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Gata6 Induces Wnt6 Expression During Primitive Endoderm Differentiation

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Abstract

Mouse F9 teratocarcinoma cells in culture differentiate into primitive endoderm when treated with retinoic acid and into parietal endoderm with subsequent treatment with dibutyryl cAMP. This *in vitro* model has been studied extensively as these chemically induced events mimic one of the earliest epithelial-to-mesenchymal transitions in mouse embryogenesis. During differentiation to primitive endoderm *Wnt6* expression is up-regulated by RA, and the result is the activation of the canonical Wnt/β-catenin signaling pathway. The factor(s) responsible for the activation of the *Wnt6* gene is not known, but *in silico* analysis reveals that its promoter region contains a putative binding site for the transcription factor GATA6. In this study, the expression of *Gata6* following retinoic acid treatment was examined and found to be up-regulated during primitive and parietal endoderm differentiation. Overexpression of *Gata6* alone induced biochemical, molecular, and morphological markers of primitive endoderm and was sufficient in up-regulating the expression of *Wnt6*. Furthermore, this up-regulation was accompanied by the activation of the canonical Wnt/β-catenin signaling pathway, as evident by the increase in phospho-GSK3β levels. *Gata6* expressing cells were also capable of completing the epithelial-to-mesenchymal transition and differentiating into parietal endoderm when treated with dibutyryl cAMP. Together, these results show that *Gata6* overexpression is sufficient to up-regulate the expression of *Wnt6*, a signaling molecule previously reported by our lab to activate the canonical Wnt/β-catenin pathway, and provides new insight to the signaling mechanisms involved in the specification of primitive endoderm.

Keywords: F9, primitive endoderm, parietal endoderm, extraembryonic endoderm, differentiation, Gata6, Wnt6, EMT

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LIST OF ABBREVIATIONS

^{O}C – degrees Celsius

APC – Adenomatous polyposis coli

BSA – Bovine serum albumin

db – Dibutyryl

cAMP – Cyclic adenosine-monophosphate

CCD – Charge-coupled device

cDNA – Complimentary deoxyribonucleic acid

C. elegans – Caenorhabditis elegans

CMV – Cytomegalovirus promoter

CO_2 – Carbon dioxide

DAPI - 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

Dvl – Dishevelled

EC – Embryonal carcinoma

EMT – Epithelial-to-mesenchymal transition

ES – Embryonic stem

ExE – Extraembryonic endoderm

FBS – Fetal bovine serum

Fig – Figure

Frz – Frizzled

G418 – Neomycin sulfate

GSK – Glycogen synthase kinase

hr – Hour

hrs – Hours

ICM – Inner cell mass

LRP – Lipoprotein-related protein

M – Molar

mA - Milliamperes

min – Minute

ml - Milliliter

mm – Millimeter

mM – Millimolar

mRNA – Messenger ribonucleic acid

NaF – Sodium fluoride

Na_3VO_4 – Sodium orthovanodate

PBS-T – Phosphate buffered saline with 0.1% Triton X-100

PCP – Planar cell polarity

PCR – Polymerase chain reaction

PE – Parietal endoderm

PrE – Primitive endoderm

PS – Penicillin-streptomycin

RA – Retinoic acid

RNA – Ribonucleic acid

RT – Reverse transcriptase

s – Seconds

SDS – Sodium dodecyl sulfate

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

Tcf/Lef – T-cell specific transcription factor/Lymphoid enhancer binding factor

TE – Trophectoderm

TF – Transcription factor

TRITC - Tetramethyl Rhodamine Isothiocyanate

TS – Trophoblast stem

µg – Microgram

µl – Microliter

VE – Visceral endoderm

Wnt – Wingless/integrated

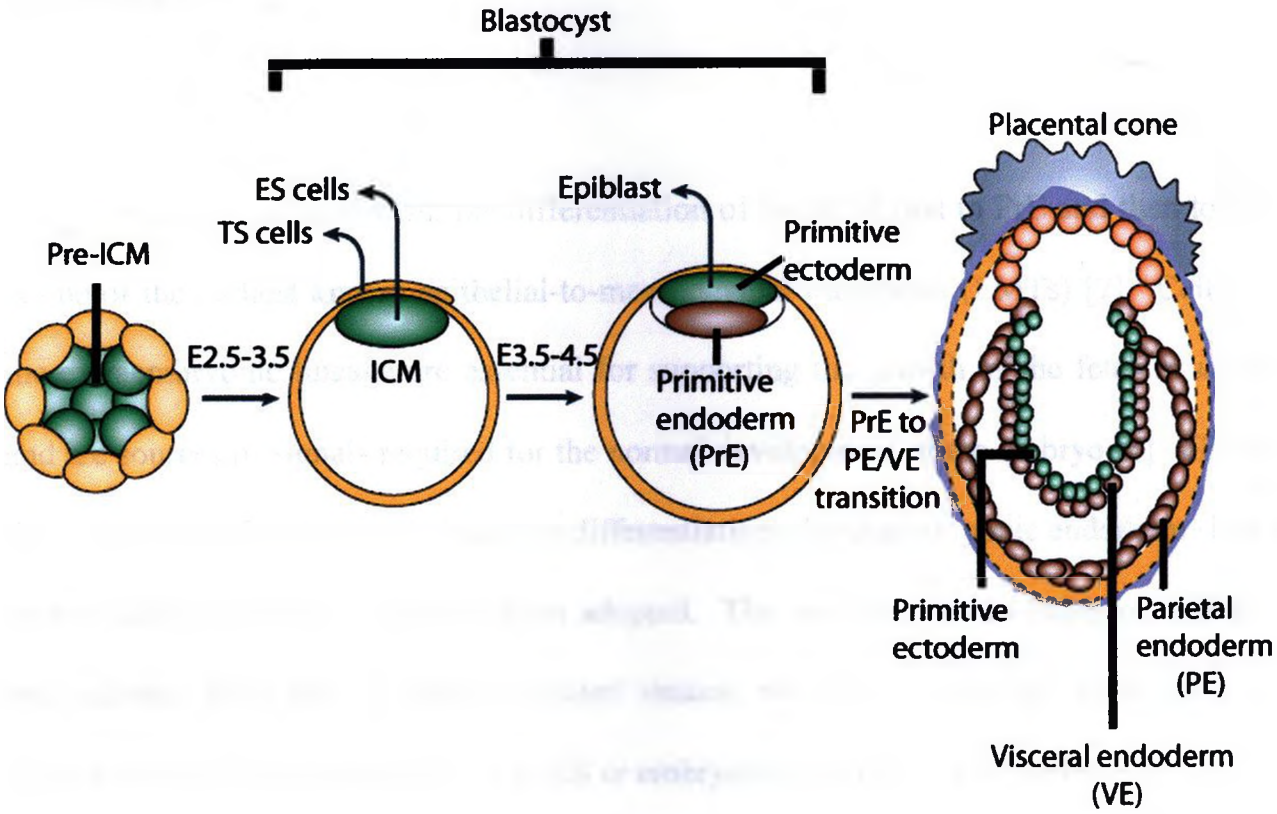
CHAPTER 1

INTRODUCTION

1.1 *Mouse Early Development*

In mammals, the fertilization of the egg occurs in the oviduct through complex processes that are not yet fully understood. Following successive rounds of mitosis a blastocyst forms and around the time of implantation, it is comprised of three cell types: the epiblast, derived from the inner cell mass (ICM), contains embryonic stem (ES) cells that gives rise to the entire fetus (embryo proper); the trophectoderm (TE), which contains trophoblast stem (TS) cells, gives rise to the placenta; and the primitive endoderm (PrE), which eventually forms the extraembryonic layers of the parietal and visceral endoderms (PE and VE, respectively; Fig. 1.1). The specification of these extraembryonic tissues (TE and PrE), the first to occur during development and well before any specification that takes place within the embryo proper, is of paramount importance for normal development [1-3]. The trophoblast cells of the TE, in direct contact with the ICM, attaches to the uterine epithelium to initiate implantation [2], and the placenta forms shortly thereafter [4]. The PrE further differentiates into PE, which migrates beneath the TE to form the parietal yolk sac, and the VE, which forms the visceral yolk sac [1]. The proper segregation and development of the extraembryonic tissues are crucial for the survival and patterning of the embryo proper [5, 6]. Given the

Figure 1.1 **Mouse early development.** The extraembryonic endoderm (primitive, parietal, and visceral endoderm) is derived around the time of implantation from embryonic stem cells of the inner cell mass of the blastocyst. Formation of the extraembryonic endoderm, which later contributes to the yolk sac, is necessary for the proper development of the epiblast. The trophoblast stem cells play an important role in the proper implantation and formation of the placenta. Inner cell mass (ICM); ES (embryonic stem); TS (trophoblast stem); E (embryonic days post-coitus); PrE (primitive endoderm); PE (parietal endoderm); VE (visceral endoderm). Modified from Boiani and Scholer (2005).


Blastocyst
Pre-ICM
ES cells
TS cells
ICM
E2.5-3.5
E3.5-4.5
Epiblast
Primitive ectoderm
Primitive endoderm (PrE)
PrE to PE/VE transition
Placental cone
Primitive ectoderm
Parietal endoderm (PE)
Visceral endoderm (VE)

importance of these tissues arising early in development, elucidating the signaling events responsible for establishing these lineages will be instrumental in better understanding the mechanisms responsible for patterning the mammalian embryo.

1.2 *F9 Teratocarcinoma Cells As A Model For Extraembryonic Endoderm Differentiation*

In mouse development, the differentiation of the ICM first to PrE and then to PE is one of the earliest known epithelial-to-mesenchymal transitions (EMTs) [7]. Cells of the extraembryonic lineage are essential for supporting the growth of the fetus *in utero* and are sources of signals required for the normal development of the embryo [8]. Given the technical difficulties in studying the differentiation of extraembryonic endoderm (ExE) *in vivo,* alternative strategies have been adopted. The first relies on isolation of primary cell cultures from pre- or post-implanted tissues, whereas the second, most favored approach relies on established lines of ES or embryonal carcinoma (EC) cells.

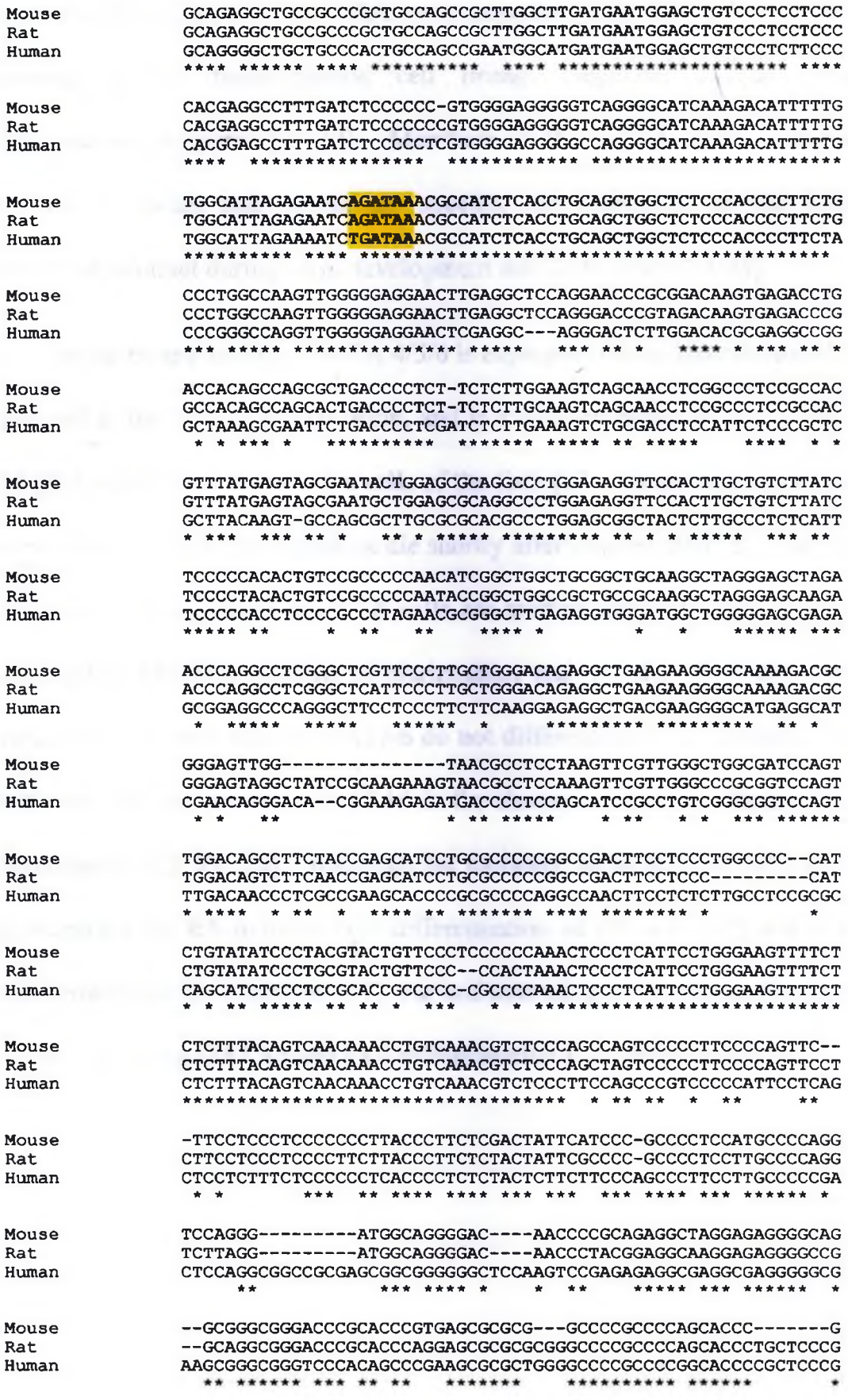
The F9 teratocarcinoma EC line was established by transplanting a 6 day old male embryo into a host testis of mouse strain 129/Sv [9]. F9 cells grow in culture as tightly packed colonies that appear predominantly homogenous [10]. The addition of retinoic acid (RA) to the culture induces morphological and biochemical changes and differentiate the cells into PrE [10]. PrE cells remain competent and can be induced to differentiate into PE and complete the EMT by subsequent treatment with dibutyryl

cyclic adenosine monophosphate (db-cAMP) or cAMP elevating agents which serve to elevate the activity of protein kinase A [11]. PrE and PE cells that differentiate from the parental F9 cells express and secrete many of the same factors found in the developing mouse embryo [12, 13]. This recapitulation that similar or identical signal transduction pathways are activated during the differentiation of ExE *in vitro* and *in vivo* has over the last 30 years established the F9 cell line as an ideal model to study ExE differentiation. Towards that end, recent evidence indicates Wnt6 signaling, through the canonical Wnt/β-catenin pathway, is sufficient to induce PrE, but not PE differentiation [7]. The mechanism, however, that leads to the up-regulation of the *Wnt6* gene and its expression remains to be elucidated. Evidence indicates that the differentiation into PrE is regulated by the activation and inhibition of genes by transcription factors that are up-regulated in response to RA. One of these is *Gata6,* which is a direct target gene of RA signaling [14] and which *in silico* analysis has revealed, could bind to a region in the *Wnt6* promoter (Fig. 1.2).

1.3 *GATA6 And Development*

The GATA family of transcription factors play pivotal roles in embryonic development and maintenance of cell differentiation in adults [15, 16]. This family has been conserved throughout evolution with members identified in yeast, *C. elegans*, *Drosophila*, zebrafish, Xenopus, chick, mouse, and human [17-19]. There are six members of the family in vertebrates, each containing two conserved zinc finger motifs

Figure 1.2 **Sequence identity between the mouse, rat, and human putative Wnt6 promoters.** The *Wnt6* promoter regions of mouse, rat, and human (Accession numbers: Mouse – NT_039170.7; Rat – NW_047816.2; Human – NT_005403.1L) were aligned using Lasergene (DNASTAR Inc.). Conserved nucleotides are shown by asterisks (*). The putative GATA binding site is highlighted in yellow.


Mouse GCAGAGGCTGCCGCCCGCTGCCAGCCGCTTGGCTTGATGAATGGAGCTGTCCCTCCTCCC
Rat GCAGAGGCTGCCGCCCGCTGCCAGCCGCTTGGCTTGATGAATGGAGCTGTCCCTCCTCCC
Human GCAGGGGCTGCTGCCCACTGCCAGCCGAATGGCATGATGAATGGAGCTGTCCCTCTTCCC
Mouse CACGAGGCCTTTGATCTCCCCCC-GTGGGGAGGGGGTCAGGGGCATCAAAGACATTTTTG
Rat CACGAGGCCTTTGATCTCCCCCCCGTGGGGAGGGGGTCAGGGGCATCAAAGACATTTTTG
Human CACGGAGCCTTTGATCTCCCCCTCGTGGGGAGGGGGCCAGGGGCATCAAAGACATTTTTG
Mouse TGGCATTAGAGAATCAGATAAACGCCATCTCACCTGCAGCTGGCTCTCCCACCCCTTCTG
Rat TGGCATTAGAGAATCAGATAAACGCCATCTCACCTGCAGCTGGCTCTCCCACCCCTTCTG
Human TGGCATTAGAAAATCTGATAAACGCCATCTCACCTGCAGCTGGCTCTCCCACCCCTTCTA
Mouse CCCTGGCCAAGTTGGGGGAGGAACTTGAGGCTCCAGGAACCCGCGGACAAGTGAGACCTG
Rat CCCTGGCCAAGTTGGGGGAGGAACTTGAGGCTCCAGGGACCCGTAGACAAGTGAGACCCG
Human CCCGGGCCAGGTTGGGGGAGGAACTCGAGGC---AGGGACTCTTGGACACGCGAGGCCGG
Mouse ACCACAGCCAGCGCTGACCCCTCT-TCTCTTGGAAGTCAGCAACCTCGGCCCTCCGCCAC
Rat GCCACAGCCAGCACTGACCCCTCT-TCTCTTGCAAGTCAGCAACCTCCGCCCTCCGCCAC
Human GCTAAAGCGAATTCTGACCCCTCGATCTCTTGAAAGTCTGCGACCTCCATTCTCCCGCTC
Mouse GTTTATGAGTAGCGAATACTGGAGCGCAGGCCCTGGAGAGGTTCCACTTGCTGTCTTATC
Rat GTTTATGAGTAGCGAATGCTGGAGCGCAGGCCCTGGAGAGGTTCCACTTGCTGTCTTATC
Human GCTTACAAGT-GCCAGCGCTTGCGCGCACGCCCTGGAGCGGCTACTCTTGCCCTCTCATT
Mouse TCCCCCACACTGTCCGCCCCCAACATCGGCTGGCTGCGGCTGCAAGGCTAGGGAGCTAGA
Rat TCCCCTACACTGTCCGCCCCCAATACCGGCTGGCCGCTGCCGCAAGGCTAGGGAGCAAGA
Human TCCCCCACCTCCCCGCCCTAGAACGCGGGCTTGAGAGGTGGGATGGCTGGGGGAGCGAGA
Mouse ACCCAGGCCTCGGGCTCGTTCCCTTGCTGGGACAGAGGCTGAAGAAGGGGCAAAAGACGC
Rat ACCCAGGCCTCGGGCTCATTCCCTTGCTGGGACAGAGGCTGAAGAAGGGGCAAAAGACGC
Human GCGGAGGCCCAGGGCTTCCTCCCTTCTTCAAGGAGAGGCTGACGAAGGGGCATGAGGCAT
Mouse GGGAGTTGG---------------TAACGCCTCCTAAGTTCGTTGGGCTGGCGATCCAGT
Rat GGGAGTAGGCTATCCGCAAGAAAGTAACGCCTCCAAAGTTCGTTGGGCCCGCGGTCCAGT
Human CGAACAGGGACA--CGGAAAGAGATGACCCCTCCAGCATCCGCCTGTCGGGCGGTCCAGT
Mouse TGGACAGCCTTCTACCGAGCATCCTGCGCCCCCGGCCGACTTCCTCCCTGGCCCC--CAT
Rat TGGACAGTCTTCAACCGAGCATCCTGCGCCCCCGGCCGACTTCCTCCC---------CAT
Human TTGACAACCCTCGCCGAAGCACCCCGCGCCCCAGGCCAACTTCCTCTCTTGCCTCCGCGC
Mouse CTGTATATCCCTACGTACTGTTCCCTCCCCCCAAACTCCCTCATTCCTGGGAAGTTTTCT
Rat CTGTATATCCCTGCGTACTGTTCCC--CCACTAAACTCCCTCATTCCTGGGAAGTTTTCT
Human CAGCATCTCCCTCCGCACCGCCCCC-CGCCCCAAACTCCCTCATTCCTGGGAAGTTTTCT
Mouse CTCTTTACAGTCAACAAACCTGTCAAACGTCTCCCAGCCAGTCCCCCTTCCCCAGTTC--
Rat CTCTTTACAGTCAACAAACCTGTCAAACGTCTCCCAGCTAGTCCCCCTTCCCCAGTTCCT
Human CTCTTTACAGTCAACAAACCTGTCAAACGTCTCCCTTCCAGCCCGTCCCCCATTCCTCAG
Mouse -TTCCTCCCTCCCCCCCTTACCCTTCTCGACTATTCATCCC-GCCCCTCCATGCCCCAGG
Rat CTTCCTCCCTCCCCTTCTTACCCTTCTCTACTATTCGCCCC-GCCCCTCCTTGCCCCAGG
Human CTCCTCTTTCTCCCCCCTCACCCCTCTCTACTCTTCTTCCCAGCCCTTCCTTGCCCCCGA
Mouse TCCAGGG---------ATGGCAGGGGAC----AACCCCGCAGAGGCTAGGAGAGGGGCAG
Rat TCTTAGG---------ATGGCAGGGGAC----AACCCTACGGAGGCAAGGAGAGGGGCCG
Human CTCCAGGCGGCCGCGAGCGGCGGGGGGCTCCAAGTCCGAGAGAGGCGAGGCGAGGGGGCG
Mouse --GCGGGCGGGACCCGCACCCGTGAGCGCGCG---GCCCCGCCCCAGCACCC-------G
Rat --GCAGGCGGGACCCGCACCCAGGAGCGCGCGCGGGCCCCGCCCCAGCACCCTGCTCCCG
Human AAGCGGGCGGGTCCCACAGCCCGAAGCGCGCTGGGGCCCCGCCCCGGCACCCCGCTCCCG

that bind DNA with an A/TGATA/G core sequence [15]. The GATA 1/2/3 subfamily, expressed in the hematopoietic cell lineage, regulates unique lineage-specific developmental programs [20-22]. Members of the GATA 4/5/6 subfamily, initially identified in avian and amphibian species, are expressed in the heart and the gastrointestinal tract during early development and in the adult [23-25].

In the mouse embryo, GATA 4/5/6 is expressed before gastrulation [12]. GATA6, is induced at the time of implantation, and is expressed randomly in the ES cells of the ICM [26], which later become the cells of the ExE [27]. Gene targeting experiments has also revealed that GATA6 null mice die shortly after implantation [27]. *In vitro*, GATA6 expression is up-regulated when ES cells are treated with RA and this is sufficient to down-regulate Oct-3/4, a marker of pluripotency and to induce ExE differentiation [12]. Furthermore, ES cells null for GATA6 do not differentiate in the presence of RA, while transfection and expression of GATA6 in the absence of RA is sufficient to induce ExE differentiation [12, 27, 28]. Although the evidence indicates that GATA6 is sufficient and necessary for RA-induced ExE differentiation of ES cells [12] and is required for normal embryonic development [27], our understanding of the mechanisms and signaling pathways involving GATA6 and ExE differentiation is far from complete.

1.4 *Wnt Signaling Pathways*

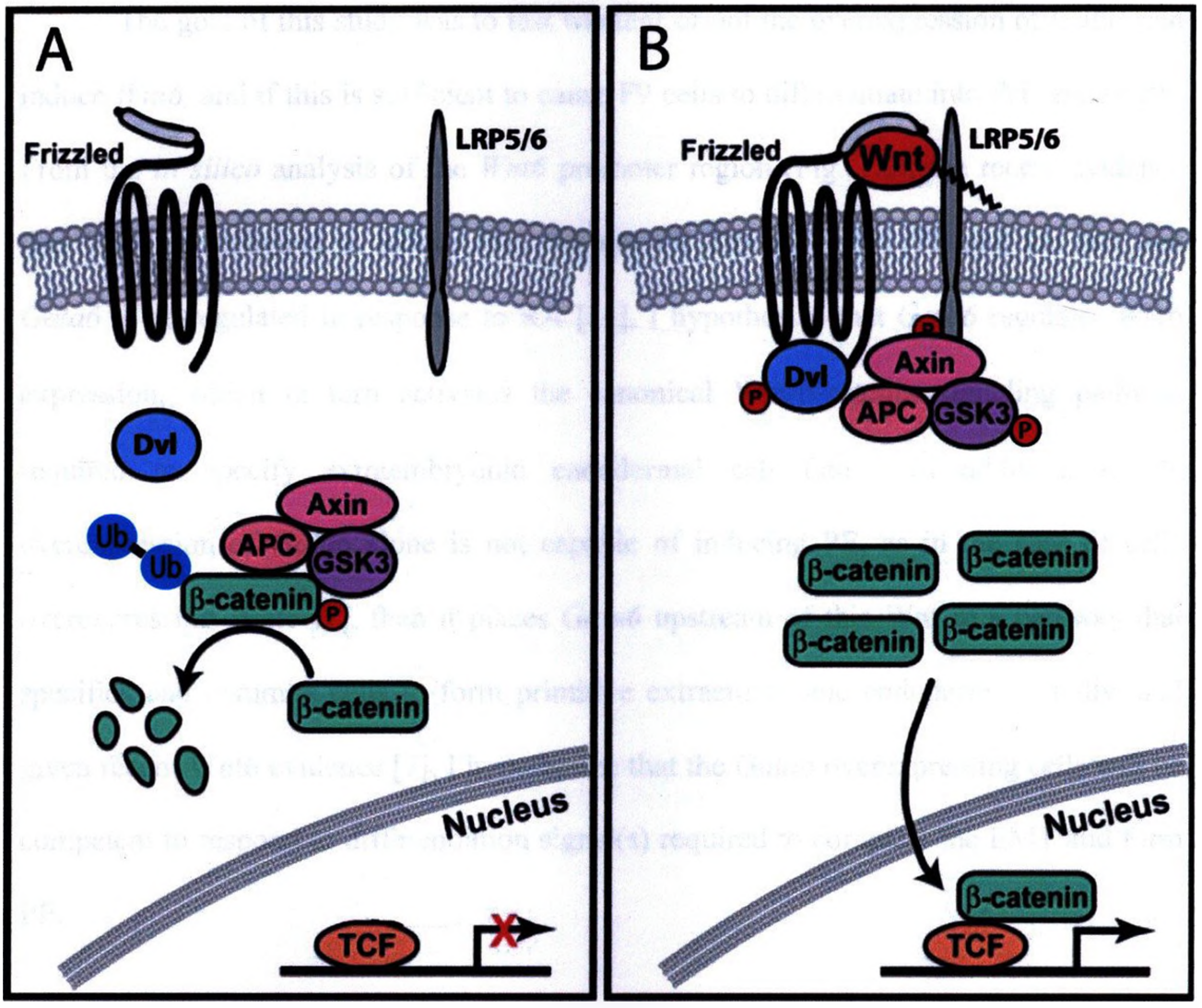
Wnts are secreted lipid-modified glycoproteins initially identified as genes affecting wing development in *Drosophila* [29]. Since the identification of the first mammalian Wnt [30], another 18 Wnt genes have been discovered [31]. Wnts are first detected in the ICM and the cells surrounding the blastocyst cavity shortly after fertilization during pre-implantation development, and their expression patterns continue throughout gastrulation, organogenesis, and into adult life [32-34]. Despite the presence of Wnts during the pre-implantation stages, the signaling pathway may not be active because active β-catenin, a key effector of the Wnt signaling pathway, is not detected at the pre-implantation stages [35]. However, recent evidence suggests that the first active Wnt signaling may occur at the time of implantation during PrE differentiation [7].

Historically, Wnts have been grouped into two classes based on their activity in *in vitro* and *in vivo* assays: canonical and non-canonical Wnts. The canonical Wnt/β-catenin signaling pathway, involved in the regulation of cell differentiation, proliferation, and self-renewal of stem and progenitor cells, is conserved from nematodes to mammals [36-38]. Under normal circumstances, the pathway is activated by a secreted member of the Wnt family, which serves as a ligand for one or more members of a group of seven-transmembrane Frizzled (Frz) receptors [26]. In the absence of Wnt, a complex composed of glycogen synthase kinase 3 (GSK3), casein kinase 1α, Axin and adenomatous polyposis coli (APC), targets β-catenin for phosphorylation, ubiquitination and ultimately degradation in the proteasome. In the presence of Wnt, the interaction

between Wnt, Frz, and a co-receptor lipoprotein-related protein 5 (LRP5) or LRP6, activates one or more of the Dishevelled (Dvl) cytoplasmic phosphoproteins, which results in the phosphorylation and inhibition of GSK3. This inactivation of GSK3 destabilizes the degradation complex, thereby allowing cytoplasmic β-catenin levels to increase. β-catenin, free of its association with the degradation complex, can then translocate to the nucleus where it binds to T-cell factor-lymphoid enhancer factor (TCF/LEF) thereby allowing transcriptional activation of several target genes (Fig. 1.3).

It is important to note that not all Wnts are involved in the canonical signaling pathway; two β-catenin independent or non-canonical pathways also utilize Frz and/or Dvl. In the planar cell polarity pathway (PCP), the binding of Wnt to Frz recruits Dvl to the plasma membrane, which results in the activation of the Jun-N-terminal kinase-Rho-Rac pathway. This activation is necessary to induce the changes to the cytoskeleton that are needed by cells during gastrulation [39-41]. The second, and least understood non-canonical Wnt/Calcium pathway involves the activation of Frz, G-proteins, calcium/calmodulin-dependent kinase II and protein kinase C [42], and much like the PCP pathway, seems to play a fundamental role in body axis specification and cellular movements during embryogenesis [43, 44]. For the purpose of my study, I will focus on the canonical pathway involving Wnt6 and β-catenin.

Figure 1.3. **Canonical Wnt/β-catenin signaling pathway. (A)** In the absence of a Wnt signal, a protein complex comprised of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3) initiates the phosphorylation of β-catenin. Phosphorylated β-catenin is then ubiquitinated (Ub) and targeted for degradation. **(B)** When present, the Wnt ligand interacts with its receptor Frizzled and a co-receptor lipoprotein-related protein 5/6 (LRP5/6) to recruit and activate the phosphoprotein Dishevelled (Dvl). Active Dvl prevents the degradation complex from forming thereby allowing cytoplasmic levels of β-catenin to increase. β-catenin translocates to the nucleus, where it interact with transcription factors of the T-cell/lymphoid enhancing factor family (TCF), to activate expression of target genes. Modified from Gordon and Nusse (2006).


A
Frizzled
LRP5/6
Dvl
Axin
Ub
Ub
APC
GSK3
β-catenin
P
β-catenin
Nucleus
TCF
B
Frizzled
Wnt
LRP5/6
Dvl
Axin
APC
GSK3
P
β-catenin
β-catenin
β-catenin
β-catenin
Nucleus
β-catenin
TCF

1.5 *Objectives of Study and Hypothesis*

The goal of this study was to test whether or not the overexpression of *Gata6* can induce *Wnt6,* and if this is sufficient to cause F9 cells to differentiate into PrE and/or PE. From the *in silico* analysis of the *Wnt6* promoter region (Fig. 1.2), the recent evidence that Wnt6 is sufficient to induce PrE, but not PE differentiation [7], and the fact that *Gata6* is up-regulated in response to RA [14], I hypothesize that *Gata6* regulates *Wnt6* expression, which in turn activates the canonical Wnt/β-catenin signaling pathway required to specify extraembryonic endodermal cell fate. In addition, if the overexpression of *Gata6* alone is not capable of inducing PE, as in the case of cells overexpressing Wnt6 [7], then it places *Gata6* upstream of this Wnt in a pathway that specifies and commits cells to form primitive extraembryonic endoderm. Finally, and given recent Wnt6 evidence [7], I hypothesize that the *Gata6* overexpressing cells will be competent to respond to differentiation signal(s) required to complete the EMT and form PE.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Cell Culturing, Transfection, and Protein Extraction*

Mouse F9 teratocarcinoma cells (ATCC) were cultured in tissue culture treated 60mm plates (BD Falcon) for protein isolation or in 35mm plates (Nunc) with 0.5% gelatin coated coverslips for immunofluorescence. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) supplemented with 5% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin antibiotic (PS; Lonza). When the cells were approximately 40% confluent, they were treated with either 10^{-7} M retinoic acid (RA all trans; Sigma) or 10^{-7}M RA plus 1mM dibutyryl cyclic adenosine monophosphate (db-cAMP; Sigma). Control and treated cells were incubated at 37°C and 5% CO_2 for 5 days.

The *Gata6* coding sequence was excised from *pCMVTag2B-GATA6* (generously provided by Dr. E. E. Morrisey, University of Pennsylvania) with BamHI and XhoI and sub-cloned into BamHI and XhoI digested *pcDNA.3.1+*. Three microliters of Lipofectamine 2000 (Invitrogen) was used to transfect 0.8µg of *pcDNA3.1-Gata6* into F9 cells grown to approximately 50% confluency. After 6 hrs, the transfected cells were treated with 400 µg/ml G418 and grown under the conditions described above. After 1

week of G418 selection, the cells were processed for immunofluorescence or lysed in 300µl of 2% sodium dodecyl sulfate (SDS) lysis buffer containing 50mM sodium fluoride (NaF; Sigma), 1mM sodium orthovanodate (Na_3VO_4; Sigma), and 1% Halt Protease Inhibitor Cocktail (Thermo Scientific) and extracts were collected for immunoblot analysis. Transfections with the *pcDNA3.1* empty vector or with Lipofectamine 2000 by itself, followed by 1 week of G418 selection served as controls.

2.2 *Reverse-Transcription PCR*

Oligodeoxynucleotide primers were designed to the mouse *Wnt6* (Accession # M89800) and *Gata6* (Accession # AK142381) nucleotide sequences. *Wnt6* sense (5' GCG GTA GAG CTC TCA GGA TG) and antisense (5' AAA GCC CAT GGC ACT TAC AC) and *Gata6* sense (5' CTC TGC ACG CTT TCC CTA CT) and antisense (5' GTA GGT CGG GTG ATG GTG AT) primers were designed to amplify partial *Wnt6*, and *Gata6* cDNAs. RNA was isolated from F9 cells treated with RA, RA and db-cAMP, F9 cells transfected with *pcDNA3.1-Gata6* or the empty vector, and converted into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen). The cDNAs were used as a template for PCR under the following reaction conditions: *Wnt6* – 35 cycles of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C; *Gata6* – 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. Primers to *L14* sense 5' GGG AGA GGT GGC CTC GGA CGC and antisense 5' GGC TGG CTT CAC TCA AAG GCC was used to amplify a constitutively expressed ribosomal gene. The number of cycles used in each of the PCR

reactions was empirically derived and were within the linear range of amplification. PCR products were analyzed on 1% agarose gels, stained with ethidium bromide, DNA visualized using a FluorChem 8900 Gel Doc System (Alpha Innotech), and the sequence of the amplified products were confirmed by DNA sequencing (Robarts Research Institute).

2.3 *Immunoblot Analysis*

Protein concentrations from treated and transfected F9 cells were determined using the Bradford Assay (Bio-Rad). Approximately thirty micrograms of protein were separated on denaturing 10% polyacrylamide gels for 2 hrs at a constant current of 400mA. Following electrophoresis, the proteins were electrophoretically transferred for 1 hr to nitrocellulose membranes (Biotrace; Pall Corp.) using tris/glycine transfer buffer with 20% metahnol. The membranes were blocked in 5% skim milk in tris buffered saline with 0.1% Tween 20 (TBS-T) for 1 hr at room temperature, probed with primary antibodies overnight at 4°C followed by 3 washes in TBS-T, probed with secondary antibody(s) for 2 hrs at room temperature followed by 3 washes in TBS-T, and the signals were detected using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce) and X-Omat Blue XB-1 Film (Kodak). The primary antibodies were directed against TROMA-1 (1:50; Developmental Studies Hybridoma Bank), β-Actin (1:10000; Santa Cruz), Phospho-GSK3β (1:1000; Cell Signaling), and GSK3β (1:1000; Cell Signaling).

Secondary antibodies were HRP-conjugated goat anti-rat, anti-mouse, and anti-rabbit (1:10000; Pierce).

2.4 *Immunofluorescence*

Cells were fixed for 10 min in phosphate buffered saline (PBS) containing 4% paraformaldehyde and then washed 3 times for 5 min each in PBS with 0.1% Triton X-100 (PBS-T). Cells were blocked in PBS-T containing 4% goat serum for 1 hr at room temperature, and then incubated with TROMA-1 antibody (1:50) for 2 hrs at room temperature. After the incubation with the primary antibody, the cells were washed 3 times with PBS-T, and then incubated in TRITC-conjugated anti-rat secondary antibody (1:100; Pierce). Cells were washed once in PBS-T containing DAPI (1:4000), twice in PBS-T, and then mounted on slides using ProLong Gold antifade reagent (Invitrogen).

2.5 *Photomicroscopy*

For fluorescence microscopy, cells were examined using a Zeiss Imager Z1 microscope and images were captured using a Zeiss Axiocam MRm CCD camera. For light microscopy, cells were examined using a Zeiss Axio Observer A1 inverted

microscope and images were captured using a QImaging Retiga CCD camera. All images were assembled as plates using Adobe Photoshop CS3 and Adobe Illustrator CS3.

2.6 *Statistical Analysis*

For densitometric analyses of immunoblots and reverse transcription PCR, data were compiled from 3 independent biological replicate experiments performed on separate occasions. Comparisons of data between the control and treated or transfected groups were performed using a Student's *t*-Test assuming unequal variances. The *P* values were one-sided and considered statistically significant at the 0.05 level.

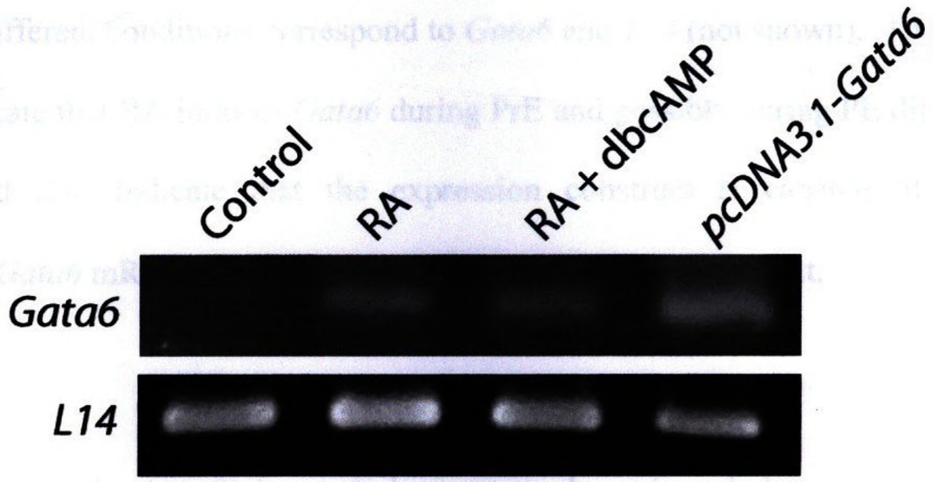
CHAPTER 3

RESULTS

3.1 *Gata6 mRNA regulation during extraembryonic endoderm differentiation*

Mouse F9 teratocarcinoma cells grown in monolayer differentiate into primitive endoderm (PrE) and parietal endoderm (PE) when exposed to retinoic acid (RA) or RA and dibutyryl cyclic adenosine monophosphate (db-cAMP), respectively. Since members of the GATA family of transcription factors are known to be up-regulated in F9 cells during PE differentiation [45], are expressed in ES cells following RA induction to PrE [46] and have been shown to play a role in formation of extraembryonic endoderm *in vivo* [28], it was hypothesized that *Gata6* would be up-regulated in F9 cells when exposed to RA to form PrE or PE following exposure to RA and db-cAMP. To test whether or not *Gata6* is expressed in F9 cells induced to form PrE or PE, total RNA was collected from cells treated with RA and RA and db-cAMP and reverse transcribed into first strand cDNA. Messenger RNA was also collected and reverse transcribed from cells transfected with *pcDNA3.1-Gata6* or the empty vector (control) to test the fidelity of the expression construct and to rule out the possibility that *Gata6* was expressed in undifferentiated cells. PCR amplification using *Gata6* primers revealed an amplicon corresponding to *Gata6* in RA and RA and db-cAMP treated cells, but not in the empty vector transfected control cells (Fig. 3.1). The same size amplicon was also detected in

Figure 3.1. ***Gata6* mRNA is up-regulated during RA-induced differentiation.** Total RNA from cells treated with retinoic acid (RA) to induce primitive endoderm or RA and dibutyryl cAMP (db-cAMP) to induce parietal endoderm, and cells transfected with empty vector (control) or *pcDNA3.1-Gata6* and selected with G418, was collected and reverse transcribed into first strand cDNA for PCR. Oligodeoxynucleotide primers for *Gata6* were expected to yield an amplicon of 233 bp. This amplicon was seen in the RA (primitive endoderm) and RA + db-cAMP (parietal endoderm) lanes, and as expected in cells ectopically expressing *Gata6*, but was not present in the control. Primers to the constitutively expressed ribosomal gene *L14* was used in the PCR reaction to ensure cDNAs were present in all samples and an amplicon of the expected 300 bp was seen in each lane.


Control
RA
RA + dbcAMP
pcDNA3.1-Gata6
Gata6
L14

cells expressing the *pcDNA3.1-Gata6* construct. As a control, PCR amplification of the *L14* ribosomal gene, which is constitutively expressed during all developmental stages, revealed an amplified product in all lanes as expected (Fig. 3.1). PCR reactions containing template cDNA from first strand cDNA synthesis in the absence of *Reverse Transcriptase* revealed no amplification indicating the absence of genomic DNA contamination (not shown). Sequencing results confirmed that the amplicons appearing under the different conditions correspond to *Gata6* and *L14* (not shown). Together, these results indicate that RA induces *Gata6* during PrE and possibly during PE differentiation. Data would also indicate that the expression construct is capable of ectopically expressing *Gata6* mRNA, which set the stage for the next experiment.

3.2 *Gata6 expression is sufficient to induce extraembryonic endoderm*

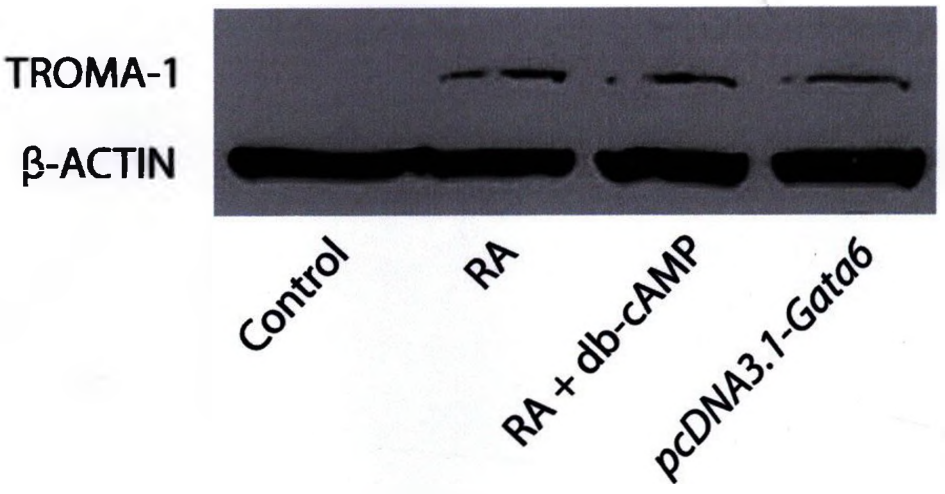
Since *Gata6* was detected in response to RA- and RA and db-cAMP-induced differentiation of F9 cells, it seemed logical to suggest that its overexpression would induce PrE and/or PE in the absence of RA and cAMP elevating agents. Immunoblot analysis showed that TROMA-1, a marker of extraembryonic endoderm, was expressed in cells treated with RA and RA and db-cAMP, but not in undifferentiated controls (Fig. 3.2A), thus confirming a previous study in my lab [7]. TROMA-1 was also expressed in cells transfected with the *pcDNA3.1-Gata6* construct, adding further evidence that *Gata6* is a key player in the differentiation of extraembryonic endoderm. Densitometric analysis of data from several independent experiments confirmed that the relative levels

of TROMA-1 induced by chemical treatment and by *Gata6* overexpression were significantly higher (P=0.013, P=0.006, and P=0.006) than that in undifferentiated F9 cells (Fig. 3.2B).

TROMA-1 is routinely used as a marker of extraembryonic endoderm, and its appearance on blots is highly suggestive that differentiation has occurred [47]. To be certain, however, the cytoA intermediate filament protein detected by the TROMA-1 antibody must assemble into the recognizable filaments that form prior to the epithelial-to-mesenchymal transition. To address this, cells were treated with RA or transfected with either the *pcDNA3.1-Gata6* construct or the empty vector, selected with G418 and then processed for immunocytochemical analysis to examine for the presence of TROMA-1–positive intermediate filaments (Fig. 3.3). Results confirm the immunoblot data and show the characteristic intermediate filament staining pattern that forms when cells are treated with RA. A similar staining pattern was seen in cells transfected with *pcDNA3.1-Gata6* (Fig. 3.3). It should be noted that the staining pattern was comparable in several fields of individual or groups of cells expressing *pcDNA3.1-Gata6* (not shown). A control for the non-specific binding of the secondary antibody alone showed no TROMA-1 staining (inset, Fig. 3.3). These results indicate that the expression of *Gata6* alone is sufficient to induce extraembryonic endoderm.

Figure 3.2. **Overexpression of *Gata6* induces extraembryonic endoderm.** Protein lysates from cells treated with RA to induce primitive endoderm or RA and db-cAMP to induce parietal endoderm, and cells transfected with the empty vector (control) or *pcDNA3.1-Gata6* and selected with G418 for 7 days were collected and processed for immunoblot analysis using antibodies to TROMA-1, a marker for extraembryonic endoderm differentiation and β-actin as a loading control. **(A)** TROMA-1 signals are seen in RA and RA + db-cAMP treated cells, and in those transfected with *pcDNA3.1-Gata6*. Data are representative of 3 independent experiments. **(B)** Analysis of the average integrated densitometric values between TROMA-1 and β-ACTIN reveals that there is a significant increase in TROMA-1 expression in chemically-induced or *pcDNA3.1-Gata6* transfected cells relative to the empty vector transfected control.

A


TROMA-1
β-ACTIN
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6

B

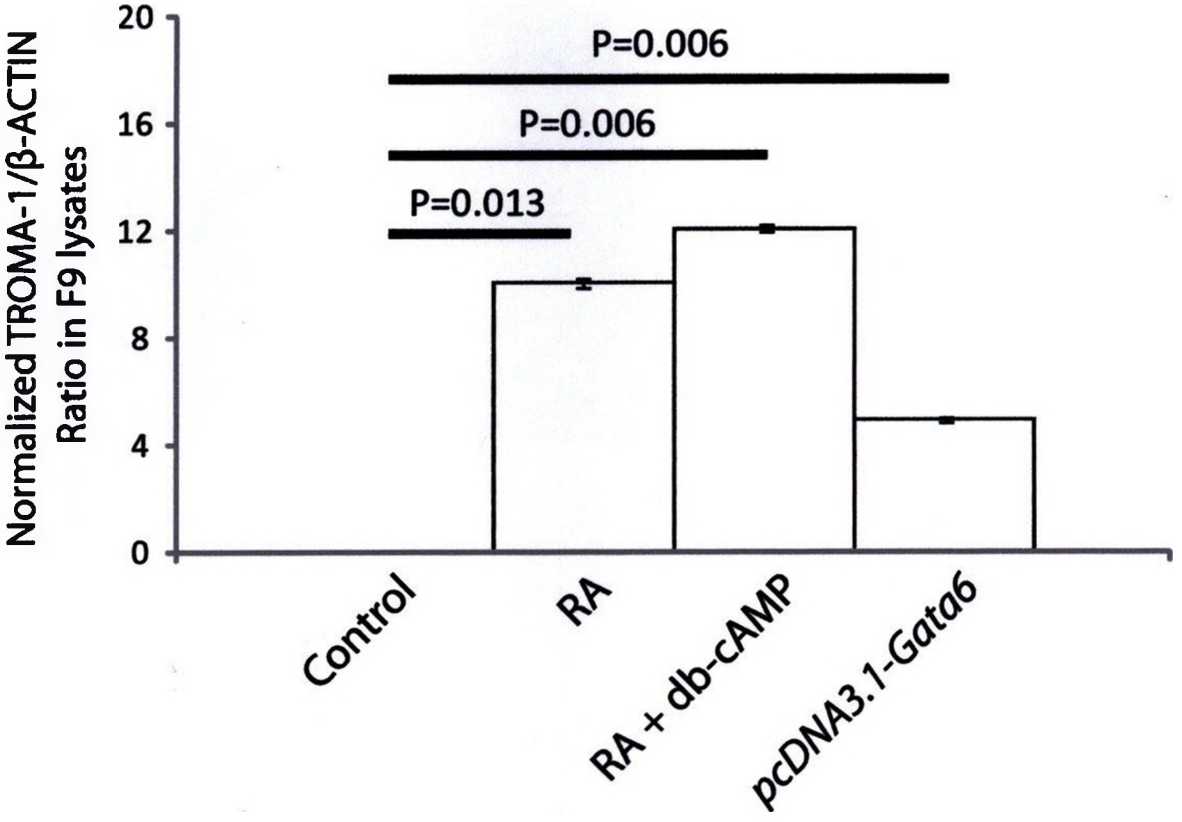
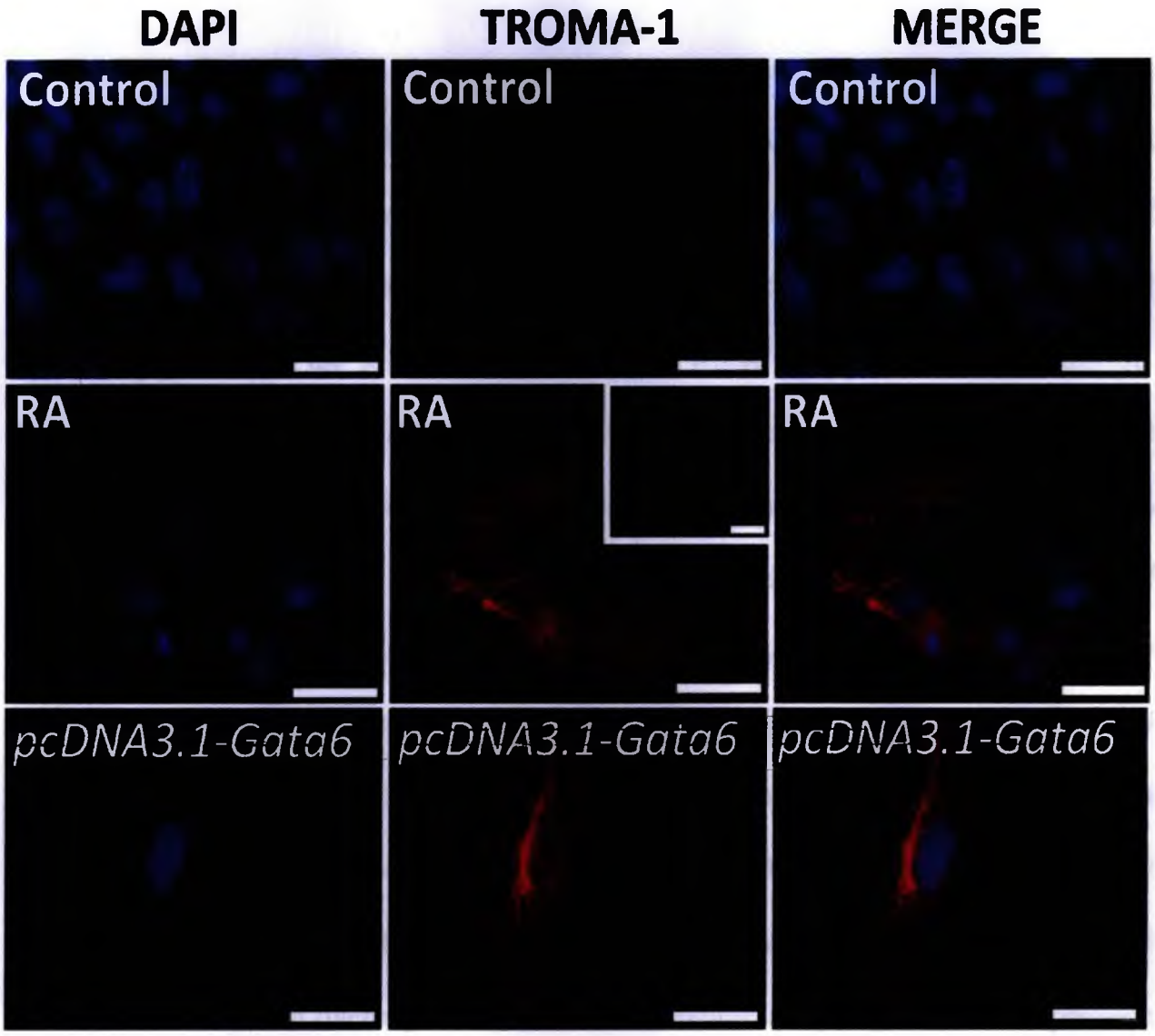

Normalized TROMA-1/β-ACTIN Ratio in F9 lysates
20
16
12
8
4
0
P=0.006
P=0.006
P=0.013
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6

Figure 3.3. **TROMA-1 positive staining intermediate filaments form as a result of *Gata6* overexpression.** Cells were treated with RA to induce primitive endoderm or transfected with the empty vector (control), or with the *pcDNA3.1-Gata6* construct and selected with G418, were fixed and processed for immunocytochemistry using the TROMA-1 monoclonal antibody to detect endo A cytokeratin filaments. TROMA-1 filaments relative to the DAPI-positive nuclei are seen in RA treated and *pcDNA3.1-Gata6* transfected cells, but not in the control cells. **(Inset)** A control for the non-specific binding of the secondary antibody alone showed no TROMA-1 staining. Data are representative of 3 independent experiments. Scale bar = 15μm.


DAPI
TROMA-1
MERGE
Control
Control
Control
RA
RA
RA
pcDNA3.1-Gata6
pcDNA3.1-Gata6
pcDNA3.1-Gata6

3.3 *Wnt6 mRNA is expressed during Gata6-induced differentiation of extraembryonic endoderm*

With the evidence that *Wnt6* is up-regulated in F9 cells in response to RA and can promote PrE differentiation when overexpressed in F9 cells [7], and given the presence of a putative GATA binding site in a region of the conserved *Wnt6* promoter (Fig. 1.2), it was hypothesized that the overexpression of *Gata6* would up-regulate *Wnt6* expression. To test this hypothesis, total RNA was collected and reverse transcribed into first strand cDNA from cells treated with RA and RA and db-cAMP, and from cells transfected with the *pcDNA3.1-Gata6* or the empty vector (control); the latter two following 7 days of G418 selection. PCR results with cDNAs and *Wnt6* or *L14* primers showed relatively equal levels of *L14* expression under all treatments (Fig. 3.4). In contrast, a *Wnt6* amplicon was not seen in undifferentiated cells and the relative amount of signal was, as previously reported by Krawetz and Kelly (2008), reduced in cells treated with RA and db-cAMP. The presence of the *Wnt6* amplicon in cells transfected with *pcDNA3.1-Gata6* is evidence that *Gata6* overexpression directly or indirectly up-regulates the expression of *Wnt6*. Densitometric analysis of the RT-PCR data was also performed and results showed that the relative levels of *Wnt6* compared with *L14* seen in RA treated and in *pcDNA3.1-Gata6* transfected cells are statistically significant when compared to the control (P=0.024 and P=0.048, respectively; Fig. 3.4B). In contrast, the relative level between RA and db-cAMP treated cells and the control was not significantly different (P=0.241, Fig. 3.4B). Taken together, these results support my hypothesis that in the

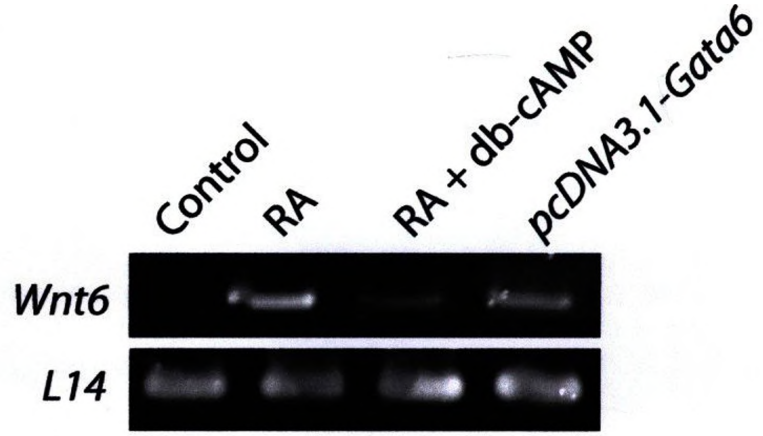
absence of RA, ectopic expression of *Gata6* directly or indirectly up-regulates the *Wnt6* gene during PrE differentiation.

3.4 *Gata6 signals through the canonical Wnt/β-catenin signaling pathway*

Since *Gata6* up-regulates *Wnt6* (this study) and *Wnt6* activates the canonical Wnt/β-catenin pathway [7], then *Gata6* in the absence of RA, should activate the canonical Wnt/β-catenin pathway. To confirm this hypothesis, cells were treated with RA, RA and db-cAMP, or transfected with the *pcDNA3.1-Gata6* construct or the empty vector (control) followed by G418 selection, and then processed for immunoblot analysis with antibodies against GSK3β and phospho-GSK3β. An increase in the level of phospho-GSK3β, the form of the protein that is incapable of degrading β-catenin, is indicative that the canonical pathway is active. Results show that cells treated with RA, RA and db-cAMP, or transfected with *pcDNA3.1-Gata6*, have intense phospho-GSK3β (P-GSK3β) staining relative to the control (Fig. 3.5A). It is interesting to note the relatively comparable intensity in staining between the RA treated and *pcDNA3.1-Gata6* transfected cells. Furthermore, by comparison, the weaker intensity of the signal in the RA and db-cAMP treated cells may be the result of a down-regulation in *Wnt6* expression in PE. Densitometric analysis of the immunoblot data showed that the normalized levels of P-GSK3β relative to GSK3β in RA, RA and db-cAMP treated and in *pcDNA3.1-Gata6* transfected cells were significantly different when compared to the levels seen in the

Figure 3.4. **Overexpression of *Gata6* induces *Wnt6* mRNA expression.** Total RNA from cells treated with RA to induce primitive endoderm or RA and db-cAMP to induce parietal endoderm, and cells transfected with empty vector (control) or *pcDNA3.1-Gata6* selected with G418 was collected and reverse transcribed into first strand cDNA for PCR. For PCR, oligodeoxynucleotide primers were designed to detect *Wnt6* or the constitutively expressed ribosomal gene *L14*. **(A)** The *Wnt6* amplicon, corresponding to 700 bp, is seen in the RA and RA + db-cAMP lanes, and in cells transfected with *pcDNA3.1-Gata6*. A *Wnt6* signal was not detected in the control. In contrast, the *L14* amplicon is present in all lanes. Data are representative of 3 independent experiments. **(B)** Analysis of the average integrated densitometric values between the *Wnt6* and corresponding *L14* signal reveals that there is a significant increase in *Wnt6* expression in RA treated and *pcDNA3.1-Gata6* transfected cells relative to the control. Analysis also reveals that the level of *Wnt6* signal in RA + db-cAMP treated cells is not significantly different from that in controls (P=0.241).

A


Control
RA
RA + db-cAMP
pcDNA3.1-Gata6
Wnt6
L14

B

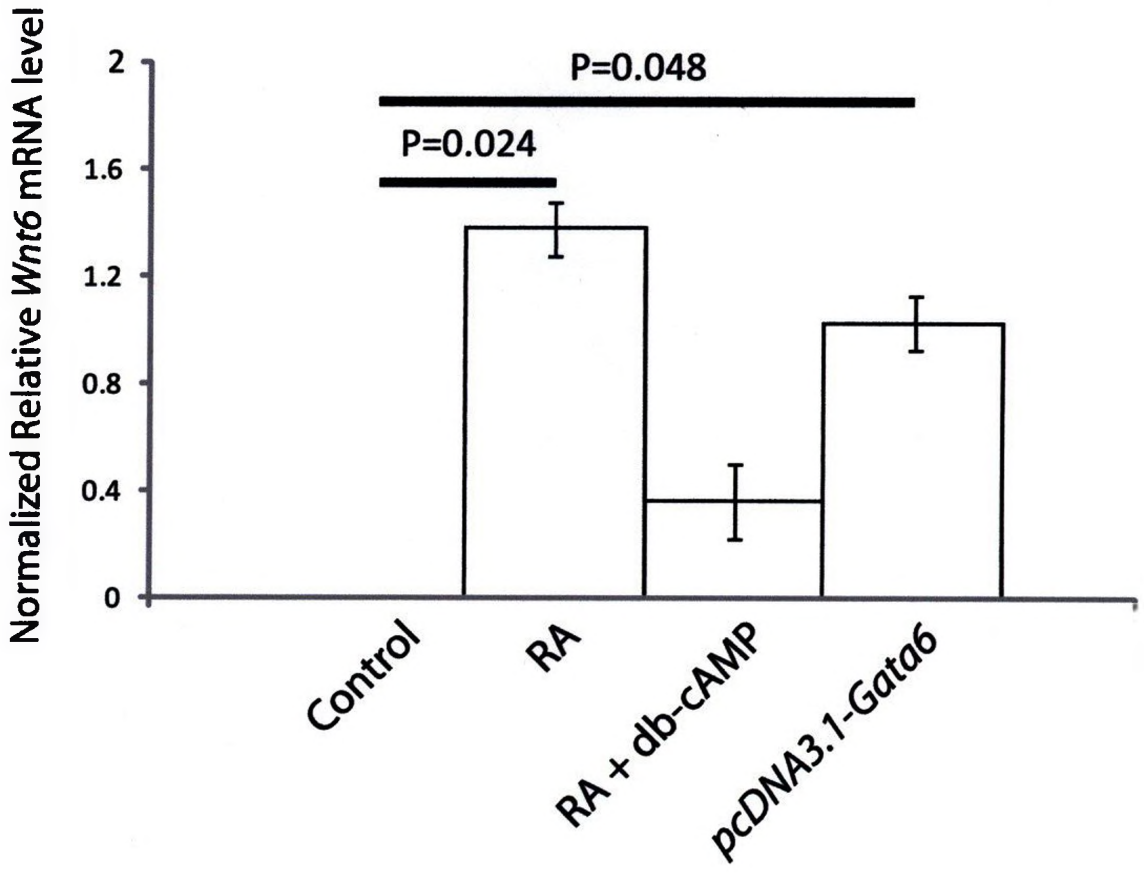
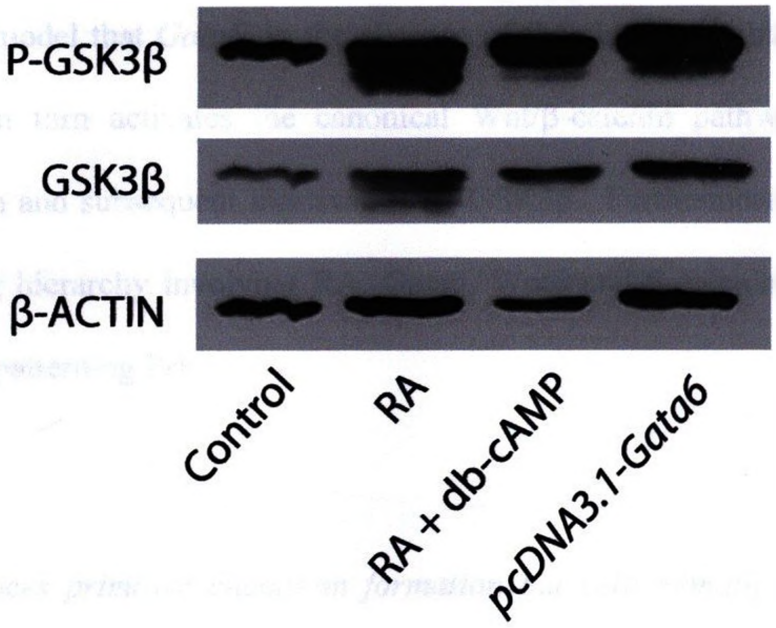
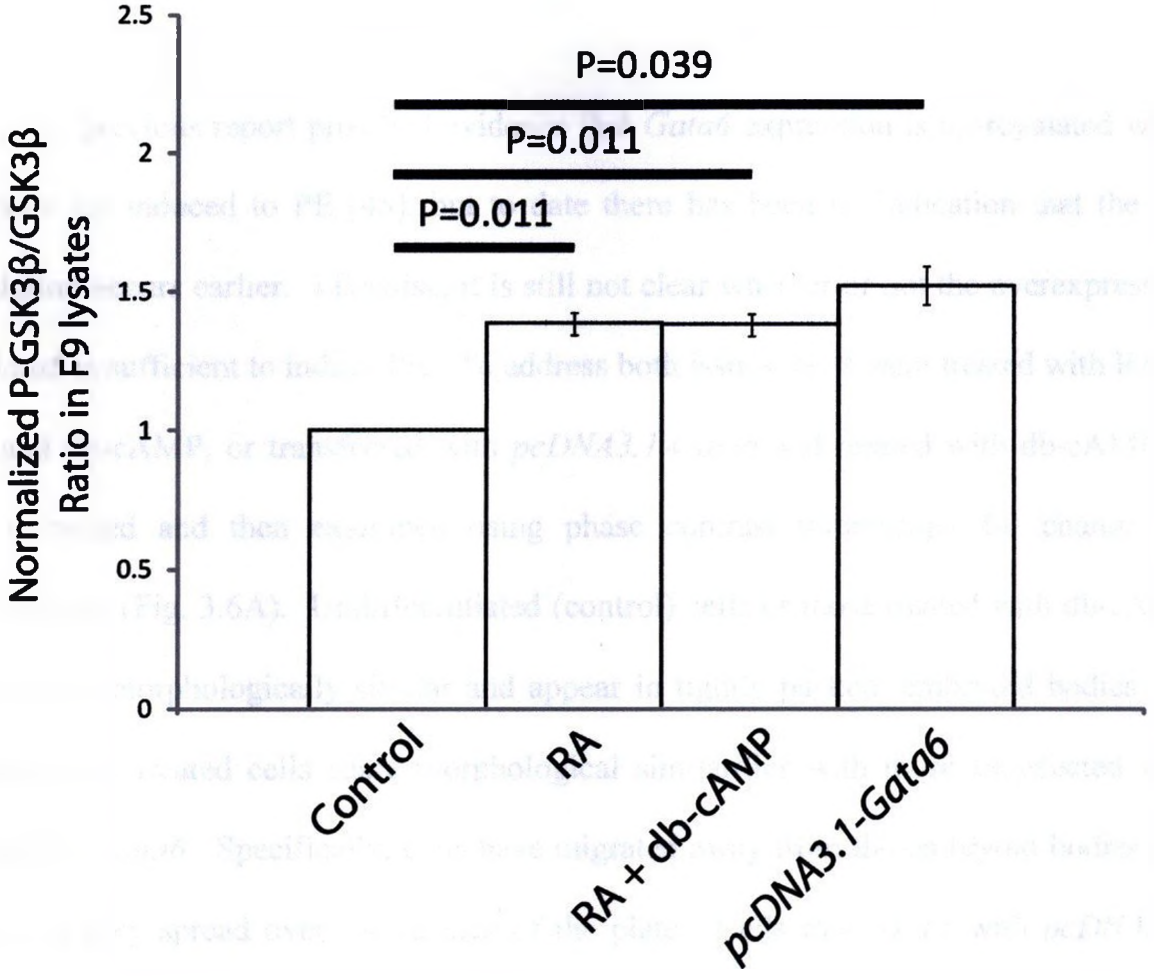

Normalized Relative Wnt6 mRNA level
2
1.6
1.2
0.8
0.4
0
P=0.048
P=0.024
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6

Figure 3.5. **Overexpression of *Gata6* activates the canonical Wnt/β-catenin pathway.** Protein lysates from cells treated with RA to induce primitive endoderm or RA and db-cAMP to induce parietal endoderm, and cells transfected with the empty vector (control) or *pcDNA3.1-Gata6* and then selected with G418 for 7 days, were collected and processed for immunoblot analysis using antibodies to phospho-GSK3β (P-GSK3β), total GSK3β (GSK3β), and β-actin as a loading control. To detect the differences in the levels of GSK3β versus the inactive P-GSK3β, blots were first probed with the phospho-specific antibody and signals revealed by enhanced chemiluminesence, and then stripped and reprobed with the GSK3β antibody. **(A)** Although the phospho-GSK3β signals are seen in all lanes, the relative intensity in the RA and RA + db-cAMP and the *pcDNA3.1-Gata6* transfected samples appears greater than that in the control. Data are representative of 3 independent experiments. **(B)** Analysis of the average integrated densitometric values between P-GSK3β and total GSK3β proteins reveals that compared to the control, there is a significant increase in the levels of P-GSK3β in RA and RA + db-cAMP treated cells and in *pcDNA3.1-Gata6*.

A


P-GSK3β
GSK3β
β-ACTIN
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6

B


Normalized P-GSK3β/GSK3β Ratio in F9 lysates
2.5
2
1.5
1
0.5
0
P=0.039
P=0.011
P=0.011
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6

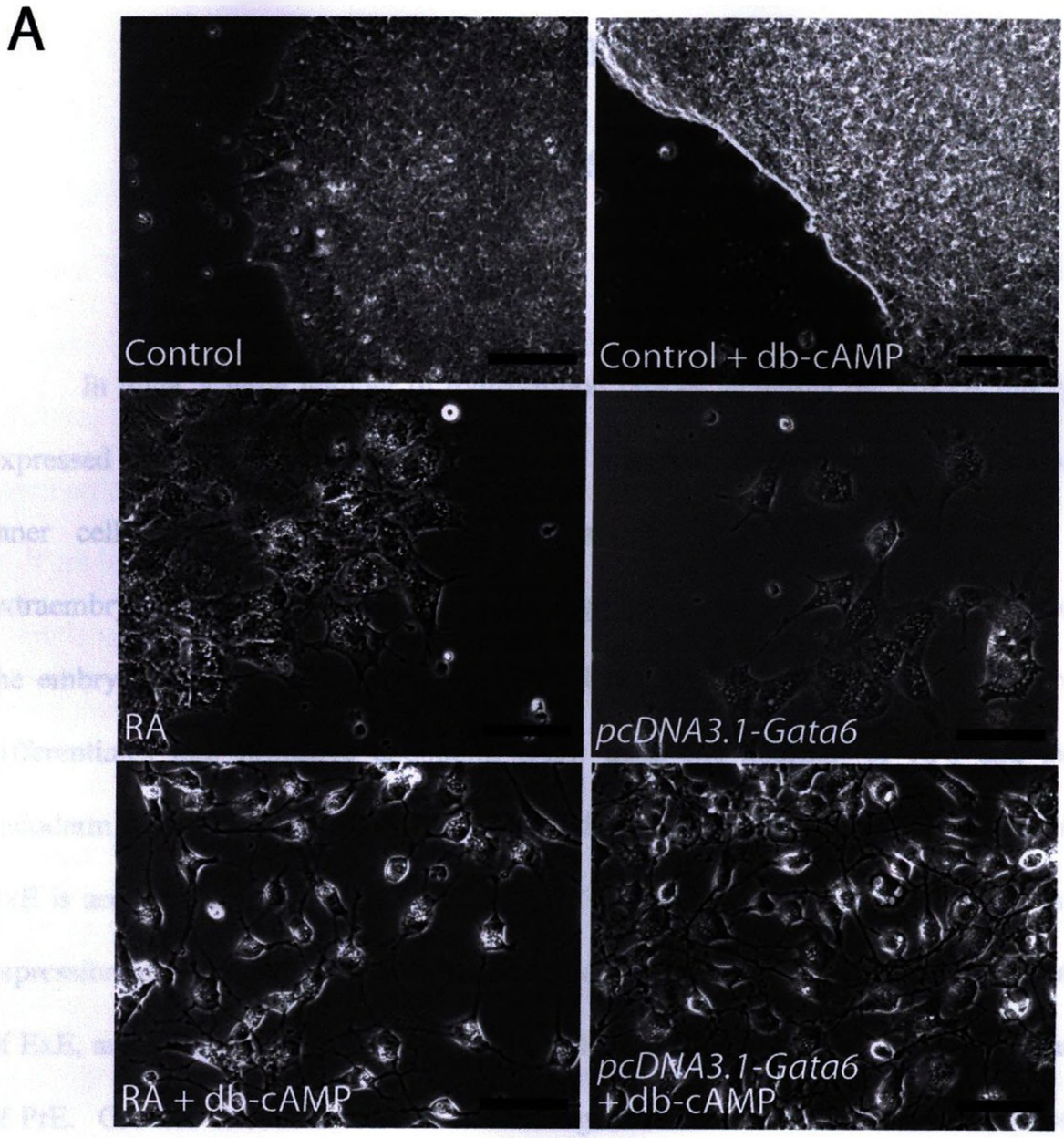
control (P=0.011, P=0.011, and P=0.039, respectively; Fig. 3.5B). These results lend support to the model that *Gata6*, in the absence of RA, is responsible for up-regulating *Wnt6*, which in turn activates the canonical Wnt/β-catenin pathway leading to the phosphorylation and subsequent inactivation of GSK3β. Furthermore, the data suggests that a signaling hierarchy involving RA, Gata6, Wnt6 and β-catenin is responsible for specifying and patterning PrE.

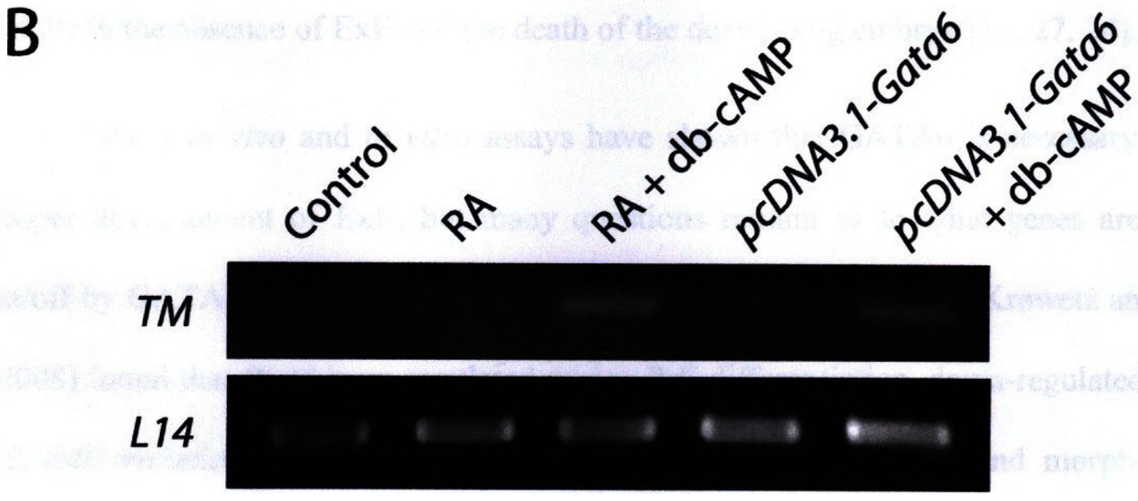
3.5 *Gata6 induces primitive endoderm formation but cells remain competent to form parietal endoderm*

A previous report provided evidence that *Gata6* expression is up-regulated when F9 cells are induced to PE [45], but to date there has been no indication that the up-regulation occurs earlier. Likewise, it is still not clear whether or not the overexpression of *Gata6* is sufficient to induce PE. To address both issues, cells were treated with RA or RA and db-cAMP, or transfected with *pcDNA3.1-Gata6* and treated with db-cAMP or left untreated and then examined using phase contrast microscopy for changes in morphology (Fig. 3.6A). Undifferentiated (control) cells or those treated with db-cAMP alone were morphologically similar and appear in tightly packed, embryoid bodies. In contrast, RA treated cells share morphological similarities with those transfected with *pcDNA3.1-Gata6.* Specifically, cells have migrated away from the embryoid bodies and flatten as they spread over the surface of the plate. Cells transfected with *pcDNA3.1-*

Gata6 and then treated with db-cAMP resembled the RA and db-cAMP treated cells. Under these conditions, cells appeared to have lost their stellate shape, and instead have rounded up and become more refractile. In addition, long slender filopodia were prominent in cells treated with RA and db-cAMP. These changes in morphology prompted further investigation using molecular markers to determine the fate of the cell resulting from the individual treatments. Total RNA was collected from these cells and reverse transcribed into cDNA for PCR analysis using primers to thrombomodulin (*TM*), a marker of PE. Results showed that *TM* was expressed, as expected in the RA and db-cAMP-induced PE (Fig. 3.6B). It was, however, not expressed in undifferentiated cells, or in RA- or *Gata6*-induced PrE. The presence of the *L14* amplicon in all lanes indicated that cDNAs were equally reverse transcribed for PCR amplification. Interestingly, a *TM* amplicon was seen in *pcDNA3.1-Gata6* expressing cells that were treated with db-cAMP (Fig. 3.6B). Taken together, these results indicate that *Gata6* is sufficient to induce PrE differentiation, and like *Wnt6* [7], these cells are competent, but do not further differentiate into PE unless supplemented with a signal (db-cAMP).

Figure 3.6. ***Gata6*-induced primitive endoderm is competent to form parietal endoderm.** **(A)** Cells that have been transfected with the empty vector (control) alone or transfected with the empty vector then subsequently treated with db-cAMP (control + db-cAMP) have similar morphology. Likewise, cells treated with RA to induce primitive endoderm are morphologically similar to those transfected with *pcDNA3.1-Gata6*. In each case, cells migrated from the embryoid bodies, characteristic of the undifferentiated cells, and adopted a stellate shape with numerous filopodia. Finally, cells transfected with *pcDNA3.1-Gata6* and treated with db-cAMP showed morphological similarities to RA and db-cAMP treated cells. Under these conditions the cells appeared more ovoid, exhibited contacting refractile bodies, and possessed relatively long filopodia. **(B)** Total RNA from cells treated with RA or RA and db-cAMP, and cells transfected with the empty vector (control), *pcDNA3.1-Gata6*, or transfected with *pcDNA3.1-Gata6* and treated with db-cAMP was collected and reverse transcribed into first strand cDNA for PCR. Oligodeoxynucleotide primers for PCR were used to detect Thrombomodulin (*TM*) expression, indicative of parietal endoderm, or *L14*, a constitutively expressed ribosomal gene. *TM* expression is only seen in cells treated with RA and db-cAMP and in those transfected with *pcDNA3.1-Gata6* then treated with db-cAMP. The presence of the *L14* amplicon indicates that cDNAs were present under all conditions. The control and *pcDNA3.1-Gata6* transfected cells were selected with G418 for 7 days or selected with G418 for 5 days then treated with db-cAMP for 4 days with continual G418 selection. Scale bars in **(A)** = 20 μm.


A
Control
Control + db-cAMP
RA
pcDNA3.1-Gata6
RA + db-cAMP
pcDNA3.1-Gata6
+ db-cAMP


B
Control
RA
RA + db-cAMP
pcDNA3.1-Gata6
pcDNA3.1-Gata6
+ db-cAMP
TM
L14

CHAPTER 4

DISCUSSION

In mice, a large number of transcription factors including the GATA family, are expressed shortly after fertilization. GATA6 is initially expressed in some cells of the inner cell mass at the time of implantation and these cells eventually form extraembryonic endoderm (ExE), a cell lineage required for the proper development of the embryo proper [13, 27]. My study used the F9 teratocarcinoma cell line, which differentiates into primitive endoderm (PrE) upon the addition of RA, and parietal endoderm (PE) upon treatment with RA and db-cAMP, to show that differentiation into ExE is accompanied by the up-regulation of *Gata6*. Furthermore, results show that the expression of *Gata6* alone is sufficient to induce biochemical and morphological markers of ExE, and is the first to report that in F9 cells, *Gata6* is expressed during the induction of PrE. GATA6 is considered as a master regulator of ExE differentiation because it is the first ExE-specific transcription factor to be expressed *in vivo*, and its loss-of-function results in the absence of ExE and the death of the developing embryo [13, 27, 28].

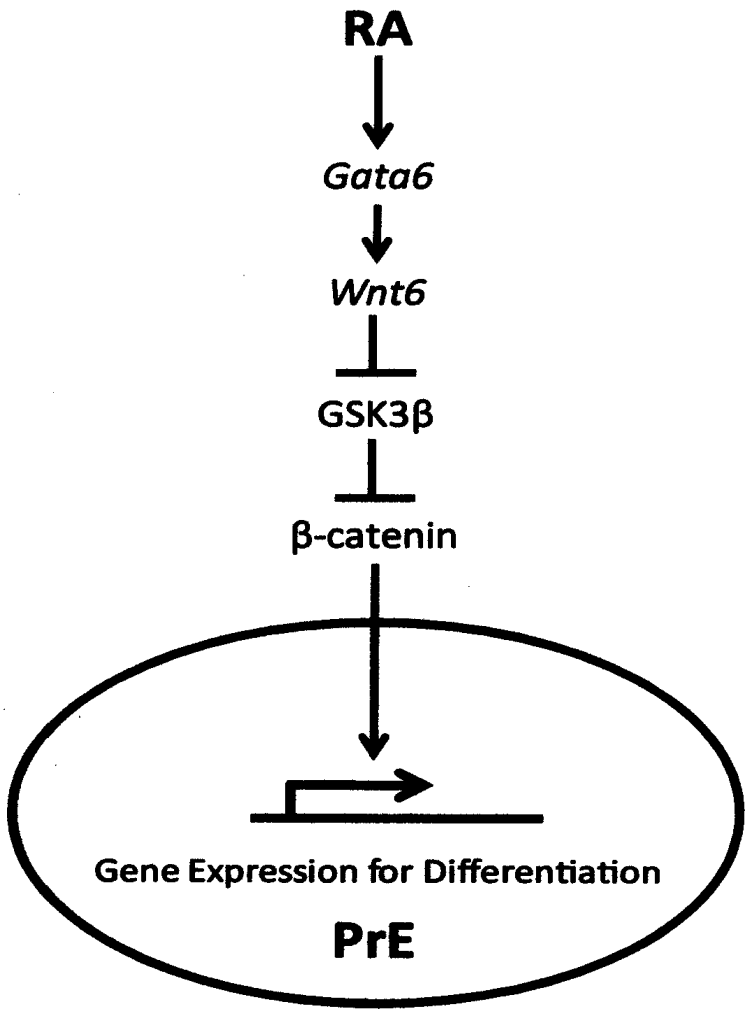
Many *in vivo* and *in vitro* assays have shown that GATA6 is necessary for the proper development of ExE, but many questions remain as to what genes are turned on/off by GATA6 during differentiation [12, 27, 28, 46]. A study by Krawetz and Kelly (2008) found that *Wnt6* is up-regulated during PrE differentiation, down-regulated during PE differentiation, and sufficient by itself to induce biochemical and morphological

markers of PrE. The authors also found that the expression of *Wnt6* leads to the activation of the canonical Wnt/β-catenin pathway, allowing for the accumulation and translocation of β-catenin into the nucleus, where it then interacts with TCF/LEF to regulate the genes necessary for PrE specification [7]. My study is the first to indicate that during PrE specification, *Gata6* regulates the expression of *Wnt6* mRNA, which leads to the activation of the canonical Wnt/β-catenin pathway and subsequent inactivation of GSK3β. Currently, efforts are underway to determine if the up-regulation of *Wnt6* by *Gata6* is direct or indirect. Furthermore, the question remains whether or not Wnt6 is necessary for the induction of PrE or if GATA6 is capable of up-regulating other genes that directly up-regulate Wnt6, or other Wnts that, like Wnt6, are sufficient to induce differentiation. In fact, recent evidence from this lab has also implicated one member of another family of transcription factors that may be involved in regulating *Wnt6* expression: the Forkhead Box A subfamily (FoxA). The vertebrate FoxA subfamily is comprised of *FoxA1, A2,* and *A3* genes and the proteins encoded by these transcripts are known to play important roles in early development, organogenesis, metabolism, and homeostasis [48]. Early in development, evidence exist that *FoxA2* is a downstream target of GATA6 [27] and similar to GATA6 nulls, embryos carrying a targeted deletion of *FoxA2* lack a definitive node or notochord and die from ExE defects [49, 50]. Interestingly, electrophoretic mobility shift assays show that FOXA2 binds to the *Wnt7b* promoter and co-transfection assays demonstrate that FOXA2 can activate the *Wnt7b* promoter [51]. Although the induction of *Wnt6* by the overexpression of *FoxA2* in F9 cells and the activation of the canonical Wnt/β-catenin pathway during PrE differentiation (unpublished data) are consistent with *Gata6*, PCR and microarray studies

have revealed that Wnt*1, 2b, 3, 3a, 4, 5a, 6, 7a, 7b, 10b, and 11* are expressed at pre-implantation stages of development. Since *Wnt1, 2b, 3, 3a, 6, 7b* are also known to activate the canonical Wnt/β-catenin pathway [52-57], a knockdown of *Wnt6* using specific siRNAs in the presence of the *pcDNA3.1-Gata6* expression construct is necessary to address the importance of this Wnt in the differentiation process.

With the aforementioned information and previous findings from the lab [7], a model has been proposed for PrE differentiation (Fig. 4.1). In F9 cells, RA up-regulates the expression of *Gata6*, which in turn up-regulates the expression of the *Wnt6* gene. The expression of *Wnt6* leads to the activation of the canonical Wnt/β-catenin pathway through the destabilization of the degradation complex including GSK3β, which then allows for the accumulation and translocation of β-catenin into the nucleus. β-catenin, together with TCF/LEF is now able to activate and repress the genes necessary for PrE differentiation. The proposed model, however, is far from complete. Considering that *Gata6* and *FoxA2* are transcription factors with multiple target genes, there is a distinct possibility that there may be more than one signaling pathway involved during the induction of ExE. Given the fact that many of these pathways employ identical proteins to mediate signaling, the possibilities exists that crosstalk between pathways is used to amplify or abridge the signal(s) required for differentiation. Elucidating these different pathways and the players involved is the subject of future investigations.

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. Following transcription and translation, the Wnt6 ligand signals to neighbouring cells, recruiting and activating Dishevelled, which dismantles the GSK3β degradation complex. The inability of GSK3β to negatively regulate β-catenin allows cytoplasmic β-catenin levels to increase and eventually translocate to the nucleus, where with Tcf/Lef transcription factors, it activates/represses the genes required for primitive endoderm differentiation.


RA
Gata6
Wnt6
GSK3β
β-catenin
Gene Expression for Differentiation
PrE

CHAPTER 5

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