



BRD4 plays an antiaging role in the senescence of renal tubular epithelial cells

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Background: Age-related kidney failure is often induced by a decrease in the bioavailability of tubular epithelial cells in elderly chronic kidney disease (CKD) patients. BRD4, an epigenetic regulator and a member of the bromodomain and extraterminal (BET) protein family, acts as a super-enhancer (SE) organizing and regulating genes expression during embryogenesis and cancer development. But the physiological function of BRD4 in normal cells has been less studied. This study aimed to research certain biological roles of BRD4 in the process of normal cell aging and discuss the potential mechanisms.

Methods: In this study, we investigated the biological functions of BRD4 proteins in the aging of renal tubular cells. At first, we used a D-galactose (D-gal) and BRD4 inhibitor (Abbv-075) to replicate kidney senescence *in vivo*. D-gal and Abbv-075 were then used to measure the aging-related changes, such as changes in cell cycle, β -galactosidase activity, cell migration, and p16 protein expression *in vitro*. At last, we knocked down and over-expressed BRD4 to investigate the aging-related physiological phenomena in renal tubular cells.

Results: *In vitro*, D-gal treatment induced noticeable aging-related changes such as inducing cell apoptosis and cell cycle arrest, increasing β -galactosidase activity as well as up-regulating p16 protein expression in primary human tubular epithelial cells. In the aging mice model, D-gal significantly induced renal function impairment and attenuated BRD4 protein expression. At the same time, the BRD4 inhibitor (Abbv-075) was able to mimic D-gal-induced cell senescence. *In vivo*, Abbv-075 also decreased kidney function and up-regulated p21 protein expression. When we knocked down the expression of BRD4, the senescence-associated β -galactosidase (SA- β -gal) activity increased dramatically, cell migration was inhibited, and the proportion of cells in the G0/G1 phase increased. Additionally, the knockdown also promoted the expression of the senescence-related proteins p16. When the renal tubular cells were overexpressed with BRD4, cell aging-related indicators were reversed in the D-gal-induced cell aging model.

Conclusions: BRD4 appears to have an active role in the aging of renal tubular cells *in vivo* and *in vitro*. The findings also suggest that BRD4 inhibitors have potential nephrotoxic effects for oncology treatment. BRD4 may be a potential therapeutic biomarker and drug target for aging-related kidney diseases, which warrants additional studies.

Keywords: BRD4; senescence; renal tubular epithelial cells

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Introduction

Aging, a natural process leading to organ function deterioration, is driven by multiple molecular mechanisms, such as cellular senescence (1). Senescence was initially described by Leonard Hayflick as a condition in which the proliferative capacity of non-immortalized human cells in culture was arrested in response to various stimuli. Cellular senescence is a state of irreversible cycle arrest in which the cell becomes resistant to growth-promoting stimuli (2). Similar to autophagy and apoptosis, senescence restricts the propagation of defective cells and maintains tissue integrity and function (3). Common stimuli of senescence include oncogenic activation, cytokine release, reactive oxygen species (ROS) overproduction, DNA damage, and nucleotide depletion (4). D-galactose (D-gal) is an aldohexose. At high levels, it can be converted into aldose and hydroperoxide under the catalysis of galactose oxidase, producing ROS. D-gal is most preferred due to its convenience, less side effects, and the higher survival rate throughout the experimental period, and it has been proven to artificially induce cellular senescence *in vitro* and *in vivo* for anti-aging therapeutic interventions (5).

Aging renders the kidneys more susceptible to internal toxins (6). End-stage renal disease results from progressive structural and functional deterioration of renal tubular epithelial cells (7). During the senescence of renal tubular epithelial cells, mitochondrial dysfunction and DNA repair deficiency lead to cell cycle arrest, apoptosis and renal fibrosis (8). The senescence of renal tubular epithelial cells can be regulated to treat aging-related kidney disease. Therefore, targeting the senescence of renal tubular

epithelial cells has become a therapeutic approach for treating kidney injury (9).

In recent years, the role of epigenetic regulation in cellular senescence has garnered much attention (10). Upon stimulation, epigenetic regulation initiates cell cycle arrest, DNA repair deficiency, senescence, and apoptosis.

The bromodomain and extraterminal (BET) family of bromodomains (BRDs) are well-known drug targets for many human diseases. BRD4, a member of the BET protein family, has been shown to be a transcriptional and epigenetic regulator in the organization and expression of genes (11). Inhibition of BRD4 can block the communication between the super-enhancer (SE) and the promoter region of the target genes, with subsequent cell-specific repression to induce cell death (12). Cells with BRD4 knockdown grow slowly and are arrested at the G0/G1 phase; however, reintroduction of BRD4 rescues G1 gene expression and promotes the cells to S phase (13).

Currently, the role of BRD4 in senescence is largely unexplored. Emerging evidence reveals that BRD4 maintains genomic integrity by reducing DNA damage (14). Lee *et al.* reported that BRD4 is required for adipogenesis and myogenesis due to its maintenance of cell-specific gene expression (15). BRD4 can also activate the DNA damage checkpoint and maintain telomeres in a non-transcriptional manner, thus trapping cells into senescence or apoptosis. Herein, we investigated the biological function of BRD4 in the aging of kidneys in cells and animals and figured out related molecular mechanisms. We present this article in accordance with the ARRIVE and MDAR reporting checklists (Available at <https://tau.amegroups.com/article/view/10.21037/tau-24-214/rc>).

Highlight box

Key findings

- BRD4 suppressed age-related kidney failure via ameliorating D-galactose (D-gal)-induced renal injury *in vivo* and *in vitro*. BRD4 inhibitors could promote renal aging and impair renal function.

What is known and what is new?

- Aging leads to a significant decrease in BRD4 expression in renal tubular cells.
- BRD4 has the potential to reverse some aging-related physiological phenomena induced by D-gal in renal tubular cells.

What is the implication, and what should change now?

- Activation of BRD4 can prevent the aging of renal tubular cells. So, BRD4 is a promising drug target for treating aging-related kidney injury.

Methods

Culture of renal tubular epithelial cells

Human proximal tubular epithelial cells (HRPTEpiCs) were purchased from ScienCell (Carlsbad, CA, USA) and cultured in medium at 37 °C in 95% air with 5% CO₂.

Cell transfection

The cells were incubated with lentiviral vectors (Genechem LLC, Shanghai, China) according to the manufacturer's protocol. First, cells were seeded in 70–90% confluent in 24-well. Second, the supernatant was removed, medium was replaced without fetal bovine serum (FBS) and the

lentiviral vectors were added. At last, after incubation for 12 hours, the culture supernatant was removed and replaced with complete growth medium. The cells were collected 48 hours after transfection for other analyses.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined by MTT assay. First, the cells were seeded in 96-well plates (5×10^3 /well) for 24 hours and then stimulated with D-gal (Selleck Chemicals LLC, Houston, TX, USA) in different concentrations (50, 100, 150, 200 mM) for 24 hours. MTT solution (5 mg/mL) was added and incubated for 4 hours, and dimethyl sulfoxide (DMSO) was added to dissolve the crystals. Absorbance was measured by the plate reader at a wavelength of 492 nm.

Cell migration assay

Cell migration was assayed in 24-well, 6.5-mm-internal-diameter transwell plates (8.0 μ m pore size; Costar Corp., Corning, NY, USA). Cells were placed on the upper chambers, and the lower chamber was filled with medium containing FBS. Cells were allowed to migrate. After 24 hours, cells on the upper surfaces of the chambers were removed by cotton swabs and the migrated cells on the undersides of the chambers were fixed with methanol, stained with crystal violet, and photographed by microscope.

Annexin V-propidium iodide (PI) staining assay for apoptosis analysis

Apoptosis of the cells was evaluated using Annexin V-PI staining. The cells were treated with various concentrations of D-gal for 24 hours, collected, washed with phosphate-buffered saline (PBS), stained with Annexin V-PI for 5 minutes in the dark, and finally analyzed by fluorescence-activated cell sorting (FACS).

PI staining assay for cell cycle analysis

Apoptosis of the cells was evaluated using PI staining (Thermo Fisher Scientific, Waltham, MA, USA). The cells were treated with various concentrations of D-gal for 24 hours, collected, washed with PBS, stained with PI for 5 minutes in the dark, and finally analyzed by FACS.

Senescence-associated β -galactosidase (SA- β -gal) staining

Senescence was evaluated according to SA- β -gal activity in cultured cells, a typical indicator of senescence. The cells were treated with various concentrations of D-gal for 24 hours on 35 mm dish in a 5% carbon dioxide (CO₂) incubator. After removing the culture medium, the cells were washed with PBS. Two mL of 4% paraformaldehyde (PFA)/PBS solution was added to the dish and the cells were incubated at room temperature. The supernatant was removed, and the cells were washed with PBS for three times. The cells were stained with SA- β -gal staining (Beyotime Biotechnology, Shanghai, China) working solution and incubated at 37 °C incubator without CO₂ for 30 minutes. After removing the supernatant, the cells were washed with PBS and observed by microscope.

Western blotting (WB)

Protein content was measured using the Bradford method. After sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was incubated with anti-p16 (Abcam, ab189034, 1:1,000; Abcam, Cambridge, MA, USA), BRD4 (Cell Signaling Technology, 13440, 1:1,000, Danvers, MA, USA) and anti-GAPDH (Cell Signaling Technology, 5174, 1:1,000) antibodies at 4 °C overnight. After washing, the membrane was incubated with the secondary antibody (Cell Signaling Technology, 7074/7076, 1:5,000) at 1:10,000 for 2 hours and then washed again. Substrate ChemStation scanning (Agilent, Santa Clara, CA, USA) for color analysis was performed with Tanon Gis software (Tanon, Shanghai, China).

Mouse model

All male mouse experiments were conducted under protocols approved by the Animal Care and Use Committee of Jiangsu Province Academy of Nanjing Medical University (No. 2201040), in compliance with Nanjing Medical University guidelines for the care and use of animals. A protocol was prepared before the study without registration. We obtained 8-week-old C57BL/6 mice from the Aniphe BioLab Company (Nanjing, China). The animals were kept at 22 \pm 1 °C in a 12-hour light-dark cycle

(6–18 hours) with free access to water and food. The physical and mental parameters of the animals were recorded on a daily basis.

Experimental design

The mice were randomly assigned to three experimental groups: a control group (CTL) that received intraperitoneal (IP) saline solution (NaCl 0.9%), a D-gal group (DGAL) that received D-gal at 150 mg/kg via IP (n=8 animals/per group). The mice in these first two groups were subjected to daily treatment for 8 weeks. The third group was DbBETi (Abbv-075) that received Abbv-075 1.5 mg/kg by oral gavage (16) for 10 days (n=8 animals/per group). The IP was standardized as a volume less than or equal to 2 mL/kg. D-gal was dosed at 150 mg/kg (as reported in the literature) to induce aging in the mice.

Biochemical analysis

At the end of week 1 and 2, blood samples were collected immediately after sacrificing the mice. The blood was centrifuged at 1,500 g and 4 °C for 15 minutes, and then the contents of blood urea nitrogen (BUN), serum creatinine (sCr), uric acid (UA), and cystatin C (Cys C) were measured using a Beckman II Analyzer (Beckman Coulter Instruments, Fullerton, CA, USA).

Histopathological analysis

The mouse kidney tissues were fixed in paraformaldehyde and then embedded in paraffin. Immunohistochemistry (IHC) assays were performed as previously described (17). The renal tissue sections were incubated with antibodies against BRD4 and P21 (Santa Cruz Biotechnology, SC-6246, 1:100, Dallas, TX, USA) at 4 °C overnight, followed by incubation with a secondary antibody. Finally, the sections were counterstained with Mayer's hematoxylin.

Statistical analysis

SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Single-factor analysis of variance (ANOVA) was used to compare data among the experimental groups. The results were expressed as the mean ± standard deviation. P<0.05 was considered statistically significant.

Results

D-gal induces HRPTEpiC senescence and renal injury

Cell viability measured by MTT assay showed that D-gal suppressed the proliferation of HRPTEpiC in a time- and concentration-dependent manner (*Figure 1A*). Next, we measured the levels of senescence-related markers. The level of SA-β-gal-positive cells among HRPTEpiCs after D-gal treatment was significantly higher than that in the control group (*Figure 1B*). Cell cycle arrest, a hallmark of cellular senescence (18), was induced by D-gal, implying that D-gal inhibited the proliferation of HRPTEpiCs (*Figure 1C*). Additionally, D-gal remarkably upregulated the expression levels of senescence-related proteins p16 in HRPTEpiCs (*Figure 1D*). *In vivo*, D-gal significantly increased the levels of renal function-related markers, such as Cys C, BUN, and sCr (*Figure 1E*).

A BRD4 inhibitor induces HRPTEpiC senescence and renal injury

BRD4 is a pivotal transcriptional and epigenetic regulator during embryogenesis and development (19). Here, we focused on whether it plays an essential role in the senescence of tubular epithelial cells. We quantified the protein expression of BRD4 in D-gal-treated cells and a mouse senescence model. D-gal significantly decreased the protein expression of BRD4, which was significantly decreased by D-gal *in vivo* (*Figure 2A,2B*). It was further revealed that Abbv-075 (Selleck Chemicals LLC), an inhibitor of BRD4, could inhibit the protein expression of BRD4 in HRPTEpiCs (*Figure 2C*). The SA-β-gal activity of HRPTEpiCs increased notably after treatment with Abbv-075 (*Figure 2D*). In addition, Abbv-075 notably aggravated 24-hour albuminuria and decreased the weight of C57/BL6 mice (*Figure 2E*). IHC results also showed that Abbv-075 increased the expression levels of senescence-related proteins (*Figure 2F*). These results verified the involvement of BRD4 in D-gal-induced cellular senescence.

Knockdown of BRD4 induces HRPTEpiC senescence

To further prove that inhibiting BRD4 could induce cell senescence, the expression of BRD4 was knocked down by short hairpin RNA (shRNA). The SA-β-gal activity in HRPTEpiCs increased dramatically after BRD4 shRNA treatment (*Figure 3A*). Senescent cells are generally believed

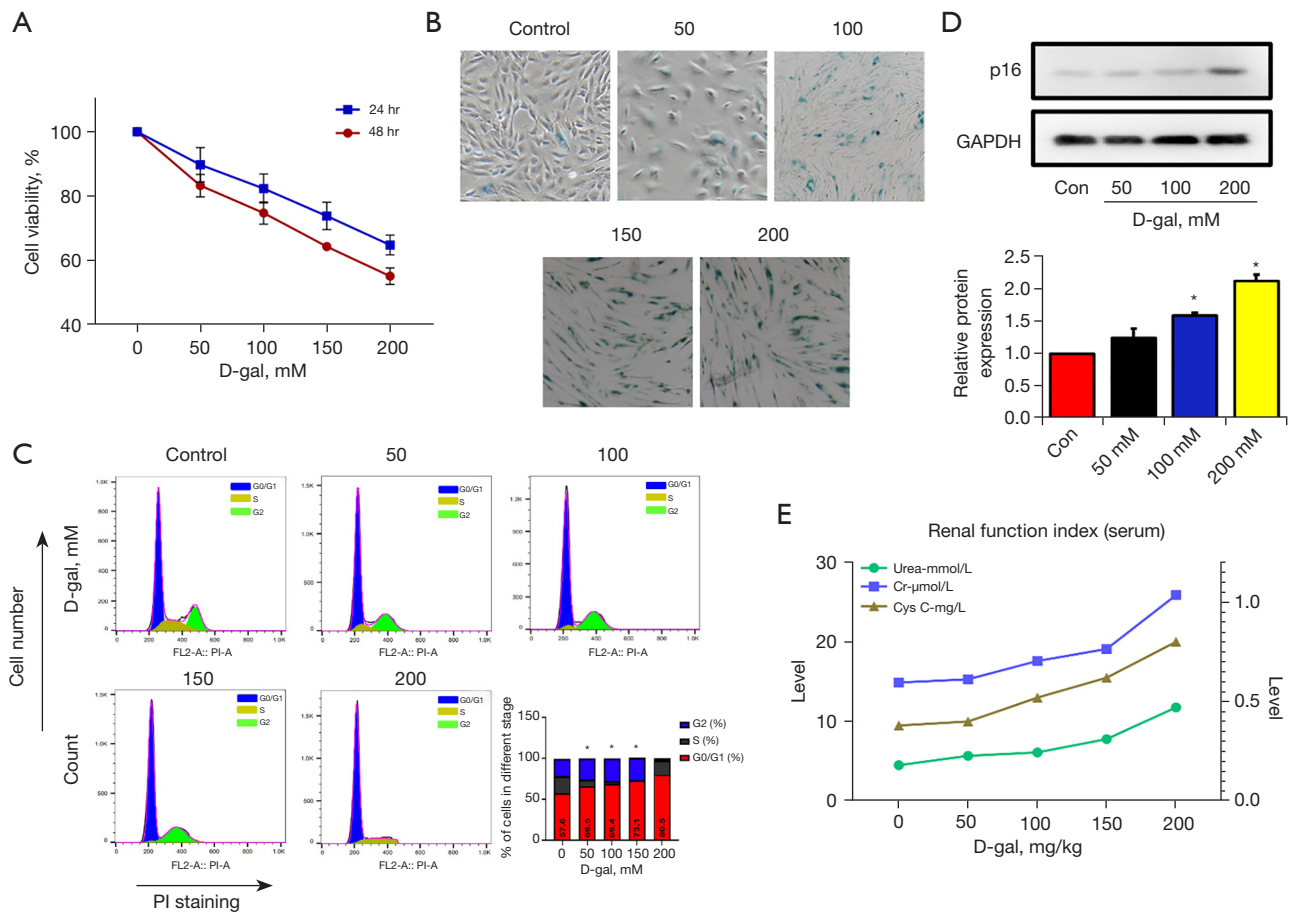


Figure 1 D-gal induces HRPTEpiC senescence and accelerates kidney function injury. (A) D-gal inhibits the proliferation of primary human tubular epithelial cells; (B) D-gal increases SA- β -gal staining *in vitro* (200 \times); (C) D-gal induces G0/G1 cell cycle arrest *in vitro*; (D) D-gal upregulates the relative expression levels of senescence-related proteins *in vitro*; (E) D-gal induces renal injury *in vivo*. The values are expressed as the means \pm SD; n=3. *, P<0.05, control vs. D-gal-treated. D-gal, D-galactose; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Con, control; Cr, creatinine; Cys C, cystatin C; HRPTEpiC, human proximal tubular epithelial cells; SD, standard deviation.

to exert an irreversible arrest in the G0/G1 stage of the cell cycle. Knockdown of BRD4 also inhibited cell migration and increased the proportion of cells in the G0/G1 phase (Figure 3B,3C). Additionally, the knockdown also promoted the expression of the senescence-related proteins p16 (Figure 3D). In summary, the knockdown of BRD4 could induce the senescence of HRPTEpiCs.

Overexpression of BRD4 rescues D-gal-induced HRPTEpiC senescence

Next, we designed an experiment to confirm whether the overexpression of BRD4 could alleviate cell senescence. As

BRD4 was overexpressed, the migration and viability of HRPTEpiCs were enhanced, as shown in the 3D migration assay (Figure 4A). As expected, D-gal-induced apoptosis and cell cycle arrest were rescued (Figure 4B,4C). Taken together, BRD4 reversed the senescence of D-gal-treated tubular epithelial cells.

BRD4 downregulates the P16 protein expression to alleviate D-gal-induced HRPTEpiC senescence

The p16 tumor suppressor (CDKN2A) is also involved in the cell cycle and senescence (20). We focused on p16 because of its well-established role in controlling cell

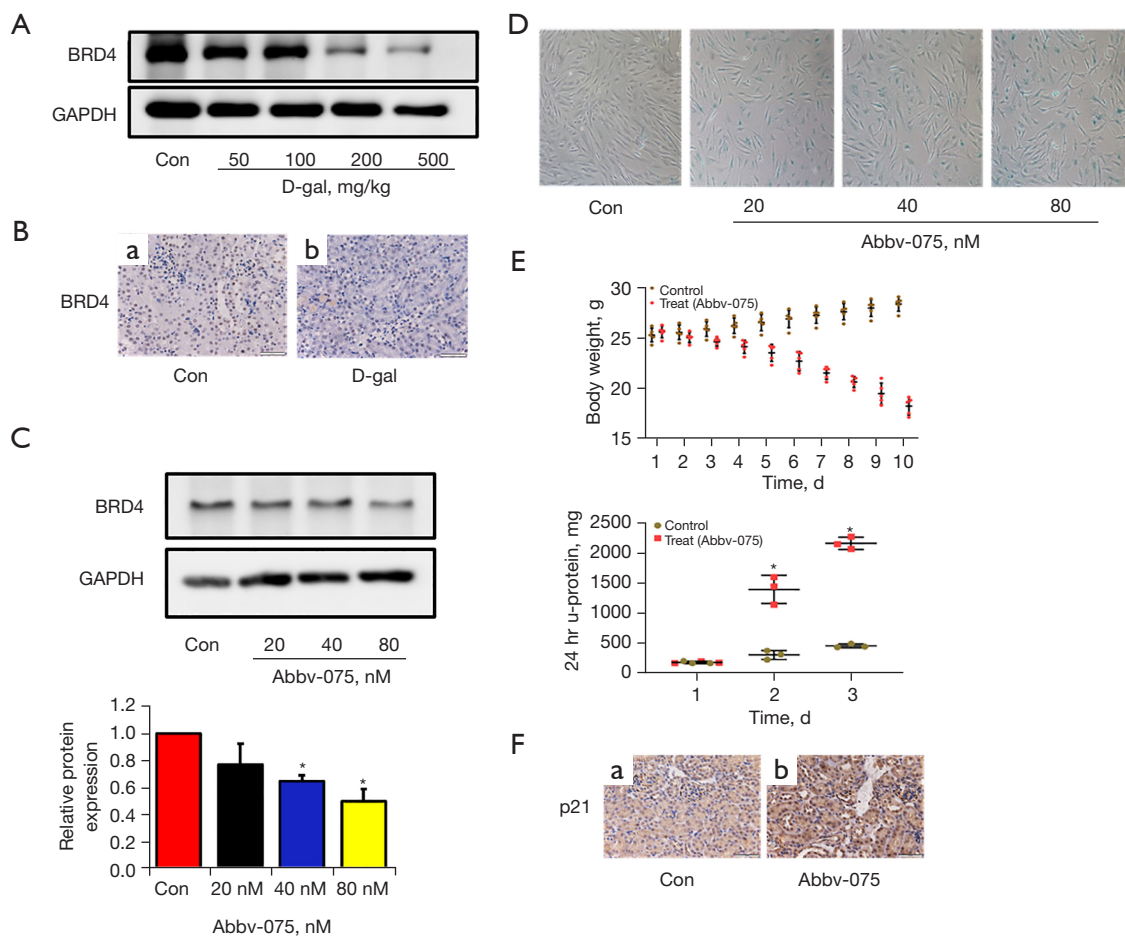


Figure 2 A BRD4 inhibitor induces HRPTEpiC senescence and kidney function injury. (A) D-gal decreases BRD4 protein expression *in vivo*; (B) D-gal decreases BRD4 protein expression *in vivo* (IHC) (100×); (C) a BRD4 inhibitor (Abbv-075) decreases BRD4 protein expression in HRPTEpiCs by WB; (D) a BRD4 inhibitor increases SA-β-gal staining *in vitro* (100×); (E) a BRD4 inhibitor induces renal injury *in vivo*; (F) a BRD4 inhibitor upregulates the expression levels of senescence-related proteins *in vivo* (IHC) (100×). The values are expressed as the means ± SD; n=3. *, P<0.05, control vs. treated with a BRD4 inhibitor. BRD, bromodomain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Con, control; D-gal, D-galactose; HRPTEpiC, human proximal tubular epithelial cells; WB, western blotting; IHC, immunohistochemistry; SD standard deviation.

proliferation, survival, and senescence. We found that overexpression BRD4 did not downregulate the protein expression of p16, but it could reverse the down-expression of p16 induced by D-gal (Figure 4D). Therefore, BRD4 attenuated D-gal-induced renal tubule senescence.

Discussion

Worldwide, life expectancy has reached an unprecedented peak. By the middle of the 21st century, the global population aged over 65 years is expected to exceed 1.5 billion (21). Aging is the major risk factor causing

the molecular and physiological changes that lead to impairment of the renal function and regenerative potential (22). With the growth of the aging population, one of our challenges is to understand the basic biological mechanisms underlying these changes that accompany aging, as these changes increase the risk of developing acute kidney injury (AKI) and chronic kidney disease (CKD) (23). A research has shown that specific cellular biologic events and signal pathways of lipotoxicity, oxidative stress, and inflammation are involved in the complex process of kidney aging (24). In this study, we highlight experimental findings about the molecular mechanisms of cellular senescence in

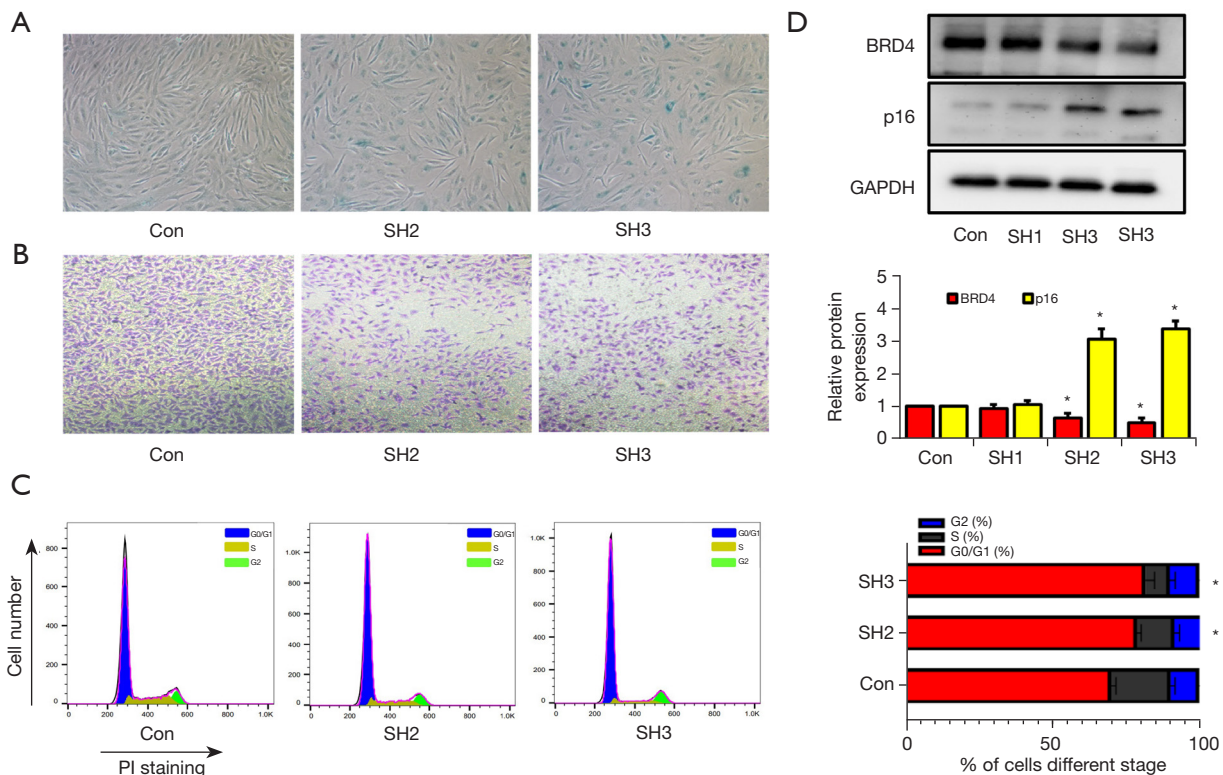


Figure 3 Knockdown of BRD4 induces HRPTEpiC senescence. (A) Knockdown of BRD4 intensified SA- β -gal staining *in vitro* (100 \times); (B) knockdown of BRD4 decreased cell migration *in vitro* by crystal violet staining (100 \times); (C) knockdown of BRD4 induced G0/G1 cell cycle arrest *in vitro*; (D) knockdown of BRD4 regulated the expression levels of senescence-related proteins *in vivo* (WB). The values are expressed as the means \pm SD; n=3. *, P<0.05, control vs. shRNA-BRD4 treated. SH1, BRD4-Sh1; SH2, RD4-Sh2; SH3, RD4-Sh3. Con, control; PI, propidium iodide; BRD, bromodomain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRPTEpiC, human proximal tubular epithelial cells; WB, western blotting; SD, standard deviation; shRNA, short hairpin RNA.

renal aging that pose challenges in therapeutic aspects of senescence. This would provide the great potential of anti-senescent therapies for alleviating the harmful senescent to avoid age-related AKI and CKD.

In the present study, we found that BRD4 attenuated D-gal-induced senescence of renal tubular cells, which contributes to aging-related renal diseases. Inspired by this finding, more in-depth studies and even potential therapeutic options can be expected.

Kidney tubular epithelial cells undergo pathological changes crucial for the development of aging-related CKD, but the related molecular mechanisms and interventional strategies remain obscure (7). BRD4 acts as a genetic reader of histone acetyl-lysine residues to regulate gene transcription. BRD4 has shown therapeutic potential in a variety of human diseases, including cancer, inflammation, nervous system disorders, and cardiovascular

diseases (25). Recently, Zhang *et al.* reported that degradation and inhibition of BRD4 exacerbate Alzheimer's disease-related neuropathy in cell models by increasing the levels of BACE1 (26). BACE1, which is also expressed in renal tubular epithelial cells, increases renal damage in aging mice (27). In this study, we found that BRD4 functioned actively in the senescence of kidney tubular epithelial cells.

D-gal is considered an authoritative agent in inducing aging in animal models (28). D-gal, an aldohexose and a monosaccharide sugar, is a reducing sugar that is present naturally in the body and in different kinds of food such as milk, yogurt, cherries, and celery (29). After ingestion, the maximal daily dose of D-gal that can be metabolized and excreted by the body is 50 g within about 8 hours for a healthy adult (30). Amounts in excess of this limit can increase the level of ROS and cause oxidative stress, inflammation, mitochondrial dysfunction, and

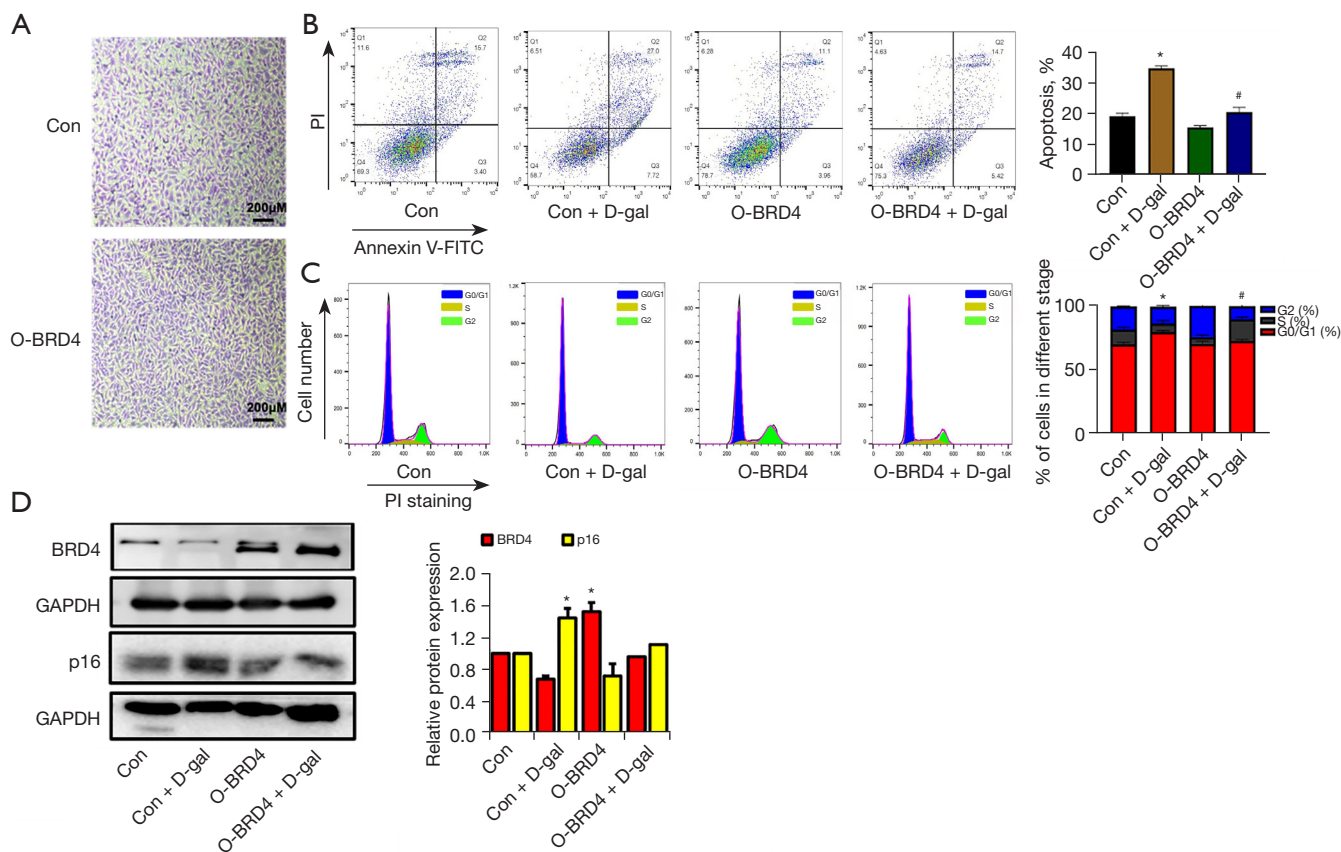


Figure 4 Overexpression of BRD4 rescues D-gal-induced HRPTEpiC senescence by de-activating the p16 function. (A) Overexpression of BRD4 increases cell migration *in vitro* by crystal violet staining; (B) overexpression of BRD4 alleviates D-gal-induced cell apoptosis *in vitro*; (C) overexpression of BRD4 alleviates D-gal-induced cell cycle arrest *in vitro*; (D) overexpression of BRD4 induces p16 protein expression *in vitro*. The values are expressed as the means \pm SD; n=3. *, P<0.05, control *vs.* control + D-gal; #, P<0.05, control + D-gal *vs.* D-gal + BRD4 overexpression. Con, control; PI, propidium iodide; D-gal, D-galactose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BRD, bromodomain; HRPTEpiC, human proximal tubular epithelial cells; SD, standard deviation.

apoptosis (31). In China, the first researchers using D-gal for the study of senescence discovered that injections of it could reduce longevity in rodents (5). Since this discovery, a study has utilized this model and it has been well confirmed to mimic the changes of some aging markers such as advanced glycation end (AGE) products, telomere length shortening, senescence-associated genes (P16, P21, P53), and senescence-associated SA- β -gal staining (32). The increased AGEs may give rise to age-related kidney failure (33). In this study, we used D-gal to mimic the senescence to investigate the possible underlying mechanisms of aging-related kidney injury.

According to our results, D-gal inhibited the expression of BRD4 and induced aging in tubular epithelial cells *in vivo* and *in vitro*. The pan-BRD4 inhibitor (Abbv-075) could

also mimic D-gal-induced aging. To further confirm this conclusion, downregulated protein expression of BRD4 may lead to tubular epithelial cell injury and subsequent renal dysfunction. To explore its role in tubular epithelial cell senescence, we overexpressed BRD4 in primary cells with a lentiviral vector. To a certain extent, upregulation of BRD4 alleviated D-gal-induced senescence of renal tubular epithelial cells. In contrast, downregulating BRD4 with the inhibitor directly induced primary tubular epithelial cell senescence and G0/G1 cell cycle arrest, an effect similar to that of D-gal. G0/G1 phase cycle arrest is a critical step in cellular senescence (34). P16, a 16 kDa protein encoded by the *CDKN2A* gene and a member of the INK family of CDKIs, can regulate the entry of cells to the G1-S-phase transition checkpoint (35). In addition, p16 inhibits the

function of CDK4/6-cyclin D, a protein complex catalyzing the progression to G1 phase (36). It also downregulates the phosphorylation of retinoblastoma (RB), which promotes the formation of the RB-E2F complex to induce the cell-cycle arrest (37). Therefore, upregulation of p16 may initiate the process of senescence by arresting cell growth. In the current study, we found that as BRD4 overexpression down-regulated the protein expression of p16 induced by D-gal, the senescence of kidney tubular epithelial cells induced by D-gal was remarkably alleviated.

Conclusions

In recent studies, the importance of BET protein BRD4 has been investigated and characterized in the senescent processes of the kidney. In this study, we showed that BRD4 inhibitors could promote renal senescence and impair renal function. And the biological function of BRD4 declines in elderly patients with chronic kidney disease. Therefore, restoring the normal physiological function of BRD4 in these patients can help slow the progression of chronic kidney disease. So, BRD4 may be a potential therapeutic biomarker for aging-related kidney diseases.

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Footnote

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Data Sharing Statement: Available at <https://tau.amegroups.com/article/view/10.21037/tau-24-214/dss>

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Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at <https://tau.amegroups.com/article/view/10.21037/tau-24-214/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2201040) granted by the Animal Care and Use Committee of Jiangsu Province Academy of Nanjing Medical University, in compliance with Nanjing Medical University guidelines for the care and use of animals.

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