Silencing P12^{CDK2AP1} with a lentivirus promotes HaCaT cell proliferation

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Abstract. The tumor suppressor P12^{CDK2AP1} negatively regulates cyclin-dependent kinase 2 (CDK2) activities and suppresses DNA replication. Notably, P12^{CDK2AP1} is known to be downregulated in head and neck squamous cell carcinomas (HNSCCs). Silencing of specific gene expression by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) using expression vectors and retroviruses has become a powerful tool for the genetic analysis of mammalian cells. In the present study, we utilized lentivirus-mediated shRNA for functional gene knockdown in normal human skin keratinocytes (HaCaT) cells in order to assess the potential role of P12^{CDK2AP1} in HNSCCs. Lentivirus-mediated RNA interference (RNAi) effectively reduced endogenous P12^{CDK2AP1} expression in HaCaT cells and significantly promoted HaCaT cell proliferation *in vitro*. Lentiviral vectors have the ability to infect dividing and non-dividing cells as well as to achieve long-term multilineage gene expression. Thus, additional studies are needed to investigate the use of such vectors as a therapeutic tool for the delivery of siRNAs.

Introduction

The various types of of head and neck cancer represent ~5% of all the cancer types, and 80-90% of these tumors constitute squamous cell carcinomas. Despite the rapid progress in the diagnosis and treatment of this disease, the overall 5-year survival of this type of cancer is among the lowest of the most common tumor types (1). Most types of human oral cancer (63.8%) exhibit either a loss or significant reduction of P12^{CDK2AP1} expression. Decreased P12^{CDK2AP1} expression has been shown to correlate with increased tumor invasion, risk of lymph node metastases, and decreased survival in patients with oral squamous cell carcinoma (2).

Human cyclin-dependent kinase 2 associated protein 1 (*CDK2AP1)* is a highly conserved gene. No mutations have been found in oral and esophageal types of cancer (3,4). P12^{CDK2AP1}, originally known as deleted in oral cancer-1 (DOC-1), is identified and cloned from the Syrian hamster oral cancer model (5) and acts as a growth suppressor by negatively regulating the activity of cyclin-dependent kinase 2 (CDK2) (6). Human P12^{CDK2AP1} associates with DNA polymerase α/primase and mediates the phosphorylation of the large p180 catalytic subunit, suggesting that it is a potential regulator of DNA replication in the S phase of the cell cycle (3). Moreover, the ectopic expression of P12^{CDK2AP1} in culture cells leads to growth suppression, arrests cells in the G1 phase of the cell cycle and suppresses DNA replication (6,7). Several functional studies support the role of P12^{CDK2AP1} as a growth suppressor (3,5,6,8).

RNA interference (RNAi) is an evolutionarily conserved mechanism of sequence-specific post-transcriptional gene silencing mediated by double-stranded RNA molecules that match the sequence of the target gene (9). Investigators have focused on HIV-1-derived lentivirus vectors due to their ability to deliver therapeutic transgenes into a wide range of difficult-to-transfect target tissues without inducing significant humoral immune responses (10-12). In this study, we constructed a lentivirus vector for the expression of small interfering RNAs (siRNAs) in mammalian cells and demonstrated its ability to efficiently downregulate the expression of CDK2AP1 protein. The present study was undertaken to elucidate the role of P12^{CDK2AP1} in cell proliferation. Results

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demonstrated that P12^{CDK2AP1} silencing promotes cell proliferation *in vitro*.

Materials and methods

Cells, cell culture and materials. HEK293T and normal human skin keratinocytes (HaCaT) cells were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Karlsruhe, Germany) and incubated in a humidified 37˚C, 5% CO_2 incubator. The primary antibody of P12^{CDK2AP1} was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). GAPDH antibody was purchased from Millipore (Billerica, MA, USA). SYBR-Green PCR Master mix for the quantification of *CDK2AP1* mRNA was obtained from Takara (Kyoto, Japan) and oligonucleotides from Sangon (Shanghai, China).

Construction of plasmid vector for the expression of siRNA. The sequence of short hairpin RNA (shRNA) targeting the human *CDK2AP1* gene was 5'-GATCTCAGTTCTGCGT TTA-3', corresponding to the coding region positions of *CDK2AP1* mRNA. The control plasmid was obtained from GenePharma Co., Ltd. (Shanghai, China). The human U6 promoter was amplified from genomic DNA and cloned in the *Age*I and *Eco*RI sites of the pGCSIL-GFP plasmid (GeneChem, Shanghai, China). The oligonucleotides which were previously shown to induce RNAi against *CDK2AP1* were cloned downstream of the U6 promoter. The sequence of the oligonucleotide (top strand) was: 5'-CcggtaGATCTCAGT TCTGCGTTTATTCAAGAGATAAACGCAGAACTGAGA TCta-TTTTTg-3'. The underlined sequence corresponds to nucleotides of human open reading frame for *CDK2AP1*. The sequence of the construct was confirmed by automated fluorescent sequencing.

Construction of lentivirus vector for siRNA delivery. A self-inactivating lentivirus vector containing a cytomegalovirus (CMV)-driven enhanced green fluorescent protein (EGFP) reporter and a U6 promoter upstream of cloning restriction sites (*Age*I and *Eco*RI) was purchased from GeneChem Co., Ltd. A control shRNA unrelated to human gene sequences was used as a negative control. We constructed 3 si-*CDK2AP1* lentiviruses, KD-1, KD-2 and KD-3, targeting human *CDK2AP1* mRNA. The following custom primers containing *Age*I and *Eco*RI sites at their 5' termini were used for PCR amplification: 5'-CCTATTTCCCATGATTCCT TCATA-3' and 5'-GTAATACGGTTATCCACGCG-3'. The resulting PCR fragment was digested with *Age*I and *Eco*RI and cloned into *Age*I- and *Eco*RI-digested pLenti vector. Oligonucleotides for constructing the negative control and 3 si-*CDK2AP1* lentiviruses were synthesized from Sangon. Correct insertions of shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing. KD-3 was identified as the most efficient and was selected for this study.

Lentiviral vector infection in cultured HaCaT cells. Cells were replated at 5x10^3 cells/well in 96-well plates along with recombinant lentivirus encoding for shRNA against *CDK2AP1* at different multiplicity of infections (MOIs) in serum-free growth medium containing 5 μg/ml polybrene at 37˚C and 5% CO_2. After 4 h, serum-containing growth medium was added to the cells, and there was complete replacement of growth medium after 48 h. After 3 days of post-infection, reporter gene expression was examined using fluorescent microscopy.

Table I. Primer sequences and the length of products for real-time RT-PCR.

Gene name	Oligonucleotide sequence	Product size (bp)
CDK2AP1	F: AAGAGCAACCCACCAAACC	92
	R: ATCAACTTACAATAAACGCAGAAC	
β-actin	F: GGCGGCACCACCATGTACCCT	202
	R: AGGGGCCGGACTCGTCATACT	

Real-time reverse transcription (RT)-PCR assay. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The expression of *CDK2AP1* mRNA was detected using the SYBR-Green miRNA assay (Takara) (13), and normalized using the $2^{-\Delta\Delta Ct}$ method (14) relative to human *β-actin* (Sangon). Primer sequences and the size of the products are shown in Table I. To ensure specificity of the PCR product amplification, the melting curves for standard and sample products were analyzed. All the real-time RT-PCR assays were performed three times.

Western blot analysis. Cells were seeded in 6-well plates and infected after 12 h. The cells were harvested 3 days following infection, washed once in phosphate-buffered saline (PBS) and lysed. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Aliquots (60 μg) were separated on a 15% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with the specific antibody followed by peroxidase-conjugated secondary antibodies. An enhanced chemiluminescence detection solution was then applied (Pierce). The relative protein level in different groups was normalized to the signal intensity of GAPDH. Quantitative analysis of the blots was performed using the NIH-ImageJ program.

Measurement of cell growth using the methyl thiazolyl tetrazolium (MTT) assay. HaCaT cells were seeded at a density of 2x10^3 cells/well in 96-well plates containing 0.2 ml DMEM (with 7% FBS) and cultured for 9 days. During this period, the medium was replaced with fresh complete medium every 3 days. Six wells from each group were selected randomly for the MTT assay each day. After 4 h of incubation, the reaction was stopped by adding 150 μl of dimethyl sulfoxide (DMSO; Sigma) to each well, followed by a 10-min incubation. The percentage of viable cells was determined by measuring the absorbance at 490 nm on a multiscanner reader (TECAN-spectra mini; Tecan Austria GmbH, Grödig, Austria). Cell growth curves were drawn by using average absorbance at 490 nm from 3 independent experiments.

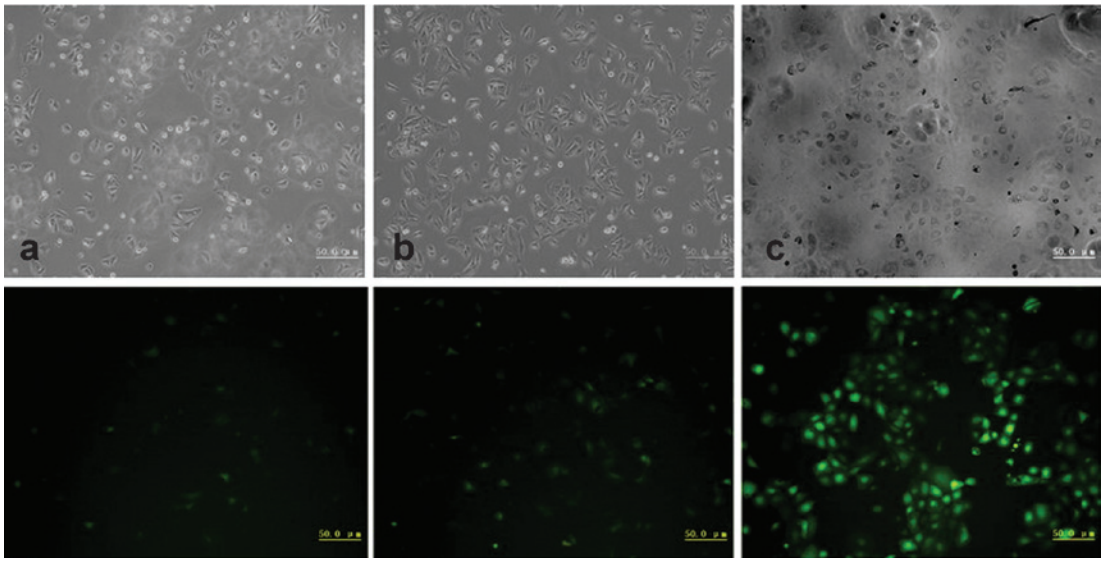

Figure 1. Determination of lentiviral infection efficiency in HaCaT cells. Infection efficiency was estimated 3 days following infection at the indicated MOIs. EGFP expression was observed under light (top panels) or fluorescence microscopy (bottom panels). (a) MOI=1; (b) MOI=20; (c) MOI=40. Scale bar, 50 μm. HaCaT cells were demonstrated to have increased EGFP expression that peaked when MOI=40.

Flow cytometric analysis of cell cycle. The cells (72 h) were harvested, washed twice with ice-cold PBS, fixed with 70% ethanol overnight at 4˚C, washed and resuspended in 100 μl of PBS containing a final concentration of 50 μg/ml RNase A for 30 min at room temperature. The cells were then stained with 20 μg/ml propidium iodide in a final volume of 300 μl for 20 min. DNA content and cell cycle were analyzed using a flow cytometer (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA), and ModFit and CellQuest software. The experiments were conducted three times.

Statistical analysis. The experiments were performed in triplicate and standard deviations were calculated. The statistical analysis was performed by a two-tailed paired Student's t-test or one-way ANOVA using SPSS 11.5 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Infection efficiency of lenti-shCDK2AP1 in HaCaT cells. To determine the lentiviral infection efficiency in HaCaT cells, EGFP expression was examined by microscopy at different MOIs 3 days after infection. The efficiency of lentiviral vector infection in HaCaT cells was >90% at an MOI of 40 (Fig. 1). The percentage of EGFP-expressing cells remained relatively unchanged when MOI was >40. Based on these results, an MOI of 40 was chosen. Our data suggested that lentivirus shRNA vector pGCSIL-GFP had high efficiency for infecting HaCaT cells.

CDK2AP1 mRNA and P12^{CDK2AP1} expression is efficiently inhibited in lenti-shCDK2AP1-infected HaCaT cells. The subconfluent cells were infected with either negative control or sh*CDK2AP1* lentivirus. Two days post-infection, the cells were collected and lysed for analysis. Real-time RT-PCR results showed the mRNA level of *CDK2AP1* in HaCaT cells infected with lenti-sh*CDK2AP1* to be ~80% lower compared to that of HaCaT control cells (Fig. 2A). As shown in Fig. 2B and C, the level of P12^{CDK2AP1} expression was markedly reduced in HaCaT cells infected by lenti-sh*CDK2AP1* compared to that in the control cells, a fact which was consistent with the downregulation of *CDK2AP1*. This study indicated that lentiviral siRNA was able to provide highly efficient and specific *CDK2AP1* knockdown in HaCaT cells.

P12^{CDK2AP1} knockdown significantly promotes HaCaT cell growth in vitro. To determine whether silencing P12^{CDK2AP1} by RNAi had a stimulative effect on HaCaT cell growth, determination of cell proliferation was performed using the MTT assay. After 3 days of infection, lenti-sh*CDK2AP1* did not positively affect HaCaT cell proliferation. However, lenti-sh*CDK2AP1* significantly increased HaCaT cell proliferation 4-9 days following infection (Fig. 3A). Notably, the suppression of P12^{CDK2AP1} led to HaCaT cell growth promotion in a time-dependent manner and this stimulative effect was not observed before day 3.

P12^{CDK2AP1} knockdown affects HaCaT cell cycle transition. The different proliferation rates of HaCaT cells infected with lenti-sh*CDK2AP1* vs. control HaCaT cells may partly be due to differences in cell cycle regulation. Therefore, cell cycle distribution was detected using flow cytometry. On day 7, a lower number of HaCaT cells infected by lenti-sh*CDK2AP1* were arrested in the G0/G1 compared to the S and G2/M phases of the cell cycle (Fig. 3B).

Discussion

P12^{CDK2AP1} is crucial in a variety of responses, including S-phase suppression associated with growth suppression (6,7), tumor progression (15), malignant transformation (5),

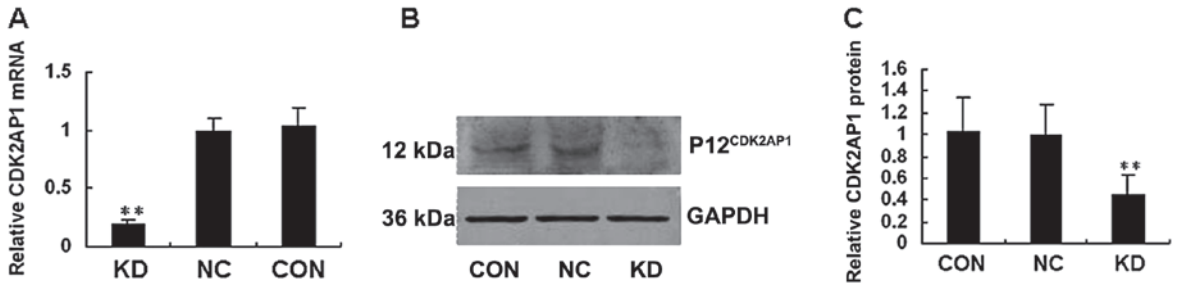

Figure 2. Stable inhibition of P12^{CDK2AP1} expression in HaCaT cell lines by lentivirus-delivered siRNA. (A) *CDK2AP1* mRNA expression was significantly decreased in the KD group (detected using real-time RT-PCR). Total RNA was isolated from HaCaT cells and HaCaT cells transfected with either *CDK2AP1* siRNA expression vector or control siRNA vector. Real-time RT-PCR was performed to measure the relative copies of *CDK2AP1* mRNA. The amount of each product was normalized to the housekeeping gene *β-actin*. *CDK2AP1* mRNA in HaCaT cells decreased significantly following infection with the lentiviral shRNA vector targeting the *CDK2AP1* gene. Each bar represents values from independent experiments performed in triplicate. (B) HaCaT cells were infected with pLenti/CON (lane 1), NC (lane 2) and pLenti/KD (lane 3) viruses and transduced cells were selected with Blasticidin. Western blot analysis demonstrated significant downregulation of P12^{CDK2AP1} expression in pLenti/KD-infected cells. GAPDH was used as an internal control. (C) Western blot analysis using NIH-ImageJ also indicated that the gray value of P12^{CDK2AP1} band in the KD group was significantly lower compared to that in the CON group. Each bar represents values from independent experiments performed in triplicate. **P<0.01. CON, HaCaT cells uninfected with lentivirus; NC, HaCaT cells infected with control lentivirus; KD, HaCaT cells infected with lentivirus carrying si*CDK2AP1*.

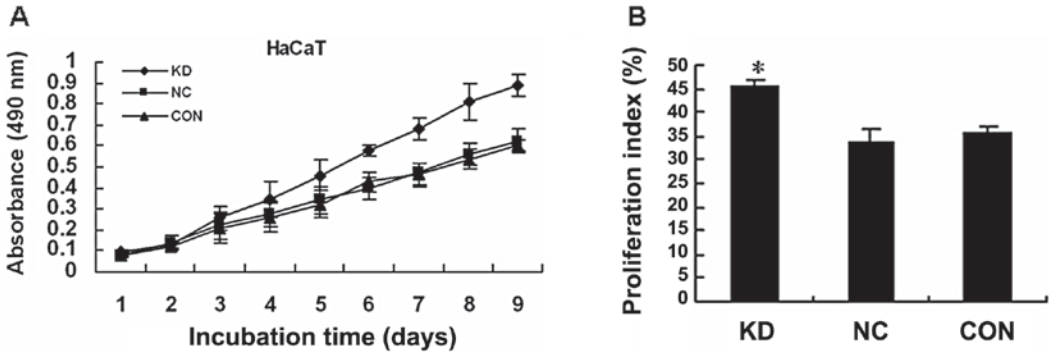

Figure 3. Knockdown of P12^{CDK2AP1} expression by RNAi promotes HaCaT cell proliferation. (A) Proliferation curve of HaCaT cells. Cell proliferation was analyzed using MTT cell proliferation assay kits. The proliferation of HaCaT cells transfected with *CDK2AP1* siRNA vector (KD) was significantly increased in a time-dependent manner, compared to cells without transfection (CON) or cells transfected with control siRNA vector (NC). (B) The flow cytometric analysis of HaCaT cells showed that the proliferation index was significantly increased in the KD group. *P<0.05.

apoptosis and oral carcinogenesis (16). It has also been shown to be reduced or absent in various types of human oral cancer and it has been found to be a positive prognostic indicator (2). Several findings have suggested that P12^{CDK2AP1} loss or reduced expression contribute to the multistep nature of oral carcinogenesis, and that P12^{CDK2AP1} loss may constitute an event associated with tumor progression (2,6,7,17). This may raise the question as to whether blocking P12^{CDK2AP1} is beneficial for promoting HaCaT cell growth, and whether targeting P12^{CDK2AP1} signaling may serve as a novel therapeutic strategy for the treatment of HNSCCs expressing downregulated P12^{CDK2AP1}.

Stable downregulation of gene expression via retroviral-mediated siRNA delivery has recently been described by several scientific groups (18-20). Lentiviral vector is a novel tool for human gene therapy. It has been shown to be an effective vehicle for stable and long-term gene expression. The results of this study demonstrated that the rate of lentiviral vector transducing to HaCaT cells was >90%. The real-time RT-PCR and western blot analyses indirectly indicated that the HaCaT cells transduced with the lentiviral shRNA vector expressed siRNA-targeting *CDK2AP1* gene. In this study, *CDK2AP1* siRNA was demonstrated to inhibit, not only P12^{CDK2AP1} expression, but also the activity of P12^{CDK2AP1} in HaCaT cells.

Previous data have demonstrated that *CDK2AP1* is crucial in cancer cell proliferation (2,5,21). In this study, in order to determine whether *CDK2AP1* regulated HaCaT cell proliferation and the cell cycle, transduced HaCaT cells were analyzed. The results showed that RNAi-targeting *CDK2AP1* promoted the proliferation of HaCaT cells. Flow cytometric analysis showed that the inhibition of P12^{CDK2AP1} expression in HaCaT cells, resulted in a lower number of cells in the G1 phase of the cell cycle.

Although significant downregulation of P12^{CDK2AP1} expression was achieved, complete gene silencing was not achieved. It is believed that residual P12^{CDK2AP1} expression in the mass culture cells is due to the presence of a few clones that have completely escaped the effect of siRNA. The incomplete gene silencing of P12^{CDK2AP1} observed in this study could be attributed to the relatively weak expression from Pol III promoters (18-20). However, it is believed that even incomplete gene silencing would be of therapeutic utility for a number of human disease conditions. Studies are underway aiming to

investigate the mechanisms underlying the incomplete gene silencing and a number of strategies could be established to improve the efficiency of this procedure.

In this study, a lentiviral vector was constructed for the delivery of siRNAs and its ability to efficiently downregulate the expression of a target gene in the infected cells was demonstrated. It was also shown that $P12^{CDK2AP1}$ silencing promotes the proliferation of cultured cells. Based on the well-known ability of lentiviruses to infect dividing and non-dividing cells as well as to achieve long-term gene expression, they are an attractive option for the *in vitro* and *in vivo* delivery of siRNAs in cultured cells.

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