AKINDAHUNSI, OLUWOLE O., M.S. Evidence of Functional Distinction between WNT5A Isoform A and Isoform B in Osteosarcoma Cells. (2014)
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WNT5A is a secreted ligand involved in differentiation, proliferation, cell movement, and apoptosis. Various studies have shown WNT5A misregulation in cancer and that it can act as both a tumor suppressor, and oncogene. The *WNT5A* gene has two transcription sites, producing mRNA transcripts that code for unique protein isoforms, termed Isoform A and Isoform B in this lab. In a recent study, Isoforms A and B were found to differentially effect proliferation, indicating the isoforms are functionally distinct. The focus of this study was on the functional distinctions between the WNT5A Isoform A and Isoform B in the osteosarcoma cell line SaOS-2. In osteosarcoma, levels of Isoform A expression are higher than normal in normal osteoblast, whereas Isoform B expression is nonexistent, indicating it is functioning as a tumor suppressor. Stable lines of SaOS-2 were generated expressing Isoform B, overexpressing Isoform A and expressing GFP, as a control. The cell lines were confirmed to expresses the expected isoform mRNA at higher levels than that of the control and to have correspondingly higher levels of WNT5A protein. These cell lines were used in assays for proliferation, migration, invasion, and apoptosis. Results show that Isoform A overexpression stimulates migration and apoptosis resistance, whereas increased Isoform B had no effect. This indicates that Isoform A and Isoform B each stimulate separate non-canonical WNT signaling pathways independent from one another. Increased Isoform B expression slightly inhibited invasiveness, but the results are still inconclusive. Both Isoform A and Isoform B both had no effect on proliferation, relative to the GFP control. Overall, these

results suggest that Isoform A and Isoform B are functionally distinct, and that the two different isoforms activate different non-canonical pathways.

EVIDENCE OF FUNCTIONAL DISTINCTION

BETWEEN WNT5A ISOFORM A

AND ISOFORM B IN

OSTEOSARCOMA

CELLS

by

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Approved by

_____
Committee Chair

To my family and friends, for their love, support and encouragement through this journey.

APPROVAL PAGE

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

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Lastly, I would like to thank my family (Dad, Mom, siblings, etc) for their support and encouragement in my present and future endeavors.

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CHAPTER I

INTRODUCTION

WNT5A, a secreted protein of the WNT signaling pathway, is well known for its

role in the cellular differentiation and organ development, as well as proliferation and

cellular migration. Studies have shown that it plays a role in many human cancers, acting

as both a tumor suppressor and oncogene. WNT5A was found to be over expressed in

osteosarcoma, melanoma, lung, and breast cancers and silenced in colorectal and gastric

cancers. The *WNT5A* gene has two main transcription start sites, promoter A and

promoter B. These promoters give rise to two transcripts that code for distinct protein

isoforms, identified as Isoform A and Isoform B. These proteins differ at the N-terminus;

Isoform A has an additional 15 amino acids in comparison to Isoform B. In

osteosarcoma, Isoform A is overexpressed, whereas Isoform B is not expressed at all.

Moreover, both Isoform A and Isoform B are expressed in normal osteoblasts. This leads

to the question of whether Isoform A has a distinct function from that of Isoform B. It is

possible the distinct functions of the WNT5A isoforms are responsible for the oncogenic

or tumor suppressor activity of WNT5A in different cancers, including osteosarcoma.

Many different proteins have isoforms, which show distinct functional differences.

Considering WNT5A's importance in cancer and normal cellular functions, it was

important to analyze and characterize the functional distinctions between WNT5A isoforms A and B.

WNT Proteins and Function

The WNT proteins are a large family of cysteine-rich proteins, within the size ranged of 40 kDa. They are a class of proteins that function as secreted ligands. There are a total of 19 WNT ligands, each defined by amino acid sequence rather than their functional properties (Clevers & Nusse, 2012). WNTs are important in the orchestration of a large range of developmental and physiological processes. These include proliferation, differentiation, apoptosis, survival, migration, and polarity (Logan & Nusse, 2004). WNTs achieve these functions by activating multiple intracellular signaling cascades.

As secreted proteins, WNTs must pass through the endoplasmic reticulum (ER)-Golgi system. WNT proteins undergo palmitoylation of cysteine and serine in the endoplasmic reticulum (Li et al., 2010). They are eventually secreted out of the cell through the Golgi apparatus. Palmitoylation activates WNTs but also accounts for the proteins hydrophobicity and poor solubility. This WNT modification is performed primarily by two specific proteins, Porcupine and Wntless (Burrus & McMahon, 1995; Tanaka et al., 2002). Porcupine (Porc) (multipass transmembrane O-acyltransferase) modifies the serine 209 via palmitoylation. Wntless (Wls), putative G-protein coupled receptor, transports the modified WNT from the Golgi in vesicles to the plasma membrane, where it is released.

WNT Signaling

WNTs function by binding to a cell surface receptor. WNT receptor is a heterodimeric receptor complex consisting of two parts, Frizzled and LRP5/6. Frizzled (Fz) seven-transmembrane (7TM) receptors have large extracellular N-terminal cysteine-rich domains (CRD) that provide a primary platform for WNT binding (Bhanot et al., 1996). A single WNT can bind multiple Fz proteins and vice versa. LRP5 is essential for embryonic development, whereas LRP6 is important for adult bone homeostasis (Clevers & Nusse, 2012; MacDonald, et al., 2009). The formation of the WNT/Frizzled/LRP (5/6) complex is sufficient to activate β-catenin signaling. However, certain WNTs may activate β-catenin pathway (also known as canonical pathway) and/or non-canonical pathways depending on the receptor complement (van Amerongen et al., 2008). Fz function is involved in both the β-catenin and non-canonical pathways, whereas the LRP5/6 is only associated with the β-catenin pathway.

β-catenin (canonical) Pathway

The β-catenin pathway has a WNT-on state, and a WNT-off state (Logan & Nusse, 2004; MacDonald et al., 2009). In the WNT-off state (where no WNT ligand is present), cytoplasmic β-catenin is regulated by the APC/Axin Destruction Complex (Kimelman & Xu, 2006). Axin, a scaffolding protein, acts as the main component of the destruction complex, interacting with β-catenin, the tumor suppressor proteins APC and WTX (MacDonald et al., 2009). Also included are CKIα/δ and GSK3a/β, two constitutively active serine-threonine kinases. Although the specific molecular activity

for APC remains unresolved, it is essential for the destruction complex function. Axin uses separate domains to interact with CKIα/δ, GSK3a/β and β-catenin. Due to no WNT ligand present Fz/LRP receptors are not engaged, β-catenin is essentially bound to the Axin/APC Destruction Complex, and is phosphorylated by CKIα/δ and GSK3a/β. The phosphorylated β-catenin is then recognized by the F-box/WD repeat protein β-TrCP, part of an E3 ubiquitin ligase complex. This leads to protein degradation of β-catenin by a proteasome (Clevers & Nusse, 2012).

In the WNT-on state (where the WNT ligand is present), the formation of the WNT /Frizzled/LRP(5/6) complex results in a ligand-induced conformational change. This leads to the LRP tail undergoing phosphorylation by the kinases, CKIα/δ and GSK3a/β. The phosphorylation leads to binding of Axin to the cytoplasmic tail of LRP6 (Mao et al., 2001). Fz receptor interacts with Dishevelled (Dsh/Dvl), a cytoplasmic scaffolding protein, which seems critical for LRP phosphorylation. WNT binding of Fz and LRP promotes direct interaction between Axin and Dsh/Dvl through their DIX domains, reconfiguring the destruction complex that regulates β-catenin levels in the cell (Schwarz-Romond, et al., 2005). In short, the APC/Axin Destruction Complex is prevented from forming, thus preventing β-catenin degradation. The β-catenin moves into the nucleus and interacts with the DNA-binding protein Lymphoid Enhancer-binding Factor 1/T Cell-specific transcription Factor (LEF/TCF)(Logan & Nusse, 2004). The β-catenin- LEF/TCF complex activates WNT target genes.

β-catenin independent (non-canonical) Pathways and WNT5A signaling

There are two types of WNT pathways that do not involve β-catenin. These include the PCP/CE (Planar Cell Polarity/Convergent Extension) and the Ca^{2+} signaling pathways (Figure 1). Of the several non-canonical WNT pathways, the most extensively studied pathway is the PCP/CE (Wang, 2009). Planar cell polarity is derived from the study of tissue polarity necessary to generate polarization within the plane of the epithelium. Non-canonical-WNTs, such as WNT5A, activate the PCP/CE pathway by binding to the 7-transmembrane Fz receptor, which recruits the cytoplasmic scaffold protein Dsh/Dvl to the plasma membrane (Simons & Mlodzik, 2008) (Figure 1). This causes a downstream effect in promoting JNK, ROCK, and Rho GTPases (Rho and Rac), which eventually promotes cell polarity and migration (Wang, 2009). Due to the PCP/CE pathway's role in cell adhesion and mobility, WNT5A has been implicated in the role of WNTs in metastasis (Yamaguchi et al., 2005). In fact, increased WNT5A expression has been associated with metastasis of melanoma, gastric cancer, and osteosarcoma cancer cells (He et al., 2008; Kurayoshi et al., 2006; Weeraratna et al., 2002).

The WNT Ca^{2+} signaling pathway is the least studied non- β-catenin pathway. The binding of WNT ligand to Fz receptor leads to a short-lived increase in the concentration of certain intracellular signaling molecules, specifically inositol 1, 4, 5-tri-phosphate (IP3), 1, 2 diacylglycerol (DAG). Both can lead to increased Ca^{2+} levels, which in turn activate Ca^{2+}/Calmodulin-dependent Protein Kinase II (CaMK-II) and Protein Kinase C (PKC) (Kohn & Moon, 2005). They, in turn, can activate nuclear

transcription factors NF-κB and CREB, which activate gene expression. WNT5A has

been shown to activate the Ca^{2+} signaling pathway (Figure 1).

WNT5A can bind to receptor-like tyrosine kinase (Ryk) and Fz receptors family

members Fz3, Fz4, Fz5, Fz7 and Fz8, mediating phosphorylation of Dvl and influencing

β-catenin signaling (Kikuchiet al., 2012; Takada et al., 2007). The PDZ domain of Dvl

directly binds to the Fz's C-terminus KTxxxW motif. There may be heterotrimeric G

proteins that might be coupled with WNT signaling, but at the moment, there is no

evidence to sustain this (Schulte & Bryja, 2007). WNT5A can also bind to receptor

tyrosine kinase-like orphan receptor 2 (Ror2) (Nishita, et al., 2010). Ror2 functions as a

co-receptor, and is more involve in WNT5A signaling. In one study, WNT5A knockout

mice phenotypes were similar to those lacking Ror2, rather than those lacking Fz (Oishi

et al., 2003). Ror2 have been shown to form a ternary complex with WNT5A and Fz

receptors (Fz5, Fz6 or Fz7). It has been also suggested that Ror1 is also a receptor for

WNT5A (De, 2011).

Other than the two non-canonical pathways, there are recent findings showing

other signaling cascades that are activated by WNT5A. These include Dvl activating

atypical PKC, generating a downstream signaling resulting in axon differentiation. This is

done probably through the Partitioning Defective Homologue 3 (PAR3)-PAR6- αPKC

complex(X. Zhang et al., 2007). WNT5A has also been shown to activate

phosphatidylinositol-3 kinase (PI3K)/ Protein kinase B (AKT) signaling. The activation

of PI3K/AKT signaling pathway resulted in a resistance to apoptosis and cell movement (Anastas et al., 2014; A. Zhang et al., 2014).

WNT5A is also shown to interact with the canonical β-catenin pathway as well (Li et al., 2010; McDonald & Silver, 2009). It has been shown that WNT5A affects the β-catenin pathway either in an agonistic or antagonistic approach (X. He, 1997; Torres et al., 1996). The result of this interaction induces changes in cell migration, cell polarity, differentiation, as well as proliferation. It is possible that the WNT5A Isoform A and Isoform B are differentially affecting the non-canonical pathways.
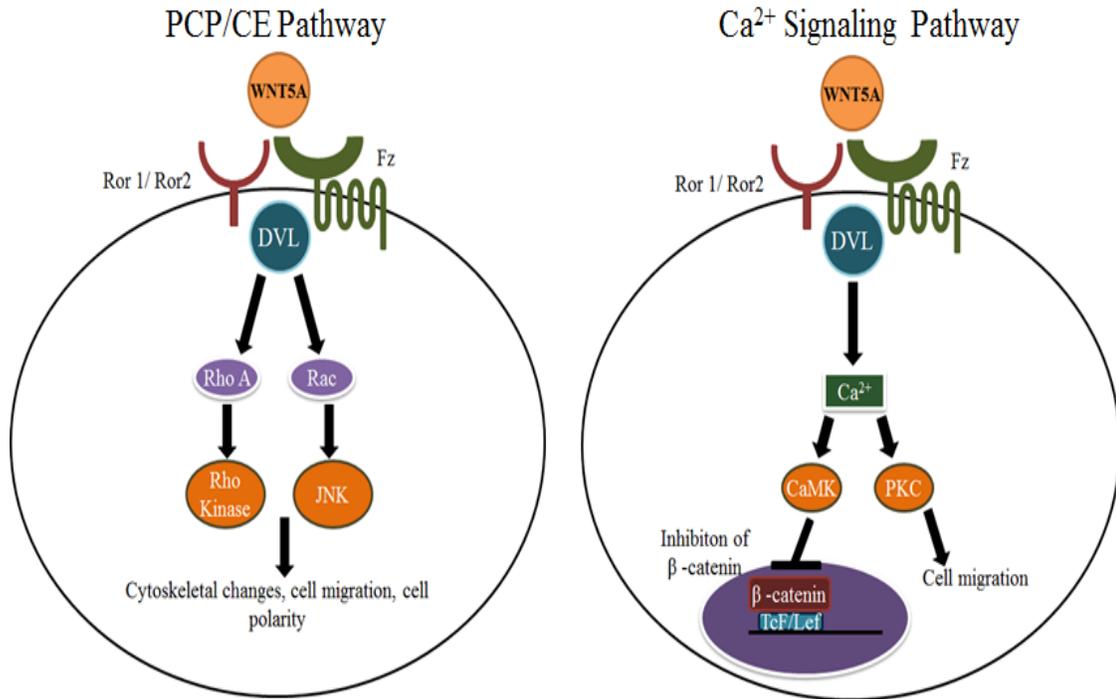
Figure 1. WNT5A Non-canonical Signaling Pathways. A. The planar cell polarity (PCP)/convergent extension (CE) pathway. WNT5A signaling cascades through this pathway affect cytoskeletal changes, cell migration and cell polarity via Rho and JNK kinases. B. The Calcium (Ca2+) pathway. WNT5A signals through this pathway inhibit β-catenin and gene expression. This leads to cell migration. CaMK, Ca2+/calmodulin-dependent kinase; Dvl, disheveled; Fz, Frizzle; PKC, protein kinase C; JNK, c-Jun N-terminal kinase; Tcf/Lef, T-cell factor/lymphoid enhancer factor. Based on diagrams in Kikuchi et al. (2012) and Nishita et al. (2010).

WNT5A: Gene, Protein Structure and Function

The WNT5A gene is found on Chromosome 3, band p13.3 between 55,499,744 and 55,521,331 base pairs (Ensembl WNT5A EN5600000114251). In humans, there are eight WNT5A transcripts, three of which give rise to complete proteins. Two of these transcripts, termed promoter A and promoter B, are being analyzed in this lab. Promoter A is the most characterized *WNT5A* transcript. The promoter B transcriptional start site is

located in intron 1 of promoter A transcription unit (Figure 2). The promoter A transcript

includes exons 1, 2, 3, 4, and 5. The promoter B transcript has a unique exon 1β and

shares exons 2, 3, and 4 and exon 5 with the promoter A transcript.
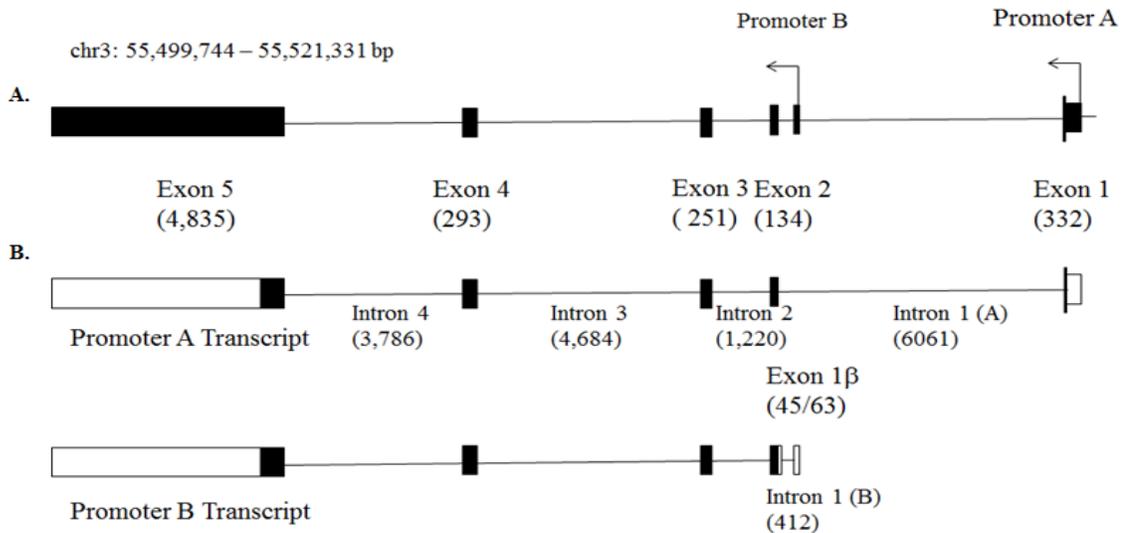
Figure 2. *WNT5A* **Gene Transcripts. A**. Promoter A and Promoter B transcription start
sites and exons, located on Chromosome 3. **B.** Promoter A and Promoter B primary
transcripts. Black boxes are coding regions; open boxes are untranslated regions. Sizes in
nucleotides of the exons and introns are shown in parenthesis. Exon 1 is unique to
Promoter A, whereas Exon 1β is unique to Promoter B. This diagram is based on the
Ensembl EN5600000114251, Bauer et. al, 2013, and Vaidya et al, submitted.

Translation of these two transcript give rise to two protein isoforms, labeled as

Isoform A (380 amino acids) and Isoform B (365 amino acids). Isoform B differs from

Isoform A, as it lacks the first 15 amino acids at the N-terminus peptide sequence (Figure

3). The two isoforms have been recently purified and characterized (Bauer et al., 2013).

They found that the isoforms have different ER signal sequences and after processing

differ by 18 amino acids on their N-terminus. The two processed WNT5A isoforms,

WNT5A-long (WNT5A-L) and WNT5A-short (WNT5A-S) correspond to the preprocessed Isoform A (long) and Isoform B (short).

As with other WNT proteins, post- translational modification of WNT5A includes palmitoylation and glycosylation (Burrus & McMahon, 1995). The protein is glycosylated at specific asparagine-linked sites. The possible sites where glycosylation occur are Asn114, Asn120, Asn311, and Asn325. Palmitoylation occurs at Ser-244. This modification allows efficient binding to frizzled receptors. Palmitoylated Ser-244 leads to the subsequent palmitoylation at Cys-104. Biochemical analysis of the purified WNT5A isoforms indicate that both have the same hydrophobicity (Bauer et al., 2013).

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              10            20           30           40            50           60
MKKSIGILSP   GVALGMAGSA  MSSKFFLVAL   AIFFSFAQVV   IEANSWWSLG  MNNPVQMSEV

              70            80           90          100           110          120
YIIGAQPLCS   QLAGLSQGQK  KLCHLYQDHM  QYIGEGAKTG   IKECQYQFRH   RRWNCSTVDN

             130           140          150          160           170          180
TSVFGRVMQI   GSRETAFTYA  VSAAGVVNAM SRACREGELS    TCGCSRAARP   KDLPRDWLWG

             190           200          210          220           230          240
GCGDNIDYGY   RFAKEFVDAR  ERERIHAKGS   YESARILMNL   HNNEAGRRTV  YNLADVACKC

             250           260          270          280           290          300
HGVSGSCSLK   TCWLQLADFR  KVGDALKEKY   DSAAAMRLNS  RGKLVQVNSR  FNSPTTQDLV

             310           320          330          340           350          360
YIDPSPDYCV   RNESTGSLGT  QGRLCNKTSE  GMDGCELMCC GRGYDQFKTV  QTERCHCKFH

             370           380
WCCYVKCKKC  TEIVDQFVCK
```

Figure 3. Human WNT5A Amino Acid Sequence for Isoform A and Isoform B. The
15 AA's unique to Isoform A is highlighted in gray. The unhighlighted amino acids are
common to both Isoform A and Isoform B

Bauer et al., (2013) determined the role of WNT5A-L (Isoform A) and WNT5A-S

(Isoform B) on the canonical WNT signaling pathway and on proliferation in various

cancer cell lines. Using a TOPFLASH- Luciferase assay, they were able to show that

both isoforms antagonized the β-catenin pathway in MDA-MB-231 (derived from a

breast carcinoma) and Human Embryonic Kidney 293 cells (HEK293) in a similar

manner. In MDA-MB-231, HeLa (cervix carcinoma) and SH-SY5Y (neuroblastoma)

cells, proliferation and viability were shown to be inhibited by WNT5A-L (Isoform A),

but promoted by WNT5A-S (Isoform B). However, they did not assay for the effects of

the isoforms on cell migration, invasiveness, or differentiation, nor on the activation of the non-canonical pathways.

Studies from this lab also indicate that isoform A and B have distinct functions. It was found that promoters A and B are differentially regulated by TNF-α (Katula et al., 2012) and during differentiation of 3T3L1 cells into adipocytes (Hsu, 2012). Moreover, promoter B was found to be silenced in the osteosarcoma cell line SaOS-2, whereas both promoters A and B are active in normal osteoblast cells (Vaidya, 2013). These studies strongly support the conclusion that WNT5A Isoform A and Isoform B have distinct functions.

WNT5A Cellular Functions

WNT5A is involved in cell migration, which includes convergent extension movement. It has been shown that the receptor Ror2 bound by WNT5A induces cell migration in embryonic fibroblast (Nishita et al., 2010). Ror2 mediates the formation of filopodia by associating with the actin-binding protein filamin A. In melanoma cells and T cells, it also has been proposed that WNT5A controls cell polarity and directional migration through the recruitment of Fz3, actin, and myosin IIB with Melanoma Cell Adhesion Molecule (MCAM, also known as MUC18 and CD146) into intracellular structure (Witze et. al., 2008). WNT5A has been suggested to cooperate with integrin signaling in cell migration/adhesion regulation though Fz receptors. WNT5A stimulation forms the Dvl/APC complex, which sequentially bind to FAK (focal adhesion kinase) and paxillin, both which are also involved in the stabilization of microtubules at the cell

periphery (Matsumoto et al., 2010). This induces disassembly of the focal adhesion, stimulating cell migration.

WNT5A is important in development and cellular differentiation, particularly of mesenchymal cells. Related to its function in cell movement, WNT5A has a role in gastrulation (Hardy et al., 2008), and limb formation (Gros et al., 2010). WNT5A knockout mice lack distal digits, as well as having a truncation of the proximal skeleton (Oishi et al., 2003). Mesenchymal stem cells can differentiate into adipocytes, chondrocytes, and osteoblast. Evidence suggests that WNT5A promotes chondrocyte differentiation (Bradley & Drissi, 2010) and suppress formation of adipocytes and chondrocyte hypertrophy(Bradley & Drissi, 2010). And, more recently, in one study WNT5A-induced non-canonical signaling was shown to cooperate with WNT/β-catenin signaling to achieve proper osteoblast differentiation and bone formation (Okamoto et al., 2014). WNT5A may also have a role in the differentiation of haematopoietic stem cells and the function in axonal outgrowth (Li et al., 2013).

WNT5A in Cancer

Most observations of WNT5A in cancer show it as a oncogene, but it has been suggested to act as an tumor suppressor as well. WNT5A is highly expressed in several cancers, such as osteosarcoma, melanoma, lung cancer, gastric cancer and breast cancer (Kurayoshi et al., 2006; Weeraratna et al., 2002). In melanoma, WNT5A induces an epithelial mesenchymal transition though protein kinase C (PKC)(Weeraratna et al., 2002). The involvement of PKC has been linked to increased cell mobility in several

types of cells, including melanocytes. In gastric cancer and prostate cancer cases, those that were positive for WNT5A showed higher tumor aggressiveness (Kurayoshi et al., 2006). In fact, WNT5A was used as a prognostic indicator in gastric cancer patients, being helpful in determining proper therapeutic strategy.

In this lab, the WNT5A isoforms have been analyzed in two types of cancers. Isoform A and Isoform B transcripts were found to be greatly reduced in the colorectal cell line HCT-116, indicating both promoters A and B were silenced (unpublished data). In contrast, in the osteosarcoma cell line SaOS-O2, promoter A is active, whereas promoter B was entirely inactive (Hsu, 2012). Bauer et al (2013) found that WNT5A-S (Isoform B) and WNT5A-L (Isoform A) are expressed in the three cancer cell types they examined; MDA-MB-231 (breast carcinoma), HeLa (cervix carcinoma), and SH-SY5Y (neuroblasoma), but that only in HeLa was WNT5A-S (Isoform B) expressed at a higher level than WNT5A-L (Isoform A). Those findings suggest that WNT5A promoters A and B are differently regulated during cellular transformation.

WNT5A also plays a role in metastasis and invasion. In one study, metastasis was severely suppressed when WNT5A was knockdown in gastric cancer cells (Yamamoto et al., 2009). WNT5A signaling might be linked to the expression of matrix metalloproteases (MMP) genes, a common target gene in cancer invasion (Yamamoto et al., 2010). In osteosarcoma, WNT5A activates c-Src, through Ror2. This leads to the expression of MMP-13 (Enomoto et al., 2009). Laminin $\gamma2$, another gene involved in

invasion and metastasis, was also stimulated by WNT5A signaling. The distinct function of the WNT5A isoforms in metastasis and invasion is not known.

Project Overview

WNT5A, a non-canonical WNT protein, is important in the regulation of differentiation, proliferation, and development in general. It is modified in cancer. WNT5A exists as two different isoforms, A and B. The promoters of each appear to be regulated differentially. This suggests that the two isoforms A and B are functionally distinct. My hypothesis is that WNT5A Isoform A regulates those three traits in a one fashion, whereas WNT5A Isoform B regulates the traits in an opposite fashion.Indeed, recent published data supports this hypothesis (Bauer et al., 2013). The goal of this project was to discover and establish the functional differences between WNT5A isoforms A and B, based on assays for proliferation, migration and invasion. We also assayed for effects on chemical induced apoptosis. To accomplish this goal, I completed the following specific aims:

1. Generated and characterized stable lines of human SaOS-2 osteosarcoma cells overexpressing WNT5A Isoform A and Isoform B.

2. Determined if overexpression of WNT5A Isoform A and Isoform B in SaOS-2 alters migration, invasion, proliferation, and apoptosis.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Mouse L-Cells (CRL-2648) and SaOS-2 (HTB-85) osteosarcoma cells were obtained from the American Type Culture Collection (ATCC). The mouse L-Cells were grown in ATCC Hybri-Care Medium (#46-X with 1.5 g/l sodium bicarbonate and 10% fetal bovine serum). The SaOS-2 cells were grown in McCoy's 5a Modified medium containing 15% fetal bovine serum and penicillin/streptomycin (50 I.U. /50 µg per ml) (referred to as 15% Complete McCoy's 5a). Both mouse L-Cell and SaOS-2 cells were grown at 37°C in a 5% CO_2 humidified cell culture incubator.

DNA Transfection

SaOS-2 and mouse L-cells were divided in to three groups and plated at 5-10 x 10^6 cells per plate in 6 x 60 mm dishes (2 plates per group). Each group was transfected with a specific expression vector containing cDNA for the WNT5A isoforms (Figure 4). One group was transfected with WNT5A Isoform A expression vector, pWNT5AisoA, containing the WNT5A Isoform A cDNA sequence NM_003392, which was purchased from Origene (SC126838). The second group was transfected with WNT5A isoform B expression vector (Clone ID MESAN2003376) purchased from the National Institute of

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Technology and Evaluation (NITE; Chiba, Japan). This vector contains the cDNA

sequence AK290869. The third group was transfected with a vector that expresses Green

Fluorescent Protein (GFP), pRNATinH1.2/Hygro.
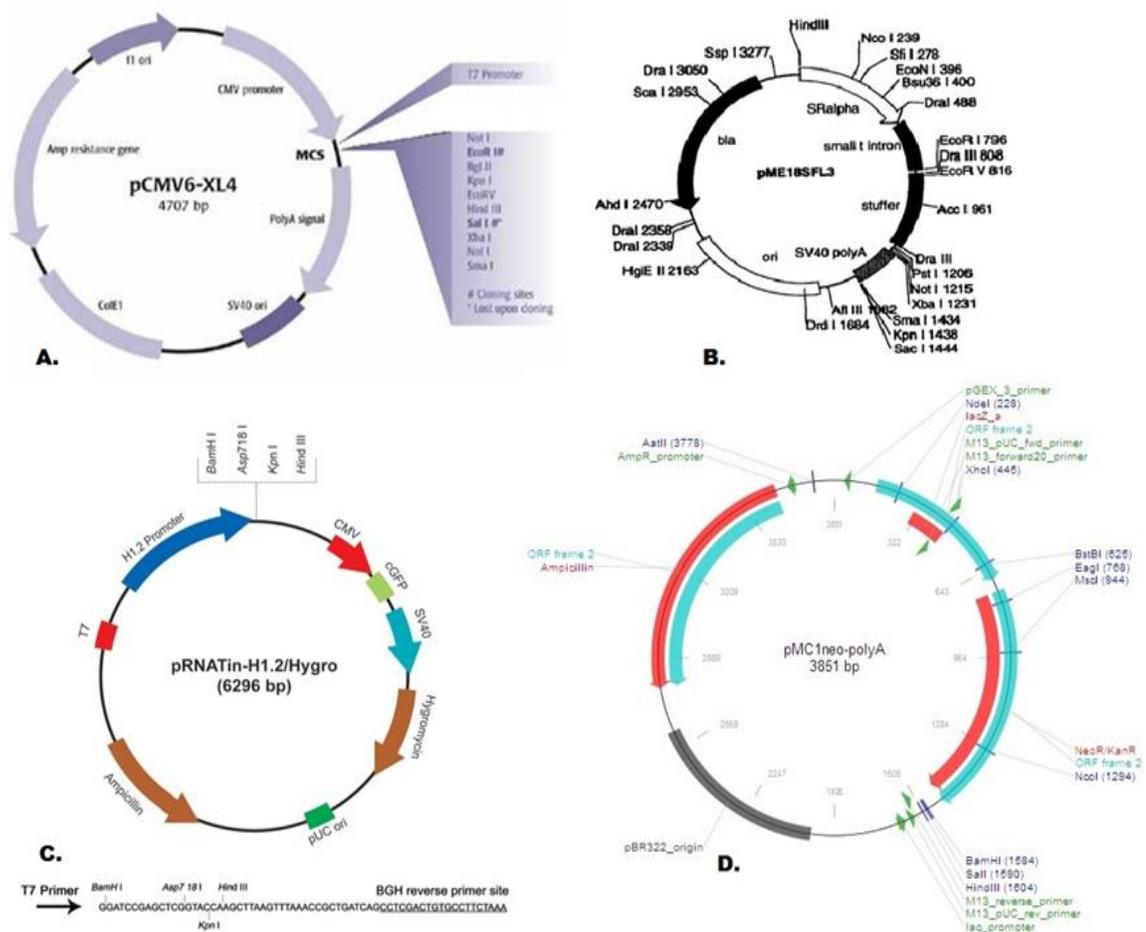
Figure 4. Vectors Used for Transfection

- A. Expression Vector pCMV6-XL4. Isoform A cDNA (NM_003392) cloned into Not 1 – Not 1 (OriGene)
- B. Expression Vector pME18SFL3. Isoform B cDNA (AK290869) cloned into the Dra III and Dra III (re place stuffer region) (NITE)
- C. Expression Vector pRNATin-H1.2/Hygro, with cGFP (GenScript)
- D. Expression Vector pMC1neopolyA (Invitrogen)

Transfection was accomplished using NanoJuice (Novagen 71902-3). Vector

DNA (pWNT5AIsoA, pWNT5AIsoB, and pGFP) was mixed at a molar ratio of 5:1 with

the selection vector pMC1neopolyA (pNeo) (Invitrogen). 72 hours after transfection,

cells were viewed, collected by trypsinization and replated in 100 mm dishes in 10 ml of

fresh medium. G418, final concentration of 0.44 mg/ml, was added to the medium.

Selection of cells was continued until cell death stopped or diminished. The resulting

resistant clones were collected by trypsinization and replated again in medium containing

G418 (0.44 mg/ml). After further growth, the resistant cells were collected and frozen (-

80°C) in complete medium containing 5% dimethyl sulfoxide (DMSO).

RNA Isolation/qRT-PCR

Cells from each stable lines (SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP) were

washed in 5 ml cold 1x phosphate buffered saline (PBS). Another 5 ml 1x PBS were

added and the cells were removed with scraping. The cells were pelleted by

centrifugation. RNA was isolated from cell PELLETS using SV Total RNA Isolation

System (Promega, Z3105). The concentration and purity of the RNA was determined by

reading the samples at 260 and 280 nm. cDNA samples were generated from 1 μg of

isolated RNA using QuantiTect Reverse Transcription Kit (Qiagen, 205313).

Quantitative RT-PCR was conducted using Applied Biosystems Taqman primer-probes.

Custom primer-probes unique to Promoter A and Promoter B were used to detect

transcripts derived from each promoter (Katula et al, 2012). A commercial TaqMan

primer-probe to actin (Applied Biosystems, 4331182) served as an internal control for

standardization. Each reaction consisted of 1x TaqMan buffer, cDNA and specific

primer-probes for a total of 10 μl. Reactions were run in triplicate. Standard instrument

amplification conditions were used: 95°C for 15 seconds, 65°C for 1 min and 40 cycles.

Western Blot

Cell pellets were collected as previously described for RNA isolation. Cells were

then lysed using Cell Extraction Buffer (Invitrogen, FNN0011) containing Protease

Inhibitors (Roche, 11777700). Protein concentration was determined using Pierce 660 nm

Protein Assay Reagent (Thermo #2260). 20-50 μg of proteins was separated on a 10%

SDS–PAGE gel and transferred to a nitrocellulose filter paper. WNT5A was detected

using Abcam anti-rabbit WNT5A primary antibody (ab174100, Abcam). The amount of

primary antibody used was 6 ml of 6μl of primary antibody in 1 % milk. The secondary

antibody used was a goat anti-rabbit IgG horseradish peroxidase (HRP). The amount of

secondary antibody used was 6 ml of 1μl of secondary antibody in 1 % milk Detection

was done with SuperSignal West Pico Chemiluminescent Substrate (Thermo# 34080).

The bands were visualized on a Bio-Rad Chemi Doc System. The expected molecular

weight of WNT5A is 41 kDa.

Cell Proliferation Assay

Stable lines of SaOS-2 expressing WNT5A Iso A, Iso B and GFP were plated in

a 96-well plate at a density of 5 x 10^3 cells per well. Cells were assayed for proliferation

using CyQUANT ® NF Cell Proliferation Assay (Invitrogen) over a 4 day period. A

group of cells were selected on a specific day. The medium was removed from the cells

and 50 µl of 1x HBSS/CyQUANT NF Dye reagent mix was added per well. The cells were then incubated 30 min at 37°C. DNA florescence was recorded using a BioTeck Synergy 2 plate reader, measuring at 485 nm excitation, 530 nm emission for the selected wells. Control wells for background contain no cells. The levels of emitted fluorescence minus background were recorded for each day. One day after plating the cells was considered Day 0.

Although cells numbers should be equal on Day 0, this was not always the case. To standardize the cell lines, the percent change in cell number was plotted by dividing each day's fluorescence values by day 0 for that cell line. The standard error for each sample was determined for each day using replica samples.

Migration Assay

Cell mobility of stably transfected SaOS-2cells expressing WNT5A Isoform A, Isoform B, and GFP was assessed with a scratch/wound assay (O'Connell, 2008). Cells (1.4×10^6 /well) were plated into 6-well plate and grown to about 80–90% confluency. 24 hours later, the confluent cell monolayers were carefully "wounded". A 200 µl yellow plastic pipette, melted in a blue flame, was used to make five scratches vertical in each well. Each culture was washed twice with 1x Hank's balanced salt solution to remove cellular debris and fresh medium was then added. The wounded cell monolayers were photographed under a phase-contrast light microscope at 0, 24, 45, and 63 hours after. The movement of cells into the scratch area was quantified using Image Pro (Media Cybernetic) to measure the thickness (width) of the scratches at each time points.

The thickness of each scratch were then used to calculate the distance cells traveled from the starting point (0%) where the cells were initially after the scratch, to the endpoint (100%) where the cells have reach the middle of the scratch.

Invasion Assay

Invasiveness stably transfected SaOS-2cells expressing WNT5A Isoform A, Isoform B, and GFP were assayed using CytoSelect™ 24-Well Cell Migration and Invasion Assay (CELL BIOLABS, INC, # 100-C). Cells were suspended to 0.5-1.0 x 10^6 cells/ml in serum free media. A cell culture insert, containing a polycarbonate membrane (8 μm pore size), was placed into a well on a 24- well plate and warmed to room temperature for 10 minutes. The basement membrane of the upper chamber of the insert was then rehydrated with serum-free medium. 500 μl of media containing 15% fetal bovine serum was added to well itself, rehydrating the outer membrane. 300 μl of the sample cell suspension was then placed in the upper chamber, where the cells that are invasive can pass through the basement membrane. After a 12- 48 hour incubation, the non-invasive cells in the top chamber were then removed with a Q-tip, whereas the invasive cells that have passed through the membrane are stained with Cell Staining Solution. After staining, the cells were washed with dd H_2O and extracted using Cell Extraction Solution. 100 μL of each sample was transferred to a 96-well microtiter plate and the absorbance read at OD 560 nm measured using a BioTeck Synergy 2 plate reader.

Apoptosis Assay (Cell Viability Assay)

Stable lines of transfected SaOS-2cells expressing WNT5A Isoform A, Isoform B, and GFP were assayed for apoptosis using a doxorubicin (DOX)-induced apoptosis assay (Tsang, 2005). The cells were plated at a density of 3.5×10^4 into a 24-well plate. Each cell line was then divided into 3 groups based on the amount of DOX that was to be administered to each group (0 μM, 0.1μM, and 0.2 μM). The next day, the appropriate amount of DOX was administered to each group per well. After 3 days, the cells were then collected and counted for survivability. The percent of cells that survived was calculated based on the control, untreated as 100%.

CHAPTER III

RESULTS

Establishment of Stable Lines

As one approach to determine the functions of the WNT5A Isoforms A and
Isoform B, stable lines of SaOS-2 overexpressing A and B were generated. SaOS-2 cells
essentially do not express Isoform B, but express Isoform A at a slightly higher level than
normal osteoblasts (unpublished, this lab). Expression vectors for each isoform were
obtained (see Figure 4) and transfected into SaOS-2, along with the selection vector,
pMC1neopolyA. A control cell line was also made, transfected with a GFP expression
vector pRNATinH1.2/Hygro. Cells were selected in G418, as described in Materials and
Methods, and collected as a heterogeneous group.

The stable SaOS-2 cell lines were characterized to confirm that the expected
isoform RNA was being made and that the level of WNT5A protein was increased. RNA
was isolated from the three stable cell lines, SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2
GFP and analyzed by qPCR using custom Taqman primer-probes (Figure 5A). Results
show that in SaOS-2 Iso B, Isoform B mRNA expression was extremely high, in
comparison to control cells that showed little or no expression of Isoform B. The level of
Isoform A mRNA in SaOS-2 Iso B cells was nearly the same as in the control SaOS-2

GFP cell line, as expected. Isoform A expression in SaOS-2 Iso A cells was also high, in comparison to control cells, which express some Isoform A.

Protein lysates were prepared from the three stable lines, SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP, and analyzed by western blot. The antibody used will detect both isoforms, so it was not possible to determine if only the A or B isoform was being made. However, when compared to the GFP control cells, the amounts of WNT5A protein increased, with SaOS-2 Iso B having the highest amount (Figure 5B). WNT5A protein also increased in the SaOS-2 Iso A cells, but not to the same level as Iso B cells. This correlates with the detected RNA levels (Figure 5A).
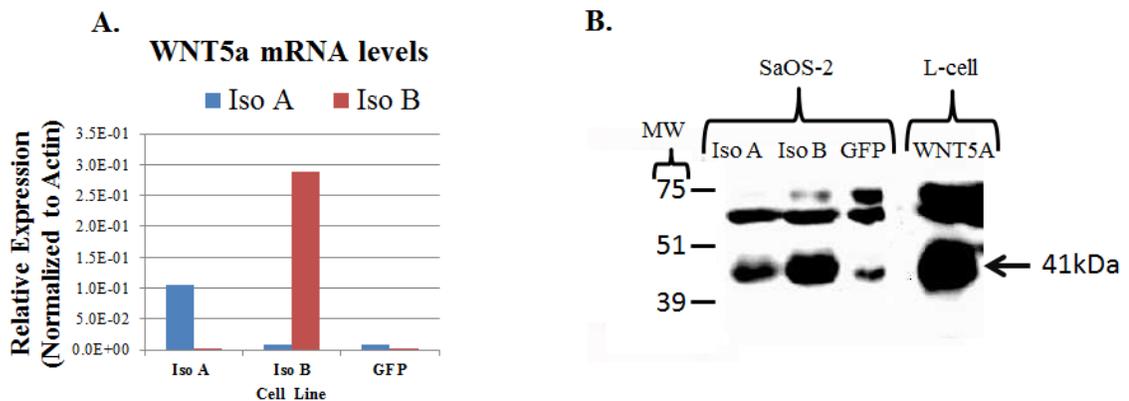
Figure 5. Expression of WNT5A mRNA and Protein in SaOS-2 Stable Lines. A. WNT5A Isoform A and Isoform B transcript levels were quantified in the SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP cell lines by qPCR. Transcript levels were normalized relative to actin levels transcript. **B.** WNT5A protein in the same cell lines were characterized by immunoblot. 100 μg of total cell lysate was loaded per lane. The arrow indicates the position of the WNT5A protein at approximately 41 kDa. An unknown non-specific band is always detected at 72 kDa. The L-cell WNT5A is a purchased mouse cell line, expressing the WNT5A Isoform A, as a positive control.

No Distinction between the Two Isoform A and Isoform B in Proliferation

There is evidence that WNT5A Isoform A inhibits proliferation, whereas Isoform B increases proliferation (Bauer et al., 2013). We wanted to know if over expression of Isoform A and Isoform B had the same effect in SaOS-2 cells. This might not be the case, since the expression of Isoform B is decreased in SaOS-2. This suggests that Isoform B acts as a tumor suppressor. In the cancer types Bauer et al. used in their study, Isoform B was expressed and in one cancer cell line, at higher levels than Isoform A.

In this experiment, each cell line (Iso A, Iso B, and GFP) were plated at an equal number in a 96 well plate. The next day (Day1), the cell number was quantified by measuring DNA content with a fluorescent DNA binding dye. Then DNA content was quantified for the next three days (2, 3, and 4). Result showed there was no distinction in cell proliferation between three SaOS-2 cell lines (Figure 6). These data contradicts that Bauer et al (2013). The assay was performed multiple times. Although there were some variations, from assay to assay, for example, the reduction at Day 3 for GFP and Isoform A was inconsistent; the results indicated no efforts of increased Isoform A or B on SaOS-2 proliferation.
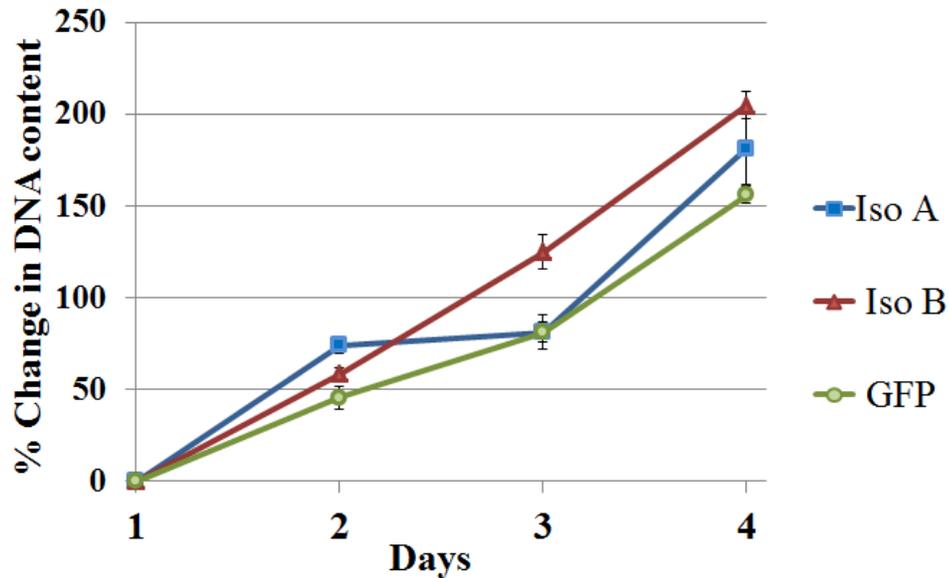
Figure 6. WNT5A Isoforms Effects on Cell Proliferation. The cell lines SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP were assayed for increase in DNA content, as a measure of cell number over a three day period. Change in DNA content was expressed as a percent of increase from Day 1. Error bars represent standard error with n=5.

Isoform A Promotes Greater Cell Migration

We then decided to test the effect of increased Isoform A and Isoform B expression on cell migration. Our hypothesis was that SaOS-2 cells expressing increased Isoform B will migrate and travel at a slower rate than the SaOS-2 cells not expressing isoform B. This is based on the fact that Isoform B is not expressed in SaOS-2 cells. Our stable lines, SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP, were assayed for migration using the scratch-wound assay over a 63 hour period. Each scratch was photographed at four time intervals, 0, 24, 45, and 63 hours post scratch (Figure 7A). Our results show that SaOS-2 cells expressing more Isoform A traveled a greater distance per unit time, in comparison to cells expressing Isoform B and the control cells with less Isoform A over

three time intervals (Figure 7B). This assay was repeated once with similar results. This correlates with both SaOS-2 Iso B cells and the control SaOS-2 cells having roughly the same levels of Isoform A transcripts. These data indicate that Isoform B does not play a role in cell migration, but that Isoform A enhances migration as previously shown (Enomoto et al., 2009).
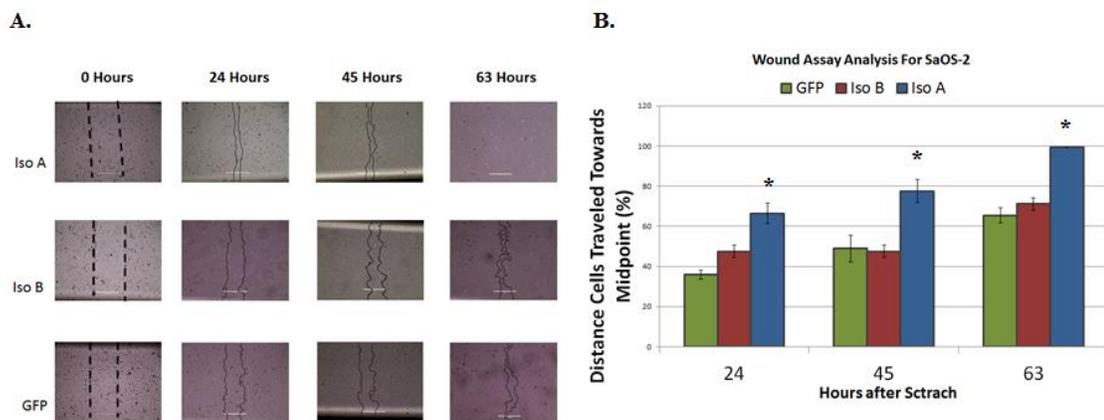
Figure 7. Effects of Increased WNT5A Isoforms A and B Expression on Cell Migration
A. Vertical scratches were created (0 hours) and photographed under phase-contrast microscopy. The added lines outline the edges of the scratch or migrating cells. **B.** The distance the cells traveled from the starting point (edge of the scratch at time 0 hrs) to the midpoint at each time point was measured. The percent distance traveled was graphed. Error bars represent standard error with n=10. * $p < 0.005$ comparing Iso A to GFP.

Increased Isoform B Expression Decreases Cell Invasion

Next, we assayed the expressing SaOS-2 cells to determine the effect of increased Isoform A and B had on cell invasiveness. Our hypothesis was that SaOS-2, cells expressing Isoform B would be less invasive than the SaOS-2 cells, expressing little or no Isoform B. The transfected cells were assayed using a commercial cell invasion assay. The assay uses inserts, each with a coated basement membrane, where only invasive cells can pass through.

For this experiment, the cells were hypothetically plated at an equal number in each insert, where the invasive cells would pass though the basement membrane over a 48 hour period. Unfortunately, it proved difficult to plate the cell lines at equal numbers, resulting in one cell line having more cells than the others. Moreover, it was not possible to quantify cell numbers after plating into the inserts. Our approach was to plate a presumed equal amount of cells, using the same cell stocks as for the invasion assay, in 96-well plates at the same time the cells are plated for the invasion assay. The next day, the DNA content of the cells in the 96-well plates were quantified. This value was used to normalize the results of the invasion assay. After the 48 hours, the cells that had invaded the insert membrane were stained, extracted and the extracts read at OD560 nm. These values were normalized for "cell number plated" based on DNA content, as just described. The OD560 /unit DNA serves to quantify the number of invasive cells.

The results suggest that SaOS-2 cells expressing more Isoform B were less invasive, in comparison, to those expressing little to no Isoform B, although significance

could not be determined (Figure 8). SaOS-2 Iso B cells had 1.45x less the amount of cells

invading the insert compare to that of the control cells. There was also a slight difference

between SaOS-2 Iso A and GFP cells, with SaOS-2 Iso A cells having 1.19x less invasive

cells than SaOS-2 GFP cells. This assay will obviously have to be repeated in order to

confirm these results. Our current data supports our hypothesis that cells expressing

Isoform B would be less invasive than the cells expressing little to no Isoform B.
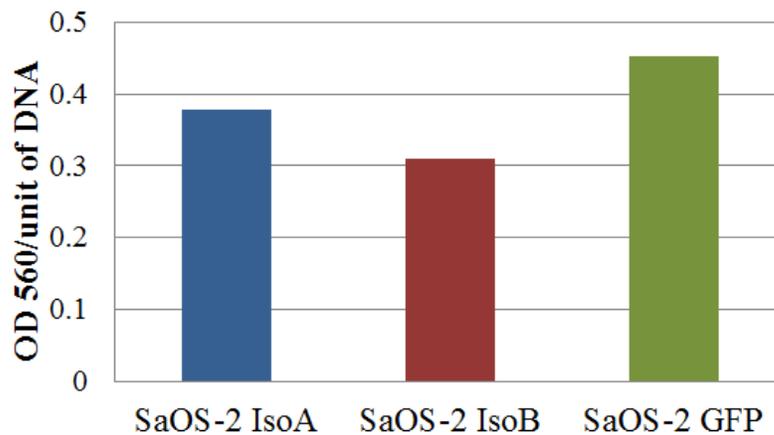
Figure 8. Effects of Increased WNT5A Isoforms Expression on Cell Invasiveness.

The cell lines SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP were used in a cell invasion assay. Invasive cells were stained, extracted, and the extracts read at OD560. Values were standardized to cell number plated based on DNA content as described. Average of two determinates are shown.

Increase of Isoform A Provides some Resistance against Drug-Induced Apoptosis

Transfected SaOS-2 cells expressing increased Isoform A and Isoform B were assayed for cell viability undergoing apoptosis induced by a pharmaceutical drug known as doxorubicin (DOX). Our hypothesis was that SaOS-2 cells expressing increased Isoform B would be more susceptible to apoptosis, and would have fewer living cells than SaOS-2 cells expressing little to no Isoform B when induced by DOX.

Our stable lines (SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP) were plated and divided into three groups between each cell line. Each group was treated with a specific concentration of DOX, 0 μM, 0.1μM, and 0.2 μM, based on a previous study (Tsang et al., 2005). After three days, the number of cells in each group was counted, as a measure of survivability. Our results showed that SaOS-2 cells with increase Isoform A expression are more resistant to DOX-induced cell death (Figure 9). SaOS-2 Iso B cells had a similar survivability percentage to that of SaOS-2 GFP cells (8.4% vs 8.9%, respectively). These data indicate that Isoform B does not influence cell death, but that increased Isoform A makes cells more resistance to apoptosis. Further assays will be performed to determine if increased Isoform A alters the the signaling pathway for apoptosis, such as caspase activity or annexin A5 affinity.
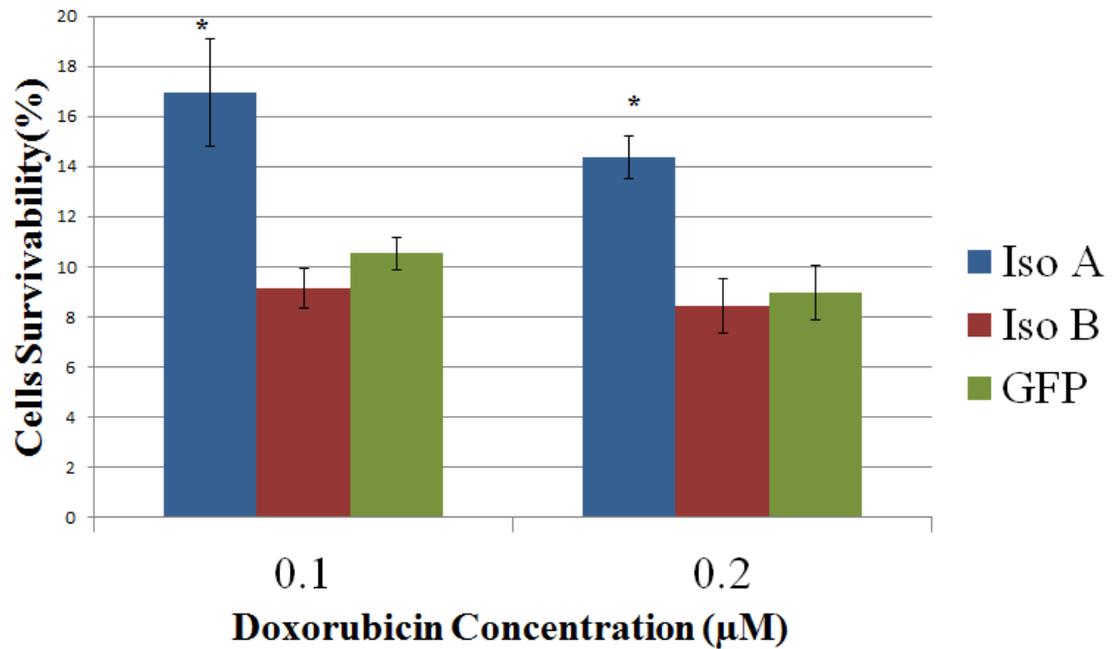
Figure 9. Effects of Increased WNT5A Isoform A and B Expression on Cell Survivability after Treatment with Doxorubicin. The cell lines SaOS-2 Iso A, SaOS-2 Iso B, and SaOS-2 GFP were treated for three days with doxorubicin to induce apoptosis. The percent of surviving cells relative to the untreated cells was determined. Cell Survivability serves to quantify the number of cells that did not undergo apoptosis. Error bars represent standard error with n=4. * $p<0.005$, ** $p<0.01$ comparing Iso A to GFP.

CHAPTER IV

DISCUSSION

Summary of Major Findings

In this study, the functional distinction between the WNT5A isoforms A and B were analyzed. Stable lines of SaOS-2 cells were generated to have an increased WNT5A protein Isoform A and Isoform B expression. The cells were characterized through qPCR and immunoblott to confirm that Isoform A and Isoform B mRNA and protein expression were higher than the control cells, transfected with GFP expression vector. These cells were assayed to observe the protein isoforms effects on cell proliferation, migration, invasion, and apoptosis. Our results showed that Isoform B in SaOS-2 has no effect on proliferation, migration, and apoptosis, with a slight inhibitory effect on invasiveness. An increase of Isoform A expression simulated migration, whereas an increase of Isoform B expression had no effect on migration. Similarly, an increase in Isoform A expression made cells more resistance to cell death due to drug treatment, whereas increased Isoform B expression had no effect. Increased Isoform B and Isoform A expression had a small effect on invasion, although more assays will need to be conducted to verify these findings. These results suggest that the WNT5A protein isoforms distinctively stimulate the non-canonical WNT pathways.

Proliferation in SaOS-2 cells is not affected by either WNT5A Isoform Protein

A previous study showed that WNT5A isoforms A and B had different effects on proliferation (Bauer et al., 2013). The effects of the WNT5A-L (Isoform A) and WNT5A-S (Isoform B) were analyzed in three cancer cell lines, MDA-MB-231 (breast carcinoma), HeLa (cervical carcinoma), and SH-SY5Y (neuroblasoma). All three cell lines expressed both isoforms, but only in HeLa is WNT5A-S (Isoform B) expressed at a higher level than WNT5A-L (Isoform A). In the experiment, each cell line was transfected with an expression vector containing cDNA for one of the isoforms and selected for six days. The cells were then plated and counted at three time intervals over a six day period. The results showed that increased WNT5A-S (Isoform B) expression in each cell line stimulates proliferation, whereas increase WNT5A-L (Isoform A) expression suppresses proliferation. This contradicts our data, where increased Isoform A or Isoform B expression showed no distinction from the control cell line, SaOS-2 GFP (Figure 6). This indicates that the pathway controlling proliferation is independent from the WNT5A non-canonical pathways in SaOS-2. It was expected that Isoform B would have acted in an inhibitory fashion in SaOS-2 cells, as the gene promoter for Isoform B is inactivated and little or no Isoform B is expressed. It is also possible that the WNT5A Isoform B doesn't have an effect on SaOS-2 cells due to the lack of a receptor for Isoform B in SaOS-2 cells. We also expected that Isoform A would stimulate proliferation, as it is active in SaOS-2 cells. In comparing our results to Bauer et al (2013), they support the general notion that WNT5A has distinct activities in *different* cell cancer types.

Stimulation of Migration by Increased WNT5A Isoform A

The overexpression of WNT5A Isoform A was shown to stimulate migration in SaOS-2 cells. The overexpression of Isoform B, however had no effect on migration compared to the control. This suggests that Isoform B does not play a role in cell migration. These results differ from that of our expectations of Isoform B, where Isoform B would inhibit migration, in comparison to the control. WNT5A Isoform A is known to function in cell migration. Considering the opposite effects Bauer et al (2013) observed on proliferation for the isoforms and the lack of Isoforms B expression in SaOS-2 cells, it seemed a reasonable hypothesis that isoform B would have a negative effect on migration.

Our results for Isoform A are consistent with other published results. In one study, the role of WNT5A/Ror2 signaling on cell migration was investigated (Nishita et al., 2010). They used various cell types to show that the Receptor Tyrosine Kinase (RTK) Ror2 is essential for WNT5A induction of migration and that Ror2 is mediating actin reorganization. This would support the idea that Isoform A is binding to Ror2 in osteosarcoma. Another studied looked at WNT5A role in PI3 Kinase/AKT signaling (A. Zhang et al., 2014). In that study, Isoform A was shown to stimulate migration by enhancing phosphorylation of phosphatidylinositol-3 kinase (PI3K)/AKT. They treated osteosarcoma MG-63 cells with different doses (0, 50, 100, and 200 ng/ml) of recombinant Wnt5a (rWnt5a), and assay for the migration rate via wound healing assays and Boyden chamber assays. Results from the assays showed that WNT5A stimulation

increases migration. They then ran an immunoblot for PI3K and AKT phosphorylation on MG-63 cells that were treated with 100 ng/ml of WNT5A.They showed activation of both PI3K and AKT at 15 min post treatment. Since only Isoform A and not Isoform B affected cell migration in our assay, we conclude, based on the Zhang et al (2014) findings, that Isoform A is stimulating a non-canonical pathway leading to PI3K and AKT phosphorylation and that Isoform B is not activating this same pathway. Either, as previously mentioned, there are no receptors for Isoform B to mediate PI3K and AKT phosphorylation or instead Isoform B activates a non-canonical pathway not linked to cell migration.

The Role of WNT5A Isoforms in Cell Invasiveness is still Inconclusive

A previous study showed that WNT5A/Ror2 signaling plays a role in the regulation of cell invasiveness in SaOS-2 cells and U2OS cells, another osteosarcoma cell line (Enomoto et al., 2009). They transfected both SaOS-2 and U2OS cells with siRNA (control siRNA, Ror2 siRNA, or WNT5A siRNA) and assayed them for cell invasiveness using a matrigel consisting of extracellular matrixes (ECM). The invasive cells were stained and counted. Results showed that cells transfected with either Ror2 siRNA, or WNT5A siRNA were less invasive. They also ran an immunoblot for matrix metalloproteinase 13 (MMP-13), a protein upregulated in metastatic cells. They found that MMP-13 levels were decreased in the absence of WNT5A/Ror2 signaling. Overall, the study showed SaOS-2 and U2OS cells displayed invasive like behavior by activating WNT5A/Ror2 signaling. This further supports the notion that Isoform A is affecting a

non-canonical pathway involving the Ror2 receptor. However, our results indicate that Isoform B may have a role on invasiveness. SaOS-2 cells that overexpressed Isoform B were the least invasive, compared to that of SaOS-2 cells that overexpressed Isoform A and the control GFP. Also, compared to the control, cells that overexpressed Isoform A were somewhat less invasive, which is contrary to the result of Enomoto et al (2009).

However, it was not possible to determine the significance of these results as only a limited number of trials were conducted. This assay would need to be repeated to statistically confirm the role of WNT5A isoforms on invasiveness. Also, the assay was difficult to perform due to problems with plating the cells at the same cell number. As described, we standardized cell number based on DNA content using cells plated in separate wells. An alternative approach would be to use condition medium expressing the WNT5A isoforms to treat the SaOS-2 cells. However, this approach has its own problems, particularly in standardizing the amount of active isoform protein in the conditioned medium. Also, it should be mentioned that the cell assay plates are expensive (12 inserts costing approximately $400), limiting the number of replicas.

Role of WNT5A in Apoptosis Resistance

A recent study looked into how WNT5A plays a role in melanoma cells resisting a therapeutic drug known as BRAF inhibitors (BRAFis) (Anastas et al., 2014). They transfected 2 BRAFis resistant melanoma cell lines (A375 and MEL624), with siRNA to silence either WNT5A or BRAF expression. Results showed that cells transfected WNT5A siRNA showed little resistance to cell death when treated with BRAFis. This result suggest that WNT5A has a role in resistance to appoptosis. We based our experiment on another study showing that SaOS-2 cells were susceptible to apoptosis in the presence of doxorubicin (Tsang et al., 2005). In that study, SaOS-2 cells were treated with different concentrations of doxorubicin. They determined that 0.1 µM of doxorubicin induced DNA degradation after 72 hours of treatment. We based our assay on these findings. As cancer cells are known to avoid apoptosis, we decided to assay for cell survivability, as cell death is an endpoint for apoptosis. This was a cost cutting move, compared to the use of other assays measuring apoptosis. Initially, we wanted to determine if there was any reason to extend our analysis of apoptosis to these more precise assays. Our results showed increased Isoform A expression lead to a higher cell survivability percentage, in comparison to that of increased Isoform B expression and the control. This is not an unexpected result as increased WNT5A would be expected to increase survivability. Isoform B showed little to no effect on cell survivability. This is an unexpected result. If Isoform B is functioning as a tumor suppressor, then the expressing protein should sensitize the cells to apoptosis, resulting in a decrease in survivability, relative to the control.

Conclusion and Future Studies

Our results suggest the WNT5A isoforms A and B are functionally distinct. We provided evidence that Isoform B decreases invasion, whereas it had no effect on proliferation, migration, or apoptosis in SaOS-2. In contrast, Isoform A, as previously stated, appears to increase migration and survivability to drug induced apoptosis. The differences in response are likely due to stimulation of different non-canonical signaling pathways. To confirm these results, it will be necessary to repeat the invasion assay.

To further our study of the functional distinctions of the WNT5A protein isoforms on cell migration, invasion, and apoptosis, we would like to use HCT-116, a colorectal cancer cell line, as a new template to assay for these characteristics. Both Isoform A and Isoform B are not expressed in HCT-116. In fact, two attempts to generate these lines were unsuccessful for different reasons. This makes it a better candidate than SaOS-2, where Isoform A is expressed, but Isoform B is not. The further analysis of SaOS-2 cells on apoptosis and invasiveness will be conducted as well. Another approach to understanding the functional distinction between the two isoforms would be the use of condition medium on SaOS-2 cells. As WNT5A is a secreted protein ligand, the cells would be treated with condition medium enriched with one of the WNT5A isoforms. Our data suggest that Isoform B is signaling through a non-canonical pathway that is different from Isoform A. An approach to investigate this question would be to analyze each of the downstream signaling proteins of PCP/CE and Ca^{2+} pathways in SaOS-2 cells stimulated by either WNT5A Isoform A or Isoform B, such as MMP-13. It could also be possible

that Isoform B plays a role in cell development and differentiation. If this is so, a different experiment system would be required, such as mouse embryos.

Also the role of WNT5A Isoform A and Isoform B in the stimulation of the PI3K/AKT signaling pathway is also another pathway to be studied, as both migration and apoptosis resistance are influenced by it. In the study done by Anastas et al (2014), PI3K and AKT protein levels were used as a marker, when assaying melanoma cells for apoptosis resistance. Zhang et al (2014) also used PI3K and AKT as markers when studying WNT5A's role on migration This could indicate that WNT5A Isoform A binds to Ror2, initiating PI3K/AKT signaling, leading to migration and apoptosis resistance (Figure 10).

We have assayed the role of WNT5A protein Isoforms on apoptosis, but we mainly focused on the endpoint. In future studies, we would like to look at other aspects along the apoptotic pathway to have a further understanding on how the WNT5A Isoforms affect earlier apoptotic markers. Based on what is known about Isoform A, it will be important to analyze the levels of activated AKT and phosphorylated Bad in SaOS-2 cells. Also, annexin A5 affinity assay could be used to confirm that the cells are actually undergoing apoptosis. These methods could give us a better understanding on how WNT5A is affecting apoptosis.
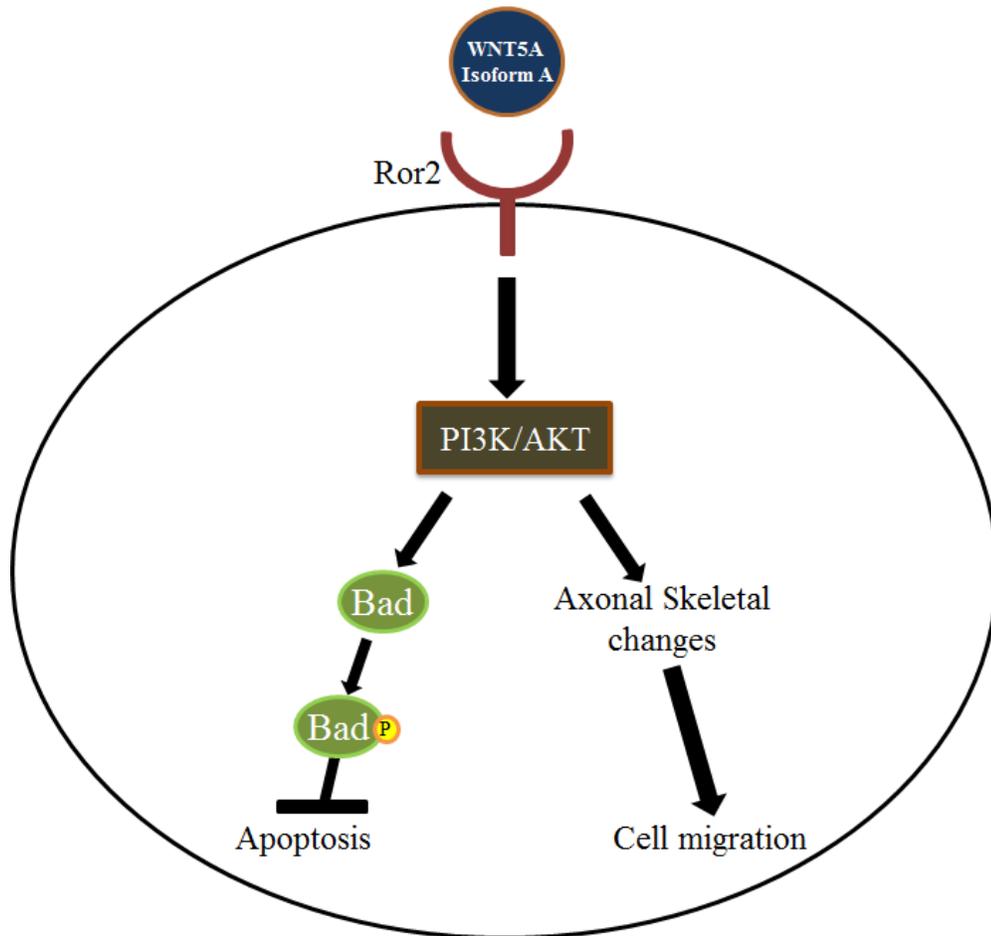
Figure 10. Model for PI3K/AKT Stimulated by WNT5A Isoform A/Ror2.

Both apoptosis and cell migration are influence by PI3K/AKT signaling due to WNT5A Isoform A. PI3K/AKT signaling leads to the phosphorylation of Bad, a member of the Bcl-2 family, rendering it inactive and preventing apoptosis. The axonal skeleton is influenced by PI3K/AKT signaling, thus influencing cell movement.

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