

## $10^{-7}$ M $17\beta$ -oestradiol enhances odonto/osteogenic potency of human dental pulp stem cells by activation of the NF- $\kappa$ B pathway

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### Abstract

**Objectives:** Oestrogen has been proven to significantly enhance osteogenic potency, while oestrogen deficiency usually leads to impaired osteogenic differentiation of mesenchymal stem cells. However, little is known concerning direct effects of oestrogen on differentiation of human dental pulp stem cells (DPSCs).

**Materials and methods:** In this study, human DPSCs were isolated and treated with  $10^{-7}$  M  $17\beta$ -oestradiol (E2). Alkaline phosphatase (ALP) assay and alizarin red staining were performed.

**Results:** Alkaline phosphatase and alizarin red showed that E2 treatment significantly enhanced ALP activity and mineralization ability of DPSCs, but had no effect on cell proliferation. Real-time RT-PCR and western blot assay demonstrated that odonto/osteogenic markers (ALP, RUNX2/RUNX2, OSX/OSX, OCN/OCN and DSPP/DSP) were significantly upregulated in the cells after E2 treatment. Moreover, phosphorylation of cytoplasmic I $\kappa$ B $\alpha$ /P65 and expression of nuclear P65 were enhanced in a time-dependent manner following E2 treatment, suggesting activation of NF- $\kappa$ B signaling. Conversely, inhibition of the NF- $\kappa$ B pathway suppressed E2-mediated upregulation of odonto/osteogenic markers, indicating that the NF- $\kappa$ B pathway was pivotal for E2-mediated differentiation.

**Conclusion:** These findings provide evidence that  $10^{-7}$  M  $17\beta$ -oestradiol promoted odonto/osteogenic

differentiation of human DPSCs *via* activation of the NF- $\kappa$ B signaling pathway.

### Introduction

Dental pulp stem cells (DPSCs) have multiple differentiation capacity and represent a cell source for regenerative medicine and tissue engineering, specially for tooth and pulp regeneration. However, both limited number of available DPSCs in vital pulp and gradual loss of their differentiation capacity following *in vitro* expansion, significantly restrict their use in clinical applications (1). Effective improvement of their odonto/osteogenic capacity is necessary for upcoming applications of DPSC-based tissue engineering. To date, there are many factors including pro-inflammatory cytokines (2), growth factors (3), mechanical stretch (4) and donor age (5), which have been implicated in regulating proliferation and odonto/osteogenic capacity of DPSCs. These results offer an opportunity to reconstruct the extrinsic microenvironment necessary for efficient differentiation of DPSCs, and eventually facilitate their application in tissue regeneration.

A natural steroid hormone, oestrogen has profound impact on the skeletal system. However, direct effects of it on dental tissues/cells, have up to now, remained unclear. Consistent with the fact that composition and calcification of dentine are similar in many ways to intramembranous bone formation, earlier studies have indicated that oestrogen causes similar changes in ground substance of dentine as it does for bone. Moreover, exogenous oestrogen can enhance osteogenic capacity of human bone marrow stromal cells and adipose-derived stromal cells (6–9), whereas oestrogen deficiency leads to impaired osteoblastic differentiation of bone marrow stromal cells and periodontal ligament stem cells (10,11). Previous study has revealed that oestrogen deficiency *in vivo* can bring about downregulation of committed differentiation of rat DPSCs (12);

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however, little is known about direct impact of oestrogen on biological features of human DPSCs.

In this study, we investigated effects of oestrogen on odonto/osteogenic differentiation of human DPSCs *in vitro*. Human DPSCs were isolated from premolars and treated with exogenous  $17\beta$ -oestradiol (E2 – oestradiol), the most important form of oestrogen in the body. Differentiation and involvement of the NF- $\kappa$ B pathway in E2-treated DPSCs were evaluated *in vitro*. Our findings demonstrated that  $10^{-7}$  M  $17\beta$ -oestradiol enhanced odonto/osteogenic differentiation of DPSCs by activation of the NF- $\kappa$ B pathway.

## Materials and methods

### Cell isolation and identification

Non-carious human premolars ( $n = 12$ ) were freshly extracted from six young female patients requiring orthodontic treatment, at the age of 12/13, in the Oral Surgery Department of Jiangsu Provincial Stomatological Hospital, after informed consents were obtained. Pulp were carefully separated, minced into  $1\text{ mm}^3$  and treated with a solution containing 3 mg/ml collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/ml dispase (Sigma), for 60 min at  $37^\circ\text{C}$ . Single cell suspensions were obtained and cultured in phenol red free, low-glucose Dulbecco's minimum essential medium (phenol red free L-DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Multi-colony-derived stem cells were isolated as previously described (13). To determine their origin from mesenchymal stem cells, DPSCs were immunostained with antibody against STRO-1 (1:200, Novus Biologicals, Littleton, CO, USA). Stem cells from different patients were mixed, subcultured and then utilized for subsequent experiments.  $17\beta$ -oestradiol (E2, Ehrenstorfer GmbH, Augsburg, Germany) was dissolved in absolute ethyl alcohol at  $10^{-3}$  M, and stored at  $-20^\circ\text{C}$  in the dark. Cells at passages 2–4 were incubated in phenol red free L-DMEM containing  $17\beta$ -oestradiol (E2 group) or 0.01% (v/v) ethyl alcohol as control (control group). Culture media of the E2 group were replaced every other day to maintain constant E2 concentration.

### MTT assay

Dental pulp stem cells were seeded at  $2 \times 10^3$  cells/well into 96-well plates (Nunc, Roskilde, Denmark). After 24-h serum starvation in serum-free media, these stem cells were cultured in complete media supplemented with  $10^{-7}$  M  $17\beta$ -oestradiol. For 11 consecutive

days, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,5-tetrazoliumbromide) assay was performed as previously reported (12). OD values at 490 nm were detected using a microtitre plate reader (Titertek, Helsinki, Finland). Experiments were performed in triplicate and data are presented as mean  $\pm$  SD.

### Alkaline phosphatase activity assay and alizarin red staining

Dental pulp stem cells in different groups were respectively seeded into 96-well plates at  $2 \times 10^3$  cells/well, and 24-well plates at  $1 \times 10^4$  cells/well, and then cultured in complete media with or without  $10^{-7}$  M  $17\beta$ -oestradiol. Alkaline phosphatase (ALP) activity assay at days 3/5 and alizarin red staining at day 14 were performed as previously described (1,14). Nodule staining was then eluted by 10% cetylpyridinium chloride (pH 7.0) and calcium concentrations were determined by absorbance measurement at 526 nm, using a universal microplate reader (BioTek Instruments Inc., Winooski, VT, USA) (14). Data are presented as mean  $\pm$  SD of three independent experiments.

### Real-time reverse transcription polymerase chain reaction

Total cell RNA in different groups was isolated using TRIzol reagent (Invitrogen, New York, NY, USA) according to the manufacturer's protocols. Then, cDNA was produced using a reverse transcription kit (TaKaRa Biotechnology, Dalian, China). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed in a single tube using SYBR Premix Ex Taq™ kit (TaKaRa Biotechnology) and ABI 7300 real-time PCR system. Primers used in the experiment were as follows: *OSX*, 5'- CCTCCTCAGCTCACCTTCTC -3' (forward) and 5'- GTTGGGAGCCCAAATAGAAA -3' (reverse); *OCN*, 5'- AGCAAAGGTGCAGCCTTTGT -3' (forward) and 5'- GCGCCTGGGTCTCTTCACT -3' (reverse); *RUNX2*, 5'- TCTTAGAACAAATTCTGCCCTT -3' (forward) and 5'- TGCTTTGGTCTTGAAATCACA -3' (reverse); *ALP*, 5'- GACCTCCTCGGAAGACACTC -3' (forward) and 5'- TGAAGGGCTTCTTGTC TGTG -3' (reverse); *DSPP*, 5'- ATATTGAGGGCTGGAATGGGGA -3' (forward) and 5'- TTTGTGGCTCCAGCATTGTCA -3' (reverse); *GAPDH*, 5'- GAAGGTGAAGGTCGGAGTC -3' (forward) and 5'- GAGATGGT GATGGGATTTC -3' (reverse). *GAPDH* served as the internal control. Data processing was performed by the method of  $2^{-\Delta\Delta\text{Ct}}$  as previously reported (15). The experiment was repeated three times and data were described as mean  $\pm$  SD.

### Western blot analysis

To explore effects of  $17\beta$ -oestradiol on odonto/osteogenic differentiation and expression of NF- $\kappa$ B pathway proteins, DPSCs in different groups were collected, washed twice in 0.01 M PBS and lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Nanjing, China) containing 1 mM phenylmethanesulfonyl fluoride. Western blot assay was performed as previously reported using 30  $\mu$ g protein per lane (12,14). Primary antibodies used were to proteins listed below: RUNX2, 1:1000, Abcam, Hong Kong, China; OSX, 1:1000, Abcam; OCN, 1:1000, Abcam; DSP, 1:500, Santa Cruz, Dallas, TX, USA; phosphor-P65, 1:1000, Cell Signaling; P65, 1:1000, Cell Signaling Boston, MA, USA; phosphor-I $\kappa$ B $\alpha$ , 1:1000, Cell Signaling; I $\kappa$ B $\alpha$ , 1:1000, Cell Signaling;  $\beta$ -ACTIN, 1:1000, Bioworld, Minneapolis, MN, USA; H1, 1:1000, Cell Signaling. Semi-quantitative analysis of western blotting was performed with Image-Proplus 5.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). This experiment was repeated three times.

### Statistics

Two-sample *t*-testing was performed to compare means of two independent samples. For multiple comparisons between experimental groups and control groups, Dunnett's test was used to check significant differences. Two-tailed *P*-values less than 0.05 were considered statistically

significant. All statistical analysis was performed with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

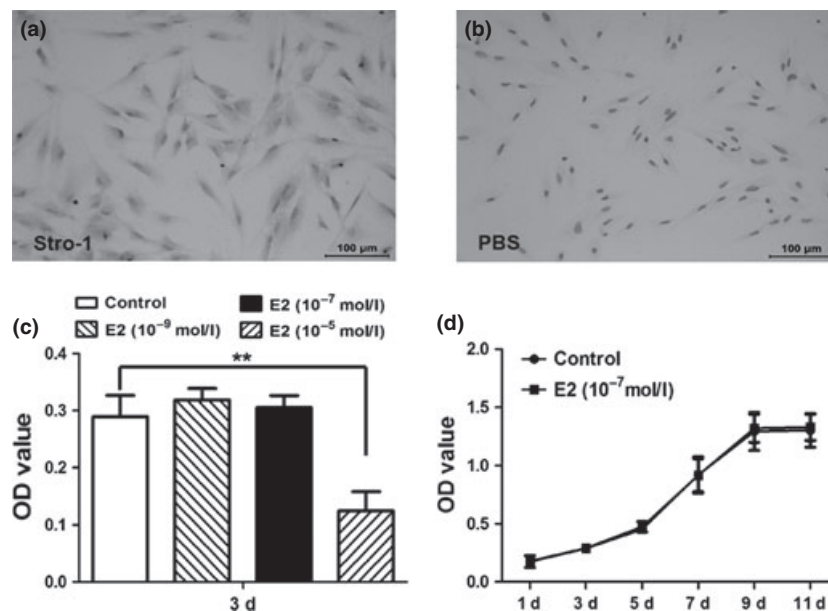
## Results

### Effects of $17\beta$ -oestradiol on proliferation of DPSCs

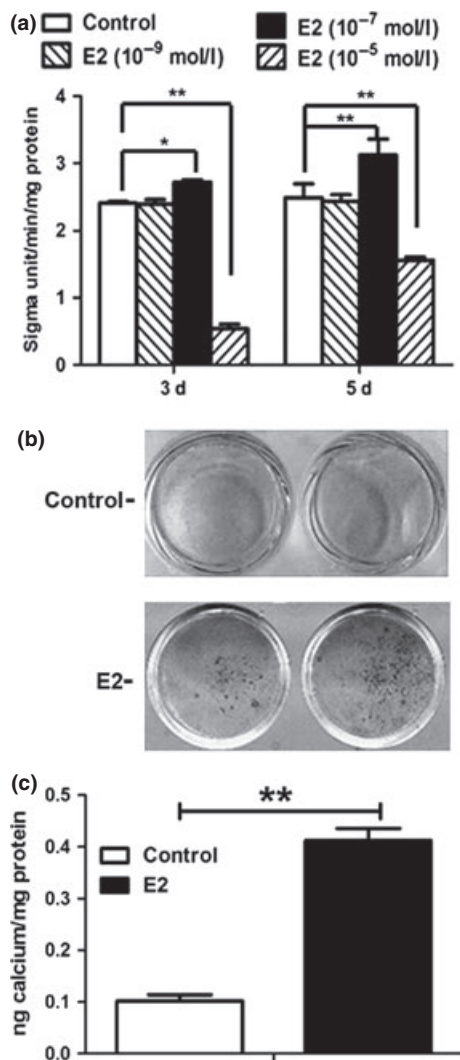
The cells had typical fibroblast- or spindle-like morphology and stained positively for putative mesenchymal stem-cell marker STRO-1 (Fig. 1a). Physiologically relevant concentrations of  $17\beta$ -oestradiol ( $10^{-9}$  and  $10^{-7}$  M; Fig. 1c,d) exerted no effect on cell proliferation, while  $10^{-5}$  M  $17\beta$ -oestradiol significantly inhibited the DPSCs' proliferation (Fig. 1c;  $P < 0.01$ ).

### Effects of $17\beta$ -oestradiol on odonto/osteogenic differentiation of DPSCs

$10^{-9}$  M  $17\beta$ -oestradiol did not change ALP activity in DPSCs (Fig. 2a;  $P > 0.05$ ), while  $10^{-7}$  M  $17\beta$ -oestradiol significantly upregulated ALP level respectively at day 3 ( $P < 0.05$ ) and day 5 ( $P < 0.01$ ), whereas  $10^{-5}$  M  $17\beta$ -oestradiol noticeably downregulated ALP activity (Fig. 2a;  $P < 0.01$ ) mostly due to inhibition of cell proliferation (Fig. 1c). Thus,  $10^{-7}$  M  $17\beta$ -oestradiol was selected to be the optimal concentration and was used for the following experiments. Alizarin red staining assay revealed that cells in E2 group produced more calcified nodules than those in control groups (Fig. 2b).



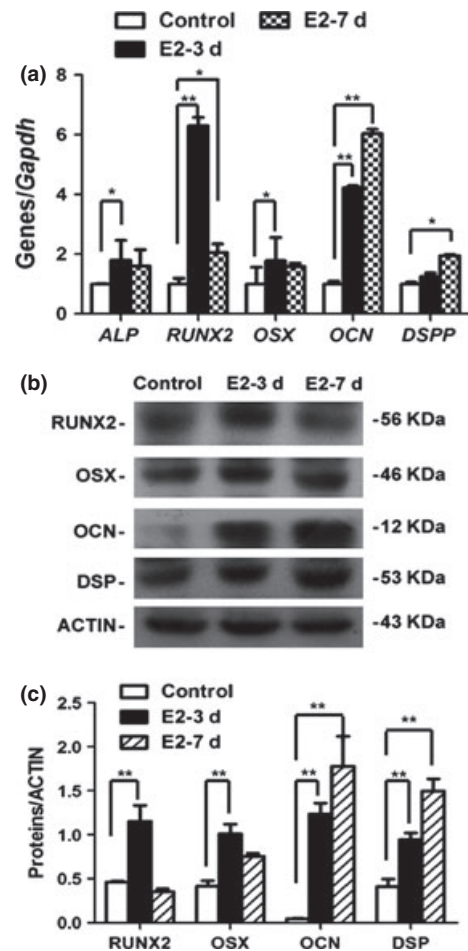
**Figure 1.** Proliferation features of dental pulp stem cells (DPSCs) treated by  $17\beta$ -oestradiol. (a) Isolated DPSCs were spindle-like cells with a positive staining for STRO-1 by immunocytochemistry. (b) PBS served as a negative control. (c) High concentration ( $10^{-5}$  M) of  $17\beta$ -oestradiol (E2) had negative effects on the proliferation of DPSCs, while the physiological concentrations ( $10^{-7}$  and  $10^{-9}$  M) of E2 did not affect the cell growth. (d) MTT assay showed that  $10^{-7}$  M E2 had no effects on the proliferation of DPSCs *in vitro*.



**Figure 2.** Alkaline phosphatase (ALP) activity and calcium deposition in E2-treated dental pulp stem cells (DPSCs). (a)  $10^{-9}$  M E2 did not affect the ALP activity of DPSCs ( $P > 0.05$ ) and  $10^{-7}$  M E2 significantly upregulated the ALP levels of DPSCs at day 3 and day 5 ( $P < 0.05$  or  $P < 0.01$ ), while  $10^{-5}$  M E2 significantly downregulated the ALP activity ( $P < 0.01$ ), as compared with control group. Data were described as mean  $\pm$  SD,  $n = 6$ . \* $P < 0.05$ , \*\* $P < 0.01$ . (b) Alizarin red staining assay showed that DPSCs in E2 group produced more calcified nodules than those in control group. (c) Quantitative calcium assay demonstrated the stronger calcium deposition in E2 group as compared with control group. Values were presented as mean  $\pm$  SD,  $n = 3$ . \*\* $P < 0.01$ .

Cetylpyridinium chloride quantitative calcium assay demonstrated higher calcium deposition in E2 group than in control groups (Fig. 2c;  $P < 0.01$ ), indicating that  $17\beta$ -oestradiol enhanced mineralization capacity of human DPSCs.

Real-time RT-PCR showed that expressions of odonto/osteogenic genes (*ALP*, *RUNX2*, *OSX*, *OCN*, and *DSPP*) were remarkably enhanced in DPSCs (Fig. 3a) following



**Figure 3.** Gene and protein expression in E2-treated dental pulp stem cells (DPSCs). (a) Real-time RT-PCR demonstrated that the expressions of *ALP*, *RUNX2*, *OSX*, *OCN*, and *DSPP* were significantly enhanced in E2-treated DPSCs at day 3 or day 7 in comparison with control group. Values were described as mean  $\pm$  SD. \*\* $2^{-\Delta\Delta Ct} > 2$ ,  $P < 0.01$ ; \* $1 < 2^{-\Delta\Delta Ct} < 2$ ,  $P < 0.01$ ;  $n = 3$ . (b) Western blot results revealed that the odonto/osteogenic proteins (*RUNX2*, *OSX*, *OCN*, and *DSP*) were significantly upregulated after 3-day or 7-day E2 stimulation, as compared with control DPSCs. (c) Semi-quantitative analysis confirmed that the expressions of *RUNX2*, *OSX*, *OCN*, and *DSP* were stronger in E2-treated DPSCs than those in control group.

E2 treatment. In particular, expressions of *ALP*, *RUNX2*, *OCN* and *OSX* were significantly higher on day 3, while expressions of *RUNX2*, *OCN* and *DSPP* were upregulated by day 7. Western blot findings further confirmed upregulation of odonto/osteogenic proteins (*RUNX2*, *OSX*, *OCN*, and *DSP*) following E2 treatment (Fig. 3b,c;  $P < 0.01$ ).

#### *NF-κB pathway involvement in E2-mediated odonto/osteogenic differentiation of DPSCs*

To determine potential involvement of the NF-κB pathway in E2-mediated differentiation of DPSCs, we inves-

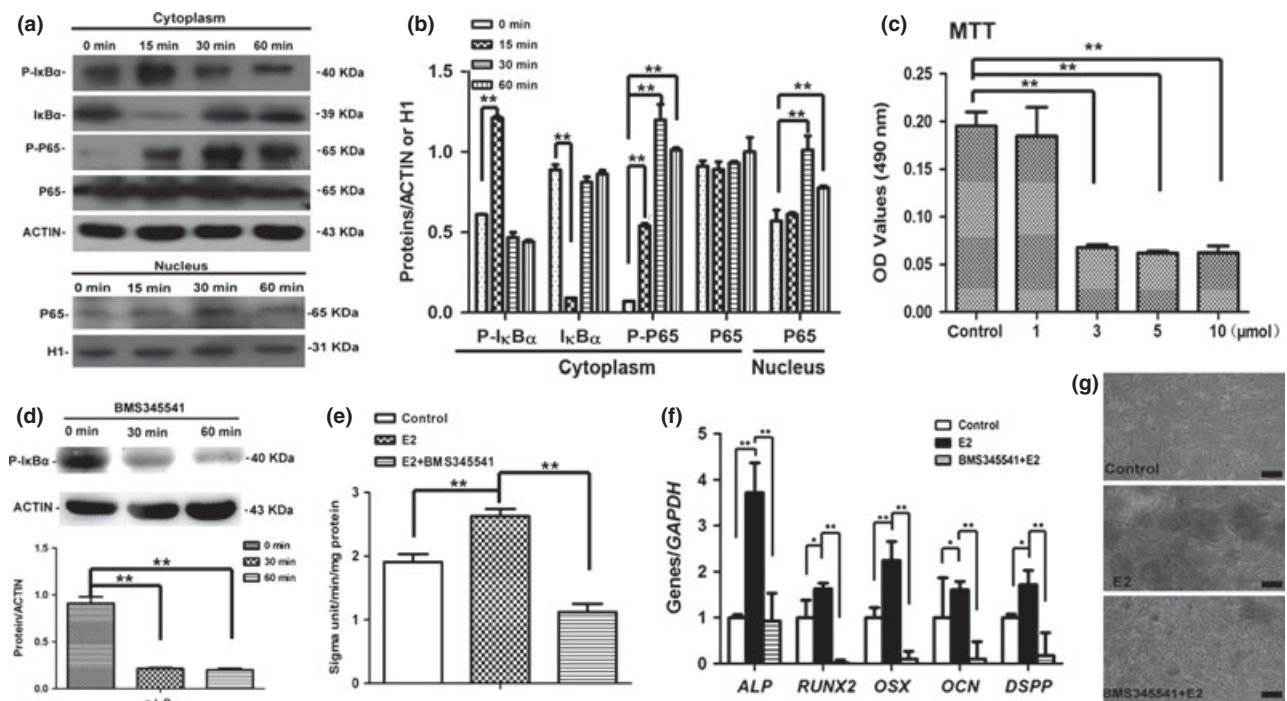
tigated phosphorylation and expression of I $\kappa$ B $\alpha$  and P65 by western blot analysis. Phosphorylation of cytoplasmic I $\kappa$ B $\alpha$  was significantly higher by minute 15 then gradually decreased between 30 and 60 min (Fig. 4a,b), while phosphorylation of cytoplasmic P65 gradually upregulated between 15 and 30 min then downregulated by 60 min following E2 treatment. In addition, expression of nuclear P65 was enhanced by 30 min and then fell by 60 min after E2 treatment.

To further determine the role of the NF- $\kappa$ B pathway in E2-mediated differentiation of DPSCs, BMS345541 (a specific IKK inhibitor) was used to suppress activity of NF- $\kappa$ B signaling for 30 min prior to 17 $\beta$ -oestradiol treatment (16–18). BMS345541 at higher concentrations (3, 5 and 10  $\mu$ M) significantly reduced proliferative activity, whereas 1  $\mu$ M BMS345541 did not affect viability of DPSCs (Fig. 4c;  $P < 0.01$ ). Moreover, 1  $\mu$ M BMS345541 significantly inhibited expression of P-I $\kappa$ B $\alpha$  and was thus used as the optimal dosage in the

following experiments (Fig. 4d;  $P < 0.01$ ). Real-time RT-PCR results demonstrated that odonto/osteogenic markers (ALP/ALP, RUNX2, OSX, OCN and DSPP) were significantly downregulated in the E2+BMS345541 group in comparison to the E2 group (Fig. 4e,f). Furthermore, BMS345541 clearly inhibited E2-mediated mineralization in DPSCs (Fig. 4g).

## Discussion

Effects of oestrogen on bone tissues/cells have been extensively studied *in vitro* and *in vivo*. However, little information has been available concerning its influence on tooth structures/cells. In a previous study our group revealed that DPSCs of an oestrogen-deficient rat model exhibited reduced differentiation towards the osteo/odontogenic cell lineages (12). To date, effects of exogenous oestrogen at physiological concentrations on proliferation and differentiation of human DPSCs had remained



**Figure 4.** NF- $\kappa$ B pathway involvement in E2-mediated odonto/osteogenic differentiation of dental pulp stem cells (DPSCs). (a) The expression of P-I $\kappa$ B $\alpha$  increased at 0–15 min and gradually decreased at minutes 30/60 in E2-treated DPSCs, whereas the expression of P-P65 gradually increased at 0–30 min after E2 treatment and decreased at minutes 60. Moreover, the expression of nuclear P65 was upregulated at 0–30 min and downregulated at minutes 60.  $\beta$ -ACTIN and Histone 1 (H1) served as internal controls respectively for cytoplasmic and nuclear proteins. (b) Semi-quantitative analysis confirmed the upregulated expression of P-I $\kappa$ B $\alpha$  and P-P65 after E2 treatment.  $**P < 0.01$ . (c) BMS345541 at high concentrations (3, 5 and 10  $\mu$ M) significantly reduced the proliferative activity, while 1  $\mu$ M BMS345541 did not affect the viability of human DPSCs.  $**P < 0.01$ ,  $n = 6$ . (d) Western blot results for P-I $\kappa$ B $\alpha$  expression in BMS345541-treated DPSCs at different time points. 1  $\mu$ M BMS345541 significantly inhibited the expression of P-I $\kappa$ B $\alpha$ . (e) Alkaline phosphatase (ALP) activity in E2-treated DPSCs after inhibition of NF- $\kappa$ B signaling with BMS345541.  $**P < 0.01$ . (f) Real-time RT-PCR results revealed that the odonto/osteogenic markers (ALP, RUNX2, OSX, OCN, and DSPP) in DPSCs were significantly downregulated in BMS345541+E2 group, as compared with E2 group.  $**2^{-\Delta\Delta Ct} > 2$ ,  $P < 0.01$ ;  $*1 < 2^{-\Delta\Delta Ct} < 2$ ,  $P < 0.01$ ;  $n = 3$ . (g) Alizarin red staining in E2-treated DPSCs after inhibiting the NF- $\kappa$ B pathway with BMS345541. Calcified nodules in BMS345541+E2 group were less than those in E2-treated DPSCs. Scale bars = 100  $\mu$ m.

unexplored (12). In the present study, the physiologically minor concentration,  $10^{-5}$  M  $17\beta$ -oestradiol, significantly inhibited proliferation of DPSCs, while physiologically relevant concentrations  $10^{-7}$  and  $10^{-9}$  M  $17\beta$ -oestradiol, had no effect.  $17\beta$ -oestradiol may regulate cell proliferation in a dose-dependent manner *via* oestrogen receptor (OR) pathways (19–21) and its high concentration can significantly inhibit proliferation of OR-positive cells (22). In particular, high dose  $17\beta$ -oestradiol can repress cancer cell proliferation, such as in carcinoma of the breast and colorectal cancer, in postmenopausal women receiving hormonal replacement therapy (22,23).

To elucidate impacts of oestrogen on cell differentiation,  $17\beta$ -oestradiol should be able to trigger differentiation without inhibiting proliferation of DPSCs. Under such conditions, stem cells can retain stable growth capacity to guarantee their sustainable differentiation. In the current study, we used a physiological concentration of  $17\beta$ -oestradiol ( $10^{-7}$  M) (24) to stimulate differentiation of DPSCs *in vitro*. Clearly,  $10^{-7}$  M E2 enhanced ALP activity, mineralization capacity and odonto/osteogenic potential of the cells, without affecting their proliferation. Understanding the effects of  $17\beta$ -oestradiol on differentiation of DPSCs provides us with the opportunity to test whether  $17\beta$ -oestradiol could facilitate the general process of tissue calcification and pulp regeneration.

As odontoblast-specific markers, DSP protein and *DSPP* mRNA have been reported to be expressed only in secretory odontoblasts (25,26). Thus, increased expression of *DSPP*/DSP in E2-treated DPSCs indicates that  $10^{-7}$  M  $17\beta$ -oestradiol could promote odontoblastic differentiation. Additionally, RUNX2 and OSX are early-stage markers of osteoblastic differentiation (27–29). RUNX2 overexpression can induce mesenchymal stem cells to differentiate into osteoblast lineages, enhance new bone formation and even drive pre-adipocytes into bone-forming cells, *in vitro* (30,31). OSX is the downstream gene of the BMP-2/Smad/Runx2 signaling pathway and is highly expressed in functional odonto/osteoblasts (32). OCN mainly appears in late stages of osteoblastic differentiation (15,28). Thus, upregulation of RUNX2, OSX and OCN after  $17\beta$ -oestradiol treatment suggests that  $17\beta$ -oestradiol induced biological changes in DPSCs, similar to osteoblastic differentiation and matrix mineralization.

In addition,  $17\beta$ -oestradiol treatment has resulted in activation of several signaling pathways including those of JNK, ORK, P38, the OR, PI3K-AKT-dependent pathways and NF- $\kappa$ B (33–38). The NF- $\kappa$ B pathway has been reported to be extensively involved in E2-mediated osteoblastic differentiation and mineralization

(12,24,39,40) and canonical NF- $\kappa$ B pathway is regulated by inhibition of the  $\kappa$ B kinase complex (IKK- $\alpha$ , IKK- $\beta$  and IKK- $\gamma$ ) (41). The IKK complex phosphorylates/degrades I $\kappa$ B and releases NF- $\kappa$ B subunits, mainly p65 and p50, for nuclear translocation and DNA binding, which subsequently regulate diverse biological processes including apoptosis, cell survival, cell division, cell differentiation and innate immunity as well as cell responses to stimuli (42). In this study, cytoplasmic P-I $\kappa$ B $\alpha$ /P-P65 and nuclear P65 were upregulated in a time-dependent manner following  $17\beta$ -oestradiol treatment, suggesting that the NF- $\kappa$ B pathway was activated in E2-treated DPSCs. Activation of NF- $\kappa$ B can promote the odontoblastic phenotype and stimulate odonto/osteogenic differentiation of dental pulp-derived stem cells (2,43,44). Furthermore, inhibition of the NF- $\kappa$ B pathway dramatically suppressed odonto/osteogenic differentiation of E2-treated DPSCs, as indicated by downregulation of several odonto/osteogenic markers and reduced mineralization capacity. Based on the present findings, it can be inferred that the NF- $\kappa$ B pathway plays a pivotal role during committed differentiation of E2-treated DPSCs.

In our previous study, DPSCs from the oestrogen deficiency rat model also exhibited activated NF- $\kappa$ B pathway, but with reduced odontogenesis/osteogenesis (12). Oestrogen deficiency usually leads to excessive expression of TNF- $\alpha$  which definitively causes activation of NF- $\kappa$ B (12,39,45,46). TNF- $\alpha$ , a potent pro-inflammatory cytokine, inhibits osteoblastogenesis and stimulates osteoclastogenesis under diverse inflammatory conditions (47–50). Here, it is possible that  $17\beta$ -oestradiol may induce odonto/osteoblastic differentiation of human DPSCs *via* activation of OR- $\alpha$ /NF- $\kappa$ B, but not by the canonical TNF- $\alpha$ /NF- $\kappa$ B pathway, and accordingly trigger differentiation-related gene expression and mineralization (9,39). Data accumulated here suggest that activation of the NF- $\kappa$ B pathway in DPSCs from different species may cause distinctive differentiation potency *via* different upstream and downstream signaling pathways. More intensive studies are required to explore upstream and downstream cell signaling of the NF- $\kappa$ B pathway during  $17\beta$ -oestradiol or oestrogen deficiency-mediated differentiation of DPSCs.

In conclusion,  $17\beta$ -oestradiol promoted odonto/osteogenic differentiation of human DPSCs *via* activation of the NF- $\kappa$ B pathway. These findings provide strong clues for therapeutic application of  $17\beta$ -oestradiol in reconstruction of the inductive microenvironment necessary for composing of bio-teeth, bio-pulp and even bio-bone. However, further studies are warranted to investigate other potential mechanisms associated with E2-mediated odonto/osteogenic differentiation of DPSCs.

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