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The RAGE/STAT5/autophagy axis regulates senescence in mesangial cells

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ABSTRACT

Renal aging and associated functional decline are associated with an increase in cellular senescence. Previous studies show a direct correlation between advanced glycation end products (AGEs) accumulation and renal aging, chronic kidney disease (CKD) and other nephropathies, although the underlying molecular mechanisms remain largely unclear. We found elevated levels of the receptor of advanced glycation end product (RAGE) as well as STAT5 in aged human kidneys, as well as in human mesangial cells aged artificially through AGEs. Furthermore, genetic and pharmacological ablation of STAT5 significantly downregulated p16 levels and the percentage of β-Gal-positive senescent cells in mesangial cells and kidneys of SD rats, indicating that AGEs-induced senescence depends on STAT5 signaling. The aged kidney tissues (both in patients and SD rats) and mesangial cells show low levels of LC3 (both LC3-II and LC3-II/I), and cultured mesangial cells also show fewer autolysosomes, autophagosomes, and autophagic vacuoles, which can be partially restored upon STAT5 inhibition. This indicates that AGEs accumulation also obliterates the protective effects of autophagy against aging via the RAGE/STAT5 axis. Direct inhibition of autophagy via 3-methyladenine (3-MA) increases the phenotype of renal aging without activating RAGE, it is inhibition of autophagy caused by RAGE/STAT5 that leads to mesangial aging. In conclusion, we found AGEs induced inhibition of autophagy and cellular senescence in mesangial cells via the RAGE/STAT5 pathway. Moreover, we found that RAGE/STAT5 acts as a key link between autophagy and senescence in the process of mesangial aging in vivo and in vitro.

1. Introduction

Aging refers to the progressive functional decline of multiple organs along with an increase in chronological age. Mechanistically, it is parallel to cellular senescence, a state of permanent growth arrest which depletes the pool of dividing cells in an organ and impairs tissue regeneration, eventually leading to age-related organ damage [1]. The aging kidney undergoes significant anatomical changes, such as decreased cortical mass, glomerulosclerosis, tubular atrophy and interstitial fibrosis, which corresponds with reduced glomerular filtration and increased renal vascular resistance in the elderly [2]. The gradual loss of renal function increases the risk of chronic kidney disease (CKD), renal cancers, and even acute kidney failure. Although CKD can occur at any age, the risk increases significantly with age, either due to normal age-related decline or a higher prevalence of risk factors, such as hypertension and diabetes, among the older individuals [3,4]. Regardless of the exact etiology, due to a globally aging population, it is imperative to determine the molecular basis of CKD, in order to identify novel therapeutic targets.

Multiple mechanisms have been implicated in CKD, including inflammation, oxidative stress and more recently, senescence [5]. The cardinal feature of senescence is prolonged cell cycle arrest induced by DNA damage repair (DDR) signaling in response to telomere attrition, oxidative stress or ionizing radiation [6,7]. Senescent cells are characterized by the accumulation of cell cycle inhibitors, such as p21 and p16, DNA damage markers, such as p53 and γH2AX, reactive oxygen species (ROS), and the senescent-associated β-galactosidase (SA-β-Gal), which have been comprehensively reviewed by Matjusaitis et al. [8]. Based on these biomarkers, studies have reported of the significant accumulation of senescent cells in aged and injured tissues. For example, high numbers of p16+ and SA-β-Gal+ cells have been detected in the aged human and rodent kidneys, and are associated with increased fibrosis and atrophy [9]. In addition, senescence biomarkers are also overexpressed in the kidneys of patients with CKD, diabetic nephropathy (DN) and hypertension, and co-localized with areas showing pathological changes [5,10,11].

Advanced glycation end products (AGEs), compounds resulting from the non-enzymatic glycation of proteins, lipids and nucleic acids, have gained attention in recent years as biomarkers of cellular senescence. AGEs accumulation is seen in aged human and rodent tissues, and is significantly higher in patients with diabetes and DN due to the hyperglycemic milieu [12]. The AGEs bind to a specific multi-ligand

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receptor known as RAGE, which is expressed in macrophages, endothelial cells, renal mesangial cells and neurons, and is activated in diabetes-associated nephropathies and vasculopathies [13]. There is evidence indicating that Janus kinases/signal transducers and activators of transcription (JAK/STAT) pathway, which lie upstream of senescence-inducing factors, such as p21 and p53, are also involved in renal aging and trauma [14,15]. In a previous study, we showed the direct role of the JAK/STAT pathway in renal aging for the first time, with increased activation of JAK2/STAT1 and JAK2/STAT3 pathways during hyperglycemia/Ang II/H_2O_2-induced senescence in the human glomerular mesangial cells (HGMCs) [16]. Another study showed that attenuating telomere shortening, downregulating p53 and p21 via the JAK2 inhibitor AG490, and silencing STAT1 significantly delay the artificial aging of HGMCs [17]. In addition, long-term hyperglycemic stimulation of MCs increase AGEs accumulation and RAGE levels, which induce senescence via STAT5 and p21 [18].

AGEs binding to RAGE are also known to induce autophagy via the Akt/mTOR pathway [19]. Autophagy is a highly conserved process that maintains cellular homeostasis, and its dysregulation is the pathogenic basis of various age-related diseases, including renal degeneration. For example, individuals with glomerulosclerosis have fewer autophagic vacuoles in their podocytes compared with healthy controls [20], and mice lacking the autophagy markers Beclin 1 and LC3 show severe renal fibrosis [21]. In addition, studies on diabetic patients and rodent models show that autophagy protects glomerular podocytes against hyperglycemia-induced apoptosis [22]. However, it is not completely clear whether autophagy affects renal cell senescence in response to AGEs accumulation. The aim of this study is to determine the association between autophagy and senescent mesangial cells, and the potential role played by STAT5. Our findings provide further insights into the mechanisms of renal aging and identify STAT5 as a novel therapeutic target for renal aging.

2. Methods

2.1. Patient tissue collection

Unilateral renal tissue specimens were collected from 20 patients aged 25–87 (median age is 59 years old) who had undergone radical nephrectomy for urological tumors or trauma at the China Medical University First Hospital. The patients were divided into two groups: young group (< 65 years old, 10 patients age 25–58 years old, median age is 38 years old) and aged group (≥ 65 years old, 10 patients age 66–87 years old, median age is 68.5 years old). The inclusion criteria for the patients were as follows: 1) having undergone radical nephrectomy due to urinary tumors or trauma, 2) willingness to provide signed informed consent, 3) a minimum surgical margin of 5 cm from the tumor, and 4) samples were confirmed to be normal tissue by intraoperative rapid pathological testing and inflamed, injured, tumor or other diseased tissues were excluded. Patients with 1) obstructive nephropathy, 2) other kidney diseases or renal insufficiency, 3) diabetes, hypertension, proteinuria or other systemic conditions involving the kidney, 4) pre-surgery radiotherapy or chemotherapy, and 5) a long history of medication were excluded. This work was approved by the Ethics Committee of China Medical University and was conducted in accordance with the ICH guidelines for Good Clinical Practice and the Declaration of Helsinki. Detailed information of patients is shown in Table 1.

2.2. Human primary mesangial cell culture

Human mesangial cells were purchased from Sciencell Research Laboratories (Cat. 4200, Sciencell, US). As recommended these cells were cultured in Mesangial Cell Medium (MCM, Cat. 4201, Sciencell, US), supplemented with 5% fetal bovine serum (FBS), 1% mesangial cell growth supplement (Cat. 4252, Sciencell, US), 1% penicillin/streptomycin solution (Cat.0503, Sciencell, US) at 37 °C under 5% CO_2. Once the cells reach a 80% confluent state, the normal medium was replaced with the serum-free medium and the cells were cultured for another 24 h. The AGEs-induced aging model was established by treating the synchronized cells with AGEs-BSA (250 mg/l) for 72 h and named as the AGEs group. Cells cultured in a normal medium with an equal volume of BSA for 72 h were named as the control group (C group). Cells transfected with negative control shRNA was named as NC-shRNA group. Cells transfected with STAT5-shRNA or RAGE-shRNA (shown in Section 2.10) and then treated with AGEs-BSA for 72 h were named as the AGEs + STAT5-shRNA or AGEs + RAGE-shRNA group, respectively. Cells treated with AGEs-BSA and losartan (1 μg/μl,Y0001062, Sigma, GER) were named as the AGEs + losartan group. Cells treated with 3-MA (5 mmol/l, M9281, Sigma, GER) were named as the 3-MA group. Cells treated with 3-MA and losartan or 3-MA and STAT5-shRNAwere named as the 3-MA + losartan group or 3-MA + STAT5-shRNA group, respectively.

2.3. Natural aging rat model

Six month old male Sprague-Dawley (SD) weighing 180–200 g were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China), and reared in specific pathogen-free (SPF) conditions in strict accordance with the guidelines of the Ethics Committee of China Medical University. They were randomized into the following three groups: 1) young group (n = 10) that were fed normally for two weeks; 2) aged group (n = 10) that were fed normally for 18 months; 3) losartan-treated aged group (n = 10) that were fed normally and additionally perfused with 30 mg/kg losartan daily for 18 months before being sacrificed. Their kidneys were resected for further processing and analyses.

2.4. Masson trichome staining

The kidney samples of the patients were fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with Masson trichome stain as per standard protocols. The stained tissue sections, each with a minimum of 20 glomeruli, were analyzed under an inverted microscope (Eclips 80i, Nikon, Japan) with 400× magnification. 20 glomeruli per section were selected randomly and a semi quantitative index from 0 to 5 was used for glomerular mesangial expansion, which was graded as follows: 0, normal; 1, slight glomerular damage and the mesangial matrix and/or hyalinosis with focal adhesion involving 10% of the glomerulus; 2, sclerosis of 10–20%; 3, sclerosis of 20–30%; 4, sclerosis of 30–40%; 5, sclerosis over 40%. The analysis was performed in a blind manner.

2.5. Transmission electron microscopy (TEM)

The kidney tissues or the mesangial cells were washed, fixed in 2% glutaraldehyde at 4 °C for 2 h, embedded in epoxy resin, and sectioned

Table 1
Clinical characteristics of the patients included in the groups.

Item	Young group (mean ± SD)	Aged group (mean ± SD)	p value	Significance
N	10	10	–	No
Age (year)	40.2 ± 9.20	71.20 ± 9.61	< 0.001	Yes
Weight (kg)	75.13 ± 14.91	65.2 ± 13.63	0.317	No
Height (cm)	166.90 ± 8.02	166.89 ± 10.35	0.733	No
Scr (μmol/L)	63.90 ± 18.27	69.10 ± 9.86	0.374	No
Proteinuria	All negative	All negative	–	No
Gender ratio (male: female)	1:1.5	1:0.67	0.371	No

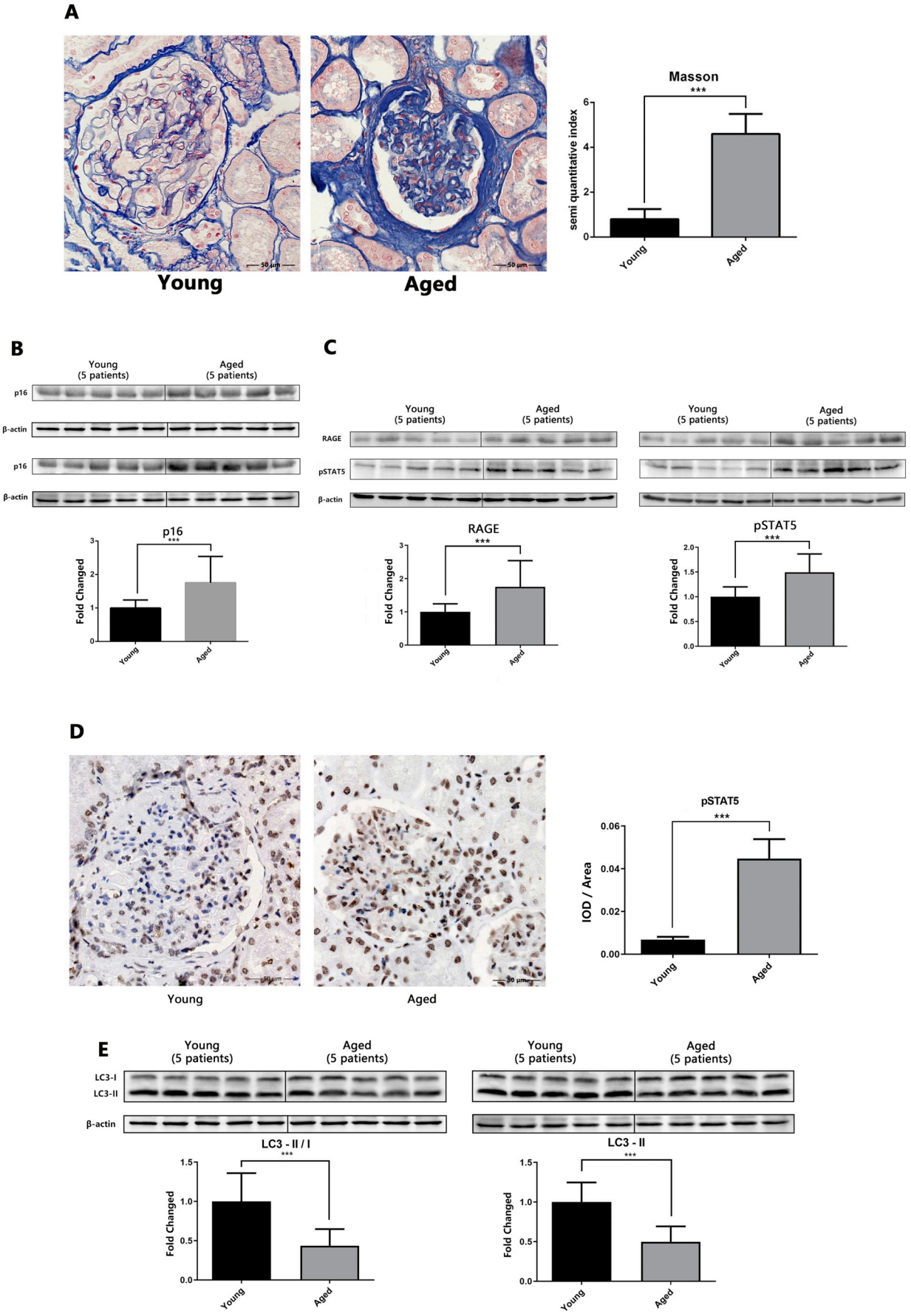

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Fig. 1. Aged kidney tissues show decreased LC3 levels, elevated RAGE and STAT5 levels. A. Masson staining images of young and aged human kidney tissues. B. Immunoblots showing the level of p16 in young and aged kidney tissues. C. Immunoblots showing the level of RAGE and pSTAT5 in young and aged kidney tissues. D. IHC images of kidney sections showing in situ activation of pSTAT5. E. Immunoblots showing levels of LC3II and LC3II/I in young and aged kidney tissues. *** $p < 0.001$ vs. young.

into ultra-thin slices. The specimens were observed under a TEM (H7650, Hitachi, JNP), and the number of autophagic vacuoles were counted in 10 random fields by two independent observers.

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

Each of the treated cells (control, NC-shRNA, AGEs, STAT5-shRNA + AGEs or Losartan + AGEs) were seeded into 500 μl serum-free and antibiotic-free MCM medium at 2×10^4 cells per well. The seeded cells were then infected with the mRFP-GFP-LC3 adenovirus (#AP2100001, Hanheng Biotechnology, CHN) at 200 MOI. After 3 h, the medium was changed into the normal medium and the culture was continued for 6 h. Finally, the results were observed under fluorescence confocal microscopy (FV1000, OLYMPUS, JPN). The number of autophagosomes (yellow puncta) and autolysosomes (rad puncta) in the field of each randomly selected cell were counted.

2.7. Senescence-associated β-galactosidase (β-gal) staining

Senescence was detected in situ, as well as in vitro in cultured mesangial cells using the β-gal staining kit (#9860, CST, US), according to the manufacturer's instructions. The paraffin sections were dewaxed, hydrated, washed thrice with PBS and fixed using the fixative included in the kit for 15 min at room temperature. The slides were washed and air-dried, and the following solutions were sequentially added: 10 μl of β-gal staining solution A, 10 μl of β-gal staining solution B, 930 μl of β-gal staining solution C and 50 μl of X-Gal. The sections were incubated for 12 h at 37 °C, in the absence of CO_2, and washed thrice with PBS before being observed under an inverted microscope. The total number of cells and the number of blue-green senescent cells were counted in each field, and three random fields were observed per slide. The mesangial cells were stained according to the instructions of the β-gal staining kit and cultured without CO_2 for another 2 h. The number of senescent cells were counted in 6 random fields, and their percentage was calculated.

2.8. Immunohistochemistry (IHC)

IHC was performed on kidney sections using the ZSGB-bio kit (ZSGB-BIO, Beijing, China), according to the manufacturer's instructions. In brief, ethylenediaminetetraacetic acid (EDTA) was used for antigen retrieval, with the tissue sections being incubated at 4 °C with an anti-STAT5 antibody (Ab32364, Abcam, UK), overnight. The secondary antibody used was rabbit anti-mouse IgG (PV9000, ZSGB-BIO, China), which was incubated with the tissue sections for 25 min at 37 °C. The color was developed using freshly-prepared 3,3′-Diaminobenzidine (DAB), and after rinsing with distilled water, the sections were counterstained with hematoxylin, dehydrated, and sealed. The immuno-positive brownish yellow cells in the glomeruli (excluding renal tubules and blood vessels) were counted and analyzed using the inverted microscope (Eclipse 80i, Nikon, Japan). IOD of each positive stain was analyzed using Image Pro Plus 6.0.

2.9. Western blotting

The kidney tissues/mesangial cells were lysed and homogenized in a cold RIPA buffer on ice for 30 min, and centrifuged at 8900*g* for 10 min. The cleared lysates were collected, and protein concentration was determined using the BCA method. Equal amount of protein (60 μg) per sample was resolved using SDS-PAGE and transferred into PVDF membranes. The latter were blocked with 5% BSA for 2 h, followed by overnight incubation with primary antibodies against pSTAT5 (1:500) (ab32364, Abcam, UK), LC-3 (1:500) (L7543, Sigma, US), p16 (1:1000) (SAB4500072, Sigma, US), RAGE (1:1000) (ab216329, Abcam, UK) and the internal control β-actin (1:2000) (ZSGB-BIO, China) at 4 °C. After washing with TBST, the blots were incubated with the corresponding HRP-labeled secondary antibody for 1 h at room temperature. The blots were washed again, and the bands were developed using an ECL chemiluminescence reagent for 30 s − 10 min. The blots were scanned, and the IOD of the bands were analyzed using a Gelpro analyzer 4.0.

2.10. Cell transfection

The mesangial cells were transfected with STAT5-shRNA, RAGE-shRNA or NC-shRNA using Lipofectamine 3000. The target sequences were as follows: STAT5 (5′- CTCAGGAGTACTTCATCAT - 3′), RAGE (5′- CACTGGTGCTGAAGTGTAA - 3′), NC (5′- TTCTCCGAACGTGTCACGT - 3′). In brief, Lipofectamine 3000 was diluted 1:50 in serum-free MCM and allowed to stand for 5 min. Equal volumes of the diluted Lipofectamine 3000 and shRNA were mixed and allowed to stand for 30 min, and the mixture was diluted 3 times with serum-free MCM. The mixture was added to 70% confluent mesangial cells that were cultured for another 24 h. The medium was aspirated 6 h later, and after washing the cells twice with PBS, complete medium with AGEs-BSA (250 mg/l) was added and the cells were incubated for 72 h.

2.11. Statistical analysis

The data were analyzed using IBM SPSS 23.0 program and are presented as mean ± standard deviation ($\bar{x} \pm s$) of three independent experiments. The continuous variables were analyzed using a Mann-Whitney *U* test. A p value of < 0.05 was considered as statistically significant.

3. Results

3.1. Aging increases RAGE/pSTAT5 and decreases LC3 levels in human kidneys

In order to determine the effect of aging on renal tissues, specimens were collected from 10 young (age < 65 years) and 10 old (≥65 years) patients and were subjected to histological and molecular analyses. We analyzed the clinical characteristics of the patients to confirm that the only variable and influencing factor between the young and old groups was age (Table 1). Masson staining showed excessive collagen deposition and kidney fibrosis in elderly individuals, consistent with age-related structural changes seen in renal tissues (Fig. 1A). Furthermore, the tissues from the aged group expressed significantly higher levels of p16 compared to that of young individuals (Fig. 1B), indicating senescence at a cellular level. Furthermore, the aged kidneys also showed elevated levels of RAGE and pSTAT5, indicating a possible role of the RAGE/STAT5 pathway in the process of kidney aging (Fig. 1C, D). The autophagy marker LC3-IIandLC3-II/I level (Fig. 1E) and autophagic vacuoles (Supplementary fig. 1) were significantly lower in aged

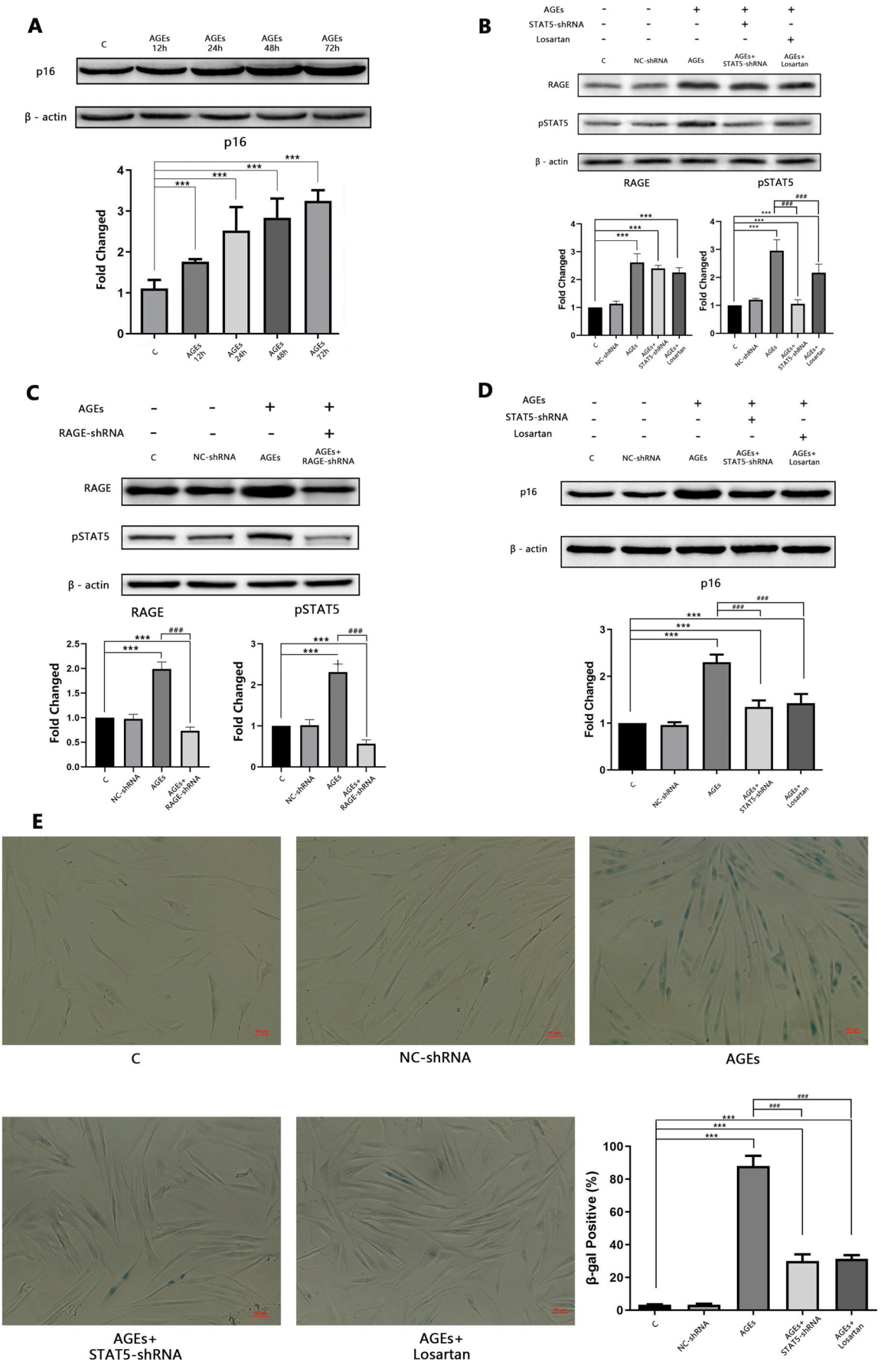

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Fig. 2. AGEs-induced senescence in mesangial cells through activating RAGE/STAT5. A. Immunoblot showing p16 protein levels in mesangial cells cultured with AGEs for varying durations. B. Immunoblot showing RAGE and pSTAT5 levels in differentially-treated mesangial cells. C. Immunoblot showing RAGE and pSTAT5 levels in differentially-treated mesangial cells. D. Immunoblot showing p16 levels in differentially-treated mesangial cells. E. Images showing percentage of senescent cells stained by SA-β-Gal in differentially-treated mesangial cells. $^{***}p < 0.001$ vs. control, $^{\#\#\#}p < 0.001$ vs. AGEs.

individuals compared with that of the young individuals, suggesting a protective role of autophagy against senescence. Based on these findings, we hypothesize that activation of the RAGE/STAT5 pathway is involved in the process of human renal aging and plays a key role in autophagy suppression.

3.2. AGEs induce senescence in mesangial cells by activating STAT5

In order to validate the above hypothesis, we established an in vitro model of AGE-induced senescence using primary human mesangial cells. As shown in Fig. 2A, exposure to AGEs significantly increased levels of p16 protein in the mesangial cells in a time-dependent manner, resulting in senile cells. Based on the preliminary results, we treated the cells for 72 h in the subsequent experiments. Consistent with the findings in the aged human kidney tissues, AGE-treated mesangial cells showed a significant elevation in levels of RAGE and pSTAT5, compared with that of the control group or NC-shRNA group (Fig. 2B). To determine the probable causative role of STAT5 in the process of senescence, we inhibited STAT5 in the mesangial cells either via shRNA mediated STAT5 silencing or treatment with losartan. Both genetic ablation and losartan treatment decreased pSTAT5 levels but had no effect on RAGE (Fig. 2B), further validating the finding that RAGE acts as an upstream regulator of STAT5 signaling. To determine the association between RAGE and pSTAT5, we increased the level of RAGE through exposure to AGEs, and decreased the level of RAGE using RAGE-shRNA. With RAGE, the level of pSTAT5 changed showing a similar trend, indicating that RAGE acts as the trigger of pSTAT5 (Fig. 2C). AGEs treatment significantly increased the percentage of β-Gal positive senescent mesangial cells, as well as the level of p16, which can be decreased by both STAT5 silencing and losartan-mediated inactivation (Fig. 2D, E), indicating the losartan may inhibit senescence via targeting STAT5, but not RAGE.

3.3. AGEs-mediated autophagy inhibition in mesangial cells is dependent on STAT5

The AGEs-induced senile mesangial cells show a significant reduction in autophagy compared with that of the control group and NC-shRNA group, in terms of a decrease in LC3-II protein levels and LC3-II/I (Fig. 3A), fewer autophagic vacuoles (Fig. 3B), autolysosomes puncta and autophagosome puncta (Fig. 3C), indicating that autophagy likely acts as a protective role in mesangial cells and is repressed during the process of senescence. However, shRNA silencing or losartan inactivation of STAT5 partially restores these decreased indices of autophagy (Fig. 3A, B and C), indicating that the RAGE/STAT5 pathway is responsible for the inhibition of autophagy in the senescent mesangial cells. In order to investigate whether there is a causal link between a decrease in autophagy and an increase senescence mediated by the RAGE/STAT5 pathway, we treated mesangial cells with 3-MA, a specific inhibitor of class III phosphatidylinositol 3-kinases (PI3K), without RAGE/STAT5 pathway involvement. Interestingly, direct inhibition of autophagy by 3-MA led to increased levels of p16 without activating RAGE (Fig. 3D). This indicates that the activation of the RAGE/STAT5 pathway leads to various changes including autophagy, moreover, it is the effect of autophagy suppression that acts as the mechanism of mesangial aging. Next, we investigated the role of STAT5 and autophagy in AGE-induced senescent cells. When AGE-induced senescent cells were treated with losartan or STAT5-shRNA alone, the expression of LC3 (both LC3-II and LC3II/I) increased and the expression of p16 decreased, which shows a restored function of autophagy and weakened senescence phenotype. However, there was no significant difference in the senescence phenotype before and after losartan or STAT5-shRNA treatment with autophagy inhibitor 3-MA, as well as in the level of LC3 (Fig. 3E and F). Therefore, we suggest that STAT5 mediates AGE-induced cellular senescence by inhibiting the autophagy pathway.

3.4. Losartan partly alleviates RAGE/STAT5-mediated autophagy and senescence

In order to determine the physiological relevance of the RAGE/STAT5 axis in kidney aging, we established a natural aging model in rats, and analyzed their renal tissue for the markers. As shown in Fig. 4A, B, C, D and E, the tissue of the aged rats had significantly higher levels of p16, RAGE, pSTAT5 and increased staining of β-Gal, which can be alleviated through losartan-mediated STAT5 inactivation. The kidneys of aged rats had significantly lower levels of the LC3-II protein, LC3II/I and autophagic vacuoles (Supplementary fig. 2), suggesting that losartan treatment restores renal autophagy. The degree of glomerulosclerosis shown through Masson staining was significantly higher in aged rats and can be restored through losartan treatment. These results are consistent with the results of the experiments on cultured mesangial cells. Taken together, our study shows that the RAGE/STAT5 pathway mediates the process of renal aging by down-regulating autophagy and acts as a target of losartan.

4. Discussion

Both normal age-related and nephropathic changes gradually lead to the loss of renal function and increase the risk of CKD at the cellular level, while this structural and functional decline is associated with both replicative and stimuli-induced senescence. A significantly higher number of p16 and SA-β-Gal positive senescent cells have been observed in the aged kidneys of both humans and rodents, and this has been found to be correlated with tubular fibrosis and glomerulosclerosis. In addition, animal models of premature aging and DN also show increased levels of p16, p21 and SA-β-Gal in their renal tissues. Accumulation of senescent cells in nephropathies, such as DN, CKD, IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS), are significantly associated with the extent of histopathological and functional changes [5,10,11]. Consistent with these findings, we found that the kidneys of elderly individuals have significant collagen deposition, interstitial fibrosis, and glomerular hardening compared with that of younger patients. In addition, the aged tissues also showed high expression of p16, which plays a vital role in inducing apoptosis or senescence, depending on its cellular level.

Considerable attention has been focused in recent years on the role of advanced glycation end-products or AGEs, a heterogeneous group of non-enzymatically glycated macromolecules, in chronic and age-related pathologies. AGEs accumulation is the result of increased oxidative

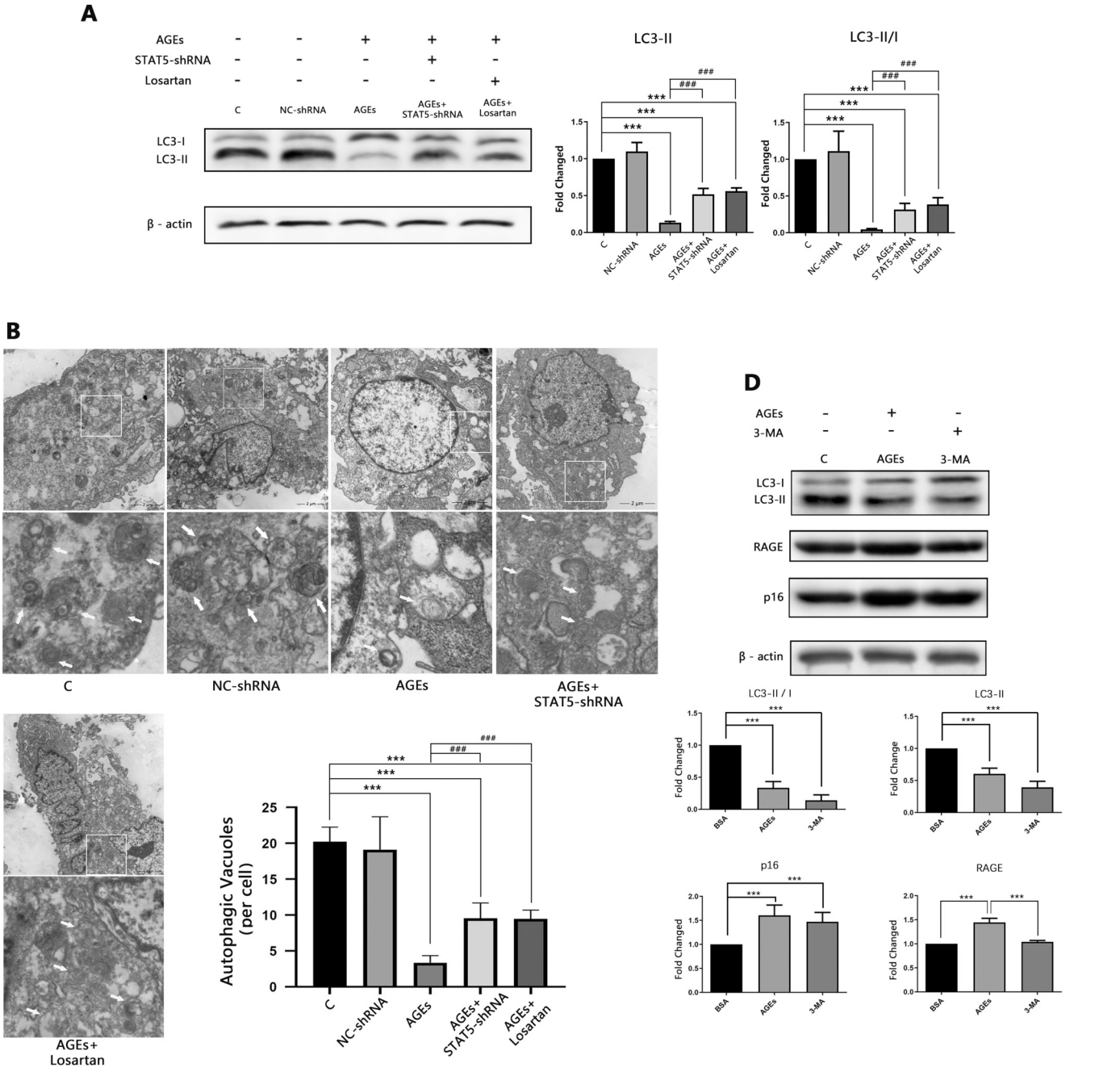

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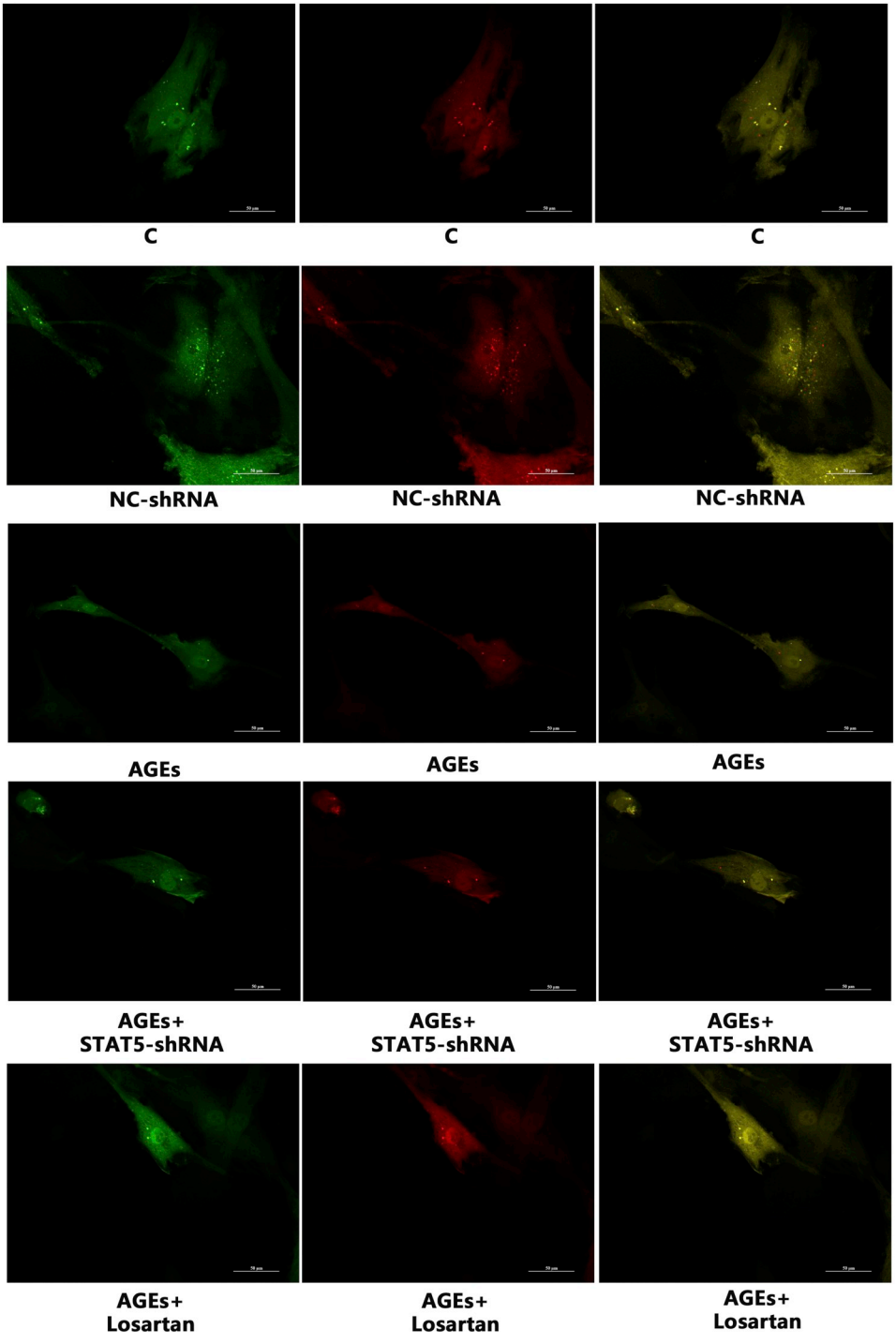
Fig. 3. STAT5 inhibition restores autophagy in senescent mesangial cells. A. Immunoblot showing LC3-II and LC3II/I levels in differentially treated mesangial cells. B. Transmission electron microscopy showing autophagic vacuoles in differentially treated mesangial cells. C. Fluorescent images obtained under confocal microscopy showing autophagosomes (yellow puncta) and autolysosomes (red puncta) in differentially treated mesangial cells. D. Immunoblot showing levels of LC3-II, LC3II/I, RAGE and p16 in AGEs-induced senescent cells and 3-MA treated cells. E. Immunoblot showing levels of LC3-II, LC3II/I and p16 in AGE-induced mesangial cells with 3-MA and Losartan treatment. F. Immunoblot showing levels of LC3-II, LC3II/I and p16 in AGE-induced mesangial cells with 3-MA and STAT5-shRNA treatment. ***$p < 0.001$ vs. control, ###$p < 0.001$ vs. AGEs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stress and inflammation, a sugar-rich diet and decreased serum clearance, which increases with age [12]. Recent studies have shown found elevated AGEs accumulation in various nephropathies. For example, diabetic patients have high AGEs levels in their kidneys, regardless of the presence of CKD. In addition, AGEs accumulation is associated with the increased severity of DN, as well as early signs of renal dysfunction [13]. Formation and accumulation of AGEs lead to increased levels of its specific receptors, of which RAGE is the most widely studied. The AGE/RAGE interaction can activate multiple signaling cascades, including JAK/STAT, TGF-β/Smad, AKT/mTOR and ERK/NF-κB/p21, which culminate in oxidative stress, apoptosis, senescence and autophagy [23]. The JAK/STAT is a pleiotropic signaling pathway that regulates essential cellular functions, such as proliferation, differentiation, apoptosis, senescence or migration via multiple downstream effectors. Currently 4 JAKs (TYK2, JAK1, JAK2 and JAK3) and 7 STATs (STAT-1, −2, −3, −4, −5a, −5b and −6) are known in mammalian cells, and have been found to be stimulated by various ligands and their specific receptors [24]. The growth hormone (GH), which mediates JAK2/STAT5 signaling is essential for maintaining homeostasis in the kidney, and is impaired in juvenile CKD patients and corresponding rodent models [25]. In addition, the JAK2/STAT1 and JAK2/STAT3 pathways have been found to be activated in hyperglycemia-aged HGMCs in vitro [16], while the direct inhibition of JAK by AG490 and STAT1 knockdown significantly reverses HGMC senescence [17]. In another study, MCs exposed to hyperglycemic conditions were found to show significant accumulation of AGEs, along with upregulation of RAGE, STAT5 and p21 [18]. Consistent with these findings, we observed elevated RAGE and pSTAT5 levels in the aged kidney tissues relative to that of younger specimens. It is important to note that we detected phosphorylated STAT5 in this study, since pSTAT5 moves from cytoplasm into nucleus and activates several genes. Taken together, the RAGE/STAT5 axis was found to be clearly associated with the senescent/aging phenotype in the renal tissues.

To further dissect the role of STAT5 in renal senescence, we established an in vitro AGE-induced aging model in primary human mesangial cells. These senile cells showed intense SA-β-Gal staining, which is consistent with the renal biopsies, where elevated levels of both RAGE and pSTAT5 were found. Furthermore, STAT5 silencing and losartan-mediated inhibition significantly downregulates pSTAT5 and decreases the number of senescent cells, without affecting RAGE levels. We also established a murine model of natural aging, and detected significantly higher levels of RAGE, pSTAT5 and p16 in the renal tissues of the aged animals. Long-term losartan treatment inhibit pSTAT5 and alleviates the senescent phenotype. Taken together, RAGE was found to be upstream of STAT5 and to trigger senescence in renal cells both in vitro and in vivo by activating the JAK/STAT5 pathway.

Senescence is accompanied by an increased accumulation of damaged organelles and mis-aggregated proteins in tissues, which are routinely cleared by autophagy. Not surprisingly therefore, autophagy is dysregulated in age-related and degenerative disorders. Studies have shown that the level of autophagy gradually decreases along with senescence and inhibits the accumulation of damaged proteins [26,27]. In senescent brain tissue, expression of autophagy marker LC3 is reduced and autophagy is decreased [28]. In senescent cardiomyocytes, enhanced autophagy improves cardiac dysfunction, cardiac hypertrophy and myocardial fibrosis. Autophagy is also closely related with maintaining normal kidney function [29]. In the senescent kidney, the expression of autophagy-related genes, Atg8 and LC3, were significantly decreases, and the expression of p62/SQSTM1 increases in the senescent kidney, suggesting that autophagy is reduced in the senescent kidney [30]. The AGE/RAGE interaction can also induce autophagy through the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that is responsive to nutrient levels and redox status [19]. Since glomerular podocytes and mesangial cells are post-mitotic cells with limited regeneration, they are dependent on autophagy to clear damaged organelles. Healthy podocytes have a high basal level of autophagy [31], while those from FSGS patients show significantly fewer autophagic vacuoles [20]. Murine models of type I and type II DN also show a deficiency of autophagy in their glomeruli [21,22]. Consistent with this finding, both human and murine aged renal tissues in our study showed significantly lower levels of LC3-II and LC3-II/I proteins. In addition, human mesangial cells treated with AGEs also show lower levels of LC3-II and LC3II/I, and decreased autophagosomes, autolysosomes and autophagic vacuoles, compared with that of the untreated controls. Thus, autophagy may play a protective role and can be inhibited during kidney aging.

We found that inhibition of the pSTAT5-mediated signaling partially restores autophagy in the mesangial cells in vitro, indicating that the RAGE/STAT5 signaling pathway is instrumental in blocking autophagy. A recent study showed that AGEs-mediated disruption of autophagolysosome formation in the tubular epithelial cells were found to aggravate symptoms of DN in a rat model [32]. To the best of our knowledge, this is the first study to show a direct link between the RAGE/JAK/STAT cascade and autophagy in mesangial cells. Based on these results, we initially hypothesized that RAGE/STAT5-mediated inhibition of autophagy is responsible for the senescent phenotype. Indeed, one study has shown that pharmacological induction and inhibition of autophagy, respectively, protects and sensitizes mice to puromycin/aminonucleoside-induced FSGS [20,33]. Furthermore, inhibition of autophagy in murine podocytes upregulates the cell cycle inhibitor, p27 [34], while induction of autophagy in murine DN models occur via caloric restriction and resveratrol alleviated renal inflammation and glomerulosclerosis [22]. Moreover, direct inhibition of autophagy by 3-MA possibly promotes senescent phenotype. Therefore, similar to our hypothesis, it was found that RAGE/STAT5-induced senescence is a direct result of blocking autophagy, and both phenomenons are independently regulated by the STAT5 cascade. Furthermore, the reno-protective role of autophagy has mainly been demonstrated in podocytes, while no reports are available on a similar function in mesangial cells. An intriguing study on phenomenons of lupus nephritis show autophagy in B-cells triggered inflammation, whereas targeted ablation of autophagy in these cells alleviate pathological symptoms [35], indicating the cell type-specific role of autophagy in renal senescence. In addition, 3-MA inhibits PI3K, which lies upstream of



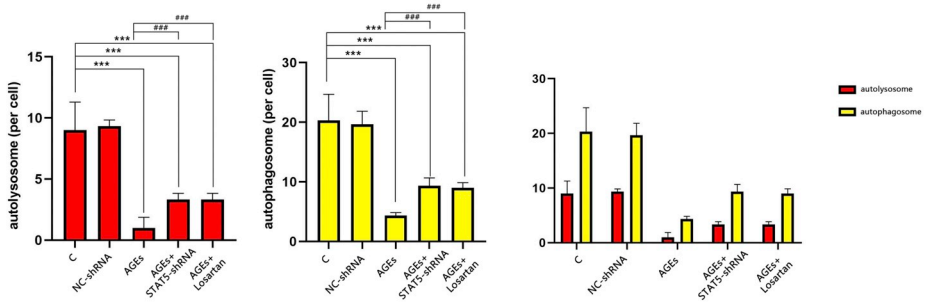


Fig. 3. (*continued*)

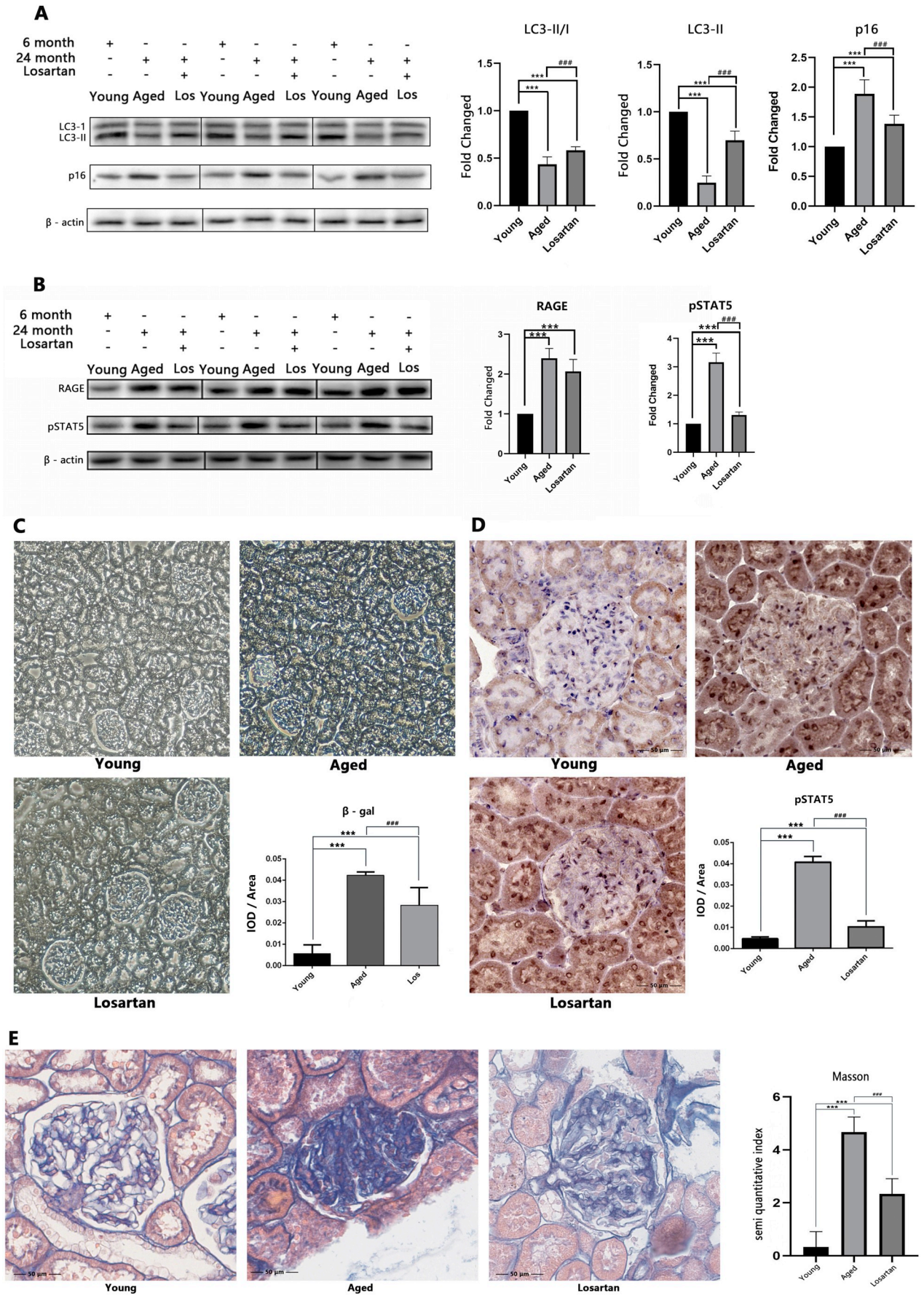


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Fig. 4. Losartan alleviates renal senescence and restores autophagy in natural aging rats. A. Immunoblots showing levels of LC3II, LC3II/I, and p16 in the kidneys of young, aged and losartan-treated aged rats. B. Immunoblots showing levels of RAGE and pSTAT5 in the kidney tissues of young, aged and losartan-treated aged rats. C. Images showing SA-β-Gal staining in the kidneys of young, aged and losartan-treated aged rats. D. IHC showing pSTAT5 levels in the kidneys of young, aged and losartan-treated aged rats. E. Masson staining images of kidney tissues from young, aged and losartan-treated aged rats. ***$p < 0.001$ vs. young, $^{###}p < 0.001$ vs. aged.

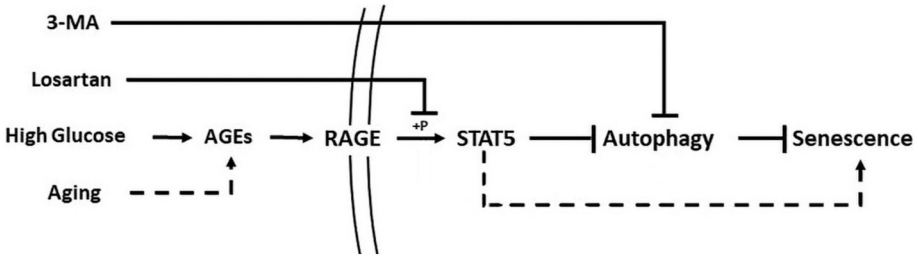

Fig. 5. Proposed mechanism of the RAGE/STAT/autophagy axis regulating senescence in mesangial cells in this study.

several signaling pathways. Therefore, we cannot rule out the possibility of a novel pathway that is independent of STAT5 linking autophagy and senescence in mesangial cells (see proposed mechanism in Fig. 5).

To conclude, our findings indicate that senescence in mesangial cells occurs via the RAGE/STAT5 pathway, moreover, we found that RAGE/STAT5 acts as a key link between autophagy and senescence in the process of mesangial aging in vivo and in vitro.

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Author contributions

Professor Lining Wang designed this research; Shuang Yang, Xinwang Zhu, Da Sun and Dan Sun performed research; Xue Jiang and Congxiao Zhang analyzed data; Mai Shi and Lining Wang wrote the manuscript.

Conflict of interests

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.05.019.

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