DDIT3 overexpression increases odontoblastic potential of human dental pulp cells

Y. Wu¹, H. Sun¹, F. Song, D. Fu and J. Wang

The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, Hubei, 430079, China

Received 21 November 2013; revision accepted 18 January 2014

Abstract

Objectives: Human dental pulp cells (HDPCs) with multi-potential differentional capacity can undergo odontoblastic differentiation when stimulated with proinflammatory cytokines. However, factors linking proinflammatory stimuli and their odontoblastic differentiation have, as yet, not been completely understood. As an apoptotic transcription factor, DDIT3 plays a crucial role in the inflammatory reaction and in osteogenic differentiation. Thus, we hypothesized that DDIT3 may participate in odontoblastic differentiation of HDPCs.

Materials and methods: Immunofluorescent staining was used to detect expression of DDIT3 in HDPCs and effects of TNF α , on its nuclear accumulation. HDPCs that overexpressed DDIT3 were developed and their proliferation and odontoblastic differentiation abilities were examined. qRT-PCR was employed to detect mineralization-related genes, including *ALP*, runt-related transcription factor-2 (*Runx2*), osterix (*OSX*), dentin sialophosphoprotein (*DSPP*), dentin matrix acidic phosphoprotein 1 (*DMP1*) and osteocalcin (*OCN*). Western blot analysis was performed to detect expression of DSPP protein.

Results: DDIT3 was expressed in HDPCs. TNF α treatment enhanced mRNA expression as well as nuclear accumulation of DDIT3 (slightly). DDIT3 overexpression reduced HDPC proliferation, how-

ever, it increased their calcium nodule formation and expression of *OSX*, *DSPP*, *DMP1* and *OCN*.

Conclusions: DDIT3 may be a factor that links proinflammatory stimuli and differentiation of HDPCs.

Introduction

Similar to mesenchymal stem cells, human dental pulp cells (HDPCs) are a heterogeneous cell population possessing self-renewal ability and undergoing multi-lineage differentiation, thereby giving rise to various cell types such as odontoblasts, osteoblasts, chondrocytes, adipocytes, myocytes and neurocytes (1–4). The dentine-pulp complex is an important part of the tooth, where it participates in various biological activities. Dentine located outside the dental pulp can protect soft dental pulp tissue, whereas dental pulp cells can regenerate dentine and provide oxygen, nutrition and innervation (5). Differentiation of HDPCs to a specific cell lineage mainly depends on interaction between external stimulation and the internal microenvironment, including presence of proinflammatory cytokines, receptor molecules, signaling molecules and transcription factors.

Odontoblasts derived from HDPCs form a continuous layer that surrounds the dental pulp, each odontoblast having a long process that extends into a dentinal tubule, through which odontoblasts can sense external stimuli (6–8). Various noxious stimuli such as infection, ischaemia, trauma and chemicals can result in inflammatory responses of dental pulp tissue and stimulate migration, proliferation and differentiation of progenitor cells, into odontoblasts (9). Moderate inflammatory stimuli can promote regenerative processes of the dentine-pulp complex, including cell recruitment, differentiation and dentine secretion (10). For instance, TNF α has been identified as a mediator that stimulates differentiation of dental pulp cells towards an odontoblastic phenotype *via* p38, under proinflammatory stimuli (11). However,

Correspondence: J. Wang, The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory for Oral Biomedical Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, Hubei 430079, China. Tel.: +86 27 87686130; Fax: +86 27 87873260; E-mail: wangjwei@hotmail.com

¹These authors contributed equally to this work.

other factors that link proinflammatory stimuli and odontoblastic differentiation of HDPCs are not completely understood.

DNA damage-inducible transcript 3 (DDIT3), also known as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), is a member of the C/EBP family of transcription factors that participates in immune reactions and osteogenic differentiation (12,13). As an apoptotic transcription factor, DDIT3 expression can be induced in response to endoplasmic reticulum (ER) stress, and plays a crucial role in pathogenesis of inflammation through induction of caspase-11 (14). Inhibiting activation of transcription factor 4 (ATF4)-DDIT3 pathway reduces inflammatory cytokine expression by repressing nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) proinflammatory signalling (15). However, the role of DDIT3 in osteogenic differentiation remains controversial. DDIT3 can promote osteoblast differentiation of stromal cells, partially by forming heterodimers with C/EBP α and C/EBP β and by enhancing Smad signalling (12). Additionally, *in vivo*, DDIT3 is essential for normal expression of the osteoblast phenotype, identified in that DDIT3 knockout mice have impaired bone formation ability (16). In contrast, DDIT3 is reported to be a dominant-negative inhibitor of C/EBP β , inhibiting osteoblast differentiation by blocking interaction between C/EBP β and Runx2 *in vitro* (17). *In vivo*, DDIT3 overexpression in the bone microenvironment of mice reduces osteoblast function and results in osteoporosis (18). These observations suggest that the role of DDIT3 in the inflammatory response is indisputable, whereas its role in osteogenesis remains controversial and even contradictory. However, it has never previously been investigated whether DDIT3 is expressed in HDPCs and how DDIT3 would affect odontoblast differentiation of HDPCs.

In the present study, we hypothesized that DDIT3 overexpression would increase odontoblastic differentiation of HDPCs.

Materials and methods

Culture of primary HDPCs

To obtain HDPCs, ten healthy premolars and impacted third molars extracted for orthodontic reasons were collected from six human donors (18–28 years) after informed patient consent was obtained. Pulp tissues were collected from sectioned teeth, minced into small pieces, then placed in culture dishes. Cells were incubated in complete medium containing α -MEM (Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin

(P/S, v/v), in humidified atmosphere of 5% CO₂ at 37 °C. Cells that migrated out of graft pieces were trypsinized, harvested, transferred into new culture dishes, and used for further study at passages 3–5.

Treatment with TNF α and immunofluorescence staining

Cells were seeded on coverslips in 24-well plates at 1.5×10^4 cells/cm², treated with 10 ng/ml recombinant human TNF α (Novoprotein, Shanghai, China) and after 24 h, cells from both experimental and control wells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After washing in PBS, cells were treated with 0.5% Triton X-100 in PBS for 15 min at room temperature, and incubated with normal goat serum for 60 min at 37 °C. Samples were subsequently incubated in rabbit anti-human DDIT3 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. Secondary antibodies conjugated to fluorescent TRITC tag (1:150; Cwbio, Beijing, China) were employed for visualization. Nuclei were stained with DAPI for 3 min at room temperature and staining was observed using a fluorescence microscope (Zeiss, Jena, Germany) and photographed.

Plasmid construction

Total RNA was extracted from the cells and reverse transcribed using first-strand complementary DNA synthesis kit (Fermentas, Shanghai, China). Entire cDNA encoding human DDIT3 was amplified by polymerase chain reaction (PCR) using PrimeSTAR Max DNA (Takara, Dalian, China), following primers being used: forward: 5'-CCGGAATTCATGGAGCTTGTCCAGCC-3' and reverse: 5'-CGCGGATCCTCATGCTTGGTG-CAGATTC-3' containing extra *EcoRI* and *BamHI* restriction sites to facilitate cloning (underlined); purified PCR products were subsequently cloned into a pLVX plasmid (Clontech, Mountain View, CA, USA). Obtained lentiviral construct was confirmed by sequencing, and designated pLVX-human-DDIT3.

Lentivirus package and cell transfection

Generation of lentiviral vectors was accomplished using a three-plasmid transfection procedure. Briefly, expression vectors, pLVX-human-DDIT3 or pLVX-IRES-GFP, were transfected into 293T packaging cells, along with pspax2 and pMD2G plasmids (Addgene, Cambridge, MA, USA). Lentiviruses were collected after 48 h and used to infect HDPCs with polybrene (4 μ g/ml), in complete medium. After 6 h, transduction medium was discarded and cells were ready for experimentation.

Infected cells were analysed using a fluorescence microscope. Expression of DDIT3 was quantified by qRT-PCR and Western blotting.

Cell proliferation assay

For the cell proliferation assay, HDPCs were cultured in 96-well plates at 2×10^3 cells/well. Twenty microlitres MTT (5.0 mg/ml) was added for different durations (24, 48, 72 and 96 h) and incubated for another 4 h at 37 °C. Subsequently, supernatant was removed and dimethylsulphoxide (DMSO) was added to the wells; optical density (OD) was measured using a microplate reader scanning at 570 nm.

Odontoblastic differentiation

Three groups of HDPCs were seeded respectively into 12-well plates and expanded in α -MEM (Hyclone) supplemented with 10% FBS and 1% P/S. When HDPCs were grown to confluence, medium was changed to odontoblastic differentiation medium consisting of α -MEM supplemented with 10% FBS, 50 μ g/ml L-ascorbate phosphate (Sigma, USA), 10 mM β -glycerophosphate (Sigma, Shanghai, China) and 10 nM dexamethasone (Sigma). Cells were maintained in fresh odontoblastic differentiation medium every two days from day 0 to 14.

ALP staining

Cells were cultured in 12-well plates for 7 days in odontoblastic differentiation medium being fixed in 4% paraformaldehyde for 15 min and washed twice in PBS. ALP staining solution naphthol AS-MX phosphate and fast red violet LB salt (Sigma) were added to cultures and incubated at room temperature for 30 min. Staining solution was removed and cells were washed in distilled water. ALP-positive cells were then visualized using a light microscope.

Alizarin red and von Kossa staining

After odontoblastic induction in differentiation medium for 14 days, mineral deposition was assessed by alizarin red staining. Three kinds of HDPC plated in triplicate in 12-well plates were fixed in 4% paraformaldehyde for 15 min at room temperature then washed in distilled water. One percent alizarin red solution was added, incubated for 10 min at room temperature followed by several washes in distilled water, and viewed under a light microscope. Staining was then desorbed with 10% cetylpyridinium chloride (Sigma) for 1 h and solutions

were collected and distributed in 96-well plates at 200 μ l/well. Absorbance was read at 590 nm using a spectrophotometer (PowerWave XS2; BioTek, Winooski, VT, USA). Alizarin red levels were normalized to total protein content. Mineral deposition was also assessed by the von Kossa method on day 14.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol (Invitrogen, Shanghai, China). Then, 1 μ g RNA was used to transcribe first-strand cDNA using M-MuLV reverse transcriptase (Fermentas, Shanghai, China) following the manufacturer's protocol. qRT-PCR was performed with the SYBR Premix Ex Taq™ (Takara), in triplicate on ABI-7500 apparatus. Thermal cycling conditions were as follows: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Primer sequences are shown in Table 1 and all quantifications were normalized to the endogenous control *GAPDH*. Comparative $2^{-\Delta\Delta C_t}$ method was used to calculate relative gene expression (19).

Western blotting

Cell lysates were collected using RIPA medium (Beyotime, Shanghai, China) and protein concentrations were determined using a BCA protein assay kit (Beyotime). Protein samples were boiled for 5 min then separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on to PVDF membranes. Blocked with 5% non-fat milk in a Tris-buffered saline Tween-20 (TBST) buffer, membranes were then

Table 1. Primer sequences for qRT-PCR

Genes	Primer sequence (5'–3') (forward/reverse)	Product size (bp)
DDIT3	CCTCACTCTCCAGATTCCA AGCCGTTTCATTCTCTTCAG	185
ALP	GGACCATTCCCACGCTTTCAC CCTTGTAAGCCAGGCCATTG	137
Runx2	CATGGTGAGATCATCGC ACTCTTGCCCTCGTCCACTC	286
OSX	CCTCTGCGGACTCAACAAC AGCCCATAGTGCTTGTAAGG	128
DSPP	CAACCATAGAGAAAGCAAACGCG TTTCTGTTGCCACTGCTGGGAC	120
DMP1	ATGCCTATCACAACAAACC CTCCTTTATGTGACAACTGC	100
OCN	ATGAGAGCCCTCACACTCCTC GCCGTAGAAGCGCCGATAGGC	294
GAPDH	CATCACCATCTTCCAGGAG AGGCTGTTGTCACTTCTC	214

incubated with primary antibodies to: DDIT3 (1:500; Santa Cruz Biotechnology), DSPP (1:500; Santa Cruz Biotechnology) and β -actin polyclone antibody (1:2000; Santa Cruz Biotechnology) overnight at 4 °C. Blots were incubated in horseradish peroxidase-conjugated secondary antibody (1:5000; Cwbio) for 1 h. Protein bands were detected using ECL reagent, and specific bands were visualized using X-ray film. Western blotting experiments were repeated in at least triplicate to confirm results.

Statistical analysis

All experiments were repeated in at least triplicate. Quantitative results were expressed as mean $\pm$ SD. Data were analysed by one-way analysis of variance using SPSS 16.0 software (Chicago, IL, USA). P -values <0.05 were considered statistically significant.

Results

Endogenous DDIT3 expression in HDPCs

Immunofluorescent staining revealed no signal when cells were incubated in secondary antibody only (Fig. 1a-1–3). Immunofluorescence using rabbit anti-human DDIT3 antibody demonstrated that DDIT3 was expressed, primarily located in the cytoplasm of HDPCs (Fig. 1b-1–3). Further studies revealed that $\text{TNF}\alpha$ treatment increased nuclear accumulation of DDIT3 (Fig. 1c-1–3). In addition, qRT-PCR confirmed that cells treated with $\text{TNF}\alpha$ increased mRNA expression of *DDIT3* and some inflammatory cytokines such as *IL-6* and *IL-1 β* (Fig. 1d). Compared to the control group without induction, HDPCs had significant upregulation of *DDIT3* mRNA and protein at day 14, when chal-

Figure 1. Immunofluorescent staining and endogenous DDIT3 expression in human dental pulp cells (HDPCs). (a) Immunofluorescent staining of DDIT3 in HDPCs without primary antibody (a-1–3: DAPI staining, TRITC-conjugated secondary antibody staining and merge). (b) Immunofluorescent staining of DDIT3 in wild-type HDPCs (b-1–3: DAPI staining, TRITC-conjugated secondary antibody staining and merge). (c) Immunofluorescent staining showed increased nuclear accumulation of DDIT3 in $\text{TNF}\alpha$ -challenged HDPCs (arrow, c-1–3: DAPI staining, TRITC-conjugated secondary antibody staining and merge). (d) mRNA expression of IL-6, IL-1 β and DDIT3 in HDPCs after $\text{TNF}\alpha$ treatment. (e) Endogenous DDIT3 mRNA expression during odontoblastic differentiation detected by qRT-PCR. (f) Endogenous DDIT3 protein expression during odontoblastic differentiation detected by Western blot. *Indicates a significant difference between two groups ($P < 0.05$). Scale bar = 50 μm .

lenged with odontoblastic differentiation medium ($P < 0.05$, Fig. 1e,f).

DDIT3 overexpression

A lentiviral vector system was used to efficiently overexpress DDIT3 in primary HDPCs (HDPCs-DDIT3-overexpression) to levels $>90\%$, quantified by GFP expression area/cell area (Fig. 2c,f). In addition, relative amount of DDIT3 expression was confirmed by qRT-PCR (Fig. 2g) and Western blot analysis (Fig. 2h). It was found that DDIT3 mRNA was overexpressed over 38-fold (Fig. 2g). Negative control cells transfected with GFP-expressing vector and wild-type HDPCs were designated as HDPCs-GFP and HDPCs-WT respectively (Fig. 2b,e and 2a,d).

Inhibition of HDPC proliferation by DDIT3 overexpression

MTT assay revealed DDIT3 overexpression reduced HDPC proliferation by 72 and 96 h compared to control groups ($P < 0.05$, Fig. 3a). No significant difference was observed between HDPCs-WT and HDPCs-GFP groups ($P > 0.05$, Fig. 3a).

Effect of DDIT3 on HDPC odontoblastic differentiation

Results revealed that compared to control groups, DDIT3 overexpression led to no significant difference in ALP staining area after 7 days culture in odontoblastic medium ($P > 0.05$, Fig. 4a–d). However, DDIT3 over-

expression enhanced calcium deposition by day 14, as examined by alizarin red and von Kossa staining (Fig. 4e–g,i–k). Absorbance at 590 nm and mineral nodule area in the DDIT3 overexpression group were 1.6 times and 1.8 times higher than those in control groups by alizarin red and von Kossa staining respectively ($P < 0.05$, Fig. 4h,l).

qRT-PCR results revealed that DDIT3 overexpression did not affect *ALP* and *Runx2* mRNA levels ($P > 0.05$, Fig. 3b,c); however, it significantly increased *OSX*, *DSPP*, *DMP1* and *OCN* mRNA levels by day 14 ($P < 0.05$, Fig. 3d–g).

Compared to control groups, Western blot analysis revealed that DSPP protein levels were higher in the DDIT3 overexpression group (Fig. 3h).

Discussion

DDIT3 is widely distributed in cells of various tissues, including bone, cartilage, heart and muscle (17). In the present study, for the first time, we confirmed presence of DDIT3 in HDPCs. Through immunofluorescence assays, DDIT3 was found to be primarily located in the cytoplasm of HDPCs. To further evaluate the role of DDIT3 in their odontoblastic differentiation, compared to controls, endogenous DDIT3 expression was found to be significantly upregulated after 14 days culture in odontoblastic differentiation medium. These results indicate that DDIT3 may function in odontoblastic differentiation of HDPCs.

Under non-stressed conditions, DDIT3 has been reported to be present in cytoplasm, however, ER stress

Figure 2. Lentiviral infection of human dental pulp cells (HDPCs) and DDIT3 expression. (a and d) Cell image of HDPCs-WT and GFP-positive cells. (b and e) Cell image of HDPCs-GFP and GFP-positive cells. (c and f) Cell image of HDPCs-DDIT3-overexpression and GFP-positive cells. (g) DDIT3 mRNA expression determined by qRT-PCR in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups. (h) DDIT3 protein expression determined by Western blot in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups. *Indicates a significant difference between HDPCs-GFP and HDPCs-DDIT3-overexpression groups ($P < 0.05$). Scale bar = 200 μ m.

Figure 3. Proliferation ability and mineralization-related genes expression in human dental pulp cells (HDPCs). (a) Cell proliferation. (b–g) ALP, Runx2, OSX, DSPP, DMP1 and OCN mRNA expression during odontoblastic differentiation at day 0, 7 and 14. (h) DSPP protein expression in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups by Western blot at day 14. The histogram shows the quantification of gene expression and is presented as fold changes compared with HDPCs-GFP group. *Indicates a significant difference between HDPCs-GFP and HDPCs-DDIT3-overexpression groups ($P < 0.05$).

leads to its induction and nuclear accumulation (20). Thus, to study nuclear translocation of DDIT3, HDPCs were treated with $\text{TNF}\alpha$. It was found that $\text{TNF}\alpha$ treatment increased mRNA expression of *DDIT3* and some inflammatory cytokines such as *IL-1 β* and *IL-6*. Furthermore, immunofluorescence staining revealed that such treatment slightly increased nuclear accumulation. $\text{TNF}\alpha$ can induce the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent manner and induce ER stress by activating tumour necrosis factor receptor 1 (TNFR1) and IRE1 α in the hypothalamus (21,22). TNFR1 is the main receptor for TNF-induced

diverse cellular events and plays a crucial role in ER stress (23). $\text{TNF}\alpha$ may cause ER stress in HDPCs then increase DDIT3 expression and nuclear accumulation. However, further studies are required to highlight detailed molecular mechanisms by which $\text{TNF}\alpha$ would increase DDIT3 expression.

There is increasing interest in the area of dental pulp regeneration in identifying factors that induce dental pulp stem cells to differentiate into odontoblasts for restoring dentine defects after injury (24,25). Dental caries or trauma can lead to exposure and inflammation of dental pulp tissue, characterized by accumulation of

Figure 4. DDIT3 overexpression increased the human dental pulp cells (HDPCs) odontoblastic differentiation. (a–c) ALP staining at day 7 in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups. (e–g) Alizarin red staining of the cultured HDPCs at day 14 in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups. (i–k) Von Kossa staining of the bone nodules formed at day 14 in HDPCs-WT, HDPCs-GFP and HDPCs-DDIT3-overexpression groups. (d) The percentage of ALP-positive cells area. (h) Alizarin red absorbance at 590 nm. (l) Mineralized matrix areas. *Indicates a significant difference between HDPCs-GFP and HDPCs-DDIT3-overexpression groups ($P < 0.05$). Scale bar = 500 μ m.

inflammatory cells releasing host inflammatory cytokines, including $\text{TNF}\alpha$. Relatively moderate inflammation exerts beneficial effects on cell differentiation and mineralization processes of dental pulp stem/progenitor cells (26). Because DDIT3 participates in the inflammatory response and control of osteoblast differentiation in appropriate stages (27,28), we propose that DDIT3 may be a factor that links proinflammatory stimuli and odontoblastic differentiation of HDPCs. To test this hypothesis, we established DDIT3 overexpressing HDPCs by transfecting cells with a lentiviral construct, and detected mRNA expression of some key odontoblastic factors. It was found that early mineralization-related genes (29,30), including *ALP* and *Runx2*, had no significant differences compared to control groups. However, mRNA levels of *OSX*, which acts downstream of *Runx2* and is necessary for odontoblast differentiation (31,32), were significantly upregulated by day 14. This may be due to that functions of *Runx2* and *OSX* are independent in regulating odontoblastic and osteoblastic differentiation are independent of each other (32). At later odontoblastic differentiation stages of tooth development, *Runx2* expression was reduced, whereas *OSX* expression was still intense in odontoblasts (32). In osteoblastic differentiation of *Runx2*-

deficient mesenchymal cells, BMP2 can promote *OSX* expression through msh homoeobox 2 (*MSX2*) independent of *Runx2* (33). Moreover, late odontoblastic differentiation markers (34,35), *DSPP*, *DMP1* and *OCN*, were dramatically upregulated here in the HDPCs-DDIT3-overexpression group, by day 14. These results imply that DDIT3 may mainly function in late odontoblastic differentiation phases. Alizarin red and von Kossa staining corroborated this result as increased calcium nodule formation was detected in the DDIT3 overexpression group. It has been demonstrated that $\text{TNF}\alpha$ -challenged HDPCs have increased mineralization and expression of *DSPP*, *DMP1* and *OCN* (11). Thus, it is reasonable to speculate that $\text{TNF}\alpha$ enhances mineralization of HDPCs, at least partially, through upregulation of DDIT3.

DDIT3 is known to play different or even opposite roles during osteogenic differentiation. For example, it promotes osteoblastic differentiation of ST-2 cells, a multi-potential mesenchymal progenitor cell line, both by inhibiting binding of C/EBPs to their consensus sequence, by sensitizing Smad and Wnt/ β -catenin signalling pathways (12). As we know, HDPCs have multiple differentiation potentials with self-renewal capacity and can differentiate into odontoblasts, adipocytes, and

neural-like cells during injury or inflammation, associated with reparative dentinogenesis (36). The final cell phenotype requires precise orchestration of extracellular and intracellular signals, by such as Wnt, Notch and BMP (37,38). In dental mesenchyme, β -catenin conditional knockout mice have absence of erupted molar roots and thin incisors, indicating that Wnt/ β -catenin signalling is essential for odontoblastic differentiation (39). Thus, we speculate that the role of DDIT3 in HDPCs is the same as that in ST-2 cells. On the one hand, enhanced odontoblastic differentiation of HDPCs may be due to direct activation of the Wnt/ β -catenin signalling pathway. On the other hand, overexpressed DDIT3 can bind to C/EBPs, functioning as a dominant-negative inhibitor, thereby reducing adipogenic potential of HDPCs, and indirectly favouring odontoblast differentiation (12). DDIT3 has the potential to become a therapeutic target for promoting odontoblastic differentiation of HDPCs. However, further studies are required to clarify mechanisms of DDIT3 involved in odontoblastic differentiation.

In summary, our results demonstrate that DDIT3 is present in HDPCs, it may correlate with their late odontoblastic differentiation, and it may be a factor that links proinflammatory stimuli and their differentiation.

Acknowledgements

This work was financially supported by The Fundamental Research Fund for the Central Universities of China (2012304020207), grant from The Office of Science and Technology of Hubei Province (2011CDB470), and grants from The National Natural Science Foundation of China (81200812, 81271179).

References

- Park SJ, Li Z, Hwang IN, Huh KM, Min KS (2013) Glycol chitin-based thermoresponsive hydrogel scaffold supplemented with enamel matrix derivative promotes odontogenic differentiation of human dental pulp cells. *J. Endod.* **39**, 1001–1007.
- Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J *et al.* (2008) Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. *Stem Cells Dev.* **17**, 1175–1184.
- Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT (2010) iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev.* **19**, 469–480.
- Zhao X, Gong P, Lin Y, Wang J, Yang X, Cai X (2012) Characterization of alpha-smooth muscle actin positive cells during multilineage differentiation of dental pulp stem cells. *Cell Prolif.* **45**, 259–265.
- Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E (2011) Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. *Braz. Dent. J.* **22**, 91–98.
- Allard B, Magloire H, Couble ML, Maurin JC, Bleicher F (2006) Voltage-gated sodium channels confer excitability to human odontoblasts: possible role in tooth pain transmission. *J. Biol. Chem.* **281**, 29002–29010.
- Farges JC, Keller JF, Carrouel F, Durand SH, Romeas A, Bleicher F *et al.* (2009) Odontoblasts in the dental pulp immune response. *J. Exp. Zool. B Mol. Dev. Evol.* **312B**, 425–436.
- Magloire H, Maurin JC, Couble ML, Shibukawa Y, Tsumura M, Thivichon-Prince B *et al.* (2010) Topical review. Dental pain and odontoblasts: facts and hypotheses. *J. Orofac. Pain* **24**, 335–349.
- Wei X, Liu L, Zhou X, Zhang F, Ling J (2012) The effect of matrix extracellular phosphoglycoprotein and its downstream osteogenesis-related gene expression on the proliferation and differentiation of human dental pulp cells. *J. Endod.* **38**, 330–338.
- Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ (2010) Inflammation-regeneration interplay in the dentine-pulp complex. *J. Dent.* **38**, 687–697.
- Paula-Silva FW, Ghosh A, Silva LA, Kapila YL (2009) TNF-alpha promotes an odontoblastic phenotype in dental pulp cells. *J. Dent. Res.* **88**, 339–344.
- Pereira RC, Delany AM, Canalis E (2004) CCAAT/enhancer binding protein homologous protein (DDIT3) induces osteoblastic cell differentiation. *Endocrinology* **145**, 1952–1960.
- Esposito V, Grosjean F, Tan J, Huang L, Zhu L, Chen J *et al.* (2013) CHOP deficiency results in elevated lipopolysaccharide-induced inflammation and kidney injury. *Am. J. Physiol. Renal Physiol.* **304**, F440–F450.
- Endo M, Mori M, Akira S, Gotoh T (2006) C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J. Immunol.* **176**, 6245–6253.
- Rao J, Qin J, Qian X, Lu L, Wang P, Wu Z *et al.* (2013) Lipopolysaccharide preconditioning protects hepatocytes from ischemia/reperfusion injury (IRI) through inhibiting ATF4-CHOP pathway in mice. *PLoS One* **8**, e65568.
- Pereira RC, Stadmeier L, Marciniak SJ, Ron D, Canalis E (2006) C/EBP homologous protein is necessary for normal osteoblastic function. *J. Cell. Biochem.* **97**, 633–640.
- Shirakawa K, Maeda S, Gotoh T, Hayashi M, Shinomiya K, Ehata S *et al.* (2006) CCAAT/enhancer-binding protein homologous protein (CHOP) regulates osteoblast differentiation. *Mol. Cell. Biol.* **26**, 6105–6116.
- Pereira RC, Stadmeier LE, Smith DL, Rydziel S, Canalis E (2007) CCAAT/Enhancer-binding protein homologous protein (CHOP) decreases bone formation and causes osteopenia. *Bone* **40**, 619–626.
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408.
- Ghosh AP, Klocke BJ, Ballesta ME, Roth KA (2012) CHOP potentially co-operates with FOXO3a in neuronal cells to regulate PUMA and BIM expression in response to ER stress. *PLoS One* **7**, e39586.
- Denis RG, Arruda AP, Romanatto T, Milanski M, Coope A, Solon C *et al.* (2010) TNF-alpha transiently induces endoplasmic reticulum stress and an incomplete unfolded protein response in the hypothalamus. *Neuroscience* **170**, 1035–1044.
- Xue X, Piao JH, Nakajima A, Sakon-Komazawa S, Kojima Y, Mori K *et al.* (2005) Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. *J. Biol. Chem.* **280**, 33917–33925.

- 23 Yang Q, Kim YS, Lin Y, Lewis J, Neckers L, Liu ZG (2006) Tumour necrosis factor receptor 1 mediates endoplasmic reticulum stress-induced activation of the MAP kinase JNK. *EMBO Rep.* **7**, 622–627.
- 24 Chai Y, Slavkin HC (2003) Prospects for tooth regeneration in the 21st century: a perspective. *Microsc. Res. Tech.* **60**, 469–479.
- 25 Duailibi MT, Duailibi SE, Young CS, Bartlett JD, Vacanti JP, Yelick PC (2004) Bioengineered teeth from cultured rat tooth bud cells. *J. Dent. Res.* **83**, 523–528.
- 26 Lee DH, Lim BS, Lee YK, Yang HC (2006) Effects of hydrogen peroxide (H₂O₂) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biol. Toxicol.* **22**, 39–46.
- 27 Gutierrez S, Javed A, Tennant DK, van Rees M, Montecino M, Stein GS *et al.* (2002) CCAAT/enhancer-binding proteins (C/EBP) beta and delta activate osteocalcin gene transcription and synergize with Runx2 at the C/EBP element to regulate bone-specific expression. *J. Biol. Chem.* **277**, 1316–1323.
- 28 Pereira RC, Delany AM, Canalis E (2002) Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression. *Bone* **30**, 685–691.
- 29 Hadzir SN, Ibrahim SN, Abdul Wahab RM, Zainol Abidin IZ, Senafi S, Ariffin ZZ *et al.* (2013) Ascorbic acid induces osteoblast differentiation of human suspension mononuclear cells. *Cytotherapy*. doi: 10.1016/j.jcyt.2013.07.013. (in press).
- 30 Long F (2012) Building strong bones: molecular regulation of the osteoblast lineage. *Nat. Rev. Mol. Cell Biol.* **13**, 27–38.
- 31 Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR *et al.* (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17–29.
- 32 Chen S, Gluhak-Heinrich J, Wang YH, Wu YM, Chuang HH, Chen L *et al.* (2009) Runx2, osx, and dspp in tooth development. *J. Dent. Res.* **88**, 904–909.
- 33 Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H *et al.* (2008) BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J. Biol. Chem.* **283**, 29119–29125.
- 34 Wang Y, Zheng Y, Wang Z, Li J, Zhang G, Yu J (2013) 10 m 17beta-oestradiol enhances odonto/osteogenic potency of human dental pulp stem cells by activation of the NF-kappaB pathway. *Cell Prolif.* **46**, 677–684.
- 35 Aubin JE (1998) Bone stem cells. *J. Cell. Biochem. Suppl.* **30–31**, 73–82.
- 36 Gronthos S, Brahimi J, Li W, Fisher LW, Cherman N, Boyde A *et al.* (2002) Stem cell properties of human dental pulp stem cells. *J. Dent. Res.* **81**, 531–535.
- 37 Bluteau G, Luder HU, De Bari C, Mitsiadis TA (2008) Stem cells for tooth engineering. *Eur. Cell Mater.* **16**, 1–9.
- 38 Cai X, Gong P, Huang Y, Lin Y (2011) Notch signalling pathway in tooth development and adult dental cells. *Cell Prolif.* **44**, 495–507.
- 39 Kim TH, Bae CH, Lee JC, Ko SO, Yang X, Jiang R *et al.* (2013) beta-catenin is required in odontoblasts for tooth root formation. *J. Dent. Res.* **92**, 215–221.