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The role of Pin1 protein in aging of human tendon stem/progenitor cells

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ABSTRACT

Aging of tendon stem/progenitor cells (TSPCs) can lead to tissue degeneration and subsequent injury. However, the molecular mechanisms controlling TSPC aging are not completely understood. In the present study, we investigated the role of Pin1 in aging of human TSPCs. Pin1 mRNA and protein expression levels were significantly decreased during prolonged *in vitro* culture of human TSPCs. Furthermore, overexpression of Pin1 delayed the progression of cellular senescence, as confirmed by downregulation of senescence-associated β-galactosidase, increased telomerase activity and decreased levels of the senescence marker, $p16^{INK4A}$. Conversely, Pin1 siRNA transfection promoted senescence in TSPCs. In addition, miR-140-5p regulated Pin1 expression at the translational level via directly targeting its 3′UTR. Our results collectively demonstrate that Pin1 acts as an important regulator of TSPC aging.

1. Introduction

Tendon stem/progenitor cells (TSPCs) are adult stem cells found in human and mouse tendons [1]. TSPCs are capable of self-renewal with multi-lineage differentiation potential, and therefore provide a potent cell source for regeneration and replacement of damaged tendons that are currently poorly responsive to more conventional treatments [2,3]. However, human TSPC senescence may lead to age-related tendon disorders and impair tendon regeneration and replacement capacity. While several previous studies have demonstrated that proliferation and differentiation potentials of TSPCs decline with senescence [4,5], the molecular mechanisms governing TSPC aging remain unclear at present.

Pin1 is a highly conserved peptidyl-prolyl isomerase (PPI) that regulates the stability, activity, and subcellular localization of proteins by modulating the three-dimensional structure of phosphoproteins through alterations in *cis-trans* isomerization upon recognition of a major regulatory phosphorylation motif (Ser/Thr-Pro) [6,7]. Pin1 has been shown to participate in diverse cellular processes, including cell cycle progression, cell survival, immune responses and lineage commitment [8–10]. Recent studies have proposed that Pin1 is intimately involved in the regulation of adult stem cells. Lee et al. [9] showed that PIN1 mRNA and protein levels are upregulated during adipogenic differentiation and steadily decline during odontogenic differentiation of human dental pulp stem cells [9]. Loss of Pin1 causes cell cycle arrest and senescence, whereas Pin1 overexpression increases differentiation and inhibits senescence of cardiac progenitor cells [7]. However, the role of Pin1 in human TSPC aging is yet to be established.

MicroRNAs (miRNAs) are a class of non-coding RNAs that regulate gene expression post-transcriptionally via complementary base pairing with the 3′-untranslated region (UTR) of target mRNAs, causing their degradation or suppression of translation [11]. miRNAs modulate multiple biological processes, including aging [12]. miR-766-mediated post-transcriptional regulation of SIRT6 has been implicated in human aging [13]. miR-17, miR-19b, miR-20a, and miR-106a are downregulated in various tissues with age in correlation with increased transcript levels of specific established target genes, particularly the cdk inhibitor, p21/CDKN1A [14]. Accordingly, we investigated the potential involvement of miRNAs in Pin1 expression.

In this study, prolonged *in vitro* culture of human TSPCs induced a decrease in Pin1 expression. Overexpression of Pin1 delayed the progression of cellular senescence, and conversely, Pin1 knockdown promoted TSPC senescence. Furthermore, we obtained evidence of miR-140-5p-mediated post-transcriptional regulation of Pin1. Our results confirm an anti-aging role of Pin1 in TSPCs,

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highlighting its potential as a molecular target for antagonizing senescence.

2. Materials and methods

2.1. Isolation and culture of human TSPCs

Human TSPCs were isolated and characterized as described previously [1]. These cells were cultured in Dulbecco's Modified Eagle's Medium plus 10% fetal bovine serum, 1% penicillin–streptomycin, and 2 mM L-glutamine. TSPCs were subcultured and split at 80–90% confluence every 3 days. We employed two TSPC lines isolated from achilles tendons obtained from different donors (28 and 31 years), designated TSPC-1 and TSPC-2, respectively. Two TSPC lines at early (P4) and late (P14) passages were used for all experiments. The procedure was approved by the Institutional Review Board of the Third Military Medical University, and informed consent obtained from all donors.

2.2. Lentivirus and siRNA

Lentiviruses containing human Pin1 or GFP were generated as described previously [15]. Briefly, lentiviral vectors were transfected into 293T cells, along with the lentiviral packaging mix. After 48 h, viral supernatants were collected, filtered and concentrated. TSPCs were transduced with viral particles, and purified 48 h after transduction via puromycin selection. Pin1 siRNA (Invitrogen, Foster city, CA) was transfected into TDSCs using HiPerfect (Qiagen, Düsseldorf, Germany). TSPCs were transfected with miR-140-5p mimics or controls using Lipofectamine 2000 (Invitrogen).

2.3. RNA preparation and quantitative RT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reverse-transcribed (2 μg) using oligo (dT) primers and M-MLV reverse transcriptase (Promega, Madison WI). Real-time RT-PCR of Pin1 was performed with SYBR Premix Ex Taq II (Takara, Japan) with β-actin as the endogenous normalization control. For miR-140-5p, total RNA was isolated using the *miR*vana miRNA isolation kit (Ambion, Grand Island, NY, USA) and reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen). U6 small nuclear RNAs were used as an internal control for normalization. Real-time RT-PCR was assayed with SYBR green master mix (Applied Biosystems, USA). Primer sequences were as follows: forward: 5′-CAGCGGCAACAGCAGCAGT-3′, reverse: 5′-TTCTGGATGTAGCCGTTGA-3′ for Pin1; forward: 5′-GCATCCTCACCCTGAAGTAC-3′, reverse: 5′-GGCTGGGGTGTTGAAGGTCT-3′ for β-actin; RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCA-3′, forward: 5′-CAGTGGTTTTACCCTA-3′, reverse: 5′-GTGCAGGGTCCGAGGT-3′ for miR-140-5p; RT: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATG-3′, forward: 5′-GCGCGTCGTGAAGCGTTC-3′, reverse: 5′-GTGCAGGGTCCGAGGT-3′ for U6. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method. All samples were examined in triplicate.

2.4. Senescence-associated β-galactosidase staining

The β-Galactosidase (β-gal) assay was performed using the SA-β-gal staining kit (Beyotime, China), according to the manufacturer's instructions. TSPCs were washed twice with PBS and fixed for 15 min. After removing the fixative, cells were washed twice with PBS and stained overnight with β-gal solution. The percentages of β-gal-positive cells (blue color) were calculated by counting 150–200 cells in six microscopic fields.

2.5. Telomerase activity

Telomerase activity was determined using the TeloTAGGG Telomerase PCR ELISA kit (Roche Applied Science, Penzberg, Germany) according to established protocols [16]. Absorbance of samples was measured at 450 nm within 30 min after adding the stop reagent. Lysis buffer and HEK-293 cells served as negative and positive controls, respectively. All samples were examined in triplicate.

2.6. Western blot analysis

Total proteins from cells were extracted with RIPA buffer. Protein concentrations were quantified using the BCA reagent kit (BioTek, China). Proteins were separated via 10% SDS-PAGE, and transferred to PVDF membrane. After 1 h blocking with 5% non-fat milk, membranes were incubated with anti-Pin1 (Cell Signaling, Danvers, MA), anti-$p16^{Ink4A}$ (Abcam, Cambridge, MA) or anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Following TBST washes, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37 °C, and protein bands detected using an enhanced chemiluminescence detection kit(Pierce, Rockford, IL, USA).

2.7. Luciferase assay

The full-length 3′UTR sequence of human Pin1 was amplified via PCR and cloned into the pGL3-control (Promega) vector. A mutant construct in the miR-140-5p binding sites of Pin1 3′UTR region was generated using QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA). Co-transfection of Pin1 3′UTR or mut Pin1 3′UTR plasmid with miR-140-5p mimics into TSPC-1 early-stage cells was accomplished using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were harvested and lysed. Luciferase activity was measured using the dual-luciferase assay kit (Promega), using Renilla luciferase for normalization.

2.8. Statistical analysis

All experiments were performed at least in triplicate, and experimental data expressed as mean values ±SD. Two-group differences were analyzed with the Student's *t*-test using SPSS version 13.0 software. *P* values <0.05 were considered significant.

3. Results

3.1. Pin1 expression is associated with TSPC senescence

TSPC-1 and TSPC-2 cells isolated from the achilles tendons of two different donors were characterized *in vitro* to confirm their stem/progenitor characters. As shown in Supplemental Fig. S1, they were positive for CD44, CD90, Stro-l, Collagen I, alpha-smooth muscle actin (α-SMA), cartilage oligomeric protein (Comp) and Tenascin C, and negative for CD34. In addition, they showed osteogenic, adipogenic and chondrogenic multidifferentiation potential. These cells were continuously passaged until they lost the ability to divide. Both cells at early (P4) and late (P14) passages were used. First, cellular senescence was assessed based on senescence-associated β-galactosidase (SA-β-gal) activity. The number of SA-β-gal-positive cells was significantly increased with prolonged culture in TSPC-1 and TSPC-2 cells (Fig. 1A). Given that telomeres are essential for maintaining cellular proliferative capacity and their loss is implicated in senescence [17], telomerase activity was detected in early and late passage cell lines of TSPC-1 and TSPC-2. Our results disclosed a significant decrease in

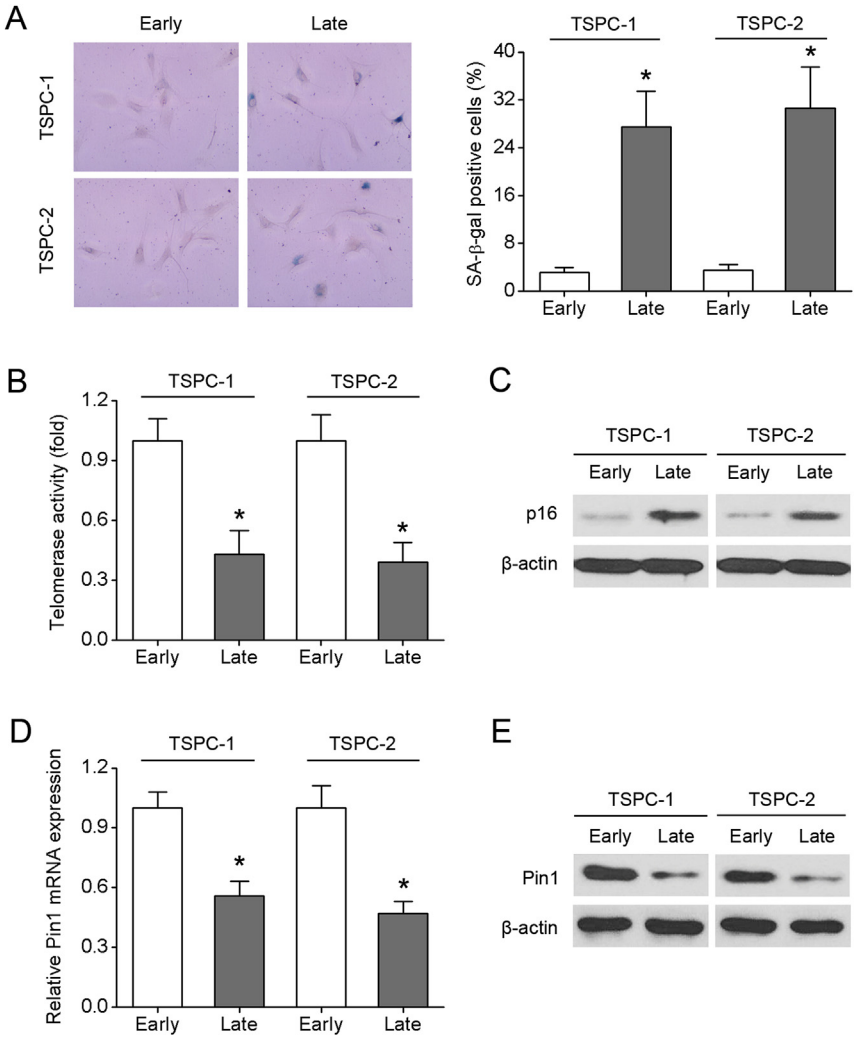

Fig. 1. Pin1 is downregulated in senescent TSPCs. (A) TSPC-1 and TSPC-2 cells obtained from two different donors were divided into early-stage (P4) and late-stage (P14) cell groups. Cells were stained with SA-β-gal, and the percentage of SA-β-gal-positive cells determined. (B) Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA kit. (C) Expression levels of the senescence marker, $p16^{INK4A}$, were assessed via western blot. (D) Pin1 mRNA levels were determined using qRT-PCR. (E) Protein levels of Pin1 were determined using western blot. All data were obtained from three independent experiments ($^*P < 0.05$).

telomerase activity in late cells (Fig. 1B). In contrast, $p16^{INK4A}$, known to be associated with cellular senescence [18], was markedly increased in TSPC-1 and TSPC-2 late-stage cells (Fig. 1C). These results suggest that senescence progresses considerably during later passages.

Since Pin1 is critically involved in cellular senescence, we speculated whether Pin1 expression is also associated with TSPC senescence. Accordingly, Pin1 mRNA levels were determined via qRT-PCR. Compared with early-stage cells, prolonged culture of TSPC-1 and TSPC-2 cells resulted in a significant decrease in Pin1 mRNA expression (Fig. 1D). Western blot analysis showed that Pin1 protein levels are markedly downregulated in late-stage cells (Fig. 1E), confirming the link between Pin1 expression and TSPC senescence.

3.2. *Pin1 overexpression delays the progression of senescence in TSPCs*

Given the negative correlation between Pin1 and senescence, we investigated whether forced overexpression of Pin1 delays TSPC senescence. Late-stage TSPC-1 and TSPC-2 cells were stably transduced with lentiviruses encoding Pin1 (Pin1) or GFP (GFP). After 48 h, elevation of Pin1 was confirmed via western blot analysis (data not shown). Next, we performed SA-β-gal staining to compare the senescence states of Pin1-TSPCs and GFP-TSPCs. The results demonstrated that Pin1 overexpression induced a significant decrease in the number of SA-β-gal-positive cells (Fig. 2A), supporting its senescence-delaying potential. Additionally, we also observed that exogenous Pin1 induced a significant increase in telomerase activity (Fig. 2B) and decrease in expression of the senescence marker, $p16^{INK4A}$, compared with GFP controls (Fig. 2C). Taken together, these results indicate that Pin1 overexpression delays the progression of senescence in TSPCs.

3.3. *Knockdown of Pin1 promotes senescence in TSPCs*

To further investigate the role of Pin1 in regulation of TSPC senescence, we conducted inhibition studies using Pin1 siRNA in early-stageTSPC-1 cells. As shown in Fig. 3A, compared with siRNA control cells, siPin1-transfected TSPCs showed a significant increase in the percentage of SA-β-gal-positive cells, suggesting that Pin1 knockdown accelerates TSPC senescence. In addition,

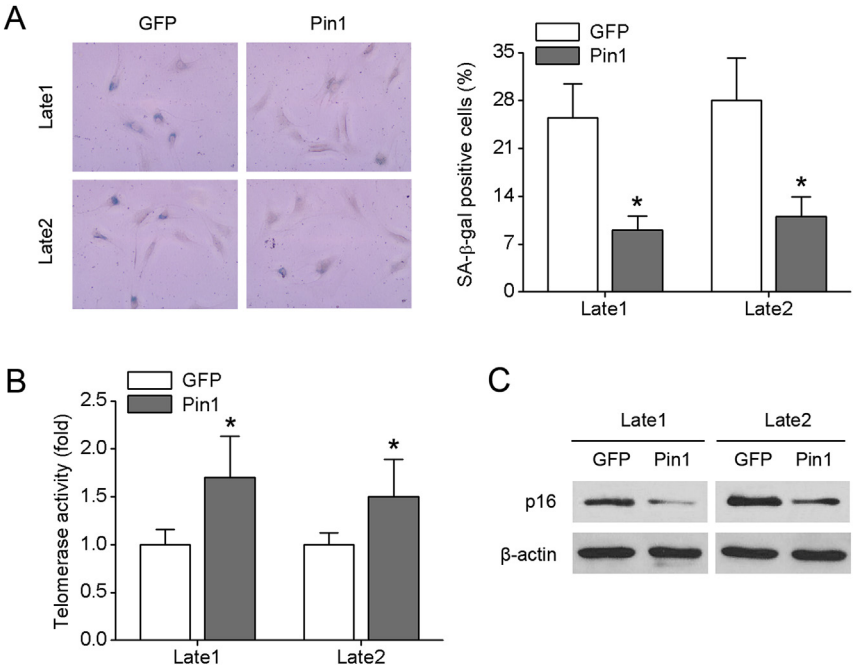

Fig. 2. Exogenous Pin1 delays senescence in TSPCs. Late-stage cells of TSPC-1 (Late1) and TSPC-2 (Late2) were transduced with lentiviruses overexpressing GFP (GFP) or Pin1. (A) Cells were stained with SA-β-gal. The percentage of SA-β-gal-positive cells is shown. (B) Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA kit. (C) Expression of the senescence marker, $p16^{INK4A}$, was measured using western blot. All data were obtained from three independent experiments (*$P < 0.05$).

telomerase activity and $p16^{INK4A}$ expression were determined in siRNA-transfected cells. Results demonstrated that Pin1 deletion led to a clear decrease in telomerase activity (Fig. 3B) and marked increase in $p16^{INK4A}$ expression (Fig. 3C). These data collectively suggest that Pin1 knockdown induces TSPC senescence.

3.4. miR-140-5p regulates Pin1 expression at the translational level

To date, little is known about the molecular mechanisms involved in regulation of Pin1. Here, we examined the hypothesis that miRNAs are involved in Pin1 regulation. Analysis via three

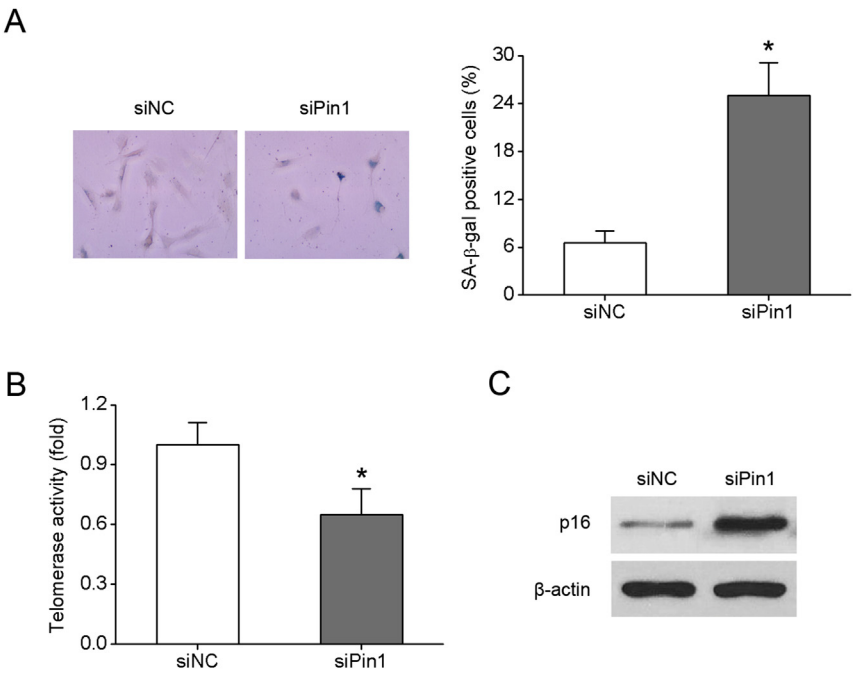

Fig. 3. Knockdown of Pin1 promotes senescence in TSPCs. TSPC-1 early-stage cells were treated with Pin1-specific or control siRNA. (A) Cells were stained with SA-β-gal. The percentage of SA-β-gal-positive cells is shown. (B) Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA kit. (C) Expression of the senescence marker, $p16^{INK4A}$, was measured via western blot. All data were obtained from three independent experiments (*$P < 0.05$).

different bioinformatic algorithms (TargetScan, pictar and miRanda) indicated that Pin1 is the theoretical target gene of miR-140-5p (Fig. 3A), a miRNA previously reported to be highly upregulated in senescent mesenchymal stem cells [19]. We also found significantly increased expression levels of miR-140-5p when TSPCs became senescent (Supplemental Fig. S2). This led us to examine whether miR-140-5p affects Pin1 mRNA and protein expression in TSPCs. To this end, miR-140-5p mimics were transiently transfected into TSPC-1 and TSPC-2 early-stage cells, and Pin1 expression levels analyzed. As shown in Fig. 4B, miR-140-5p overexpression led to a substantial decrease in Pin1 protein expression in TSPC-1 and TSPC-2 cells. However, no significant alterations in mRNA levels were observed, suggesting that the suppressive effect of miR-140-5p on Pin1 occurs mainly through translational repression. To further establish whether miR-140-5p directly targets the 3′UTR region of Pin1, full-length 3′UTR of Pin1 was subcloned into a luciferase reporter vector. Dual luciferase reporter assays (Fig. 4C) revealed that miR-140-5p significantly attenuates luciferase activity of a reporter vector with the WT 3′UTR of Pin1. This effect was abrogated upon mutation of the 3′UTR binding site. Based on these results, we suggest that miR-140-5p affects Pin1 expression at the translational level.

4. Discussion

Aging is a major risk factor for tendon injury and impaired tendon healing, but the mechanisms regulating this process remain largely unknown. Here, we demonstrated a critical role of Pin1 in TSPC aging. Firstly, Pin1 expression was significantly decreased during replicative senescence. Secondly, overexpression of Pin1 effectively delayed the progression of cellular senescence in late-stage TSPCs. Thirdly, Pin1 knockdown promoted senescence to a marked extent in early-stage TSPCs. We further showed that miR-140-5p regulates Pin1 expression at the translational level by directly targeting its 3′UTR.

Pin1 is involved in the regulation of cellular aging. Inhibition of Pin1 leads to senescence in late-passage human fibroblasts, while ectopic expression rescues cells from BTG2-induced senescence [20]. Additionally, Pin1-deficient mice display widespread premature aging phenotypes. For example, the phenotype of Pin1 knockout mice recapitulates premature aging observed in telomerase-deficient mice [21]. Pin1 knockout in mice causes progressive age-dependent neuropathy characterized by motor and behavioral deficits, tau hyperphosphorylation, tau filament formation and neuronal degeneration [22]. While Pin1 has been established as a crucial anti-aging molecule, its role in human TSPC aging remains to be determined. In the current study, we investigated the role of Pin1 by modulating its expression in TSPCs and analyzing the effects on subsequent senescence.

The stem cell aging process involves specific tumor suppressor pathways, such as $p16^{INK4A}$ [18,23]. In hematopoietic stem cells of aged mice, the cyclin-dependent kinase inhibitor, $p16^{INK4A}$, accumulates and modulates a number of age-associated functions [24]. A significant increase in expression of $p16^{INK4A}$ was also observed in adipose tissue derivedmesenchymal stem cells from aged donors [25]. Aging $p16^{INK4A}$-deficient mice showed a considerably smaller decline in subventricular zone proliferation, olfactory bulb neurogenesis, and frequency and self-renewal potential of multipotent progenitors [26]. These findings highlight the prominent role of $p16^{INK4A}$ in aging and suggest that targeting the mechanisms underlying the $p16^{INK4A}$ switch in aged tissues may be potentially applied to ameliorate tissue aging and degeneration [27]. Therefore, we selected $p16^{INK4A}$ as a marker for cellular senescence. As telomere loss has been implicated in aging, telomerase activity was additionally detected as a senescence marker. Importantly, a previous study reported that Pin1 inhibition enhances TRF1 binding to telomeres and leads to gradual telomere loss in human cells and mice [21]. Data from the current investigation showed that exogenous Pin1 expression in late-stage TSPCs suppresses SA-β-gal activity and $p16^{INK4A}$ expression and promotes telomerase activity, whereas knockdown of Pin1 in early-stage TSPCs enhances SA-β-gal activity and $p16^{INK4A}$ expression and decreases telomerase activity, clearly supporting a critical role in TSPC aging.

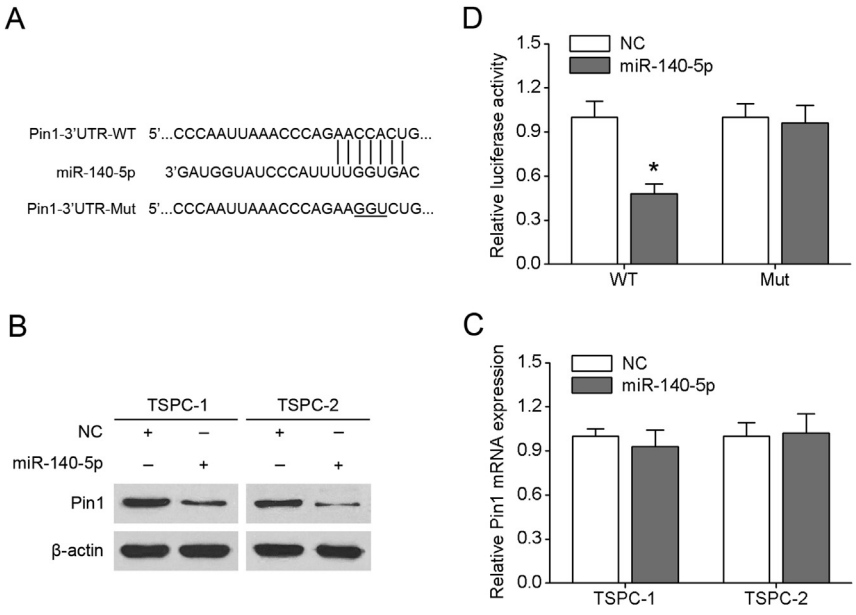

Fig. 4. miR-140-5p directly targets Pin1. (A) Nucleotide sequence of miR-140-5p binding sites within the Pin1 3′UTR region. (B) Western blot analysis of Pin1 expression in TSPC-1 and TSPC-2 early-stage cells transfected with control or miR-140-5p mimics. (C) qRT-PCR analysis of Pin1 expression in the indicated cells. (D) TSPC-1 early-stage cells were co-transfected with the Pin1 3′UTR construct and control or miR-140-5p mimics, and luciferase activity measured 48 h later. All data were obtained from three independent experiments (*$P < 0.05$).

Limited information is currently available on the mechanisms underlying regulation of Pin1 expression. However, increasing evidence has highlighted the importance of miRNAs in regulating gene expression and aging [12]. Recent studies disclosed the involvement of miR-766 and miR-106a in regulation of aging through specific target genes (SIRT6, p21/CDKN1A) [13,14]. Considering these findings, we investigated the possibility that miRNAs are involved in Pin1 downregulation. miR-140-5p was identified as the strongest candidate, following analysis of different databases to predict potential Pin1-targeting miRNAs. Luciferase assay confirmed that miR-140-5p directly targets the 3′UTR of Pin1. Additionally, miR-140-5p mimics significantly inhibited the protein expression of Pin1. However, Pin1 mRNA expression was not obviously affected, indicating that miR-140-5p regulates Pin1 at the translational level. Interestingly, miR-296-5p and miR-200b were identified as regulators of Pin1 in recent studies [28,29]. Since a single gene can be regulated by many miRNAs, we propose that miR-140-5p is another bona fide modulator of Pin1.

In conclusion, Pin1 plays a critical role in TSPC aging and is regulated by miR-140-5p at the translational level. Pin1 and its upstream miRNA may therefore present promising targets to prevent TSPC aging.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.163.

Conflict of interest

The authors (Lei Chen, Junpeng Liu, Xu Tao, Guodong Wang, Qing Wang, Ximing Liu) declare no conflict of interest.

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