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The role of STAT3/mTOR-regulated autophagy in angiotensin II-induced senescence of human glomerular mesangial cells

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ABSTRACT

The kidney is one of the fastest-aging organs, and renal senescence has become a major disease affecting human health. Renal cellular senescence is regulated by the joint action of multiple signal transduction pathways. The previous study by our research group found that the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway was involved in angiotensin II (Ang II)-induced senescence of human glomerular mesangial cells. However, the unique role of STAT3 activation in Ang II-induced senescence of human glomerular mesangial cells and the underlying mechanisms remain unclear. The present study revealed that Ang II induced premature senescence, promoted autophagy and activated oxidative stress responses in human glomerular mesangial cells. Autophagy mediates the senescence-inducing effect of Ang II on human glomerular mesangial cells. Inhibition of oxidative stress with *N*-acetylcysteine (NAC) or interference with STAT3/mechanistic target of rapamycin (mTOR) activity with S3I-201 or STAT3-siRNA suppressed autophagy to a certain extent, which was conducive to delaying the senescence of glomerular mesangial cells. The antioxidant probucol reduced autophagy in human glomerular mesangial cells and alleviated the aging process of these cells by regulating STAT3/mTOR. These findings identify a role of STAT3/mTOR-regulated autophagy in Ang II-induced senescence of human glomerular mesangial cells and may provide a theoretical basis for anti-senescence treatment in clinical practice.

1. Introduction

Aging refers to the systemic manifestation of age-related body function decline and physiological dysfunction [1]. The kidney is one of the fastest-aging organs, and renal aging has an especially important effect on human health [2]. In particular, the incidence of chronic kidney disease (CKD) among the elderly population has been increasing annually. The U.S. Renal Data System 2015 Annual Data Report showed that patients with stage 1–4 CKD constituted approximately 47.7% of the elderly population [3]. Thus, renal aging has become a major disease affecting national health.

Cell senescence refers to the decline in the physiological functions of cells, including a decreased cell proliferative capability, irreversible cell cycle arrest, reduced sensitivity to stressors and the accumulation of senescence-related genes and proteins [4]. Cell senescence is induced once various biochemical events in the microenvironment (i.e. telomere shortening, DNA damage response, oxidative stress and senescence-associated secretion phenotype (SASP)) reach a certain threshold [4]. This effect can be amplified from the cellular level to the tissue level, ultimately resulting in organ aging [5]. Therefore, cell senescence is an important breakthrough point for studies of renal aging. The kidney has a high local concentration of angiotensin II (Ang II), which plays an important role in renal pathophysiological changes [6]. The mitogenic effect of Ang II on intrinsic renal cells activates the cyclin-dependent kinase inhibitors, resulting in cell cycle arrest and cell senescence [7]. The previous study by our research group found that the Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT) pathway was involved in Ang II-induced senescence of human glomerular mesangial cells [8–10]. However, the unique role of STAT3 activation in Ang II-induced senescence of human glomerular mesangial cells and the underlying mechanisms remain unclear. The present study found that during the senescence process, the STAT3/mechanistic target of rapamycin (mTOR) activity activity and autophagy in human glomerular mesangial cells was altered. Autophagy is an important senescence-related mechanism that has been identified recently. The effect of autophagy on cell senescence varies in different environments. The present study showed that Ang II induced premature senescence in human glomerular mesangial cells and promoted autophagy [11]. Inhibition of oxidative stress and interference with STAT3/mTOR activity suppressed the autophagy level to a certain extent, which was

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conducive to delaying cell senescence. The antioxidant probucol reduced autophagy in human glomerular mesangial cells and alleviated the aging process of human glomerular mesangial cells by regulating STAT3/mTOR.

2. Materials and methods

2.1. Reagents and antibodies

Ang II (#ab120183) was purchased from Abcam (Cambridge, UK). 3-Methyladenine (3MA, #S2767), rapamycin (RAP, #S1039) and S3I-201 (#S1155) were purchased from Selleck Chemicals (Houston, TX, USA). *N*-acetylcysteine (NAC, #S0077) was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). The rabbit anti-LC3B (#7543), anti-p62/sequestosome-1 (SQSTM1) (#0067) and anti-p21 (#SAB4500065) antibodies were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The rabbit anti-STAT3 (#4904), anti-phospho-STAT3 (p-STAT3) (#9145), anti-p53 (#2524), anti-mTOR (#2983) and anti-phospho-mTOR (p-mTOR) (#5536) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The β-Galactosidase Staining Kit and the Reactive Oxygen Species Detection Assay Kit (#C0602) were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Small interfering RNAs (siRNAs) were purchased from GenePharma (Suzhou, China). Lipofectamine 2000 (#11668019) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). The mRFP-GFP-LC3 adenovirus (#AP2100001) was purchased from Han Heng Biotechnology (Shanghai, China).

2.2. Culture of human glomerular mesangial cells and establishment of an senescence model

Human glomerular mesangial cells (#4200) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were cultured in specific mesangial culture medium containing 2% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37 °C in a 5% CO_2 incubator. Once 80% confluence was reached, the cells were harvested using the standard trypsin digestion procedure and passaged at a split ratio of 1:2. The senescence model was established by treatment of the human glomerular mesangial cells with 10^{-6} mol/L Ang II for 72 h.

2.3. Beta-galactosidase staining

The cells were cultured in 6-well plates. After aspiration of the culture medium, the cells were washed once with phosphate-buffered saline (PBS) and fixed in 1 mL of fixative solution (which was included in the β-Galactosidase Staining Kit) at room temperature for 15 min. The cell fixative solution was removed, and the cells were washed 3 times with PBS for 3 min per wash. Subsequently, 1 mL of cell staining working solution (prepared by mixing 10 μL of β-galactosidase staining solution A, 10 μL of β-galactosidase staining solution B, 930 μL of β-galactosidase staining solution C and 50 μL of X-Gal solution together) was added to each well. After incubation overnight at 37 °C in the absence of CO_2, the cells were observed under an optical microscope. The senescent cells displayed a bluish-green color.

2.4. Transmission electron microscopy

The cells were fixed overnight with 2.5% glutaraldehyde and then post-fixed with 1% osmium tetroxide. After washing with PBS, the cells were dehydrated in gradient ethanol and acetone. Subsequently, the cells were subjected to epoxy resin infiltration and embedment. The embedded cells were sectioned and examined by transmission electron microscopy. During transmission electron microscopy study, 10 random fields were captured in a grid and the numbers of autophagic vacuoles and cells were counted per field.

2.5. Infection of the cells with the mRFP-GFP-LC3 adenovirus

The cells were seeded into 24-well plates at a density of 4×10^4 cells per well. Subsequently, the virus was added to the cells at a multiplicity of infection (MOI) of 200. At 6 h after addition of the virus, the virus-containing medium was replaced with fresh medium. Seventy-two hours later, the cells were washed with PBS and fixed with 4% paraformaldehyde. The viral infection results were examined using confocal microscopy using a GFP and RFP filter to detect autophagosomes (yellow puncta) and autolysosomes (red puncta).

2.6. Western blotting analysis

The sodium dodecyl sulfate (SDS)-polyacrylamide gel used in the western blotting analysis was composed of a 12% separating gel and a 5% stacking gel. Approximately 40–80 μg of proteins was loaded onto the gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk, incubated with the primary antibodies overnight and then incubated with the corresponding secondary antibodies for 2 h. The target proteins were visualized using Millipore Immobilon Western Chemiluminescent HRP Substrate (ECL) Kit (#WBKLS0100), and the images were captured using the MicroChemi 4.2 automatic gel imaging and analysis system.

2.7. Transfection

Prior to transfection, the cells were cultured in serum-containing antibiotic-free medium. Once the cells reached 50% confluence, the serum-containing medium was replaced with serum- and antibiotic-free culture medium. To transfect cells cultured in 6-well plates, 5 μL of Lipofectamine 2000 was diluted in 500 μL of serum- and antibiotic-free medium and incubated at RT for 5 min. Subsequently, 5 μL of siRNA (20 μmol/L) was dissolved in 500 μL of serum- and antibiotic-free medium. The diluted Lipofectamine 2000 was mixed thoroughly with the siRNA. The mixture was incubated at RT for 20 min, which allowed the formation of the siRNA-Lipofectamine 2000 complex. The siRNA-Lipofectamine 2000 complex was added slowly to the cells in a dropwise manner. The plates were shaken gently to ensure complete mixing. The cells were placed at 37 °C in a 5% CO_2 incubator. After 4 h of incubation, the culture supernatant was replaced with serum-containing culture medium, and the cells continued to be cultured. The STAT3-siRNA sequence was as follows: sense strand, 5′-GGG ACC UGG UGU GAA UUA UTT-3′, and antisense strand, 5′-AUA AUU CAC ACC AGG UCC CTT-3′. The sequence of the Con-siRNA (negative control) was as follows: sense strand, 5′-UUC UCC GAA CGU GUC ACG UTT-3′, and antisense strand, 5′-ACG UGA CAC GUU CGG AGA ATT-3′.

2.8. Detection of reactive oxygen species (ROS)

After removal of the culture medium, the cells were overlaid with 2′, 7′-dichlorodihydrofluorescein diacetate solution (DCFH-DA, 10 μmol/L) and incubated in a 37 °C incubator for 60 min. Subsequently, the cells were washed 3 times with serum-free medium. The cellular fluorescence intensity was determined by flow cytometry.

2.9. Statistical methods

The measurement data are expressed as the mean ± S.E.M. The data were analyzed with one-way analysis of variance (ANOVA) using the SPSS 19.0 statistical software. A p value $< .05$ indicates that the difference is significant.

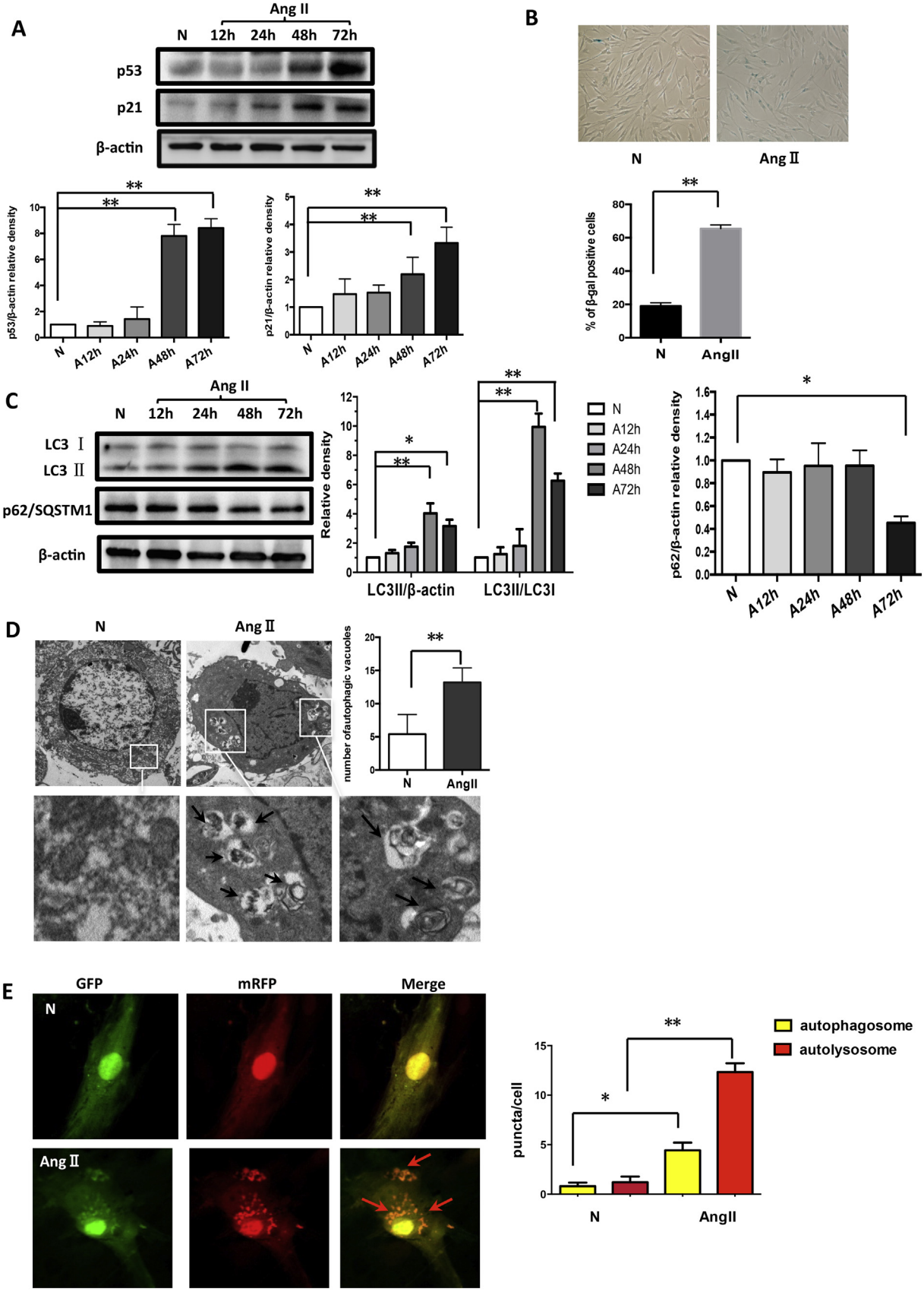

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Fig. 1. Angiotensin II (Ang II) induces the senescence of human glomerular mesangial cells and promotes autophagy.
(A) Western blot analysis of the senescence-related proteins p53 and p21 after treatment of the human glomerular mesangial cells with 10^{-6} mmol/L Ang II for 0–72 h.
(B) Senescence-associated β-galactosidase staining of cells that had been treated with 10 − 6 mmol/L Ang II for 72 h.
(C) Western blot analysis of the autophagy-related proteins LC3 II and p62/SQSTM1 after treatment of the human glomerular mesangial cells with 10 − 6 mmol/L Ang II for 0–72 h.
(D) Transmission electron microscopy showed autophagic vacuoles in cells that had been treated with 10 − 6 mmol/L Ang II for 72 h. Autophagic vacuoles are indicated by arrows. Bar = 2 μm.
(E) Images of fluorescent LC3 puncta were shown in cells that had been treated with 10–6 mmol/L Ang II for 72 h. Number of autophagosomes represented by yellow puncta and autolysosomes represented by red puncta in merged images.
The data are presented as the mean ± S.E.M. from at least three independent experiments. Statistical significance is indicated at *P < .05 **P < .01.

3. Results

3.1. Ang II induces the senescence of human glomerular mesangial cells and promotes autophagy

As shown in Fig. 1 A, compared with the normal control group, the western blotting analysis showed that the expression levels of the senescence-related proteins p53 and p21 rose gradually as the duration of the 10^{-6} mmol/L Ang II treatment increased. The rate of positive β-galactosidase staining reached 65.43 ± 1.31% after treatment of Ang II for 72 h, which was significantly higher than the rate obtained in the normal control group (19.00 ± 1.15%, Fig. 1B, $p < .01$). The human glomerular mesangial cells displayed a senescent phenotype after exposure to Ang II for 72 h.

In addition to inducing senescence, Ang II affected autophagy in the human glomerular mesangial cells. The expression of the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3)-phosphatidylethanolamine conjugate (LC3II) was increased in the Ang II-stimulated cells in a time-dependent manner. LC3II expression reached a peak value after treatment with Ang II for 48 h. LC3II expression was slightly decreased at 72 h but remained significantly higher than the expression in the normal control group. Moreover, compared with the normal control group, the Ang II-stimulated cells showed a significantly elevated LC3II/LC3I ratio at 48–72 h (Fig. 1C). Since the LC3-II protein expression level only reflected the number of autophagosomes, we examined the autophagic degradation of the p62/SQSTM1 substrate, which fully reflected the autophagic activity. The p62 expression level gradually decreased as the duration of Ang II stimulation increased. P62 expression began to decrease markedly at 48 h and reached its minimum level at 72 h (Fig. 1C). Additionally, transmission electron microscopy showed that the number of autophagic vacuoles was significantly increased in the Ang II-stimulated cells compared to the normal control group (13.2 ± 0.97 vs. 5.4 ± 1.33, $p < .01$, Fig. 1D). To dynamically observe the changes in the autophagic flux, human glomerular mesangial cells were infected with the mRFP-GFP-LC3 adenovirus. The yellow dots represent autophagosomes, and the red dots represent autolysosomes. The numbers of yellow and red dots were drastically increased under Ang II stimulation compared with the control group (Fig. 1E). The finding indicates that Ang II promotes autophagic activity in human glomerular mesangial cells.

3.2. Autophagy mediates the senescence-inducing effect of Ang II on human glomerular mesangial cells

To investigate the effect of autophagy on the Ang II-induced senescence of human glomerular mesangial cells, the autophagy inhibitor 3MA and autophagy inducer RAP were employed to treat senescent human glomerular mesangial cells. The cells were pretreated with 5 mmol/L 3MA or 500nmmol/L RAP for 1 h and then subjected to 10^{-6} mmol/L Ang II stimulation. Compared with the group exposed to Ang II alone, 3MA pretreatment markedly reduced Ang II-induced LC3II expression, decreased the LC3II/LC3I ratio, promoted p62 protein expression and reduced p53 and p21 protein expression (Fig. 2A). 3MA pretreatment reduced the rate of positive β-galactosidase staining and ameliorated the senescence induced by Ang II (34.95 ± 5.30 vs. 68.52 ± 3.04, $p < .01$, Fig. 2C). Additionally, 3MA significantly inhibited Ang II-induced autophagic vacuoles formation (4.83 ± 0.31 vs. 13.17 ± 1.80, p < .01, Fig. 2D) and both yellow and red dots of GFP-RFP-LC3 in cells (Fig. 2E). RAP further promoted Ang II-induced LC3II expression, increased the LC3II/LC3I ratio, but had no significant effect on p62 (Fig. 2B). However, RAP did not affect p53 and p21 protein expression and β-galactosidase staining in the human glomerular mesangial cells stimulated with Ang II (68.25 ± 3.45 vs. 68.52 ± 3.04, $p > .05$, Fig. 2B–C). RAP treatment in combination with Ang II increased the number of autophagic vacuoles and red dots compared with Ang II alone (Fig. 2D–E). The findings indicated that inhibition of autophagy by 3MA alleviated cell senescence in human glomerular mesangial cells exposed to Ang II stimulation.

3.3. The STAT3/mTOR pathway mediates the regulatory effect of Ang II on the autophagy and senescence of human glomerular mesangial cells

Our previous studies had shown that STAT3 was activated during Ang II -induced human glomerular mesangial cell senescence [8–10], so we further explored the regulatory role and mechanism of STAT3 on senescence. We treated human glomerular mesangial cells with the STAT3-specific inhibitor S3I-201 and the STAT3-siRNA to inhibit STAT3 at the drug and gene levels, respectively, and then examined the effect of STAT3 inhibition on autophagy and senescence. S3I-201 selectively inhibits the DNA binding activity of STAT3 and blocks the activation of STAT3 at a specific tyrosine site, thereby inhibiting STAT3-dependent transcriptional activity. Pretreatment with S3I-201 (50 μmol/L) and STAT3-siRNA treatment resulted in significantly reduced Ang II-induced LC3II expression and LC3II/LC3I ratio, drastically increased p62 protein expression and decreased expression of the senescence-related proteins p53 and p21 compared with the Ang II group (Fig. 3A, B). Compared with the group exposed to Ang II alone, both S3I-201 and STAT3-siRNA treatment reduced the positive rate of β-galactosidase staining in human glomerular mesangial cells exposed to Ang II stimulation respectively (50.26 ± 7.24% vs. 68.52 ± 3.04%, $p < .05$, and 53.65 ± 3.90% vs. 68.52 ± 3.04%, p < .05, Fig. 3C). Additionally, both pretreatment with S3I-201 and STAT3-siRNA attenuated the Ang II-induced increase in the number of autophagic vacuoles (4.80 ± 0.80 vs 13.17 ± 1.80, $p < .01$, and 5.40 ± 0.51 vs 13.17 ± 1.80 p < .01, Fig. 3D), increased the amount of yellow dots and reduced red dots (Fig. 3E). The results indicated that inhibition of STAT3 activity with S3I-201 and silencing of STAT3 expression with STAT3-siRNA attenuated the Ang II-induced autophagy activation and reduced cell senescence.

Western blotting showed that the mTOR pathway activity gradually decreased with the activation of the STAT3 pathway in the process of human glomerular mesangial cells senescence induced by Ang II. The p-mTOR activity was almost completely lost at 72 h (Fig. 4A). S3I-201 alone did not significantly reduce STAT3 and p-STAT3 expression. Pretreatment with S3I-201 and STAT3-siRNA markedly reduced Ang II-induced STAT3 and p-STAT3 expression (Fig. 4B, C). Inhibition of STAT3 activity with S3I-201 or silencing of STAT3 expression with

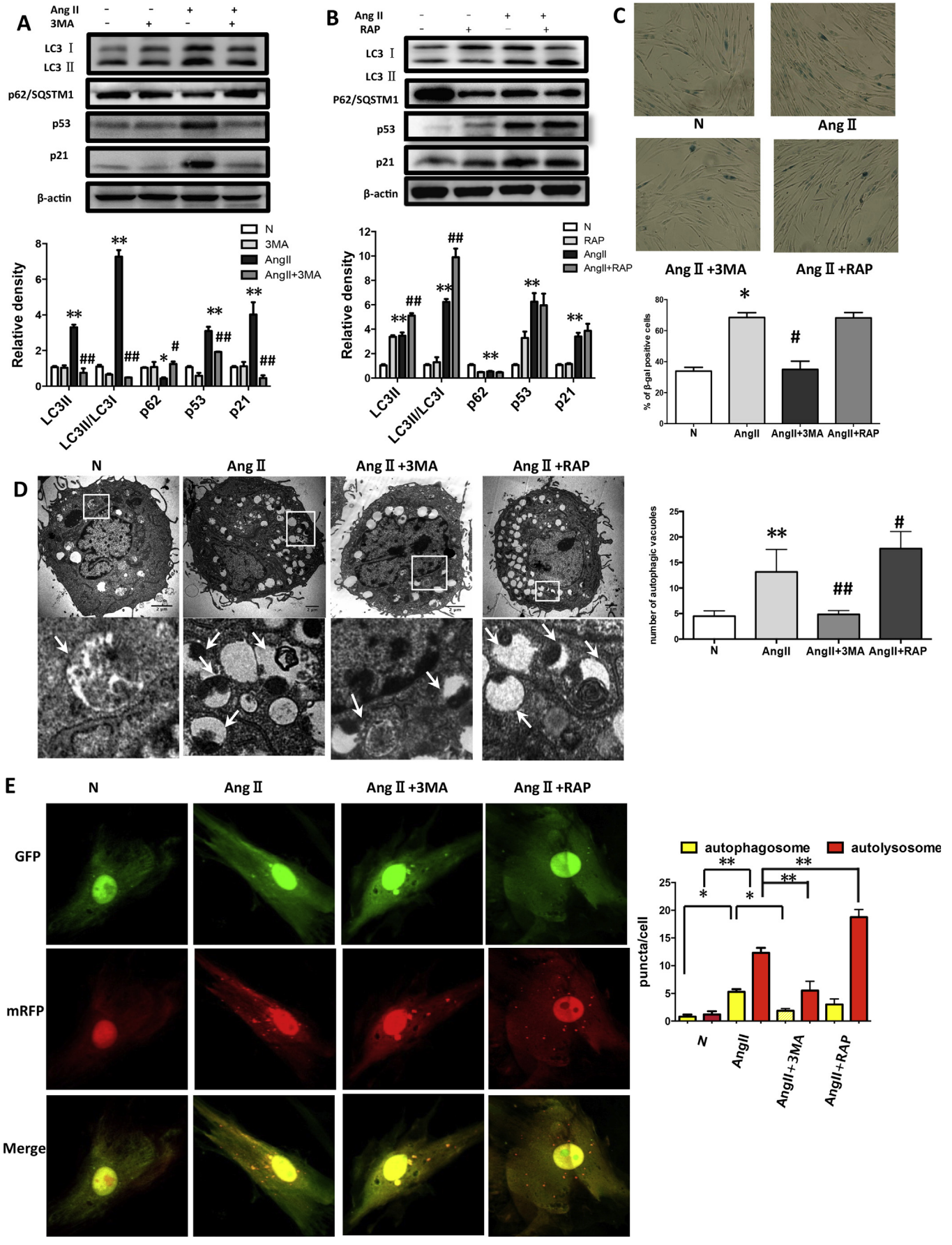

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Fig. 2. Autophagy mediates the senescence-inducing effect of Angiotensin II on human glomerular mesangial cells.
The cells were pretreated with 5 mmol/L 3-Methyladenine (3MA) or 500nmmol/L rapamycin (RAP) for 1 h and then subjected to $10-6$ mmol/L Ang II.
(A) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without 3MA treatment and incubated with Ang II for 72 h.
(B) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without RAP treatment and incubated with Ang II for 72 h.
(C) Senescence-associated β-galactosidase staining of cells that had been treated with 3MA or RAP for 1 h and then subjected to Ang II stimulation.
(D) Transmission electron microscopy showed autophagic vacuoles in cells that had been treated with 3MA or RAP for 1 h and then subjected to Ang II stimulation. Autophagic vacuoles are indicated by arrows. Bar = 2 μm.
(E) Images of fluorescent LC3 puncta were shown in cells that had been treated with 3MA or RAP for 1 h and then subjected to Ang II stimulation for 72 h. Number of autophagosomes represented by yellow puncta and autolysosomes represented by red puncta in merged images.
The data are presented as the mean ± S.E.M. from at least three independent experiments. Statistical significance is indicated at *P < .05 vs normal, **P < .01 vs normal, $^{\#}$P < .05 vs Ang II, $^{\#\#}$P < .01 vs Ang II.

STAT3-siRNA reversed the Ang II-imposed inhibition of the mTOR pathway in human glomerular mesangial cells (Fig. 4B, C). Therefore, inhibition of the mTOR pathway may represent one molecular mechanism by which STAT3 affects autophagy.

3.4. ROS are involved in the autophagy- and senescence-promoting activities of Ang II

Since oxidative stress is involved in the autophagy and senescence of a variety of cells under various circumstances, the effect of Ang II on autophagy and senescence may be related to its ability to induce oxidative stress. Therefore, we examined the intracellular production of ROS. Human glomerular mesangial cells were pretreated with the antioxidant NAC (10 mmol/L) and then exposed to 10^{-6} mmol/L Ang II stimulation. These cells served as the positive controls for the examination of the antioxidative effect. Compared with the normal control group, the intracellular ROS content was significantly increased after exposure to Ang II for 72 h ($p < .01$, Fig. 5A). Ang II stimulation activated the intracellular oxidative stress responses. NAC pretreatment significantly inhibited the intracellular production of ROS (p < .01, Fig. 5A), reduced the Ang II-induced increase in LC3II expression, decreased the LC3II/LC3I ratio and reduced p53 and p21 expression (Fig. 5B). NAC pretreatment also reduced the rate of positive β-galactosidase staining (41.21 ± 3.73% vs. 68.52 ± 3.04%, p < .01, Fig. 5C) and ameliorated the excessive autophagic vacuoles (5.17 ± 0.98 vs 13.17 ± 1.80, p < .01, Fig. 5D) and fluorescent red dots respectively, increased the amount of yellow dots (Fig. 5G). The results indicate that ROS are involved in the autophagy- and senescence-promoting activities of Ang II. Therefore, we applied the commonly used antioxidative drug probucol in the subsequent experiments in an attempt to explore the anti- senescent effect of probucol and its mechanism of action. Similar to NAC, probucol inhibited Ang II-induced STAT3 activation. The effects of NAC and probucol on STAT3 and its phosphorylation were shown in Fig. S1.

3.5. Probucol reduces Ang II-induced autophagy and senescence in human glomerular mesangial cells by alleviating oxidative stress and regulating STAT3/mTOR activity

Our previous study show that probucol attenuates the activation of STAT3 [12], therefore, we intend to investigate the effect of probucol on autophagy and senescence in human glomerular mesangial cells. Pretreatment with 40 μmol/L probucol for 1 h markedly reduced the intracellular ROS content (Fig. 5A), indicating that probucol successfully exerted its antioxidative effect. Probucol pretreatment also significantly decreased the rate of positive β-galactosidase staining in human glomerular mesangial cells exposed to Ang II (47.17 ± 3.07% vs. 68.52 ± 3.04%, $p < .01$, Fig. 5C) and reduced the number of autophagic vacuoles in the cells (6.00 ± 0.31 vs. 13.17 ± 1.80, p < .01, Fig. 5D) and fluorescent mRFP-LC3 puncta respectively (Fig. 5G). Additionally, probucol significantly inhibited the Ang II-induced expression of the senescence-related proteins p53 and p21 (Fig. 5E). The results indicated that probucol alleviated Ang II-induced senescence and autophagy in human glomerular mesangial cells. Probucol pretreatment significantly inhibited STAT3 activation and restored mTOR activity (Fig. 5F), which might represent the mechanism by which probucol reduced the excessive autophagy and alleviated the senescence induced by Ang II.

4. Discussion

Activation of the renin-angiotensin-aldosterone system (RAAS) plays a key role in the development and progression of kidney diseases, including hypertension, glomerulosclerosis, interstitial fibrosis, cell proliferation, apoptosis and cell senescence [13]. As the most important effector molecule in the RAAS system, Ang II plays a central role in the pathophysiological changes in the kidney. A variety of renal cells synthesize Ang II, including mesangial cells, podocytes and renal tubular epithelial cells. The damaging effect of Ang II on the kidney is related to local hemodynamic changes. Additionally, Ang II stimulates the growth, apoptosis and senescence of the intrinsic renal cells and promotes the release of proinflammatory factors, thereby exerting its kidney-damaging effect. Ang II also has a mitogenic effect and promotes the cell cycle transition from the G1 to S phase, leading to cell senescence and the development and progression of age-related diseases [7, 14–16]. The present study showed that Ang II induced oxidative stress, resulting in an increase in the level of intracellular ROS. Ang II also induced premature senescence of human glomerular mesangial cells via the p53/p21 pathway.

As the ultimate driving factor behind organ aging, cell senescence is the central event connecting microscopic cell damage and macroscopic organ aging and may serve as a breakthrough point for studies of organ aging [4,17]. A variety of mechanisms are involved in the senescence process, including telomere shortening, oxidative stress, mitochondrial damage, DNA damage, inflammation, SASP and abnormal regulation of energy metabolism [4,18,19]. Autophagy is an important mechanism responsible for the regulation of senescence. Autophagy refers to the process by which the cell self-degrades its damaged organelles and macromolecular substances through lysosomes. The effect of autophagy on senescence is complex and remains controversial. The cell senescence process is often accompanied by a decreased autophagy level. Autophagy deficiency leads to the accumulation of intracellular metabolic wastes, which accelerates the senescence process [10,20]. However, our study found that Ang II not only acted as an adverse stimulus factor to induce the senescence of human glomerular mesangial cells but also activated autophagy simultaneously. In senescent glomerular mesangial cells, the number of autophagic vacuoles was increased, the expression of the autophagy-related protein LC3 II was elevated, the LC3II/LC3I ratio was increased, and p62 expression was reduced, suggesting that the autophagic activity was enhanced in senescent cells exposed to Ang II stimulation. The above results indicate that both autophagy and senescence are cellular responses to cytotoxic stress.

To determine whether autophagy mediates the senescence-promoting effect of Ang II, human glomerular mesangial cells were treated

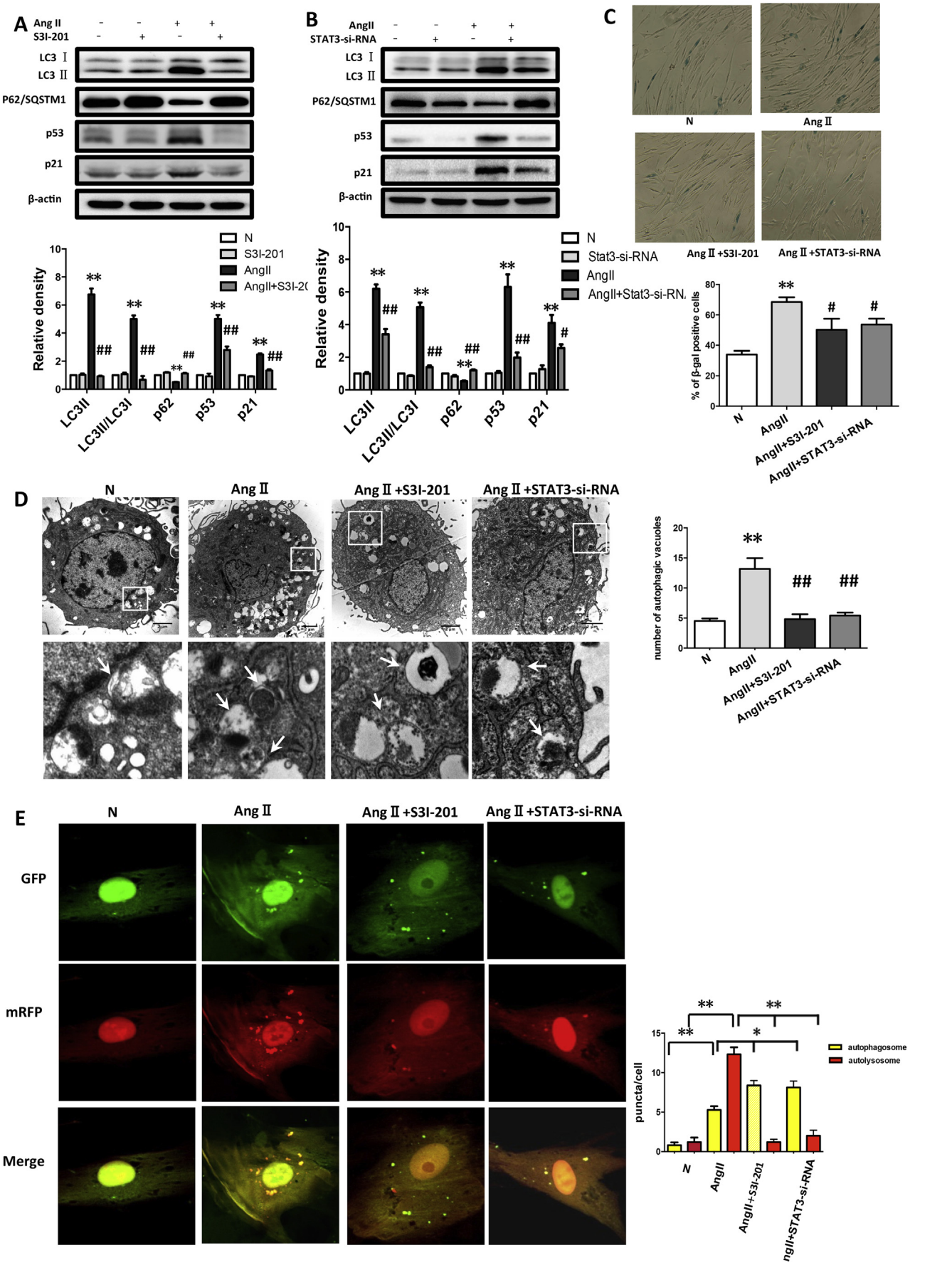

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Fig. 3. S3I-201 and STAT3-siRNA attenuats the Angiotensin II-induced autophagy activation and reduced cell senescence.
(A) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without S3I-201 (50 μmol/L) treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(B) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without STAT3-siRNA treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(C) Senescence-associated β-galactosidase staining of cells that had been treated with S3I-201 (50 μmol/L) or STAT3-siRNA and then subjected to 10 − 6 mmol/L Ang II stimulation for 72 h.
(D) Transmission electron microscopy showed autophagic vacuoles in cells that had been treated with S3I-201 (50 μmol/L) or STAT3-siRNA and then subjected to 10 − 6 mmol/L Ang II stimulation for 72 h. Autophagic vacuoles are indicated by arrows. Bar = 2 μm.
(E) Images of fluorescent LC3 puncta were shown in cells that had been treated with S3I-201 (50 μmol/L) or STAT3-siRNA and then subjected to 10 − 6 mmol/L Ang II stimulation for 72 h. Number of autophagosomes represented by yellow puncta and autolysosomes represented by red puncta in merged images.
The data are presented as the mean ± S.E.M. from at least three independent experiments. Statistical significance is indicated at *P < .05 vs normal, **P < .01 vs normal, #P < .05 vs Ang II, ##P < .01 vs Ang II.

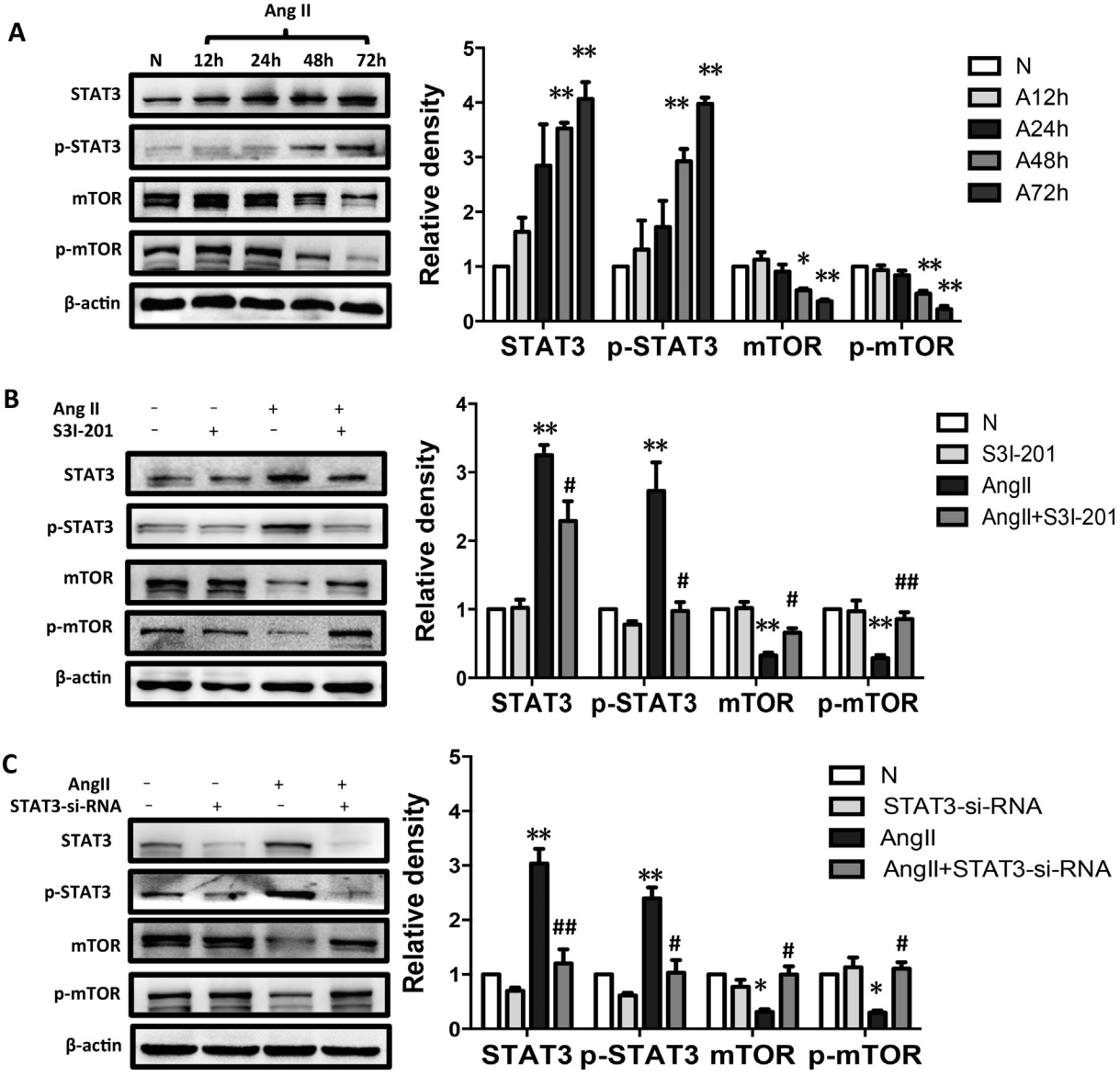

Fig. 4. The STAT3/mTOR pathway mediates the regulatory effect of Ang II on the autophagy and senescence of human glomerular mesangial cells.
(A) Western blot analysis of STAT3, p-STAT3, mTOR and p-mTOR after treatment of the human glomerular mesangial cells with 10 − 6 mmol/L Ang II for 0–72 h.
(B) Western blot analysis of STAT3, p-STAT3, mTOR and p-mTOR from human glomerular mesangial cells pretreated with or without S3I-201 (50 μmol/L) treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(C) Western blot analysis of STAT3, p-STAT3, mTOR and p-mTOR from human glomerular mesangial cells pretreated with or without STAT3-siRNA treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
The data are presented as the mean ± S.E.M. from at least three independent experiments. Statistical significance is indicated at *P < .05 vs normal, **P < .01 vs normal, #P < .05 vs Ang II, ##P < .01 vs Ang II.

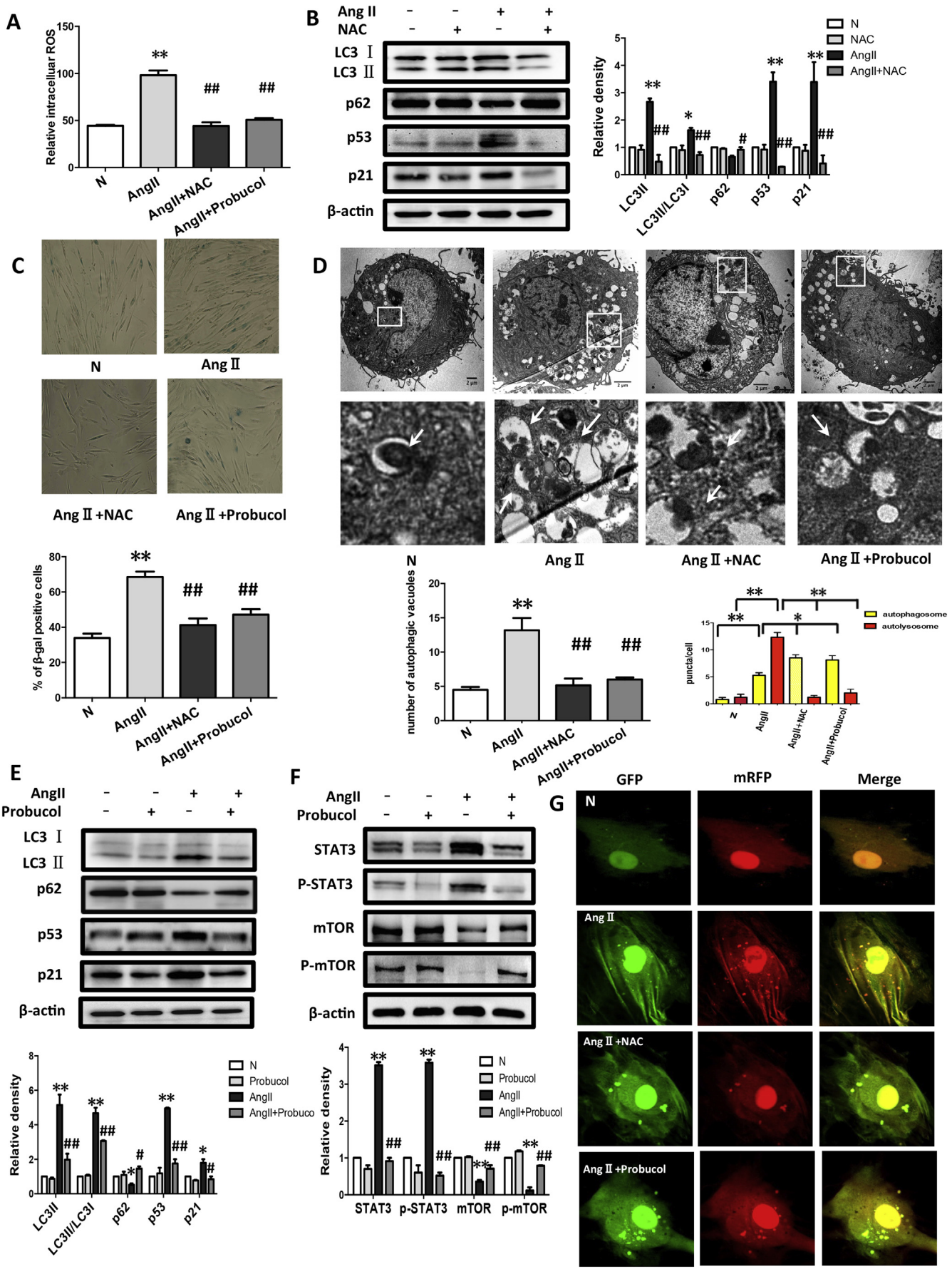

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Fig. 5. Probucol reduces Ang II-induced autophagy and senescence in human glomerular mesangial cells by alleviating oxidative stress and regulating STAT3/mTOR activity.
(A) The generation of ROS from human glomerular mesangial cells pretreated with or without NAC (10 mmol/L) or probucol (40 μmol/L) treatment and then incubated with 10 − 6 mmol/L Ang II for 72 h.
(B) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without NAC (10 mmol/L) treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(C) Senescence-associated β-galactosidase staining of cells that had been treated with or without NAC (10 mmol/L) or probucol (40 μmol/L) treatment and then incubated with 10 − 6 mmol/L Ang II for 72 h.
(D) Transmission electron microscopy showed autophagic vacuoles in cells that had been treated with or without NAC (10 mmol/L) or probucol (40 μmol/L) treatment and then incubated with 10 − 6 mmol/L Ang II for 72 h. Autophagic vacuoles are indicated by arrows. Bar = 2 μm.
(E) Western blot analysis of LC3 II, p62/SQSTM1, p53 and p21 from human glomerular mesangial cells pretreated with or without probucol (40 μmol/L) treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(F) Western blot analysis of STAT3, p-STAT3, mTOR and p-mTOR from human glomerular mesangial cells pretreated with or without probucol (40 μmol/L) treatment and incubated with 10 − 6 mmol/L Ang II for 72 h.
(G) Images of fluorescent LC3 puncta were shown in cells that had been treated with with or without NAC (10 mmol/L) or probucol (40 μmol/L) treatment and then incubated with 10 − 6 mmol/L Ang II for 72 h. Number of autophagosomes represented by yellow puncta and autolysosomes represented by red puncta in merged images.
The data are presented as the mean ± S.E.M. from at least three independent experiments. Statistical significance is indicated at $^{*}P < .05$ vs normal, $^{**}P < .01$ vs normal, $^{\#}P < .05$ vs Ang II, $^{\#\#}P < .01$ vs Ang II.

with the autophagy inhibitor 3MA in the present study. 3MA is a selective phosphatidylinositol 3-kinase (PI3K) inhibitor capable of blocking autophagosome formation. 3MA downregulated the autophagy level in human glomerular mesangial cells exposed to Ang II stimulation. Additionally, 3MA downregulated p53 and p21 expression, decreased the positive rate of β-galactosidase staining and reduced the senescence-inducing effect of Ang II. Our findings were consistent with the experimental results reported by Yong et al. [21]. Yong et al. studied the induction of the senescence by the Ras oncogene in human embryonic lung diploid cells and found that overexpression of the autophagy-related unc-51-like kinase 3 (ULK3) gene enhanced autophagic activity and accelerated senescence. In contrast, knockout of autophagy-related protein 5 (ATG5) or autophagy-related protein 7 (ATG7) inhibited autophagy and alleviated senescence [21]. Therefore, we concluded that autophagy mediated the senescence-promoting effect of Ang II on human glomerular mesangial cells and that inhibition of autophagy could antagonize the senescence-promoting effect of Ang II. Our study seemed to yield opposing results. The contradiction may be related to the differences in the cell type and the microenvironment surrounding the cells [22,23]. For example, in a high-glucose environment, a decrease in the autophagic capability of cardiomyocytes protected the cells from senescence and apoptotic injury [24]. In contrast, high glucose-induced activation of autophagy in podocytes promoted apoptosis [25]. Therefore, autophagy acts like a double-edged sword when regulating the fate of cells. As for the in-depth mechanism, Kang and colleagues reported that this may be related to GATA [26]. GATA4 is degraded by p62-mediated selective autophagy, but this regulation is suppressed during senescence, thereby stabilizing GATA4. GATA4 in turn activates the transcription factor NF-κB to initiate the SASP and facilitate senescence. Appropriate enhancement of autophagic activity removes damaged organelles, reduces the intracellular accumulation of abnormal proteins, promotes pathogen clearance and thus contributes to cell survival. However, excessive autophagy may induce cell senescence and apoptosis. The senescence-promoting effect of autophagy activation may be related to an increased autophagic flux. Enhancement of the autophagic flux induces the production of large amounts of amino acids and metabolites, which are conducive to the synthesis of SASP factors. Once synthesized, SASP factors promote senescence [27]. Moreover, the senescence-promoting effect of autophagy activation may be related to the induction of excessive ROS production by autophagy. Ang II induces oxidative stress in a variety of renal cells [28–30]. As second messengers, ROS activate downstream signaling molecules of Ang II, such as the tyrosine kinase, mitogen-activated protein kinases (MAPKs), STAT and nuclear factor-κB (NF-kB) families of transcription factors [31–33]. Eventually, ROS evoke a series of cytological effects, including autophagy and senescence [30]. Our study found that exposure of human glomerular mesangial cells to Ang II enhanced the intracellular production of ROS. The antioxidant NAC reduced the production of intracellular ROS. Additionally, NAC inhibited the transformation of LC3I to LC3 II, decreased the number of autophagosomes and reduced Ang II-induced autophagy. NAC also improved the senescence-associated phenotypic changes induced by Ang II. The above results confirmed the critical role of oxidative stress in inducing autophagy and promoting senescence.

The regulation of senescence is a complex process that involves multiple signal transduction pathways and a variety of life phenomena. As the age increases and the internal and external environments change, STAT3, which is a member of the JAK/STAT pathway, mediates extracellular signal transduction by interacting with polypeptide receptors on the cell surface. Studies have shown that STAT3 is involved in the aging processes of various organs and cells, including the heart, the brain, the liver, skeletal muscle, fibroblasts, T lymphocytes and tumor cells [34–36]. Additionally, STAT3 activates or inhibits autophagy in a transcriptional or non-transcriptional manner in various cell types and in different environments [37,38]. Our study showed that inhibition of STAT3 expression by drug or gene silencing reduced the autophagic activity, which was reflected by the reduction of the Ang II-induced LC3II expression, an increase in p62 expression and a decrease in the number of autophagosomes. Furthermore, inhibition of STAT3 expression by drug or gene silencing allevted the senescence of human glomerular mesangial cells. Antagonism of excessive autophagy with the autophagy inducer 3MA yielded similar results. Therefore, the present study demonstrates that the excessive autophagy induced by Ang II promotes the occurrence of cell senescence. Appropriate mitigation of autophagy by interfering with STAT3 activity may alleviate cell senescence-related pathological changes to a certain extent.

To date, > 30 autophagy-related genes have been discovered. mTOR is a key protein that regulates autophagy. Under a variety of physiological and pathological conditions, a decrease in mTOR activity activates autophagy [39]. Therefore, we examined the expression status of mTOR in an attempt to identify targets in the STAT3-mediated regulation of autophagy. We found that the Ang II-induced autophagy and senescence of human glomerular mesangial cells were accompanied by decreased mTOR activity. Once STAT3 expression was inhibited by drug or gene silencing, the originally repressed mTOR protein activity was restored and the autophagic activity was decreased. The findings indicate that STAT3/mTOR is involved in the Ang II-mediated regulation of autophagy. Activated mTOR activates the ribosomal p70S6 kinase (SK61) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), initiates mRNA translation and inhibits the activation of transcription of the autophagy-related genes ATG and ULK1, eventually resulting in a decrease in the autophagy level and the

alleviation of senescence [40]. Therefore, STAT3/mTOR pathway-related autophagy may represent one molecular mechanism involved in the regulation of mesangial cell senescence. Due to the central role of STAT3, inhibition of STAT3 activation may serve as an approach to alleviate the senescence-promoting effect of Ang II. Therefore, probucol, which is a clinical drug capable of inhibiting STAT3 activation, was used in the present study in an attempt to develop an effective strategy for clinical anti-aging treatments. The present study found that probucol alleviated the Ang II-induced premature senescence of human glomerular mesangial cells. Similar to NAC, probucol also exhibited antioxidative activity and inhibited Ang II-induced STAT3 activation. Therefore, probucol may inhibit STAT3 activation by alleviating oxidative stress, and other underlying mechanisms need further study. Both probucol and NAC pretreatment significantly inhibited the intracellular production of ROS induced by Ang II. Similar to NAC, probucol inhibited Ang II-induced STAT3 activation. Therefore, we conjecture that probucol may inhibit STAT3 activation by alleviating oxidative stress, thereby inhibiting the excessive activation of autophagy and reducing the p53/p21 expression induced by Ang II. Probucol may exert an anti-senescence effect through the mechanisms described above.

In conclusion, our study established a critical role of STAT3/mTOR-regulated autophagy in Ang II-induced senescence of human glomerular mesangial cells and may provide a theoretical basis for anti-senescence treatment in clinical practice. The present study detected changes in mTOR expression while studying the mechanisms underlying the STAT3-mediated regulation of autophagy.

However, the interaction between STAT3 and mTOR and the potential mechanism of autophagy on senescence needs to be further explored.

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Author contributions

LNW designed the research; SY, DS, XYW, MS, XJ and XRG performed research; SY and LNW analyzed data; SY and LNW wrote the manuscript.

Conflict of interest

None declared.

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