JOYNER-POWELL, NICOLE BUNA, M.S. Characterization and Regulation of *Wnt5a* Alternate Promoters A and B. (2011)
Directed by Dr. Karen S. Katula. 76 pp.

WNT5A is an extracellular glycoprotein that activates several Wnt signaling pathways important in cancer. Significantly, *Wnt5a* expression is altered in numerous cancers. Little is known about *Wnt5a* gene regulation but current data indicate that misregulation of *Wnt5a* expression involves non genetic changes. The goal of this study was to characterize transcriptional regulation from two alternate *Wnt5a* promoters, A and B. To analyze the relative level of expression from the human *Wnt5a* alternative promoters, promoter-luciferase reporter constructs containing different amounts of upstream sequence from promoters A and B were transiently transfected into NIH3T3 and Caco-2 cell lines. The relative level of promoter activity was compared by measuring luciferase activity in the transfected cells. Both *Wnt5a* promoters A and B were functional but found to have differential expression patterns in NIH3T3 and Caco-2 cells, indicating both positive and negative regulatory sequences. The transcription factor NFkappaB and the MAPK signaling pathway were studied to determine if they influence the transcriptional activity of *Wnt5a* promoters A and B. Stable lines of NIH3T3 cells with *Wnt5a* promoters A and B were treated for 6 and 24 hours with TNFalpha, a known inducer of NFkappaB activity, and inhibitors of MAPK pathway kinases (MEK1/2 and ERK). The cells were collected and assayed for firefly luciferase activity (relative light units) and standardized to DNA content. TNFalpha slightly increased promoters A and B activity at 6 hrs. TNFalpha had little or no effect on promoter A at 24 hours, whereas promoter B activity increased between 1.28 and 2.84

fold. The NFkappaB inhibitor, JSH-23, confirmed that NFkappaB is involved in the response of promoter B but not promoter A to TNFalpha. MEK1/2 inhibitor had inconsistent effects on promoter A, whereas promoter B activity decreases at both time points. ERK inhibitor increased promoter A activity for both 6 and 24 hours, whereas activity only increased for promoter B at 24 hours. These findings were further examined by measuring the relative contribution of *Wnt5a* alternative promoter A and B endogenous transcripts in both NIH3T3 and GM03349. Custom primers that amplify and detect human and mouse *Wnt5a* promoters A and B specific transcripts were created to analyze the relative amount of each transcript by qRT-PCR. Results show that promoter A generates more transcripts than promoter B and that TNFalpha increases the activity of both promoters in mouse and human fibroblasts. Overall, these data indicate that *Wnt5a* promoters A and B are differentially regulated and that NFkappaB influences transcriptional activity of *Wnt5a* promoter B rather than promoter A.

CHARACTERIZATION AND REGULATION OF *WNT5A* ALTERNATE PROMOTERS A AND B

by

Nicole Buna Joyner-Powell

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

Greensboro
2011

Approved by

_____
Committee Chair

To my husband, Adrian, who sacrifices and supports me so I can realize my dreams.

APPROVAL PAGE

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

Committee Chair_____

Committee Members _____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGMENTS

I would first like to thank my advisor and committee chair Dr. Karen Katula. Without her help and guidance this could not have been possible. Her patience, support and knowledge were key components in completing this project and I am grateful for her hard work and dedication.

I would also like to thank my committee members Dr. Yashomati Patel and Dr. Amy Adamson for their suggestions and time. I would also like to acknowledge Dr. Parke Rublee for his suggestions and thoughts. I would also like to thank present and former members of the lab, especially Amit Patel and Chia-Chi Hsu, for their contributions to this research.

Lastly, I would like to thank my family and friends for their support and encouragement in this project and my future endeavors.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Statement of Problem

Cancer continues to be prevalent in the US with an estimated 11.7 million people living with the illness (American Cancer Society, 2007). It is a complex disease that results from both genetic and nongenetic molecular alterations in numerous cellular pathways involved in cell cycle and growth control, apoptosis, DNA repair, cell movement and cell adhesion. One pathway often altered in transformed cancer cells is the Wnt signaling pathway. In this study, the focus was on the Wnt ligand, WNT5A. WNT5A, a member of the Wnt protein family, is an extracellular glycoprotein that activates both the canonical and noncanonical pathways (McDonald and Silver, 2009). *Wnt5a* expression has been found to be altered in numerous cancers including lung, breast, thyroid, osteosarcoma, gastric, colorectal, leukemias, skin, pancreatic and prostate (reviewed in Katoh and Katoh, 2009). While *Wnt5a* has been shown to be down regulated in several cancers such as colon and breast cancer (Ying et al., 2008; Leris et al., 2005), it has also been shown to be over expressed in gastric and pancreatic cancer (Kurayoshi et al., 2006; Ripka et al., 2007). Thus, it can function as either an oncogene or tumor suppressor. Significantly, over expression of *Wnt5a* has been associated with

metastasis in pancreatic cancer (Ripka et al., 2007) and melanoma (Weeraratna et al., 2002). Current published data indicate that changes in *Wnt5a* expression during cancer progression do not involve genetic (DNA) changes such as gene mutation, amplification, or deletion but rather non genetic, epigenetic changes and alterations in various signaling pathways.

Little is known about how the *Wnt5a* gene is regulated, however, there is some evidence that NFkappaB can influence *Wnt5a* expression. NFkappaB is a transcription factor that mediates many genes that influence growth and inflammation and has been shown to be upregulated in cancer (Basseres and Baldwin, 2006), possibly due to its proinflammatory nature. In this study we determined if *Wnt5a* expression changes with increased NFkappaB activity. In addition, we examined the role of the MAPK pathway in *Wnt5a* expression, as MAPK signaling is often altered in transformed cells. For these studies, we analyzed transcription from two of the *Wnt5a* alternative promoters in both mouse and human cells to determine the relative contribution of each promoter to total *Wnt5a* transcript levels. These alternative promoters were also characterized using promoter- reporter vectors. Results from these studies have increased understanding of *Wnt5a* transcriptional regulation and its altered expression in transformed cells.

Wnt Signaling and Wnt5a

Wnts are a class of 19 extracellular secreted proteins that play a role in development, proliferation and apoptosis (Nishita et al., 2010). They are hydrophobic signal peptides with no transmembrane domain. Wnts are typically ligands for the

Frizzled (Fzd) receptor and low density lipoprotein receptor-related proteins (LPR) but do not solely signal through these receptors. There are two classes of Wnt proteins: canonical, which results in an increase of beta-catenin through a signal transduction pathway, and non-canonical, which is any pathway that does not result in an increase of beta-catenin. Wnt activation through the canonical pathway is vital for normal animal development, while deregulation of this pathway can result in cancer cell proliferation and cell migration. Non-canonical Wnts are known to antagonize canonical Wnts and control morphogenic movements through activation of the planar cell polarity (PCP) pathway and the Wnt Ca^{2+} signaling pathway (McDonald and Silver, 2009). Although other Wnts have been studied and linked to cancer, including Wnt 1, 3, and 7, WNT5A has more recently been shown to be altered in transformed cells (Pukrop and Binder, 2008). WNT5A has traditionally been known to signal through the non-canonical pathway; however, recently WNT5A has also been shown to signal in canonical pathways (Nishita et al., 2010; McDonald and Silver, 2009). Because WNT5A seems to signal multiple pathways, establishing it to be a cancer suppressor or proliferator has been difficult.

Wnt5a's Involvement in Cell Function and Development

WNT5A has several normal physiological roles that may indicate its role in cancer development and progression. WNT5A plays a role in mesenchymal differentiation, colon epithelial development, cell movement and morphogenesis (Pukrop and Binder, 2008). Yamaguchi et al. (1999) explored the importance of *Wnt5a*

expression in the outgrowth structures of mouse embryos. They detected *Wnt5a* in the distal parts where limbs, digits and face outgrowths are located. They also created mice with an inactive *Wnt5a* gene, which died shortly after being born. These embryos were examined and were found to have truncated limbs and underdeveloped digits as a result of decreased progenitor cell proliferation. Thus, *Wnt5a* may function as a regulator of cell division during development, providing evidence for its role in cancer development and progression. More recently, *Wnt5a* has also been shown to be involved in adipogenesis and promote adipocyte differentiation (Nishizuka et al., 2008). Interestingly, obese adipose tissues have higher levels of *Wnt5a* secretion resulting in increased insulin resistance through activated JNK1 and proinflammatory cytokines release from active macrophages (Oh and Olefsky, 2010). *Wnt5a* also contributes to central nervous system maturation by regulating midbrain morphogenesis (Andersson et al., 2008), stimulating synaptic differentiation (Varela-Nallar et al., 2010), inducing cortical axon outgrowth (Li et al., 2010), and promoting interneuron progenitors differentiation (Paina et al., 2011). *Wnt5a* has also been shown to be involved in prostate gland development (Huang et al., 2009), gastrulation (Hardy et al., 2008), intestinal elongation (Cervantes et al., 2009), and chondrocyte proliferation (Yang et al., 2003).

Wnt5a and Cancer

Wnt5a expression has been found to be altered in numerous cancers including lung, breast, thyroid, osteosarcoma, gastric, colorectal, leukemias, skin, pancreatic and

prostate (reviewed in Katoh and Katoh, 2009). While *Wnt5a* has been shown to be down regulated in several cancers such as colon and breast cancer (Ying et al., 2008; Leris et al., 2005), it has also been shown to be over expressed in gastric and pancreatic cancer (Kurayoshi et al., 2006; Ripka et al., 2007). Thus, *Wnt5a* can function as either an oncogene or tumor suppressor. Significantly, over expression of *Wnt5a* has been associated with metastasis in pancreatic cancer (Ripka et al., 2007) and melanoma (Weeraratna et al., 2002). In one particular article, Weeraratna et al. (2002) examined the role of *Wnt5a* in melanoma metastasis. Melanoma cells from a patient were transfected with vectors that constitutively express *Wnt5a*. These cells showed increased PKC (protein kinase C) activity, a protein known to play a role in cytoskeleton changes and cell motility. In addition to measuring PKC activity, Weeraratna et al. also studied the effects of reduced Wnt pathway activity. Using an antibody against Frizzled-5, the WNT5A receptor, they were able to disrupt Frizzled-5/WNT5A interaction in the *Wnt5a* transfected cells and in other cells that have high endogenous *Wnt5a* expression. The cells treated with the antibody were shown to have decreased motility in scratch assays and in the Boyden chamber invasion assay. The cells that were treated with the Frizzled-5 antibody were also shown to have decreased activity of PKC. These studies strongly suggest a role of *Wnt5a* in cell movement during metastases.

In osteosarcoma cells, signaling by WNT5A through the noncanonical pathway Ror2 receptor was shown to be responsible for invasiveness of the osteosarcoma cells (Ren et al., 2011; Enomoto et al., 2009). Suppressed expression of either WNT5A or Ror2 in the osteosarcoma cells inhibited invasiveness, including decreased invadopodia

formation (Enomoto et al., 2009). In another study, WNT5A was shown to be bound to Frizzled2 at the edge of migrating cells and to regulate focal adhesion (Matsumoto et al., 2010). Also, *Wnt5a* expression is upregulated during epithelial-mesenchymal transition in human squamous carcinoma cells (Taki et al., 2003). These studies suggest that *Wnt5a* contributes to metastasis by regulating adhesion junctions, invasiveness, and regulating the epithelial-mesenchymal transition.

It is not clear, however, why *Wnt5a* expression is upregulated in some cancers and downregulated in others. One suggestion is that the tumor cell type and microenvironment coupled with the complex regulation of *Wnt5a*, involving multiple pathways and epigenetic mechanisms, leads to these different responses of *Wnt5a* (Pukrop and Binder, 2008). It is also possible that *Wnt5a* expression varies during transformation such that during progression, *Wnt5a* is upregulated when the tumor cells become metastastic. Regardless, a more complete analysis of the *Wnt5a* gene regulatory region and its transcriptional regulation will provide insights in to how the gene is misregulated during tumorigenesis.

Wnt5a Gene Structure and Regulation

The gene for human *Wnt5a* is located between 55,499,744 and 55,521,331 base pairs on the p14.3 band of chromosome 3 (Entrez; Gene ID 7474). Multiple transcripts are generated from the *Wnt5a* transcription unit due to alternative splicing and distinct starts of transcription (Ensembl; figure 1). One well described transcription unit has 5 exons and 4 introns, producing an mRNA approximately 5,855 nucleotides long (Ensembl;

ENST00000264634, figure 1). The protein derived from this transcript has 380 amino acids. The more well characterized promoter is located upstream of exon 1 of this *Wnt5a* transcription unit. There is, however, evidence of another promoter located in the first intron of this transcript (ENST00000497027, figure 1). These two promoters have different exon 1's, referred to as 1a and 1b, but have exon 2, 3, 4 and 5 in common (figure 2).

Figure 1: Human *Wnt5a* Transcripts. The two transcription units analyzed in this study are boxed. The ENST00000264634 transcript correlates with *h*promoter A. The ENST00000497027 transcript correlates with *h*promoter B.

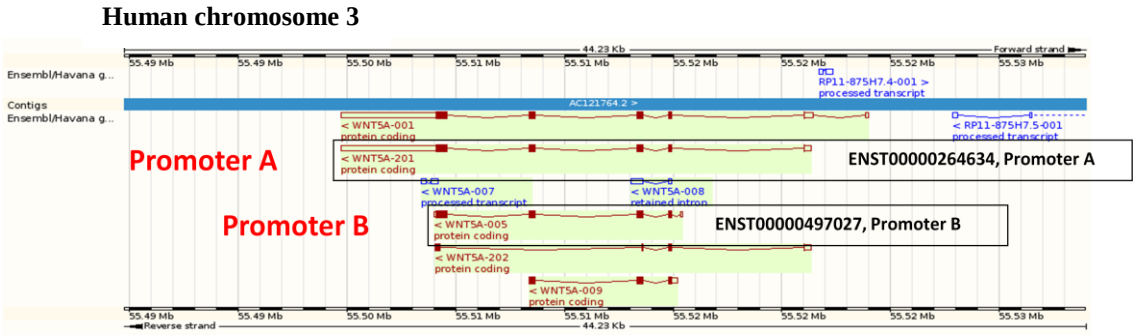
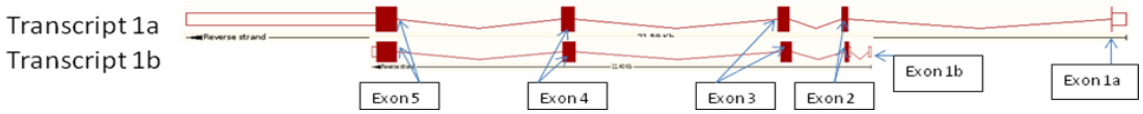


Figure 2: Human *Wnt5a* Transcripts 1a and 1b. Exon 1b is located in the intron of transcript 1a. Exons 2, 3, 4, and 5 are the same for both transcripts.



In this study, the promoter associated with the transcript beginning at exon 1a is referred to as *h*promoter A (h=human), whereas the promoter associated with exon 1b will be referred to as *h*promoter B. Distinct *Wnt5a* cDNAs have been isolated for transcripts derived from *h*promoter A (NM_003392) and *h*promoter B (AK290869), providing support for transcription from the alternate start sites. The protein derived from the *h*promoter B transcript lacks the first 15 amino acids of the *h*promoter A protein (figure 3). However, the final protein product is identical due to removal of the endoplasmic reticulum (ER) signal sequence found in both proteins. Thus, the two alternate transcripts result in the same protein after translation and post translational modifications.

Further evidence of a functional significance of alternative promoters is the homology between the human and mouse *Wnt5a* genes. The mouse *Wnt5a* gene is located on chromosome 14 and has 5 exons as well. The mouse *Wnt5a* has a similar gene structure as the human with multiple transcripts derived from both alternative splicing and transcription starts (figure 4). The 4273 base pair mRNA also results in a 380 amino acid protein. There are two transcripts, #1a (Ensembl; ENSMUST00000063465) which is similar to the *h*promoter A transcript, and #2 (Ensembl; ENSMUST00000112272) which is similar to *h*promoter B transcript. The promoter associated with the transcript #1a will be referred to as *m*promoter A and promoter associated with the transcript #2 will be referred to as *m*promoter B. Characteristics of the human and mouse *Wnt5a* gene are listed in table 1.

Figure 3: Human Wnt5a Protein Sequences. Isoform B lacks the first 15 amino acids found in isoform A. However, these 15 unique amino acids are cleaved off during protein modification resulting in identical proteins.

Isoform A – Human Wnt5a Promoter A (NM_003392)

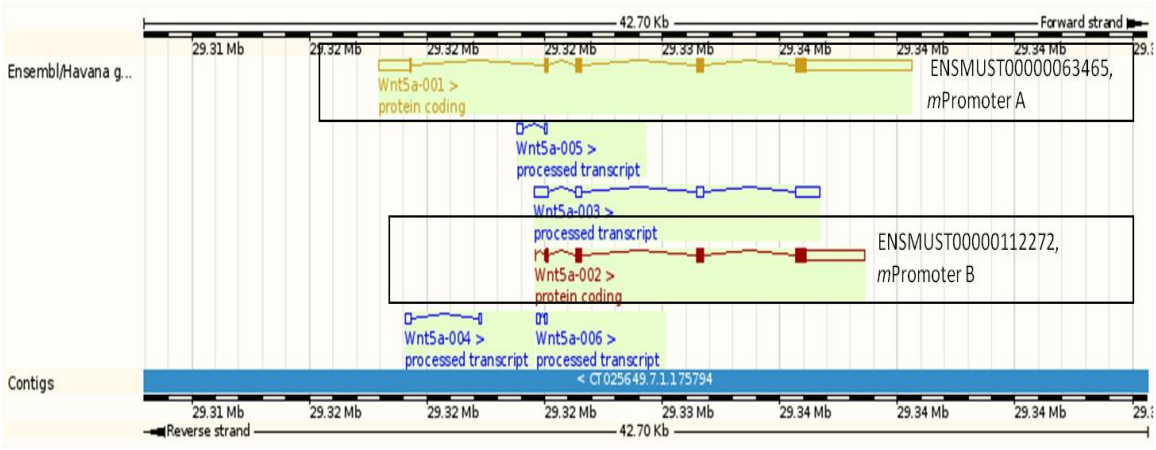
MKKSIGILSPGVALGMAGSAMSSKFFLVALAIFFS….

Unique to Isoform A

Isoform B – Human Wnt5a Promoter B (AK290869)

MAGSAMSSKFFLVALAIFFS

Figure 4: Mouse *Wnt5a* Transcripts. The two transcription units analyzed in this study are boxed. The ENSMUST00000063465 mouse transcript (#1A) correlates with *h*promoter A, which we refer to as *m*promoter A. The ENSMUST00000112272 (#2) mouse transcript correlates with *h*promoter B which we refer to as *m*promoter B.



A comparison of the 4000 bp sequences upstream of the *h*promoter A transcription unit to 4000 bp sequences upstream to the mouse transcript #1a start of transcription reveals little homology (figure 5). However, the mouse exon 1 is larger than the human exon 1 and extends further upstream. Consequently, a comparison was made between 1400 bp upstream of the ATG, start of translation, in exon 1 of mouse and human *Wnt5a* transcript (figure 6). There appears to be greater homology between a 500 bp region upstream of the human exon 1 and a region contained within mouse exon 1. A comparison was also made between intron 1 of the mouse and human promoter A transcript. This would include the promoter B exon 1b (figure 7). The region of highest homology appears in the region including the promoter B exon 1 and near the splice junctions at both ends. Together, these comparisons support the conclusion that the mouse and human *Wnt5a* share conserved regulatory sequences in the region of promoters A and B. This suggests that the *h*promoters A and B and the *m*promoters A and B are functionally important and that they share regulatory sequences.

Table 1. Comparison of Mouse and Human *Wnt5a* Genes [1]

	Mouse	Human
Location	Chromosome 14 29,317,936-29,340,633	Chromosome 3 55,499,743-55,523,973
Number of Transcripts	7	7
Direction of Transcription	Forward	Reverse
Transcript size (bases) and ID [2]	7009 basepairs (ID-063465) 3650 basepairs (ID-112272)	6042 basepairs (ID-474267) 1299 basepairs (ID-497027)
Proteins Produced	2	5
Protein Length (AA) and ID [3]	380 residues (ID-064878) 360 residues (ID-107891)	380 residues (ID-417310) 365 residues (ID-420104)
Number of Exons [4]	5 (ID-063465) 5 (ID-112272)	5 (ID-474267) 5 (ID-497027)

[1]Source: Mouse *Wnt5a* ENSMUSG00000021994 and Human ENSG00000114251. [2] For the two transcripts analyzed in this study. Transcript ID is preceded by ENSMUST000000. [3] Derived from the two transcripts analyzed.

Figure 5: No Homology Between Human and Mouse Promoters A and B 4000 bp Upstream. This graph was generated using the MacVector Pustnell DNA matrix application. Homologous regions are indicated by the dark lines or spots on the diagonal.

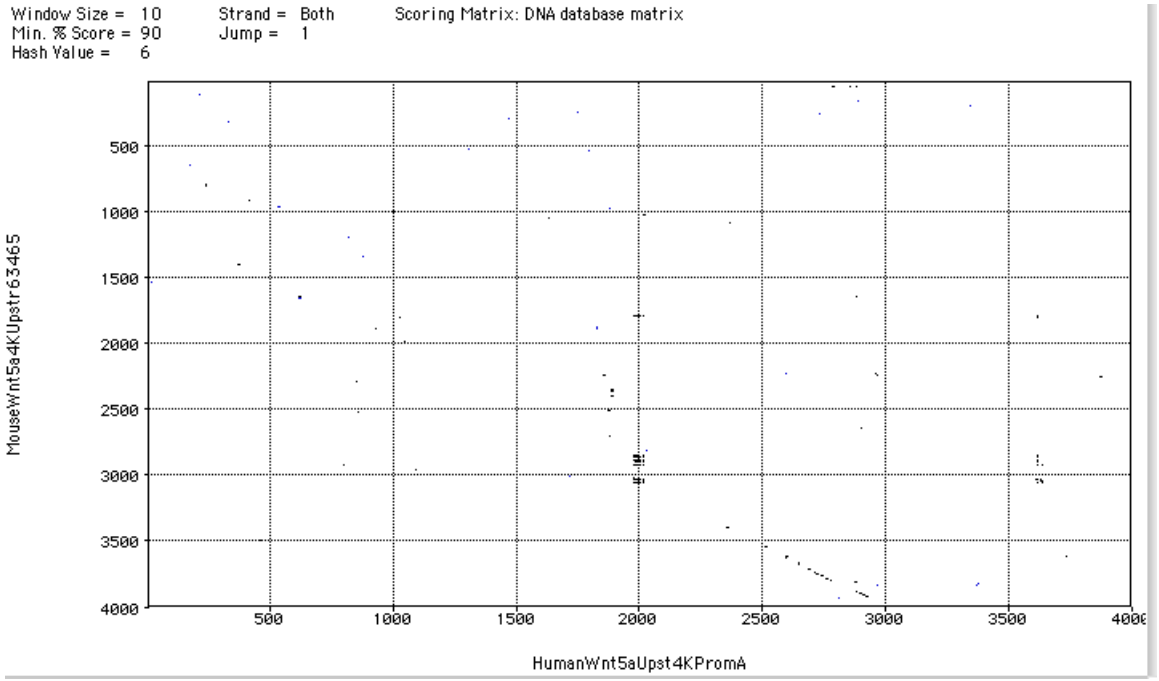


Figure 6: Homology Between Human and Mouse Promoter A. Comparison between 1400 bp upstream of the ATG start of translation of mouse and human *Wnt5a* corresponds with Promoter A. This graph was generated using the MacVector Pustnell DNA matrix application. Homologous regions are circled and indicated by the dark lines or spots on the diagonal.

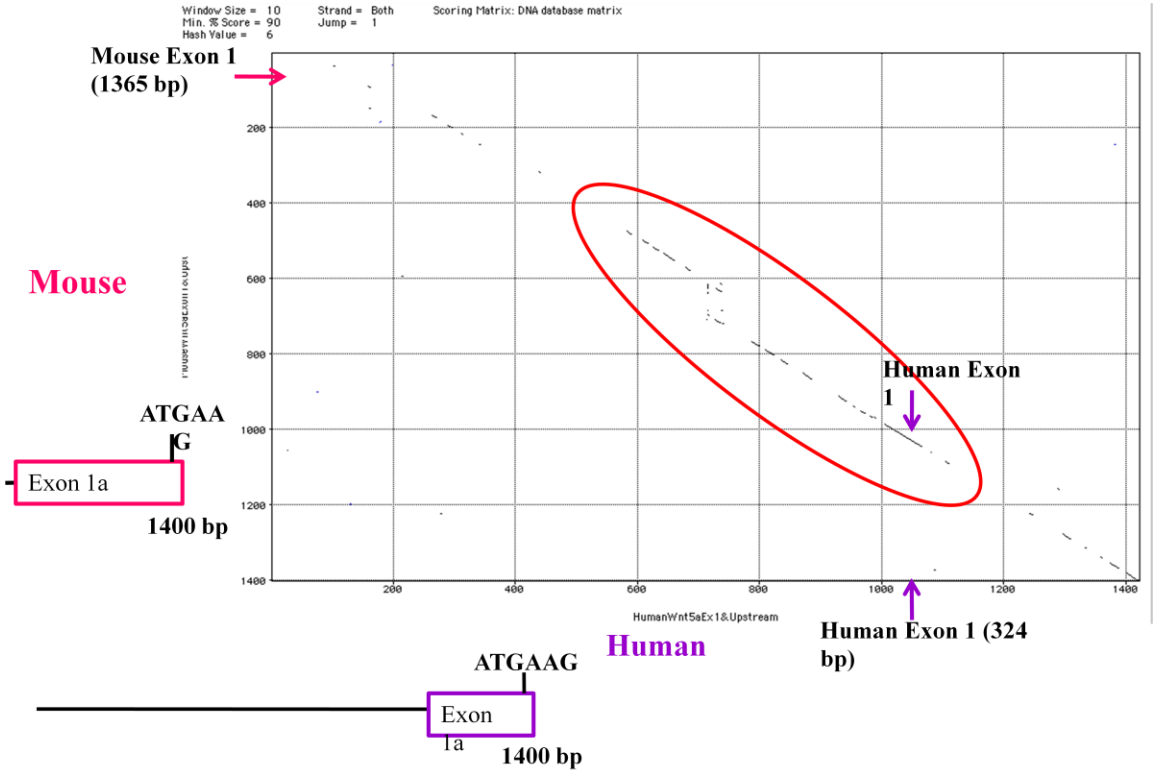
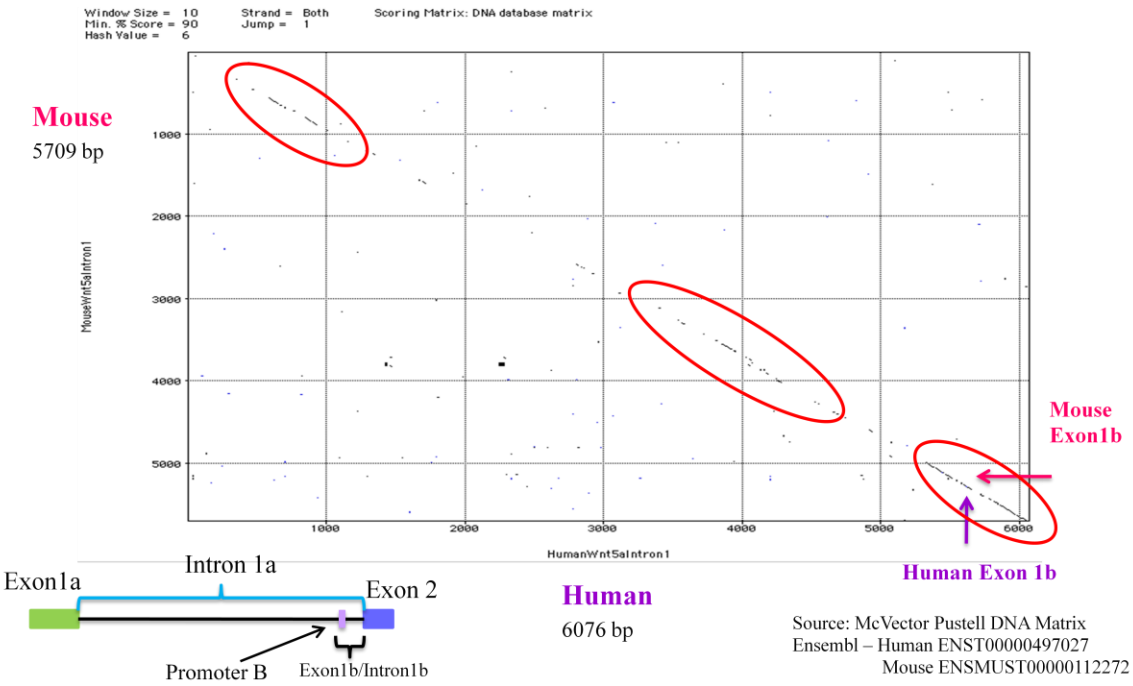


Figure 7: Homology Between Human and Mouse Promoter B. Comparison between intron 1 of mouse and human *Wnt5a* corresponds with Promoter B. These graphs were generated using the MacVector Pustnell DNA matrix application. Homologous regions are circled and indicated by the dark lines or spots on the diagonal.



Alterations in Wnt5a Expression

Current published data indicate that changes in *Wnt5a* expression during cancer progression do not involve genetic (DNA) changes such as gene mutation, amplification, or deletion but rather non genetic, epigenetic changes and alterations in various signaling pathways (Ying et al., 2008). The *Wnt5a* gene has been shown to be both hypo- and hypermethylated in various cancer types. Hypermethylation of the *Wnt5a* gene has been shown in acute myeloid and lymphoblastic leukaemia (Martin et al., 2010; Roman-Gomez et al., 2007) and colorectal cancer (Ying et al., 2008, Hibi et al., 2009), whereas hypomethylation has been shown in prostate cancer (Wang et al., 2007). *Wnt5a* expression is also regulated by various transcription factors and signaling pathways. Transcription factors CUTL1 and PAX2 have been shown to bind to the *Wnt5a* promoter (Ripka et al., 2007; Tamimi et al., 2008) and activated Ras, a member of the mitogen activated protein kinase (MAPK) signaling pathway, has been shown to decrease *Wnt5a* expression (Bui et al., 1997). In another study, SW480 cells, a human colon cancer cell line, underwent amino acid deprivation to induce the MAPK pathway (Wang and Chen, 2009). There was an increase of phosphorylated ERK1/2, which also led to a downregulated *Wnt5a* expression. There is some limited evidence that NFkappaB influences *Wnt5a* expression. NFkappaB is a transcription factor that mediates many genes that influence growth and inflammation and has been shown to be upregulated in cancer (Basseres and Baldwin, 2006), possibly due to its proinflammatory nature. Ge et al. (2011) found that overexpression of NFkappaB induces *Wnt5a* expression. Furthermore, ChIP analysis showed NFkappaB has the ability to bind to the *Wnt5a*

promoter. *Wnt5a* expression was also upregulated by TNFalpha, a known inducer of NFkappaB, in MKN45 cells derived from gastric cancer (Saitoh and Katoh, 2002). These studies were either focused on the *Wnt5a* regulatory sequences upstream of *h*promoter A exon 1 or general *Wnt5a* mRNA expression. Recent bioinformatic analyses have identified some possible NFkappaB binding sites in both *h*promoters A and B (Katoh and Katoh, 2009). However, in the literature there has been little exploration of *h*promoter B and its associated regulatory sequences in relation to *h*promoter A.

Study Overview and Specific Aims

Wnt5a is important in development and differentiation and is involved in regulation of cell growth control, differentiation and movement. Expression of *Wnt5a* is often altered during tumorgenesis, due to nongenetic mechanisms. In spite of its importance, current understanding of *Wnt5a* gene regulation is limited and questions remain. In this study, I addressed some of these questions. In particular, transcription from two of *Wnt5a* alternate promoters were analyzed from the endogenous gene and using a promoter-reporter vector. Also, the role of the transcription factor NFkappaB and MAPK signaling pathway was examined. Specifically, I had three aims: (1) analyze the relative level of expression from the human *Wnt5a* alternative *h*promoters, A and B using luciferase reporter vectors; (2) determine the contribution of the transcription factor NFkappaB to transcription from *Wnt5a* alternative *h*promoters A and B and involvement of the MAPK signaling pathway; and (3) determine the relative contribution of transcripts

from *Wnt5a* alternative promoters A and B to *Wnt5a* mRNA levels from the genomic *Wnt5a* gene sequences.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

NIH3T3 mouse fibroblasts originally obtained from the American Type Culture Collection (ATCC, Manassas, VA) were plated in a T-75 flask (Falcon, Franklin Lakes, NJ) using DMEM (Mediatech, Herndon, VA), supplemented with 10% calf serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (5000 I.U./mL and 5000 ug/mL) (MP Biomedicals, Solon, OH). This complete medium is referred to as DMEM-C. The cells were grown in a 37ºC and 5% carbon dioxide humidified incubator. The cells were collected by trypsinization when at 80% confluency. Essentially, the cells were washed with Ca^{2+}-Mg^{2+} free Hank's (400 mg/L KCl, 60 mg/L KH_2PO_4, 350 mg/L $NaHCO_3$, 8 g/L NaCl, 90 mg/L $Na_2HPO_4 \cdot 7H_2O$, 1 g/L D-glucose) and 4-5 mL 0.05% Trypsin and 0.53 mM EDTA was added. After one minute, the cells were microscopically examined. Once released from the plate, an equal volume of DMEM-C was added and the cell mixture transferred to a sterile 15 mL tube. The cells were pelleted by centrifugation, and resuspended in 5 mL DMEM-C. The cell density (cells/mL) was determined using a hemocytometer. Cells were re-plated at different densities depending on the experiment.

Caco-2, a human colorectal adenocarcinoma derived cell line, was obtained from ATCC. The Caco-2 cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin (5000 I.U./mL and 5000 ug/mL) (MP Biomedicals, Solon, OH). The cells were cultured in a 37ºC and 5% carbon dioxide humidified incubator. Caco-2 cells were maintained at sub-confluence densities. Cells were re-plated at $1x10^4$ cells per cm^2. Cells were collected as described for NIH3T3 with the following exceptions: The trypsin solution was 0.25% Trypsin and 1.0 mM EDTA and trypsinization was at 37ºC for 3 to 5 minutes.

GM03349 cells are normal human dermal fibroblast cells obtained from National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository (Corriell, Camden, NJ). They are from a 10 year old male. They were grown in Minimum Essential Medium (MEM) (Mediatech, Inc, Manassas, VA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 2 mM L-glutamine and 1% penicillin/streptomycin (5000 I.U./mL and 5000 ug/mL) (MP Biomedicals, Solon, OH). The cells were cultured in a 37ºC and 5% carbon dioxide humidified incubator.

Transient Transfections and Dual Luciferase Assays

NIH3T3 cells (6×10^4 cells/well) and Caco-2 cells (4×10^4 cells/well) were plated in 24-well plates (AGadvangene, Lake Bluff, IL) and incubated for 24 hours. The *h*promoter A and B constructs (0.5 ug) and the parental plasmid pGL4.17 (Promega,

Madison, WI) (0.5 ug) were transiently transfected into NIH3T3 cells using TransIT-LT (Mirus, Madison, WI) and Caco-2 using Nanojuice (Novagen, San Diego, CA), along with 10 ng of a *Renilla* control vector (pHRL-SV40) according to instructions. Each construct was transfected into four wells of cells. After 24 hours the cells were collected. Essentially, the media was removed and the cells were washed with 1x Phosphate Buffered Saline (PBS) (NaCl 81%, Na_2HPO_4 ≈14%, KH_2PO_4 ≈3%, KCl ≈2%) (Fisher Scientific, Cat# BP661, Pittsburgh, PA). The PBS was removed and 1x Passive Lysis Buffer (Promega, Madison, WI) was added to each well. The samples were incubated at 37ºC for 15 minutes and the entire plate placed at -80ºC. When assayed, the plates were removed from the freezer and the samples allowed to thaw to room temperature. 20 uL of lysate from each sample was added to a black-sided 96 well plate (Corning Inc, Corning, NY) to be assayed for luciferase activity on a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont) using the Dual-Luciferase Assay System (Promega, Madison, WI). The Dual-Luciferase Assay system was used to determine firefly and *Renilla* luciferase activity in the same sample. The raw firefly RLU values for each sample was divided by the *Renilla* RLU values to control for DNA uptake during transfection. The average firefly RLU/*Renilla* RLU value was graphed for each construct. The standard error was determined for the four values for each construct. The transfection was repeated three independent times using newly plated cells.

Treatment of Wnt5a Promoter A and B Stable Cell Lines

NIH3T3 cell lines stably transfected with the full length *Wnt5a h*promoters A p2178and B p1981 reporter constructs were utilized in these studies. These cells were plated in 48 well plate (AGadvangene Lifescience Plasticware, Lake Bluff, IL) at two different densities for the different treatments: $2x10^4$ cells per well for 6 hour treatments and 1.5 $x10^4$ cells per well for 24 hour treatments. The next day, the cells were treated with TNFalpha (Thermoscientific, Rockford, IL), dimethyl sulfoxide (DMSO) (ATCC), NFkappaB Activation Inhibitor II (JSH-23) (Santa Cruz Biotechnology, Santa Cruz, CA), 1-fluoro-2-[2-(4-methoxyphenyl)ethenyl]-benzene (NFkappaB inhibitor CAY105512) (Cayman Chemical, Ann Arbor, MI), 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione,HCl (ERK inhibitor) (Calbiochem, Gibbstown, NJ) or U01296 (MEK 1/2 Inhibitor) (Promega, Madison, WI). TNFalpha and the various inhibitors were added to tubes of fresh medium to their final concentrations. The medium was removed from the cells and replaced with the appropriate medium plus TNFalpha and\or inhibitors. The stock and final concentrations used in these studies were TNFalpha (1 ng/ul; 5 ng/ml), JSH-23(40 mM; 30 uM), CAY105512 (10 mM; 1 uM), ERK inhibitor (40 mM; 25uM), U01296 (10 mM; 10 uM). All compounds were prepared in DMSO with the exception of TNFalpha which was prepared in 1% BSA in PBS. Control cells were either left untreated in studies of TNFalpha only or DMSO at the same percent concentration as the inhibitors. Incubation was for 6 and 24 hours. The cells were collected by removing the media and washing with 1x PBS. 100 ul of 1x Cell Lysis Buffer (Promega, Madison, WI) was added to each well and the entire plate placed at -

80ºC. When assayed, the plates were removed from the freezer and the samples allowed to thaw to room temperature. 20 ul of lysate from each sample was added to a black-sided 96 well plate (Corning Inc, Corning, NY) to be assayed for luciferase activity (relative light units or RLUs) on a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont) using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Total DNA content was determined as a measure of cell number by assaying 20 ul of lysate using the CyQUANT NF Cell proliferation Assay (Invitrogen, Carlsbad, CA). The RLUs were standardized to DNA content and results were graphed for comparison between the different treatments. Each study was independently replicated 4-6 times. Standard error values were determined and significance using the student t-test ($p<0.05$) for replicas.

RNA Isolation and Real Time PCR

NIH3T3 cells and GM03349 cells were plated in 100 mm dishes. After reaching 80% confluency, the cells were treated with 5 ng/ml TNFalpha (Thermoscientific, Rockford, IL), ERK inhibitor (25 uM) (Calbiochem, Gibbstown, NJ) or MEK 1/2 inhibitor U01296 (10 uM) (Promega, Madison, WI) for 6 and 24 hours as previously described. The cells were collected by first washing them twice with cold PBS. 5 ml of cold PBS was added and the cells were scraped off the plate. The cell suspension was transferred to a 15 ml conical tube and centrifuged at 3,000 RPM (1,000 x g) at 4 ºC for 5 minutes in an IEC Centra GP8R centrifuge (ThermoScientific, Asheville, NC). The

supernatant was removed and the cell pellet quick froze in liquid nitrogen and placed at -80 ºC. The total RNA was isolated from the cells using the SV Total RNA Isolation Kit (Promega, Madison, WI). The concentration and 260/280 ratio was determined using the Take3 module on a Synergy 2 multi-mode microplate reader (BioTek, Winooski, Vermont). 1-3 ug of RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) or the Maxima Reverse Transcriptase (Fermentas, Glen Burnie, Maryland).

Quantitative RT-PCR was conducted using the Applied Biosystem TaqMan Assay (Applied Biosystems, Foster City, CA). For NIH3T3 cells, primers used for amplification included mouse *Wnt5a* (Mm03053674_s1) and mouse cyclin D1 (Mm00432358), which served as a positive control in the case of TNFalpha stimulation. In addition, custom primers were prepared that amplified mouse *Wnt5a* transcripts derived specifically from promoter A (AILJICZ) and promoter B (AIMSGI7). For the human GM03349 cells, human *Wnt5a* (Hs00180103) and human cyclin D1 (Hs00765553) were used, as well as custom human *Wnt5a* derived transcripts from promoter A (AIFARB5) and promoter B (AID1S5X). Each amplification included internal control primers either GAPDH (mouse-Mm03302249_g1; human- Hs99999905), or ribosomal RNA (mouse-Mm01974474_gH; human-Hs01562845_s1). A master mix for each target was prepared containing the 1x TaqMan Gene Expression Master Mix and primers. This was aliquoted into separate tubes for each cDNA sample. Sample cDNA was added and 10 ul pipetted into 3 wells of either a 48 or 96 well plate. A test

amplification was generally conducted to determine the dilution factor for the cDNA. cDNA was diluted 1:2- 1:5 for most experiments.

Quantitative RT-PCR was conducted on the Applied Biosystems StepOne Real Time PCR System thermal cycling block. Amplification conditions were 95ºC for 15 seconds and 60ºC for 1 min for 40 cycles. The C_T ($\Delta\Delta C_T$) value was determined for each cDNA sample using Microsoft Excel formula provided by R. Paules (National Institute of Environmental Health Sciences). C_T ($\Delta\Delta C_T$) is a measure of the relative level of transcripts, standardized to the endogenous control.

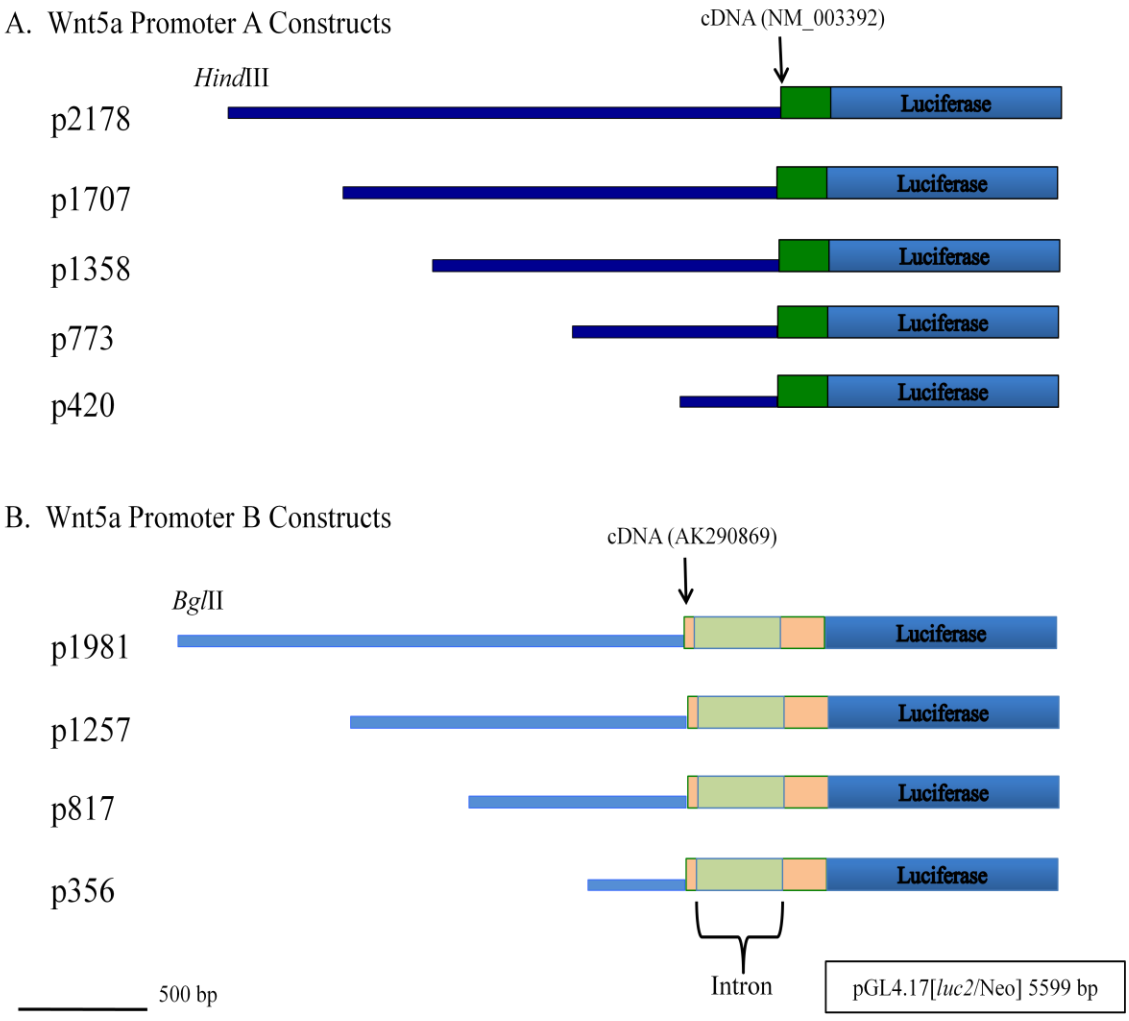
CHAPTER III

RESULTS

Relative Expression Levels of the Human Wnt5a Alternative Promoters, A and B

To examine the activity and regulation of the human *Wnt5a* alternative promoters A and B, deletion constructs previously created in the lab were used. *Wnt5a h*promoter A constructs containing fragments of 2178 bp, 1707 bp, 1358 bp, 773 bp and 425 bp were cloned upstream of the firefly luciferase gene of the pGL4.17-basic vector (figure 8). Likewise, similar constructs were created for *h*promoter B containing fragments of 1981 bp, 1257 bp, 817 bp and 356 bp (figure 8). The location of these sequences within the *Wnt5a* genomic region and the primers used to generate the fragments for cloning are shown in Appendix A. The *h*promoter A and B deletion constructs and the empty pGL4-basic were transiently transfected, along with a *Renilla* vector as a control for DNA uptake, into NIH3T3 (mouse fibroblast cells) and Caco-2 (human colorectal epithelial adenocarcinoma cells). After 24-48 hours, the cells were collected and assayed for firefly and *Renilla* luciferase activity. The firefly luciferase activity or relative light units (RLUs) was standardized to the *Renilla* RLUs for the same sample and graphed for comparison of each deletion construct's relative expression in both cell types.

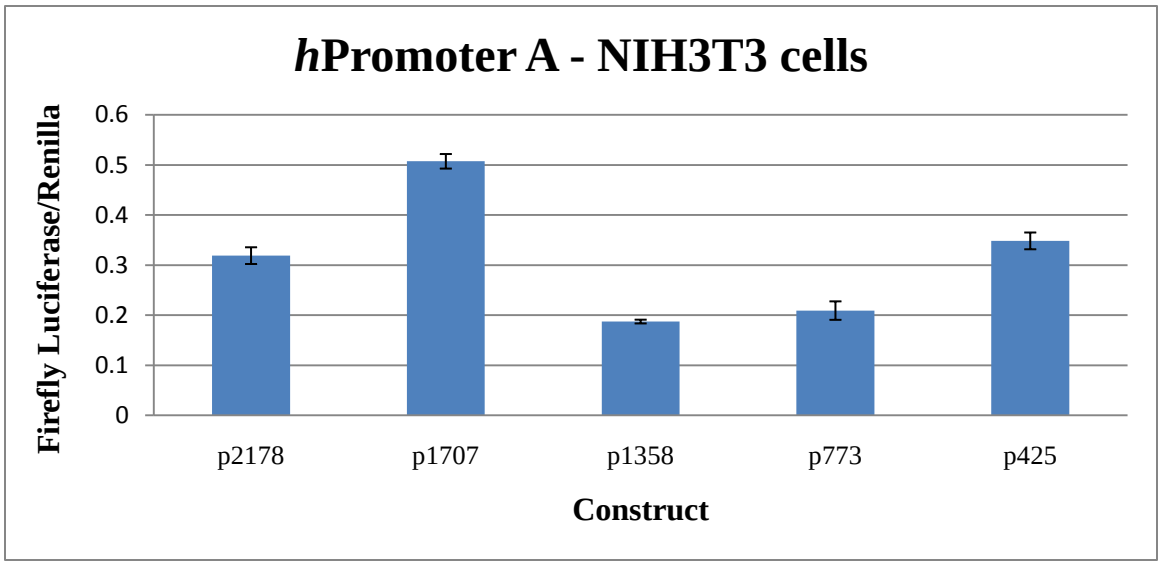
Figure 8. *Wnt5a h*Promoters A and B Luciferase Reporter Constructs. The numbers are base pairs (bp) upstream from the first nucleotide of the cDNA as indicated. The *h*promoter A fragments were cloned into pGL4.17 using a *Hind* III site, whereas *Bgl* II was used for *h*promoter B fragments. *h*Promoter B constructs contain a 412 bp intron. The scale bar applies only to the *Wnt5a* upstream sequences. The sequence of the upstream regions can be found in Appendix A.

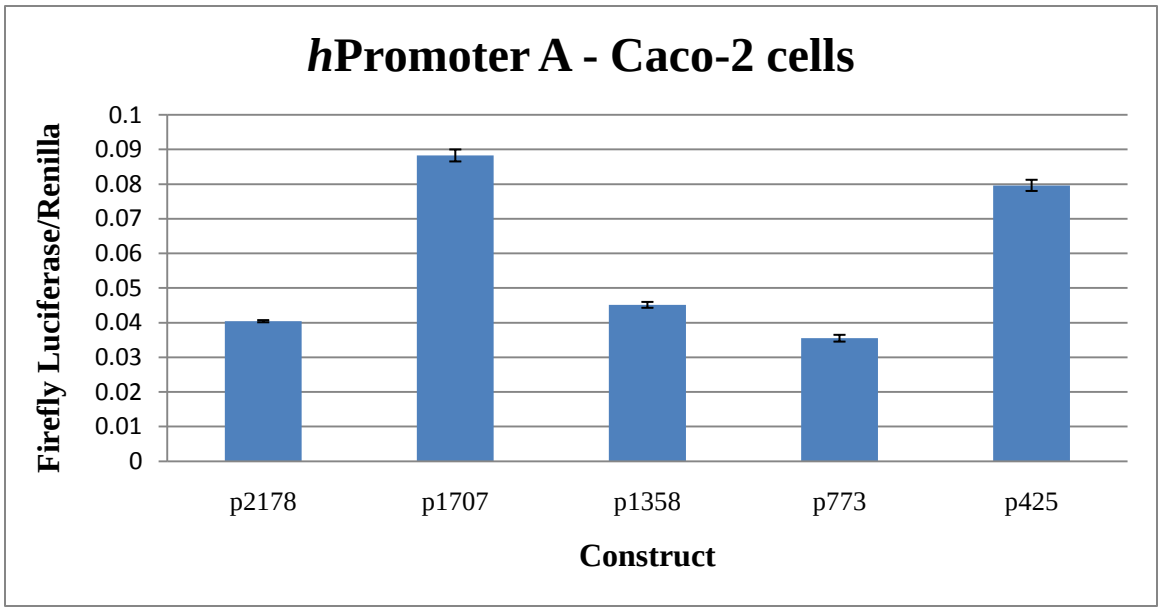


It is important to note that by cloning the *h*promoter A and B gene regulatory regions separately into the luciferase reporter vector the transcriptional activity of these sequences are being analyzed independently. Genomically, these sequence regions are linked.

*h*Promoters A and B are active in both cell types at relatively the same level of activity. A low level of activity was not detected for any of the *h*promoter A and B constructs, indicating that 425 bp (*h*promoter A) and 356 bp (*h*promoter B) of sequence are sufficient for transcriptional initiation and relatively high level of expression. However, the level of activity varies between different cell types and amount of upstream sequence. The *h*promoter A constructs displayed a similar pattern of luciferase activity in both NIH3T3 and Caco-2 cells (figure 9). The increased activity observed comparing the p2178 and p1707 constructs suggests that a negative acting sequence is contained in the removed 471 base pairs. The p1358 and p773 constructs had similar levels of activity in both cell types that was 2-3 fold less than the p1707 construct. This suggests the removal of a positive acting sequence in this region. There was an increase in luciferase activity for the p425 construct, indicating the presence of a negative acting sequence in the 348 base pair region between p773 and p425.

Figure 9. Transient Expression of *Wnt5a h*Promoter A Deletion Constructs in NIH3T3 and Caco-2 Cells. The cells were transfected with the *h*promoter A deletion constructs (figure 8), and an empty PGL4-basic, along with a *Renilla* vector as a control for DNA uptake. After 24-48 hours, the cells were collected and assayed for firefly luciferase and *Renilla* activity. The firefly luciferase activity or RLUs were divided by the *Renilla* RLUs for the same sample and graphed for comparison of each deletion construct's relative expression in both cell types. Bars are the means +/- standard error. N=4

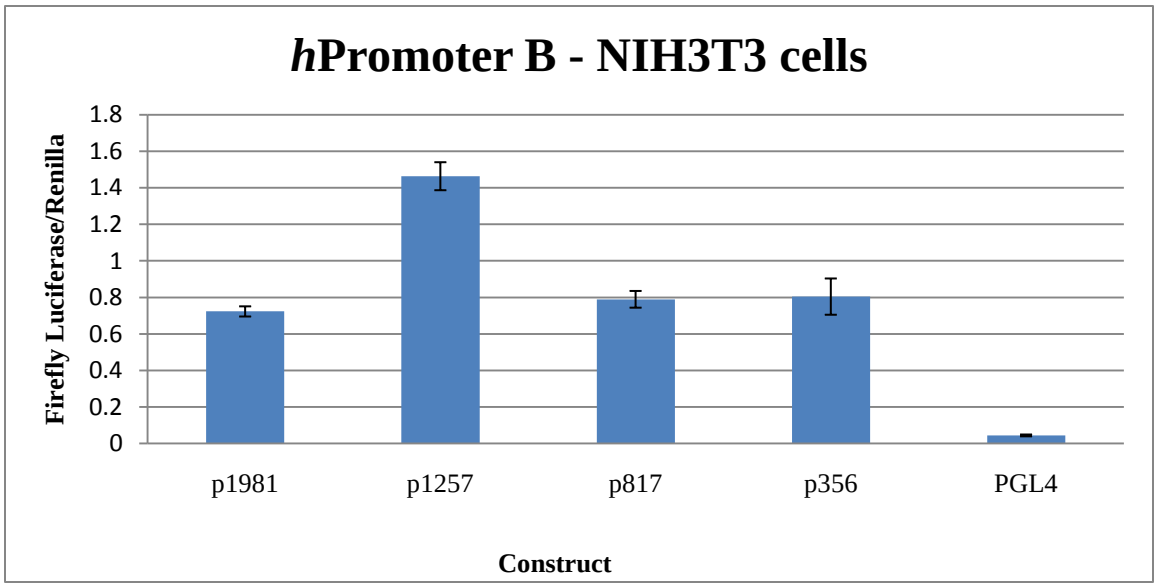


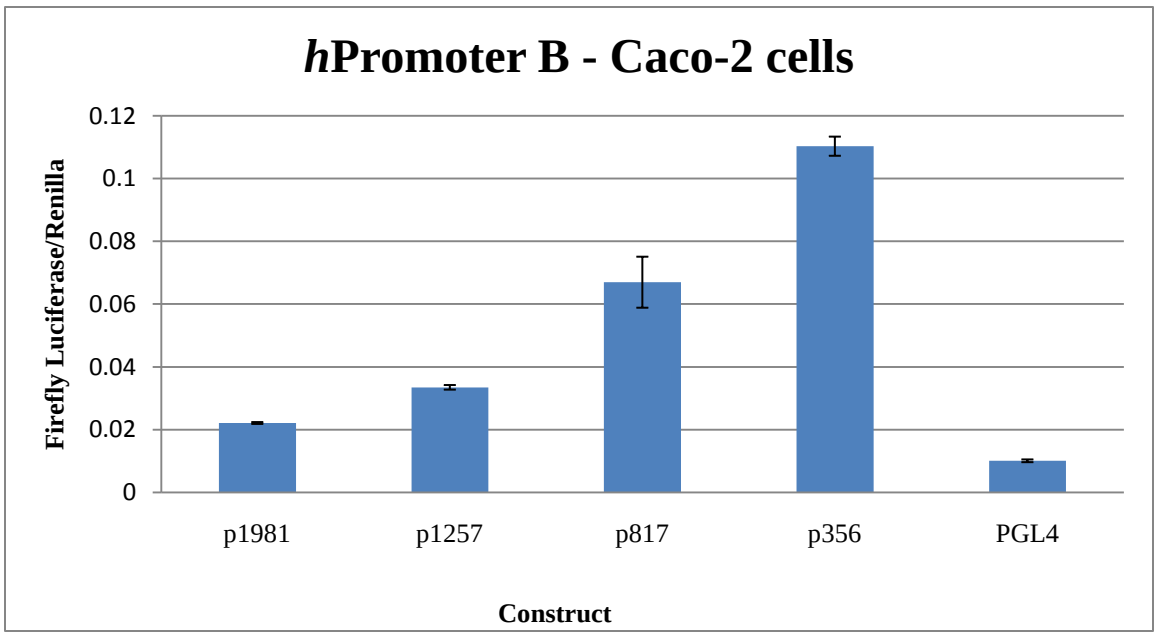


The *h*promoter B constructs displayed distinctive patterns of activity in the NIH3T3 and Caco-2 cells (figure 10). In the NIH3T3 cells, the pattern is more similar to *h*promoter A. There was an increase in activity of approximately 2-fold after removing 724 base pairs (comparing constructs p1981 and p1257), that indicates the presence of a negative regulatory sequence in this region. Removal of additional sequences (constructs p817 and p356) reduced activity to the level of the p1981 construct, suggesting that a positive sequence was removed between 1257- 817 base pairs of the total promoter. In contrast to the *h*promoter A p425 construct, the *h*promoter B p356 construct did not display an increase relative to the longest, p1981 construct.

In the Caco-2 cells there was increasing activity as more of the *h*promoter B upstream sequences were removed, with maximal activity measured with the shortest construct, p356. The level of activity for the p356 construct was approximately 5-fold greater than the largest construct, p1981. This suggests that negative acting regulatory sequences are present in these three regions: 1981-1257, 1257-817, and 817-356. As expected, there was minimal activity from the control vector (empty pGL4.17) for both cell types.

Figure 10. Transient Expression of *Wnt5a h*Promoter B Deletion Constructs in NIH3T3 and Caco-2 Cells. The cells were transfected with the *h*promoter B deletion constructs (figure 8), and an empty PGL4-basic, along with a *Renilla* vector as a control for DNA uptake. After 24-48 hours, the cells were collected and assayed for firefly luciferase and *Renilla* activity. The firefly luciferase activity or RLUs were divided by the *Renilla* RLUs for the same sample and graphed for comparison of each deletion construct's relative expression in both cell types. Bars are the means +/- standard error. N=4





These transfections were repeated four times in both cells. *h*Promoter B constructs gave very consistent results. *h*Promoter A results were more variable, but the data in figures 9 and 10 are representative.

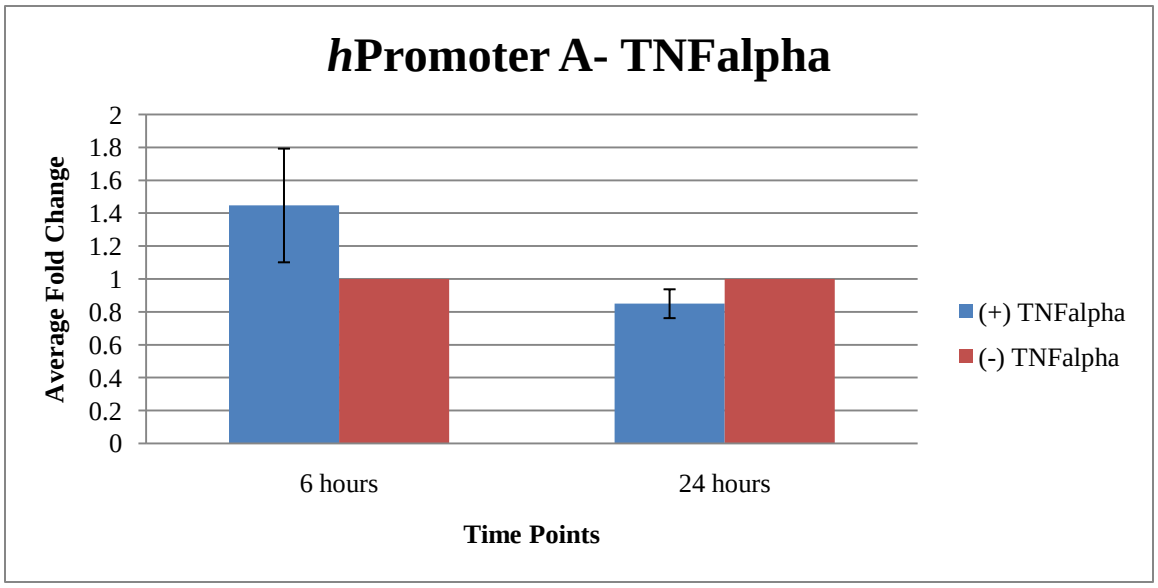
Overall, these data suggest there is complex regulation of both *Wnt5a* alternative *h*promoters A and B, involving both positive and negative sequences. Also, there are differential expression patterns in NIH3T3 and Caco-2 cells. *h*Promoters A and B activities are more similar in NIH3T3 mouse fibroblast cells, whereas there is more distinction between *h*promoters A and B in Caco-2 human intestinal epithelial cells.

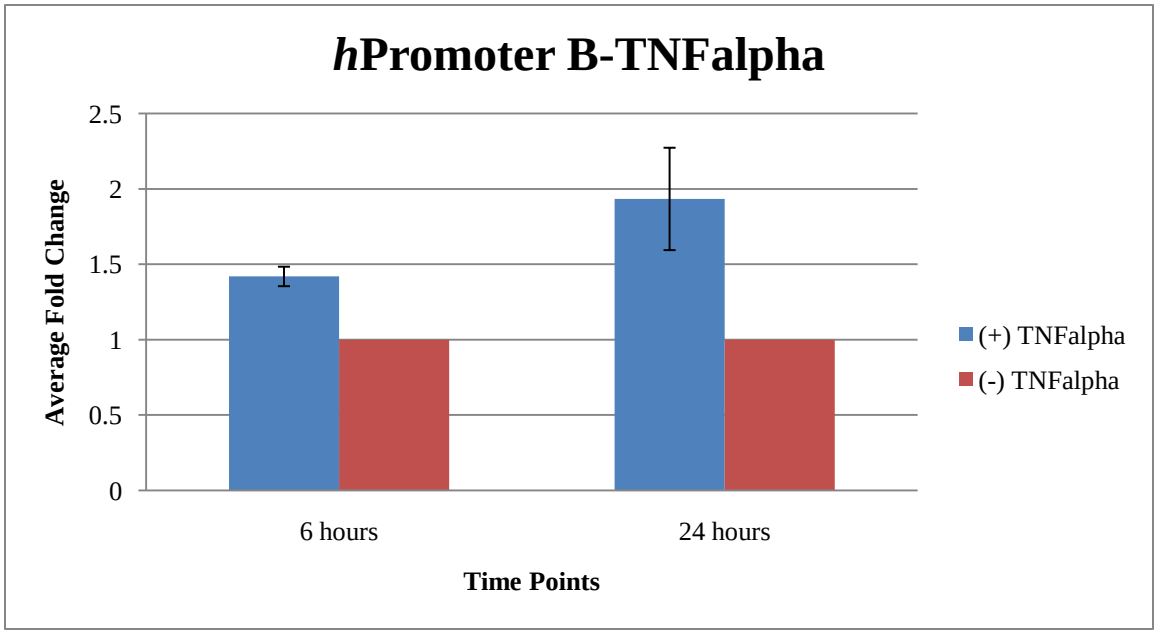
Contribution of the Transcription Factor NFkappaB to Transcription From Wnt5a Alternative Promoters A and B

Previous literature has shown that there are potential NFkappaB binding sites in both *Wnt5a* promoters (Katoh and Katoh, 2009; Ge et al., 2011) and that TNFalpha increases *Wnt5a* expression (Saitoh and Katoh, 2002). However, no one has confirmed the effect of TNFalpha on *Wnt5a* gene expression nor if both promoters A and B are affected. This is important as NFkappaB is often over expressed in transformed cells and may alter *Wnt5a* transcription. Stable lines of NIH3T3 cells with *Wnt5a h*promoter A p2178 and *h*promoter B p1981constructs were utilized in these studies. The cells were plated in multi-well plates and treated for 6 and 24 hours with 5 ng/ml of TNFalpha, a known inducer of NFkappaB expression. The cells were collected and assayed for firefly luciferase activity or RLUs. The RLUs were standardized to DNA content, which is a measure of cell number. This TNFalpha treatment experiment was repeated four times. The TNFalpha treated cells were standardized to control cells and the average fold change for all four trials was determined and graphed.

*h*Promoter A stable lines showed an increase in activity after 6 hours TNFalpha stimulation, indicating a more direct effect of NFkappaB binding on promoter activity (figure 11). However, there was a slight decrease in activity 24 hours after treatment. It is important to note that because the luciferase enzyme has a 2 hour half life, luciferase activity is a reflection of how active the promoter is at the 24 hour time point, not an accumulation of activity over a 24 hour period.

Figure 11. TNFalpha Effects on *h*Promoter A and B Activity. Stable lines of NIH3T3 cells containing either the *h*promoter A p2178 luciferase reporter construct or the *h*promoter B p1981 luciferase reporter construct were treated with 5ng/ml of TNFalpha for 6 and 24 hours. The cells were lysed and assayed for luciferase activity and DNA content determined. Activity is expressed as RLU/DNA. The TNFalpha treated cells were standardized to control cells and the average fold change for all four trials was determined and graphed. Bars are the average fold change +/- standard error. N=6.





*h*Promoter B activity showed an increase in activity at both 6 and 24 hours. The average fold change was determined for the four trials. At 6 hours there was average fold increase of 1.42 in *h*promoter B activity. At 24 hours the average increase in activity was 1.93 fold.

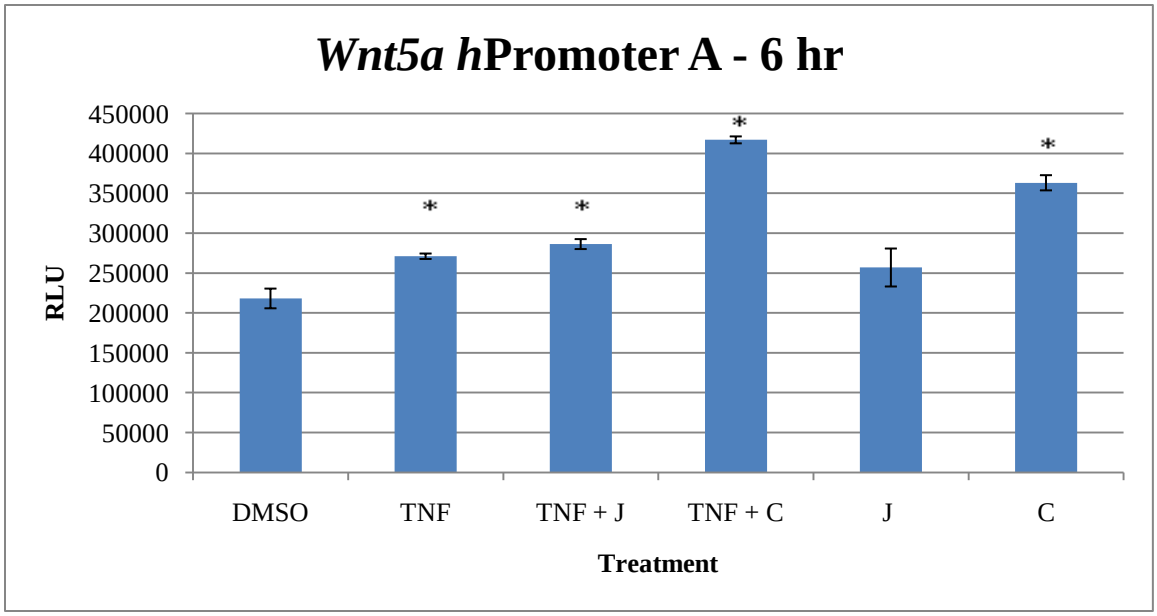
*h*Promoter A showed a more variable response to TNFalpha. At 6 hours the average fold increase was 1.44. At 24 hours, *h*promoter A showed an average slight decrease of 0.85 fold. These data suggest that *h*promoter A and B both respond to TNFalpha at 6 hours but the effect is prolonged for *h*promoter B over a 24 hour period.

The TNFalpha signaling pathway can activate multiple pathways, in addition to the NFkappaB pathway. To determine if NFkappaB activation is involved in the response of the *Wnt5a h*promoters A and B to TNFalpha stimulation we used inhibitors of NFkappaB activation. The *Wnt5a h*promoter A and B luciferase reporter lines were treated with and without TNFalpha in the presence and absence of inhibitors JSH-23 (J) and CAY10512 (C). J prevents NFkappaB translocation into the nucleus following its activation. C is a polyphenolic trans-stilbene resveratrol analog. It has been shown to block TNFalpha activation of NFkappaB, although the exact mechanism is not known. After 6 and 24 hours, the cells were collected and assayed for luciferase activity. Values were plotted as RLUs. *Wnt5a h*promoter B had consistent and similar results at 6 and 24 hours (figure 13). As previously observed (see figure 11), TNFalpha causes an increase in luciferase at both 6 and 24 hrs. Addition of the inhibitor JSH-23 reduced RLU's to approximately the level of the DMSO control (figure 13). This result suggests that the

response of *h*promoter B to TNFalpha is due to NFkappaB. Unexpectedly, inhibitor C increased RLU's both alone and with TNFalpha, although the increase was higher with TNFalpha. The increase was approximately 2-fold at 6 hrs for TNFalpha plus C inhibitor and 1.5-fold with C only. At 24 hr, the increase was approximately 4 fold with TNFalpha plus C inhibitor and 2 fold with C inhibitor only.

There was a slight increase in RLU's in the *h*promoter A reporter cell lines treated with TNFalpha (figure 12). Addition of J inhibitor did not reduce luciferase activity to control levels, as seen for *h*promoter B but slightly increased activity. This result suggests that NFkappaB is not responsible for the observed increase in *h*promoter A activity by TNFalpha stimulation. The response of the *h*promoter A reporter cell line to inhibitor C was similar to *h*promoter B; an increase in activity above DMSO treated control cells was detected in cells treated with TNFalpha plus C and C only treated cells.

Figure 12. TNFalpha and NFkappaB Inhibitors Do Not Affect *h*Promoter A Activity. Stable lines of NIH3T3 cells containing the *h*promoter A p2178 luciferase reporter construct were treated with and without 5ng/ml TNFalpha in the absence and presence of 30 uM inhibitor JSH-23 (J) and 1 uM inhibitor CAY10512 (C) for 6 and 24 hours. The cells were lysed and assayed for luciferase activity and DNA content determined. Activity is expressed as RLU/DNA. Bars are +/- standard error. The asterisk indicates a significant difference between the treated and control (DMSO) ($p<0.05$) N=6.



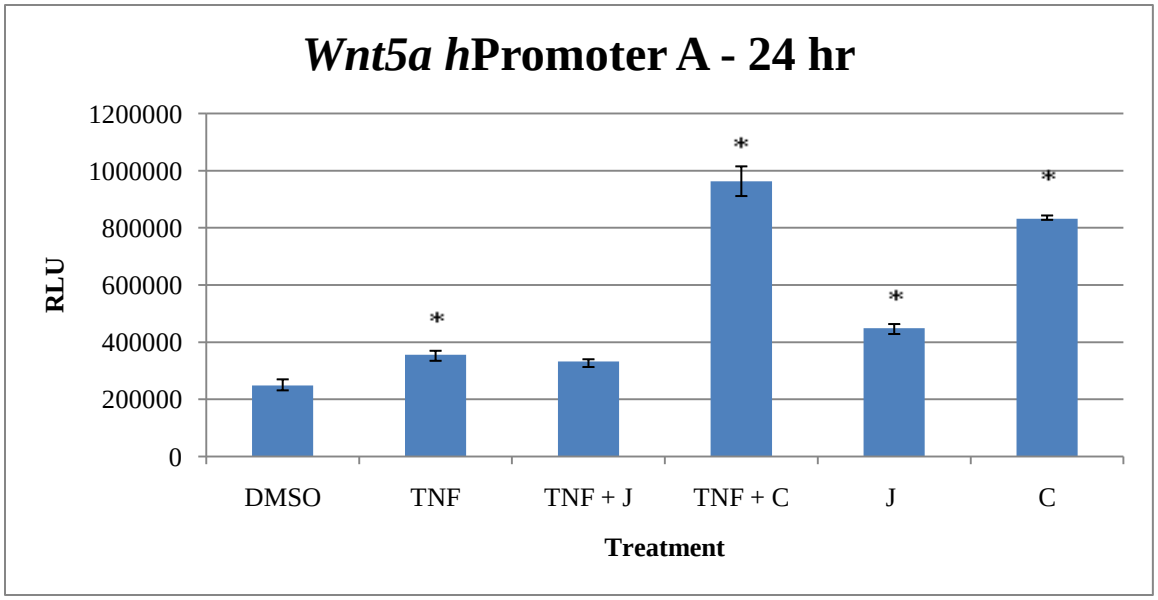
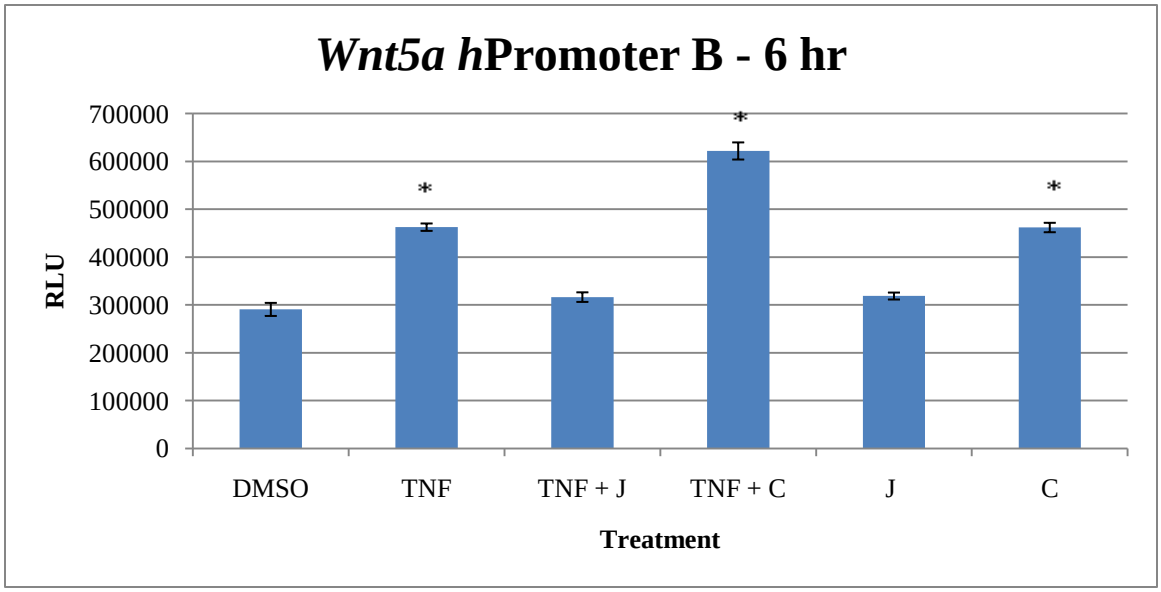
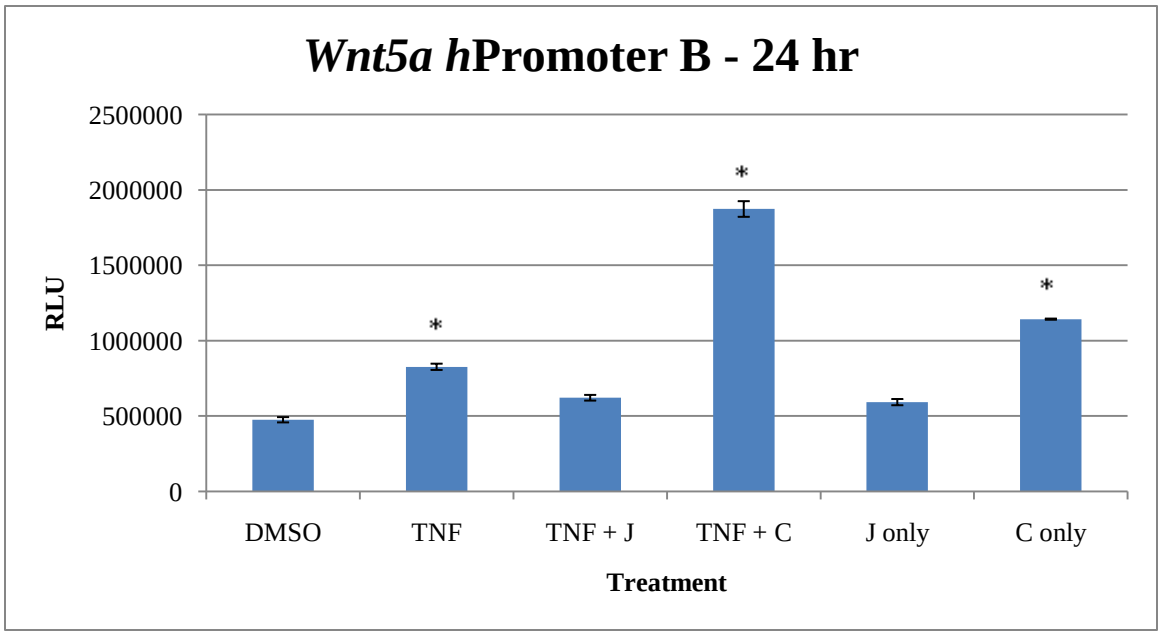


Figure 13. TNFalpha and NFkappaB Inhibitors Affect *h*promoter B Activity. Stable lines of NIH3T3 cells containing the *h*promoter B p1981 luciferase reporter construct were treated with and without 5ng/ml TNFalpha in the absence and presence of 30 uM inhibitor JSH-23 (J) and 1 uM inhibitor CAY10512 (C) for 6 and 24 hours. The cells were lysed and assayed for luciferase activity and DNA content determined. Activity is expressed as RLU/DNA. Bars are +/- standard error. The asterisk indicates a significant difference between the treated and control (DMSO) ($p<0.05$) N=6





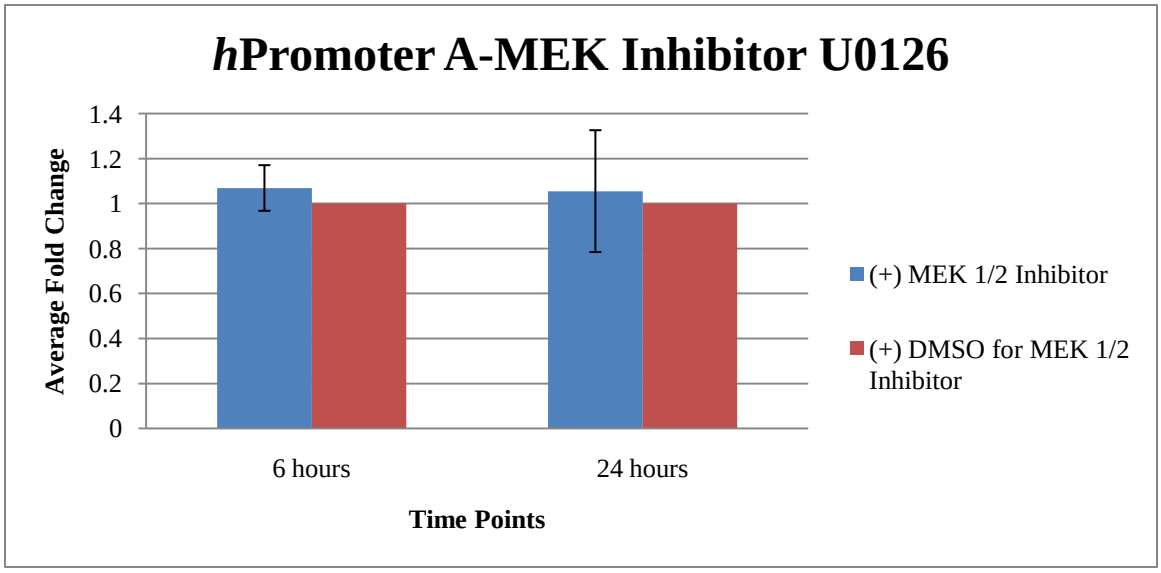
MAPK Signaling Pathway Involvement in Wnt5a Alternative Promoters A and B Activation

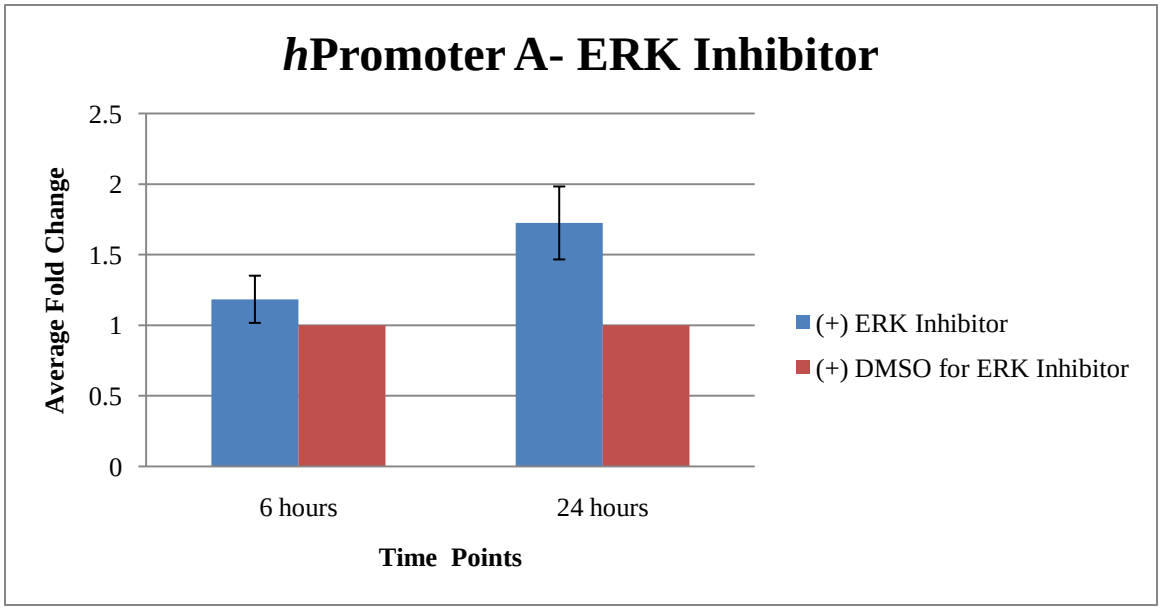
The MAPK pathway is often over activated in transformed cells due to genetic modification of one or more pathway components and there is limited evidence *Wnt5a* is regulated by this pathway (Bui et al., 1997; Wang and Chen, 2009). To determine if *Wnt5a* transcription from its alternate promoters is linked to the MAPK signaling pathway, stable lines of NIH3T3 cells with human *Wnt5a h*promoter A p2178 and *h*promoter B p1981constructs were utilized. The cells were plated in multi-well plates and treated for 6 and 24 hours with ERK and MEK 1/2 (U0126) inhibitors along with a control treatment of DMSO. The cells were collected and assayed for firefly luciferase activity or RLUs. The RLUs were standardized to DNA content, which is a measure of cell number. This treatment experiment was repeated at least four times. The inhibitor treated cells were standardized to control cells and the average fold change for all trials were determined and graphed. The assumption in this study is that the "constitutively" expressed *Wnt5a* in the fibroblast cells are responding to growth factor stimulation via MAPK signaling pathway. Our working hypothesis is that inhibition of the MAPK pathway will cause a decrease in *Wnt5a* transcription from both or either alternative promoter.

Treatment of *h*promoter A p2178 NIH3T3 cell lines with the MEK 1/2 inhibitor U0126 gave variable results. At 6 and 24 hours, there was essentially no effect on *h*promoter A activity (figure 14). However, ERK inhibitor treatment slightly increased

*h*promoter A activity at 6 hours (average fold change 1.18). At 24 hours there was a larger average fold increase in *h*promoter A activity after ERK inhibitor exposure of 1.72.

Figure 14. MEK 1/2 and ERK Inhibitor Effects on *h*Promoter A Activity. Stable lines of *h*promoter A p2178 NIH3T3 cells were plated and treated with either 10 uM MEK 1/2 inhibitor U0126 or 25 uM ERK inhibitor for 6 and 24 hours. The cells were lysed in cell lysis buffer and assayed for luciferase activity. Values were standardized to DNA content. Activity is expressed as RLU/DNA. The inhibitor treated cells were standardized to control cells and the average fold change for all trials were determined and graphed. Bars are the average fold change +/- standard error. N=6.

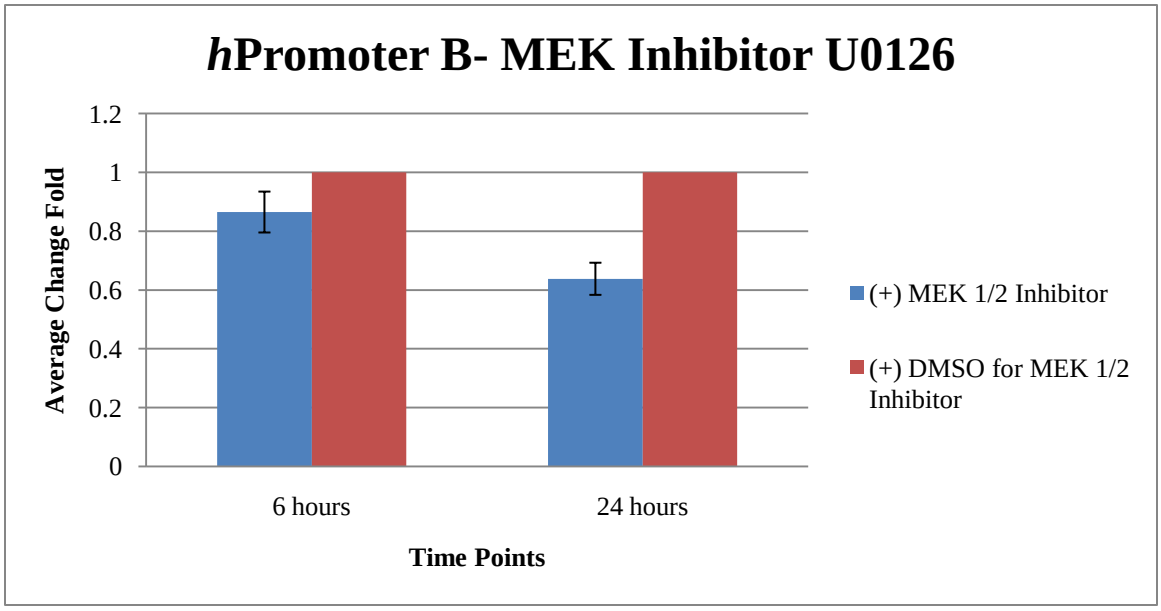


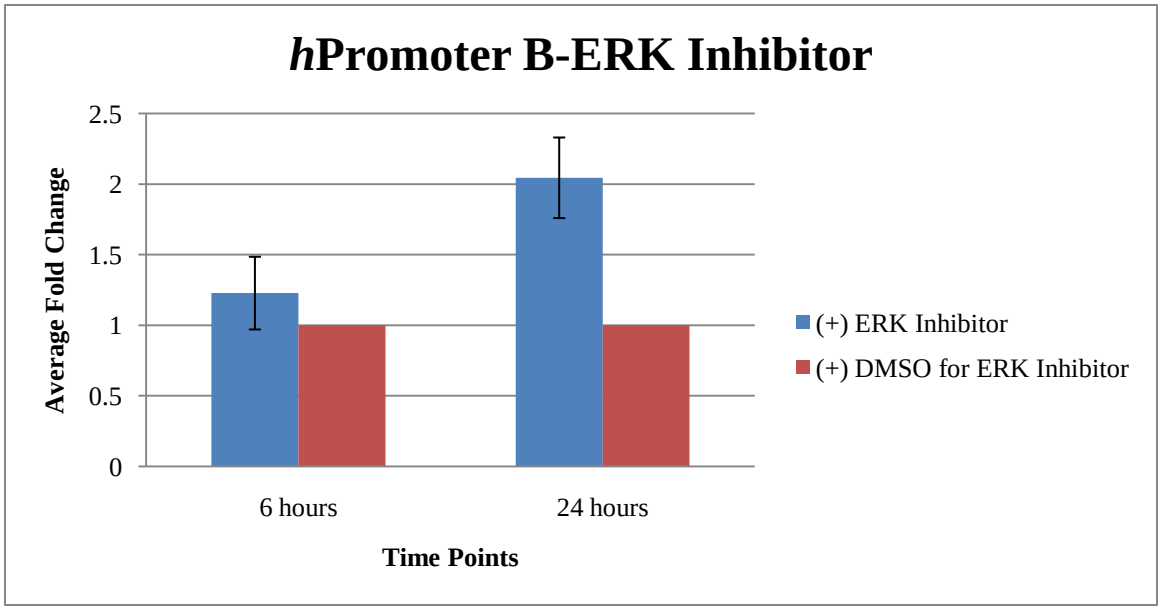


MEK 1/2 inhibition also influenced *h*promoter B activity. At 6 hours there was a slight decrease in activity with an average fold change of 0.87 (figure 15). At 24 hours there was also a large decrease in *h*promoter B activity with an average fold change of 0.64.

*h*Promoter B activity in 6 hours ERK inhibitor treated cells essentially showed no change. However, at 24 hours *h*promoter B activity increased in ERK inhibitor treated cells with an average fold change of 2.04.

Figure 15. MEK 1/2 and ERK Inhibitor Effects on *h*promoter B Activity. Stable lines of *h*promoter B p1981 NIH3T3 cells were plated and treated with either 10 uM MEK 1/2 inhibitor U0126 or 25 uM ERK inhibitor for 6 and 24 hours. The cells were lysed in cell lysis buffer and assayed for luciferase activity. Values were standardized to DNA content. Activity is expressed as RLU/DNA. The inhibitor treated cells were standardized to control cells and the average fold change for all trials were determined and graphed. Bars are the average fold change +/- standard error. N=6.





Relative Contribution of Transcripts from Wnt5a Alternative Promoters A and B in Treated and Untreated Cells

We used *h*promoter A and promoter B luciferase reporter vectors to analyze the activity from these alternative promoters independently of each other. Genomically, the promoters are linked and it is possible that transcription from the two promoters are influenced by each other's presence and additional genomic sequences. For example, promoter A may predominate over promoter B and sequences located upstream promoter B, which is intron 1 for the promoter A transcript, may influence the activity of promoter A. Thus, it is important to analyze the activities of promoter A and promoter B in situ from the genomic sequence.

As previously discussed, there are *Wnt5a* transcript variants produced by the two different promoters. Transcripts from *h*promoter A includes exons 1a, 2, 3, 4, and 5, whereas transcripts from *h*promoter B includes exons 1b, 2, 3, 4, and 5 (figure 2). The mouse produces similar transcripts (figure 4). Thus, the different isoforms of *Wnt5a* mRNA can be individually detected using transcript specific primers. We designed TaqMan primer probes that would amplify and detect human and mouse *Wnt5a* promoter A and promoter B specific transcripts. The location of the primers and probes in the cDNA sequences are shown in figures 16 and 17 for human and figures 18 and 19 for mouse. The sequences of the primers and probe are in table 2. The *h*promoter A and B transcript specific primers were designed such that the forward and reverse primers flank a splice junction. The *h*promoter A primers amplify a 77 bp fragment including sequences in exon 1a and exon2. The *h*promoter B primers amplify a 71 bp fragment

including most of exon 1b and sequence in exon 2. The *m*promoter A and B primers are designed similarly to the human primers. The promoter A primers generate a 85 bp product and promoter B primers a 62 bp product (table 2). The correct product sizes were confirmed by DNA agarose gel electrophoresis for the mouse (C. Hsu, personal communication; data not shown).

Figure 16. Human *Wnt5a* Promoter A Exon 1a-Exon 2 Fused Sequences. The forward primer is shown in red, the probe in purple, and the reverse primer in blue. The splice junction is denoted by an asterisk**.**

```
        10         20         30         40         50         60
AGTTGCCTGC GCGCCCTCGC CGGACCGGCG GCTCCCTAGT TGCGCCCCGA CCAGGCCCTG
TCAACGGACG CGCGGGAGCG GCCTGGCCGC CGAGGGATCA ACGCGGGGCT GGTCCGGGAC

        70         80         90        100        110        120
CCCTTGCTGC CGGCTCGCGC GCGTCCGCGC CCCCTCCATT CCTGGGCGCA TCCCAGCTCT
GGGAACGACG GCCGAGCGCG CGCAGGCGCG GGGGAGGTAA GGACCCGCGT AGGGTCGAGA

       130        140        150        160        170        180
GCCCCAACTC GGGAGTCCAG GCCCGGGCGC CAGTGCCCGC TTCAGCTCCG GTTCACTGCG
CGGGGTTGAG CCCTCAGGTC CGGGCCCGCG GTCACGGGCG AAGTCGAGGC CAAGTGACGC

       190        200        210        220        230        240
CCCGCCGGAC GCGCGCCGGA GGACTCCGCA GCCCTGCTCC TGACCGTCCC CCCAGGCTTA
GGGCGGCCTG CGCGCGGCCT CCTGAGGCGT CGGGACGAGG ACTGGCAGGG GGGTCCGAAT
                                        Forward Primer
       250        260        270        280        290        300

ACCCGGTCGC TCCGCTCGGA TTCCTCGGCT GCGCTCGCTC GGGTGGCGAC TTCCTCCCCG
TGGGCCAGCG AGGCGAGCCT AAGGAGCCGA CGCGAGCGAG CCCACCGCTG AAGGAGGGGC
      Probe
       310        320     *  330        340        350        360
CGCCCCCTCC CCCTCGCCAT GAAGAAGTCC ATTGGAATAT TAAGCCCAGG AGTTGCTTTG
GCGGGGGAGG GGGAGCGGTA CTTCTTCAGG TAACCTTATA ATTCGGGTCC TCAACGAAAC
                                        Reverse Primer
       370        380        390        400        410        420
GGGATGGCTG GAAGTGCAAT GTCTTCCAAG TTCTTCCTAG TGGCTTTGGC CATATTTTTC
CCCTACCGAC CTTCACGTTA CAGAAGGTTC AAGAAGGATC ACCGAAACCG GTATAAAAAG

       430        440        450
TCCTTCGCCC AGGTTGTAAT TGAAGCCAAT TCTTGGTG
AGGAAGCGGG TCCAACATTA ACTTCGGTTA AGAACCAC
```

Figure 17. Human *Wnt5a* Promoter B Exon 1b-Exon 2 Fused Sequences. The forward primer is shown in red, the probe in purple, and the reverse primer in blue. The splice junction is denoted by an asterisk**.**

```
      Forward Primer                        Probe              *
        10         20         30         40         50         60
CTCCTCTCGC CCATGGAATT AATTCTGGCT CCACTTGTTG CTCGGCCCAG AAGTCCATTG
GAGGAGAGCG GGTACCTTAA TTAAGACCGA GGTGAACAAC GAGCCGGGTC TTCAGGTAAC
        70         80         90        100        110        120
GAATATTAAG CCCAGGAGTT GCTTTGGGGA TGGCTGGAAG TGCAATGTCT TCCAAGTTCT
CTTATAATTC GGGTCCTCAA CGAAACCCCT ACCGACCTTC ACGTTACAGA AGGTTCAAGA
Reverse Primer
       130        140        150        160        170        180
TCCTAGTGGC TTTGGCCATA TTTTTCTCCT TCGCCCAGGT TGTAATTGAA GCCAATTCTT
AGGATCACCG AAACCGGTAT AAAAAGAGGA AGCGGGTCCA ACATTAACTT CGGTTAAGAA

GGTG
CCAC
```

Figure 18. Mouse *Wnt5a* Promoter A Exon 1a-Exon 2 Fused Sequences. The forward primer is shown in red, the probe in purple, and the reverse primer in blue. The splice junction is denoted by an asterisk.

```
             Forward Primer                Probe           *
         10          20          30          40          50          60
CTTCGCTCGG GTGGCGACTT CCTCTCCGTG CCCCCTCCCC CTCGCCATGA AGAAGCCCAT
GAAGCGAGCC CACCGCTGAA GGAGAGGCAC GGGGGAGGGG GAGCGGTACT TCTTCGGGTA
         70          80          90         100         110         120
TGGAATATTA AGCCCGGGAG TGGCTTTGGG GACCGCTGGA GGTGCCATGT CTTCCAAGTT
ACCTTATAAT TCGGGCCCTC ACCGAAACCC CTGGCGACCT CCACGGTACA GAAGGTTCAA
                      Reverse Primer
       130         140         150         160         170         180
CTTCCTAATG GCTTTGGCCA CGTTTTTCTC CTTCGCCCAG GTTGTTATAG AAGCTAATTC
GAAGGATTAC CGAAACCGGT GCAAAAAGAG GAAGCGGGTC CAACAATATC TTCGATTAAG

TTGGTG
AACCAC
```

Figure 19. Mouse *Wnt5a* Promoter B Exon 1b-Exon 2 Fused Sequences. The forward primer is shown in red, the probe in purple, and the reverse primer in blue. The splice junction is denoted by an asterisk.

```
 Forward Primer   *           Probe
        10          20          30          40          50          60
ACTTGTTGCT CCGGCCCAGA AGCCCATTGG AATATTAAGC CCGGGAGTGG CTTTGGGGAC
TGAACAACGA GGCCGGGTCT TCGGGTAACC TTATAATTCG GGCCCTCACC GAAACCCCTG
                                                  Reverse Primer
        70          80          90         100         110         120
CGCTGGAGGT GCCATGTCTT CCAAGTTCTT CCTAATGGCT TTGGCCACGT TTTTCTCCTT
GCGACCTCCA CGGTACAGAA GGTTCAAGAA GGATTACCGA AACCGGTGCA AAAAGAGGAA
       130         140         150
CGCCCAGGTT GTTATAGAAG CTAATTCTTG GTG
GCGGGTCCAA CAATATCTTC GATTAAGAAC CAC
```

Table 2. Sequence of Primers and Probes for qRT-PCR.

	Sequence (5'-3')	Sequence Length	Product Size (bp)
Human Promoter A	Forward Primer- TCGGGTGGCGACTTCCT	17	77
	Reverse Primer-CAACTCCTGGGCTTAATATTCCAAT	25	
	Probe-CGCCCCCTCCCCCTCGCCATGAAG	24	
Human Promoter B	Forward Primer- CCTCTCGCCCATGGAATT	18	71
	Reverse Primer- GGGCTTAATATTCCAATGGACTTC	24	
	Probe- CTGGCTCCACTTGTTGCTCGGCC	23	
Mouse Promoter A	Forward Primer- GTGGCGACTTCCTCTCCGT	19	85
	Reverse Primer- CGGTCCCCAAAGCCACT	17	
	Probe- CCCCTCGCCATGAAGAAGCCCA	22	
Mouse Promoter B	Forward Primer-ACTTGTTGCTCCGGCCC	17	62
	Reverse Primer-CGGTCCCCAAAGCCACT	17	
	Probe- AGAAGCCCATTGGAATATTAAGCCCGG	27	

Our first study was to determine if TNFalpha treatment affects the level of human and mouse endogenous promoters A and B specific transcript using our custom TaqMan specific primers. In addition, we wanted to determine the relative levels of promoters A and B transcripts in treated cells. Both mouse fibroblasts (NIH3T3) and normal human fibroblasts (GM03349) were used to analyze the mouse and human *Wnt5a* genes. The NIH3T3 and GM03349 cells treated with TNFalpha (5 ng/ml) for 6 and 24 hours. Total RNA was isolated from cells and converted into cDNA for quantitative RT-PCR. Each cDNA sample was amplified in triplicate using the following Applied Biosystems TaqMan primer sets. Primers for the NIH3T3 cells include mouse *Wnt5a*, mouse cyclin D1 as a positive control of TNFalpha stimulation, custom mouse *Wnt5a* promoter A and promoter B derived transcripts primers, and ribosomal RNA as an internal control. Primers for the human GM03349 cells, include human *Wnt5a* and human cyclin D1 as a positive control of TNFalpha stimulation, custom human *Wnt5a* promoter A and promoter B derived transcripts primers, and GAPDH as an internal control. These primers were used to analyze the relative amounts of each isoform by qRT-PCR using the Taqman system (Applied Biosystems). The relative amount of each isoform was compared to the total amount of *Wnt5a* and to itself for treated and untreated samples. The C_T ($\Delta\Delta C_T$) value was determined for each cDNA sample. This provides a measure of the relative level of transcripts, standardized to the endogenous control. For example, *Wnt5a* relative transcript levels were measured in TNFalpha treated cells relative to untreated cells, using GAPDH or ribosomal RNA as internal controls. A C_T ($\Delta\Delta C_T$) value of > 1 indicates an increase; $=1$ indicates no change and < 1 indicates a decrease.

First, we noted the cycle numbers for the *Wnt5a*, promoter A and promoter B transcripts in the NIH3T3 and GM03349 cells (table 3). These results suggest that there are a greater number of promoter A than promoter B transcripts. In mouse, the promoter A cycle was one less than promoter B, indicating two times (2^1) the number of template. In GM03349 cells, the cycle number difference is greater, two, resulting in four times (2^2) more promoter A transcripts than promoter B. As expected, *Wnt5a* total was larger, indicated by a lower cycle number than either promoter A or promoter B.

Next, we examined the effect of TNFalpha treatment on transcript levels. In the NIH3T3 cells, there was essentially no change in *Wnt5a* transcript, and promoter A and B transcripts at 6 hours (table 4). This is in contrast to the NIH3T3 stable line treatment where there was an increase in promoter A and B activity (figure 10). At 24 hours, there was an increase in all the target transcripts: total *Wnt5a*, cyclin D1, promoter A and promoter B transcripts (table 4). Cyclin D1 transcription is known to be increased by TNFalpha stimulation and is a positive control. The increase in total *Wnt5a* would be expected, along with an increase in either or both promoters A and B. Both promoter A and B transcripts increased by about 2 fold (figure 20).

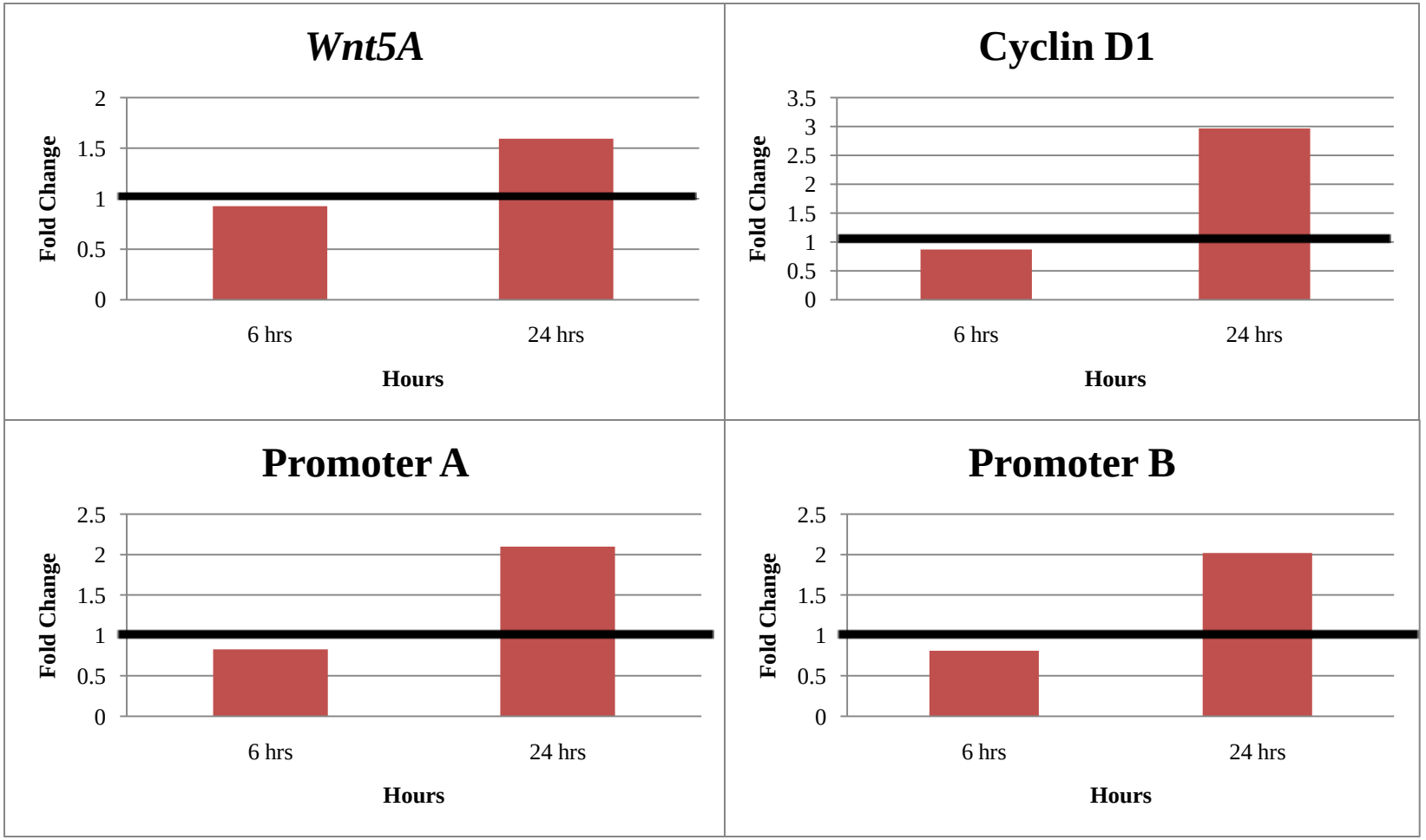
Table 3. qRT-PCR Cycle Number of *Wnt5a* Promoters A and B Transcripts in NIH3T3 and GM03349 Cells.

	Wnt5a	Promoter A	Promoter B
NIH3T3 cells (6 hours untreated)	26.34	28.31	29.27
GM03349 cells (6 hours untreated)	28.59	28.90	30.90

Table 4. Fold Changes of qRT-PCR Targets in TNFalpha Treated NIH3T3 Cells.

	Fold Change	
	6 hours	24 hours
Wnt5a	0.91	1.59
Promoter A	0.83	2.10
Promoter B	1.30	2.02
Cyclin D1	0.87	2.97

Figure 20. qRT-PCR of NIH3T3 Cells Treated with TNFalpha. Custom primers for mouse Wnt5a promoters A and B transcripts were used along with primers for Wnt5a and cyclin D1. Cycles were normalized to the ribosomal protein cycle numbers and treated values compared to the untreated, normal cells. The horizontal bar indicates no change relative to the control.

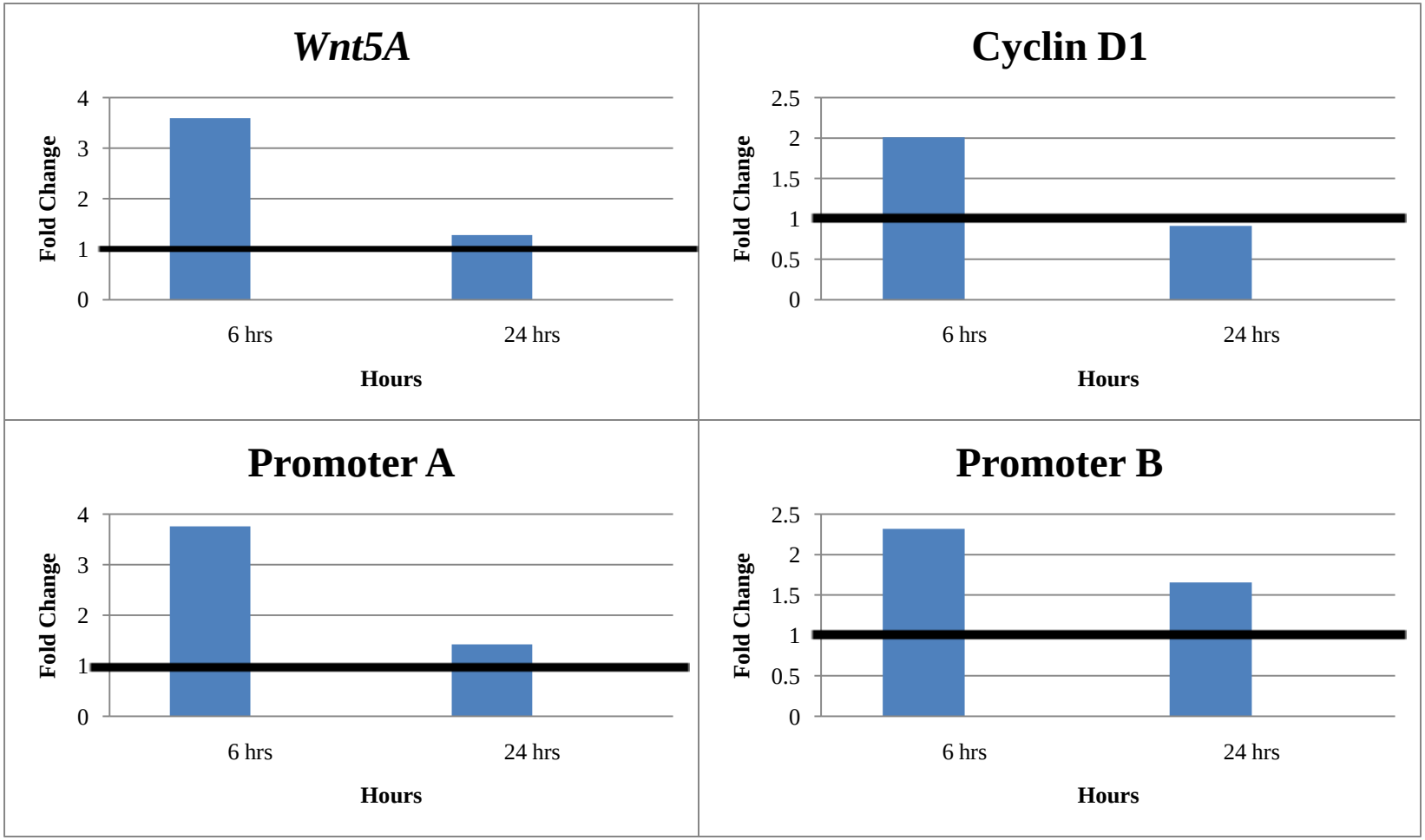


In the GM03349 cells there was an increase in *Wnt5a*, specific promoter A and B and cyclin D1 transcripts at 6 hours (figure 21). The calculated fold changes are shown in table 5. At 24 hours the relative transcript levels decreased for all but cyclin D1, but were slightly higher than controls. These results support our findings with the reporter cell lines; both promoter A and B are affected by TNFalpha treatment.

Table 5. Fold Changes of qRT-PCR Targets in TNFalpha Treated GM03349 Cells.

	Fold Change	
	6 hours	24 hours
Wnt5a	3.60	1.28
Promoter A	3.75	1.42
Promoter B	2.32	1.65
Cyclin D1	2.01	0.91

Figure 21. qRT-PCR of GM03349 Cells Treated with TNFalpha. Custom primers for human Wnt5a promoters A and B transcripts were used along with primers for *Wnt5a* and cyclin D1. Cycles were normalized to the ribosomal protein cycle numbers and treated values compared to the untreated, normal cells. The horizontal bar indicates no change relative to the control.



CHAPTER IV

DISCUSSION

Both Wnt5a Promoters A and B are Transcriptionally Active in Distinct Cell Types

Human *Wnt5a* alternative *h*promoters A and B were analyzed separately using luciferase reporter vectors that were transiently transfected to determine their relative levels of transcriptional activity and map potential regulatory sequences by deletion analysis. Our data, indicated by the similar values for firefly RLU/*Renilla* RLU for the different constructs, show that both *h*promoters are equally active when assayed separately (figures 9 and 10). This result suggests that *h*promoter B has equal transcriptional potential in comparison to *h*promoter A. However, it is possible that when genomically linked within the *Wnt5a* transcription unit, the promoters are differentially utilized. In fact, qRT-PCR analysis of mouse and human promoter A and B transcripts indicates that promoter A is utilized slightly more than promoter B. In mouse NIH3T3 cells, promoter A in general had one less cycle than B, indicating twice as many promoter A transcripts than B (table 3). In the human GM03349 cells there was a two cycle difference, thus four times as many promoter A transcripts as B. However, in the cells treated with TNFalpha for 6 hours, promoter A was 2.7 cycles less promoter B, resulting in 6.5 more promoter A transcripts than promoter B. This observation is significant because the modulation of total *Wnt5a* in cancer cells may actually be due to differential promoter activity rather than other epigenetic factors.

To confirm the actual level of promoter A and B transcripts in situ it will be necessary to conduct a standard curve qRT-PCR.

Deletion analysis of the *h*promoter A and B upstream sequences support the conclusion that there are positive and negative acting sequences within the approximately 2000 bp of upstream sequence being analyzed and that 350 to 400 bp is sufficient for a high level of expression (figures 9 and 10). In general, the changes in expression levels for the different constructs varied only 2 to 3 fold for *h*promoters A and B in NIH3T3 cells and *h*promoter A in Caco-2 cells. These observations and the fact that the *Wnt5a* is activity transcribed in NIH3T3 cells, as shown by our qRT-PCR analysis, indicates that the gene is constitutively expressed in a fibroblast cell. Regulation of *Wnt5a* in these cells is likely to involve slight modulations UP or DOWN in transcription rates rather than ON and OFF. Based on our data, the *Wnt5a* gene is responding to negative and positive factors, which may fluctuate depending on the growth state of the cell population. Induction of *Wnt5a* gene transcription from an OFF to an ON state is more likely during development. As previously discussed, *Wnt5a* is involved in numerous developmental events, including limb, brain, intestine, and prostate formation. To identify the regulatory sequences and ultimately transcriptions factors that are responsible for the cell-specific expression of *Wnt5a* during development would require alternative approaches.

One unexpected result is that *h*promoter B displays a different pattern of expression in Caco-2 than in NIH3T3. We found that removal of upstream sequences

lead to continual increase of expression. This finding suggests that negative regulatory sequences are being removed between the 1981-356 region of promoter B. Although Caco-2 is a human cell and the *Wnt5a* reporter constructs are human, this does not explain the similar patterns of expression for *h*promoter A in Caco-2 and NIH3T3 nor the similarity between *h*promoters A and B in NIH3T3 cells. A comparison of human promoter A and B upstream sequences show no homology. It is possible, however, that similar transcription factor binding sites are found in each promoter that are not detected by a sequence homology comparison.

Activated NFkappaB Increases Promoter B Activity

Collectively, our results from *Wnt5a* promoter A and B luciferase reporter stable cell lines and qRT-PCR plus other preliminary data support the conclusion that activated NFkappaB increases promoter B activity (figure 13), whereas NFkappaB does not appear to be involved in TNFalpha stimulation of promoter A activity (figure 12). Using *Wnt5a* promoter A and B stable luciferase reporter lines, we found that TNFalpha treatment lead to increased *Wnt5a* promoter activity at 6 hours for both promoter A and B constructs, whereas only promoter B showed an increase at 24 hours (figure 11). qRT-PCR analysis of *Wnt5a* promoter A and B specific transcripts in TNFalpha treated mouse NIH3T3 fibroblasts and normal human fibroblast GM03349 confirmed TNFalpha increases both promoter A and B derived transcripts (tables 4 and 5/figures 20 and 21). However, the increase was observed primarily at 6 hrs in the GM03349 cells and at 24 hours in the NIH3T3 cells. At 24 hours in GM03349 cells, transcripts decreased but were slightly

higher than controls. We do not have a clear explanation for the differences observed between the cell types and between the qRT-PCR results and stable reporter cell lines. One possibility is that TNFalpha signaling in normal human fibroblasts is more rapidly activated and terminated in comparison to the immortalized mouse fibroblasts, causing an earlier and less sustained increase in transcript levels. The lack of *Wnt5a* promoter A and promoter B transcript increases in the NIH3T3 cells at 6 hours by qRT-PCR, in comparison to the luciferase reporter stable lines is less clear. One explanation is the likely presence of concatenated promoter A and B reporter constructs in the stable lines, leading to greater sensitivity and detection by 6 hours after TNFalpha treatment. Nevertheless, our data does show that both human and mouse cell types are affected by TNFalpha.

Because TNFalpha can also active MAPK signaling pathways, our results suggest but do not confirm the role of NFkappaB in *Wnt5a* promoter A and B transcription. To verify that NFkappaB is responsible for the increase, we used inhibitors of NFkappaB. Addition of the NFkappaB inhibitor JSH-23 to promoter B NIH3T3 stable cell lines treated with TNFalpha reduced activity to control levels at both 6 and 24 hours (figure 13). Moreover, analysis of promoter B specific transcript levels by qRT-PCR in NIH3T3 cells treated with TNFalpha alone and with JSH-23, confirmed these results; promoter B transcript levels increased in TNFalpha treated cells but were reduced to control levels in TNFalpha plus JSH-23 treated cells (preliminary data, not shown). In contrast, JSH-23 did not reduce the increased promoter A luciferase reporter activity or transcript levels to control levels in TNFalpha treated cells (figure 12). These results suggest that the

increase in *Wnt5a* promoter A activity may be due to other pathways activated by TNFalpha rather than NFkappaB activation. Surprisingly, we found NFkappaB inhibitor CAY10512 to increase *h*promoter A and B activity rather than decrease activity as seen in NFkappaB inhibitor JSH-23. One possibility for this result is the concentration is too high and pathways other than NFkappaB are being affected. However, 1 uM is the same concentrations used in other studies in the literature. A dose dependent assay is needed to determine if our results are due to high concentrations of NFkappaB inhibitor CAY10512.

We have examined the upstream region of both the mouse and human promoter A and intron 1 of the human and mouse promoter A transcript for potential NFkappaB binding sites. Promoter A intron 1 includes promoter B exon 1, intron 1, and upstream sequences. Using the consensus sequence GGGRNYYYCC (R=A or G; N=A,T,C,G; Y=C or T) (Chen et al., 1998), three NFkappaB sites were identified in the mouse intron 1 at positions 2585, 3190, and 3410 of the 5709 bp intron (#1 bp = 5' nucleotide of the intron). Two sites were identified in the human intron 1 at positions 1870 and 4260 of the 6076 bp intron. Site 4260 is contained within the human promoter B luciferase reporter construct p1987. No NFkappaB sites were identified in 4000 bp upstream of exon 1 or within exon 1 of the mouse *Wnt5a* promoter A. One site was identified at position 2450 in a 4000 bp region upstream of exon 1 of human promoter A and no sites were identified in exon 1. This analysis confirms that the *Wnt5a* gene includes NFkappaB binding sites in intron 1, which potentially can affect both promoter A and promoter B, although our data indicates that promoter B is most affected.

In a recent study, Ge et al., (2011) showed that interleukin-1β stimulation of normal chondrocytes caused an increase in both *Wnt5a* transcript and protein levels. This increase was reduced by an inhibitor of IκBa phosphorylation, required for NFkappaB activation. In their study, the qRT-PCR primers detected total *Wnt5a* transcript levels and did not differentiated between the different *Wnt5a* isoforms (i.e. promoter A and B transcripts). Thus, it is possible that the change in *Wnt5a* transcript levels in their study was due to increased transcription from promoter B. They identified an NFkappaB binding site immediately upstream of exon 1 of the human promoter A transcript unit. However, this sequence does not match the consensus NFkappaB binding sequence or slightly altered versions of it. However, by ChIP analysis they did find NFkappaB enrichment at this sequence. None of the potential NFkappaB binding sites within intron 1 were analyzed. There is one other published report of increased WNT5A protein levels in TNFalpha treated MKN45 gastric cancer cells (Saitoh and Katoh, 2002). These studies are consistent with our findings.

No Clear Evidence of MAPK Pathway Involvement in Wnt5a Transcription

It is not possible from our results to conclude if *Wnt5a* promoters A and/or B transcription are affected by the MAPK signaling pathway. In our experiment, NIH3T3 cells stably transfected with the human *Wnt5a* promoter A and B constructs were treated with Ras pathway inhibitors and the level of luciferase activity determined in treated and untreated cells as a measure of promoter A and B activity. The inhibitors blocked activity of two adjacent effector kinases in the Ras pathway, MEK1/2 and ERK. Thus, similar

results would be expected using the two inhibitors. However, we found that the MEK1/2 inhibitor U0126 did not affect promoter A activity and decreased luciferase activity B cell lines at 24 hours, whereas the ERK inhibitor consistently increased activity (figures 14 and 15). One possibility for this discrepancy is that the concentration of the U0126 (10 uM) is high enough to affect other cellular kinases. We also examined the level of mouse promoter A and B transcripts in NIH3T3 cells treated with both MEK1/2 and ERK inhibitors by qRT-PCR and found no effect on transcript levels for total *Wnt5a* and promoter A and B transcripts (preliminary data, not shown). This result indicates that, at least in a dividing cell population, inhibition of the Ras signaling pathway does not lead to alterations in *Wnt5a* transcription from either *Wnt5a* alternative promoters. To further resolve this question we plan to serum starve cells and then treat them with an activator of the Ras pathway such as epidermal growth factor and assay for changes in transcript levels from the *Wnt5a* promoters A and B. Overall, the results presented and preliminary data do not clearly support involvement of Ras signaling in *Wnt5a* transcription. However, not tested in this study were the p38 and JNK pathways.

Summary and Conclusions

Our study increased understanding of *Wnt5a* alternative promoters A and B transcriptional regulation. Using the human *Wnt5a* alternative promoter- reporter vectors we found both *Wnt5a* Promoters A and B were transcriptionally active in NIH3T3 and Caco-2 cells. However, we found both mouse and human promoter A to be more transcriptionally active than promoter B when assayed endogenously by qRT-PCR. One

of our project goals was to determine if *Wnt5a* expression changes with increased NFkappaB activity. We determined NFkappaB, a factor increased in cancer, may play a role in promoter B regulation rather than promoter A. However, there is no clear evidence that the MAPK pathway is involved in *Wnt5a* transcription. These studies are significant because they provide insight into the normal regulation of *Wnt5a* expression and how its expression might be altered in cancer.

REFERENCES

American Cancer Society. 2010. American Cancer Society, Inc. 30 October 2010 <http://www.cancer.org/cancer/cancerbasics/cancer-prevalence>.

Andersson ER, Prakash N, Cajanek L, Minina E, Bryjova L, Yamaguchi TP, Hall AC, Wurst W, Arenas E. (2008) Wnt5a regulates ventral midbrain morphogenesis and the development of A9-A10 dopaminergic cells in vivo. PLoS ONE 3(10): e3517

Basseres DS and Baldwin AS. (2006) Nuclear factor-κB and inhibitor of κB kinase pathways in oncogenic initiation and progression. Oncogene 25:6817-6830.

Bui TD, Tortora G, Ciardiello F, Harris AL. (1997) Expression of Wnt5a is downregulated by extracellular matrix and mutated c-Ha-ras in the human mammary epithelial cell line MCF-10-A. Biochemical and Biophysical Research Communications 239:911-917.

Cervantes S, Yamaguchi TP, Hebrok M. (2009) Wnt5a is essential for intestinal elongation in mice. Developmental Biology 326(2):285-294.

Enomoto M, Hayakawa S, Itsukushima S, Ren DY, Matsuo M, Tamada K, Oneyama C, Okada M, Takumi T, Nishita M, Minami Y. (2009) Autonomous regulation of osteosarcoma cell invasiveness by Wnt5a/Ror2 signaling. Oncogene 28:3197-3208

Ge XP, Gan YH, Zhang CG, Zhou CY, Ma KT, Meng JH, Ma XC. Requirement of the NF-κB pathway for induction of Wnt-5A by interleukin-1β in condylar chondrocytes of the temporomandibular joint: functional crosstalk between the Wnt-5A and NF-κB signaling pathways. Osteoarthritis Cartilage 19(1):111-117.

Hardy KM, Garriock RJ, Yatskievych TA, D'Agostino SL, Antin PB, Krieg PA. (2008) Non-canonical Wnt signaling through Wnt5a/b and a novel Wnt11 gene, Wnt5b, regulates cell migration during avian gastrulation. Developmental Biology 320(2):391-401.

Hibi K, Mizukami H, Goto T, Kitamura Y, Sakata M, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y. (2009) WNT5A gene is aberrantly methylated from the early stages of colorectal cancer. Hepato-Gastroenterology 56: 1007-1009.

Huang L, Pu Y, Hu WY, Birch L, Luccio-Camelo D, Yamaguchi T, Prins GS. (2009) The role of Wnt5a in prostate gland development. Developmental Biology 328(2):188-199.

Katoh M and Katoh M. (2009) Transcriptional mechanisms of Wnt5a based on NF-κB, hedgehog, TGFβ, and Notch signaling cascades. International Journal of Molecular Medicine 23: 763-769.

Kurayoshi M, Oue N, Yamamoto H, Kishida M, Inoue A, Asahara T, Yasui W, Kikuchi A. (2006) Expression of Wnt5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Research 66(21): 10439-10448.

Leris AC, Roberts TR, Jiang WG, Newbold RF, Mokbel K. (2005) Wnt5a expression in human breast cancer. Anticancer Research 25(2A): 731-734.

Li L, Hutchins BI, Kalil K. (2010) Wnt5a induces simultaneous cortical axon outgrowth and repulsive turning through distinct signaling mechanisms. Science Signaling 3(147):pt2

Martin V, Valencia A, Agirre X, Cervera J, San Jose-Eneriz E, Vilas-Zornoza A, Rodriguez-Otero P, Sanz M, Herrera C, Torres A, Prosper F, Roman-Gomez J. (2010) Epigenetic regulation of the non-canonical Wnt pathway in acute myeloid leukemia. Cancer Science 101(2): 425-432.

Matsumoto S, Fumoto K, Okamoto T, Kaibuchi K, Kikuchi A. (2010) Binding of APC and disheveled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells. The EMBO Journal 29(7):1192-1204.

McDonald SL and Silver A. (2009) The opposing roles of Wnt5a in cancer. British Journal of Cancer 101(2): 209-214.

Nishita M, Enomoto M, Yamagata K, Minami Y. (2010) Cell tissue trophic functions of Wnt5a signaling in normal and cancer cells. Trends in Cell Biology 20(6): 346-354.

Nishizuka M, Koyanagi A, Osada S, Imagawa M. (2008) Wnt4 and Wnt5a promote adipocyte differentiation. FEBS Letters 582 (21-22): 3201-3205.

Oh DY and Olefsky JM. (2010) Wnt fans the flames in obesity. Science 329 (5990):397-398.

Paina S, Garzotto D, DeMarchis S, Marino M, Conti L, Cattaneo E, Perera M, Corte G, Calautti E, Merlo GR. (2011) Wnt5a is a transcriptional target of DIx homeogenes and promotes differentiation of interneuron progenitors in vitro and in vivo. The Journal of Neuroscience 31(7):2675-2687.

Pukrop T and Binder C. (2008) The complex pathways of Wnt5a in cancer progression. Journal of Molecular Medicine 86: 259-266.

Ren D, Minami Y, Nishita M. (2011) Critical role of Wnt5a-Ror2 signaling in motility and invasiveness of carcinoma cells following Snail-mediated epithelial-mesenchymal transition. Genes to Cells 16(3):304-315.

Ripka S, Konig A, Buchholz M, Wagner M, Sipos B, Kloppel G, Downward J, Gress TM, Michl P. (2007) Wnt5a- target of Cutl1 and potent modulator of tumor cell migration and invasion in pancreatic cancer. Carcinogenesis 28(6): 1178-1187.

Roman-Gomez J, Jimenez-Velaso A, Cordeu L, Vilas-Zornoza A, San Jose-Eneriz E, Garate L, Castillejo J, Martin V, Prosper F, Heiniger A, Torres A, Agirre X. (2007) WNT5A, a putative tumour suppressor of lymphoid malignancies, is inactivated by aberrant methylation in acute lymphoblastic leukaemia. European Journal of Cancer 43: 2736-2746.

Saitoh T and Katoh M. (2002) Expression and regulation of WNT5A and WNT5B in human cancer: up-regulation of WNT5A by TNFalpha in MKN45 cells and up-regulation of WNT5B by beta-estradiol in MCF-7 cells. International Journal of Molecular Medicine 10(3): 345-349.

Taki M, Kamata N, Yokoyama K, Fujimoto R, Tsutsumi S, Nagayama M. (2003) Down-regulation of Wnt-4 and up-regulation of Wnt5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells. Cancer Science 94(7):593-597.

Tamimi Y, Ekuere U, Laughton N, Grundy P. (2008) WNT5A is regulated by PAX2 and may be involved in blastemal predominant Wilms tumorigenesis. Neoplasia 10(12): 1470-1480.

Varela-Nallar L, Alfaro IE, Serrano FG, Parodi J, Inestrosa NC. (2010) Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. Proceedings of the National Academy of Sciences of the United States of America 107(49): 21164-21169.

Wang Q, Willamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J, Hubank MJF, Ahmed A, Masters JR. (2007) Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. Oncogene 26: 6560-6565.

Wang Z and Chen H. (2009) Amino acid limitation induces down-regulation of WNT5a at transcriptional level. Biochemical and Biophysical Research Communications 378(4):789-794.

Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM. (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 1(3): 279-288.

Yamaguchi TP, Bradley A, McMahon AP, Jones S. (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126 (6): 1211-1223.

Yang Y, Topol L, Lee H, Wu J. (2003) Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 130(5):1003-1015.

Ying J, Li H, Yu J, Ng KM, Poon FF, Wong SC, Chan AT, Sung JJ, Tao Q. (2008) Wnt5a exhibits tumor-suppressive activity through antagonizing the Wnt/β- Catenin signaling, and is frequently methylated in colorectal cancer. Clinical Cancer Research 14(1): 55-61.

APPENDIX A.

PRIMER LOCATIONS FOR HUMAN WNT5A PROMOTER A LUCIFERASE CONSTRUCTS

Human Wnt5a Promoter A Exon 1a and 2000 bp Upstream Sequence

TGTGTGTGTGCGCGCGTGTGTGTGTCTTCACCTTTGACAGCTGGAGAATGCAGACGGCG
GAAAATAACTATTACGCTCCAGTGTGAGGCACTGTTTGTGGGGTGGGGCCCTCGGCGTG
CTGGAGGTAGCGGACACAAATCCACCAACCCTGCCCCTCCGGAGTCCCCAAAACCACGG
GCATCTGGAAGCGTCTAGAAATTCCGGTGCCCGGAACCCGGTTTTGCCAAACTTCGCAA
TCTCCTCACCTGAGGGCACCCGCCAAGATCTCCGTGGCAGGACCACGCATCGCGCCTCA
TGCTCTGGGCCGGAGCCAGATGAGCAATCATGCATCCCACTACCCAAGTCCCTACTACT
CTGGGGACTTTGCAGGAGGTGAGCGCCTAACCCTATCCATCAGCCCCAGTGCTTAAGTA
TCCTCCCCCAGAGGTAAGAATGAGGAGGTCCCCCATGAGGTTCCATGTCAAGGCAGCAG
TCTCCGTGGAGCCCCAGTGGGAGCGGTCTGGGATACGCGGGCGAGCCAAGGGGTCCAGG
CGGGGAAGTTGGGGTTCAGCCTCCCGCCGGTCGGTGGCTCTGGAAGGAGCGCAGACAAC
CAGGCGGCAGGCAGGCAGCGGCTGCGGAGTGGACCTCTCTCTTTCAGCAGATGCCGCGG
GGGATGGGGAACTTAGAGAAGGGGAGACAAGGAGAGTGTACCCCGCCTCCCGGCACCCC
AGCCCGTAGTACGGCTTTAAAATCCTGCAGAGTTTGTCATAGGCCGGCCTCACCACGCA
TGCGCATAGCAAAGTTGAGTTTTTTCTTTCTTCCCCCCCCTTCCCTCTCTCTTTCCAGC
CGCCCCTAGTTCTGAAACTTTAAAGAACTTTTTTCTTTCTTTCTTTCTTTCTTTTTTTT
TTTTTCTTCCTTCTGAGAACACCAGGGGAAAGAGGAGTCTGGAGAGAGGGAGGGAGAGA
GTAAGGCAGTTGGCCAGAAGGGCTGGGGGGTGGGAGGGGGGGACGCGTCCCCGCGGTCG
GTCAGTCTGTTTGCGGGGCACAGGATCGCGTGGAAATCTGCTGCGAGCTCCACGGAGCC
GCCTCCGCCGCCCAGAGCCGGGGTCCGTGCGGCAGCTGCCCAGCGAGCCCTTCCGCCGC
CGCCGTTGTGCCCGGGACCGCCCGCCTCCCGCCGGCCCGCTCTCCCGCCCCTCGGACCC
GGGCGCCAAACGAGGAGGTGGGCAGCTTCGCCGGCCATGGACCGCCGGAGCTGAGTCGC
CGCCGCGGAGCTCCGAGCTCTCGCCCTTCCCCAGCCTCGCGCCCGCGGTGCACTCGCTG
CTCTTCGCCCTTCGCCCTGGAAAAACGGGGAAAAGAGAGATCAACCCGAAGAGAGAAAG

AACGGGATACGTGGTGGGGGGGGGGAGCCCGCGTCTCCCCTGGGTTAGTCGCACCCCTC
CAGCTCACCGCACAGCAATAAGTTCCGGGGCGACTTTCACCTATTTTGCTCCCCGTTTT
TGCCGAACCCTAAAATGAAAGGACACAATTTGGGGCTGACTCTTGCAGCTCAGATGGCG
ACTTGTGCGTTTTCAGCGGCATCTCCCGGAGAAAAAGCCATGCGCGCCTGATTGTTCTG
TGGCCCCAAAGCTGCAGCGGGGGTGTGTGTGTTGGGGTGTGTGTCTGTGTGTGTACAGA
GACGTCCACACTAACTCGCGGCTGCAGGATCAGCGTCTGGAAGCAGACGTTTCGGCTAC
AGACCCAGAGAGGAGGAGCTGGAGATCAGGAGGCGTGAGCCGCCAAGAGTTTGCAGAAT
CTGTGGTGTGAATGAACTGGGGGCACCTGGGCGCACAGATCGCCCCCCTTCCCCCGCCC
CGGGCCACAGTTGAGTAGTGGTACATTTTTTTCACCCTCTTGTGAAGAATTTCTTTTTA
TTATTATTTGTCGTAAGGTCTTTTGCACAATCACGCCCACATTTGGGGTTGGAAAGCCC
TAATTACCGCCGTCGCTGATGGACGTTGGAAACGGAGCGCCTCTCCGTGGAACAGTTGC
CTGCGCGCCCTCGCCGGACCGGCGGCTCCCTAGTTGCGCCCCGACCAGGCCCTGCCCTT
GCTGCCGGCTCGCGCGCGTCCGCGCCCCCTCCATTCCTGGGCGCATCCCAGCTCTGCCC
CAACTCGGGAGTCCAGGCCCGGGCGCCAGTGCCCGCTTCAGCTCCGGTTCACTGCGCCC
GCCGGACGCGCGCCGGAGGACTCCGCAGCCCTGCTCCTGACCGTCCCCCCAGGCTTAAC
CCGGTCGCTCCGCTCGGATTCCTCGGCTGCGCTCGCTCGGGTGGCGACTTCCTCCCCGC
GCCCCCTCCCCCTCGCC**ATG**

Black = upstream sequence

Red=Exon 1

ATG = start of translation
Underlined Blue = Forward PCR Primers
Underlined Red = Reverse PCR Primer

APPENDIX B.

PRIMER LOCATIONS FOR HUMAN WNT5A PROMOTER B LUCIFERASE CONSTRUCTS

Human Wnt5a Upstream of Exon1b and Exon 2(Promoter B)

TTGCACTGGGATTGAAGAGGGAAGAGGGCCAAGGTGTTTCCGGGCAAGCGGCGGGGTTA
AGTGGAGATGCGACTCGTGAGGCTCTCCTTTCCGATCCCCCTTTGGGACACCCTCTGCC
TACCTCTACCCTGGAGCCAGGGAGACCCAAGTCTTGGTGACCGGATGGGCCCGCTCTCA
GTTGGCCTGGGCTCTGGGAACTGGTGGACTCTCCCTGGGGGCTTCGGGCTGGGAGTGGG
TTCGGTTTGTGTGGCTTCGGCTCTAACAAAGAGATCCGCTGTAATCCGCCGAATCTGTT
ATCAATTTCTCTGCTGCTTGAGCCCCGCCCCACGCGCCCCGCTCGCCGCGAAGCTTGGA
AAGTGCACGCGGCCAGCACCAATCTGGGCCGCTGACTCGGAAACATGTCGCAGCGTGTG
TGTCTATGCACGCGTGTGAGTGTGTAAATGTGCACGAGTGTGAATGTGTATGATGTGTG
TGCACGCGGCATCGGCTGCCCTTGGGGAGAGTTGACTTTGCAGCCTGGGCTGCGCGAGA
AGCAGACTTTGCAGCCCACTCCCTCCCCTGGAGGAAATTTGACACTTAGGGCGGGGGTG
GGGAGATAGCCGGAGCCTTCTCTCTCCTAGCTGGGGAAACCCCAGATTTCCATTCTCCA
GGATGCGCCCCCCAGCTTTGCAGCGTTTTGGGGACAACTGGGCCTTGGGTGTGGAGCCC
TGCTTAGCAGGCGCTGGGGACCACATAAGCATTTCCTCTTTGAGAAGCCCCGAAGCGTC
CAGGCCAAAGGGGCGGCTCACGGAGAAAAACCTTGGCACGCCCCTGAGCCCCATAGCTT
TACCAGGGCTGCCTAGGTCCCCTCTGCCCCTTTTACGGCACAGGTTCCAAGCCAGGCTC
TTCCACCGCCTTAAAGAGGCTCACCTTTCTTTTCTTTTCTGTGGAAGGGGCTCCTTCAG
GGGCTATGGGCGATGCAGTGCGGCAGGGTTAGACTTACGTGTAAGGGGATTTTTAAAAC
CCGCTCCTCCCACCCGCACCCGCCACCTACTCGCTCCGCCGCCGCCTACAGGTGGAGAA
GTCACCAGTGGGGAGGAACGGCAGCGGAAGCTTCCAAGGCCAACTCCTACCCCTGAAAT
TCTTCAGGAAGGGAACCTTCGCCGCTGGGGGCCTCTTTGCCCTGGAATCGATGCGCCCA
GCTGCGGCTCGGAAGCCAGCGCCTCTGGCCCCGTCTGGACTCATCTGCAAGGGCTCTGG
CCTCGCCCCGCACCCCCACCTTTCGGCACTGACCGAACCAAGTCTGAGTTGGGCTGGAG
AGGCTAGACTGGAGGCAGGGTGGCAGAGTTCCAACGACAGGCTCGCAGTGCCGCGATGG
CAAAGTGGCCCACAACCCCAGATCAGGACCAAGGGAAACTGGAGTCTCTCTGGGCCTCC
CATCTCCTCCCTCCCTGGCAGCTGCCAGGTGTGGGGTGGGAGGGAGAGTGAAAAATCAG
AATTTGGGAGAAAGCTGTGGGGAGGGCAGGGAAGGGATCTTCTCCCCGGGGAAGCGAGA
CCCAGACTCCCTTCTTTCCTCTAGGGTTCCATCCCTTCTCTCAGTCCGTGGAAGAGGCC
ACAGGCGACGCGGGCGAGGGTGGCACTCTTTTCCAGTTTCCTTGGTTGGGAGACCCGAC
CTCTCTCTCCATTATCCCCTAGGGCCCCCATCTCCTTCTCCCCTCCCTAGTCTGGCTGA

AGAACGTCCTTAAGGAAATCCGGGCTGCTCTTCCCCATCTGGAAGTGGCTTTCCCCACA
TCGGCTCGTAAACTGATTATGAAACATACGATGTTAATTCGGAGCTGCATTTCCCAGCT
GGGCACTCTCGCGCGCTGGTCCCCGGGGCCTCGCCCCCCACCCCCTGCCCTTCCCTCCC
GCGTCCTGCCCCCATCCTCCACCCCCCGCGCTGGCCACCCCGCCTCCTTGGCAGCCTCT
GGCGGCAGCGCGCTCCACTCGCCTCCGTGCTCCTCTCGCCCATGGAATTAATTCTGGCT
CCACTTGTTGCTCGGCCCAGGTTGGGGAGAGGACGGAGGGTGGCCGCAGCGGGTTCCTG
AGTGAATTACCCAGGAGGGACTGAGCACAGCACCAACTAGAGGGGGGCCAGGGGGTGCG
GGACTCGAGCGAGCAGGAAGGAGGCAGCGCCTGGCACCAGGGCTTTGACTCAACAGAAT
TGAGACACGTTTGTAATCGCTGGCGTGCCCCGCGCACAGGATCCCAGCGAAATCAGATT
TCCTGGTGAGGTTGCGTGGGTGGATTAATTTGGAAAAAGAAACTGCCTATATCTTGCCA
TCAAAAAACTCACGGAGGAGAAGCGCAGTCAATCAACAGTAAACTTAAGAGACCCCGGA
TGCTTCCGTTGTTTAAACTTGTATGCTTGAAAATTATCTGAGAGGCAATAAACATCTGC
TCCTTTCTTCCCTCTCCAGAAGTCCATTGGAATATTAAGCCCAGGAGTTGCTTTGGGGA
TGGCTGGAAGTGCAATGTCTTCCAAGTTCTTCCTAGTGGCTTTGGCCATATTTTTCTCC
TTCGCCCAGGTTGTAATTGAAGCCAATTCTTGGTG

Black = upstream
Red = exons
Green = intron
ATG = start of translation
Underlined Blue = Forward PCR Primers
Underlined Red = Reverse PCR Primer