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MiR-335-3p/miR-155-5p Involved in IGFBP7-AS1–Enhanced Odontogenic Differentiation

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ABSTRACT

Background: The differentiation of stem cells from exfoliated deciduous teeth (SHEDs) into odontoblasts determines the regeneration of dentin-pulp complex. Non-coding RNAs (ncRNAs), including microRNA (miRNA) and long non-coding RNA (lncRNA), participate in many multiple biological processes, but the specific miRNAs involved in odontogenesis are incompletely defined. It was confirmed that lncRNA IGFBP7-AS1 could positively regulate odontogenetic differentiation in SHEDs. To investigate the downstream mechanisms of this process, miR-335-3p and miR-155-5p were found to be closely related with SHED odontogenic differentiation through whole-genome sequencing. The aim of the current study was to determine the role of miR-335-3p/miR-155-5p in IGFBP7-AS1–enhanced SHED differentiation and explore the potential mechanism of IGFBP7-AS1–mediated odontogenesis.

Methods: Putative miR-335-3p/miR-155-5p binding sites within IGFBP7-AS1 were identified by bioinformatics analysis, and the binding of miR-335-3p/miR-155-5p to these sites was confirmed by dual-luciferase reporter gene assays. The effects of miR-335-3p/miR-155-5p in odontogenesis were detected by tissue nonspecific alkaline phosphatase staining, Alizarin red staining, quantitative real-time polymerase chain reaction (qRT-PCR) analyses, and western blot testing. The molecular mechanisms of miR-335-3p/miR-155-5p involved in IGFBP7-AS1–mediated odontogenesis were analysed by qRT-PCR and western blot testing.

Results: Dual-luciferase reporter gene assays showed that miR-335-3p/miR-155-5p could directly bind to IGFBP7-AS1. MiR-335-3p and miR-155-5p both could down-regulate dentin sialophosphoprotein expression, and both miRNAs could inhibit IGFBP7-AS1–mediated SHED odontogenetic differentiation via suppression of the extracellular signal-regulated kinase (ERK) pathway.

Conclusions: Both miR-335-3p and miR-155-5p were negative regulators to IGFBP7-AS1-enhanced odontogenic differentiation of SHED through suppression of the ERK pathway.

Introduction

Stem cell from human exfoliated deciduous teeth (SHED) has been demonstrated to be a promising cell source for regenerative endodontic strategies due to the higher rate of proliferation *in vitro*,[1] greater differentiation capacity,[2,3] and easier acquisition with fewer ethical concerns.[4] The differentiation of SHEDs into odontoblasts is a complex process; our previous study reported that lncRNA IGFBP7-AS1 could positively regulate odontogenetic differentiation in SHEDs without activation of osteogenic differentiation, which was important in regenerative endodontics.[5] However, the exact mechanism of IGFBP7-AS1–enhanced SHED odontogenesis remains unknown.

MicroRNAs regulate complex processes in many different biological systems. MiRNAs are small, highly conserved, noncoding RNAs, ~21 to 23 nucleotides in length, that regulate specific target gene expression through degradation of mRNA or inhibition of translation.[6,7] Computational predictions of miRNA targets indicate that each miRNA potentially regulates hundreds of mRNAs and that approximately one-third of all mammalian protein-coding

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genes are regulated by miRNAs.[8] Epigenetic modifier enzymes regulate a wide variety of critical biological functions, including development, differentiation, organogenesis, and apoptosis, and are themselves regulated by miRNAs. MiRNAs that regulate epigenetic enzymes, including miR-335[9–11] and miR-155[12,13], act as pivotal determinants of cell fate. However, there is few studies concerning the odontogenetic inducibility of miR-155 or miR-335.

Approaches to modify gene regulation by miRNA include inhibitors of miRNAs or the use of miRNA "sponges," which mimic the effects competing endogenous RNA (ceRNA) to deplete the available pool of miRNAs that can regulate endogenous gene expression. lncRNAs can act as ceRNAs and molecular sponges for miRNAs[14], associated with the pathogenesis of a number of diseases[15] as potential therapeutic targets. Furthermore, each lncRNA could target several miRNAs, influencing multiple biological behaviours[16] and creating a complicated network. Once clarified, lncRNA could be a potential amplifier in miRNA-based interventions.

In our previous research, miR-335-3p and miR-155-5p were found to be associated with IGFBP7-AS1—enhanced differentiation via whole-genome sequencing. The purpose of this study is to determine the potential role of miR-335-3p and miR-155-5p in SHED differentiation, investigate the interaction of lncRNA/miRNA, and clarify the mechanism of IGFBP7-AS1—mediated odontogenesis.

Materials and methods

Cell culture

All experimental protocols were approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China (Ethical approval number: LA2020063). SHEDs were obtained from retained primary teeth extracted at the Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology. Briefly, the dental crown was moved off at the cervical part and pulp tissue was isolated from the teeth. The pulpal tissue was cut into pieces and then dissociated in a mixture of 3 mg/mL type I collagenase (Sigma-Aldrich) and 4 mg/mL dispase (Sigma-Aldrich) for 1 hour at 37 °C. Next, the suspensions of SHEDs were passed through a 70-μm strainer (Falcon) to separate the cells. The single-cell suspensions were cultured in α-MEM (GIBCO/BRL), supplemented with 10% fetal bovine serum, (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin, and were incubated at 37 °C with 5% CO_2. SHEDs between 4 and 6 passages were used for subsequent experiments.

Plasmid construction and transfection

The gene sequence of miR-335-3p (No. MIMAT0004703) and miR-155-5p (No. MIMAT0000646) were obtained from the miRBase database. To overexpress IGFBP7-AS1 in SHEDs, an IGFBP7-AS1 overexpression plasmid and control empty plasmid were synthesised. The corresponding plasmids and the lentiviral packaging vectors were first mixed and then used to transfect SHEDs. The transfection efficiency was examined, and the transfected cells underwent selection. MiR-335-3p/miR-155-5p mimic and inhibitor were transfected into IGFBP7-AS1—overexpressing SHEDs using Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific, Inc.). All oligonucleotides were synthesised purified by high-performance liquid chromatography by Sangon Biotechnology Co., Ltd. Twenty-four hours after transfection, cells were incubated and harvested for further analyses. Negative mimic/inhibitor transfected cells served as another control.

Histologic analysis

SHEDs and induced SHEDs were seeded in 6-well plates at 2×10^4 cells per well. Cells were then treated with miR-335-3p/miR-155-5p mimic or inhibitor in odontogenetic-inducing medium for 7 days. After 7 days, cells were fixed and alkaline phosphatase (ALP) levels were evaluated using a tissue nonspecific alkaline phosphatase (TNAP) histochemical staining kit (Cwbiotech) according to the manufacturer's protocol. The Alizarin red staining (Cwbiotech) was applied after 14 days, following the manufacturer's instruction. The integrated density of histochemical slides was processed by ImageJ, and data were analysed.

Construction of luciferase reporter vector and dual-luciferase reporter gene assays

The wild-type IGFBP7-AS1-3'miRNA response element (MRE)-wt plasmid and mutant IGFBP7-AS1-3'MRE-mut plasmid were designed and synthesised (Table). Briefly, 293T cells were transfected with a firefly luciferase reporter pmirGLO vector containing *Renilla* luciferase (Promega Corporation), which harboured the wild-type or mutant IGFBP7-AS1-3'-MRE1 towards miR-335-3p and IGFBP7-AS1-3'-MRE2 towards miR-155-5p and the control vector with miR-335-3p/miR-155-5p mimics using Lipofectamine® 3000. Transfected cells were cultured in an incubator for 6 hours (Thermo Fisher Scientific) and were then transferred into new culture medium containing 10% fetal bovine serum and cultured for an additional 48 hours. The dual luciferase activity was evaluated according to instructions provided by Promega (Promega Corporation). In brief, the cell culture solution was removed from the 96-well plate, and 100 μl phosphate buffer solution was added to wash the cells. Then, 100 μL lysis solution was added to each well, and the plate was shaken for 15 minutes at room temperature to collect the cell lysate. Next, 20 μL cell lysate was mixed with 100 μL luciferase assay reagent II. A 30IOC chemiluminescence analyser was used to evaluate firefly luciferase activity. Subsequently, 100 μL of 1 × Stop & Glo reagent was added to detect the activity of the *Renilla* luciferase. Reporter gene activity was presented as the ratio of the firefly luciferase activity to the *Renilla* luciferase activity.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR analysis was performed using a previously published method.[17] In brief, cells were harvested, and total RNA was extracted from treated SHEDs using TRIzol reagent (Introgen). Total RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RTase, Promega). qRT-PCR analysis was performed on a total volume

Table – The designed sequences of the IGFBP7-AS1-3'MRE plasmid.

IGFBP7-AS1-MRE1-wt	GAGCTCGAGCAGCGCGGGGCGAGGCCGCCGCGGACGCCCCGTCGGATGTGCCCTTCGCTGGGCCGAGCGGCGCAGGGTTGGAGAGG GAAGCGCTCGTGCCCACCTTGCTCGCAGGTGCCCTTGCTGACCTGGGTGATGGCCTTCTCCCCGCGGCTCTCGGCCCTCTGGCTGGC GG CGCGCAGCGTCGAC
IGFBP7-AS1-MRE1-mut	GAGCTCGAGCAGCGCGGGGCGAGGCCGCCGCGGACGCCCCGTCGGATGTCGGGAACGCTGGGCCGAGCGGCGCAGGGTTGGAGAG GGAAGCGCTCGTGCCCACCTTGCTCGCAGGTCGGGAAGCTGACCTGGGTGATGGCCTTCTCCCCGCGGCTCTCGGCCCTCTGGCTG GCGGCGCGCAGCGTCGAC
IGFBP7-AS1-MRE2-wt	GAGCTCTACTTGACAAAGATCAGAACCCTGAGCAATCAAGGGCAGGAAACATAGGCTAGCTGAGCTTTGAAGGAAGTGAAAAGAAA AAGAGGCAGCAAAGATGGGAACATGGAGGACAGTCTGAGAAAACAAGCTCAGGGTTGAAGGGGGTGTTCCAGTGAAGGGGTGAT GAGTGGGACCTCACACATGGAGCAGATAACATTGGTGCATCAGTTGATTTAAATGAGCAGAATGGAAAGTGTCTAAAAGAGAGAT GTGGAAATGAGTAGAAACATCCCTTTCCTGTTGGGCCAGCTATTGTCTATGGTTAGTCCTTTTCCGTCGAC
IGFBP7-AS1-MRE2-mut	GAGCTCTACTTGACAAAGATCAGAACCCTGAGCAATCAAGGGCAGGAAACATAGGCTAGCTGAGCTTTGAAGGAAGTGAAAAGAAA AAGAGGCAGCAAAGATGGGAACATGGAGGACAGTCTGAGAAAACAAGCTCAGGGTTGAAGGGGGTGTTCCTCACTTCCCGTGAT-GAGTGGGACCTCACACATGGAGCAGATAACATTGGTGCATCAGTTGATTTAAATGAGCAGAATGGAAAGTGTCTAAAAGAGAGAT GTGGAAATGAGTAGAAACATCCCTTTCCTGTTGGGCCAGCTATTGTCTATGGTTAGTCCTTTTCCGTCGAC

of 20 μL in SYBR® Green master mix (Rox, Roche Applied Science), with 0.5 μL cDNA and 200 nM primers. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), dentin
sialophosphoprotein (DSPP), ALP, and dentin matrix acidic phosphoprotein (DMP) 1 were designed by Primer3 and synthesised commercially (Sangon). qRT-PCR amplification was performed using the following thermal cycling conditions: 50 °C for 2 minutes, then 95 °C for 10 minutes, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for 1 minute. An ABI PRISM 7500 Sequence Detection System (Applied Biosystems) was used to perform the qRT-PCR reactions. All data were analysed using PRISM6 software (one-way analysis of variance [ANOVA] and least significant difference [LSD] comparison test).

Western blot analysis

Cells were incubated in inducing medium for 7 days, then were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein was extracted and quantified using a BCA Protein Assay (Pierce). Briefly, 40 μg protein from each sample was separated on 10% SDS-PAGE gels then transferred to PVDF membranes (Millipore) at 100 V for 60 minutes. The membranes were incubated in blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, pH 7.4) for 1 hour and then incubated with antibodies: DSPP (ab216892, Abcam, China/sc-73632, Santa Cruz Biotechnology), DMP1 (sc-6551, Santa Cruz Biotechnology), extracellular signal-regulated kinase (ERK) 1/2 (ab17942, Abcam), phos-ERK1/2 (ab201015, Abcam), and β-actin (D6A8, 8457T, Cell Signaling Technology) in 1:1000 dilutions at 4 °C overnight. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (PV9001, PV9002, ZSJQ) for 1 hour at room temperature. Protein bands were visualised using a Fusin Fx assay (Vilber Lourmat).

Statistical analysis

The staining results were processed, and the relative integrated densities were measured by ImageJ. Statistical analysis was performed using one-way ANOVA and LSD comparison test using PRISM6. A P value <.05 was considered to indicate a statistically significant difference between groups.

Results

miR-335-3p and miR-155-5p are involved in odontogenetic differentiation of SHED

The levels of miR-335-3p and miR-155-5p were upregulated by transfection with miR-335-3p and miR-155-5p mimics, and miR-335-3p and miR-155-5p expression was suppressed by transfection with miRNA inhibitors (Figure 1A). TNAP staining demonstrated negative regulation of miR-335-3p and miR-155-5p (Figure 1B, 1D), and alizarin red staining showed no significant difference (Figure 1C, 1E). Downregulation of miR-335-3p or miR-155-5p significantly increased the mRNA expression of DMP1 (Figure 1A). The expression of DSPP was influenced by miR-155-5p but not miR-335-3p (Figure 1A).

Western blot protein analysis demonstrated that inhibition of miR-335-3p or miR-155-5p in SHED increased protein expression of DSPP and DMP1, and transfection with miRNA miR-335-3p or miR-155-5p mimics suppressed the expression of DSPP and DMP1 (Figure 2A). These data suggest that miR-335-3p and miR-155-5p negatively regulate odontogenetic differentiation of SHED.

IGFBP7-AS1 directly regulates miR-335-3p and miR-155-5p expression

IGFBP7-AS1 has been suggested to be a regulator in SHED differentiation,[5] and miR-335-3p and miR-155-5p are predicted to be downstream targets of IGFBP7-AS1. Based on bioinformatic prediction analysis (Figure 3A, 3B), a miR-335-3p-binding site and a miR-155-5p-binding site were mapped to the 3'-MRE of IGFBP7-AS1 (Figure 3C). The luciferase activity in cells transfected with a wild-type IGFBP7-AS1-3'MRE vector containing miR-335-3p and miR-155-5p mimics was significantly reduced compared to cells transfected with the mutated IGFBP7-AS1-3'MRE vector (Figure 3D, 3E), suggesting that expression of IGFBP7-AS1 was inhibited by miR-335-3p and miR-155-5p.

miR-335-3p/miR-155-5p reverse IGFBP7-AS1–mediated DSPP expression through ERK pathway regulation

To verify the regulation of IGFBP7-AS1 by miR-335-3p and miR-155-5p, western blot assay was carried out to test the level of target genes in IGFBP7-AS1–overexpressing SHED.

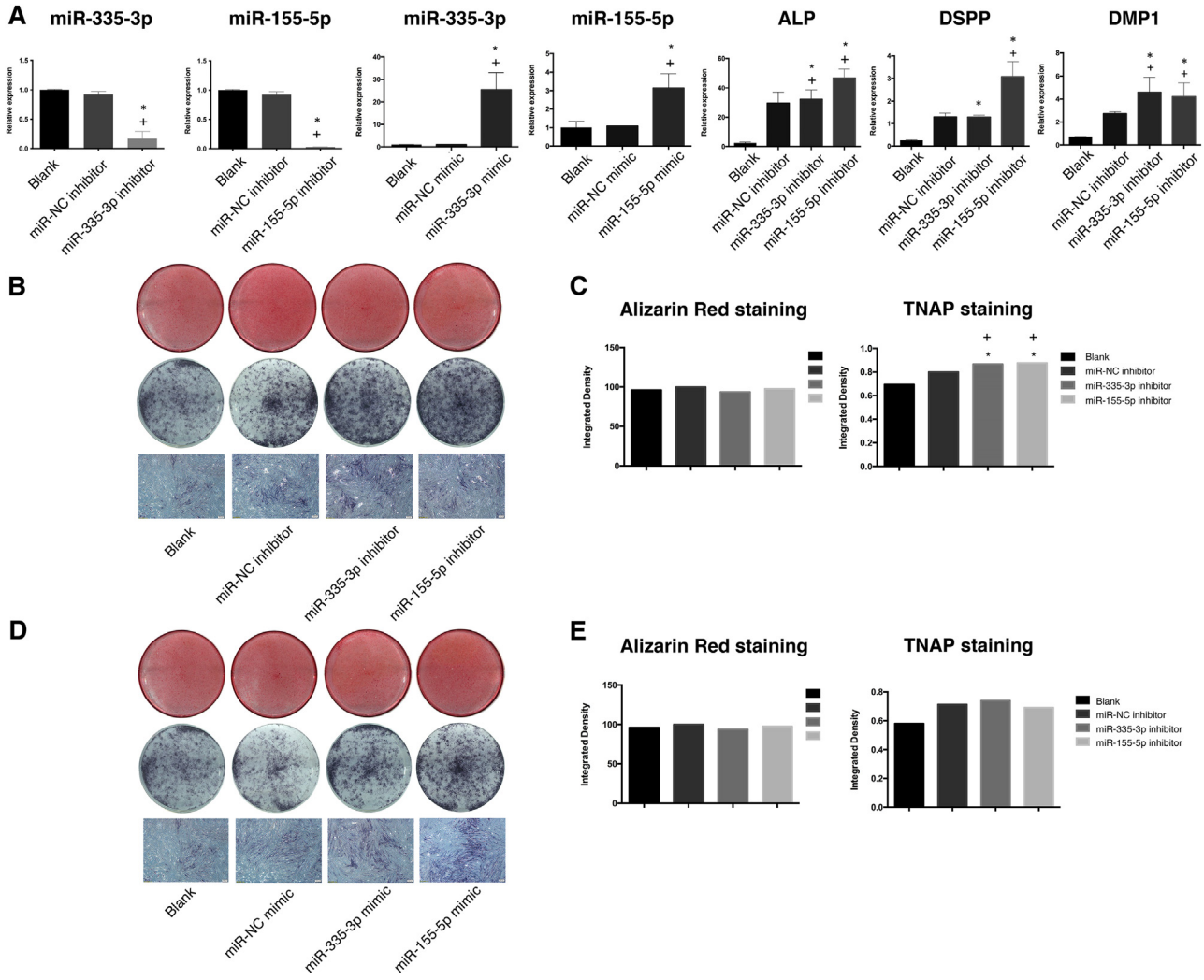

Fig. 1 – Quantitative analysis of the stem cell from exfoliated deciduous teeth (SHED) odontogenic differentiation regulated by miR-335-3p/miR-155-5p. A, Quantitative real-time polymerase chain reaction showed that the expression of miR-335-3p/miR-155-5p were inhibited by miRNA inhibitor and enhanced by miRNA mimic. The expression of alkaline phosphatase (ALP), and dentin matrix acidic phosphoprotein 1 were upregulated by the miR-335-3p/miR-155-5p inhibitor, and DSPP was enhanced by miR-155-5p inhibitor. B–E, SHEDs transduced with the mimic or inhibitor of miR-335-3p/miR-155-5p were cultured for 2 weeks in a medium containing osteogenic factors. Alizarin red staining showed no significance between miR-335-3p/miR-155-5pinhibited SHED and the negative control. The TNAP staining in low and high power fields (scale bars, 200 μm) showed that ALP activity of miR-335-3p/miR-155-5p-inhibited SHEDs were higher than the negative control. Alizarin red staining showed no significance between miR-335-3p/miR-155-5p upregulated SHED and the negative control. The TNAP staining in low and high power fields (scale bars, 200 μm) showed that ALP activity of miR-335-3p/miR-155-5p–upregulated SHEDs were higher than the negative control (B, D). The grey density of both staining results was analysed by ImageJ (C, E). Representative images are shown. N ≥ 3. Values were normalised to the mean of control healthy individuals. Results were expressed as mean ± standard error of the mean. $^{*}P < .05$ vs blank; $^{+}P < .05$ vs control empty vector. One-way analysis of variance, LSD.

The mRNA and protein level of DSPP were elevated by IGFBP7-AS1 overexpression, and miR-335-3p or miR-155-5p upregulation by miRNA mimic inhibited IGFBP7-AS1 –induced expression of DSPP (Figure 4A, 4B). These data suggest that IGFBP7-AS1/miR-335-3p/miR-155-5p participate in the odontogenic differentiation of SHED. Furthermore, phosphorylated ERK1/2 was suppressed by miR-335-3p or miR-155-5p mimics (Figure 4B), suggesting that these noncoding RNAs are involved in regulating the ERK pathway.

Discussion

The formation of pulp–dentin complex is strategic for the development of immature root and generation of reparative

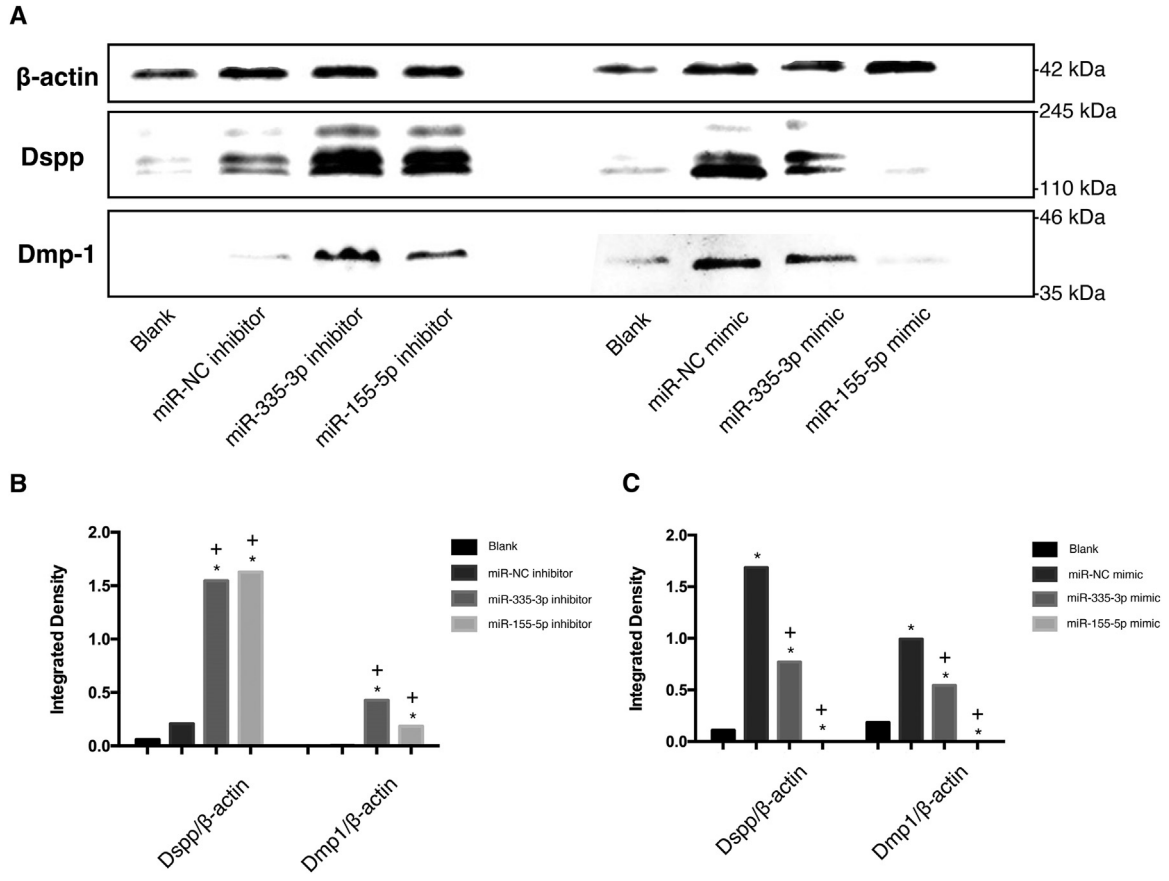

Fig. 2 – The expression levels of odontogenic markers in transduced stem cell from exfoliated deciduous teeth (SHED) were examined by western blot assay. A, Dentin sialophosphoprotein and dentin matrix acidic phosphoprotein 1 expression levels in miR-335-3p/miR-155-5p–inhibited SHEDs and miR-335-3p/miR-155-5p–upregulated SHEDs were examined at day 7 after induction by western blot analysis. B, The grey density of blotting bands was analysed by ImageJ. C, The grey density of blotting bands was analysed by ImageJ. $^{*}P < .05$ vs blank; $^{+}P < .05$ vs control empty vector. One-way analysis of variance, LSD.

dentin. During odontogenic differentiation, multipotent stem cells respond to specific signals and differentiate into odontoblasts, then produce functional dentin, a procedure which relies on the collaboration of multiple growth factors, including the expression of many noncoding RNAs and miRNAs.

More than 300 protein-coding genes are involved in odontogenesis,[18] and nearly 60% of these genes are regulated by miRNAs,[19] with an average of 200 gene targets per miRNA.[20,21] miRNAs can regulate genes at the posttranscriptional level, and miR-21, miR-140-5p, and miR-143-5p have been reported to regulate odontoblast differentiation of dental pulp cells.[22,23] The expression of miR-155 is controlled by multiple signaling pathways, including transforming growth factor β (TGF-β).[24,25] miR-155-5p is the predominant functional miR-155[16] gene. Potential miR-335 targets in human mesenchymal stem cells are enriched in transcription regulators of cell movement and differentiation, including RUNX2, a transcription factor essential for osteogenic differentiation, which was validated as a direct miR-335 target.[26] More than 200 genes are predicted to be targeted by miR-335, and many of these targets are involved in signaling pathways, including the p53, MAPK, TGF-β, and Wnt pathways.[27] It was reported that both miR-335 and miR-155 were involved in the process of cellular differentiation, and our research confirmed that miR-335-3p as well as miR-155-5p could influence the odontogenic differentiation of SHED. ceRNAs can regulate target genes and miRNAs by competing for MREs and shifting the balance between target genes and miRNAs in physiologic and pathophysiologic processes.[28,29] Amongst these, lncRNAs form more complicated structures that regulate an array of cellular processes,[30,31] including genomic imprinting, chromosome inactivation,[32] differentiation, transcription, and translation,[33] including odontoblasts and osteoblast differentiation in dental pulp stem cells.[34,35] In this study, we demonstrated that both miR-335-3p and miR-155-5p could bind to the 3'MRE of IGFBP7-AS1, interfering with IGFBP7-AS1-mediated DSPP expression by suppressing the ERK1/2 pathway, speculating that IGFBP7-AS1 might amplify the odontogenic differentiation as an "enhanced sponge," gathering miR-335-3p/miR-155-5p to regulate DSPP. It is critical to improve our understanding of how multiple miRNAs work synergistically

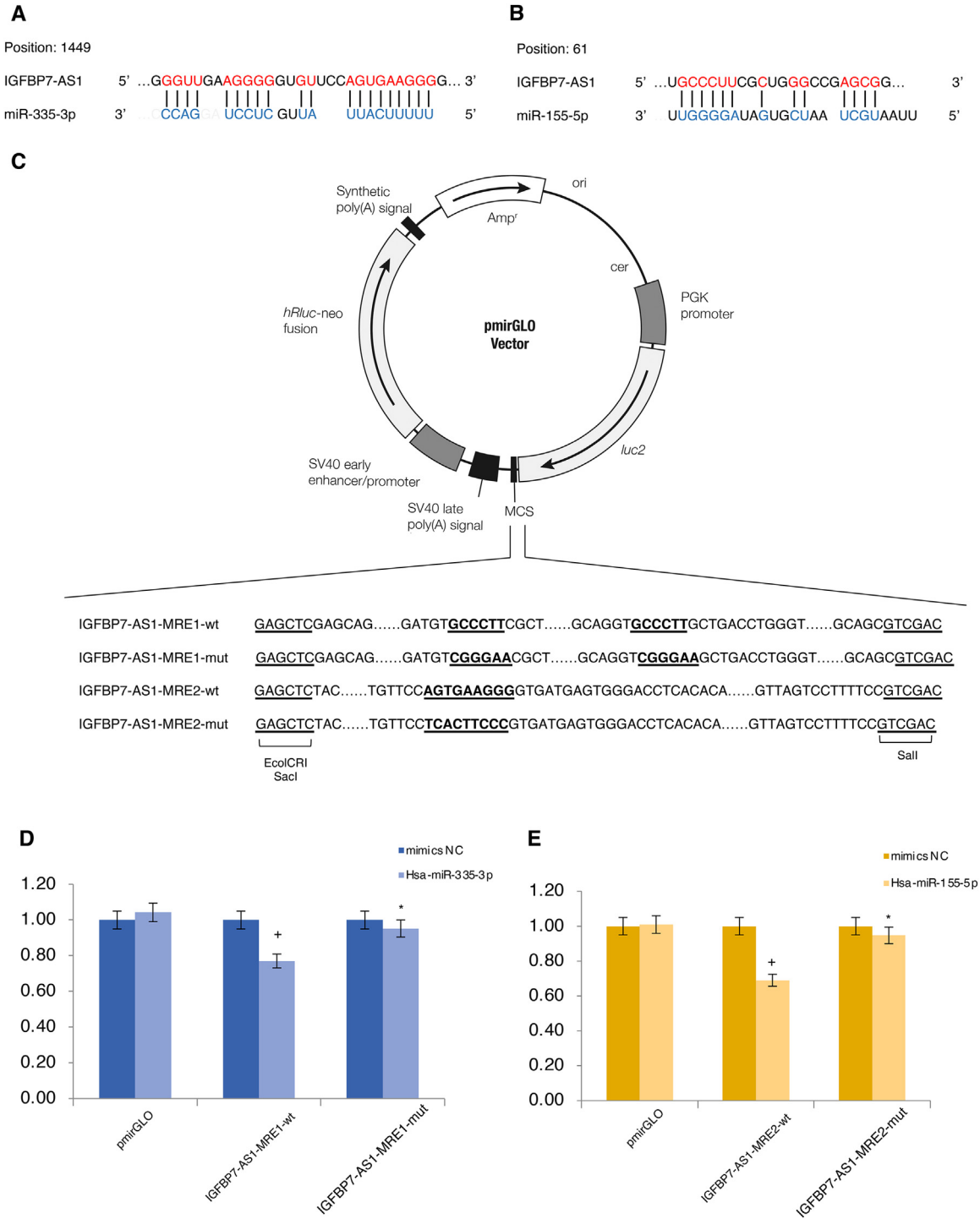

Fig. 3 – miR-335-3p/miR-155-5p could directly bind to IGFBP7-AS1. A, Predicted duplex formation between miR-335-3p and the wild-type IGFBP7-AS1 binding site is indicated. B, Predicted duplex formation between miR-155-5p and the wild-type IGFBP7-AS1 binding site is indicated. C, The plasmid was designed and synthesised. D, Luciferase activity of the constructs containing the wild type or mutant IGFBP7-AS1 reporter gene in 293T cells co-transfected with negative control or miR-335-3p. Relative *Renilla* luciferase activity was determined and normalised against the firefly luciferase activity. E, Luciferase activity of the constructs containing the wild-type or mutant IGFBP7-AS1 reporter gene in 293T cells co-transfected with negative control or miR-155-5p. Relative *Renilla* luciferase activity was determined and normalised against the firefly luciferase activity. $^{*}P < .05$ vs wild type; $^{+}P < .05$ vs control vector. MCS, modulating and coding sequences.

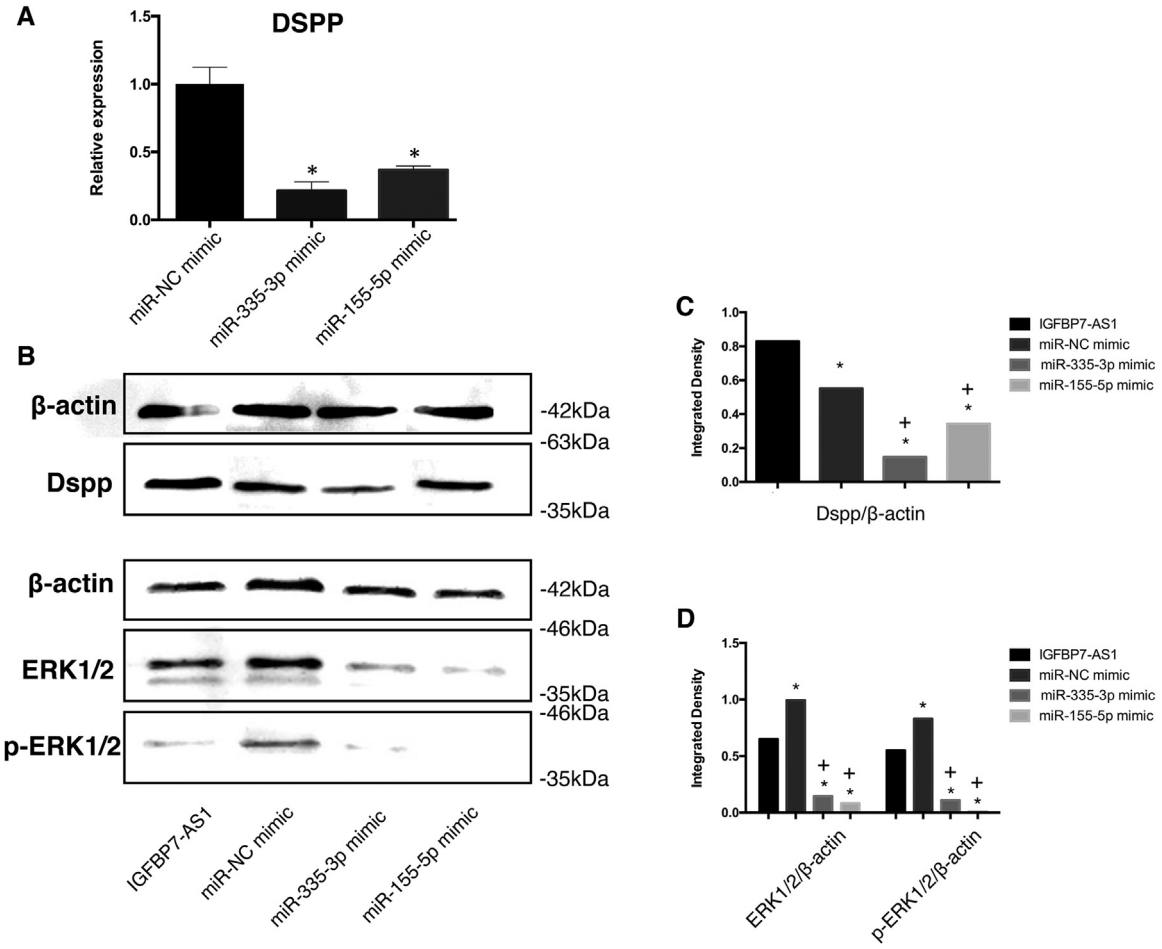

Fig. 4 – Reverse validation assay. A, Quantitative real-time polymerase chain reaction analysis of the expression of dentin sialophosphoprotein (DSPP) in IGFBP7-AS1–overexpressed stem cells from exfoliated deciduous teeth (SHEDs) transfected with miR-335-3p/miR-155-5p mimics. B, Western blot analysis in IGFBP7-AS1–overexpressed SHEDs transfected with miR-335-3p/miR-155-5p mimics. C, Grey density analysis of the bands of DSPP in IGFBP7-AS1–overexpressed SHEDs transfected with miR-335-3p/miR-155-5p mimics. D, Grey density analysis of the bands of extracellular signal-regulated kinase (ERK) 1/2 and phos-ERK1/2 in IGFBP7-AS1–overexpressed SHEDs transfected with miR-335-3p/miR-155-5p mimics. *P < .05.

to regulate a single mRNA target, as is the case with the IGFBP7-AS1 regulation of DSPP expression by miR-335-3p and miR-155-5p. Except for the ERK pathway, there are several pathways involved in odontoblast differentiation, and both miRNAs could activate other pathways to affect more physiologic functions.[26,27] In previous studies, the improvement of a physiologic effect mostly focused on certain effector, not continuous reaction series. Considering that some miRNAs can co-regulate their targeted genes,[36] it is possible to regulate a single target to initiate cascade reactions, just as IGFBP7-AS1 could accommodate miR-335-3p as well as miR-155-5p, influencing their different downstream effector molecules. Our results raised a possibility that single lncRNA could modulate multiple miRNAs to manipulate certain complicated process such as pulpal regeneration, which needs further elucidation about the complete mechanism to consummate this potential strategy to improve clinical dental pulp regeneration.

Conclusions

This study reported miR-335-3p and miR-155-5p represented as important regulators during SHED differentiation and both involved in IGFBP7-AS1–mediated odontogenesis via the ERK1/2 pathway. These data suggest that IGFBP7-AS1 may be a productive gene target that could lead to the development of miRNA co-regulation in odontogenesis of SHED and as an enhanced regulator for its diverse binding ability. Our findings illuminate the binding interactions between IGFBP7-AS1 and miR-335-3p/miR-155-5p and underscore the significance of IGFBP7-AS1/miR-335-3p/miR-155-5p as novel targets for therapeutic intervention in dentin–pulp complex regeneration.

Conflict of interest

None disclosed.

Acknowledgements

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.identj.2022.07.008.

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