MicroRNA-374a promotes osteogenic differentiation of periodontal ligament stem cells through directly targeting APC/Wnt/β-catenin signaling pathway

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Abstract: MicroRNAs (miRNAs) have a significant regulatory effect on the differentiation of human stem cells, such as human adipose tissue-derived stem cells (hADSCs), human mesenchymal stem cells (hMSCs) and embryonic stem cells (ESCs). However, little is known about the role of miRNAs in osteogenic differentiation of periodontal ligament stem cells (PDLSCs). Therefore, the aim of the present study is to determine the role of miRNAs and to elucidate its regulatory mechanism in osteogenic differentiation of PDLSCs. The miRNA expression profile before and after the osteoblastic differentiation of PDLSCs was assessed by microarray analysis and selected miRNAs were validated by quantitative RT-PCR (qRT-PCR). The gain- and loss-of-function experiments were performed to investigate the underlying function of selected miRNA in osteogenic differentiation of PDLSCs. Regulation of putative selected miRNA targets was determined by dual-luciferase reporter assays. In addition, the effects of selected miRNA on the Wnt/β-catenin signaling pathway were investigated by TOP/FOP flash luciferase assays and Western blot. Based on the miRNA expression profiling, we identified that 50 miRNAs were deregulated during the osteoblastic differentiation. Among these miRNAs, miR-374a was chosen for further study because miR-374a was significantly upregulated during osteogenic differentiation. Gain- and loss-of-function studies showed that overexpression of miR-374a significantly enhanced osteogenic differentiation of PDLSCs whereas miR-374a inhibition attenuated cell differentiation. Mechanically, we identified that adenomatous polyposis coli (APC), an antagonist of Wnt/β-catenin signaling, was the target gene of miR-374a. Furthermore, down-regulation of miR-374a led to inhibition of Wnt/β-catenin signaling pathway during differentiation. Our results demonstrate that miR-374a promoted osteogenic differentiation of PDLSCs by targeting APC through Wnt/β-catenin signaling pathway and highlighted the potential of forward miRNA-based therapy to promote periodontal ligament regeneration of humans.

Keywords: Periodontal ligament stem cells, osteogenic differentiation, microRNA-374a, APC, Wnt/β-catenin signaling pathway

Introduction

Human periodontal ligament stem cells (PDLSCs) are known as a population of multi-potent progenitor cells which can differentiate into various types of cells such as osteoblasts, chondrocytes, adipocytes, and myoblasts [1]. For clinical use of progenitor cells, PDLSCs play important roles in the maintenance and regeneration of periodontal tissues [2-4]. However, how to regulate and control the potency of osteogenic differentiation of PDLSCs remains to be an unsolved problem.

Several signaling pathways such as Wnt/β-catenin, TGF-β and BMP, have been implicated in regulating osteogenic differentiation of mesenchymal stem cells (MSCs) [5, 6]. In particular, Wnt/β-catenin signaling plays a vital role in the differentiation of human mesenchymal stem cells into osteoblasts [6]. Recent observations showed that Wnt/β-catenin signaling was crucial in the differentiation of MSCs, including osteogenic, chondrogenesis, adipogenesis and myogenesis differentiation [7-10]. However, limited studies have paid attention on the functions and underlying mechanisms of Wnt/β-catenin signaling in the differentiation PDLSCs.

MicroRNAs (miRNAs) are a class of short (18-25 nucleotides) RNAs that repress the expression of target genes by degrading mRNA or prevent-
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As we know, miRNAs have been reported to play indispensable roles in many biological processes, including proliferation, differentiation and apoptosis [13, 14]. Recently, Wei et al. found that miR-21 regulated stretch-induced osteogenic differentiation of PDLSCs by targeting ACVR2B [15]. Another recent study found that miRNA-218 was downregulated in differentiated PDLSCs and identified as targeting RUNX2 to control osteogenic maturation [16]. Therefore, more extensive investigations on the identification and the functions of any other miRNAs that are deregulated in the differentiation of PDLSCs are necessary.

In this study, miR-374a was recognized as one of the highly upregulated miRNAs during PDLSCs differentiation development. Then, we found that miR-374a overexpression promoted osteoblastic differentiation, whereas miR-374a inhibition suppressed osteoblastic differentiation of PDLSCs. Furthermore, we found that miR-374a activated Wnt signaling through accumulation of β-catenin by suppressing APC expression, leading to cell differentiation. Our findings provided new insights into the molecular function of miR-374a/Wnt signaling pathway in PDLSCs differentiation.

Materials and methods

Cell culture

PDLSCs were obtained from seven donors aged 10-14 years (mean age: 12.2), who underwent molar extraction, and cultured by the methods as described previously [17], and the experiments used third-passage PDLSCs. Written informed consent for use of periodontal tissue was obtained from all donors. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Jinan University. To detect osteogenic differentiation, cells were cultured in the presence of 100 μM L-ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 10 nM dexamethasone for 3 weeks.

MiRNA microarray

The miRNA microarray was performed as described previously [18, 19]. Briefly, total RNA of different PDLSCs groups was isolated by a miRNAeasy mini kit (Qiagen), and followed by labeling and hybridization with the miRCURYTM LNA Array (v.16.0, Exiqon). The feature extraction software (Agilent Technologies) was used to quantify the fluorescent intensity of each spot of microarray images, and signal intensities >10 were considered positive expression. The statistical significance of upregulated or downregulated miRNAs was analyzed by t-test. MEV software (v4.6, TIGR) was used to perform hierarchical clustering.

Real-time reverse transcription polymerase chain reaction (PCR)

To measure miR-374a and mRNA levels of runt-related transcription factor 2 (Runx2), Alkaline phosphatase (ALP), osteocalcin (OCN) and bone sialoprotein (BSP), total RNA was isolated from different PDLSCs groups using an RNeasy® mini kit (Qiagen GmbH, Hilden, Germany). Concentration and purity were determined using a NanoDrop 1000™ spectrophotometer (Thermo Fisher, Dubuque, IA, USA). Real-time PCR was performed using two-step Stemaim-it miR qRT-PCR Quantitation Kit (SYBR Green) (Novland, Shanghai, China) on Bio-RAD IQ5 real-time PCR instrument. U6 and β-actin were used as references for miRNAs and RNAs, respectively. All reactions were conducted in triplicate. The 2^ΔΔCt method was used to determine the relative quantitation of gene expression levels.

Transfection

MiR-374a mimic, miR-374a inhibitor and controls were purchased from Shanghai GenePharma (Shanghai, China). Cells were plated into six-well plates and grown to 30-50% confluence after 24 hours of incubation and were then transfected with miRNA mimics, miRNA inhibitor and negative control using siLentFect™ Lipid reagent (Life Science Research). The cells were then diluted in DMEM/F12 without serum (GeneChem, Shanghai, China). After 4 h of incubation in a CO2 incubator at 37°C, the medium was changed to 10% FBS containing DMEM. At indicated time, cells were collected for further protein extraction.

Alizarin red staining and quantification

Cells were seeded into 24-well plates at a density of approximately 1×10^5 cells per well separately. After cells reached 80% confluence, the culturing medium was changed into standard osteogenic differentiation induction medium. After 21 days of culturing, cells were stained
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Figure 1. miR-374a is significantly upregulated during PDLSCs osteogenic differentiation. A. The hierarchical clustering of miRNAs differentially expressed between differentiated and non-differentiated PDLSCs. B-E. qRT-PCR assay was performed to determine the expression of miR-761, miR-374a, miR-133a and miR-663b at 0 d, 10 d and 21 d after PDLSCs osteogenic differentiation. U6 was used as endogenous control. Data are shown as means ± SD of three separate experiments. **P<0.01 vs non-differentiated group.
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Figure 2. Overexpression of miR-374a promotes osteoblastic differentiation of PDLSCs. PDLSCs were transiently transfected with miR-374a mimic and then the conditioned medium was changed every 3 days. A. After 21 days of culturing, the cells were stained with alizarin red (pH = 4.1), and PDLSCs transected with miR-374a mimic formed more mineralized nodules than the control groups. B-E. PDLSCs were transduced with miR-214 mimic. Total RNA was analyzed by real time qRT-PCR for mRNA expression profile of bone marker genes (ALP, BSP, Runx2 and osteocalcin) at d10 and d21. The data are the mean ± SD of three independent experiments. **P<0.01 vs mimics NC.

with Alizarin red staining solution (Sigma, St. Louis, MO, USA). After washing in PBS, the cells were observed using an inverted microscope and quantified according to the methods previously published.

Luciferase assays

To confirm the direct regulating relationship between miR-374a and APC, the full-length 3'-UTR of the APC mRNA and a mutant variant were amplified by PCR and cloned into a pmirGlo Dual-luciferase miRNA Target Expression Vector to form the reporter vector APC-wildtype (APC-WT) (GenePharma). To mutate the putative binding site of miR-374a in the 3'-UTR-containing vector, the sequence of putative binding site was replaced as indicated and was named as APC-mutated-type (APC-MUT). pmirGLO-APC-Mt or pmirGLO-APC-WT was co-transfected with miR-374a mimics, miR-374a inhibitor or miR-374a NC into PDLSCs using Lipofectamine 2000. Renilla and firefly luciferase activities were measured with the Dual-Luciferase Reporter system (Promega) at 24 h after transfection. All assays were independently performed in triplicate.

Western blots

Total cellular proteins were extracted using RIPA lysis buffer containing proteinase inhibitor (Sigma, USA). Equal amounts of total protein were loaded into a 10% SDS-PAGE transferred onto a PVDF membrane probed with indicated primary antibody for β-catenin, APC, TCF4 and β-actin (Abcam; 1:1000 dilution). After incubation with secondary antibody, antibody-bound protein complex in the membrane was detected with chemiluminescence reagent. Relative band intensities were determined by densitometry using Scion image software (version 4.0).

Plasmid construction

TOPflash and FOPflash reporters contain wild-type (WT) and mutated TCF-4 consensus bind-
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Statistics

All experimental conditions were replicated at least three times. Data are expressed as mean ± SD. Student’s t-test or one-way analysis of variance was made for multiple comparisons. *P-values <0.05 will be considered to be statistically significant.

Results

Identification of differentially expressed miRNAs after osteogenic differentiation of PDLSCs

In this study, we first studied the dysregulated miRNAs during PDLSCs differentiation by miRNA microarray. As shown in Figure 1A, hierarchical clustering showed systematic variations in the expression of 50 miRNAs between differentiated and non-differentiated PDLSCs. Among them, 4 selected miRNAs during osteogenic differentiation, including miR-761, miR-374a, miR-133a and 663b were validated by quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that miR-761 and miR-374a were gradually increased after osteogenic differentiation of PDLSCs, while miR-133a and 663b decreased (Figure 1B-E). A recent study showed that miR-374a regulated dexamethasone-induced differentiation of primary cultures of porcine adipocytes [21]. Therefore, we focused our attention on miR-374a for further study.

Upregulation of miR-374a promotes the osteogenic differentiation of PDLSCs

Given the up-regulation of miR-374a in PDLSCs, we predicted that miR-374a may promote the osteogenic differentiation of PDLSCs. To verify our hypothesis, miR-NC and miR-374a mimics were transfected into PDLSCs. After 21 days culturing, Alizarin red staining and quantification sites, respectively, and are widely used to evaluate β-catenin dependent signaling events that drive the expression of TCF. These reporters have been described previously [20].

Figure 3. Inhibition of miR-374a suppresses osteoblastic differentiation of PDLSCs. PDLSCs were transiently transfected with miR-374a inhibitor and then the conditioned medium was changed every 3 days. A. After 21 days of culturing, the cells were stained with alizarin red (pH = 4.1), and PDLSCs transfected with miR-374a inhibitor formed fewer mineralized nodules than the control groups. B-E. PDLSCs were transduced with miR-374a inhibitor. Total RNA was analyzed by real time qRT-PCR for mRNA expression profile of bone marker genes (ALP, BSP, Runx2 and osteocalcin) at d10 and d21. The data are the mean ± SD of three independent experiments. **P<0.01 vs inhibitor NC.
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Figure 4. miR-374a directly targets APC. miR-374a binds to the predicted site of the 3’-UTR of APC. Dual-luciferase reporter gene analysis of APC 3’-UTR in HEK293T cells following co-transfection of miR-374a mimics, miR-374a inhibitors or miR-NC with pmiRGlo constructs containing WT- or MUT-3’-UTR of APC. A. The APC 3’-UTR region containing the wild type or mutant binding site for miR-374a. B. Relative luciferase activities are the ratios of Renilla luciferase normalized to the control mimics. C and D. Protein levels of APC in the indicated cells transfected with 20 nM miR-374a mimic, miR-374a inhibitor or miR-NC were measured by Western blot assays. β-actin was used as internal controls. Data are shown as means ± SD of three separate experiments. **P<0.01 vs miR-NC.

Downregulation of miR-374a inhibits the osteogenic differentiation of PDLSCs

To further investigate the physiological role of miR-374a in PDLSCs differentiation, PDLSCs were transfected with miR-374a inhibitor to down-regulate miR-374a. After 3 weeks of osteogenic induction, Alizarin red staining and quantification results showed that inhibition of miR-374a significantly suppressed the formation of mineralized nodules compared with the control group (Figure 3A). We also analyzed the mRNA expression of ALP, osteocalcin, BSP and Runx2 [22] by qRT-PCR. Results showed that knockdown of miR-374a decreased the expression level of all these genes (Figure 3B-E). These data indicated that down-regulated miR-374a inhibited the osteogenic differentiation of PDLSCs.

APC is a direct target of miR-374a

To explore the molecular mechanism by which miR-374a functions in the osteogenic differentiation of PDLSCs, TargetScan and PicTar algorithms were used to identify the potential target genes of miR-374a. Adenomatous polyposis coli (APC), an antagonist of Wnt/β-catenin signaling, were predicted as the functional target...
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of miR-374a (Figure 4A). To further validate whether APC was a direct target of miR-374a, a luciferase activity assay was conducted. The results showed that co-transfection of miR-374a mimic and pmirGLO-3-APC-wt significantly decreased the luciferase activity, whereas co-transfection of miR-374a inhibitor and pmirGLO-3-APC-wt increased the luciferase activity (Figure 4B). Likewise, cells co-transfected with miR-374a mimic, miR-374a inhibitor and pmirGLO-3-APC-mut showed no obvious change in luciferase activity (Figure 4B). In addition, Western Blot analysis showed that miR-374a mimic decreased the levels of APC protein expression while inhibited miR-374a expression exhibited the opposite effects (Figure 4C, 4D). These results indicated that APC was a direct target of miR-374a in PDLSCs.

Figure 5. Effects of miR-374a on the activity of Wnt/β-catenin signaling pathway. A and B. Luciferase activity of TOPFlash/FOPFlash in PDLSCs treated with miR-374a mimic and miR-374a inhibitor. Knockdown of miR-374a resulted in the inactivation of TopFlash luciferase activity, while overexpression of miR-374a had an opposite result. C. PDLSCs were divided into non-differentiation group and differentiation group. In differentiation group, PDLSCs were transfected with a miR-374a inhibitor for 24 h. An inhibitor NC (negative control) was used as a negative control. Thereafter, the cells were incubated with basal medium (BM) or osteogenic medium (OM) until the indicated times. Western blot showed the protein expressions of β-catenin and TCF-4 in differentiation group were increased compared with non-differentiation group. But their expressions were decreased after miR-374a inhibitor transfected. All data represent the mean ± SD results of three independent experiments. **P<0.01 vs miR-NC.

miR-374a promotes activity of the Wnt/β-catenin signaling pathway

Wnt/β-catenin signaling is an important signaling factor implicated in the control of differentiation of human mesenchymal stem cells into osteoblasts [7, 13]. Based on our results that miR-374a promoted osteogenic differentiation of PDLSCs by functionally targeting APC, a negative regulator of Wnt/β-catenin signaling, we wondered whether miR-374a play an important role in regulating the activity of the Wnt/β-catenin pathway. To verify our hypothesis, we first employed TOPflash and FOPflash reporters, which are widely used to evaluate β-catenin-dependent signaling activity, to evaluate the effects of miR-374a on Wnt/β-catenin signaling. As expected, Wnt/β-catenin signaling was activated when the level of miR-374a was up-regulated, whereas blocked when the level of miR-374a was down-regulated (Figure 5A, 5B). Subsequently, we analyzed the expression levels of β-catenin and TCF-4 proteins that were transcriptional mediators of activated Wnt signaling [23]. As shown in Figure 5C, protein levels of β-catenin and TCF4 were drastically enhanced after differentiation compared with that in non-differentiation PDLSCs, but decreased by miR-374a inhibitor treatment, indicating that miR-374a inhibition was able to block the Wnt signaling. These data suggested that miR-374a promoted osteogenic differentiation of PDLSCs by activating Wnt/β-catenin signaling.
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Discussion

In the present study, differentially expressed miRNAs after differentiation were identified and miR-374a was found gradually upregulated during PDLSCs osteogenic differentiation. The loss- and gain-of-function results confirmed that miR-374a promoted osteogenic differentiation of PDLSCs. Importantly, we found that miR-374a downregulated the expression of APC, the negative modulator of Wnt/β-catenin signaling, thereby activated Wnt/β-catenin pathway, as the consequence, promoting the osteogenic differentiation.

Increasing evidence demonstrated that miRNAs play an important role in the control of osteoblastic differentiation [13, 14]. For example, miR-200c and miR-375 have been reported to regulate osteoblast differentiation by targeting various osteoblast genes [24, 25]. Thus, more evidence for the roles of miRNAs in regulating osteogenic differentiation is needed [26]. In this study, we established the profile of miRNAs before and after differentiated PDLSCs for the first time and finally focused on miR-374a which was previously reported up-regulated during adipocyte differentiation for the further study. Moreover, upregulation of miR-374a promoted PDLSCs osteogenic differentiation and enhanced the expression of ALP, BSP, osteocalcin, and Runx2, while inhibition of miR-374a had an opposite results. Therefore, we concluded that miR-374a positively regulates PDLSCs osteogenic differentiation. However, it remains unknown how miR-374a functions in osteogenic differentiation.

It has been demonstrated that miRNAs modulate gene expression by binding to target mRNAs, thereby inhibiting their translation. Previous studies reported that miR-374a exhibited its functions on the progression of a variety of tumors through targeting numerous key genes [27-29]. In recent years, APC has been reported to play important roles in regulating osteoblast differentiation [30]. Tao Wang et al. demonstrate that miR-27 regulates osteoblast differentiation by targeting APC [31]. Here, we reported that APC, a regulatory molecular in Wnt/β-catenin signaling, is a direct target of miR-374a in PDLSCs. Our data further demonstrated that miR-374a inhibition significantly decreased TOPFlash activity. However, TOPFlash activity in cells treated with miR-374a mimic was increased, indicating that Wnt signaling was potentiated. In addition, miR-374a inhibition significantly decreased total and activated β-catenin expression. These results suggest that miR-374a-mediated downregulation of APC leads to activation of Wnt/β-catenin signaling in PDLSCs, and subsequently promoted osteogenic differentiation.

In conclusion, this study identified miR-374a as a positive regulator of osteogenic differentiation, acting by targeting APC and activating Wnt/β-catenin signaling in PDLSCs. Our findings suggest that miR-374a may be considered as a new molecular target for tooth regeneration and oral tissue engineering in the future.

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Disclosure of conflict of interest

None.

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