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Circ-VANGL1 promotes the progression of osteoporosis by absorbing miRNA-217 to regulate RUNX2 expression

L. YANG[1], Z. ZENG[2], N. KANG[1], J.-C. YANG[1], X. WEI[3], Y. HAI[1]

[1]Department of Orthopedics, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China.
[2]Department of Orthopedics, Beijing Tiantan Hospital, Capital Medical University, Beijing, China.
[3]Department of Orthopedics, Aerospace Center Hospital, Beijing, China.

Abstract. – OBJECTIVE: This study aims to investigate whether circ-VANGL1 can promote the progression of osteoporosis (OP) by absorbing miRNA-217 to regulate RUNX2 expression.

PATIENTS AND METHODS: The serum levels of circ-VANGL1, miRNA-217 and RUNX2 in OP patients and non-OP patients were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Their expression levels in human bone marrow mesenchymal stem cells (hBMSCs) at different time points of osteogenesis differentiation were determined as well. The expression levels of RUNX2 and osteogenic proteins (BSP, OCN, OPN) in hBMSCs were detected by Western blot. Dual-Luciferase reporter gene assay was performed to verify the relationship among circ-VANGL1, miRNA-217 and RUNX2. Alkaline phosphatase (ALP) staining was conducted to evaluate the degree of osteogenic differentiation influenced by circ-VANGL1 and miRNA-217.

RESULTS: OP patients presented a higher serum level of miRNA-217 and lower serum levels of circ-VANGL1 and RUNX2 relative to non-OP patients. Circ-VANGL1 accelerated osteogenic differentiation by absorbing miRNA-217 to regulate RUNX2 expression. Moreover, miRNA-217 inhibited osteogenic differentiation by degrading RUNX2 by targeting to RUNX2 3'UTR. The overexpression of circ-VANGL1 upregulated expressions of RUNX2, BSP, OCN, and OPN. Meanwhile, ALP activity increased in hBMSCs overexpressing circ-VANGL1. However, co-overexpression of circ-VANGL1 and miRNA-217 did not alter RUNX2 expression. ALP activity in hBMSCs co-overexpressing circ-VANGL1 and miRNA-217 slightly increased, but had no difference with controls.

CONCLUSIONS: Circ-VANGL1 promotes the development of OP via binding to miRNA-217 to downregulate RUNX2 expression.

Key Words:
OP, Circ-VANGL1, MiRNA-217, RUNX2.

Introduction

Osteoporosis (OP) is a metabolic and systemic skeletal lesion. The major pathological manifestations of OP include decreased bone mass, impaired bone microstructure and increased bone fragility, ultimately leading to decreased bone strength and increased fracture risks. OP patients are prone to experience pathological fractures and lumbago, especially in middle-aged and elderly people[1,2]. With the acceleration of the aging, senile osteoporosis and its complications have achieved great attention. Globally, more than 9 million fracture cases result from OP, of which approximately 2 million cases occur in the United States, directly contributing to 17 billion medical expenses. Therefore, effective therapeutic approaches for OP are urgently needed to improve the life quality of the elderly and alleviate the economic burden[3].

CircRNA is a special non-coding RNA with a cyclic structure and exerts an important regulatory role in many life activities[4]. However, circRNA was previously thought to be a non-functional waste formed by incorrect splicing of gene transcripts[5]. Recent studies have shown that circRNA is widely involved in tissue regeneration and stem cell differentiation. Some key circRNAs participate in the priming phase of rat liver regeneration[6]. CircRNAs derived from pluripotent stem cells contribute to cardiomyocyte derivation[7]. Cir-

Corresponding Author: Yong Hai, MD; e-mail: prof.haiyong@yahoo.com

cRNAs are widely expressed during human epidermal stem cell differentiation[8]. BMP2 induces osteogenic differentiation of MC3T3-E1158 cells relying on circRNA functions[9]. Circ-VANGL1 is derived from reverse splicing of exon 3 and exon 4 of VANGL1 mRNA. VANGL1 is a highly conserved transmembrane protein in the VANGL family, which contains two homologous genes VANGL1 and VANGL2[10]. The homology of these two members is as high as 73.1%, allowing their similarities not only in structures but also in functions[11]. So far, the regulatory effect of circ-VANGL1 on the development of OP remains unclear.

CircRNAs can be used as miRNA sponges to regulate expressions of parental genes, thus involving in the occurrence and development of diseases, including tumors, cardiovascular diseases, nervous system diseases, endocrine diseases, osteoarthritis, etc.[12]. Some circRNAs are capable of binding to miRNAs and interacting with miRNAs[13]. They exert important regulatory roles at the transcriptional or post-transcriptional level[14]. For example, the activation of ciRS-7 (a circRNA sponging miR-7) derived from the CDR1 antisense transcript reduces abundances of mRNAs containing the binding sites of miR-7, further demonstrating that ciRS-7 could competitively bind to miR-7[15,16].

Explorations on the biological roles of miRNAs in osteogenic differentiation have now progressed. Kahai et al[17] found that miR-378 can increase the activities of galnt-7 and adiponectin, thereby accelerating osteoblast differentiation. Eskildsen et al[18] found that miR-138 attenuates the osteogenic differentiation of hBMSCs partially by inhibiting the focal adhesion kinase pathway. Therefore, it is important to study the relationship between circ-VANGL1 and corresponding target miRNAs, to further elucidate their potential roles in the development of OP.

RUNX2, also known as core binding factor α1 (Cbfα1), is a key regulator of osteoblastization in vertebrates and specifically expressed during the differentiation of mesenchymal stem cells into osteoblasts19. As a specific cis-acting element, RUNX2 can activate the transcription and expressions of osteogenesis-related genes (such as collagen I, alkaline phosphatase, osteopontin, etc.), further controlling osteogenesis. We believed that RUNX2 exerts a crucial role in osteogenic differentiation.

This study explored the expression levels of circ-VANGL1, miRNA-217 and RUNX2 in OP patients, and their relationship in regulating the progression of OP.

Patients and Methods

Sample Collection

Fifteen patients who were clinically diagnosed with OP and 15 non-OP subjects were enrolled. Under a fasting state, 5 mL of venous blood was harvested in each subject in the morning and set for 30 min, followed by centrifugation at 4°C, 3000 g for 10 min. Harvested supernatant was centrifuged again at 4°C, 13500 g for 15 min. The supernatant serum was stored at -80°C. This study was approved by the Ethics Committee of our Hospital. Signed informed consents were obtained from all participants before the study.

Cell Culture and Induction of Osteogenic Differentiation

Adult hBMSCs were purchased from Cyagen Biotechnology Co., Ltd. (Cat. HUXMA-01001, Nanjing, China). hBMSCs were cultured in a 25 cm2 culture flask at 37°C, 5% CO2, and the culture medium was changed every 3-4 days. Until cell adherence to 80-90%, hBMSCs were washed with Phosphate-Buffered Saline (PBS) twice and digested with 0.3 mL of 0.25% trypsin containing 0.02% Ethylene Diamine Tetraacetic Acid (EDTA) (Thermo Fisher Scientific, Waltham, MA, USA). The 3rd to 5th generation of hBMSCs were inoculated in a 6-well plate pre-coated with gelatin at a density of 1×106 cells/mL. After 24 hours, 2 mL of osteogenic differentiation medium (DMEM; Dulbecco's Modified Eagle Medium) with 10% FBS (fetal bovine serum), 100 U/mL penicillin-streptomycin, 1% glutamine, 10 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerol phosphate (Gibco, Grand Island, NY, USA) were supplied in each well. Osteogenic differentiation medium was replaced every 3 days. Cells were harvested at day 0, 7, 14, and 21, respectively.

Transfection

hBMSCs were planted in 6-well plates for 24 hours before transfection. Until 50%-60% confluence, cells were transfected with miRNA-217 mimic, miRNA-217 inhibitor, circ-VANGL1 overexpression plasmid or circ-VANGL1 siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and miRNA extraction kit (Qiagen, Hilden, Ger-

Table I. Primer sequences used for qRT-PCR.

Genes	Primer sequences
Circ-VANGL1	F: 5′-GTCCGCTCCACCGATGGCGA-3′ R: 5′-CTGAACTTCCTCTGTCCGAGT-3′
VANGL1	F: 5′-GACGGCAGAGGGTCAGAAAA-3′ R: 5′-TCCTGAACTTCCTCTGTCCGA-3′
GAPDH	F: 5′-ATGTTGCAACCGGGAAGGAA-3′ R: 5′-AGGAAAAGCATCACCCGGAG-3′

many). Subsequently, complementary deoxyribose nucleic acid (cDNA) was synthesized using a miScript Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR was performed according to miScript SYBR Green PCR Kit (Life Technologies, Gaithersburg, MD, USA) and miScript/QuantiTect Primer assay (Qiagen, Hilden, Germany). Primers used in this study were listed in detail (Table I).

Western Blot

Total protein was extracted from the cell lysate for determining protein expression. The protein sample was quantified by bicinchoninic acid (BCA; Pierce, Waltham, MA, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by chemiluminescence.

Dual-Luciferase Reporter Gene Assay

After transfection for 48 h, cells were washed with PBS twice, lysed in 100 μL of PLB and shaken for 15 min. The lysate was transferred into a 1.5 mL Eppendorf (EP; Eppendorf, Hamburg, Germany) tube and centrifuged at 12000 rpm for 5 min. 20 μL of cell lysate was taken to incubate with 100 μL of LARII, followed by Luciferase intensity determination at 10 s (Firefly Luciferase) using the GloMaxTM luminescence detector. Subsequently, 100 μL of Stop&Glo was added to terminate the reaction, and Luciferase intensity at 10 s was recorded as Renilla Luciferase (Promega, Madison, WI, USA). Cells in each group were introduced with phRL-null as an internal control. Average Luciferase intensity of each sample was calculated as the ratio of Firefly Luciferase to Renilla Luciferase from three independent records.

Alkaline Phosphatase (ALP) Staining

hBMSCs at 14 days of osteogenic differentiation were harvested for ALP staining (BioLegend, San Diego, CA, USA). Incubation solution was added on the slides placed in the 6-well plate for 15 min at 37°C. ALP staining was performed for 5 min, followed by washing with running water. Images were observed and captured using an inverted microscope.

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing the differences between the two groups. The differences among multiple groups were analyzed using the one-way ANOVA, followed by Least Significant Difference as its post-hoc test. Correlation analysis was conducted using Spearman's correlation coefficient for ranked data. $p<0.05$ was considered statistically significant.

Results

Circ-VANGL1 and RUNX2 Were Lowly Expressed in OP

QRT-PCR was performed to detect the serum levels of circ-VANGL1 and RUNX2 in OP patients and non-OP subjects. OP patients had a lower serum level of circ-VANGL1 relative to non-OP subjects (Figure 1A). Similarly, the serum level of RUNX2 was lower in OP patients compared with that of non-OP subjects (Figure 1B). Spearman correlation analysis showed a negative correlation between the serum levels of circ-VANGL1 expression and RUNX2 in 15 OP patients (Figure 1C). To sum up, circ-VANGL1 and RUNX2 showed low serum levels in OP patients, and were negatively correlated with each other.

MiRNA-217 Contained Targets for Circ-VANGL1 and RUNX2

We predicted the binding sites of miRNA-217 to circ-VANGL1 and RUNX2 by Target Scan 7.1. It is found that circ-VANGL1 and RUNX2 contai-

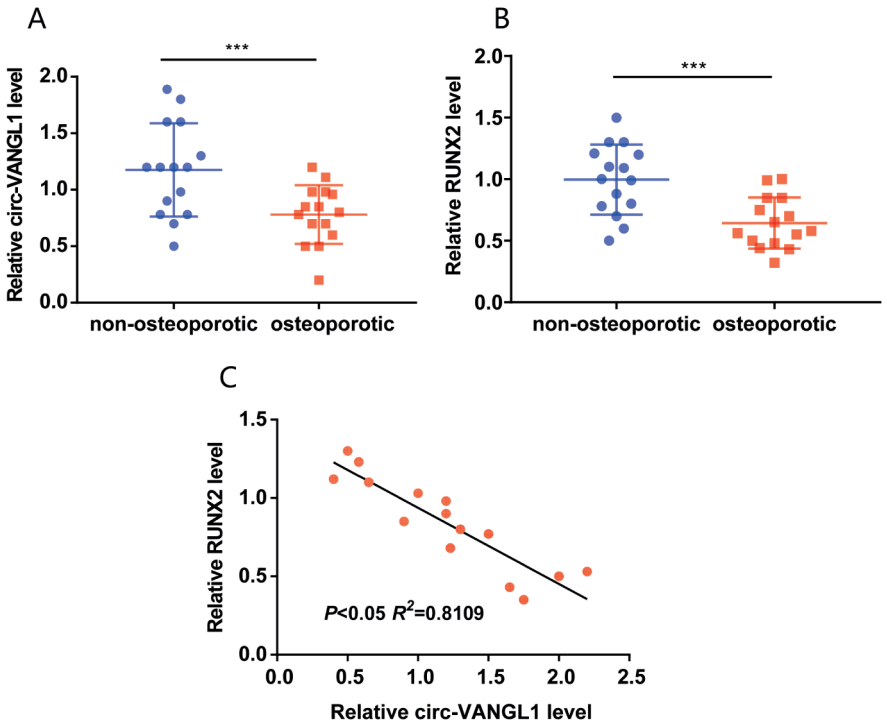

Figure 1. Circ-VANGL1 and RUNX2 were lowly expressed in OP. ***A,*** QRT-PCR showed that OP patients had a lower serum level of circ-VANGL1 relative to non-OP subjects. ***B,*** QRT-PCR showed that OP patients had a lower serum level of RUNX2 relative to non-OP subjects. ***C,*** Spearman correlation analysis showed a negative correlation between serum levels of circ-VANGL1 expression and RUNX2 in 15 OP patients (p<0.05, R2=0.8109). $^{***}p$<0.001.

ned the same miRNA-217 binding region, UACGUCA (Figure 2A). Furthermore, target binding between miRNA-217 and circ-VANGL1/RUNX2 was verified by Dual-Luciferase reporter gene assay. Our data revealed that the knockdown of miRNA-217 resulted in fluorescence quenching of wild-type circ-VANGL1 and wild-type RUNX2, confirming the binding condition between miRNA-217 with circ-VANGL1/ RUNX2 (Figure 2B). QRT-PCR clarified a high serum level of miRNA-217 in OP patients (Figure 2C). In addition, Spearman correlation analysis identified a negative correlation between miRNA-217 and RUNX2 (Figure 2D). The above results indicated that miRNA-217 exhibited high expression in OP patients, which targeted circ-VANGL1 and RUNX2.

Circ-VANGL1/MiRNA-217/RUNX2 Axis in Ontogenetic Differentiation

During the osteogenic differentiation of hBMSCs, the expression levels of circ-VANGL1, miRNA-217 and RUNX2 were measured at day 0, 7, 14, and 21, respectively. It is shown that miRNA-217 expression gradually decreased with the prolongation of osteogenic induction (Figure 3B). On the contrary, the expression levels of circ-VANGL1 and RUNX2 increased in a time-dependent manner (Figure 3A, 3C). To further investigate the association among circ-VANGL1, miRNA-217 and RUNX2, qRT-PCR was used to determine their expressions after altering expressions of circ-VANGL1 and miRNA-217. The overexpression of circ-VANGL1 downregulated miRNA-217 but upregulated RUNX2 at mRNA levels. The transfection of si-circ-VANGL1 obtained the opposite results (Figure 3D). Moreover, miRNA-217 overexpression inhibited RUNX2 expression, but had no influence on circ-VANGL1 expression (Figure 3E). We may conclude that RUNX2 was regulated by circ-VANGL1 and miRNA-217.

Circ-VANGL1 Regulated Osteogenic Differentiation of hBMSCs via Targeting MiRNA-217

To further elucidate the role of circ-VANGL1/miRNA-217/RUNX2 axis in ontogenetic differentiation, hBMSCs were divided into the NC group, circ-VANGL1 overexpression group, miRNA-217 overexpression group, and circ-VANGL1+miRNA-217 co-overexpression group. QRT-

PCR results confirmed that the overexpression of miRNA-217 downregulated RUNX2 expression, while the overexpression of circ-VANGL1 upregulated RUNX2 expression. No significant change in RUNX2 expression was observed by co-overexpression of circ-VANGL1 and miRNA-217 (Figure 4A). Subsequently, Western blot analysis was used to detect the expression levels of RUNX2 and osteogenesis-related genes. The overexpression of circ-VANGL1 upregulated expressions of RUNX2, BSP, OCN, and OPN, which were inhibited by co-overexpression of circ-VANGL1 and miRNA-217 (Figure 4B). After several days of osteogenic differentiation of hBMSCs, ALP activity was greatly elevated in hBMSCs overexpressing circ-VANGL1, which was inhibited in those overexpressing miRNA-217. We did not see a significant change in ALP activity between the NC group and circ-VANGL1+miRNA-217 co-overexpression group (Figure 4C). It is concluded

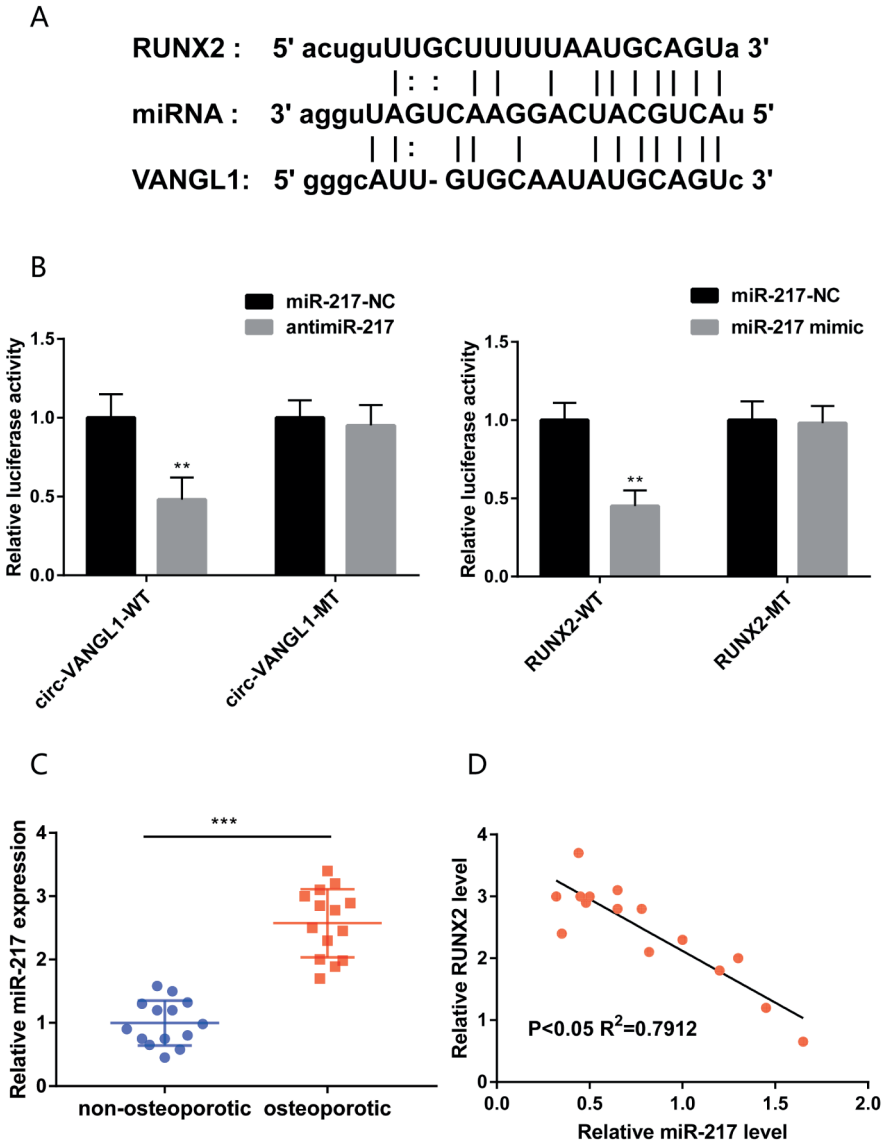

Figure 2. MiR-217 contained targets for circ-VANGL1 and RUNX2. ***A,*** Circ-VANGL1 and RUNX2 contained the same miR-217 binding region, UACGUCA. ***B,*** Knockdown of miR-217 resulted in fluorescence quenching of wild-type circ-VANGL1 and wild-type RUNX2, confirming the binding condition. ***C,*** QRT-PCR showed that OP patients had a higher serum level of miR-217 relative to non-OP subjects. ***D,*** Spearman correlation analysis identified a negative correlation between miR-217 and RUNX2 ($p<0.05$, R2=0.7912). $^{**}p<0.01$, $^{***}p<0.001$.

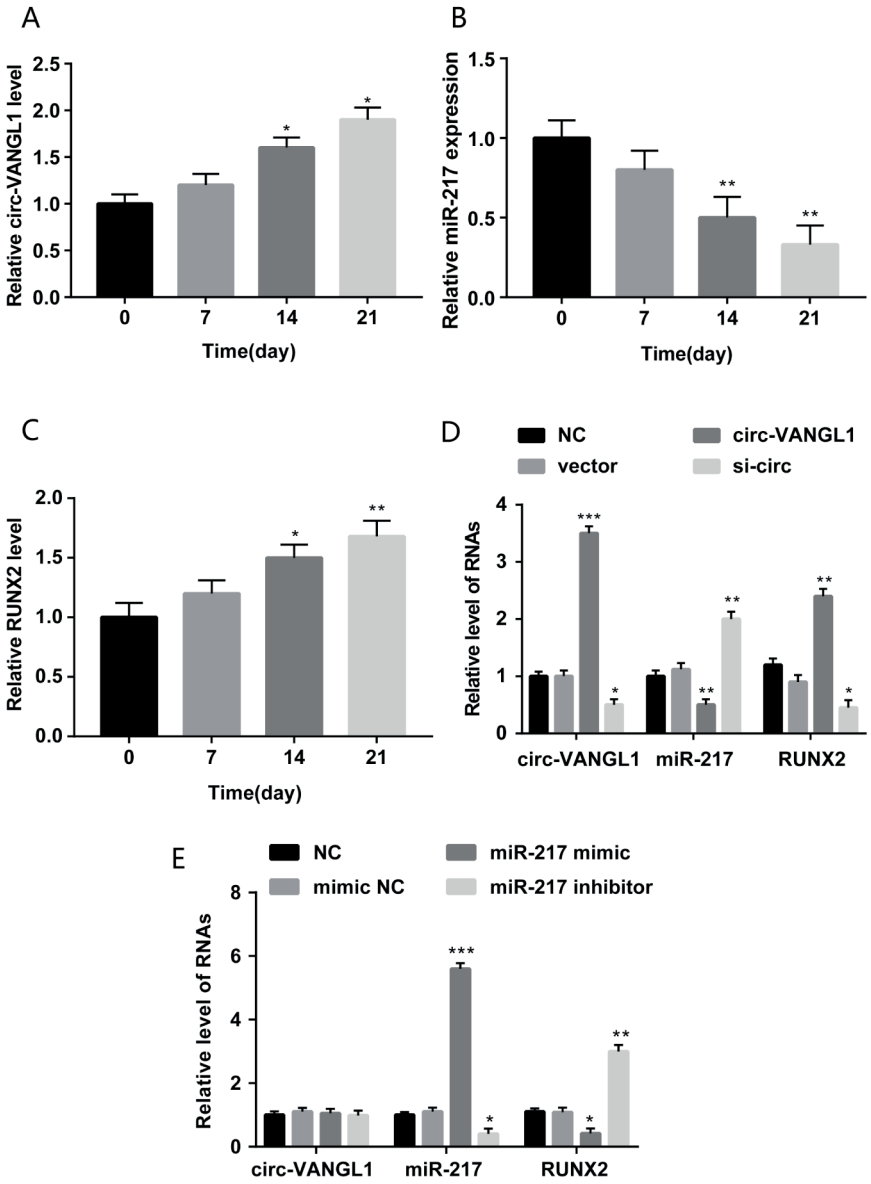

Figure 3. Circ-VANGL1/miR-217/RUNX2 axis in ontogenetic differentiation. ***A,*** QRT-PCR showed that circ-VANGL1 expression gradually increased with the prolongation of osteogenic induction. ***B,*** QRT-PCR showed that miR-217 expression gradually decreased with the prolongation of osteogenic induction. ***C,*** QRT-PCR showed that RUNX2 expression gradually increased with the prolongation of osteogenic induction. ***D,*** QRT-PCR showed expressions of circ-VANGL1, miR-217 and RUNX2 in hBMSCs with circ-VANGL1 overexpression or knockdown. ***E,*** QRT-PCR showed expressions of circ-VANGL1, miR-217 and RUNX2 in hBMSCs with miR-217 overexpression or knockdown. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

that the correlation between circ-VANGL1 and miRNA-217 regulated osteogenic differentiation by affecting RUNX2.

Discussion

With the continuous advancement of RNA sequencing technology and bioinformatics technology, large-scale analyses of transcriptome data have identified the formation of circRNAs by nonlinear reverse splicing or gene rearrangement. These circRNAs present great abundances and high stability, exerting great functions in the disease regulation. For example, INK4a/ARF is involved in the development and progression of atherosclerosis, which could be inhibited by polycomb-group genes[20]. cANRIL could influen-

ce the expression of INK4a/ARF through differentially binding to the polycomb-group genes[21]. The expression of hsa_circ_002059 remains low in gastric cancer tissues. Plasma levels of certain circRNAs that are differentially expressed before and after surgery are correlated to metastasis, TNM stage, age and sex of gastric cancer patients[22]. Current studies also pointed out the crucial function of circRNA in OP. Yin et al[23] found that circ-RUNX2 can prevent OP by regulating has-miR-203 and RUNX2. This work showed lower serum level of circ-VANGL1 in OP patients compared with that of non-OP subjects.

MiRNA-217 is a small ribonucleic acid located on chromosome 2p16.1. Accumulating evidence has proved its important role in the

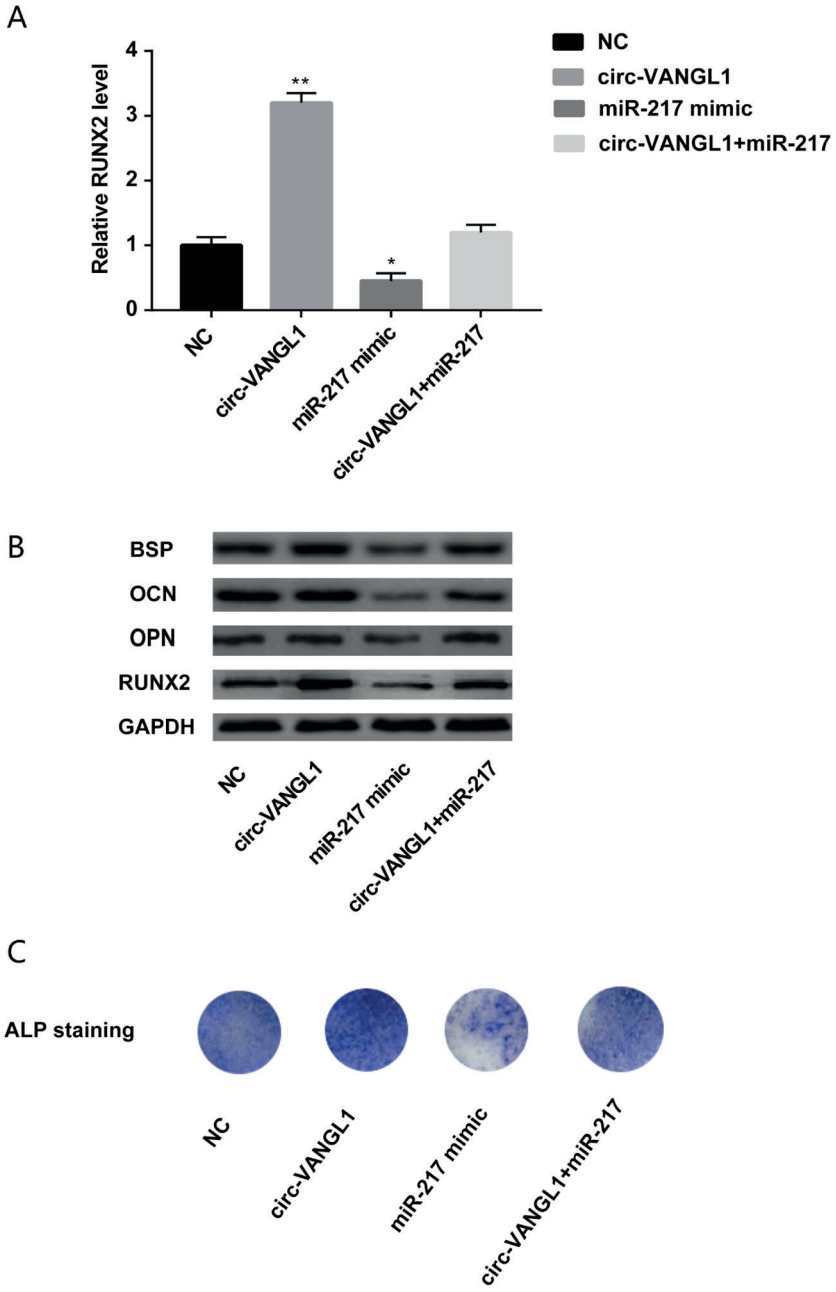

Figure 4. Circ-VANGL1 regulated osteogenic differentiation of hBMSCs via targeting miR-217; hBMSCs were divided into the NC group, circ-VANGL1 overexpression group, miR-217 overexpression group and circ-VANGL1+miR-217 co-overexpression group. ***A,*** QRT-PCR showed relative expression of RUNX2 in each group. B, Western blot analyses of BSP, OCN, OPN and RUNX2 in each group. ***C,*** ALP staining in each group. $^{*}p<0.05$, $^{**}p<0.01$.

occurrence and development of tumors. MiRNA-217 participated in the development of various tumors, such as osteosarcoma[24], gastric cancer[25], pancreatic cancer[26], and clear cell renal cell carcinoma[27]. Some studies have reported that miRNA-217 expression gradually decreases during osteogenic differentiation of rat MSCs. MiRNA-217 inhibits osteogenic differentiation by directly inhibiting the expression of RUNX2, thereby downregulating the expressions of osteoblast-associated genes[28]. RUNX2 belongs to the RUNX protein family and is a key transcription factor responsible for inducing differentiation and maturation of hBMSCs into chondrocytes and osteoblasts. Functionally, RUNX2 is greatly involved in bone repair and reconstruction[29,30]. This work pointed out that OP patients had a high serum level of miRNA-217. With the prolongation of osteogenic differentiation, miRNA-217 expression in hBMSCs gradually decreased. Further functional experiments confirmed that miRNA-217 was capable of inhibiting osteogenic differentiation by degrading RUNX2 via binding to RUNX2 3'UTR.

Many studies have shown that circRNAs are involved in the regulation of biological processes as miRNA sponges. CircRNA CDRIas binds to miRNA-7 by competition with Pax6 and Myrip, thus preventing diabetes by enhancing insulin transcription and secretion[31]. CircRNA HRCR sponges and negatively regulates the activity of miRNA-223 as a ceRNA, thus upregulating the expression of the target gene ARC. It also inhibits cardiomyocyte apoptosis by upregulating the expression of parental gene HRCR, thereafter preventing cardiac hypertrophy and heart failure[32]. CircRNA RUNX2 is capable of accelerating osteogenic differentiation by targeting miR-203 to regulate RUNX2[33]. In this study, we aimed to elucidate the correlation between circ-VANGL1 and miRNA-217, as well as their functions in regulating osteogenic differentiation. Our results revealed a positive correlation between circ-VANGL1 and miRNA-217. Importantly, we verified potential binding sites between them, further suggesting their interacted functions in regulating osteogenic differentiation. The overexpression of circ-VANGL1 increased expressions of RUNX2 and osteogenesis-related genes, as well as ALP activity, which was reversed by co-overexpression with miRNA-217. Hence, we believed that circ-VANGL1 could inhibit the osteogenic differentiation by absorbing miRNA-217 to regulate RUNX2 expression, thus promoting the progression of OP.

Conclusions

We showed that circ-VANGL1 promotes the development of OP via binding to miRNA-217 to downregulate RUNX2 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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