

Effects of neurotrophin receptor-mediated MAGE homology on proliferation and odontoblastic differentiation of mouse dental pulp cells

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Abstract

Objectives: This study aimed to investigate effects of neurotrophin receptor-mediated melanoma antigen-encoding gene homology (NRAGE) on proliferation and odontoblastic differentiation of mouse dental pulp cells (mDPCs).

Materials and methods: Mouse dental pulp cells were infected with recombinant lentivirus to stably knockdown expression of NRAGE, and biological effects of NRAGE on the cells were detected. Proliferation and odontoblastic differentiation of mDPCs were observed. Simultaneously, mRNA and protein levels of NRAGE and nuclear factor- κ B (NF- κ B) protein expression were detected. Immunofluorescence assay was used to detect expression and location of NRAGE and NF- κ B.

Results: NRAGE mRNA and protein levels reduced significantly after mDPC odontoblastic induction. Knockdown of NRAGE inhibited the proliferation of mDPCs. However, knockdown of NRAGE enhanced their odontoblastic differentiation with up-regulated ALPase activity. It also promoted mineral nodule formation as well as mRNA and protein expressions of ALP, DSPP and DMP1. Protein levels of NF- κ B/p50 significantly increased, whereas NF- κ B/p105 protein expression decreased in the mDPC/shNRG group. Immunofluorescence revealed that relocation of NF- κ B was similar to that of NRAGE during

odontoblastic induction, in which NF- κ B translocated from the cytoplasm to the nucleus.

Conclusion: NRAGE is a potent regulator of proliferation and odontoblastic differentiation of mDPCs, which might be *via* the NF- κ B signalling pathway.

Introduction

Dental pulp is composed of ectomesenchymal components with neural crest-derived cells, and contains mixed cell types including fibroblasts, odontoblasts and undifferentiated mesenchymal cells; it plays important roles in dentinogenesis (1,2). Dental pulp cells (DPCs) can proliferate and differentiate into odontoblasts, thereby creating reparative dentin in response to appropriate stimuli (3,4). This process involves migration, proliferation and differentiation of DPCs into odontoblast/odontoblast-like cells (5). A variety of key growth factors and signalling molecules have been tested in this process. For example, nuclear factor I-C regulates dentin sialophosphoprotein (DSPP) and E-cadherin by control of Kruppel-like factor 4 during dentinogenesis (6). The TGF- β /BMP family of growth factors induce odontoblast differentiation and reparative dentin synthesis (7). HMGB1 promotes proliferation and differentiation of human DPCs. microRNA-27 promotes differentiation of odontoblastic cells by activating Wnt/ β -catenin signalling (8). However, up to now, precise mechanisms underlying proliferation and differentiation of DPCs has remained unclear.

Neurotrophin receptor-interacting melanoma antigen-encoding gene homology (NRAGE) is also denominated as MAGE-D1 or Dlxin-1. It is a member of the Type II melanoma-associated antigen (MAGE) family of proteins characterized by presence of a unique region of about 200 amino acids known as the MAGE homology domain. It has been identified as a molecule interacting with low affinity nerve growth factor receptor p75NTR

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(9,10). Previous studies of NRAGE have mainly concerned apoptosis, the cell cycle and cell differentiation (11,12). NRAGE plays an essential role in developmental apoptosis of sympathetic neurons *in vivo* (11), which may be involved in prion diseases (13). NRAGE suppresses metastasis of melanoma and pancreatic cancers *in vivo* and in *in vitro* studies (14); NRAGE also promotes proliferation of oesophageal carcinoma cells (15). Meanwhile, NRAGE provides endogenous regulation of neuronal proliferation and differentiation of PC12 cells (16). According to previous studies, NRAGE modulates function of *Dlx/Msx* to promote myogenic differentiation (17) and strong NRAGE mRNA signals have been found in cell layers surrounding cartilaginous elements in bone rudiments, during digit formation in embryogenesis. Further study has proven that NRAGE may act as a regulator of *Dlx* family members in bone formation (12). *Dlx3* also plays a key role in odontoblast polarization and dentin formation (18). NRAGE participates in activating non-canonical and alternative bone morphogenetic protein (BMP) signalling pathways (19,20). Meanwhile, BMP signalling pathways play an important role in tooth development (21–23) and regulating proliferation and differentiation of DPCs (24,25). Odontoblasts and osteoblasts share many similarities. Thus, we conjectured that NRAGE may exert effects on mouse DPCs (mDPCs) during tooth development.

In the present study, expression of NRAGE during mDPC differentiation was investigated using quantitative real-time polymerase chain reaction (RT-PCR) and western blot analysis. NRAGE was stably knocked down in mDPCs to determine its effects on their proliferation and odontoblastic differentiation.

Materials and methods

This study was performed according to the informed protocol approved by the Ethics Committee of Shanghai Tenth People's Hospital, Tongji University, Shanghai.

Isolation and culture of primary mDPCs

Primary cultured mDPCs were isolated from incisors of healthy 4-week-old mice. Pulp tissues were gently separated from teeth and cut into small pieces (~1 mm³) using ophthalmic scissors. Tissue pieces were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Gibco-BRL Life Technologies, Paisley, UK) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin, Gibco-BRL) in a humidified atmosphere of 5% CO₂ at 37 °C. When the cells reached confluence, they were digested

by trypsinization (0.2% trypsin and 0.02% ethylenediaminetetraacetic acid; Gibco) and cultured. Cultures between the third and sixth passages were used in the study, and culture medium was changed every 3 days.

DMEM supplemented with 10% FBS, antibiotics, 50 µg/ml ascorbic acid, 10 mmol/l sodium β-glycero-phosphate and 10 nmol/l dexamethasone (Sigma, St Louis, MO, USA) was used as odontoblastic induction medium. mDPCs were plated in six-well plates (Corning Inc., Corning, NY, USA) at 2×10^5 cells/well and then cultured in odontoblastic induction medium.

Plasmid construction and interfering RNA transfection

Lentiviral plasmids carrying small hairpin NRAGE interference (shNRG), targeting AAGATGAAAGTGCTGAGATTC sequence (hNRAGE, a human neurotrophin receptor interacting MAGE homologue) or non-specific sequence (shCon), were constructed using the vector pLKO.1-puro (Sigma), as reported in previous work (26). In brief, shCon-plasmid and shRNA-plasmid cotransfection were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. shNRG or shCon were packaged in 293T and infected mDPCs using polybrene (Sigma) for 24 h, then 2 µg/ml puromycin (Sigma) was added to select positive transfected cells, for 4–7 days. A monoclonal population of stably infected cells was prepared by limiting dilution assay, and verified by RT-PCR and western blotting.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo Kagaku Co., Kumamoto, Japan) was used to analyse effects of NRAGE on mDPC proliferation, according to the manufacturer's protocols. Briefly, wild-type, shCon- or shNRG-infected mDPCs were seeded at 5×10^3 cells/well in four 96-well plates (Corning Inc.), and then cultured overnight. Subsequently, fresh medium with 10% FBS was changed every day. After cells had been cultured for 1, 3, 5 and 7 days, numbers of cells were assessed using the cell counting kit. Absorbance was measured using a microplate reader at 450 nm to determine number of viable cells in each well. A well containing medium and CCK-8 solution, but without cells, was used as blank control. Cell proliferation was represented as mean ± SD of absorbance for five wells from each group.

Alizarin red and alkaline phosphatase (ALP) staining

Wild-type, shCon- or shNRG-infected mDPCs were incubated under odontoblastic induction for 14 days.

Induction medium was replaced every 3 days. Mineralization was assessed by alizarin red staining. Briefly, 1% alizarin red (Sigma) was prepared in distilled water, adjusted to pH 4.3 and applied to cells in six-well plates, for 30 min at 37 °C. For ALP staining, plates were harvested at 14 days. ALP colour development kit (Beyotime Institute of Biotechnology, Shanghai, China) was used, according to the manufacturer's protocols. All cells were washed three times in distilled water, then observed using phase contrast microscopy.

ALPase activity assay

ALPase activity was determined using cell lysates. Briefly, 220 µl 0.9 mM 2-amino-2-methyl-1-propanol buffer was added to 5 µl samples or standards. Subsequently, 25 µl 15 mM p-nitrophenyl phosphate was added and mixtures were incubated at 37 °C for 30 min. ALP activity was measured at 405 nm using an ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). ALPase activity (U/mg) was defined as release of 1 mol p-nitrophenol per milligram of total cell protein.

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA of wild-type, shCon- and shNRG-infected mDPCs cultured in odontoblastic induction medium for different times (0, 7 and 14 days) was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. cDNA synthesized using Prime-Script RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan), was used as PCR template. Odontogenic differentiation of cells was monitored by analysing odontoblast-related marker *dental matrix protein 1* (*dmp1*), *dspp* and *alp*. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used to normalize RNA expression. Sequences of specific primers used were as follows: *dspp* (forward: 5'-TCGGTTACCGGTTGACATGG-3', reverse: 5'-GCAGG CCCTGTTTCCTCAT-3'); *dmp1* (forward: 5'-ACAGCCTGAACACAT TCTCC-3', reverse: 5'-ATGTTCTTGG GACGGATGT C-3'); *alp* (forward: 5'-TTCTCTCTTGGCAGGCAAG-3', reverse: 5'-ACCCCGCTATTCCAAACAG G-3'); *nrage* (forward: 5'-GGCATACT GGGAACGAC CAA-3', reverse: 5'-CCAGAGCATCCAAGGCTTC A-3'); and *gapdh* (forward: 5'-TGGGTGTGAACCATGAGAAGT-3', reverse: 5'-TGAGTCCTTCCAC GATACCAA-3'). Real-time PCR reaction was amplified with SYBR Premix Ex Taq II (Takara Bio) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (27).

Mean Ct value of the target gene was normalized to its averaged Ct values of *gapdh*, to obtain a ΔCt value, which was then normalized to control samples to obtain a $\Delta\Delta Ct$ value. Each measurement was assessed in triplicate. Gene expression ratio is shown as mean \pm SD from three independent experiments.

Western blot analysis

Wild-type, shCon- and shNRG-infected mDPCs cultured in odontoblastic induction medium for 0, 7 and 14 days were lysed using a protein extraction kit (Pierce, Rockford, IL, USA). Protein concentrations were determined employing a bicinchoninic acid protein assay kit (Pierce). An equal amount of protein was separated, then transferred on to nitrocellulose membranes (Millipore Corporation, Billerica, MA, USA). After blocking, primary antibodies to mouse anti-mouse NRAGE (Santa Cruz Biotechnology Inc., Dallas, TX, USA), rabbit anti-mouse DMP1 (Bio Vision Research Products, Mountain View, CA, USA), rabbit anti-mouse DSP (Santa Cruz Biotechnology Inc), rabbit anti-mouse nuclear factor- κ B (NF- κ B) (Epitomics, Inc., Burlingame, CA, USA) and rabbit anti-mouse β -actin (Santa Cruz Biotechnology, Inc) were used. Then, secondary antibodies goat anti-mouse immunoglobulin G (Licor Co., Lincoln, NE, USA) and goat anti-rabbit immunoglobulin G (Licor Co.) were used. After the final wash, membranes were visualized using the Odyssey LI-CDR system.

Immunofluorescence

Wild-type mDPCs were seeded in chambers of 24-well plates, then incubated overnight at 37 °C, in a humidified atmosphere of 5% CO₂. Subsequently, medium was changed to odontoblastic induction for 0, 3, 7, 11 and 14 days. Cells in chambers were fixed in 4% paraformaldehyde at 4 °C for 25 min, permeabilized with 0.2% Triton-X 100 (Sigma) for 15 min at room temperature and blocked in 5% bovine serum albumin in PBS. Subsequently, samples on slides were incubated with relevant primary antibodies in 2% bovine serum albumin overnight at 4 °C. They were washed and incubated with appropriate secondary antibodies for 1 h at 37 °C. 1:200 dilution mouse anti-mouse NRAGE antibody (Santa Cruz Biotechnology, Inc), 1:300 rabbit anti-mouse NF- κ B antibody (Epitomics, Inc.), goat anti-mouse immunoglobulin G fragment (Alexa Fluor 488 Conjugate) (Cell Signaling Technology, Inc., Boston, MA, USA) and goat anti-rabbit immunoglobulin G fragment (Alexa Fluor 555 Conjugate) (Cell Signaling Technology, Inc.) were used.

After sample nuclei were stained for 10 min in Hoechst solution (Dojindo Laboratories, Kumamoto, Japan), they were visualized using confocal fluorescence microscopy (Carl Zeiss, German).

Statistical analysis

Experiments were performed in triplicate, and data are presented as mean \pm SD. Results were evaluated by one-way ANOVA using SPSS software (Version 10.0; SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Expression of NRAGE during odontoblastic differentiation of mDPCs

To detect expression of NRAGE in cell cultures, mRNA and protein levels of DMP-1, DSPP, ALP and NRAGE were measured using real-time PCR and western blot analysis during odontoblastic induction of mDPCs (Fig. 1). mRNA and protein levels of DMP-1, DSPP and ALP increased during induction (Fig. 1a–c, f) and ALP activity exhibited the same trend as *alp* mRNA expression (Fig. 1d). In contrast, mRNA and protein levels of NRAGE were down-regulated during odontoblastic differentiation (Fig. 1e, f).

Stable knockdown of NRAGE in mDPCs

Stably transfected cell populations of mDPC/shCon and mDPC/shNRG were constructed. mRNA and protein levels of NRAGE were clearly lower in mDPC/shNRG than those in mDPC/wt and mDPC/shCon groups, after infection (Fig. 2). Further verification by RT-PCR and western blotting demonstrated that expression of NRAGE remained down-regulated until day 14 (Fig. 2). These results showed that NRAGE was stably knocked down in mDPC/shNRG.

Inhibition of mDPC proliferation after knockdown of NRAGE

We measured cell proliferation using CCK-8 and results of the assay indicated that proliferation level of NRAGE-depleted NRAGE mDPCs was much lower than that of mDPCs/wt and mDPC/shCon groups ($P < 0.01$) on days 1, 3, 5 and 7, whereas no significant difference was observed between mDPCs/wt and mDPCs/shCon groups (Fig. 3). All results demonstrated that cell population growth of NRAGE-depleted mDPCs was greatly inhibited.

Promotion of odontoblastic differentiation of mDPCs after knockdown of NRAGE

Mineralized nodule formation, ALP staining and ALP activity assay were performed to directly reveal effects of NRAGE on odontoblastic differentiation. After 14 days culture in odontoblastic induction medium, mineralized nodule formation was observed, using phase contrast microscopy. Amounts of nodules formed in the mDPC/shNRG group by 14 days were significantly higher than in mDPC/wt and mDPC/shCon groups (Fig. 4a). ALP staining had the same trend as mineralization in the three groups (Fig. 4b). ALP activity was also significantly higher in mDPC/shNRG group than in mDPC/wt and mDPC/shCon groups (Fig. 5d). To further document effects of endogenous NRAGE on regulating odontoblastic differentiation, mineralization-related markers were selected. mRNA levels of *dmp1*, *dspp* and *alp* were significantly higher in mDPC/shNRG group than those in mDPC/wt and mDPC/shCon groups ($P < 0.05$; Fig. 5a–c). Western blot analysis indicated that DMP1 and DSP protein expression levels were much higher in mDPC/shNRG than those in mDPC/wt and mDPC/shCon groups (Fig. 5e). These results indicate that knockdown of NRAGE promoted odontoblastic differentiation of mDPCs.

Possible role of the NF- κ B pathway in regulating odontoblastic differentiation of mDPCs

Western blot analysis revealed two bands for NF- κ B protein. NF- κ B/p105 was down-regulated in mDPC/shNRG group, while NF- κ B/p50 was up-regulated (Fig. 6). Immunofluorescence staining showed that NRAGE was located homogeneously in the nucleus and cytoplasm of mDPCs without any induction, while NF- κ B was mainly located in the cytoplasm (Fig. 7). Under odontoblastic induction, NRAGE gradually translocated to nuclei of mDPCs, but was still found in both cytoplasm and nucleus (Fig. 7). Meanwhile, NF- κ B translocated from the cytoplasm into nuclei of mDPCs during the same process (Fig. 7). Moreover, locations of NRAGE and NF- κ B were almost the same after induction for 14 days (Fig. 7). These results suggest that NRAGE regulated odontoblastic differentiation of mDPCs via the NF- κ B pathway.

Discussion

Development, morphology and repair of damaged dental pulp tissue are dependent on proliferation and differentiation of DPCs. Proliferation and odontoblastic differentiation of dental pulp involves various growth factors,

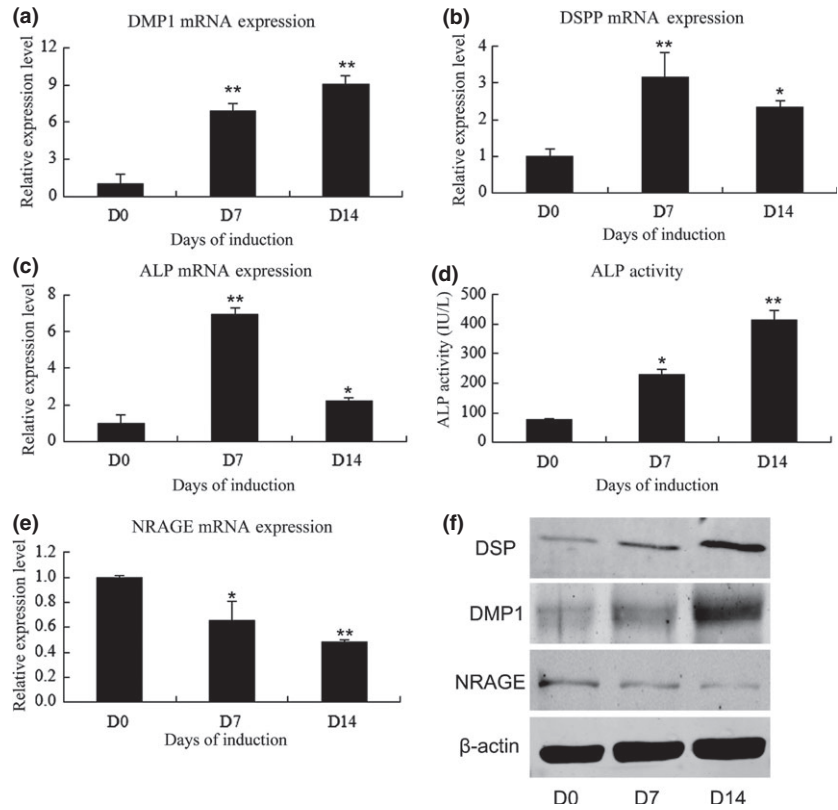


Figure 1. Expression of NRAGE during odontoblastic differentiation of mDPCs. mRNA expression of mineralization-related markers and NRAGE on days 0, 7 and 14 after induction: (a) DMP1, (b) DSPP, (c) ALP, (d) ALP activity and (e) NRAGE. Glyceraldehyde-3-phosphate (GAPDH) was used as normalization control. (f) DSP, DMP1 and NRAGE protein levels on days 0, 7 and 14 after induction. *Significant difference ($P < 0.05$) versus day 0, **Significant difference ($P < 0.01$) versus day 0.

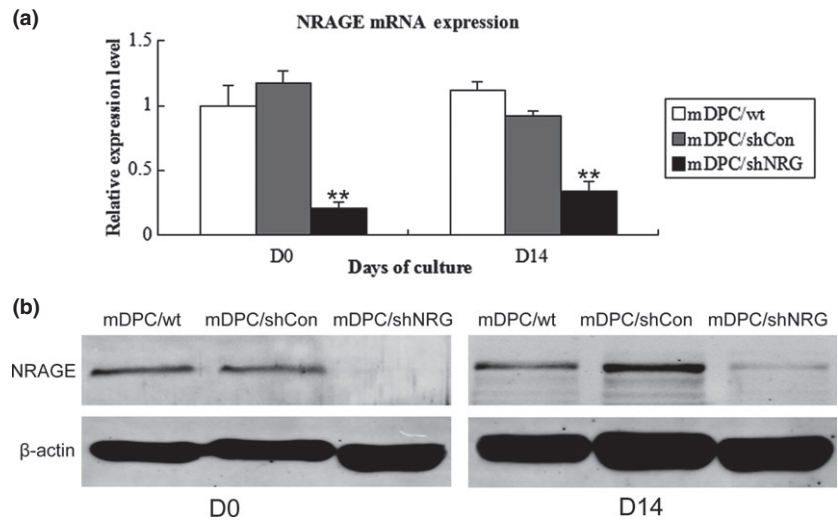


Figure 2. Stable knockdown of NRAGE in mDPCs. mRNA level (a) and protein level (b) on days 0 and 14 after mDPC infection. **Significant difference ($P < 0.01$) versus control.

signalling molecules and transcription factors, such as BMP-2 and BMP-4 (28,29), MSX1 (30), Dlx1/Dlx2 (31), HMGB1 (32) and more. This present study is the first to analyse function of NRAGE during proliferation and odontoblastic differentiation of mDPCs.

DMP1, ALP and DSPP are commonly used as mineralization markers in odontoblast/odontoblast-like differentiation of DPCs (33). In particular, DSP protein and DSPP mRNA have been reported to be

odontoblast-specific markers (34,35). In the current study, ALP expression and ALP activity increased during odontoblastic induction. DMP1 and DSPP were up-regulated during the same procedure. These results demonstrate that mDPCs differentiated into odontoblast-like cells; meanwhile, mRNA and protein levels of NRAGE were significantly down-regulated in the process. Thus, we concluded that NRAGE possibly regulated odontoblastic differentiation of mDPCs.

NRAGE has been identified as a binding partner of the intracellular domain of p75 neurotrophin receptor, thereby facilitating apoptosis in neural progenitors (36). NRAGE has been investigated for participating in neurone apoptosis (36), cell cycle regulation (37), renal branching morphogenesis (38) and bone formation (20). It is expressed in most developing and adult tissues (39) and plays important roles in activating the BMP MAPK and p38^{MAPK} pathways (39) and in tooth development, proliferation and differentiation of DPCs involves numerous overlapping tracts, including BMP MAPK (21) and p38^{MAPK} pathways (40). Thus, we presumed

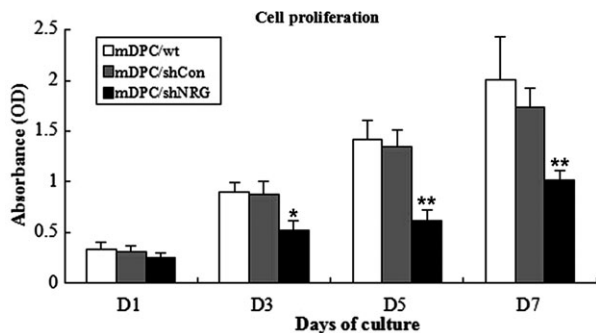


Figure 3. Effect of NRAGE knockdown on mDPC proliferation. *Significant differences ($P < 0.05$) versus control and **Significant difference ($P < 0.01$) versus control. OD, optical density.

that NRAGE possibly participated in tooth development and reparative dentin formation.

Lentiviral vectors provide efficient gene knockout *in vitro* and infect non-dividing cells. Results of CCK-8 demonstrate that cell proliferation of the mDPC/shNRG group was significantly inhibited. No significant differences between mDPC/wt and mDPC/shCon groups were observed in terms of either proliferation or differentiation. These findings indicate that recombinant lentivirus infection did not disturb the properties of mDPCs. NRAGE knockdown inhibited proliferation of mDPCs, identical to the previously published effect of NRAGE on oesophageal squamous cell carcinomas (26).

In this study, real-time PCR and western blotting showed that odontoblastic differentiation markers, including DMP1, DSP and ALP, were up-regulated in the mDPC/shNRG group, and to confirm this result, ALP activity, ALP staining and mineralization assays were performed. ALP activity and ALP staining increased with ALP mRNA level in the mDPC/shNRG group. The mineralization assay showed that NRAGE knockdown promoted mineralization potential of mDPCs. These results suggest that NRAGE knockout forced odontoblastic differentiation of mDPCs, which shows a potential role for NRAGE in tooth development and reparative dentin formation.

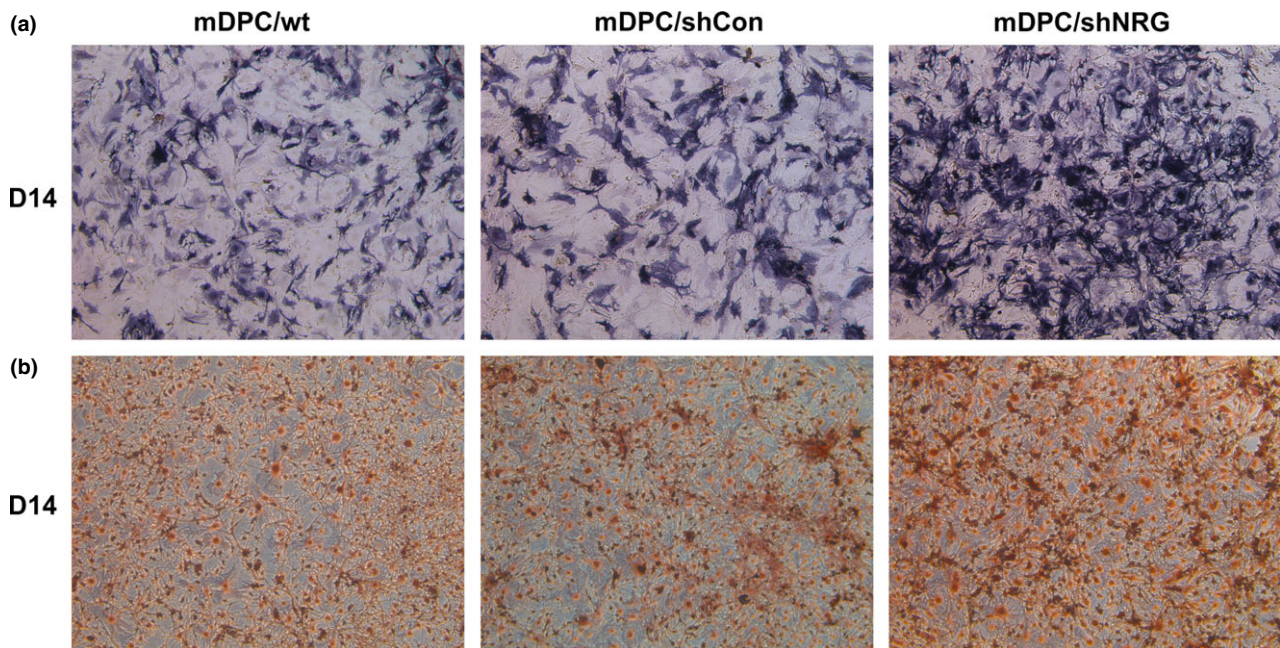


Figure 4. Mineralization and ALP staining of mDPC/wt, mDPC/shCon and mDPC/shNRG after odontoblastic induction for 14 days. (a) Result of ALP staining was higher in the mDPC/shNRG group than in mDPC/wt and mDPC/shCon groups (original magnification 50 \times). (b) Formation of mineralization nodules was much more in mDPC/shNRG group than in mDPC/wt and mDPC/shCon groups (original magnification 50 \times).

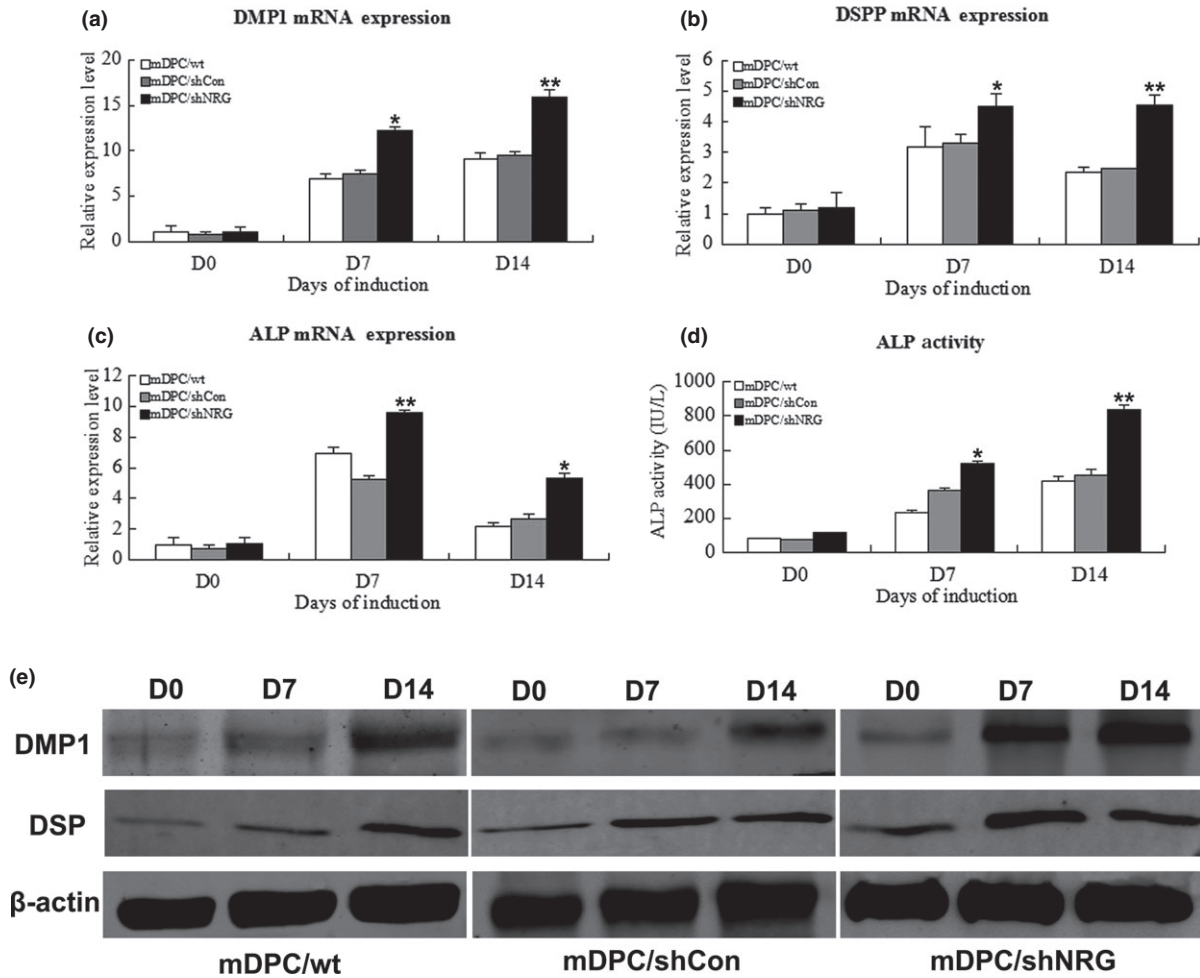


Figure 5. Effects of NRAGE knockdown on odontoblastic differentiation of mDPCs. Effect of NRAGE knockdown on mRNA level of odontoblastic marker genes in mDPCs on days 0, 7 and 14: (a) DMP1, (b) DSPP and (c) ALP. GAPDH was used as normalization control. (d) ALP activity in mDPC/shNRG group after 0, 7 and 14 days induction. Protein levels of DMP1 and DSP by western blot analysis on days 0, 7 and 14. *Significant difference ($P < 0.05$) versus control, **Significant difference ($P < 0.01$) versus control.

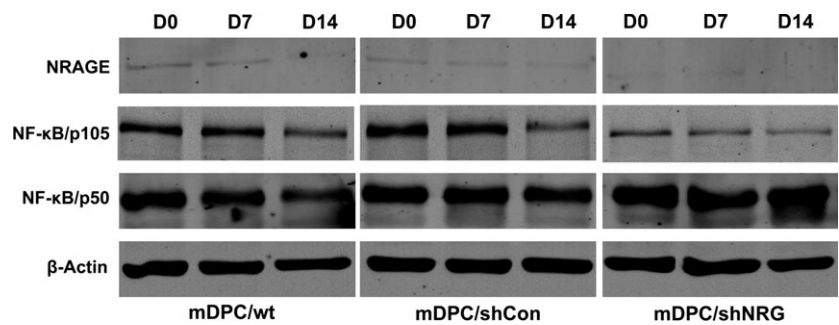


Figure 6. Protein levels of NRAGE and NF-κB after odontoblastic induction for 0, 7 and 14 days in mDPC/wt, mDPC/shCon and mDPC/shNRG groups. NRAGE was stably knocked down during induction in the mDPC/shNRG group. Protein levels of NF-κB/p50 and NF-κB/p105 by western blot analysis.

The BMP pathways regulate tooth development through two representative methods, namely, canonical and non-canonical pathways. In the non-canonical BMP pathway, NRAGE facilitates TAK1–TAB 1–XIAP complex, and drives BMP receptor and p38 activation (41).

Previous researchers have shown that NF-κB pathway links to non-canonical BMP pathway *via* formation of the TAK1–TAB 1–XIAP complex (39,42–44). NRAGE has been reported to regulate neuronal progenitor cell differentiation by up-regulating NF-κB activity and

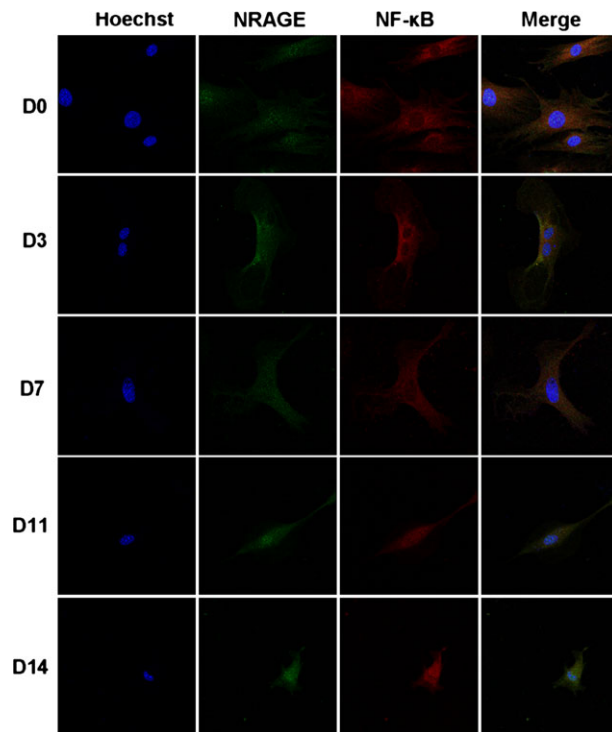


Figure 7. Immunofluorescence staining of NRAGE and NF- κ B relocation in mDPCs during odontoblastic induction for days 0, 3, 7, 11 and 14 (400 \times magnification). Immunofluorescence staining with anti-NRAGE antibody (green), anti-NF- κ B antibody (red) and Hoechst (blue) was performed. Negative control staining was performed with immunoglobulin G control antibody.

activating the BMP pathway (20). NF- κ B pathway is activated at sites of injury, and regulates apoptosis and the cell cycle (19). Activation of NF- κ B promotes odontoblastic differentiation against oxidative stress in DPCs (45) and furthermore, β -oestradiol enhances odonto/osteogenic potency of DPSCs *via* activation of NF- κ B (46). Most importantly, Lee *et al.* have shown that NF- κ B is activated on promotion of proliferation, migration and differentiation in recombinant human DSP-treated DPCs (47). Thus, the NF- κ B pathway may also regulate reparative dentin formation. However, activation of NF- κ B inhibits odonto/osteogenic differentiation of DPSCs in oestrogen-deficient rats (48). These data suggest that activation of NF- κ B in different cell types may result in distinctive differentiation potency through different upstream and downstream signalling pathways.

In unstimulated cells, NF- κ B is sequestered in the cytoplasm, and translocates to nuclei when activated (39). Generally, NF- κ B/p105 is retained in the cytoplasm to inhibit activation of transcription. In response to stimuli, NF- κ B/p50 is released rapidly and translocates to the nucleus, whereas NF- κ B/p105 is degraded in proteasomes. These observations are in accordance with our

results. Immunofluorescence revealed that NF- κ B gradually translocated from cytoplasm to nuclei during odontoblastic induction, and locations of NRAGE and NF- κ B were similar on day 14 after induction. Western blotting demonstrated that protein level of NF- κ B/p105 was down-regulated compared with that of controls, whereas protein level of NF- κ B/p50 was up-regulated. Thus it seems that, NRAGE possibly regulated odontoblastic differentiation of mDPCs by activation of the NF- κ B pathway, in our study. More intensive investigations are necessary to elucidate relationships between NRAGE and the NF- κ B pathway in odontoblastic differentiation.

In summary, NRAGE regulated odontoblastic differentiation of mDPCs, which seemed to depend on the NF- κ B pathway. Our studies showed that NRAGE could be a new regulation marker for use in regenerative dentistry. We will further elucidate the signalling pathway of NRAGE on proliferation and odontoblastic differentiation of DPCs; primary human DPCs will be used in our future studies.

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