Circular RNA CircFgfr2 Promotes Osteogenic Differentiation of Rat Dental Follicle Cells via Sponging MicroRNA miR-133a-3p and Up-regulating Distal-less Homeobox DLX3

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Abstract

Background

As promising seed cells of periodontal tissue regeneration, dental follicle cells (DFCs) can differentiate into cementoblasts, osteoblasts and fibroblasts upon the regulation of signaling pathways. Emerging evidence shows that circular RNA (circRNA) may participate in tooth regeneration, however its effect on DFCs remains unclear. Our early research found that the expression of circFgfr2 was significantly upregulated when the rat DFCs were incubated by mineral induction medium for 4 weeks, while the expression of miR-133 was down-regulated. It has been widely reported that circRNA may exert as a miRNA sponge to target genes. Therefore, the function of circFgfr2 in osteogenesis of rDFCs was further identified in this study.

Methods

CircFgfr2 was identified by Sanger sequencing and agarose gel electrophoresis. The intracellular location of circFgfr2 in rDFCs was analyzed by fluorescence hybridization (FISH). Furthermore, we constructed circFgfr2 overexpressed rDFCs via pLC5-ciR plasmid. RNA high-throughput sequencing was carried out to investigate the differently expressed messenger RNA (mRNAs) between circFgfr2 overexpressed (OE) groups and control groups. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to further explore the role of circFgfr2 in rDFCs. Moreover, the binding sites of miR-133a-3p and miR-133a-5p with circFgfr2 were predicted by bioinformatics and detected by dual-luciferase reporter (DLR) assay and Chromatin Isolation by RNA Purification (ChIRP). Bioinformatics analysis and the luciferase reporter assay were also performed to investigate the interaction between miR-133a-3p, miR-133a-5p and Distal-Less Homeobox 3 (DLX3). We also constructed miR-133a-3p overexpressed rDFCs via miR-133a-3p mimics. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine their expression of circFgfr2, miR-133a-3p, miR-133a-5p and bone-related genes including bone morphogenetic protein2 (BMP2), BMP6, Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), Osterix (OSX) and distal-less homeobox DLX3. Alkaline phosphatase (ALP) activity detection and staining along with Alizarin red staining and were performed to evaluate the mineralization ability of rDFCs. Western blot were performed to analyse RUNX2 protein expression.

Results

CircFgfr2 primarily had a cytoplasmic location. The mRNA sequencing results illustrated that 361 and 391 mRNAs were upregulated and downregulated respectively. GO and KEGG analysis indicated several pathways and processes which are important for osteogenic differentiation. Furthermore, circFgfr2 acted as a molecular sponge for miR-133a-3p which inhibited DLX3 expression. Overexpression of circFgfr2 increased expression levels of markers of osteogenesis including RUNX2, OCN, OSX, BMP2, BMP6 and DLX3 and decreased the expression of miR-133a-3p and miR-133a-5p. In addition, miR-133a-3p was shown to decrease mineralization in rDFCs. Moreover, circFgfr2 protected DLX3 and Runx2 from miR-133a-3p mediated suppression.
Conclusion

We demonstrated that circFgfr2 promoted the osteogenic differentiation of rDFCs via circFgfr2 / miR-133a-3p / DLX3 / RUNX2 axis. CircFgfr2 has the potential to be the molecular target for the bone tissue engineering.

1. Introduction

The dental follicle is a loose connective tissue surrounding the tooth germ responsible for cementum, periodontal ligament, and alveolar bone formation in tooth development. Dental follicle cells (DFCs) are a group of heterogeneous cells from dental follicle and have the capacity for self-renewal and the potential for multi-directional differentiation[1,2]. In 2002, dental follicle cell was firstly isolated by Zhao et al.[3] from the mice molar region and differentiated toward osteoblast in vitro with exogenous bone morphogenetic protein 2 (BMP2) induction. In 2005, Morsczeck et al.[4] found precursor cells isolated from dental follicle of human third molar teeth expressed putative stem cell markers Notch Receptor 1 (Notch1) and Nestin. In 2010, Honda et al.[5] reported new bone formation after DFCs transplantation in calvarial defects created in immunodeficient rats. Tsuchiya et al.[6] found that there were no statistically significant differences between DFCs and Mesenchymal stem cells (MSCs) with respect to capacity for osteogenic ability. Compared with other dental-derived stem cells like dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from exfoliated deciduous teeth and stem cells from apical papilla, DFCs exhibited strong proliferative capacity, superior pluripotency, and high immunosuppressed effect[7]. Due to these properties, DFCs has the potential for clinical application in bone regeneration treatment.

Circular RNAs (circRNAs) are produced by a specific splicing called “backsplicing” which is different from the canonical splicing of linear RNAs[8]. Unlike linear RNAs, circRNAs lack 5’caps and 3’tails and form closed loop structures[9]. CircRNAs almost located in the cytoplasm and are highly conserved and tissue-specific[10]. In 1976, Sanger et al.[11] firstly found circRNAs in RNA viruses. CircRNAs were originally thought to be errors in RNA splicing. On account of high-throughput sequencing technologies and bioinformatics progression, large numbers of circRNAs are reported to be endogenous, abundant, conserved and stable in cells. Researchers found circRNAs function as competing endogenous RNAs or miRNAs sponges and modulation the expression of target genes[12]. CircRNAs can competitively bind with miRNAs to reverse the inhibition of miRNAs on target genes. The mechanism is called “competing endogenous RNAs (ceRNAs)”. It is becoming evident that circRNAs may be involved in many diseases[13] such as diabetes[14], cardiovascular diseases[15], cancers[16], etc.

There is increasing evidence that circRNAs function during osteogenic differentiation of periodontal tissue (or dental pulp) derived progenitor cells[17]. Chen et al.[18] found 43 upregulated circRNAs and 144 downregulated circRNAs during the differentiation process of human dental pulp stem cells (hDPSCs). Ji et al.[19] revealed that has_circ_0026827 was upregulated during osteogenic differentiation of hDPSCs in
vitro and overexpression of has_circ_0026827 promoted bone formation in vivo. Gu et al.[20] found that circRNA BANP and circRNA ITCH may interact with miR-34a and miR-146a to promote PDLSCs osteogenic differentiation via the mitogen-activated protein kinase (MAPK) pathway. Hence, we proposed to explore the difference in circRNA expression during osteogenic differentiation of rDFCs. We have previously found that the level of circFgfr2 significantly increases after osteogenic differentiation induction for 28 days in rDFCs[21]. Therefore, our target is to further investigate the mechanism of circFgfr2 in rDFCs osteogenesis.

2. Materials And Methods

Ethics Statement

The study was conducted in accordance with the National Institutes of Health guidelines on the ethical use of animals. All experiments were performed with the approval of the Ethics Committee of Sun Yat-sen University. The experimental rats were purchased from the Laboratory Animal Center of Southern Medical University (License No: SCXK [Yue] 20160041).

Isolation, culture and identification of rDFCs

Sprague-Dawley rats (postnatal 5-7 days) were euthanatized by pentobarbital sodium and bathed in 75% ethanol for 10 min. rDFCs were carefully isolated from the tooth germs of the first and second mandibular molars of each rat and were cultured in α-modified Eagle's medium (Gibco, Guangzhou, China) supplemented with 20% fetal bovine serum (FBS) (Gibco, Guangzhou, China) and 1% penicillin/streptomycin (Gibco, Guangzhou, China). rDFCs were incubated under 5% CO₂ at 37°C. The medium was changed every 3 days until the cells reached ~80% confluence. Flow cytometry (FCM) was used to identify rDFCs with anti-CD29, CD34, CD44, CD45 and CD90 (1:200; Abcam Inc.).

Sanger Sequencing and Agarose gel Electrophoresis

According to the circRNA circularization site, specific divergent primers were designed. Sanger sequencing were carried out on the amplified products of divergent primers. Total RNA of rDFCs was extracted by Trizol reagent with or without RNaseR to obtain cDNA through inverse transcription of arbitrary primers. Then, cDNA was amplified by convergent primers and divergent primers. Agarose gel electrophoresis of the PCR product was carried out.

Fluorescence in situ Hybridization (FISH)

The probe labeled with CY3 were constructed for the circFgfr2 sequence and used for in situ hybridization. rDFCs were fixed with 4% paraformaldehyde for 5 min at 4 °C, treated with 0.5% Triton X-
100 15 min at room temperature, incubated with 100% alcohol for 1 min. Dehydration and air drying. The probe was prepared and the hybridization buffer with the probe was denatured at 88 °C for 5 min. After hybridization, the slide was washed with 2×SSC, 5 min, at 42 °C and then 2×SSC was washed at room temperature, 5 min×2. Inhale 50 µL DAPI-Antifade solution on the slide, cover the slide and incubate at dark room temperature for 20 min. Observation under fluorescence microscope.

**Cell transfection**

The full-length cDNA of circFgfr2 was amplified from rDFCs cloned into the specific vector pLC5-ciR (Geneseed, Guangzhou) between the BamHI and EcoRI sites for circFgfr2 over-expression (pLC5-ciR-circFgfr2). The mock plasmid pLC5-ciR (NC) without the circFgfr2 cDNA served as a control. Immunofluorescence was carried out for the verification of transfection according to the manufacturer's instructions. To over-express miR-133a-3p, miR-133a-3p mimics and NC-mimics were purchased from ThermoFisher. The rDFCs were transfected with the above plasmids and mimics according to the requirements of each experiment by using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. After 48h, the rDFCs were harvested and used for further research.

**RNA high-throughput sequencing**

Total RNA was extracted from rDFCs of the circFgfr2 overexpression group and control groups. Then, the messenger RNA was purified by polyA selection, chemically fragmented and converted into single-stranded cDNA via random hexamer priming. After that, the second strand is generated to create double-stranded cDNA. TruSeq libraries were prepared using TruSeq DNA Library Preparation Kits (illumina, San Diego, CA) following with quality control using software Fastp (Shenzhen, China). During quality control, the sequence of the joint and the three bases at the beginning and end were removed. Then, 4bp width window was used to retrieve the sequencing read segments, and the base quality lower than Q30 was removed. Finally, the read segment with at least 50bp length is selected for the downstream analysis process. The data after quality control was compared to the Rat genome (HISAT2 index: Ensembl rnor_6.0 Genome_TRAN) using software HISAT2 V.2.0.5 ([https://ccb.jhu.edu/software/hisat2/index.shtml](https://ccb.jhu.edu/software/hisat2/index.shtml)). The result (BAM format file) is then compared and sorted by chromosome position. Through StringTie V.1.3.3b software ([http://ccb.jhu.edu/software/stringtie/](http://ccb.jhu.edu/software/stringtie/)), we detected and quantified the known protein encoding gene transcripts. Differentially expressed genes were analyzed using edgeR software ([https://bioconductor.org/packages/release/bioc/html/edgeR.html](https://bioconductor.org/packages/release/bioc/html/edgeR.html)). Firstly, the relative expression level was generated according to the original count of the gene, and the unit was CPM (Counts Per Million). Then differentially expressed genes were were calculated and considered statistically significant when P-value<0.05 and |log(FC)|≧1. Metascape gene annotation and analysis resource ([https://metascape.org/gp/#/main/step1](https://metascape.org/gp/#/main/step1)), the Kyoto Encyclopedia of Genes and Genomes (KEGG,
http://www.genome.jp/kegg/) and Cytoscape V3.6.0 software (The Cytoscape Consortium, San Diego, CA) were used for gene annotation, enrichment and pathway analysis.

**Real-time quantitative polymerase chain reaction (qRT-PCR)**

Total RNA was extracted through Trizol reagent (Invitrogen). Total RNA was reverse transcribed to cDNA using Geneseed® II First Strand cDNA Synthesis Kit (Geneseed, China). Relative mRNA and miRNA expression levels were detected by qRT-PCR using Geneseed® qPCR SYBR® Green Master Mix (Geneseed, China). GAPDH or U6 was used as an internal control. All reactions were run in triplicates and fold expression changes calculated via the comparative $2^{-\Delta\Delta Ct}$ method. **Table 1** lists PCR primers used in this study. Primers were synthesized by Forevergen Co, Ltd (Guangzhou, China).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>circFgfr2</td>
<td>GTCCCATCAGACAAAGGCA</td>
<td>GCAGGGACACGATGGAG</td>
</tr>
<tr>
<td>miR-133a-3p</td>
<td>CTTTGGTCCCCTTCA</td>
<td>TGTTGTCGTGGAGTCG</td>
</tr>
<tr>
<td>miR-133a-5p</td>
<td>GCCGAGAGCTGGTAAAATGG</td>
<td>TGTTGTCGTGGAGTCG</td>
</tr>
<tr>
<td>RUNX2</td>
<td>AACCAAGTGCCAGGTTCAA</td>
<td>GGATGAGGAATGCCTAA</td>
</tr>
<tr>
<td>OCN</td>
<td>AGCTCAACCCCAAATGTGAC</td>
<td>AGCTGTGCGGCTCATATT</td>
</tr>
<tr>
<td>Osterix</td>
<td>GTCTTGGGCAACACTCCTACC</td>
<td>GGGCAAAGTCAGACGCTTA</td>
</tr>
<tr>
<td>BMP-2</td>
<td>CCAGCTGACACTTTATATATTTCCA</td>
<td>TGTTGTCGTGGAGGTCA</td>
</tr>
<tr>
<td>BMP-6</td>
<td>GGAGATCCTGTCGGTGTTGG</td>
<td>GATCCAGCATGAAGAGCGGA</td>
</tr>
<tr>
<td>DLX3</td>
<td>GCCTCACACAAACACAGGTGA</td>
<td>GTGGTACCAGGGTTTGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCCATGGCACCAGTCAAG</td>
<td>CAGCGTCTTCCTCAGG</td>
</tr>
<tr>
<td>U6</td>
<td>CTCGCTTCGGCCAGCACA</td>
<td>AAGGCTTCACGCTTTCG</td>
</tr>
</tbody>
</table>

**CircRNA Analysis and Target Prediction**

The genomic loci of the FGFR2 gene and circFgfr2 were analyzed by NCBI database (https://www.ncbi.nlm.nih.gov/). The binding sites of miRNAs with circFgfr2 were predicted by RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/). Downstream genes of miRNAs were predicted by miRDB.
Dual-luciferase reporter assay

PsiCHECK2.0 dual luciferase reporter vector containing wide type (WT) circFgfr2 (circFgfr2 WT) and WT DLX3 3’UTR (DLX3 WT) along with their mutant forms (circFgfr2 MUT 1, circFgfr2 MUT 2, circFgfr2 MUT 3 and DLX3 MUT, respectively), was constructed by Geneseed (Guangzhou, China). To determine whether miR-133a-5p directly targets circFgfr2, 293T cells were co-transfected with circFgfr2 WT or circFgfr2 MUT 1 together with miR-133a-5p mimics or NC. To determine whether miR-133a-3p directly targets circFgfr2, 293T cells were co-transfected with circFgfr2 WT or circFgfr2 MUT 2 or/and circFgfr2 MUT 3 together with miR-133a-3p mimics or NC. In order to verify the interaction between miR-133a-3p and DLX3 gene, another method of Dual-Luciferase reporter assay was employed. DLX3 WT or DLX3 MUT was co-transfected with miR-133a-3p mimics and NC mimics into 293T cells.

48h after the co-transfection, luciferase activity was measured via Dual-Glo@Luciferase Assay System E2940 (Promega, USA) according to the manufacturer’s instructions. Firefly luciferase activities were normalized to Renilla luminescence in each well. All the experiments were independently repeated three times.

Chromatin Isolation by RNA Purification (ChIRP)

circFgfr2 anti-sense DNA probes with biotin labeling at 3-prime end were designed and produced by Geneseed Biotech (Guangzhou, China): 1. GTGCTCCAACAACATCAAGG-Bio; 2. GTACGGTGCTCCAACAACAT-Bio. ChIRP analysis was performed according to protocols published by Chu et al.[22]. LacZ probes were used as a non-specific probe. Total RNA was collected, and qRT-PCR was performed to confirm circFgfr2 probes and interaction of circFgfr2 with miR-133a-3p and miR-133a-5p.

Alkaline phosphatase activity detection and staining

ALP activity was assessed using AKP/ALP test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to manufacturer’s protocol. Briefly, cells were seeded in 96-well plates, cell lysates were collected, samples were incubated at room temperature for 5-10 min, and dilution buffer was added to 100 µL of the sample in a 96-well plate. Finally, fluorescence was measured at OD 405 nm. rDFCs were fixed in 4% paraformaldehyde for 10 min, and then ALP staining was performed by ALP staining kit (Beyotime, Shanghai, China) according to the manufacturer’s protocol.

Alizarin red staining
RDFCs were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min, and then stained with Alizarin red staining solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 30 min at room temperature.

**Western blot analysis**

Radioimmunoprecipitation assay lysis buffer (Fude, China) was used to extract proteins from cells. Protein concentrations were measured using a BCA protein assay kit (Abcam, USA), according to manufacturer’s protocols. Each sample (20 μg) was mixed with 5x loading buffer (Cell Signaling Technology, USA), and boiled for 10 min. After separation using SDS-PAGE and 4%-20% gradient gels, the proteins were transferred to 0.22 μm polyvinylidene difluoride membranes (Millipore, USA). The nonspecific binding sites were blocked with 5% (wt/vol) skim milk for 120 min. The membranes were incubated with the primary antibody of RUNX2 (1:1000, abcam, USA) at 4 °C overnight. Subsequently, the membranes were incubated with horseradish peroxidase AffiniPure goat anti-mouse IgG secondary antibody (Emabio, Beijing, China) at room temperature for 1 h. An enhanced chemiluminescence kit (Millipore Corp, Bedford, MA) was used for imaging.

**Statistical analysis**

Results are presented as mean ± standard deviation. Statistical significance was determined using two-tailed t-test. All data were analyzed using Prism 7.04 (GraphPad Software, USA). *P*<0.05 was considered statistically significant.

3. Results

**Culture and Characterization of rDFCs and Location of circFgfr2 in rDFCs**

The rDFCs had a polygon or long spindle morphology (Figure 1A). Flow cytometry analysis showed rDFCs were positive for MSC markers CD29, CD90, CD44, but were negative for CD34 and CD45 (Figure 1B). CircFgfr2 derived from FGFR2 gene exons5 and exons6 is located on chr1: 200648164-200658087 and 9630 bp in length (Figure 1C). Sanger sequencing and agarose gel electrophoresis confirmed the closed loop structure of circFgfr2 and resistance to RNaseR digestion (Figure 1D). The FISH examination showed that circFgfr2 primarily had a cytoplasmic location in rDFCs (Figure 1E).

**Differentially expressed mRNAs and function enrichment analysis suggest circRNAs participant in osteogenic differentiation of rDFCs**
CircFgfr2 overexpression vector (pLC5-ciR-circFgfr2) was constructed and rDFCs were transfected with the vector which confirmed by immunofluorescence (Figure 2A). The mRNA sequencing results illustrated that 361 mRNAs were upregulated, and 391 mRNAs were downregulated (Figure 2B, C). Furthermore, GO analysis revealed that differentially expressed mRNAs are associated with several biological process including cellular process, biological regulation, metabolic process, multicellular organisms process, development process and so on (Figure 3A). GO pathway enrichment analysis revealed 181 pathways and process including IL-17 signaling, response to mechanical stimulus, positive regulation of MAPK and biomineralization. Heatmap was plotted by http://www.bioinformatics.com.cn, a free online platform for data analysis and visualization (Figure 3B, C, D). Moreover, KEGG pathway analysis indicated that 54 pathways were related to the differentially expressed mRNAs, including MAPK signaling, cAMP signaling, Calcium signaling and TNF signaling which are important for osteogenic differentiation (Figure 3E). Thus, function enrichment analysis indicated that circFgfr2 may play a important role in osteogenesis.

CircFgfr2 Promotes the Osteogenic Differentiation of rDFCs and Functions as a Sponge of miR-133a-3p in rDFCs

Increasing evidence indicated that circRNAs can suppress the levels of functional miRNA by serving as miRNA sponges. The expression levels of miR-133a-3p and miR-133a-5p in rDFCs after transfection with pLC5-ciR-circFgfr2 were examined by qRT-PCR. Upregulated circFgfr2 significantly inhibited miR-133a-3p and miR-133a-5p mRNA levels \( (P < 0.001) \) (Figure 4A). In order to identify the overexpression of circFgfr2, qRT-PCR was used to detect the expression levels of circFgfr2 in rDFCs. Compared with the group transfected with pLC5-ciR-circFgfr2, the overexpression (OE) group expressed a higher level of circFgfr2 \( (P < 0.001) \) (Figure 4B). We previously found\(^{[21]} \) circFgfr2 was remarkably increased during the osteogenic differentiation of rDFCs and speculated that circFgfr2 might stimulate the osteogenic differentiation. So, we evaluated the levels of osteogenesis markers including Runx-related transcription factor 2 (RUNX2), osteocalcin (OCN), Osterix (OSX), bone morphogenetic protein2 (BMP2) and BMP6. The mRNA expression levels of RUNX2, OCN and Osterix were upregulated significantly in circFgfr2 over-expression groups, when compared with NC group (Figure 4C). Moreover, the data showed that overexpression of circFgfr2 significantly upregulated BMP2 and BMP6 mRNA levels \( (P < 0.01) \) (Figure 4D). These data imply that circFgfr2 promotes the osteogenic differentiation of rDFCs and positively regulates the expression of osteogenesis-related genes. Therefore, we investigated the potential mechanism by which circFgfr2 promoted the osteogenesis of rDFCs. MiR-133a has been proven to inhibit the osteogenic differentiation of vascular smooth muscle cells\(^{[23]} \) and we found the levels of miR-133a-3p and miR-133a-5p decreased in circFgfr2 overexpressed rDFCs. Bioinformatics analysis revealed that miR-133a-3p has two binding sites and miR-133a-5p has one binding site for circFgfr2 (Figure 4E). The potential interaction models between miR-133a-3p, miR-133a-5p and circFgfr2 were predicted via RegRNA (Figure 4F). DLR assay showed that both miR-133a-3p and miR-133a-5p can effectively inhibit circFgfr2 luciferase activity \( (P < 0.001) \) and the inhibitions disappeared when the binding sites are mutated (Figure 4G, H). However, ChIRP assay illustrated that compared with LacZ, only miR-133a-3p was obviously enriched in the pellet pulled
down by circFgfr2 probes ($P < 0.05$, Figure 4I). Hence, DLR and ChIRP assay showed that circFgfr2 functioned as sponge to miR-133a-3p.

### miR-133a-3p Suppresses the Osteogenic Differentiation by targeting gene DLX3 in rDFCs and Overexpressing circFgfr2 Reversed the Inhibition

It is well accepted that miRNAs function mainly via directly targeting mRNAs. Downstream potential targets of miR-133a-3p were predicted by miRDB (http://mirdb.org/index.html), microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) and Targetscan (http://www.targetscan.org/vert_71/) databases (Figure 3A). TOP 10 Downstream targets of miR-133a-3p were shown in Figure 3B and the second is DLX3 which was proved to participated in osteogenesis. Hence, we performed Dual luciferase reporter assays to investigate whether miR-133a-3p interacts directly with DLX3 mRNA. The luciferase reporter assays showed that compared to miR-NC, miR-133a-3p overexpression significantly reduced the activity of the luciferase reporter containing the DLX3 WT; but this effect disappeared when the miR-133a-3p binding sites were mutated ($P < 0.001$; Figure 3C). QRT-PCR revealed that miR-133a-3p overexpression significantly reduced DLX3 mRNA level in rDFCs, compared with NC group ($P < 0.01$; Figure 3D). These data indicated that DLX3 is the target of miR-133a-3p. Moreover, circFgfr2 overexpression significantly improved DLX3 mRNA level but co-transfection with miR-133a-3p mimics reversed the improvement ($P < 0.001$; Figure 3E). Next, we investigated whether miR-133a-3p influenced the osteogenic differentiation of rDFCs. Overexpression of miR-133a-3p significantly reduced the activity of ALP, compared with NC groups ($P < 0.01$; Figure 3F). ALP staining (Figure 3G) and ARS staining (Figure 3H) indicated that the miR-133a-3p mimics suppressed osteogenic differentiation of rDFCs. Collectively, these results indicated that miR-133a-3p inhibited the osteogenic differentiation of rDFCs by targeting DLX3. Moreover, it is well known that RUNX2 play essential roles in osteogenesis and its transcription is activated by DLX3$^{[24]}$. Therefore, we evaluated the level of RUNX2 to verify the effect of circFgfr2 and miR-133a-3p. Western blot analysis showed that circFgfr2 overexpression upregulated RUNX2 protein level. Furthermore, miR-133a-3p overexpression significantly inhibited RUNX2 protein level but co-transfection with circFgfr2 overexpression vector significantly reversed the inhibition ($P < 0.001$; Figure 3I, J).

### 4. Discussion

In recent years, researchers have reported the participation of circRNAs in the progression of many diseases$^{[13]}$. However, the role and mechanism of circRNAs in osteogenesis stilled remain to be clarified. In this study, we found that circFgfr2 overexpression promoted the osteogenesis and circFgfr2 can act as
a sponge for miR-133a-3p and reverse the inhibition of miR-133a-3p on DLX3 and RUNX2 in rDFCs. Thus, circFgfr2 has the potential to be the molecular target for the bone tissue engineering.

We previously found circFgfr2 upregulated 7.7 times in rDFCs during osteogenic differentiation via RNA high-throughput sequencing\(^2\). In order to further investigate the function of circFgfr2 in osteogenesis, plasmids containing circFgfr2-overexpression fragments were transfected into rDFCs and mRNA high-throughput sequencing analysis was carried out. The mRNA sequencing results illustrated that 361 mRNAs were upregulated, and 391 mRNAs were downregulated. GO analysis revealed that differentially expressed mRNAs in circFgfr2 overexpressed rDFCs are associated with several biological processes. Moreover, GO and KEGG pathway analyses revealed that mitogen-activated protein kinase (MAPK) signaling was enriched which consistent with our prior studies. The MAPK signaling pathway involves a variety of cellular biological processes, including proliferation, differentiation, and migration. ERK, JNK, and p38 MAPK are some of the main sub-families\(^2\). Jin et al.\(^\text{26}\) reported that ERK/p38 MAPK signaling pathway is an important factor for regulating osteogenic differentiation in hPDLSCs. The activation of the MAPK signaling pathway by phosphorylation of p38 and ERK1/2 with BMP9 increases ALP activity and calcium deposition in rDFCs\(^\text{27}\). Meng et al.\(^\text{28}\) revealed the inhibition of JNK and p38 MAPK pathways decreased the expression of osteoblast-related genes (ALP, OPN, RUNX2) in dental follicle cells. Along with former studies, our results demonstrated that circFgfr2 participates in MAPK pathway during osteogenic differentiation, however, the exact mechanism needs further research.

Studies have demonstrated that miRNAs act as a regulator of osteogenic differentiation. Zhao et al.\(^\text{29}\) transfected miR-129-5p mimics into BMSCs and found the expression of osteogenic genes (Runx2, Bmp2, and OCN) improved which indicated miR-129-5p promoted osteogenic differentiation of BMSCs and bone regeneration. On the contrary, Hu et al.\(^\text{30}\) transfected BMSCs with miRNA-132-3p mimics or inhibitors. The upregulation of miRNA-132-3p expression inhibited osteogenic differentiation, whereas the downregulation of miRNA-132-3p expression enhanced osteogenic differentiation. Moreover, miR-100-5p overexpression inhibited the osteogenesis of hBMSCs and silencing miR-100-5p expression rescued the reduction in osteogenesis\(^\text{31}\). MiRNAs regulate gene expression via inhibiting mRNA translation or promoting mRNA degradation following binding to the 3’ untranslated of target genes\(^\text{32}\). We speculated whether miRNAs played a positive or negative role in osteogenesis is determined by downstream targets. In this study, we present evidence supporting DLX3 as a target of miR-133a-3p using bioinformatics analysis. DLX3 is a member of the DLX family transcription factors and plays an important role in osteogenic differentiation. DLX3 mRNA is expressed at relatively high levels in osteoblasts and can stimulate osteoblastic differentiation\(^\text{33}\). Northern blot showed transduction with DLX3 containing virus improved the expression of bone sialoprotein (BSP) and osteocalcin (OC) in MSCs. Sun et al.\(^\text{34}\) revealed that DLX3 overexpression promoted the expression of ALP, RUNX2, OSX and OCN and induced the formation of calcified matrix in BMSCs. However, Duverger et al.\(^\text{35}\) provided the opposite evidence in vivo. Neural crest deletion of DLX3 increased bone formation and mineralization in craniofacial bones which suggested DLX3 inhibited osteoblastic differentiation. Consistent with our study, Qadir et al.\(^\text{24}\) found two binding sites for miR-133a in DLX3 3’-UTR by performing bioinformatics analysis and
luciferase reporter assays. Then C2C12 premyoblast cells were transfected with miR-133a mimics in the myogenic medium and western blotting indicated that DLX3 protein expression decreased. We found miR-133a-3p overexpression suppressed DLX3 expression and osteogenesis in rDFCs, and miR-133a-3p inhibition by circFgfr2 overexpression reversed the suppression. Therefore, miR-133a-3p plays a negative role in the regulation of osteogenic differentiation via targeting DLX3.

RUNX2 is a BMP response gene essential for early bone formation\[^{[36]}\]. DLX3 regulates the transcription of RUNX2 by binding to its promoters during osteogenic differentiation\[^{[24]}\]. Issac et al.\[^{[37]}\] carried out RNA sequencing and chromatin immunoprecipitation-Seq analyses and found DLX3 regulated RUNX2 which is crucial for bone formation. In this study, transfection of miR-133a-3p mimics suppressed the osteogenic differentiation and RUNX2 protein expression in rDFCs. Zhou et al.\[^{[38]}\] inhibited the expression of miR-133a with ibandronate and found the mRNA level of ALP, type I collagen (COL-1), osteoprotegerin (OPG), OCN, and Runx2 enhanced in PDLSCs. Liao et al.\[^{[23]}\] revealed overexpression of miR-133a suppressed the osteogenic differentiation of vascular smooth muscle cells and caused a decrease in alkaline phosphatase activity, osteocalcin secretion and Runx2 expression. Zhang et al.\[^{[39]}\] carried out 3'UTR luciferase reporter, immunoblotting, and mRNA stability assays and found Runx2 protein accumulation reduced when transfected with miR-133a in osteoblasts and chondrocytes. Hence, miR-133a-3p inhibits RUNX2 expression via regulating DLX3 which regulates RUNX2 transcription.

Recent studies revealed that circRNAs have been involved in mandiseases such as diabetes mellitus\[^{[40]}\], neurological disorders\[^{[41]}\], cardiovascular diseases\[^{[42]}\] and cancer\[^{[43]}\]. Abundant circRNAs exert important biological functions by acting as microRNA or protein inhibitors (‘sponges’), by regulating protein function or by being translated themselves. However, most of circRNAs which have investigated were proposed to act as miRNA sponges\[^{[44]}\]. Liu et al.\[^{[45]}\] found circ_AFF4 stimulated Irisin expression through complementary binding to its downstream target molecule miR-135a-5p in BMSCs. In our study, FISH analysis showed that circFgfr2 primarily had a cytoplasmic location. Furthermore, using dual-luciferase reporter assay and ChIRP assay, we found circFgfr2 bond to miR-133a-3p and regulated its function. Chen et al.\[^{[46]}\] revealed that circFgfr2 overexpression accelerated the myogenic differentiation and the formation of myotubes via eliminating the inhibition effect of miR-133a-5p and miR-29b-1-5p in chicken myoblasts. We found circFgfr2 overexpression promoted the osteogenic differentiation by sponging miR-133a-3p in rDFCs which indicated circFgfr2 was a positive regulator during osteogenic differentiation. However, whether circFgfr2 expedites osteogenesis and bone formation in vivo need to be confirmed in future studies.

This study indicates circFgfr2 acts as a sponge for miR-133a-3p and positively regulates the osteogenic differentiation in rDFCs. The circFgfr2 / miR-133a-3p / DLX3 / RUNX2 axis may be a new therapeutic strategy for the treatment of bone regeneration.

5. Conclusion
We concluded that circFgfr2 promoted the osteogenic differentiation of rDFCs via sponging miR-133a-3p. Our study indicated that a circFgfr2 / miR-133a-3p / DLX3 / RUNX2 axis may serve as a potential therapeutic target for improving bone regeneration. Therefore, circFgfr2 has the potential to be the molecular target for the bone tissue engineering.

**Abbreviations**

**DFCs:** dental follicle cells

**rDFCs:** rat dental follicle cells

**circRNA:** circular RNA

**FISH:** fluorescence hybridization

**mRNAs:** messenger RNAs

**OE:** overexpressed

**GO:** Gene Ontology

**KEGG:** Kyoto Encyclopedia of Genes and Genomes

**DLR:** dual-luciferase reporter

**ChIRP:** Chromatin Isolation by RNA Purification

**DLX3:** Distal-Less Homeobox 3

**qRT-PCR:** Quantitative real-time polymerase chain reaction

**BMP2:** bone morphogenetic protein 2

**BMP6:** bone morphogenetic protein 6

**RUNX2:** Runt-related transcription factor 2

**OCN:** osteocalcin

**OSX:** Osterix

**ALP:** alkaline phosphatase

**Notch1:** notch Receptor 1

**MSCs:** mesenchymal stem cells
Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the National Institutes of Health guidelines on the ethical use of animals. All experiments were approved by the Ethics Committee of Sun Yat-sen University.

Consent for publication

Not applicable.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions
CX carried out the molecular biological studies, participated in the high-throughput sequencing and drafted the manuscript. GL participated in the design of the study and performed the statistical analysis. JL participated in the high-throughput sequencing. HJ, YD, YN conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

References


Figures
Figure 1

Characteristics of rDFCs and circFgfr2. (A) Typical fibroblastic-like morphology of rDFCs at 0 days, 5 days and passage 3. (B) Flow cytometry analysis of the surface markers in rDFCs. (C) The genomic loci of the FGFR2 gene and circFgfr2. (D) Agarose gel electrophoresis assay of circFgfr2 under RNaseR digestion. (E) Fluorescence in situ hybridization (FISH) analysis of circFgfr2 in rDFCs.
Figure 2

Transfection of circFgfr2 over-expression vector and high-throughput sequencing for mRNA expression profiles. (A) Construction and immunofluorescence of pLC5-ciR-circFgfr2 in rDFCs. (B) Heat map of all differentially expressed mRNAs. (C) Volcano plot assessing the significant differentially expressed mRNAs between the overexpression groups and NC groups. OE, overexpression.
Figure 3

Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. (A) Gene ontology analysis in biological processes, cellular components and molecular functions. (B) GO chord diagram using Gene ontology enrichment. (C) Network of enriched terms colored
by cluster ID, where nodes that share the same cluster ID are typically close to each other. (D) Network of enriched terms colored by P-value, where terms containing more genes tend to have a more significant P-value. (E) Bubble diagrams via KEGG Pathway enrichment. BP, biological processes; CC, cellular components; MF, molecular functions; MAPK, mitogen-activated protein kinase; cAMP, cyclic adenosine monophosphate; TNF, tumor necrosis factor.

Figure 4
circFgfr2 promotes the osteogenic differentiation of rDFCs and functions as a miRNA sponge and negatively regulates miR-133a-3p in rDFCs. (A) The expression levels of miR-133a-3p and miR-133a-5p of rDFCs after transfection with pLC5-ciR-circFgfr2. (B) qRT-PCR was used to determine the expression levels of circFgfr2. (C) qRT-PCR was used to determine the expression levels of RUNX2, OPN and osterix. (D) qRT-PCR was used to determine the expression levels of BMP2 and BMP6. (E) The potential binding site sequence of miR-133a-3p, miR-133a-5p on circFgfr2 and mutant sequence for DLR. (F) The potential interaction model between miR-133a-3p, miR-133a-5p and circFgfr2 from RNAhybrid. (G) Relative luciferase activity in rDFCs was detected with luciferase assays after transfection with miR-133a-3p mimics. (H) Relative luciferase activity in rDFCs was detected with luciferase assays after transfected with miR-133a-5p mimics. (I) ChIRP assay was conducted to prove the interaction between miR-133a-3p or miR-133a-5p and circFgfr2 in rDFCs and followed by qRT-PCR to detect circFgfr2 expression level. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5

miR-133a-3p inhibits the osteogenic differentiation of rDFCs in vitro and circFgfr2 over-expression reverses the inhibition. (A) Downstream potential targets of miR-133a-3p predicted by miRDB (http://mirdb.org/index.html), microT-CDS databases: (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) and Targetscan (http://www.targetscan.org/vert_71/) databases. (B) TOP 10 Downstream targets of miR-133a-3p. (C)
Binding relation of miR-133a-3p and DLX3 was verified through dual-luciferase reporter gene assay. (D) The expression levels of DLX3 of rDFCs after transfection with miR-133a-3p mimics or NC. (E) The expression levels of DLX3 of rDFCs after transfection with pLC5-ciR-circFgfr2 and/or miR-133a-3p mimics. (F, G) ALP activity and staining of rDFCs transfected with miR-133a-3p mimics or NC on the 7th day. (H) Alizarin red staining after rDFCs were transfected with miR-133a-3p mimics or NC on the 21th day. (I) Western blot analysis of RUNX2 in rDFCs transfected with miR-133a-3p mimics and/or pLC5-ciR-circFgfr2. (J) Quantitative western blot analyses of the relative expression of RUNX2. *P < 0.05, **P < 0.01, ***P < 0.001.