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miR-21 Downregulates the Tumor Suppressor P12^{CDK2AP1} and Stimulates Cell Proliferation and Invasion

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ABSTRACT

The present study was undertaken to investigate the regulation of P12^{CDK2AP1} by miRNAs. A conserved target site for miR-21 within the *CDK2AP1*-3′-UTR at nt 349–370 was predicted by bioinformatics software and an inverse correlation of miR-21 and *CDK2AP1* protein was observed. Highly specific amplification and quantification of miR-21 was achieved using real-time RT-PCR. Transfection of HaCaT cells with pre-miR-21 significantly suppressed a luciferase reporter including the *CDK2AP1*-3′-UTR, whereas transfection of Tca8113 with anti-miR-21 increased activity of this reporter. This was abolished when a construct mutated at the miR-21/nt 349–370 target site was used instead. Anti-miR-21-transfected Tca8113 cells showed an increase of *CDK2AP1* protein and reduced proliferation and invasion. Resected primary tumors and tumor-free surgical margins of 18 patients with head and neck squamous cell carcinomas demonstrated an inverse correlation between miR-21 and P12^{CDK2AP1}. This study shows that P12^{CDK2AP1} is downregulated by miR-21 and that miR-21 promotes proliferation and invasion in cultured cells. J. Cell. Biochem. 112: 872–880, 2011.

KEY WORDS: *CDK2AP1*; miR-21; HNSCCS; INVASION; PROLIFERATION

Human *CDK2AP1* is a highly conserved cellular gene. It has been mapped to chromosome 12q24 and no mutations have been found in oral and esophageal cancers [Daigo et al., 1997; Tsuji et al., 1998]. The full-length human *CDK2AP1* cDNA are 1.6 kb, P12^{CDK2AP1}, originally named for deleted in oral cancer-1 was identified and cloned from the Syrian hamster oral cancer model [Todd et al., 1995]. P12^{CDK2AP1} encodes a 115-amino-acid peptide with a molecular mass of 12.4 kDa (pI of 9.62). It is a specific CDK2-associated protein, which is thought to negatively regulate CDK2 activity by sequestering monomeric CDK2, and targeting CDK2 for proteolysis. P12^{CDK2AP1} was found to also interact with DNA polymerase alpha/primase and mediate the phosphorylation of the large p180 subunit, which suggested the regulatory role in DNA replication during S phase of the cell cycle. A similar gene in hamster was isolated from, and functions as a growth suppressor of normal keratinocytes. Several functional studies support the growth suppressor role of P12^{CDK2AP1} [Todd et al., 1995; Tsuji et al., 1998; Shintani et al., 2000].

miRNAs are non-coding small RNAs that regulate gene expression by Watson–Crick base pairing to target mRNA. They

Jun Zheng and Hui Xue contributed equally to this work.

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are involved in the majority of biological and pathological processes, and they have also been shown to control gene expression by binding to specific motifs in the 3′-UTR of target mRNAs, causing translational repression or degradation [Bartel, 2004; Berezikov et al., 2005; Zamore and Haley, 2005]. The highly critical role miRNAs play in gene regulation is widely accepted and well documented [Esquela-Kerscher and Slack, 2006]. 3′-UTRs have been shown to contain several regulatory motifs which modify mRNA stability, localization, and degradation, thereby influencing gene expression [Mazumder et al., 2003].

The majority of human oral cancers (63.8%) exhibit either a loss or significant reduction of $P12^{CDK2AP1}$, but the mechanism of the tumor-associated loss of $P12^{CDK2AP1}$ expression is currently unclear. 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 [Shintani et al., 2001]. Recent data suggest that although transcriptional activity of *CDK2AP1* is detected in normal and tumor cell lines and tissues [Tsuji et al., 1998], $P12^{CDK2AP1}$ was only detected in normal tissues and cell lines [Shintani et al., 2000], thus post-translational regulation might exist. miRNAs have been discovered as naturally occurring non-coding RNAs, controlling gene expression via specific sites at the 3′-UTR of target mRNAs, causing translational repression or degradation. However, little is known of the different molecular mechanisms of regulation mediated by miRNA-3′-UTR RNA duplexes in *CDK2AP1*. Further mechanisms leading to downregulation of this important tumor suppressor in cancer need to be elucidated.

MATERIALS AND METHODS

CELL LINES, ANTIBODIES, AND MATERIALS

OKF4, OKF6 (normal human oral keratinocyte lines), HaCaT (human normal skin keratinocytes), SCC9, SCC15 (malignant human oral keratinocyte lines), and Tca8113 (human tongue cancer cell) cell lines were purchased from the China Center Type Culture Collection. Primary antibody against $P12^{CDK2AP1}$ was purchased from Santa Cruz (CA). GAPDH antibody was purchased from Millipore. FBS/media were purchased from Invitrogen (Karlsruhe, Germany), Transwell chambers (8 μm pores) and Matrigel from Becton Dickinson (Bedford, MA), and pre-miR21, control-miR, anti-miR-21, and control-anti-miR from GenePharma Co., Ltd (Shanghai, P.R. China). SYBR Green PCR Master Mix for quantification of *CDK2AP1* mRNA and miR-21 was from TaKaRa (Kyoto, Japan). Oligonucleotides were from Sangon (Shanghai, P.R. China). Tissue specimens (tumors and tumor-free surgical margins) of 18 patients with head and neck squamous cell carcinomas (HNSCCs) were collected after informed consent and verification by a pathologist, and immediately frozen in liquid nitrogen.

WESTERN BLOT ANALYSIS

Cells were seeded in six-well plates and transfected after 12 h. The cells were harvested 2 days after transfection, washed once in phosphate-buffered saline (PBS) and lysed in lysis buffer. Fifty to 100 mg tissues were homogenized using a dismembrator, and proteins were extracted. Protein concentration was determined by BCA (Pierce, Rockford, IL). 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 (goat anti-rabbit IgG) and an enhanced chemiluminescence (ECL) detection solution was applied (Pierce). The relative protein level in different cell lines was normalized to the signal intensity of GAPDH as an internal control.

QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Total RNA from cells and tissues was extracted using Trizol reagent (Invitrogen, CA). Expression of mature miRNAs was detected by the SYBR Green-miRNA-assay [Chen et al., 2005] (TaKaRa), and normalized using the $2^{-\Delta\Delta Ct}$ method [Pfaffl, 2001] relative to human U6-snRNA. The primer sequences and PCR product sizes are shown in Supplementary Table 1, and the PCR reaction system works well (Supplementary Fig. 1). *CDK2AP1* mRNA was quantified by SYBR Green-qRT-PCR and normalized to β-actin (Sangon). To ensure specificity of the PCR product amplification, the melting curves for standard and sample products were analyzed. All qRT-PCRs were performed three times.

VECTOR CONSTRUCTION AND REPORTER ASSAYS

The 3′-UTR of *CDK2AP1* (nt 579) was amplified using cDNA from HaCaT cells using the following primer sequences: FW, 5′-GCTCTAGACTGCCTTGTTGGTTTTGAAGGATTT-3′ and RV, 5′-CGGAATTCCTTG TTAGGTTTGGTGGGTTGCTCT-3′. It was then cloned into a modified pGL3 vector (Promega, Madison, WI) downstream of the luciferase gene, checked for orientation, sequenced and named Luc-*CDK2AP1*Wt. The modified pGL3 vector was constructed as reported [Meng et al., 2006]. The quick change-mutagenesis kit (Stratagene, Heidelberg, Germany) was used to introduce specific point mutations into the seed region of Luc-*CDK2AP1*Wt, generating Luc-*CDK2AP1*Mut. For reporter assays, cells were seeded in 96-well plates and transfected with 50 nM miRNA, 120 ng of luciferase vector (pGL3 constructs), and 25 ng of Renilla vector (pRL-TK) (as indicated in Fig. 3) using Lipofectamine 2000 (Invitrogen). Thirty-six hours after transfection, cells were harvested and luciferase activity was measured using the Dual-Glo luciferase assay (Promega).

TRANSWELL INVASION ASSAY

Transwell insert chambers (Becton Dickinson, Franklin Lakes, NJ) were precoated with a final concentration of 1.5 mg/ml of Matrigel (Becton Dickinson). Cells were transfected with 50 nM anti-miR-21, control-anti-miR, pre-miR-21, or control-miR. Cells were trypsinized 48 h post-transfection, then 1×10^4 cells were seeded in the upper chambers with 200 μl serum-free DMEM and the lower wells were filled with 500 μl DMEM with 10% FBS as a chemoattractant [Valster et al., 2005]. After 24 h, non-invading cells were removed with cotton swabs. Invading cells were examined by microscope after mounting on a slide. The cells that migrated onto the lower surface of the filter were examined by microscope after mounting on a slide. A total of eight random high-power microscopic fields (200× magnification) per filter were photographed and the numbers of cells were counted.

SOFT-AGAR ASSAY

Cells (1×10^4) were added to 3 ml of DMEM (supplemented with 10% FBS) with 0.3% agar and layered onto 6 ml beds of 0.5% agar in 60 mm dishes. Cells were cultured for 2 weeks and then the colonies were photographed. Colonies larger than 50 μm in diameter were counted as positive for growth. Assays were conducted three times.

FLOW CYTOMETRIC ANALYSIS OF CELL CYCLE

Cells were harvested 48 h post-transfection, washed twice with ice-cold PBS, fixed with 70% ethanol overnight at 4°C, then washed and resuspended in 100 μl of PBS with a final concentration of 50 μg/ml RNase A for 30 min at room temperature. The cells were then stained with 20 μg/ml PI in a final volume of 300 μl for 20 min. DNA content and cell cycle were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA), using the MODFIT and CELLQUEST software. The experiments were conducted three times.

MEASUREMENT OF CELL GROWTH BY METHYL THIAZOLYL TETRAZOLIUM ASSAY (MTT)

HaCaT and Tca8113 cells were seeded at a density of 2×10^3 cells/well in 96-well plates containing 0.2 ml DMEM (with 7% FBS) and cultured for 7 days. During this period, the cells were given fresh complete medium every 3 days. Six wells from each group were randomly selected daily for the MTT assay (Sigma-Aldrich Co., St. Louis, MO); 50 μg was taken from each well. After 4 h of incubation the reaction was stopped by adding 150 μl of dimethyl sulfoxide (DMSO, Sigma) to each well and incubating for 10 min. The percentage of viable cells was determined by measuring the absorbance at 490 nm on a multiscanner reader (TECAN-spectra mini Grodig, Austria). Cell growth curves were drawn by using average absorbance at 490 nm from three independent experiments.

STATISTICAL ANALYSIS

All experiments were performed three times and standard deviations were calculated. The statistics were performed using SPSS 11.5 (Chicago, IL). Differences/correlations between groups were calculated with Student's *t*-test or Pearson test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

THE 3′-UTR OF *CDK2AP1* MIGHT BE A TARGET SEQUENCE FOR miR-21

To predict the miRNA target of *CDK2AP1*, the nt 743-3′-UTR was scanned for complementary sequences of known miRNAs by bioinformatics searches (miRGator, http://genome.ewha.ac.kr/miRGator/ miRNAexpression.html; http://www.ebi.ac.uk/enright-srv/microcosm/ cgi-bin/targets/v5/detail_view.pl?transcript_id=ENST00000261692) [Enright et al., 2003; Griffiths-Jones et al., 2006, 2008]. A full-match target sequence for miR-21 at nt 349–370 of *CDK2AP1*-3′-UTR was found. As shown in Figure 1A, the minimum free energy predicted for hybridization with the *CDK2AP1*-3′-UTR and miR-21 at this target site was −14.2 kcal/mol, determined by RNAhybrid software (Version: RNAhybrid 2.2, and the URL: http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) analysis [Rehmsmeier et al., 2004; Kruger andRehmsmeier, 2006], and it is consistent with authentic miRNA targeting [Doench and Sharp, 2004]. We compared the target sequence for interspecies homology, and found that the miR-21 target sequence at nt 349–370 of the *CDK2AP1*-3′-UTR is highly conserved (MicroCosm Targets Version 5, http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/detail_view.pl?transcript_id=ENST00000261692) between Sapiens and Mulatta (Fig. 1B). We also found another conserved target site for miR-26b, but in the initial reporter results we found that it was not validated by functional experiments. So we conducted our studies on miR-21.

$P12^{CDK2AP1}$ AND miR-21 EXPRESSION CORRELATE INVERSELY IN TESTED CELL LINES

We detected endogenous expression levels of miR-21 and $P12^{CDK2AP1}$ (mRNA and protein) in three normal (OKF4, OKF6, and HaCaT) and three malignant (SCC9, SCC15, and Tca8113) cell lines (Fig. 2). Tca8113, SCC9, and SCC15 cell lines had high endogenous miR-21 expression (Fig. 2C, lanes 4, 5, 6) as measured by miRNA qRT-PCR, and low levels of $P12^{CDK2AP1}$ at 12 kDa were observed in these cells (Fig. 2A,B, lanes 4, 5, 6); OKF4, OKF6, and

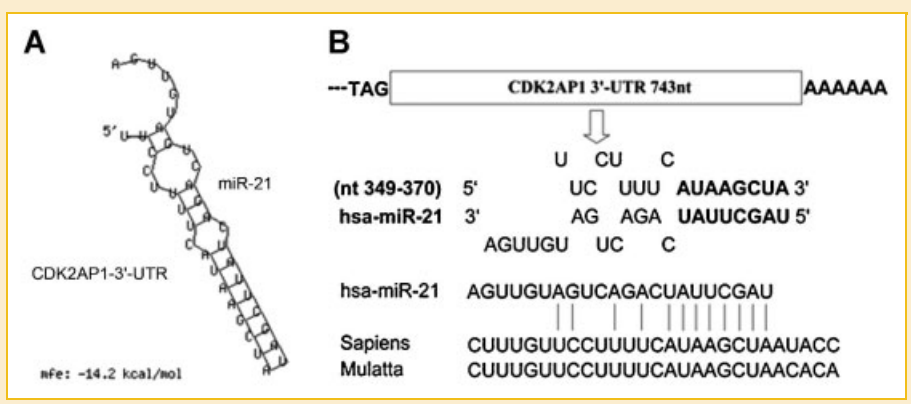

Fig. 1. miR-21 target site was predicted at nt 349–370 of the *CDK2AP1*-3′-UTR, and it is highly conserved between Sapiens and Mulatta. A: Predicted hybridization of miR-21 and *CDK2AP1*-3′-UTR by using the RNAhybrid software. The minimum free energy (mfe) required for RNA hybridization is shown. B: The predicted binding of miR-21 with the 3′-UTR of *CDK2AP1* mRNA and comparison of nucleotides between miR-21 seed sequence and its target.

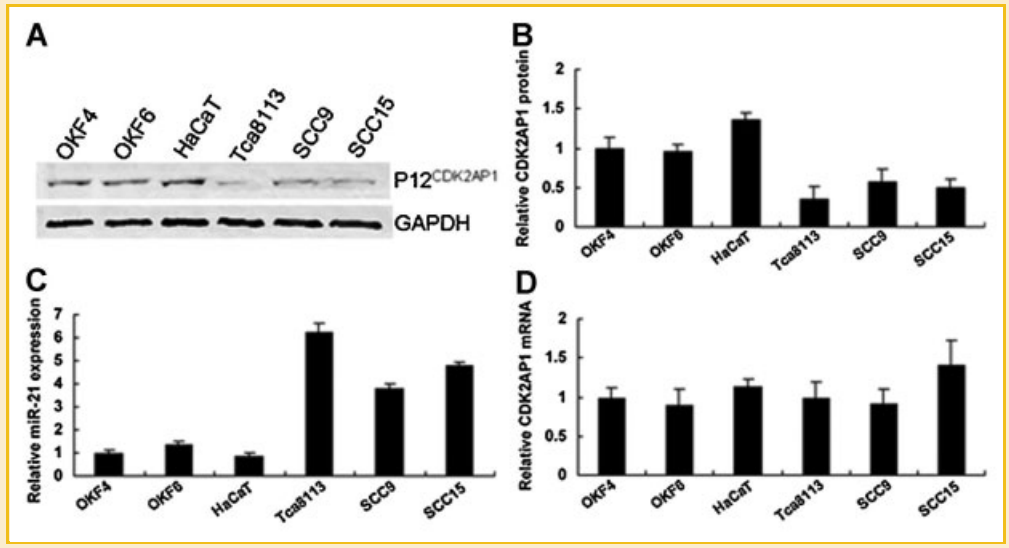

Fig. 2. miR-21 and $P12^{CDK2AP1}$ correlate inversely in tested cell lines. A: Western blots showing $P12^{CDK2AP1}$ expression in three normal and three malignant cell lines. GAPDH was used as an internal control. B: Western-blot analysis by NIH-ImageJ also indicated that the gray value of the $P12^{CDK2AP1}$ band in normal cells was significantly higher than that in malignant cells. Each bar represents values from independent experiments were performed three times (the same to below). C: miR-21 expression was detected by qRT-PCR as shown. The malignant cell lines have higher endogenous miR-21 than that in normal cell lines. D: *CDK2AP1* mRNA expression as indicated (detected by qRT-PCR), and there is no significant differences in fold change.

HaCaT cell lines with low miR-21 expression (Fig. 2C, lanes 1, 2, 3) showed high amounts of $P12^{CDK2AP1}$ (Fig. 2A,B, lanes 1, 2, 3). Across the cell lines tested, we found a significant inverse correlation between miR-21 and $P12^{CDK2AP1}$ protein levels ($P < 0.01$). There was no significant association with miR-21 and *CDK2AP1* mRNA, and the fold differences in *CDK2AP1* mRNA (Fig. 2D) were less than that in *CDK2AP1* protein. These primary data suggest that miR-21 might downregulate *CDK2AP1* expression at the post-transcriptional level.

THE *CDK2AP1*-3′-UTR/NT 349–370 IS A TARGET FOR miR-21

We cloned a reporter plasmid containing potential miR-21 binding sites within the 3′-UTR of *CDK2AP1* for further validation by

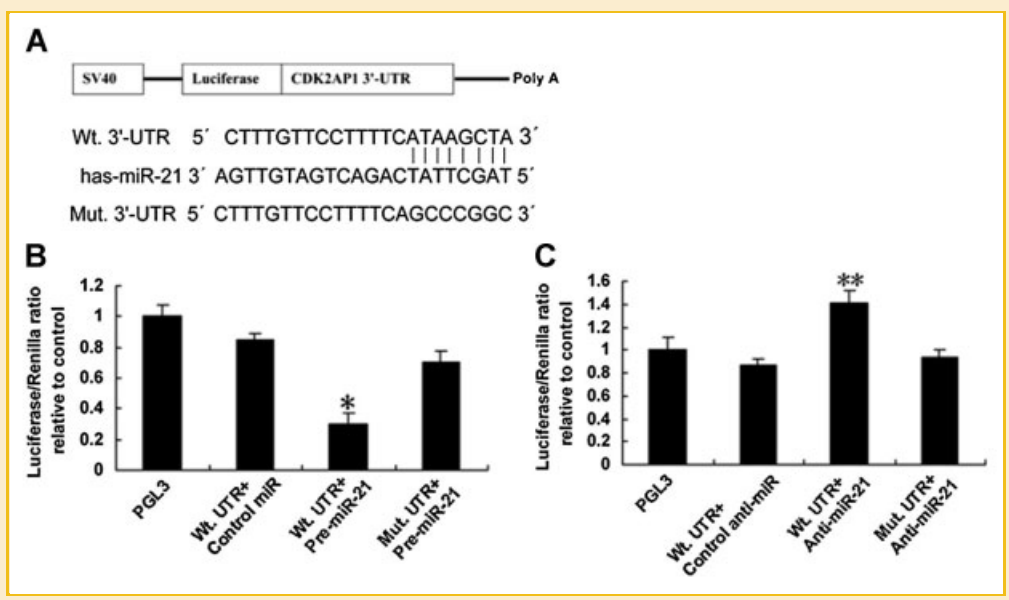

Fig. 3. Validation of miR-21 target site by reporter assay. A: The diagram shows that *CDK2AP1*-3′-UTR reporter constructs. Mut. 3′-UTR: contains 7-base-mutation at the miR-21 target region, abolishing its binding site. B: Firefly luciferase reporter genes with constructs holding 3′-UTR sequences (wt-UTR or mut-UTR) from the *CDK2AP1* gene were cotransfected into HaCaT cells along with a Renilla luciferase transfection control plasmid either alone or together with pre-miR-21 or control-miR. Relative luciferase values normalized to transfections without miRNA are shown. HaCaT cells were transfected with the wt-UTR reporter construct and pre-miR-21 exhibited a significant decrease of reporter activity, while the activity of the reporter construct mutated at miR-21 target site at nt 349–370 was almost unaffected. C: Firefly luciferase reporter assays of Tca8113 cells as in B, with control anti-miR and anti-miR-21. When Tca8113 cells were transfected with anti-miR-21 and the wt-UTR-reporter construct a significant increase in activity of the wild-type reporter was observed. It was abolished when the reporter construct was mutated at the miR-21 target site instead. $^{*}P < 0.05$, $^{**}P < 0.01$.

luciferase reporter assays. Five hundred seventy-nine-base pair fragments of the 3′-UTR were cloned into a modified pGL3 vector downstream of the luciferase gene and driven by the SV40 basal promoter (wt-UTR, Fig. 3A). In parallel, we cloned another reporter plasmid in which the conserved targeting region UAAGCUA of miR-21 within nt 349–370 was specifically mutated; this was predicted to abolish the binding site (mut-UTR, Fig. 3A). Transient transfection of HaCaT cells, with a low endogenous miR-21 expression, with the wt-UTR reporter construct and pre-miR-21, led to a significant decrease of reporter activity as compared to the control (Fig. 3B). However, the activity of the mutant reporter construct was almost unaffected by cotransfection with pre-miR-21. Similar results were observed in HeLa cells (data not shown), and miR-21 did not affect luciferase mRNA steady state levels (Supplementary Fig. 2). Next the experiments were performed using anti-miR-21, which binds to endogenous miR-21 and antagonizes its activity [Meister et al., 2004; Asangani et al., 2008]. When Tca8113 cells, with high endogenous miR-21 expression (Fig. 2C, lane 1), were transfected with anti-miR-21 and the wt-UTR-reporter construct a significant increase in activity of the wild-type reporter was observed. It was abolished when the same cell lines were transfected with the mutant reporter construct (Fig. 3C). Similar results were not seen when HaCaT and HeLa cells were transfected with reporter constructed pre-miR-26b, and there was no additional effect which transfected with pre-miR-21 (Supplementary Fig. 3). These experiments suggest that the *CDK2AP1*-3′-UTR/nt 349–370 is a functional target site for miR-21.

miR-21 DOWNREGULATES P12^{CDK2AP1} AND PROMOTES HaCaT CELL PROLIFERATION

In the next experiment, we investigated whether transfection of cell lines with pre-miR-21 or anti-miR-21 would affect P12^{CDK2AP1} expression and proliferation. In Tca8113 cells, with high miR-21 expression, downregulation of endogenous miR-21 with anti-miR-21 (Fig. 4A) resulted in a significant increase in P12^{CDK2AP1} (Fig. 4C) almost without any change in *CDK2AP1* mRNA (Fig. 4B). To determine whether overexpression of miR-21 in low miR-21 expressing cells will have the opposite effect on P12^{CDK2AP1} expression and proliferation, HaCaT cells were transiently transfected with pre-miR-21, control-miR, and mock control (Fig. 4). The transfection was efficient with an almost 80-fold expression of miR-21 compared to the control (Fig. 4D). Whereas *CDK2AP1* mRNA was almost unaltered in the pre-miR-21 transfected cells (Fig. 4E), there was a significant reduction of P12^{CDK2AP1} expression in pre-miR-21 transfected cells (Fig. 4F). In MTT assays and flow cytometric analysis we found a significant increase in proliferation capacity in pre-miR-21 transfected cells (Fig. 4G,H). These data suggest that miR-21 specifically downregulates *CDK2AP1* at the post-transcriptional level, and that miR-21 positively regulates proliferation of HaCaT cells in vitro.

Anti-miR-21 TRANSFECTION RESULTS IN DECREASED INVASION AND PROLIFERATION

To study the effect of anti-miR-21 treatment on invasion and proliferation we employed transwell invasion (Soft-Agar Assay) and MTT assays. Tca8113 cells were transfected with anti-miR-21, control-anti-miR, and mock control, seeded in the upper chambers with 200 μl serum-free DMEM, and the lower wells were filled with 500 μl DMEM with 10% FBS as an inducer of cell migration. After 24 h incubation, significantly fewer invasions were observed in the anti-miR-21 treated group (Fig. 5A,B). We then evaluated the effect of P12^{CDK2AP1} suppression on anchorage independent colony formation in soft agar as an additional assessment of invasion. The number of colonies significantly decreased in the anti-miR-21 treated group (Fig. 5C,D). In the MTT assay the growth of Tca8113 cells was notably inhibited in a time-dependent manner (Fig. 5E). The flow cytometric analysis of Tca8113 cells showed that the Proliferation Index (PrI = S + G2/M) was decreased with transfection of anti-miR-21 (Fig. 5F). These observations clearly confirm the role of miR-21 in the invasion and proliferation potential of Tca8113 cells.

miR-21 AND P12^{CDK2AP1} ARE INVERSELY EXPRESSED IN RESECTED PATIENT TUMORS IN VIVO

Resected primary tumors and tumor-free surgical margins of 18 patients with HNSCCs were analyzed for *CDK2AP1* protein, miR-21 and *CDK2AP1* mRNA. Representative examples are shown in Figure 6A. A comprehensive analysis of miR-21, *CDK2AP1* mRNA and P12^{CDK2AP1} for all patients is shown in Figure 6B–D. The clinicopathologic characteristics in 18 patients with HNSCCs are given in Supplementary Table 2. As reported from other tumor entities [Meng et al., 2007; Hiyoshi et al., 2009], we observed that all of the tumors investigated showed an expression of miR-21 higher than that in the tumor-free surgical margins (Fig. 6A,B). This increase of miR-21 expression in tumor tissues was highly significant (Fig. 6C). Within all resected primary tumors and tumor-free surgical margins, we observed a highly significant negative correlation between P12^{CDK2AP1} and miR-21 ($P < 0.01$), high expression of miR-21 being associated with low amounts of P12^{CDK2AP1} in all cases (Fig. 6B,C). Even though we observed a weak negative correlation ($0.01 < P < 0.05$) between miR-21 and *CDK2AP1* mRNA in resected primary tumors, there was no significant difference in *CDK2AP1* mRNA levels between tumor and tumor-free surgical margins (Fig. 6D, $P > 0.05$). This in vivo data support the notion that P12^{CDK2AP1} is negatively regulated by miR-21 at the post-transcriptional level.

DISCUSSION

P12^{CDK2AP1} is a suppressor of S-phase associated growth [Matsuo et al., 2000; Shintani et al., 2000], tumor progression [Todd et al., 1997], malignant transformation [Todd et al., 1995], apoptosis and oral carcinogenesis [Kohno et al., 2002]. It has also been shown to be reduced or absent in human oral cancers and is found to be a positive prognostic indicator [Shintani et al., 2001]. Recently, Kim et al. [2009] demonstrated that *Cdk2ap1* is an essential gene in the proper development of mouse embryos, and a specific deletion of *Cdk2ap1* leads to the aberrant craniofacial development. Expression of P12^{CDK2AP1} is consistently reduced or lost in transformed oral keratinocytes. It even could not be detected in malignant human keratinocyte cell lines or in freshly resected oral cancers. miR-21 has

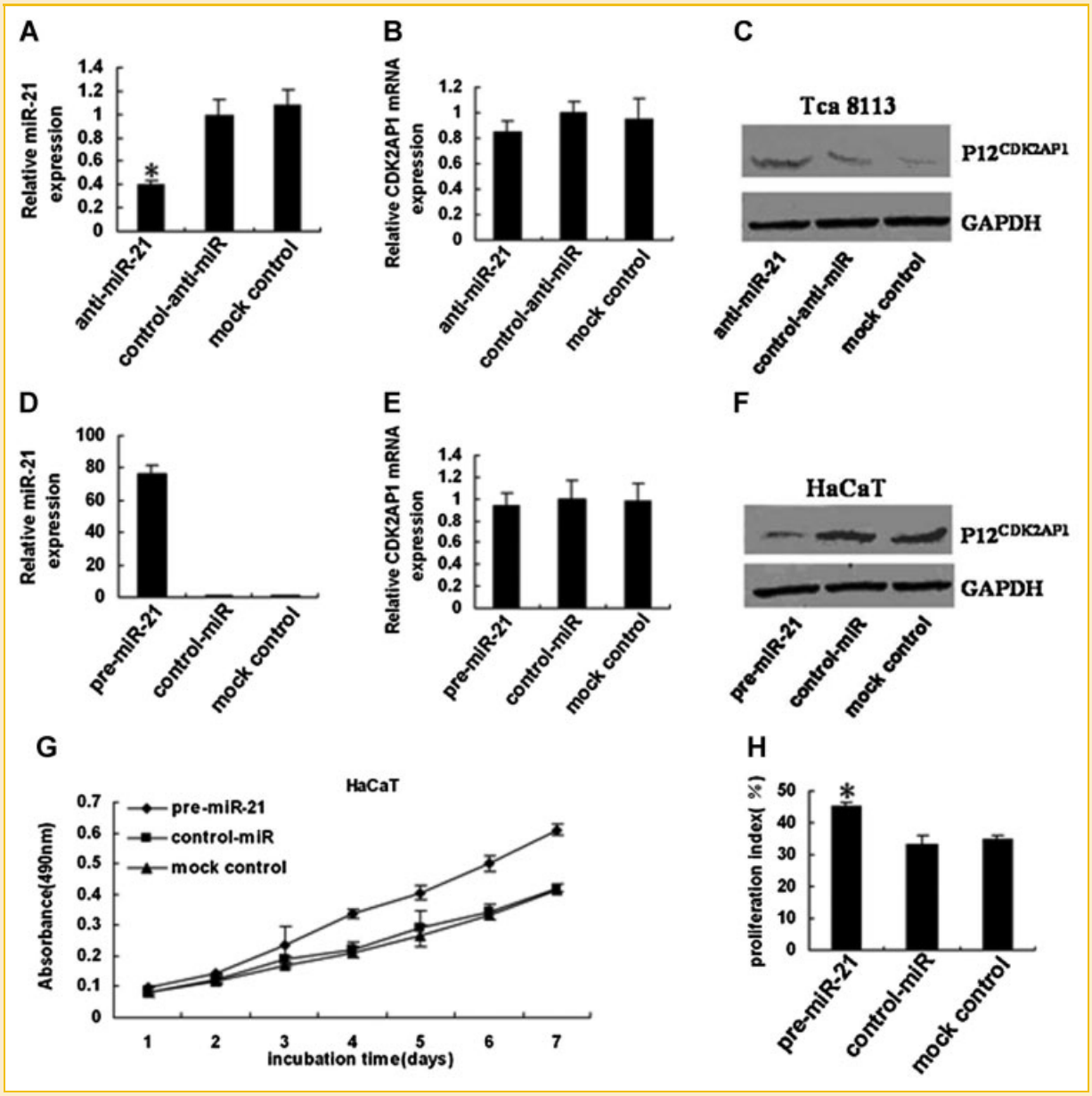

Fig. 4. miR-21 negatively regulates *CDK2AP1* expression at the post-transcriptional level and promotes cell proliferation. A–C: Tca8113 cells were transfected with 50 nM anti-miR-21, control-anti-miR and mock control; then analyzed for miR-21, *CDK2AP1* mRNA, and protein expression. Fold differences in *CDK2AP1* protein are higher than for *CDK2AP1* mRNA, and inversely correlate with miR-21, in contrast to *CDK2AP1* mRNA. D–F: HaCaT cells were transfected with 50 nM pre-miR-21, control-miR and mock control; analyzed for miR-21, *CDK2AP1* mRNA, and protein expression. *CDK2AP1* protein expression was down regulated significantly, and miR-21 expression was significantly increased, while *CDK2AP1* mRNA was barely changed. G: MTT assay of HaCaT cells transfected as indicated. Cell growth was notably promoted in a time-dependent manner. H: The flow cytometric analysis of HaCaT cells showed that the Proliferation Index was significantly increased with transfection pre-miR-21. $^{*}P < 0.05$.

been shown to act as an anti-apoptotic factor in glioblastoma cells [Chan et al., 2005]. Other studies have shown that miR-21 also acts as an activator of tumor cell proliferation [Roldo et al., 2006; Si et al., 2007; Asangani et al., 2008]; this effect was also observed in our experiments (Fig. 4G,H). Our present study shows that miR-21 is a negative regulator of $P12^{CDK2AP1}$. It is attractive to additionally speculate that miR-21 might promote proliferation, at least in part, through downregulating $P12^{CDK2AP1}$. Knockdown experiment for $P12^{CDK2AP1}$ by siRNA in examined cells also supports this conclusion (Supplementary Fig. 4A). For miRNAs study, the current challenge is to identify biologically relevant targets that are regulated by individual miRNAs. Further complicating this task is the fact that every miRNA can potentially bind and regulate many mRNA targets and each mRNA can be bound and regulated by several miRNAs [Cahill et al., 2006]. Therefore, it is reasonable to deduce that other miRNAs may also be involved in $P12^{CDK2AP1}$ regulation. The focus of our study is to test whether and how miR-21 regulates the expression of $P12^{CDK2AP1}$. In our transfection

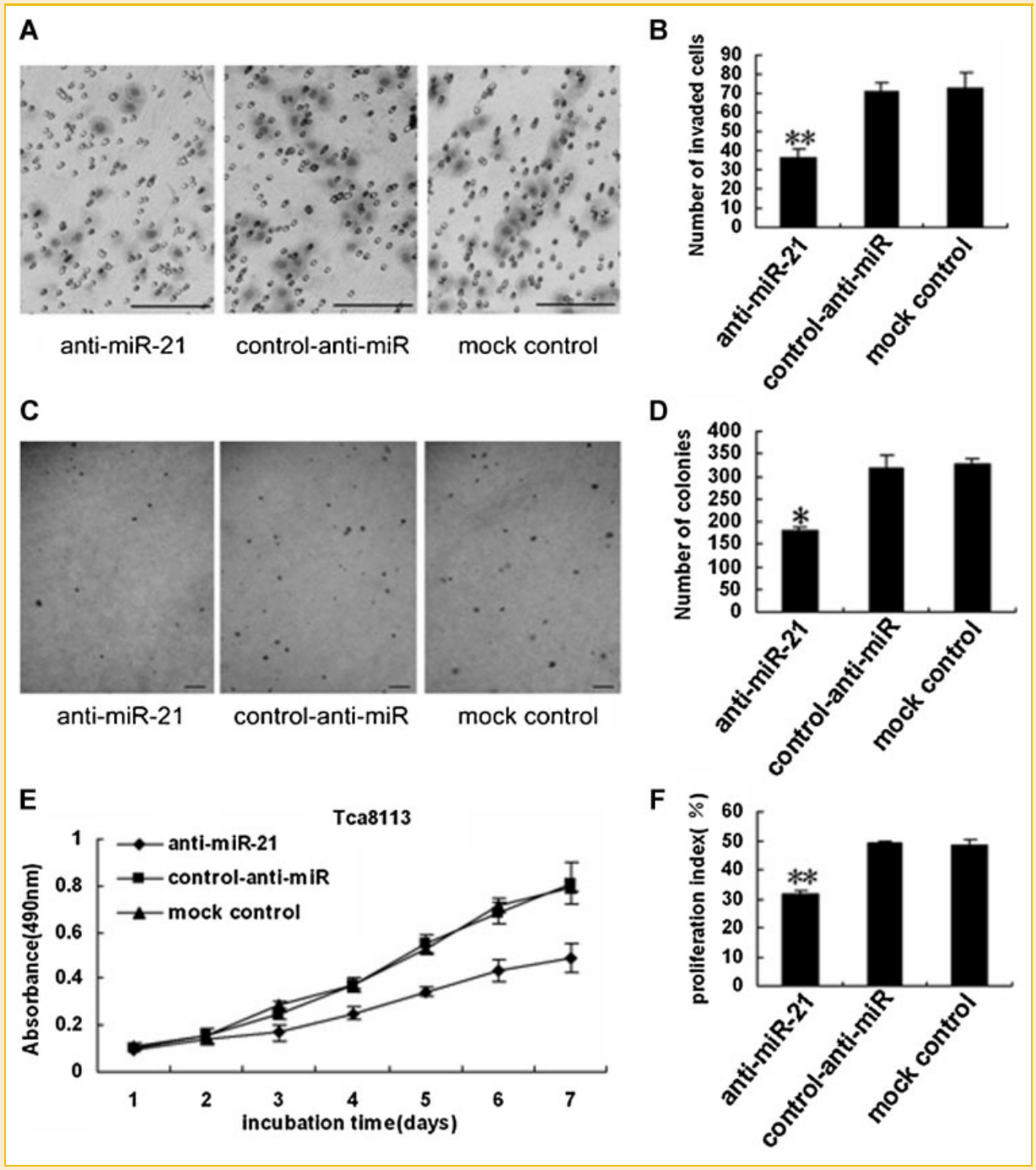

Fig. 5. Anti-miR-21 transfection decreased Tca8113 tongue cancer cell invasion (tumorigenicity) and proliferation. A,B: Transwell assay of Tca8113 with transfection as indicated. Significantly fewer invaded cells were observed for the anti-miR-21 treated group. Scale bar: 50 μm. C,D: The number of colonies formed was significantly decreased in anti-miR-21 treated cells. Scale bar: 1.0 mm. E: MTT assay of Tca8113 with transfection as indicated. The growth of Tca8113 cells was inhibited notably in a time-dependent manner. F: The flow cytometric analysis of Tca8113 cells showed that the Proliferation Index was decreased with transfection of anti-miR-21. $^{*}P < 0.05$, $^{**}P < 0.01$.

experiments (Fig. 4) *CDK2AP1* mRNA was almost unaltered, although $P12^{CDK2AP1}$ protein levels decreased dramatically. We therefore suppose that the main mechanism of miR-21-induced *CDK2AP1* suppression is post-transcriptional. But we could not exclude the possibility that additional means of *CDK2AP1*-regulation such as CpG methylation can alter expression of the gene [Tsuji et al., 1998], and add to miR-21 induced *CDK2AP1* suppression.

$P12^{CDK2AP1}$ has been shown to decrease S phase and increase apoptosis [Yuan et al., 2003]. Downregulated $P12^{CDK2AP1}$ expression by siRNA in Tca8113 promote cell invasion (Supplementary Fig. 4B). We demonstrate that miR-21 is an effective stimulator of the

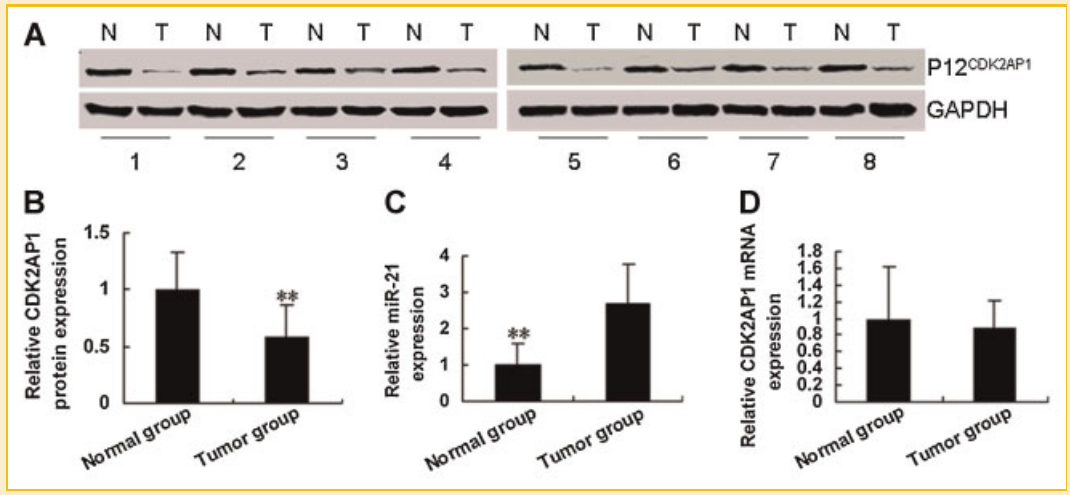

Fig. 6. miR-21 expression is negatively associated with $P12^{CDK2AP1}$, and is significantly upregulated in 18 resected HNSCCs. A: Western blots showing $P12^{CDK2AP1}$ expression in the 8 of 18 matched normal/tumor tissues investigated. GAPDH was used as internal control. The level of $P12^{CDK2AP1}$ was significantly lower in the tumors group than that in the normal group. B: The autoradiographic densities of each protein band were quantified using NIH-ImageJ. The results were standardized against the levels of GAPDH and were presented as the relative density. C: Relative miR-21 amounts were determined in 18 cases (SYBR Green-miRNA-assay) and expressed as fold change after normalization to U6-snRNA. D: Relative *CDK2AP1* mRNA was determined by SYBR Green-qRT-PCR and expressed as fold change after normalization (β-actin-mRNA). $^{**}P < 0.01$ (Student's *t*-test).

invasion capacity of Tca8113 cells, possibly by downregulating $P12^{CDK2AP1}$, but in our study the effect on invasion was not as prominent as the stimulating effect of miR-21 on proliferation in HaCaT cells. Our present work on miR-21 does not exclude other important mechanisms of $P12^{CDK2AP1}$ regulation in tumors, for example, the regulation by phosphorylation or histone deacetylation or DNA methylation induced inactivation of $P12^{CDK2AP1}$ [Tsuji et al., 1998; Terret et al., 2003; Hu et al., 2004; Le Guezennec et al., 2006; Iorio et al., 2007].

miR-21 is involved in the genesis and progression of human malignancies. It has been implicated in the promotion of anti-apoptosis [Chan et al., 2005], response to gemcitabine-based chemotherapy [Meng et al., 2006], proliferation [Roldo et al., 2006], and tumor growth [Si et al., 2007]. Several studies have shown that miR-21 is overexpressed in different tumor types: Dillhoff et al. [2008] found that miR-21 is significantly over-expressed in pancreatic cancers as detected by in situ hybridization. miR-21 is also up-regulated in breast cancer [Iorio et al., 2005], glioblastoma [Chan et al., 2005], lung cancer [Yanaihara et al., 2006], and esophageal squamous cell carcinoma [Hiyoshi et al., 2009]. Chang et al. [2008] screened for altered microRNA expression in HNSCCs primary tissue and cell lines, and found that miR-21 is overexpressed and validated by qRT-PCR. It might mean that miR-21 is upregulated in HNSCCs primary tissue while $P12^{CDK2AP1}$ expression is downregulated. This inverse correlation between miR-21 and $P12^{CDK2AP1}$ expressions in primary samples is parallel with our results. On the whole, these studies and our results support the hypothesis that miR-21 might be one of the oncogene-like factors [Esquela-Kerscher and Slack, 2006] amid the class of miRNAs. Our observations also show that $P12^{CDK2AP1}$ was not regulated by miR-26b, although a potential binding site for this miRNA was found within the 3′-UTR of *CDK2AP1* mRNA. This supported the specificity of our findings for miR-21. It also emphasizes that the presence of a putative miRNA-target sequence does not have to imply functionality.

Our whole study suggests that the tumor suppressor $P12^{CDK2AP1}$ is downregulated at the post-transcriptional level by miR-21 through a specific target motif at nt 349–370 of the *CDK2AP1*-3′-UTR. Furthermore, miR-21 promotes invasion/proliferation in vitro, demonstrating that *CDK2AP1* is an important functional target of miR-21 in this model. Our studies on miR-21 and $P12^{CDK2AP1}$ indicate that interfering with the miR-21/*CDK2AP1* interaction, downregulating miR-21 expression or promoting $P12^{CDK2AP1}$ expression might be an effective new rationale for therapeutic applications in HNSCCs in the future.

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