Advanced glycation end products promote the proliferation and migration of primary rat vascular smooth muscle cells via the upregulation of BAG3

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Abstract. The present study was aimed to investigate the role of reactive oxygen species (ROS) on advanced glycation end product (AGE)-induced proliferation and migration of vascular smooth muscle cells (VSMCs) and whether Bcl-2-associated athanogene 3 (BAG3) is involved in the process. Primary rat VSMCs were extracted and cultured *in vitro*. Cell viability was detected by MTT assay and cell proliferation was detected by EdU incorporation assay. Cell migration was detected by wound healing and Transwell assays. BAG3 was detected using qPCR and western blot analysis. Transcriptional and translational inhibitors (actinomycin D and cycloheximide, respectively) were used to study the effect of AGEs on the expression of BAG3 in VSMCs. Lentiviral plasmids containing short hairpin RNA (shRNA) against rat BAG3 or control shRNA were transduced into VSMCs. Cellular ROS were detected by 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. Mitochondrial membrane potential was detected by tetramethylrhodamine methyl ester (TMRE) staining. AGEs significantly increased the expression of BAG3 in a dose-and time-dependent manner. Furthermore, AGEs mainly increased the expression of BAG3 mRNA by increasing the RNA synthesis rather than inhibiting the RNA translation. BAG3 knockdown reduced the proliferation and migration of VSMCs induced by AGEs. BAG3 knockdown reduced the generation of ROS and sustained the mitochondrial membrane potential of VSMCs. Reduction of ROS production by *N*-acetylcysteine (NAC), a potent antioxidant, also reduced the proliferation and migration of VSMCs. On the whole, the present study demonstrated for the first time that AGEs could increase ROS production and promote the proliferation and migration of VSMCs by upregulating BAG3 expression. This study indicated that BAG3 should be considered as a potential target for the prevention and/or treatment of vascular complications of diabetes.

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Introduction

Vascular smooth muscle cells (VSMCs) are one of the major cellular components of the blood vessel wall, and are mainly responsible for the regulation of blood flow distribution and blood pressure (1,2). Under physiological conditions, VSMCs maintain an extremely low proliferation rate, but they are highly plastic and can convert from a differentiated phenotype to dedifferentiated phenotype as adaptive responses to environmental changes (2-4). In the process of phenotypic modulation, VSMCs are characterized by an increased abilities of proliferation and migration, as well as an increase in extracellular matrix protein deposition, which collectively can accelerate atherosclerosis, hypertension, and diabetic vascular complications (5,6). An increasing number of studies have demonstrated a variety of factors including growth factors, cytokines, mitogens, cell adhesion, cell-cell contact, mechanical influences, extracellular matrix interactions that may control the phenotypic modulation of VSMCs (7). In diabetics, accumulating evidence has demonstrated that the production and accumulation of advanced glycation end products (AGEs) play an important role in regulating the proliferation and migration of VSMCs (8-11), indicating that AGEs are an important mediator in various vascular diseases, particularly diabetic vascular complications.

AGEs result from a slow nonenzymatic glycation reaction between sugars and amine groups present in proteins, lipids or DNA, and can form and accumulate in diabetics (12). AGE formation can activate the receptor (RAGE) and furthermore lead to an aberrant activation of multiple signaling pathways including nuclear factor-κB (NF-κB) (11), mitogen-activated protein kinases (MAPK) (12) and PI3K/AKT (13). Our previous study also indicated that AGEs could promote proliferation and suppress autophagy via reduction of cathepsin D

in VSMCs (14). Notably, several pathways are involved in oxidative stress via an increased production of reactive oxygen species (ROS) (14-17), suggesting that oxidative stress could be an important contributor to the proliferation and migration of VSMCs induced by AGEs. However the underlying mechanisms are so complex that there is still much to be explored.

Bcl-2-associated athanogene 3 (BAG3) is a member of the BAG family and plays an important role in diverse cellular behaviors including cell apoptosis, autophagy, proliferation, adhesion, migration, and differentiation (18-20). As a previous study summarized, BAG3 expression could be upregulated in a varieties of human primary tumors (21). Normal tissues seldom express BAG3, except for cardiomyocytes and skeletal muscle cells, but its expression is induced upon exposure to various stressful stimuli (22). In recent years, accumulating evidence indicates that BAG3 is also associated with various cardiovascular diseases such as myocardial hypertrophy, dilated cardiomyopathy, Takotsubo cardiomyopathy and chronic heart failure (23-26). To date, the role of BAG3 in the proliferation and migration of VSMCs has not been explored.

Therefore, the present study was aimed to investigate the role of ROS in AGE-induced proliferation and migration of VSMCs and whether BAG3 is involved in the process.

Materials and methods

Ethics statement. Animals used in this study were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The procedures were in accordance with the Ethical Standards of the Committee on Animal Experimentation of China Medical University (project identification code, SCXK-2013-0001).

Materials. The Real-Time polymerase chain reaction (qPCR) system was purchased from Applied Biosystems (Foster City, CA, USA). Click-iT Nascent RNA Capture kit was purchased from Invitrogen (Carlsbad, CA, USA). Western blot analysis-related equipment was purchased from Invitrogen. EdU Alexa Fluor 555 Imaging kit was purchased from Invitrogen. Tetramethylrhodamine methyl ester (TMRE) was purchased from Molecular Probes (Eugene, OR, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA), *N*-acetylcysteine (NAC), cycloheximide (CHX), and actinomycin D were purchased from Sigma (St. Louis, MO, USA). BSA and AGEs-BSA were obtained from Merck-Millipore (Darmstadt, Germany). A fluorescence microscope (CKX41-F32FL) was purchased from Olympus (Tokyo, Japan). Microchemi 4.2 was purchased from DNR Bio-Imaging Systems, Ltd. (Jerusalem, Israel). Transwell-related equipment (8-μm pore) was purchased from BD Biosciences (Franklin Lakes, NJ, USA) . Microplate reader was purchased from Bio-Rad (Hercules, CA, USA). Antibodies for BAG3 (sc-292154) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-47724) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Lentiviral vectors were purchased from GeneChem (Shanghai, China).

Isolation and culture of primary rat VSMCs. Neonatal rats (1-2 days old) were sacrificed by cervical dislocation, disinfected with 75% alcohol, and then moved to a clean bench. The thoracic aorta was excised and the inner/outer layers of blood vessels were removed. Primary neonatal rat VSMCs were then isolated as described in our previous study (14). VSMCs were cultured in complete medium including 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml-100 μg/ml) at 37˚C, in 5% CO_2 and a humidified atmosphere as previously described (14,27). Media were changed every other day. After primary cells achieved 80-90% confluence, 0.25% trypsin was added into the culture plate for digestion. Thirty seconds later, serum-containing medium was used to terminate the digestion. Subsequently, a part of the cells was moved into a new culture dish. After attachment to the wall, the cells were incubated with complete medium, which was passage 2 of VSMCs. Using the methods, cells between passages 2 to 8 were obtained and applied for the next experiments.

Construction of BAG3 plasmid and cell transfection. The construction of the BAG3 plasmids was carried out by GeneChem. The cells were transfected with Lipofectamine 2000 reagent (Invitrogen) as previously described (28). The lentiviral plasmids which contained short hairpin RNA (shRNA) against rat BAG3 or control shRNA were labelled with green fluorescent protein (GFP) and used in the knockdown experiments. There were five shRNA oligonucleotides specific for rat BAG3, i.e. shBAG3#1, #2, #3, #4 and #5. The titers of control shRNA and shBAG3#1, #2, #3, #4 and #5 were $8x10^8$, $4x10^8$, $4x10^8$, $3x10^8$, $3x10^8$ and $4x10^8$ TU/ml, respectively. Cells were seeded into 6-well plate and incubated with vector supernatants at a multiplicity of infection (MOI) of 100 for 12 h. Then the old culture medium was removed and replaced with DMEM with 10% FBS. After being cultured for 2 days, the cells were observed under fluorescence microscopy and transduction efficiency was calculated using the following formula: Transduction efficiency = GFP^+ cells/total cells. After digestion, transduced cells were cultured for another 2 days. To confirm that the transduction was successful, the mRNA and protein expression levels of BAG3 were analyzed by quantitative PCR (qPCR) and western blot analysis, respectively.

Western blot analysis. Western blot analysis was performed as described in our previous studies (14,29). Briefly, the cells were solubilized in a radio-immunoprecipitation assay (RIPA) lysis buffer for 30 min, and then total protein concentrations were measured by a BCA protein assay kit (Beyotime, Shanghai, China). After heat denaturation, the samples were analyzed on a 12 or 14% Tris-glycine gradient gel, and then transferred to PVDF membranes and blocked with 5% nonfat milk in Tris-buffered solution (TBS) for 1.5 h at room temperature. The membranes were incubated with primary antibody overnight at 4˚C. After washing three times with TBS, the membranes were incubated with secondary antibodies for 1.5 h at room temperature. After the washing steps, immunoreactive binding was detected with enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ, USA). The band intensity was quantified using ImageJ 1.47 software and GAPDH served as a control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was used to determined cell viability. VSMCs were seeded in a 96-well plate at a density

of $4x10^3$ cells/well. After being cultured for 48 h, the cells were incubated with MTT solution (final concentration, 5 mg/ml) for 4 h at 37˚C. Then the culture media containing MTT were removed and replaced with 100 μl DMSO. Then the plate was gently rotated on a linear and orbital shaker for 5 min to completely dissolve the precipite. The absorbance was measured with a microplate reader at a wavelenght of 570 nm. The percentage of cell viability was calculated according to the following formula: Cell viability (%) = optical density (OD) of the treatment group/OD of the control group x100%.

EdU incorporation analysis. As described in our previous studies (14,30) and according to the manufacturer's instructions, the DNA synthesis rate in VSMCs was determined by EdU incorporation analysis using Click-iT™ EdU Alexa Fluor 555 Imaging kit. Briefly, the cells were incubated with EdU-labeling solution for 8 h at 37˚C, and then fixed with 4% cold formaldehyde for 30 min at room temperature. After permeabilization with 1% Triton X-100, the cells were reacted with Click-iT reaction cocktails (Invitrogen) for 30 min. Subsequently, the DNA contents of the cells were stained with Hoechst 33258 for 30 min. Finally, EdU-labeled cells were counted using ImageJ 1.47 software and normalized to the total number of Hoechst-stained cells. At least 500 cells in each experiment were counted, and EdU-positive cells are expressed as a percentage of the total cells.

Wound-healing assay. Cells at 80-90% confluence were wounded with a 200-μl pipette tip and incubated with BSA or AGEs for 24 h. Multiple views of the leading edge of the scratch were photographed under a microscope at 0 and 24 h. The experiments were performed three times independently.

Transwell migration assay. For the Transwell migration assay, cells were seeded at a density of $2x10^6$ cells in the upper chamber. The lower chamber was filled with BSA or AGEs. After being cultured for 24 h, the cells on the upper chamber were removed by gentle abrasion with a cotton swab, and the cells on the lower chamber were fixed and stained with Hoechst 33258. Three experiments were performed independently. The cells that had passed through the filter were photographed under a fluorescence microscope with ultraviolet light. Hoechst-labeled cells in five representative microscopic fields were counted using ImageJ 1.47 software.

RNA extraction and qPCR. The total RNA was extracted using the Qiagen RNeasy Mini kit. After the determination of concentration, the synthesis of cDNA was performed. With a primer design software we synthesized the sense and antisense primers of each fragment: BAG3 sense, 5'-CATCCAGGAGT GCTGAAAGTG-3' and antisense primer, 5'-TCTGAACCT TCCTGACACCG-3'; GAPDH sense, 5'-GCACCGTCAAGG CTGAGAAC-3' and antisense primer, 5'-TGGTGAAGACGC CAGTGGA-3'. qPCR was run and analyzed with the 7500 Real-Time-PCR system. Results were normalized against those of GAPDH and are presented as arbitrary unit.

Labeling and capture of nascent RNA. Click-iT Nascent RNA capture kit was used to detect newly synthesized RNA according to the manufacturer's instructions. 5-Ethymyl uridine (EU) is an alkyne-modified uridine analog and it is efficiently and naturally incorporated into nascent RNA. Cells were incubated in 0.2 mM of EU for 4 h and total RNA labeled with EU was isolated using Trizol reagent (Invitrogen). Then EU-labeled RNA was biotinylated in a Click-iT reaction buffer with 0.5 mM of biotin azide and subsequently captured on streptavidin magnetic beads.

Measurement of mitochondrial membrane potential. We used TMRE (Ex/Em, 549/573 nm) to detect changes in mitochondrial membrane potential, as described in our previous study (31). Briefly, unfixed live cells were incubated with 100 nM TMRE in the dark for 30 min at 37˚C in 5% CO_2. After being washed, the cells were analyzed under a fluorescence microscope. The fluorescence intensity of TMRE staining was quantified using ImageJ.

Measurement of intracellular ROS. The formation of intracellular ROS was measured with the DCFH-DA (Ex/Em, 485/530 nm) method, as described in our previous study (31). DCFH-DA transforms into the fluorescent compound dichlorofluorescein (DCF) upon oxidation by ROS. Briefly, the cells were incubated with DCFH-DA at a final concentration of 5 mM at 37˚C in 5% CO_2 in darkness for 40 min. After being washed, the cells were analyzed under a fluorescence microscope. The fluorescence intensity of DCFH-DA staining was quantified using ImageJ.

Statistical analysis. Data were obtained from at least three individual experiments. Continuous variables were expressed as the mean ± SD and tested by one-way ANOVA or Student's t-test. All the statistical analyses were performed using SPSS statistics for Windows (version 17.0; SPSS, Chicago, IL, USA), and p-values <0.05 were considered statistically significant.

Results

AGEs increase the expression of BAG3 in primary rat VSMCs. The cells were treated with different concentrations of AGEs (25, 50, 100 and 200 μg/ml) and BSA (2.5, 5, 10 and 20 μg/ml) for 24 h, respectively. We found that AGEs increased the BAG3 mRNA (Fig. 1A) and protein (Fig. 1B) expression in the VSMCs in a dose-dependent manner using qPCR and western blot analysis, respectively. Next, we addressed whether transcriptional and translational inhibitors (actinomycin D and CHX, respectively) could modulate the effect of AGEs on the expression of BAG3 in VSMCs. The results of qPCR showed that actinomycin D significantly reduced the expression of BAG3 mRNA induced by AGEs while CHX had no effects (Fig. 1C), which indicated that AGEs mainly increased the expression of BAG3 mRNA by increasing the RNA synthesis rather than inhibiting the RNA translation. Next, to further support these observations, we evaluated the effect of AGEs on BAG3 mRNA expression using Click-iT Nascent RNA capture kit to isolate newly synthesized RNA. The results of qPCR indicated that AGEs significantly increased BAG3 mRNA synthesis at 24 h with the maximal stimulation effect at a concentration of 100 μg/ml (Fig. 1D). Then, the cells were incubated with actinomycin D and 100 μg/ml AGEs or 10 μg/ml BSA for different

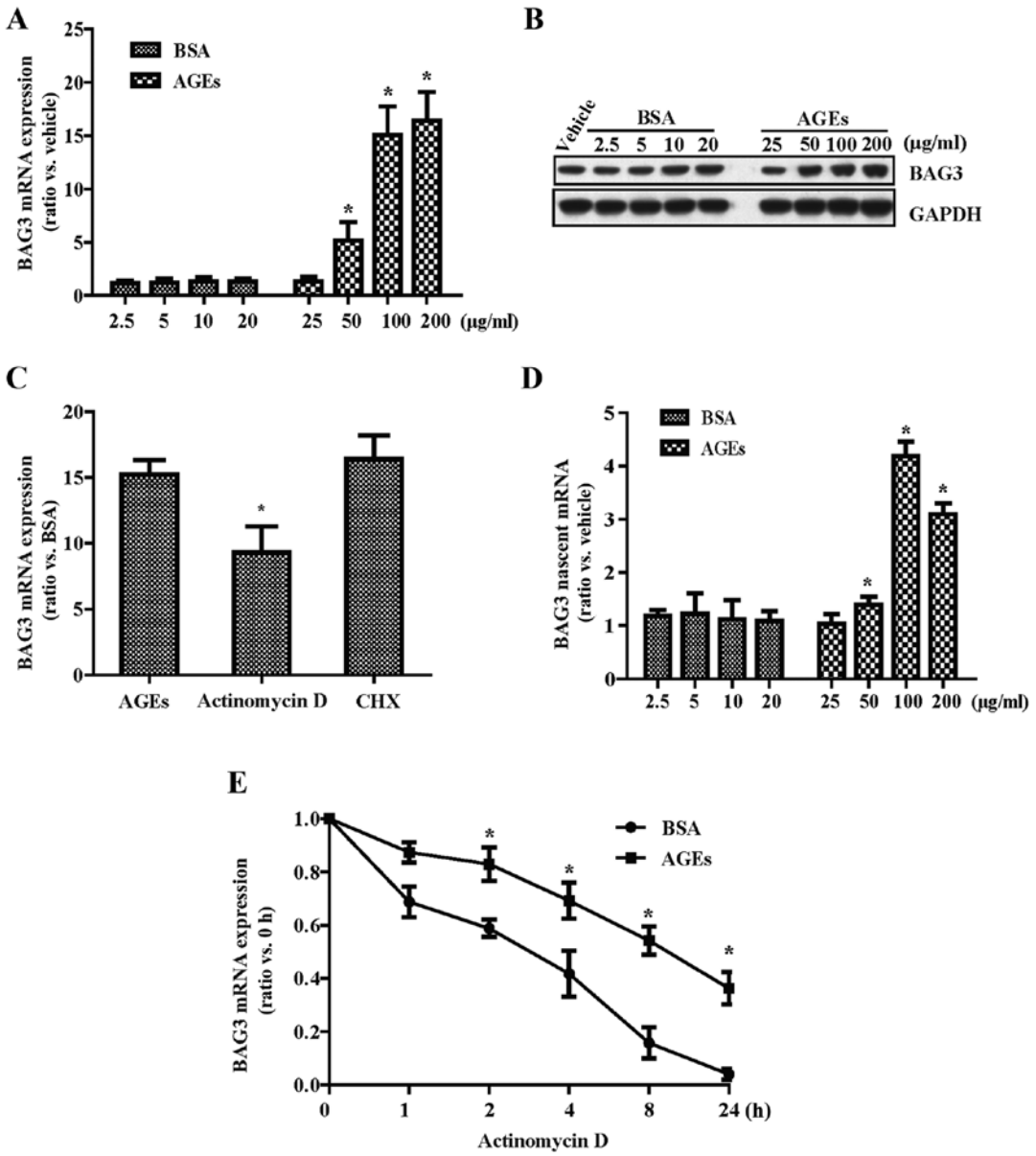

Figure 1. Advanced glycation end products (AGEs) increase the expression of Bcl-2-associated athanogene 3 (BAG3) in cultured primary rat vascular smooth muscle cells (VSMCs). Cells were treated with different concentrations of AGEs (25, 50, 100 and 200 μg/ml) and BSA (2.5, 5, 10 and 20 μg/ml), respectively. The mRNA and protein expression levels of BAG3 were detected by (A) RT-PCR and (B) western blotting, respectively. (C) Actinomycin D (10 μg/ml), a transcriptional inhibitor, and cycloheximide (CHX) (20 μg/ml), a translational inhibitor, were used to study the effect of AGEs on the mRNA expression of BAG3. (D) Click-iT nascent RNA capture kit was used to label and isolate newly synthesized RNA. (E) Cells were incubated with 10 μg/ml actinomycin D and 100 μg/ml AGEs or 10 μg/ml BSA for different times (0, 1, 2, 4, 8 and 24 h), and then the mRNA expression of BAG3 was detected by RT-PCR. The experiments were repeated three times with reproducible results. *p<0.05 compared with the control.

times (0, 1, 2, 4, 8 and 24 h). We found that AGEs increased the expression of BAG3 nascent mRNA in a time-dependent manner (Fig. 1E). Therefore, in the next experiment, we used the dose (100 μg/ml AGEs/10 μg/ml BSA) and time (24 h) to determine the molecular mechanism of AGE-induced proliferation and migration of VSMCs.

BAG3 promotes the proliferation of primary rat VSMCs. To further investigate the involvement of BAG3 in VSMC proliferation, we generated lentiviral vectors containing shRNAs against BAG3 (shBAG3) to knock down BAG3 expression in the VSMCs. Measurement of the GFP$^+$ cells under fluorescence microscopy demonstrated that the transduction efficiency by lentiviral vectors at 100 MOI was 80-90% (Fig. 2A). The results of qPCR (Fig. 2B) and western blot analysis (Fig. 2C) demonstrated that two shRNAs (shBAG3#3 and shBAG3#5) significantly decreased the mRNA and protein expression of BAG3 in VSMCs, respectively. Importantly, results from MTT assay (Fig. 2D) and EdU staining (Fig. 2E and F) consistently demonstrated that forced knockout of BAG3 significantly reduced the cell viability and proliferation of VSMCs.

AGEs promote the proliferation of primary rat VSMCs via BAG3. To investigate the potential involvement of BAG3 in the proliferation of VSMCs induced by AGEs, VSMCs containing

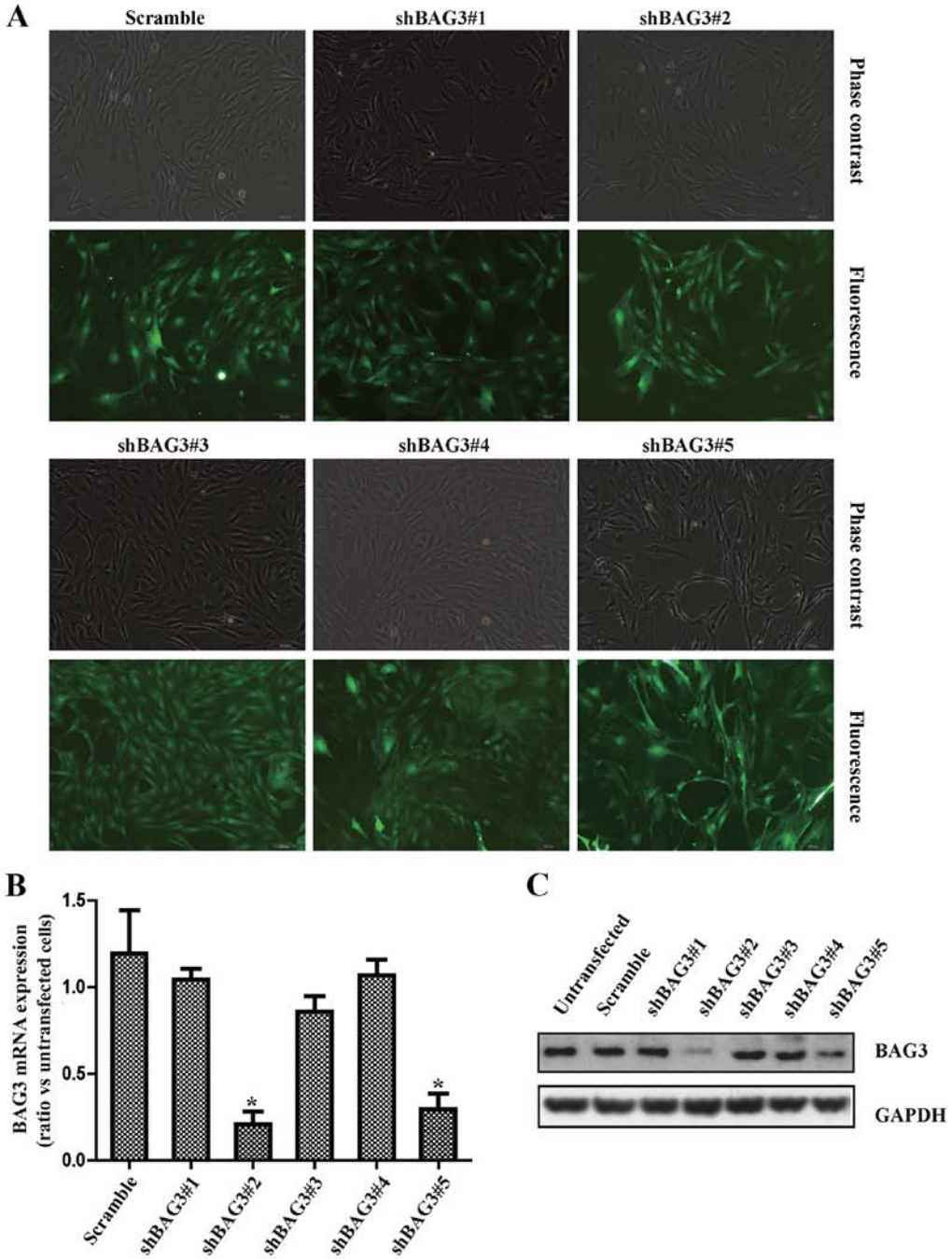

Figure 2. Effects of Bcl-2-associated athanogene 3 (BAG3) on the proliferation of primary rat vascular smooth muscle cells (VSMCs). (A) We generated lentiviral vectors containing shRNAs against BAG3 (shBAG3) to knock down BAG3 expression in VSMCs. The mRNA and protein expression levels of BAG3 were detected by (B) RT-PCR and (C) western blotting, respectively. *p<0.05 compared with the control.

shRNA against BAG3 were treated with 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. The results of the MTT assay (Fig. 3A) and EdU staining (Fig. 3B) demonstrated that BAG3 knockout could significantly decrease the proliferation of VSMCs induced by AGEs.

AGEs promote the migration of primary rat VSMCs via BAG3. Cells were treated with 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. The wound-healing assay showed that AGEs significantly increased the migration of VSMCs compared with that noted in control group (Fig. 4A). The Transwell assay demonstrated that the number of invasive cells in the AGE-treated group was significantly higher than the number in the control group (Fig. 4B and C). We then continued to investigate the potential involvement of BAG3 in the migration of VSMCs. The results of the wound-healing assay (Fig. 4D) and Transwell assay (Fig. 4E) demonstrated that BAG3 knockout significantly decreased the migration of the VSMCs.

Knockout of BAG3 reduces the oxidative stress and maintains the mitochondrial membrane potential of VSMCs. The results of DCHF-DA assay indicated that BAG3 knockout obviously reduced the ROS generation in the VSMCs (Fig. 5A and B). ROS mainly result from mitochondrial respiratory chain complexes

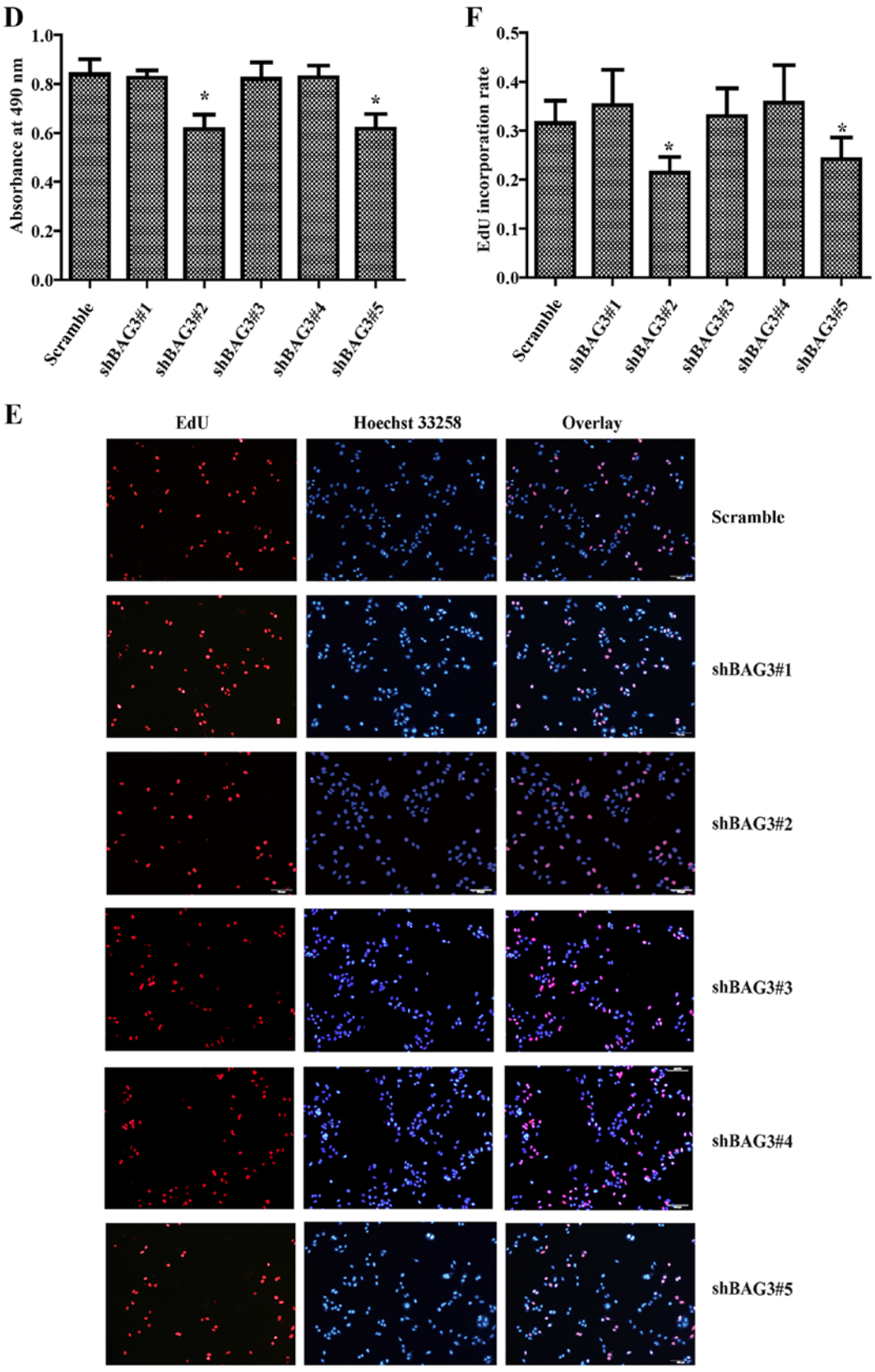

Figure 2. Continued. (D) Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell proliferation was determined by (E) EdU staining and (F) EdU incorporation was calculated as EdU^+ cells/total cells, quantified by ImageJ. The experiments were repeated three times with reproducible results. $^*p<0.05$ compared with the control.

in mitochondria (32). We then investigated the potential effect of BAG3 on the mitochondria. The results of TMRE showed that BAG3 knockout reversed the decrease in mitochondrial membrane potential induced by AGEs (Fig. 5C and D).

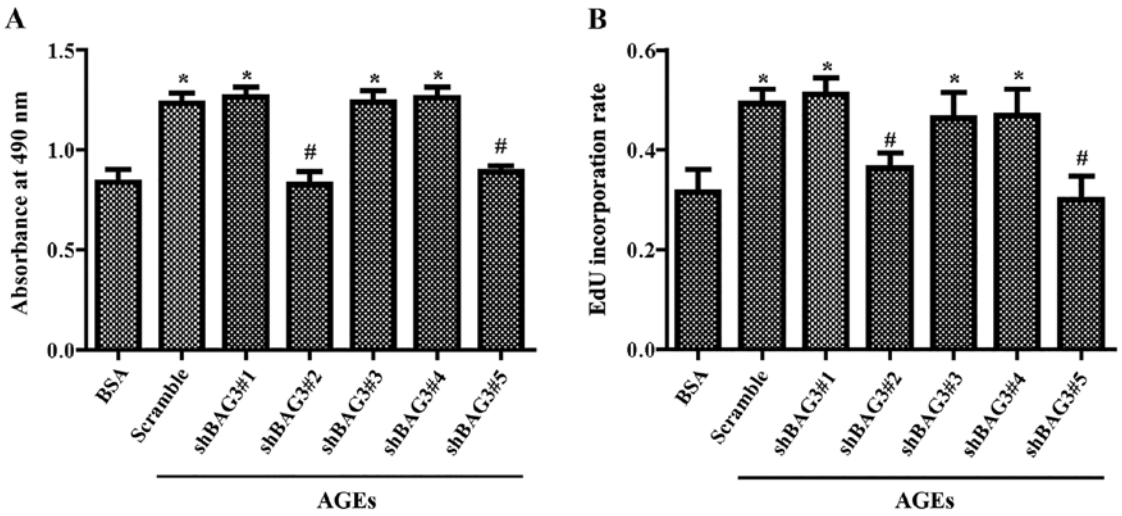

Figure 3. Advanced glycation end products (AGEs) promote the proliferation of primary rat vascular smooth muscle cells (VSMCs) via Bcl-2-associated athanogene 3 (BAG3). VSMCs transfected with shRNAs against BAG3 (shBAG3) were treated with 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. (A) Cell viability was determined by MTT assay. (B) Cell proliferation was determined by EdU staining and EdU incorporation was calculated as EdU$^+$ cells/total cells, quantified by ImageJ. The experiments were repeated three times with reproducible results. *p<0.05 compared with the control; $^\#$p<0.05 compared with the scramble + AGEs group.

AGEs promote the proliferation and migration of primary rat VSMCs via oxidative stress. ROS, such as superoxide anions and hydrogen peroxide, play a crucial role in regulating the proliferation and migration of VSMCs. We tested the hypothesis that the stimulative effect of AGEs on the proliferation and migration of VSMCs involved ROS production. We next used NAC, a potent antioxidant, to investigate the potential effect of oxidative stress on the VSMCs. Cells were incubated with 100 μg/ml NAC and 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. The results of the DCFH-DA assay indicated that NAC could completely prevented the generation of the intracellular ROS level after AGE stimulation (Fig. 6A and B). Furthermore, the results from the EdU staining (Fig. 6C and D) and MTT assay (Fig. 6E) consistently demonstrated that NAC significantly reduced the proliferation of VSMCs induced by AGEs. Results from the wound healing (Fig. 6F) and Transwell assays (Fig. 6G and H) consistently demonstrated that NAC significantly reduced the migration of VSMCs induced by AGEs. Overall, our results indicated that intracellular ROS generation has an essential role in AGE-induced proliferation and migration of VSMCs.

Discussion

The present study demonstrated a novel role for BAG3 in regulating the proliferation and migration of VSMCs induced by AGEs. We found that AGEs promoted the proliferation and migration of VSMCs via upregulation of BAG3 expression, in which ROS played an important role.

In vivo, AGEs slowly form in hyperglycemic environments and during aging, and play role in a variety of microvascular and macrovascular complications in diabetes (13). Accumulating evidence suggests that AGEs promote the proliferation and migration of VSMCs, which accelerate atherosclerosis and restenosis after percutaneous coronary intervention (9,33,34). Our results were consistent with these previous studies. The interaction between AGEs and its receptor RAGE plays an important role in AGE-induced cell injury. It is well accepted that overexpression of RAGE is activated by AGEs in vascular dysfunction and resulting in apoptosis, oxidative stress and inflammation responses (35). There is a growing body of evidence that shows that AGE-RAGE interaction with a positive feedback loop is related to the dysfunction of VSMCs (10,36-38). A previous study found that 100 μg/ml AGEs enhanced vascular calcification through a RAGE/oxidative stress pathway (38). In the process of vascular calcification, the proliferation and migration of VSMCs play a crucial role. Based on these previous studies, the present study continued to explore the mechanisms underlining the promotion of the proliferation and migration of VSMCs by AGEs. We found that AGEs significantly promoted the expression of BAG3 and knockout of BAG3 reduced the proliferation and migration of VSMCs induced by AGEs.

BAG3 plays an important role in a series of cellular processes, including cell proliferation, migration, apoptosis, autophagy, adhesion and cell cycle progression (18-20,39). Several lines of evidence suggest that the expression of BAG3 is elevated in various tumors including glioblastoma, acute lymphoblastic leukemia, and prostate carcinoma (40-42). However, normal tissues seldom express BAG3, except for cardiomyocytes and skeletal muscle cells (22). The expression of BAG3 can be induced by a variety of stimuli such as the early growth responsive gene-1 (Egr-1) (43), heat shock factor-1 (HSF-1) (44), proteasome inhibitors (45), heavy metals as well as heat stress (46,47). BAG3 is seldom expressed in normal tissues, but its expression is induced upon exposure to various stressful stimuli, which appears to be a protective mechanism (22). Previous studies indicated that BAG3 involved in various CVD such as myocardial hypertrophy, dilated cardiomyopathy, Takotsubo cardiomyopathy and chronic heart failure (23-26). However, the relationship between the expression of BAG3 and the proliferation and migration of VSMCs is still unclear.

This study, for the first time, found the dynamic alterations of BAG3 expression in VSMCs treated with AGEs and demonstrated that shBAG3 could reduce the proliferation and

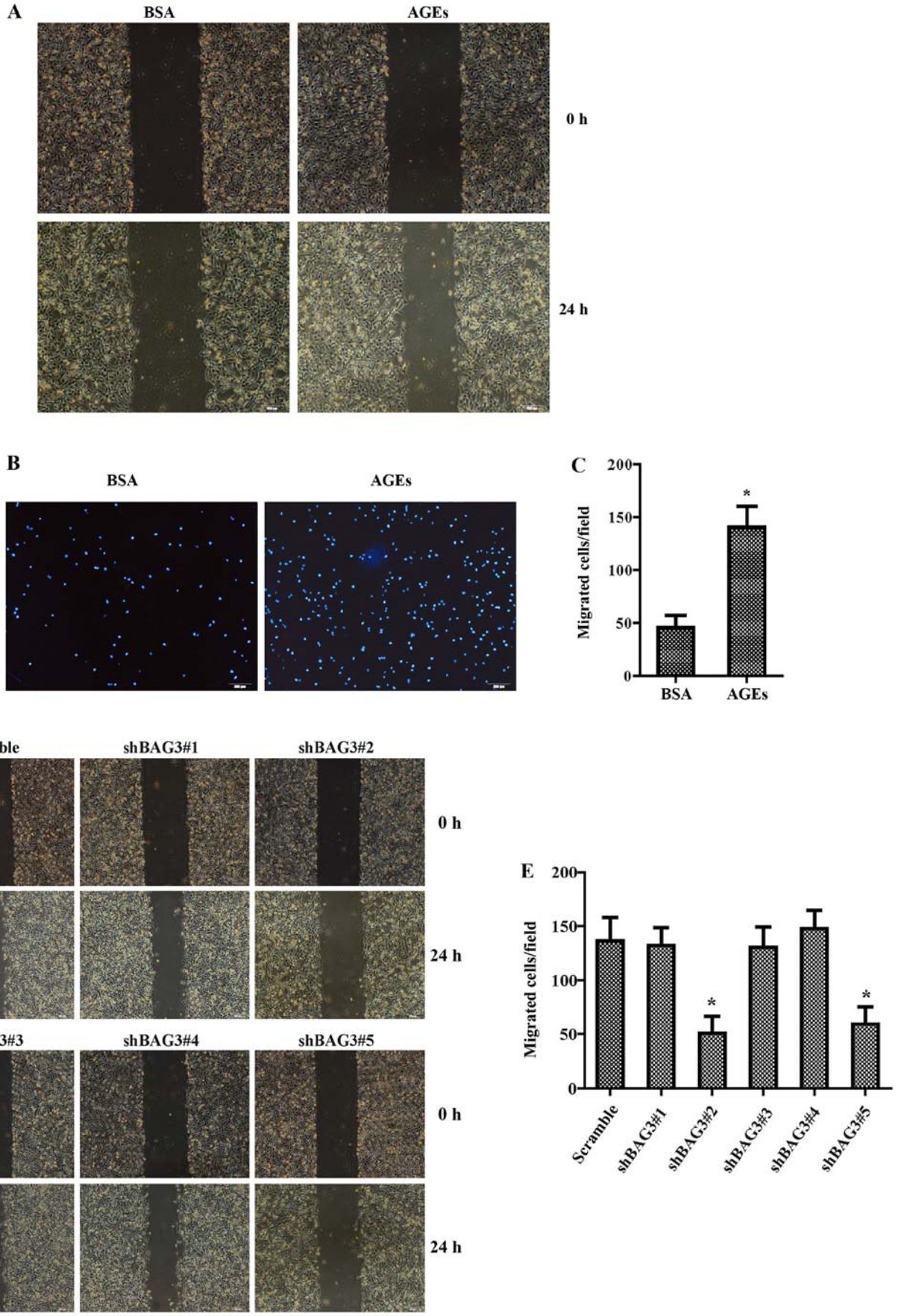

Figure 4. Advanced glycation end products (AGEs) promote the migration of primary rat vascular smooth muscle cells (VSMCs) via Bcl-2-associated athanogene 3 (BAG3). VSMCs were treated with 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. (A) Cell migration was detected by wound healing assay and (B) Transwell assay. (C) Migrated cells were quantified by ImageJ. Then the migration of VSMCs transfected with shRNAs against BAG3 (shBAG3) was detected by (D) wound healing assay and (E) Transwell assay. The experiments were repeated three times with reproducible results. $^{*}p<0.05$ compared with the control.

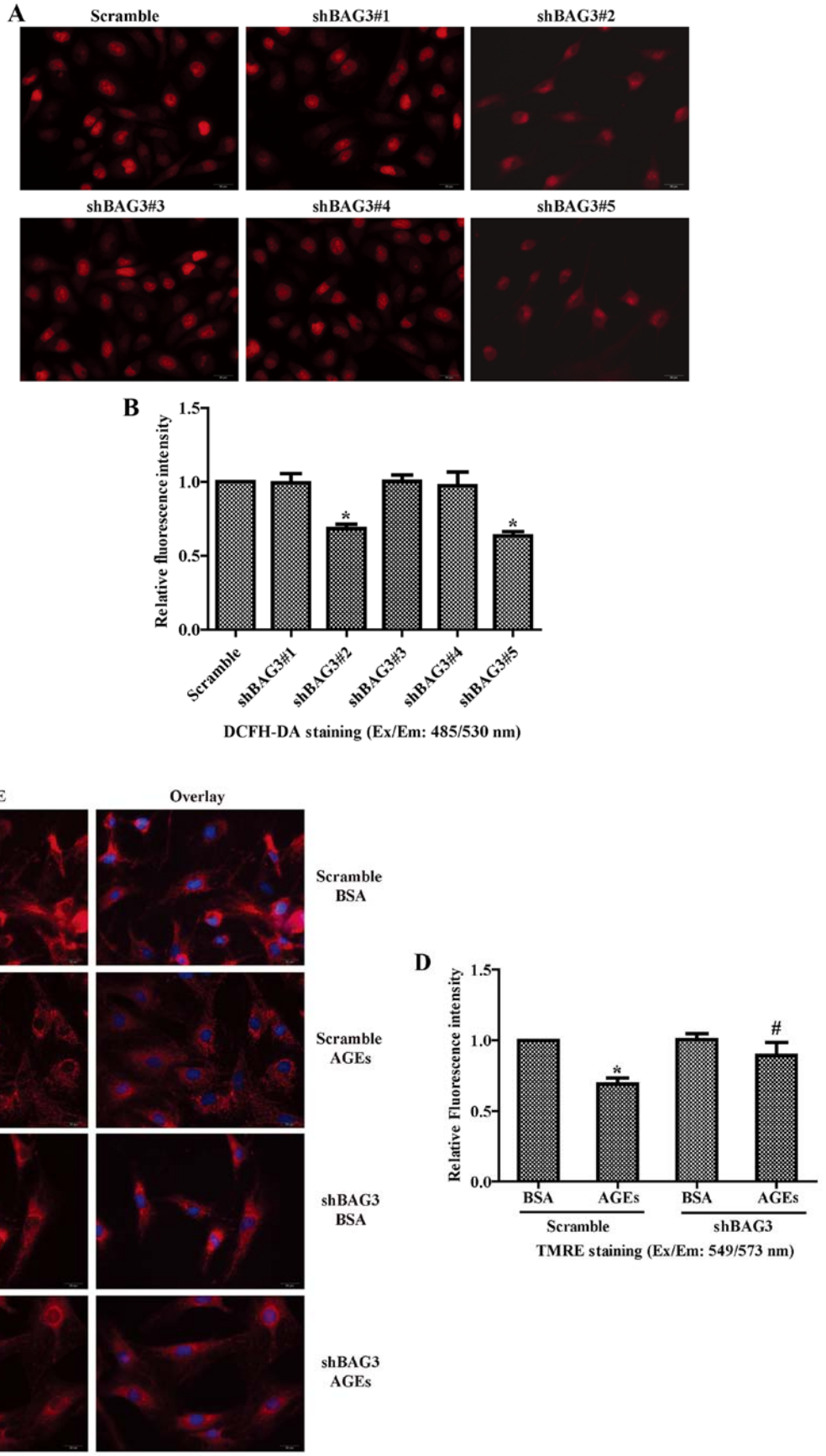

Figure 5. Effect of Bcl-2-associated athanogene 3 (BAG3) on the oxidative stress and mitochondrial membrane potential of vascular smooth muscle cells (VSMCs). (A) The cells were labeled with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Ex/Em, 485/530 nm) to detect reactive oxygen species (ROS) and analyzed with a fluorescence microscope (scale bar, 20 μm). (B) The fluorescence intensity of DCFH-DA staining was quantified using ImageJ, normalized by the scramble group, *p<0.05 vs. the scramble group. (C) The mitochondria were labeled with tetramethylrhodamine methyl ester (TMRE) (Ex/Em, 549/573 nm) to detect mitochondrial membrane potential and analyzed with a fluorescence micro-scope (scale bar, 20 μm). (D) The fluorescence intensity of TMRE staining was quantified using ImageJ, normalized by the scramble group, *p<0.05 vs. the scramble group; $^{\#}$p<0.05 vs. the scramble + advanced glycation end product (AGE) group.

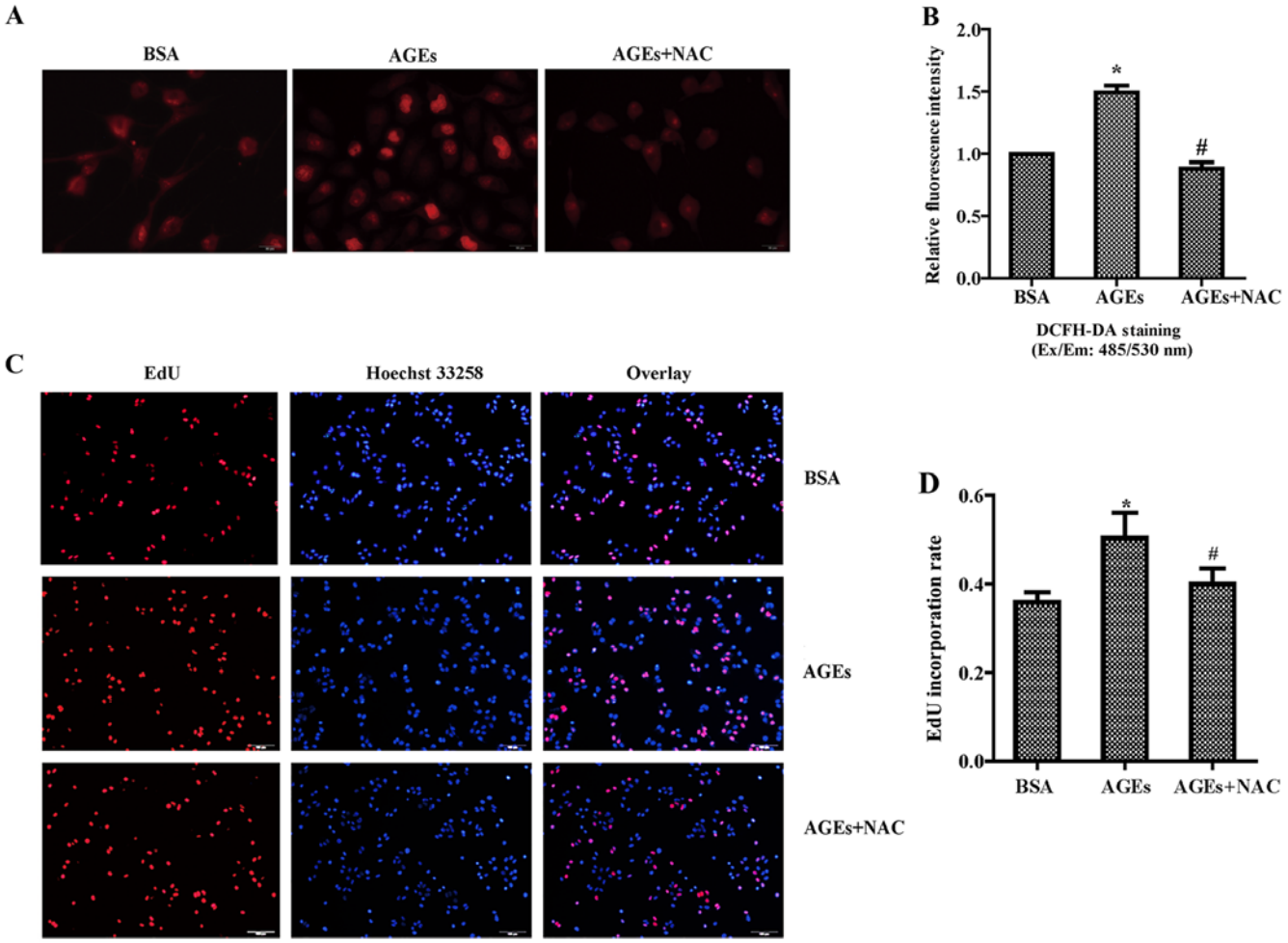

Figure 6. Advanced glycation end products (AGEs) promote the proliferation and migration of vascular smooth muscle cells (VSMCs) via oxidative stress. Cells were incubated with 100 μg/ml *N*-acetylcysteine (NAC) and 100 μg/ml AGEs or 10 μg/ml BSA for 24 h. (A) Reactive oxygen species (ROS) were detected by DCHF-DA assay. (B) The fluorescence intensity of 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining was quantified using ImageJ, normalized by the scramble group. (C and D) Cell proliferation was detected by EdU assay. *p<0.05 and $^{\#}$p<0.05 compared with the BSA group.

migration of VSMCs induced by AGEs. As far as we know, currents there are scarce data on AGEs and BAG3. However, it is reasonable to speculate that unfolded protein response (UPR) is a main mediator. Endoplasmic reticulum (ER) is responsible for the post-translational modification, folding and trafficking of approximately one-third of all cellular proteins (48). Under physiological conditions, ER can maintain a balance between folded and misfolded proteins. However, when unfolded/misfolded protein accumulation impairs ER homeostasis, ER stress occurs, which could further activate UPR (48). As summarized (49), AGEs induce the UPR in different cell types including endothelial, neuronal, pancreatic cells and podocytes, suggesting this crosstalk as an underlying pathological mechanism that contributes to metabolic diseases. At the same time, BAG3, as a molecular chaperones, plays a major role in protein quality control and could sense misfolded proteins and direct them to protein degradation systems (50). Therefore, the increased expression of BAG3 seems to protect against cell death under extreme stimuli. However, the exact mechanism by which AGEs induce the expression of BAG3 in VSMCs warrants further investigation.

In addition, our data demonstrated that shBAG3 reduced the proliferation and migration of VSMCs and ROS production; while reducing ROS production by NAC also inhibited the proliferation and migration of VSMCs. These results indicated that BAG3 is a regulator of ROS. A previous study (51) found that BAG3 overexpression significantly decreased lipid peroxidative product MDA content but increased SOD and GSH-Px activity (two important anti-oxidases) in cardiomyocytes after anoxia/reoxygenation injury, which indicated that BAG3 plays an important role in reducing ROS generation of cardiomyocytes. The difference between these two studies indicate that the mechanism by which BAG3 regulates ROS may be different in different cell type and is due to various signaling pathways. In VSMCs, ROS are mainly produced by NOX activity and mitochondrial respiratory electron transport chain during oxidative respiration (32). ROS include superoxide anion, hydroxyl radicals and hydrogen peroxide, which are the destructive feature of oxidative stress (17). Previous research indicates that ROS are involved in various vascular cell signaling via modulating redox-sensitive transcription and transduction pathways (52). Furthermore, increasing evidence has demonstrated that ROS accumulation plays an important role in the proliferation and migration of VSMCs (15-17). Thereby, attenuating ROS production may be a promising

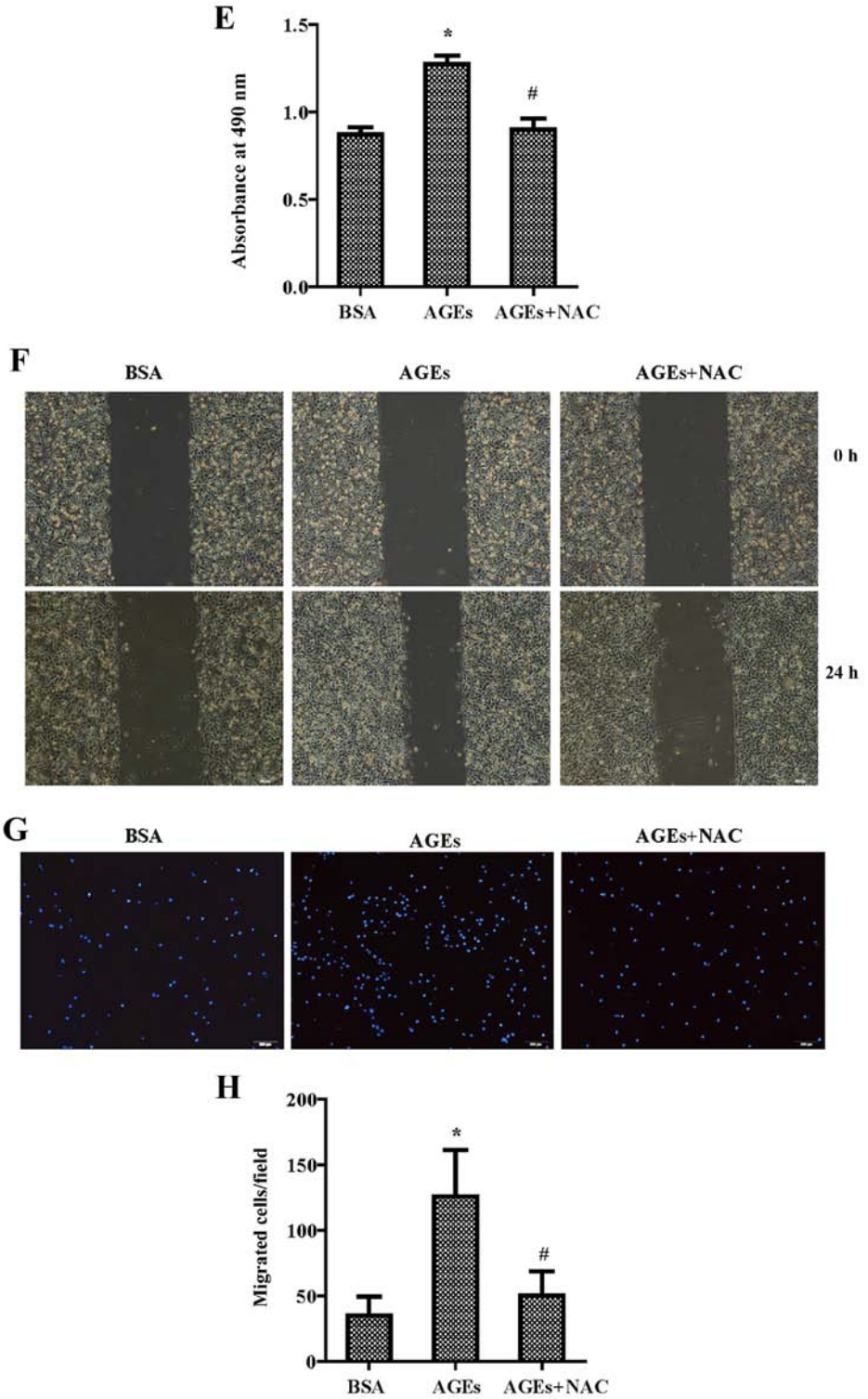

Figure 6. Continued. (E) Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell migration was detected by (F) wound healing assay and (G and H) Transwell assay. The experiments were repeated three times with reproducible results. $^{*}p<0.05$ compared with the BSA control.

therapeutic stategy for preventing the proliferation and migration of VSMCs in the process of vascular complications of diabetes.

In conclusion, the present study demonstrated for the first time that AGEs increase ROS production and promote the proliferation and migration of VSMCs by upregulating BAG3 expression. This study suggests that BAG3 is a potential target for the prevention and/or treatment of the vascular complications of diabetes.

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