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Cul4A is an oncogene in malignant pleural mesothelioma

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Abstract

Cullin 4A (Cul4A) is important in cell survival, development, growth and the cell cycle, but its role in mesothelioma has not been studied. For the first time, we identified amplification of the *Cul4A* gene in four of five mesothelioma cell lines. Consistent with increased *Cul4A* gene copy number, we found that Cul4A protein was overexpressed in mesothelioma cells as well. Cul4A protein was also overexpressed in 64% of primary malignant pleural mesothelioma (MPM) tumours. Furthermore, knockdown of Cul4A with shRNA in mesothelioma cells resulted in up-regulation of p21 and p27 tumour suppressor proteins in a p53-independent manner in H290, H28 and MS-1 mesothelioma cell lines. Knockdown of Cul4A also resulted in G0/G1 cell cycle arrest and decreased colony formation in H290, H28 and MS-1 mesothelioma cell lines. Moreover, G0/G1 cell cycle arrest was partially reversed by siRNA down-regulation of p21 and/or p27 in Cul4A knockdown H290 cell line. In the contrary, overexpression of Cul4A resulted in down-regulation of p21 and p27 proteins and increased colony formation in H28 mesothelioma cell line. Both p21 and p27 showed faster degradation rates in Cul4A overexpressed H28 cell line and slower degradation rates in Cul4A knockdown H28 cell line. Our study indicates that Cul4A amplification and overexpression play an oncogenic role in the pathogenesis of mesothelioma. Thus, Cul4A may be a potential therapeutic target for MPM.

Keywords: Cul4A • amplification • mesothelioma • p21 • p27

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive cancer that originates mostly from the pleura of the lung and is associated with occupational exposure to asbestos, simian virus-40 (SV40) infection or genetic predisposition. About 3000 patients are diagnosed annually in the United States [1], mostly at the relatively late stage, making curative resection difficult. Despite aggressive treatment with radiation therapy or chemotherapy, the prognosis of MPM has remained poor in the past decades, with a median survival time of about 8–18 months [2]. Therefore, identifying molecular targets in MPM and developing new treatments are urgent needs.

Cul4A belongs to the family of evolutionally conserved cullin proteins, including seven related cullins (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5 and Cul7) [3]. In human cells, Cul4A forms a part of the multifunctional ubiquitin-protein ligase E3 complex by interacting with ring finger protein and damaged DNA binding protein (DDB1) [4]. Cullin4A is an important component of E3 ubiquitinin ligase family and ubiquinates a large number of substrates that are transferred to the 26S proteosoma for degradation. For example, Cul4A is a target molecule for neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), an ubiquitin-like protein [5] and ubiquitylates xeroderma pigmentosum group C and histone H2A [6]. Cul4A is an important regulator of the cell cycle and cell growth. Inactivation of Cul4A is associated with G1 cell-cycle arrest both in *Drosophila* and human cells [7], and overexpression of Cul4A increases cell growth in irradiated mammary

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epithelial cells [8]. Cul4A is also a critical gene for hematopoietic cell survival and development [9].

The *Cul4A* gene is amplified in breast [10] and liver [11] cancers and is associated with poor prognosis in node-negative breast cancer [12]. In addition, Cul4A overexpression may contribute to tumorgenesis and cancer development in cancer cells, because Cul4A has been observed in the ubiquitination and proteolysis of tumour suppressors, such as p21 [13], p27 [14], DDB2 [15] and p53 [16]. However, the role of Cul4A in mesothelioma has not been studied.

In this study, using fluorescence *in situ* hybridization (FISH) and Western blot analysis we observed that the *Cul4A* gene is amplified and the Cul4A protein is overexpressed in mesothelioma cell lines and MPM tissues. We also showed that knockdown of Cul4A with shRNA increases p21 and p27 proteins, and also induces cell-cycle arrest and growth inhibition in mesothelioma cells. The cell-cycle arrest and growth inhibition are reversed by siRNA down-regulation of p21 and/or p27 levels in Cul4A shRNA transfected mesothelioma cells.

Materials and methods

Cell lines and cell culture

Human mesothelioma cell lines were obtained as follows: NCI-H28, MSTO-211H from American Type Culture Collections (ATCC, Manassas, VA, USA), REN as a generous gift from Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA), NCI-H290 and MS-1 from NIH (Frederick, MD, USA). All mesothelioma cell lines were cultured in RPMI 1640 complete medium supplemented with 10% foetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml). LP9, a normal mesothelial cell line as described previously [17], was obtained from the Cell Culture Core Facility at Havard University (Boston, MA, USA). LP9 was cultured in M199 containing 15% FBS, 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, penicillin (100 IU/ml) and streptomycin (100 μg/ml). HCT116 p53-null ($^{-/-}$) colon cancer cells were cultured in McCoy's 5A complete medium supplemented with 10% FBS. All cells were cultured at 37°C and 5% CO2 in a humid incubator.

Tissues

Fresh MPM and adjacent normal pleural tissues were obtained from MPM patients undergoing surgical resection of the primary tumour after institutional review board approval and patients' signed consent were obtained. Tissue samples were kept at −180°C liquid nitrogen freezers before use, and final pathologic diagnosis was confirmed by a pathologist in the University of California, San Francisco, USA.

Fluorescence *in situ* hybridization analysis

FISH analysis was performed on metaphase slides of normal lymphocyte (Vysis, Downers Grove, IL, USA), LP9 cells and mesothelioma cell lines with a bacterial artificial chromosome (BAC) clone (RP11-391H12) targeting Cul4A gene at the chromosome 13q34 as described previously [11]. The BAC was labelled by nick translation with spectrum red deoxyuridine Triphosphate (dUTP) and hybridized to metaphase slides overnight. The chromosomes were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Genomic copy numbers of Cul4A were determined by digital image microscopy after FISH.

Cloning Cul4A shRNA into a retroviral vector

The shRNA sequences were designed from a pre-designed and pre-validated Cul4A siRNA (Ambion, Austin, TX, USA). The forward and reverse sequences were 5′-GATCCCCGGTTTATCCACGGTA AAGA TTCAAGAGATCTTTACCGTGGATAAACCTTTTTGGAAA-3′ and 5′-AGCT TTTCCAAAAAGGTTTATCCACGGTAAAGATCTCTTGAATCTTTA CCGTGGATAAACCGGG-3′, respectively. Steps for cloning oligonucleotides into pSUPER.retro.puro vector (Oligoengine, Seattle, WA, USA) were carried out according to manufacturer's protocol (www.oligoengine.com). After the forward and reverse oligonucleotides were annealed, they were then ligated into BglII and HindIII cleavage sites within the pSUPER.retro.puro vector digested with the same restriction enzymes. Recombinant vectors containing inserts were then transformed into One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA, USA). After selection in LB agar plate containing 50 μg/ml of ampicillin, colonies were examined for the presence of recombinant vectors *via* direct DNA sequencing analysis.

Retroviral production and transduction

Cul4A shRNA retroviral vectors were then transfected into the HEK 293 Phoenix ampho packaging cells (ATCC) by using Fu-GENE6 transfection reagent (Roche, Lewes, UK) to produce retroviral supernatants. Forty-eight hours after transfection, the supernatant was filtered through a 0.45 μm syringe filter. Retroviral infection was performed by adding filtered supernatant to mesothelioma cell lines cultured on 10 cm dishes with 50% confluent in the presence 8 ug/ml of polybrene (Sigma, St. Louis, MO, USA). Six hours after infection, medium was changed with fresh medium and infected cells were allowed to recover for 48 hrs. Infected cells were selected by adding 1 μg/ml puromycin (Sigma) to the culture medium for 48 hrs and then maintained in complete medium with 0.5 μg/ml puromycin. Empty retroviral-infected stable cell lines were also produced by the above protocols. Down-regulation of Cul4A expression was confirmed by RT-PCR and Western blot analysis.

Western blot analysis

Whole protein was extracted by M-PER mammalian protein extraction reagent from cell lines and T-PER tissue protein extraction reagent (Pierce, Rockfold, IL, USA) from MPM tissues, added with Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA, USA) and Complete Protease Inhibitor Cocktails (Roche) according to manufactures' protocols. Proteins were separated on 4–15% gradient SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The following primary antibodies were used: Cul4A (Abcam, Cambridge, MA, USA), p27, p21 (Santa Cruz, CA, USA), p53 and β-actin (Sigma). After antigen antibody complexes were bound to indicated secondary antibodies, an enhanced chemiluminescence blotting analysis system (GE Healthcare Life Sciences, Piscataway, NJ, USA) was used to detect antigen-antibody complexes.

Cell cycle analysis

H290, H28 and MS-1 stably transfected cells were synchronized by serum starvation for 48 hrs, and then incubated with complete medium for 0, 2 and 8 hrs. They were harvested after treatment with 10 μM of 5-bromo 2-deoxyuridine (BrdU) for 30 min. at the end of each incubation time. BrdU Flow Kit (BD, San Jose, CA, USA) was used for cell cycle analysis according to the manufacture's protocol. FITC-conjugated BrdU antibody was used to label BrdU-incorporated cells and 7-amino-actinomycin D for total DNA content. Accuri's C6 Flow Cytometer(tm) System and CFlow Plus Software (Accuri Cytometers, Ann Arbor, MI, USA) were used for flow analysis.

Colony formation assay

Stably transfected H290, H28 and MS-1 cells (5×10^2) were plated in 10 cm culture dishes and incubated in complete medium for 14 days. The colonies were then stained with 0.1% crystal violet, and colonies with more than 50 cells were counted. Results were expressed as relative colony formation: percentage of the number of colonies relative to the empty vector transfected controls. Three independent experiments were performed.

Transfection of siRNA

Pre-designed and validated anti-p21 and anti-p27 siRNAs were purchased from Daharmacon (Arvada, CO, USA). Universal negative control siRNA was purchased from Invitrogen. Transfection was performed using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's manual. Cells were plated in 60 mm dishes in antibiotic-free media and transfection was performed with cells at 60% confluence with a final concentration of 30 nM for each siRNA. At 72 and 96 hrs after transfection, cells were analysed for protein expression and cell cycle change.

Cloning Cul4A gene into a retroviral vector

The pBabe-puro retroviral vector was used to transduce the *Cul4A* gene. The cDNA of wild-type Cul4A was amplified by PCR using SuperScript™ One-Step RT-PCR kit (Invitrogen), cloned into pcDNA3.1$^+$/myc-His vector (Invitrogen) *via* EcoRV and XhoI restriction sites. The EcoRV and PmeI fragment was then inserted into the SnaBI site of pBabe-puro. The Cul4A sequence was confirmed by direct DNA sequencing analysis. The upstream and downstream PCR primers for cloning are 5′-CCGATATC ACCATGGCG-GACGAGG-3′ and 5′-GATGTCGACAGGC CACG TAGTGGTAC-3′, respectively. Retroviral production and transduction were performed as described in 'Materials and methods'.

Protein degradation assay

The stably transfected H28pBabe (Cul4A overexpression) and H28pSuper (Cul4A knockdown) cells were treated with 50 μg/ml cycloheximide and then harvested at 0, 1, 2 and 4 hrs. The cellular lysates were then analysed by Western blot analysis.

Statistical analysis

The data shown represent mean values ± S.D. Student's t-test was used to compare results between control and experimental groups with respect to cell cycle analysis and colony formation assay. Statistical analysis was carried out using SPSS (version 10.0, Chicago, IL, USA). Two-sided *P*-values <0.05 were considered significant.

Results

Amplification of the Cul4A gene in mesothelioma cells

To determine the gene copy numbers of Cul4A in normal and mesothelioma cells, we carried out FISH analysis using a BAC probe (RP11-391H12) that contains the *Cul4A* gene. The precise foci of the BAC probe were confirmed by FISH to normal metaphase chromosomes of human lymphocyte (Fig. 1A). Two FISH signals were seen in normal lymphocytes and normal mesothelial (LP9) cells and three FISH signals were seen in MS-1(Fig. 1B) and H290 malignant mesothelioma cells (Fig. 1C). Of the five mesothelioma cell lines evaluated by FISH, four (MS-1, H290, 211H and H28) showed increased gene copy numbers of Cul4A compared to normal lymphocyte and mesothelial cells (Fig. 1D).

Cul4A is overexpressed in mesothelioma cells and MPM tissues

Since gene copy numbers of Cul4A were increased in mesothelioma cells, we evaluated the expression of Cul4A mRNA by Western blot analysis. Expression levels of Cul4A protein were also higher in the four malignant mesothelioma cell lines than in LP9 cells by Western blot analysis (Fig. 2A). We further examined Cul4A protein expression in eight primary MPM tumours using LP9 cells and normal pleural tissue from one of the eight patients as normal controls. CUL4A protein expression was increased in 9 of 14 (64.3%) specimens of MPM (Fig. 2B).

Down-regulation of Cul4A with shRNA in mesothelioma cells up-regulates p21 and p27 proteins

To further study the possible mechanism of Cul4A on mesothelioma development, we down-regulated Cul4A expression in H290, H28 and MS-1 mesothelioma cells using Cul4A specific shRNA. The lysates from these stably transfected cells were analysed for expression of p53, p21 and p27 proteins, which are important regulators of the cell cycle. The shRNA effectively reduced Cul4A protein level in H290, H28 and MS-1 cells (Fig. 3A–C). Down-regulation of Cul4A increased the levels of p21

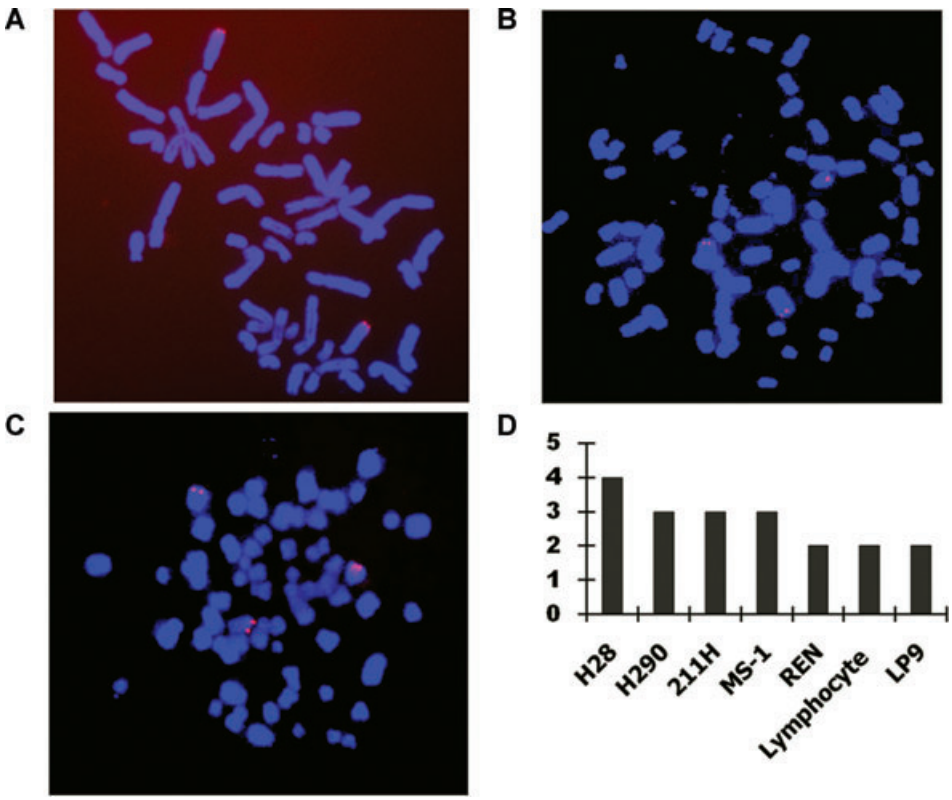

Fig. 1 Cul4A gene is amplified in mesothelioma cells. Repre-sentative pictures of FISH assay for the copy numbers of the *Cul4A* gene. The probe is mapped on the chromosome 13q34 (red dots). (**A**) Normal lymphocyte. (**B**) MS-1 mesothelioma cells. (**C**) H290 mesothelioma cells. (**D**) The copy numbers of the *Cul4A* gene of five mesothelioma cell lines, one normal mesothelial cell line (LP9) and lymphocytes were expressed as a histogram.

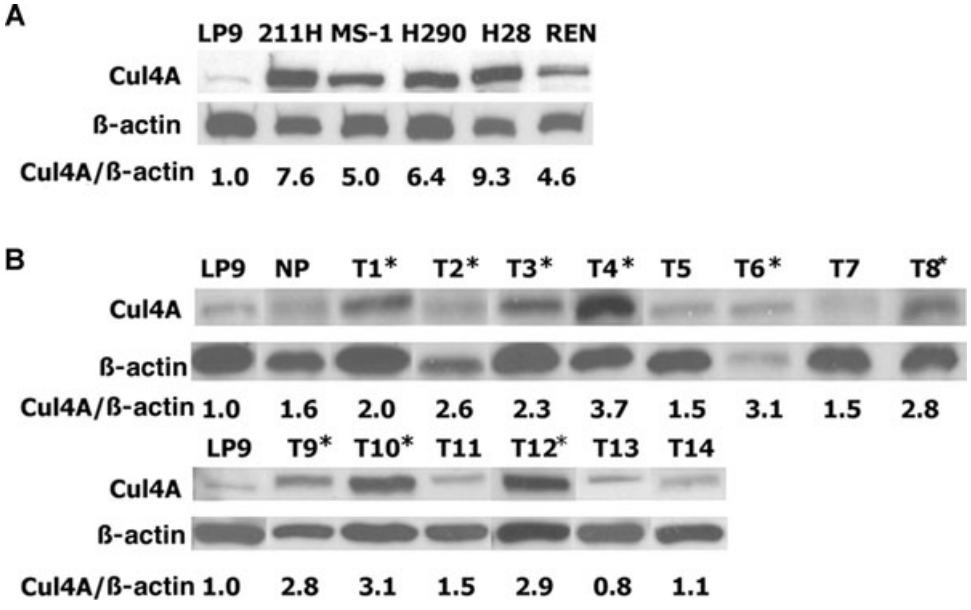

Fig. 2 Cul4A is overexpressed in mesothelioma cells and MPM tumors. (**A**) Western blot analysis results of Cul4A protein expression in normal pleural cell line (LP9) and five mesothelioma cell lines. (**B**) Western blot analysis results of Cul4A protein expression in 14 malignant mesothelioma tissue samples. Internal control: β-actin. Density of Cul4A bands was quantified by normalization to β-actin using LP9 as a normal control. *Denotes higher expression of Cul4A compared to normal pleural tissue. NP, normal pleural tissue; T, mesothelioma tissue.

and p27 protein expression compared to protein levels in empty vector transfected cells in all cell lines studied (Fig. 3A–C). The level of p53 protein was only increased in MS-1 cells (Fig. 3C), suggesting Cul4A regulates p21 and p27 in a p53-indendent manner in p14ARF-null H290 and H28 cells. To confirm this, HCT116 p-53 null ($^{-/-}$) colon cancer cells were transfected with Cul4A shRNA, and increased levels of p21 and p27 proteins were also noted after Cul4A shRNA knockdown (Fig. 3D).

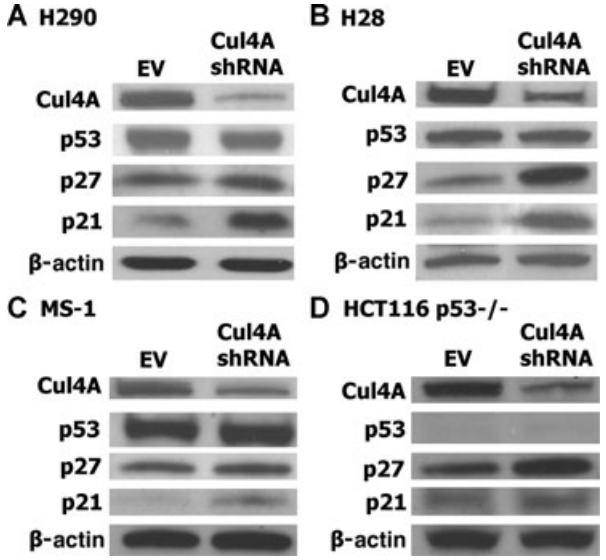

Fig. 3 Down-regulation of Cul4A with shRNA in mesothelioma cells up-regulates p21 and p27 proteins. Western blot analysis of Cul4A, p53, p21, p27 and β-actin proteins after Cul4A shRNA knockdown. (**A**) H290 mesothelioma cells. (**B**) H28 mesothelioma cells. (**C**) MS-1 mesothelioma cells. (**D**) HCT116 $p53^{-/-}$ colon cancer cells. EV, empty vector.

Down-regulation of Cul4A induces cell cycle arrests in G0/G1 and inhibits mesothelioma cell growth

Next, we examined whether down-regulation of Cul4A affects cell cycle and proliferation. Cell cycle analysis was carried out using flow cytometry in H290, H28 and MS-1 cells with Cul4A shRNA knockdown. A time-course study showed significantly more G0/G1 cells in Cul4A knockdown H290 and H28 cells at 0 hr, 2 hrs and 8 hrs and Cul4A knockdown MS-1 cells at 8 hrs (Fig. 4A). The effects of Cul4A knockdown on the growth of mesothelioma cells were further evaluated by anchorage-dependent colony formation assays on H290, H28 and MS-1 cells. Significantly lower colony numbers were noted in Cul4A knockdown H290, H28 and MS-1 mesothelioma cells than in controls (Fig. 4B–D).

G0/G1 arrest after Cul4A down-regulation is reversed by siRNA knockdown of p21 and/or p27 in mesothelioma cells

To determine roles of p21 and p27 in G0/G1 cell cycle arrest after Cul4A down-regulation, cell cycle analysis was performed at 72 and 96 hrs after transient transfection of p21 and/or p27 siRNA in H290 cells transfected with Cul4A shRNA. Both p21 and p27 siRNA resulted in more than an 80% decrease in p21 and p27 protein levels at 72 hrs after transfection (Fig. 5A). Compared to the cell cycle of negative control siRNA transfected cells, G0/G1 phases were significantly decreased in p21 knockdown and both p21 and p27 knockdown cells after 72 hrs (Fig. 5B) and 96 hrs (Fig. 5C), and in p27 knockdown cells after 96 hrs (Fig. 5C). S phases were significantly increased in p21 knockdown cells, p27 knockdown cells, and both p21 and p27 knockdown cells after 96 hrs (Fig. 5C). Notably, compared to the cell cycle of empty vector transfected H290 cells, H290 cells transfected with Cul4A shRNA showed partially reversed effects of G0/G1 arrest at 96 hrs after p21 and/or p27 siRNA transfection (Fig. 5C).

Overexpression of ectopic Cul4A in mesothelioma cells down-regulates p21 and p27 proteins and promotes cells growth

To further confirm the role of Cul4A in mesothelioma, we overexpressed ectopic *Cul4A* gene in stably transfected H28 mesothelioma cells. In the contrary to Cul4A knockdown, overexpression of ectopic Cul4A in H28 mesothelioma cells down-regulates p21 and p27 proteins (Fig. 6A). Furthermore, significantly more colony numbers were noted in Cul4A overexpressed stable H28 cells than in controls (Fig. 6B).

Cul4A promotes degradation of p21 and p27 proteins in mesothelioma cells

We next tried to evaluate the role of Cul4A on the degradation of p21 and p27 proteins in mesothelioma cells. In Cul4A overexpressed H28 cells (H28 pBabe Cul4A), both p21 and p27 proteins showed faster degradation rates compared to empty vector transfected controls (H28 pBabe EV) (Fig. 7A), following the addition of cycloheximide. In the contrary, in Cul4A knockdown H28 cells (H28 pSuperCul4A), both p21 and p27 proteins showed slower degradation rates compared to empty vector transfected controls (H28 pSuper EV) (Fig. 7B).

Discussion

In this study, we showed for the first time that the *Cul4A* gene is amplified in human mesothelioma cell lines. Consistent with gene amplification, overexpression of Cul4A protein was observed in mesothelioma cell lines and MPM tissues. Further knockdown of Cul4A by shRNA in mesothelioma cells also inhibited cells growth. In the contrary, overexpression of ectopic Cul4A in mesothelioma cells promoted cells growth. Thus, our results indicate that amplification of *Cul4A* gene may be an important oncogenic event in mesothelioma development.

We also observed that down-regulation of Cul4A with shRNA causes cell cycle arrest and growth inhibition through up-regulation

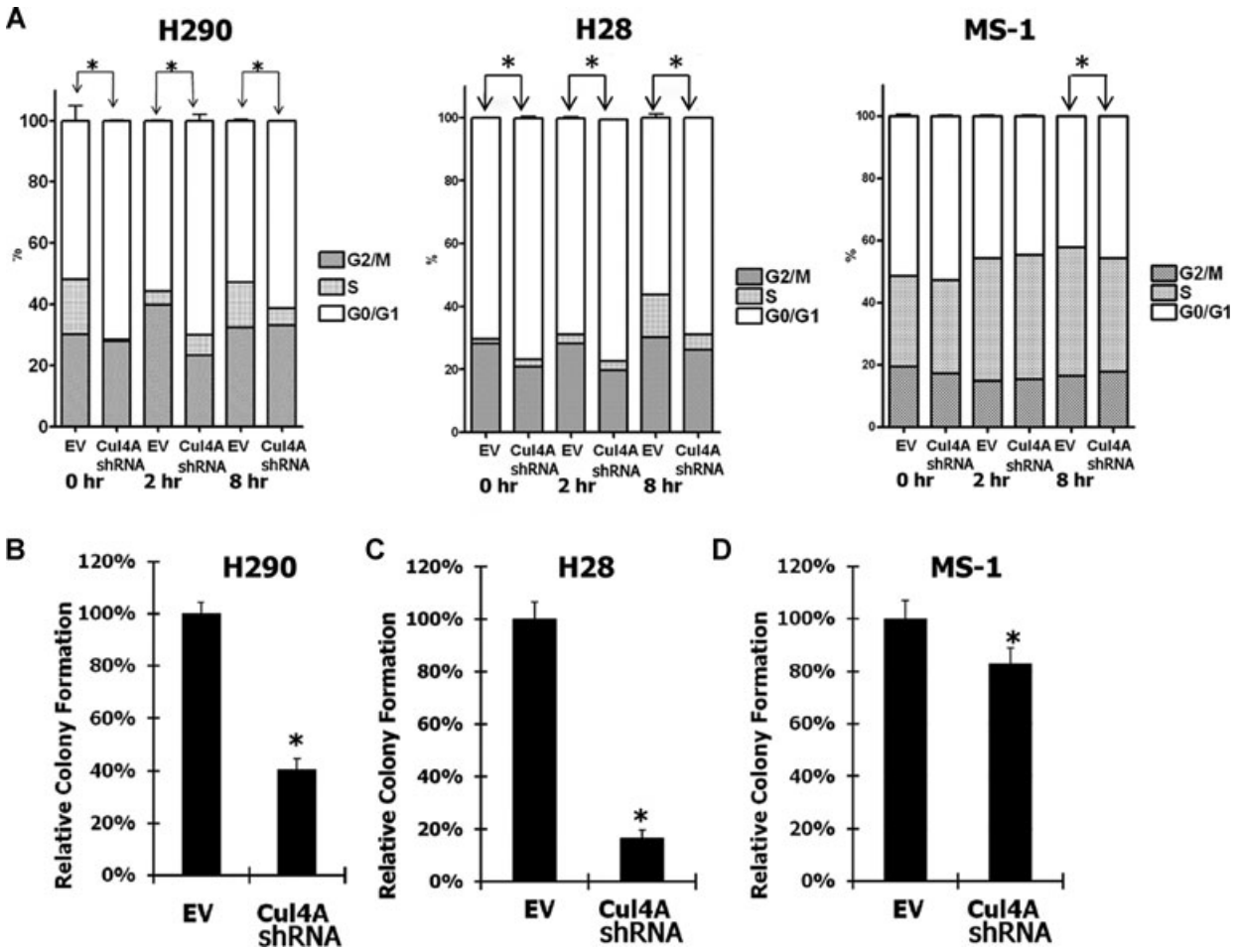

Fig. 4 Down-regulation of Cul4A induces cell cycle arrests in GO/G1 and inhibits mesothelioma cell growth. (**A**) Cell cycle analysis results in H290, H28 and MS-1 mesothelioma cells after Cul4A shRNA knockdown. (**B–D**) Colony formation assay of H290, H28 and MS-1 mesothelioma cells. Relative colony formation in both cell lines was expressed as percentage normalized to empty vector transfected control group (bar ± S.D.) in triplet experiments. *Denotes $P < 0.05$. EV, empty vector.

of p21 and p27 proteins in a p53-independent manner in mesothelioma cells. Several lines of evidence support this hypothesis. First, down-regulation of Cul4A with shRNA increased the levels of p21 and p27 in the p14ARF-null mesothelioma cell lines (H290 and H28) we studied, whereas p53 protein levels were unchanged in these cell lines. Second, down-regulation of Cul4A in the p53-null HCT-116 cells increased p21 and p27 protein levels. Third, a very recent report provided the evidence of direct degradation of p21 through a Cul4-DDB1^{Cdt2} pathway [13], suggesting that up-regulation of p21 may be due to decreased proteolysis of p21 after Cul4A knockdown without participation of p53. Previous work has also shown that Cul4A-DDB1 associates with SCF^{SKP2}, and participates in p27 degradation between G1/S and S phase [18]. In short, these studies suggest that Cul4A can regulate p21 and p27 protein levels in a p53-independent manner, probably through direct ubiquitination and proteolysis of p21 and p27 proteins. Furthermore, the results of our colony formation assay provide additional evidence that the p14ARF-null mesothelioma cell lines are more dependent on Cul4A regulation than is the mesothelioma cell line (MS-1) with an intact p14ARF-p53 pathway. More dramatic inhibition of colony formation was noticed in the two p14ARF-null cell lines: H290 and H28, whereas only modest inhibition was noticed in the MS-1 cell line. MS-1 cells, which still retain expression of p14ARF [19], showed increased p53 and p21 after knockdown of Cul4A, indicating that up-regulation of p21 may occur in both a p53-dependent and p53-independent manner after knockdown of Cul4A. Taken together, our findings suggest that Cul4A shRNA may be more effective in cancer cells that lack the intact p14ARF-p53 pathway. Since most human cancer cells lack the

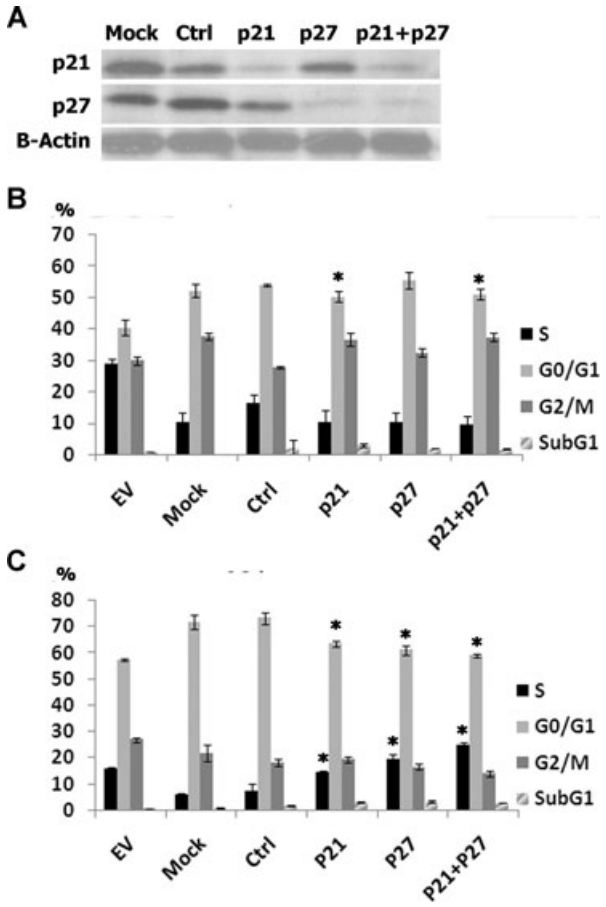

Fig. 5 G0/G1 arrest after Cul4A down-regulation is reversed by siRNA knockdown of p21 and/or p27 in mesothelioma cells. (**A**) Western blot analysis of p27, p21 and β-actin proteins at 72 hrs after siRNA transfection in Cul4A shRNA stably knockdown H290 cells. (**B**) Cell-cycle analysis at 72 hrs after siRNA transfection and (**C**), at 96 hrs after transfection. Percentages of cells in S, G0/G1, G2/M and sub-G1 phases were expressed as bar ± S.D. in triplet experiments. *Denotes $P < 0.05$. EV: empty vector. Mock: without siRNA. Ctrl: universal negative control siRNA. p21, p21 siRNA; p27,p27 siRNA; p21+p27, p21 and p27 siRNA. Cells transfected with universal negative control siRNA were used as control groups.

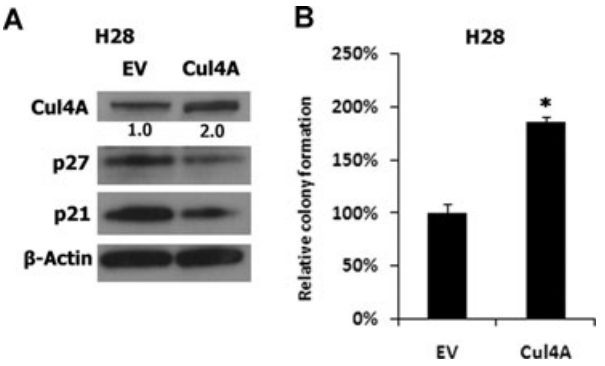

Fig. 6 Overexpression of ectopic Cul4A in mesothelioma cells down-regulates p21 and p27proteins and promotes cells growth. (**A**) Western blot analysis of Cul4A, p27, p21 and β-actin proteins in ectopic Cul4A stably transfected H28 mesothelioma cells. Density of Cul4A bands was quantified by normalization to β-actin using empty vector transfected group as a normal control. (**B**) Colony formation assay of H28 mesothelioma cells. Relative colony formation was expressed as percentage normalized to empty vector transfected control group (bar ± S.D.) in triplet experiments. *Denotes $P < 0.05$. EV, empty vector.

intact pathway, the Cul4A complex may be a potential interesting target for cancer therapy.

In this study, up-regulation of p21 and p27 proteins after Cul4A knockdown and down-regulation of p21 and p27 proteins after expression of ectopic Cul4A were observed. Our data suggests that both p21 and p27 proteins are at least partially regulated by Cul4A. Both p21 and p27 are important regulators of cell cycle and cell growth. p21 is a cyclin-dependent kinase inhibitor encoded by the growth inhibitory gene *p21*$^{waf1/cip1}$, and exerts its roles in cell-cycle arrest. Deficiency of p21 protein is associated with abrogation of cells that undergo G1 arrest after DNA damage in p21 knockout mice [20] and colon cancer cells [21]. Overexpression of p21 in hamster BHK21 cells causes cell cycle arrest in G1 phase, and reduces cell growth and DNA synthesis [22]. In addition to p21 overexpression, we noted up-regulation of p27 after Cul4A knockdown. p27 is also a cyclin-dependent kinase inhibitor, and its expression inversely correlates with poor patient prognosis in a large variety of cancers [23–25]. In mouse models, p27 has been noted to be a haplo-insufficient tumour suppressor [26]. It is tightly regulated in G0, G1 and S phases, and is elevated in G0 phase or growth factor-depleted cells. In contrast, degradation of p27 has been noted as cells are released from G1 and reaches the lowest levels in the late G1 and S phases [27]. Furthermore, overexpression of p27 was found to prevent activation of cyclin-dependent kinases and entry into the S phase of the cell cycle [28]. In replicating cells, the well-known p27 regulatory pathway involves proteasomal degradation of p27, which requires polyubiquitination by the SCF^{skp2}-E3 ligase in late G1 and early S phase [29]. Previous work has shown that inactivation of the Cul4A ubiquitin E3 ligases pathway by siRNA knockdown of Cul4A is associated with p27 stabilization and p27-dependent G1 cell cycle arrest in human cancer cells [7]. However, the association of Cul4A knockdown and p21 up-regulation in human cancer cells has not been elucidated.

Our study showed that down-regulation of Cul4A causes G0/G1 cell cycle arrest and then growth inhibition in mesothelioma cells, and that siRNA knockdown of p21 and/or p27 restored G0/G1 cell cycle arrest in Cul4A knockdown mesothelioma cells. Interestingly, knockdown of p21 and/or p27 at least partially reversed in Cul4A stably knockdown mesothelioma cells. Importantly, up-regulation of p21 seems also to have effects on G0/G1 arrest in Cul4A knockdown mesothelioma cells, because restoration effects of G0/G1 arrest appear earlier and

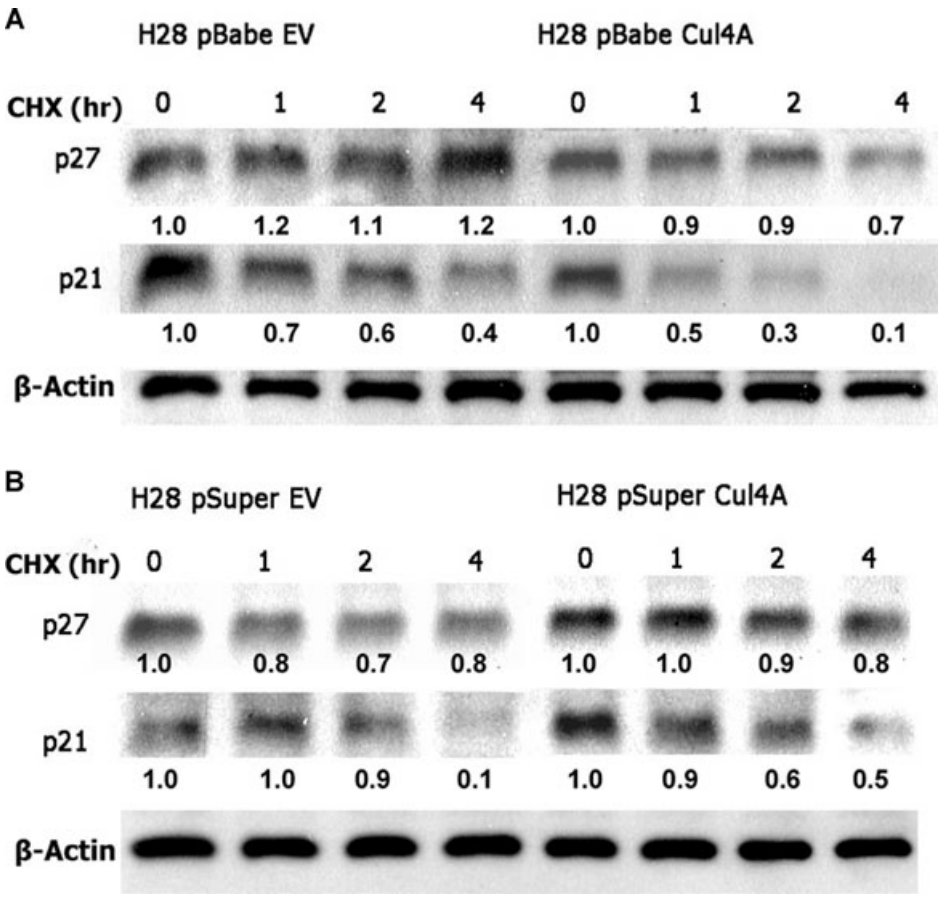

Fig. 7 Cul4A promotes degradation of p21 and p27 proteins in mesothelioma cells. (**A**) Western blot analysis of p27, p21 and β-actin proteins in ectopic Cul4A stably transfected H28 mesothelioma cells (H28pBabeCul4A) and empty vector transfected controls (H28pBabeEV). (**B**) Western blot analysis of p27, p21 and β-actin proteins in Cul4A stably knockdown H28 mesothelioma cells (H28pSuperCul4A) and empty vector transfected controls (H28pSuperEV). Cells were treated with cycloheximide for the time periods indicated. Densities of p21 and p27 bands were quantified by normalization to β-actin using the 0 hr group as a normal control.

stronger after siRNA knockdown of p21 than after siRNA knockdown of p27. Our results imply that Cul4A may have a role in promoting cell renewal by avoiding G1 phase through regulation of both p21 and p27.

In summary, we have shown for the first time that the *Cul4A* gene is amplified and overexpressed in mesothelioma cells. Through shRNA knockdown and overexpression of ectopic Cul4A studies, we also showed that Cul4A controls the cell cycle and cell growth through p21 and p27 tumour suppressors in a p53-independent manner in mesothelioma cells. Our study demonstrates that the amplification of Cul4A may be an oncogenic event in mesothelioma development. Future studies on Cul4A as prognostic and therapeutic targets in MPM are warranted.

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