2014년 2월
석사학위논문

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조선대학교 대학원

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이 논문을 치의학 석사학위신청 논문으로 제출함.

2013년 10월

조선대학교 대학원

치의학과

박 민 경

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2013년　11월

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ABSTRACT in KOREAN

MicroRNA-27의 adenomatous polyposis coli 표적작용을 통한 상아질모세포 분화 촉진

박 민 경

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상아질은 치아의 가장 중요한 구성 성분으로 치관과 치근의 대부분을 구성하며, 상아질모세포는 제Ⅰ형과 Ⅱ형 교원질과 같은 유기기질과 당단백 및 dentin sialophosphoprotein을 합성하고 분비한다. 상아질의 기질이 침착됨에 따라 상아질모세포가 세포질 돌기들을 원심 쪽으로 내어 상아세관에 묻히게 되고, 결과적으로 상아질모세포는 치수 가장자리 상아질의 내면에 위치하여 상아질을 유지하게 된다. 그러나 상아질모세포의 분화기전과 상아질의 형성과정을 조절하는 인자 그리고 그와 관련된 분자생물학적 기전은 아직까지 명확히 알려져 있지 않다. 생체에서 내인성으로 존재하는 microRNA(miRNA, miR)는 종간에도 잘 보존되어 있으며, 21-25개의 염기서열로 구성된 매우 작은 noncoding RNA 분자로 알려져 있다. miRNA는 RNA 간섭을 통하여 기능을 나타내며 주로 표적 유전자의 3’-UTR 결합부위에 상보적인 염기서열을 가지면서 그 표적유전자의 단백합성을 억제하거나 촉진시킨다. 또한 miRNA는 세포 내

다양한 유전자를 조절함으로써 세포분화, 성장과 증식 등 대부분의 생명현상에 관여하는 것으로 보고되고 있다. 본 연구에서는 상아질모세포로서 MDPC-23 세포주의 분화과정에서 miR-27에 의한 상아질모세포 분화의 촉진과 그 분자적 기전을 밝히고자 하였다.

상아질모세포의 분화과정에서 miR-27에 의한 상아질모세포의 분화기전을 분석하기 위해, 생쥐 치유두세포 유래의 상아질세포 전구세포주인 MDPC-23 세포주에서 분화유도와 miR-27을 처리한 후, miRNA array 분석, 정량 PCR 분석, Alizarin red S 염색, 세포증식 분석, immunoblotting, luciferase activity 분석 및 단백질 핵 전이 분석 등을 시행하여 다음과 같은 결과를 얻었다.

1. MDPC-23 세포주의 분화과정에서 miR-27의 발현은 증가하였다.
2. miR-27은 MDPC-23 세포주의 석회화를 촉진시켰다.
3. miR-27은 MDPC-23 세포주의 증식에는 영향을 주지 않았다.
4. miR-27은 MDPC-23 세포주의 분화과정에서 adenomatous polyposis coli(APC)의 발현을 억제하였다.
5. miR-27은 MDPC-23 세포주의 분화과정에서 luciferase 활성을 감소시켰다.
6. miR-27은 MDPC-23 세포주의 분화과정에서 세포질 β-catenin을 핵 내로 이동시켰다.

본 연구의 결과로서 상아질모세포 MDPC-23 세포주의 분화과정에서 그 발현이 증가된 miR-27은 APC의 활성을 억제시켜 β-catenin을 핵 내로 이동시킴으로서 상아질모세포의 분화를 촉진시키는 것으로 사료된다.

중심어: miR-27, 상아질모세포, 세포분화, β-catenin, adenomatous polyposis coli

I. 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 that becomes mineralized and forms dentin, the main hard tissue of a tooth. Dentin sialophosphoprotein and dentin matrix protein-1 synthesized and secreted by odontoblast are regarded as odontoblast differentiation markers [2]. 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]. Indeed, the exquisite balance between conserved signaling pathways and transcription factors is important for all aspects of odontoblast differentiation [2,3]. However, the exact molecular mechanisms underlying odontoblast differentiation are unclear.

Canonical Wnt signaling is very important for the differentiation of several cells including odontoblasts [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 complexes such as adenomatous polyposis coli (APC), anaphase-promoting complex, and glycogen synthase kinase 3b (GSK3b) [4]. In the absence of Wnt signaling, β

-catenin is constitutively phosphorylated by GSK3 on N-terminal residues and then targeted by ubiquitination [4]. The inhibition of GSK3 activity by Wnt results in the translocation of β-catenin to cell nuclei from the cell cytoplasm [6]. Nuclear β-catenin then functions as a transcriptional co-activator for the TCF/LEF family of transcription factors, and activates transcription of genes necessary for differentiation [6]. Wnt signaling must be tightly regulated for proper differentiation. However, the exact molecular mechanisms underlying odontoblast differentiation are unclear.

MicroRNA (miRNA) is an endogenous, non-protein-coding sequence that regulates the expression of genes, either by inhibiting or inducing mRNA transcription [7]. miRNA have been profiled by their typical mechanisms of transcriptional regulation in a variety of cells and cellular systems. Moreover, numerous cellular process of miRNA itself have been examined, including cellular differentiation [8], organism development [9], proliferation [10], and apoptosis [11]. Therefore, current research focuses on the utility of miRNA as diagnostic and prognostic tools as well as potential therapeutic targets. Although many studies related to miRNA are underway, their biological functions or cellular mechanisms are largely unknown.

There are a few studies indicating that post-transcriptional regulation of gene expression, mediated by miRNAs, is important in the control of odontoblast differentiation [2,12,13]. Gong et al. demonstrate that the differential expression miRNAs may be involved in governing human dental pulp cells (hDPCs)

odontogenic differentiation [12]. Liu et al. showed that the miR-143 and miR-145 control odontoblast differentiation and dentin formation through Klf4 and Osx transcriptional factor signaling pathways [13]. Sun et al. reported that miR-338-3p promote odontoblast differentiation through targeting Runx2 [2]. miR-27 could regulate adipogenesis, myeloblast differentiation, skeletal muscle development, and osteoblast differentiation [4,14,15]. However, the role of miR-27 in regulating odontoblast differentiation remains unknown.

Based on these findings, we investigated the molecular mechanism of miR-27 promoting odontoblast differentiation in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells. We found that increased expression of miR-27 contributed to nuclear accumulation of β-catenin by repressing APC expression, resulting in MDPC-23 cell differentiation.

II. MATERIALS AND METHODS

1. Cell culture

MDPC-23 odontoblastic cells provided by Dr. J.E. Nö r (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 and antibiotic -antimycotics (Invitrogen, Carlsbad, CA, USA) in a 5% CO2 atmosphere at 37℃. To induce cell differentiation and mineralized nodule formation, confluent MDPC-23 cells were treated with 50 μg/ml ascorbic acid and 10 mM ß-glycerophosphate for up to 10 days.

2. miRNA isolation and Affymetrix miRNA array analysis

Total RNAs including miRNAs from MDPC-23 cells of day 0, day 4 and day 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 RNAs were quantified using the Nano-Drop® ND-1000 ultraviolet Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The miRNA array was scanned using an Affymetrix GeneChip Platefom (DNA link, Seoul, Korea). For each sample, total RNAs were subjected to a tailing reaction (2.5 mM MnCl2, ATP, Poly A Polymerase - incubation for 15 min at 37℃) 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 adding of stop solution. Each sample was hybridized to a GeneChipH miRNA Array at 48℃ and 60 rpm for 16 hrs, then washed and stained on Fluidics Station 450 and scanned on a GeneChip® Scanner3000 7G (Affymetrix, Fremont, CA, USA). The image data were analyzed with the miRNA QC Tool software for quality control.

3. Quantitative real time-PCR (qRT-PCR) and quantitative PCR (qPCR)

Reverse transcription of the miRNA was performed using a miScript Reverse Transcription kit (Qiagen, Valencia, CA, USA) starting from 1 ㎍ 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 [16], were normalized against an internal control (U6 RNA), and the comparative threshold cycle method ($\Delta\Delta$Ct) 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 separate experiments. To perform qPCR, 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 ㎍ 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'-GTCTTCTCCA

CCGTGGGTCT-3'), type I collagen (Col I) (Col I-F, 5' -GTGAACCTGGCAAAC AAGGT-3' ; Col I-R, 5' -CTGGAGACCAGAGAAGCCAC-3') and GAPDH (GAPDH-F, 5' -TGCATCC TGCACCACCAACT-3' ; GAPDH-R, 5' -CGCCTGCTTCACCACCTTC-3') induction were measured by qPCR, and visualized by DNA agarose gel electrophoresis. The differences in expression are presented as a histogram after densitometry using a VersaDocTM imaging system (Biorad, Hercules, CA, USA).

4. 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-APC-3' UTR were transfected into cultured MDPC-23 cells using LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer' s instruction. The experimental group cells were treated with 10 ng/ml miRNAs for 2 days.

5. 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% NH4OH at pH 4.2-4.4. The mineralization assays were performed by staining MDPC-23 cells treated 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℃. Cell lysis 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.

6. Cell proliferation assay

The cells were seeded at a density of 4×10^4 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 LipofectamineTM 2000. The cells were incubated with miR-27 at a defined concentration (2, 20, and 200 ng/ml) for 1, 2 and 3 days at 37° C. After incubation under the defined conditions, the cells were incubated for a further four hrs in 20 μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). To dissolve the formazan transformed from MTT, the cells were resuspended in 150 μl dimethyl sulfoxide (DMSO) and the optical density (OD) of the solution was determined using a spectrometer at an incident wavelength of 495 nm. The experiments were repeated four times, independently.

7. Immunoblotting

To determine the level of APC 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-APC (1:500 dilution; Abcam, Cambridge, UK) 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) was used to magnify the signal.

8. Vector construction and luciferase activity assay

The 3' UTR of APC was amplified from genomic DNA, isolated from MDPC-23 cells using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instruction, using APC-3' UTR forward primer (5' -AAGAGAGGAAGAATGAAACTAAGAAA-3') and APC-3' UTR reverse primer (5' -TGCTGATCTCCATTGTTTATGGAA-3'). pGL3-promoter-APC-3' UTR was constructed by cloning the 3' UTR of APC (2,113 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-APC-3' UTR, 5 ng Renilla for normalization, and 0.5 μg of miR-27-WT or same amount of synthesized mimic miR-27. Luciferase activity was measured two days after transfection using the Dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instruction.

9. Nuclear translocation

To observe the β-catenin nuclear translocation, MDPC-23 cells transfected with miR-27 were fixed with 1% paraformaldehyde, permeabilized in 0.2% Triton X-100, and washed with phosphate buffered saline, and nonspecific signals were blocked with normal goat serum. The cells were incubated for overnight at 4℃ with rabbit anti-β-catenin antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with goat anti-rabbit IgG-heavy and light chain antibody DyLight® 488 (Bethyl). For nuclear counterstain, all cells were incubated with 4’,6-diamidino-2-phenylindole. Using Nikon Eclipse E2000 microscope (Nikon Instruments, Melville, NY, USA), nuclear images and cellular fluorescent stain were visualized with an ultraviolet filter and green filter, respectively.

10. Cell fractionation

The cells were washed twice with ice-cold PBS and harvested by scraping with a rubber policeman in lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl^2$, 0.5 mM DTT, 5 mM EDTA, 1 mM Na^3 VO^4 , 1 mM PMSF and 50 mM β-glycerophosphate). After incubation on ice for 15 min, cells were homogenized by passing through a 22 1/2G needle 20 times. The homogenate was centrifuged at 3,500 rpm for 5 min to sediment the nuclei. This supernatant was then centrifuged at 16,000 × g for 20 min and the subsequent supernatant was harvested as the non-nuclear fraction. The nuclear pellet was washed three times

with lysis buffer to remove contamination with cytoplasmic membranes. To extract nuclear proteins, isolated nuclei were re-suspended in lysis buffer and sonicated briefly. Nuclear lysates were collected after centrifugation. Immunoblotting was then performed with β-catenin antibody (1:1000 dilution; Santa Cruz Biotechnology) and with β-actin (cytosolic protein) and Lamin B (nuclear protein) as the internal controls.

11. Statistical analysis

All experiments were performed at least in triplicate. The results were presented as the mean ± SEM. Statistical significance was calculated using Student's t-test for two groups and one-way analysis of variance for multi-group comparisons using StatView version 5.0 for Windows. Null hypotheses of no difference were rejected if p-values were less than .05.

Ⅲ. RESULTS

1. miR-27 is significantly up-regulated during MDPC-23 odontoblastic cell differentiation

To determine the expression pattern of miRNA during MDPC-23 cell differentiation, we performed a miRNA microarray on cell samples of day 0, day 4 and day 7 of differentiation (n=4 per each cells) using affymetrix Genechip. In this analysis, miR-27 in MDPC-23 cells of day 4 and day 7 of differentiation was significantly up-regulated approximately three-fold compared with 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 differentiation medium (Fig. 1B). This shows that the miR-27 was significantly up-regulated during MDPC-23 cell differentiation and suggests that over-expressed miR-27 increases cell differentiation in MDPC-23 cells.

2. miR-27 accelerates mineralization in MDPC-23 odontoblastic cells

We measured the effect of miR-27 on odontoblastic cell mineralization. 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 seven days of culture. The miR-27 transfected MDPC-23 cells showed mineralized nodules after four days, and the mineralized nodules increased with time during the culture period (Fig. 2A). To study the potential role of miR-27 in MDPC-23 differentiation, the cells transfected with miR-27 were analyzed. qPCR showed that miR-27 enhanced MDPC-23 odontoblastic differentiation, indicated by higher expression of the odontoblast marker gene ALP and lower expression of Col I, accompanied by increased ALP activity and decreased Col I activity in comparison to cells transfected with control (Fig. 2B). As shown in Fig. 2C, the up-regulated miR-27 did not alter the cell proliferation in the MDPC-23 cells. These results suggest that up-regulation of miR-27 levels positively regulated MDPC-23 cell differentiation.

3. Upregulated miR-27 in MDPC-23 cells promotes differentiation by targeting APC and activating Wnt/β-catenin signaling

To investigate the molecular mechanisms by which miR-27 promotes MDPC-23 cells differentiation, putative miR-27 targets were predicted using TargetScan, a target prediction program. The results of miR-27 target prediction revealed that the 3' UTR of APC mRNA contains a complementary site for the miR-27. Therefore, we performed western blot to observe the alteration of APC expression in the MDPC-23 cells after the transfection of miR-27. As shown in Fig. 3A, the over-expression of miR-27 significantly decreased the expressional levels of APC compared with control in a time-dependent manner. To determine

whether APC is a direct target gene of miR-27, the 3' UTR of APC containing the miR-27 binding sites was subcloned into a downstream of the luciferase reporter gene in pGL3 promoter vector as shown as Fig 3B. pGL3 reporter vector subcloned with 3' UTR of APC was co-transfected with miR-27 or mimic miR-27 into MDPC-23 cells followed by luciferase activity assay. The luciferase activity assay showed that the relative luciferase activity decreased significantly by miR-27 compared with pGL vector only, pGL3-APC 3' UTR, and mimic miR-27 (Fig. 3C). Based on these results, we hypothesized that the down-regulated APC expression by miR-27 targeting induced the activation of Wnt/β-catenin signaling to promote the differentiation of MDPC-23 cells via degradation of β-catenin complex in the cytosol. Therefore, we observed the nuclear translocalization of β-catenin after transfection of miR-27 into MDPC-23 cells. As shown in Fig. 3D, the cytosolic β-catenin of MDPC-23 cells were significantly translocated toward the nucleus by miR-27 transfection. Furthermore, cytosolic β-catenin decreased in a time dependent manner after miR-27 transfection, whereas the nucleus β-catenin increased significantly (Fig. 3E).

Ⅳ. DISCUSSION

The role of miRNA is a reseach focus in biological events, including cell differentiation [8], development [9], proliferation [10], tumorigenesis [17] and apoptosis [7]. In addition, miRNAs regulate adipogenesis [18], myeloblast differentiation [14], skeletal muscle development [15], and recently noted as regulating osteoblastogenesis [19]. On the other hand, miRNA mechanisms in regulating odontoblast differentiation are unclear. We report here that miR-27 promoted odontoblast differentiation by modulating Wnt signal pathways.

The function of miR-27 was recently reported associated with the regulation of several biological processes [4,14,20]. Wang and Xu reported that miR-27 is an important mediator of osteoblast differentiation, thus offering a new target for the development of preventive or therapeutic agents against osteogenic disorders [4]. In addition, Feng et al. suggested that miR-27 enhances differentiation of myeloblasts into granulocytes via post-transcriptional down-regulation of Runx1 [14]. Lin et al. showed that the miR-27 gene family is down-regulated during adipogenic differentiation, and over-expressed miR-27 specifically inhibits adipocyte formation [20]. However, the physiological role of miR-27 in the regulation of odontoblast differentiation is not entirely clear. We concluded that miR-27 might regulate odontoblast differentiation.

In the present study, miR-27 levels were significantly up-regulated during MDPC-23 odontoblastic cell differentiation (Fig. 1). In addition, over-expressed

miR-27 accelerated mineralization in MDPC-23 odontoblastic cells with an increase of ALP mRNA and decrease of Col I mRNA, while over-expressed miR-27 did not alter cell proliferation in the MDPC-23 cells (Fig. 2). These results suggest that up-regulation of miR-27 levels positively regulated MDPC-23 cell differentiation without increasing cell proliferation.

Although the roles of miRNAs associated with cell differentiation are described for various cell types, miR-27 induced odontoblastic cells differentiation is not fully understood. To investigate the cellular mechanism of miR-27-induced odontoblastic cell differentiation, we performed the miR-27 target gene prediction associated with differentiation using TargetScan program. The results of miR-27 target gene prediction revealed that APC was a prime molecule to promote ondontoblast differentiation.

In the canonical Wnt/β-catenin pathway, APC is a key regulator associated with several cellular processes including development and proliferation [21]. However, the binding of Wnt protein on fizzled seven transmembrane receptor induces the formation of dimeric receptors with low-density lipoprotein receptor-related protein (LRP) to initiate the activation of Wnt/β-catenin signaling. These dimeric receptors lead to intact destruction complex through binding to phosphorylated LRP. The β-TrCP phosphorylates β-catenin on the destruction complex is captured on LRP without ubiquitination to prevent the degradation by proteasome. At the end of the path, Wnt/β-catenin signaling pathway is activated via the translocation and accumulation of newly synthesized β-catenins in the nucleus [22]. Furthermore, the mutation or expressional down-regulation of component of destruction complex could lead to mimicking activation of Wnt/β

-catenin signaling pathway without Wnt protein [23,24]. Wang and Xu recently 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, hFOB [4]. We also showed that miR-27 significantly suppressed the expressional level of APC in MDPC-23 cells, suggesting that down-regulated APC by miR-27 is induced by mimicking the activation of Wnt/β-catenin signaling pathway (Fig. 3A and C). Furthermore, we showed that β-catenin significantly translocated and accumulated nuclear translocation and accumulation of β-catenin in the MDPC-23 cells. These data clearly suggest that up-regulated miR-27 in MDPC-23 cells promotes odontoblastic cell differentiation by targeting APC and activating Wnt/β-catenin signaling (Fig. 4). Therefore, miR-27 might be a critical candidate as an odontoblastic differentiation molecular target for the development of miRNA based therapeutic agents in dental medicine.

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Ⅵ. FIGURE LEGENDS

Fig. 1. 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 ß-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. *$p<0.05$ or ***$p<0.001$ vs. day 0.

Fig. 2. 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 mineralization was evaluated by Alizarin red S staining (upper panel). The mineralization was quantified by colorimetric spectrophotometry (lower panel). Each data point represents the mean ± SEM of three experiments. ***$p<0.001$

vs. control (the control cells were measured in the absence of miR-27 transfection). (B) Effect of miR-27 on ALP and Col I mRNA expressions. The percentage of ALP and Col I mRNA expressions was calculated as a ratio of GAPDH band (lower panel). (C) Measurement of MDPC cell proliferation by up-regulated miR-27. The cell proliferation was determined by MTT assays after miR-27 transfection.

Fig. 3. Activation of Wnt/β-catenin signaing through the down-regulation of APC by miR-27.

(A) The expressional level of APC was suppressed by miR-27. MDPC-23 cells were transfected with miR-27 according to the defined condtions. The expressional level of APC was assayed by western blotting according to standard methods. (B) The schematic diagram to generate the reporter vector. The 3' UTR of APC was cloned by PCR and subclond into the downstream of luciferase gene in the pGL3 promoter vector. (C) The luciferase activity of generated reporter vector containing 3' UTR of APC was siginificantly down-regulated by miR-27. MDPC-23 cells were co-transfected with generated reporter vector containing 3' UTR of APC, miR-27, and mimic miR-27 according to the defined condition. Luciferase activity was assayed by manufacturer' s instruction. Each data point represents the mean ± SEM of three experiments. (D) Nuclear translocation of β-catenin. After miR-27 transfection into MDPC-23 cells, the nuclear translocation of β-catenin was visualized by β-catenin

antibody conjugated with 2nd FITC antibody and DAPI staining for nucleus. (E) Accumulation of β-catenin in the nucleus of MDPC-23 cells. Cytosolic and nuclear protein were obtained by methods described in M&M. β-catenin was assayed by western blotting. β-actin and Lamin B was used as internal normalization for cytosolic and nucleus protein, respectively.

Fig. 4. Cellular mechanism of miR-27-induced promoting odontoblastic cell differentiation.

Ⅶ. FIGURES

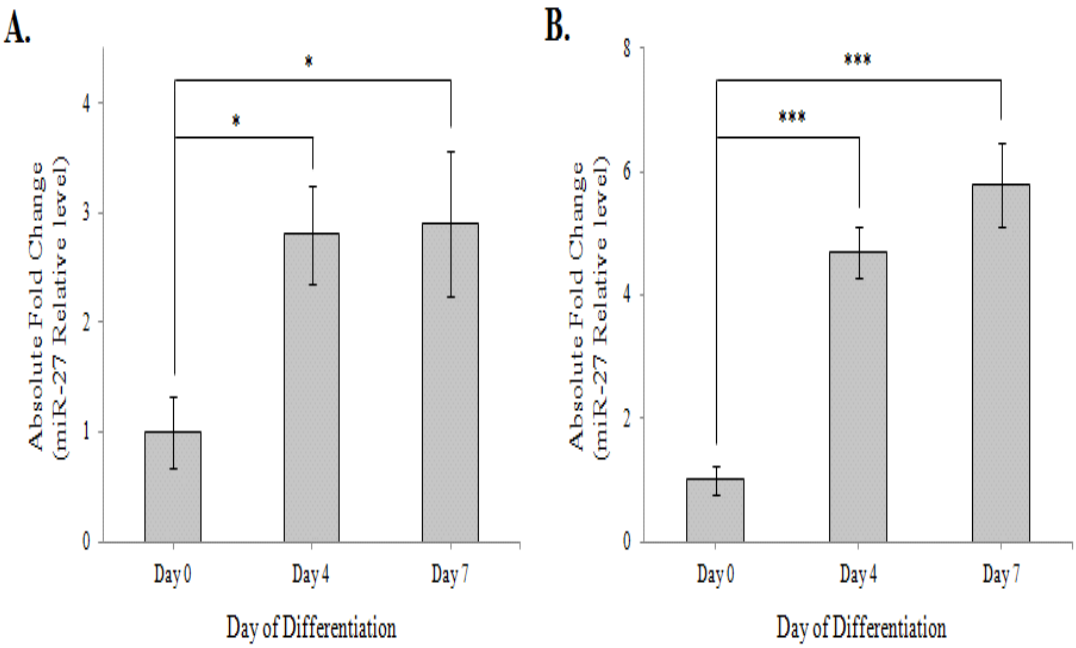
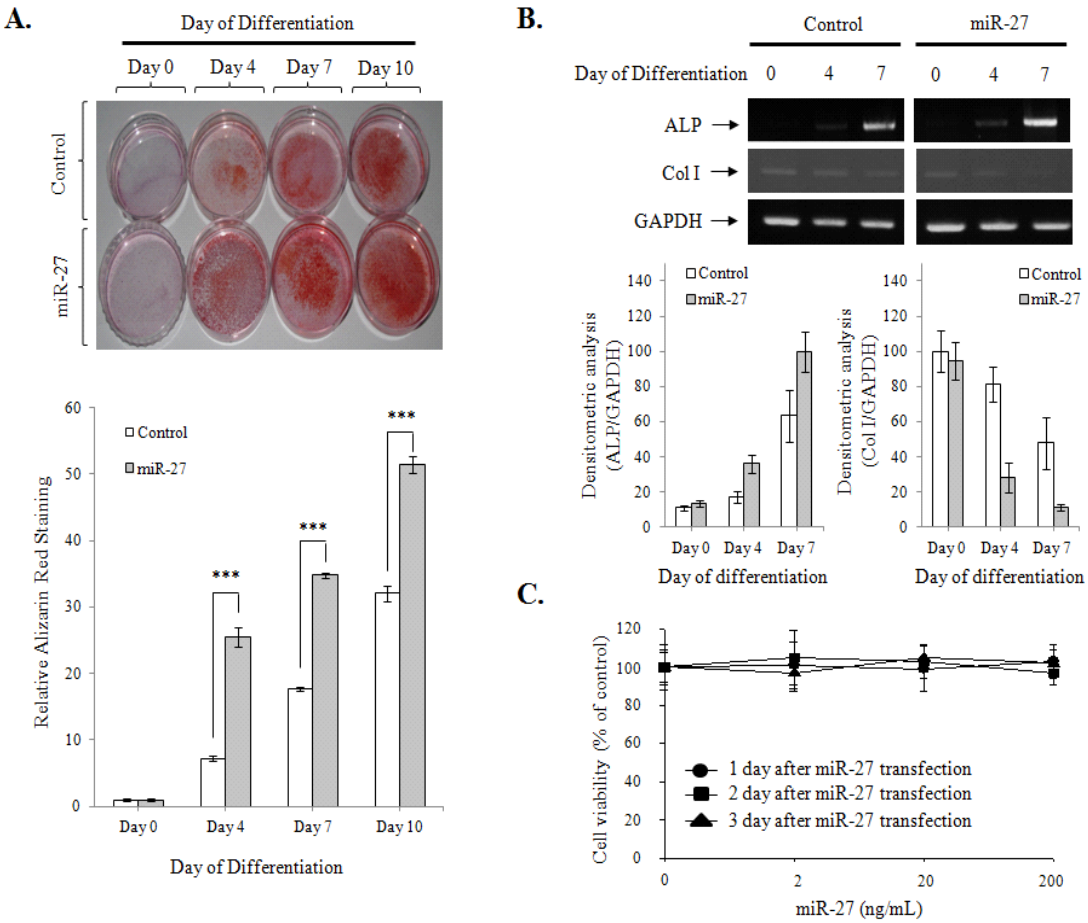


Fig. 1. Relative expression of miR-27 during MDPC-23 odontoblastic cell differentiation.

Ⅶ. FIGURES



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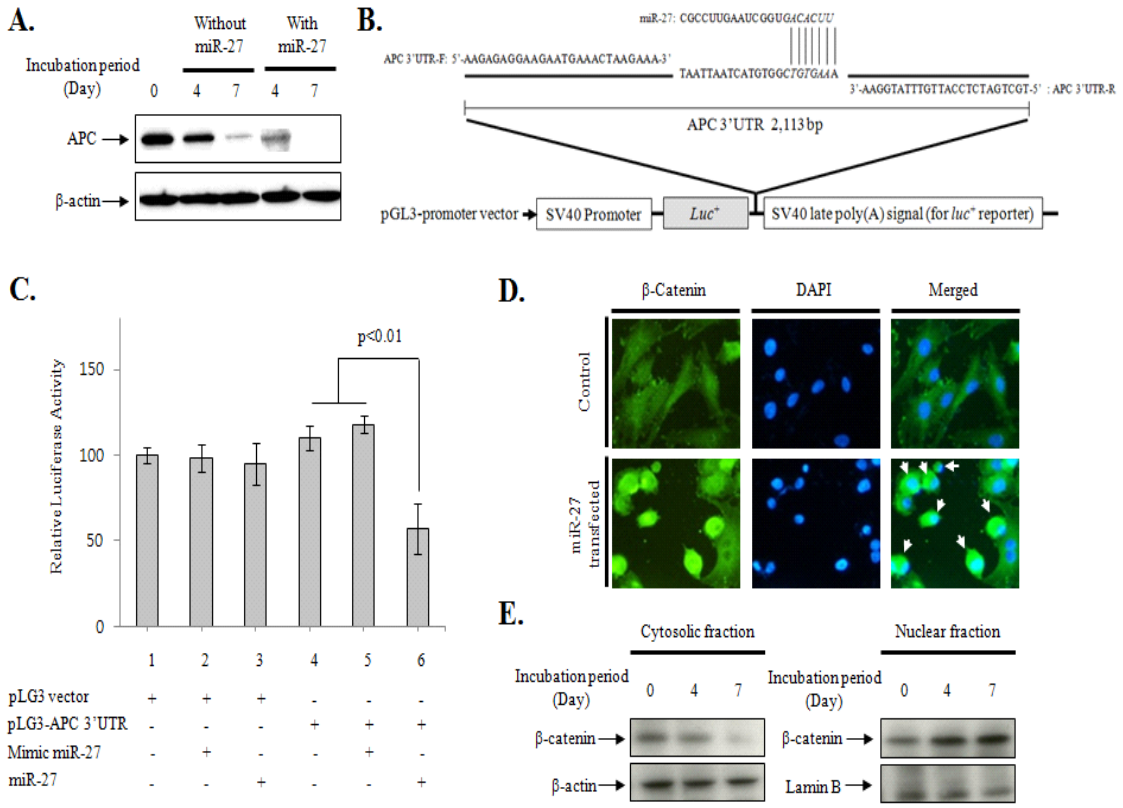
Ⅶ. FIGURES



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Ⅶ. FIGURES

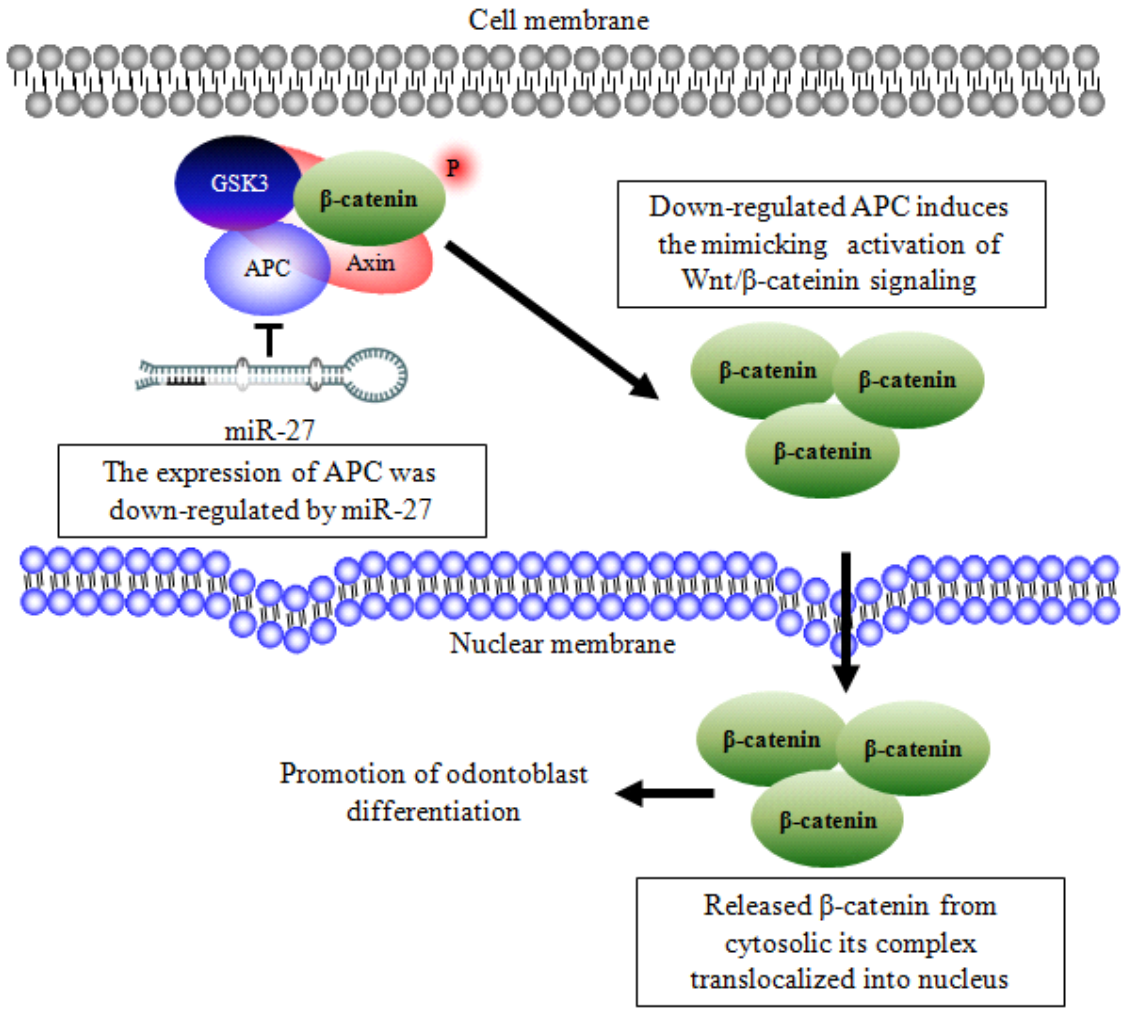

Fig. 4. Cellular mechanism of miR-27-induced promoting odontoblastic cell differentiation.

ABSTRACT

MicroRNA-27 promotes odontoblastic cell differentiation by targeting APC and activating Wnt/β-catenin signaling

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MicroRNAs (miRNAs) are essential in regulating cell differentiation either by inhibiting mRNA translation or by inducing its degradation. However, the role of miRNAs in odontoblastic cell differentiation is largely unknown. In the present study, we demonstrate that the expression of miR-27 increased significantly during MDPC-23 odontoblastic cell differentiation. Furthermore, the up-regulation of miR-27 promotes the differentiation of MDPC-23 odontoblastic cells and accelerates mineralization without cell proliferation. In addition, our target gene prediction results revealed that the mRNA of adenomatous polyposis coli (APC) associated with Wnt/β-catenin signaling pathway has an

miR-27 binding site in its 3' UTR and is suppressed by miR-27. Subsequentially, the down-regulated APC by miR-27 triggered the activation of Wnt/β-catenin signaling through accumulation of β-catenin in the nucleus. Our findings suggest that miR-27 promotes MDPC-23 odontoblastic cell differentiation by targeting APC and activating Wnt/β-catenin signaling. Therefore, miR-27 is a critical candidate as an odontoblastic differentiation molecular target for the development of miRNA based therapeutic agents in dental medicine.

Keywords: miR-27, odontoblasts, differentiation, β-catenin, adenomatous polyposis coli