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WNT5A is a secreted glycoprotein that binds to both canonical and non-canonical Wnt receptors and has important roles in morphogenesis (e.g., anterior-posterior axis elongation and limb formation) and differentiation (e.g., bone and cartilage). On a cellular level WNT5A functions in proliferation, adhesion, migration, and cell polarity. Altered WNT5A expression is associated with various human diseases, particularly cancer, but has also been linked with the inflammatory response. WNT5A has two isoforms that are derived from distinct promoters; the proteins isoforms referred to as L(A) and S(B) differ by 18 amino acids. In this project I examined the functional differences between the proteins isoforms L(A) and S(B) and the regulation of their promoters. The isoforms may have differential affinity for non-canonical receptors, selectively activating particular signaling pathways. They may also display distinct patterns of expression in particular cells, during differentiation and in development, as a consequence of their unique promoters. Conditioned medium (CM) was prepared from CHO cells expressing either isoform L(A) or S(B). Using a TOPFlash system the CM-L(A) and CM-S(B) were shown to be active. The CM was used to analyze the effects of the isoforms on the non-canonical Wnt signaling pathways  $Ca^{2+}$  and PCP/CE in HCT 116 (colon cancer) and hFOB1.19 (normal human osteoblast) cell lines by measuring levels of phospho (p)PKC and phospho (p)JNK, downstream targets of each pathway. Results showed that the CM-S(B) activated both pPKC and pJNK in HCT 116 cells whereas CM-L(A) had less of an effect. There was little or no effect of both CM's in hFOB1.19 cells.

AP-1 and NFAT luciferase reporter assays in HCT 116 cells confirmed the effect of CM-S(B) on pJNK. Next the effect of the CM's on apoptosis, proliferation, and migration were analyzed. Neither CMs affected the level of apoptosis in HCT 116 cells. Both isoforms were found to decrease proliferation in HCT 116 cells but CM-S(B) had a more consistent effect. CM-S(B) was found to increase migration and CM-L(A) decrease migration in HCT 116 cells. In a mouse embryonic fibroblast (MEF)-with a PORCN gene knock-out, both CMs caused a decrease in migration. Next, isoform L(A) and S(B) luciferase promoter constructs were transfected into HCT 116 and hFOB1.19 cells. The pattern of expression in the two cell types was similar for promoter L(A) and promoter S(B) but promoter S(B) showed a higher level of expression. 1707bp of upstream sequence showed a maximal expression for promoter L(A) and 1257 bp showed a maximal expression for promoter S(B) in both proliferating cell types. Analysis of the promoter L(A) and promoter S(B) reporter constructs during hFOB1.19 differentiation indicated that promoter L(A) is more highly activated than promoter S(B). Sequences within 420bp and 187bp for promoter L(A) and promoter S(B), respectively, were sufficient for activation. Putative transcription factor binding sites were identified in promoter L(A) and promoter S(B) upstream sequences. Some of these factors are known to be involved in osteogenesis. In summary, my results suggest that the WNT5A isoform proteins have distinct and cell type dependent. The isoform promoters have distinct sequences and include similar and unique putative transcription factor binding sites but both promoters are activated during osteogenesis.

ARE THE WNT5A ISOFORMS FUNCTIONALLY DISTINCT?- PROMOTER AND  
SIGNALING PATHWAY ANALYSES

by

Ahmed G. Elshaarawi

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Committee Chair

Dedicated to my lovely wife, Hadeer, my son Adam, my passed away Dad, Prof. Dr.  
Gamaleldin Elshaarrawi, also to my beloved Mom, Bothina and sisters Magy and Omnia  
for their love, support and understanding in all my endeavors.

APPROVAL PAGE

This thesis written by Ahmed G. Elshaarrawi 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

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## TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vii
LIST OF FIGURES .....	viii
CHAPTER	
I. INTRODUCTION .....	1
Wnt Signaling .....	1
Canonical Wnt Signaling .....	2
Non-Canonical Wnt Signaling.....	3
WNT5A Gene and Proteins Structure.....	5
The WNT5A Protein Processing and Secretion.....	7
WNT5A and Cancer .....	8
WNT5A Isoform Functions .....	9
WNT5A and Osteogenesis.....	10
Project Overview and Significance.....	11
II. MATERIALS AND METHODS.....	13
Cells and Cell Culture.....	13
Maintaining the HCT 116 and hFOB1.19 Cell Lines .....	14
Osteoblast Differentiation.....	15
Preparation of Conditioned Medium (CM).....	15
Characterization of the CM.....	16
Western Blot .....	16
TOPFlash and FOPFlash Assays .....	17
Transfecting hFOB1.19 and HCT 116 Cells.....	18
AP-1 and NFAT Reporter Assays.....	18
Analysis of Wnt Non-Canonical Signaling by Flow Cytometry .....	19
Apoptosis Assay.....	21
Luciferase Assay and Analysis .....	22
Proliferation Assay.....	22
Transfection and Analysis of Isoform L(A) and Isoform S(B)	
Promoter-Luciferase Constructs .....	23
Transcription Factor Analysis and Sequence Comparison .....	24
Migration Assay.....	25
III. RESULTS .....	27

Preparation and Characterization of WNT5A Conditioned Medium.....	27
Analysis of WNT5A-Isoform L(A) and S(B) Activation of Non-Canonical Signaling by Flow Cytometry .....	32
pPKC- HCT 116 .....	33
pJNK-HCT 116.....	36
pPKC-hFOB1.19.....	39
pJNK-hFOB1.19.....	41
Mouse Embryonic Fibroblast (MEF)- PORCN Knock-Out .....	43
Analysis of WNT5A-Isoform L(A) and S(B) Activation of Non-Canonical Signaling by Reporter Assays .....	45
Cellular Effects of the WNT5A Isoforms A and B – Apoptosis, Proliferation, and Migration.....	48
Apoptosis Assay.....	48
Cell Proliferation Assay.....	49
Migration Assay.....	51
Analysis of the WNT5A Isoform Promoters in Dividing Cells and During Osteoblast Differentiation.....	53
Transcription Factor Binding Site Analysis.....	57
Promoter A.....	57
Promoter B.....	60
IV. DISCUSSION.....	65
REFERENCES .....	78



## LIST OF TABLES

	Page
Table 1. Antibody List used in Flow Cytometry Analysis .....	20
Table 2. TOPFlash and FOPFlash Assay Treatment Combinations.....	28
Table 3. Promoter A TF's .....	59
Table 4. Promoter B TF's .....	61

## LIST OF FIGURES

	Page
Figure 1. Wnt Signaling Overview .....	2
Figure 2. The Overall Exon- Intron Structure of the WNT5A Gene.....	6
Figure 3. Amino Acid Sequence Alignment for Isoform A and B .....	7
Figure 4. AP-1 Reporter Vector and NFAT Reporter Vector.....	19
Figure 5. Effect of Conditioned Medium (CM) NIH3T3 .....	29
Figure 6. Effect of CM HEK293T .....	31
Figure 7. Conditioned Medium Western Blot.....	32
Figure 8. Flow Cytometry Analysis Outline.....	33
Figure 9. Trial 1 HCT 116 Flow Cytometric Results of pPKC .....	35
Figure 10. Trial 2 HCT 116 Flow Cytometric Results of pPKC. ....	36
Figure 11. Trial 1 HCT 116 Flow Cytometric Results of pJNK.....	38
Figure 12. Trial 2 HCT 116 Flow Cytometric Results of pJNK.....	39
Figure 13. Trial 1 hFOB1.19 Flow Cytometric Results of pPKC .....	40
Figure 14. Trial 2 hFOB1.19 Flow Cytometric Results of pPKC .....	41
Figure 15. Trial 1 hFOB1.19 Flow Cytometric Results of pJNK.....	42
Figure 16. Trial 2 hFOB1.19 Flow Cytometric Results of pJNK.....	43
Figure 17. MEK PORCN Flow Cytometric Results of pPKC.....	44
Figure 18. MEK PORCN Flow Cytometric Results of pJNK .....	45
Figure 19. HCT 116 Transfected with AP-1, NFAT and CMV Luciferase Constructs .....	47

Figure 20. HCT 116 Apoptosis Assay. ....	48
Figure 21. HCT 116 Proliferation Assay .....	50
Figure 22. hFOB1.19 Proliferation Assay .....	51
Figure 23. HCT 116 Migration Fluorescent Assay.....	52
Figure 24. MEF-PORCN Migration Assay .....	53
Figure 25. WNT5A Promoter Constructs .....	55
Figure 26. Promoter Constructs Expression in hFOB1.19 and HCT 116.....	56
Figure 27. Promoter Constructs Expression During Differentiation hFOB1.19 .....	57
Figure 28. Generalized Map of Transcription Factors Promoter A .....	63
Figure 29. Generalized Map of Transcription Factors Promoter B .....	64

# CHAPTER I

## INTRODUCTION

### **Wnt Signaling**

Wnt signaling is important to variety of developmental processes and is altered in various diseases. The name *WNT* is derived from the combination of the *Drosophila* Wingless gene (*Wg*) and the mammalian integration 1 gene (*Int1*). The mammalian homolog was discovered in 1982 in mammal (Nusse and Varmus, 2012). The *WNT* family consists of 19 genes leading to 12 evolutionarily conserved *WNT* subfamilies throughout the animal kingdom. The Wnt family of secreted glycoproteins function as short or long range signaling molecules.

Over the past years, our understanding of Wnt signaling has greatly increased. The Wnt signaling pathways are divided into canonical and non-canonical. The canonical pathway involves  $\beta$ -catenin, whereas the non-canonical doesn't involve  $\beta$ -catenin.

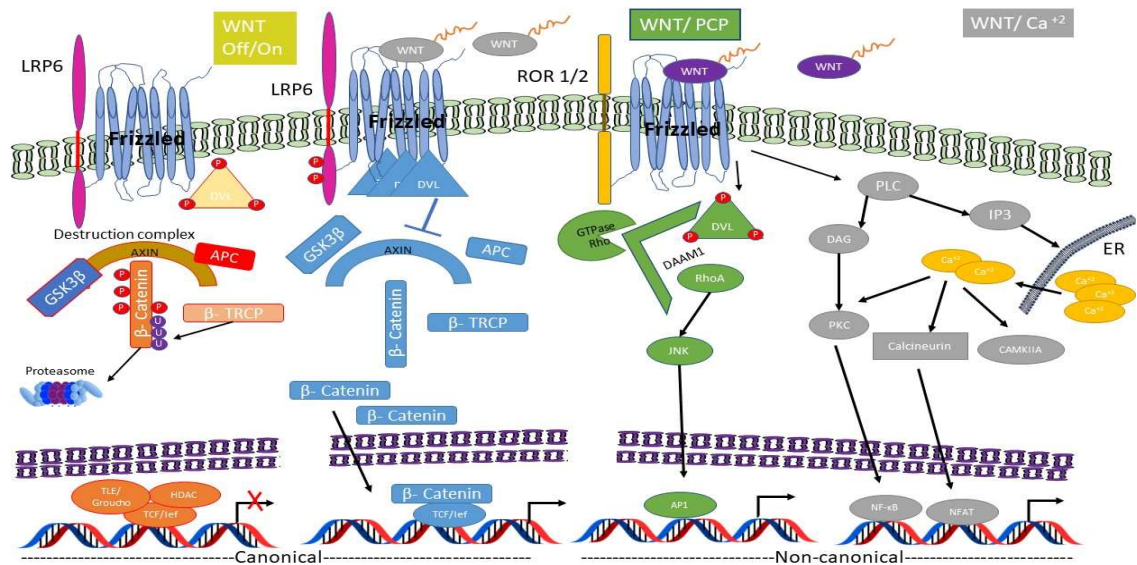


Figure 1. *Wnt Signaling Overview. Canonical on and off state (left and middle) non-canonical PCP and Ca<sup>2+</sup> (right) modified from Zhan et al., (2017).*

The focus of this research is WNT5A, a secreted ligand involved in activating non-canonical pathways. It has been established that WNT5A binds to several receptors and co-receptors; the Frizzled (Fz) family of receptors, Lipoprotein receptor-related protein (LRP) family, and Receptor tyrosine kinase kinase-like orphan receptor 2 (ROR2) (Figure 1).

### *Canonical Wnt Signaling*

Canonical Wnt signaling includes a single central pathway that involves the translocation of β-catenin to the nucleus, where it acts as a transcription factor (Figure 1). In the absence of the Wnt ligands such as WNT3A, Dishevelled (Dvl) is phosphorylated. Dvl blocks the intrinsic activation of destruction complex. The destruction complex, consisting of Glycogen synthase kinase 3β (GSK-3β), the scaffold protein Axin and Adenomatous polyposis coli (APC), phosphorylates β-catenin. Phosphorylated β-catenin

can then be dissociated from the complex and be ubiquitinated by  $\beta$ -Trcp (F-box/WD repeat-containing protein), which targets  $\beta$ -catenin for proteasome degradation. In the absence of  $\beta$ -catenin, the repression complex of T-cell factor/lymphoid enhancer factor (TCF/LEF) and Transducing like enhancer protein (TLE/Groucho) recruits Histone deacetylase complexes (HDACs) to repress target genes. In the ON status Wnt ligands bind to Fz receptor and LRP 6 co-receptors are phosphorylated. Dishevelled (Dvl) proteins are recruited to the plasma membrane where they polymerize and are activated. Consequently, the destruction complex is not formed. These events will cause the release, stabilization and translocation to the nucleus of  $\beta$ -catenin (Rao and Kuhl, 2010; Zhan et al., 2017). Activation of  $\beta$ -catenin leads to transcription of numerous genes including c-myc, cyclin D, Runx2 and EGF receptor (Nusse and Varmus, 2012).

### *Non-Canonical Wnt Signaling*

Planar cell polarity / cell elongation (PCP/CE) and  $\text{Ca}^{2+}$  signaling are the two chief non-canonical Wnt signaling pathways (Figure 1). The PCP/CE pathway is initiated when a Wnt ligand such as WNT5A binds to a Fz receptor and the Receptor tyrosine kinase-like orphan receptor (ROR) co-receptor to form a receptor complex. This binding recruits and activate Dvl. The Dvl activates G protein Rho (GTPase family) and reactivates the scaffolding protein, Dvl associated activator of morphogenesis 1 (DAAM 1). Ras homolog gene family member A (RhoA) becomes activated, followed by triggering of GTPase family members, Rho-associated coiled-coil-containing protein kinase (ROCK) and Jun amino-terminal kinase (JNK). This leads to a cytoskeleton and/or transcriptional response (Martinez et al., 2015; Yang et al., 2016b; Zhan et al., 2017).

The Wnt /Calcium ( $\text{Ca}^{2+}$ ) Pathway is initiated by WNT ligand binding to the Fz receptor and co-receptor ROR. This activates G proteins, which elicits Phospholipase C (PLC) activity and synthesis of Inositol trisphosphate (IP3) and Diacylglycerol (DAG). This leads to the mobilization of free intracellular  $\text{Ca}^{2+}$  that regulates many calcium dependent processes including the cytoskeleton, cell motility and transcriptional regulation. IP3 diffuses to the cytosol causing  $\text{Ca}^{2+}$  release and binding to Calmodulin, which activates Calcium calmodulin- dependent protein Kinase II (CAMKII). The increase in  $\text{Ca}^{2+}$  also leads to Protein Kinase C (PKC) activation. This mainly leads to the translocation of Nuclear factor kappa-light-chain-enhancer of activated beta cells (NF-kB) and Nuclear factor of activated T cells (NFAT) transcription factors to the nucleus. The released  $\text{Ca}^{2+}$  binds PKC, Calcineurin and CAMKII. Both CAMKII and PKC phosphorylate and activate NF-kB and NFAT translocation to the nucleus (Chow et al., 2011; De, 2011; Zhan et al., 2017)

Activation of the non-canonical pathways will lead to transcription factor activation and changes in gene expression (Figure 1) One transcription factor previously mentioned is NFAT. NFATs is comprise a family of transcription factors found in many cell types and controls several processes, including angiogenesis and osteogenesis. It is a therapeutic target for modulation of bone remodeling. It has been reported that NFAT relay signaling in the control of osteogenesis and osteoporosis (Piva, 2011).

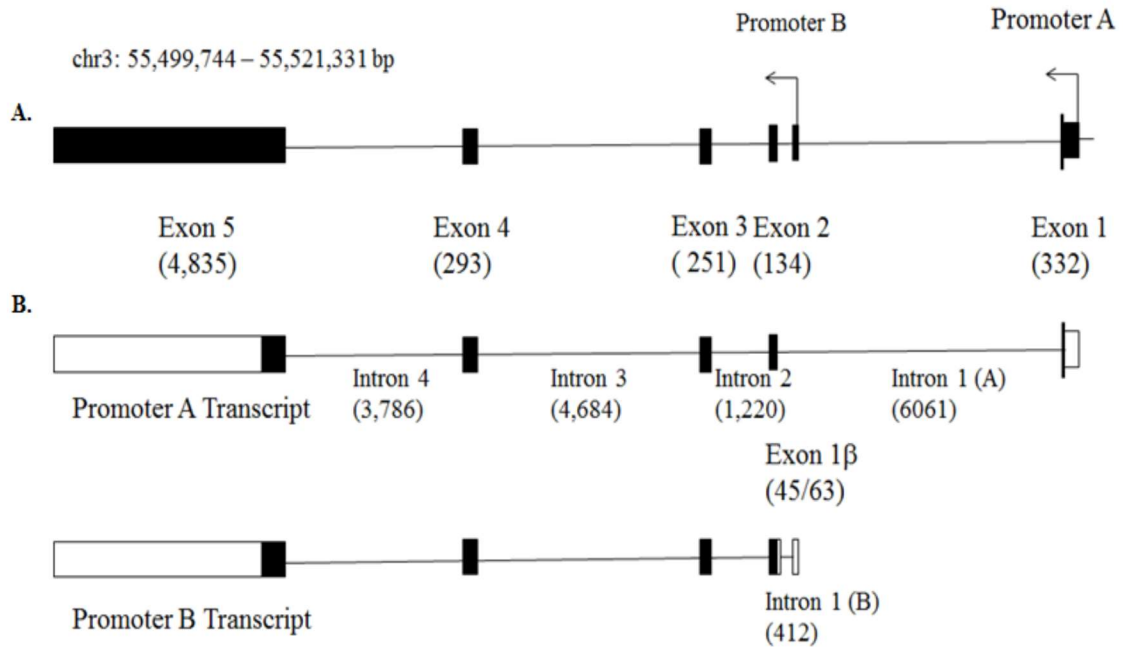
Another WNT transcription factor target is Activator Protein-1 (AP-1), which is downstream of the Planer cell polarity/ cell elongation (PCP/CE) pathway. It exerts numerous effects on transformation, stress response, differentiation, proliferation,

development and apoptosis. AP-1 overactivation has been reported in colorectal cancer but the specific role of activated AP-1 in colorectal cancer are not totally understood (Ashida et al., 2005; Kang et al., 2012).

### **WNT5A Gene and Proteins Structure**

The human *WNT5A* gene is located on chromosome 3 (3p14-p21) and it generates five distinct transcripts. This thesis is focused on two of these transcripts, WNT5A 201 [Isoform L(A)] and WNT5A 205 [(Isoform S(B))]. These transcripts are composed of 5 exons (Figure 2). The promoter A transcript includes a unique exon 1, whereas the promoter B transcripts includes a unique exon 1 $\beta$ . Exon 1 $\beta$  sequences are found in the first intron of the promoter A transcript but are spliced out. Exons 2, 3, 4,5 are common to both transcripts.





*Figure 2. The Overall Exon- Intron Structure of the WNT5A Gene. A) Black box represents exons, lines represent introns, and arrows represent transcription start sites. B) Promoter A and Promoter B primary transcripts. Black boxes are coding regions; open boxes are untranslated regions. Intron and exon lengths are indicated in parentheses. Exon 1 is unique to Promoter A, whereas Exon 1 $\beta$  is unique to Promoter B (Katula et al., 2012). The orientation of the gene is shown as found in the genome.*

These transcripts are translated into two protein isoforms; WNT5A Isoform L(A) and Isoform S(B). Which are named Long (L) and Short (S) by (Bauer et al., 2013), whereas, our lab has named them A and B. The isoforms differ in length by 15 amino acids at the N-terminus. After processing in the endoplasmic reticulum (ER) the isoforms differ in length by 18 amino acids at the N-terminus. Isoform S(B) is essentially a shortened version of isoform L(A) (Figure 3) (Bauer et al., 2013).



Figure 3. Amino Acid Sequence Alignment for Isoform A and B. A and B upper row and lower row respectively. Red box is the AA before processing and the green box is the AA after processing (Bauer et al., 2013; Manner, 2016).

### The WNT5A Protein Processing and Secretion

Wnt processing and secretion are a highly regulated processes. The secreted Wnt is hydrophobic and mostly found associated with cell membranes and extracellular matrix. Mass spectroscopy and mutational analyses showed that Wnt is lipid modified and N-linked glycosylated. It has been shown that the role of palmitoylation of Wnt is important for glycosylation, secretion, membrane targeting and its function (Mikels and Nusse, 2006a; Port and Basler, 2010).

WNT5A is palmitoylated at Cys<sup>104</sup>; this is necessary for receptor binding but not necessary for secretion (Willert et al., 2003). It is glycosylated at Asn<sup>114</sup>, Asn<sup>120</sup>, Asn<sup>311</sup>, and Asn<sup>325</sup> (Kikuchi et al., 2012).

The enzyme Porcupine (PORCN) is important for post-translational modification of all of the Wnt's, including WNT5A. PORCN is a member of the membrane-bound O-acyltransferase family (MBOAT) situated in the endoplasmic reticulum. PORCN adds a palmitoyl group to Wnt, which is an essential lipid-modifying step required for Wnt signaling ability and secretion. Recent studies have demonstrated that PORCN inhibitors may serve as promising drug targets of the Wnt signaling pathway, as they show

specificity to Wnt production (Boone et al., 2016; Tian et al., 2017). Various inhibitors of PORCN have been identified and characterized as inhibitors of Wnt production (IWPs), such as IWP-1, IWP-2 and LGK974, (Novellademunt et al., 2015). These inhibitors are also being used in to block Wnt signaling.

### **WNT5A and Cancer**

The connection of Wnt pathway with cancer was first shown via the activation of WNT1 either by pro-viral insertion or transgenic overexpression. This resulted in mammary hyperplasia and tumors. In 1991, mutations in the Adenomatous polyposis (APC) gene were discovered to be associated with colon cancer. The APC gene inhibits the canonical Wnt signaling by formation of the  $\beta$ -catenin destruction complex (Zhan et al., 2017).

WNT5A mis-regulation has been associated with cancer, colorectal carcinoma (Ying et al., 2008), neuroblastoma (Blanc et al., 2005), hepatocellular carcinoma (Bi et al., 2014), leukemia (Yu et al., 2017), lung (Yang et al., 2016), pancreatic (Bo et al., 2013), thyroid (Wang et al., 2016), melanoma (Sadeghi et al., 2018), and osteosarcoma (Zhang et al., 2014). WNT5A has been found to be altered (Fernandez-Cobo et al., 2007) and it is governed by the accessibility of receptors and intercellular interaction with assorted cell types in breast cancer (Zeng et al., 2016). However, It appears that WNT5A serves as a tumor suppressor in some cancers, being inactivated or down-regulated, and as an oncogene in others, in which WNT5A is up-regulated.

It is was found that in melanoma and gastric cancer cells WNT5A signaling induces metastasis and invasion (Shojima et al., 2015; Weeraratna et al., 2002). In

hepatocellular carcinoma (HCC) WNT5A is activated in a well differentiated, e.g. HepG2 cell line (less aggressive form of HCC) and it is repressed in poorly differentiated, e.g. SNU398 cell line (more aggressive form of HCC) (Yuzugullu et al., 2009). Hence, understanding WNT5A regulation and function in cancer progression is of medical and societal importance.

### **WNT5A Isoform Functions**

The two WNT5A isoforms L(A) and S(B) have distinct functions. It is possible that the different isoforms account for WNT5A acting as either a tumor suppressors or oncogene. Bauer et al., (2013) found WNT5A isoform levels vary in different cancers. WNT5A-L(A) and WNT5A-S (B) varied in MDA-MB-231 (breast carcinoma), HeLa (cervix carcinoma) and SH-SY5Y (neuroblastoma) cell lines. In a comparative study, isoform B increased the proliferation rate, whereas overexpression of isoform A decreased proliferation rate. Additionally, it was showed that both isoforms inhibited the canonical ( $\beta$ -catenin) pathway to the same degree.

Huang et al., (2017) studied the two WNT5A isoforms in colorectal cancer, specifically in HCT116 cell line. Our lab has found that this cell line has a very low or no expression of both WNT5A isoforms (data unpublished). In contrast, Huang et al., state that both isoforms are expressed and WNT5A-S(B) has a higher level of expression than that of WNT5A-L(A). Silencing of the WNT5A-S(B) isoform but not WNT5A-L(A) increased apoptosis and reduced colony formation. The WNT5A-S(B) correlated with a high  $\beta$ -catenin level, which indicates a positive feedback. These results suggest that the WNT5A isoforms are functionally distinct. However, it is unclear how meaningful the

results can be by knocking out expression of genes transcribed at very low levels, particularly for WNT5A-L(A).

Katula et al., (2012) reported that the WNT5A isoforms promoters show differential regulation in NIH3T3 and Caco-2 cells. Promoter B of WNT5A-S(B) exhibited a distinct level of expression in these cell lines. The promoter A of WNT5A-L(A) demonstrated higher expression than the promoter B in fibroblast cells. Treatment of the cells with TNF- $\alpha$  revealed the differential responsiveness and activity level of each promoter. In another study, WNT5A isoform B was found to be decreased in osteosarcoma cells, relative to isoform A and this was due to promoter DNA methylation and histone modification (Vaidya et al., 2016).

### **WNT5A and Osteogenesis**

The two major pathways of bone formation are direct conversion of mesenchymal tissue into bone and indirect conversion where a cartilage intermediate is formed. These modes of osteogenesis are called intramembranous ossification and endochondral ossification (Van Amerongen et al., 2012). Osteoblast cells are responsible for secretion of an extracellular matrix (collagen-proteoglycan) that is able to bind calcium salt, i.e. start of mineralization that is found in the intramembranous osteogenesis (Gilbert, 2000). In this project, I focus on the intramembranous ossification pathway that involves committed bone precursor cells or osteoblast cells.

A high level of Wnt / $\beta$ -catenin was shown to inhibit the osteogenic differentiation of embryonic mesenchymal cells and induce osteogenesis in mature osteoblast, i.e. it is needed only at earlier activation of the pathway. WNT5A overexpression was found to

induce the Wnt / $\beta$ -catenin pathway in mesenchymal cells, which had hindering effects on ossification (Van Amerongen et al., 2012). It was also illustrated that recombinant WNT5A expression can enhance osteogenesis and osteogenic differentiation (Keller et al., 2016). WNT5A knock-out mice show an array of phenotypes including skeletal and internal organs defects, dwarfism, craniofacial defects, limb abnormalities and intestinal elongation defects (Keller et al., 2016). These studies support the conclusion that WNT5A has a functional role in osteogenesis. However, the specific functions of the WNT5A isoforms in osteogenesis is not known. Research in our lab has shown that transcript levels of both WNT5A isoforms increase during osteogenesis (unpublished data). As such, osteogenesis is a good model to investigate both the functions of the WNT5A isoforms and their transcriptional regulation.

### **Project Overview and Significance**

WNT5A is conserved across many species and is essential in early organismal development, cell homeostasis, stem-cell biology, and cancer progression. In cancer, WNT5A appears to act as either an oncogene or tumor suppressor, however the function of WNT5A in cancer is not thoroughly understood. In particular, the functions of the two WNT5A isoforms L(A) and S(B) need further investigation. In osteogenesis, there is a distinct level of expression of the isoforms during the bone formation process. Elucidation of the cellular functions, activation of the non-canonical signaling pathways by the two isoforms, and the differential regulation of the isoform promoters is significant as it will increase understanding of the role and regulation of WNT5A in cancer cell behavior and the osteogenesis process. Results from this project may allow for

manipulation of WNT5A expression and activities. In order to address these questions, I completed the following specific aims:

1. Determined which Wnt non-canonical signaling pathway is being activated by the WNT5A isoforms L (A) and S (B).
2. Determined the effects of the WNT5A isoforms on cell proliferation, migration, and apoptosis.
3. Identified the regulatory sequence regions responsible for increased expression of the WNT5A isoforms during osteogenesis
4. Identified putative consensus transcription factor binding sites in promoters and osteoblast regulatory regions of the WNT5A isoforms L (A) and S (B)

## CHAPTER II

### MATERIALS AND METHODS

#### **Cells and Cell Culture**

All the cell lines were obtained from the American Type Culture Collection (ATCC) and grown following the ATCC protocol specific for each cell line (see below). The cells were grown in an incubator with the CO<sub>2</sub> level at 5% and 37°C unless stated otherwise.

L cell (*CRL-2647*) are fibroblastic mouse cells. They were grown in Dulbecco Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1x penicillin (pen) /streptomycin (strep) using a 100x stock (10,000 unit's penicillin/10,000µg streptomycin).

L Wnt3A (*CRL-2647*) are fibroblastic mouse cells stably transfected with an expression vector for the human Wnt3A protein. The cells secrete the Wnt3A into the medium. They were grown in DMEM with 10% fetal bovine serum (FBS) and 1x pen/strep using a 100x stock (10,000 unit's penicillin/ 10,000µg streptomycin).

HEK293T (*CRL-3216*) are epithelial human embryonic kidney cells. They were grown in DMEM with 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% bovine calf serum (FBS) and 1x pen/strep using a 100x stock (10,000 unit's penicillin/ 10,000µg streptomycin).



NIH/3T3 (*CRL-1658*) are fibroblastic mouse embryonic cells. They were grown in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% bovine calf serum (FBS) and 1x pen/strep using a 100x stock (10,000 unit's penicillin/ 10,000µg streptomycin).

HCT 116 (*CRL-247*) are epithelial human colon cancer cells. They were grown in McCoy's 5a with 1.5 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) and 1x pen/strep using a 100x stock (10,000 unit's penicillin/ 10,000µg streptomycin).

hFOB1.19 (*CRL-11372*) are normal human osteoblast cells. The cells are immortalized due to expression of the SV40 Large-T. They were grown in DMEM/Hams's F12 1:1 with 2.5 mM L-glutamine without phenol red, 0.3 mg/ml G418, 10% fetal bovine serum (FBS) and 1x pen/strep using a 100x stock (10,000 unit's penicillin/ 10,000µg streptomycin).

#### *Maintaining the HCT 116 and hFOB1.19 Cell Lines*

The HCT 116 cells were grown to 80-90% confluence then collected using Trypsin-EDTA (0.25% w/v- 0.53mM). Cells were counted using a hemocytometer and re-plated in a T-75 flask at a lower density.

hFOB1.19 cells were grown to 80-90% confluence at 34.4°C, then collected using Trypsin-EDTA (0.25% w/v- 0.53mM). The cells are counted using a hemocytometer and re-plated in T-75 flask. The medium is changed every 3-4 days.

## **Osteoblast Differentiation**

Osteoblast hFOB1.19 cells are grown to confluency at 34.4°C. The cells were induced to differentiate by the addition of differentiation media to the cells and incubating the cells at 39.4°C. Differentiation medium is the hFOB1.19 complete medium supplemented with  $\beta$ -glycerol phosphate ( $5 \times 10^{-3}$  mol/L), ascorbic acid (0.1 g/L), Menadione ( $10^{-8}$  mol/L), 1, 25(OH) $_2$ D $_3$  (vitamin D) ( $10^{-7}$  mol/L). Cells were maintained in this media during the length of the study involving differentiating cells.

## **Preparation of Conditioned Medium (CM)**

Chinese Hamster Ovary (CHO) cells stably transfected to express Isoform L(A) or S(B) and the parental CHO line were a gift from Dr. Karl Willert (University of California, San Diego). The CHO cells were grown in DMEM (D1145, ATCC), supplemented with 10%FBS, 1x penicillin / streptomycin using a 100x stock (10,000 units penicillin/ 10,000 $\mu$ g streptomycin) and L-Glutamine (0.584 g/L), then G418 (0.4 mg/ml), Blasticidin (3 $\mu$ g/ml) and Doxycycline (5ng/ml) were added per 12 mL of complete medium in a T-75 flask. The cells were grown to 80-90% confluence in an incubator with CO $_2$  level 5% and 37°C. The cells were collected and re-plated in 10 cm plates and grown to confluency. Protein expression was induced by adding Doxycycline (250ng/ml). No Blasticidin or G418 was added. The media was collected from the cells at 3 days and stored at 4°C. Fresh medium was added along with doxycycline. The medium was collected after another 4 days of incubation and combined with the previously collected medium. The medium was filter sterilized, first through a 0.4 $\mu$ m filter and then through 0.25 $\mu$ m filter. The WNT5A isoform L(A) CM is referred to as CM L(A) and

isoform S(B) CM as CM S(B). The CM was stored at 4°C for further use in various experiments.

## **Characterization of the CM**

### *Western Blot*

To determine isoform L (A) and isoform S (B) protein levels in the CM, 50 µl aliquots of the CM were prepared for western blot by adding 10 µl of a 5x SDS Sample Buffer and heating the samples for 5 min at 95°C. The samples were run on a 10% SDS-PAGE gel at 40 mA constant current for one gel. The gel was blotted onto nitrocellulose overnight using a BIO-RAD Mini Trans-Blot Cell apparatus at 30V constant voltage. The next day the blot was removed, washed in Tris-buffered saline (pH 8.0), 0.1% Tween 20 (TTBS) and protein detected by Ponceau S staining by adding Ponceau S staining solution to the blot for 5 min and de-staining in water. The stained blot was photographed and washed in TTBS to remove the stain. The blot was blocked in 5% dried milk-TTBS for one hour. The primary antibody (Cell Signaling -WNT5a/b (C27E8) Rabbit mAb) was added at 1:1000 in 1% dried milk-TTBS and incubated at 4°C overnight with shaking. The next day the antibody was removed, and the blot washed (3x for 5 min. and 1x for 15 min.) in TTBS. The secondary anti-mouse IgG-HRP (Cell Signaling) was added at 1:10,000 diluted in 1% milk-TTBS and incubated for one hour. The blot was washed as before and then detected using the detection solution (SuperSignal West Pico Chemiluminescent Substrate #34087- Thermo Scientific). The level of WNT5A protein band on the blot was quantified using the BioRad Documentation System.

### *TOPFlash and FOPFlash Assays*

The activity of the WNT5A isoforms in the prepared CHO-CM was assayed using a luciferase reporter of canonical Wnt signaling. This assay required the preparation of Wnt3A conditioned medium. Wnt3A activates the canonical Wnt signaling pathway. Wnt3A was prepared from the L Wnt3A cell line. The L cell Wnt3A and control L cells were grown in T-75 flasks, re-plated in 10 cm plates and grown to confluency. After 3 days the medium was removed and saved. Fresh medium was added and 4 days later the medium was removed and combined with the saved medium. The medium was filtered sterilized, aliquoted and stored at -20°C. The CM was labeled as L-CM and L Wnt3A-CM.

HEK293T cells were plated at  $1 \times 10^6$  cells per well in a 6 well plate. The next day cells was transfected with M50 Super 8x TOPFlash and M51 Super8x FOPFlash, which were gifts from Randell Moon (Addgene Plasmid#12456; Addgene Plasmid #12457) with the FuGENE6<sup>®</sup> transfection reagent (Promega; E2691) at a ratio of 3:1 reagent to DNA. The next day the cells were collected and re-plated in 96 well plate at  $1 \times 10^4$  cells per well. After one day the cells were treated as described in the results with the different conditioned mediums.

The following day cells was collected in Lysis Buffer 1X (Promega) and luminescence was measured using the Luciferase Assay Kit (Promega; E1500) for luciferase using a Bertholt LB 9501 luminometer.

### **Transfecting hFOB1.19 and HCT 116 Cells**

FuGENE 6<sup>®</sup> transfection reagent (Promega; E2691) was used in transfection of the cells. A ratio of 3:1 of reagent to DNA for hFOB1.19 and HCT 116 were determined in the lab to give the best transfection efficiency. The amount of DNA/Plasmid added is equal to 1.25 $\mu$ g/well for a 6- well plate. *Renilla* vector DNA was mixed with the reporter vector (1ng/well) in some experiments as an internal control for the transfection. *Renilla* vector DNA was added at 1.2 ng per 6-well plate. The cells were transfected one day after plating.

### **AP-1 and NFAT Reporter Assays**

hFOB1.19 and HCT 116 cells were used to assay for AP-1 and NFAT activity after treatment with CM L(A) and CM S (B). Assays were conducted in the absence and presence of a PORCN inhibitor. PORCN is an endoplasmic reticulum bound O-acyl transferase required for palmitoylation of Wnt ligands. Inhibition of PORCN prevents secretion of Wnt ligands. Assay without PORCN inhibitor: hFOB1.19 and HCT 116 cells were plated in 6-well culture dishes. The next day the cells were transfected with AP-1 (PGL4.4[luc2P/AP1 RE/Hygro] 6052 pb) (Promega) and NFAT (PGL3-NFAT luciferase 5049 bp) (Addgene) luciferase reporter vectors, in addition to a *Renilla* vector (control for transfection efficiency) using Fugene 6<sup>®</sup>, as previously described. The vectors are shown in Figure 4. The cells were grown for another day, collected, counted and re-plated in 48- well cell culture dishes at  $5 \times 10^4$  cells per well. The next day the medium was removed and replaced with the following: 1) Fresh medium; 2) 1:1 Fresh medium:

CM L(A); 3) 1:1 Fresh medium: CM S(B) CM; 4) 1:1 Fresh medium: CM P, (see experimental methods for generation and characterization of conditioned medium).

The treated cells were grown another day and lysed in Passive Lysis Reagent (Promega) and assayed for Luciferase and *Renilla* luciferase activity (see below). Or, in one study the cells were treated for a 6 hr and 12 hr time period.

With PORCN inhibitor: The protocol is same as above but the PORCN inhibitor (WNT-C59, Selleck) was added at 30 $\mu$ M after re-plating the cells in 48-well dish. The cells were grown one day in PORCN inhibitor before treating with CM.

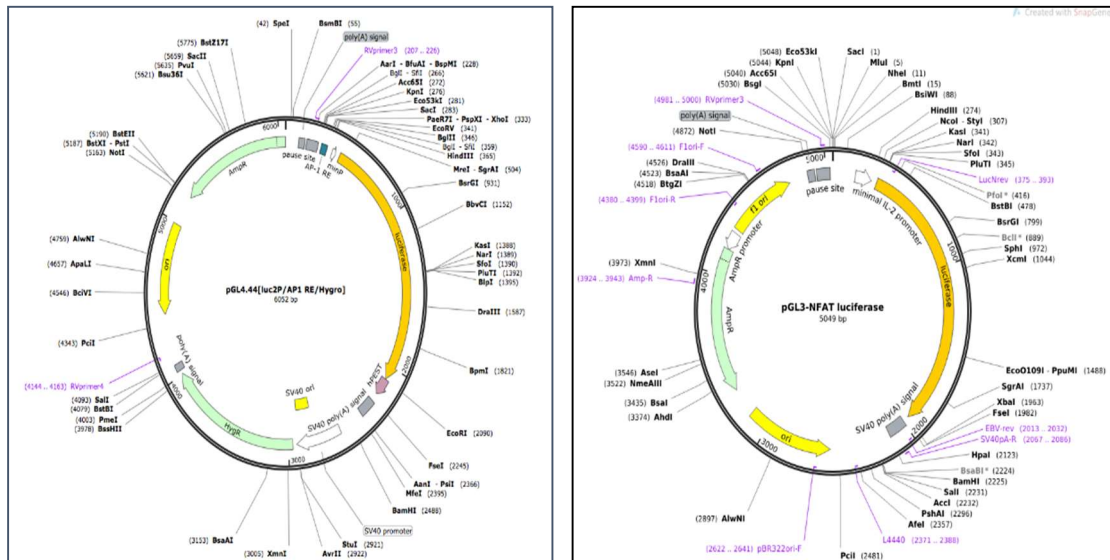


Figure 4. AP-1 Reporter Vector and NFAT Reporter Vector. AP-1 (image from Promega) (left) and NFAT (image from Addgene)(right).

### Analysis of Wnt Non-Canonical Signaling by Flow Cytometry

Flow cytometry was used to assay for changes in phosphor-JNK and phosphor-PKC levels in HCT 116 and hFOB1.19 cells after treatment with CM-L(A) and CM-

S(B). Cells were plated in 24-well plates and treated with CM-P, CM-L(A), and CM-S(B) as previously described but the exposure times varied and included 1 hr, 2 hr and 6 hr. Treated cells were harvested using Trypsin-EDTA, washed in Phosphate Buffered Saline 1X (PBS) and fixed for 10 min in 60% acetone in PBS on ice. The cells were pelleted by centrifugation and washed with 0.3% Bovine Serum Albumin (BSA) in PBS. The cell pellets were taken up in 100µL of primary antibody solution containing 0.3%BSA in PBS, 0.1% Triton X-100, 5% normal goat serum. The primary antibodies used in this study and amounts added are listed (Table 1). The cells in antibody solution were incubated at R/T for 1 hour, pelleted by centrifugation, and washed in 0.3% BSA in PBS.

*Table 1. Antibody List used in Flow Cytometry Analysis.*

Name	Company	Catalogue number	Dilution	Amount per 1x10 <sup>6</sup> cells per 100 µL
Phospho-PKC alpha (Thr638) polyclonal Rabbit Ab	Thermo Scientific	44-962G	1:250	0.4µL
Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb	Cell Signaling	9255	1:400	0.25µL
Total JNK (D-2)	Santa cruz	SC-7345	1:100	5µL
Total PKC α (H-7)	Santa cruz	SC-8393	1:500	0.2µL
Alexa Fluor <sup>®</sup> 488 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson Immunoresearch	715-545-150	1:200	0.5µL
Alexa Fluor <sup>®</sup> 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson Immunoresearch	711-545-152	1:200	0.5µL

The pelleted cells were re-suspended in the secondary antibody solutions containing 0.3%BSA/PBS and 0.5µl of the antibody Alexa Fluor<sup>®</sup> 488 anti-Rabbit or

Alexa Fluor<sup>®</sup> 488 anti-Mouse Ab (Table 1) were used. The cell antibody solution was incubated for 1 hr at rt then pelleted by centrifugation and washed with 0.3% BSA/PBS. The final cell pellet was re-suspended in 500 µl PBS. Counts of fixed and stained cells were acquired via Guava Easy Cyte 6-2L benchtop flow cytometer at 3000 to 5000 events (cells counted). The raw data was analyzed via Flowing Software v2.5.1 (Perttu Terho, Turku Centre for Biotechnology, Finland; [www.flowingsoftware.com](http://www.flowingsoftware.com)). From the generated graphs (counts vs Green fluorescence).

### **Apoptosis Assay**

Treated HCT 116 cells were assayed for apoptosis using the FITC Annexin V kit (BD Pharmingen). HCT 116 cells were plated in 24 well plates at  $3 \times 10^5$ . The next day the cells were treated with and without camptothecin (0.25 µM final concentration) and with the following conditioned mediums mixed 1:1 with fresh HCT 116 medium: CM-P, CM-L(A), and CM-S(B). The next day the cells were collected using Trypsin-EDTA. The cell pellets were washed in PBS, pelleted by centrifugation, and re-suspended in 400 µl of Annexin Binding Buffer. 50 µl of cells in binding buffer was removed to a new microfuge tube. 2.5 µl of FITC anti-annexin V antibody and 0.5 µl of propidium iodide solution (100 µg/ml stock). The samples were incubated at rt for 15 min in the dark. 400 µl of Annexin Binding Buffer was added and the cells analyzed by flow cytometry using the Guava Easy Cyte 6-2L benchtop flow cytometer at 5000 events. The data was collected as FITC (x-axis) vs PI (y-axis). The gate was adjusted using the CM-P treated, without camptothecin sample. Early (bottom right quadrant) and late (top right quadrant)



apoptosis values were summed and used for total apoptosis. Each sample was read three times and the experiment repeated four independent times. The average total apoptosis values were each sample was determined from the three technical replicas. The % change in total apoptosis of treated samples [CM-L(A) and CM-S(B)], with and with camptothecin, was determined relative to the CM-P control for each trial. These values were averaged for the four trials and standard error and significance determined.

### **Luciferase Assay and Analysis**

Dual-Luciferase<sup>®</sup> Reporter Assay System Kit (Promega; E1910) was used to assay for luciferase activity and *Renilla* activities. The media was removed from the each well and the cells washed once with PBS. 100 µl of 1x Passive Lysis Buffer (PLB) was added to the cells in the wells and incubate for 15 minutes at rt. Samples were stored at -80°C. 30µl of the PLB lysate was transferred to a 96-well plate and the activities of firefly and *Renilla* luciferase were read using the Synergy 2- BioTek plate reader with dual injectors. 45 µl of each reagent was injected. The firefly luciferase readings for each sample was divided by the *Renilla* readings for that same sample to obtain the L/R ratio. The averages of 4 to 6 replicate values and standard error were determined, and these values plotted in a bar graph.

### **Proliferation Assay**

HCT 116 and hFOB1.19 cells were plated at  $3 \times 10^3$  and  $1 \times 10^3$  cell per well of 96 well plates. The next day, day 0 cells were assayed. CyQuant<sup>™</sup> Proliferation Assay (Invitrogen) was used to quantify the DNA each day from day 0 to day 7. Following the CyQuant<sup>™</sup> protocol the media was removed from the wells of the cells being assayed

and 50  $\mu$ l of the CyQuant™ dye reagent was added, and the plate incubated for 30 min in the CO<sub>2</sub> cell culture incubator. The wells being assayed were read using microplate reader Synergy 2- BioTek with 420nm/580nm excitation and emission wave length. For testing the effects of CM-L(A), CM-S(B) and the control CM-P on proliferation, on day 0 the different CM's were mixed 1:1 with fresh complete medium and added to the appropriate number of wells for 7 days of assays. On day 0, the cells were re-treated with CM-medium (1:1). Each day the appropriate number of well for each treatment group were assayed using the CyQuant™ Proliferation Assay. The data were analyzed by determining the average value for each day and treatment. A student T-test was carried to determine significance ( $p < 0.05$ ) between samples and plotted in a graph.

### **Transfection and Analysis of Isoform L(A) and Isoform S(B) Promoter-Luciferase Constructs**

The upstream genomic sequences of the WNT5A isoform A and B were used to generate promoter constructs with a luciferase reporter (Figure 25) (Katula et al., 2012). The number beside each construct indicates the base pair upstream of each isoform cDNA sequence. These constructs were transfected into hFOB1.19 and HCT 116 cells.

hFOB1.19 cells were plated in 96-well cell culture dishes plate at  $1.1 \times 10^4$  cell per well to ensure 70-80% confluence. The next day the cells are transfected with each isoform promoter-luciferase constructs at 0.1  $\mu$ g DNA per well, along with 1ng of *Renilla* (Control vector) using FuGENE 6® as previously described. The next day, osteoblast differentiation medium was added to each well. This was considered Day 0. Cells were collected on Days 0 and 3 in PLB and the lysates stored at -80 °C. All samples are

assayed at the same time for firefly and *Renilla* luciferase activity, as previously described.

HCT 116 cells were plated in a 96-well culture plate at  $5 \times 10^4$  cell per well to ensure approximately 70-80% confluency. The next day the cells were transfected using the same protocol as for hFOB1.19. Approximately 24 hr later the cells were collected on day 0 and 3 in PLB, as previously described, the samples stored at  $-80^{\circ}\text{C}$ . The samples were assayed and analyzed as previously described.

### **Transcription Factor Analysis and Sequence Comparison**

DNA sequences of potential regulatory significance were identified in regions of WNT5A promoter A and B based on results of the transfections assay. The sequences required for maximal expression in differentiating hFOB1.19 were analyzed for potential transcription factor binding sites using various bioinformatics tools. The upstream sequences were obtained from Ensembl ([ENSG00000114251](http://ensembl.org/Homo_sapiens/Transcript/View?g=ENSG00000114251)). The sequences were analyzed in the ALGGEN (<http://algggen.lsi.upc.es/>) via PROMO analysis tool. PROMO is a virtual laboratory used to identify the transcription factors that can bind to specific sequence, which are extracted from TRANSFAC public database (<http://gene-regulation.com/pub/databases.html#transfac>). The initial list of possible transcription factors for a particular sequence was large. The stringency tool was adjusted to reduce the number of identified factors. The locations and number of particular binding sites was mapped onto the gene regulatory region of each isoform. Specific transcription factors were further analyzed via a PubMed search to determine their relationship to osteogenesis.

WNT5A Promoter A and B upstream sequences were compared using an alignment software (MUSCLE).

### **Migration Assay**

Mouse embryonic fibroblast (MEF)-PORCN knock-out cells (gift of K. Saito-Diaz, Vanderbilt University) were plated at  $1 \times 10^6$  and  $2 \times 10^6$  cells per well in a 6 well plate. Next, a razor blade was used to mark the bottom of the plate; this serves as a guide for four scratches in the cell layer. The scratches were made with a steady and fast pace with a sterile tip of yellow 200 $\mu$ l tip that had been flamed closed. Subsequently, the well was washed with Hank's solution two times to remove the cell debris and de-attracted cells. Then, each well was treated with the CMs (L(A), S(B), and P). Snapshots were taken with a AMG EVOS XL Microscope (Advanced Microscopy Group, Bothell, WA) at different time points and saved for analysis via ImageJ imaging software for percentage closure.

HCT 116 migration was assayed using an Oris™ Cell migration assay kit (Platypus technologies). Essentially, “inserts” were placed into the wells of a 96-well plate. HCT 116 cells growing in a 6-well plate were first stained with a fluorescence vital Calcein, AM, cell-permeant dye (Thermo Scientific, C3100MP) The HCT 116 cells were then collect and counted.  $1 \times 10^5$  cells were added in 100  $\mu$ l of medium into the “slot” of the insert. The cells were grown overnight. The next day the inserts were removed, leading a perfect, cell-free area in the middle of the confluent cell layer in each well of the 96-well plate. CM mediums L(A), S(B), and P were mixed 1:1 with fresh medium. The medium was carefully removed from the wells and the CMs added. The cell

migration was measured in two ways: First, a “mask” was placed on the bottom of the plate that should block the confluent cell layer and making visible only the exclusion area. The 96-well plate was read in the multi-plate reader at 485 /528 nm excitation / emission filters, sensitivity 55 nm. at the different time points (Times 0, 30, 46, 71 and 112). Second, images of the exclusion zone were taken at different times. The images were analyzed to determine the area value of the remaining open or cell free region of the exclusion zone. These values were subtracted from the exclusion zone area value at time 0. This value was divided by the exclusion zone area value at time 0 to give the % cells that have moved into the exclusion zone at each time point. The averages of these value (n=28) for each treatment groups and standard deviation were determined. The Student T-test was used to compare the different treatment groups.

## CHAPTER III

### RESULTS

#### **Preparation and Characterization of WNT5A Conditioned Medium**

These studies make use of WNT5A isoform L(A) and S(B) generated from engineered CHO cells provided by Dr. Karl Willet (UCSD). The isoform proteins are secreted into the cell culture medium after induction and the medium saved as conditioned medium (CM); isoform L(A) [CM-L(A)], isoform S(B) [CM-S(B)] or Parental (CM-P). The control is Parental CHO without expression of WNT5A. After the CM was prepared it was characterized by western blot and functionally analyzed using the TOPFlash reporter system.

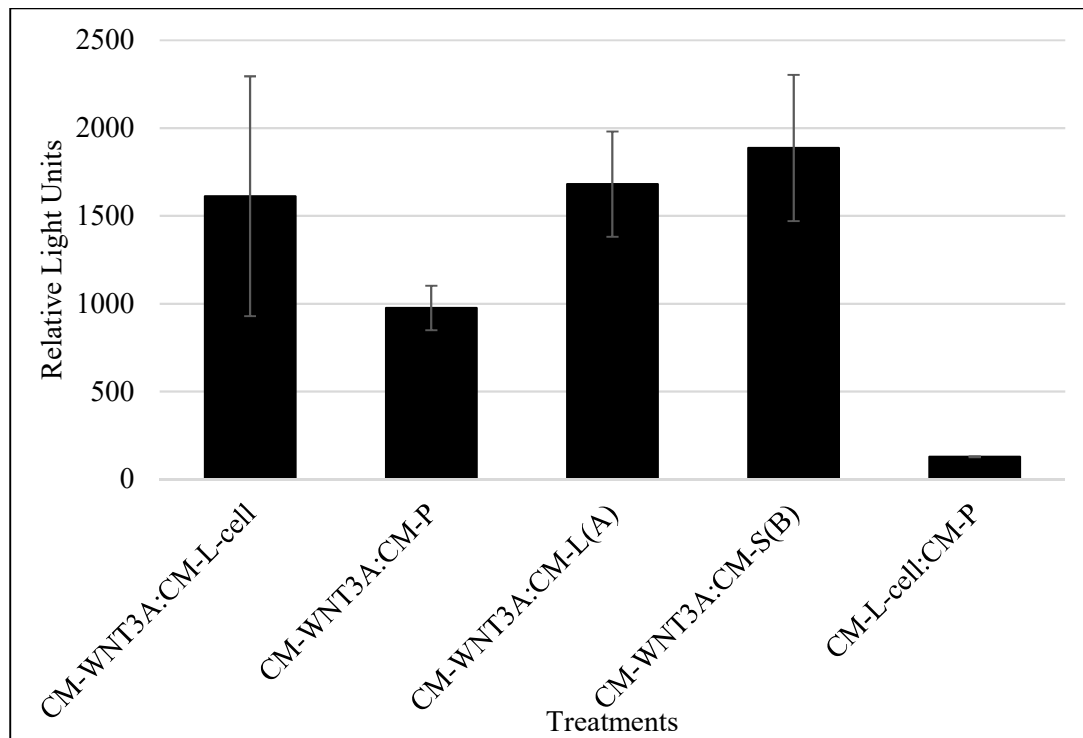
It is known that the WNT3A ligand induces the canonical Wnt signaling pathway and the WNT5A inhibits canonical Wnt signaling via noncanonical signaling pathway induction. Moreover, the WNT5A isoforms have been shown to have a similar inhibitory effect on the canonical pathway using a TOPFlash reporter system (Bauer et al., 2013). The TOPFlash is a luciferase plasmid having a consensus site wild-type TCF/LEF1 binding region for the  $\beta$ -catenin transcription factor; this serves as a promoter for the firefly luciferase gene. It is induced when  $\beta$ -catenin is present and activated. Activation is achieved by treating the TOPFlash cells with WNT3A. The FOPFlash is the same as TOPFlash but with a mutation in the  $\beta$ -catenin consensus site. FOPFlash is acting as a

negative control for the TOPFlash. For optimum determination of WNT5A protein activity I used combinations of CM listed in Table 2.

*Table 2. TOPFlash and FOPFlash Assay Treatment Combinations. The different CM's were mixed 1:1 in the combinations represented by the letters.*

1:1	CM L cell	CM-P	CM L Wnt3A	CM-L(A)	CM-S(B)
CM L cell	A				
CM-P	B	D	F		
CM L Wnt3A	C	E	G		
CM-L(A)			J	H	
CM-S(B)			K		I

Initially, I used NIH3T3 cells stably transfected with the TOPFlash and FOPFlash vectors, however neither CM-L(A) or CM-S(B) inhibited the increase in luciferase induced by WNT3A (Figure 5). After doing a literature search I found one study showing that the TOPFlash vector in NIH3T3 cells didn't respond to the non-canonical ligand WNT 11 (Maye et al., 2004). Consequently, I decided to use HEK293T (human embryonic kidney cell line), which shows a response to both the WNT3A and WNT5A.



*Figure 5. Effect of Conditioned Medium (CM) NIH3T3. WNT5A isoforms- L(A) and S(B) have no effect on the canonical pathway in NIH3T3 cells stably transfected with a TOPFlash vector. The cells were treated with different conditioned media, alone and in combination, as indicated.*

The HEK293T were transiently transfected with the TOPFlash and FOPFlash vectors and treated with the different CM's (Table 2). As expected, treatment with CM-WNT3A caused an increase in luciferase activity (Figure 6). It should be noted that CM-WNT3A is prepared from a WNT3A expressing mouse L Cell line. HEK293T treated with CM-L Cell, CM-P, and a mixture of both did not induce luciferase activity, as expected. A mixture of CM-WNT3A and CM-P showed an increase identical to CM-WNT3A alone, indicating the CHO medium has no effect on WNT3A activity. CM-L(A) and CM-S(B), alone, caused no increase, which indicated neither ligand can activate the



canonical pathway. Of most importance, CM-L(A) and CM-S(B) in combination with CM-WNT3A, showed a significant decrease in activity relative to the CM-WNT3A alone. This result confirms that the WNT5A-L(A) and WNT5A-S(B) in the CM is active.

HEK293T cells transfected with the FOPFlash were treated with similar combinations of CM. There was no induction of luciferase activity by CM-WNT3A and no effect of CM-L(A) and CM- S(B), alone, or in combination with CM-WNT3A (Figure 6-B).

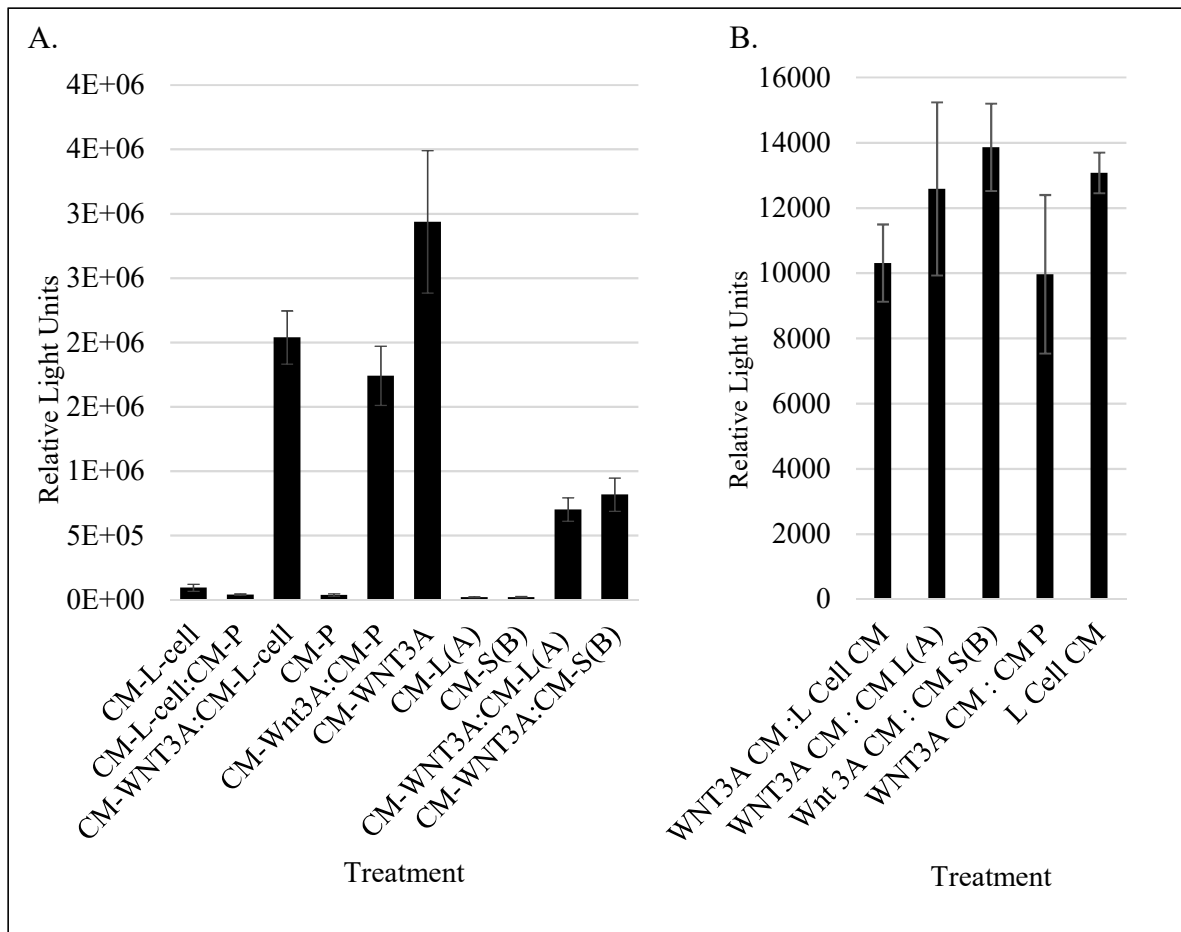
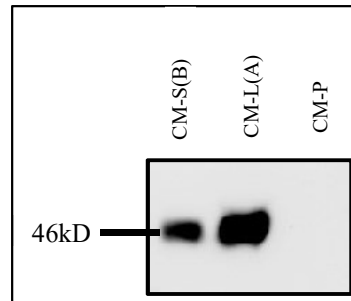


Figure 6. Effect of CM HEK293T. WNT5A isoform-L(A) and S(B) inhibit canonical Wnt signaling in HEK293T cells. HEK293T cells were transiently transfected with TOPFlash (A) or FOPFlash (B). The cells were collected and re-plated in multi-well plates. The following day, cells were treated as indicated.

Next, we wanted to confirm that WNT5A isoforms were, indeed, secreted into the medium from the CHO expressing cells. Aliquots from the CM-P, CM-L(A) and CM-S(B) were analyzed by western blot, using an antibody that detects both isoforms. As shown in Figure 7, there is a high level of WNT5A protein in both CM-L(A) and CM-S(B). I have consistently found that there is more CM-L(A) than CM-S(B) per unit volume medium; this is likely due to the different growth properties of the CHO cells.

Quantification suggests there is approximately 2.7-fold more CM-L(A) than CM-S(B). In CM-P, as expected, no WNT5A was detected.



*Figure 7. Conditioned Medium Western Blot. CM-S(B), CM-L(A) and CM-P.*

### **Analysis of WNT5A-Isoform L(A) and S(B) Activation of Non-Canonical Signaling by Flow Cytometry**

There are two major non-canonical Wnt pathways; calcium ( $\text{Ca}^{2+}$ ) and Planer cell polarity/ Cell elongation (PCP/CE). The  $\text{Ca}^{2+}$  leads to PKC phosphorylation and activation, whereas the PCP/CE pathway results in JNK phosphorylation and activation. My goal in this study was to determine which pathways are activated by the WNT5A isoforms A and B. The difficulties with this type of analysis include the choice of appropriate cell type, period of exposure, and quantification. I found that western blot analysis was not suitable for this analysis as it is not so sensitive for the slight change in activation. Instead I choose to use antibody staining with flow cytometry. Basically, cells were treated with CM-L(A), CM-S(B) and CM-P for different time periods, collected, fixed and immunostained with a primary antibody (anti-pPKC, anti-pJNK, and total PKC and JNK) (Figure 8) followed by a fluorescent secondary antibody. Cells were then

analyzed by flow cytometry. A positive effect (phosphorylation) would be seen as a shift right and increased mean fluorescence. A negative effect (possibly de-phosphorylation) would be a shift left and reduced mean fluorescence. Effects of CM-L(A) and CM-S(B) on pPKC and pJNK were analyzed in two cell types, HCT 116 (colorectal cancer) and hFOB1.19 (normal human osteoblast) at different time points.

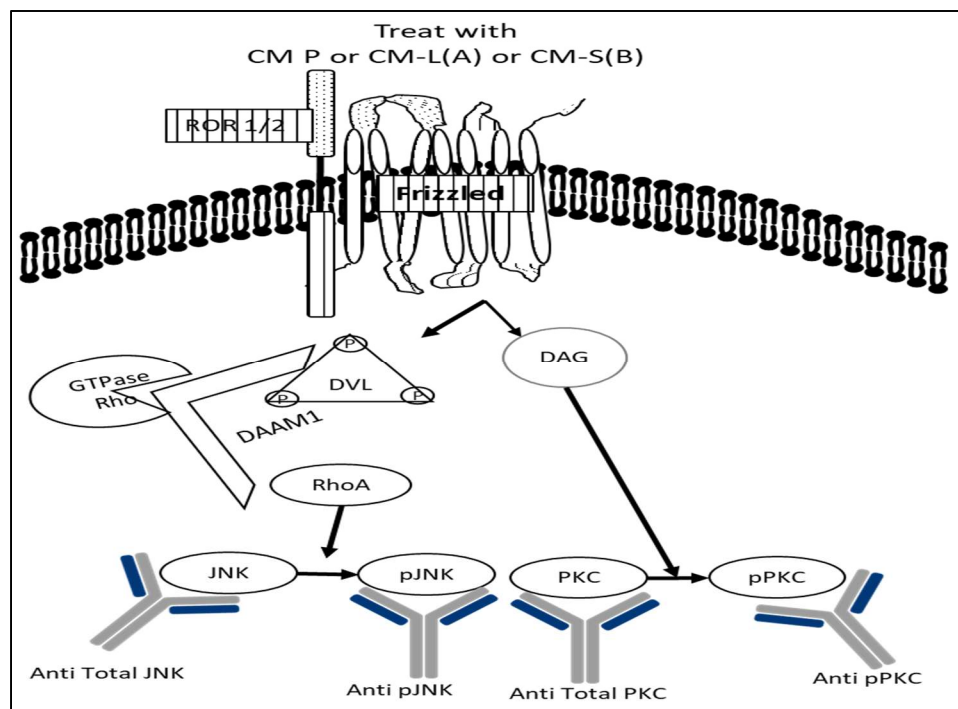


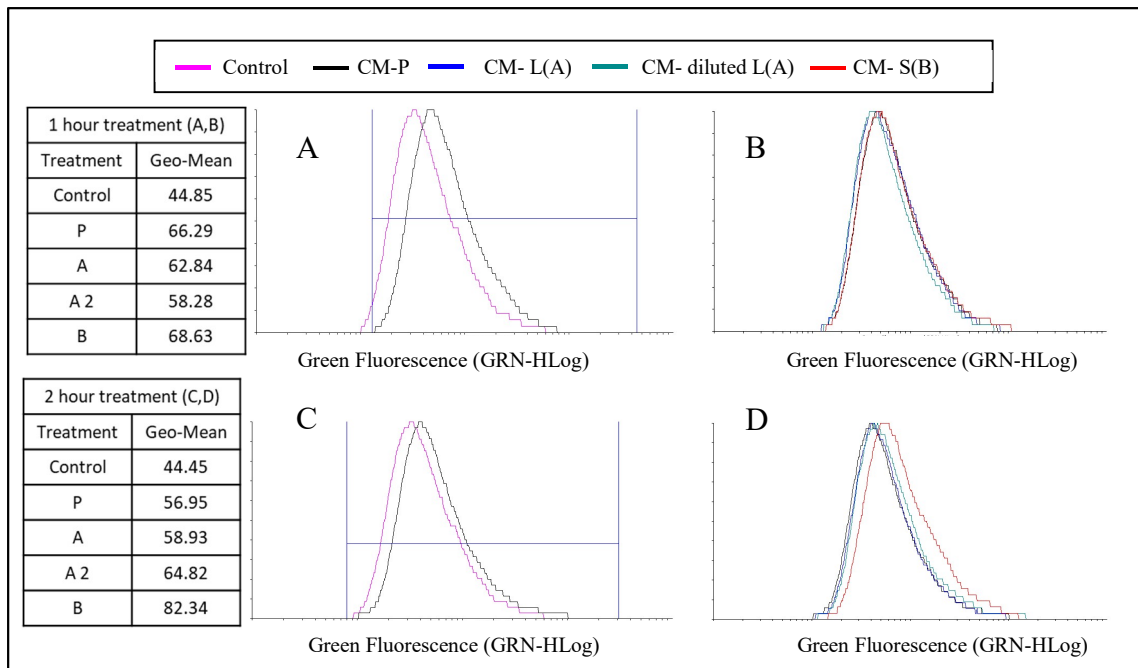
Figure 8. Flow Cytometry Analysis Outline. Modified from Zhan et al., (2017).

### pPKC- HCT 116

Overall, the level of pPKC in untreated HCT 116 is relatively low (Figure 9-A+C). The level of activated pPKC was the same at 1hr treatment in cells treated with CM-L(A) and CM-S(B) and CM-P (Figure 9-B). The Geo-metric mean for CM-P, CM-L(A) and CM-S(B) were higher than the control and varied slightly. At 2hr, CM-S(B)

shifted more to the right (Figure 9-D). The Geo-mean value for CM-S(B) was higher than CM-P (82.34 compared to 56.95). CM-L(A) did not shift right and had a similar Geo-mean to the CM-P. The diluted (1:2) CM-L(A)-A2 showed a slight increase in Geo-mean.

The experiment was repeated in HCT 116 cells at 2hr and 6hr (Figure 10). The results for CM-S(B) was confirmed at 2hr; a slight shift right and an increase in Geo-mean was detected (Figure 10-B). Again, CM-L(A) showed no effect. At 6hr, either CM-S(B) or CM-L(A) had an effect; there was no shift and Geo-mean was similar to CM-P. These results suggest that WNT5A isoform S(B) is activating the  $\text{Ca}^{2+}$  non-canonical pathway and leading to pPKC but the effect is transient. Isoform A has less of an effect.



*Figure 9. Trial 1 HCT 116 Flow Cytometric Results of pPKC. CM treatment was done for 1hr and 2hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are CM-P and negative control (minus primary antibody), whereas while B and D represent treatment with CM-L(A), CM-L(A2), CM-S(B) and CM-P, standard for pJNK. CM-L(A2) is a 1:2 dilution of CM-L(A)*

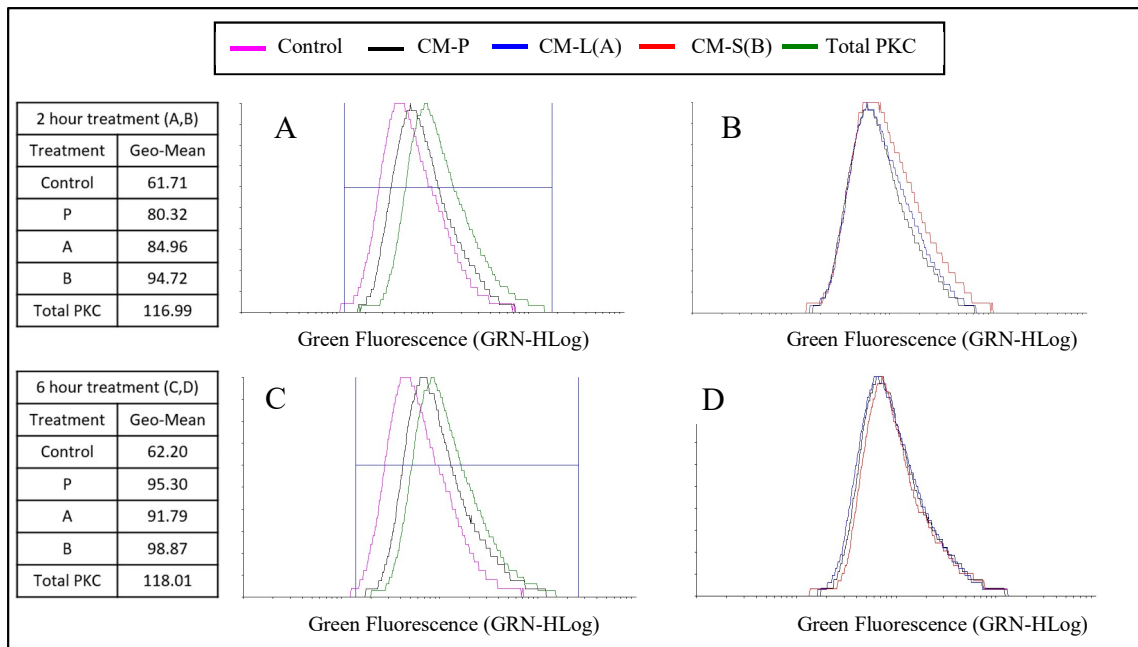


Figure 10. Trial 2 HCT 116 Flow Cytometric Results of pPKC. CM treatment was done for 2hr and 6hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are control CM-P, negative control (minus primary antibody) and total PKC. B and D represent the treatment with CM-P, CM-L(A) and CM-S(B).

### pJNK-HCT 116

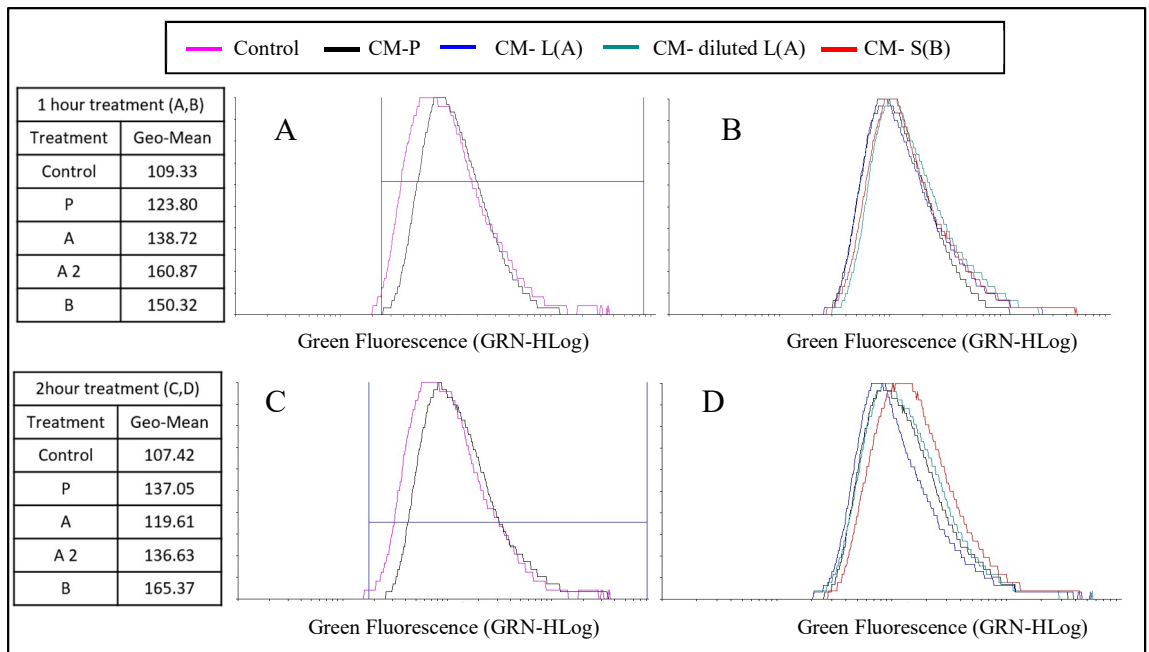
The level of activated pJNK was slightly higher at 1hr treatment in cells treated with CM-L(A) and CM-S(B) (Figure 11-B). The Geo-mean for CM-L(A) was slightly higher than CM-P (138.72 compared to 123.80). For the diluted CM-(A)- A2 it was higher than CM-P (160.87 compared to 123.80 compared), and for CM-S(B) it was higher than CM-P (150.32 compared to 123.80). At 2hr, CM-S(B) shifted more to the right and CM-L(A) shifted left (Figure 11-D). The Geo-mean value for CM-S(B) was higher than CM-P (165.37 compared to 137.05). CM-L(A) shifted left and had a lower Geo-mean compared to the CM-P (119.61 compared to 137.05). The diluted CM-L(A)-A2 did not shift right and had a similar Geo-mean to the CM-P. These results suggest that WNT5A

isoform B stimulates the PCP/CE pathway, leading to increased pJNK. The results suggest that isoform A at higher concentrations may reduce pJNK levels.

The experiment was repeated in HCT 116 cells at 2hr and 6hr (Figure 12) The results for CM S(B) was partially confirmed at 2hr; there was no clear shift right and but a slight increase in Geo-mean was detected. CM-L(A) showed no shift left but increased in Geo-mean. At 6hr, CM S(B) had no shift but the Geo-mean was slightly higher than CM P (102.97 compared to 90.83) while CM L(A) had no shift but the Geo-mean was slightly higher than CM P (96.20 compared to 90.83).

It is possible that the second trial had problems with the pJNK staining, as the control and the pJNK were closer together than in the first trial.





*Figure 11. Trial 1 HCT 116 Flow Cytometric Results of pJNK. CM treatment was done for 1hr and 2hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are CM-P and negative control (minus primary antibody), whereas B and D represent treatment with CM-L(A), CM-L(A2),CM-S(B) and CM-P, standard for pJNK. CM-L(A2) is a 1:2 dilution of CM-L(A).*

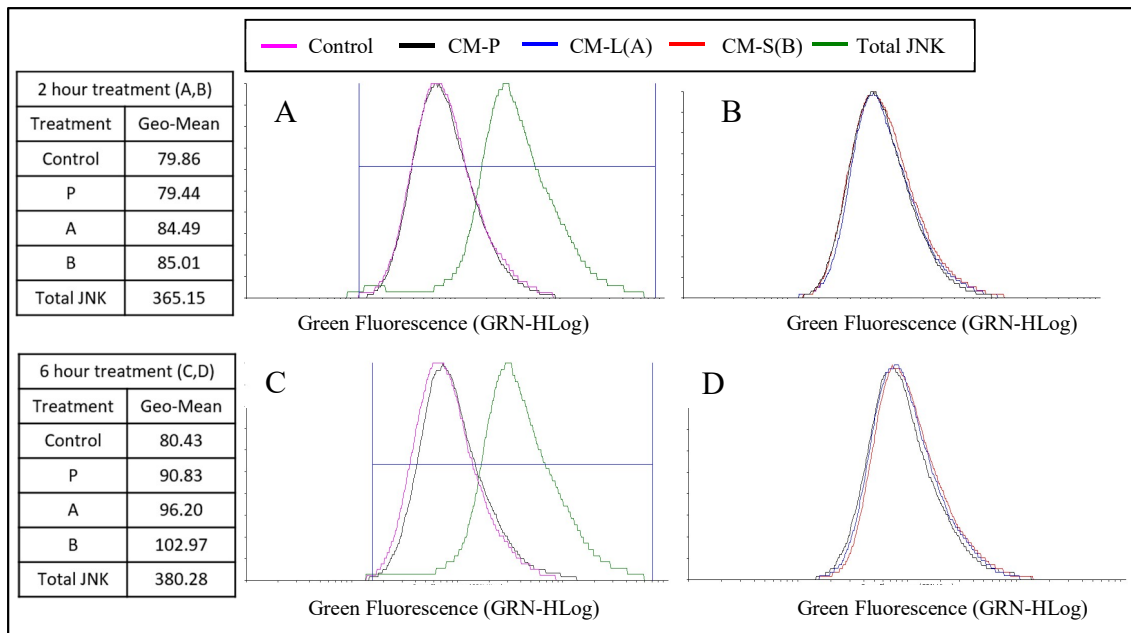


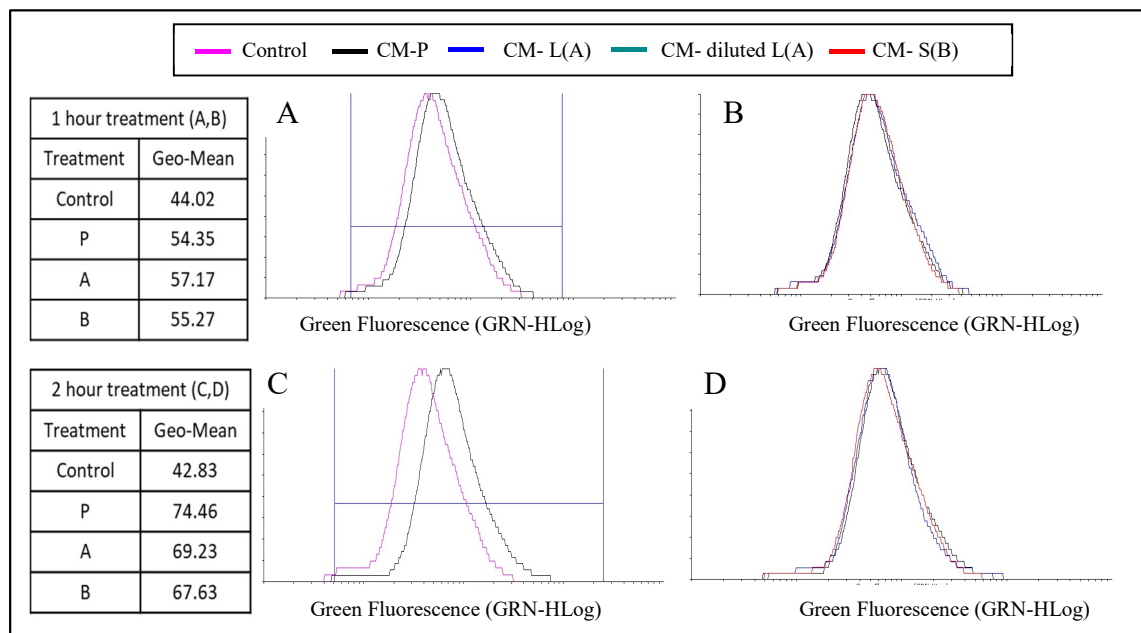
Figure 12. Trial 2 HCT 116 Flow Cytometric Results of pJNK. CM treatment was done for 2hr and 6hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are control CM-P, negative control (minus primary antibody) and total PKC. B and D represent the treatment with CM-P, CM-L(A) and CM-S(B).

### pPKC-hFOB1.19

The level of activated pPKC was the same at 1hr treatment in cells treated with CM-L(A) and CM-S(B) and CM-P (Figure 13-B). The Geo-mean for CM-P, CM-L(A) and CM-S(B) were higher than the control and varied slightly. At 2hr, CM-L(A) and CM-S(B) did not shift (Figure 13-D). The Geo-mean values for both CM-L(A) and CM-S(B) were slightly lower than CM-P (69.23 and 67.63 compared to 74.46).

The experiment was repeated in hFOB1.19 cells at 2hr and 6hr. The level of activated pPKC was higher (Figure 14). At 2hr, CM-L(A) showed a slight shift right and the Geo-mean value was slightly higher than CM-P (120.55 compared to 94.54). While CM-S(B) showed a slight shift right and the Geo-mean value was slightly higher than

CM-P (101.35 compared to 94.54). At 6hr, CM-S(B) and CM-L(A) had left shifted; the Geo-means were lower than CM-P (121.99 and 129.42 compared to 130.36). These results suggest that WNT5A isoform A effects the Ca<sup>2+</sup> pathway to a greater degree than isoform B. however, the results in hFOB1.19 are variable and the effect is transient.



*Figure 13. Trial 1 hFOB1.19 Flow Cytometric Results of pPKC. CM treatment was done for 1hr and 2hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are CM-P and negative control (minus primary antibody), whereas B and D represent treatment with CM-L(A), CM-S(B) and CM-P, standard for pPKC.*

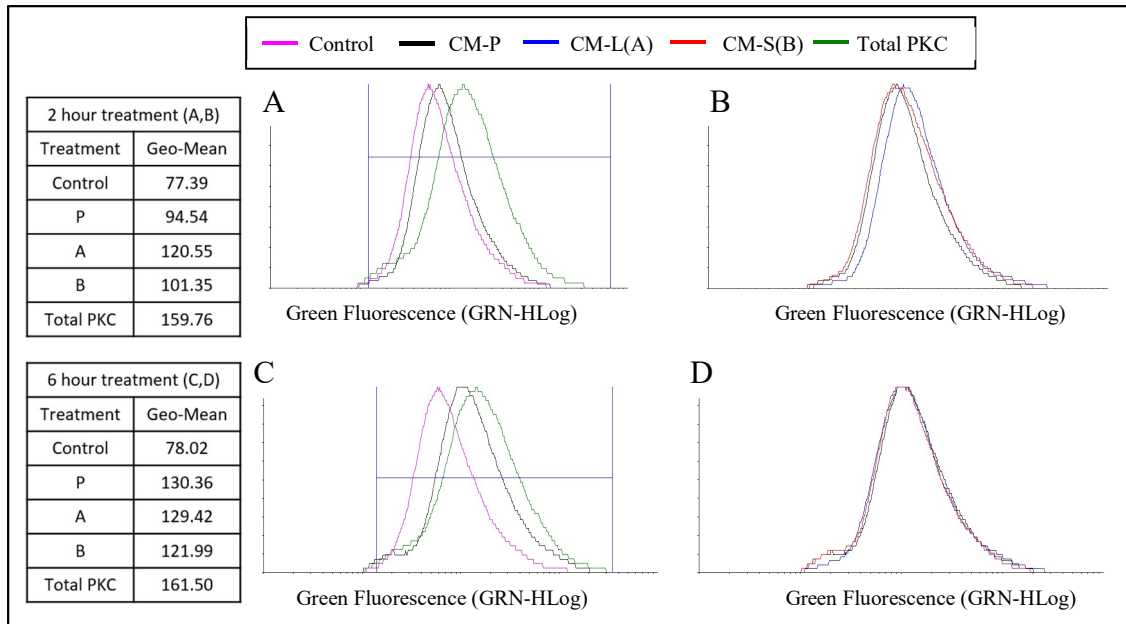


Figure 14. Trial 2 hFOB1.19 Flow Cytometric Results of pPKC. CM treatment was done for 2hr and 6hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are control CM-P, negative control (minus primary antibody) and total PKC. B and D represent the treatment with CM-P, CM-L(A) and CM-S(B).

### pJNK-hFOB1.19

The level of activated pJNK was similar at 1hr treatment in cells treated with CM-P to and CM-L(A), while for CM-S(B) it was lower compared to CM-P with a slight shift left (Figure 15-B). The Geo-mean for CM-L(A) was slightly higher than CM P (194.50 compared to 190.19). And for CM- S(B) the Geo-mean was lower than CM-P (150.94 compared to 190.19). At 2hr, CM-S(B) shifted slightly to the left (Figure 15-D). The Geo-mean value for CM-S(B) was lower than CM-P (189.96 compared to 227.80). CM-L(A) also shifted and had a lower Geo-mean compared to the CM-P (196.61 compared to 227.80).

The experiment was repeated in hFOB1.19 cells at 2hr and 6hr (Figure 16). There was an increase in activated pJNK for CM-L(A) and CM-S(B) at 2hr; a slight shift right and a slight increase in Geo-mean was detected. At 6hr, CM-P, CM-L(A) and CM-S(B) showed a similar shift, but the Geo-mean for CM-L(A) and CM-S(B) were lower than CM-P (99.89 and 94.27 compared to 112.97). Overall, these results suggest that either the CM-L(A) or CM-S(B) activate pJNK in hFOB1.19 cells. There is some evidence that both CM decrease pJNK.

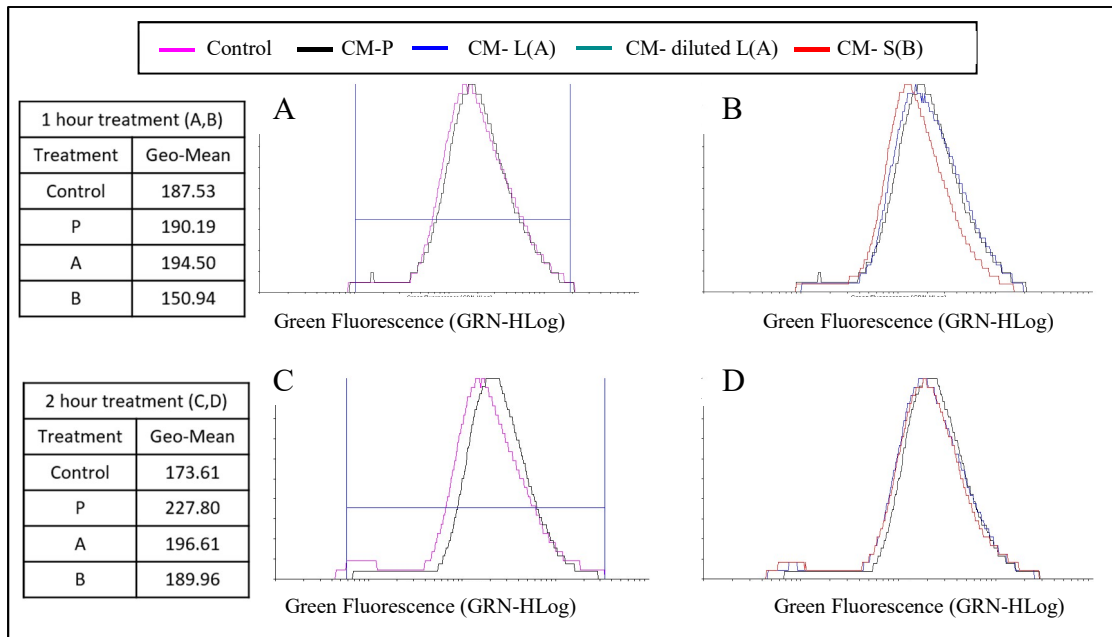
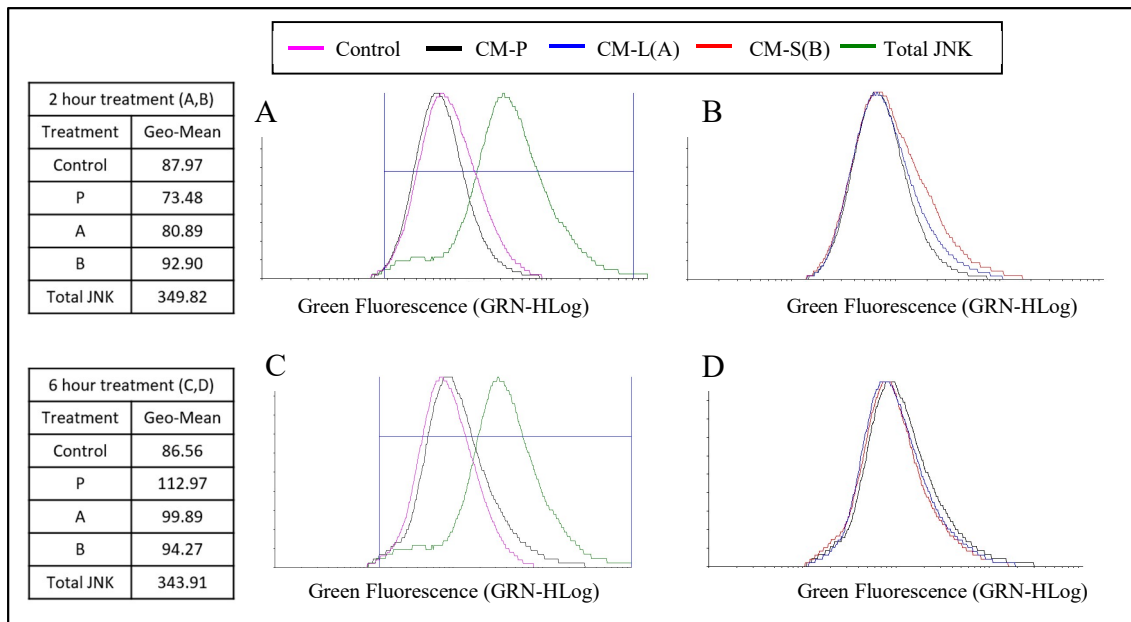


Figure 15. Trial 1 hFOB1.19 Flow Cytometric Results of pJNK. CM treatment was done for 1hr and 2hr. Cell counts (Y-axis) versus green fluorescence (X-axis). A and C are CM-P and negative control (minus primary antibody), whereas while B and D represent treatment with CM-L(A), CM-S(B) and CM-P, standard for pJNK.



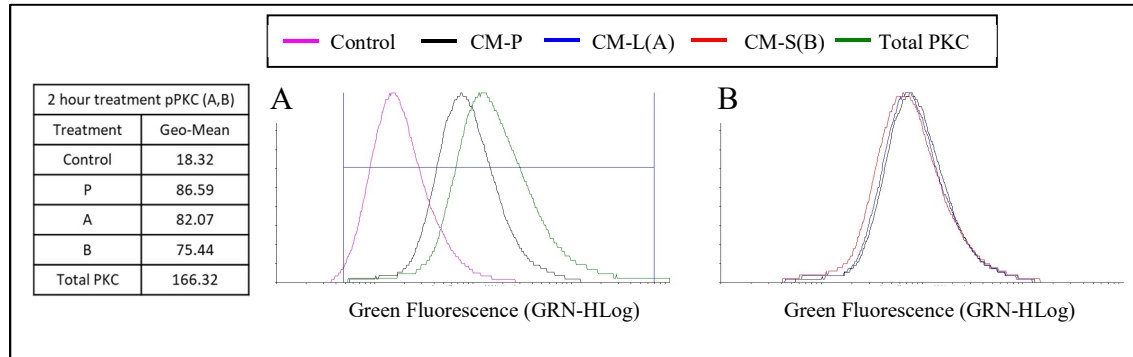
*Figure 16. Trial 2 hFOB1.19 Flow Cytometric Results of pJNK. CM treatment was for 2hr and 6hr. Cell counts (Y- axis) versus green fluorescence (X-axis). A and C are control CM-P, negative control (minus primary antibody) and total JNK, B and D represent the treatment with CM-P, CM-L(A) and CM-S(B).*

#### *Mouse Embryonic Fibroblast (MEF)- PORCN Knock-Out*

We also tested the effect of the CM's on a MEF cell line that has a knock-out in the PORCN gene. As such, these cells do not secrete any Wnt ligands. This cell line may allow for a cleaner analysis of the effects of CM-L(A) and CM-S(B) on non-canonical Wnt signaling. The level of activated pPKC was found to be lower in cells treated with CM-L(A) and CM-S(B) at 2hr compared to CM-P. The Geo mean for CM-P, CM-L(A) and CM-S(B) were higher than the control and varied slightly. CM-S(B) shifted slightly to the left (Figure 17-B). The Geo-mean value for CM-S(B) was lower than the CM-P (75.44 compared to 86.59). CM-L(A) shifted slightly to left (Figure 17-B). The Geo-mean value for CM-L(A) was slightly lower than CM-P (82.02 compared to 86.59).

These results suggest that CM-L(A) and CM-S(B) have little or a negative effect on pPKC levels in MEF-PORCN cells.

The level of activated JNK was found to be similar in cells treated with CM-P, CM-L(A) and CM-S(B) at 2hr. The Geo-mean for CM-P, CM-L(A) and CM-S(B) were higher than the control and varied slightly. CM-S(B) shift was similar to CM-P (Figure 18-B), the Geo-mean value for CM-S(B) was slightly lower than CM-P (91.28 compared to 95.97). CM-L(A) shift was similar to CM-P (Figure 18-B); the Geo-mean value for CM-L(A) was slightly higher than CM P (98.33 compared to 95.97). These results suggest that the CM-L(A) and CM-S(B) have little or may be a slightly negative effect on pJNK in MEK cells.



*Figure 17. MEK PORCN Flow Cytometric Results of pPKC. CM treatment 2hr, counts (Y-axis) versus green fluorescence (X-axis). A) negative control (minus primary antibody). CM-P treated (pPKC) and total PKC. B) treatment with CM-P, CM-L(A) and CM-S(B) stained with pPKC.*

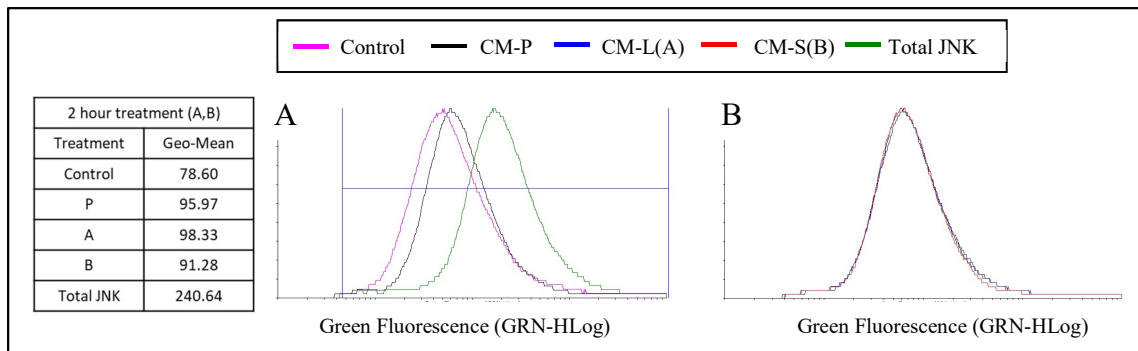


Figure 18. MEK PORCN Flow Cytometric Results of pJNK. CM treatment 2hr, counts (Y- axis) versus green fluorescence (X-axis). A) negative control (minus primary antibody). CM-P treated (pJNK) and total JNK. B) treatment with CM-P, CM-L(A) and CM-S(B) stained with pJNK.

### Analysis of WNT5A-Isoform L(A) and S(B) Activation of Non-Canonical Signaling by Reporter Assays

I used another approach to analyze the possible effects of isoforms L(A) and S(B) on the non-canonical pathways. HCT 116 cells were transfected with reporter vectors for the transcription factors, AP-1 and NFAT. AP-1 is a downstream target of pJNK and the PCP/CE Wnt non- canonical pathway. NFAT is one of the targets of the Ca<sup>+2</sup> non-canonical pathway. HCT 116 has been shown to express AP-1 (Lee and Lim, 2007; Miao et al., 2017) and NFAT (Zhou et al., 2017).

I transfected the reporter vectors separately and the next day, re-plated the cells in 96-well plates. The cells were treated with a PORCN inhibitor for 24hr and then treated with the CM's for 6hr and 12hr. Luciferase activity was determined in cell lysates. The AP-1 expression level was significantly (P<0.05) upregulated when treated with CM-S(B) relative to CM-L(A) and CM-P for 6hr (Figure 19-A), whereas at 12hr there was no significant difference between the CM treatments (Figure 19-B). The expression levels



from the NFAT promoter were low and there was no significant difference between the treatment groups (Figure 19-B). The control CMV reporter showed little expression.

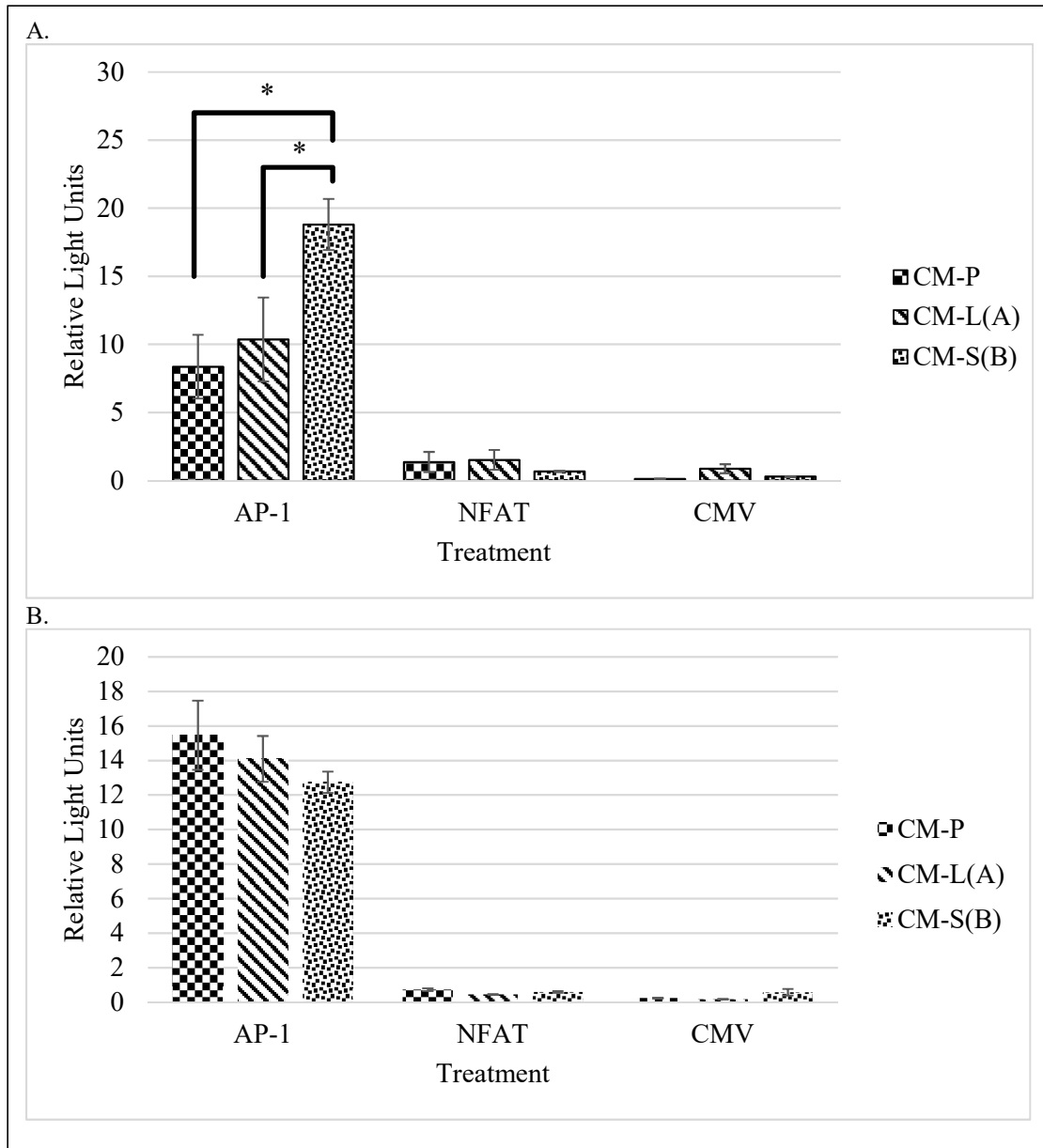


Figure 19. HCT 116 Transfected with AP-1, NFAT and CMV Luciferase Constructs. Treatment with CM-P, CM-L(A) and CM-S(B) for 6hr(A) and 12hr(B). Cells were transfected with reporter vectors, collected and re-plated and cells were treated with PORCN and CMs. The (\*) asterisk represents significance ( $P < 0.05$ ), by utilizing Student T-test.  $n = 8$  for each AP-1 and NFAT,  $n = 4$  for CMV.

## Cellular Effects of the WNT5A Isoforms A and B – Apoptosis, Proliferation, and Migration

I decided to use the CM-L(A) and CM-S(B) to determine if the WNT5A isoforms have the same or different cellular effects. I tested for the effects of the CM's on apoptosis, proliferation, and migration using HCT 116, hFOB1.19, and MEK-PORCN cells.

### *Apoptosis Assay*

HCT 116 were exposed to CM-P, CM-L(A), and CM-S(B) for 18-24 hours, with and without 0.25  $\mu$ M camptothecin. Camptothecin is a topoisomerase inhibitor and inducer of apoptosis. The cells were collected and analyzed for apoptosis, using the annexin V assay. The results of four independent trials show there was no significant difference between the control cells (CM-P treated) and the experimental [CM-L(A) and CM-S(B)] with or without the induction of apoptosis (Figure 20).

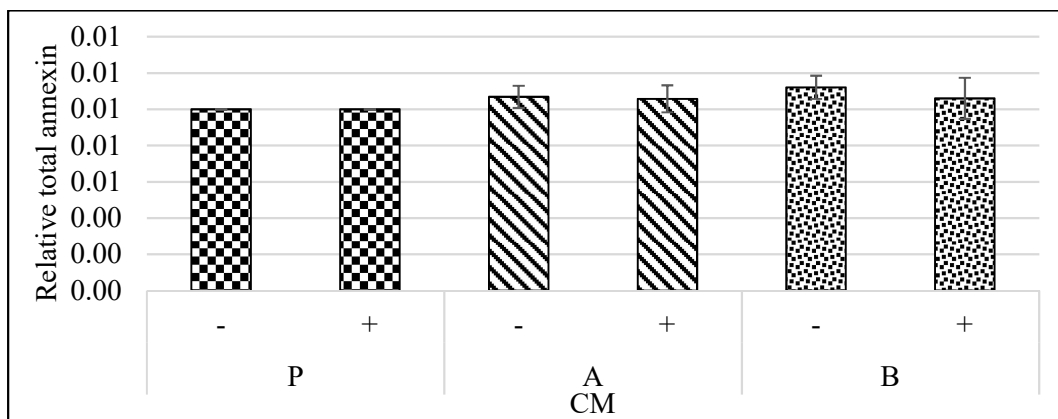


Figure 20. HCT 116 Apoptosis Assay. No effect of CM-L(A) and CM-S(B) treatment on apoptosis in HCT 116 cells. P=CM-P; A=CM-L(A), B=CM-S(B). Minus (-) and Plus (+) camptothecin. Error bars are standard error derived from 4 independent assays. The % total annexin was determined relative to the control, CM-P for each trial.

### *Cell Proliferation Assay*

Bauer et al., (2013) found that the WNT5A isoforms have opposite effects on proliferation of various cancer cell lines; over expression of isoform-S(B) increased proliferation, whereas isoform- L(A) decreased proliferation. We tested effect of CM-L(A) and CM-S(B) on proliferation in two cell types, HCT 116 and hFOB1.19.

#### HCT 116

Two independent trials were conducted in HCT 116 cells. I found that treatment with isoform B conditioned medium, CM-S(B), resulted in a significant ( $p < 0.05$ ) decrease in cell density at day 4, 5 and 6, relative to control, CM-P, for trial 1 (Figure 21-A). The trend continued to day 7. The results were similar for trial 2 (Figure 21-B); there was a significant difference at days 3, 4 and 5. Isoform A conditioned medium, CM-L(A) was not significantly different from the control, CM-P at any days for trial 1, although there was a trend toward a decrease at days 6 and 7 (Figure 21-A). However, in trial 2 there was a significant difference at day 3 and 4 between CM-L(A) and CM-P (-B). These results suggest that WNT5A isoform B has an inhibitory effect on proliferation in HCT 116 cells (Figure 21). Further studies are necessary to confirm this result and the results of isoform A.

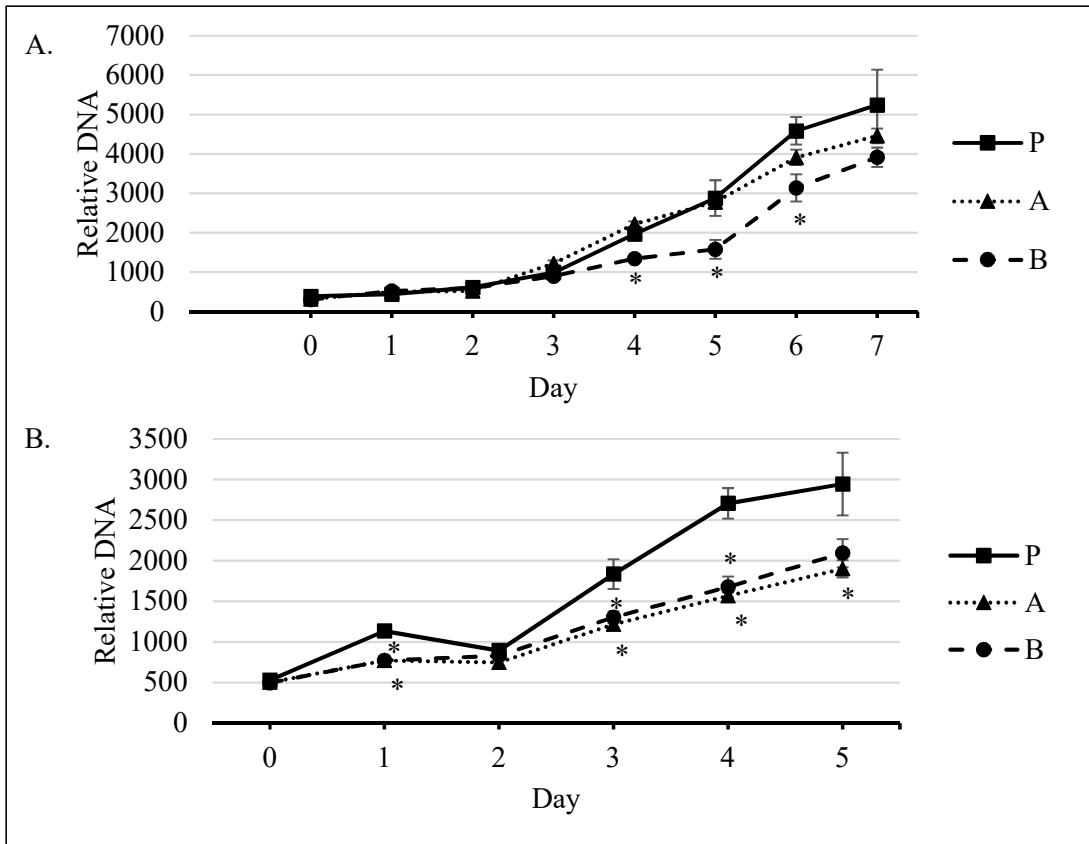


Figure 21. HCT 116 Proliferation Assay. HCT 116 proliferation is reduced by treatment with WNT5A CM-S(B). HCT 116 cells were grown at a low density in the presence of CM-P, CM-L(A) and CM-S(B). At each day DNA content was measured by a dye binding assay. A) proliferation measured over 7 days for the treatment groups. Bars are standard error. B) A replica of A. but only carried out for 5 days. The (\*) asterisk represents significance ( $P < 0.05$ ), by utilizing student T-test.

#### hFOB1.19

I found hFOB1.19 treated with isoform A conditioned medium, CM-L(A), showed a significant increase at days 4,6,7 and 8. Cell proliferation in CM-S(B) treated cells was not significantly different from the control CM-P (Figure 22).

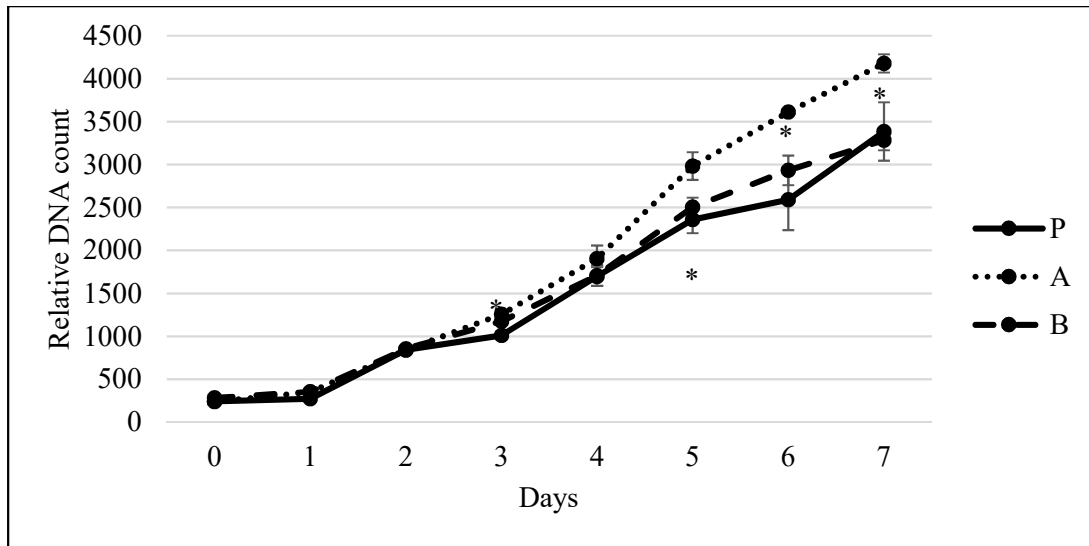


Figure 22. *hFOB1.19 Proliferation Assay. hFOB1.19 proliferation is increase by treatment with WNT5A isoform A, CM-L(A). hFOB1.19 cells were treated with CM-P, CM-L(A) and CM-S(B). Proliferation was measured over 7 days for the three treatment groups. Bars are standard error. The (\*) asterisk represents significance ( $P < 0.05$ ), by utilizing student T-test.*

Overall, those results suggest that WNT5A isoforms have different effects on proliferation and that the effects of the isoforms are cell type specific.

#### *Migration Assay*

#### HCT 116

I found that treatment with CM-L(A) decreased the migration rate relative to CM-P and media alone. There was a significant difference between CM-L(A) and CM-P at T46, T71 and T112. As for CM-S(B) there was a trend toward an increase in the migration rate relative to CM-P and media (Figure 23). There is a significance between

CM-S(B) and CM-P at T112. There was a significance between CM-L(A) and CM-S(B) at each time point.

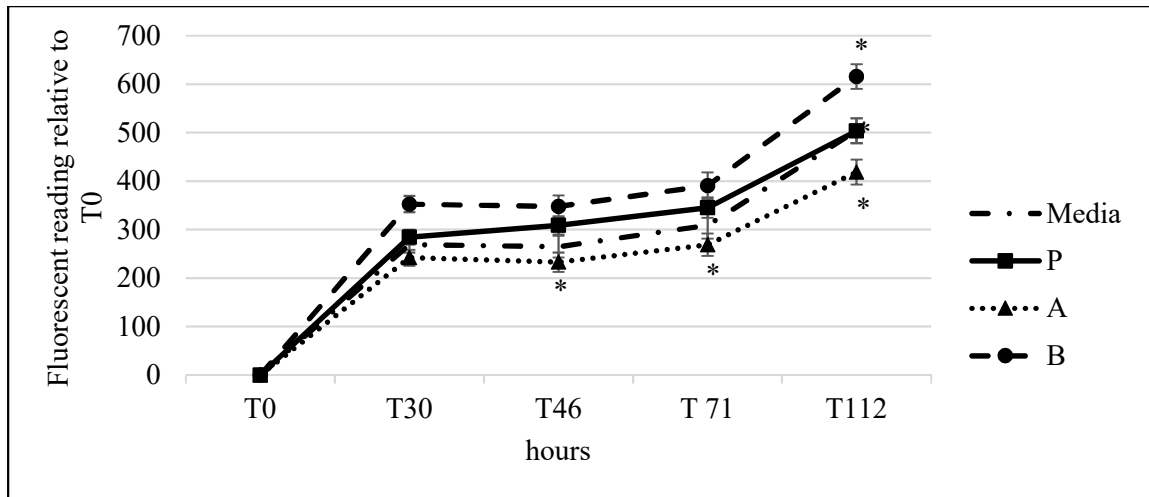


Figure 23. HCT 116 Migration Fluorescent Assay. The (\*) asterisk represents significance ( $P < 0.05$ ), utilizing Student T-test  $n = 28$  for CM-P,  $n = 32$  for CM-L(A), CM-S(B) and  $n = 4$  for media.

#### MEF-PORCN

In MEF-PORCN cells I found a decrease in migration rate when treated with CM-L(A) and CM-S(B) relative to control CM-P (Figure 24). However, while there was no significant difference between the two isoform CM's, there is a significance at T6 and T11 between CM-L(A) and CM-P.

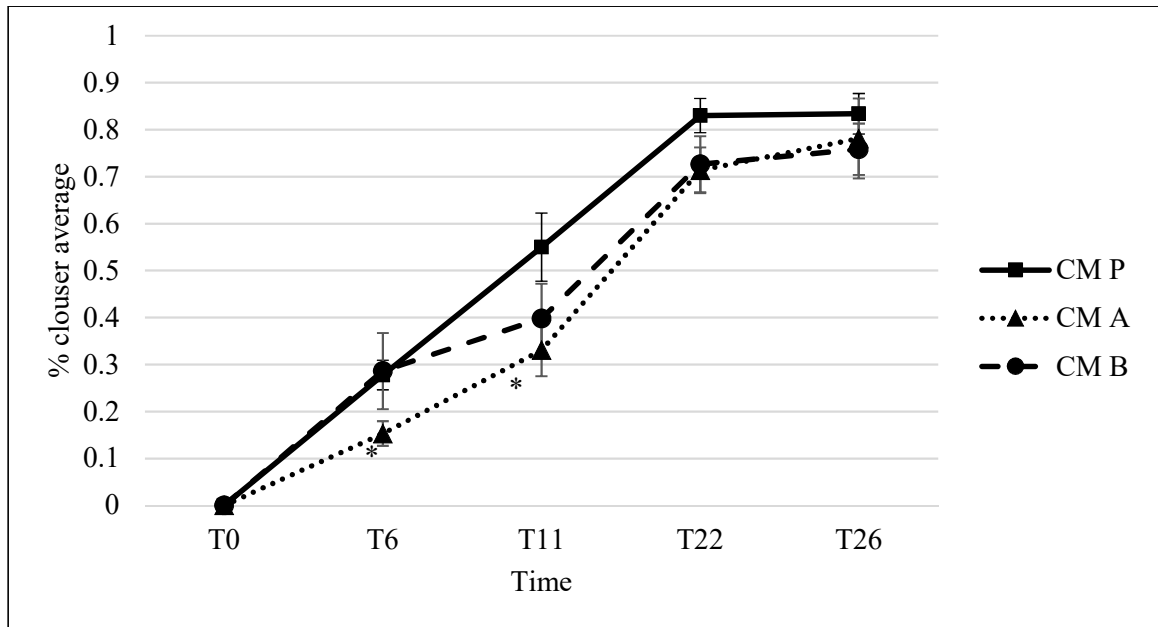


Figure 24. MEF-PORCN Migration Assay. MEF-PORCN migration scratch assay analysis. The (\*) asterisk represents significance relative to control, by utilizing Student T-test.  $n=8$  for each CM.

### Analysis of the WNT5A Isoform Promoters in Dividing Cells and During Osteoblast Differentiation

I hypothesized that the WNT5A isoform A and B promoters are differentially regulated. A comparison was made between 550 bp upstream of exon 1 (Promoter A) and 550 bp upstream of exon 1 $\beta$  (Promoter B) using the alignment program MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>). The identity value was 44%, indicating the sequences are not very similar. An alignment of each region to the upstream 550 bp of another un-related gene gave a similar value of 45%.

I examined the sequences of promoters A and B by transfecting luciferase promoter constructs into HCT 116 and hFOB1.19 cells. This allows for identification of



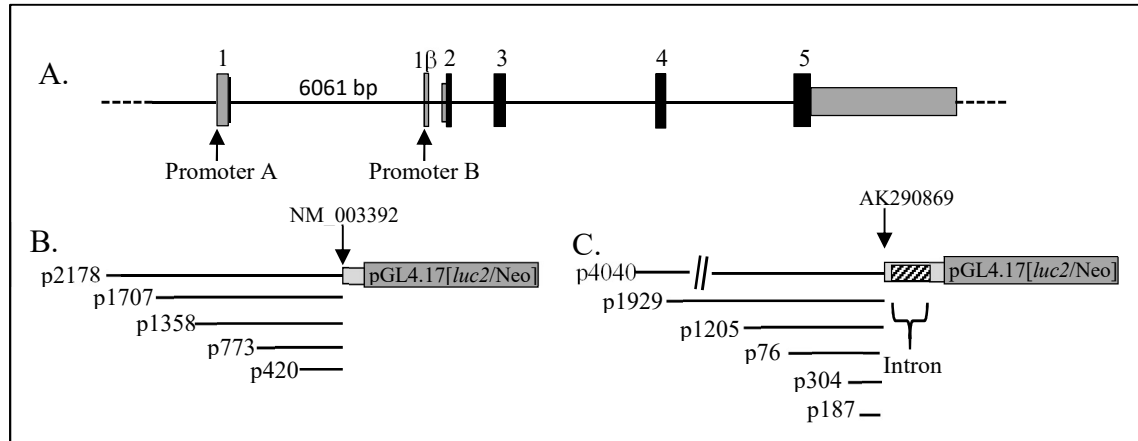
sequences required for expression in dividing cells. Next, I used the same constructs and examined their expression during osteoblast differentiation. It has been shown in our lab that both A and B transcripts increase during osteoblast differentiation (unpublished data). The constructs used for this analysis are shown in Figure 25. In hFOB1.19 cells, 1707 bp of promoter A upstream sequence was necessary for maximal expression in dividing cells. Removal of additional sequences decreased activity. Promoter B constructs were expressed at a higher level than promoter A. 1257 bp was required for maximal expression and further decreases down to 187 bp only reduced expression by about 30% (Figure 26-A).

The pattern of activity in HCT 116 for both promoter A and promoter B constructs was similar to results in hFOB1.19 with some differences. Maximal expression for both was observed with the same promoter constructs (PA 1707 and PB 1257). Again, in hFOB1.19, promoter B was expressed at a higher level than promoter A (Figure 26-B). The transfections were repeated using newly prepared luciferase reporter plasmids and results were nearly identical (data not shown). These results suggest that both promoters are active in HCT 116, that promoter B expresses at a higher level, and that there are both negative and positive regulators of expression.

Next, I analyzed the same constructs during hFOB1.19 differentiation (Figure 27) Fold-change in promoter activity was determined relative to day 0, before addition of differentiation medium. The highest fold-change for promoter A was obtained with PA420 (approximately 10-fold), the lowest PA1707 (approximately 5.5-fold). These results suggest that 420 bp is sufficient for induction and that there are inhibitory

sequence further upstream (e.g. between 1707bp and 1358bp). Promoter B constructs showed similar levels of induction, although the lowest was PB187 (approximately 4.57). This may indicate that additional sequences are required for maximal induction (e.g. PB817 and PB1981). Also, in general, promoter A was increased to a higher level than promoter B. For example, for the two constructs of comparable size the promoter A PA420 increased approximately 10X, whereas the promoter B construct PB 354 increased only 5X. PA1707 (5.5X) only are promoter A construct was in the same range of 5.5-6.5X as promoter B constructs.

Overall, these results illustrate how the two isoforms promoter are differentially regulated. Also, a cell type specific regulation for each promoter was found.



*Figure 25. WNT5A Promoter Constructs. A) The overall structure of the human WNT5A gene, showing the exons (boxes), introns (lines) and relative positions of the isoform promoters A and B. Promoter A (B) and Promoter B (C) luciferase constructs. The members represent base pair upstream of the beginning of the cDNA sequence as indicated (image from Vaidya et al., 2016).*

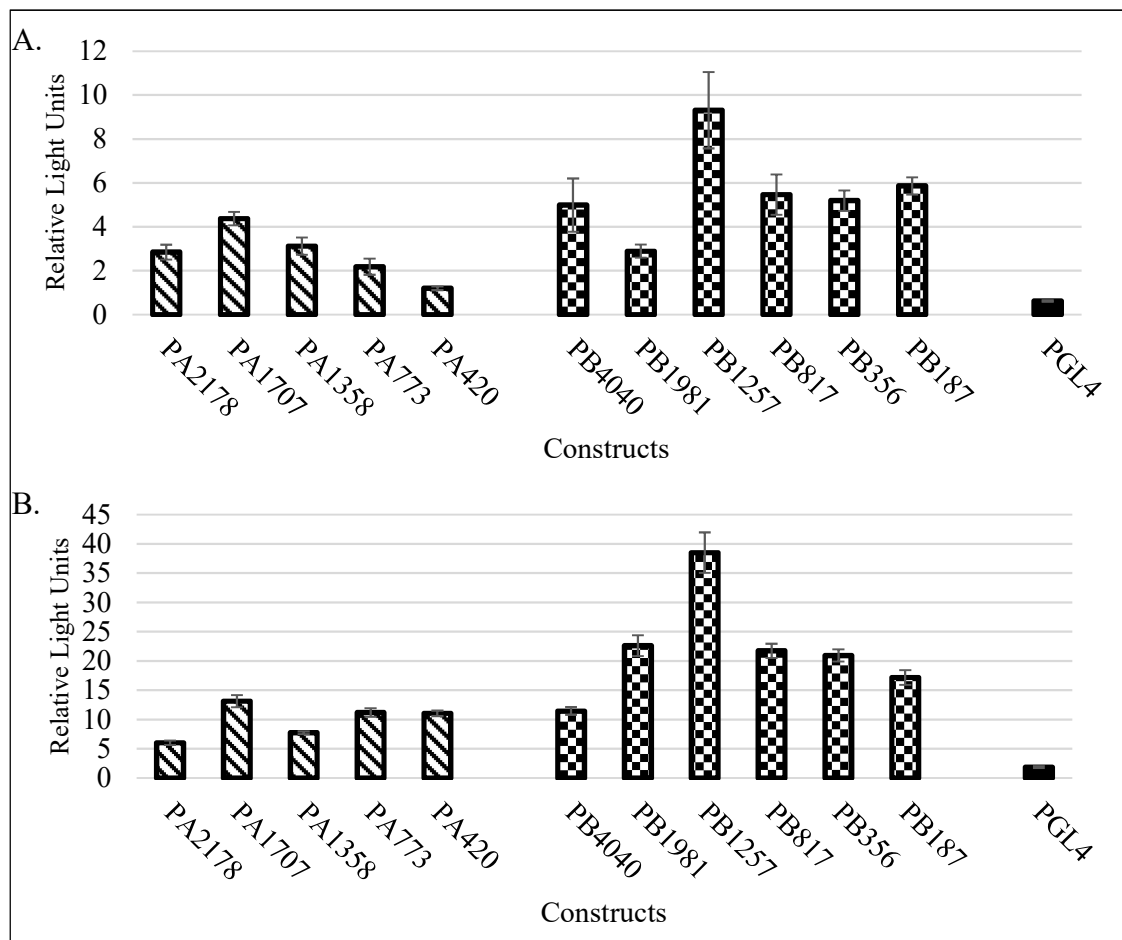


Figure 26. Promoter Constructs Expression in hFOB1.19 and HCT 116. The luciferase constructs were transfected in hFOB1.19 (A) and HCT 116 (B) along with a control Renilla vector. The next day, the cells were collected and assay for both luciferase and Renilla, values of luciferase were scandalized to Renilla luciferase. Error bars are standard error, n=5.

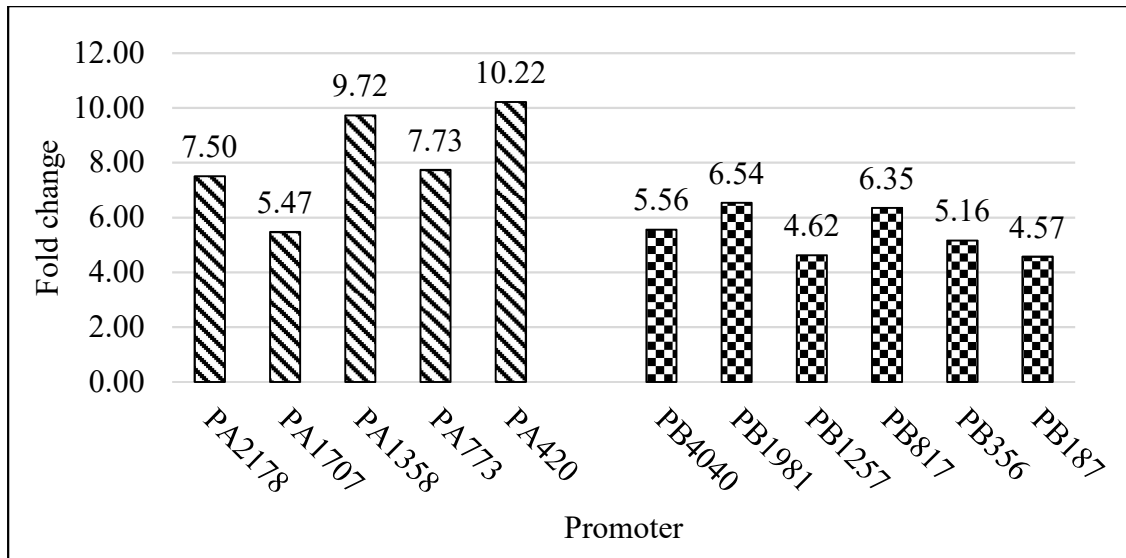


Figure 27. Promoter Constructs Expression During Differentiation hFOB1.19. WNT5A promoter A and B are upregulated during differentiation. hFOB1.19 cells were transfected with the constructs shown in Figure 25 and the next day treated with differentiation medium. At day 0 and 3, cells were collected. Lysates were analyzed for luciferase activity. Values are fold-change day 3 relative to day 0.

### Transcription Factor Binding Site Analysis

The promoter analysis suggest that specific sequence regions are required for the upregulation of promoter A and B during osteogenesis. To identify these, the sequences were analyzed using various online analysis tools (see materials and methods). I identified putative transcription factors (TF) for the investigated constructs that may have negative and positive effects on promoter activity during hFOB1.19 differentiation. These are summarized in (Table 3 and 4).

#### Promoter A

The PA420 construct was sufficient for maximal increase in promoter A activity during hFOB1.19 differentiation (Table 3)( Figure 28 and 27). The TF's identified within

this regions include C/EBPalpha [T00105], C/EBPbeta [T00581], GR-alpha [T00337], GR-beta [T01920], PXR-1:RXR-alpha [T05671], Sp1 [T00759], TFII-I [T00824] and YY1 [T00915] (Table 3). PXR-1:RXR-alpha [T05671], enhances bone formation and hinder bone resorption (Azuma et al., 2010). Sp1 [T00759], it has been shown to be significantly related to bone mass and osteoporotic fracture (Braga et al., 2002).

My results suggest that sequences between 1707-1358 bp have a negative effect on promoter A transcription. Analysis of this regions identified a variety of TF's (Table 3). Those unique to this region include AP-2alphaA [T00035], c-Ets-1 [T00112], Elk-1 [T00250], FOXP3 [T04280], and TCF-4E [T02878]. FOXP3 [T04280] is a Forkhead box protein P3 (FOXP3). FOXP3 transgenic mice display a defective osteoclast differentiation that is reflected in the increased bone mass (Zaiss et al., 2010). c-Ets-1 [T00112], is involved in development of bone, and osteogenesis, specifically at differentiation and mineralization phases (Raouf and Seth, 2001).

Region 1707-2178 appears to have a positive effect on promoter A transcription; this region include unique sites for p53 [T00671], PR A [T01661] and PR B [T00696]. All other were found in one or the other or both regions analyzed.

One TF of particular interest that was located in all three regions is TFII-I [T00824]. This factor was shown to interconnect with Runx2, a bone specific TF, and inhibit activation of genes associated with osteogenesis (Lazebnik et al., 2009). And, c-Ets-1 [T00112] is involved in the development of bone and osteogenesis, specifically at the differentiation, and mineralization phase.

Table 3. Promoter A TF's. Name [Accession Number]; Matrix width

420pb	Repeats	1358bp-1707bp	Repeats	1707bp -2178bp	Repeats
		AP-2alphaA [T00035]; 6	4		
C/EBPalpha [T00105]; 7	1			C/EBPalpha [T00105]; 7	1
C/EBPbeta [T00581]; 4	5	C/EBPbeta [T00581]; 4	4	C/EBPbeta [T00581]; 4	6
		c-Ets-1 [T00112]; 7	1		
		Elk-1 [T00250]; 9	1		
		FOXP3 [T04280]; 6	1		
GR-alpha [T00337]; 5	2	GR-alpha [T00337]; 5	5	GR-alpha [T00337]; 5	2
GR-beta [T01920]; 5	6			GR-beta [T01920]; 5	2
				p53 [T00671]; 7	1
		Pax-5 [T00070]; 7	1	Pax-5 [T00070]; 7	2
				PR A [T01661]; 7	1
				PR B [T00696]; 7	1
PXR-1:RXR-alpha [T05671]; 8	1				
		RXR-alpha [T01345]; 7	2	RXR-alpha [T01345]; 7	1
Sp1 [T00759]; 10	1				
		TCF-4E [T02878]; 7	1		
		TFIID [T00820]; 7	1		
TFII-I [T00824]; 6	1	TFII-I [T00824]; 6	2	TFII-I [T00824]; 6	1
YY1 [T00915]; 4	1	YY1 [T00915]; 4	1	YY1 [T00915]; 4	3

Along with the unique TF's the number of repeated TF's were higher for some, which may affect the decrease or increases in expression level. Lower number of repeats

were found in 1358 -1707bp region compared to 420bp and 1707-2178bp regions.

Whereas for 1707- 2178bp there were higher repeats number relative to 420bp.

### *Promoter B*

187bp of upstream sequence was sufficient for an approximately 4.5-fold increase during osteogenesis. Thus, region includes numerous TF binding sites (Table 4) (Figure 29). Of these AhR:Arnt [T05394], GATA-1 [T00306], GCF [T00320], GR [T05076] and STAT4 [T01577] were not identified in promoter A. No TFs appeared to be unique to only the 187bp region in comparison to the 817bp-187bp region. In fact, this region appears very similar to the 187bp region in the types of TF binding sites. A distinction is the presence of binding sites for ER-alpha [T00261], HNF-1A [T00368], p53 [T00671] and TFII-I [T00824]. The Estrogen Receptor-alpha (ER-alpha) [T00261] was found to be expressed in primary human osteoblast. It is known that estrogen control bone homeostasis by hindering the bone turnover via enhancing osteoblast bone formation and diminish osteoclast bone resorption (Deroo et al., 2014). Hepatocyte Nuclear Factor- 1 Alpha [T00368], it also called Transcription Factor 1 (Tcf-1). It was found lower in Wnt16 <sup>-/-</sup> mice; these mice develop spontaneous fractures (Movérare-Skrtic et al., 2014). TFII-I [T00824] is unique to this region of promoter B but is also found in all three regions analyzed in promoter A. To reiterate, TFII-I interconnects with Runx2 and contributes to inhibition of genes responsible for gene regulation in osteogenesis (Lazebnik et al., 2009).

Overall, both promoters A and B include similar and distinct transcription factor binding sites, and some of these TF's are known to be involved in osteogenesis. These

include c-Ets-1, ER-alpha and TFII-I that are putative binding sites common to both promoters.

*Table 4. Promoter B TF's. Name [Accession Number]; Matrix width.*

187bp	Repeats	187bp-817bp	Repeats
AhR:Arnt [T05394]; 10	1		
AP-1 [T00029]; 9	1		
AP-2alphaA [T00035]; 6	1		
C/EBPbeta [T00581]; 4	9	C/EBPbeta [T00581]; 4	4
c-Ets-1 [T00112]; 7	1	c-Ets-1 [T00112]; 7	1
c-Jun [T00133]; 7	1		
Elk-1 [T00250]; 9	1		
		ER-alpha [T00261]; 5	1
FOXP3 [T04280]; 6	1	FOXP3 [T04280]; 6	1
GATA-1 [T00306]; 6	2		
GCF [T00320]; 9	1	GCF [T00320]; 9	1
GR [T05076]; 7	1		
GR-alpha [T00337]; 5	8	GR-alpha [T00337]; 5	10
GR-beta [T01920]; 5	9	GR-beta [T01920]; 5	4
		HNF-1A [T00368]; 8	1
		p53 [T00671]; 7	3
Pax-5 [T00070]; 7	1	Pax-5 [T00070]; 7	4
PR A [T01661]; 7	1		
PR B [T00696]; 7	1		
STAT4 [T01577]; 6	1	STAT4 [T01577]; 6	2
TFIID [T00820]; 7	2		



		TFII-I [T00824]; 6	2
YY1 [T00915]; 4	4	YY1 [T00915]; 4	7

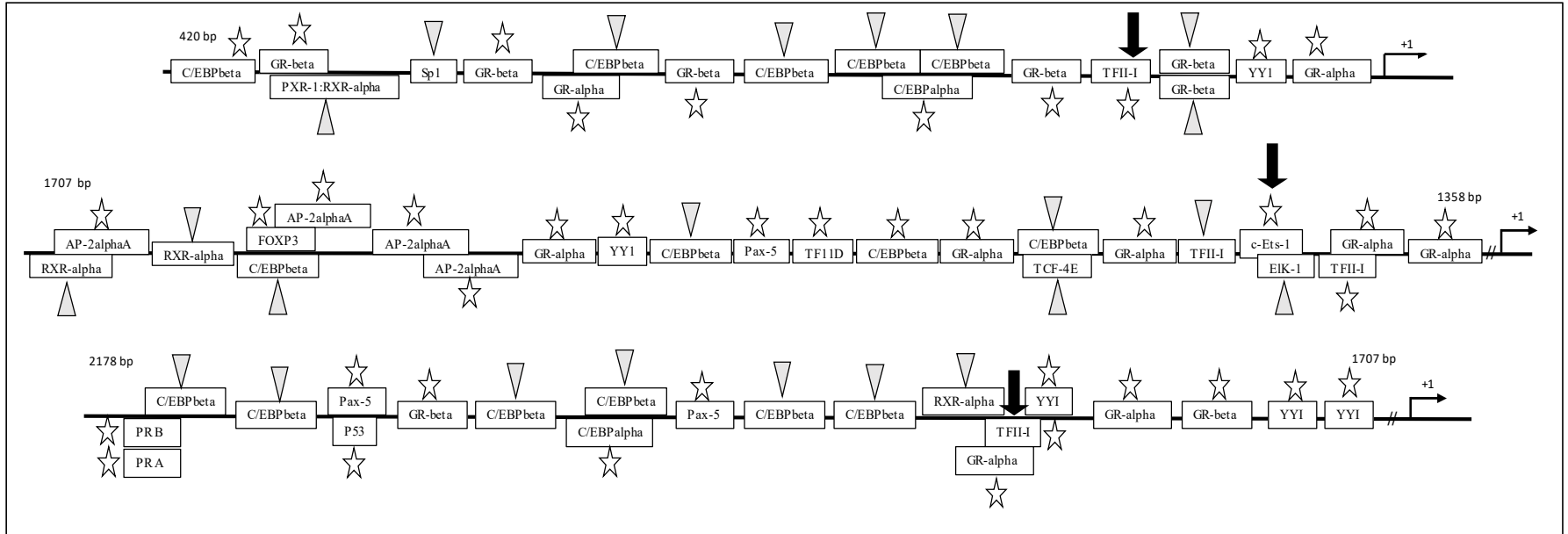


Figure 28. Generalized Map of Transcription Factors Promoter A. The stars are common TFs to Promoter A and B, while the triangles are unique to Promoter A. The black arrows are TFs involved in osteogenesis. Images not to scale.

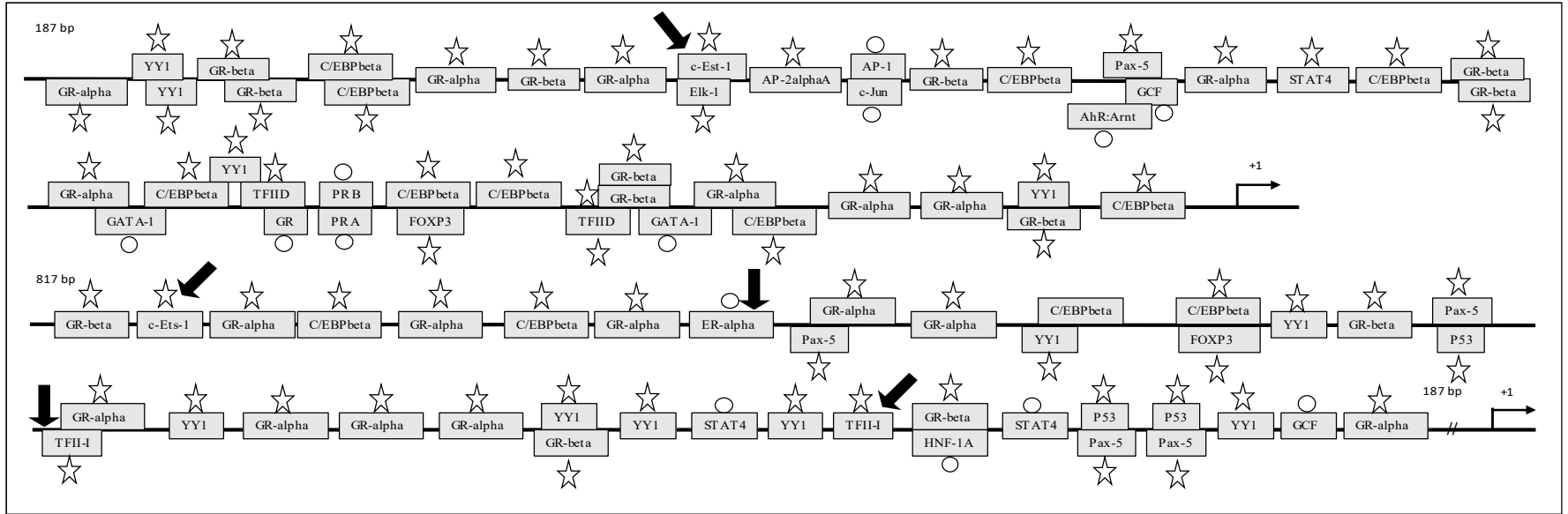


Figure 29. Generalized Map of Transcription Factors Promoter B. The stars are common TFs to Promoter B and A, while the circles are unique to Promoter B. The black arrows are TFs involved in osteogenesis. Images not to scale.

## CHAPTER IV

### DISCUSSION

The goal of this research study is to determine if there are functional differences between the WNT5A isoforms L(A) and S(B). The isoforms differ by only 18 amino acids on the N-terminus and have distinct proximal and upstream promoter sequences. The isoforms are evolutionarily conserved, having been identified in chimpanzee, mouse, chicken, and zebrafish (Bauer et al., 2013). Moreover, a sequence comparison of the promoter and upstream sequence regions of the mouse and human isoforms indicate 66% promoter A and 76% promoter B similarities (MUSCLE), suggesting these putative regulatory sequences have been maintained. These observations suggest that the WNT5A isoforms are distinct at the protein levels and are differentially regulated at the promoter level.

I chose to analyze the proteins by generating a conditioned medium that contains either isoform L(A) or S(B) from engineered CHO cells. This conditioned medium was used in a variety of assays to analyze non-canonical signaling, downstream signaling pathway targets, proliferation, apoptosis, and migration. In addition, the gene regulatory regions of the isoforms were analyzed in two cell types using luciferase-promote constructs. There are some difficulties and concerns associated with these studies. First, the isoforms in the conditioned medium must be active.

To confirm activity, I used the TOPFlash assay via transiently transfected the HEK293T cells with TOPFlash reporter, followed by treatment with conditioned medium of WNT3A and WNT5A. I confirmed that the WNT5A isoform CM-L(A) and CM-S(B) inhibit the canonical Wnt signaling (induced by WNT3A) via non-canonical signaling. These results conform to those of Bauer et al. (2013), who also showed that WNT5A isoforms inhibit canonical Wnt signaling. I also confirmed that the isoform proteins are present in the medium, however it was not possible to generate the same concentration of the isoforms in the conditioned medium. The difference varied from approximately 2.7 to 1.3-fold more L(A) than S(B). Although it is possible that this difference could affect the response of the cells to the isoforms, the TOPFlash assay results were the same for the isoforms. Another concern is the high levels of the isoforms in the medium, which may lead to unexpected results. Regardless, these experiments represent first trials using the conditioned medium.

One hypothesis is that the WNT5A isoforms have a preference for a particular non-canonical pathway. PCP/CE pathway outcomes are a cytoskeleton and/or transcriptional response. The conveyance of signal is initiated by the formation of a receptor complex by binding of WNT5A to Fz 3 or 4 receptor and ROR co-receptor. This receptor complex recruits and activate Dvl. DAAM 1 scaffolding protein and G protein Rho are being activated by Dvl. DAAM 1 and G protein Rho activates RhoA, which leads to downstream triggering of GTPase family members ROCK and JNK, this leads to the cellular response. One hypothesis is that the WNT5A isoforms have a preference for a particular non-canonical pathway. PCP/CE pathway outcomes are a cytoskeleton and/or

transcriptional response. The conveyance of signal is initiated by the formation of a receptor complex by binding of WNT5A to Fz 3 or 4 receptor and ROR co-receptor. This receptor complex recruits and activate Dvl. DAAM 1 scaffolding protein and G protein Rho are being activated by Dvl. DAAM 1 and G protein Rho activates RhoA, which leads to downstream triggering of GTPase family members ROCK and JNK, this leads to the cellular response of PCP/CE. The  $Ca^{2+}$  pathway outcomes are cytoskeletal, cell motility and/or transcriptional response conveyed via the translocation of NF- $\kappa$ B and NFAT. This pathway is triggered by binding to WNT5A to the Fz 2, 3, 4, 6 or 5 receptor and ROR co-receptor. This will lead to downstream mobilization of free  $Ca^{2+}$  via activated G proteins through PLC, IP3 and DAG activation.  $Ca^{2+}$  released cause activation of PKC and it binds to calmodulin that activated CAMKII. All these events of activation leads to the cellular response of the  $Ca^{2+}$  pathway. Hence, it is possible and likely that the WNT5A isoforms have a preference for a particular Fz-ROR receptor complex, leading to greater activation of one or the other non-canonical Wnt pathway.

I used epithelial human colon cancer cells (HCT 116), normal human osteoblast cells (hFOB1.19) and mouse embryonic fibroblast (MEF) with a knock-out of the PORCN gene (MEF-PORCN) in this study. It is known that the HCT 116 cells have low or nearly undetectable levels of the WNT5A isoforms. It is also known that HCT 116 has a mutated  $\beta$ -catenin gene, which changes a C-to-A in codon 33. This results in the loss of a serine involved in an interaction with GSK 3 $\beta$  and higher  $\beta$ -catenin activity (Ilyas et al., 1997). hFOB1.19 expresses both WNT5A isoforms and the levels of the isoforms increase during osteoblast differentiation (unpublished data, this lab). The MEF-PORCN

cells do not secrete any Wnt ligands. The MEF are known to have receptors for WNT5A (Barrott et al., 2011) so I would expect to see a clear effect from each treatment. Also, because the MEF-PORCN cells lack background/ internal interference from any secreted WNT5A.

The response of each cell type to pathway and isoform treatment are reflected in the results of each experiment conducted as follows: HCT 116 showed a higher level of pPKC and pJNK activation from the isoform S(B) treatment. While for isoform L(A) treatment showed less of an effect on pPKC and pJNK. However, it appeared that diluting CM-L(A) 1:2 gave a greater activation for pPKC and pJNK compared to the undiluted CM-L(A). Thus the high concentration of the WNT5A-L(A) in the CM had inhibitory effects. In addition, I found that the effect of the CM's on the levels of pPKC and pJNK in HCT 116 was time-dependent and apparently transient; higher levels of activation were generally observed at 2hr compared to 1hr and 6hr.

I also assayed for non-canonical activation using a AP-1 and NFAT reporter system. These transcription factors can be activated by the distinct non-canonical pathways. The level of AP-1 was higher in cells treated with isoform B relative to isoform A and control at a 6hr time point while for NFAT there was no significant difference between isoforms treatment and control. I conclude that the HCT 116 has the AP-1 transcription factor, which is downstream of the non-canonical PCP/CE pathway and pJNK. Since I found that pJNK was activated in HCT 116 cells by CM-S(B), it would be expected that AP-1, if present, would be activated. The AP-1 has oncogenic function (Ashida et al., 2005). As for NFAT it is dephosphorylated and activated via the

Ca<sup>2+</sup> pathway through the activation of calcineurin, a phosphatase. It is not clear why NFAT activation was not detected. It is possible that a particular form of NFAT doesn't bind to the luciferase reporter; there are five NFAT family members NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5.

In hFOB1.19 my results showed a slight pPKC activation when the cells were treated with CM-L(A) but no effects on pJNK were detected. As for the CM-S(B) no clear effects on either pathway were detected. Assays using the AP-1 and NFAT reporters in hFOB1.19 were consistent with these results, as no activation of the reporters were detected with treatment of the transfected cells with either CM-L(A) or CM-S(B) (data not shown). I conclude that the effects of the CMs in proliferating hFOB1.19 cells may be transient and at a low level and any transcription factors (AP-1 and NFAT) in hFOB1.19 are not activated. MEF showed little or negative levels of pJNK and pPKC when treated with WNT5A isoforms. It is possible that proliferating fibroblasts such as MEF and hFOB1.19 have low levels of non-canonical receptors.

WNT5A has been shown to increase pPKC and pJNK in other systems. Treatment of a non-small-cell lung cancer cell line A549 (adenocarcinoma) with WNT5A increased "stemness" of the cells and this effect involved PKC signaling (Yang et al., 2016). Mesenchymal stem cell differentiation into alveolar epithelial cells (*in vitro*) involved WNT5A activation of JNK and PKC (Liu et al., 2014). The activation of AP-1 via WNT5A required Ror2/Fz7 receptor complex to convey the signal via dishevelled in mouse fibroblast L cells (Nishita et al., 2010). AP-1 is responsible for the activation of Matrix Metalloproteinase (MMP-13) via WNT5A/Ror2/JNK signaling pathway in



osteosarcoma cells (Yamagata et al., 2012). In epithelial ovarian cancer, WNT5A induced epithelial mesenchymal transition (EMT) and vasculogenic mimicry (VM) via the activation of pPKC $\alpha$  (QI et al., 2014). WNT5A was shown to play a major role in differentiation and migration of mesenchymal stem cell to type II alveolar epithelial cells; this was conveyed through PCP/CE pathway activation of pJNK alone or with Ca<sup>2+</sup> pathway activation of pPKC. In these studies, the WNT5A represented the L(A) isoform.

I performed proliferation studies using our two cell lines, HCT 116 and hFOB1.19. In HCT 116 both isoforms showed a decrease in proliferation rate with indistinct proliferation rate difference between isoform L(A) and S(B). Further replica studies need to confirm a distinct difference between the two isoforms. In hFOB1.19 isoform L(A) shows an increase in proliferation whereas isoform S(B) shows no significant difference relative to control. In a previous study, the WNT5A isoforms were shown to have differential effects on proliferation in three different cancer cell lines (Bauer et al., 2013); isoform L(A) decreased proliferation rate and isoform S(B) increase proliferation rate. In a recent study, HCT 116 cell line showed an increase of foci in a colony forming assay with WNT5A isoform L(A) knock-down (siRNA), whereas siRNA knock-down of WNT5A isoform S(B) decreased the number of foci (Huang et al., 2017). Assuming this effect is due to altered proliferation, the results are opposite for isoform S(B) from my results in HCT 116. Again, I found that isoform S(B) decreased proliferation. However my results for isoform L(A) correspond to those of Huang et al., (2017). There are a number of studies indicating that WNT5A decreases proliferation.

These studies involved only isoform L(A). In one study, overexpression of

WNT5A via an expression vector in FTC-133 thyroid tumor cell line lead to reduced proliferation (Kremenevskaja et al., 2005). HepG2 hepatocyte cells treated with human recombinant WNT5A had decreased proliferation (Yang et al., 2015). In cortical neurons overexpressing WNT5A isoform L(A) showed increased neuronal survival by suppression of cyclin D1 expression and reduced proliferation (Zhou et al., 2017). A fetal liver specific knock-out of WNT5A (isoform L(A) in mice lead to an increase in the B cell numbers, indicating that *lack* of isoform L(A) increases proliferation (Liang et al., 2003). The molecular pathway involved a reduction of cyclin D1. A suppression of cell proliferation was caused by overexpression of WNT5A isoform L(A) in HaCaT keratinocytes cells (Wang et al., 2018). However, in another study using the same cell line, knock-down with siRNA of WNT5A isoform L(A) caused a suppression of cell proliferation (Zhang et al., 2015). These results indicate that WNT5A isoform L(A) is acting to enhance proliferation. In fact, in other studies using different cell types, WNT5A has been found to increase proliferation. In PanINs pancreatic cancer cells overexpression of WNT5A isoform L(A) lead to increased proliferation (Ripka et al., 2007). My overall conclusion is that altering levels of WNTA isoforms either by knock-down or overexpressed have different effects on proliferation and the response depends on the cell type.

I analyzed the effects of the isoform on apoptosis in HCT 116, with and without an inducer of apoptosis. The experiment was conducted in four independent trials. I found significant difference between the isoforms treatment and the control. In a recent study, Isoform L(A) and S(B) were knocked-down by siRNA for 48hr and analyzed for

apoptosis via annexin V staining. They found an increase in apoptosis for S(B) knock-down siRNA but no effect of siRNA knock-down of L(A) (Huang et al., 2017). However, as previously mentioned, the knock-down of isoform L(A), which is already at low and almost undetectable levels, is unlikely to lead to effect. It is not clear why the results for isoform S(B) are different from ours, although the fact they used siRNA knock-down and we used CM treatment must be considered. Other studies showed a contradiction in overexpression and knock-down of WNT5A cellular behavior in keratinocytes (Wang et al., 2018; Zhang et al., 2015). This may be clarified by repeating the experiment with different time points and dosages of the WNT5A conditioned media. It is possible that at the higher dosages such as might be found in the conditioned medium, other signaling pathways are being activated that normally wouldn't.

I analyzed the effects of the isoform on migration in two different cell lines, HCT 116 and MEF-PORCN. HCT 116 is epithelial human colon cancer cell line. I found the isoform L(A) decreased migration rate relative to control, whereas for isoform S(B) there was a trend of increased migration relative to control. In previous studies, HCT 116 WNT5A isoform L(A) knock-down decreased migration rate, whereas exogenous WNT5A addition enhances migration. They further confirmed these results by generating a stably expressing WNT5A cell line. The results was consistent with the exogenous addition of WNT5A (Bakker et al., 2013). In MG-63 osteosarcoma human cell line, the cells were treated with recombinant WNT5A (isoform L(A) and found an increased cell migration via PCP/CE pathway through P13K and Akt (Zhang et al., 2014). MEF-PORCN is a fibroblastic mouse embryonic cell line (PORCN-knock-out) making it a

“clean” system. I found that in these cells treatment with both isoforms slightly decreased migration. Again, these results suggest that the isoforms have distinct effects and that they are cell-type specific.

Different cells likely have different receptors and non-canonical pathways. For example, NIH3T3 doesn't respond to WNT11. This might be a receptor difference. Also, the Wnt signaling is not regulated by the protein itself but by the availability of the receptor. This can lead to a distinct signaling pathway (Mikels and Nusse, 2006).

I analyzed the gene regulatory regions of the WNT5A isoforms in two different cell types HCT 116 and hFOB1.19. This was accomplished by transfecting different luciferase promoter constructs and assaying for luciferase activity. The basis of the study is that different gene sequences will contain distinct regulatory elements. First, promoter activity was compared in proliferating cells of the two cell lines HCT 116 and hFOB1.19.

My results suggest that the promoters of both L(A) and S(B) contain positive and negative regulators. Promoter L(A) showed maximal expression with 1707bp of upstream sequence; removal of additional sequences decreased expression. And, there appears to be a negative regulator between 2178bp and 1701bp, as removal of this region increases promoter activity to the maximum. I found that this region contains three unique transcription factor binding sites; PR A, PR B, and p53. P53 has been shown to act as a negative regulator (Molchadsky et al., 2008), however, it is unlikely that p53 will be functional, as it generally is induced by stress. PR A and PR B are progesterone receptors (isoforms A and B; T01661 and T00696) and would be expected to respond only in the presence of the hormone progesterone. Thus, based on my analysis, it is not clear what

factor or factors are responsible for the negative effect of this region. It could be epigenetic modifications - histone methylation that affects the chromatin structure.

Within the 1707 bp region there are a number of unique transcription factor binding sites that may contribute to high level of expression. These include AP-2alphaA, c-Ets-1, Elk-1, FOXP3, and TCF-4E.

Promoter S(B) showed maximal expression with 1257bp of upstream sequence in proliferating cells, both HCT 116 and hFOB1.19. While this region was not analyzed completely for transcription factors, I found that the 817bp region contained the following unique sites: ER-alpha, HNF-1A, and p53. In general, the promoter S(B) upstream sequences and proximal promoter region seem to include a greater number of putative transcription factor binding sites than the sequence regions of promoter L(A).

Next, I analyzed the promoters during hFOB1.19 differentiation to identify gene regulatory regions required for increased isoform promoter activity during osteogenesis. Data from our lab has shown that both isoform promoter activity increases during hFOB1.19. Increased promoter activity at day 3 of hFOB1.19 differentiation was measured for the shortest constructs for each promoter; 420bp for promoter L(A) and 187bp for promoter S(B). The level of activation was maximal for the 420bp of promoter L(A) (over 10-fold). 817bp was sufficient for over 6-fold increased expression for promoter S(B), whereas the 187bp gave approximately a 4.5-fold increased expression. Both promoters L(A) and S(B) contain putative transcription factors shown to be involve in osteogenesis in some capacity. These include c-Ets-1, ER-alpha and TFII-I that are putative binding sites common to both promoters. It is possible that these factors are

involved in the increased activity of both promoters L(A) and S(B). There is one report that the transcription factor RUNX1 is regulating the WNT5A [isoform L(A)] promoter during osteogenesis (Liang et al., 2011). There is only an abstract for this article, as it is written only in Chinese. It is stated that the RUNX1 binds to the WNT5A sequences. Overexpression of RUNX1 in mesenchymal stem cells increased WNT5A [isoform L(A)] transcripts. I analyzed the upstream regions of the promoters L(A) and S(B) for the consensus, core binding site for RUNX1 (5'-TGTGGTNNN-3'). I also analyzed the sequences for RUNX2 (5'-PYGPGYGGT -3') that is known to be involve in osteogenesis. The results show that 4000bp of promoter L(A) upstream sequences contain five RUNX1 and four RUNX2 putative binding sites. Single RUNX1 and RUNX2 sites were located within the first 300bp or included on the PA420 construct. In contrast, promoter S(B) contained two RUNX1 and two RUNX2 sites but none of these sites were located within the first 500bp upstream of exon1 $\beta$ . And, the PB187 construct shows increased activity on day 3 of osteogenesis. The functional importance of these putative RUNX1 and 2 sites needs to be determined.

In summary, my findings suggest that WNT5A isoforms L(A) and S(B) are redundant in some settings, functionally distinct in others and both are cell-type specific. Also, my results contradict other studies. An example of cell-type specificity is the finding that isoform L(A) and S(B) tended to decrease proliferation in HCT 116 cells but isoform S(B) increased proliferation in hFOB1.19, whereas Isoform L(A) had no effect. Using conditioned media, I found no effect of either isoform on apoptosis in HCT 116, contradicting what was found using siRNA to modulate levels. Similarly, I found a

decrease in migration with isoform L(A) treatment and increase with isoform S(B) treatment, contradicting to what found in the literature. Isoform S(B) had a greater effect on PCP/CE and  $Ca^{+2}$  pathways than isoform L(S). In contrast, in hFOB1.19 isoform L(A) had more effect on PCP/CE pathway than isoform S(B).

Wnts and FZ have a distinct affinity pairing leading to individual related interactions (Van Amerongen et al., 2008). I would expect that there is a receptor affinity of each WNT5A isoforms. I could not find any articles to prove a distinct receptor affinity regarding the isoforms. The Frizzled (Fz) family of receptors [5], Lipoprotein receptor-related protein (LRP) family [2], and Receptor tyrosine kinase kinase-like orphan receptor (ROR) family [2]. The combination of all there are 25 and 25 possible outcomes. Due to these different receptors isoform L(A) may have a greater affinity for one receptor-co receptor combination than isoform S(B).

One major challenge was determining the correct time window for treating cells for analysis of the signaling pathways. This variable could be different for each signaling pathway and cell type. Also, as previously mentioned, contradictory results have been reported for the WNT5A isoforms using either an siRNA approach versus overexpression. And, conditioned medium may add another variable since as discussed, the concentration of the WNT5A isoforms in the medium is likely much higher than usually found in the cell environment. My explanation on the temporal difference in detection after treatment, is this has to do with cells adjusting to pathway stimulation. Maybe when the non-canonical pathway is turned on, there are inhibitors of the pathway

that are activated and down regulate it. This is true for the  $\beta$ -catenin pathway,  $\beta$ -catenin turns on DKK1, which inhibits the pathway (Niida et al., 2004).

To further my study, detecting other PCP/CE and  $\text{Ca}^{+2}$  pathway downstream targets to more accurately confirm which pathway is being activated may be important. These may include STAT4, ROCK, and CAMKII. Also, the use of specific activators of the target proteins such as pJNK and pPKC to confirm the flow cytometry will be helpful. Additionally, it would be informative to conduct a TOPFlash assay first on each cell type that I will use to confirm a functional noncanonical signaling system. I would also run a dose-dependent assay to equalize the effect of each isoform on each treatment.

My results suggest that the WNT5A promoters L(A) and S(B) are similarly regulated during osteogenesis and that this regulation includes shared and distinct transcription factors. Also, I found that both promoters are active in HCT 116 cells that express the WNT5A isoforms at low levels. I identified a collection of putative TFs binding sites in each isoform promoter sequence. These TFs can act as an enhancer or suppressor for the differential expression of each isoform. A potential problem with these results is that it is based on a bioinformatic analysis and not experimental. I need to experimentally confirm these results. To further my study regarding the transcription factor analysis, I would narrow them down via Systemic evaluation of ligands by exponential enrichment (SELEX), Chromatin Immunoprecipitation (ChIP) and Gel shift.



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