Transcriptional regulation of *CDKN2A/p16* by sirtuin 7 in senescence

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Abstract. Cell senescence is a state of limited cell proliferation during a stress response or as part of a programmed process. When a senescent cell stops dividing, maintaining metabolic activity contributes to cellular homeostasis maintenance. In this process, the cell cycle is arrested at the G0/G1 phase. $p16^{INK4A}$ protein is a key regulator of this process via its cyclin-dependent kinase inhibitor (CDKI) function. *CDKI 2A* (*CDKN2A)/p16* gene expression is regulated by DNA methylation and histone acetylation. Sirtuins (SIRTs) are nicotinamide dinucleotide (NAD^+)-dependent deacetylases that have properties which prevent diseases and reverse certain aspects of aging (such as immune, metabolic and cardiovascular diseases). By performing quantitative PCR, Western blot, ChIP, and siRNAs assays, in this study it was demonstrated that *CDKN2A/p16* gene transcriptional activation and repression were accompanied by selective deposition and elimination of histone acetylation during the senescence of MRC5 cells. Specifically, significant H3K9Ac and H3K18Ac enrichment in cells with a senescent phenotype concomitant with *CDKN2A/p16* gene overexpression was demonstrated compared with the non-senescent phenotype. Furthermore, the presence of H3K18Ac in deacetyl-transferase SIRT7 knockdown MRC5 cells allowed *CDKN2A/p16* promoter activation. These results suggested that SIRT7 served as a critical component of an epigenetic mechanism involved in senescence mediated by the *CDKN2A/p16* gene.

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Introduction

Cell senescence is defined as the irreversible cell cycle arrest state that occurs in response to various stress and damage signals (1,2). The senescence phenotype is characterized by cell cycle arrest, resistance to apoptotic stimuli (3,4), the release of inflammatory cytokines and chemokines (5), endoplasmic reticulum stress (6), metabolism dysregulation (7,8), genomic instability due to DNA damage and chromatin changes affecting transcription (9,10).

The molecular mechanism of senescence involves proteins such as $p16^{INK4A}$ and $p21^{WAF1}$, which serve as key cell cycle regulators via their function as cyclin-dependent kinase inhibitors (CDKIs) (11). $p16^{INK4A}$ mediates senescence via the retinoblastoma signaling pathway, inhibiting CDKs and leading to G1 cell cycle arrest (12). Regulation of *CDKI 2A (CDKN2A)/p16* gene expression involves transcription factors and epigenetic mechanisms such as histone post-translational modification. Specifically, histone acetylation is performed by histone acetyltransferases and deacetylases, which are responsible for the addition and removal, respectively, of acetyl groups from lysine residues on the N-terminal tails of histones (13). Acetylation at histone lysine residues causes neutralization of its positive charge and a decrease in DNA-nucleosome affinity associated with transcriptional activation (13). Deacetylation is associated with compacted chromatin and a transcriptionally silent state (14).

Sirtuins (SIRTs) are a conserved family of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases. SIRTs act in different cell compartments. In the nucleus, SIRTs deacetylate histones and regulate expression. In the mitochondria, SIRTs are components of the metabolic machinery. In the cytoplasm, SIRTs modulate cytoskeletal and signaling molecules (15). Collectively, SIRTs modulate metabolic processes such as energy availability, stress response, protein aggregation, inflammation, and genome stability (15). A total of seven sirtuins have been identified in mammals (16). They share structural homology, particularly in their highly conserved catalytic and NAD^+-binding domains (16). SIRT1 is a deacetylase that contains both nuclear and exporting sequences and therefore serves as a key regulator of certain

proteins such as NF-κB, peroxisome proliferators-activated receptor γ and its coactivator peroxisome proliferator-activated receptor gamma coactivator 1-α, protein tyrosine phosphatase, forkhead transcriptional factors, adenosine monophosphate activated protein kinase, CRE-binding protein-regulated transcription coactivator 2, endothelial nitric oxide synthase, p53, myogenic differentiation, liver X receptor and Transcription factor E2F1 (17). SIRT2 is a deacetylase with cytosolic localization (18-20). SIRT3-5 are mitochondrial SIRTs containing mitochondrial-targeting sequences and SIRT6-7 are predominantly localized in the nucleus (18-20).

In the SIRT family, SIRT1 is the most studied in cellular senescence and is reported as specifically catalyzing the removal of acetylation at residues H3K9, H3K14, H3K56, H4K16, and H1K26 and regulating transcription-associated genes, such as *CDKN2A/p16* and *CDKN1A/p21* (21-24). In the present study, the regulatory role of the histone deacetylase enzymes SIRT1, SIRT2, SIRT6, and SIRT7 on gene expression of *CDKN2A/p16* were evaluated in human MRC5 cells, which were used as a model of replicative cellular senescence.

Materials and methods

Cell culture. Primary human lung fibroblast MRC5 cells (derived from 14-week-old male fetus normal lung tissue) were purchased from American Type Culture Collection. MRC5 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were maintained in a humidified atmosphere at 37˚C and 5% CO_2. Cells were cultured for 8 weeks and harvested at different time points (2, 4, 6 and 8 weeks) to perform the assays.

Senescence-associated β-galactosidase (SA-β-gal) activity. The endogenous SA-β-gal activity in MRC5 culture was assessed at 2, 4, 6 and 8 weeks using a Senescence Detection kit (cat. no. ab65351; Abcam) according to the manufacturer's protocol. Positive cells were imaged and counted in inverted white light microscope (KERN OCM 161) at 40x magnification Percentage of SA-β-galactosidase-positive tissue area was calculated and plotted with GraphPad Prism.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from culture cells was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. An equal amount of each sample (2 μg) was used for RT using a ProtoScript® First Strand cDNA Synthesis kit (New England BioLabs, Inc.) following to the manufacturer's protocol. qPCR was performed using a FastStart Essential DNA green Master kit (Roche Diagnostics) using LightCycler® Nano (Roche Diagnostics). The reaction conditions were as follows: Initial denaturation for 10 min at 95˚C, followed by 45 cycles of denaturation for 10 sec at 95˚C; annealing for 15 sec at 62˚C for *P16INK4α* and *Laminin B* primers, and 60˚C for *SIRT7* and *GAPDH* primers; ending with 20 sec of elongation at 72˚C, The results were quantified using the $2^{-\Delta\Delta Cq}$ method (25). Data are presented as relative mRNA expression levels normalized to *GAPDH* mRNA expression levels. The sequences of the primers used to amplify genes of interest are presented in Table SI.

Nuclear extract and western blotting. Nuclear extracts were prepared from MRC5 cultures with buffer containing 420.0 mM NaCl, 25.0% glycerol, 0.2 mM EDTA, 1.0 mM DTT, 20.0 mM HEPES (pH 7.9) and 1.5 mM $MgCl_2$ using the Dignam method (26). Total protein was quantified using the Bradford technique (27). A total of 25 μg protein/lane was separated using 10% SDS-PAGE. Subsequently, the proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk solution in TBS-Tween (0.1%) for 1 h at room temperature. Then, the membranes were incubated at 4˚C overnight with primary anti-SIRT7 (1:500; anti-rabbit; cat. no. D2K5A Cell Signaling Technology, Inc.) and anti-transcription factor IIB (TFIIB), dilution 1/100 anti-rabbit (C-18 sc-225 Santa Cruz Biotechnology) were used as a control. Goat anti-Rabbit IgG Poly-HRP was used as secondary antibody, dilution 1/5000, incubation 2 h (32260 Thermo Fisher Scientific), at room temperature. The immunoblots were visualized in CL-Xposure Film using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Inc.).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed in cells at 4 and 8 weeks to identify regulatory components that mediated epigenetic changes associated with CDKN2A/p16 transcriptional control during replicative senescence in MRC5 cells. Cross-linked chromatin samples were prepared as described previously by Rojas *et al* (28). Chromatin was sheared using a Bioruptor® Pico sonication device (Diagenode SA) to obtain ≤500 bp fragments and stored at -80˚C; one aliquot was used for quantification using A260 assessment. Chromatin size was confirmed by electrophoretic analysis. Cross-linked extracts were resuspended in sonication buffer to a final volume of 500 μl. The samples were precleared by incubation with 2-4 μg normal IgG and 40 μl protein A/G PLUS-agarose beads (Santa Cruz Biotechnology Inc.sc-2003) for 1.5 h at 4˚C with agitation. Samples were centrifuged at 4,000 x g for 5 min, at 4˚C. The supernatant was collected and immunoprecipitated with specific antibodies (Table SII) for 12-16 h at 4˚C. The immunocomplexes were recovered with the addition of 50 μl protein A (for rabbit antibodies) or G agarose beads (for mouse antibodies), followed by incubation for 1 h at 4˚C with gentle agitation. Immunoprecipitated complexes were washed once with sonication buffer (50 mm HEPES, pH 7.9, 140 mm NaCl, 1 mm EDTA, 1% Triton X-100, 0.1% deoxycholate acid, 0.1% SDS, and a mixture of proteinase inhibitors), twice with LiCl buffer (100 mM Tris-HCl; pH 8.0; 500 mM LiCl; 0.1% Nonidet P40 and 0.1% deoxycholic acid) and once with Tris-EDTA (50 mM Tris-HCl, pH 8.0, and 2 mM EDTA) for 5 min each at 4˚C, followed by centrifugation at 4,000 x g for 5 min at 4˚C. The protein-DNA complexes were eluted by incubation with 100 μl elution buffer (50 mM $NaHCO_3$ and 1% SDS) for 15 min at 65˚C. Extracts were centrifuged at 10,000 x g for 5 min at room temperature. The supernatant was collected and incubated for 12-16 h at 65˚C to reverse cross-linking. Proteins were digested with 100 μg/ml proteinase K for 2 h at 50˚C and the DNA was recovered using a ChIP DNA Clean & Concentrator kit (cat. no. D5201; Zymo Research Corp.). qPCR was performed as aforementioned. The qPCR primers used to evaluate the human *CDKN2A/p16* promoter region are presented in Table SI.

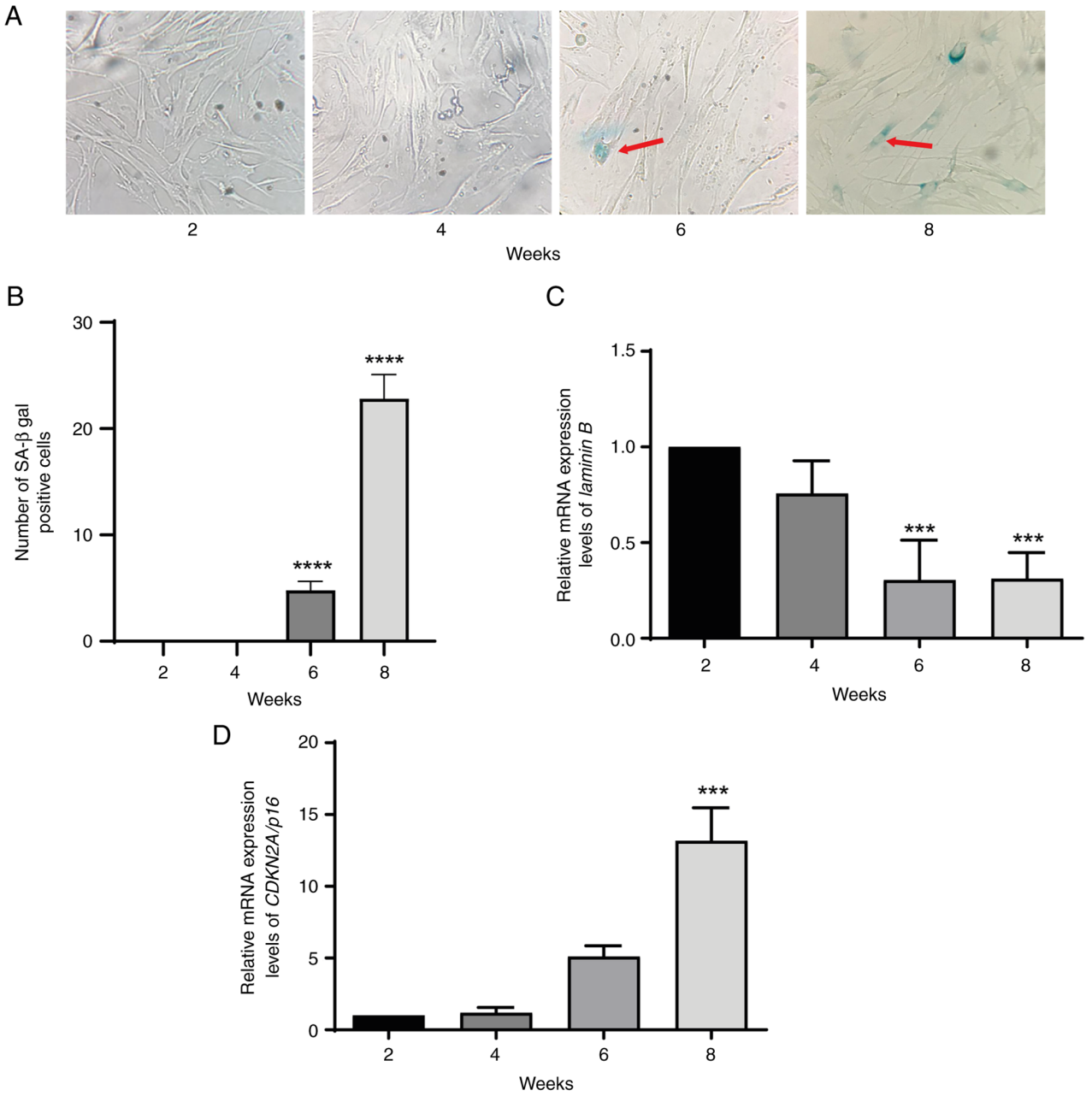

Figure 1. Senescence markers in MRC5 cells. MRC5 cells were cultured for 2, 4, 6 and 8 weeks to induce replicative senescence *in vitro*. (A) SA-β-galactosidase staining of non-senescent (2 and 4 weeks) and senescent MRC5 cells (6 and 8 weeks). Arrows indicate positive blue staining for SA-β-galactosidase. After staining, cells were imaged using phase-contrast microscopy (40x magnification). (B) Quantitative analysis of SA-β-stained cells. Each measurement was performed in triplicate. Total mRNA expression levels of (C) *lamin B* and (D) *CDKN2A/p16* were assessed by reverse transcription-quantitative PCR. Values were normalized to *GAPDH* mRNA expression. ***P<0.0002 and ****P<0.0001 vs. 2 weeks. SA-β-gal, senescence-associated β-galactosidase; CDKN2A, cyclin dependent kinase inhibitor 2A.

Small interfering RNA (siRNA) knockdown. MRC5 cells cultured for 2 weeks (non-senescent cells) were plated on 6-well plates at 50% confluence overnight and transfected with 50 μM siRNA oligonucleotides targeting *SIRT7* (siSIRT7; cat. no. sc-63030; Santa Cruz Biotechnology, Inc.). siSIRT7 is a mix of three target-specific 19-25 nt siRNAs designed to knock down *SIRT7* gene expression. Control siRNA (siCtrl), a non-targeting 20-25 nt siRNA (cat. no. sc-37007; Santa Cruz Biotechnology, Inc.), was used as a negative control. siRNA sequences are presented in Table SIII. Transfection was performed using transfection reagent according to the manufacturer's protocol (sc-29528 Santa Cruz Biotechnology, Inc.). Briefly, 1ug of siRNA duplex was diluted into 100 μl siRNA transfection Medium (sc-36868; Santa Cruz Biotechnology, Inc.). For each transfection, 4 μl of siRNA transfection reagent were used. This transfection reagent mixture was overlayed onto the washed cells and incubated for 6 h at 37˚C in CO_2 incubator. Finally, transfection medium was replaced with fresh 1X normal growth medium. Subsequent experiments were performed 48 h later.

Statistical analysis. ChIP assay results and mRNA expression levels were analyzed by one-way ANOVA followed by Dunnett's post hoc test to assess significant changes between

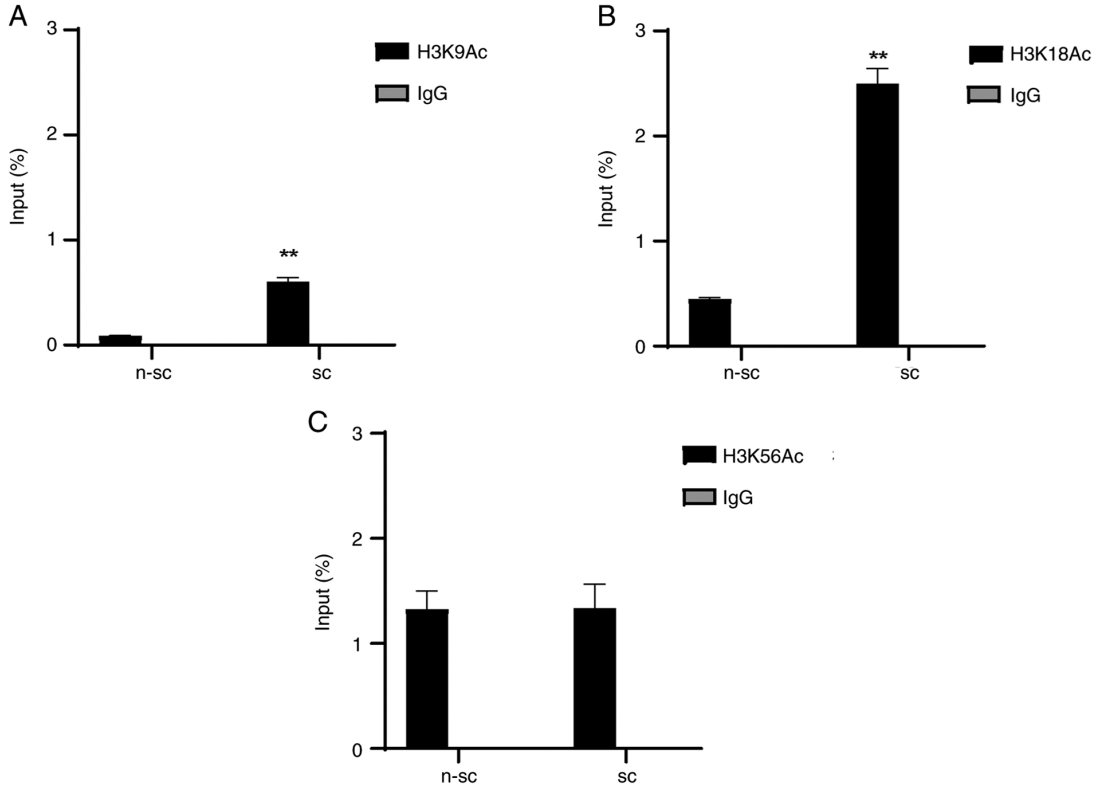

Figure 2. Histone acetylation of *CDKN2A/p16* promoter region changes in senescent MRC5 cells. MRC5 cultured cells at 4 (n-sc) and 8 weeks (sc) were collected and chromatin immunoprecipitation assay was performed using antibodies against (A) H3K9Ac, (B) H3K18Ac and (C) H3K56Ac. Data are presented as % input ± SEM using normal IgG as a specific control. **P<0.01 vs. n-sc. CDKN2A, cyclin dependent kinase inhibitor 2A; sc, senescent cell; n-sc, non-sc.

three or more samples and the control, while the Mann-Whitney assay was applied to establish differences between two nonparametric data. Data are presented as mean ± SD. P<0.05 was considered to indicate a statistically significant difference. All statistical analyzes were performed with GraphPad Prism version 8 (GraphPad Software, Inc.). All experiments were performed in triplicate.

Results

CDKN2A/p16 expression in MRC5 human fibroblast cells and during activation of senescence. Replicative senescence limits somatic cell proliferation in culture and may reflect cellular aging *in vivo* (26). The most widely used biomarker for senescent and aging cells is SA-β-gal, assessed as the level of β-gal activity at pH 6.0 in senescent cells (29). mRNA expression levels of *CDKN2A/p16*, a cell cycle regulator, and *lamin B*, a nuclear morphology factor, were analyzed as senescent cells demonstrate an increase and decrease in these genes, respectively (30). A time course experiment was used to assess changes during senescence. MRC5 cells were cultured for 2, 4, 6 and 8 weeks and used in SA-β-gal and RT-qPCR assays to evaluate the *in vitro* senescence model.

MRC5 cells were assessed for SA-β-gal activity using X-gal at pH 6.0. β-gal activity was demonstrated at 6 and 8 weeks of cell culture (Fig. 1A and B). The β-gal activity level in cells cultured for eight weeks was 4-5 times higher than that in cells cultured for 6 weeks. RT-qPCR was used to assess *lamin B* and *CDKN2A/p16* mRNA expression levels in MRC5 culture cells at 2, 4, 6 and 8 weeks. The results demonstrated that *lamin B* was significantly downregulated in cultured cells at 6 and 8 weeks compared with 2 weeks (Fig. 1C). *Lamin B* downregulation was associated with *CDKN2A/p16* mRNA expression levels, which were significantly increased at 8 weeks compared with 2 weeks (Fig. 1D). These results demonstrated that MRC5 cells entered senescence by 6 weeks.

Epigenetic changes in histone modifications are associated with CDKN2A/p16 gene expression in senescent cells. Histone acetylation is associated with transcriptional activation (13). To determine whether epigenetic modifications participated in *CDKN2A/p16* transcriptional control in senescence, changes in histone modification were assessed using ChIP assay using 4 (non-senescent) and 8-week cultured cells (senescent).

The results demonstrated that senescent cells exhibited histone H3 post-translational modifications characteristic of transcriptionally active genes. Specifically, 8-week-old cultured cells presented enrichment levels of H3K9Ac (0.8%) and H3K18Ac (2.5%) in the *CDKN2A/p16* gene promoter region (Fig. 2A-C). These enrichment percentages were significantly increased in senescence cells compared with non-senescence cells.

However, enrichment of 1,2% were detected in H3K56Ac in non-senescent cells. These results suggested that H3K9Ac and H3K18Ac may be involved in *CDKN2A/p16* gene overexpression in senescent cells.

SIRT7 weakly binds to the CDKN2A/p16 promoter in senescent MRC5 cells. To identify the regulatory components that mediated the epigenetic changes associated with

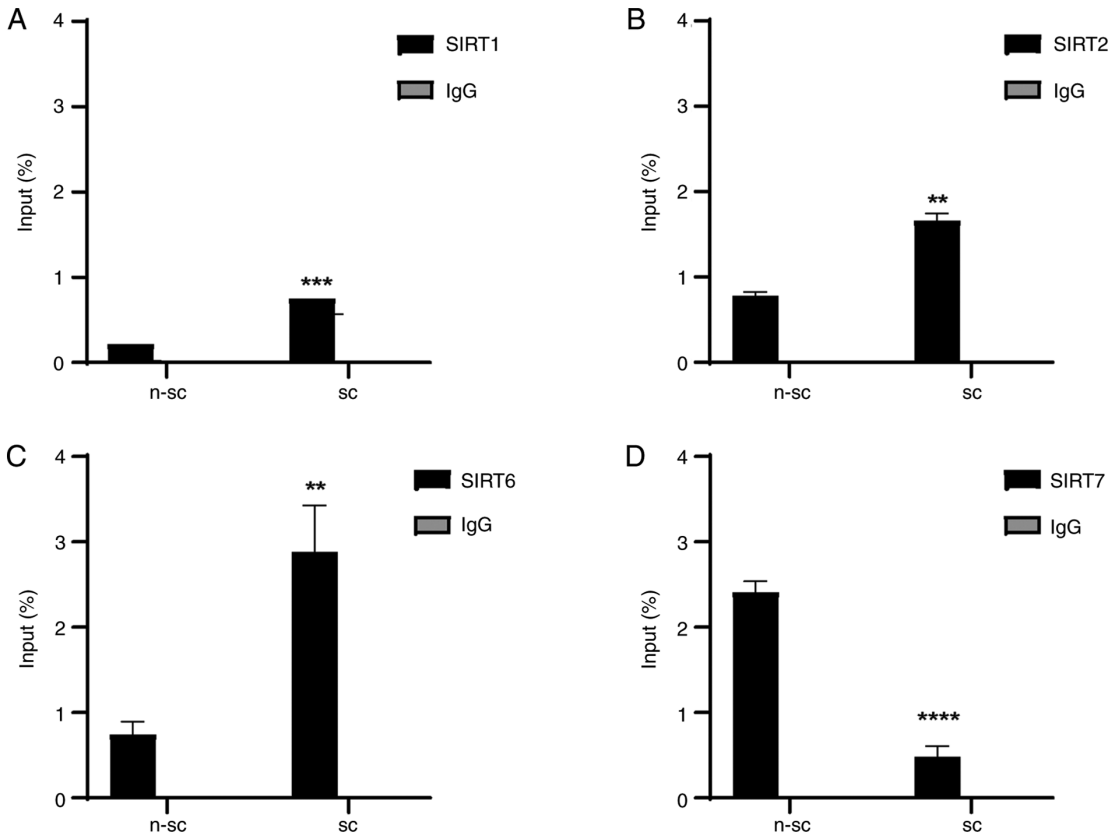

Figure 3. SIRT7 binding to the *CDKN2A/p16* promoter is lost in senescent MRC5 cells. MRC5 cells were cultured at 4 (n-sc) and 8 weeks (sc) and chromatin samples were collected. Chromatin immunoprecipitation assay was performed using antibodies against (A) SIRT1, (B) SIRT2, (C) SIRT6 and (D) SIRT7 chromatin-modifying proteins. Data are presented as % input ± SEM using normal IgG as a specific control. **P<0.01, ***P<0.001, ****P<0.0001 vs. n-sc. sc, senescent cell; n-sc, non-sc; SIRT; sirtuin.

transcriptional control of the *CDKN2A/p16* promoter during senescence, ChIP assay was performed for epigenetic suppressors (SIRT1, SIRT2, SIRT6, and SIRT7) that modulate post-translational histone modification.

ChIP assay with senescent cells demonstrated that SIRT1, SIRT2 and SIRT6 were significantly enriched in the *CDKN2A/p16* promoter (Fig. 3A-C) compared with non-senescent cells. SIRT7 association with the *CDKN2A/p16* promoter was significantly decreased in senescent compared with non-senescent cells (Fig. 3D). This may indicate *CDKN2A/p16* transcriptional activation.

mRNA expression levels *of SIRT1, SIRT2, SIRT6,* and *SIRT7* were assessed using RT-qPCR at 2, 4, 6 and 8 weeks. Low mRNA expression levels of *SIRT1* and *SIRT2* were demonstrated and mRNA expression levels of *SIRT6* and *SIRT7* were markedly higher at all times. (Fig. S1). SIRT1 expression levels decreased significantly compared with non-senescent cells. Additionally, SIRT7 protein expression levels were analyzed by western blot at different times evaluated (2,4,6 and 8 weeks). Fig. S2) show that the levels of protein expression in the different times didn't change.

SIRT7 is an epigenetic regulator of CDKN2A/p16 gene in replicative senescence. ChIP and RT-qPCR assay in non-senescent cells demonstrated SIRT7 binding in the promoter region (Fig. 3D) and low *CDKN2A/p16* mRNA expression levels (Fig. 1D). The role of SIRT7 in epigenetic control of *CDKN2A/p16* gene was assessed. siRNA-mediated knockdown of SIRT7 in non-senescent cells (2 weeks) was performed. *SIRT7* mRNA expression levels were significantly downregulated and protein expression levels were markedly downregulated in cells transfected with siSIRT7 at 48 h compared with cells transfected with siCtrl when assessed using qPCR (Fig. 4A) and western blotting (Fig. 4C). Furthermore, this decrease was associated with a significant increase in *CDKN2A/p16* expression in cells transfected with siSIRT7 at 48 h compared with cells transfected with siCtrl (Fig. 4B). However, SIRT7 knockdown demonstrated a significant decrease in SIRT7 binding to the *CDKN2A/p16* promoter sequence (Fig. 4D) and significant enrichment of H3K18Ac compared with siCtrl (Fig. 4E).

Discussion

Histone modification is an epigenetic mechanism that regulates gene expression. These modifications are catalyzed by enzyme complexes that act on the N-terminal ends of histones that form nucleosomes, mediating the removal or aggregation of chemical marks such as acetylation, methylation and phosphorylation (16). SIRTs are enzymes that regulate gene expression and biological activities, such as cell senescence (16). This is mediated by removing acetyl groups from histones, favoring compaction of chromatin and therefore mediating gene repression (31). A total of seven SIRTs has been reported in mammals,

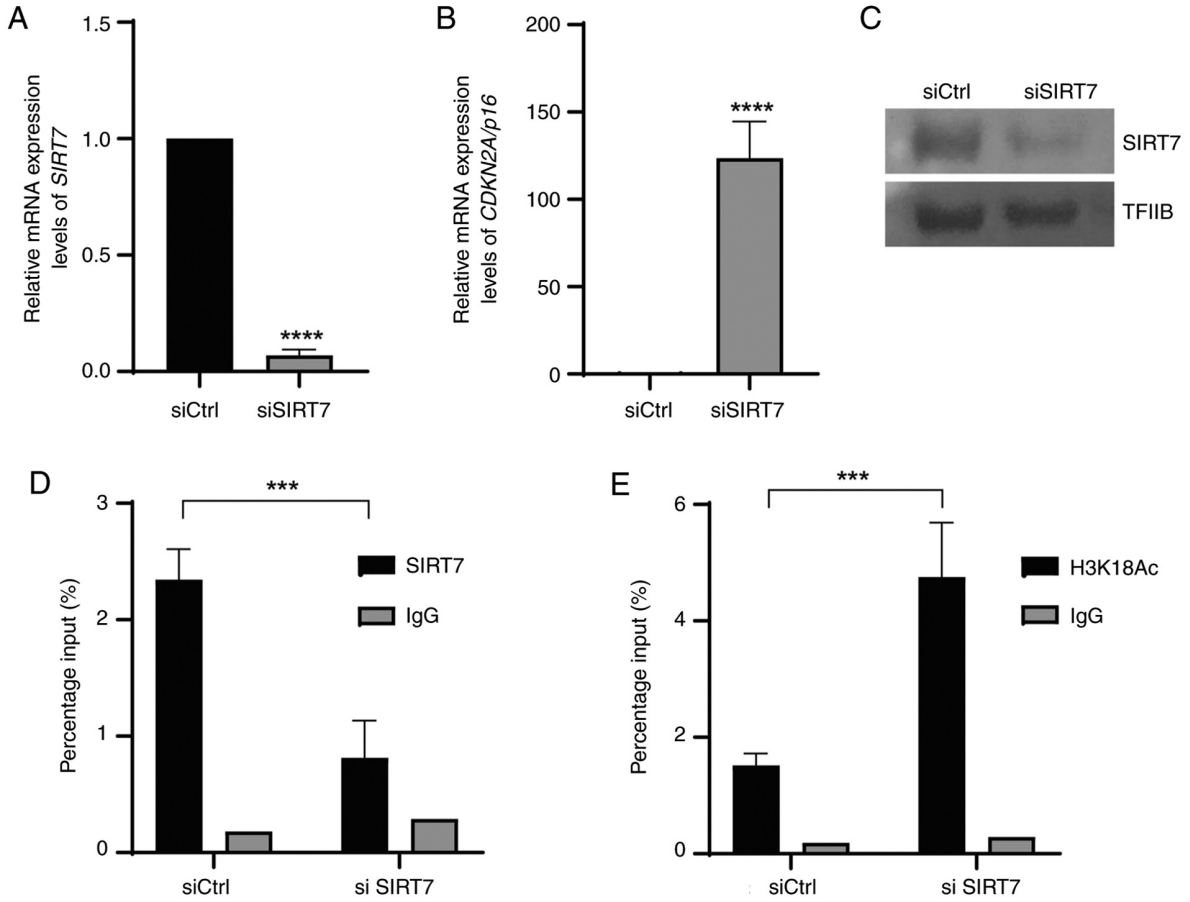

Figure 4. SIRT7 knockdown deacetylates the *CDKN2A/p16* promoter and upregulates *p16* mRNA expression levels in senescent MRC5 cells. Non-senescent MRC5 cultured cells (2 weeks) were transfected with si-SIRT7. Effective downregulation was demonstrated using RT-qPCR and western blotting 48 h post-transfection. (A) *SIRT7* and (B) *CDKN2A/p16* mRNA expression levels were assessed using qPCR 48 h after transfection and normalized against *GAPDH* mRNA expression levels. ****P<0.0001 (C) SIRT7 Protein expression was assessed by Western blot. TFIIB protein was used as a loading control. Chromatin immunoprecipitation assay was performed using antibodies against (D) SIRT7 or (E) H3K18Ac. Data are presented as % input ± SEM using normal IgG as a specific control. ***P<0.001 vs. siCtrl. SIRT; sirtuin; CDKN2A, cyclin dependent kinase inhibitor 2A; si, small interfering; Ctrl, control; RT-qPCR, reverse transcription-quantitative PCR; TFIIB, transcription factor IIB).

SIRT1-7 (16). SIRT1 and SIRT2 participate in cell senescence and SIRT2 ortholog overexpression extends lifespan in a range of lower eukaryotes (32). Previous reports showed that extra copies of SIR2, a member of Sirtuin in budding yeast Saccharomyces cerevisiae, extended the lifespan by 30% by preventing the formation of extrachromosomal DNA circles. Caenorhabditis elegans has four Sirtuins (sir-2.1, sir-2.2, sir-2.3, and sir-2.4), where sir-2.1 is the most similar to the S. cerevisiae SIR2. On the other hand, Drosophila melanogaster has five Sirtuins (dSirt1, dSirt2, dSirt4, dSirt6, and dSirt7), of which Sirt1 (better known as dSir2) is most similar to S. cerevisiae SIR2, and high levels are found in the nuclei and/or cytoplasm of neurons and fat bodies (33).

However, Huang *et al* (32) demonstrated that under stress, SIRT1 overexpression contributes to cell proliferation and prevents senescence in human diploid fibroblasts. The SIRT1-mediated delay of senescence is associated with $P16^{INK4A}$/Rb pathway downregulation and ERK/S6K1 signaling activation (32). However, the role of SIRT7 in cell senescence is unknown.

To elucidate the role of SIRT7 in senescence, a senescent cell model was developed *in vitro*. Pulmonary fibroblast MRC5 cells were cultured for 2, 4, 6 and 8 weeks. β-gal activity was assessed at 6 and 8 weeks, allowing acquisition of the senescent phenotype (3). In general, induction of the senescent phenotype induces expression of transcription factor EB, which increases lysosomal biogenesis, leading to overproduction of lysosomes and a decrease in their elimination (31). These lipid vesicles contain the enzyme SA-β-gal and when a chromogenic substrate is added to senescent cells, SA-β gal releases the chromogen from galactose, which resulted in formation of a blue coloration that demonstrates the increase in lysosomal content, is therefore an indicator that cells exhibit the senescent phenotype (3).

MRC5 cells cultured for 6 or 8 weeks demonstrated a significant decrease in *lamin B* mRNA expression, a biomarker of senescence, compared with 2-week cells. Senescent cells have a distinct gene expression profile, often accompanied by spatial redistribution of heterochromatin into senescence-associated heterochromatic foci (SAHFs) (34). Previously, a genome-wide mapping study reported that *lamin B1* is depleted during senescence, preferentially at central regions of lamin-associated domains, and Lys9 trimethylation on histone H3 (H3K9me3) is enriched (30). *Lamin B1* knockdown facilitates the spatial re-localization of perinuclear H3K9me3-positive heterochromatin, thus promoting SAHF formation, which is inhibited by ectopic lamin B expression (34).

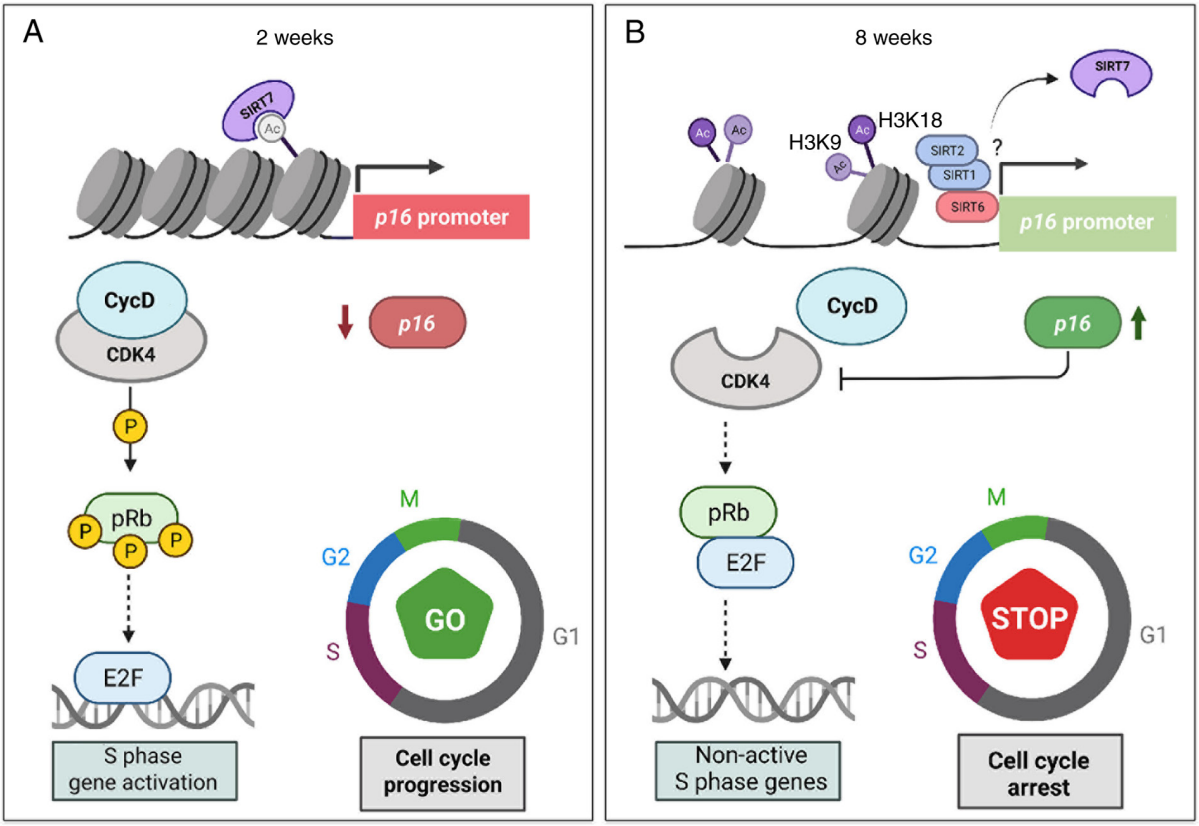

Figure 5. Proposed model of *CDKN2A/p16* gene epigenetic regulation in cellular senescence. (A) Non-senescent cell state. Cells demonstrated SIRT7 enrichment in the *CDKN2A/p16* promoter region. SIRT7 binding was associated with activity as a suppressor enzyme mediating H3K18Ac removal and inhibiting *CDKN2A/p16* expression. As there were low cellular $p16^{INK4A}$ levels, CDK and the CycD complex phosphorylate Rb, favoring release of transcription factor E2F and inducing protein synthesis necessary for DNA replication and cell cycle progression. (B) Senescent cell model. Lack of suppressor enzyme SIRT7 binding in the *CDKN2A/p16* promoter region allows its transcriptional activation. The presence of $P\text{-}16^{INK4A}$ protein in senescent cells inhibits CDK4/CycD binding, avoiding Rb phosphorylation and inhibiting release of transcription factor E2F, thus leading to cell cycle arrest. SIRT; sirtuin; CDK, cyclin-dependent kinase; CDKN2A, CDK inhibitor 2A; p, phosphorylated; Rb, retinoblastoma protein; E2F, E2 factor; CycD, cyclin D.

In the present study, MRC5 cells at 6 and 8 weeks demonstrated a marked increase in *CDKN2A/p16* mRNA expression levels. It has been previously reported that expression of *CDKN2A/p16* is crucial for CDKI activation-dependent senescence (3). Induction of senescence phenotype causes the cell to express other typical senescence characteristics, such as proinflammatory protein synthesis, resistance to apoptosis, active metabolism, endoplasmic reticulum stress, *p53* overexpression, *lamin B1* downregulation and increased lysosomal content (3). The present study demonstrated that senescence started at 6 weeks in cultured cells, which validated this senescent cellular model. Several studies have reported that MRC5 cells are an ideal biological model to study senescence and analyze molecular changes as such as nuclear laminin-associated protein, lamin B1 and loss of the epigenetic suppressive marker tri-methylation of Lys 27 on histone H3 in chromatin (35-38).

The epigenetic mechanisms involved in *CDKN2A/p16* transcriptional activation, were evaluated using ChIP assay, which demonstrated significant enrichment of H3K9Ac and H3K18Ac accompanied by a significant decrease in SIRT7 in the promoter region in cells with a senescent compared with non-senescent phenotype. A decrease in SIRT7 expression has been reported in aging tissue (39) and SIRT7 enzyme loss in mice leads to a decrease in embryonic viability and lifespan, aging-associated pathology and the loss of regenerative potential hematopoietic stem cells (40,41). Previous studies have reported increased levels of the senescence marker *CDKN2A/p16* in SIRT7-deficient cell cultures and increased *CDKN2A/p16* mRNA expression levels in splenocytes and fibroblasts obtained from SIRT7-negative mice (40,41).

The present study demonstrated that SIRT7 knockdown markedly increased *CDKN2A/p16* mRNA and protein expression levels compared with siCtrl in cells cultured for 2 weeks. This enhanced *CDKN2A/p16* expression was associated with *CDKN2A/p16* gene changes, which demonstrated a 2-fold H3K18Ac enrichment on the promoter. These results demonstrated that the SIRT7 deacetylase enzyme participated in cell senescence via transcriptional regulation of *CDKN2A/p16*. Previous studies reported that SIRT7 serves key roles in cell senescence and aging, SIRT7-deficient mice demonstrate a shortened lifespan and aging-associated phenotypes (39,40,42-45) and overexpression of SIRT7 in senescent-induced cells suppresses expression of senescence markers such as p53 and p21 (39,46).

In terms of the epigenetic mechanisms that control *CDKN2A/p16* expression, DNA methylation of its promoter region has been reported in pathology, such as cancer (47). However, in normal cells of young mammals, the *INK4/ARF* locus remains silenced (embryonic and fetal stem cells) due to suppressive Polycomb complexes PRC1 and PRC2 action (48).

However, the *INK4/ARF* locus responds to oncogenic stress signals when stem cells lose self-renewal and differentiation capacity. In these cases, alterations in PRC1 and PRC2 complex member proteins (Chromobox 7, BMI1 proto-oncogene polycomb ring finger and enhancer of zeste 2 Polycomb repressive complex 2 subunit) that produce loss of suppressor markers in trimethylated lysine 27 of histone H3 (H3K27). This activates *CDKN2A/p16* expression to induce senescence (48). The aim of the present study was to assess the role of the epigenetic enzyme SIRT7 in transcriptional control of CDKN2A/P16. In future, the protein expression profiles of P16 and lamin B1 should be assessed in biological models of cellular senescence such as fibroblasts or culture of neurons.

The present study demonstrated that in non-senescent cells, transcriptional suppression of *CDKN2A/p16* gene was mediated by binding of SIRT7 and low levels of H3K18Ac in its promoter region. In this context, there were low levels of cellular *CDKN2A/p16* RNA messenger. If this mRNA low levels translate into low protein levels, it could be proposed that CDK and the CycD complex to phosphorylate the retinoblastoma protein, favoring transcription factor E2F release and inducing the protein synthesis necessary for DNA replication and cell cycle progression (Fig. 5A). However, in the senescent cell model, there was no evidence of the repressor enzyme SIRT7 binding in the *CDKN2A/p16* promoter region, which allowed its transcriptional activation. It was hypothesized that $p16^{INK4A}$ protein in senescent cells would therefore inhibit CDK4/CycD binding, avoiding retinoblastoma protein phosphorylation and inhibiting the release of the transcription factor E2F, thus leading to cell cycle arrest (Fig. 5B). However, the present study did not perform protein expression assay; this is required in future works.

The present study assessed epigenetic parameters regulating *CDKN2A/p16* transcription during senescence. These results validated the MRC5 cell line as a model of senescence. Furthermore, it was demonstrated that SIRT7 decreased H3K18Ac in the *CDKN2A/p6* promoter region and was directly associated with suppression of this gene.

In the present study, the regulatory effect of the histone deacetylase enzyme SIRT7 on the gene expression of *CDKN2A/p16* was evaluated in human MRC5 cells used as a model of replicative cell senescence. The results demonstrated that *CDKN2A/p16* transcriptional repression was regulated by SIRT7 via direct binding of the promoter region and deacetylation of the activating epigenetic marker H3K18Ac.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BH and AR conceived and designed the experiments. SR, LGB, CB, and DG performed the experiments, analyzed the data and wrote the manuscript. AC and TMR analyzed data and designed experiments. AR and BH supervised the study. AR and BH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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