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Downregulation of *adenomatous polyposis coli* by microRNA-663 promotes odontogenic differentiation through activation of Wnt/beta-catenin signaling

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ABSTRACT

MicroRNAs (miRNAs) regulate cell differentiation by inhibiting mRNA translation or by inducing its degradation. However, the role of miRNAs in odontogenic differentiation is largely unknown. In this present study, we observed that the expression of miR-663 increased significantly during differentiation of MDPC-23 cells to odontoblasts. Furthermore, up-regulation of miR-663 expression promoted odontogenic differentiation and accelerated mineralization without proliferation in MDPC-23 cells. In addition, target gene prediction for miR-663 revealed that the mRNA of the *adenomatous polyposis coli* (APC) gene, which is associated with the Wnt/β-catenin signaling pathway, has a miR-663 binding site in its 3′-untranslated region (3′UTR). Furthermore, APC expressional was suppressed significantly by miR-663, and this down-regulation of APC expression triggered activation of Wnt/β-catenin signaling through accumulation of β-catenin in the nucleus. Taken together, these findings suggest that miR-663 promotes differentiation of MDPC-23 cells to odontoblasts by targeting APC-mediated activation of Wnt/β-catenin signaling. Therefore, miR-663 can be considered a critical regulator of odontoblast differentiation and can be utilized for developing miRNA-based therapeutic agents.

1. Introduction

Dentin, which forms the bulk of the tooth, is a mineralized tissue composed of 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, which subsequently mineralize to form dentin, the main hard tissue of a tooth. Dentin sialophosphoprotein and dentin matrix protein-1, which are synthesized and secreted by odontoblasts, 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 Runt-related transcription factor 2 (Runx2) are involved in odontoblast differentiation [2–4]. Indeed, the balance between conserved signaling pathways and transcription factors is important for all aspects of odontoblast differentiation [2,3]. However, the molecular mechanism underlying odontoblast differentiation remains unclear.

Canonical Wnt signaling is very important for the differentiation of several cells including odontoblasts [4,5]. Wnt ligands bind to a heterodimeric complex formed by the LRP5/6 co-receptor and a member of the frizzled receptor family [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 for 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

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necessary for differentiation [6]. Wnt signaling must be tightly regulated for proper differentiation.

MicroRNA (miRNA) is an endogenous, non-protein-coding RNA that regulates the expression of genes, either by inhibiting or by promoting mRNA transcription [7]. miRNAs have been profiled by their typical mechanisms of transcriptional regulation in a variety of cells and cellular systems. Moreover, numerous cellular processes of miRNAs 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. Despite the numerous studies on miRNAs, their biological functions or mechanisms of action are not well understood.

Few studies indicated that post-transcriptional regulation of gene expression mediated by miRNAs is important to control the differentiation of odontoblast [2,12,13]. Gong et al. demonstrated that the differential expression miRNAs may be involved in governing odontogenic differentiation of human dental pulp cells (hDPCs) [12]. Liu et al. showed that the miR-143 and miR-145 control odontoblast differentiation and dentin formation through Kruppel-like factor 4 (Klf4) and Osteoblast-specific transcription factor Osterix (Osx) mediated signaling pathways [13]. Sun et al. reported that miR-338-3p promotes odontoblast differentiation through targeting Runx2 [2]. On the other hand, miR-663 could be associated with cellular senescence, immunity, inflammation and cancer [14,15]. In addition, many studies suggested that miR-663 acts as a tumor suppressor [14,15]. Nevertheless, at present, the role of miR-663 in regulating odontoblast differentiation remains unknown.

Based on these findings, we investigated the molecular mechanism of miR-663 promoting odontoblast differentiation in MDPC-23 odontoblastic cells derived from mouse dental papilla. We found that increased expression of miR-663 led to the nuclear accumulation of β-catenin by repressing APC expression, resulting in MDPC-23 cell differentiation.

2. Materials and methods

2.1. Cell culture

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 and antibiotic–antimycotics (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37 °C. To induce cell differentiation and mineralized nodule formation, confluent MDPC-23 cells were treated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for up to 10 days.

2.2. 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-Drop® ND-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 °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 °C and 60 rpm for 16 h, 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.

2.3. 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 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 [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-663 (5′-AGGCG GGGCGCCGCGGGACCGC-3′) was measured by qRT-PCR.

The deviations in the samples represent four independent 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 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′-GTCTTCT CCACCGTGGGTCT-3′), type I collagen (Col I) (Col I-F, 5′-TAAGTTGC-CAAGAACGTGCC-3′; Col I-R, 5′-AATTGAAAGCCAGGAGGCAT-3′), bone sialoprotein (BSP) (BSP-F, 5′-AAGAAAATGGAGACGGCGAT-3′; BSP-R, 5′-CACCTGCTTCAGTGACGCTT-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′-ATAGCACCAACCATGAGGCT-3′; DSPP-R, 5′-CTTT TGTTGCCTTTGTTGGG-3′) and GAPDH (GAPDH-F, 5′-TGCATCCTGCA CCACCAACT-3′; GAPDH-R, 5′-CGCCTGCTTCACCACCTTC-3′) induction were measured by qPCR, 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).

2.4. miRNAs and transfection

miR-663 and scrambled miR-663 were purchased from Ambion (Austin, TX, USA). The miR-663, mimic miR-663 and pGL3-promoter-APC-3′ untranslated region (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.

2.5. 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% 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 °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.

2.6. 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-663 using Lipofectamine™ 2000. The cells were incubated with miR-663 at various defined concentrations (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 another 4 h 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. Four independent experiments were performed.

2.7. Immunoblotting

To determine the level of APC in MDPC-23 cells transfected with miR-663, 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, Rockford, IL, USA) was used to magnify the signal.

2.8. 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 (2113 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-663-WT or same amount of synthesized mimic miR-663. Luciferase activity was measured after two days of transfection using the Dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instruction.

2.9. Nuclear translocation

To observe the nuclear translocation of β-catenin, MDPC-23 cells transfected with miR-663 were fixed with 1% paraformaldehyde and permeabilized in 0.2% Triton X-100. The cells were then washed with phosphate buffered saline and nonspecific signals were blocked with normal goat serum. The cells were incubated overnight at 48 °C with 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 Laboratories, Inc., Montgomery, TX, USA). For nuclear counterstain, all cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI). 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.

2.10. 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_3VO_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 3500 rpm for 5 min to sediment the nuclei. The supernatant was then centrifuged at 16,000g 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 any 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) antibodies as the internal controls.

2.11. 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 0.05.

3. Results

3.1. miR-663 expression was significantly up-regulated during MDPC-23 cell differentiation to odontoblast

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-663 in MDPC-23 cells from days 4 and 7 of differentiation was significantly up-regulated over fourfold compared to control cells of day 0 (Fig. 1A). The miRNA array data was verified by examining the expression pattern of miR-663 by miRNA qRT-PCR. In miRNA qRT-PCR analysis, miR-663 levels were increased in MDPC-23 cells maintained in odontoblastic differentiation medium (Fig. 1B). These results showed that miR-663 was significantly up-regulated during MDPC-23 cell differentiation and over-expression of miR-663 enhanced the cell differentiation process in MDPC-23 cells.

3.2. miR-663 accelerates mineralization in MDPC-23 odontoblastic cells

The effect of miR-663 on odontoblastic cell mineralization was measured. MDPC-23 cells were cultured for 10 days in differentiation media transfected with miR-663 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-663 transfected MDPC-23 cells showed mineralized nodules after four days that increased with time during the culture period (Fig. 2A). The potential role of miR-663 in MDPC-23 cell differentiation was evaluated by analyzing the cells transfected with miR-663. The qPCR result showed that miR-663 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. 2B). Furthermore, BSP, a bone marker gene, was

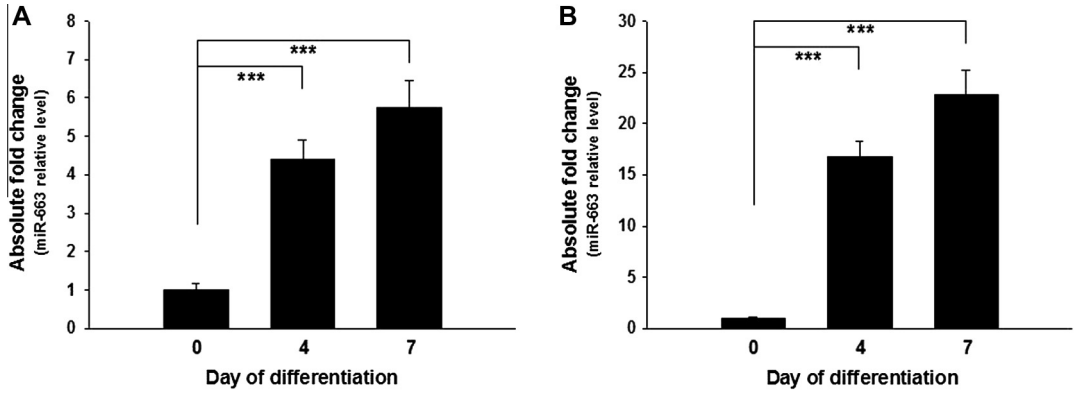

Fig. 1. Relative expression of miR-663 during MDPC-23 differentiation to odontoblasts. Confluent cultures of MDPC-23 cells were maintained in complete medium with the addition of differentiation cocktail (50 μg/ml ascorbic acid and 10 mM β-glycerophosphate). Cells were harvested following 0, 4 and 7 days of incubation and endogenous expression level of miR-663 was measured. The relative expression of miR-663 was determined by miRNA array using Affymetrix Genechip (A) and by qRT-PCR (B). Each data point represents the mean ± SEM of four experiments. $^{***}p < 0.001$ vs. day 0.

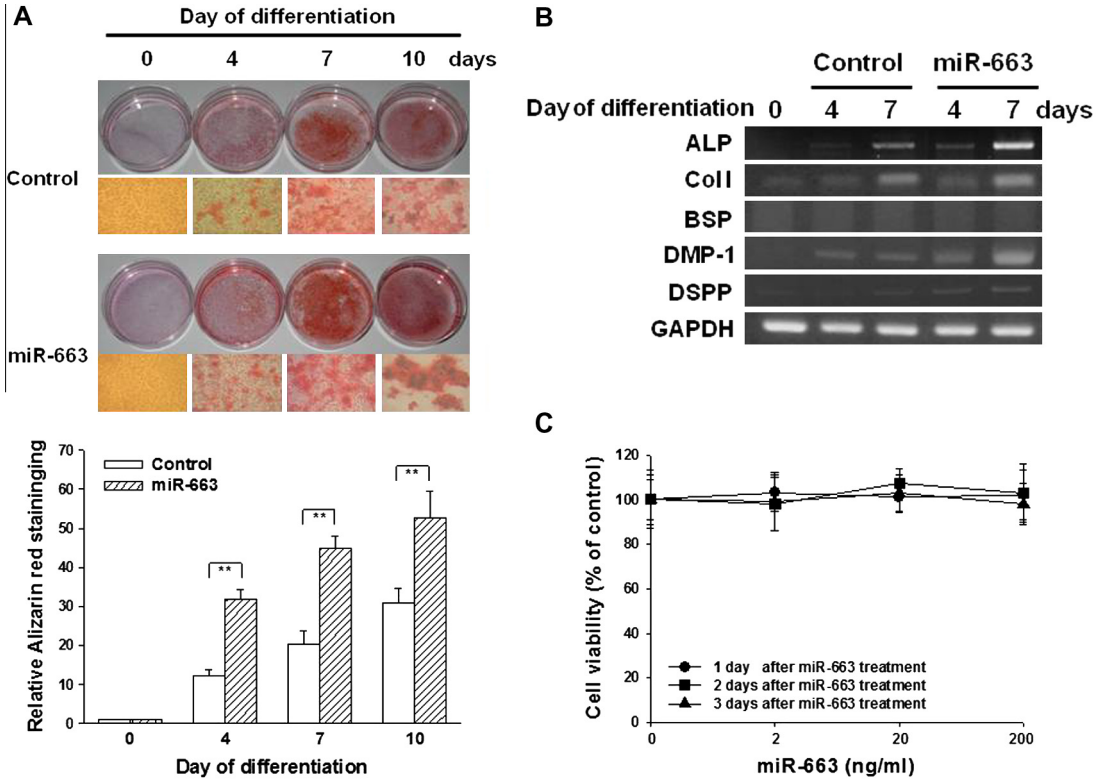

Fig. 2. Effect of miR-663 on mineralization in MDPC-23 cells. MDPC-23 odontoblast-like cell mineralization was altered by over-expressed miR-663. (A) Mineralized nodule formation in MDPC-23 cells. MDPC-23 cells were transfected with miR-663 for 10 days, and mineralization was evaluated by Alizarin red S staining (upper panel). The mineralization was quantified by colorimetric spectrophotometry (lower panel). Each data point represents the mean ± SEM of three experiments. $^{**}p < 0.01$ vs. control (the control cells were measured in the absence of miR-663 transfection). (B) Effect of miR-663 on ALP, Col I, BSP, DMP-1 and DSPP mRNA expressions. (C) Measurement of MDPC cell proliferation by up-regulated miR-663. Cell proliferation was determined by MTT assays after miR-663 transfection.

not expressed at any experimental group (Fig. 2B). Additionally, up-regulation of miR-663 did not alter the proliferation of the MDPC-23 cells (Fig. 2C), suggesting that up-regulation of miR-663 positively regulated the differentiation of MDPC-23 cells.

3.3. miR-663 upregulation in MDPC-23 cells promotes differentiation by targeting APC and activating Wnt/β-catenin signaling

The molecular mechanisms by which miR-663 promotes differentiation of MDPC-23 cells were investigated. Putative miR-663 targets were predicted using a target prediction program, TargetScan, which revealed that the 3′UTR of APC mRNA contains a complementary site for the miR-663. Accordingly, Western blotting was performed to observe the alteration of APC expression in the MDPC-23 cells after the transfection of miR-663. As shown in Fig. 3A, the over-expression of miR-663 significantly decreased the expression levels of APC compared to control in a time-dependent manner. The 3′UTR of APC, containing the miR-663 binding sites, was subcloned downstream of a luciferase reporter gene in pGL3 promoter vector and co-transfected with miR-663 or mimic

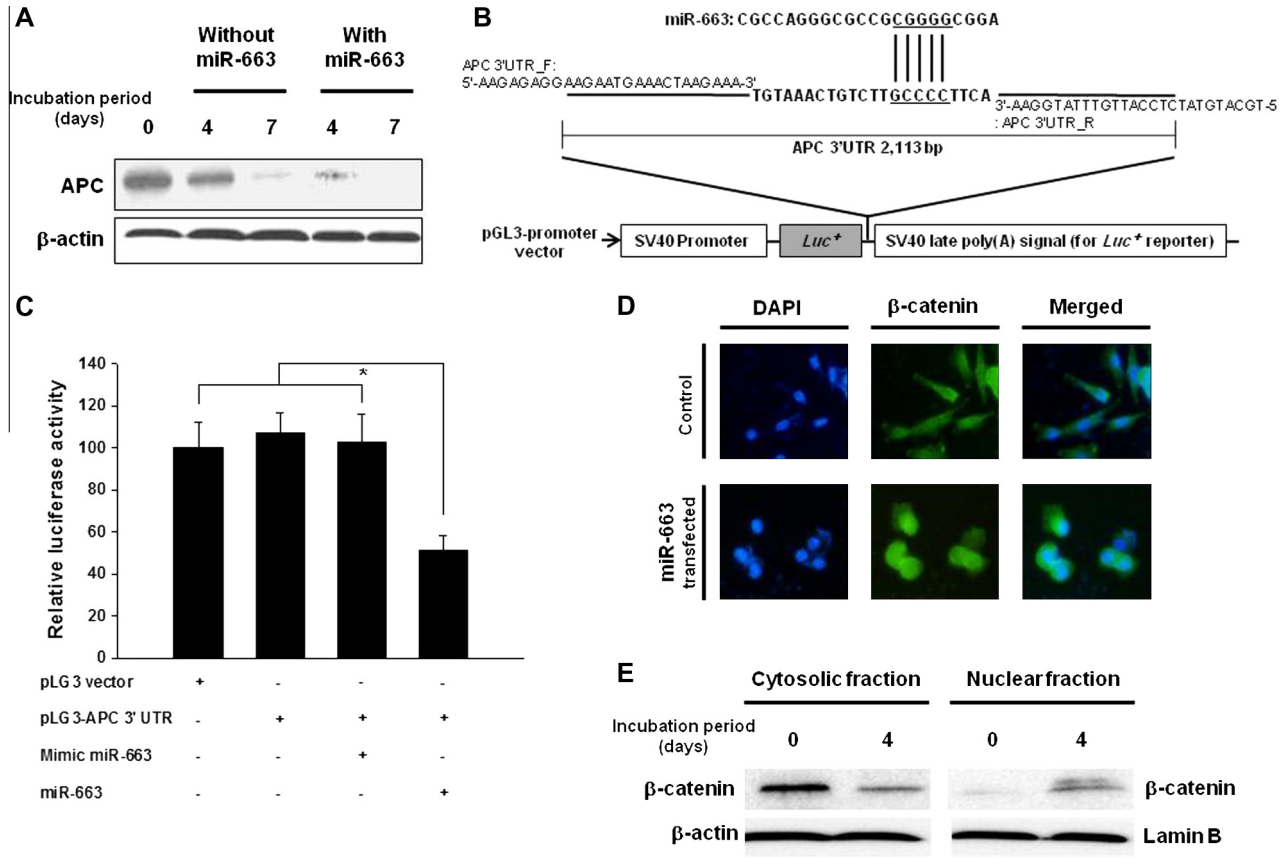

Fig. 3. Activation of Wnt/β-catenin signaling through down-regulation of APC by miR-663. (A) The expression of APC was suppressed by miR-663. MDPC-23 cells were transfected with miR-663 according to the defined conditions. The expression level of APC was assayed by Western blotting according to standard methods. (B) The schematic representation of the construction of reporter vector. The 3′UTR of APC was cloned by PCR and subcloned into the downstream of luciferase gene in the pGL3 promoter vector. (C) The luciferase activity of the generated reporter vector containing 3′UTR of APC was significantly down-regulated by miR-663. MDPC-23 cells were co-transfected with the generated reporter vector containing 3′UTR of APC, miR-663 and mimic miR-663 according to the defined conditions. Luciferase activity was measured as per manufacturer's instruction. Each data point represents the mean ± SEM of three experiments. (D) Nuclear translocation of β-catenin. After miR-663 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 of nucleus. (E) Accumulation of β-catenin in the nucleus of MDPC-23 cells. Cytosolic and nuclear proteins were obtained by methods described in Section 2. Relative level of β-catenin was determined by Western blotting using β-actin and Lamin B as internal controls for cytosolic and nuclear proteins respectively.

miR-663 into MDPC-23 cells followed by luciferase activity assay in order to determine whether APC is a direct target of miR-663 (Fig. 3B). The luciferase activity assay showed that the relative luciferase activity decreased significantly by miR-663 compared to pGL vector alone, pGL3-APC 3′UTR and mimic miR-663 (Fig. 3C). These results hypothesized that the down-regulated APC expression by miR-663 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. Thus, the nuclear translocation of β-catenin was studied after transfection of miR-663 into the MDPC-23 cells. The cytosolic β-catenin of MDPC-23 cells was significantly translocated toward the nucleus by miR-663 transfection (Fig. 3D). Furthermore, cytosolic β-catenin was decreased after miR-663 transfection, whereas the nuclear β-catenin increased significantly (Fig. 3E).

4. Discussion

The role of miRNA is in the focus of biological research, including cell differentiation [8], development [9], proliferation [10], tumorigenesis [17] and apoptosis [7]. In addition, miRNAs regulate adipogenesis [18] and have recently been shown to regulate osteoblastogenesis as well [19]. However, the regulatory role of miRNAs in odontoblast differentiation is unclear. In this study, we report that miR-663 promotes odontoblast differentiation by modulating Wnt signaling pathway.

Many studies show that miR-663 has anti-inflammatory effects and functions as a tumor suppressor [14,15]. In addition, Jian et al. suggested that miR-663 induced significant HL-60 cell differentiation [20]. Taken together, these reports suggest that the down-regulation of miR-663 expression might be helpful to promote tumorigenesis. However, the physiological role of miR-663 in the regulation of odontoblast differentiation is not entirely known. Thus, our aim was to investigate the physiological role of miR-663 associated with odontogenic differentiation in MDPC-23 cells.

Our study shows that, miR-663 levels were significantly up-regulated during differentiation of MDPC-23 cells to odontoblasts (Fig. 1). In addition, while over-expressed miR-663 accelerated mineralization in MDPC-23 odontoblastic cells with increase in ALP and Col I mRNAs, well known markers of odontoblastic differentiation [21], over-expressed miR-663 did not alter the cell proliferation in the MDPC-23 cells (Fig. 2). Furthermore, to determine whether miR-663 induces the odontogenic differentiation in MDPC-23, we measured the expression levels of DSPP and DMP-1, well known representative markers of odontogenic differentiation [21]. Our qPCR results show that the expression of DSPP and DMP-1 were gradually up-regulated in MDPC-23 cells transfected with miR-663. Besides, the expression of osteogenic differentiation marker gene, BSP, was not observed in MDPC-23 cells

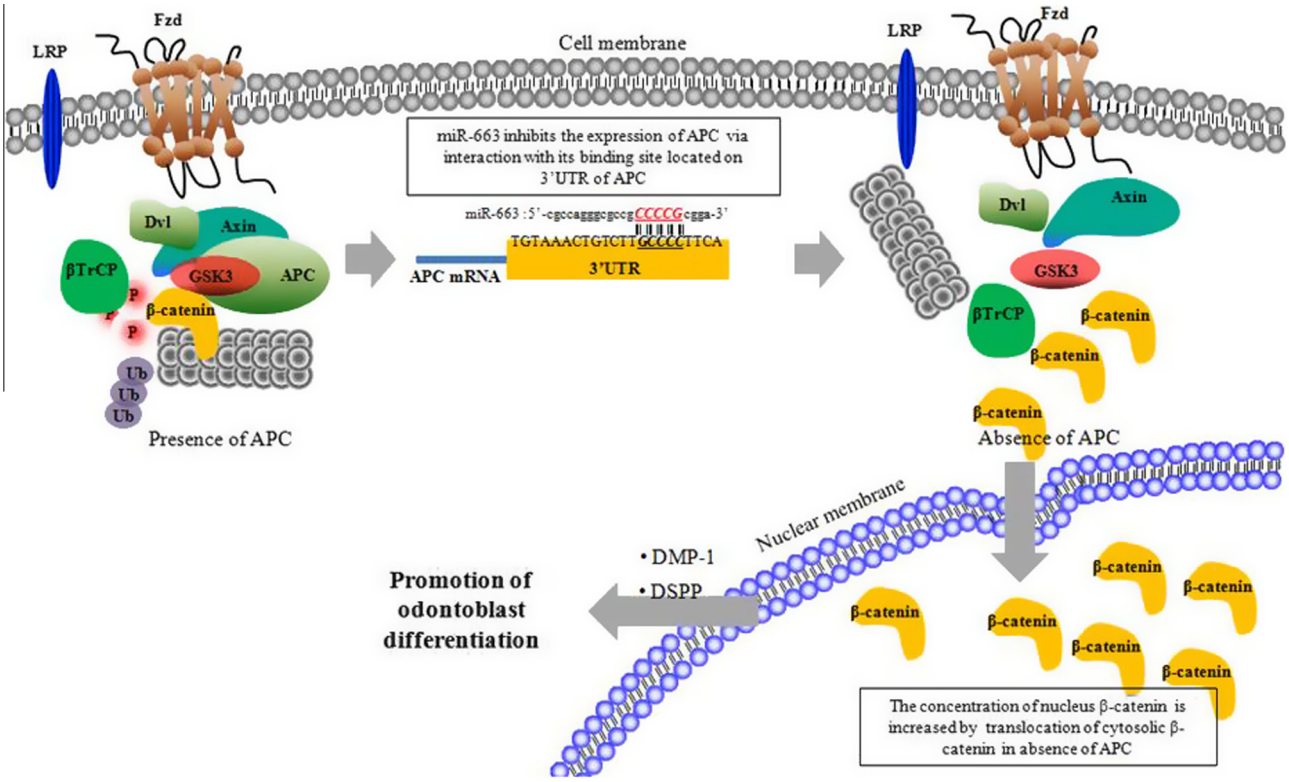

Fig. 4. Mechanism of miR-663-mediated promotion of odontoblast differentiation.

after miR-663 transfection. Taken together, these data suggest that miR-663 may positively accelerate the differentiation of MDPC-23 cells compared to control without inducing cell proliferation.

Although, the functions of miRNAs associated with cell differentiation have been described for various cell types, miR-663 induced odontoblastic cells differentiation is not fully understood. To investigate the cellular mechanism of miR-663-induced odontoblastic cell differentiation, we performed the miR-663 target gene prediction associated with differentiation using TargetScan program. The target gene prediction revealed that APC was a prime target of miR-663 to promote odontoblast differentiation.

In the canonical Wnt/β-catenin pathway, APC is the key regulator associated with several cellular processes including development and proliferation [22]. However, the binding of Wnt protein on frizzled 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 an intact destruction complex through binding of phosphorylated LRP. The β-TrCP phosphorylates β-catenin on the destruction complex captured on LRP without ubiquitination to prevent the degradation by proteasome. Wnt/β-catenin signaling pathway is activated via the translocation and accumulation of newly synthesized β-catenins in the nucleus [23]. Furthermore, mutation or down-regulated expression of components of destruction complex could lead to mimic the activation of Wnt/β-catenin signaling pathway without Wnt protein [24,25]. We showed that miR-663 significantly suppressed the expression level of APC in MDPC-23 cells, suggesting that down-regulation of APC by miR-663 is induced by mimicking the activation of Wnt/β-catenin signaling pathway (Fig. 3). Furthermore, we demonstrated that β-catenin significantly translocated and accumulated in nuclei of the MDPC-23 cells. Similar to our results, Hu et al., reported that MicroRNA-142-3p promoted Wnt signaling through inhibition of APC, leading to accumulation and nuclear translocation of β-catenin in the human fetal osteoblastic 1.19 (hFOB1.19) [26]. These data clearly suggest that miR-663 in MDPC-23 cells promotes odontoblastic cell differentiation by targeting APC and activating Wnt/β-catenin signaling pathway (Fig. 4). Therefore, miR-663 can be considered as a critical molecular target in odontoblastic differentiation for the development of miRNA based therapeutic agents in dental medicine. To the best of our knowledge, this is the first report describing that the up-regulated miR-663 promotes odontoblastic cell differentiation by targeting APC and activating Wnt/β-catenin signaling.

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