Depletion of EREG enhances the osteo/dentinogenic differentiation ability of dental pulp stem cells via p38 MAPK and Erk pathway in inflammatory microenvironment

Running head: EREG inhibits osteo/dentinogenic differentiation of DPSC

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Abstract

Background

Epiregulin (EREG) is an important component of EGF, which was demonstrated to promote the osteo/dentinogenic differentiation of stem cells from SCAPs. Whether it could stimulate the osteo/dentinogenic differentiation of DPSCs in inflammatory environment is not clear. The purpose of the present study was to investigate the role of EREG on the DPSCs’ osteo/dentinogenic differentiation ability in inflammatory environment.

Methods

DPSCs were isolated from human third molars. Short hairpin RNAs (shRNAs) was used to knock down the EREG expression in DPSCs. Recombinant human EREG protein (rhEREG) was adopted in the rescue experiment. TNF-α was employed to mimic the inflammatory environment in vitro. Alkaline phosphatase (ALP) staining, Alizarin red staining, quantitative calcium analysis, and real time RT-PCR was used to detect the osteo/dentinogenic differentiation markers and related signaling pathways under normal and inflammatory environment.

Results

EREG depletion promoted ALP activity and mineralization ability of DPSCs. Expression of BSP, DMP-1, and DSPP were also enhanced. Besides, 50ng/ml rhEREG treatment weakened the osteo/dentinogenic differentiation potential. 10 ng/mL TNF-α
treatment for 4h increased the expression of EREG in DPSCs. However, knockdown of EREG rescued the impaired osteo/dentinogenic differentiation ability caused by TNF-α treatment. Further mechanism study showed that, EREG depletion activated p38 MAPK and Erk signaling pathways in DPSCs under normal and inflammatory environment.

**Conclusions**

Our results demonstrated that EREG could inhibited the osteo/dentinogenic differentiation potential of DPSCs via p38 MAPK and Erk signaling pathways in normal and inflammatory environment.

**Keywords:**

Epiregulin (EREG); dental pulp stem cells (DPSCs); osteo/dentinogenic differentiation; TNF-α; inflammatory environment

**Background**

Pulpitis is a common disease which contribute to non-vital teeth, and teeth missing. Traditional methods include vital pulpotomy, apexification, revascularization, and root canal treatment, however, neither of them can solve the problems such as cessation of root development, fragile root canal walls and a series of complication.(1) Therefore, it is of great significance to regenerate dentin-pulp complex so as to maintain normal physiological function. With the development of tissue engineering, increasing researches have shed light on the dentin-pulp regeneration based on mesenchymal stem cells.(2) However, during the process of tissue engineering, MSCs are engrafted to the sites of injury and inflammation and the engraftment efficiency is largely influenced by the local microenvironment, among which, inflammation is one of the most important
factors affecting the regeneration efficiency of MSCs for pulpitis. (3)

Both trauma and microbes, which result in pulpitis, can lead to an inflammatory microenvironment, characterized by an accumulation of inflammatory cells, which release host proinflammatory cytokines, including tumor necrosis factor-α (TNF-α). (4, 5) Thus, TNF-α has been documented in the acute inflammatory phase which stimulates the immunosuppressive ability of MSCs. (6) TNF-α was also shown to affect osteoclastogenesis and bone formation. (7) Studies have demonstrated that an increased apoptotic signaling with a compromised longevity of DPSCs upon short term exposure to inflammatory factors. (8) Besides, in the diseased pulpal tissue, DPSCs displayed weak and hyperproliferative ability with a diminished mineralization potential. (9) Consequently, it is critical to study the functional changes and to identify suitable growth factors to promote the function of DPSCs in inflammatory microenvironment.

Recently, researchers have attempted to identify the underlying molecules involving in inflammatory microenvironment in terms of mediated growth factors and signaling pathway. With the identification, epidermal growth factor (EGF) has been reported as a potent regulator. (10) The EGF family comprises multiple mediators such as transforming growth factor-α, amphiregulin, heparin binding-EGF, and epiregulin (EREG), which are crucially in regulating fundamental functions in mammalian cells including survival, migration, and proliferation. (11) Besides, results of inflammation also indicated the important role of EGF family, such as amphiregulin, (12) HB-EGF, (13) TGF-α, (14) especially EREG. (15, 16) Like the other members of EGF family, EREG binds to several EGF receptors, thus couple to numerous signaling cascades, most notably the MAPK kinase (Erk) signaling pathway, the phospholipase C gamma pathway, and the PI3 kinase/Akt signaling pathway to regulate series of cellular physiological and pathological functions, (17) such as vascular remodeling, (18) liver
regeneration(19) and cutaneous wound healing.(20) Our previous studies have shown that EREG could improve the proliferation and osteo/dentinogenic differentiation ability of SCAPs by accelerating phosphorylation of MAPK-ERK kinase (MEK)/Erk and c-Jun Nterminal-kinase (JNK).(21, 22) In addition, EREG improved the migration and chemotaxis ability of ADSCs depending on the activation of MAPK signaling pathways, including p38 MAPK, JNK, and Erk1/2.(23) Other studies have also demonstrated that EREG takes part in the development of bronchial infection and rheumatoid arthritis.(24) Intervention of EREG may provide an effective way to control the progress of infection.(25) Besides, epieregulin plays a role in skin inflammation and cutaneous wound healing by regulating Toll-like receptor pathway and angiogenesis.(20, 26) However, it is unclear whether EREG has an effect on MSCs function under inflammatory microenvironment.

In the present study, we aim to investigate the role of EREG on MSCs function under inflammatory microenvironment. DPSCs were the first dental mesenchymal stem cells isolated from the dental pulp of permanent teeth and contribute to dentinogenesis and show good performance in colony units forming, surviving time, and osteo/dentinogenesis ability.(27) Hence, DPSCs were considered an ideal tool to regain lost dental tissues and to re-engineer the root canal system. Therefore, in this study, DPSCs were used to investigate the function of EREG and the underlying mechanism. We adopted TNF-α to mimic inflammatory environment and performed a loss-gain-function assay to investigate the role of EREG on the osteo/dentinogenic differentiation ability of DPSCs. Our results demonstrated that EREG has a significant role in regulating the osteo/dentinogenic differentiation potential of DPSCs under inflammatory environment depending on MAPK signaling pathway.

Methods
**Human dental pulp stem cells (DPSCs) culturing**

According to ISSCR “Guidelines for the Conduct of Human Embryonic Stem Cell Research, DPSCs were cultured as described in our previous studies.(22) Wisdom teeth were acquired from Beijing Stomatological Hospital, Capital Medical University, and all procedures followed the rules approved by Beijing Stomatological Hospital, Capital Medical University. Informed agreement was obtained from all patients. Briefly, the tissues were gently separated from the dental pulp and then digested in a solution of 3mg/mL collagenase type I (Worthington Biochemical Corp, Lakewood, NJ, USA) and 4mg/mL dispase II (Roche Diagnostics Corp, Indianapolis, IN, USA) for 1h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-mm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA). DPSCs were grown in a humidified, 5% CO2 incubator at 37°C by using Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum, 2mmol/L glutamine, 100U/mL penicillin, and 100mg/mL streptomycin(Invitrogen, Carlsbad, CA, USA ). The culture medium was changed every 3 days. in the present study, 10 ng/mL tumour necrosis factor-alpha (TNF-α) (R&D Systems, Minneapolis, MN, USA) and human recombinant EREG protein (Abcam, Cambridge, MA, USA) were used to stimulate the DPSCs. The p38 MAPK-specific inhibitor, SB203580 (Med Chem Express, NJ, USA), and the Erk-specific inhibitor, PD98059 (Merck, Darmstadt, Germany), were used to treat the DPSCs.

**Plasmid construction and viral infection**
Plasmids were constructed using standard methods; all structures were verified by appropriate restriction digestion. Short hairpin RNAs (shRNA) with complementary sequences of the target genes were subcloned into the pLKO.1 lentiviral vector (Addgene, Cambridge, MA, USA). For viral infections, DPSCs were plated overnight, then infected with retroviruses in the presence of 6 mg/ml polybrene (Sigma–Aldrich, St. Louis, MO, USA) for 6 h. After 48 h, infected cells were selected with 2 mg/mL puromycin for 7 days. Scramble shRNA (Addgene, Cambridge, MA, USA) was used as control. The target sequences for the shRNA were as follows: EREG shRNA: 5′-actactgcaggtgaagt-3′;

Real-Time Reverse Transcriptase-PCR (Real-time RT-PCR)

Total RNA was isolated from DPSCs using Trizol reagents (Invitrogen, Carlsbad, CA, USA ). From 2μg RNA aliquots, we integrated cDNA by using oligo (dT) and reverse transcriptase, according to the manufacturer's protocol(23) (Invitrogen, Carlsbad, CA, USA ). Next, Real-time RT-PCR reactions were performed according to the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) and an IcycleriQ Multi-colour Real-time RT-PCR Detection System.(23). Primers for specific genes used in this study are protected by copyright. All rights reserved. The list of primer sequences is presented in Supplementary Table 1.

Alkaline phosphatase (ALP) activity assay and Alizarin red detection

DPSCs were cultured in osteogenic-inducing medium containing α-MEM, 15% fetal
bovine serum, 100 μM/mL ascorbic acid, 2 mM β-glycerophosphate, 1.8 mM KH2PO4 and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured for 5 days and the ALP activity was measured using an ALP activity kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.(28) After osteogenic induction of DPSCs for 2 weeks, 70% ethanol and 2% Alizarin red (Sigma-Aldrich, St. Louis, MO, USA) were used to fix and stain the cultured cells. The plates were then detained for 30 minutes at room temperature with 10% cetylpyridinium chloride. (28) The absorbance of the cell cultures was measured at 562 nm on a multi-plate reader, and the final calcium level was normalized according to the total protein concentration in duplicate plates.

**Western blot analysis**

The total protein extraction and SDS-polyacrylamide gel electrophoresis tests were performed in previous study. (22) The primary antibodies used in this study were anti-EREG (Cat No. 93815; Cell Signaling Technology, Boston, MA, USA), mouse monoclonal anti-HA (Clone No. C29F4; Cat No. MMS-101P; Covance, Princeton, NJ, USA), anti-phospho-p38 MAPK (Cat No. 4631; Cell Signaling Technology, Boston, MA, USA), anti-p38 MAPK (Cat No. 8690; Cell Signaling Technology, Boston, MA, USA), anti-phospho-Erk1/2 (Cat No. 4377S; Cell Signaling Technology, Boston, MA, USA), anti-Erk1/2 (Cat No. 4695S; Cell Signaling Technology, Boston, MA, USA). The primary monoclonal antibodies for the housekeeping proteins was the monoclonal antibody against β-actin (Cat No. C1313; Applygen, Beijing, China).
**Statistical analysis**

Each experiment was independently conducted at least three times. Results are expressed as mean ± standard deviation (SD). Significant differences were assessed with the Student’s t test (two-tailed), one-way ANOVA with the post hoc Bonferroni test. \( P \leq 0.05 \) was considered significant.

**Results**

*Knockdown of EREG enhanced the osteo/dentinogenic differentiation of DPSCs*

To investigate the function of EREG on DPSCs, we knocked down the EREG expression in DPSCs using lentiviral vector infection. The knockdown efficiency was verified by Western blot (Figure.1A). After 5 days of culturing in osteogenic-inducing medium, the result indicated that EREG depletion enhanced the ALP activity of DPSCs compared with the Scramsh group (Scramsh group) (Figure.1B). Two weeks after induction, alizarin red staining and quantitative calcium measurements revealed increased mineralization in vitro in EREGsh DPSCs compared with Scramsh group (Figure.1C-D). Real-time RT-PCR was conducted on day 7 and 14 during the osteo/dentinogenic induction and the results revealed that the osteo/dentinogenic marker gene BSP significantly increased on day 7 and day 14 (Figure.1E) in EREGsh DPSCs compared with Scramsh DPSCs. Besides, the DMP-1 expression level elevated evidently on day 14 (Figure.1F) and the DSPP on day 7 (Figure.1G).
**EREG recombinant protein inhibited the osteo/dentinogenic differentiation of DPSCs**

To further verify the effect of EREG on the DPSCs osteo/dentinogenic potential, 50 ng/ml human recombinant EREG protein was added during the osteo/dentinogenic induction and mineralization ability was determined. After 5 days of osteogenic induction, the ALP activity assay result showed that 50 ng/mL EREG decreased the ALP activity (Fig. 2A). Two weeks later, Alizarin red staining and the quantitative calcium analysis results showed that 50 ng/mL EREG impaired the mineralization ability of DPSCs (Figure.2B-C). In the meantime, real-time RT-PCR results showed that DSPP expression was significantly decreased on day 7 (Figure.2D), the BSP expression decreased on day 14 (Figure.2E) and DMP-1 significantly decreased on both day 7 and 14 (Figure.2F) when comparing EREG treated DPSCs with control DPSCs.

**EREG depletion activated the p38 MAPK and Erk signaling pathways in DPSCs**

In the following study, we explored the mechanism of EREG on the osteo/dentinogenic potential of DPSCs. Western blot was carried out to evaluate the protein levels of critical members of MAPK pathway. The results showed that silencing of EREG up-regulated the expression of phosphorylated p38 MAPK and Erk1/2 in DPSCs, since the levels of phosphorylated p38 MAPK and Erk1/2 were increased while the level of total p38 MAPK and Erk1/2 were not affected (Figure 3A-B). To further validate our findings, Erk1/2 or p38 MAPK was inhibited with specific inhibitor PD98059 or SB203580, respectively. DPSCs with EREG depletion were pretreated with 20 μmol/L inhibitors
for 1 hour separately. Erk1/2-specific inhibitor PD98059 effectively suppressed the expression of phosphorylated Erk1/2 in EREGsh DPSCs (Figure 3C-D). p38 MAPK-specific inhibitor SB203580 displayed similar results (Figure 3E-F). These results indicated that effect of EREG on the osteo/dentinogenic differentiation of DPSCs was probably depending on the regulation of p38 MAPK and Erk signaling pathways.

**EREG depletion enhanced the osteo/dentinogenic differentiation potential of DPSCs after stimulation with TNF-α**

We then studied the effect of EREG on DPSCs under inflammatory microenvironment. 10ng/mL TNF-α was used to mimic inflammatory microenvironment in vitro and EREG level was detected. The results showed that EREG expression increased at 2, 4 and 8 hours after 10 ng/mL TNF-α treatment as shown by real-time RT-PCR (Figure 4A). Furthermore, we investigated the function of EREG in DPSCs upon 10 ng/mL TNF-α treatment. With 10 ng/mL TNF-α treatment, EREG knockdown could promote the ALP activity and mineralization ability (Figure 4B-D). 2 weeks after osteogenic induction, the real-time RT-PCR results also showed that the expressions of DSPP, DMP-1 and BSP were increased in EREG knockdown group compared with the control group (Figure 4E-G).

**EREGsh activated the p38 MAPK and Erk1/2 signaling pathway that were inhibited by TNF-α stimulation**

We further investigated the effect of EREG on p38 MAPK or Erk signaling pathways
under inflammatory conditions. Western blot revealed that the levels of phosphorylated p38 MAPK and phosphorylated Erk1/2 expression were decreased in DPSCs after 10 ng/mL TNF-α stimulation (Figure 5A). Knockdown of EREG rescued the decreased levels of phosphorylated p38 MAPK and phosphorylated Erk1/2 caused by 10 ng/mL TNF-α treatment.

Discussion

Pulp-dentin regeneration based on MSCs brings hope for the treatment of pulpitis and functional reconstruction of pulp-dentin complex. However, local inflammatory microenvironment is one of the key factors affecting the efficiency of stem cell-mediated tissue regeneration.

In the present study, we found that EREG depletion promoted the osteo/dentinogenic differentiation potential of DPSCs. Consistently, rhEREG showed an opposite effect. The result is contrary to our previous findings. Previous study indicated that EREG could improve the osteogenic differentiation of SCAPs and ADSCs.(22, 23) Similarly, a recent study has also illustrated that EREG could enhance the odontoblastic differentiation of DPSCs.(29) The reasons why EREG has different functions in different studies may lie on the following aspects. Firstly, different cells may respond differently to EREG. Furthermore, even for the specific cell type, the performance of EREG is depending on the concentration. High concentrations (100 ng/mL) increased the osteogenic differentiation activity of DPSCs in Cui’s study, while lower concentration (50 ng/mL) of EREG inhibited the osteogenic differentiation of DPSCs in the present study. Thus, we speculated that EREG is a concentration-sensitive cytokine. Last but not least, personalized experimental tools may also lead to different
results. In the present study, primer sequence for EREG shRNA is 5′-actactgcaggtgaagt-3′; while, in Cui’s study, the primer sequence is 5′-ggctttgaccgtgatttat-3′. Therefore, individual sequences may result in different knockout efficiency of EREG, or different functional regions may bring out different outcomes. In fact, as a growth factor, EREG could bind with EGFR to regulate various biological processes, including cell growth, motility, proliferation and differentiation.(30, 31) It can be released from vascular smooth muscle cells, and acts as a major autocrine/paracrine factor for dedifferentiation.(32) EREG could also stimulate the proliferation of fibroblasts, hepatocytes, smooth muscle cells;(33, 34) conversely, inhibit expansion of several types of tumor-derived epithelial cells.(35, 36) Thus, EREG may display diverse effects depending on the dose and the different cell types.

In the following study, we further investigated the effects of EREG on DPSCs under inflammatory condition. Studies have shown that inflammatory factors such as TNF-α, IL-6(37) and IL-8(38) is closely related to the development of pulpitis(39) and TNF-α could enhance paracrine regulation of mesenchymal stem cells.(40) In a clinical research, the amount of TNF-α in dental pulp tissue of pulpitis is much higher than that in the normal pulp tissue.(41) In our study, TNF-α was used to mimic inflammatory condition. Studies have revealed that TNF-α inhibits osteoblastogenesis through several mechanisms.(42) However, it has also been reported that TNF-α promotes osteoblastogenesis.(43) These discrepancies may depend on the cellular types, the concentration, and the timing and duration of TNF-α administration. In our study, under the stimulation of 10 ng/mL TNF-α for 4 h, EREG expression was found to increase and the osteo/dentinogenic differentiation of DPSCs was decreased. EREG depletion could rescue the osteo/dentinogenic differentiation of DPSCs that was impaired by
TNF-α. The present study indicated that EREG may act as a new target to regulate the osteo/dentinogenic differentiation of DPSCs in inflammatory condition.

The development of pulpitis is tied to the accumulation of inflammatory mediators including TNF-α. A previous study found that TNF-α stimulation repressed the p38 MAPK, JNK and Erk1/2 signaling pathways in PDLSCs.(28) Still, other studies demonstrated that TNF-α mediates p38 MAPK activation and negatively regulates bone formation,(44) which is consistent with our findings. Our investigation showed that TNF-α repressed the p38 MAPK and Erk1/2 signaling pathways, however, EREG depletion rescued the change. EREG is one of the most potent ligands that binds the EGF receptors,(14) thus couple to numerous signaling cascades, including MAPKS. MAPK signaling, which is consist of p38 MAPK, JNKS, and Erk1/2, involving a set of serine/threonine kinases, plays a crucial part in various physiological functions.(45) p38 MAPK and Erk1/2 can be stimulated in the inflammatory, hypoxia process and environment stresses changed. Studies have highlighted the involvement of EREG and MAPK-Erk1/2 signaling pathway activated by Streptococcus suis serotype 2, which subsequently initiates and mediates the inflammatory response in the brain and finally the CNS dysfunction.(46) Besides, during the process of rhinovirus infection, the RV16 infection could rapidly promote induction of EREG, thus increase IL-8 and ICAM-1 levels through p38 MAPK and Erk1/2 pathway.(25) Under the stimulation of pro-inflammatory cytokine, PGE2, human granulosa cells may induce the biosynthesis of EREG, which further activated the MAPK pathways.(47) In addition, the activation of Erk1/2 signaling is required during cytokines mediated osteogenic differentiation in PDLSC,(48) SCAPs,(22) and BMSCs,(49) as well as stem cell homing/migration.(23) In consist with our study, the present study demonstrated the involvement of both Erk1/2 and p38 MAPK signaling in the osteo/dentinogenic differentiation of DPSCs.
Studies have shown that Erk signaling pathway was a downstream molecule of p38 MAPK signaling pathways. Other studies about the effects of Erk1/2 and p38 on the osteo/dentinogenic differentiation of DPSCs indicates that inhibition of p38 MAPK is able to inhibit the osteogenic differentiation of DPSCs whereas inhibition of Erk1/2 demonstrates the opposite effect. (43) In the present study, we used the specific inhibitors to block p38 MAPK or Erk 1/2 signaling pathway separately. When blocked each of them, p38 or Erk signaling pathways was inhibited individually. Different chemical and physical stimuli, cell types, culture methods, times of inhibitor administration and the dosage of the inhibitor may influence the effect of Erk1/2 and p38 on the differentiation of MSCs.

**Conclusion**

Our study demonstrated that EREG negatively regulated the osteo/dentinogenic differentiation potential of DPSCs via p38 MAPK and Erk pathway in inflammatory microenvironment. These results provide insights into the mechanisms underlying functional regulation of DPSCs and indicate that EREG has a significant role in maintaining the osteo/dentinogenic differentiation potential of DPSCs.

**Abbreviation**

| Abbreviation | Description |
|--------------|-------------|
| ALP          | Alkaline phosphatase |
| α-MEM        | α minimal essential medium |
| BMSCs        | Bone marrow stromal cells |
| BSP          | Bone sialoprotein |
| CNS          | Central nervous system |
| DPSCs        | Dental pulp stem cells |
| Acronym   | Full Form                                      |
|-----------|-----------------------------------------------|
| DMSO      | Dimethyl sulfoxide                            |
| DSPP      | Dentin sialophosphoprotein                    |
| DMP-1     | Dentin matrix protein-1                       |
| EGF       | Epidermal growth factor                       |
| EREG      | Epiregulin                                    |
| Erk       | Extracellular signal-regulated protein kinases |
| FBS       | Fetal bovine serum                            |
| GAPDH     | Glyceraldehyde-3-phosphate dehydrogenase      |
| HB-EGF    | Heparin-binding epidermal growth factor       |
| ICAM-1    | Intercellular cell adhesion molecule-1         |
| IL-6      | Interleukin-6                                 |
| IL-8      | Interleukin-8                                 |
| JNK       | C-Jun N-terminal kinase                       |
| MAPK      | Mitogen-activated protein kinase              |
| MSCs      | Mesenchymal stem cells                        |
| OD        | Optical density                               |
| PCR       | Polymerase chain reaction                     |
| PDLSCs    | Periodontal ligament stem cells               |
| PGE2      | Prostaglandin E2                              |
| rhEREG    | Recombinant human EREG protein                |
| RT-PCR    | Reverse transcriptase–polymerase chain reaction|
| SCAPs     | Apical papilla                                |
| shRNA     | Short hairpin RNAs                            |
| TGF-α     | Transforming growth factor alpha               |
| TNF-α     | Tumor necrosis factor alpha                   |
Declarations:

Ethics approval and consent to participate

All researches involving human DPSCs complied with the International Society for Stem Cell Research “Guidelines for the Conduct of Human Embryonic Stem Cell Research”. Written informed consent was obtained from each patient before human impacted third molar were collected, under approved guidelines set by Beijing Stomatological Hospital, Capital Medical University. All human experiments were approved by the Ethics Committee of Beijing Stomatological Hospital, Capital Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author’s contribution

Ran ran performed the molecular biological studies, participated in data analysis and interpretation, and drafted the manuscript. Haoqing Yang acquired, analyzed, interpreted and drafted the manuscript; Yangyang Cao designed, acquired, analyzed and drafted the manuscript; Wanhao Yan analyzed and interpreted the data and drafted the manuscript; Ying Zheng and Luyuan Jin conceived, designed and drafted the data and critically revised the manuscript.

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**Figure legends**

**Figure1. The knockdown of EREG enhanced the osteo/dentinogenic differentiation ability of DPSCs.** Short hairpin RNAs were used to silence the expression of EREG (EREGsh) and scramble shRNA was used as control (scramsh). (A): Western blot results for expression of EREG in EREGsh and Scramsh DPSCs (B): ALP activity results of EREGsh and Scramsh DPSCs on day 5. (C, D): Alizarin Red staining and calcium quantitative analysis results for both groups after 2 weeks of osteogenic
induction. (E-G): Real-time RT-PCR for expression of osteo/dentinogenic differentiation markers BSP, DMP-1 and DSPP. GAPDH was used as the internal control. Error bars represent the SD (n=3). The Student’s t test was used to test statistical significance. *P≤0.05. **P≤0.01.

Figure 2. The rhEREG inhibited the osteo/dentinogenic differentiation ability of DPSCs.

(A): ALP activity results of control and EREG-treated groups on day 5. (B, C): Alizarin Red staining and calcium quantitative analysis results for both groups after 2 weeks of osteogenic induction. (D-F): Real-time RT-PCR for expression of osteo/dentinogenic differentiation markers DSPP, BSP and DMP-1. GAPDH was used as the internal control. Error bars represent the SD (n=3). The Student’s t test was used to test statistical significance. *P≤0.05. **P≤0.01.

Figure 3. The knockdown of EREG activated the p38 MAPK and Erk signaling pathways in DPSCs.

(A-B): Western blot result and quantitative analysis for the expression of phosphorylated p38 MAPK and phosphorylated Erk in EREGsh and Scramsh DPSCs. β-actin was used as an internal control. (C-D): Western blot results and quantitative analysis for the expression of phosphorylated Erk in DPSCs after treatment with 20 μmol/L Erk-specific inhibitor PD98059 for 1 hour in DPSCs. β-actin was used as an internal control. (E, F) Western blot and quantitative analysis for the expression of
phosphorylated p38 MAPK in DPSCs after treatment with 20 μmol/L p38 MAPK-specific inhibitor SB203580 for 1 hour in DPSCs. β-actin was used as an internal control. Error bars represent the SD (n=3). The Student's t test and one-way ANOVA was used to test statistical significance. *P≤0.05. **P≤0.01.

**Figure 4. The knockdown of EREG improved the osteo/dentinogenic differentiation ability of DPSCs under TNF-α stimulation.**

DPSCs were treated with 10 ng/mL TNF-α. (A): Real-time RT-PCR results for the expression of EREG at 1, 2, 4 and 8 h after 10 ng/mL TNF-α treatment in DPSCs. (B): ALP activity results of EREGsh and Scramsh DPSCs under TNF-α stimulation. (C-D): Alizarin Red staining and calcium quantitative analysis results for EREGsh and Scramsh DPSCs under TNF-α stimulation. (E-G): Real-time RT-PCR results for the expressions of DSPP, BSP and DMP-1 in EREGsh and Scramsh DPSCs under TNF-α stimulation. GAPDH was used as the internal control. Error bars represent the SD (n=3). The Student's t test and one-way ANOVA was used to test statistical significance. *P≤0.05. **P≤0.01.

**Figure 5. EREG depletion restored the decreased phosphorylation of p38 MAPK and Erk1/2 in TNF-α stimulated DPSCs.** 10 ng/mL TNF-α was used to treat DPSCs for 4 h. (A): Western blot and quantitative protein analysis for the expression of phosphorylated p38 MAPK and Erk1/2. β-actin was used as an internal control. Error bars represent the SD (n=3). The Student's t test and one-way ANOVA was used to test
statistical significance. *P≤0.05. **P≤0.01.