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miR-143 suppresses the osteogenic differentiation of dental pulp stem cells by inactivation of NF-κB signaling pathway via targeting TNF-α

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ABSTRACT

Background: Dental pulp stem cells (DPSCs) are multipotent and play an important role in repairing damaged and/or defective dentinogenesis/osteogenesis. Recent studies have documented the implication of miR-143 in osteogenic differentiation of DPSCs. Nevertheless, the detailed mechanisms of miR-143 involved in the osteogenic differentiation of DPSCs remain to be further elaborated.

Methods: Isolated DPSCs were incubated with osteogenic differentiation medium to induce osteogenic differentiation. qRT-PCR and western blot were performed to determine the expressions of miR-143 and tumor necrosis factor α (TNF-α). Luciferase reporter assay was used to confirm whether TNF-α was a target of miR-143. Osteogenic differentiation of DPSCs was evaluated by alkaline phosphatase (ALP) activity assay, ALP staining, and western blot analyses of osteogenic-markers including bone morphogenetic protein 2 (BMP2), ALP, runt-related transcription factor 2 (RUNX2) and collagen type I (COLI).

Results: miR-143 was downregulated and TNF-α was upregulated during osteogenic differentiation of DPSCs. miR-143 posttranscriptionally regulated TNF-α expression in DPSCs by binding to its 3′UTR. miR-143 overexpression suppressed osteogenic differentiation of DPSCs, as demonstrated by the decrease of ALP activity, ALP positive cell ratio, as well as BMP2, ALP, RUNX2, and COLI expressions. Moreover, miR-143 reversed TNF-α-induced osteogenic differentiation of DPSCs. Finally, the osteogenic differentiation of DPSCs induced by miR-143 inhibitor was attenuated following inactivation of nuclear factor kappa B (NF-κB) signaling pathway.

Conclusion: miR-143 suppressed the osteogenic differentiation of DPSCs by blockade of NF-κB signaling pathway via targeting TNF-α.

1. Introduction

Dental pulp stem cells (DPSCs) have been identified as a type of mesenchymal stem cells (MSCs) that were originally isolated from adult human dental pulp tissues (Estrela, Alencar, Kitten, Vencio, & Gava, 2011). DPSCs have been demonstrated to exhibit their potential of multilineage differentiation and self-renewal, implying that they can differentiate into various lineages, such as osteoclasts, chondrocytes, adipocytes, cardiomyocytes, liver cells, neuronal cells and so on (Grottkau, Purudappa, & Lin, 2010). Accumulating studies have demonstrated that DPSCs, with great potential to generate dentin and pulp tissues under appropriate conditions, play an important role in repairing damaged and/or defective dentinogenesis/osteogenesis (Goto & Fujimoto, 2016; Gronthos, Mankani, Brahim, Robey, & Shi, 2000). Accordingly, DPSCs are increasingly being recognized as valuable sources of stem cells for tissues engineering and regenerative medicine for the treatment of dental and other diseases (Alraies, Alaidaroos, Waddington, Moseley, & Sloan, 2017).

Considerable research has revealed that multiple pro-inflammatory cytokines can influence the biological behavior of DPSC and play vital physiological roles in the regeneration of pulp and dentin (He et al., 2017). It was previously documented that tumor necrosis factor α (TNF-α), a marker of early inflammation, facilitated the proliferation, migration, and multipotent differentiation of DPSCs (Ueda et al., 2014). Also, TNF-α has been shown to affect osteoclastogenesis and bone formation (Lisignoli et al., 2004). A previous study confirmed that TNF-α promoted osteogenic differentiation of DPSCs by activating the nuclear factor kappa B (NF-κB) pathway (Feng et al., 2013). However, the molecular mechanisms of TNF-α in osteogenic differentiation of DPSCs remain largely unknown.

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Nomenclature

DPSCs	Dental pulp stem cells
TNF-α	Tumor necrosis factor α
BMP2	Bone morphogenetic protein 2
ALP	Alkaline phosphatase
RUNX2	Runt-related transcription factor 2
COLI	Collagen type I
NF-κB	Nuclear factor kappa B
MSCs	Mesenchymal stem cells
miRNAs	microRNAs
qRT-PCR	Quantitative real-time PCR
PVDF	Polyvinylidene difluoride
ELISA	Enzyme-linked immunosorbent assay
TIONFH	Trauma-induced osteonecrosis of the femoral head

microRNAs (miRNAs), a class small noncoding RNAs with 17–25 nucleotide, regulate gene expression at a posttranscriptional level via base pairing to complementary sequences in the 3′-untranslated region (UTR) of target mRNAs. miRNAs display critical regulatory effects in various stem cell-related physiological and pathologic processes, such as cell proliferation, apoptosis, self-renewal, differentiation and carcinogenesis (Lujambio & Lowe, 2012). Recently, miRNAs have been identified as important regulators of stem cell differentiation and function (Hatfield & Ruohola-Baker, 2008). Moreover, recent studies have reported the involvement of miRNAs in osteoblast differentiation of DPSCs (Gay et al., 2014; Lian et al., 2012). For example, miR-26a and miR-125b have been discovered to negatively regulate osteoblastic differentiation (Luzi et al., 2008; Mizuno et al., 2008), while miR-210 and miR-29b have been found to positively regulate osteoblastic differentiation (Li et al., 2009; Mizuno et al., 2009). miR-143, a miRNA located in chromosome 5q33, was reported to suppress osteogenic differentiation by targeting osterix (Li, Zhang, Yuan, & Ma, 2014). However, the exact molecular mechanism of miR-143 involved in osteogenic differentiation of DPSCs remains to be further explored.

According to our bioinformatics analyses, 3′UTR sequence of TNF-α contained the complementary binding sites in miR-143. Thus, the present study aimed to explore the relationship between miR-143 and TNF-α in DPSCs and the molecular mechanism by which miR-143 regulated the osteogenic differentiation of DPSCs.

2. Materials and methods

2.1. Isolation and culture of DPSCs

All experiment protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The informed consents were obtained from all subjects. Healthy human impacted third molars were collected from patients aged from 17 to 23 years (n = 9) with no carious lesions and oral infection who were undergoing treatment at the Dental Department of the First Affiliated Hospital of Zhengzhou University. Fresh isolated tooth was cleaned and the pulp chamber was exposed using sterilized dental fissure burs. The pulp was immersed into the following digestive solutions: 3 mg/mL collagenase type I (Invitrogen, Carlsbad, CA, USA) and 4 mg/mL dispase (Roche Diagnostics, Mannheim, Germany) at 37 °C for 1 h. Single-cell suspensions were obtained by passing the digested tissues through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ). Cell suspensions of dental pulp were seeded into 25-cm^2 tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), and 1% penicillin/streptomycin at 37 °C in 5% CO_2. The culture medium was replaced every 2 days. Cells at the third passage were used for subsequent experiments.

2.2. Osteogenic differentiation of DPSCs

To induce osteogenic differentiation, DPSCs at a density of 2×10^4 cells/cm^2 were incubated with osteogenic differentiation medium, which composed of DMEM (Sigma), 0.1 μM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), 50 μg/mL L-ascorbic acid (Sigma), and 10% FBS. DPSCs were differentiated for 1, 3, and 7 day(s). Osteogenic differentiation medium was replaced every 3 days. In addition, DPSCs were pretreated with 100 μM NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma) for 2 h in order to inactivate the NF-κB pathway.

2.3. Plasmids and oligonucleotides transfection

To enhance TNF-α expression, the full-length sequences of TNF-α was amplified by PCR and cloned into pcDNA3.1 plasmids (Invitrogen), named as pcDNA-TNF-α (TNF-α). The pcDNA empty plasmids were used as the control. miR-143 mimic, anti-miR-143, miRNA negative control (miR-con), or miRNA inhibitor control (anti-miR-con) were purchased from GenePharma (Shanghai, China). Transient transfection with plasmids or oligonucleotides was performed using Lipofectamine 2000 reagent (Invitrogen). Cells were collected at 48 posttransfection for further analysis.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from DPSCs cultured in osteogenic differentiation medium using TRIzol (Takara, Bio, Otsu, Japan) at day 0, 1, 3, and 7 according to the manufacturer's instructions. The concentration of RNA samples was quantified by NanoDrop mass spectrometry (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For the detection of mRNA expression, the first-strand cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and RT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) on an ABI Prism 7500 qPCR system (Applied Biosystems). For the detection of miRNA expression, cDNA was synthesized using the Primescript RT reagent (Takara) and RT-PCR was conducted using TaqMan miRNA (Applied Biosystems) on an ABI Prism 7500 qPCR system (Applied Biosystems). GAPDH and U6 were used as the normalization for mRNA and miRNA, respectively. The relative gene expression level was calculated using the $2^{-D\Delta Ct}$ method.

2.5. Western blot

Total protein from DPSCs was extracted using RIPA Lysis buffer (Beyotime, Nantong, China) and quantified using a bicinchoninic acid Protein Assay Kit (Beyotime). Denatured proteins (30 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 for 2 h at room temperature, membranes were probed overnight at 4 °C with primary antibodies against TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), bone morphogenetic protein 2 (BMP2) (Santa Cruz Biotechnology), alkaline phosphatase (ALP) (Santa Cruz Biotechnology), runt-related transcription factor 2 (RUNX2) (Santa Cruz Biotechnology), collagen type I (COLI) (Santa Cruz Biotechnology), IκBα (Santa Cruz Biotechnology), phosphorylated-IκBα (*p*-IκBα) (Santa Cruz Biotechnology), p65 (Santa Cruz Biotechnology), phosphorylated-p65 (p-p65) (Santa Cruz