Circular RNA atlas in osteoclast differentiation with and without alendronate treatment

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Abstract

Background: Alendronate (AL) is the most widely used bisphosphonate in the treatment of osteoporosis (OP). However, the role of circular RNAs (circRNAs) in the treatment of OP with AL remains unclear.

Methods: In this study, we showed that osteoclast (OC) precursors (OPCSs) could be induced into OCs with macrophage colony-stimulating factor (MCSF) and receptor activator of nuclear factor-κB ligand (RANKL) treatment. Subsequently, the OCs were treated with AL. OC differentiation-related biomarkers including RANK, tartrate-resistant acid phosphatase (TRAP), and cathepsin K (CTSK) were analyzed with TRAP staining, quantitative real-time (qPCR), and western blotting. Differentially expressed circRNAs (DECs) were identified among the OPCS, OC, and OC + AL groups. In addition, the expression levels of 10 DECs related to OC differentiation were verified by qPCR.

Results: TRAP staining showed that MCSF and RANKL treatment effectively induced OPCSs to differentiate into OCs. In addition, qPCR and western blot analysis revealed that the three biomarkers of OC (RANK, TRAP, and CTSK) were expressed significantly more in the OC group than those in the OPCS group. In contrast, the mRNA and protein expression levels of these three biomarkers decreased significantly in OCs treated with AL compared with those non-treated OCs. GO analysis of the DECs in the OPCS group vs. the OC group revealed that their functions were mainly related to cell, cell part, binding, and single-organism terms. KEGG analysis of the top 20 DECs in a comparison between the OPCS and OC groups showed that genes involved in mitogen-activated protein kinase signaling were the most common. Results of functional analyses of DECs in an OC vs. OC + AL comparison were similar to those in the OPCS vs. OC comparison. Finally, qPCR showed that, in the OC + AL vs. OC group comparison, the expression levels of seven and three DECs significantly decreased and increased, respectively.

Conclusions: Having successfully induced OPCSs to differentiate into OCs, we showed that AL suppresses the differentiation of OPCS into OC and that 10 DECs were involved in the regulation of this process. This indicates that these DECs might be important to the treatment of OP.

Keywords: Alendronate, Osteoporosis, MCSF, RANKL
**Introduction**

Osteoporosis (OP) is among the most common bone diseases worldwide. It is characterized by a reduction in bone tissue volume and bone density per unit volume, and it is caused by the loss of bone calcium and bone matrix [1]. Because the average age of populations is generally increasing, OP is becoming a worldwide health problem. Indeed, according to the International Osteoporosis Foundation, more than 200 million people worldwide have OP, with the most common type being postmenopausal OP [2] (which about 30% of postmenopausal women suffer from). The most important mechanisms of postmenopausal OP are the excessive activation of osteoclasts (OCs) and the inhibition of osteoblasts caused by estrogen deficiency. Moreover, attenuation of osteoblast formation and/or increased bone resorption by OCs is a key mechanism of pathogenesis in OP; thus, OC dysfunction is an important OP pathogenic factor [3, 4]. Although previous studies have revealed multiple molecular mechanisms for OC dysfunction, few have investigated the roles of circular RNAs (circRNAs) during drug-related regulation of OC differentiation.

Osteoblasts and OCs are the main functional cells of bone formation: osteoblasts are responsible for new bone formation, whereas OCs are responsible for aged bone resorption [5]. Thus, these cells maintain a dynamic balance of formation and resorption; however, when this balance is disrupted, the function or architecture of the bone becomes abnormal [6]. Consequently, drugs for the treatment of OP mainly include bone resorption inhibitors and bone formation promoters. One such drug is alendronate (AL), which is a bisphosphonate widely used for OP treatment. AL has multiple effects in OP patients including inhibiting OC activity, increasing bone density, and reducing the incidence of vertebral and non-vertebral fractures [7, 8].

Previous studies have suggested that bisphosphonates’ mechanism of action is dependent on three processes: (1) altering the morphology and structure of the OCs to inhibit their function; (2) inhibiting the production of osteoblast-mediated interleukin (IL)-6 and tumor necrosis factor-α, thereby decreasing OC genesis; and (3) physically and chemically bonding with the bone matrix, thereby interfering with bone resorption [9]. However, the role of circRNAs in the underlying molecular mechanisms of AL’s effects in OP remains to be elucidated.

circRNAs are covalently closed RNA molecules that are generated through a process named back-splicing [10] and are involved in the regulation of various biological processes. For example, circRNA.014511 inhibits P53 expression by binding to miR-29b-2-5p, and it reduces the sensitivity of bone marrow mesenchymal stem cells by regulating cell apoptosis and the cell cycle [11]. Additionally, circRNA.33186-knockdown inhibits apoptosis and promotes proliferation in chondrocytes treated with IL-1β [12]. Also, circRNA.28313 functions in macrophage colony-stimulating factor (MCSF) + receptor activator of nuclear factor-kB ligand (RANKL)-induced OC differentiation via the regulation of miR-195a expression; this affects bone absorption in mice [13]. Moreover, at different stages of mouse OC growth, the expression levels of various circRNAs are different [14].

Unfortunately, OCs are difficult to separate from cortical bone in vitro: they are terminally differentiated cells lacking proliferation abilities [15]. Therefore, OCs cannot be isolated for culture in vitro. However, THP-1 cells can differentiate into OCs when stimulated by phorbol-12 myristate-13 acetate (PMA), RANKL, and MCSF [16, 17]. Thus, to investigate the involvement of circRNAs in the AL treatment of OP, THP-1 cells were used in the present study to differentiate into OCs for further functional analysis. Specifically, we probed the potential circRNA atlas involved in AL-induced OC differentiation. From our results, we were able to draw a circRNA–miRNA–mRNA network of AL-induced OC differentiation. Our results provide insights that could be used for further clinical studies of OP treatment.

**Materials and methods**

**Cell treatment**

THP-1 cells were purchased from ATCC (VA, USA) and maintained in RPMI 1640 with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (HyClone, USA). Cell lines were maintained in a humidified chamber at 5% CO₂ and 37°C. THP-1 cells were induced into OC precursors (OPCSs) through stimulation with 100 ng/mL PMA for 3 days. The detailed experimental procedure for this process was previously described by Takashiba et al. [18]. Once OPCSs were available, they were uniformly inoculated into 96-well plates (5000 cells/well); they were induced over 7 days into OCs using a final concentration that contained 100 ng/mL RANKL and 50 ng/mL MCSF. Every 72 h during induction, the culture medium was changed and the state of OC differentiation was evaluated. During OC differentiation, 10⁻⁸ M AL was either added or not added (depending on the treatment group) to the induction culture on day 4. After 7 days, the treated cells were harvested and divided into three groups: the OPCS group, OC group, and OC + AL group.

**Tartrate-resistant acid phosphatase (TRAP) staining**

Here, TRAP staining was performed by modifying the method of Oshima et al. [19]. Briefly, OCs were incubated for 15 min at 37°C in freshly prepared 0.1 M Tris buffer (pH 5.0) that contained 1.35 mM naphthol AS-MX phosphate (Sigma); 0.362 M N, N-dimethylformamide; 3.88 mM Violet LB salt (Sigma); and 25 mM N,N-dimethylformamide (N,N-dimethylformamide).
### Table 1 Primer sequences of circRNAs

| CircBase ID       | Sequence (5' to 3')                                      | Product length | Gene ID |
|-------------------|----------------------------------------------------------|----------------|---------|
| hsa_circ_0000284  | TCTCGGTACTACAGGTATGGC ACCTTATTGAGGAGATGAGA TGCCACCTTGTGACATTGAGA | 193            | HIPK3   |
| hsa_circ_0000638  | CGGGAAAAATAGTGACACACAGC TCTCTCTTCCATTTCGTCCTGACGC | 173            | ETFA    |
| hsa_circ_0000994  | TGAATTTGTTAGTTGAGACTGCTGTTGTCCTCGAC | 158            | SLC8A1  |
| hsa_circ_0001776  | CAAGGAACCTTCCGGTGTTGATGAGA TCCCTTCTTTCATTTCTGTCTCAGC | 143            | ESYT2   |
| hsa_circ_0002922  | TGAGGCGAAGACTCCTAATCTCGGATGAGA TGCCTGCTTTCCTCCTGGTCGC | 165            | ZNF124  |
| hsa_circ_0003249  | AATCATTCCTGTGAGACTCCTAATCTCGGATGAGA TGAGGACCTTCCATTTCGTCCTGACGC | 186            | LRP11   |
| hsa_circ_0007710  | CCAGAAGGGAAAGACACTTGCTGTTGATGAGA AGTCAATAGAGATGGAGTGGAGT TCCCTTCTTTCATTTCTGTCTCAGC | 173            | ELF2    |
| hsa_circ_0094798  | ATGAGAATGGCATCTAAGAATGAAG TCCCTTCTTTCATTTCTGTCTCAGC | 163            | CWF19L2 |
| hsa_circ_0101874  | CCAGAAGGGAAAGACACTTGCTGTTGATGAGA AGTCAATAGAGATGGAGTGGAGT TCCCTTCTTTCATTTCTGTCTCAGC | 165            | ZNF124  |
| hsa_circ_0113954  | AATCATTCCTGTGAGACTCCTAATCTCGGATGAGA TGAGGACCTTCCATTTCGTCCTGACGC | 186            | LRP11   |
|                   |                                                          |                |         |

### Table 2 Primer sequences of qPCR

| Gene   | Primer       | Sequence (5' to 3')                                      | Product length (bp) |
|--------|--------------|----------------------------------------------------------|---------------------|
| HIPK3  | H-HIPK3-F    | CAGCTCTTCCTTCTCCGGACCTGCCCTCC | 166                 |
|        | H-HIPK3-F    | CTCTCTTCTCCGGACCTGCCCTCC | 196                 |
| ETFA   | H-ETFA-F     | CTCTCCAGCTAATCCACCT | 181                 |
|        | H-ETFA-F     | TGGACCTGTGGATCTGTTAAG | 120                 |
| SLC8A1 | H-SLC8A1-F   | CATCGAAGGGACTGCCAGAG | 180                 |
|        | H-SLC8A1-F   | TCCACTCATCTCCACCCAGGC | 151                 |
| ESYT2  | H-ESYT2-F    | AAGGAACCTTCCGTACGG | 160                 |
|        | H-ESYT2-F    | TCCACACAGGTTCATTTG | 121                 |
| ZNF124 | H-ZNF124-F   | CTCTCTTCCTCCTCCTCCGG | 195                 |
|        | H-ZNF124-F   | TCCACACACAGGCCTCCCTCC | 182                 |
| LRP11  | H-LRP11-F    | TGAGTCAAGGGATGGAGGAGG | 191                 |
|        | H-LRP11-F    | TAGTCGGCATGCAACCATGA | 171                 |
| ELF2   | H-ELF2-F     | TCCCTTCTCCTCCTTGAGGC | 180                 |
|        | H-ELF2-F     | TCCCTTCTCCTCCTTGAGGC | 151                 |
| CWF19L2| H-CWF19L2-F  | TGAGTCAAGGGATGGAGGAGG | 195                 |
|        | H-CWF19L2-F  | TGGAGTCAAGGGATGGAGGAGG | 182                 |
| FKB2P3 | H-FKB2P3-F   | AGTAAAGCGGAGGAGGAGG | 151                 |
|        | H-FKB2P3-F   | TGGTTATATATATATATATATAT | 182                 |
| MIER1  | H-MIER1-F    | TGGTTATATATATATATATATAT | 182                 |
|        | H-MIER1-F    | TGGTTATATATATATATATATAT | 182                 |
sodium tartrate. Slides were rinsed for 10 min and counterstained with hematoxylin. One-micromolar sections were cut using a Sorvall Porter-blum MT-2B ultra microtome. New bone formation was visualized using fluorescence microscopy (Nikon, Tokyo, Japan).

**Real-time quantitative PCR (qPCR)**

Reverse transcription of mRNA from the three treatment groups was carried out using a final volume of 100 μL from 400 ng total RNA and with a High-capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer’s instructions. mRNA levels were determined by qPCR, and the following primers were used: RANK forward 5′-TCTGCTTCTCTTCGCGTCTG-3′; RANK reverse 5′-AGCCTCATTGATCCAGTGCC-3′; TRAP forward 5′-GATCCCCCAGACCAATGTGTC-3′; TRAP reverse 5′-CCAGCAGCTAGTCTCCTCCT-3′; cathepsin K (CTSK) forward 5′-TCTGCTTCTCCTCTCTGTTTTGAC-3′; CTSK reverse 5′-GTCATGTAGCCTCCAC-3′. Reactions were performed in 50-μL volumes containing SYBR Green PCR master mix (PerkinElmer Biosystems). qPCR was performed using 96-well optical plates in a GeneAmp PCR System 9600 (Perkin-Elmer Biosystems). Thermal cycling conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. Data were collected using an ABI analytical thermal cycler. mRNA
expression levels were calculated on the basis of a relative standard curve and using the $2^{-\Delta\Delta Ct}$ method. In addition, we designed 20 primer pairs to identify the linear DNA and corresponding circRNAs; the primer sequences for these are listed in Tables 1 and 2. PCR products were analyzed using agarose gel electrophoresis (1.5%).

Western blot analysis
Total cellular protein from the three treatment groups was isolated by the addition of 1% PMSF and RIPA lysis buffer (containing 50 mM Tris–HCl at pH 7.4, 150 mM NaCl, 1% NP-40, and 0.1% SDS). After boiling with SDS-PAGE sample buffer for 5 min, the samples were subjected to SDS-PAGE. The proteins were then transferred to a PVDF membrane (Millipore, USA). After blocking for 1 h at room temperature, the membrane was incubated with a 1:1000 dilution of rabbit polyclonal anti-mouse RANK, TRAP, CTSK, or GAPDH antibodies (ABGENT, USA) overnight. Before detection with an enhanced chemiluminescence detection kit (Advansa, USA), proteins were incubated with the corresponding secondary antibody for 1 h at room temperature. The bands were obtained by GeneGnome 5 (Synoptics Ltd., UK).

**Fig. 3** Differentially expressed circRNAs and their functions in the OC group and OPCS group. **a** Differentially expressed circRNAs in the OC group and OPCS group. **b** GO analysis of differentially expressed circRNAs. All GO items could be divided into three main subgroups: cellular components, molecular functions, and biological processes. **c** Heatmap analysis of the differentially expressed circRNAs in the OC and OPCS groups. **d** KEGG analysis of the top 20 differentially expressed circRNAs in the OC and OPCS groups. OCs, osteoclasts; OPCSs, osteogenic precursor cells.
Bioinformatics
Differentially expressed circRNAs (DECs) were determined using the following parameters: $P$ value $\leq 0.05$ and $|\log_{2}\text{Ratio}| \geq 2$. GO enrichment analysis and KEGG analysis of DECs were carried out using the online database KOBASE3.0 (http://kobas.cbi.pku.edu.cn/) with the default parameters maintained. In addition, a hypergeometric test was used to calculate the major biochemical metabolic pathways in which the DECs were involved.

Data analysis
SPSS V16.0 software (IBM, USA) was used for statistical analyses. Statistical differences between groups were calculated using Tukey's test. Where $P$ was $< 0.05$, this indicated a significant difference.

Results
OC differentiation and drug induction
In order to investigate the OC differentiation and AL induction of OCs, THP-1 cells were induced into OPCSs by PMA treatment. Subsequently, OPCSs were further induced into OCs by MCSF and RANKL treatment. Finally, AL was used to treat OC. TRAP staining showed that MCSF and RANKL treatment effectively induced OPCS to differentiate into OCs (Fig. 1a). Moreover, qPCR showed that the expression levels of the OC differentiation-related biomarkers RANK, TRAP, and CTSK were significantly increased in OCs compared with OPCSs ($P < 0.05$) (Fig. 1b). Western blot analysis confirmed these results ($P < 0.05$) (Fig. 1c). In the AL induction of OCs, qPCR showed that the expression levels of RANK, TRAP, and CTSK were significantly decreased in AL-treated OCs compared with those in untreated OCs ($P < 0.05$) (Fig. 2a). Similar results were observed in western blot analysis (Fig. 2b). Thus, AL apparently inhibited the differentiation of OCs.

DECs in an OPCS group vs. OC group comparison
We identified the circRNAs that were differentially expressed in the OPCS and OC groups. Figure 3a shows that the number of upregulated DECs (1394) was higher than the number of downregulated DECs (214) in the OPCS group vs. the OC group. GO analysis revealed that the functions of these DECs were mainly distributed into three categories: cellular component, molecular function, and biological process (Fig. 3b). In the cellular component category, “cell,” “cell part,” and “organelle” were the three most enriched items. In the molecular function category, “binding” was the most enriched item. Finally, in the biological process category, “cellular process” and “single-organism process” were the most enriched items. We also produced a heat map to show the cluster relationships among these DECs (Fig. 3c). KEGG analysis of the top 20 DECs showed that enrichment was mostly in metabolic pathways and in the mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 3d and Table 3).

### Table 3 Top 20 differential expression circRNA list between the OC group and OPCS group

| CircBase_ID | Gene ID | OC-TPM | OPCS-TPM | Log2 ratio (OPCS/OC) | Up-downregulation | $P$ value | FDR |
|-------------|---------|--------|----------|----------------------|-------------------|-----------|-----|
| hsa_circ_0003307 | TALDO1 | 11.919 | 26.6945 | 4.5 | Up | 2.05E–07 | 1.77E–05 |
| hsa_circ_0007761 | ATXN7 | 29.796 | 629.171 | 4.4 | Up | 3.76E–15 | 1.14E–12 |
| hsa_circ_0007674 | MCM3 | 35.756 | 629.171 | 4.137 | Up | 1.75E–14 | 5.21E–12 |
| hsa_circ_0007220 | C5orf42 | 11.919 | 202.234 | 3.822 | Up | 1.42E–05 | 0.000626866 |
| hsa_circ_0004012 | PDXDC2P | 17.878 | 269.645 | 3.915 | Up | 8.83E–07 | 6.07E–05 |
| hsa_circ_0003260 | RAP1B | 17.878 | 269.645 | 3.915 | Up | 8.83E–07 | 6.05E–05 |
| hsa_circ_00030213 | LRC11 | 11.919 | 179.763 | 3.915 | Up | 5.66E–05 | 0.001799064 |
| hsa_circ_0017065 | B3GALNT2 | 11.919 | 179.763 | 3.915 | Up | 5.66E–05 | 0.001795578 |
| hsa_circ_0008884 | ZNF367 | 23.837 | 337.056 | 3.822 | Up | 5.60E–08 | 5.45E–06 |
| hsa_circ_0008883 | CAPN15 | 11.919 | 157.293 | 3.722 | Up | 0.000222228 | 0.005249134 |
| hsa_circ_0003535 | NNT | 476.741 | 44.941 | – 3.407 | Down | 1.74E–06 | 0.000102458 |
| hsa_circ_0017077 | LYST | 435.026 | 44.941 | – 3.275 | Down | 7.63E–06 | 0.000353697 |
| hsa_circ_0001541 | ANKH1 | 417.148 | 44.941 | – 3.214 | Down | 1.43E–05 | 0.000631106 |
| hsa_circ_0001613 | SENP6 | 613.804 | 67.411 | – 3.187 | Down | 9.68E–08 | 8.75E–06 |
| hsa_circ_0001346 | RNF13 | 1501.734 | 179.763 | – 3.062 | Down | 5.76E–17 | 2.48E–14 |
| hsa_circ_0002394 | SNX27 | 309.882 | 44.941 | – 2.786 | Down | 0.000577398 | 0.012403449 |
| hsa_circ_00046999 | CEP192 | 280.085 | 44.941 | – 2.64 | Down | 0.00156603 | 0.024002196 |
| hsa_circ_0000099 | AMY2B | 280.085 | 44.941 | – 2.64 | Down | 0.00156603 | 0.023979743 |
| hsa_circ_0001432 | MANBA | 965.401 | 157.293 | – 2.618 | Down | 6.75E–10 | 8.84E–08 |
| hsa_circ_0001508 | MSH3 | 274.126 | 44.941 | – 2.609 | Down | 0.001908118 | 0.028164097 |
DECs in an OC group vs. OC + AL group comparison

We also identified the circRNAs that were differentially expressed in the OC and OC + AL groups. Figure 4a shows that the number of upregulated DEC (1434) was higher than the number of downregulated DEC (219) in this OC group vs. OPCS group comparison. As with the previous GO analysis, the functions of these DECs could be largely separated into the three categories cellular component, molecular function, and biological process (Fig. 4b). The most enriched items in the cellular component (“cell”, “cell part”, and “organelle”), molecular function (“binding”), and biological process (“cellular process” and “single-organism process”) categories were respectively identical to those in the OPCS group vs. OC group comparison. The heat map in Fig. 4c shows the cluster relationships among these DECs (Fig. 4c). As with the OPCS group vs. OC group comparison, KEGG analysis of the top 20 DECs showed that enrichment was mostly in metabolic pathways and in the MAPK signaling pathway (Fig. 4d and Table 4).

Network of circRNA–miRNA–mRNA

To further examine the function of DECs, we screened the overlap of DECs among the three treatment groups: 110 circRNAs were differentially expressed among these groups.
Of the 110 DECs, 95 and 15 were upregulated and downregulated after AL treatment, respectively (Fig. 5a, b). In addition, we constructed a circRNA–miRNA–mRNA network. The circRNAs related to OC differentiation were selected using the following criteria: (1) circRNAs were sourced from exons, and (2) the length of circRNAs ranged from 300 to 1200 bp. Figure 5 shows that hsa_circ_0002922 bound to hsa-miR-181b-5p to regulate the expression of MAP2K1 and hsa_circ_0007710 bound to hsa-miR-197-3p to regulate the expression of MAPK1, and it also bound to hsa-miR-20a-5p to regulate the expression of MAPK9. The top 20 DECs are shown in Table 5. Figure 5c shows that

### Table 4 Top 20 differential expression circRNA list between OC group and OC + AL group

| CircBase_ID       | Gene ID | OC-TPM  | OC_AL-TPM | Log2 ratio (OC_AL/OC) | Up-downregulation | P value     | FDR         |
|-------------------|---------|---------|-----------|-----------------------|-------------------|-------------|-------------|
| hsa_circ_0033144  | BCL11B  | 11.919  | 2248.509  | 7.56                  | Up                | 9.34E−43    | 1.14E−39    |
| hsa_circ_0009125  | HABP4   | 35.756  | 2981.719  | 6.382                 | Up                | 1.92E−52    | 3.05E−49    |
| hsa_circ_0083766  | EPK2    | 11.919  | 830971    | 6.123                 | Up                | 1.37E−15    | 2.14E−13    |
| hsa_circ_0007426  | FNBP1   | 11.919  | 782.09    | 6.036                 | Up                | 1.13E−14    | 1.53E−12    |
| hsa_circ_0005035  | IGF1R   | 17.878  | 977.613   | 5.773                 | Up                | 1.67E−17    | 3.15E−15    |
| hsa_circ_0063809  | CELSR1  | 17.878  | 830971    | 5.539                 | Up                | 8.57E−15    | 1.19E−12    |
| hsa_circ_0005660  | NFIX    | 11.919  | 488806    | 5.358                 | Up                | 3.16E−09    | 1.94E−07    |
| hsa_circ_0033476  | MARK3   | 11.919  | 488806    | 5.358                 | Up                | 3.16E−09    | 1.93E−07    |
| hsa_circ_0003489  | CDK8    | 11.919  | 488806    | 5.358                 | Up                | 3.16E−09    | 1.92E−07    |
| hsa_circ_0001730  | EPHB4   | 53.633  | 2150.748  | 5.326                 | Up                | 1.63E−34    | 1.23E−31    |
| hsa_circ_0000566  | VRK1    | 1853.331| 97.761    | 4.245                 | Down              | 3.16E−13    | 3.86E−11    |
| hsa_circ_0000826  | ANKRD12 | 3629.191| 244.403   | 3.892                 | Down              | 7.83E−24    | 2.53E−21    |
| hsa_circ_0005615  | NFATC3  | 3259.717| 244.403   | 3.737                 | Down              | 5.80E−21    | 1.59E−18    |
| hsa_circ_0004658  | EMLIN2  | 1251.445| 97.761    | 3.678                 | Down              | 1.66E−08    | 8.44E−07    |
| hsa_circ_0000284  | HIPK3   | 2330.667| 1906.345  | 3.612                 | Down              | 1.52E−13    | 6.05E−16    |
| hsa_circ_0086735  | UBP2    | 2407.542| 244.403   | 3.3                   | Down              | 1.85E−14    | 2.44E−12    |
| hsa_circ_0000994  | SLC8A1  | 7472.915| 879.851   | 3.086                 | Down              | 2.15E−40    | 2.13E−37    |
| hsa_circ_0002538  | KLHL8   | 709.152 | 97.761    | 2.859                 | Down              | 0.000201768 | 0.003278317 |
| hsa_circ_0006156  | FND3C1  | 4058.258| 635.448   | 2.675                 | Down              | 7.46E−20    | 1.77E−17    |
| hsa_circ_0006595  | ST3GAL3 | 1489.816| 244.403   | 2.608                 | Down              | 9.13E−08    | 3.79E−06    |
| CircBase_ID   | Gene ID | OC-TPM   | OC_AL-TPM | OPCS-TPM | Log₂ ratio (OPCS/OC) | OPSC/OC Log₂ (OC_AL/OC) | Up-downregulation (OPCS/OC) | P value  | FDR   | Log₂ ratio (OC_AL/OC) | Up-downregulation (OC_AL/OC) | FDR   |
|---------------|---------|----------|-----------|----------|----------------------|-------------------------|----------------------------|----------|-------|----------------------|-------------------------------|-------|
| hsa_circ_0008883 | CAPN15  | 11.919   | 439.926   | 157.293  | 3.722                |                         | Up                         | 0.000222228 | 0.003249134 | 5.206                | Up                              | 2.46E-08 |
| hsa_circ_0007761 | ATXN7   | 29.796   | 782.09    | 629.171  | 4.4                  |                         | Up                         | 3.76E-15   | 1.14E-12        | 4.714                | Up                              | 1.26E-12 |
| hsa_circ_0000053 | STK40   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033387027    | 4.621                | Up                              | 9.62E-08 |
| hsa_circ_0004137 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
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| hsa_circ_0000053 | RBM23   | 17.878   | 439.926   | 134.822  | 2.915                |                         | Up                         | 0.00232928 | 0.033125964    | 4.258                | Up                              | 1.26E-06 |
the top 10 DECs played key roles in the circRNA–miRNA–mRNA network. Furthermore, the expression levels of 10 DECs in the key networks (hsa_circ_0000284, hsa_circ_0000638, hsa_circ_0000994, hsa_circ_0001776, hsa_circ_0002922, hsa_circ_0003249, hsa_circ_0007710, hsa_circ_0094789, hsa_circ_0113954, and hsa_circ_0101874) were also validated in the three treatment groups using qPCR (Fig. 6). The expression levels of hsa_circ_0000284, hsa_circ_0000638, hsa_circ_0000994, hsa_circ_0001776, hsa_circ_0002922, hsa_circ_0007710, and hsa_circ_0113954 were significantly increased in the OC group compared with those in the OPCS group (P < 0.05). However, the expression levels of these DECs were significantly decreased in the OC + AL group compared with those in the OC group (P < 0.05). Furthermore, the expression levels of hsa_circ_0003249, hsa_circ_0094789, and hsa_circ_0101874 were significantly decreased in the OC group compared with those in the OPCS group (P < 0.05). In contrast, the expression levels of these DECs were significantly increased in the OC + AL group compared with those in the OC group (P < 0.05). Finally, we confirmed the identity of the top 10 DECs via agarose gel electrophoresis (Fig. 6).

Discussion

OCs are the main functional cells involved in bone resorption during bone remodeling. Their differentiation and activity are regulated by a variety of hormones and cytokines, including MCSF and RANKL: these two cytokines are critical for the regulation of OC-like cell differentiation in vitro [20]. Therefore, we selected both MCSF and RANKL to induce THP-1 cells into OCs and showed that they were functionally able to induce OC differentiation, which is a finding consistent with that of a previous study [21].

The anti-bone resorption effects of AL are mainly achieved via the inhibition of OCs. AL binds to non-hydrolyzable ATP analogs on the surface of OC membranes and inhibits the action of ATP-dependent intracellular enzymes, which in turn significantly inhibits the effects of OCs [22]; specifically, OCs lose the ability to absorb bone, which affects bone resorption and bone turnover rate [23, 24]. Bisphosphonates such as AL are currently the most commonly used drugs for the treatment of OP; they can specifically bind to hydroxyapatite in the bone so are strong inhibitors of bone resorption but have little effect on other tissues [25]. Srisubut et al. found that AL promotes bone formation in bioactive glass grafting in rats [26], and Jensen et al. demonstrated that orally administering AL to dogs with prosthesis implantations promotes bone formation around a good prosthesis [27]. These studies indicate that AL plays a role in promoting local bone formation; similarly, we found that AL effectively inhibits OC differentiation.

We also found that 1394 and 214 circRNAs were differentially upregulated and downregulated, respectively, in the OPCS and OC groups, whereas 1434 and 219 circRNAs were differentially upregulated and downregulated, respectively, in the OC and OC + AL groups. Notably, GO and KEGG analysis revealed that gene functions and signaling pathways were similarly enriched between these two groups of DECs. This indicates that the same genes might participate in both OC...
differentiation and inhibition. For example, the MAPK signaling pathway was enriched in both sets of DECs. The MAPK pathway is important in the signaling network of eukaryotic cells: it transduces extracellular mitosis signals to the cell nuclei [28]. Activated MAPK regulates a variety of cellular physiological processes such as growth, division, differentiation, and apoptosis through phosphorylation and ubiquitination [29]. Previous studies have shown that the MAPK pathway regulates the osteoprotegerin/RANKL intracellular pathway involving in the balance of bone metabolism.

The MAPKs associated with OC differentiation mainly comprise three subfamilies: extracellular signal-regulated protein kinase (ERK), p38MAPK, and c-Jun amino-terminal kinase [30]. The MAPK pathway is also considered to be an important pathway in inflammatory bone injury [31]. Other studies have shown that the p38 inhibitor SB203580 and the ERK inhibitor U0126 significantly reduce the activity of TRAP during OC formation and thereby inhibit the formation and function of OCs [32]. In addition, the circRNA CDR1as can inhibit the expression of miR-7, which releases the negative regulation of miR-7 on growth differentiation factor 5, further activates the p38MAPK pathway and pSmad1/5/8, and promotes osteogenesis of periodontal ligament stem cells [33]. These results are consistent with our finding that circRNAs might bind to miRNA to regulate the MAPK pathway during differentiation of OCs treated with AL.

To date, there is a lack of research on circRNAs in the skeletal system. Some early studies have reported the expression of long non-coding RNAs during chondrocyte differentiation and osteoblast differentiation [34]; however, the expression and function of circRNAs in the process of OC formation had previously not been demonstrated. Thus, our study is the first to report the expression profiles of circRNAs during the differentiation of OCs treated with AL.

Conclusions
In summary, here, we successfully induced OPCSs to differentiate into OCs, showed that AL could suppress OPCSs from differentiating into OCs, and identified 10 DECs that were involved in the regulation of this process. These 10 circRNAs might be important to the treatment of OP; this speculation requires further enquiry.

Abbreviations
OP: Osteoporosis; OC: Osteoclast; circRNAs: Circular RNAs; AL: Alendronate; IL: Interleukin; MCSF: Macrophage colony-stimulating factor; RANKL: Receptor activator of nuclear factor-κB ligand; PMA: Phorbol-12-myristate-13 acetate; OPCS: Osteoclast precursor; TRAP: Tartrate-resistant acid phosphatase; CTSK: Cathepsin K; DEC: Differentially expressed circRNA; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated protein kinase

Acknowledgements
Not applicable.

Authors’ contributions
Guofeng Huang and Zhenqi Ding conceived and designed the study and critically revised the manuscript. Jianbiao Lin, Shaofeng Ma, and Cong Zh performed the experiments, analyzed the data, and drafted the manuscript. Changqing Chen, Weibin Lin, and Canbin Lin participated in study design, study implementation, and manuscript revision. All authors read and approved the final manuscript.

Funding
This work was supported by the Natural Science Foundation of Fujian Province of China [grant number 2016J01243], Military Youth Training Program of China [grant number 19QNP046], Scientific Research Projects on Military Logistics [grant number CNJ16C013], and Youth Training Program of 18th Sub-region.

Availability of data and materials
Any information used and analyzed during this study is available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 6 November 2019 Accepted: 20 May 2020
Published online: 01 July 2020

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