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MiR-148a-3p Regulates Stem Cell Osteogenic Differentiation and Enamel Development by Targeting Runt-Related Transcription Factor 2 and E-cadherin via the Wnt1/β-catenin Signaling Pathway

Huaiguang Chang1, Tingting Jiang2, Liang Kou1, Duo Li3, Xinchen Yu1, Youqin Li1 and Lei Zhang1

1. Department of Stomatology, Ningbo University of Health Sciences, Ningbo, Zhejiang Province, China
2. Department of Prosthodontics, Yinzhou Stomatology Hospital, Ningbo, Zhejiang Province, China
(accepted for publication, March 11, 2022)

Abstract: We aimed to evaluate the regulatory effects of miR-148a-3p on stem cell osteogenic differentiation and enamel development by targeting runt-related transcription factor 2 (RUNX2) and E-cadherin, respectively. TargetScan software was utilized to predict the binding sites between miR-148a-3p and osteogenic marker gene RUNX2 or E-cadherin. The changes in miR-148a-3p expression during osteogenic differentiation of epidermal stem cells were detected. After transfection with miR-148a-3p mimics or miR-148a-3p inhibitor, epidermal stem cells were induced towards osteogenic differentiation, and the changes in RUNX2 expression were measured. The changes in miR-148a-3p expression during enamel development regulated by epidermal stem cells were determined. After liposome-mediated transfection with miR-148a-3p mimics or miR-148a-3p inhibitor, epidermal stem cells were induced towards ameloblast development. Cell proliferation and apoptosis abilities were tested using methyl thiazolyl tetrazolium assay and flow cytometry, respectively. The expression of miR-148a-3p was detected by RT-PCR. The protein expressions of Wnt1, β-catenin, RUNX2 and E-cadherin were measured by Western blotting. Epidermal stem cells differentiated into osteoblasts through osteogenic induction culture. On 5, 12, 15 and 30 d, epidermal stem cells gradually differentiated into osteoblasts through epithelial aggregation and depression, mesenchymal aggregation, and dentin and enamel secretion. After transfection, compared with negative control (NC) group, the cell viability of miR-148a-3p group significantly decreased (P<0.05), and the apoptosis rate increased (P<0.01). The viability of miR-148a-3p inhibitor group significantly increased (P<0.05), while the apoptosis rate reduced (P<0.01). The dual-luciferase reporter assay showed that miR-148a-3p targeted Wnt1. Compared with NC group, the expression of miR-148a-3p group significantly increased (P<0.001), and the protein expression levels of Wnt1, β-catenin, RUNX2 and E-cadherin significantly decreased. Compared with NC group, the expression of miR-148a-3p inhibitor group significantly decreased (P<0.05), and the protein expression levels of Wnt1, β-catenin, RUNX2 and E-cadherin significantly increased. MiR-148a-3p is highly expressed in and regulates osteogenic differentiation and enamel development through targeting RUNX2 and E-cadherin respectively via the Wnt1/β-catenin pathway, which plays an important role in cell differentiation, stem cell proliferation and enamel development.

Key words: miR-148a-3p, Stem cell, Osteogenic differentiation, Enamel development, Runt-related transcription factor 2, E-cadherin, Wnt1, β-Catenin

Introduction

Stem cells are the progenitor cells of osteoblasts, and their ability to differentiate into osteoblasts is directly proportional to bone growth, hence their osteogenic differentiation ability has a crucial influence on the tooth development15. Enamel formation disorders, such as amelogenesis imperfecta, can be caused by signal interference and mutation during development. Tooth enamel composed of orderly-arranged hydroxyapatite crystals is the hardest mineralized tissue in the human body, which possesses the functions of chewing, protection and aesthetics3. The differentiation of ameloblasts and the secretion and mineralization of enamel matrix are accompanied in enamel development5. Currently, bone marrow mesenchymal stem cells or epidermal stem cells, as previously reported, can differentiate into ameloblasts and odontoblasts and secrete matrix, thereby forming the tooth body4. Therefore, searching for the target genes for regulating stem cell osteogenic differentiation and enamel development and exploring the molecular mechanism of enamel development are of great significance for improving the enamel development. Micro ribonucleic acids (miRNAs) are a class of non-coding small RNAs widely existing in eukaryotes, which are not only implicated in cell proliferation, differentiation and apoptosis, but also closely associated with the formation and repair of tooth enamel56.

The Wnt/β-catenin signaling pathway, which widely exists in invertebrates and vertebrates, is highly conserved in the evolution of species. This pathway can regulate and repair dentin formation and dental germ development. Additionally, the osteoblast, chondrocyte and adipocyte differentiations of stem cells are regulated by β-catenin, and RUNX2 is an important regulator of osteoblast and odontoblast differentiations7.
MiR-148a-3p is one of the widely-studied miRNAs in recent years, and it is an important player in such physiological and pathological processes as inflammation, bone formation and tumorigenesis. Disorders of miR-148a-3p can be found in multiple tumors, such as pancreatic cancer, gastric cancer, non-small cell lung cancer, breast cancer and nasopharyngeal cancer\(^1\). MiR-148a-3p suppresses the metastasis and invasion of gastric cancer through inhibiting the expression of DNA methylase I. Moreover, miR-148a-3p can, through guiding DNA methylation, regulate the expression of cell surface estrogen receptors in breast cancer, and it also has a close association with the proliferation and apoptosis of osteoblasts and osteoecteals\(^2\). At present, the mechanism of miR-148a-3p in regulating stem cell osteogenic differentiation and enamel development has not been widely reported. Thus, this study aimed to investigate the effects of overexpression and inhibition of miR-148a-3p on stem cell osteogenic differentiation and enamel development and probe into its mechanisms, thus exploring the possible targeting mechanism of miR-148a-3p.

Materials and Methods

Cell lines and main reagents

Human epidermal stem cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640 medium, special medium for osteogenic differentiation, serum-free medium (SFM), phosphorylated ascorbic acid (ASAP), fetal bovine serum (FBS) and 0.25% trypsin were supplied by Gibco (Waltham, MA, USA). TRIzol reagent and Lipofectamine 2000 were bought from Invitrogen (Carlsbad, CA, USA). Reverse transcription kits and SYBR Green fluorescence quantitative polymerase chain reaction (PCR) kits were provided by TaKaRa (Shiga, Japan). Methyl thiazolyl tetrazolium (MTT) assay kit, phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Saint Louis, MO, USA). Runt-related transcription factor 2 (RUNX2), E-cadherin, Wnt1, β-catenin and β-actin primary antibodies and horseradish peroxidase (HRP)-labeled goat anti-rabbit or anti-mouse immunoglobulin G (IgG) secondary antibody were bought from Abcam (Cambridge, UK).

Cell culture and transfection

The epidermal stem cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO\(_2\) incubator at 37°C. Then the well-grown epidermal stem cells in the logarithmic phase were inoculated into a 96-well plate (1×10\(^5\) cells/well) with RPMI 1640 medium without antibiotics. Upon reaching about 60% confluence, the cells were transfected in accordance with the instructions of Lipofectamine 2000 Reagent. The cells were divided into 3 groups, i.e., miR-148a-3p group (transfected with miR-148a-3p), miR-148a-3p inhibitor group (transfected with miR-148a-3p inhibitor) and NC group (negative control), with 3 replicates in each group.

Epidermal stem cell osteogenic differentiation and enamel development

The cells were cultured for 72 h under the conditions of 37°C, 5% CO\(_2\) and saturated humidity. After the medium was discarded, the cells were washed twice with 0.1 mol/l PBS, and cultured with 1 ml/1 DMSO. The medium was replaced every 72 h. Upon reaching more than 80% confluence, osteogenic induction was performed by 0.1 mol/l β-glycerophosphate sodium, 10\(^{-7}\) mol/l dexamethasone and 50 mg/l ASAP (Sigma, Saint Louis, MO, USA) for 8 d.

According to the instructions, FGF8a (Invitrogen, Carlsbad, CA, USA) was dissolved with 100 μl of sterile PBS and used to induce the epidermal stem cells. Then the epidermal stem cells were scraped and recombined with those with the ability to induce odontogenesis to form a chimera. Finally, the chimera was incubated on an organ culture dish overnight.

Detection of miR-148a-3p expression in cells by qPCR

Total RNA was extracted from cells using TRizol kits, and reversely transcribed into cDNA according to the instructions of reverse transcription kits. Then qPCR was performed using a qPCR system (Applied Biosystems 7600, ABI, USA) under the following conditions: pre-denaturation at 95°C for 15 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 30 s. With U6 as an internal reference, the expression levels of miR-148a-3p in tissues and cells were calculated by 2\(^{-\Delta\Delta CT}\) method. Primer sequences involved: miR-148a-3p F: 5’-CAAACATAGGAA-3’, R: 5’-CAAGGACTAACCCGAAA-3’; U6 F: 5’-CTCGCTTCGGCAAGCACA-3’, R: 5’-AACGCTTCAGAATTTTGCGT-3’.

Detection of cell proliferation ability by MTT assay

After transfection, the cells in each group were inoculated into a 96-well plate, with 3 replicates for each well. Then 100 μl of fresh medium was added into each well for culture. After 24, 48, 72 and 96 h, the original medium was replaced with fresh medium. Then 20 μl of MTT reagent was added into each well for incubation in an incubator for another 4 h. After the MTT reagent was discarded, the precipitate was fully dissolved with 150 μl of DMSO, and the optical density (OD) value was measured at a wavelength of 490 nm using a microplate reader (Thermo Fisher, Waltham, MA, USA). With OD value as the ordinate and time as the abscissa, the growth curve was plotted.

Detection of cell apoptosis by flow cytometry

After transfection, the cells in each group were digested, resuspended in binding buffer, mixed evenly and added with 5 μl of Annexin V, followed by staining for 10 min away from light. Then 50 ml/PI staining solution was added for reaction at room temperature for 5 min away from light. Finally, the proportion of Annexin V-positive cells was detected using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to determine the apoptosis rate.

Dual-luciferase reporter assay

According to the predicted binding site between miR-148a-3p and Wnt1, the Wnt1 mutant sequence fragment was designed. Partial fragments of wild-type (WT) or mutant (MUT) Wnt1 were cloned into a luciferase vector (E1330). The WT or MUT constructs of Wnt1 reporter plasmid were transfected into cells with miR-148a-3p mimics. The transfected cells were maintained for 48 h, and luciferase activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Detection of relevant protein expressions of osteogenic differentiation and enamel development by Western blotting

Total protein was extracted using the total protein extraction kit, and its concentration was measured via BCA method. After SDS-PAGE, the protein was transferred onto a membrane, blocked with PBS containing 5% skim milk powder at room temperature for 1 h, and incubated with RUNX2, E-cadherin, Wnt1, β-catenin and β-actin primary antibodies (1:1,000) at 4°C overnight. The next day, after the membrane was washed, the protein was incubated again with HRP-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (1:2,000) at room tem-
temperature for 2 h, followed by color development with ECL in a dark room, and image analysis using ImageJ software (NIH, USA). The ratio of gray value of target protein band to that of internal reference β-actin indicated the relative expression of the target protein.

**Statistical analysis**

QPCR, MTT assay, flow cytometry and Western blotting were repeated 3 times. SPSS 26.0 software (IBM, New York, NY, USA) was used for statistical analysis. Normally distributed measurement data were expressed as (x±s). One-way analysis of variance was performed for comparison among groups, and LSD-t test for pairwise comparison. Numerical data were subjected to χ² test. P<0.05 was considered to be statistically significant.

**Results**

**Induction of osteogenic differentiation of epidermal stem cells and regulation of enamel development**

Epidermal stem cells could differentiate into osteoblasts through osteogenic induction culture. At 5, 12, 15 and 30 d, epidermal stem cells gradually differentiated into osteoblasts, and went through such processes as epithelial aggregation and depression, mesenchymal aggregation, and dentin and enamel secretion, ultimately forming the tooth body (Fig. 1).

**Epidermal stem cell viability and apoptosis**

After transfection, compared with NC group, the cell viability of miR-148-3p group significantly decreased (P<0.05), and the apoptosis rate increased (P<0.01). The viability of miR-148-3p inhibitor group significantly increased (P<0.05), while the apoptosis rate reduced (P<0.01) (Figs. 2 and 3).
Changes in miR-148a-3p expression

Compared with NC group, the expression of miR-148a-3p significantly rose in miR-148a-3p group (P<0.001), while declined in miR-148a-3p inhibitor group (P<0.05) (Fig. 4).

Targeting relationship between miR-148a-3p and Wnt1 determined by dual-luciferase reporter assay

Fig. 5A exhibits the specific binding sites between miR-148a-3p and Wnt1, indicating that Wnt1 may be targeted by miR-148a-3p. The luciferase activity of miR-148a-3p mimics in the cells with Wnt1-WT reporter plasmid was significantly attenuated, but showed no difference in the cells with Wnt1-MUT (Fig. 5B). Thus, Wnt1 was targeted by miR-148a-3p.

Changes in RUNX2, E-cadherin, Wnt1 and β-catenin protein expression levels

Compared with NC group, the protein expression levels of Wnt1, β-catenin, RUNX2 and E-cadherin in miR-148a-3p group significantly decreased, whereas those in miR-148a-3p inhibitor group significantly increased. The expression of RUNX2 was significantly higher in miR-148a-3p group but significantly lower in miR-148a-3p inhibitor group than that in NC group (P<0.05). Compared with NC group, miR-148a-3p group had a significantly increased expression of E-cadherin, while miR-148a-3p inhibitor group exhibited a significantly decreased expression of E-cadherin (P<0.05) (Fig. 6).

Discussion

Teeth comprise cementum, dentin and enamel. Cementum is present in the root of a tooth to maintain its position, and dentin is a bone-like matrix necessary for supporting enamel. Enamel composed of less than 1% organic matter and a large amount of inorganic components is a kind of highly mineralized hard tissues in the human body. The densely arranged hydroxyapatite crystals gather into clusters to form a staggered structure of enamel fibers and interenamel fibers, which provide a material and structural basis for enamel hardness and elasticity. The enamel matrix is dependent on ameloblast production and secretion. The death of ameloblasts, however, limits enamel renewal during tooth eruption. The ability of bone marrow mesenchymal stem cells and epidermal stem cells to develop into osteocytes and tooth enamel, thus generating the tooth body, has been proven. Therefore, it is of great significance to search for new highly-specific molecular targets for improving the repair of tooth enamel and ameliorating the prognosis of patients\(^\text{9,10}\). Recently, numerous studies have demonstrated that miRNAs are important regulators in cell development, metabolism, senescence, death and other important life activities\(^\text{11,12}\). The research on miRNAs contributes to clarifying the mechanism of disease occurrence and development and offering new targets. In recent years, RT-qPCR, miRNA microarray and high-throughput sequence analysis have been conducted to compare the spatio-temporal characteristics of miRNA expression in mouse tooth germ tissues and each stage of development, uncovering the role of miRNAs in different stages of tooth development. Therefore, measuring the changes in specific miRNA expression is highly important for regulating stem cell osteogenic differentiation and enamel development, and
clarifying its molecular mechanism possesses high academic and guidance value.

There is a low expression of miR-148a-3p in malignant tumor cell lines. Overexpression of miR-148a-3p is capable of inhibiting cancer cell invasion, metastasis and transformation. In this study, TargetScan software was used to predict the binding site between miR-148a-3p and osteogenic marker gene RUNX2, and the binding site between miR-148a-3p and E-cadherin. RUNX2, also known as core binding factor α1 (CBFα1), is a transcription factor of the Runt domain family and regulates protein expression at the transcriptional level. As a pivotal transcription factor in bone and tooth development, RUNX2 can bind to specific binding sites on DNA promoters, thereby regulating target gene transcription and participating in organ formation. It has been found that as a specific transcription factor regulating osteogenic differentiation, RUNX2 has the ability to directly induce the differentiation of mesenchymal stem cells into osteoblasts, and also acts as an important player in the repair and reconstruction of adult bone tissues. Cadherin is a kind of calcium-dependent cell-cell adhesion molecule, which is classified into E-cadherin, N-cadherin and P-cadherin based on the distribution and structure. E-cadherin distributed in epithelial tissues is a calcium-dependent cell-cell glycoprotein adhesion molecule implicated in tumor occurrence, metastasis and embryonic growth and development, and has important effects in the morphological formation and function of tissues, which is an important regulatory substance in tooth development. However, the mechanism of miR-148a-3p in regulating stem cell osteogenic differentiation and enamel development through targeting RUNX2 and E-cadherin has not been deeply explored.

In this study, epidermal stem cells could differentiate into osteoblasts through osteogenic induction culture. At 5, 12, 15 and 30 d, epidermal stem cells gradually differentiated into osteoblasts, and went through such processes as epithelial aggregation and depression, mesenchymal aggregation, and dentin and enamel secretion. It can be seen that the epidermal stem cells can differentiate into osteocytes and regulate the enamel development. The results of MTT assay showed that after transfection with si-miR-148a-3p, the proliferation of epithelial stem cells was significantly enhanced, and the apoptosis rate reduced, indicating that miR-148a-3p participated in the biological behavior and related functions of these cells. MiR-148a-3p also has high expression in the differentiation of epithelial stem cells, and its downstream regulation can arrest stem cell proliferation, inhibit growth and prevent differentiation. In addition, miR-148a-3p acts as a tumor suppressor gene in malignancies. Hence, the results of this study confirmed that miR-148a-3p facilitated the osteogenic differentiation of epidermal stem cells and regulated enamel development, with significantly higher expression in cells transfected with miR-148a-3p than that in blank cells.

In this study, the expressions of RUNX2 and E-cadherin were significantly lower in miR-148a-3p group but higher in miR-148a-3p inhibitor group than those in NC group (P<0.05). RUNX2 has been linked to skeletal development regulation. MiRNAs regulate transcription factors, while transcription factors regulate miRNAs in the co-regulatory network established by transcription factors and miRNAs, resulting in a complex interaction network. E-cadherin is involved in tooth germ development and is essential in tooth morphogenesis and cell differentiation, particularly in the formation of the tooth body. It can be demonstrated that miR-148a-3p regulates osteogenic differentiation and enamel development by targeting RUNX2 and E-cadherin, respectively, and that the miRNA-protein regulatory network also modulates tooth development. The regulatory effect of miRNAs on downstream signal factors has been validated in animal models of enamel developmental disorders. For example, it was found by genomic DNA microarray that the RUNX2 signal expression in tooth germ significantly rises, whereas the RUNX2 gene expression declines after miRNA knockout in Pitx2-Cre/Dicer1flox/flox mice. In miR-200c/141 transgenic mice, the expression of E-cadherin in presecretory/secretory ameloblasts is remarkably raised, and the protein expressions of E-cadherin and AMELX are also increased. A previous study manifested that Pitx2 is able to activate miR-200a-3p and negatively regulate E-cadherin expression in pre-ameloblasts, and Pit2/miR-200a-3p can enhance mesenchymal cell transition into dental epithelioid cells through modulating the Wnt/-catenin signaling pathway. Therefore, miR-148a-3p may regulate osteogenic differentiation and enamel development simultaneously by targeting RUNX2 and E-cadherin, respectively.

Our study confirmed that Wnt1 was the target gene of miR-148a-3p. Wnt1 belongs to the Wnt family, and the Wnt/β-catenin pathway plays essential roles in physiological processes such as organ formation and tissue regeneration. Moreover, this pathway has been widely reported to participate in cell proliferation, fibrosis and differentiation. The Wnt1/β-catenin pathway is also involved in the odontoblast differentiation of DPSCs and can be regulated by miR-140-5p. Likewise, Western blotting herein revealed that miR-148a-3p overexpression suppressed the expressions of Wnt1 and β-catenin, while inhibiting miR-148a-3p expression exerted the opposite effect. Therefore, miR-148a-3p affected the osteoblast differentiation of epithelial stem cells and enamel development by targeting the Wnt1/β-catenin pathway. However, more research is needed to find out whether the specific interaction between its molecules and related proteins affects osteogenic differentiation and enamel development.

In conclusion, miR-148a-3p has a high expression in differentiation of epithelial stem cells, which may regulate osteogenic differentiation and enamel development through targeting RUNX2 and E-cadherin respectively via the Wnt1/β-catenin pathway. Thus, miR-148a-3p can serve as a potential target for dental restoration and treatment.

Acknowledgments
This study was financially supported by Ningbo Municipal Natural Science Foundation (No. 202003N4187), Zhejiang Provincial Science and Technology Innovation Project for College Students (No. 2021R464002) and Science Foundation of Ningbo Oral Health Research Institute.

Competing Interests
The authors declare that they have no competing interests.

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