**Changes of mitochondrial respiratory function during odontogenic differentiation of**

**rat dental papilla cells**

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**Abstract** Dental papilla cells (DPCs) belong to precursor cells differentiating to odontoblasts and play an important role in dentin formation and reproduction. This study aimed to explore the changes and and involvement of mitochondrial respiratory function during odontogenicdifferentiation. Primary DPCs were obtained from ﬁrst molar dental papilla of neonatal rats and cultured in odontogenic medium for 7, 14, 21 days. DPCs, which expressed mesenchymal surface markers CD29, CD44 and CD90, had the capacity for self-renewal and multipotent differentiation. Odontoblastic induction increased mineralized matrix formation in a time-dependent manner, which was accompanied by elevated alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1) expression at mRNA and protein levels. Notably, odontogenic medium led to an increase in adenosine-5'-triphosphate (ATP) content and mitochondrial membrane potential (MMP), whereas a decrease in intercellular reactive oxygen species (ROS) production and NAD+/NADH ratio. Furthermore, odontogenicdifferentiation was significantly suppressed by treatment with rotenone, an inhibitor of mitochondrial respiratory chain. These results demonstrate that enhanced mitochondrial function is crucial for odontogenicdifferentiation of DPCs.

**Key words** dental papilla cells ∙ odontogenicdifferentiation ∙ mitochondrial function ∙ reactive oxygen species

**Introduction**

The formation of dentin, known as dentinogenesis, is initiated by differentiation of dental papilla cells (DPCs) into odontoblasts, which is the result of the reciprocal interactions between ectoderm-derived oral epithelium and cranial neural crest cells (Kollar and Baird 1970; Ruch et al. 1995; Thesleff 2006; Shi et al. 2016). DPCs have the potential to differentiate into odontoblasts under odonto/osteogenic conditions in vitro (Begue-Kirn et al. 1998; Kikuchi et al. 2004). Alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1) are selected commonly as speciﬁc markers to detect the odontogenic potential (Lin et al. 2013; Yang et al. 2013; Yang et al. 2014; Lee et al. 2016; Lian et al. 2016). DPCs can also differentiate into other kinds of cell, such as adipocytes (Bai et al. 2010; Chen et al. 2015), nerve cell (Chen et al. 2015), endothelial cell and epithelial cells (Dogan et al. 2015). Due to its outstanding multiple differentiation potency, DPCs are used not only as a promising resource for dental regeneration, but also as a source for cell therapy of liver disease (Ikeda et al. 2008) and cardiovascular disease (Dogan et al. 2015).

Mitochondria are known for producing adenosine-5'-triphosphate (ATP) via oxidative phosphorylation (OXPHOS) in eukaryotic cells and inﬂuence how the cell responds to environmental cues, thus playing a critical role in cell fate (Kasahara and Scorrano 2014). Accumulating evidence has revealed that the switch of energy supply from glycolysis to aerobic metabolism and upregulation of mitochondrial functions are essential for successful differentiation of mesenchymal stem cells (MSCs) (Chen et al. 2008; Zhang et al. 2013; Hsu et al. 2016; Li et al. 2017). It was reported that differentiation of MSCs, such as bone marrow-derived mesenchymal stem cells (BMSCs) (Chen et al. 2008; Pietila et al. 2012; Sanchez-Arago et al. 2013) and placenta-derived mesenchymal stem cells (PDMSCs) (Solis et al. 2016), is accompanied by increase in activity of respiratory enzyme complexes, mitochondrial membrane potential (MMP) and intracellular ATP content. Futhermore, mitochondrial inhibitors could retard the differentiation of MSCs, suggesting increased mitochondrial activity played an essential role in MSC differentiation (Chen et al. 2008; Zhang et al. 2013). The data about the changes and involvement of mitochondrial function were obtained during the differentiation of BMSCs and PDMSCs, such a mechanism could also be operative in the DPCs (MSCs in dental tissues). Thus the aim of this study was to explore how mitochondrial respiratory function responded to odontogenic induction and to study whether mitochondrial function could impact odontogenic differentiation of DPCs.

**Materials and Methods**

**Cell cultures**

All experiments were approved by the Ethics Committee of Guanghua School and Hospital of Stomatology, Sun Yat-sen University, China (ERC-2013-15). The DPCs were primary cultured after isolated from the ﬁrst molar dental papilla of neonatal Sprague-Dawley (SD) rats (purchased from Sun Yat-sen University, Guangzhou, China). Brieﬂy, rats were sacrificed using by an overdose of 10% chloralhydrate solution. Dental papilla tissues were carefully isolated using a dental explorer under the stereomicroscope (Stemi2000, Zeiss, Jena, Germany), minced into small pieces (1 mm3), plated on 25 cm2 ﬂask, and incubated in α-Modiﬁed Eagle’s Medium (α-MEM) with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 ug/mL streptomycin in a humidiﬁed atmosphere containing 95% O2 and 5% CO2 at 37 °C. The culture medium was changed every 3 days. Cells were digested using 0.25% trypsin with 0.25 mM EDTA when they reached 80-90% conﬂuence and distinct digestion method was applied to purify cells. Passages 3-5 were used for the following research. DPCs were cultured in an osteo/odontogenic medium (OS, 10% FBS, 0.1 mM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate; Sigma-Aldrich, St. Louis, MO, USA) with or without rotenone (0.1-10 nM, Sigma-Aldrich, St. Louis, MO, USA), a mitochondrial complex I inhibitor. Rotenone was ﬁrst dissolved in dimethyl sulphoxide (DMSO), and then diluted in medium at a working concentration. The ﬁnal concentration of DMSO in medium was no more than 0.1%. All the above reagents and media were purchased from Gibco (Grand Island, NY, USA) unless otherwise stated.

**Immunocytochemical staining of cytokeratin and vimentin**

DPCs were fixed in ice-cold 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 for 10 min, incubated 0.3% hydrogen peroxide (H2O2) for 30 min to block endogenous peroxidase activity and 5% goat serum for 30 min at 37 °C to eliminate nonspecific background staining. The cells were incubated with mouse anti-cytokeratin antibody (1:100, BOSTER, China) or mouse anti-vimentin antibody (1:100, BOSTER, China) for 2 h at 37 °C. HEK293T cells stained for cytokeratin served as positive control and primary antibodies replaced by PBS served as negative control. The samples were then incubated with rabbit anti-mouse immunoglobulin (IgG H + L, 1:100, BOSTER, China) for 1 h at 37 °C and stained with 3,3'- diaminobenzidine（DAB）for 3 min and haematoxylin for 1 min. Photographs were captured using an inverted microscope (Axio Observer Z1; Carl Zeiss AG, Oberkochen, Germany).

**Multipotential differentiation**

DPCs were induced in an odonto/osteogenic medium (OS) or adipogenic medium (10% FBS, 1 mM dexamethasone, 200 mM indomethacin, and 0.5 mM 3-isobutyl-1-methylxanthine; Sigma-Aldrich, St. Louis, MO, USA) for 14 days. Cells were fixed in ice-cold 4% paraformaldehyde and stained with 1% Alizarin Red S solution (Cyagen, China) for osteogenesis assay or 3 mg/mL oil red O (Sigma-Aldrich, St. Louis, MO, USA) for adipogenesis analysis. The images were viewed with an inverted microscope (Axio Observer Z1; Carl Zeiss AG, Oberkochen, Germany). For quantitative evaluation, osteogenesis stain was extracted with 100 nM cetylpyridinum chloride (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature, and the absorbance of the supernatant was measured at 562 nm.

**Colony-forming assay**

100 DPCs were seeded in 10-cm-diameter culture dishes (Corning, NY, USA). After 14 days of culture, cells were stained with 0.1% crystal violet for 20 min. The number of colonies per dish was determined visually under a light microscope. Colonies containing more than 50 cells were considered as one colony. Colony-forming efﬁciency was calculated by dividing the number of total colonies by the number of seeded cells.

**Flow cytometric analysis**

Passage-3 DPCs were prepared as single-cell suspensions using trypsin/EDTA buffer and resuspended in PBS solution containing 2% FBS. Cells were incubated with several mesenchymal markers CD29, CD34, CD44, CD45 and CD90 (BD Biosciences, San Diego, CA, USA) for 30 min at 4 °C. Cell samples were analyzed by ﬂow cytometer (Beckman-coulter, CA, USA).

**Real-time quantitative PCR analysis**

Total mRNA was extracted using TRIzol (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s protocol. cDNA was synthesized by using RNA and transcriptor first strand cDNA synthesis kit (Roche Applied Science, Penzberg, Bayern, Germany). Real-time PCR reactions were performed in a LightCycler using SYBR Green I Master (Roche Applied Science, Penzberg, Bayern, Germany). Thermocycling conditions were as follows: initial denaturation at 95 °C for 5 min, 45 cycles at 95 °C for 10 sec, at 60 °C for 20 sec, at 72 °C for 20 sec, followed by 1 cycle at 95 °C for 5 sec, at 65 °C for 1 min, at 97 °C, and then a final 10 sec cooling at 40 °C. The primers used were detailed in Table 1. PCR products were analyzed on a 2% agarose gel.

**Quantitative assay of** **ALP activity**

ALP activity was measured using a commercial ALP kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. The cells were lysed or the supernatants were collected, and then samples were incubated with 0.5 mg/mL p-nitrophenyl phosphate solution. The production of p-nitrophenol in the presence of ALP was measured by monitoring light absorbance at 520 nm using an ELISA plate reader (Tecan, Grodig, Austria) and then divided by protein concentrations.

**Western blotting**

Total protein was extracted from the cells and the protein concentration was measured using the BCA protein assay (Beyotime, Shanghai, China). Equal amount of proteins were loaded and separated on 10% SDS-PAGE gels by electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were immunoblotted with the following primary antibodies: monoclonal mouse anti-DSPP antibody (1:500, Santa Cruz, CA, USA), polyclonal rabbit anti-DMP1 antibody (1:500, Novus Biologicals, Littleton, CO, USA) and monoclonal mouse anti-β-actin (1:1000, Beyotime, Shanghai, China) overnight at 4 °C, followed by incubation with a horse-radish-peroxidase-conjugated goat anti-mouse/rabbit (1:1000, Beyotime, China) at room temperature for 1 h. Immunoreactive bands were revealed by enhanced chemiluminescence (Supersignal West Dura extended duration substrate, CWBiotech, China) and visualized using an ImageQuant Las 4000 mini imaging system and software according to the manufacturer’s instructions. Band intensities were quantified by using ImageJ 1.36b (NIH Freeware, USA)

**Measurement of intracellular ATP content**

The DPCs were cultured in the basal medium or in odontogenic medium for 7 days, and then ATP level was measured by ATP Colorimetric/Fluorometric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. Briefly, 1×106 cells were lysed in 100 μL of ATP Assay Buffer and deproteinized using a 10 kDa MWCO spin filter. 50 μL samples were incubated with 50 μL ATP Assay Mix at room temperature for 30 min and measured the absorbance at the fluorescence (FLU, λex = 535/ λem = 587 nm) by Promega GloMax Multiplus Plate Reader (Promega Corporation, Wisconsin, USA). The luminescence intensity was then divided by total cell number.

**Measurement of MMP**

MMP was analyzed using Mitochondria Membrane Potential Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. Brieﬂy, 8×104 cells were incubated with 100 μL Dye Loading Solution containing 1× Mitochondrial Potential Dye in a 5% CO2, 37 °C incubator for 30 min. Then samples were then added with 50 μL Assay Buffer B and incubated for 30 min. The fluorescence intensity (λex = 540/λem = 590 nm) was measured by Promega GloMax Multiplus Plate Reader (Promega Corporation, Wisconsin, USA) and then divided by total cell number.

**NAD(P)+/NAD(P)H assay**

Intracellular NAD+ and NADH or NADP+ and NADPH levels were measured by NAD+/NADH Quantification Kit or NADP+/NADPH Quantification Kit, respectively (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. Brieﬂy, 2×106 cells were extracted with 400 μL Extraction Buffer by freeze/thawing for 2 cycles (20 min on dry ice followed by 10 min at room temperature) and deproteinized using a 10 kDa MWCO spin filter. Intracellular NAD(P)+ was decompose to NAD(P)H by taking out 200 μL samples into new microcentrifuge tubes and heating to 60 °C for 30 min. Total NAD(P)t and NAD(P)H were detected following the instruction in a 96-well plate. Absorbance was measured at 450 nm wavelength in an ELISA plate reader (Tecan, Grodig, Austria). NAD(P) +/ NAD(P)H ratio is calculated as: [NAD(P)t – NAD(P)H]/NAD(P)H.

**Measurement of intracellular reactive oxygen species (ROS)**

The DPCs were prepared as single-cell suspensions using trypsin/EDTA and resuspended in α-MEM containing 2% FBS. Cells were incubated in 5 μM CellROX® Green Reagent (Invitrogen, Grand Island, NY, USA) at 37 °C for 60 min and then washed with PBS. Fuorescence intensity of 1×104 cells was recorded on a ﬂow cytometer (Beckman-coulter, CA, USA).

**Cell viability assay**

Cell viability was analyzed by Cell Counting Kit-8 (CCK8, Dojindo, Japan). The DPCs were seeded in 96-well plates at a density of 2×103 cells/well and allowed to adhere for 24 h prior to supplementation with varying concentrations of rotenone (0.1-100 nM) or 0.1% DMSO. After 48 h, cells were treated with 10% CCK-8 in new media for 2 h at 37 °C. Absorbance at 450 nm was measured in an ELISA plate reader (Tecan, Grodig, Austria). Each experiment was performed in three parallel wells, and the mean optical density (OD) values were measured.

**Statistical analysis**

All data are expressed as mean ± standard deviation (SD). The two-tailed Student's t-test was used for the two-subgroup comparisons. One-way ANOVA was used to compare the means between multiple groups and least significant difference (LSD) was used for post hoc analysis. *P*<0.05 was considered to be significant. Statistical analysis was performed using SPSS 19.0 (IBM Corp, USA).

**Results**

**Isolation and identiﬁcation of DPCs**

Small pieces of dental papilla were cultured in 25 cm2 ﬂask, and DPCs migrated from the pieces at days 2 (Fig. 1A (i)). DPCs were homogenous, large, polygonal ﬁbroblastic cells with abundance cytoplasm and centrally localized single nuclei. Cells were subcultured and further purified by distinct digestion in vitro, then used for functional experiments once they grew to near 90% conﬂuence (Fig. 1A (ii)). DPCs were found to be positive for mesenchymal cell marker vimentin (Fig. 1B (i)) and negative for epithelial cell marker cytokeratin (Fig. 1B (ii)), which was consistent with the properties of stromal cell. Negative control and positive control for cytokeratin were shown in Fig. 1B (iii) and (iv) respectively. After osteogenic or adipogenic induction, DPCs were found to form calcium nodules (Fig. 1C (i)) or lipid droplets (Fig. 1C (ii)). In order to determine the proliferative properties of DPCs, a colony-forming assay was performed. After 14 days of culture, DPCs formed colonies (Fig. 1D (i)) and were found to be spindle-shaped, and arranged in clusters under a microscope (Fig. 1D (ii)). The colony-forming efficiency was ~25% (±3.6%; n = 3). DPCs were positive for mesenchymal markers including CD29 (98.58%), CD44 (79.69%), and CD90 (99.82%), while negative for hematopoietic markers CD34 and CD45 using flow cytometric analysis (Fig. 1E).

**DPCs differentiated to odontoblast upon odontogenic induction**

To determine whether DPCs have odontoblast differentiation potential, the cells were cultured in odontogenic medium for different periods of time ranging from 7 to 21 days. Fig. 2A shows that odontogenic induction increased ALP, DSPP and DMP1 mRNA expression on 7 day treatment. The ALP activity of cell lysates increased significantly (*p*<0.05, Fig. 2B) in odontogenic medium when compared with cells exposed to growth medium. But odontogenic medium did not significantly increase ALP activity in cell supernatants (Fig. 2B). The effect of odontogenic induction on DSPP and DMP1 protein levels was time-dependent, and maximum stimulation (4-fold increase for DSPP and 3-fold increase for DMP1) was observed at 14 days (Fig. 2C and D). Similar findings were observed in calcium nodules that were detected using alizarin red staining in DPCs with or without odontogenic induction (Fig. 2E). Quantitative analysis showed red-stained calcium nodules significantly increased in a time-dependent manner (Fig. 2F).

**Changes of mitochondrial respiratory function upon odontogenic induction**

To assess the respiratory function of DPCs during odontogenic induction, we firstly assayed intracellular ATP content and MMP. As illustrated in Fig. 3A, odontogenic media significantly led to a 1.7-fold increase in the ATP content compared with the control, indicating the upregulation of aerobic mitochondrial metabolism. To conﬁrm the energy requirements furtherly, MMP was also assayed in DPCs, whereas there was no significant difference between experimental and control group (*p*＞0.05), odontogenic induction upregulated MMP (Fig. 3B). NAD(P)H and its oxidized form NAD(P)+ have been known as classic molecules involving in oxidative metabolism and mitochondrial respiratory function, which are the major electron donors for the electron transport chain. So the NAD(P)+/NAD(P)H ratio is one of the modulators of OXPHOS, which can significantly influence mitochondrial functions (Ying 2008). Consistent with the increase of ATP and MMP, a 30% reduction in the NAD+/NADH ratio was observed during the process (Fig. 3C). But NADP+/NADPH ratio remained unchanged (Fig. 3D). Since enhanced mitochondrial respiratory function was associated with odontogenic differentiation, we further investigated the change of ROS, which are mainly produced from OXPHOS in mitochondria during ATP synthesis (Valko et al. 2007; Chen et al. 2008). Interestingly, quantitative analysis revealed a ~25% (±8%; n = 3, *p* < 0.05) decrease in ROS levels compared with that of the control (Fig. 3E).

**Inhibition of odontogenic differentiation by rotenone**

Based on the observations of enhanced mitochondrial respiratory function, we speculated that mitochondria play an important role in odontogenic differentiation. Therefore, we investigated whether alterations of mitochondrial function by addition of the inhibitor would affect odontogenic differentiation of DPCs. Rotenone is an inhibitor of mitochondrial complex I, thus affecting OXPHOS system and ATP generation (Kwak et al. 2010). Firstly, we examined the concentration-dependent toxicities of rotenone. Cell viability of DPCs was significantly reduced in the presence of 100 nM rotenone for 48h (*p* < 0.01), whereas no obvious change in cell viability was observed in the presence of 0.1-10 nM rotenone or 0.1% DMSO (Fig. 4A). Accordingly, rotenone in the concentration range from 0.1 to 10 nM was used for further experiments. Fig. 4B showed that rotenone had no effect on ALP, DSPP and DMP1 mRNA expression in undifferentiated DPCs. But when DPCs were incubated with odontogenic medium for 7 days, rotenone signiﬁcantly inhibited mRNA expression of these genes in a concentration-dependent manner (Fig. 4B). The inhibitory effect of rotenone was further confirmed by decreased ALP activity of cell lysates (Fig. 4C). These results indicate that decreased mitochondrial respiratory function could hamper the odontogenic differentiation of DPCs.

**Discussion**

DPCs are the cell type which has the ability to differentiate into odontoblasts, and an ectomesenchymal cell population which is acquired unique roles during dentinogenesis stages (Kollar and Baird 1970; Hao et al. 1997; Du J et al. 2016). But the molecular basis for the initiation of odontogenic differentiation is still not clear. In order to keep studies on the mechanisms, DPCs were acquired successfully from different species, including human (Hao et al. 1997; Peng et al. 2010; Yang et al. 2013), mouse (Thesleff 1986; Lin et al. 2013; Wang et al. 2013) and rat (Webb et al. 1995; Kikuchi et al. 2004; Reynolds and Jahoda 2004; Liu et al. 2013; Chen et al. 2014). Considering the convenience of experiment and ethical dilemmas, DPCs obtained from ﬁrst molar of rat have the advantage that they are easier to get more tissue block of dental papilla and to be cultured successfully in vitro (Reynolds and Jahoda 2004; Bai et al. 2010; Ge et al. 2013; Liu et al. 2013; Chen et al. 2014). In previous studies, dental papillae were acquired from different periods of ﬁrst molar ranging from embryonic (E)-20 to postnatal (PN) 3 weeks (Reynolds and Jahoda 2004; Bai et al. 2010; Ge et al. 2013; Liu et al. 2013; Chen et al. 2014). But tooth germ of ﬁrst molar was first observed by E-14 and at the bell stage by E-18 (Webb et al. 1995). Notably, Layers of dentin were first seen by PN 3-4 days (Webb et al. 1995; Chen et al. 2014). The periods between E-18 and PN 3-4 days is more reasonable time to acquire dental papillae. Thus DPCs in our team were isolated from the ﬁrst molar of neonatal or 1-day- PN rat (Liu et al. 2013).

MSCs are adherent ﬁbroblast-like multipotent cells, and were initially discovered in bone marrow. The Mesenchymal and Tissue Stem Cell Committee of International Society for Cellular Therapy has deﬁned MSCs based on the following criteria (Dominici et al. 2006): first, MSCs must be plastic-adherent and ﬁbroblast-like cells; second, MSCs must have multipotent differentiation potential; third, MSCs must have specific surface antigen expression. MSCs in dental tissues have been acquired in the periodontal ligament (Zhu et al. 2013; Wang et al. 2017), gingiva (Zhang et al. 2009), dental follicles (Tomic et al. 2011), dental pulp (Tomic et al. 2011; Liu et al. 2017), apical papilla (Sonoyama et al. 2008; Zhou et al. 2017), and human exfoliated deciduous teeth (Wang et al. 2010; Nowwarote et al. 2015; Rossato et al. 2017). The DPCs of this study are obtained from dental papillae and found to be ﬁbroblastic stromal cell, positive for CD29, CD44 and CD90, whereas negative for hematopoietic markers CD34 and CD4. The DPCs also have the capacity for self-renewal and multilineages differentiate to osteoblasts, adipocytes and odontoblasts. These results revealed the stromal origin of DPCs with MSC characteristics and the absence of hematopoietic contamination, which are consistent with previous ﬁndings (Huang et al. 2009; Chen et al. 2015).

Odontogenesis is similar to osteogenesis in many ways; so many bone markers are the markers of odontoblasts, such as ALP and extracellular matrix mineralization. Odontoblastic differentiation is characterized by upregulating ALP expression and followed by a deposition of collagenous matrix (Hao et al. 1997; Peng et al. 2010; Liu et al. 2013). DSPP and DMP1, extracellular matrix proteins, traditionally dentine-speciﬁc genes, play an important role in dentine mineralization, which are developmentally expressed by odontoblasts (Lin et al. 2013; Chen et al. 2016; Lee et al. 2016; Lian et al. 2016). Our data showed that odontogenic media induced ALP, DSPP and DMP1 expression, as well as matrix mineralization. These results suggested that DPCs can differentiate into odontoblasts via odonto/osteogenic induction in vitro.

Variations in the functions of mitochondria in different cell types adapt to different distinct demands and environmental cues (Li et al. 2017). There is a substantial body of evidence indicating that mitochondria are maintained at a relatively low activity level in primitive cells, whereas the switch of energy supply from glycolysis to aerobic metabolism and upregulation of mitochondrial respiratory function have been certain during the differentiation of MSCs, in response to a higher energy demand (Chen et al. 2008; Pietila et al. 2012; Sanchez-Arago et al. 2013; Solis et al. 2016). Our data showed that dentinogenic induction also led to an increase of ATP level and MMP, whereas a decrease of NAD+/NADH ratio in DPCs, which suggested that mitochondrial respiratory function is enhanced and energy supply switches to aerobic metabolism during dentinogenesis. Apart from respiratory function and energy supply, mitochondria are also the major source of ROS, which are oxygen-derived small molecules, including superoxide (O2-), H2O2 and hydroxyl radical (-OH) (Valko et al. 2007; Li et al. 2017). A numerous of observations have confirmed that higher levels of ROS promote differentiation in different types of cells, such as neural stem cell (Santos et al. 2013), vascular smooth muscle cell (Su et al. 2001) and in neuroblastoma cells (Oravecz et al. 2002). Interestingly, in our study ROS levels were decreased during odontoblastic differentiation of DPCs. Excessive amounts and unregulated levels of ROS can cause cellular damage (Valko et al. 2007; Chen et al. 2008) and there is an array of defense systems antioxidants to protect cells from the attack of ROS (Valko et al. 2007; Chen et al. 2008). Moreover, intracellular NADH level has been considered as an effective antioxidant (Ying 2008). Consistent with inhibition in ROS, we noted a decrease in the NAD+/NADH ratio, suggesting a relatively increase of NADH. Chen et al. (2008) also reported a dramatic decline of ROS during osteogenic differentiation of hMSCs and some antioxidant enzymes, such as catalase and MnSOD, were increased to reduce ROS, which are in line with our findings. Meanwhile, Lee et al. (2006) and Tahara et al. (2009) both have found that H2O2 reduced the activity of ALP, suggesting that excess ROS impairs osteogenic differentiation. It's worth mentioned that elevated ROS levels were involved in the adipogenic differentiation of MSCs (Zhang et al. 2013; Wang et al. 2015). So decreased ROS may be involved in odontoblast differentiation at the expense of adipocyte differentiation.

To assess the involvement of mitochondria in odontogenic differentiation, we differentiated DPCs in the presence of rotenone. Rotenone is an inhibitor of mitochondrial complex I (NADH dehydrogenase), which is part of OXPHOS system and couples electron transfer from NADH to ubiquinone with proton translocation across the energy-transducing inner membrane (Kwak et al. 2010; Vinothkumar et al. 2014). Rotenone speciﬁcially binds to the ubiquinone binding site of complex I and prevents electron transfer, thus affecting ATP generation (Kwak et al. 2010). Previous studies have showed that high concentration ranged from 1 to 100 μM of rotenone causes cell apoptosis and toxicity in nervous cells via mitochondrial impairment and excess ROS (Swarnkar et al. 2012; Cabezas et al. 2015). Our study found that treatment with 100 nM rotenone significantly decreased the cell viability of DPCs. Further experiments were performed by application of rotenone with lower concentrations to exclude the possibility that the inhibition of differentiation was due to cytotoxicity. The results found that rotenone could hamper the dentinogenic differentiation, demonstrating that increasing mitochondrial function was a prerequisite for dentinogenesis to occur. These data revealed that rotenone could help to maintain DPCs in undifferentiated state, result in an inhibition of differentiation. Kato et al. (2017) also found that rotenone could delay neuronal differentiation of stem cell from human exfoliated deciduous teeth, which are consistent with our ﬁndings.

In summary, this study demonstrated that DPCs showed properties similar to those of MSCs, which can differentiate into odontoblasts in vitro, and mitochondrial respiratory function is elevated during the process of dentinogenesis. Though there are sufficient researches studying the regulation of mitochondria functions in the differentiation of MSCs, we show for the first time that metabolic change and upregulation of mitochondrial funtion are crucial for dentinogenic differentiation, which suggested that mitochondria may be involved in dentine formation and tooth development. Our finding might give some new insights into the mechanism of regulating DPCs to achieve dentine regeneration. However, further studies are needed to identify specific molecular mechanisms by which mitochondrial respiration affects dentinogenesis.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

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**Figure legends**

**Fig. 1** The isolation, culture and identification of DPCs (**A**) Primary culture: cells were migrated from dental papilla tissue pieces after 2 days’ culture (**i**) and DPCs grew to near confluent state after 7 days (**ii**). (**B**) Identification of DPCs by immunocytochemical staining: DPCs were positive for vimentin (**i**) and negative for cytokeratin (**ii**). Negative control (**iii**) and positive control for cytokeratin (**iv**) were shown. (**C**) Identification of DPCs by osteogenic/adipogenic induction: Mineralized nodules were detected under osteogenic conditions using alizarin red staining (**i**). Lipid clusters were stained with oil red O (**ii**). (**D**) Identification of DPCs by colony-forming assay: gross appearance (**i**) and microscopic appearance (**ii**) of cell colonies. (**E**) Cell surface markers of DPCs: cells were positive for CD29, CD44 and CD90, while negative for CD34 and CD45. Scale bars =100 μm

**Fig. 2** Dental papilla cells could diﬀerentiate into dontoblasts (**A**) The DPCs were exposed in odontogenic medium for 7 days. mRNA levels of ALP, DSPP and DMP1 were determined by real-time PCR analysis. Data are expressed as the means ± SD; n = 4. (\**p*<0.05, \*\**p*<0.01) (**B**) ALP activity in supernatants and lysates of DPCs under odontogenic treatments was determined by colorimetric assay on day 7. Each value is presented as mean ± SD; n = 4. (\**p*<0.05) (**C, D**) Effect of odontogenic medium on protein levels of DSPP and DMP1 in DPCs. Representative immunoblots are shown (**C**), and β-actin was used as the internal control. The optical density was examined by Image J (**D**), and each value is presented as mean ± SD; n = 3. (\**p*< 0.05,\*\**p*< 0.01) (**E, F**) Cells were incubated with basal growth medium (upper), or odontogenic medium (lower) for 7, 14 and 21 days. (**E**) Gross appearance is displayed at the top-left corner of microscopic image. Scale bars = 100 μm. (**F**) Quantitative evaluation of alizarin red staining for DPCs. Each value is presented as mean ± SD; n = 4. (\*\**p*< 0.01, \*\*\**p*< 0.001 *vs*. basal growth medium for 14 or 21 days, respectively)

**Fig. 3** Changes in mitochondrial function upon odontogenic induction (**A**) Increase of the intracellular ATP content at day 7. (**B**) Odontogenic induction upregulated MMP, but had statistical difference compared with the control. (**C**) Decrease of NAD+/NADH ratio upon odontogenic medium. (**D**) NADP+/NADPH ratio remained constant during the induction. (E, F) ROS production that was determined by ﬂow cytometry is displayed (**E**). (**F**) Quantitative evaluation of ROS in DPCs. Each value is presented as as mean ± SD; n = 3. (\**p*<0.05,\*\**p*< 0.01\*\*\*, *p*< 0.001)

**Fig.4** Inhibitory effects of rotenone on odontogenic differentiation of DPCs (**A**) Cells were treated with various concentrations of rotenone or 0.1% DMSO alone for 48 h. Cells viability measured by CCK8 was significantly reduced in the presence of 100 nM rotenone. (**B, C**) The DPCs were exposed in basal growth or odontogenic medium in the absence or presence of rotenone for 7 days. (**B**) Rotenone decreased ALP, DSPP and DMP1 mRNA levels of differentiated DPCs in a concentration-dependent manner. (**C**) ALP activities of cell lysates were decreased in the presence of rotenone and odontogenic medium. Each value is presented as mean ± SD; n = 4. \**p*<0.05,\*\**p*< 0.01, \*\*\**p*< 0.001 *vs*. basal growth medium; #*p*<0.05, ##*p*< 0.01*vs*. osteo/odontogenic medium (OS).

**Table 1 Real-time PCR primers**

|  |  |  |  |
| --- | --- | --- | --- |
| Gene abbr. | Species | Forward primers, 5’-3’ | Reverse primers, 5’-3’ |
| ALP | Rat | GACAAGAAGCCCTTCACAGC | ACTGGGCCTGGTAGTTGTTG |
| DSPP | Rat | ACGCCACTAACGACGATTC | CCTCCTACGGCTATCGACTC |
| DMP 1 | Rat | ACCAAAATACTGAATCTGAAAGCTC | TGCTGTCCGTGTGGTCACTA |
| β-actin | Rat | CAGCACTGTGTTGGCATA | CGGTCAGGTCATCACTATC |