Expression profiles of the Wnt/β-catenin signaling-related extracellular antagonists during proliferation and differentiation in human osteoblast-like cells

ALMA Y. PARRA-TORRES[1], JUANA ENRÍQUEZ[2], ROGELIO F. JIMÉNEZ-ORTEGA[1],
NELLY PATIÑO[3], MANUEL DE JESÚS CASTILLEJOS-LÓPEZ[4], LUZ M. TORRES-ESPÍNDOLA[5],
ERIC G. RAMÍREZ-SALAZAR[6] and RAFAEL VELÁZQUEZ-CRUZ[1]

[1]Genomics of Bone Metabolism Laboratory, National Institute of Genomic Medicine (INMEGEN), Mexico City 14610;
[2]Department of Reproduction Biology Carlos Gual Castro, National Institute of Medical Sciences and Nutrition Salvador
Zubirán (INCMNSZ), Mexico City 14080; [3]Subdirection of Clinical Applications Development, National Institute of
Genomic Medicine (INMEGEN), Mexico City 14610; [4]Epidemiological Surveillance Unit, National Institute of
Respiratory Diseases (INER) 'Ismael Cosío Villegas', Mexico City 14080; [5]Pharmacology Laboratory,
National Institute of Pediatrics (INP), Mexico City 04530; [6]National Council for Science and Technology
(CONACYT)-National Institute of Genomic Medicine (INMEGEN), Mexico City 14610, Mexico

Abstract. Bone formation is a dynamic process directed by osteoblast activity. The transition from the proliferation to differentiation stage during osteoblast maturation involves the downregulation of the Wnt/β-catenin signaling pathway, and extracellular antagonists are important for the regulation of Wnt signaling. However, the expression levels of Wnt antagonists in these stages of human osteoblast maturation have not been fully elucidated. Therefore, the aim of the present study was to investigate the expression levels of extracellular Wnt antagonists during proliferation and differentiation in osteoblast-like cell lines. The results demonstrated an overlap between the differential expression of secreted Frizzled-related protein (*SFPR*)*2*, *SFRP3*, *SFRP4* and Dickkopf *(DKK) 2* genes during the differentiation stage in the MG-63 and Saos-2 cells. Furthermore, high expression levels of *DKK3* in MG-63 cells, Wnt inhibitory factor 1 (*WIF1*) in Saos-2 cells and *DKK4* in hFOB 1.19 cells during the same stage (differentiation), were observed. The upregulated expression levels of Wnt antagonists were also correlated with the high expression of *anxin 2* during the differentiation stage. These findings suggested that Wnt-related antagonists could modulate the Wnt/β-catenin signaling pathway. By contrast, *DKK1* was the only gene that was found to be upregulated during the proliferation stage in hFOB 1.19 and Saos-2 cells. To the best of our knowledge, the present study provides, for the first time, the expression profile of Wnt antagonists during the proliferation stage and the initial phases of differentiation in osteoblast-like cell lines. The current results offer a basis to investigate potential targets for bone-related Wnt-signaling modulation in bone metabolism research.

Introduction

Bone remodeling is a dynamic process orchestrated by bone-forming osteoblasts and bone-resorbing osteoclasts (1). The bone formation process involves the activation of different signaling pathways, which modulate multiple cellular and molecular events during osteoblast proliferation, differentiation and mineralization stages (1). Genetic studies in both humans and mice have revealed that the Wnt/β-catenin signaling pathway is an important mechanism for stimulating osteoblasts function (2).

The Wnt/β-catenin signaling pathway is activated by the binding of Wnt proteins with the Frizzled (Fzd)/low-density lipoprotein receptor-related protein (Lrp) (5/6) receptor complex. The formation of this receptor complex induces the cytoplasmic accumulation of β-catenin molecules and their nuclear translocation for the interaction with the T-cell specific transcription factor (Tcf)/lymphoid-enhancer binding factor (Lef) transcription factors, as well as the induction of

---

expression of target genes, such as *Lef1, Tcf7, NKD inhibitor of WNT signaling pathway 2* and *anxin 2 (AXIN2)* (2,3). In addition, the induction of *AXIN2*, a negative regulator of the signaling pathway, has been proposed to act at the transcriptional level and may create a negative feedback loop to silence the Wnt/β-catenin signaling pathway (4).

Under basal conditions, when the Wnt pathway is inactive, β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3), which forms part of a complex integrated by AXIN2 and the protein of the colon APC regulator of WNT signaling pathway (APC) gene, to be subsequently degraded in the proteasome (5). Therefore, the intracellular levels of β-catenin are kept relatively low. However, when the Wnt pathway is activated by the binding of the Wnt ligands to the Fxd/Lrp5/6 receptor complex, the decomposition of the intracellular AXIN2-APC-GSK3 complex is activated, which results in the inhibition of the phosphorylation of β-catenin (5). The hypophosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it regulates gene expression via the activation of various transcription factors, such as Tcf/Lef1 (6). The Wnt/β-catenin signaling pathway is also regulated by several different extracellular antagonists, such as the family of secreted Fzd-related proteins 1-4 (SFRPs 1-4), the four members of the Dickkopf family (DKK 1-4) and the inhibitor Wnt inhibitory factor 1 (WIF1) (7). These molecules can act as decoys to compete with the Wnt ligands to bind with the receptors; for example, some SFRPs are secreted into the extracellular medium. Furthermore, members within the DKK protein family, in which type 1 (DKK-1) is particularly important in bone, can antagonize Wnt signals by binding with the Lrp5/6 co-receptors (4).

The roles of the Wnt signaling-related antagonists and their effect on bone metabolism and osteoblasts activities have not yet been fully elucidated. However, previous research suggests that they are involved in the regulation of osteoblast functions. For instance, studies in mice have reported that deletion of *SFRP1* or elimination of one allele of *DKK1*, stimulates osteoblast proliferation and bone mass formation via the activation of the Wnt signaling pathway (8,9). Another study revealed that administration of recombinant SFRP2 or SFRP4 proteins enhanced the alkaline phosphatase (ALP) activity in mouse mesenchymal C3H10T1/2 cells, suggesting a role for SFRPs in osteoblastogenesis (9). Moreover, the overexpression of *WIF1* in murine embryonic mesenchymal cells, inhibits osteoblast differentiation (10), and knockdown of *DKK1* and *DKK2* decreases matrix mineralization in KS483 mesenchymal stem cells (11). Similarly, clinical studies have observed an association between genetic variants in *SFRP1* and *SFRP4* and bone mass content in postmenopausal women (12,13). Taken together, these findings indicate that the Wnt signaling-related extracellular antagonists influence the osteoblast maturation process and bone formation, and that they can serve as potential targets to prevent the loss of bone mass. However, the expression levels of Wnt antagonists during the human osteoblast proliferation and differentiation stages remain unknown.

The aim of the present study was to investigate the dynamics of gene expression of extracellular antagonists, *SFRP 1-4, DKK 1-4* and *WIF1,* during the proliferation and cell-differentiation stages of osteoblasts maturation. The hFOB 1.19 normal osteoblasts and the MG-63 and Saos-2 osteosarcoma cell lines were used as a model system. ALP activity and the expression levels of osterix (*OSX*) and RUNX family transcription factor 2 (*RUNX2*), which are markers of the early stage of osteoblast differentiation, were measured (14). In addition, *AXIN2* expression was investigated to determine the activation status of the Wnt/β-catenin signaling pathway during the transition of osteoblasts, from proliferation to differentiation stage.

Materials and methods

Cell culture. The hFOB 1.19 (cat. no. CRL-11372), MG-63 (cat. no. CRL-1427) and Saos-2 (cat. no. HTB-85) cell lines were purchased from the American Type Culture Collection. hFOB 1.19 cells were maintained in DMEM/F-12 culture medium without phenol red (Sigma-Aldrich; Merck KGaA) and supplemented with 10% FBS (Biowest) and 0.3 mg/ml G418 (Sigma-Aldrich; Merck KGaA) at 37˚C and 5% CO_2. MG-63 and Saos-2 cells were maintained in EMEM and McCoy's 5A (both Sigma-Aldrich; Merck KGaA), respectively, supplemented with 10% FBS (Biowest) and an antibiotic solution (penicillin and streptomycin, both 100 mg/ml; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2.

Cell viability and ALP activity assays. Cell viability and ALP activity were assessed in the hFOB 1.19, MG-63 and Saos-2 cell lines at 1, 3, 8, 15 and 21 days of cell culture. Cells were plated at a density of $70x10^4$ cells/dish in their respective medium and cultured at 37˚C in a humidified incubator with 5% CO_2 for the aforementioned time points. The culture media were removed and replaced with fresh medium every other day. The media were replaced with osteogenic media (100 μg/ml ascorbic acid and 5 mM β-glycerol phosphate) to maintain the osteoblast phenotype from day 8 onwards. Previous studies have reported that the decline in viability, which occurs after 8 days of cell culture, is essential to stimulate the osteoblast differentiation-related activities (15). Subsequently, the medium for hFOB 1.19 cells was replaced with culture medium supplemented with 0.01 μM menadione, 100 μg/ml ascorbic acid and 5 mM β-glycerol phosphate (16,17) (all Sigma-Aldrich; Merck KGaA). The medium for the MG-63 and Saos-2 cell lines was replaced by their respective medium supplemented with 100 μg/ml ascorbic acid and 5 mM β-glycerol phosphate (all Sigma-Aldrich; Merck KGaA).

In order to test the cell viability, the cells were harvested at the indicated times using a 0.05% trypsin/EDTA solution (GIBCO) for 2-3 min at 37˚C to detach the cells from the adherent substrate, the cell suspension was washed twice and resuspended in 1 ml of cold phosphate buffer saline (PBS) (4˚C). One part of the cell suspension was mixed with one part of 0.4% trypan blue, incubated for 3 min at room temperature and then visually examined to determine whether cells take up or exclude the dye, for direct identification and enumeration of live (unstained) and dead (blue) cells in a given population. and counted on a light inverted microscope using the 20x objective. The experiments were performed in triplicate and the cell viability was expressed as the cell number.

To determine the ALP activity, at the aforementioned time points, the cells were lysed with a solution containing 0.1 M Tris-HCl and 0.1% Tween-20 (pH 7.5) for 2 min on ice.

Cell lysates were freeze-thawed (-70°C/ice) twice and enzyme activity was determined using the Lowry method (18) using *p*-nitrophenyl phosphate (Sigma-Aldrich; Merck KGaA) as the substrate. Protein concentration was determined using the Bradford method, using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.) with dilution from 0.2 to 0.9 mg/ml of BSA for the standard curve, following the manufacturer's protocol. All the experiments were performed three times in triplicate and results are expressed as enzymatic activity U/mg protein/min.

Gene expression studies. At the end of each incubation time point (1, 3, 8, 15 and 21 days of cell culture), the expression levels of *RUNX2*, *OSX* and *AXIN2,* as well as those of the extracellular antagonists were determined using reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's instructions. cDNA was transcribed from 1 µg total RNA using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions under the following conditions: 5 min at 65°C, 2 min at 4°C, 30 min at 37°C, and 5 min, 95°C. qPCR was performed using the following TaqMan gene expression assays (Applied Biosystems; Thermo Fisher Scientific, Inc.): *RUNX2* (assay ID, Hs00231692_m1), *OSX* (assay ID, Hs01866874_s1) *SFRP1* (assay ID, Hs00610060_m1), *SFRP2* (assay ID, Hs00293258_m1), *SFRP3* (assay ID, Hs0017350_m1), *SFRP4* (assay ID, Hs00180066_m1), *DKK1* (assay ID, Hs00183740_m1), *DKK2* (assay ID, Hs00205294_m1), *DKK3* (assay ID, Hs00183740_m1), *DKK4* (assay ID, Hs00205290_m1), *WIF1* (assay ID, Hs00183662_m1) and *AXIN2* (assay ID, Hs00610344_m1) under universal cycling conditions (10 min at 95°C; 15 sec at 95°C, 1 min 60°C, 40 cycles). PCR amplification was performed in triplicate using a QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Gene expression was normalized using *GAPDH*, and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Statistical analysis. Normal distribution was analyzed using the Shapiro-Wilk test and homogeneity of variance was determined using the Levene's test. Statistical differences were examined using a one-way ANOVA followed by Tukey's post hoc test. Correlations were evaluated using the Spearman correlation coefficient (r_s). $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS v20.0 software (IBM Corp). All assays were performed in triplicate.

Results

Cell viability and differentiation. To distinguish between the osteoblast proliferation and differentiation stages, the viability of the hFOB 1.19, MG-63 and Saos-2 osteoblastic cell lines during 21 days of cell culture was determined. The three cell lines exhibited distinct viability rates (Fig. 1A; Tables SI and SII). As expected, the Saos-2 cell line exhibited the highest viability rate (25.4-fold), followed by MG-63 (17.5-fold), while the hFOB 1.19 cell line had the lowest rate

(13.5-fold). After day 8, the densities of the three osteoblastic cell lines increased significantly as compared with the respective day 1, in which the hFOB 1.19 and MG-63 cells reached saturation densities after 15 days of culture. It was observed that the cellular proliferation between day 15 and 21 was not significant.

To identify the osteoblast differentiation stage, ALP activity and the expression levels of the transcription factors *RUNX2* and *OSX*, which are three well-known markers of osteoblast differentiation (20,21), were assessed during 21 days of cell culture. The ALP activity at the different time points is presented in Fig. 1B. It was found that the levels of ALP activity in the hFOB1.19, MG-63 and Saos-2 cell lines were correlated with their corresponding cells in terms of viability (Tables SI and SII). The highest level of ALP activity, expressed as units of enzyme activity, was found in the Saos-2 cell line since early days of culture and was maintained until day 21. Notably, the ALP activity level was similar in the hFOB 1.19 and MG-63 cell lines during 21 days of culture, in which the enzymatic activity increased in a time-dependent manner, reaching maximal levels at day 15 of cell culture compared to the respective day 1. However, the enzyme activity in Saos-2 cells was much higher compared with that other two cell lines, which had lower viability (Fig. 1B).

The expression levels of *RUNX2* and *OSX* in the hFOB 1.19, MG-63 and Saos-2 cells at days 1, 3, 8, 15 and 21 of cell culture are illustrated in Fig. 1C and D, respectively. The expression profiles of *RUNX2* in MG-63 and Saos-2 cells demonstrated a strong positive correlation between both cell lines, and the expression levels increased significantly in a time-dependent manner during the 21 days of culture, when compared with the day 1 of culture. By contrast, there was no significant increase in the expression levels of *RUNX2* during the same days of culture in the hFOB 1.19 cell line (Fig. 1C). With respect to *OSX*, similar expression profiles were observed in the three cell lines (Table SIII).

High expression levels of *OSX* were observed at day 15 and reached maximum levels at day 21 in the three osteoblastic cell lines (Fig. 1D). These results indicated that the differentiation stage in osteoblast-like cell lines can be established from day 15 under the conditions used in the present study. The potential relationship existing between osteoblast markers is presented in Table SI.

Gene expression levels of Wnt-related antagonists
SFRPs genes. The expression levels of *SFRP1*, *SFRP2*, *SFRP3* and *SFRP4* in the hFOB 1.19, MG-63 and Saos-2 cell lines, during 21 days of cell culture are shown in Fig. 2. The expression levels of *SFRP1* and *SFRP3* were increased from day 3 until day 21 in the hFOB 1.19 cell line, compared with those on day 1. By contrast, the expression levels of *SFRP2* and *SFRP4* genes decreased from day 3 until day 21, compared with those on day 1 (Fig. 2A).

In the MG-63 cell line, there was an increase in the expression of *SFRP1* from day 3 until day 21, while high expression levels of *SFRP2*, *SFRP3* and *SFRP4* were detected after day 15 of cell culture, reaching maximum levels at day 21 (Fig. 2B). The Saos-2 cell line had higher expression levels of *SFRP2*, *SFRP3* and *SFRP*4 compared with those in the hFOB 1.19 and MG-63 cell lines (Fig. 2C). These high levels were detected
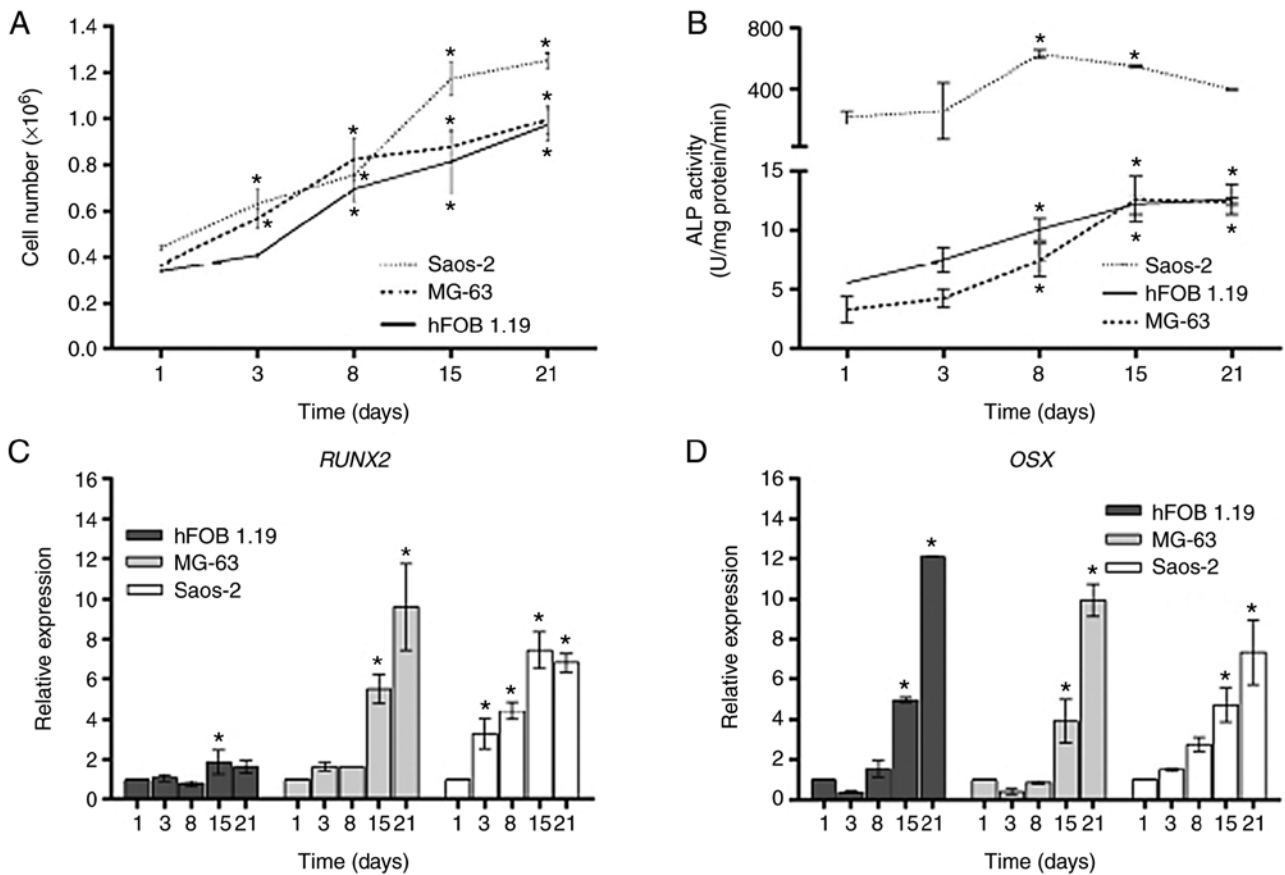
Figure 1. Profiles of osteoblast markers of proliferation (evaluated as cell viability) and differentiation in normal hFOB 1.19 osteoblasts and MG-63 and Saos-2 osteosarcoma cell lines. (A) Cell number, (B) ALP activity and the expression levels of (C) *RUNX2* and (D) *OSX* were determined at different time points during 21 days of cell culture. Data are presented as the mean ± SD of three experiments, in triplicate. *P<0.001 vs. respective day 1 of culture. *RUNX2*, RUNX family transcription factor 2; *OSX*, osterix; ALP, alkaline phosphatase.

from day 15 and the maximum levels were detected at day 21. Notably, there were no significant differences in the expression levels of *SFRP1* during the 21 days of culture of Saos-2 cells (Fig. 2C).

DKK genes. The expression levels of *DKK1*, *DKK2*, *DKK3* and *DKK4* in the hFOB 1.19, MG-63 and Saos-2 cell lines at days 1, 3, 8, 15 and 21 of cell culture are presented in Fig. 3. The hFOB 1.19 cell line demonstrated an increase in the expression of *DKK1* after 3 days of culture, after which the expression decreased to levels below the basal line to day 21. The expression of *DKK4* decreased at day 8 of cell culture compared with that on day 1. At day 15, there was a statistically significant increase in the expression of *DKK4*. With respect to *DKK2*, there was a decrease in its expression level during the 21 days of culture compared with that on day 1. There were no significant changes in *DKK3* expression during the 21 days of cell culture (Fig. 3A).

In the MG-63 cell line, the expression levels of *DKK2* and *DKK3* increased in a time-dependent manner, reaching maximum levels at day 21 of cell culture. On the other hand, the expression levels of *DKK1* and *DKK4* were decreased below their basal levels during the 21 days of cell culture, as compared with day 1 (Fig. 3B). By contrast, there was high expression of *DKK1* on day 3 in the Saos-2 cell line, which gradually decreased to levels below its basal level at day 21

of culture. Furthermore, the expression of *DKK2* increased, reaching maximum levels on day 21, and there were no differences in the expression levels of *DKK3* and *DKK4* during 21 days of cell culture. The expression of *DKK4* was not observed on day 21 (Fig. 3C).

WIF1 gene. *WIF1* is an important negative regulator factor of the Wnt/β-catenin signaling pathway, and is structurally different from the SFRP and DKK families (22). WIF1 inhibits the activity of the Wnt signaling pathway by directly binding to Wnt proteins (22). Moreover, it is well-known that WIF1 can act as a tumor suppressor and its downregulation is associated with the development of various types of cancer (23). The expression of *WIF1* in the hFOB 1.19, MG-63 and Saos-2 cell lines during the 21 days of culture is presented in Fig. 4. There was an increment on day 3, which remained constant until day 21 in the hFOB 1.19 cell line, while in Saos-2 cells, higher levels of *WIF1* were observed from day 15 and reached maximum levels on day 21 when compared with day 1. By contrast, there were no changes in the expression of *WIF1* in the MG-63 cell line.

AXIN2 gene. AXIN2 is a negative intracellular regulator of the Wnt signaling pathway, which forms a complex with APC and GSK3, and results in the inhibition of the phosphorylation of β-catenin (5). The expression of *AXIN2* in the hFOB 1.19,
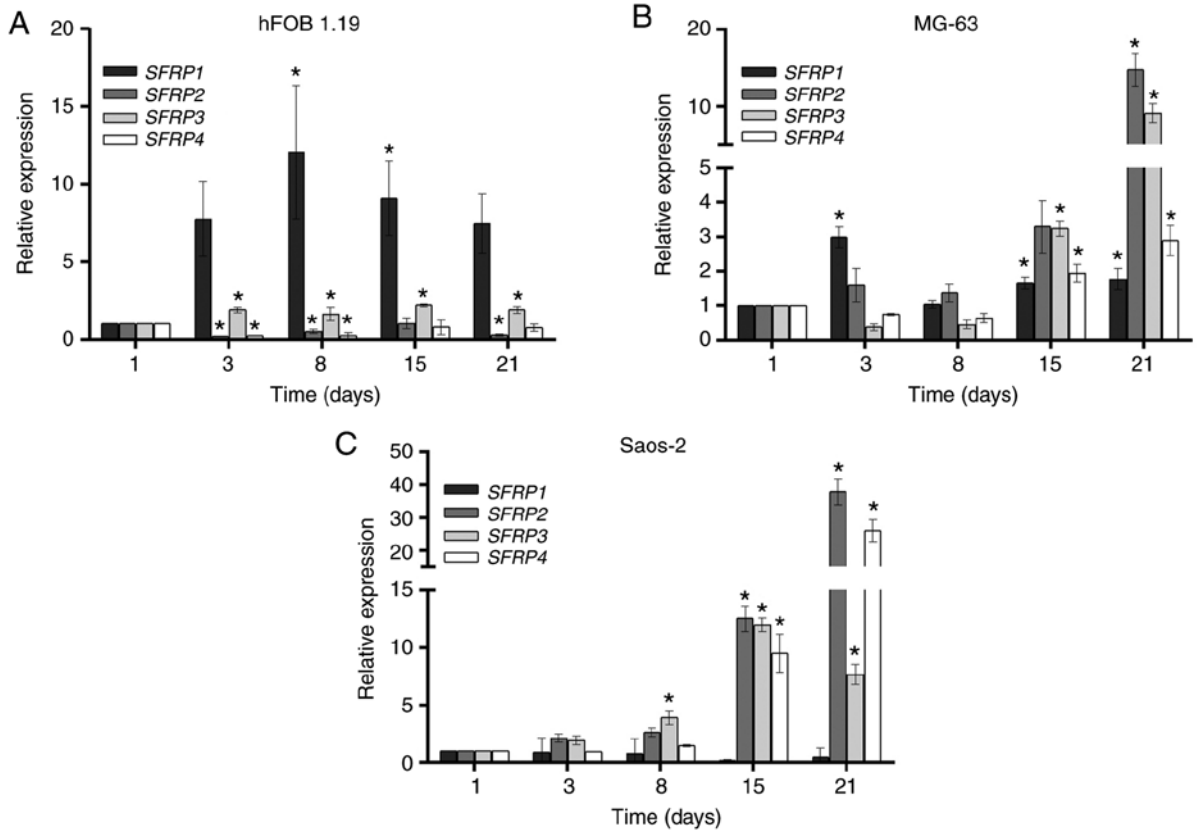
Figure 2. Expression profiles of *SFRP1*, *SFRP2*, *SFRP3* and *SFRP4* in the three osteoblast-like cell lines. (A) hFOB 1.19, (B) MG-63 and (C) Saos-2 cell lines were cultured for 21 days. Data are presented as the mean ± SD of three experiments, in triplicate. *P<0.05 vs. cells incubated at day 1. *SFRP*, secreted Frizzled-related protein.
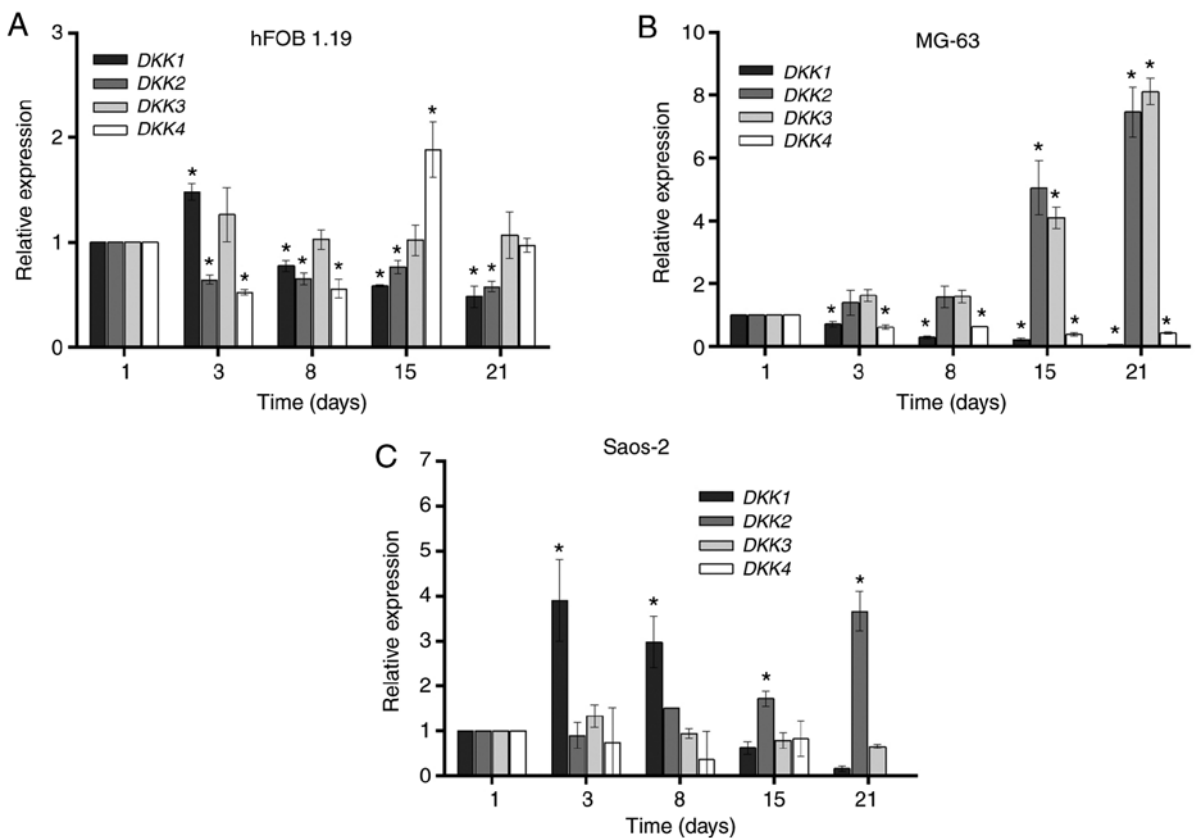

Figure 3. Expression profiles of *DKK1*, *DKK2*, *DKK3* and *DKK4* in three osteoblastic cell lines. Expression profiles in (A) hFOB 1.19, (B) MG-63 and (C) Saos-2 cell lines. Data are presented as the mean ± SD of three experiments, in triplicate. *P<0.001 vs. respective day 1 of culture. *DKK*, Dickkopf.
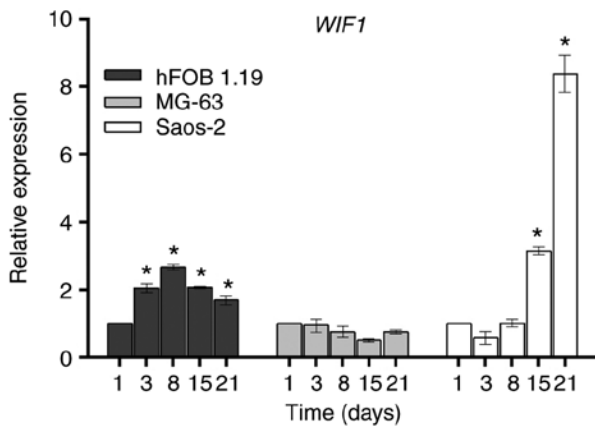
Figure 4. Expression of *WIF1* in hFOB 1.19, MG-63 and Saos-2 cell lines during 21 days of cell culture. Data are presented as the mean ± SD of three experiments, in triplicate. *P<0.05 vs. cells incubated at day 1. *WIF1*, Wnt inhibitory factor 1.
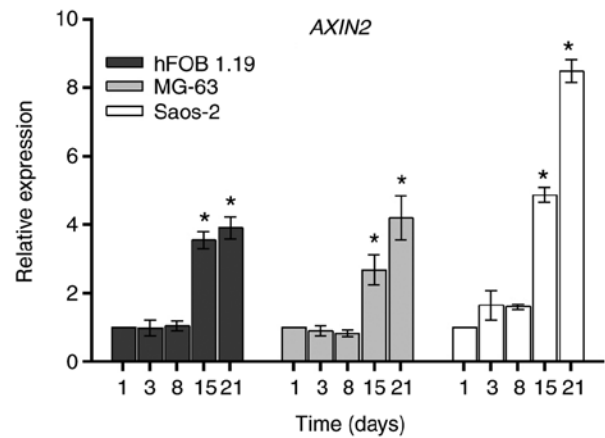
Figure 5. Expression of *AXIN2* in the hFOB 1.19, MG-63 and Saos-2 cell lines during 21 days of cell culture. Data are presented as the mean ± SD of three experiments in triplicate. *P<0.001 vs. cells incubated at day 1. *AXIN2*, axin 2.

MG-63 and Saos-2 cell lines during days 1, 3, 8, 15 and 21 of cell culture is illustrated in Fig. 5. The three cell lines had a similar expression profile of AXIN2 (Table SIII). The results identified that *AXIN2* was constantly expressed during the proliferative stage and there was a significant increase on day 15, which reached maximum levels on day 21 of culture in all three osteoblastic cell lines, as compared with the respective day 1. The relationship between *AXIN2* and osteoblastic markers is presented in Tables SI and SII. A positive correlation was observed between the *AXIN2* expression profile and cell markers of osteoblast proliferation (cell viability) and differentiation stages in hFOB 1.19, MG-63 and Saos-2 cell lines. On the other hand, a strong correlation between the mean of *AXIN2* gene expression and differentially expressed extracellular Wnt antagonists during differentiation stage in the three cell lines was observed (Tables SIV-SVI).

Discussion

The results from the present study provide evidence of the differential expression of certain bone-related Wnt antagonists, during the proliferation (evaluated as cell viability) or differentiation stages in human osteoblast-like cells. The roles of these Wnt antagonists in the control of Wnt signaling have been only partially described, which is due to the complexity of their functions and the manner in which they have been investigated (4).

In the present study, to evaluate the dynamics of gene expression of the Wnt antagonists in the human osteoblasts, the transition from proliferation to differentiation stage was firstly determined in the hFOB 1.19, MG-63 and Saos-2 cells lines. The differentiation stage was defined by high confluency in cell culture, increased ALP activity and high expression levels of *RUNX2* and *OSX* (20,21). The results demonstrated that arrest of cellular proliferation, judged by cell confluency at day 15 of cell culture, was associated with high levels of ALP activity and the expression of *RUNX2* and *OSX*. The present results support the findings observed in previous studies by Stein *et al* (24) and Owen *et al* (25), in which high mRNA and protein expression levels of *ALP*, as

well as no detectable expression of *RUNX2* and *OSX*, were identified before day 12 of cell culture in rodent osteoblasts. Furthermore, in another study, high levels of ALP activity and osteocalcin, a differentiation marker, were observed at day 15 of cell culture in a neonatal rat calvarial osteoblasts model (26). Based on the present results, it was suggested that day 15 of cell culture was the beginning of the differentiation stage in the three osteoblast-like cell lines. To determine the expression levels of Wnt antagonists during the early differentiation stage, the present study was limited to days 15-21 of cell culture, when ALP reached maximum levels of activity. Previous studies have reported a significant decrease in enzyme activity when cell cultures progress into the mineralization stage (after day 25 of cell culture) (24,25). However, the association between the Wnt-pathway antagonists and the mineralization process was beyond the scope of the present research.

To characterize the expression profiles of the Wnt antagonists in the three osteoblast-like cell lines during the proliferation and differentiation stages, the cells were cultured for 21 days. Distinctive expression patterns were identified during both the proliferation and differentiation stages for each cell line. The differential expression patterns of Wnt antagonists suggested there was a possible balance between the temporal and spatial expression of Wnt-pathway antagonists during the progression of proliferative stage towards the differentiation of the osteoblasts (27,28). The present results demonstrated there was an overlap between *SFPR2*, *SFRP3*, *SFRP4* and *DKK2* gene expression levels in MG-63 and Saos-2 cell lines. The analysis of their expression patterns identified high levels on days 15 and 21 of cell culture, suggesting that these antagonists were upregulated during the differentiation stage in osteosarcoma cell lines. In addition, high expression of *DKK3* in MG-63, but not in Saos-2 cells, was observed during the differentiation stage. On the other hand, in the hFOB1.19 cells, high expression of *DKK4* was found during the differentiation stage (day 15). Several studies in murine osteoblasts cells have reported that the expression levels of *SFRP2*, *SFRP4* (9,29) and *SFRP3*, activated by the β-catenin-independent pathway (30), can promote osteoblast
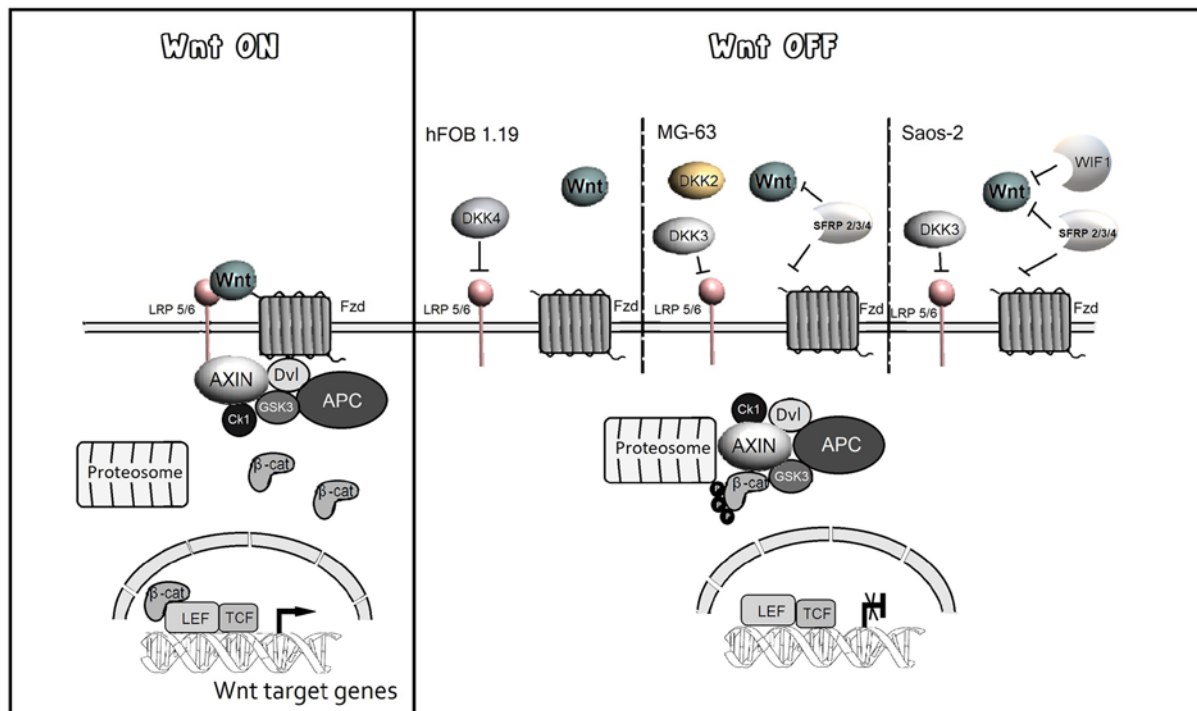
Figure 6. Schematic diagram of the effects of extracellular antagonists in the Wnt/β-cat signaling pathway during the proliferation and differentiation in human osteoblast. Activation of the Wnt signaling induces the expression of genes that promote cell proliferation. In the transition to differentiation, the expression of extracellular antagonists decreases Wnt signaling and promotes the expression of differentiation markers. During the differentiation, the upregulation of *SFRP2*, *SFRP3*, *SFRP4* and *DKK2* is observed in the Saos-2 and MG-63 cell lines. *DKK3* and *WIF1* are upregulated in MG-63 and Saos-2 respectively, while *DKK4* is uniquely upregulated in the hFOB1.19 cell line. *AXIN2*, anxin 2; *WIF1*, Wnt inhibitory factor 1; *DKK*, Dickkopf; *SFRP*, secreted Frizzled-related protein; *Fzd*, Frizzled; LRP, Low-density lipoprotein receptor-related protein; GSK3, glycogen synthase kinase 3; CK1, casein kinase 1; β-cat, β-catenin; Tcf, T-cell specific transcription factor; Lef, lymphoid-enhancer binding factor; Dvl, Dishevelled.

differentiation by decreasing cell proliferation and inducing ALP activity. It has also been revealed that increased expression levels of *DKK2* (31) or *WIF1* (32) in murine osteoblasts and Saos-2 cells, promote *in vitro* mineralization. Moreover, *DKK3,* in Saos-2 and mesenchymal cells (33,34) and *DKK4* in MC3T3-E1 cells (35), can increase cell proliferation and decrease or inhibit osteogenic differentiation.

WIF1 is a negative regulator, which acts upstream of the Wnt signaling pathway, and can inhibit the activation of the pathway by directly binding with the Wnt signaling proteins (22). WIF1 has also been found to act as a tumor suppressor protein (23). The current expression analysis of *WIF1* identified that the MG-63 and Saos-2 cell lines exhibited low levels during the proliferative stage, and these were constantly low during the differentiation stage in the MG-63 cells, but not in Saos-2 cells. In the Saos-2 cell line, *WIF1* was upregulated in a time-dependent manner from days 15 to 21. In normal hFOB1.19 cells, the expression of *WIF1* was constant in both the proliferation and differentiation stages, but its levels were higher compared with those in MG-63 cells. These findings suggested that the activation of the Wnt/β-catenin signaling pathway may be associated with cell proliferation. A recent study reported an association between the decreased mRNA and protein expression levels of *WIF1* and the increased levels of β-catenin and cyclin D1 expression in tumor tissues, compared with that in healthy tissues (36).

AXIN2 is a known intracellular negative regulator of the Wnt/β-catenin signaling pathway, which acts by preventing spontaneous signal transduction in the absence of a Wnt signal (5). At the same time, AXIN2 expression is repressed by the activation of the Wnt/β-catenin signaling pathway, which creates a negative feedback loop between the two (6). Several studies have revealed that high expression of *AXIN2* is associated with the inhibition of the Wnt/β-catenin signaling pathway (6,37). The results from the present study demonstrated that high mRNA expression levels of *AXIN2* during differentiation stage was associated with the overexpression of the Wnt antagonists. The expression levels of *SFRP2*, *SFRP3*, *SFRP4* and *DKK2* were upregulated in the Saos-2 and MG-63 cell lines, *DKK3* was upregulated in the MG-63 cell line and *WIF1* was upregulated in the Saos-2 cell line, while *DKK4* was upregulated only in the hFOB 1.19 cell line. These results indicated that extracellular and intracellular antagonists could modulate the Wnt/β-catenin signaling pathway to decrease cell proliferation and promote osteoblast differentiation (37). However, additional studies are required to further assess this hypothesis.

The number of upregulated Wnt antagonists during cell proliferation is limited (17). The present study identified that *DKK1* was the only gene that had a high expression in the proliferation stage in the hFOB 1.19 and Saos-2 cell lines. However, the underlying mechanism of this high expression is currently unknown. A previous study revealed that overexpression of *DKK1* in the MG-63 and Saos-2 cell lines decreased the lag time prior to rapid exponential growth during cell proliferation (38).

The present results suggested that majority of the Wnt antagonists were downregulated during proliferation and/or differentiation stages; for instance, *SFRP2*, *SFRP4*, *DKK1* and *DKK2* in the hFOB 1.19 osteoblasts, *DKK1* and *DKK4* in the MG-63 cells and *SFRP1* and *DKK1* in the Saos-2 cells. The mechanism underlying the observed gene repression of the Wnt antagonist was not determined; however, recent studies have proposed a role for the small non-coding RNAs in the control of these genes (39,40). For example, overexpression of microRNA(miR)-29 modulates the intracellular mRNAs expression levels of *DKK1* and *SFRP2*, thus promoting the differentiation of human osteoblasts (17). Furthermore, it has been shown that the high expression of miR-940 activates the Wnt/β-catenin signaling pathway by downregulating *SFRP1* in human osteosarcoma tissue (41). Thus, the expression profile of these small non-coding RNAs in the progression of proliferation and differentiation in osteoblasts and osteosarcoma cell lines requires further research.

To the best of our knowledge, the current study provides for the first time, the distinctive and characteristic expression patterns of several Wnt antagonists during the proliferation and differentiation stages of human osteoblast cell lines (Fig. 6). However, the association between the gene expression levels and the protein levels, as well as their functional roles during both stages are still require further investigation. Moreover, other technologies, such as next-generation sequencing (seq), including RNA-seq and small-RNAseq, could be used to analyze additional aspects of RNA biology to identify the changes in the expression levels of the Wnt antagonists.

In conclusion, the present results provide novel insights into the expression levels of Wnt antagonists during proliferation and differentiation stages in human osteoblast-like cell lines. In addition, the results offer a basis to evaluate novel potential targets for bone-related Wnt-signaling modulation and provide an additional area of research into Wnt-signaling in bone metabolism.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AYPT and RVC conceived and designed the study. JE, EGRS, RFJO and NP carried out the experiments, acquisition of data, analysis and interpretation of data. LMTE and MDJCL contributed to the statistical analysis and interpretation of data. AYPT, JE, EGRS, MDJCL and RVC drafted, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

Miss Alma Y. Parra-Torres is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 421295 from CONACYT.

References

1. Raggatt LJ and Partridge NC: Cellular and molecular mechanisms of bone remodeling. J Biol Chem 285: 25103-25108, 2010.
2. Karner CM and Long F: Wnt signaling and cellular metabolism in osteoblasts. Cell Mol Life Sci 74: 1649-1657, 2017.
3. Kobayashi Y, Uehara S, Udagawa N and Takahashi N: Regulation of bone metabolism by Wnt signals. J Biochem 159: 387-392, 2016.
4. Boudin E, Fijalkowski I, Piters E and Van Hul W: The role of extracellular modulators of canonical Wnt signaling in bone metabolism and diseases. Semin Arthritis Rheum 43: 220-240, 2013.
5. Nusse R and Clevers H: Wnt/β-catenin signaling, disease, and emerging therapeutic modalities. Cell 169: 985-999, 2017.
6. Jho E, Zhang T, Domon C, Joo CK, Freund JN and Costantini F: Wnt/beta-catenin/Tcf signaling induces the transcription of axin2, a negative regulator of the signaling pathway. Mol Cell Biol 22: 1172-1183, 2002.
7. Bodine PVN, Zhao W, Kharode YP, Bex FJ, Lambert AJ, Goad MB, Gaur T, Stein GS, Lian JB and Komm BS: The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. Mol Endocrinol 18: 1222-1237, 2004.
8. Morvan F, Boulukos K, Clément-Lacroix P, Roman SR, Suc-Royer I, Vayssière B, Ammann P, Martin P, Pinho S, Pognonec P, *et al*: Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21: 934-945, 2006.
9. Cho SW, Her SJ, Sun HJ, Choi OK, Yang JY, Kim SW, Kim SY and Shin CS: Differential effects of secreted frizzled-related proteins (sFRPs) on osteoblastic differentiation of mouse mesenchymal cells and apoptosis of osteoblasts. Biochem Biophys Res Commun 367: 399-405, 2008.
10. Cho SW, Yang JY, Sun HJ, Jung JY, Her SJ, Cho HY, Choi HJ, Kim SW, Kim SY and Shin CS: Wnt inhibitory factor (WIF)-1 inhibits osteoblastic differentiation in mouse embryonic mesenchymal cells. Bone 44: 1069-1077, 2009.
11. van der Horst G, van der Werf SM, Farih-Sips H, van Bezooijen RL, Löwik CWGM and Karperien M: Downregulation of Wnt signaling by increased expression of Dickkopf-1 and -2 is a prerequisite for late-stage osteoblast differentiation of KS483 cells. J Bone Miner Res 20: 1867-1877, 2005.
12. Sims AM, Shephard N, Carter K, Doan T, Dowling A, Duncan EL, Eisman J, Jones G, Nicholson G, Prince R, *et al*: Genetic analyses in a sample of individuals with high or low BMD shows association with multiple Wnt pathway genes. J Bone Miner Res 23: 499-506, 2008.

13. Fujita M, Urano T, Shiraki M, Momoeda M, Tsutsumi, O, Hosoi T, Clinic K, Orimo H, Ouchi Y and Inoue S: Association of a single nucleotide polymorphism in the secreted frizzled-related protein 4 (sFRP4) gene with bone mineral density. Geriatrics Gerontol Int 4: 175-180, 2004.

14. Maehata Y, Takamizawa S, Ozawa S, Kato Y, Sato S, Kubota E and Hata RI: Both direct and collagen-mediated signals are required for active vitamin D3-elicited differentiation of human osteoblastic cells: Roles of osterix, an osteoblast-related transcription factor. Matrix Biol 25: 47-58, 2006.

15. Coelho MJ and Fernandes MH: Human bone cell cultures in biocompatibility testing. Part II: Effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. Biomaterials 21: 1095-1102, 2000.

16. Harris SA, Enger RJ, Riggs LB and Spelsberg TC: Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 10: 178-186, 1995.

17. Kapinas K, Kessler C, Ricks T, Gronowicz G and Delany AM: miR-29 modulates wnt signaling in human osteoblasts through a positive feedback loop. J Biol Chem 285: 25221-25231, 2010.

18. Waterborg JH and Matthews HR: The lowry method for protein quantitation. Methods Mol Biol 1: 1-3, 1984.

19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

20. Hoemann CD, El-Gabalawy H and McKee MD: In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization. Pathol Biol (Paris) 57: 318-323, 2009.

21. Landim de Barros T, Brito VGB, do Amaral CCF, Chaves-Neto AH, Campanelli AP and Oliveira SHP: Osteogenic markers are reduced in bone-marrow mesenchymal cells and femoral bone of young spontaneously hypertensive rats. Life Sci 146: 174-183, 2016.

22. Kerekes K, Bányai L, Trexler M and Patthy L: Structure, function and disease relevance of Wnt inhibitory factor 1, a secreted protein controlling the Wnt and hedgehog pathways. Growth Factors 37: 29-52, 2019.

23. Rubin EM, Guo Y, Tu K, Xie J, Zi X and Hoang BH: Wnt inhibitory factor 1 decreases tumorigenesis and metastasis in osteosarcoma. Mol Cancer Ther 9: 731-741, 2010.

24. Stein GS, Lian JB and Owen TA: Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4: 3111-3123, 1990.

25. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB and Stein GS: Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143: 420-430, 1990.

26. Enríquez J, Lemus AE, Chimal-Monroy J, Arzate H, García GA, Herrero B, Larrea F and Pérez-Palacios G: The effects of synthetic 19-norprogestins on osteoblastic cell function are mediated by their non-phenolic reduced metabolites. J Endocrinol 193: 493-504, 2007.

27. Vaes BL, Dechering KJ, van Someren EP, Hendriks JM, van de Ven CJ, Feijen A, Mummery CL, Reinders MJ, Olijve W, van Zoelen EJ and Steegenga WT: Microarray analysis reveals expression regulation of Wnt antagonists in differentiating osteoblasts. Bone 36: 803-811, 2005.

28. Eijken M, Meijer IMJ, Westbroek I, Koedam M, Chiba H, Uitterlinden AG, Pols HAP and van Leeuwen JPTM: Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner. J Cell Biochem 104: 568-579, 2008.

29. von Marschall Z and Fisher LW: Secreted frizzled-related protein-2 (sFRP2) augments canonical Wnt3a-induced signaling. Biochem Biophys Res Commun 400: 299-304, 2010.

30. Chung YS, Baylink DJ, Srivastava AK, Amaar Y, Tapia B, Kasukawa Y and Mohan S: Effects of secreted frizzled-related protein 3 on osteoblasts in vitro. J Bone Miner Res 19: 1395-1402, 2004.

31. Li X, Liu P, Liu W, Maye P, Zhang J, Zhang Y, Hurley M, Guo C, Boskey A, Sun L, et al: Dkk2 has a role in terminal osteoblast differentiation and mineralized matrix formation. Nat Genet 37: 945-952, 2005.

32. Baker EK, Taylor S, Gupte A, Chalk AM, Bhattacharya S, Green AC, Martin TJ, Strbenac D, Robinson MD, Purton LE and Walkley CR: Wnt inhibitory factor 1 (WIF1) is a marker of osteoblastic differentiation stage and is not silenced by DNA methylation in osteosarcoma. Bone 73: 223-232, 2015.

33. Hoang BH, Kubo T, Healey JH, Yang R, Nathan SS, Kolb EA, Mazza BA, Meyers PA and Gorlick R: Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-beta-catenin pathway. Cancer Res 64: 2734-2739, 2004.

34. Aslan H, Ravid-Amir O, Clancy BM, Rezvankhah S, Pittman D, Pelled G, Turgeman G, Zilberman Y, Gazit Z, Hoffmann A, et al: Advanced molecular profiling in vivo detects novel function of dickkopf-3 in the regulation of bone formation. J Bone Miner Res 21: 1935-1945, 2006.

35. Hiramitsu S, Terauchi M and Kubota T: The effects of dickkopf-4 on the proliferation, differentiation, and apoptosis of osteoblasts. Endocrinology 154: 4618-4626, 2013.

36. Tang Q, Zhao H, Yang B, Li L, Shi Q, Jiang C and Liu H: WIF1 gene inhibition and Wnt signal transduction pathway activation in NSCLC tumorigenesis. Oncol Lett 13: 1183-1188, 2017.

37. Yan Y, Tang D, Chen M, Huang J, Xie R, Jonason JH, Tan X, Hou W, Reynolds D, Hsu W, et al: Axin2 controls bone remodeling through the beta-catenin-BMP signaling pathway in adult mice. J Cell Sci 22: 3566-3578, 2009.

38. Gregory CA, Singh H, Perry AS and Prockop DJ: The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. J Biol Chem 278: 28067-28078, 2003.

39. Nakasa T, Yoshizuka M, Andry Usman M, Elbadry Mahmoud E and Ochi M: MicroRNAs and bone regeneration. Curr Genomics 16: 441-452, 2015.

40. Wang J, Liu S, Li J, Zhao S and Yi Z: Roles for miRNAs in osteogenic differentiation of bone marrow mesenchymal stem cells. Stem Cell Res Ther 10: 197, 2019.

41. Lin ZW, Zhang W, Jiang SD, Wei WB and Li XF: Inhibition of microRNA-940 suppresses the migration and invasion of human osteosarcoma cells through the secreted frizzled-related protein 1-mediated Wnt/β-catenin signaling pathway. J Cell Biochem: Oct 15, 2018 (Epub ahead of print). doi: 10.1002/jcb.27580.