SUMOylation and deacetylation affect NF-κB p65 activity induced by high glucose in human lens epithelial cells

Xiao Han¹, Xiao-Xuan Dong², Ming-Yu Shi¹, Li Feng¹, Xin-Ling Wang¹, Jin-Song Zhang¹, Qi-Chang Yan¹

¹Department of Ophthalmology, the Fourth Affiliated Hospital of China Medical University; Key Laboratory of Lens Research of Liaoning Province; Eye Hospital of China Medical University, Shenyang 110005, Liaoning Province, China
²Department of Ophthalmology, the Fourth People’s Hospital of Shenyang, Shenyang 110031, Liaoning Province, China

Correspondence to: Qi-Chang Yan. Department of Ophthalmology, the Fourth Affiliated Hospital of China Medical University; Key Laboratory of Lens Research of Liaoning Province; Eye Hospital of China Medical University, 11Xinhua Road, Heping District, Shenyang 110005, China. cmu4h_yqc@163.com

Received: 2018-12-25 Accepted: 2019-05-07

Abstract

AIM: To explore the effects of IκBα SUMOylation and NF-κB p65 deacetylation on NF-κB p65 activity induced by high glucose in cultured human lens epithelial cells (HLECs).

METHODS: HLECs (SRA01/04) were cultured with 5.5, 25, and 50 mmol/L glucose media for 24h, and with 50 mmol/L glucose media for 0, 12, and 24h respectively. SUMO1 and SIRT1 expressions were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot (WB). IκBα and NF-κB p65 expressions were detected by WB. With NAC, DTT, MG132 or Resveratrol (RSV) treatment, SUMO1 and SIRT1 expressions were detected by WB. Protein expression localizations were examined by immunofluorescence and co-immunofluorescence. The effects of SUMO1 or SIRT1 overexpression, as well as MG132 and RSV, on the nuclear expression and activity of IκBα and NF-κB p65 were analyzed by immunoblot and dual luciferase reporter gene assay.

RESULTS: SUMO1 and SIRT1 expressions were influenced by high glucose in mRNA and protein levels, which could be blocked by NAC or DTT. SUMO1 was down-regulated by using MG132, and SIRT1 was up-regulated under RSV treatment. IκBα nuclear expression was attenuated and NF-κB p65 was opposite under high glucose, while IκBα and NF-κB p65 location was transferred to the nucleus. SUMO1 or SIRT1 overexpression and MG132 or RSV treatment affected the nuclear expression and activity of IκBα and NF-κB p65 under high glucose condition.

CONCLUSION: IκBα SUMOylation and NF-κB p65 deacetylation affect NF-κB p65 activity in cultured HLECs under high glucose, and presumably play a significant role in controlling diabetic cataract.

KEYWORDS: SUMOylation; deacetylation; NF-κB; IκBα; diabetic cataract; high glucose; lens epithelial cells

DOI:10.18240/ijo.2019.09.01

INTRODUCTION

Diabetic cataract (DC) is a common complication of type 1 diabetes mellitus[1]. High blood glucose can obviously accelerate cataract progression in age-related cataract patients[2] and promote cataract formation in type 2 diabetes mellitus patients[3]. High glucose treatment can cause oxidative stress to damage the human lens epithelial cells (HLECs) and form cataractogenesis[4]. Meanwhile, it is widely acknowledged that oxidative stress is one of major mechanisms of DC[5-8]. In previous studies, it is clarified that oxidative stress can motivate various signal pathways, as well as posttranslational modification (PTM), such as SUMOylation[9-11] and deacetylation[12-13]. Nuclear factor κB (NF-κB) as a sensitive transcriptional factor plays an important role in regulating oxidative stress which contributes to DC[12-13]. However, whether SUMOylation and deacetylation has relationship with NF-κB signal pathway involving in DC in HLECs is still unknown.

SUMOylation is one of PTM, which has been validated to protect target proteins from degradation by ubiquitination[14-15]. There are four various isoforms of SUMO grouped into three classes, SUMO1, SUMO2/3 and SUMO4 mediating SUMOylation in mammals[16]. SUMO1, the most well-known SUMO family member, is involved in kinds of cellular processes and resulted in different diseases. It establishes an essential role of regulating transcriptional factors[17], protecting cell[18], inhibiting oxidative stress reaction[19], repairing DNA damage[20], preventing apoptosis[21] and so on. Nevertheless, there is few study of cataract to date have focused on SUMO1 and SUMOylation.
SIRT1, the most crucial sirtuin family member, modifies deacetylation (another significant PTM) and brings on various physiological and pathological processes. SIRT1, as a longevity gene, is participated in reducing oxidative stress\cite{22}, preventing apoptosis\cite{23}, resisting aging\cite{24} and all that. It has demonstrated the relationship between SIRT1 and age-related cataract in others studies\cite{25-26}. Increasing evidence suggests that Resveratrol (RSV), a famous antioxidant and anti-aging agent, can accelerate the expression and activity of SIRT1\cite{27}. But the influence of SIRT1 and RSV in HLECs under high glucose remains poorly understood.

NF-κB is a significant stress responsive transcriptional factor located in the cytoplasm in nonactivated state and its activity can be affected by various external stimuli. NF-κB p65, as a prominent member of NF-κB family, is concerned with various stimuli stress, especially oxidative stress\cite{28}. When stimulated, NF-κB is activated and translocated into the nucleus. Its transcriptional activity is controlled by inhibitor IκB proteins\cite{29} and IκBα is a main member of IκBs. NF-κB activity is determined by degradation of IκB which is mediated by ubiquitin-proteasome pathway (UPP). Therefore, in another study, it has verified the proteasome inhibitor MG132 reversed IκBα degradation and decreased NF-κB expression and activity which was induced by high glucose in rat mesangial cells\cite{30}. MG132 treatment could also accumulate the conjugations of SUMO and target proteins\cite{31}. Liu et al\cite{32} found that ubiquitin and SUMO competed for the same target lysine on K11/22 of IκBα in previous study. It was cleared that acetylation sites of NF-κB p65 were found on lysines K221, K310, and K122/123, although there were different effects on different lysines\cite{33}. This study is the first to demonstrate whether high glucose could induce SUMO1 and SIRT1 expression owing to oxidative stress in cultured HLECs, and whether IκBα SUMOylation and NF-κB p65 deacetylation could affect NF-κB p65 activity in vitro. The results showed SUMO1 or SIRT1 overexpression could influence the nuclear expression of IκBα and NF-κB p65, as well as the activity of NF-κB p65 in cultured HLECs. Meanwhile, it was the first time to investigate the effects of MG132 and RSV on protecting lens transparency from oxidative damage induced by high glucose through regulation of NF-κB p65 activity in HLECs.

**MATERIALS AND METHODS**

**Cell Culture and Treatments** Human lens epithelial cells (SRA01/04) were gifts from Key Lens Laboratory of Lens Research of Liaoning Province. The cells were cultured in Dulbecco’s modified Eagle’s media (DMEM; Hyclone) with 5.5 mmol/L glucose, 10% fetal bovine serum (FBS; Invitrogen), 100 mg/mL streptomycin (Hyclone) and 100 IU/mL penicillin (Hyclone) in a 5% CO2 humidified atmosphere at 37°C. The SRA01/04 cells were grown to 75%-80% confluence and divided randomly into several groups: normal control glucose group (NC; media with 5.5 mmol/L glucose), high glucose 1 group (HG1; media with 25 mmol/L glucose), high glucose 2 group (HG2; media with 50 mmol/L glucose), and osmotic pressure control group (OP; media with 50 mmol/L mannitol). N-acetyl cysteine (NAC; Sigma) 5 mmol/L for 4h or the thiol-reducing agent dithiothreitol (DTT; Sigma) 10 mmol/L for 1h was as antioxidant addressed in high glucose media. MG132 (Sigma) 10 µmol/L as the proteasome inhibitor added in media for 4h. RSV (Sigma) 10 µmol/L as SIRT1 activator was participated in media for 4h.

**Reverse Transcription-Polymerase Chain Reaction** Total RNA from SRA01/04 cells was extracted using TRIzol (TaKaRa) and reverse transcribed using M-MLV First Kit (Invitrogen) to get cDNA, which was amplified using Taq DNA polymerase Recombinant Kit (Invitrogen). The results were determined using chemiluminescent gel imaging system and normalized to β-actin gene expression. The primer sequences were as followed: SUMO1 (forward: 5’-tgg aca gga tag cag tga ga-3’; reverse: 5’-tct tcc tcc att ccc agt tgt-3’; product size: 174 bp), SIRT1 (forward: 5’-cca gcc atc tgt cca ca-3’; reverse: 5’-tcc tgc tag cgc atc aca gt-3’; product size: 193 bp), β-actin (forward: 5’-cat ccg taa aga cct cta tgc caa c-3’; reverse: 5’-atg gag cca ccg ac tca cac a-3’; product size: 171 bp).

**Western Blot** Total proteins from SRA01/04 cells were extracted using RIPA lysis buffer with PMSF and protease inhibitor cocktail set (Calbiochem, Germany). Nuclear proteins from SRA01/04 cells were extracted with nuclear protein extraction kit (Beyotime, China). The lysates were separated by NuPAGE 10% Bis-Tris Gel (Invitrogen), and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Primary antibodies against SUMO1 (Abcam), SIRT1 (Abcam), NF-κB p65 (Bios, China), IκBα (Bios, China), and β-actin (Proteintech, USA) were used, as well as peroxidase-conjugated affinipure goat anti-rabbit IgG and peroxidase-conjugated affinipure goat anti-mouse IgG secondary antibodies (Jackson immunoResearch, USA). The proteins were detected by enhanced hemagglutinin (Thermo), quantified by chemiluminescent gel imaging system, and normalized to β-actin protein expression.

**Immunofluorescence** The SRA01/04 cells were cultured on cover lips in 24-well plates and were treated with 5.5 mmol/L (NC) and 50 mmol/L (HG2) glucose in media for 24h. The cells were fixed with 4% paraformaldehyde (PFA, Invitrogen) solubilized in 0.1% Triton×100-PBS for 20min, and were blocked with 5% BSA-PBS (Sigma) for 1h. The cells were incubated with anti-IκBα and anti-NF-κB p65 antibodies in 2% BSA-PBS overnight at 4°C. Alex Fluor 596 goat anti-rabbit IgG (H+L) (Invitrogen) in 2% BSA-PBS was as secondary antibody incubated for 1h in the dark room. DAPI (Beyotime,
China) used to stain the nucleus for 1 min. Images were taken with fluorescence microscope.

**SUMO1 or SIRT1 Overexpression and Immunoblot Analysis**

GFP-SUMO1 (gift from Prof. Chen [34]), GFP-SIRT1 (gift from Doctor Jiang) and empty GFP-vector (Invitrogen) were transfected with lipofectamin 2000 (Invitrogen) into cells for 6 h. After cultured 40 h, the cells were incubated with 5.5 mmol/L (NC) and 50 mmol/L (HG2) glucose media respectively for 24 h. The nuclear protein of transected SUMO1 or SIRT1 cells was extracted and detected the nuclear protein expressions of IκBα and NF-κB p65 by immunoblot. The cells transfected with empty GFP-vector were as a blank group.

**MG132 or RSV Treatment and Immunoblot Analysis**

SRA01/04 cells were cultured with normal (NC) or high glucose (HG2) media for 24 h. Then, cells were treated with 10 µmol/L MG132 or 10 µmol/L RSV for 4 h respectively. The nuclear proteins of treated cells were extracted and the nuclear expression of IκBα and NF-κB p65 was detected by immunoblot.

**Dual Luciferase Reporter Gene Assay**

SRA01/04 cells were cultured in 6 well plates and transiently transfected with pNF-κB-TA-luc, the control pGL6-TA (Beyotime, China) reporters, GFP-SUMO1, GFP-SIRT1, GFP-vector, and together with Renilla luciferase control plasmid (pRL-TK) as internal control plasmid. After 24 h co-transfection, cells were treated with or without high glucose, and MG132 or RSV 10 µmol/L treatment for 4 h. Absolute luminescence was measured according to the Dual-Luciferase Reporter Assay protocol (Beyotime, China). The relative NF-κB dual luciferase activities were measured and firefly values were normalized by Renilla values.

**Statistical Analysis**

All data were presented as the mean±SD for at least 3 independent experiments and statistical analysis was evaluated using one-way ANOVA of SPSS program version 19.0. *P*<0.05 was considered statistically significant.

**RESULTS**

**SUMO1 and SIRT1 Expression Influenced by High Glucose-Induced Oxidative Stress**

The relative SUMO1 expression of HG1 and HG2 groups were higher than that of NC group in both mRNA and protein levels. In contrast, the expression of SIRT1 was decreased in HG1 and HG2 groups, which compared with NC group in both mRNA and protein levels (Figure 1A, 1C). Compared with 0 h group, SUMO1 expression in mRNA and protein levels were enhanced in 12 h and 24 h group. There were also had different results for SIRT1 (Figure 1B, 1D). Importantly, it was confirmed that SUMO1 and SIRT1 expression changed by high glucose owing to oxidative stress rather than osmotic pressure stress (compared with NC, *P*>0.05, Figure 1A, 1C). The increase in SUMO1 and SIRT1 expression was influenced by high glucose treatment. The relative NF-κB dual luciferase activities were measured and firefly values were normalized by Renilla values.

![Figure 1](image-url)
protein could be blocked by NAC or DTT (antioxidant) treatment under high glucose condition. In the same way, the decrease in SIRT1 protein could be reversed by NAC or DTT addition in high glucose media (Figure 1E).

MG132 and RSV Influenced SUMO1 and SIRT1 Expression

The SUMO1 expression was decreased in whether normal (NC) or high glucose (HG1) condition when it was treated with MG132 (Figure 2A). As shown in Figure 2B, SIRT1 had an opposite situation. After RSV was participated in normal or high glucose media, the expression of SIRT1 was enhanced obviously.

High Glucose Affected the Nuclear Expression of IκBα and NF-κB p65

High glucose could attenuate IκBα nuclear expression, while increase the nuclear expression of NF-κB p65 (Figure 3A). However, osmotic pressure had little effect on the nuclear expressions of IκBα and NF-κB p65 (compared with NC, \( P > 0.05 \); Figure 3A). Immunofluorescence (Figure 3B, 3C) showed the locations of IκBα and NF-κB p65 were transferred to nucleus from cytoplasm induced by high glucose.

SUMO1 or SIRT1 Overexpression Influenced IκBα Nuclear Expression and NF-κB p65 Activity

SRA01/04 cells were highly efficient transfected with GFP-SUMO1 or SIRT1 respectively and cultured with high glucose media for 24h. Compared with transfected empty GFP-vector cells, IκBα nuclear expression was increased and NF-κB p65 nuclear expression was decreased in transfected SUMO1 or SIRT1.
cells under high glucose condition (Figure 4A). SRA01/04 cells were highly efficient transfected with GFP-SUMO1, GFP-SIRT1, GFP-vector, pNF-κB-TA-luc, pGL6-TA, and pRL-TK respectively and cultured with high glucose media for 24h. Compared with transfected empty GFP-vector cells, NF-κB p65 activity was decreased in transfected SUMO1 or SIRT1 cells under high glucose condition (Figure 4B). There was no obvious change in pGL6-TA control groups \((P>0.05)\; \text{(Figure 4B)}\).

**MG132 and RSV Influenced IκBα Nuclear Expression and NF-κB p65 Activity** After cultured with or without high glucose for 24h, the nuclear expression of IκBα and NF-κB p65 was changed by MG132 and RSV treatment. Both MG132 and RSV could enhance IκBα nuclear expression; in contrast, reduce the nuclear expression of NF-κB p65. The effects of MG132 and RSV were just like SUMO1 and SIRT1 overexpression on the nuclear expression of IκBα and NF-κB p65 in HLECs (Figure 5A). SRA01/04 cells were highly efficient transfected with GpNF-κB-TA-luc, pGL6-TA and pRL-TK respectively. After cultured with or without high glucose for 24h, the activity of NF-κB p65 was changed by MG132 and RSV treatment. Compared with HG2 group, both MG132 and RSV could reduce the activity of NF-κB p65 under high glucose condition. The effects of MG132 and RSV were just like SUMO1 and SIRT1 overexpression on the activity of NF-κB p65 in HLECs (Figure 5B). There was no obvious change in pGL6-TA control groups \((P>0.05)\; \text{(Figure 5B)}\).

**DISCUSSION**

To date, there was no previous experimental evidence for the function of SUMOylation and deacetylation in HLECs or pathology of diabetic cataract. In the previous work, we have discussed the expression and function of SUMO1-4 and SUMO E3 (Cbx4 and PIASy) under high glucose environment in HLECs\(^{[35]}\). The increasing studies have reported the regulation of SUMOylation and deacetylation by cellular stress, suggesting a key role of SUMOylation and deacetylation in the cellular response. Therefore, it is significant to explore study the relative proteins of SUMOylation and deacetylation under stress in HLECs.

For the first time, this finding was demonstrated that SUMO1 and SIRT1 expression was influenced by high glucose in mRNA and protein levels in HLECs. They were also changed time-dependently in mRNA and protein levels. Huang et al\(^{[30]}\) study clarified that SUMO1-3 expression was also enhanced by high glucose in rat mesangial cells. High glucose-induced oxidative stress represses SIRT expression and increases histone acetylation leading to neural tube defects\(^{[36]}\). We tried to explore the reason for the changes of SUMO1 and SIRT1 under high glucose microenvironments, because osmotic pressure stress had little effect on regulating SUMO1 and

**Figure 4 SUMO1 or SIRT1 overexpression influenced the nuclear expression of IκBα and affected the expression and activity of NF-κB p65 in HLECs**

- A: The cells transfected with SUMO1 or SIRT1 were cultured with high glucose media to detect the nuclear expression of IκBα and NF-κB p65. Compared with HG2+vector group, \(P<0.05\). The data were normalized to β-actin and expressed as mean±SD of triplicates in an independent experiment, which was repeated at least 3 times with the same results. B: The cells transfected with GFP-SUMO1, GFP-SIRT1, GFP-vector, pNF-κB-TA-luc, pGL6-TA and pRL-TK respectively were cultured with high glucose media to detect the relative NF-κB luciferase activity. Compared with transfected HG2+GFP-vector group, \(P<0.05\). There was no obvious change in pGL6-TA control groups. The value of fluorescence was normalized by Renilla values and expressed as mean±SD of triplicates in an independent experiment, which was repeated at least 9 times with the same results. NC: Media with 5.5 mmol/L glucose; HG2: Media with 50 mmol/L glucose.

SIRT1 expression in HLECs. We found the change of SUMO1 and SIRT1 expression under high glucose could be blocked and reversed by anti-oxidants, NAC or DTT. It was guessed that high glucose could regulate SUMO1 and SIRT1 protein expression owing to oxidative stress reaction, which allowed for a new SUMOylation/deSUMOylation and acetylation/deacetylation balance in response to oxidative stress. We
hypothesized that high glucose-mediated oxidative stress might decline the conjugation of SUMO1 and its target proteins, leading to endogenous free SUMO1 proteins increase. In our results, MG132 (as antioxidant) might improve SUMO1 conjugating with target proteins, which could lead to decrease of endogenous free SUMO1 under MG132 treatment in HLECs. In another study, MG132 could induce accumulation of SUMO2/3 conjugates, while reduce the expression of free endogenous SUMO2/3 in HEK 293T cells. According to the change of SIRT1 under high glucose, we hypothesized that oxidative stress might reduce SIRT1 expression and activity mediated deacetylation under high glucose. It has been recognized that H\textsubscript{2}O\textsubscript{2}-mediated oxidative stress reduces the expression and activity of SIRT1 in human lung epithelial cells. In our study, it was striking that RSV was still SIRT1 activator in HLECs.

In addition to regulating SUMO1 and SIRT1, oxidative stress is also regulated by SUMO1 and SIRT1. Researchers have revealed that SUMO1 conjugated to proteins involving in the regulation of diverse cellular events, including transcriptional regulation, stress resistance, cellular senescence, apoptosis, responses to extracellular stimuli, and especially oxidative stress. SUMOylated lysines cannot be ubiquitinated, which contribute to the stabilization of target proteins. Deacetylation of target proteins mediated by SIRT1 involved in activity/inactivity of substance, which also contributes to regulating aging, inflammation, metabolic processes, oxygen sensing, redox-dependent cellular processes, among others. SUMO1 mediated SUMOylation to prevent degradation of I\textkappa B\textalpha from ubiquitin-proteasome pathway, and to stabilize I\textkappa B\textalpha of I\textkappa B by SUMO, which results in the inhibition of NF-\kappa B transcriptional activity. Moreover, the activation of NF-\kappa B p65 was reduced through deacetylation mediated by SIRT1. SIRT1 interacts with NF-\kappa B p65 leading to its deacetylation and resulting in decreased NF-\kappa B-dependent transcription. As a transcriptional factor, NF-\kappa B has function for many target genes that control various cellular responses such as apoptosis, stress and inflammation. More importantly, clinical and laboratory studies have demonstrated NF-\kappa B pathway participated in diverse human diseases, such as myocardial disease, diabetic nephropathy, cataract and cancer. A crucial observation made in this study was NF-\kappa B p65 nuclear expression induced by high glucose in HLECs, while I\textkappa B\textalpha nuclear expression was opposite. It was confirmed that the changes of NF-\kappa B p65 and I\textkappa B\textalpha was due to oxidative stress rather than osmotic pressure stress. The finding suggested NF-\kappa B p65 and I\textkappa B\textalpha were mainly located in the cytoplasm normally, but were transferred into nucleus by high glucose mediated oxidative stress. It also evidenced that high glucose activated and translocated NF-\kappa B p65 and I\textkappa B\textalpha protein. Redox derived by high glucose led to covalent post-translational modifications, SUMOylation and deacetylation. SUMOylated I\textkappa B\textalpha as inhibitor of NF-\kappa B has been demonstrated in attenuating NF-\kappa B activation. It was verified SUMO1 overexpression promoted I\textkappa B\textalpha SUMOylation and attenuated the activity of NF-\kappa B p65 in HLECs. We also recognized that SIRT1 overexpression induced NF-\kappa B p65 activity in HLECs.
activity of SIRT1 maintained the expression level of IκBα was discovered that RSV inhibited IκBα phosphorylation to inhibit the degradation of IκBα in our study. The mechanism to affect NF-κB p65 activity in HLECs. RSV could also response through kinds of pathway. In our study, as SIRT1 with NF-κB p65, and inhibit the activation of NF-κB p65. We therefore, stabilized IκBα can promote its conjugating through UPP. Meanwhile, MG132 can improve the conjugation of SUMO1 and IκBα. Therefore, stabilization of IκBα can promote its conjugating with NF-κB p65, and inhibit the activation of NF-κB p65. We also found RSV as antioxidant to treat HLECs under normal or high glucose condition. The fact proved that MG132 and RSV, both could influence the activity of NF-κB p65 and IκBα. In addition, MG132 is one kind of proteasome inhibitor which inhibits modification of ubiquitination and accumulates conjugations of SUMO and its target proteins. It is universal that IκBα degradation is attenuated through UPP. Meanwhile, MG132 can improve the conjugation of SUMO1 and IκBα. Therefore, stabilization of IκBα can promote its conjugating with NF-κB p65, and inhibit the activation of NF-κB p65. As an antioxidant, RSV might influence cellular stress response through kinds of pathway. In our study, as SIRT1 activator, RSV might regulate SIRT1 mediated deacetylation to affect NF-κB p65 activity in HLECs. RSV could also inhibit the degradation of IκBα in our study. The mechanism was discovered that RSV inhibited IκBα phosphorylation to maintain the expression level of IκBα[40]. The relationship of MG132, RSV, IκBα and NF-κB p65 was described as shown in Figure 6. In previous studies, there have been reports to identified that MG132 or RSV was involved in controlling high glucose in diabetes respectively[51-52]. It was full proof that MG132 and RSV as antioxidant might play a significant role in protecting lens from DC.

In conclusion, the study was first to found that SUMO1 and SIRT1 expression was influenced by high glucose due to oxidative stress in cultured HLECs. SUMO1 or SIRT1 overexpression could enhance the modifications of IκBα SUMOylation and NF-κB p65 deacetylation, and then influence the activation of NF-κB p65. In the same time, MG132 and RSV as antioxidant also regulated NF-κB p65 activity through influencing SUMOylation and deacetylation respectively. They had the potential to protect HLECs from oxidative damage and maintain lens transparency. This study supported that IκBα SUMOylation and NF-κB p65 deacetylation may be involved in the pathogenesis of DC through affecting NF-κB p65 activity under high glucose conditions. SUMO and SIRT signaling molecules may be potential therapeutic targets for the treatment of DC.

ACKNOWLEDGEMENTS

We are grateful to Prof. Chen of Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education (Shanghai Jiao-Tong University School of Medicine) and Dr. Zhong-Xiu Jiang of Department of Oncology (Shengjing Affiliated Hospital of China Medical University) supporting plasmids for the gifts.

Foundations: Supported by the National Natural Science Foundation of China (No.81170836; No.81570838).

Conflicts of Interest: Han X, None; Dong XX, None; Shi MY, None; Feng L, None; Wang XL, None; Zhang JS, None; Yan QC, None.

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NF-κB p65 activity in HLECs


