UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of Receptor Mediated Interactions with Wnt Proteins

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

Tyler Louis Sloan

Committee in charge:

Professor Karl Willert, Chair
Professor David Traver, Co-Chair
Professor Colin Jamora

2011

The Thesis of Tyler Louis Sloan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

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Acknowledgements

I would first like to thank Dr. Karl Willert for his support as the chair of my committee. Dr. Willert was integral in the design and guidance of my entire masters project. He was a tremendous mentor and inspiration during my time working in his laboratory. I would like to thank my committee members for their time and support. I would also like to thank the members of the Willert lab who were a constant source of help and always available to assist me in any way possible. In particular I would like to thank Antonio Fernandez for his guidance and help along the way, Matt Bauer for his help and purifying the fusion proteins, and Ian Higgins for being a constant source of information and proof reading this thesis. Lastly, I would like to thank my parents Mike and Anne, my brother Daniel, and my grandparents Stan the Man and Sheila for supporting me throughout this entire process.

Abstract of the Thesis

Characterization of Receptor Interactions with WNT Proteins

by

Tyler Louis Sloan

Master of Science in Biology

University of California, San Diego, 2011

Professor Karl Willert, Chair

The processes of differentiation and self renewal in human pluripotent stem cells (hPSCs) is regulated by a host of extracellular factors, many of which act through cell surface receptors and trigger signaling pathways to control gene expression. A key class of developmental signaling modules regulating stem cell behavior is the Wnt signaling pathway. Our understanding of how Wnt signaling proteins regulate the choice between self-renewal and differentiation is inadequate. Complicating these studies is the fact that the genome contains 19 WNT genes and at least 13 WNT receptors encoded by the FRIZZLED, ROR and RYK genes, which bind and transduce signaling inputs of the various Wnt proteins.

My research has explored the interactions between two Wnt proteins, Wnt3a and Wnt5a, and a subset of Wnt receptor: Frizzled 5 (FZD5), Frizzled 7 (FZD7), Frizzled 10 (FZD10), and ROR2. Both FZD and ROR receptors have a conserved domain on the extracellular portion on the receptor known as the cysteine-rich domain (CRD), which is responsible for binding Wnts. I constructed fusion genes comprised of the CRD region of these Wnt receptors and a tag, the constant region of an immunoglobulin (Ig). Upon stable transduction, selection, and clonal expansion of cells carrying these chimeras, I isolated and purified the fusion proteins. Finally, I used these recombinant proteins to interfere with Wnt signaling in cell-based assays. My results to date demonstrate that these CRD-Ig fusion proteins antagonizeWnt3a signaling in a standard Wnt reporter assay. These studies provide proof-of-principle that this approach can be utilized to specifically affect Wnt signaling in cell-based assays and can potentially be exploited to regulate stem cell behavior.

Chapter 1 Introduction

1.1Wnts

Wnt genes are found in all phyla of the animal kingdom (Srivastava et al., 2008). Roeland Nusse and Harold Varmus discovered the first Wnt gene, then called Int-1. In their study, they infected mice with mouse mammary tumor virus (MMTV), a retro-virus that causes mammary gland tumors. Analysis of the genomic DNA in the tumor cells indicated that MMTV provirus sequences specifically integrated in a locus called "integration site-1" orInt-1 for short. Sequencing of this locus identified the presence of a gene, which we now know to be Wnt-1. (Nusse et al., 1984). Subsequently a homolog of Int-1 was identified in *Drosophila,* a gene called Wingless (Wg). The name "Wnt" comes from a portmanteau of Wg and Int-1. Since the discovery of these first two genes, many other Wnt genes were discovered and sequencing of the human genome revealed the presence of at least 19 distinct Wnt genes.

Wnts are involved in normal physiological processes in adult animals but are typically known for their roles in embryogenesis, cancer, and self-renewal of hematopoietic stem cells (Lie et al., 2005, Reya et al. 2003). Wnt proteins are secreted lipid-modified signaling proteins ranging from 350 to 400 amino acids that activate various pathways in the cell, often classified as canonical and non-canonical pathways, that will be discussed in section 1.3 (Cadigan et al., 1997; Nelson et al., 2004). As secreted proteins, Wnts contain a signal sequence which is cleaved upon translation into the endoplasmic reticulum. The signal sequence is followed by300-400 amino acids with a large number (22-24) of interspersed and invariantly spaced cysteine residues, many of

which are thought to form disulfide bridges and maintain a globular protein structure. In the case of Wnt3a, one of these cysteine residues was shown to be a palmitoylation site. This post-translational modification gives Wnts their notorious hydrophobicity (Willert et al., 2003).

Wnt proteins play a variety of important roles in embryonic development, cell proliferation, differentiation, polarity and migration. These biological processes are regulated by both the canonical and non-canonical signaling pathways (Logan et al., 2004). Of the 19 Wnt proteins I will be focusing on the interaction of Wnt 3a and Wnt5a with the four receptor CRD-Ig fusion proteins that I have generated. Future experiments will expand this approach to include other Wnt ligands and receptors to determine binding specificities.

1.2 Wnt Receptors

Wnt receptors likewise play crucial roles in governing embryonic development, cell proliferation, differentiation, polarity and migration, and many other processes in developing and adult organisms (reviewed in Huang and Klein, 2004). The human genome contains a total of 19 Wnt genes which encode secreted lipid modified signaling molecules that interact with cell surface receptors encoded by the FZD (1-10), ROR (1 & 2), Ryk, and co-receptor genes.

Studies in Drosophila have led to many important discoveries in Wnt receptors. One such gene is the *frizzled* gene. In *Drosophila,* the protein encoded by this gene is known to direct a process called planar cell polarity where the cytoskeletons of epidermal cells in the wing produce a parallel array of hairs; in the wild-type wing, all hairs are

aligned and point towards the distal tip of the wing. In *frizzled* mutants, the individual hairs are disorganized. (Adler, et al., 1990). Mutations in a second Frizzled gene in flies, *frizzled2*, when combined with *frizzled* mutations produce a segment polarity phenotype, which is identical to mutations in the fly Wnt homolog *wingless*. This provides evidence that the *frizzled* genes in flies mediate at least the segment polarity and the planar polarity pathways through Wnt signaling.

I have been working with the Frizzled and ROR receptors, two classes of Wnt receptors. *Frizzled* genes, in flies to humans, encode integral membrane proteins with seven transmembrane (TM) domains whereas ROR has just one TM domain. In addition to this membrane structure, these Wnt receptors contain extracellular and cytoplasmic domains, both of which are critical for signaling (Adler et al., 1989). In particular, the extracellular domains carry conserved Cysteine Rich Domains (CRDs), which are critical for Wnt binding and signaling. CRDs are also found in multiple other proteins, including the secreted frizzled related proteins (Sfrp), several receptor Tyrosine kinases, and others (see Xu and Nusse 1998).

Amongst the various CRD containing cell surface receptors, FZD and ROR have been shown to act as bonafide Wnt receptors. The structure of the CRD is largely composed of alpha helices and contains ten conserved cysteines that form five disulphide bridges (Saldanha et al., 1998, Hofmann et al., 1998). Little is known about the specificity of particular Wnt receptors to particular Wnts, however, it is known that the CRD mediates Wnt/ β-catenin signaling (Umbhauer et al., 2000). This claim was further substantiated with the discovery of a secreted Frizzled related proteins which were shown

to carry CRDs that interact with Wnt proteins and thereby prevent them from engaging the Wnt receptors. Therefore, Sfrps act as Wnt antagonists that modulate Wnt signaling activities during development (Finch et al., 1997). These data provides strong evidence that the extracellular CRD of Wnt receptors are necessary for Wnt signaling.

1.3 Receptor Background

I chose to study Wnt receptors that have displayed critical roles in the development of hESCs. FZD7 has been implicated in the self-renewal of hESCs and in cancer (Melchoir et al., 2006, Ueno et al, 2008).FZD7 mRNA levels in human ES cells are upregulated up to two hundred fold when compared to differentiated cell types. When FZD7 was knocked down by shRNA, human ES cells showed a striking change in colony morphology and expression levels of germ layer-specific marker genes, as well as a rapid loss of the expression of OCT4, an ES cell-specific pluripotency marker. This evidence suggests that FZD7 has a large role in the maintenance of ES cell self-renewal (Melchoir et al., 2006). Another reason FZD7 was chosen was its connection to cancer. FZD7 is predominantly expressed in colon cancer cells and is implicated in aberrant canonical Wnt signaling in colon cancer cells with APC or β-catenin mutations (Ueno et al, 2008). Furthermore, other findings revealed that Wnt ligands or inhibitors affect the growth and survival of colon cancer cells in spite of the presence of APC or β-catenin mutations (Bafico et al, 2004; He et al, 2005). These findings suggest that Wnt ligands and receptors that function upstream of APC might have a vital role in the development of colorectal cancers (CRCs). Indeed, Ueno et al. showed that down regulation of Frizzled-7

expression by shRNA decreases survival, invasion and metastatic capabilities of colon cancer cells.

ROR2 is critical for primitive gut tube formation in mice. It has also been shown to inhibit canonical Wnt signaling. ROR2 is an orphan tyrosine kinase with one transmembrane domain. ROR2, in conjunction with Wnt5a, is responsible for inhibiting the canonical pathway through either the degradation of β-catenin (Topol et al., 2003) or through another mechanism that blocks the interaction of β -catenin with Tcf. Furthermore, Wnt5a and ROR2 have overlapping expression patterns, and their knockout phenotypes are similar, providing evidence that Wnt5a/Ror2 act in the same pathway (Mikels & Nusse, 2006). Wnt5a has also been discovered to be essential for the development and elongation of the small intestine from the midgut region. Mice lacking Wnt5a showed decreased cell proliferation and re-intercalation of post-mitotic cells into the elongating gut tube epithelium was disrupted. In addition, the mice had small intestine that was severely shortened and duplicated, causing a bifurcated lumen to form instead of a single tube. This evidence suggests that Wnt5a serves as a significant regulator of midgut formation and morphogenesis in mammals, making it an intriguing candidate for further research (Cervantes et al., 2009).

FZD5 has also been specifically implicated Wnt5A/non canonical Wnt signaling and has a wide range of effects at different stages of development. Ishikawa et al. used homologous recombination in embryonic stem cells to generate *Fzd5* knockout mice Though heterozygotes were viable and appeared normal, homozygous embryos died in utero around 10.75 days post coitus, due to defects in yolk sac angiogenesis. (Ishikawa et

al., 2001). Other studies have also implicated FZD5 in mammalian ocular development (Liu & Nathans, 2008).

Lastly, FZD10 was chosen due to its association with cancer and because it is highly expressed in a variety of different cell types discussed below. FZD10 also signals through the canonical pathway (Wang et al., 2005). FZD10is highly expressed in placenta, fetal kidney, fetal lung and brain. In the adult brain, FZD10 mRNA is highly expressed in the cerebellum (Koike et al., 1999). FZD10 has also been shown to be highly up regulated in primary colorectal cancers (Terasaki et al., 2002).

The properties of these receptors make them appealing to characterize due to the fact that once their function and capacities are fully established, better protocols can developed for the maintenance of pluripotency. This may be especially true for FZD10, which is expressed in multiple tissue types. The other Wnt receptors encoded by the Frizzled gene family, ROR1 and Ryk also play critical roles in development and most likely in stem cell biology. A long term goal of the Willert lab is to study all of these Wnt receptors.

1.4Wnt Signaling

The two most well known Wnt signaling pathways are the canonical and non-canonical pathways, with the chief difference that the canonical pathway requires β-catenin as a downstream signaling molecule. In the canonical pathway (Figure 1) Wnt proteins act on target cells by binding to the Frizzled /low density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to several intracellular proteins including Dishevelled (Dsh), Glycogen Synthase

Kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin. Cytoplasmic β-catenin levels are usually kept at low concentrations by continuous proteasome-mediated degradation, which is controlled by GSK-3, APC, and Axin, collectively known as the degradation complex. In canonical signaling, Wnt binds a Frizzled receptor which then inhibits the degradation of β-catenin, allowing it to relocate to the nucleus. Transcription factors of the lymphoid enhancer-binding factor and1/T cell-specific transcription factor (LEF/TCF) family interact with β-catenin to regulate transcription. A large number of Wnt targets have been identified that include members of the Wnt signal transduction pathway itself, such as Axin2 and DKK1, which provide negative feedback control during Wnt signaling (Logan & Nusse, 2004, review).

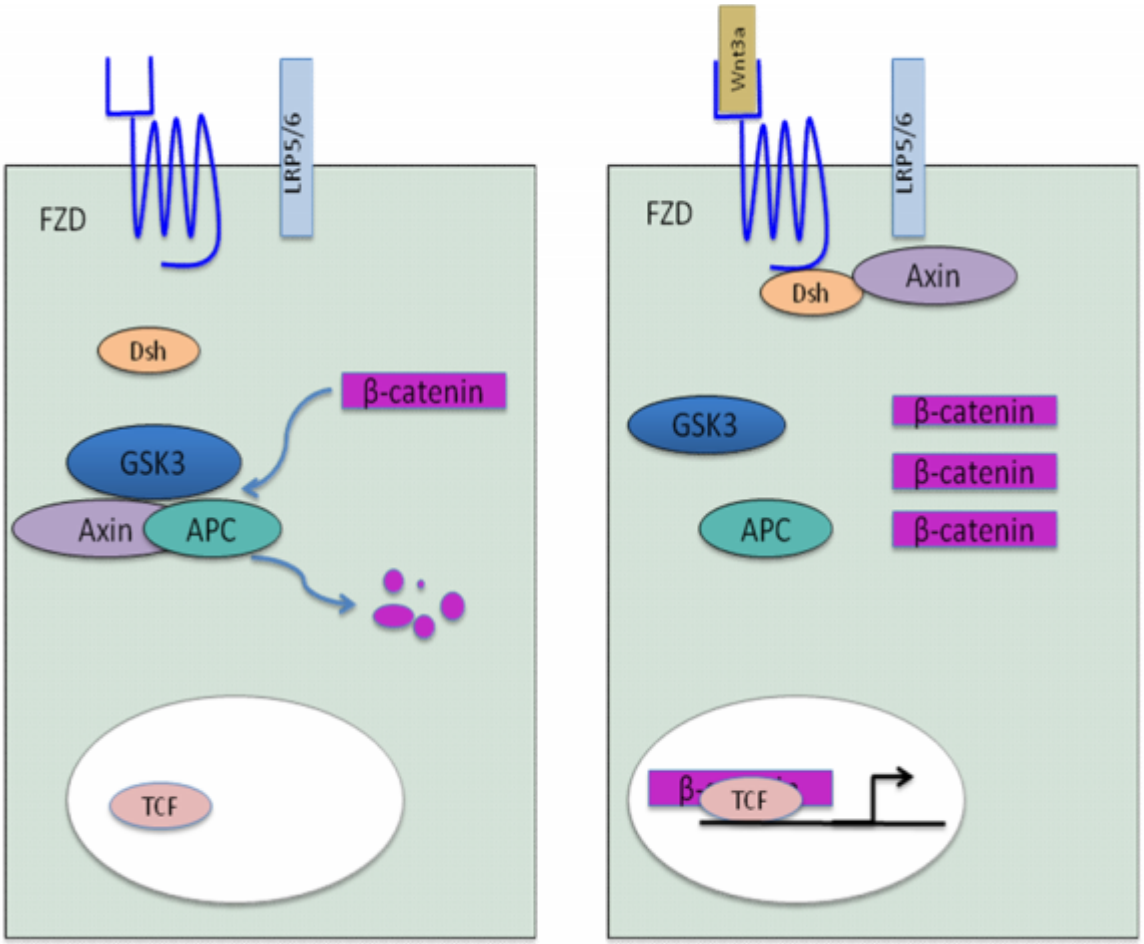

Figure 1: Canonical Wnt signaling pathway. In the absence of Wnt signaling (left diagram) cytosolic β-catenin is continuously degraded by GSK3, APC and Axin. In this destruction complex, GSK3 phosphorylates the amino terminus of β-catenin thereby targeting it for proteosomal degradation. When a cell receives a Wnt signaling input (right panel), the Dishevelled protein, as well as several components of the degradation complex (GSK3 and Axin), are re-located to the membrane where they interact with the Wnt receptor complex. This disassembly of the degradation complex releases β-catenin so that it accumulates in the cytoplasm and subsequently can enter the nucleus where it activates Wnt target genes together with TCFs.

In contrast to the canonical Wnt signaling pathway, significantly less is known about non-canonical pathways. From studies in Drosophila we know that Frizzled can act in both canonical (segment polarity) and non-canonical (planar cell polarity) signaling pathways. Whether individual Frizzled genes act in multiple pathways in mammalian

cells is not currently well established. Several non-canonical Wnt signaling pathways have been described. All of these appear to involve Wnt, Frizzled and the intracellular signal transducer Disheveled, abbreviated Dsh in Drosophila and DVL1, 2, and 3 in mammalian systems. The following non-canonical Wnt pathways have been described:

1. The Wnt/Ca pathway: In this signaling pathway, Wnts bind to FZD receptors to activate Dvl, but the downstream pathways activated by this binding do not signal GSK-3β or β-catenin. This pathway was observed to be active when Wnt5a or Wnt11 are overexpressed in *Xenopus* oocytes, which leads to an increase in intracellular calcium while β-catenin levels remained constant (Kuhl et al. 2000, Slusarski et al. 1997). Following studies demonstrated that certain combinations of Wnts and FZDs can activate calcium/calmodulin-dependent kinase (CAMKII) and protein kinase C (PKC) (Kuhl et al. 2000, Sheldahl et al. 1999).
2. The Wnt/G protein pathway: Some data suggests that FZDs activate G proteins, which then activate phosphodiesterase and phospholipase C (Liu et al. 2001,Wang & Malbon 2003). However, to date no experiments have clearly demonstrated GTP-GDP exchange upon Wnt signaling and the role of G proteins in Wnt signal transduction remains controversial.
3. The Wnt/planar cell polarity (PCP) pathway. This pathway also activates a number of different molecules that regulate the generation of planar cell polarity (PCP) (Fanto & McNeill 2004, Mlodzik 2002). This pathway is critical for the polarized organization of cells within an epithelial sheet, and mutations in PCP components, such as Frizzled and Dsh, disturb the organization of highly

organized epithelial cells. Additionally, activation of DSH leads to the activation of small GTPases, G proteins, and, in some cases, C-Jun N-terminal kinase (JNK) which activates the PCP pathway (Montcouquiol et al., 2006).

4. The Wnt-ROR pathway. Amongst the non-canonical Wnt pathways this particular pathway is of greatest interest for my research. Wnt5a has been shown to inhibit canonical Wnt reporter activation and signal through Jun Kinase (JNK) (Oishi et al., 2003). One hypothesis for the inhibition of canonical signaling postulated that Wnt5a outcompetes Wnt3a when binding to receptors, however, it has since been determined that ROR2 is responsible for the inhibition of canonical signaling. Mikels and Nusse showed that β-catenin levels remain similar with or without the addition Wnt5a in the absence of Wnt3a (2006). Furthermore, a different study done in 2003 discovered that this inhibition of canonical signaling could be due destruction of β-catenin although the mechanism for this has yet to be determined (Topol et al.)

Only Wnt-ROR will be shown in Figure 2 because it relates most directly to my project.

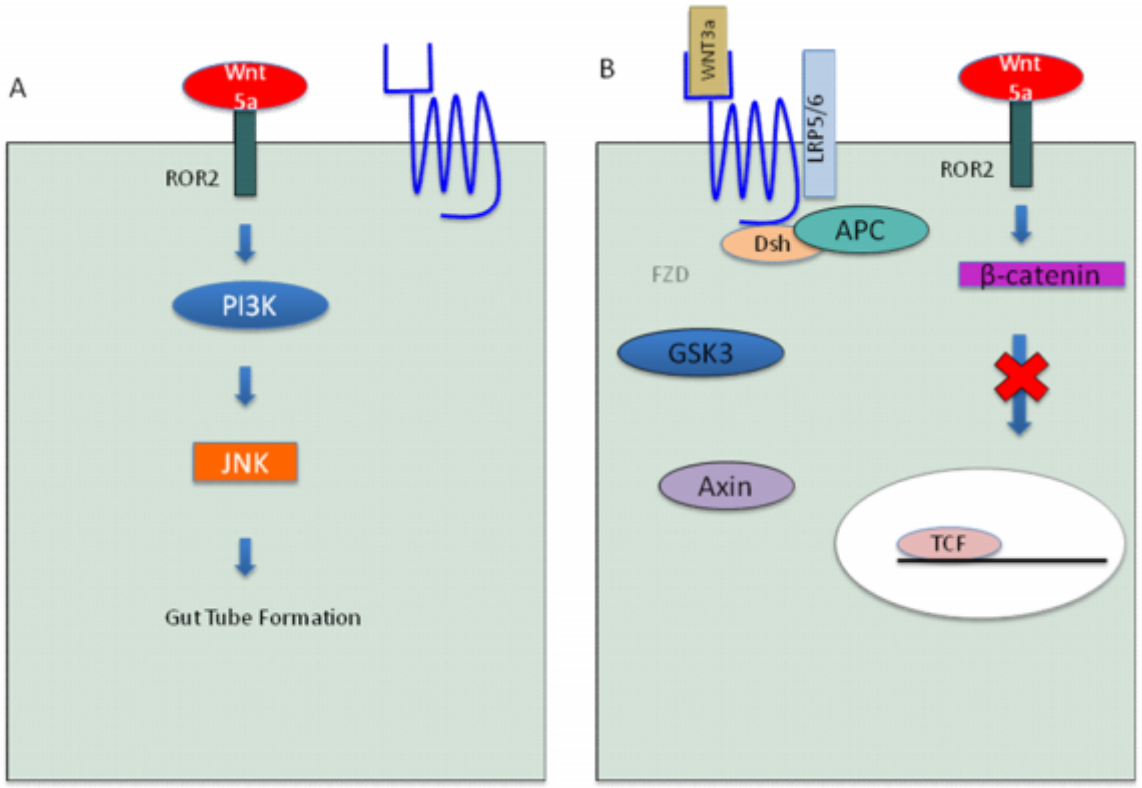

Figure 2: Non-Canonical Wnt Signaling pathway. Panel A represents non-canonical signaling pathways without the presence of Wnt3a. Panel B represents inhibition of canonical Wnt signaling by Wnt5a binding to ROR2 in the presence of Wnt3a and other FZD receptors.

In the past, Wnt genes were categorized to be either canonical or non-canonical based on their ability or inability to elicit β-catenin dependent signaling. However, it has been shown that so called non-canonical Wnts can act in a canonical manner if the appropriate FZD receptor is present. For example, it has been shown Wnt5a can also activate β-catenin signaling in the presence of Frizzled 4. Thus, a single Wnt ligand can initiate different signaling pathways depending on which receptors are available (Mikels& Nusse, 2006). The extent to which this is true in other Wnt/Wnt receptor combinations is unknown. The ability of FZD to signal into either the canonical or non-

canonical pathways is believed to be mediated by co-receptors: for example, a Wnt receptor complex comprised of FZD and LRP5/6 will transduce a Wnt signal into the β-catenin pathway.

1.5Approach

Human pluripotent stem cells (hPSCs) have the potential to become any type of cell in the body, a property that can be exploited to generate appropriate cells for cell replacement therapies of diseased, damaged, or dead tissues. In addition, these cells have proven useful in drug screening and disease modeling. A major challenge in the study of hPSCs is to direct their differentiation into the cell type of interest. The best way to achieve this is to mimic the events that control embryonic development by manipulating the cellular microenvironment. This cellular microenvironment, also referred to as the niche, consists of, but isn't limited to, extracellular matrix components, growth factors, cell-cell interactions, mechanical forces, and small molecules.

In my research project, I focused on one of these major components of the cellular microenvironment, the Wnt proteins and their receptors, which together control and regulate many cellular processes. The specific interaction between the various Wnt proteins and the FZD and ROR receptors are still undefined and there are many questions that have yet to be answered. For instance, it is unclear which Wnts bind what Wnt receptor, whether a single Wnt can interact with multiple receptors, and whether a single receptor can interact with multiple Wnts. In addition, we don't understand how these individual interactions result in distinct signaling outputs, including the activation of either the canonical or non-canonical pathway. If we can characterize these Wnt-receptor

interactions, we will be better able to control Wnt signaling and thereby exploit these properties to develop protocols for the directed differentiation of hPSCs. For example, protocols to differentiate hPSCs into definitive endoderm, a precursor cell population to the formation of such tissues as the lung, pancreas, stomach and intestine, require the activation of the canonical Wnt signaling pathway. However, these protocols are quite inefficient at generating endoderm. A better understanding of which Wnt-receptor interactions mediate this difference will likely improve these efficiencies.

The Willert laboratory has developed methods for the isolation and purification of WNT proteins. My approach to determining these Wnt-receptor interactions will be to construct, express, and purify fusion proteins that carry CRD of various Wnt receptors and a tag, the constant region of an immunoglobulin (Ig). After purification, these recombinant proteins can be used to study Wnt-receptor interactions, and interfere with Wnt signaling in cell-based assays. To date, I have constructed vectors that express the Wnt binding domain of FZD7, ROR2, and FZD5 fused to the constant region (Fc) of the immunoglobulin (Ig) heavy chain. Additionally, I am constructing a similar vector carrying the CRD of FZD10. Using an affinity purification method, I have successfully purified both the FZD7-Ig fusion protein, FZD5-Ig fusion protein, and ROR2-Ig fusion protein. These fusion protein receptors are critical reagents to investigate Wnt-receptor interactions and to specifically modulate Wnt signaling during the process of hPSC proliferation and differentiation. Figure 3 depicts how a Wnt reporter cell line, called 293HEK TOP-Flash, is activated by Wnt signaling and Figures 4 and 5 depicts the

strategy I will employ to block Wnt signaling using CRD fusion proteins in a Wnt reporter cell line.

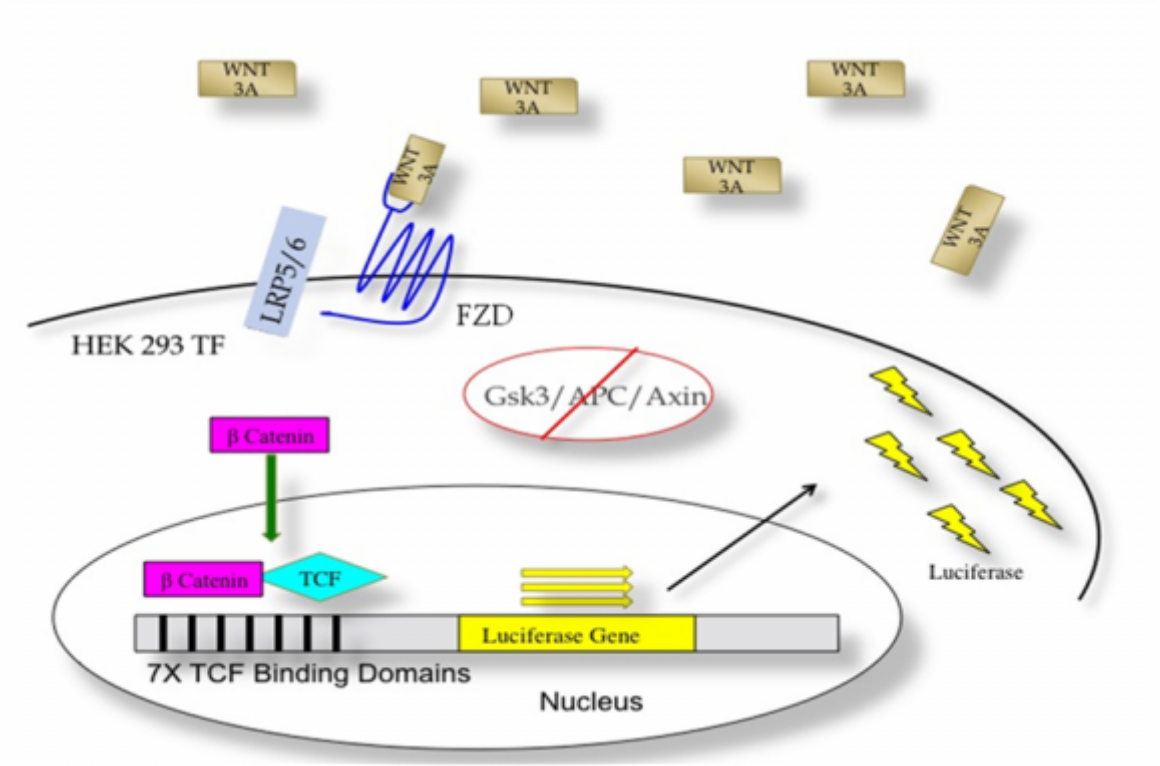

Figure 3: Cartoon Model of Wnt signaling in Wnt reporter cells. HEK293 cells stably transduced with a Wnt reporter, called TOP-Flash, activate expression of a luciferase reporter gene upon addition of Wnt3a protein. Wnt3a leads to the stabilization of β-catenin, which acts together with TCF to activate expression of the luciferase gene under control of a Wnt responsive promoter called TOP (TCF optimal promoter).

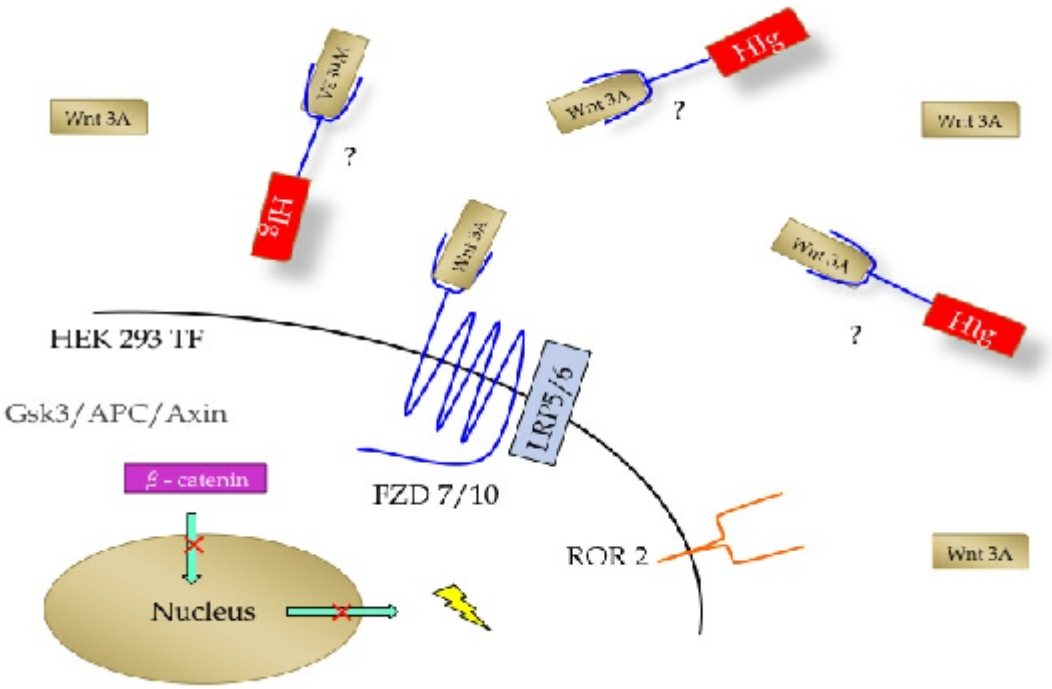

Figure 4: Cartoon Model of Inhibition of Wnt signaling. Upon addition of the CRD-Ig fusion proteins, Wnt proteins are bound thus preventing their interaction with the cell surface receptors. Consequently, the Wnt signal is not transduced into the cell and the reporter construct is not activated. A cell receiving a Wnt signal will express high levels of luciferase, however, addition of these fusion proteins will interfere with expression of this reporter.

Addition of Wnt3a activates the Wnt reporter. In contrast, Wnt5a has no effect on this Wnt reporter activity. However, Wnt5a will block the Wnt3a activity in these reporter cells. Upon addition of the ROR2-CRD-Igfusion protein to reporter cells treated with both Wnt3a and Wnt5a, I expect that the reporter activity will be increased. The proposed model for this effect is illustrated in Figure 5.

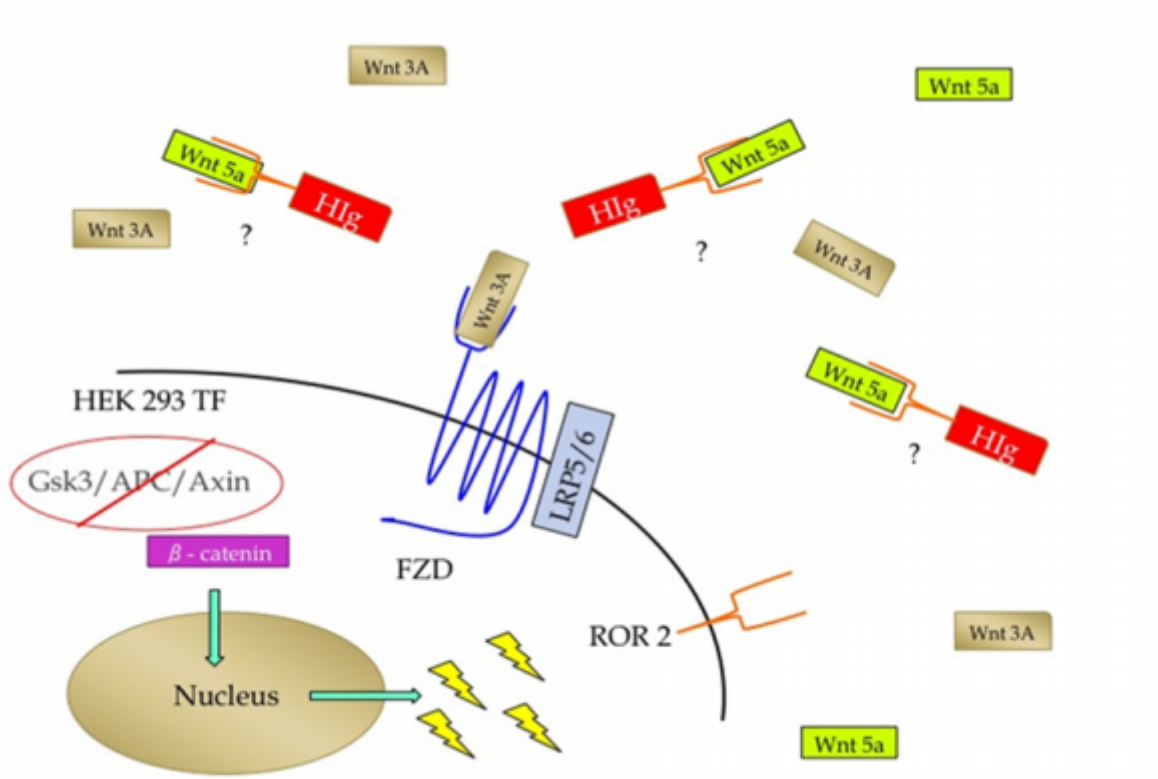

Figure 5: Inhibition of ROR2 signaling. ROR2 activation by Wnt5aleads to the inhibition of canonical Wnt3a signaling and addition of the ROR2-CRD-Ig protein will alleviate this inhibition. Consequently, adding exogenous ROR2-CRD-Ig fusion protein to the media should sequester Wnt5a, thereby leaving less Wnt5a to bind to the CRD of ROR2 on HEK293 TOP-Flash cells. If less ROR2 is being activated in the Wnt reporter cells, Wnt3a signaling should be restored along with luciferase activity.

Chapter 2 Materials and Methods

2.1 Generation of CRD Plasmids

The following cloning strategy was used to construct vectors for expression of the FZD7-CRD-Ig, FZD5-CRD-Ig, FZD10-CRD-Ig, and ROR2-CRD-Ig fusion proteins. Any special techniques or modifications that deviated from the standard strategy will be discussed appropriately.

1. Sequencing and PCR amplification of the CRD. The following primers were used to sequence and amplify the CRD of Wnt receptors. APEX PCR Taq polymerase was used for PCR of the CRD.

Oligo Name	Oligo Sequence	Application
Frizzled 5	TCGTACAGGTAGCAGGCC	Sequencing Primer 1
	CCCGAGCGCATGAGCTGC	Sequencing Primer 2
	CAGGGGCGCCGGCCT	Sequencing Primer 3
	GGCATTCGCCCCCCGA	Sequencing Primer 4
	GCCGAATTCGCGAGGACACG TCCAACGCCAGC	PCR Forward Primer with EcoRI to generate 576 bp fragment encompassing the CRD portion of FZD5
	GGCGTCGACGCCCCTGGCGG GCCTGGAAGG	PCR Reverse Primer with SalI to generate 576 bp fragment encompassing the CRD portion of FZD5
Frizzled 10	CCGCCACCCTGCGCATGACC	Sequencing Primer 1
	AGCCAACAGCAGCTACTTCC	Sequencing Primer 2
	AAGCACCACATCTTAGCTCC	Sequencing Primer 3

Frizzled10	GCCGAATTCAGGACACGTCC AACGCCAGCATG	PCR Forward Primer with EcoRI to generate 490 bp fragment encompassing the CRD portion of FZD10
	GCCGTCGACCGCTGCGGCCG GAACAGC	PCR Reverse Primer with SalI to generate 490 bp fragment encompassing the CRD portion of FZD10
Frizzled 7	CGGGAATTCACTCGCGGCCG GCG ATG CGG	PCR Forward Primer with EcoRI to generate 577 bp fragment encompassing the CRD portion of FZD7
	CGGGTCGACGCGGT AGG GTAGGCAGTGG	PCR Reverse Primer with SalI to generate 577 bp fragment encompassing the CRD portion of FZD7
ROR 2	CGCGAATTCGCCTTGGACGC ATCGTAG	PCR Forward Primer with EcoRI to generate 1090 bp fragment encompassing the CRD portion of ROR2
	CGGGTCGACGTTCCTCTGTA ATCCATGCC	PCR Reverse Primer with SalI to generate1090 bp fragment encompassing the CRD portion of ROR2

Table 1: Primers used for sequencing and PCR. The forward and reverse primers included restriction sites to allow ligation of the PCR product into the mammalian Ig fusion protein expression vector, called pFuse-hIgG1. The first three bases CGG, are added to protect the 5' ends of each oligo and reduce the risk of the restriction sites being destroyed.

The restriction site SalI in the reverse primer was designed so that the CRD coding sequence would be in frame with that of the Ig coding sequence of pFuse hIgG. The products of the PCR were analyzed on a 1% agarose gel and the DNA fragment with the size indicated in the table was excised from the gel and isolated into 50ul of elution buffer using a QIAquick Gel Extraction Kit by Qiagen.

2. Generation and verification of a vector carrying the PCR product encoding the CRD region. The CRD fragment of FZD7 was ligated into a vector named Pcr-

BluntII-TOPO (Invitrogen). The CRDs of FZD5, FZD10 and ROR2 were ligated into a vector named pGEM-T. pGEM-T is provided with the PCR cloning kit (pGEM®-T Vector System I by Promega). The linearized vector provides an A overhang so that PCR products generated using Taq polymerase, which typically adds a T to the end of PCR products, are efficiently ligated into the vector. The ligation mixture was composed of, 1 µl of T4 DNA ligase, 1 µl of 10x Ligase buffer, 1 µl of Pcr-BluntII-TOPO or pGEM-T and 7 ul of the CRD fragment. A negative control was used composed of the same mixture except water was used instead of the CRD Ig fragment. The ligation mixtures were incubated at room temperature for one to two hours before proceeding to the next step.

3. Bacterial Transformation. Two 50 µl aliquots of chemically competent DH5α were taken from the -80°C freezer and thawed on ice. Once thawed, 5 µls of each ligation mixture was added to the thawed bacteria and mixed gently by swirling. The cells were put back on ice for an additional 30 minutes. The bacteria were then heat shocked at 42°C for 45 seconds. One ml of LB was added to the cells and they were placed in a 37°C shaker to recover for approximately one hour. After recovery, 10 µl of the transformation(both negative control and ligation) was spread on an agar-ampicillin plate using glass beads. The remaining volume was spun down for 3 minutes at 5,000 rpm to pellet the bacteria. All but 100 µls was removed from the Eppendorf tubes, the pellets were resuspended in the remaining volume, and spread on agar-ampicillin-plates. The plates were placed upside in a 37°C incubator overnight.

4. Expansion and Miniprep. Multiple colonies were picked and inoculated into 3 ml of LB media with ampicillin. Bacterial plates were wrapped with parafilm for storage at 4°C in case more colonies needed to be picked. The inoculated LB media was placed in the 37°C shaker at 225 rpms overnight. The plasmid DNA was then isolated using a QIAprep Spin Miniprep Kit by Qiagen.
5. DNA detection and purity. Purity and quantity of plasmid DNA extracted was determined using a Thermo Scientific Nano-Drop. Purity was assessed by A260/A280. All of the samples had to have an A260/280 ratio of at least 1.7 before continuing. In most cases the ratio was 1.8 to 1.9. The A260 measurement was used to determine the concentration.
6. Confirmation of sequences. Restriction digestion was used to identify clones that carried an insert of the appropriate size. The enzymes used to determine if the CRD insert had been ligated into the vector were SalI and EcoRI. Clones carrying an insert were submitted for sequencing (Eton). To sequence I used T7 and SP6 primers which flank the insert and are part of the cloning vectors pGEM-T and pCR-Blunt-TOPO. DNA sequences received from Eton Biosciences Inc. were analyzed by comparing them to the published sequences available through NCBI to ensure that no errors were incorporated during the PCR reaction. I used a software package called Lasergene to analyze the sequences and assemble maps of the final constructs.
7. Large scale plasmid isolation. Once a positive clone was identified without errors, the plasmid was re-transformed and isolated using the same processes outlined in

steps 3-5, except a Midi-prep was done using 50 ml of LB instead of 3 mls. After determining the DNA purity and concentration using the Nano-drop, the concentration was diluted to 1 µg/µl.

8. Digestion and extraction of CRD fragment. The CRD containing fragment was cut from Pcr-BluntII-TOPO or pGEM by digesting with EcorI and SalI at 37°C for two hours. The samples were then loaded onto a 1% agarose gel and the DNA fragment was recovered with the QIAgen gel extraction kit.
9. Ligation of CRD containing fragment into pFUSE-hIgG1-Fc1 (pFUSE). pFUSE was linearized by cutting with restriction enzymes EcoRI and XhoI, subsequently it was run on a 1% agarose gel and purified using the QIAgen gel extraction kit. To ligate I added 1 µl of the linear pFuse at 75 ng/ul, 1 µl of 10X Ligation buffer, 1 µl of T4 DNA ligase, and 7 µls of the CRD containing DNA fragment (insert) at 100 ng/ul. The ligation was incubated overnight at room temperature.
10. Final Steps. Subsequent to the ligation of the insert into pFUSE, I repeated steps 3-7. However instead of a midi-prep in step 7 a Qiagen QIAprep Maxi-prep kit was used to generate more DNA. Also, instead of Ampicillin, I used the antibiotic Zeocin because the pFUSE vector carries a Zeocin resistance gene.
11. A special case for FZD5. A cDNA for human FZD5 was obtained from Open Biosystems (pCR-BluntII-TOPO_hFZD5, clone ID# 40011265). To confirm that the received plasmid carried the proper gene I submitted the plasmid for sequencing using T7 and SP6 primers. All sequencing reactions were performed by Eton Inc. (San Diego, Ca). This analysis revealed that the FZD5 cDNA carried

a nonsense mutation at nucleotide position 1701 (counting from the start ATG), changing a C/G to an A/T thus producing a TAG stop codon rather than the expected TCG codon encoding Serine. This cDNA is expected to produce a shortened FZD5 protein that truncates at amino acid 567 (position relative to first Met). This mutation was observed in the clone we received from Open Biosystems but not in several other clones listed on the NCBI website including clone ID# 7855 for reference. This suggests that this change was likely acquired as a mistake during the cloning of this cDNA. Since this mutation is expected to have detrimental consequences, I used the QuikChange II XL Site-Directed Mutagenesis Kit (Cat# 200521 by Agilent Technologies) to revert the mutation and restore the complete open reading frame. To do so, I designed the following two complementary oligos: FZD5 forward primer: CAGGGGCGCCGGCCT**C**GGGGGGCGAATGCC and FZD5 reverse primer: GGCATTCGCCCCCC**G**AGGCCGGCGCCCCTG. The bolded nucleotide indicates the nucleotide that was changed from the incorrect T nucleotide. Using the high fidelity DNA polymerase Pfu, these two primers and the plasmid carrying the FZD5 cDNA, I used PCR to amplify the entire plasmid. The products of the PCR reaction were then digested with DpnI, a restriction enzyme that cleaves only methylated DNA. Since only the original input plasmid DNA was methylated, this restriction digestion removed all plasmid DNA carrying the mutant FZD5 cDNA. The DpnI digested PCR products were then transformed using the same process as described in step 3.

2.2 CRD Ig Overexpression in Mammalian Cells

The following conditions and procedures were used to express the CRD Ig fusion protein in mammalian cells.

1. Cell culture. For all transfections and cell culture, human embryonic kidney (HEK) 293 cells were cultured in DMEM with 10% fetal bovine serum (FBS), Penicillin, Streptomycin, and Glutamate, referred to from here on as "media".
2. Transient Transfection. HEK 293 cells were grown to 80% confluence and the newly generated plasmids (ROR2 CRD Ig, FZD7 CRD Ig, and FZD5 CRD Ig). 12.5 micro grams of the CRD Ig plasmid was added to 500 µls of DMEM without FBS in 1.5 ml Eppendorf tube. Subsequently, 50.2 µls of PEI, the reagent responsible for weakening the cell membrane and allowing the plasmid DNA to penetrate, was added to the media and allowed to incubate at room temperature for 5 minutes. The media was then aspirated and replaced after washing the cells with PBS. The transfection mixture was added in a drop wise fashion to the HEK 293, mixed by gentle shaking, and placed back in the 37°C incubator. The media was replaced after five hours with fresh media. The HEK 293 cells were then placed back in the 37°C incubator for 72 hours. After the incubation period, the conditioned media and cell lysate was collected from the transient transfection plates.
3. Stable Transfection. The same process was used for the stable transfections as detailed above however after 72 hours the transfected HEK 293 cells were split 1:10 by washing the cells with PBS and then dissociating them in 1 ml of

.05% trypsin. Once the cells were dissociated from the bottom of the dish, 9 mls of DMEM were added to the cells and pipetted up and down to resuspend the cells. 1 ml of this mixture was then added to a new 10 cm plate with 9 mls of fresh media. Next, 40 µls of Zeocin was added to the mix to bring the total concentration of Zeocin to 400 µg/ml. Fresh media supplemented with Zeocin were added very three days until cells reached confluency.

4. Generating single cell clones. After approximately two and a half weeks of Zeocin selection, the pool of stable drug selected cells were serially diluted in 96 well plates so that some wells would receive one cell. To do so, 200 µls of media with Zeocin at 400 µg/ml was added to every well of the 96 well plate. The pool of stable clones was split 1:10 in the same fashion as before however, an additional 1 ml was added to a reservoir with 10 mls of DMEM . This was and mixed by pipetting up and down. Using a multi-channel pipette, 50 µls of the cell mixture was added to the first row of 12 wells of the prepared 96 well plate. Subsequently, 50 µls of this first row of wells was transferred to the next row of wells thereby yielding a 1:4 dilution (= 50 µl into 200 µl). This was repeated to the subsequent rows of wells. The wells were monitored daily and after about two weeks, the wells that contained colonies from a single cell were expanded into a 48 well plates. The cells were expanded further into 24 well, 6 well and eventually 10 cm plates.

2.3 Protein Detection and Purification

The following conditions and procedures were used to collect and detect the CRD-Ig fusion proteins in the conditioned media (CM).

1. Collection of conditioned media. The CM from transiently transfected or Zeocin selected clones was filtered by passing it through a .22um syringe filter and stored at 4°C. 100 µls of the filtered CM was set aside.
2. Addition of PGS. The Ig portion of the fusion proteins can be efficiently bound and precipitated using Protein G Sepharose (PGS). In order to detect the fusion proteins, I added 40µls of 1:1 slurry of PGS beads in PBS to the filtered conditioned media (about 10ml) and rotated for one hour at 4°C. This allows the fusion proteins to bind to the PGS beads.
3. Washing. The beads were pelleted in a tabletop centrifuge for 10 min at 3500 rpms. The supernatant was carefully removed without disturbing the pelleted PGS beads and transferred to a 15 ml conical tube. The pelleted beads were then washed three times in TNT (1% Triton X100, 150mM NaCl, and 50mM Tris-Cl, pH7.5). The supernatant (CM) was then subjected to the same protocol described in steps 2 and 3. This was done to determine if the beads were depleting all of the fusion proteins. The CM/beads used in this step were handled the exact same way as the original beads from this point on.
4. Protein denaturing. After washing, 40µls of 1X protein loading buffer was added to the beads. Also, 10 µls of 2X protein loading buffer was added to 10 µls of the CM. All of the samples (PGS beads with CM precipitate (PPT) and

PGS depleted PPT) were boiled at 95°C in a heating block for 5 minutes. This step was done to denature and release the proteins from the beads. The samples

5. Sample loading and transfer. 20µls of the samples were loaded onto a SDS-polyacrylamide gel and after electrophoresis proteins were transferred to a nitrocellulose membrane using a standard transfer box in transfer buffer.
6. Wash and blocking buffer. After transfer the nitrocellulose membrane was washed in water for 5 minutes and proteins were visualized using Ponceau S stain. Subsequently, the membrane was washed in TBST (20mM Tris-Cl, 150mM NaCl, 0.2% Tween 20) and then blocked in blocking buffer (3% non-fat dried milk and 1% BSA in TBST) on a shaker for 30 minutes.
7. Antibody hybridization. 20 mls of fresh blocking buffer was added to the nitrocellulose membrane along with 1.5 µl of the antibody α-hIgG HRP (Promega, W403B) and incubated at room temperature for 1-2 hours on the shaker.
8. Wash. The nitrocellulose was then washed again 3 times in TBST for five minutes.
9. ECL detection. 1 ml of ECL mixture (500µl of each substrate in the Super Signal West Pico Chemi Kit, Pierce) was placed on top of the nitrocellulose and repeatedly pipetted on top of the membrane. The nitrocellulose membrane was placed in cellophane and taped to the inside of the photo cassette. In the dark room, the X-ray film was placed on the nitrocellulose paper for varying

amounts of time and the protein was detected using the film-processing machine.

The following conditions and procedures were used to collect and detect the CRD Ig fusion protein in the cell lysate.

1. Cell preparation. The cells expressing the fusion protein were first washed in PBS. One ml of PBS was then added and the cells were collected using a cell scraper.
2. Spin. The cells were then spun down for 3 minutes at 2000 rpm.
3. Lysis. The cells were then lysed in TNT containing a cocktail of protease inhibitors (AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, and Pepstatin A) and incubated on ice for 10 minutes.
4. Re-spin. The cells lysate was then centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant, or lysate, was transferred to a fresh tube.
5. Protein analysis and sample loading. The protein concentration of the lysate was assayed using a Bradford Assay and the OD595 was measured using a Perkin Elmer Envision Multimode Plate Reader. Using this quantization, 20 µg of protein in protein loading buffer was analyzed by SDS-PAGE.
6. Repeated steps 5-11 as described above in the CM section.

After single cell clones were made of the fusion proteins, a large amount of conditioned media was generated so that the proteins could be purified using FPLC.

1. Expansion. A clone was selected that produced a high amount of the fusion protein. It was split and expanded into twenty 20 cm plates.
2. Collection. After the cells had reached confluency, the media was collected and filtered using a .22 μm vacuum filter. More media was added to the cells and collected 72 hours later using another vacuum filer.
3. Purification. Approximately one liter of CM was loaded onto a 1 ml PGS column using an AKTA purifying system. The column was then washed using PBS and bound proteins were eluted with0.1 M glycine, pH 2.5. The fractions were collected into 75 μl of Trizma to neutralize the pH.
4. Finally the fractions were analyzed by detecting with an α-hIgG HRP by western blot as described above in steps 5-11 in the CM section.

2.4 Cell Based Assays

The following procedures and assays were used to determine if the fusion proteins had the ability to inhibit canonical Wnt signaling in the reporter cell line HEK293_TOP-Flash (TF) cells.

1. Plating. The TF cells were plated in a 96 well dish and allowed to reach 80% confluency.
2. Wnt3a dilutions. Wnt 3a was diluted to 1:100 and 1:400 and CRD-Ig fusion proteins were added to make 1:1, 1:2, 1:4, and 1:10 dilutions.
3. 200 μls of each Wnt3a/CRD Ig dilution was added to the TF cells in three different wells to make triplicates.
4. Incubation. The cells were put back in the incubator for 24 hours at 37°C.

5. Aspirate and Lysis. The media was aspirated from the cells and were lysed in 25 µls of lysis buffer (100mM K-PO4, pH 7.8, 0.2% Triton X-100).
6. Analyze. 20 µls of the cell lysate was added to a black-walled 96 well plate with a clear bottom. Luciferase assays were performed in a Perkin-Elmer Envision plate reader. The amount of luciferase activity is directly representative of the amount of Wnt activity present in the media. Addition of CRD Ig fusion proteins is predicted to inhibit this activity.

Chapter 3 Results

3.1 Cloning Results

The first step in generating the fusion protein was to design primers that amplify the CRD region of the Wnt Receptors. Sequences of FZD7 and ROR 2 were previously confirmed by other members of the lab. The PCR product shown below (Figure 6) was generated using the FZD7-CRD primers listed in Table 1 and two distinct plasmids carrying FZD7 cDNA. The other three fusion proteins were generated using the same method and therefore will not be shown.

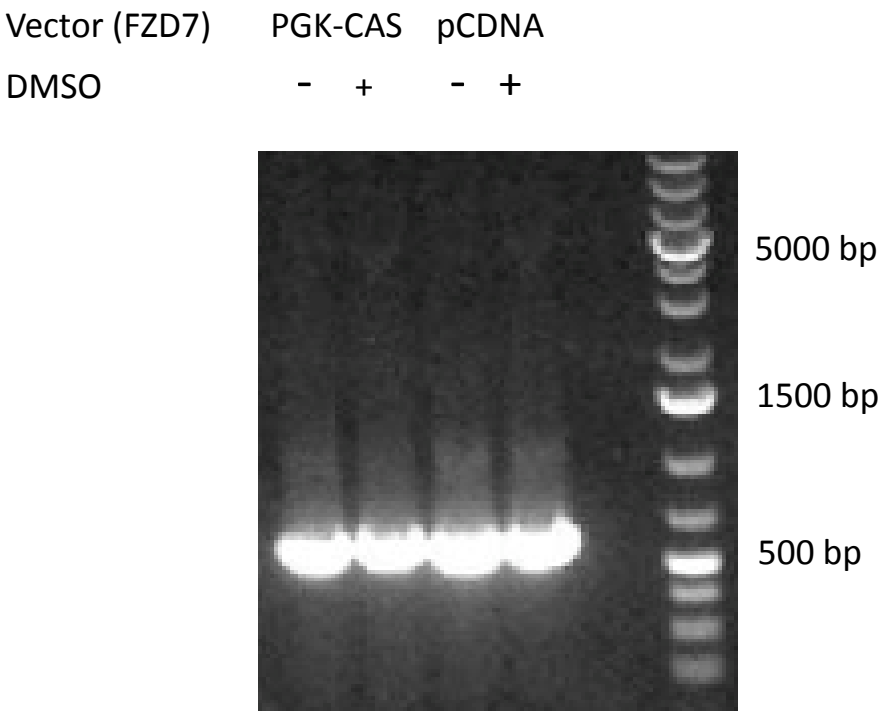

Figure 6: PCR of FZD7 CRD Region. Gel shown is a 1% Agarose gel. Addition of DMSO often times improves yield of PCR products. In this case, DMSO had no appreciable effect on the PCR.

The ligation into Pcr-BluntII-TOPO and subsequent transformation was extremely inefficient for FZD7-CRD and ROR2-CRD, yielding only a small number of colonies. The clones were digested with SalI and EcoRI and isolated using a gel DNA extraction kit. After several failed attempts trying to ligate the FZD7 CRD fragment into pFuse-hIgG1-Fc1 I had the fragment sequenced and discovered that the fragment that I had isolated from the Pcr-BluntII- OPO was not in fact the correct sequence. The SalI site had not been incorporated into the DNA fragment during PCR. The reason I still obtained the right size fragment was due to the fact that the Pcr-BluntII-TOPO vector has EcoRI sites on the ends of the linearized form. Consequently, regardless of what is inserted into the vector the fragment, it will be cut out with EcoR1. Given that I was already cutting with EcoRI to extract my CRD fragment, a band that was approximately the right size was isolated but not the correct FZD7 CRD fragment. I've illustrated the FZD7-CRD fragment below with the relevant restriction sites in Figure 7.

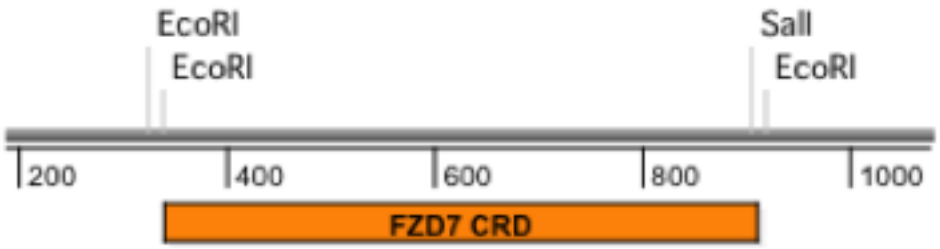

Figure 7: FZD7 CRD in Pcr-BluntII-TOPO with Restriction Sites.

At this point I started over from the beginning to generate a new Pcr-BluntII-TOPO FZD7 CRD shuttle vector. Again I had trouble generating a clone and continued to get no results before finally getting seven positive clones. I ran several diagnostic

digestions to determine if any of the clones contained the correct sequence. I also had the plasmid sequenced in order to confirm before moving on. Yet again the fragment I had obtained was the correct size but still incorrect, this time containing a completely wrong sequence of E.coli chromosomal DNA. The third attempt was also difficult. I had wrongly assumed there was an error in my technique or that my protocol wasn't sufficient for generating clones. I altered ligation protocol several times several times throughout the process but continued to get no or very few colonies. At a later point I discovered that the batch of bacteria I was using had very poor transformation efficiency. After switching bacteria, I was able to generate the other CRD clones with much more ease. After the third ligation into the Pcr-BluntII-TOPO vector, I obtained four clones. A mini-prep was performed on all four clones obtained and subsequently digested EcoRI as well as sequenced. Of the four clones only clone four contained the correct insert (Figure 8).

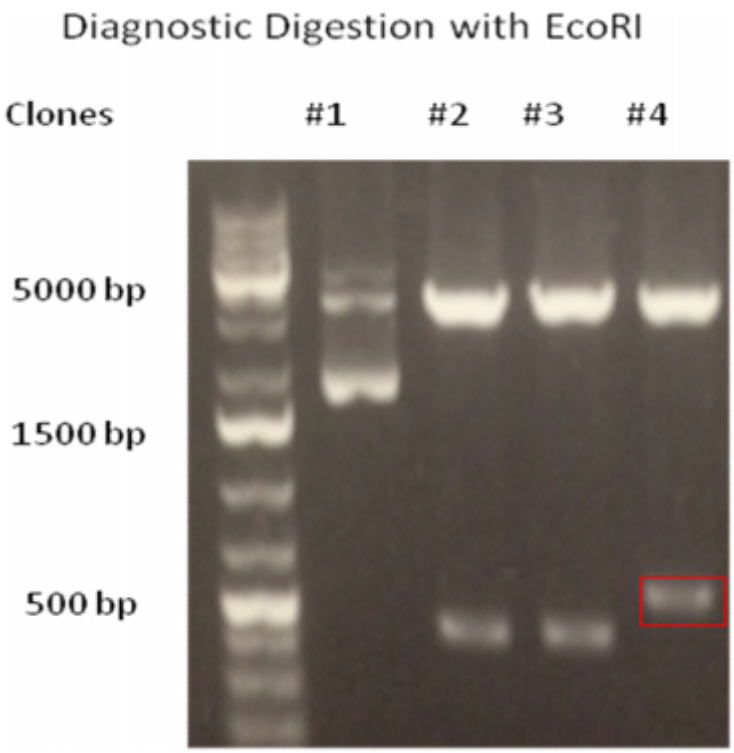

Figure 8: Diagnostic Digestion of Clones with Pcr-BluntII-TOPO FZD7 CRD. The clone in lane 4 has the correct band at approximately 570 bp. Gel shown is a 1% Agarose gel.

Following digestion with the restriction enzymes, clone 4 was re-transformed and a midi prep was performed to generate more DNA. The plasmid was cut with EcoRI and SalI and the fragment was then isolated and ligated into pFUSE hIgG-Fc. Figure 8shows another diagnostic digestion using FZD7 CRD Ig as an example with EcoRI and NcoI after insertion into pFUSE.

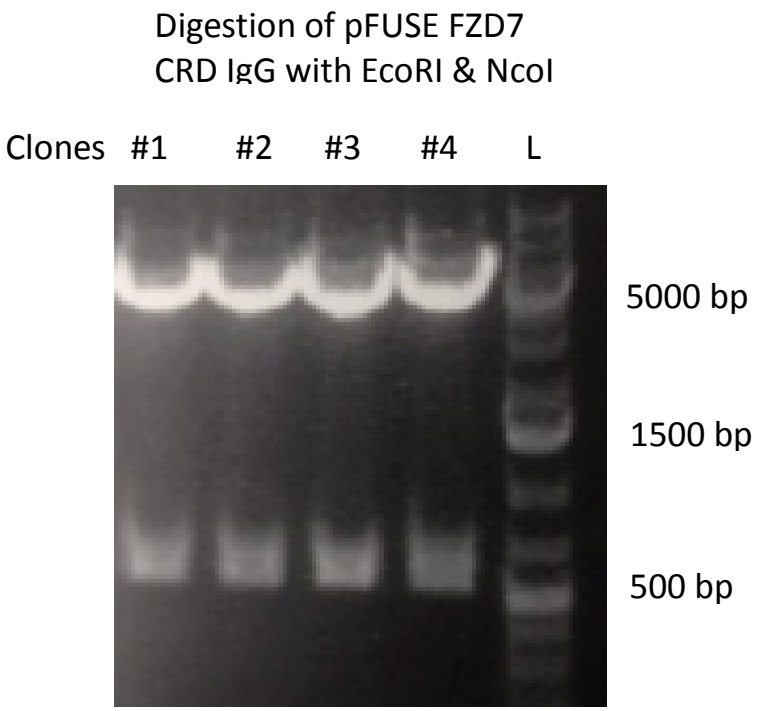

Figure 9: Diagnostic Digest of Clones 1-4 of FZD 7 CRD Ig. All clones have the correct insert and were digested with EcoRI and NcoI. Gel is a 1% Agarose gel.

The following table includes all constructs made.

Construct Name	Vector Name	CRD Size From ATG	CRD plus Ig	Expected MW of CRD-Ig	Number of amino acids for CRD-Ig
FZD7 CRD Figure 1	pFuse-hIgG1	551 bp	1.26 Kb	45.5 Kda	417
FZD10 CRD Figure 10	pFuse-hIgG1	457 bp	1.15 Kb	42.8Kda	381
FZD5 CRD Figure 10	pFuse-hIgG1	530 bp	1.23 Kb	44.2 Kda	402
ROR2 CRD Figure 10	pFuse-hIgG1	983 bp	1.69 Kb	62.7 Kda	561
Constructs	Vector Name	CRD size with restriction sites	Total Size		
FZD7CRD	Pcr-BluntII-TOPO	577 bp	4.09 Kb		
FZD10 CRD	pGEM-T	487 bp	3.49 kb		
FZD5 CRD	pGEM-T	576 bp	3.58 Kb		
ROR2 CRD	Pcr-BluntII-TOPO	1084 bp	4.60 Kb		

Table 2: Plasmids Constructed. All CRD Ig fusion plasmids as well as intermediate plasmids that have been constructed or in the process of constructing.

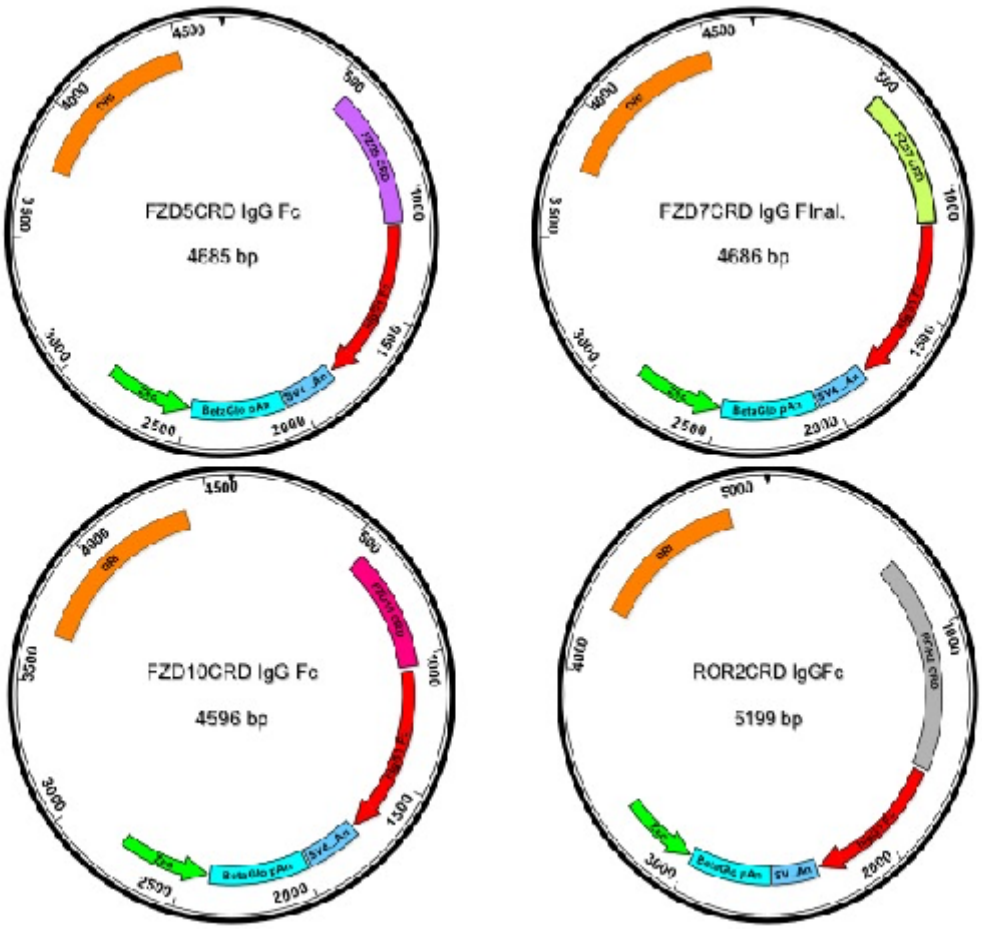

Figure 10: Maps of plasmids carrying fusion protein genes between CRD and Ig. FZD10 is still in the process of being made.

3.2 Protein Detection and Purification Results

The next stage of the project was to express, analyze, and purify the fusion proteins. To do so, I transfected the plasmids into the mammalian cell line HEK293, as described in Chapter 2.2. Although all three fusion receptors were eventually expressed there was some difficulty in detecting the CRD Ig for some of the receptors. For example,

FZD7 could not initially be detected and ROR2 still expresses a higher concentration of the fusion protein in the cell lysate compared to the conditioned media (CM). However, after a few trials I was able to get all of the fusion proteins in 10 ml conditioned media obtained from both a stable pool and clonal cell line (see section 2.2). As stated in chapter 2.3, the proteins were collected either through PGS beads or through FPLC purification. The Western Blot in Figure 10 shows the detection of the CRD Ig fusion protein isolated from various sources.

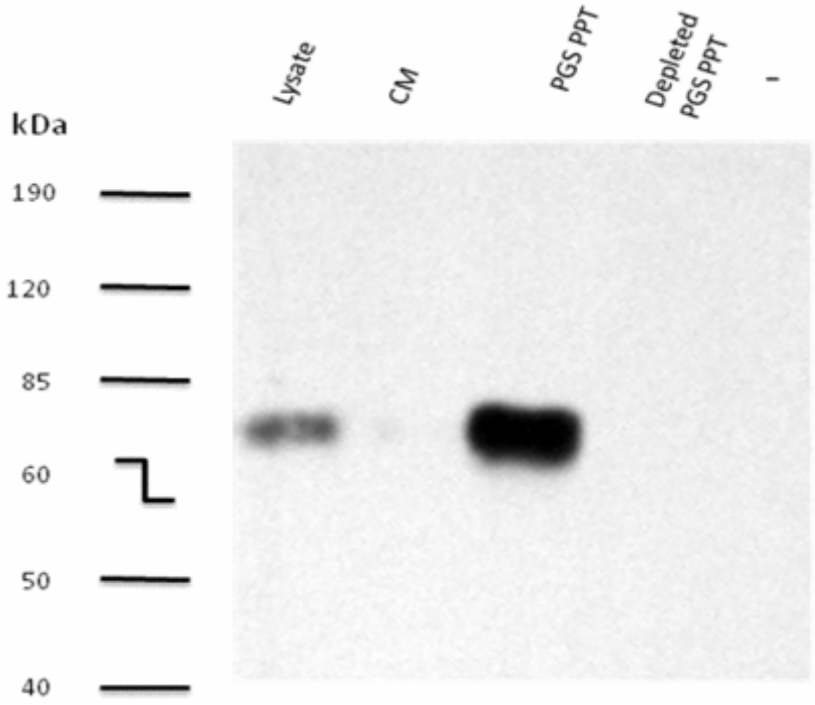

Figure 11: Western Blot Testing Stable Pool of FZD7 CRD Ig. Detected using α-hIgG HRP antibody at a 1:5000 dilution. The negative control was HEK 293 lysate.

After detecting the fusion protein in a stable pool, single cell clones were isolated and tested by western blot to determine if they were still producing the CRD Ig protein.

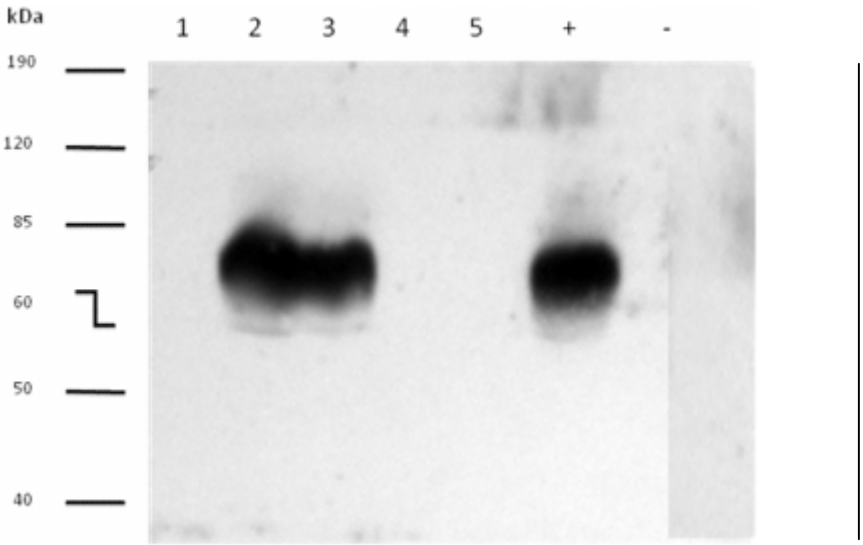

Figure 12: Clones Tested for the Presence of the FZD7 CRD Ig Protein. This is a western blot using α-hIgG HRP antibody at a 1:5000 dilution. Lane six was a positive control using CM purified with PGS from a pool of stable clones. The negative control was HEK 293 CM, PGS was also used to concentrate the CM.

A positive clone was picked and expanded to generate about a liter of conditioned media. The conditioned media was purified using FPLC and the two fractions with the majority of proteins were pooled. Figure 12 shows the detection of the purified material.

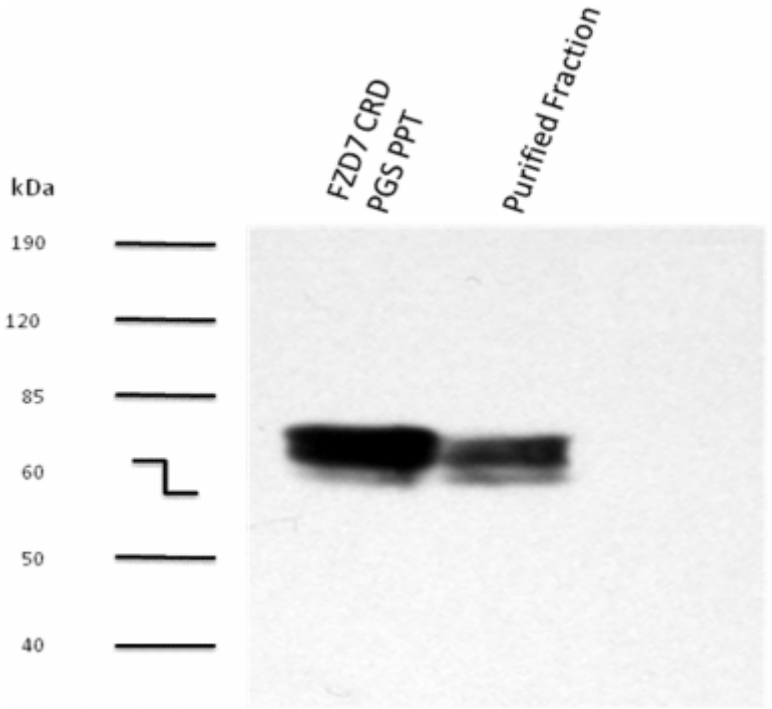

Figure 13: Western Blot of Purified Fusion Protein by FPLC. The negative control was HEK 293 CM purified by PGS.

Lastly, Figure 13 depicts a western blot of all the fusion proteins I have generated.

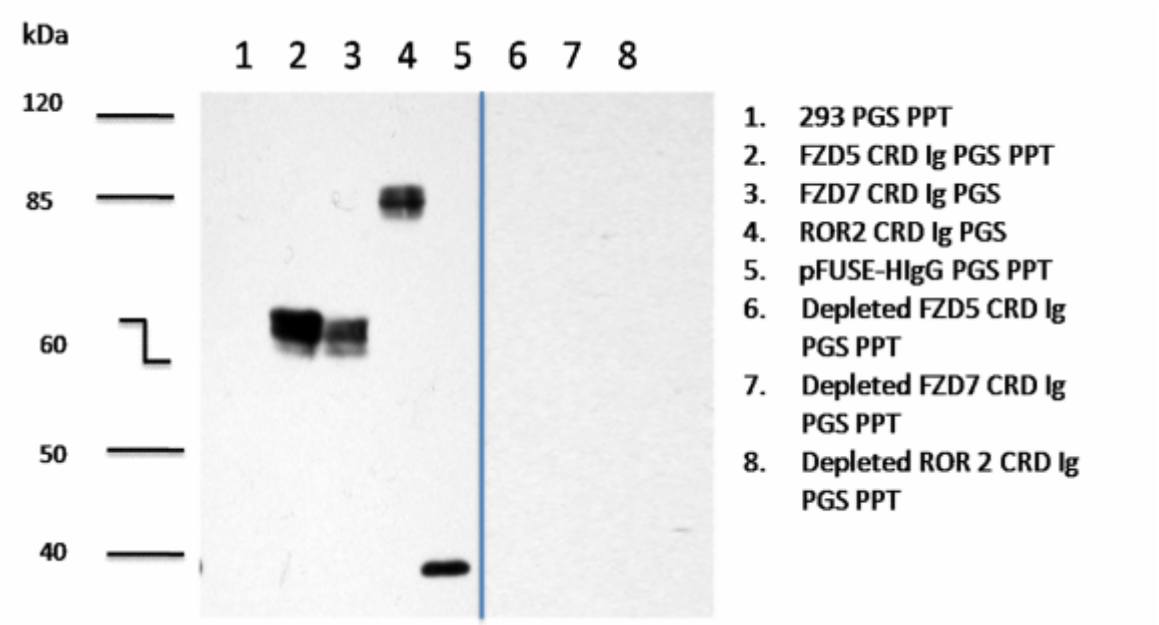

Figure 14.Western Blot of all CRD Ig Fusion Proteins. Lane 1 served as a negative control using CM from normal HEK 293 Cells. Lane 5 contained pFUSE HIgG with no insert to serve as a positive control. Lanes 5,6, and 7 was the same CM as samples 2,3, and 4 subject to the same process of PGS enrichment as detailed in section 2.3.

3.3 Cell Based Assay Results

The last step of my project was to determine if the fusion proteins were able to inhibit WNT signaling. Before I ran assays with the fusion proteins in Wnt reporter lines, I needed to determine if Wnt3a can signal the reporter cell line, HEK 293 TF, and drive the expression of luciferase. In figure 15, we see that as we add increasing concentrations of Wnt3a, the amount of luciferase activity increases accordingly. It was also necessary to determine if Wnt5a can inhibit canonical signaling by Wnt3a. In figure 16 we see that Wnt5a inhibits luciferase activity as increasing concentrations are added, presumably by activating the ROR2 pathway and inhibiting β-catenin.

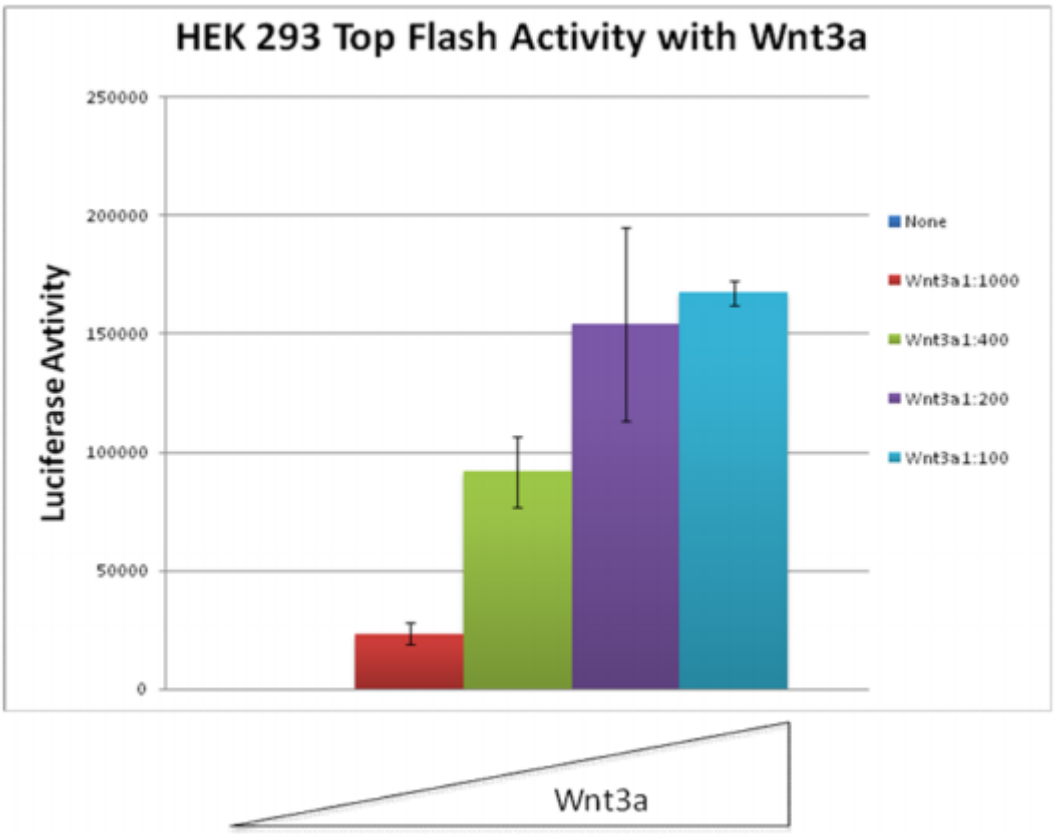

Figure 15: HEK 293 Top Flash Activity with Wnt3a. Luciferase expression driven by Wnt 3a increases as it is added in higher concentrations. Zero expression was seen when no Wnt3a was added.

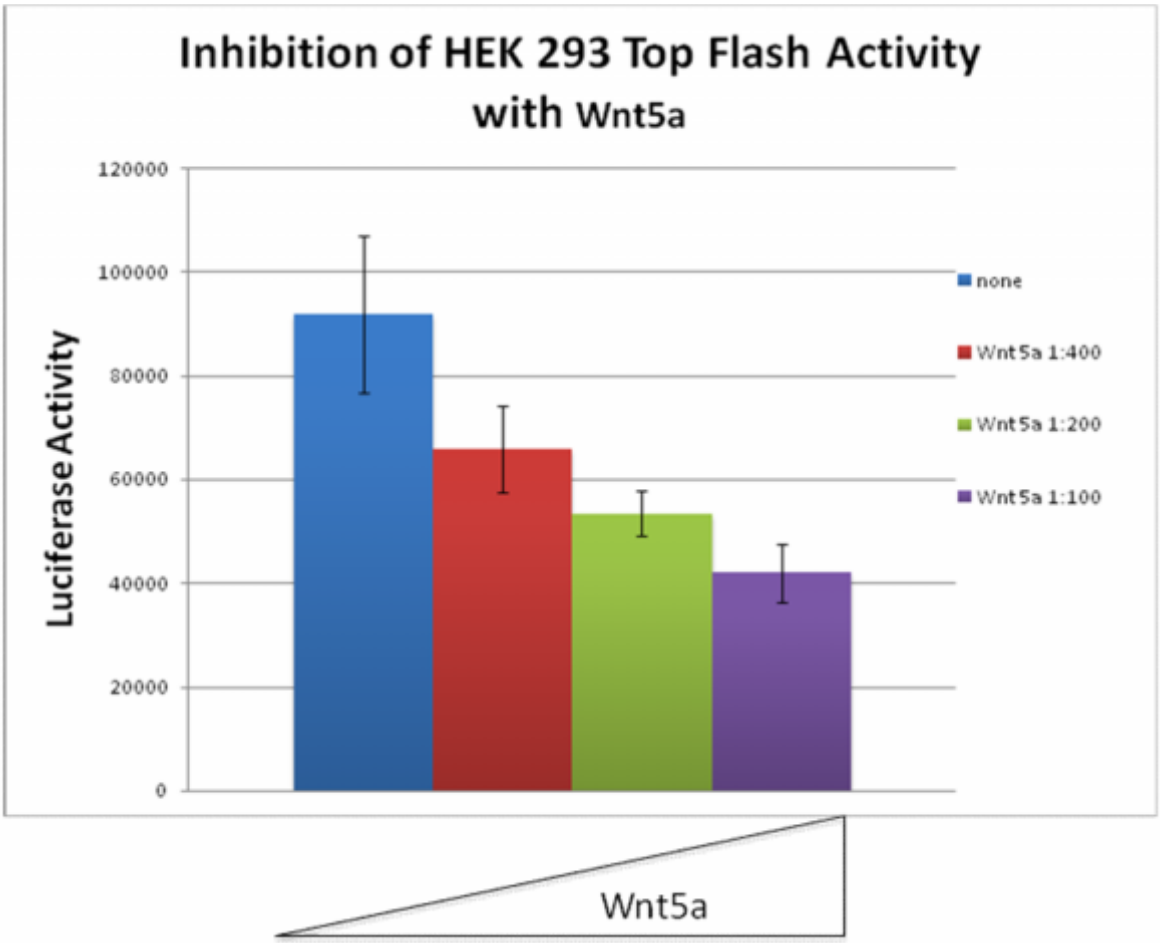

Figure 16: Inhibition of HEK 293 Top Flash Activity with Wnt5a. As the concentration of Wnt5a is increased we see a steady drop in luciferase activity. This assay was done with Wnt3a present at a 1:400 dilution.

The next step was to demonstrate that the fusion proteins could be used to inhibit Wnt signaling. In figures 17 and 18, filtered conditioned media from a FZD5 CRD Ig HEK 293 clone was added at 1:1, 1:2, 1:4, and 1:10 dilution to Wnt3a at 1:100 and 1:400 concentrations, respectively. These dilutions of WNT3a/FZD5 CM were then added to a new 96 well plate of HEK 293 TF cells and assayed as described in the methods section.

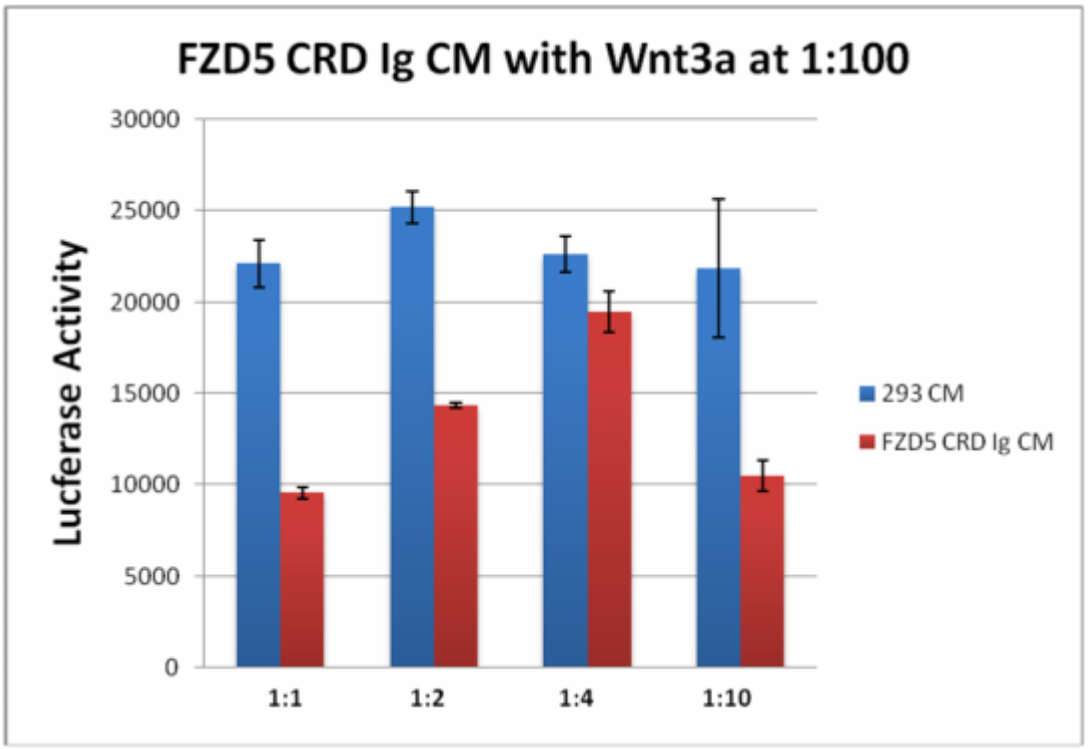

Figure 17: FZD5 CRD Ig CM with Wnt3a at 1:100. 293 CM was used as a control. The experiment was done in triplicate in a 96 well plate. The data suggests that the FZD5 CRD Ig effectively inhibits Wnt signalling

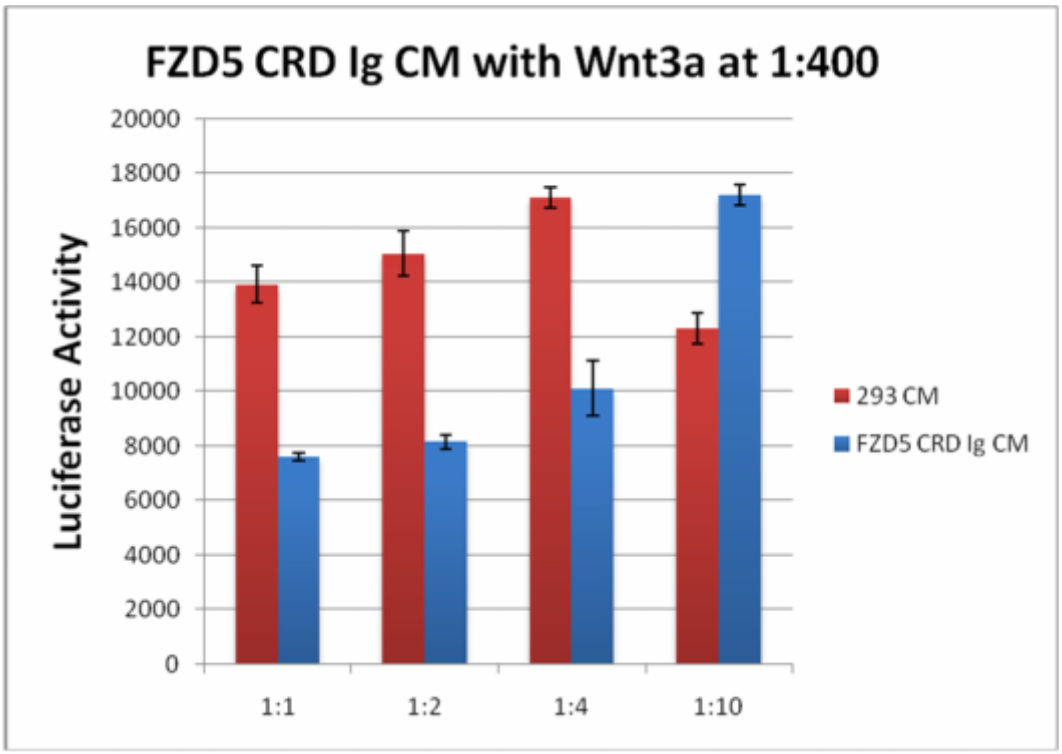

Figure 18: FZD5 CRD Ig CM with Wnt3a at 1:400. The experiment was done in triplicate in a 96 well plate. The data suggests that the FZD5 CRD Ig effectively inhibits Wnt signalling

As shown in Figures 17 and 18, the fusion proteins were able to inhibit WNT signaling when using fresh conditioned media. When looking at figure 17, inhibition of signaling returns at the 1:10 dilution of the fusion protein. I can not provide a solid explantion as to why this effect is seen. One possible explanation is that fusion proteins aggregate together at higher concentrations and don't inhibit signaling effectively. Although, if this were the case an opposite trend would be seen in the graph as more of the fusion protein was added. Another possibility is human error, it is possible that I accidentally aspirated more cells than usual in these particular wells when removing the media, thereby leaving less lysate and luciferase in the wells. However, it is most likely that that this was just due to random error such as a poor read by the plate reader.

Regardless of the origin of the error, it can be ruled out as an outlier. Early on I saw robust inhibition at higher concentrations of the fusion protein and little inhibition at low concentrations. However, there was quite a bit of error at every concentration. When the experiment was repeated at a later date I noticed that the level of inhibition had significantly decreased as well as that the overall value of the TOP-Flash activity had diminished. After several test runs, I determined that the TOP-Flash cells had significantly declined in luciferase production, even under constant selection. I was also able to determine that the fusion proteins lost their inhibitory ability after being stored for as little as one month (figure 19).

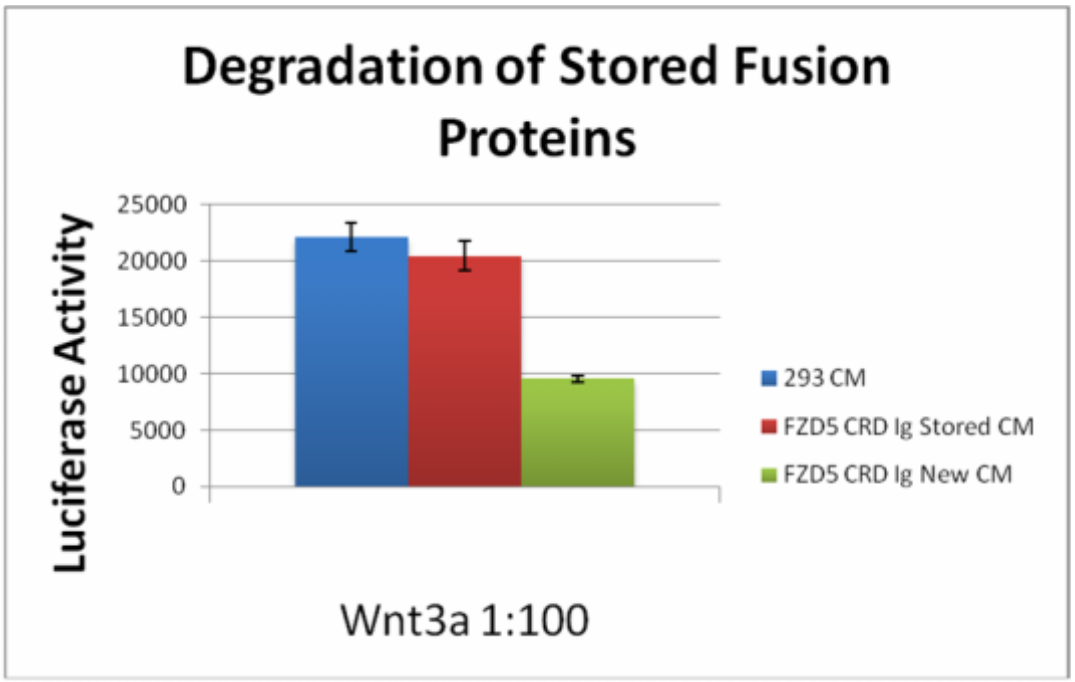

Figure 19:HEK 293 Top Flash Assay with Stored FZD5 CRD Ig CM. This data suggests that FZD5 CRD Ig CM detereates after about a month or storage due to its similar results as regular 293 CM, the control conditioned media,

Figure 19 provides clear evidence that inhibitory abilities of the fusion proteins degrade after an unknown amount of time. The FZD CRD Ig CM media used in this

assay was approximately one month old, stored at 4°C and its ability to inhibit has most likely been completely diminished. Nonethess, Figures 17 and 18 clearly illustrate that FZD5 CRD Ig fusion protein can effectively inhibit Wnt 3a signalling (as does FZD7 CRD Ig).

Chapter 4 Conclusion and Future Directions

In conclusion, I have demonstrated a strategy and method to generate CRD-Ig fusion proteins. My preliminary data provides evidence that these fusion proteins can be detected and purified as well as inhibit WNT signaling but are limited by rapid degradation in storage. These fusion proteins can be used to study WNT receptor interactions and to disrupt WNT signaling in cell culture models therebyclarifying the mechanism of receptor-ligand specificity and interaction. Much remains to be done in the future to further characterize these fusion proteins, including testing them with different Wnt proteins as well as constructing FZD10 CRD Ig. Also, it still remains to be proven whether ROR2 CRD Ig can impede the inhibition of Wnt signalling by binding exogenous Wnt5a and restoring Wnt3a signaling in HEK 293 TOP-Flash cells.

By characterizing these Wnt-receptor interactions, we have gained a better understanding of the control of Canonical and Non-Canoncial Wnt signaling. These properties can be exploited in the future to develop protocols for the directed differentiation of hPSCs. It will also be beneficial to study the effect of CRD-Ig chimeras on hPSC proliferation and differentiation by using them to interfere with Wnt-dependent aspects of proliferation and pluripotency. Additionally, these fusion proteinshave potential in other multipotent cell types. Perhapsthey can be used to block differentiation towards definitive endoderm orto modulate neural progenitor cell proliferation and pluripotency. There are a wealth of possibilities that have yet to explored. These CRD Ig fusion proteins can be employed with a staggering number of applications and could possibly lead to a plethora of breakthroughs in the stem cell biology.

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