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Signaling Events During Extraembryonic Endoderm Differentiation

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ABSTRACT

Mouse F9 cells differentiate into primitive endoderm (PrE) when treated with retinoic acid (RA) and into parietal endoderm (PE) following subsequent treatment with dibutyryl cAMP. *Wnt6* is up-regulated in PrE cell, and although it is sufficient to induce differentiation by signaling through the canonical WNT/β-catenin pathway, the mechanism by which the *Wnt6* gene is regulated is not known. In addition to WNT signaling, PrE differentiation is accompanied by an increase in reactive oxygen species (ROS). ROS have been implicated in regulating the canonical WNT/β-catenin signaling pathway through Nucleoredoxin (NRX), but whether they are sufficient to induce extraembryonic endoderm *in vitro* is not known. In F9 cells the overexpression of *Gata6* or *Foxa2*, which are two integral members responsible for patterning extraembryonic endoderm, induces biochemical and morphological markers of PrE by directly up-regulating the expression of *Wnt6*, and activating the canonical WNT/β-catenin signaling pathway. Treating cells with H_2O_2, or knocking down the expression of *Nrx* also activates canonical WNT/β-catenin signaling leading to the induction of these markers. Treating cells with antioxidants, however, impedes the ability of RA to induce PrE. Furthermore, and regardless as to how F9 cells are induced, these PrE cells remain competent and differentiate into PE when treated with db-cAMP. Together, these results indicate that *Gata6* and *Foxa2* are responsible for initiating the canonical WNT/β-catenin pathway in F9 cells and ROS, impinging on NRX, regulate the pathway necessary for PrE differentiation.

Keywords: F9, primitive endoderm, parietal endoderm, extraembryonic endoderm, differentiation, GATA6, FOXA2, WNT6, ROS, canonical WNT/β-catenin

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LIST OF ABBREVIATIONS

^{O}C – degrees Celsius

μM – Micromolar

Afp – Alpha fetoprotein

APC – Adenomatous polyposis coli

ATCC – American Type Cell Culture

BARL – β-catenin activated reporter luciferase

BD – Becton Dickinson

BSA – Bovine serum albumin

BS/KS – Bluescript KS-

Dab2 – Disabled homolog 2

db – Dibutyryl

Ca^{2+} – Calcium

cAMP – Cyclic adenosine-monophosphate

CCD – Charge-coupled device

cDNA – Complimentary deoxyribonucleic acid

C. elegans – Caenorhabditis elegans

ChIP – Chromatin immunoprecipitation

CK1γ – Casein kinase 1 gamma

CMV – Cytomegalovirus promoter

CO_2 – Carbon dioxide

CS – Creative suites

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

DCF – Dichlorodihydrofluorescein

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

DPI – Diphenyleneiodonium chloride

Duox – Dual oxidase

DVL – Dishevelled

EC – Embryonal carcinoma

EMT – Epithelial-to-mesenchymal transition

ES – Embryonic stem

ExE – Extraembryonic endoderm

FBS – Fetal bovine serum

Fig – Figure

FZD – Frizzled

G418 – Neomycin sulfate

GFP – Green fluorescence protein

GSK – Glycogen synthase kinase

H_2O_2 – Hydrogen peroxide

HCl – Hydrogen chloride

HNF – Hepatocyte nuclear factor

$HO^{\cdot}$ – Hydroxyl radical

hr – Hour

HRP – Horseradish peroxidase

hrs – Hours

HSD – Honest significant difference

ICM – Inner cell mass

JNK – c-Jun N-terminal kinase

Kb – Kilobase

LEF – Lymphoid enhancer factor

LRP – Lipoprotein-related protein

M – Molar

mA - Milliamperes

min – Minute

ml - Milliliter

mm – Millimeter

mM – Millimolar

mRNA – Messenger ribonucleic acid

NAC – N-acetyl cysteine

NADPH – Nicotinamide adenine dinucleotide phosphate

NaF – Sodium fluoride

Na_3VO_4 – Sodium orthovanodate

ng – Nanograms

nM – Nanomolar

Nox – NADPH oxidase

NRX – Nucleoredoxin

O_2^- – Superoxide anion

ON – Ontario

PBS – Phosphate buffered saline

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

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

ROS – Reactive oxygen species

RNA – Ribonucleic acid

RNAi – Ribonucleic acid interference

RT – Reverse transcriptase

s – Seconds

sc – Scrambled

SDS – Sodium dodecyl sulfate

sh – Short hairpin

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

TC – Tissue culture

TCF – T-cell specific transcription factor

TE – Trophectoderm

TF – Transcription factor

TM – Thrombomodulin

TRITC – Tetramethyl Rhodamine Isothiocyanate

Trolox – 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

TROMA-1 – Trophectodermal monoclonal antibody-1

TRX – Thioredoxin

TS – Trophoblast stem

µg – Microgram

µl – Microliter

VE – Visceral endoderm

Wnt/WNT – Wingless/integrated

XEN – Extraembryonic endoderm

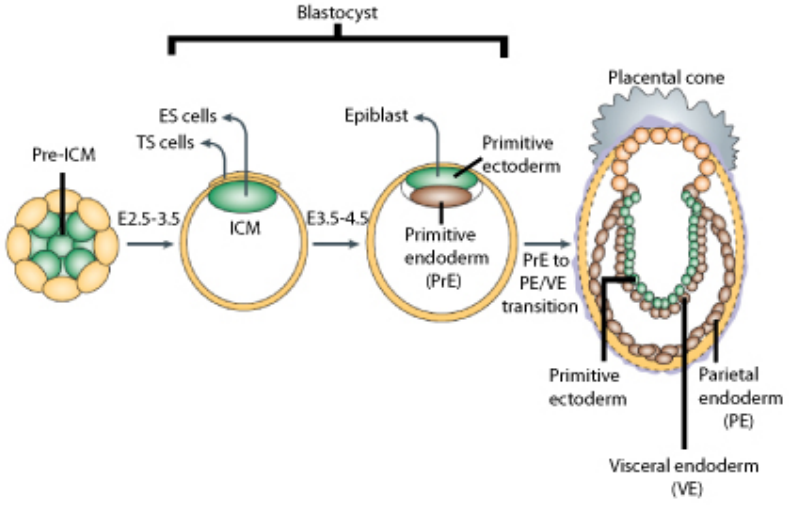
CHAPTER 1

GENERAL INTRODUCTION

1.1 Early mouse embryogenesis

In mammals, the fertilization of the egg occurs in the oviduct through complex processes that are not yet fully understood. Following successive rounds of cell divisions, a blastocyst forms and around the time of implantation, it is comprised of three cell layers: the epiblast, derived from the inner cell mass (ICM), contains embryonic stem (ES) cells expressing Oct4 and Nanog [1, 2] that give rise to the entire fetus (embryo proper); the trophectoderm (TE), which contains trophoblast stem (TS) cells expressing Cdx2 [3] that gives rise to the placenta; and Gata6 and Foxa2 expressing primitive endoderm (PrE) [4, 5], 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 embryogenesis and well before any cell fate decisions take place within the embryo proper, is of paramount importance for normal embryonic development [6-8]. The cells of the TE, in direct contact with the ICM, attaches to the uterine epithelium to initiate implantation [7] and the placenta forms shortly thereafter [9]. The PrE further differentiates into PE, which migrates beneath the TE to form the parietal yolk sac and the VE, which forms 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
Placental cone
ES cells
TS cells
Epiblast
Primitive ectoderm
Pre-ICM
E2.5-3.5
ICM
E3.5-4.5
Primitive endoderm (PrE)
PrE to PE/VE transition
Primitive ectoderm
Parietal endoderm (PE)
Visceral endoderm (VE)

visceral yolk sac [6]. The proper segregation and development of the extraembryonic tissues are crucial for the survival and patterning of the embryo proper [10, 11]. Given that these tissues arise early during development and are responsible for subsequent developmental processes, 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 the earliest epithelial-to-mesenchymal transitions (EMTs) in mouse development. An EMT, a process by which cells lose their polarity and cell-cell adhesion, and gain migratory and invasive properties, have been shown to be critical for the development of tissues and organs in the developing embryo [12]. 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 [13]. 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 more favored and second 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 the testis of a 129/Sv mouse [14]. F9 cells grow in culture as tightly packed colonies that appear homogenous [15]. The addition of retinoic acid (RA) to the culture induces morphological and biochemical changes that result in the differentiation of cells into PrE [15]. 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 [16]. PrE and PE cells that differentiate from the parental F9 cells express and secrete many of the same factors found in extraembryonic tissues of the developing mouse embryo [10, 17]. These factors activate numerous signal transduction pathways including those that rely on the Wnt ligand and β-catenin [18].

1.3 WNT signaling pathways

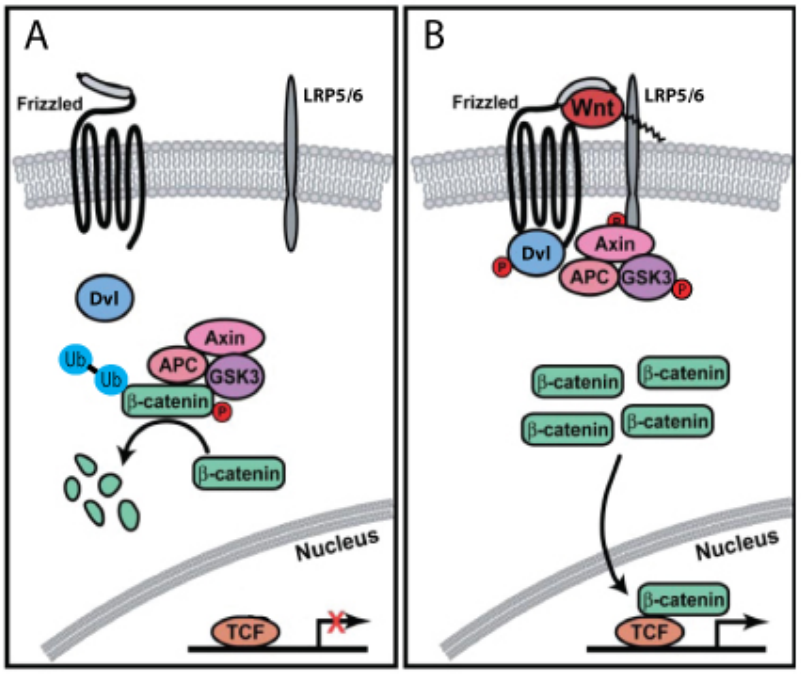
Wnt genes were first identified in *Drosophila* [19] and in mammals, there are 19 Wnt genes all encoding secreted lipid-modified glycoproteins [20, 21]. In mouse, *Wnts* are first detected in the ICM and the cells surrounding the blastocyst cavity shortly after fertilization. That their expression continues throughout gastrulation, organogenesis, and into adult life underscores their involvement in a number of developmental processes [22-24]. Despite the presence of *Wnts* during the pre-implantation stages, the signaling pathway may not be active; active β-catenin is not detected at the pre-implantation stages

[25], however, recent evidence suggests that the first active WNT signaling may occur at the time of implantation during PrE differentiation [18].

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 [26-28]. Under normal circumstances, the pathway is activated by a secreted member of the WNT family, serving as a ligand for one or more members of a group of seven-transmembrane Frizzled (FZD) receptors [4]. In the absence of WNT, a degradation complex composed of glycogen synthase kinase-3β (GSK-3β), casein kinase 1α, Axin, and adenomatous polyposis coli (APC), targets β-catenin for phosphorylation, ubiquitination, and ultimately proteasomal degredation. In the presence of WNT, however, the interaction between WNT, FZD, and a co-receptor lipoprotein-related protein 5/6 (LRP5/6), activates one or more of the Dishevelled (DVL) cytoplasmic phosphoproteins, which recruits Axin away from the destruction complex, which now is no longer active. This inactivation allows cytoplasmic β-catenin levels to increase. β-catenin is now positioned to translocate to the nucleus, where with T-cell factors-lymphoid enhancer factors (TCFs-LEFs) serves as a transcriptional co-activator of many target genes (Fig. 1.2).

As noted above, not all WNTs are involved in the canonical signaling pathway; two β-catenin independent or non-canonical pathways also utilize FZD and/or DVL. In the planar cell polarity pathway (PCP), the binding of WNT to FZD recruits DVL to the plasma membrane, which results in the activation of the Jun-N-terminal Kinase-Rho-Rac

Figure 1.2. **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
APC
GSK3
Ub
Ub
β-catenin
P
β-catenin
Nucleus
TCF
B
Frizzled
Wnt
LRP5/6
P
Dvl
Axin
APC
GSK3
P
β-catenin
β-catenin
β-catenin
β-catenin
Nucleus
β-catenin
TCF

pathway. This activation is necessary to induce the changes to the cytoskeleton that are needed by cells during gastrulation [29-31]. The second or least understood non-canonical WNT-Calcium pathway involves the activation of FZD, G-proteins, calcium/calmodulin-dependent-kinase II, and protein kinase C [32], and much like the PCP pathway, seems to play a fundamental role in body axis specification and cellular movements during embryogenesis [33, 34].

1.4 Reactive oxygen species

In addition to classical signaling pathways, recently the impact of other factors has been shown to have important roles during early mouse embryogenesis. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and superoxide anion (O_2^-), consist of radical and non-radical oxygen species. Historically, ROS are considered by-products from the incomplete reduction of oxygen through cellular respiration resulting in oxidative stress. This oxidative stress results in ROS-mediated damage of nucleic acids, lipids, and proteins, and has been implicated in age-related and vascular diseases like neurodegeneration and atherosclerosis, respectively [35-38]. Although ROS are known for their deleterious effects on various targets, convincing studies have shown that the specific production of ROS, especially by membrane bound NADPH oxidases (NOX), is beneficial where the products are used for a variety of physiologically and developmentally relevant purposes [39].

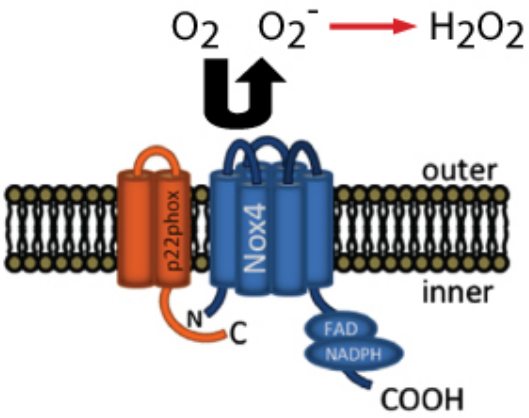
1.5 NADPH oxidase

NADPH oxidase or Nox proteins are membrane-associated, multi-unit enzymes that catalyze the reduction of oxygen using NADPH as an electron donor. Nox proteins produce O_2^- radicals via a single electron reduction. Previous studies have reported on Nox involvement in a respiratory burst in phagocytes of the innate immune system [40]. Over the last decade, however, NOX family members and the ROS they produce have been identified as contributors to many important signaling pathways [41]. In mammals, the NOX family consists of isoforms 1-5, and Dual oxidase (DUOX) 1 and 2 [42-48]. Structurally, all of the NOX members contain both transmembrane and cytosolic domains, and share many of the regulatory subunits necessary for their activities [41]. A detailed review of Nox enzymes by Brown and Griendling [41] discuss recent literature on distribution, localization, and activation. For the purpose of my thesis, I will focus on Nox4, as outlined below.

1.6 NOX4

NOX4 originally described as Renox from its expression levels in the kidney, is unique among the catalytic NOX subunits, since it only requires the membrane subunit p22phox for ROS-producing activity (Fig. 1.3) [49]. Unlike NOX1, 2, and 3 whose activity depends on the presence of activator or organizer subunits [50] NOX4 is active in cells that do not express these cytoplasmic subunits [51]. Since its discovery in the

Fig. 1.3. **NOX4 produces hydrogen peroxide.** Oxidase activity occurs when NADPH binds to Nox on the cytosolic side and transfers electrons to FAD and then to oxygen (O_2) on the outer membrane surface, resulting in oxygen anion (O_2^-) formation. Since O_2^- is highly unstable, hydrogen peroxide (H_2O_2) is formed rapidly. The transmembrane subunit p22phox associates with the catalytic Nox4 subunit regardless of enzymatic activity. N: amino-terminus; C: carboxy terminus; COOH: carboxylic acid; FAD: flavin adenine dinucleotide; NADPH: nicotinamide adenine dinucleotide phosphate. Modified from Brown and Griendling (2009).


O2
O2-
H2O2
outer
inner
p22phox
Nox4
N
C
FAD
NADPH
COOH

kidneys, *Nox4* has been shown to be expressed in endothelial cells, fibroblasts, and embryonic stem cells [52-54]. The observations that *Nox4* is an inducible NOX, that its ability to produce ROS in the form of H_2O_2 is proportional to NOX4 protein expression [49], and its ubiquitous tissue distribution compared to other NOX homologues [55], has implicated NOX4 as the prime candidate for my thesis research.

1.7 NRX: The link between ROS and WNT

It has been recently reported that when F9 cells are treated with RA to induce differentiation to PrE, there is an accompanied increase in ROS levels and in the absence of RA, F9 cells treated with H_2O_2 differentiate to PrE [56]. This work also highlighted the importance of ROS in the differentiation process as reducing ROS levels by using antioxidants attenuated differentiation [56]. The mechanism(s) by which ROS act on PrE differentiation is/are not known, but evidence from other models suggests the involvement of Nucleoredoxin (NRX). Amino acid sequence comparisons indicate that NRX is part of the Thioredoxin (TRX) family of redox sensor proteins [57]. TRX, a ubiquitously expressed and evolutionarily conserved protein, catalyzes NADPH-dependent reductions of disulfide bridges and functions as a disulfide oxidoreductase [57]. Under oxidizing conditions, the thiol functional group on the two cysteine residues form a disulfide bridge capable of changing protein function or modulating protein-protein interactions [58].

Evidence from whole-mount *in situ* hybridization studies using mouse embryos show strong *NRX* expression in growing limb buds and somites [57], but nothing until our report was known regarding a role for NRX in ExE formation [59]. The inaugural report by Funato et al., using NIH3T3 cells, showed that canonical WNT/β-catenin signaling is redox regulated resulting from the interaction between NRX and DVL [60]. The observation that the interaction between NRX and DVL increases under reduced conditions and decreases when subjected to oxidizing conditions suggests the redox state of the cell regulates these signal transduction events [60]. Under reducing conditions, the interaction between NRX and DVL augments the negative regulation of β-catenin in the WNT pathway via the destruction complex, while under oxidizing conditions NRX dissociates from DVL, allowing the latter to destabilize the destruction complex in the presence of a WNT ligand, leading to β-catenin dependent gene activation [60].

1.8 Objectives of study and hypotheses

The goal of this study was to investigate whether the canonical Wnt signaling pathway is involved in the differentiation of ExE. As mentioned earlier in relation to the mouse model system, ExE differentiation is the first EMT to occur during mouse development. Since this EMT occurs *in utero* at the peri-implantation stage of development, I used the F9 model cell line to investigate how this process occurs *in vivo*. RA-induction of F9 cells mimic morphological and biochemical characteristics of primitive endoderm. Since RA induces many target genes ultimately leading to canonical

WNT/β-catenin signaling necessary for PrE differentiation [18], I hypothesized that Gata6 and Foxa2, two regulators of extraembryonic endoderm, act in a positive fashion during these inductive events. Furthermore, the noted increase in ROS induced by RA leading to PrE and its cytosolic source led to the additional hypothesis that NOX proteins played a role in ExE differentiation [56]. Pre-treating cells with DPI, a NOX inhibitor, attenuated PrE formation [56]. Lastly, I tested the hypothesis that canonical WNT/β-catenin signaling which is obligatory for ExE formation [56], is influenced by crosstalk imparted by ROS and provide evidence that this influence is dependent on NRX. Together, this new data provide the requisite foundational studies required to address questions pertaining to WNT signaling and ExE formation *in vivo*.

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CHAPTER 2

GATA6 AND FOXA2 REGULATE WNT6 EXPRESSION DURING EXTRAEMBRYONIC ENDODERM FORMATION

2.1 Introduction

2.1.1 Extraembryonic endoderm

In mouse development the differentiation of cells in the inner cell mass (ICM) to primitive and then to parietal extraembryonic endoderms (PrE and PE, respectively), is one of the earliest epithelial-to-mesenchymal transitions (EMTs) [1, 2]. 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 [3, 4]. The F9 teratocarcinoma cell line is an ideal model to study how extraembryonic endoderm (ExE or XEN) differentiates *in vitro*. The addition of retinoic acid (RA) to these cells induces morphological and biochemical changes, leading to the formation of PrE [5]. 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) [6]. Gene profiling studies on F9 cells have shown that RA regulates the expression of many genes [7, 8]. Furthermore, our lab and others have reported on specific proteins that are

sufficient to induce cells to form PrE [9, 10], and many of these are linked to the canonical WNT/β-catenin signaling pathway [11-13].

2.1.2 Canonical WNT signaling

WNTs are secreted glycoproteins that are involved in a plethora of developmental processes [14-17]. Wnt expression is first detected in the ICM and in cells surrounding the blastocoele cavity, [18-20]. In humans, one or more of the 19 different WNTs are expressed normally throughout gastrulation, organogenesis, and into adulthood, but they can also be expressed inadvertently, as evident in a variety of dissimilar cancers [21-23]. Historically, WNTs have been classified based on their ability to signal through either the canonical β-catenin or non-canonical pathways. The canonical WNT pathway is activated when a WNT ligand binds to one of a group of seven-transmembrane Frizzled (FZD) receptors [2]. The pathway involves a complex of proteins that are regulated by post-translational modifications. In the absence of WNT, β-catenin is recruited to a destruction complex of proteins including APC and AXIN, where it is phosphorylated by GSK-3β and CK1γ. Phosphorylation primes β-catenin for ubiquitination, leading to its degradation in a proteasome-dependent manner. When WNT is present, however, it binds to its FZD receptor and LRP5/6 co-receptor, which recruits the multi-domain containing protein Dishevelled (DVL) to the plasma membrane where it binds to FZD. GSK-3β and CK1γ now phosphorylate LRP5/6, which together with DVL facilitates the translocation and binding of AXIN to DVL and LRP5/6. AXIN can no longer participate

as part of the destruction complex, allowing β-catenin to accumulate in the cytoplasm. Subsequent translocation of β-catenin into the nucleus facilitates its interaction with TCF/LEF transcription factors to impart changes in gene expression.

2.1.3 Non-canonical WNT signaling

In the case of either the planar cell polarity (PCP) or WNT/Ca^{2+} non-canonical pathways, signaling occurs via WNT, FZD and DVL, but further downstream events are independent of β-catenin [24]. In the PCP pathway, WNT binding to FZD recruits DVL to the plasma membrane, which results in the activation of Rho-Rac-JNK pathway. This activation is necessary to induce the changes to the cytoskeleton that are needed for coordinated cell movement [25-27]. In the WNT/Ca^{2+} pathway, the activation of FZD, Knypek, Ror2, and G-proteins, trigger downstream effectors including calcium/calmodulin-dependent kinase IIα, and PKC [24]. Much like the PCP pathway, the WNT/Ca^{2+} pathway influences cell polarity, cell adhesion, cell shape, as well as the nuclear factor of activated T-cells (NF-AT) [14, 28, 29]. The involvement of the WNT/Ca^{2+} pathway during ExE differentiation is not well understood, but there is evidence that p38 MAPK activation in F9 cells engineered to express rat FZD2, occurs in a DVL-independent manner following WNT5a stimulation [30]. In contrast, the canonical WNT and the PCP signaling pathways are known to play an important role during PrE differentiation.

2.1.4 Primitive endoderm differentiation

RA treatment of P19 cells activates RhoA, Rac1, and JNK in the PCP pathway, which is sufficient to induce PrE differentiation [31]. RA also up-regulates the expression of *Wnt6* in F9 cells, which activates the canonical β-catenin pathway, leading to PrE formation [12]. The same is true for WNT3a, when applied to F9 cells ectopically expressing rat FZD1 [32]. Although the link between RA and the differentiation of ExE *in vitro* is clear, that between RA and WNT is not. Towards that end and before implementing *in vivo* studies, we decided to elucidate the mechanism(s) responsible for the RA-dependent induction of *Wnt6* that initiates PrE formation *in vitro*. Numerous studies have identified possible candidates as regulators involved in PrE differentiation [7, 33]. *Gata6*, a direct target gene of RA signaling [7], and *Foxa2*, a target gene of GATA6 [34], are well known players in endoderm formation. In the mouse embryo GATA6 is expressed in some ES cells of the ICM [1, 2], which later become the cells of the ExE [35]. Gene targeting experiments has revealed that *Gata6* null mice die shortly after implantation [35]. Furthermore, *in vitro* studies show that *Gata6* expression is up-regulated when ES cells are treated with RA and this is sufficient to down-regulate the pluripotency marker *Oct-3/4*, and to induce ExE differentiation [34]. Interestingly, *Gata6* null ES cells 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 [34-36]. Although the evidence indicates that GATA6 is sufficient and necessary for RA-induced ExE differentiation of ES cells [34], and has also been proposed to bind to the rat and

human *WNT6* promoters [37], it is not known whether or not it signals directly or indirectly through WNT6 to induce ExE.

FOXA2 (HNF3β), initially identified as a liver-specific transcription factor [38], is another regulator of mouse visceral and definitive endoderm formation [39-42]. The visceral endoderm, a derivative of PrE [43], has been classified as extraembryonic tissue required for supporting the proper growth of the embryo [44]. FOXA2 expression is also essential as *Foxa2* null mice die between 6.5 and 9.5 days post fertilization due to a lack of a definitive node and notochord, and severe constriction at the embryonic-extraembryonic junction [40, 45]. Furthermore, these authors noted that mutant embryos often develop outside of the yolk sac, with defects in axial elongation and anterior development. Although FOXA2 is considered a marker of visceral endoderm [46], it is also expressed in PrE as evident in F9 cells induced by RA [47], and like GATA6, may bind to the rat and human *WNT6* promoters [37]. This information, together with that for GATA6 was enough to warrant further investigation on how *Wnt6* is regulated during extraembryonic endoderm formation.

2.1.5 Objectives of study

Here we provide new evidence for a role for GATA6 and FOXA2 regulating a canonical WNT/β-catenin signaling pathway involved in the differentiation of F9 cells to primitive extraembryonic endoderm. In this study we show that overexpression of *Gata6* or *Foxa2* in the absence of RA was accompanied by an increase in *Wnt6* expression and

corresponding changes in cell shape that are hallmarks of PrE derived from F9 cells. Immunoblot and immunocytochemistry revealed that in the absence of RA, F9 cells overexpressing *Gata6* or *Foxa2* induced the appearance of TROMA-1 intermediate filaments characteristic of ExE. *Gata6* or *Foxa2* overexpressing F9 cells also had elevated levels of TCF-dependent transcription, indicative of active canonical WNT/β-catenin signaling. ChIP analysis showed that GATA6 and FOXA2 could bind to the *Wnt6* promoter and when *Gata6* or *Foxa2* is overexpressed, there was increased activity in gene expression of a *Wnt6* reporter construct. Furthermore, these *Gata6* or *Foxa2* expressing F9 cells, when treated with db-cAMP, were competent to complete the epithelial-to-mesenchymal transition leading to parietal extraembryonic endoderm. Together these results highlight a signaling hierarchy between RA, GATA6, FOXA2, and WNT6 during the specification of primitive endoderm.

2.2 Materials and methods

2.2.1 Plasmids and reagents

pCMV-*Gata6* was provided by Dr. E. E. Morrisey (University of Pennsylvania), pBS/KS-*Foxa2* by Dr. K. H. Kaestner (University of Pennsylvania), pRL-*TK* by Dr. Rodney DeKoter (University of Western Ontario), and pGL3-*BARL* by Dr. S. Anger (University of Toronto). *Gata6* and *Foxa2* were subcloned into pcDNA3.1 (Invitrogen). *All trans* RA and db-cAMP were from Sigma, and neomycin (G418) from Calbiochem.

2.2.2 Cell culture, transfection, and treatment

Mouse F9 teratocarcinoma cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (Lonza). Cells were transfected with empty vector, pcDNA3.1-*Gata6*, pcDNA3.1-*Foxa2*, pGL3-*Wnt6*, pGL3-BARL, and pRL-*TK* constructs using FuGENE6 according to the manufacturer's recommendations (Roche). Briefly, 3 µl of FuGENE6 reagent was mixed with a total of 1.5 µg of expression construct to transfect cells grown to 60% confluence in 35 mm TC dish (BD Falcon); for co-transfection experiments, equal amounts of each of the constructs were used with FuGENE6 reagent to DNA ratio of 3:1.5. Cells transfected with pcDNA3.1-

Gata6, pcDNA3.1-*Foxa2* or the empty vector control, were passed to 60 mm TC dishes (BD Falcon) 24 hours post transfection and treated with 400 µg/ml G418. Cells were treated with 0.05% DMSO (vehicle control), 10^{-7} M RA or 10^{-7} M RA and 1 mM db-cAMP. All cells were incubated at 37°C and 5% CO_2.

2.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Oligodeoxynucleotide primers were designed to the mouse *Wnt6* (Accession # M89800), *Gata6* (Accession # AK142381), *Foxa2* (Accession # AL845297), and *Thrombomodulin* (*TM)* (Accession # BC019154) nucleotide sequences. *Wnt6* sense (5' GCG GTA GAG CTC TCA GGA TG) and antisense (5' AAA GCC CAT GGC ACT TAC AC), *Gata6* sense (5' CTC TGC ACG CTT TCC CTA CT) and antisense (5' GTA GGT CGG GTG ATG GTG AT), *Foxa2* sense (5' ACC TGA GTC CGA GTC TGA GC) and antisense (5' CAT GGT GAT GAG CGA GAT GT), and *TM* sense (5' CCA GGC TCT TAC TCC TGT A) and antisense (5' TGG CAC TGA AAC TCG CAG TT) primers were designed to amplify partial *Wnt6*, *Gata6*, *Foxa2*, and *TM* cDNAs. RNA was isolated from F9 cells treated with RA, RA and db-cAMP, or F9 cells transfected with pcDNA3.1-*Gata6*, pcDNA3.1-*Foxa2* 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 45 s at 72°C; *Gata6* – 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; *Foxa2* – 35 cycles of 94°C, 30 s at 58°C, and 30 s at 72°C; *TM* – 32 cycles

of 94^{o}C, 30 s at 60^{o}C, and 30 s at 72^{o}C. Primers to *L14* sense (5' GGG AGA GGT GGC CTC GGA CGC) and antisense (5' GGC TGG CTT CAC TCA AAG GCC) were used to amplify a constitutively expressed ribosomal gene. Samples were run on 1% agarose gels containing ethidium bromide and visualized using the FluorChem 8900 gel imaging station (Alpha Innotech). Amplicons from all PCRs were sequenced at the Robarts Research Sequencing Facility (London, ON) to confirm their identity.

2.2.4 Immunoblot analysis

Total cell lysates were prepared in 1% SDS lysis buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% Mercapto-2-ethanol, and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentrations were quantified using a Bradford protein assay (Bio-Rad) and equal amounts were separated on denaturing 10% polyacrylamide gels and transferred to nitrocellulose membranes (Biotrace; Pall Corp.). The membranes were blocked in 5% skim milk, probed with antibodies, and the signals were detected using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce). The primary antibodies were directed against TROMA-1 (1:50; Developmental Studies Hybridoma Bank), and β-ACTIN (1:10000; Santa Cruz). Secondary antibodies were HRP-conjugated goat anti-rat and anti-mouse (1:10000; Pierce).

2.2.5 Immunofluorescence and light microscopy

Cells were fixed in 4% paraformaldehyde in PBS, blocked with 4% goat serum and then incubated with TROMA-1 antibody (1:50). After the incubation with the primary antibody, the cells were incubated in Alexa488-conjugated anti-rat secondary antibody (1:200; Invitrogen). Cells were mounted on slides using ProLong Gold antifade reagent (Invitrogen), examined using a Zeiss Imager Z1 microscope, and images were captured using a Zeiss Axiocam MRm digital video camera. For light microscopy, cells were examined using a Zeiss Axio Observer A1 and images were captured using a QImaging Retiga digital video camera. All images were assembled as plates using Adobe Photoshop CS3 and Adobe Illustrator CS3.

2.2.6 TCF/LEF reporter assay

Cells transfected with pGL3-*BARL* and then treated with 0.05% DMSO (vehicle control) or 10^{-7} M RA, or co-transfected with pGL3-*BARL* and pcDNA3.1 empty vector, pcDNA3.1-*Gata6*, or pcDNA3.1-*Foxa2* in equal amounts, were prepared 24 h post-treatment or post-transfection using the Dual Luciferase Assay Kit as per manufacturer's instructions (Promega). Luciferase expression was quantified using the GloMax Multi Detection System (Promega). Cells were also co-transfected with pRL-*TK* to normalize luciferase levels.

2.2.7 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP-IT kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Anti-GATA6 and anti-FOXA2 antibodies (sc-7244 and sc-6554, respectively) were from Santa Cruz (Santa Cruz, CA). PCR analysis was performed on DNA isolated by ChIP, using the following primers to amplify the GATA6 binding and the FOXA2 binding regions within the mouse *Wnt6* promoter: GATA6-F, 5' TGT TCT CCG TTT CCA CTT CT; GATA6-R, 5' AGT GCA GAG GGA CAG GTG; FOXA2-F, 5' CAG TTG GAC AGC CTT CTA CC; FOXA2-R, 5' CGG GAT GAA TAG TCG AGA AG. Cycling temperatures were as follows: GATA6 – 35 cycles of 30 s at 94^{o}C, 30 s at 52^{o}C, and 30 s at 72^{o}C; FOXA2 – 35 cycles of 30 s at 94^{o}C, 30 s at 58^{o}C, and 30 s at 72^{o}C. Samples were separated on 1.5% agarose gels containing ethidium bromide and visualized using the FluorChem 8900 gel imaging station. Amplicons were sequenced at the Robarts Research Sequencing Facility (London, ON) to confirm their identity.

2.2.8 *Wnt6* promoter assay

The promoter region of the *Wnt6* gene was cloned into the pGL3-luciferase vector (pGL3-*Wnt6*) and was co-transfected with pcDNA3.1-*Gata6* or pcDNA3.1-*Foxa2* in equal amounts. Cells were prepared using the Luciferase Assay Kit as per

manufacturer's instructions (Promega). Luciferase expression was quantified using the GloMax Multi Detection System (Promega). Cells co-transfected with pGL3-*Wnt6* and pcDNA3.1 served as control.

2.2.9 Statistical analysis

Data from all experiments are representative of at least three independent biological replicates performed on separate occasions. Densitometry data were obtained using a FluorChem 8900 Chemiluminescence and Gel Image (Alpha Innotech). Analysis of data between control and treated or transfected groups was performed using a Student's *t*-Test assuming unequal variances (Excel, Microsoft Corp., Redmond, WA). *P* values were one-sided and considered statistically significant at the 0.05 level. Statistical data is presented as the mean ± S.E.

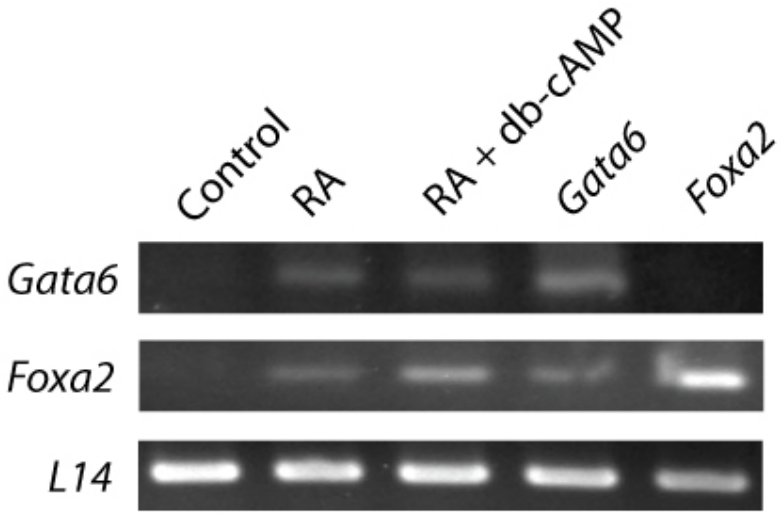
2.3 Results

2.3.1 *Gata6* and *Foxa2* up-regulation by RA leads to *Wnt6* expression in F9 cells

Mouse F9 teratocarcinoma cells grown in monolayer differentiate into primitive endoderm (PrE) and parietal endoderm (PE) when exposed to RA or RA and db-cAMP, respectively. Although *Gata6* and *Foxa2* were previously reported to be up-regulated in RA and RA and db-cAMP-treated F9 cells, respectively [48, 49], for our purposes it was necessary to re-examine their expression profiles in PrE cells, which were induced by treating F9 cells with RA alone. *Gata6* was not expressed in pcDNA3.1-containing cells (control), but was present in RA- and RA and db-cAMP-treated cells (Fig. 2.1). The expression of *Foxa2* was detected in RA-treated cells, confirming an earlier report [47] and in RA and db-cAMP-treated cells. cDNA from *Gata6*-transfected cells was also used in a PCR with *Foxa2* primers, to test the notion that *Foxa2* is regulated by GATA6 in F9 cells, as it is in ES cells [34]. Likewise, cDNA from *Foxa2*-transfected cells was used in a PCR with *Gata6* primers. Results from these experiments revealed an amplicon corresponding to *Foxa2* in *Gata6*-expressing cells (Fig. 2.1). Thus, the hierarchy at this point would indicate that RA up-regulates *Gata6* and *Foxa2* expression, the latter either directly or indirectly via GATA6.

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 [12], and given the putative RA-responsive transcription factor (GATA6 and FOXA2) binding sites in a region of the

Figure 2.1. ***Gata6* and *Foxa2* mRNA are up-regulated during RA-induced differentiation.** Total RNA from cells treated with RA or RA and db-cAMP, and cells transfected with empty vector (control), *Gata6* or *Foxa2*, and selected with G418, was collected and reverse transcribed into first strand cDNA for PCR. A *Gata6* amplicon was seen in RA and RA and db-cAMP lanes, and as expected in cells ectopically expressing *Gata6*, but not in the control or cells ectopically expressing *Foxa2*. A *Foxa2* amplicon was seen in RA and RA and db-cAMP lanes, and in cells ectopically expressing *Gata6* or *Foxa2*, but not in the control. The *L14* positive control amplicon was seen under all conditions. Representative results from five independent experiments are shown.

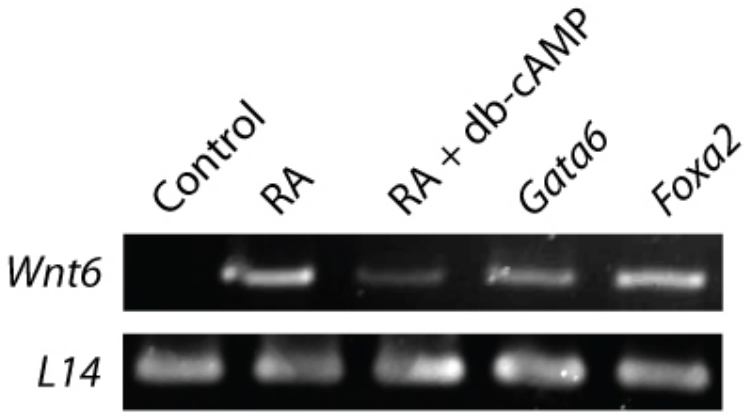

Control
RA
RA + db-cAMP
Gata6
Foxa2
Gata6
Foxa2
L14

conserved human and rat *WNT6* promoters [37], we hypothesized that in the absence of RA, the overexpression of *Gata6 or Foxa2* would up-regulate *Wnt6* expression in F9 cells. To test this, total RNA was collected and reverse transcribed into cDNA from cells treated with RA and RA and db-cAMP, and from cells transfected with pcDNA3.1-*Gata6*, pcDNA3.1-*Foxa2*, or the empty vector (negative control); the latter three 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. 2.2). For *Wnt6,* no amplicon was seen in controls, but was as previously reported [12], present in cells induced to form PrE and to some extent in those treated with RA and db-cAMP to induce PE. Results also showed a *Wnt6* amplicon of the expected size in F9 cells transfected with the *Gata6* expression construct (Fig. 2.2). Experiments were repeated for the analysis of *Foxa2* over-expressing cells and results were similar to that seen for *Gata6* (Fig. 2.2). Sequencing confirmed the identity of the amplicon as being *Wnt6* and the appearance of the *L14* amplicon confirmed that cDNAs were present under all conditions. Taken together, these results would indicate that in the absence of RA, *Gata6* or *Foxa2* overexpression was sufficient to up-regulate the endogenous *Wnt6* gene in F9 cells.

2.3.2 *Gata6* and *Foxa2* expression is sufficient to induce extraembryonic endoderm

Since *Gata6* expression was RA-responsive and could up-regulate a *Wnt* known for its ability to induce PrE [12], we hypothesized *Gata6* overexpression would induce PrE in the absence of RA. The appearance of TROMA-1, a cytokeratin A intermediate

Figure 2.2. **Overexpression of *Gata6* or *Foxa2* induces *Wnt6* expression.** Total RNA from cells treated with RA to induce PrE or RA and db-cAMP to induce PE, and cells transfected with empty vector (control), *Gata6* or *Foxa2* and selected with G418, was collected and reverse transcribed into first strand cDNA for PCR. Oligodeoxynucleotide primers for PCR were designed to detect *Wnt6* or the constitutively expressed ribosomal gene *L14*. A *Wnt6* amplicon was seen in the RA and RA and db-cAMP lanes, in cells transfected with *Gata6* or *Foxa2*, but not in the control. The *L14* amplicon was present in all lanes. Representative results from three independent experiments are shown.

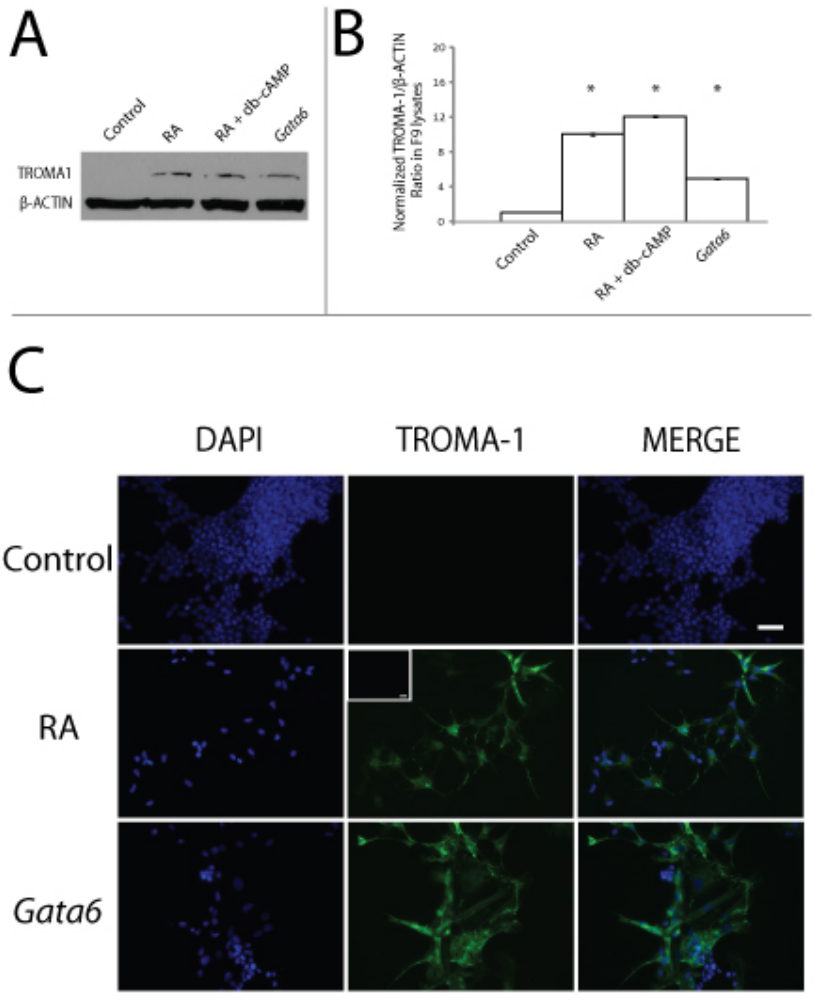

Control
RA
RA + db-cAMP
Gata6
Foxa2
Wnt6
L14

filament, was used as a definitive marker of extraembryonic endoderm [50]. Immunoblot analysis showed that TROMA-1 was expressed in cells treated with RA and RA and db-cAMP, but not in empty vector transfected controls (Fig. 2.3A). TROMA-1 was also expressed in cells transfected with the *Gata6* construct, providing evidence for a role in ExE differentiation (Fig. 2.3A). Densitometry analysis confirmed that the relative levels of TROMA-1 induced by chemical treatment and by *Gata6* overexpression were significantly higher than that in untreated F9 cells (Fig. 2.3B).

The assembly of TROMA-1-positive cytokeratin A filaments was also used as a molecular readout of PrE and PE formation. To examine for the presence of TROMA-1-positive intermediate filaments, cells were treated with RA or transfected with either the *Gata6* or the empty vector construct, selected with G418 and then processed for immunocytochemical analysis (Fig. 2.3C). The extensive network of intermediate filaments that formed when F9 cells were treated with RA corroborates the immunoblot data. A similar staining pattern was seen in cells transfected with the pcDNA3.1-*Gata6* plasmid and selected using G418 (Fig. 2.3C). A control for the non-specific binding of the secondary antibody alone showed no TROMA-1 staining (inset, Fig. 2.3C). Together, these results indicate that the overexpression of *Gata6* alone is sufficient to increase the levels of cytokeratin A, which in turn get assembled into intermediate filaments indicative of extraembryonic endoderm.

Having established the expression pattern of *Gata6* following RA treatment, the ability of *Foxa2* overexpression to induce cells to form PrE was examined. Furthermore, since *Gata6* overexpression induced markers of differentiation (Fig. 2.3), caused changes in cell morphology, and could induce the expression of *Foxa2* (Fig. 2.1), then by

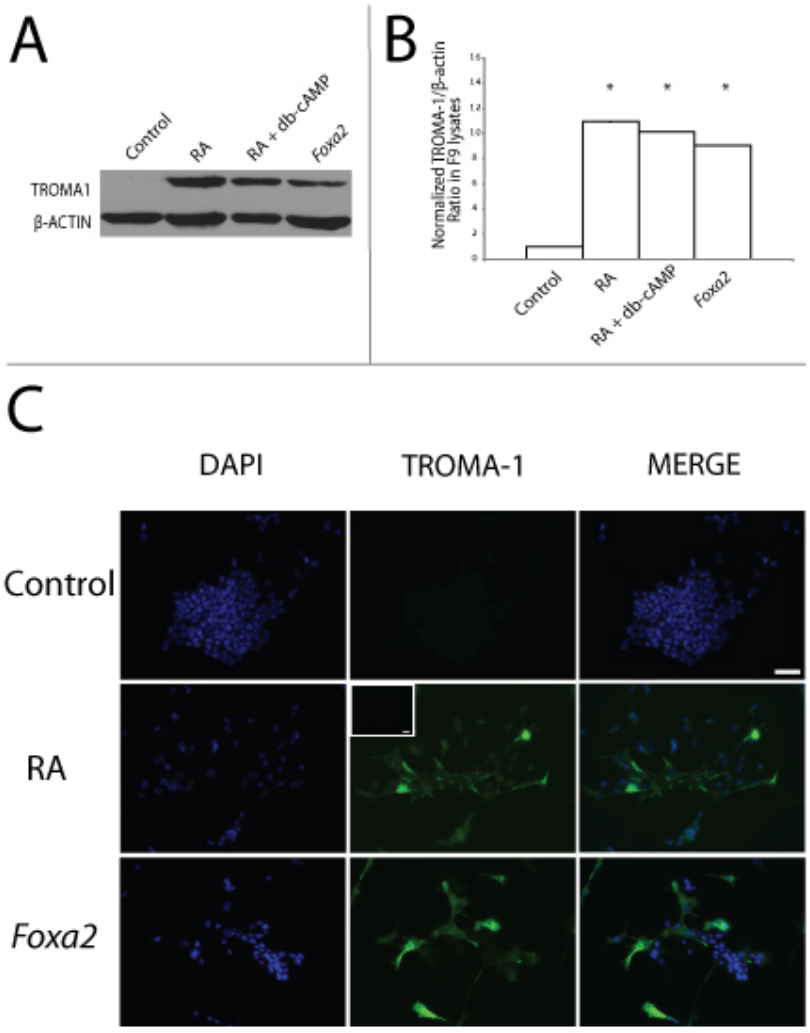
Figure 2.3. **Overexpression of *Gata6* induces extraembryonic endoderm. (A, B)** Protein lysates from cells treated with RA or RA and db-cAMP, and cells transfected with the empty vector (control) or *Gata6* and selected with G418, were collected and processed for immunoblot analysis using antibodies to TROMA-1 and β-ACTIN. **(A)** Representative blot showing TROMA-1 signals in RA and RA and db-cAMP-treated cells, and in those transfected with *Gata6*. **(B)** Analysis of the average integrated densitometric values between TROMA-1 and β-actin from three independent blots showed that there was a significant increase in TROMA-1 expression in RA- or RA and db-cAMP-induced or *Gata6*-transfected cells relative to the control. * $P<0.05$. **(C)** Cells treated with RA, or transfected with the empty vector (control) or with *Gata6* and selected with G418, were fixed and processed for immunocytochemistry using the TROMA-1 antibody. TROMA-1 filaments surrounding DAPI-positive nuclei were seen in RA-treated and *Gata6*-transfected cells, but not in the controls. (Inset) A control for non-specific binding of the secondary antibody alone showed no staining. Scale bar = 50 μm.


A
Control
RA
RA + db-cAMP
Gata6
TROMA1
β-ACTIN
B
Normalized TROMA-1/β-ACTIN Ratio in F9 lysates
20
16
12
8
4
0
*
*
*
Control
RA
RA + db-cAMP
Gata6
C
DAPI
TROMA-1
MERGE
Control
RA
Gata6

inference we predicted that *Foxa2* overexpression would also induce PrE in the absence of RA. Immunoblot analysis showed that TROMA-1 was expressed in cells treated with RA and RA and db-cAMP, but not in empty vector transfected controls (Fig. 2.4A). TROMA-1 was also expressed in cells transfected with the *Foxa2* construct (Fig. 2.4A), and densitometry analysis confirmed that the relative levels of TROMA-1 induced by chemical treatment and by *Foxa2* overexpression were significantly higher than that in the controls (Fig. 2.4B). Immunocytochemistry was also used to confirm that the *Foxa2*-dependent increase in TROMA-1 levels would translate into the assembly of cytokeratin A intermediate filaments (Fig. 2.4C). Cells were treated with RA or transfected with either the *Foxa2* or the empty vector construct, and then selected with G418 before being processed for immunocytochemistry with the TROMA-1 antibody. Results confirmed the immunoblot data and the appearance of intermediate filaments in cells transfected with pcDNA3.1-*Foxa2* was reminiscent of that in the positive control cells treated with RA (Fig. 2.4C). A control for the non-specific binding of the secondary antibody alone showed no TROMA-1 staining (inset, Fig. 2.4C). These results indicated that the overexpression of *Gata6* or *Foxa2* alone was sufficient to induce a marker of primitive and parietal endoderm, and to cause changes in the morphology of these cells, both of which parallel the effects when F9 cells are treated with RA.

2.3.3 *Gata6* and *Foxa2* signal through the canonical WNT/β-catenin signaling pathway

Figure 2.4. **Overexpression of *Foxa2* induces extraembryonic endoderm. (A, B)** Protein lysates from cells treated with RA or RA and db-cAMP, and cells transfected with the empty vector (control) or *Foxa2* and selected with G418 were collected and processed for immunoblot analysis using antibodies to TROMA-1 and β-ACTIN. **(A)** Representative blot showing TROMA-1 signals in RA and RA and db-cAMP treated cells, and in those transfected with *Foxa2*. **(B)** Analysis of the average integrated densitometric values between TROMA-1 and β-actin from three independent blots indicated that there was a significant increase in TROMA-1 expression in the induced and transfected cells relative to the control. * $P<0.01$. **(C)** Cells treated with RA, or transfected with the empty vector (control) or with *Foxa2* and selected with G418, were fixed and processed for immunocytochemistry using the TROMA-1 antibody. TROMA-1 filaments were seen in RA-treated and *Foxa2*-transfected cells, but not in the controls. (Inset) A control for non-specific binding of the secondary antibody showed no staining. Scale bar = 50 μm.

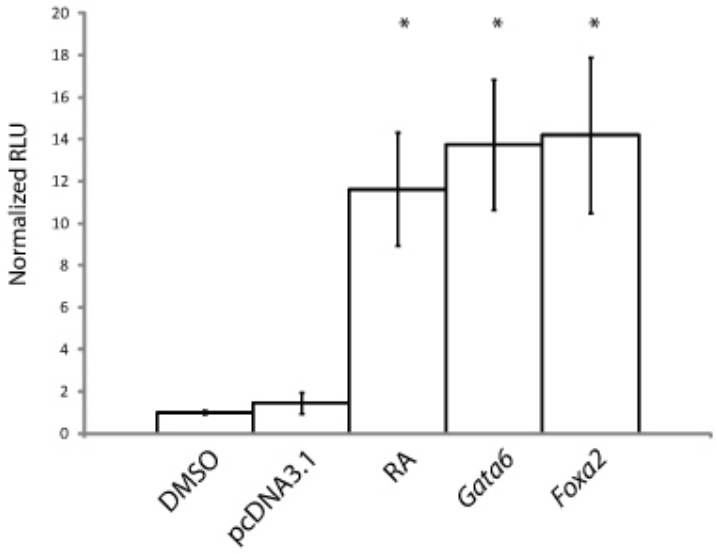

A
Control
RA
RA + db-cAMP
Foxa2
TROMA1
β-ACTIN
B
Normalized TROMA-1/β-actin Ratio in F9 lysates
Control
RA
RA + db-cAMP
Foxa2
C
DAPI
TROMA-1
MERGE
Control
RA
Foxa2

Since *Gata6* or *Foxa2* were found to up-regulate *Wnt6* (Fig. 2.2A) and WNT6 activates the canonical WNT signaling pathway [12], then one would expect that *Gata6* or *Foxa2* overexpression should activate canonical WNT/β-catenin signaling. The activation of the canonical pathway and β-catenin/TCF/LEF-dependent transcription was tested directly using a pGL3-*BARL* reporter assay [51]. F9 cells co-transfected with pGL3-*BARL* and a *Renilla* luciferase construct (pRL-*TK*) and then treated with DMSO (vehicle control), had 10 fold less luciferase activity relative to that in RA-treated cells (Fig. 2.5). F9 cells were also co-transfected with pGL3-*BARL* and pcDNA3.1-*Gata6*, pGL3-*BARL* and pcDNA3.1-*Foxa2*, or pGL3-*BARL* and pcDNA3.1 empty vector, and then assayed for luciferase activity after 24 h. Luciferase activity in cells overexpressing *Gata6* or *Foxa2* was significantly higher (12 fold) than the controls. Together, this evidence allows us to place GATA6 and FOXA2 between RA and β-catenin/TCF/LEF dependent signaling in a hierarchy responsible for the induction of F9 cells to form ExE.

2.3.4 GATA6 and FOXA2 bind the *Wnt6* promoter and regulate its activity leading to primitive endoderm formation

To assign a direct link between GATA6 and FOXA2 and WNT6 expression during ExE differentiation, it was first necessary to demonstrate that GATA6 and FOXA2 could bind to the endogenous *Wnt6* promoter. Once established, the next question was to ask whether or not binding was sufficient to drive the expression of a *Wnt6* reporter construct. To begin, DMSO- and RA-treated F9 cells were processed for

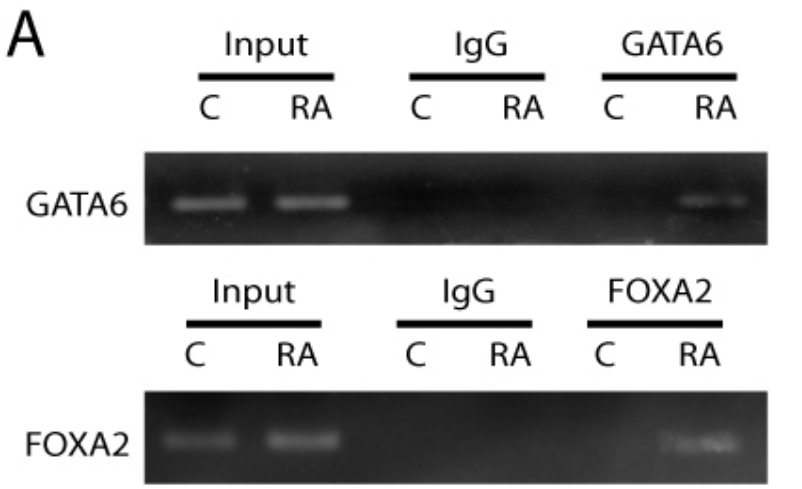
Figure 2.5. ***Gata6* and *Foxa2* activate canonical WNT/β-catenin/TCF dependent transcription.** Cell lysates from cells transfected with pGL3-*BARL*, then treated with RA or DMSO vehicle, and cells co-transfected with pGL3-*BARL* and the empty vector control, *Gata6* or *Foxa2*, were collected and processed for luciferase activity 24 hr post RA treatment or transfection. Cells treated with RA had a 12 fold increase in luciferase activity relative to the DMSO-treated controls. *Gata6*- or *Foxa2*-transfected cells exhibited a 9 fold increase in luciferase activity relative to the transfected empty vector controls. Data are representative of three independent experiments. Bars represent mean fold change in relative luciferase units (RLU) ± S.E., normalized for *Renilla* luciferase activity. * $P<0.05$.

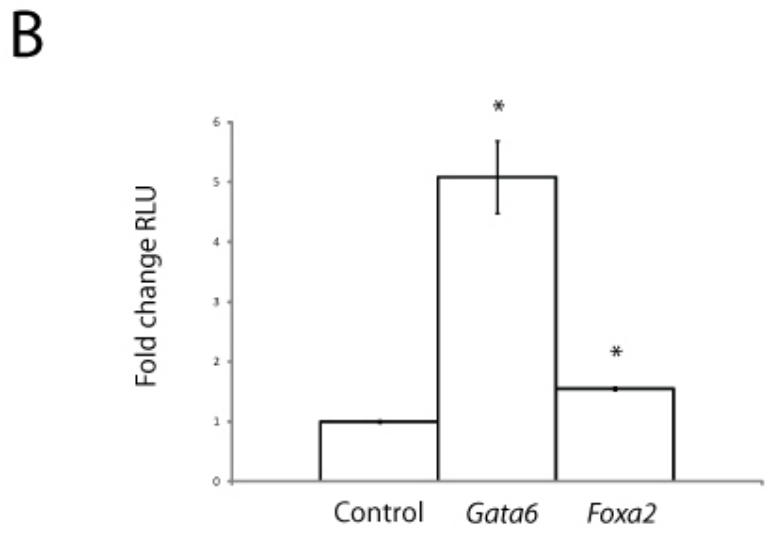

Normalized RLU
20
18
16
14
12
10
8
6
4
2
0
*
*
*
DMSO
pcDNA3.1
RA
Gata6
Foxa2

chromatin immunoprecipitation using antibodies against GATA6 and FOXA2. IgG served as a negative control. PCR, using primers spanning the putative GATA6 and FOXA2 binding sites within the *Wnt6* promoter [37], was first performed on sheared DNA prior to immunoprecipitation (input, Fig. 2.6A). Results revealed amplicons of the expected size in DMSO- and RA-treated cells, and sequencing confirmed their identity. PCR following immunoprecipitation with antibodies against GATA6 or FOXA2 produced similar results, but only in RA-treated cells (Fig. 2.6A). Amplicons were not seen in DMSO-treated cells, or when the immunoprecipitation was performed with IgG. Together, the data indicated that both transcription factors were capable of binding the *Wnt6* promoter in F9 cells.

Satisfied with the data from the ChIP analysis, we next employed a luciferase reporter assay using approximately 1.2 Kb of sequence of the mouse *Wnt6* promoter upstream of the ATG start site. COS-7 cells were co-transfected with the pGL3-*Wnt6* reporter and either pcDNA3.1-*Gata6* or pcDNA3.1-*Foxa2*, and then assayed for luciferase activity. The cell line was chosen since it does not express *Wnt6* (data not shown), *Foxa2* [52], or any significant levels of *Gata* factors [53]. Furthermore, evidence also exists that *Foxa2* expression is not enhanced in COS cells transfected with *Gata4* [54], which has a similar consensus binding site found in GATA6 and plays a role during PrE differentiation [55, 56]. Results revealed that when either the *Gata6* or *Foxa2* constructs were present, the luciferase activity was significantly higher relative to the empty vector control (Fig. 2.6B). Thus, GATA6 and FOXA2 acted directly on the *Wnt6* promoter and although reports indicate that the activation of these transcription factors is

Figure 2.6. **GATA6 and FOXA2 bind to and activate the *Wnt6* promoter. (A)** PCR of sheared DNA from DMSO- and RA-treated F9 cells prior to immunoprecipitation (input) revealed amplicons corresponding to the putative GATA6 and FOXA2 binding sites within the *Wnt6* promoter. Similar results were seen in RA-treated cells following immunoprecipitation with antibodies against GATA6 or FOXA2. No amplicons were seen following GATA6 or FOXA2 immunoprecipitation of DMSO-treated cells, or from cells of either treatment regimen following immunoprecipitation with IgG. **(B)** Cell lysates from COS-7 cells co-transfected with pGL3-*Wnt6* and *Gata6*, or *Foxa2* were collected and processed for luciferase activity 24 hours post transfection. Overexpression with *Gata6* or *Foxa2* showed a 5 fold and 1.5 fold increase in luciferase activities, respectively, relative to the transfected empty vector control. Data are representative of three independent experiments. Bars represent mean fold change in relative luciferase units (RLU) ± S.E. * $P<0.005$.


A
Input
IgG
GATA6
C
RA
C
RA
C
RA
GATA6
Input
IgG
FOXA2
C
RA
C
RA
C
RA
FOXA2

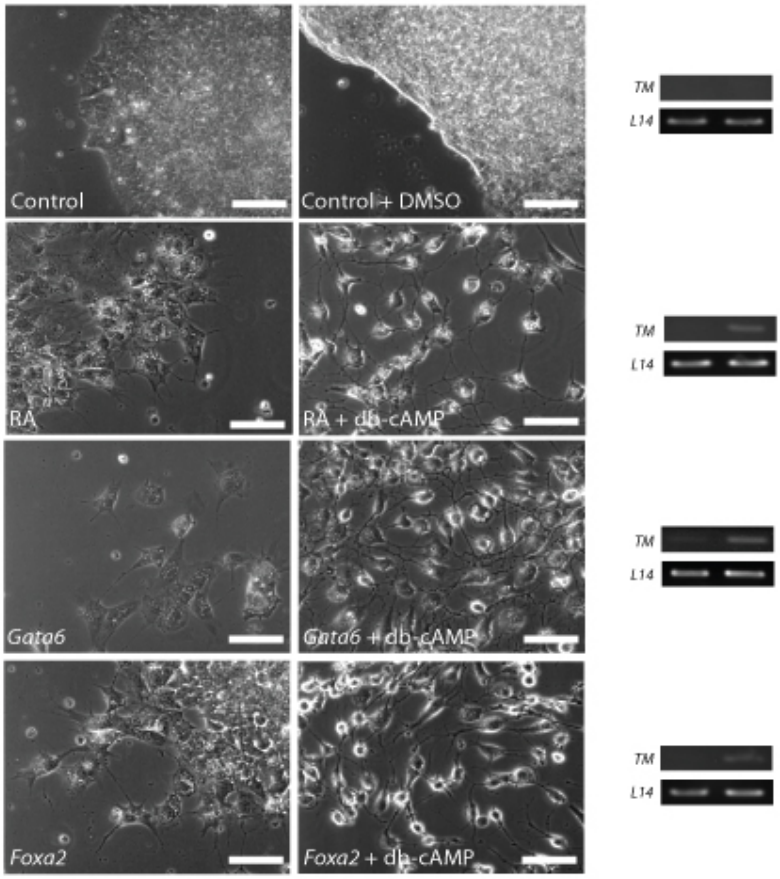

B
Fold change RLU
0
1
2
3
4
5
6
*
*
Control
Gata6
Foxa2

sufficient to induce cells to form PE [1, 49], our previous work would argue that the up-regulation of *Wnt6* is only sufficient to induce cells to form PrE.

2.3.5 *Gata6*- and *Foxa2*-expressing cells are competent to form parietal endoderm

We demonstrated previously that WNT6 was sufficient to induce F9 cells to form PrE, but not PE [12], whereas the expression of constitutively active Gα13 permitted cells to proceed through to PE [9]. A previous study has also reported that *Gata6* and *Foxa2* expression is up-regulated when F9 cells are induced to PE and *Gata6* overexpression in *Sox7*-silenced cells was also able to induce PE [49]. Thus, it is still not clear whether or not the overexpression of *Gata6* or *Foxa2* alone is sufficient to induce PE. To address this issue cells were treated with RA or RA and db-cAMP, or transfected with pcDNA3.1-*Gata6* or pcDNA3.1-*Foxa2* and then treated with db-cAMP or left untreated, and then examined for changes in morphology using phase contrast microscopy (Fig. 2.7). Empty vector transfected and DMSO-treated cells (controls) were morphologically similar as compact bodies (top panels Fig. 2.7). Morphologically, these cells were indistinguishable from cells treated with db-cAMP (data not shown). In contrast, RA-treated cells shared morphological similarities with those transfected with pcDNA3.1-*Gata6* or pcDNA3.1-*Foxa2*. Specifically, cells had migrated away from the compact bodies and flattened out over the surface of the plate. Cells transfected with pcDNA3.1-*Gata6* or pcDNA3.1-*Foxa2* and then treated with db-cAMP resembled the RA and db-cAMP-treated positive control. Under these conditions, cells appeared to

Figure 2.7. **Gata6- or Foxa2-induced primitive endoderm is competent to complete the EMT and form parietal endoderm.** Untreated or DMSO-treated cells showed similar morphology. Likewise, cells treated with RA to induce primitive endoderm were morphologically similar to those transfected with *Gata6* or *Foxa2*. In each case, cells migrated from the compact bodies, characteristic of the undifferentiated cells, and adopted a stellate shape with numerous filopodia. Cells transfected with *Gata6* or *Foxa2,* and then treated with db-cAMP, showed morphological similarities to RA and db-cAMP treated cells. Under these conditions, the cells were more spherical in shape, were highly refractile and possessed relatively long filopodia. Total RNA from cells treated with DMSO, RA, or RA and db-cAMP, and cells transfected with *Gata6*, *Foxa2*, or transfected with *Gata6* or *Foxa2* and then 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 *Gata6* or *Foxa2*, and then treated with db-cAMP. The presence of the *L14* amplicon indicated that cDNAs were present under all conditions. The *Gata6*- or *Foxa2*-transfected cells were selected with G418 for 7 days or selected with G418 for 5 days and then treated with db-cAMP for 4 days under continual G418 selection. Scale bars in = 20 μm.


Control
Control + DMSO
RA
RA + db-cAMP
Gata6
Gata6 + db-cAMP
Foxa2
Foxa2 + db-cAMP
TM
L14
TM
L14
TM
L14
TM
L14

have lost their stellate shape and instead have rounded up, developed long slender filopodia and became more refractile. These changes in morphology prompted further investigation using molecular markers to determine the fate of the cell resulting from the individual treatments. PCR analysis was performed with primers to *Thrombomodulin* (*TM*), a PE marker that increases 4 fold over the barely detectable levels in PrE [9, 57]. Results showed that *TM* was not detected in empty vector transfected or DMSO-treated cells or when cells were treated with RA alone. As expected, *TM* was expressed in the positive controls, which were those cells induced to form PE by RA and db-cAMP. Interestingly, *TM* amplicons were not seen in *Gata6*- or *Foxa2*-overexpressing cells, although cDNA was available for amplification as evident by the *L14* amplicons in all lanes. In contrast, *TM* amplicons were seen in *Gata6*- or *Foxa2*-transfected cells that were treated with db-cAMP. Thus, we are proposing that these transfected cells had developed into PrE and remained competent to form PE and complete the EMT following the appropriate stimulation to augment PKA activity. Taken together, our study provides new evidence that GATA6 and FOXA2 signal through a canonical WNT/β-catenin signaling pathway in F9 cells to induce PrE, but like WNT6 [12], neither one of these transcription factors permit cells to differentiate into PE. Furthermore, GATA6 appears to have a dual role, inducing the expression of *Wnt6* as well as *Foxa2*.

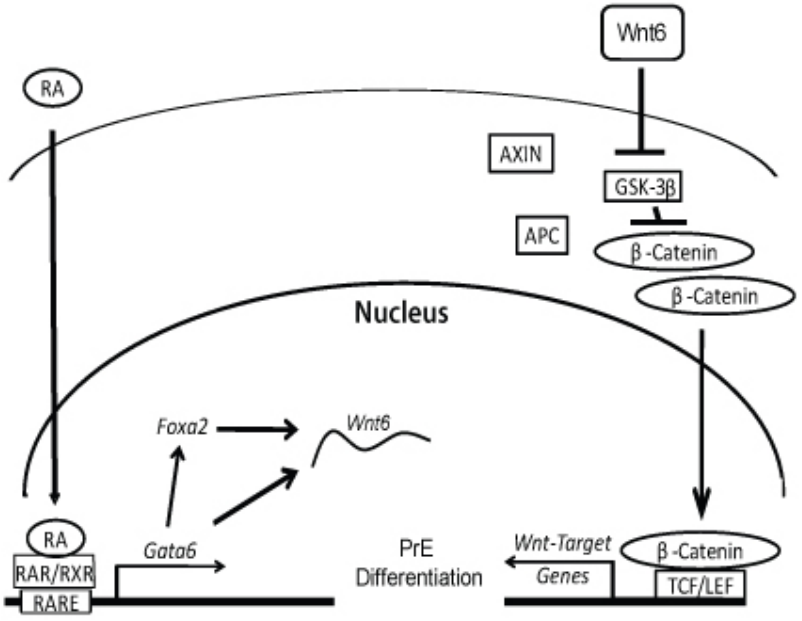
2.4 Discussion

In the mouse embryo several transcription factors including those of the SOX, GATA and FOX (HNF) families are expressed shortly after fertilization [58]. GATA6 is expressed initially in some cells of the inner cell mass at the time of implantation, while FOXA2 expression is only apparent in cells of the ExE [1, 35, 42, 47, 59]. My study with the F9 teratocarcinoma cell line, which differentiates into PrE and PE following treatment with RA and RA and db-cAMP, respectively, confirmed that RA-induced differentiation into ExE was accompanied by the up-regulation of the *Gata6* and *Foxa2* genes (Fig. 2.1). Furthermore, results indicated that the overexpression of *Gata6* or *Foxa2* alone was sufficient to induce biochemical and morphological markers of ExE, specifically PrE (Figs. 2.3 and 2.4). TROMA-1 serves as a useful marker of extraembryonic endoderm [50], but it does not discriminate between PrE and PE. To distinguish between the two, a PCR-based assay was employed to detect changes in the expression of *Nanog*, *Sox2*, *Utf-1* and others candidates (data not shown). Unfortunately, we were unable to find a marker unique to PrE (Ren, Caraher and Kelly, unpublished). In contrast, *TM* expression is used to distinguish between PrE and PE [57], and its presence together with the refractile appearance of the cells (this study) and changes to their morphology [6], indicated that cells ectopically expressing *Gata-6* and treated with db-cAMP had differentiated into PE (Fig. 2.7). That these changes were not seen in cells ectopically expressing *Gata-6* alone would indicate that PE differentiation is also dependent on other factors (Fig. 2.7). Not to undermine its importance, GATA6 is a master regulator of ExE differentiation. Furthermore, it is one of the first ExE-specific

transcription factors to be expressed *in vivo*, and its loss-of-function results in the absence of ExE and *Foxa2* expression, and the death of the developing embryo [1, 35, 36, 60]. Compelling evidence indicates that *Gata6* expression is indicative of and required for the formation of the primitive endoderm lineage that precedes the parietal and/or visceral endoderm lineages [2, 33, 36, 61, 62]. The ability of GATA6 to induce the expression of *Foxa2* (Fig. 2.1) might explain the absence of the latter reported for the *Gata6* nulls. That FOXA2 is not required for early embryogenesis and visceral endoderm forms in its absence [40, 42, 63, 64], is also indicative of the placement of GATA6 in the hierarchy of coordinated signaling required during ExE patterning and formation. Irrespective of this placement and despite the numerous *in vivo* and *in vitro* assays showing the necessity for GATA6 and FOXA2 in the proper development of ExE, many questions remain as to what genes are regulated by these transcription factors during differentiation, and what subsequent impact do the proteins they encode have on embryonic patterning. Our previous study found that one of these genes is *Wnt6,* which is up-regulated during PrE differentiation and down-regulated during PE differentiation [12]. Furthermore, we also reported that the overexpression 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 can interact with TCF/LEF to regulate the genes necessary for PrE specification. The present study is the first to report that the overexpression of *Gata6* or *Foxa2* can regulate the expression of the mouse *Wnt6* gene (Fig. 2.2), and the ChIP analysis provided evidence to indicate that this regulation is directly the result of GATA6 or FOXA2 binding to the *Wnt6* promoter (Fig. 2.6). Interestingly, this regulation may be mammalian-specific as in *Xenopus* WNT6 appears to regulate GATA4 and 6 during heart

development [65]. Despite this difference between model systems, we provide evidence that in a feed-forward manner, GATA6 and FOXA2 activate the WNT/β-catenin pathway, which leads to ExE differentiation. Inactivation of GSK-3β by phosphorylation is one of the hallmark indicators that this pathway has been activated [17]. Likewise, the presence of nuclear β-catenin is highly suggestive of active WNT signaling, but this localization does not necessarily equate to transcriptional activation [66]. To clarify this distinction in the case of ExE, we provide direct evidence for TCF/LEF-dependent transcriptional activity resulting from ectopic expression of either *Gata6* or *Foxa2* (Fig. 2.5). Thus, we are confident that the expression of these transcription factors, are responsible for elevating the expression of a *Wnt* gene involved in canonical β-catenin signaling. Although differentiation is temporarily halted at the PrE stage, as evident by the fact that these cells do not express *TM*, a marker of PE, and they appear similar morphologically to PrE, these cells nevertheless remain competent to form PE when PKA activity is increased (Fig. 2.7). Again, this evidence corroborates our earlier work that *Wnt6* expression is only sufficient to induce cells into the PrE lineage [11, 12], and puts into context the limitations in inductive capabilities of GATA6 and FOXA2. Finally, that the *Wnt6* reporter construct is activated by *Gata6* overexpression (Fig. 2.6B), puts *Wnt6* in the ever-growing list of genes including *Dab2*, *laminin*, *Afp*, *HNF4* and others, regulated by GATA6 during endoderm formation [35, 67, 68]. That *Foxa2* overexpression also has the ability to induce the *Wnt6* reporter (Fig. 2.6B), points to a complex signaling hierarchy involved in a transcriptional network controlling the specification of PrE in the very early development of the mouse (Fig. 2.8). This complexity, as evident from the fact that embryos carrying targeted deletions in either *Foxa2* [40, 45] or *Gata6* [2, 35] die

Figure 2.8. **Model of the signaling hierarchy during primitive endoderm specification in F9 cells.** Retinoic acid induces the expression of *Gata6* and GATA6 in turn, induces the expression of *Foxa2*, two transcription factors that directly up-regulate the *Wnt6* gene. WNT6 signals to neighbouring cells by destabilizing the GSK-3β degradation complex, which allows cytoplasmic β-catenin levels to increase and eventually translocate to the nucleus, where with TCF/LEF transcription factors, activates/represses the genes required for primitive endoderm differentiation.


Wnt6
RA
AXIN
GSK-3β
APC
β -Catenin
β -Catenin
Nucleus
Foxa2
Wnt6
RA
RAR/RXR
RARE
Gata6
PrE
Differentiation
Wnt-Target
Genes
β -Catenin
TCF/LEF

from ExE defects, underscores the importance of each protein in regulating the expression of a number of genes and gene families, including those encoding WNT proteins, required for embryogenesis.

The question pertaining to the WNT family is whether or not WNT6 is the key member involved in establishing the ExE lineage *in vivo*. *Wnt6* mRNA is expressed at the correct time [18, 69], but studies have also revealed that *Wnt1*, *2b*, *3*, *3a*, *4*, *5a*, *7a*, *7b*, *10b*, and *11* are also expressed at early stages of mouse development [70, 71], and many as protein ligands activate the canonical WNT/β-catenin pathway [72-76]. Based on the readout of their overexpression in F9 cells, care must be exercised when extrapolating how these WNTs function *in vivo*. For instance, WNT3a will induce F9 cells ectopically expressing rat FZD1 to form ExE [32], however, in RA-induced wild type F9 cells, WNT3a treatment blocks differentiation [77]. It is also interesting to note that *Wnt6* is dispensable for embryonic development [78], which means that another WNT is likely to act in a functionally redundant manner to ensure embryo viability. Identifying the WNT(s) compensating for the loss of WNT6 during ExE formation will require a systematic *in vitro* approach using si/shRNAs, but to begin it may be more advantageous to continue focussing on how these genes are regulated at the transcriptional level. For instance, GATA6 and FOXA2 are also known to bind to and activate the *Wnt7b* promoter [79], a WNT that also signals through the canonical pathway [80]. While we are currently using the F9 model to test the likely possibility that more than one WNT is involved in ExE formation, a concerted effort to create mice with double gene knock-outs will be necessary to provide evidence for this *in vivo*.

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CHAPTER 3

REDOX REGULATION OF CANONICAL WNT SIGNALING AND EXTRAEMBRYONIC ENDODERM FORMATION

3.1 Introduction

3.1.1 Extraembryonic endoderm

During mouse embryogenesis the differentiation of a distinct population of cells in the inner cell mass (ICM) to primitive endoderm and then subsequently into parietal endoderm (PrE and PE, respectively) is one of the earliest epithelial-to-mesenchymal transitions (EMTs) [1, 2]. Cells of the PrE and PE lineages, collectively termed extraembryonic endoderm (ExE), are essential for fetal development *in utero* and are major signaling sources required for proper development of the embryo [3, 4]. Due to the difficulties of studying this process *in vivo*, F9 teratocarcinoma cells have been used as a model to study ExE differentiation *in vitro*. The addition of retinoic acid (RA) to these cells induces cellular changes required for the formation of PrE [5]. To complete the EMT, these competent PrE cells are treated with dibutyryl cyclic-adenosine monophosphate (db-cAMP) to induce PE [6]. Genetic studies on F9 cells have shown that RA regulates the expression of many genes [7, 8], some of which encode for proteins that

are sufficient to induce PrE formation [9, 10], and many linked to the canonical WNT/β-catenin signaling pathway [11-13].

3.1.2 Canonical WNT signaling

The canonical WNT pathway, activated when a Wnt ligand binds to the Frizzled (Fzd) receptor [14], facilitates the localization of Dishevelled (DVL) to the plasma membrane, where it interacts with Axin, a protein that serves in a destruction complex with APC and GSK-3β. In the absence of WNT, the destruction complex phosphorylates β-catenin leading to its ubiquitination and proteasomal degredation. When WNT is present, however, formation of the destruction complex is prevented leading to the accumulation of β-catenin in the cytoplasm, and eventual translocation into the nucleus where it interacts with TCF-LEF transcription factors to induce changes in gene expression. DVL, a key component in the WNT signaling pathway, contains three major functional domains allowing for Dvl to interact with over 60 proteins [15]. Within the list of DVL-interacting proteins, nucleoredoxin (NRX) is of particular interest in this study.

3.1.3 Nucleoredoxin

NRX is a thioredoxin family member that is sensitive to reactive oxygen species (ROS). Funato *et al.*, reported that NRX when bound to DVL acts as an inhibitor of the

WNT signaling pathway [16, 17]. In the presence of ROS, however, NRX is displaced from DVL, which lifts the inhibition on canonical Wnt signaling in the absence of a WNT ligand. In this regard, the WNT pathway is considered to be redox regulated and ROS, which are not just highly reactive and dangerous by-products of cellular metabolism, actually functions as mediators in this signaling pathway. An expanding body of evidence indicates that the specific production of ROS, at levels below those that can damage proteins, nucleic acids, and lipids, to affect gene expression and cell fate determination, are products of membrane bound NADPH oxidases (NOX) [18]. In reference to EMTs, ROS in the form of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), trigger responses such as differentiation and the expression of mesenchymal markers in epithelial-like cells [19]. Furthermore, these ROS are also accepted as being physiologically relevant mediators of developmental processes, likely to impact embryonic redox homeostasis [20-22].

3.1.4 Objectives of study

In light of these recent evidence, I investigated the role of ROS signaling during differentiation of F9 cells in order to test the hypothesis that ROS participate with canonical WNT signaling to initiate extraembryonic endoderm formation. I found that cells treated with H_2O_2 induced the morphological and molecular characteristics of PrE, and these cells were competent to form PE following db-cAMP treatment. Culturing the cells in the presence of H_2O_2 induced canonical Wnt signaling while pre-treating these

cells with N-acetyl cysteine or Trolox attenuated the ability of RA to induce PrE. Real time PCR revealed that *Nox1-4* and *Duox2* mRNAs, candidates encoding proteins responsible for producing ROS, were up-regulated in response to RA. Moreover, ectopic expression of *Gata6*, a master regulator of extraembryonic endoderm, induced the expression of *Nox4* and ROS, while ectopic expression of *Nox4* alone induced ROS necessary for PrE formation. Since Funato *et. al.*, showed Nucleoredoxin (NRX) impinges on canonical WNT signaling through DVL in NIH3T3 cells [16], I hypothesized that it plays a role during RA-induced PrE formation. Although NRX mRNA and protein expression did not change in response to RA, reducing *NRX* mRNA levels by RNAi induced morphological and molecular markers of PrE, with a significant increase in canonical WNT signaling. Furthermore, these NRX-depleted cells completed the EMT and differentiated into PE when treated with db-cAMP. Together, these results indicate that NRX is involved in maintaining F9 cells in an undifferentiated state, while an up-regulation in *Nox* expression by RA, and the subsequent increase in ROS leads to conditions favourable for NRX dissociation from DVL and activation of canonical WNT signaling required for PrE formation.

3.2 Materials and methods

3.2.1 Cell culture, transfection, and treatment

Mouse F9 teratocarcinoma cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10 % fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 mg/mL streptomycin (Lonza). Chemical treatments used are as follows: dimethyl sulphoxide (DMSO; Caledon Labs) as vehicle control; 10^{-7} M retinoic acid (RA all-trans; Sigma), 50 or 100 µM hydrogen peroxide (H_2O_2; EMD); 50 µM dibutyryl cyclic adenosine monophosphate (db-cAMP; Sigma); 1 mM N-acetyl cysteine (NAC; Sigma); 0.1 mM 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma). Cells were transfected with pcDNA empty vector, pcDNA-*Gata6*, pcDNA-*mNox4* (gift from Dr. M. Jaconi, University of Geneva), sc-*NRX* (scrambled control *NRX*), sh-*NRX* (short hairpin *NRX*), pGL3-*BARL*, or pRL-*TK* constructs using Lipofectamine 2000, according to the manufacturer's recommendations (Invitrogen). Treated and transfected cells were grown at 37^{o}C and 5% CO_2.

3.2.2 Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was collected using the RNeasy kit (Qiagen) and converted into first strand cDNA using reverse transcriptase qScript cDNA Supermix (Quanta BioSciences) according to the manufacturer's recommendations. RNA was isolated following treatment, transfections, or transfections then treatments of F9 cells as mentioned in the results and figure legends. Primers were designed to: *L14* sense (5'GGG AGA GGT GGC CTC GGA CGC) and antisense (5'GGC TGG CTT CAC TCA AAG GCC), a constitutively expressed gene in F9 cells used as an internal control; *NRX* sense (5'TCT GCT CAC CAT TCT GGA CA) and antisense (5'ACA CGC TGG AAA AGT CCA AG); *Gata6* sense (5'CTC TGC ACG CTT TCC CTA CT) and antisense (5'GTA GGT CGG GTG ATG GTG AT); *Foxa2* sense (5'ACC TGA GTC CGA GTC TGA GC) and antisense (5'CAT GGT GAT GAG CGA GAT GT); *Wnt6* sense (5'GCG GTA GAG CTC TCA GGA TG) and antisense (5'AAA GCC CAT GGC ACT TAC AC); *Thrombomodulin TM* sense (5'CCA GGC TCT TAC TCC TGT A) and antisense (5'TGG CAC TGA AAC TCG CAG TT); *Nox1* sense (5'AAT GCC CAG GAT CGA GGT) and antisense (5'GAT GGA AGC AAA GGG AGT GA); *Nox2* sense (5'ACC TTA CTG GCT GGG ATG AA) and antisense (5'TGC AAT GGT CTT GAA CTC GT); *Nox3* sense (5'TGT CAT GCC GGT GTG CTG GA) and antisense (5'CCC GTA GGC AAC GAG TTT GTG GA); *Nox4* sense (5'GAT CAC AGA AGG TCC CTA GCA) and antisense (5'GTT GAG GGC ATT CAC CAA GT); *Duox1* sense (5'AAA ACA CCA GGA ACG GAT TGT) and antisense (5'AGA AGA CAT TGG GCT GTA GG); *Duox2* sense (5'AGC TGG CTG AGA AGT TCG AC) and antisense (5'CCT GTG GAT GGA CTT CCT GT). Primers were used with first strand cDNA template for RT-PCR and the following conditions: *NRX* – 35 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C, and 30 s at 72^{o}C;

Gata6 – 35 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C, and 30 s at 72^{o}C; *Foxa2* – 35 cycles of 30 s at 94^{o}C, 30 s at 58^{o}C, and 30 s at 72^{o}C; *Wnt6* – 35 cycles of 30 s at 94^{o}C, 30 s at 62^{o}C, and 45 s at 72^{o}C; *TM* – 32 cycles of 30 s at 94^{o}C, 30 s at 60^{o}C, and 30 s at 72^{o}C; all *Nox* and *Duox* – 35 cycles of 30 s at 94^{o}C, 30 s at 58^{o}C, and 30 s at 72^{o}C. RT-PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide and images were captured using a FluorChem 8900 gel imaging station (Alpha Innotech). Amplicons were also sequenced (Robarts Research Inst., London, ON) to confirm their identity. For qRT-PCR, analysis was carried out in triplicate using the Corbett Research Rotar Gene RG-300. Each reaction contained 500 nM concentrations of each primer, 1X PerfeCTa SYBR Green FastMix (Quanta BioSciences), and 50 ng of cDNA. Gene expression analysis was performed using the comparative cycle threshold (delta/delta C_T) method, normalized to *L14* expression, and fold changes were calculated relative to the DMSO vehicle-treated or pcDNA empty vector transfected control cells.

3.2.3 Immunoblot analysis

Total cell lysates were prepared in 2% sodium dodecyl sulfate lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% mercapto-2-ethanol, and 1X Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentrations were quantified using a Bradford protein assay (Bio-Rad), and equal amounts were separated on denaturing 10% polyacrylamide gels and transferred to nitrocellulose membranes (Biotrace; Pall Corp.). The membranes were blocked in 5% skim milk and probed with

antibodies, and the signals were detected using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce). The primary antibodies were directed against TROMA-1 (1:25; Developmental Studies Hybridoma Bank), and β-Actin (1:10,000; Santa Cruz). Secondary antibodies were HRP-conjugated goat anti-rat and anti-mouse (1:10,000; Pierce).

3.2.4 Microscopy and intracellular reactive oxygen species detection

For microscopy, cells were observed with a Zeiss Axio Observer A1, and images were captured using a QImaging Retiga digital video camera. Intracellular ROS generation was determined using 5-(and-6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Life Technologies, Invitrogen) in conjugation with fluorescence microscopy. CM-H_2DCFDA was prepared in DMSO according to the manufacturer's recommendations. Following treatment or transfection, cells were incubated with 2 μM CM-H_2DCFDA in PBS for 10 min at 37^{o}C and 5% CO_2. Immediately after incubation, cells were washed twice with PBS and images were captured as mentioned above. All images were assembled as plates using Adobe Photoshop and Adobe Illustrator (CS5.1).

3.2.5 sh-*NRX* and RNAi

The following sequence derived from the mouse *NRX* cDNA was used for the knockdown studies: sh-*NRX* (AA) GAT CAT TGC CAA GTA CAA; for the scrambled control the following sequence was used: sc-*NRX* (AA) GAT CAT TGC ACA GTA CAA A [16]. Oligonucleotide primers designed to amplify this sequence was used with PCR to clone the *NRX* sh-RNA into the pRS sh-RNA vector (a gift from Dr. Robert Cumming, UWO). Cells were transfected with sh-*NRX*, sc-*NRX*, or CMV-GFP constructs, the latter two serving as negative and transfection efficiency controls, respectively.

3.2.6 TCF/LEF reporter assay

Cells transfected with pGL3-*BARL* and then treated with DMSO (vehicle control) or 10^{-7} M RA, or co-transfected with *pGL3-BARL* and sc-*NRX* or sh-*NRX* in equal amounts, were prepared 24 h post-treatment or post-transfection using the Dual-Glo Luciferase Assay System as per the manufacturer's recommendations (Promega). Luciferase expression was quantified using the GloMax Multi Detection System (Promega). Cells were also co-transfected with pRL-*TK* to normalize luciferase levels.

3.2.7 Statistical analysis

Data from all experiments are representative of at least three independent biological replicates performed on separate occasions. Densitometry data were obtained

using a FluorChem 8900 Chemiluminescence and Gel Image Doc (Alpha Innotech). Analysis of all data was performed using a one way ANOVA and Tukey's HSD post-hoc test (SPSS PASW Statistics v. 19). *P* values were considered statistically significant at the 0.05 level. Statistical data are presented as the mean ± S.E.

3.3 Results

3.3.1 H_2O_2 induces primitive endoderm and these cells are competent to form parietal endoderm

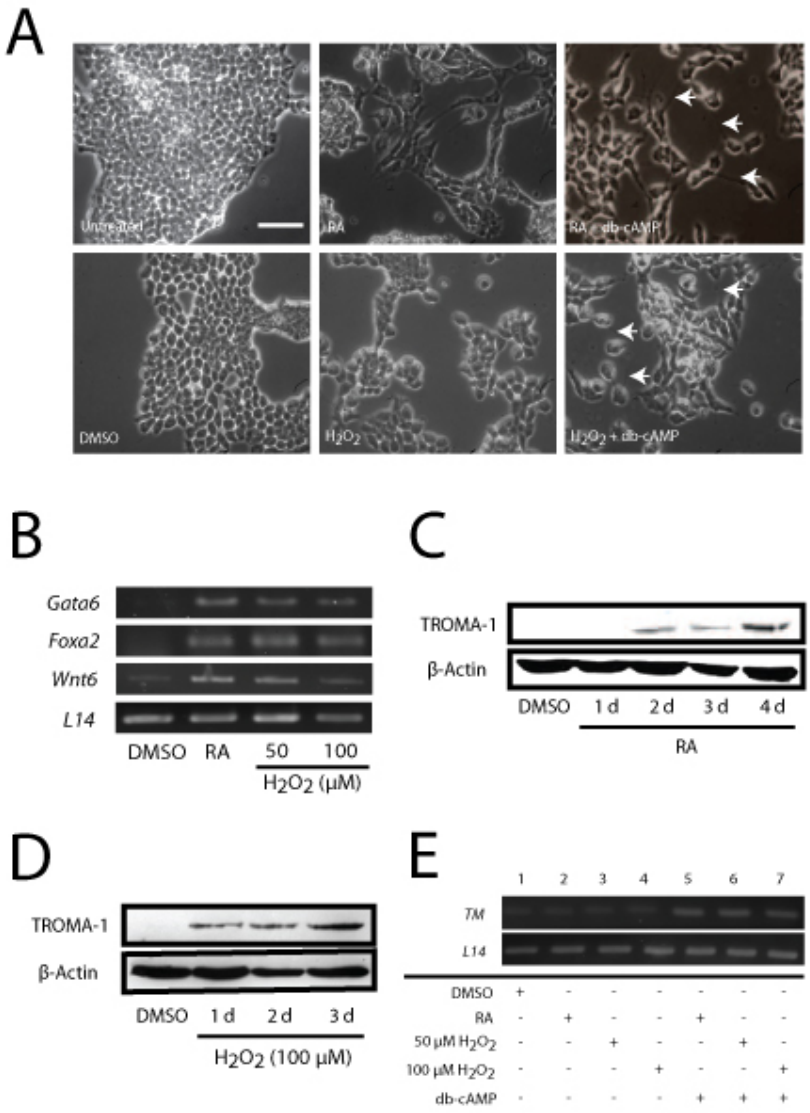
The ability of RA to induce differentiation of F9 cells into PrE has been documented previously [5]. Recently, work in our lab demonstrated that when F9 cells are induced by RA to form PrE, there is an increase in ROS levels [23]. To test if H_2O_2, a ROS, is sufficient to induce molecular and morphological features of PrE in the absence of RA, cells were first treated with RA or H_2O_2 and then examined for hallmark changes of PrE differentiation (Fig. 3.1). Untreated cells or those treated with DMSO vehicle were similar in morphology, appearing round in shape and growing in compact bodies (Fig. 3.1A). RA-treated cells adopted a more flattened morphology and had spread out over the plate (Fig. 3.1A). Cells treated with H_2O_2 shared the morphological features seen in RA treatment, suggesting they had differentiated into PrE (Fig. 3.1A). This was confirmed by RT-PCR analysis of *Gata6*, *Foxa2* and *Wnt6*, which are markers of PrE differentiation (Fig. 3.1B) and immnoblot analysis for TROMA-1, an endo-A cytokeratin present in differentiated ExE cells (Fig. 3.1C-D). An up-regulation of *Gata6*, *Foxa2* and *Wnt6* with a positive TROMA-1 signal was detected in RA-, and H_2O_2-treated cells, with little to no signal in DMSO-treated controls (Fig. 3.1B and Fig 3.1C-D, respectively). Confident that ROS has the ability to induce PrE differentiation, the next step was to determine if these cells remained competent to complete the EMT and form PE. Although the ability to

chemically induce F9 cells to form PE is well known [6], no report has documented whether H_2O_2 in the presence of db-cAMP would have the same outcome. To test this, cells were treated with RA and then db-cAMP as a control to induce PE, or with H_2O_2 and db-cAMP, and examined by phase contrast microscopy and using RT-PCR for *Thromobomodulin* (*TM*), a marker of PE [24] (Fig. 3.1A and 3.1E, respectively). My previous work reported that subtle morphological differences exist between PrE and PE, specifically the refractile nature of PE cells and the presence of filopodia [25]. Both of these features were obvious when cells were treated with RA and db-cAMP, or H_2O_2 and db-cAMP (Fig. 3.1A, arrows). Amplicons for *TM* were detected in the RA and db-cAMP positive control (Fig. 3.1E lane 5), as well as in the H_2O_2 treatments containing db-cAMP, but not in the DMSO negative control, or in samples from cells treated with either RA or H_2O_2 alone (Fig. 3.1E). Together, these results indicated that in the absence of RA, cells treated with H_2O_2 had differentiated into PrE and were competent to form PE when treated with db-cAMP.

3.3.2 H_2O_2 activates canonical WNT/β-catenin/TCF signaling

Activation of the WNT/β-catenin/TCF pathway in F9 cells is an obligatory step for RA-induced PrE differentiation [11, 13, 25] and evidence indicates that ROS interact at the level of DVL to positively regulate this pathway [16, 26]. Furthermore, my lab has shown that the addition of RA to cells pre-treated with antioxidants N-acetyl cysteine (NAC) or Trolox attenuated RA's ability to induce PrE [23]. Together with the ability of

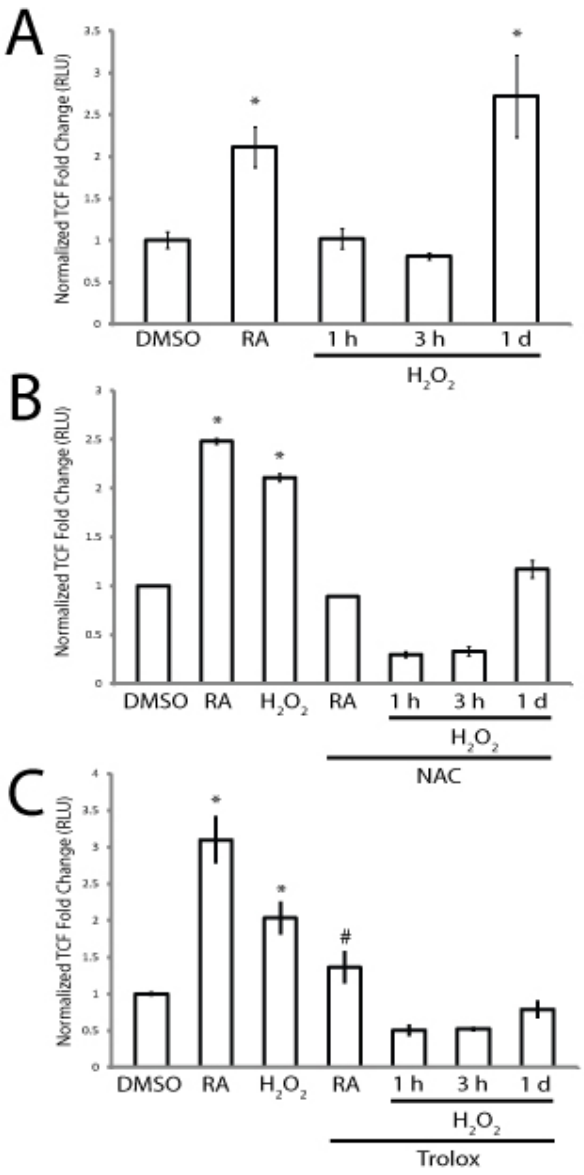
Figure 3.1. **H_2O_2 induces morphological and molecular markers of primitive endoderm and these cells are competent to complete the EMT to parietal endoderm.** **(A)** Untreated or DMSO-treated F9 cells showed similar morphology. Likewise, cells treated with RA to induce primitive endoderm were morphologically similar to those treated with H_2O_2. RA-induced primitive endoderm cells treated with db-cAMP were morphologically similar to those treated with H_2O_2 and db-cAMP. These cells had long protrusions (white arrows), and were more refractile in appearance than those treated with RA or H_2O_2 alone. Scale bar = 50 µm. **(B)** RT-PCR analysis showed the up-regulation *Gata6*, *Foxa2* and *Wnt6* following RA or H_2O_2 treatments. **(C)** Immunoblots with the TROMA-1 antibody to detect the primitive endoderm marker endo-A cytokeratin showed an increase in endo-A protein levels from day 2 through 4 post-RA treatment. **(D)** H_2O_2-treated cells also expressed endo-A cytokeratin. **(E)** RT-PCR analysis showed *Thrombomodulin* (*TM*), a marker of parietal endoderm, was up-regulated in cells treated with RA and db-cAMP (lane 5). Up-regulation was also detected in cells treated with H_2O_2 (two concentrations) and db-cAMP (lanes 6 and 7). H_2O_2 alone had no obvious effect on *TM* expression. **(A-E)** Data are representative of three independent experiments.


A
Untreated
RA
RA + db-cAMP
DMSO
H2O2
H2O2 + db-cAMP
B
Gata6
Foxa2
Wnt6
L14
DMSO
RA
50
100
H2O2 (μM)
C
TROMA-1
β-Actin
DMSO
1 d
2 d
3 d
4 d
RA
D
TROMA-1
β-Actin
DMSO
1 d
2 d
3 d
H2O2 (100 μM)
E
1
2
3
4
5
6
7
TM
L14
DMSO
RA
50 μM H2O2
100 μM H2O2
db-cAMP

RA and H_2O_2 to induce markers of PrE, the correlative changes in ROS levels following RA treatment [23], and evidence linking ROS to canonical WNT/β-catenin signaling [16, 26], I hypothesized that H_2O_2 would activate the canonical WNT/β-catenin pathway. A β-catenin/TCF-responsive luciferase reporter assay was used to test this hypothesis (Fig. 3.2). The reporter activity in cells treated with DMSO vehicle control was comparable to that recorded in cells treated for 1 or 3 h with H_2O_2 (Fig. 3.2, top). This activity increased significantly in cells exposed for 1 day with H_2O_2, as well as in those treated with RA (Fig. 3.2). When cells were pre-treated with NAC, however, and then treated with RA or H_2O_2 for 1 day, there was no significant difference in luciferase activity compared to the DMSO controls (Fig. 3.2B). The same was true for cells pre-treated with Trolox and then treated for 1 day with H_2O_2 (Fig. 3.2C). In contrast, cells pre-treated with Trolox and then with RA showed a small increase in luciferase activity compared to the controls. Despite this increase, the activity was significantly less when compared to cells treated with RA alone (Fig. 3.2C). These results indicated that H_2O_2 was responsible for increasing the readout of active canonical WNT/β-catenin signaling and this was independent of RA. Furthermore, that antioxidants impede the ability of RA or H_2O_2 to activate canonical WNT/β-catenin signalling, would suggest ROS are necessary in the RA-induced induction of PrE.

3.3.3 RA induces NADPH-oxidase (*Nox*) expression

Figure 3.2. **Antioxidants attenuate RA- or H_2O_2-induced canonical WNT/β-catenin/TCF signaling. (A)** Cells transfected with the pGL3-*BARL* TCF-luciferase reporter construct were treated 24 h post-transfection with DMSO or RA for 1 day, or H_2O_2 for 1 h, 3 h or 1 day. **(B, C)** Cells were pre-treated for 1 h with either: **(B)** N-acetyl cysteine (NAC) or **(C)** Trolox and then treated as described above in **(A)**. Following all treatments, cells were lysed and luciferase activity determined using a GloMax luminometer. **(A-C)** Cells treated with RA or H_2O_2 for 1 day showed a significant increase in TCF reporter activity compared to DMSO controls (* $P<0.05$). **(B, C)** Cells pre-treated with **(B)** NAC or **(C)** Trolox and then treated with RA or H_2O_2 showed no difference in TCF-luciferase activity compared to DMSO-treated control. **(C)** Cells pre-treated with Trolox and then treated with RA showed a slight increase in TCF-luciferase activity, but this was significantly less when compared to cells treated with RA alone (# $P<0.05$). **(A-C)** Data are representative of three independent experiments.

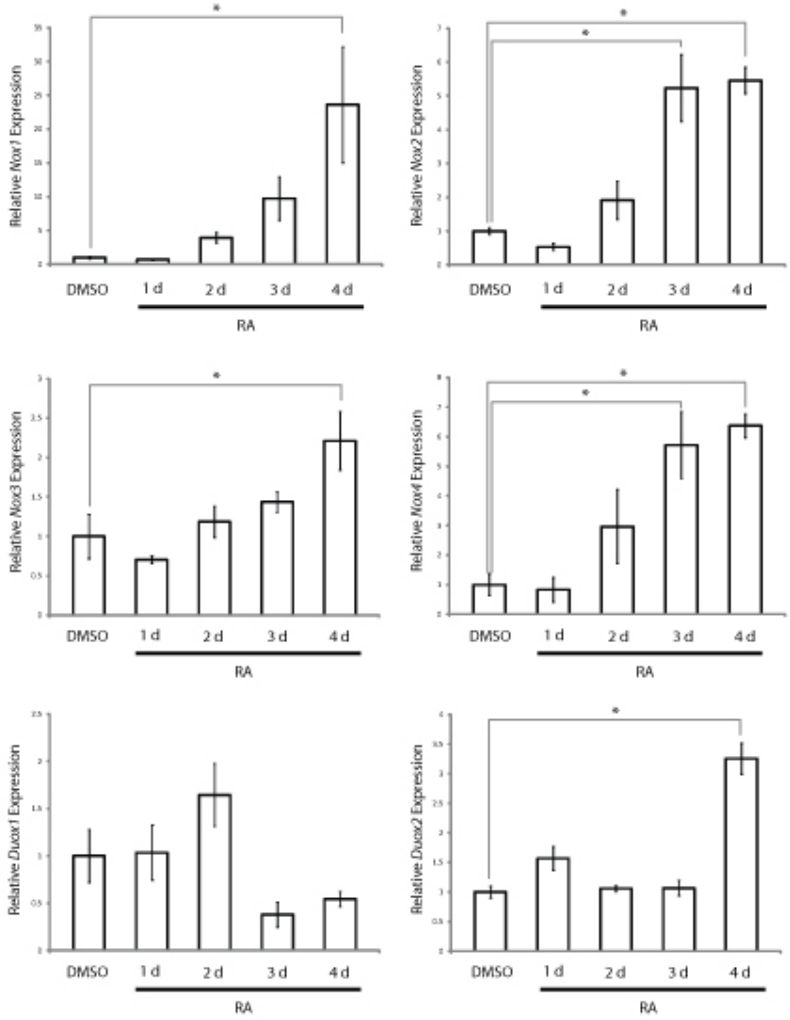

A
Normalized TCF Fold Change (RLU)
DMSO
RA
1 h
3 h
1 d
H2O2
B
Normalized TCF Fold Change (RLU)
DMSO
RA
H2O2
RA
1 h
3 h
1 d
H2O2
NAC
C
Normalized TCF Fold Change (RLU)
DMSO
RA
H2O2
RA
1 h
3 h
1 d
H2O2
Trolox

The mitochondria and the cytoplasm are two major sources of ROS production in a cell. Since previous work in my lab has shown that mitochondrial ROS may not play a significant role in RA-induced increase in ROS levels [23], my focus turned to the NOX family of proteins, which are known for their widespread expression patterns and their role in producing ROS that affects various biological processes [27]. To test if *Nox* genes are expressed in F9 cells and more importantly if their regulation is in response to RA, *Nox1-4* and *Duox1* and *2*, levels were investigated by qRT-PCR (Fig. 3.3). Results showed that all but the *Duox1* gene was RA responsive relative to the DMSO vehicle control. It is interesting to note that significant differences were seen beginning on day 3 for *Nox2* and *4*, whereas it took one additional day for these differences to be seen for *Nox1*, *3* and *Duox 2* (Fig. 3.3). These results indicated *Nox* transcripts are up-regulated in response to RA and presumably, the proteins these transcripts encode participate in complexes that produce the ROS.

3.3.4 *Gata6* induces *Nox4* expression, leading to increases in ROS and primitive endoderm differentiation

Although Nox activity is regulated at the level of the protein subunits that comprise the functional ROS producing complex, changes in gene expression of the various *Nox* isoforms have also been reported to be critical for NOX activity [28-30]. To investigate the latter I used an *in silico* analysis of the promoter regions of the various *Nox* genes and found putative binding sites for GATA6 (data not shown). Since GATA6

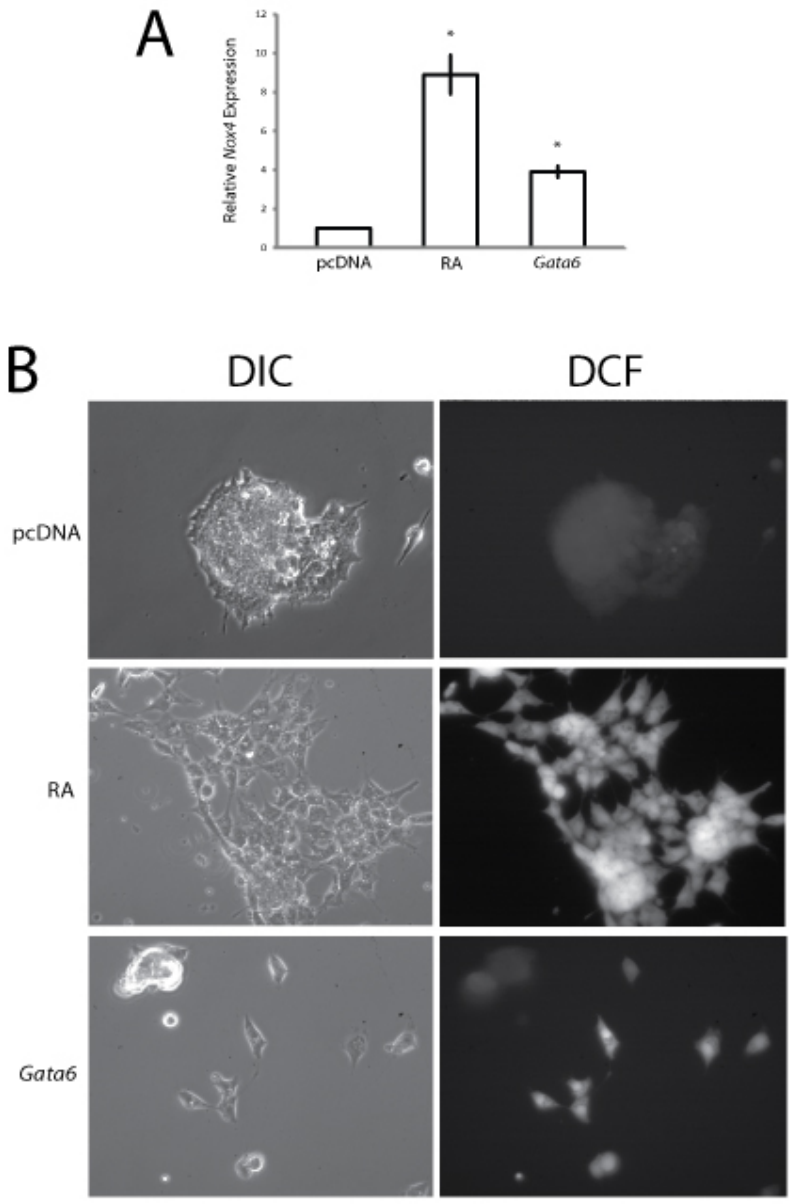
Figure 3.3. **RA induces expression of *Nox* mRNAs.** Total RNA isolated from F9 cells treated with RA for 1-4 days or from DMSO-treated cells grown for 4 days, was reverse transcribed into cDNA and then processed for real time PCR. The expression of *Nox* mRNAs was quantified relative to the expression of the constitutively expressed *L14* gene. The expression of *Nox1-4* and *Duox2* increased after RA treatment. Levels of *Duox1* did not change appreciably over time. * denotes significant difference ($P<0.05$) between treatment and DMSO control. Data are representative of three independent experiments.


Relative Nox1 Expression
Relative Nox2 Expression
Relative Nox3 Expression
Relative Nox4 Expression
Relative Duox1 Expression
Relative Duox2 Expression
DMSO
1 d
2 d
3 d
4 d
RA
*

is a master regulator of PrE formation [1, 31] and is up-regulated in response to RA (Fig. 3.1B), I hypothesized that ectopic expression of *Gata6* in the absence of RA would induce *Nox* expression. To test this, total RNA from cells transfected with pcDNA-*Gata6* were collected and reverse transcribed into first strand cDNA for qRT-PCR analysis (Fig 3.4A). Although RA induced the expression of *Nox1-4* and *Duox2* (Fig. 3.3), *Gata6*-transfected cells only induced the expression of *Nox1* and *Nox4* (data not shown and Fig. 3.4A, respectively). The ability of *Gata6* to induce *Nox* expression prompted me to determine whether or not the ectopic expression of *Gata6* alone was sufficient to induce ROS in the absence of RA. DCF, a ROS indicator, was used in conjunction with fluorescence microscopy to analyze cells that were treated with RA, transfected with pcDNA empty vector, or transfected with pcDNA-*Gata6*. Results showed that cells transfected with pcDNA-*Gata6* exhibited a prominent increase in fluorescence similar to those treated with RA, while the empty vector transfected controls showed no fluorescence (Fig. 3.4B). Taken together, the data would suggest that in the absence of RA, *Gata6* induces the expression of *Nox1* and *4*, which led to the increase in the levels of ROS detected by DCF.

Nox4 was chosen to be investigated further since its promoter contains a putative binding site for GATA6 (data not shown), a master regulator of ExE. Furthermore, the ROS produced by NOX4 is in the form of extracellular H_2O_2 and the activity of NOX4 is determined by *Nox4* mRNA expression [32]. To address whether or not ectopic expression of *Nox4* would induce ROS, cells were transfected with pcDNA-*Nox4* or pcDNA empty vector and then loaded with DCF. Results showed that cells transfected with pcDNA-*Nox4* exhibited an increase in fluorescence, while the empty vector

Figure 3.4. **Overexpression of *Gata6* induces *Nox4* expression and increases intracellular ROS levels. (A)** Total RNA isolated from F9 cells treated with RA or transfected with the empty vector (pcDNA) or *Gata6* (pcDNA-*Gata6*) were reverse transcribed to cDNA and processed for real time PCR. Both RA-treated and *Gata6*-transfected cells showed an up-regulation of *Nox4* mRNA compared to the empty vector-transfected controls (* $P<0.05$). **(B)** Cells treated with RA or transfected with pcDNA-*Gata6* or pcDNA empty vector were exposed to CM-H_2DCFDA (DCF), an indicator of intracellular ROS. DCF fluorescence was evident in RA-treated and *Gata6*-transfected cells, but not in the pcDNA-transfected controls. The morphology of cells following the different treatment regimens was examined using differential interference contrast (DIC) microscopy. **(A, B)** Data are representative of three independent experiments.


A
Relative Nox4 Expression
12
10
8
6
4
2
0
*
*
pcDNA
RA
Gata6
B
DIC
DCF
pcDNA
RA
Gata6

transfected control showed little fluorescence (Fig. 3.5). It is interesting to note that the flattened, stellate shaped morphology of the *Nox4*-transfected cells resembled that seen when cells were treated with RA or transfected with *Gata6* (Fig. 3.5 and Fig. 3.4B, respectively). Taken together, these data suggests a signaling hierarchy between RA, *Gata6* and *Nox4*, leading to the increase in ROS, an activation of the canonical WNT/β-catenin pathway and changes in cell morphology that accompany PrE formation.

3.3.5 NRX negatively regulates primitive endoderm formation

NRX is a redox-regulated protein that modulates the WNT/β-catenin pathway in NIH3T3 cells and *Xenopus* embryos [16, 17, 33]. Given the ROS data described above, and its apparent link to WNT signaling, it seemed logical to propose that NRX may also be involved in PrE formation. To address this, *NRX* was knocked down in F9 cells using an sh-*NRX* approach, and the efficiency of the knockdown was evaluated by qRT-PCR (Fig. 3.6A). Treating cells with DMSO or RA had no effect on *NRX* levels and the same was true for RA and db-cAMP (Fig. 3.6A, lanes 1-3). In contrast, *NRX* amplicons were absent when cells were transfected with sh-*NRX* (Fig. 3.6A, lanes 6 and 7), which contrasts that seen in cells transfected with the sc-*NRX* scrambled control or sc-*NRX* and db-cAMP (Fig. 3.6A, lanes 4 and 5, respectively).

Confident that the sh-*NRX* would effectively knock down endogenous expression, the effects on cell morphology were examined by phase contrast microscopy (Fig. 3.6B). Cells transfected with the sc-*NRX* control appeared morphologically similar to those

Figure 3.5. **Overexpression of *Nox4* induces intracellular ROS.** Cells transfected with pcDNA-*mNox4* or the pcDNA empty vectors were exposed to CM-H_2DCFDA (DCF), an indicator of intracellular ROS. DCF fluorescence was evident in *mNox4*-transfected cells, but not in pcDNA-transfected controls. Differential interference contrast (DIC) microscopy highlights the morphological similarities between the different treatment regimens. Data are representative of three independent experiments.

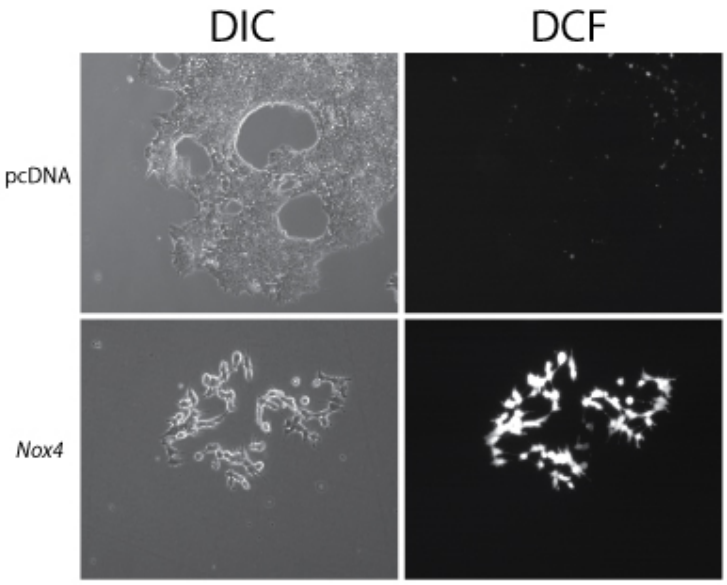
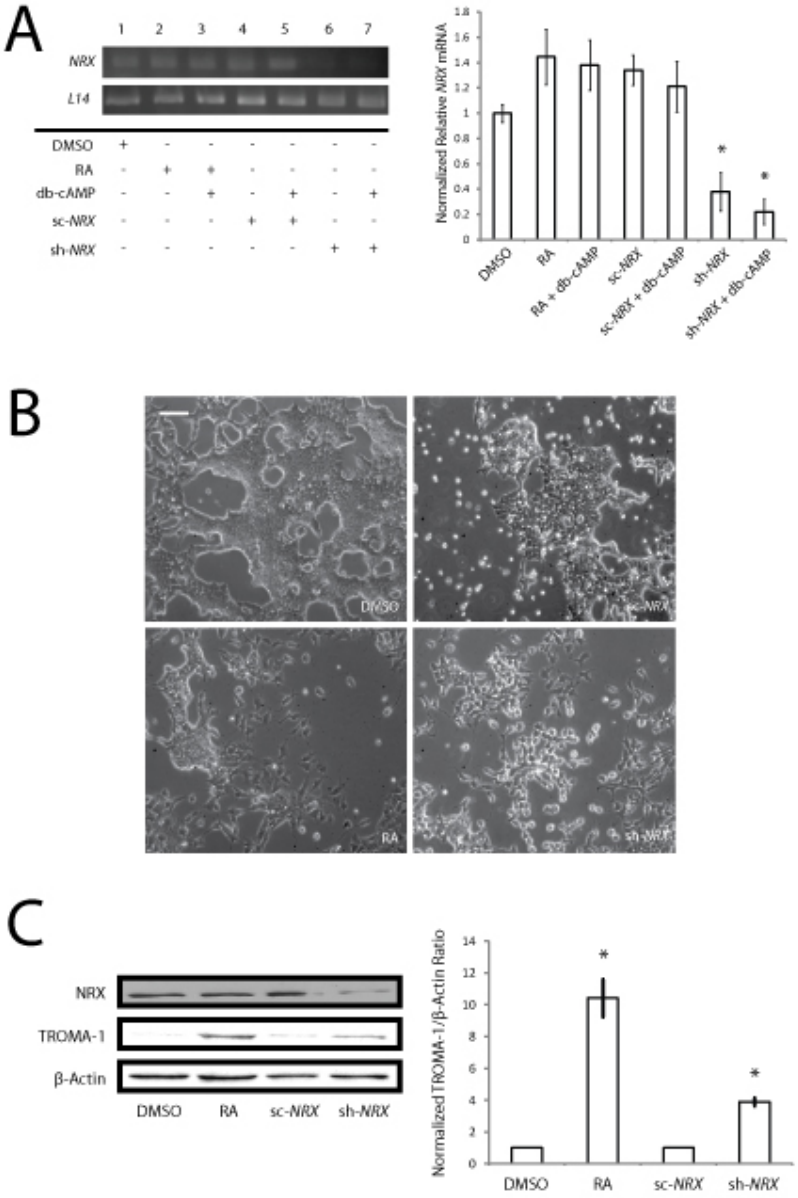

DIC
DCF
pcDNA
Nox4

Figure 3.6. **Depletion of *NRX* induces primitive endoderm formation. (A)** Total RNA from F9 cells transfected with sc- or sh-*NRX* (sc: scrambled control; sh: short hairpin), or transfected and then treated with db-cAMP, were collected, reverse transcribed into first strand cDNA, and then used as a template for PCR and qRT-PCR with primers specific to *NRX* and *L14* (control). An *NRX* amplicon was present in all lanes except those from cells that were transfected with the sh-*NRX* plasmid (lanes 6 and 7). qRT-PCR confirmed that the decrease in *NRX* expression, as a result of the sh-*NRX* transfection, was significant. **(B)** F9 cells transfected with sc-*NRX* were morphologically similar to those treated with DMSO, appearing in tightly compact groups reminiscent of undifferentiated cells. In contrast, F9 cells transfected with sh-*NRX* showed a flattened, more elongated morphology, comparable to RA-induced primitive endoderm. Scale bar = 50 μm. **(C)** F9 cells were transfected with sc- or sh-*NRX*, or treated with DMSO or RA, and protein lysates collected to detect the primitive endoderm marker endo-A cytokeratin. Immunoblot analysis with the TROMA-1 antibody, which detects endo-A cytokeratin, showed little to no signal in the DMSO control or sc-*NRX* control. Signals were seen in RA-treated or sh-*NRX*-transfected cells. An antibody to NRX confirmed the efficacy of the knockdown, while one to β-Actin served as the loading control. Densitometry analysis confirmed that there was a significant increase in TROMA-1 positive signal when cells were treated with RA compared to DMSO, and the same was true for endo-A levels in cells transfected with sh-*NRX* compared to sc-*NRX*. **(A-C)** Data are representative of three independent experiments. **(A, C)** * denotes $P<0.05$. **(C)** Densitometric data represents the mean ± S.E.


A
1 2 3 4 5 6 7
NRX
LI4
DMSO + - - - - - -
RA - + + - - - -
db-cAMP - - + - + - +
sc-NRX - - - + + - -
sh-NRX - - - - - + +
Normalized Relative NRX mRNA
DMSO
RA
RA + db-cAMP
sc-NRX
sc-NRX + db-cAMP
sh-NRX
sh-NRX + db-cAMP
B
DMSO
sc-NRX
RA
sh-NRX
C
NRX
TROMA-1
β-Actin
DMSO RA sc-NRX sh-NRX
Normalized TROMA-1/β-Actin Ratio
DMSO
RA
sc-NRX
sh-NRX

treated with DMSO. Specifically, cells were round in shape and grew in compact masses (Fig. 3.6B). In contrast, those transfected with sh-*NRX* were elongated and flattened out, which are characteristic changes in morphology associated with RA-induced PrE cells [25]. The ability of NRX depleted cells to differentiate into PrE was confirmed by immunoblot analysis (Fig. 3.6C). DMSO or RA treatment had no affect on the levels of NRX, while cells transfected with sh-*NRX* showed reduced NRX levels. Analysis with the TROMA-1 antibody to detect the endo-A marker of PrE showed, as expected, a strong signal in the RA lane compared to those treated with DMSO (Fig. 3.6C). The signal also appeared higher in cells transfected with sh-*NRX* compared to the sc-*NRX* transfected controls. Densitometry data confirmed these results and showed there was not only a significant increase in TROMA-1 levels in RA-treated cells compared to those seen in DMSO treatments, but also a significant increase resulting from the *NRX* knockdown. Together, the molecular and morphological data confirmed that the *NRX* knockdown in the absence of RA was sufficient to induce F9 cells to form PrE.

3.3.6 Loss of NRX correlates with an increase in canonical WNT signaling

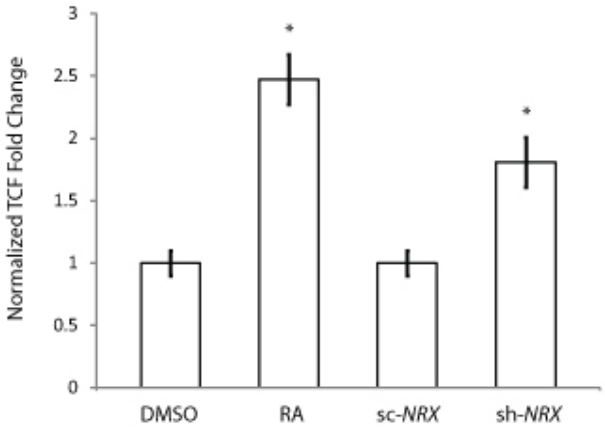
The evidence I have shown so far appears to be in agreement from an earlier study showing NIH3T3 cells having elevated β-catenin/TCF activity when NRX is depleted [16]. I used a TCF-luciferase assay to confirm my hypothesis that an increase in TCF-mediated activity, as a result of NRX depletion, contributes to PrE formation (Fig. 3.7). Cells were co-transfected with pGL3-*BARL* and a *Renilla* luciferase construct and

then treated with RA, or co-transfected with sc-*NRX* or sh-*NRX*. Results showed that RA induced a 2.5-fold increase in luciferase activity in cells depleted of NRX relative to those transfected with the sc-*NRX* control (Fig. 3.7). Thus, depleting cells of NRX is sufficient to activate canonical WNT/β-catenin signaling.

3.3.7 NRX-depleted cells are competent to form parietal endoderm

PrE is an intermediate step and the completion of the EMT is required for cells to adopt the parietal fate [11]. The data above would indicate that depleting cells of NRX was sufficient to induce cells to form PrE, but the questions remained as to whether or not these cells were in fact PrE in nature and if so, were they competent to complete EMT and differentiate into PE. To address these questions, cells transfected with either sc-*NRX* or sh-*NRX* were treated with db-cAMP and their morphology and molecular characteristics were assessed by phase contrast microscopy and qRT-PCR, respectively (Fig. 3.8). Those transfected with sc-*NRX* and treated with db-cAMP were morphologically indistinguishable from those treated with DMSO (data not shown). In contrast, cells transfected with sh-*NRX* and treated with db-cAMP were morphologically similar to those treated with RA and db-cAMP (Fig. 3.8A). Further analysis using RT-PCR/qRT-PCR showed the up-regulated expression of the PE marker *TM* in cells transfected with sh-*NRX* and treated with db-cAMP (Fig. 3.8B). Although the increase was not as dramatic as that seen in PE cells induced by RA and db-cAMP, the amount was significant compared to that in the other treatments (Fig. 3.8B). Thus, although the

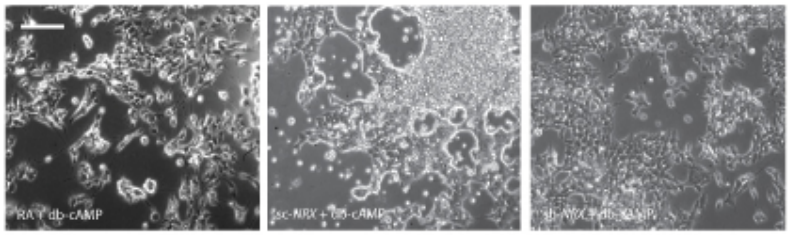
Figure 3.7. **Depletion of NRX induces canonical WNT/β-catenin signaling in the absence of RA.** Protein lysates from cells transfected with pGL3-*BARL* and then treated with RA, and from cells co-transfected with pGL3-*BARL* and sc- or sh-*NRX* (sc: scrambled control; sh-short hairpin) plasmid, were collected and processed for luciferase activity 24 h post-RA treatment or transfection. Cells treated with RA had a 2.5-fold increase in TCF-luciferase activity relative to DMSO-treated controls, while sh-*NRX*-transfected cells showed a 1.8-fold increase relative to the sc-*NRX* controls. Data represents the mean fold change ± S.E. of three independent experiments. * denotes $P<0.05$.


Normalized TCF Fold Change
3
2.5
2
1.5
1
0.5
0
*
*
DMSO
RA
sc-NRX
sh-NRX

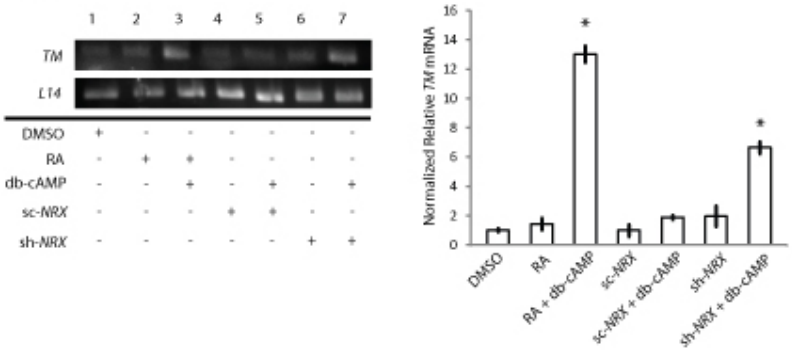
effect of knocking down NRX in the presence of db-cAMP on PE formation may not be as robust as treating cells with RA and db-cAMP, it is nevertheless sufficient to induce the competent PrE cells to differentiate into PE.

Figure 3.8. **Cells transfected with sh-*NRX* are competent to form parietal endoderm. (A)** F9 cells treated with RA and db-cAMP to form parietal endoderm have flattened out over the dish and are more refractile with multiple protrusions. In contrast, cells transfected with sc-NRX (scrambled control) and then treated with db-cAMP grow in tightly compact groups, similar to that seen when cells were treated with DMSO alone (Fig. 3.6B). Cells transfected with sh-*NRX* and then treated with db-cAMP share morphological features in common with RA and db-cAMP-induced parietal endoderm, specifically the protrusions and the refractile appearance [23, 25]. Scale bar = 50 μm. **(B)** Total RNA from F9 cells treated with DMSO, RA, or RA and db-cAMP, transfected with sc- or sh-*NRX*, or transfected with sc- or sh-*NRX* and then treated with db-cAMP, was isolated, reverse transcribed into cDNA, and then used as templates for PCR analysis with primers designed to the parietal endoderm marker, *Thrombomodulin* (*TM*). The constitutively active *L14* gene was used as a control. RT-PCR analysis showed that *TM* was up-regulated in parietal endoderm cells that were induced by RA and db-cAMP (lane 3). A slight increase was also seen in cells transfected with sh-*NRX* and then treated with db-cAMP (lane 7). qRT-PCR analysis confirmed that these increases in *TM* expression seen in lanes 3 and 7 were significant. * denotes $P<0.05$. Data represents the mean ± S.E. of three independent experiments.

A


RA + db-cAMP
sc-NRX + db-cAMP
sh-NRX + db-cAMP

B


1 2 3 4 5 6 7
TM
LT4
DMSO + - - - - - -
RA - + + - - - -
db-cAMP - - + - + - +
sc-NRX - - - + + - -
sh-NRX - - - - - + +
Normalized Relative TM mRNA
16
14
12
10
8
6
4
2
0
*
*
DMSO
RA
RA + db-cAMP
sc-NRX
sc-NRX + db-cAMP
sh-NRX
sh-NRX + db-cAMP

3.4 Discussion

The canonical WNT/β-catenin signaling pathway has been elucidated in great detail and numerous reports underlie its importance in key events required for normal embryonic development and those in adult life [34]. Abnormal WNT signaling, however, is at the root of several human diseases and its importance in maintenance of embryonic, adult, and cancer stem cells has gained notable attention in the last few years [35]. For these reasons, it is important to identify how WNT pathways are regulated under normal conditions in the effort of being able to target these regulatory mechanisms to prevent the onset and/or progression of disease states. Towards that end, I have used the F9 teratocarcinoma stem cell model to study how the activation of canonical WNT/β-catenin signaling in naïve cells leads to the initiation of EMTs required to pattern extraembryonic endoderm in the mouse embryo. An obligatory step required for the differentiation of F9 cells into PrE is the activation of TCF/LEF-dependent transcription [13], which is the downstream readout of active canonical WNT/β-catenin signaling. Previous work from my lab has shown that *Wnt6* is at the center of this EMT [11] and the transcription factors responsible for *Wnt6* expression have been identified (Chapter 2). Given that RA causes an increase in ROS [23] and the ability of ROS to impinge on the WNT pathway [36], it seemed plausible that ROS was initiating and/or modulating the WNT response in F9 cells.

Several pieces of evidence led to the proposal that ROS, like RA, was influencing the WNT signaling pathway involved in PrE formation. For instance, cell morphology

changed in response to both agents, as did the expression of molecular markers of differentiation, and the competency of both RA- and ROS-treated cells to further differentiate into PE in the presence of db-cAMP (Fig. 3.1). Given the significant increase in ROS within F9 cells following RA treatment [23], the question remained as to the link between RA and ROS. Increase in ROS levels may reflect either an increase in ROS production and/or decrease in antioxidant capacity or perhaps both as the two events are not mutually exclusive. Although intracellular antioxidant status was not investigated, it is interesting to note that during mouse embryonic stem cell differentiation, the redox status shifts to an oxidative state and antioxidant supplementation delays differentiation [37]. While an investigation to address the state of various redox-sensitive proteins would have been insightful, the focus first turned to identifying whether or not ROS were acting on the canonical WNT signaling pathway, which is integral in PrE formation, and to detect the source of the ROS.

Funato *et. al.* [16] was first to report that ROS signaling in the form of H_2O_2 displaces NRX from DVL, activating canonical WNT/β-catenin signaling. That exogenous H_2O_2 activates TCF/LEF-dependent transcription independent of a WNT ligand in NIH3T3 and HEK293 cells [16] was sufficient evidence to propose that the same phenomenon exists in F9 cells. This hypothesis was tested and the fact that H_2O_2 treatment resulted in the activation of TCF/LEF-dependent transcription, while pre-treatment of cells with the antioxidants NAC and Trolox attenuated the ability of H_2O_2 to induce TCF/LEF-dependent activity (Fig. 3.2) supports this notion. It is reasonable to speculate that ROS signaling *in vivo* has a direct role in mediating the WNT signaling

that directs ExE differentiation. While studies are underway to address this, one matter that remained was to identify the non-mitochondrial source of ROS.

The NOX family of enzymes became the focus of attention because they are known to catalyze the reduction of oxygen by using NADPH as its electron donor and produce ROS, which stimulates the post-translational modification of proteins to influence cell signaling, differentiation and regulation of gene expression [27, 38]. Furthermore, recent evidence exists to indicate that RA signals through NOX to induce cell differentiation [39]. It was first necessary to determine if *Nox* genes are expressed in F9 cells and more importantly, if RA regulates them. Towards that end, I found that the expression of *Nox1-4* and *Duox2* increased in response to RA (Fig. 3.3). *Nox4* was chosen to be investigated further since its promoter contains a putative binding site for GATA6 (data not shown), a master regulator of ExE. Furthermore, the ROS produced by NOX4 is in the form of extracellular H_2O_2 [32], which if formed by F9 cells under RA stimulation, would be available to signal in a paracrine fashion. I found that the expression of *Nox4* is up-regulated in response to ectopic expression of *Gata6* (Fig. 3.4A), followed by concomitant increase in ROS (Fig. 3.4B and Fig. 3.5). Although the type of ROS produced by ectopic expression of *Gata6* or *Nox4* alone was not determined, the morphological features between those ectopically expressing *Gata6* or *Nox4*, and those treated with RA were strikingly similar. Coupled with the notion that an increase in the level of ROS is obligatory for differentiation [23], leading to active canonical WNT/β-catenin signaling (Fig. 3.2), my attention turned on how ROS modulates canonical WNT/β-catenin signaling during ExE differentiation.

NRX is a member of the thioredoxin (TRX) family of evolutionarily conserved and ubiquitously expressed proteins that contain a redox sensitive active site motif [40]. TRX catalyzes NADP-dependent reductions of disulfide bridges and functions as a disulfide oxidoreductase. Under oxidizing conditions the thiol functional group on the two cysteine residues form a disulfide bridge capable of changing protein function or interactions [41]. The importance of TRX in embryogenesis is evident from the studies showing that the knockout of either of the two *TRX* genes in mouse embryos is lethal with $TRX1^{-/-}$ embryos dying shortly after implantation [42, 43]. It is also interesting to note that although $NRX^{-/-}$ pups show embryonic defects, they die later around birth [36]. Although the whole mount *in situ* hybridizations studies indicate that NRX plays a role in patterning tissues and regions during mouse embryogenesis [40, 44], nothing until now has been reported on its involvement in the early events associated with ExE development. My data showing the knockdown of *NRX* leads to the molecular and morphological changes that recapitulate those that occur as a result of RA treatment would indicate NRX acts to negatively regulate WNT signaling during ExE formation (Fig. 3.6). That *NRX* depleted cells have increased TCF/LEF-mediated transcriptional activity (Fig. 3.7) lends support to the idea that NRX serves as a negative regulator of the WNT pathway. It is interesting to note that a decrease in GSK-3β activity also occurs when cellular H_2O_2 levels increase [45] and under these conditions, β-catenin levels would also be expected to increase. This has not been examined in reference to F9 cells, but together it is easy to envision how the redox state of the cell can influence multiple signaling events. In the case of ExE formation, these changes led to PrE formation with

cells remaining competent to complete the EMT and further differentiate into PE under the appropriate conditions (Fig. 3.8).

3.5 References

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CHAPTER 4

GENERAL DISCUSSIONS

4.1 Introduction

This thesis detailed the crosstalk imparted on a signal transduction pathway involved in the differentiation of extraembryonic endoderm. The goal of my thesis was to investigate the canonical WNT signaling pathway involved in extraembryonic endoderm formation with the hypothesis that GATA6 and FOXA2, two transcriptions factors necessary for primitive endoderm differentiation, can initiate two signaling events that were once perceived independent. Within the chapters of this thesis, the data on how canonical WNT/β-catenin signaling is initiated by GATA6 and FOXA2 (Chapter 2) and how ROS positively modulates WNT signaling (Chapter 3), provide new insight into how extraembryonic endoderm differentiates in the mammalian embryo.

4.2 Markers of extraembryonic endoderm

Pre-implantation development in the mouse is characterized by the presence and differentiation of the extraembryonic lineages, as well as the pluripotent cells of the epiblast that gives rise to the embryo proper [1-4]. Positional cues direct the outer

extraembryonic cells to form trophectoderm, which surrounds the ICM at the blastocyst stage [5]. The mechanisms governing primitive endoderm differentiation from the epiblast is not very well understood, but involves the regulation of many genes followed by cell sorting and death [6-8]. Around the time of implantation, the morphologically distinct primitive endoderm expresses markers like *Gata6*, *Gata4*, *Sox17*, and *Pdgfra* and separates the epiblast from the blastocyst cavity [1, 8-10]. *In vivo*, *Gata6* is required for primitive endoderm formation as well as for the expression of *Gata4* and *Foxa2* [1, 8, 11-16]. In the F9 model system, the requirement for *Gata6* can be by-passed by ectopically expressing *Foxa2* (Chapter 2). These *Foxa2* expressing cells form primitive endoderm in the absence of RA by directly up-regulating *Wnt6* expression and canonical WNT/β-catenin signaling, and are competent to complete the EMT and form parietal endoderm with subsequent treatment with db-cAMP (Chapter 2). Although many markers of primitive endoderm have been identified, it is difficult to establish independent markers for parietal and visceral endoderms due to the fact that they both share a common lineage precursor, the primitive endoderm.

4.3 Non-mitochondrial induction of ROS

Over the last decade ROS have gained much attention as a modulator of signaling pathways involved in diverse biological processes including cell growth, differentiation, apoptosis, and angiogenesis [17, 18]. Specifically, the NOX family of catalytic enzymes are recognized as the proteins responsible for the non-mitochondrial production of ROS

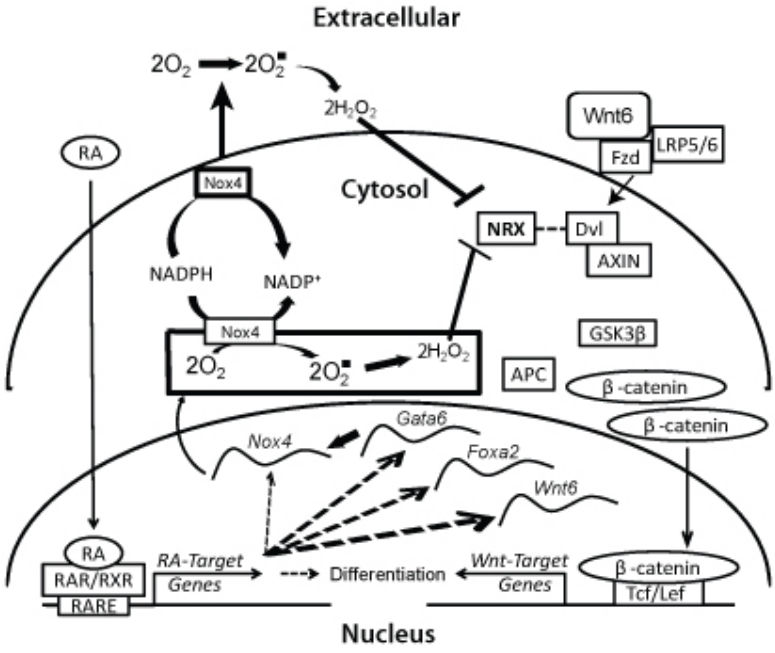
[18]. NOX enzymes, which are transmembrane proteins with binding sites for NADPH and FAD, are responsible for transferring an electron to molecular oxygen to generate ROS [17, 18]. Due to the cellular compartmentalization and tissue-specific expression profiles of the seven different isoforms in humans, it is believed that each NOX enzyme plays very specific biological roles with distinct and overlapping regulatory systems. In addition to the regulation of NOX activity by their subunits, changes in gene expression levels of the Nox isoforms have been reported. For example, the expression of *Nox2* in phagocytes is induced by IFNγ [19], while *Nox1* expression is stimulated by PDGF and angiotensin II in vascular smooth muscle cells [20]. More recently, *Nox1* was shown to be up-regulated directly by GATA6, HNF1α, Cdx1 and Cdx2 in colon cancer cells [21, 22]. My results showing the RA-induction of *Nox1-4* and *Duox2* in F9 cells (Chapter 3) is in agreement with other reports highlighting the transcriptional regulation of *Nox* genes in other systems. Furthermore, ectopic expression of *Gata6* in the absence of RA induced *Nox1* and *Nox4* expression (data not shown and Chapter 3, respectively), but had no effect on the expression of genes encoding the other NOX isoforms (data not shown). Although the expression levels of the NOX regulatory subunits were not examined during my studies, work is currently underway to determine their involvement on ROS production during extraembryonic endoderm differentiation. Moreover, the question still remains as to whether or not one or a combination of several Nox isoforms are necessary for the increase in ROS required for this differentiation.

4.4 Model for primitive endoderm differentiation

The induction of extraembryonic endoderm requires a complex interplay of many elements that regulate gene transcription and protein function. The literature as well as this thesis indicates that RA, WNT and ROS signaling are ideally suited to regulate each or a combination of the aforementioned elements required for primitive differentiation (Chapters 2 and 3). I have identified a novel mechanism and a signaling hierarchy leading to the induction of primitive endoderm in F9 cells. My working model proposes that in undifferentiated F9 cells, NRX prevents aberrant activation of the Wnt/β-catenin pathway. RA treatment induces differentiation where changes in gene expression including the up-regulation of *Gata6* and *Foxa2*, leads to *Wnt6* and *Nox4* expression and subsequent generation of ROS. An increase in the ROS load promotes the dissociation of NRX from Dishevelled, which essentially primes the pathway in preparation for full activation when the WNT ligand appears. When this occurs, the resulting increase in β-catenin levels leads to the subsequent activation of TCF/LEF-dependent regulation of WNT target genes, which encode proteins that promote the EMT associated with primitive endoderm formation (Fig. 4.1). Thus, the data in this thesis further delineates how a signal transduction pathway required for extraembryonic endoderm differentiation is initiated and as important, how it can be modulated by products that were once considered harmful to cells.

4.5 Future Directions

Figure 4.1. **A model for the differentiation of primitive endoderm.** RA acts through RA receptors which bind RA response elements in the promoter regions of target genes. In undifferentiated F9 cells, NRX bound to Dvl, prevents Dvl from destabilizing the β-catenin destruction complex. RA treatment induces differentiation where changes in gene expression, including *Gata6*, *Foxa2*, *Wnt6*, and *Nox4*, leads to the generation of ROS. An increase in ROS levels promotes the dissociation of Dvl from NRX, while Wnt6 binding to its Fzd receptor and LRP5/6 co-receptor ensures complete activation of the canonical Wnt pathway and regulation of Wnt target genes that promote the EMT associated with primitive endoderm formation. RA: retinoic acid; RAR/RXR: retinoic acid receptor/retinoid X receptor; RARE: retinoic acid response elements; O_2: oxygen; O_2^-: supoxide anion; H_2O_2: hydrogen peroxide; NADPH: nicotinamide adenine dinucleotide phosphate; Nox: NADPH oxidase; Fzd: frizzled; LRP5/6: lipoprotein related protein; NRX: nucleoredoxin; Dvl: dishevelled; GSK3β: glycogen synthase kinase; APC: adenomatous polyposis coli; Tcf/Lef: T-cell factor/lymphoid enhancer factor.


Extracellular
$2O_2$
$2O_2^{\bullet}$
$2H_2O_2$
Wnt6
Fzd
LRP5/6
RA
Nox4
Cytosol
NRX
Dvl
AXIN
NADPH
NADP+
Nox4
GSK3β
$2O_2$
$2O_2^{\bullet}$
$2H_2O_2$
APC
β -catenin
β -catenin
Gata6
Nox4
Foxa2
Wnt6
RA
RAR/RXR
RARE
RA-Target
Genes
Differentiation
Wnt-Target
Genes
β -catenin
Tcf/Lef
Nucleus

Although the data presented throughout this thesis (Chapters 2 and 3) supports the hypothesis that the induction of primitive endoderm requires a complex interplay of many elements that regulate gene transcription and protein function, several questions remain to be elucidated: 1) If both GATA6 and FOXA2 induce primitive endoderm and GATA6 induces FOXA2 (Chapter 2), is GATA6 necessary for primitive endoderm formation in the presence of FOXA2? This can be addressed by using an RNAi approach to knock down *Gata6* expression while ectopically expressing *Foxa2*; 2) *Wnt6* is sufficient to induce the canonical WNT signaling required for primitive endoderm formation, but is *Wnt6* necessary for this differentiation? An RNAi approach to knock down the expression of *Wnt6* in the presence of retinoic acid could be used. This task may prove to be difficult because in vertebrates, there are 19 different *Wnt* genes, and any one or combinations of them can be functionally redundant; 3) Both *Nox1* and *Nox4* are up-regulated by GATA6 (Chapter 3), however, whether this up-regulation is direct or indirect, and which NOX is sufficient and/or necessary for the production of ROS and induction of primitive endoderm remains unanswered. The direct or indirect relationship between GATA6 and *Nox1* or *Nox4* expression can be addressed using chromatin immunoprecipitation. To address if NOX1 and/or NOX4 is/are sufficient and/or necessary, selective chemical inhibitors of NOX1 or NOX4 or an RNAi approach to knock down *Nox1* or *Nox4* while ectopically expressing the other can be used; 4) Lastly, it would be very interesting to test whether the induction of primitive endoderm via the signaling crosstalk between WNT and ROS (Fig. 4.1) occurs *in vivo*.

4.6 References

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