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Dihydromyricetin enhances the osteogenic differentiation of human bone marrow mesenchymal stem cells *in vitro* partially via the activation of Wnt /β-catenin signaling pathway

Running Head: Dihydromyricetin enhances the osteogenesis

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ABSTRACT

Substantial evidence has demonstrated that the decreased osteogenic differentiation of bone mesenchymal stem cells (BMSCs) is closely related to bone metabolic diseases. Thus, it is very important to develop several potentially useful therapeutic agents to enhance BMSC osteogenesis. Flavonoids show promise in enhancing bone mass. Dihydromyricetin (DMY), a type of flavonoid, has not yet been investigated regarding its effects on BMSC osteogenesis. To investigate the effects of DMY on osteogenesis, human BMSCs were induced with or without DMY. We found that DMY (0.1 μM to 50 μM) exhibited no cytotoxic effect on proliferation, but increased alkaline phosphatase activity, osteoblast-specific gene expression, and mineral deposition. It also enhanced active β-catenin expression and reduced dickkopf-1(DKK1) and sclerostin expression. The Wnt/β-catenin signaling pathway inhibitor (DKK1 and β-catenin-specific siRNA) decreased the enhanced bone mineral formation caused by DMY. Taken together, these findings

reveal that DMY enhances osteogenic differentiation of human BMSCs partly through Wnt /β-catenin *in vitro*.

Keywords Dihydromyricetin, mesenchymal stem cells, osteogenic differentiation, Wnt/β-catenin signaling pathway

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (BMSCs) retain their self-renewal capability and have the potential to differentiate into bone tissue, making them attractive candidates for clinical applications for the repair or reconstruction of bone defects. Moreover, osteoporosis has been mainly attributed to the decreased osteogenic differentiation of BMSCs [1, 2]. Accordingly, it is essential to identify strategies to enhance BMSC osteogenesis [3, 4].

Ampelopsis grossedentata, an edible plant from southern China, is a traditional Chinese medicinal herb [5]. Dihydromyricetin (DMY), also known as ampelopsin, is the major bioactive component extracted from *Ampelopsis grossedentata*. As a type of flavonoid, it has numerous biological and pharmacological actions, including improvement in insulin resistance, and anti-inflammatory, antibacterial, antioxidant, and antitumor effects [5-10]. According to previous reports, flavonoids are members of the catechin family with the strongest bone loss prevention effects [11-17]. Recently, several studies have reported that the biological effects of DMY were correlated with activation of β-catenin canonical Wnt signaling [12, 18].

The Wnt signaling pathway plays an essential role in the balance between adipogenesis and osteogenesis [19]. Canonical Wnt pathway, or WNT/βcatenin pathway, these components consist of a heterotrimeric complex composed of different ligand Wnt, the single-pass transmembrane co-receptor LRP and the seven transmembrane signalling

receptor Frizzled [20]. Wnt ligands bind to Frizzled and LRP5/6 receptors, and induce the stabilization of cytoplasmic β-catenin [21]. β-catenin accumulates in the cytoplasm and translocates into the nucleus to regulate target gene transcription. The receptor-binding WNT protein in canonical pathways mainly included Wnt 3a and Wnt 10b [20]. The binding of dickkopf-1 (DKK1) to the LRP5/6 or Frizzled coreceptor was shown to block Wnt/β-catenin signaling [22, 23]. Noncanonical Wnt pathway also regulates the differentiation state of BMSCs [24]. It is well known that Wnt 5a activates both Wnt–Ca^{2+} and Wnt–JNK noncanonical pathways to regulate bone formation [25]. Moreover，Yun et al. suggest that flavonoids might promote osteogenic differentation of BMSCs through the activation of Wnt/β-catenin signaling [18].

To the best of our knowledge, the pharmacological actions of DMY in bone-forming cells have not yet been defined. Accordingly, we hypothesized that DMY promotes osteogenic differentiation of BMSCs via activation of the Wnt/β-catenin signaling pathway. In this study, by assessing the expression levels of specific genes and calcium deposition, we reveal that DMY enhances osteogenic differentiation of human BMSCs partly via the Wnt/β-catenin signaling pathway *in vitro*.

MATERIALS AND METHODS

Cells and reagents

Human BMSCs, as reported previously [26], were purchased from Cyagen Biosciences (Guangzhou, China). These cells can differentiate into osteoblasts, adipoblasts, and chondrocytes under specific inductive conditions. Adherent cells were trypsinized and passaged after reaching 80% confluence. Cells from passages 5-9 were used in subsequent experiments.

DMY was purchased from Selleckchem (S2399, Houston, TX, USA). According to the data of cell counting kit-8 (CCK-8), 0.1 μM and 50 μM DMY were added to osteogenic induction medium. Recombinant DKK1 was purchased from PeproTech (Rocky Hill, NJ, USA). In accordance with a previous study, the applied concentration of DKK1 was 0.5

μg/mL [18]. A stealth small interference RNA (siRNA) specific to total β-catenin (5'-CCCUCAGAU GGUGUCUGCCAUUGUA-3') (200 pmol/L) [12, 27] and a negative control siRNA (5'-CCACTACCTGAGCACCCAGTT-3') were prepared by Invitrogen (USA).

CCK-8

To assess the effect of DMY on the proliferation of BMSCs, the cells were seeded into a 96-well plate (5000/well) and allowed to adhere for 24 h. After 24 h, the medium was removed, and the cells were treated with 10% CCK-8 (Dojindo, Kumanoto, Japan) in 100 μL low-sugar Dulbecco's modified Eagle's medium (L-DMEM) without FBS for 3 h at 37°C. Absorbance at 450 nm, which is directly proportional to cell proliferation, was measured by a microplate reader (ELX808, BioTek, Winooski, VT, USA).

Osteogenic differentiation protocol

BMSCs were cultured in growth medium [L-DMEM, 10% FBS (1495527, Gibco, USA), 100 IU/mL penicillin/streptomycin] in 6 or 12-well cell culture plates (Corning, Shanghai, China), at a density of $8\times10^4/cm^2$, and incubated for 48 h at 37°C under 5% CO_2. The cells were subsequently cultured in osteogenic induction medium (L-DMEM, 10% FBS, 100 IU/mL penicillin/streptomycin, 100 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerophosphate). The cells were maintained by the addition of fresh osteogenic induction medium every 2-3 days.

Measurement of ALP activity

For the measurement of ALP activity, cells were lysed in RIPA lysis buffer (Beyotime, Shanghai, China), and the lysate (10 μL) was incubated with 90 μL fresh solution containing p-nitrophenyl phosphate substrate at 37°C for 30 min. The reaction was stopped by the addition of 0.5 N NaOH (100 μL), and the absorbance was measured at 405 nm using a microplate reader (ELX808, BioTek). The total protein concentration was measured using a BCA protein assay kit (KeyGen BioTECH, Nanjing, China). The relative ALP activity is expressed as the percentage change in optical density (OD) per unit time per milligram protein (OD/15 min/mg protein) ×100.

Alizarin red staining (ARS)

After the induction of osteogenic differentiation, mineral deposition was assessed by ARS (Cyagen Biosciences). Cells were fixed in 4% paraformaldehyde (Sangon Biotech, Shanghai,

China) for 5 min at room temperature, and then washed with distilled water twice. A 1% solution of alizarin red was added and incubated for 10 min at room temperature, followed by rinsing with distilled water. The solution was collected and 200 μL were plated on 96-well plates. OD was measured at 560 nm using a mircoplate reader (ELX808, BioTek, USA). The readings were normalized to the total protein concentration.

Immunofluorescence

Cells were cultured in induction medium in a 12-well plate, runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN), and β-catenin were detected using a fluorescence microscope (EU5888, Leica, Wetzlar, Germany). Briefly, cells were fixed in 4% paraformaldehyde for 30 min, permeabilized, and blocked for 30 min in 0.02% Triton X-100 and 2% bovine serum albumin. Fixed cells were washed and incubated overnight with anti-RUNX2 (1:1600; Cell Signaling Technology, Shanghai, China), OCN (1:100; abcam, Shanghai, China), or β-catenin (1:100; Cell Signaling Technology, Shanghai, China). Cells were incubated with a fluorescence-conjugated secondary antibody (Beyotime, Shanghai, China) for 120 min, and nuclei were stained with 4', 6-diamidino-2-phenylindole (KeyGen Biotech, Nanjing, China) for 3 min. It was observed under a fluorescence microscope (Leica, Germany).

RNA isolation and qPCR

Total cellular RNA was isolated using RNAiso reagent (Takara, Dalian, China) and quantified by measuring the absorbance at 260 nm (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (≤1000 ng) was reverse-transcribed into cDNA in a reaction volume of 20 μL using the a Double-Strand cDNA Synthesis Kit (Takara, Dalian, China). One microliter of cDNA was used as the template for qPCR reaction. All gene transcripts were quantified by qPCR using the Power SYBR® Green PCR Master Mix (Takara) on the ABI StepOnePlus System (Applied Biosystems, Warrington, UK). The mRNA of the target genes and the housekeeping gene (18S) were quantified in separate tubes. All primers were synthesized by Sangon Biotech (Shanghai, China). The primer sequences used are shown in Table 1. The cycle conditions were as follows: 95°C for 30 s and then 40 cycles of 95°C for 5

s and 60°C for 30 s. The relative target gene levels expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were lysed in RIPA lysis buffer (Beyotime) with a proteasome inhibitor (Beyotime). Total proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). After blocking in 5% non-fat milk for 2 h, the membranes were incubated overnight at 4°C with antibodies specific to GAPDH (1:1500; Cell Signaling Technology), collagen I (COL1A1) (1:5000; abcam, USA), active β-catenin (1:1000, Cell Signaling Technology) , RUNX2 (1:1000; Cell Signaling Technology), or β-catenin (1:1000; Cell Signaling Technology). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000; Cell Signaling Technology) was applied as a secondary antibody for 2 h at room temperature. The immunoreactive bands were detected using an enhanced chemiluminescent detection reagent (Millipore). Signal intensity was measured using a Bio-Rad XRS chemiluminescence detection system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (IBM, Armonk, NY, USA). All experiments were performed at least in triplicate, and the data are presented as means ± standard deviations. Statistical significance was determined using a two-tailed Student's *t*-test when comparing two groups, and one-way ANOVA followed by Bonferroni's *post hoc* test when comparing more than two groups. A *P* value of 0.05 or less was considered to reprsent a statistically significant difference.

RESULTS

Effects of DMY on BMSC proliferation

The chemical structure of DMY is shown in Figure 1A. To determine the cytotoxic potential of DMY, its effects on BMSC proliferation were evaluated by CCK-8 detection. No significant cytotoxic effects were observed between groups pretreated with 0.1 or 50 μM DMY and the control group (Figure 1B). However, DMY at 100 μM significantly decreased the proliferation rate of BMSCs (Figure 1B).

DMY enhanced alkaline phosphatase (ALP) activity and calcium deposit formation

ALP activity was increased at day 7 in the presence of 0.1 or 50 μM DMY (Figure 1C).

When BMSCs were treated with DMY (0.1 or 50 μM), significantly more calcium deposits were detected by alizarin red staining (Figures 1D–I).

DMY increased the levels of osteo-specific genes and proteins

To assess the role of DMY in the osteogenic differentiation of BMSCs, levels of osteo-specific genes and proteins, including ALP, RUNX2, osteocalcin (OCN), and COL1A1, were determined by qPCR. qPCR analysis revealed that ALP, RUNX2, OCN, and COL1A1 mRNA levels were significantly higher at day 7 in BMSCs in the presence of DMY (0.1 or 50 μM) than in its absence ($P < 0.05$, Figures 2A–D).

We also used immunofluorescence to confirm the expression of RUNX2 and OCN proteins, and showed that the expression levels of these proteins increased at day 7 in the BMSCs treated with DMY (Figure 3).

DMY activated the Wnt/β-catenin signaling pathway

To confirm the above findings suggesting that Wnt/β-catenin signaling is involved in the observed phenomena, the expression of Wnt 3a, Wnt 5a, β-catenin, DKK1 and SOST was determined by qPCR. Western blot analysis and immunofluorescence were also performed to examine the expression of β-catenin. A higher level of Wnt 3a expression in BMSCs with DMY than without it (Figures 2H). DMY did not affect the expression of Wnt 5a mRNA (Figures 2I). The results of qPCR and Western blot analyses demonstrated lower expression of total β-catenin and active β-catenin in BMSCs without DMY (Figures 2E, J, L). Compared with those in the control group, DKK1 and SOST levels were significantly reduced due to DMY addition (Figures 2F, G). Moreover, using immunofluorescence, we found higher levels of total β-catenin accumulation due to DMY (Figure 4).

The increased osteogenic differentiation of BMSCs due to the presence of DMY was partially rescued by the addition of Wnt/β-catenin signaling inhibitors (DKK1 and total β-catenin siRNA)

To confirm the involvement of the Wnt/β-catenin signaling pathway, we investigated the inhibitory effect of this signaling pathway on the osteogenic differentiation of BMSCs. Here, 0.1μM DMY was added to osteogenic induction medium. After the addition of DKK1 for 1 h, osteo-specific genes and proteins were examined. As shown in Figure 5, lower expression levels of the osteo-specific genes (ALP, RUNX2, OCN, and COL1A1) were identified in the DMY + DKK1-treated cells than in cells treated with DMY alone.

Higher ALP activity was also detected in BMSCs with DMY than in the DMY + DKK1 group (Figure 6A). In addition, there was less matrix mineralization at day 12 of osteogenic differentiation in the DMY + DKK1 group than in the DMY only group (Figures 6B–I).

In addition, after the knockdown of total β-catenin and active β-catenin by the specific siRNA, the markers of osteogenesis and mineral deposition were inhibited due to the presence of DMY (Figure 5, 6).

DISCUSSION

In this study, for the first time, we found that DMY, at a concentration of 0.1 or 50 μM, had a positive effect on osteoblast-specific gene expression and mineral deposition *in vitro*, and did not cause any significant side effects. DMY enhanced β-catenin expression. In addition, it decreased DKK1 and SOST in BMSCs. The Wnt/β-catenin signaling pathway inhibitors, including DKK1 and β-catenin siRNA partly rescued the enhanced matrix mineralization induced by DMY. These findings indicate that DMY promotes the osteogenic differentiation of BMSCs, at least partly through activation of the Wnt/β-catenin signaling pathway.

Mounting evidence has demonstrated that flavonoids, as a class of phytochemicals, have promise in protecting against bone loss [11]. DMY is a major flavonoid component derived from *Ampelopsis grossedentata.* However, at present, its effects on osteogenesis have not been revealed. Thus, we assessed its effects on the osteogenic differentiation of BMSCs. We found that RUNX2 was upregulated due to DMY at concentrations of 0.1 and 50 μM. RUNX2 is a key transcription factor involved in osteogenic differentiation [28]. The expression of early (ALP) and late (OCN, COL1A1, OPN) markers of osteogenic differentiation was also increased due to DMY addition. Furthermore, it significantly enhanced mineral deposition and ALP activity. Meanwhile, DMY had no adverse effects on the proliferation of BMSCs at concentrations of 0.1 and 50 μM. These results indicate that DMY enhances the osteogenic differentiation of BMSCs *in vitro*.

Several previous studies have reported that flavonoids promote the differentiation of BMSCs into the osteogenic lineage through the activation of Wnt/β-catenin signaling [11, 12]. It is well-known that Wnt/β-catenin signaling is an important signaling pathway in osteogenesis [21, 29]. It leads to the cellular accumulation of Wnt/β-catenin, followed by nuclear translocation of β-catenin and the activation of other target genes [30]. Canonical Wnt protein could induce β-catenin translocation from cell-cell contacts into the cytosol and nucleus, thereby activating canonical Wnt/β-catenin signalling [31, 32]. It increases osteogenesis by directly stimulating RUNX2 expression [33]. Noncanonical Wnt pathway (Wnt5a, which signals mainly through the Wnt/calcium) also regulates the osteogenic differentiation of BMSCs [24]. In the present study, we detected higher expression of β-catenin and Wnt 3a following the addition of DMY during osteogenesis. Meanwhile, lower levels of DKK1 and SOST were observed, which inhibited Wnt/β-catenin by combining with LRP5/6 via interference of Wnt/LRP/Fz trimolecular complex formation [22]. These results were similar with previous studies [34-36]. Meike Simann et al. revealed that Heparins promoted the osteogenesis of hMSCs via the activation of Wnt/β-catenin pathway, moreover, the expression of SOST and DKK1 were decreased [34]. Rybchyn et al. also demonstrated that strontium increased mineralization and decreased the expression of SOST [36]. However, González-Sancho JM et al. reported that activation of the Wnt/β-catenin pathway initiated a negative feedback loop in human colon cancer cells, thus increasing the expression of DKK-1 [37]. A possible reason for this issue is that the role of Wnt/β-catenin pathway varies between distinct tissues and according to the pathophysiological status.

In addition, in our study, the increased osteogenesis of BMSCs by DMY overexpression was partly decreased by an inhibitor of Wnt/β-catenin (DKK1). Those results are also similar to previous studies [18, 38]. Likewise, Yun et al. also reported that 4,2',5'-trihydroxy-4'-methoxychalcone, a type of flavonoids, promoted osteogenic differentiation of BMSCs via Wnt/β-catenin signaling pathway and did not influence the

expression of Wnt 5a [18]. These findings indicate that DMY regulates the canonical Wnt/β-catenin signaling pathway during osteogenic differentiation of BMSCs.

There are some limitations to the present study. First, several studies have suggest that Wnt/β-catenin signalling alone is not sufficient to induce osteogenesis, unless other stimulatory signals are present [31, 39]. Although our results indicate that DMY regulates the canonical Wnt/β-catenin signaling pathway to enhance BMSC osteogenesis, the underlying other molecular mechanism remains unclear. Further studies are needed to investigate the regulation of DMY on other signaling pathways. Second, the results obtained *in vitro* are unlikely to be fully representative of the effects *in vivo*; therefore, the translational relevance of these findings should be confirmed *in vivo*.

CONCLUSION

Based on our data, we found that DMY enhances the osteogenic differentiation of BMSCs, partly through activation of the Wnt/β-catenin signaling pathway. DMY may be a promising therapeutic agent for conditions such as osteoporosis and fracture nonunion.

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CONFLICTS OF INTEREST

All authors have contributed significantly and are in agreement with the content of the manuscript. All authors have no relevant financial relationships to disclose.

AUTHOR CONTRIBUTION

ZP and XG contributed design and funding sources to this study. WZ and SW drafted the manuscript. WZ, DX, EC, QZ, and SW did all the *in vitro* parts of the study. QZ and XG carried out statistical work. All authors have contributed significantly. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Effects of dihydromyricetin (DMY) on cytotoxicity and osteoblast differentiation in human bone marrow mesenchymal stem cells (BMSCs) (A) Chemical structure of DMY. (B) The cytotoxic effects of DMY on BMSCs were detected using Cell Counting Kit-8. Data are expressed as the mean ± standard deviation (SD), n = 3. *, $P < 0.05$ vs. BMSCs without DMY. (C) Alkaline phosphatase activity at day 7 during osteogenic differentiation of BMSCs. (D–I) Alizarin red staining at day 12 of osteogenic differentiation. Scale bar = 500 μm. Data are expressed as the mean ± SD, n = 3. * $P < 0.05$ vs. BMSCs treated with osteogenic induction medium alone.

Figure 2. Effects of dihydromyricetin on the levels of osteoblast-specific gene expression and the Wnt/β-catenin signaling pathway during osteogenesis of bone marrow mesenchymal stem cells (BMSCs). mRNA expression of (A) alkaline phosphatase, (B) RUNX2, (C) osteocalcin, (D) COL1A1, (E) β-catenin, (F) DKK1, (G) sclerostin, (H) Wnt 3a, and (I) Wnt 5a was determined by quantitative reverse transcription polymerase chain reaction (qPCR) at day 7 during osteogenic differentiation. (J–L) The expression of COL1A1, RUNX2, active β-catenin (A-βCN) and total β-catenin (T-βCN) protein were determined by Western blot analysis after osteogenic differentiation for 3 days. mRNA expression levels were normalized to 18S ribosomal RNA. Protein expression levels were normalized to GAPDH. Data are expressed as the mean ± standard deviation (SD) of three independent experiments, and one of three independent experiments is shown. Data

are expressed as the mean ± SD, n = 3. * $P < 0.05$ vs. BMSCs treated with osteogenic induction medium alone.

Figure 3. Immunofluorescence staining showing that the protein levels of RUNX2 and osteocalcin are upregulated by the addition of dihydromyricetin (0.1 or 50 μM) at day 7 of osteogenic differentiation. Osteocalcin is stained green. RUNX2 is stained red. Nuclei are stained with 4′, 6-diamidino-2-phenylindole (blue). Scale bar = 200 μm.

Figure 4. Immunofluorescence staining showing that the protein levels of β-catenin are upregulated by dihydromyricetin addition (0.1 or 50 μM) at day 3 of osteogenic differentiation. β-catenin are stained red. Nuclei are stained with DAPI (blue). Scale bar = 50 μm.

Figure 5. Effects of the wnt/β-catenin signaling inhibitors (DKK1 and β-catenin-specific siRNA) on the enhanced expression of an osteoblast-specific gene of bone marrow mesenchymal stem cells by dihydromyricetin (DMY). (A) The expression of ALP, RUNX2, and OCN in control, control+DKK1, DMY (0.1μM), and DMY (0.1μM)+DKK1 groups was determined by quantitative reverse transcription polymerase chain reaction (qPCR). (B) The expression of ALP, RUNX2, and OCN in control, control+siRNA control, control+β-catenin siRNA, DMY (0.1μM), DMY (0.1μM)+siRNA control, and DMY (0.1μM)+β-catenin siRNADKK1 groups was determined by qPCR. (C-E) The expression of COL1A1, RUNX2, active β-catenin (A-βCN), and total β-catenin (T-βCN) were assessed by Western blot analysis. DKK1 (0.5 μg/ml) was applied for 1 h, followed by culture in osteogenic induction medium with DMY for 1 days.Prior to the addition of osteogenic induction medium, the specific siRNA was used to downregulate the expression of β-catenin. (F, G) Before the osteogenic differentiation, Wnt/β-catenin

signaling inhibitors, including DKK1 and siRNA, were applied. the Alkaline phosphatase activity at day 7 during osteogenic differentiation of BMSCs. mRNA expression levels were normalized to 18S ribosomal RNA. Protein expression levels were normalized to GAPDH. Data are expressed as the mean ± standard deviation (SD) of three independent experiments, and one of three independent experiments is shown. Data are expressed as the mean ± SD. * $P < 0.05$ vs. group with osteogenic induction medium alone. $^{\#}$ $P < 0.05$ vs. DMY group.

Figure 6. Effects of Wnt/β-catenin signaling inhibitors (DKK1 and β-catenin siRNA) on the enhanced osteogenesis of bone marrow mesenchymal stem cells (BMSCs) by dihydromyricetin (DMY). Alizarin red staining at day 12 of osteogenic differentiation. Scale bar = 500 μm.

Table 1

Primer sequences for quantitative real-time polymerase chain reaction

Genes	Primer Sequences (5' to 3') (forward/reverse)	Genbank Accession
ALP	GACACGCTGAGCCTCGTCACT CCTGGACCGTTTCCGTATAGG	NM_001632.3
COL1A1	CCTGCTGGCAAGAGTGGTGAT GAAGCCACGGTGACCCTTTATG	NM_000088.3
OCN	GGAGGGCAGCGAGGTAGTGAA	NM_199173.4

	GCCTCCTGAAAGCCGATGTGGT	
Wnt 3a	AGCTACCCGATCTGGTGGTC CAAACTCGATGTCCTCGCTAC	NM_033131
Wnt 5a	ATTCTTGGTGGTCGCTAGGTA CGCCTTCTCCGATGTACTGC	NM_003392
RUNX2	CCTCAGGCATGTCCCTCGGTAT CCTCAGGCATGTCCCTCGGTAT	NM_001024630.3
18S	GACTCAACACGGGAAACCTCAC CCAGACAAATCGCTCCACCAAC	NR_003286

Abbreviations: ALP, alkaline phosphatase; COL1A1, collagen, type I, alpha 1; OCN, osteocalcin; RUNX2, Runt-related transcription factor 2; 18S, 18S ribosomal RNA.