

Silencing PinX1 Compromises Telomere Length Maintenance As Well As Tumorigenicity in Telomerase-Positive Human Cancer Cells

Bin Zhang,¹ Yun Xiu Bai,¹ Hang Hang Ma,¹ Feng Feng,² Rui Jin,¹ Zhi Long Wang,¹ Jian Lin,¹ Shi Peng Sun,¹ Pingxun Yang,¹ Xiao Xiong Wang,² Pei Tang Huang,¹ Cui Fen Huang,¹ Ying Peng,³ Yang Chao Chen,⁴ Hsiang-fu Kung,⁴ and Jun Jian Huang¹

¹Laboratory of Tumor and Molecular Biology, Beijing Institute of Biotechnology, ²Department of Urology, General Hospital of People's Liberation Army, Beijing, P.R. China; ³Department of Neurology, The Second Affiliated Hospital, Sun Yat-Sen University, Guangzhou, P.R. China; and ⁴Stanley Ho Center for Emerging Infectious Diseases, Li Ka-Shing Medical Institute, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, P.R. China

Abstract

The nucleolar protein PinX1 has been proposed to be a putative tumor suppressor due to its binding to and inhibition of the catalytic activity of telomerase, an enzyme that is highly expressed in most human cancers in which it counteracts telomere shortening–induced senescence to confer cancer cell immortalization. However, the role of PinX1 in telomere regulation, as well as in cancer, is still poorly understood. In this study, we showed that the PinX1 protein is constitutively expressed in various human cells regardless of their telomerase activity and malignant status. Most interestingly, we found that silencing PinX1 expression by a potent short hairpin RNA construct led to a robust telomere length shortening and growth inhibition in telomerase-positive but not in telomerase-negative human cancer cells. We further showed that silencing PinX1 significantly reduced the endogenous association of telomerase with the Pot1-containing telomeric protein complex, and therefore, could account for the phenotypic telomere shortening in the affected telomerase-positive cancer cells. Our results thus reveal a novel positive role for PinX1 in telomerase/telomere regulations and suggest that the constitutive expression of PinX1 attributes to telomere maintenance by telomerase and tumorigenicity in cancer cells. [Cancer Res 2009;69(1):75–83]

Introduction

Telomerase is a specialized reverse transcriptase used by most eukaryotes to synthesize the terminal (telomeric) DNA sequences of chromosomes to ensure complete genome replication during cell division. In humans, telomerase expression is suppressed in most somatic cells. These normal cells suffer from a progressive telomere shortening in each round of cell division due to the end-replication problem of chromosomes and eventually fall into senescence when their telomeres shorten to critical points (1, 2). Bypassing this telomere length shortening–induced senescence is essential for human cancer formation and >85% of human cancer cells acquire this ability by reactivating telomerase (3, 4).

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Requests for reprints: Jun Jian Huang, Beijing Institute of Biotechnology, 27 Taiping Road, Hai Dian District, Beijing, 100850 China. Phone: 86-10-6693-1323; Fax: 86-10-6824-8045; E-mail: huangjun_j@yahoo.com.

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Telomerase is a ribonucleoprotein complex minimally composed of a single-stranded RNA (hTR) and a catalytic protein subunit (hTERT), which reverse transcribes and adds a template region of the hTR RNA onto chromosomal ends as the telomeric DNA sequences (5, 6). The hTERT expression is the rate-limiting step for cellular telomerase activity (6). Oncogenic proteins such as c-Myc and SP1 have been identified as transcriptional activators of the *hTERT* gene, whereas multiple tumor suppressors have also been found to suppress *hTERT* gene expression (7). Therefore, aberrant hyperactivity of oncogenic transcription factors and loss of function of tumor suppressors during carcinogenesis may account for *hTERT* derepression and telomerase reactivation in various cancer cells.

After gene expression, the hTERT protein is subjected to a highly programmed subcellular transportation that has important implications in directing the biogenesis of telomerase ribonucleoprotein (i.e., the combination of hTERT protein and hTR RNA) and the access of telomerase to telomeres to meet its default substrates (8–11). The mechanisms involved in these processes are still poorly understood. Notably, a particular mechanism in regulating the hTERT nucleolar translocation seems to be widely disturbed by human cellular transformation. It has been shown that the expressed hTERT displays a predominant nucleolar localization in normal cells but exhibits a nucleolar exclusion distribution in various types of cancer cells (12). We have recently shown that the nucleolar localization of hTERT is unrelated to its telomerase function in human cells (13). Whether and how the deregulation of hTERT nucleolar localization confers advantages to cancer cell growth is still elusive.

PinX1 is a nucleolar protein evolutionarily conserved from the yeasts to the human being. Both the human and the yeast PinX1 have been experimentally shown to be able to bind to and suppress telomerase enzymatic activity (14). The human PinX1 contains two hTERT-binding sites separately enclosed within its extreme NH₂- and COOH-terminal regions, with the COOH-terminal region being characterized as the telomerase-inhibitory domain (TID; ref. 14). In their original report, Zhou and colleagues showed that ectopic overexpression of the PinX1 COOH-terminal TID fragment could block telomerase activity, shorten telomeres, and suppress tumor growth, whereas down-regulation of PinX1 expression via antisense cDNA transfection could result in increased cellular telomerase activity, increased telomere length, and enhanced tumorigenicity in the telomerase-positive HT1080 cancer cell line, thereby suggesting PinX1 as an intrinsic telomerase/telomere inhibitor and a putative tumor suppressor in human cells (14).

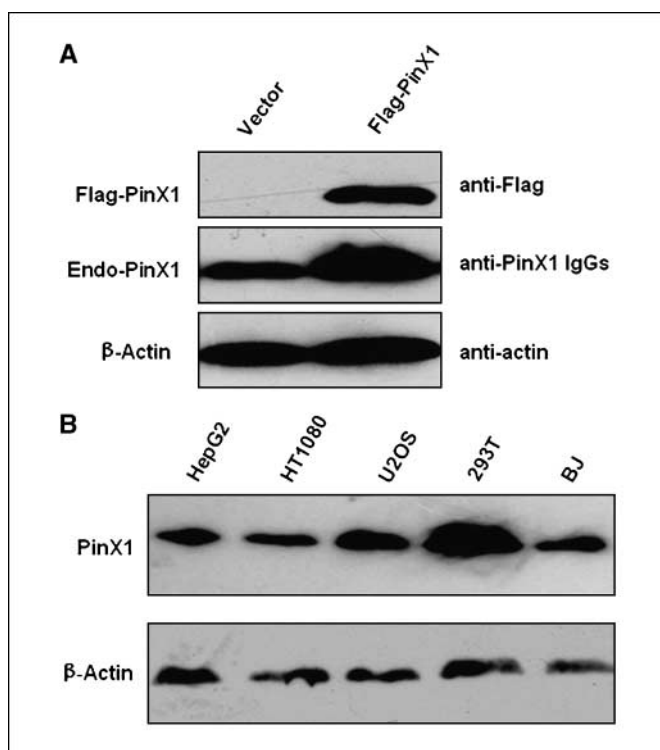


Figure 1. PinX1 protein expression in cultured human cell lines. **A**, characterization of the prepared anti-PinX1 IgGs. Cellular extracts (40 μ g) from either the vector or the Flag-PinX1 lentivirally infected HT1080 cells were subjected to SDS-PAGE. The transferred membrane was first detected with the F12 anti-Flag monoclonal antibody (sigma) and reprobed with the anti-PinX1 antibodies, confirming the performance of the prepared antibodies upon detection of the cellular expressed PinX1. **B**, Western blotting with the prepared anti-PinX1 antibodies showed that the expression of endogenous PinX1 proteins was constitutively present, without obvious signs of down-regulation, in all tested malignant cell lines as compared with the normal BJ fibroblasts. β -Actin for loading control.

Although with these facts, the roles of PinX1 in telomerase/telomere regulations and in cancers still remains elusive. Analyzing studies indicated that the expression of the PinX1 mRNA transcript is present in most tested human clinical tumors (15–18). We also found that the PinX1 protein is constitutively expressed, without any signs of down-regulation, in multiple human cancer cell lines (Fig. 1B). Meanwhile, although its COOH-terminal polypeptide sequences encode a potent TID, ectopic overexpression of the full-length PinX1 only modestly affects cellular telomerase activities and is insufficient to induce obvious telomere length shortening in telomerase-dependent cancer cells (14). Even more, depletion of PinX1 expression has been reported to shorten telomere length and inhibit proliferation in yeast cells (19). In fact, in addition to telomerase interaction, PinX1 has also been found to associate with telomeres but without functional elucidation (14). These evidences suggest that PinX1 may have other unknown roles in telomeres' biology.

To clarify the biological significance of PinX1 expression in human cancers, we investigate the cellular effects of silencing PinX1 on various human cancer cell lines in this study. We found that depletion of PinX1 expression by stable transfection of a potent PinX1 short hairpin RNA (shRNA) resulted in telomere length shortening associated with the reduction of tumorigenicity as well as the enhancement of apoptotic response to chemo-

therapeutics in the two telomerase-positive HT1080 and HepG2 cancer cell lines but not in the telomerase-negative U2OS tumor cell line. We further provided evidence that depletion of PinX1 expression significantly reduced the endogenous association of telomerase with Pot1, a constitutive telomeric protein component that has important roles in regulating telomerase access to and in elongating telomeres (20–22). Our data thus clearly revealed a novel role for PinX1 in positively regulating telomerase-mediated telomere maintenance, possibly by facilitating the recruitment of telomerase function on telomeres in human cancer cells. Because depletion of PinX1 specifically impairs telomere maintenance by telomerase, our results also suggest that this PinX1-dependent telomere-maintaining pathway might represent a novel target for the antitelomerase cancer therapy that is currently being used by many studies (23–25).

Materials and Methods

Cell culture. All experimental cell lines used in this study were described in the text. Cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS (HyClone) and penicillin/streptomycin, and maintained in an incubator with 5% CO₂ at 37°C.

PinX1-expressing plasmids. PinX1 cDNA was obtained by reverse transcription-PCR from 293T cells total RNA. For *Escherichia coli* expression, the PinX1 cDNA was inserted into either the pGEX-4T-1 vector (expressed as the GST-PinX1 fusion protein; Invitrogen) or the pET28a vector (expressed as His-PinX1 fusion protein; Stratagen). For stable cell expression, the fusion gene encoding Flag-PinX1 was cloned into the lentiviral vector pSL6 (26). The production of recombinant lentivirus and their use in cell infection were performed according to ref. 26.

PinX1 shRNA. For constructing PinX1 shRNA, the two oligos, 5'-GATCGGAGCTACCATCAATAATGAATATGTGTAATACGCTTCATTATTGATGGTAGCTCCCTTTT-3' and 5'-AGCTAAAAAGGAGCTACCATCAATAATGAAGACGTATTACACATATTCATTATTGATGGTAGCTCC-3', were synthesized, annealed, and cloned into the vector pSilencerU6 (Ambion). The performance of this shRNA on specifically targeting the PinX1 sequence was confirmed by its cotransfection with plasmids in which cDNAs for PinX1 or other known telomerase/telomeric protein components were individually inserted downstream of the stop codon of GFP into 293T cells. For stable expression, the U6 promoter-PinX1 shRNA-expressing cassette was prepared by PCR from the pSilencerU6-PinX1 shRNA plasmid and inserted into the lentiviral vector pSL6. The recombinant virus was produced as previously described (26), and used for cell infection.

Preparation of PinX1 antibodies. The fusion protein GST-PinX1 produced and purified from *E. coli* was used as the antigen to immunize BALB/c mice. Serum collected from immunized mice were used to isolate anti-PinX1 antibodies with the His-PinX1-conjugated affinity column. The performance of the affinity-purified IgGs on the detection of both ectopic and endogenous PinX1 proteins was characterized by Western blotting assay.

Telomerase activity assays. Cellular extracts were prepared with 1 \times CHAPS lysis buffer. Telomerase activity was measured with the TRAPeze telomerase detection kit (Chemicon). PCR products were separated by electrophoresis on a 12.5% nondenaturing polyacrylamide gel, visualized by silver staining, and semiquantitated according to the instruction of the manufacturer.

Telomere length analysis. Two micrograms of genomic DNA, prepared from the indicated stable cells, were incubated with excess amounts of *Hinf*I and *Rsa*I restriction enzymes overnight at 37°C. The DNA products of the double-enzyme digestion were separated by electrophoresis on a 0.8% agarose gel and transferred onto a nylon membrane. The membrane was then blocked and probed with digoxin-labeled (TTAGGG)₃ oligos. After extensively washing thrice with the washing buffer, the hybridization signal was detected by the horseradish peroxidase-conjugated antidigoxin antibodies (Roche) and imaged by the enhanced chemiluminescence reagent.

Etoposide treatment. The indicated stable cells [~ 20 population doubling (PD)] were seeded into six-well plates at 10^6 cells/well, 24 h before treatment with the indicated dosages of etoposide (Sigma). After 24 h of treatment, cellular apoptosis was analyzed with the Hoechst staining assay (27). Apoptotic cells were identified by their distinct condense nuclear staining as observed under fluorescent microscopic analysis and represented as a percentage of 500 cells counted for each group. For detecting the treatment-induced caspase 3 and PARP degradations, cellular extracts (40 μ g/sample) were fractionated by SDS-PAGE and probed with anti-caspase 3 (Santa Cruz Biotechnology) and anti-PARP (Abcam) using instructions provided by the manufacturers.

Telomere dysfunction-induced foci immunofluorescent assay. Cells grown on coverslips were fixed in 2% paraformaldehyde. After permeabilization for 10 min with PBST (PBS with 0.2% Triton X-100), cells were blocked for 1 h and incubated with antibodies diluted in blocking buffer. For double-staining, cells were first stained with the TRF1 monoclonal antibody (1:100 dilution; Abcam) for 1 h, washed extensively with PBST, incubated with TRITC-labeled secondary anti-

bodies for 30 min, and subsequently fixed for 15 min with 2% paraformaldehyde in PBS. The formaldehyde was inactivated for 15 min using 25 mmol/L of glycine in PBS. Samples were then washed and stained with the anti- γ H2AX (Ser-139) monoclonal antibody (1:200 dilution; Upstate) and subsequently by the FITC-labeled secondary antibodies, and again fixed as described above. Images were observed under fluorescence microscopy and processed by Adobe Photoshop. Blue 4',6-diamidino-2-phenylindole was used for nuclear staining. Senescence-associated β -galactosidase (SA- β -gal) staining was done as previously described (13).

Null mice xenografting experiments. Cells (~ 25 PD) were collected and assessed for viability using trypan blue staining. For comparison, the indicated shRNA-stable cells and their corresponding vector control cells were injected s.c. at the bases of either the right or left flanks of the same individual BALB/c inbred null mice ($n = 6$ for each group, 2.5×10^6 viable cells/site). Tumor growth was monitored weekly for 2 months. Tumor diameters were measured with calipers and volume calculated by the formula (volume = width² \times length / 2).

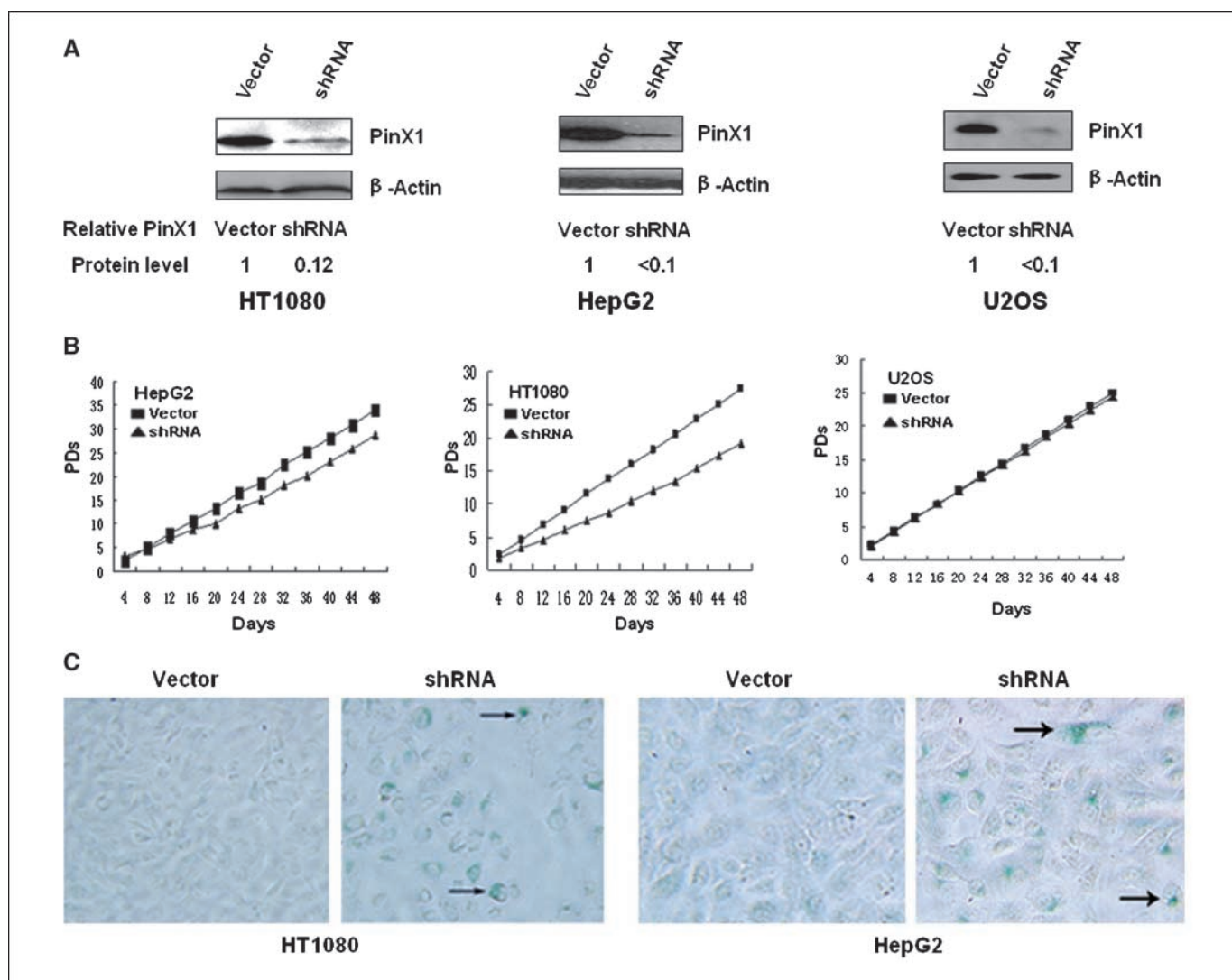


Figure 2. Silencing PinX1 affects the proliferation of the two telomerase-positive HT1080 and HepG2 cancer cell lines. **A**, the PinX1 shRNA was stably transfected into the indicated cell types through recombinant lentiviral infection. The endogenous PinX1 expression status in these stable cell masses (collected at 10 PDs) was analyzed by immunoblotting assays with the anti-PinX1 antibodies. The relative PinX1 protein expression levels in vector and shRNA cells normalized to each β -actin loading control (*bottom*). **B**, growth curve analysis showed that the proliferation rates for both shRNA-stable HepG2 and HT1080 cells were constantly slow as compared with their according vector control cells, but no differences in the proliferation rate were found between the shRNA and the vector-stable U2OS cells when these cells were cultured under the 3T3 format. **C**, SA- β -gal assay shows senescent cells (blue) in both the shRNA HT1080 and HepG2 cultures (analyzed at 35 PDs).

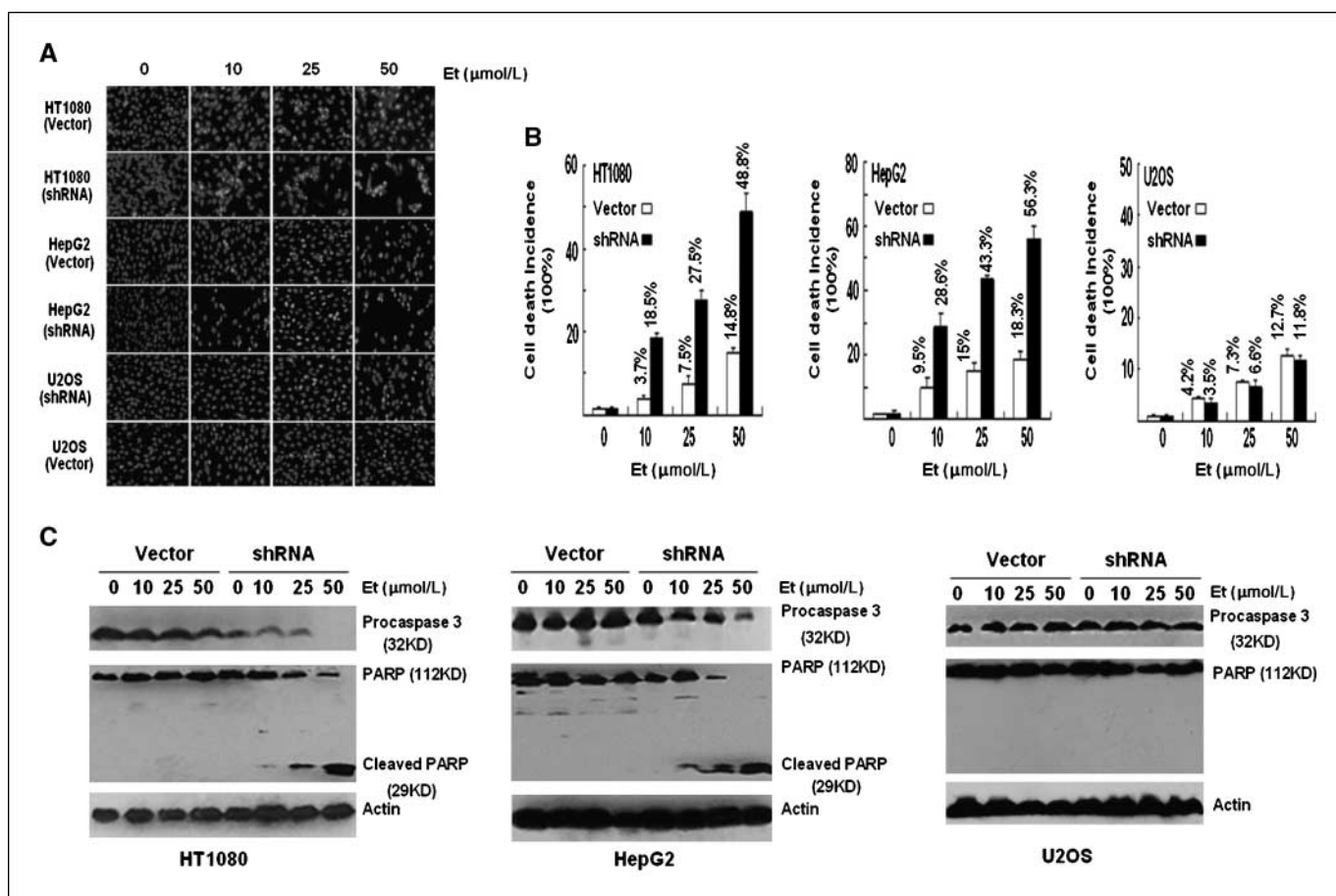


Figure 3. Silencing PinX1 enhances DNA damage-induced apoptotic response in the two telomerase-positive HT1080 and HepG2 cancer cells. *A*, the etoposide-induced cell death in each stable cell culture was analyzed by Hoechst staining assay. *Arrows*, apoptotic cells identified with condense nuclear staining under fluorescent microscopic observation (magnification, $\times 100$). The average incidence of inducible cell death for these cells from three independent experiments (*B*). *C*, immunoblotting assays show that caspase 3 degradation and PARP cleavage were readily induced by the treatment of etoposide with a dose-dependent manner in both the shRNA-stable HT1080 and HepG2 cells, but not in the shRNA-stable U2OS cells as compared with their corresponding vector control cells under treatment. *Et*, etoposide. β -Actin was used for loading controls in immunoblotting assays.

Results

PinX1 expression in human cell lines. To analyze the PinX1 expression status in human cancer cell lines, we immunized BALB/c mice with the *E. coli*-produced GST-PinX1 fusion protein and purified the IgGs from immunized sera. The performance of the prepared antibodies on PinX1 detection was analyzed by Western blotting assay using the nuclear extracts of the Flag-PinX1-stable HT1080 cells (Fig. 1A). Endogenous PinX1 protein expression was then examined in various cells including the two telomerase-positive HT1080 and HepG2 cancer cell lines, the telomerase-negative U2OS cancer cell line, the telomerase-positive transformed 293T cell line, and the telomerase-negative normal diploid BJ fibroblasts. As shown in Fig. 1B, the PinX1 protein was readily detectable in all tested cell types and without any signs of down-regulation in transformed and cancerous cells as compared with the normal BJ cells. Other earlier studies had also indicated that the expression levels of PinX1 mRNA transcripts were not affected in multiple clinical cancers as compared with their corresponding normal tissues (15–18). We suggest that PinX1 is ubiquitously expressed in human cells regardless of their telomerase activity or malignant status.

Silencing PinX1 induces senescence in telomerase-positive cancer cells. To elucidate the cellular biological significance of PinX1 expression in cancer cells, we designed a shRNA construct based on the PinX1 mRNA sequence. The specificity and potency of this shRNA on targeting the PinX1 encoding sequences was initially confirmed through its transient cotransfection with the GFP-PinX1 fusion gene in 293T cells (Supplementary Fig. S1). The shRNA construct was then introduced into three different human cancer cell lines (HT1080, HepG2, and U2OS) for stable expression via the recombinant lentiviral infection. After 4 weeks of the 800 $\mu\text{g}/\text{mL}$ neomycin drug selection, endogenous PinX1 expression in stable transfectant masses was monitored by Western blotting. As shown in Fig. 2A, stable transfection of this shRNA significantly reduced endogenous PinX1 expression in all of these tested cell types. The expression levels of endogenous PinX1 proteins were reduced by more than 8-fold in HT1080, 10-fold in HepG2, and 10-fold in U2OS shRNA-stable cells as compared with their corresponding vector control cells (Fig. 2A). When these stable cells were propagated in culture with the 3T3 format, the overall growth rates for both the shRNA-stable HT1080 and HepG2 cells were constantly slower as compared with their controls cells (Fig. 2B). Microscopic observations indicated that there was a substantial fraction of

cells (~35% in the shRNA-stable HT1080 and ~25% in the shRNA HepG2 cultures, in a total of 400 cells counted, respectively) which showed positive staining following the SA-β-gal assay (Fig. 2C). However, no differences in the proliferation rate were observed between the shRNA and the vector control U2OS cells under normal culture conditions (Fig. 2B), and these cells also showed negative SA-β-gal staining in their cultures (data not shown). These data suggest that silencing PinX1 inhibits the proliferation of telomerase-positive cancer cells by inducing these cells' senescence.

Depletion of PinX1 expression sensitizes telomerase-positive cancer cells to the DNA damage-induced apoptosis. We next determined whether silencing PinX1 affects cancer cell DNA damage responses. The aforementioned stable cells were treated with the indicated dosages of the DNA-damaging reagent etoposide for 24 hours. The inducible cell death event was detected by the Hoechst staining assay. As shown in Fig. 3A and B, the incidences of inducible cell death were significantly exhibited at a dose-dependent fashion in both the shRNA HT1080 (from 18.5% at 10 μmol/L to 48.8% at 50 μmol/L of the etoposide treatment) and HepG2 cells (from 28.6% at 10 μmol/L to 56.3% at 50 μmol/L of the etoposide treatment), and only modestly manifested in their

corresponding control cells (from 3.7% and 9.5% at 10 μmol/L to 14.8% and 18.3% at 50 μmol/L of the etoposide treatment for the control HT1080 and HepG2 cells, respectively). Consistently, the dose-dependent effects of etoposide on inducing caspase 3 degradation and PARP cleavage, the two established molecular indicators for cellular apoptosis (28), were readily detected in both shRNA-stable HT1080 and HepG2 cancer cells, but not obviously manifested in their control cells (Fig. 3C). Again, we found that there were no differences between the shRNA and vector-stable U2OS cells in their responses to etoposide-induced cell death under the same experiments (Fig. 3A-C). Taken together, our data suggested that silencing PinX1 might specifically affect a telomerase-dependent pathway that is important for cancer cell growth under both normal and DNA damage stress conditions.

Loss of PinX1 causes telomere length shortening and telomere dysfunction in telomerase-positive cancer cells. An established role of PinX1 is that it functions as an intrinsic inhibitor of telomerase enzymatic activity (14). Consistently, TRAP assays showed that telomerase activities were increased in both the shRNA-stable HT1080 cells (~2.5-fold) and HepG2 cells (~3.1-fold) as compared with their control vector cells (Fig. 4A). However,

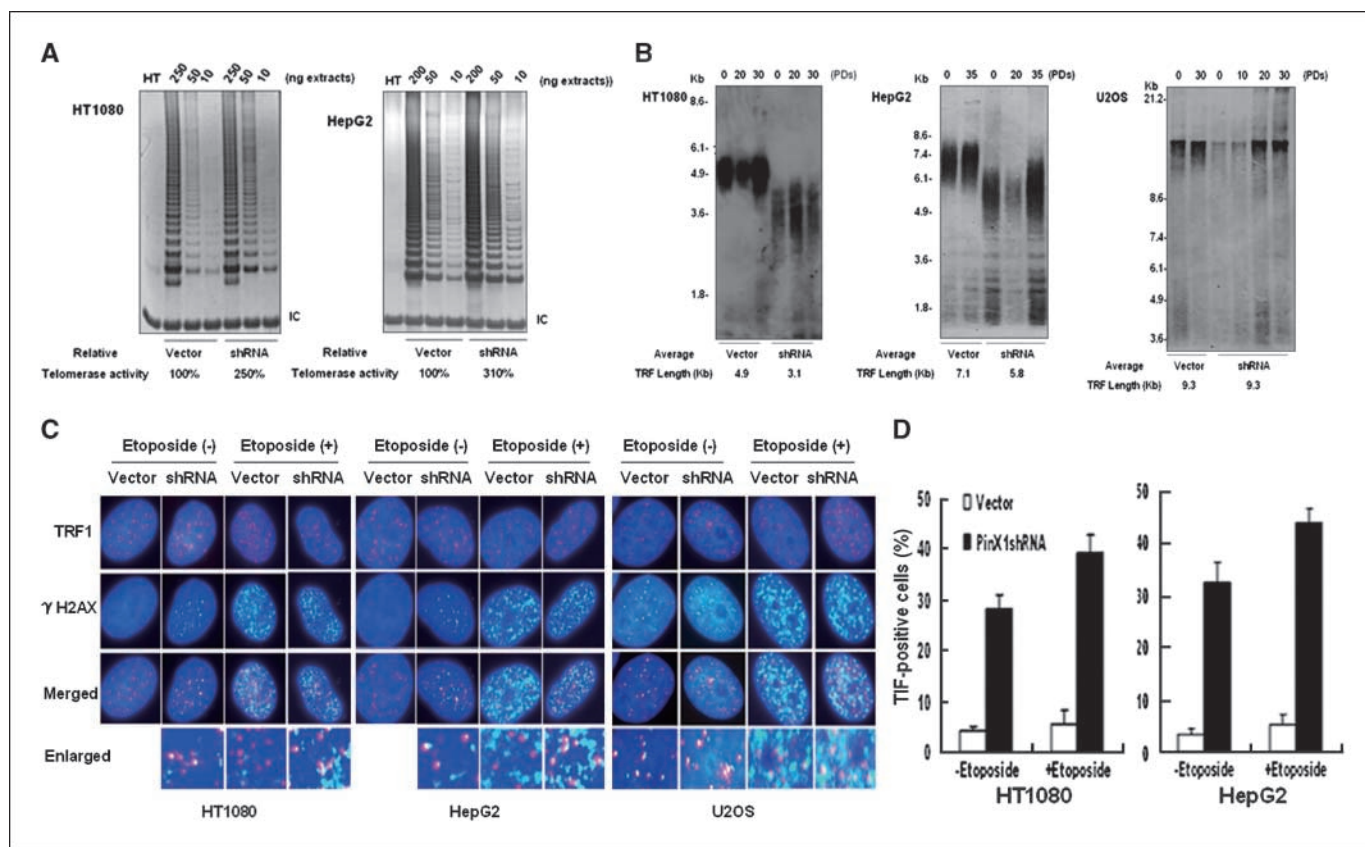


Figure 4. Silencing PinX1 results in telomere shortening and telomere damage-induced foci (TIF) in the two telomerase-positive HT1080 and HepG2 cells. **A**, nuclear extracts prepared from either HT1080 or HepG2-stable cell masses at ~20 PD were subjected to the TRAP assay and the relative telomerase activities in vector control and shRNA-stable cell masses (bottom). **HT**, heat-treated 250 ng nuclear extracts from vector cells as the negative control; **IC**, internal controls for the TRAP assays. **B**, genomic DNA (2 μg) from each indicated stable cell mass at various PDs were digested with *Hinf1* and *Ras1* restriction enzymes followed by Southern blot analysis using a TTAGGG repeat as a probe. The average of the overall telomere length (Kb) for each type of stable cell mass (bottom). **C**, PinX1 depletion results in γH2AX-positive TIF formation in both the shRNA-stable HT1080 and HepG2 cells. Immunofluorescence assays with the anti-TRF1 antibody (1:100 dilution; Abcam) for telomere markers (red), and the anti-phosphorylated histone H2A.X (Ser-139) monoclonal antibody (1:200 dilution; Upstate) for the DNA damage-activated γH2AX foci (green). Enlarged views show the merged images for the colocalizations of TRF1 and γH2AX foci; TIF incidence was constitutively presented in a large fraction (~50%) of cells and without significant differences between the vector and the shRNA-stable U2OS culture before and after etoposide treatment. **D**, quantification of γH2AX-TIF-positive cells in HT1080 and HepG2-stable cultures (at ~35 PDs) before (-) and after (+) the etoposide treatment (columns, mean of triplicate experiments; bars, SD; percentages were calculated with >400 total cells counted for each group).

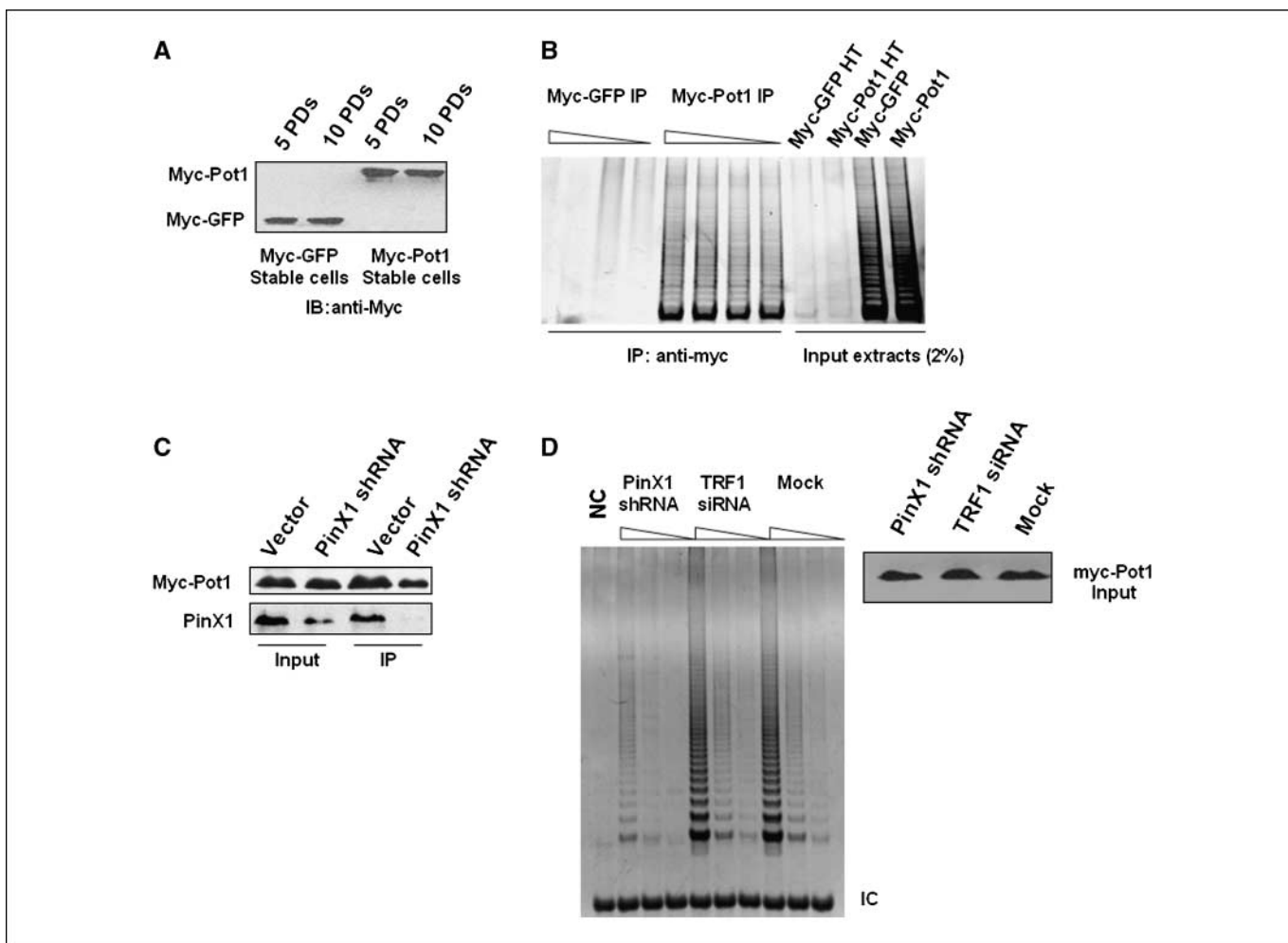


Figure 5. PinX1 depletion suppresses the endogenous telomerase association with the Pot1-containing telomeric protein complex. *A*, extracts from Myc-Pot1 or Myc-GFP–stable HepG2 cells at the indicated time points after their establishment were subjected to immunoblotting assay to confirm stable expressions of the ectopically expressed fusion proteins. *B*, TRAP assay shows that telomerase enzymatic activity was specifically present in the Myc-Pot1, but not the Myc-GFP, immunoprecipitates. *HT*, heat-treated inactivation of the sample to exclude nonspecific results of the assay. *C*, immunoprecipitation with the anti-myc tag antibody shows that the endogenous PinX1 can be coprecipitated with Myc-Pot1 and that the Myc-Pot1–associated PinX1 can be significantly reduced by shRNA transfection. *D*, transfection of the PinX1 shRNA significantly reduced telomerase enzymatic activity in the Myc-Pot1 immunoprecipitates. As a control, transfection of the TRF1 siRNA does not affect the Myc-Pot1–associated telomerase activity. *Mock*, Myc-Pot1–stable HepG2 cells without transfection. *Left*, immunoblotting shows equal amounts of the input Myc-Pot1 precipitates from each indicated cell type for the TRAP assay.

when these cells were subjected to telomere length analysis, a significant reduction of the overall telomere lengths was observed in both the shRNA-stable HT1080 cells and HepG2 cells (Fig. 4B). As expected, we found that PinX1 depletion had no effect on telomere length in the telomerase-negative U2OS cell line (Fig. 4B). These results suggest that PinX1 might specifically affect telomere length maintenance in the two telomerase-expressing HT1080 and HepG2 cancer cells.

We further determined if PinX1 depletion induces telomere dysfunction in telomerase-expressing cancer cells, which could account for the observed phenotypes of growth inhibition and chemotherapeutic hypersensitivity for these affected cells. We found that the telomere dysfunction–induced foci (TIF), evidenced by the colocalizations of the DNA damage factor γ H2AX with the constitutive telomeric protein component TRF1 (Fig. 4C), were readily detectable in a substantial fraction of cells in both shRNA-stable HT1080 and HepG2 cultures (~28% and ~38% in a total of 400 cells counted, respectively) but was not observed in almost all

of their control cells (Fig. 4C and D). These positive cells in the shRNA cultures usually contained more than 5 γ H2AX-TIFs, with some cells even containing 15 to 20 TIFs, far pronounced compared with that of the scarcely observed positive cells in their control cell populations (with a mean of 3 in the range of 1–5 TIFs/cell). When these cells were subjected to the 10 μ mol/L etoposide treatment that caused an obvious apoptotic response in shRNA-stable cell types, but not in their vector control cells (Fig. 3), the TIF incidences could be further increased in both the shRNA HT1080 and HepG2 cultures (from ~28% to ~40% and ~32% to ~43% before and after the treatment, respectively; Fig. 4C and D). However, the TIF incidence almost remained unchanged in their control cultures following the same treatment (Fig. 4D). Interestingly, we repeatedly found that a remarkable fraction (~50%) of cells in the vector-stable U2OS cell cultures (and also observed in the parental U2OS cell line; data not shown) displayed multiple obvious γ H2AX-TIF signals under normal growth conditions (Fig. 4C), suggesting that these telomerase-negative tumor cells may undergo a constitutive

telomere dysfunction in their physiologic cellular environment even though with a content of much longer overall telomere lengths. However, no detectable increase of the TIF event was found in the shRNA-stable U2OS culture and the percentages of TIF-positive cells in these two stable U2OS cultures also remained unchanged before and after the etoposide treatment (data not shown). Therefore, the constitutive telomere dysfunction in the telomerase-negative U2OS cells seems to be unrelated to their PinX1 expression status. Based on these observations, we proposed that PinX1 depletion may specifically affect the telomere length maintenance by telomerase, which in turn resulted in telomere dysfunction that could account for the phenotypes of growth inhibition and DNA-damaging hypersensitivity observed in the two telomerase-positive HT1080 and HepG2 cancer cell lines.

Depletion of PinX1 reduces the endogenous association of telomerase with the Pot1 telomeric protein component. We proceeded to finding how PinX1 depletion affects telomere length maintenance in telomerase-expressing cancer cells. To elongate telomeres, telomerase needs to make contact with its default substrate—the 3'-overhanged single-stranded telomeric DNA that is naturally occupied by the telomeric protein Pot1 (29), which in turn can associate with other telomeric protein components to form diverse protein complexes to protect telomere stability (30). Recent studies suggest that a Pot1-containing telomeric protein complex may function as the telomeric receptor for recruiting and guiding telomerase access (21). But how telomerase is loaded onto the Pot1 protein complex is still poorly understood. We wonder if PinX1 is involved in the process of telomerase telomere recruitment given that this factor has been found with the capacity of binding with both telomerase and telomeres (14). To test this hypothesis, we first introduced a Myc-tagged Pot1 fusion protein into the HepG2 cell line for stable expression (Fig. 5A). Immunoprecipitation with the specific anti-Myc tag antibody 9E10 (Sigma) showed that robust telomerase activities could be detected in the precipitates from extracts of the Myc-Pot1-stable cells, but not in the precipitates from extracts of the control Myc-GFP-stable cells (Fig. 5B), confirming an endogenous association between telomerase and the Pot1 protein complex in cells. As the predicted, the Myc-Pot1 precipitates also contained detectable PinX1 proteins (Fig. 5C). We then transfected the PinX1 shRNA into Myc-Pot1-stable cells to knock down the endogenous PinX1 expression. We found that suppressing the endogenous PinX1 expression, thereby reducing the amount of the Pot1-associated

PinX1 (Fig. 5C), was accompanied with a significant reduction of the associated telomerase activities in the Myc-Pot1 immunoprecipitates (Fig. 5D). As another control, we also transfected the Myc-Pot1-stable cells with small interfering RNA (siRNA), specifically against the dominant telomeric protein TRF1 (Supplementary Fig. S2) and showed that suppression of TRF1 expression did not affect the Pot1-associated telomerase activities (Fig. 5D). Based on these results, we interpreted that silencing PinX1, thereby suppressing the telomerase association with the Pot1-containing telomeric protein complex, consequently blocks the telomere length maintenance in telomerase-positive cancer cells.

Depletion of PinX1 expression compromises the tumorigenicity of both HT1080 and HepG2 cells. We finally examined whether depletion of PinX1 expression would also affect the tumorigenicity of telomerase-positive cancer cells. The shRNA or vector-stable HT1080 and HepG2 cells were injected into the right and the left sides of the same individual BALB/c inbred null mice ($n = 6$ for each group, 2.5×10^6 viable cells/per site). Tumor production was monitored weekly for 2 months after the injection. As shown in Fig. 6 and Table 1, the tumor growth capacity of both the shRNA HepG2 and HT1080 cancer cells in the xenografted null mice was significantly suppressed as compared with that of their control cells. In animals tested with the HepG2 cell lines, although all injected sites apparently had tumors at ~ 2 weeks after the injection, tumors produced by the shRNA cells grew much slower and smaller than those produced by the vector cells (with an average volume of $174 \pm 44 \text{ mm}^3$ for the shRNA tumors versus $1,224 \pm 280 \text{ mm}^3$ for the vector tumors at the end of the experiment; $P < 0.05$). For the group of animals injected with the HT1080-stable cell lines, all sites (six of six) injected with the vector cells produced tumors at ~ 3 weeks and reached a size of $1,094 \pm 238 \text{ mm}^3$ on average at the end of the 2-month observation period. But only two sites (two of six) with the shRNA cells injection became tumor-bearing at 4 weeks, and with an average final volume of $17 \pm 5 \text{ mm}^3$ at the end of the experiment. Therefore, depletion of PinX1, thereby suppressing telomerase function on telomere length maintenance, compromises the tumorigenicity in telomerase-positive human cancer cells.

Discussion

This study reveals that the ubiquitously expressed nucleolar protein PinX1 plays a positive role in regulating telomerase-mediated

Figure 6. Silencing PinX1 expression impairs tumorigenicity in both HT1080 and HepG2 cells. Cells at ~ 25 PD in their culture were harvested and injected s.c. in BALB/c inbred null mice ($n = 6$ for each group). For each animal, the left flanks were used for vector control cell injections and the right flanks for shRNA cell injections (2.5×10^6 viable cells/site). Mice were monitored weekly for the appearance of tumors at injection sites and tumor size was determined by the formula (volume = width² \times length / 2) 2 mo after injection. Two representative xenografting tumor growths in null mice (red cycle for the control tumors and the green cycle for the shRNA tumors) are shown and the details of the experimental information are summarized in Table 1.

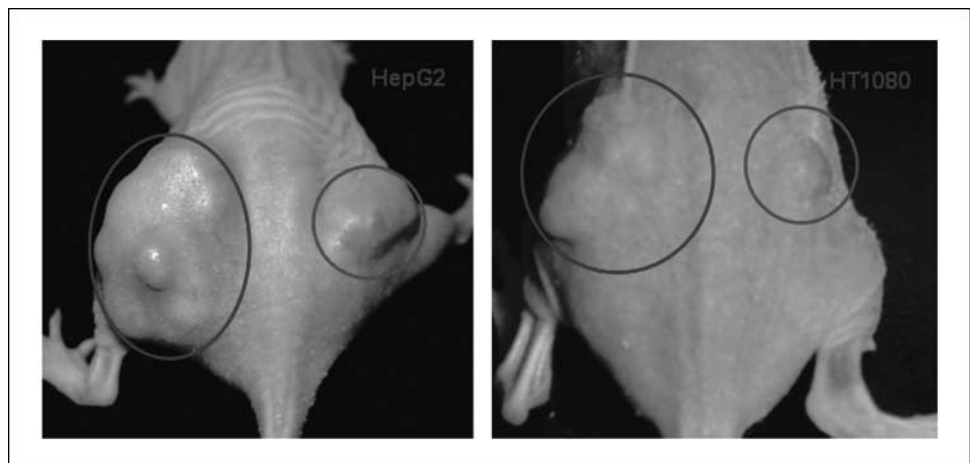


Table 1. PinX1 depletion impairs tumorigenicity in both HT1080 and HepG2 cells

Cell type	Tumor frequency*	Latency (wk) [†]	Sizes (mm ³) [‡]
HepG2 Vector	6/6	~ 2	1,224 ± 280 (1,484, 1,666, 1,080, 1,008, 1,008, 1098)
HepG2 shRNA	6/6	~ 2	174 ± 44 (162, 220, 198, 220, 126, 125)
HT1080 Vector	6/6	~ 3	1,094 ± 238 (1,352, 1,080, 968, 1,352, 1,089, 725)
HT1080 shRNA	2/6	~ 4	17 ± 5 (13, 21, 0, 0, 0, 0)

NOTE: Null mice were injected s.c. with 2.5×10^6 viable cells of indicated HepG2 or HT1080 cell types ($n = 6$ for each group). For each animal, the right flank was injected with the shRNA cells and the left flank was injected with the corresponding vector control cells.

*Data represent the number of tumor formations/the number of injected site at the end of the 2-mo observation.

[†]Data represent the time of tumor appearance after the injection.

[‡]Values are mean ± SD. Numbers in parentheses indicate the size of individual tumor masses at each injected site.

telomere length maintenance in human cancer cells. The role of PinX1 in this process seems to be functionally linked to mediating the association of telomerase with the Pot1-containing telomeric protein complex *in vivo*, consequently facilitating telomerase access to and elongating telomeric DNA (21). Because genetic ablation of PinX1 expression in yeast cells was also previously reported to shorten telomeres in the affected yeast cells (19), we further suggest that this positive role of PinX1 on telomere length regulation may be evolutionarily conserved between humans and yeasts.

Together with previous findings that PinX1 also possesses an intrinsic telomerase-inhibitory activity (14), we prefer to suggest this factor as a dual-role regulator of telomerase. This nature may help to explain why PinX1 overexpression and its severe depletion could show similar phenotypic effects on telomere lengths in telomerase-dependent cancer cells. We interpret that the physiologic expression levels of PinX1 is sufficient to fully load telomerase onto the Pot1-containing telomeric protein complex because only 96 Pot1 molecules are available at telomeres for the 48 chromatins of a human cell. Therefore, up-regulation of PinX1 expression provides little help to further stimulate telomerase access to telomeres. Instead, the overdosed PinX1 proteins would enhance its intrinsic negative effect on cellular telomerase enzymatic activity and thereby resulting in a telomere-shortening phenotype. However, because the negative effect of PinX1 overexpression on telomerase activity *in vivo* is modest and not complete (ref. 14, and also observed in our multiple experiments with various cancer cell lines), its effect on telomere shortening is also expected to be far less significant compared with PinX1 depletion, which acts by interrupting telomerase access to telomeres, even though robust telomerase activity exists under this situation. Notably, Zhou and colleagues, in their original report, showed that attenuation of PinX1 expression by antisense cDNA transfection results in telomere lengthening in the HT1080 cancer cell line (14), in disagreement with the report here. We consider that such a discrepancy might be related to the difference in the depletion level of PinX1 expression among the cell lines. In that report, the suppression of PinX1 expression was moderate (~ 3-fold), and was not as significant as ours (8-fold to 10-fold). We consider that such a moderate down-regulation of PinX1 might affect little, if any, of the telomerase telomere trafficking processes due to the still relative abundance of PinX1 present in those affected cells. These results could be reflected by

our observations that, although severe depletion of PinX1 in both the shRNA-stable HT1080 and HepG2 cells caused an acute telomere shortening, these shortened telomeres could be stably retained during their continual propagation (Fig. 4B), implying that the remnant PinX1 in those shRNA-stable cells could still work to maintain telomere length at a relatively limited content and suggesting a dominant role for this factor in positively regulating telomerase-mediated telomere length maintenance. However, because down-regulation of PinX1 expression could substantially increase cellular telomerase catalytic activities that could stimulate its action effectively, an overt telomere lengthening in those stable cells would be expected. These observations also raise an interesting question of whether regulating PinX1 expression, thereby regulating the telomerase/telomere function, would be a pathway with biological significance under certain cell growth conditions.

How PinX1 loads telomerase onto the Pot1-containing telomeric protein complex is still elusive. TRF1 is the only known telomeric protein that PinX1 can bind directly (14). Furthermore, TRF1 can also associate with Pot1 via the protein interactions of Pot1-TPP1-Tin2-TRF1 (30). However, because we found that siRNA knockdown of TRF1 does not affect the Pot1-associated telomerase activity (Fig. 5D), it seems less likely that the putative PinX1-TRF1 interaction would function as the molecular linker to couple telomerase with the particular Pot1-containing telomeric protein complex. It is therefore worthwhile to explore if PinX1 could interact with other known telomeric proteins in addition to the TRF1.

According to our findings, disruption of the PinX1-dependent telomere maintenance pathway is able to compromise tumorigenicity as well as enhancing chemotherapeutic sensitivity in telomerase-positive human cancer cells. This may be beneficial for those currently undergoing telomerase-based anticancer therapy. Telomerase has been suggested as a plausible anticancer target due to its critical role in cancer cells for telomere length maintenance (23, 25, 31). Many current studies aim to suppress either telomerase expression or its enzymatic activity. However, it has been shown that telomerase is in fact expressed at an extremely low level in normal human somatic cells that were previously thought to be telomerase-negative (32). Most importantly, suppressing telomerase expression or its enzymatic activity was found to result in normal human cells prematurely aging before their telomeres displayed obvious shortening (32). This

finding suggests that normal cells actually require a trace of telomerase activity for keeping their physiologic proliferation capacity, although the function of telomerase expression in normal cells is not for the purpose of counteracting telomere shortening. Because silencing PinX1 could interrupt the telomerase-mediated telomere maintenance whereas sparing the need for suppressing telomerase expression or its enzymatic activity per se, it is worthwhile to further investigate if targeting PinX1 would represent a novel avenue for telomerase-based anticancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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