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miR-182 targets CHL1 and controls tumor growth and invasion in papillary thyroid carcinoma

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ABSTRACT

In this study, we investigated the role and underlying mechanism of action of miR-182 in papillary thyroid carcinoma (PTC). Bioinformatics analysis revealed close homolog of LI (CHL1) as a potential target of miR-182. Upregulation of miR-182 was significantly correlated with CHL1 downregulation in human PTC tissues and cell lines. miR-182 suppressed the expression of CHL1 mRNA through direct targeting of the 3′-untranslated region (3′-UTR). Downregulation of miR-182 suppressed growth and invasion of PTC cells. Silencing of CHL1 counteracted the effects of miR-182 suppression, while its overexpression mimicked these effects. Our data collectively indicate that miR-182 in PTC promotes cell proliferation and invasion through direct suppression of CHL1, supporting the potential utility of miR-182 inhibition as a novel therapeutic strategy against PTC.

1. Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, and papillary thyroid carcinoma (PTC) the most prevalent histology subtype accounting for approximately 80% of all TC cases [1,2]. Currently, most PTCs can be managed successfully with a combination of radioiodine and levothyroxine after complete thyroidectomy [3]. However, about 10% of cases present recurrence in local/regional and distant sites, which is associated with increasing mortality [4]. Therefore, there is an urgent medical need to elucidate the molecular mechanisms of PTC progression to facilitate the development of novel targeted therapies.

MicroRNAs (miRNAs) are a class of small non-coding RNAs ~22 nucleotides in length that regulate gene expression by binding to the 3′ untranslated regions (3′-UTR) of target mRNAs in a sequence-specific manner, resulting in gene silencing or/and translational repression [5,6]. Accumulating reports support critical roles of miRNAs in the progression of human cancers, where they function as either onco-miRNAs or tumor suppressors [7]. Several miRNAs have been shown to be dysregulated in PTC, including miR-146b, miR-155, miR-34a and miR-199a-3p, which participate in tumor initiation and progression [8–11]. miR-182 is clearly upregulated and exhibits oncogenic properties in many cancers [12]. A recent study reported overexpression of miR-182 in PTCs, compared with adjacent normal tissues [13]. However, the specific role of miR-182 in acquisition of the malignant phenotype of PTC cells and underlying mechanisms remain to be established.

Close homolog of L1 (CHL1) is a transmembrane protein influencing the development and regeneration of the nervous system [14]. It is also a critical factor in the development and progression of cancers. For instance, CHL1 overexpression leads to suppression of proliferation and invasion of cervical cancer and breast cancer cells *in vitro* and tumorigenicity *in vivo* [15–17]; however, its knockdown in oral squamous cell carcinoma contributes to malignant transformation [18]. Findings to date indicate a putative tumor suppressor function of CHL1 in cancers.

In the current study, bioinformatics analysis using TargetScan and miRanda led to the identification of CHL1 as a candidate target of miR-182. The main aim of this investigation was to ascertain the effect of miR-182 on CHL1 in PTC cells and explore the role of this mechanism. Our results clearly suggest that the oncogenic role of miR-182 is mediated via CHL1 suppression in PTC.

2. Materials and methods

2.1. Tumor tissue samples

Ten pairs of PTC and adjacent normal thyroid tissues were obtained from Shanghai Pudong New Area Gongli Hospital, China.

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Tissues were immediately stored at −80 °C and histologically confirmed. Informed consent was obtained from all patients, and the study approved by the Human Research Ethics Committee of Shanghai Pudong New Area Gongli Hospital.

2.2. Cell culture and transfection

PTC-derived thyroid cell lines, TPC-1 and BCPAP, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Antisense oligonucleotide of miR-182 (anti-182) and negative oligonucleotide control (anti-neg) were purchased from Applied Biosystems (Foster City, CA, USA). siRNA against CHL1 (si-CHL1) and siRNA control (si-control) were acquired from GenePharma (Shanghai, China). Transient transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of tissues and cell lines was extracted with TRIzol Reagent (Invitrogen). qRT-PCR analysis of miRNA expression was performed on LightCycler 480 (Roche Diagnostics, Germany) with a TaqMan MicroRNA Assay kit, and analysis of mRNA with the SYBR Green PCR master mix (Applied Biosystems). All reactions were run in triplicate. U6 small nuclear RNA and β-actin were used as endogenous controls for detection of miRNA and mRNA, respectively. For data analysis, the $2^{-\Delta\Delta Ct}$ method was used to calculate fold change.

2.4. Vector construction and transfection

The 3′-UTR of CHL1 containing the putative miR-182 binding site was amplified and cloned into the pGL3 control vector (Promega, Madison, WI). Mutations in the miR-182-binding site of CHL1 3′-UTR were introduced with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The coding sequence of CHL1 was subcloned into pcDNA3.1 to construct the CHL1 expression plasmid (pcDNA3.1-CHL1). Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Control lentiviruses and those encoding the antisense sequence for miR-182 were purchased from GeneChem (Shanghai, China). Lentivirus infection to establish anti-miR-182-expressing stable clones (TPC-1/anti-miR-182) was performed according to the manufacturer's instructions.

2.5. Cell proliferation assay

Cells (2000 cells/well) were plated in 96-well plates. Following transient transfection at 48 h, cells were continually cultured for 96 h. At 24, 48, 72 and 96 h, 10 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was added to each well. Cells were incubated at 37 °C for 4 h, the medium removed, and precipitated formazan dissolved in 100 μl DMSO. After shaking for 20 min, absorbance was detected at 490 nm on a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, USA).

2.6. Cell cycle analysis

Cells were harvested during the exponential growth phase, and single-cell suspensions containing 2×10^5 cells fixed with 70% alcohol overnight at 4 °C. The cell cycle segment was determined using propidium iodide (PI) staining for nuclei. Fluorescence of DNA-bound PI in cells was measured using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.7. Luciferase activity assay

Luciferase assays were carried out as described previously [19]. Briefly, cells were co-transfected with wild-type or mutant luciferase reporter constructs, anti-miRNA and Renil using Lipofectamine 2000 (Invitrogen), and Firefly and Renilla luciferase activities measured after 48 h with a Dual-Luciferase® Reporter Assay System (Promega) using a luminometer.

2.8. Transwell invasion assay

For invasion assays, cells (5×10^4) were resuspended in 100 μl serum-free DMEM at 48 h post-transfection and added to the upper compartments of chambers coated with Matrigel (Clontech, Mountain View, CA). The lower compartments were filled with 600 μl DMEM containing 10% FBS. After incubation at 37 °C for 24 h, cells remaining on the upper surface of the membrane were removed. Cells on the lower surface of the membrane were fixed, stained with crystal violet, and counted under a light microscope.

2.9. Western blot analysis

Protein lysate preparation and SDS–PAGE were performed as described previously [20]. Briefly, cell lysates were separated via SDS–PAGE, transferred to nitrocellulose membranes and blocked in phosphate-buffered saline/Tween-20 containing 5% non-fat milk. Membranes were incubated with primary antibodies against CHL1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, followed by HRP-labeled secondary antibodies for 2 h. Protein expression was assessed via enhanced chemiluminescence and exposure to film (Fujifilm, Tokyo, Japan).

2.10. Tumor growth assay

Four-week-old male BALB/c nu/nu mice were purchased and bred at the Animal Laboratory Center, Fudan University. All animal protocols were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of Fudan University. Anti-miR-182-expressing stable TPC-1 cells (TPC-1/anti-182) and control cells (TPC-1/vector) were harvested, washed with PBS, and resuspended in medium. An aliquot of 2×10^6 cells was independently injected subcutaneously into 4-week-old male BALB/C-nu/nu nude mice. Tumors were measured every 3 days after they were visible to the naked eye. Tumor volume was calculated using the following formula $V = 0.5 \times D \times d^2$, where V represents volume, D the longitudinal diameter, and d the latitudinal diameter. All mice were sacrificed, tumors dissected, and the tumor weights measured.

2.11. Statistical analysis

Statistical analyses were performed using the SPSS 16.0 statistical software package. Experiments were repeated independently at least three times, and data presented as means ± SD. The association between miR-182 and CHL1 was analyzed using Spearman's correlation test. Comparisons between groups for statistical significance were conducted with Student's paired two tailed t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-182 and CHL1 expression patterns are negatively correlated

We initially analyzed the expression of miR-182 and CHL1 mRNA in PTC specimens and adjacent normal thyroid tissues.

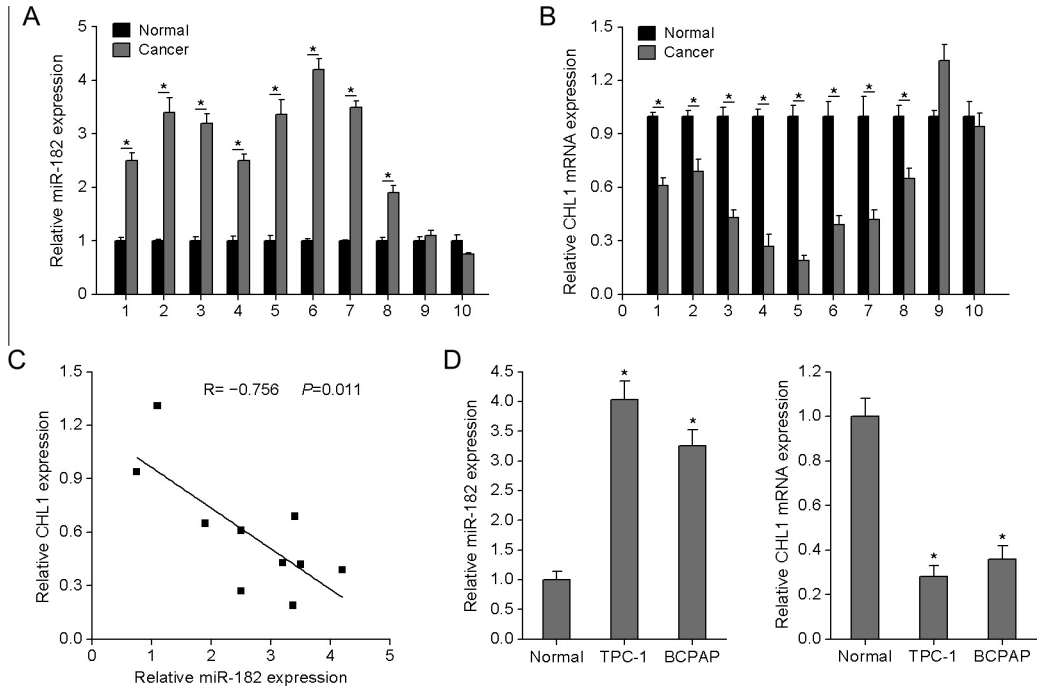

Fig. 1. miR-182 and CHL1 expression are negatively correlated in PTC tissues and cell lines. (A) Relative expression of miR-182 in 10 PTC specimens and adjacent normal thyroid tissues determined using qRT-PCR and normalized against endogenous control (U6 snRNA). (B) qRT-PCR analysis of CHL1 mRNA with β-actin as an internal control. (C) Spearman's correlation analysis of expression of miR-182 and CHL1 mRNA in PTC tissues. (D) Relative expression of miR-182 and CHL1 mRNA in the human PTC cell lines TPC-1 and BCPAP. All data are shown as means ± SD. $^{*}P < 0.05$.

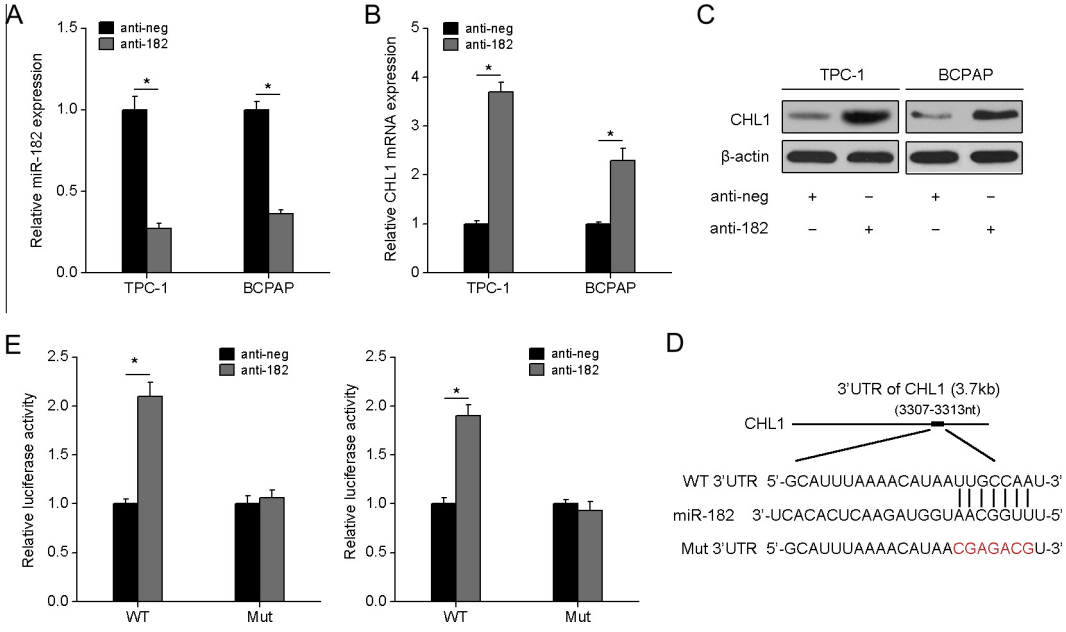

Fig. 2. miR-182 directly targets CHL1 in PTC cells. (A) TPC-1 and BCPAP cells were transfected with anti-miR-182 (anti-182) or anti-negative control (anti-neg) and subjected to qRT-PCR analysis. qRT-PCR (B) and western blot (C) analysis of CHL1 expression in indicated cells. (D) Schematic representation of the 3′-UTR of CHL1 with the predicted target site for miR-182. (E) Luciferase reporter assays in PTC cells following co-transfection with wild-type or mutant 3′-UTR of CHL1 and anti-182, as indicated. Data are presented as means of three independent experiments. All data are shown as means ± SD. $^{*}P < 0.05$.

qRT-PCR analysis revealed significantly higher miR-182 levels in PTC than matched normal tissues. In contrast, CHL1 mRNA levels were lower in tumor tissues (Fig. 1A and B). Expression of miR-182 was significantly negatively correlated with that of CHL1 in PTC tissues ($R = -0.756$; $P = 0.011$; Fig. 1C). Analysis of miR-182 expression in different cell lines confirmed this finding (Fig. 1D).

3.2. CHL1 is a direct target of miR-182 in PTC cells

To determine whether miR-182 targets CHL1, we performed transient transfection by introducing an antisense oligonucleotide for miR-182 into TPC-1 and BCPAP cells. The efficiency of anti-miR-182 was confirmed via qRT-PCR (Fig. 2A). The effect of miR-182 on endogenous CHL1 was confirmed using qRT-PCR and

western blot analyses. As shown in Fig. 2B and C, transfection of anti-miR-182 led to significant upregulation of both CHL1 mRNA and protein. To further determine whether miR-182 binding to the predicted site in the 3′-UTR region of CHL1 is responsible for regulation of its expression, the luciferase reporter assay was performed. The predicted wild-type (WT) target sequence of miR-182 was cloned into pGL3 control vector (Fig. 2D), and co-transfected into TPC-1 and BCPAP cells with anti-miR-182. Luciferase activities of cells transfected with anti-miR-182 were significantly increased, compared with that of the negative control. However, mutation of the putative miR-182-binding site (Mut) in the CHL1 3′-UTR region blocked anti-182-induced elevation of luciferase activity (Fig. 2E). Our results collectively demonstrate that miR-182 directly targets the 3′-UTR of CHL1 in PTC cells.

3.3. miR-182 suppression inhibits PTC cell growth and invasion

To investigate the potential role of miR-182 in PTC, we performed loss-of-function experiments. MTT data showed significantly reduced proliferation rates in TPC-1 and BCPAP cells transfected with anti-miR-182, compared to cells transfected with the negative control (Fig. 3A). In addition, miR-182 inhibition blocked cell cycle progression, as evident from the increased percentage of G0/G1 phase and reduced S phase cell population (Fig. 3B). PTC cell invasion was significantly suppressed in anti-182-transfected cells, compared with anti-neg-transfected cells (Fig. 3C). We further examined the potential activity of miR-182 in tumorigenesis using a TPC-1 xenograft model. Tumors grew slower in the TPC-1/anti-182 group than the TPC-1/vector group (Fig. 3D). At the end of the study (day 22), a significant decrease in tumor size and weight was observed in mice injected with TPC-1/anti-182, compared to the group injected with TPC-1/vector (Fig. 2E and F), supporting the theory that miR-182 functions as an oncogene in PTC.

3.4. CHL1 is involved in miR-182-mediated cell behavior

To determine the influence of CHL1 on growth and invasion of PTC cells, the pCDNA3.1 vector encompassing the CHL1 sequence was transfected into TPC-1 and BCPAP cells. Western blot analysis disclosed that transfection with pCDNA3.1-CHL1 leads to a significant increase in CHL1 expression in PTC cells, compared with control cells (Fig. 4A). In MTT and transwell assays, CHL1 overexpression mimicked the effects of anti-miR-182, resulting in decreased cell proliferation and invasion (Fig. 4B and C). To further evaluate whether the effects of miR-182 on cell growth and invasion are mediated through interactions with CHL1, PTC cells were transfected with CHL1 siRNA (si-CHL1) or control siRNA (si-control) in the absence or presence of anti-miR-182 (Fig. 4D). As shown in Fig. 4E and F, inhibition of cell proliferation and invasion by anti-miR-182 was counteracted upon CHL1 silencing. These findings collectively reinforce the concept that CHL1 is a functional target of miR-182.

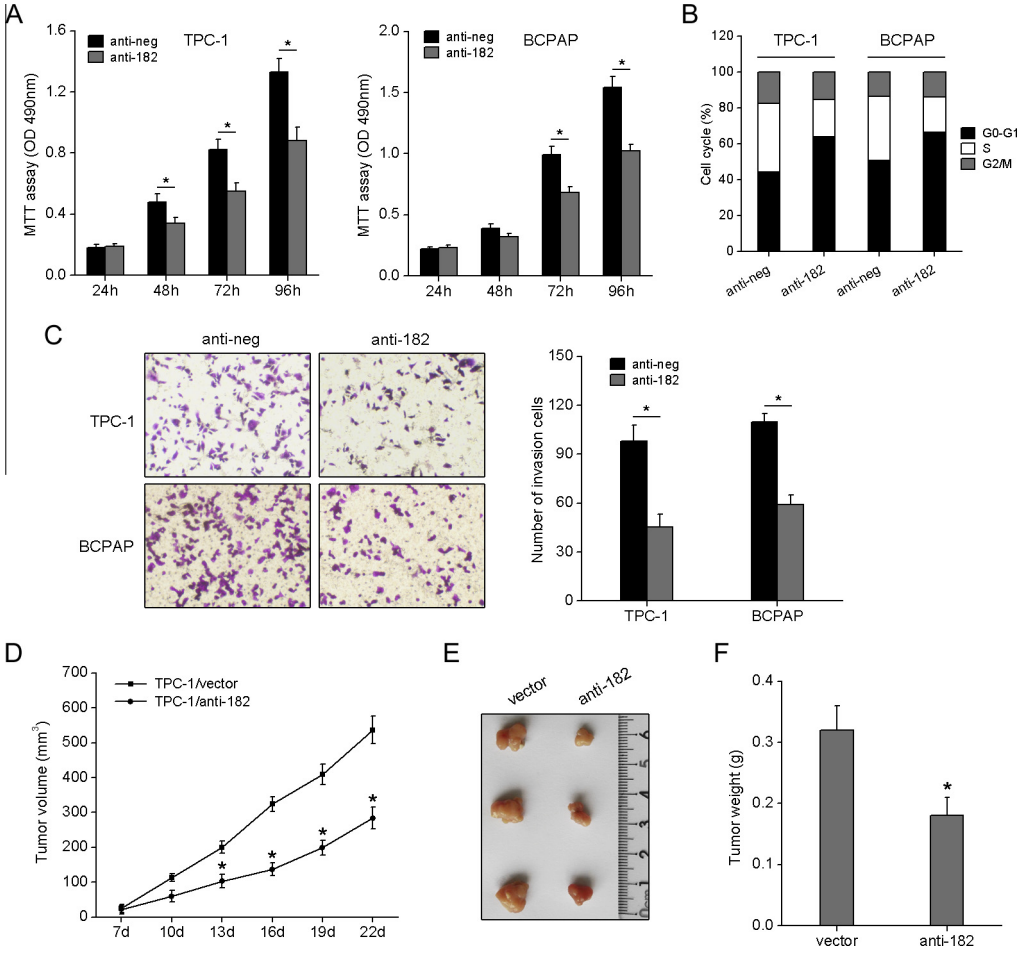

Fig. 3. miR-182 suppression inhibits proliferation and invasion of PTC cells *in vitro* and *in vivo*. TPC-1 and BCPAP cells were transfected with anti-182 or anti-neg. Forty-eight hours later, proliferation (A), cell cycle (B) and invasion (C) were evaluated with the MTT assay, flow cytometry analysis and transwell assay, respectively. (D-F) Effect of anti-miR-182 on tumor growth *in vivo*. TPC-1 cells infected with anti-182 lentivirus were injected subcutaneously into nude mice. Mice were killed 22 days after anti-182 treatment, and tumors assessed. Anti-182 caused a decline in tumor volume (D), size (E) and weight (F). All data are shown as means ± SD. $^{*}P < 0.05$.

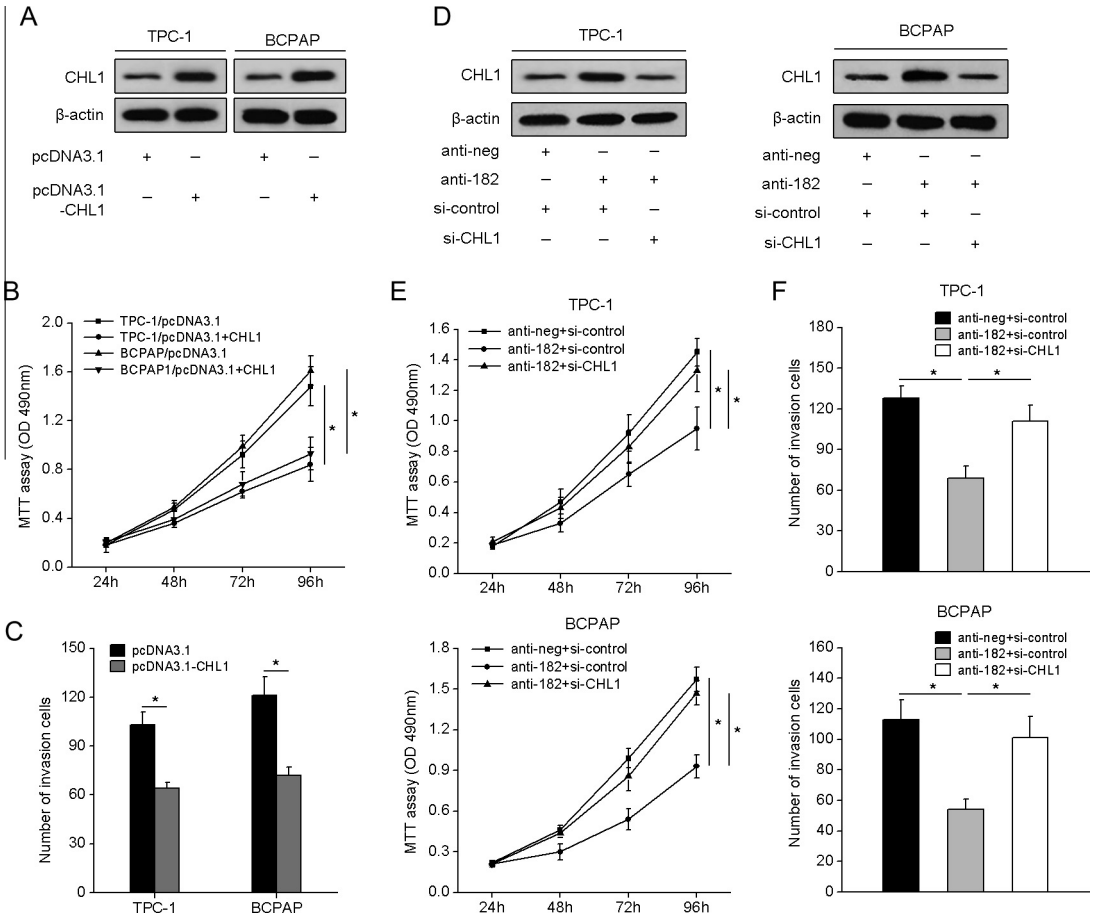

Fig. 4. CHL1 is involved in miR-182-mediated cell behavior. (A) TPC-1 and BCPAP cells were transfected with pCDNA3.1-CHL1 or pCDNA3.1, and CHL1 expression measured via western blot. Cell proliferation (B) and invasion (C) were evaluated with the MTT and transwell assays in each group. TPC-1 and BCPAP cells were transfected with CHL1 siRNA (si-CHL1) or control siRNA (si-control) in the absence or presence of anti-miR-182. CHL1 expression (D), cell proliferation (E) and invasion (F) were detected in all groups. All data are shown as means ± SD. $^{*}P < 0.05$.

4. Discussion

Emerging evidence has revealed that aberrant miRNA expression is correlated with a wide range of human cancers, functioning as tumor suppressors or oncogenes with important roles in the initiation, promotion and progression of cancers [21,22]. A number of miRNAs contributing to cell proliferation, migration and invasion in PTC have been identified to date. For instance, upregulation of miR-146b significantly promotes cell migration and invasiveness and increases resistance to chemotherapy-induced apoptosis in PTC [3]. miR-155 promotes tumor growth of PTC by targeting APC and activating Wnt/β-catenin signaling [9]. Conversely, miR-199a-3p has been shown to display a tumor suppressor function in papillary thyroid carcinoma [11]. Data from the current study provide evidence that miR-182 targeting of CHL1 promotes proliferation and invasion in PTC.

miR-182, a member of the miR-183 cluster located in the 7q32 region, plays a critical role in cancer development [12]. In 2009, Segura et al. [13] reported that miR-182 is upregulated and promotes melanoma metastasis by suppressing FOXO3 and microphthalmia-associated transcription factor. Subsequently, overexpression of this miRNA was detected in several other cancers, including glioma, lung cancer, ovarian carcinoma and breast cancer, and additional target genes identified, such as FOXO1, MTSS1 and MIM, resulting in promotion of tumorigenesis and metastasis [23–27]. However, miR-182 was significantly downregulated in human gastric adenocarcinoma and exerted tumor suppressor activity by targeting cAMP-responsive element-binding protein 1 (CREB1) in another study [28]. Data from the current investigation confirmed that miR-182 expression is significantly upregulated in PTC tissues than normal thyroid tissue and negatively correlated with that of CHL1.

CHL1 is a type I transmembrane protein belonging to the L1 family of neural cell adhesion molecules [15]. CHL1 is expressed in normal tissue beside the brain as well as a variety of human cancer cell lines and primary tumor tissues [29]. Senchenko et al. [30] reported that CHL1 is frequently downregulated in 11 cancer types, including PTC, implying its involvement in disease progression. Our experiments further disclosed that CHL1 is a direct target of miR-182. Data from qPCR and western blot analyses showed that upon repression of endogenous miR-182, CHL1 expression is enhanced at both the mRNA and protein levels. Luciferase reporter assays confirmed direct and negative regulation of CHL1 gene expression by miR-182 through binding to the 3′-UTR region.

The effects of miR-182 on PTC proliferation and invasion were investigated, in view of the critical role of CHL1 in cancer development [16,17]. Silencing of miR-182 led to significant suppression of cell proliferation, invasion and tumor growth *in vivo*. Furthermore, CHL1 knockdown partially, but significantly reversed the suppressive effect of miR-182 inhibition, suggesting that CHL1 is a downstream mediator of miR-182 activity in PTCs.

In summary, our study has revealed the importance of regulation of miR-182 and its target CHL1 in PTC progression. The results collectively support an oncogenic role of miR-182 in PTC cell

proliferation and invasion through downregulation of CHL1 expression. The newly identified miR-182/CHL1 link in PTC should be useful in clarifying the mechanism underlying progression of PTC, and facilitate the development of appropriate therapeutic targets.

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