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Original Article

The Effect of NRAGE on cell cycle and apoptosis of human dental pulp cells and MDPC-23

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Abstract: Objectives: Neurotrophin receptor-mediated melanoma antigen-encoding gene homology (NRAGE) is an important regulator of proliferation, cell cycle arrest and apoptosis. Our previous study showed that NRAGE is an important regulator of proliferation and odontogenic differentiation of mouse dental pulp cells. This study aimed to investigate the effects of NRAGE on the cell cycle and apoptosis on human dental pulp cells (hDPCs) and MDPC-23. Materials and methods: Cells were infected by recombinant lentivirus to stably knockdown the expression of NRAGE, then the biological effects of NRAGE on the MDPC-23 was detected. The cell cycle distributions and apoptosis of hDPCs and MCPC-23 were performed by flow cytometric analysis. Simultaneously, the cell cycle and apoptosis were also detected after cells treated with IKK inhibitor. Results: The mRNA and protein levels of NRAGE decreased significantly after infected by recombinant lentivirus. Knockdown of NRAGE inhibited the apoptosis in hDPCs and MCPC-23. Knockdown of NRAGE show significantly G0G1 arrest in hDPCs, while no significantly difference in MDPC-23. Meanwhile, Knockdown of NRAGE activated the NF-κB signaling pathway. After treated with IKK inhibitor, the effect of NRAGE knockdown on apoptosis was reversed in both hDPCs and MDPC-23. Conclusion: NRAGE is a potent regulator for cell cycle and apoptosis of hDPCs. Knockdown of NRAGE inhibited apoptosis of hDPCs and MDPC-23 through the NF-κB signaling pathway.

Keywords: NRAGE, human dental pulp cells, MDPC-23, cell cycle, apoptosis, NF-κB signaling

Introduction

Dental pulp is composed of ectomesenchymal components with neural crest-derived cells, which contains mixed cells types including fibroblasts, odontoblasts and undifferentiated mesenchymal cells; and dental pulp plays important roles in dentinogenesis [1, 2]. Dental pulp cells (DPCs) can proliferate and differentiate into odontoblasts, thereby creating reparative dentin in response to the appropriate stimuli [3, 4]. In the progress of multicellular organism development, cellular homeostasis and cellular response to stimuli, cell cycle arrest and apoptosis play essential roles. Many molecular regulates the tooth development via cell cycle [5, 6]. Cell cycle arrest can protect DPCs against lethal heat shock following by restorative procedures [7] and irradiation induced damage [8].

Cell death regulated by a genetic program is called programmed cell death including apoptosis, necroptosis, and autophagy. This is a fundamental mechanism of tissue homoeostasis. Apoptosis, which is critical to many multicellular organisms, is an evolutionarily conserved mode of programmed cell death. Apoptosis also takes place in mature dental pulp when it is exposed to extrinsic stimuli such as bacterial infection, ischemia, mechanical stimuli, or dental material [9-11]. Appropriate apoptosis of DPCs is beneficial to tissue regeneration.

Neurotrophin receptor-interacting MAGE homolog (NRAGE), a member of type II melanoma antigen-encoding gene family, is widely expressed in many normal tissues [12] and is called MAGE-D1 or Dlxin-1 [13]. NRAGE contains 25 repeats of a WQXPXX hexapeptide domain in the middle region, suggesting that

NRAGE performs unique functions compared with other proteins belonging to the MAGE family [14]. Studies have shown that NRAGE is involved in cellular functions such as cell proliferation [15, 16], cell motility [17], cell cycle arrest [16, 18] and apoptosis [18, 19]. NRAGE plays an essential role in development apoptosis in vivo through NF-kB pathway [20], which could be regulated by NRAGE Interaction with UNC5H1 [21], TAK1-TAB1-XIAP complex [22], Che-1 [23], and so on. In our previous study, we found that NRAGE is an important regulator of proliferation and odontogenic differentiation of mouse dental pulp cells, which might via NF-kB signaling pathway [24]. However, the effect underlying the cell cycle distributions and apoptosis caused by NRAGE in DPCs is unknown.

In the present study, the cell cycle distributions and apoptosis were investigated by Flow cytometry. NRAGE was stably knocked down to determine its effects on cell cycle distribution and apoptosis of hDPCs and MDPC-23.

Materials and methods

Isolation and culture of primary human dental pulp cells (hDPCs)

The primary cultured hDPCs were isolated from the premolar of healthy people undergoing tooth extraction for orthodontic treatment and were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL Life Technologies, Paisley, UK, USA) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin, Gibco-BRL, USA) in a humidified atmosphere of 5% CO_2 at 37°C. Cell cultures between the third and sixth passages were used in the study and the culture medium was changed every 3 d.

Odontoblast-like MDPC-23 cells provided by Dr. Guan Yang (Molecular Laboratory for Gene Therapy and Tooth Regeneration, Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, Capital Medical University School of Stomatology, Beijing, P. R. China) were maintained in DMEM/high glucose (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS (Gibco-BRL Life Technologies, Paisley, UK) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin, Gibco-BRL, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Plasmid construction and interfering RNA transfection

Lentiviral plasmids carrying the small hairpin NRAGE interference (shNRG) was reported in our previous study [24]. In brief, shCon-plasmid and shRNA-plasmid cotransfection were performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. shNRG or shCon were packaged in 293T and infected hDPCs and MDPC-23 using polybrene (Sigma) for 24 h, then 2 and 8 ug/ml puromycin (Sigma) was added to select the positive transfected hDPCs and MDPC-23 for 4-7 d, respectively. The hDPCs transfected by shNRG and shCON were short for H-shNRG and H-shCON, respectively. MDPC-23 transfected by shNRG and shCON were short for M-shNRG and M-shCON, respectively.

Cytotoxity ananlysis of NF-kB pathway inhibitor on hDPCs and MDPC-23

The Cell Counting Kit-8 (CCK-8; Dojindo Kagaku Co, Kumamoto, Japan) was used to analyze the cytotoxicity of NF-kB pathway inhibitor (BMS345541) on hDPCs and MDPC-23 according to the manufacturer's protocols. Briefly, cells were seeded at a density of 5×10^3 cells/well in a 96-well plate (Corning Inc, Corning, NY, USA) and then were cultured overnight. Subsequently, fresh medium with 10% FBS containing BMS345541 with different concentrations was added to replace the culture medium. The cells were cultured for another 3 d, and the number of cells was assessed using a cell counting kit. The method to analyze the results was reported in our previous study [24].

RNA Isolation and semi-quantitative RT-PCR analysis

Total RNA of hDPCs (H-mock), H-shCon and H-shNRG; MDPC-23(M-mock), M-shCON and M-shNRG were isolated with TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. cDNA synthesized using a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) was used as a template in PCR. The mRNA level of NRAGE was analyzed. 18 S was used to normalize the RNA expression. The

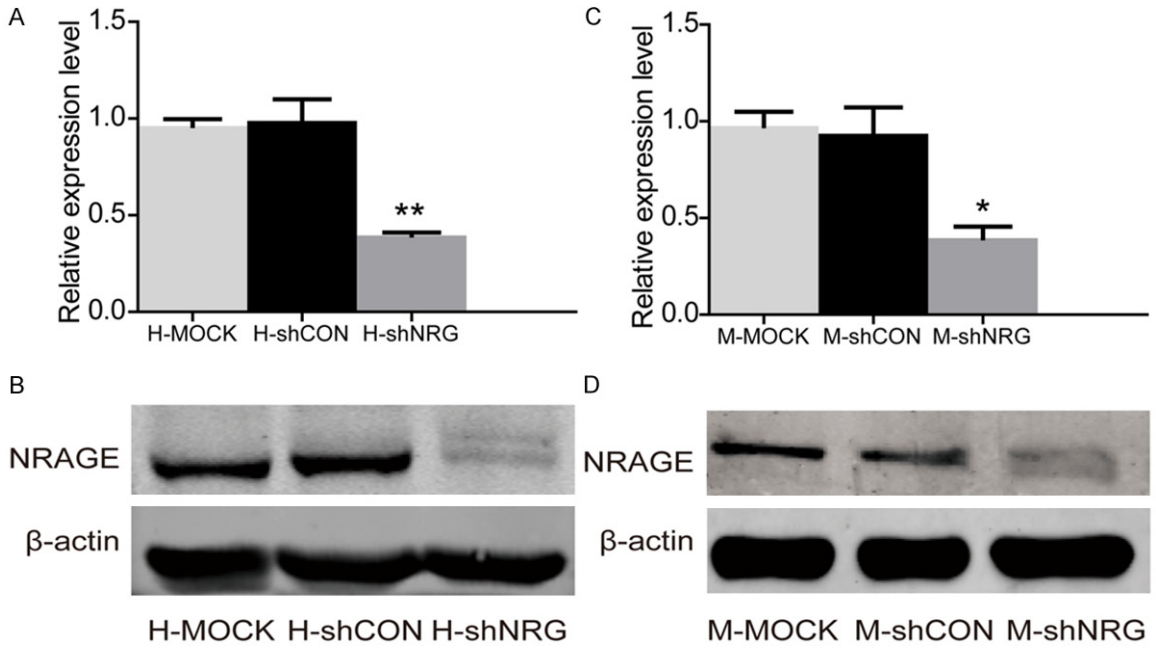


Figure 1. Stable knockdown of NRAGE in hDPCs and MDPC-23. (A) mRNA level and (B) protein level of NRAGE after hDPCs infection. (C) mRNA level and (D) protein level of NRAGE after MDPC-23 infection. Mock represents for the untreated cells. Data represents three independent experiments with similar results *(*P<0.05 and **P<0.01).*

sequences of the specific primers used in this study were as follows: NRAGE (mouse) (forward: 5'-GGCATACTGGGAACGACCAA-3', reverse: 5'-C-CAGAGCATCCAAGGCTTCA-3'), NRAGE (human) (forward: 5'-GCTCGGTCTCCTCTTGGT GATTC-3', reverse: 5'-GGCACTCGTCTGTAGTCCA-GGTATT-3'); 18S: forward, 5'-CCTGGATACCGCA-GCTAGGA-3'; reverse, 5'-GCGGCGCAATACGAAT-GCCCC-3'. Real-time PCR reaction was amplified with SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated using the comparative 2-ΔΔCt method. Each measurement was assessed in triplicate. The gene expression ratio was shown as mean ± SD from three independent experiments.

Immunofluorescence

The H-shNRG, H-shCON, M-shNRG and M-shCON were seeded on chambers in 24-well plates, and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. The method was reported in our previous study [24]. A 1:300 dilution of rabbit anti-mouse NF-κB (p105/p50) antibody (abcam, Camb., UK), goat anti-rabbit immunoglobulin G Fragment (Alexa Fluor® 555 Conjugate) (Cell Signaling Technology, Inc., USA) were used in this study.

Western blot

The H-mock, H-shCon and H-shNRG; M-mock, M-shCON and M-shNRG cultured into 60 mm cell culture dishes were lysed in a protein extraction kit (Piece, Rockford, IL, USA). Meanwhile, the protein of hDPCs and MDPC-23 were extracted after treated by appropriate dose of BMS345541 for 0 h, 1 h and 2 h. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Piece, Rockford, IL, USA). An equal amount of protein was separated and then transferred onto nitrocellulose membranes (Millipore Corporation, Billerica, MA, USA). After blocking, the primary antibodies of mouse anti-mouse NRAGE (Santa Cruz Biotechnology, Inc, USA), rabbit anti-Phospho-p65 (p-p65) (1:1000; Cell Signaling, Beverly, MA, USA) and rabbit anti-mouse β-actin (Santa Cruz Biotechnology, Inc., USA) were used. Then, secondary antibodies of goat anti-mouse immunoglobulin G (Licor Co., Lincoln, NE, USA) and goat anti-rabbit immunoglobulin G (Licor Co., Lincoln, NE, USA) were used. After the final wash, the membranes were visualized using the Odyssey LI-CDR system.

Flow cytometry

For Flow cytometric analysis, cells were seeded into 60 mm cell culture dishes in routine cul-

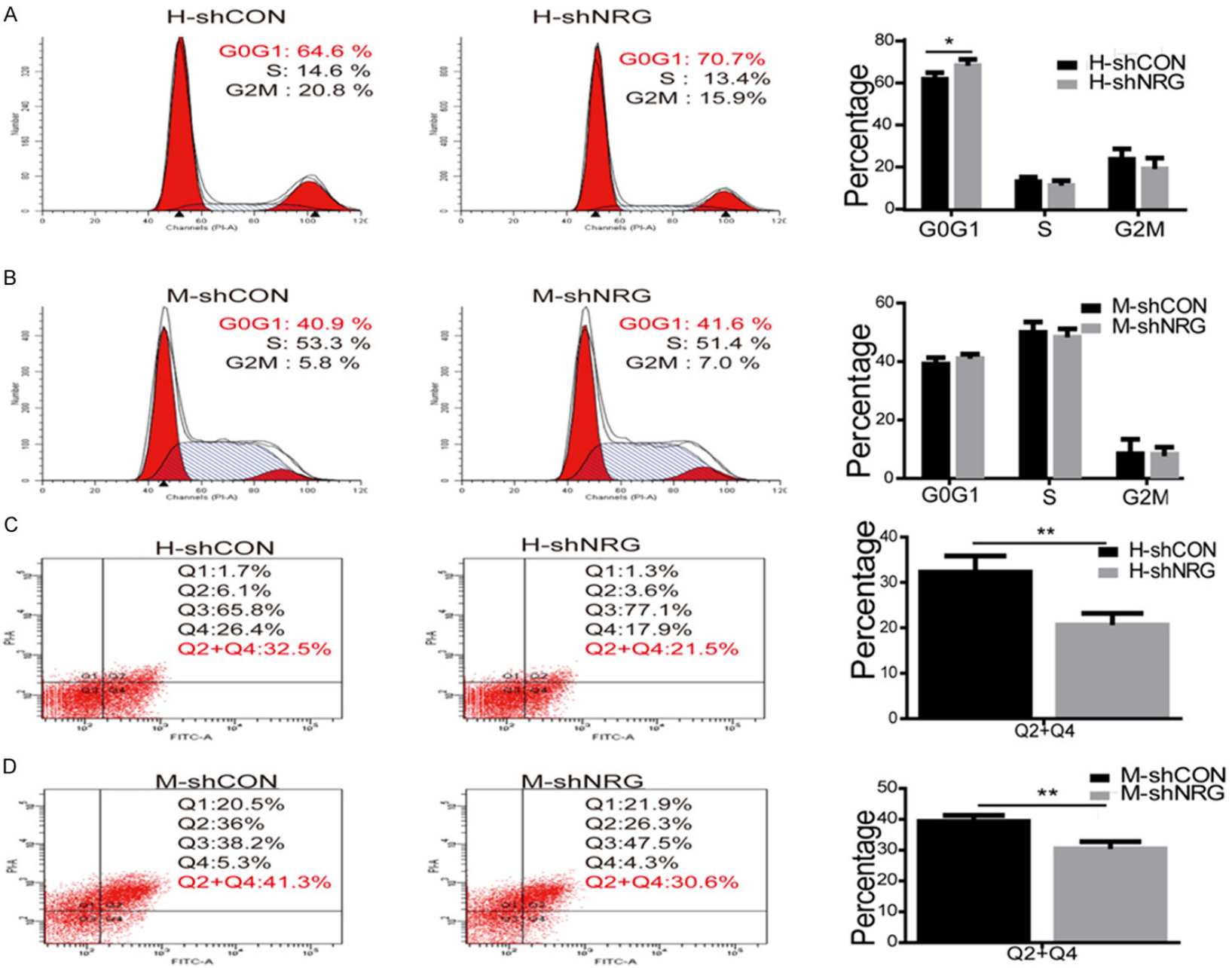

A
H-shCON
G0G1: 64.6 %
S: 14.6 %
G2M : 20.8 %
H-shNRG
G0G1: 70.7%
S : 13.4%
G2M : 15.9%
Percentage
H-shCON
H-shNRG
G0G1
S
G2M
B
M-shCON
G0G1: 40.9 %
S: 53.3 %
G2M : 5.8 %
M-shNRG
G0G1: 41.6 %
S: 51.4 %
G2M : 7.0 %
M-shCON
M-shNRG
C
H-shCON
Q1:1.7%
Q2:6.1%
Q3:65.8%
Q4:26.4%
Q2+Q4:32.5%
H-shNRG
Q1:1.3%
Q2:3.6%
Q3:77.1%
Q4:17.9%
Q2+Q4:21.5%
Q2+Q4
D
M-shCON
Q1:20.5%
Q2:36%
Q3:38.2%
Q4:5.3%
Q2+Q4:41.3%
M-shNRG
Q1:21.9%
Q2:26.3%
Q3:47.5%
Q4:4.3%
Q2+Q4:30.6%

Figure 2. Effect of NRAGE knockdown on cell cycle and apoptosis of hDPCs and MDPC-23. Flow cytometric assay was used to analyze cell cycle distribution. A. H-shNRG groups (70.7%) show significantly G0G1 arrest compared with the H-shCON groups (64.6%). B. No significant difference on MDPC-23. C and D. NRAGE knockdown significantly inhibited the apoptosis (later apoptosis (Q2)+early apoptosis (Q4)) in both hDPCs and MDPC-23. The data shown are from three independent experiments *(*P<0.05 and **P<0.01).*

ture media supplemented with 10% FBS for 24 h. After 24 h starvation with serum-free conditions, serum-contained medium with or without BMS345541 was added to the cultures, and cells were permitted to grow for an additional 0, 24, or 48 h. The cells were then collected and fixed in 75% ethanol at 4°C for 30 min in the dark. The samples were centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellets were washed with PBS. After adding 200 μl propidium iodide (PI), the samples were incubated for 30 min in the dark and then cell cycle distributions were analyzed with a flow cytometer (BD Biosciences). For cell apoptosis the pellets were resuspended in stained with Annexin V (BD Pharmingen, Franklin Lakes, USA) and propidium iodide (PI) (BD Pharmingen, Franklin Lakes, USA) according to the manufacturer's instructions. Apoptosis cell fractions and cell cycle distributions were analyzed by FACScan cytometry (Becton-Dickinson, SanJose, CA, USA). In our studies, the early apoptotic cells (Q2: Annexin V+/PI− staining) and the late apoptotic cells (Q4: Annexin V+/PI+ staining) were considered to be undergoing apoptosis, and the proportion of these cells out of the total number of cells analyzed were determined.

Statistical analysis

Experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). All statistical analyses were evaluated by one-way ANOVA and Turkey post hoc testing using the SPSS software (Version 13.0; SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Stable knockdown of NRAGE in hDPCs/MDPC-23

Stable transfected cell populations of H-shCon, H-shNRG, M-shCon and M-shNRG were constructed. The mRNA levels (**Figure 1A** and **1C**) and protein levels of NRAGE (**Figure 1B** and **1D**) were obviously lower in the H-shNRG and M-shNRG than those in the H-shCON and M-shCON. The results showed that NRAGE was stably knocked down in H-shNRG and M-shNRG.

The cell cycle distribution after knockdown of NRAGE in hDPCs and MDPC-23

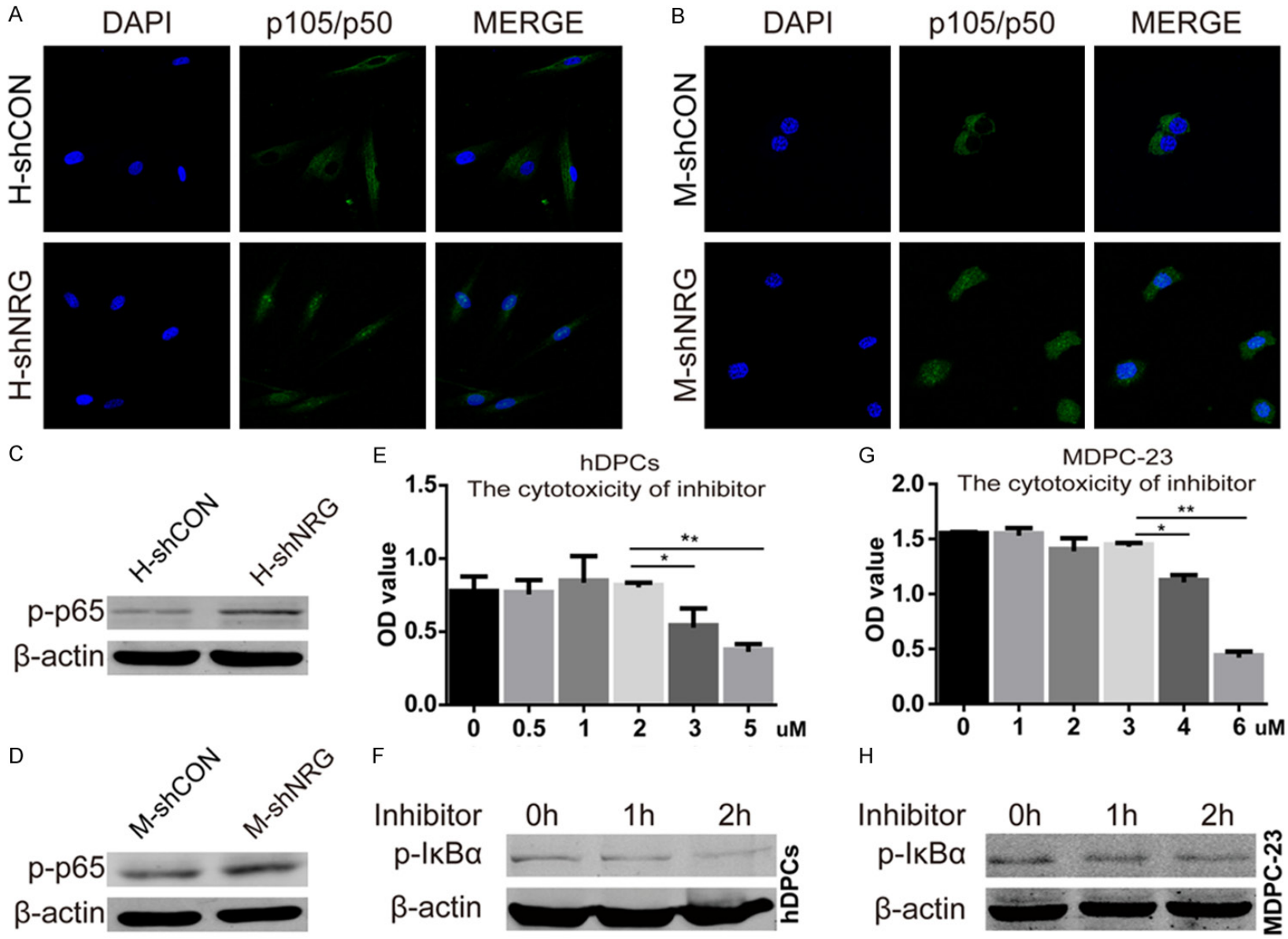
To determine the role of NRAGE in cell cycle distribution, flow cytometric analysis was performed. The results showed that H-shNRG group (70.7%) showed significantly G0G1 arrest compared with the H-shCON gruop (64.6%) (**Figure 2A**), while there are no significant difference between M-shCON and M-shNRG (**Figure 2B**) (**P<0.05*).

Knockdown of NRAGE inhibited the apoptosis of hDPCs and MDPC-23

The relative number of differently stained cells is shown using flow cytometry dot plots (**Figure 2C** and **2D**). We detected the percentage of apoptotic cells (Q2+Q4) in the shNRG groups and shCON groups of hDPCs (**Figure 2C**) and MDPC-23 (**Figure 2D**). **Figure 2C** showed that the percentage of apoptotic cells in H-shNRG group (21.5%) was significantly lower than in H-shCON group (32.5%). **Figure 2D** presented the same trend in MDPC-23 (30.6% in M-shNRG *VS* 41.3% in M-shCON). The results indicated that NRAGE knockdown significantly inhibited the apoptosis in both hDPCs and MDPC-23. (***P<0.01*).

The NF-κB signaling pathway was activated after the knockdown of NRAGE in hDPCs and MDPC-23

After knockdown of NRAGE, we detected the translocation of p105/p50 using Immunofluorescence (**Figure 3A** and **3B**), meanwhile we analyzed the protein expression of p-p65 by western blot (**Figure 3C** and **3D**). P105/p50 was detected transfer from cytoplasm to nuclear after NRAGE knockdown in hDPCs (**Figure 3A**), and the similar results also found in MDPC-23 (**Figure 3B**). The expression of p-p65 in H-shNRG was higher than in H-shCON (**Figure 3C**), which was similar in MDPC-23 (**Figure 3D**). Those results indicated that NF-κB pathway


A
DAPI
p105/p50
MERGE
H-shCON
H-shNRG
B
DAPI
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M-shCON
M-shNRG
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H-shCON
H-shNRG
p-p65
β-actin
D
M-shCON
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The cytotoxicity of inhibitor
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p-IκBα
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MDPC-23

Figure 3. Knockdown of NRAGE stimulated NF-κB signaling pathway in hDPCs and MDPCS-23. A and B. Immunofluorescence showed that p105/p50 translocated from cytoplasm into nuclear after NRAGE knockdown in hDPCs (A. magnification: 400×) and MDPC-23 (B. magnification: 630×) (DAPI: blue; p105/p50: green). C and D. Protein level of p-p65 were enhanced in H-shNRG and M-shNRG groups. E and G. Cytotoxicity of NF-kB inhibitor BMS345541 on hDPCs and MDPC-23 at different concentration was analyzed by CCK-8. 2 um and 3 um BMS345541 are appropriate for hDPCs and MDPC-23, respectively. F and H. The inhibition effect of appropriate concentration of BMS345541 on hDPCs (2 um) and MDPC-23 (3 um). Western Blot showed that P-IkBα was downregulated at different time points. Data are shown as mean ± SD from three independent experiments. *(*P<0.05 and **P<0.01).*

was activated after knockdown of NRAGE in hDPCs and MDPC-23.

NF-κB inhibitor could rescue the effect of NRAGE on the apoptosis in hDPCs and MDPC-23

To further examine the role of NF-κB pathway in NRAGE mediated apoptosis of hDPCs and MDPC-23, a specific IKK inhibitor (BMS345541) was used to suppress the activity of NF-κB pathway during inducing apoptosis. The appropriate concentration of BMS345541 was chosen according to the results of CCK-8. We found that higher concentrations (3 and 5 uM BMS345541) significantly reduced the viability of hDPCs, whereas 2 uM BMS345541 did not affect viability of hDPCs (**Figure 3E**). Meanwhile, 2 uM BMS345541 can significantly inhibited expression of p-I-B α (**Figure 3F**) in hDPCs which showed NF-kB pathway was inhibited. The similar results of 3uM BMS345541 on MDPC-23 was detected (**Figure 3G** and **3H**). Thus 2 uM and 3 uM BMS345541 were chosen as the optimal dose for hDPCs and MDPC-23 in the following experiments, respectively. Flow cytometric analyses demonstrated that the apoptosis was reversed after H-shNRG and M-shNRG treating by optimal dose of BMS-345541 (**Figure 4A** and **4B**). Those results indicated that NRAGE regulated apoptosis through the NF-κB signaling pathway. However, Flow cytometric analyses demonstrated that there was no significantly change on cycle distribution on H-shNRG (**Figure 4C**). Which means that NRAGE affect the cycle distribution might be through other signaling pathway, not NF-κB pathway.

Discussion

Dental pulp cells (DPCs) can form reparative dentin in response to the appropriate stimuli [3, 4], which including odontoblastic differentiation, cell cycle arrest and apoptosis. Cell cycle arrest is an important protection for DPCs response to stimuli. DPCs protect the pulp dentin complex by regulating cell cycle arrest response to heat shock during restorative procedures [7]. Dental pulp stem cells (DPSCs) respond to irradiation induced damage by permanent cell cycle arrest [8]. Meanwhile, the homeobox, msh-like 1 (MSX1) regulates cell cycle arrest to participate tooth germ development [5]. Nuclear factor I-C regulates the cell cycle of DPSCs in tooth root development [6]. Apoptosis is necessary for odontoblasts to maintain an appropriate dentin deposition rate [25] and tooth development [26]. The difference of apoptosis and necrosis is that necrosis triggers inflammation, while apoptosis prevents it. Moreover, the modulation of autophagy and apoptosis plays a role in progression of periapical lesions [27]. Cell epithelial proliferation of radicular and dentigerous cyst is regulated by apoptosis [28]. Apoptosis is critical to tissue development and recovery response to internal and external stimuli [9, 11, 25]. Therefore, apoptosis is believed to prevent tissue damage [29] and may be play a important role in the minimization of dental pulp.

NRAGE mainly participated in cell apoptosis, cell cycle and cell differentiation [13, 20]. NRAGE plays an essential role in developmental apoptosis of sympathetic neurons in vivo [20], which maybe involve in Prion diseases [30]. Meanwhile, NRAGE is an endogenous regulation for neuronal proliferation and differentiation of PC12 cells [31]. According to our previous studies, NRAGE promotes odontoblastic differentiation of mouse dental pulp cells [24]. Therefore, it is important to explicit the effect of NRAGE on cell cycle distribution and apoptosis of DPCs. This is the first report to evaluate the effects of NRAGE on cell cycle distribution and apoptosis of hDPCs and MDPC-23.

In the present study, NRAGE was knocked down by recombinant lentivirus (**Figure 1**). Flow cytometry were used to detect cells undergoing apoptosis and cell cycle distribution. There was a significantly difference in cell cycle after knockdown of NRAGE in hDPCs. In H-shNRG

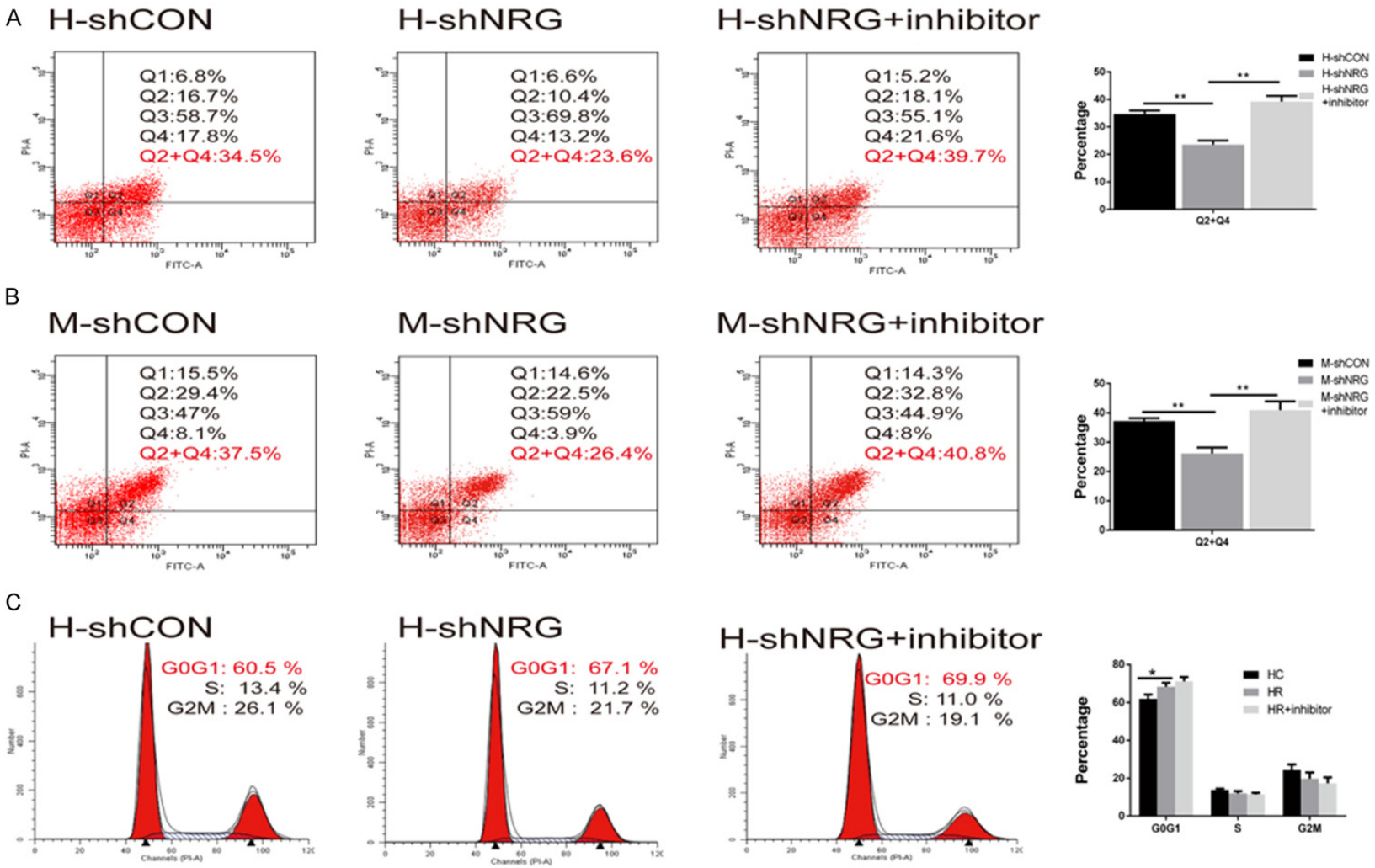

Figure 4. Effect of NF-kB inhibitor on cell apoptosis and cell cycle distribution of H-shNRG and M-shNRG groups. A. The percentage of apoptotic cells in inhibitor treated H-shNRG (39.7%) was significantly higher than H-shNRG (23.6%). B. The percentage of apoptotic cells in inhibitor treated M-shNRG (40.8%) was significantly higher than M-shNRG (26.4%). C. Cell cycle distribution was not affected after H-shNRG treated by optimal dose of NF-kB inhibitor. The data shown are from three independent experiments. (**P<0.05 and **P<0.01*).

group, cells were arrested in G0G1 phase compared with H-shCON group (**Figure 2A**). Meanwhile, the relative numbers of apoptotic cells were lower in H-shNRG groups than in H-shCON groups, as well as MDPC-23. There were significant differences in the relative numbers of apoptosis cells between shNRG groups and shCON groups. These results suggested that knockdown of NRAGE inhibited the apoptosis of hDPCs and MDPC-23, which is consist with other studies. NRAGE protects cells from neuronal damage induced by apoptosis [23]. NRAGE plays an essential role in developmental apoptosis of sympathetic neurons in vivo [20].

The NF-κB pathway is activated at sites of injury, and regulates apoptosis and the cell cycle. Previous researchers have proven that NRAGE interaction with Chi-1, XIAP and UNC5H regulated the apoptosis through NF-κB pathway. NF-κB is a family of transcription factors that involved in many aspects of normal cellular functions [32] and have five original members such as p65 (Rel A), p50 (NF-κB1), p52 s(NF-κB2), RelB, and c-Re l [33]. NF-κB pathway is an important regulator in cell apoptosis, which was the same as our present results. In this study, the protein level of p-p65 were upregulated and p105/p50 translocated from cytoplasm to nuclear after knockdown of NRAGE, suggesting that the NF-κB pathway was activated after knockdown of NRAGE in hDPCs and MDPC-23. Moreover, after H-shNRG and M-shNRG treated by the optimal dose of NF-κB inhibitor (BMS345541), the relative number of apoptosis cells were enhanced. It means that the effect of NRAGE on hDPCs and MDPC-23 were rescued by the NF-kB inhibitor, which suggested that knockdown of NRAGE inhibited apoptosis via activating NF-κB pathway in hDPCs and MDPC-23. While there are no differences on cell cycle distribution after cells treated by NF-κB inhibitor. Apoptosis plays an important role in the formation of reparative dentin by providing room for new dentin and preventing stimuli [25]. Reparative and reactionary dentin is critical to dental pulp response to tooth injury and dental caries [34]. More intensive investigations are necessary to elucidate relationships between NRAGE mediated apoptosis and odontoblastic differentiation of DPCs responds to external and internal stimuli in vivo.

In summary, NRAGE regulated cell apoptosis of hDPCs and MDPC-23 via activation of NF-κB pathway. While NRAGE regulated cell cycle distribution in hDPCs through other signaling pathway, not NF-κB pathway. Furthermore, cell cycle of MDPC-23 was not regulated by NRAGE. Therefore, further studies are warranted to investigate other potential mechanisms associated with NRAGE-mediated biology effects of DPCs and MDPC-23.

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Disclosure of conflict of interest

None.

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