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# Role of tea polyphenols in delaying hyperglycemia-induced senescence in human glomerular mesangial cells via miR-126/Akt-p53-p21 pathways

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## Abstract

**Purpose** The aim of this study was to investigate the effects and possible mechanism of tea polyphenols (TPs) on the senescence of human glomerular mesangial cells (HGMCs) under high glucose conditions.

**Methods** HGMCs were divided into the normal group (NG, 5.5 mmol/L glucose), mannitol group (MNT, 5.5 mmol/L glucose and 24.5 mmol/L mannitol), TP group (TP, 30 mmol/L glucose and 5 μg/mL TP) and high-dose D-glucose group (HG, 30 mmol/L glucose). The effects of TP on the cell morphology of HGMCs; the percentage of cells positive for senescence-associated β-galactosidase (SA-β-gal); the ratio of G1 phase of cell cycle; telomere length; and the expression of p-Akt, p53, p21 and Rb proteins of the Akt-p53-p21 signaling pathway and the expression miR-126 were examined.

**Results** High glucose led to premature senescence of HGMCs, as evident from the increase in the percentage of SA-β-gal-positive cells, decrease in telomere length, cell cycle arrest at G1 phase,decrease in the expression of miR-126 and p-Akt and increase in the expression of p53, p21 and Rb proteins in the HG group. In contrast, in the TP group, these effects of high glucose treatment were abrogated; this indicates that TP had a protective effect on HGMCs.

**Conclusions** High glucose induces the senescence of HGMCs in vitro via the miR-126 and Akt-p53-p21 signaling pathways. TP can delay the high glucose-induced senescence of HGMCs by regulating the activity of these signaling pathways. Thus, the polyphenols present in tea may have potential for the treatment of diabetic nephropathies associated with premature senescence.

**Keywords** Diabetic kidney disease · Tea polyphenols · Mesangial cell senescence · miR-126 · Akt-p53-p21 signaling pathway

## Introduction

Diabetic kidney disease (DKD), a common microvascular complication of type 2 diabetes, has become the leading cause of chronic kidney disease as a result of the increase in the incidence of type 2 diabetes mellitus in developed countries. DKD is characterized by the senescence of kidney and renal cells. In particular, hyperglycemia has been demonstrated to be associated with renal cell senescence in DKD [1, 2]. Type 2 diabetic nephropathy is associated with an accelerated senescence phenotype in defined renal cell types, which mainly include tubule cells and mesangial cells [3]. Similar senescence patterns have been observed in proximal tubule cells cultured in high-glucose medium [3]. Therefore, strategies that target renal cell senescence might be highly useful for the treatment of DKD.

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Telomeres are nucleoprotein caps that flank DNA. Shortening of telomeres is associated with cellular senescence. As an indicator of oxidative stress and senescence, telomere length is hypothesized to be a biomarker of aging [4]. In our previous study, we found that patients with DKD had shorter telomere length than normal individuals. Therefore, telomere length may be a useful biomarker of cellular senescence in renal cells under hyperglycemic conditions. It has been reported that the development of cell senescence is regulated by several signal pathways, including the p16/MAPKAPK-2/Rb and DDR-mediated p53/p21/Rb signaling pathways. Recently, the Akt-p53-p21-Rb pathway has been receiving increased attention for its role in cell senescence [5]. Specifically, the Akt-p53-p21-Rb pathway activates the p53-p21-Rb signaling pathway to induce replicative senescence. Therefore, apart from telomere length, the other known biomarkers of cell senescence are p53 (tumor suppressor gene), the cell-cycle kinase dependent inhibitors p16 and p21, cell cycle regulator (retinoblastoma protein, Rb), and p38. In addition, senescence-associated β-galactosidase (SA-β-Gal) was the biomarker of senescence to be discovered. Recent research also found that miR-126 was associated with renal injury and endothelial senescence; this means that it could be a potential biomarker of renal cell senescence in patients with DKD [6].

Tea, which has been consumed as a beverage for well over 2000 years, has been found to contain substances called polyphenols, which have a protective effect on DNA [7]. In particular, it has been shown that green tea can contribute to increasing the lifespan of Drosophila by the regulation of mitoferrin and reduction of mitochondrial iron [8–11]. Given these protective effects of tea polyphenols (TPs) on aging, tea polyphenols could serve as a novel strategy for the treatment of DKD. However, the role and possible mechanisms of TPs in renal cell senescence is still poorly understood, especially with regard to the senescence of human glomerular mesangial cells (HGMCs) induced by high glucose. Therefore, in the present study, we cultured HGMCs exposed to high glucose levels with TPs, in order to explore the possible benefits of TPs in delaying high glucose-induced renal cell senescence and the underlying mechanisms.

## Materials and methods

### Reagents

TP was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (polyphenol content, ≥ 98%) and stored in a − 20 °C freezer. Before the experiment, TP was diluted with the culture solution to the required concentration. Roswell Park Memorial Institute (RPMI-1640) culture fluid was purchased from GIBCO; fetal bovine serum (FBS) was purchased from HyClone; CCK-8 (Cell Counting Kit-8) was purchased from Biyuntian Institute of Biotechnology. *Hin*fI restriction enzyme was from Sigma; λDNA/*Hin*dIII, from GiBCO; mouse anti-human p21 antibody, rabbit anti-human p53, Rb, and p-Akt antibody, from Santa Cruz Biotechnology; and mirVana miRNA isolation kit, TaqMan® MicroRNA Reverse Transcription kit, and TaqMan Univsersal PCR Master Mix II, from Thermo Fisher Scientific.

### Cell culture and treatment

HGMCs were purchased from Shanghai Institute of Cell Biology and cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin in a 5% $CO_2$ incubator at 37 °C. CCK-8 was used to determine the effect of different concentrations of TP on the proliferation of HGMCs. The cells were diluted in cell suspension to a concentration of $2 \times 10^5$ cells/mL. Approximately $2 \times 10^4$ cells per well (100 μL) were plated in 96-well plates, to which TP was added at concentrations of 0, 5, 10, 20, 40, 80, or 160 μg/mL. The cells were cultured for 72 h, after which 10 μL CCK-8 solution was added per well and the culture was continued for 2 h. Absorbance (*A*) was measured at 450 nm with an enzyme marker, and the mean value of each concentration group was calculated.

HGMCs were used in the experiments after three passages. The cells were divided into the normal group (NG, 5.5 mmol/L glucose), mannitol group (MNT, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), TP group (TP, 30 mmol/L glucose plus 5 μg/mL TP), and the high-dose glucose group (HG, 30 mmol/L glucose).

### SA-β-gal staining

Cells were washed twice with PBS, fixed at room temperature with 2% formaldehyde and 0.2% glutaraldehyde for 5 min, washed with PBS three times for 5 min each time, and fixed with freshly prepared SA-β-gal stain (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate pH 6.0 buffer, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$) by incubation at 37 °C (without $CO_2$) for 12–16 h. The stain started developing at 2–4 h, and the highest staining intensity was observed between 12 and 16 h. There were three samples in each group, and six fields of vision were randomly selected for each sample to calculate the staining intensity.

### Cell cycle analysis

The cells were washed with PBS, detached with 0.25% trypsin, and fixed with 75% ethanol overnight, then were resuspended in PBS and stained with propidium iodide in the dark for 30 min. The DNA contents were measured with

a fluorescence-activated cell sorting flow cytometry system (FACS II, Becton–Dickinson Biosciences).

### Telomere length

Southern blot analysis was used to detect the telomere length of mesangial cells in each group. Mesangial cells were grown to 80%, digested with trypsin and transferred to a 1.5-mL Eppendorf tube. DNA was extracted by the phenol–chloroform method, and UV spectrophotometer was used for quantitation and electrophoresis. For the electrophoresis analysis, 10 μg of DNA was digested with the restriction endonuclease *Hin*fI and electrophoresed. After electrophoresis was performed for 15 h at a voltage of 25 V, the gel was gently shaken in 0.25 M HCl for 10 min until the bromophenol blue was discolored, denatured and neutralized. The gel was then transferred to a membrane and cross-linked by UV via incubation with (TTAGGG) 5 oligonucleotide probe (100 μmol/L) and 1 μL of probe label in a water bath at 37 °C for 2 h, after which 2 μL of EDTA was added to terminate the reaction. The pre-hybridization solution was preheated at 42 °C, pre-hybridized for 2 h, labelled with human isotopes, hybridized for 24 h and washed. The membranes were then placed in X-ray cassettes and autoradiographed 48–72 h later. With the help of image line integration optical density scanning, telomere restriction fragment length (TRF) was calculated using the following formula: average TRF = $\sum$OD$i$/$\sum$ (OD$i$/L$i$), where OD$i$ is the absorbance value of point $i$ and L$i$ is the marker length of point $i$.

### Western blot analysis

Mesangial cells were lysed in protein lysis buffer, and the protein concentration was measured using a previously published method. Immunoblotting was performed with primary antibodies against p53 (1:500), p21 (1:1000), Rb (1:500), and p-Akt (1:1000). The blots were visualized with the Amersham ECL Detection System (Amersham, Buckinghamshire, UK). Densitometric analysis was performed using the Quantity One software (Bio-Rad).

### miR-126 expression in mesangial cells

Total miRNA was extracted from cells that had reached 80% confluence with the help of the Trizol kit. miRNA-specific stem-loop primers were reverse transcribed to cDNA according to the instructions of the miRNA reverse transcription kit. RNU6B was used as an internal reference. Blank controls were used to ensure that the PCR product was not contaminated. The miR-126 line was analyzed by real-time fluorescence quantitative PCR (qRT-PCR) using the Taqman probe method. The following PCR protocol was used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The results were calculated using the sequence detection software of Thermo Fisher Scientific. The $2^{-\Delta\Delta C_t}$ method was used to determine the relative expression of miR-126 with RNU6B as the standard. The above experiments were performed in triplicate.

### Statistical analysis

All results are presented as the mean ± SE. Data between groups were compared using one-way analysis of variance, and data among more than two groups were compared by the Kruskal–Wallis test. SPSS 16.0 was used for all statistical analyses. A value of $P < 0.05$ was considered to indicate statistical significance.

## Results

### Effect of TP on HGMC proliferation

We cultured HGMCs with either the vehicle or different doses of TP (0, 5, 10, 20, 40, 80, and 160 μg/mL) for 72 h after high glucose stimulation. As shown in Fig. 1, the CCK-8 assay showed that TP doses of 5–20 μg/mL had almost no effect on HGMC proliferation, while high doses of TP (40–80 μg/mL) inhibited the activation of HGMC proliferation induced by high glucose. Based on these findings, we chose the 5 μg/mL dose to observe the protective effect of TPs on high glucose-induced HGMCs and to elucidate the underlying mechanism.

### Effect of TP on high glucose-induced senescence in HGMCs

In the HG group, the cell body of HGMCs was enlarged and the amount of cytoplasmic particles was increased in comparison with the NG group. Further, the HG group had

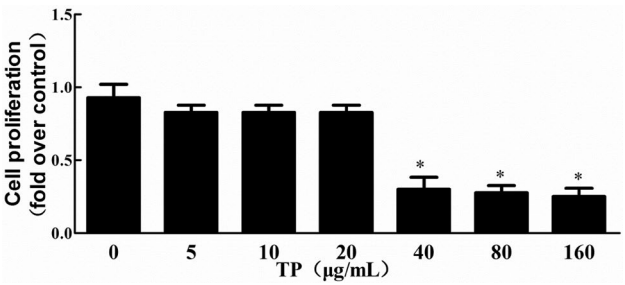


**Fig. 1** Effects of TP on the activation of HGMC proliferation. HGMCs were grown on six-well plates until 80% confluence and then treated with different dose of TP (0, 5, 10, 20, 40, 80, 160 μg/mL). CCK-8 was used to determine the effect of different concentrations of TP on the proliferation of HGMCs. The results are presented as means ± SE ($n = 6$). *$P < 0.05$ vs. controls (0 dose of TP)

a significantly higher percentage of SA-β-Gal-positive cells than the NG group ($P < 0.05$). However, the percentage of SA-β-Gal-positive cells was not significantly different between the MNT and NG group ($P > 0.05$, Fig. 2).

There was no difference in G1 phase ratio between NG and MNT group ($P > 0.05$). Cell cycle arrest was observed in G1 phase in the HG group (Fig. 3). Similarly, telomere length was not significantly different between the NG and

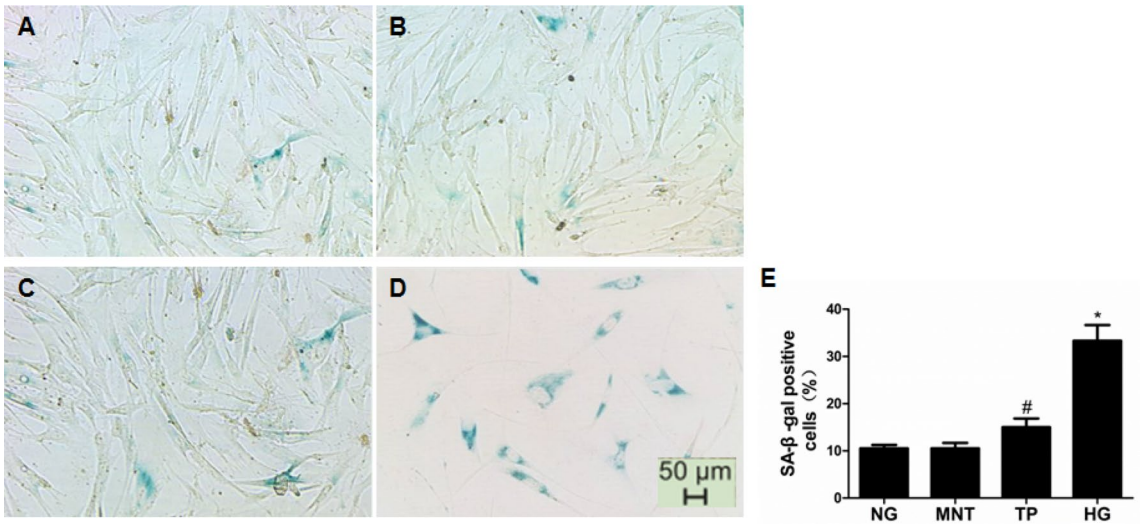


**Fig. 2** Effects of TP on cellular morphology and SA-β-gal staining of HGMCs exposed to high glucose. HGMCs were grown on six-well plates until 80% confluence and then treated with glucose or TP. **a** Normal group (NG, 5.5 mmol/L glucose), **b** mannitol group (MNT, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), **c** TP group (TP, 30 mmol/L glucose plus 5 μg/mL TP), and **d** the high-dose glucose group (HG, 30 mmol/L glucose). After 24 h, SA-β-gal stain was performed with treated cells. **e** The quantification data for SA-β-gal staining. The results are presented as means ± SE ($n = 6$). *$P < 0.05$ vs. NG. #$P < 0.05$ vs HG group

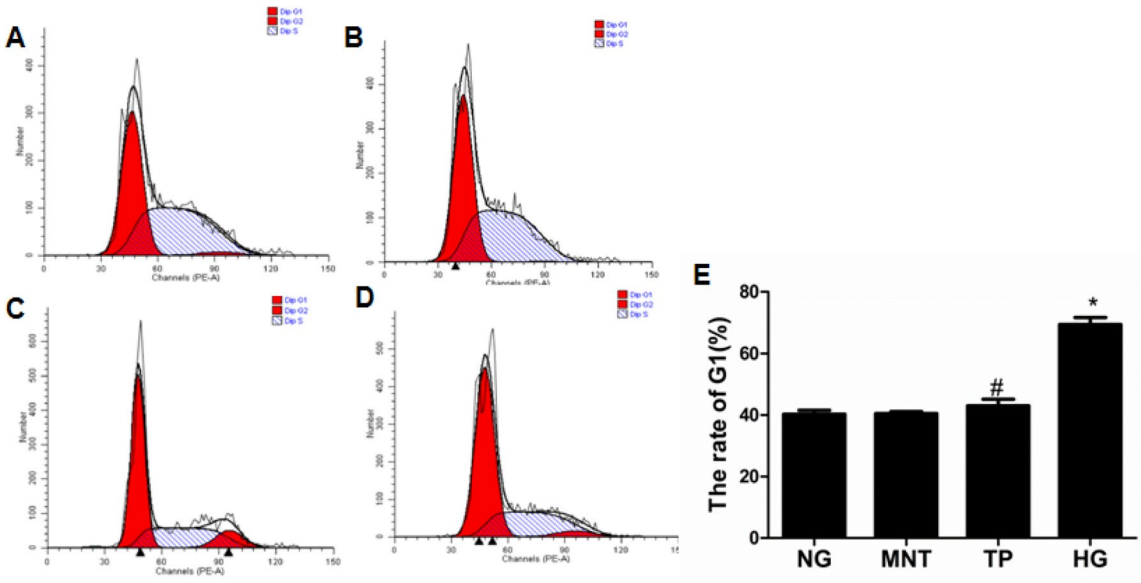


**Fig. 3** Effects of TP on the cell cycle in HGMCs exposed to high glucose. HGMCs were grown on 6-well plates until 80% confluence and then treated with glucose or TP. NG (normal group, 5.5 mmol/L glucose), MNT (mannitol group, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), TP (TP group, 30 mmol/L glucose plus 5 μg/mL TP), and HG (the high-dose glucose group, 30 mmol/L glucose). Flow cytometry was used to detect the cell cycles of mesangial cells in each group. **a** Representative the cell cycle of NG group by flow cytometry. **b** Representative the cell cycle of MNT group by flow cytometry. **c** Representative the cell cycle of TP group by flow cytometry. **d** Representative the cell cycle of HG group by flow cytometry. **e** The quantification data analysis. The results are presented as means ± SE ($n = 6$). *$P < 0.05$ vs. NG. #$P < 0.05$ vs HG group

MNT group ($P > 0.05$). In contrast, the telomere length of HGMCs in the HG group was significantly shorter than that in the NG group ($P < 0.05$) (Fig. 4).

With regard to the effect of TP on high glucose-induced cell senescence, we found that the percentage of SA-β-Gal-positive cells was significantly lower in the TP group than in the HG group ($P < 0.05$, Fig. 2). The rate of G1 phase was lower in the TP group than in the HG group ($P < 0.05$, Fig. 3). Further, the telomere length of HGMCs in the TP group was also significantly longer than that in the HG group ($P < 0.05$, Fig. 4). Thus, TP treatment was associated with lower rate of G1 phase,longer telomere length as well as lower SA-β-Gal staining intensity.

## Effect of TP on activation of the Akt-p53-p21 signaling pathway in HGMCs exposed to high glucose

According to the results of western blot analysis, the expression of p53, p21, and Rb in HGMCs was significantly higher in the HG group than in the NG group ($P < 0.05$, Fig. 5). In contrast, p-Akt expression was significantly lower in the HG group ($P < 0.05$).

With regard to the effect of TP, the expression of p-Akt was significantly higher and the expression of p53, p21 and Rb was significantly lower in the TP group than in the HG group ($P < 0.05$, Fig. 5). These findings indicate that the mechanism via which TP affects senescence of HGMCs involves the Akt-p53-p21 signaling pathway.

## Effect of TP on the expression of miR-126 in HGMCs exposed to high glucose

MiR-126 expression in the HG group was significantly lower than that in the NG group ($P < 0.05$, Fig. 6). In the TP group, the expression of miR-126 was significantly higher than that in the HG group ($P < 0.05$). These results indicate that TP can restore the low expression level of miR-126 in HGMCs induced by high glucose.

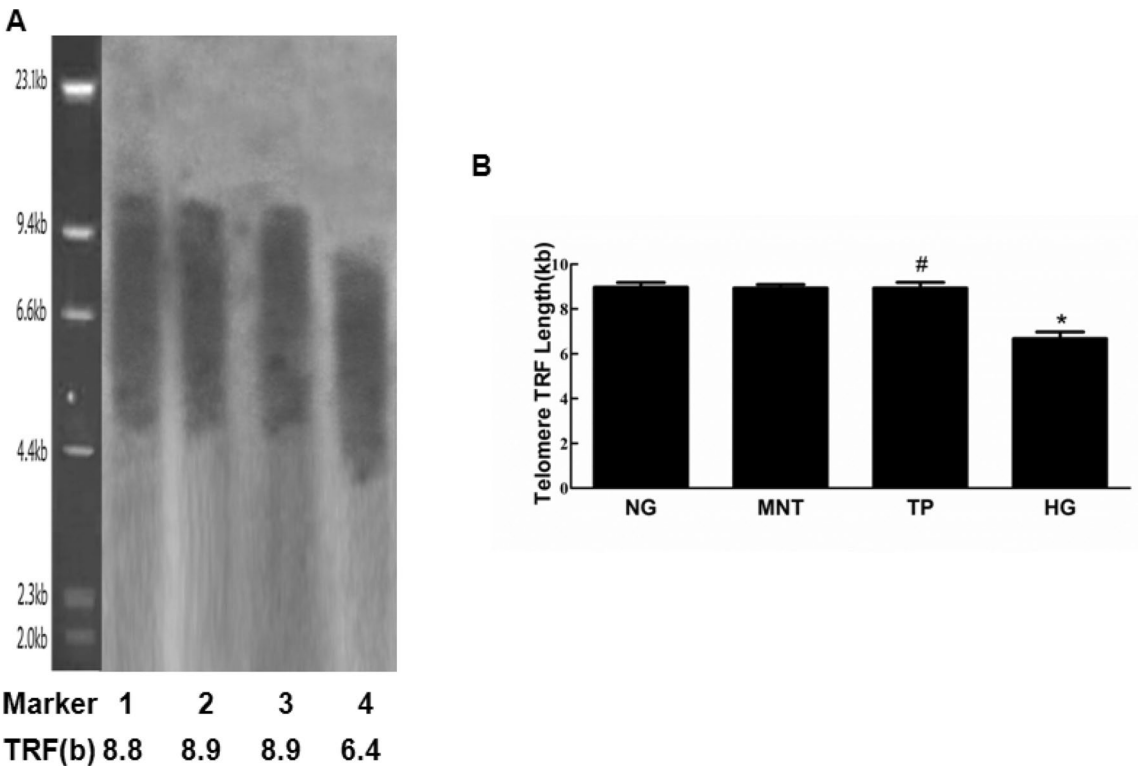


**Fig. 4** Effects of TP on telomere length in HGMCs exposed to high glucose. HGMCs were grown on six-well plates until 80% confluence and then treated with glucose or TP. NG (normal group, 5.5 mmol/L glucose), MNT (mannitol group, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), TP (TP group, 30 mmol/L glucose plus 5 μg/mL TP), and HG (the high-dose glucose group, 30 mmol/L glucose). Southern blot analysis was used to detect the telomere length of mesangial cells in each group. **a** Representative immunoblots of telomere length by Southern blotting analysis. **b** Densitometric analysis. The results are presented as means ± SE ($n = 6$). *$P < 0.05$ vs. NG. #$P < 0.05$ vs HG group

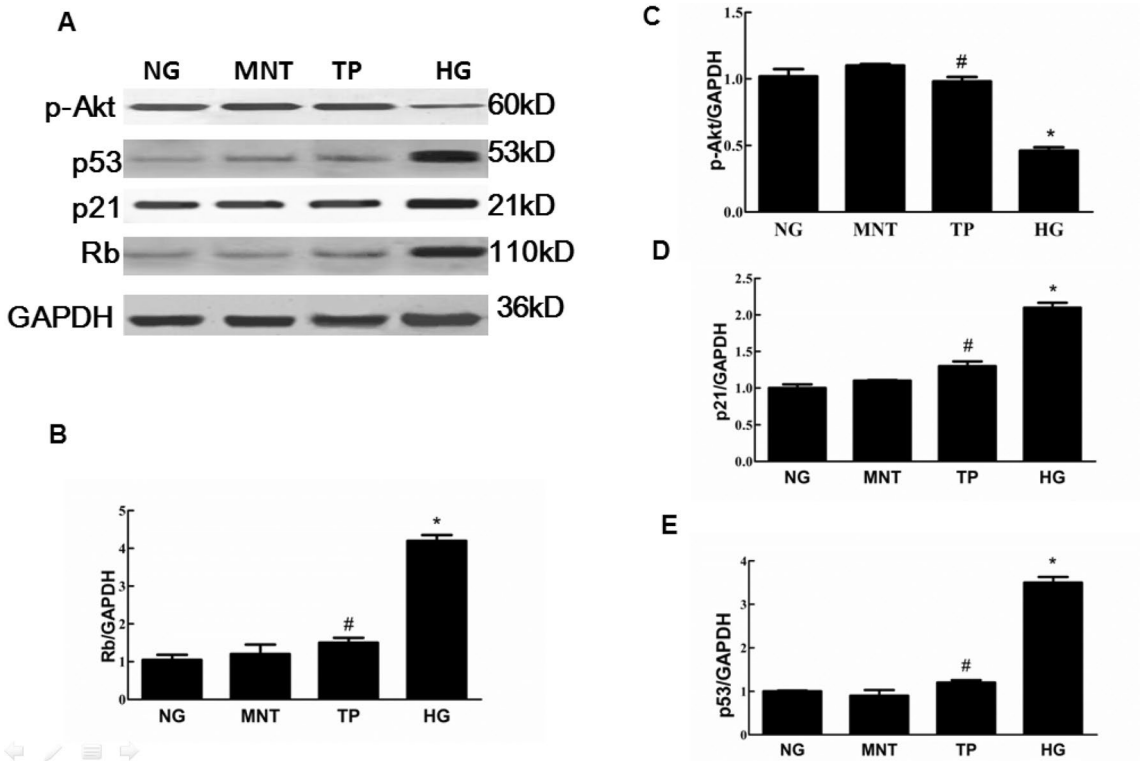


**Fig. 5** Effects of TP on the activation of Akt-p53-p21 signaling pathway in HGMCs exposed to HG. HGMCs were grown on six-well plates until 80% confluence and then treated with glucose or TP. NG (normal group, 5.5 mmol/L glucose), MNT (mannitol group, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), TP (TP group, 30 mmol/L glucose plus 5 μg/mL TP), and HG (the high-dose glucose group, 30 mmol/L glucose). **a** Western blotting analysis for p-Akt, P53, P21, Rb expression. qRT-PCR analysis for Rb (**b**), p-Akt (**c**), P21 (**d**), P53 (**e**). The results are presented as means ± SE ($n = 6$). $^{*}P < 0.05$ vs. NG. $^{\#}P < 0.05$ vs HG group

## Discussion

In the present study, we showed that tea polyphenols had a protective effect against high glucose-induced proliferation and cellular senescence in HGMCs. In particular, TP resulted in a decrease in the proportion of SA-β-Gal-positive cells and restored telomere length in high glucose-treated HGMCs, in comparison to cultured HGMCs that were exposed to high glucose levels and not treated with TP. With regard to the underlying mechanism, TP was found to inhibit the activity of the Akt-p53-p21 signaling pathway, as well as restore the expression of miR-126 in HGMCs.

In our study, we found high glucose can promote HGMCs senescence. Maeda M showed that constant high glucose increased senescence-associated-β-galactosidase (SA-β-gal) activity and activated telomerase and shortened telomere length, which suggested replicative senescence [12]. Gurung demonstrated that short leukocyte telomere length predicts albuminuria progression in DKD [13]. With regard to the underlying mechanism, it has been proposed that oxidative stress, high sugar levels and other factors lead to shortening of telomere length, which induces an increase in p53 expression. This in turn results in an increase in the expression of the downstream p21 gene, which inhibits the activity of the corresponding protein kinase and inhibits Rb phosphorylation.

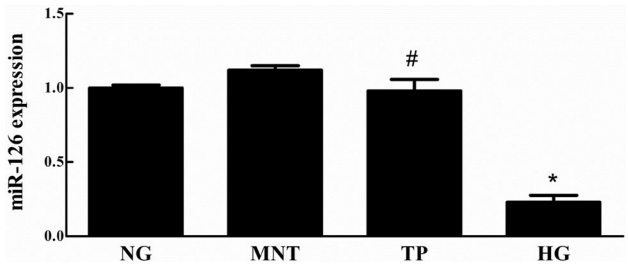


**Fig. 6** Effects of TP on the expression of miR-126 in HGMCs exposed to high glucose. HGMCs were grown on six-well plates until 80% confluence and then treated with glucose or TP. NG (normal group, 5.5 mmol/L glucose), MNT (mannitol group, 5.5 mmol/L glucose plus 24.5 mmol/L mannitol), TP (TP group, 30 mmol/L glucose plus 5 μg/mL TP), and HG (the high-dose glucose group, 30 mmol/L glucose). The miR-126 level was analyzed by real-time fluorescence quantitative PCR (qRT-PCR) using the Taqman probe method. The results are presented as means ± SE ($n = 6$). $^{*}P < 0.05$ vs. NG. $^{\#}P < 0.05$ vs HG group

Tea polyphenols (TP) is one of the most used antioxidants and exerts beneficial effect on aging diseases. In recent years, the anti-aging effect of TP has been recognized, and its anti-aging role is gradually attracting attention [7, 14–16]. The studies also demonstrated TP has anti-cancer effect in multiple cancer types [17, 18]. Accumulating evidence suggests that tea polyphenols confer protective effects on cell senescence. Onishi found TP prevented high-fat diet-induced muscle weight loss in senescence-accelerated mouse [19]. Consistent with previous reports, our study also found that TP can delay the senescence of glomerular mesangial cells induced by high glucose, mainly by inhibiting the activity of Akt-p53-p21 pathway.

MiR-126, which is detected in glomerular and peritoneal mesothelial cells, is considered as a biomarker of DKD [20]. In particular, the GHADA study [6] has shown that peripheral blood miR-126 is decreased in patients with type 2 diabetes and DKD. In our study, we detected a decrease in miR-126 that was associated with senescence of mesangial cells induced by high glucose; this implies that miR-126 is involved in the process of senescence of glomerular mesangial cells induced by high glucose levels. MiR-126 may play a key role in the pathogenesis of diabetic nephropathy, but the specific mechanism remains to be elucidated. Further, TP inhibited the decrease in miR-126 induced by high glucose, suggesting that the protective effect of TP on the renal endothelium and glomeruli may be via its effect on the expression of miR-126.

In summary, the polyphenols present in tea may have a beneficial effect on senescence in HGMCs induced by exposure to high glucose by protecting telomeres against shortening and restoring miR-126 expression. Further, the mechanism by which the polyphenols bring about these effects is via the Akt-p53-p21 signaling pathway. Further exploration into the underlying mechanisms of these polyphenols could help identify therapeutic targets for the treatment of DKD.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interests regarding the publication of this paper.

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