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Cell Signaling Crosstalk and Differentiation of F9 Cells into Extraembryonic Endoderm

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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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ABSTRACT

Mouse F9 cells differentiate into primitive endoderm (PrE) when treated with retinoic acid (RA). During PrE differentiation the canonical Wnt signaling pathway plays an integral role in the process, along with implications of the involvement of the Hedgehog (Hh) pathway. Previous results show the Indian Hedgehog (*Ihh*) gene is upregulated during RA-induced differentiation; however details of Hh signaling during PrE differentiation remain undiscovered. A *Gli*-luciferase construct indicated that Hh signaling increases during RA-induced differentiation, implicating Hh involvement in PrE formation. Inhibiting Hh signaling impeded RA-induced differentiation, revealing that Hh is required for PrE differentiation. Despite being required, Hh signaling alone was unable to facilitate differentiation. Overexpression of *Gata6*, a master regulator of PrE, was found to increase expression of *Ihh* and *Gli* activity; furthering the involvement of the Hh pathway during development. Additionally, I found that there is signaling crosstalk between the Hh and Wnt pathways. Induced Wnt signaling was found to increase the activity of *Gli*, while inhibition of Hh impeded the ability of RA to increase Wnt signaling. Together, these results indicate that the Hh signaling pathway plays an important role in embryogenesis.

Keywords: F9, primitive endoderm, extraembryonic endoderm, differentiation,
Hedgehog, IHH, canonical WNT/ β -catenin, crosstalk, GATA6

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LIST OF ABBREVIATIONS

- APC – Adenomatous polyposis coli
- BARL - β -catenin activated reporter luciferase
- BSA – Bovine serum albumin
- Ca²⁺ - Calcium
- cAMP – Cyclic adenosine-monophosphate
- cDNA – Complimentary deoxyribonucleic acid
- ChIP – Chromatin immunoprecipitation
- CO₂ – Carbon dioxide
- db – Dibutyl
- Dhh – Desert Hedgehog
- DMEM – Dulbecco's Modified Eagle Medium
- DMSO – Dimethylsulfoxide
- DNA – Deoxyribonucleic acid
- DVL – Dishevelled
- EMT – Epithelial-to-mesenchymal transition
- ExE – Extraembryonic Endoderm
- FBS – Fetal Bovine Serum
- Fig – Figure
- GSK – Glycogen synthase kinase
- Hh – Hedgehog
- Hrs – Hours
- ICM – Inner cell mass

Ihh- Indian Hedgehog
Kb – Kilobase
LEF – Lymphoid enhance factor
LRP – Lipoprotein-related protein
 μg - Microgram
 μl - Microliter
M – Molar
ml – Milliliter
mm – Millimeter
mM – Millimolar
mRNA – Messenger ribonucleic acid
ng – Nanograms
nM – Nanomolar
PBS – Phosphate buffer saline
PCP – Planar cell polarity
PCR – Polymerase chain reaction
PE – Parietal endoderm
PKC – Protein kinase C
PrE – Primitive endoderm
PS – Penicillin-streptomycin
q – Quantitative
RA – Retinoic Acid
RL/TK – Renilla Luciferase TK

RNA – Ribonucleic acid

RT – Reverse transcriptase

SDS – Sodium dodecyl sulfate

Shh – Sonic Hedgehog

TBS-T – Tris buffered saline with 0.1% Tween 20

TCF – T-cell specific transcription factor

TE –Trophectoderm

TROMA-1 – Trophectodermal monoclonal antibody-1

VE – Visceral Endoderm

Wnt/WNT – Wingless/integrated

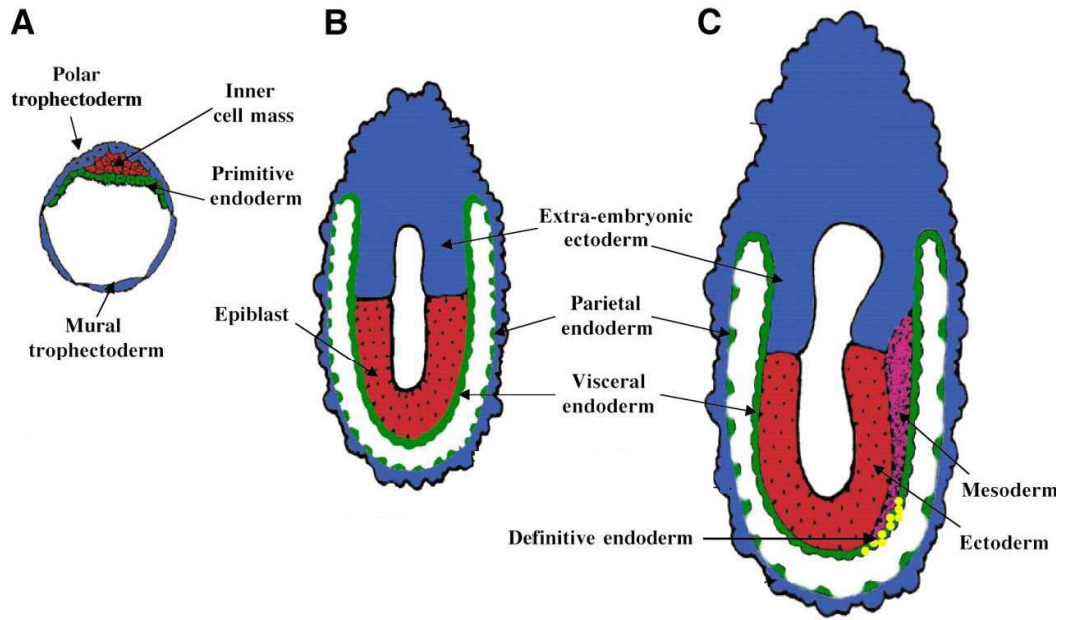
Chapter 1

Introduction

1.1 Early Mouse Development and Extraembryonic endoderm formation

The development of the mouse embryo involves a series of regulated cellular divisions of the single-celled zygote. Successive divisions lead to the formation of the morula, which undergoes compaction when there is an 8-cell to 16-cell mass of blastomeres [1]. The majority of cells of the morula begin to establish cell polarity, a key step in the first lineage differentiation as the blastocyst develops [1]. The blastocyst, in preparation for implantation, is made up of three cell types: the epithelial trophoctoderm (TE), which contributes to the formation of the placenta; the pluripotent, non-polarized cells of the inner cell mass (ICM), that are committed to becoming the embryo proper; and the cells of the primitive endoderm (PrE), which is the initial cell type in the extraembryonic endoderm (ExE) cell lineage [2]. ExE formation begins during the initial implantation phase of the blastocyst, where pluripotent cells in the ICM differentiate to become PrE [3]. PrE cells will continue to differentiate, giving rise to two cell types, parietal endoderm (PE) and visceral endoderm (VE), which give rise to the parietal and visceral yolk sacs, respectively (Figure 1.1) [4]. Both yolk sacs are essential for proper embryonic development, as they are involved in nutrient absorption as well as protection against mechanical damage to the embryo proper [5].

Figure 1.1. **Extraembryonic Endoderm Formation.** The early embryo is made up of three distinct cell types: the epiblast, the primitive endoderm and the trophoectoderm. During the time of implantation, the tissues of the extraembryonic endoderm (primitive, parietal, and visceral endoderm) are derived from embryonic stem cells present in the inner cell mass. The tissues of the extraembryonic endoderm are necessary for correct development of the growing embryo. Modified from Tam, P., and Rossant, J. 2003. *Development* **1309**(25): 6155-63.



1.2 Utilizing the F9 Teratocarcinoma Cell Line as a Model for Extraembryonic Endoderm Differentiation

In mammalian embryos, one of the earliest epithelial-to-mesenchymal transitions (EMT) results in the establishment of the ExE [6]. An EMT is a phenotypic change that takes place during tissue remodeling, wound healing, tumour invasion/metastasis and embryonic development [7]. The transition itself involves epithelial cells losing their apical-basal polarity, various cellular junctional complexes and becoming more loosely organized within the three-dimensional extracellular matrix similar to motile mesenchymal cells [8]. Studying how the ExE forms, and the associated EMTs, in a live embryo is difficult, not only due to the fact that mammalian development generally occurs *in utero*, but also because ExE differentiation occurs very early in development, when the embryo is typically no larger than 100µm. Due to these and other constraints, some investigators have turned to the F9 teratocarcinoma cell line to study ExE development. The F9 cell line was established from a teratocarcinoma that formed after investigators implanted a 6-day old male mouse embryo into the testes of a 129/Sv adult mouse [9]. When F9 cells are cultured *in vitro* they adopt a homogenous morphology, growing into clumped and compacted colonies [11]. These cells can be chemically induced to differentiate into ExE-like cell types following treatment with retinoic acid (RA) [10]. RA-induced PrE cells are further induced to differentiate into PE by subsequent treatment with dibutyryl cyclic adenosine monophosphate (db-cAMP), which activates PKA activity necessary to inhibit the MAPK signaling regulated initially by RA. These chemically-induced differentiated states of PrE and PE mimic events seen in the developing mouse embryo, thus making the F9 cell line an excellent model for

studying the development of mammalian ExE [11].

Although details regarding differentiation and investigations with the F9 cell model over the last 35 years have been exhaustive, a complete understanding as to the mechanism(s) as to how an EMT is responsible for patterning the ExE remain largely unknown. Previous work conducted in our lab has shown that the canonical Wnt signal transduction pathway plays an integral role in the process [12], and data in the present study would indicate that Hedgehog signaling is also involved.

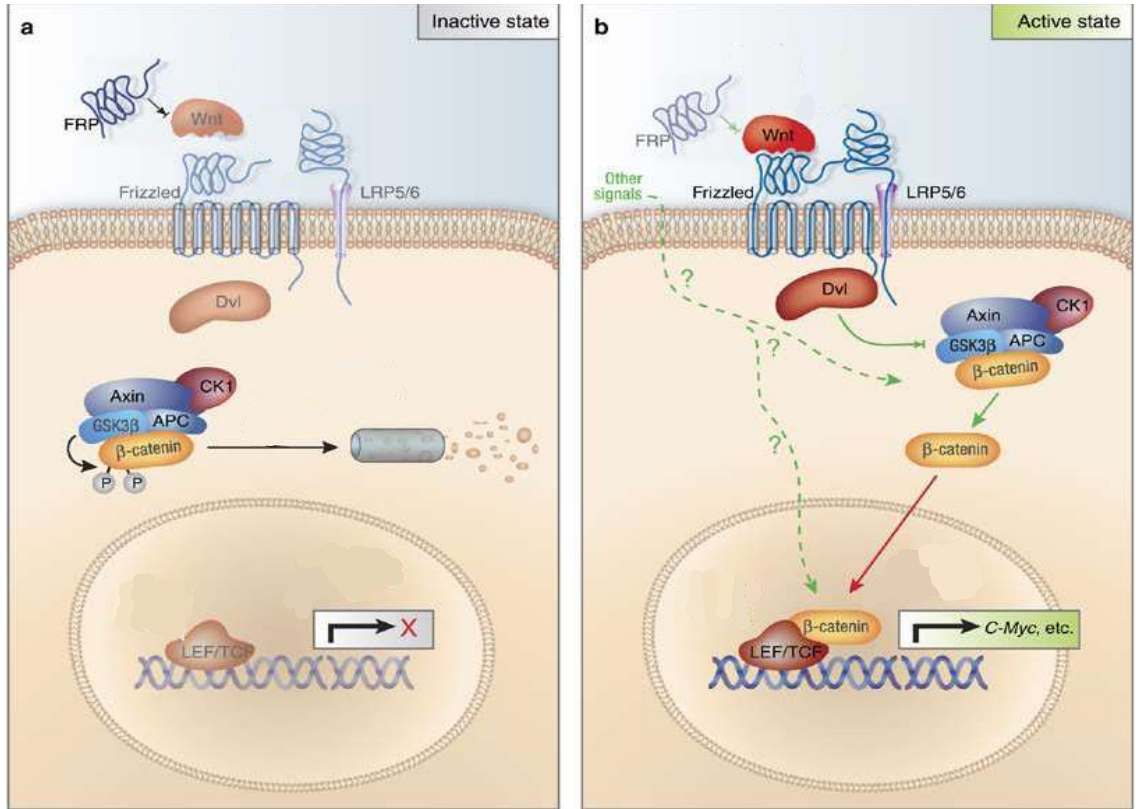
1.3 *Wnt signaling pathways*

Wnt signaling pathways play an integral role in many different vertebrate and invertebrate developmental events. Wnt signaling is able to regulate cell proliferation, cell survival, cell behavior, and cell fate decisions in both embryos and adults [13]. There are nineteen unique vertebrate *Wnt* genes that encode for specific ligands that will use members of the Frizzled family of receptors and other co-receptors to initiate cellular signaling [14]. Wnt ligands signal and activate at least three different pathways: the canonical- β -catenin, the planar cell polarity (PCP) and the Wnt-Ca²⁺-cGMP pathways [12]. In the case of the vertebrate canonical- β -catenin pathway, initiation occurs when Wnt binds to one of several Frizzled receptors and low-density lipoprotein related protein (LRP), which recruit Dishevelled and G proteins to disassemble a multimeric protein destruction complex [15]. The destruction complex is made up of five different proteins: Axin, adenomatous polyposis coli (APC) tumour suppressor protein, casein kinase 1- α (CK1), glycogen synthase kinase-3 β (GSK-3 β), and β -catenin [15]. The disassembly of this destruction complex allows for β -catenin to accumulate in the

cytoplasm and eventually translocate into the nucleus. In the nucleus, β -catenin is able to interact with the transcription factors lymphoid-enhancing factors (LEF) and T cell factors (TCF), in order to initiate the transcription of Wnt target genes [13]. In the absence of a Wnt ligand, however, the destruction complex leads to the ubiquitination and degradation of β -catenin lowering the steady state levels of β -catenin and preventing the regulation of Wnt target genes. [13] (Figure 1.2).

Signaling through the canonical β -catenin pathway is implicated in many cell fate decisions during early development [14]. In F9 cells, increased Wnt6 activity leads to the stabilization of β -catenin, which promotes the translocation of the latter to the nucleus [14]. In the nucleus and again in conjunction with TCF-LEF, β -catenin regulates the expression of target genes required for PrE formation [16]. Together, these results demonstrate the importance of the Wnt signaling pathway in PrE formation, but other pathways are involved and understanding those additional pathways was a goal of this thesis. One of the potential pathways investigated was the Hedgehog (Hh) signaling pathway.

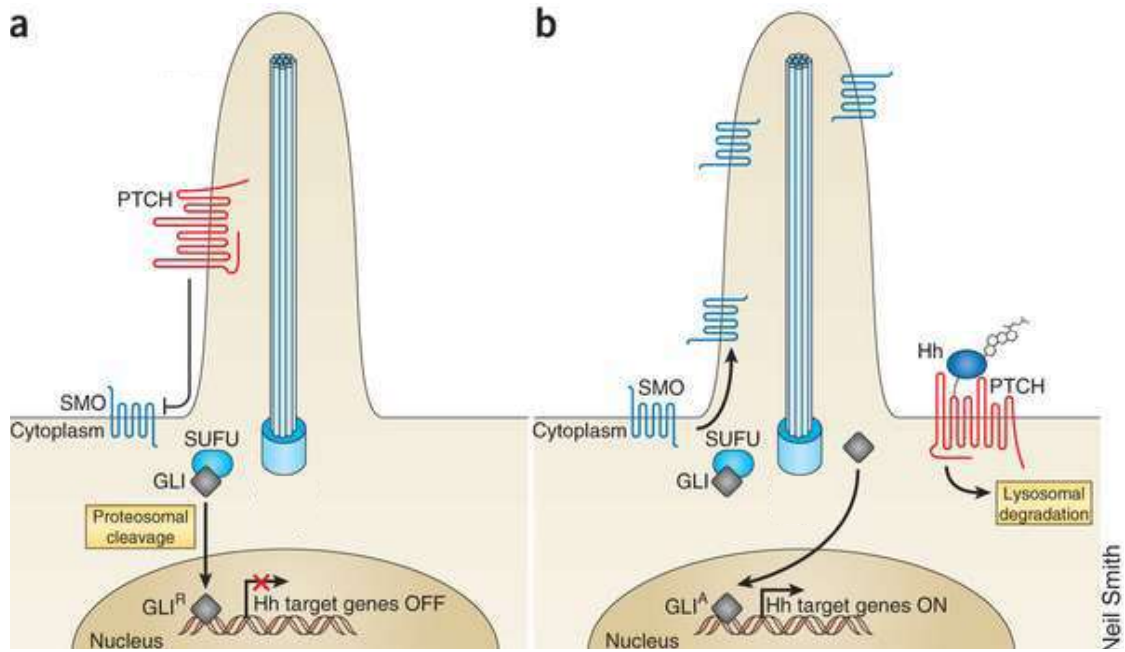
Figure 1.2. **Canonical Wnt/Beta-Catenin signaling pathway.** **(a)** In the absence of a Wnt ligand the proteins Axin, adenomatous polyposis coli (APC), casein kinase 1- α (CK1), and glycogen synthase kinase-3 β (GSK-3 β) form a destruction complex enclosing the Wnt signaling transcription factor β -catenin. The destruction complex causes the ubiquitination of the β -catenin protein, leading to its degradation in the proteasome, effectively preventing the activation of Wnt target genes. **(b)** When a Wnt ligand is present, the ligand will bind to the Frizzled receptor and the co-receptor lipoprotein-related protein 5/6. This interaction causes the recruitment and activation of the Dishevelled protein, which acts to disassemble the destruction complex. This allows β -catenin to translocate to the nucleus, where it interacts with T-cell/lymphoid enhancing factor family transcription factors to activate expression of Wnt target genes. Modified from Luo, J. et al. 2007. *Laboratory Investigation* **87**, 97-103.



1.4 *Hedgehog signaling pathway*

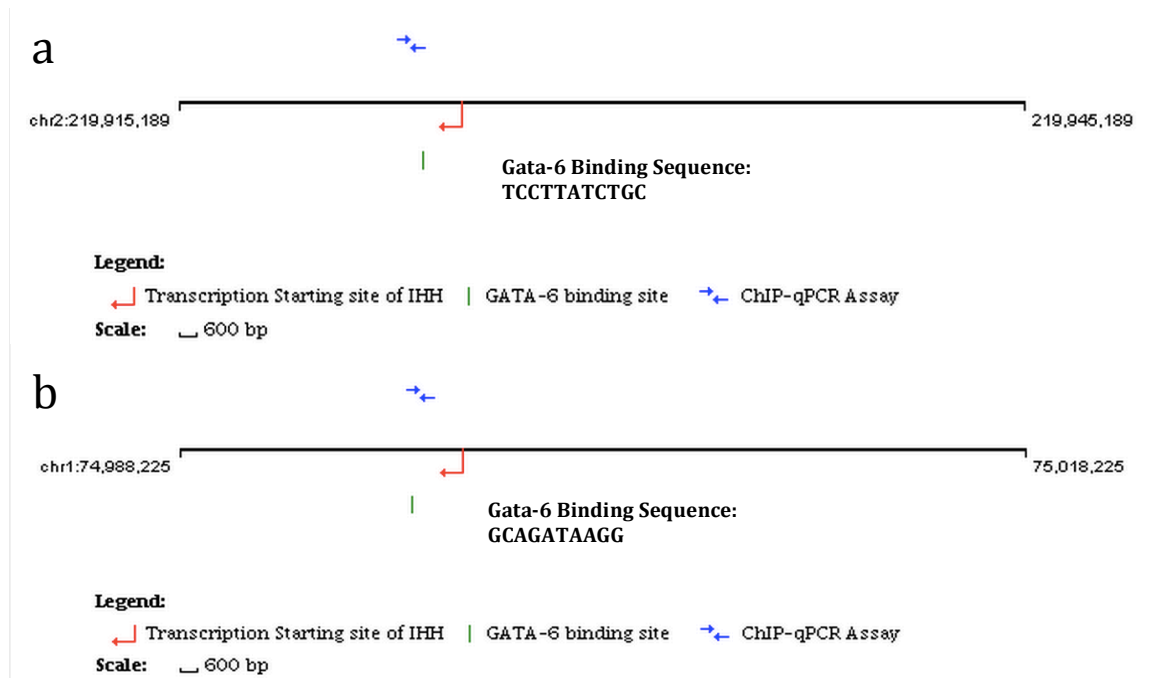
The Hh signaling pathway plays a key role in tissue and organ development in several different invertebrate and vertebrate species [17]. In mammals there are 3 Hh ligands, Sonic (Shh), Indian (Ihh) and Desert (Dhh) [18], and elucidating how these ligands perform a multitude of duties in embryogenesis is complicated by the fact that they can act as morphogens, function as mitogens, or serve as inducible factors [19]. Despite this complexity, the signaling pathway activated by Hh has been characterized extensively [20]. The pathway consists primarily of a series of repressive interactions, which start with the patched (Ptc) protein [19]. Ptc is a 12-pass transmembrane receptor that represses target gene expression when the Hh ligand is absent [20]. Ptc does this by inhibiting the function of smoothened (Smo), a 7-pass transmembrane protein [20]. Ptc inhibition in the presence of Hh, through an unknown mechanism, allows Smo to become activated [18]. Once active, Smo inhibits another negative regulator of the Hh pathway, Suppressor of Fused (SuFu), which in turn and working through the activation of three Gli transcription factors, Gli 1, 2, and 3, leads to the subsequent activation of Hh-specific target genes [18] (Figure 1.3).

Figure 1.3. Mammalian Hedgehog Signaling Pathway. (a) The Hedgehog signaling pathway is kept inactive through a series of protein repression events. With no ligand present, the Patched protein (PTCH) is active and causes the repression of the Smoothed protein (SMO). With SMO repressed the Suppressor of Fused protein keeps the GLI proteins inactive, essentially halting the transcription of Hedgehog target genes. (b) In the pathway's active form, a Hedgehog ligand binds and inactivates the PTCH protein, eventually causing its lysosomal degradation. With PTCH degraded, active SMO is able to negatively regulate SUFU, disassembling and degrading the SUFU protein. This event frees the GLI proteins from negative regulation and allows them to activate the transcription of Hedgehog target genes. Modified from Amakye, D., Jagani, Z., and Dorsch, M. 2013. *Nat Med* **19**(11): 1410-22.



In vitro studies would suggest that in the mouse embryo, Hh signaling plays a role in the differentiation events leading to ExE [21]. These studies reported that when F9 cells are treated with RA, the expression of the *Ihh* ligand increases as the cells differentiate into a PrE state [21]. This correlative evidence suggests that *Ihh* may function in a regulatory manner when F9 cells begin to differentiate into the ExE lineage. Furthermore, through the use of *in silico* analysis, our lab discovered that the promoter region for the *Ihh* gene has a putative binding site for GATA6 (Figure 1.4) (unpublished observation), a major transcriptional regulator of embryonic and extraembryonic endoderm [22]. *Gata6* is up-regulated during RA-induced differentiation of F9 cells and it is known to regulate the expression of *Wnt6* [12]. Thus, like *Wnt6*, *Ihh* signaling is linked to PrE formation, but whether or not the two pathways are linked through signaling crosstalk remains to be determined. Thus, elucidating how Hh signals during PrE formation may provide a better understanding of the already complex network that regulates ExE differentiation.

Figure 1.4. Sequence Identity Gata6 binding sites in human and mouse *Ihh* promoters. The *Ihh* promoter sequences of human (a) and mouse (b) were analyzed using the QIAGEN Champion ChIP Transcription Factor Search portal based on SABiosciences' database DECODE. The green line marks Gata6 binding sites and binding sequences are stated.



1.5 *Wnt and Hedgehog Signaling Crosstalk*

Crosstalk within cell signaling cascades allows for greater regulation, maintenance, and control of specific developmental events. The Hh and Wnt signaling pathways have been both found to play key individual roles in embryonic development [23]. However, the potential for crosstalk between these two major pathways during development has yet to be thoroughly investigated, especially during ExE differentiation. Evidence for signaling crosstalk between the Hh and Wnt pathways is known in the developing neural tube [24], where the inhibition of Hh signaling leads to a reduction in Wnt-mediated transcriptional activation [24]. When the Hh pathway is inactive, the Gli3 protein, a transcriptional regulator, takes on its repressor form, Gli3R which is an amino-terminal fragment of the full-length Gli3 protein; and this leads to the repression of Hh-regulated target genes [25]. Gli3R specifically causes this reduction in Wnt signaling by physically interacting with the carboxy-terminal domain of β -catenin, which directly antagonizes the active form of β -catenin [24]. Thus, Wnt-target genes cannot be activated under these conditions.

Signaling crosstalk between the Wnt and Hh pathways also has a major role in cancer metastasis involving EMTs. In hepatocellular carcinomas (HCC), cellular metastasis hinges on the joint activation of both the Wnt and Hh signaling pathways [26]. More specifically, an EMT driven by aberrant TGF β signaling leads to the activation of the Hh pathway via Shh. In this case, activation leads to increased Gli activity, which in turn activates the Wnt- β -catenin pathway [26]. Once active, feedback from the Wnt pathway signals back to the Hh pathway through TCF/LEF, establishing crosstalk activity that leads to the stabilization of active Gli [26]. Through the activation of both pathways,

HCC cell lines then undergo EMT, producing cells exhibiting mesenchymal-like characteristics [26]. This ability of interconnected and interdependent signaling between two or more pathways, found to promote and stabilize EMT in HCC cells, is just another example lending precedence that a similar form of crosstalk may influence the EMT in ExE formation.

1.6 Objectives of Study and Hypothesis

One goal of this study was to determine if the Hh signaling pathway is necessary and/or sufficient to induce ExE differentiation. Another goal was to determine if this signaling impacts on the canonical Wnt/ β -catenin pathway that is obligatory for PrE formation. Given the *in silico* analysis of the *Ihh* promoter region (Figure 1.4), implying that the gene may be regulated by GATA6, a master regulator of ExE formation, and the fact that the *Ihh* gene is up-regulated in response to RA [21], I hypothesize that the Hh signaling pathway is necessary for the formation of the ExE, particularly when transitioning to the PrE state. Experiments were also designed to test whether or not activating the Hh pathway was sufficient to induce ExE. Finally, with the preexisting information that Wnt signaling plays a major role in ExE formation [16] and is able to interact with several components of the Hh pathway [24], I hypothesize that both the Wnt and Hh signaling pathways act in a network to regulate and influence the formation of the ExE.

Chapter 2

Materials and Methods

2.1 Cell Culture, Transfection, and Treatment

Mouse F9 teratocarcinoma cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) enriched with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin antibiotic (PS; Lonza). The cells were seeded in tissue culture (TC) treated 60mm plates (BD Falcon) for subsequent protein isolation. When cells were approximately 40% confluent they were treated with either 10^{-7} M retinoic acid (RA; Sigma) or with 2.5-10 μ M Cyclopamine (Cyc; EMD Millipore) overnight and then subsequently treated with 10^{-7} M RA. Control and treated cells were incubated at 37°C and 5% CO₂ for 4 days.

Cyc is a plant-derived steroidal alkaloid that binds directly and specifically to the smoothed protein in the Hh signaling pathway, and inhibits its function; effectively inhibiting all Hh signaling in cells treated with the chemical [28].

Cells were transfected with empty vector, pcDNA3.1-*Gata6*, pcDNA-*Foxa2*, pGL3-*BARL*, pGL3-*Gli*, and pRL-*TK* constructs, respectively, using Lipofectamine2000 according to the manufacturer's recommendations (ThermoFisher Scientific). Briefly, 10 μ L of Lipofectamine2000 was mixed with a total of 4 μ g of expression constructs to transfect cells grown to 60% confluence in 35mm tissue culture treated plates (BD Falcon); for co-transfection experiments, equal amounts of each construct were used with Lipofectamine2000 to DNA ratio of 10:4. Transfected cells were then passaged 24hr later

to 60mm TC dishes. Cells were treated with 0.05% Dimethyl sulfoxide (DMSO) (vehicle control), 10^{-7} M RA, 7.5 μ M Cyc, 5nM-0.5 μ M 6-Bromoindirubin-3'-oxime (BIO, Sigma-Aldrich), 5nM-10nM Smoothened Agonist (SAG, EMD Millipore), 7.5 μ M Cyc and 10^{-7} M RA, or 5nM-0.5 μ M BIO and 10^{-7} M RA. All cells were incubated at 37°C and 5% CO₂.

SAG is a synthetic small molecule activator that binds directly to the smoothened protein and causes its activation, thereby activating the Hh signaling pathway within cells [36].

BIO is a pharmacological inhibitor that specifically inactivates GSK3 protein activity, and in doing so facilitates efficient Wnt pathway activation within treated cells [33].

2.2 Reverse-Transcription PCR

Oligodeoxynucleotide primers were designed to the mouse *Shh* (Accession # NM009170), *Dhh* (Accession # NM007857), *Ihh* (Accession # NM010544), *Gata6* (Accession # AK142381) and *Foxa2* (Accession # AL845297) nucleotide sequences. *Shh* sense (5' CCA CTG TTC TGT GAA AGC AGA G) and antisense (5' CAG CGT CTC GAT CAC GTA GAA G), *Dhh* sense (5' AGC CGG ATT CGA CTG GGT CTA C) and antisense (5' GGT CCA GGA AGA GCA GCA CTG), *Ihh* sense (5' TAT CAC CAC CTC AGA CCG TGA C) and antisense (5' ACC CGG TCT CCT GGC TTT ACA G), *Gata6* sense (5' CTC TGC ACG CTT TCC CTA CT) and antisense (5' GTA GGT CGG GTG ATG GTG AT), and *Foxa2* sense (5' ACC TGA GTC CGA TGA GC) and antisense (5' CAT GGT GAT GAG CGA GAT GT) primers were designed to amplify

Shh, *Dhh*, *Ihh*, *Gata6*, and *Foxa2* cDNAs. RNA was isolated from treated or transfected F9 cells and converted into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR was conducted with the cDNAs as templates under the following reaction conditions: *Shh*, *Dhh*, and *Ihh* – 35 cycles of 30s at 94°C, 30s at 57°C, and 30s at 72°C. *Gata6* – 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30s at 72°C; *Foxa2* – 35 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C. Primers to constitutively expressed ribosomal gene *L14*, sense (5' GGG AGA GGT GGC CTC GGA CGC) and antisense (5' GGC TGG CTT CAC TCA AAG GCC) were used as controls. PCR amplified samples were run on 1% agarose gels containing RedSafe (FroggaBio) nucleic acid staining solution and visualized using the FluorChem 8900 gel imaging station (Alpha Innotech).

2.3 Immunoblot Analysis

Cells were lysed in 300µL of 1% sodium dodecyl sulfate (SDS) buffer containing 62.5mM Tris-HCL pH 6.8, 10% glycerol, 5% Mercapto-2-ethanol, and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentrations of lysates were measured using a Bradford assay (Bio-Rad) and equivalent amounts were separated on denaturing 10% polyacrylamide gels and transferred to nitrocellulose membranes (Biotrace; Pall Corp.). Membranes were blocked in 5% skim milk and then probed with primary antibodies directed against TROMA-1 (1:50; Developmental Studies Hybridoma Bank), and β -actin (1:10,000; Pierce). After extensive washes, membranes were probed with the appropriate secondary antibodies, washed and then signals detected using the SuperSignal West Pico Chemiluminescent Kit (Pierce).

2.4 TCF/LEF and Gli Reporter Assay

Cells transfected with either pGL3-*BARL* or pGL3-*Gli* and then treated with 0.05% DMSO (vehicle control), 10^{-7} M RA, 7.5 μ M Cyc, 5nM-0.5 μ M BIO or 5nM-10nM SAG, or 7.5 μ M Cyc and 10^{-7} M RA, or 5nM-0.5 μ M BIO and 10^{-7} M RA were prepared 48hr after treatment using the Dual Luciferase Assay Kit as per manufacturer's instructions (Promega). Cells transfected with either pGL3-*BARL* or pGL3-*Gli* and then co-transfected with pcDNA3.1 (empty vector control), pcDNA3.1-*Gata6*, or pcDNA3.1-*Foxa2* were also prepared 48hr post-transfection using the Dual Luciferase Assay Kit. Luciferase expression was quantified using the GloMax Multi Detection System (Promega). Cells were also co-transfected with pRL-*TK* to normalize luciferase levels.

2.5 Statistical Analysis

Data from all experiments are representative of at least three independent biological replicates performed on separate occasions. Data comparisons between the control and treated groups were performed using a Student's t-Test assuming unequal variances. P values were one-sided and considered statistically significant at the 0.05 value.

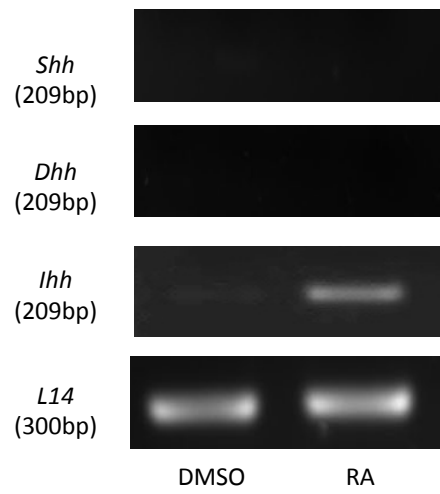
CHAPTER 3

RESULTS

3.1 Hedgehog gene expression during RA-induced Primitive Endoderm Differentiation

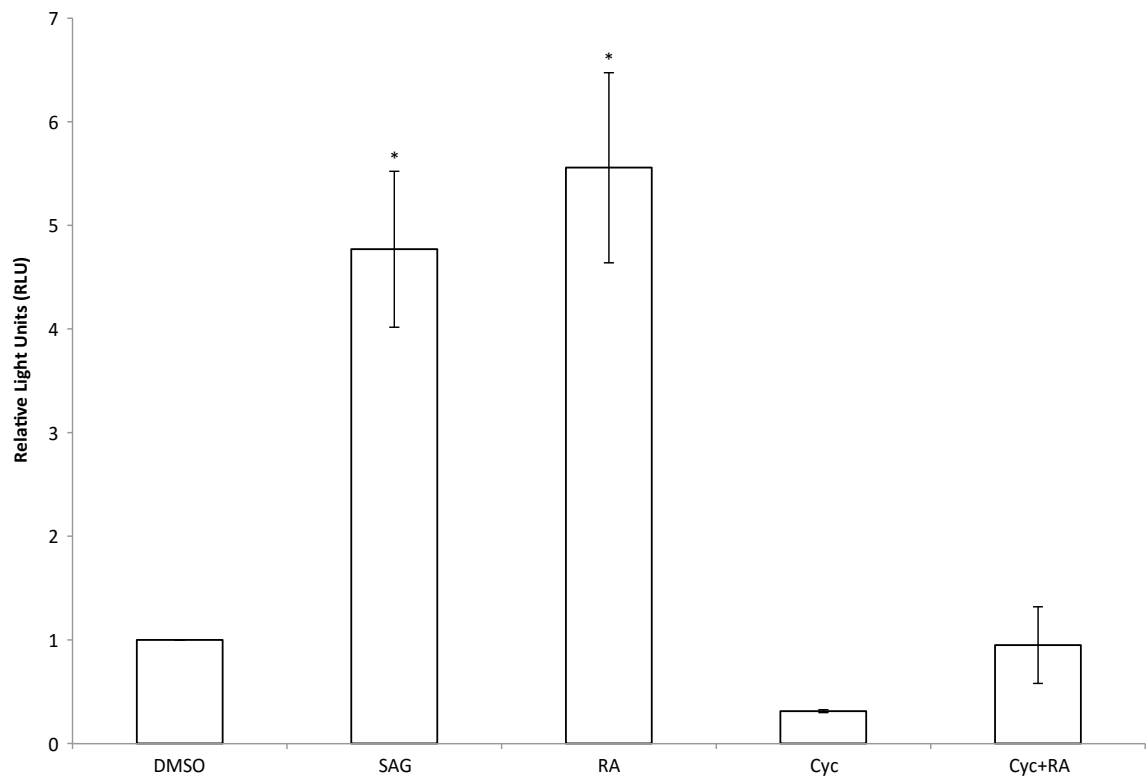
Mouse F9 teratocarcinoma cells treated with RA differentiate into PrE [27], and this is accompanied by an increase in the expression of the Indian Hedgehog (*Ihh*) gene [21]. For my study, the expression profiles of all three Hh genes, Indian (*Ihh*), Sonic (*Shh*), and Desert Hedgehog (*Dhh*) were examined during PrE induction. Towards that end, total RNA was collected from cells treated with dimethyl sulfoxide (DMSO) (vehicle control) or with RA (10^{-7} M). RNA was reverse transcribed into first strand cDNA and used for PCR amplification using primers specific for each *Hh* gene (Fig. 3.1). No Hh amplicons were present in the DMSO treated controls. Transcript signals were detected using the *Ihh* primer set, confirming previous results [21] (Fig. 3.1). Expression of *Shh* and *Dhh*, was not detected in either DMSO-treated or RA-treated cells. Transcript signals corresponding to the amplification of the constitutively expressed *L14* ribosomal gene were seen at equal levels within all samples (Fig. 3.1). These results would indicate that *Ihh* is the only *Hh* gene that is expressed in response to RA addition and strongly supports the idea that *Ihh* is the ligand involved in the differentiation of PrE.

Figure 3.1 ***Ihh* mRNA is upregulated in response to Retinoic Acid induced PrE differentiation.** Total RNA was extracted from F9 cells treated with retinoic acid (RA) to induce primitive endoderm. An amplicon corresponding to *Ihh* was only seen in cells treated with RA. Primers designed to the constitutively expressed ribosomal *L14* (300BP) gene amplified a product in the control and treated cells. Data are representative of three independent experiments.



After confirming that *Ihh* expression is up regulated in response to RA during the differentiation of PrE, I tested whether this increase in gene activity resulted in the subsequent activation of a Hh signaling cascade. To investigate this, changes in Gli-mediated transcriptional activity were examined in a reporter assay. A pGL3-*Gli* luciferase reporter and a *Renilla* luciferase construct (pRL-*TK*) were co-transfected into F9 cells and then subsequently treated with DMSO (vehicle control) or RA. Treatment of RA led to a significant 5.56-fold increase in luciferase activity relative to the controls (DMSO) (Fig. 3.2). Cells were also treated with SAG, a smoothened-agonist that would activate Hh signaling [28], and function as a positive control for the *Gli* luciferase reporter. Treatments with SAG led to a 4.47-fold increase in luciferase activity, an increase that was significantly different from the DMSO control (Fig 3.2). Experiments were repeated with Cyc to block Hh signaling and ensure that this RA-induced increase in Gli activity was due to activation of the Hh pathway itself. Cyc binds to and inhibits the heptahelical Smo protein thereby blocking the Hh pathway [28]. Treating F9 cells with Cyc, followed by RA resulted in only a 0.95-fold increase in luciferase activity relative to controls cells [Fig. 3.2]. That Cyc was able to attenuate the RA-induced increase in Gli reporter activity would indicate that RA activates the Hh pathway in cells destined to form PrE. Together these results provide evidence for *Ihh* and the Hh pathway in the RA induction of extraembryonic endoderm.

Figure 3.2 Retinoic acid increases Gli-mediated transcriptional activity through canonical Hedgehog signaling. Lysates collected from F9 cells transfected with pGL3-*Gli* and subsequently treated with DMSO, Smoothed Agonist (SAG), retinoic acid (RA), Cyclopamine (Cyc), or RA and Cyc, were collected 48 hours after treatment. Cells treated with RA had a 5.56-fold increase ($P = 0.038$) in luciferase activity relative to the control (DMSO-treated cells). Cells treated with SAG had a 4.47-fold increase ($P = 0.038$) in luciferase activity, again a value significantly different from the DMSO control. F9 cells treated with Cyc had a 0.31-fold increase in luciferase activity relative to the control, while those treated with RA and Cyc had a 0.95-fold increase in luciferase activity relative to the control. Data are representative of three independent experiments. Bars represent mean fold changes in relative light units (RLU) \pm S.E., normalized against *Renilla* luciferase activity. * = $P < 0.05$.

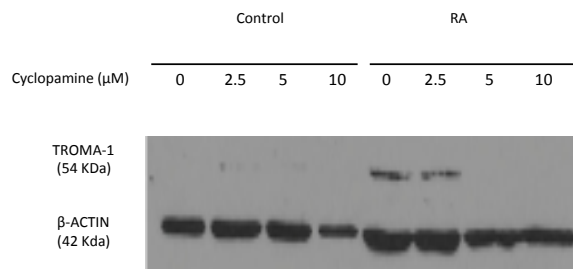


3.2 Hedgehog signaling is necessary, but not sufficient for Primitive Endoderm Differentiation

That *Ihh* expression and Gli activity increased during RA-induced PrE formation, and that activity was attenuated by Cyc indicated the involvement of the Hh pathway in PrE formation, but more importantly it intimated that this pathway as being necessary for differentiation. To confirm the necessity of a functioning Hh pathway during differentiation, cells were treated with RA, RA and Cyc, and DMSO (control), and then protein was isolated and processed for immunoblot analysis using the TROMA-1 antibody to detect cytokeratin 8, an intermediate filament protein expressed in ExE [29]. While immunoblot analysis with the TROMA-1 antibody revealed a protein signal in cells treated with RA, no signals were seen in cells treated with Cyc and RA (Fig. 3.3). Thus, the data would suggest that inhibiting Hh signaling had blocked the ability of RA to induce F9 cells to differentiate, support the hypothesis that active Hh signaling is necessary for PrE differentiation.

Since the data pointed to active Hh signaling being required during the induction of PrE, the next question was to determine if the pathway was sufficient to induce F9 cells to differentiation into the ExE lineage. Previous reports from our lab [14, 16] and others [27, 30] have demonstrated the role of canonical Wnt/b-catenin in this process, but no evidence exists that it also requires active Hh signaling. To test for sufficiency, F9 cells were treated with the Smo agonist, SAG, at either 5nM or 10nM and protein lysates collected for immunoblot analysis to detect the TROMA-1 marker.

Figure 3.3 **Hedgehog signaling is required for RA-induced primitive endoderm differentiation.** Protein lysates were collected from F9 cells treated with cyclopamine or retinoic acid (RA) and Cyclopamine, and then processed for immunoblot analysis with antibodies to TROMA-1. An anti- β -actin antibody was used to ensure equal loading. TROMA-1 signals were not detected in cells treated with Cyc alone; however, they were seen in the RA positive control and in cells treated with RA and 2.5 μ M Cyc. Higher concentrations of Cyc blocked the TROMA-1 signal despite the cells having been treated with RA. Data are representative of three independent experiments.

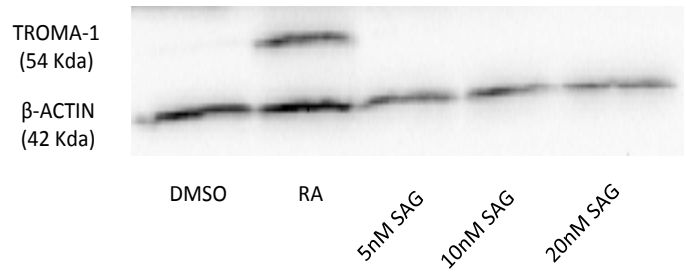


Unexpectedly, however, and unlike the RA positive control, SAG was unable to produce TROMA-1 signals (Fig. 3.4). This was not because SAG had lost its activity because subsequent experiments found that, at the concentrations used, it activated Gli-mediated transcription (Fig. 3.2). Thus, the data would suggest that the activation of Hh signaling, by itself, is not sufficient to induce embryonic cells to differentiate to PrE.

3.3 Overexpression of *Gata6* upregulates Indian Hedgehog and activates Hh Signaling

Gata6 and *Foxa2* encode transcription factors that are master regulators of embryonic and extraembryonic endoderm differentiation [22, 31, 32]. Previous work from our lab has shown that *Gata6* is a direct target of RA signaling and *Foxa2* is up-regulated in response to increased GATA6 [12]. Furthermore, the overexpression of each gene in F9 cells is sufficient to induce PrE via Wnt6 activation of the canonical Wnt-b-catenin pathway [12]. Considering the involvement of GATA6 and FOXA2 in PrE formation, and the identification of a putative GATA6 binding site in the *Ihh* promoter (this study, Fig. 1.4), it was hypothesized that the overexpression of *Gata6* and *Foxa2* would increase *Ihh* expression. In order to investigate the hypothesis that *Gata6* and *Foxa2* overexpression increases *Ihh* expression, F9 cells were transfected with pcDNA3.1 (empty vector control), pcDNA3.1-*Gata6*, or pcDNA3.1-*Foxa2*, and 48hrs later total mRNA was collected and reverse transcribed into cDNA for PCR analysis with *L14* and *Ihh* primers. Results showed that *Ihh* expression was increased in cells transfected with the pcDNA3.1-*Gata6* overexpression vector, while cells transfected with either the empty

Figure 3.4 **Hedgehog signaling is unable to induce primitive endoderm differentiation.** F9 cells were treated with DMSO (vehicle control), retinoic acid (RA), or smoothened agonist (SAG), and protein lysates were collected for immunoblot analysis. Antibodies against TROMA-1 and β -actin were used to detect differentiation of F9 cells to a primitive endoderm state, and as a loading control, respectively. TROMA-1 signals were only detected in cells treated with RA. Data are representative of three independent experiments.



vector or pcDNA3.1-*Foxa2* vector had no detectable changes in *Ihh* expression (Fig. 3.5; qRT-PCR completed by T.N. Cuthbert). These results, although indicating that GATA6 signaling is able to directly or indirectly regulate the expression of *Ihh*, do not address whether this increase in expression translates into activation of the Hh pathway. To test this, F9 cells were co-transfected with pcDNA3.1 (empty vector control), or pcDNA3.1-*Gata6*, and the *Gli* and pRL-*TK* luciferase reporter construct described above. Despite *Ihh* not being induced by the *Foxa2* construct, cells were also transfected with the reporter constructs and pcDNA3.1-*Foxa2* to determine if the Hh pathway could be activated downstream of the ligand. Lysates were collected 48hrs post transfection and the luciferase activity was analyzed and compared between treatments. Results showed that the pcDNA3.1-*Gata6* plasmid caused a 2.29-fold change in the *Gli* reporter activity, which was significantly different when compared to the change seen in the empty vector control. Transcription of the pcDNA3.1-*Foxa2* vector caused a no significant change in *Gli* luciferase activity (Fig. 3.6). Together, these results show that increased *Gata6* expression, and not *Foxa2* expression, serves to up-regulate the *Ihh* gene, and thus results in the increase in Gli-dependent transcription.

Figure 3.5 **Overexpression of *Gata6* up-regulates *Ihh* expression.** F9 cells were transfected with empty vector (control), pcDNA3.1-*Gata6*, or pcDNA3.1-*Foxa2*. Total RNA was collected 48hrs later and analyzed by PCR using primers designed to amplify *Ihh* cDNA. Amplicons (209bp) were only detected in cells transfected with pcDNA3.1-*Gata6*. Primers designed to the *L14* gene (300bp) were utilized in the PCR to ensure equal loading between treatments.

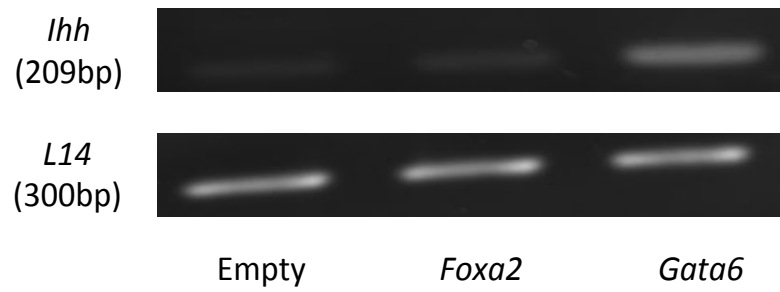
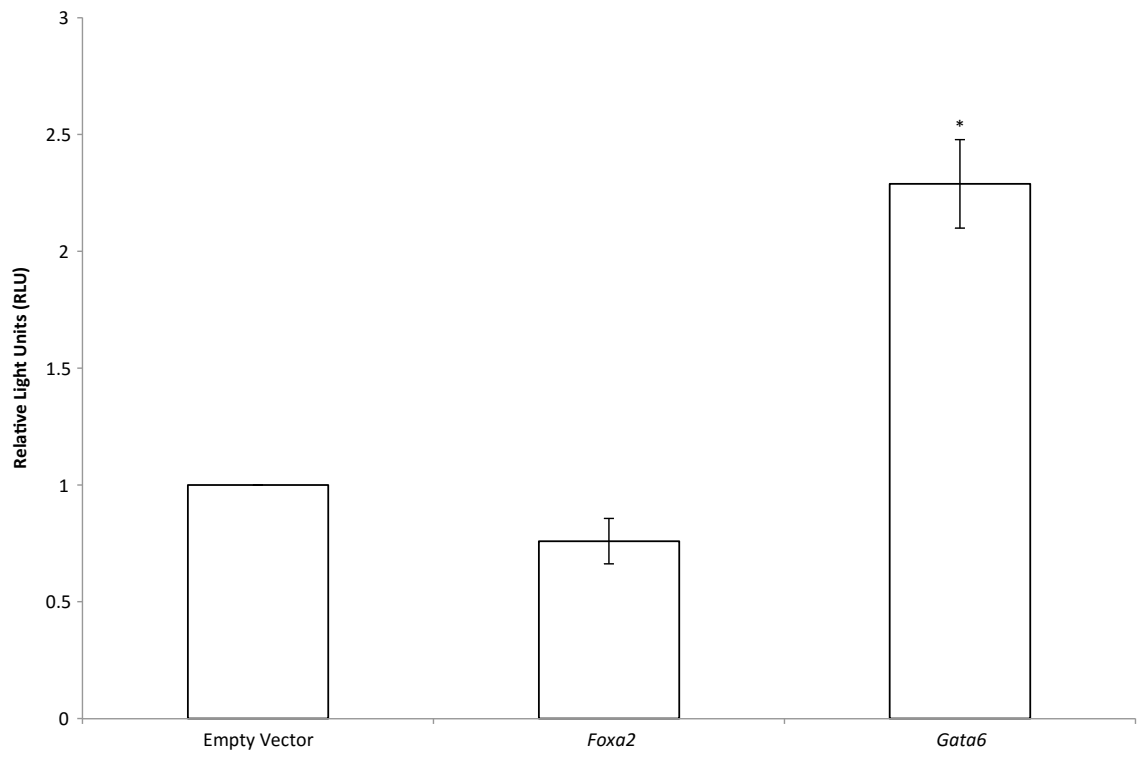


Figure 3.6 ***Gata6*, but not *Foxa2*, overexpression alters *Gli* Reporter activity.** F9 cells co-transfected with pGL3-*Gli* and either the empty vector control, pcDNA3.1-*Foxa2* or pcDNA3.1-*Gata6* were collected 48hrs post transfection and lysates processed to measure *Gli*-dependent luciferase activity. Cells transfected with the *Foxa2* vector showed a 0.76-fold increase in activity relative to the control (empty vector), which was not statistically significant from the empty vector control. *Gata6* overexpression, however, produced a 2.29-fold increase ($P = 0.013$) in luciferase activity that was significantly different from that seen in the empty vector control. Data are representative of three independent experiments. Bars represent mean fold changes in relative light units (RLU) \pm S.E., normalized against *Renilla* luciferase activity. * = $P < 0.05$.



3.4 *Wnt Signaling Impacts the Hedgehog Pathway during Extraembryonic Endoderm Differentiation*

Hh, like Wnt signaling, is involved in PrE differentiation and messages encoding both ligands in their respective signaling pathways are up-regulated in response to *Gata6* over-expression [this study; 12]. Given these relationships, we next wanted to examine if the pathways were working independent of each other, or if there was crosstalk between them. Previous work has established that both pathways form integrative signaling webs, however, the specifics of signaling crosstalk is poorly understood [24, 26]. To address the possible crosstalk between the two pathways, cells were transfected with either pGL3-*Gli* or pGL3-*BARL* reporter constructs, readouts indicative of active Hh and Wnt signaling, respectively, and with a *Renilla* luciferase construct to normalize the luciferase data. To determine if the Hh pathway was able to signal to the Wnt pathway, F9 cells were transfected with the pGL3-*BARL* construct and treated with DMSO (vehicle control), RA, or with 5nM or 10nM SAG. Cells were also treated with BIO, a GSK-3 β inhibitor [33], which acted as a positive control for the TCF/LEF reporter construct. Results indicated that the 5nM and 10nM concentration of SAG only caused no significant increase in *BARL* activity compared to the DMSO vehicle control (Fig. 3.7). Interestingly, results showed that when F9 cells were treated with Cyc and then treated subsequently with RA, there was no significant increase in luciferase activity compared to control treatments (Fig. 3.8), in contrast to the significant increase in reporter activity caused by the RA treatment alone (Fig. 3.8).

Figure 3.7 Activation of the Hedgehog pathway does not affect TCF/LEF transcriptional activity. F9 cells were transfected with pGL3-*BARL* and then treated with either DMSO, BIO, retinoic acid (RA), or 5nM or 10nM SAG, and lysates collected 48hrs later to measure luciferase activity. Treatment with BIO caused a significant 8.05-fold increase ($P = 0.002$) in luciferase activity relative to DMSO treated cells. As expected, RA caused a significant increase (8.62-fold ($P = 0.014$)) in luciferase activity compared to the control (DMSO). In contrast, neither SAG treatment affected the TCF/LEF reporter, having only caused 1.23 and 1.20-fold, respectively, increases relative to the control. Data are representative of three independent experiments. Bars represent mean fold changes in relative light units (RLU) \pm S.E., normalized against *Renilla* luciferase activity. * = $P < 0.05$.

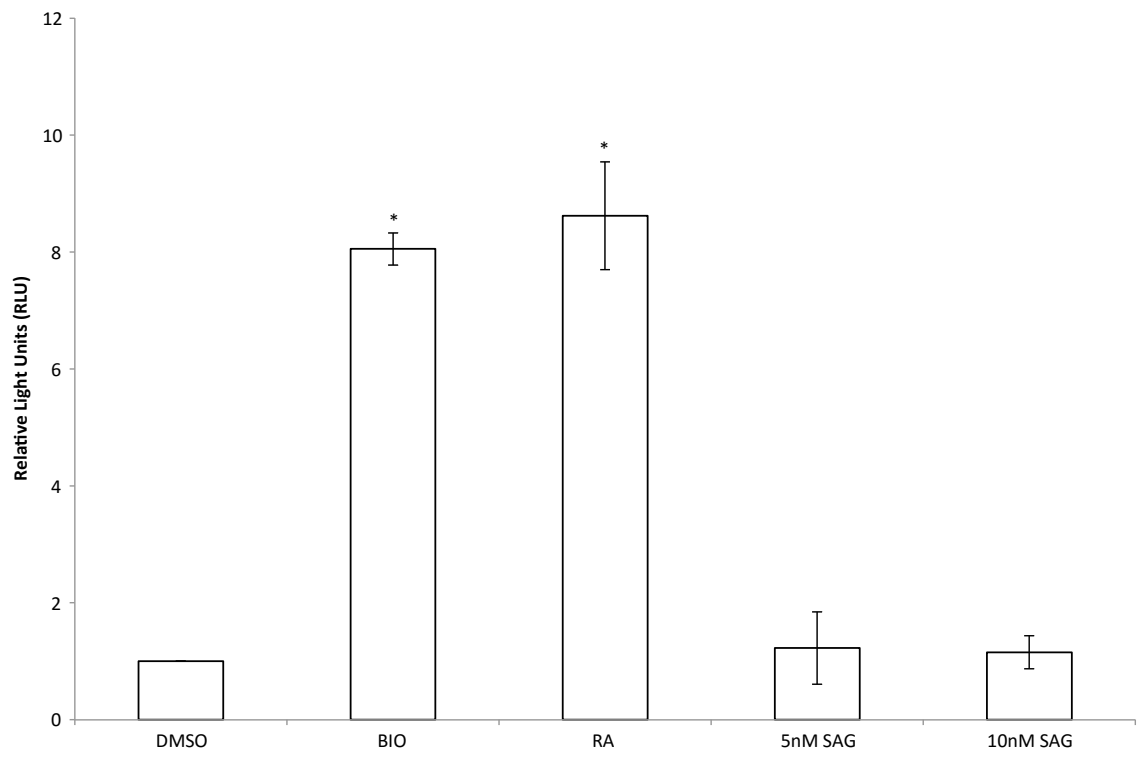
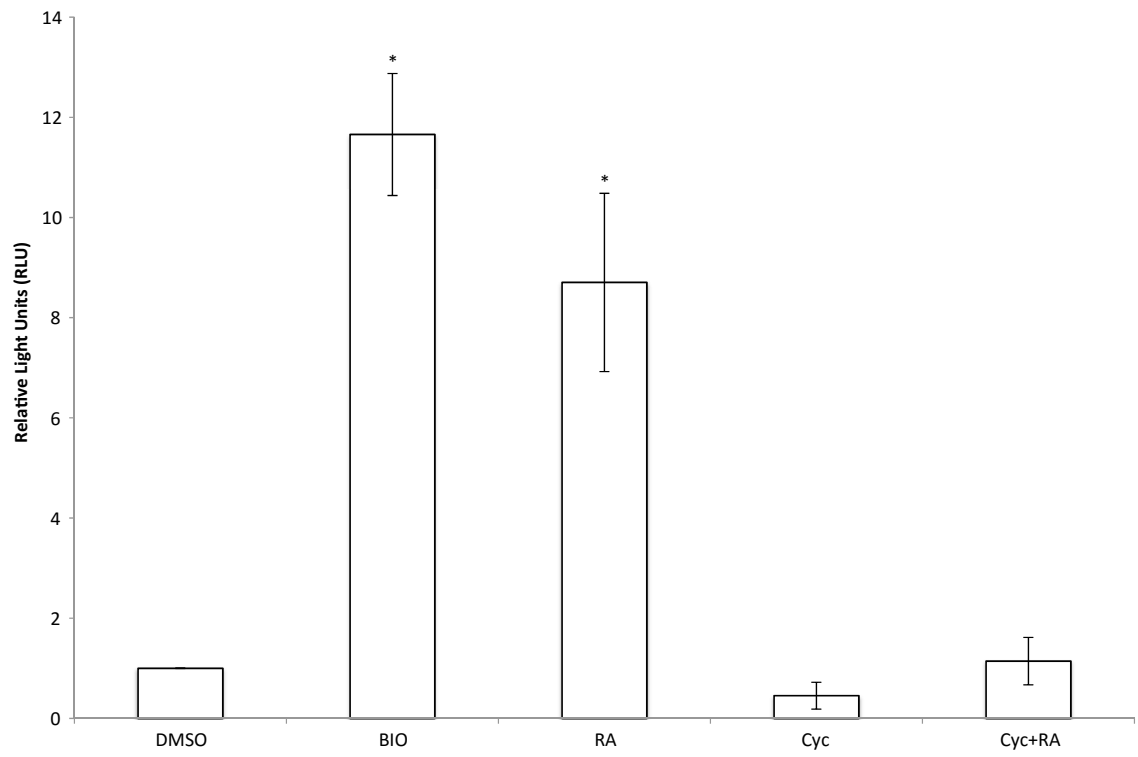


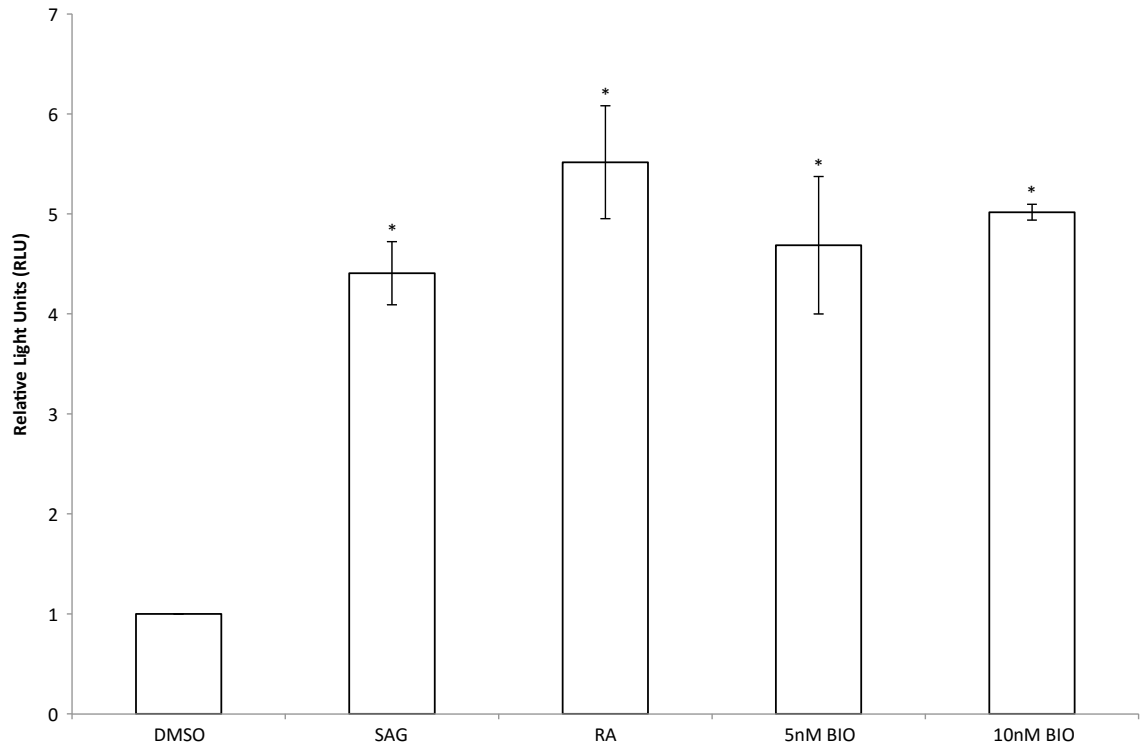
Figure 3.8 Inhibiting the Hedgehog pathway prevents the RA-dependent increase in TCF/LEF transcriptional activity. F9 cells were transfected with pGL3-*BARL* and then treated with DMSO, BIO, retinoic acid (RA), Cyclopamine (Cyc), or RA and Cyc, and lysates collected 48hrs post treatment to measure luciferase activity. Treatment with BIO induced a 11.66-fold increase ($P = 0.013$) in luciferase activity, whereas RA treatment caused a 8.7-fold increase ($P = 0.049$), both of which were significantly different compared to the control (DMSO). Cyc treatment had little effect on TCF/LEF activity, causing only a 0.45-fold increase. Interestingly, when cells were treated with Cyc and RA, there was only a 1.14-fold increase in reporter activity. Data are representative of three independent experiments. Bars represent mean fold changes in relative light units (RLU) \pm S.E., normalized against *Renilla* luciferase activity. * = $P < 0.05$.



These results would suggest that while active Hh signaling alone did not increase TCF/LEF transcriptional activity, Smoothed must not be inhibited in order for RA to increase this activity.

Converse experiments tested whether or not the inhibition of GSK3, which stems from the activation of the canonical Wnt-b-catenin pathway, had any effect on the canonical Hh pathway. F9 cells were transfected with the pGL3-*Gli* reporter construct and then treated with DMSO (vehicle control), SAG (positive control), RA, or with 5nM BIO or 10nM BIO, a GSK-3 β inhibitor [33]. SAG treatment caused no significant increase in *Gli* transcription activity (Fig. 3.9). Interestingly, the 5nM and 10nM BIO treatments caused a significant increase in *Gli*-dependent activity, compared to the DMSO vehicle control (Fig. 3.9). Together, these results indicate that while activating the Hh pathway has no detectable effect on b-catenin-dependent transcription, inhibiting GSK3b, which normally accompanies the activation of the canonical Wnt pathway, impacts positively on the activity of *Gli*-mediated transcription.

Figure 3.9 Inhibition of GSK3 activity increases *Gli*-mediated transcription. F9 cells were co-transfected with pGL3-*Gli* and *Renilla* luciferase vectors. After transfection, cells were treated with either DMSO, Smoothened Agonist (SAG), retinoic acid (RA), 5nM or 10nM of BIO and lysates collected 48hrs later. RA treatment caused a 5.52-fold increase ($P = 0.015$) relative to the control (DMSO). Treatment with SAG also caused a significant increase (4.41-fold ($P = 0.008$)) in luciferase activity compared to DMSO treated cells. Interestingly, 5nM and 10nM BIO caused a 4.69-fold ($P = 0.033$) and 5.02-fold ($P = 0.0001$) increase, respectively, compared to the control. Results from the RA, SAG, and BIO treatments were significantly different from the control samples. Data are representative of three independent experiments. Bars represent mean fold changes in relative light units (RLU) \pm S.E., normalized against *Renilla* luciferase activity. * = $P < 0.05$.



Chapter 4

Discussion

In the mouse embryo several signaling pathways play major roles in regulating different developmental processes and events; one of those pathways involves the Hedgehog morphogen [17]. Hedgehog (Hh) signaling plays a regulatory role in the formation of early developmental structures such as the notochord, neural tube, lung bud, hindgut, and limb buds [34]. Despite the many studies documenting these events, little is known of the involvement Hh has in extraembryonic endoderm (ExE) differentiation. My study was designed towards better understanding the role of Hh signaling during these events of ExE differentiation. By utilizing the F9 teratocarcinoma cell line, which models ExE differentiation to primitive endoderm (PrE) through chemical treatment with retinoic acid (RA), my study extended from those of previous studies showing Indian Hedgehog involvement in PrE formation [21] (Fig. 3.1). That no detectable changes in the expression of the other two *Hh* genes encoding the Sonic and Desert ligands, would indicate that *Ihh* has the potential to be involved in ExE differentiation (Fig. 3.1). To further solidify the importance of these results, a fellow student within the Kelly lab, Nicole Cuthbert, conducted a real-time quantitative PCR analysis of the expression patterns of each Hh ligand. Her results showed that *Ihh* expression significantly increased in F9 cells after RA treatment; also it was the only Hh isoform to show this increase, therefore corroborating the initial results of this study. Further investigation into Hh signaling in response to RA treatment led me to show that RA activates the Hh signaling

cascade through the increase in Gli-dependent transcriptional activity (Fig. 3.2). Antagonizing the pathway with cyclopamine confirmed that the RA-induced changes in this Gli-dependent activity were specific to the Hh pathway (Fig. 3.2). Thus, this study has established that RA-induced ExE differentiation is accompanied by an increase in *Ihh* expression and the subsequent activation of the canonical Hh signaling cascade.

Once I established a link between the Hh signaling cascade and PrE formation, I demonstrated that cyclopamine treatment effectively blocked the ability of RA to induce the differentiation of F9 cells into the PrE state (Fig. 3.3). Surprisingly, however, and despite this requirement, activating the Hh pathway was not sufficient to induce differentiation (Fig 3.4). Together, this study has provided evidence that a functional Hh signaling pathway is required for proper ExE development to occur, and adds to the overwhelming evidence that Hh plays numerous roles in development and is essential for the survival of an embryo [35]. In fact, this data may indicate the earliest time point in mouse development when the Hh pathway requiring a Hh ligand is required. The importance of this pathway to embryonic development is underscored by studies showing that it interacts with other signaling networks [24, 25, 26], and in the final part of this study, I provide evidence for such crosstalk in ExE formation. Specifically, I discovered an involvement between Hh and Wnt signaling, which is linked to GATA6 and FOXA2. GATA6 and FOXA2 are transcription factors that are master regulators of embryonic and extraembryonic endoderm [22, 31, 32]. *Gata6* expression increases directly in response to RA, and precedes the up-regulation in *Foxa2*, which is linked to increased GATA6 activity [12]. Therefore, investigating the effects of increased *Gata6* and *Foxa2* expression on the *Ihh* gene was examined. Using an *in silico* analysis, I found that the *Ihh*

promoter contains GATA6 binding sites, suggesting that GATA6 regulates directly or indirectly the expression of *Ihh* (Fig 1.4). To test this, F9 cells were transfected with either *Gata6* or *Foxa2* vectors to overexpress the protein, and when PCR analysis was conducted with primers designed to amplify *Ihh* cDNA, results showed that expression increased only in response to the *Gata6* vector (Fig. 3.5). Subsequent experiments using cells co-transfected with *Gata6* and a *Gli* luciferase reporter were done to test if increased *Gata6* expression could activate the Hh pathway, specifically in relation to Gli-dependent transcriptional activation. Results shows that overexpression of *Gata6* led to a significant increase in *Gli* reporter activity (Fig. 3.6), which now places GATA6 in the signaling hierarchy upstream of Hh signaling in ExE formation. Together, this study is the first to show that GATA6 plays a role in modulating the Hh pathway required for PrE formation.

The behaviour and characteristics of the Hh pathway during ExE differentiation are similar in part to the Wnt signaling pathway. Akin to the canonical Hh pathway, messages that encode the Wnt6 ligand are increased in response to RA [16], and to increases in *Gata6*, where regulation of the former is direct since GATA6 binds directly to the *Wnt6* promoter [12]. How Hh signaling impacts on Wnt signaling was tested using chemical activators for each pathway. Results showed that while active Wnt signaling increased the transcriptional activity of a *Gli* reporter construct (Fig. 3.9), active Hh signaling at the level of Smoothened was unable to change TCF/LEF reporter activity (Fig. 3.7). Interestingly, inhibiting the Hh pathway, also at the level of Smoothened, diminished the ability of RA to increase TCF/LEF reporter activity (Fig 3.8). These results would suggest that RA activates Wnt and Hh signaling, but blocking the latter affects the ability of both pathways to signal to the Gli transcription factors. The question

remains as to the node of intersection between the two pathways and for that reason, given the data with BIO (Figs. 3.8 and 3.9), I hypothesize it is at the level of GSK3 β . As described earlier, GSK3 β is a constitutively active kinase of the destruction complex present in the canonical Wnt/b-catenin pathway. When active, GSK3 β ensures b-catenin is phosphorylated and primed for ubiquitination and subsequent proteasomal degradation, thereby reducing levels of the latter to a point where it cannot function as a co-transcriptional activator of Wnt target genes. GSK3 β is also able to regulate the activity of the Gli proteins within the Hh pathway, either indirectly to influence the ability of b-catenin to bind to Gli3, or directly as it can phosphorylate Gli transcription factors to influence post-translational modifications that determine if Gli will become an activator (Gli2) or repressor (Gli3) [24]. Unfortunately, I have no evidence from my thesis to test this hypothesis, but my work has set the stage and is being continued in the Kelly lab.

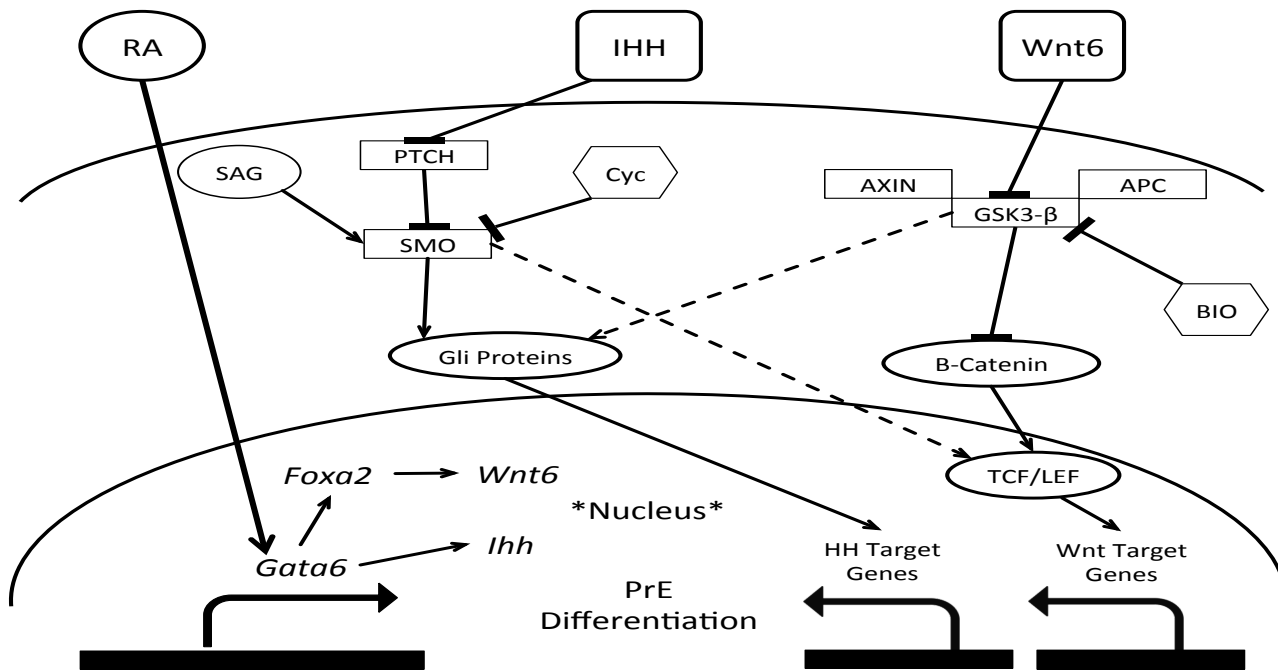
My research builds on the results of several studies that have shown the importance of Wnt signaling during PrE differentiation, along with studies that have demonstrated the ability of GATA6 to act as a regulator during early extraembryonic development [12, 14, 16]. Towards that end I propose a model for PrE differentiation that incorporates the predicted crosstalk between the Hh and Wnt pathways (Fig 4.1). These novel findings are the first step towards understanding how complex the crosstalk is between these pathways in ExE formation.

Further investigation is required in order to pinpoint the node(s) as to where and how these pathways communicate during these early events in development, and if in fact one pathway (Wnt) is more dominant than the other (Hh). More specifically, to determine the checks and balances each pathway imposes on each other. To that end a possible

ChIP analysis can be conducted in order to determine whether GATA6 directly binds to the *Ihh* promoter, confirming the initial findings of their interaction within this study. Along with this further investigation into the importance of the Hh signalling pathway in early embryonic differentiation is required; therefore avenues of future research could look into the ability of the specific GLI proteins, GLI 1, 2, and 3, and determine the particular effects these proteins have in changing the signalling environment.

Finally one of the big leads that can be followed from this investigation is the importance of GSK3- β as signalling node between both the Hh and Wnt signalling pathways. By chemically inhibiting GSK3- β through the use of BIO, this study has shown that both Hh and Wnt signalling are significantly affected, therefore further investigation into how and where GSK3- β specifically interacts in the ExE signalling network will provide valuable information on understanding embryonic development. The novel findings in this study have provided the initial steps into thoroughly understanding the complex crosstalk network involved in ExE differentiation.

Figure 4.1. A model for primitive endoderm differentiation in F9 cells. Retinoic acid induces the expression of *Gata6*, a transcription factor that up-regulates the *Wnt6* gene through the up-regulation of the *Foxa2* and *Ihh* genes. After transcription and translation of the gene products of the *Ihh* and *Wnt6* genes, their respective ligands, Ihh and Wnt6, signal to and activate their particular pathways. Once active, both pathways signal downstream, which culminates in the respective transcription factors of each pathway translocating to the nucleus, and activating (or repressing) genes required for primitive endoderm (PrE) differentiation. Dashed lines represent possible crosstalk interactions between the two pathways.



Chapter 5

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