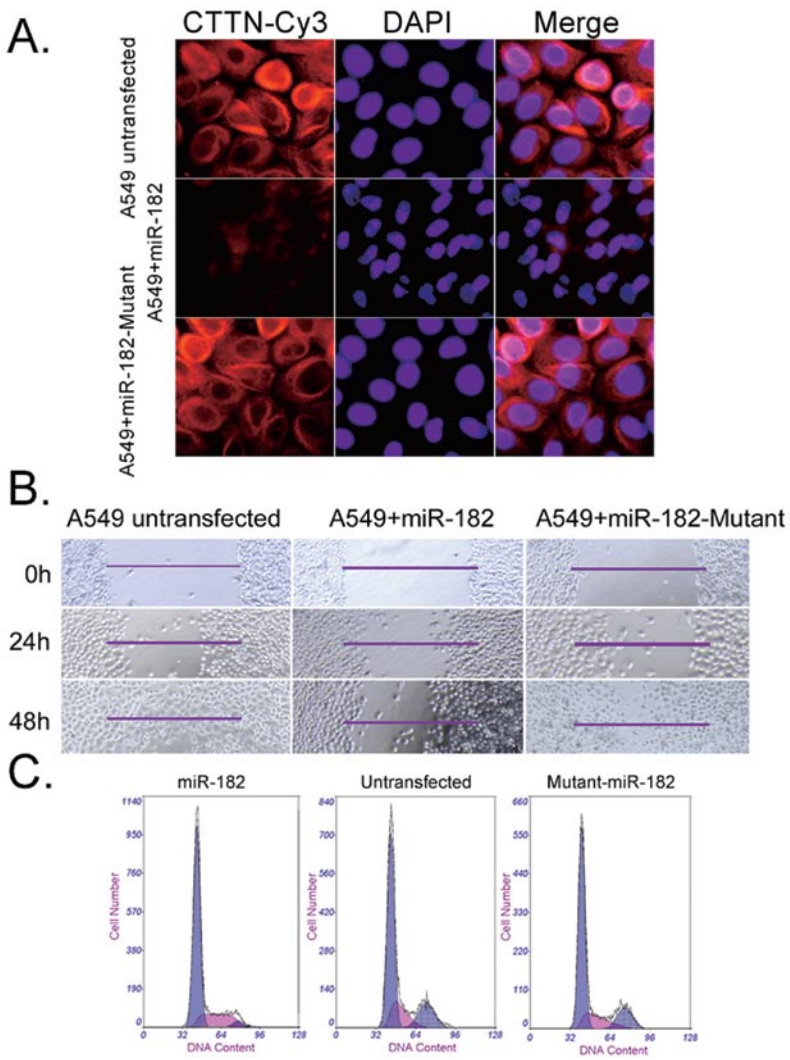

Figure 3. Exogenous miR-182 can not only influence the cortactin expression, but also the proliferation and cell cycle of the A549 cell line. (A) Immunofluorescence (IF) staining showing that exogenous miR-182 influences the expression of cortactin. Cortactin protein expression is decreased in the A549 cell line transfected with the miR-182 expression plasmid, compared with those cells transfected with the mutant-miR-182 expression plasmid or with untransfected cells. (B) Wound-healing assay showing that exogenous miR-182 can influence proliferation of A549 cells. The wound-healing assay showing that the miR-182 transfected A549 cells migrate more slowly than the mutant-miR-182 transfected and the untransfected cells. (C) Flow cytometry showing that exogenous miR-182 can influence the cell cycle of the A549 cell line. It shows that most of the miR-182 transfected cells were arrested in the G_0/G_1 phase of the cell cycle and the percentage of cells in the S and G_2/M phases were markedly decreased.

Discussion

There is increasing evidence supporting that cortactin is a prominent substrate of Src-protein-tyrosine kinase (18,26), and that tyrosine phosphorylation of cortactin occurs frequently in response to various extracellular stimuli, including growth factors, bacterially-mediated phagocytosis, cell shrinkage and membrane injury. Although studies have found that the introduction of a mutant cortactin deficient in tyrosine phosphorylation impairs cell motility, tumor invasion, invadopodia, and podosome formation (18,20,27,28), and that cortactin plays important roles in tumor growth, there have been few reports on the role of cortactin regulation in tumors by epigenetic modification. Studies have however, increasingly shown that miRNAs play important roles in tumor occurrence, proliferation and invasion by interference with the expression of certain target cancer inhibitor genes. In addition, Sun *et al* indicated that the expression of endogenous RGS17 factor is regulated by miR-182 in human lung cancer cell lines; furthermore, ectopic expression of miR-182 significantly inhibits lung cancer cell proliferation and anchorage-independent cell growth (3). When bioinformatic tools were used to search for candidate genes related to miR-182, it was surprisingly found that miR-182 could putatively interfere with the expression of *CTTN* through sites located in the CTTN 3'-UTR region. We therefore, hypothesized that miR-182 may suppress human lung cancer A549 cells through down-regulation of *CTTN* expression.

Putative miRNA target sites in the 3'-UTR of CTTN mRNA were used to construct a miR-182 expression vector, which was then transfected into the human lung cancer cell line, A549. The luciferase activity assay indicated that the activity of the CTTN 3'-UTR site was significantly inhibited by miR-182, while that of the mutated CTTN 3'-UTR site was unchanged, which suggests that miR-182 targets *CTTN*. IF staining, qRT-PCR and Western blotting showed that cortactin protein expression decreased in the A549 cell line that was

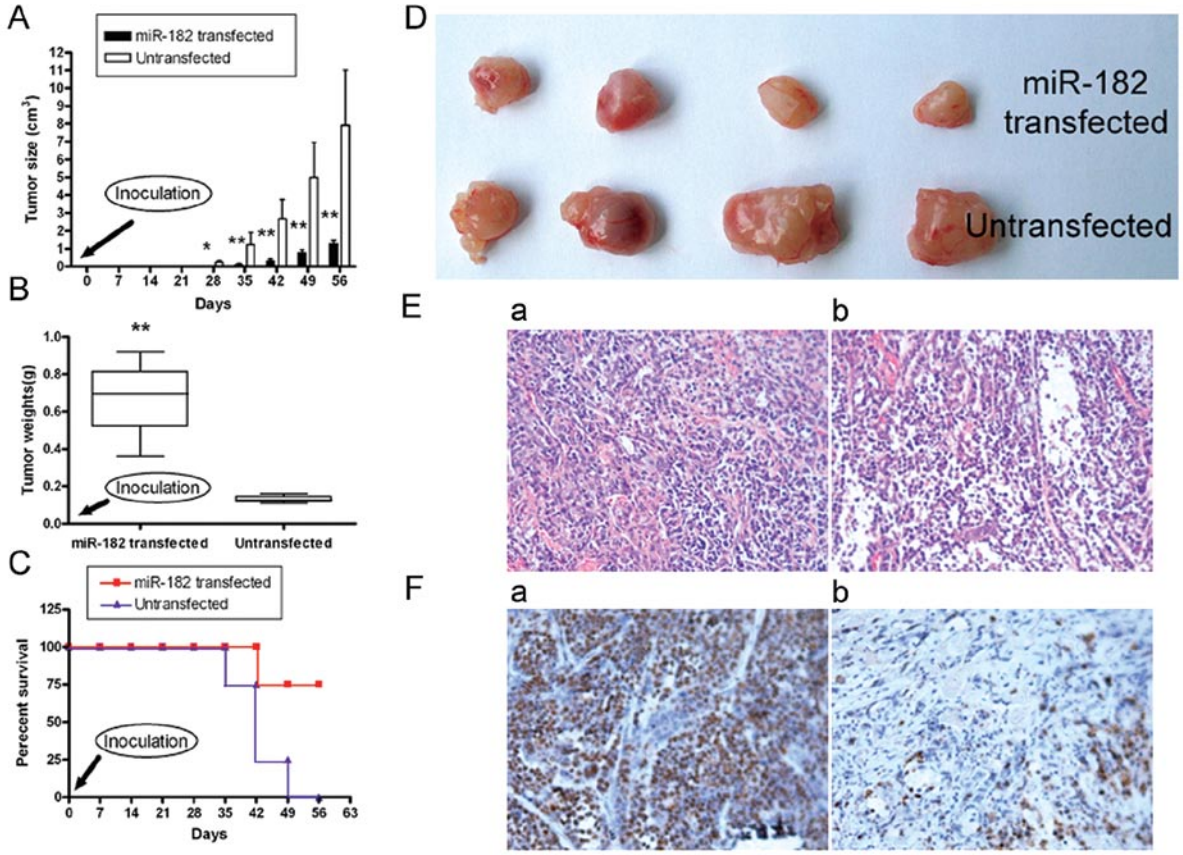

Figure 4. The results of xenograft experiments. (A) All mice of the untransfected group developed tumors approximately 28 days after injection. The tumors formed by the miR-182 transfected cells grew more slowly (*P<0.05 and **P<0.01 vs. untransfected cells; n=4). (B) The tumor weight of the untransfected group is heavier than those of the miR-182 transfected group, and miR-182 expression leads to a decrease in the tumor volume (**P<0.01 vs. untransfected; n=4). (C) Kaplan-Meier survival curves of nude mice inoculated with A549 cell lines showing marked differences between the miR-182 transfected and untransfected groups. (D) Macroscopic appearance of the excised tumors in the transfected miR-182 and untransfected groups. (E) Hematoxylin and eosin staining reveals the cellular heterogeneity in pathological sections of the excised tumor tissue (a) untransfected group; (b) miR-182 transfected group (original magnification, x100). (F) Immunohistochemistry shows that the tumors formed by (a) untransfected A549 cells are positive or strongly positive with Ki-67 stain; whereas (b) the tumors formed by miR-182 transfected cells are weakly positive (original magnification, x100).

transfected with the miR-182 expression plasmid, compared with those transfected with the mutant miR-182 expression plasmid or untransfected cells. Flow cytometry revealed that most of the miR-182 transfected cells were arrested in the G_0/G_1 phase of the cell cycle with reduced percentages in the S and G_2/M phases, which suggested that miR-182 expression could affect cell cycle regulation of lung cancer cells *in vitro*. Similarly, the MTT assay indicated that exogenous miR-182 expression inhibited the growth of the A549 cell line *in vitro* and the wound-healing assay showed that the miR-182 transfected cells migrated more slowly. Lastly, the results of xenograft experiments indicated that miR-182 expressed in the A549 cell line also suppressed tumor growth *in vivo* by promotion of apoptosis.

In contrast to a previous report that exogenous miR-182 expression in human lung cancer cell lines inhibited endogenous RGS17 protein expression, in this study it was found that *CTTN* expression was decreased in the miR-182 transfected cell line. The results not only suggest that the expression of cortactin was increased in the cell line, but also that miR-182 expression could influence endogenous *CTTN* expression. Taken together, these results suggest that the proliferation and invasion of human lung cancer cells may be related to miR-182 expression. Inhibition or loss of miR-182 expression may result in excessive cortactin expression, and thus, influence tumor growth. Further studies will be required to fully clarify the function of miR-182, but it may potentially play an important role in human lung cancer.

Acknowledgements

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