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Wnt Signalling During F9 Cell Differentiation

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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WNT SIGNALLING DURING F9 CELL DIFFERENTIATION

(Thesis format: Monograph)

by

Gregory Golenia

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Mouse F9 cells differentiate into primitive endoderm (PrE) when treated with retinoic acid (RA) and this is accompanied by the upregulation of Wnt6 and activation of the canonical WNT/β-catenin pathway. Previous studies have demonstrated the necessity of β-catenin-TCF/LEF transcription for primitive endoderm differentiation, however the Frizzled (FZD) receptor responsible for binding WNT6 and activating the canonical WNT pathway is not known. It was hypothesized that FZD7 is responsible for binding and transducing the WNT6 signal. Fzd7 mRNA is detected in undifferentiated and primitive endoderm cells, and its expression does not change significantly in response to RA. Moreover and contrary to my hypothesis, the knockdown of endogenous Fzd7 with siRNA does not attenuate differentiation. Other Fzd receptors are expressed in F9 cells and notably Fzd1, 4 and 8 are significantly upregulated by RA, and it is possible that one or more of these are serving to induce the WNT6 signal. That said, this study supports the notion that canonical WNT signaling activated through FZD receptors is necessary for PrE differentiation as the inhibitor Dickkopf-1 (Dkk1) is upregulated in response to RA, and expressing human DKK1 in undifferentiated F9 cells or treating cells with DKK1 conditioned medium impedes the ability of RA to induce PrE. Together, this data indicates that FZD receptors are involved in WNT6 signal transduction in F9 cells and attenuating canonical WNT signaling using DKK1 blocks the ability of RA to induce PrE.

Keywords

F9, Extraembryonic endoderm, Primitive endoderm, Parietal endoderm, Frizzled, Wnt, Dickkopf, Embryonal carcinoma cells, Differentiation, Retinoic acid
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# Table of Contents

<table>
<thead>
<tr>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page ........................................................................................................</td>
</tr>
<tr>
<td>Abstract ............................................................................................................</td>
</tr>
<tr>
<td>Acknowledgments ...............................................................................................</td>
</tr>
<tr>
<td>Table of Contents ..............................................................................................</td>
</tr>
<tr>
<td>List of Figures ..................................................................................................</td>
</tr>
<tr>
<td>List of Appendices .............................................................................................</td>
</tr>
<tr>
<td>List of Abbreviations .........................................................................................</td>
</tr>
<tr>
<td>Chapter 1 ............................................................................................................</td>
</tr>
<tr>
<td>1 Introduction ....................................................................................................</td>
</tr>
<tr>
<td>1.1 Early mouse development and epithelial-to-mesenchymal transitions ..........</td>
</tr>
<tr>
<td>1.2 F9 teratocarcinoma cells .............................................................................</td>
</tr>
<tr>
<td>1.3 Canonical Wnt/β-catenin signalling pathway ............................................</td>
</tr>
<tr>
<td>1.4 WNT-Frizzled signalling during development and in F9 cell differentiation ...</td>
</tr>
<tr>
<td>1.5 Frizzled receptor structure and WNT-Frizzled selectivity..........................</td>
</tr>
<tr>
<td>1.6 Secreted antagonists of WNT signalling ....................................................</td>
</tr>
<tr>
<td>1.7 Rationale, objective and hypothesis ..........................................................</td>
</tr>
<tr>
<td>2 Materials and Methods ...................................................................................</td>
</tr>
<tr>
<td>2.1 Cell culture, reverse transfection, and chemical treatments .....................</td>
</tr>
<tr>
<td>2.2 Reverse-transcription PCR and quantitative reverse-transcription PCR ........</td>
</tr>
<tr>
<td>2.3 Immunoblot Analysis .....................................................................................</td>
</tr>
<tr>
<td>2.4 TCF/LEF reporter assay ...............................................................................</td>
</tr>
<tr>
<td>2.5 MYC-DKK1 conditioned medium ...................................................................</td>
</tr>
</tbody>
</table>
2.6 Statistical analysis

Chapter 2

3 Results

3.1 Frizzled7 expression

3.2 Frizzled family members Fzd3 and Fzd9 are not detected in F9 cells

3.3 qRT-PCR analysis of Fzd transcripts in F9 cells

3.4 Knockdown of Fzd7 does not inhibit RA-induced primitive endoderm differentiation

3.5 Overexpression of Fzd7 activates canonical Wnt/β-catenin signalling in F9 cells but is not sufficient to induce PrE

3.6 Dkk1 mRNA expression is induced by RA treatment and Wnt6 overexpression

3.7 Ectopic expression of canonical WNT antagonist Dickkopf-1 attenuates primitive endoderm differentiation

3.8 MYC-DKK1 conditioned medium attenuates PrE formation

Chapter 3

4 Discussion

4.1 Transcriptional response of Frizzled genes during PrE differentiation

4.2 Knockdown of Fzd7 does not attenuate PrE differentiation

4.3 Overexpression of Fzd7 is not sufficient to induce PrE

4.4 Role of Dkk1 in ExE differentiation

4.5 Conclusion

Literature Cited

Appendices

Curriculum Vitae
List of Figures

Figure 1.1 *Mouse early embryonic development* ................................................................. 2

Figure 1.2 *The canonical Wnt/β-Catenin signalling pathway* ........................................... 7

Figure 3.1 *Fzd7 is expressed in undifferentiated and differentiated F9 cells and its expression is unaffected by RA exposure* ................................................................. 21

Figure 3.2 *Eight of the ten known Fzd genes are expressed in F9 cells* ......................... 24

Figure 3.3 *Fzd receptor expression in response to RA treatment* ..................................... 27

Figure 3.4 *F9 cells transfected with Fzd7 siRNA are able to form primitive endoderm* . 31

Figure 3.5 *Overexpression of Fzd7 activates β-catenin/TCF/LEF-dependent transcription in F9 cells, but does not alter levels of the PrE marker Cytokeratin A* ....................... 35

Figure 3.6 *Dkk1 is induced by overexpression of Wnt6* ......................................................... 40

Figure 3.7 *Cells transfected with MYC-DKK1 show decreased expression of PrE markers* ............................................................................................................................... 44

Figure 3.8 *Ectopically expressed MYC-DKK1 attenuates RA-induced PrE differentiation of F9 cells* ......................................................................................................................... 48
List of Appendices

Appendix 1- Primer sequences and annealing temperatures ............................................. 66

Appendix 2- Fold change Fzd mRNA expression in response to RA (relative to DMSO-treated cells) .................................................................................................................. 67

Appendix 3- Fold change TCF/LEF luciferase activity relative to DMSO ...................... 68

Appendix 4- Fold change Dkk1 mRNA expression in response to RA or Wnt6 (relative to DMSO-treated or empty vector transfected cells, respectively) .................. 68

Appendix 5- Fold change Dab2 mRNA expression in response to transfection with empty vector or MYC- DKK1 and treatment with DMSO or RA (relative to pcDNA3.1 +DMSO) .................................................................................................................. 69
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-TM</td>
<td>seven-transmembrane domain receptor</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>β</td>
<td>beta</td>
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<td>BARL</td>
<td>β-Catenin activated reporter luciferase</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>Dab2</td>
<td>Disabled homolog 2</td>
</tr>
<tr>
<td>db</td>
<td>dibutyryl</td>
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<td>Ca^{2+}</td>
<td>calcium</td>
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<td>cAMP</td>
<td>cyclic adenosine-monophosphate</td>
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<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
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<td>CK1</td>
<td>casein kinase 1</td>
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<td>CO₂</td>
<td>carbon dioxide</td>
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CRC  colorectal cancer
CRD  cysteine-rich domain
Ct  cycle threshold
Cthrc1  collagen triple helix repeat-containing protein 1
Daam1  Dishevelled-associated activator of morphogenesis 1
DMEM  Dulbecco’s Modified Eagle Medium
DMSO  dimethylsulfoxide
DNA  deoxyribonucleic acid
Dvl  Dishevelled
EC  embryonal carcinoma
EMT  epithelial-to-mesenchymal transition
ExE  extraembryonic endoderm
F9  mouse F9 embryonal carcinoma (teratocarcinoma)
FBS  fetal bovine serum
Fig  figure
Fzd  Frizzled
G418  Neomycin, disulfate salt
GPCR  G-protein-coupled receptor
GSK3  glycogen synthase kinase-3
h  hours
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<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<td>human embryonic kidney</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HSD</td>
<td>honestly significant difference</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>kb</td>
<td>kilobase</td>
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<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>KTXXXW</td>
<td>lysine-threonine-any amino acid (3)-tryptophan</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LEF</td>
<td>lymphoid enhancer factor</td>
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<td>LRP</td>
<td>lipoprotein receptor-related protein</td>
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<td>mitogen-activated protein kinase</td>
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<td>MW</td>
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<td>MYC-DKK1</td>
<td>myc-tagged human Dickkopf homolog 1</td>
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<tr>
<td>nM</td>
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<tr>
<td>NP-40</td>
<td>nonyl phenoxypolyethoxyethanol</td>
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</tr>
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<td>NT2/D1</td>
<td>NTERA2/D1 cell line</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
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<td>PCP</td>
<td>planar cell polarity</td>
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<td>PDZ</td>
<td>post synaptic density-95/discs large/zonula occludens 1</td>
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<td>PE</td>
<td>parietal endoderm</td>
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<td>PrE</td>
<td>primitive endoderm</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>p-value</td>
<td>probability value</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<td>qRT-PCR</td>
<td>quantitative reverse-transcription polymerase chain reaction</td>
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<td>RA</td>
<td><em>all-trans</em> retinoic acid</td>
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<td>RAR</td>
<td>retinoic acid receptor</td>
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<td>retinoic acid response element</td>
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<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<td>RL</td>
<td><em>Renilla</em> luciferase</td>
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<tr>
<td>RNAi</td>
<td>ribonucleic acid interference</td>
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<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
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<td>ROR</td>
<td>receptor tyrosine kinase-like orphan receptor</td>
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<td>RT-PCR</td>
<td>reverse-transcription polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>s</td>
<td>seconds</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SFRP</td>
<td>secreted frizzled-related protein</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline with 0.1% Tween 20</td>
</tr>
<tr>
<td>TC</td>
<td>tissue culture</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell specific transcription factor</td>
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<tr>
<td>TE</td>
<td>trophectoderm</td>
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<tr>
<td>TNBC</td>
<td>triple-negative breast cancer</td>
</tr>
<tr>
<td>TROMA-1</td>
<td>trophectodermal monoclonal antibody-1</td>
</tr>
<tr>
<td>VE</td>
<td>visceral endoderm</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless/MMTV integrated site</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Early mouse development and epithelial-to-mesenchymal transitions

A fertilized mouse egg undergoes several cleavage events that partition the cellular components asymmetrically such that by the end of the 8-cell morula stage, the resulting cells are restricted to different developmental fates [1]. Cells of the morula give rise to two distinct lineages that contribute to the early blastocyst: the trophoblast and the inner cell mass (ICM) [2]. Signals received from the underlying ICM by the trophoblast cells trigger the proliferation and differentiation of the trophoblast into extraembryonic ectoderm, the primary role of which is to contribute to embryonic implantation and placental development. The ICM differentiates into the epiblast and extraembryonic endoderm, the former giving rise to the embryo proper and the latter to the primitive endoderm (PrE) [1, 2] (Fig. 1.2).

The PrE in turn differentiates into the parietal endoderm (PE) and visceral endoderm (VE), which give rise to the parietal and visceral yolk sacs, respectively [3]. These structures are not part of the embryo proper, but physically connect the embryo to the uterine wall to provide protection and nourishment during development [1]. The process that leads to the development of the PrE and subsequently PE from the ICM is considered to be the first of many epithelial-to-mesenchymal transition (EMT) events in the developing mouse embryo [4]. The changes in cell morphology and gene expression that characterize an EMT include a loss of E-cadherin expression, an increase in cell motility, and a change in cell polarity. EMTs are of significant importance in the developing embryo as without them the formation of important structures such as the neural crest would not occur [4]. EMTs also underlie the basis of metastatic cancer, thus studying them in these models provides insight into the mechanisms that control normal embryogenesis and development.
Figure 1.1 Mouse early embryonic development. The extraembryonic endoderm (ExE) consists of the primitive, parietal, and visceral endoderms. The primitive endoderm (PrE) segregates from the inner cell mass (ICM) at the blastocyst stage. PrE further differentiates into parietal and visceral endoderm, which give rise to the parietal and visceral yolk sacs, respectively. PrE cells that remain attached to the basement membrane of the ICM become visceral endoderm (VE) while other cells undergo an epithelial-to-mesenchymal-transition to become parietal endoderm (PE). The PE and VE lineages are highly important for proper development of the epiblast as together they are responsible for foetal-maternal nutrient/waste exchange prior to placentation. Adapted from Anne Grapin-Botton (2008).
Mouse

![Diagram of mouse developmental stages](image)

**E4.5 mouse**
- Primitive endoderm (PrE)
- Inner cell mass (ICM)
- Trophectoderm (TE)

**E6 mouse**
- Epiblast
  - Future definitive endoderm
  - Future prechordal plate
  - Future mesoderm
  - Future ectoderm

**Extraembryonic**
- Anterior visceral endoderm (m, EmVE)
- Posterior visceral endoderm (m, EmVE)
- Extraembryonic visceral endoderm (m, ExVE)
- Parietal endoderm (PE, m)
- Extraembryonic mesoderm (c?, m)
- Extraembryonic ectoderm (ExE)
1.2 F9 teratocarcinoma cells

The EMT that results in PE formation occurs at the time of embryonic implantation, approximately 4.5 days after fertilization in the mouse, and is therefore technically difficult to study in vivo. The use of certain in vitro models has allowed researchers to circumvent the issue of inaccessibility to the early mouse embryo and therefore allowed the study of the signalling events involved in the EMT associated with extraembryonic endoderm (ExE) formation [5]. One such in vitro model is the F9 cell line which was derived from an experimentally induced teratocarcinoma, generated by ectopic implantation of a six-day old embryo into the testes of a 129/J mouse. Under normal culture conditions, F9 cells undergo spontaneous differentiation at a very low frequency. However when treated with physiological concentrations (10^{-7} M) of the vitamin A derivative retinoic acid (RA), F9 cells undergo phenotypic changes that resemble formation of PrE [6]. The effect of RA is mediated by binding to RA receptors (RARs) and retinoid X receptors (RXRs), which form homo- or heterodimers that bind the RA response element (RARE) to regulate the transcription of target genes [7]. Many of these genes encode cytoskeletal proteins, which play an integral role during the morphogenesis that accompanies EMTs.

In response to RA, some F9 cells will remain in groups and adopt a polygonal morphology, while others move apart from each other forming large cytoplasmic processes and adopting a stellate appearance [8]. As F9 cells undergo this transformation, intermediate filament proteins assemble and can be identified with TROMA-1, a TROphectodermal Monoclonal Antibody. TROMA-1 binds endoA-positive cytokeratin intermediate filaments (cytokeratin-8) that are synthesized in TE cells, but not in those of the ICM cells [9]. The induction of F9 cells from the undifferentiated state into PrE is the first step of the EMT; however, it is not complete until the cells have differentiated further into PE. RA on its own is sufficient to induce cells to form PrE, but further signalling is necessary to induce cells to differentiate into PE.

The addition of RA to F9 cells causes wide scale changes in the expression profile of many hundreds of genes [10]. There is also an increase in the activation of the mitogen
activated protein kinase (MAPK) pathway, and its subsequent downregulation is necessary for the F9 cells to become PE and complete the EMT [11]. This downregulation is accomplished by the addition of dibutyl cyclic adenosine monophosphate (db-cAMP). The elevation of intracellular cAMP activates Protein Kinase A (PKA), which negatively regulates the activity of the MAPK pathway, allowing the cells to complete the PrE to PE transition [11]. Another pathway that is activated in response to RA and central to my studies involves the canonical Wnt/β-catenin pathway [12, 13].

1.3 Canonical Wnt/β-catenin signalling pathway

WNTs are a family of secreted glycoproteins that elicit a multitude of cellular responses in embryos, neonates and adults. The effects of a WNT ligand binding to one of its seven transmembrane (7-TM) spanning Frizzled (FZD) receptors are pleiotropic and include stimulation of cell proliferation, cell motility and control of cell fate/differentiation [14]. Although in the fully developed individual, WNTs are important for normal tissue maintenance, abnormal WNT signalling promotes osteoarthritis as well as neurodegenerative diseases and cancer [15]. There are 19 known mammalian WNT proteins that have the potential to signal through one or more of three different WNT pathways: the canonical/β-catenin, and the non-canonical WNT/Ca\(^{2+}\) and planar cell polarity (PCP) pathways [14, 15]. WNT ligands activate the canonical pathway and alter cell fate/proliferation, whereas activation of either of the two non-canonical pathways tends to affect cell polarity, adhesion and motility through cytoskeletal reorganizations [16]. In the case of F9 cells, RA treatment leads to an up-regulation of Wnt6, which then activates the canonical Wnt/β-catenin pathway [12].

The canonical WNT/β-catenin pathway is the most well understood of the WNT pathways. In the absence of a WNT ligand, the intracellular levels of β-catenin are maintained at a low level through association with a protein complex known as the “β-catenin destruction complex” that consists of the proteins AXIN, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK-3), and casein kinase 1 (CK1) [15]. The interaction of β-catenin with the destruction complex results in its initial and subsequent
phosphorylation by CK1 and GSK-3, respectively, and its eventual proteasome-mediated degradation. When WNT is present, however, it can bind to its serpentine FZD receptor at the cell surface along with the low density lipoprotein (LDL) receptor-related protein 5/6 (LRP5/6) [15]. Binding leads to activation of the FZD receptor and recruitment of the scaffold proteins Dishevelled (DVL) and AXIN to the plasma membrane. The disruption of this destruction complex prevents the phosphorylation of β-catenin, allowing it to accumulate in the cytosol and translocate to the nucleus, where it interacts with the T-cell-factor-lymphoid enhancer factor (TCF-LEF) family of transcription factors to activate transcription of WNT target genes [15]. Thus, the interaction of β-catenin with TCFs-LEFs converts them from transcriptional repressors to activators, which promotes the expression of genes that allow the induction of the PrE phenotype (Fig. 1.2).

1.4 WNT-Frizzled signalling during development and in F9 cell differentiation

As noted earlier, RA treatment of F9 cells leads to an up-regulation of Wnt6, which when translated activates the canonical WNT/β-catenin pathway. Research from our laboratory has shown that treatment of undifferentiated F9 cells with WNT6 conditioned medium, or exogenous expression of Wnt6, is sufficient for PrE induction [12]. As canonical WNT signalling is mediated through FZD and its co-receptor LRP5/6, then one or more FZD receptor types, present on the surface of undifferentiated F9 cells, likely bind to WNT6 and transduce the signal. Incidentally, F9 cells require the activation of more than one WNT signalling pathway to complete differentiation into PE: canonical WNT/β-catenin signalling for differentiation of F9 cells into PrE, and the non-canonical/PCP pathway to mediate proper migration and orientation of PE cells required for PE differentiation and the completion of EMT [13] [17]. Therefore, a receptor that has the potential to activate both of these WNT signalling pathways would serve as an attractive candidate for inducing differentiation of F9 cells into ExE. Among the ten members of the FZD receptor family, FZD7 has been shown to mediate both canonical
Figure 1.2 The canonical Wnt/β-Catenin signalling pathway.  

(A) In the absence of a WNT ligand, cytoplasmic β-catenin is phosphorylated by a complex of proteins known as the “destruction complex” composed of axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 phosphorylate the amino terminal of β-catenin, resulting in the ubiquitination of β-catenin and its subsequent degradation. Canonical WNT target genes are thereby repressed by transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family.  

(B) When WNT is present and bound to FZD and its low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor, the Disheveled (DVL) protein is recruited to the cell membrane resulting in LRP5/6 phosphorylation and disassembly of the destruction complex. β-catenin accumulates in the cytosol and subsequently translocates to the nucleus, where it complexes with TCF/LEF and causes target gene activation. Adapted from MacDonald, Tamai and He (2009). Reprinted with permission from Elsevier.
and non-canonical WNT signals in several different cell types [18]. One potential candidate involved in the differentiation of ExE is encoded by Fzd7. It is the most predominantly expressed Fzd receptor in triple negative breast cancer cells and its overexpression is associated with increased metastatic potential and cell proliferation through activation of the canonical WNT pathway [10]. In colorectal cancer, FZD7 is reported to be involved in both motility and invasiveness through activation of the non-canonical/PCP pathway and increased cell survival through canonical WNT/β-catenin signalling [19]. Particularly of interest is the role of FZD7 in Xenopus (Xfz7) endoderm progenitor cells, where WNT binding leads to activation of either the canonical/β-catenin or non-canonical/PCP pathway and the pathway activated is dependent on the dose of WNT that the cells received, which was modulated by the soluble inhibitor of Wnt signaling Secreted Frizzled-Related Protein 5 (SFRP5). This latter example thus demonstrates that not only can a single FZD receptor activate more than one WNT pathway in the same cell type, but also that the WNT dosage can specify activation of one pathway over another [18]. In addition to regulating proper foregut development, Xfz7 is also required for the formation of the neural crest, which is dependent on an EMT, and accompanied by the activation of the canonical/β-catenin pathway [20]. As EMTs are fundamental processes that control morphogenesis in multicellular organisms, many of the mechanisms and signal transduction pathways involved in their regulation are well-conserved [21]. FZD7 has been shown to activate WNT signalling in several developmental EMTs and also in cancer cell models where EMTs mediate progression of the malignant phenotype, thus pointing to its role in controlling these processes [19]. Thus, FZD7 is capable of interacting with multiple WNT ligands and activating multiple pathways, but whether or not it serves as a receptor for WNT6 during F9 cell differentiation remains to be determined. A link between WNT6 and FZD7 has been proposed in the developing chick, where WNT6 signalling through the canonical β-catenin pathway is responsible for maintaining the epithelial structure of somites [22]. It is interesting to note from this study that WNT6 might maintain the expression of FZD7 as blocking the WNT6 signal emanating from ectoderm overlaying the somites results in a loss of FZD7 expression in the somites. Although this may not be a general phenomenon and may only be specific to the developing chick, it does provide evidence
linking WNT6 and *FZD7* in a developmental model. Preliminary data from our lab would also indicate that FZD7 is sufficient to cause differentiation, as evident from the results from the transient over-expression of Fzd7 in undifferentiated F9 cells, showing that PrE is induced and is accompanied by phosphorylation and hence inactivation of GSK-3β, a negative regulator of canonical Wnt/β-catenin pathway activation (Benjamin Cadesky, unpublished data).

Sequence homology between Fzd7 and the other Fzd receptors may account for functional redundancy in various developmental processes regulated by WNT signalling. Homology analysis of Fzd receptors indicates that they share approximately 20-40% sequence identity, which is higher within certain clusters [23]. Specifically, Fzd7 is grouped within the same cluster as Fzd1 and Fzd2 and shares 75% sequence identity with these receptors [23]. Liu et al. demonstrated that ectopic expression of rat Fzd1 or Fzd2 β-adrenergic chimeras in mouse F9 cells is sufficient to induce PrE. These results, however, may not be physiologically relevant as both Fzd1 and Fzd2 constructs used in these studies generated chimeric receptors, the result of fusing the sequence encoding the extracellular and transmembrane domains of the β-adrenergic receptor with the intracellular components of rFzd1/2 [24, 25]. Finally, there is a link noted between the up-regulation of WNT6 with FZD1 and FZD2 in human breast cancer cells, but whether or not this occurs in the context of F9 cell differentiation remains to be determined [26].

### 1.5 Frizzled receptor structure and WNT-Frizzled selectivity

Frizzled proteins were first identified as receptors for WNT ligands in *D. melanogaster* [27]. Frizzled receptors display many similarities to classical G-protein coupled receptors (GPCRs), yet are distinct enough to be grouped into their own class within this superfamily [28]. It was initially postulated that FZD receptors, despite demonstrating the 7-TM spanning hydrophobic domains characteristic of GPCRs, were not coupled to G-proteins and used other means to transduce signals downstream. G-proteins were introduced into the mechanistic explanation of FZD signalling when it was
shown that the WNT-Ca\(^{2+}\) signalling pathway in zebrafish embryos could be inhibited by the addition of pertussis toxin [29]. Historically, the WNT/β-catenin and WNT-PCP pathways were considered to be independent of heterotrimeric G-proteins. However, a growing body of evidence indicates that G-proteins, interacting with DVL, a key player in canonical and non-canonical WNT signalling, play a more central role in the transduction of WNT signals [29].

Structurally, FZD receptors consist of seven hydrophobic alpha helical segments, which comprise the transmembrane domains, three extracellular and three intracellular loops, an extracellular N-terminus, and an intracellular C-terminal tail [30]. Within the C-terminus of FZD receptors is a completely conserved KTXXXW (X= any amino acid) motif, which is responsible for the interaction of FZD with the PDZ domain of DVL [31]. This interaction recruits AXIN to the cell membrane, which effectively inactivates the β-catenin destruction complex. The N-terminus of FZD contains a signal sequence peptide that ensures proper trafficking and membrane insertion of the receptor into the membrane, as well as a cysteine-rich domain (CRD). The CRD contains ten highly conserved cysteine residues that are thought to be the main WNT ligand binding domain [30]. Dimerization of the FZD receptors through disulfide bond formation within the CRDs occurs, and in some instances this dimerization alone is sufficient to activate WNT/β-catenin signalling [32].

The mechanisms by which signalling specificity is imparted between the nineteen WNT ligands and ten FZD receptors are poorly understood. The combinatorial and temporal expression of the different FZD receptors, co-receptors, WNT ligands and extracellular antagonists such as secreted frizzled-related proteins (SFRPs), are likely to be responsible for activation of different signalling pathways by the same FZD receptor. A “WNT switch” mechanism has been proposed to explain the activation of multiple WNT pathways by the same FZD receptor, with lower concentrations of WNT ligand activating the WNT-Ca\(^{2+}\) pathway and higher concentrations responsible for β-catenin signalling [33]. However it is not known if such a model is responsible for PrE formation in F9 cells.
1.6 Secreted antagonists of WNT signalling

There are several secreted proteins that modulate WNT signaling during development by preventing the interaction of WNT ligands with their FZD receptors and/or co-receptors [34]. These extracellular antagonists of WNT signalling are divided into two functional classes: the secreted Frizzled-related protein (sFRP) class and the Dickkopf (DKK) class. Members of the sFRP class include the sFRP family, WNT inhibitory factor 1 (WIF-1), and Cerberus. This class of inhibitors antagonizes WNT signalling by directly binding to WNT and preventing WNT from interacting with its receptors [34]. The DKK family inhibits canonical WNT/β-catenin signalling by binding to the LRP5/6 co-receptor and prevents formation of a WNT-FZD-LRP5/6 ternary complex. Thus, in theory, the sFRP class will inhibit both canonical and non-canonical WNT signalling, whereas the DKK class specifically inhibits canonical WNT/β-catenin signalling [34]. The Dkk family encodes secreted proteins and consists of four members, Dkk 1-4 [35]. DKK1 was the founding member of the family and it was discovered to block WNT signalling which is required for head induction during Xenopus development [36]. Since its initial discovery, DKK1 has been shown to inhibit canonical WNT/β-catenin signalling in many cell types and vertebrate species [35]. A negative feedback loop involving DKK1 was discovered in WNT signalling when four TCF/LEF binding elements were identified within the mDkk1 promoter [35]. Indeed, the expression of Dkk1 is upregulated in many diseases/cancers in which WNT signalling is constitutively active [37, 38]. As aberrant activation of the WNT pathway is the hallmark of many cancers, initiation of the expression of negative feedback regulators that control the duration and intensity of the WNT signal is an important mechanism to maintain WNT signalling homeostasis in cellular processes that utilize this pathway [39].

There is evidence that F9 cells express inhibitors of WNT signalling that may be used “turn off” the WNT pathway once cells have differentiated. In a microarray profiling study using F9 cells, Gudas et al. identified two RA-target genes Dab2 and sFRP-5, both of which encode inhibitors of WNT signalling [40]. Additionally, in F9 cells expressing a chimeric rFzd1-β-adrenergic receptor, stimulation of the chimeric receptor with β-agonist to activate FZD signalling induces PrE differentiation and this is
accompanied by an upregulation of sFRP-1 expression [41]. As well, F9 cells induced to differentiate into VE are not responsive to WNT ligands, suggesting a blockade in WNT signalling accompanies differentiation [42]. Taken together, these results may indicate the presence of a negative feedback loop involving both secreted and intracellular antagonists of the WNT pathway that regulate WNT signalling during F9 cell differentiation.

1.7 Rationale, objective and hypothesis

The primary goal of my research is to address the role of the FZD7 receptor in the process of primitive endoderm formation. Previous work from our laboratory has shown that transient overexpression of Wnt6 or Fzd7, as well as treatment of undifferentiated F9 cells with WNT6 conditioned media, is sufficient to induce PrE. Thus, increasing WNT6 or FZD7 levels on their own has the potential to activate a signalling cascade that allows naïve cells to adopt a new fate. Further research, however, is needed to determine if WNT6 can only induce differentiation in the presence of the FZD7 receptor. Based on the information above, I hypothesize that FZD7 is the receptor that binds to WNT6 and transduces its signal during RA-induced differentiation of F9 cells. If this is true, then Fzd7 mRNA and protein should be present in undifferentiated cells, awaiting the WNT6 ligand that is seen following RA treatment. Another possibility is that other Fzd transcripts are present in undifferentiated F9 cells, and these encode receptors that act in a functionally redundant manner to FZD7. To test my hypothesis I first used qRT-PCR to catalogue the expression of the different Fzd receptors in undifferentiated and RA-induced F9 cells, and then conducted knockdown experiments of Fzd7 mRNA and treated cells with RA to determine the effect on differentiation. If my hypothesis is correct then there will be no PrE differentiation in the absence of Fzd7. If on the other hand PrE markers appear in cells depleted of Fzd7, the qRT-PCR data will provide evidence as to the FZD receptor(s) that may be responsible for transducing the WNT6 required for differentiation. The secondary goal of my research is to address the role of the secreted canonical WNT signalling antagonist DKK1, and whether it is involved in a
negative feedback loop regulating WNT signalling during F9 cell differentiation. To address this, the expression of Dkk1 was examined in F9 cells treated with RA or expressing Wnt6 using qRT-PCR. Additionally, F9 cells overexpressing human DKK1 or treated with DKK1 conditioned medium were induced with RA to examine the effect of ectopic DKK1 on RA-induced differentiation. As other pathways have been shown to crosstalk with the canonical WNT/β-catenin pathway during RA-induced differentiation [43], these experiments will determine the effect of inhibiting the canonical WNT pathway at the level of the receptor and co-receptor.
2 Materials and Methods

2.1 Cell culture, reverse transfection, and chemical treatments

Mouse F9 teratocarcinoma cells (ATCC) were cultured in Dulbecco’s Modified Eagle medium (DMEM) (Gibco) supplemented with 1% penicillin-streptomycin (P/S) (Gibco) and 10% fetal bovine serum (FBS) (Gibco) and incubated at 37 °C and 5% CO₂. Cells were cultured in 35 mm plates for transfection or 60 mm plates (Nunc Thermoscientific or Sarstedt) for protein and/or RNA isolation. Cells were transfected with empty vector, pcDNA3.1-Fzd7, pcDNA3.1-Wnt6, pcDNA3.1-MYC-DKK1, pBARL and pRL-TK constructs using Lipofectamine 2000 (Life Technologies) according to manufacturer’s recommendations. Briefly, 10 uL of Lipofectamine 2000 were combined with a total of 4 µg of plasmid DNA in Opti-MEM reduced serum medium (Life Technologies) and incubated for 20 min at RT. Fzd7 was subcloned into pcDNA3.1 (Invitrogen) by Benajmin Cadesky. pRL-TK was provided by Dr. Rodney Dekoter (University of Western Ontario). pBARL was provided by Dr. S. Angers (University of Toronto). pcDNA3.1-MYC-DKK1 was provided by Dr. Yonghe Li (Southern Research Institute, Birmingham, Alabama, USA).

For Fzd7 knockdown, three 27-mer antisense RNA sequences (Origene) were pooled together at 10 nM each. Approximately 2.5 x 10⁵ cells were added to 2 mL DMEM with serum and antibiotics and seeded directly into 35 mm dishes containing the Opti-MEM transfection mix. Medium was replaced after 6 h and approximately 1 x 10⁵ cells were passaged into 60 mm dishes after 24 h and selected for antibiotic resistance with 1.75 mg/mL G418 sulfate (Sigma). Cells transfected with pcDNA3.1-MYC-DKK1 or treated with MYC-DKK1 conditioned medium were treated with DMSO or 5 x 10⁻⁸ M RA for 4 days. For all other experiments, cells were treated with DMSO or 10⁻⁷ M RA for 3 or 4 days.
2.2 Reverse-transcription PCR and quantitative reverse-transcription PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and used as a template for first-strand cDNA synthesis, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with 1 µg RNA. RNA was isolated following treatment of F9 cells with DMSO, RA (1, 2, 3, and 4 days post-treatment) or RA and dibutyryl-cyclic Adenosine Mono Phosphate (db-cAMP). For RT-PCR and qRT-PCR with *Fzd* primers, an on-column DNase digestion was performed on all RNA samples using the RNase-Free DNase kit (Qiagen) according to manufacturer’s protocol. Purity and concentration of RNA was analyzed using the NanoDrop2000C Spectrophotometer (Thermo Scientific). PCR reactions were analyzed on 1% agarose gels containing RedSafe Nucleic Acid Stain (iNtRON Biotechnology) and amplicons were visualized using a FluorChem 8900 Gel Doc System (Alpha Innotech). All reactions were performed using the following thermo-cycling conditions for 35-40 cycles: 94 °C for 30 s, the primer-specific annealing temperature for 30 s and 72 °C extension for 30 s. All amplicons were sequenced (Robarts Research Institute, Western University) and aligned to annotated sequences using the NCBI Basic Local Alignment Search Tool (BLAST) to confirm their identity. Primers sequences and annealing temperatures are listed in Appendix 1.

For qRT-PCR analysis, total RNA was isolated and reverse-transcribed into first-strand cDNA as described above. For *Fzd* expression analysis, qRT-PCR was performed using the Rotor-Gene 3000 (Corbett Research) and Power SYBR Green Master Mix (Applied Biosystems) according to manufacturer’s protocol. For all other qRT-PCR reactions, the CFX Connect Real-Time PCR Detection System (Bio-Rad) was used with the SensiFAST SYBR No-ROX Kit (Bioline) according to manufacturer’s protocol. Each qRT-PCR reaction contained 10 µL of Power SYBR Green Master Mix or SensiFAST SYBR, 500 nM of each primer described above, and 1 uL of cDNA template. Amplification plots were analyzed using Rotor-Gene software or CFX Manager Software. Gene expression was quantified using the comparative cycle threshold (Ct) method and displayed as gene expression relative to the *L14* (*2^ΔCt*) or as fold-change (*2^-ΔCt*).
ΔΔCt) relative to DMSO or empty vector control (Appendix 2, 4 and 5). *LI4* encodes the constitutively expressed 60S ribosomal protein L14

### 2.3 Immunoblot Analysis

Protein lysates were prepared in radioimmunoprecipitation assay buffer (RIPA) containing 150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl and 1x Halt Protease inhibitor cocktail (Thermo Scientific). Protein concentrations were quantified using the DC (Detergent Compatible) protein assay (Bio-Rad) and Modulus II Microplate Multimode Reader (Turner Biosystems). Approximately 20 µg of protein were separated on 10% denaturing polyacrylamide gels for 2.5 h at 100 V. Following electrophoresis, the proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation) overnight at 4 °C and 20 V in Tris-glycine transfer buffer containing 20% methanol. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk for 1 h at room temperature. Membranes were probed overnight at 4 °C with the following primary antibodies diluted in 5% bovine serum albumin (BSA) containing TBS-T: TROMA-1 (1:5; 55kDa, Developmental Studies Hybridoma Bank), β-Actin (1:5000; 47kDa, Santa Cruz) and c-Myc (9E10) (1:50, approximately 39 kDa for MYC-DKK1 detection, Santa Cruz). Membranes were washed in TBS-T (three washes for five minutes each) and then probed with either HRP-conjugated goat anti-rat or anti-mouse secondary antibody(s) in 5% skim milk in TBS-T for 2 h at room temperature. Following three additional five minute washes with TBS-T, signals were detected using the Supersignal West Pico Chemiluminescent Detection Kit (Pierce), then captured with Molecular Imager Gel Doc XR System (Bio-Rad) and imaged with Quantity One Software. Densitometry analysis was performed using ImageJ software.

### 2.4 TCF/LEF reporter assay

Cells transfected with *pBARL* (β-Catenin-activated reporter luciferase) and then treated with DMSO or 10⁻⁷ M RA, or co-transfected with *pBARL* and *pcDNA3.1-EV*
(empty vector) or pcDNA3.1-Fzd7 in equal amounts, were collected 48 h post-treatment or post-transfection. Cells were also co-transfected with pRL-TK (Renilla luciferase) to normalize luciferase levels. Lysates were collected by gently shaking cells in 1 X Passive Lysis Buffer (PLB, Promega) for 15 min at room temperature. Luciferase activity was quantified using the Dual Luciferase Assay Reporter System (Promega) and the Modulus II Multimode Microplate Reader (Turner Biosystems). Briefly, 100 µL of Luciferase Assay Reagent was added to 75 µL of PLB lysate in a 96-well plate and firefly luciferase activity was quantified. 100 µL of Stop & Glo Reagent was added to each well and Renilla luciferase activity was then quantified. Firefly luciferase values were normalized to Renilla luciferase values.

2.5 MYC-DKK1 conditioned medium

HEK-293 cells were grown to 90% confluence in 60 mm culture dishes and maintained in DMEM with 10% FBS and 1% P/S. Cells were transfected using 10 µL Lipofectamine 2000 with 4 µg pcDNA3.1-EV (empty vector) or pcDNA3.1-MYC-DKK1. Medium was replaced with complete DMEM after 6 hours and replaced again with serum-free medium containing 1% P/S after 24 h. The medium was collected after 48 h and centrifuged at 10,000 x g for 10 min at 4 °C to pellet cellular debris. The supernatant was then filter sterilized using 0.2 µm Acrodisc Syringe Filters (Pall Corporation) and concentrated using Amicon Ultra-4 Centrifugal Filter Units (10,000 nominal molecular weight limit-EMD Millipore) according to manufacturer’s instructions. Approximately 1.2x10^4 F9 cells were seeded in 12-well plates and co-treated with 5x10^-8 M RA and 10 µL of media concentrate from pcDNA3.1-EV or pcDNA3.1-MYC-DKK1 transfected cells. Cells were supplemented with 10 µL of concentrated medium every day for a total of 4 days. For immunoblot analysis of concentrated conditioned medium, 5, 10 or 15 µL of medium from pcDNA3.1-EV or pcDNA3.1-MYC-DKK1 transfected cells were resolved by electrophoresis on 10% denaturing polyacrylamide gels, transferred to PVDF membranes and probed using the 9E10 antibody (Santa Cruz).
2.6 Statistical analysis

Densitometric analyses of immunoblots, qRT-PCR data, and Luciferase assay data were compiled from three independent biological replicates performed on separate occasions. Comparisons of data between the control and treated, or transfected groups were performed using a one-way ANOVA and Tukey’s honest significant difference (HSD) post-hoc test or a Student’s t-Test (SPSS Statistics for Windows Version 19.0, IBM Corp. Released 2010, Armonk, NY). One-way ANOVA followed by Tukey’s HSD test was used for statistical analysis of Luciferase assay data, Dkk1 and Dab2 qRT-PCR data, and densitometry data of TROMA-1 immunoblots from Fzd7 knockdown experiments. Student’s t-Test was used for statistical analysis of all other data. P-values were one-sided and considered statistically significant at the 0.05 level. Statistical data are presented as the mean ± S.E.
Chapter 2

3 Results

3.1 Frizzled7 expression

F9 teratocarcinoma cells differentiate into primitive endoderm when exposed to retinoic acid (RA) and to parietal endoderm when exposed sequentially to RA and dibutyryl cyclic adenosine monophosphate (db-cAMP). RA-induced differentiation is accompanied by an increase in Wnt6 expression, and WNT6 by itself induces differentiation [12]. Although a receptor for WNT6 in F9 cells has not been identified, FZD7 is a candidate and while there is a link between it and WNT6 in developing chick somites [22], more importantly its overexpression in F9 cells has been reported to induce primitive endoderm (Benjamin Cadesky, unpublished). RT-PCR was used to address if Fzd7 is expressed in F9 cells. Total RNA was collected from F9 cells and those treated with either RA for one to four days, or with RA and db-cAMP, and then reverse-transcribed into first-strand cDNA for PCR. Results show the presence of an amplicon in all treatments, including the DMSO-treated controls (Fig. 3.1A). Amplicons were sequenced and all were found to be identical to the Fzd7 sequence appearing in the NCBI Nucleotide database.

The presence of a Fzd7 amplicon regardless of treatment prompted the need to use qRT-PCR to detect subtle changes in Fzd7 expression. Total RNA was collected from undifferentiated F9 cells and cells treated with RA for 3 days, and then reverse-transcribed into cDNA for qRT-PCR with Fzd7-specific primers. Results showed that there was no significant change in Fzd7 mRNA abundance due to RA treatment (Fig. 3.2B). To corroborate these results and to ensure that RA had induced differentiation, end-point PCR was used with Gata6-specific primers. That a Gata6 amplicon was only present in RA-treated cells indicated that RA had successfully induced the cells to form PrE (Fig 3.2C). To extend these studies, the expression of other members of the Fzd family was examined to determine if they were present in F9 cells and if so, whether their expression changed during RA-induced differentiation.
Figure 3.1 *Fzd7 is expressed in undifferentiated and differentiated F9 cells and its expression is unaffected by RA exposure.* (A) F9 cells were treated with either DMSO (control), RA for one to four days, or with RA and db-cAMP for four days (with db-cAMP treatment after 48 h of RA exposure) and total RNA was collected, reverse-transcribed into cDNA and used as a template for RT-PCR with primers designed to *Fzd7*. A *Fzd7* amplicon was amplified from first strand cDNA derived from all treatments. The presence of the positive control, *L14* amplicon, indicated that cDNAs were present in all treatments. (B) qRT-PCR analysis of *Fzd7* in cells treated with RA (10^{-7} M) for 3 days shows that *Fzd7* expression in RA-treated cells is not significantly different from expression in undifferentiated (DMSO-treated) F9 cells. (C) Agarose gel showing the presence of a *Gata6* amplicon in RA-treated, but not DMSO-treated control cells, indicating that the RA pathway had been successfully activated. Data are representative of three independent experiments. * denotes significance (p<0.05).
3.2 Frizzled family members *Fzd3* and *Fzd9* are not detected in F9 cells

Given the results for *Fzd7*, the focus expanded to investigating other members of the *Fzd* family in F9 cells, specifically their expression levels in undifferentiated and RA-induced PrE cells. Although it has been reported that 18 of the 19 *Wnt* genes and all 10 members of the *Fzd* family are expressed in F9 cells [44], our laboratory has shown that only *Wnt6* is expressed [12]. To re-evaluate the *Fzd* data [44], F9 cells were treated with RA to induce PrE and total RNA was isolated after 3 days and reverse-transcribed into cDNA for end-point PCR using primers for *Fzd1* through *Fzd10*. Results show amplicons for eight of the ten *Fzd* family members in both undifferentiated and RA-treated F9 cells (Fig 3.2A). The expression of *Fzd3* and *Fzd9* was not detected even after 40 cycles of PCR or by altering annealing temperatures. To corroborate that these two latter genes were not expressed in F9 cells, mouse tissues known to express these *Fzd* genes were obtained and used in a PCR analysis. Total RNA was collected from mouse tissues known to express *Fzd3* and *Fzd9* (dermis and hippocampus, respectively) and reverse-transcribed into cDNA to be used for end-point PCR with *Fzd3* (Fig 3.2B) and *Fzd9* primers (Fig. 3.2C). That the primers amplify a product in the cDNA of their respective positive control samples, but not in cDNA from F9 cells, indicates that these two *Fzd* genes were not detected by end-point PCR in F9 cells and are likely not expressed by this cell line.
Figure 3.2 Eight of the ten known Fzd genes are expressed in F9 cells. Total RNA was collected from F9 cells treated with either RA or DMSO for 3 days (A) or from mouse dermis (B) and hippocampus (C) and used as template for RT-PCR. (A) Agarose gels showing amplicons corresponding to the predicted size of each Fzd amplicon. Amplicons are present in both undifferentiated (DMSO) cells and in cells treated with RA. Between 35-40 PCR cycles were used to detecte the Fzd transcripts. Amplicons were purified and sequenced to confirm their identity. (B) Fzd3 amplicons were detected in mouse dermal tissue isolated from three mouse pups of the same litter. The dermis is known to express Fzd3 during development and was used as a positive control for the Fzd3 primers tested with the F9 cells. (C) A Fzd9 amplicon was detected in mouse hippocampal tissue. The mouse hippocampus is known to express Fzd9 and was used a positive control for the Fzd9 primers tested with the F9 cells. Data are representative of three independent experiments.
3.3 qRT-PCR analysis of Fzd transcripts in F9 cells

Detecting expression of eight of the 10 Fzd genes in F9 cells prompted further analysis to determine if the genes were RA-responsive. The Fzd amplicons shown in Fig. 3.2 were detected using 40 cycles of PCR, and as such subtle differences in expression of each gene between the undifferentiated and RA-treated cells would not be detectable at such a high cycle number. qRT-PCR analysis was therefore necessary to determine if the Fzds were responsive to RA. To address this, F9 cells were treated with DMSO or RA for 3 days and total RNA was isolated and reverse-transcribed into cDNA for qRT-PCR. Results show that Fzd1, 4, and 8 were significantly upregulated by RA treatment, showing a 9.82, 4.86, and 3.69-fold increase in expression relative to control, respectively (Fig 3.3, Appendix 2). Fzd2 and Fzd6 showed a slight increase in expression after exposure to RA, but neither was statistically significant. The expression of Fzd5 and Fzd10 was lower following RA treatment, showing a 0.52 and 0.61 fold-decrease relative to control, respectively, however this decrease in expression was not statistically significant (Fig. 3.3). Thus, while it appears Fzd1, 4 and 8 genes are RA-responsive, Fzd 2, 5, 6, 7 and 10 were not. Interestingly, Fzd7 is the most abundantly expressed of the Fzd mRNAs detected in undifferentiated F9 cells.
Figure 3.3 *Fzd* receptor expression in response to RA treatment. (A) qRT-PCR analysis was used to determine the gene expression profile of *Fzd* receptors relative to the control gene *L14* in response to RA. Results show *Fzd1*, 4 and 8 are significantly upregulated by RA at 3 days (9.82, 4.86, and 3.69 fold up-regulation relative to DMSO control cells, respectively). The expression of *Fzd2*, 5, 6, 7 and 10 does not change significantly at 3 days of RA exposure. *Fzd7* is the most abundantly expressed *Fzd* gene in undifferentiated F9 cells. Asterisks denote significant difference (p≤0.05). Data represents the mean and standard error of three independent experiments. For fold-changes in *Fzd* mRNA expression relative to DMSO, see Appendix 2.
A

![Graph showing normalized Fzd mRNA expression](image)

- Y-axis: Normalized Fzd mRNA Expression ($2^{-\Delta Ct}$)
- X-axis: Fzd1, Fzd2, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, Fzd10
- Legend: DMSO, RA

* Significant difference compared to DMSO
3.4 Knockdown of Fzd7 does not inhibit RA-induced primitive endoderm differentiation

qRT-PCR analysis of the Fzd receptors detected in F9 cells showed that Fzd1, 4, and 8 are upregulated in response to RA, with Fzd1 showing the greatest increase in this group after three days of RA treatment. Although Fzd7 was not significantly upregulated by RA, its expression in undifferentiated cells relative to the endogenous control indicates that Fzd7 is the most abundantly expressed Fzd and this is in agreement with the expression profile of Fzd7 in NT2/D1 embryonal carcinoma cells [45]. Given this relatively high expression of Fzd7 in F9 cells, and the fact that WNT6 conditioned medium differentiates F9 cells into PrE, I predicted that FZD7 was the WNT6 receptor, which when activated initiates canonical WNT/β-catenin signalling in F9 cells. To test this, it was necessary to determine if cells could be induced to differentiate into PrE in the absence of Fzd7. FZD7 was also chosen as a candidate receptor involved in PrE differentiation because of its role in activation of both the canonical and non-canonical WNT pathways in the same cell type [18]. As both canonical WNT and WNT/PCP signalling have been reported as necessary for ExE formation [13, 17] I was anticipating that FZD7 was involved in activation of both pathways during the differentiation process. If my hypothesis was correct and FZD7 binds WNT6 and transduces a differentiation signal, then depleting cells of Fzd7 should reduce PrE differentiation.

To address the role of FZD7 in PrE differentiation, F9 cells were transfected with Fzd7 short interfering RNA (siRNA) to knock down endogenous Fzd7 transcripts. F9 cells were transfected with Fzd7 siRNA or non-targeting control siRNA and treated with RA for 3 days. Protein lysates were harvested for immunoblot analysis using the TROMA-1 antibody as a marker for PrE differentiation. The TROMA-1 antibody binds to Cytokeratin A, an intermediate filament protein that is used as a molecular readout for extraembryonic endoderm differentiation [9]. Immunoblot analysis with the TROMA-1 antibody indicates that cytokeratin-A positive intermediate filaments were present at approximately equal amounts in all RA-treated cells, including those transfected with Fzd7 siRNA or non-targeting control siRNA (Fig 3.4A). Densitometry analysis indicates that TROMA-1 levels were not significantly different due to Fzd7 siRNA transfection
qRT-PCR was used to confirm that *Fzd7* mRNA levels were reduced in *Fzd7* siRNA-transfected cells relative to non-targeting control siRNA transfected cells (Fig 3.5C). The results indicate that reducing the message encoding the FZD7 receptor using a siRNA approach does not have a detrimental effect on PrE differentiation as indicated by the strong TROMA-1 signal. Taken together, it would appear that these results do not support the hypothesis that FZD7 is a receptor necessary for PrE differentiation. Nevertheless, given the functional redundancy issue reported for the Fzd receptors [46], it is entirely possible Fzd7 is the receptor, but when absent one or more other receptors can serve in its place. Alternatively, knockdown of *Fzd7* may not be an effective means of reducing FZD7 protein and this is discussed in Chapter 4.2
Figure 3.4 *F9 cells transfected with Fzd7 siRNA are able to form primitive endoderm.* F9 cells were transfected with *Fzd7* siRNA or non-targeting control siRNA and treated with RA for 3 days. Protein lysates were collected from each for immunoblot analysis with the TROMA-1 antibody (A and B). Total RNA was also collected from cells transfected with *Fzd7* siRNA and non-targeting control siRNA, and RNA was reverse transcribed into cDNA for qRT-PCR analysis to confirm that *Fzd7* siRNA specifically targets the *Fzd7* transcript (C). (A) Immunoblot results show that TROMA-1 levels in *Fzd7* siRNA transfected cells were approximately equal to those in the non-targeting control siRNA transfected cells after treatment with RA, and cells treated with RA alone. Antibodies against β-actin were used to ensure equal loading in all lanes. (B) Densitometry analysis shows that TROMA-1 levels were not significantly different in RA-treated cells transfected with *Fzd7* siRNA compared to non-targeting control-transfected cells treated with RA. Densitometry data was collected by comparing the TROMA-1/β-actin ratio in each sample. (C) qRT-PCR analysis indicates that *Fzd7* mRNA expression was significantly reduced relative to levels in non-targeting control siRNA transfected cells. Data represent the mean and standard error of three independent experiments (p<0.05).
A

TROMA-1

β-Actin

DMSO  RA  Fzd7 siRNA +RA  Non-targeting siRNA +RA

B

![Bar graph showing TROMA-1/β-Actin Ratio](image)

RA  Fzd7 siRNA +RA  Non-targeting siRNA +RA

C

![Bar graph showing Normalized Fzd7 mRNA Expression](image)

Non-targeting siRNA  Fzd7 siRNA
3.5 Overexpression of Fzd7 activates canonical Wnt/β-catenin signalling in F9 cells but is not sufficient to induce PrE

The previous results indicate that knock down of Fzd7 does not attenuate RA-induced PrE differentiation. If in F9 cells FZD7 is the only receptor for WNT6, and Wnt6 is the only Wnt detected in these cells during RA-induced differentiation, then knocking down Fzd7 should have a detrimental effect on the appearance of PrE differentiation markers. However this was not the case, as immunoblot results with the TROMA-1 antibody indicate that Cytokeratin A levels were not reduced by the Fzd7 siRNA treatment. This raised the question as to whether or not Fzd7 was actually the receptor involved in differentiation. Since previous results from the Kelly lab suggested that Fzd7 overexpression was sufficient to induce PrE and this is accompanied by inactivation of GSK3, I decided to confirm these results and show that overexpression of Fzd7 induces differentiation.

If overexpression of Fzd7 is sufficient to activate canonical Wnt signalling required for PrE formation, then increased β-catenin/TCF/LEF transcriptional activity should be detected in cells overexpressing Fzd7. To test this, F9 cells transfected with Fzd7 were analyzed for β-catenin/TCF/LEF-dependent transcription using the Dual-Luciferase® reporter assay system. Cells were co-transfected with pBARL (β-Catenin-activated reporter luciferase) to detect activation of TCF/LEF-dependent transcription and pRL-TK (Renilla luciferase) plasmids, and either Fzd7 or pcDNA3.1-empty vector (EV). Cells co-transfected with pBARL and pRL-TK and treated with RA or DMSO were used as positive and negative controls, respectively. pRL-TK was used to normalized luciferase values. Relative to the DMSO-treated cells, results indicate that cells treated with RA or transfected with Fzd7 showed a significant increase in luciferase activity (4.32 and 3.06-fold higher luciferase activity, respectively). DMSO and empty-vector transfected cells both exhibit approximately equal levels of luciferase activity (Fig. 3.5A). Thus, the results would indicate that overexpressing Fzd7 is sufficient for activation of β-catenin/TCF/LEF-dependent transcription in F9 cells.
Although reducing Fzd7 mRNA with siRNA does not inhibit PrE differentiation, overexpression of Fzd7 activates the canonical Wnt/β-catenin signalling pathway and therefore should induce PrE in a manner similar to Wnt6. To test this F9 cells were transfected with Fzd7 or pcDNA3.1 and selected with G418 for 5 days, or treated with RA or DMSO as positive and negative controls for differentiation, respectively. Protein lysates were collected and used for immunoblot analysis with the TROMA-1 antibody as a marker for PrE differentiation. Total RNA was also collected, reverse-transcribed into cDNA, and used for qRT-PCR with Fzd7 primers to verify that Fzd7 was being overexpressed. qRT-PCR results show that Fzd7 mRNA is expressed 163-fold higher in Fzd7-transfected cells relative to empty-vector control (Fig. 3.7B). Immunoblot analysis indicated that levels of TROMA-1 in Fzd7 transfected cells did not change significantly and were similar to those detected in DMSO-treated and pcDNA3.1-transfected cells (Fig 3.7C). Densitometry was not performed as TROMA-1 levels in DMSO, Fzd7, and pcDNA3.1 lysates were not detectable above the background noise. That a TROMA-1 signal was detected in lysates of RA-treated cells, but was barely visible in DMSO-treated cells, would indicate that F9 cells had successfully differentiated into PrE due to RA treatment and that the TROMA-1 antibody was functioning as expected.
Figure 3.5 Overexpression of Fzd7 activates β-catenin/TCF/LEF-dependent transcription in F9 cells, but does not alter levels of the PrE marker Cytokeratin A. (A) F9 cells were co-transfected with pBARL (β-catenin activated reporter luciferase) and pRL-TK (Renilla luciferase) plasmids, as well as either Fzd7 or pcDNA3.1-empty vector, or treated with RA or DMSO for 48 hours. Lysates were collected and processed for luciferase activity. Cells treated with RA or Fzd7 show a 4.32 and a 3.06 fold-increase in luciferase activity respectively, relative to DMSO-treated cells (Appendix 3). (B) F9 cells were transfected with pcDNA3.1 or Fzd7, or treated with DMSO or RA and total RNA or protein lysate was collected after 5 days. Total RNA from pcDNA3.1 and Fzd7-transfected cells was collected, reverse-transcribed, and used for qRT-PCR analysis with primers for Fzd7. Results show that Fzd7 mRNA is expressed 163-fold higher in Fzd7-transfected cells relative to pcDNA3.1-transfected cells. (C) Representative blot showing a TROMA-1 signal in RA-treated, but not in DMSO-treated, Fzd7 or pcDNA3.1-transfected cells. Data represent the mean and standard error of three independent experiments (p<0.05). Luciferase activity was calculated by normalizing firefly luciferase activity to Renilla activity. For fold changes in TCF/LEF luciferase activity relative to DMSO, see Appendix 3.
A

TCF/LEF Luciferase RLU

DMSO      RA      pcDNA3.1  Fzd7

B

Normalized Fzd7 mRNA Expression (2^{-ΔCt})

pcDNA3.1  Fzd7

C

TROMA-1

β-Actin

DMSO  RA  Fzd7  pcDNA3.1
3.6 **Dkk1 mRNA expression is induced by RA treatment and Wnt6 overexpression**

As previously mentioned, studies from the Kelly lab have demonstrated that ectopic expression of *Fzd7* in F9 cells is sufficient to induce PrE differentiation and also to inactivate GSK-3 (Benjamin Cadesky, unpublished). This is supported by the results from the β-catenin/TCF/LEF luciferase assay, where overexpression of *Fzd7* induced β-catenin/TCF/LEF-dependent transcription (Fig 3.5A). However, these results are not corroborated by the *Fzd7* knockdown (Fig 3.4A) or the overexpression studies (Fig. 3.5C). Together, it would appear that these results do not support the hypothesis that FZD7 is a receptor necessary for binding WNT6 during RA-induced differentiation.

Nevertheless, the hypothesis may still hold true if the *Fzd7* knockdown was compensated by another Fzd receptor acting in a functionally redundant manner, and/or the fact that my overexpression experiments were ineffective in producing enough protein necessary to induce differentiation. Whatever the case, canonical Wnt signalling in F9 cells is necessary and sufficient for ExE formation and thus inhibiting the interaction between the ligand and the FZD receptor should inhibit the differentiation process. In addition, TCF/LEF-dependent transcription is necessary for F9 cells to differentiate in response to RA, and as other pathways can crosstalk with the Wnt pathway and cause β-catenin stabilization downstream of FZD receptors [43], I wanted to confirm the necessity of the pathway by blocking its activation at the level of the receptors. As attempts to inhibit differentiation by targeting *Fzd7* were unsuccessful, a broader approach was taken to inhibit WNT signalling at the cell surface. Antibodies that specifically target FZD receptors are currently in development, and are not available. The lack of Frizzled-specific antibodies/chemical inhibitors prompted me to employ proteins known to antagonize WNT signalling [47].

In a microarray profiling study using F9 cells, Gudas et al. identified two RA-target genes *Dab2* and *sFRP5*, both of which encode inhibitors of WNT signalling [40]. Interestingly, F9 cells induced to differentiate into VE are not responsive to WNT ligands, suggesting a blockade in WNT signalling accompanies differentiation [42]. Thus, it is plausible that F9 cells upregulate the expression of inhibitors of WNT
signalling in response to RA. To address this I examined Dickkopf-1 (DKK1), a specific inhibitor of canonical Wnt signalling for the following reasons. First, DKK1 is a secreted antagonist of WNT signalling, and therefore inhibits signalling at the level of the receptor and its ligand. Second, DKK1 is reported to specifically inhibit canonical WNT signalling as it binds a WNT co-receptor obligatory for canonical pathway activation [13]. In addition to binding FZD receptors, WNT ligands also bind to the single-span transmembrane LDL receptor-related protein 5/6 (LRP5/6) co-receptor to form a WNT-FZD-LRP5/6 ternary complex [39]. This interaction between WNT, FZD, and an LRP co-receptor is obligatory for activation of the canonical WNT pathway [48]. Third, Dkk1 expression is regulated by the β-catenin/TCF-LEF transcriptional complex in many cell lines, and several TCF-binding element (TBE) consensus sites have been identified within a highly conserved region 1kb upstream of the transcription start site in the human and mouse Dkk1 promoters [35]. Finally, Dkk1 is reportedly expressed in F9 cells induced to differentiate into VE [42].

With the above in mind, I sought to determine if Dkk1 expression is upregulated during PrE differentiation. To address this, the expression level of Dkk1 was analyzed over a four day period of RA-treatment. F9 cells were treated with 10^{-7} M RA for 1-4 days, or DMSO for 4 days and total RNA was collected and reverse-transcribed into cDNA for qRT-PCR analysis. qRT-PCR results indicate that Dkk1 expression is significantly increased at day 3 and 4 of RA treatment relative to DMSO control cells (Fig 3.6A). Expression of Dkk1 mRNA increased by 118 and 611-fold in cells treated with RA for 3 and 4 days, respectively, compared to DMSO control cells (Fig 3.6A). These results would indicate that the Dkk1 gene is RA-responsive, and is either a direct target of the RA pathway, or is regulated by a transcription factor that is downstream of RA signalling.

As Wnt6 is upregulated in F9 cells in response to RA, and Wnt6 overexpression or WNT6 conditioned medium is sufficient to induce PrE through activation of canonical WNT signalling [12], I decided to determine if overexpression of Wnt6 could also cause an increase in Dkk1 expression similar to that induced by RA. F9 cells were transfected with Wnt6 or pcDNA3.1, treated with G418 for 4 days, and total RNA was collected and
reverse-transcribed into cDNA to be used as template for qRT-PCR analysis. Results show that Dkk1 expression increased by 2.92-fold in cells transfected with Wnt6 compared to empty vector-transfected cells (Fig 3.6B). To confirm that Wnt6 was being overexpressed, the same cDNAs used in qRT-PCR with Dkk1 primers were also used for end-point PCR with primers for Wnt6. Results show the presence of a Wnt6 amplicon in cells transfected with pcDNA3.1-Wnt6 (Fig 3.6C). An amplicon corresponding to Wnt6 was not detected in cDNA from empty-vector transfected cells, however, the presence of an L14 amplicon indicated that cDNA was available for amplification. Taken together, these results demonstrate that the Dkk1 gene is induced by RA treatment and by overexpression of Wnt6 alone.

That Dkk1 expression is greatly increased in the presence of RA, and only slightly increased due to Wnt6 overexpression, prompted subsequent analysis of the mouse Dkk1 promoter region for conserved Retinoic Acid Response Elements (RARE) binding sites, the presence of which might explain the 611-fold upregulation of Dkk1 by RA. The Ensembl Genome Browser Regulatory Build was used to identify putative regulatory regions upstream of the Dkk1 transcription start site. A 961bp sequence was predicted as the Dkk1 promoter region and this sequence was used to identify potential RARE consensus DNA motifs. That two conserved RARE nucleotide sequences were found within this region, spaced 9bp apart (Fig3.6D), would suggest the potential for regulation of the Dkk1 gene by RAR-RXR homo/heterodimers.
Figure 3.6 Dkk1 is induced by overexpression of Wnt6. Total RNA was isolated from cells treated with RA (positive control) for 1-4 days and reverse-transcribed into cDNA (A). Total RNA was also isolated from F9 cells transfected with pcDNA3.1-Wnt6 and reverse-transcribed into cDNA (B and C). qRT-PCR was performed using primers for Dkk1 (A) and qRT-PCR and end point-PCR were performed using primers for Wnt6 (B and C). (A) qRT-PCR results indicate the expression of Dkk1 is significantly increased at 3 and 4 days after RA treatment compared to DMSO-treated cells or cells treated with RA for 1 day. Expression of Dkk1 at day 2 of RA treatment is not significantly different compared to DMSO-treated cells or cells treated with RA for 1 or 3 days but was significantly different from Dkk1 expression at 4 days post-RA treatment. $2^{-\Delta\Delta Ct}$ analysis indicates that compared to DMSO-treated cells, Dkk1 levels were increased by 119 and 611-fold at 3 and 4 days post-RA treatment, respectively (Appendix 4). Expression of Dkk1 mRNA was normalized to the expression of L14 mRNA. (B) qRT-PCR analysis indicates that Dkk1 mRNA expression increased by 2.92-fold in Wnt6-transfected cells compared to the empty vector control. (C) End-point PCR using the same cDNA samples in (B) show the presence of a Wnt6 amplicon in cells transfected with pcDNA3.1-Wnt6 compared to empty vector control. (D) In silico analysis of the predicted Dkk1 promoter region. The presence of two conserved RARE motifs within the predicted promoter region suggests possible regulation by RAR-RXR nuclear transcription factors. Data represent the mean and standard error of three independent experiments. Letters and * denote significance (p<0.05). For fold changes in Dkk1 mRNA expression relative to DMSO or empty vector treatments, see Appendix 4.
Mouse dickkopf homolog 1 (Xenopus laevis)
ENSEMBL Regulatory Region ENSMUSR00000425059—Positive strand

RARE Consensus Motif:
5’-AGGTCA-3’

Normalized Dkk1 mRNA (2-ΔCt)

DMSO  Day 1 RA  Day 2  Day 3  Day 4

Normalized Dkk1 mRNA (2-ΔCt)

pcDNA3.1  Wnt6

Wnt6  L14

pcDNA3.1  Wnt6

Mouse dickkopf homolog 1 (Xenopus laevis)
ENSEMBL Regulatory Region ENSMUSR00000425059—Positive strand

CTCTC CAAAGC CCACTT TAAAG CAGTGAT TCCCA AACAC CACCC AAA
TCTC ACAGAC CTGAT CTTG AGAC CCCG TCGGAT CAGCA CCCCT TTATGG
TAGTTG CAAAGGCT TTGCT ATTGCTGCGCCCTATGAGAATCTCCGGCGC CACTG
ACAGCCAGGCCGGCTGGCCCCACACCAAGGCTGGGGCAGTTCTGAGTCCGCG
TTGGAATG ATGAA AACTGT GCTCAAGGCTCAGG CACTGGCT CGCTCA GAGGACGTCAG
AGGCCACAT ATTGTA AACACGGCC AAGAAC CGCTGCGCCCTTATGACAAACCATC
ATCTCCGA AAGC CAAACAGGCA AAAGGTCAGG AAGAAA AGAGAGT GACTTTG CAGGCGGCG
TGCTGCAGAGCTGGAGAATCCCCA AACTTGGGACCCGGCTGGTGAGTCTCTG
GCTGCAGGCGACACGT CGCCAC CGCCACCTTGGCTCCTTAATATCGGCGC
TCCACCGCCTCACGCTCTGCAAGGGAGCAACAAAGGCGGATGGATTTCAAGCTTT
GGAGAGGAGCTGGAGGGGAGTTTTGTTGTTGGAGAGGAGGAGCATTTTGGACACTTTC
CCTCCTGGCCCTCGTTTGACTGTTGGTCCCTTGCGACCTGACCTTTAGAGTGTTGGTGAC
TAGACGCTAGACCTAGACCTAGACCTAGCCCCAAAACTTTTAAATAAAATAAAATAAA
TAAATGGCGGGAGGGTGCACTCTGTGAGCTTGAGTCTGTGCTGGGCTGGAGTTAGTT
CTGTCTGAAATCCTTGTCAGAGTTAAAAGTGATAGAA

CTCTTCAAAAGC CCACTT TAAAG CAGTGAT TCCCA AACAC CACCC AAA
TCTC ACAGAC CTGAT CTTG AGAC CCCG TCGGAT CAGCA CCCCT TTATGG
TAGTTG CAAAGGCT TTGCT ATTGCTGCGCCCTATGAGAATCTCCGGCGC CACTG
ACAGCCAGGCCGGCTGGCCCCACACCAAGGCTGGGGCAGTTCTGAGTCCGCG
TTGGAATG ATGAA AACTGT GCTCAAGGCTCAGG CACTGGCT CGCTCA GAGGACGTCAG
AGGCCACAT ATTGTA AACACGGCC AAGAAC CGCTGCGCCCTTATGACAAACCATC
ATCTCCGA AAGC CAAACAGGCA AAAGGTCAGG AAGAAA AGAGAGT GACTTTG CAGGCGGCG
TGCTGCAGAGCTGGAGAATCCCCA AACTTGGGACCCGGCTGGTGAGTCTCTG
GCTGCAGGCGACACGT CGCCAC CGCCACCTTGGCTCCTTAATATCGGCGC
TCCACCGCCTCACGCTCTGCAAGGGAGCAACAAAGGCGGATGGATTTCAAGCTTT
GGAGAGGAGCTGGAGGGGAGTTTTGTTGTTGGAGAGGAGGAGCATTTTGGACACTTTC
CCTCCTGGCCCTCGTTTGACTGTTGGTCCCTTGCGACCTGACCTTTAGAGTGTTGGTGAC
TAGACGCTAGACCTAGACCTAGACCTAGCCCCAAAACTTTTAAATAAAATAAAATAAA
TAAATGGCGGGAGGGTGCACTCTGTGAGCTTGAGTCTGTGCTGGGCTGGAGTTAGTT
CTGTCTGAAATCCTTGTCAGAGTTAAAAGTGATAGAA
3.7 Ectopic expression of canonical WNT antagonist Dickkopf-1 attenuates primitive endoderm differentiation

The previous results indicate that Dkk1 is expressed in F9 cells and is upregulated during RA differentiation. After determining the expression profile of Dkk1 during PrE differentiation, the next step was to assess the effect ectopically expressing Dkk1 would have on F9 cells induced to differentiate by RA. As canonical Wnt/β-catenin signalling has been reported as necessary for F9 cell differentiation [13], it is expected that if Dkk1 is expressed early enough during the differentiation process, it would inhibit canonical Wnt signalling and subsequently attenuate/inhibit differentiation.

To determine the effect of Dkk1 on PrE differentiation, F9 cells were transfected with a myc-tagged human Dickkopf-1 (MYC-DKK1) construct and selected with G418, then treated with RA for 4 days to induce PrE. Protein lysates were collected and processed for immunoblot analysis with the TROMA-1 antibody to determine if cells had differentiated into PrE. Immunoblot analysis showed that Cytokeratin A was expressed in the positive controls treated with RA or transfected with empty vector and treated with RA (Fig. 3.7A). Cytokeratin A levels were reduced in cells transfected with the MYC-DKK1 construct and treated with RA (Fig 3.8A). Densitometry analysis confirmed this significant decrease in TROMA-1 levels in cells transfected with MYC-DKK1 and treated with RA compared to cells transfected with empty vector and treated with RA (Fig. 3.7B). The apparent reduction in TROMA-1 signal in cells transfected with empty vector and treated with RA compared to cells treated with RA alone would indicate that transfection had a detrimental effect on differentiation. Therefore, all further experiments were conducted using transfected positive (ie. pcDNA3.1 +RA) and negative controls (i.e. pcDNA3.1 +DMSO) to control for these transfection effects. To corroborate the TROMA-1 immunoblot data, this experiment was repeated using another marker of PrE differentiation [49]. Thus, since ectopic MYC-DKK1 expression can inhibit differentiation as evident by a reduction in Cytokeratin A levels, then its overexpression should also negatively impact Dab2 expression. Total RNA was collected and reverse-transcribed into cDNA and used for qRT-PCR analysis with Dab2 primers. qRT-PCR analysis showed that relative to controls, Dab2 was upregulated by 141-fold in empty
vector transfected and RA-treated cells and by 118-fold in cells transfected with MYC-DKK1 and treated with RA (Fig 3.7C). Taken together, these results indicate that ectopically expressing MYC-DKK1 in undifferentiated F9 cells reduces their ability to differentiate into PrE when treated with RA.
Figure 3.7 Cells transfected with MYC-DKK1 show decreased expression of PrE markers. (A) F9 cells were transfected with pcDNA3.1 or MYC-DKK1 and treated with DMSO or RA, or treated with DMSO or RA alone, and protein lysates were collected for immunoblot analysis with the TROMA-1 antibody. Immunoblot analysis shows a TROMA-1 signal in cells transfected with pcDNA3.1 and treated with RA and cells treated with RA alone. This signal is also present in cells transfected with MYC-DKK1 and treated with RA, however, its intensity is reduced in comparison to the positive controls (RA and pcDNA3.1+RA), and is closer to that seen in Lane1 (DMSO –treated cells). β-actin served as a loading control. (B) Densitometry analysis confirmed that there was a significant reduction in TROMA-1 levels in cells transfected with MYC-DKK1 and treated with RA, compared to cells transfected with empty vector and treated with RA. (C) Total RNA was collected from F9 cells transfected with MYC-DKK1 and treated with RA or transfected with empty vector and treated with DMSO or RA, and qRT-PCR analysis was performed using Dab2 primers. Results show that Dab2 mRNA expression was significantly reduced (by approximately 23-fold) in MYC-DKK1 transfected, RA-treated cells compared to empty vector transfected, RA-treated cells (Appendix 5). Data represent the mean and standard error of three independent experiments. * denotes significance (p<0.05). For fold changes in Dab2 expression relative to empty vector transfected, DMSO-treated control, see Appendix 5.
A

TROMA-1

β-Actin

DMSO  RA  pcDNA3.1 +RA  MYC-DKK1 +RA

B

TROMA-1/β-Actin Ratio

pcDNA3.1 +RA  MYC-DKK1 +RA

C

Normalized mDob2 mRNA Expression ($2^{\Delta \Delta CT}$)

pcDNA3.1 +DMSO  pcDNA3.1 +RA  MYC-DKK1 +RA

*
3.8 MYC-DKK1 conditioned medium attenuates PrE formation

The results from the overexpression of MYC-DKK1 indicate that ectopically expressing a construct encoding a secreted antagonist of canonical WNT/β-catenin signalling in undifferentiated F9 cells attenuates the ability of RA to induce the PrE phenotype. This confirms an earlier study that reported that canonical WNT/β-catenin signalling was necessary for RA-induced PrE differentiation [13]. Furthermore, my results show that differentiation is blocked when WNT is prevented from forming a WNT-FZD-LRP5/6 receptor complex on the plasma membrane. To corroborate this data, MYC-DKK1 conditioned medium was generated using HEK-293 cells to examine whether ectopically applying the protein to F9 cells mimics the effect of overexpression of MYC-DKK1 on differentiation.

Briefly, HEK-293 cells were transfected with pcDNA3.1 or MYC-DKK1, and medium was collected after 48 hours, filter sterilized, and concentrated. HEK-293 protein lysates were also collected for immunoblot analysis with the anti-c-Myc antibody (9E10) to confirm that the cells were translating the MYC-DKK1 transcripts. A band at approximately 39kD corresponding to the MW of MYC-DKK1 was detected in HEK-293 cells transfected with the MYC-DKK1 construct, but not in cells transfected with the empty-vector (Fig. 3.9A). To confirm that the HEK-293 cells were secreting the protein, the medium generated from these cells was concentrated and used for immunoblot analysis. If the MYC-DKK1 protein is being translated and secreted by the cells, then the 9E10 antibody should recognize the MYC-epitope both in cell lysates and in the medium. The concentrated medium was used for immunoblot analysis and a 39kD band was detected in the medium from cells transfected with MYC-DKK1, but not the empty vector (Fig. 3.8B). The MYC-DKK1 conditioned medium was applied to F9 cells in the presence of DMSO or RA to see if it would mimic the effect of expressing MYC-DKK1 on differentiation. Protein lysates were collected for immunoblot analysis and results showed a TROMA-1 signal present in cells treated with the empty vector-conditioned medium and RA, but absent in cells treated with this conditioned medium and DMSO. Thus, the conditioned medium did not contain factors that could induce differentiation on
its own (Fig. 3.8C). A TROMA-1 signal was also present in cells treated with \textit{MYC-DKK1} conditioned medium and treated with RA, however, its intensity was reduced compared to that seen in cells treated with the empty vector-conditioned medium and RA (Fig 3.8C). Densitometry analysis confirmed that the reduction of TROMA-1 seen in cells treated with MYC-DKK1 conditioned medium and RA was significant compared to cells treated with the empty vector-conditioned medium and RA (Fig. 3.8D). Taken together, these results indicate that the HEK-293 cells were a useful vehicle for the production and secretion of the MYC-DKK1 protein. More importantly, applying the ectopically expressed protein on F9 cells in the presence of RA attenuates the ability of RA to induce PrE differentiation.
Figure 3.8 Ectopically expressed MYC-DKK1 attenuates RA-induced PrE differentiation of F9 cells. HEK-293 cells were transfected with pcDNA3.1 or MYC-DKK1, and protein lysates and media were collected for immunoblot analysis with the 9E10 antibody to determine if the cells were translating (A) and secreting (B) the Myc-tagged DKK1 protein. (A) Immunoblot analysis of lysates with the 9E10 antibody shows the ectopic DKK1 protein present in cells transfected with MYC-DKK1, but not pcDNA3.1 (N=2). (B) Conditioned medium collected from these cells was filter sterilized, concentrated and used for immunoblot analysis with the 9E10 antibody to confirm that the MYC-DKK1 protein was secreted into the medium. The unorthodox bands pattern is likely an artifact of the concentration process, perhaps due to increased salt concentration (N=2). (C) A representative blot showing TROMA-1 levels are reduced in cells treated with the MYC-DKK1 CM and RA compared to pcDNA3.1-CM and RA. F9 cells co-treated with the concentrated CM from HEK-293 cells and either DMSO or RA were collected for immunoblot analysis with the TROMA-1 antibody. (D) Densitometry analysis confirmed that there was a significant decrease in TROMA-1 signal (0.39-fold) in cells co-treated with MYC-DKK1 CM and RA, compared to cells co-treated with pcDNA3.1 CM and RA. Data is representative of three independent experiments, except for (A) and (B) (N=2).* denotes significance (p<0.05).
Chapter 3

4 Discussion

The WNT signalling pathway is ancient and evolutionarily conserved in Metazoans and one that is required for proper embryonic development, differentiation of stem cells, organogenesis and tissue patterning. The result of a WNT ligand binding to its cognate FZD receptor is the initiation of one or several intracellular signalling cascades, which include the canonical WNT/β-catenin-dependent pathway and the non-canonical, β-catenin-independent Planar Cell Polarity (PCP) and WNT/Ca²⁺ pathways. There are 10 different Fzd receptors and 19 Wnts that have been identified in vertebrates, allowing for a remarkable number of receptor-ligand combinations that complicates the understanding of how one particular WNT pathway can be activated preferentially over another. The activation of one signalling branch over another is thought to depend on several factors such as the intrinsic properties of WNTs and FZDs themselves, as well as expression of receptors and co-receptors in the cellular context of study [28].

In this study, F9 cells were used to investigate the role of the canonical WNT/β-catenin signalling pathway, and specifically the role of FZD receptors, in specifying a set of naïve cells to adopt an epithelial state known as primitive endoderm. F9 cells also utilize the PCP and WNT/Ca²⁺ pathways during differentiation; however these pathways were not investigated in this study. The PCP pathway is reported to control the oriented migration of PE cells [17], where WNT binding to FZD, like that in canonical β-catenin signalling, initiates DVL activation. Unlike, β-catenin-dependent signalling, however, this binding leads to activation of RHO-ROCK and RAC-JNK signalling, which causes cytoskeletal changes that mediate cell polarity and cell movements. Although the PCP pathway was not investigated in this study, its importance should not be underrated as it is key to specifying gastrulation, one of the most important developmental and morphogenetic processes [14]. The other known PCP pathway involves Ca²⁺ signalling, and was discovered when some WNT and FZD receptors were found to stimulate intracellular calcium ion release from the endoplasmic reticulum [50]. WNT/Ca²⁺ signalling is thought to depend on the action of G-proteins, Ca²⁺/calmodulin-dependent
protein kinase, and Protein Kinase C to regulate convergent extension movements along the anterior-posterior axis during gastrulation, but again this pathway was not investigated in this study.

The most studied of the WNT pathways and one that was the focus of this study is the canonical WNT/β-catenin-dependent pathway. F9 cells differentiate into PrE when treated with RA, and this differentiation is accompanied by the upregulation of Wnt6 and activation of the canonical WNT/β-catenin pathway [12]. Wnt6 is upregulated by the transcription factors GATA6 and FOXA2. Gata6 is a direct target gene of RA, and FoxA2 expression is induced by GATA6. Both GATA6 and FOXA2 bind the promoter of Wnt6 in F9 cells to activate its transcription [51]. Thus, Wnt6 is upregulated during RA induced differentiation; however the receptor responsible for binding WNT6 and transducing this differentiation signal is heretofore unknown.

4.1 Transcriptional response of Frizzled genes during PrE differentiation

Since RA causes an increase in the expression of Wnt6, which in turn activates canonical WNT signalling, and if FZD7 is the receptor that binds WNT6 to activate this pathway, then I would expect Fzd7 mRNA to be present in undifferentiated cells, awaiting translation and trafficking to the cell membrane in order to receive the WNT signal. That Fzd7 mRNA was detected in undifferentiated F9 cells and throughout differentiation into both PrE and PE supports this idea. Data from the Kelly laboratory has shown that Fzd7 expression is significantly increased in F9 cells following RA treatment (Benjamin Cadesky, unpublished) however these results were not confirmed in this study and is attributed to differences in the days that cells were exposed to RA. This prompted investigation of other members of the Fzd family to determine if they are expressed in F9 cells and if there is a particular member that is preferentially increased in response to RA. Okoye et al. report that all Fzds and 18 out of 19 Wnts are expressed in undifferentiated F9 cells. This, however, contradicts the Fzd expression data in this study.
and with previous findings from the Kelly lab, which show that only eight out of the ten Fzds are expressed in F9 cells, and that Wnt6 is the only expressed Wnt. These contradictory results are likely due to amplification of genomic material/contaminants leading to false positive signals seen in the Okoye et al. report.

My results show that three of the eight Fzd genes were significantly upregulated by RA. This transcriptional response to RA has also been shown in NTERA-2 human embryonal carcinoma cells (NT2/D1) [45, 52]. Interestingly, treatment of human embryonal carcinoma NT2/D1 cells with RA causes upregulation of several Wnt and Fzd genes, most notably being Fzd7 [45], which is in agreement with my results. The transcriptional response to RA indicates that the RA pathway regulates Fzd receptor encoding genes, but there may be further, more refined regulation involving canonical WNT/β-catenin signalling. Both positive and negative regulation of Fzd expression by the canonical WNT/β-catenin signalling pathway was reported when human embryonal carcinoma cells were treated with WNT-3A conditioned medium [53, 54]. Thus, it is tempting to speculate that the observed change in Fzd receptor expression during PrE differentiation is the indirect result of RA signalling and the direct result caused by TCF/LEF target gene activation induced by Wnt6.

Regardless of regulation, the transcriptional response is the upregulation of these genes and subsequent translation of the message and trafficking of the receptor to the cell membrane, which would be present to receive WNT6. Binding would initiate activation of the canonical WNT/β-catenin signalling pathway and facilitate the transition from the undifferentiated state into PrE. That WNT6 conditioned medium is sufficient to activate canonical WNT/β-catenin signalling and differentiate cells into PrE [12], would suggest that receptors are already in place in undifferentiated cells and that transcriptional regulation following WNT6 to bind the F9 cell surface induces other Fzd genes to activate other signalling pathways needed for differentiation. The results showing Fzd1, 4 and 8 are upregulated in PrE cells compared to undifferentiated cells would indicate that these receptors are preferentially utilized in the PrE state, and may mediate the transition from PrE to PE. If that is the case, future studies should focus on blocking the expression
of Fzd1, 4 and 8 and determining if this down-regulation would have an effect on the transitioning of cells from PrE to PE.

4.2 Knockdown of Fzd7 does not attenuate PrE differentiation

The Frizzled gene family encodes receptors for WNT ligands and these receptors are therefore the lynchpins in transducing the signals that specify numerous events during embryonic, neonate and adult development. Specific FZD receptors and the downstream pathways they are coupled to have been studied in various contexts. In the case of the β-catenin pathway in F9 cells, WNT binding to a FZD receptor and LRP5/6 co-receptor initiates this pathway in response to WNT6. Subsequent TCF/LEF target genes are then activated that specify cells to adopt a PrE fate. The question remains, however, as to the identity of the FZD receptor that initiates this process, especially given the considerable promiscuity between WNTs and their FZD receptors [15]. Despite the fact that the original hypothesis was that FZD7 is the primary receptor responsible for binding WNT6 in F9 cells, given that Fzd7 siRNA did not lower Cytokeratin A levels would suggest that other FZD receptors can bind WNT6 or that the siRNA did not effectively reduce FZD7 protein levels. If FZD7 was the sole receptor for WNT6, or the only Fzd expressed in F9 cells, then knocking it down would attenuate differentiation. However, this was not the case, and could be explained by the functional redundancy among FZD receptors or by an ineffective knockdown. Towards the end of functional redundancy, it has been shown that Drosophila fz and Dfz2 are functionally redundant for canonical WNT/β-catenin signalling, as fz loss-of-function mutant flies do not show disrupted canonical signalling [46]. Phylogenetic analysis of human Fzd genes groups them into four clusters: Fzd1/2/7, Fzd3/6, Fzd5/8 and Fzd4/9/10. As Fzd1, 2 and 7 share 75% sequence identity with each other, it is not surprising that there is considerable functional redundancy within this particular cluster [23]. Fzd7−/− mice demonstrate a 15% penetrant cardiac phenotype compared to Fzd2−/−;Fzd7−/− embryos, which display a 100% penetrant convergent extension defect and mid-gestational lethality [55]. Fzd7 and Fzd5 are also functionally redundant for canonical WNT/β-catenin signalling in cell culture, as ectopic expression
of Fzd5 in human mesenchymal stem cells (hMSCs) harboring a Fzd7 knockdown can rescue TCF/LEF luciferase reporter activity. Conversely, ectopic expression of Fzd7 can rescue the same reporter activity in Fzd5 knockdown hMSCs [56]. Interestingly, Fzd5 was the second most abundantly expressed Fzd in F9 cells and given that it can substitute for Fzd7 to fulfill the role of a canonical WNT signalling receptor in another stem cell model, its knockdown in combination with Fzd7 in F9 cells may have a detrimental effect on differentiation. It is also important to note that Fzd1 displayed the greatest increase in expression after RA treatment, and overexpression of chimeric rFz1 and rFz2 receptors in F9 cells is sufficient for primitive endoderm differentiation [24, 25]. Due to functional redundancy, it is likely that multiple Fzd receptors need to be knocked down simultaneously to prevent WNT6 from activating canonical WNT/β-catenin signalling and attenuate differentiation into PrE. Thus, it would be interesting to determine if knocking down the entire cluster consisting of Fzd1, 2 and 7 in an attempt to abolish redundant pathway activation would have an effect on differentiation. In this study, it is also possible that Fzd7 mRNA levels were not sufficiently reduced to cause a subsequent knockdown of FZD7 protein, as FZD7 protein levels were not assessed in this study using an anti-FZD7 antibody, this scenario should not be ruled out. Also, the doubling time of F9 cells is approximately eight hours [8] and as a result siRNA could be diluted below an effective amount due to cell division. To circumvent this issue, a selectable short-hairpin RNA (shRNA) vector could be utilized as a more potent method of RNA interference. shRNAs confer several advantages over siRNAs, as they allow for constitutive expression of the antisense RNA and continual knockdown of the Fzd in question, both circumventing potential problems associated with inadequate knockdown time. Additionally, cells that have taken up the vector can be selected, eliminating cells that were not successfully transfected. Alternatively, siRNA may not be an effective strategy for reducing protein levels and inhibiting differentiation if FZD7 is a stable protein. If this is the case, the reduction in protein translation and accumulation that follows degradation of the endogenous Fzd7 transcript would not have a significant effect on FZD7 protein levels, and it could still be present at sufficient levels to bind WNT6 and induce differentiation. As FZD7 protein expression was not examined following siRNA transfection, this possibility should not be ruled out.
4.3 Overexpression of Fzd7 is not sufficient to induce PrE

The overexpression of Fzd7 has been reported in many types of human cancer. Notably, overexpression of Fzd7 is reported in colorectal cancer, hepatocellular carcinoma (HCC), and triple-negative breast cancer (TNBC). Knockdown of Fzd7 in colorectal cancer cells decreases β-catenin/TCF/LEF activity, invasion, and migration. Similarly in HCC and TNBC cells, knockdown or inhibition of Fzd7 causes a similar reduction in β-catenin activity resulting in reduced cell motility and cell proliferation [19]. Thus, Fzd7 is thought to confer malignancy via its ability to activate the canonical WNT/β-catenin signalling pathway. In this study, Fzd7 was overexpressed in F9 cells to determine if it could activate the canonical WNT/β-catenin pathway and if so, whether this activation would be sufficient for PrE differentiation. It was expected that Fzd7 overexpression would cause activation of canonical signalling, and the results showing activation of the TCF/LEF luciferase reporter assay were encouraging. However, that no effect was observed on Cytokeratin A levels in an immunoblot assay after five days of culturing would suggest the overexpression of Fzd7 was not sufficient to induce PrE. Several reasons to explain this discrepancy could be proposed, including the possibility that the reporter assay was sensitive to detect activation, but that the overexpression was not adequate for the requisite activation of endogenous genes needed to induce these naïve cells. Although the TCF/LEF luciferase reporter was activated in cells transfected with Fzd7 in comparison to empty vector transfected cells, it is possible that there are other explanations for this increase in reporter activity and without demonstrating increased FZD7 protein expression transfected cells, alternative explanations for the observed reporter activity should not be ruled out. Future studies would, however, need to be employed to test these ideas.

Why overexpression of Fzd7 did not induce PrE differentiation is not known despite the fact that it causes activation of the canonical WNT/β-catenin pathway. Activation of WNT signalling due to Fzd overexpression is accompanied by the recruitment of DVL to the plasma membrane [46]. In Xenopus, certain FZDs are capable of activating the canonical WNT pathway/β-catenin when injected alone (e.g., Xfz3),
while others require the co-injection of a Wnt for pathway activation (e.g., Xfz4 and Xfz7) [31]. Thus, it seems that some FZDs activate WNT signalling in a ligand-independent fashion when overexpressed, while others require the presence of an appropriate ligand. Other studies show that FZDs exist as homodimers when overexpressed and this dimerization is associated with activation of the canonical WNT/β-catenin pathway [32]. Whether this dimerization is necessary for DVL recruitment is not known [32], but two cysteine residues conserved among FZDs and located in the first and second extracellular loops, would facilitate intermolecular disulfide bond formation needed for dimer formation [14]. Indeed, receptor dimerization and activation has been shown for several GPCRs but whether dimerization is necessary and sufficient and occurs in F9 cells is not known. Likewise, Fzd7 was not expressed with a Wnt in this study, which may have had an effect. Nonetheless overexpression of Fzd7 alone was sufficient to activate TCF/LEF-dependent transcription demonstrating ligand-independent pathway activation.

4.4 Role of Dkk1 in ExE differentiation

Since overexpression of Fzd7 did not induce PrE, and its knockdown in the presence of RA did not inhibit PrE formation, a more widespread approach was taken to inhibit canonical WNT/β-catenin signalling during differentiation. Rather than attempt to inhibit WNT6 from interacting with a single FZD receptor, DKK1 was used to inhibit WNT-FZD-LRP5/6 complex formation, which is indispensable for activation of the canonical WNT pathway [48]. DKK1 modulates the output of the canonical WNT pathway by binding to LRP5/6 and blocking the LRP5/6-WNT interaction, thus preventing complex formation between WNT, FZD and LRP5/6 [57]. In addition, DKK1-mediated inhibition of WNT signalling is enhanced by the presence of the single-pass transmembrane protein Kremen1/2 (KRM1/2). KRM2 forms a ternary complex with DKK1 and LRP6 and causes rapid endocytosis of LRP6 from the cell membrane [58]. The hallmark function of the DKK family of proteins is their ability to modulate WNT signalling. Given the highly important role of WNT signalling pathways during development, the maintenance of WNT signalling homeostasis by DKK proteins is crucial. DKK1 is required for normal antero-posterior axial patterning, limb and vertebral formation where Dkk1−/− mice display embryonic lethality showing deletion of anterior
head structures, postaxial polysyndactyly, and fused vertebrae. In addition, misexpression of $Dkk1$ is reported in several types of cancer such as Wilm’s tumour, colorectal, and HCC [59].

The significant upregulation in expression of $Dkk1$ mRNA in RA-treated F9 cells suggests that it plays a role primarily in the PrE state, as it is highly upregulated after four days of RA treatment. This expression pattern is concurrent with other inhibitors of WNT signalling, such as $Dab2$. DAB2 inhibits WNT/$\beta$-catenin signalling by binding LRP6 and promoting its internalization, as well as by interacting with and stabilizing AXIN, preventing destruction complex disassembly [60]. Increased levels of $Dab2$ and $Dkk1$ induced by RA in F9 cells would suggest that canonical WNT/$\beta$-catenin signalling is inhibited in PrE cells at more than one level of the WNT pathway. This blockade in canonical WNT/$\beta$-catenin signalling has been demonstrated in F9 cells induced to differentiate into visceral endoderm (VE). These cells are not responsive to WNTs, and it was proposed that a blockade to WNT signalling is necessary in order for F9 cells to differentiate into VE, and that WNT signalling inhibits RA-induced differentiation [42]. The authors identify $Dkk1$ as an inhibitor upregulated by F9 cells induced to differentiate into VE, but did not observe the effect of ectopic expression of $Dkk1$ on differentiation [42]. In the current study, the data from the $Dkk1$ overexpression and conditioned medium experiments indicating that DKK1 can attenuate differentiation into PrE supports the notion that the canonical WNT/$\beta$-catenin pathway is necessary for this event to occur. Thus, differences in the role of the WNT pathway in differentiation may be due to differences in activation of cellular pathways associated with VE compared to PrE.

$Dkk1$ is reportedly regulated by the $\beta$-catenin/TCF/LEF transcriptional complex in several cell lines, and there are four TCF/LEF DNA binding motifs located within the human and mouse $Dkk1$ promoter [14]. As $Wnt6$ is upregulated by GATA6 and FOXA2 during RA-induced differentiation [51], it was predicted that $Dkk1$ would be upregulated downstream of $Wnt6$. $Dkk1$ expression was strongly induced by RA-treatment, and furthermore overexpression of $Wnt6$ induced $Dkk1$ expression, albeit to a much lesser extent than RA. This upregulation of $Dkk1$ by $Wnt6$ overexpression supports the notion that $Dkk1$ is regulated by TCF/LEF in F9 cells. However, the significant upregulation of
Dkk1 by RA treatment brought into question the possibility of direct Dkk1 regulation by the RA pathway. *In silico* analysis of the Dkk1 promoter revealed the presence of a conserved retinoic acid response element (RARE), consisting of a direct repeat of the AGGTCA sequence separated by 9bp [61]. The conserved RARE motif is typically spaced 1, 2, or 5bp apart, however, other arrangements have been reported in the literature [61]. Thus, it seems likely that the Dkk1 gene is regulated by both the RA and β-catenin signalling pathway, and therefore participates in negative feedback regulation of canonical WNT/β-catenin signalling in F9 cells. Further analysis using ChIP assays would be required to confirm if one or both of the pathways are involved in negative feedback of the WNT pathway by DKK1. Nevertheless, there is precedence for this in the literature [14].

Thus, in our model of F9 cell differentiation, expression of Dkk1 in PrE cells would inhibit WNT6 from binding to LRP5/6, but WNT6 can still bind FZD receptors present on the plasma membrane. This would reduce canonical WNT/β-catenin pathway output or inhibit it entirely, preventing β-catenin from stabilizing and translocating to the nucleus to stop the activation of TCF/LEF target genes that was necessary for PrE differentiation. Thus, WNT6-FZD-LRP complex formation in the presence of DKK1 would be minimal or non-existent, while association of WNT6 with FZD and non-canonical WNT receptors, such as ROR2, would still be possible. With this in mind, the apparent reduction in canonical signalling due to interference with WNT6-LRP interactions at the cell surface may place F9 cells in a situation where WNT6 is now able to activate non-canonical PCP signalling. Indeed, PCP signalling is reported as necessary for oriented migration of PE cells through DAAM1/RHO/ROCK signalling. Treatment of F9 cells with SFRP perturbs the actin cytoskeleton and causes a disorganized pattern of PE cell migration, and β-catenin was found to play no role in this process [28]. Thus, it seems that WNT signalling is active in PE cells, but canonical WNT signalling is blocked after RA treatment, and this blockade may facilitate the activation of the non-canonical pathway due to changes in receptor availability on the plasma membrane. Yamamoto et al. (2008) speculate that β-catenin signalling is simultaneously inhibited while non-canonical PCP signalling is activated due to the enhancement of WNT-FZD-ROR2 complexes at the expense of WNT-FZD-LRP6 complexes, and this is mediated by the
collagen triple helix repeat-containing protein 1 (CTHRC1) [14]. Mikels and Nusse (2006) also found that WNT-5A can act on the canonical and non-canonical WNT pathways, and this depended on the presence of the LRP5/6 co-receptor [60]. Thus, it is tempting to speculate that F9 cells also utilize a similar mechanism that involves DKK1 and other inhibitors to help mediate a switch from canonical WNT/β-catenin signalling to non-canonical PCP signalling in order to facilitate the outgrowth and oriented migration of PE cells, thus completing the transition from PrE to PE. This phenomenon is worthy of investigation as it could help further the understanding of how WNTs activate specific downstream signalling pathways.

Finally, the detection of a signal at approximately 39 kD in conditioned medium collected from HEK-293 cells transfected with MYC-DKK1 was evidence for the presence of the MYC-DKK1 protein in the medium. However, the bands produced in the immunoblot assay were smeared and distorted and might not be reliable proof of the presence of the MYC-DKK1 protein in the medium. Rather, they might represent some artifact of the protein concentration process or the immunoblot assay. Other assays should be used in future conditioned medium studies to demonstrate the presence of the ectopically expressed protein such as an enzyme-linked immunosorbent assay (ELISA) which could be used to detect the recombinant protein and determine its concentration in the medium. It is also important to consider the fact that conditioned medium contains many factors in addition to the expressed protein of interest, and it is possible that another protein was responsible for the decrease in the TROMA-1 signal (Fig 3.8B, C). However, as the control conditioned medium did not inhibit the ability of RA to differentiate cells, this is likely not the case.

Finally, the necessity of the canonical WNT/β-catenin pathway in PrE differentiation has been demonstrated using a dominant-negative mutant of TCF4 [13]. Expression of this dominant-negative construct in F9 cells blocked the ability of RA to induce markers of PrE. My study provides additional support for the necessity of the canonical WNT/β-catenin pathway as it demonstrates that inhibition of the WNT pathway at the plasma membrane using DKK1 impedes the ability of RA to induce PrE. This study provides further evidence for the necessity of the canonical WNT/β-catenin
pathway in the differentiation of F9 cells into PrE, and highlights the importance of an inhibitor of a cell signalling pathway to allow for continued differentiation into a different cell type.

4.5 Conclusion

The differentiation of naïve F9 cells into PrE is a highly regulated event that requires the presence of canonical WNT/β-catenin signalling. The goal of this study was to determine the role of FZD receptors, specifically FZD7, in the differentiation of F9 cells into PrE. It was hypothesized that FZD7 binds WNT6 during F9 cell differentiation, but this was not supported by the data. Many Fzd receptors are expressed in F9 cells and in some cases their expression changes in response to RA. FZD7 can potentially serve as a receptor for WNT6 as its overexpression induces canonical WNT signalling. However, it is not likely the sole FZD receptor for WNT6 in this model system as FZD receptors exhibit functional redundancy and the extent of the knockdown of Fzd7 did not have a detrimental effect on differentiation. Differentiation into PrE is attenuated when the canonical WNT antagonist DKK1 is expressed in the undifferentiated state. This inhibitor is expressed endogenously by F9 cells, and is likely involved in a negative feedback loop that tightly regulates the canonical WNT signalling pathway once cells reach the PrE state. Taken together, these results help to explain the dynamics and the role of the WNT signalling pathway in mammalian extraembryonic endoderm differentiation. Given the role of the WNT pathway in cancer, it may also provide a better understanding of how metastatic cancers utilize the WNT pathway to undergo EMTs and migrate to secondary sites. The role of negative feedback loops in processes such as differentiation is exemplified by the expression and regulation of Dkk1 in this system. That its ectopic expression perturbs the differentiation into PrE highlights the importance of modulating the canonical WNT/β-catenin signalling pathway at the correct time throughout differentiation.
Literature Cited


Appendices

**Appendix 1-** Primer sequences and annealing temperatures

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing Temp. (T&lt;sub&gt;a&lt;/sub&gt;)</th>
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<tr>
<td>Fzd1</td>
<td>5'-CAAGGTTTACGGGTCTCATGT-3'</td>
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<td>Fzd2</td>
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Appendix 2- Fold change Fzd mRNA expression in response to RA (relative to DMSO-treated cells)

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<th>Fold Change Fzd mRNA expression ($2^{\Delta\Delta Ct}$)</th>
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**Appendix 3**- Fold change TCF/LEF luciferase activity relative to DMSO

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<tr>
<td>RA</td>
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<tr>
<td>pcDNA3.1</td>
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</tr>
<tr>
<td>Fzd7</td>
<td>3.06</td>
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**Appendix 4**- Fold change *Dkk1* mRNA expression in response to RA or Wnt6 (relative to DMSO-treated or empty vector transfected cells, respectively)

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<th>Treatment</th>
<th>Fold Change <em>Dkk1</em> mRNA Expression ($2^{\Delta\Delta Ct}$)</th>
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<td>27.15</td>
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<td>RA Day 3</td>
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<tr>
<td>RA Day 4</td>
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<tr>
<td>Treatment</td>
<td>Fold Change Dkk1 mRNA Expression ($2^{-ΔΔCt}$)</td>
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<td>$Wnt6$</td>
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**Appendix 5-** Fold change $Dab2$ mRNA expression in response to transfection with empty vector or $MYC-DKK1$ and treatment with DMSO or RA (relative to $pcDNA3.1$ +DMSO)

<table>
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<tr>
<th>Treatment</th>
<th>Fold Change Dab2 mRNA Expression ($2^{-ΔΔCt}$)</th>
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<tr>
<td>$pcDNA3.1$ +DMSO</td>
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<td>$MYC-DKK1$ +RA</td>
<td>118.35</td>
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</tbody>
</table>
Curriculum Vitae

Gregory Golenia

Education

2011-2015  **Master of Science**- Department of Biology, Collaborative Graduate Program in Developmental Biology. Western University, London, ON.

2008-2011  **Bachelor of Science**- Honours Specialization in Cell and Developmental Biology. Western University, London, ON.

Research Experience

2011-Current  Graduate Research, Dr. Gregory Kelly lab, Department of Biology, Collaborative Developmental Biology Program. Western University, London, ON.

- M.Sc. Thesis: Wnt Signalling During F9 Cell Differentiation

2010-2011  Undergraduate Research, Dr. Sashko Damjanovski lab, Department of Biology, Western University, London, ON.

- Biology 4999 Thesis: MT1-MMP may alter MMP-2 gene expression and activity in response to TIMP-2 treatment in MCF-7 breast adenocarcinoma cells

Honours and Awards

2011-2012  Western Graduate Research Scholarship

2011  Developmental Biology Entrance Scholarship

2011  The University of Western Ontario Gold Medal

2008-2011  Dean’s Honour List

2008  Western Scholarship of Excellence (Admission Scholarship)
Teaching Experience

Western University Teaching Assistant:

2013-2014 Biology 1001 - Biology for Sciences (Lab Manager- Winona Gadapati)-
Laboratory TA for the course, set up and maintained labs, lectured, graded
lab assignments

Biology 2290 - Scientific Methods in Biology (Lecturer-Irene Krajnyk)-
Set up and cleaned laboratory, lectured about statistical analysis of
scientific data, graded lab books and poster presentations

2012-2013 Biology 4338 (Advanced Cell Biology, Dr. Gregory Kelly) - Assisted in
preparing and running course laboratory, graded midterm and final
examinations, evaluated student presentations and papers, deliberated
student debates

Biology 4300 (Selected Topics in Cell Biology: Stem Cells, Dr. Gregory
Kelly) - Assisted in grading midterms and final exams, graded student
papers and presentations on selected topics/researchers in stem cell
biology

2011-2012 Biology 3316 (Advanced Cell Biology, Dr. Ron Podesta) - Ran tutorial
and evaluated student presentations on current topics in cell biology

Biology 3326 (Parasitology, Dr. Ron Podesta) - Gave lectures in tutorials
and wrote and graded quizzes/midterms

Contributions to Research

Poster Presentations

  receptor expression and function during extraembryonic endoderm formation.
  Biology Graduate Research Forum, Western University, London, ON.

  between Wnt and Hedgehog signalling direct extraembryonic endoderm
  formation. 17th International Congress of Developmental Biology. Cancun,
  Mexico.
Journal Reviewer

2015  Peer-reviewed a manuscript submitted to the journal PLOS ONE

Volunteer Experience

2011  UWO Violence Prevention Program- attended training sessions and lead high school classrooms in London, ON through a set of activities designed to facilitate the discussion of violence prevention

2009-2011  UWO Science Soph- upper year student volunteer position designed to help increase the quality of the orientation week experience for new students. Addressed questions, concerns, or problems, lead campus and residence tours, accompanied students to various Orientation week events, organized charity initiatives.