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The Role of NADPH Oxidase in ROS Mediated Differentiation

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Abstract

Mouse teratocarcinoma F9 cells differentiate into primitive endoderm (PrE) when treated with retinoic acid (RA) or H_2O_2 and these changes are accompanied by an up-regulation of *Wnt6* and activation of the canonical WNT/β-catenin pathway. Data from our lab shows PrE differentiation is accompanied by an increase in reactive oxygen species (ROS), which induces a conformational change in Nucleoredoxin preventing its ability to bind and inhibit Dishevelled. This in turn positively impacts on the WNT/β-catenin signaling pathway leading to differentiation. The source of endogenous ROS seen following RA treatment was investigated and members of the NADPH oxidase (NOX) family were identified as candidates as *Nox1-4* and *Duox2* genes are up-regulated by RA. This study shows *Nox1* and *Nox4* are up-regulated when Gata6 is overexpressed in F9 cells. Furthermore, the pan-NOX inhibitor VAS2870 and NOX1-specific inhibitor ML171 significantly reduced the ability of RA to induce PrE differentiation. Additionally, a knockdown of *Nox1* and/or *Nox4* attenuate RA-mediated differentiation. Overexpression of *Nox1* or *Nox4* in F9 cells increases the levels of ROS, however, this is not sufficient for differentiation. Thus, the data suggest that the ROS produced during the differentiation of F9 cells into PrE is the result of an increase in NOX1 and NOX4 activity; however, overexpressing *Nox1* or *Nox4* alone is not sufficient to induce cells to form PrE.

Keywords

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List of Abbreviations

ANOVA   Analysis of variance

CK1   Casein kinase 1

C_t cycle threshold

db-cAMP   Dibutyryl cyclic adenosine monophosphate

DPI   Diphenyleneiodonium

DAB2   Disabled-2

DNA   Deoxyribonucleic acid

DVL   Dishevelled

EMT   Epithelial-to-mesenchymal transition

FBS   Fetal bovine serum

FZD   Frizzled

G418   Neomycin, disulfate salt

GPCR   G-protein-coupled receptor

GSK-3β   Glycogen synthase kinase 3β

HSD   Honest significant difference

HRP   Horseradish peroxidase

KER8	Keratin-8
mRNA	messenger ribonucleic acid
NOX	NADPH oxidase
NRX	Nucleoredoxin
PE	Parietal endoderm
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PrE	Primitive endoderm
PKA	Protein Kinase A
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RL	*Renilla* luciferase
ROS	Reactive oxygen species
RXR	Retinoic X receptor
SDS	sodium dodecyl sulphate
SE	standard error

siRNA	short interfering ribonucleic acid
SSEA-1	Stage-specific embryonic antigen
TCF-LEF	T-cell-factors-Lymphoid enhancer factors
TROMA-1	trophectodermal monoclonal antibody-1
TM	Thrombomodulin
TBS-T	Tris buffered saline with Tween-20
VE	Visceral endoderm
WNT	Wingless/MMTV integrated site

Chapter 1

1 Introduction

1.1 Mouse early development

Fertilization of mouse eggs occurs in the oviduct, followed by dramatic architectural changes to the zygote post implantation [1]. The zygote divides to give rise to the blastocyst, which is comprised of both the trophectoderm and inner cell mass (ICM) [1]. Cells of the ICM differentiate forming the epiblast, which gives rise to the embryo proper and primitive endoderm (PrE), the first extraembryonic endoderm (XEN) population of cells [2]. Endoderm is the innermost germ layer of growing embryos and is found in all multi-layered embryos [3]. XEN, although endoderm, does not contribute to the embryo proper [3]. Cells committed towards PrE express *Gata6*, *FoxA2*, and *Dab2* among other genes [3] (Fig. 1.1). PrE gives rise to two separate cell lineages: parietal endoderm (PE) and visceral endoderm (VE) [4]. Cells of the parietal endoderm, which migrate to line the trophectoderm, are crucial for protection of the embryo from mechanical damage and absorption of nutrients through the formation of the parietal yolk sac [4]. Visceral endoderm remains in contact with the epiblast and gives rise to the visceral yolk sac [4].

The differentiation of PrE to PE is one of the earliest epithelial-to-mesenchymal transitions (EMTs) in the developing mouse embryo [4]. EMTs occur when non-motile adherent epithelial cells adopt a migratory fate characteristic of mesenchymal cells. This is accomplished through changes in gene expression that lead to the loss of cell-cell adhesion, changes in cell polarity, morphology and migration. Loss of E-cadherin is a hallmark of an EMT, which destabilizes cell-cell junctions, enabling cell migration [5]. Although EMTs play pivotal roles in developmental processes such as gastrulation,

generation of the neural crest and organogenesis, they are also linked to cancer metastasis [6].

Figure 1.1. **Mouse early development**. In the early blastocyst two cell types are present: the inner cell mass (ICM) and the trophectoderm. The ICM is characterized by the expression of *Oct4* (yellow)*, Gata6* (purple) and *Nanog* (red)*. The ICM gives rise to the epiblast, which maintains expression of *Nanog* and PrE expressing *Gata4, 6, Sox7, 17, Dab2,* and *FoxA2*. PrE (purple) is the first population of XEN and differentiates into either PE (green) cells or VE (purple). PrE cells differentiating to a PE lineage undergo an EMT lining the blastocoel surface of the trophectoderm, and are characterized by expression of *Thrombomodulin.* PE cells create the parietal yolk sac. PrE cells differentiating to VE cells remain attached to the basement membrane of the ICM and form the visceral yolk sac. The extraembryonic endoderm is important for proper development of the epiblast as they are responsible for nutrient and waste exchange prior to placentation. Adapted from Kelly and Drysdale (2015).

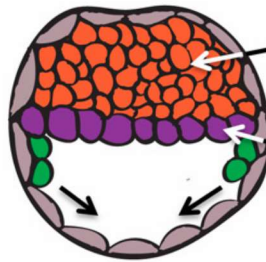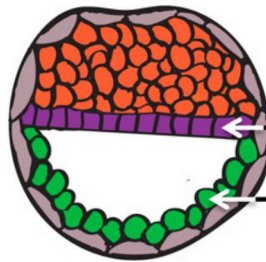**E3.5 Blastocyst (Early)**
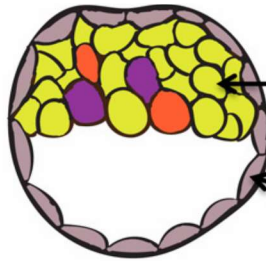
Inner Cell Mass
Oct4, Gata6, Nanog

Trophectoderm
Cdx2

E4.5 Blastocyst (Late)

Epiblast
Nanog

Primitive Endoderm
Gata 4,6, Sox7, 17, Dab2, FoxA2

Visceral Endoderm

Parietal Endoderm
Thrombomodulin

1.2 The F9 teratocarcinoma cell line model of mouse extraembryonic endoderm differentiation

The study of extraembryonic endoderm formation *in vivo* presents unavoidable difficulties; e.g., mammalian development occurs *in utero*, the small size of the embryo at these early stages, and the presence of other cell lineages in addition to XEN. To circumvent these constraints, investigators use the F9 teratocarcinoma cell line as an *in vitro* model to study XEN differentiation [6]. The F9 cell line was derived from a teratoma that formed following grafting 6-day old mouse embryos into the testes of the 129/J mouse strain [7]. Spontaneous differentiation rarely occurs in F9 cells, but differentiation to XEN can be chemically induced [8]. For instance, F9 cells treated with retinoic acid (RA) differentiate into PrE and can be further induced to parietal endoderm by subsequent treatment with dibutyryl cyclic adenosine monophosphate (db-cAMP) to augment Protein Kinase A (PKA) activity [9]. RA, a metabolite of vitamin A, crosses the plasma and nuclear membranes to bind the retinoic acid receptor (RAR) and retinoic X receptor (RXR) heterodimer [10]. There are three subtypes of RAR (RARα, β, and γ) and RXR (RXRα, β, and γ); however, it is the RXRα-RARγ heterodimer that is essential for differentiation of F9 cells into PrE [10] In the absence of RA, RAR/RXR heterodimers recruit repressive complexes inhibiting transcription [3]. However, this repressive complex is exchanged for an activating complex in the presence of RA [3]. RA is considered the limiting factor in the activation of the RAR/RXR target genes [3].

Differentiation of F9 cells is accompanied by increased synthesis of extracellular matrix proteins, cytokeratins and a loss of stage-specific embryonic antigen (SSEA-1) [8]. Increased expression of *Keratin-8* (*Ker8*), encoding an intermediate filament protein, and the accumulation of KER8 accompanies F9 cell differentiation [11, 12]. *Ker8* is not expressed in the inner cell mass, but its expression is characteristic of XEN and the trophectoderm [11]. Additionally, an increase in *disabled-2* (*Dab2*) expression is also indicative of PrE formation [13]. The differentiation from PrE to PE can be identified by

the increase of *Thrombomodulin* (*TM*), an endothelial cell receptor [14]. In addition to these changes of molecular markers, F9 cells undergo morphological changes and adopt large processes and become more rounded as they differentiate [8]. Concurrently, these cells migrate from the large colonies that develop in the undifferentiated state [8]. Our lab has shown that in addition to these molecular and morphological changes induced by RA, differentiation of F9 cells is also accompanied by activation of the canonical WNT/β-catenin pathway [9], and as described below, this pathway is modulated by Reactive Oxygen Species (ROS) [6].

1.3 Canonical WNT/β-catenin signaling

WNTs belong to a family of secreted glycoproteins involved in several important cellular processes including proliferation, differentiation and migration. There are at least three WNT signaling pathways: the canonical WNT/β-catenin pathway and two non-canonical pathways, WNT/JNK-planar cell polarity (PCP) and WNT/Ca^{2+}. Canonical WNT/β-catenin signaling plays an essential role in EMTs during embryonic development and is implicated in the progression of cancer [15]. Canonical WNT signaling occurs when the WNT ligand binds to and activates a seven-transmembrane Frizzled (FZD) receptor. As a result, Dishevelled (DVL) moves to the plasma membrane and recruits AXIN away from a destruction complex that, in the absence of the WNT ligand, functions via glycogen synthase kinase 3β (GSK-3β) and casein kinase I (CK1) to phosphorylate β-catenin, marking it for ubiquitination and degradation in the proteasome. However, when WNT is present, β-catenin is not degraded, allowing the latter to accumulate in the cytosol and translocate to the nucleus where it binds to and activates the T-cell-factors-Lymphoid enhancer factors (TCF-LEF) family of transcription factors. Together, β-catenin/TCF-LEF complex impart changes in gene expression resulting in the loss of epithelial markers (E-cadherin) and concomitant increase in mesenchymal markers (N-cadherin) [6]. Recently, we have found that DVL in undifferentiated F9 cells associates with nucleoredoxin (NRX), a redox sensitive protein of the Thioredoxin family

[16] that scavenges ROS [17]. This NRX-DVL association in F9 cells has also been shown in NIH3T3 murine fibroblast-derived cells and N1E-115 neuroblastoma cells. [18]. NRX when bound to DVL inhibits canonical WNT signaling, however, in the presence of ROS, NRX dissociates from DVL and the WNT pathway is primed awaiting the WNT ligand [17] (Fig 1.2). Support for this redox-sensitive regulation comes from NRX knockdown studies that show this is sufficient to increase TCF activity [17]. NRX, under oxidizing conditions, forms a disulphide bridge between two cysteine residues of the thiol functional group; this allows for changes in protein function and modulates protein-protein interactions of NRX [19]. Sandieson et al. (2014) reported using co-immunoprecipitation that while reduced binding of NRX and DVL occurred in F9 cells treated with the H_2O_2, increased binding was favoured under reducing conditions with dithiothreitol [17]. Furthermore, a knockdown of *NRX* in F9 cells increases β-catenin/TCF dependent transcription, allowing cells to differentiate to PrE, or to PE if treated with db-cAMP [17]. These data show an integral role of the redox sensitive protein NRX in F9 cell differentiation.

1.4 Reactive oxygen species

ROS are classically known as by-products of metabolic processes produced through the incomplete reduction of oxygen. There are two groups of ROS; oxygen-derived radicals, which include superoxide, hydroxyl, peroxyl and alkoxyl and nonradicals, which act as oxidizers or are readily converted to radicals such as hypochlorous acid, ozone, singlet oxygen and hydrogen peroxide [24]. ROS create oxidative stress on cells, damaging nucleic acids, proteins and lipids. This damage is linked to age-related and vascular diseases including neurodegeneration and atherosclerosis, respectively [20, 21, 22, 23]. Despite the negative aspects of ROS, recent studies now show cells benefitting from it to induce biological processes such as differentiation, apoptosis and proliferation [6, 20, 24]. ROS affect cell signaling through

oxidizing redox-sensitive cysteine residues on proteins, including the aforementioned NRX and its effect on the canonical WNT/β-catenin pathway [17, 24]. ROS also effects cell proliferation through the activation of the transcription factor nuclear factor kappaB [26]. Our lab has recently shown that H_2O_2 treatment alone is sufficient to induce XEN differentiation, showing both morphological and molecular changes similar to those accompanying RA-induced PrE formation [6] and interestingly, F9 cells treated with RA show elevated ROS levels in the cytosol. Furthermore, ROS is necessary for XEN to develop since differentiation is blocked when RA-treated F9 cells are either pre-treated with antioxidants to quench the levels of ROS or when diphenyleneiodonium (DPI) is used to inhibit NADPH oxidase (NOX) activity [6].

1.5 NADPH oxidase

RA-induced differentiation is accompanied and promoted by an increase in the levels of ROS [6, 17]. Due to lack of MitoTracker Red probe staining corresponding with the ROS indicator CM-H2DCFDA, Wen et al. (2012) concluded that the RA-induced ROS is not produced by the mitochondria. The cytosolic source of this ROS was investigated and the candidates identified include the members of the membrane bound NOX family, which are capable of producing superoxide that is converted to H_2O_2 by superoxide dismutase [6, 25]. NOX proteins were first found in professional phagocytes, which use superoxide to combat bacterial infections [24, 27]. Subsequent investigations have revealed homologues of the membrane bound cytochrome subunit, now delineating four mouse NOX proteins (NOX1, NOX2, NOX3, and NOX4) and two DUOX proteins (DUOX1 and DUOX2) [24, 28]. These NOX proteins work in concert with cytosolic subunits that include: p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox}, and the small GTP-binding proteins RAC1 and RAC2 [24]. NOX1 function is dependent on the membrane subunit p22^{phox}, as well as the cytosolic subunits p47^{phox} and p67^{phox}, and evidence exists that Rac is required to regulate its activity [24]. NOX4, however, is a p22^{phox}-dependent enzyme,

and does not require additional cytosolic subunits or Rac for activation and function [24]. NOX proteins have been shown localized to many different membranes including the endoplasmic reticulum, caveolae or lipid rafts, phagocytic vacuoles and nucleus [24]. As noted above, DPI, presumably acting on all NOX proteins in RA treated F9 cells, blocked ROS production and attenuated differentiation [6]. DPI has served as a general NOX inhibitor, but because it also acts on other non-NOX flavoenzymes [29], further research was done to confirm an integral role for NOX proteins in the RA mediated differentiation of F9 cells.

There is precedence for NOX proteins being involved in WNT signaling (Fig 1.2) and PrE differentiation as *Nox1-4* and *Duox2* gene expression in F9 cells is upregulated following RA treatment [6], and the *Nox1* promoter is regulated GATA6, which is involved in XEN differentiation [30, 31]. Furthermore, Brewer et al. (2006) reported that Gata6 binds directly to the *Nox1* proximal promoter and regulates gene expression. Whereas overexpressing NOX1 in IEC-6 cells treated with WNT3a increased β-catenin protein levels when compared to WNT3a treatment alone [32], increased *Nox4* expression in HEK-293 cells spontaneously increased ROS production without the need for additional stimulus [33]. Additionally, NOX1 can post-transcriptionally increase the levels of Keratin 18 [34], a fetal-type cytokeratin encoded by transcripts that increase during RA-induced F9 cell differentiation [12]. Wen et al. (2012) also show NOX1 to be upregulated higher than the other NOX subunits. NOX4 however, has been linked to cell proliferation, cytoskeletal reorganization, migration and stem cell differentiation [35, 36, 37]. Ellmark et al. (2005) showed that an increase in ROS occurs with an increase in *Nox4* mRNA. For these reasons I have specifically selected NOX1 and NOX4 to interrogate as the major candidates involved in the ROS production that is sufficient and necessary for RA-induced PrE formation.

Figure 1.2. **The canonical WNT/β-catenin signaling pathway and the role of ROS**. When WNT ligand is present it binds to its receptor FZD. DVL then moves to the plasma membrane, recruiting AXIN away from the destruction complex, inhibiting GSK3-β from marking β-catenin for degradation. However, when NRX is present it inhibits DVL from recruiting AXIN inhibiting the WNT/β-catenin pathway. NOX1 generates ROS through the activation of Rac1 and when NRX is in the presence of ROS it dissociates from DVL. This enables DVL to recruit AXIN in the presence of the WNT ligand. Adapted from Kajla et al (2012).
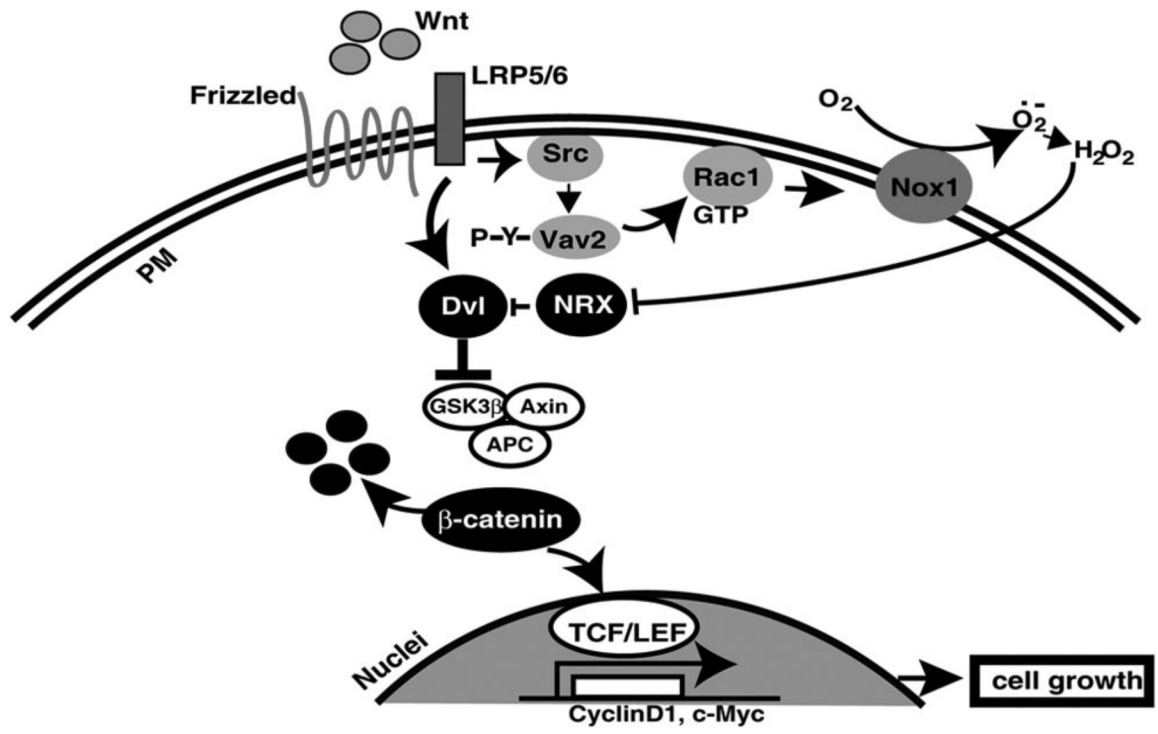
1.6 Hypothesis and Objectives:

Given what we know about the redox regulation of the WNT signaling pathway in a number of cell types including F9 cells, I hypothesize that the activity of NADPH oxidase 1 and/or 4 is/are responsible for producing the ROS that are necessary and sufficient to induce F9 cells to differentiate.

My objectives to address this hypothesis are:

1. To identify components of the signaling pathway(s) that regulate the expression of *Nox1* and *4* genes

2. To determine if NOX1 or 4 activity is necessary for XEN formation

3. To determine if NOX1 or 4 activity is sufficient for XEN formation

Chapter 2

2. Materials and Methods

2.1 Cell Culture

Mouse teratocarcinoma F9 cells (ATCC) were cultured in Dulbecco's modified Eagles medium (Lonza), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Lonza), and incubated at 37°C and 5% CO_2. Cells were treated with 10^{-7} M retinoic acid (RA all-trans; Sigma Aldrich), dimethyl sulfoxide (DMSO; Caledon) as a negative control, or 100 μM H_2O_2. DMSO was the solvent for all chemicals used in this study. Cells were treated or co-treated with the pan-NOX inhibitor VAS2870 (1 μM) (Sigma) and RA 24 hours after seeding and grown for 3 days. Cells were treated or co-treated with the NOX1 specific inhibitor ML171 (250 nM) (Tocris) and RA immediately post seeding and grown for 4 days as described above. Cells were treated with H_2O_2 prior to ROS detection.

2.2 Plasmids

The following plasmids were transfected into cells for overexpression: pcDNA3.1-*EV* (empty vector) as a transfection control, pcDNA3.1-*Gata6;* pcDNA-*mNox4* (a gift from Dr. M. Jaconi*,* University of Geneva), pcDNA3.1-*mNox1* (Addgene # 58340), pRL-*TK*, a gift from Dr. R. DeKoter (Western University), and pGL3-*BARL*. The following plasmids were transfected into cells for knockdown of target genes: piLenti-siRNA-GFP-*Nox1* "CTATTTAACTTCGAACGCTACAGAAGAAG", piLenti-siRNA-GFP-*Nox1* "TGCTTCCATCTTGAAATCTATCTGGTACA", piLenti-siRNA-GFP-*Nox4* "ACATTTGGTGTCCACTTTAAAGTAGTAGG", piLenti-siRNA-GFP-*Nox4*

"TCCAGTGGTTTGCAGATTTACTCTGTGTG" (Applied Biological Materials,
Richmond BC).

2.3 Cell Transient Transfection

F9 cells were reverse transfected using the transfection reagent Lipofectamine
2000 (Life Technologies) according to manufacturer's recommendations. Approximately
2.5×10^5 cells were added to a 35 mm dish already containing a total of 4 µg of DNA
plasmid and 10 µl of Lipofectamine 2000 that were diluted in Opti-MEM reduced serum
medium (Life Technologies). After 6 h medium was replaced with fresh culture medium
and cells grew overnight. Approximately 1×10^5 cells were then passaged onto 60 mm
dishes containing 1.75 mg/ml G418 sulfate (sigma) or 1 µg/ml puromycin, depending on
the resistance inferred by the vector and grown for 4 days. This selection allowed only
cells that were transfected and actively transcribing the vector to survive.

2.4 Immunoblot analysis

Immunoblot analysis was used to determine presence or absence, as well as
relative amount, of protein present in cells at the time of lysis. Protein lysates of cells
were collected in RIPA buffer containing 150 mM sodium chloride, 1.0 % Triton X-100,
0.5 % deoxycholate, 0.1 % SDS and 50 mM Tris pH 8.0. Protein concentrations were
determined using the DC^{TM} Protein Assay (Bio-Rad), and 20 µg of protein lysate was
mixed 1:2 with 3X SDS loading buffer containing 10 % β-mercaptoethanol and separated
on 10 % polyacrylamide gels by electrophoresis for 2 h with 100 V at 4°C. Following
electrophoresis, the proteins were wet-transferred electrophoretically to Immunoblot
PVDF membrane (Bio-Rad) for 16 h with 20 V at 4°C using a Tris-glycine transfer

buffer containing 20 % methanol. Membranes were incubated in Tris buffered saline with 0.1 % Tween-20 (TBS-T) containing 10% w/v skim milk powder for 1 h shaking at room temperature. Membranes were then incubated with primary antibody overnight at 4°C, followed by 3 washes 5 min each in TBS-T, incubated with secondary antibody for 2 h at room temperature followed by 3 washes for 10 min each in TBS-T. SuperSignal West Pico Chemiluminescent Detection Kit (Thermo Scientific) was used to detect the presence of secondary antibodies conjugated to horseradish peroxidase (HRP). Signals were captured using a Molecular Imager Gel Doc XR system (Bio-Rad) with Quantity One Software. The primary antibodies used were directed against TROMA-1 (1:10; 55 kDa, Developmental Studies Hybridoma Bank) and β-actin (1:10000; 47 kDa, Santa Cruz) dissolved in 3 % Bovine Serum Albumin w/v in TBS-T. TROMA-1 targets the intermediate filament KER8 found in differentiated F9 cells. β-actin was used as a protein loading control. Secondary anti-rat (1:1000) and anti-mouse (1:1000) antibodies were HRP-conjugated and dissolved in 3 % skim milk w/v in TBS-T.

2.5 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was used to determine relative steady state levels of mRNA in cells. Total RNA from treated and/or transfected cells after 4 days of growth was isolated and collected using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed into first strand cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the manufacturer's recommendations. The CFX Connect Real-Time PCR Detection System (Bio-Rad) was used for qRT-PCR analysis. Each reaction contained 500 nM of each primer, SensiFAST SYBR Mix (FroggaBio), and 1 µL of cDNA. Primers sequences are listed in Appendix 1. Analysis of steady state mRNA levels was determined using the comparative cycle threshold (Δ/ΔCt) method. Steady state mRNA levels were normalized to *L14* mRNA and relative values were normalized

by comparing treatments to DMSO-treated and/or control plasmid transfected control cells to determine fold change.

2.6 TCF/LEF reporter assay

Cells were transfected with pGL3-*BARL*, which is used as a readout for TCF activity and pRL-*TK* (transfection control). Cells were then treated with DMSO (vehicle control), 10^{-7} M RA, or co-transfected with *Nox1* or *Nox4* expression plasmids in equal amounts of DNA. Protein lysates were collected 3 days post-treatment or post-transfection with a passive lysis buffer (Promega) contained in the Dual-Glo Luciferase Assay System and according to manufacturer's recommendations (Promega). Luciferase amount was quantified using the GloMax Multi Detection System (Promega) by measuring luminescence after treatment with Luciferase Assay Substrate for firefly luminescence and treatment with Stop & Glo® Reagent for Renilla luminescence. Values were presented as relative luminescence derived from the quotient of firefly luminescence (pGL3-*BARL*) and Renilla luminescence (pRL-*TK*).

2.7 ROS detection

Intracellular ROS was detected using 5-(and-6-)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate prepared in DMSO (CM-H2DCFDA; Life Technologies, Invitrogen) and fluorescence microscopy. This ROS indicator works after cleavage of acetate groups within cells, and then oxidation of the nonfluorescent CM-H2DCFDA produces the highly fluorescent 2',7;dichlorofluroescein (DCF). Following treatment or transfection, cells were incubated with 2 µM CM-H2DCFDA and 10 µg/ml Hoechst 33342 (Molecular Probes, Life Technologies) in Hank's Balanced Salt Solution

(Life Technologies, Invitrogen) for 15 min at 37°C and 5% CO_2. Immediately after incubation, cells were rinsed twice with PBS and images were captured using a Zeiss Axio Observer A1 inverted microscope with a QImaging Retiga CCD.

2.8 Statistical Analysis

Data from qRT-PCR, densitometric analysis of immunoblots and Luciferase assays were gathered from three independent biological replicates. Comparisons of data between control and experimental groups were performed using a one-way ANOVA with Tukey's honest significant difference (HSD) post-hoc test or a Student's t-Test (SPSS Statistics for Windows Version 19.0, IBM Corp. Released 2010, Armonk, NY). Student's t-Test was used for statistical analysis of data when comparing control to only one experimental data set and * is used to denote significant difference. All other data was analyzed using a one-way ANOVA followed by Tukey's HSD test and letters are used to indicate significant differences. P-values were considered statistically significant at the 0.05 level. Statistical data are presented as the mean ± S.E.

Chapter 3

3. Results

3.1 Overexpression of *Gata6* increased *Nox1* and *Nox4* mRNA levels

We have previously shown an increase in ROS levels, accompanied by increased expression of *Nox1-4*, during RA-mediated F9 cell differentiation [6]. Additionally, *Gata6,* a regulator of endoderm and extraembryonic endoderm formation, was upregulated in cells treated with RA, and when overexpressed alone, was sufficient to induce differentiation [38]. Furthermore, Adachi et al. (2008) showed in CaCo-2 cells that Gata6 was responsible for increased *Nox1* transcription. This evidence led me to propose that GATA6 was responsible for the upregulation of *Nox1* and *Nox4* in F9 cells, leading to differentiation of PrE. To address this, I transiently overexpressed *Gata6* and then analyzed *Nox1* and *Nox4* mRNA levels 4 days post transfection using qRT-PCR (Fig 3.1). *L14* primers were used to ensure equal loading control, and all data was analyzed using the delta/delta CT method to determine relative mRNA levels. As expected, *Gata6* steady state levels of mRNA were increased following *Gata6* overexpression ($P<0.05$) (Fig 3.1A), and this was also sufficient to significantly increase ($P < 0.05$) the levels of *Nox1* (Fig 3.1B) and *Nox4* (Fig 3.1C) mRNA. Note that these levels were comparable and not significantly different to those induced by RA.

Figure 3.1. **GATA6 overexpression increased steady state levels of *Nox1* and *Nox4* mRNAs.** Total RNA was collected from F9 cells transfected with pcDNA3.1-*EV* (control) and treated with either DMSO or RA or transfected with pcDNA3.1-*Gata6* and cultured for 4 days with 1.75 mg/ml G418. Collected RNA was reverse transcribed into cDNA and then processed for real time PCR. **(A)** Cells transfected with pcDNA3.1-*Gata6* showed increased mRNA of *Gata6* when compared with *EV* transfected cells. **(B)** Cells transfected with pcDNA3.1-*Gata6* and cells transfected with *EV* and treated with RA showed increased *Nox1* mRNA when compared with DMSO treated cells transfected with *EV*. **(C)** Cells transfected with pcDNA3.1-*Gata6* and cells transfected with *EV* with RA treatment showed increased *Nox4* mRNA when compared with DMSO treated cells transfected with *EV*. Data are representative of three independent experiments. * denotes significance ($P<0.05$) and means with different letters were significantly different ($P<0.05$).
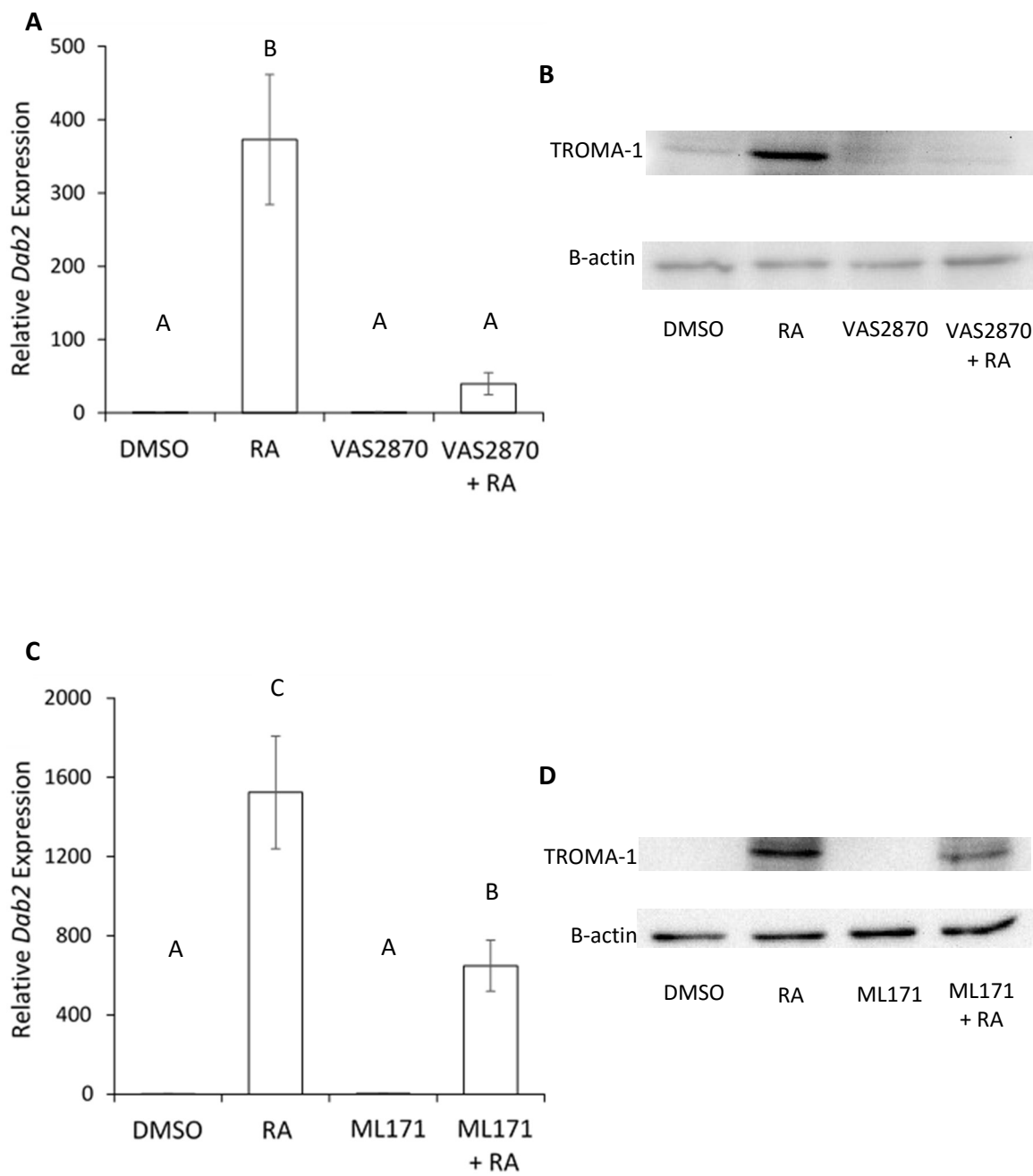
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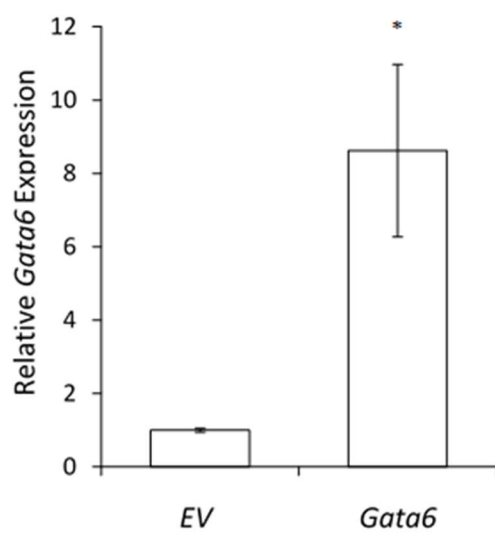
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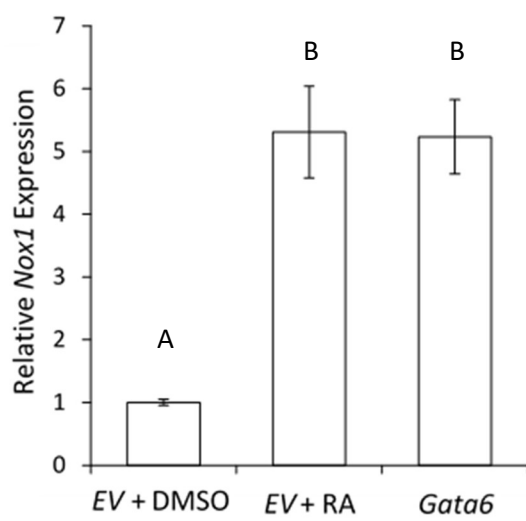
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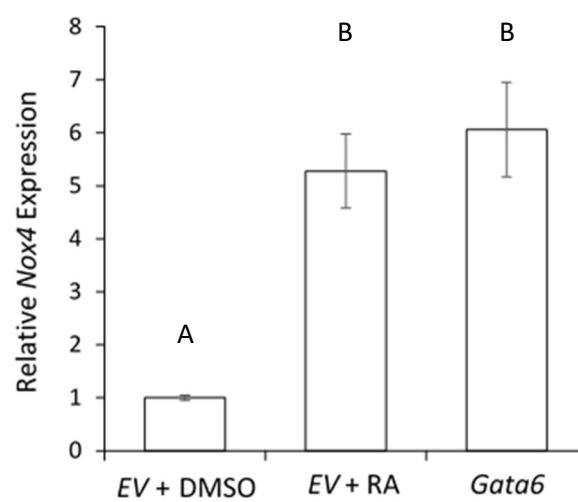
3.2 Chemical inhibition of NOX attenuated F9 cell differentiation

A role for ROS in PrE development was established in our lab, where it was shown that Diphenyleneiodonium chloride (DPI), a flavoenzyme inhibitor of ROS, attenuates RA-induced PrE differentiation [6]. DPI is frequently used to inhibit ROS production, but given the fact that it acts on numerous oxidoreductases, including the NOX family of proteins, an approach specifically targeted to the NOX family was needed [39]. Despite the fact that *Nox* genes in F9 cells are RA-responsive, the source of the ROS may not only come from the NOX proteins. To test the hypothesis that the activity of the NOX proteins is necessary for PrE differentiation, F9 cells were treated with NOX-specific inhibitors and the mRNA knocked down using siRNAs specific to *Nox1* and/or *4*. First, the pan-NOX inhibitor, VAS2870 (Sigma) [40] was used to determine if members of the NOX family were necessary for F9 cells to differentiate to PrE. VAS2870 can inhibit all NOX isoforms, but it specifically targets NOX1, NOX2 [41] and NOX4 [42]. Moreover, VAS2870 does not serve as an antioxidant because it has no O_2^- scavenging effects [43]. Cells treated with DMSO, RA, or VAS2870 (1 μM) or co-treated with VAS2870 and RA (10^{-7} M) were analyzed using qRT-PCR for changes in *Dab2*, a marker of PrE (Fig 3.2). Results of *Dab2* mRNA, relative to the loading control *L14,* showed no significant difference between DMSO, VAS2870 or VAS2870 and RA. The mRNA level of *Dab2* in cells treated with RA was significantly different from the other three treatments ($P<0.001$) (Fig 3.2A). Immunoblot analysis with the TROMA-1 antibody was used to detect increased KER8 levels characteristic of F9 cell induced to differentiate into PrE [44]. TROMA-1 signals on immunoblots were only seen following RA treatment (Fig 3.2B). Thus, inhibiting the NOX family of proteins attenuated RA-mediated differentiation, and would indicate that NOX proteins were necessary for complete differentiation.

I extended these results to specifically examine the necessity of NOX1 and NOX4. The NOX1 specific inhibitor ML171 (Tocris) was used at the reported NOX1 inhibition IC_{50} of 250 nM [43]. ML171 was reported to have no ROS scavenging effects

[43], making it an ideal candidate for determining specific involvement for NOX1 in F9 cell PrE differentiation. The mRNA level of *Dab2* in DMSO and ML171 treated cells was not significantly different from each other, but both were significantly different from RA treated cells ($P<0.001$) and cells co-treated with RA and ML171 ($P<0.05$) (Fig 3.2C). Furthermore, the *Dab2* mRNA level in co-treated cells was also significantly different from that in RA treated cells ($P<0.05$) (Fig 3.2C). Immunoblot analysis with the TROMA-1 antibody was used to determine if ML171 treatment could block or reduce differentiation caused by RA. Unlike the RA treated controls, TROMA-1 was not detected in DMSO or ML171 treated cells (Fig 3.2D). Cells co-treated with ML171 and RA show reduced TROMA-1 signal (Fig 3.2D), indicating that the inhibition of NOX1 activity attenuated RA-induced differentiation of PrE. NOX4 was not targeted since there was no known specific chemical inhibitor(s) that did not have ROS scavenging effects or additionally target other NOX proteins [43].

Figure 3.2. **PrE markers are attenuated following co-treatment of VAS2870 or ML171 with RA. (A)** Total RNA was collected from F9 cells treated with DMSO, RA and/or VAS2870 (1 μM) 1 day post plating and cultured an additional 3 days. Collected RNA was reverse transcribed into cDNA and then processed for real time PCR. RA-treated cells showed increased *Dab2* mRNA when compared with cells treated with DMSO, VAS2870 or VAS2870 with RA. **(B)** Protein lysates were collected from F9 cells treated with DMSO, RA and/or VAS2870 (1 μM). Although immunoblot analysis with the TROMA-1 antibody showed signals in RA-treated cells, no signals were detected in cells treated with DMSO, VAS2870 alone or co-treated with RA and VAS2870. β-actin was used as a loading control. **(C)** Total RNA was collected from F9 cells treated with DMSO, RA and/or ML171 (250 nM) and cultured for 4 days. RA-treated cells showed increased *Dab2* mRNA when compared with cells treated with DMSO, ML171 or ML171 with RA. Cells co-treated with ML171 and RA showed a significant increase in *Dab2* mRNA when compared with DMSO and ML171 treated cells. **(D)** Protein lysates were collected from F9 cells treated with DMSO, RA and/or ML171. Immunoblot analysis with the TROMA-1 antibody showed signals in RA-treated cells, but not in cells treated with DMSO and ML171 alone. Cells co-treated with RA and ML171 show reduced TROMA-1 signals. β-actin was used as a loading control. Data are representative of three independent experiments. Means with different letters were significantly different (*P*<0.05).

A

B

TROMA-1

B-actin

DMSO   RA   VAS2870   VAS2870
                                    + RA

C

D

TROMA-1

B-actin

DMSO   RA   ML171   ML171
                                + RA

3.3 Knockdown of *Nox1* and/or *Nox4* attenuated F9 cell differentiation

To corroborate the chemical inhibitor data, F9 cells were transfected with an siRNA vector to knockdown *Nox1* mRNA. Cells were also transfected with an siRNA vector to target *Nox4,* or transfected with a scrambled siRNA and treated with DMSO or RA as a negative and positive controls, respectively. Additionally, to test if knocking down *Nox1* and *Nox4* together would have a greater effect on blocking differentiation than the individual knockdowns, both siRNA vectors were transfected into cells. The results of the *Nox1* knockdown are shown in Fig 3.3A, the steady state mRNA levels induced by RA was significantly lower in cells containing the siRNA targeting *Nox1* transcript ($P<0.05$). A significant reduction in *Nox4* mRNA induced by RA was also seen ($P<0.05$) (Fig 3.3B). qRT-PCR analysis revealed that there was no significant difference in *Dab2* mRNA between the scrambled siRNA transfected cells treated with DMSO, individual knockdowns treated with RA or *Nox*1 and *Nox4* knocked down together and treated with RA (Fig 3.4A). As expected, there was a significant increase in *Dab2* mRNA ($P<0.05$) in scrambled siRNA transfected cells treated with RA (Fig 3.4A). Finally, the knockdown of *Nox1* and *Nox4* together treated with RA show no significant difference in TROMA-1 levels when compared to the individual knockdowns treated with RA, or the scrambled siRNA treated with DMSO control and TROMA-1 levels were significantly higher in scrambled siRNA transfected cells treated with RA ($P<0.05$) (Fig 3.4B and Fig 3.4C). Thus, the results would indicate that Nox1 and Nox4 were required for differentiation.

Figure 3.3. **Knockdown of *Nox1* and *Nox4*.** Total RNA was collected from F9 cells transfected with si-*scr*, si-*Nox1* and/or si-*Nox4* and cultured 4 days with RA treatment and 1.0 µg/ml puromycin for selection. Collected RNA was reverse transcribed into cDNA and then processed for real time PCR. **(A)** Cell transfected with si-*Nox1* and both si-*Nox1* and si-*Nox4* showed significantly decreased *Nox1* mRNA when compared cells transfected with si-*scr*. **(B)** Cell transfected with si-*Nox1* and both si-*Nox1* and si-*Nox4* showed significantly decreased *Nox4* mRNA when compared cells transfected with si-*scr*. Data are representative of three independent experiments. Means with different letters were significantly different ($P<0.05$).

	A	B
RA	+ + +	+ + +
si-*scr*	+ - -	+ - -
si-*Nox1*	- + +	- - +
si-*Nox4*	- - +	- + +
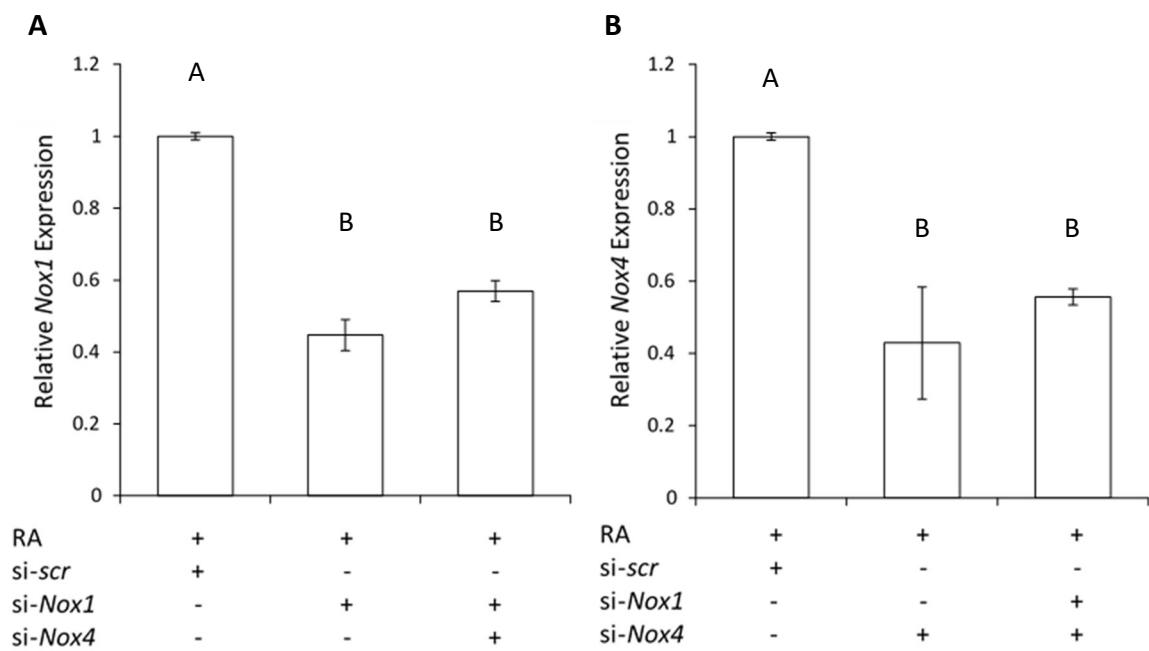
Figure 3.4. **Knockdown of *Nox1* and *Nox4* reduced the appearance of RA-mediated PrE markers.** Total RNA and protein were collected from F9 cells transfected with si-*scr*, si-*Nox1* and/or si-*Nox4* and cultured 4 days with RA treatment and 1.0 µg/ml puromycin for selection. Collected RNA was reverse transcribed into cDNA and then processed for real time PCR. **(A)** Relative *Dab2* mRNA was significantly decreased in RA-treated cells transfected with *si-Nox1* and/or si-*Nox4* when compared with RA-treated si-*scr* transfected cells. *Dab2* mRNA in DMSO-treated cells transfected with si-*scr* was not significantly different from RA-treated cells transfected with *si-Nox1* and/or si-*Nox4*. **(B, C)** Protein lysates were collected from F9 cells transfected with si-*scr*, si-*Nox1* and/or si-*Nox4* and treated with DMSO or RA for 4 days. Immunoblot analysis with the TROMA-1 antibody shows significantly decreased signal in RA-treated cells transfected with *si-Nox1* and/or si-*Nox4* when compared with RA-treated si-*scr* transfected cells. TROMA-1 levels in DMSO-treated cells transfected with si-*scr* were not significantly different from RA-treated cells transfected with *si-Nox1* and/or si-*Nox4*. β-actin was used as a loading control. Data are representative of three independent experiments. Means with different letters were significantly different ($P<0.05$).

A

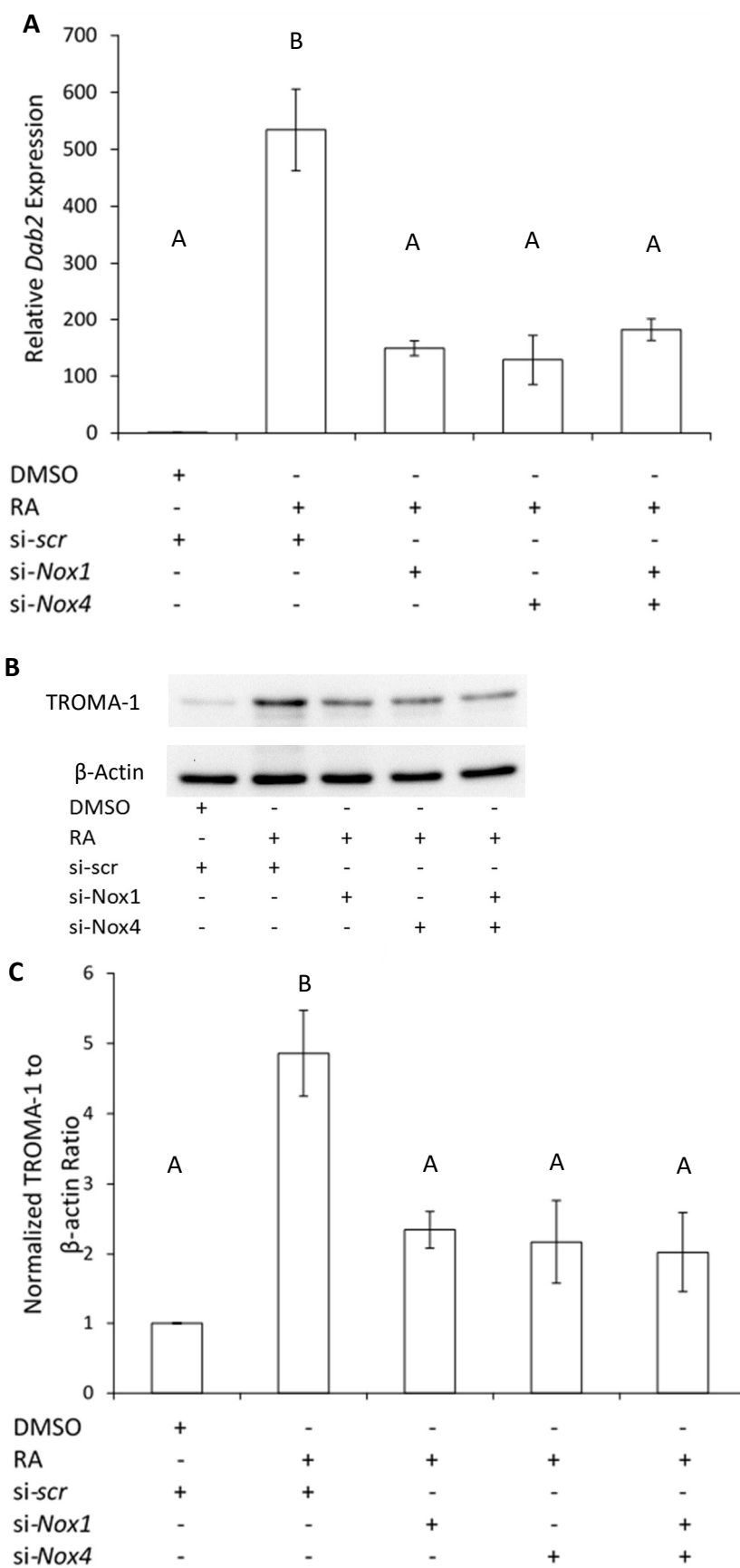| DMSO | + | - | - | - | - |
RA	-	+	+	+	+
si-*scr*	+	+	-	-	-
si-*Nox1*	-	-	+	-	+
si-*Nox4*	-	-	-	+	+

B

DMSO	+	-	-	-	-
RA	-	+	+	+	+
si-scr	+	+	-	-	-
si-Nox1	-	-	+	-	+
si-Nox4	-	-	-	+	+

C

| DMSO | + | - | - | - | - |
RA	-	+	+	+	+
si-*scr*	+	+	-	-	-
si-*Nox1*	-	-	+	-	+
si-*Nox4*	-	-	-	+	+

3.4 Overexpression of *Nox1* and *Nox4* did not induce F9 cell differentiation

Given these results, together with the fact that H_2O_2 treatment induced F9 cells to differentiate into PrE [6, 17], it seemed logical to propose that *Nox* overexpression should also induce differentiation. To test whether or not *Nox1* or *Nox4* overexpression could induce differentiation, F9 cells were transfected with pcDNA3.1-*EV* and treated with either DMSO or RA or transfected with pcDNA3.1-*Nox1* or *4*. The relative degree of overexpression was determined by qRT-PCR, and results show an approximate 700 and 1800 fold increase for *Nox1* (Fig 3.5A) and *Nox4* (Fig 3.5B), respectively. Despite the massive increase in *Nox1* and *Nox4* mRNA there was no obvious increase in cell death, although the latter was never measured. Once established that there was a significant increase in the levels of *Nox1* and *4* mRNA in cells, it was necessary to examine if this increase translated into increased ROS levels. Transfected F9 cells were incubated with 2 μM CM-H2DCFDA, as a ROS indicator, and 10 μg/ml Hoechst 33342 (Molecular Probes, Life Technologies) for nuclear staining. Cells transfected with pcDNA3.1-*EV* and treated with DMSO show little ROS fluorescence, whereas RA-treated cells and those transfected with either *Nox1* or *Nox4* show high levels of ROS (Fig 3.5C). As another positive control, cells were transfected with pcDNA3.1-*EV* and treated with H_2O_2 (Fig 3.5C). As expected, these CM-H2DCFDA-containing cells show very strong ROS staining. Together, these data confirmed that ectopic expression of *Nox1* or *Nox4* increased ROS levels in F9 cells. To determine if these increased levels in ROS could activate canonical WNT-β-catenin signaling, cells were co-transfected with *Nox1* or *Nox4*, and the firefly luciferase reporter construct, pGL3-*BARL*, which contains 12 TCF-binding sites upstream of a firefly luciferase gene, used as a readout for active canonical WNT signaling [45]. All cells were transfected with the *Renilla* luciferase construct, TK-*RL*, to normalize for transfection efficiency of pGL3-*BARL*. Relative TCF activity can be determined by comparing the measured luminescence produced by the firefly luciferase activity relative to the luminescence produced by the transfection control *Renilla*

luciferase activity. As expected, results from RA-treated F9 cells transfected with pcDNA3.1-*EV*, showed a significant increase in TCF/LEF activity over the negative DMSO control (Fig 3.5D). Cells overexpressing *Nox1* or *Nox4* showed an increase in TCF/LEF activity, however, this increase was less than that seen in RA-treated cells, and was not significant from either RA or DMSO treatments (Fig 3.5D). Encouraged by the readout results for increased ROS and a trend for active WNT signaling, cells overexpressing *Nox1* or *Nox4* were then analyzed for PrE differentiation. qRT-PCR and immunoblot analysis, using *Dab2* and TROMA-1, respectively, were used to confirm the differentiation of F9 cells to PrE. As expected, *Dab2* mRNA level relative to the *L14* loading control was significantly higher in RA treated cells than DMSO treated cells ($P<0.001$) (Fig. 3.6A and Fig 3.6B). *Dab2* mRNA, however, resulting from either the overexpression of *Nox1* (Fig 3.6A) or *Nox4* (Fig 3.6B) was not significantly different from that in DMSO treated cells ($P>0.05$), suggesting that differentiation had not occurred. That TROMA-1 signals were not seen in DMSO treated, *Nox1* overexpressing, or *Nox4* overexpressing cells, but were present in RA treated cells (Fig 3.6C), confirmed that PrE had not formed as a result of *Nox* overexpression. Thus, despite the fact that overexpressing *Nox1* or *Nox4* in F9 cells produces ROS, this did not lead to a significant increase in canonical WNT signaling, or a significantly appearance of PrE markers suggested that either the levels of ROS were not sufficient to induce differentiation or that other factors are required.

Figure 3.5. **Overexpression of *Nox1* and *Nox4* increased intracellular ROS levels, but did not significantly increase TCF/LEF activation.** Total RNA was collected from F9 cells transfected with an empty vector (*EV*) control, pcDNA3.1-*Nox1* or pcDNA3.1-*Nox4* and cultured for 4 days with G418 selection at 1.75 mg/ml. Collected RNA was reverse transcribed into cDNA and then processed for real time PCR. **(A)** Cells transfected with pcDNA3.1-*Nox1* showed increased *Nox1* mRNA when compared to cells transfected with the *EV*. **(B)** Likewise, cells transfected with pcDNA3.1-*Nox4* showed increased *Nox4* mRNA when compared to cells transfected with the *EV*. **(C)** F9 cells were transfected with pcDNA3.1-*Nox1*, or pcDNA3.1-*Nox4* or transfected with an *EV* control and treated with DMSO or RA were cultured for 4 days, and then imaged using 2 µM CM-H2DCFDA to examine intracellular ROS and 10 µg/ml Hoechst 33342 (Molecular Probes, Life Technologies) to image nuclei. Cells transfected with *EV* were also treated with 100 µM H_2O_2 as a positive control and imaged following CM-H_2DCFDA treatment. Cells transfected with the *EV* and treated with either RA or H_2O_2 and cells transfected with either pcDNA3.1-*Nox1* or pcDNA3.1-*Nox4* all show increased intracellular ROS when compared with cells transfected with the *EV* and treated with DMSO. **(D)** *EV* and pGL3-*BARL* transfected cells treated with RA show a significant increase in luciferase activity compared to that when cells were treated with DMSO. TCF activation in cells transfected with pcDNA3.1-*Nox1* or pcDNA3.1-*Nox4* showed no significant difference when compared to both RA and DMSO treated *EV* transfected cells. Data are representative of three independent experiments. Scale bar (30 µm). * denotes significance ($P<0.05$) and means with different letters were significantly different ($P<0.05$).

D
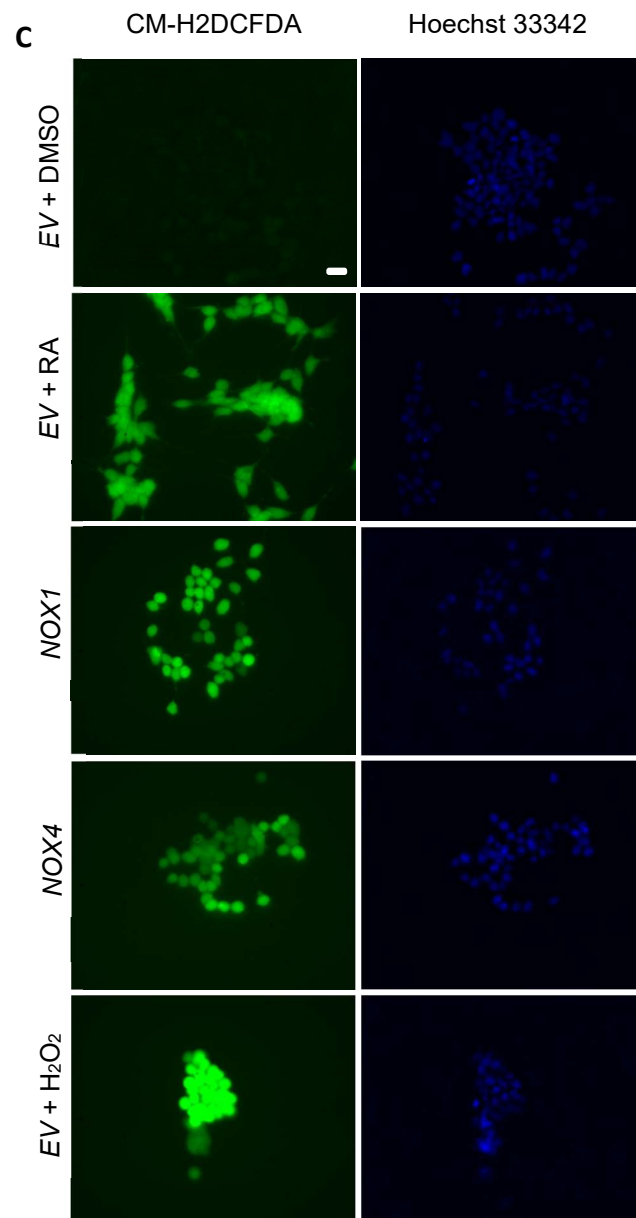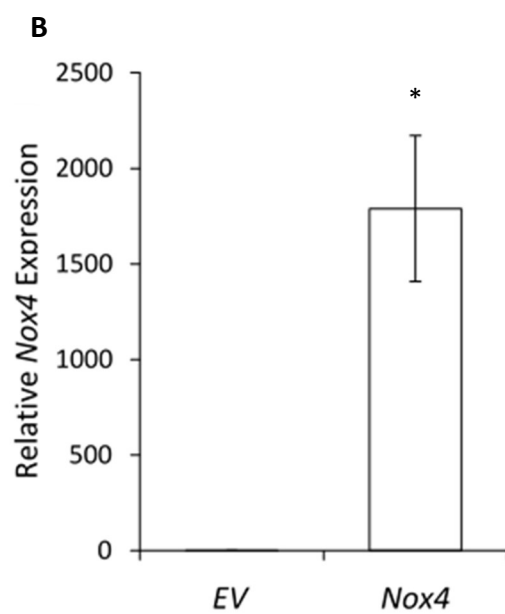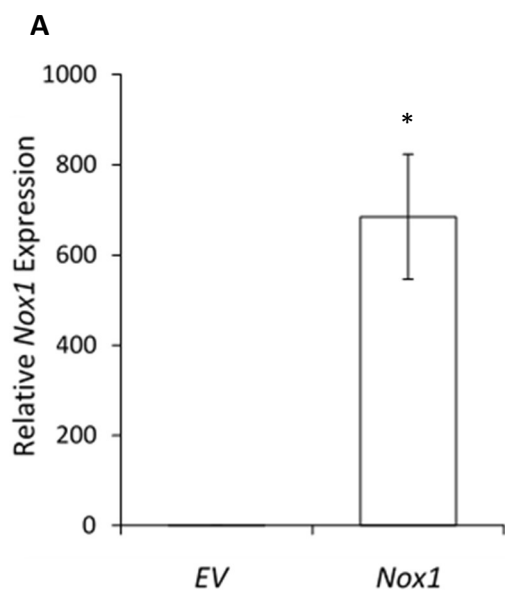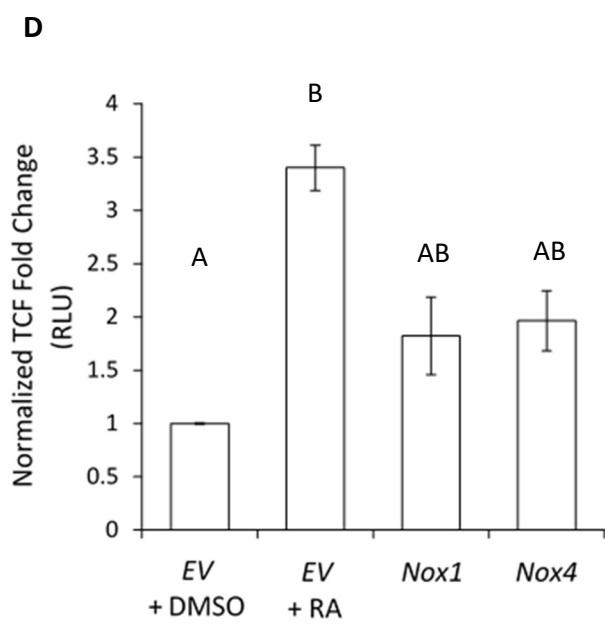
3.6 Overexpression of *Nox1* and *Nox4* did not induce markers of PrE differentiation.
Total RNA and protein were collected from F9 cells transfected with an empty vector
(*EV*) control, pcDNA3.1-*Nox1* or pcDNA3.1-*Nox4* and cultured for 4 days with G418
selection at 1.75 mg/ml. Collected RNA was reverse transcribed into cDNA and then
processed for real time PCR. **(A)** Cells transfected with the *EV* and treated with RA
showed increased *Dab2* mRNA when compared with DMSO treated cells transfected
with the *EV* and pcDNA3.1-*Nox1* constructs. *Dab2* mRNA in control cells was not
significantly different from that seen in *Nox1* transfected cells. **(B)** Cells transfected with
the *EV* and treated with RA showed increased *Dab2* mRNA when compared with that in
DMSO treated cells transfected with the *EV* and pcDNA3.1-*Nox4* constructs. *Dab2*
mRNA in control cells was not significantly different from that seen in *Nox4* transfected
cells. **(C)** Protein lysates were collected from F9 cells transiently transfected with the *EV*,
Nox1, or *Nox4* or from cells treated with DMSO or with RA. Immunoblot analysis with
the TROMA-1 antibody only shows signal in RA-treated cells. β-actin was used as a
loading control. Data are representative of three independent experiments. Means with
different letters were significantly different ($P<0.05$).
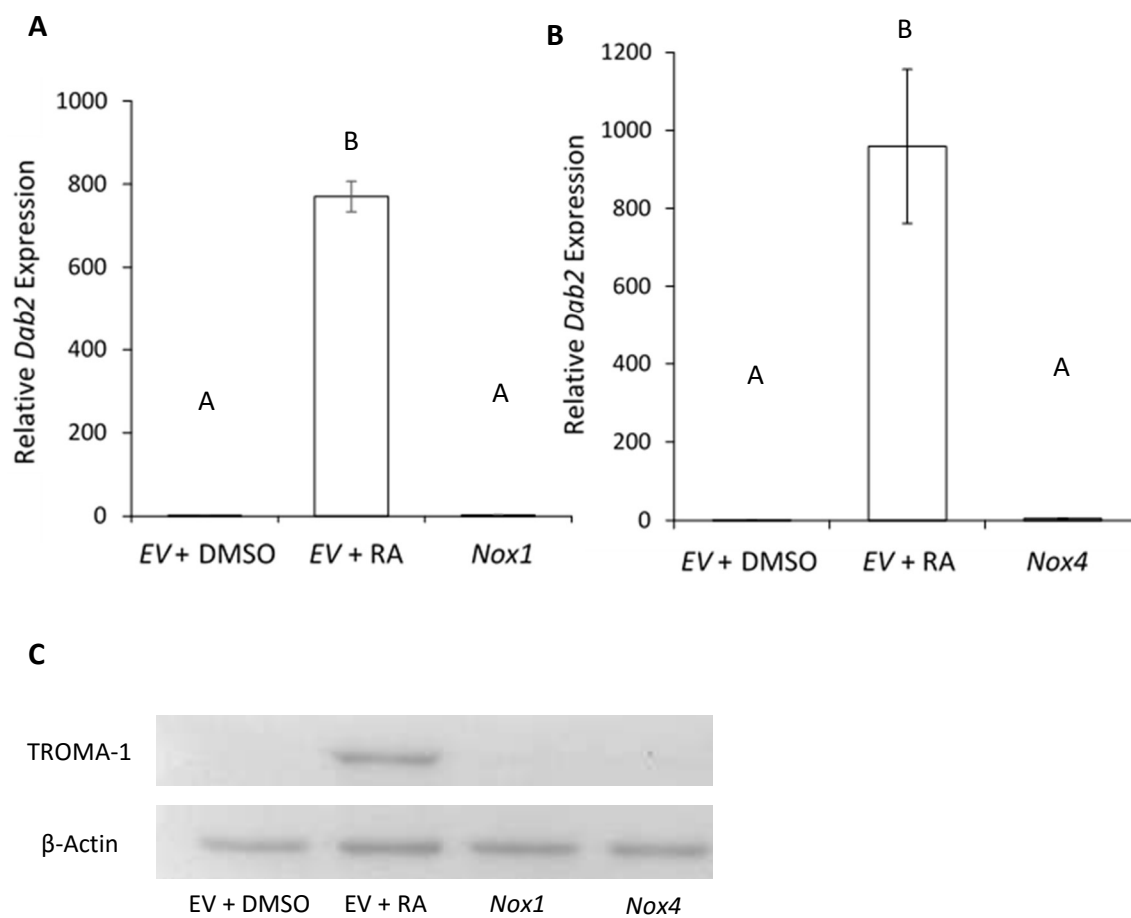
Chapter 4

4 Discussion

4.1 WNT signaling and ROS in Differentiation

The differentiation of F9 teratocarcinoma cells is dependent on the presence of ROS, the redox protein NRX, and the WNT signaling pathway [6, 9, 17]. Differentiation can be induced through direct manipulation of the aforementioned signaling molecules or proteins, but can also be induced through RA treatment or *Gata6* overexpression [6, 9, 17]. Additionally, *in vivo* cells of the ICM expressing *Gata6* differentiate into PrE on E4.5 in the late blastocyst [3]. ROS and NRX work in tandem to augment the WNT pathway [18] and manipulation of them can affect the ability of F9 cells to differentiate [6, 9, 17]. F9 cells are used to recapitulate the differentiation of XEN *in vitro* [3, 6]. If the molecular changes seen when F9 cells differentiate *in vitro* also occur *in vivo*, we can reason that the ROS producing NOX family of proteins are important for the differentiation of the population of ICM cells that differentiate to PrE.

WNT is important in three WNT pathways as well as being implicated in crosstalk with a variety of signaling pathways. The three WNT pathways are the WNT/β-catenin pathway, Planar Cell Polarity (PCP) pathway, and WNT/Ca^{2+} pathway. DVL is present in all three WNT pathways, therefore NRX and ROS are negative and positive regulators respectively of PCP and WNT/Ca^{2+} pathways [6]. The WNT/β-catenin pathway is characterized by increased TCF/LEF activity due to β-catenin nuclear translocation, which is facilitated by FZD and DVL inhibiting a destruction complex in the presence of the WNT ligand. This pathway is implicated in both embryonic development and the progression of cancer [15, 45]. In PCP signaling, WNT activates the RHO-RAC-JNK pathway, through FZD and DVL, which occurs independently of β-catenin [47]. The PCP pathway affects the cytoskeleton, and therefore cell morphology and migration [47]. Additionally, the PCP pathway directs the migration of F9 cells to a
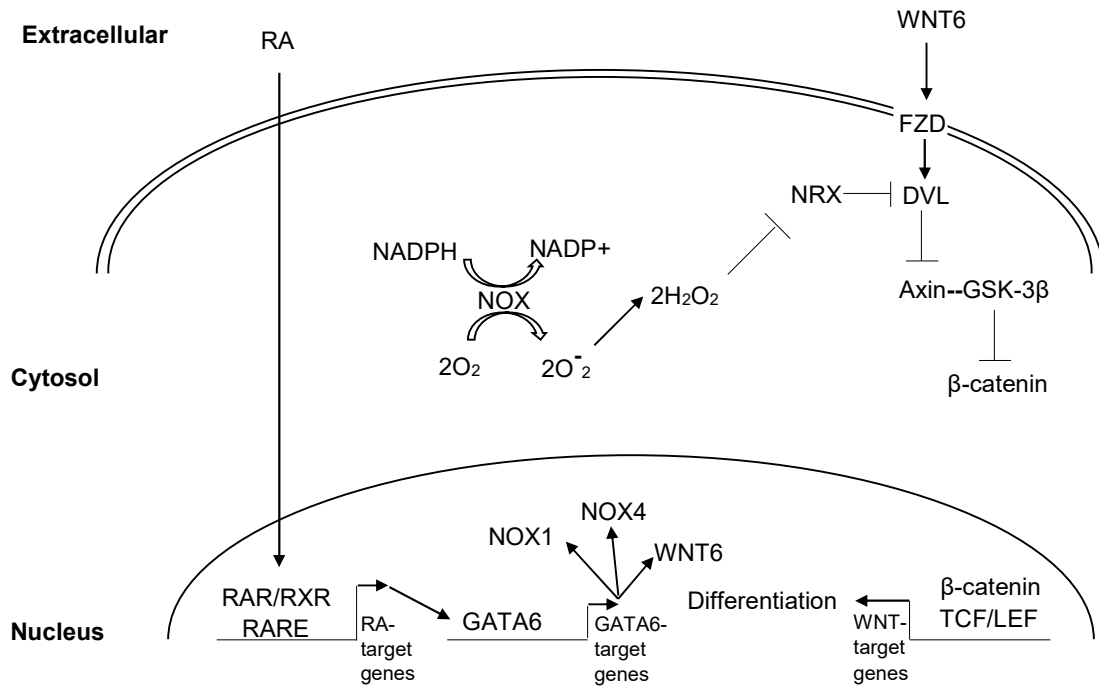
PE-like lineage [48]. Investigation of the role of the PCP pathway in F9 cell differentiation was beyond the scope of my project, but given the information noted above, further investigation is warranted. Lastly, the WNT/Ca^{2+} is characterized by Ca^{2+} release from the endoplasmic reticulum due to activation of phospholipase C mediated through FZD and DVL [47]. Increased intracellular Ca^{2+} activates calcium/calmodulin-dependent kinase II (CAMKII), which in turn leads to activation of Nemo-like kinase (NLK) antagonizing β-catenin/TCF-LEF signaling [47]. Despite these two additional, and possibly interacting pathways, the focus of my project was on the canonical WNT/β-catenin pathway and the role of the NOX family of proteins.

Research in our lab, as well as that from others, shows that ROS have an intimate role in regulating the WNT/β-catenin pathway [17, 18]. WNT/β-catenin is redox regulated at the level of NRX binding to DVL and subsequently inhibiting WNT/β-catenin signaling in the absence of ROS [17, 18]. In F9 cells, treatment with RA induces PrE differentiation and an increase in cytosolic ROS is observed [6]. Wen et al. (2012) show non co-localizing MitoTracker Red and CM-H2DCFDA, concluding that the source of ROS was likely not mitochondrial. *Nox* expression is also upregulated with RA treatment, providing a potential candidate for the cytosolic ROS production noted above [6]. Additionally, co-treatment with antioxidants or the general flavoenzyme inhibitor DPI attenuates RA-mediated PrE differentiation [6]. Together, this data indicates that ROS is integral to F9 cell PrE differentiation due to the interaction of NRX and DVL, and that NOX proteins could potentially be a factor in the production of ROS.

Our working hypothesis prior to this study proposed that RA activates WNT/β-catenin signaling through many facets. First, RA upregulates *Gata6*, a transcription factor, which in turn leads to increased WNT6 and potentially NOX proteins, and an increase in ROS [6, 38, 49]. Increased ROS causes a dissociation of NRX from DVL [17, 18], allowing the latter to be recruited to FZD at the plasma membrane and facilitate the disassembly of GSK-3β, AXIN, and the APC destruction complex. This disassembly allows β-catenin to accumulate and translocate to the nucleus where it associates with TCF-LEF to cause activation of WNT target genes necessary for F9 cell differentiation [38] (Fig 4.1). One question that arises, however, is why the need for negative regulation

of the WNT/β-catenin in the absence of the WNT ligand? Although many explanations are plausible, since an overexpression of *Dvl* can activate TCF-LEF and knockdown of *Nrx* induces PrE differentiation of F9 cells [17], we believe that NRX inhibiting DVL is a fail-safe mechanism to maintain low levels of WNT signaling. Additionally, G-protein coupled receptors (GPCRs), which includes FZD, have basal activity in the absence of ligand, indicating a need for negative regulation to prevent aberrant signaling [50]. This study aimed to determine if NOX1 and NOX4 play a role in augmenting the WNT/β-catenin and F9 cell differentiation.

Figure 4.1. **A model of extraembryonic endoderm differentiation of F9 cells**. RA binds RAR and RXR heterodimers, which in turn bind RA-response elements in the promoter region of RA-target genes. RA induces the expression of *Gata6* leading to increased expression of *Wnt6*, *Nox1* and *Nox4*. The increase in *Nox1* and *Nox4* leads to increased production of cytosolic ROS. This ROS oxidizes NRX, causing a dissociation of NRX and DVL. This dissociation, along with the presence of WNT6, enables FZD to recruit DVL, thereby inhibiting the AXIN-GSK-3β destruction complex leading to an accumulation of β-catenin. β-catenin translocates to the nucleus where it binds to TCF/LEF to alter gene expression sufficient to differentiate F9 cells into extraembryonic endoderm.

Extracellular

RA

WNT6

FZD

NRX —| DVL

$NADPH$ $NADP+$

NOX

$2H_2O_2$

Axin--GSK-3β

Cytosol

$2O_2$ $2O^-_2$

β-catenin

Nucleus

NOX4

NOX1   WNT6

Differentiation   β-catenin

RAR/RXR
RARE   RA-target genes   GATA6   GATA6-target genes   WNT-target genes   TCF/LEF

4.2 NOX1 and NOX4 in PrE differentiation

During RA-mediated F9 cell differentiation there is an increase in cytosolic ROS with a concomitant increase in the expression of *Nox1-4* and *Duox2* [6]. NOX4 is associated with cell proliferation, cytoskeletal reorganization, migration and stem cell differentiation [35, 36, 37]. Similarly, NOX1 has links to the WNT/β-catenin pathway and can post-transcriptionally increase the levels of Keratin 18 [32, 34]. Therefore, NOX1 and NOX4 were chosen as candidates for this study. Previous research has also shown that GATA6 directly binds the *Nox1* proximal promoter [51] and an overexpression of *Gata6* increases *Nox4* expression in F9 cells [49]. My results show that an overexpression of *Gata6* significantly increases *Nox1* steady state mRNA levels when compared to DMSO treated control cells, and is not significantly different from RA treated cells. Furthermore, I confirmed that overexpressing *Gata6* significantly increases *Nox4* steady state mRNA levels when compared to DMSO treated control cells [49]; however, this increase was not significantly different from RA treated cells. Future studies involving ChIP analysis are needed to determine if GATA6 is bound to the *Nox* promoters and then by using PCR and DNA sequencing to analyze the prospective sites we can determine whether or not GATA6 is solely responsible for the increase in *Nox1* and *Nox4* expression in F9 cells. If that is not the case, and to further prove that GATA6 is responsible for *Nox* upregulation, *Gata6* could be overexpressed and WNT signaling blocked using chemical inhibitors to determine if another pathway, independent of WNT, acts downstream of GATA6.

4.3 NOX dependent differentiation of F9 cells

A previous study in the Kelly lab showed that ROS is necessary for PrE differentiation of F9 cells [6]. Unfortunately, a comprehensive NOX gene expression is

missing for most major model systems during development [51]. Weaver et al. (2016) showed that NOX proteins are expressed in zebrafish as early as 12h post-fertilization; however, XEN evolved in amniotes (reptiles, birds and mammals) [53]. Treating F9 cells with antioxidants or the non-specific flavoenzyme inhibitor DPI attenuates RA-mediated differentiation [6]. My study employs a more specific approach to targeting NOX proteins and the subsequent effect on PrE differentiation. My results showed that specifically inhibiting the NOX family, with VAS2870, attenuated RA-mediated TROMA-1 and *Dab2* steady state mRNA levels. Thus, this data confirms that the NOX family is necessary for PrE differentiation and indicates it is most likely the major contributor of ROS production after RA treatment. Results in this study with the chemical inhibitor ML171 also revealed that NOX1 specifically is necessary for F9 cell differentiation. Furthermore, the genetic approach with siRNAs showed that the knockdown of *Nox1* and *Nox4* both individually and together resulted in attenuated differentiation, further adding support that NOX1 and NOX4 are necessary for F9 cells to form PrE. Although this data would indicate that the presence and functionality of both NOX1 and NOX4 are necessary for F9 cell PrE differentiation, further research into the roles of NOX2, NOX3, and DUOX2 in PrE differentiation is necessary to elucidate the specificity of ROS production by other NOX family members during differentiation. Also, additional studies are needed to determine if ROS, and potentially NOX1 and NOX4, play a role in PE differentiation.

4.4 NOX1 and NOX4 produce ROS but not differentiated phenotype

The results of my study show an overexpression of either *Nox1* or *Nox4* produce ROS. Overexpressing *Nox4* increases ROS in F9 cells [49] as well ROS production is known to be proportional to *Nox4* expression [54]. Interestingly, it was previously shown that overexpressing *Nox1* alone was sufficient to induce ROS production [55], but

subsequent studies have brought these results into question [24, 56]. This inconsistency in the literature could be cell line specific as evident in Banfi et al. (2003), who showed that co-overexpression of *Nox1*, *p47phox* and *p67phox* in HEK-293 cells produces superoxide following phorbol ester PMA treatment, but *Nox1* overexpression alone with PMA treatment does not produce ROS. Additionally, the contradiction in the data for ROS production by *Nox1* overexpression may also be due to the method of detection. DCFDA is a non-discriminatory ROS indicator, capable of detecting intracellular H_2O_2. Banfi et al. (2003) measured superoxide production and the possibility exists that the cytosolic superoxide produced by NOX1 was converted to H_2O_2 by superoxide dismutase and therefore not detected. In support, Suh et al. (1999) show *Nox1* overexpression can produce superoxide and that it is reduced when in the presence of superoxide dismutase. Whether or not H_2O_2 was produced as a direct or indirect result of the *Nox1* overexpression was not the focus of my research. Nevertheless, this opens new avenues of research to determine if the expression patterns of additional NOX subunits prior to RA treatment change and if manipulating their expression, or their function, affects differentiation.

Although the *Nox1* and *Nox4* overexpression in F9 cells produced ROS, it did not induce differentiation of these cells, which is unlike the treatment with exogenous H_2O_2 previously shown by Wen et al. (2012). This could be explained by the concentration of ROS as the NOX proteins would produce ROS overtime, whereas the exogenous treatment would occur in a singular burst. Additionally, the overexpression of *Nox1* and *Nox4* produced an increased amount of TCF/LEF activity, however, this increase is not significant from either the positive control (RA) or negative control (DMSO) treated cells. This would indicate that there is a potential for the WNT/β-catenin pathway to be affected, at least to some degree, by the overexpression of *Nox1* and *Nox4* in F9 cells as shown in IEC-6 cells [32]. Perhaps the amount of ROS produced by overexpressing *Nox1* and *Nox4* was not sufficient to oxidize enough NRX to induce differentiation. Previous research shows a knockdown of NRX is sufficient for differentiation [17]. Since the overexpression of *Nox1* and *Nox4* was not sufficient to induce differentiation, it is possible the threshold of basal GPCR activity of the FZD receptor was inhibited because NRX-DVL binding was maintaining cells in the undifferentiated state. Exogenous

treatment of H$_2$O$_2$ at 100 μM was, however, sufficient to facilitate DVL-NRX dissociation [6] and this I believe would allow basal FZD signaling to activate WNT/β-catenin signaling required to differentiate F9 cells. In this regard, our model would suggest that in GATA6- and RA-mediated differentiation, the ROS produced by NOX1 and NOX4 is sufficient to prime the WNT pathway and the presence of the WNT ligand in concert with ROS production would induce cells to form PrE. This could be tested if I were to overexpress *Nox1* or *Nox4* and then apply either sub-optimal concentrations of RA or Wnt6-conditioned media.

4.5 Relationship of WNT and NOX in F9 cells

Overall, the data in this thesis provides cogent evidence that NOX1 and NOX4 play an important role in WNT/β-catenin dependent F9 cell differentiation to PrE. In our model, WNT/β-catenin signaling is negatively regulated by NRX, at the level of DVL, and this fail-safe mechanism is removed in the presence of ROS [17]. The ROS producing proteins NOX1 and NOX4 were shown in this study to be downstream of the transcription factor GATA6 and necessary for PrE differentiation. That aberrant WNT signaling is prevalent in cancer biology [15, 46] underpins the importance of better understanding the regulation of WNT signaling and the role of ROS in both development and cancer research. My study not only helps to elucidate the regulation of how extraembryonic endoderm differentiates in mouse early development and the potential role of NOX proteins, but it also furthers our understanding of the crosstalk imparted by NOX proteins and ROS on the WNT/β-catenin pathway.

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Appendices

Appendix 1- Primer sequences

	Forward	Reverse
Dab2	5'GGAGCATGTAGACCATGATG	5'AAAGGATTTCCGAAAGGGCT
Gata6	5'ATGGCGTAGAAATGCTGAGG	5'TGAGGTGGTCGCTTGTGTAG
L14	5'GGGAGAGGTGGCCTCGGACGC	5'GGCTGGCTTTCACTCAAAGGCC
Nox1	5'AATGCCCAGGATCGAGGT	5'GATGGAAGCAAAGGGAGTGA
Nox4	5'GATCACAGAAGGTCCCTAGCA	5'GTTGAGGGCATTCACCAAGT

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