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Original Article

MiR-633 promotes cell proliferation and differentiation by targeting MEPE in human adult dental pulp stem cells

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Abstract: Increasing evidence have showed that abnormal microRNAs (miRNAs) expression is involved in the influence on myogenic differentiation of human adult dental pulp cells (DPSCs). Recently, up-regulation of miR-633 in DPSCs has been reported, however, the function and mechanism of miR-633 in DPSCs are not quite clear. In this study, we reveal that the increased expression levels of miR-633 in human DPSCs, meanwhile, the expression levels of miR-633 in stem-cell-enriched side population (SP) cells from human DPSCs was lower than the differentiated main population (MP) cells. *In vitro,* miR-633 over expression significantly increased cell proliferation and differentiation in DPSCs. Furthermore, miR-633 decreased the expression levels of Matrix extracellular phosphoglycoprotein (MEPE) by directly interact with 3'-UTR-MEPE. The loss of MEPE could promote DPSCs proliferation and differentiation. Moreover, the expression of miR-633 and MEPE was a strong negative correlation in DPSCs. In conclusion, miR-633 plays an important role in the promotion of DPSCs proliferation and differentiation by directly targeting MEPE, and probably is a regulating molecular biomarker for the differentiation of DPSCs.

Keywords: microRNA-633, Matrix extracellular phosphoglycoprotein (MEPE), human adult dental pulpcells (DPSCs), proliferation, differentiation

Introduction

Stem cells are undifferentiated cells, which have self-renewal, high proliferation and multilineage differentiation capabilities [1, 2]. The advantages of dental pulp stem cells (DPSCs) possess the characteristics of stem cells and can be effectively be obtained from iatro-waste products (such as impacted wisdom tooth and the extracted teeth for orthodontic reason), cultured and refrigerated [3, 4]. Meanwhile, DPSCs are the important sources of stem cells for tissue engineering and regenerative medicine research [5]. Research of these stem cells will create broader space for tissue engineering and regenerative medicine and will have important values in translational research [6]. However, the underlying mechanisms about the proliferation and differentiation acknowledged as important odontogenic differentiation attributes for DPSCs are largely unknown. The deeper understanding of the biology of stem cells of DPSCs is important to get great promise in the field of regenerative medicine about tooth.

MicroRNAs (miRNAs) as a class of 22 nt noncoding RNAs participate in a large amount of protein-coding genes expression in animals and plants [7, 8]. It has been confirmed that miRNAs have an important role in many stem cells-related processes involved in cell growth, proliferation, differentiation and apotosis [9]. Recently, growing evidences showed that miRNAs are tightly associated with DPSCs proliferation and differentiation [10, 11]. An increasing miRNAs were proved to be involved in the proliferation and differentiation of DPSCs, such as miR-143, miR-720, miR-218, miR-7-5p, miR-516a-3p, miR-433, miR-424, miR-146a [10-16].

In previous studies, they found miR-633 was decreased expression in glioblastoma cells, and it inhibits the proliferation, migration and invasion of glioblastoma cells via targeting TGF-β1 [17]. Although growing studies focus on the role of miR-633 in cell biology [18, 19], the

exact mechanisms and function of miR-633 up-regulation in DPSCs are still largely not clear.

In this study, we validated that the expression of miR-633 in DPSCs were significantly higher than PDLCs and the expression levels ofmiR-633 in stem-cell-enriched side population (SP) cells from human DPSCs was lower than the differentiated main population (MP) cells. *In vitro*, miR-633 over expression significantly increased cell proliferation and differentiation in DPSCs. Furthermore, we found that miR-633 directly down-regulated the expression of MEPE by binding to MEPE-3'-UTR and MEPE was negatively correlated with the expression of miR-633 in DPSCs. Our findings indicate that miR-633 might be a potential biomarker to promote DPSCs proliferation and differentiation and be targeted for novel therapeutic.

Materials and methods

Cell lines, and transfection

DPSCs and PDLCs were isolated from third molars or premolars extracted from at least 4 adults under the approved guidelines and protocol (Third Military Medical University Xinqiao Hospital Ethics Committee) with written informed consent obtained from all subjects. The isolation and cultivation of human DPCs and PDLCs were performed according to a previously reported method [20]. Cells were cultured in a-Modified Essential Medium (Boster Biology Co. Wuhan, China) supplemented with 15% fetal bovine serum, 100 mM Lascorbicacid 2-phosphate (Boster Biology Co. Wuhan, China), 2 mM L-glutamine (Life Technologies TM), 1% penicillin and streptomycin (Sigma, USA) under the condition of 5% CO_2 and 95% humidity if not specified in the text. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA) based on the manufacturer's instruction. The scrambled oligonucleotide was chose as negative control.

RNA extraction and real-time PCR for MEPE mRNA and miR-633 expression

Total RNA was extracted and isolated from cells and tissues using Trizol reagent (Roche, Germany). qRT-PCR assays for MEPE and miR-633 were performed were performed using SYBR Green Reagents (TaKaRa, Tokyo, Japan). Primers used were as following: MEPE forward, 5'-CCGCTCGAGCTGAAGACCTCGTCACCT-3' and reverse, 5'-CGACGCGTCATAGAAGGCATTTGTGA-3'; GAPDH forward, 5'-TCAACGACCACTTTGTCA-AGCTCA-3', and reverse, 5'-GCTGGTGGTCCAG-GGGTCTTACT-3'. Primers for U6 and miR-633 were purchased from GeneCopoeia (Ribobio). MEPE level was normalized with GAPDH, and miR-633 level was normalized with U6.

Cell proliferation assay

MP and SP cells, transfection of miR-NC, miR-633, negative control or cotransfection of miR-633 and MEPE vector, were seeded in triplicate to each well of 96-well plates at a density of 10^3 per well, respectively. Microplate reader (Bio-Rad, USA) was performed to examine the cell viability by absorbance at 570 nm.

Cell differentiation assays

In differentiation assays, 1.0×10^5 cells/ml of MP and SP cells were transfected with miR-NC, or miR-633 for 24 h, respectively. In addition, 1.0×10^5 cells/ml of MP and SP cells were transfected with miR-NC or miR-633, or cotransfection with miR-633 and MEPE vector. After cultured 0, 3, 7 and 14 days, the relative mRNA expression of collagen I, OPN and OCN were detected in MP and SP cells, respectively.

Vector constructand luciferase assay

In order to prove that miR-633 regulates the expression of the human gene MEPE through directly targeting its 3'-UTR, flanking *Not*I *and Xba*I restriction enzyme digestion sites were used to synthesize wild-type or mutant 3'-UTR of MEPE. Luciferase report vectors were built by pMIR-REPORT™ Luciferace vectors. Flanking *EcoR* I and *Hind* III restriction enzyme digestion sites were adopted toamplify the product that cDNA clone for MEPE transcribed, and followed DNAs were inserted into pcDNA3.1 vector. Renilla luciferase control vector, mutant pMIR-miR-633-3'UTR or wide-type pMIR-miR-633-3'UTR using Lipofectamine 2000 were transfected into HEK293T cells, and then miR-NC or miR-633 were transfected into the cells. Dual-Luciferase reporter assay system (Promega, USA) and an LB 960 Centro XS3 luminometer (Berthold, USA) was used to examine luciferase activity after transfection 48 h. Renilla luciferase activity was regarded as normalised firefly luciferase activity.

Lentivirus packaging and transduction

MiR-control and miR-633 precursor sequenceswere amplified and into the lentiviral vector

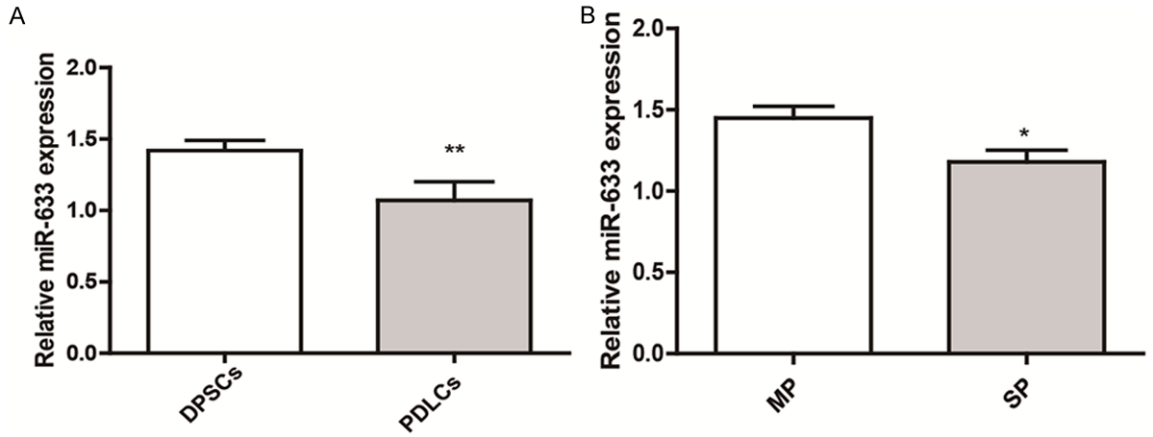


Figure 1. The increased expression of miR-633 in DPSCs. A. The expression levels of miR-633 in DPSCs and PDLCs was detected. B. The expression levels of miR-633 in MP and SP cells was detected. *P<0.05, **P<0.01 compared with the control. All assays were performed in duplicates.

pLVX-shRNA1 (Invitrogen, USA). HEK293T cells received virus packaging. pLV-miR-control or pLV-miR-633 and Lenti-X HTX Packaging Kitusing the Xfect transfection reagent were co-transfected. pLV-miR-control or pLV-miR-633 were transduced into MP cells. About 24 h after injection, 2 ug/ml of puromycin was added to the media for two weeks to select the cells infected with the lentivirus. The control vector cell line and cell linestably expressed miRNA-633 were named LV-miR control-MP and LV-miR633-MP, respectively.

Western blot assay

Cells were first lysed in prepared buffer containing 10 mM Tris, 0.1% SDS and 5 mM EDTA. The pH was regulated to 7.2. 20 μg of samples were separated by SDS-PAGE. PVDF membranes were chosen for transfer. After blocking in 5% nonfat dry milk in PBS, the membranes were incubated with antibodies against MEPE (1:1000) and GAPDH (1:1000) overnight at 4°C. Then the membranes were washed by TBST and incubated with secondary antibody (1:2000) for by 1 h at room temperature. The immune blot signals were visualized using the Easy See Weatern Blot Kit (Transgen, China). The protein bands were detected by densitometric scanning (Tanon-1600 gel image system, China).

Statistical analysis

All data were analyzed by SPSS (ver. 21.0) software and the results were showed by mean ± SD unless otherwise noted. Pearson's correlation was used to analyse the relationship of miR-633 and MEPE mRNA. Two-way analysis of variance (ANOVA) and student's t-test were used to assess statistical significance. A $P \leq 0.05$ was regarded as significant.

Results

The increased expression of miR-633 in DPSCs and MP cells

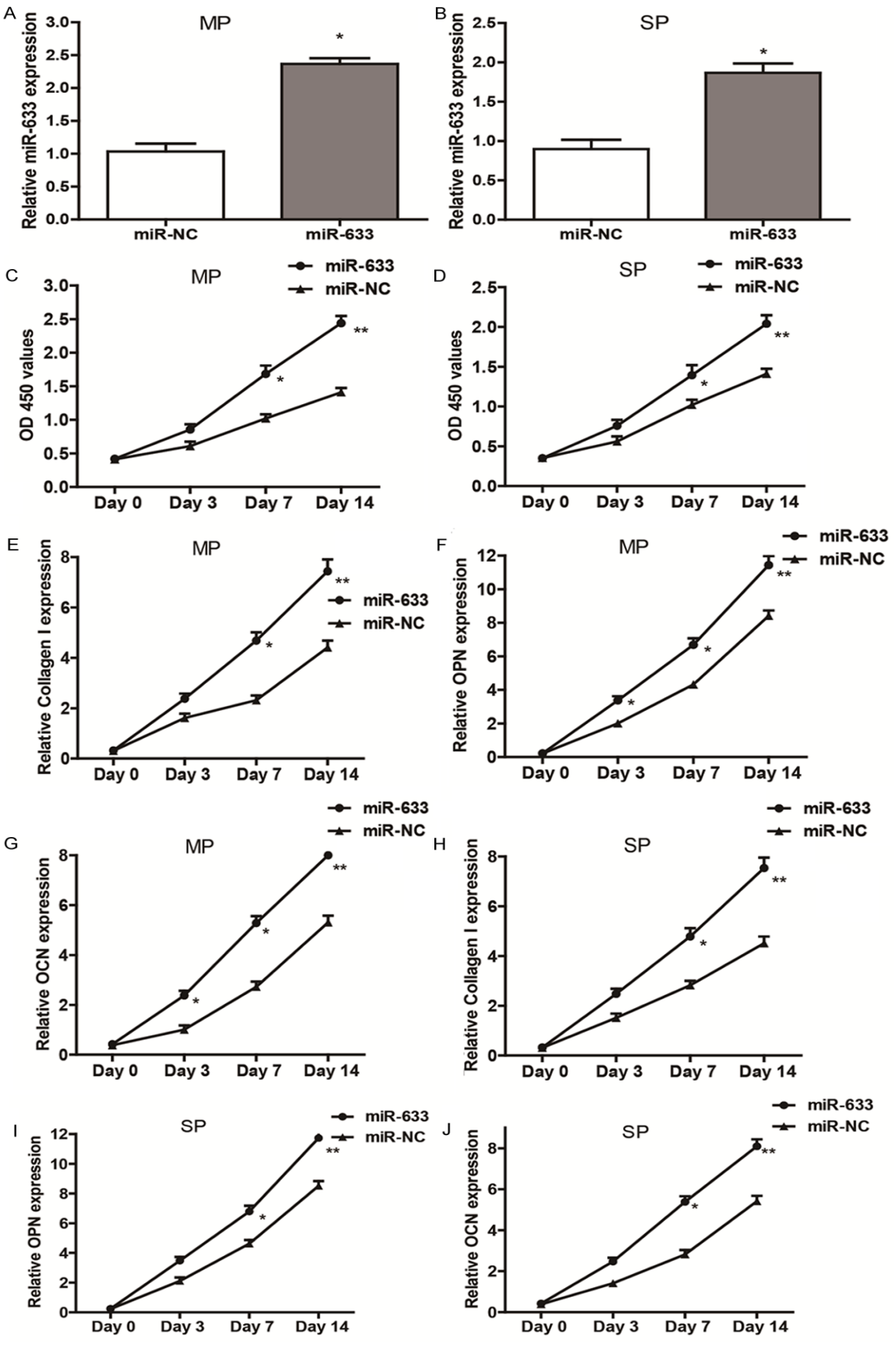
The expression levels of miR-633 in DPSCs was significantly increased compared with PDLCs (**Figure 1A**). Furthermore, miR-633 expression in SP cells was significantly lower than the MP cells (**Figure 1B**).

MiR-633 promoted DPSCs proliferation and differentiation

In order to examine the effect of miR-633 on DPSCs proliferation and differentiation MP and SP cells were transfected with miR-NC or miR-633. We found that miR-633 expression in MP and SP cells transfection of miR-633 was significantly increased by qRT-PCR (**Figure 2A**, **2B**) and miR-633 significantly promoted the proliferation (**Figure 2C**, **2D**) and differentiation (**Figure 2E-J**) of MP and SP cells. Therefore, miR-633 ectopic expression promoted proliferation and differentiation of DPSCs.

MEPE was a target of miR-633 in DPSCs

In order to detect whether MEPE is a directly target of miR-633, the mutant 3'-UTR and wild-


A
MP
Relative miR-633 expression
3.0
2.5
2.0
1.5
1.0
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miR-NC
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SP
Relative miR-633 expression
2.5
2.0
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Figure 2. MiR-633 promoted DPSCs proliferation and differentiation. (A, B) The expression levels of miR-633 in MP and SP cells transfected with miR-NC or miR-633 was detected. After transfection, MP and SP cells were used to perform the proliferation (C, D), differentiation (E-J) assays. (E-G) In vitro differentiation assay of MP cells with miR-633, the relative expression levels of collagen I, OPN and OCN were detected. (H-J) In vitro differentiation assay of SP cells with miR-633, the relative expression levelsof collagen I, OPN and OCN were detected. *P<0.05 and **P<0.01 compared with the control. All assays were performed in duplicates.

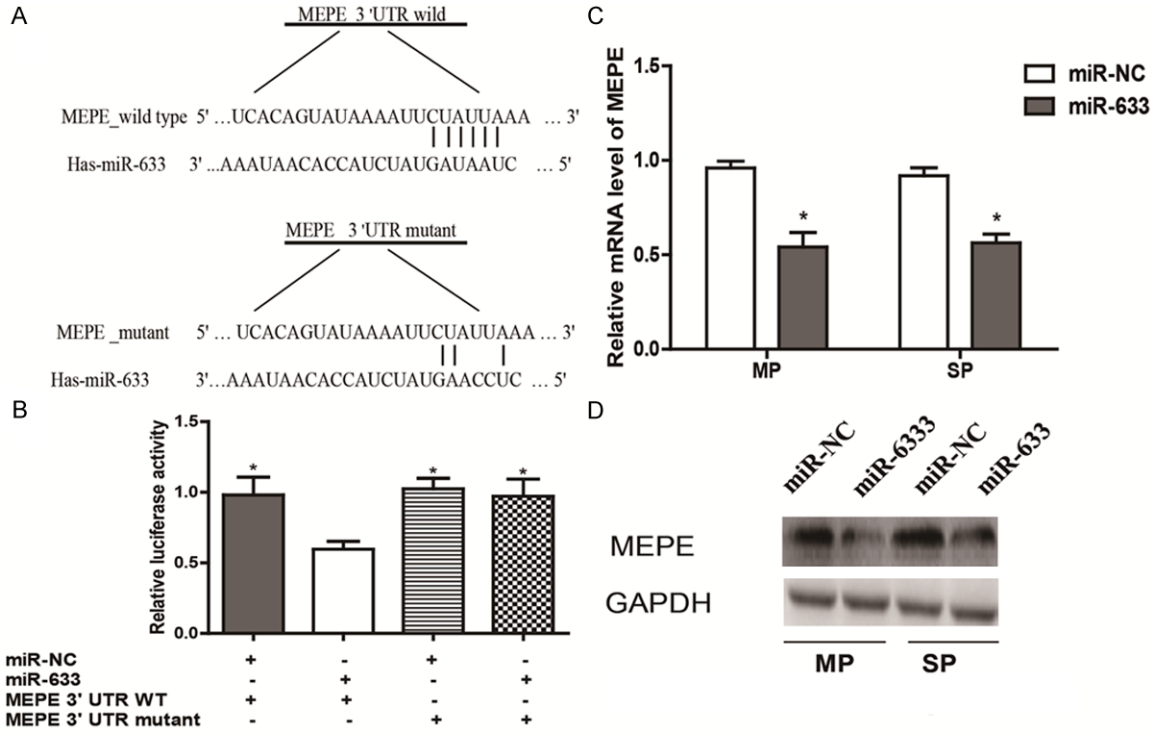

Figure 3. MEPE was atarget of miR-633 in DPSCs. (A) The putative binding sequences of miR-633 in the 3'-UTR of MEPE. (B) The expressions of MEPE mRNA (C) and MEPE protein (D) in MP and SP cells after transfection of miR-NC or miR-633. *P<0.05 compared with the control. All assays were performed in duplicates.

type 3'-UTR of MEPE vectors were constructed. The putative miR-633-binding sites 3'-UTR of MEPE was fused downstream of a firefly luciferase gene (**Figure 3A**). The wild-type and mutant were cotransfected into HEK293T cells with miR-NC or miR-633. The relative luciferase activity was significantly decreased in miR-633 transfected with wild-type 3'-UTR of MEPE and not significantly changed in miR-633 transfected mutant 3'-UTR of MEPE (**Figure 3B**). Moreover, the mRNA and protein expression of MEPE were significantly decreased by miR-633 (**Figure 3C**, **3D**). Together, miR-633 down-regulated the expression of MEPE by directly targeting the 3'-UTR of MEPE.

MiR-633 promoted DPSCs proliferation and differentiation by directly targeting MEPE

In order to explore the effect of miR-633 on DPSCs proliferation and differentiation by directly targeting MEPE, the MP and SP cells were transfected with miR-NC, miR-633, or cotransfected with miR-633 and MEPE vector. Function investigation showed that the proliferation and differentiation of DPSCs were significantly promoted after transfected with miR-633, while the restoration of MEPE not significantly promoted the proliferation (**Figure 4A**, **4B**) and differentiation (**Figure 4C-H**) enhancement. Together, miR-633-induced the loss of MEPE promoted the cell proliferation and differentiation by targeting MEPE.

Negative correlation between the expression of miR-633 and MEPE in DPSCs

In order to examinethe relationship between the expression of miR-633 and MEPE in DPSCs, The expression of MEPE in DPSCs and PDLCs was detected. We found that the average level of MEPE mRNA in DPSCs was significantly

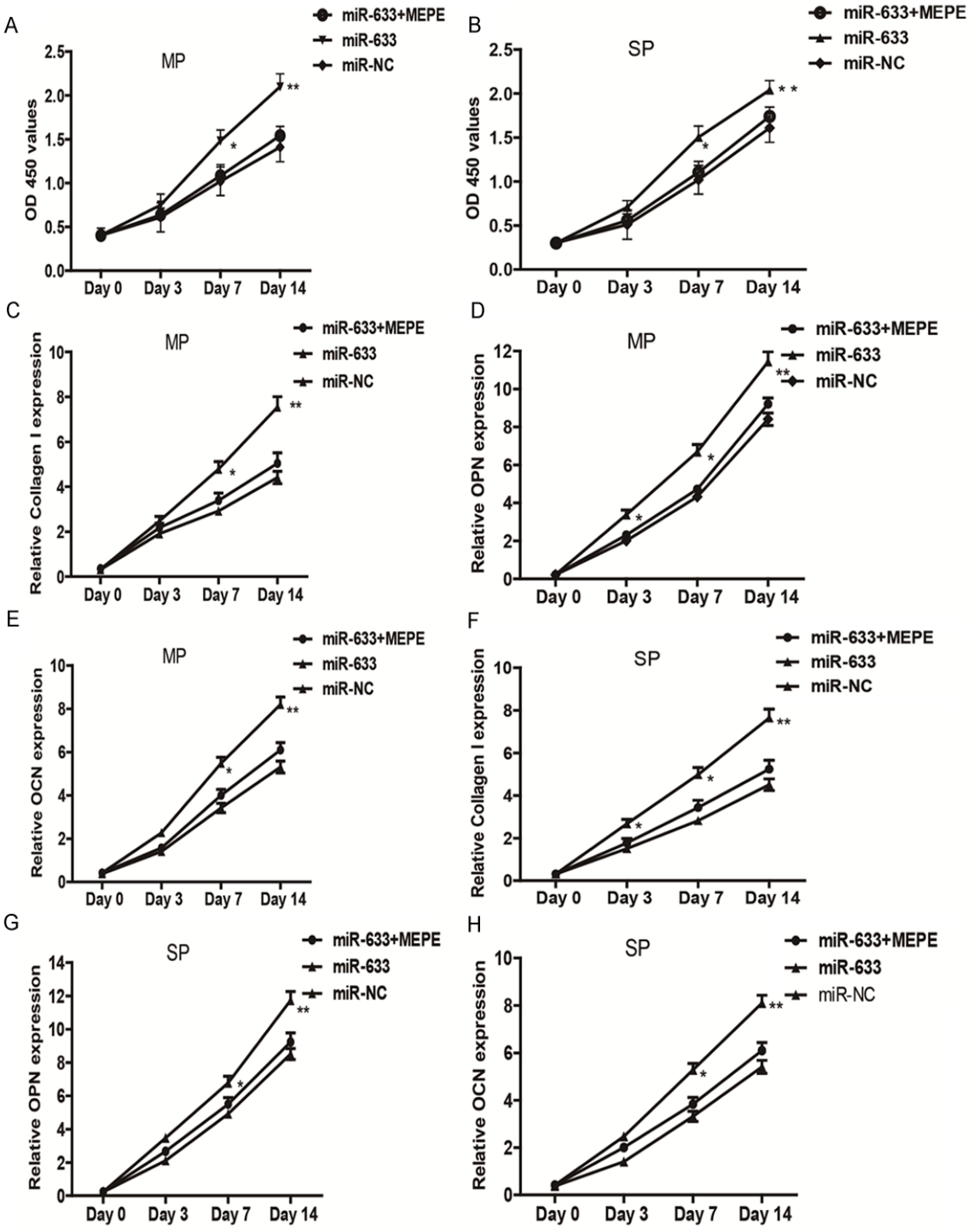

Figure 4. MiR-633 promoted DPSCs proliferation and differentiation by directly targeting MEPE. A, B. MP and SP cells were cotransfected with miR-633 and pcDNA3.1-MEPE or the vector. CCK8 assay was used to measure proliferation. C-E. In vitro differentiation assay of MP cells with miR-633 and pcDNA3.1-MEPE or the vector, the relative expression levels of collagen I, OPN and OCN were detected. F-H. In vitro differentiation assay of SP cells with miR-633 and pcDNA3.1-MEPE or the vector, the relative expression levelsof collagen I, OPN and OCN were detected. *P<0.05 and **P<0.01 compared with miR-NC group. Data were drawn from three independent experiments.

decreased compared with PDLCs (**Figure 5A**). Furthermore, the mRNA expression of MEPE was negatively correlated with the mRNA expression of miR-633 (**Figure 5B**).

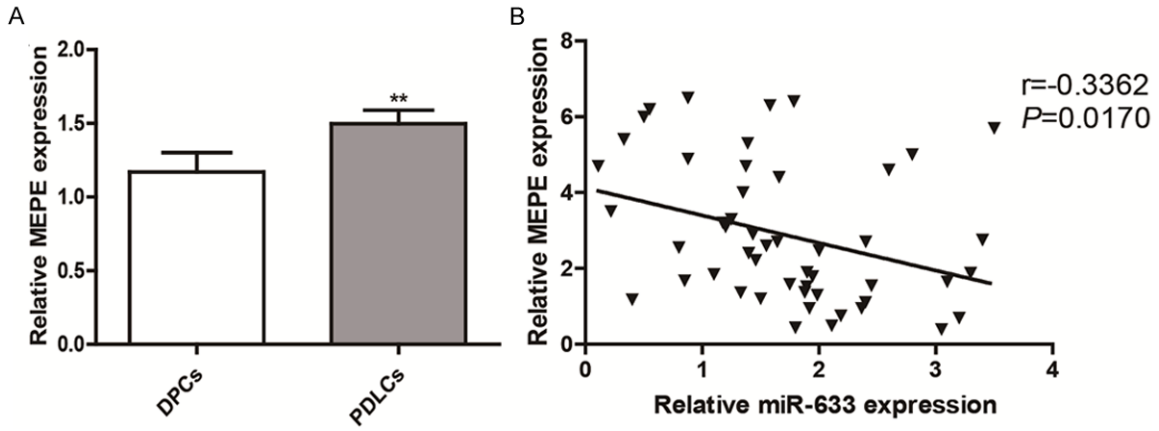

Figure 5. Negative correlation between the expression of miR-633 and MEPE in DPSCs. A. Expression of MEPE in DPSCs and PDLCs was detected. B. Correlation analysis between miR-633 and MEPE mRNA level in DPSCs (r=-0.3362; P=0.0170).

Discussion

In this study, we showed that miR-633 was significantly increased in DPSCs, compared with PDLCs. The expression levels of miR-633 in SP cells from human DPSCs was lower than the differentiated MP cells. miR-633 over expression significantly increased cell proliferation and differentiation in DPSCs. MiR-633 was associated with enhancing of MEPE by a luciferase reporter assay. Furthermore, our observation fora strong negative correlation between the expression of miR-633 and MEPE in DPSCs fills this important void in literature for the missing experimental evidence for the function of miR-633 and MEPE in DPSCs. In conclusion, our study showed miR-633 might be a potential biomarker to promote DPSCs proliferation and differentiation and be targeted for novel therapeutic in the future.

More and more evidences reveal that the miRNAs have an important role in the regulation of genes during DPSCs proliferation and differentiation. Many miRNAs3 has been reported to associate with DPSCs, including miR-143, miR-720, miR-218, miR-7-5p, miR-516a-3p, miR-433, miR-424, miR-146a [10-16]. Emilio et al reported miRNA-720 controls stem cell phenotype, proliferation and differentiation of human dental pulp cells [11]. Gay et al showed miRNA-218 controls DPSCs osteogenic differentiation by targeting RUNX2 [10]. However, the exact mechanisms and function of miR-633 up-regulation in DPSCs are still largely not clear.

In this study, we showed that miR-633 was increased in DPSCs compared with PDLCs. Moreover, we suggested that miR-633 ectopic expression promoted the cell proliferation and differentiation of DPSCs. It showed miR-633 can control DPSCs proliferation and differentiation to transform human odontoblast cells, at least to some extent.

MEPE a non-collagenous extracellular matrix phosphorylated glycoprotein, are both important proteins involved in osteogenic signaling pathway [21, 22]. In recent years, it has been found that BMP can regulate the expression levels of MEPE, and play an important role in phosphate metabolism, osteoblast proliferation and differentiation [23-25]. The function of MEPE is multiple and complicated, phosphate homeostasis regulation, skeletal mineralization, the development and formation of bone and dental stem cells and the effect of cellular sensitivity to radiation are also related to biological function of MEPE [26-28]. MEPE has been reported frequently amplified or overexpressed in bone formation and one tumour [29-31]. Consistent with these evidences, our data showed the average level of MEPE mRNA in DPSCs was significantly decreased compared with PDLCs, furthermore, MEPE were impoverished in the DPSCs that negatively correlated to miR-633 levels. MEPE ectopic expression restores the effects of miR-633 on the cell proliferation and differentiation in DPSCs. It demonstrated that MEPE contributes to cell proliferation and differentiation in DPSCs and is a direct target of miR-633.

In conclusion, we confirmed that miR-633 is a potent promoting differentiation in the DPSCs through targeting MEPE. Meanwhile, our study demonstrated that the miR-633/MEPE axis regulates the cell proliferation and differentiation of DPSCs. These findings provide a better understanding pathogenesis and differentiation of DPSCs and may be an important potential biomarker to promote DPSCs proliferation and differentiation and be targeted for novel therapeutic.

Disclosure of conflict of interest

None.

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