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The human WNT5A isoforms display similar patterns of expression but distinct and overlapping activities in normal human osteoblasts

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Abstract

WNT5A activates noncanonical Wnt signaling pathways and has critical functions in early development, differentiation, and tissue homeostasis. Two major WNT5A protein isoforms, which in this study we term WNT5A-L(A) and WNT5A-S(B), have been identified that differ by 18 AA at their amino terminus. Functional differences between the isoforms have been indicated in studies utilizing cancer cell lines but the activities of the isoforms in normal cells and during differentiation have not been explored. We examined the WNT5A isoforms in the normal osteoblast cell line hFOB1.19. WNT5A-L(A) and WNT5A-S(B) transcripts increased from Days 3 to 21 of differentiation but WNT5A-S(B) showed a greater fold-change. In undifferentiated cells, there are 2-fold more WNT5A-L(A) than WNT5A-S(B) transcripts. Total intracellular WNT5A protein increased up to 3-fold during differentiation. siRNA knockdown of total WNT5A leads to a decrease in the expression of the differentiation markers, osteocalcin and *RUNX2*. Conditioned medium containing the isoform proteins [CM-L(A) and CM-S(B)] was used to analyze the effects of the isoforms on β-catenin and noncanonical signaling, proliferation, gene expression, and alkaline phosphatase (ALP) activity. Treatment with both CM-L(A) and CM-S(B) reduced β-catenin signaling. CM-L(A) but not CM-S(B) significantly increased the proliferation of nondifferentiated hFOB1.19 cells. CM-L(A) enhanced osteocalcin transcripts over 2-fold in differentiating cells, whereas CM-S(B) had no effect. Analysis of differentiating cells up to Day 21 revealed no significant effect of treatment with CM-L(A) or CM-S(B) on ALP activity or osteocalcin gene expression. pJNK levels were unaffected in proliferating cells by treatment with neither isoform. pPKC increased slightly in CM-L(A)-treated cells at 15 min but by 2 h pPKC levels were less than the control. CM-S(B) had a more robust effect on pPKC levels that continued up to 2 h. Together these

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results suggest that the WNT5A isoforms have distinct and overlapping functions in normal osteoblasts.

KEYWORDS

hFOB1.19, isoform, noncanonical Wnt signaling, osteoblast, osteogenesis, WNT5A

1 | INTRODUCTION

WNT signaling includes both the canonical or β-catenin-dependent pathway and the noncanonical or β-catenin independent pathway. The β-catenin pathway is initiated by Wnt ligand (e.g., Wnt1, Wnt3, Wnt3a) binding to Frizzled (Fzd) receptors, of which there are 10 subtypes, and low-density lipoprotein receptor-related protein (Lrp) 5 or Lrp6.[1-3] Consequently, β-catenin levels increase and are activated by phosphorylation. The activated β-catenin translocates into the nucleus where it complexes with the T-cell factor 1/lymphoid enhancer factor (Tcf/Lef1) and CREB-binding protein and induces specific gene transcription. In contrast, the noncanonical pathways offer more varied outcomes. WNT5A is one of the major ligands involved in noncanonical signaling. It binds Fzd and receptor tyrosine kinase-like orphan receptor 1/2 (Ror 1/2) receptors, leading to Disheveled (Dvl) binding to the receptor complex and its activation.[4] WNT5A has been shown to bind Fzd receptor subtypes 2–8, allowing for activation of different non-canonical signaling pathways.[1,2] These include the planar cell polarity (PCP) and convergent extension (CE) pathway (PCP/CE), which results in activation of the small GTPases, RhoA and Rac. RhoA activates Rho-associated kinase (Rho-kinase), whereas Rac activates c-Jun N-terminal kinase (JNK). Alternatively, the Ca^{2+} pathway is initiated when WNT5A binding leads to the release of Ca^{2+} followed by downstream activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) kinases. Importantly, noncanonical WNT5A signaling can have positive and negative effects on the β-catenin signaling pathway, depending on the cell and its receptor subtypes.[2] Both WNT3A and WNT5A bind to Fzd receptors 2–8, allowing for their direct interactions. WNT5A has been shown to directly compete with WNT3A for binding to Fzd2 in some cell types, inhibiting canonical activation.[5] Alternatively, WNT5A noncanonical signaling can indirectly inhibit β-catenin signaling[6] or in another cellular context, indirectly increase β-catenin signaling by upregulating LRP5/6 expression.[7] These and other studies highlight the complexities of WNT5A cellular activities in the context of multiple receptor subtypes, distinct noncanonical signaling pathways, and cross-talk with the Wnt canonical pathway.

Activation of noncanonical signaling by WNT5A has been found to be critical in early development, tissue differentiation, and tissue homeostasis.[2,4] Most relevant to this study, WNT5A functions in bone formation and dynamics.[8-12] Mutations in WNT5A (and Ror2) are associated with Robinow syndrome, a human genetic disease, that is, in part, characterized by major skeletal defects.[13] Early studies of Wnt5a knockout mice revealed various bone-related phenotypes, including dwarfism, craniofacial abnormalities, short limbs, and tails.[14] Osteoblast-lineage cells derived from Wnt5a-deficient mice were found to have reduced Wnt/β-catenin signaling, impaired osteoblast differentiation, and lower mineralization.[15] And, expression of genes regulating osteogenesis was shown to be decreased in Wnt5a-/- osteoblast cell lines.[16] Moreover, WNT5A secreted by mature osteoblasts enhances osteoclastogenesis by increasing receptor activator of nuclear factor κ B (RANK) receptor in pre-osteoclast macrophage cells.[15] When secreted by mature osteoclasts, the WNT5A promotes bone resorption.[17]

The human WNT5A gene generates multiple transcripts by alterative and distinct transcription start sites (Ensembl WNT5A ENSG00000114251). The most characterized and studied WNT5A isoform corresponds to Ensembl CCDS46850 or Uniport A0A384N611 and is derived from the WNT5A-201 (Ensemble ENST00000264634.8) transcript. The other isoform WNT5A-205 (Ensemble ENST00000497027.5; CCDS58835) is generated by an alternative promoter located within the first intron of WNT5A-201. The unprocessed protein derived from the WNT5A-201 transcript is 15 AA longer than the WNT5A-205 transcript but 18 AA longer after processing in the ER.[18] The only protein-coding transcripts of the WNT5A gene in the mouse are these same two isoforms. In this study, we refer to the longer WNT5A isoform as WNT5A-L(A) and the shorter as WNT5A-S(B).

The distinct functions of the WNT5A isoforms remain obscure. This is an important question considering WNT5A′s varied and critical activities. This lab has analyzed the promoters of the two isoforms and their expression patterns in osteosarcoma cells. We provide evidence that the promoters can function independently and are differentially regulated.[19] In osteosarcoma cell lines, the WNT5A-L(A) is expressed at a higher level in comparison to WNT5A-S(B), whereas in normal osteoblasts derived from bone tissue, both transcripts are expressed at a high level but WNT5A-S(B) transcripts are approximately 11-fold higher than WNT5A-L(A).[20,18]

analyzed the isoforms termed WNT5A-L (for long) and WNT5A-S (for short) and showed that overexpression of WNT5A-S increased proliferation in three different cancer cell lines, whereas WNT5A-L decreased expression.[21] analyzed the isoforms in colorectal cancer cell lines, showing that the isoforms differentially affect apoptosis. To date, the functional role of the WNT5A isoforms has only been studied in transformed cells.

To further explore the functional distinctions between the WNT5A isoforms we chose normal human osteoblast differentiation as a model, using the cell line hFOB1.19. This cell line is immortalized due to the expression of SV40 large T but is considered normal and can differentiate in culture. The effects of the WNT5A isoforms on the proliferation of undifferentiated osteoblasts, the expression of osteoblast differentiation markers in both proliferating and differentiating osteoblasts, and canonical and noncanonical signaling were examined. Based on these analyses, we provide evidence that the WNT5A isoforms display distinct and overlapping activities.

2 | MATERIALS AND METHODS

2.1 | Cells and Cell Culture

Except where indicated the cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC cell-specific protocols (see below). With one exception (hFOB1.19, see below) the cell lines were grown in a 5% CO_2, 37°C incubator. The different cell line-specific mediums were supplemented with 10% fetal bovine serum (FBS) (Gibco, 10437028) and 1× penicillin/streptomycin using a 100× stock (10 000 units penicillin/10 000 μg streptomycin) (PSL01; Caisson Labs). All cell lines were collected using 0.25% Trypsin/0.53 mM EDTA. The cells were passaged as suggested by ATCC and the medium changed every 3–4 days.

Mouse fibroblast L-cells (*CRL-2648*) and L-Wnt3a (stably transfected with a Wnt3a expression vector) (*CRL-2647*) were grown in Dulbecco Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D2906). HEK293T (*CRL-3216*), an epithelial human embryonic kidney cells, were grown in DMEM containing 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. NIH-3T3 (*CRL-1658*), a mouse embryonic fibroblast cell line, was grown in DMEM with 4 mM L-glutamine and 1.5 g/L sodium bicarbonate.

Chinese Hamster Ovary (CHO) cells stably transfected to express WNT5A-L(A) or WNT5A-S(B) and the parental CHO line were a gift from Dr. Karl Willert (University of California).[18] The CHO cells were grown in DMEM lacking phenol red, supplemented with L-Glutamine (0.584 g/L) to which Geneticin Sulfate or G418 (0.4 mg/ml) (sc-29065A; Santa Cruz Biotechnology), Blasticidin (3 μg/ml) (15205; Sigma-Aldrich), and Doxycycline (5 ng/ml) (10692-13; Sigma-Aldrich) were added.

hFOB1.19 (*CRL-11372*), considered a normal human osteoblast cell, is immortalized due to expression of the SV40 Large-T. The cells were grown in DMEM/Hams's F12 1:1 (D2906; Sigma-Aldrich) with 2.5 mM L-glutamine, without phenol red, and 0.3 mg/ml G418. Proliferating and non-differentiating hFOB1.19 were grown at 34.4°C in a 5% CO_2 incubator. To induce osteogenic differentiation, the hFOB1.19 cells were grown to confluency at 34.4°C, shifted to the restrictive temperature (39°C), and grown in normal hFOB1.19 medium supplemented with β-glycerol phosphate $(5 \times 10^{-3}$ mol/L) (50020; Sigma-Aldrich), ascorbic acid (0.1 g/L) (A1417; Sigma-Aldrich), Menadione $(10^{-8}$ mol/L) (M5625; Sigma-Aldrich), 1, $25(OH)_2D_3$ (vitamin D) $(10^{-7}$ mol/L) (D1530; Sigma-Aldrich).

2.2 | Preparation of conditioned medium (CM)

The three CHO cell lines (WNT5A-L(A), WNT5A-S(B), and parental) were grown to 80%–90% confluency, collected and re-plated in 10 cm plates, and grown to confluency. Protein expression was induced by adding doxycycline (250 ng/ml), without the addition of blasticidin or G418. The media was collected from the cells at 3 days and stored at 4°C. Fresh medium was added along with doxycycline. The medium was collected after another 4 days of incubation, combined with the previously collected medium and filter sterilized. The WNT5A-L(A) CM is referred to as CM-L(A), the WNT5A-S(B) CM as CM-S(B), and parental as CM-P. The CM was stored at 4°C for use in various experiments.

2.3 | RNA isolation and qPCR

Cells were washed in 1× PBS, lysed in RNA Lysis Buffer, and RNA isolated as instructed using either the SV Total RNA Isolation System (Z3100; Promega) or Quick-RNA™ Mini Prep (R1054; Zymo Research). Approximately 0.2–1 μg of RNA was used for cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit (K1671; Thermo Fisher Scientific) or Superscript III First Strand synthesis Kit (11752; Invitrogen). For qPCR, a master mix was prepared for each target. Three technical replicas were performed for each target and sample. β-actin was used as the internal control. The following

Applied Biosystems Taqman primers were used: β-actin (Hs00357333 g1), *RUNX2* (Hs01047973 m1), osterix (*SP1*) (Hs01866874 s1), and osteocalcin (*BGLAP*) (Hs01587814 g1). Custom-prepared Taqman primers for WNT5A-L(A) and WNT5A-S(B) were designed and verified[19]; the sequences are included in Table S1. Reactions were run on a StepOne Applied Biosystem realtime thermocycler. Reactions were repeated three times.

Relative fold-change was determined using a Microsoft Excel generated formula. The averages of the C_t values were calculated from the technical, triplicate values for a given sample (target gene and treatment group) and the actin control C_t values for that sample. The actin average C_t value was subtracted from the matching sample average C_t value. This generated a ΔC_t value for both the control sample (which varied, depending on the experiment) and a particular experimental sample (e.g., different day or treatment). A $\Delta\Delta C_t$ value was determined by subtracting the control sample ΔC_t value from the experimental sample ΔC_t value. The fold-change (comparing the experimental sample to the control sample) was calculated by determining the 2-ΔΔCt value.

2.4 | Transcript number quantification

For analysis of isoform-specific transcript numbers, a standard curve of C_t value versus transcript number was generated for both isoforms. Purified and quantified (ng/µl) WNT5A-L(A) and WNT5A-S(B) PCR products were used to generate standard curves (Figure S1). Transcript numbers were calculated from the molecular weight of the primer sequence, Avogadro's number, and PCR product concentration. The C_t values versus transcript numbers were plotted and the line equation was obtained. RNAs isolated from differentiating hFOB1.19 cells at days 0, 3, 7, 14, and 21 were converted to cDNA and analyzed by qPCR using WNT5A-L(A) and WNT5A-S(B)-specific primers (Table S1). The resulting C_t values were adjusted using the actin control values for each day. The adjusted C_t value was used to solve for "x" or transcript number using the line equations. This value was adjusted to transcript number per µg RNA, based on the amount of RNA used in the cDNA reaction and the amount of cDNA used in each qPCR reaction. Standard curves were generated along with qPCR amplification of the samples for each analysis.

2.5 | WNT5A western blot

WNT5A protein was detected by western blot. To determine the presence of WNT5A-L(A) and WNT5A-S(B)

protein levels in the CM, 50 µl aliquots of the CM were run on a 10% SDS-PAGE gel and blotted onto nitrocellulose. After blocking in 5% dried milk-TTBS (Tris-Tween Buffered Saline; 0.01 M Tris, pH 8.0, 0.15 M NaCl, 0.01% Tween 20), primary WNT5A antibody (Ab174100; Abcam) was added at 1:1000 in 1% dried milk-TTBS and then blot incubated at 4°C overnight. After washing, the secondary antibody (Abcam-ab97051; goat Antirabbit IgG-HRP) diluted 1:10 000 in 1% dried milk-TTBS was added and incubated for one hour. The blot was detected using Super-Signal West Pico PLUS Chemiluminescent Substrate (p134577; Thermo Fisher Scientific). The detected protein bands were quantified using the BioRad documentation system. WNT5A in protein lysates were prepared from differentiating hFOB1.19 using the Cell-Lytic M reagent (C3228; Sigma-Aldrich), containing various protease inhibitors. Protein concentrations were determined using the Bio-Rad Protein Assay Dye Reagent (#5000006) with bovine serum albumin (BSA) as the standard. Equal amounts of protein were loaded on the SDS-PAGE gel. The WNT5A was detected as described for the CM. The blots were stripped and reprobed for β-actin using an Antimouse actin monoclonal antibody (Developmental Studies Hybridoma Bank; DSHB-JLA20). β-actin served as a loading control.

2.6 | TOPFlash and FOPFlash assays

The activity of the WNT5A isoforms in the prepared CM was assayed using a luciferase reporter of canonical Wnt signaling. This assay required the preparation of the Wnt3a-conditioned medium. Wnt3a activates the canonical Wnt signaling pathway. Wnt3a and control conditioned medium were prepared from the L-Wnt3a and L-cell cell lines as described previously for the CHO expressing cells. The CM was labeled as CM-L-cell and CM-Wnt3a and stored at −20°C

HEK293T and hFOB1.19 cells were plated at 1×10^6 and 5×10^5 cells, respectively, per well in a 6-well plate. The next day, cells were transfected with M50 Super8x TOPFlash and M51 Super8x FOPFlash, which were gifts from Randell Moon (Addgene Plasmid #12456; Addgene Plasmid #12457) with the FuGENE6 transfection reagent (Promega-E2691) at a ratio of 3:1 reagent to DNA for both cell lines. The next day, the cells were collected and re-plated in a 96-well plate at 1×10^4 (HEK293T) and 5×10^4 cells per well of a 48-well plate (hFOB1.19). After 1 day, the cells were treated as described in the results with the different CM's. The following day, cells were collected in 1× Cell Lysis Buffer (E1500; Promega) and luminescence was measured using the Luciferase Assay Kit (E1500; Promega) for luciferase in a Bertholt LB 9501

luminometer or a Synergy 2-BioTek multipurpose microplate reader.

2.7 | Proliferation assay

hFOB1.19 cells were plated at 1×10^3 cell per well of 96-well plates. The next day (Day 0) and on various days over a 7-day period, cells were assayed for DNA content using the CyQuant Proliferation Assay (C7026; Invitrogen/Thermo Fisher Scientific). Fluorescence was measured per well using a microplate reader (Synergy 2-BioTek) set at 420 nm/580 nm. For testing the effects of CM-L(A), CM-S(B), and the control CM-P on proliferation, on Day 0 the different CM's were mixed 1:1 with fresh complete medium and added to the appropriate number of wells for 7 days of assays. On Day 4, the cells were re-treated with 1:1, CM:Medium. Each day the selected wells for each treatment group were assayed. Six replicas were assayed for each time point and treatment. The assay was repeated three independent times.

2.8 | Analysis of Wnt noncanonical signaling by flow cytometry

Flow cytometry was used to assay for changes in phospho-JNK (pJNK) and phospho-PKC (pPKC) levels in proliferating hFOB1.19 cells after treatment with CM-L (A) and CM-S(B). Cells were plated in 24-well plates and treated with CM-P, CM-L(A), and CM-S(B) as previously described. To generate positive controls for pJNK and pPKC, cells were treated with $1.13\,\mu$M anisomycin (SC-3524; ChemCruz) and $1\,\mu$M phorbol 12, 13-diacetate (Santa Cruz Biotechnology, SC-202283), respectively. Cells were collected at 15 min, 30 min, 1 h, and 2 h using Trypsin-EDTA. The released cells were washed with 1× PBS and the cell pellets were taken up in 60% acetone and incubated on ice for 10 min. The cells were pelleted by centrifugation and washed with 0.3% BSA in 1× PBS. The cell pellets were taken up in $100\,\mu$l of primary antibody solution containing 0.3% BSA in 1× PBS, 0.1% Triton X-100, and 5% normal goat serum. The primary antibodies source and dilutions were as follows: (1) rabbit anti-Phospho-PKC alpha (Thr638) rabbit polyclonal (Thermo Fisher Scientific (44-962G), 1:250); (2) Phospho-SAPK/JNK (Thr183/Tyr185 mouse mAb (Cell Signaling (9255), 1:400); Total JNK (Santa Cruz Biotechnology (SC-7345), 1:100); Total PKC α (Santa Cruz Biotechnology (SC-8393), 1:500). Cells in antibody solution were incubated at room temperature for 1 h, pelleted by centrifugation, and washed in 0.3% BSA in 1× PBS. The pelleted cells were re-suspended in the secondary antibody solutions containing 0.3% BSA/PBS and $0.5\,\mu$l of the secondary antibody: Alexa FluorR 488 Donkey Antimouse IgG (H+L) ([Jackson ImmunoResearch (715-545-150), 1:200); Alexa FluorR 488 Antirabbit IgG (H+L), (Jackson ImmunoResearch (711-545-152), 1:200]. The cell antibody solution was incubated for 1 h at room temperature, then pelleted by centrifugation and washed with 0.3% BSA in 1× PBS. The final cell pellet was re-suspended in $500\,\mu$l 1× PBS. Counts of fixed and stained cells were acquired via Guava Easy Cyte 6-2L benchtop flow cytometer at 3000–5000 events (cells counted). The raw data were analyzed using Flowing Software v2.5.1.[22]

2.9 | Alkaline phosphatase assay (ALP)

Alkaline phosphatase was measured using the Bio Vision Alkaline Phosphatase Activity Colorimetric Assay Kit (K412-500; BioVision). All buffers and components used for the assay were supplied in the kit. For the collection of cells for the ALP assay, cells were initially washed with 1× PBS and the plates with the washed cells were stored at −80°C. On the day of the assay, frozen cells were taken up in Alkaline Phosphatase Buffer and homogenized by pipetting up and down. A p-nitrophenyl phosphate (pNPP) substrate tablet was taken up in 2.7 ml Alkaline Phosphatase Buffer. A total of $80\,\mu$l of each sample was pipetted into each of four wells of a 96-well plate. At least three control wells with buffer only were included on each plate. After all the samples were pipetted, $50\,\mu$l of the pNPP substrate was added. The samples were incubated at room temperature in the dark for 60 min. Stop Solution ($20\,\mu$l) was added to each well at the end of the incubation time. The absorbance was measured using the Synergy 2 BioTek multipurpose microplate reader at 405 nm.

2.10 | Knockdown of WNT5A by siRNA transfection

Short interfering RNA (siRNA) for WNT5A isoforms L (A) and S(B), total WNT5A, and nonsilencing control sequences used for knockdown assay were identical to those designed and used by ref[18] (Table S2). siRNAs were purchased from Dharmacon. A $10\,\mu$M stock of each siRNA was prepared in sterile distilled water. The siRNAs were transfected into hFOB1.19 cells in 96-well plates, 48-well plates, and 24-well plates at seeding densities of 1×10^4, 5×10^4, and 1×10^5 cells per well, respectively. siRNA was transfected using Lipofectamine RNAiMAX (13778-030; Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions.

Cells were transfected when approximately 60%–80% confluency was achieved, which generally took 1 day after plating. The medium was changed to Opti-MEM reduced serum medium (11058-021; Gibco by Life Technologies) before transfecting the cells. For a 48-well plate, siRNA/Lipofectamine RNAiMAX mix was prepared according to the 24-well protocol. During transfection, the siRNA-lipid transfection mix was halved so the wells were transfected with 25 µl of the transfection mix. For a 96-well, the siRNA was made according to the protocol and 10 µl of the transfection mix was added to the wells. The cells were incubated at 34°C post-transfection in Opti-MEM medium. When the cells reached 90%–100% confluency (generally the following day or two of transfection), it was considered Day 0 of the assessment. On Day 0, the cells in 96-well plates were processed for ALP assay, as previously described, and 24-well plate for RNA isolation. On Day 0, the medium was also changed to the differentiation medium and the remaining cells were induced to differentiate by incubating at 39°C. The cells were harvested at 2 and 3 days after inducing differentiation for ALP activity and RNA extraction. The ALP activity was measured to evaluate differentiation and qPCR was conducted to examine transfection efficacy and to determine the effect on the molecular markers of osteoblast differentiation. qPCR analysis was performed and fold changes were determined as previously described.

2.11 | Statistics

A two-tailed and two-typed (equal variance) Student t test was used to determine significance ($p < 0.05$) comparing sample values. These values represented three to four biological replicas, depending on the experiment, as indicated in the figure legend.

3 | RESULTS

3.1 | The WNT5A L(A) and S(B) isoform transcripts increase during hFOB1.19 differentiation

The pattern of WNT5A isoform expression during osteogenesis may provide insights into their functional distinctions. Undifferentiated hFOB1.19 cells were grown to confluency (Day 0) and induced to differentiate. Cells collected at different days up to Day 21 were analyzed for
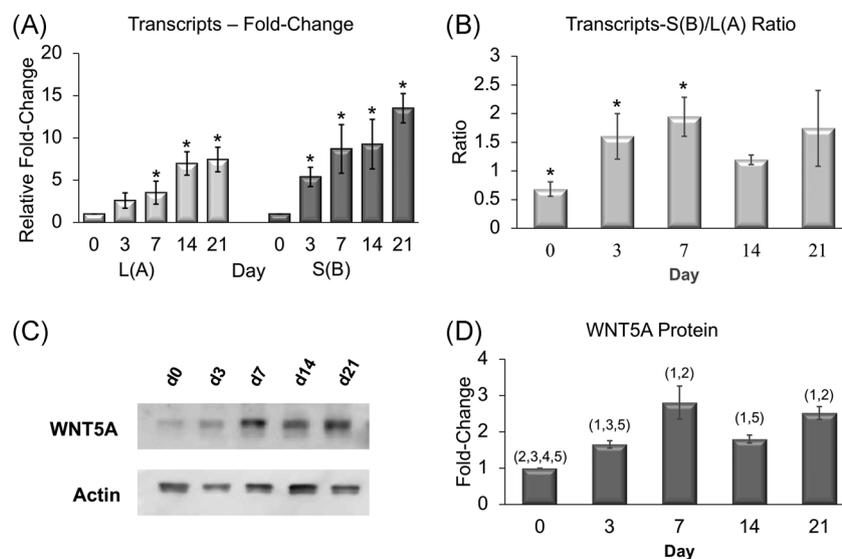

FIGURE 1 WNT5A-L(A) and WNT5A-S(B) transcripts and total WNT5A protein increase during hFOB1.19 osteoblast differentiation. Relative fold-change values represent the 2-ΔΔCt values, derived from the C_t values at each day and C_t values of Day 0, which were normalized to actin. Bars represent ±standard error derived from three biological replicas. Asterisks indicate a significant difference ($p < 0.05$) compared to Day 0. (B) WNT5A L(A) and WNT5A-S(B) transcript numbers were determined at the indicated days and the S(B)/L(A) transcript number ratio was calculated. Values are the average of three biological replicas. Bars are ±standard error. Asterisks indicate significance ($p < 0.05$), comparing each value to the L(A)/L(A) transcript number ratio (or 1). (C) Total WNT5A protein was detected by western blot at the indicated days. 50 µg of total cellular protein was loaded per lane. β-actin served as a loading control. (D) Quantification of WNT5A protein levels. The relative protein amounts in each band were standardized to β-actin. Average fold-change values were derived from four biological replicas. Bars are ±standard error. Numbers above the bars indicate sample pairs showing a significant difference ($p < 0.05$)

WNT5A isoform-specific transcripts. Transcript levels relative to Day 0 for both isoforms increased (Figure 1A). WNT5A-L(A) increased 6–7-fold at Day 21, whereas WNT5A-S(B) showed an average 15-fold increase. Both isoforms increased by Day 3 after induction. Determination of absolute transcript levels (per µg RNA) revealed that WNT5A-S(B) transcript numbers are approximately 0.6× less than WNT5A-L(A) at Day 0 (Figure 1B). This, in part, accounts for the higher relative fold increase in WNT5A-S(B) transcripts compared to WNT5A-L(A). The ratio of WNT5A-S(B) to WNT5A-L(A) transcript numbers was determined (Figure 1B). Up to Day 7 there are approximately 1.5 to 2× more WNT5A-S(B) than WNT5A-L(A) transcripts, whereas at Days 14 and 21 differences in transcript numbers between the isoforms were insignificant ($p > 0.05$) (Figure 1B).

We measured the level of *total* WNT5A protein during hFOB1.19 differentiation to confirm that increasing WNT5A transcript levels correspond to increased WNT5A protein levels. Isoform-specific antibodies were unavailable. *Total* WNT5A protein increased during osteoblast differentiation (Figure 1C). Quantification relative to β-actin showed that WNT5A protein increases 1.5 to 3-fold over the 21-day period (Figure 1D). The fact that WNT5A is a secreted protein likely accounts for the modest increase in protein levels, compared to increases in transcript levels.

To correlate WNT5A expression with hFOB1.19 cell differentiation, we analyzed the transcript levels of *RUNX2*, Osterix (*SP1*), and osteocalcin (*BGLAP*). As shown in Figure 2A–C, transcripts for all three differentiation markers increased by Day 3, relative to levels in undifferentiated Day 0 control cells. Osteocalcin displayed the largest increase,

varying from 100 to 350× on different days up to Day 21. *RUNX2* increased approximately 3-fold by Day 3 then decreased to Day 0 levels from Days 7 to 21. Osterix increased 2-fold, decreased to control levels at Day 10, and again increased to over 4-fold at Days 17 and 21. Together these data confirm that increases in WNT5A-L(A) and WNT5A-S(B) transcripts and *total* WNT5A protein are associated with hFOB1.19 cell differentiation.

3.2 | WNT5A has a positive role in osteoblast differentiation

We attempted to knock down the individual isoforms in the hFOB1.19 cells but were unsuccessful using published siRNA sequences[18] (Tables S2 and S3). siRNA for *total* WNT5A did achieve sufficient knockdown during early periods of differentiation (Table S3). Since the differentiation markers (*RUNX2*, osterix, osteocalcin) and WNT5A protein increased by Day 3 (see Figures 1 and 2), *total* WNT5A knockdown should provide insight into the role of WNT5A during this critical time period. *Total* WNT5A knockdown decreased *RUNX2* and osteocalcin transcript levels (Figure 3A). By Day 3, however, *RUNX2* and osteocalcin returned to control levels and unexpectedly, osterix transcript levels increased (Figure 3B, C). We also assayed for levels of ALP activity, another differentiation marker, and found that *total* WNT5A knockdown reduced ALP activity at Days 2 and 3 (Figure 3D). These results are consistent with the conclusion that WNT5A enhances markers of osteogenesis. However, we were unable to assess the role of the individual WNT5A isoforms in early osteoblast differentiation.
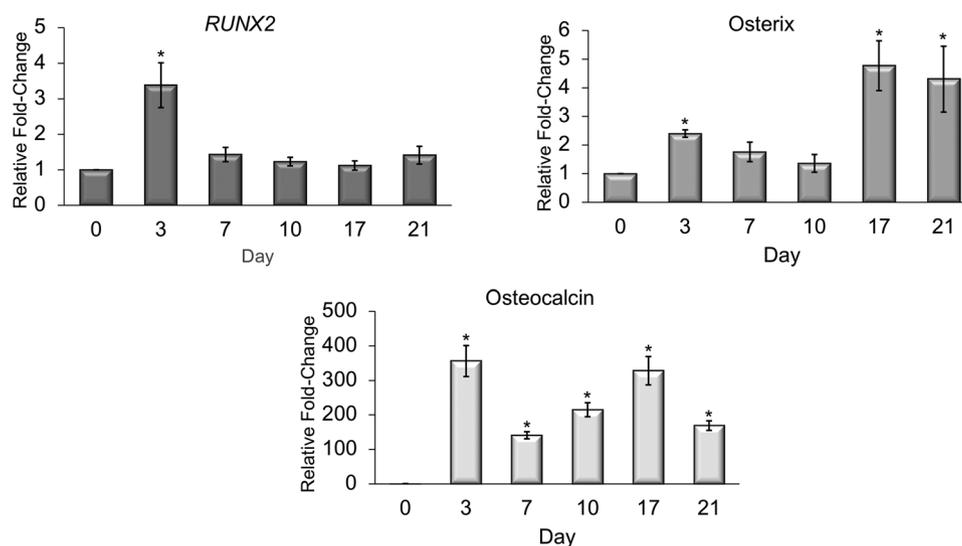

FIGURE 2 Transcript levels of known osteoblast differentiation markers (A) *RUNX2*, (B) osterix, and (C) osteocalcin were determined by qPCR at the days indicated. Average fold-change values were determined from three biological replicas. Bars are ±standard error. Asterisks indicate a significant difference ($p < 0.05$) relative to Day 0 (set to 1)
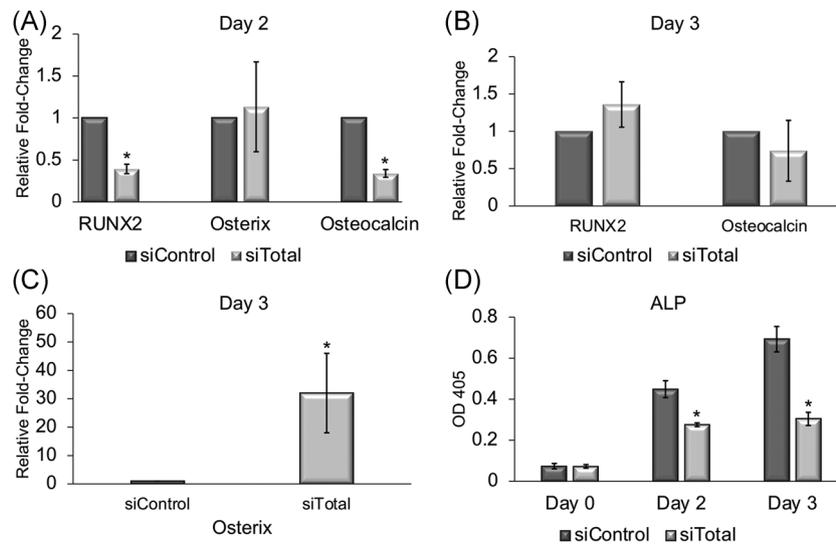
FIGURE 3 Knockdown of *total* WNT5A during early stages of hFOB1.19 cell differentiation. hFOB1.19 cells were transfected with a control siRNA (siControl) and a siRNA specific to total WNT5A (siTotal). Transfected cells were grown under differentiating conditions (confluent/39°C/with differentiation reagents). Expression of differentiation markers *RUNX2*, osteocalcin, and osterix was determined at (A) Day 2 and (B, C) Day 3 of differentiation. d) ALP activity was measured in siRNA-transfected cells at Days 0, 2, and 3. Average fold-change values were derived from three biological replicas. Bars are ±standard error. Asterisks indicate a significant difference ($p < 0.05$) between siTotal and siControl

3.3 | Effects of increased WNT5A-L(A) and WNT5A-S(B) on β-catenin signaling in HEK292T and hFOB1.19 cells

It has been reported that both WNT5A isoforms inhibit β-catenin signaling in HEK292T cells.[18] We next determined if the isoforms have a similar effect in hFOB1.19. We prepared conditioned medium (CM) from engineered CHO cells that contained WNT5A-L(A) (CM-A) or WNT5A-S(B) (CM-B). The control CM (CM-P) was prepared from parental CHO not expressing the isoforms. The presence of the isoform proteins in the CM was confirmed by western blot (Figure 4A). We used a TOPFlash assay system to determine the effect of the CM's on Wnt3a-induced β-catenin activity. Treatment of TOPFlash-transfected HEK293T cells with CM-Wnt3a (derived from Wnt3a expressing mouse L-cells) increased TOPFlash activity (Figure 4B). Treatment with a 1:1 mixture of CM-P:CM-Wnt3a reduced TOPFlash activity but the difference in relative light units compared to the
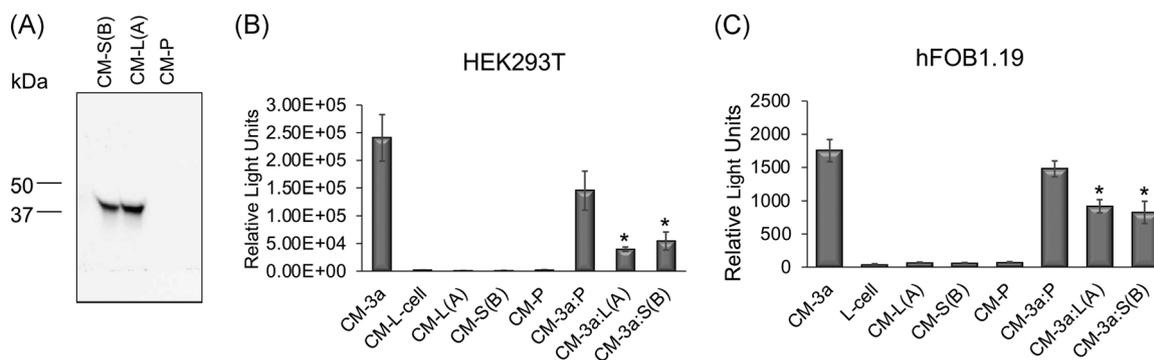

FIGURE 4 WNT5A-L(A) and WNT5A-S(B) reduce canonical β-catenin signaling in hFOB1.19 and HEK293T cell lines. (A) WNT5A protein was detected in the prepared conditioned medium CM-L(A) and CM-S(B) by western blot. No WNT5A was detected in the control CM-P. (B, C) HEK293T and hFOB1.19 cells transfected with a TOPFlash vector were treated as indicated. Asterisks indicate a significant difference ($p < 0.05$) relative to the control-treated cells (CM-3a:P). Data represents one trial of two biological replicas. The reduction in the activity of CM-3a:P compared to CM-3a was not significant ($p > 0.05$). Bars are ±standard errors derived from four technical replicas. CM-3a, Wnt3a containing conditioned medium; L-cell, medium from mouse L-cells; CM-3a:P, CM-3a:L(A), and CM-3a:S(B), 1:1 mixture of CM-3a, and the indicated CM's

control CM-Wnt3a was insignificant ($p > 0.05$). Treatment with 1:1 CM-Wnt3a:CM-L(A) or CM-S(B) caused a significant reduction ($p < 0.05$) compared to the control, CM-P:CM-Wnt3a (Figure 4B). There was no effect of the CM's on FOPFlash (contains mutated β-catenin-binding sites) (data not shown). These results confirmed that WNT5A protein in the CM-L(A) and CM-S(B) was active. Similar results were found using hFOB1.19 cells; both CM-L(A) and CM-S(B) significantly ($p < 0.05$) reduced luciferase (β-catenin) activity compared to the control CM-P. The level of reduction was less than measured in HEK293T. These results support the conclusion that both WNT5A isoforms can reduce canonical β-catenin signaling in proliferating hFOB1.19 cells and have redundant activities.

3.4 | Effects of increased WNT5A-L(A) and WNT5A-S(B) on hFOB1.19 proliferation and differentiation

We used the characterized CM's to determine if *increased* levels of the WNT5A isoforms differentially affect hFOB1.19 proliferation and differentiation markers. Cells were plated at a low cell density and treated with 1:1, hFOB1.19 medium: CM-L(A), CM -S(B), or CM-P. DNA content was determined at specific days, up to days 6 or 7, as a measure of cell number. We found that treatment of the cells with CM-S(B) had no effect on proliferation, relative to the control (CM-P), whereas treatment with CM-L(A) had a slight but significant positive effect measured at Days 5, 6, and 7 ($p < 0.05$ comparing CM-L(A) to CM-P and CM-S(B)) (Figure 5). These results suggest that the isoforms have distinct activities in proliferating hFOB1.19 cells.

Next, we analyzed the effect of increased isoforms on osteoblast differentiation markers in both proliferating hFOB1.19 cells grown at 34.4°C (nondifferentiating conditions) and confluent cells grown at 39°C (differentiating conditions) (Figure 6). The differentiation reagents were not included as we wanted to judge the effects of the isoforms, alone. hFOB1.19 will differentiate once confluent at 39°C, without the added reagents. *RUNX2*, osterix, and osteocalcin transcripts levels were analyzed on Day 2 and 3 (Figure 6A, B). *RUNX2* transcripts levels were unaffected by CM-S(B) on both days. CM-L(A) had no effect on *RUNX2* at Day 2 but caused a slight decrease on Day 3. CM-L(A) had no effect on osterix transcripts at Days 2 and 3, whereas CM-S(B) significantly ($p < 0.05$) decreased osterix transcript levels relative to CM-L(A) and CM-P on Day 2. On Day 3, CM-L(A) and CM-S(B) did not significantly affect osterix transcript levels. CM-L(A) increased osteocalcin at both days 2 and 3 by approximately 2- to 3-fold. CM-S(B) had no effect on osteocalcin transcript levels. These data suggest that WNT5A-L(A)

has a positive effect on osteoblast differentiation in hFOB1.19 cells grown under differentiating conditions by increasing osteocalcin but not directly affecting *RUNX2* and osterix. In contrast, WNT5A-S(B) had no effect on *RUNX2* or osteocalcin but a negative effect on osterix.

There were no significant effects of CM-L(A) and CM-S(B) treatment on the differentiation markers *RUNX2*, osterix, and osteocalcin in non-differentiating, proliferating osteoblast cells (Figure 6C). There was a trend toward an increase in osteocalcin by treatment with both CM-L(A) and CM-S(B) and an increase in osterix by treatment with CM-S(B).

We also analyzed the effect of increasing the isoform levels over 21 days of differentiation (Figure 7). hFOB1.19 cells grown in differentiation medium plus CM-L(A) and CM-S(B) were assayed for the differentiation marker osteocalcin and ALP activity. No significant differences ($p > 0.05$) were detected at any time point, comparing CM-L(A) and CM-S(B) treated cells to the control (CM-P) for either osteocalcin or ALP. There was a trend, however, suggesting that CM-L(A) increases osteocalcin at Days 3, 14, and 21.

3.5 | The WNT5A isoforms have differing effects on PKC activation in proliferating osteoblasts

Our results suggest that the WNT5A isoforms L(A) and S (B) have differing effects in proliferating and differentiating hFOB1.19 osteoblasts. One explanation is that the isoforms have a preference for the Ca^{2+} or the PCP/
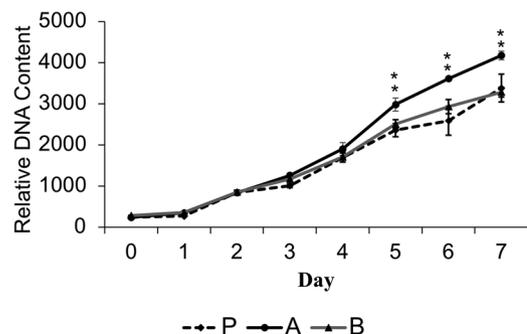

FIGURE 5 Effect of increased WNT5A-L(A) and WNT5A-S(B) on hFOB1.19 proliferation. hFOB1.19 cells were plated at a low density, treated with CM-L(A), CM-S(B), and CM-P-conditioned mediums, and DNA content determined over the indicated days. The results shown represent one example of three biological trials. CM-L(A) has a slightly positive but significant effect on proliferation compared to CM-P and CM-S(B). CM-S(B) had no effect on proliferation compared to CM-P. The asterisks indicate significance ($p < 0.05$) comparing CM-L(A) to CM-P and CM-S(B). Bars are ±standard error
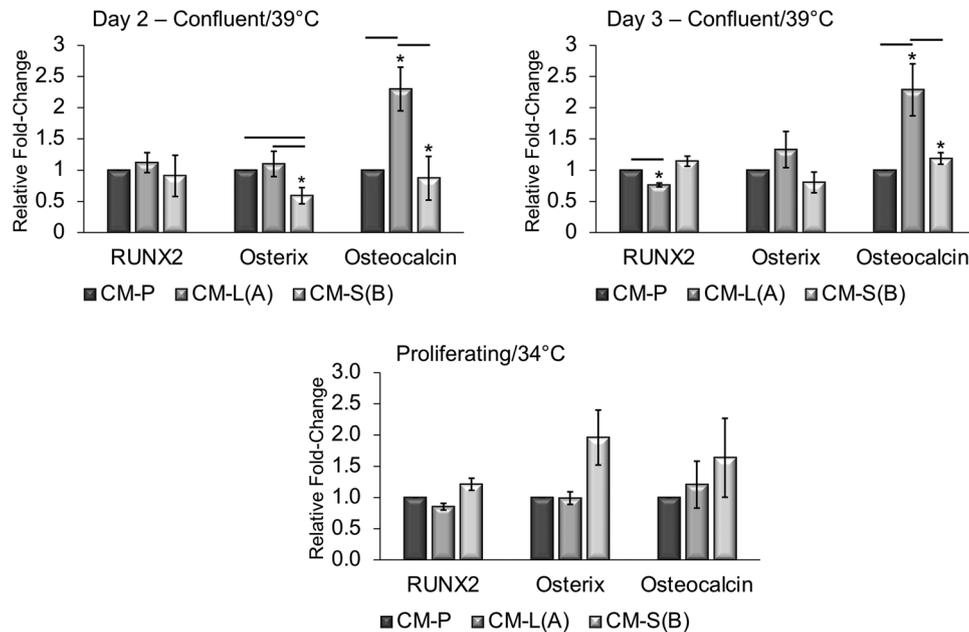
FIGURE 6 Effects of increased WNT5A-L(A) or WNT5A-S(B) on osteoblast differentiation. hFOB1.19 cells were grown under proliferating/nondifferentiating or confluent/differentiating conditions and treated with CM-P, CM-L(A), and CM-S(B). *RUNX2*, osterix, and osteocalcin transcripts levels were analyzed at days 2 and 3 in differentiating cells and at Day 2 after CM addition to proliferating cells. The average relative fold-change values (compared to CM-P, set to 1) were derived from three biological trials. Vertical bars are ±standard error. Asterisks indicate a significant difference ($p < 0.05$) of the indicated pairings (horizontal bars)
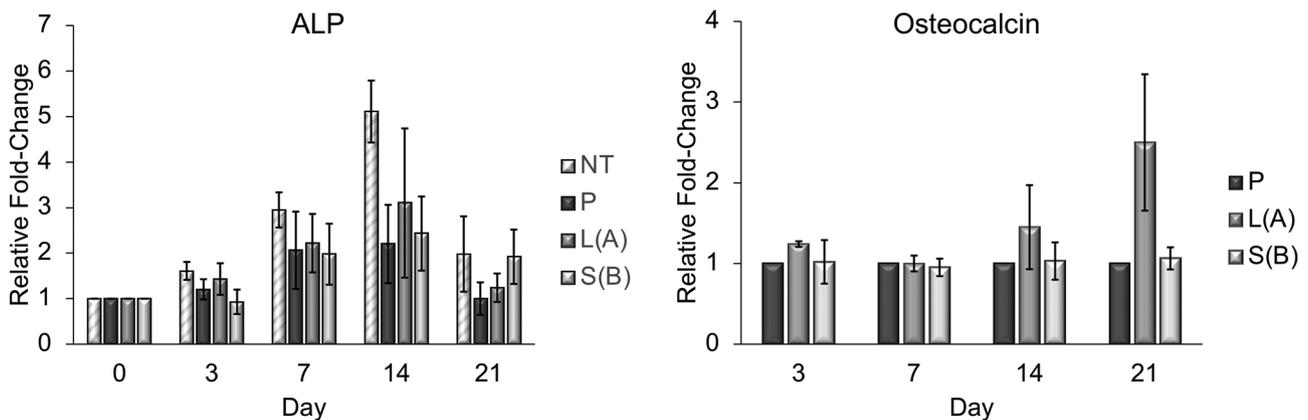

FIGURE 7 Effects of increased WNT5A-L(A) or WNT5A-S(B) on ALP activity and osteocalcin transcript levels over 21 days. hFOB1.19 cells were grown under differentiating conditions in 1:1, hFOB1.19 medium: CM-P, CM-L(A), or CM-S(B). At the days indicated cells were assayed for ALP activity and osteocalcin transcript levels. Average fold-change values relative to the nontreated (NT) for ALP activity and to the CM-P control for osteocalcin transcripts were determined from three biological replicas. Bars are ±standard error. No significant differences ($p > 0.05$) were detected among the treatment groups and controls

CE noncanonical pathways. To address this question we treated proliferating hFOB1.19 osteoblasts with CM-L(A) and CM-S(B) and at different time points the cells were analyzed for pJNK (PCP/CE) and pPKC (Ca²⁺ pathway) by flow cytometry. There was no effect of either CM-L(A) or CM-S(B) on pJNK levels in these cells (Figure 8A). CM-L(A) treatment slightly increased pPKC at 15 min (compare Geo-Means: P - 136 to L(A) - 154). At 30 min and 1 h there were no detectable changes in pPKC due to

CM-L(A) treatment. However, at 2 h in the CM-L(A) treated cells, the pPKC curve shifted to the left and the Geo-Mean was less than the value for the control CM-P, indicating that WNT5A-L(A) enhances *dephosphorylation* of the pPKC with time.

CM-S(B) treatment had a different effect on pPKC levels. At 15 min the Geo-Mean for the treated cells was slightly higher than in the control cells, similar to isoform L(A). However, by 1 h, levels of pPKC were higher
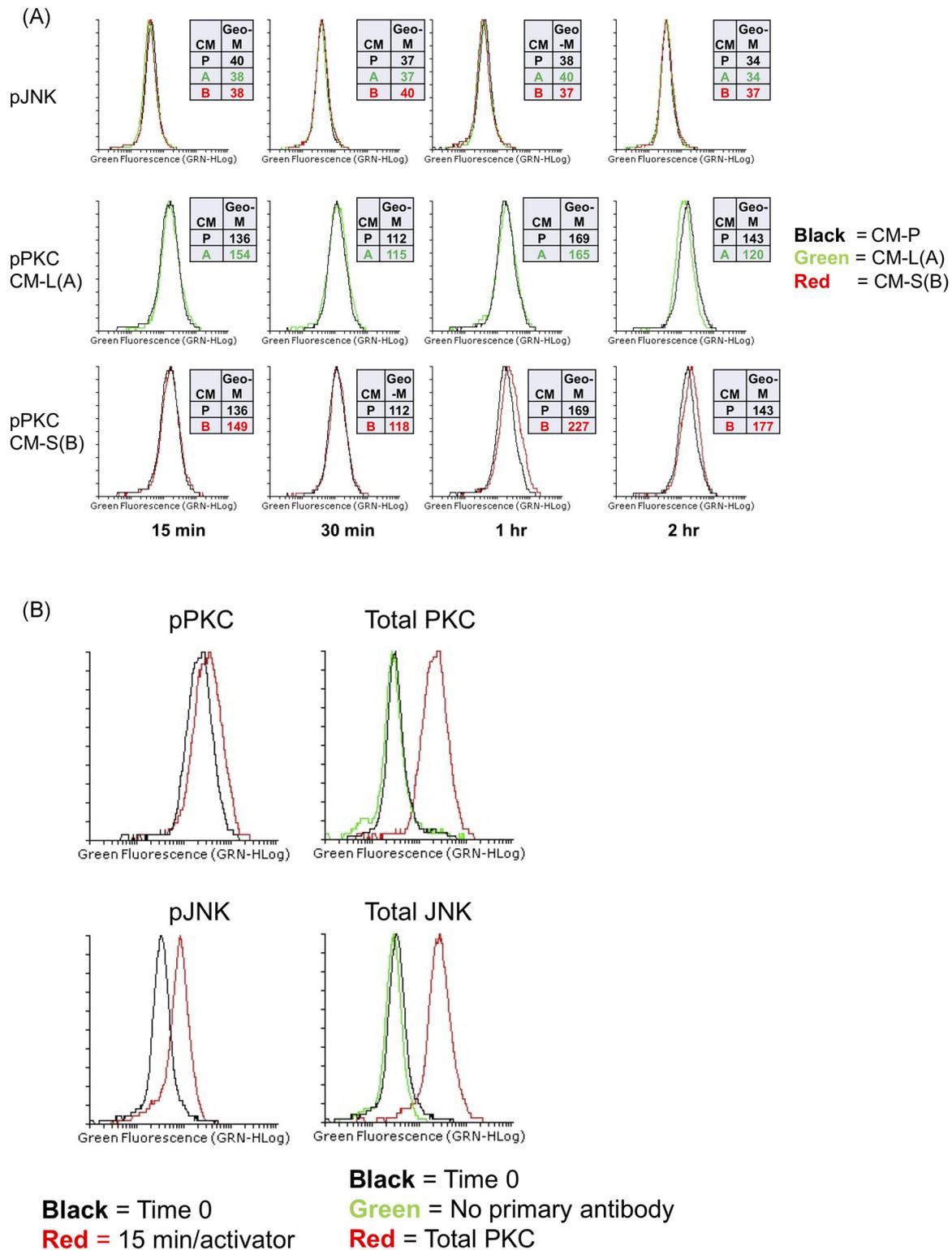
FIGURE 8 Effect of WNT5A- L(A) and WNT5A-S(B) on activation of JNK and PKC in proliferating hFOB1.19 cells. Cells were treated with 1:1, hFOB1.19 medium: CM-P, CM-(L)A, or CM-S(B) for the indicated times. Cells were immunostained with primary antibodies anti-phospho-JNK (pJNK) and anti-phospho-PKC (pPKC) and analyzed by flow cytometry. The Geo-Mean (Geo-M) indicates total fluorescence under the curve. The results shown are one of two biological replicas. (A) Results of pJNK and pPKC immunostaining of CM treated hFOB1.19 cells for the indicated times. (B) Control results from hFOB1.19 cells treated with PKC and JNK activators and immunostained for pPKC and pJNK and for total JNK and PKC proteins

compared to control cells (Geo-Means, P-169 and S(B)-227). At 2 h, the level of pPKC remains higher in the CM-S(B) treated cells compared to the control (Geo-Means, P-143 and S(B)-177). These results suggest that the isoforms differentially affect PKC activation.

We used known activators of pPKC and pJNK and detected total PKC and JNK as controls (Figure 8B). These results confirm that levels of pPKC and pJNK are easily detectable by flow cytometry and that hFOB1.19 cells contain pools of both PKC and JNK proteins.

4 | DISCUSSION

WNT5A has critical roles in early embryogenesis and is involved in cell differentiation and homeostasis. Thus, understanding the functional roles of its two major isoforms is of importance. We selected normal osteoblasts as a model to address this question, based on published data supporting the involvement of WNT5A in osteogenesis. Our results indicate that the isoforms display distinct and overlapping functions. This conclusion is supported by four findings: (1) WNT5A-L(A) has a positive effect on osteoblast proliferation, whereas WNT5A-S(B) had no effect; (2) Excess WNT5A-L(A) increased osteocalcin transcript levels in osteoblasts grown under differentiating conditions, whereas WNT5A-S(B) had no effect on osteocalcin; (3) In proliferating, non-differentiating osteoblasts, WNT5A-S(B) and WNT5A-L(A), were found to have distinct effects on PKC activation by phosphorylation; and (4) WNT5A-L(A) and WNT5A-S(B) both reduce β-catenin signaling in hFOB1.19 cells.

We detected more WNT5A-L(A) transcripts than WNT5A-S(B) in proliferating undifferentiated hFOB1.19 cells by approximately 2-fold. During osteoblast differentiation, WNT5A-S(B) transcripts are at a slightly higher or equal level to WNT5A-L(A). On Day 21, WNT5A-L(A) and WNT5A-S(B) transcript levels varied between 1×10^7 to 1×10^9 (per µg RNA). In our previous study, we found that normal human osteoblasts had approximately 11-fold more WNT5A-S(B) transcripts than WNT5A-L(A).[20] One explanation for this difference is that the hFOB1.19 are immortalized, whereas the human osteoblasts were obtained from normal bone tissue. In contrast, both WNT5A-L(A) and WNT5A-S(B) transcript levels were reduced in osteosarcoma tumor tissue samples from three patients by 10^2–10^3 fold, compared to normal osteoblasts.[20] In all three osteosarcoma tissue samples the levels of WNT5A-S(B) were lower than WNT5A-L(A). These findings indicate that the WNT5A isoforms are reduced in osteosarcoma tissue. We previously showed that increased DNA methylation and altered histone modifications are the likely cause of this decrease.[20]

Our finding that WNT5A-L(A) has a positive effect on proliferation in hFOB1.19 cells is contrary to the report of Bauer et al.[18] They found that overexpression of WNT5A-L(A) (referred to as WNT5A-L in their study) significantly decreased cell growth in breast, cervical, and neuroblastoma cancer cell lines, whereas overexpression of WNT5A-S(B) (referred to as WNT5A-S) dramatically increased growth. We found that increased WNT5A-S(B) isoform had no effect on hFOB1.19 growth. One possible explanation for this discrepancy is the use of the conditioned medium in our study versus endogenous overexpression of the isoforms in the Bauer et al. study. In addition, all three cancer cell lines most certainly have multiple cell cycle checkpoint alterations, which may allow for distinct effects on proliferation by the isoforms.

We found that knockdown of *total* WNT5A leads to decreased expression of three markers for osteoblast differentiation, *RUNX2*, osteocalcin, and ALP activity, at Day 2 of differentiation. Unfortunately, we were unable to individually knock down the WNT5A isoforms during osteogenesis. These results are consistent with the known involvement of WNT5A in osteogenesis.[7,15] Most relevant is the study of Guo et al.[16] in which they analyzed the differentiation of mouse Wnt5a −/− derived calvarial osteoblast cells. In comparison to wild-type cells, the Wnt5a defective cells had decreased expression of *Runx2*, osterix, and alkaline phosphatase. These results are similar to our findings of *total* WNT5A knockout with the exception that we found that osterix levels did not decrease but were significantly increased by Day 3. A more definitive answer to the distinct roles of the isoforms in the osteoblasts may be achieved by knocking out the individual isoforms using CRISPR technology.

Excess WNT5A-L(A) was found to *enhance* osteocalcin expression in nonproliferating, confluent hFOB1.19 cells, suggesting that noncanonical cell signaling initiated by WNT5A-L(A) leads to increased transcription of the osteocalcin gene. This finding is consistent with our results showing that *total* WNT5A knockout caused a decrease in osteocalcin transcripts. Excess WNT5A-L(A) only slightly decreased levels of *RUNX2* transcripts and had no effect on osterix. In contrast, excess WNT5A-S(B) had no effect on *RUNX*2 or osteocalcin but significantly decreased osterix transcript levels at Day 2. We found no significant effects of excess WNT5A-L(A) or WNT5A-S(B) on ALP activity or osteocalcin expression at later periods of osteoblast differentiation. This lack of a robust effect may reflect the limited involvement of WNT5A at later stages of differentiation or the fact the cells already generate sufficient WNT5A. Regardless, these results support the conclusion

that excess WNT5A-L(A) enhances osteocalcin transcript levels and WNT5A-S(B) does not.

We examined the ability of the isoforms to activate either the Ca^{2+} or PCP/CE noncanonical pathways in proliferating, undifferentiated hFOB1.19 osteoblasts by measuring phosphorylation of downstream effectors PKC and JNK using flow cytometry. No phosphorylation of JNK was detected by either WNT5A-L(A) or WNT5A-S(B). Significantly, the isoforms had different effects on PKC phosphorylation. WNT5A-S(B) produced a more robust and sustained response with pPKC, still detectable at 2 h. In contrast, WNT5A-L(A) appeared to *decrease* PKC phosphorylation over time, following some activation at 15 min. There are few studies characterizing WNT5A signaling in proliferating or differentiating osteoblasts. In one study of human osteoarthritis osteoblasts, WNT5A was shown to increase phosphorylation of JNK and to a lesser extent, PKC in osteoarthritis-derived osteoblasts compared to normal osteoblasts.[23] They found that added recombinant WNT5A [assumed to be WNT5A-L(A)] did not activate pJNK in the *normal* human osteoblasts nor was there a significant increase in pPKC. In this study, only a 15-min time point was examined. These results are in agreement with our findings of WNT5A-L(A). In another study, added WNT5A slightly increased pPKC in MC3T3-E1 osteoblasts but less than 1.5-fold above controls and at 9 min of treatment.[24] In mouse embryo stem cells that were induced to differentiate by addition of recombinant Wnt5a [assumed to be WNT5A-L(A)], only a 1.5 fold increase was detected in PKC activity at 15 and 45 min, whereas no change was detected in JNK1.[25] We detected only a slight transient increase in pPKC levels at 15 min in hFOB1.19 cells treated with WNT5A-L(A). Overall, these studies support our findings regarding WNT5A-L(A). There are no published reports on WNT5A-S(B) and its effects on PKC and JNK.

We showed, however, that both isoforms were able to reduce β-catenin signaling in hFOB1.19 and HEK293T cells, suggesting that the isoforms are acting through a similar mechanism. These findings are consistent with those of Bauer et al.[18] According to our flow cytometry results, the pathway would not involve noncanonical pathway activation of JNK or PKC. More likely, the WNT5A isoforms are reducing β-catenin pathway activity by competing with Wnt3a for binding to Fzd2.[5]

One important consideration is the likely involvement of the WNT5A isoforms in both autocrine and paracrine signaling within bone tissue. Restricting our assays to hFOB1.19 cells overlooks this possibility. WNT5A secreted from osteoblasts is known to function in a paracrine manner by binding to ROR2/Fzd receptors in preosteoclasts and activating JNK.[11,12,26,27] This leads to RANK receptor expression and osteoclast differentiation due to the binding of a RANK ligand (RANKL) also secreted from the osteoblast.[15,17] One conceivable model is that the WNT5A isoforms are preferentially functioning either in an autocrine or paracrine fashion within the bone tissue during osteoblast and osteoclast differentiation.

Osteoblasts and osteoclasts express multiple Fzd receptors (1-9) and Ror2.[11,12] The WNT5A isoforms may show preferential binding to particular Fzd/Ror2 pairs, based on their N-terminal sequences, restricting their effects to either the osteoblast or osteoclast. In addition, there is evidence that WNT5A is post translationally modified by phosphorylation and this modification is cell type-specific.[28] In this study, Wnt5a was shown to undergo serine-phosphorylation in osteoclasts but not osteoblast cells, and that the phosphorylated Wnt5a acts in a paracrine manner affecting osteoblast cells. Perhaps the unique N-terminal sequences determine if the WNT5A isoform is posttranslationally modified, directing the protein to act either in a paracrine or autocrine fashion. The 18 amino acids unique to WNT5A-L(A) include three serine residues. It will be important to examine the phosphorylation state of the WNT5A isoforms secreted from both osteoblasts and osteoclast cells.

Recent studies provide insights into the possible secretory pathways of Wnt proteins and adds further support for the idea that the WNT5A isoforms are differentially directed to either an autocrine or paracrine pathway. Moti et al.[29] eloquently showed that Wnt-containing secretory vesicles can associate with specialized, signaling filopodia called cytonemes, which deliver the Wnt-containing vesicle to the receiving cell. Alternatively, the Wnt-containing Golgi transport vesicle can fuse with the plasma membrane, releasing the Wnt ligand to act in an autocrine fashion. This model is supported by the finding that Wnt8a through Ror2/Fzd receptor binding and noncanonical PCP pathway activation leads to induction of cytoneme formation, allowing for paracrine Wnt signaling via cytonemes from that same cell.[30] Perhaps one of the isoforms, based on its unique N-terminus, activates the production of the cytonemes through Ror2/Fzd autocrine signaling, whereas the other isoform is transported in a secretory vesicle via cytonemes to neighboring cells.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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