BHANDARI, DRISTI., M.S. Role of WNT5A Isoform L(A) and Isoform S(B) During Osteoblast Differentiation. (2018)
Directed by Dr. Karen Katula. 78 pp.

WNT signaling has been characterized as a critical signaling cascade in many aspects of embryogenesis, cell differentiation and tissue homeostasis. Appropriate WNT signaling requires dynamic expression of WNTs along with their receptors, ensuring a proper balance between cell differentiation and proliferation. As a result, perturbation in WNT signaling by aberrant expression underlies various congenital malfunctions, cancer and other diseases. This study is focused on one family of WNT ligands called WNT5A. WNT5A plays a central role in primary axis formation and limb bud extension in development and has regulatory functions in mesenchymal cell differentiation, including bone formation. Moreover, WNT5A exhibits tumor suppressive as well as oncogenic properties in a wide range of cancers. Notably, the WNT5A gene encodes for two protein isoforms, isoform L(A) and isoform S(B). There is evidence that the isoforms are functionally distinct in cancer. However, there is a critical gap in understanding of the functional roles of the WNT5A isoforms in normal cell function. In this study, I investigated the functional contributions of the WNT5A isoforms during the process of normal osteoblast differentiation using the human fetal osteoblast cell line, hFOB1.19. The results show an increase in transcripts of both isoforms for normal differentiating osteoblasts. A trial examined for 21 days revealed that isoform L(A) and S(B) exhibit similar pattern during differentiation where both show gradual increase with progressing days. In addition, we identified an increase in expression of the WNT5A protein in hFOB1.19 cell line. The increase in isoform expression was correlated with molecular

markers of osteoblast differentiation including RUNX2, osterix and osteocalcin and alkaline phosphatase (ALP) activity. Increasing the individual isoforms L(A) and S(B) in hFOB1.19 cells did not inhibit increases in RUNX2 and osteocalcin expression. Our results suggest a slight decrease in osteocalcin levels with treatment of L(A) and S(B)-conditioned medium (CM) on day 3 compared to the control. In contrast, RUNX2 was increased at day 2 and decreased at day 3 in isoform-CM treated cells. ALP activity decreased at day 7and 10 for both L(A)-CM and S(B)-CM treated cells, but only L(A)-CM showed a significant change at day 10. Next, I attempted to knockdown the individual isoforms and total WNT5A transcripts using siRNA techniques. Knockdown was achieved total WNT5A. Results show that by knocking down WNT5A, the early differentiation marker, RUNX2 decreases significantly by day 3 whereas the later differentiation marker, osteocalcin increases significantly by day 3. Taken together, these findings suggest that WNT5A isoform L(A) and S(B) appear to be involved in osteogenesis and the onset and increases of differentiation molecular markers show correlations with increases in the isoforms. However, as both WNT5A isoforms displayed similar patterns of expression during normal differentiation and after alteration of the individual isoform levels, a functional distinction between the isoforms during osteoblast differentiation cannot be asserted.

ROLE OF WNT5A ISOFORM L(A) AND ISOFORM S(B) DURING OSTEOBLAST DIFFERENTIATION

by

Dristi Bhandari

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2018

Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis written by Dristi Bhandari has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Karen Katula and my committee members Dr. Ramji Bhandari and Dr. Zhenquan Jia for their patience, expertise, and continued support throughout my project. I would like to thank my former lab members, especially Ahmed Gamaleldin Abdelhamed Elsayed Elshaarrawi, for their help and friendly guidance during this research. I am also thankful for my family and friends who provided constant support and encouragement throughout the project. Finally, I want to thank the UNCG biology department for funding my research.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

WNT Signaling

WNTs are a highly conserved family of 19 secreted cysteine-rich glycoproteins involved in regulation of embryogenesis and tissue homeostasis (Angers and Moon, 2009). The name "WNT" is derived from a combination of two homologous genes, wingless (Wg) gene in drosophila and Int gene in mouse mammary tumor virus (MMTV) (Sethi and Vidal-Puig, 2010). WNT receptors bind to the amino-terminal cysteine-rich domain of the Frizzled (Fz) receptor, thereby eliciting a cascade of signal transduction events which include the canonical or β-catenin dependent pathway and the non-canonical or β-catenin independent pathway. The canonical WNT pathway involves nuclear translocation of β-catenin for transcriptional activation of target genes. Alternatively, the non-canonical pathway functions independently of β-catenin and is further branched into two major signaling cascades, planar cell polarity (PCP) and WNT/Ca^{2+}, to promote cytoskeletal organization and migration initiated by WNT-receptor interaction. Frizzled (Fz) family of receptors act as a common receptor for both pathways which function along with several co-receptors including the low-density-lipoprotein receptor-related protein 5/6 (LRP5/6) and receptor tyrosine kinase-like orphan

receptor ½ (Ror ½) in β-catenin dependent and β-catenin independent pathway, respectively (Huang and Klein 2004). WNT-Fz mediated activation of downstream pathway is dependent upon cellular context and the particular combination of receptor and co-receptors utilized (Niehrs, 2012).

Canonical WNT Signaling

The key event of the canonical WNT pathway is the cytoplasmic stabilization and nuclear translocation of the adherens junction associated-protein β-catenin (Komiya and Habas, 2008). In its active form, β-catenin functions as a transcriptional activator for the T cell factor (TCF)/lymphoid enhancer factor 1 (LEF-1) family of DNA binding proteins which can activate genes involved in cell proliferation such as c-myc and cyclin D1. β-catenin is regulated by a cytoplasmic pentameric complex or the destruction complex involving the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). In the absence of WNT, cytoplasmic β-catenin is phosphorylated at the amino-terminal by CK1 and targeted by GSK3 via phosphorylation for ubiquitination and subsequent proteasomal degradation, thereby inhibiting β-catenin mediated upregulation of target genes (De, 2011).

In the presence of WNT ligands (e.g. WNT3a), GSK3 is inactivated, resulting in an un-phosphorylated but an active β-catenin. This event is stimulated upon binding of WNT to its seven-transmembrane receptor Fz and LRP5/6 (McDonald and He, 2012).

The WNT-fzd-LRP5/6 trimeric complex causes recruitment of the intracellular protein dishevled (Dvl) and GSK3 to the membrane, thereby signaling LRP5/6 phosphorylation by CK1. This leads to inhibition of the β-catenin destruction complex, thereby resulting in the cytoplasmic stabilization of β-catenin followed by its nuclear translocation where multiple WNT target genes are activated via β-catenin binding to TCF/LEF transcription factors (Niehrs, 2012). The genes activated by this pathway are associated with embryogenesis, tissue function and disease. Alterations in this pathway that result in an aberrant WNT activation are implicated in several types of cancer.

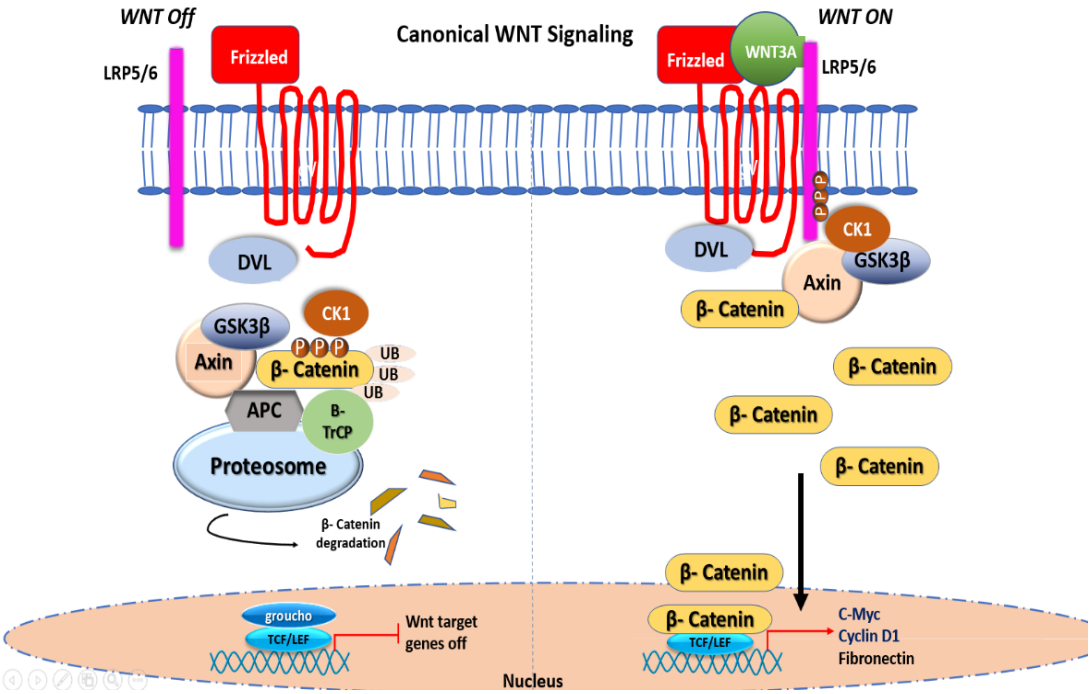


Figure 1. Canonical/ β-dependent WNT Signaling Pathway. (Left Panel) In the absence of WNT ligand, β-catenin is targeted for proteasomal degradation, representing the off state of WNT canonical signaling. (Right Panel) Nuclear translocation of β-catenin in the presence of a WNT ligand, turning on WNT target genes.

Non-canonical WNT PCP Signaling

The non-canonical WNT signaling pathway is independent of β-catenin and involves two different mechanisms of signaling. The highly characterized and evolutionary conserved of the two pathways is known as the planar cell polarity pathway/cell elongation pathway (PCP/CE) or the WNT/JNK signaling pathway. This pathway mediates the polarization of the individual cells along the axis of polarity. The role of WNT PCP/CE pathway in regulation of cell adhesion, motility and movement in various developmental morphogenesis has implications in tumor metastasis during altered signaling (Wang, 2009).

PCP signaling is initiated by the binding of non-canonical WNT proteins (WNT5A and WNT11) to the Fzd-receptor, causing subsequent recruitment of the cytoplasmic scaffold protein, Dvl to the plasma membrane. Downstream effectors of Dvl protein includes dischevelled associated activator of morphogenesis 1 (DAAM1) and rac family small GTPase 1 (Rac1) which elicit varying signal transduction events for cytoskeletal reorganization in polarity and cell movements. DAAM1 forms a complex with Dvl and ras homolog gene family, member A (RhoA) by binding to Dvl protein at its carboxy- terminus and RhoA at its amino- terminus (Wang, 2009). This in turn causes activation of Rho associated kinase, ROCK and signals cytoskeletal rearrangement (Sato et al., 2006). Dvl-Rac1 complex leads to an activation of the c-Jun N-terminal Kinase (JNK)-type mitogen-activated protein (MAP) kinase cascade, resulting in propagation of cell movement and polarity signals (Wang, 2009).

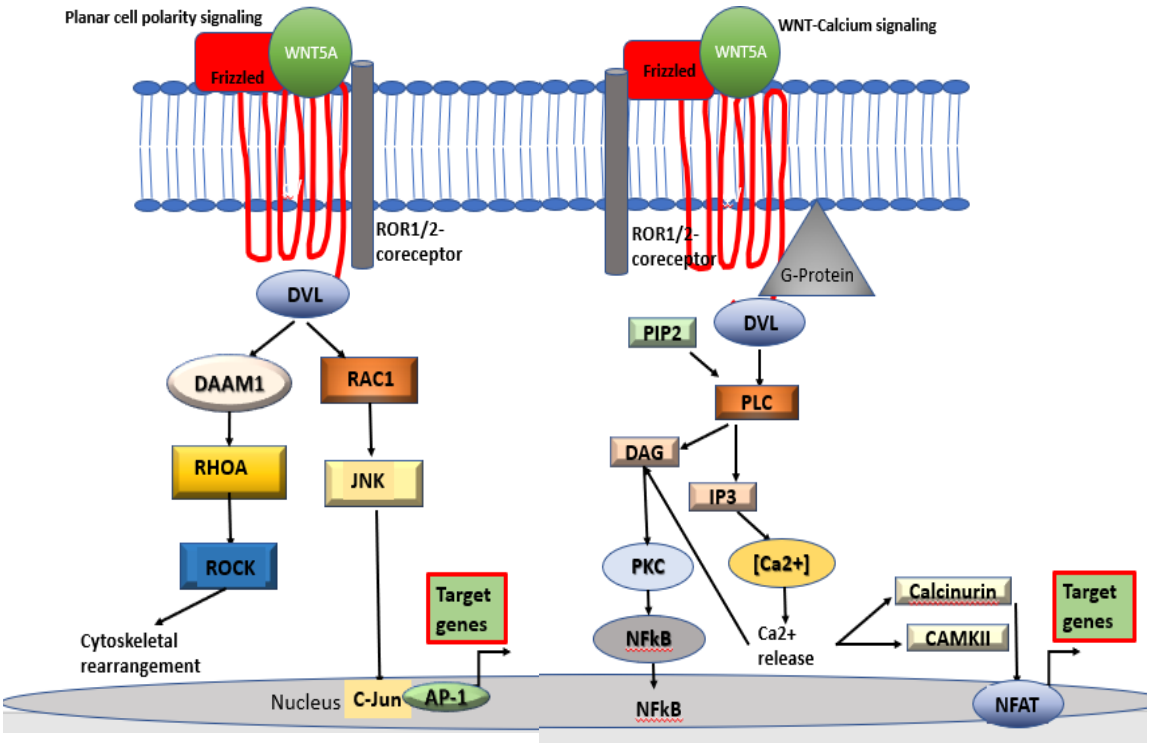


Figure 2. Non-canonical WNT Signaling Pathways. (Left Panel) Planar cell polarity pathway. (Right Panel) WNT-Ca^{2+} signaling pathway.

Non-canonical WNT/Calcium Pathway

This pathway involves the mobilization of free intracellular Ca^{2+} which regulates many cellular processes including the actin cytoskeleton and cell motility. Pathway is initiated by WNT ligand binding to the Fz receptor and co-receptor, receptor tyrosine kinase-like orphan receptor-1 and -2 (ROR1/2) that causes recruitment of Dvl to the membrane. Synthesis of inositol trisphosphate (IP3) and diacylglycerol (DAG) is initiated. IP3 and DAG are derived from the membrane-bound phospholipid phosphatidyl inositol 4,5-bisphosphate (PIP2) via the action of the membrane bound enzyme,

phospholipase C (PLC) located on the plasma membrane. IP3 diffuses through the cytosol and via interaction with the calcium channels present on the membrane of the endoplasmic reticulum, causing calcium ions to be released. Calcium ions bind and activate calmodulin which activates calcium calmodulin- dependent protein kinase II (CAMKII) and calcinurin (Cn), a protein phosphatase. In a second pathway, the released Ca^{2+} binds DAG which activates protein kinase C (PKC). Both CAMKII and PKC phosphorylate and activate the various regulatory proteins, nuclear factor kappa-light-chain-enhancer of activated beta cells (NFkB) and cAMP response element-binding protein (CREB), which are nuclear transcription factors. Calcinurin (Cn) can activate cytoplasmic protein nuclear factor associated with T cells (NFAT) via de-phosphorylation. Activated NFAT, CREB and NFkB translocate to the nucleus and may boost the expression of several genes in neurons, cardiac and skeletal muscle cells, and pro-inflammatory genes in lymphocytes. In addition, WNT-Fz interaction may activate phosphodiesterase 6 (PDE6) in a calcium-dependent manner, leading to a decrease in cyclic guanosine monophosphate (cGMP) (De, 2011).

WNT5A Gene and Protein Structure

WNT family member 5A protein is encoded by the WNT5A gene. The cytogenic location for WNT5A gene is on the short arm of chromosome 3 at position 14.3 (NIH US National Library of Medicine, Genetics Home Ref). Genomically, the WNT5A gene is mapped to 55,465,715-55,490,539 bp of chromosome 3. (Ensembl:ENSG00000114251). This gene consists of 5 conserved exons which initiate distinct WNT5A transcripts.

Among the five, this study is focused in the proteins generated from two of these transcripts (Ensembl:ENST00000234634.8 and ENST00000497027.5). The transcripts for each of these proteins are derived from alternative transcriptional start sites. The upstream sequences for the transcripts are referred to as promoter A and promoter S(B), which generate two isoforms termed WNT5A-Long (L) and WNT5A-Short (S), respectively. These isoforms hereafter will be described as Isoform L(A) for WNT5A-Long and Isoform S(B) for WNT5A-Short. Isoform L(A) transcription is initiated at exon1, whereas isoform S(B) transcript is initiated at exon 1B, located within the first intron of isoform L(A) (Figure 3). Consequently, the primary transcripts of isoforms A and B differ in size. The transcripts have unique first exons but share exons 2,3, 4 and 5. The truncated proteins generated by the mature mRNA are 380 AA [Isoform L(A)] and 365 AA [Isoform S(B]), differing by 15 AA. However, as WNT5A is a secreted protein, it is processed in the endoplasmic reticulum by cleavage of the N-terminus. Bauer et al (2013) showed that the isoform L(A) and S(B) proteins are cleaved at unique sites, generating isoforms that now differ by 18 AAs (Figure 4).

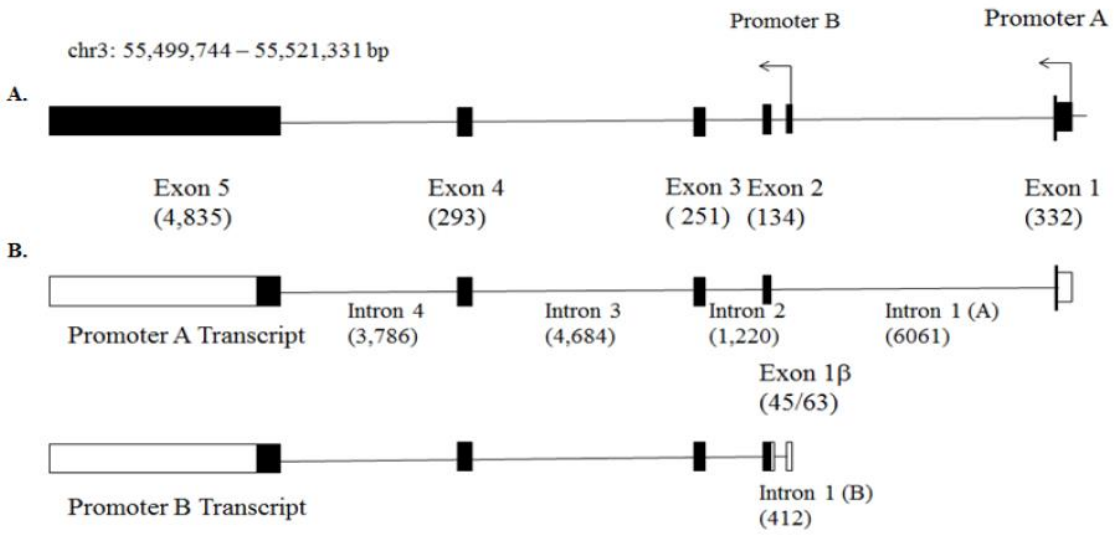


Figure 3. WNT5A Gene Structure.(A) Exon Intron Transcriptional start site. **(B)** Promoter L(A) and Promoter S(B) primary transcripts. Black boxes represent protein coding regions; open boxes are untranslated regions. The parenthesis indicate intron and exon lengths. Isoform L(A) is initiated at exon 1 and Isoform S(B) is initiated at Exon 1β. The diagram is based on Katula et al. 2012.

Both WNT5A Isoforms L(A) and S(B) are secreted lipids that act as signaling molecules in WNT pathways. WNT proteins are globular, α-helix rich and highly hydrophobic as a result of post-translational lipid modification. WNT5A is palmitoylated at Cys^{104} which enables binding of WNT to Frizzled receptor and is glycosylated at Asn^{114}, Asn^{120}, Asn^{311} and Asn^{325} for proper protein folding and secretion. Hence, both palmitoylation and glycosylation are essential lipid modifications that ensure secretion of WNT5A in its active form (Kurayoshi et al., 2007). The posttranslational palmitoylation of WNT5A (and all WNT ligands) occurs in the ER due to the activity of the enzyme,

porcupine (PORCN). Inhibitors of PORCN are being implicated to block regulation and function of WNT5A isoforms (Liu et al., 2013).

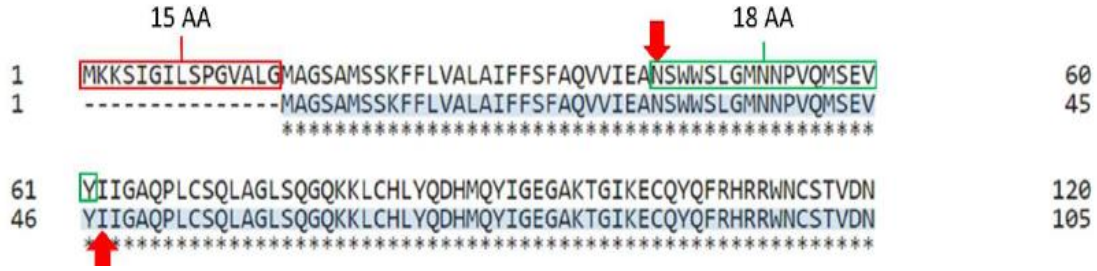


Figure 4. Amino Acid Sequence Alignment of WNT5A Isoforms L(A) and S(B). The blue highlighted region represents the Isoform S(B) residues and the un-highlighted region represents Isoform L(A). Before processing, Isoform L(A) is 15 amino acids longer than Isoform S(B) at the N-terminus denoted by the red box. Post-processing N-terminal residue is denoted by the red arrow for each isoform. Thus, the N-terminal sequence for Isoform L(A) post processing is NSWWSLGM… while the N-terminal sequence for Isoform S(B) is IIGAQPLC… (Bauer et al., 2013 and Carl Manner III 2016). Post-processing, the two proteins differ in length by 18 amino acids indicated by the green box. Thus, Isoform L(A) consists of 337 amino acids post-processing and Isoform S(B) consists of 319 amino acids post-processing. The figure and information are derived from Manner III (2016)

Protein Isoforms and Differential Function

There are an estimated 19,000-20,000 genes within the human genome. The general question is, “how can so few genes generate such a complex organism”? In reality, the complexity of genetic information is increased by alternative splicing and distinct promoters, generating multiple isoforms. It is predicted that many of these isoforms have distinct functions (Park and Graveley, 2007). Examples of such important

isoforms for WNT5A signaling in osteogenesis are RUNX and peroxisome proliferator-activated receptors (PPAR).

Runx2 is an important transcription factor for osteoblast differentiation and bone development. Studies have shown that Runx2 encodes three major isoforms, Runx2-type I, Runx2-type II, and Runx2-type III. Among the three, Runx2-type I and Runx2-type II are present in mice and humans. Both Runx2-1 and Runx2-2 are expressed in osteoblasts but Runx2-1 has been shown to also exist in undifferentiated mesenchymal stem cells, pre-osteoblasts and precursors of chondrocytes. Lines of evidence support that the Runx2 isoforms are functionally distinct, and one of the contributing factors to that difference is the difference in the N-terminal amino acids between Runx2-I and Runx2-II; Runx2-I and Runx2-II differ by 19 unique amino acids (Park et al., 2001). Moreover, it is established that Runx2-II is derived from distal promoter 1, whereas Runx2-I is derived from proximal promoter 2 (Stock and Otto, 2005).

Peroxisome proliferator-activated receptor, PPAR, is a ligand-dependent nuclear receptor that has implications in many biological processes including cell differentiation and cell-fate determination. To date, three isoforms of PPAR named PPAR-α, PPAR-Δ and PPAR-γ have been described which are encoded by distinct genes (Blitek and Szymanska, 2017). Although all the isoforms bind to the same response element, they exhibit distinct tissue distribution and biological function (Shi et al., 2001). PPAR-α is predominantly expressed in the liver and has lower expressions in muscle heart and bone. PPAR- γ is expressed in endothelial cells and vascular muscle cells and is known to play

a role in energy metabolism (Tyagi et al., 2011). PPAR- Δ is expressed ubiquitously in the whole body and is known to be upregulated in colorectal carcinogenesis (Takayoma et al., 2006).

The finding of these studies for Runx2 and PPAR are significant as they further elucidate the importance of multiple protein isoforms and differential functions that are associated with them. The studies provide evidence that generation of the isoforms, due to alternative splicing, and distinct trans-activation sites can have differential regulatory roles across tissues and during embryogenesis. Moreover, it can be asserted that individual isoforms may have specific spatial and temporal roles. Due to their implications in normal physiological functions as well as wide number of pathologies, it is important to elucidate the roles of the various isoforms, including WNT5A. This study should provide insights on the pattern of the WNT5A isoforms L(A) and S(B) during osteoblast differentiation which can help understand mechanisms of pathologies that affect the bone.

WNT5A Isoforms and Evidence for Differential Function

WNT5A isoforms are functionally distinct in cancer cells. Bauer et al. (2013) used three different cancer cell lines, MDA-MB-231 (breast carcinoma), HeLa (cervix carcinoma) and SH-SY5Y (neuroblastoma tumors) to investigate the functional differences in the isoforms. First, they characterized the isoform protein sequences before and after processing in the ER. Importantly, they identified the ER signal sequence

cleavage site for each isoform and showed that the processed isoforms differ by 18 AAs at the N-terminus (See Fig. 3). Next, they overexpressed and knocked down each isoform in the cancer cell line. Results indicated that isoform L(A) decreases proliferation whereas isoform S(B) increases proliferation. They further showed that isoform L(A) was downregulated while Isoform S(B) was overexpressed in 15% of breast carcinomas. Both isoform L(A) and S(B) were expressed in normal uterine cervix epithelium. However, in cervix carcinoma, they found isoform L(A) to be downregulated while isoform S(B) was upregulated. Moreover, elevated isoform S(B) was associated more frequently with high risk neuroblastoma compared to low risk neuroblastoma. Overall, they show that in the selected human cancer, isoform L(A) and S(B) behave as a tumor suppressor and an oncogene, respectively (Bauer et al., 2013).

In recent paper, it was reported that isoform S(B) transcript was relatively high in comparison to isoform A transcript in colorectal cancer (CRC) lines, HCT116, which showed positive correlation with tumor depth of the CRC patients. Knocking down isoform A led to an increase in proliferation while knocking down of isoform S(B) inhibited proliferation. In a colony-forming assay, they detected significant increase in colony numbers and sizes in HCT116 that had isoform A knockdown and decrease in colony numbers and sized in cells with isoform S(B) knockdown. Next, they determined the effect of each isoform on spheroid formation of HCT116 cells and found that with reduced isoform S(B) mRNA levels, the cells formed smaller colonies as compared to the control siRNA and reduced isoform A mRNA, hinting that the isoforms exert distinct

regulatory functions in CRC cell growth. They also determined whether the cancer promoting activity of isoform S(B) was mediated by cell cycle effect or apoptosis. Results of this assay showed that HCT116 cells transfected with siRNA of isoform S(B) caused a decrease in G_0/G_1 phase which resulted in an accumulation of G_2/M phase. They further showed that transfection with siRNA of isoform S(B) caused a significant increase in HCT116 cell apoptosis compared to control siRNA through expression of the death receptor, FAS and downregulation of the tumor necrosis factor (TNF). In addition, knockdown of isoform A led to CDK4 accumulation, hence, caused an increase in proliferation of the CRC cell lines (Hunag et al., 2017).

There is evidence that WNT5A isoforms are differentially regulated at the promoter level, allowing for fine tuning of WNT5A expression. Research from this lab using NIH3T3 mouse fibroblasts and Caco-2 (epithelial cells), showed that promoter L(A) is more active in humans and mouse. Interestingly, promoter S(B) was reported to display a distinct pattern of expression in Caco-2 cells in which removing the upstream sequences of promoter S(B) resulted in a continual increase in expression. This effect was not displayed in the mouse fibroblasts, suggesting that removal of the negative regulatory sequences of promoter S(B) rendered them active in Caco-2 cells but not in NIH3T3 cells. Furthermore, treating the NIH3T3 cells with tumor necrosis factor (TNF)-alpha caused an upregulation of promoter L(A) and S(B) activities, with S(B) showing a stronger response. Using inhibitors to block TNF signaling, they found that p38 and MEk1/2 kinases appear to be involved in the activities of promoter L(A) and S(B) and

may play a role in TNF-alpha stimulation of activity (Katula et al., 2012). This study provides further support to the idea that the WNT5A isoforms play a critical role in the distinct functionalities of WNT5A. Thus, characterizing the differential expression and function of the WNT5A isoforms in development is of critical importance due to its high involvement in embryogenesis and pathologies ranging from developmental malfunctions to cancer.

WNT5A Cell Signaling and Role in Osteogenesis

Osteogenesis is a skeletal developmental process that involves the differentiation of mesenchymal stem cells to osteoblasts or specialized fibroblasts. Osteoblasts are the pre-cursors to bone forming cells called osteocytes (Milona et al., 2003). Osteoblasts function in secreting and mineralizing the bone matrix. The differentiation of osteoblasts from mesenchymal stem cells is mediated by a complex set of transcription factors and signaling proteins such as bone morphogenetic proteins (BMPs), Indian hedgehog (Ihh), RUNX2, Osterix, and proteins involved in WNT signaling (Zhang et al. 2011). Characterization of osteoblast differentiation can involve three stages: cell proliferation, matrix maturation and matrix mineralization (Rutkovskiy et al., 2016).

Indian hedgehog (Ihh) is required for activation of Runx2, which plays a major role in mesenchymal differentiation to pre-osteoblasts. *Osterix* or Sp7 is a downstream gene of RUNX2 and an osteoblast specific transcription factor. Sp7 is critical for commitment of pre-osteoblastic cell differentiation into mature osteoblasts (Chang et al.

2011). Hence, it is required for expression of the osteoblast-specific markers, including type I collagen, osteocalcin, osteonectin and osteopontin (Rutkovskiy et al., 2016).

RUNX2 is a transcription factor that has a crucial role during osteogenesis. RUNX2 requires a co-transcription factor, Cbf-beta, for DNA binding of RUNX2 and RUNX2 dependent osteoblast differentiation and bone formation (Komori, 2006). Initially, RUNX2 is expressed in pre-osteoblasts and highly expressed in immature osteoblasts which significantly decreases in mature osteoblasts. Hence, RUNX2 is an indicator of early stages of osteoblasts.

RUNX2 contains a transactivation domain and has been linked to turning on certain genes. Another gene that can be activated by RUNX2 includes osteocalcin. Osteocalcin is another important biomarker of osteoblast differentiation. Osteocalcin is a hormone secreted by active osteoblasts as they reach a stage of maturation. Hence, this marker is known to appear later in the differentiation process as matrix mineralization takes place (Huang et al., 2007).

WNT proteins have been implicated in regulation of osteogenesis through the canonical β-catenin dependent and the non-canonical β-catenin independent pathways. WNT5A-induced activation of non-canonical WNT signaling has been associated with suppression of adipogenesis, thereby, promoting osteoblast differentiation from mesenchymal stem cells. Interestingly, there seems to be a cross talk between non-canonical ligand, WNT5A and canonical receptor, LRP5/6, inducing differentiation of

osteoblasts. A study found that WNT5A-increases BMP, leading to increased Sp7. They provide evidence that Sp7 upregulates the expression of LRP5/6 by binding to the Sp1 site in the proximal LRP5 promoter. (Okamoto et al., 2014). An *in vitro* study using mouse embryonic stem cell (ESC) discovered WNT5A to be expressed early during osteogenesis (Keller et al., 2016).

The canonical WNT pathway has been extensively studied in association with osteogenesis. It is also known that WNT5A has a role in osteoblast differentiation by activation of the non-canonical WNT pathway. The non-canonical pathway activation by WNT5A is shown to be Ror2 mediated in cultured pre-osteoblasts, MC3T3-E1 cells (Nemoto et al., 2012). Taken together the studies linking WNT5A with osteogenesis, it can be asserted that WNT5A activates canonical and the non-canonical pathway, depending on the receptor, cell type and even BMPs. The dual role of WNT5A in osteogenesis can also be due to the differential expression and function of the isoforms of WNT5A. There is lack of literature investigating the role of WNT5A isoforms A and B in activation of either canonical or non-canonical WNT pathway, leading to promotion or inhibition of osteogenesis. It is of importance that the function of isoforms be analyzed to understand the mechanisms leading to differentiation of osteoblasts as this has implications in tissue engineering and repair.

Project Overview and Specific Aims

WNT5A has a critical role in regulating normal process of embryogenesis, including differentiation, proliferation, stem-cell renewal, cell polarity and disease. Alteration in WNT5A signaling, resulting in aberrant activation or inhibition of non-canonical WNT pathway, have been associated with cancer progression, in which it has been shown to function as an oncogene and a tumor suppressor (Zhou et al., 2017). A potential explanation to such contradictory functions of WNT5A can be due to functional distinctions between WNT5A isoforms L(A) and S(B), which may be mediated by the differential activation of non-canonical signaling pathways. Previous studies in this lab have shown distinct patterns of expression of the two isoforms during mouse development (Manner III 2016). Moreover, the promoter sequences of the isoforms are distinct and results from this lab indicate that the promoters are differentially regulated (Katula et al., 2012).

Based on these and other data, it is likely that the WNT5A isoforms display distinct patterns of expression and the proteins are functionally distinct. To investigate these questions, I analyzed the role of the WNT5A isoforms in osteoblast differentiation. My hypothesis was that WNT5A isoforms till have distinct patterns and levels of expression during osteogenesis. I predicted that both isoforms will increase in differentiating osteoblasts but that the levels of the isoforms will differ. To test my hypothesis, I completed the following aims:

Aim 1. Determine the pattern of WNT5A isoform A and B expression during osteoblast differentiation.

Aim 2. Determine the effects of altering WNT5A isoform A and isoform S(B) levels in osteoblast differentiation.

CHAPTER II

MATERIALS AND METHODS

Cell Line and Culture Techniques

The SV40 immortalized human fetal osteoblastic cell line hFOB1.19 (CRL-11372) were purchased from American Type Culture Collection (ATCC). hFOB1.19 cells provide a model system for studying human osteoblast differentiation and function. Frozen cells were stored in liquid nitrogen until required. The frozen cell vials were allowed to thaw via gentle agitation in a 37°C water bath. The thawed-hFOB1.19 cells were cultured in high glucose, DMEM/F12 without phenol red (Invitrogen) medium supplemented with 10% fetal bovine serum and 0.3 g/L G418 and 1x penicillin/streptomycin (100x stock; 10,000 units penicillin and 10,000 μg of Streptomycin per ml). Cells were cultured in 75 cm^2 flasks (T75) at a permissive temperature (34°C) in a humidified incubator with 5% CO_2.

Osteoblast Differentiation and Cell Collection

To induce *in vitro* osteogenic differentiation, hFOB1.19 cells were grown to complete confluency. The cells were plated in different well sizes depending on the experiment. For RNA and protein isolation, cells were plated in 60 mm, 6-well or 24-well plates. For alkaline phosphatase assays, the cells were plated in 48-well. Cells were

plated at 1 x 10^6 (60mm), 4.4 x 10^5 (6-well), 4.4 x 10^4 (48-well), 1 x 10^4 (96-well) to allow for 2-3 days to confluency. Once confluent, the cells were shifted to a restrictive temperature (39.4°C) in the normal/osteogenic medium (see above) supplemented with ascorbic acid (0.1g/L), β-glycerol phosphate ($5x10^{-3}$ mol/L), $25(OH)_2D_3$(10^{-7} mol/L) and menadione (10^{-8} mol/L). Cells were collected at indicated times for each experiment. For time points beyond day 3 after induction, the medium (supplemented with differentiation components) was changed every 3-4 days. Cells were collected by first washing with 1x phosphate buffered saline (PBS), and then scraping the cells in 1x PBS. The cell suspension was centrifuged for 2-3 minutes and the cell pellet quick frozen in liquid nitrogen and stored at -80°C.

RNA Isolation

Total RNA was isolated using the stored cell pellets according to the SV Total RNA Isolation System (Promega) or Quick-RNA Mini Prep (ZYMO RESEARCH). Absorbance for RNA samples were measured at 260/280 nm using the Synergy 2 multi-mode microplate reader (BioTek) and RNA concentration (ng/µl) was calculated by the instrument. Samples with low concentrations were evaporated using Savant SpeedVac Concentrator (SVC100H) to achieve a concentration sufficient for cDNA synthesis.

Conversion to cDNA and qPCR

Approximately 0.1-1 µg of mRNA was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, K1671) for qPCR. For each assay

set, the same amount of RNA was added per reaction. For qPCR, a master mix was prepared in a 1.5 ml microfuge tubes for each primer set with nuclease-free water, 2x Taqman buffer and the associated primer. Then, 29.7 µl of the Master Mix was added to smaller microfuge tubes for each RNA sample being assayed. 3.3 µl of each cDNA sample was added to its assigned tubes and mixed thoroughly by gently pipetting up and down. 10 µl of each sample reaction mix was added in triplicates in a 48 or 96 well plate and centrifuged at 4000 rpm for 5 minutes before running qPCR. The following Applied Biosystems Taqman primers were used: β-Actin (Hs00357333 g1), RUNX2 (Hs01047973 m1), osterix (Hs01866874 s1), osteocalcin (Hs01587814 g1). The custom prepared Taqman primers for WNT5A isoform L(A) and S(B) are shown in Table 1. These primers were designed and verified in this lab (Katula et al., 2012).

qPCR analysis of differentiation was performed using Microsoft Excel. The averages of the C_t values were calculated from the technical triplicates values. The three C_t values for each sample (days 0, 3, 7, 10, 17 and 21) for a given target genes (isoform L(A), isoform S(B), RUNX2, osterix and osteocalcin) were standardized to the actin value which was used as the housekeeping gene. This generated a ΔC_t value (gene - actin) for both control (day 0) and a particular experimental group (one of the other days being analyzed). A $\Delta\Delta C_t$ value was generated by subtracting the control group from the treated. Fold-change relative to control or $2^{(-\Delta\Delta Ct)}$) was calculated from the $\Delta\Delta C_t$ value.

Table 1. Taqman Primers for WNT5A Isoforms L(A) and S(B)

	Sequence (5' → 3')	**Length (base)**	**Product Size (bp)**
Human Isoform L(A)			
Forward	TCGGGTGGCGACTTCCT	17	77
Reverse	CGCCCCCTCCCCCTCGCCATGAAG	17	
Probe	AGAAGCCCATTGGAATATTAAGCCCGG	27	
Human Isoform S(B)			
Forward	CCTCTCGCCCATGGAATT	18	71
Reverse	CTTCAGGTAACCTTATAATTCGGG	24	
Probe	CTGGCTCCACTTGTTGCTCGGCC	23	

Alkaline Phosphatase Assay (ALP)

Alkaline phosphatase was measured using the Bio Vision Alkaline Phosphatase Activity Colorimetric Assay Kit (Bio Vision Cat# K412-500). All buffers and components used for the assay were supplied in the kit. For collection of cells for the ALP assay, cells were initially washed with 1 X PBS and then scraped in 1 X PBS. Aliquots of the suspended cells were made for ALP and RNA isolation in separate tubes. The cells were pelleted by centrifugation, the supernatant was removed, and the pellets were stored at -80° C (see below for details). Frozen cell pellets were taken up in Alkaline Phosphatase Buffer and homogenized by pipetting up and down. The samples were centrifuged at 13000 g for 3 min in a microfuge tube to remove insoluble material. A p-nitrophenyl phosphate (pNPP) substrate tablet was taken up in 2.7 ml Alkaline

Phosphatase Buffer. 60 µl of each sample was pipetted into each of four wells of a 96-well plate to which 20 µl Alkaline Phosphatase Buffer as added. At least three control wells with buffer only were included on each plate. After all the samples were pipetted, 50 µl of the pNPP substrate as added. The samples were incubated at room temperature in the dark for 60 minutes. Stop solution was added to each well at the end of the incubation time. The absorbance was measured using the Synergy 2 multi-mode microplate reader (BioTek) at excitation 485 nm and emission 528 nm.

Western Blot

Determination of Protein Concentration of Cell Lysates

Cells were lysed in Cell Lytic Reagent (Sigma-Aldrich, C2978) containing protease inhibitors (Pierce, NL 181839) and incubated on ice for 15 min with periodic mixing. The samples were centrifuged for 10 min at top speed in a microcentrifuge. The supernatant was removed and transferred to a clean tube. Protein concentrations were determined by colorimetric assay using bovine serum albumin (BSA) as the standard at 0, 5, 10, 15, 20, 25, and 30 µg. Each standard was assayed in duplicate. 5µl of cell lysate was added to one well of a 96-well plate, repeated in duplicate. 150 µl of Protein Assay Reagent dye (Pierce® ThermoScientific, 22660) was added to each well, standards and samples. The plate was incubated for 5-10 mins with shaking at 660 nm wavelength using the BioTek microplate reader. The background absorbance (0µg BSA) was subtracted from all values. Averages were determined for duplicate samples and standards. A standard curve of protein concentration vs 660 nm absorbance was

generated from the BSA values and a trend-line generated using Microsoft Excel. From the line equation, the protein concentrations of the samples were determined, using their 660 nm absorbance values (minus background).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein levels of WNT5A during osteoblast differentiation was assessed using western blot. Proteins were separated by molecular weight on a 10% polyacrylamide gel. The running gel consisted of 10% acrylamide, 0.375 M TRIS-HCL (pH 8.8), 0.1% SDS, 0.06% ammonium persulphate (APS) and 0.08% N, N, N', N'-Tetramethylethylenediamine (TEMED). Immediately, the running gel mix was added to the pre-made gel chambers. A thin layer of water saturated isobutanol was added to the top of the gel mixture. After polymerization, a stacking gel was prepared consisting of 3.75% acrylamide, 0.125 M TRis-HCL (pH 6.8), 0.1% SDS, 0.06% APS and 0.125% TEMED. The polymerized running gel was washed with distilled water and the stacking gel added. A well-forming comb was immediately added and the gel was left to polymerize. After polymerization, the comb was removed and the wells were washed with running buffer after filling the wells with running buffer (0.025M TRIS/ 0.1M Glycine, 0.1% SDS). Approximately 50µg of cell lysate protein for each sample was loaded per well. This was prepared by mixing the determined volume of sample (=50 µg) and adjusting all the samples to the same volume with Cell Lytic Reagent. 5X Lane Marker Reducing Sample Buffer (Thermo Scientific #39000) was added to each sample for a 1X concentration. Samples were heated to 90°C for 5 minutes and loaded onto the

polyacrylamide gel along with a molecular weight markers (BIO-RAD Precision Plus Protein Dual Xtra Standards Cat# 161-0377) for reference. The gel was electrophoresed at constant 40 mA for approximately 1 hour. After the tracking dye reached the bottom of the gel, the gel was removed from the apparatus and prepared for blotting (see next section).

Western Blotting

Proteins were transferred onto nitrocellulose membrane from the gel using a BIO-RAD Mini Trans-Blot Cell (Cat#015214) apparatus. The transfer cassette was prepared by stacking the following in order on the cassette dark side: two sponges, Whatman blotting paper, SDS-PAGE gel, nitrocellulose membrane, Whatman blotting paper, and two sponges. Each layer (except for the gel) was pre-immersed in transfer buffer (0.025M TRIS/ 0.19M Glycine, 20% methanol). The cassette was closed and placed into the transfer chamber, filled with transfer buffer, a cold pack added, and transferred overnight at constant 30V.

Immunodetection

The blotted nitrocellulose membrane was washed briefly in TTBS (Tris-Tween-Buffered-Saline; 0.01 M Tris (pH 8.0), 0.15 M NaCl, 0.01% Tween 20) and stained with 10% Ponceau-S stain for 15 minutes. Residual Ponceau-S stain was removed by washing with distilled water and the blot with revealed protein bands photographed. Stain was removed from the nitrocellulose membrane by washing in TTBS. The membrane was

blocked in 3% dried powdered milk in TTBS by shaking for 1 hour. The blocked membrane was incubated overnight with shaking at 4°C in primary antibody anti-WNT5A wnt5a/b (Cell signaling, Cat#C27E8) (6 µl in 6 ml of 1% TTBS). The following day, 1° antibody was removed, and the membrane washed with TTBS (3x for 5 min and 1x for 15 min). The membrane was incubated for 1 hour at room temperature in secondary antibody, Goat anti-Rabbit IgG (Abcam, Cat#ab97051). This was followed by washing the membrane in the same manner as described previously with TTBS. The blot was detected using super-signal® West Pico Chemiluminescent Substrate (Thermo Scientific Prod# 34087) and the signal detected using the BioRad (ChemiDoc Documentation Imaging System).

The same blot used to detect WNT5A was used for tubulin detection. The nitrocellulose membrane was re-blocked in 3% milk in TTBS for 1 hour. This was followed by incubation of the membrane with primary mouse anti-tubulin antibody (E7-S Developmental Studies Hybridoma Bank, U, of Iowa), which was composed of 119 µl antibody in 5 ml 1% milk TTBS for 1 hour at room temperature. The membrane was washed in TTBS as mentioned above and incubated for 1 hour by shaking in secondary antibody, goat anti-mouse (Abcam, Cat#ab97023) composed of 1 µl antibody in 15 ml 1% TTBS milk (1: 15,000 dilution). The blot was detected using the Super-signal West Pico Chemiluminescent Substrate as previously described.

The intensities of the bands were determined using BioRad (ChemiDoc™ Documentation) Imaging System by background subtraction method. The density

(INT/mm2) of the bands for both WNT5A and tubulin were determined by selecting the volume of the bands. Using Microsoft Excel, the WNT5A/ α-tubulin densities were calculated for normalization of the protein levels across the blot.

Treatment of hFOB1.19 Cells with "Conditioned Medium" (CM) Expressing Isoform L(A) or Isoform S(B)

For ALP assay, hFOB1.19 cells were grown to confluency in 48-well dishes at a seeding density of 4.4×10^4 per well. The cells were incubated at 34°C for three days (time required to achieve confluency). 4-6 wells of cells were collected on day 0 when confluency was achieved and before addition of CM or differentiation components. The remaining cells were treated with the following conditioned mediums containing the WNT5A isoforms CM mixed 1:1 with the hFOB1.19 differentiation medium: 1) Control CM prepared from the parental CHO cells (P-CM); 2) Isoform L(A) CM (L(A)-CM); and 3) Isoform S(B) CM (S(B)-CM). Each treatment was repeated in quadruplet wells for the following time points: day 0, 3, 7, and 10. Following treatment with differentiation medium plus conditioned medium, cells were shifted to 39.4°C. Cells were collected at day 3 for ALP using the following protocol: Medium was aspirated from the wells and 100 µl of Alkaline Phosphatase Buffer was added to each well. This was followed by scraping the cells with a small pipet tip and transferring the cells in buffer to a pre-labeled 1.5 ml microfuge tubes. Cells were frozen in the Alkaline Phosphatase Buffer at -80°C. Cells were collected for ALP assay on days 7 and 10 in the same manner.

For isolation of RNA, hFOB1.19 were grown in 24-well dishes to confluency and treated with differentiation medium and the different conditioned medium as described in the previous paragraph. Cells were collected and RNA was isolated as previously described. qPCR analysis was performed and fold-changes were generated as described earlier. For CM treated, day 0 P-CM was used as the control and samples were analyzed for the differentiation markers, RUNX2 and osteocalcin.

Knockdown of WNT5A Isoform L(A) and S(B) by siRNA Transfection

Short interfering RNA (siRNA) for WNT5A isoforms L(A) and S(B), total WNT5A and non-silencing control sequences used for knockdown assay were identical to those used by Bauer et al. (2013). These siRNAs were purchased from Dharmacon, ThermoScientific. The sequences are shown in Table 2. A 10 μM stock of each siRNA was prepared in sterile distilled water. The siRNA's 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 (Invitrogen, Thermo Fisher Scientific, 13778-030) according to the manufacturer's instructions. Cells were transfected when approximately 60 to 80% confluency was achieved, which generally took one day after plating. The medium was changed to Opti-MEM reduced serum medium (Gibco® by Life Technologies, 11058-021) prior to transfecting the cells. For 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 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 were harvested from 96-well plate for ALP, 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 again on day 2 (2 days after inducing differentiation) and day 3 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 biomolecular markers of differentiation. qPCR analysis was performed and fold-changes generated as described previously.

There were two approaches used for analysis: 1) Values for siRNA-T, siRNA-L(A) and siRNA-S(B) were determined relative to the control, siRNA-S from each day. If the siRNA exerted no effect, then the values should be "1". If the siRNA decreased transcript levels, then the values should be less than "1". 2) RUNX2 and osteocalcin fold-changes were calculated relative to day 0 of each siRNA. This analysis will indicate if the fold-change pattern of RUNX2 and osteocalcin on days 2 and 3 are the same or different in the four siRNA treatment groups.

Table 2. Sense and Antisense Strand Sequences for Short Interfering RNAs (siRNA's) Used During Transfection (Obtained from Bauer et al., 2013)

	Sense Strand	**Antisense Strand**
siLUC (siRNA targeting firefly luciferase)- Non-silencing control siRNA	5'-CGUACGCGGAAUACUUCGA**dTdG**-3'	5'-UCGAAGUAUUCCGCGUACG**dTdG**-3'
siWNT5A-LS(AB) (pan isoforms- siRNA targeting human *WNT5A* mRNA, exon 3)	5'-CCCUGUUCAGAUGUCAGAA**dGdT**-3'	5'-UUCUGACAUCUGAACAGGG**dTdT**-3'
siWNT5A-L(A) (long isoform- siRNA targeting human *WNT5A* mRNA, exon 1)	5'-CCCGGUCGCUCCGCUCGGA**dTdT**-3'	5'-UCCGAGCGGAGCGACCGGG**dTdT**-3'
siWNT5A-S(B) (short isoform- siRNA targeting human *WNT5A* mRNA, exon 1β)	5'-UUCUGGCUCCACUUGUUGC**dTdC**-3'	5'-GAACCAAGUGGAGCCAGAA**dTdT**-3'

CHAPTER III

RESULTS

Previous studies have shown that WNT5A is critically involved in the process of cellular differentiation and there is strong evidence that WNT5A is involved in osteogenesis. However, the roles of the WNT5A isoforms L(A) and S(B) in osteogenesis have not been investigated. This study aims to investigate the functions of WNT5A isoforms, isoform L (A) and isoform S (B) during osteoblast differentiation by completing the following: 1) measuring the levels of WNT5A isoforms L(A) and S(B) during normal differentiation; 2) determining the effect of overexpression of isoform L(A) and S(B); and 3) determining the effect of knockdown of isoform L(A) and S(B) transcripts/proteins on osteoblast differentiation. For these studies, I used the normal human fetal osteoblast cell line, hFOB1.19. These studies should provide critical insights into the functional distinctions, if any, between the WNT5A isoforms.

Pattern of WNT5A Isoform A and B Expression During Normal Osteoblast Differentiation

To investigate the pattern of the WNT5A isoforms, hFOB1.19 cells were grown to confluency and treated with differentiation media. Cells were harvested before inducing differentiation as a control and at specific time-points during the differentiation

period. Gene expression analysis was performed by qPCR to determine any differences in the levels of isoform L(A) and isoform S(B) transcripts during osteoblast differentiation.

Three independent trials of days 0, 3, 7, and 10 were carried out and analyzed (Table 3, Figure 5). An average fold-change for isoform L(A) and isoform S(B) was determined for this analysis (Table 3). The results indicate that both isoforms L(A) and S(B) transcripts increase during osteogenesis. By day 3 of differentiation, an increase was detected for both WNT5A isoforms; on average, isoform L(A) showed a 3-fold increase and isoform S(B) a 5-fold increase (Table 3). On day 10, isoform S(B) showed a greater fold-increase by approximately 2x compared to isoform L(A). Although there was an increase at all three days, there was no significant difference between the days for either isoform L(A) or isoform S(B). However, in two of the trials, both isoforms L(A) and S(B) transcript levels were higher at days 7 and 10 in comparison to day 3 (Table 3). On-average the fold-changes ranged from 6 to 13 (Table 3).

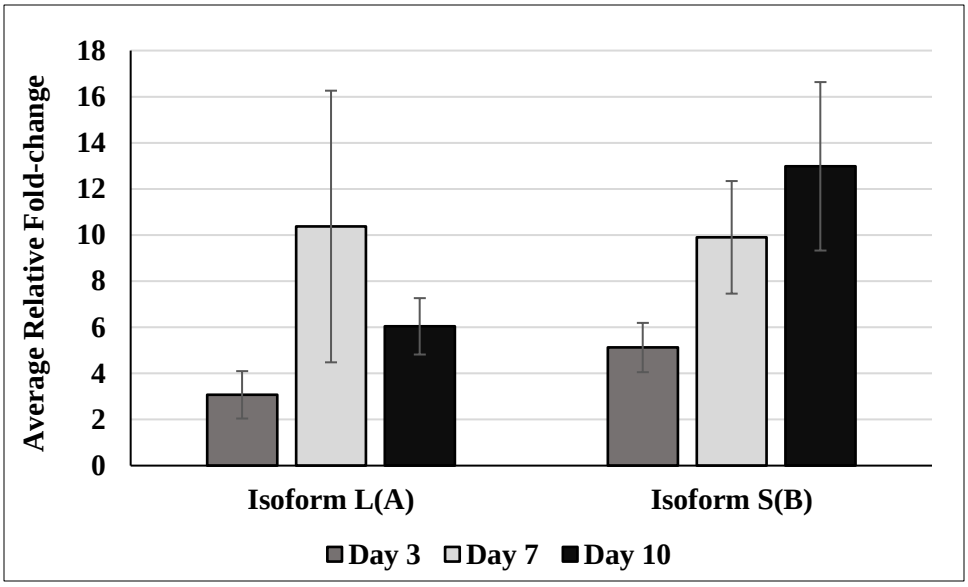


Figure 5. Fold-change in WNT5A Isoform L(A) and S(B) Transcripts During Normal Osteoblast Differentiation. Fold-change in transcript levels were determined at days 3, 7 and 10 of hFOB1.19 differentiation relative to day 0. Actin was used as the housekeeping gene to standardize the C_t values for each sample. The values shown are the averages of three independent trials. The bars indicate standard error. There was no significant difference ($P \leq 0.05$) comparing the different days using the student T-test.

Table 3. Average Fold-changes of WNT5A Isoform L(A) and S(B) Derived From Three Independent qPCR Trials

	Days	Trial 1	Trial 2	Trial 3	Average
Isoform L(A)	**3**	5.12	2.25	1.86	3.08
	7	7.25	21.76	2.08	10.36
	10	6.3	8.01	3.80	6.04
Isoform S(B)	**3**	7.25	4.22	3.89	5.12
	7	13.96	10.22	5.52	9.90
	10	15.71	5.75	17.49	12.98

For trial 3, the assay was extended to days 17 and 21. As shown in Figure 6a and b, both isoform transcripts display further increases at days 17 and 21. The isoform L(A) fold-increase at day 21 was 5x more compared to day 3. There was a 4x increase in fold-change at day 21 compared to day 3 for isoform S(B) and 5x fold-increase at day 17 compared to day 3. At day 10, isoform S(B) fold-change was 4.6x higher than isoform L(A) on the same day. Overall, the fold-increases for trial 3 were greater for WNT5A isoform S(B) than isoform L(A), although replicas of this assay must confirm these results. These data confirm that both isoforms L(A) and S(B) transcripts begin increasing during early osteogenesis and continue to increase at later stages.

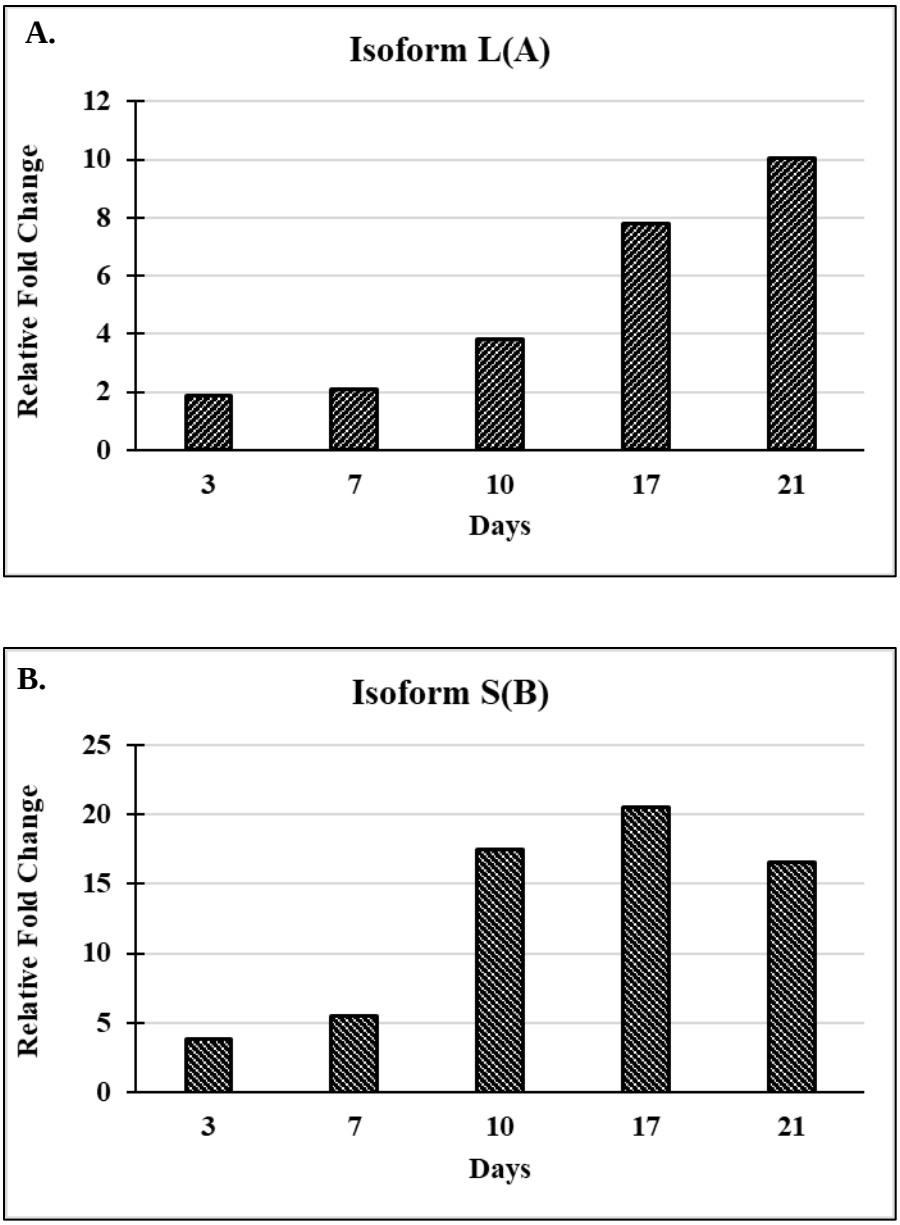


Figure 6. WNT5A Isoform Transcript Levels up to 21 Days of Osteogenesis. hFOB1.19 cells were induced to differentiate and cells collected at the indicated time points. RNA was isolated and used for qPCR analysis of isoform transcript levels. The fold-change is relative to day 0. A) Isoform L(A) and (B) Isoform S(B).

The actual transcript numbers of isoform L(A) and isoform S(B) were determined by generating standard curves using purified and quantified isoform L(A) and S(B) PCR products (see Materials and Methods) and using the line equation of the standard curve to calculate number of molecules of cDNA template in the qPCR reaction (Figure 7a and b). From these values, the number of transcripts per µg RNA was determined for each day (0, 3, 7, 10, 17 and 21) (using the data in Figure 5C.) For days 0, 3, 7, 10 and 17, 0.0225 µg equivalence RNA was used per qPCR reaction whereas for day 21, 0.0093 µg equivalence RNA was used.

The results reveal that the number of transcripts for both WNT5A isoforms L(A) and S(B) increase from day 3 to day 21. This would be expected based on the calculations of relative fold-change. However, at day 0, there was approximately 2.2x greater number of isoform L(A) transcripts than isoform S(B). By day 3, the number of transcripts were essentially the same for the two isoforms. At days 7, 10 and 17, there were slightly more isoform S(B) transcripts with values of (B)/(A) of 1.3, 1.6 and 1.25. At day 21, the value of B/A was 0.75, indicating fewer isoform S(B) transcripts than isoform L(A). The lower number of isoform S(B) transcripts at day 0, combined with the higher number of transcripts at other time points, likely explains the greater fold-change in isoform S(B) transcripts when determined relative to day 0 (fold-change). Overall, these results suggest that the difference in transcript numbers of isoform L(A) and S(B) is relatively small during the course of osteoblast differentiation and that both isoforms show an increase in transcript numbers during osteogenesis.

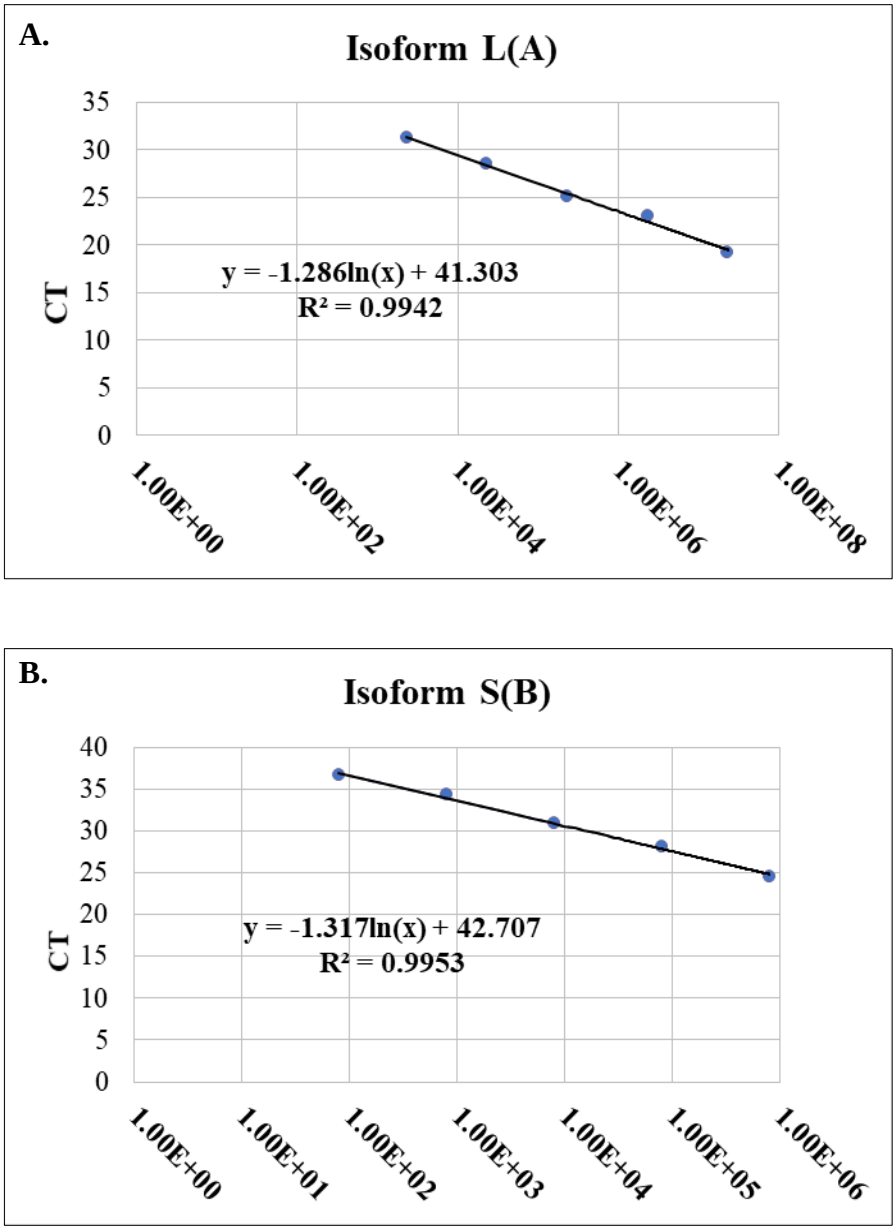


Figure 7. qPCR Standard Curves for A) Isoform L(A) and B) Isoform S(B). Purified and quantified PCR products for isoform L(A) and S(B) at 10-fold dilutions were amplified. The C_t values of each transcript number was graphed and the line equation was determined as shown.

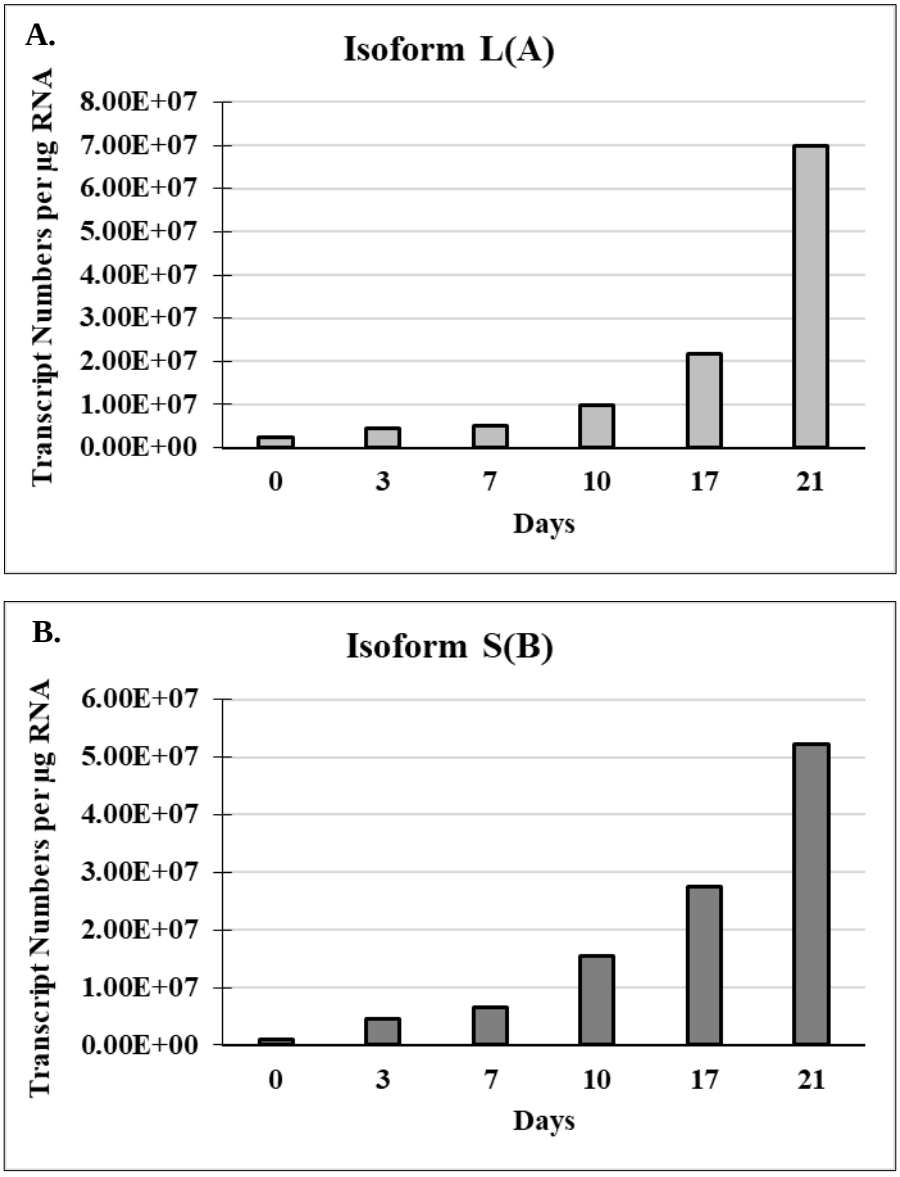


Figure 8. Isoform L(A) and S(B) Transcript Numbers During Osteoblast Differentiation. C_t values for each of the days shown were used to determine actual transcript numbers from the equations in Figure 7. These values were converted to "transcript numbers per µg RNA", based on the amount of RNA used for the cDNA reactions. A) isoform L(A) transcript numbers and B) isoform S(B) transcript numbers.

Expression of Differentiation Markers During Osteogenesis

Next, I determined the expression pattern for the osteogenesis differentiation markers, to allow for comparison to the WNT5A isoform expression patterns. The expression of molecular markers of osteoblast differentiation were determined by qPCR analyses, which included RUNX2, osterix and osteocalcin. An Alkaline Phosphatase (ALP) assay was performed to further confirm differentiation of the hFOB1.19 cell line.

RNA isolated from differentiating hFOB1.19 cells used for the analysis in Figure 6 was also analyzed by qPCR for the three differentiation markers (Figure 9). RUNX2 transcripts showed an approximately 4-fold increase by day 3, relative to day 0. At day 7 RUNX2 displayed a 7x increase in fold-change compared to day 3 and approximately 26x fold-change increase relative to day 0. This level was maintained with a slight decrease at days 10 and 17. By day 21, RUNX2 transcript levels showed a fold-change of approximately 1.9, which is less than the fold-change at day 3, indicating that RUNX2 levels are reduced at later stages of osteogenesis. RUNX2 is a primary transcription factor required for osteogenesis. An early increase in RUNX2 would be expected.

Similar to RUNX2, osterix is a transcription factor required for osteogenesis and bone formation. The fold-changes for osterix over the course of 21 days were lower than for RUNX2 (ranging between 2 and 6.5). Osterix showed some increase at day 17 and 21 (an approximately 3x increase in fold-change) compared to days 3, 7 and 10. These results show that osterix transcripts increase in differentiating osteoblasts and that this

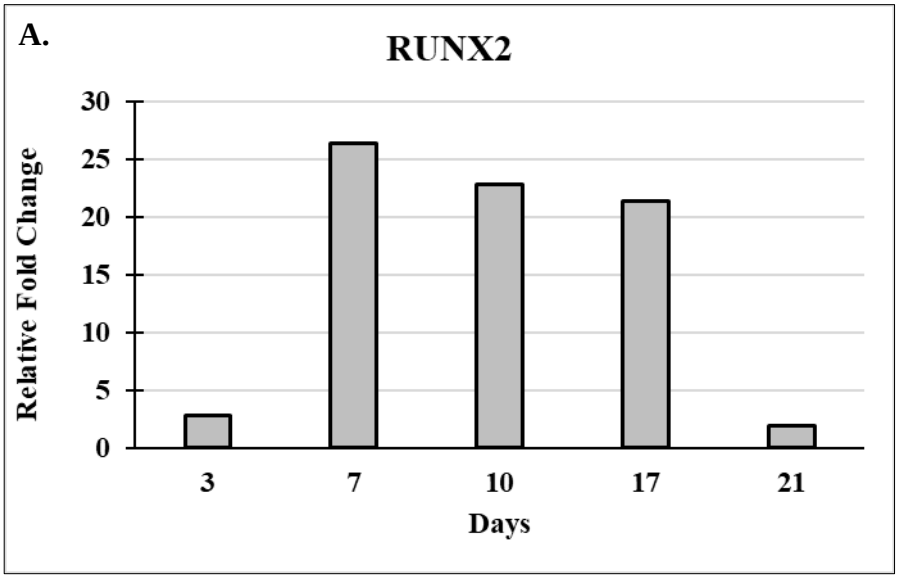
increase continues to later stages of osteogenesis. The molecular expression of osterix has yet to be fully delineated. However, it has been suggested that RUNX2 is involved in regulation of osterix (Huang et al., 2007).

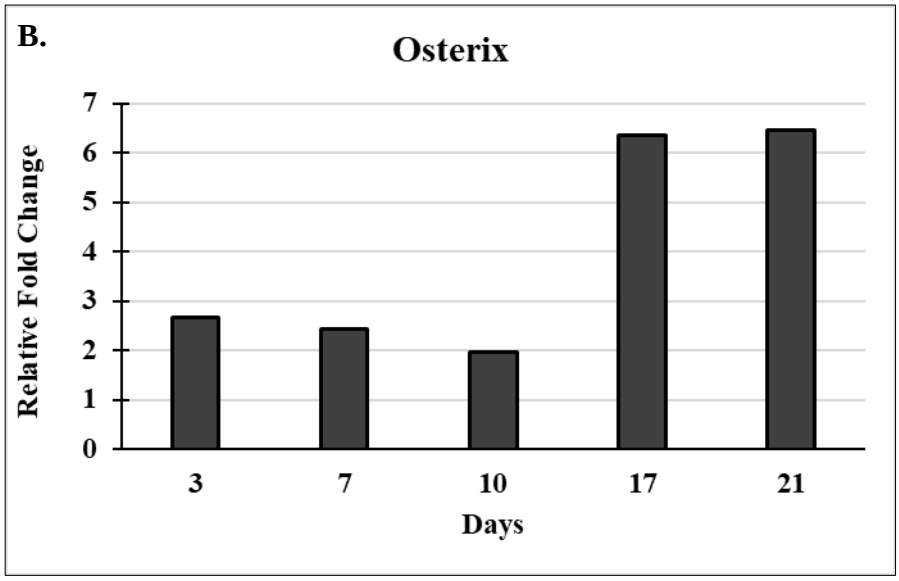
Relative changes in osteocalcin transcript levels were also measured. Osteocalcin is an osteoblast secreted hormone that is involved in bone mineralization and calcium homeostasis. It plays a greater role in the later events of osteogenesis. Our data shows a large fold-change in osteocalcin transcript levels, relative to day 0 at all the days analyzed. The fold-changes were higher for osteocalcin than for RUNX2 and osterix. The increases in fold-change ranged from 200 to 1000- fold. At day 3, there was an approximately 450 fold-change. There is a decrease at day 7 to approximately 230-fold and then increases again at day 10, 17 and 21 to approximately 750, 800 and 1000-fold respectively. These results correspond to the known involvement of osteocalcin at later days of differentiation.

Other qPCR trials assaying for the differentiation markers at days 3, 7 and 10 were conducted (data not shown). The trend was similar in that RUNX2 showed a decrease with progression of differentiation. Osterix showed an increase and osteocalcin had the highest increase, especially at day 10. The results were as expected and consistent with the data derived for day 0 to 21 qPCR trial. These results confirm hFOB1.19 differentiation during a period in culture up to 21 days. The increases in osterix and osteocalcin at later days of differentiation, correspond to increases in WNT5A isoform L(A) and S(B) transcript levels (Figure 8).

ALP activity has been shown to increase during osteogenesis and is a distinct marker of differentiation. ALP activity was analyzed at days 0, 3, 7 and 10 during hFOB1.19 differentiation (Figure 10). A significant increase ($p<0.05$) in activity at day 3 of approximately 5-fold compared to day 0 was detected. On day 7, ALP activity showed a decrease when compared to activity at day 3 and was not significantly different than at day 0. On day 10, ALP activity was significantly higher than at day 3 with a p-value less than 0.001. The decrease at day 7 was not expected. In another ALP assay (Figure 14), ALP activity was found to increase when assayed at days 7 and 10 for the control but differentiating cells. It is likely that the cell numbers for day 7 in Figure 10 were less than for the other days. To control for this, we determined the amount of DNA in the samples as a measure of cell numbers. Expressed as OD-ALP/OD-DNA, the fold-change value for day 7 is greater than days 0 and 3. However, the fold-change for day 10 is reduced. Since completing this assay, a modified approach has been used to ensure equal cell numbers. This approach was used for all other ALP assays. In comparison to day 0 however, all the observed time points showed a greater ALP activity. In general, these results further confirm that differentiation is occurring in the induced hFOB1.19 cells.

Based on the increases in differentiation markers, RUNX2, osterix, osteocalcin and ALP activity, it was confirmed that the hFOB1.19 induced cells are differentiating. The fold-changes and increases in transcript numbers of WNT5A isoforms L(A) and S(B) transcripts correlate with these changes.


A.
RUNX2
Relative Fold Change
30
25
20
15
10
5
0
3
7
10
17
21
Days


B.
Osterix
Relative Fold Change
7
6
5
4
3
2
1
0
3
7
10
17
21
Days

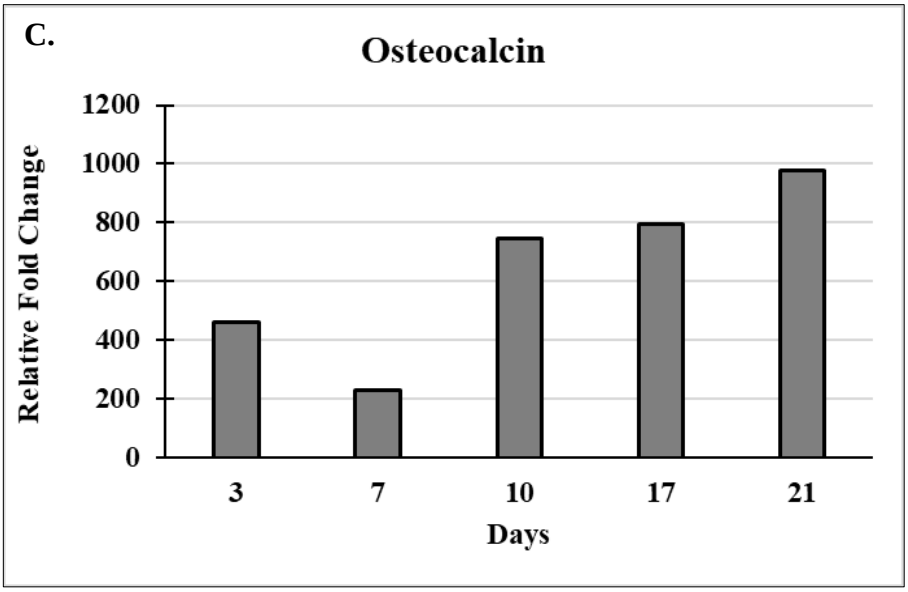


Figure 9. Fold-change of Differentiation Molecular Markers, A) RUNX2, B) Osterix, and C) Osteocalcin in hFOB1.19 Undergoing 21 Days of Osteogenesis. A) RUNX2= Early differentiation marker; B) Osterix= Regulated by RUNX2; and C) Osteocalcin= Later differentiation marker. The fold-changes are relative to the control, day 0 without induced differentiation. The indicated days were assayed for RT-qPCR.

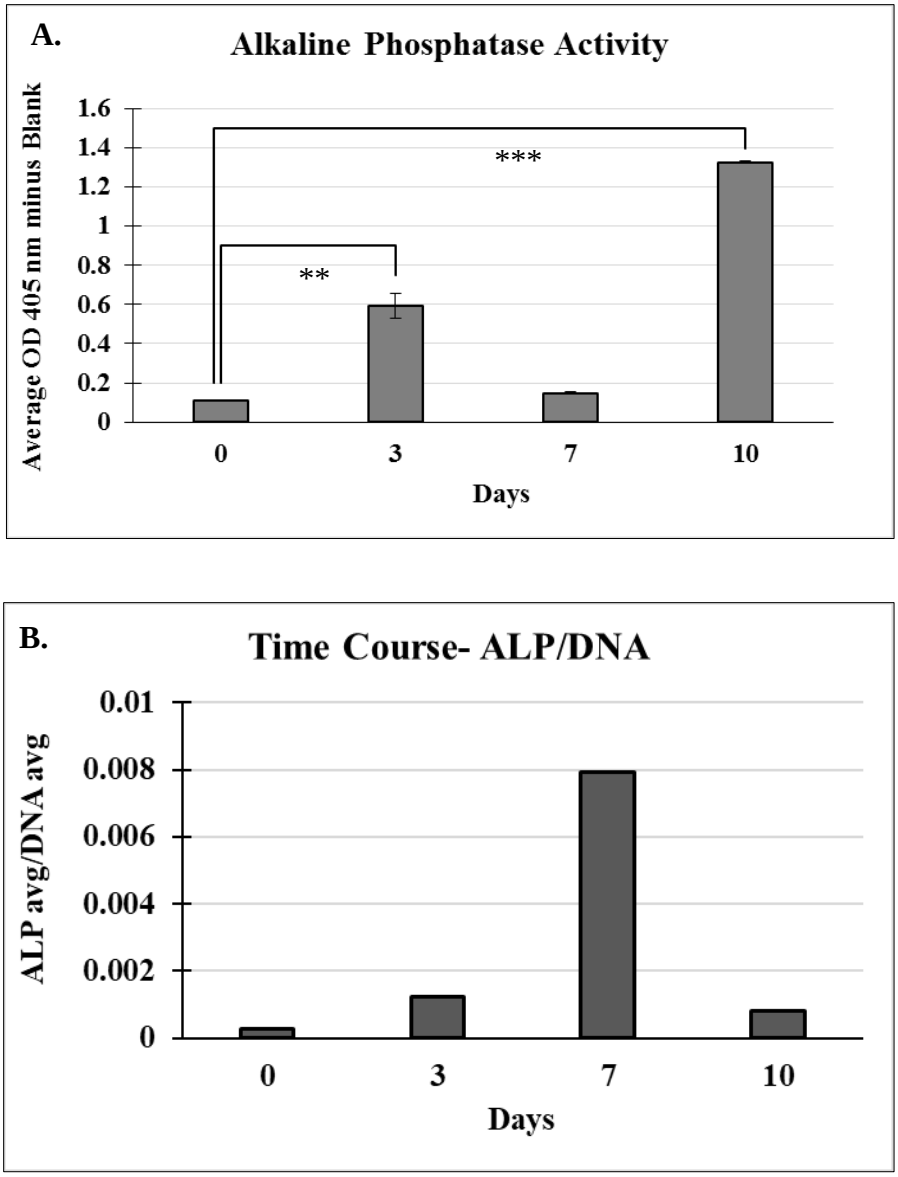


Figure 10. Alkaline Phosphatase Activity in Differentiating hFOB1.19. hFOB1.19 cells were induced to differentiate. A) Cells were collected at the indicated days and cell pellets assayed for ALP activity. Day 0 serves as the control without induced differentiation. The values are averages of the optical density at 405 nm minus the background (n=6). **= P value ≤ 0.01; ***= P value ≤ 0.001; B) ALP/DNA content. Averages of ALP activity were standardized to the averages of hFOB1.19 DNA content as a means to measure the cell numbers for each day.

WNT5A Protein Determination During Normal Osteoblast Differentiation

WNT5A is characterized as a critical signaling molecule during osteogenesis through β-catenin dependent canonical and β-catenin independent non-canonical pathways (Okamoto et al., 2014). Due to its substantial role during osteoblast differentiation, we performed a western blot analysis on days 0, 3, 7, 10 and 17 to confirm that our hFOB.19 cells expressed WNT5A protein. Day 0 is used as the control at which the cells are undifferentiated. Days 3, 7, 10 and 17 include the time points when differentiated hFOB1.19 were harvested for analysis. Shown on Figure 11a are the results of the western blot analysis for both WNT5A and tubulin. The signal of WNT5A was normalized to the amount of tubulin for each sample. Although the same amount of protein was loaded per well, the amount of tubulin showed a decrease with increasing days. This result is not what would be expected. One likely explanation is that the amount of extracellular matrix is increasing as the hFOB1.19 differentiate, which contributes to the "protein mass" per cell. Hence, loading 50 μg of protein at day 3 represents more cells than at day 10 or 17, accounting for the reduction in tubulin at these later days. The normalized western blot results indicate that the level of WNT5A protein increases with osteoblast differentiation (Figure 11B). The highest level was detected at day 17, which is approximately 9x greater than at 0. This increase in WNT5A protein correlates with the increase in WNT5A isoform transcripts (Figure 8). No increase in WNT5A protein levels relative to day 0, was detected at day 3.

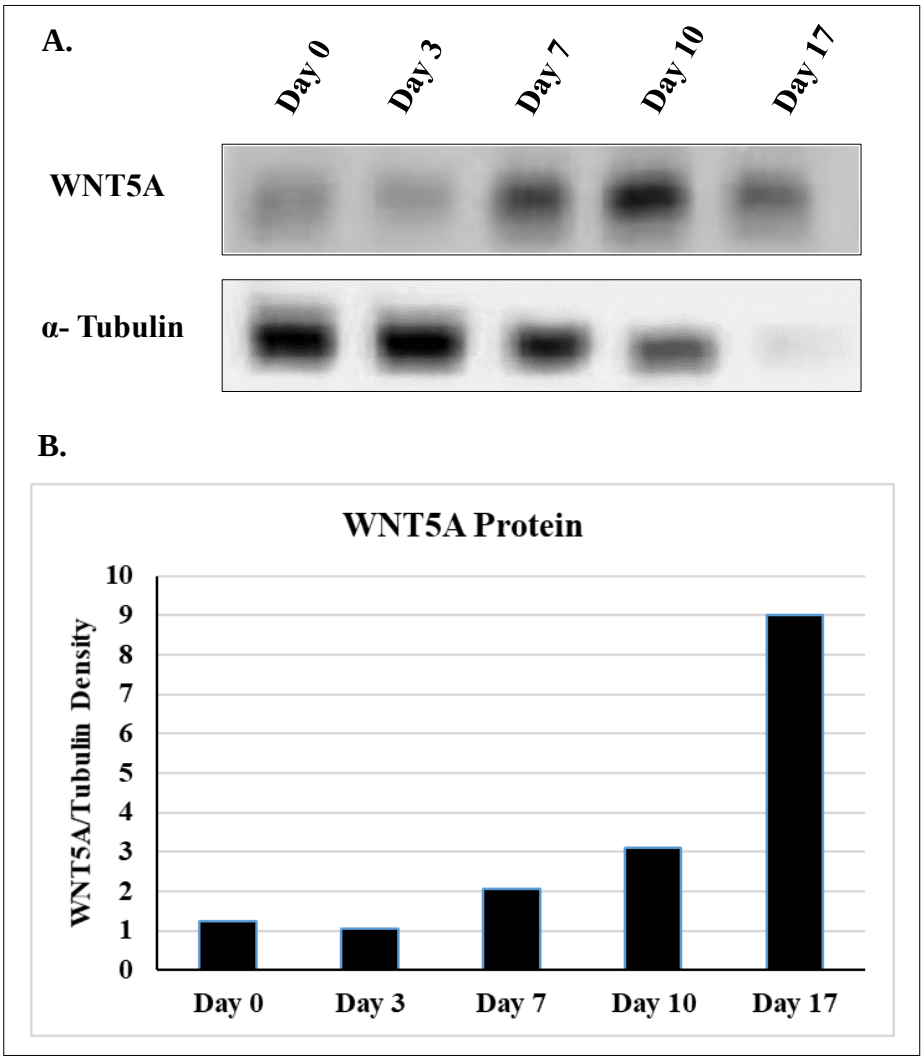


Figure 11. WNT5A Protein Detection in Differentiating hFOB1.19. A) Western Blot for WNT5A and α- Tubulin on days 0, 3, 7, 10 and 17; B) Quantification of the western blot showing WNT5A/Tubulin Density for the indicated time points.

Effects of Altering WNT5A Isoform L(A) and Isoform S(B) in Osteoblast Differentiation

Overexpressing WNT5A Isoforms L(A) and S(B)

As one approach to determining if there is a functional difference between the WNT5A isoforms, I observed if an increase in the level of extracellular WNT5A isoforms L(A) or S(B) had an effect on hFOB1.19 differentiation. This experiment was performed by growing hFOB1.19 cells to confluency and treating the cells with differentiation medium and conditioned medium (CM) containing either isoform L(A) or isoform S(B). The CM was prepared by collecting media from Chinese hamster ovary (CHO) cells expressing WNT5A isoform L (A) or isoform S (B). The control CM was prepared from parental CHO cells without any expression vector. The cells were treated with the following conditions: 1) isoform L(A)-CM, 2) isoform S(B)-CM, and 3) control, P-CM. Cells were harvested at indicated time points and analyzed for WNT5A isoforms transcript levels, molecular osteoblast differentiation markers, and ALP activity.

We previously showed (Figure 8) that both WNT5A isoforms increase during differentiation of hFOB1.19 cells. This would suggest that both isoforms have a positive role in osteogenesis. In this experiment, I increased the levels of the isoforms during hFOB1.19 differentiation and determined the effect on osteogenesis. Based on our findings, I would hypothesize that increasing one or the other WNT5A isoform enhances osteogenesis. However, due to the complexities of the non-canonical pathways, overexpression of the WNT5A isoform may lead to unexpected results.

I measured relative fold-changes in two differentiation markers, RUNX2 and osteocalcin at days 0, 2, 3 and 7 in CM treated and control cells. The qPCR results showed a fold-change increase in RUNX2 of approximately 1.6 and 2.1 in hFOB1.19 treated with isoforms L(A)-CM and S(B)-CM at day 2 relative to day 0. On day 2, the control cells treated with P-CM was equal to the control day 0 levels. These results indicate that treatment with either isoform L(A)-CM or isoform S(B)-CM slightly increased the fold-change in RUNX2 transcript. At day 3, there was a slight decrease in fold-change for RUNX2 in both L(A)-CM and S(B)-CM treated cells, compared to control, P-CM. At day 7, there is essentially no difference in the fold-change of RUNX2 between the P-CM treated cells and the isoform L(A) or S(B)-CM treated cells. These results confirm that RUNX2 transcripts increase during the early stages of differentiation. The effects of increasing the isoforms appears transient and complex, as there was an initial positive effect (increased fold-change) and then negative effect (decreased fold-change) in RUNX2 transcripts. Both isoforms had similar effects, indicating no functional differences.

Another important molecular marker for differentiation, osteocalcin, was also measured. On day 2, the transcripts for P-CM isoform, L(A)-CM and isoform S(B)-CM treated cells showed no difference in fold-changes. As expected, the fold-change in osteocalcin transcript levels increased in comparison to day 0. At day 3-4, the P-CM showed a fold-change of approximately138 relative to day 0. The osteocalcin fold-change in isoform L(A)-CM treated cells was approximately 33x relative to day 0. This is an

approximately 4x decrease in comparison to the control P-CM fold-change at day 3. Isoform S(B)-CM treated cells had a fold-change of 103 relative to day 0 which is less but closer to the level in the P-CM cells. By day 7, the P-CM treated cells showed a fold-change of approximately 265 relative to day 0. Isoform L(A)-CM treated cells displayed a fold-change of approximately 350 and isoform S(B) a fold-change of approximately 185 relative to day 0. These results suggest that increasing the WNT5A isoforms has an effect on osteocalcin transcript levels during differentiation.

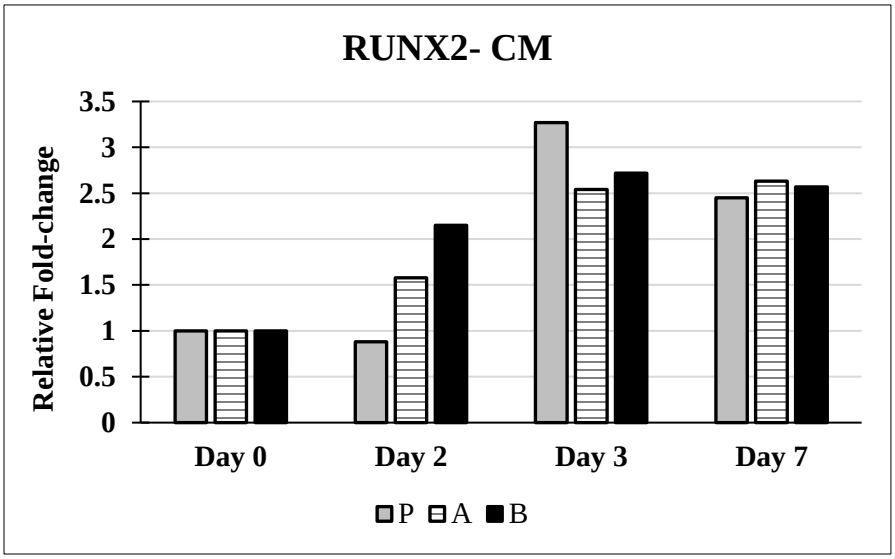


Figure 12. Fold-change for RUNX2 in CM Treated hFOB1.19 Cells Undergoing 7 Days of Osteogenesis. Day 0 serves as the control without induction of differentiation and without any CM treatments. The fold-changes determined from qPCR for days 2, 3 and 7 are relative to day 0 values. Day 0 is relative to itself (=1). Treatment conditions: P= P=CM. A= isoform L(A)-CM. and B= isoform S(B)-CM.

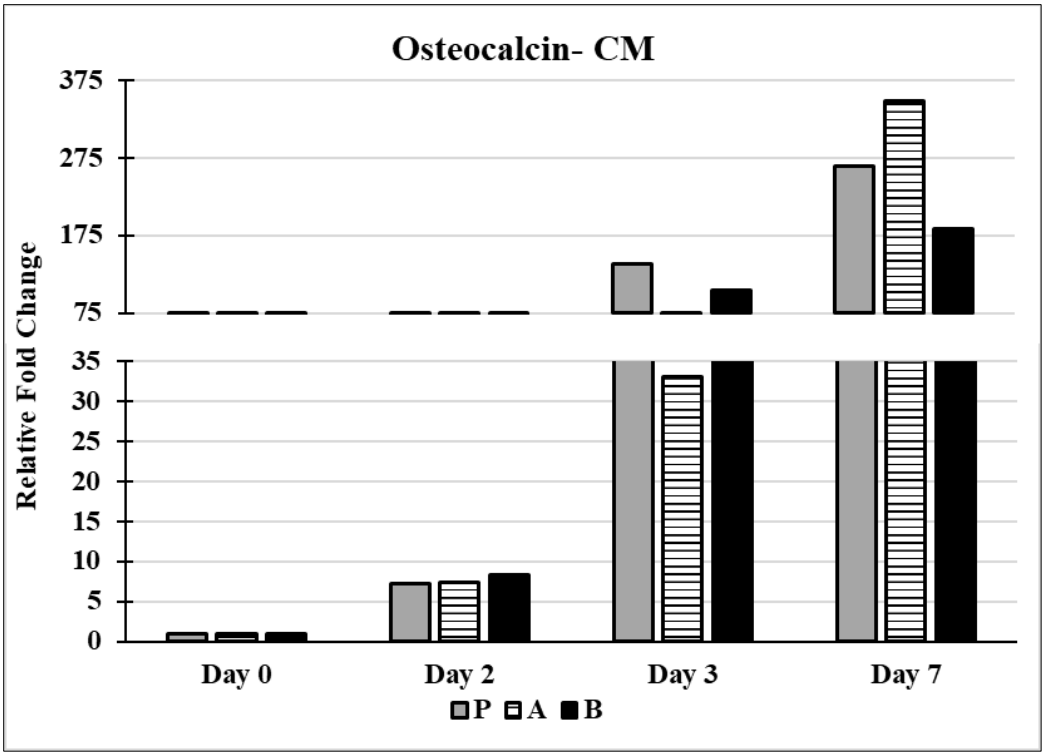


Figure 13. Fold-change in Osteocalcin Transcripts in CM Treated hFOB1.19 Cells Undergoing 7 Days of Osteogenesis. Day 0 serves as the control without induction of differentiation. The fold-changes determined from qPCR for days 2, 3 and 7 are relative to day 0 values. Day 0 is relative to itself (=1). Treatment conditions: P= P-CM. A=isoform L(A)-CM. B= isoform S(B)-CM.

ALP activity was assessed in hFOB1.19 cells treated with isoforms L(A)-CM and S(B)-CM. The results showed that ALP activity in the isoform L(A)-CM and isoform S(B)-CM were not statistically different from the control P-CM at the days assayed with two exceptions. On day 2, ALP activity for P-CM, isoform L(A)-CM and isoform S(B)-CM are increased in comparison to day 0. At day 2, ALP activity in isoform S(B)-CM treated cells showed a slight but significantly ($p \leq 0.01$) higher value compared to the control, P-CM. And, at day 10, isoform L(A)-CM treated cells had a slightly lower but significant ALP activity than the P-CM cells. The isoform S(B)-CM treated cells at day 10 trended to a decrease. These results suggest that treatment of differentiating hFOB1.19 with WNT5A isoform-CM affects ALP activity by day 7 and that the isoforms are not distinct in their effects.

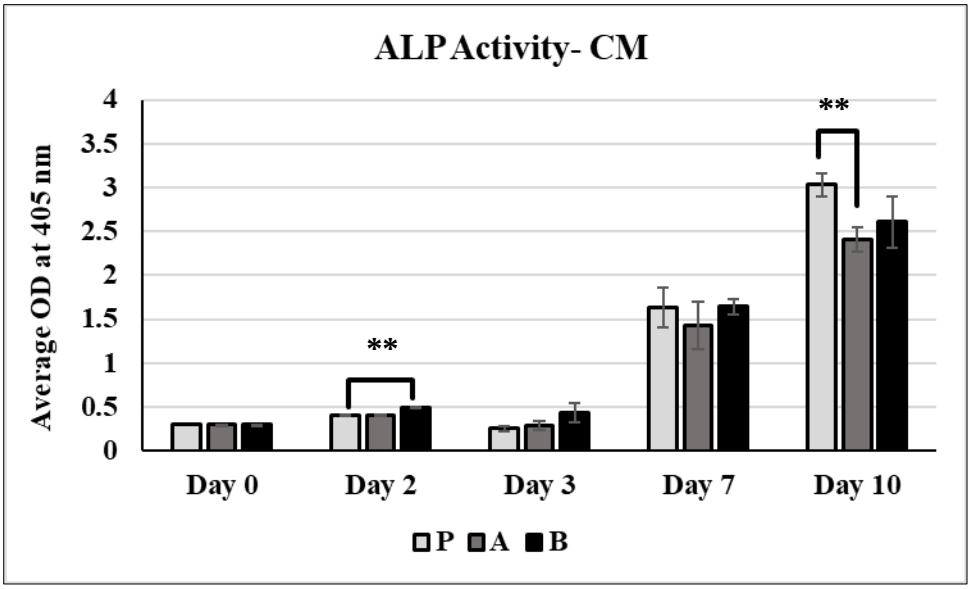


Figure 14. Alkaline Phosphatase Activity (ALP) in Conditioned Medium (CM) Treated hFOB1.19. Treatment conditions: P= P-CM. A= isoform L(A)-CM. B= isoform S(B)-CM. **= P value ≤ 0.01. Day 0, 2, 3, 7, and 10 indicate the days hFOB1.19 were harvested for ALP assay.

Knocking Down WNT5A Isoform L(A), Isoform S(B) and Total WNT5A in hFOB1.19 Cell Line

Next, we wanted to determine the effect on osteogenesis of knocking down the WNT5A isoforms. For this assay, the hFOB1.19 cells were transfected with specific short interfering (si)RNA's when they reached 60 to 80% confluency and then were allowed to grow for 2 days before adding the differentiation medium. The short interfering RNA's used for this assay included: 1) siRNA-L(A), 2) siRNA-S(B), 3) siRNA-T (total, specific to both WNT5A isoforms), and siRNA-S (scrambled siRNA control). The sequences of the siRNA are shown in Table 2. The cell pellets were harvested at days 0, 2, and 3. The effect of the siRNA's on WNT5A isoform transcripts and differentiation markers were determined by qPCR.

It would be expected that siRNA for each of the isoforms would reduce the specific isoform transcript level, whereas siRNA for total WNT5A would cause a reduction in both isoform levels. After the knockdown was confirmed, the levels of differentiation markers (RUNX2, osterix and osteocalcin) and ALP activity were measured to determine effects on the process of osteoblast differentiation.

Initially, I determined if the siRNA was causing the predicted knockdown in undifferentiated hFOB1.19 cells. siRNA transfected hFOB1.19 cells were grown for 2 days and processed for qPCR analysis. Results revealed that siRNA was able to achieve approximately 20% knockdown of isoform L(A) transcripts (Figure 15A). This small

change does not verify knockdown. Isoform L(A) transcript levels were slightly higher (1.2 fold) in cells transfected with siRNA-S(B) (Figure 15A) but likely indicates no effect, which is the expected result. In contrast, isoform L(A) transcript levels were reduced by approximately 66% in cells transfected with siRNA-T, which is the expected result.

Analysis of isoform S(B) transcripts after knockdown assay revealed approximately 15% decrease in siRNA-S(B) transfected cells. In siRNA-L(A) transfected cells, the fold-change was similar to siRNA-S(B) transfected cells. As displayed for isoform L(A), the total WNT5A knocked out cells (siRNA-T), had the greatest effect in terms of knocking down the isoform levels. Transfecting with siRNA-T caused a 50% decrease of isoform S(B); a reduction would be expected.

Overall, these results indicate that transfection of the siRNA for the individual isoforms is not working to reduce isoform transcripts, whereas the siRNA-T is reducing both isoform L(A) and S(B) transcript levels.

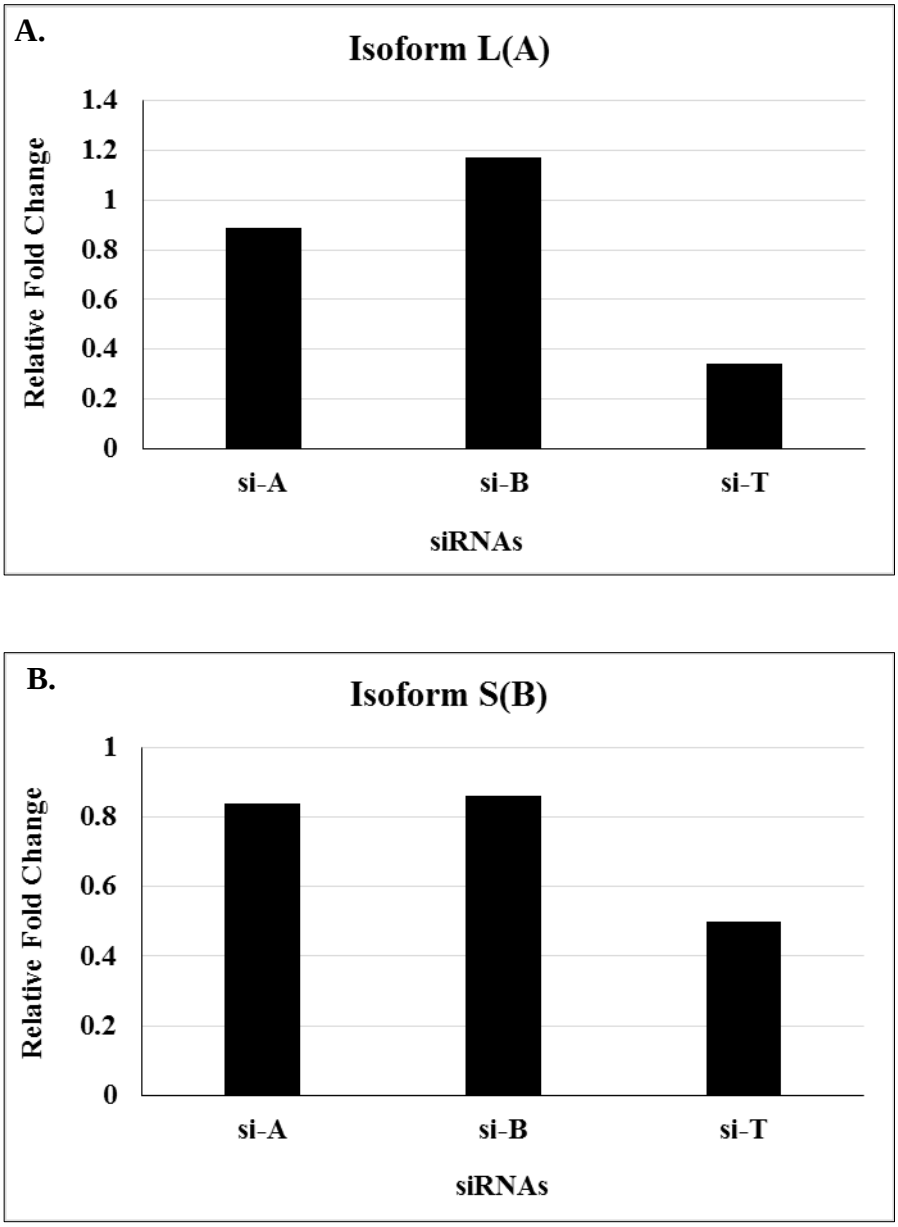


Figure 15. Fold-change of A) Isoform L(A) and B) Isoform S(B) Transcripts 48 Hours After Transfection. The following siRNAs were transfected**:** siA= cells transfected with siRNA-L(A). siB= cells transfected with siRNA-S(B). siT= cells transfected wth siRNA-T. The hFOB1.19 cells were undifferentiated. The fold-change shown is relative to the control siRNA (siRNA-S).

The 48 hours analysis confirmed that knockdown was achieved at the highest rate when transfected with the siRNA-T for total WNT5A [L(A)+S(B)]. My next goal was to measure the levels of WNT5A isoforms L(A) and S(B) when transfected with the same siRNA conditions except, this time, in differentiating hFOB1.19 cells. In this trial, the transfected cells were assayed at days 0, 2, and 3. Day 0 cells were harvested on the day of confluency and before inducing differentiation. Although I didn't find an effect on transcript using the individual isoform siRNA's in the initial analysis, I included these siRNA's in the analysis. The relative fold-change for a given transcript was compared to the siRNA-S control at each day assayed. If the siRNA [L(A), S(B) or T] had no effect on transcript level, the value would be expected to equal "1" when measured relative to the control siRNA-S. If the siRNA [L(S), S(B) or T] had an effect on transcript levels, then the value would be expected to be less than 1.

When transfected with siRNA-L(A), there is essentially no difference in the transcript levels of isoform L(A) at days 0 and 2 relative to the control, siRNA-S (non-silencing control) (Figure 16). There is a slight increase on day 3 but only by 0.4 compared to days 0 and 2. Transfecting with siRNA-S(B) showed almost no effect on the levels of isoform S(B) transcripts, which indicates that the siRNA-S(B) had no effect on the transcript levels (Figure 16). Relative to the control-S, transfecting hFOB1.19 with siRNA-T induced approximately a 50% decrease at both days 0 and 2 in isoform L(A) transcripts (Figure 16). By day 3, there was an increase in the isoform L(A) transcript levels when transfected with siRNA-T. Levels of isoform S(B) was assessed similarly

which showed approximately 45% decrease at day 0 when transfected with siRNA-T. The S(B) levels increased by day 2 and 3 which follows the observed pattern. These results are consistent with the results in Figure 15 showing a knock down only with siRNA-T. The results confirm that siRNA-T knocks down both isoform transcripts during hFOB1.19 differentiation and indicate that by day 3, the effects of the siRNA are diminishing.

I also wanted to determine if in the siRNA-S transfected cells there is an increase in the isoform L(A) and S(B) transcripts during differentiation> The data used for Figure 16 was re-analyzed; relative fold-changes (compared to day 0) of isoform L(A) and S(B) transcripts were calculated for siRNA-S transected cells (Figure 17). It would be expected that fold-change increases for both isoform L(A) and S(B) transcripts at least by day 3, based our results in Figure 5 and 6. I found that isoform L(A) transcript fold-change increased at both days 2 and 3, whereas isoform S(B) transcript fold-change increased at day 3 only. These results suggest that transfection with the siRNA's is not having a non-specific effect on hFOB1.19 cells as they differentiate.

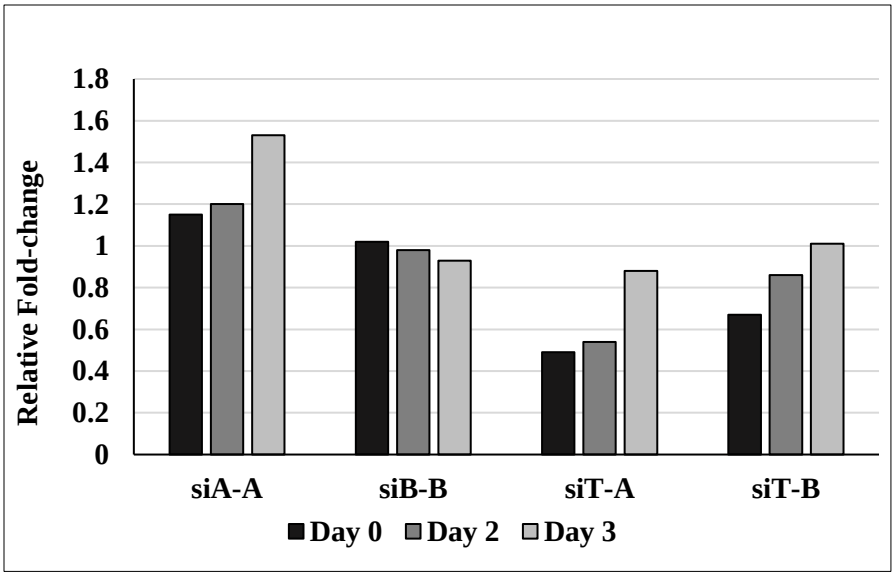


Figure 16. Fold-change of Isoform L(A) and S(B) Transcripts in siRNA Transfected Differentiating hFOB1.19 Cells. Fold-change is relative to the control, si-S or non-silencing control. siRNA-S or non-silencing control. siA-A= isoform L(A) transcripts measured in siRNA-A knocked down cells; siB-B= isoform S(B) transcripts measured in siRNA-B knocked down cells; siT-A= isoform L(A) transcripts measured in siRNA-T knocked down cells; and siT-B= isoform S(B) transcripts measured in siRNA-T knocked down cells. The fold-changes are relative to the control, siRNA-S for each days of assay.

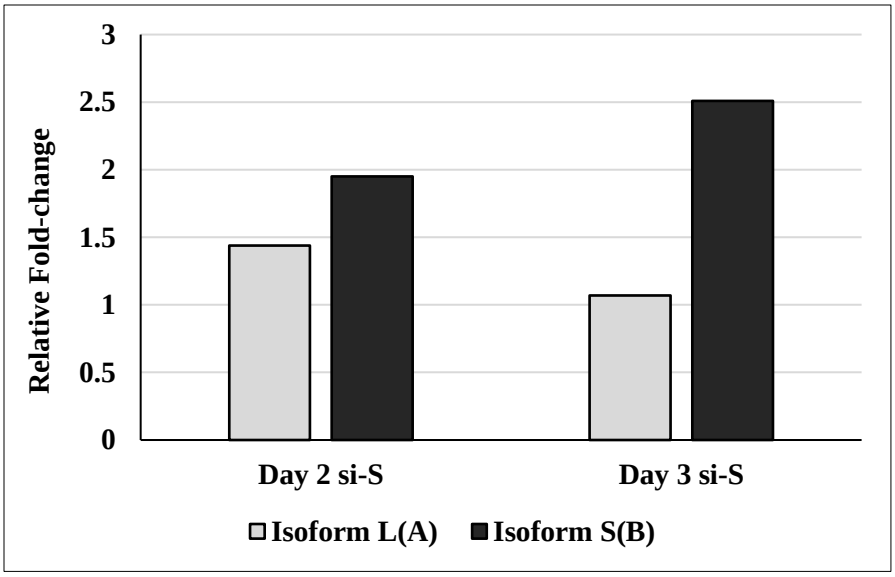


Figure 17. Fold-change of Isoform L(A) and S(B) Transcripts in siRNA-S Knockdown hFOB1.19 Cells. Day 2 and 3 si-S indicate isoform L(A) and S(B) fold-changes when the non-silencing control was transfected at each given day. The values graphed are relative to day 0 si-S. Day 0 represents the immediate day after transfection.

Effect on Differentiation Molecular Markers of siRNA Knockdown of Isoform L(A) and S(B) Transcripts in Differentiating hFOB1.19 Cells

Next, I determined the change in differentiation molecular markers in WNT5A knock down of isoform L(A) and S(B) transcripts in differentiating hFOB1.19. This analysis was completed using cDNA that generated Figures 16 and 17. The markers examined were RUNX2, a transcription factor and early differentiation marker, and osteocalcin, a later differentiation marker and ALP activity. I found that the siRNA0T both isoforms) had an effect on WNT5A isoform transcript levels but the individual isoform siRNAs did not. Hence, it was expected that only in the siRNA-T cells, would any effect on differentiation markers be detected. Hence, only the siRNA-T and S transfected cells were used for this analysis.

For qPCR analysis, the relative fold-change for RUNX2 and osteocalcin was calculated by comparing the siRNA-T C_t values to those of the siRNA-S control for each day analyzed. If there was no difference between control, siRNA-S, versus treated, siRNA-T, then the value would be 1. A value of less than 1 would indicate a negative effect on the markers. If the value was higher than 1, than that would indicate a positive effect on the markers.

There was no effect on RUNX2 transcripts on day 0 in siRNA-T cells compared to siRNA-S (Figure 18a). Similarly, there was barely any effect on osteocalcin (value of 0.8 compared to 1). This result is as expected because on day 0, the hFOB1.19 had not

been induced to differentiate. The results suggest that on day 2, there was an increase in RUNX2 (approximately by 0.4) and osteocalcin (value of approximately 0.6) in comparison to the control. On day 3, there was a slight increase in RUNX2 compared to the control (value > 1). Overall, RUNX2 was higher on days 2 and 3 in comparison to day 0, relative to the control. In contrast, osteocalcin showed a decrease at day 3 (by 0.3 relative to 1). These results are suggestive that knocking down both WNT5A isoforms together did not induce any negative effects on the differentiation markers. Moreover, the results are rather indicate an enhancement of the molecular markers at any early point in differentiation (day 2) but this effect is transient. This transient effect might be expected, as the effect of the siRNA-T was found to be diminished at day 3 (Figure 18).

The relative fold-changes were also calculated to assess the *pattern* of the differentiation molecular markers on siRNA-T knock down in hFOB1.19 (Figure 19). In this analysis, the fold-changes were derived relative to day 0 for each set of siRNAs (siRNA-S and siRNA-T). For example, the fold-change in day 2 and 3 of RUNX2 and osteocalcin in siRNA-S transfected group was calculated relative to the day 0 C_t value of si-S for each specific molecular markers. Similarly, fold-change in day 2 and 3 of both markers in siRNA-T transfected group was derived relative to the day 0 C_t value of siRNA-T for each of the differentiation markers.

The results showed that RUNX2 levels were approximately 1-fold higher in the siRNA-T group in comparison to the siRNA-S group at day 2 (Figure 19). The T/S value

was approximately 1.6 for this day (day 2). On day 3, both groups decreased to the same level (fold-change ~0.5- 0.6) relative to each of their control (day 0).

For osteocalcin, the fold-change was higher on day 2 for both the siRNA-T and siRNA-S transfected groups but the value by approximately 1.6x for siRNA-T. On day 3, the fold-change was doubled for siRNA-S in comparison to itself on day 0 for osteocalcin. There was no difference in the fold-change of siRNA-T treated group for day 3 in comparison to itself on day 2. However, compared to siRNA-S on day 3, the levels of osteocalcin were slightly lower (T/S value of 0.8).

Overall, these results suggest that knock down of both WNT5A isoforms may be enhancing differentiation but the effect is transient. Knockdown did not eliminate the increases expected in RUNX2 and osteocalcin transcripts.

Next, I conducted an ALP assay to determine the effect of knocking down WNT5A isoforms on ALP activity, another marker of differentiation. For all three days assayed, siRNA-S and siRNA-T samples revealed an increase in ALP activity, as would be expected (Figure 20). On day 2, there was a significant decrease in ALP activity in si-Total group compared to the si-control group. This decrease was statistically significant with $p \leq 0.01$. On day 3, the increase in ALP activity was significantly higher ($p \leq 0.001$) for siRNA-S sample compared to siRNA-T. These results for ALP activity are not as expected as the differentiation molecular markers displayed an enhancement with sIRNA-T knock down (Figure 19).

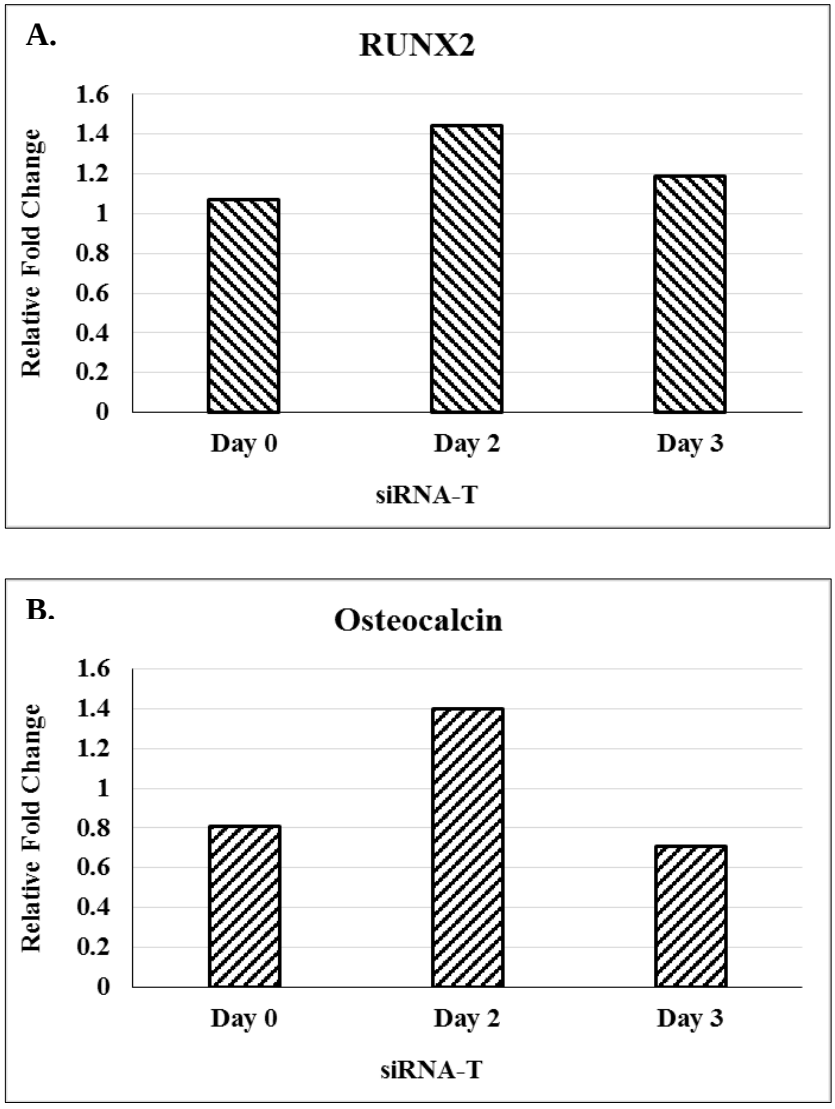


Figure 18. Fold-change in Differentiation Molecular Markers in siRNA-T, Total WNT5A Knocked Down Differentiating hFOB1.19 Cells. Cells were transfected with siRNA-T and siRNA-S and 2 days later treated with differentiation medium. Cells were collected at the days indicated and transcript levels analyzed by qPCR. Day 0 cells represent confluent but undifferentiated hFOB1.19 cells. siRNA-T= siRNA for total WNT5A isoforms L(A) and S(B). Fold-changes for days 0, 2 and 3 are relative to siRNA-S or the non-silencing control for each indicated day. A) RUNX2 fold-change and B) Osteocalcin fold-changes.

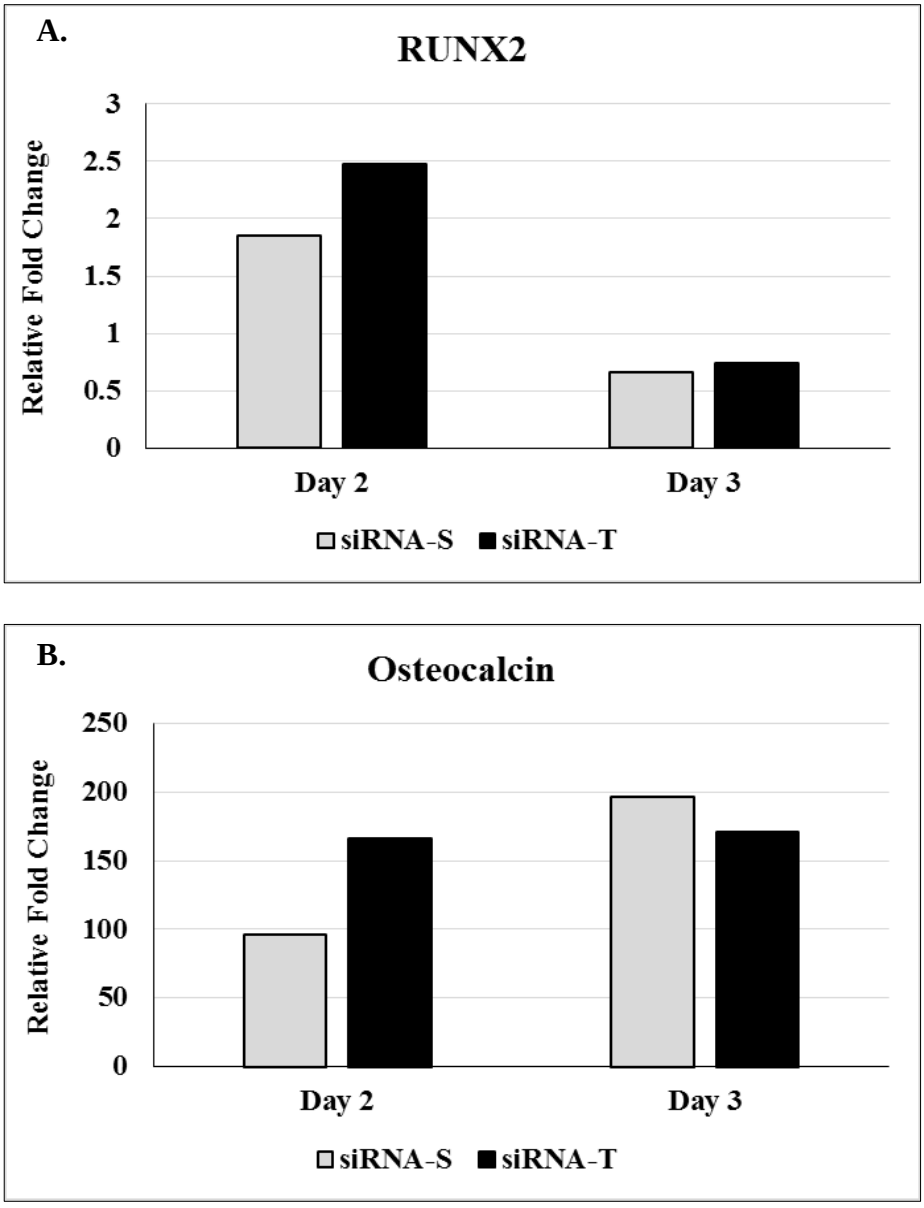


Figure 19. Fold-change for A) RUNX2 and B) Osteocalcin in siRNA-S and siRNA-T Transfected hFOB1.19 Cells. siRNA-S= non-silencing control; siRNA-T= siRNA for total WNT5A [L(A)+S(B)]. The fold-change for si-S day 2 and 3 are relative to siRNA-S day 0. Fold-change for siRNA-T day 2 and 3 are relative to si-T day 0. A) Fold-change of RUNX2 transcripts in siRNA-S and siRNA-T knock down cells; B) Fold-change of osteocalcin transcripts in siRNA-S and siRNA-T knock down cells.

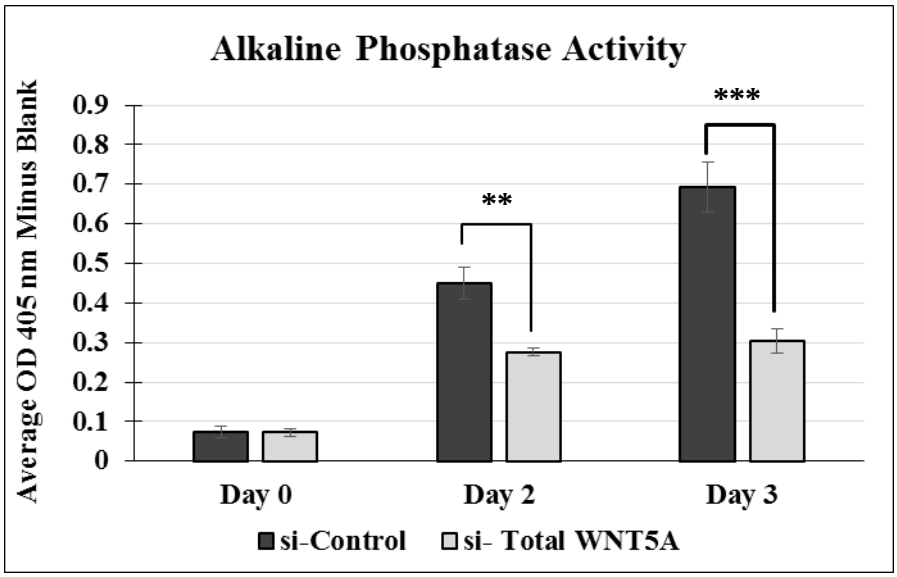


Figure 20. ALP Activity in siRNA-S and siRNA-T Knockdown hFOB1.19 Cells. Cells were grown in 48-well and transfected with siRNAs. The next day, the confluent cells were treated with differentiation medium. At the days shown, cells were collected from each well and assayed for ALP activity. si-Total WNT5A= siRNA-T knock down cells; isoforms L(A) and S(B) are silenced; si-Control= siRNA-S knock down cells. (n=4) **= P value ≤ 0.01; ***= P value ≤ 0.001

CHAPTER IV

DISCUSSION

In this study, we report for the first time that both WNT5A isoforms L(A) and S(B) transcript levels increase during differentiation of the osteoblast cell line, hFOB1.19. The increase in the isoforms correlated with the molecular markers of differentiation including RUNX2, osterix, osteocalcin and ALP activity. Western blot analysis also revealed an increase in total WNT5A protein during hFOB1.19 differentiation. Altering the amount of WNT5A isoforms by knocking down the isoforms and increasing the protein isoforms demonstrated an effect on the molecular differentiation markers. Our results suggest that the WNT5A isoforms are involved in osteoblast differentiation. However, the data do not suggest that the WNT5A isoforms L(A) and S(B) are functionally different.

Investigating the pattern of expression of the WNT5A isoform L(A) and S(B) levels revealed that both isoforms increased in comparison to day 3 relative to the control, day 0. Isoform S(B), was higher on day 3 compared to isoform L(A) and on day 7 of evaluation, both isoforms on average had the same fold -change relative to day 0 (approximately a fold-change of 10). On day 10, isoform S(B) displayed a higher fold-

change than isoform L(A) of about 13 in comparison to a fold-change of 6 for L(A). Inducing hFOB1.19 to differentiate for 21 days generated the same results; both isoform L(A) and S(B) levels overall showed an increase throughout the differentiation period. It appeared that isoform L(B) had an overall greater fold increase than L(A). However, calculation of the actual transcript numbers of the WNT5A isoforms (for the 21 day assay) revealed that there were fewer isoform S(B) transcripts than L(A) at day 0. This difference in transcript numbers at day 0 may account for the greater fold increase of isoform S(B).

These increases in isoform levels were associated with osteogenesis. This was shown by measuring molecular markers of osteogenesis, which were RUNX2, osterix, osteocalcin and ALP activity. RUNX2, an initial marker of osteoblast lineage, showed increases in fold-change on days 3 and 7 which began declining on day 10 and 21. Osterix was lower on the initial days and showed increases at days 17 and 21. This is expected because it is a biomarker for distinguishing pre-osteoblasts and differentiated osteoblasts. Osteocalcin showed high fold-changes (800-1000 fold), especially at the later days. As the osteoblast cells secrete the extracellular matrix and reach a maturation stage, osteocalcin protein levels increase, so this result once again was expected. ALP activity also increased with increasing days indicating that our fetal osteoblast cells were differentiating and maturing. These changes in osteogenesis differentiation markers are documented.

L(A) and S(B) levels overall showed an increase throughout the differentiation period. It appeared that isoform L(B) had an overall greater fold increase than L(A). However, calculation of the actual transcript numbers of the WNT5A isoforms (for the 21 days assay) revealed that there were fewer isoform S(B) transcripts than L(A) at day 0. This difference in transcript numbers at day 0 may account for the greater fold increase of isoform S(B).

One suggestion for a functional distinction between the isoforms is that the pattern of expression of the isoforms show variation during differentiation. My data indicates the opposite, that both isoforms have a similar pattern of expression. It is possible that the isoforms have a different functional role during osteogenesis. To evaluate these functional differences, I used two approaches: overexpression with conditioned medium (CM) and knockdown with WNT5A siRNA. The overexpression assay showed that on day 2, there were no differences in osteocalcin levels between the P-CM, isoform L(A)-CM and isoform S(B)- CM treated cells. However, on day 3, both L(A)-CM and S(B) CM treated cells had lower fold-changes in osteocalcin in comparison to the control P-CM. On day 7, L(A)-CM treated cells displayed approximately a fold-change that was 1.3 x greater than P(A)-CM, whereas S(B)-CM treated cells had a fold-change 0.6 x less. The significance of this increase in levels of osteocalcin on L(A)-CM treated cells for this particular day has not been determined; additional biological replicas will need to be completed.

RUNX2 transcript levels were also affected by increased isoform L(A) and S(B). Both isoform L(A)-CM and S(B)-CM treated cells had a greater fold-change in RUNX2 than P-CM treated cells on day 2 of differentiation. On day 3 and 7, the levels of RUNX2 were higher than the previous day for both isoform L(A)-CM and S(B)-CM treated cells. However, on day 3, fold-changes were decreased in both L(A) and S(B)-CM treated cells, relative to the control P-CM and by day 7, fold change was the same for treated and control groups. Again, additional studies are needed to statistically confirm these results. It should be noted that CM treated cells still express the endogenous WNT5A isoforms, hence the cells are exposed to an increase in one or the other isoform. The results with RUNX2 suggest that the effects of increasing the isoforms are similar for both L(A) and S(B). compared to P-CM, the isoforms treated cells had lower fold-changes on day 3. There is some variation for osteocalcin. One study reported that osteocalcin expression was unaffected by WNT5A, indicating that although WNT5A had a direct effect on the secretion of osteocalcin, it does not regulate the transcription of osteocalcin (Martineau et al., 2017). This is in contrast to our results, showing a decrease in osteocalcin transcript fold-change levels at day 2 in CM treated cells. Overall, the results of the CM experiments suggest that increasing the isoforms can slightly alter the transcript levels of differentiation markers, but these effects appear transient. There are potential limitations to increasing WNT5a protein levels using conditioned medium because adding much higher levels of either WNT5A isoform L(A) or isoform S(B) may lead to complex and unexpected results.

In a second approach for analyzing the functional roles of the WNT5A isoforms in osteogenesis, I attempted to knock down each of the isoforms in differentiating hFOB1.19 cells using siRNA. Unfortunately, I was unable to individually knock down the isoforms and more preliminary studies are needed to refine the siRNA transfection conditions. However, I was able to knock down both isoforms using an siRNA to total WNT5A. My results suggest that the knock down was partially successful at day 0 and 2 but did not extend to day 3 of differentiation (5 days after transfection). And, the siRNA-T produced a greater knockdown of isoform L(A) then isoform S(B). Also, it will be important to confirm that the WNT5A protein levels were also knocked down. Regardless, my results suggest that knock down of total WNT5A causes a slight *increase* in the differentiation markers, RUNX2 and osteocalcin at day 2 (4 days after transfection). At day 3, the effect is diminished, which would be expected as the siRNA effect was found to be diminished. This increase in osteocalcin fold-change as a consequence of a knock down of the WNT5A is what might be expected, as an increase in both isoforms in the CM-treated cells caused a *decrease* in osteocalcin fold-change at day 3. However, RUNX2 results showed more inconsistency. In contrast to our results, an analysis performed using human mesenchymal stem cells (HMSc), reported that knock down of WNT5A caused a *down regulation* of the differentiation markers when knocking down WNT5A (Guo et al., 2008). Overall, our knock down studies were ubanle to provide insights into the functional differences between the WNT5A isoforms L(A) and S(B). Moreover, since the knockdown was incomplete, it was not possible to confirm if

WNT5A, in general, is necessary for hFOB1.19 differentiation. And, the slight effects observed must be re-confirmed.

There are three other studies, previously detailed in the introduction, that have addressed the functional distinctions between the WNT5A isoforms. In the Bauer et al. (2013) study, the authors used only transformed, cancer cell lines to investigate the isoform. In general, they found that isoform S(B) increased cell proliferation when overexpressed and isoform L(A) decreased proliferation. In the study by Huang et al. (2017), they analyzed the isoforms in primarily one cancer cell line, HCT116, a colorectal cancer cell line. They provide evidence that isoform S(B) reduces apoptosis, whereas isoform L(A) had no effect on apoptosis. And, isoform S(B) when knocked down, reduced colony formation, compared to L(A), indicating isoform S(B) promotes cell proliferation. However, there is some concern about the results of this study, considering they used knock down to decrease already reduced levels of WNT5A in the HCT116 cell line. In fact, based on results in this lab, it appears that the WNT5A transcripts are barely detectable in HCT116 cells. In a third study, Katula et al. (2012) analyzed the promoters of the isoforms and provided evidence that isoform S(B) promoter is differentially regulated by NF-κB. This result suggests that differential promoter regulation could provide an explanation for the two distinct isoforms. I chose to analyze the isoforms in a normal cell line during differentiation of a mesenchymal cell type, in which WNT5A is known to be expressed and functional. In general, my results suggest no functional differences between the isoforms during hFOB1.19 differentiation.

I found that both isoform transcript levels increase during hFOB1.19 differentiation and are at their highest levels at later periods of differentiation. However, increases also took place during early differentiation by day 3 of differentiation. In addition, I confirmed that total WNT5A protein levels are increasing. It is possible and likely that WNT5A is performing distinct functions at different periods of hFOB1.19 differentiation. This could be due to changes in receptor types that appear during differentiation. There are various studies that support this possibility. It is known that bone formation is regulated by WNT via β-catenin dependent and independent pathway (Okamoto et al., 2014). In a mechanistic study elucidating this cooperation between the pathways, it was reported that WNT5A is important for WNT/β-catenin signaling as an increase in WNT5A was shown to increase LRP5/6, a WNT canonical receptor, in osteoblast lineage cells. As expected, a knock down of WNT5A was shown to reduce the expression of LRP5/6 receptor. LRP5/6 signaling has been identified as critical for formation of bone in cells of osteoblastic lineage. The same study reported that knocking out WNT5A *in vivo* caused an impairment in mineralization in osteoblast-lineage cells from mice (Okamoto et al., 2014). These studies may indicate that during early differentiation, WNT5A function is to increase β-catenin activity by increasing LRP5/6 receptors.

WNT5A has been reported to promote bone-resorbing activity of osteoclasts via induction of receptor activation of NF-κB ligand (RANKL) (Uehara et al., 2017). A study by Maeda et al. (2012) suggests that osteoblast-lineage cells secrete WNT5A and the

expression of RANKL is upregulated by WNT5A, which in turn induces osteoclast formation via ROR2 signaling (non-canonical pathway). These results may provide an explanation for the further level of WNT5A at later stages of hFOB1.19 differentiation. It is possible that the WNT5A secreted from highly differentiated hFOB1.19 cells is serving a paracrine functions and *in vivo* binding to receptors on pre osteoclasts within the bone marrow. It has been suggested in one model that WNT5A blocks the effects of WNT16 on osteoclast differentiation, and also stimulates differentiation of the pre-osteoclasts to mature osteoclasts (Kobayashi et al., 2015). It is not possible in the hFOB1.19 cell line system to analyze the role of the WNT5A secreted from the mature osteocytes as the osteoblast to osteocyte transition involves a complex network.

There are various studies suggesting that WNT5a plays a role in defining mesenchymal stem cell fate to pre-osteoblasts. In one study, WNT5A directed the fate of embryonic stem cells to osteoblasts (Keller et al., 2016). In two other studies, WNT5A was important for defining the fate of adipocyte stem cells to an osteoblast lineage (Santos et al., 2010; Zhang et al., 2014). These studies suggest that WNT5A may be functioning prior to osteoblast determination. The hFOB1.19 cell line used in this study are already pre-determined as osteoblasts and not mesenchymal stem cells. Our results indicate that both WNT5A isoform L(A) and S(B) transcripts are at a relatively low level in this cell line. Hence, this system only allows for the analysis of hFOB1.19 differentiation to osteocytes.

In conclusion, my data suggests that WNT5A is not required for the observed *increase* of the biomolecular markers of osteoblast differentiation. My results show a correlation between increased WNT5A isoform expression and increased hFOB1.19 osteoblast differentiation. I provide some but still unverified evidence that WNT5A expression levels can modify the expression of differentiation markers. Future studies will be important to confirm or affirm functional distinctions between the isoforms. In this study, the knockdown was not achieved with individual isoforms so, in future, achievement of complete knockdown will prove critical for understanding each isoform's role in osteoblast differentiation. Assays increasing amount of the WNT5A isoforms could be improved ensuring the same levels of isoform proteins are being used and that both isoforms are increased together [L(A) + S(B)]. The levels of LRP5/6 receptors should be determined in CM treated and knock down cells and the activity of β-catenin evaluated.

REFERENCES

1. Angers, S., and Moon, R. T. (2009). Proximal events in WNT signal transduction. *Nature Reviews Molecular and Cell Biology*, 10, 468-477.

2. Bauer, M., Bénard, J., Gaasterland, T., Willert, K., and Cappellen, D. (2013) WNT5A Encodes Two Isoforms with Distinct Functions in Cancers. *PLoS ONE*, 8(11): e80526.

3. Blitek, A., and Szymanska, M. (2017). Peroxisome proliferator-activated receptor (PPAR) isoforms are differentiatlly expressed in prei-implantation procine conceptuses. *Theriogenology*, 101, 53-61.

4. De, A. (2011). WNT/Ca^{2+} signaling pathway: a brief overview. *Acta Biochimica Biophysica Sinica*, 43(10), 745-756.

5. Guo, J., Jin, J., and Cooper, L-F. (2008). Dissection of sets of genes that control the character of wnt5a-deficient mouse cavalier cells. *Elsevier*, 43, 961-971.

6. K, H.-C., & Klein, P. S. (2004). The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biology*, *5*(7), 234.

7. Huang, T. C., Lee, P-T., Wu, M-H., Huang, C-C., Ko, C-Y., Lee, Y-C., Lin, D-Y., Cheng, Y-W., and Lee, K-H. (2017). Distinct rols and differential expression levels of WNT5A mRNA isoforms in colorectal cancer cells. *PLoS ONE*, 12(8): e0181034.

8. Huang, W., Yang, S., Shao, J., AND Li, Y.-P. (2007). Signaling and transcriptional regulation in osteoblast commitment and differentiation. Frontiers in Bioscience : A Journal and Virtual Library, 12, 3068–3092.

9. Katula, K. S., Joyner-Powell, N. B., Hsu, C.-C., and Kuk, A. (2012). Differential Regulation of the Mouse and Human *WNT5A* Alternative Promoters A and B. *DNA and Cell Biology*, *31*(11), 1585–1597.

10. Keller, K.C., Ding, H., Tieu, R., Sparks, N. R. L., Ehnes, D. D., and Zur-Nieden, N. I. (2016). WNT5A support osteogenic lineage decisions in embryonic stem cells. *Stem Cells and Development*, 25(13), 1020-1032.

11. Komiya, Y., and Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, 4(2), 68–7

12. Komori, T. (2006). Mechanism of transcriptional regulation by Runx2 in osteoblasts, *Clinical Calcium*, 16(5), 801-807.

13. Kurayoshi, M., Yamamoto, H., Izumi, S., and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochemical Journal*, 402(Pt 3), 515–523.

14. Liu, J., Pan, S., Hsieh, M. H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A. G., Cheng, D., Li, J., Tompkins, C., Pferdekamper, A., Steffy, A., Cheng, J., Kowal, C., Phung, V., Guo, G., Wang, Y., Graham, M. P., Flynn, S., Brenner, J. C., Li, C., Villarroel, M. C., Schultz, P. G., Wu, X., McNamara, P., Sellers, W. R., Petruzzelli, L., Boral, A. L., Siedel, H. M., McLaughlin, M. F., Che, J., Carey, T. E., Vanasse, G., and Harris, J. L. (2013). Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proceedings of the National Academy of Sciences*, 110(50), 20224-20229.

15. Maeda, K., Kobayashi, Y., Udagawa, N., Uehara, S., Ishihara, A., Mizoguvhi, T., Kikuchi, Y., Takada, I., Kato, S., Kani, S., Nishita, M., Marumo, K., Martin, T-J., Minami, Y., and Takahashi, N. (2012). Wnt5a-Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. Nature Medicine, 18(3), 405-412.

16. Manner III, C. (2016). Functional distinctions between two isoforms of WNT5A. *UNCG Library*, 53 pp.

17. Martineau X, Abed É, Martel-Pelletier J, Pelletier J-P, Lajeunesse D. (2017). Alteration of Wnt5a expression and of the non-canonical Wnt/PCP and Wnt/PKC-Ca2+ pathways in human osteoarthritis osteoblasts. *PLoS ONE* 12(8): e0180711.

18. Milona, M., Gough, J. E., and Edgar, A. J. (2003). Expression of alternatively spliced isoforms of human Sp7 in osteoblast-like cells. *BMC Genomics, 4*, 43.

19. Nemoto, E., Ebe, Y., Kanaya, S., Tsuchiya, M., Nakamura, T., Tamura, M., & Shimauchi, H. (2012). WNT5A signaling is a substantial constituent in bone morphogenetic protein-2-mediated osteoblastogenesis. *Biochemical and Biophysical Research Communications,422*(4), 627-632.

20. Niehrs, C. (2012). The complex world of WNT receptor signaling. *Nature Reviews Molecular Cell Biology*, 13, 767-779.

21. Okamoto, M., Kobayashi, Y., Kato, H., Maeda, K., Minami, Y., Nakamichi, Y., Saito, N., Shunsuke, U., Takahashi, N., Udagawa, N., Uehara, S., and Yamashita, T. (2014). Noncanonical Wnt5a enhances Wnt/β-catenin signaling during osteoblastogenesis. *Scientific Reports*, 4, 4493.

22. Park, M-H., Choi, J-Y., Kim, H-J., Kim, Y-J, Nam, S-H., and Shin, H-I. (2001). Differential expression patterns of Runx2 isoforms in cranial structure morphogenesis. *Journal of Bone and Mineral Research*, 16(5), 885-892.

23. Park, J. W., and Graveley, B. R. (2007) Complex alternative splicing. *Advances in Experimental Medicine and Biology*, 623, 50-63.

24. Rutkovskiy, A., Stensløkken, K-O., and Vaage, I. J. (2016). Osteoblast differentiation at a glance. *Medical Science Monitor Basic Research*, 22, 95-106.

25. Santos, A., Bakker, A-D., Blieck-Hogervorst, J., and Klein-Nulend, J. (2010). WNT5A induces osteogenic differentiation of human adipose stem cells via rho-associated kinase Rock. *Cytotherapy*, 12, 924-932.

26. Sato, A., Khadka, D. K., Liu, W., Bharti, R., Runnels, L. W., Dawid, I. B., & Habas, R. (2006). Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation. *Development, 133*(21), 4219-4231.

27. Sethi, J. K., and Vidal-Puig, A. (2010). Wnt signalling and the control of cellular metabolism. *The Biochemical Journal, 427*(1), 1–17.

28. Shi, Y., Hon, M., and Evans, R. M. (2001). The peroxisome proliferator-activated receptor δ, an integrator of transcriptional repression and nuclear receptor signaling. *Proceedings of the National Academy of Sciences*, 99(5), 2613-2618.

29. Stock, M., and Otto, F. (2005), Control of RUNX2 isoform expression: The role of promoters and enhancers. *Journal of Cellular Biochemistry*, 95: 506–517.

30. Takayama, O., Yamamoto, H., Damdinsuren, B., Sugita, Y., Ngan, C. Y., Xu, X., Tsujino, T., Takemasa, I., Ikeda, M., Sekimoto, M., Matsuura, N., and Monden, M. (2006). Expression of PPAR*δ* in multistage carcinogenesis of the colorectum: implications of malignant cancer morphology. *British Journal of Cancer*, *95*(7), 889–895.

31. Tyagi, S., Gupta, P., Saini, A. S., Kaushal, C., and Sharma, S. (2011). The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *Journal of Advanced Pharmaceutical Technology & Research*, *2*(4), 236–240.

32. Uehara, S., Yamashita, T., Udagawa, N., Takahashi, N., Nishita, M., Nakamura, T., Murakami, K., Mukai, H., Minami, Y., Maeda, K., Ishihara, A., and Kobayashi, Y. (2017). Protein kinase N3 promotes bone resorption by osteoclasts in response to Wnt5a-Ror2 signaling. Science Signaling, 10(494).

33. Wang, Y. (2009). Wnt/Planar cell polarity signaling: A new paradigm for cancer therapy. *Molecular Cancer Therapeutics*, *8*(8), 2103-2109.

34. Zhang, C., Tang, W., Li, Y., Yang, F., Dowd, D. R., and Macdonald, P. N. (2011). Osteoblast-Specific Transcription Factor Osterix Increases Vitamin D Receptor Gene Expression in Osteoblasts. *PLoS ONE*, *6*(10): e26504.

35. Zhang, X., Guo, J., Zhou, Y., and Wu, G. The roles of bone morphogenetic proteins and their signaling in the osteogenesis of adipose-derived stem cells. *Tissue Engineering*, 20(1), 84-92.

36. Zhou, Y., Kipps, T. J., and Zhang, S. (2017). WNT5A Signaling in Normal and Cancer Stem Cells. *Stem Cells International*, 2017, Article ID 5295286, 6 pages