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p75NTR-Mediated Odonto/Osteogenic Differentiation of EMSCs: The Upstream Regulatory Role of circRNA

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ABSTRACT

Neural crest-derived ecto-mesenchymal stem cells (EMSCs), as the progenitor cells of odontogenic stem cells, may be the suitable seed cell for regenerative dentistry. p75NTR, as the neural crest stem cell marker, plays a crucial role during the teeth development of EMSCs. Nevertheless, the interaction networks p75NTR connecting with odonto/osteogenic differentiation and mineralization are still poorly studied. Circular RNAs (circRNAs) regulate life processes mainly by performing as a competitive endogenous RNA (ceRNA) to prevent the process that microRNAs (miRNAs) bind with their target mRNAs. But the roles of p75NTR-related circRNAs in EMSCs are largely unknown. We used circRNA-seq to examine the differentially expressed circRNAs between WT and p75NTR (-/-) EMSCs and three of them were selected for qRT-PCR verification. GO, KEGG and Reactome enrichment analyses showed that differentially expressed circRNAs interact with cell proliferation, locomotory behavior, cell differentiation and are mainly involved in Wnt, JAK/STAT, Hippo and TGF-β signaling pathways. Then, CCK8, transwell assay and ALP staining assay were performed to verify the result of enrichment analyses. Then, the circRNA-miRNA interaction networks were constructed by using bioinformatics analysis. A new circRNA, mmu_circ_0001380 and mmu_circ_0013536 were selected to predict potential target miRNAs. Besides, with TargetScan, we noticed that these three circRNAs may influence the expression of DSPP and RUNX2 and qRT-PCR was performed for verification. Therefore, these three circRNAs are inclined to be vital in developing EMSCs and be novel core molecules for the further understanding of odontogenesis related to p75NTR.

INTRODUCTION

Neural crest-derived ecto-mesenchymal stem cells (EMSCs) have the potential to differentiate into smooth muscle cells, glial cells, and osteoblasts, respectively (Deng et al. 2003). With these unique characteristics, neural crest-derived EMSCs may be suitable seed cell for regenerative dentistry. Neurotrophic factor low- affinity receptor p75NTR, as a member of the tumor necrosis factor superfamily, can mediate multiple biological processes, such as odontogenesis (Shan et al. 2022; Wang et al. 2021). Besides, p75NTR can serve as the marker protein of neural crest-derived EMSCs, which indicates that p75NTR is vital in the odontogenesis of EMSCs (Wen et al. 2012). However, the tooth formation includes at least two crucial processes odonto/osteogenic differentiation and mineralization. Therefore, understanding whether p75NTR promotes or inhibits these two processes is the foundation for further studies. As for odonto/osteogenic differentiation, two researchers have opposing ideas that p75NTR promotes differentiation in dental pulp stem cells (DPSCs) of permanent teeth while inhibiting in DPSC of deciduous teeth (Mikami et al.2011; Waddington et al.2009). Besides, there is a debate about the mineralization effect of p75NTR: p75NTR negatively regulates mineralization in DPSC while positively in EMSC (Mikami et al.2011; Yang et al.2017). Due to the complex biological functions of p75NTR in different cells, we acquire p75NTR (-/-) EMSCs to explore functions of p75NTR in EMSCs.

There are complex signaling pathways related to p75NTR, and researchers attempt to reveal the mechanisms by studying classical signaling pathways and in the aspect of the downstream molecule. Microarray assay was adopted to demonstrate differential gene expression profiles between p75 (-/-) EMSCs and WT EMSCs, and Smad4 was found to be essential in the determination of odonto-differentiation potential (Xing et al. 2016). Wang selected PI3K/Akt through RNA-seq analysis between p75NTR (-/-) EMSCs and WT EMSCs and further discovered that p75NTR could promote osteogenesis differentiation via regulating PI3K/Akt/β-catenin (Wang et al. 2020). However, these studies failed to cast light on the upstream molecule and mechanisms underlying comprehensively. Based on the research above, therefore, circRNA-seq, the new method

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that focuses on the upstream molecule and reveals gene expression pattern, is urgently needed to supplement signaling pathway networks. CircRNAs may participate in many life processes and they have high abundance, stability, evolutionary conservation and cell type specificity (Qu et al. 2017). However, the complex interplay between circRNAs and p75NTR is poorly understood. Circular RNAs (circRNAs) play a biological role mainly in several ways, such as performing as a competitive endogenous RNA (ceRNA), binding to RNA-binding protein (RBP), protein scaffold, transcription regulator and translation (Kristensen et al. 2019). Among them, ceRNA is the most common research direction. CeRNA mechanism means circRNAs bine with miRNAs to regulate their target mRNAs (Li et al. 2018). Zheng analyzed the circRNA expression profiles of periodontal ligament stem cells and discovered circRNAs participate in the process of osteogenesis, which leads to the further understanding of miR-7 and p38 MAPK signaling pathways (Zheng et al. 2017; Li et al. 2018). Besides, research demonstrates that circRNA-vgll3 promotes the construction of new bone and osteogenic differentiation of ADSCs (Zhang et al. 2021). Therefore, we inferred that p75NTR-related circRNAs might lead to the difference between p75NTR (-/-) EMSCs and WT EMSCs and may be the biomarker for regulating the teeth development of EMSCs.

We analyzed the comprehensive circRNA expression pattern in WT EMSCs and p75NTR (-/-) EMSCs to gain a preliminary understanding of circRNAs. Then, the circRNA-miRNA interaction networks were constructed by miRanda, and functional analyses were applied to analyze the host gene of the differentially expressed circRNAs. Besides, CCK8, transwell assay and ALP staining assay were performed to verify the functions of circRNAs. Moreover, miRNA-mRNA networks were drawn based on the TargetScan. After that, qRT-PCR was applied to detect the expression level of potential target mRNAs. Hopefully, our study could identify the crucial circRNAs correlated with p75NTR and provide new clues for understanding the mechanism of p75NTR-mediated biological effects in EMSCs.

MATERIALS AND METHODS

Ethics statement

We followed the rules of the Medical Ethics Committee of the Wenzhou Medical University (NO. wydw2019-0224) to design experimental protocols in this study and we complied with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health to take all experimental steps.

Acquisition of WT and p75NTR (-/-) mice

p75NTR (+/-) mice were bred in the Animal Center of Wenzhou medical university (Wenzhou, Zhejiang Province, China). Then, the embryos were removed from the female mice at 16.5 days of pregnancy. Subsequently, the genotypes of the mice's tails were determined by PCR amplification.

Cell culture and RNA extraction

We isolated the embryonic mandibular of six embryos. Half of them are from p75NTR (-/-) mice, and others are from WT mice. Then, EMSCs were extracted and cultured in Dulbecco's modified eagle medium: F12 (DMEM/F12) (Gibco, Thermo Fisher Scientific, MA, USA), 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, MA, USA), 1% antibiotics (100 ug/ml penicillin and 100 μg/mL streptomycin), and subsequently they were cultured in a humidified atmosphere containing 5% CO2 for three generations at 37°C. Then they were put in mineralized induction fluid for seven days. RNA from EMSCs was acquired by Trizol (Invitrogen, Thermo Fisher Scientific, MA, USA).

CircRNA-sequencing

After the EMSCs samples were qualified, the linear RNA was removed by RNase R, and then the gene library was constructed using KAPA RNA HyperPrep Kit with RiboErase (HMR) for Illumina®. After that, Qubit3.0 and Agilent 2100 Bioanalyzer were applied to detect the size range of the gene library. After the qualification of the gene library meeting the standard, gene libraries are obtained by pooling based on the requirements of effective concentration and target on-machine data quantity. After that, sequencing is conducted by Nova-seq 6000 PE150 mode with the sequencing data quantity of 10G.

Identification and expression quantification of circRNAs

we obtain clean reads by removing the joint sequences, sequences with more N base and low-quality reads from Raw reads to obtain high-quality data (clean reads). Then clean reads and reference genomes (UCSC transcript set) were mapped for the identification of circRNAs. The STAR and DCC were used for comprehensive identification of circRNAs.

Construction of circRNA-miRNA networks

The interactions between circRNA and miRNA were predicted using miRanda (http://www.microrna.org/microrna/home.do/). Then we left these with scores ≥ 140 for further research. Because the more the number of miRNA binding sites (mreFreq) on the circRNA sequence, the more likely miRNA is to bind to circRNA, we retained the top 1000 miRNA-circRNA relationships with the largest total score. In the end, we applied Cytoscape_v3.7.0 to draw networks.

GO, KEGG and Reactome enrichment analyses

GO (http://www.geneontology.org/), Reactome (https://reactome.org/) and KEGG (https://www.genome.jp/kegg/pathway.html) pathway enrichment analyses were performed by ClusterProfiler ofR/bioconductor(http://enrich.shbio.com/index/ga.asp) to predict the function of circRNAs.

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

We use NanoDrop (Thermo Fisher Scientific, MA, USA) to examine RNA quality. Then, the total RNA (1μg) was reverse-transcribed to cDNA using HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China) with random primers. Quantification of circRNA was performed using StepOneplus Real-Time Pcr System (Applied Biosystems, Thermo Fisher Scientific, USA). Primers used for real-time PCR were as follows: GAPDH: F5'CAAGGTCATCCATGACAACTTTG3', R5'GTCCACCACCCTGTTGCTGTAG3'.
mmu_circ_0008240:
F5'CGGAAAGTGGATTGTCA3',
R5'CCCGGTCTGCTGCTGCTGTT3'.
mmu_circ_0008720:
F5'CGTGACAACAGACGGCAGC3',
R5'CGATGACCTTCTCCAGCACG3'mmu_circ_0011879: F5'CCAGGCTTGCCATCTCCA3', R5'CTGGTCCTTAATGTCAGCTTCACT3'.

DSPP, 5'- GGCTCCGAGTCAATACATGTA-3' and 5'-CTCC

TTGGTGTCCATTGCTAT-3'; runt-related transcription factor 2 (Runx2), 5'-CTGCCACCTCTGACTTCTGC-3' and 5'-GATGAAATGCCTGGGAACTG-3'; DSPP, 5'-GGCTCCGAGTCAATACATGTA-3' and 5'-CTCC TTGGTGTCCATTGCTAT-3'.

CCK8 assay

We seeded WT and p75NTR (−/ −) EMSCs in 96-well plates. After that, we added 200ul 10%FBS and 1%DMEM-F12 into each well and incubated EMSCs for 12 hours. Then, CCK8 solution (CCK8, Dojindo, Japan) was added to each well. After incubating for 2 hours at 37°C, we measured the absorbance at 450 nm with a microplate reader.

Transwell assay

We cultivated WT and p75NTR (−/−) EMSCs in a transwell chamber (Corning, USA) for 24 hours. Then, PBS and paraformaldehyde were fixed to wash cells and the cells was stained with 0.1% crystal violet. After cleaning the upper part of the chamber with a cotton swab, we observed cells in the lower part of the membrane under a microscope (Leica DM750, Germany) and washed them with 3% acetic acid. Subsequently, the solution was collected and transferred to a 96-well plate. Then, we measured the absorbance at 490 nm with a microplate reader.

Alkaline phosphatase

We seeded WT and p75NTR（−/−） EMSCs onto 24-well plates. Then HAT-CM was gradually added to take place of the medium. The resolution was changed every 3 days. After cultivating for 7 days, we washed the cells for three times with PBS and fixed them in paraformaldehyde for 30 minutes. Thereafter, they were stained with an ALP kit (Beyotime, China) for 30 minutes. Subsequently, we washed the fixed cells with double-distilled twice and applied a microscope to observe.

Statistical analysis

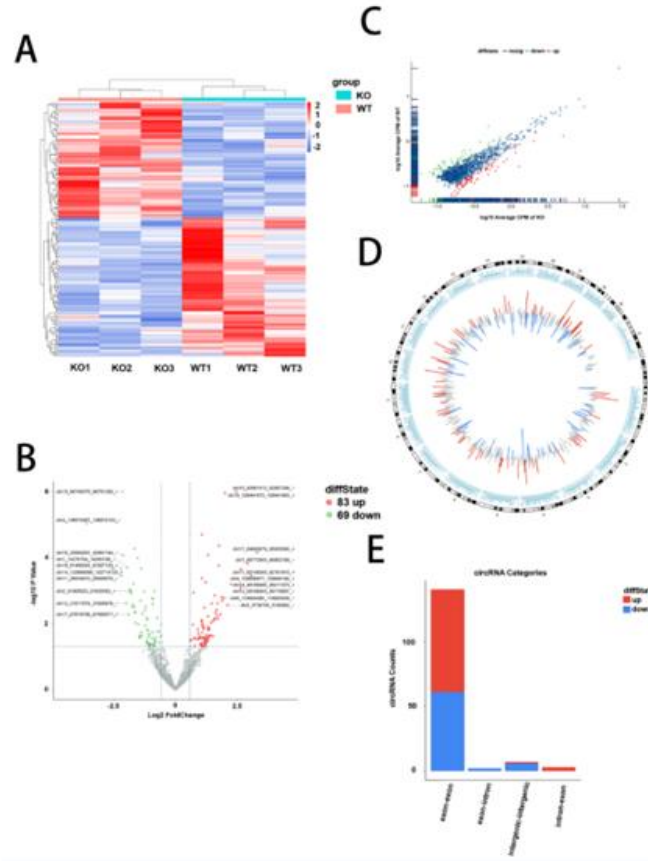
All analyses were used GraphPad Prism software. All data includes means and standard deviations. And independent-samples t-tests, matched t-test and two-way analysis of variance (ANOVA) were performed. P-values < 0.05 was considered statistical significance.

RESULTS

Expression profiles of circRNAs between p75NTR (-/-) EMSCs and WT EMSCs

For the identification of p75NTR-related circRNAs, analysis of the expression pattern of circRNAs was taken in p75NTR (-/-) EMSCs and WT EMSCs. Based on the standard of p-value < 0.05, 152 significantly differently expressed circRNAs were detected, including 83 up-regulated and 69 down-regulated circRNAs in p75NTR (-/-) EMSCs compared with WT EMSCs. Among them,34 circRNAs were first discovered in this study. Scatter plots, volcano diagrams and hierarchical clustering analyses were employed to show differentially expressed circRNAs (Fig. 1A-C). The top 10 up- and down-regulated circRNAs in the p75NTR (-/-) EMSCs were selected (Supplementary. Table 1). Among them, chr17_84993679_85005566_+ and mmu_circ_0011313 rank first among up-regulated and down-regulated genes, respectively. Furthermore, the differently expressed circRNAs were distributed on almost all chromosomes, especially in chromosome 1, chromosome 4, chromosome 10 and chromosome 18, which offers a preliminary understanding of the function of circRNAs (Fig. 1D). We also classified the circRNAs based on the ring-forming characteristics. And most of them are exon-exon, which means they were derived from the DNA sequence that codes protein (Fig. 1E).

Figure 1: Differentially expressed circRNAs in p75NTR (-/-) EMSCs and WT EMSCs.

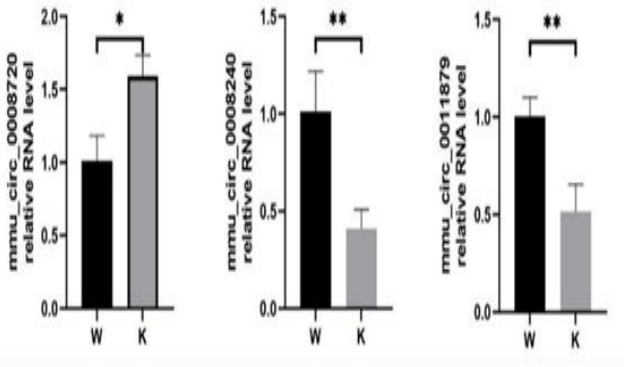


A Hierarchical cluster analysis of circRNAs chosen between two groups (P < 0:05; red means upregulation; green means downregulation). **B** Volcano plot showed the selected circRNAs between the two groups. **C** Scatter diagram means the same way as that in the volcano plot. **D** The distributions of the differentially expressed circRNAs in chromosomes. E Categories of differentially expressed circRNAs. WT, WT EMSCs samples; KO, p75NTR (-/-) EMSCs samples.

qRT-PCR validation of differentially expressed circRNAs

One up-regulated circRNAs (mmu_circ_0008720) and two down-regulated circRNAs (mmu_circ_0011879, mmu_circ_0008240) with higher FCs, lower p-values and suitable primer were performed qRT-PCR to verify the correctness of the results of circRNA-seq. The results of circRNA-seq showed that the log2FC values of mmu_circ_0008720, mmu_circ_0011879 and mmu_circ_0008240 are 1.63, -1.81 and -1.95, respectively. Meanwhile, the result of q-PCR demonstrated the differences between two groups of each circRNA were statistically significant, and the expression level in the qRT-PCR was coordinated with the RNA-seq results, suggesting the validity of the transcriptomics data (Fig. 2).

Figure 2: Results of the qRT-PCR data for candidate circRNAs.



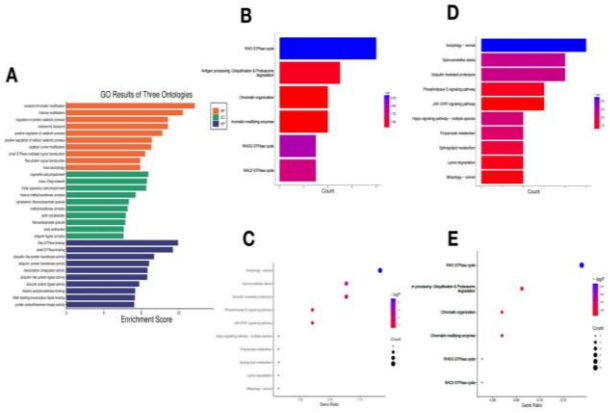
mmu_circ_0008720, mmu_circ_0011879 and mmu_circ_0008240 were performed qRT-PCR for the verification of circRNA-seq. W refers to WT EMSCs, while K refers to p75NTR (-/-) EMSCs. This data consists of the means ± standard deviations. Value > 1 means up-regulated circRNAs, and values < 1 means the opposite. *p < 0.05, **p < 0.01. W, WT EMSCs samples; K, p75NTR (-/-) EMSCs samples.

GO, KEGG pathway and Reactome analysis of p75NTR (-/-) and WT EMSCs.

To understand the possible functions of the differentially expressed circRNAs, GO, KEGG and Reactome enrichment analyses for their host genes were carried out in ClusterProfiler. 122 farther genes were enriched in 130 GO terms. We depicted the differently expressed circRNAs in three parts: Molecular function (MF), biological process (BP) and cellular component (CP) (Fig. 3A). For BP, the host genes of circRNAs are closely related to the regulation of the Wnt signaling pathway (GO:0030111), neuroblast proliferation (GO:0007405) and locomotory behavior (GO:0007626). For MF, the term with the most genes are phosphoprotein phosphatase activity (GO:0004721) and phosphatase activity (GO:0016791). For CC, the host genes of circRNAs are closely related to nuclear speck (GO:0016607) and integral component of the endoplasmic reticulum membrane (GO:0030176). Among the multiple GO terms, we noticed that many GO terms are related to Wnt, JAk/STAT signaling pathways, cell proliferation and cell differentiation, suggesting these may be vital in the odontogenesis of EMSCs (Supplementary. Table 2). Significantly, KEGG analysis revealed 11 significant pathways (Fig. 3B and Fig. 3C). Among them, Hippo signaling pathway(KEGG:mmu04630) and JAK/STAT signaling pathway(KEGG: mmu04392) are most relative to odonto/osteogenic differentiation and mineralization. Besides, Reactome analysis showed the host genes are related to GTP (Reactome: R-MMU-9012999), Antigen processing (Reactome: R-MMU-983168)

and Chromatin (Reactome: R-MMU-3247509) (Fig. 3D and Fig. 3E).

Figure 3: GO, KEGG pathway and Reactome analysis of p75NTR (-/-) and WT EMSCs.



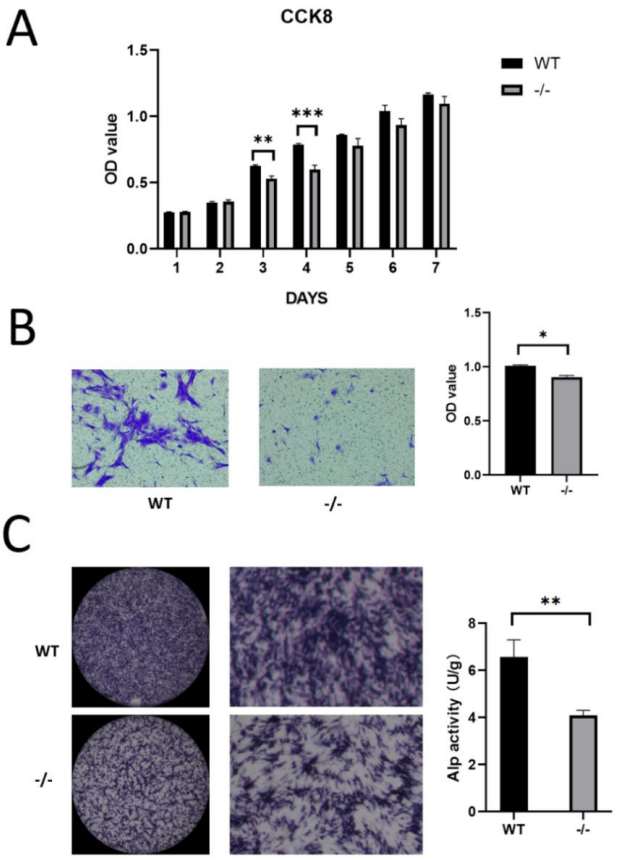
A Go analysis in three parts: molecular function, biological process and cellular component. **B** The bar chart shows the annotation of the KEGG pathways via ClusterProfile. **C** The dot chart shows the annotation of the KEGG pathways. **D** The bar chart shows the Reactome analysis of selected circRNAs. **E** The dot chart shows the Reactome analysis of selected circRNAs.

Verification of functions of circRNAs by CCK8, transwell assay and ALP staining assay in EMSCs.

Among the multiple function predictions of differently expressed circRNAs, we focused on cell proliferation (GO:1902692), cell migration (GO:0007626), cell differentiation (GO:0045664), and mineralization (GO:0030111). Therefore, we applied CCK8, transwell assay and ALP staining assay to further understand the functions of p75NTR-related circRNAs in EMSCs. The column of CCK8 results demonstrated that the proliferation of WT EMSCs is better than p75NTR (−/−) EMSCs overall, and the difference is the most credible in day3 and day4 (Fig.4A). In the transwell assay, the image result indicated that the quantity of cells in the WT groups is more compared with p75NTR (−/−) EMSCs (Fig.4B). Along with the quantitative analysis, these results suggested that WT EMSCs had a higher migrative ability than p75NTR (−/−) EMSCs. The result of two groups under microplate reader of ALP staining assay, along with the p-value<0.01 and FC>1.5, showed

that ALP level in WT EMSCs was significantly higher than that in p75NTR (-/-) EMSCs (Fig.4C). All these findings further signified the p75NTR-related circRNAs might be close to the development of EMSCs.

Figure 4: CCK8, Transwell assay and ALP staining assay of WT EMSCs and p75NTR (-/-) EMSCs.



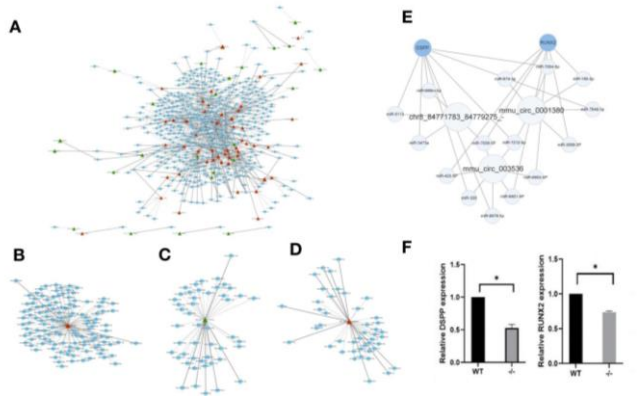
A Growth condition of WT and p75NTR−/− EMSCs. Scale bar, 50 μm. **B** Images of the transwell assay and quantitative measurement of the migration rate of EMSCs. **C** Images of ALP staining assay of WT and p75NTR (−/−) EMSCs under induction for 7 days and quantitative measurement of ALP activities of EMSCs. Scale bar, 100 μm. The data are presented as mean ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < .001$.

Construction of circRNA-miRNA crosstalk networks and qRT-PCR verification of target mRNAs.

CircRNA-miRNA relationships were predicted by miRanda. The top 1000 circRNA-miRNA relationships with the largest total score were retained to draw the circRNA-miRNA regulatory network by Cytoscape_v3.7.0（Fig. 5A）. The ceRNA network of circRNA-miRNA interactions includes 74 circRNAs and 484 miRNAs (Supplementary. Image 1). To find the potential target miRNAs related to odonto/osteogenic differentiation and mineralization, chr8_84771783_84779275_-, mmu_circ_0001380 and

mmu_circ_003536 were selected to draw circRNA-miRNA network of each circRNA respectively (Fig. 5B-D). miR-6971-5p, miR-574-5p and miR-882 are the most potential target miRNAs of chr8_84771783_84779275_-. Mmu_circ_0001380 may bind with miR-874-3p and miR-17-5p, while miR-7212-5p, miR-326-5p and miR-1249-5p may be the targets of mmu_circ_003536. These results suggest that chr8_84771783_84779275_-, mmu_circ_0001380 and mmu_circ_0013536 may participate in the odontogenesis of EMSCs via interactions with miRNAs. Through TargetScan, we gained more understanding of the miRNA-mRNA interaction. Based on the known key protein, circRNAs mentioned above have a close relationship with DSPP and RUNX2 which are both odonto/osteogenic genes (Fig.5E). Therefore, qRT-PCR was applied to compare the expression quantity of DSPP and RUNX2 between two kinds of EMSCs. Further, quantitative analysis showed that the expression level of DSPP in WT EMSCs is 1.9 times more than that in p75NTR (-/-) EMSCs, while 1.4 times for RUNX2 both with p-value <0.05(Fig.5F). Besides, miR-7212-5p is the common target of mmu_circ_0001380 and mmu_circ_0013536 while miR-7058-5p is the common target of chr8_84771783_84779275_- and mmu_circ_0013536. Thus, in all probability, these two miRNAs have more complex functions in regulating biological processes.

Figure 5: Prediction of the circRNA-miRNA interaction networks of differently expressed circRNAs.



A The miRNA-circRNA relations with the high total score. Triangular represents circRNAs while circle means miRNA (Triangles represent circRNAs, circles represent miRNAs, red represents up-regulation, and green represents down-regulation). B The ceRNA network of mmu_circ_003536. C The ceRNA network of chr8_84771783_84779275_-. D The ceRNA network of mmu_circ_0001380. E The circRNA-miRNA-mRNA networks related to DSPP and RUNX2. F The mRNA expression of DSPP and Runx2 were examined by RT-PCR. The data are presented as mean ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < .01$, $^{***}P < .001$.

DISCUSSIONS

p75NTR can serve as the marker protein of EMSCs and is responsible for cell differentiation, osteogenesis and rebuilding of bone tissue (Shan et al. 2022; Mikami et al. 2011; Douillard et al.2016; Bougault et al. 2015). It is well acknowledged that circRNAs have covalently linked ends which can bind to miRNA to regulate gene expressions (Wang et al. 2016). And we assumed that p75NTR-related circRNAs could be vital in the odontogenesis of EMSCs.

Therefore, circRNA-seq and bioinformatic analysis were implied to explore more about p75NTR-related circRNAs between the p75NTR (-/-) and WT EMSCs.

Then, functions of differently expressed circRNAs were predicted by GO, KEGG and Reactome analysis. Among the multiple GO terms, rhythmic process (GO:0048511), regulation of circadian rhythm (GO:0042752), cell differentiation (GO:0045661, GO:0045663, GO:0045445, GO:0045664), negative regulation of cell cycle (GO:0045786), locomotory behavior (GO:0007626) and regulation of Wnt signaling pathways (GO:0016055) are of high p-value and may have a close relationship with the development of EMSCs.

Regarding biological rhythm (GO:0048511, GO:0042752), p75NTR was reported to be the essential protein of biological rhythm when enamel and dentin are formed (Nirvani et al. 2017).

There are five circRNAs related to circadian rhythm, and mmu_circ_0013317 is the only down-regulated circRNA among them with the highest p-value, which indicated that mmu_circ_0013317 may be important in regulating the biological rhythm in the development of EMSCs. The correctness of functions predicted by software in EMSCs is uncertain.

To further understand the role of circRNAs in proliferation, invasion and mineralization, CCK8, transwell assay and ALP staining assay were performed.

The results suggested that WT EMSCs and p75NTR (-/-) EMSCs differed much in these three aspects. Nevertheless, among these multiple differently expressed circRNAs, which one plays the crucial role remains unclear. As for the functions of cell proliferation and invasion, the possible regulating circRNAs are mmu_circ_0000639 and mmu_circ_0011313 with p-value <0.003 and their host genes are Lrrk2 and Kdm1a, respectively.

Recently, it has been reported that Lrrk2 may regulate the invasion and migration of papillary thyroid cancer cells (Li et al. 2021). Kdm1a have a close relationship with both proliferation and invasion (Cai et al. 2018; Wang et al. 2022). Regarding the new research on these two circRNAs' host genes, we could infer the importance of the two circRNAs and silence each circRNAs separately to explore further. To sum up, function predictions were confirmed in EMSCs and the functions of selected circRNAs' host genes were confirmed in other cells. Therefore, we could research further in EMSCs based on these findings.

When it comes to differentiation, GO items are all about neuron and myoblast differentiation. The particular stage when the embryo was acquired determined that Neural crest-derived EMSCs play an essential role in the formation of nerve, muscle, melanin and so on. Wen et al. verified this result in the vitro culture of EMSCs and the identification of multi-directional differentiation of EMSCs (Wen et al. 2012). At present, the osteogenesis and mineralization characteristics of ectoblastic mesenchymal cells have aroused high interest among researchers. As for osteogenic differentiation, our research focused on the signaling pathways related to Wnt. In this research, 7 GO terms associated with Wnt were selected, and the functions of WNT have been partially confirmed in previous studies. Carole Bougault reports that Wnt5a may negatively regulate mineralization in chondrocytes and enthesis (Bougault et al. 2015). Besides, the involvement of BRD4 related to Wnt/β-catenin signaling pathway significantly influences the process of BMSC osteogenic differentiation (Wang et al. 2021). Combining the result of circRNA-seq and the research about Wnt, we supposed that p75NTR-related circRNAs might affect mineralization through Wnt pathways. Besides, we also noticed that in addition to the classic WNT path, mmu_circ_0004400, as the transcript of PIK3R1, may influence the donto/osteogenic differentiation progress of EMSCs. Recently research that PIK3R1 exhibits an osteoprotective effect via regulating osteoblast differentiation supports this idea (Zhu et al. 2021). These findings can further enrich the molecular network of p75NTR in osteogenic differentiation.

Interestingly, the KEGG analysis suggests that differently expressed circRNAs were enriched in the JAK/STAT signaling pathway (KEGG: mmu04630) and Hippo signaling pathway (KEGG: mmu04392).

And then, GO analysis indicates that differently expressed circRNAs were related to JAK/STAT (GO:1904892: regulation of receptor signaling pathway via STAT; GO:0046425: regulation of receptor signaling pathway via JAK/STAT). We noticed that Hippo, JAK/STAT, and TGF-β signaling pathways are crucial in the stem cells during osteogenesis (Wang et al. 2019; Zhang et al. 2020; Zhou et al. 2016). Besides, a non-coding RNA study reported that lncRNA SNHG1 could cause IFNGR1 to express more by binding miR-320b and impact the JAK/STAT signaling (Wang et al. 2020). lncRNA and circRNA can both serve as ceRNA to influence the abundance of the target mRNA (Salmena et al. 2020). Thus, we suspected JAK-STAT and Hippo signaling pathways are important during the development of EMSCs, and p75NTR-related circRNAs may function through these two pathways.

A new circRNA (chr8_84771783_84779275_-) and the most likely potential target miRNAs for chr8_84771783_84779275_- include miR-6971-5p, miR-574-5p and miR-882. It is noteworthy that miR-574-5p can release Rheumatoid Arthritis by exerting an impact on TLR 7/8 to induce osteoclast differentiation (Hegewald et al. 2020). Besides, melatonin inhibits osteoclastogenesis in osteoblasts via the miR-882/Rev-Erbα axis (Tian et al. 2022). The result of circRNA-seq showed chr8_84771783_84779275_- was down-regulated in p75NTR (-/-) EMSCs, which indicates that chr8_84771783_84779275_- may promote the activity of osteoclast during the development of EMSCs by suppressing miR-574-5p and miR-882. Furthermore, miR-149-3p targets FTP to regulate whether BMSCs begin adipogenic differentiation or osteogenic differentiation (Li et al. 2019). And miR-149-3p is the only possible target miRNA of mmu_circ_0003625, which suggests that mmu_circ_0003625 may be vital in the incidence of odonto/osteogenic differentiation in EMSCs.

The most likely potential target miRNAs for the mmu_circ_0001380 are miR-874-3p and miR-17-5p. miR-17-5p and PTEN are highly relevant to osteoclast differentiation, while miR-874-3p inhibits osteoporosis by targeting leptin (LEP) (Wang et al. 2021; Mei et al. 2021). In circRNA-seq, mmu_circ_0001380 was up-regulated in p75NTR (-/-) EMSCs, which indicates that mmu_circ_0001380 may inhibit the odontogenesis of EMSCs by suppressing miR-874-3p and miR-17-5p.As for mmu_circ_0013536, these miRNAs are miR-1249-, miR-6945-5p, miR-326-5p, miR-665-5p and miR-346-3p. miR-326-5p negatively regulates adipose-derived mesenchymal stem cells (ADSCs) osteogenesis by targeting Itga5(Zhang et al. 2021). Besides, miR-326-5p can also stimulate the expression of osteogenesis-related mRNAs in bone marrow stem cells (BMSCs) (Li et al. 2021). Interestingly, the role of miR-326-5p differs between ADSCs and BMSCs. MiR-1249-5p regulates PI3K/Akt signaling pathway to promote ADSCs into the process of osteogenesis by targeting PDX1 and influences osteogenesis of MC3T3-E1 and MDPC23 cells (Yang et al. 2021; Wu et al. 2020). miR-149-3p can promote osteogenic differentiation in hADSCs and

BMSCs (Li et al. 2019; Lai et al. 2021). In circRNA-seq, mmu_circ_003536 was up-regulated in p75NTR (-/-) EMSCs. Therefore, we suspect mmu_circ_003536 may inhibit the onto/osteogenic differentiation and mineralization in EMSCs by suppressing miR-1249-5p and miR-149-3p. But the role of miR-326-5p needs further studies.

Based on the prediction of circRNA-miRNA, we selected three circRNAs as potential fundamental molecules in the circRNA-miRNA- mRNA network.it is reported that the miR-185-5p inhibits mRNA expression levels of Runx2 in hPDLCs (Li et al. 2022). This finding was accordant with the circRNA-miRNA-mRNA network (Fig.5E) and suggested that chr8_84771783_84779275_- and miR-185-5p may be vital in osteo/dentinogenic differentiation of EMSCs. Molecular biology studies have demonstrated that these three circRNAs can differentially affect the expression abundance of DSPP and RUNX2 in stem cells, which represent ossification/osteogenesis levels. Furthermore, qRT-PCR was applied to verify the prediction. The expression levels of DSPP and RUNX2 differed much between WT EMSCs and p75NTR (-/-) EMSCs. This result is not only consistent with the prediction, but also similar to the previous research on odonto-differentiation potential of p75NTR (-/-) EMSCs (Xing et al. 2016). Besides, our research verified this result in the perspective of bioinformatics and therefore was more credible. Moreover, the researcher reported that miR-874-3p upregulation promoted RUNX2 mRNA expression, cell proliferation, cell differentiation and calcium deposition in hBMSCs (Mei et al. 2021). These three biological functions play an essential role during the development of EMSCs, and RUNX2 is the target of miR-874-3p according to the TargetScan. Therefore, we could silence miR-874-3p in further research about each specific miRNA functions in EMSCs. Besides, Fu reported that SNHG1 bind to miR-328-3p to regulate the mRNA expression of DSPP (Fu et al. 2022). Along with the mmu_circ_003536- miR-328-3p-DSPP axis (Fig.5E), we supposed that mmu_circ_003536 might function the same way in EMSCs as we expected in networks. To sum up, the interaction networks we drew based on the results of circRNA-seq were accordant with abundant research about other cells. However, the specific role of each circRNA or miRNA in EMSCs needs further investigations.

CONCLUSIONS

In summary, based on the research of p75NTR (-/-) EMSCs, we identified the comprehensive circRNAs expression pattern in WT EMSC and p75NTR (-/-) EMSC during the odonto/osteogenic differentiation. Then we used GO, KEGG and Reactome to predict the functions of p75NTR-related circRNAs to understand the mechanisms about p75NTR further. After that, CCK8, transwell assay and ALP staining assay were performed to verify the functions of potential key circRNAs. Then the prediction of ceRNA regulation networks and bioinformatics analysis both revealed that p75NTR had the biggest impact on WNT pathway. Besides, JAK was presumably involved in regulating the signaling pathway. Then ceRNA network prediction and bioinformatics analysis could provide a comprehensive understanding of a new circRNA (chr8_84771783_84779275_-), mmu_circ_0001380 and mmu_circ_0013536. Subsequently, based on the known proteins, circRNA-miRNA-mRNA networks indicated that these three circRNAs might regulate the expression of donto/osteogenic related genes DSPP and RUNX2 and qRT-PCR was applied for verification. However, the interactions and cascade signaling pathways of these three circRNAs and related miRNAs in osteogenic/osteogenic differentiation and mineralization of EMSCs need further study.

DECLARATIONS

Author Contributions

Qing Yuan: Investigation, Methodology, Software, Data curation, Writing the original draft. Leyu Wan: Investigation, Software, Formal analysis, Writing the original draft. Yuanyi Li: Resources, Validation. Yanyan Zhang: Visualization. Xiaoke Zeng and Keyu Wang: Software. Xin Nie: Project administration.

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Data Availability

The datasets used during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1.Deng MJ, Jin Y, Shi JN, et al. 2004 Sep-Oct. Multilineage differentiation of ectomesenchymal cells isolated from the first branchial arch. Tissue Eng. 10(9-10):1597-606.

2.Shan P, Wang X, Zhang Y, et al. 2022 Apr. P75 neurotrophin receptor positively regulates the odontogenic/osteogenic differentiation of ectomesenchymal stem cells via nuclear factor kappa-B signaling pathway. Bioengineered. 13(4):11201-11213.

3.Wang K, Zhao Z, Wang X, et al. 2021 Oct. BRD4 induces osteogenic differentiation of BMSCs via the Wnt/β-catenin signaling pathway. Tissue Cell. 72:101555.

4.Wen X, Liu L, Deng M, et al. 2012 Oct 12. Characterization of p75(+) ectomesenchymal stem cells from rat embryonic facial process tissue. Biochem Biophys Res Commun. 427(1):5-10.

5.Mikami Y, Ishii Y, Watanabe N, et al. 2011 May. CD271/p75(NTR) inhibits the differentiation of mesenchymal stem cells into osteogenic, adipogenic, chondrogenic, and myogenic lineages. Stem Cells Dev, 20(5):901-13.

6.Waddington RJ, Youde SJ, Lee CP, et al. 2009. Isolation of distinct progenitor stem cell populations from dental pulp. Cells Tissues Organs. 189(1-4):268-74.

7.Yang K, Wang Y, Ju Y, et al. 2017 Feb. p75 neurotrophin receptor regulates differential mineralization of rat ectomesenchymal stem cells. Cell Prolif. 50(1): e12290.

8.Xing Y, Nie X, Chen G, et al. 2016 Apr. Comparison of P75 NTR-positive and -negative etcomesenchymal stem cell odontogenic differentiation through epithelial-mesenchymal interaction. Cell Prolif. 49(2):185-94.

9.Wang Y, Yang K, Li G, et al. 2020 Apr. p75NTR-/- mice exhibit an alveolar bone loss phenotype and inhibited PI3K/Akt/β-catenin pathway. Cell Prolif. 53(4):e12800.

10.Qu S, Zhong Y, Shang R, et al. 2017 Aug 3. The emerging landscape of circular RNA in life processes. RNA Bio. 14(8):992-999.

11.Kristensen LS, Andersen MS, Stagsted Lvw, et al. 2019 Nov. The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet. 20(11):675-691.

12.Li Y, Huo C, Lin X, et al. 2018. Computational Identification of Cross-Talking ceRNAs. Adv Exp Med Biol. 1094:97-108.

13.Zheng Y, Li X, Huang Y, et al. 2017 Sep. The Circular RNA Landscape of Periodontal Ligament Stem Cells During Osteogenesis. J Periodontol. 88(9):906-914.

14.Li X, Zheng Y, Zheng Y, et al. 2018 Aug 31. Circular RNA CDR1as regulates osteoblastic differentiation of periodontal ligament stem cells via the miR-7/GDF5/SMAD and p38 MAPK signaling pathway. Stem Cell Res Ther. 9(1):232.

15.Zhang D, Ni N, Wang Y, et al. 2021 Jan. CircRNA-vgll3 promotes osteogenic differentiation of adipose-derived mesenchymal stem cells via modulating miRNA-dependent integrin α5 expression. Cell Death Differ. 28(1):283-302.

16.Douillard T, Martinelli-Kläy CP, Lombardi T. 2016 Jun. Nerve Growth Factor Expression and Its Receptors TrkA and p75NTR in Peri-Implantitis Lesions. Implant Dent. 25(3):373-9.

17.Bougault C, Briolay A, Boutet MA, et al. 2015 Dec. Wnt5a is expressed in spondyloarthritis and exerts opposite effects on enthesis and bone in murine organ and cell cultures. Transl Res. 166(6):627-38.

18.Wang F, Nazarali AJ, Ji S. 2016 Jun 1. Circular RNAs as potential biomarkers for cancer diagnosis and therapy. Am J Cancer Res. 6(6):1167-76.

19.Nirvani M, Khuu C, Utheim TP, et al. 2017 Mar. Circadian rhythms and gene expression during mouse molar tooth development. Acta Odontol Scand. 75(2):144-153.

20.Li ZY, Liu WZ, Guang H, et al. 2021 Jan-Feb. Silencing of long noncoding RNA H19 inhibited invasion and migration of papillary thyroid cancer cells via microRNA-138-dependent regulating of LRRK2. J Biol Regul Homeost Agents. 35(1):253-258.

21.Cai L, Chen Q, Fang S, et al. 2018 Apr. MicroRNA-329 inhibits cell proliferation and tumor growth while facilitates apoptosis via negative regulation of KDM1A in gastric cancer. J Cell Biochem. 119(4):3338-3351.

22.Wang T, Zhang F, Sun F. 2022 Feb. ORY-1001, a KDM1A inhibitor, inhibits proliferation, and promotes apoptosis of triple negative breast cancer cells by inactivating androgen receptor. Drug Dev Res. 83(1):208-216.

23.Wang K, Zhao Z, Wang X, et al. 2021 Oct. BRD4 induces osteogenic differentiation of BMSCs via the Wnt/β-catenin signaling pathway. Tissue Cell. 72:101555.

24.Zhu H, Chen H, Ding D, et al. 2021 Oct 14. Overexpression of PIK3R1 Promotes Bone Formation by Regulating Osteoblast Differentiation and Osteoclast Formation. Comput Math Methods Med. 2021:2909454.

25.Wang B, Lin J, Zhang Q, et al. 2019 Nov. αCGRP Affects BMSCs' Migration and Osteogenesis via the Hippo-YAP Pathway. Cell Transplant, 28(11):1420-1431.

26.Zhang B, Yang L, Zeng Z, et al. 2020 Apr 15. Leptin Potentiates BMP9-Induced Osteogenic Differentiation of Mesenchymal Stem Cells Through the Activation of JAK/STAT Signaling. Stem Cells Dev. 29(8):498-510.

27.Zhou N, Li Q, Lin X, et al. 2016 Oct. BMP2 induces chondrogenic differentiation, osteogenic differentiation and endochondral ossification in stem cells. Cell Tissue Res. 366(1):101-11.

28.Wang Y, Niu H, Liu Y, et al. 2020 Nov. Promoting effect of long non-coding RNA SNHG1 on osteogenic differentiation of fibroblastic cells from the posterior longitudinal ligament by the microRNA-320b/IFNGR1 network. Cell Cycle. 19(21):2836-2850.

29.Salmena L, Poliseno L, Tay Y, et al. 2011 Aug 5. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 46(3):353-8.

30.Hegewald AB, Breitwieser K, Ottinger SM, et al. 2020 Oct 14. Extracellular miR-574-5p Induces Osteoclast Differentiation via TLR 7/8 in Rheumatoid Arthritis. Front Immunol. 11:585282.

31.Tian Y, Ming J. 2022 Jul. Melatonin inhibits osteoclastogenesis via RANKL/OPG suppression mediated by Rev-Erbα in osteoblasts. J Cell Mol Med. 26(14):4032-4047.

32.Li Y, Yang F, Gao M, et al. 2019 Sep 6. miR-149-3p Regulates the Switch between Adipogenic and Osteogenic Differentiation of BMSCs by Targeting FTO. Mol Ther Nucleic Acids. 17:590-600.

33.Wang M, Zhao M, Guo Q, et al. 2021 Nov 1. Non-small cell lung cancer cell-derived exosomal miR-17-5p promotes osteoclast differentiation by targeting PTEN. Exp Cell Res. 408(1):112834.

34.Mei L, Li M, Zhang T. 2021 Dec. MicroRNA miR-874-3p inhibits osteoporosis by targeting leptin (LEP). Bioengineered. 12(2):11756-11767.

35.Li X, Chen R, Lei X, et al. 2021 Feb 15. Quercetin regulates ERα mediated differentiation of BMSCs through circular RNA. Gene. 769:145172.

36.Yang XM., Song YQ, Li L, et al. 2021 Jan 6. miR-1249-5p regulates the osteogenic differentiation of ADSCs by targeting PDX1. J Orthop Surg Res. 16(1):10.

37.Wu J, Ren W, Zheng Z, et al. 2020 Sep. Mmu_circ_003795 regulates osteoblast differentiation and mineralization in MC3T3 E1 and MDPC23 by targeting COL15A1. Mol Med Rep. 22(3):1737-1746.

38.Lai AN, Zhou R, Chen B, et al. 2021 Dec. MiR-149-3p can improve the osteogenic differentiation of human adipose-derived stem cells via targeting AKT1. Kaohsiung J Med Sci. 37(12):1077-1088.

39.Li D, Zhu Y, Zhang L, et al. 2022 Aug. MZB1 targeted by miR-185-5p inhibits the migration of human periodontal ligament cells through NF-κB signaling and promotes alveolar bone loss. J Periodontal Res. 57(4):811-823.

40Mei L, Li M, Zhang T. 2021 Dec. MicroRNA miR-874-3p inhibits osteoporosis by targeting leptin (LEP). Bioengineered. 12(2):11756-11767.

41.Fu T, Liu Y, Huang X, et al. 2022 Jul 15. lncRNA SNHG1 regulates odontogenic differentiation of human dental pulp stem cells via miR-328-3p/Wnt/β-catenin pathway. Stem Cell Res Ther. 13(1):311.