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WNT5A is a secreted protein ligand with important roles in development, adult tissue homeostasis, and many basic cellular functions. In cancers, aberrant WNT5A signaling is often observed. This study focused on potential functional differences between two isoforms of this protein, on potential signaling differences between these two isoforms, and on differential expression of these isoforms in the context of mouse embryonic development. This study found that these isoforms both had no effect on either cellular migration or invasion in HCT116, contrary to reports published in the literature. This study also found that both these isoforms had no detectable effect on activation of terminal effector molecules in the PCP/CE pathway or in the Wnt/Ca^{2+} pathway. In a murine model of embryonic development, we observed that WNT5A isoforms were differentially regulated. Overall, the findings of this study suggest that WNT5A encodes functionally distinct protein isoforms which may play a role in enabling the orchestration of a complex series of biological events during embryonic development.

FUNCTIONAL DISTINCTIONS BETWEEN

TWO ISOFORMS OF WNT5A

by

Carl J. Manner III

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the Faculty of The Graduate School at
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Approved by

_____
Committee Chair

To my family and friends, for their love, support and understanding in trying times, and to Penny, Cynthia, and Mary.

APPROVAL PAGE

This thesis written by Carl J. Manner III 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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CHAPTER I

INTRODUCTION

Statement of Problem

In the United States, approximately one third of women and one half of men will develop cancer at some point in their lifetime (American Cancer Society, 2014). The term 'cancer' encompasses many diseases, unified by a common cause – the abnormal growth of cells. These cells proliferate uncontrollably and indefinitely and acquire other "hallmarks of cancer", including tissue invasion and metastasis. The root cause of these acquired traits is a variety of genetic and non-genetic changes in a single parental cell. Misregulation of the signaling protein WNT5A has been shown to play a role in well over a dozen different cancers including colorectal, breast, osteosarcoma and pancreatic. In some cancers, WNT5A acts as an oncogene, while in others, it appears to act as a tumor suppressor. Significantly, overexpression of WNT5A has been associated with tumor metastasis. WNT5A also plays critical roles in embryonic development and adult tissue homeostasis. Importantly, WNT5A is required for gastrulation, limb formation, and directional extension of the anterior-posterior growth axis – all, like metastasis, processes of cell movement. The *WNT5A* gene codes for multiple protein isoforms, two of which have been shown to be active and may be functionally distinct. The mechanism by which any functional distinction is achieved, however, is not understood. These isoforms are highly conserved among vertebrates, but in the absence of data pointing to a strong functional distinction between these isoforms, the reason for this evolutionary conservation is unclear. This study sought to elucidate what, if any, functional differences there might be between isoforms of

WNT5A in a cancer model system, whether the isoforms might be differentially expressed in a model of embryonic development, and through which of the Wnt signaling pathways the putative functional differences between isoforms might be operating. Answers to any one of these questions could point to a potential reason for the evolutionary conservation of WNT5A protein isoforms.

WNT5A Gene Structure and Protein Isoforms

The *WNT5A* gene is located on chromosome 3 from 55,499,743-55,521,670 bp. It is composed of 5 exons. The *WNT5A* gene generates five distinct transcripts which are protein coding (Ensembl: ENS600000114251). This study is focused on the proteins generated from two of these transcripts (Ensembl: ENST00000497027 and ENST00000264634). The primary transcripts for these proteins are derived from unique transcription start sites, each with distinct promoters (A and B; Figure 1). The promoter A primary transcript includes a unique Exon 1, whereas the Promoter B transcript includes Exon 1β. Exon 1β sequences are found in the first intron of the Promoter A transcript but are spliced out. Exons 2, 3, 4, and 5 are common to both transcripts.

These transcripts are translated into two protein isoforms – termed isoform A and isoform B, which differ in length by 15 amino acids at the N-terminus (Figure 2). Post-processing, the isoforms differ in length by 18 amino acids at the N-terminus (Bauer, Bénard, Gaasterland, Willert, & Cappellen, 2013). Isoform B is essentially a truncated form of isoform A (Figure 2).

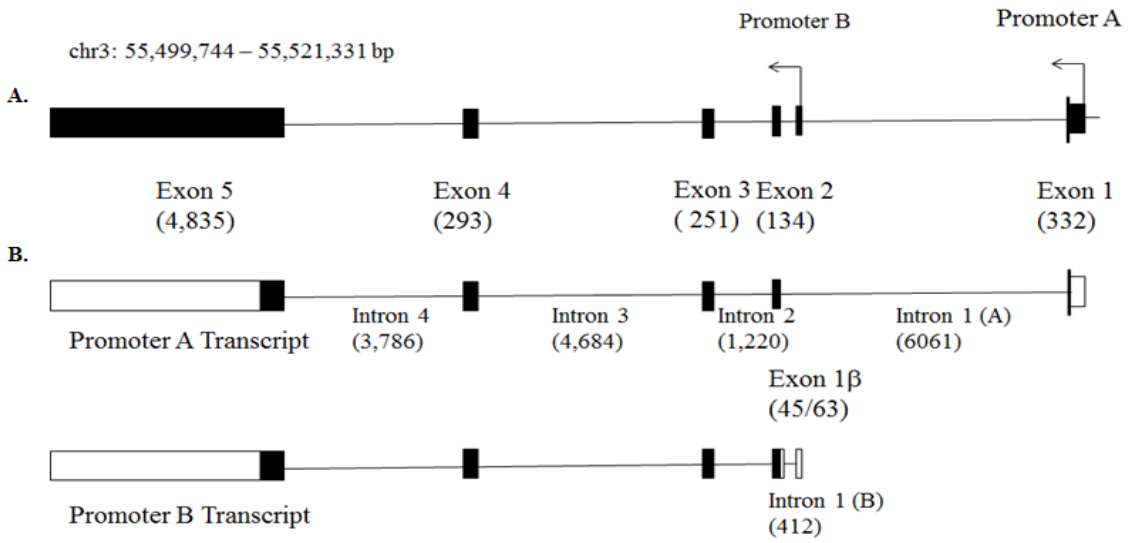


Figure 1. *WNT5A* Gene Structure. (A) *WNT5A* is located on chromosome 3. Promoters A and B are shown. Black boxes represent exons, lines represent introns, and arrows represent transcription start sites. **(B)** Promoter A and Promoter B primary transcripts. Black boxes are coding regions; open boxes are untranslated regions. Intron and exon lengths are indicated in parentheses. Exon 1 is unique to Promoter A, whereas Exon 1β is unique to Promoter B. This diagram is based on the Ensembl EN5600000114251, Bauer et al., 2013, and Vaidya et al.

WNT5A isoforms A and B are both secreted ligands affecting the Wnt signaling pathways. These proteins are globular, α-helix rich, and highly hydrophobic (Figure 3). They are glycosylated, palmitoylated and cleaved prior to secretion (Bauer et al., 2013; Kurayoshi, Yamamoto, Izumi, & Kikuchi, 2007). WNT5A is palmitoylated at Cys^{104} and glycosylated at Asn^{114}, Asn^{120}, Asn^{311}, and Asn^{325}; palmitoylation is required for binding of Frizzled family receptors, and glycosylation is required for secretion (Kurayoshi et al., 2007). As of yet, no solved tertiary structures exist for WNT5A, and *in silico* structures for WNT5A isoforms A and B are uninformative with respect to the differences between isoforms.

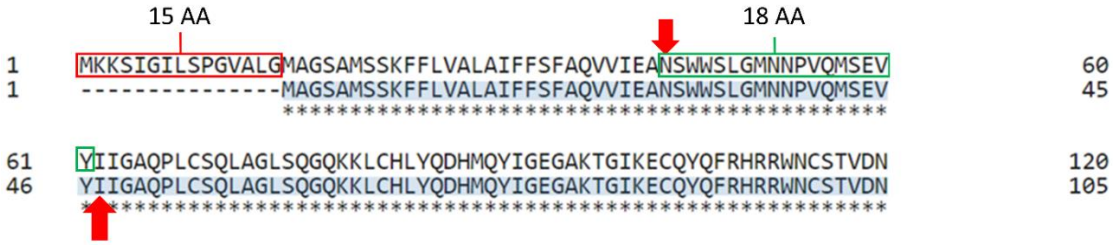


Figure 2. Amino Acid Sequence Alignment for Isoforms A and B of WNT5A. Before processing, isoform A is 15 amino acids longer than isoform B at its N-terminus (red box). Isoform B sequence is highlighted in blue. For each isoform, the N-terminal residue post-processing is indicated with a red arrow. The N-terminal sequence for isoform A post-processing is NSWWSLGM…, while the N-terminal sequence for isoform B post-processing is IIGAQPLC… (Bauer et al., 2013). The mature proteins differ in length by 18 amino acids (green box). Isoform A is 380 amino acids before processing and 337 amino acids after processing. Isoform B is 365 amino acids before processing and 319 amino acids after processing. The omitted C-terminal sequence is identical for both isoforms.

WNT5A and Cell Signaling

Wnt proteins have historically been divided into two subtypes: the canonical Wnt ligands, which signal through a well characterized β-catenin dependent (canonical) pathway, and the non-canonical Wnt ligands, which signal through a variety of other, less characterized pathways. WNT5A is the paradigmatic non-canonical Wnt ligand, though it has been demonstrated that given an appropriate – and arguably artificial – receptor context, WNT5A can also stimulate the canonical pathway (Mikels & Nusse, 2006; van Amerongen, Fuerer, Mizutani, & Nusse, 2012).

Figure 3. WNT5A Isoform B *in silico* Protein Structure. WNT5A is globular, predominantly α-helical, and highly hydrophobic. The putative binding domain lies in the crevice between the two largest pairs of β-pleated sheet, top center. The N-terminus is marked in this figure with a blue arrow. An 18 amino acid extension at this N-terminus would account for any observed differences in function between the isoforms. Post translational modifications are not shown in this model, but are factored into structure prediction. The model for isoform B is identical to that for isoform A; the prediction algorithms used are incapable of modeling the extra N-terminal residues, as no suitable homologies exist in presently solved structures. Structure generated by RaptorX (Ma, Peng, Wang, & Xu, 2012; Ma, Wang, Zhao, & Xu, 2013; Peng & Xu, 2009, 2010, 2011a, 2011b).

Canonical Wnt Signaling

Canonical Wnt signaling involves a single central pathway that terminates in the translocation of β-catenin from the cytosol to the nucleus, where it acts as a transcription factor. In the absence of activating canonical Wnt ligands, a β-catenin destruction complex consisting of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), protein phosphatase 2A (PP2A), Axin, and WTX (Wilms tumor gene on X chromosome) associates with β-catenin, and recruits Casein kinase 1 (CK1) and GSK-3β which phosphorylate β-catenin at Ser45, Ser33,

Ser37, (CK1) and Thr41(GSK-3β) (C. Liu et al., 2015). Phosphorylated β-catenin then dissociates from the destruction complex and is polyubiquitinated by β-TrCP (F-box/WD repeat-containing protein). Polyubiquitination signals β-catenin for proteasomal degradation (Aberle, Bauer, Stappert, Kispert, & Kemler, 1997).

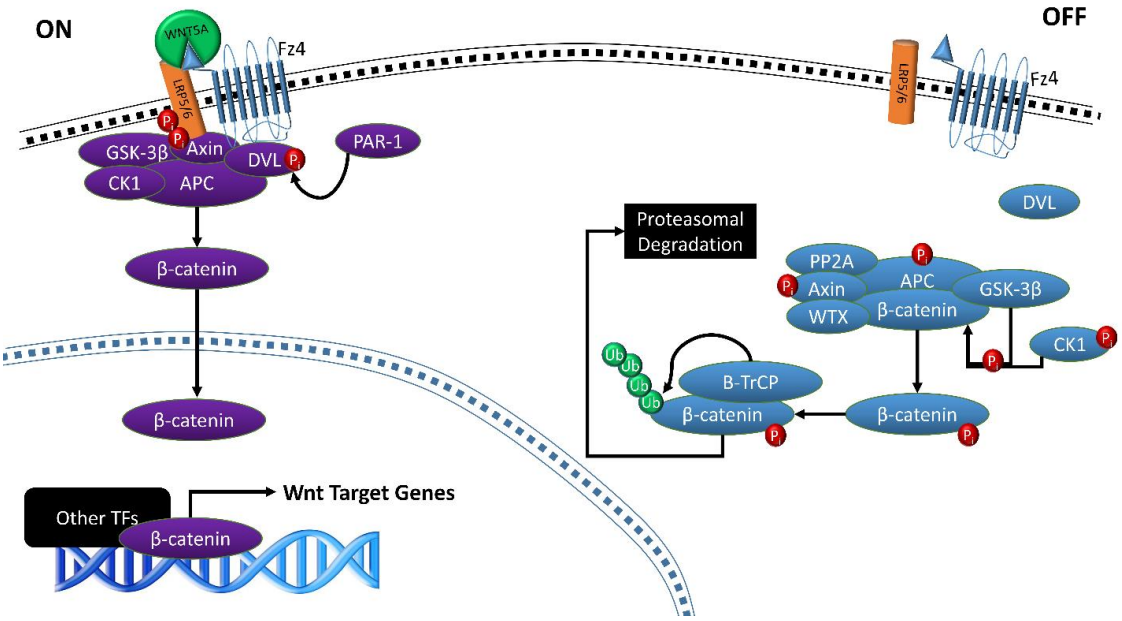


Figure 4. Canonical Wnt Signaling Pathway. Left side (purple), canonical signaling in the "on" state results in the translocation of β-catenin to the nucleus. Right side (blue), canonical signaling in the "off" state results in the degradation of β-catenin. Red circles represent phosphate groups. Green circles represent ubiquitin.

When a canonical Wnt ligand binds to a frizzled receptor, disheveled (Dvl) is phosphorylated by PAR-1 (Protease-activated receptor 1), and the destruction complex associates with Dvl (Sun et al., 2001). The Dvl in the complex binds the frizzled receptor, and GSK-3β and Axin bind and phosphorylate the Wnt coreceptor LRP5/6 (Wong et al., 2003). The destruction complex then dissociates, causing the release and stabilization of β-catenin, which builds up in the cytosol and translocates to the nucleus, where it acts as a transcription factor (Cong &

Varmus, 2004; Henderson & Fagotto, 2002; Wu et al., 2008). There, it associates with other transcription factors and activates transcription of multiple Wnt target genes (*c-myc, c-jun, cycD,* etc.), many of which are associated with embryonic development and disease (Mann et al., 1999). There are multiple Wnt ligands capable of activating the canonical Wnt signaling pathway; WNT5A in particular has been shown to activate canonical signaling pathways in the presence of Fz4 and LRP5 (Mikels & Nusse, 2006).

Non-Canonical Wnt/JNK Signaling

Non-canonical Wnt signaling encompasses several distinct Wnt signaling pathways, all of which begin with the binding of a Wnt ligand to its Fzd receptor, and none of which utilize β-catenin as a downstream effector. The most thoroughly characterized of these pathways is the Wnt/JNK signaling pathway, also referred to as the planar cell polarity/cell elongation pathway (PCP/CE).

Wnt/JNK signaling is initiated by the binding of a Wnt ligand – typically *WNT5A, WNT5B,* or *WNT11* – to its receptor-coreceptor pair. FZD3 or FZD6, and ROR1, ROR2 or PTK7 coreceptors are most commonly associated with Wnt/JNK signaling (Katoh & Katoh, 2005a, 2005b; Kirikoshi et al., 2000; Lu et al., 2004; Tokuhara, Hirai, Atomi, Terada, & Katoh, 1998). Binding of the ligand/receptor/coreceptor pair results in signal transduction through Dvl1/2/3 to Rac1. Rac1 initiates a MAPK signaling cascade that results in the activation of JNK, which influences cell survival and gene expression. Concurrently, Dvl1/2/3 activates DAAM1/2, which activates RhoA (Habas, Kato, & He, 2001). RhoA activates ROCK, which influences cytoskeletal rearrangements (Dyberg et al., 2014).

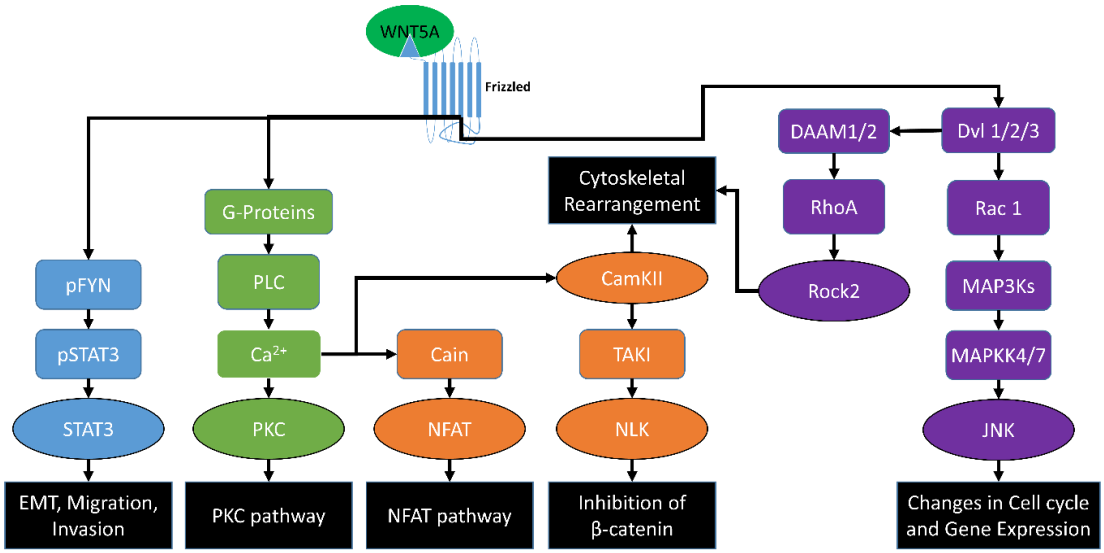


Figure 5. Non-Canonical Wnt Signaling Pathways. Wnt/STAT3 pathway is depicted in blue. Wnt/PKC pathway is depicted in green. Other Wnt/Ca^{2+} pathways are shown in orange. PCP/CE pathway is shown in purple. Cellular responses are shown in black. Signal transduction components are rounded rectangles, effector molecules are ovals, and black boxes are cellular responses.

Non-Canonical Wnt/PKC Signaling

Wnt/PKC signaling is a non-canonical Wnt signaling pathway that affects cellular responses through PKC (Protein Kinase C). This pathway is part of a larger Wnt signaling pathway referred to as the Wnt/Ca^{2+} pathway. In this pathway, a Wnt ligand binds Fz, which transduces a signaling cascade through associated heterotrimeric GTP-binding proteins, as evidenced by pertussis toxin (PTX) sensitivity (Wang & Malbon, 2004). These GTP binding proteins activate phospholipase C (PLC) and phosphodiesterases (PDEs) which causes the release of intracellular calcium (Slusarski, Corces, & Moon, 1997). Intracellular calcium release

activates PKC (Sheldahl, Park, Malbon, & Moon, 2015), the activity of which directly affects invasion in melanomas (Weeraratna et al., 2002).

Non-Canonical Wnt/STAT3 Signaling

The non-canonical Wnt/STAT3 pathway is the most recently discovered Wnt signaling pathway. In this pathway, a non-canonical Wnt ligand – such as WNT5A or WNT5B – binds Fzd2, which transduces a signal through pFyn (a sarc family kinase) and pSTAT3. STAT3 (signal transducer and activator of transcription 3) is a transcription factor that is the final effector of the cellular response in this pathway, and triggers metastasis, cell migration, and epithelial to mesenchymal transition (EMT) (Gujral et al., 2014).

WNT5A and Development

WNT5A is required for a host of developmental processes, including the outgrowth of limbs, face, ears and genitals, (Yamaguchi, Bradley, Mcmahon, & Jones, 1999) female reproductive tract development, (Mericskay, Kitajewski, & Sassoon, 2004) directional extension of the palate and other craniofacial features along the anterior-posterior axis, (He et al., 2008; Lin et al., 2011; H.-X. Liu et al., 2012; Ruest, Xiang, Lim, Levi, & Clouthier, 2004; Xiao, Zhu, Nagatsuka, Gunduz, & Li, 2006) chondrocyte differentiation and bone development (Church, 2002; Hosseini-Farahabadi et al., 2013; Hu et al., 2005; Yang, 2003), hair follicle development (Andl, Reddy, Gaddapara, & Millar, 2002; Logan & Nusse, 2004), and other processes. The normal processes governed by WNT5A during early development are not unlike cancer metastasis in that a majority involve cell movement. WNT5A plays a multifaceted role in embryonic development; and Wnt5A knockout mice exhibit complex phenotypes (Logan & Nusse, 2004; Xiao et al., 2006).

WNT5A and Cancer

In cancers, WNT5A plays a bifurcated role, operating in some cancers as a tumor suppressor and in others as an oncogene (Axelsson et al., 2009; Bachmann, Straume, Puntervoll, Kalvenes, & Akslen, 2005; Holcombe et al., 2002; Huang et al., 2005; Ying et al., 2008). In melanoma and in gastric cancers, WNT5A expression promotes cell invasion and correlates positively with tumor grade and patient outcomes (Da Forno et al., 2008; Kurayoshi et al., 2006; Weeraratna et al., 2002). In pancreatic cancer, WNT5A promotes cell invasion and migration and confers apoptotic resistance (Griesmann et al., 2013; Ripka et al., 2007). In some colon cancers, WNT5A acts as a tumor suppressor, inhibiting proliferation and EMT (Cheng et al., 2014; Ying et al., 2008). Contrary to these findings, Bakker et al. (2013) found that WNT5A promotes migration and invasion in human colon cancers, but that WNT5A expression does not promote metastasis or malignancy of human colon cancers in mice. In leukemias and lymphomas, WNT5A acts as a tumor suppressor (Liang et al., 2003; Ying et al., 2007). While receptor context certainly plays a large role in the duality of WNT5A function (Mikels & Nusse, 2006), it is also possible that functional distinctions between WNT5A isoforms play a role in the complex, sometimes paradoxical nature of WNT5As role in cancer. Bauer et al. (2013) report that WNT5A isoform A acts as a tumor suppressor in various cancer cell lines, inhibiting proliferation, while WNT5A isoform B acts as an oncoprotein, enhancing proliferation in various cancer cell lines.

Project Overview

WNT5A plays critical roles in embryonic development, tissue differentiation, adult tissue homeostasis, and cancer progression – particularly with respect to metastasis. There exists a wealth of scientific literature regarding the function of WNT5A in both development and disease. In cancers, WNT5A can act as either an oncogene or a tumor suppressor. There are a variety of potential explanations for this puzzling fact, one of which is that the WNT5A isoforms A and B

have distinct functions and that these functions are mediated by the differential activation of non-canonical signaling pathways. Functional differences between these proteins would also explain the broad evolutionary conservation of these isoforms in vertebrates. Assuming that these isoforms are indeed functionally distinct, it is reasonable to assume that these isoforms would display distinct temporospatial patterns of expression in development. To address these questions regarding the function of WNT5A, the following specific aims were completed:

1. Determine if WNT5A isoform A and isoform B differentially affect cell migration and invasion in the colorectal cancer cell line HCT-116.

2. Quantify the extent to which WNT5A isoform A and isoform B activate the Wnt/JNK, and Wnt/PKC non-canonical pathways.

3. Determine if WNT5A isoform A and isoform B are differentially regulated in embryonic development using a mouse model.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

HCT 116 (CCL-247), L Wnt-5A (CRL-2814), L Wnt-3A (CRL-2647), and L Cells (CRL-2648) were obtained from the American Type Culture Collection (ATCC). HEK 293T/17 (ATCC CRL-11268) cells were graciously provided by Dr. Ron Morrison (UNCG, Department of Nutrition). HCT116 are human colorectal cancer cells. L Wnt-5A are mouse fibroblasts stably transfected with a WNT5A isoform A expression vector, L Wnt-3A are mouse fibroblasts stably transfected with a WNT3A expression vector, and L Cells are parental mouse fibroblasts. HEK293T/17 are human embryonic kidney cells that constitutively express the simian virus 40 (SV40) large T antigen. The HCT116 cells were grown in McCoy's 5a Modified medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (P/S) at 50 I.U. /50 µg per ml (hereafter, Complete McCoy's 5a). In migration assays, HCT 116 cells were grown in Cell Cycle Arrest Medium (CCAM). CCAM was composed of McCoy's 5A base medium supplemented with 5% FBS and 5µg/mL (+)-aphidicolin (Cayman Chemical 14007). (+)-aphidicolin arrests the cell cycle at the G_1/S phase transition by inhibiting the activity of DNA polymerases α, δ, and ε. The L cells and their derivative lines – L Wnt-5A and L Wnt-3A - were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and P/S at 50 I.U. /50 µg per ml (hereafter, DMEM-C). DMEM-C was also used to culture HEK 293T/17. HEK 293T/17 cells were maintained on plates treated with poly-L-lysine to facilitate attachment. Plates were treated with 1ml/25cm^2 of 1 mg/mL filter sterilized poly-L-lysine solution in ddH_2O. The solution was allowed to incubate for 5 minutes at room temperature, at which point it was

aspirated off. The plates were then washed twice with sterile ddH_2O and allowed to dry for a minimum of 2 hours. All cells were grown at 37°C in a 5% CO_2 humidified cell culture incubator. Cells were passaged using a solution of 0.25% (w/v) Trypsin- 0.53mM EDTA. Trypsin was neutralized with an equal volume of complete media, cells were centrifuged into a pellet, supernatant was removed, and cells were resuspended in complete media. Cell concentration (cells/mL) was determined using a hemocytometer.

Transfection

Cells were transfected using Fugene 6 (Promega E2691). A target DNA to reagent ratio of 3:1 was used for all transfections. Cells were transfected during log phase growth in 6-well plates. For each transfection, serum-free medium (SFM) was added to a sterile microfuge tube such that the total volume after addition of transfection reagent and DNA would be 300µL. 18µL of room temperature Fugene 6 was added to the center of the tube, mixed immediately, and allowed to incubate at room temperature for 5 minutes. 6µg of sterile, endotoxin-free plasmid DNA (19:1 target:pEGFP) was added to the reagent/SFM mixture, mixed immediately, and incubated at room temperature for 15 minutes. The entire volume of this mixture was then added directly to the cultured cells, dropwise, with swirling. Cells were incubated at 37°C in a 5% CO_2 humidified cell culture incubator for 24-48 hours prior to collection. Transfection efficiency was monitored by confocal microscopy.

Stable Transfectant Selection

Stable cell lines were generated using the aforementioned transfection protocol. Target DNA was mixed at a 5:1 molar ratio with a neomyosin resistance plasmid (pMC1neopolyA, Invitrogen) prior to transfection. Cells were grown for two days prior to selection by the addition of Genticin (G418) to the growth medium at a concentration of 400µg/mL. Medium was changed

and G418 refreshed every two days until all remaining cells were G418 resistant. The resulting lines were maintained in G418 thereafter.

Luciferase Assay

To determine canonical Wnt signaling pathway activation, we used the TOPFlash reporter. M50 Super 8x TOPFlash and M51 Super 8x FOPFlash (TOPFlash mutant) were gifts from Randall Moon (Addgene plasmid #12456; Addgene plasmid #12457, respectively)(Veeman, Slusarski, Kaykas, Louie, & Moon, 2003). TOPFlash is a luciferase reporter plasmid that contains multiple copies of wild-type TCF/LEF1 binding regions, which function as a promoter for a firefly luciferase gene. When canonical signaling is activated, β-catenin translocates to the nucleus and associates with TCF/LEF1 to activate transcription of firefly luciferase. FOPFlash is a reporter plasmid that functions as a negative control. It contains mutated TCF/LEF1 binding regions upstream of firefly luciferase. In order to quantify canonical pathway activity, stable NIH3T3 cell lines containing the TOPFlash reporter or FOPFlash reporter seeded at a concentration of 6×10^4 cells per well in a 24-well plate and grown for 24 hours. Cells were then treated with conditioned medium or purified protein for 24 hours, and were assayed using the Promega Luciferase Assay System (Promega E4030). Luminescence was measured using a Berthold Lumat LB 9501 luminometer. After initial optimization, all assays were conducted in triplicate. Increased relative luciferase activity (luminescence) corresponds to increased canonical Wnt signaling, and loss of luminescence corresponds to inhibition of canonical Wnt signaling.

Conditioned Medium Generation and Characterization

L Wnt-3A, L-cells, L-cells^{+IsoB}, or L Wnt-5A cells were grown in DMEM-C + G418 in a 75cm^2 flask until nearly confluent. Cells were passaged 1:10 into 10 new 100mm tissue culture dishes in DMEM-C without G418. Cells were grown for four days, first-batch medium was harvested and stored at 4°C, cells were grown three more days in fresh DMEM-C, and second-batch medium was harvested. The batches were combined and filter sterilized using ultra low protein binding 0.22 µm filters. Conditioned medium was aliquoted and frozen at -20°C. L Wnt-3A conditioned medium was used 1:1 with DMEM-C to activate canonical Wnt signaling in TOPFlash and FOPFlash reporter cell lines. L cell conditioned medium 1:1 with DMEM-C was used as a negative control. Characterization attempts for Wnt5A Isoform A and Wnt5A Isoform B conditioned media were done by supplementing the WNT3A (activating) conditioned medium with either Isoform A of Isoform B conditioned media, which inhibit canonical signaling (to which the TOPFlash reporter responds). The amount of fresh DMEM and WNT3A conditioned medium in each well was kept constant in the course of characterizing the WNT5A isoform A and WNT5A isoform B conditioned medium.

Purified Protein Treatment

Purified WNT5A isoform B protein was graciously provided by Dr. Karl Willibert (UCSD, Cellular and Molecular Medicine, Stem Cell Program). Cells were treated with purified protein by direct application to the growth medium. TOPFlash and FOPFlash cells were treated with WNT3A conditioned medium and varying concentrations of purified WNT5A isoform B protein. Robust inhibition of canonical Wnt singaling occurred at a treatment concentration of 1:50. This treatment concentration was used thereafter for the treatment of HEK293T/17 cells. All purified protein treatments were conducted using buffer only controls. Buffer composition was 1M NaCl with 1% v/v CHAPS in 1X PBS.

Migration Assay

HCT 116 cells were stably transfected with expression vectors for Isoform A, Isoform B, and GFP (hereafter, HCT-A, HCT-B, and HCT-G, respectively). HCT-A, HCT-B, and HCT-G cells were plated in complete McCoy's 5A +G418 at a density of $1x10^6$ cells/well in a 6-well plate. Once the cells had adhered to the plate and had reached 70% confluence (about 18 hours), the normal growth medium was replaced with CCAM and incubated an additional 12 hours. Following this incubation, the underside of each well was scored with a razor blade to create a reference line, and the cell monolayer was scratched using a yellow 200µL polystyrene pipet tip that had been sealed at the end in a Bunsen burner flame and re-sterilized by incubation in 95% ethanol. Five scratches were made perpendicular to and across the reference line. The scratches were imaged at the same location 12 hours, 24 hours, and 36 hours following the scratching of the monolayer. The average width of each scratch at each time point was assessed using the trace and vertical thickness functions in the Imaris ImagePro software, which was graciously provided by Dr. Paul Steimle (UNCG Department of Biology).

Invasion Assays

An Oris Cell Migration Assembly Kit – FLEX (Platypus Technologies, CMAUFL4) was adapted modified to assess cellular invasion.

Collagen I Coating

Oris compatible 96 well plates were first coated with Collagen I. 3.28mL of 20mM sterile acetic acid was added to a sterile 15mL centrifuge tube along with 33.17µL of Cultrex 3-D Culture Matrix Rat Collagen I (Trevigen 3447-020-01) (hereafter, Collagen), and mixed. 33.17µL of the resulting coating solution was added to each well of an Oris compatible 96 well plate, and the plate was incubated at room temperature for 1 hour. The coating solution was

aspirated from the side of each well, and each well was washed three times with 1X PBS. The plate was then air-dried at 4°C for 24 hours.

Cell Seeding Stopper Population

Oris Cell Seeding Stoppers were added to the Collagen I coated plate. The stoppers were inserted into the wells to half-depth two-by-two such that each column of 8 wells on the plate was populated with stoppers at the same time. Stoppers were adjusted after placement such that they were vertical with respect to the well, and then were firmly pressed into the wells. Stopper sealing was visually inspected under indirect light, and stoppers were adjusted until all were properly seated.

Cell Seeding and 3D Culture

HCT-A, HCT-B, and HCT-G were grown to 70% confluence in complete McCoy's 5A +G418. Cells were labeled with CellTracker Green CMFDA (ThermoFisher C2925) (hereafter, CTG). CTG was diluted to 1mM in DMSO, aliquoted and frozen at -20°C. For staining, an aliquot of CTG was thawed and protected from light. To 35.8 mL of pre-warmed SFM, 180µL of CTG was added and thoroughly mixed to make a 5µM working solution. Growth medium was removed from the cells and replaced with the working solution. Cells were incubated for 30 minutes, the working solution was removed, and cells were washed with 1X PBS. The cell monolayer was treated with 4mL of Trypsin-EDTA solution for 5 minutes to release the cells. 4mL of complete growth medium was added and the cells were pelleted by centrifugation. The supernatant was removed, and cells were resuspended in CCAM. On ice, 550µL 10X PBS, 41.25µL 7.5% (w/v) sodium bicarbonate solution, 508.75µL sterile ultra-pure water, and 3mL Collagen were added to a 15mL sterile centrifuge tube, vortexed and vigorously pipetted to mix. The solution was centrifuged at 4°C for 5 minutes to degas. 1.46mL of collagen mixture was added to each of three sterile culture tubes, and 367µL of cell suspension – containing 3.75×10^6

cells/mL in CCAM – was incorporated by pipetting, with care taken not to introduce air bubbles, to make the seeding solution. 20µL of seeding solution was pipetted, using positive pressure and a slender gel-loading pipette tip, into each of the two side ports of each well. 32 wells contained HCT-A, 32 wells contained HCT-B, and 32 wells contained HCT-G. The plate was centrifuged twice for 20 seconds at 100x g in order to encourage the seeding solution to settle evenly. The plate was incubated at 37°C/100% RH/5% CO_2 for 1 hour allowing the collagen matrix to form a sturdy gel.

2D Cell Seeding and Overlay

The assay was also repeated using a traditional 2D culture and a simple collagen I overlay. HCT-A, HCT-B, and HCT-G cells were grown to 70% confluence in 100mm dishes in complete McCoy's 5A + G418 and were stained with CTG as described above. Cells were plated at a density of 5×10^5 cells/well and were allowed to attach.

Stopper Removal and Creation of Detection Zone

Oris Cell Seeding Stoppers were removed following seeding of the wells using the manufacturer provided stopper tool. The stopper tool was washed twice in 95% ethanol and allowed to dry in an aseptic environment prior to use. Care was taken to remove the stoppers slowly and vertically with respect to the well. Removal of the stoppers left behind a cylindrical detection zone (in the 3D assay) which was subsequently filled with a collagen gelling solution. In the 2D assay, a circular detection zone was created. In a 15mL sterile centrifuge tube, 150µL 10X PBS, 11.25µL 7.5% (w/v) sodium bicarbonate, 138.75µL sterile ultra-pure water, 900µL Collagen I, and 300µL CCAM supplemented with 5% extra FBS were mixed by vortexing to create the gelling solution. The gelling solution was centrifuged at 4°C for 5 minutes to degas. 15µL of gelling solution was pipetted using positive pressure into the center bottom of the detection zone through a slender gel-loading pipette tip for the 3D assay. For the 2D assay, wells

were washed gently with 1X PBS and cells were overlaid with 50 µL of gelling solution. Care was taken not to disturb the cells already seeded into the wells. The plate was centrifuged twice for 20 seconds at 100x g in order to remove any air bubbles. The plate was then incubated 37°C/100% RH/5% CO_2 for 1 hour allowing the collagen matrix in the detection zone to form a sturdy gel. 100µL CCAM supplemented with an additional 5% FBS was added to each well after the collagen matrix had congealed.

Data Collection

Immediately following the addition of media to each well in the 96 well plate, a detection mask was clipped onto the underside of the plate. The detection mask is an opaque plastic insert that shields cells outside of the detection zone from any light originating from the underside of the plate, while leaving the detection zone itself exposed. The fluorescence (492nm excitation/517nm emission) of each well was subsequently read using a BioTek Synergy 2 microplate reader. Fluorescence corresponds to the number of CTG-stained cells that had invaded through the collagen matrix at the edges of the well into the matrix in the center of the well. Subsequent readings were taken every 4 hours for five days in the initial assay. After the time it took for cells to completely invade into the detection zone was determined, readings were taken every 4 hours for 36 hours.

Western Blot Analysis

Protein Lysate Preparation

Cultures were prepared either of parental HEK293T/17 cells, or of HEK293T/17 cells transfected with one of WNT5A Isoform A, WNT5A Isoform B, or EGFP expression vectors. Parental HEK293T/17 cultures were treated with purified WNT5A Isoform B protein or buffer-only control for 2 minutes, 10 minutes, 90 minutes, or 0 minutes. At the time of collection, cells were washed with ice-cold 1X PBS, and complete lysis buffer was added directly to the

monolayer. Cells were shaken at 4°C for 15 minutes, scraped off of the plates, and lysates were transferred to a clean microcentrifuge tube. Lysates were spun at 15,000 X g at 4°C for 15 minutes, and the supernatant was aliquoted to fresh microcentrifuge tubes. Samples were stored at -80°C until use. Complete lysis buffer consisted of either Invitrogen Cell Extraction Buffer (ThermoFisher FNN0011) or Sigma-Aldrich CelLytic M (C2978) supplemented with 13mg/mL crushed Pierce Protease Inhibitor Tablets, EDTA Free (ThermoFisher 88266), and 2% (v/v) each of Sigma-Aldrich Phosphatase Inhibitor Cocktail 2 and Phosphatase Inhibitor Cocktail 3 (P5726-1ML and P0044-1ML, respectively). Complete lysis buffer was stored on ice immediately prior to use, and was freshly prepared for each use.

Protein Determination

Pierce 660nm Protein Assay Reagent (ThermoFisher 22660) was used to determine protein concentration of samples. BSA protein standards were prepared at concentrations of 0 µg/µL, 0.5 µg/µL, 1 µg/µL, 1.5 µg/µL, 2 µg/µL, 2.5 µg/µL, and 3 µg/µL in complete lysis buffer. Wells of a 96-well plate were loaded with 10µL of each standard in duplicate. 10µL of each sample was also loaded, also in duplicate. Each standard and sample well received 150 µL of Pierce 660nm Protein Assay Reagent, and the plate was shaken for 5 minutes at room temperature. Absorbance at 660nm was read using the BioTek Synergy2 microplate reader. Absorbance values of the BSA protein standards were plotted against their concentrations, and a linear regression was performed to generate a standard curve. Only standard curves with an R^2 value above 0.95 were used. The equation for the linear regression was solved for protein concentration and used to determine the concentration of the protein lysates based on their absorbance in the 660nm protein assay.

Sample Preparation, SDS-PAGE, and Transfer

Volumes of samples containing 110µg of protein were added to clean microcentrifuge tubes. 20µL of 5X SDS sample buffer was added to each tube, along with complete lysis buffer to bring the total volume of each tube to 110µL. Samples were incubated at 95°C for 5 minutes. 50µL of each sample was loaded in duplicate on Precise 10% Tris-Glycine Precast Gels (ThermoFisher 25261). Samples were run at 80V/gel constant voltage for 90 minutes. Protein was transferred from the gel to either a nitrocellulose or PVDF membrane. Both gels and membranes were equilibrated in fresh transfer buffer for a minimum of 5 minutes. Transfers were done at 40V constant voltage for 2 hours. Successful transfer and even loading was confirmed by Ponceau S staining.

Immunoblotting

Membranes were cut in half after transfer to produce two identical blots. Blots were blocked for 1 hour in 5% (w/v) BSA in TTBS with 0.01% Phosphatase Inhibitors 2/3, incubated in primary antibody solution for 1 hour at 4°C with shaking, incubated in secondary antibody for 1 hour at room temperature with shaking, and detected using a SuperSignal West Pico Chemiluminescent Substrate kit (Thermo# 34080). Bands were documented using the Bio-Rad Chemi Doc System. At each step between blocking and detection, blots were washed thoroughly in TTBS.

Antibodies

Primary antibodies raised against STAT3 (Rockland Antibodies & Assays, 600-401-GH6), pSTAT3Y710 (Rockland Antibodies & Assays, 600-401-C64), JNK (Santa Cruz Biotechnology, sc-7345), pJNKT183/185 (Santa Cruz Biotechnology, sc-6254), PKCα (Santa Cruz Biotechnology, sc-8393) pPKCαS657 (Santa Cruz Biotechnology, sc-12356), and WNT5A (Abcam, ab174100) were used. Primary antibodies were diluted in a solution of 1% (w/v) BSA in

TTBS with 0.01% (v/v) phosphatase inhibitors 2/3. Primary antibodies against STAT3, pSTAT3Y710, pJNKT183/185, and WNT5A were diluted at a ratio of 1:1000, and all other primary antibodies were diluted at a ratio of 1:2000. Antibody-HRP conjugates raised against goat IgG (Abcam, ab97110), rabbit IgG (ThermoFisher, #31466), and mouse IgG (Abcam ab97023) were used as secondary antibodies and were diluted at a ratio of 1:10,000 in 1% (w/v) BSA in TTBS with 0.01% (v/v) phosphatase inhibitors 2/3.

Quantitative Real Time-PCR (qPCR)

Staged CD1 mouse embryo RNA was obtained from Zyagen (MR-104-005). RNA was from E10, E11, E12, E13, and E14 embryos. RNA was converted into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, 205313). Each reaction mixture consisted of 1µg of RNA, reverse transcriptase, reverse transcriptase buffer, primers, and nuclease free water to give a final 20 µl reaction. The reaction mixtures were incubated for 5 minutes at 42 °C and then at 95 °C for 3 minutes.

Wnt5A Promotor A and *Wnt5A* Promotor B transcripts were quantified from this cDNA by qPCR. Each reaction was run in triplicate with three target primer-probes; Promoter A, Promoter B, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (internal control) (Applied Biosystems HS99999905_m1) for cDNA from each mouse embryo stage. Quantified Promoter A and Promoter B PCR products were run in triplicate as standards from which absolute transcript numbers were determined. The equation of a logarithmic regression of standard C_t values plotted against standard concentrations was solved for concentration, and sample C_t values were converted into absolute transcript numbers using this equation. The sequence of Promoter A and Promoter B primer-probes are given in Table 1.

Table 1. *Wnt5a* Mouse Primer-Probe Sets

	Sequence (5' → 3')	**Length (base)**	**Product Size (bp)**
Mouse Promoter A			
Forward	GTGGCGACTTCCTCTCCGT	19	85
Reverse	AGTGGCTTTGGGGACCG	17	
Probe	CCCCTCGCCATGAAGAAGCCCA	22	
Mouse Promoter B			
Forward	ACTTGTTGCTCCGGCCC	17	62
Reverse	CGGTCCCCAAAGCCACT	17	
Probe	AGAAGCCCATTGGAATATTAAGCCCGG	27	

(Hsu, 2011)

CHAPTER III

RESULTS

The Role of WNT5A Isoforms in Human Colorectal Carcinoma Migration and Invasion

WNT5A Isoforms A and B do not Influence Migration in HCT116 Colorectal Carcinoma Cells

WNT5A is known to have an effect on cellular migration in many cellular models of cancer, and previous studies in this lab have sought to determine if WNT5A isoforms A and B differentially affect migration in SaOS-2, a human osteosarcoma cell line. Additionally, some studies in the literature suggest that WNT5A promotes migration in human colorectal cancers, while others suggest that WNT5A inhibits migration in human colorectal cancers. In order to determine if this apparent duality might be due to properties specific to a particular isoform, we conducted experiments to determine if WNT5A isoforms A and B differentially affect migration in HCT116. HCT116 was chosen because previous unpublished studies in this lab indicate that HCT116 expresses neither WNT5A isoform A nor WNT5A isoform B.

HCT116 cells were stably transfected with expression vectors for WNT5A isoform A (Figure 6A), WNT5A isoform B (Figure 6B), and EGFP (Figure 6C). Following selection, expression was confirmed by qPCR and western blot (Figure 7). qPCR indicated that in HCT-A and HCT-B, *WNT5A* was expressed at a high level, nearly equivalent with actin. qPCR results indicated that *WNT5A* is not expressed in HCT-G. Western blot analysis revealed very little WNT5A protein in HCT-A and HCT-B cells, and no WNT5A protein in HCT-G cells. We suspect that the western blot analysis is not indicative of the amount of WNT5A present in HCT-A and HCT-B cells, given that WNT5A is secreted post-processing.

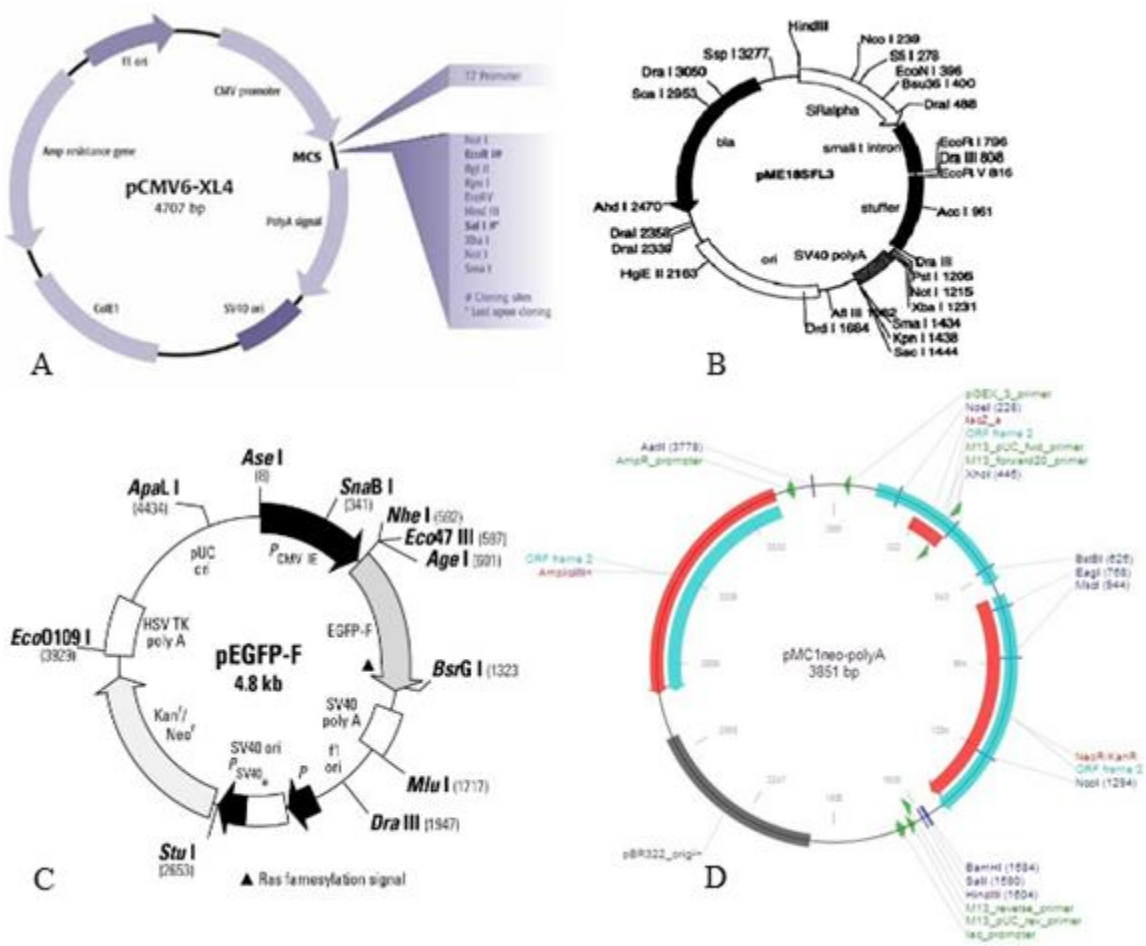
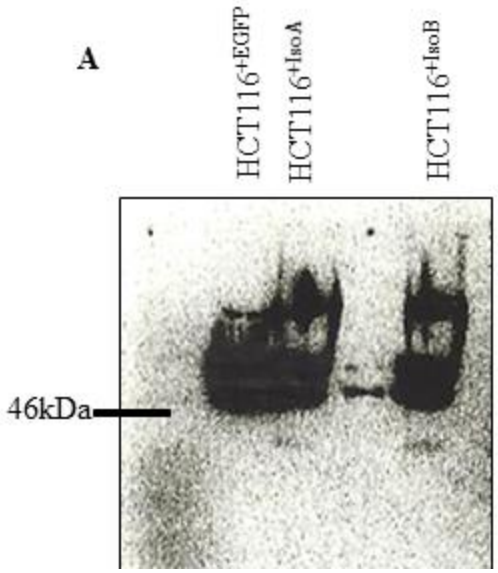


Figure 6. Expression Vectors Used for Transfection. (A) pCMV6-XL4 expression vector with Isoform A cDNA (NM_003392)cloned into Not 1 – Not 1 (OriGene). **(B)** Expression Vector pME18SFL3. Isoform B cDNA (AK290869) cloned into Dra III – Dra III (replacing stuffer region) (NITE). **(C)** pEGFP-F expression vector (Clontech). **(D)** pMC1neo-polyA expression vector (Invitrogen).

A



B

Expression (Cycle Threshold Values)			
	Isoform A	**Isoform B**	**Actin**
HCT116 GFP	38*	35.63313	19.87127
HCT116 Iso A	**25.1637**	37.6401	23.51643
HCT116 Iso B	37.6792	**24.3464**	21.84393

Figure 7. Expression of WNT5A Isoforms in HCT116 Stable Lines. (A) Western blot of HCT116 stable line lysates probed against WNT5A. Dark Bands are non-specific. Faint band just below 46kDa corresponds to WNT5A. HCT-A and HCT-B express WNT5A, whereas HCT-G does not. **(B)** Cycle threshold values from qPCR. Values are log scale; lower values correspond to higher expression. HCT-A expresses isoform A nearly on par with actin, but does not express isoform B. HCT-B expresses isoform B nearly on par with actin, but does not express isoform A. HCT-G expresses neither isoform.

A wound healing assay was used to assess migration in HCT-A, HCT-B, and HCT-G cells (Figure 8A). We found no significant difference between HCT-A and HCT-B cell lines in terms of the rate of migration, nor was there a difference between either HCT-A or HCT-B and HCT-G (Figure 8B). The rates of HCT-A migration and HCT-B migration are expressed relative to control (HCT-G) rates and are the average of four independent experiments, each with a sample size for each treatment of n=9.

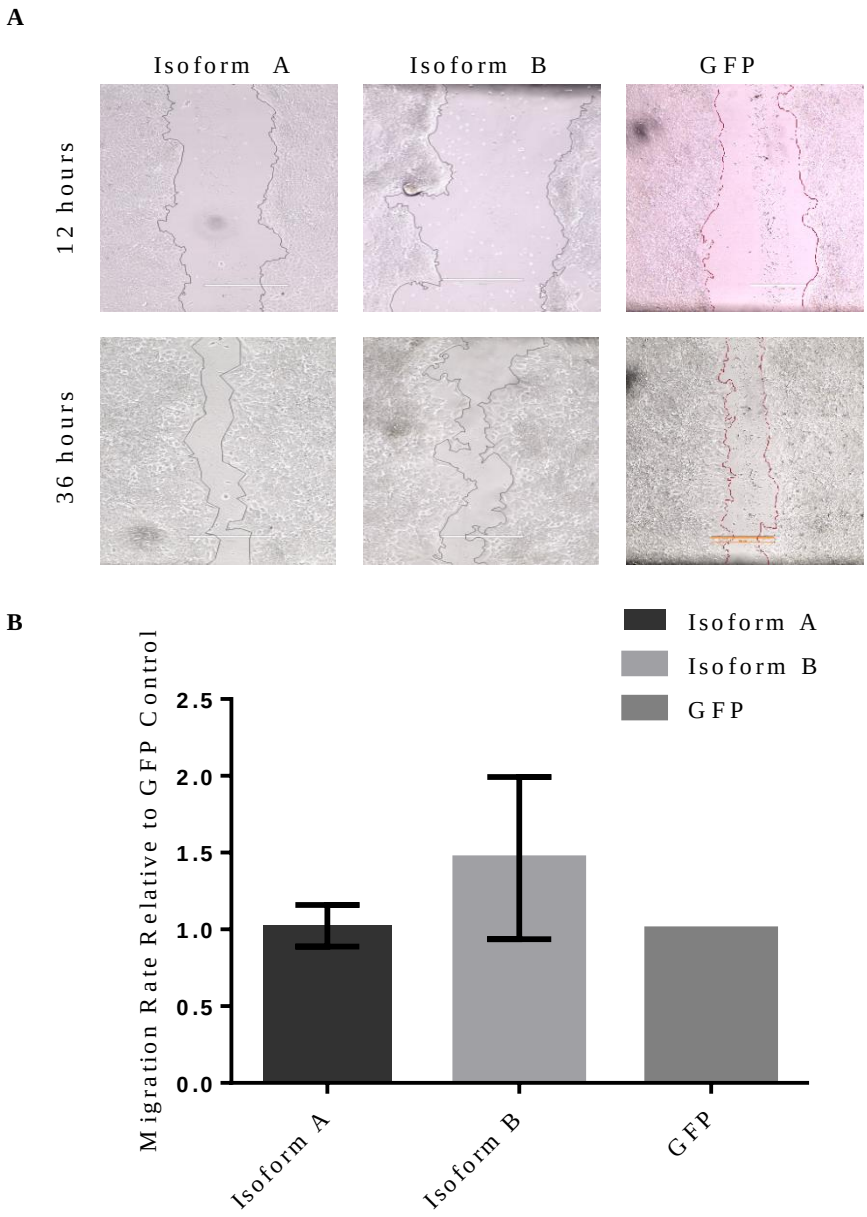


Figure 8. Migration in HCT-A, HCT-B, and HCT-G Stable Lines. (A) Images of scratched cell monolayers 12 hours after scratching (before migration), and 36 hours after scratching (after migration). Vertical traces show the leading edge of migrating cells. Average distance between traces before and after migration was used to compute the distance migrated. **(B)** Rates of migration of HCT-A, and HCT-B cells relative to migration of HCT-G cells. Error bars represent 95% confidence interval for the mean. There is no significant difference between HCT-A migration of HCT-B migration and the control (HCT-G migration).

WNT5A Isoforms A and B do not Influence Invasion in HCT116 Colorectal Carcinoma Cells

We also assayed the stable HCT116 cells stably expressing WNT5A isoforms A and B for cellular invasion. WNT5A is known to have an effect on cellular invasion in many cellular models of cancer, and previous studies in this lab have sought to determine if WNT5A isoforms A and B differentially affect invasion in SaOS-2, a human osteosarcoma cell line. A 3-dimensional invasion assay was conducted in duplicate (Figure 9), and a 2-dimensional invasion assay was conducted in triplicate (Figure 10). We found no difference between WNT5A isoforms A and B with respect to invasion, nor did we find that either isoform significantly affected cellular invasion compared to GFP control using the 2D assay. The 3D invasion assay yielded similar results.

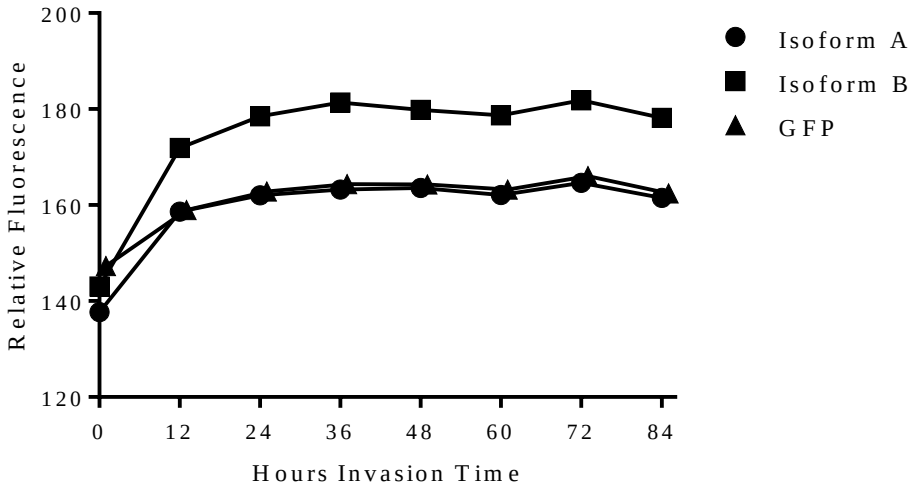


Figure 9. Invasion in HCT-A, HCT-B, and HCT-G Stable Lines in a 3D Culture System. Invasiveness of HCT-A, HCT-B, and HCT-G was assessed using a modified Oris Cell Migration Assay and 3D Culture System. Invasiveness was quantified by amount of fluorescence in the exclusion zone, which is directly proportional to the number of cells that invaded. Most invasion occurred within 12 hours. Marker location is the mean of two replicates.

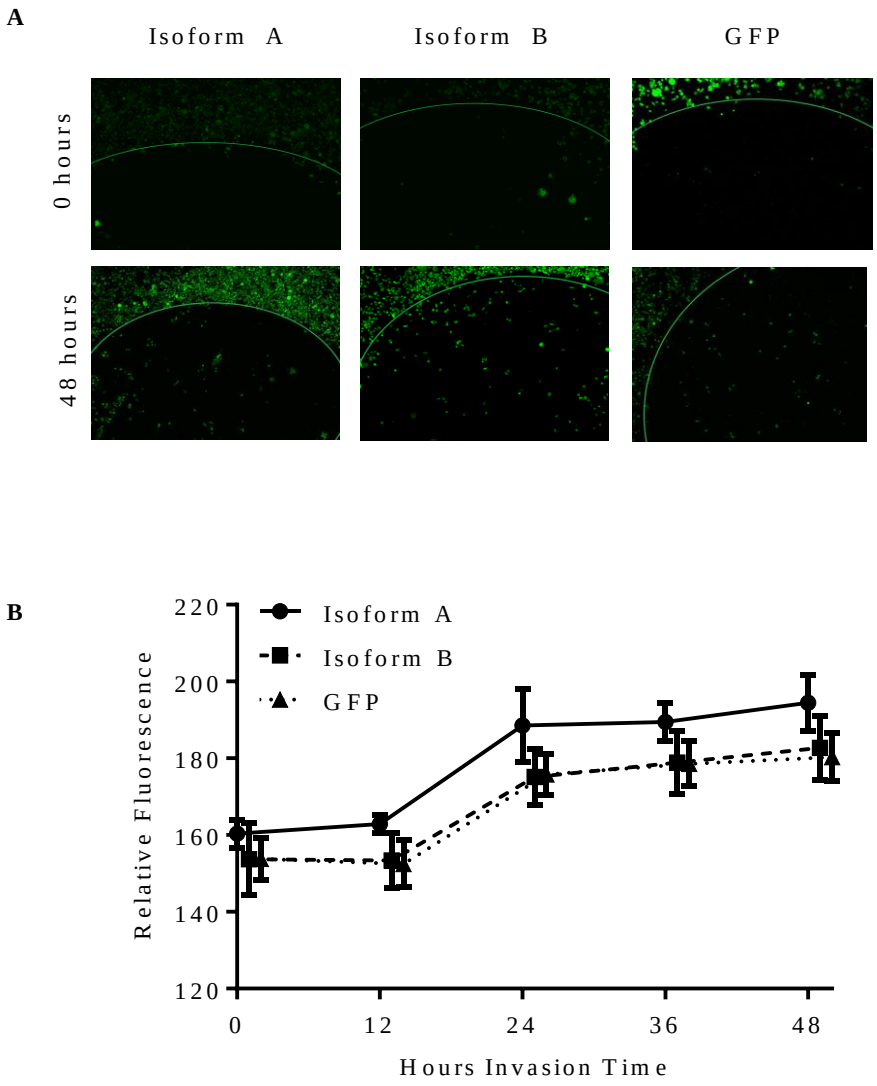


Figure 10. Invasion in HCT-A, HCT-B, and HCT-G Stable Lines. Invasiveness of HCT-A, HCT-B, and HCT-G was assessed using a modified Oris Cell Migration Assay. **(A)** Confocal images of cells both before and after invasion. 48 hours in, cells have invaded into the collagen overlay and the center of the exclusion zone. **(B)** Invasiveness was quantified by the amount of fluorescence in the exclusion zone, which is directly proportional to the number of cells that invaded. Most invasion occurred between 12 and 24 hours after plating of the collagen overlay and creation of the detection zone.

The Role of WNT5A Isoforms A and B in Wnt Signaling

WNT5A is the paradigmatic non-canonical Wnt ligand, in that it generally activates the non-canonical Wnt signaling pathways and only the non-canonical Wnt signaling pathways. These pathways consist of the PCP/CE pathway, the Wnt/Ca^{2+} pathway, and the Wnt/STAT3 pathway. The terminal effector molecules in these pathways are JNK, PKC, and STAT3, respectively. We investigated the possibility that specific isoforms of WNT5A could differentially activate these pathways. Specifically, we looked at the activation of JNK and PKC by assessing the prevalence of their phosphorylated forms by western blot analysis. HEK293T/17 cells were transfected with expression vectors for WNT5A isoform A, WNT5A isoform B, or GFP. Transfection efficiency was nearly 100% as determined by confocal microscopy. Protein lysates were generated 48 hours following transfection. These lysates were resolved by SDS-PAGE and transferred to nitrocellulose or PVDF for western blotting. Expression of WNT5A isoforms was confirmed by western blot (Figure 11). Control cells – HEK293T/17 transfected with pEGFP – express some WNT5A. Cells transfected with Isoform A or Isoform B expression vectors express WNT5A at a much higher level than control cells. We also sought to determine if WNT5A isoform B was activating the STAT3, JNK, and PKC pathways using purified WNT5A isoform B treatment.

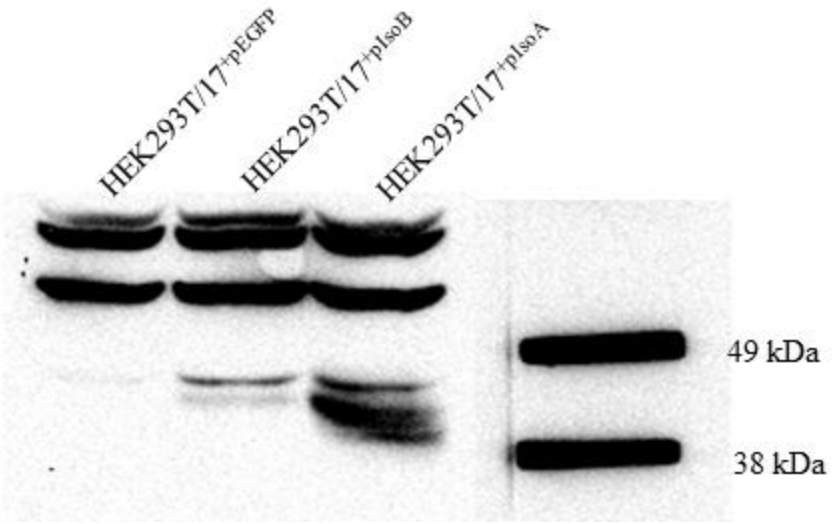


Figure 11. Overexpression of WNT5A Isoforms A and B in HEK293T/17. Western blot of HEK293T/17 lysates transfected with expression vectors for isoform A, isoform B, or EGFP. HEK293T/17 cells transfected with pEGFP express very little WNT5A, while cells transfected with either Isoform A or Isoform B expression vectors express much more WNT5A. WNT5A band is at approximately 42 kDa; bands above 49kDa marker are nonspecific, and serve as an internal loading control.

Effect of WNT5A Isoform Overexpression on JNK Pathway Activation

In order to determine if the JNK pathway was being stimulated by overexpression of WNT5A isoforms A and B, we blotted for JNK and phosphoJNKThr183 (Figure 12). JNK collectively refers to ten protein isoforms that run at either 46 or 54 kDa in SDS-PAGE. We used the ratio of phosphorylated JNK to total JNK as a measure of JNK pathway activity. We found no significant difference in JNK pathway activity between cells transfected with WNT5A isoform A expression vector and cells transfected with WNT5A isoform B expression vector, nor did we find a significant difference between cells overexpressing either isoform and control cells (Figure 12).

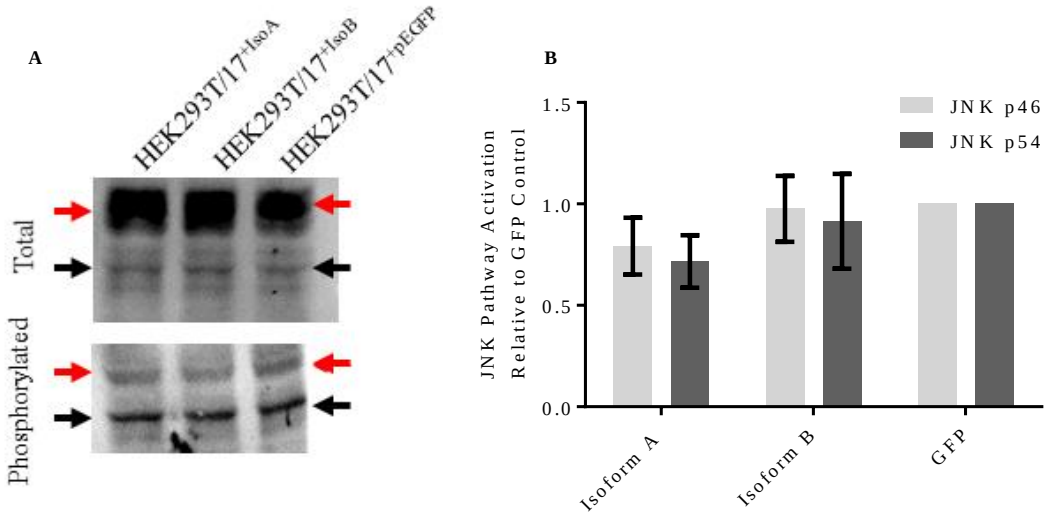


Figure 12. JNK Pathway Activation in Response to Overexpression of WNT5A Isoforms A and B in HEK293T/17. Western blots of lysates from HEK293T/17 transfected with Isoform A, Isoform B, or GFP expression vectors. Upper image was immunostained against total JNK protein. Red arrow indicates p54 JNK isoform, black arrow indicates p46 JNK isoform. Lower image was immunostained against phosphoJNKThr183. Red arrow indicates p54 JNK isoform, black arrow indicates p46 JNK isoform. Samples were run on a single SDS-PAGE gel There is dramatically more p54 JNK than p46 JNK, and a much larger proportion of p46 JNK is phosphorylated compared to p54 JNK. **(B)** Graph of western blot analysis results. Relative JNK pathway activation is ratio of phosphoJNKThr183 to total JNK. Values shown are relative to GFP control. Error Bars represent standard deviation. We observed no significant change in JNK pathway activation in response to overexpression of either WNT5A isoform.

Effect of WNT5A Isoform Overexpression on PKC Pathway Activation

In order to determine if the PKC pathway was being activated in response to overexpression of WNT5A isoforms A and B, we blotted for PKCα and phosphoPKCαSer657 (Figure 13). PKC has 16 distinct isotypes. PKCα is one of the isotypes most commonly involved in non-canonical Wnt signaling. We used the ratio of phosphorylated PKCα to total PKCα as a measure of PKC pathway activity. We found no significant difference in PKC pathway activity between cells overexpressing WNT5A isoform A and cells overexpressing WNT5A isoform B,

nor did we find a significant difference between cells overexpressing either isoform and control cells (Figure 13).

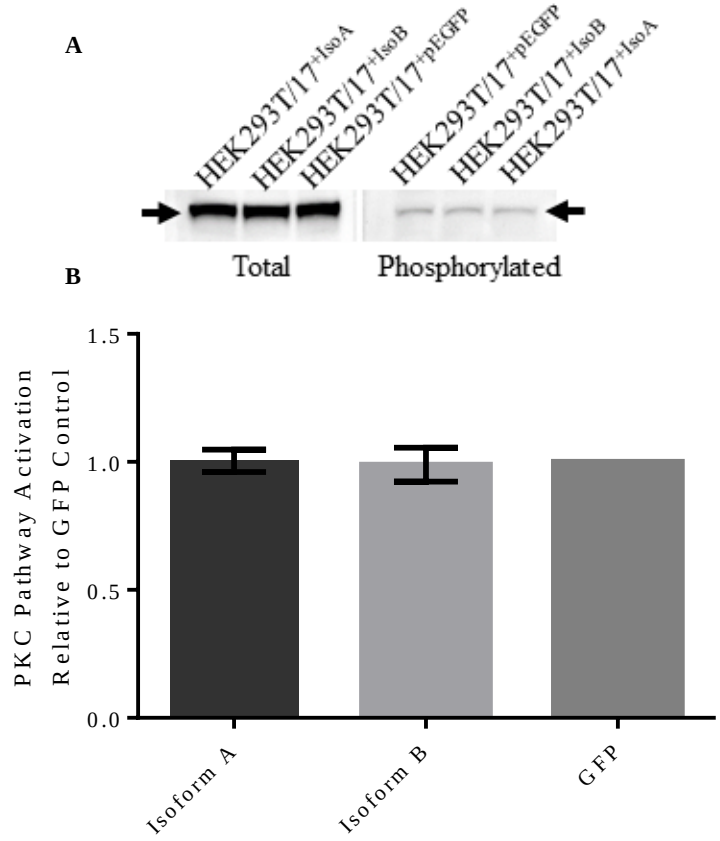
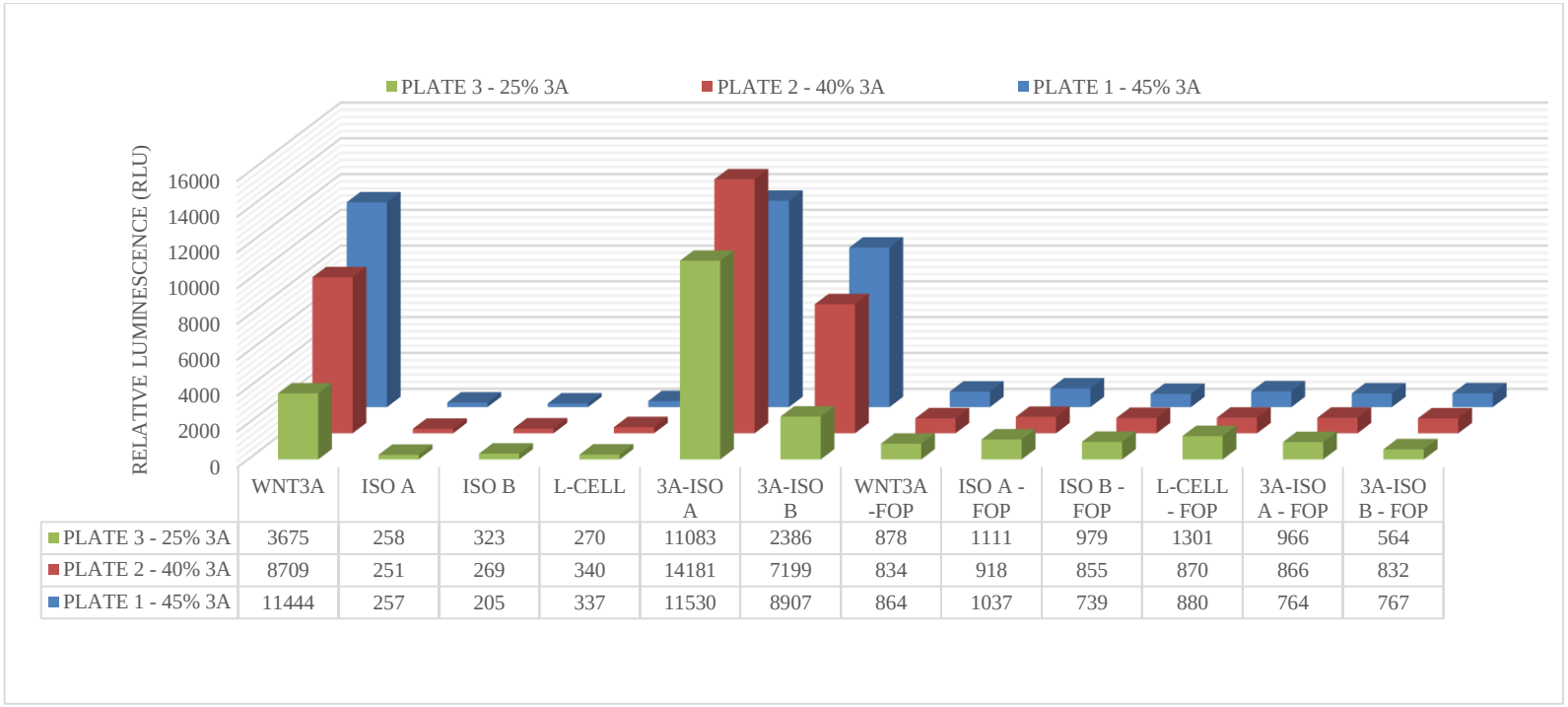


Figure 13. PKC Pathway Activation in Response to Overexpression of WNT5A Isoforms A and B in HEK293T/17. (A) Western blots of lysates from HEK293T/17 transfected with Isoform A, Isoform B, or GFP expression vectors. Blot on left was immunostained against total PKC; blot on right was immunostained against phosphoPKCSer657. Arrows indicate PKC and phospho-PKCSer657 location. Each sample contains approximately the same amount of total PKC, and approximately the same amount of phosphoPKCSer657. **(B)** Graph of western blot analysis results. PKC pathway activation is ratio of phosphoPKCSer657 to total PKC. Results are shown relative to GFP control. Error bars represent standard deviation. We observed no significant change in PKC pathway activation in response to overexpression of either WNT5A isoform.

Canonical Pathway Inhibition in Response to WNT5A Isoform B, but not Isoform A, Conditioned Medium Treatment

Prior to determining JNK and PKC pathway activation by overexpression, we had sought to utilize a conditioned medium based approach for this work. This approach required that we characterize the medium in order to ensure equal activity from batch to batch. In order to do this, we sought to replicate a finding from Bauer et al. (2013), where equal amounts of active WNT5A equally inhibited canonical Wnt signaling as determined using the TOPFlash reporter. We generated WNT5A isoform A conditioned medium, WNT5A isoform B conditioned medium, WNT3A conditioned medium, and conditioned medium from parental L-cells. We then sought to characterize this medium with respect to its ability to activate or inhibit the canonical Wnt signaling pathways using TOPFlash (Figure 14). WNT3A conditioned medium activated the canonical Wnt signaling pathways in a dose-dependent fashion, as expected. Isoform A conditioned medium, Isoform B conditioned medium, and L-cell conditioned medium all failed to activate the canonical Wnt signaling pathway on their own, as expected. Isoform B conditioned medium, used in conjunction with WNT3A conditioned medium, inhibited canonical signaling in a dose-dependent fashion, consistent with our expectations. Isoform A conditioned medium, used in conjunction with WNT3A conditioned medium, produced synergistic activation of the canonical Wnt signaling pathway. We abandoned the conditioned medium approach to analyzing Wnt signaling pathways and opted instead for a transfection based approach on the basis of this result.



	WNT3A	ISO A	ISO B	L-CELL	3A-ISO A	3A-ISO B	WNT3A -FOP	ISO A - FOP	ISO B - FOP	L-CELL - FOP	3A-ISO A - FOP	3A-ISO B - FOP
PLATE 3 - 25% 3A	3675	258	323	270	11083	2386	878	1111	979	1301	966	564
PLATE 2 - 40% 3A	8709	251	269	340	14181	7199	834	918	855	870	866	832
PLATE 1 - 45% 3A	11444	257	205	337	11530	8907	864	1037	739	880	764	767

Figure 14. Canonical Pathway Inhibition in Response to WNT5A Isoform B, but not Isoform A, Conditioned Medium Treatment. TOPFlash (left side) or FOPFlash(right side) cells were treated with conditioned medium (CM) as indicated. Each bar represents the mean of three replicates. Where either WNT3A or 3A is indicated, cells received WNT3A CM at the concentration (v/v) indicated. Iso A and Iso B correspond to Isoform A CM and Isoform B CM, respectively. WNT3A conditioned medium activated canonical signaling in a dose-dependent manner (far left), but neither Iso A, nor Iso B, nor L-cell CM alone did (2nd, 3rd, and 4th rows from left). The addition of Iso B CM inhibited WNT3A-induced canonical signaling (6th row from left). The addition of Iso A CM enhanced WNT3A-induced canonical signaling (5th row from left). FOPFlash was not stimulated by any treatment, indicating low minimal promoter activity (7th through 12th rows from left). Table below graph shows mean RLU values for each treatment condition.

Effect of Purified WNT5A Isoform B Treatment on Canonical Wnt Signaling

Purified WNT5A isoform B protein was graciously provided by Dr. Karl Willibert (UCSD, Cellular and Molecular Medicine, Stem Cell Program). In order to determine if the protein was active, and in order to determine the minimum concentration at which the purified WNT5A isoform B would exert its effects, we sought to characterize this protein using the TOPFlash reporter system (Figure 15). We observed significant inhibition of the canonical Wnt signaling pathway in response to treatment with purified WNT5A isoform B at a concentration of 1:50 (v:v).

Effect of Purified WNT5A Isoform B Treatment on Non-Canonical Wnt Signaling

We assumed that WNT5A Isoform B was inhibiting the canonical pathway through one of the known non-canonical pathways. In order to determine which of the known non-canonical signaling pathways were being activated by WNT5A isoform B, HEK293T/17 cells were treated with purified WNT5A Isoform B. Cells were treated at either 1:50 (v:v) or 1:100 (v:v) with 100ng/μL WNT5A isoform B. The cells responded to the purified protein buffer by detaching from the plate and clumping, even with Poly-L-lysine coated plates. After about 60 minutes, the cells reattached to the plates. We blotted for several terminal effector molecules (PKC, JNK, and STAT3) and their corresponding phosphoproteins as a measure of non-canonical Wnt signaling pathway activation (Figure 16). We suspended this approach after finding that HEK293T/17 tolerated the protein buffer poorly. As such, the sample sizes do not permit statistical analysis. These results are, however, consistent with the results we obtained by

overexpression of WNT5A over a 48 hour period, in that we found no indication of an effect dramatic enough to be reliably detected by western blot analysis.

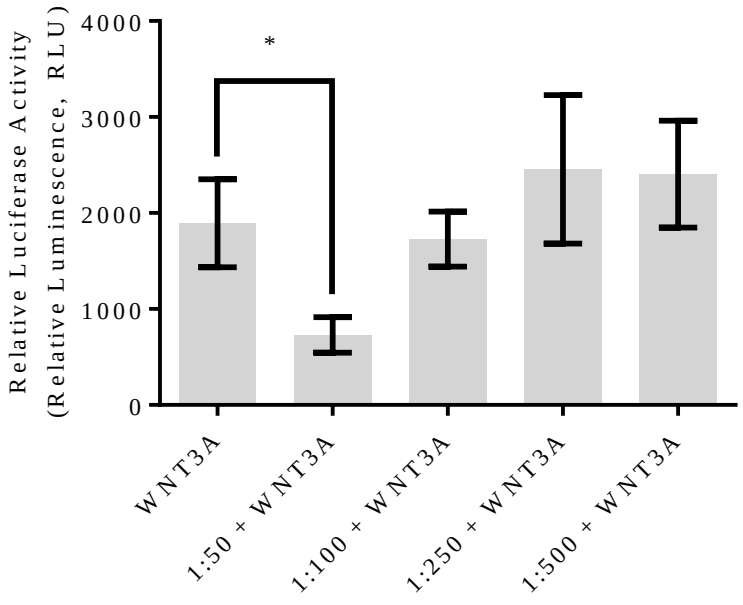


Figure 15. Canonical Pathway Inhibition in Response to WNT5A Isoform B Purified Protein Treatment. TOPFlash cells were treated 1:1 with WNT3A conditioned medium and fresh complete growth medium to activate the canonical signaling pathway. Cells were then treated with purified WNT5A Isoform B at a concentration of 1:50, 1:100, 1:250, or 1:500 (v:v) by direct application to the growth medium. Significant inhibition of canonical Wnt signaling was achieved at a treatment concentration of 1:50. FOPFlash was used as a negative control (data not shown).

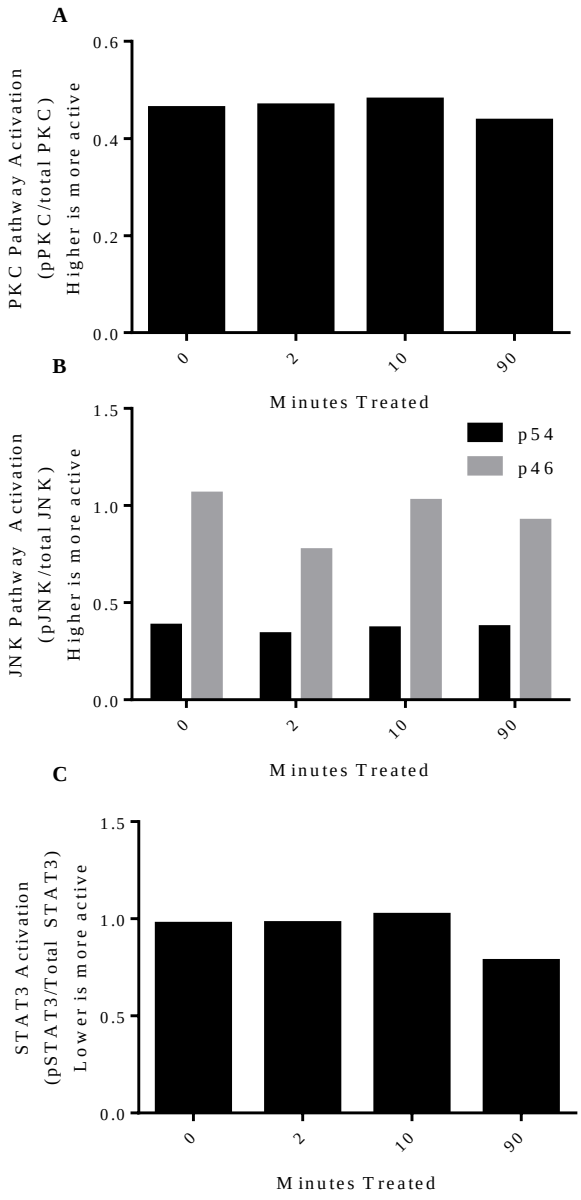


Figure 16. Non-Canonical Pathway Activation in Response to WNT5A Isoform B Purified Protein Treatment. (A-C) Graphs of western blot analysis results. We observed no dramatic activation of the **(A)** PKC pathway, **(B)** the JNK Pathway, **(C)** or the STAT3 pathway in response to WNT5A isoform B treatment.

Temporal Regulation of WNT5A Isoforms A and B Expression During Mouse Embryonic Development

We sought to investigate whether WNT5A isoforms A and B might be differentially regulated during mouse embryonic development. Because the isoforms are highly conserved, they must in some way be distinct, and that such a distinction might be more apparent in the context of embryonic development. To determine if expression of these isoforms was differentially regulated during embryonic development, we performed qPCR for both isoforms using RNA collected from staged mouse embryos (Figure 17). We chose RNA from embryos that were 10, 11, 12, 13, and 14 days past conception, because limb bud formation and outgrowth, a process in which WNT5A plays a major role, occurs within this window. We observed a dramatic increase in the number of Promoter A, but not Promoter B, transcripts at 11 days past conception. This increase in expression of Promoter A transcripts is in line with what is known about *WNT5A* expression during embryonic development. We also observed a decrease in the prevalence of Promoter A and Promoter B transcripts at 12 days past conception. The decrease in Promoter B transcripts at 12 days past conception is previously unreported.

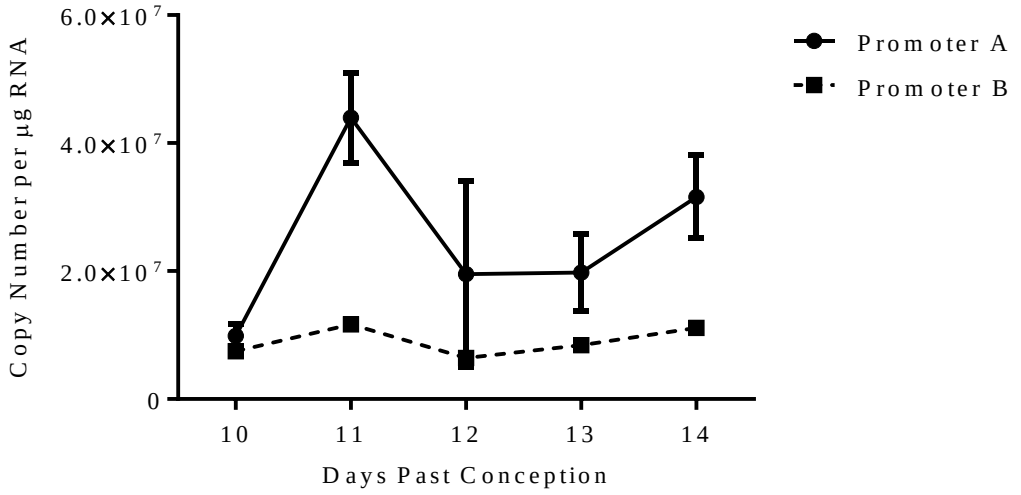


Figure 17. Expression of *WNT5A* Alternative Transcripts During Mouse Embryonic Development. Absolute copy numbers for mouse *WNT5A* promoter A and B primary transcripts from 10 days past conception through 14 days past conception. Error bars represent 95% confidence intervals for the mean. Promoter A differs significantly from Promoter B at 11, 13, and 14 days past conception.

CHAPTER IV

DISCUSSION

Summary of Major Findings

This study investigated both the functional differences between WNT5A isoforms A and B, and the mechanisms by which putative functional differences might be mediated. The effect of WNT5A isoform A and B overexpression on cellular invasion and migration was investigated using HCT116 cell lines stably overexpressing WNT5A isoforms A or B, or GFP. Overexpression was confirmed by qPCR and western blot, both of which showed that HCT-A and HCT-B lines express WNT5A isoforms A and B, respectively, at a higher level than HCT-G control cells. Our results indicate that neither WNT5A isoform A or B significantly affects cellular invasion or migration in HCT116. These results conflict with at least two published studies (Bakker et al., 2013; Cheng et al., 2014). Our results indicate that WNT5A isoforms A and B do not stimulate the canonical Wnt signaling pathway, nor do they stimulate the PKC or JNK pathways when overexpressed over a 24 hour period. Our results do indicate that WNT5A isoform B inhibits canonical Wnt signaling, and that this inhibition is likely not mediated via the Wnt/Ca^{2+} pathway (given that Wnt/PKC pathway is convergent with the Wnt/Ca^{2+} pathway). We were unable to determine if WNT5A isoforms A and B stimulate the non-canonical Wnt/STAT3 pathway. We determined that during mouse embryonic development, WNT5A isoforms A and B are expressed at different levels, and that their level of expression fluctuates significantly with time. These results collectively suggest that at a cellular level, either WNT5A isoforms A and B are functionally redundant, or functional differences between WNT5A isoforms A and B are

smaller than what is detectable using our assays. These results further suggest that the isoforms of WNT5A may function as a mechanism to achieve nuanced regulation of overall WNT5A production during embryonic development. The possibility also remains that these isoforms are differentially regulated not only in a temporal fashion during development, but also spatially. Finally, these results point towards the possible existence of an as-of-yet undiscovered non-canonical Wnt signaling pathway that inhibits canonical signaling by some mechanism independent of Wnt/Ca^{2+} signaling.

Migration in HCT116 is not Affected by WNT5A Isoforms A or B

Neither overexpression of WNT5A isoform A nor overexpression of WNT5A isoform B had any detectable effect on cellular migration in HCT116. These results differ from our expectations, which were that isoform A would promote migration and isoform B would inhibit migration.

In a variety of cell types, WNT5A isoform A is known to affect migration. WNT5A isoform A was shown by Bakker et al. (2013) to promote migration in HCT116. The methodology employed in that paper differs significantly from the methodology we used, and is likely far more sensitive. Whereas we used a standard wound-healing assay, Bakker et al. employed an approach based on time-lapse microscopy that measures multiple cell movement parameters. Their finding did not translate into increased metastasis in a murine model of colorectal cancer.

Our result, that WNT5A has no effect on cell migration in HCT116, also conflicts with a study by Cheng et al. (2014), which found that WNT5A *inhibits* migration in HCT116. Cheng et al. also assert that HCT116 expresses endogenous WNT5A, which conflicts with findings from this lab.

Parental HCT116 cells express neither endogenous WNT5A isoform A nor WNT5A isoform B. When we tested our stable lines for WNT5A expression using a qPCR based approach, we observed expression of each isoform nearly equivalent to actin. When we tested our stable lines for WNT5A expression by western blot, we saw very little WNT5A protein. There are a few potential explanations for this. It is possible that WNT5A is modified and secreted rapidly following translation, thus accumulating in the media and preventing accumulation in the cells (and hence detection by western blot). It is also possible that HCT116 cells lack some of the machinery required to create mature WNT5A protein. The former explanation does not change the interpretation of our results, while the latter explanations casts doubt on our results.

While the assertion that migration in HCT116 is not affected by WNT5A is not particularly exciting, it does make sense, given that there are findings in the published literature that stand in direct contradiction to one another. We had expected to explain the contradictory nature of WNT5A in HCT116 migration by demonstrating functional distinctions between isoforms; instead, we note that even using a highly optimized assay system, we were unable to discern an effect, and that this is itself a novel finding.

Invasion in HCT116 is not Affected by WNT5A Isoforms A or B

Neither overexpression of isoform A nor overexpression of isoform B had an effect on the invasiveness of HCT116. This result differs from our expectation, which was that Isoform A would promote invasion and that isoform B would inhibit invasion. Power analyses conducted prior to experimentation suggested our sample size (n = 32) was sufficient to allow detection of greater than 95% of differences greater than or equal to the standard deviation of the dataset.

Our results conflict with reports in the literature. Previous studies have concluded that WNT5A both promotes (Bakker et al., 2013) and inhibits (Cheng et al., 2014) invasion in

HCT116. We could find no reports in the literature suggesting that WNT5A does not affect invasiveness in HCT116; in that capacity, ours is a novel finding.

There are a multitude of potential reasons that HCT116 might not respond to WNT5A overexpression insofar as cellular migration and invasion are concerned. It could be that HCT116 cells lack an appropriate receptor context to transduce a signal in response to WNT5A, or that HCT116 cells lack an appropriate receptor context to allow WNT5A to activate pathways related to cell migration or invasion. It also is possible that HCT116 lack some of the cellular machinery required to modify Wnt proteins such that they are fully active upon secretion.

WNT5A Isoform A and B Overexpression Stimulates neither the JNK Pathway nor the PKC Pathway in HEK293T/17

In order to determine if WNT5A isoforms A and B stimulate distinct Wnt signaling pathways, we transiently transfected HEK293T/17 with Isoform A, Isoform B, or GFP and analyzed JNK and PKC pathway activation by western blot analysis. HEK293T/17 was chosen because it is exquisitely transfectable and because it expresses a wide variety of Wnt receptors. We found that overexpression of isoforms A and B did not result in an increase in phosphorylated JNK or PKC relative to total JNK or PKC. This is rather surprising, given that these are by far the two most characterized non-canonical Wnt signaling pathways and that WNT5A is the paradigmatic non-canonical Wnt ligand.

It is highly unlikely that no Wnt signaling pathway is being activated in HEK293T/17 overexpressing WNT5A, given that we know the cells are producing WNT5A protein, and given that HEK293T/17 express a large number of putative Wnt receptors. One potential reason that no change in Wnt signaling was detected in these cells in response to overexpression of WNT5A isoforms is that overexpression simply activated a pathway whose downstream effector we did not analyze. It is also possible that the downstream effectors we did analyze were somehow

isolated from the Wnt signaling cascade. It is possible that WNT5A isoforms A and B did stimulate the Wnt signaling pathways, but that feedback and receptor internalization tempered the measurable response. Finally, it is possible that a signal was transduced, but that western blot analysis was unable to detect it. It is worth noting that we obtained results using a purified protein based approach that support our results obtained via overexpression.

WNT5A Isoform A and B are Differentially Regulated in Mouse Embryonic Development

Because WNT5A performs a broad variety of functions during embryonic development, and because of the extreme coordination of events required for embryonic development to be successful, we reasoned that an embryonic system would be fertile ground for detecting functional differences between isoforms. We sought to investigate first if isoform A and isoform B were expressed at different levels during mouse embryonic development, and if the level of expression of each changed over time in a distinct fashion for each isoform. We obtained RNA from staged mouse embryos, converted the RNA to cDNA, and used qPCR to determine expression levels for both isoform A and isoform B. Our results show that isoform A and isoform B are indeed regulated differently during mouse embryonic development. Isoform A expression is the same as isoform B expression at 10 and 12 DPC. Isoform A expression increases dramatically at 11 DPC, falls back to approximately its 10 DPC level at 12 DPC, and rises gradually at 13 and 14 DPC. Isoform B expression, meanwhile, stays relatively more stable from 10 DPC through 14 DPC. Isoform B expression does increase slightly at 11 DPC, but not as dramatically.

That WNT5A isoforms A and B are differentially regulated during embryonic development is a finding unreported elsewhere in the literature. Additionally, this constitutes a functional difference of sorts between the isoforms. This finding points to the possibility that these isoforms may exist, and may be conserved, in order to finely tune overall WNT5A expression during embryonic development. This study determined only a temporal pattern of

expression during the window from 10 DPC to 14 DPC, and did not look at spatial patterns of expression. It is possible that these isoforms are differentially regulated in a spatiotemporal fashion.

Conclusion and Future Studies

Our results suggest that WNT5A isoforms A and B are redundant in some contexts, and functionally distinct in others. We provide evidence that these isoforms have no effect on migration or invasion in HCT116, contrary to other reports in the literature. We show that overexpression of WNT5A isoforms A and B alone is not enough to stimulate terminal effectors in the PCP/CE pathway (JNK) or in the Wnt/Ca^{2+} pathway (PKC) in HEK293T/17. We also demonstrated that WNT5A isoforms A and B are differentially regulated during embryonic development, their expression levels changing over time independently of one another.

To further our study of the functional distinctions between isoform A and B of WNT5A with respect to invasion and migration, we would like to repeat all of our assays using recombinant purified protein. The use of recombinant protein abrogates questions of the suitability of HCT116 for transfection and Wnt protein expression entirely.

Additionally, we would like to conduct a signaling pathway analysis using cells treated with recombinant protein. The use of ELISA in place of western blot, particularly for targets where there is no single high grade antibody (e.g., STAT3), is a potential solution to what may well be a problem of assay sensitivity.

To further our study of the functional distinctions between WNT5A isoforms A and B in the context of embryonic development, we would like to perform several experiments. Firstly, we would like to determine if the isoform A and isoform B mRNAs are equally stable. Secondly, we would like to determine if WNT5A isoforms A and B are differentially regulated in a spatiotemporal fashion at the protein level. To do this, we would like to develop a transgenic

mouse expressing GFP driven by the isoform A promoter, and expressing RFP (red fluorescent protein) driven by the isoform B promoter. Staged embryos could then be harvested from these mice, cleared using a tissue clearing technique like CLARITY or 3DISCO, and imaged by confocal microscopy to determine the spatiotemporal pattern of expression for each isoform.

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