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Original Article

Silencing of microRNA-340 promotes the odontogenic differentiation of induced pluripotent stem cells through upregulating BMP4

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Abstract: Purpose: This study aimed to evaluate the specific roles of microRNA-340 (miR-340) on the odontogenic differentiation of induced pluripotent stem cells (iPSCs), and reveal the potential regulatory mechanisms relating with bone morphogenetic protein 4 (BMP4). Methods: Rat iPSCs were induced with 50 ng/mL BMP4, and transfected with siRNA-BMP4 (si-BMP4), miR-340 mimic, and miR-340 inhibitor, respectively. The odontogenic differentiation of iPSCs was detected by Alizarin red S staining. The expression of BMP4, ameloblastin (AMBN) and dentin matrix protein 1 (DMP-1) in iPSCs was detected by quantitative real-time PCR (qRT-PCR), Western blot, and immunofluorescence. The interaction between BMP4 and miR-340 was identified by dual luciferase reporter gene (DLR) assay. Results: The intervention of BMP4, and si-BMP4 significantly upregulated, and downregulated AMBN and DMP-1 in iPSCs, repectively. The tranfection of miR-340 inhibitor significantly increased the percentage of positive stained cells, as well as the expression of BMP4, AMBN and DMP-1 in iPSCs ($P < 0.05$). The tranfection of miR-340 mimic significantly decreased the percentage of positive stained cells in BMP4 group ($P < 0.05$). In addition, BMP4 was identified as a target of miR-340. MiR-340 inhibitor significantly reversed the inhibitory effect of si-BMP4 on the expression of AMBN and DMP-1 in iPSCs. Conclusions: Silencing of miR-340 promoted the odontogenic differentiation of iPSCs through upregulating BMP4.

Keywords: microRNA-340, bone morphogenetic protein 4, induced pluripotent stem cells, odontogenic differentiation

Introduction

Tooth damage is a common oral disease that seriously influences the life quality of patients of all ages [1]. Diverse pathologies contribute to the occurrence of tooth damage, such as caries, pulpitis, mechanical damage, natural shedding, and genetic alterations [2]. Synthetic materials, such as amalgam, resin and guttapercha have been widely applied in tooth restoration [3]. However, these materials could not restore the biological function of the original tooth tissues [4]. With the development of stem cell-based tissue engineering, dental pulp stem cells (DPSCs) have been demonstrated to be an ideal cell source for tooth regeneration [5]. Since the clinical application of DPSCs is greatly limited by insufficient source, induced pluripotent stem cells (iPSCs) are considered a promising alternative in tooth regeneration [6].

IPSCs are a kind of pluripotent stem cell that are generated from adult cells by reprogramming [7]. Similar with embryonic stem cells, iPSCs also exhibit the properties of self-renal, and multiple differentiation potentials [8]. The differentiation potential of iPSCs towards tooth-like structures has been identified by diverse research. For examples, iPSCs induce the formation of dental pulp-like structure, and osteo-

pontin is observed in the apical part of tooth-like structure [9]. IPSCs-derived epithelial sheets differentiate into enamel-secreting ameloblasts in tooth-like structures, exhibiting consistent elastic modulus and hardness like teeth [10]. The odontogenic differentiation of iPSCs depends on the intervention of recombinant growth factors, and differentiation-associated factors [11, 12]. Bone morphogenetic protein 4 (BMP4) is an important growth factor that is involved in bone formation [13]. Previous studies have proved that BMP4 promotes the differentiation of iPSCs into ameloblast- and odontoblast-like cells, and upregulates related genes, such as dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), ameloblastin (AMBN), and amelogenin (AMGN) [12, 14, 15]. However, the specific regulatory mechanisms of BMP4 on odontogenic differentiation of iPSCs are still not fully revealed.

MicroRNAs (miRs) are a class of endogenous noncoding RNAs that play important roles in the regulation of cell differentiation [16]. A pervious study has shown that 12 miRs are up-regulated and 10 miRs are down-regulated in human dental pulp cells (hDPCs) during odontogenic differentiation [17]. These miRs play diverse roles in the regulation of odontogenic differentiation. It has been reported that miR-675 promotes the odontogenic differentiation of hDPCs through inhibiting DNA methyltransferase 3 beta-mediated methylation of distal-less homeobox [18]. MiR-663 promotes the differentiation of odontoblast-like MDPC-23 cells into odontoblasts through adenomatous polyposis coli-mediated activation of Wnt/β-catenin signaling [19]. miR-720 promotes the odontogenic differentiation of hDPCs, and upregulates alkaline phosphatase and osteopontin [20]. MiR-340 is known as a tumor suppressor that is involved in diverse tumors, such as osteosarcoma [21], hepatocellular carcinoma [22], endometrial carcinoma [23], and mastadenoma [24]. A pervious study has shown that miR-340 inhibits the osteoclast differentiation of bone marrow-derived macrophages [25]. However, the specific roles of miR-340 on odontogenic differentiation of iPSCs are still unclear.

In this study, the specific effects of miR-340 on odontogenic differentiation of iPSCs, and on the expression of AMBN and DMP-1 were evaluated. The potential regulatory mechanisms of miR-340 relating with BMP4 were further analyzed. Our findings may reveal the specific role of miR-340 on odontogenic differentiation, and provide guidance for iPSCs-based tooth regeneration.

Methods

Cell culture and treatments

Rat iPSCs were purchased from Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences (Guangzhou, China), and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing a bottom-layer of trophoblast cells (mitomycin-treated mouse fibroblasts containing 0.1% gelatin). Cells were maintained in an incubator at 37°C with 95% humidity and 5% CO_2. Cells in logarithmic growth phase were used for treatment and tansfection.

BMP4 treatment

The odontogenic differentiation of iPSCs was induced by the intervention of BMP4. Simply, cells were digested with pancreatin, and then induced with 50 ng/mL BMP4 (Abbkine, USA) for 48 h (BMP4 group). Normal iPSCs without treatment were used as the control (Blank group).

Cell transfection

In order to silence BMP4 in iPSCs, iPSCs were transfected with si-BMP4 (Thermo Fisher Scientific, USA) using lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instruction. iPSCs transfected with si-RNA negative control (NC) were used as the control (siRNA NC group). In addition, iPSCs were transfected with miR-340 mimic, and miR-340 inhibitor to downregulate, and upregulate miR-340 in iPSCs, respectively (miR-340 mimic group, and miR-340 inhibitor group). IPSCs transfected with miR-340 NC were used as the control (miR-340 NC group). After 48 h of transfection, transfected cells were used for further assay.

Alizarin red S staining

Alizarin red S staining was performed to identify the odontogenic differentiation of iPSCs. Simply, iPSCs of different groups were fixed in

Table 1. The sequences of specific primers used in quantitative real-time PCR (qRT-PCR)

Primers	Sequences
miR-340-F	5'-GCGGTTATAAAGCAATGAGA-3'
miR-340-R	5'-GTGCGTGTCGTGGAGTCG-3'
BMP4-F	5'-AAGCGTAGCCCTAAGCATCA-3'
BMP4-R	5'-GGCTTTGGGGATACTGGAAT-3'
AMBN-F	5'-CCTTGAGACAATGAGACAGTTGGG-3'
AMBN-R	5'-CCTGGGTAAAAAGCGGATGC-3'
DMP-1-F	5'-TCAGGACAGTAGCCGATCCAAAG-3'
DMP-1-R	5'-ATGGGTTTGTTGTAAGCATCA-3'
GAPDH-F	GTCGATGGCTAGTCGTAGCATCGAT
GAPDH-R	TGCTAGCTGGCATGCCCGATCGATC

4% paraformaldehyde for 20 min, and then stained with 1% alizarin red S (pH 4.2, Abbkine) for 20 min at room temperature. Positive stained cells were visualized and counted under a microscope (Nikon, Japan).

Quantitative real-time PCR (qRT-PCR)

QRT-PCR was performed to detect the expression of BMP4, AMBN and DMP-1 in iPSCs at a mRNA level. Total RNAs were extracted from iPSCs of different groups using Trizol reagent (Fermentas, USA), and then reverse transcripted using First Strand cDNA Synthesis Kit (Fermentas). QRT-PCR was performed on Option2 (MJ Research) using special primers (**Table 1**). GAPDH was used as an internal control. The PCR program included 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 34 s. The relative expression of target genes was calculated according to the $2^{-\Delta\Delta Ct}$ method [26].

Western blot

Western blot was performed to detect the expression of BMP4, AMBN and DMP-1 in iPSCs at protein level. Total proteins of iPSCs of different groups were extracted in lysis buffer. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene-fluoride membrane (Millipore, USA). After blocked with 5% skim milk for 1 h, the membrane was incubated with primary antibodies (anti-BMP4, anti-AMBN, anti-DMP-1, 1:1,000, Santa Cruz Biotechnology, USA) for 12 h at 4°C. Then membrane was washed with TBST three times, and incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit, 1:5000, Santa Cruz Biotechnology) for 2 h at 25°C. The protein brands were visualized using a Gel imaging analyzer (Vitber tourmat, France).

Dual luciferase reporter gene (DLR) assay

DLR assay was performed to identify the interaction between BMP4 and miR-340. Simply, cells were co-transfected with luciferase plasmids carrying BMP4 wildtype (BMP4 WT)/ BMP4 mutant (BMP4 MT) and miR-340 mimic/ miR-340 NC. After 48 h of incubation, the fluorescence was visualized using a DLR kit according to the manufacturer's instruction (Thermo Fisher Scientific), and the fluorescence intensity was detected by a Microplate Reader (Thermo Fisher Scientific).

Immunofluorescence

Immunofluorescence was performed to detect the expression of AMBN and DMP-1 in iPSCs at the protein level. IPSCs of different groups were fixed in 2.5% glutaraldehyde for 15 min, and incubated in 0.25% Triton X-100 for 15 min. After being blocked with 4% lowlenthal serum at room temperature for 30 min, cells were incubated with primary antibody (anti-AMBN, anti-DMP-1, 1:100, IQBio, China) at 4°C for 12 h. Then cells were washed with PBS three times, and incubated with rhodamine-labeled secondary antibody (1:200, ZSGB-Bio, China) at 37°C for 40 min. Followed by staining with DAPI (4,6-diamino-2-phenylindole) for 5 min, postive stained cells were visualized and counted under a fluorescent microscope (Nikon).

Statistical analyses

All data were expressed as mean ± standard deviation (SD) and repeated three times. Statistical analysis was performed by SPSS version 18.0 (SPSS Inc., Chicago, IL). Comparison between different groups was determined by one-way ANOVA compared with Least Square Deconvolution. A *P*-value less than 0.05 represented data being significantly different.

Results

BMP4 upregulated AMBN and DMP-1 in iPSCs

IPSCs were induced with BMP4 for 48 h. qRT-RCR showed that the expression of AMBN was

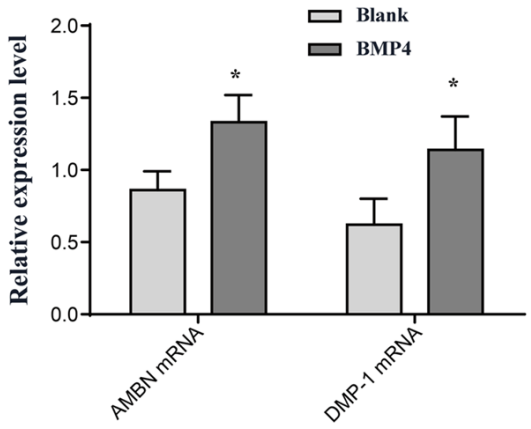

Figure 1. The effects of bone morphogenetic protein 4 (BMP4) intervention on the expression of ameloblastin (AMBN) and dentin matrix protein 1 (DMP-1) in induced pluripotent stem cells (iPSCs) at the mRNA level (qRT-PCR). BMP4, iPSCs induced BMP4; Blank, normal iPSCs without treatment. Data were expressed as mean ± standard deviation and repeated for three times. *P < 0.05 vs. Blank.

significantly higher in the BMP4 group than in the blank group (1.33 ± 0.19 *vs.* 1.14 ± 0.23, P < 0.05). In addition, the expression of DMP-1 in iPSCs was also significantly increased by the intervention of BMP4 (0.86 ± 0.13 vs. 0.62 ± 0.18, P < 0.05) (**Figure 1**).

Silencing of BMP4 downregulated AMBN and DMP-1 in iPSCs

BMP4 was silenced in iPSCs by si-BMP4 tranfection. As shown in **Figure 2**, the expression of BMP4 in iPSCs was significantly inhibited by si-BMP4 tranfection at both the mRNA and protein levels (P < 0.05, **Figure 2A**). In adiition, the expression of AMBN and DMP-1 was significantly lower in the si-BMP4 group than in the blank group at both the mRNA and protein level (P < 0.05). No significant different on the expression of AMBN and DMP-1 was observed between si-BMP4 and siRNA NC group (**Figure 2B**).

Downregulation of miR-340 promoted the odontogenic differentiation of iPSCs

The odontogenic differentiation of iPSCs was evaluated by alizarin red S staining. As shown in **Figure 3**, cells in the BMP4 group exhibited significantly higher percentage of positive stained cells than the blank group (P < 0.05). The tranfection of miR-340 inhibitor significantly increased the percentage of positive stained cells, while the tranfection of miR-340 mimic significantly decreased the percentage of positive stained cells (P < 0.05). In addition, the percentage of positive stained cells was significantly lower in the miR-340 mimic + BMP 4 group than in the BMP4 group (P < 0.05). No significant differences on the percentage of positive stained cells were observed between blank and miR-340 NC group.

Downregulation of miR-340 increased the expression of BMP4, AMBN and DMP-1 in iPSCs

MiR-340 was upragulated, and downregulated in iPSCs by the tranfection of miR-340 mimic and miR-340 inhibitor, respectively. As shown in **Figure 4**, the expression of BMP4, AMBN and DMP-1 was significantly lower in the miR-340 mimic group than in the blank group at both the mRNA and protein levels (P < 0.05). The tranfection of miR-340 inhibitor significantly increased the expression of BMP4, AMBN and DMP-1 in iPSCs at both the mRNA and protein levels (P < 0.05). No significant different on the expression of BMP4, AMBN and DMP-1 was observed between blank and miR-340 NC group (**Figure 4A**, **4B**).

BMP4 was a target of miR-340

A miR-340 binding site at 3'-UTR of BMP4 was observed by an online target gene prediction software (Target Scan) (**Figure 5A**). Then the specific interaction between BMP4 and miR-340 was identified by DLR assay. As shown in **Figure 5B**, cells co-transfected with BMP4 WT + miR-340 mimic (WT + mimic) exhibited significantly lower fluorescence intensity than those co-transfected with BMP4 WT + miR-340 NC (WT + NC), BMP4 MT + miR-340 mimic (MT + mimic), and BMP4 MT + miR-340 NC (MT + NC) (P < 0.05) (**Figure 5B**).

Downregulation of miR-340 reversed the inhibitory effect of si-BMP4 on the expression of AMBN and DMP-1 in iPSCs

The regulatory mechanism of miR-340 relating with BMP4 was further identified in iPSCs. QRT-PCR, Western blot, and immunofluorescence showed that si-BMP4 significantly downregulated AMBN and DMP-1 in iPSCs at both the mRNA and protein levels (P < 0.05). MiR-340 inhibitor significantly upregulated AMBN and

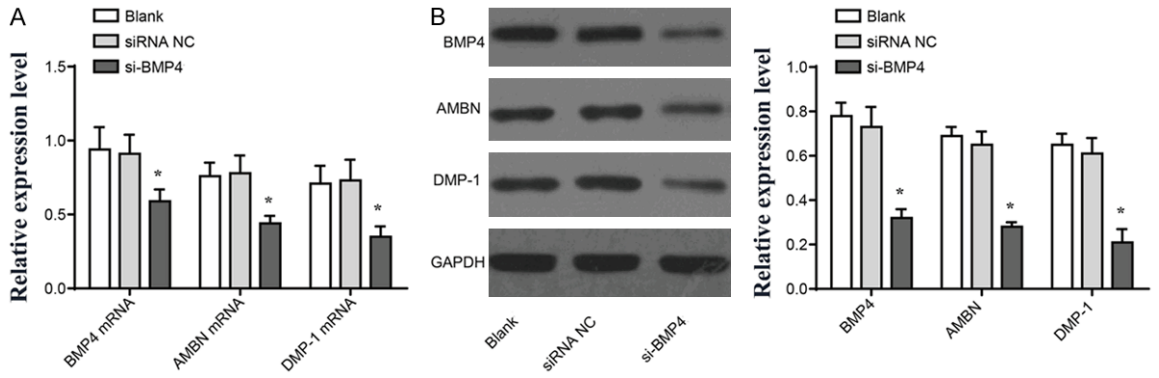

Figure 2. The effects of siRNA-bone morphogenetic protein 4 (si-BMP4) tranfection on the expression of ameloblastin (AMBN) and dentin matrix protein 1 (DMP-1) in induced pluripotent stem cells (iPSCs). A. Relative expression at mRNA level (qRT-PCR); B. Relative expression at protein level (Western blot). si-BMP4, iPSCs tranfected with siRNA-BMP4; siRNA NC, iPSCs tranfected with siRNA-negative control; Blank, normal iPSCs without treatment. Data were expressed as mean ± standard deviation and repeated for three times. *P < 0.05 vs. Blank or siRNA NC.

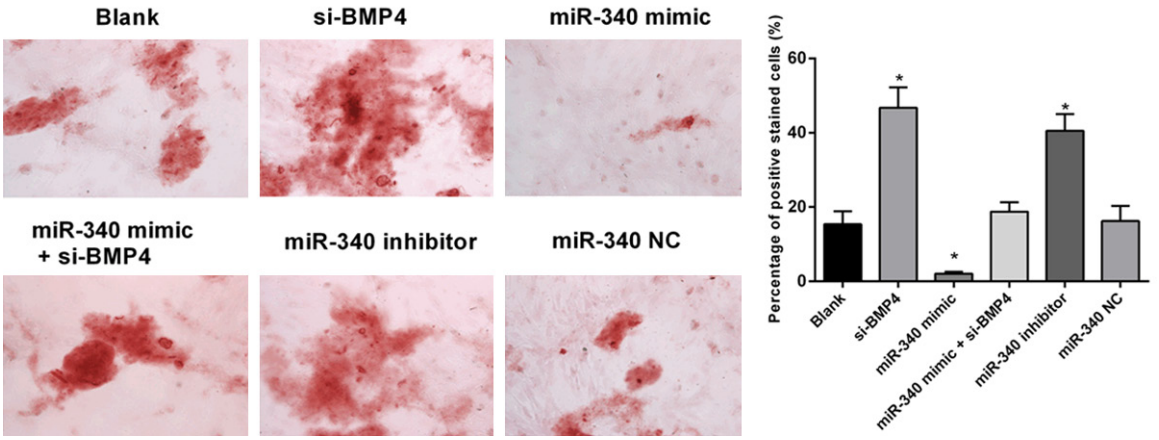

Figure 3. Alizarin red S staining of induced pluripotent stem cells (iPSCs). Cells were observed under a microscope and the percentage of positive stained cells were calculated. BMP4, iPSCs induced with bone morphogenetic protein 4 (BMP4); miR-340 mimic, iPSCs tranfected with microRNA-340 (miR-340) mimic; miR-340 inhibitor, iPSCs tranfected with miR-340 inhibitor; miR-340 mimic + BMP4; iPSCs tranfected with miR-340 inhibitor, and induced with BMP4; miR-340 NC, iPSCs tranfected with miR-340-negative control; Blank, normal iPSCs without treatment. Data were expressed as mean ± standard deviation and repeated three times. *P < 0.05 vs. blank and miR-340 NC.

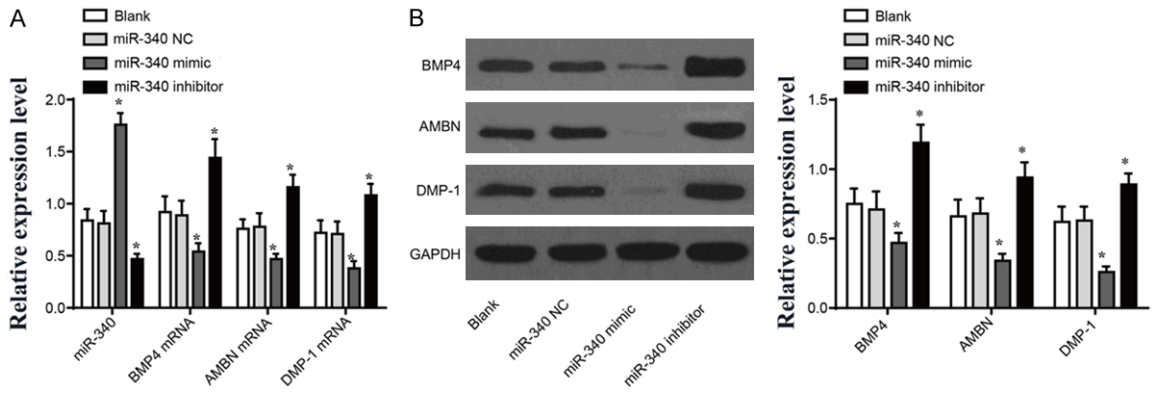

Figure 4. The effects of microRNA-340 (miR-340) on the expression of bone morphogenetic protein 4 (BMP4), ameloblastin (AMBN) and dentin matrix protein 1 (DMP-1) in induced pluripotent stem cells (iPSCs). A. Relative expression at mRNA level (qRT-PCR); B. Protein expression and relative expression at protein level (Western blot). MiR-340

mimic, iPSCs tranfected with microRNA-340 (miR-340) mimic; miR-340 inhibitor, iPSCs tranfected with miR-340 inhibitor; miR-340 NC, iPSCs tranfected with miR-340 negative control; Blank, normal iPSCs without treatment. Data were expressed as mean ± standard deviation and repeated three times. *P < 0.05 vs. Blank or miR-340 NC.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	PCT
Position 241-247 of BMP4 3'UTR hsa-miR-340-5p	5' ···UUUUGACAAAAUAUAUUUAU AC··· 3' UUAGUCAGAGUAACGAAAUAUU	7mer-A1	-0.06	94	-0.06	2.908	N/A

B

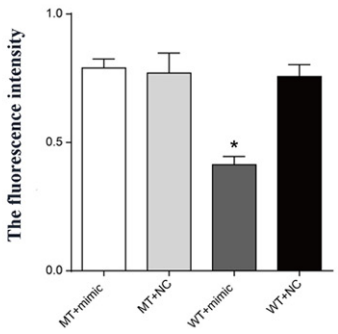

Figure 5. The interaction between bone morphogenetic protein 4 (BMP4) and microRNA-340 (miR-340). A. A miR-340 binding site at 3'-UTR of BMP4 predicted by Target Scan; B. The fluorescence intensity of co-transfected cells detected by dual luciferase reporter gene (DLR) assay. WT + mimic, BMP4 wildtype (WT) + miR-340 mimic; WT + NC, BMP4 WT + miR-340 negative control (NC); MT + mimic, BMP4 mutant (MT) + miR-340 mimic; MT + NC, BMP4 MT + miR-340 NC. Data were expressed as mean ± standard deviation and repeated three times. *P < 0.05 vs. WT + NC, MT + mimic or MT + NC group.

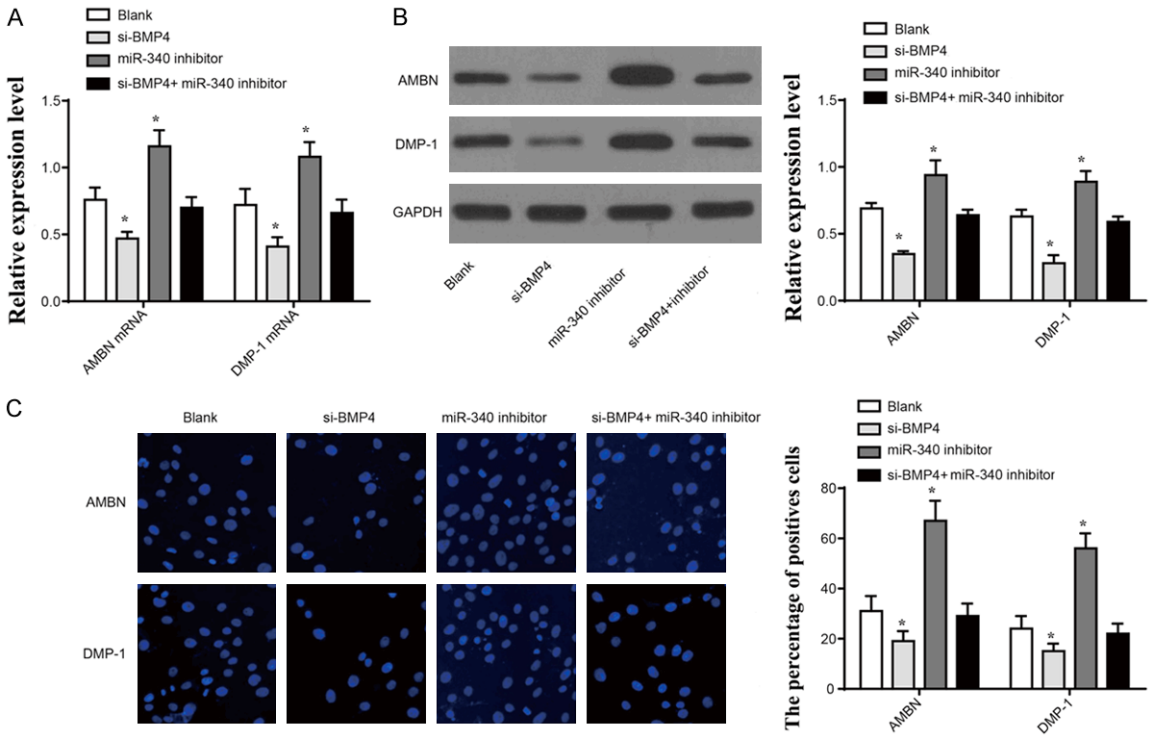

Figure 6. The effects of microRNA-340 (miR-340) inhibitor on the expression of ameloblastin (AMBN) and dentin matrix protein 1 (DMP-1) in induced pluripotent stem cells (iPSCs). A. Relative expression at the mRNA level (qRT-PCR); B. Protein relative expression at protein level (Western blot); C. The observation of cells under microscope and the percentage of positive stained cells. Si-BMP4, iPSCs tranfected with siRNA-bone morphogenetic protein 4 (si-BMP4); miR-340 inhibitor, iPSCs tranfected with microRNA-340 (miR-340) inhibitor; si-BMP4 + miR-340 inhibitor, iPSCs co-tranfected with siRNA BMP4 and miR-340 inhibitor; Blank, normal iPSCs without treatment. Data were expressed as mean ± standard deviation and repeated three times. *P < 0.05 vs. blank or si-BMP4 + miR-340 inhibitor group.

DMP-1 in iPSCs at both the mRNA and protein levels (P < 0.05). Note worthily, the expression of AMBN and DMP-1 in si-BMP4 + miR-340 inhibitor group was significantly higher than in

the si-BMP4 group, and was significantly lower than in the miR-340 inhibitor group ($P < 0.05$). The expression of AMBN and DMP-1 in the si-BMP4 + miR-340 inhibitor group was relatively close to that in blank group (**Figure 6A-C**).

Discussion

Stem cell-based tissue engineering provides a promising strategy to achieve tooth regeneration [27]. Nowadays, increasing evidence has proved that miRs play important roles in odontogenic differentiation of stem cells. However, the specific role of miR-340 on odontogenic differentiation, as well as the potential regulatory mechanisms are still unclear. In this study, we found that the tranfection of miR-340 inhibitor significantly promoted the odontogenic differentiation of iPSCs. In addition, the regulatory effect of miR-340 on odontogenic differentiation of iPSCs was closely related with BMP4.

BMP4 is a vital regulatory gene that is involved in mesoderm induction, tooth development, and bone formation [28]. The specific inducing role of BMP4 on odontogenic differentiation has been identified by many studies. It has been reported that recombinant human BMP4 promotes the differentiation of adult pulp cells into odontoblasts [29]. Ameloblasts serum-free conditioned medium supplemented with BMP4 promotes the odontogenic differentiation of iPSCs [14]. Exogenous BMP4 increases the expression of DMP1 and DSPP in iPSC-derived neural crest-like cells [12]. In this study, we found that the intervention of BMP4 significantly increased the expression of AMBN and DMP-1 in iPSCs, while the tranfection of si-BMP4 significantly decreased the expression of AMBN and DMP-1. Since AMBN and DMP1 is necessary in ameloblast differentiation and dentin mineralization during tooth development, AMBN and DMP1 are considered as marker genes in odontogenic differentiation [30]. The upregulation of AMBN and DMP-1 indicate that BMP4 can promote the odontogenic differentiation of iPSCs. Our findings are consistent with previous studies, and further illustrate that BMP4 is an inducing factor of odontogenic differentiation.

MiRs are key regulators involved in diverse cellular processes, including differentiation [16]. According to a pervious study based on miR microarray, 12 miRs are up-regulated in hDPCs during odontogenic differentiation, including miR-20b, -34a, -937, -130b, -100, -335, -944, -17, -562, -338-5p, -122, and -521; while 10 miRs are down-regulated, including miR-542-5p, -1224-5p, -382, -431, -203, -1225-5p, -486-3p, -517c, -135b, and -371-3p [17]. In addition, previous studies have shown that miR-675, -663, and -720 can promote odontogenic differentiation [18-20]. However, the specific role of miR-340 on odontogenic differentiation is rarely reported. In this study, we found that the transfection of miR-340 inhibitor significantly increased the percentage of positive cells stained with alizarin red S, as well as the the expression of AMBN and DMP-1 at both the mRNA and protein levels. Our findings illustrate that the downregulation of miR-340 promotes the odontogenic differentiation of iPSCs. The promoting effects of miR-340 inhibition on odontogenic differentiation are consistent with previous studies on miR-675, -663, and -720 [18-20]. However, the regulatory mode of miR-340 is contrary to miR-675, -663, and -720.

Since BMP4 is a promoter of odontogenic differentiation, the regulatory mechanisms of miR-340 relating with BMP4 were further identified. We found that miR-340 mimic, and miR-340 inhibitor significantly decreased, and increased the expression of BMP4 in iPSCs, respectively. These results indicate that miR-340 can regulate the expression of BMP4 in iPSCs. The specific regulatory role of miR-340 on BMP4 is consitent with previous studies. It has been reported that miR-340-5p promotes thyroid cancer proliferation by inhibiting BMP4 [31]. MiR-340-5p inhibits the neuronal differentiation of neural stem cells via downregulating BMP4 [32]. Since a miR-340 binding site at 3'-UTR of BMP4 was predicted by Target Scan, DLR assay was performed to further identify the specific relation between miR-340 on BMP4. Note worthily, BMP4 was identified as a target of miR-340. Therefore, we suspect that miR-340 inhibition may promote the odontogenic differentiation of iPSCs via upregulating BMP4. This hypothesis has also been identified in our study. Our further further research showed that miR-340 mimic significantly decreased the percentage of positive stained cells in the BMP4 group, and miR-340 inhibition significantly reversed the inhibitory effect of si-BMP4 on the expression of AMBN and DMP-1 in iPSCs.

In conclusion, silencing of miR-340 promoted the odontogenic differentiation of iPSCs, and upregulated AMBN and DMP-1 in iPSCs via upregulating target BMP4. Silencing of miR-340 may be used as a promising strategy in induction of odontogenic differentiation during tooth regeneration. However, this study is still limited at the cellular level. Further research on the specific roles of miR-340 in animal models are still needed.

Disclosure of conflict of interest

None.

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