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PCDH10 Interacts With hTERT and Negatively Regulates Telomerase Activity

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Abstract: Telomerase catalyzes telomeric DNA synthesis, an essential process to maintain the length of telomere for continuous cell proliferation and genomic stability. Telomerase is activated in gametes, stem cells, and most tumor cells, and its activity is tightly controlled by a catalytic human telomerase reverse transcriptase (hTERT) subunit and a collection of associated proteins.

In the present work, normal human testis tissue was used for the first time to identify proteins involved in the telomerase regulation under normal physiological conditions.

Immunoprecipitation was performed using total protein lysates from the normal testis tissue and the proteins of interest were identified by microfluidic high-performance liquid chromatography and tandem mass spectrometry (HPLC-Chip-MS/MS). The regulatory role of PCDH10 in telomerase activity was confirmed by a telomeric repeat amplification protocol (TRAP) assay, and the biological functions of it were characterized by in vitro proliferation, migration, and invasion assays.

A new in vivo hTERT interacting protein, protocadherin 10 (PCDH10), was identified. Overexpression of PCDH10 in pancreatic cancer cells impaired telomere elongation by inhibiting telomerase activity while having no obvious effect on hTERT expression at mRNA and protein levels. As a result of this critical function in telomerase regulation, PCDH10 was found to inhibit cell proliferation, migration, and invasion, suggesting a tumor suppressive role of this protein.

Our data suggested that PCDH10 played a critical role in cancer cell growth, by negatively regulating telomerase activity, implicating a potential value in future therapeutic development against cancer.

(*Medicine* 94(50):e2230)

Abbreviations: GAPDH = glyceraldehyde 3-phosphate dehydrogenase, HPLC-Chip-MS/MS = microfluidic high-performance liquid chromatography and tandem mass spectrometry, hTERT = human telomerase reverse transcriptase, hTR = human telomerase RNA, PCDH10 = protocadherin 10, RT-PCR = reverse transcription-polymerase chain reaction, TRAP = telomeric repeat amplification protocol.

Editor: Patrick Wall.
Received: January 26, 2015; revised and accepted: November 11, 2015.
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This work was supported by the National Natural Science Foundation of China (No.81302333 and cstc2013jcyjA1705).
The authors have no conflicts of interest to disclose.

ISSN: 0025-7974
DOI: 10.1097/MD.0000000000002230

INTRODUCTION

Telomeres are repetitive guanine-rich sequences located at the ends of eukaryotic chromatids and they protect chromatids from deterioration and inappropriate recombination. Telomeres shorten after each round of cell division in normal human somatic cells, eventually limit cell replications, and cause replicative senescence.[1] However, telomere shortening in immortalized cells is alleviated by telomerase, a special ribonucleoprotein enzyme that maintains telomere homeostasis by synthesizing and elongating telomeric repeats.[2]

In normal physiology telomerase is only expressed in a limited number of cells, such as gametes, activated lymphocytes, and stem cells where natural replication is essential.[3] However, over 90% of tumor cells express telomerase, making it a common phenotypic feature among different malignancies.[4] The activation of telomerase has been found to be a pivotal step in carcinogenesis and its down-regulation is associated with the differentiation of tumor cells. Thus, the telomerase regulation mechanism is crucial for cancer cell survival.[5]

Human telomerase reverse transcriptase (hTERT) is a catalytic component of the human telomerase complex and also the rate-limiting factor of telomerase activity.[6] Its transcription is directly controlled by c-myc, SP1, p53, and Wilms tumor (WT)-1.[7] In addition, telomerase activity can also be mediated at the post-translational level. Reversible phosphorylation of hTERT at serine/threonine or tyrosine resides, as a result of the activation of multiple kinases or phosphatases, is important for its structure, localization, and catalytic activity.[8] Identification of molecules and proteins involved in the telomerase complex is, therefore, a prerequisite to understanding the molecular mechanism underlying the delicately controlled elongation of telomeres under both physiological and pathological conditions.

Systematic proteomics is a powerful tool for screening protein–protein interactions, and its application in tumor models with overexpressed hTERT facilitates the identification of upstream regulators of telomerase. Using yeast 2-hybrid systems, the association of telomerase with HSP90, p23, Ku, and 14-3-3 signaling proteins was uncovered, improving our understanding of assembly of telomerase complex and its access to telomeric DNA ends.[9–11] However, given that the human telomerase complex has an estimated mass of 1000 kDa, it is predicted that there are additional hTERT-associated proteins that remain to be identified.[12]

While telomerase regulation in cancer cells has been well characterized, much less is known about the telomerase complex in normal biology. In healthy tissues, the telomerase is largely inactive due to the transcriptional repression of hTERT prior to birth, except for germinal tissues such as testis[13] and

ovary,[14–16] lymph nodes,[17] and some hyperplastic tissues.[18,19] This dormant state of telomerase presents an ideal opportunity for the discovery of telomerase-suppressive factors, which may represent keys to future therapeutic development targeting telomerase activity.

In this study, we discovered a new hTERT-interacting protein, protocadherin 10 (PCDH10), in normal human testis tissues using immunoprecipitation followed by a microfluidic-based high-performance liquid chromatography and tandem mass spectrometry (HPLC-Chip-MS/MS), a powerful approach in the study of protein–protein interactions.[20–22] The interaction of PCDH10 with hTERT was confirmed by reciprocal immunoprecipitation, and the inhibitory effects of this interaction on telomerase activity were characterized by a telomeric repeat amplification protocol (TRAP) assay. Moreover, the observations that overexpression of PCDH10 inhibits cancer cell proliferation, adhesion, migration, and invasion support a potential tumor suppressor role for PCDH10.

METHODS

Ethics Statement

Fresh normal testis tissues were collected from patients who received surgical treatment for prostate cancer at the Department of Urology, Southwest Hospital, The Third Military Medical University, China. All the patients were informed of the purpose and procedure of this study and written consent was obtained before surgery. The protocols used for this study were approved by the Research Ethics Committee of Southwest Hospital affiliated to The Third Military Medical University, Chongqing, China.

Immunopurification of hTERT-Associated Complex and HPLC-CHIP-MS-MS

A Pierce immunoprecipitation kit (Thermo Scientific, Waltham, MA) was used following the manufacturer's instructions. Briefly, total protein was isolated with an immunoprecipitation lysis/wash buffer from fresh testis tissue diced to small pieces, in the presence of a complete protease inhibitor cocktail (Roche Diagnostics, Shanghai, China). Total protein concentration was determined by a bicinchoninic acid assay. The lysate was then precleared with the control agarose in a Pierce spin column before incubating overnight at 4°C with 20 μg of anti-hTERT polyclonal antibody (Abcam, Cambridge, UK) or an IgG control coupled to an AminoLink Plus Coupling resin. The resin was washed with the immunoprecipitation lysis/wash buffer before the immunoprecipitates were eluted with the elution buffer.

The immunoprecipitates were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel stained with 0.25% Coomassie blue R-250 in 10% acetic acid/45% methanol for at least 3 hours with gentle shaking. After thoroughly destaining in 50% methano1 and 10% ethanol, the gel was stored in distilled water at room temperature until HPLC-Chip-MS/MS analysis.

The bands of interest were excised from the SDS-PAGE gel and then were destained in ammonium bicarbonate/acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide, and dehydrated in acetonitrile. Automatic in-gel protein digestion was performed with a MassPrep station (Waters, Milford, MA) and 12.5 ng/μL trypsin in 50-mM ammonium bicarbonate for 5 hours at 37°C.

Digested peptides were separated using an Agilent 1200 series Nanoflow HPLC system (Agilent Technologies, Santa Clara, CA). The sample in solvent A (0.1% formic acid in water) was trapped in a Zorbax 300SB C18 enrichment column for 1.5 minutes and then was applied to an analytical column with a biphasic gradient of solvent B (90% acetonitrile, 10% water with 0.1% formic acid) ranging from 3% to15% for 2 minutes, then from 15% to 50% for 70 minutes, with a fixed flow rate of 0.3 mL/min. Mass spectrometry was performed with an Agilent 1100 Series LC/MSD Trap MS with an Agilent orthogonal nanoelectrospray source. MS/MS analysis was controlled by a data-dependent setting. The nanoelectrospray source was operated at 2000 V with a Pico Tip EMITTER (New Objective, Woburn, MA). Due to statistical fluctuations in the peptide precursor selection during MS/MS acquisition, LC-MS/MS assays were performed in triplicate for each sample to achieve appropriate proteome comparison.

The identification of peptides and proteins was automatically performed by Spectrum Mill software (Agilent) using the Swiss-Prot protein database (Geneva, Switzerland) and screened for tryptic peptides restricted to *Homo sapiens*.

Immunoprecipitation and Western Blotting

Total protein isolated from normal testis tissue was incubated with an anti-hTERT or anti-PCDH10 polyclonal antibody (Santa Cruz, Dallas, TX) coupled to an AminoLink Plus Coupling resin. Resin without antibody was used as a control. The resulting immunoprecipitates were separated on SDS(10%)-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% skim milk in phosphate-buffered saline (PBS), the membrane was incubated with the appropriate primary antibodies overnight at 4°C with gentle agitation, followed by 2-hour incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz) at room temperature. The signal was visualized by using a SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

Cloning of the PCDH10 Gene

The full-length coding region of *PCDH10* was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA isolated from testis using TRIzol reagent (Thermo Scientific) according to the manufacturer's protocol. RT-PCR was performed using a Real-Time PCR Master Mix (Toyobo, Japan). Specific primers for *PCDH10* were designed according to the *PCDH10* sequence registered in Genbank (NM_001098170) and synthesized by Shanghai Bioasia Biotech (Shanghai, China). The sequences of the forward and reverse *PCDH10* primers were 5′-GGGGTACCATGATTGTGCTATTATTGTTTGCCT-3′ and 5′-GCTCTAGACTAGCATATCCTTTTCCGTGTCA-3′, respectively. The PCR product was ligated into a pTA2 vector (Toyobo), which was transformed into DH5α competent *Escherichia coli* (American Type Culture Collection, Manassas, VA). The sequence of *PCDH10* was confirmed by Sanger sequencing at Sangon Biotech (Shanghai, China).

Construction of an Adenovirus-Based PCDH10 Expressing Vector

An adenovirus-based PCDH10 expressing vector was constructed by DNA recombination using a pAdTrack-CMV and pAdEasyTMxL Adenoviral Vector System (Agilent Technologies). The pTA2-PCDH10 and pAdTrack-CMV shuttle plasmids were each digested by *Kpn* I and *Xba* I and ligated. The resulting recombined shuttle plasmids were transformed into DH5α *E. coli*, selected, and linearized by *Pme* I. The pAdTrack-

TABLE 1. Primers Sequences of hTERT and hTERT-Interacting Protein Genes

Target	Forward Primer	Reverse Primer
hTERT	5′- TATGCCGTGGTCCAGAAGG-3′	5′- CAAGAAATCATCCACCAAACG-3′
hTR	5'-CTGGGAGGGGTGGTGGCCATTT-3'	5'-CGAACGGGCCAGCAGCTGACAT-3'
PCDH10	5′-GCACATATGGTTTATTACCCAG-3′	5′-CAGGATCCTTAAAAAAAGTGGAGG-3′
GAPDH	5′- CGTCTTCACCACCATGGAGA-3′	5′-CGGCCATCGCCACAGTTT-3′

CMV-PCDH10 obtained was then transformed into BJ5183 *E. coli* in which pAdEasy-PCDH10 was generated. Linearized by *Pac* I, the recombinant adenovirus plasmid, pAdEasy-PCDH10, was transfected into 293T cells using lipofectamine (Thermo Scientific) to generate and propagate the recombinant adenovirus, Ad-PCDH10. Virus titer was detected by using a tissue culture infective dose assay.

Cell Culture and Virus Transduction

The pancreatic cancer cell line, HS 776T (JENNIO Biological Technology, Guangzhou, China), was maintained in RPMI 1640 medium (Thermo Scientific) supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL) and streptomycin (100 units/mL) at 37°C in a humidified 5% CO_2 atmosphere. For virus transduction, 1×10^6 cells were seeded and cultured to 80% confluence in 75 cm^2 flasks, washed with PBS and antibiotic-free, serum-free medium. The virus containing medium (1×10^9 plaque-forming units (PFU)/mL) was added to cover the HS 776 T cells and was incubated with the cells for 2 hours at 37°C before the medium was replaced with fresh RPMI 1640 medium containing 10% FBS. The cells were allowed to recover for 4 hours before being examined microscopically for the expression of green fluorescent protein at different time points.

Real-Time PCR

Real-time PCR was performed using a SYBR Green Realtime PCR Master Mix (Toyobo) following the manufacturer's instruction. Briefly, total RNA was extracted from cultured cells with TRIzol reagent and used as a template for complementary DNA synthesis driven by avian myeloblastosis virus (AMV) reverse transcriptase with oligo (dT) primers. Real-time PCR was performed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. The primers used were designed based on the sequences registered in GenBank and synthesized by Shanghai Bioasia Biotech (Table 1). A melting curve was obtained to determine product purity. The threshold cycle (Ct) of the target mRNAs were normalized to that of GAPDH and used to calculate relative levels of the target transcripts.

Telomerase Assay

The telomerase activity was assessed by a telomeric repeat amplification protocol (TRAP) assay using a Telomerase Detection Kit (Millipore, Billerica, MA). Briefly, immunoprecipitation with an anti-PCDH10 antibody was performed using HS 776T cells that were transduced by PCDH10 expressing viruses for 72 hours. TRAP lysis buffer were used as a negative control and cell extracts from telomerase-active 293 cells were used as a positive control. After 30 minutes of telomerase extraction, 30 minutes of primer elongation at 23°C, 3 minutes of telomerase inactivation at 94°C, depuration, and 30 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 90 s) were performed. From each RT-PCR sample, 25 μL aliquots were separated on a 12% nondenaturing polyacrylamide gel, which was then stained and visualized under ultraviolet (UV) light. A semiquantitative densitometric evaluation was conducted using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). All of these experiments were performed in triplicate.

Cell Proliferation Assay

Cell proliferation was assessed by monitoring cell viability after PCDH10 manipulation. Cells were seeded into 96-well plates (1×10^4 cells/well) and transduced with the PCDH10-expressing or the empty control viruses. At various time points, 10 μL of CCK-8 solution was added to each well. After another 1 to 4 hours, absorbance at 450 nm was measured. The experiment was performed 3 times with 6 replicates for each sample.

Wound Healing Assay

A monolayer wound healing assay was performed to assess the impact of PCDH10 on cell mobility. HS 776T cells with stable expression of PCDH10 were grown in 35-mm dishes to form a monolayer, on which scratches were then carefully made using sterile tips. After 2 washes with fresh medium, the cells were cultured under normal conditions and imaged at 24 and 48 hours under a phase-contrast microscope. The experiments were repeated for 3 times.

Matrigel Invasion Assay

The effect of PCDH10 on cell invasion was assessed in a 24-well plate fitted with invasion chambers that were sealed with PET membrane (8-μm pore size) at the bottom and coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ). HS 776T cells with and without stable PCDH10 expression were washed 3 times, and seeded into the top chamber at 4×10^4 cells/well in 100-μL fresh medium containing 1% FBS, while in the bottom chamber 600-μL medium supplemented with 5 mg/mL fibronectin (Sigma) was added. The cells were incubated for 24 hours at 37°C, and those migrating through the Matrigel-coated membrane were stained with Gentian Violet and counted. The experiment was repeated 3 times.

Statistical Analysis

All values are expressed as the mean ± standard deviation (SD). Correlations between the expression of PCDH10, expression of hTERT, and telomerase activity in the cells examined were analyzed. Differences between experimental groups were assessed with a 2-tailed unpaired Student *t* test. Statistical analysis was performed using a SPSS 16.0 package (IBM, Armonk, NY) and $P < 0.05$ was considered to be statistically significant.

RESULTS

Identification of Novel hTERT-Binding Proteins

The hTERT-binding protein complex was immunoprecipitated from human testis tissue extracts using an hTERT antibody. The complex was separated by SDS-PAGE and was compared with human testis extracts that were incubated with an IgG control antibody. Two bands were detected in the former and not the latter (Bands 1 and 2, Fig. 1A) and they had molecular weights of ~120 and ~110 kDa. The 120-kDa band was confirmed to be hTERT in the Western blotting analysis (Fig. 1B) run in parallel using the same batch of immunoprecipitate samples as in Figure 1A. hTERT was not detected in the immunoprecipitate of IgG control, indicating the specificity of the hTERT antibody used in the experiment. Band 2 (~110 kDa) was unknown and subjected to HPLC-Chip-MS/MS analysis after reduction, alkylation, and trypsinization.

After HPLC-Chip-MS/MS, peptide mass fingerprinting was performed by searching against Swiss-Prot protein database. The protein, PCDH10, was consistently identified as one of the top 5 binding partners of hTERT in the 3 HPLC-Chip-MS/MS analyses performed (Fig. 1, C and D).

Confirmation of the Interactions Between PCDH10 and hTERT

To confirm the association of hTERT with the newly identified binding partner, PCDH10, reciprocal immunoprecipitation was performed. Total protein isolated from testis tissue was used for immunoprecipitation with anti-hTERT and anti-PCDH10, with isotype matched IgG as controls, and detected by Western blotting for PCDH10 and hTERT, respectively. The results are shown in Figure 2A and B. PCDH10 was detected in the immunoprecipitate of the anti-hTERT antibody, and hTERT was detected in the immunoprecipitate of the anti-PCDH10 antibody. These data demonstrate for the first time that hTERT associates with PCDH10 in vivo.

Construction of Recombinant Adenovirus Encoding Human PCDH10 Protein

The full-length coding region of *PCDH10* was cloned from normal human testis tissue using RT-PCR (Fig. 3A). The amplified fragment was inserted into adenovirus shuttle vector pAdTrack-CMV. The obtained pAdTrack-CMV-PCDH10 and adenovirus genome-containing pAdEasy-1 plasmid underwent homologous recombination in *E. coli* BJ5183 cells (Fig. 3B). Finally, the recombinant adenovirus Ad-PCDH10 was packed and propagated after pAdEasy-PCDH10 was transfected into 293T cells. The adenovirus of high purity was obtained after 5 passages and named as Ad-PCDH10 (Fig. 3C and D).

Effect of PCDH10 Overexpression on Telomerase Functions

The interaction of PCDH10 with hTERT in vivo directed us toward examining its function in telomerase regulation. PCDH10 was overexpressed in HS 776T cells using the recombinant adenovirus, Ad-PCDH10, and cells treated with empty Ad vector and untransduced cells were used as controls. Western blotting and RT-PCR confirmed significantly higher levels of PCDH10 in cells transduced with Ad-PCDH10 adenovirus compared with the mock and vector controls (Fig. 4, A–D). Of note, the exogenously expressed PCDH10 had no effect on the expression of hTERT on both mRNA and protein levels and the abundance of telomerase RNA (hTR) (Fig. 4, A–D).

To assess the effect of PCDH10 on telomerase activity, immunoprecipitation was performed with an anti-PCDH10 antibody using cell extracts collected from cells overexpressing PCDH10 or the control cells. The immunoprecipitates were then used for TRAP assay. Unsurprisingly, more hTERT was pulled down with the anti-PCDH10 antibody in cells overexpressing PCDH10 (Fig. 4E, bottom panel). The TRAP assay results indicated a remarkable reduction in telomerase activity in the HS 776T cells with PCDH10 overexpression compared with the mock and vector controls (Fig. 4E and F). Taken together, these data demonstrate that PCDH10 was physically involved in the telomerase complex through its interactions with hTERT in vivo, and this association inhibits telomerase activity.

Effect of PCDH10 on Cell Proliferation, Migration, and Invasion

PCDH10 was overexpressed in HS 776T cells by transduction with adenovirus Ad-PCDH10 using the empty Ad vector and untransduced cells as controls. At 24, 48, 72, 96, 120, and 144 hours posttransduction, a CCK-8 assay was performed to assess the cell viability. A gradual decrease in cell proliferation was observed at the 96, 120, and 140 hours time points for the cells that overexpressed PCDH10. Specifically, cell proliferation was decreased by 12.73% ($P < 0.05$), 19.87% ($P < 0.01$), and 30.14% ($P < 0.001$), respectively, compared with the untransduced control (Fig. 5A). Meanwhile, the cells treated with empty virus appeared unaffected. These data suggest that PCDH10 overexpression strongly inhibits tumor cell proliferation.

We next assessed the effect of PCDH10 overexpression on cell migration by a wound healing assay on the HS 776T cell monolayer. Cell motility was found to be unchanged for the HS 776T cells that were transduced with empty virus compared with untransduced cells ($P > 0.05$), whereas the cells overexpressing PCDH10 exhibited significantly slower motility across the scratch mark (Fig. 5B). The relative migration of the PCDH10 overexpressing cells was deceased by 30.07% after 24 hours ($P < 0.001$) and by 52.95% after 48 hours ($P < 0.001$), compared with the controls (Fig. 5C). These results suggest that PCDH10 inhibits tumor cell migration.

Furthermore, a matrigel invasion assay was also performed to examine the invasive potential of HS 776T cells with and without exogenous PCDH10 expression. As shown in Figure 5D, upregulation of PCDH10 significantly reduced the number of cells that migrated through the matrigel-coated membranes compared with the untranduced cells ($P < 0.001$), whereas the empty virus had no effect. In combination, these results suggest that PCDH10 overexpression potently inhibits the invasion of HS 776T cells.

DISCUSSION

The dormant state of telomerase in the majority of normal tissues suggests that hTERT-associated proteins may coordinately function as inhibitors of telomerase, and play a critical role in the restriction of cell replication to maintain tissue homeostasis. In the present investigation, we screened for hTERT interacting proteins in the normal testis by HPLC-Chip-MS/MS analysis of proteins immunoprecipitated with an hTERT antibody. This approach allowed us to more directly identify proteins interacting with hTERT at the physiological level, rather than using yeast hybrid systems which rely on in

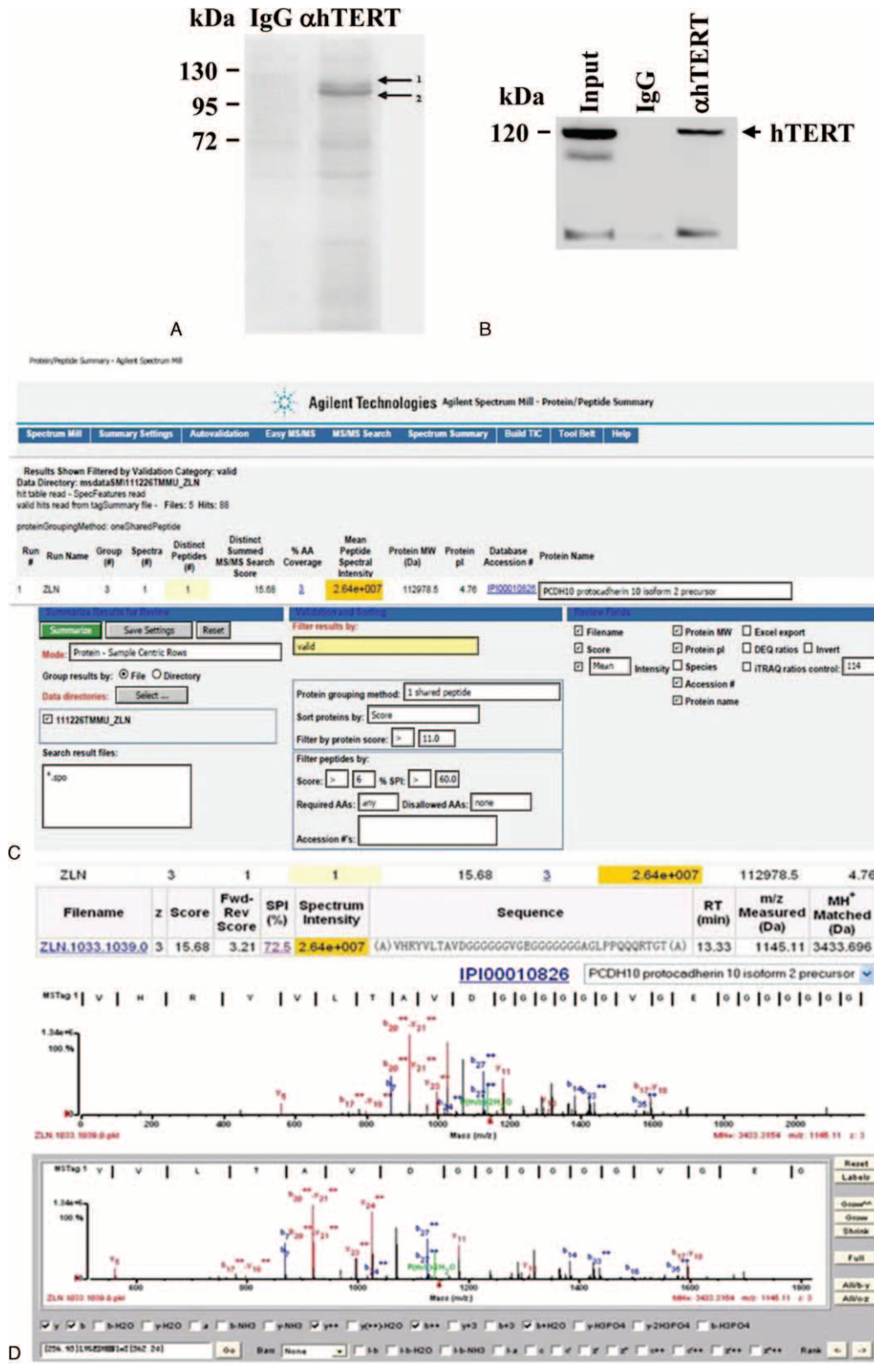

FIGURE 1. Immunoprecipitation and HPLC-Clip-MS/MS analysis of hTERT-interacting proteins. A, Proteins interacting with hTERT were immunoprecipitated by an hTERT antibody from extracts collected from normal testis tissues. Immunoprecipitation reactions using IgG as a control were performed as a comparison. B, Specificity of the immunoprecipitation was confirmed by Western blotting. C and D, Peptide mass fingerprinting identified the PCDH10 protein.

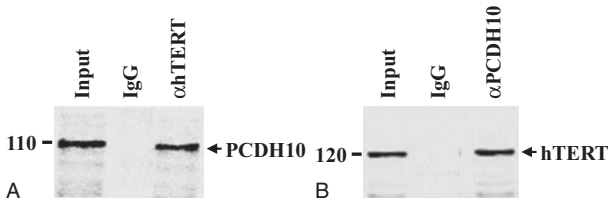

FIGURE 2. hTERT and PCDH10 proteins are associated with each other in vivo. Reciprocal immunoprecipitations were performed to examine the association. A, Western Blotting analysis of PCDH10 in proteins immunoprecipitated with anti-hTERT antibody. B, hTERT protein was detected in immunonprecipitate by an anti-PCDH10 antibody.

vitro protein–protein interactions. To our knowledge, this study was the first to demonstrate that PCDH10 as an hTERT-interacting protein which negatively regulates telomerase activity and inhibits cancer cell proliferation, migration, and invasion.

The protein identified, PCDH10, belongs to the protocadherin subfamily of cadherins.[23] This subfamily of proteins contain 6 extracellular cadherin domains, a transmembrane domain, and a cytoplasmic tail that differs them from the classical cadherins.[23] As a newly discovered protein, the function of PCDH10 remains unclear. Accumulating evidence links it with signal transduction and growth control during development of the central nervous system.[24,25] Importantly for this study, recent data indicated frequent transcriptional silencing and promoter methylation of *PCDH10* in most carcinoma cell lines, including 82% of nasopharyngeal carcinoma, and 42% to 51% of other carcinomas, but not in normal tissues,[25–28] although the molecular reason is unknown. In this study, we overexpressed PCDH10 in cancer cells where its expression is

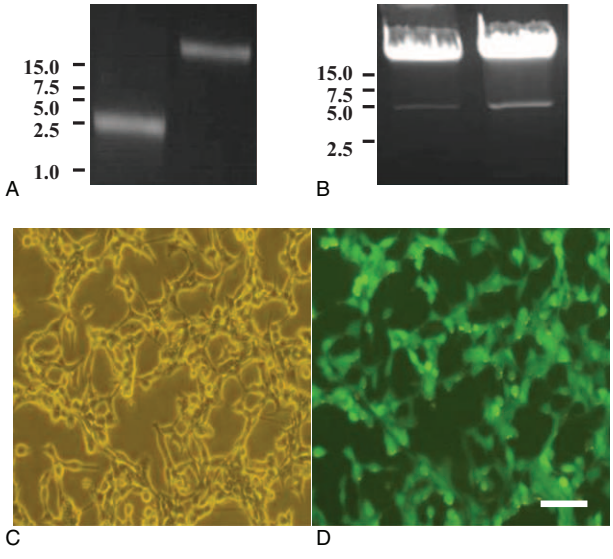

FIGURE 3. Construction of a recombinant adenovirus expressing human PCDH10. A, Amplification of the PCDH10 open reading frame by RT-PCR from complementary DNA synthesized with RNA isolated from normal testis tissue. B, Verification of the recombinant adenovirus plasmid for overexpression of PCDH10, pAdEasy-PCDH10, by *Pac* I digestion. C and D, Imaging of 293T cells transfected with pAdEasy-PCDH10 under an inverted microscope (C) and a fluorescence microscope (D). Scale bar: 40 μm.

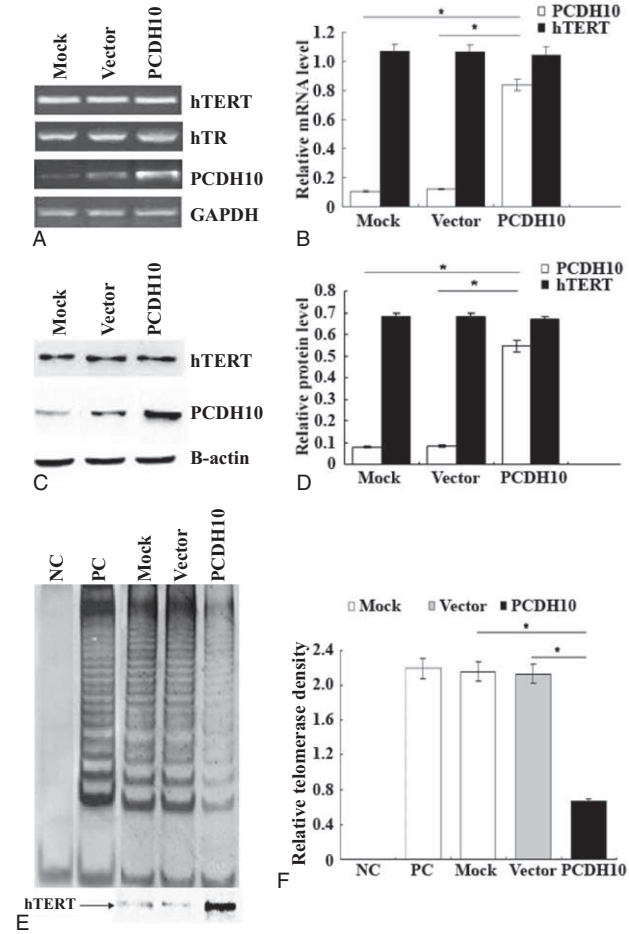

FIGURE 4. Effect of PCDH10 on hTERT expression and telomerase activity. PCDH10 was exogenously expressed in an HS 776T cell line, expressions of hTERT and PCDH10 were assessed at both the transcriptional (A, B) and translational levels (C, D) by RT-PCR and Western blotting, respectively. Untransfected and empty virus-transduced cells (Mock and Vector, respectively) were analyzed in parallel as controls. Levels of telomerase RNA (hTR) were also examined by RT-PCR. β-actin and GAPDH were used as loading controls, respectively. A densitometric analysis was performed for both agarose gel (A) and Western blot (C) images, and data from a minimum of 3 replicates ± SD are summarized in (B) and (D), respectively. E, A TRAP assay was performed using immunoprecipitate by an anti-PCDH10 antibody from PCDH10-overexpressing HS 776T cells, and the result presented as the telomeric ladders generated by PCR amplification, using a telomeric sequence primer, of the oligonucleotide repeats resulted from the telomerase activity. NC = no cells control; PC = parental cells control. The Western blot analysis to detect hTERT is shown below the panel. F, A densitometric analysis was performed for 3 independent TRAP assays, and the results ± SD are presented in the bar graph. $^{*}P < 0.01$ between the 2 groups.

usually suppressed, and found significant decrease in telomerase activity (Fig. 4E). Our data provide the first experimental evidence that PCDH10 negatively regulates telomerase activity, explaining that, by silencing and methylating PCDH10 gene, cancer cells hijack telomerase activity to achieve telomere elongation and continuous proliferation capacity.

Marked inhibition of cell proliferation, migration, and matrigel invasion were observed with overexpression of

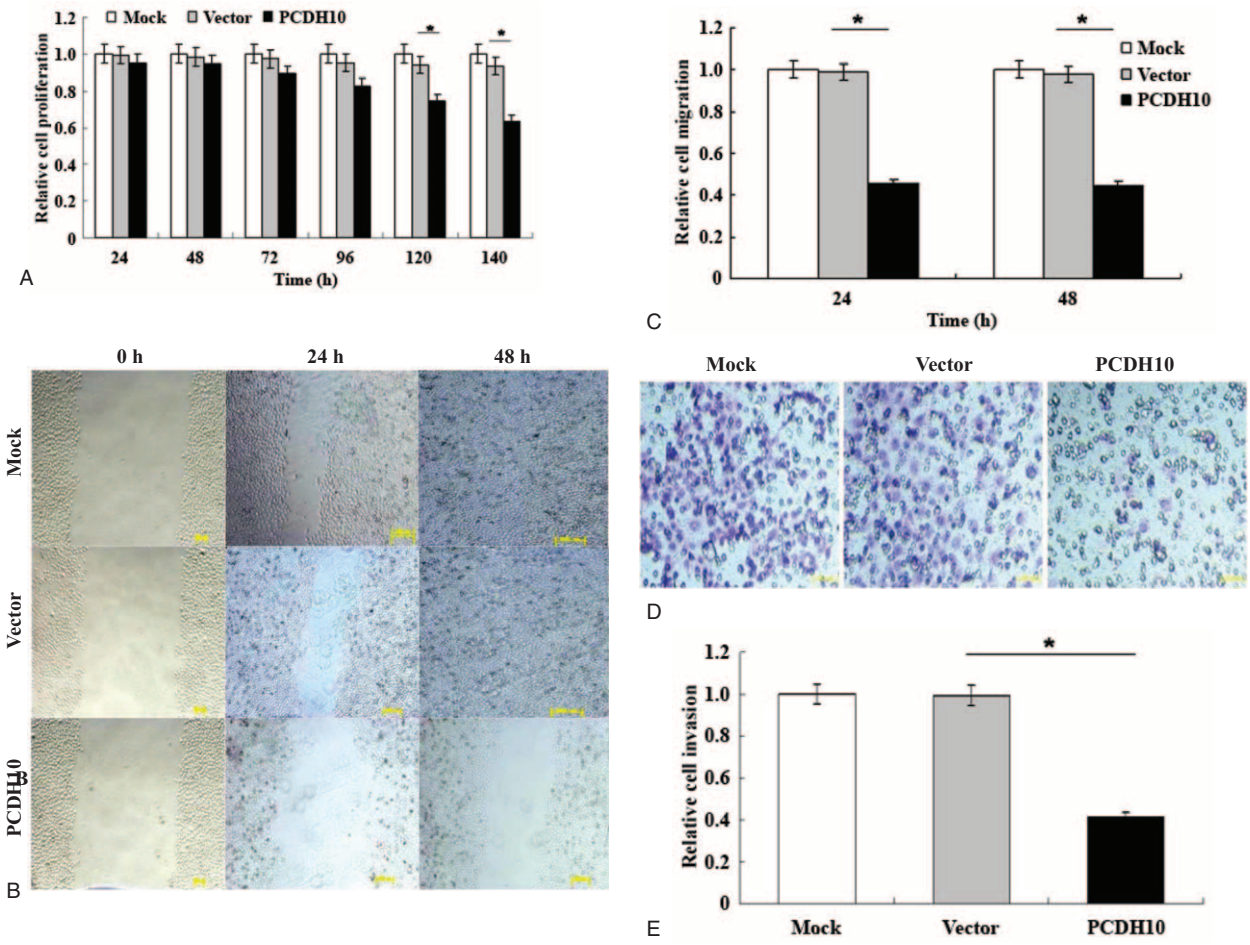

FIGURE 5. Ectopic PCDH10 expression suppresses the proliferation, migration, and invasion of pancreatic carcinoma cells. HS 776T cells were transduced with an adenovirus expressing PCDH10 (PCDH10), whereas untransduced and empty adenovirus-transduced cells (Mock and Vector, respectively) served as controls. A, CCK-8 assay was used to monitor cell proliferation at various time points up to 140 h after transduction. The mean ± standard error values are shown ($^{*}P < 0.01$). B, C, Motility of HS 776T cells was assessed by wound migration assays. The migration of Mock, Vector, and PCDH10 cells across the wound is imaged in B, and relative migration is quantified in C ($^{*}P < 0.01$). D, The invasive capacity of Mock, Vector, and PCDH10 cells was assessed by matrigel invasion assays. The cells that migrated were stained with hematoxylin and eosin and imaged (D). E, The cells migrated were quantified and the data averaged from 3 independent experiments. $^{*}P < 0.01$.

PCDH10 in a pancreatic cancer cell line (Fig. 5). These data correlate with an inhibitory role for PCDH10 in telomerase regulation, and are consistent with previous reports that PCDH10 may be a candidate tumor suppressor gene.[25] Accordingly, inactivation of PCDH10 may permit tumor growth and expansion, and its ectopic expression in carcinoma cell lines dramatically inhibits tumor cell growth, migration, invasion, and colony formation.[28]

It is uncertain whether protocadherins such as PCDH10 are functionally redundant to classic cadherins. PCDH10 is unlikely a major regulator for cell–cell junction considering that mouse PCDH10 only weakly mediates cell aggregation.[25] It may not interfere with the Wnt/β-catenin/Tcf signaling pathway like cadherins[29] because protocadherins, including PCDH10, do not bind β-catenin.[23] In this study, we identified PCDH10 as a negative factor of telomerase. RT-PCR and Western Blotting results exhibited that hTERT was expressed in the presence of PCDH10, suggesting that suppression of telomerase activity by PCDH10 is mediated through the interactions between PCDH10 and hTERT, rather than an inhibition of hTERT transcription (Fig. 4, A–D). Therefore, we speculate that interactions between PCDH10 and hTERT may directly inhibit telomerase activity. PCDH10 is a typical protocadherin that contains a cytoplasmic motif, CM-2, which is homologous to the laminin-type EGF-like domains.[24] CM-2 is also similar to the C_2HC-type zinc finger or zinc knuckle finger motif. Since some zinc fingers have been shown to mediate protein–protein interactions, the CM-2 motif in PCDH10 may mediate its interactions with other proteins. Previously, it has been shown that the introduction of *hTERT* into normal cells induces telomerase activity and confers a continuous replication capacity, whereas alterations in the C-terminus of hTERT disrupt its ability to maintain telomerase activity to immortalize a cell.[30] Taken together, these observations suggest that the C-terminus of hTERT interacts with other essential factors for telomerase activity, and it will be interesting to investigate whether the C-terminus of hTERT interacts with the CM-2 domain of PCDH10.

In the present study, we screened for hTERT interacting proteins in the normal testis tissue and identified PCDH10 as a novel hTERT interacting partner. Our data suggest that interactions between hTERT and PCDH10 contribute to the regulation of telomerase activity in the cell, since overexpression of PCDH10 significantly inhibited the telomerase activity, as well as the proliferation, migration, and invasion activity of a pancreatic cell line. To our knowledge, these findings provide the first-line evidence for an inhibitory role for PCDH10 in telomerase activity in normal tissue. Further studies are needed to improve our understanding of the regulatory pathways of telomerase which will eventually contribute to the development, prevention, diagnosis, and therapy of cancer.

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