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Reactive Oxygen Species Signaling in Extraembryonic Endoderm Differentiation

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Reactive Oxygen Species Signaling in Extraembryonic Endoderm Differentiation

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Abstract

Development of extraembryonic endoderm is essential for proper placenta formation and mammalian embryogenesis. Formation of primitive endoderm from cells of the inner cell mass is one of the earliest cell fate decisions made in the developing embryo. However, the signals and mechanisms involved in orchestrating this developmental process are not completely understood and are difficult to study *in vivo*. The mouse embryonic carcinoma (F9) cell line can be chemically re-programmed to mimic cells of the inner cell mass, thereby serving as an *in vitro* model to study extraembryonic endoderm differentiation. F9 cells treated with retinoic acid (RA) differentiate into primitive endoderm. Similarly, activation of the Wnt-β-catenin pathway is also sufficient to induce differentiation. Reactive oxygen species (ROS), including H_2O_2 have been identified as key regulators of normal cell physiology. In the present study, a sustained increase in the levels of ROS was found when F9 cells were treated with RA. In addition, an increase in TCF-LEF transcriptional activity, a read out of Wnt-β-catenin signaling, was also seen in response to exogenous H_2O_2 treatment. Immunoblot and immunofluorescence analyses using molecular markers of differentiation confirmed that H_2O_2-treated F9 cells had formed primitive endoderm. In contrast, differentiation was blocked when RA-treated cells were exposed to the antioxidants N-Acetyl Cysteine or Trolox. In a survey to identify the source of the ROS, a candidate gene encoding the NADPH oxidase *NOX4,* was identified as being up-regulated in response to RA. Furthermore, diphenyleneiodonium chloride a general NADPH oxidase inhibitor, was shown to attenuate the RA-induced differentiation, implicating NADPH oxidases and possibly

NOX4, as the source of H_2O_2. Together, these results strongly suggest that ROS impinge on the Wnt-β-catenin signaling that is required for the cell fate decision making process(es) that lead to the formation of extraembryonic endoderm.

Keywords

ROS, H_2O_2, F9, Primitive endoderm, Extraembryonic endoderm, Differentiation, NOX4

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List of Abbreviations

°C	Degrees Celsius
µM	Micromolar
APC	Adenomatous Polyposis Coli
bp	Base pair
BSA	Bovine Serum Albumin
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CO_2	Carbon Dioxide
DAPI	4′,6-Diamidino-2-phenyindole
db-cAMP	dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPI	Diphenyleneiodonium chloride
DVL	Disheveled
EC	Embryonal Carcinoma
EMT	Epithelial-Mesenchymal Transition
ESC	Embryonic Stem Cell
F9	Mouse F9 Embryonal Carcinoma (Teratocarcinoma)
FBS	Fetal Bovine Serum
Fig.	Figure
FZD	Frizzled

G418	Neomycin
GSH	(Reduced) Glutathione
GSK3β	Glycogen Synthase Kinase 3β
GSSG	(Oxidized) Glutathione Disulfied
H_2O	Water
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
hrs	Hours
ICM	Inner Cell Mass
JNK	c-Jun-N-terminal Kinase
kDa	Kilodalton
LDL	Low density lipoprotein
LRP	LDL Receptor-related Protein
mins	Minutes
mM	Millimolar
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
MTR	MitoTracker Red
NAC	N-Acetyl Cysteine
NOX	NADPH Oxidase
NRX	Nucleoredoxin
O_2^-	Superoxide
PAGE	Polyacrylamide Gel Electrophoresis

PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween-20
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PE	Parietal Endoderm
PKC	Protein Kinase C
PrE	Primitive Endoderm
RA	*all-trans* Retinoic Acid
RAR	Retinoic Acid Receptors
RARE	Retinoic Acid Response Elements
RBP	Retinol Binding Protein
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
RXR	Retinoid X Receptors
s	Seconds
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
TB	Trypan Blue
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20
TCF-LEF	T-Cell specific transcription factor/Lymphoid Enhancer binding factor
TE	Trophectoderm
TRITC	Tetramethyl Rhodamine Isothiocyanate

Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TROMA-1	Trophectoderm Monoclonal Antibody-1
V	Volts
VE	Visceral Endoderm
WNT	Wingless / MMTV Integrated site
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
ZP	Zona Pellucida

Chapter 1 – Introduction

1.1 *Mammalian embryogenesis and cell fate decisions*

Mammalian development begins when the ovum is fertilized by a sperm cell to create the zygote. The zygote within the zona pellucida (ZP) begins to develop by initiating a series of mitotic cell divisions followed by compaction. As mitosis continues, a solid sphere of cells called the morula is formed. Subsequently, the embryo is faced with its first cell fate decision: to become cells of the inner cell mass (ICM) or trophoblast stem cells. The former develops into the embryo proper while the latter forms the trophectoderm (TE) replacing the ZP as protection for the developing blastocyst. The TE in conjunction with extraembryonic endoderm contributes to form the placenta (Rossant, 2004; Ralston & Rossant, 2005; Chen *et al.*, 2010).

Proper segregation and development of extraembryonic tissues are essential for proper embryonic patterning and survival. While studies have provided insight into the steps involved during embryogenesis, the signaling mechanisms responsible for establishing embryonic cell lineages are poorly understood.

1.2 *Extraembryonic endoderm – cell fate decisions during embryonic development*

The extraembryonic endoderm acts as an essential scaffold to support embryonic development by providing a connection between maternal and embryonic tissue and supplying crucial signals required for proper embryonic patterning (Tam & Loebel, 2007).

The extraembryonic endoderm lineages include the primitive (PrE), parietal (PE) and visceral (VE) endoderms (Dziadek, 1979). Each lineage is unique in both form and function. The first lineage to arise is the PrE, after differentiating outward from the surface of the ICM (Nadijcka & Hillman, 1974). The PrE plays a pivotal role in extraembryonic endoderm formation because it is the precursor which gives rise to both PE and VE (Gardner & Rossant, 1979; Gardner, 1982). PrE spans the luminal surface of the TE, while VE encloses the epiblast. Regardless of their position, both cell types play essential roles during embryonic development by mediating signaling cascades and nutritional exchange (Rossant, 2004).

Formation of the PrE is the second cell fate decision made by the developing mammalian embryo (Zernicka-Goet *et al.*, 2009). Cells of the ICM, in contact with the blastocyst cavity, relinquish their totipotency and differentiate into PrE. As development advances, PrE cells progressively commit themselves towards the subsequent extraembryonic endoderm lineages. PrE connected to the TE at the lateral boarders of the ICM differentiates into PE, which then migrates along the inner surface of the TE, enveloping the parietal yolk sac (Fig. 1.1). Migration of PE is recognized as the first migratory event in mammalian embryogenesis (LaMonica *et al.*, 2009; Ralston & Rossant, 2005).

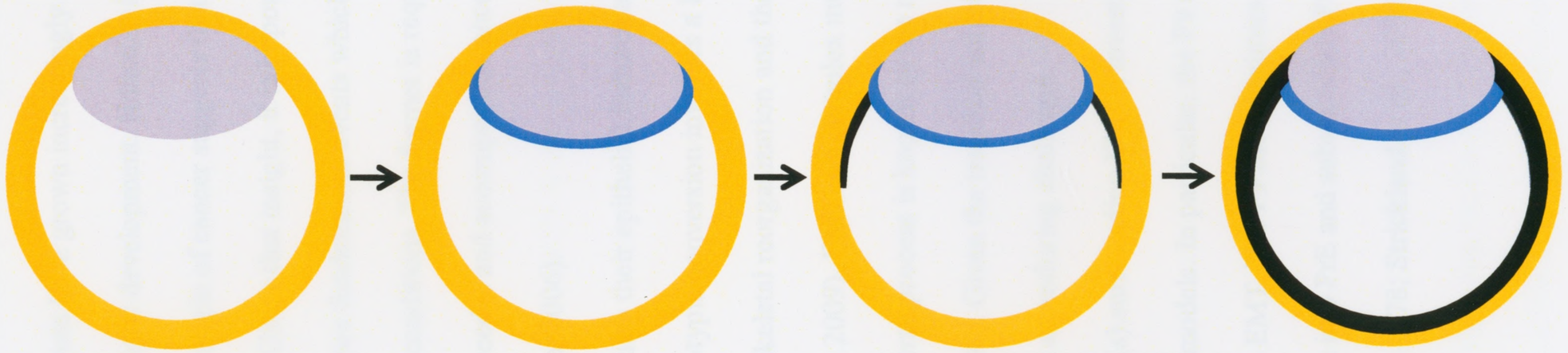
Migratory cell types acquire a unique phenotype, and these cell fate decisions must be highly regulated to ensure proper embryonic development. Although the importance of these crucial decisions has been recognized for years, their mechanisms have yet to be completely defined. More specifically, the cellular signals that govern the

transition of a stationary cell type into a migratory phenotype are not completely understood.

Figure 1.1 ***Extraembryonic endoderm development during mouse embryogenesis.*** A developing embryo typically reaches the blastocyst stage around E3.5. Around the same time, the first lineage of extraembryonic endoderm begins to form and cells within the inner cell mass in proximity to the blastocoel differentiate into primitive endoderm (PrE). Shortly after its establishment, PrE extends along the blastocoel surface, giving rise to parietal endoderm (PE); the first migratory cell type. PE migrates along the luminal surface of the trophectoderm (TE), forming the parietal yolk sac. Together, the TE and PE surround the developing conceptus.

Blastocyst

E3.5 ——————————————————————————— E4.5



Inner Cell Mass → Embryo Proper

Trophectoderm → Placenta Tissue

Primitive Endoderm → Extraembryonic Endoderm

Parietal Endoderm → Extraembryonic Endoderm

1.3 *Epithelial-Mesenchymal Transitions*

Over the years developmental biologists have grown increasingly aware of the striking parallels between normal and neoplastic development. Hence, it is not surprising that one of the most popular theories on the origin of cancer suggests that oncogenesis is essentially normal development gone awry. Given that insight, it is important to note that epithelial-mesenchymal transitions (EMTs) are fundamental events which occur under both normal physiological and pathological conditions. The former is required for proper embryonic development, while the latter precedes and accompanies cancer metastasis (Hay, 1995; Thiery 2002; Thiery & Sleeman, 2006).

During EMTs, epithelial cells relinquish their epithelial features and adopt a motile-mesenchymal phenotype. This phenotypic conversion involves a loss of cell-cell adhesion concomitantly with dramatic cytoskeletal reorganization and the acquisition of a migratory phenotype (Moreno-Bueno *et al.*, 2009). EMT is a complex multistep program that is both transient and reversible; the reverse process is known as the mesenchymal-epithelial transition (Thiery & Sleeman, 2006). Given the transient nature of EMTs, its study *in vivo* has presented researchers with an enduring challenge.

In order to elucidate the mechanism(s) surrounding EMTs, investigators have traditionally favored the use of *in vitro* cell models. In particular, the F9 model is used as a model of choice to recapitulate an *in vitro* EMT. The F9 model mimics the progression of cells within the ICM as they differentiate into PrE and subsequently PE; the first migratory cell type (Strickland & Mahdavi, 1978; Strickland *et al.*, 1980).

1.4 *F9 Embryonal Carcinoma – a model for studying cellular differentiation in vitro*

The mouse F9 embryonal carcinoma (EC) cell line provides an excellent platform for studying fate decisions *in vitro*. It is also a prime model for investigating the molecular mechanisms which regulate differentiation during extraembryonic endoderm development. EC cells can be experimentally engineered by grafting early embryos into ectopic sites within a host animal. F9 cells were derived from the OTT6050 line of EC cells that were originally established by grafting an E6 stage mouse embryo into the testes of a 129/Sv mouse, a laboratory strain known for their high incidence of spontaneous testicular teratomas (Bernstine *et al.*, 1973). It was noted that F9 cells had stem cell like properties *in vitro*; they were able to undergo continuous self-renewal without overt differentiation. Later, studies further demonstrated that F9 cells also shared markers and morphological features reminiscent of early embryos, highlighting their potential as a model system for studying early embryogenesis (Martin, 1980; Lehtonen *et al.*, 1989; Solter, 2006).

1.5 *F9 cell model system*

EC cells have numerous traits in common with embryonic stem cells (ESC), most notably their ability to undergo continuous self-renewal as well as pluripotency. Moreover, when injected into a mouse blastocyst, ECs renounce their malignant feature and participate in normal development, giving rise to a viable chimeric offspring (Papaioannou *et al.*, 1975; Papaioannou *et al.*, 1978). Hence, the F9 model provides an *in vitro* platform for studying the myriad of signaling mechanisms that orchestrate cellular development. Furthermore, the F9 cell line offers several advantages over

traditional ESCs. Firstly, F9 cells require relatively low maintenance when compared to ESC lines. F9 cells can be grown rapidly and cultured indefinitely, without the support of feeder layers. Their short doubling time (8 -10hrs; Alonso *et al.*, 1991) also makes them excellent candidates for proliferation studies. In addition, F9 cells can be maintained in an undifferentiated state and undergo continuous self-renewal when cultured in conventional growth media. Finally, one of its greatest attributes is the wealth of scientific literature on this cell line, dating back to the 1970's when it was discovered that F9 cells can be induced to differentiate into extraembryonic endoderm (Strickland & Mahdavi, 1978; Strickland *et al.*, 1980). These and subsequent studies have established the F9 cell line as an ideal model to investigate the molecular mechanisms that regulate extraembryonic endoderm differentiation.

1.6 *Differentiation is accompanied by morphological changes to F9 cells*

F9 cells undergoing differentiation develop unique morphological characteristics that are reminiscent of the extraembryonic endoderm phenotype. These changes are easily identified using conventional light microscopy. Prior to the chemical induction of differentiation, F9 cells are spherical and, depending on the density during seeding, grow rapidly into embryoid bodies. F9 cells exposed to retinoic acid (RA) differentiate into PrE, characterized by loss of cell-cell adherence and dissipation of the embryoid body as individual cells migrate outward onto the collagen substrate. PrE cells adopt a flat, stellate-shape cell body surrounded by numerous filopodia. Treating these cells with dibutyryl cyclic adenosine monophosphate (db-cAMP) leads to further differentiation as cells adopt the PE fate and they complete the EMT. PE cells have a similar appearance to

PrE, however, when examined by phase contrast microscopy they are highly refractile in nature, the reason of which is unknown. Overall, the differentiation pathway of F9 cells in their transformation from a cohesive cluster of cells in the embryoid body, to individual motile cells is reminiscent of that taken by cells undergoing an EMT (Alonso *et al.*, 1991).

1.7 *Retinoic acid signaling pathway in development and differentiation*

RA, a biologically active derivative of vitamin A, plays a critical role in vertebrate embryonic development and for that reason, altering its level can be teratogenic. In eutherians, maternally derived retinol is the major source of RA (Niederreither & Dolle, 2008). In maternal plasma, retinol is bound by retinol binding protein (RBP), which is endocytosed by cell-surface RBP-receptors. Within the cytoplasm, retinol is converted to retinaldehyde by alcohol dehydrogenase or retinol dehydrogenase. Retinaldehyde is then oxidized by retinaldehyde dehydrogenase to yield RA, which is a ligand for the nuclear RA-receptors (RAR) and retinoid X receptors (RXR). The mechanism of action initiated by RA is twofold. First, the binding of RA results in a conformational change within the heterodimerized RAR/RXR complex, which is bound to specific DNA sequences known as RA response elements (RARE) located upstream of RA-target genes. This change to the ligand-binding domain leads to the release of bound co-repressors that interact with histone deacetylases and methyl transferases, thereby alleviating repression by restoring access to target-gene sequences. Second, RA-bound RAR/RXR facilitates the transcriptional activation of target-genes by recruiting co-activators that work to assists in chromatin remodeling that facilitates

transcription. Ultimately, numerous genes become transcriptionally active and although their numbers are vast and beyond the scope of this thesis, it is important to note that ones central to my study include, but are not limited to, those encoding CyclinD2, Gata6, FoxA2 and Wnt6 (Liu *et al.*, 2000; Li *et al.*, 2004; Krawetz & Kelly, 2008; Hwang & Kelly, unpublished).

RA induced differentiation of F9 cells to form PrE is preceded by a series of molecular changes. Specifically, there is a decrease in the expression of the stem cell markers SSEA-1, Nanog and Oct4 (Solter & Knowles, 1978; Chen *et al.*, 2006). In contrast, there is an increase in the expression of differentiation markers including cytokeratin endo-A and plasminogen activator (Strickland & Mahdavi, 1978; Strickland *et al.*, 1980; Alonso *et al.*, 1991). The accumulation of cytokeratin endo-A, a component of intermediate filaments, is particularly useful in determining F9 differentiation as it reflects the obligatory cytoskeletal changes that precede and accompany the morphological changes (Kurki *et al.*, 1989).

RA also acts upstream of the Fox and Gata family of transcription factors that play key roles in embryogenesis (Fujikura *et al.*, 2002; Su & Gudas, 2008). In particular, these transcription factors activate expression of the Wnt family of secreted glycoproteins, which facilitate the development of extraembryonic endoderm. Members of the Fox family of transcription factors are known to play important roles in embryonic development, organogenesis, metabolism and tissue homeostasis (Carlsson & Mahlapuu, 2002; Papanicolaou *et al.*, 2008). Likewise, the Gata family of transcription factors also act as key signaling mediators involved in embryogenesis and tissue maintenance (Orkin, 1992; Simon, 1995). Given the similarity in function of these transcription factors during

development, it is not surprising to find evidence of crosstalk between their signaling pathways. In particular, it has been shown that FoxA2 cooperates as a downstream target of Gata6 (Morrisey *et al.*, 1998), which is a key factor for the specification of extraembryonic endoderm. It has also been recently elucidated that Wnt6 is an important regulator of extraembryonic endoderm, as it has been previously shown that Wnt6 induces the specification and epithelialization of F9 cells into PrE (Krawetz & Kelly, 2008). Recent evidence from our lab also implicates Wnt6 as a downstream target of Gata6 and FoxA2 in F9 cells (Hwang & Kelly, unpublished).

1.8 *Wnts in Development*

In 1982, while studying the genetic mechanism of oncogenesis induced by the mouse mammary tumor virus (MMTV), Nusse and Varmus discovered that this particular retrovirus preferentially integrated itself within the site of a gene which they called *MMTV integrated site 1* (*int1*; Nusse & Varmus, 1982). Sequence analysis of *int1* revealed that it encoded a secreted protein that was homologous to *wingless* (*wg*) in *Drosophila*, a regulatory ligand that controls segment polarity during larva development. Based on the high degree of sequence homology, it was eventually realized that these genes are evolutionarily linked (McMahon & Moon, 1989). Consequently, the nomenclature was harmonized and *wg*/*int1* became unified as *Wnt1*; the first identified member of a family of signaling molecules crucial for the regulation of both development and disease.

Over the span of the past 20 years, roughly 20 Wnts have been identified in vertebrates, 19 of which have been confirmed within the mouse genome (for a

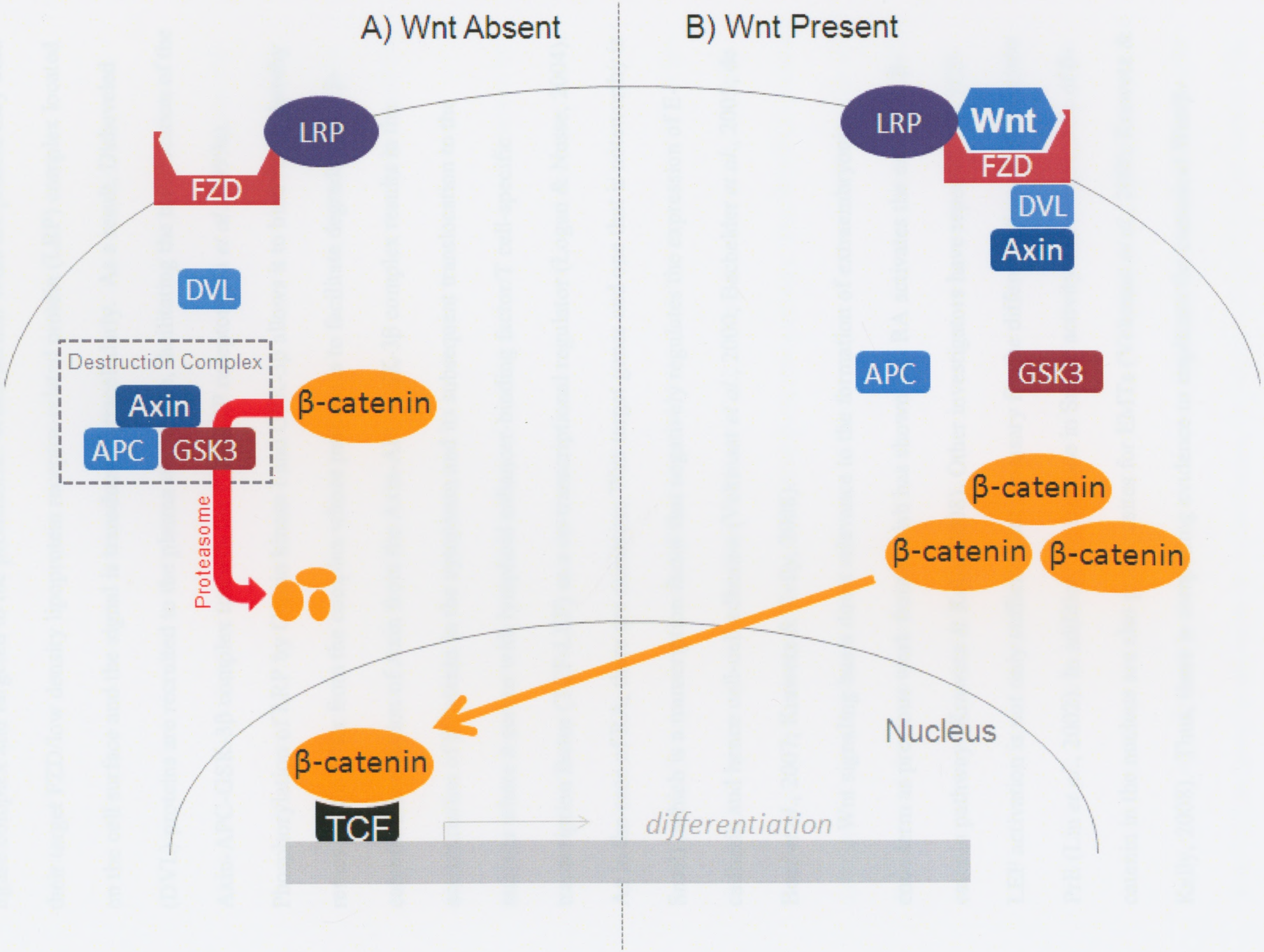
comprehensive list see: *http://www.stanford.edu/~rnusse/wntwindow.html*). Wnt molecules have been shown to be important morphogenetic signals responsible for numerous developmental events such as embryo patterning, as well as a number of human cancers and disease. While it has been shown that different Wnts are localized to specific regions within the developing embryo and trigger different cellular pathways, a common theme shared amongst all Wnts is that they are secreted signaling molecules (Witte *et al.*, 2009). Wnt proteins can function as signaling molecules in an autocrine, paracrine, or endocrine fashion (Zecca *et al.*, 1996; Cadigan *et al.*, 1998; Strigini & Cohen 2000) acting as key mediators of development in embryogenesis as well as adult tissue homeostasis.

1.9 *Wnt Signaling*

The Wnt proteins that participate in the developmental events described above serve as ligands for the Frizzled (FZD) family of G-protein-coupled receptor-like receptors. Wnt signaling is pleiotropic and cell-type specific, acting through the canonical β-catenin, planar cell polarity (PCP), or the Wnt-Ca^{2+} pathway (Veeman *et al.*, 2003; Logan & Nusse, 2004; Clevers, 2006). The former two have relevance to my thesis and will be described further beginning with the canonical pathway.

β-catenin, originally identified as a protein that plays a structural role anchoring the plasma membrane-bound cadherins to actin microfilaments, is the key effector downstream of Wnt signaling (Fig. 1.2). In the absence of Wnt, β-catenin gets degraded because in a complex with Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β), it is phosphorylated, ubiquitinated by an E3 ubiquitin

Figure 1.2 ***Canonical Wnt/β-catenin signaling.*** A) In the absence of Wnt, β-catenin is associated with a complex consisting of Axin, Adenomatous polyposis coli (APC) and Glycogen synthase kinase 3β (GSK3), where it is phosphorylated and subsequently ubiquitinated leading to its degradation by the proteasome. Continuous turnover of cytoplasmic β-catenin effectively suppresses the Wnt/β-catenin signaling pathway. B) Conversely, the Low-density lipoprotein receptor-related protein (LRP)-Frizzled (FZD) receptor is activated by the Wnt ligand, recruiting Disheveled (DVL) to the plasma membrane. Once in position, DVL recruits Axin to the membrane, preventing the formation of the Axin-APC-GSK3 complex. As a result, β-catenin degradation is halted, leading to the accumulation of cytoplasmic β-catenin. Stabilization of cytoplasmic β-catenin leads to its nuclear translocation. In the nucleus, β-catenin associates with the DNA-bound TCF-LEF transcription factors to regulate the Wnt-target gene expression.


A) Wnt Absent
B) Wnt Present
LRP
FZD
DVL
Destruction Complex
Axin
APC
GSK3
β-catenin
Proteasome
LRP
Wnt
FZD
DVL
Axin
APC
GSK3
β-catenin
β-catenin
β-catenin
Nucleus
β-catenin
TCF
differentiation

ligase complex and targeted to the proteasome. When certain Wnts are present they bind their target FZD/low density lipoprotein receptor-related protein (LRP) complex located on the cell surface and the signal is transduced intracellularly. As a result, Disheveled (DVL) proteins are recruited to the plasma membrane, facilitating the translocation of the Axin-APC-GSK-3β complex towards the FZD-LRP receptors (Li *et al.*, 1999a). Phosphorylation of LRP by Casein kinase I and GSK-3β allows it to bind Axin, thereby removing the latter from the complex whose purpose is to facilitate degradation of β-catenin. Dissociation of Axin from the Axin-APC-GSK-3β complex results in the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus where it serves with lymphoid enhancer-binding factor/T cell-specific transcription factor (TCF-LEF) as a co-transcriptional regulator (Logan & Nusse, 2004). As in the case of RA, there are numerous Wnt-target genes and one that is noteworthy is Snail1, which is a transcription factor that negatively regulates the expression of E-cadherin and hence cell-cell adhesion (Veltmaat *et al.*, 2000; Bachelder *et al.*, 2005; de Boer *et al.*, 2007; Krawetz & Kelly, 2008).

Wnt signaling has a direct relevance to the formation of extraembryonic endoderm as previous work from our lab has shown that RA activates the canonical β-catenin pathway (Krawetz & Kelly, 2008). Other investigators have reported that TCF-LEF activation is not only sufficient but necessary for the differentiation of F9 cells into PrE (Liu *et al.*, 2002). In addition, the increase in Snail1 activity and the presence of β-catenin in the nucleus are also prerequisites for EMTs (Veltmaat *et al.*, 2000; Krawetz & Kelly, 2008). Thus, there is compelling evidence to implicate the canonical Wnt/β-

catenin signaling pathway as an integral mechanism in the differentiation program of F9 cells into PrE.

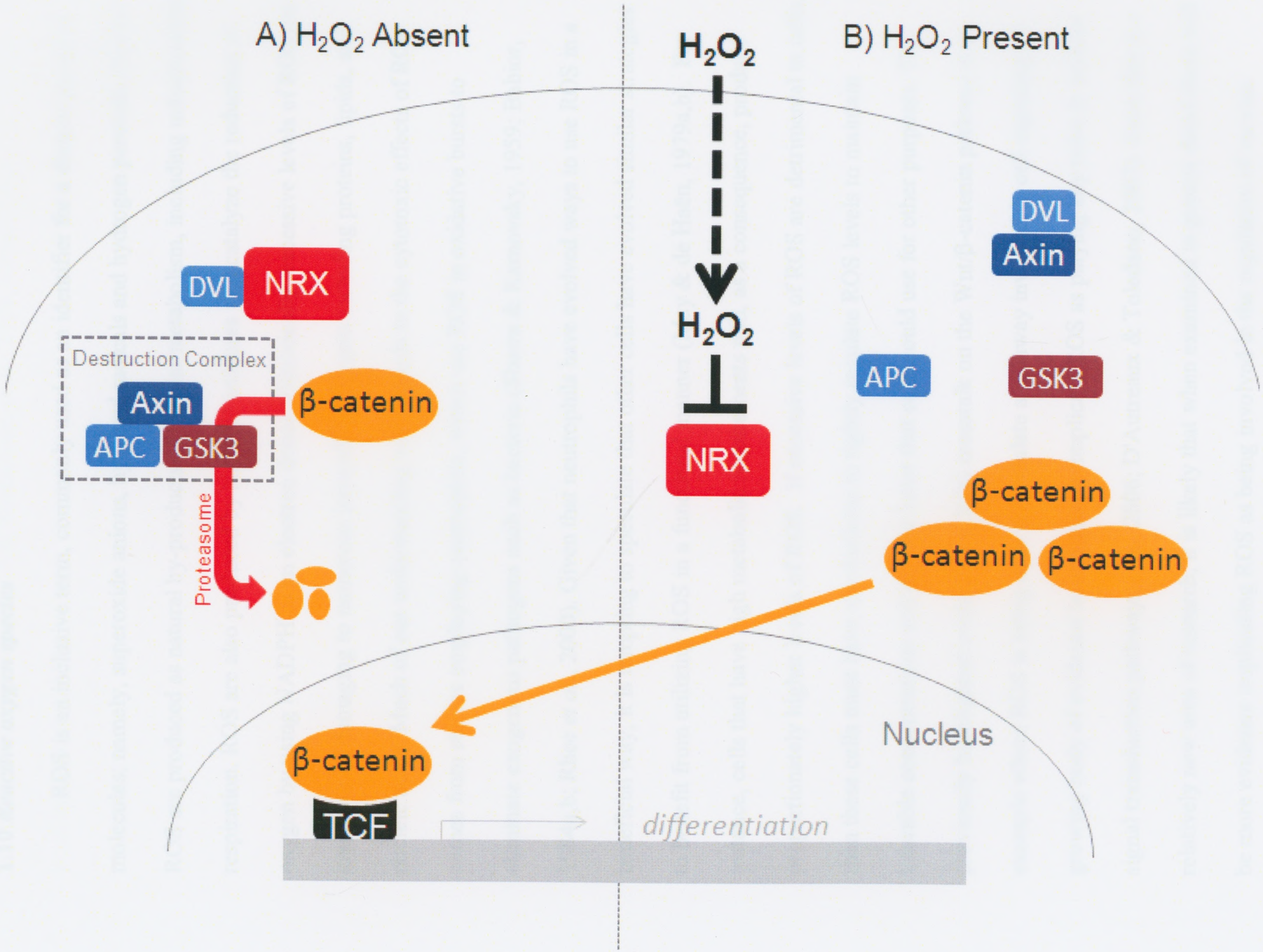
While it is now accepted that canonical Wnt/β-catenin signaling plays a part in PrE differentiation, the PCP pathway is also involved. The Wnt/PCP pathway stands apart from the canonical Wnt/β-catenin pathway because it operates independently of β-catenin. However, the Wnt/PCP pathway still retains two important components of the canonical signaling cascade that were previously described: namely FZD and DVL. As suggested by its name, the Wnt/PCP pathway is heavily involved in the regulation of cell polarity. The Wnt/PCP pathway regulates polarity by exerting its influence over cytoskeletal components (Gao & Chen, 2010). The PCP pathway has been shown to be involved in the regulation of cytoskeleton-associated cell movements during gastrulation by influencing changes in cytoskeletal proteins resulting in changes in cell shape, polarity and motility during embryogenesis (Veeman *et al.*, 2003; LaMonica *et al.*, 2009). While the Wnt/PCP pathway retains its use of FZD and DVL, further downstream effectors deviate greatly from that of the canonical Wnt/β-catenin pathway. The Wnt/PCP pathway can signal through c-Jun-N-terminal kinase (JNK) as well as the small GTPases Rho and Rac to control cytoskeletal remodeling (Ridley *et al.*, 1992). Signaling through Rho leads to the activation of protein kinase C which in turn signals to the protein cell division cycle 42 resulting in changes in the cell cycle as well as cytoskeleton (Gordon & Nusse, 2006; Wang & Nathans, 2007). Furthermore, JNK as previously mentioned, is a crucial mediator of convergent-extension movements during embryogenesis, and is also involved in Wnt/PCP signaling (Choi & Han, 2002). Evidence for the involvement of JNK in the Wnt/PCP pathway comes from the observation that expression of DVL can induce JNK

activity in mammalian cells. Moreover, a loss of downstream JNK effectors can suppress the *Drosophila* PCP phenotype caused by DVL overexpression. While it has been suggested that JNK can be activated by Rac, the mechanism of action regarding Wnt/PCP-JNK pathway has yet to be fully elucidated (Minden *et al.*, 1995; Coso *et al.*, 1995).

Interestingly, a new perspective on Wnt signaling is emerging to suggest that perhaps the two pathways (canonical and PCP) are intertwined. Evidence for this signaling crosstalk originates from studies of the Wnt/β-catenin signaling pathway in F9 cells. Bikkavilli and colleagues (2008a,b) showed that chemical inhibitors of JNK attenuated Wnt/β-catenin signaling in F9 cells during differentiation, suggesting an intimate connection between the two pathways previously thought to be separate signaling cascades. Similarly, Li *et al.* (1999b) showed that DVL activated JNK and c-Jun-target genes in addition to its ability to regulate β-catenin signaling.

Although much is known about the players and the mechanisms involved in PrE differentiation, recent evidence indicates that signaling crosstalk modulates the activities of both Wnt pathways. This crosstalk comes in the form of reactive oxygen species (ROS) and the protein implicated is nucleoredoxin (NRX), a known DVL binding protein. Although details are just emerging, two reports have demonstrated that NRX binds to and inhibits the actions of DVL. Alleviating this binding by NRX oxidation or by knocking down NRX using siRNAs is sufficient to activate β-catenin signaling in the absence of Wnt (Funato *et al.*, 2006; Funato *et al.*, 2010). Thus, this data provides evidence to suggest that the canonical Wnt/β-catenin pathway is redox-regulated by ROS (Fig. 1.3).

Figure 1.3 ***Redox regulation of the Canonical Wnt/β-catenin signaling pathway.*** A) In the absence of H_2O_2, Nucleoredoxin (NRX), a known Disheveled (DVL) binding partner inhibits the action(s) of DVL, which facilitates the destruction of β-catenin, thereby ensuring the Wnt/β-catenin signaling cascade remains in the "off" state. B) Conversely, treatment with exogenous H_2O_2, which diffuses readily across cell membranes, promotes oxidation of NRX and its dissociation from DVL, thereby activating the Wnt/β-catenin signaling pathway in the absence of Wnt (seen here).

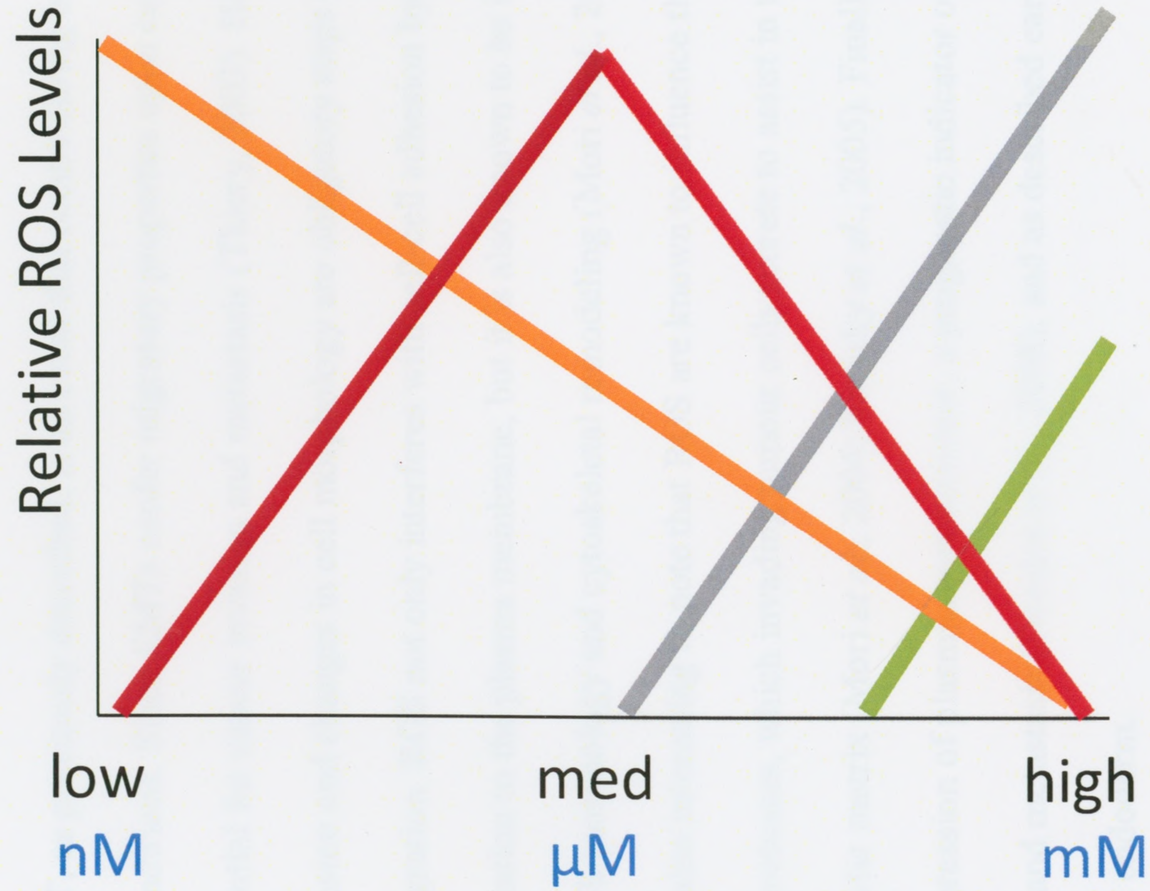

A) H_2O_2 Absent
B) H_2O_2 Present
H_2O_2
H_2O_2
DVL
NRX
Destruction Complex
Axin
APC
GSK3
β-catenin
Proteasome
DVL
Axin
APC
GSK3
NRX
β-catenin
β-catenin
β-catenin
Nucleus
β-catenin
TCF
differentiation

1.10 *Reactive oxygen species*

ROS is an inclusive term, commonly used as an identifier for a diverse group of molecules; namely, superoxide anions, hydroxyl radicals and hydrogen peroxide (H_2O_2). ROS are produced as natural by-products of cellular metabolism, including mitochondrial respiration. ROS are also produced by NADPH oxidases that catalyze the reduction of oxygen by using NADPH as an electron donor. Historically, excessive levels of ROS are known to be damaging to numerous intracellular targets including proteins, lipids, and nucleic acids. Much of our understanding with regards to the cytotoxic effects of ROS comes from studies employing neutrophils, which use ROS in oxidative bursts to eliminate exogenous pathogens such as bacteria (Sbarra & Karnovsky, 1959; Babior, 1984a,b; Rhee *et al.*, 2000). Given that neutrophils have evolved ways to use ROS in a beneficial way, it is tempting to speculate that other cells have evolved similar strategies to benefit from utilizing ROS in a functional manner (May & de Haën, 1979a,b). For instance, cells that have high metabolic requirements will, as a consequence, produce proportionately higher levels of ROS. If excessive levels of ROS are detrimental to cells, then these cells must have mechanisms to tightly regulate ROS levels to maintain tolerable concentrations and it is the latter that cells could use for other purposes. The previously highlighted evidence for ROS crosstalk on the Wnt/β-catenin pathway is an example where ROS is being used to modulate a pathway involved in cell signaling. A growing body of evidence is emerging to implicate ROS as playing key roles in various signal transduction pathways (Wu, 2006; D'Autreaux & Toledano, 2007). Since this is a relatively new area of research, it is likely that when examined in greater detail there will be more evidence implicating ROS as being involved in the regulation of various

developmental processes (Fig. 1.4; Sundaresan *et al.*, 1995; Ushio-Fukai *et al.*, 1999; Yoshizumi *et al.*, 2000).

Figure 1.4 ***Perspectives regarding the influence of intracellular redox environment on cell development.*** When stimulated with low levels (nanomolar range) of reactive oxygen species (ROS), cells are likely to respond by proliferating, whereas exposure to intermediate levels (micromolar range) will favor differentiation. Higher levels (millimolar range) of ROS induce apoptosis and necrosis.



Adapted from
Dennery, 2007

1.11 *ROS in EMT and rationale for further analysis*

An EMT, as previously described, is an event that occurs during both normal and pathological conditions. Since EMTs confer migratory properties upon cells, they are considered essential for cancer invasion and metastasis (Thiery, 2003). However, the loss of cell-cell adhesion and changes in cell morphology are obligatory steps that must precede cell migration. ROS not only interferes with cell-cell adhesion by decreasing cadherin localization to the plasma membrane, but it is also known to be involved in the regulation of cell morphology and cytoskeletal remodeling (Mori *et al.*, 2004; Inumaru *et al.*, 2009). It is also interesting to note that ROS are known to influence the expression of matrix metalloproteases, which invading tumour cells secrete to assist in the breakdown of the extracellular matrix (Mori *et al.*, 2004; Radisky *et al.*, 2005). Finally, ROS can up-regulate the expression of plasminogen activator, a prognostic indicator of tumour aggressiveness and metastasis (Oszajca *et al.,* 2008), and as described earlier, a marker of extraembryonic endoderm.

Overall, when RA-treated F9 cells differentiate they acquire morphological, molecular and biochemical characteristics of PrE and previous reports from our lab have detailed that differentiation is accompanied by an up-regulation of Gata6 and FoxA2. We also have evidence to indicate that the overexpression of either of these transcription factors is sufficient to up-regulate the expression of Wnt6 (Hwang & Kelly, unpublished). Furthermore, in the absence of RA, differentiation occurs when cells are treated with Wnt6-conditioned media and/or when the canonical Wnt/β-catenin signaling pathway is activated (Krawetz & Kelly, 2008). Since ROS are known to converge on the Wnt/β-catenin signaling pathway and can alter cell morphology, cytoskeleton, and cell adhesion,

there is precedence to suggest that it has a role in EMTs, and taken one step further, a contribution in the mechanism(s) involved in differentiation of PrE.

1.12 *Hypothesis & Objectives*

While there have been a plethora of studies involving ROS and their detrimental and beneficial roles on normal and abnormal cell physiology, its role in extraembryonic endoderm formation has not been examined *in vitro* or *in vivo*. Given the ability of ROS to modulate the activity of DVL, I hypothesize that ROS play a key role in the RA signaling pathway that specifies F9 cells to form extraembryonic endoderm. I will investigate the role of ROS signaling in the differentiation of F9 cells by the following three objectives: 1) Analyze the intracellular ROS levels of F9 cells undergoing RA-induced differentiation. 2) Determine whether or not exogenous ROS in the form of H_2O_2 is sufficient to recapitulate differentiation. 3) Reduce ROS levels using antioxidants to determine if ROS is necessary for differentiation.

Chapter 2 – Materials & Methods

2.1 *Cell culture & Transfection*

All experiments were performed using the F9 mouse embryonal carcinoma cell line (ATCC). F9 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (FBS; PAA), penicillin (100units/ml), and streptomycin (100μg/ml; Gibco). F9 cell cultures were maintained in a humidified environment at 37°C with 5% CO_2.

Differentiation of F9 cells was induced using 0.1μM all-*trans* retinoic acid (RA; Sigma) prepared in dimethyl sulfoxide (DMSO; Caledon Labs). ROS treatment of F9 cells was done using H_2O_2 (EMD) diluted in Milli-Q grade H_2O to the experimental concentrations indicated.

N-acetyl cysteine (NAC; Sigma) was prepared in cell culture media on the day of treatment. 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma) was prepared by first dissolving in DMSO, which was added to media and heated until completely dissolved. Diphenyleneiodonium chloride (DPI; Sigma) was also prepared in DMSO.

Transfections were performed as per the manufacturer's instructions using FuGene 6 transfection reagent (Roche). Antibiotic selection was performed using Neomycin Sulphate (G418; Calbiochem) at a concentration of 800μg/ml and 400μg/ml for selection and maintenance, respectively.

2.2 *Plasmids*

The SuperTOPflash β-catenin-responsive luciferase reporter construct was generously provided by Dr. Stephane Angers (University of Toronto). The neomycin selectable pcDNA 3.1+ vector was purchased from Invitrogen and used as transfection control.

2.3 *RNA extraction, cDNA synthesis & PCR conditions*

Cell cultures were lysed using RLT buffer (Qiagen). Whole cell lysates were homogenized using QIAShredder (Qiagen), and total RNA was extracted from homogenates using an RNeasy kit (Qiagen) as per the manufacturer's instructions. Isolated RNA was immediately used for cDNA synthesis using a Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. Resultant cDNA was used as a template for amplification by polymerase chain reaction (PCR) or stored at -20°C. PCR was performed under the following conditions: 35 cycles of 30s at 94°C, 30s at 58°C and 30s at 72°C. PCR products were separated by electrophoresis on 2% agarose gels. Electrophoresis was performed at 60V for 3hrs. Products were resolved by staining in ethidium bromide and images were captured using a FluorChem 8900 gel imaging station (Alpha Innotech).

2.4 *PCR primers*

Mouse *NOX4* primers (Fwd 5'-GATCACAGAAGGTCCCTAGCAG & Rev 5'-GTTGAGGGCATTCACCAAGT; 134bp) were designed by Sauer *et al.* (2008). Mouse

ribosomal protein *L14* primers were (Fwd 5'-GGGAGAGGTGGCCTCGGACGC & Rev 5'-GGCTGGCTTCACTCAAAGGCC; 300bp) used to amplify cDNA corresponding to a constitutively expressed gene.

2.5 *Cell viability assay*

Cytotoxicity was assessed using a Trypan Blue (TB; 0.4% in PBS; Allied Chem) dye exclusion test. Cells were first trypsinized (TrypLE; Invitrogen) and resuspended in complete DMEM. TB was added to an aliquot of cell suspension in a 1:1 (vol/vol) ratio, the mixture was then incubated at room temperature for 5mins. The TB-cell suspension mixture was loaded onto a hemocytometer and cells were counted to provide an estimation of cell viability.

Cell density was visualized using 0.2% Coomassie blue (Pierce). Cells were first fixed on ice with cold methanol for 5mins then aspirated. Coomasie blue solution was added and allowed to stain for 30mins at room temperature. Subsequently, staining solution was aspirated and cells were gently rinsed with water and dishes were air dried for image capture.

2.6 *WST-1 cell proliferation assay*

Proliferation was assessed using a WST-1 colorimetric assay (Roche) as per manufacturer's instructions. Briefly, WST-1 reagent was added directly to treated cell cultures (1/10 dilution) and incubated for 30mins in 37°C and 5% CO_2. Treated cultures

were then analyzed using a spectrophotometic microplate reader (BR3550; Bio-Rad) with an endpoint measurement at 450nm and a reference wavelength of 655nm. Resultant data was recorded using Microplate Manager 4.0 software (Bio-Rad).

2.7 *Immunoblotting*

Lysate preparation

Whole cell lysates were prepared in RIPA buffer (Sigma) supplemented with a protease cocktail inhibitor mix and EDTA (PIC; Thermo Scientific). Cells were lysed in cold RIPA-PIC buffer for 5mins on ice. Lysates were scrapped into chilled micro-centrifuge tubes and homogenized by sonication on ice. Homogenates were then cleared by centrifugation at 14,000 RPM for 5mins. The supernatant was collected for immunoblot analysis or stored at -20°C.

Protein assay

Protein concentrations were quantified using a Bradford protein assay (Bio-Rad). All samples and protein standards were prepared in either duplicates or triplicates and analyzed using a microplate absorbance reader (BR3550; Bio-Rad). Absorbance data were gathered using an endpoint measurement at 595nm and protein concentrations were calculated using Microplate Manager 4.0 software (Bio-Rad).

Polyacrylamide gel electrophoresis

Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes. Briefly,

lysates were mixed with 3X SDS blue loading buffer (NEB) and boiled for 5mins, then cooled on ice. Lysates were loaded and resolved on 10% SDS-PAGE gels; proteins separation occurred under constant voltage (100V) for 2hrs. Proteins were transferred onto nitrocellulose membranes using constant voltage (100V) for 1hr. Membranes were washed using Tris Buffered Saline with 0.1% Tween-20 (TBST; pH 7.5) and blocked in 5% skim milk for 1hr at room temperature. Membranes were probed with rat anti-cytokeratin endo-A (TROMA-1; 1/50; University of Iowa Developmental Studies Hybridoma Bank) and mouse anti-β-actin (1/10,000; Santa Cruz) overnight at 4°C. Following primary antibody incubation, membranes were washed in TBST and subsequently probed with horseradish peroxidase (HRP)-conjugated goat anti-rat, anti-mouse antibodies (1/10,000; Pierce) overnight at 4°C. After secondary antibody incubation, the membrane was washed extensively in TBST and incubated in SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) as per the manufacturer's instructions. X-Ray films (Kodak) were scanned and HRP-signal intensities were quantified using a FluorChem 8900 Imager (Alpha Innotech) equipped with densitometric analysis software.

2.8 *Immunofluorescence microscopy*

Cells were seeded on 0.2% gelatin coated glass cover slips in 35mm dishes (BD). After treatment, cells were washed with phosphate buffered saline (PBS; Lonza) and fixed on ice with cold methanol:acetone (1:1) for 10mins. Following fixation, blocking was done at room temperature with 1% bovine serum albumin (BSA; EMD) in PBS (with 0.1%

Triton X-100; PBST) for 30mins. Cells were incubated with TROMA-1 (1/20; diluted in 3% BSA/PBS) overnight at 4°C, and then rinsed in PBS and incubated with TRITC-conjugated goat anti-rat IgG (1/100; Sigma) at room temperature for 1 hour. Following several washes with PBS, cover slips were mounted onto glass slides using Prolong Gold mounting reagent with DAPI (Molecular Probes). Images were captured using an Axio Imager A1 (Zeiss) microscope with Northern Eclipse 7.0 software (Empix Imaging) and processed using Photoshop CS3 (Adobe) as described previously (Paddock et al., 1997).

2.9 *Detecting intracellular reactive oxygen species*

Intracellular ROS generation was determined in F9 cells using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes) in conjunction with fluorescence microscopy. CM-H_2DCFDA was prepared in DMSO as described previously (Eruslanov & Kusmartsev, 2010). Additionally, mitochondria were labeled using MitoTracker Red (MTR; Molecular Probes) prepared in DMSO. F9 cells were grown and treated on 0.2% gelatin coated glass cover slips. Following treatment, cells were incubated with 1μM CM-H_2DCFDA, 0.1μM MTR and 10μg/ml Hoechst stain in PBS for 10mins at 37°C and 5% CO_2. Immediately after incubation, cells were washed twice with PBS and mounted for live cell imaging. Images were captured using an Axio Imager A1 (Zeiss) fluorescence microscope with Northern Eclipse 7.0 software (Empix Imaging) and processed using Photoshop CS3 (Adobe).

2.10 *β-catenin-responsive luciferase reporter assay*

Transfections were performed as described above. F9 cells were grown to 50% confluency and co-transfected with SuperTOPflash β-catenin-responsive luciferase reporter mixed (1:1) with pcDNA 3.1+. After 48hrs, cells were plated in selection media for 1 week in G418. Surviving colonies were once again split, passaged and maintained in G418 for the entire duration of treatment. Following treatments, cells were lysed in lysis reagent (CCLR; Promega) and assayed for luciferase expression as per the manufacturer's instructions. Lysate luciferase expression was quantified using a Modulus II Multimode Microplate reader (Promega).

2.11 *Statistical analysis*

All assays were performed and repeated independently at least three times. Densitometry data were obtained using a FluorChem 8900 Chemiluminescence and Gel Imager (Alpha Innotech) and analyzed using Microsoft Excel 2007. Results were analyzed using Student's t-test and data is presented as mean ± standard error of the mean (SEM). Statistical significance was acknowledged if $P<0.05$.

Chapter 3 – Results

As previously noted, ROS signaling has been implicated in RA, Wnt and G-protein signaling, all of which are known to play crucial roles in mediating F9 differentiation. Although ROS have been studied in the context of cellular damage, their role in signal transduction in F9 cells has not been reported. Towards that end, the present study examined the role of ROS signaling, and in particular the effect of H_2O_2 on F9 cells and their ability to differentiate into extraembryonic endoderm.

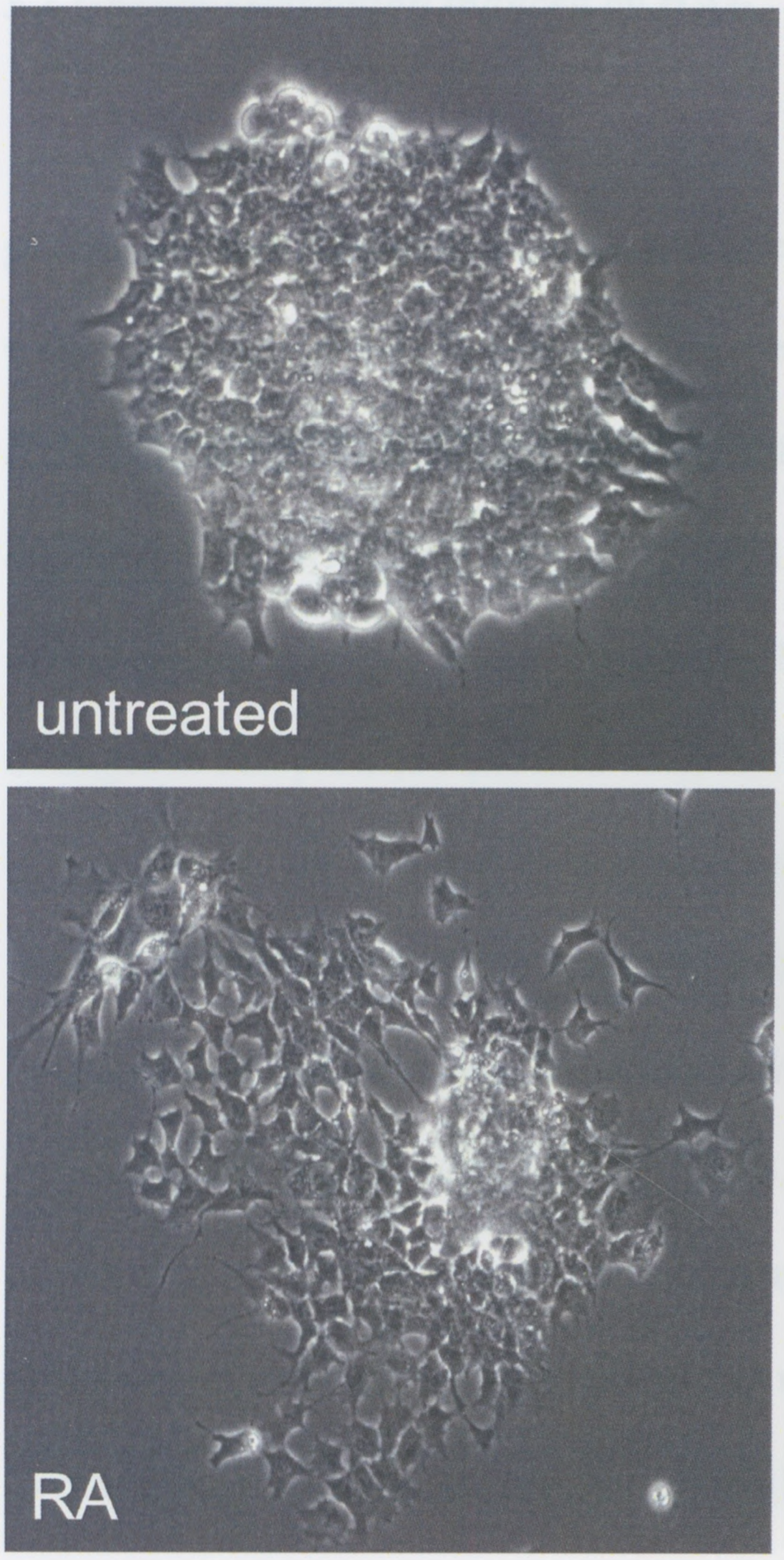
3.1 *RA induced the differentiation of F9 cells*

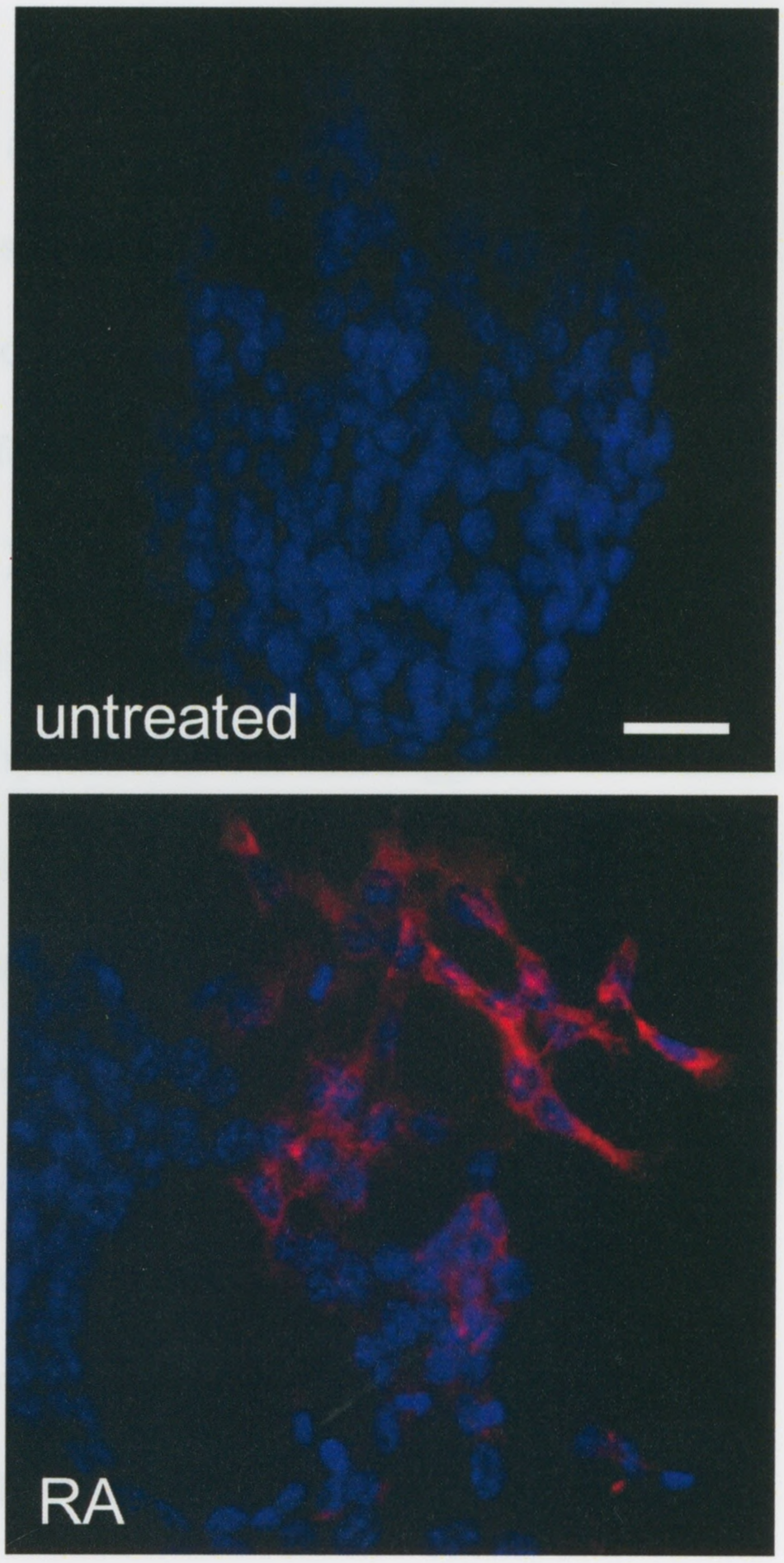
It is known that RA induces the differentiation of F9 cells into extraembryonic endoderm (Strickland & Mahdavi, 1978). As expected, treatment with RA induced dramatic morphological changes in F9 cells (Fig. 3.1A). In contrast to the tightly grown embryoid bodies seen in untreated cells, RA treated cells appeared scattered, flatten and elongated with stellate-shaped cell bodies. An increase in intermediate filament expression is also a signature of differentiated F9 cells (Kurki *et al.*, 1989; Krawetz & Kelly, 2008). Consistently, RA treated cells showed a robust increase in intermediate filament expression (Fig. 3.1B-C) suggesting that they have adopted an extraembryonic endoderm cell fate.

Figure 3.1 ***Morphological changes and intermediate filament expression accompany F9 cell differentiation.*** F9 cells were untreated or treated with RA (0.1μM) for three days. A) Morphological analysis of cells using phase-contrast microscopy. Untreated cells grow in tightly grouped embryoid bodies, whereas those treated with RA adopt an elongated stellate-shape. B) Immunofluorescence microscopy with the TROMA-1 (red) antibody, to detect endoA cytokeratin filaments, shows increased intermediate filament expression in response to RA. DAPI (blue) was used to counter-stain nuclei. C) Immunoblot analysis confirmed the immunofluorescence data, and showed increase TROMA-1 detection two days following RA exposure. DMSO, the delivery vehicle for RA had no effect on cytokeratin filament expression. β-Actin was included as a loading control. Scale bar (white; 30μm). Note: cells in panel A) are from a different field of view than panel B).

A Morphology

B Immunofluorescence





C Immunoblotting

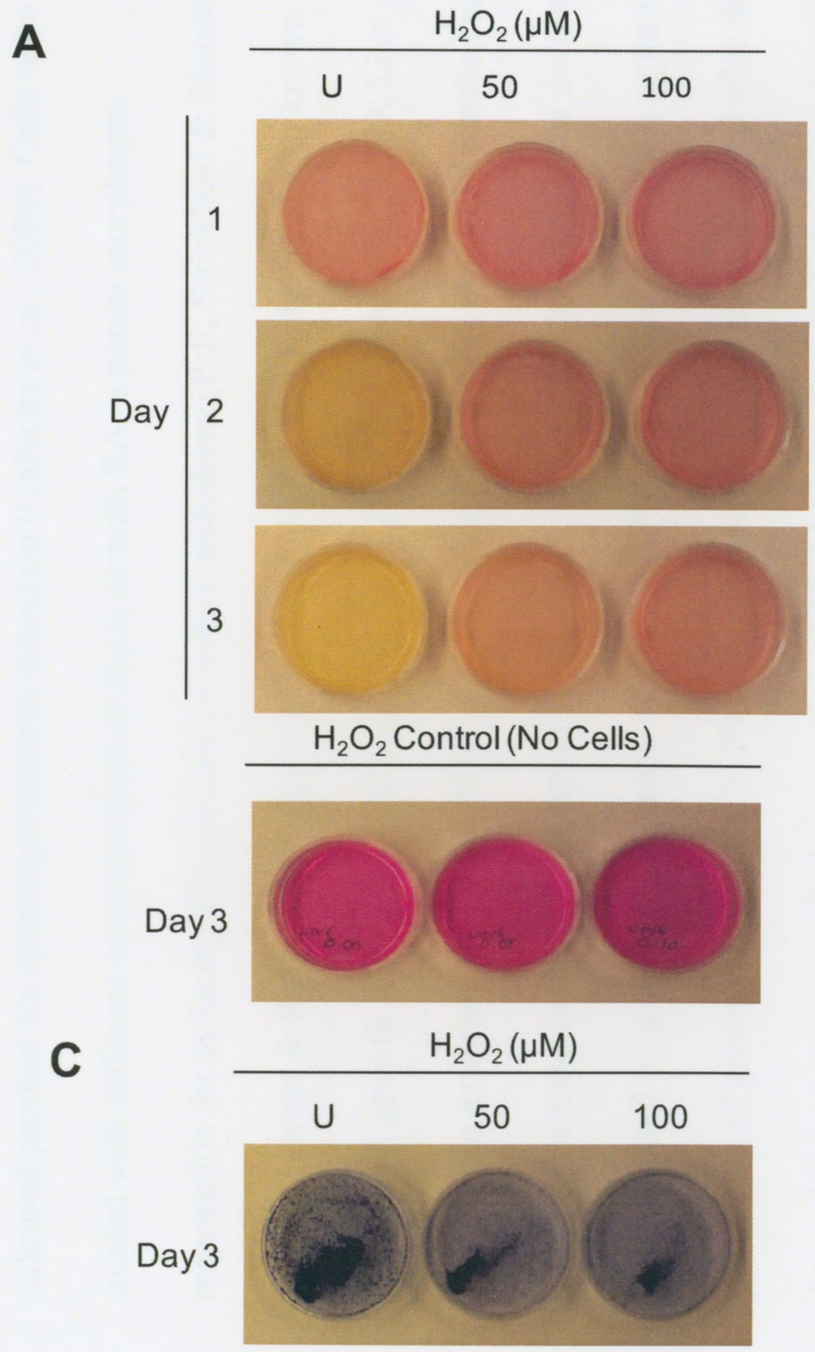
untreated
DMSO
1 day
2 days
3 days
RA

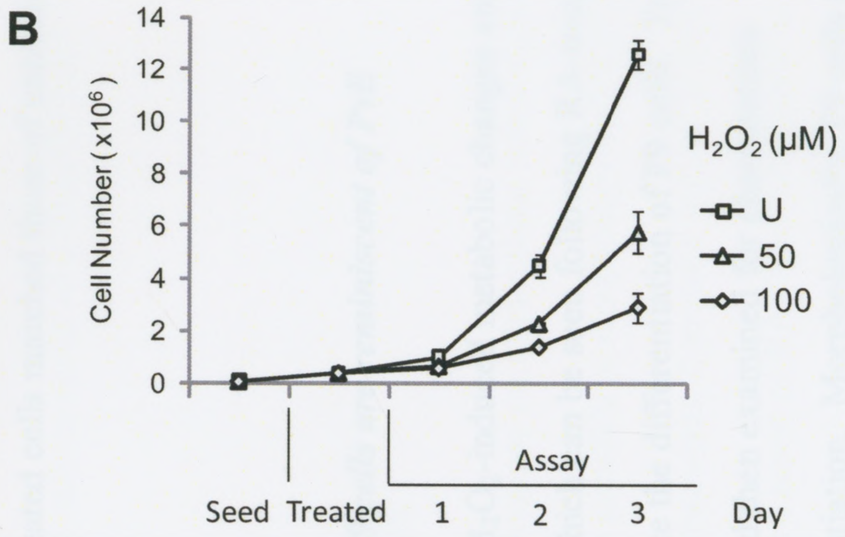
3.2 *Growth retardation induced by H_2O_2*

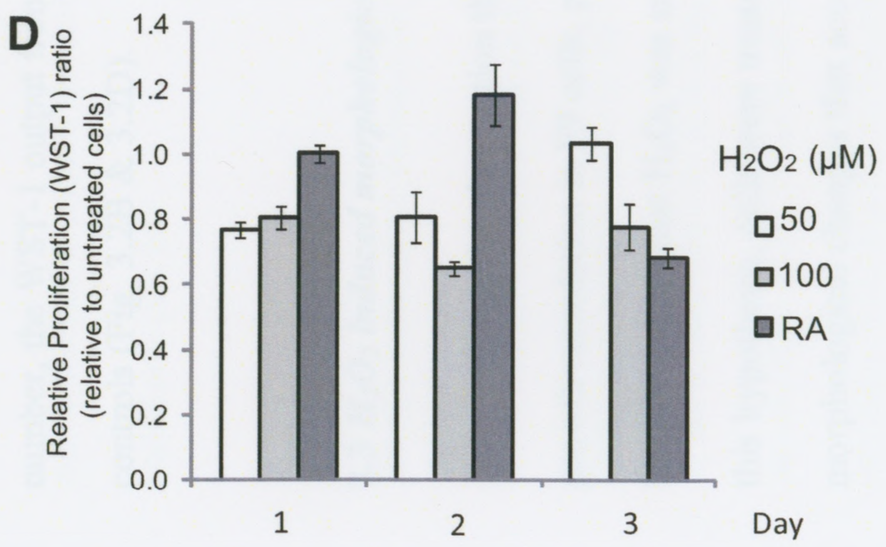
The pH of cell culture media serves as an informative, basic indicator of cell growth. Proliferating cells produce and release metabolites, such as lactic acid, causing a visible decrease in the pH of the culture media, changing phenol red to yellow (Masters & Stacey, 2007). F9 cells have a high proliferative rate complemented by a rapid doubling time (8-10hrs; Alonso *et al.*, 1991). Under standard culturing conditions this robust proliferation of F9 cells is mirrored by a rapid decline in media pH (Fig. 3.2A). Changes in cell proliferation following H_2O_2 treatment was first suggested when different concentrations of H_2O_2 were tested as a prelude to investigating whether or not it affects the ability of F9 cells to differentiate into extraembryonic endoderm. Results showed that F9 cells displayed a decrease in proliferative capacity in response to increasing concentrations of H_2O_2 (50, 100μM; Fig. 3.2A). The observed decrease in proliferation in response to H_2O_2 did not appear to be due to increased cytotoxicity as there was no difference in viability between treated and untreated cultures when evaluated using the Trypan Blue dye exclusion test (not shown). Consistently, cell counts from H_2O_2 treatment did not decrease below the initial seeding density (Fig. 3.2B). Cell counts corroborated the qualitative data, revealing that H_2O_2-treated cells had slowed their growth relative to untreated cells (Fig. 3.2B-C). These results are in agreement a previous study showing that H_2O_2-induced growth retardation in mouse embryonic stem cells, particularly in the 50-100μM range (Guo *et al.*, 2010).

Since H_2O_2 at the concentrations tested appeared to retard growth, rather than increase cell death, the next series of experiments were designed to compare the proliferative capacity of H_2O_2 treated cells to RA treated cells. WST-1 is a widely used

Figure 3.2 ***Effects of H_2O_2 on F9 cell proliferation.*** Cells were incubated for one day prior to treatment then treated with 50 or 100μM H_2O_2 daily for three days. Untreated (U) cells served as controls. Cell growth was monitored for a total of four days. A) Changes in the phenol red pH indicator over time. Dishes containing untreated cells displayed changes in the color of the phenol red indicator. These changes did not occur as rapidly when cells were treated with either concentration of H_2O_2. H_2O_2 concentrations alone had no effect on the pH of the media when cells were absent. B) Cells treated as in A) were counted and the data (± S.E.M.) presented in a growth curve. C) Coomassie blue staining three days following H_2O_2 treatment shows cell density differences. D) Cell proliferation under RA and H_2O_2 treatments was analyzed using the WST-1 assay. Graph represents the relative proliferation (WST-1) ratio of RA (0.1μM) or H_2O_2-(50 or 100μM) treated cells relative to untreated cells ± S.E.M. Data was collected from at least three independent experiments.


A
H_2O_2 (μM)
U
50
100
Day
1
2
3
H_2O_2 Control (No Cells)
Day 3
C
H_2O_2 (μM)
U
50
100
Day 3


B
Cell Number (x10^6)
14
12
10
8
6
4
2
0
H_2O_2 (μM)
U
50
100
Assay
Seed
Treated
1
2
3
Day


D
Relative Proliferation (WST-1) ratio
(relative to untreated cells)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
H_2O_2 (μM)
50
100
RA
1
2
3
Day

proliferation assay. WST-1 reflects proliferation by measuring the relative metabolic activity of viable cells using the cleavage of colourless tetrazolium salts which produces coloured product for spectrophotometry detection (Galluzzi *et al.*, 2009). Cells were treated with different concentrations of H_2O_2 or with RA, a potent morphogen responsible for inducing F9 cells into primitive endoderm (PrE; Strickland & Mahdavi, 1978; Krawetz & Kelly, 2008; Krawetz & Kelly, 2009), and a chemical also known to inhibit cell growth in multiple cell lines including F9 cells (Kurki *et al.*, 1989; Li *et al.*, 2004), then examined using the WST-1 assay. As expected, RA treatment resulted in a marked decrease in WST-1 detection by day three, although, interestingly the decrease was preceded by a transient increase on day two (Fig. 3.2D). Similarly, H_2O_2 reproduced the effects of growth retardation in F9 cells comparable to the level observed after three days of RA treatment, with the exception of 50μM H_2O_2. Despite being lower in cell number, the WST-1 output from 50μM H_2O_2 treated cells matched those of untreated controls (Fig. 3.2B & 3.2D).

3.3 *H_2O_2 induced morphological changes in F9 cells are reminiscent of PrE*

Given the observation that some of the H_2O_2-induced metabolic changes and growth retardation in F9 cells, parallel to that which can be seen following RA-treatment, I hypothesized that H_2O_2 was sufficient to induce the differentiation of F9 cells. To test this hypothesis, cells were treated with H_2O_2 and then examined for characteristic morphological changes that accompany differentiation. Morphologically, F9 cells treated with H_2O_2 (100μM) appeared strikingly different when compared to untreated cells

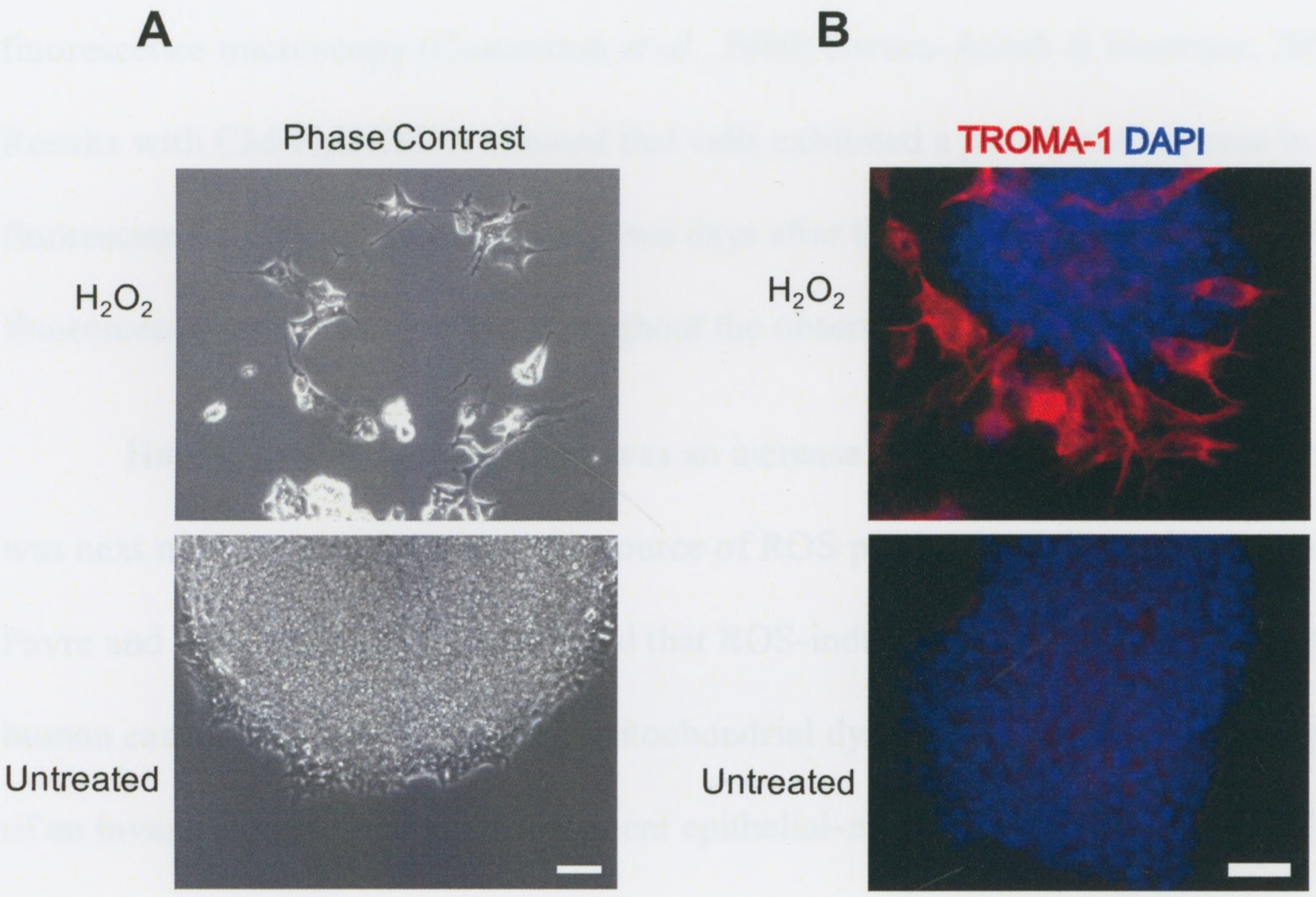
which remained in aggregates after three days. The observed phenotype in response to H_2O_2 treatment appeared similar to the morphological features induced by RA treatment. In contrast to untreated cells, cells treated with H_2O_2 displayed a flatten-elongated stellate-shaped phenotype (Fig. 3.3A). Thus, phenotypic similarities prompted me to examine the cells for molecular markers of differentiation.

Analysis using the TROMA-1 antibody, used to detect extraembryonic endoderm (Krawetz & Kelly, 2008) in conjunction with immunofluorescence microscopy, revealed the presence of distinct endo-A cytokeratin filaments in cells that had been treated with H_2O_2 (Fig. 3.3B). As expected, there was no obvious TROMA-1-positive staining in untreated cells. This H_2O_2-dependent increase in endo-A cytokeratin was also detected by Western blot analysis (Fig. 3.5; see below). Together, the morphological and intermediate filament changes would indicate that in the absence of RA, H_2O_2 treatment was sufficient to induce the differentiation of F9 cells towards an extraembryonic endoderm cell fate.

3.4 *RA induced an increase in intracellular ROS*

With evidence that retinoid signaling converges on intracellular redox signaling (Nitti *et al.*, 2010), and my data highlighting the parallels in morphology and expression of molecular markers following RA and H_2O_2 treatments, it seemed logical to predict that intracellular ROS levels in cells may increase as a result of RA exposure. However, the literature with regards to the redox response triggered by RA is contradictory, and RA can act as either a pro or antioxidant depending on the cell type examined (Choudhary *et*

Figure 3.3 ***Morphological changes and intermediate filament expression in F9 cells treated with H_2O_2.*** A) Phase contrast images of untreated cells and after three days of treatment with H_2O_2 (100μM). H_2O_2 treated cells appear elongated with stellate-shaped cell bodies. B) Immunofluorescence microscopy with the TROMA-1 antibody shows prominent endo-A staining in cells treated with H_2O_2 but not in untreated cells. DAPI (blue) was used to counter-stain nuclei. Panels show representative images from at least three independent experiments. Scale bar (white; 30μm). Note: cells in panel A) are from a different field of view than panel B).

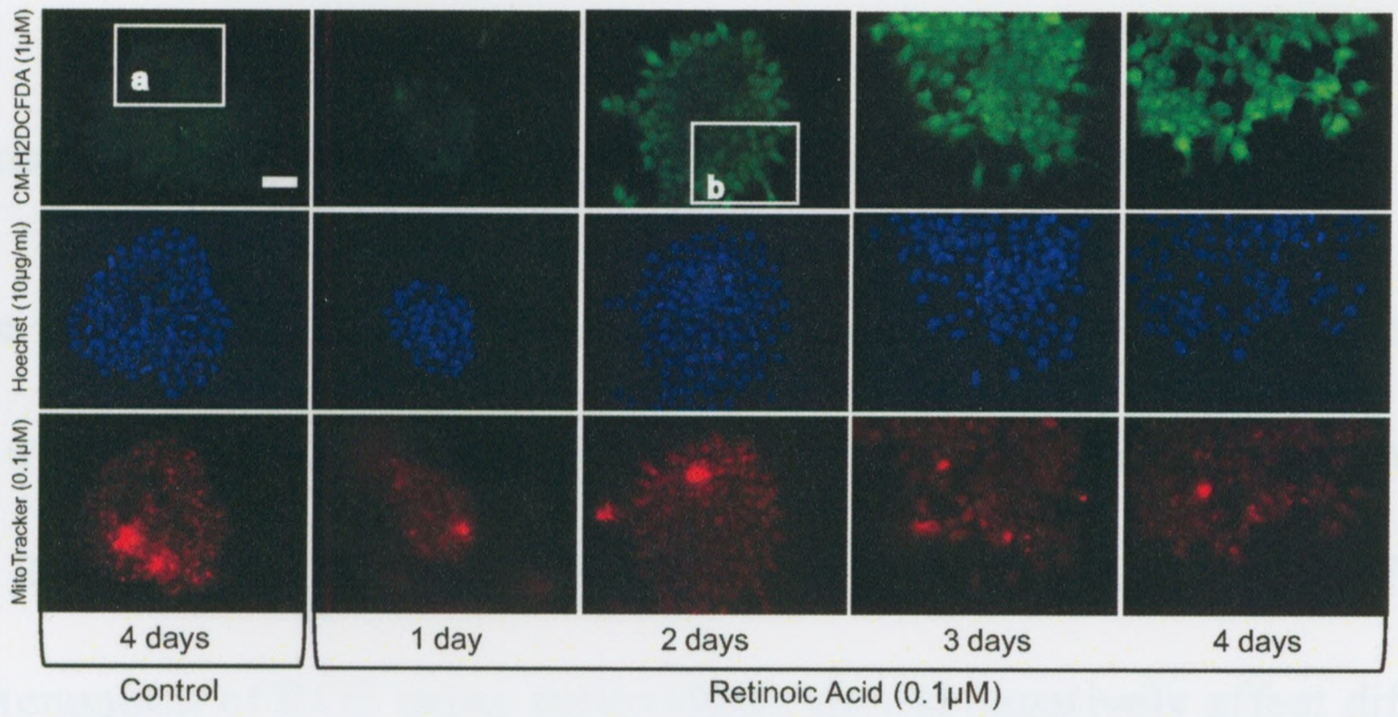

A
Phase Contrast
H_2O_2
Untreated
B
TROMA-1 DAPI
H_2O_2
Untreated

al., 2008; Konopka *et al.*, 2008; Miyoshi *et al.*, 2010). Thus, CM-H_2DCFDA was used to investigate the intracellular redox status of F9 cells during RA-induced differentiation. CM-H_2DCFDA is a non-fluorescent, uncharged, lipophilic probe that rapidly diffuses across cell membranes. Inside the cell its diacetate group is cleaved by nonspecific intracellular esterases and is retained by the intact plasma membrane. Following oxidation by intracellular ROS the resultant non-fluorescent anion is converted to the highly fluorescent 2',7'-dichlorofluorescein that is easily visualized using conventional fluorescence microscopy (Cossarizza *et al.*, 2009; Owusu-Ansah & Banerjee, 2009). Results with CM-H_2DCFDA showed that cells exhibited a prominent increase in fluorescence intensity approximately two days after the addition of RA (Fig. 3.4A). This fluorescence remained elevated throughout the observational period.

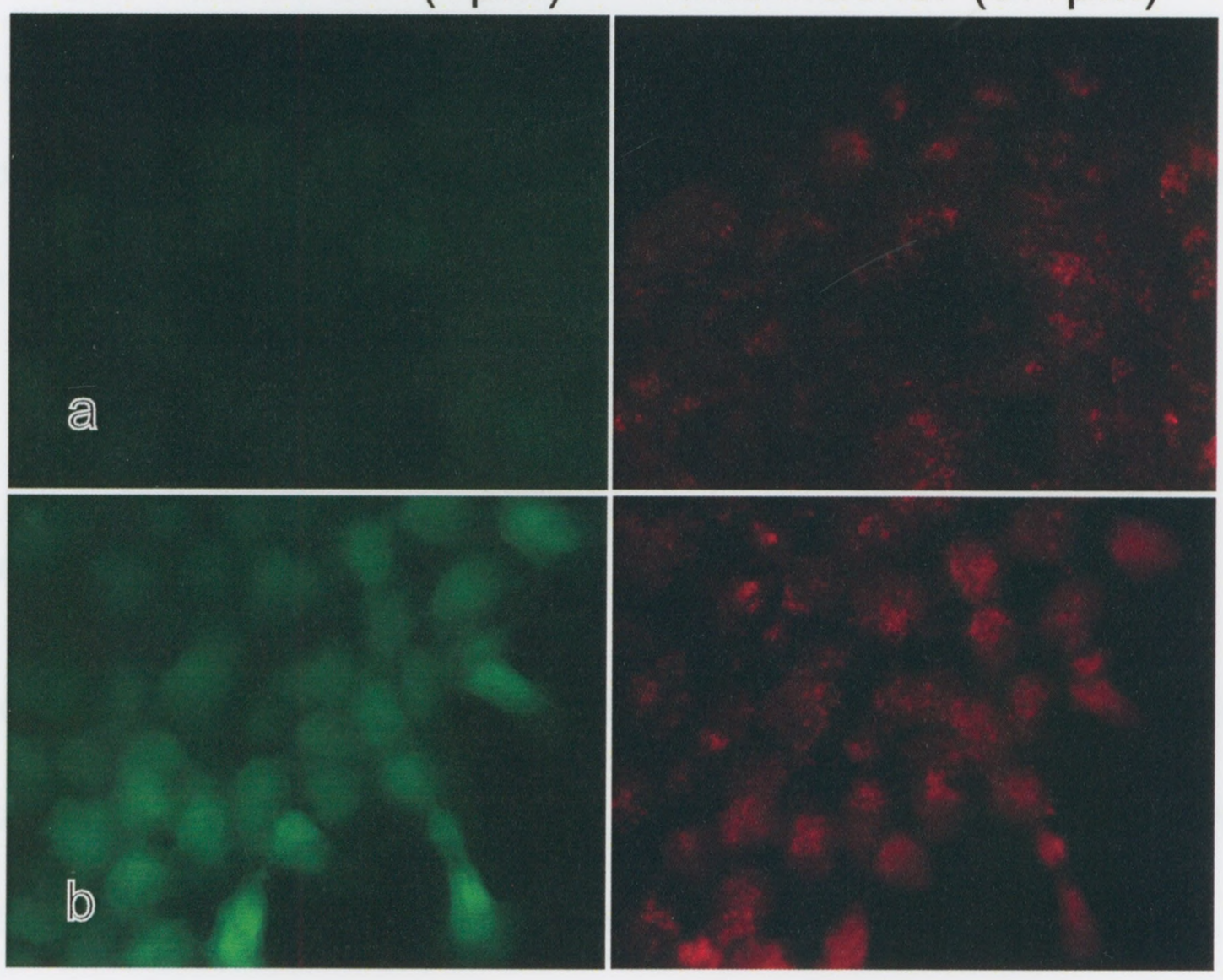
Having established that there was an increase of ROS following RA treatment it was next necessary to determine the source of ROS production. A previous report by Favre and colleagues (2010) suggested that ROS-induced differentiation in several human cancer cell lines arises from mitochondrial dysfunction, leading to the appearance of an invasive phenotype and subsequent epithelial-mesenchymal transition (EMT). Since the differentiation of F9 cells into PrE is one step towards an EMT, it seemed logical to predict mitochondria as the source of the intracellular ROS. To test this theory, the MitoTracker Red (MTR) probe, which specifically accumulates in respiring mitochondria, was used. Results using MTR showed punctate staining in both treated and untreated cells (Fig. 3.4B). MTR staining patterns were similar between treated and untreated cells and did not appear to correlate with CM-H_2DCFDA intensive regions, as one would expect if mitochondria was indeed the source responsible for ROS production

Figure 3.4 ***RA treatment of F9 cells increases intracellular levels of ROS.*** Untreated cells or those treated with RA (0.1μM) for up to 4 days were stained. A) Fluorescence detection of CM-H_2DCFDA (green) as an indicator of intracellular (cytoplasmic) ROS and MTR (red) to detect mitochondrial ROS showed RA treatment induced a sustained increase in cytoplasmic ROS from days 2-4. Nuclei were counterstained with Hoechst (blue). B) Higher magnification of areas denoted in A) revealed similar dense punctate MTR staining in both untreated and treated cells, whereas intracellular CM-H_2DCFDA was only seen in RA-treated cells. Images are representative of results obtained from at least three independent experiments. Scale bar (30μm).

A


CM-H2DCFDA (1μM)
Hoechst (10μg/ml)
MitoTracker (0.1μM)
a
b
4 days
1 day
2 days
3 days
4 days
Control
Retinoic Acid (0.1μM)

B


CM-H2DCFDA (1μM)
MitoTracker (0.1μM)
a
b

(Favre *et al.*, 2010). Thus, it would appear that the source of ROS was not necessarily from mitochondria as first predicted.

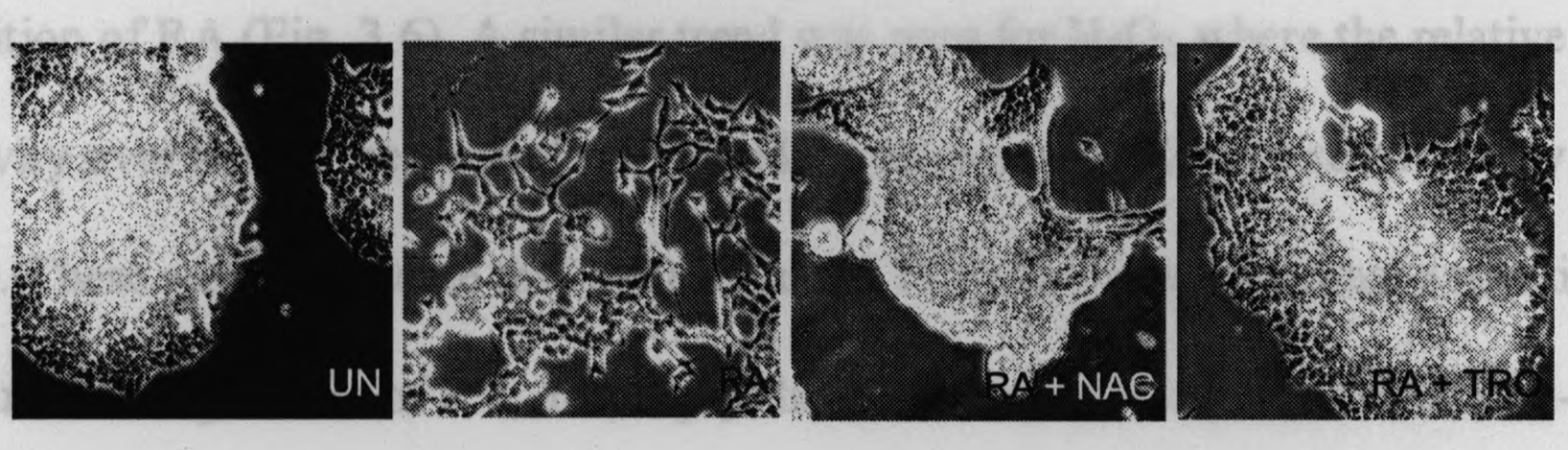
3.5 *Antioxidants attenuate RA-induced F9 cell differentiation*

The phenotypic similarities between RA and H_2O_2-induced differentiation, and the ability of RA to increase the ROS levels, would suggest that H_2O_2 converges on or is part of the RA signaling pathway. If H_2O_2 is important for RA-induced differentiation, then the attenuation of ROS using antioxidants should negatively affect differentiation. The antioxidants N-Acetyl Cysteine and Trolox were chosen for this particular experiment due to their proven efficacy in ameliorating ROS-induced signaling in both *in vitro* and *in vivo* applications (Aruoma *et al.*, 1989; Wu *et al.*, 1991; Zafarullah *et al.*, 2003; Wang *et al.*, 2008). Results clearly show that the RA-dependent appearance of endo-A cytokeratin decreased when cells were co-treated with either antioxidant (Fig. 3.5A & 3.5B). Taken together, the evidence points to a role for redox signaling in the RA-induced differentiation of F9 cells, however, the mechanism(s) influenced directly or indirectly by ROS remained unclear.

3.6 *Activation of TCF-LEF transcription activity by H_2O_2*

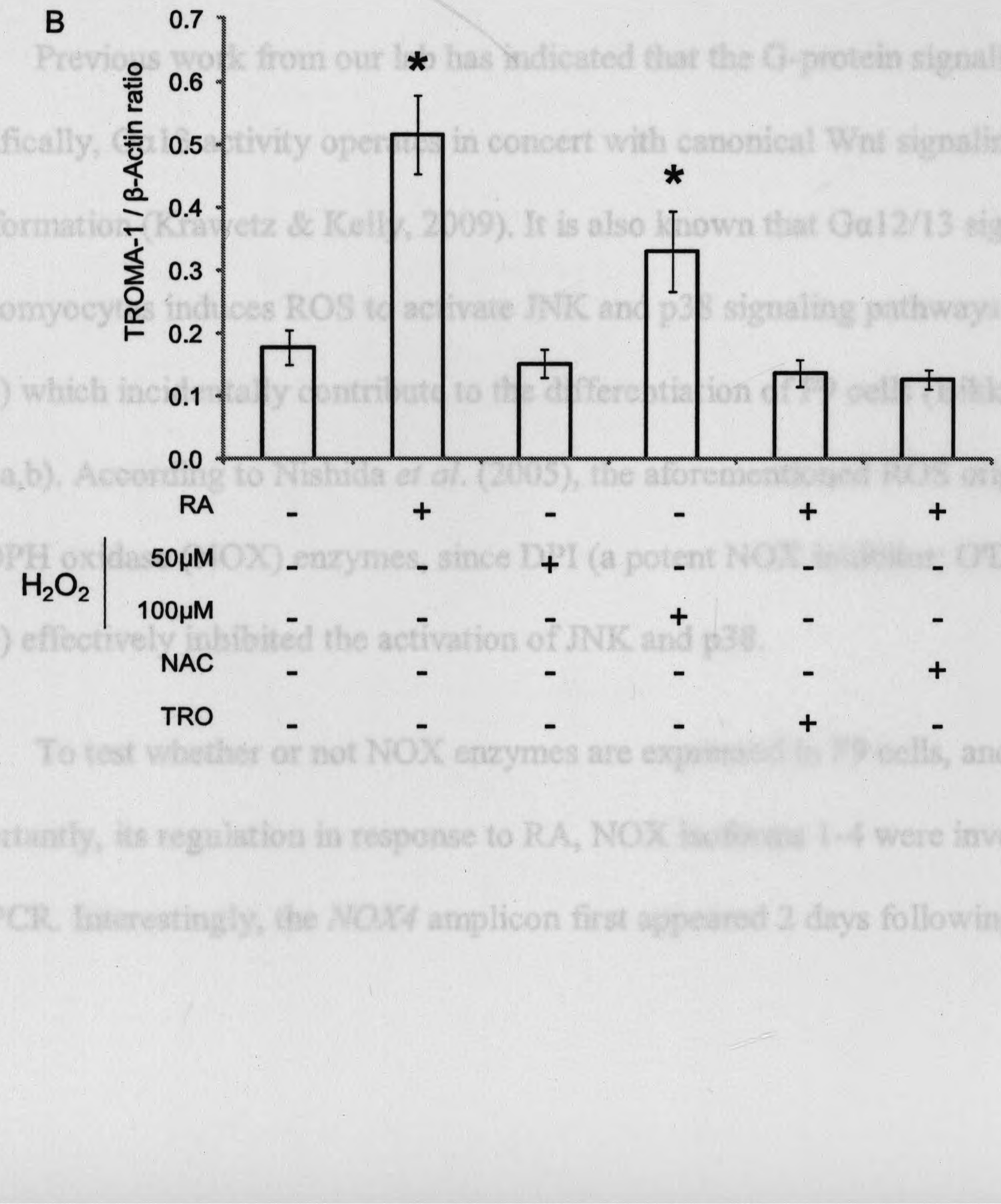
Activation of the β-catenin/TCF-LEF pathway is an obligatory step required for the RA-induced differentiation of F9 cells into PrE (Liu *et al.*, 2002; Krawetz & Kelly, 2008) and evidence indicates that ROS interact at the level of Disheveled to positively

Figure 3.5 ***Effects of RA, H_2O_2 and antioxidants on endo-A intermediate filament expression in F9 cells.*** A) Cells were either untreated or treated with RA (0.1μM), H_2O_2 (50, 100μM), RA plus NAC (1mM) or RA plus Trolox (0.1mM) for three days, and then prepared for immunoblot analysis. B) Densitometry data showing the relative ratio of TROMA-1 normalized to the β-Actin loading control ± S.E.M from three independent experiments. Asterisks denote significant difference ($P<0.05$) reported by Student's t-test of treatment relative to untreated control.


UN
RA
RA + NAC
RA + TRO

A


TROMA-1
β-Actin
55 kDa
43 kDa


B
TROMA-1 / β-Actin ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
RA - + - - + +
H2O2 50μM - - + - - -
100μM - - - + - -
NAC - - - - - +
TRO - - - - + -

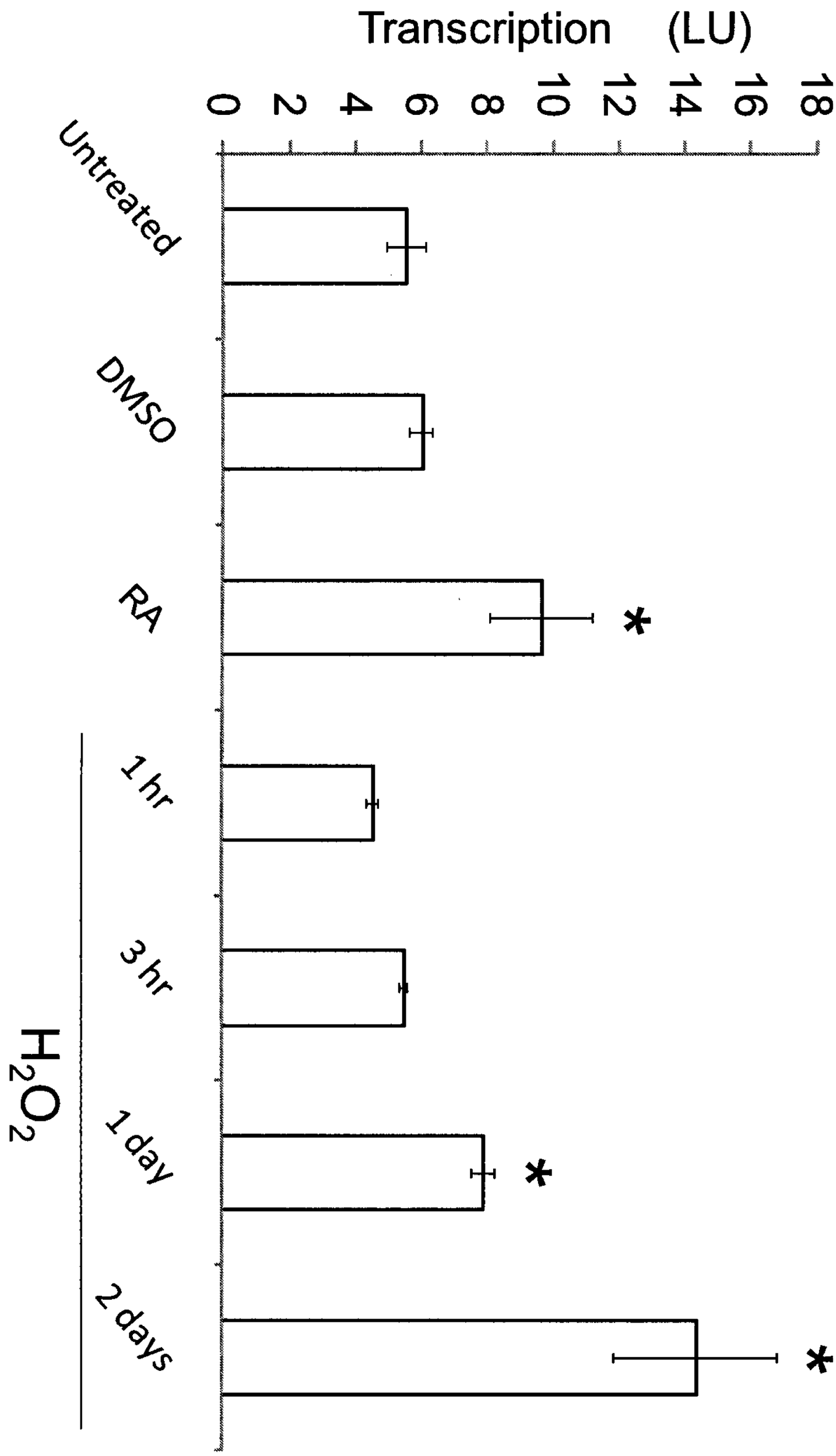
regulate canonical Wnt-β-catenin signaling (Funato *et al.*, 2006). To test whether or not a similar situation exists for F9 cells, an assay was performed using a β-catenin/TCF-LEF responsive luciferase reporter construct. As expected, F9 cells transfected with this reporter showed a marked increase in TCF-LEF transcription activity one day after the addition of RA (Fig. 3.6). A similar trend was seen for H_2O_2, where the relative levels in activity over that in the controls increased on day one and even more so on day two (Fig. 3.6). These results indicate that H_2O_2-induced TCF-LEF transcriptional activation occurred independent of RA, and imply that ROS can influence canonical Wnt-β-catenin signaling in the F9 model system.

3.7 *RA induced the expression of NOX4*

Previous work from our lab has indicated that the G-protein signaling, specifically, Gα13 activity operates in concert with canonical Wnt signaling to induce PrE formation (Krawetz & Kelly, 2009). It is also known that Gα12/13 signaling in cardiomyocytes induces ROS to activate JNK and p38 signaling pathways (Nishida *et al.*, 2005) which incidentally contribute to the differentiation of F9 cells (Bikkavilli *et al.*, 2008a,b). According to Nishida *et al.* (2005), the aforementioned ROS originate from NADPH oxidase (NOX) enzymes, since DPI (a potent NOX inhibitor; O'Donnell *et al.*, 1993) effectively inhibited the activation of JNK and p38.

To test whether or not NOX enzymes are expressed in F9 cells, and more importantly, its regulation in response to RA, NOX isoforms 1-4 were investigated by RT-PCR. Interestingly, the *NOX4* amplicon first appeared 2 days following RA

Figure 3.6 ***Chemical activation of TCF-LEF transcription in F9 cells.*** Cells co-transfected with a SuperTOPflash TCF-LEF transcription reporter and selection vector pcDNA 3.1+ was exposed to G418 (800ug/ml) and selected for one week. Transfected cells were treated with DMSO (1 day), RA (0.1μM; 1day) or H_2O_2 (100μM; 1hr, 3hrs, 1 day, 2 days) or left untreated. Following treatment, cells were lysed and luciferase expression levels were assayed using a Thermo-Scientific Modulus II luminometer. Bars indicate averaged light units (LU) ± S.E.M. of TCF-LEF transcription activity from three independent experiments. Student's t-tests were performed comparing respective treatments against untreated controls. A significant difference ($P<0.05$) between treatment and control is denoted by an asterisk.


Transcription (LU)
0
2
4
6
8
10
12
14
16
18
Untreated
DMSO
RA
*
H_2O_2
1 hr
3 hr
1 day
*
2 days
*

treatment, which coincides with the prominent increase in ROS production described above. *NOX4* amplicons were also seen in cDNA samples from days 3 and 4, but not in untreated controls (Fig. 3.7). Another approach, using DPI to inhibit NOX activity was taken to corroborate these results. To test the hypothesis that NOX activity produces the ROS responsible for influencing the decision making process of F9 cells to form extraembryonic endoderm, cells treated with DPI should not exhibit molecular markers of differentiation. Results from TROMA-1 immunoblot analysis show that this was indeed the case, where treatment with DPI in the presence of RA reduced the levels of endo-A cytokeratin (Fig. 3.8). Thus, this data point to a hierarchy where RA increases Gα13 activity (Jho & Malbon, 1997; Krawetz & Kelly, 2006), leading to the up-regulation of NOX4, consequently producing the ROS that impacts on the canonical Wnt-β-catenin and JNK and p38 signaling pathways required for extraembryonic endoderm differentiation (Krawetz & Kelly 2008; Bikkavilli *et al.*, 2008a,b).

Figure 3.7 ***RA induces the expression of NOX4 mRNA.*** Total RNA was isolated from F9 cells treated with RA (0.1μM) for 1-4 days or from untreated cells grown for 4 days and reverse transcribed to cDNA. Results from the PCR analysis of these cDNAs with NOX4-specific primers show an amplicon of the expected size first appearing by day 2. Bands of similar size are also seen in the day 4 sample, and when the intensity is increased, in the day 3 sample. RA treatment had no effect on the constitutively expressed L14 gene which was used as PCR control.

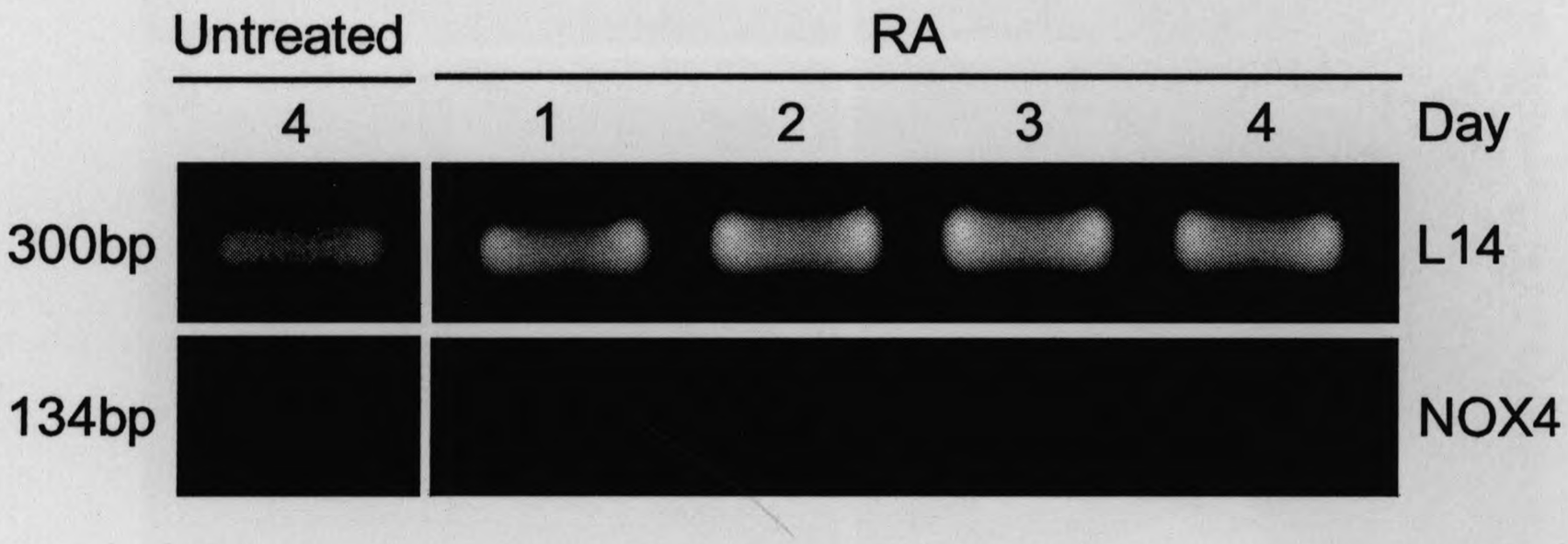
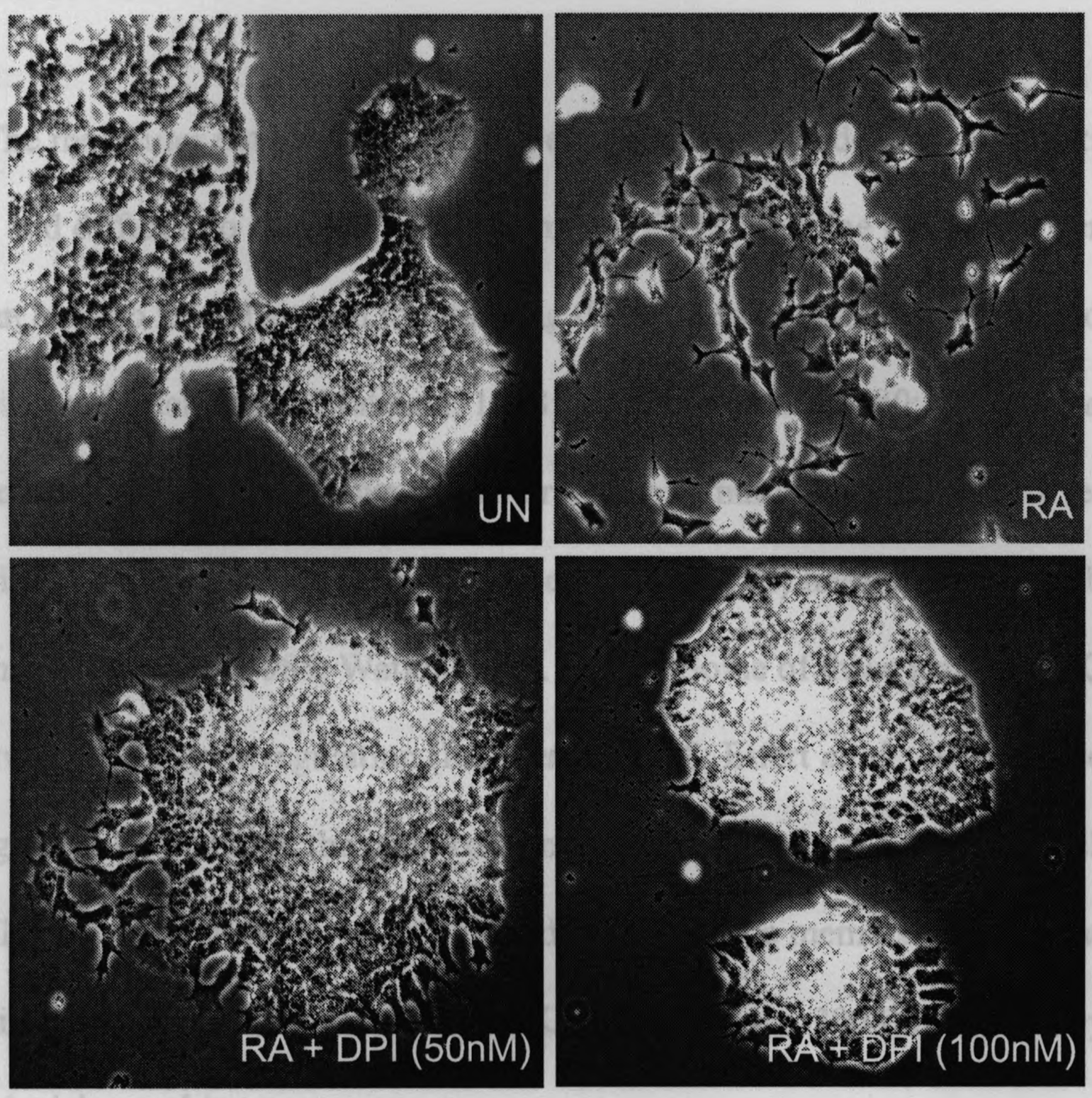
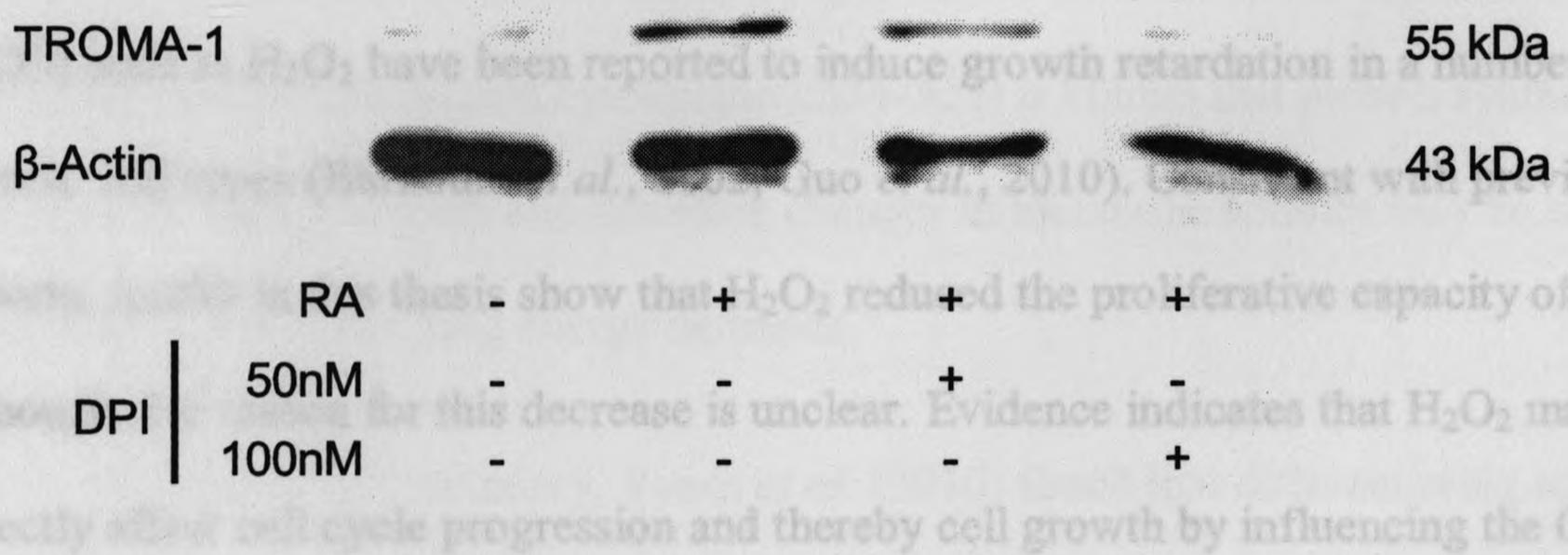

Untreated
RA
4
1
2
3
4
Day
300bp
L14
134bp
NOX4

Figure 3.8 ***NOX inhibition blocks RA-induced differentiation of F9 cells.*** Immunoblot analysis of endo-A cytokeratin expression in untreated cells, or after 3 days of treatment with either RA or RA plus DPI- (50 or 100nM). β-Actin was included as loading control. DPI, at both concentrations tested, was effective in reducing TROMA-1 expression.


UN
RA
RA + DPI (50nM)
RA + DPI (100nM)


TROMA-1
55 kDa
β-Actin
43 kDa
RA - + + +
DPI 50nM - - + -
DPI 100nM - - - +

Chapter 4 - Discussion

The molecular pathways underlying extraembryonic endoderm differentiation are complex and difficult to study *in vivo*. The mouse F9 EC cell line has been extensively used as an *in vitro* model system for studying the process of extraembryonic endoderm differentiation (Strickland & Mahdavi, 1978; Strickland *et al.*, 1980; Lehtonen *et al.*, 1989). Several studies have shown that RA regulates a number of genes involved in the differentiation of F9 cells into the primitive endoderm (PrE) lineage of extraembryonic endoderm (Wang *et al.*, 1985; Watkins *et al.*, 1992). Some of these genes, like Cyclin D3 are involved in cell cycle regulation (Faria *et al.*, 1998; Eifert *et al.*, 2006), those including Gelsolin that alter the cytoskeleton in preparation for the dramatic cell shape and motility changes (Eifert *et al.*, 2006) and others encode members of the Wnt family of signaling molecules (Eifert *et al.*, 2006; Krawetz & Kelly, 2008) that play key roles in cell fate decision making processes.

RA is known to induce the effect of growth retardation in several cell lines, including F9 cells (Liu *et al.*, 1996; Li *et al.*, 2004). Similarly, reactive oxygen species (ROS) such as H_2O_2 have been reported to induce growth retardation in a number of diverse cell types (Barnouin *et al.*, 2002; Guo *et al.*, 2010). Consistent with previous reports, results in this thesis show that H_2O_2 reduced the proliferative capacity of F9 cells, although, the reason for this decrease is unclear. Evidence indicates that H_2O_2 may directly affect cell cycle progression and thereby cell growth by influencing the Cyclin family of cell cycle regulators. Specifically, Barnouin and colleagues (2002) reported that

H_2O_2 induced a rapid decline in Cyclin D3 protein expression levels in mouse fibroblasts. Interestingly, Faria *et al.* (1998) reported that the onset of growth retardation following RA treatment of F9 cells also coincided with reduced levels of Cyclin D3 expression. Although, it is tempting to speculate that H_2O_2 directly regulates Cyclin D3 levels in the F9 system, further investigation is necessary confirm this speculation.

In contrast, analysis of proliferation using the WST-1 assay revealed a transient, but unexpected increase in WST-1 detection in RA treated cells, suggesting an increase in proliferation in response to RA. Although evidence in the literature does not support this claim (Linder *et al.*, 1981; Lehtonen *et al.*, 1989), thus, the exact cause of this increase is unclear. In principle, the WST-1 assay operates by the enzymatic cleavage of colourless tetrazolium salts, mainly by NAD(P)-dependent dehydrogenase into coloured products, by metabolically active cells (Wittstock *et al.*, 2001). This process is dependent on glycolytic activity which may reflect metabolic alterations rather than proliferation (Galluzzi *et al.*, 2009). Studies have shown that numerous genes are up-regulated in response to RA (Wang *et al.*, 1985; Liu *et al.*, 2000; Eifert *et al.*, 2006; Su & Gudas, 2008). Therefore, RA treatment is likely to enhance protein synthesis. For example, it was shown in this report that RA induces a prominent increase in the expression of intermediate filament protein cytokeratin endo-A. It is known that protein synthesis is an energy demanding process and therefore changes in metabolic activity may be necessary to accommodate increasing energy demands.

In support of this theory, Yanes *et al.* (2010) found that differentiating mouse embryonic stem cells (ESCs) have a different profile of metabolites than their

differentiated counterparts, indicating that metabolic changes accompany the process of differentiation. Taken together, I speculate that the transient increase in WST-1 processing is a reflection of metabolic changes rather than proliferation. Though, further investigation into the metabolic profile of F9 cells undergoing differentiation is necessary to confirm this speculation.

Additionally, Yanes *et al.* (2010) also noted a drop in antioxidant capacity during ESC differentiation, suggesting that intracellular redox status might play a role in mediating ESC fate. My data supports this contention as tipping the intracellular redox balance to an oxidative state by the exogenous addition of H_2O_2 induced the differentiation of F9 cells. The ability of ROS such as H_2O_2 to participate in physiological processes as second messengers has been well documented across multiple model systems both *in vitro* and *in vivo* (Sundaresan *et al.*, 1995; Yoon *et al.*, 2002; Radisky *et al.*, 2005; Niethammer *et al.*, 2009; Owusu-Ansah & Banerjee, 2009); yet, ROS signaling mechanisms have not been investigated in F9 cells. A growing body of evidence exists to implicate ROS as signaling molecules involved in the regulation of cell growth and differentiation (D'Autreaux & Toledano, 2007; Funato & Miki, 2010; Hernandez-Garcia *et al.*, 2010).

RA treatment in the present study induced a sustained increase in ROS in F9 cells which may reflect either an increase in ROS production and/or decrease in antioxidant capacity or perhaps both as the two events are not exclusive. Although intracellular antioxidant status was not investigated in my study, others have used the ratio of reduced (GSH) and oxidized (GSSG) glutathione to examine changes in intracellular redox status

during mouse ESC differentiation (Yanes *et al.*, 2010). GSH is a naturally produced intracellular antioxidant, mainly used as a cofactor during the reduction of H_2O_2 to H_2O by glutathione peroxidase. By using mass spectrometry to analyze the ratio of GSH/GSSG during ESC differentiation, Yanes *et al.* (2010) found that the redox status of differentiating cells shifted to an oxidative state indicated by a sharp and linear decline in GSH/GSSG ratio. They also reported that antioxidant supplementation delayed the onset of ESC differentiation. Given the fact that I also found antioxidants attenuated the ability of RA-treated F9 cells to differentiate, together, this suggests that redox status may play an integral role in the ability of a cell to differentiate under normal or pathological conditions.

As previously mentioned, it is well established that ROS such as H_2O_2 play distinct roles as second messengers involved in the activation and regulation of signaling pathways. Correspondingly, it was found that exogenous addition of H_2O_2 activated TCF-LEF-dependent transcription in F9 cells. Activation of the TCF-LEF transcription pathway, resulting from the upstream activation of the Wnt/β-catenin pathway (Logan & Nusse, 2004; Clevers, 2006; Gordon & Nusse, 2006), is an obligatory step required for the differentiation of F9 cells into PrE (Liu *et al.*, 2002). Previous work from our lab has shown that during PrE formation, Wnt6 is up-regulated in response to RA (Krawetz & Kelly, 2008). Treatment with Wnt6 conditioned media alone is sufficient to induce the canonical Wnt/β-catenin cascade leading to the appearance of intermediate filaments that accompany differentiation of F9 cells (Krawetz & Kelly, 2008).

The Wnt family of secreted glycoproteins plays essential roles in cell signaling during adult and embryonic development. In canonical Wnt signaling, Wnt associates with its low-density lipoprotein receptor-related protein and binds to Frizzled to activate downstream signaling effectors Disheveled (DVL) and Casein kinase I, which leads to the dissociation of the Axin, Adenomatous polyposis coli, and Glycogen synthase kinase 3β complex. Without the complex in place, β-catenin is not targeted for proteasome degradation, but instead becomes stabilized in the cytoplasm. Eventually, β-catenin translocates to the nucleus, where it binds to and activates the TCF-LEF transcriptional machinery to regulate numerous TCF-LEF-target genes (Logan & Nusse, 2004; Clevers, 2006; Gordon & Nusse, 2006).

Interestingly, Funato and colleagues (2006) reported that ROS signaling is also involved in modulating the Wnt/β-catenin signaling cascade. Nucleoredoxin (NRX) is a member of the thioredoxin family of evolutionarily conserved proteins, and is expressed in both embryonic and adult mouse tissue (Kurooka *et al.*, 1997). It is interesting to note that NRX is a known binding partner and negative regulator of DVL. Funato and colleagues (2006) showed that the association of NRX to DVL is inhibited by H_2O_2. Furthermore, exogenous addition of H_2O_2 activated TCF-LEF-dependent transcription independent of a Wnt ligand. These results indicate that ROS may promote a post-translational modification of NRX, in the same way that ROS can modulate thioredoxin, thereby leading to NRX's dissociation from DVL, ultimately allowing DVL to signal downstream effectors leading to TCF-LEF activation.

Although evidence points to ROS having a positive influence on canonical Wnt signaling, other reports have shown that H_2O_2 attenuates Wnt/β-catenin signaling through the down-regulation of β-catenin itself (Shin *et al.*, 2004). One thing, however, that should be noted from their report, is that no transfection controls were used and no statistical analysis was performed. Thus, a varied response in TCF-LEF activity could simply reflect variability in transfection efficiency, and although a drop in nuclear β-catenin levels was reported, its significance is inconclusive and questionable. Taken together, data presented in this report in conjunction with evidence from the literature strongly suggests that ROS converges on the Wnt/β-catenin signaling pathway that is required for the cell fate decisions leading to the differentiation of F9 cells.

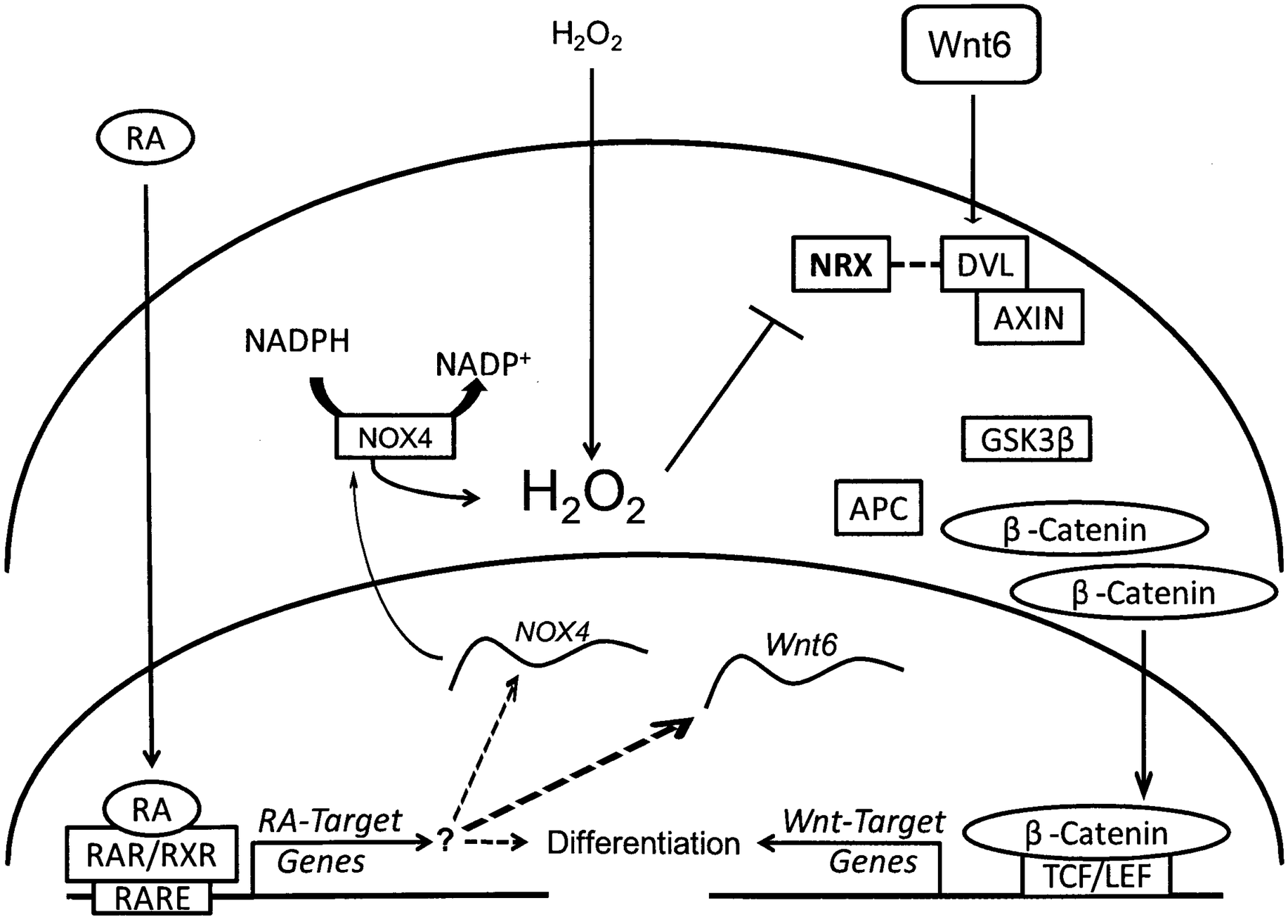
One caveat concerning the statement above pertains to the cause or source of ROS. In the case of human ESC, differentiation was reported to be accompanied by an increase in mitochondria (Cho *et al.*, 2006). Although mitochondrial density was never quantified in the present study, staining with MitoTracker Red (MTR) would suggest that ROS production from the mitochondria did not change in response to RA treatment. Additionally, although evidence exists to indicate that RA may be directly acting as an intracellular oxidant (Koga *et al.*, 1997; Conte da Frota *et al.*, 2006), the initial delay I noted in ROS detection in response to RA does not support this claim. Instead, I am in favour of the notion that RA induced changes in gene expression that subsequently led to the observed increase in ROS. In support of this theory, and in light of the profiling studies highlighting the extensive number of genes up-regulated in F9 cells in response to RA (Wang *et al.*, 1985; Liu *et al.*, 2000; Eifert *et al.*, 2006; Su & Gudas, 2008), a reported candidate (*NOX4),* became the focus of interest. However, it is not yet known

whether RA up-regulated *NOX4* transcription directly or indirectly in F9 cells. NADPH oxidases (NOX) are a family of enzymes that produce ROS by catalyzing the reduction of oxygen by using NADPH as its electron donor (Brown & Griendling, 2009). Unlike other NOX enzymes, which produce O_2^-, NOX4 produces H_2O_2 (Serrander *et al.*, 2007). This, together with the timing of *NOX4* expression in RA-treated F9 cells which correlated with the onset of ROS detection, suggests that NOX4 may be the source of ROS production required for differentiation. Additionally, NOX4 has been suggested to be involved in the regulation of the cytoskeleton and differentiation in various cell lines (Cucoranu *et al.*, 2005; Li *et al.*, 2006; Clempus *et al.,* 2007; Lyle *et al.,* 2009). Although it is not known if NOX4 is essential for the cytoskeletal remodeling of F9 cells undergoing differentiation, it is tempting to implicate it considering its role in multiple ROS-mediated signaling pathways (Mahadev *et al.*, 2004; Cucoranu *et al.*, 2005; Lyle *et al.*, 2009). Finally, if NOX-dependent production of ROS is key to the differentiation of extraembryonic endoderm then blocking its activity should have grave consequences. The NOX inhibitor DPI was used to test this hypothesis since it was shown previously to block RA-induced differentiation of P19 embryonal carcinoma cells (Konopka *et al.*, 2008). As expected, DPI attenuated the RA-induced differentiation of F9 cells, but because DPI is a general NOX inhibitor, further studies using NOX-specific siRNAs are required to confirm that NOX4 is the enzyme in question.

Overall, the present data provides compelling evidence that ROS signaling converges on the RA-induced activation of the Wnt pathway required to induce extraembryonic endoderm in F9 cells. With this information, I would like to suggest a model of F9 differentiation (Fig. 4.1) where RA induces the expression of NOX4, which

Figure 4.1 ***A model for the differentiation of F9 cells into extraembryonic endoderm.***

RA diffuses across the plasma membrane and is transported inside the nucleus where it binds to RA receptors (RAR/RXR) on RA response elements (RAREs) of target genes. Although RA induces the expression of *NOX4*, it is not known if it is activated directly or indirectly (question mark, dashed arrow). NOX4 produces intracellular H_2O_2, which blocks the association of NRX to DVL (Funato *et al.*, 2006), permitting the accumulation of β-catenin and subsequent activation of TCF/LEF-dependent transcription. Wnt6, another positive regulator of the β-catenin pathway, is also up-regulated in response to RA (Krawetz & Kelly, 2008). Together, H_2O_2 serves to intensify the Wnt6 signal and in concert, act to induce the differentiation of F9 cells into extraembryonic endoderm. Upper and lower crescents represent plasma and nuclear membranes respectively.


H_2O_2
Wnt6
RA
NRX
DVL
AXIN
NADPH
NADP+
NOX4
GSK3β
H_2O_2
APC
β -Catenin
β -Catenin
NOX4
Wnt6
RA
RAR/RXR
RARE
RA-Target
Genes
?
Differentiation
Wnt-Target
Genes
β -Catenin
TCF/LEF

consequently leads to the production of H_2O_2. Accumulation of intracellular H_2O_2 subsequently alleviates the negative inhibitory binding of NRX to DVL (Funato *et al.*, 2006). This derepression, together with the up-regulation of Wnt6, activates the canonical β-catenin signaling pathway required to stimulate TCF-LEF transcriptional activity needed to alter gene expression that precedes the induction of extraembryonic endoderm.

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