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Exploring the Mechanism of CircRNA-vgll3 in Osteogenically Differentiated Human Bone Marrow Mesenchymal Stem Cells

Yajie Huo, Yu Mao, Fang Luo, Fengjiao Zhang, Lifang Xie, Xiaoke Zhang, Kai Liu, Ling Sun, Hongmei Liu, Lige Song, Huanhuan Wang, Zhiqiang Kang*

Department of Endocrinology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou 450007, Henan Province, China

***Corresponding author:** Zhiqiang Kang, kangzhiqiangzx@sina.cn

Abstract: *Objective:* To explore the mechanism of circRNA-vgll3 in osteogenic differentiation of human bone marrow mesenchymal stem cells. *Methods:* BMSCs cells were transfected with circRNA-vgll3, and divided into circRNA-vgll3 high-level group, circRNA-vgll3 low-level group, and negative control group (circRNA-vgll3 not transfected) according to the amount of transfection. The proliferation and apoptosis of BMSCs osteoblasts in each group were analyzed, and the alkaline phosphatase (ALP) activity, type I collagen gray value, bone morphogenetic protein 2 (BMP-2), Runx2 protein, and mRNA expression levels were detected. *Results:* The circRNA-vgll3 low-level group had a significant inhibitory effect on the proliferation of BMSCs osteoblasts, and the apoptosis rate of the circRNA-vgll3 low-level group was significantly higher than that of the circRNA-vgll3 high-level group ($P < 0.05$); ALP activity, type I collagen gray value, BMP-2, Runx2 protein, and mRNA expression levels in the high-level circRNA-vgll3 group were significantly higher than those in the low-level circRNA-vgll3 group, and the difference was statistically significant ($P < 0.05$). *Conclusion:* Overexpression of circRNA-vgll3 can promote the osteogenic differentiation ability of BMSCs, while low expression of circRNA-vgll3 can inhibit the osteogenic differentiation ability of BMSCs. The main mechanism of action is that circRNA-vgll3 can affect osteogenic differentiation by regulating the Runx2 protein.

Keywords: CircRNA-vgll3; Osteogenic differentiation; Human bone marrow mesenchymal stem cells; Mechanism of action

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1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) were first discovered in bone marrow by Friedenstein et al. Because BMSCs have the potential for multidirectional differentiation, under specific induction conditions, they can develop into osteoblasts, fat cells, chondrocytes, osteoblasts, fibroblasts, and even differentiate into myoblasts, are therefore defined as pluripotent cells. In addition, BMSCs can also self-renew and produce immunomodulatory responses [1-2]. Studies have shown that BMSCs can differentiate into multiple lineages, including tissues other than their origin, such as neurons, liver cells, and skeletal muscle cells [3]. BMSCs are easy to obtain, easy to expand *in vitro*, and still have good differentiation potential after being isolated from adult bone marrow. BMSCs have become extremely important seed cells in gene therapy, tissue engineering, cell replacement therapy, and regenerative medicine because of their functions of multilineage differentiation, self-renewal, and immune regulation. Circular RNAs (circRNAs)

are a class of non-coding RNAs widely present in eukaryotic cells. Unlike canonical linear RNAs, circRNAs form covalently closed continuous loops without 5' or 3' polarity [4]. CircRNAs are abundantly expressed in cells and tissues, are highly conserved and relatively stable during evolution, and are generally considered to be by-products of mis-splicing or messenger RNA processes. With the rapid development of high-throughput RNA sequencing (RNA-seq) technology and bioinformatics methods, a large number of circular RNAs have been discovered and identified in many species, such as circ_28313, circ_0016624, circ_0006393, circ_0076906, and circ_0048211. A large number of studies [5-7] have shown that circRNAs play an important role in the osteogenic differentiation of bone marrow mesenchymal stem cells. Engineering and clinical treatments provide new theoretical and experimental basis.

2. Materials and methods

2.1. Experimental materials

Human BMSCs were purchased from Shanghai Lianmai Bioengineering Co. Ltd. Dulbecco's modified eagle medium (DMEM) medium and fetal bovine serum from Gibco Company (US), flow cytometer from BD Company (US), CCK - 8 kit from MyBioSource Company (US), mRNA reverse transcription kit and Trizol reagent from Invitrogen Company (US) were used in this study.

2.2. Methods

2.2.1. BMSCs cell culture

BMSCs cells were placed in DMEM medium with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, incubated statically in a cell culture incubator at 5% CO_2 and 37°C, and the medium was changed every 3 days. The 3–5 generations of cells with good growth status were taken for subsequent experiments.

2.2.2. CircRNA-vgll3 transfected BMSCs cells

The cell density of BMSCs with good growth status was adjusted to 5×10^4 cells/well, seeded on a 6-well plate, and when the cells grew to 60%, BMSCs were transfected with circRNA-vgll3. According to the amount of transfection, they were divided into circRNA-vgll3 high-level group, circRNA-vgll3 low-level group, and negative control group (circRNA-vgll3 not transfected), and the transfection was observed using flow cytometry.

2.2.3. Analysis of proliferation of BMSCs osteoblasts

The number of cells was observed under a microscope, the concentration was adjusted, and they were then inoculated into 96-well plates, placed in an incubator, and cultured until they adhered to the wall. The culture time is 12, 24, and 48 h respectively. Ten μL of cell counting kit-8 (CCK-8) cell proliferation reagent was added under light-shielded conditions, and the photometric optical density value (OD value) by BMSCs osteoblasts at 450 nm of each sample were detected after 2 hours.

2.2.4. Analysis of apoptosis of BMSCs osteoblasts

After the cells in the incubator were taken out, they were digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin, centrifuged at 1500 rpm for 10 min, washed with phosphate buffer saline (PBS) solution, added to the centrifuge tube with diluted Binding Buffer solution, and stained with propidium iodide (PI) in the dark for 15 min, using 10,000 cells were collected by flow cytometry and the apoptosis rate was calculated.

2.2.5. Analysis of BMSCs osteoblast alkaline phosphatase (ALP) activity

The BMSCs osteoblasts were placed on a 24-well cell culture plate, and after 48 h of culture, the alkaline phosphatase (ALP) detection kit was used to measure their activity in the 4th week, the 8th week, and the 12th week.

2.2.6. Gray value analysis of BMSCs osteoblast type I collagen

BMSCs osteoblasts were taken, the cell concentration was adjusted to 1×10^4 and seeded in cell culture plates, and the gray value of type I collagen in each group of cells was detected by using the cell differentiation into osteocyte type I collagen immunohistochemical staining kit.

2.2.7. Expression levels of bone morphogenetic protein 2 (BMP-2) and Runt-related transcription factor 2 (Runx2) in BMSCs osteoblasts

BMSCs osteoblasts were placed on a 24-well cell culture plate, and after 48 hours of culture, the cells were lysed with RIPA lysis solution, and the cells were repeatedly blown to fully release the protein. The obtained protein was blocked with 3% H_2O_2 for 10 min, washed 3 times with PBS, 3 min each time; after the primary antibody was dropped overnight at 4°C, washed 3 times with PBS, 3 min each time; after adding the secondary antibody, incubate at 37°C for 20 min, wash with PBS 3 times, 3 min each time. After that, 1,3-dimethylbarbituric acid (DBA) was added dropwise for color development, washed with PBS, counterstained with hematoxylin, washed with water and transparent, and mounted. Ten fields of view were randomly selected to observe and measure the optical density at a magnification of 400 times [3].

2.2.8. BMP-2 and Runx2 mRNA expression levels in BMSCs osteoblasts

Trizol reagent was added to extract total RNA from BMSCs osteoblasts. RNA concentration and purity were determined by spectrophotometry and 1% agarose gel electrophoresis. The isolated RNA was reverse-transcribed into cDNA using PrimeScript RT kit and oligonucleotide primers and analyzed by real-time quantitative polymerase chain reaction (RT PCT). With 3-phosphoglyceraldehyde dehydrogenation as an internal reference, the $2^{-\triangle\triangle Ct}$ method was used to analyze BMP-2 and the expression level of Runx2 mRNA [8].

2.3. Statistical analysis

All the data in this study were processed by SPSS20.0 statistical analysis software (IBM, USA); the measurement data were represented by mean ± standard deviation (SD), and the comparison between groups was by independent sample *t*-test; the count data were by percentage (%), the comparison between groups was analyzed by χ^2; $P < 0.05$ means that the difference is statistically significant.

3. Results

3.1. Analysis of BMSCs osteoblast proliferation

The proliferation of BMSCs osteoblasts in each group was compared, and the difference was statistically significant ($P < 0.05$). Among them, the OD value of the negative control group was the largest, and the OD value of the circRNA-vgll3 low-level group was the smallest, and with the prolongation of the intervention time, the OD value became larger and larger. Therefore, the results showed that the circRNA-vgll3 low-level group significantly inhibited the proliferation of BMSCs osteoblasts effect, that is, there is a greater impact on bone formation. See **Table 1**.

Table 1. BMSCs osteoblast proliferation analysis (mean ± SD, %)

Group	12 hours	24 hours	48 hours	*f*	*P*
CircRNA-vgll3 high-level group	0.489 ± 0.003	0.524 ± 0.011	0.639 ± 0.005	3.426	0.041
CircRNA-vgll3 low-level group	0.425 ± 0.024	0.513 ± 0.038	0.601 ± 0.031	4.884	0.024
Negative control group	0.519 ± 0.012	0.623 ± 0.009	0.802 ± 0.027	5.464	0.035
t	23.277	12.664	13.029	-	-
P	< 0.001	< 0.001	< 0.001	-	-

3.2. Analysis of BMSCs osteoblast apoptosis

Compared to the apoptosis of BMSCs osteoblasts in each group, the difference was statistically significant ($P < 0.05$). Among them, the BMSCs osteoblasts in the negative control group were treated for 12 h to 48 h, and the apoptosis rate increased from 3.28 ± 2.66 to 5.98 ± 0.25. With the increase of circRNA-vgll3 transfection level, the apoptosis rate was obvious. The apoptosis rate of the circRNA-vgll3 low-level group was significantly higher than that of the circRNA-vgll3 high-level group, as shown in **Table 2**.

Table 2. Analysis of apoptosis of BMSCs osteoblasts (mean ± SD, %)

Group	12 hours	24 hours	48 hours	*f*	*P*
CircRNA-vgll3 high-level group	4.12 ± 0.58	4.89 ± 1.69	5.44 ± 0.14	7.797	0.001
CircRNA-vgll3 low-level group	6.39 ± 1.25	8.25 ± 0.26	10.22 ± 0.36	8.835	0.001
Negative control group	3.28 ± 2.66	4.12 ± 0.69	5.98 ± 0.25	9.323	0.001
t	25.808	16.967	19.626	-	-
P	< 0.001	< 0.001	< 0.001	-	-

3.3. BMSCs osteoblast ALP activity analysis

Table 3 showed that the ALP activity in each group increased significantly over time ($P < 0.05$). At each time point, the ALP activity of both the circRNA high-level and low-level groups was significantly higher than that of the negative control group ($P < 0.05$); and the ALP activity of the circRNA-vgll3 high-level group was significantly higher than that of the circRNA-vgll3 low-level group ($P < 0.05$).

Table 3. BMSCs osteoblast ALP activity analysis (mean ± SD)

Group	Week 4	Week 8	Week 12	*f*	*P*
CircRNA-vgll3 high-level group	25.56 ± 2.57	33.08 ± 4.29	40.72 ± 3.49	8.529	0.001
CircRNA-vgll3 low-level group	20.98 ± 2.23	25.98 ± 4.50	37.32 ± 3.14	8.935	0.001
Negative control group	15.62 ± 1.72	16.62 ± 3.51	17.37 ± 3.42	5.358	0.001
t	16.894	15.992	15.932	-	-
P	< 0.001	< 0.001	< 0.001	-	-

3.4. Gray value analysis of BMSCs osteoblast type I collagen

Table 4 showed that the gray value of type I collagen in each group increased significantly ($P < 0.05$). At each time point, the gray value of type I collagen in both the circRNA-vgll3 high-level and low-level groups was significantly higher than that in the negative control group ($P < 0.05$); and the gray value of Type I collagen in the circRNA-vgll3 high-level group was significantly higher than the circRNA-vgll3 low-level group ($P < 0.05$).

Table 4. BMSCs osteoblast type I collagen gray value analysis (mean ± SD)

Group	Week 4	Week 8	Week 12	*f*	*P*
CircRNA-vgll3 high-level group	28.34 ± 5.65	42.23 ± 5.09	44.37 ± 3.42	6.932	0.001
CircRNA-vgll3 low-level group	21.49 ± 3.91	31.76 ± 3.56	20.98 ± 4.50	4.994	0.001
Negative control group	19.06 ± 2.23	21.49 ± 3.43	25.62 ± 3.51	7.983	0.001
t	21.720	12.854	17.663	-	-
P	< 0.001	< 0.001	< 0.001	-	-

3.5. BMP-2 and Runx2 protein expression levels in BMSCs osteoblasts

The bone morphogenetic protein 2 **(**BMP-2) and Runt-related transcription factor 2 (Runx2) protein expression levels of BMSCs osteoblasts in each group were compared, and the difference was statistically significant ($P < 0.05$). The protein expression levels of BMP-2 and Runx2 in the high-level and low-level circRNA-vgll3 groups were significantly higher than those in the negative control group ($P < 0.05$), and the protein expression levels of BMP-2 and Runx2 in the high-level circRNA-vgll3 group were significantly higher in the circRNA-vgll3 low-level group ($P < 0.05$). See **Table 5**.

Table 5. BMP-2 and Runx2 protein expression levels in BMSCs osteoblasts (mean ±SD)

Group	BMP-2 protein	Runx2 protein
CircRNA-vgll3 high-level group	21.37 ± 2.04	35.59 ± 4.28
CircRNA-vgll3 low-level group	20.34 ± 3.34	34.60 ± 4.34
Negative control group	12.46 ± 2.25	32.57 ± 3.69
t	10.984	14.445
P	< 0.001	< 0.001

3.6. BMP-2 and Runx2 mRNA expression levels in BMSCs osteoblasts

The BMP-2 and Runx2 mRNA expression levels of BMSCs osteoblasts in each group were compared, and the difference was statistically significant ($P < 0.05$). The expression levels of BMP-2 and Runx2 mRNA in the high-level and low-level circRNA-vgll3 groups were significantly higher than those in the negative control group ($P < 0.05$), and the protein expression levels of BMP-2 and Runx2 in the high-level circRNA-vgll3 group were significantly higher in the CircRNA-vgll3 low-level group ($P < 0.05$), as shown in **Table 6**.

Table 6. BMP-2 and Runx2 mRNA expression levels in BMSCs osteoblasts (mean ± SD)

Group	BMP-2 mRNA	Runx2 mRNA
CircRNA-vgll3 high-level group	0.402 ± 0.109	0.638 ± 0.241
CircRNA-vgll3 low-level group	0.369 ± 0.121	0.604 ± 0.139
Negative control group	0.146 ± 0.025	0.343 ± 0.108
t	12.435	12.325
P	<0.001	0.001

4. Discussion

In humans, osteoblasts involved in bone formation are inseparable from the differentiation of BMSCs. Studies have shown that circRNAs play an important role in the osteogenic differentiation of BMSCs, and

different circRNAs can promote or inhibit the osteogenic differentiation of BMSCs. Wu et al found differentially expressed circRNAs in patients with osteoporosis and found that 237 circRNAs were up-regulated and 279 circRNAs were down-regulated, which also confirmed the important role of circRNAs in the osteogenic differentiation of BMSCs [9]. Another study found that circRNAs were differentially expressed in patients with traumatic femoral head necrosis, and found that 234 circRNAs were upregulated and 148 circRNAs were downregulated [10]. Zou et al found that circRNAs such as circ_28313, circ_0016624, circ_0006393, circ_0076906, and circ_0048211 were differentially expressed in patients with osteoporosis and played an important role in the differentiation, proliferation, and apoptosis of bone marrow mesenchymal stem cells [3].

The results of this study showed that the circRNA-vgll3 low-level group had a significant inhibitory effect on the proliferation of BMSCs osteoblasts, and the apoptosis rate of the circRNA-vgll3 low-level group was significantly higher than that of the circRNA-vgll3 high-level group ($P < 0.05$). Similarly, Song et al found that the expression of circRNA was significantly upregulated in the peripheral blood of patients with traumatic femoral head necrosis by reverse transcription-quantitative PCR [1]. Further experiments found that circRNA can promote cell proliferation and invasion by promoting the expression of micro-RNA (miRNA / miR)-134-3p, to inhibit the apoptosis of BMSCs and osteoclast-like cells. Arshad et al found that circRNAs inhibited the osteogenic differentiation process of BMSCs while silencing circRNA promoted the differentiation of osteoblasts, which also indicated that circRNA-miRNA interaction contributed to the osteogenic differentiation of BMSCs [11].

BMP-2 and Runx2 are early markers of osteoblasts, which reflect the differentiation ability of osteoblasts, and can carry out directional differentiation, transcription, and translation of related osteogenic genes of BMSCs. New evidence shows that circRNAs can act as miRNAs and regulate gene transcription, and some circRNAs can be translated into proteins or peptides [12,13]. CircRNAs have miRNA sponge binding sites, which act as competitive endogenous RNAs to inhibit miRNAs from binding to their targets, thereby inhibiting mRNA translation. CircRNAs are abundantly present in the nucleus and can bind RBP, especially transcription-related factors, including RNA polymerase II and transcription factors, and recruit them to parental genes, thereby affecting the expression of parental genes and regulating the transcription process. Runx2 belongs to the Runx family, and the DNA binding domain is Runt, which consists of Runx1, Runx2, and Runx3 [14]. This study found that ALP activity, type I collagen gray value, BMP-2, and Runx2 protein and mRNA expression levels in the circRNA high-level group were significantly higher than those in the circRNA low-level group, and the difference was statistically significant ($P < 0.05$). Similarly, studies have shown that Runx2 plays a crucial role in the osteogenic differentiation of bone marrow mesenchymal stem cells [15]. Zong et al found that the expression of circRNA was reduced in bone marrow mesenchymal stem cells of patients with osteosarcoma, and lentivirus experiments found that overexpression of circRNA can promote the osteogenic differentiation of bone marrow mesenchymal stem cells, and circRNA combined with miRNA-942-5p can regulate the expression of Runx2 and vascular endothelial growth factor (VEGF) in bone marrow mesenchymal stem cells [16]. The results of this study indicate that circRNA plays an important role in the osteogenesis process and may become a new target for the treatment of senile osteoarthritis. BMP-2 is a member of bone morphogenetic proteins (BMPs), which belongs to the transforming growth factor-β (TGF-β) superfamily, and plays a role in various cellular processes such as cell proliferation, differentiation, apoptosis, angiogenesis, migration, and extracellular matrix remodeling plays a crucial role. More than 20 BMPs have been found in human tissues, and as the most well-studied one, BMP-2 is widely used in bone formation due to its strong osteogenic ability and has been approved by the US Food and Drug Administration (FDA) for orthopedics and dental applications [17].

In summary, overexpression of circRNA-vgll3 can promote the osteogenic differentiation ability of BMSCs, while low expression of circRNA-vgll3 can inhibit the osteogenic differentiation ability of BMSCs.

The main mechanism of action is that circRNA-vgll3 can affect osteogenic differentiation by regulating the Runx2 protein. Bone marrow mesenchymal stem cells are an important source of osteogenic seed cells in tissue engineering, and circRNA-vgll3 plays a key role in the osteogenic differentiation of bone marrow mesenchymal stem cells. It is of great significance to elucidate its mechanism of action. In addition, this study also provides new theoretical and experimental basis for bone tissue engineering and clinical treatment.

Disclosure statement

The authors declare no conflict of interest.

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