**University of Alberta**

# MECHANISM OF ACTION OF THE CDH-INDUCING TERATOGEN NITROFEN

by

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**Dedication**

To my family, who has always supported me in every possible way,

And Kelly Adams, who's encouragement has been my foundation.

## ABSTRACT

Congenital diaphragmatic hernia (CDH) is a life-threatening condition with an unknown etiology. The well-studied nitrofen model of CDH has many similarities to the human condition. **Objective:** Test hypotheses regarding the teratogenic action of nitrofen. **Results:** Nitrofen does not affect thyroid signaling, but does interfere with retinoid signaling leading to a decrease in retinoic acid (RA) levels. Retinal and vitamin A are able to rescue the nitrofen induced decrease in RA, however the antioxidants, vitamin C and E, cannot. Quantitative PCR shows that nitrofen does not decrease the expression of important retinoid signaling genes, Raldh2, CRABP II, RARα, RARβ and Cyp26A1. Immunohistochemical staining of E11.0 embryos from Wnt1-Cre/R26R-LacZ mutant mice demonstrates that the amuscular cells of the diaphragm are not of neural crest origin. **Summary:** Collectively, these data refute some longstanding hypotheses that required systematic testing and provide further impetus toward the focus on retinoid signaling and related developmental processes.

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## LIST OF ABBREVIATIONS

The following abbreviations, definitions and units have been used throughout the thesis.

| | |
|---|---|
| ADH | Alcohol Dehydrogenase |
| AF-1 | Activation Funtion-1 |
| ALDH | Aldehyde Dehydrogenase |
| β-gal | β-galactosidase |
| CDH | Congenital Diaphragmatic Hernia |
| COUP TF II | Chick Ovalbumin Upstream Promoter-Transcription Factors II |
| CRABP | Cellular Retinoic Acid Binding Protein |
| CRBP | Cellular Retinol Binding Protein |
| DIT | Diiodotyrosine |
| ECMO | Extracorporeal Membrane Oxygenation |
| HRE | Hormone Response Element |
| HFOV | High Frequency Oscillatory Ventilation |
| HGF/SF | Hepatocyte Growth Factor/Scatter Factor |
| iNO | inhaled Nitric Oxide |
| LBD | Ligand Binding Domain |
| LRAT | Lecithin:Retinol Acyltransferase |
| MIT | Monoiodotyrosine |
| NCAM | Neural Cell Adhesion Molecule |
| PPF | Pleuroperitoneal Fold |
| PPHT | Persistent Pulmonary Hypertension |
| PSA-NCAM | Polysialylated Neural Cell Adhesion Molecule |
| PTFE | Polytetraflurouethylene |
| Raldh | Retinal Dehydrogenase |
| RA | Retinoic Acid |
| RAR | Retinoic Acid Receptor |
| RARE | Retinoic Acid Response Element |

| | |
|---|---|
| RBP | Retinol Binding Protein |
| RXR | Retinoid X Receptor |
| SDR | Short-chain Dehydrogenase/Reducdase |
| TBG | Thyroxine-Binding Globulin |
| TBPA | Thyroxine-Binding Prealbumin |
| $T_3$ | Triiodothyronine |
| $T_4$ | Thyroxine |
| TG | Thyroglobulin |
| TR | Thyroid Receptor |
| TRE | Thyroid Response Element |
| TSH | Thyroid Stimulating Hormone |
| TTF-1 | Thyroid Transcription Factor-1 |
| TTR | Transthyretin |
| VAD | Vitamin A Deficient |
| WT1 | Wilms' Tumor 1 |

# CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

## *1.1 Objectives*

The main objective of this work was to test various hypotheses regarding the actions of the teratogen nitrofen, which induces the most commonly used model of congenital diaphragmatic hernia (CDH). It has been hypothesized that the teratogenic effects seen with this model of CDH are due to: i) nitrofen acting as a thyromimetic; ii) nitrofen's oxidizing ability; iii) nitrofen interfering with retinoids or their downstream targets. It has also been suggested that the affected amuscular diaphragm cells may be of neural crest cell origin. The work in this thesis provides a systematic examination of these issues.

Chapter 1 provides a general introduction to the topics of diaphragm development, the clinical aspects and animal models of CDH, and both retinoid and thyroid signaling. This provides the basis for the research and supports the notion that understanding the actions of nitrofen will guide us towards an improved knowledge of the pathogenesis and/or etiology of the human condition.

Chapter 2 outlines the methods used during individual experiments, while Chapter 3 presents the results of my studies. Chapter 4 addresses the major points for discussion and the conclusions that can be drawn from my work as a whole.

# INTRODUCTION

## *1.2 Diaphragm Embryogenesis*

Traditional theories of the development of the diaphragm came from extrapolations based on anatomical studies. In recent years, molecular and advanced anatomical techniques have been able to dispel dogmas and bring to light a more accurate picture of the embryogenesis of the diaphragm.

There are two main stages of development of the diaphragm, the formation of the primordial diaphragm, the pleuroperitoneal fold (PPF), and the expansion of the PPF and associated cells to form the diaphragm. In the first stage, phrenic nerve axons and muscle precursor cells migrate to the PPF. Both the phrenic and brachial cervical axons begin to emerge from the spinal cord on E11 and reach the brachial plexus by about E12.5. The phrenic nerve then defasiculates from the brachial nerve and begins to travel ventrally towards the PPF using pioneering axons as guides. This process of phrenic axon separation is in part mediated by the expression of the polysialylated form of neural cell adhesion molecule (NCAM) that acts as an anti-adhesive. The identity of other attractive and repellent molecules guiding phrenic axon elongation remains unknown. The migration of phrenic axons to the PPF is completed by E13 (Allan and Greer, 1997). The population of muscle precursor cells that migrate to the PPF during the first stage were initially thought to be derived from adjacent populations of cells including the lateral body wall, septum transversun and/or esophageal mesenchyme. In reality, diaphragmatic muscle cells are derived from the lateral dermamyotome of cervical somites (Babiuk, et al., 2003). These muscle precursor cells express the receptor c-met, which they use to follow a path of hepatocyte growth factor/scatter factor (HGF/SF)

signals to the PPF, where they coalesce. Undoubtedly, there are further, as yet unidentified, guidance mechanisms controlling myogenic cell migration to the PPF. At E13, when both the phrenic axons and muscle precursor cells have reached the PPF, the triangular shaped tissue extends medially from the body wall, and fuses ventrally with the septum transversum.

During the second stage of diaphragm development, muscle precursor cells and phrenic axons branch out from the PPF to populate the dorsolateral costal, sternal costal and crural regions of the developing diaphragm. This occurs between E13.5 and E17.5 (Babiuk, et al., 2003). Within the diaphragm, there is a spatiotemporal correlation between nerve branching and the distribution of myoblasts and myotubes, suggesting a connection between their respective migration and development (Bennett and Pettigrew, 1974; Harris, 1981). This is also suggested by the lack of phrenic nerve migration within the amuscular diaphragm of c-met null mice. In fact, immunohistochemical images show that there is a radiation of myotube elongation from the point of the nerve innervation towards the central tendon and the lateral edges of the diaphragm (Greer, et al., 1999).

At E17.5, the pattern of innervation and muscle architecture has formed a functional diaphragm. At this gestational age the cervical dorsal root ganglia have also reached the phrenic motoneuron dendrites. This coincides with the inception of the respiratory drive transmission in utero, which can be seen as early as E17. Post E17, there is increasing maturation of muscle contractile properties and neuromuscular transmission (Martin-Caraballo and Greer, 2000). After birth, the phrenic nerve retracts some of its polysynaptic neuromuscular terminals and the diaphragm progressively matures (Prakash et al., 2000). The fully developed and functioning diaphragm muscle is

essential for breathing and contracts in response to signals from the phrenic nerve. The diaphragm also separates the thoracic and abdominal cavities, which is vital for breathing and proper lung development. If the development of this muscle is abnormal it can result in a condition known as congenital diaphragmatic hernia (CDH).

## *1.3 Congenital Diaphragmatic Hernia*

### *1.3.1 Pathophysiology of CDH*

Congenital Diaphragmatic Hernia (CDH) was first described by Riverius over 400 years ago, however it wasn't until almost 200 years later that an anatomist from Prague, Bochdalek, described the form of CDH which still bears his name (Puri and Wester, 1997). Although the Bochdalek type hernia occurs in approximately 95% of CDH cases, there are three other types that exist (Torfs, et al., 1992): the Morgagni hernia, eventration of the diaphragm and the central tendon or septum transversum hernia.

All forms of CDH, except eventration, are characterized by a hole in the diaphragm. In the Bochdalek type hernia the defect is located in the posteriorlateral aspect of the diaphragm. It is more commonly found in the left hemidiaphragm (80-95%) than on the right (10-15%) and in rare occasions (~1%) the defect is bilateral (Butler and Claireaux, 1962; Skandalakis, 1994). The size of the missing diaphragm can vary from a few inches to an entire side. Alternatively, the Morgagni type of hernia describes the condition where the parasternal aspect of the diaphragm is missing. This type of CDH occurs in about 1-2% of infants born with CDH (Carter et al., 1962; Stokes, 1991). Even less common is the central tendon hernia. In this type of defect there is an opening in the medial part of the diaphragm, known as the central tendon, and is thought to occur as a secondary rupture of the septum transversum during development

(Wesselhoeft and De Luca, 1984). In these three types of CDH, the diaphragm does not form a complete barrier between the abdominal and thoracic cavities, therefore abdominal contents are able to protrude into the thoracic cavity and impair lung growth and development. In eventration of the diaphragm, pressure is exerted on the developing lungs by the extension of the diaphragm and abdominal viscera creating a balloon that expands into the thoracic cavity. This type of hernia may be due to a thinner than normal muscle or to the complete lack of muscle cells on the affected hemidiaphragm (Skandalakis et al., 1994). Although the 4 types of CDH differ in location and incidence, all types are life threatening and occur in approximately 1 in 3000 live births (Harrison et al., 1995). These facts demonstrate the importance of the investigation of this congenital abnormality.

### *1.3.2 Diagnosis and Treatment of CDH*

On average, 50% of cases of CDH can be detected during pregnancy using ultrasound or radiograph between weeks 16 and 24 of gestation (Lewis et al., 1997). Prenatal diagnosis corresponds to a decreased chance of survival after birth (Stege et al, 2003; Colvin et al, 2005), because larger herniations, related to an increased risk, are more frequently and more easily detected. In cases where prenatal diagnosis did not occur, CDH is suggested by respiratory distress and persistent pulmonary hypertension (PPHT) soon after birth.

At birth, the main concern for the CDH patient is pulmonary hypertension, due to pulmonary hypoplasia. The combination of hypertension and hypoplasia, as well as other possible congenital abnormalities, makes the treatment of CDH more complex than for pulmonary hypertension alone. Many strategies developed for treating infants born with

pulmonary hypertension are employed to treat CDH patients, however they fail to show benefit for this particular treatment group.

In the 1980's and 1990's, a number of new treatments became available for the CDH patient. These therapies include extracorporeal membrane oxygenation (ECMO), high frequency oscillatory ventilation (HFOV), inhaled nitric oxide (iNO), exogenous surfactant and permissive hypercapnia. Case reports and retrospective reviews provide debate over which methods are beneficial, and which may possibly be harmful. In particular, the use of exogenous surfactant has not been shown to improve survival (Van Meurs, 2004), and HFOV has actually been shown to worsen survival due to increased barotrauma (Kays et al., 1999). Barotrauma increases the risk of mortality for CDH patients, as it decreases the limited amount of mature lung tissue. In order to limit barotruama, clinicians employ gentle ventilation and permissive hypercapnia. In recent years, the most widely used ventilation goal has become permissive hypercapnia, while the use of hyperoxia and induced alkalosis as a management strategies have been reduced (Lally, et al., 2006). The utilization of ECMO has not been shown to increase survival rates and has also been declining in recent years (Lally, et al., 2006; Khan, and Lally, 2005). However it may still be beneficial in instances where there are associated cardiovascular abnormalities. Alternatively, iNO has seen an increase is use, although it too has not been shown to be beneficial in trials (Finer, and Barrington, 2006).

Recent treatment strategies also include in utero surgery in order to either repair the diaphragm or induce lung growth. Repairing the diaphragm in utero prevents future interference of the viscera on lung development and provides a permissive environment for accelerated lung development before birth. In utero, diaphragm repair also removes

the requirement for surgery at birth in cases of isolated herniation. However, neither randomized control nor retrospective studies of complete in utero diaphragm repair have been shown to improve outcomes (Harrison et al., 1993). The other technique, tracheal occlusion, obstructs the normal egress of lung fluid and stimulates lung growth (Deprest, et al., 2004; Deprest, et al., 1997). It was originally preformed using an external clip, but the use of an inflatable balloon has now become more common (Harrison et al., 2003). In the normal fetus, the lungs produce a fluid that flows outward through the upper airway and into the amniotic fluid. Occluding the trachea prevents the egress of the fetal lung fluid and causes the lungs to expand in order to accommodate the increase in volume. This lung growth can then push invading abdominal contents back into the abdomen. Although there was initial evidence to suggest that tracheal occlusion may be effective, this technique has not been shown to improve survival over standard strategies (Keller et al., 2004).

The treatment strategy for the diaphragmatic defect has moved towards delayed surgical repair. This permits full assessment and stabilization of the patient before the stress of surgery is undertaken. In most cases, a primary repair (using the remaining part of the affected side of the diaphragm) is sufficient to close the defect, however in cases where there is little to no diaphragm left, a patch is used. The most common patch material is polytetraflurouethylene (PTFE), a non absorbable synthetic, while other possible patch material includes Gortex, Dexon, or biological material such as another muscle or lypopylized dura (Clark et al., 1998; Sydorak, et al., 2003; Smith, et al., 2004). Although these methods succeed in separating the thoracic and abdominal cavities, reherniation occurs in 50% of cases when a prosthetic patch is used (Moss, et al., 2001;

Hajer, et al., 1998). Associated issues with the use of a prosthetic patch include the fact that it does not grow with the child and that the area does not become innervated by the phrenic nerve or populated by muscle cells. The only exception are a few reported cases of successful phrenic nerve activity in the ipsilateral hemidiaphragm in which the reverse latissimus dorsi muscle flap was used for repair (Sydorak, et al., 2003).

With an improvement in reported mortality rates there is subsequently an increasing need to address associated long-term morbidity in this patient group. Patients with CDH suffer from a long list of associated problems including a failure to thrive, increased caloric requirements, gastroesophageal reflux, increased work required to breath, impaired diaphragm motility, thoracic scoliosis and recurrence of the hernia (Arena, et al., 2005; Vanamo, et al., 1996; Muratore, et al., 2001; Hajer, et al., 1998). All of these issues warrant increased consideration and study.

Treatment strategies for CDH are complex and expensive. Improving the understanding of the etiology and pathogenesis of CDH will be important to develop new methods of early diagnosis and prevention.

### *1.3.3 Genetics/Associated Malformations*

Although some cases of CDH are genetically linked, the majority are idiopathic. Reports on gender differences vary between studies, with earlier studies suggesting a greater number of females and later studies suggesting a greater number of males are affected (Butler and Claireaux, 1962; David, 1976; Robert et al. 1997; Benjamin et al., 1988). However, regardless of gender, CDH is associated with other malformations with a frequency of 40-50% (Fauza and Wilson, 1994; Torfs et al., 1992). While cardiovascular anomalies are the most common (Greenwood et al., 1976; Migliazza et al., 1999), other

frequently occurring malformations include craniofacial, central nervous system, gastrointestinal and urogenital (Bedoyan et al., 2004; Harmath, et al., 2006).

Approximately 9-15% of isolated cases of CDH are associated with chromosomal abnormalities. This percentage increases when CDH is present with another congenital defect (Bollmann et al., 1995; Enns et al., 1998; Witters et al., 2001). Some common chromosomal trisomies and congenital syndromes associated with CDH include trisomy 18, trisomy 21, trisomy 13, Turner syndrome (XO), Fryns syndrome and DiGeorge syndrome (Tibboel, and Gaag, 1996). Reports of CDH have been associated with all chromosomes (Lurie, 2003) however, there is growing evidence that the long arm of chromosome 15 may play a crucial role in the development of CDH and some of its associated abnormalities (Biggio, et al., 2004; Klaassens et al., 2005; Castiglia et al., 2005). Although there have been genetic links with CDH, familial cases are rare, with an incidence of no more than 2% (Czeizel and Kovacs, 1985; Lipson and Williams, 1985; Torfs et al., 1992). A multifactorial mode of inheritance of familial CDH, as suggested by Wolff in 1980, is most likely, as it is the only theory that can account for the variations found among cases. There is no clear environmental or common genetic connection between all cases of CDH. Therefore uncovering its developmental origin has been difficult.

### *1.3.4 Animal Models of CDH*

The etiology and pathogenesis of CDH is unknown, therefore to study these aspects models of the diseases are necessary. The most commonly used models of CDH are the lamb and rabbit surgical models, as well as the teratogen induced rodent model. These paradigms are useful to study different aspects of CDH. The lamb and rabbit models are

utilized primarily to examine potential treatments for fetuses and newborns with CDH and to investigate the effect of diaphragm herniation and protruding viscera on lung development. The teratogen model is employed to develop an understanding of the etiology and pathogenesis of CDH. Other rodent models include the vitamin A deficient (VAD) rat model, and more recently, mutant mouse models (discussed below).

In the lamb model of CDH, a diaphragmatic defect is surgically produced on day 90 of gestation, with term being 145 days. This results in delayed lung development and hypoplasia, but lacks the invading viscera common in human cases (deLorimier et al., 1967; Kent et al., 1972). Therefore, a second lamb model was developed in order to stimulate lung compression due to pressure from the viscera. In this case a conical silicon rubber balloon is gradually inflated within the thoracic cavity beginning at day 100 of gestation (Harrison et al., 1980). The similarities between this model and the anatomical and clinical defects seen in human infants with CDH indicate that it is a good model to study these aspects of CDH. In fact, due to the large size of the animal, these techniques are particularly useful in examining treatments that may improve lung growth during fetal development, such as tracheal occlusion and prenatal surgical treatments, while providing physiological measurements of these treatments (Hellmeyer et al., 2006). However, there are a number of disadvantages including, the high cost of animals, the long gestational time, and the fact that it is an isolated, artificial condition created relatively late in gestation, unlike the human condition. In order to address some of these issues, the rabbit model can be used. Similar to the lamb model, a hernia is surgically induced on embryonic day 24, with term being 31 days (Fauza et al., 1994). Advantages include larger litters and a relatively short gestation time, however the

smaller size makes surgery difficult. While this model does result in lung hypoplasia, other associated malformations do not occur, and it, as well as the lamb model, cannot be used to study the pathogenesis of CDH.

The rodent model most often used to study CDH is induced by the teratogen nitrofen. Toxicology studies of this herbicide demonstrated that giving a single dose of nitrofen to pregnant dams between embryonic day eight (E8) and twelve (E12) produce offspring with malformed diaphragms similar in size and location to the human condition. The associated defects observed also parallel those seen in the human pathology, including the protrusion of viscera into the thoracic cavity, lung hypoplasia and various organ abnormalities (Losty et al., 1999; Iritani, 1984; Tenbrinck et al., 1990; Kluth et al., 1990). The incidence of hernias in Sprague-Dawley rats with nitrofen administered between E8-10 of gestation is approximately 45%, with the majority of hernias occurring on the left side. Occasionally hernias are seen on the right side or bilaterally, again similar to the human condition (Costlow and Manson, 1981; Kluth et al., 1990; Allan and Greer, 1997, Francis, et al., 1999). Interestingly, nitrofen treatment on E8 produces a majority of left-sided hernias, while treatment on E11 produces preferentially right-sided defects. This could be due to differences in expression of left-right patterning genes during development. In CD1 mice the incidence of hernias is 25-30% when nitrofen treatment is given on E8 (Wickman et al., 1993; Cilley et al., 1997). Different rat and mouse strains require different amounts of nitrofen or a combination of nitrofen and other CDH inducing teratogens to induce hernias (Burke-Hurt et al., 1983; Babiuk and Greer, 2002). This strain specific difference suggests that each strain has a different susceptibility to the teratogens effects and provides strength to the proposed

hypothesis that development of CDH is due to the interplay of a number of genetic factors as oppose to single gene abnormalities.

One clue to factors that may play a role in the pathogenesis of CDH came during vitamin A deficiency (VAD) studies. Offspring born to VAD dams had a number of abnormalities, including malformations of the eye, genito-urinary tract, the heart and major arteries and, importantly, diaphragmatic hernia (Wilson et al., 1953). This model of CDH has been developed in recent years and is the only known dietary model of CDH. Other important rodent models have arisen from gene knockout studies. Bochdalek hernias are observed in mice with double knockout RARα/RARβ (Mendelsohn et al., 1994; Lohnes et al., 1995) and MyoR/Capsulin (Lu et al., 2002), as well as single knockout Wilms' Tumor 1 (WT1) (Kreidberg et al., 1993) and COUP TFII inactivated in Nkx3-2-expressing tissue (You et al., 2005). All of these genes are directly involved in transcription and are active during development.

### *1.4 Pathogenesis of Nitrofen Induced CDH*

The nitrofen model of CDH has been used extensively in an attempt to identify the pathogenesis of CDH. From these studies a number of lessons have been learned. First, nitrofen causes a diaphragmatic defect independent of both lung formation and myogenesis (Babiuk and Greer, 2002). This indicates that nitrofen perturbs the development of the amuscular cells that populate the diaphragm and that this effect is not due to abnormally developing lungs. It is generally considered that the invading viscera are responsible for the lung hypoplasia associated with CDH. However, nitrofen may also be acting directly on the developing lungs, as lung hypoplasia is seen with nitrofen treatment, without the presence of a diaphragmatic hernia. The majority of

associated malformations in both the nitrofen induced and human cases of CDH are linked by their neural crest (NC) cell origin. It has therefore been hypothesized that the amuscular cells of the diaphragm are also NC cell derived.

The primary defect caused by nitrofen in the development of CDH is the diaphragmatic hernia. In fact, the abnormal development of the diaphragm can be traced back to an earlier stage of growth. Careful analysis shows that there is a defect in the posterolateral area of the PPF in three separate models of CDH (Greer, et al., 2000; Clugston, et al., 2006). The abnormal PPF common to the teratogen induced, VAD, and WT1 models of CDH provides evidence that all three models share the same pathogenesis. A common point of origin implies that valid hypotheses with respect to the pathogenesis of human CDH can be drawn based on rodent models. This suggests that studying the actions of nitrofen is relevant to understanding the human pathogenesis.

### *1.4.1 Teratogenic Effects of Nitrofen*

There have been a number of different hypotheses put forward as to the mechanism of action of nitrofen. One hypothesis is that nitrofen may be acting as the thyromimetic, due to a common stereochemical structure with the thyroid hormones ($T_4$ and $T_3$). Nitrofen treatment of pregnant dams causes a decrease in $T_4$ and TSH serum levels in the dam and a decrease in fetal $T_4$ levels at term in their offspring. Co-administration of $T_4$ reduced nitrofen mediated malformations, other than a herniated diaphragm, by 70% (Manson, et al., 1984). At high levels, nitrofen decreases binding of $T_3$ to both the alpha and beta thyroid hormone receptors (TR) via a non-competitive interaction (Brandsma, et al., 1994). Alternatively, at low doses, nitrofen significantly increases thyroid receptor hormone activity in HeLa cells transfected with the human TR$\alpha$1 (Yamada-Okabe, et

al., 2005). It remains unclear what role, if any, thyroid signaling has in the pathogenesis of nitrofen induced CDH.

An alternative hypothesis based on the oxidizing properties of nitrofen has recently been given attention. Prenatal treatment of rats with doses of nitrofen plus an antioxidant vitamin, such as A, C and E, partially rescues nitrofen-induced heart defects and lung hypoplasia. As well, these vitamins rescue the decrease in cell proliferation normally associated with nitrofen induced CDH (Gonzalez-Reyes, et al., 2005; Gonzalez-Reyes, et al., 2003). Nitrofen treated cultured human pneumocytes show a rescue of the expression of genes involved in lung development, such as thyroid transcription factor 1 (TTF-1) and surfactant protein B (SP-B) when antioxidants were co-administered. Antioxidants are also able to normalize the levels of proliferation in the cultured human pneumocyte cell line H441, decreased by nitrofen treatment (Gonzalez-Reyes, et al., 2006).

Finally, the most widely accepted theory to explain the teratogenic effects of nitrofen is the Retinoid Hypothesis. This hypothesis suggests that abnormalities in retinoid signaling, or in factors influenced by retinoids, lead to the development of the primary defect in CDH (Babiuk and Greer, 2002; Greer, et al., 2003). Both vitamin A and its functional derivative, retinoic acid (RA), reduce the number of embryos with CDH from dams treated with nitrofen (Thebaud et al., 1999; Thebaud, et al., 2001; Babiuk et al., 2004). As well, mice with LacZ expression regulated by RA levels show a decrease in β-galactosidase staining after exposure to nitrofen. This effect is reversed by RA treatment (Chen et al., 2003). In vitro experiments have deciphered one point in the retinoid signaling pathway that is affected by nitrofen, and three other CDH inducing

teratogens. Specifically, these teratogens are able to interfere with the enzymatic activity of Retinal Dehydrogenase 2 (Raldh2), the enzyme responsible for the generation of RA from Retinal (discussed below). Importantly, Raldh2 is present within the PPF (Mey et al., 2003). The VAD and RARα/RARβ knock out models of CDH also provide strong support for the role of retinoids in the pathogenic process of CDH. There is additional evidence for a role for retinoids in human CDH cases. In a small study of infants born with CDH and their mothers, levels of vitamin A (retinol) and retinol binding protein (RBP) were measured. The results showed that both umbilical cord blood retinol and RBP levels in CDH patients is about 50% those of normal patients, whereas maternal retinol and RBP levels are increased in mothers of CDH babies (Major, et al., 1998). This suggests that there may be an impairment of the transfer of retinol from mother to child.

There are a number of different hypotheses regarding the mechanism of action of nitrofen. The relevance of the nitrofen model to the human condition provides more than adequate reason to explore these varying hypotheses.

## *1.5 Retinoid and Thyroid Signaling*

### *1.5.1 Retinoids*

Retinol is a fat soluble vitamin that is essential for skeletal growth, vision, normal reproductive function and the maintenance of epithelial tissue. It is also of critical importance to the development of organisms. The effects of retinol are almost exclusively due to its main functional derivative, retinoic acid (RA). During development RA has many vital roles and even small perturbations in RA levels can lead to severe developmental abnormalities.

Vitamin A is found in the diet in two principle precursor forms, as fatty acid retinyl esters, and as certain carotenoids, known as provitamin A. These molecules are found in milk, eggs, fish oil and butter, and in leafy green vegetables and brightly yellow coloured fruits and vegetables, respectively (Fraser and Bramley, 2004; Harrison, 2005). The form of carotenoid found in fruits and vegetables is β-carotene and is converted to retinol when the body is low in vitamin A.

About 70%-90% of vitamin A from the diets is absorbed in the intestine in a process that requires bile salts, pancreatic enzymes and dietary fat. Inside the intestinal lumen, retinyl esters and provitamin A are broken down into retinal, which is then reduced to retinol by retinal reductases (Li and Tso, 2003). Retinol is packaged into micelles, which are then absorbed by the enterocytes of the intestine, where they are bound to cellular retinol binding protein II (CRBP-II) (Crow and Ong, 1985). Inside the enterocytes retinol is converted into retinyl esters by lecithin:retinol acyl-transfease (LRAT) (Herr and Ong, 1992). The retinyl esters are then packaged into chylomicrons (Blomhoff et al., 1982) and delivered to the lymphatic system for transport (Blomhoff et al., 1990). The chylomicrons enter circulation via the thoracic duct, and while circulating, lipoprotein lipase cause the chylomicrons to release triglycerides leaving chylomicron remnants, which still contain almost all the original retinyl ester (Blomhoff et al., 1990). These remnants travel to the liver and are taken up by the parenchymal cells (Blomhoff et al., 1982) where the retinyl esters are converted back into retinol. The parenchymal cells are also a major site of retinol binding protein (RBP) production. The binding of retinol to RBP initiates the secretion of this complex into the plasma (Ronne et al., 1983). This complex is also involved in the transport of retinol to stellate cells for

storage. In these cells retinol is again esterified and stored with droplets of lipid until required by the body. Approximately 50-80% of the bodies total vitamin A content is stored in the stellate cells of the liver (Senoo, 2004; Blomhoff et al., 1985). A small portion of retinyl esters are also stored in adipose cells in other tissues throughout the body, including the lungs, kidneys and intestine (Nagy et al., 1997; Blomhoff and Wake, 1991). When the body needs vitamin A, it is removed from storage and converted to retinol. Retinol then binds to RBP (Kanai et al., 1968) and about 95% of this vitamin A-RBP complex also binds to transthyretin (TTR) (Peterson, 1971). The function of RBP is to protect retinol and reduce the cellular up-take of retinol, in order to prevent toxicity (Zanotti and Berni, 2004). The addition of TTR to this complex prevents retinol from being lost by excretion due to glomerular filtration (Peterson, 1971).

Once retinol has reached its target tissue, it is again bound to CRBP in the cytosol of the target cell. Retinol undergoes a reversible conversion to retinal by the oxidizing action of members of either the alcohol dehydrogenase (ADH) or short-chain dehydrogenase/reductase (SDR) enzyme families. Aldehyde dehydrogenase (ALDH), also known as retinal dehydrogenase (Raldh), enzymes are responsible for the rate limiting, nonreversible oxidation of retinal to RA. Retinoic acid is the main functional derivative of retinol. It is found in the cell bound to one of two cellular retinoic acid binding proteins (CRABP I or CRABP II). This complex prevents RA from binding to its nuclear receptors and promotes its catabolism via the Cyp26 enzymes, Cyp26A1, Cyp26B1 and Cyp26C1 (Boylan and Gudas, 1991; Boylan and Gudas, 1992; Swindell et al., 1999; White et al., 2000; Taimi et al., 2004). These enzymes belong to the cytochrome P450 family of enzymes and act to break down RA into polar metabolites

that can be further conjugated to form water-soluble molecules able to be excreted. In order to regulate levels of exogenous RA, the expression of many of the proteins involved in retinoid processing are dependent on the amount of RA within the cell. This forms an autoregulatory feedback loop that tightly controls RA levels.

### *1.5.2 Thyroid Hormone*

Thyroid hormone has a number of effects in the human body including increasing cardiac output, heart rate, ventilation rate, and metabolic rate. It can also induce thickening of the endometrium and is involved in a number of developmental processes including the development of the brain (Bernal, 2005).

The thyroid hormones, thyroxine ($T_4$) and triiodothyronine ($T_3$) are produced by the follicular cells of the thyroid gland. Both of these hormones are formed by attaching iodine atoms to the ring structure of tyrosine molecules. Iodine is actively absorbed from the bloodstream by the thyroid gland and sequestered in the follicular cells. There iodine is captured by either free tyrosine or the tyrosine residues of the protein thyroglobulin (TG). Hydrogen peroxide, generated by the enzyme thyroid peroxidase, is linked to the 3' and 5' sites of the benzene ring of the tyrosine residue and captures free iodine via the enzyme thyroperoxidase. This reaction forms monoiodotyrosine (MIT) and diiodotyrosine (DIT). Linking two moieties of DIT produces thyroxine, while linking one MIT and one DIT produces triiodothyronine. The production of thyroid hormone is regulated by thyroid-stimulating hormone (TSH), which is released by the anterior pituitary. These two hormones act in a negative feedback loop, where TSH production suppresses $T_4$ and vice versa.

The thyroid hormone secreted by the thyroid gland is 90% $T_4$ and 10% $T_3$. Almost all thyroid hormone circulates bound to one of three proteins, thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) or albumin (Davis et al., 1970). The protein TBPA is also known as TTR, which also binds the circulating retinol-RBP complex. When thyroid hormone is bound to one of its binding proteins it is not active. When unbound, thyroxine enters cells and is converted to the active $T_3$ by deiodinases.

### *1.5.3 Nuclear Receptors*

Both RA and $T_3$ are ligands for receptors that are members of the steroid/hormone receptor superfamily, which also includes receptors for various steroids as well as vitamin D. These receptors activate or repress the transcription of specific genes by binding as dimers to small cis-linked DNA sequences termed hormone response elements (HRE). These HRE are composed of direct or inverted repeats of a hexanucleotide consensus sequence (Glass, 1994; Leid et al., 1992). Receptors in this superfamily have common functional domains. The N-terminus is composed of the autonomous activation function (AF-1) in the A/B region, which is highly variable. Another functional domain is the DNA binding domain, which is made up of two zinc fingers, is highly conserved within each group of nuclear receptors (Berg, 1989; Klug and Schwabe, 1995). The C-terminal half of the receptor contains the ligand-binding domain (LBD), which ensures the specificity of the response to the appropriate ligand.

The nuclear receptors can be broadly divided into four classes (Stunnenberg, 1993; Mangelsdorf et al., 1995). Class I receptors function as ligand induced homodimers and bind DNA half-sites as inverted repeats. Class II receptors heterodimerize with retinoic X receptors (RXR) and bind direct repeats. Class III

receptors bind primarily to direct repeats as homodimers, while Class IV receptors bind the core sites as monomers. Both RA and $T_3$ receptors, retinoic acid receptors (RARα, RARβ, RARγ) and thyroid hormone receptors (TRα, TRβ) belong to Class II and bind to their respective HRE, retinoic acid response element (RARE) and thyroid response element (TRE), as heterodimers with RXR (Force et al., 1994; Mangelsdorf and Evans, 1995). In order to activate the nuclear receptors, ligands bind to their appropriate LBD. In the majority of cases, this binding causes a conformational change in the receptor, which allows it to release co-repressor proteins and to bind transcriptionally permissive ligand-dependent co-activator proteins (Horwitz et al., 1996). These co-activator proteins bridge the nuclear receptor and the transcription initiation apparatus and allow transcription to proceed (Tjian and Maniatis, 1994; Roeder, 1996). In other instances, ligand binding can be inhibitory towards transcription, depending on the nuclear receptors present, as well as the nucleotides of the HRE, however, this situation is less well studied.

Both retinoic acid and thyroid hormone are able to regulate the transcription of many genes important during development. It is the action of the CDH-inducing teratogen nitrofen in relation to these signaling pathways that is the focus of my work.

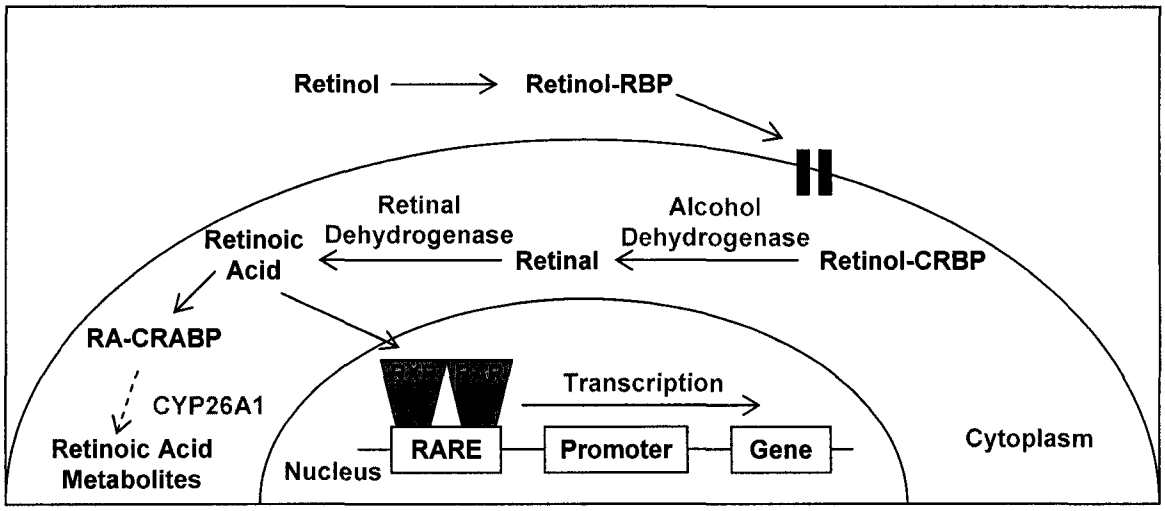



**Figure 1.1: Simplified Overview of the Retinoid Signaling Pathway**

Retinol is taken-up by the cell and is internally bound to CRBP. Within the cytoplasm alcohol dehydrogenases converts retinol into retinal in a reversible reaction. Retinal dehydrogenase then produces retinoic acid from retinal in a nonreversible, rate limiting enzymatic reaction. Retinoic acid can either translocate to the nucleus and bind to its nuclear receptor (RAR), or can remain in the cytoplasm bound to CRABP. In the nucleus, RAR heterodimerizes with RXR and bind to RARE upstream of the promoter region of a given gene, and initiates transcription. In the cytoplasm, Cyp26 enzymes preferentially break down RA bound to CRABP into polar metabolites.

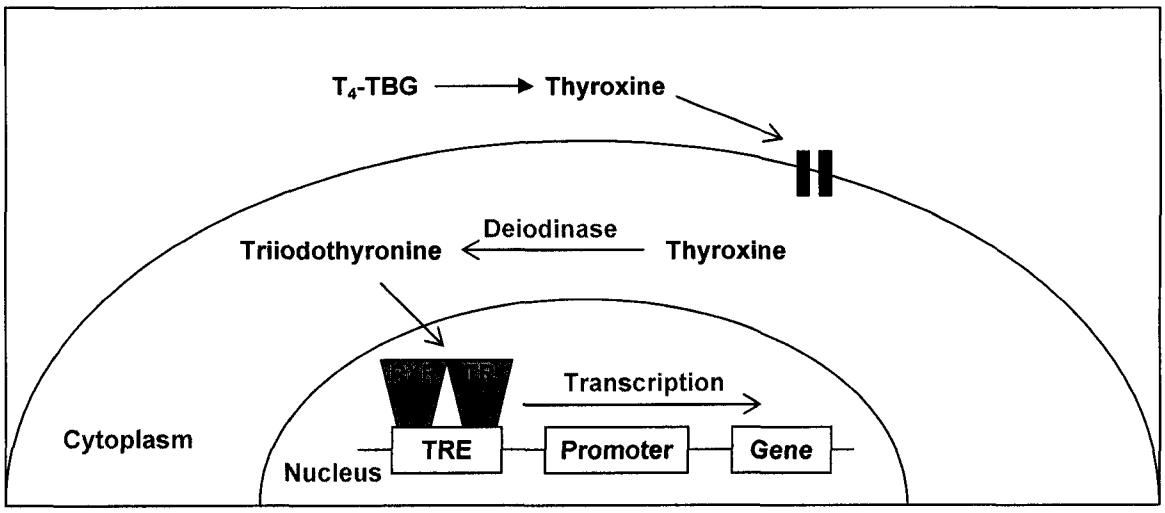



**Figure 1.2: Simplified Overview of Thyroid Signaling**

Thyroxine ($T_4$) is taken-up by cells and converted to triiodothyronine ($T_3$) by deiodinases within the cytoplasm. $T_3$ then translocates to the nucleus and binds its nuclear receptor (TR). Within the nucleus TR binds to RXR to form a heterodimer, which can activate transcription of genes with a TRE upstream of its promoter region.

# CHAPTER 2. MATERIALS AND METHODS

### *2.1.1 Cell Culture*

P19 cells (from American Type Culture Collection), which are derived from an embryonal carcinoma induced in a C3H/He mouse, were grown in Minimal Essential Medium alpha with 7.5% Bovine Calf serum, 2.5% Fetal Bovine serum, 100U Penicillin and 100μg Streptomycin. Cells were grown under standard conditions (37°C, 5% $CO_2$ and 95% relative humidity), and in flasks coated with 0.1% gelatin.

### *2.1.2 Plasmids*

Plasmids were transformed into DH5α competent bacteria. Glycerol stocks were made by adding 300μl of 50% glycerol to 700μl of bacteria culture grown overnight, and stored at -80°C. Bacteria from glycerol stocks were grown in LB medium with the appropriate selective antibiotic for 8 hours at 37°C with vigorous shaking (~300 rpm). The starter culture was diluted 1/500 in 50ml LB medium and grown for 12 hours under the same conditions. The bacteria were then harvested by centrifugation at 6000g for 15 minutes at 4°C. The medium was removed and the pellet was stored at -20°C. The plasmids were purified using the HiSpeed Plasmid Midi Kit from Qiagen. Briefly, the bacteria pellet was resuspended and the bacteria were lysed. Lysate was filtered and then washed. Plasmids were eluted, precipitated and then cleaned before being resuspended in TE buffer (a solution containing Tris and EDTA) to protect them from degradation. Plasmid solutions were then cleaned and plasmids concentrated as follows. First, 2 times the original volume of 95% ethanol and 0.1 times the original volume of 3M sodium acetate was added to the plasmid solution. This was placed at -80°C for at least 30 minutes. The solution was centrifuged at 14,000 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet was cleaned using 75% ethanol and vortexing.

The tubes were again centrifuged at 14,000 rpm at 4°C for 10 minutes, and afterwards the supernatant was removed. Pellets were resuspended in TE buffer and concentrations were determined by spectrometry. Plasmid solutions were stored at -20°C.

### *2.1.3 Luciferase Assay*

P19 cells were cultured in 12-well plates overnight. They were transfected with plasmids and 8 hours later treated as described below. At 24 hours after treatment the cells were washed with 1X PBS for 5 minutes and then lysed using Passive Lysis Buffer from Promega for 20 minutes (Figure 2.1). The plates were then wrapped in parafilm and placed at -80°C. Transfections always included either RARE-Luciferase or TRE-Luciferase and pRLSV40 and were co-transfected with either pSG5 (control), Raldh1, Raldh2, Raldh3 or TRα as specified. Transfections were performed using FuGene6 Reagent and the accompanying protocol from Roche. Treatments included Nitrofen (10nM-100μM), 100nM Retinal, 100nM Retinoic Acid, 10μM $T_3$, 60μM Vitamin A, 30μM Vitamin C or 30μM Vitamin E alone or in combination as stated. After lysis, a 50μl sample from each well was placed into a 96-well plate to determine the amount of Renilla and Flirefly Luciferase present. Renilla Luciferase (pRLSV40) and Firefly Luciferase (RARE- or TRE-Luciferase) were detected using Stop n' Glow buffer and Luciferase Assay Buffer II respectively. Data were collected using the Fluoroskan Ascent FL from Thermo Labsystems and Ascent Software Version 2.4.2. The results were then collected in excel format, and normalized for transfection efficiency. One experiment, performed in triplicate, consists of transfection, treatment and collection of results for each variable tested within the figure. A minimum of five separate experiments were combined for each graph, unless otherwise stated. In order to combine

results from each experiment the values were normalized to the mean of the appropriate control. Results were analyzed using GraphPad Prism 4. All results (mean +/- SD) were tested for a Gaussian distribution using a Kolmogorov-Smirnov test. For experiments including only one type of transfection, a one-way ANOVA was performed with a Bonferroni post hoc test. In experiments in which there were multiple transfections and treatments a two-way ANOVA was done with a Bonferroni post hoc test.

### *2.1.4 RNA Isolation*

P19 cells were plated in 60mm dishes coated with 0.1% gelatin. Cells were grown overnight. The next day the media was replaced with media containing increasing concentrations of Nitrofen (10nM-10μM), 100nM RA, vehicle control or media alone. After 24 hours cells were lysed and total RNA was isolated using TRIzol reagent from Invitrogen and the accompanying protocol. Briefly, cells were lysed with 2ml of TRIzol and then mixed with chloroform. The aqueous phase containing the RNA was removed to a new tube. The RNA was then precipitated using isopropanol and washed with 75% ethanol. The RNA was then resuspended in DEPC treated water and stored at -70°C.

### *2.1.5 Reverse Transcription*

Reverse transcription of RNA was performed using SuperScript III Reverse Transcriptase from Invitrogen. The accompanying protocol was followed. Briefly, a mixture of oligo-dT primers, RNA, dNTP and water was heated to 65°C for 5 minutes then placed on ice for at least 1 minute. Next, 5x buffer, 0.1M DTT and SuperScript III RT was added and the mixture was heated to 50°C for 45 minutes. The reaction was

inactivated by heating to 70°C for 15 minutes. Concentrations of cDNA were determined using mass spectrometry.

### *2.1.6 Quantitative PCR*

Quantitative PCR was performed using cDNA concentrations of 50ng/μl and TaqMan expression assays from Applied Biosystems. Standard curves were done for all genes to determine their efficiency. All genes examined had standard curves of 85% or higher. The expression of 18S was used as a control for the amount of cDNA present. The threshold cycle was determined and used for analysis. PCR for each sample was performed in triplicate, with the mean threshold cycle used for further comparison. The ratio of the mean value for the expression of the gene of interest versus 18S, corrected for the efficiency, was used to determine expression levels in relation to control. The PCR was performed by the iCycler from BioRad Laboratories Inc. and data were collected using the iCycler iQ Optical System Software version 3.1. Results were analyzed using GraphPad Prism 4. All results (mean +/- SD) were tested for a Gaussian distribution using a Kolmogorov-Smirnov test. A one-way ANOVA, with a Bonferroni post hoc test, was performed to determine significance.

### *2.1.7 Tissue Preparation*

Embryonic tissue from E11.0 Wnt1-Cre/R26R-LacZ (Jiang et al., 2000) mice fixed in 0.2% gluteraldehyde was kindly provided by Dr. Sucov's Lab at the University of Southern California, LA. This tissue was dehydrated in increasing concentrations of ethanol and then xylene. The tissue was next placed in paraffin wax at 42°C for 4 days. Afterwards, tissue was oriented in the paraffin and cooled to room temperature so that

the paraffin solidified the tissue in place. The tissue was cut into 7μm slices using a microtome and these slices were mounted onto Superfrost glass slides.

### *2.1.8 Immunohistochemical Labeling*

Staining for β-gal was used to define expression patterns of neural crest derived cells in E11.0 Wnt1-Cre/R26R-LacZ tissue. Paraffin was removed from sliced tissue using xylene. The slices were then hydrated by 5 minutes incubations with decreasing concentrations of ethanol and then PBS. To detect β-galactosidase activity the A11132 primary antibody from Invitrogen was used. The secondary antibody was a Biotin labeled donkey anti rabbit antibody. Briefly, the epitope was unmasked using 0.1M sodium citrate buffer (pH6.0) and heating on high in the microwave for 5 minutes. After cooling the tissue was permeablized with PBS, 0.4% Triton and 1% $H_2O_2$ and then washed with PBS. Primary antibody (anti β-gal, rabbit IgG) was diluted 1:1000 in PBS, 0.4% Triton and 0.1% BSA and left on the slides overnight. The next morning, slides were washed and the secondary antibody was applied (1:200 Biotin labeled donkey anti rabbit, PBS, 0.4% Triton, 0.1% BSA). After another wash in PBS, ABC reaction was performed with a 1:100 dilution of A and B in PBS, 0.4% Triton and 0.1% BSA. The subsequent DAB reaction took approximately 40 minutes. Stained tissue was then dehydrated with increasing concentrations of ethanol followed by xylene and mounted using Entellan rapid mounting medium for microscopy from EMD. The tissue was examined to determine where staining occurred. This was done using an Olympus light microscope and images were taken with the Spot camera model 1.2.0 from Diagnostic Instruments Inc. The SPOT Advances software version 4.0.6 for windows was used to view images.

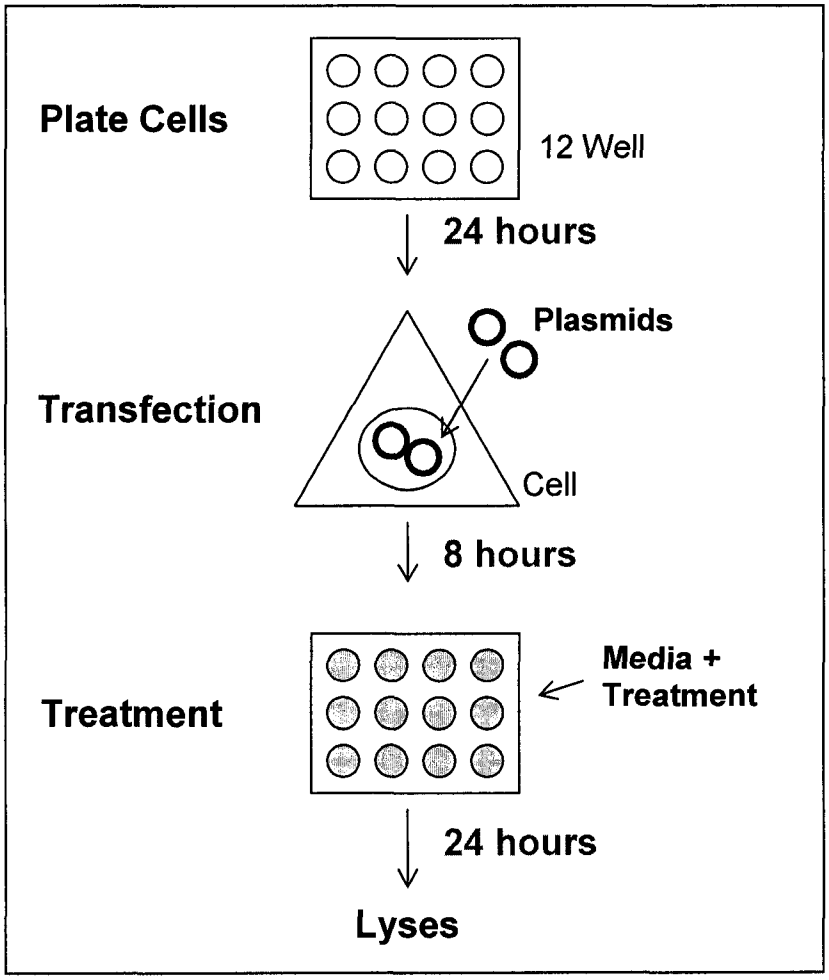



**Figure 2.1: Simplification of Luciferase Assay experiments**

P19 cells were plated in 12 well plates overnight. The next morning cells were transfected with appropriate plasmids and 8 hours later media was exchanged for media containing specific treatments. Twenty-four hours after treatment the cells were lysed and then stored at -80°C until needed for the luciferase assay.

# CHAPTER 3. RESULTS

All work was performed by me except: Figure 3.6 part A and B.

### *3.1* ***Nitrofen and Retinoid Signaling***

I employed the dual luciferase assay system and RARE-Luciferase as an indirect measure of retinoic acid (RA) levels in P19 cells. When RA is present it acts as a ligand for its nuclear receptors and activates transcription of the luciferase gene. To ensure this system could detect changes in RA levels, I treated cells with retinal or RA (Figure 3.1). The results show RA is more potent at inducing the expression of luciferase. I also wanted to ensure the system responded to transfection with plasmids containing the RA synthesizing enzyme retinal dehydrogenase (Raldh). Figure 3.1 shows that Raldh2 transfection results in an increase in luciferase expression versus control when cells are treated with retinal. To determine if Raldh2 transfection plus retinal treatment resulted in maximal Luciferase activation, I compared it to treatment with RA alone. Results in Figure 3.1 show that Raldh2 transfection plus retinal treatment increases luciferase expression levels above retinal treatment alone, but not to a level greater than RA treatment. This demonstrates that Raldh2 transfection plus retinal treatment does not saturate the retinoic acid receptors (RAR's) ability to activate synthesis of luciferase.

To ensure that none of the treatments used altered the expression or detection of either the luciferase or renilla enzymes, all treatments were tested in cells transfected with the control plasmid (pSG5) during each experiment. Results for all treatments, Nitrofen 10nM-10μM, Retinal 100nM, RA 100nM, show that none had any effect on relative RARE-Luciferase activity (Figure 3.2 for nitrofen and data not shown).

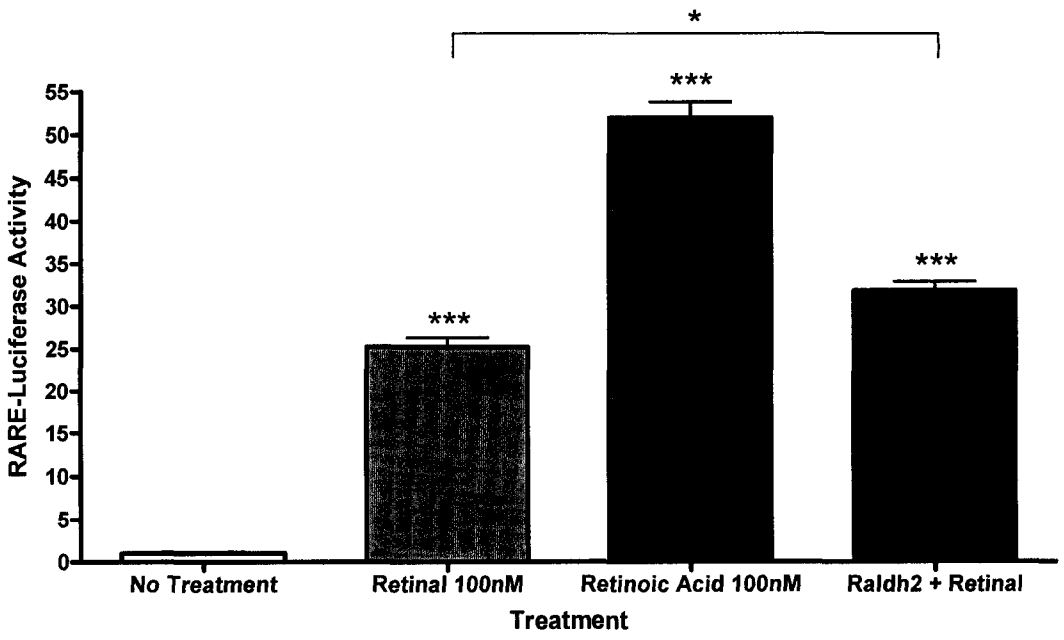



**Figure 3.1: RARE-Luciferase Assay Showing Appropriate Responses to Retinoids**

In the first three bars cells are transfected with control plasmid (pSG5) and treated as indicated. For bar four, cells are transfected with Raldh2 and treated with 100nM retinal. Retinoic acid treatment results in a greater increase in relative luciferase activity than retinal. When cells are transfected with Raldh2, luciferase activity is increased compared to control transfection when both are given retinal treatment ($p<0.05$). These results are from one experiment performed in triplicate. (One-way ANOVA *$p<0.05$, ***$p<0.001$ versus No Treatment)

After ensuring the system could detect changes in RA levels, I tested the ability of nitrofen to interfere with retinoid signaling. Nitrofen had no effect with control transfections therefore it does not produce a detectable change in endogenous RA levels.

To determine if nitrofen altered RA levels by interfering with Raldh2, as has been previously reported in a biochemical assay (Mey et al., 2003), cells were transfected with Raldh2 and treated with increasing concentrations of nitrofen. A clear dose dependent decrease in RA levels is seen with increasing concentrations of nitrofen (Figure 3.2). The addition of 100nM of retinal rescues the effect of nitrofen to control levels. These results confirm previous data showing that nitrofen causes a decrease in RA by inhibiting Raldh2, and validates the use of this paradigm to examine the actions of nitrofen in altering retinoid levels in a cell culture system.

It was also important to determine if nitrofen could interfere with Raldh1 and Raldh3, or if its action was specific for Raldh2. Results show that nitrofen also causes a dose dependent decrease in RA when the cells are transfected with Raldh1 or Raldh3 (Figure 3.3). There is no significant difference in the degree to which nitrofen is able to interfere with the three enzymes. However, the mean inhibition of nitrofen at 10μM compared to control transfection is 58%, 71% and 59% for Raldh1, Raldh2 and Raldh3, respectively.

I also wanted to know if nitrofen was affecting retinoid signaling by altering the expression of genes involved in the retinoid pathway. Results of quantitative PCR for Raldh2, CRABP II, RARα, RARβ and Cyp26A1, show no changes after nitrofen treatment (Figures 3.4). There was a significant increase in expression of CRABP II, RARβ, Cyp26A1 after treatment with 100nM of RA, as has been previously reported.

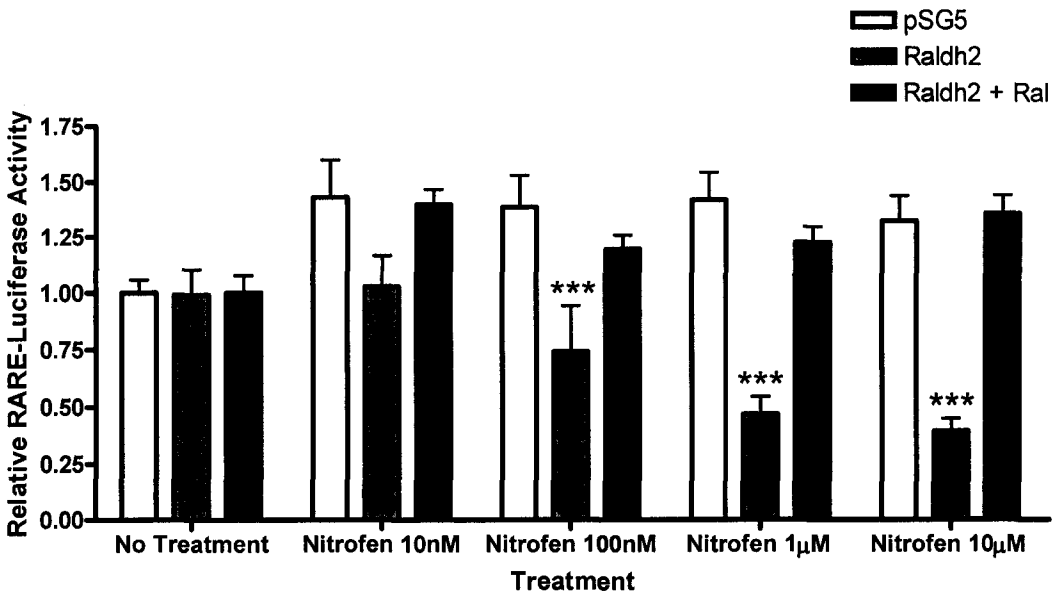



**Figure 3.2: RARE-Luciferase Activity with Raldh2 transfection**
Cells were transfected with either control (pSG5) or Raldh2 plasmids. Treatment included increasing concentrations of nitrofen (10nM-10μM), and shows that with Raldh2 transfection there is a significant decrease in RARE-Luciferase expression compared to control transfection. The black bars are the results for cells transfected with Raldh2 and also treated with Retinal (100nM) at the same time as nitrofen treatment. There was no significant difference between the pSG5 transfected cells and the Raldh2 transfected cells also treated with retinal, indicating that retinal rescues the decrease in relative RARE-Luciferase activity due to nitrofen back to control levels. (Two-way ANOVA ***$p<0.001$ versus pSG5 transfection for each treatment)

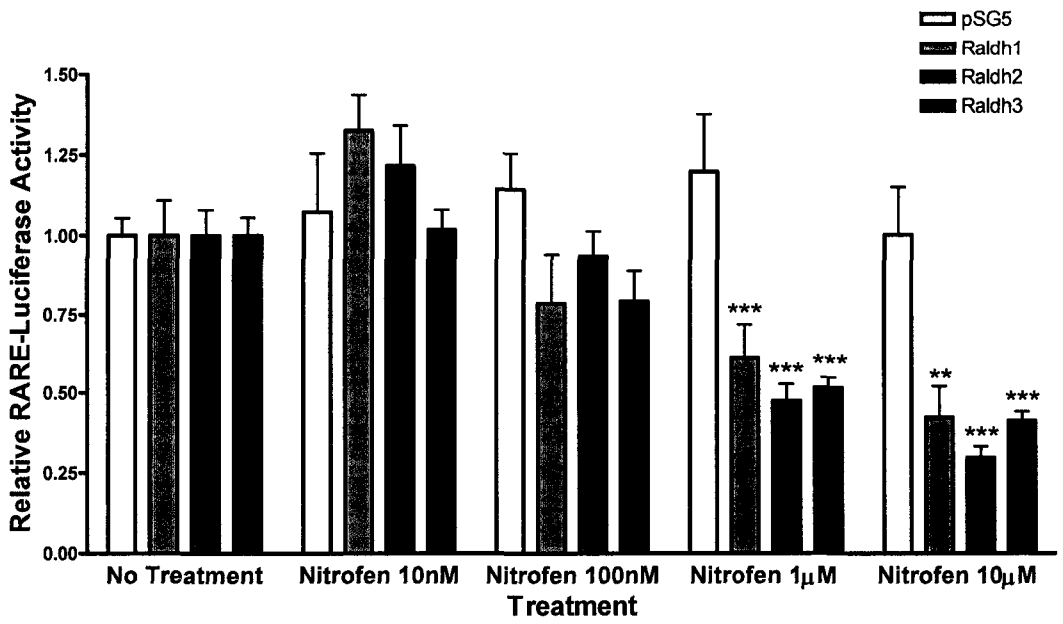
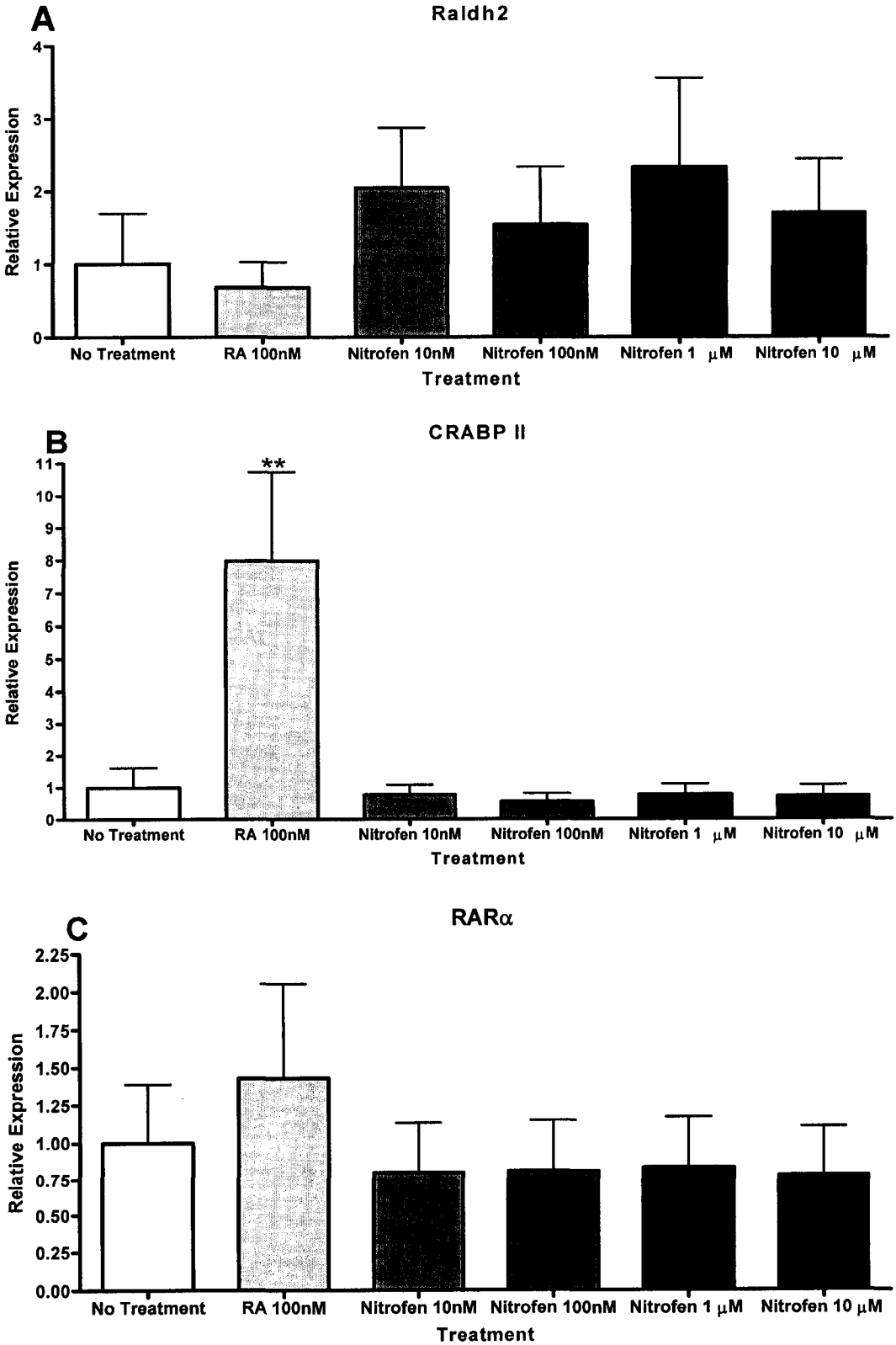



**Figure 3.3: RARE-Luciferase assay Nitrofen with either Raldh1, 2 or 3 Transfection**

Cells were transfected with either control (pSG5), Raldh1, Raldh2 or Raldh3 plasmids. Cells were then treated with various concentrations of nitrofen (10nM-10μM). A clear dose dependent decrease in relative RARE-Luciferase activity is seen with increasing doses of nitrofen for all three Raldh enzyme transfections. (Two-way ANOVA **$p<0.01$, ***$p<0.001$ versus pSG5 transfection for each treatment)



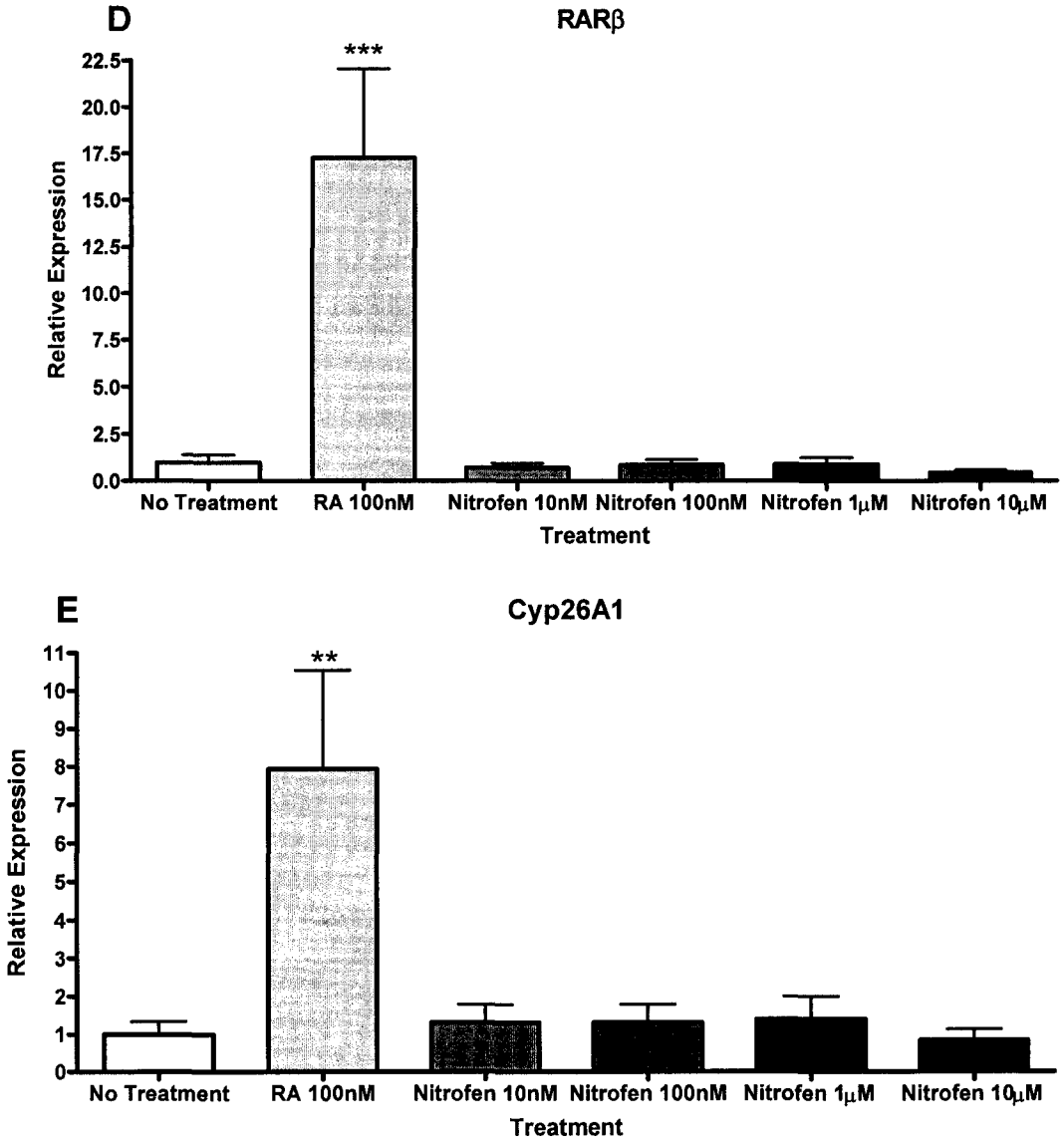



**Figure 3.4: Quantitative PCR for Raldh2, CRABP II, RARα, RARβ and Cyp26A1**
Cells were treated with RA (100nM) or nitrofen (10nM-10μM) for 24 hours. The expression of (A) Raldh2, (B) CRABP II, (C) RARα, (D) RARβ and (E) Cyp26A1 were determined using quantitative PCR. There were no significant changes in expression of any of the genes due to nitrofen treatment. There was a significant increase in CRABP II, RARβ and Cyp26A1 expression with RA treatment (One-way ANOVA **$p<0.01$, ***$p<0.001$). Two separate experiments were performed in triplicate.

To test the hypothesis that the mechanism of action of nitrofen is due to its oxidizing properties, I tested the ability of vitamin A, C, and E to rescue the nitrofen induced decrease in RA levels. Cells were transfected with Raldh2 and treated with nitrofen or a combination of nitrofen and either vitamin A, C or E. The concentration of vitamins used was based on those reported to improve nitrofen's effects on cultured pneumocytes. Results demonstrate that vitamin A is able to rescue the effects of nitrofen however vitamin C and E do not have any rescuing ability (Figure 3.5). This is consistent with the hypothesis that it is retinoid signaling that is being inhibited and does not support the role of nitrofen as an oxidant in the pathogenesis of CDH.

Nitrofen treated embryos that were also given vitamin A treatment were shown to have improvements in the percentage and degree of organ abnormalities, specifically in neural crest derived tissue. Therefore, I examined the possibility that the primordial diaphragm is also of neural crest cell origin. I used E11.0 embryos collected from Wnt1-Cre/R26R-LacZ mice that express β-galactosidase (β-gal) in all neural crest derived cells. Examination of immunohistochemical staining for β-gal within the pleuroperitoneal fold (PPF) reveals that there is a small amount of staining surrounding the phrenic axons and that it is not present at all rostral-caudal levels of the PPF (Figure 3.6 and data not shown). When compared to images of the PPF with phrenic nerve staining, it is clear that the β-gal expressing cells are not the amuscular cells of the PPF. Therefore I conclude that the cells of the PPF are not of neural crest cell origin.

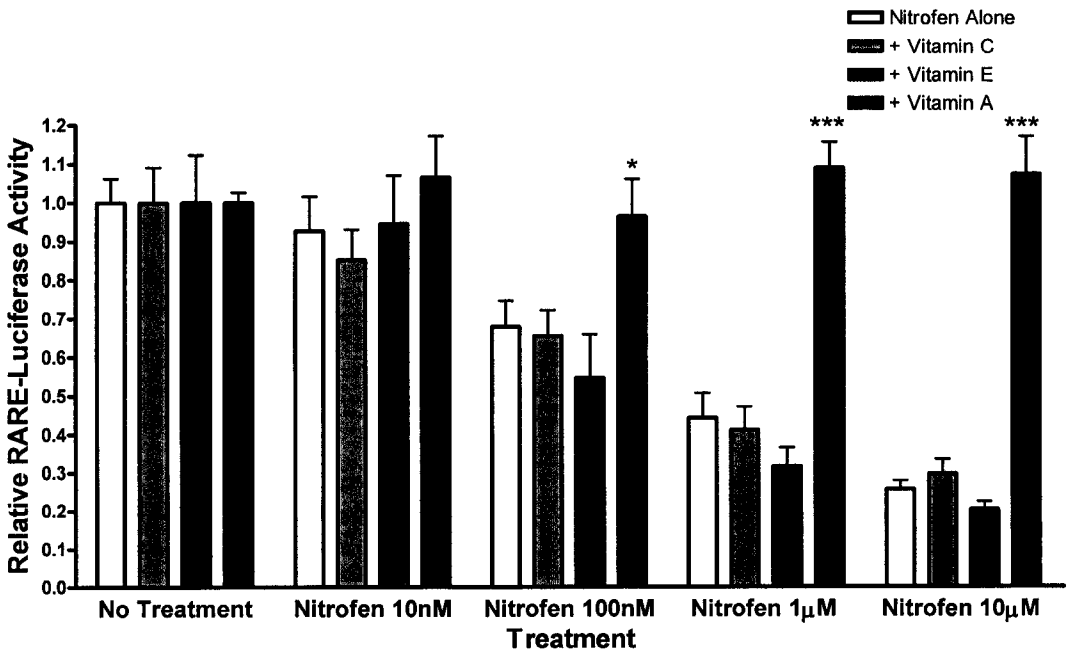



**Figure 3.5: RARE-Luciferase Assay Showing Treatment of Nitrofen Alone or with Vitamin A, C or E**

Cells were transfected with Raldh2 and treated with nitrofen (10nM-10μM) alone or in combination with vitamin A (60μM), vitamin C (30μM) or vitamin E (30μM). Vitamin A was able to rescue the effects of nitrofen at all concentrations, while no changes were seen between nitrofen treatment alone and nitrofen plus with vitamin C or E. (Two-way ANOVA *$p<0.05$, ***$p<0.001$ versus Nitrofen alone within each treatment)

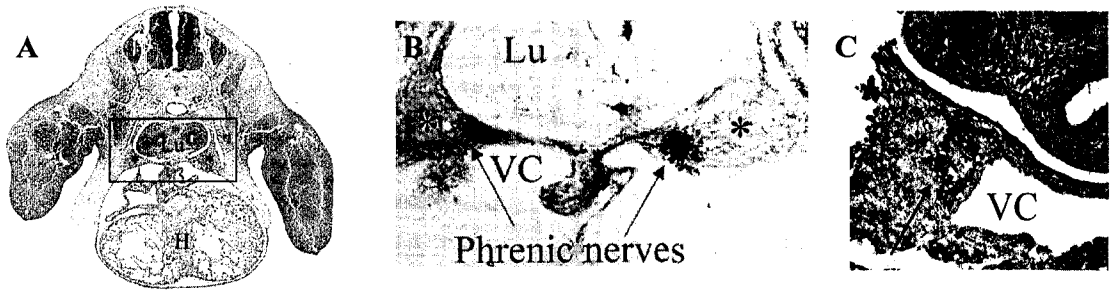



**Figure 3.6: Images of the PPF**
Transverse section of E13.5 rat (A and B) or E11.0 Wnt1-Cre/R26R-LacZ mouse (C) embryos. A: Shows the location of the PPF (*) with respect to surrounding tissue (hematoxylin and eosine staining). (B): A higher magnification of the PPF region (*) with immunohistochemical staining for the low-affinity nerve growth factor (p75) receptor which labels the phrenic nerve and primordial muscle cells within the PPF. C: Immunolabeling for β-gal within the PPF identifying neural crest derived cells. These neural crest derived cells are clearly located within the area corresponding to the phrenic nerve and muscle precursor cells, and cells throughout the PPF. SC – Spinal Cord, Lu – Lung, VC – Vena Cava, H – Heart

### 3.2 *Nitrofen as a Thyromimetic*

An alternative hypothesis for the teratogenic effect of nitrofen is that it is acting as a thyromimetic. The similarity in structure between $T_3$ and nitrofen suggest that nitrofen may be acting directly on thyroid receptors or by interfering with $T_3$ binding to its receptor to alter thyroid signaling. To test this hypothesis, cells were transfected with both TRE-luciferase and pRLSV40 as well as either TRα or control (pSG5) plasmids. Treatment with $T_3$ consistently resulted in a small, non-significant decrease in luciferase levels, which became significant when cells were transfected with TRα (Figure 3.7). Although this was unexpected, thyroid hormone has been shown to have an inhibiting effect on expression of TRE regulated genes (Nygard et al., 2006; Wei et al., 1997; Carr and Wang, 1994; Koller et al., 1987; Lechen and Kakucska, 1992).

Treatment with nitrofen and/or $T_3$ with control transfections were performed with each experiment as controls. Results show that neither treatment alone or in combination altered the detection of either the luciferase or renilla proteins (Figure 3.7). The decrease in relative luciferase expression at 100μM of nitrofen for all test groups suggests that this treatment may be having a general effect on the cells, possibly causing cell death. This is suggested by the decrease in Renilla expression when cells are treated with 100μM nitrofen, as well as a study showing nitrofen causes cell death in P19 cells at 100μM after 24 hours (Kling et al., 2005).

Cells transfected with TRα or pSG5 do not have changes in relative TRE-Luciferase activity when exposed to nitrofen, nor does nitrofen alter relative TRE-Luciferase levels when co administered with $T_3$ in the presence or absence of TRα (Figure 3.7). These results suggest that first, nitrofen does not interact directly with TRα,

and that second, nitrofen does not interfere with $T_3$ binding to TRα. None of the results showed any nitrofen induced effect on thyroid signaling compared to control.

It has been shown that at high doses $T_3$ is able to inhibit the activity of Raldh1 and Raldh3 in vitro (Graham, et al., 2006; Yamauchi et al., 1999). I wanted to determine if this inhibition could be detected using the dual luciferase reporter assay system, and if $T_3$ was also able to inhibit Raldh2 activity. Cells were transfected with RARE-Luciferase and either pSG5, Raldh2 or Raldh3 containing plasmids. Using $T_3$ concentrations previously reported to inhibit Raldh3 activity, the luciferase assay showed that $T_3$ treatment does result a decrease in RA levels with either Raldh2 or Raldh3 transfection (Figure 3.8). Since $T_3$ and nitrofen have similar structures, these results suggest the possibility that nitrofen may be inhibiting the Raldh enzymes via the same mechanism as $T_3$.

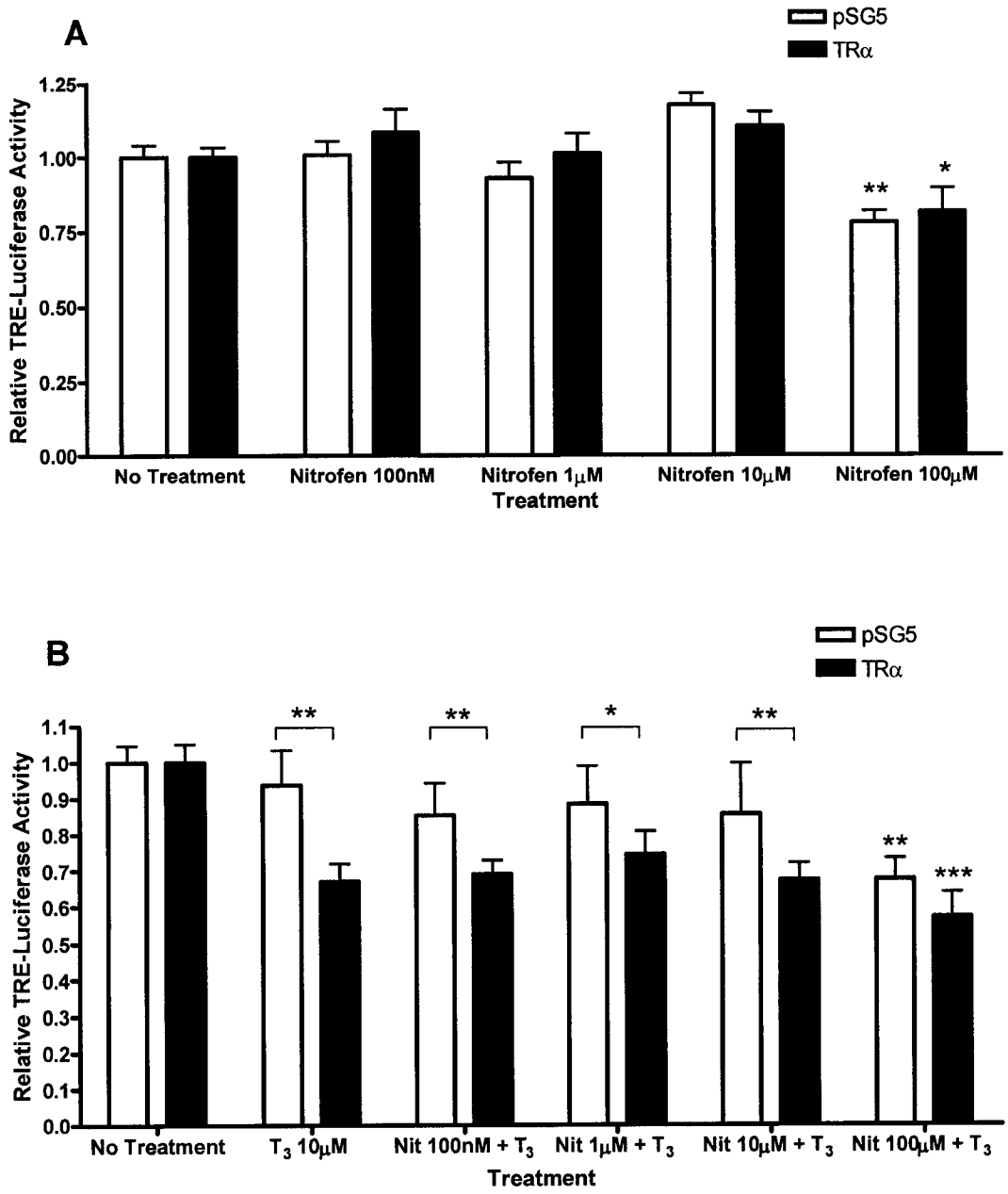



**Figure 3.7: TRE-Luciferase Assay with Nitrofen Treatment**
Cells were transfected with either control (pSG5) or TRα plasmids. Treatment consisted of either nitrofen alone (A), or in combination with $T_3$ (B). A: With 100μM Nitrofen treatment there was a significant decrease in luciferase expression compared to No Treatment. No other significant differences were observed. B: Treatment with $T_3$ consistently shows a decrease in relative luciferase activity which was significant when the cells were transfected with TRα, which is not affected by Nitrofen. Again at 10μM Nitrofen cells show a significant decrease in luciferase expression for both transfections. (Two-way ANOVA *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus pSG5 transfection within each treatment)

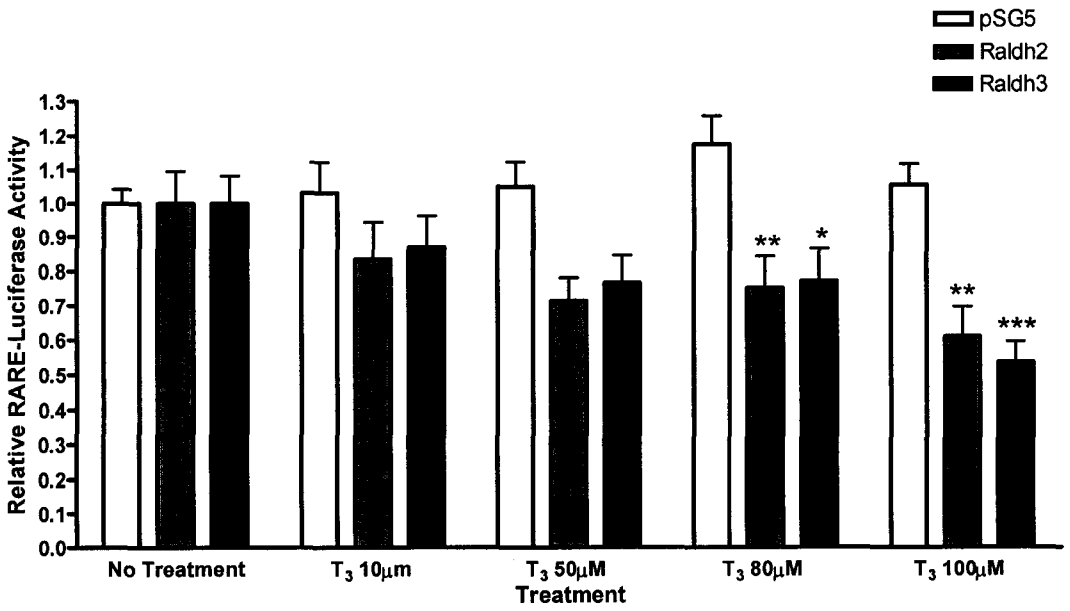



**Figure 3.8: RARE-Luciferase Assay with $T_3$ Treatment**
Treatment with $T_3$ caused a decrease in relative luciferase expression when cells were transfected with Raldh2 and Raldh3, but not control (pSG5) at 80μM and 100μM $T_3$. (Two-way ANOVA *$p<0.05$, **$p<0.001$, ***$p<0.0001$ versus pSG5 transfection within each treatment)

# CHAPTER 4. DISCUSSION

## *4.1 General Discussion*

There has been substantial progress made in understanding the development of the diaphragm, as well as the anatomical changes that occur in the lungs and diaphragm during the pathogenic process of CDH. However what is lacking is significant insight into the cellular and molecular processes involved in the development of these physical changes. There have been a number of studies focusing on possible mechanisms of the thoroughly studied nitrofen model of CDH. A discussion of my results in light of these hypotheses will be examined and future directions will be discussed.

## *4.2 Amuscular PPF Cells*

The rodent model of CDH is induced when pregnant dams are treated with a single dose (100mg/ml olive oil) of nitrofen between embryonic day 8 (E8) and embryonic day 10 (E10). Administration of vitamin A or retinoic acid (RA) reduces the incidence of CDH, with treatment being most effective when given ~E10 (Thebaud et al., 2001). Nitrofen is shown to reach the embryo within 2 hours after oral administration, and peaks by 4-6 hours (Costlow and Manson, 1983). Levels remain high to the last tested time-point of 72 hours (Brown and Manson, 1986). These data indicate that nitrofen is most likely having its teratogenic effect between E8.5 and E12, and that ~E10 is a critical period for RA levels in the embryo. During this stage of development the PPF is in the process of forming, and cannot begin to be identified until E12.5. Nitrofen causes a defect in the amuscular PPF, and since the critical period of nitrofen exposure occurs before the PPF forms, the teratogen is most likely acting on the precursor PPF cells. The identity of these cells and their origin is presently unknown.

Many of the heart and great vessel defects seen in nitrofen induced and human CDH involve structures composed of cells of neural crest (NC) origin (Migliazza et al, 1999). As well, nitrofen has effects on other NC cell derived organs such as the thymus, parathyroid and thyroid (Yu et al., 2002). The expression of two genes important to NC communication and movement, connexin 43 (Lo, et al., 1997) and Pax3 (Li et al., 1999) respectively, are abnormally expressed in the hearts of nitrofen treated embryos (Gonzalez-Reyes et all 2006c; Gonzalez-Reyes et al., 2005). It has been purposed that retinoids play a role in CDH pathogenesis (discussed below), and the retinoid, vitamin A, was shown to produce at least a small improvement in the nitrofen induced outcomes described above. Another drug known to interfere with retinoid signaling is citral. It inhibits the action of Raldh enzymes, which are critical for RA synthesis, and has been shown to increase NC cell death (Schuh et al., 1993). These data all purpose that nitrofen is acting preferentially on NC derived cells and suggests the possibility that the amuscular cells of the PPF may also be of NC cell origin.

The Wnt1-Cre/R26R-LacZ mouse line expresses β-galactosidase in all neural crest cell derivatives (Poelmann et al., 2004). This mouse line was used to determine if any of the cells in the PPF are of NC cell origin. My results clearly show that the only β-gal positive staining in the PPF is seen in the area that corresponds to the precursor Schwann cells, known to be of NC origin, and not in the main area of the PPF. Since these cells are known to be of neural crest origin, the staining is expected. This demonstrates that the cells of the amuscular PPF are not of neural crest cell origin.

***Future Directions***

It will be important to determine the linage of the amuscular PPF cells, as they are affected by nitrofen and are the root cause of the diaphragmatic defect. It is possible that these amuscular cells are mesothelial cells, which are a type of cell that surrounds serosal cavities and the majority of internal organs. These cells express both epithelial and mesenchymal characteristics. It has been observed that cuboidal mesothelial cells exist on the peritoneal side of the diaphragm (Wang, 1998). In order to determine if amuscular PPF cells are in fact mesothelial cells a number of different approaches can be utilized. First, examining these cells under scanning electron microscopy (SEM) would provide important information in order to correctly identify these cells. As well expression of mesothelial markers, such as E-, P- and predominately N-cadherin (Simsir et al., 1999) could be used as molecular markers. The mutant mouse line lacking Pax3 expression in the somites, but not neural crest cells, produce homozygous offspring that have amuscular diaphragms (Engleka et al., 2005). Homozygous mutant embryos from these mice could be used to examine the amuscular cells of the diaphragm specifically. These will be important experiments in determining the pathogenesis of CDH.

A possible means to locate the origin of the cells that form the amuscular PPF may employ the use of RARE-LacZ mice. A decrease in the staining for β-galactosidase is seen in whole embryos at E9.5 of RARE-LacZ mice after dam treatment with nitrofen (Chen et al, 2003). A more detailed study of the cells with reduced staining may suggest the location at an early stage of development of the cells that form the amuscular PPF, and may also provide insight into cell type. The laboratory has a colony of these mice and studies are underway.

### *4.3 Thyroid Hormone and Nitrofen*

Thyroid hormone is known to be important to the development of the fetus. At embryonic day 11 (E11) in the rat, the thyroid rudiment has just begun to form, while trapping iodide and synthesis of thyroid hormone does not begin until E16-E17 (Dussault and Labrie, 1975). Interestingly, thyroid hormone is detected in the fetus before the onset of fetal thyroid function and thyroid hormone receptors are expressed in fetal tissue from E13 onward. This suggests that embryos have the potential to respond to $T_3$ as early as E13 (Obregon et al. 1984), well before endogenous synthesis of the hormone. It is also important to note that the fetal hypothalamic-pituitary-thyroid axis develops and functions independently of the maternal system (Dussault and Labrie, 1975), that and maternal thyroid stimulating hormone (TSH) does not cross the placenta. As well, exogenously administered thyroid hormone is broken down by the placenta, preventing intact thyroid hormone from reaching the developing embryo (Roti et al., 1982; El-Zaheri et al., 1981). However, recently it has been shown that at least some $T_4$ is transferred from the mother to the fetus (Vulsma et al., 1989). Taken together this suggests that changes in the maternal levels of thyroid hormone would have a minimal effect on the fetus. Original studies examining the possible link between thyroid hormone and nitrofen showed that nitrofen treatment on embryonic day 11 (E11) caused a decrease in TSH and $T_4$ serum levels, however these had returned to control levels by 24 and 48 hours after treatment, respectively (Manson et al., 1984). Possibly more relevant to the pathogenesis of CDH, the Manson et al., (1984) study also found that $T_4$ levels were decreased at term in rat fetuses from nitrofen treated dams. Both TSH and $T_3$ levels in these fetuses were normal. They also showed that associated heart and kidney

anomalies were decreased when $T_4$ was co-administered with nitrofen, however diaphragmatic abnormalities remained constant between the two groups. Another group has since shown that plasma levels of both $T_3$ and $T_4$ are decreased in nitrofen exposed fetuses, while TSH levels remained unchanged (Tovar et al., 1997). These results suggest that nitrofen may act as a thyromimetic, but does not provide much evidence on a mechanism of action.

Thyroid hormone has been shown to be particularly important to lung development during both the prenatal and postnatal period. Maturation of the lungs during the perinatal period is impaired by thyroidectomy (Erenberg et al., 1979) and accelerated with thyroxine injections (Wu et al., 1973). As well, fetal and neonatal lung maturation is delayed in mice with primary hypothyroidism (DeMello et al., 1994). In support of a role for nitrofen in lung hypoplasia, the growth of lungs in nitrofen treated fetuses is significantly decreased at various doses and regardless of association with CDH (Gray et al., 1983). One study suggests that early exposure of the fetus to a thyroid active compound may stimulate putative $T_3$ responses, as seen with an enhanced amplification of the β-adrenergic signals of the heart (Lau and Slotkin, 1979). However, in rats $T_3$ administration accelerates the process of septation and results in a greater alveolar surface area (Massaro et al., 1986), while in nitrofen exposed embryos, tracheo-broncheal branching is reduced in cultured lung explants (Guilbert et al., 2000; Keijzer et al., 2000). These data suggest that nitrofen may be acting to inhibit the action of $T_3$, and that it does not act as a thyroid active compound.

Both TRα1 and TRβ2 are expressed in the lungs during fetal growth and are essential for lung development (Rajatapiti et al., 2006; Weinberger et al., 1986). One

study reported that at term, fetuses exposed to nitrofen, both with and without CDH, have decreased expression of TRα1 and TRβ1 in the lung compared to controls (Teramoto et al., 2001). A more recent study showed the level of expression of TRα and TRβ in the lung are not altered compared to controls, nor was their spatial-temporal expression (Rajatapiti et al., 2006). An in vitro experiment illustrated that nitrofen decreased the ability of $T_3$ to bind to both TRα and TRβ via non-competitive inhibition (Brandsma et al., 1994). Although the concentrations of nitrofen used in this experiment were above levels those that have been shown to reach the embryo (Manson, 1986), it suggests the possibility that nitrofen may be acting directly on the thyroid receptors to inhibit the action of $T_3$.

The treatments used during my experiments had no effect on the detection of the luciferase enzymes. The decrease in luciferase expression when cells where transfected with TRα and treated with $T_3$ was unexpected. However, the interaction and corresponding result of ligand binding to thyroid receptors is reliant upon the expression of other nuclear receptors, the presence or absence of various co-activators and co-repressor as well as the exact sequence of the TRE and its flaking nucleotides (Force et al., 1994; Hsu et al., 1995; Wu et al., 2001; Suen et al., 1994). Also, there are a number of genes that have been identified that are negatively regulated by thyroid hormone (Nygard et al., 2006; Shupnik et al., 1985; Shupnik et al., 1987; Koller et al., 1987; Lechen and Kakucska, 1992). The consistent decrease during each experiment demonstrates that changes in the ability of TRα to initiate luciferase expression could be detected using this assay system.

Nitrofen was not able to alter the levels of TRE-Luciferase expression when cells were transfected with TRα with or without $T_3$ co-administration. My results indicate that nitrofen does not act directly on TRα, nor does it interfere with the interaction between TRα and $T_3$. A previous study showed no change in $T_3$ or $T_4$ levels in lung tissue between control and nitrofen treated, or between control and nitrofen treated with CDH animals (Tovar, et al., 1997). As well, the role of thyroid hormone in lung development occurs during later stages, whereas nitrofen is having its molecular effects on the lung during early lung development (Kejizer, et al., 2000). It is also important to note that initial in vivo experiments examining the possible interaction between nitrofen and thyroid hormone, dams were treated with nitrofen at E11.0, which is after the critical point for CDH induction. Taken together, these data cast doubt on the possibility that nitrofen is having an effect via the thyroid signaling pathway especially in lung development.

***Future Directions***

The current data points towards the conclusion that nitrofen is not acting to alter thyroid signaling by altering levels of thyroid hormone, its receptors or by interfering with the interaction of the nuclear receptors and their ligand. The results of the original study demonstrating a more systemic effect of nitrofen may warrant an examination of nitrofen's action in the maternal pituitary, however the limited ability of maternal thyroid levels to affect the fetus leads to questions regarding the relevance of such tests. As well, $T_4$ co administration with nitrofen did not reduce the incidence of diaphragmatic defects and therefore the relevance to pathogenesis of CDH, of any affect nitrofen may be having on the thyroid hormones or thyroid signaling is questionable.

In order to determine if any action on thyroid signaling by nitrofen could have an effect on diaphragm development, a study on the expression of TRα, TRβ and deiodinases within the developing diaphragm needs to be undertaken. As well, it will be important to determine if altered thyroid hormone levels are found in other genetic and teratogenic models of CDH, to determine if it is simply an artifact of the nitrofen model or if it plays a role in the pathogenesis of CDH.

### *4.4 Antioxidant Vitamins and Nitrofen*

There is an alternative hypothesis that has recently been put forward regarding the actions of nitrofen. This hypothesis is that nitrofen is acting as an oxidizing agent, and is based on data showing that antioxidants are be able to reverse or prevent some of the effects of nitrofen on the lungs and heart. Supporting data demonstrates that in the lungs, vitamins A, C and E can restore cell mass and inhibit the negative action of nitrofen on proliferation and apoptosis (Gonzalez-Reyes et al., 2006b). These vitamins have also been shown to mitigate nitrofen's suppression of genes important to lung development including TTF-1, HNF-3b and SP-B in both the lungs of nitrofen exposed embryos (Gonzalez-Reyes et al., 2006b) and cultured human pneumocytes (Gonzalez-Reyes et al., 2006a). In the heart, vitamin E has been shown to ameliorate nitrofen induced decrease in weight (Gonzalez-Reyes et al., 2003). These studies also suggest that antioxidants improve the size of the hypoplastic lung and heart by increasing proliferation. One other study that supports this hypothesis showed that in vitro exposure to the anti-oxidant agent N-acetyl-cysteine improved lung hypoplasia after in vivo nitrofen treatment (Fisher et al., 2002). The main problem with these studies is that they examine the effects of antioxidants on the late stages of lung or heart development

(vitamin treatment beginning on E16), well past the critical stage for the development of diaphragm herniation (~E10). As well, the cultured pneumocytes used by Gonzalez-Reyes et al., (2006a) contain several phenotypic features of mature type II pnuemocytes (Gazdar et al., 1990), whereas the molecular effects of nitrofen are seen in the early stages of lung development. Further, there is no evidence that antioxidant treatment, other than vitamin A, is able to reduce the incidence of nitrofen induced CDH.

The action of nitrofen on the cells of the diaphragm is most likely due to an alteration in retinoid levels, however this hypothesis is still being tested and is discussed below. My study examined whether it was the oxidizing action of nitrofen that may be causing the effects seen in retinoid signaling. The results show that only vitamin A is able to rescue the effect of nitrofen on RA levels. This is expected since vitamin A is itself a retinoid and a precursor to RA. Therefore there seems to be little connection between antioxidants and the pathogenesis of CDH.

***Future Directions***

The results from antioxidant studies suggest that vitamins A, C and E may be important in later stages of lung and heart development, as opposed to the earlier stages when the development of the diaphragmatic defect occurs. As well, my results provide evidence that the oxidizing action of nitrofen is not the mechanism by which CDH develops.

There may be a role for these vitamins in improving the later lung hypoplasia normally associated with CDH. This could be examined using the lamb or rabbit model of CDH, as it focuses on the detrimental effects of visceral protrusion into the thoracic cavity on lung development. It is also possible that antioxidants provide a benefit to heart and lung development that is independent of nitrofen.

Earlier treatment with antioxidant vitamins in the nitrofen model and examination of the incidence of CDH with vitamin co administration will provide critical information on the importance of these vitamins on the initial stages of the pathogenesis of CDH.

### *4.5 Retinoids and Nitrofen*

There is a great deal of evidence suggesting a role for retinoids in the development of CDH. The Retinoid Hypothesis proposes that abnormalities in retinoid signaling, or in factors influenced by retinoids, leads to the development of the primary defect, diaphragm herniation, in CDH (Greer et al., 2003; Babiuk and Greer, 2002). This hypothesis is based on a number of lines of evidence. First, rats fed vitamin A deficient (VAD) diets produce 20-45% of offspring with CDH (Anderson, 1941; Anderson, 1949; Warkany and Wilson, 1948; Wilson et al., 1953). The number of herniations is decreased in embryos of VAD animals with the administration of vitamin A midway through gestation. Second, vitamin A treatment also reduces the number of embryos with CDH in the commonly used nitrofen model (Thebaud et al., 1999; Thebaud et al., 2001). Further studies showed that RA was even more potent at reducing the CDH inducing effects of nitrofen (Babiuk et al., 2004). Third, RAR knock-out mice suggest RA plays a key role in CDH, as double knock-out mice (RARα and RARβ) produce some offspring with CDH like hernias (Mendelsohn et al., 1994; Lohnes et al., 1995). Fourth, studies of RARE-lacZ mice confirm that RA levels are decreased in embryos after exposure to nitrofen. This effect is reversed with RA treatment (Chen et al., 2003). The retinoid hypothesis is also relevant to humans. A small case study examining retinoid levels in

mothers and infants showed that both umbilical cord blood retinol and RBP levels in CDH patients is about 50% those of normal patients, whereas retinol and RBP levels are increased in mothers of CDH babies (Major et al., 1998). This suggests that there may be an impairment of the transfer of retinol from mother to child. Taken together, these data provide substantial support for the hypothesis that retinoids play an important part in the development of the diaphragm defect in the rodent model of CDH.

There are also a number of lines of evidence that suggest retinoids play a role in the lung and heart pathogenesis of nitrofen induced CDH. The hypoplastic lungs of nitrofen treated embryos are rescued to normal size when cultured with RA (Montedonico et al., 2006). This study examined the early factors involved in lung development, and shows that retinoids act in this process. As stated above, vitamin A also normalizes the expression of connexin 43 and Pax3, normally altered after nitrofen treatment. As well, early vitamin A treatment improves the hypoplastic effect of nitrofen on the heart and thymus (Yu et al., 2002). Vitamin A also improved, although modestly, cardiovascular defects (Yu et al., 2002). A study by Baptista et al., (2005) showed that vitamin A had positive effects on early lung hypoplasia and suggested that this was due to cell proliferation. Evidence that retinoids are important for lung development come from RARα/RXRγ and RARα/RARβ double knock out mice, which have hypoplastic or absent lungs (Mendelsohn et al., 1994; Maden, 2000; Mollard et al., 2000). The functional relevance of RA in the development of many different tissues provides further support that nitrofen induced alterations in RA levels could lead to the plethora of abnormalities associated with CDH.

The possibility that nitrofen is interfering with retinoid signaling requires that relevant genes are expressed in the affected tissues. The major enzyme responsible for RA synthesis, Raldh2, is expressed in the lung during development (Giguere, 1994). As well, all three RAR's and RXR's are expressed in both rodent and human lungs during development (Rajatepiti et al., 2006). Taken together, this data indicates that the developing lungs are able to produce and response to RA. In the diaphragm, Raldh2 (Mey et al., 2003) and RARβ2 (Mendelsohn et al., 1994) are known to be expressed and suggests that the cells of the PPF are also responsive to retinoid signaling. It is also important to note that during the period nitrofen is effective at inducing CDH, embryonic susceptibility to changes in RA levels may be increased due to a severe dip in retinol levels in the maternal blood stream (Satre et al., 1992; Takahashi et al., 1977). These factors provide further support for the role of retinoids in the pathogenesis of CDH.

With regard to the mechanism by which nitrofen may be affecting the retinoid signaling pathway, there is a hypothesis that nitrofen may alter the level of proteins involved in retinoid signaling, and therefore reduce RA signaling, by decreasing mRNA expression. However my data, along with that of others, show that this is not the case either in a cell culture system or in the intact embryo (Rejatapiti et al., 2006). The genes I examined were chosen based on their relevance to retinoid signaling within the diaphragm. Raldh2 has been shown to be expressed within the PPF (Mey et al., 2003), as has CRABP II (unpublished data), while Cyp26A1 is expressed in the developing diaphragm (Abu-Abed et al., 2002). Lastly, CDH occurs in double knock-out RARα/RARβ mice (Mendelsohn et al., 1994; Lohnes et al., 1995). There was an

increase in the expression of CRABP II, RARβ and Cyp26A1, which was expected, as they are known to contain sensitive RARE's in their promoter regions (Durand et al., 1992; Reijntjes et al., 2005; Takeyama et al., 1996).

Another possible mechanism for the action of nitrofen is that it is interfering with enzymes involved in the retinoid signaling pathway. Nitrofen, as well as three other CDH inducing teratogens, have been shown to inhibit Raldh2 activity in vitro (Mey et al., 2003). My data supports these findings in a cell culture system. When cells were transfected with the control plasmid, nitrofen had no effect on luciferase expression. This indicates that the teratogen does not interfere with the luciferase assay, nor does it cause a detectable change in RA levels. However, when the cells are transfected with Raldh2 and treated with nitrofen, there is a dose dependent decrease in RARE-Luciferase expression, which is an indirect measure of RA levels. The only change that was made was the increase in expression of Raldh2, which strongly suggests that nitrofen is interfering with this enzymes activity. As well, nitrofen caused a dose dependent decrease in RA levels when cells were transfected with Raldh1 or Raldh3. These results resolve any debate over the specificity of nitrofen regarding the Raldh enzymes.

There is the possibility that nitrofen could be also be interfering with signaling downstream of Raldh2. Results show that transfection with Raldh2 plus retinal treatment does not lead to the saturation of the retinoid receptors by RA, which could mask any possible effect nitrofen may have on the action of these receptors. The co administration of retinal with nitrofen is able to rescue the decrease in RARE-Luciferase activity to control levels. If nitrofen were acting on the retinoid receptors, retinal would not be able

to fully rescue RA levels, therefore these results suggest that nitrofen is not acting downstream of Raldh2. This is supported by a study which showed that RA, downstream of Raldh2 in the retinoid pathway, is better able to rescue the CDH inducing effects of nitrofen than vitamin A, which is found upstream of Raldh2 (Babiuk et al., 2004).

Interestingly, thyroid hormone is able to interfere with Raldh1 and Raldh3 activity (Graham, et al., 2006; Yamauchi et al., 1999). Since nitrofen and $T_3$ have similar structures, I tested the ability of thyroid hormone to interfere with Raldh2 and Raldh3 in this cell culture system. The results show that $T_3$ does in fact decrease RA levels, suggesting that it is possible that nitrofen is acting to inhibit Raldh activity via the same mechanism as $T_3$. It has been suggested that $T_3$ may play an important role in the regulation of RA synthesis due to its ability to inhibit the Raldh enzymes. Nitrofen may act in a similar fashion, or may interfere with this regulatory action of $T_3$. The identification of a possible mechanism of action of nitrofen with respect to the activity of the Raldh enzymes may provide insight into the pathogenesis of CDH.

***Future Directions***

It will be vitally important to test the retinoid hypothesis on a larger human population to determine if these data are relevant to the human case. With regard to the models of CDH it will be critical to determine possible links between the mouse models, genetic anomalies in humans and the retinoid hypothesis. One study could examine the effects of nitrofen on the expression of genes shown to be relevant to CDH, while the dual luciferase assay system could be used to determine if there is any interaction between these genes and retinoids. These two experiments may reveal links between retinoids and

CDH relevant genes that could uncover a common connection between all cases of CDH.

Another significant next step will be to define the cellular result of the nitrofen induced reduction in RA levels in vivo. Does this reduction cause a decrease in proliferation or an increase in apoptosis in the cells destined to be the amuscular cells of the diaphragm? Preliminary results show that nitrofen is inducing cell death in cultured cells, but whether that is due to a decrease in RA levels or some other action of nitrofen has yet to be determined. It will also be essential to confirm these results in the embryo.

With regard to the hypothesis that nitrofen is acting via the same mechanism as $T_3$ in inhibiting the Raldh enzymes, it will be important to examine the effects of co administration of $T_3$ and nitrofen, as well as the ability of retinal to rescue the action of $T_3$.

# CHAPTER 5. CONCLUSION

The work presented here addresses some of the main hypothesis regarding the pathogenesis of the nitrofen induced model of CDH. The suggestion that the amuscular PPF cells are of neural crest cell origin has been rejected. It will be important to determine the origin of these cells in order to develop a better understanding of the pathogenesis of CDH. I have also provided supporting evidence that nitrofen is not acting directly on TRα, nor interfering with the interaction of thyroid hormone and TRα. My work demonstrates, in a cell culture system, that nitrofen is able to decrease RA levels and that it is likely due to interference with Raldh enzymatic activity, and not a decrease in the expression of genes important to retinoid signaling. This decrease in RA was shown not to be due to nitrofen's oxidizing ability, but may act in a similar fashion to the inhibition of the Raldh enzymes by $T_3$. In summary, this work has examined and rejected a number of the proposed pathogenic mechanisms of nitrofen. It has also strengthened the retinoid hypothesis, while suggesting a new hypothesis for the mechanism of nitrofen's CDH inducing action. An understanding of the pathogenic process of CDH may lead to improved detection and a reduction in the incidence of this lethal congenital disease.

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