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TNF-α was involved in calcium hydroxide-promoted osteogenic differentiation of human DPSCs through NF-κB/p38MAPK/Wnt pathway

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It has been reported that calcium hydroxide can induce proliferation, migration, and mineralization in dental pulp stem cells (DPSCs), but the underlying molecular mechanisms are still unclear. In this study, we sought to explore the role of calcium hydroxide in the cell proliferation and directional differentiation of DPSCs and to study the regulatory effect of NF-κB, p38MAPK, and Wnt signaling on differentiation of DPSCs. CCK8 cell assay, Wound Healing Assay, and Alkaline Phosphatase Staining Assay were respectively used to determine the proliferation rate, migration and ALP expression of DPSCs. Alizarin Red Staining Assay was used to observe the mineralization of DPSCs. RT-PCR analysis and Western Blot Analysis displayed the expression of related fators at mRNA and protein level, respectively. In the present study, we found that NF-κB, p38MAPK, and Wnt signaling could abolish calcium hydroxide-induced proliferation of DPSCs. The inhibition of NF-κB, p38MAPK, and Wnt signaling suppressed the migration, ALP expression, and mineralization of DPSCs. NF-κB, p38MAPK, and Wnt signaling involved in directional differentiation of DPSCs. Moverover, calcium hydroxide could activate NF-κB, p38MAPK, and Wnt pathway by regulating TNF-α. Our study showed that NF-κB, p38MAPK, and Wnt signaling pathway were involved in calcium hydroxide-induced proliferation, migration, mineralization, and osteogenic differentiation in DPSCs. Calcium hydroxide affected NF-κB, p38MAPK, and Wnt pathway by regulating TNF-α.

1. Introduction

Pulpitis is a common oral disease leading to severe pain, which is due to G-bacteria invasion of the pulp. Lipopolysaccharide (LPS) is a G-bacillus main virulence factor, which could induce mononuclear macrophages and fibroblasts into secreting series of cytokines such as IL-1, IL-6 and IL-8. Pulpitis seriously affects health and quality of patient's life (Ershova and Dmitrieva 1987; Singh and Jadhav 2014; Trowbridge 1981). Especially in children unilateral mastication may be caused affecting the maxillary and facial morphology. When pulp is inflammated or injured, cell morphology, synaptic connections and gene expression are changed in the medullary dorsal horn, so as to participate in the process of central sensitization in inflammatory pain at medullary levels, formatting the characteristic symptoms of pulpits pain. However, its central regulatory mechanism is not fully elucidated.

As a kind of undifferentiated adult stem cells, dental pulp stem cells (DPSCs) are among the hot topics in the area of oral biology. DPSCs, originated from neural crest cells-derived mesenchyme (Wang et al. 2011), have high proliferative potential for self-renew and display strong odontogenic potential compared with bone marrow stem cells (Pierdomenico et al. 2005), which can differentiate into odontoblast/osteoblasts, neurocytes, and adipocytes *in vitro* (Ranganathan and Lakshminarayanan 2012). Moreover, several researchers have found that DPSCs could form the dentin/pulp-like complex (Gronthos et al. 2011, 2001). Therefore, DPSCs are considered as ideal seeding cells in tissue engineering.

Calcium hydroxide has been reported to have the potential to increase proliferation, migration, and mineralization of DPSCs (Ji et al. 2010), but the underlying mechanism is still unclear. NF-κB is located in the cytoplasm and participates in many pathological and physiological cellular processes (Didonato et al. 2012; Gasparini and Feldmann 2012; Kumar et al. 2004). Several studies have demonstrated that it plays an important role during the tooth organogenesis and eruption process (Courtney et al. 2005; Ohazama and Sharpe 2004). As is known, p38 regulates the expression of many cytokines. It also plays an essential role in LPS-promoted odontoblastic differentiation in DPSCs (He et al. 2015). It has been reported that the Wnt signaling pathway, the MAPK signaling pathway, and the TGF-βsignaling pathway are involved in the differentiation of BMSCs. Therefore, we sought to explore the role of calcium hydroxide in cell proliferation and directional differentiation of DPSCs and to study the regulatory effect of NF-κB, p38MAPK, and Wnt signaling on differentiation of DPSCs.

2. Investigations and results

2.1. NF-κB, p38MAPK and Wnt involved in calcium hydroxide-induced proliferation in DPSCs

CCK8 cell assay was used to determine the proliferation rate of DPSCs (Fig. 1). The results showed that the cell viability of DPSCs induced by $Ca(OH)_2$ was significantly increased ($P<0.05$) compared with the control group. However, the cell viability of DPSCs was significantly decreased ($P<0.05$) calcium hydroxide-induced proliferation by inhibitors of NF-κB, p38, and Wnt. Our results indicated that NF-κB, p38, and Wnt were involved in calcium hydroxide-induced proliferation in DPSCs.

2.2. NF-κB, p38MAPK and Wnt involved in calcium hydroxide-induced migration in DPSCs

Wound Healing Assay was used to determine the migration of DPSCs. The results showed that the migration of DPSCs induced by calcium hydroxide was significantly increased ($P<0.05$)

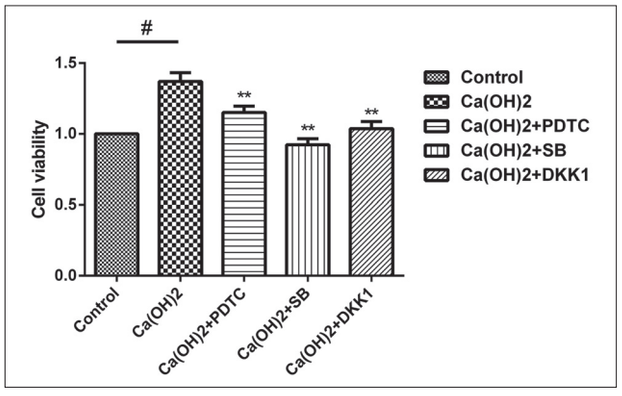

Fig. 1: NF-κB/p38 MAPK/Wnt pathway involved in calcium hydroxide-induced proliferation in DPSCs. CCK8 cell assays were used to analyze the cell viability of calcium-induced proliferation treated or untreated with the inhibitor of NF-κB (PDTC, 10 mmol/L), p38 (SB:SB203580, 10 mmol/L), and Wnt (DKK1,10 mmol/L). Error bars indicate means±SD and *indicates significant difference compared with calcium hydroxide group ($p < 0.05$), #indicates significant difference compared with control group($p < 0.05$).

compared with the control group (Fig. 2). After 24 h, the inhibition of NF-κB, p38 and Wnt all highly depressed calcium hydroxide-induced migrations ($p<0.05$). It was shown that NF-κB, p38, and Wnt were involved in calcium hydroxide-induced migration in DPSCs.

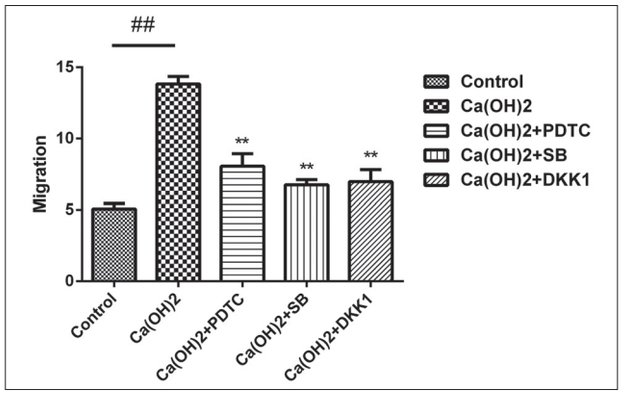

Fig. 2: NF-κB/p38 MAPK/Wnt pathway involved in calcium hydroxide-induced migration in DPSCs. Wound Healing Assay were used to analyze the cell migration of calcium-induced migration treated or untreated with the inhibitor of NF-κB (PDTC, 10 mmol/L), p38 (SB:SB203580,10 mmol/L), and Wnt (DKK1, 10 mmol/L). Error bars indicate means±SD and *indicates significant difference compared with calcium hydroxide group ($p < 0.05$), #indicates significant difference compared with control group($p < 0.05$).

2.3. NF-κB, p38MAPK and Wnt involved in calcium hydroxide-induced alkaline phosphatase expression in DPSCs

To investigate calcium hydroxide-induced osteogenesis in DPSCs, we detected ALP expression (Fig. 3). The results showed that calcium hydroxide significantly promoted ALP expression after 14 days of incubation. NF-κB, p38 and Wnt inhibitors significantly decreased calcium hydroxide-induced ALP expression. It was shown that NF-κB, p38 and Wnt played important roles in calcium hydroxide-inducedosteogenic differentiation in DPSCs.

2.4. NF-κB/p38MAPK / Wnt involved in calcium hydroxide-induced mineralization in DPSCs

Alizarin Red Staining Assay was used to observe the mineralization of DPSCs (Fig. 4). After 14 days, calcium hydroxide significantly increased mineralization ($P<0.05$), while NF-κB, p38MAPK, and Wnt inhibitors inhibited the formation of mineral nodes remark-

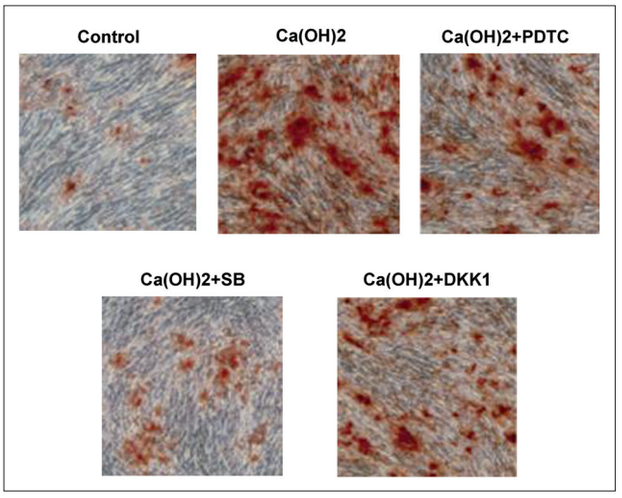

Fig. 3: NF-κB/p38 MAPK/Wnt pathway played important roles in calcium hydroxide-induced ALP expression in DPSCs. After preincubation with the inhibitor of NF-κB (PDTC, 10 mmol/L), p38 (SB:SB203580, 10 mmol/L), and Wnt (DKK1, 10 mmol/L) for 1 h, the culture medium was changed into normal medium with or without calcium hydroxide. After 14 days, the ALP staining assay was analyzed and representative data were shown.

ably compared with calcium hydroxide group ($P<0.05$). The results showed that NF-κB, p38MAPK, and Wnt were involved in calcium hydroxide-induced mineralization in DPSCs.

2.5. Calcium hydroxide induced phosphorylation of NF-κB/p38 MAPK/Wnt

RT-PCR analysis and Western Blot Analysis displayed the expression of p-IKBA, p-p38 and p-GSK3β at mRNA and protein level, respectively. As shown in Fig. 5, the expression of p-IKBA and p-p38 was significantly increased, while the expression of p-GSK3β was significantly decreased after being incubated with calcium hydroxide for 30 min. After 60 min, the expression of p-p38 was slightly decreased, but still significantly higher than in the control group. The expression of p-GSK3β was lower than that after treatment for 30 min.

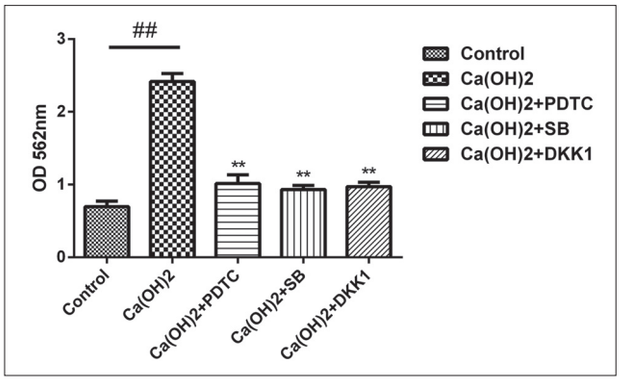

Fig. 4: NF-κB/p38 MAPK/Wnt involved in calcium hydroxide-induced mineralization in DPSCs. Alizarin red staining showed mineral nodule formation after 14 days. Representative data were shown and each result was repeated at least 3times. Error bars indicate means±SD and *indicates significant difference compared with calcium hydroxide group ($p < 0.05$), #indicates significant difference compared with control group ($p < 0.05$).

2.6. NF-κB/p38MAPK / Wnt involved in directional differentiation of DPSCs

RT-PCR analysis and western blot analysis displayed the expression of DSPP, DMP1, OPN and ALP (Fig. 6). The results showed that the NF-κB inhibitor increased calcium hydroxide-induced expression of DSPP, DMP1, OPN and ALP significantly ($P<0.05$)

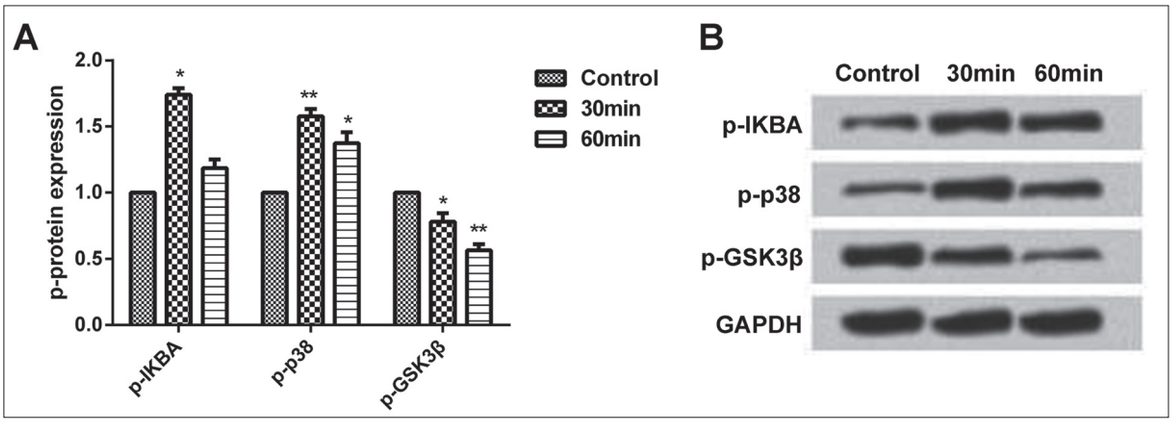

Fig. 5: Calcium hydroxide induced phosphorylation of NF-κB / p38 MAPK / Wnt. (A) the expression of NF-κB / p38 MAPK / Wnt pathway proteins was analyzed by RT-PCR at different time points. (B) the expression of NF-κB / p38 MAPK / Wnt pathway proteins was analyzed by western blot at different time points. Error bars indicate means±SD and *indicates significant difference compared with control group ($p < 0.05$).

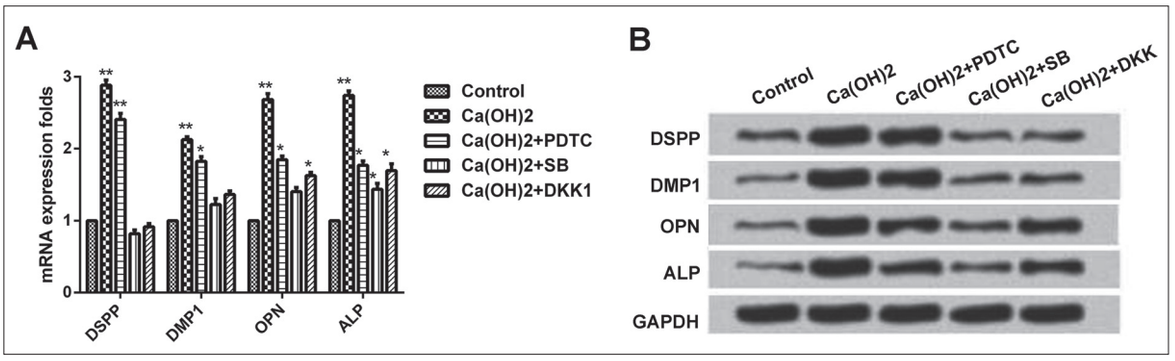

Fig. 6: NF-κB/p38 MAPK/Wnt involved in calcium hydroxide-induced odonto/osteogenic differentiation in DPSCs.. (A) odonto/ostegenic genes (ALP, DSPP, DMP-1, and OPN) were analyzed by RT-PCR. (B) odonto/ostegenic genes (ALP, DSPP, DMP-1, and OPN) were analyzed by western blot. Error bars indicate means±SD and *indicates significant difference compared with control group ($p < 0.05$).

compared with the control group. The Wnt inhibitor increased the expression of OPN and ALP significantly ($P<0.05$) compared with the control group. The p38 inhibitor increased ALP expression significantly ($P<0.05$) compared with the control group. However, the three inhibitors decreased the expression of these proteins compared with the calcium hydroxide group. It was shown that NF-κB played the most important role in directional differentiation of DPSCs, followed by Wnt and p38.

2.7. Expression of p-IKBA, p-p38 and p-GSK3β after TNF-α siRNAs transfection

As shown in Fig. 7, calcium hydroxide-induced p-IKBA and p-p38 expression were significantly decreased after TNF-α siRNAs transfection ($p<0.05$). However, calcium hydroxide-induced p-GSK3β expression was significantly increased after TNF-α siRNAs transfection ($p<0.05$). The results indicated that calcium hydroxide could activate the NF-κB and p38MAPK pathway, but not Wnt pathway by regulating TNF-α.

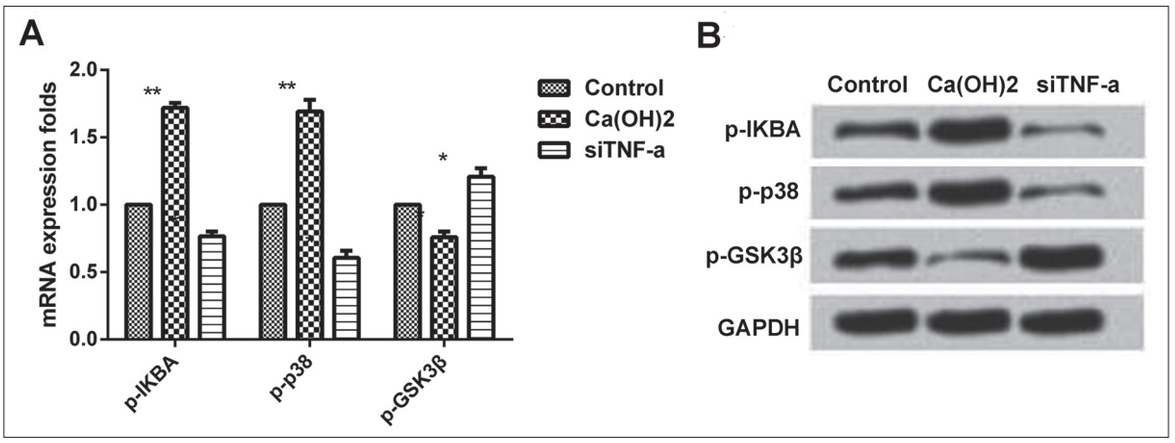

Fig. 7: The effect of calcium hydroxide on NF-κB/p38 MAPK/Wnt after siTNF-α. (A) Expression of NF-κB/p38 MAPK/Wnt pathway proteins was analyzed by RT-PCR at different time points. (B) Expression of NF-κB/p38 MAPK/Wnt pathway proteins was analyzed by western blot at different time points. Error bars indicate means±SD and *indicates significant difference compared with control group ($p < 0.05$).

3. Discussion

DPSCs are known candidates for bone and dental tissue engineering and have the characteristics of high proliferation rate, self-renewal and multi-differentiation capability. The therapeutic usefulness of DPSCs is limited by low survivability upon transplantation in situ due to the presence of various proapoptotic factors within damaged tissues (Heng et al. 2016). Various internal mechanisms and microenvironmental factors can influence their proliferation and differentiation. Inflammation leads to massive cytokines release which change the microenvironment where stem cell mediate tissue regeneration and repair (Monje et al. 2002, 2003). Several studies have shown that persistent chronic inflammation environmental stimulation can reduce the proliferation and migration ability of stem cells (Wang et al. et al. 2008; Pluchino 2008). The occurrence and development of pulpitis is a complex physiological process including various cytokines, such as TNF-α, IL-1p, and IL-8. As the gold standard of pulp capping materials in clinical vital pulp therapy, calcium hydroxide has been reported to increase cell proliferation, migration, and mineralization of DPSCs (Chen et al. 2016; Ji et al. 2010). Migration is indispensable for pulpal wound healing, which can induce DPSCs to the wounded areas before they differentiate to form a reparative dentin (Schröder 1985).

According to previous reports, NF-κB pathway plays an important role in the proliferation and committed differentiation of human stem cells from apical papilla (Li et al. 2014). Previous studies have shown that various stimuli play an important role in the committed differentiation of dental stem cells by regulating NF-κB signaling pathway (Wang et al. 2013). In the present study, we found that NF-κB pathway-inhibited DPSCs exhibited a lower proliferation and migration area and decreased odonto/osteogenic ability. p38 MAPK signaling pathway plays a favorable role in the LPS regulated the differentiation of hDPSCs (He et al. 2013). It is reported that p38 is involved in calcium hydroxide-induced migration and osteogenic differentiation in human DPSCs (Chen et al. 2016). The results are identical with our study. Wnt/β-catenin signaling pathway plays an important role in bone formation and homeostasis. A previous study has shown that inflammatory microenvironments could activate the Wnt/β-catenin pathway, which mediated the impaired osteogenic differentiation of PDLSCs (Kong et al. 2013). Our study showed that Wnt inhibitors could decrease the calcium hydroxide-induced cell proliferation, migration and odontogenic/osteogenic differentiation in DPSCs. All the analysis showed that NF-κB, p38 MAPK, and Wnt/β-catenin pathway played an essential role in calcium hydroxide-induced odontogenic/osteogenic differentiation in DPSCs.

It is well known that ALP is a marker of odontogenic/osteogenic differentiation (Shui and Scutt 2001). As another marker of odontoblasts, DSPP is highly expressed in dentin or predentin structures and essential for detinogenesis (Suzuki et al. 2012). DMP-1, an acidic extracellular matrix protein, is found in dentin and bone, which is involved in dentin mineralization and signal transduction in the process of odontogenesis (Prescott et al. 2008). OPN is an important extracellular matrix protein expressed in odontoblasts, and plays multifaceted roles in various biological and pathological processes, such as osteogenic diferentiation, tooth mineralization, and dental biofilm formation (Cao 2012; Schlafer et al. 2012). Our study showed that the inhibition of NF-κB, p38, and Wnt decreased the expression of DSPP, DMP1, OPN, and ALP compared with calcium hydroxide group. The result indicated that it attenuated calcium hydroxide-induced odontogenic/osteogenic differentiation.

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine associated with tissue destruction in periodontitis. It can activate multiple signaling pathways related to odontogenic/osteogenic differentiation. Previous studies have demonstrated that TNF-α can increase the expression of BMP-2 by activating the NF-κB pathway, leading to the mineralization of extracellular matrix (Hess et al. 2009), and that TNF-α could inhibit osteogenic differentiation of PDLSCs through activating the Wnt pathway. Our study demonstrated that the expression of p-IKBA and p-p38 was decreased, while p-GSK3β expression was increased after TNF-α siRNAs transfection. These results indicated that TNF-α played an important role in NF-κB and p38, but not in the Wnt pathway.

Our findings suggest that NF-κB, p38, and Wnt pathway are involved in calcium hydroxide-induced proliferation, migration, mineralization, and osteogenic differentiation in DPSCs. Calcium hydroxide could affect NF-κB, p38, and Wnt by regulating TNF-α. Our work revealed the mechanism of calcium hydroxide-induced DPSCs and provides a scientific foundation for future cell therapy with DPSCs.

4. Experimental

4.1. Cell isolation and culture

Human dental pulp tissues were gathered from healthy young patients (14–22 years) with extraction of the third molars for orthodontic reasons at Peking University People's Hospital, Beijing, China. The pulp tissue was digested with 3 mg/mL collagenase type I and 4 mg/mL Dispase for 1 h at 37 °C. Single cell suspensions were seeded into 96-well plates by 1 to 2 cells/well with alpha modification of Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Over 50 cells/well were scored as colonies and passaged by 0.25% trypsin. The cells between passages 3 and 5 were used in the following experiments: DPSCs characterized by flow cytometric analysis of CD44, CD146, CD29, STRO-1, CD45, and CD34. The multiple differentiation potentials of osteogenesis and chondrogenesis were also confirmed.

4.2. CCK8 cell assay

Equal amounts of cells were seeded in 96-well plates. DPSCs were pretreated with dimethyl sulphoxide (DMSO) for the control and calcium hydroxide groups or with 10 mmol/L NF-κB inhibitor (PDTC), an Wnt inhibitor (DKK1), or a p38 inhibitor SB203580) for 1 h. The medium was changed to fresh medium with or without 10 mg/mL calcium hydroxide (Amresco). After 24 h of treatment, CCK8 reagent (0.5 mg/ml) was then added to the cell culture medium and incubated at 37 °C for 1 h. The absorbance was evaluated at 450 nm using a microplate reader.

4.3. Wound healing assay

DPSCs were cultured on 6-well plastic dishes until they grew a monolayer. A cross area was scratched with a pipette tip, and the floating cells were moved by phosphate-buffered saline. DPSCs were pretreated with an MAP kinase inhibitor and changed to culture medium with or without calcium hydroxide as described previously. The scratched areas at each time point of 24 h were imaged. To estimate the migration of DPSCs, the cell-free area of each cross was calculated using DP2-BSW software (Olympus, Tokyo, Japan). Each migration result was relative to the image of the control group. The related wound area (percentage) was used to quantify the data.

4.4. Alkaline phosphatase staining assay

DPSCs were pretreated with an MAP kinase inhibitor and changed to normal medium with or without calcium hydroxide for 14 days and incubated with BCIP/NBP reagent (Beyotime) for about 10 min at room temperature. Color development was stopped by water. All staining images were taken under a microscope (IX 71, Olympus). Each experiment was performed at least three times.

4.5. Alizarin red staining assay

DPSCs were pretreated with an MAP kinase inhibitor and changed to normal medium and mineralization induction medium (50 mmol/L ascorbic acid, 10 mmol/L b-glycerophosphate, and 100 nmol/L dexamethasone) with calcium hydroxide for 14 days. The control groups were pre-incubated with the same volume of DMSO and changed with normal medium or mineralization induction medium without calcium hydroxide for 14 d. DPSCs were added to 40 mmol/L alizarin red (pH = 4.2; American MasterTech, Lodi, CA) solution and incubated at room temperature for 30 min with 50 rpm shaking. Each dish was washed and imaged. For the quantitative assay, 10 mmol/L sodium phosphate–10% acetylpyrimidium (pH = 7.0) solution was added into the alizarinred–stained samples and incubated at room temperature for 15 min. Then, the solutions were transferred to a 96-well plate and measured at 562 nm.

4.6. TNF-α siRNAs transfection

miR-132 mimic and corresponding miRNA negative control (miR-control) were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China), and were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

4.7. RealTime-PCR

Total RNA was extracted from cultured cells and used to synthesize cDNA using a SYBR Premix Ex Taq II Reagent Kit and gDNA Eraser reverse transcriptase, according to the manufacturer's instructions (Takara, Japan). For polymerase chain reaction (PCR) analysis, samples were normalized to β-actin expression by calcu-

lating ΔCT (CT target gene - CT actin). The 2-ΔΔCT method was used to calculated the relative amount of target mRNA.

4.8. Western Blot

Hippocampal neuron cells were collected, and lysed for 30 min in lysis buffer containing 1 mM phenylmethanesulfonyl fluoride. The cells were then centrifuged at 12000 rpm for 10 min at 4 °C. Protein concentrations in the cell lysates were determined by the BCA protein assay. Equal amounts of cell lysates (20 mg) were separated by 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes and blocked with 1% bovine serum albumin with 0.05% Tween 20 in PBS for 30 min, followed by incubation with Santibodies, and with b-actin as a control, at 4 °C overnight. After three washes with TBST, the membranes were incubated with alkaline phosphatase–goat anti-rabbit IgG or alkaline phosphatase-rabbit anti-goat IgG for 1 h. Western Blue stabilized substrate alkaline phosphatase was used to detect the immunoreactive signals. The air-dried membranes were imaged using an image analyzer. Band intensities were measured using the Image J v1.50 software.

4.9. Statistic analysis

Results are presented as mean±SEM. All assays were compared among three or more independent experiments. Differences among groups were analyzed using repeated measures one-way ANOVA comparison tests. A value of $p < 0.05$ was considered to be significant.

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Conflict of interests: The authors declared no conflict of interests.

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