Autophagy in homocysteine-induced HUVEC senescence

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Abstract. The senescence of vascular endothelial cells (VECs) drives the occurrence and development of cardiovascular disease (CVD). Homocysteine (HCY) is a general risk factor for age-associated CVDs. Autophagy, an evolutionarily conserved lysosomal protein degradation pathway, serves a part in VEC senescence. The purpose of this study was to investigate the role of autophagy in HCY-induced endothelial cell senescence and explore novel mechanisms and therapeutic approaches for related CVDs. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords of healthy pregnancies. Cell Counting Kit-8, flow cytometry and senescence-associated (SA) β-galactosidase (Gal) staining demonstrated that HCY induced HUVEC senescence by decreasing cell proliferation, arresting cell cycle and increasing the number of SA-β-Gal-positive cells. Stub-RFP-Sens-GFP-LC3 autophagy-related double fluorescence lentivirus revealed that HCY increased autophagic flux. Further, inhibition of autophagy using 3-methyladenine increased HCY-induced HUVEC senescence. By contrast, the induction of autophagy via rapamycin alleviated HCY-induced HUVEC senescence. Finally, the detection of reactive oxygen species (ROS) with ROS kit showed that HCY increased intracellular ROS, whereas induction of autophagy reduced intracellular ROS. In conclusion, HCY increased HUVEC senescence and upregulated autophagy; moderate autophagy could reverse HCY-induced cell senescence. Autophagy may alleviate HCY-induced cell senescence by decreasing intracellular ROS. This provides insight into the underlying mechanism of HCY-induced VEC senescence and potential treatments for age-associated CVDs.

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Introduction

Increasing age promotes the onset and development of cardiovascular disease (CVD); the increase in the incidence of age-associated CVDs causes a heavy burden on human health (1). The association between aging and CVDs is thus receiving increasing attention (1-3). Vascular endothelial cell (VEC) senescence is an important process that contributes to the pathogenesis of age-associated CVDs (4). Senescence impairs vascular endothelial repair, compromises vascular regeneration, decreases the bioavailability of nitric oxide and increases the expression of pro-inflammatory factors and coagulation molecules, contributing to the pro-inflammatory, pro-thrombotic and pro-atherogenic effects of senescence on endothelial cells (2,5).

Homocysteine (HCY) is a cytotoxic methionine metabolite and sulfur-containing amino acid and is a key independent risk factor for age-associated CVDs (6,7). HCY has been shown to induce senescence of endothelial cells by reducing telomerase activity via induction of telomerase reverse transcriptase DNA hypomethylation by decreasing telomerase expression via increases in intracellular reactive oxygen species (ROS) levels (8,9) and by inhibiting plasminogen activator inhibitor-1 and integrin β-3 (10). It is thus of importance to explore the mechanism of HCY-induced VEC senescence and therapeutic strategies for its treatment.

Autophagy, as a potential anti-aging treatment, has been found to be regulated by HCY (11). Autophagy is an evolutionarily conserved subcellular process that participates in lysosomal

degradation of proteins and damaged organelles (12). Under normal conditions, cells maintain low levels of autophagy to maintain cellular homeostasis (1). Some previous studies have found that autophagy is involved in the regulation of endothelial cell senescence (11,13,14). The biomarkers of autophagy, such as beclin-1, p62, and LC-3, are decreased in endothelial cells of the elderly (61-71 years) and autophagy induction improves the endothelial-dependent diastolic function and bioavailability of nitric oxide in elderly (27- to 28-month-old) mice (1). Compared with a control group, the senescence of aortic endothelial cells in mice lacking autophagy-related protein 5 or 7 is increased and the senescence of human umbilical vein endothelial cells (HUVECs) is induced by the drug-induced inhibition or gene knockout of autophagy-related gene 5 (13). While induction of autophagy plays a protective role, it has been suggested that it may contribute to endothelial cell injury (15). For example, the inhibition of autophagy with citrus flavones alleviates HUVEC injury induced by high-glucose- and high-fat-mediated activation of the PI3K/Akt/mTOR pathway (16). Therefore, the beneficial effects of autophagy on endothelial cells are dependent on conditions (17), and these determinants need to be explored.

HCY induces autophagy in mouse liver cells and rat brain cells (15,18) but also inhibits autophagy in mouse brain cells (19). To the best of our knowledge, the association between HCY and autophagy under different conditions has not been fully explored. Moreover, the role of autophagy in HCY-induced endothelial cell senescence has not been reported to date. The present study used HCY to establish a senescence model in HUVECs and the association between autophagy and HCY-induced cell senescence was investigated to provide a novel approach for the treatment of HCY-induced age-associated CVDs.

Materials and methods

Reagents. HCY and D-galactose (both Sigma-Aldrich; Merck KGaA) were dissolved in cell culture media [complete medium containing Dulbecco's modified Eagle's Medium/F-12 (HyClone; Cytiva), 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% endothelial cell growth supplement (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (both Hyclone; Cytiva)]. 3-Methyladenine (3-MA) and rapamycin (both Gene Chem Co., Ltd., China) were dissolved in dimethyl sulfoxide (DMSO; Beijing Solarbio Science & Technology Co., Ltd.). All of the reagents were diluted with cell culture media to experimental concentrations and were newly prepared for each experiment.

Cell isolation and culture. Primary HUVECs were immediately isolated from fresh umbilical cords of healthy pregnant individuals, as previously described (20). The present study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Urumchi, China; no. 20190225-13). Informed written consent was obtained from all participants (age, 20-25 years) from whom HUVECs were obtained. Human umbilical cords were collected into a sterile centrifuge tube containing PBS (Shanghai Sangon Biotech Co, Ltd.), 100 U/ml penicillin G and 100 ug/ml streptomycin sulfate and were isolated immediately. All procedures were performed out on a cleaning bench. Following washing with PBS, the umbilical vein was digested with 0.25% pancreatic enzyme at 37˚C for 10 min, centrifuged at 1,000 x g at 28˚C for 5 min, resuspended and cultured in Dulbecco's modified Eagle's medium/F-12 (Hyclone; Cytiva) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% endothelial cell growth supplement (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin G and 100 ug/ml streptomycin sulfate (both Hyclone; Cytiva) in a humidified atmosphere with 5% CO_2 at 37˚C. The medium was changed and the cells were passaged every 2 days. Cells obtained between three and six passages were used to ensure cell line stability.

HUVECs were treated with various concentrations (0, 25, 100 and 500 μM) of HCY at 37˚C for 24 h, followed by incubation at 37˚C for 24 h. Cells treated with 5 g/l D-galactose at 37˚C for 24 h were used as a classic senescence model control. Then, cells were treated with 100 μM 3-MA or 5.5 nM rapamycin for 1 h at 37˚C before culture with 500 μM HCY for 24 h at 37˚C to test changes in autophagy and its role in HCY-induced senescence. The final concentration of DMSO used for dilution did not exceed 0.2%.

Cell Counting Kit-8 (CCK-8) assay. CCK-8 (Dojindo Laboratories, Inc.) assay was used to assess cell proliferation. Untreated HUVECs were plated at a density of $1x10^3$/well in 96-well plates and incubated as aforementioned. Following culture period, cells were washed with PBS three times. The cells were then cultured with 100 μl fresh cell culture media and 10 μl CCK-8 solution. After 3 h incubation at 37˚C with 5% CO_2, the absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.). The cell proliferation was calculated using the following formula: Proliferation rate=[optical density $(OD)_{experimental\ group}$-$OD_{blank\ group}$]/($OD_{control\ group}$-$OD_{blank\ group}$).

Cell cycle assay. Propidium iodide (PI)/ribonuclease (RNase) staining buffer (BD Pharmingen; BD Biosciences) was used to measure cell cycle arrest. Cells in 100-mm petri dishes were cultured to 50-60% confluence and treated with HCY, D-galactose, 3-MA and rapamycin as aforementioned. Following treatment, cells were collected in 5 ml Eppendorf (EP) tubes, fixed at -4˚C for 24 h with precooled 70% ethanol and incubated at -4˚C overnight. Cells were centrifuged at 1,000 x g for 5 min at 28˚C, the supernatant was discarded and the cells were resuspended in PBS. The procedure was repeated three times. The cells were stained with PI/RNase staining buffer for 15 min at 28˚C in the dark. Each sample was analyzed using the BD LSRII Analyzer (BD Pharmingen; BD Biosciences) and Modfit version 5 (Becton, Dickinson and Company).

Senescence-associated β-galactosidase (SA-β-Gal) staining. Cells in 6-well plates were cultured to 50-60% confluence. The cells were washed with PBS once after treatment with HCY, D-galactose, 3-MA and rapamycin as aforementioned, and stained with SA-β-Gal staining reagent (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. The cells were still mounted. Blue-stained cells were manually counted in three randomly selected fields of view using light microscopy (x100 magnification).

ROS test. Cells in 6-well plates were cultured to 50-60% confluence. The cells were washed with PBS. Cells were cultured for 20 min with DCFH-DA (Beyotime Institute of Biotechnology), which was diluted 1:1,000 in cell culture media without FBS, at 37˚C and 5% CO_2 in the dark. After washing the cells three times with Dulbecco's modified Eagle's Medium/F-12, images were obtained using a Leica DMI6000B fluorescence microscope (Leica Microsystems GmbH; x100 magnification) and the mean fluorescence intensity/cell was measured using ImageJ 1.53e (National Institutes of Health).

Cell autophagic flux measurement. Cells in 96-well plates were cultured to 10-20% confluence. The cells were transfected with 100 μl mixed liquid containing 10 μl autophagy-related lentivirus, 4 μl HitransG A (both Shanghai GeneChem Co., Ltd) and 86 μl cell culture media, followed by incubation for 12-16 h at 37˚C. The Stub-RFP-Sens-GFP-LC3 autophagy-related double fluorescence lentivirus is a lentivirus expressing red fluorescent and green fluorescent protein and autophagy marker protein LC3 that was used according to the manufacturer's instructions. The Sens-GFP is an acid-sensitive protein and the Stub-RFP is a stable fluorescent protein. When the autophagic flux increases, the expression of GFP and RFP increases simultaneously. Then autophagosomes fuse with lysosomes, changing the environment to acid, which quenches green fluorescence. The fluorescence color change from yellow to red indicates autophagy flux change (21). The transfected cells were cultured in a 100-mm Petri dish for 36-72 h at 37˚C. Successfully transfected cells were cultured for 24 h at 37˚C in confocal dishes and treated with HCY, D-galactose, 3-MA and rapamycin as aforementioned. Images were captured using a confocal laser microscope (Leica Microsystems GmbH; x400 magnification). The number of autophagy-related fluorescent dots/cell was manually counted in three randomly selected fields of view.

Statistical analysis. All data are presented as the mean ± standard deviation of three independent experimental repeats. SPSS version 20.0 (IBM Corp.) was used to perform statistical analysis. Comparisons between two independent samples were performed using an unpaired t-test. One-way analysis of variance followed by Dunnett's post hoc test was used for comparisons between >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HCY induces senescent phenotype of HUVECs. D-galactose is a classical senescence inducer (4). D-galactose was used to establish the classical senescence model. The cell proliferation rate in each group was tested by CCK-8 and examined via assessing cell viability. Compared with that in the control group, the cell proliferation rate of D-galactose group was reduced by 40.87%, and that of 500 μM HCY group was decreased by 49.46%. HCY decreased cell proliferation activity significantly in a dose-dependent manner (Fig. 1A). The cell cycle arrest was tested by flow cytometry. Compared with that in the control group, the number of G_1/G_0 stage cells increased by 15.46% in the D-galactose intervention group and 11.40% in the 500μM HCY group. Cell cycle in the HCY-treated group was arrested in a dose-dependent manner and HCY significantly arrested HUVECs in the G_1/G_0 stage (Fig. 1B and C).

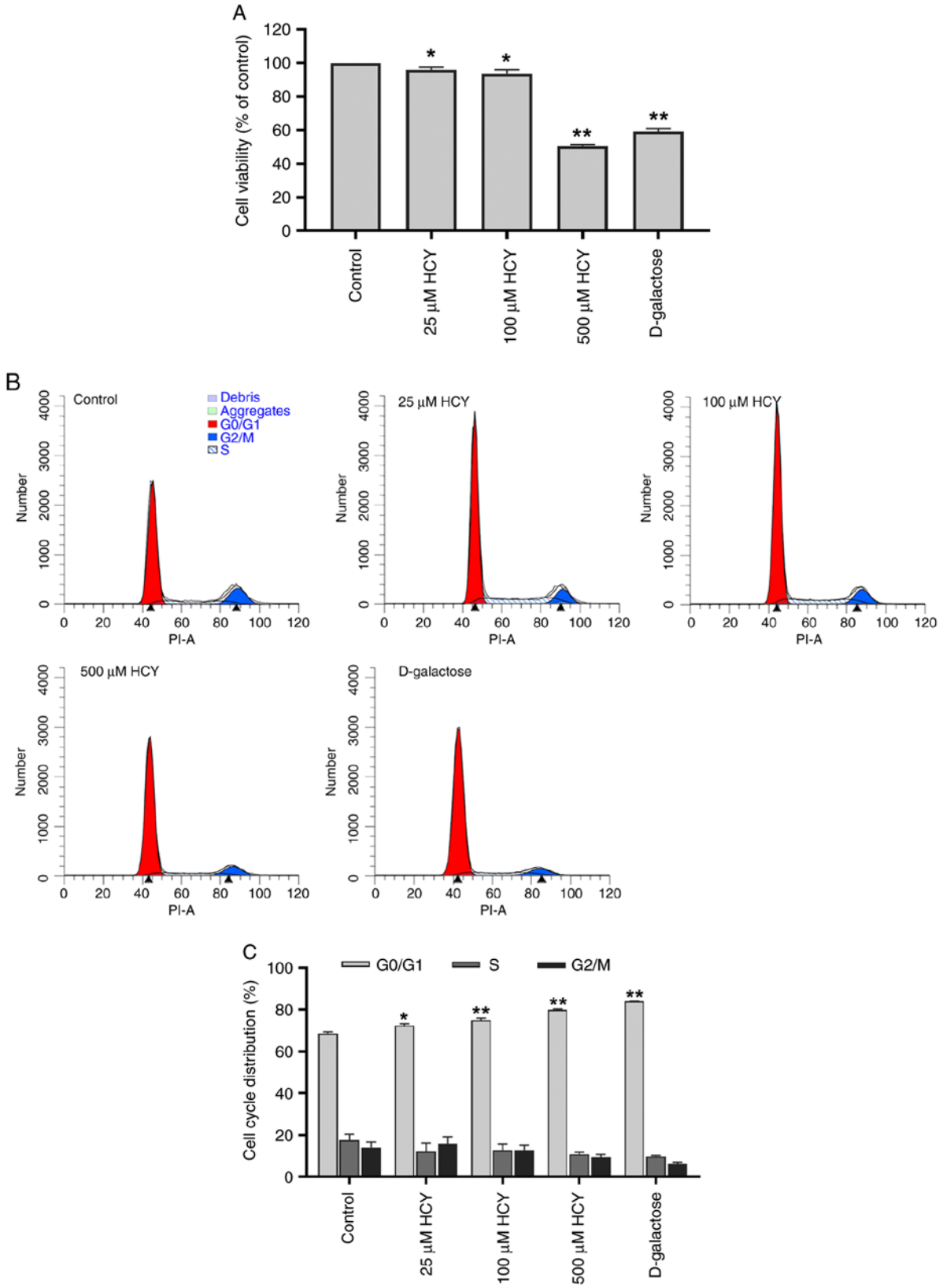
The activity of SA-β-Gal is upregulated during cell senescence and the test of SA-β-Gal activation is used as a senescence marker (9). Blue-stained cells represent senescent cells. The number of senescent cells increased significantly in HUVECs subjected to HCY treatment (Fig. 1D and E). Higher HCY concentration resulted in more senescent cells. Compared with that in the control group, the number of senescent cells increased 6.04 times in the D-galactose intervention group and 5.39 times in the 500 μM HCY group.

Autophagy alleviates the senescent phenotype of HCY-induced HUVECs. LC3 is an autophagy marker protein (22). To observe intracellular autophagic flux, the Stub-RFP-Sens-GFP-LC3 autophagy-related double fluorescence lentivirus was used. Autophagy-associated yellow dots increased significantly in HUVECs treated with HCY (Fig. 2A and B). The autophagy dots in the 500 μM HCY group increased by 2.53 times compared with those of the control group.

To investigate the effect of autophagy on HCY-induced HUVEC senescence, 3-MA, a commonly used autophagy inhibitor, and rapamycin, an autophagy inducer, were used to inhibit and induce autophagy, respectively (22). Compared with the 500 μM HCY group, 3-MA decreased the number of autophagic dots in HUVECs treated with HCY by 30.10%, while rapamycin increased these by 82.94% (Fig. 2C and D). The HVUECs treated with 3-MA exhibited decreased cell viability and cell cycle progression (Fig. 2E-G). Treatment with rapamycin increased cell viability and alleviated cell cycle arrest. Compared with the 500 μM HCY group, 3-MA reduced the cell viability of HUVECs treated with HCY by 29.13%, and increased the number of G_1/G_0 stage cells by 5.66%, while rapamycin increased the cell viability of HUVECs treated with HCY by 35.56%, and reduced the number of G_1/G_0 stage cells by 6.94%. Furthermore, compared with the 500 μM HCY group, 3-MA treatment increased the number of senescent cells in the HCY-treated group by 57.81%, while rapamycin decreased the number of senescent cells by 42.74% (Fig. 2H and I).

Autophagy alleviates HCY-induced senescence by reducing intracellular ROS. ROS are increased during cell senescence and participate in the mechanisms of senescence (1). To test ROS in HCY-treated HUVECs, an inverted fluorescence microscope was used. The mean fluorescence intensity, indicative of the ROS concentration, was significantly higher in HUVECs treated with HCY and D-galactose than in the control group (Fig. 3A and B). Compared with that in the control group, the mean fluorescence intensity in the 500 μM HCY intervention group increased by 1.61 times, and in the D-galactose intervention group, by 1.76 times.

Previous studies have shown that ROS induces autophagy, while autophagy can delay cell senescence by decreasing intracellular ROS (4,23). To investigate the role of ROS in autophagy and HCY-induced endothelial cell senescence, 3-MA and rapamycin were used. Compared with the 500 μM HCY group, exposure to 3-MA increased ROS levels in HUVECs treated with HCY by 13.46%, while exposure to rapamycin decreased ROS levels in HUVECs treated with HCY by 18.59% (Fig. 3C and D).



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Discussion

VECs are the primary cell type in blood vessels. Aging endothelial cells exhibit the decreased ability to synthesize NO, increased secretion of pro-inflammatory and pro-thrombotic factors and impaired vascular regenerative functions, which contribute to development of hypertension, atherosclerosis and the other age-associated CVDs (4). Age-associated vascular changes are promising targets for preventive therapy and lifestyle interventions, including drug therapies, must

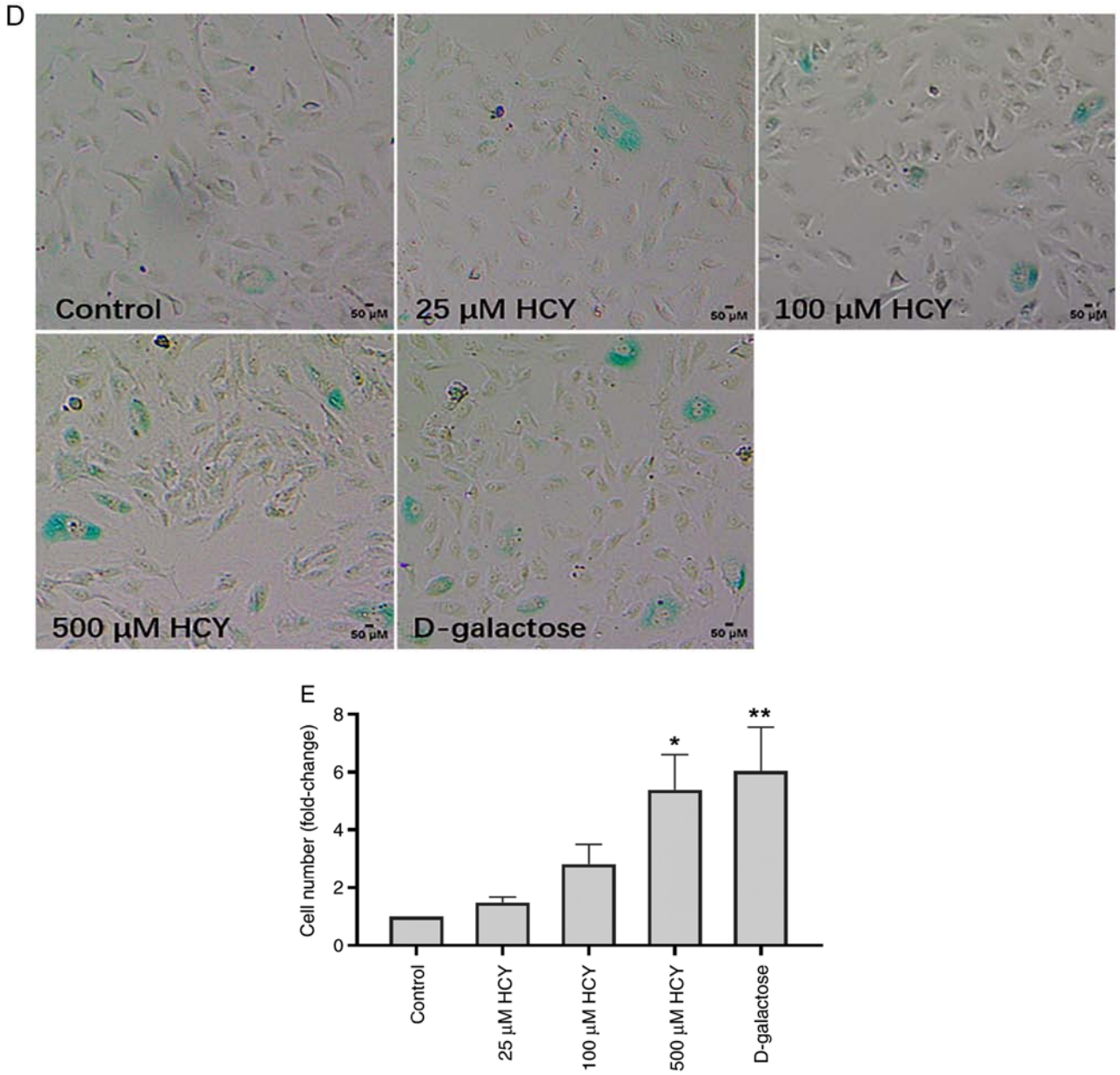
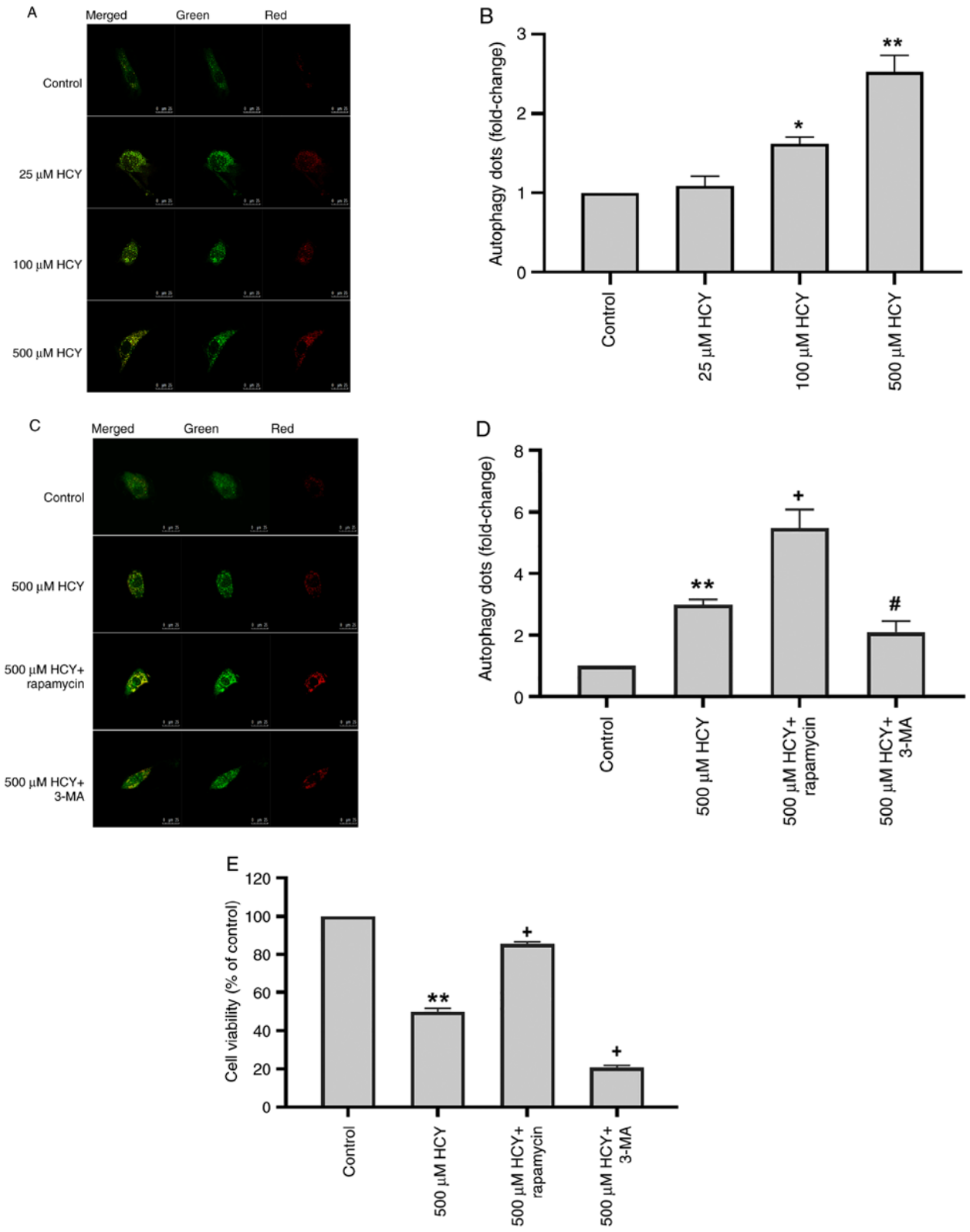


Figure 1. HCY induces senescence. (A) Changes in cell viability following treatment with HCY and 5 g/ml D-galactose for 24 h. (B) Flow cytometry image and (C) cell cycle distribution in cells treated with HCY and 5 g/ml D-galactose for 24 h. (D) Representative images of (E) SA-β-Gal staining in cells treated with HCY and 5 g/ml D-galactose for 24 h. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.001 vs. control. HCY, homocysteine; SA-β-Gal, senescence-associated β-galactosidase.

be implemented to prevent the development of subclinical manifestation of senescence before the pathology becomes apparent (3).

HCY is a non-classical independent risk factor for age-related CVDs and risk of chronic heart disease increases by ~20% for each 5 μM increase in the serum HCY concentration (7,24). The mechanism by which HCY accelerates vascular disease has not been fully clarified, but it may be related to induced endothelial dysfunction, increased platelet adhesion, induced production of low-density lipoprotein and enhanced coagulation cascade reactions (25). HCY can cause endothelial dysfunction by inducing endothelial cell senescence. For example, HCY can induce endothelial cell senescence by inducing DNA hypomethylation (8), increasing intracellular ROS levels (9) and inhibiting plasminogen activator inhibitor-1 and integrin β-3 (10). Folic acid and adenosine methionine reverse the HCY-induced upregulation of the senescence markers P21, P16 and P53 in endothelial cells, preventing HCY-induced endothelial cell senescence (8). In the present study, HCY was used to establish an endothelial cell senescence model, which demonstrated decreased cell proliferation, inhibition of the cell cycle at G_1/G_0 phase and an increase in the activation of SA-β-Gal. This was similar to the classical cell senescence model established by D-galactose.

As a widely recognized anti-aging mechanism, autophagy has received increasing attention in previous years (4,11,26). To study the association between autophagy and HCY-induced endothelial cell senescence, double-fluorescent-labeled LC3 lentivirus was used to detect autophagic flux. HCY increased autophagy in endothelial cells. Then, 3-MA and rapamycin



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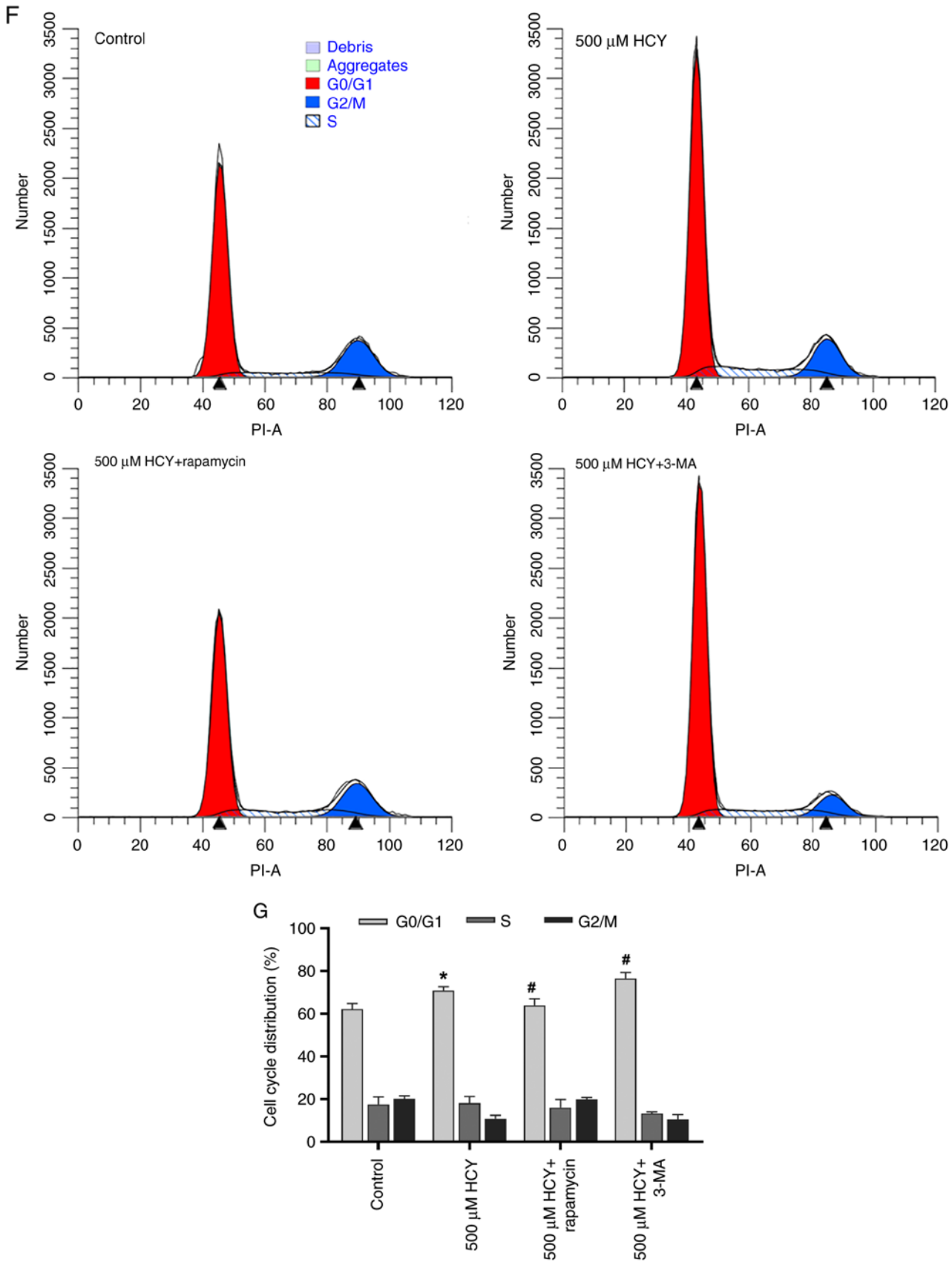
were used to inhibit and induce autophagy, respectively. The inhibition of autophagy by 3-MA increased HCY-induced endothelial cell senescence, while the upregulation of autophagy by rapamycin alleviated HCY-induced endothelial cell senescence. In previous studies, HCY has been reported to both induce and inhibit autophagy (15,19,27,28). In mouse hepatocytes, HCY inhibits expression of cystic fibrosis transmembrane conductance regulator protein via interaction between histone H3 lysine methylation and DNA methylation, thus activating mouse liver autophagy and aggravating liver injury (18). However, HCY activates mTOR in mouse brain neurons and in cultured forebrain neurons derived from



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pluripotent stem cells, inhibiting autophagy and inducing dysfunction of neuronal clearance, thus contributing to the pathogenesis of Alzheimer's disease-like spongiform neurodegeneration via accumulation of phosphorylated τ and amyloid aggregates (19). However, the reason for the discrepancy between HCY and autophagy effects is not

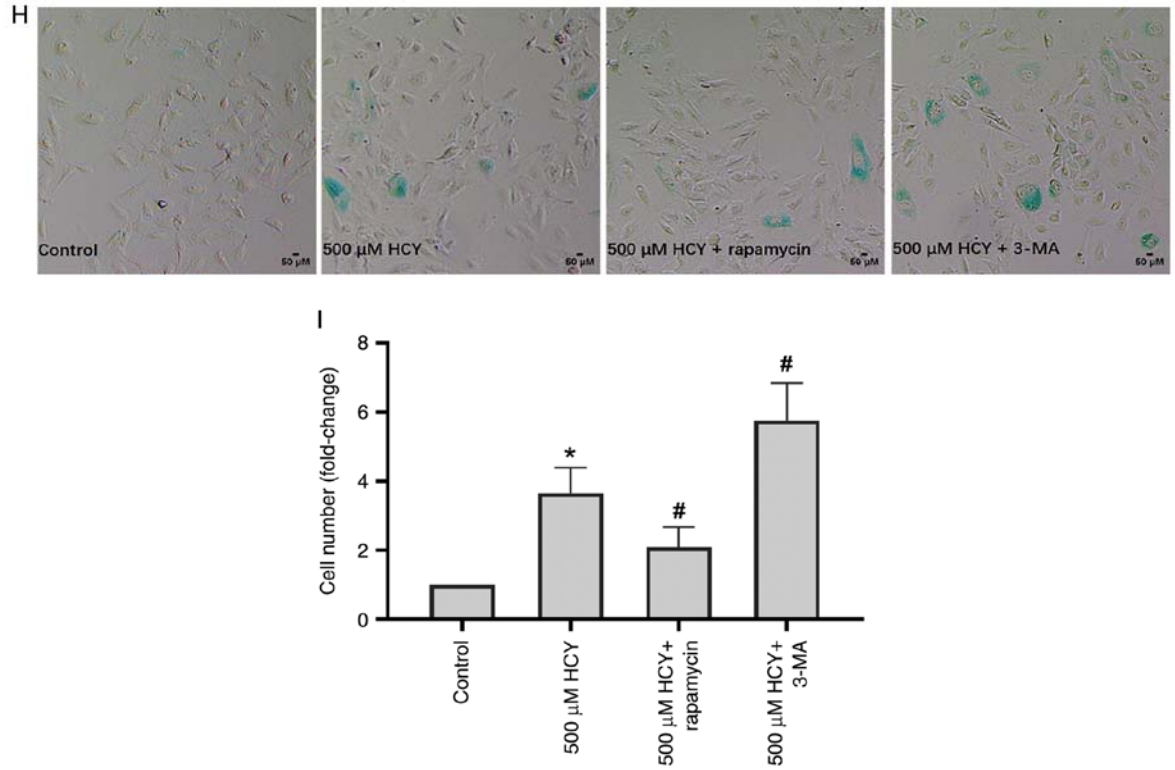

Figure 2. Effects of autophagy on HCY-induced HUVEC senescence. (A) Representative images of (B) autophagic flux in HUVECs treated with HCY for 24 h. (C) Representative images of (D) autophagy changes in cells following treatment with 3-MA and rapamycin for 1 h in addition to HCY treatment. (E) Changes in cell viability following treatment with 3-MA and rapamycin for 1 h in addition to HCY treatment. (F) Representative images of (G) cell cycle changes following 3-MA and rapamycin in addition to HCY treatment. (H) Representative images of (I) senescent cells following 3-MA and rapamycin treatment for 1 h in addition to HCY treatment. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.001 vs. control; $^{+}$P<0.001 vs. HCY; $^{\#}$P<0.05 vs. HCY. HCY, homocysteine; HUVEC, human umbilical vein endothelial cell; 3-MA, methyladenine.

known. From a macroscopic perspective, variations in experimental conditions stemming from cell type, animal model, reagents, intervention and sample collection may affect initial autophagic state of the cells, intensity of autophagic responses to stress and the degree to which the intervention influences autophagy. From the perspective of microscopic genes, proteins and other molecules, researches of autophagy in tumors may provide further mechanistic insight into the differences between autophagy effects. Different types of intracellular autophagy, substrate and autophagy receptor, time of autophagy activation and the cell sites of autophagy activation determine the final autophagic effect (26). In summary, the cytoprotective effect of autophagy is dependent on the environmental conditions (17). Considering that excessive autophagy leads to cell damage and death (29) and the complexity of the autophagy regulatory network, the specific mechanism and conditions of autophagic protection require further study. Further, a method to regulate autophagy at an appropriate level without cell damage is key to alleviate cell aging.

ROS, including superoxide and hydroxyl radicals and H_2O_2, are highly active molecules involved in signal transduction, gene expression, cell proliferation and other cellular pathophysiological processes (22). ROS are increased in senescent cells. Although the increase in ROS is not a landmark event for cell senescence, its increase is one indicator of this process and a potential key factor in inducing and maintaining cell senescence (22). HCY has been reported to induce cell senescence via induction of intracellular ROS (9). Consistent with results of previous studies (7,9,25), the present found that HCY induced HUVEC senescence by inducing the production of intracellular ROS. It was reported previously that oxidative stress promotes autophagy and autophagy might decrease oxidative damage through the engulfment and degradation of oxidative substances (23). Therefore, the present study used rapamycin to induce autophagy; autophagy decreased intracellular ROS levels, while the use of 3-MA increased intracellular ROS levels. Therefore, it was hypothesized that the decrease in ROS production is one of the underlying mechanisms by which autophagy alleviates HCY-induced HUVEC senescence.

The present study explored the relationship between autophagy and HCY-induced HUVEC senescence and confirmed that HCY increased HUVEC senescence and autophagy by inducing production of intracellular ROS. The induction of autophagy decreased production of ROS and alleviated HCY-induced HUVEC senescence. However, the present study only performed phenotypical observations based on *in vitro* cell experiments. The lack of mRNA and protein analysis of senescence and autophagy markers is a limitation of this study; these experiments should be performed in future. Manipulating autophagy *in vivo* is complicated; therefore, further experiments should be conducted to elucidate the regulatory mechanism of autophagy in tissue homeostasis. The present findings may provide insight into the underlying mechanism of HCY-induced VEC senescence and potential treatments for age-associated CVDs.

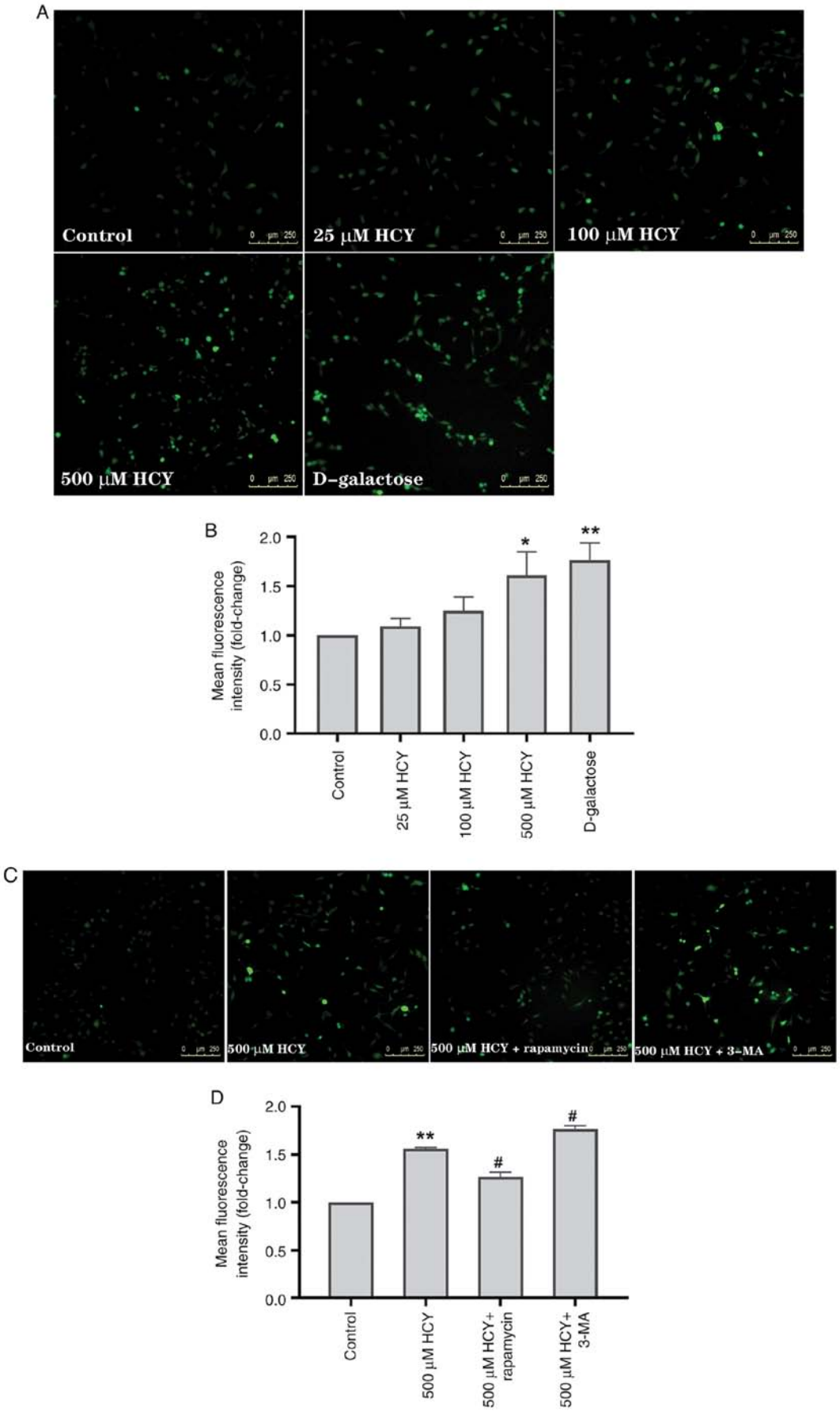

Figure 3. ROS serve as a regulatory bridge between autophagy and HCY-induced HUVEC senescence. (A) Representative images of (B) intracellular ROS levels in cells treated with HCY and 5 g/ml D-galactose for 24 h. (C) Representative images of (D) intracellular ROS levels following 3-MA and rapamycin treatment for 1 h in addition to HCY treatment. n=3. *P<0.05, **P<0.001 vs. control; $^{\#}$P<0.05 vs. HCY. ROS, reactive oxygen species; HCY, homocysteine; 3-MA, 3-methyladenine.

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Availability of data and materials

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

Authors' contributions

YZ, JO, HW and XZ contributed to the design of the present study. YZ, JO, LZ, YL, SL and YH performed the experiments and analyzed the data. HW and XZ drafted the manuscript. YZ, JO, HW and XZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval no. 20190225-13). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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