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MicroRNA-27 Promotes Odontoblast Differentiation via Wnt1 Signaling

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MicroRNA (miRNA, miR) is essential in regulating cell differentiation either by inhibiting mRNA translation or by inducing its degradation. However, the role of miRNA in odontoblastic cell differentiation is still unclear. In this study, we examined the molecular mechanism of miR-27-mediated regulation of odontoblast differentiation in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells. The results of the present study demonstrated that the miR-27 expression increases significantly during MDPC-23 odontoblastic cell differentiation. Furthermore, miR-27 up-regulation promotes the differentiation of MDPC-23 cells and accelerates mineralization without cell proliferation. The over-expression of miR-27 significantly increased the expression levels of Wnt1 mRNA and protein. In addition, the results of target gene prediction revealed that Wnt1 mRNA has an miR-27 binding site in its 3'UTR, and is increased by miR-27. These results suggested that miR-27 promotes MDPC-23 odontoblastic cell differentiation by targeting Wnt1 signaling. Therefore, miR-27 is a critical odontoblastic differentiation molecular target for the development of miRNA based therapeutic agents in dental medicine.

Key words: miR-27, odontoblasts, differentiation, Wnt1

Introduction

Dentin forms the bulk of the tooth, and is a mineralized tissue formed by odontoblasts [1]. Odontoblasts are differentiated from ectomesenchymal cells, and are involved in the secretion of the organic matrix during odontoblast differentiation [1,2]. This matrix contains a mixture of collagenous and non-collagenous proteins, and it becomes mineralized and forms dentin, the main hard tissue of a tooth [1,2]. Many researchers suggest that signaling molecules in the bone morphogenetic protein, fibroblast growth factor and wingless (Wnt) families as well as transcription factors such as Runx2 are involved in the odontoblast differentiation [2-4]. However, the exact molecular mechanisms underlying odontoblast differentiation are still unclear.

Canonical Wnt signaling is very important for the differentiation of several cells including odontoblast [4,5]. Wnt ligands bind a heterodimeric complex formed by the LRP5/6 co-receptor and a member of the frizzled receptor families [4]. Activation of the receptor inhibits the destruction complex such as *adenomatous polyposis coli* (APC), anaphase-promoting complex, and glycogen synthase kinase 3b (GSK3b) [4]. Wnt signaling must be tightly regulated for proper differentiation

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[4,5]. However, the exact molecular mechanisms of Wnt signaling underlying odontoblast differentiation are still unclear.

MicroRNA (miRNA, miR) is an endogenous, small non-coding single strand RNA that have been shown to play a role in numerous cellular process, including cellular differentiation [6], organism development [7], proliferation [8] and apoptosis [9]. Therefore, current research focuses on the utility of miRNAs as diagnostic and prognostic tools as well as potential therapeutic targets. Despite the numerous studies on miRNAs, their biological functions or mechanisms of action are not well understood.

There is a few study that post-transcriptional regulation of gene expression, mediated by miRNAs, plays an important role in the control of odontoblast differentiation [2,10,11]. Liu et al. showed that the miR-143 and miR-145 control odontoblast differentiation and dentin formation through Krüppel-like factor 4 and Osterix transcriptional factor signaling pathways [11]. Sun et al. reported that miR-338-3p promote odontoblast differentiation through targeting Runx2 [2]. miR-27 could regulate adipogenesis, myeloblasts differentiation, osteoblast differentiation, and skeletal muscle development [4,12,13]. Nevertheless, at present, the role of miR-27 in regulating odontoblast differentiation remains unknown.

In this study, therefore, the molecular mechanism of miR-27 on regulating odontoblast differentiation was investigated in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells.

Materials and Methods

Cell cultures

MDPC-23 odontoblastic cells provided by Dr. J.E. Nör (University of Michigan, Ann Arbor, MI, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37 ^{o}C. To induce cell differentiation and mineralization, confluent MDPC-23 cells were treated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for up to 10 days [14].

miRNA isolation and Affymetrix miRNA array analysis

Total RNA including miRNAs from MDPC-23 cells from days 0, 4 and 7 of differentiation were isolated with miRNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The concentration, purity and amount of total RNA were quantified using the Nano-DropND-1000 ultraviolet Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The miRNA array was scanned using an Affymetrix GeneChip Platform (DNA link, Seoul, Korea). For each sample, total RNA was subjected to a tailing reaction (2.5 mM $MnCl_2$, ATP, Poly A Polymerase, incubation for 15 min at 37 ^{o}C) followed by ligation of the biotinylated signal molecule to the target RNA sample (16 Flash Tag ligation mix biotin, T4 DNA ligase, incubation for 30 min at room temperature) and the reaction was terminated with addition of stop solution. Each sample was hybridized to a GeneChipH miRNA Array at 48 ^{o}C and 60 rpm for 16 h, then washed and stained on Fluidics Station 450 and scanned on a GeneChipScanner3000 7G (Affymetrix, Fremont, CA, USA). The image data were analyzed with the miRNA QC Tool software for quality control.

Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real time-PCR (qRT-PCR)

Reverse transcription of the miRNA was performed using a miScript Reverse Transcription kit (Qiagen, Valencia, CA, USA) starting from 1 μg of total RNA. TaqMan miRNA assays kits (ABI, Carlsbad, CA, USA) were used to examine the specific miRNA expression by qRT-PCR according to the manufacturer's protocol. The qRT-PCR results, which were recorded as threshold cycle numbers, were normalized against an internal control (U6 RNA), and the comparative threshold cycle method was used to determine the levels of miRNA expression. The level of miR-27 (5'-AGGGCTTAGCTGCTTGTGAGCA-3') was measured by qRT-PCR. The deviations in the samples represent four independent experiments.

To perform RT-PCR, the total RNA was isolated using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was carried out with 1 μg of total RNA using the TermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA). The levels of alkaline phosphatase (ALP) (ALP-F, 5'-CTCTCCGAGATGGTGGAGGT-3'; ALP-R, 5'-GTC TTCTCCACCGTGGGTCT-3'), type I collagen (Col I) (Col I-F, 5'-TAAGTTGCC AAGAACGTGCC-3'; Col I-R, 5'-AATTGAAAGCCAGGAGGCAT-3'), dentin matrix protein 1 (DMP-1) (DMP-1-F, 5'-CGGCTGGTGGTCTCTCTAAG-3';

DMP-1-R, 5'-ATCTTCCTGGGACTGGGTCT-3'), dentin sialophosphoprotein (DSPP) (DSPP-F, 5'-ATAGCACCAACCA TGAGGCT-3'; DSPP-R, 5'-CTTTTGTTGCCTTTGTTGGG −3'), Wnt1 (Wnt1-F, 5'-CAGCGTTCATCTTCGCAATCACCT-3'; Wnt1-R, 5'-AAGGTTCATGAGGAAGCGTAGGTC−3') and GAPDH (GAPDH-F, 5'-TGC ATCCTGCACCACCAACT-3'; GAPDH-R, 5'-CGCCTGCTTCACCACCTTC-3') induction were measured by RT-PCR, and visualized by DNA agarose gel electrophoresis. The differences in expression were presented as a histogram after densitometry using a VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA).

miRNAs and transfection

miR-27 and scrambled miR-27 were purchased from Ambion (Austin, TX, USA). The miR-27, mimic miR-27 and pGL3-promoter-Wnt1-3'UTR were transfected into cultured MDPC-23 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The experimental group of cells was treated with 10 ng/ml miRNAs for 2 days.

Alizarin red S staining

The cells were fixed with 70% ethanol for 20 min and stained with 1% Alizarin red S (Sigma−Aldrich Corp., St. Louis, MO, USA) in 0.1% NH_4OH at pH 4.2−4.4. The mineralization assays were performed by staining MDPC-23 cells with Alizarin red S solution. The cells were evaluated at 0, 4, 7 and 10 days. To quantify the intensity of mineralization, we measured density of stained nodules by colorimetric spectrophotometry. The stained cells were collected by centrifugation at 13,000 rpm for 10 min at 4 ^{o}C. Cell lysate was solubilized with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature. Solubilized stain (0.1 ml) was transferred to wells of a 96-well plate, and absorbance was measured at 405 nm.

Cell proliferation assay

The cells were seeded at a density of 4 X 10^4 cells/well in 24-well plates and allowed to attach to the well overnight. After incubation, the cultured cells were transfected with miR-27 using Lipofectamine™ 2000. The cells were incubated with miR-27 at various defined concentrations (2, 20 and 200 ng/ml) for 1 and 2 days at 37 ^{o}C. After incubation under the defined conditions, the cells were incubated for another 4 h in 20 $\mu\ell$ of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). To dissolve the formazan transformed from MTT, the cells were resuspended in 150 $\mu\ell$ dimethyl sulfoxide and the optical density of the solution was determined using a spectrometer at an incident wavelength of 495 nm [15]. The experiments were repeated four times, independently.

Immunoblotting

To determine the level of Wnt1 in MDPC-23 cells transfected with miR-27, the proteins were extracted. The cells were washed twice with ice-cold PBS and lysed using a RIPA buffer for protein extraction according to the manufacturer's instructions. The total protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). An equal amount of protein was resolved by 10% SDS−PAGE and transferred to a nitrocellulose membrane for immunoblotting. The anti-Wnt1 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody was used as the primary antibody. The immunoreactivity was visualized using an ECL system (Amersham Biosciences, Piscataway, NJ, USA) and a Signal Visual Enhancer system (Pierce, Rockford, IL, USA) was used to magnify the signal.

Vector construction and luciferase activity assay

The 3'UTR of Wnt1 was amplified from genomic DNA isolated from MDPC-23 cells using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instruction, using Wnt1-3'UTR forward primer (5'-CCGCTCGAGGACCCAGCCTAC CTCCCT-3') and Wnt1-3'UTR reverse primer (5'-ATAGTTTAGCGGCCGCTCGTT GATAAAAATAGATATT-3'). pGL3-promoter-Wnt1-3'UTR was constructed by cloning the 3'UTR of Wnt1 (736 bp) into the downstream of the luciferase gene in the pGL3-promoter vector for luciferase activity assay. For luciferase activity assay, MDPC-23 cells were cultured in 24-well plates and co-transfected with 5 ng pGL3-promoter-Wnt1-3'UTR, 5 ng Renilla for normalization, and 100 ng of miR-27-wild type or same amount of synthesized mimic miR-27. Luciferase activity was measure 2 days after transfection using the Dual-luciferase reporter assay system (Promega, Madisom, WI, USA) according to the manufacturer's instruction.

Statistical analysis

All experiments were performed at least in triplicate. The results were presented as the mean ± SEM. Statistical

significance was analyzed using a Student's *t*-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

miR−27 expression is significantly up−regulated during MDPC−23 odontoblastic cell differentiation.

The expression pattern of miRNA during MDPC-23 cell differentiation was determined by miRNA microarray with cell samples from days 0, 4 and 7 of differentiation (n = 4) using Affymetrix Genechip. This experiment showed that miR-27 in MDPC-23 cells from days 4 and 7 of differentiation was significantly up-regulated over 3 fold compared to control cells of day 0 (Fig. 1A). The miRNA array data was verified by examining the expression pattern of miR-27 by miRNA qRT-PCR. In miRNA qRT-PCR analysis, miR-27 levels were increased in MDPC-23 cells maintained in odontoblastic cell differentiation medium (Fig. 1B). These results showed that miR-27 was significantly up-regulated during MDPC-23 cell differentiation and increment of expression of miR-27 enhanced the cell differentiation process in MDPC-23 cells.

miR−27 accelerates mineralization in MDPC−23 odontoblastic cells.

The effect of miR-27 on odontoblastic cell mineralization was measured. MDPC-23 cells were cultured for 10 days in differentiation media transfected with miR-27 and the mineralized nodules were evaluated by Alizarin red S staining. In control MDPC-23 cells, mineralized nodules appeared after 7 days of culture. The miR-27 transfected MDPC-23 cells showed mineralized nodules after 4 days, and the mineralized nodules increased with time during the culture period (Fig. 2).

To study the potential role of miR-27 in MDPC-23 differentiation, the cells transfected with miR-27 were analyzed. The RT-PCR results showed that miR-27 enhanced the differentiation of MDPC-23 odontoblastic cells, indicated by higher expression of the odontoblast marker genes ALP, Col I, DMP-1 and DSPP, along with an increase in their activities compared to control cells (Fig. 3).

As shown in Fig. 4, the up-regulated miR-27 did not alter the cell proliferation in the MDPC-23 cells.

Taken together, these data were suggesting that miR-27 was promoting the odontogenic specific differentiation without alteration the cell proliferation in MDPC-23.

miR−27 up−regulation in MDPC−23 cells promotes differentiation by increasing the Wnt1 expression.

The molecular mechanisms by which miR-27 promotes

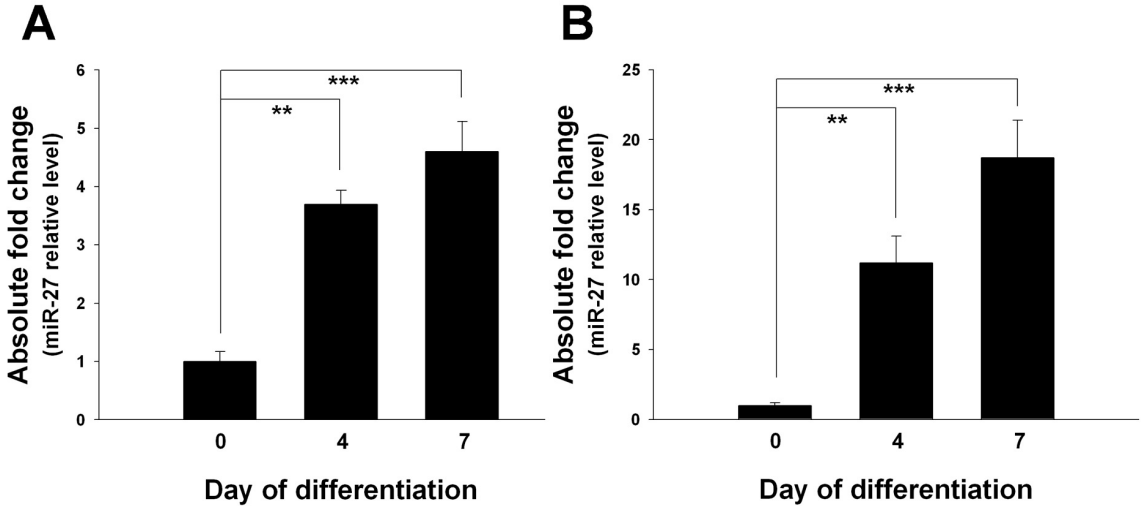

Fig. 1. The relative expression of miR-27 during MDPC-23 odontoblastic cell differentiation. Confluent cultures of MDPC-23 cells were maintained in complete medium with the addition of differentiation cocktail (50 μg/ml ascorbic acid and 10 mM β -glycerophosphate). Cells were harvested following 0, 4 and 7 days incubation and endogenous expression level of miR-27 was measured. The relative expression of miR-27 was accessed by miRNA array using affymetrix Genechip (A) and by qRT-PCR (B). Each data point represents the mean ± SEM of four experiments. $^{**}p<0.01$ or $^{***}p<0.001$ vs. day 0.

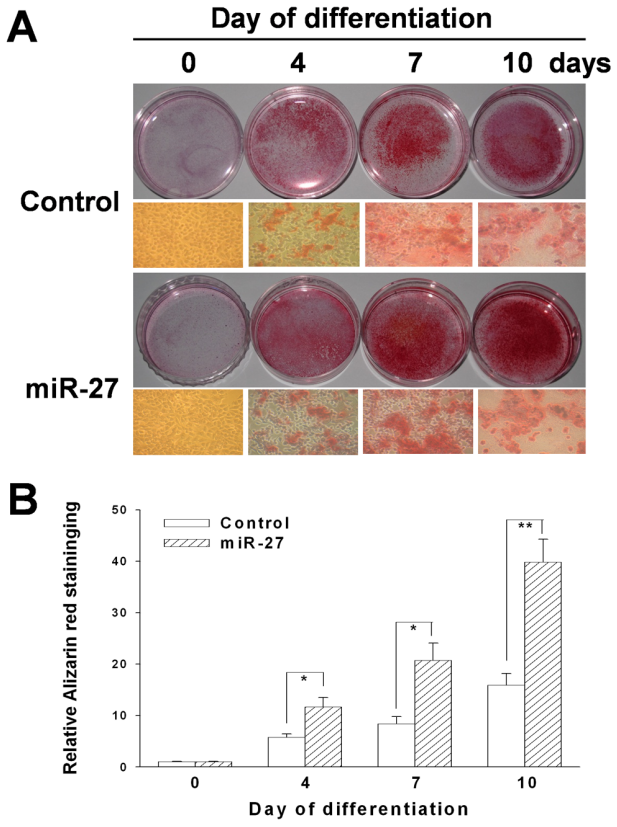

Fig. 2. The effect of miR-27 on the mineralization in MDPC-23 odontoblastic cells. MDPC-23 odontoblastic cell mineralization was altered by over-expressed miR-27. (A) Mineralized nodule formation in MDPC-23 cells. MDPC-23 cells were transfected with miR-27 for 10 days, and the mineralization was evaluated by Alizarin red S staining. (B) Quantification of mineralization was accessed by colorimetric spectrophotometry. Each data point represents the mean ± SEM of three experiments. $^{*}p<0.05$ or $^{**}p<0.01$ vs. control (the control cells were measured in the absence of miR-27 transfection).

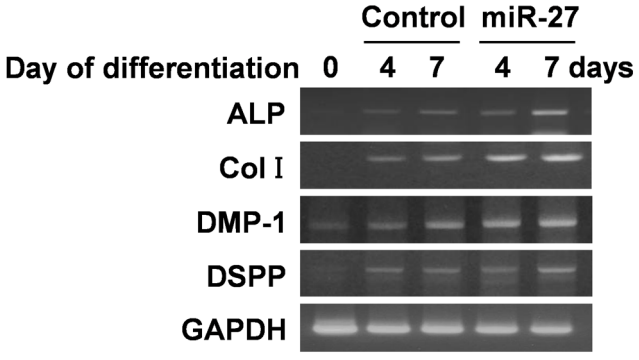

Fig. 3. The effect of miR-27 on ALP, Col I, DMP-1 and DSPP mRNA expressions. Total RNA was isolated using TRIzol reagent and reverse transcription was carried out with 1 μg of total RNA using the TermoScript RT-PCR system. The PCR products were electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide.

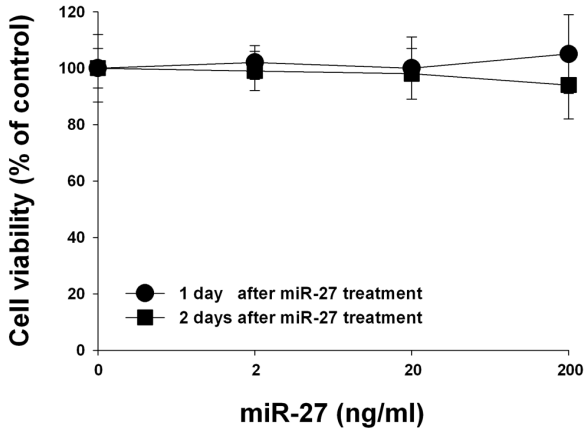

Fig. 4. The measurement of MDPC cell proliferation by up-regulated miR-27. The cells were incubated with miR-27 at various defined concentrations (2, 20 and 200 ng/ml) for 1 and 2 days at 37 ^{o}C. The cell proliferation was determined by MTT assays after miR-27 transfection. The experiments were repeated four times, independently.

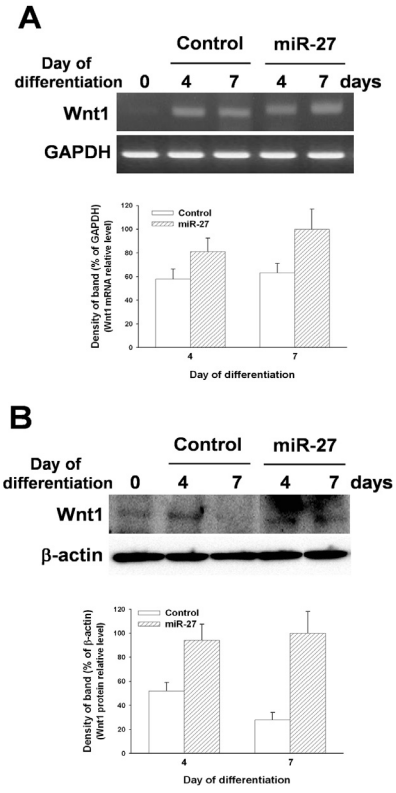

Fig. 5. The effect of miR-27 on the expression of Wnt1 during MDPC-23 odontoblastic cell differentiation. The over-expression of miR-27 significantly increased the expression levels of Wnt1 mRNA (A) and protein (B) compared to control. MDPC-23 cells were transfected with miR-27 according to the defined conditions. (Lower panel) Quantitative data for (Upper panel) were analyzed by using Imagegauge 3.12 software after GAPDH or β-actin normalization. $^{*}p<0.05$ or $^{**}p<0.01$ vs. control (the control cells were measured in the absence of miR-27 transfection).

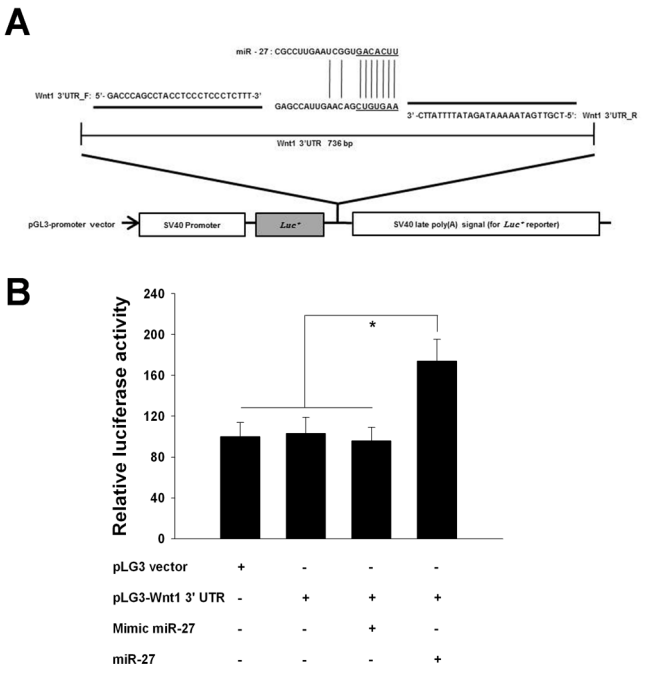

Fig. 6. The activation of Wnt1 signaling by miR-27. (A) The schematic diagram to generate the reporter vector. The 3'UTR of Wnt1 was cloned by PCR and subclond into the downstream of luciferase gene in the pGL3 promoter vector. (B) The luciferase activity of generated reporter vector containing 3'UTR of Wnt1 was significantly up-regulated by miR-27. MDPC-23 cells were co-transfected with generated reporter vector containing 3'UTR of Wnt1, miR-27 and mimic miR-27 according to the defined condition. And then luciferase activity was accessed by manufacturer's instruction. Each data point represents the mean ± SEM of three experiments. $^{*}p<0.05$ vs. other experimental groups.

differentiation of MDPC-23 cells were investigated. Putative miR-27 targets were predicted using a target prediction program, TargetScan, which revealed that the 3'UTR of Wnt1 mRNA contains a complementary site for the miR-27. Accordingly, RT-PCR and immunoblotting were performed to observe the alteration of Wnt1 expression in the MDPC-23 cells after the transfection of miR-27. As shown in Fig. 5, the over-expression of miR-27 significantly increased the expression levels of Wnt1 mRNA (Fig. 5A) and protein (Fig. 5B) compared to control.

The 3'UTR of Wnt1, containing the miR-27 binding sites, was subcloned downstream of a luciferase reporter gene in pGL3 promoter vector and co-transfected with miR-27 or mimic miR-27 into MDPC-23 cells followed by luciferase activity assay in order to determine whether Wnt1 is a direct target of miR-27 (Fig. 6A). The luciferase activity assay showed that the relative luciferase activity increased significantly by miR-27 compared to pGL vector alone, pGL3-Wnt1 3'UTR and mimic miR-27 (Fig. 6B). These results hypothesized that the up-regulated Wnt1 expression by miR-27 induces the promotion of MDPC-23 odontoblastic cell differentiation.

Discussion

In recent years, the role of miRNA has been studied in biological events, including cell differentiation [6], development [7], proliferation [8], adipogenesis [16] and osteoblastogenesis [17]. However, the physiological role of miR-27 in the regulation of odontoblast differentiation is not entirely clear. In this study, therefore, the molecular mechanism of miR-27 on regulating odontoblast differentiation was examined in MDPC-23 odontoblastic cells. This study reports here that miR-27 promoted odontoblast differentiation by modulating Wnt1 signal pathway.

In the present study, the results were shown that miR-27 was significantly up-regulated during MDPC-23 cell differentiation and over-expression of miR-27 enhanced the cell differentiation process in MDPC-23 cells (Fig. 1). In addition, over-expressed miR-27 accelerated mineralization in MDPC-23 cells (Fig. 2) with increase of ALP mRNA and Col I mRNA (Fig. 3). Furthermore, to determine whether miR-27 induces the odontogenic specific differentiation in MDPC-23, it was measured the expressional level of DMP-1 and DSPP, which are well known a representative marker to identify the odontogenic differentiation [2,10]. As shown in PCR results, the expressions of DMP-1 and DSPP were gradually up-regulated in MDPC-23 cells transfected with miR-27 (Fig. 3). On the other hand, over-expressed miR-27 did not alter the cell proliferation in the MDPC-23 cells (Fig. 4). Taken together, these data suggest that miR-27 might be accelerating the differentiation of MDPC-23 cells without increasing cell proliferating.

Although the roles of miRNAs associated cell differentiation were demonstrated in several cell types, the role of miR-27 induced odontoblastic cell differentiation is not fully understood [10-12]. However, it has been reported that Wnt/β-catenin is an upstream regulatory molecules for DSPP for dentin mineralization in mouse dental paollar cells [18]. Moreover, Koizumin et al., have been reported that the expression of DSPP and DMP-1 in odontogenic differentiating by bone morphogenic protein 2 was mediated by Wnt/β-catenin signaling pathway

[19]. Consequently, in the present study, to investigate the cellular mechanism of miR-27-induced odontoblastic cell differentiation, the miR-27 target gene prediction associated with differentiation using TargetScan program was performed. The results of miR-27 target gene prediction revealed that Wnt1 was a prime molecule to promote ondontoblast differentiation. In addition, the over-expression of miR-27 significantly increased the expression levels of Wnt1 mRNA and protein (Fig. 5 and 6), which is suggesting that up-regulated Wnt1 by miR-27. Recently, it is reported that miR-27 promotes osteoblast differentiation by modulating Wnt signaling through the expressional down-regulation of APC in the human fetal osteoblastic 1.19 cell line [4]. These data suggested that up-regulated miR-27 in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells promotes odontoblast differentiation by targeting Wnt1 signaling. However, the mechanisms of odontoblast differentiation induced by miR-27 are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of odontoblast differentiation induced by miR-27. Overall, miR-27 might be considered a critical candidate as an odontoblastic cell differentiation molecular target for the development of miRNA based therapeutic agents in the dental medicine.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflicting interest.

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