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SiRNA-HMGA2 weakened AGEs-induced epithelial-to-mesenchymal transition in tubular epithelial cells

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ABSTRACT

Diabetic nephropathy as the most common cause of end-stage renal disease accounts for a significant increase in morbidity and mortality in patients. Epithelial to mesenchymal transition (EMT) of tubular cells is associated with diabetic nephropathy. Advanced glycation end products (AGEs) are thought to be involved in the pathogenesis of diabetic nephropathy via multifactorial mechanisms. However, whether AGEs could induce EMT in Tubular epithelial cells is still unknown. In this study, we found that AGEs induced EMT and accompanied by reduced expression of the epithelial markers E-cadherin and enhanced expression of the mesenchymal markers vimentin and alpha-smooth muscle actin. Furthermore, the expression of HMGA2 was upregulated by AGEs. Far more interesting, its knockdown by short interfering RNA (siRNA) effectively reversed AGEs-induced EMT. Meanwhile, we also found that knockdown of HMGA2 inhibited high AGEs-induced generation of reactive oxygen species (ROS) and the activation of p38 MAPK. Collectively, these studies suggest that HMGA2 plays a important role in EMT during Diabetic nephropathy and more study toward HMGA2 should be played in renal pathogenesis.

1. Introduction

Diabetic nephropathy is defined as a progressive decline in glomerular filtration rate, accompanied by proteinuria and other end-organ complications. Diabetic nephropathy as one of the most important complications of diabetic patients has become the second leading cause of the end-stage renal disease after glomerulonephritis and increased by more than 50% in the last 10 years [1,2]. Previous study postulated that Diabetic nephropathy occurs as a result of the interplay of metabolic and hemodynamic factors in the renal microcirculation [3]. We believe that the pathogenesis of diabetic nephropathy is complex and multidimensional involving the interaction of many networks of abnormalities and pathways and some mechanism is unclear requiring further research.

Advanced glycation end products (AGEs) derived from reducing sugars reaction non-enzymatically constitute a diverse group of compounds formed when glucose or other reducing sugars such as galactose and fructose react with amino acids, nucleotide bases or fatty acids, forming glycated molecules [4]. AGEs play an important role in the pathogenesis of numerous diseases, including diabetic complications, aging and atherosclerosis [5,6]. Recently, considerable evidences have suggested that AGEs might contribute to diabetic nephropathy. Study found that AGEs could induce the tubular cells apoptosis and asymmetric dimethylarginine (ADMA) generation [7]. Secondly, the interaction between AGEs and their receptors could trigger oxidative stress damage which played a crucial role in the development and progression of Diabetic nephropathy [8]. Epithelial to mesenchymal transition (EMT) play an important role in cellular transdifferentiation during embryonic development, tumor invasion and metastasis and in tissue fibrosis [9]. Study showed that Epithelial-mesenchymal transition (EMT) participates in the happenings of Diabetic nephropathy [10]. However, there are little reports referring to the effect of AGEs on EMT in Tubular epithelial cells. The present study aimed to explore the role of AGEs in epithelial–to–mesenchymal transition and AGEs-induced HMGA2 (high mobility group AT-hook 2) level changes as well as the effect of SiRNA-HMGA2 on AGEs-induced EMT in vitro.

Abbreviations: HMGA2, high mobility group AT-hook 2; EMT, epithelial-to-mesenchymal transition; ROS, reactive oxygen species; α-SMA, α-smooth muscle actin; PI, propidium iodide; MTT, 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; AGEs, Advanced glycation end products; DCF-DA, 2,7-dichlorofluorescein-diacetate; TBS, tris-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

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2. Materials and methods

2.1. Reagents

AGEs,RNase-free DNaseI,DMSO, MTT and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMEM/F12 and Fetal bovine serum (FBS) were obtained from Gibco Laboratories (NY,USA). Anti-β-actin, anti–P38, anti-phospho P38, anti-vimentin were obtained were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-SMA, anti-E-cadherin were purchased from Biosciences (WA Seattle, USA). Anti-HMGA2 were obtained from Sigma (St. Louis, USA). ECL kit was purchased Pierce (thermo Co. Ltd, USA). Reactive oxygen species assay kit was purchased from Beyotime (Shanghai, China). Flow cytometer (FACSCalibur, Becton–Dickinson, USA). All reagents used were trace element analysis grade. All water used was glass distilled.

2.2. Cell culture

NRK-52E cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium DMEM/F12 medium supplemented with 10% fetal bovine serum in a humidified atmosphere consisting of 5% CO_2 at 37 °C. They were subcultured every 3 days using 0.2% trypsin plus 0.02%EDTA. For experiments, cells were cultured for 24 h to obtain monolayers in 3 ml DMEM/F12 with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged and the cells were cultured further.

2.3. Cell viability

Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 105 cells/ml in 96-well plates. Briefly, 10 μl MTT (final concentration, 5 mg/mL) was added to the medium and incubated at 37 °C for 4 h. The MTT solution was removed and 100 μl dimethyl sulfoxide (DMSO) was added to dissolve the colored formazan crystals for 15 min. The optical densities were measured using microplate reader at 490 nm. Relative cellular growth was determined by a ratio of average absorbance in treatment cells versus the average absorbance in control cells. The cell viability was calculated as the ratio of optical densities.

2.4. Detection of intracellular ROS level

ROS were detected according to the manufacturer's instructions of reactive oxygen species assay kit (Beyotime, China). Briefly, NRK-52E cells (4 × 106) were incubated with 10 μmol/L DCFH-DA probes at 37 °C for 30 min and washed with PBS for 3 times. DCFH-DA was deacetylated intracellularly by nonspecific esterase, which was further oxidized by ROS to the fluorescent compound 2,7-dichlorofluorescein (DCF). DCF fluorescence was detected by flow cytometer using a Becton–Dickinson FACS Caliber and analyzed by Cell Quest software (Becton–Dickinson, San Jose, CA).

3. RT-PCR

Total RNA from cultured cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). We used RNase-free DNaseI to eliminate genomic DNA contamination in RNA samples. The 260/280 absorbance ratio was measured for verification of the purity of RNA. The sequences of Vimentin, FN, E-cadherin, HMGA2 and GAPDH genes were obtained from the GenBank database, and specific primers for them were designed over an exon–exon junction with Primer Premier 5.0. The following primers were used: GAPDH:5′-ACCACAGTCCATGAAATCAC-3′, 5′-AGGTTTCTCCAGGCGGC ATG-3′; α-SMA: 5′-TGTGGTAG AGCAAGACTGGAG A-3′,5′- GGCAGAA G AGGGTGATACAGTT-3′; E-cadherin: 5′-CCTGGGA CTCCACCTACAGA-3′,5′-GGATGACACAGCGTGAGAGA; Vimentin: 5′- GC CATCAA-CACCGA GTTCAAG -3′, 5′- TTCGTTTGACTCCTGCTTTGC-3′. HMGA2: 5′-TCCCTCT AAAGCAGCTCAAAA-3′, 5′-ACTTGTTG TG GCC AT TTCCT-3′. PCR reactions were performed with a Gene Amp PCR system 9700 (PerkinElmer, USA) and amplified for 35 cycles. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Each product was visualized after separation and using GAPDH as an internal control. Image density was quantified with a FluoroImager SI (Amersham Pharmacia Biotech).

3.1. Western blot analysis

Samples containing 10 μg of proteins were electrophoresed and then transferred to nitrocellulose membranes. The nitrocellulose membrane was cut according the molecular weight of protein and be incubated with different protein antibody. The following primary antibodies were used: The primary antibodies used in this study include anti-β-actin (1:400), anti-P38 (1:400), anti-p-P38 (1:400), anti-vimentin (1:400), anti-α-SMA (1:400), anti-E-cadherin (1:400), anti-HMGA2 (1:400). The appropriate peroxidase-conjugated secondary antibodies were used, detection was performed using an enhanced chemiluminescence kit (Pierce), The relative amounts of various proteins were analyzed. The results were quantified by Quantity One Software.

3.2. RNA interference plasmid construction and transient transfection

Cells were placed in standard medium without antibiotics for 24 h. The HMGA2 siRNA plasmid and control siRNA plasmid were purchased from Genechem Co. (Shanghai, China). The target sequences of the HMGA2 siRNA and control HMGA2 siRNA were BLAST searched against the GenBank database. The HMGA2 targeting sequence matched exactly with partial sequences of the rat HMGA2 gene, but not with any other genes. The control siRNA did not match any known HMGA2 gene. Transient transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's specifications. After transfection, cells were harvested and analyzed at the indicated times, and the efficacy of HMGA2 inhibition was tested by RT-PCR.

3.3. Statistical analysis

Statistical analysis was performed using SPSS (Version 18). Data were expressed as mean ± standard error of the mean (SEM). Variance was homogenous for use of standard ANOVA methodology. After statistical significance was established by ANOVA, individual comparisons were made using Tukey's multiple comparison test. The level of significance was defined at $P < 0.05$.

4. Results

4.1. AGEs induces EMT in NRK-52E cells

Advanced glycation end products (AGEs) could induce cell apoptosis. But whether AGEs induced EMT is unclear. To elucidate AGEs-induced EMT, we treated the NRK-52E cells with various doses of AGEs for 24, 48 and 72 h. From the result of Fig.1A, cell viability was significantly decreased in NRK-52E cells exposed to AGEs at a dose higher than 300 μg/ml ($P < 0.01$). However, there was no significant difference in cell viability between NRK-52E cells

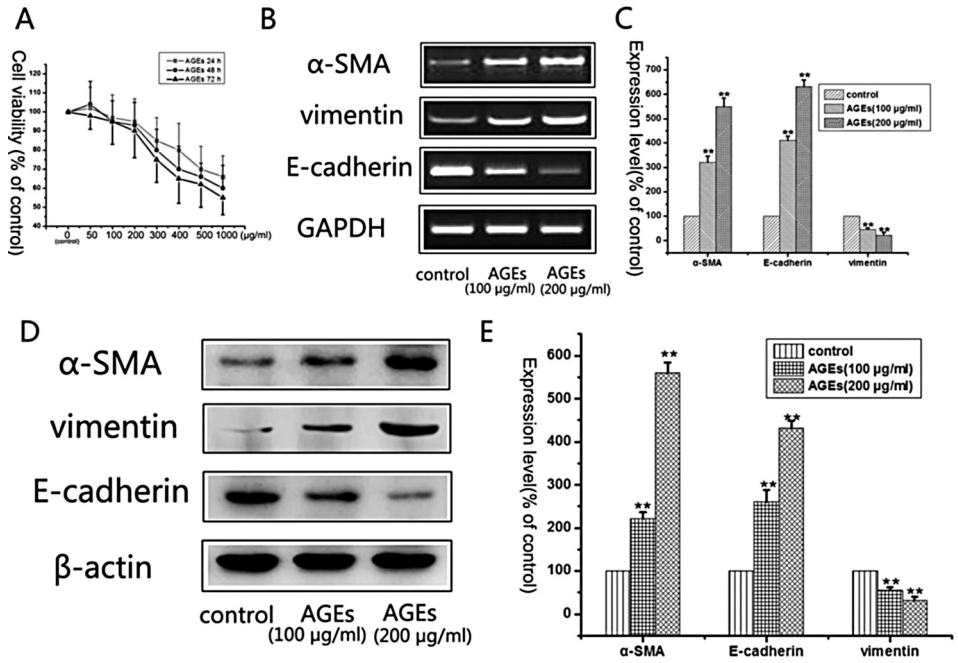

Fig. 1. Effects of AGEs on EMT in Tubular epithelial cells. (A). The NRK-52E cells were treated with various doses of AGEs (0–1000 μg/ml) for 24, 48 and 72 h, and the cell viability was analyzed by MTT assay. The data were mean ± SEM (n = 5). (B). The NRK-52E cells were treated with 0, 100 and 200 μg/ml AGEs for 24 h, and the expression of E-cadherin, vimentin and α-SMA were detected by RT-PCR. (C). The results were obtained from three independent experiments. GAPDH was as a loading control.(**$P < 0.01$ vs. control group). (D). Cells were treated as described above, and the expression level of E-cadherin, vimentin and α-SMA were detected by western-blot. (E). Each value represents mean ± SEM (n = 3). β-actin was used as loading control. (**$P < 0.01$ vs. control).

exposed to 0 or 100, 200 μg/ml AGEs ($P > 0.05$). To investigated whether the lower dose of AGEs could induce EMT, NRK-52E cells were treated with 0, 100 and 200 μg/ml AGEs for 24 h, and the expression of E-cadherin, vimentin and α-SMA were detected by RT-PCR and western-blot. RT-PCR analysis showed that AGEs treatment down-regulated E-cadherin and upregulated vimentin and α-SMA at mRNA levels (Fig. 1B and C). Similarly, western blotting analysis further confirmed the increasing expression of vimentin and α-SMA and the decreasing expression of E-cadherin at protein levels cultured with AGEs (Fig. 1D and E). Collectively, these observations suggested that NRK-52E cells had undergone an EMT after treated by AGEs.

4.2. The expression of HMGA2 was upregulated in AGEs-Induced EMT in NRK-52E cells

HMGA2 as a nonhistone nuclear-binding protein and an important regulator of cell growth and differentiation are shown to be involved in aggressive tumor growth, DNA damage response, and tumor cell differentiation [11]. However, the roles of HMGA2 in EMT are still not fully understood. To evaluate the role of HMGA2, we treated the cells with AGEs for different hours and detected the level of HMGA2 by RT-PCR and western-blot. As shown in Fig. 2A and B, NRK-52E cells were cultured under media containing various concentrations of AGEs. HMGA2 expression started to be increased by at a dose higher than 100 μg/ml AGEs. And we got a more higher level of HMGA2 in 200 μg/ml AGEs than 100 μg/ml AGEs. These observations suggested that AGEs promote the expression of HMGA2 in dose-dependent manner. Furthermore, RT-PCR and western-blot analysis showed AGEs promote HMGA2 expression in time-dependent manner (Fig. 2C and D). Taken together, these observations demonstrated that HMGA2 expression is upregulated in AGEs-induced EMT in NRK-52E cells.

4.3. The HMGA2 protein is involved in AGEs-Induced EMT in NRK-52E cells

To evaluate the role of HMGA2, we compared the degree of EMT in NRK-52E cells by modulating HMGA2 expression in NRK-52E cells through siRNA knockdown. The HMGA2 siRNA plasmid were transfected to the NRK-52E cells for 0–72 h and the expression of HMGA2 was detected by RT-PCR. From the results of RT-PCR, we found that siRNA-HMGA2 get the best effect of transfection in 48 h and the inhibition rate of siRNA-HMGA2 reached more than 50% (Fig.3A). Second, To further investigate the role of HMGA2 in AGEs-induced EMT, control cells, SiRNA-control cells and HMGA2-siRNA cells were exposed to 200 μg/ml AGEs for 24 h, and the expression level of E-cadherin, vimentin and α-SMA were detected by western-blot. As the results of Fig.3B and C revealed, siRNA-HMGA2 inhibit EMT changes. Silencing of HMGA2 attenuated AGEs-induced down-regulation of E-cadherin and up-regulation of vimentin and α-SMA which were not observed in control si-RNA-transfected cells or control cells (Fig. 3B and C). These findings demonstrated that the expression of HMGA2 may trigger EMT in NRK-52E cells. Based on these observations, we assessed that the up-regulation of HMGA2 may be crucial for AGEs-induced EMT in NRK-52E cells.

4.4. SiRNA-HMGA2 inhibit ROS/P38 pathways

The ROS/p38-MAPK signaling pathway has been reported to be involved in EMT in the different cells [12,13]. Having shown that SiRNA-HMGA2 inhibited EMT, we next explored the effect of SiRNA-HMGA2 on ROS/P38-MAPK signaling pathway in NRK-52E cells. Compared to the control group, the intracellular ROS production were not changed significantly in siRNA-control cells and HMGA2-SiRNA cells (shown in Fig.4A). However, when cultured with 200 μg/ml AGEs for 24 h, the level of intracellular ROS was

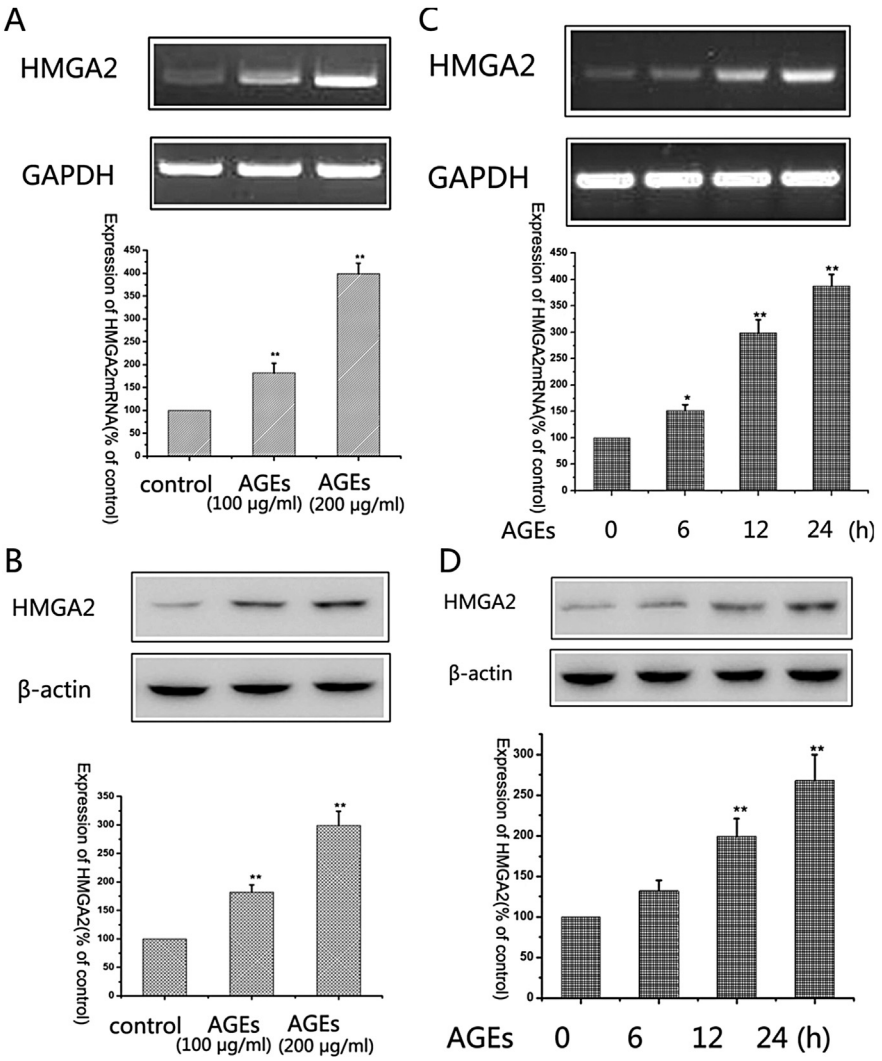

Fig. 2. Effect of AGEs on HMGA2 expression in Tubular epithelial cells. (A). The NRK-52E cells were treated with 0, 100 and 200 μg/ml AGEs for 24 h, and the expression of HMGA2 was detected by RT-PCR. The results were obtained from three independent experiments. GAPDH was as a loading control.(**$P < 0.01$ vs. control group). (B). Cells were treated as described above, and the expression level of HMGA2 was detected by western-blot. Each value represents mean ± SEM (n = 3). β-actin was used as loading control. (**$P < 0.01$ vs. control). (C). The NRK-52E cells was treated with 200 μg/ml AGEs for 0,6,12 and 24 h, and the expression of HMGA2 was detected by RT-PCR. The results were obtained from three independent experiments. GAPDH was as a loading control.(**$P < 0.01$ vs. 0 h group). (D). Cells were treated as described above, and the expression level of HMGA2 was detected by western-blot. Each value represents mean ± SEM (n = 3). β-actin was used as loading control. (**$P < 0.01$ vs.0 h group).

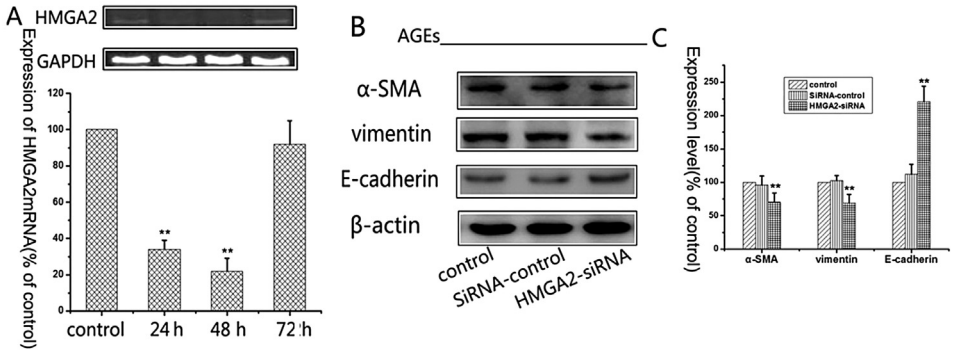

Fig. 3. SiRNA-HMGA2 inhibit AGEs-induced EMT in Tubular epithelial cells. (A). RT-PCR analysis was performed from isolated total RNA for HMGA2 mRNA after HMGA2-siRNA transfection at different times in NRK-52E cells. Each value represents mean ± SEM (n = 3). GAPDH was as a loading control. (**$P < 0.01$ vs. control). (B). NRK-52E cells were transfected with HMGA2-siRNA plasmid and the cells were allowed to recover in regular culture medium for 24 h after transfection. Then all the cells (control cells, siRNA-control cells and HMGA2-siRNA cells) were exposed to 200 μg/ml AGEs for an additional 24 h, and the expression level of E-cadherin, vimentin and α-SMA were detected by western-blot. (C). Each value represents mean ± SEM (n = 3). β-actin was used as loading control. (**$P < 0.01$ vs. control).

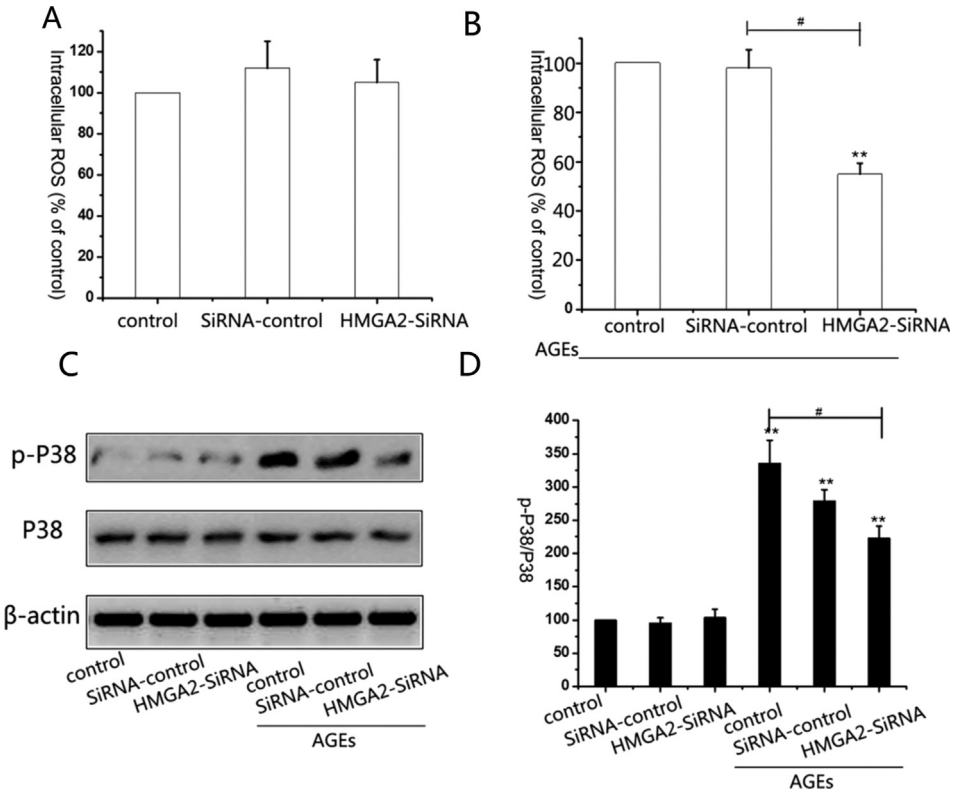

Fig. 4. Effect of HMGA2 on AGEs-induced ROS/P38 signaling pathways. (A). The cells (control cells, SiRNA-control cells and HMGA2-SiRNA cells) were stained with DCF-DA to detect the intracellular ROS production. Value represents mean ± SEM (n = 3). (B). The cells (control cells, SiRNA-control cells and HMGA2-SiRNA cells) were treated with 200 μg/ml AGEs for 24 h and stained with DCF-DA to detect the intracellular ROS production. Value represents mean ± SEM (n = 3). (**$P < 0.01$ vs. control; #$P < 0.01$ SiRNA control group vs. HMGA2-SiRNA group).(C). The cells (control cells, SiRNA-control cells and HMGA2-SiRNA cells) were treated with or without 200 μg/ml AGEs for 24 h and the expression of P38 and p-P38 were detected by western-blot. (D). Each value represents mean ± SEM (n = 3). β-actin was used as loading control. (**$P < 0.01$ vs. control; #$P < 0.01$ control group vs. HMGA2-SiRNA group).

significantly increased in control group or siRNA-control group and these changes were significantly attenuated by HMGA2-SiRNA in HMGA2-SiRNA group (Fig.4B). Theses results suggest that the expression of the HMGA2 is responsible for the activation of ROS pathway. We next measured the expression of P38 or P38 phosphorylation in NRK-52E cells. Similarly, HMGA2-SiRNA attenuated AGEs-induced up-regulation of P38 phosphorylation compared to the controls. But the level of P38 did not changed significantly (Fig. 4C and D). These results demonstrated that HMGA2 play a key role of activating ROS/P38 signaling that lead to the phosphorylation of P38 and subsequently EMT changes.

5. Disscussion

HMGA protein family consists of four members: HMGA1a, HMGA1b, HMGA1c, and HMGA2. They influence a diverse array of normal biological processes including cell growth, proliferation, differentiation and death [14]. HMGA2 as an oncofetal protein is overexpressed in embryonic tissue and many malignant neoplasms, but rarely in normal adult tissues [15]. As an architectural transcription factor, HMGA2 can modify the structure of its binding partners to generate a conformation that facilitates various DNA-dependent activities and influence a variety of biological processes including cell growth, proliferation, differentiation and death [16]. Study found that HMGA2 protein has a close relationship with tumor growth, progression, invasion and metastasis. Kumar MS found HMGA2 both as a protein-coding gene and as a non-coding RNA is highly expressed in metastatic lung adenocarcinoma, in which it contributes to cancer progression and metastasis [17]. Müller MH found that HMGA2 is expressed independently of external stimuli, whereas proliferation stimulated by growth factors is independent of further elevated HMGA2 expression [18]. The relationship between HMGA2 and EMT are unclear. And this article is to explore the relationship between AGE-induced EMT and the expression of HMGA2. First, NRK-52E cells cultured with AGEs had a significant loss of E-cadherin and increase of vimentin and had undergone an EMT after treated by AGEs (Fig. 1). Second, our observations demonstrated that HMGA2 expression is upregulated in AGEs-induced EMT in NRK-52E cells (Fig. 2). Until recently, HMGA2 has not been linked to EMT regulation. We study the relationship between EMT and HMGA2 through siRNA knockdown. Our study demonstrated that the expression of HMGA2 may trigger EMT and silencing of HMGA2 attenuated AGEs-induced EMT in NRK-52E cells (Fig. 3).

Studies have revealed that EMT can be induced by factors such as reactive oxygen species (ROS) [19]. Moreover, ROS can crosstalk with multiple signaling pathways, such as MAPK, PI3K and NF-κB [20]. It is worth to further investigating the relation between these signaling pathways and the HMGA2 protein. Our study found that HMGA2 play a key role of activating ROS/P38 signaling and SiRNA-HMGA2 attenuated AGEs-induced up-regulation of P38 phosphorylation. But the specific signaling pathways and transcription factor genes change is not particularly clear. Further characterization of how HMGA2 regulates these signaling pathways and gene expressions and their EMT function will provide new insight for our understanding of Diabetic nephropathy.

In summary, we have for the first time, to our knowledge, achieved HMGA2 upregulation in AGEs-induced EMT in NRK-52E cells.

We found that SiRNA-HMGA2 was sufficient to inhibit AGEs-induced EMT. Moreover, we also found that HMGA2 play a key role of activating ROS/P38 signaling that lead to subsequently EMT changes. Further characterization of the functional relationship between HMGA2 and AGEs-mediated EMT target gene regulation will help us understand the genesis of diabetic nephropathy.

Conflict of interest

None.

Acknowledgments

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Transparency document

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