University of Alberta

Centre for Neuroscience

Insights into the pathogenesis and etiology of congenital diaphragmatic hernia

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Edmonton, Alberta

Fall, 2008

Your file Votre référence
ISBN: 978-0-494-46299-7
Our file Notre référence
ISBN: 978-0-494-46299-7

To a mouse

By Robert Burns (1785)

Wee, sleeket, cowran, tim'rous beastie,
O, what panic's in thy breastie!
Thou need na start awa sae hasty,
Wi' bickering brattle!
I wad be laith to rin an' chase thee,
Wi' murd'ring pattle!

I'm truly sorry Man's dominion
Has broken Nature's social union,
An' justifies that ill opinion,
Which makes thee startle,
At me, thy poor, earth-born companion,
An' fellow-mortal!

Abstract

Congenital diaphragmatic hernia (CDH) is a common birth defect (incidence = 1:2500) and a source of potentially fatal neonatal respiratory distress. The pathogenesis and etiology of CDH is poorly understood; it is with this in mind that the research described in this thesis was undertaken. The experiments performed utilised rodent models of CDH and particular attention was paid to a structure of the primordial diaphragm, called the pleuro-peritoneal fold (PPF).

The research performed encompassed two major themes; experiments addressing the pathogenesis of diaphragmatic hernia and experiments addressing its etiology. Speculation in the literature regarding the pathogenesis of CDH has been widespread since the defect was first described. However, until recently, few laboratories have systematically studied this anomaly. Regarding the pathogenesis of CDH, the major findings of this thesis are: 1) normal development of the PPF in humans and rats was defined and found to be the same. 2) Teratogen-induced, dietary, and genetic models of CDH were shown to have a common mechanism of pathogenesis with parallel features found in human CDH. 3) Decreased cell proliferation was indicated as a causative factor in abnormal PPF development in the nitrofen model of CDH. Combined, these studies represent a significant advance in our understanding of normal and pathological diaphragm development in the context of CDH.

The etiology of CDH is complex, involving both genetic and environmental factors. The major findings in this thesis regarding CDH's etiology are: 1) The CDH-critical region 15q26 was found to contain several genes associated with the

development of the diaphragm. 2) Genes strongly associated with CDH were found to be preferentially expressed in the non-muscular, mesenchymal cells of the PPF. 3) CDH-inducing teratogens were shown to suppress retinoid signalling. 4) The PPF was identified as a centre of retinoid signalling. And 5) A new model to study CDH was identified utilising a pan-retinoid receptor antagonist.

Together, the studies described in this thesis represent an important contribution to the body of knowledge concerning the pathogenesis and etiology of CDH. This work has generated novel hypotheses and will inform future research into this potentially devastating birth defect.

Acknowledgements

It seems most appropriate to first acknowledge the contribution of my supervisor John J. Greer in the completion of this thesis. It was through John that I was first introduced to medical research, and under his supervision I have been allowed to develop my own ideas as my thesis has evolved. Thanks for letting me get on with it.

The Greer lab has always been full of intellectual and technical support; thanks to Wei Zhang, Randy Babiuk, Rhiannon Noble, Floriane Lenal, Jun Ren, and especially Silvia Pagliardini. Beyond the laboratory; the secretarial support of Carol Ann Johnson and Brenda Topliss has always been appreciated, and the work of laboratory animal services has been truly invaluable. There are certain faculty members at the University of Alberta who have also had a positive influence on me and deserve recognition: Drs. Pete Dickie, Kieth Bagnall, and Clayton Dickson.

My family deserves acknowledgment, if only for their patience during my Canadian sojourn and doctoral studies. As a son to Irene and David, a brother to Jonathan and Andrew, and a member of the extended Frood and Clugston families, I have laboured away in a country far removed from my Scottish homeland on a body of work they little understand but value nevertheless. Thank you for your support.

It seems that part of a University education is the constant coming and going of people, new students arrive and old students graduate. Over the years of my studies I have made countless friends. To name a few, from the homeland: Eilidh MacLean, David Inglis, and Frances Shaw. From 'the early days': Chris Ford, Grant Wickman, and Theresa Hopkins. From the '5th floor': Bryan Norrie, Lisa Guevremont, Francois Roy, Jan Kowalczeski, Dirk Everaert and Trevor Hamilton. Thank you for the good times.

There are of course a group of friends who require special mention. They have been a source of both distraction and motivation, which were welcome in equal measures: Tera Mosher, Suzanne Sjovold (nee DeBow), Travis Sjovold, Gavin Searle, Nicola Webster, Sarah Thomas, and last but not least Erin Doxsey-Whitfield. Thank you.

As part of a balanced lifestyle the following have also contributed, perhaps indirectly, in the completion of this thesis. For the exercise of body and mind: Sadie, Edmonton Overlanders Orienteering Club, Urban Uprising climbing gym, the Edmonton river valley, and the Canadian Rockies! As a purveyor of food and alcohol, and a venue to study and write: The Sugar Bowl and Muddy Waters. Thanks.

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List of Abbreviations

β-gal	β-galactosidase
ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt
ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CDH	Congenital diaphragmatic hernia
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CRL	Crown-rump length
DAB	3,3-Diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
E	Embryonic day (when followed by a number)
ECMO	Extra-corporeal membrane oxygenation
ELISA	Enzyme linked immuno-sorbent assay
FETO	Foetal endoscopic tracheal occlusion
LHR	Lung to head ratio
LRAT	Lecithin:retinol acyltransferase
MPC	muscle precursor cell
NO	Nitric oxide
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PPC	Pleuro-peritoneal canal
PPF	Pleuro-peritoneal fold
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RE	Retinyl ester
REH	Retinyl ester hydrolase

RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SEM	Standard error mean
TUNEL	terminal uridine deoxynucleotidyl transferase dUTP nick end labelling
VAD	Vitamin a deficiency
WAGR	Wilms Tumour aniridia growth retardation
WT1	Wilms Tumour 1

Note – The following convention for abbreviating genes and proteins has been used in this thesis. Human genes are written in capital letters and in italics (*GENE*), human proteins are written in capital letters, but are not italicized (PROTEIN). Rodent genes are written in italics, though only the first letter is capitalized (*Gene*). Rodent proteins are not italicized and only the first letter is capitalized (Protein).

Chapter 1: Prolegomenon

Sections of this chapter were adapted from the original publication:

Diaphragm development and congenital diaphragmatic hernia

Robin D Clugston and John J Greer

Seminars in Pediatric Surgery 2007 16(2):94-100

1.1 Introductory Remarks

In Canada, the majority of pregnant women will have a routine ultrasound examination around the 18^{th} to 22^{nd} week of gestation to assess the development of her unborn child. The majority of the time, the mother-to-be will leave the clinic with a black and white ultrasound image of her healthy foetus. In a mercifully small number of pregnancies, the ultrasound technician may notice something abnormal in the chest cavity of the foetus; perhaps the stomach, some intestines, or part of the liver. This observation, when confirmed, indicates the presence of a hole in the diaphragm and the diagnosis of congenital diaphragmatic hernia (CDH; figure 1.1). In essence, the research presented in this thesis is aimed at helping to answer those first questions of parents recently informed they are going to have a baby with CDH: Why? How?

Throughout a typical lifetime the diaphragm muscle assists in breathing; around 21,600 times a day the diaphragm involuntarily contracts, enlarging the thoracic cavity and creating a negative pressure which draws air into the lungs. Though considered to be the primary muscle of respiration, the diaphragm performs an often overlooked, yet critical, function. Simply put, it forms an anatomical barrier between the thorax and abdominal cavity. It is perhaps during gestation that this barrier function is most important, for if the diaphragm forms incompletely (as occurs in CDH) the abdominal contents can protrude into the thorax where they impede the growth of the developing lungs. Upon birth, when the newborn baby is required to breathe on its own for the first time, its poorly developed lungs are often unable to provide enough oxygen and respiratory distress rapidly ensues. After a

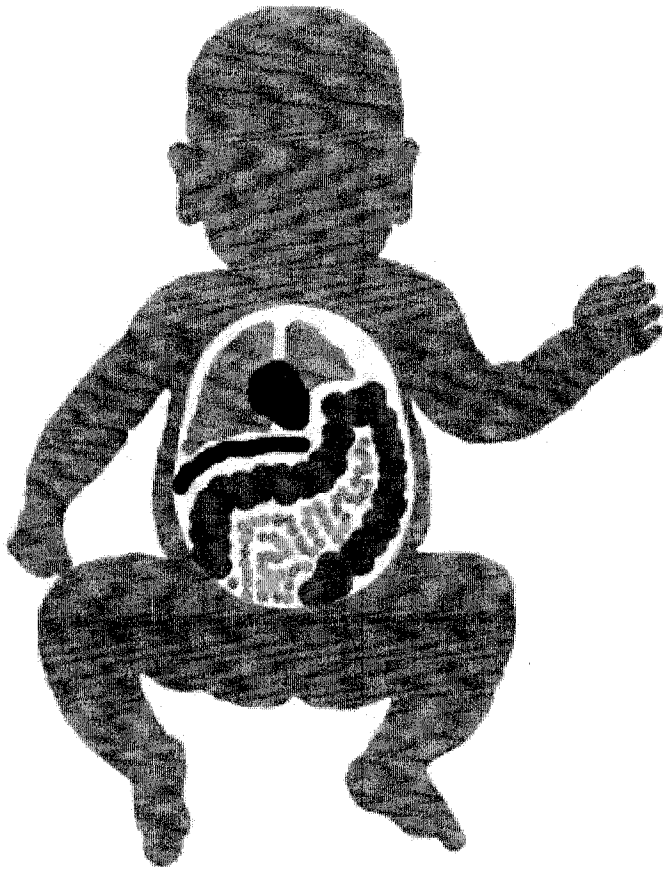

Figure 1.1 Congenital diaphragmatic hernia.

A schematic representation outlining the anatomy of a typical case of CDH is shown. A hole in the left side of the diaphragm (purple) allows the abdominal contents (green) to herniate into the thorax, compressing the left lung (pink) as it grows.

lengthy stay in the neonatal intensive care unit, and surgery to repair the hole in the diaphragm, surviving newborns can be discharged home, though long-term follow-up is required to manage the after-effects of being born with CDH.

So what are the causes of CDH and how does it develop? The search for the answer to these questions is at the centre of a large international biomedical research effort. At the time of writing, the on-line database pubmed (www.pubmed.com) listed 3771 articles on CDH (search term "congenital diaphragmatic hernia"; May 30th, 2008), 83 of which were published in 2008. The majority of CDH research focuses on the developing lungs; an understandable endeavour given the importance of these structures for respiration and the damage inflicted upon them by herniated abdominal contents. In contrast, the research presented in this thesis is directly concerned with the diaphragm abnormality itself. As discussed in the forthcoming chapters, through the use of experimental models (including cell culture, rodent models, and human pathological specimens) we have explored various aspects of how diaphragm abnormalities in CDH arise with particular regard to the normal and pathological embryogenesis of the diaphragm (chapter 3 and 5), the importance of retinoid signalling in diaphragm development (chapter 6), and recent advances in our understanding of the genetics of CDH (Chapter 4).

1.2 General objectives

The general aims of the studies presented in this thesis were to provide a greater understanding of the pathogenesis and etiology of CDH. The work encompasses three broad and inter-related themes: 1) the embryogenesis of diaphragm defects in rodent models of CDH, 2) the importance of retinoid signalling in diaphragm development, and 3) genetic origins of CDH in rodents and humans.

1.2.1 Embryogenesis of diaphragmatic hernia

One of the outstanding questions in our understanding of CDH is how the hole in the diaphragm actually forms. The entrenched, text book definition of how CDH forms was formulated approximately 60 years ago and has been perpetuated despite it having very little experimental data to support it. Modern advances in scientific investigation have allowed a re-examination of how the diaphragm forms normally and the pathogenic hallmarks of CDH. Previous work from the laboratory of Dr. John Greer using the well characterized nitrofen model of CDH in rodents has identified a specific lesion in the pleuro-peritoneal fold (PPF), a key structure in early diaphragm development, which underlies CDH. In chapter 3 the aim was to substantiate this hypothesis by integrating data from the nitrofen model with newly defined rodent models of CDH, specifically vitamin A deficient rats and *Wt1* null-mutant mice. Further, by including human post-mortem tissue in our analysis we attempted to bridge the gap between data obtained from animal models and how the defect actually appears in human cases of CDH. The results from this integrated approach support the hypothesis that diaphragm defects in CDH have their origin in

a malformed PPF and should lead to a revision of the popular understanding of how CDH develops.

In chapter 5 we took the next logical step and further studied the evolution of the PPF defect which underlies the hole in the fully formed diaphragm. In this chapter we tested specific hypotheses regarding the basic cellular mechanism that leads to the abnormal formation of the PPF. This question was addressed using *in vitro* cell culture techniques and whole animal studies. The data obtained indicates that, at least in rodents, the PPF defect originates from decreased cellular proliferation caused by nitrofen. Also in this chapter we asked more basic questions about how the PPF itself develops, paying particular regard to its neuromuscular component, as well as examining when the PPF defect first appears following nitrofen exposure. Importantly, a survey of archived human embryonic tissue allowed the timeline of PPF development in humans to be delineated, thereby identifying a critical period in diaphragm development with respect to CDH.

These studies into the embryogenesis of the diaphragm and diaphragmatic hernia expand upon existing hypotheses regarding the development of diaphragm abnormalities in CDH and provide novel perspectives on the specific cellular mechanisms that generate this defect.

1.2.2 Retinoid signalling in diaphragm development

The cause of CDH is only clear when there is a discernible genetic defect in the affected individual. However, such genetic insults are only found in a minority of cases of CDH. In the remaining instances the etiologic origin(s) of CDH is unknown and are assumed to be multifactorial. Identification of the causative

factor(s) in non-genetic cases of CDH could have a profound effect on the way cases are identified, how their prognosis is assessed, and how they are treated. One possibility that has been forwarded to explain apparently idiopathic CDH is the so-called *retinoid hypothesis*; this posits that abnormalities in retinoid signalling could underlie the formation of CDH. In consideration of this hypothesis, chapter 6 examines retinoid signalling in normal and pathological diaphragm development. In this chapter we looked at two aspects of retinoid signalling. First we addressed the effect of CDH-inducing teratogens on retinoid signalling in transgenic mice, using production of the enzyme β-galactosidase by the transgene RARE-lacZ as a proxy for retinoid signalling. Second, we aimed to identify the specific network of proteins expressed in the PPF which are involved in retinoid signalling; this included the identification of retinoic acid binding proteins, synthetic enzymes and nuclear receptors. The results of these studies lend credence to the retinoid hypothesis and provide a better understanding of retinoid signalling in the developing diaphragm including how CDH-inducing teratogens disrupt this signalling network.

1.2.3 Genetic origins of CDH

The etiology of CDH is complex and is likely to be multifactorial in nature, involving some interplay between environmental and genetic factors. While there are some cases of CDH that can be directly attributed to an obvious genetic deficit, it is also likely that more subtle genetic mutations could predispose to CDH and are modulated by environmental factors. As such, studies exploring the genetics of CDH are a key step in improving our understanding of the etiology of this birth defect, with implications for the diagnosis, determination of prognosis and treatment

of it. In recent years there has been a large increase in the number of genes identified in association with CDH. However how the proteins encoded by these genes act in diaphragm development is largely unknown. The purpose of the studies described in chapter 4 was to integrate our knowledge of genes identified from human cases of CDH, and mutant-mouse models, with our current understanding of CDH pathogenesis and diaphragm development. From the first step of determining whether these CDH-critical genes are expressed during diaphragm development, we continued to explore their specific pattern of expression and drew conclusions regarding their role in the formation of this structure. The result of this work is a clearer understanding of how a genetic abnormality can lead to CDH. Further, we were able to incorporate our expression data of CDH-critical genes into a broader understanding of diaphragm development and hypothetical models of CDH pathogenesis.

1.3 CDH: A working definition

CDH can be phenotypically characterized into several sub-types depending on the location of the defect or its nature (figure 1.2). The most common type of CDH, and the primary focus of this thesis, is the posterolateral diaphragm defect. Clinically referred to as a Bochdalek hernia, it accounts for greater than 95% of cases and is typically synonymous with the diagnosis of CDH (Torfs et al., 1992; Yang et al., 2006). There are also three other rarer types of CDH, i) eventration of the diaphragm, ii) defects of the central tendon, and iii) Morgagni hernias, which will be briefly discussed in subsequent sections.

Please note that for the purposes of this thesis the term CDH will be used in relation to Bochdalek CDH, unless otherwise specified.

In all of the above presentations of CDH, the integrity of the diaphragm as a barrier between the abdomen and thoracic cavity is diminished. This allows the abdominal viscera to protrude into the thorax, forming a space-occupying lesion in this cavity and impairing foetal breathing movements, all these factors combine to impede lung development. The section detailing the clinical perspective of CDH will outline the pathophysiology of CDH, but first we will discuss the variety of animal models available to study this birth defect.

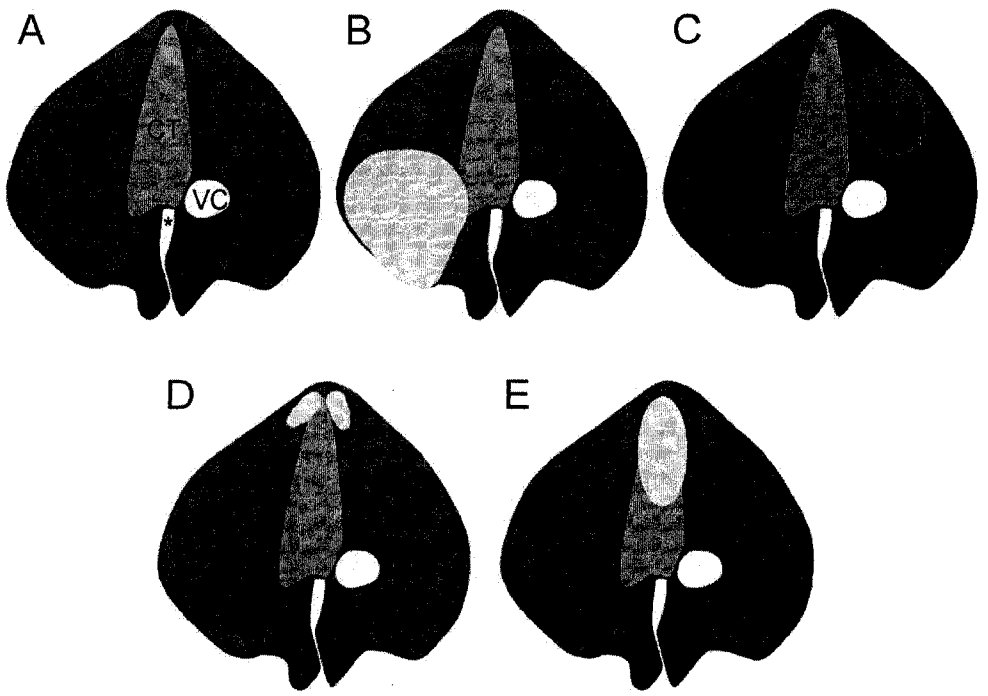

Figure 1.2 Different types of diaphragmatic hernia.

Simplified view of the mammalian diaphragm, viewed from above. A) Normal diaphragm; the passage of the vena cava (VC) through the diaphragm forms a natural hole in it. The oesophagus and abdominal aorta pass through the diaphragm between the crural muscles (*). CT: central tendon. B) Bochdalek hernia; located in the postero-lateral region of the diaphragm, typically on the left side of the body. C) Diaphragm eventration; incomplete muscularisation of the diaphragm leaves a weakened area (dotted lines) through which the abdominal contents can protrude (location variable). D) Morgagni hernia; a small defect occurring on either the left or right hand side through the foramen of Morgagni. E) Central tendon defect; incomplete development of the central tendon becomes a site of herniation.

1.4 Animal models of CDH

The ancient Greek physician Galen is commonly considered to be the *father of vivisection.* Although he frequently dissected animals for anatomical purposes, the widespread use of animals to recapitulate aspects of human disease did not become popular until the late 19th century and is now commonplace (Bynum et al., 1990). With regard to studying birth defects, animal models are effective because they provide a model for experimentation that would be ethically and practically limited in humans. Further, they can be used to provide valuable insight into the pathogenesis and etiology of birth defects, as well as providing models for pioneering therapies to be tested before their use in humans. With regard to CDH, large animals (i.e. sheep) have been used to accurately model the clinical presentation of infants with CDH, while various rodent models have been used to gain insight into the pathogenesis and etiology of CDH. A variety of different animals have been used to study CDH including rats, mice, rabbits, sheep, pigs and Tamarin monkeys. The first animal model of CDH was described in 1967; by surgically creating a diaphragmatic hernia in foetal lambs, the authors were able to study the morphologic and mechanical characteristics of lung hypoplasia caused by the herniated abdominal contents (De Lorimier et al., 1967). Subsequently the surgical lamb model was refined and has been successfully used to model various aspects of the lung defects seen in CDH (Wilcox et al., 1996). This model has also been used to pioneer various surgical interventions to prevent/revert lung damage *in utero*, including foetal surgery to repair the diaphragm defect and foetal tracheal occlusion (Harrison et al., 1981; Adzick et al., 1985; Hedrick et al., 1994; see

below). The strength of the surgical lamb model is that it accurately reflects the pathophysiology of the lung defect and cardiovascular changes observed in neonates with CDH, but because the defect in the diaphragm is artificially created it does not provide any information regarding the pathogenesis of the diaphragm defect or indicate anything about its etiology. Described below are animal models that, despite their own limitations, have been effectively used to study the pathogenesis of diaphragm defects in CDH, and in some cases have shed light on possible etiologic factors that lead to diaphragmatic hernia.

1.4.1 Nitrofen and teratogenic models of CDH in rodents

In the 1970s toxicological studies of the herbicide nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether) showed that while relatively harmless to adult rodents, nitrofen could induce developmental anomalies in the lungs, hearts, diaphragms and skeletal tissues of foetuses exposed *in utero* (Ambrose et al., 1979; Costlow and Manson, 1981). Further study showed that diaphragm defects could be induced by administering a single 100mg dose of nitrofen to pregnant rats, typically between embryonic day (E)8-11, and most significantly the defects produced were remarkably similar to those documented in human Bochdalek CDH with respect to their size, location of the defect and accompanying intrusion of the abdominal viscera into the thoracic cavity (Allan and Greer 1997; Cilley et al., 1997; Figure 1.3). In addition to its effect in rats, nitrofen has also successfully been used to create CDH in mice (Wickman et al., 1993).

Nitrofen exposure is now a widely used model to study various aspects of CDH, though the small size of the rat foetus limits physiological testing, it is well

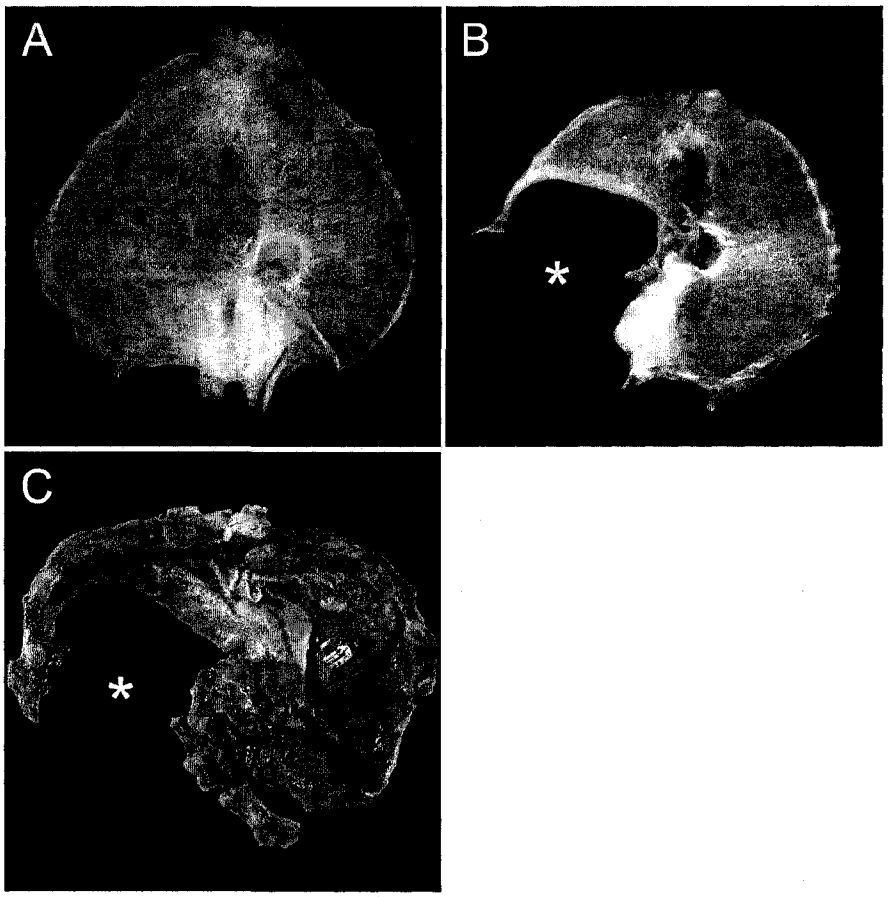

Figure 1.3 Nitrofen induced CDH.

A) Normal E16.5 rat diaphragm viewed from above, with the ventral tip pointing upwards. B) E16.5 rat diaphragm isolated from a nitrofen-exposed foetus, note the large hole (*) in the left postero-lateral corner of the diaphragm. C) Diaphragm isolated from a term-foetus with Bochdalek CDH, note the region of missing tissue (*) and compare with B. Diaphragms not to scale.

suited to anatomical and molecular biological investigations. An important caveat to studying the lungs in nitrofen exposed foetuses is that the teratogen affects multiple tissues and has a direct effect on lung development, thus careful interpretation is required to separate the effects of i) nitrofen exposure, and ii) visceral herniation and compression, on lung pathology (Keijzer et al., 2000). Despite this limitation, the nitrofen model has been successfully used to study the pathogenesis of the diaphragm defect in CDH (see below). Further, its use raises intriguing questions about how CDH develops. For example, treatment with nitrofen on E8 produces a large percentage of left-sided diaphragm defects, whereas treatment with nitrofen on E9 or later produces more right-sided defects (Kluth et al., 1990; Allan and Greer, 1997). While treatment on E8 is generally preferred because it produces more left sided defects (which occur most commonly in humans) this correlation between the time of exposure and the side of the diaphragm defect provides an opportunity to study what controls the laterality of CDH. Also, it has been observed that some strains of rat are more susceptible to nitrofen exposure suggesting that genetic background is an important factor in determining the incidence of nitrofen-induced CDH (Kang et al., 1986)

In addition to nitrofen, three other CDH-inducing teratogens have been recently identified; 4-biphenyl carboxylic acid (BPCA), bisdiamine [N,N'-octamethylenebis (dichloroacetamide)] and SB-210661 (Mey et al., 2003). BPCA is a breakdown product of a thromboxane-A_2 receptor antagonist, bisdiamine is a spermatogenesis inhibitor and SB-210661 is a benzofuranyl urea derivative developed for inhibiting 5-lipoxygenase. Although these drugs were designed for

very different purposes, they have chemical structures similar to each other, and to nitrofen, hinting at a common mechanism of action. Importantly, each of the four teratogens have their effect in the same critical period of diaphragm development and produce diaphragm defects in developing rats that are similar to those in infants with CDH.

1.4.2 Mutant mouse models of CDH

Mutant mouse models of CDH are beneficial as they provide a highly reproducible and convenient model to study the pathogenesis of CDH, as well as providing insight into the etiology of CDH by way of identifying essential genes for successful diaphragm development. As more and more genetically engineered mutant mice are created, several lines of mice have been identified which have diaphragmatic hernia. The first mutant mouse model to be associated with CDH was Wilms Tumour 1 (*Wt1*) knock-out mice (Kreidberg et al., 1993). Originally created to study urogenital development these mice were serendipitously found to have diaphragmatic hernia, thus not only did this identify *Wt1* as an essential gene for diaphragm development but it also provided a new model to study the pathogenesis of CDH free from the non-specific effects of nitrofen. Unfortunately, diaphragmatic hernia was not 100% penetrant in this line of mice and because of *Wt1*'s essential role in heart development the foetal mice died *in utero*, preventing examination of the diaphragm in late gestation and early postnatal life. More recently, another strain of mice has been characterized which overcomes some of these limitations. A strain of mice with the chick ovalbumin upstream promoter-transcription factor II (*Coup-tfII*) gene conditionally inactivated in *Nkx3-2* expressing tissues has CDH

(You et al., 2005). Though diaphragm defects are not 100% penetrant in this line of mice they survive to term and because the deletion of *Coup-tfII* is conditional, the phenotype is largely limited to CDH. In addition to *Wt1* and *Coup-tfII* mutant mice, diaphragm defects have been observed (amongst other defects) in several other strains of mutant mice including: compound retinoic acid receptor knock-out mice (see below), *MyoR*:*Capsulin* compound mutants, and *PDGRFα* null-mutant mice (Mendelsohn, et al., 1994; Lu et al., 2002; Bleyl et al., 2007). Apart from the initial observation that these mice have diaphragmatic hernia, little work has been done to characterize the pathogenesis of their diaphragm defects. The majority of strains mentioned above are limited by incomplete penetrance of the diaphragm defect and lethality of the mutations; the generation of more refined mutant mouse models has the potential to provide excellent models to study the pathogenesis of CDH.

1.4.3 Modelling rare subtypes of CDH

The majority of research in the field of CDH is concerned with the development of Bochdalek hernias of which there are several animal models available. However, largely through the discovery of mutant mice with abnormal diaphragm phenotypes, models are now becoming available to study the less common subtypes of CDH.

1.4.3.1 Eventration of the diaphragm

Diaphragmatic eventration is a relatively uncommon class of CDH. It is characterized by incomplete muscularisation of the diaphragm, allowing the abdominal contents to protrude into the thoracic cavity in the areas where no muscle has formed, and the diaphragm is subsequently weaker. The pathogenesis has not

been thoroughly studied; however the recent characterization of mice expressing a mutant form of *Fog2*, which have a phenotype consistent with diaphragmatic eventration as seen in humans, provides an excellent animal model to further study this rare sub-type of CDH (Ackerman et al., 2005).

1.4.3.2 Central tendon defects of the diaphragm

Central tendon defects are characterized by congenital herniation of abdominal contents through the central tendon of the diaphragm. The embryogenesis of this defect is poorly understood, failure to form, rupture, or stretching of the central tendon due to an underlying weakness have all been suggested to explain this defect (Skandalakis et al., 1994). Mice with a null mutation in the *Slit3* gene have central tendon defects similar to those seen in humans; shedding light on the etiology of this defect and providing a novel tool for further study (Yuan et al., 2003; Liu et al., 2003).

1.4.3.3 Morgagni hernia

This rare anterior defect of the diaphragm is variably referred to as Morgagni, retrosternal, or parasternal hernia. It accounts for only ~5% of all CDH cases. It is characterized by herniation of abdominal contents through the formanina of Morgagni; small triangular areas of the diaphragm adjacent to the lower end of the sternum. Embryologically, this area is considered congenitally weak and that this is the source of herniation when it occurs (Skandalakis et al., 1994). There is no mutant mouse model with a clear Morgagni-type phenotype. However, the Golden Lion Tamarin (*Leontopithecus rosalia*), a small endangered primate of the Atlantic coastal rainforest of Brazil, may provide a very useful model of this specific type of

CDH. The incidence of diaphragmatic Morgagni defects amongst newborn Tamarins is ~9%, with a definite heritable basis (Randolph et al., 1981; Bush et al., 1996). While the precise mechanism has not been determined, the pattern is suggestive of a simple autosomal recessive mode of inheritance. Clearly, the genetic mutation(s) that underlies the Morgagni-type diaphragmatic defect has penetrated the Tamarin genome to a striking degree and merits further investigation. Further, the incidence of Morgagni hernia amongst children with Down's syndrome may be as high as 1:1000, indicating some unknown link between these two conditions (Honore et al., 1993).

1.4.4 Summary

In summary, animal models are used to study various aspects of CDH. The mainstay into studying the pathogenesis of diaphragm defects is the nitrofen model in rodents. Mutant mouse models have indicated genes that are essential for normal diaphragm development, with important implications for our understanding of CDH etiology. Further, these mutant mouse models also provide tools to study CDH pathogenesis. An approach integrating data from human CDH with multiple animal models seems most effective at overcoming the limitations of individual models and identifying common features in the pathogenesis and etiology of CDH.

1.5 Congenital diaphragmatic hernia: a clinical perspective

1.5.1 Pathophysiology of CDH

As mentioned above diaphragm defects of the Bochdalek type are the most common manifestation of CDH, accounting for ~95% of cases (Torfs et al., 1992; Yang et al., 2006). Bochdalek hernias can be further classified by the side of the defect; approximately 80% of these types of defect are located on the left side of the body, 15-20% are right sided, and the remaining small percentage are bilateral (Torfs et al., 1992; CDH study group, 2007). What determines the sidedness of the defect is poorly understood and may be associated with subtle differences in the rate of development between the left and right side of the body. Regardless of its etiology, the sidedness of the defect can affect outcome; right-sided CDH is associated with increased morbidity and mortality compared to left-CDH. One of the largest studies of its kind focusing on right-CDH found only a 55% survival rate, compared with 77% for left-CDH (Fisher et al., 2008). Another important indicator of outcome related to the nature of the hole in the diaphragm is its actual size; such that those individuals with the largest defects have the poorest outcome. In this scenario, it is thought the size of the defect is a proxy for the extent of pulmonary hypoplasia (and concomitant pulmonary hypertension) and therefore how well the baby will be able to breathe at birth (CDH study group, 2007).

Although CDH is characterized by a hole in the diaphragm, it is the damage inflicted upon the lungs as a consequence of the diaphragm defect that often proves fatal. Pathological series have shown that the lungs of infants with CDH have multiple abnormalities, with the lung ipsilateral to the diaphragm defect being more

severely affected than the contralateral lung. The lungs of infants with CDH are hypoplastic; there is a significant reduction in lung size with respect to body weight and gestational age, and there is a decreased lung volume secondary to reduced numbers of airway generations and alveoli. With regard to the pulmonary vasculature, there is a decrease in absolute number of arteries, medial arterial wall hypertrophy, and abnormal peripheral arterial muscularisation (Areechon et al., 1963; Kitagawa et al., 1971; Reale et al., 1973; Levin, 1978; Geggel et al., 1985; Bohn et al., 1987). The net result of these structural abnormalities is a decrease in the surface area for gas exchange and an increase in the pulmonary vascular resistance, contributing to pulmonary hypertension. At the time of birth, the foetal circulation which bypasses the lungs during gestation normally closes; however in neonates with CDH their elevated pulmonary vascular resistance causes the ductus arteriosis and foramen ovale to remain open. This condition is known as persistent foetal circulation and results in a continued right to left shunt of blood through the heart, largely bypassing the lungs. Poor ventilation and perfusion, combined with a reduced surface area for gas exchange all contribute to hypoxemia. Low oxygen tension further contributes to pulmonary hypertension by causing pulmonary vasoconstriction, which is confounded by the mechanical changes to the vasculature described above. If untreated, a continued lack of sufficient oxygen can lead to shock, multiple organ failure, heart failure and ultimately death (Dibbins et al., 1974; Harrison et al., 1994). In addition to the pulmonary hypoplasia and vascular abnormalities described above, the lungs of infants with CDH are thought to have other detrimental abnormalities. For example, it has been proposed that neonates

with CDH have impaired surfactant production adversely affecting lung inflation (Lotze et al., 1994; Moya et al., 1995). Pulmonary hypertension in CDH has been associated with increased circulating levels of vasoconstrictors and decreased levels of vasodilators in affected individuals (Stolar et al., 1985; Kobayashi et al., 1994). Lastly, the lungs are not the only organs that can be affected by CDH. Infants with this defect also have reduced cardiac mass, largely reflecting hypoplasia of the left atrium and ventricle, though to result from mediastinal compression by herniated abdominal organs (Siebert et al., 1984). Equally, the right heart often becomes hypertrophied due to the increased load placed on it associated with pulmonary hypertension.

The above description of the pathophysiology of CDH describes the common clinical and pathological manifestations resulting from incomplete diaphragm formation; this well defined condition is referred to as isolated CDH. However, there are cases of CDH where other birth defects, unrelated to the hole in the diaphragm, are present. Indeed, diaphragmatic hernia is a feature of several congenital syndromes including Pallister-Killian syndrome, and Fryns syndrome, among others. Population-based studies indicate that these associated abnormalities are found in 23-55% of cases and can have a profoundly negative effect on outcome (Torfs et al., 1992; Gallot et al., 2006; Yang et al., 2006). The associated anomalies found in conjunction with CDH are wide ranging and affect almost all organ systems. The most common associated abnormalities involve the cardiovascular system (e.g. ventricular septal defects and transposition of the great vessels), the genitourinary system (e.g. renal agenesis and dysgenesis), the musculoskeletal

system (e.g. absent ribs and upper limb defects), and nervous system (e.g. hydrocephalus and spina bifida; Bollmann et al., 1995; Yang et al., 2006).

In summary the pathophysiology of CDH is complex and represents a major challenge to the health care team who manage it. The realization that CDH is more of a physiological disease than simply a surgical emergency is important when considering what management strategy to employ (Muratore and Wilson, 2000). The next two sections will describe the diagnosis of CDH, determinants of outcome, and the treatment options available.

1.5.2 Diagnosis and determination of CDH prognosis

Since the early 1980's prenatal ultrasound has been successfully used to identify CDH *in utero*, with the detection of abdominal contents within the foetal thorax making a definitive diagnosis (Chinn et al., 1983). Prenatal diagnosis of CDH is an important determinant in the outcome of CDH. Importantly, it allows the parents to be counselled regarding the nature of the birth defect. Further testing including genetic screening and detailed ultrasound assessment of lung hypoplasia and other possible abnormalities allows decisions to be made about possible termination of pregnancy or preparations for the birth to be made at a specialized care centre (Gallot et al., 2005; Graham and Devine 2005; Frenckner et al., 2007). Despite the widespread use of prenatal ultrasound testing, approximately 50% of cases of CDH go undiagnosed (Lewis et al., 1997; CDH study group 2007). Neonates with undiagnosed CDH typically present at birth with respiratory distress; a simple chest x-ray can reveal the presence of abdominal organs within the chest cavity and lead to the diagnosis of CDH. Fortunately, it is often the milder cases of

CDH that go unnoticed prenatally and the consequences are relatively less severe (Lewis et al., 1997). A corollary to this is that infants with a prenatal diagnosis of CDH have a worse outcome, reflecting the severity of their case. For example, in one recent study, survival following prenatal diagnosis was ~53%, whereas in those infants who were diagnosed after birth, survival was ~81% (Rygl et al., 2006). As such, the prenatal diagnosis of CDH is important in identifying severely affected individuals and making appropriate preparations for their birth.

With regard to evaluating the prognosis for infants with CDH there are several factors to be considered. As mentioned above, the side of the diaphragm defect and its size both indicate something about the possible outcome. Infants with larger diaphragm defects have a poorer outcome, as do those with right-sided versus left-sided hernias (CDH study group, 2007; Fisher et al., 2008). Similarly, foetal liver position is also important; foetuses whose liver has herniated into the thorax have a poorer outcome compared to those whose liver remains in the abdomen (Albanese et al., 1998; Kitano et al., 2005). Other important determinants of outcome are the presence of an associated structural abnormality or the detection of a genetic defect (see below). In order to try and quantify the extent of lung hypoplasia *in utero* and therefore predict outcome, great emphasis has been placed on the utility of measuring the lung to head ratio (LHR) of prenatally diagnosed foetuses with CDH. The LHR is defined as the ratio of the area of the contralateral lung to foetal head circumference. Initial reports suggested that it was effective in predicting outcome; for example in the series published by Metkus, foetuses with an LHR < 0.6 had 100% mortality, foetuses with an LHR between 0.6 and 1.35 had

43% mortality, and there was 100% survival in those with an LHR > 1.35 (Metkus et al., 1996). The use of LHR to predict outcome was supported in subsequent case series, however a systematic review of LHR use to predict CDH outcome concluded that in the absence of a prospective, mutli-centre trial there was insufficient evidence to support the use of LHR in predicting outcome (Lipsutz et al., 1997; Laudy et al., 2003; Ba'ath et al., 2007). Thus, there are several presentations of CDH that are associated with a poor outcome and techniques for accurately predicting outcome have still to be unequivocally proven as accurate.

1.5.3 Clinical management of CDH

As indicated above, neonates with CDH often present with severe respiratory distress which requires mechanical ventilation to provide adequate oxygenation. Recent years have seen a paradigm shift in the approach towards the management of CDH. Traditionally, immediate surgical repair of the diaphragm defect was carried out, now a more reserved approach is taken in which surgical repair is delayed and stabilization of the infant is paramount. Moreover, the commonly used approach of aggressive hyperventilation in order to establish normal blood gas levels is now thought to actually damage the lungs and is avoided (Sakurai et al., 1999; Muratore and Wilson, 2000). The use of gentle ventilation and permissive hypercapnia combined with delayed surgical repair is now recommended and has been associated with improved outcomes. (Bagolan et al., 2004). In those infants with CDH who do not respond to standard treatment, various other strategies have been employed, including inhaled nitric oxide (NO) and surfactant therapy. Inhaled NO is used to cause vasodilatation in the pulmonary vasculature and alleviate the symptoms of

pulmonary hypertension associated with CDH. However, despite the apparent success of this therapy, two randomized controlled trials and systematic reviews looking at the efficacy of inhaled NO in CDH had unremarkable results (Neonatal inhaled nitric oxide study group, 1997; Kinsella JP et al., 1997; Finer and Barrington, 2001; Logan et al., 2007). Surfactant replacement therapy is proposed to prevent alveolar collapse in instances of impaired surfactant production; neonates with CDH were proposed to have decreased surfactant production, however this finding has recently been refuted (Lotze et al., 1994; Moya et al., 1995; Boucherat et al., 2007). Early reports on the efficacy of surfactant therapy have also been questioned when larger, better controlled studies have been carried out (Bos et al., 1991; Glick et al., 1992; Lotze et al., 1994; Logan et al., 2007). In cases where CDH associated lung hypoplasia is so severe that life cannot be maintained and the risk of death is high, extra corporeal membrane oxidation (ECMO) can be used as a life saving treatment. This approach allows the blood of the neonate to be oxygenated (and carbon dioxide removed) outside the body, before it is pumped back through the circulation. In a systematic review of reports describing the use of ECMO in infants with CDH it was found to reduce short term mortality, but had no effect on long term mortality (Morini et al., 2006). An important caveat to the use of ECMO is the increased morbidity associated with its use, for example infants on ECMO are at an elevated risk for developing an intracranial haemorrhage as well as sensorineural hearing loss (Austin et al., 2004; Fligor et al., 2005).

The purpose of the above management strategies is to ensure that neonates with CDH are stabilized and they are provided with adequate oxygenation. Once

these infants are stable enough to undergo surgery they will have the hole in their diaphragm repaired. There are two possible approaches: i) repair with living tissue, typically a reflected section of latissimus dorsi, or ii) prosthetic patch repair; this approach is reserved for larger diaphragm defects and involves a prosthetic patch (typically made from gore-tex) which is stitched into place to cover the hole in the diaphragm (Rowe and Stolar, 2003).

There is one final avenue for the management of infants with CDH that has not been discussed; with prenatal diagnosis of CDH comes the opportunity for foetal intervention (reviewed in detail by Grethel and Nobuhara, 2006). Based on successful animal studies, the first serious attempts at foetal surgery in cases of CDH were carried out by a team led by Dr Michael Harrison at the University of California, San Francisco (Harrison et al., 1993). These studies were based on the hypotheses that surgical repair of the diaphragm defect would allow time for the pulmonary hypoplasia to be resolved, at least in part, before birth (Beals et al., 1992). However, *in utero* repair of the diaphragm for foetuses with CDH had no effect on infant mortality compared to conventional treatment in a controlled trial, and because the increased risks associated with this technique were considered too great it was recommended that prenatally diagnosed CDH should be treated postnatally (Harrison et al., 1997). From the failure of *in utero* surgery to repair the diaphragm defect came the next attempt at foetal intervention for CDH; foetal endoscopic tracheal occlusion (FETO). FETO works by endoscopically occluding the trachea, thereby blocking the egress of foetal lung secretions which subsequently build up. The increased pressure within the lung causes lung expansion and helps to

reduce the volume of herniated abdominal viscera. Increased lung growth and survival was seen in sheep with CDH who received tracheal occlusion and the technique was considered to be a promising approach for foetuses with a poor prognosis (Wilson et al., 1993; DiFiore et al., 1994; Hedrick et al., 1994). Early trials of FETO in foetuses with CDH had positive results and justified a randomized controlled trial comparing FETO with optimal postnatal care (Harrison et al., 2003a). Unfortunately, this trial was prematurely stopped because advances in optimal postnatal care increased survival rates in the most severely affected sub-group of CDH babies and it was believed that no statistically significant increase in survival could be achieved by FETO in a study of its size (Harrison et al., 2003b). Though this trial was prematurely halted it still provided valuable insight into using FETO to treat foetuses with CDH, particularly with regard to refining the technique and managing its complications (most notably premature birth). The authors speculated that although FETO was a feasible approach to treat CDH prenatally, its advantages were counterbalanced by the adverse effects of premature delivery that it typically caused (Harrison et al., 2003b). Further refinement of the FETO technique continues today, led by the *FETO task group* in Belgium, and there is still hope that it will prove a successful way to manage CDH, particularly for those infants with the poorest prognosis (Deprest et al., 2005; Jani et al., 2006; Peralta et al., 2008).

1.5.4 CDH outcomes: mortality and morbidity

In recent history the outcome for CDH was very poor, with survival as low as 40% (Harrison et al., 1994). However at the break of the new millennium, Muratore and colleagues described a new era of CDH management and reports from

specialized, tertiary care centres achieved survival rates in excess of 80% (Muratore and Wilson 2000; Al-Shanafey et al., 2002; Downard et al., 2003; Bagolan et al., 2004). Despite the apparent impact of new therapies on the mortality of CDH these successes were questioned, with great emphasis placed on the impact of case selection bias, leading to the conclusion that the mortality of CDH is unchanged in the face of new therapies when complete case ascertainment is achieved (Stege et al., 2003). Whether the increase in overall survival rates for CDH is genuine, as recent studies uphold, careful breakdown of mortality data shows that survival rates are only increasing in those patients with isolated CDH and that the mortality rate for infants with associated abnormalities remains relatively unchanged (Gallot et al., 2006; Logan et al., 2007). It is also important not to forget the *hidden mortality* of CDH, the number of foetuses and neonates that die *in utero* with undiagnosed CDH (Harrison et al., 1994).

As more infants with CDH apparently survive the neonatal period, the threat of life-long morbidity becomes an increasing problem. Previous studies into the quality of life of CDH survivors indicate that they have a poor quality of life during the first year of life, but by the age of 16 their quality of life is comparable with the general population (Poley et al., 2004). However, as mentioned above, with improved neonatal intensive care there is a generation of critically ill infants who might normally have died in the neonatal period surviving into later life. These infants who might have had larger holes in their diaphragm, more lung damage, or who were hypoxic for longer periods of time, could be more prone to long-term

morbidity associated with CDH. Long-term follow up of these infants is therefore essential (West and Wilson, 2005).

The long-term morbidities associated with CDH are diverse; while pulmonary morbidity occurs most commonly there are also a number of extra-pulmonary morbidities in this population. After surgical repair of the diaphragm defect, infants with CDH continue to have poor pulmonary function; however adolescents have been shown to have only mild lung abnormalities. Retrospective studies show that significant improvement in lung function occurs after the first 6 months of life and that by 2 years only mild abnormalities in lung function remain, though these seem to persist throughout later life (Koumbourlis, et al., 2006). Thus, the hypoplastic lungs of infants with CDH recover in the first 2 years of life, but normal functional capacity is not fully attained. The most commonly occurring extra-pulmonary morbidity in CDH survivors includes hernia recurrence, musculoskeletal abnormalities, gastroesophageal reflux disease and sensori-neural hearing loss. With regard to hernia recurrence, no prospective studies examining the incidence of this problem have been carried out. Its occurrence seems to depend on how the hole in the diaphragm is repaired; for example, within three years of insertion, up to half of all prosthetic patches require revision due to re-herniation, whereas the recurrence rate for primary repair has been reported at ~22% (Cohen and Reid, 1981; Moss et al., 2001). Musculoskeletal malformations, including anterior chest wall deformities and thoracic scoliosis, also frequently occur. Their pathophysiology is not fully understood but these defects may arise from patch repair of the diaphragm, which do not grow with the infant (Nobuhara et al., 1996).

Gastroesophageal reflux disease (GER) is defined as spontaneous return of gastric contents into the oesophagus. It is a common but poorly understood morbidity associated with CDH, occurring in the majority of CDH survivors (Stolar et al., 1990; Koot et al., 1993; Koivusalo et al., 2008; Arena et al., 2008). GER contributes to a general nutritional morbidity in CDH survivors, which manifests as compromised growth and low body weight (Muratore et al., 2001). Sensori-neural hearing loss is a general phenomenon in survivors of severe neonatal respiratory failure, but it is particularly prevalent in the CDH population where its incidence is ~50-60% (Cheung et al., 1999; Robertson et al., 2002; Morini et al., 2008). Its etiology has been linked to the use of mechanical ventilation, ECMO, loop diuretics, and the muscle relaxant pancuronium bromide; reflecting such risk factors as hypoxia and the use of ototoxic drugs (Lasky et al., 1998; Cheung et al., 1999; Kuga et al., 2000; Masumoto et al., 2007; Morini et al., 2008).

1.5.5 Summary

In summary, early identification of CDH is possible by ultrasound, this allows for the severity of the condition to be assessed and preparations to be made for birth in a specialized care centre. Newborns with CDH require the full care of the neonatal intensive care unit; experimental procedures are constantly being developed in order to improve the outcomes of this high-risk group of patients. However, the impact of new therapies to manage CDH will take carefully controlled and conducted studies to reveal their true significance with regard to mortality and other outcome measures. Infants with CDH who survive the neonatal period are at risk of developing a variety of morbidities which can negatively impact later life;

long-term follow up in these individuals is important so they continue to receive appropriate care. Though advances are being made in the management of infants with CDH the incidence remains unchanged. As Langham stated *...the best solution is prevention*, a sentiment later echoed by Jesuadson who thought that *prevention of the birth defect...may represent the ultimate solution* (Langham et al., 1996; Jesuadson, 2002). For advances to be made in the prevention of CDH its cause must first be understood, the next section will discuss the current understanding of the etiology of CDH.

1.6 Etiology of CDH

Large population based studies estimate that the incidence of CDH, inclusive of foetuses and live births, is approximately 1:3000 (Torfs et al., 1992; Gallot et al., 2006; Yang et al., 2006). In all of these studies, the incidence of CDH did not vary during the study period (range: 5-18 years) and despite the large number of births recorded, they did not consistently identify any sub-sets of the population that were at increased risk of developing CDH. There are approximately 350,000 births per year in Canada; 45,000 of which are in Alberta (2006/2007 data; www.statcan.ca). Accepting an incidence of 1:3000, this implies that there are ~117 cases of CDH per year in Canada, ~15 of which occur in Alberta. With so many cases of CDH in Canada each year, what is known about the cause of CDH? As outlined below, the answer to this question is that the etiology of CDH is poorly understood and certainly requires further study.

1.6.1 Familial CDH

With regard to their cause, cases of CDH can be split into three categories: 1) familial CDH, 2) those with an identifiable genetic abnormality ("genetic CDH") and, 3) those with no identifiable genetic basis or apparent cause ("idiopathic CDH"). Familial CDH is relatively rare; its exact incidence is difficult to accurately ascertain though it is certainly very low (< 1%; Czeizel et al., 1985). For example, in a large population survey of over 700,000 total births, only one familial occurrence of CDH was observed (Torfs et al., 1992). The recurrence risk of having a second child with CDH is estimated to be less than 2% (Norio et al., 1984; Narayan et al., 1993; Pober et al., 2005). The mode of inheritance of CDH in family

pedigrees is unclear; while autosomal recessive inheritance has been implied in some pedigrees, there is convincing evidence that multifactorial inheritance is more likely (Norio et al., 1984; Czeizel et al., 1985; Ding et al., 2005). The exact etiology, genetic or otherwise, in any family pedigree has yet to be determined.

1.6.2 Genetic etiology of CDH

Although it concerns only a minority of cases, great progress has recently been made in understanding the genetics of CDH. This category includes cases of CDH associated with chromosomal abnormalities (structural and numerical), as well as cases associated with point mutations within individual genes. Genetic CDH accounts for approximately 10-20% of the CDH population (Torfs et al., 1992; Yang et al., 2006), although there is great heterogeneity even within this sub-group. The most commonly occurring numerical chromosomal abnormalities (aneuploidies) associated with CDH are trisomy 13 and trisomy 18; both of which produce a spectrum of congenital anomalies, including CDH, which are incompatible with life (Thorpe-Beeston et al., 1989; Torfs et al., 1992; Yang et al., 2006). CDH is also a frequently occurring abnormality in tetrasomy 12p (Pallister Killian syndrome; Lurie et al., 2003). With regard to structural chromosomal abnormalities (e.g. translocations, deletions, and inversions) a review by Lurie highlights the heterogeneity of genetic CDH; this author found chromosomal abnormalities in association with CDH in almost every human chromosome (Lurie et al., 2003). Subsequent analysis of specific chromosomal regions, deletion of which has repeatedly been associated with CDH, led to the identification of so called CDH-critical regions. The first of the CDH-critical regions to be identified and the best

characterized is located at 15q24 (found within the long arm of chromosome 15). Deletion of this part of chromosome 15 has been estimated to account for ~1.5% of CDH cases and is associated with a very poor outcome (Lurie et al., 2003; Biggio et al., 2004; Klaassens et al., 2005). Several other regions of the human genome have also been identified as putative CDH-critical regions, including 1q41-q42, 4p16.3, 8p23.1, and 8q22-q23 (Slavotinek et al., 2006; Holder et al., 2007). Lastly, CDH has also been associated with point mutations within several individual genes, for example *WT1* and *STRA6*. The Wilms tumour associated gene (*WT1*) is mutated in several congenital syndromes that include CDH within their spectrum of abnormalities, including Denys-Drash syndrome, WAGR (Wilms Tumour Aniridia Growth Retardation) syndrome, and Meacham syndrome (Devriendt et al., 1995; Reardon et al., 2004; Scott et al., 2005; Cho et al., 2006; Antonius et al., 2008). *STRA6* encodes the cell surface receptor for retinol binding protein (RBP); mutation in this gene has recently been associated with CDH on several occasions (Pasutto et al., 2007; Chitayat et al., 2007; Golzio et al., 2007).

In summary, the genetic etiology of CDH is heterogeneous with a wide variety of different genetic insults manifesting in diaphragmatic hernia. This heterogeneity makes the study of the genetic origins complex, particularly as some defects have only been reported in a handful of cases. Further, some genetic defects that have been reported more commonly do not always produce diaphragm defects. This incomplete penetrance points toward genetic redundancy or epigenetic factors that modulate the phenotype produced. In this regard, there is a paucity of data linking genotype with phenotype in CDH; correlations have been drawn between the

deletion/mutation of certain genes and the appearance of diaphragmatic hernia, however very few studies have been carried out to explore the mechanism by which a certain genotype leads to CDH.

1.6.3 Environmental factors in idiopathic CDH

As indicated above, genetic CDH accounts for the minority of CDH cases, with the remainder being largely idiopathic in origin. While some of these cases may have an unidentified genetic component, other factors related to the *in utero* environment are likely to contribute to the development of diaphragm defects. Large population based studies have failed to consistently identify a single factor which may contribute to the development of CDH (Torfs et al., 1992; Yang et al., 2006). A recent questionnaire study of parents of children with CDH examining possible exposure to environmental risk factors during early pregnancy identified a significant association with maternal alcohol consumption and CDH, however a subsequently published, and much larger study, found no association between alcohol intake and CDH (Felix et la., 2008; Yang et al., 2008). Interestingly, a survey of nutrient intakes in women who gave birth to infants with CDH concluded that certain aspects of periconceptual diet might be associated with the occurrence of CDH. Among several nutrients examined, the authors found some evidence to suggest that decreased intake of vitamin A/retinal was associated with an increased risk for CDH; however this link has to be balanced against the absence of high incidences of CDH amongst populations who are chronically vitamin A deficient (Yang et al., 2008). Regardless, this finding is of interest because there is convincing evidence from animal models, plus some human studies, that implicate

abnormal retinoid signalling in the etiology of CDH; the following section explores this hypothesis in detail.

1.6.4 Retinoid hypothesis of CDH etiology

Retinoids are small molecules with several important physiological roles, e.g. vision, cell proliferation and differentiation, and the immune system. The hypothesis that abnormal retinoid signalling might contribute to the development of CDH was comprehensively forwarded by Greer in 2003. Drawing on experimental evidence from the previous 50 years, the retinoid hypothesis was the first which accounted for idiopathic CDH that had a strong basis in experimental and clinical studies (Greer et al., 2003; a brief overview of retinoid metabolism and signalling is provided in figure 1.4). The following paragraphs will outline the evidence to support the retinoid hypothesis drawn from vitamin A deficiency studies in rats, retinoid receptor knock-out mice, the nitrofen model of CDH, and clinical evidence from human CDH.

1.6.4.1 Vitamin A deficiency studies

Some of the earliest evidence to suggest a link between retinoids and CDH came from studying the effects of vitamin A deficiency (VAD) during pregnancy in rats. Interestingly, several studies reported an increased incidence of diaphragmatic hernia (~25%) in the pups of rats which were fed the minimum amount of vitamin A required to sustain pregnancy (Andersen et al., 1941; Andersen et al., 1949; Warkany and Wilson, 1948). Further, it was also observed that a single bolus of vitamin A delivered mid-gestation could decrease the number of hernias observed (Wilson et al., 1953). Recently this model of VAD was revisited; this refined model

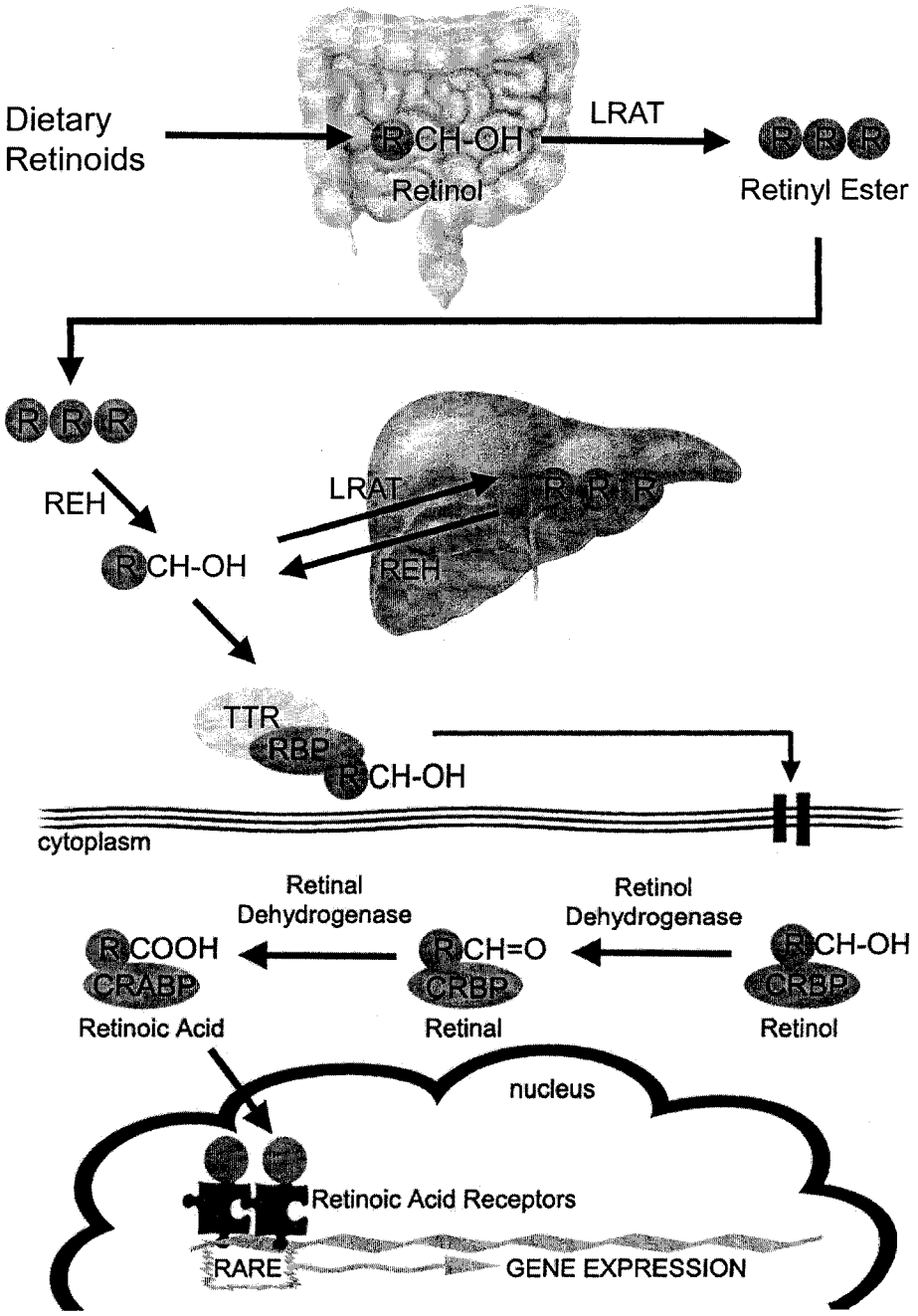

Figure 1.4 **Overview of retinoid signalling metabolism and signalling**
(figure legend overleaf)

Figure 1.4 Overview of retinoid signalling metabolism and signalling.

Retinoids are obtained from the diet in the form of retinyl ester (RE) and beta-carotene. Within the intestines these retinoids are converted into retinol then polymerized into RE by lecithin:retinol acyltransferase (LRAT) and packaged into chylomicrons for transport to the liver. Within the liver, retinol can be stored for future use in the form of RE or bound to retinol binding protein (RBP) for circulation throughout the body. The balance between bioavailable retinol and stored RE is achieved by the balance between retinyl ester hydrolase (REH) and LRAT activity. Circulating retinol-RBP is bound to the large protein transthyretin (TTR) which prevents it from being filtered out through the kidneys. Retinol is taken up by individual cells via the membrane bound RBP-receptor, STRA6. Within the cytoplasm of the cell, retinol is bound to cellular retinol binding protein (CRBP) and can be stored for future use (not shown) or converted into retinoic acid (RA). RA is synthesized by conversion of retinol to retinal (by cellular alcohol dehydrogenases) and then from retinal to RA. The conversion of retinal to RA is catalyzed by retinal dehydrogenase enzymes (RALDH) and is the rate limiting step in the synthesis of RA. Newly synthesized RA is bound to cellular retinoic acid binding protein (CRABP). Different CRABP isoforms can mediate the breakdown of RA into polar metabolites by CYP26A1 (not shown) or deliver RA to retinoic acid receptors (RARs) within the nucleus. RARs are bound to retinoic acid response elements (RAREs) within the promoters of target genes; binding of RA to RARs directly mediates the control of gene expression.

circumvented the high rate of resorption caused by VAD by providing exogenous all-trans retinoic acid during the critical period of heart development. Foetuses from mothers bred on this VAD diet (amongst an array of other abnormalities) had 100% incidence of diaphragm defects and, similar to previous studies, supplementation with retinol or retinoic acid decreased the number of observed defects (See et al., 2008).

1.6.4.2 Retinoid receptor knock-out mice

Retinoid receptors are steroid hormone nuclear receptors which mediate the action of retinoic acid by altering gene expression. There are two groups of retinoid receptors; retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both of which can be split into alpha, beta, and gamma subtypes. Combinations of different retinoid receptors bind as dimers to specific DNA sequences in the promoters of target genes, called retinoic acid response elements (RAREs), to control gene expression (Chambon, 1996). In the mid-1990's several groups set out to determine the biological function of the different retinoid receptors by targeted gene deletion and various combinations of knock-out mice were subsequently found to recapitulate all the hallmarks of VAD, including CDH (Mark et al., 1999). Careful examination of these mutant mice allows a genetic dissection of the retinoid receptors required for normal diaphragm development. For example, compound mutants for RAR_{α} and RAR_{β} have diaphragmatic hernia similar in phenotype to CDH seen in humans (Ghyselinck et al., 1997). These results suggest that RAR_{α} and RAR_{β} are both required for diaphragm embryogenesis. Diaphragm defects have also been observed in mice expressing a mutant copy of RXR_{α} which has a mutation in its ligand

binding domain preventing ligand-dependent activation of the receptor (Mascrez et al., 1998). This finding suggests that activation of RXR_{α} is required for retinoic acid dependent development of the diaphragm. Taken together these studies highlight the importance of signalling through the retinoid receptors in successful diaphragm development, with particular emphasis on RAR_{α}, RAR_{β}, and RXR_{α}.

1.6.4.3 Evidence from the nitrofen model

As described above, nitrofen exposure during pregnancy is a well established model for inducing CDH in rodents. However, nitrofen's mechanism of action has only recently been examined. Based on the hypothesis that antenatal Vitamin A treatment would improve lung growth in nitrofen exposed rat foetuses, Thebaud et al. found a decrease in the incidence of CDH in vitamin A treated litters (Thebaud et al., 1999). It was subsequently found that treatment with retinoic acid, instead of Vitamin A, was even more potent at rescuing nitrofen-induced CDH (Babiuk et al., 2004). These studies indicated that nitrofen may be acting by disrupting retinoid signalling. In a novel experiment using transgenic mice, nitrofen exposure was found to decrease activation of a RARE-lacZ transgene, which could be reversed by retinoic acid exposure, supporting the hypothesis that nitrofen suppresses retinoid signalling (Chen et al., 2003). Based on the above data it was further hypothesized that nitrofen may disrupt retinoid signalling by inhibiting production of retinoic acid by the retinoic acid-synthesizing enzyme retinal dehydrogenase (Raldh). This hypothesis was supported by *in vitro* data showing that nitrofen could inhibit retinoic acid production by Raldh2 in a dose-dependent manner (Mey et al., 2003). Further studies revealed that nitrofen could actually inhibit all three of the Raldh isoforms,

and also refuted the alternate hypothesis that it affected retinoid signalling by inhibiting receptor activity (Noble et al., 2007). Finally, high pressure liquid chromatography analysis of retinoid levels in nitrofen exposed rat embryos showed a decrease in retinoic acid levels following nitrofen exposure (Noble et al., 2007). Together these data support the hypothesis that nitrofen induces CDH by inhibiting production of retinoic acid, leading to a vitamin A deficient-like state, and decreased retinoid signalling activity. Similarly, the retinoid hypothesis suggests that CDH in humans may be caused by abnormal retinoid signalling, though there are limited clinical studies to support it.

1.6.4.4 Clinical evidence supporting the retinoid hypothesis

When it was first proposed, the primary piece of data linking abnormal retinoid signalling to CDH in humans was a small study comparing retinoid levels in infants with CDH, and their mothers, to age-matched controls (Major et al., 1998). This study found that infants with CDH had significantly lower levels of retinol and RBP compared to controls. Interestingly, the mothers of CDH babies had increased levels of retinol and RBP suggesting that maternal vitamin A deficiency was not to blame, but rather a congenital insufficiency in retinol transport across the placenta or its utilization by the foetus could have contributed to the development of CDH. A larger study looking at retinoid levels in infants with CDH is on-going and should confirm the link between decreased retinoid levels and CDH.

There is a general lack of genetic evidence from human cases of CDH to support the retinoid hypothesis; none of the CDH-critical regions of the genome so far identified contain genes directly associated with retinoid metabolism or

signalling. When one considers the absence of phenotype in single retinoid receptor knock-out mice and retinoid binding protein knock-outs, coupled with the severe phenotype of Raldh2 mutant mice, which is embryonic lethal, it is possible to conclude that similar deletions in humans would either be compensated for by genetic redundancy in the retinoid signalling pathway, or the result would be so deleterious that it was incompatible with further development (Mendelsohn et al., 1994; Lampron et al., 1995; Niederreither, et al., 1999). In saying that, there is one recent example which provides genetic evidence for a link between abnormal retinoid signalling and CDH in humans. The gene *STRA6* encodes the membrane receptor for RBP which facilitates the cellular uptake of retinol (Kawaguchi et al., 2007). There are 2 cases of infants with point mutations in *STRA6* causing multiple congenital anomalies including diaphragmatic hernia reported in the literature (Pasutto et al., 2007). As such there is some genetic evidence from humans with CDH to support a link between abnormal retinoid signalling and CDH.

1.6.5 Summary

In summary, there is a large body of evidence to support the hypothesis that abnormal retinoid signalling contributes to the development of CDH. What is clear from the above studies is that different insults to the retinoid pathway (such as vitamin A deficiency, Raldh inhibition, or retinoid receptor mutation) can all lead to abnormal diaphragm development. This supports the general concept that CDH can have multiple triggers and that a defect along any part of the retinoid synthetic and signalling pathway can lead to abnormal development. Future studies focusing on establishing the retinoid hypothesis in regard to CDH in humans will be important,

as will studies aimed at dissecting the exact role of retinoid signalling in diaphragm development, and what the downstream targets of this signalling pathway are.

1.7 Pathogenesis of CDH

The previous section was concerned with the etiology of CDH, in this section we will address the pathogenesis of CDH; specifically how the hole in the diaphragm forms. Historical hypotheses on the development of diaphragm defects will be discussed, as will current hypotheses inspired by data from animal studies. However, in order to prepare the reader we will start with a brief overview of our current understanding of normal diaphragm development.

1.7.1 Normal diaphragm development

The basic structure of the diaphragm is established early in gestation and is intimately linked with the formation of the body cavities of which it separates the thorax and abdomen. The process can be broken down into several steps which are outlined below. All of the gestational ages referred to are given for rat development unless otherwise specified.

1.7.1.1 Development of the Septum Transversum.

At embryonic day (E)8 the rat embryo begins gastrulation, forming an essentially flat trilaminar disc (3^{rd} to 4^{th} week in human gestation). Subsequent rapid growth of the cranial neural fold and invagination of the foregut radically changes the shape of the embryo. The presumptive septum transversum which was located at the anterior aspect of the embryo (where the visceral yolk sac and amnion meet) is displaced, such that its position relative to the heart changes from a rostral to a caudal position. In its final resting place, the septum transversum lies caudal to the heart and rostral to the umbilicus. Dunwoodie provides a detailed description of this

process in the developing rodent using the transcription factor *Mrg1* as a marker for the septum transversum (Dunwoodie et al., 1998).

1.7.1.2 Separation of the body cavities.

As a result of embryonic folding, the septum transversum lies in a position that partially divides the intra-embryonic cavity into the pleuro-pericardial cavity and the peritoneal cavity. In the following days, an intricate network of folds develops that separate the pleuro-pericardial cavity into distinct pleural and pericardial cavities. At this stage, the pleural cavity still communicates with the peritoneal cavity via the pleuro-peritoneal canals (PPCs; Wells et al., 1954). The separation of these two cavities occurs between E14 and E16 and represents the final stage in the formation of the basic foundation of the diaphragm. A series of scanning electron microscope images published by Kluth excellently illustrates the closure of the PPCs (Kluth et al., 1987; Kluth et al., 1996a; Kluth et al., 1996b).

The networks of folds that separate the body cavities are inter-related. In this article we use the term pleuro-peritoneal fold (PPF) to describe the transient structure formed at the union of the pleuro-pericardial folds and the septum transversum. As shown in figure 1-5, the PPFs are paired, pyramidal-shaped structures that project from the lateral body wall, fusing medially with the oesophageal mesentery. The PPF is fully formed by E13.5, which corresponds to weeks 5 to 6 in human embryogenesis. Whilst the PPF is really a structure which is bounded by the pericardium as well as the pleural and peritoneal cavity, we prefer the term PPF for its relative simplicity. Myogenic cells and phrenic axons destined to form the neuro-musculature of the diaphragm migrate to the PPF (figure 1-5), and

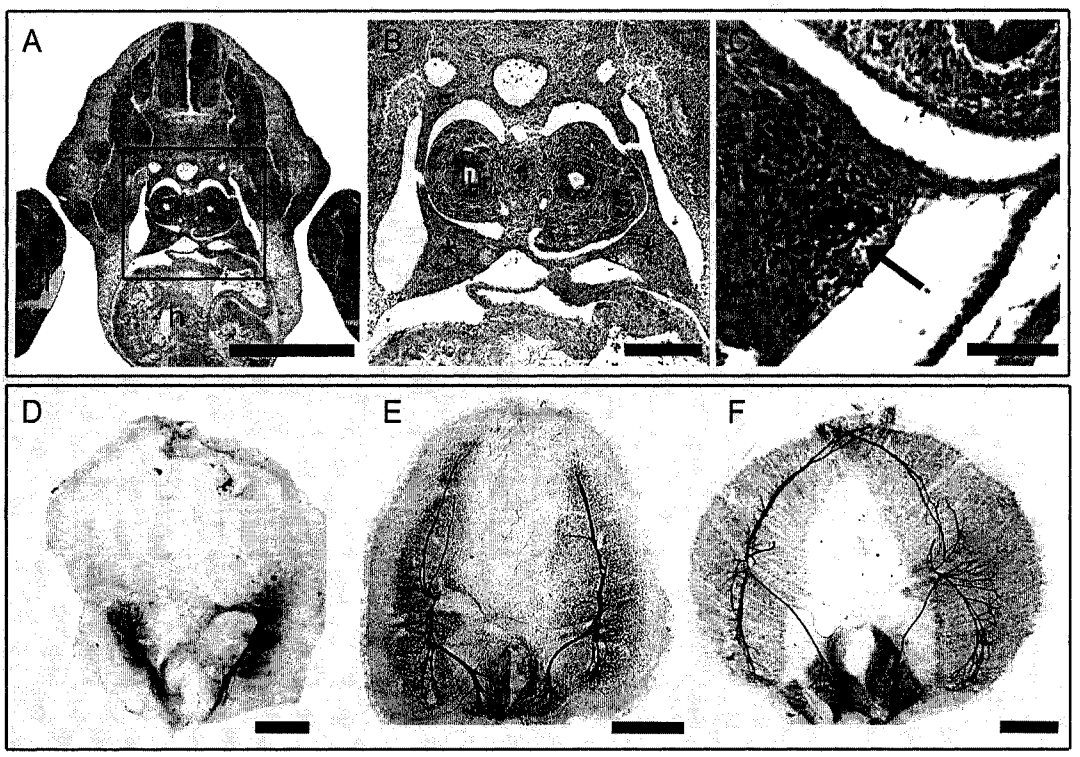

Figure 1.5 Embryological origins of the diaphragm.

A) Cervical transverse section of an E13.5 rat. B) The area enclosed by the box in A is shown at higher power illustrating the paired PPFs (*) and their triangular profile. C) Immunohistochemical staining for Pax3 and neurofilament labels muscle precursor cells and the phrenic nerve (arrow) within the PPF. D) Pax3 positive muscle precursors and the phrenic nerve can be seen in the whole diaphragm from the relative position of the PPF at E15. E) At E15.5 the neuromuscular component of the diaphragm has spread ventrally. F) At E17 the entire muscularised region of the diaphragm has been populated. (nt = neural tube, fl = forelimb, h = heart and lu = lung) Scale bars: A = 1 mm, B = 200 μm, C = 100 μm, D = 500 μm, E-F = 1 mm.

it is their proliferation and distribution from this point that contributes to the formation of the mature diaphragm (Babiuk et al., 2003).

1.7.1.3 Diaphragm Muscularisation.

This is the stage of diaphragm development that is perhaps best understood and has undergone considerable revision from earlier attempts to describe it. It was previously thought that the musculature of the diaphragm was derived from the muscular layers of the body wall, and this view still permeates the literature today (Skandalakis et al., 1994; Robertson et al., 2006). However, with the recent ability to immunologically stain developmentally controlled proteins and the widespread use of transgenic mice, it has become possible to closely follow the process of diaphragm muscularisation and elucidate its true origin. It initially became clear that the musculature of the diaphragm had a distinct origin than that of the body wall when the tyrosine kinase receptor *c-met* was inactivated in mice (Bladt et a., 1995). The receptor-protein encoded by *c-met* is essential for the delamination and migration of muscle precursor cells (MPCs) from the somites (reviewed by Birchmeier and Brohman, 2000), *c-met* null-mutant mice notably have an amuscular diaphragm, yet the muscles of the body wall are normal. The phenotype of *c-met* null-mutant mice suggested that the diaphragm was populated by a distinct, migratory, population of MPCs, this conclusion was further substantiated when it was found that diaphragm MPCs express *Lbx1*, a transcription factor only expressed in migratory MPCs (Gross et al., 2000). Thus, rather than being a derivative of the ventrally projecting part of the hypaxial myotome (which forms the body wall musculature) the diaphragm is formed by a migratory population of MPCs

originating from the lateral dermomyotmal lip, analogous to the MPCs which populate the limb (Birchmeier and Brohman, 2000). Further, immunological analysis of diaphragm muscularisation in the rat revealed no contribution of MPCs from the body wall (Babiuk et al., 2003). From this study it is apparent that muscle precursors cells which have migrated to the PPF, proliferate and radiate out from the relative position of the PPF within the diaphragm to populate the entire structure, this process takes place between E15 and E17 of rat gestation and is illustrated in figure 1-5. In parallel to the muscularisation of the diaphragm, the phrenic nerve also projects to the PPF, from which point it trifurcates and its collateral branches innervate the entire diaphragm (Greer et al., 1999).

In summary, during the process of embryonic folding and separation of the body cavities the basic structure of the diaphragm is formed. At this early age the thorax and abdomen are divided ventrally by the septum transversum and dorsally by the growing PPFs. The PPFs are subsequently populated by migratory muscle precursor cells and the phrenic nerve, it is from this relative position in the diaphragm rudiment that these components spread out to populate the entire tissue. This process is complete by E17 of rat gestation and is shortly followed by the commencement of foetal breathing movements (Kobayashi et al., 2001). Studies into the maturation of the motor units within the diaphragm during the perinatal period are described elsewhere (Mantilla et al., 2008).

1.7.2 Historical perspectives on CDH pathogenesis

As their understanding of normal diaphragm embryogenesis improved, early investigators studying CDH began to speculate on the origins of diaphragmatic

hernia. One of the earliest (and most persistent) explanations for the development of CDH was first proposed by Harrington who posited that diaphragmatic hernia forms when the abdominal contents herniate through the pleuro-peritoneal canals (PPCs) to impede lung development (Harrington, 1948). Several hypotheses were subsequently offered to explain the persistence of the PPC including: i) failure of the PPC to close properly, ii) delayed closure of the PPC allowing the returning abdominal contents entrance into the thorax, or iii) early return of the abdominal contents, preventing PPC closure (please note that early in gestation the intestinal loops grow partially outside of the abdominal cavity and are drawn back in as the abdominal cavity grows to accommodate them; Harrington, 1948; Holder et al., 1979; Skandalakis et al., 1994). Some authors went on to try and explain why the pleuro-peritoneal membranes did not grow enough to close the PPC, such as inadequate innervation via the phrenic nerve (Iritani, 1984; Kluth et al., 1996b). In one counterintuitive reversal of the dogma that a patent PPC led to herniation of the abdominal viscera and subsequent poor lung development, it has been proposed that a primary defect in early lung development could lead to abnormal diaphragm development (Iritani, 1984; Skandalakis et al., 1994). Another alternate hypothesis to the idea that CDH was caused by failure of the PPCs to close was based on the concept that muscularisation of the diaphragm was incomplete and that congenital weak-points would rupture to produce holes in the diaphragm (Gray et al., 1972).

The initial hypothesis that CDH was caused by failure of the PPC to close was based on observations of defect location and incorporated then recent advances in the understanding of normal diaphragm development. Subsequent speculation on

the cause of this failed closure and related hypotheses had little experimental basis, though the concepts persist in the literature and are found in current medical textbooks (Kluth et al., 1996b; Sadler et al., 2004; Gallot et al., 2008). The characterization of the nitrofen model and advances in modern scientific techniques has provided an opportunity for the early embryonic origins of diaphragmatic hernia to be systematically studied. As detailed below, data obtained from the nitrofen model (and more recently mutant mouse models) has refuted these traditional hypotheses and new hypotheses with a sound experimental basis have evolved.

1.7.3 Lessons from animal models and CDH in humans

It is one of the basic tenants of science that when a hypothesis is formulated it should be rigorously tested and duly upheld or found to be wanting. The use of animal models and careful observation in case reports of human CDH allowed the hypothesis that CDH arises from a failure of the PPC to close to be systematically tested.

It became obvious from initial studies into the pathogenesis of diaphragm defects in the nitrofen model that patent PPCs were not to blame. Studies using scanning electron microscopy revealed that not only were the PPCs too small to allow passage of the intestinal loops into the thorax, but it was also apparent that malformations in the postero-lateral diaphragm were anatomically distinct from the PPC (Kluth et al., 1993; Kluth et al., 1996a; Allan and Greer, 1997). Further, careful review of CDH case reports yields several instances describing the co-occurrence of diaphragmatic hernia and omphalocele (Carmi et al., 1990; Donnai and Barrow, 1993; Bird et al., 1994). It is hard to imagine the intestinal loops preventing closure

of the PPC when these loops remain outside of the abdomen, as occurs in a foetus with omphalocele. Further studies into the pathogenesis of CDH in the nitrofen model demonstrated that abnormal phrenic nerve innervation or myotube formation were not responsible for the diaphragm defect as had been previously suggested (Allan and Greer, 1997). Subsequent studies combining the nitrofen model and transgenic mice were also inconsistent with the hypothesis that the primary defect in CDH is pulmonary, such that abnormal lung development somehow induces the diaphragm defect (Babiuk and Greer, 2002).

This hypothesis implied that primordial diaphragm embryogenesis is regulated or influenced directly by the development of the adjacent lung tissue (Iritani, 1984). Transgenic mice with *Fgf10* inactivated do not develop lung tissue and therefore provided an excellent tool to address this issue (Sekine et al., 1999). Despite having essentially no lungs, *Fgf10* null-mutant mice have normal diaphragms. Further, defects in the diaphragm can also be induced by teratogen exposure in the absence of lung tissue. Thus it was concluded that diaphragm development is independent from lung organogenesis and that diaphragm defects in CDH are primary and are not a secondary result of lung hypoplasia (Babiuk and Greer, 2002; Arkovitz et al., 2005). This conclusion is supported by so-called *experiments of nature*; the literature contains numerous cases reports of lung agenesis in humans with normal diaphragm development (Podlech et al., 1995; Gabarre et al., 2005; Sharma et al., 2005).

In addition to refuting several historical hypotheses on the origin of CDH, data from the nitrofen model provided a foundation for the alternate hypothesis that

it is a malformation of the primordial diaphragm tissue, the PPF, which underlies the pathogenesis of CDH. The PPF is best visualized at E13.5 of rat gestation. In nitrofen exposed foetuses examined at this age it is clear that the postero-lateral portion of the PPF is malformed (Figure 1.6). Notably, it is the postero-lateral area of the diaphragm that is missing in older rodent foetuses, and in humans with CDH. The identification of an abnormal PPF in nitrofen exposed foetuses meant that the embryogenesis of this structure became a major focal point towards elucidating the pathogenesis of CDH. Studies examining muscle precursor migration to the PPF and subsequent proliferation and differentiation of myoblasts in nitrofen exposed rodents did not reveal any obvious abnormalities in myogenesis, suggesting that it was the non-muscular substratum of the PPF that was abnormal (Allan and Greer, 1997). This has lead to a novel hypothesis, proposed by our laboratory, that the mesenchymal component of the PPF is defective and does not provide a complete foundation for the formation of the diaphragmatic musculature. This hypothesis was tested using *c-met* null-mutant mice which have a diaphragm with no muscle and offer the opportunity to clearly visualize the formation of the non-muscular component of the diaphragm (Bladt et al., 1995). Examination of teratogen exposed *c-met* null-mutant mice demonstrated that diaphragm defects can be produced independently of myogenesis (Babiuk and Greer, 2000). As such, we believe that muscle precursor cells migrating to a malformed PPF accumulate in the remaining normal tissue and that this manifests as a thickening of the diaphragm around the defect, an observation made from the nitrofen model (Allan and Greer 1997). The hypothesis that abnormal development of the PPF underlies CDH is in contrast to

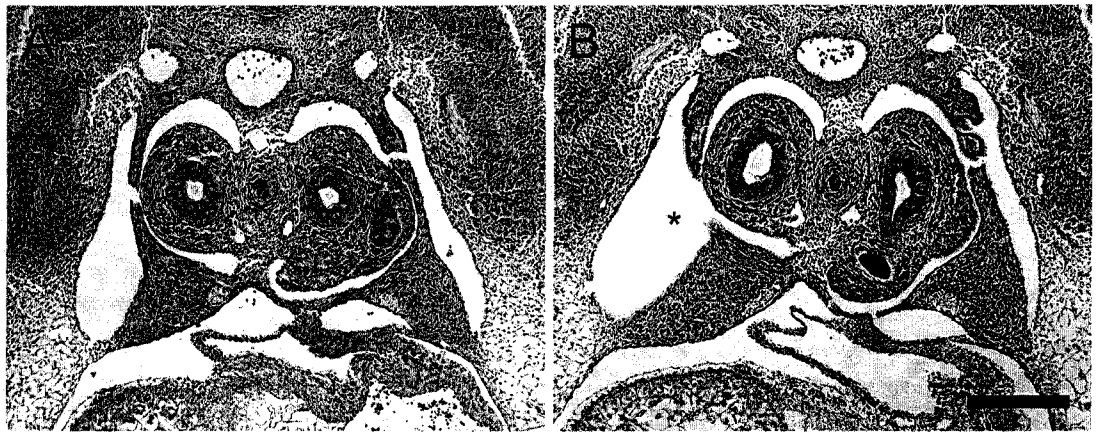

Figure 1.6 A defect in the PPF underlies nitrofen-induced CDH.

A) High magnification image of the PPFs of the rat at E13.5. B) Similar view of an E13.5 rat embryo exposed to nitrofen showing a large defect in the PPF (*). Scale bar = 200 μm.

the other theories propounded to explain the pathogenesis of CDH. However, like the hypotheses that preceded it, this new concept of CDH pathogenesis has to be tested and firm links made between observations from animal models and CDH in humans.

1.7.4 Summary

In summary, the normal embryogenesis of the diaphragm is a complicated process that occurs very early in gestation and is intimately linked with the separation of the body cavities. Early hypotheses attempting to explain the pathogenesis of CDH have not been supported by experimental observations; rather a new hypothesis which implicates a defect in the PPF has been proposed and remains to be tested.

1.8 General summary

The purpose of the preceding sections were to provide the reader with an overview of research in the field of CDH research, with particular emphasis on the pathogenesis and etiology of diaphragm defects associated with this condition. There is an extensive literature focused on the pathophysiology of lung defects in CDH described elsewhere (see the following reviews: Thebaud et al., 1998; Chinoy, 2002; Smith et al., 2002; Ackerman and Pober 2007; Kinane, 2007). It should be apparent that CDH is a serious birth defect; its frequent occurrence, resistance to treatment, and high morbidity and mortality, makes research into its development of paramount importance. While progress has been made into identifying the embryologic origins of the diaphragm defect in the nitrofen model, further research is required to determine if this phenomenon occurs in other CDH models and whether it is applicable to CDH in humans (see chapter 3). Further, as our focus shifts to the early embryogenesis of the diaphragm, the role of retinoid signalling in the developing diaphragm (chapter 6) as well as the mechanism by which diaphragm abnormalities arise (chapter 5) need to be understood. Equally, as our knowledge of the genetics of CDH in humans improves, it will be valuable to integrate this data into our animal models in order to generate and test hypotheses which will further improve our understanding of CDH (chapter 4). The following chapters will address these issues in turn.

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Chapter 2: General methods

Explanatory note: the purpose of this chapter is to describe some of the basic methodologies employed during the course of the research presented in this thesis. Those methods that were commonly used in later chapters are entered here in order to prevent repetition; subsequent chapters still contain method sections to describe those techniques unique to that particular project.

2.1 Animals and tissue collection

Timed-pregnant Sprague-Dawley rats were used for all experiments unless otherwise stated. A colony of rats was housed within the animal breeding facility at the University of Alberta and all experiments were carried out in accordance with the guidelines established by the animal welfare committee at this institution. To obtain pregnant females, animals were set up for overnight breeding. Noon of the day on which a sperm plug was observed in the breeding cage was considered as embryonic day (E) 0.5. Embryonic and fatal tissue was collected on the desired day by caesarean section. Animals were anesthetized with halothane or isoflurane (~3% delivered in 95% 0_2 and 5% CO_2) prior to surgery and a laparotomy was performed to gain access to the abdominal cavity and the gravid uterus. The crown-rump length of isolated tissue was measured to confirm gestational age; for rat tissue a previously published growth-curve was used (Angulo Y Gonzalez, 1932), for mouse tissue we generated our own growth-curve using data from multiple sources (Gruneberg, 1943; Rugh, 1964; Thieler, 1972; Rugh, 1990; Kaufman, 1995; see figure 2.1). Collected embryos were decapitated and the hindquarters removed. The remaining trunk was then fixed by immersion in 4% paraformaldehyde (PFA) for at least 24 hours at 4°C. For foetuses, a dissecting microscope (Leica Wild M3C; Wetzlar, Germany) was used to isolate the whole diaphragm which was then rinsed in phosphate-buffered saline (PBS) and fixed in 4% PFA.

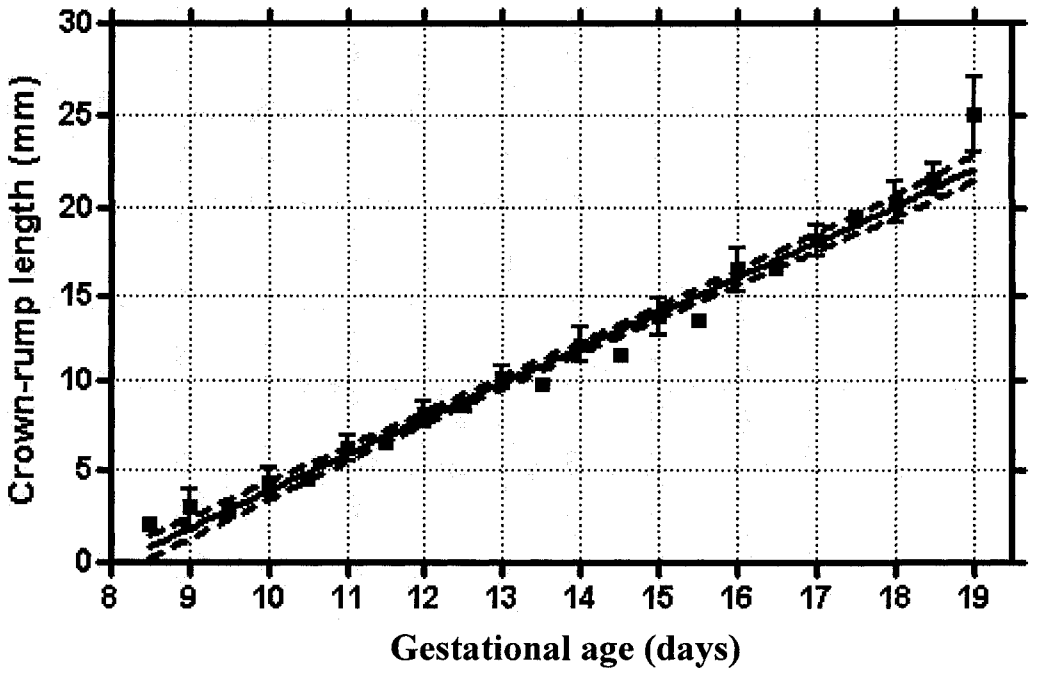

Figure 2.1 Mouse Growth-curve.

Growth-curve used to correlate crown-rump length with gestational age. Black squares represent the mean crown-rump length at a given age (n = 2-6). Black line represents a curve fit with non-linear regression (polynomial: first order [straight line]), dashed lines represent the 95% confidence interval.

2.2 Nitrofen administration

Nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether; obtained from the China National Construction Jiangsu Company [Nanjing, China]) was originally developed as a herbicide. However it was later found to induce CDH in the off-spring of pregnant rats treated with this compound between E8 and E11 (Costlow and Manson, 1981). Unless otherwise stated, we administered 100 mg of nitrofen, dissolved in 1 ml of olive oil, at noon on the 8^{th} day of gestation (~E8.5). The nitrofen was administered to lightly anesthetized rats by oral gavage.

2.3 Tissue preparation and basic histology

Isolated tissue was dehydrated in a graded series of ethanol (50%; 75%; 90%; 95%; 100% Ethanol) and cleared with xylene before being embedded in molten paraffin wax. Serial, transverse sections of paraffin embedded tissue was cut using a rotary microtome (Leica RM2135; ; Leica Microsystems, Wetzlar, Germany) at a thickness of 8 to 10 μm, which were then mounted on pre-subbed glass slides. For basic histology the slides were rehydrated and counterstained with haematoxylin and eosin. Following tissue staining, slides were dehydrated using a generated series of ethanol and cover-slipped with mounting medium.

2.4 Basic immunohistochemistry protocol

Multiple different antibodies were used in the studies described in this thesis; below is the basic immunohistochemistry protocol used in these experiments. The methods section of each chapter contains details regarding specific antibodies used. All immunohistochemistry experiments were repeated in triplicate, including negative controls, to confirm the pattern of staining, and representative examples were chosen for publication.

Sections were dewaxed in xylene and then rehydrated using a graded series of alcohol. Sections were rinsed in PBS, microwaved in 0.01 mol/L sodium citrate buffer (pH 6) at 600 W for 5 minutes, and then pre-treated with 1% hydrogen peroxide in 100% methanol for 30 minutes. Sections were treated with 1% bovine serum albumin (Sigma, St. Louis, MO) in 0.4% Triton X-100/PBS for 30 minutes before incubation with the appropriate primary antibody. All primary antibodies were diluted in PBS with 0.1% bovine serum albumin and 0.4% Triton X-100; the antibodies were left on the sections to incubate overnight at room temperature. After incubation with primary antibodies two different approaches were used to visualize primary antibody localisation: i) chromogenic visualization and ii) fluorescent visualization (preferred for double-labelling). For chromogenic visualization, sections were thoroughly washed with PBS and incubated with a biotin conjugated secondary antibody for 60 min at a 1:200 dilution. The slides were then washed in PBS and incubated with a 1:100 avidin-biotinylated peroxidase complex solution (ABC kit, PK4000, Vector Laboratories, Burlingame, CA) for 60 min. A final wash step was followed by antigen visualization via Nickel intensified 3,3-

Diaminobenzidine tetrahydrochloride (DAB) labelling (0.04% DAB, 0.04% hydrogen peroxide, 0.6% ammonium nickel sulphate, in 0.1 mol/l Tris buffer). For fluorescence, sections were washed with PBS and incubated with a fluorophore-conjugated secondary antibody diluted in PBS and 0.1% bovine serum albumin for 2 hours. After incubation with the secondary antibody, sections were further washed in PBS and cover slipped with Fluorsave mounting medium (Calbiochem, San Diego, CA). DAB-labelled tissues were visualized using bright-field microscopy and fluorescent-stained sections were visualized using confocal microscopy.

On occasions when double-labelling was being carried out for two antibodies from the same species of origin, immunohistochemistry was performed using a tyramide signal amplification kit (PerkinElmer Life Sciences, Boston, MA). In brief, sections were pre-treated in 3% H_2O_2 in PBS, washed in TNT buffer (0.1 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, and 0.05% Tween 20), and incubated in TNB-T (0.1 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, 0.5% bovine serum albumin, and 0.3% Triton X-100). Sections were incubated with the first primary antibody in TNB-T overnight. After several washes in TNT, sections were incubated for 2 hours in biotinylated donkey anti-rabbit (1:200). Biotin was revealed using the tyramide signal amplification kit. Sections were incubated with streptavidin-horseradish peroxidase (1:150) for 30 minutes followed by tyramide conjugated to fluorescein (1:75), diluted in amplification diluent, for 10 minutes. After labelling for the first primary antibody, sections were extensively washed in TNT and incubated for 30 minutes in TNB-T followed by the second primary antibody (1:250) in TNB-T overnight. The following day, sections were incubated with donkey anti-rabbit-Cy3

(1:200) in TNB for 2 hours, washed, mounted on slides, and cover slipped with Fluorsave solution.

2.5 Microscopy

Various microscope set-ups were used to examine tissue and obtain digital photographs for publication; all digital images and figures were prepared for publication using Adobe Photoshop 6.0 (Adobe Systems, Mountains View, CA).

2.5.1 Bright-field microscopy

Bright-field microscopy was used to examine and photograph free-floating tissues as well as to examine and photograph haematoxylin and eosin stained, and DAB-stained tissue sections. Free-floating diaphragm and lung tissue was examined using a dissecting microscope (Leica Wild M3C) with a digital camera attachment. Digital photographs of free-floating diaphragms were taken using a Nikon 990 digital camera (Nikon, Tokyo, Japan). Stained tissue sections were examined using a conventional optical microscope (Olympus BX40; Olympus corp., Tokyo, Japan) and photographed using the SPOT imaging suite (Diagnostic Instruments, inc.; Sterling Heights, MI).

2.5.2 Confocal microscopy

Immunostained sections were scanned with a Zeiss Axioplan microscope using an LSM 510 NLO laser configured to a computer running LSM 510 software (Zeiss, Jena, Germany). For Cy3 fluorescence, excitation (HeNe, 1 mV) was set to 543 nm, and emissions were collected using a 560-nm long-pass filter. For Cy5 excitation (HeNe, 1 mV) was set to 633 nm, and emissions were collected using a 630-nm long-pass filter. For Alexafluor 488 and fluorescein, excitation (Argon, 40 mV) was set to 488 nm, and emissions were collected with a 505 nm long-pass filter.

For DAPI, a two-photon laser was used with excitation set to 780 nm and emissions collected using a 390 nm to 465 nm band pass filter.

2.6 Statistics

As specified in the appropriate chapters, three types of statistical analysis were used in this thesis. A paired t-test was used when comparing the population mean of two matched groups obtained from paired observations from the same sample set, Student's unpaired t-test was used to compare the means of two independent populations, and a one-way analysis of variance (ANOVA) was used to test for differences amongst three or more independent groups; Tukey's multiple comparisons post-hoc analysis was used to determine where significant differences were found. For all analyses a p-value < 0.05 was considered as being statistically significant. Data were compiled using Excel (Microsoft Office Excel; Microsoft Corporation, Redmond WA) and imported into GraphPad Prism (v4; GraphPad Software Inc., San Diego, CA) for statistical analysis and to generate graphs for publication.

2.7 References

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Chapter 3: Teratogen-induced, dietary and genetic models of congenital diaphragmatic hernia share a common mechanism of pathogenesis.

This chapter was adapted from the original publication by:

Clugston RD, Klattig J, Englert C, Clagett-Dame M, Martinovic J, Benachi A, Greer JJ.

American Journal of Pathology. 2006 Nov; 169(5):1541-9.

Contributions: The majority of the experimental work described in this paper was performed by RDC, with technical assistance from Wei Zhang. JK, CE, MC-D, JM, and AB were involved in tissue collection which was sent to the Greer Laboratory for analysis. The manuscript was written by RDC and edited by JJG.

3.1 Introduction

Congenital diaphragmatic hernia (CDH) is a severe developmental defect occurring in approximately 1 in 2500 live births and accounting for ~8% of all major congenital anomalies (Tonks et al., 2004; Doyle et al., 2004). Reports of the CDH mortality rate range from 32 to 62% (Stege et al., 2003; Lally et al., 2006), with some specialized, tertiary care centres achieving survival rates in excess of 80% (Al-Shanafey et al., 2002; Downard et al., 2003; Bagolan et al., 2004). There is significant long-term morbidity among survivors (Muratore et al, 2001; Poley et al., 2004; Traschel et al., 2005). The majority of CDH cases (>95%) involve incomplete formation of the posterolateral portion of the diaphragm, clinically referred to as a Bochdalek hernia, most commonly occurring on the left side (Torfs et al., 1992). The hole in the diaphragm allows the abdominal viscera to invade the thoracic cavity, thereby impeding normal lung development. Lung hypoplasia and concomitant pulmonary hypertension contribute to the high mortality rate and morbidity associated with CDH (Harrison et al., 1994).

The pathogenesis and etiology of CDH are not well understood. Some insights have been derived from studies of teratogen-induced CDH in rodent models, particularly the nitrofen model. The defect in this model can be traced back to a malformation of the amuscular component of the primordial diaphragm, the pleuroperitoneal fold (PPF; Allan and Greer, 1997; Babiuk and Greer, 2002). However, as Gosche stressed in a recent commentary, further advances will depend on experimental data derived from additional animal models (Gosche et al., 2005).

Specifically, a case for the primary diaphragm defect being linked to a malformation of the PPF will be greatly enhanced if a common pathogenesis is discovered in different animal models. In this study, we took an important step in that direction by analyzing and comparing diaphragm defects in the well-characterized nitrofen model with vitamin A-deficient (VAD) rats and Wilm's Tumor 1 (*Wt1*) mutant mouse models of Bochdalek CDH. Furthermore, we examined post-mortem CDH diaphragm tissue to determine whether the pattern of muscularisation is consistent with the proposed pathogenic mechanism discovered in animal models.

Diaphragm defects in VAD rats were initially observed over 50 years ago (Andersen et al., 1941, Andersen et al, 1948; Warkany et al., 1948). Furthermore, data from the teratogenic CDH models provided evidence for a role of retinoid signalling in the etiology of CDH (reviewed by Greer et al., 2003). In this study, we used a modified VAD paradigm to examine the pathogenesis of the diaphragm defect in this model of CDH (White et al., 2000).

The past 10 years have seen several reports of diaphragm defects in genetically engineered mutant mice, introducing potential genetic models for the study of this disease and further insight into its genetic etiology. Bochdalek diaphragmatic hernias have been observed in Wilms' Tumor 1 (*Wt1*) null-mutant mice, retinoic acid receptor (*Rar*) α and *Rarβ2* double null-mutant mice, *MyoR* and *Capsulin* double null-mutant mice, and most recently in mice with *Coup-tfII* conditionally inactivated in *Nkx3-2*-expressing tissues (Kreidberg et al., 1993; Mendelsohn et al., 194; Lu et al., 2002; and You et al., 2005). *Wt1* null-mutant mice were originally created to study the role of *Wt1* in urogenital development,

diaphragm defects being only an incidental observation (Kreidberg et al., 1993). The *WT1* gene, mutated in 10 to 15% cases of human Wilms' Tumor, encodes a DNA-binding protein with four zinc fingers and is thought to function as a transcription factor, as well as having a role in RNA processing (Brown et al., 2001). We chose to focus on *Wt1* null-mutant mice in this study because it is expressed in the developing PPF of humans (Pritchard-Jones et al 1990; see Figure 2i) and because there have been several descriptions of congenital syndromes in humans that include CDH within their spectrum of abnormalities and have their origins in *WT1* mutations. These syndromes include Denys-Drash syndrome, Wilms tumour aniridia growth retardation (WAGR) syndrome, and Meacham syndrome (Devriendt et al., 1995; Reardon et al., 2004; Scott et al., 2005). We hypothesized that the anatomical hallmarks of the teratogenic, VAD, and *Wt1* models are the same; specifically, that the diaphragm defects are 1) in the foramen of Bochdalek, 2) can be traced back to abnormalities in the PPF, and 3) are consistent with initial defects arising in the amuscular component of the PPF.

3.2 Materials and Methods

A description of the basic methodologies concerning rat breeding, tissue collection, nitrofen treatment, basic histology, immunohistochemistry, and microscopy can be found in the relevant section of chapter 2. The following sections concern methods only employed in this study and are not described elsewhere in this thesis.

3.2.1 Vitamin A-Deficient Rats

A full description of the technique to render animals VAD has been previously described (White et al., 1998). In brief, weanling female rats (Harlan-Sprague-Dawley) were fed a special diet devoid of all forms of vitamin A to render them VAD and were subsequently recovered on a limiting amount of all-*trans* retinoic acid (atRA; 12 μg/g diet) before mating. Pregnant rats from all groups received 12 μg/g diet atRA starting at E0.5 until day E8.5. Animals in the first control group continued on a diet of 12 μg/g diet atRA, which was supplemented with 100 units/day of retinyl palmitate after E8.5. A second group of control animals received 250 μg/g diet atRA between E8.5 and E10.5 and were then transferred to a diet containing a combination of atRA and retinyl palmitate (12 μg/g diet and 100 units/day, respectively). VAD animals also received 250 μg/g diet atRA between E8.5 and E10.5 but then received only a 12-μg atRA/g diet. Note that E8.5 to E10.5 represents a critical period during rat gestation that requires a high dose of dietary atRA to support later embryonic survival (White et al., 2000). A complete description of this late embryonic VAD model will be presented elsewhere (See et al., 2008).

3.2.2 Wt1 Null-Mutant Mice

Wt1 was functionally knocked out by the insertion of the *neo*-resistance cassette into the first exon of the gene on a C57BL/6 mouse background; a full description of how these mice were generated has previously been published (Kreidberg et al., 1993). In these experiments, we used animals from a mixed MF1 × C57BL/6 background (Herzer et al., 1999).

3.2.3 Three-Dimensional Modelling

A complete series of consecutive sections spanning the rostral to caudal extent of the PPF were digitally photographed. Each section was 10 μm and was prepared in the same way as described in the histology section. Three-dimensional (3-D) models were created using Rhinoceros-3D modelling software (Seattle, WA). A curve outlining the PPF was made from each image; these curves were then collated to create a wire frame model of the PPF to which a surface was digitally added, thus creating an accurate 3-D rendering of the PPF. For clarity, the oesophageal mesentery was excluded when outlines of the PPF were being generated.

3.2.4 Immunohistochemistry

A list of antibodies used in this study can be found in table 3.1, a description of the basic immunohistochemistry protocol used can found in section 2.4.

3.2.5 Human Tissue

In accordance with French law, institutional ethics policy, and parental consent for unrestricted autopsy and genetic testing, post-mortem diaphragmatic tissues from human cases of CDH, were obtained by the Department of Embryo-

Table 3.1 Table of antibodies used

Antibody	Dilution	Source (Catalogue number)
Mouse monoclonal antibodies:		
Wt1 (6F-H2)	1:50	Dako (M3561)
Coup-tfII	1:250	PPMX (H7147)
Goat polyclonal antibodies:		
Pax3/7 (C-20)	1:100	SCBT (sc-7748)

PPMX: Perseus Proteomics, Tokyo, Japan; SCBT: Santa Cruz Biotechnology, Santa Cruz, CA; Dako Canada, Mississauga, ON

Fetopathology, Hopital Necker-Enfants Malades (Paris, France) were removed at autopsy and fixed in 10% phosphate-buffered formalin before further examination.

3.3 Results

3.3.1 Characterization of Diaphragm Defects

The first stage of the study was to characterize the defects in the diaphragm of the various CDH rodent models. E16.5 foetuses removed from nitrofen-treated rats, VAD rats, and *Wt1* null-mutant mice were used to study the whole diaphragm. These animals all appeared grossly normal compared with control embryos. Dissection of the thoracic cavity revealed hypoplastic lungs and abdominal contents protruding into the cavity through a hole in the diaphragm (data not shown). Figure 3.1 illustrates a representative selection of diaphragm defects observed, all of which were positioned posterolaterally, in the foramen of Bochdalek. No diaphragm defects were observed in control animals. Treatment with 100 mg of nitrofen on E8 yielded posterolateral diaphragm defects in ~50% of offspring, the majority of which were on the left side; this was consistent with previously published data (Allan and Greer, 1997). Diaphragm defects were observed in all of the VAD foetuses examined (n = 5), and the majority of the diaphragm defects were on the right side or were bilateral, i.e., holes in the left and right posterolateral portion of the diaphragm. Left-sided posterolateral defects were also observed in *Wt1* null-mutant tissue (n = 3); however, due to the small number of foetuses examined, an accurate incidence of diaphragmatic hernia in these mice could not be calculated, although it should be noted that not every *Wt1* null-mutant foetus had a diaphragm defect.

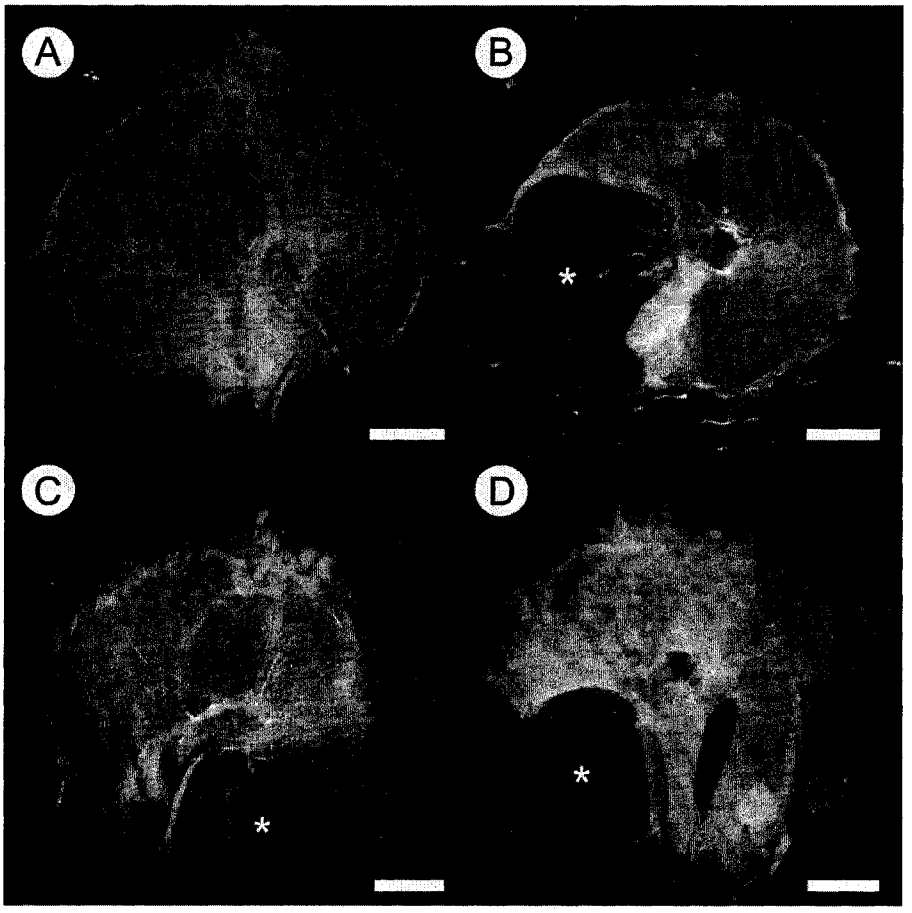

Figure 3.1 Representative examples of diaphragm defects in teratogen-induced, dietary, and genetic models of CDH.

Photomicrographs of whole diaphragms isolated from control (A), nitrofen-treated rat (B), VAD rat (C), and Wt1 null-mutant mouse (D), showing representative examples of Bochdalek diaphragm defects (*). Diaphragms are oriented such that the top of the image is anterior and the bottom of the image is posterior. Scale bar = 2 mm.

The characterization of the defects in rodent models of CDH was complemented by examination of human diaphragms collected at autopsy from foetuses of varying gestational age. A normally developed diaphragm at 35 weeks of gestation is shown for comparison with a diaphragm isolated from a foetus at 34 weeks of gestation with a large left-sided diaphragm defect; another diaphragm with a large left-sided defect is also shown after 30 weeks of gestation (Figure 3.2).

3.3.2 Characterization of PPF Defects

The next stage of the study was to examine the defects in the primordial diaphragm (PPF) in each of the rodent CDH models. Embryos from control, nitrofen-treated, and VAD rats were collected at E13.5, and *Wt1* null-mutant mouse tissue was collected at E12.5 of gestation. Transverse sections were cut through the embryos at a cervical level to examine the PPF as shown in Figure 3.3. The PPFs in control animals were triangular-shaped structures protruding out from the lateral body wall. Consistent with original reports by Allan and Greer (1997), nitrofen-treated animals commonly had an abnormal PPF structure, characterized by the absence of the dorsally projecting point of the triangular PPF. A similar abnormality was found in the PPFs of the VAD rat embryos examined (n = 6). Abnormal PPF structure was also seen in transverse sections of *Wt1* null-mutant mice (n = 3), although the defect extended to the more lateral aspect of the PPF.

3-D reconstructions of the PPFs from control, nitrofen-treated, VAD, and *Wt1* null-mutant tissue were made from serial sections of tissue cut along the rostrocaudal extent of this structure and are presented in Figure 3.4. Recreated in 3-

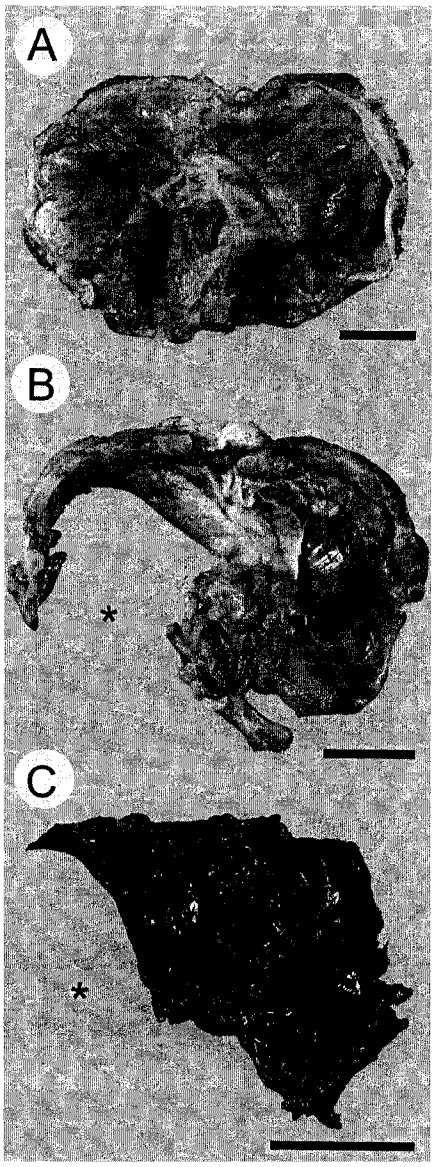

Figure 3.2 Representative example of diaphragm defects in human CDH.

Photomicrographs of human diaphragms. A normal diaphragm at 35 weeks of gestation (A) is shown for comparison against a diaphragm isolated from a foetus at 34 weeks of gestation (B) with a large left-sided diaphragm defect (*) and a diaphragm isolated from a 30-week foetus (C) also with a left-sided defect (*). Diaphragms are oriented such that the top of the image is anterior and the bottom of the image is posterior. Scale bar = 2 cm.

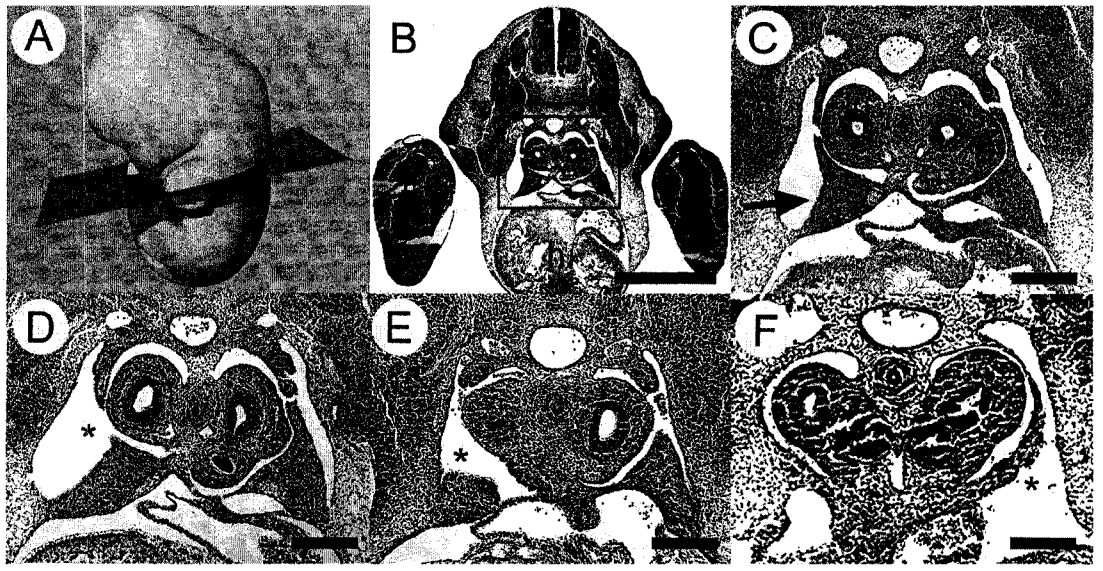

Figure 3.3 Diaphragm defects have their origin in an abnormal PPF.

A schematic representation of an E13.5 rat is provided with the plane of section shown to orient the reader (A). A transverse section through the cervical region of an E13.5 rat embryo (B) shows the neural tube (nt), forelimb (fl), and heart (h). The boxed region, when viewed at higher power (C), contains the developing lungs (lu) and the triangular PPFs (arrow). Representative PPF defects (*) are shown from nitrofen-treated (D), VAD (E), and wt1 null-mutant (F) animals. Scale bar in B = 1 mm, scale bar in C–F = 200 μm.

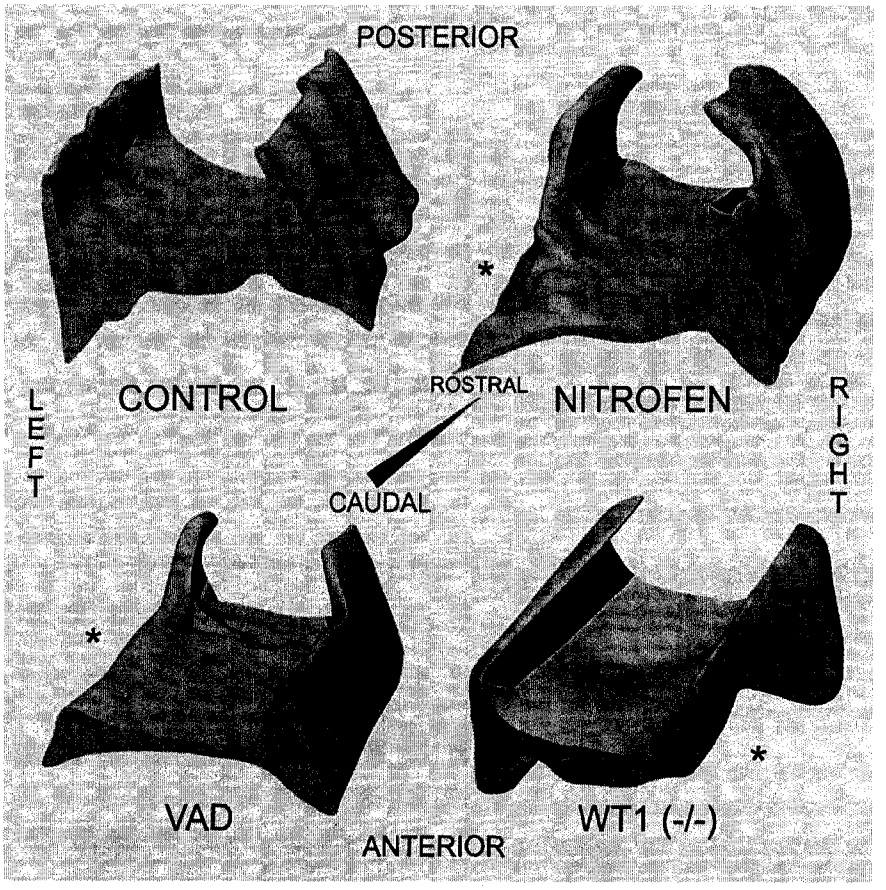

Figure 3.4 Three-dimensional reconstructions of the PPF from teratogen-induced, dietary, and genetic models of CDH.

Three-dimensional reconstruction of PPFs recreated from control (upper left), nitrofen-treated (upper right), VAD (lower left), and *Wt1* null-mutant (lower right) tissue sections. PPF defects are highlighted by an asterisk.

D, the PPFs normally look like a pair of triangular wedge-shaped tissues joined together at one of their apices. The recreated PPFs from nitrofen-treated rats look similar; however, on one side there is an area of tissue missing from the posterior and caudal-most region of the PPF. Recreation of the PPF from VAD tissue yields an almost identical picture, revealing an area of tissue missing along the posterior and most caudal aspect of the PPF.

Reconstruction of the PPFs in wild-type mice looks essentially identical to that of control rat tissue (data not shown). The structure of the PPFs from *Wt1* null-mutant mice shows that the caudal and more anterior aspect of the PPF is unilaterally missing.

3.3.3 Characterization of Muscle Architecture in Defective Diaphragms

A current model of how the dorsolaterally located PPF defect translates into the characteristic hole in the dorsolateral diaphragm states that muscle precursors normally destined for the defective region accumulate on the remaining surrounding PPF mesenchyme (reviewed by Greer et al., 2005). This, in turn, is subsequently manifest as a thickening of muscle adjacent to the herniated region of the diaphragm. To determine whether this was a common feature in the three rodent models and in human CDH, whole diaphragms were sectioned along their anteroposterior axis to study diaphragm thickness. As previously observed (Allan and Greer, 1997), diaphragms isolated from nitrofen-treated rats were thickened around the periphery of the defect compared with the intact contralateral side (Figure 3.5A). Diaphragms isolated from VAD rats ($n = 6$) also showed a similar thickening around the defect

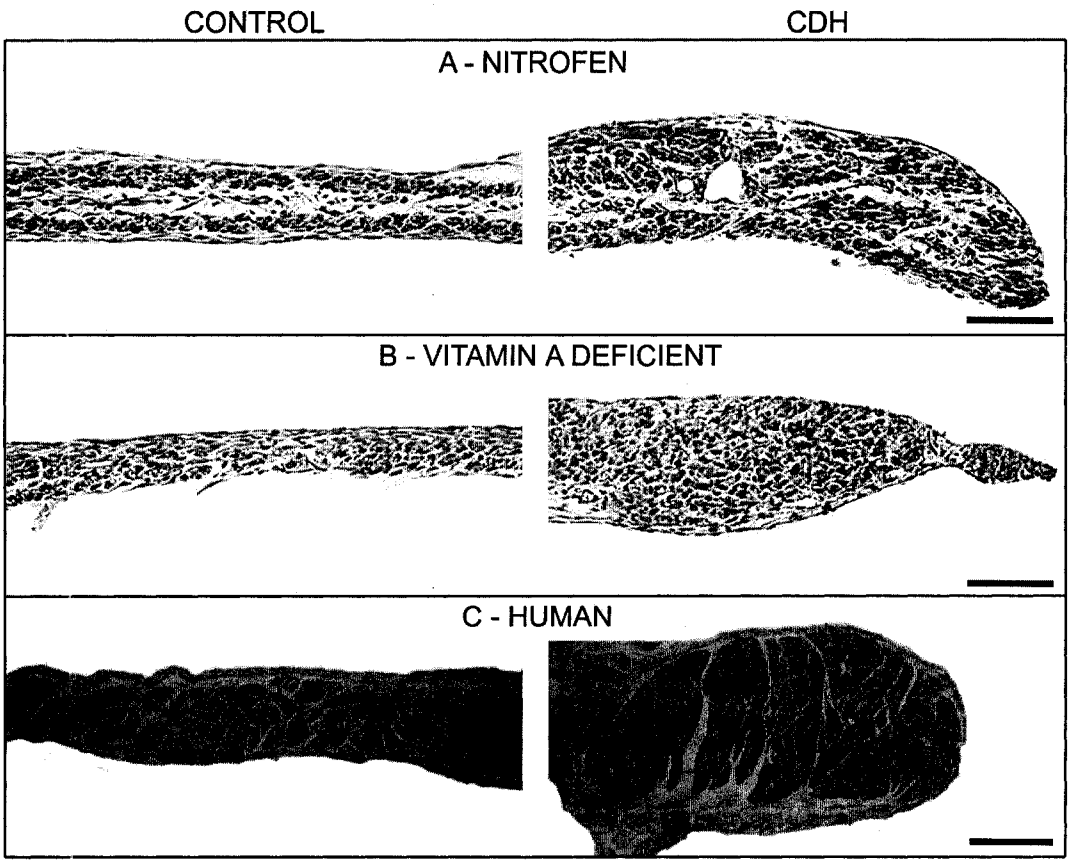

Figure 3.5 Diaphragm thickening is a hallmark of CDH.

The musculature of the diaphragm is thicker around the diaphragm defect (right column) compared with the normal contralateral side of the diaphragm (left column) in nitrofen-exposed animals (A), VAD animals (B), and in human cases of CDH (C). Scale bars in A and B = 100 μm; in C, scale bar = 2 mm.

(Figure 3.5B). Sufficient mature diaphragm was unavailable to draw any conclusions regarding diaphragm thickening in *Wt1* null-mutant mice.

A representative example of a cross-section through a human diaphragm is shown in Figure 3.5C. The study of one post-mortem diaphragm available to us from a non-CDH case indicated that the control side of the diaphragm in cases of CDH is representative of normal diaphragm thickness. The thickness of four human diaphragms with diaphragmatic hernia was measured, and the data are presented in Figure 3.6 relative to the normal, contralateral side of the diaphragm. Thickness of the normal side of the diaphragm was normalized to 100%; of the four diaphragms examined, the thickness of the defective side was 183 ± 37.2%, with this change representing a significant increase in diaphragm thickness surrounding the diaphragm defect.

3.3.4 Expression of Wt1 and Coup-tfII in the PPF

The proposed model of the PPF defect states that the pathogenesis can be traced to a malformation of the mesenchymal tissue rather than the myogenic component of the primordial diaphragm (Greer et al., 2005). Thus, we tested the hypothesis that Wt1 protein is expressed in the non-muscular component of the PPF. Furthermore, given that *Coup-tfII*-null mice have abnormal PPF structure and Bochdalek hernias (You et al., 2005), we tested the hypothesis that Coup-tfII protein would also be expressed in non-muscular cells of the PPF. Figure 3.7A demonstrates that Wt1 immunopositive cells are found throughout the PPF at E13.5 of rat gestation, but there is no colocalization with Pax3, a marker of myogenic cells in the PPF (Babiuk et al., 2003). Likewise, Coup-tfII is expressed in the PPF but not by

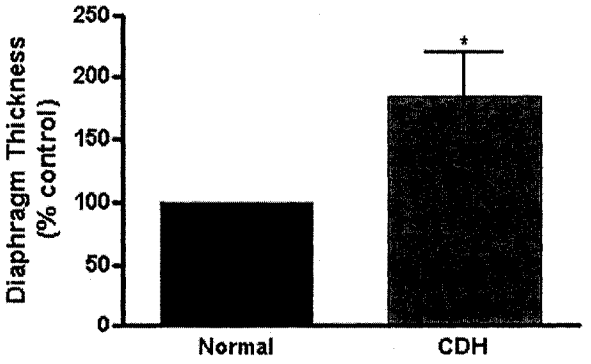

Figure 3.6 Relative thickness of normal and CDH diaphragms in humans.

Graph comparing the relative thickness of the normal hemi-diaphragm and the thickness around the periphery of the defect from infants with CDH. Error bars indicate the SD of measurements from four separate diaphragms. * $P = 0.05$, paired sample t-test.

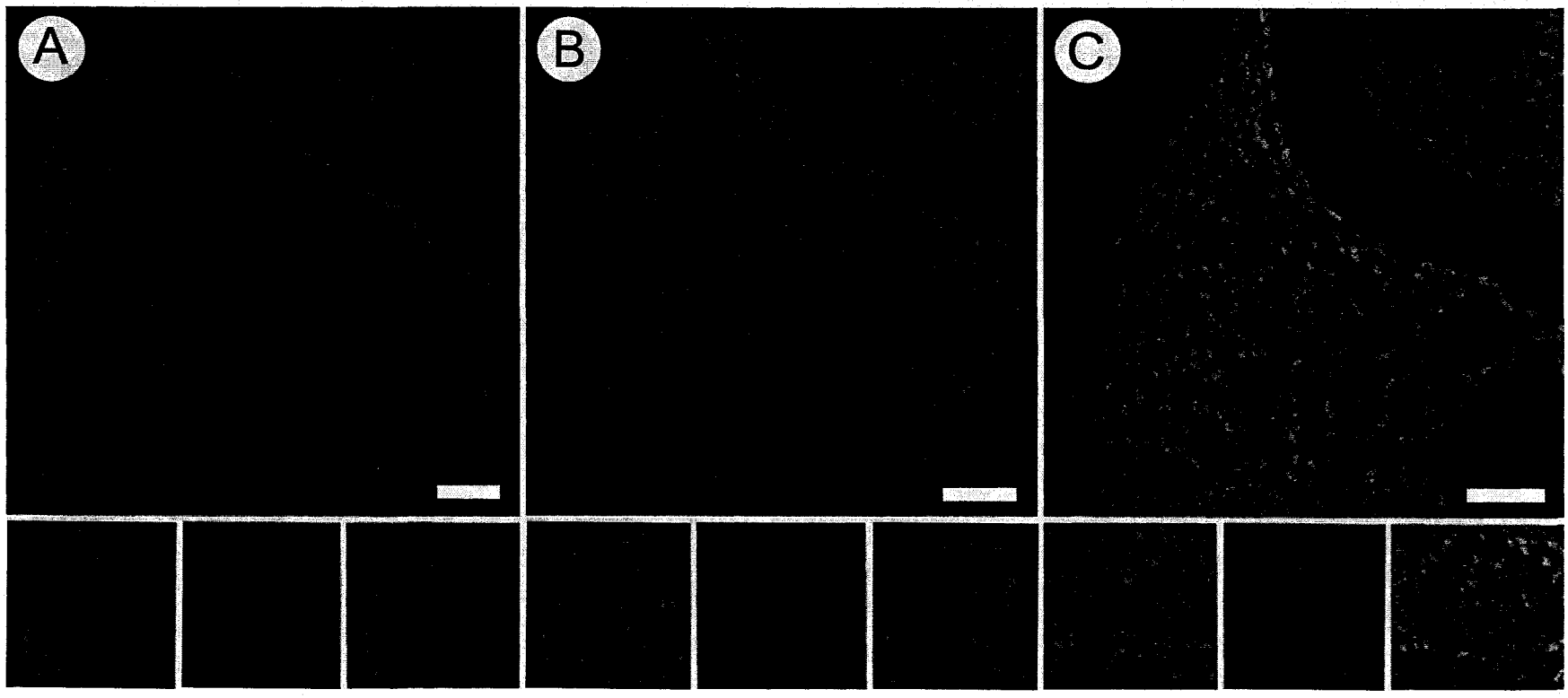

Figure 3.7 Representative immunohistochemical staining for Wt1 and Coup-tfII within the developing PPF of rats at E13.5 at X20 magnification (figure legend overleaf).

Figure 3.7 A: Double labelling for Wt1 (green) and Pax3 (red). B: Double labelling for Coup-tfII (green) and Pax3 (red). C: Double labelling for Wt1 (red) and Coup-tfII (green). Double-labelled cells appear yellow. Lower panels in A, B, and C show the red channel, green channel, and merged images from left to right, respectively. Scale bars = 100 μm.

Pax3-expressing myogenic cells (Figure 3.7B). Double immunolabelling for Coup-tfII and Wt1 shows that these two proteins colocalise within the cells of the PPF, although there is not 100% overlap of expression (Figure 3.7C).

3.4 Discussion

In recent years, significant advances have been made in the treatment of CDH; however, its incidence remains unchanged and it is still unclear how and why the diaphragm defect arises, necessitating continuing research. We have demonstrated that, in addition to nitrofen treatment, vitamin A deficiency and inactivation of the *Wt1* gene all result in the formation of Bochdalek-type diaphragmatic hernia, and, therefore, they can be collectively used as teratogenic, dietary and genetic models of CDH, respectively. Furthermore, examination of post-mortem CDH diaphragm tissue revealed parallels between the rodent models and CDH in humans.

Nitrofen-exposed, VAD, and *Wt1* null-mutant rodents all have posterolateral diaphragm defects similar in phenotype to diaphragm defects observed in infants with CDH. One of the first theories put forth to explain the pathogenesis of CDH stated that failure of the pleuro-peritoneal canals to close properly allowed persistent communication between the pleural and abdominal cavities, causing the abdominal viscera to herniate into the chest. This hypothesis was formulated on the basis of pathological specimens from infants with CDH and the study of normal diaphragm development in the embryo (Harrington, 1948; Wells, 1954); it has persisted in the literature despite the absence of supporting experimental evidence. In contrast, previous work in the nitrofen model has shown that the diaphragm defects in this model are distinct from, and occur before, pleuro-peritoneal canal closure and that a malformed PPF is the origin of the defect (Allan and Greer, 1997). Here we tested

the hypothesis that CDH in VAD rats and *Wt1* null-mutant mice is also caused by an abnormally formed PPF, independent of pleuro-peritoneal canal closure.

In cross-section, the PPFs appear as paired, triangular-shaped structures protruding from the lateral body wall. As previously described (Allan and Greer, 1997), nitrofen treatment leads to a characteristic defect in the PPF; partial loss of PPF tissue maintains communication between the abdominal and pleural cavities and forms the basis for a hole in the developing diaphragm. VAD produces an abnormal PPF that is almost identical in appearance to the defect produced by nitrofen treatment. *Wt1* null-mutant mice also have a malformed PPF. To better visualize the PPF malformations, 3-D reconstructions of the developing PPFs were made. Seen in 3-D, the paired PPFs appear as wedge-shaped structures linked at their apex and tapering off caudally. The posteriorly projecting "base" of these wedges is absent in nitrofen-treated and VAD rats. The extent of the missing tissue in *Wt1* null-mutant mice is slightly different, which may represent a subtle species difference in this stage of diaphragm development or a result of the specific mutation. Analysis of the PPF in other mutant mouse models of CDH will clarify this issue. Nevertheless, a malformed PPF underlies diaphragmatic hernia in nitrofen-treated and VAD rats and *Wt1* null-mutant mice, demonstrating that three distinct models of CDH have a common pathogenic origin.

The original description of the *Wt1* null-mutant phenotype described incomplete diaphragm formation and showed lung tissue herniating into the abdominal cavity (Kreidberg et al., 1993). In the foetuses we examined, we observed a more classic picture of CDH, with abdominal contents herniating into the

pleural cavity. It seems likely that variability in the penetrance of specific abnormalities may explain this difference; pericardial bleeding and a smaller pleural cavity may raise intrathoracic pressure to a point at which it is greater than intra-abdominal pressure, pushing the lungs downwards and through the hole in the diaphragm. This phenotype was not as prevalent in the mice we examined; thus, as is typically the case in CDH, abdominal pressure was greater than thoracic pressure, and the abdominal viscera were pushed into the thoracic cavity. As detailed in the Introduction, there are several strains of mutant mice with posterolateral diaphragm defects. Our examination of *Wt1* null-mutant mice provides a detailed description of the pathogenesis of diaphragm defects occurring in a genetic model of Bochdalek CDH. Genetic models for the less common subtypes of diaphragmatic hernia have also been characterized. For example, a mutation in *Fog2* produces mice with eventration of the diaphragm, and mice lacking *Slit3* expression have diaphragmatic central tendon defects, another rare form of CDH (Yuan et al., 2003; Liu et al., 2003; Ackerman et al., 2005).

Although it was not possible to study the developing PPF in human cases of CDH, phenotypic similarities between abnormal human and rodent diaphragms support the hypothesis that CDH in humans arises from an abnormal PPF. In addition to the location of the hole in the diaphragm, we demonstrated that the diaphragm is thicker around the defect in nitrofen-treated and VAD rats and that this is also a feature of diaphragms isolated from infants with CDH. We interpret thickening of the diaphragm around the defect as being caused by the aggregation of muscle precursor cells that would normally spread out to populate the entire

diaphragm. Accordingly, muscle precursors migrating to a malformed PPF from the somites accumulate in the remaining PPF tissue, and their subsequent proliferation and differentiation in a restricted space leads to thickening of the diaphragmatic musculature. As such, the thickening around the diaphragm defect in humans is consistent with its origins in a malformed PPF. We examined pathological diaphragm specimens, which may have produced a bias in our sample toward larger defects (and therefore thickening) since infants with CDH with large defects are more likely to die. However, diaphragm thickness, which can be measured by magnetic resonance imaging, is also increased in infants who survive the perinatal period (Dr. R. Bhargava, personal correspondence).

We could not measure diaphragm thickness in *Wt1* null-mutant mice because of the lethality of the mutation. The creation of a conditional *Wt1* null-mutant mouse might allow improved survival during late gestation, allowing diaphragm thickness to be directly measured; such a strain of mice would also allow the time period during which *Wt1* is essential for diaphragm development to be assessed. Although diaphragm thickness was not directly measured, the conditional *Coup-tfII* knockout model of CDH appears to have a thickened diaphragm around the periphery of the defect (see You et al 2005; Figure 4C). In summary, thickening of the diaphragmatic musculature around the defect in rodent models of CDH is also a feature of CDH in humans and is indicative of an earlier defect in the PPF.

In addition to the assertion that failure of pleuro-peritoneal canal closure leads to CDH, it has also been proposed that CDH arises from an abnormality in the developing muscle of the diaphragm (Biggio et al., 2004). However, exposure of *c-*

met null-mutant mice, which have an amuscular diaphragm, to nitrofen and other CDH-inducing teratogens causes diaphragm defects independent of myogenesis, supporting the hypothesis that CDH arises from an abnormality in the non-muscular, mesenchymal substratum of the developing diaphragm (Babiuk and Greer, 2002). Our immunohistochemical analysis of the PPF demonstrates that *Wt1* and *Coup-tfII*, two genes that cause CDH when inactivated, are expressed throughout the PPF, but they are not expressed in Pax3-positive muscle precursor cells. This result indicates that it is the non-muscular cells of the PPF that are affected in these strains of mice, supporting the hypothesis that CDH arises from a defect in the non-muscular component of the PPF. Furthermore, Wt1 and Coup-tfII double labelling suggests that these proteins colocalise within the PPF, highlighting the possibility that the same cells are being affected in both strains of mutant mice.

In summary, we have highlighted a common pathogenic mechanism in nitrofen-treated rats, VAD rats, and *Wt1* null-mutant mice — representing distinct teratogenic, dietary, and genetic models of CDH respectively. Anatomical similarities between these rodent models and human tissue support the hypothesis that CDH in humans arises from a defect in the developing PPF. Furthermore, the immunohistochemical data highlight the concept that it is the non-muscular, mesenchymal substratum of the PPF that is malformed. Future studies using a combination of the differing animal models will hopefully provide unique mechanistic insights into the etiology of CDH.

3.5 References

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Chapter 4: Gene expression in the developing diaphragm: significance for congenital diaphragmatic hernia

This chapter was adapted from the original publication by:

Clugston RD, Zhang W, Greer JJ.

American Journal of Physiology: Lung Cellular and Molecular Physiology

2008 Apr; 294(4):L665-75

Contributions: The majority of the experimental work described in this paper was performed by RDC, with technical assistance from WZ. The manuscript was written by RDC and edited by JJG.

4.1 Introduction

Congenital diaphragmatic hernia (CDH) is a severe birth defect causing life-threatening respiratory distress in the neonatal period. It occurs in approximately 1 in 2500 pregnancies with an overall mortality rate of 50-70%, though single specialized centres have reported significantly reduced mortality rates (Skari et al., 2000; Downard et al., 2003; Doyle et al., 2004; Tonks et al., 2004; Bagolan et al., 2004; Levison et al., 2006). In cases of Bochdalek CDH, which is the most frequent presentation of CDH, incomplete formation of the postero-lateral diaphragm allows invasion of the abdominal contents into the thoracic cavity, impeding lung development (Torfs et al., 1992; Harrison et al., 1994). The pathogenesis of CDH is poorly understood, however recent research using different animal models of the disorder is beginning to provide insight into its embryonic origins (Clugston et al., 2006; Beurskens et al., 2007; Clugston and Greer 2007). Equally, progress is also being made into understanding the etiology of CDH. In addition to the recently proposed retinoid hypothesis (Greer et al., 2003), the genetic origins of CDH are also being carefully examined, largely spurred on by the characterization of mutant mice with abnormal diaphragm phenotypes and genetic screening in infants diagnosed with CDH (Ackerman et al., 2007a; Pober et al., 2007; Slavotinek et al., 2007). In the latter case, there has been considerable attention paid toward so called CDH-critical regions; parts of chromosomes containing several genes where recurring structural abnormalities have been found in multiple cases of CDH (Lurie et al., 2003; Holder et al., 2007). These findings suggest that one or more genes in specific regions are essential for normal diaphragm development. The first critical

region to be identified and the best characterized is located at 15q26; a deletion of this part of chromosome 15 has been shown to account for ~1.5% of CDH cases and is associated with very high mortality (Schlembach et al., 2001; Biggio et al., 2004; Klaassens et al., 2005). The spectrum of anomalies produced by 15q26 deletion is phenotypically most similar to Fryns' syndrome (Slavotinek et al., 2005; Klaassens et al., 2007). Several other regions of the human genome have also been identified as putative CDH-critical regions, including 1q41-q42, 4p16.3, 8p23.1, and 8q22-q23 (Slavotinek et al., 2006). All of these regions are in the order of several mega-bases and contain numerous genes, however how these genes might contribute to diaphragm development, and their significance with regard to CDH is unknown. In fact, the need for studies focusing on the molecular origins of how genetic disruptions result in diaphragm defects has recently been highlighted (Holder et al., 2007). The aim of this study was therefore to systematically test the hypothesis that genes identified from CDH-critical regions are expressed in the developing rodent diaphragm at the time of the initial defect. We focused our expression studies on two important stages of rat diaphragm embryogenesis; at embryonic day (E) 13.5 when the pleuro-peritoneal folds (PPFs) are developing, and at E16.5 when diaphragm formation is essentially complete (Clugston and Greer, 2007). Expression patterns within the PPF are of interest as this is a key structure in the developing diaphragm, particularly in the context of CDH. Muscle precursor cells (MPCs) which form the complete musculature of the diaphragm first migrate to the PPF, and it is also the target for pioneer axons of the phrenic nerve which provides the diaphragm with its nervous input (Greer et al., 1999; Babiuk et al., 2003). With regard to CDH,

abnormal PPF development has been shown to underlie diaphragmatic hernia in nitrofen treated rats, Vitamin A deficient rats, and Wt1 null-mutant mice (Allan and Greer, 1997a; Clugston et al., 2006). Further, analyses of human CDH post-mortem tissue have generated data consistent with primary PPF defects (Clugston et al., 2006). As such, genes found to be expressed in this structure are assumed to be important for normal diaphragm development and, hypothetically, could lead to CDH when abnormally expressed or regulated. In this study we focused on the rodent orthologues of ten genes located within the region of chromosome 15q26 whose deletion is associated with CDH (table 4.1). This list includes *COUP-TFII*, which is regarded as the most important gene from 15q26 in diaphragm development (Holder et al., 2007). We also studied two other specific genes, *FOG2* and *GATA4*, identified as the most significant genes associated with CDH in the critical regions 8q22-q23 and 8p23.1 respectively (table I; Holder et al., 2007). Because of the large number of genes identified from 15q26, in the first part of this study we performed a screen of these candidate genes for their expression in the PPF. Laser capture micro-dissection was used to precisely isolate PPF tissue and RT-PCR was performed on extracted mRNA. The protein expression pattern of positively identified genes was then determined using immunohistochemistry. We also determined the protein expression pattern of FOG2 and GATA4 in the PPF. Further, because of the unusual diaphragm phenotype seen in *Fog2* and *Gata4* mutant mice we also studied the expression of these factors in the diaphragm at E16.5, in order to better understand these phenotypes (see discussion; Ackerman et al., 2005; Jay et al., 2007). Our protein expression analyses in all cases addressed two questions; first, are these

Table 4.1 Genes of interest from CDH-critical regions

Gene	*Ensembl gene ID*	*Genomic location*	*Function*
ST8SIA2	ENSG00000140557	15q26.1	Enzyme catalyzing polysialic acid synthesis
CHD2	ENSG00000173575	15q26.1	Chromatin remodelling
RGMA	ENSG00000182175	15q26.1	Repulsive guidance molecule
MCTP2	ENSG00000140563	15q26.2	Calcium-mediated signalling
COUP-TFII	ENSG00000185551	15q26.2	Transcription factor
ARRDC4	ENSG00000140450	15q26.3	Unknown
IGF1R	ENSG00000140443	15q26.3	Signalling, receptor
DMN	ENSG00000182253	15q26.3	Structural Protein
TTC23	ENSG00000103852	15q26.3	Unknown
LRRC28	ENSG00000168904	15q26.3	Unknown
FOG2	ENSG00000169946	8q23.1	Transcription factor
GATA4	ENSG00000136574	8p23.1	Transcription factor

Information source: www.ensembl.org

genes expressed in MPCs or mesenchymal cells, and second, are they co-expressed in the same cells? In our co-expression studies we also included *WT1* into our analysis; this gene is expressed in the PPF and is known to be essential for normal diaphragm development (Kreidberg et al., 1993; Clugston et al., 2006).

The etiology of CDH is diverse and complex. This study is the first to provide an in depth examination of how genes associated with CDH are expressed in the developing diaphragm, and provides an important foundation for understanding how the deletion of specific genes may contribute to abnormal diaphragm formation.

4.2 Materials and methods

A description of the basic methodologies concerning rat breeding, tissue collection, basic histology, immunohistochemistry, and microscopy can be found in the relevant section of chapter 2. The following sections concern methods only employed in this study and are not described elsewhere in this thesis.

4.2.1 Tissue collection

For RNA isolation, E13.5 embryos were either snap frozen in liquid nitrogen (for whole embryo RNA), or frozen in OCT prior to cryostat sectioning (for PPF RNA). E16.5 diaphragms were isolated immediately after caesarean section, rinsed in PBS and snap frozen in liquid nitrogen.

4.2.2 PPF Laser capture micro-dissection

Embedded embryos were sectioned at 10μm using a cryostat (Leica, CM1900) and mounted on uncoated, RNase free slides, then stored at -80°C. Prior to laser capture the frozen sections were thawed at room temperature for 30 sec, and then immersed in 75% ethanol for 30 sec. After this fixation the slides were washed for 30 sec in DEPC-treated water and stained in Mayer's haematoxylin for 1 min. The slides were then dehydrated in graded ethanol concentrations and cleared with xylene. The AutoPix automated laser capture system (Arcturus Bioscience Inc., Mountain View, CA) was used for capturing PPF tissue. Briefly, activation of the laser beam was focused on a thermoplastic film of CapSure HS LCM cap, leading to melting of the film bond to selected tissue. As illustrated in figure 4.1, we were able to specifically target the PPF and isolate it without collecting tissue from adjacent

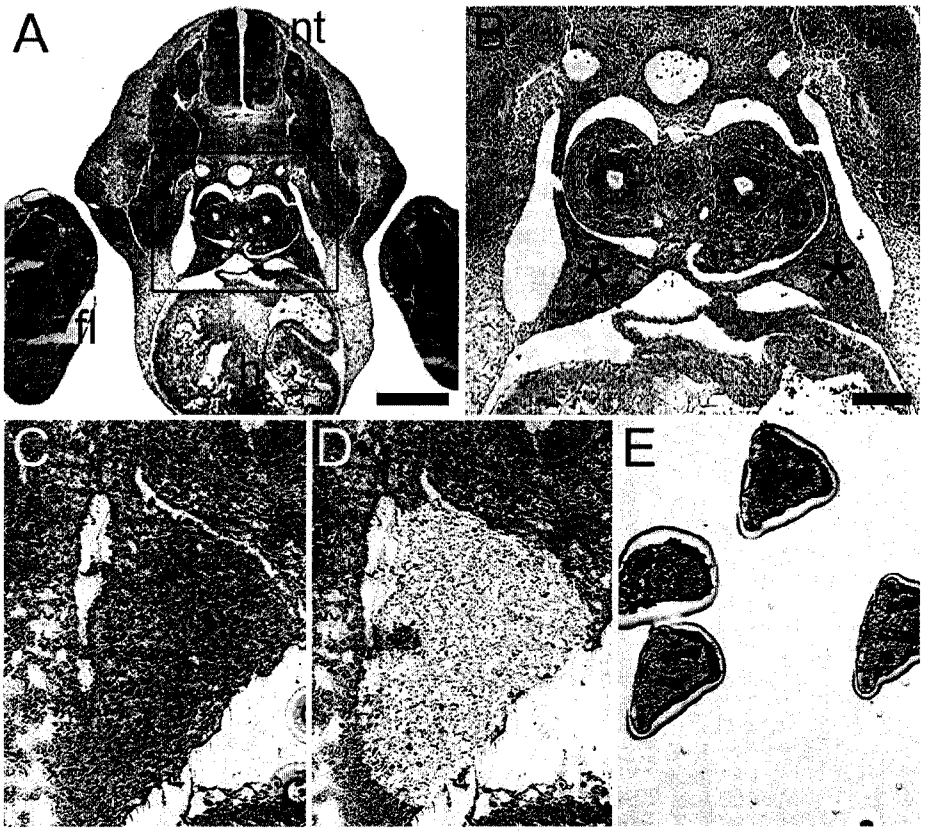

Figure 4.1 Isolation of the PPF by laser capture micro-dissection.

A: Representative image of a transverse section through an E13.5 rat embryo at the lower cervical level, showing the forelimbs (fl), neural tube (nt), and heart (h). B: Boxed region from A enlarged to show the distinctive triangular PPFs (*) and the adjacent lung buds. C: High magnification image a single PPF prior to laser capture. D: Same region as C seen after removal of the PPF. E: Several isolated PPFs following laser capture. Scale bars: A = 500 μm; B = 400 μm; C-E are not to scale.

structures. After dissection, the captured cells and assembly cap with ExtracSure Extraction Device was stored at -80°C.

4.2.3 RNA isolation

Total RNA was extracted from whole embryos (n=6) and diaphragms (n=10) using TRIzol (Invitrogen Canada Inc., Burlington, ON, Canada), according to the manufacturer's protocol. Total RNA was isolated from collected PPF fragments using the PicoPure RNA isolation kit (Arcturus Bioscience Inc.), due to the small amount of RNA harvested from each PPF, the RNA extracted from ~10 PPF's was pooled for RT-PCR analysis. For RNA extraction, 10 µl extraction buffer was added to the CapSure- ExtracSure assembly, covered in a 0.5-ml microcentrifuge tube on the top of assembly and incubated for 30 min at 42°C. After incubation, the tube was centrifuged to collect the cell extract. Before RNA isolation, the purification column was washed with condition buffer at room temperature prior to adding the mixture of cell extract and an equal volume of 70% ethanol. After centrifugation and disposal of the flow-through, RNA was bonded onto the surface membrane of the purification column. The column was washed with buffer and centrifuged to remove salt and other contaminants. The column was then transferred to a new 0.5-ml microcentrifuge tube, pure RNA was eluted from the membrane of the purification column using elution buffer and centrifugation. The concentration of RNA in all cases was measured using a spectrophotometer (NanoDrop, ND-1000) and diluted to the same concentration for RT-PCR (50ng/µl).

Table 4.2 Primer sequences, annealing temperature and amplicon size for CDH associated genes found in region 15q26.

Gene	Primer Sequence	Annealing Temperature (°C)	Amplicon size (bp)
ST8SIA2	F 5'-TGG AGA CAC AAC CAG ACG CTC-3' R 5'-GGT GCA TGG TCA CTA GGT CAG-3'	62	363
CHD2	F 5'-CAG AGT TGT ATC GAC AGC TTC C-3' R 5'-TCT GGA TGG TCT TTC CTA GGC C-3'	58	244
RGMA	F 5'-CGA GAT CTG CCA CTA TGA GAA G-3' R 5'-CAC CTT CTC TGT GAT CTT CAG G-3'	62	370
MCTP2	F 5'-TAC CTC CTC ACC ATA CAC CTG-3' R 5'-CCG CTT CCG ATT TGA CCA ACG-3'	60	411
COUP-TFII	F 5'-GCA AGA GCT TCT TCA AGC G-3' R 5'-GCT TCT CCA CTT GCT CTT GG-3'	58	612
ARRDC4	F 5'-GAC CAG AGT GTA AGA AGA GAG C-3' R 5'-GCT AAG GAG TAG TCC ACT CTG-3'	60	425
IGF1R	F 5'-GAG TAC AAC TAC CGC TGC TGG-3' R 5'-GCA GAC GTC ACA GAA TCG ATG -3'	62	446
DMN	F 5'-AGG ATG AGC TGC TTC GGA TGC-3' R 5'- GGT TGT CTG TGA GTC AAG GTG-3'	58	407
TTC23	F 5'-GCA TAT GTG AAT CTG GCT CAG G-3' R 5'-CCC ACA TTG CAG CAT CTC CTT G-3'	62	258
LRRC28	F 5'-CGC AAT CGG CTC TGG TGT GTG-3' R 5'-TGG GAG GAA CAC GGT CAG CTC-3'	62	276
β-Actin (Control)	F 5'-GTA TGC CTC TGG TCG TAC CA-3' R 5'-CTT CTG CAT CCT GTC AGC AA-3'	62	520

4.2.4 RT-PCR

RT-PCR was performed using a one-step RT-PCR Kit (Qiagen Canada Inc., Mississauga, ON, Canada). Primer sequences, annealing temperatures and amplicon sizes are presented in table 4.2. Reactions were performed in 25 µl volumes, with the final reaction mixture containing 5 µl 5X RT-PCR buffer, 5 µl "Q" solution, 1 µl dNTPs (10 mM), 1.5 µl primer solution (containing forward and reverse primers; final concentration 0.6 µM), 10.5 µl water, 1 µl enzyme mix, and 1 µl template RNA. The RT-PCR protocol used was as following: reverse transcription at 50°C for 30 min, PCR activation at 95°C for 15 min, followed by 30-35 cycles of 94°C for 30 sec denaturation, 30 sec annealing at temperature indicated in table 2, and 30 sec at 72°C extension. A final extension step of 72°C for 10 min was also carried out. Reaction products were run on a 1.2% agarose gel containing ethidium bromide and bands visualized using a 312-nm transilluminator (Fisher Scientific, Pittsburgh PA, FBTI 88). At the beginning of the experimental series, bands for each gene of interest were extracted using a gel extraction kit (Qiagen Canada Inc.) and their identity confirmed by DNA sequencing. For each reaction series, β-Actin was included as a positive control and H_2O was used instead of RNA as a negative control to assay for contaminating RNA. For each gene of interest reactions were carried out in triplicate and repeated at least two times.

4.2.5 Immunohistochemistry

A list of antibodies used in this study can be found in table 4.3, a description of the basic immunohistochemistry protocol used can found in section 2.4.

Table 4.3 Table of antibodies used

Antibody	Dilution	Source (Catalogue number)
Mouse monoclonal antibodies:		
Wt1 (6F-H2)	1:50	Dako (M3561)
MyoD1 (5.8A)	1:50	Dako (M3512)
Coup-tfII	1:250	PPMX (H7147)
Goat polyclonal antibodies:		
Pax3/7 (C-20)	1:100	SCBT (sc-7748)
Gata4 (C-20)	1:250	SCBT (sc-1237)
Rabbit polyclonal antibodies:		
Fog2 (M-247)	1:50 – 100	SCBT (sc-10755)
Rgma (C-16)	1:50	SCBT (sc-46481)
Igf1r (C-20)	1:100	SCBT (sc-713)

PPMX: Perseus Proteomics, Tokyo, Japan; SCBT: Santa Cruz Biotechnology, Santa Cruz, CA; Dako Canada, Mississauga, ON

4.3 Results

4.3.1 RT-PCR analysis of 15q26 CDH-critical genes in the developing diaphragm

A systematic RT-PCR analysis of the genes from the CDH-critical region found at 15q26 was performed. Total RNA extracted from the whole embryo, PPF and whole diaphragm was assayed for each gene of interest (Table 4.4). Consistent amplification between replicates and experiments was considered to reflect positive expression in the target tissue. The inability to produce a detectable PCR product was considered as an absence of expression. We were able to amplify mRNA for all of the genes studied from whole embryo extracts at E13.5. The following genes were found to be expressed in the PPF at E13.5: *St8sia2*, *Rgma*, *Coup-tfII*, *Arrdc4*, *Igf1r*, and *Lrrc28*. We were unable to amplify transcripts for *Chd2*, *Mctp2*, *Dmn*, and *Ttc23*, suggesting they are not expressed in this tissue. Similarly, *St8sia2*, *Rgma*, *Coup-tfII*, *Arrdc4*, *Igf1r*, and *Lrrc28* were found to be expressed in the whole diaphragm at E16.5; further *Chd2* was also found to be expressed at this age. Similar to our results for PPF RNA, we could not amplify *Mctp2*, *Dmn*, and *Ttc23* transcripts from the whole diaphragm.

4.3.2 Immunohistochemical expression of Coup-tfII, Igf1r and Rgma within the PPF

Immunohistochemical staining for Coup-tfII, Igf1r and Rgma was performed to visualize the expression pattern of these proteins within the developing PPF of the rat at E13.5 (figure 4.2). There were no commercially available antibodies available for St8sia2, Arrdc4, and Lrrc28; therefore we could not examine the expression of

Table 4.4 Results of RT-PCR analysis of CDH-associated genes

Gene	Embryonic (E13.5)	PPF (E13.5)	Diaphragm (E16.5)
ST8SIA2	+	+	+
CHD2	+	-	+
RGMA	+	+	+
MCTP2	+	-	-
COUP-TFII	+	+	+
ARRDC4	+	+	+
IGF1R	+	+	+
DMN	+	-	-
TTC23	+	-	-
LRRC28	+	+	+
β-Actin (Control)	+	+	+

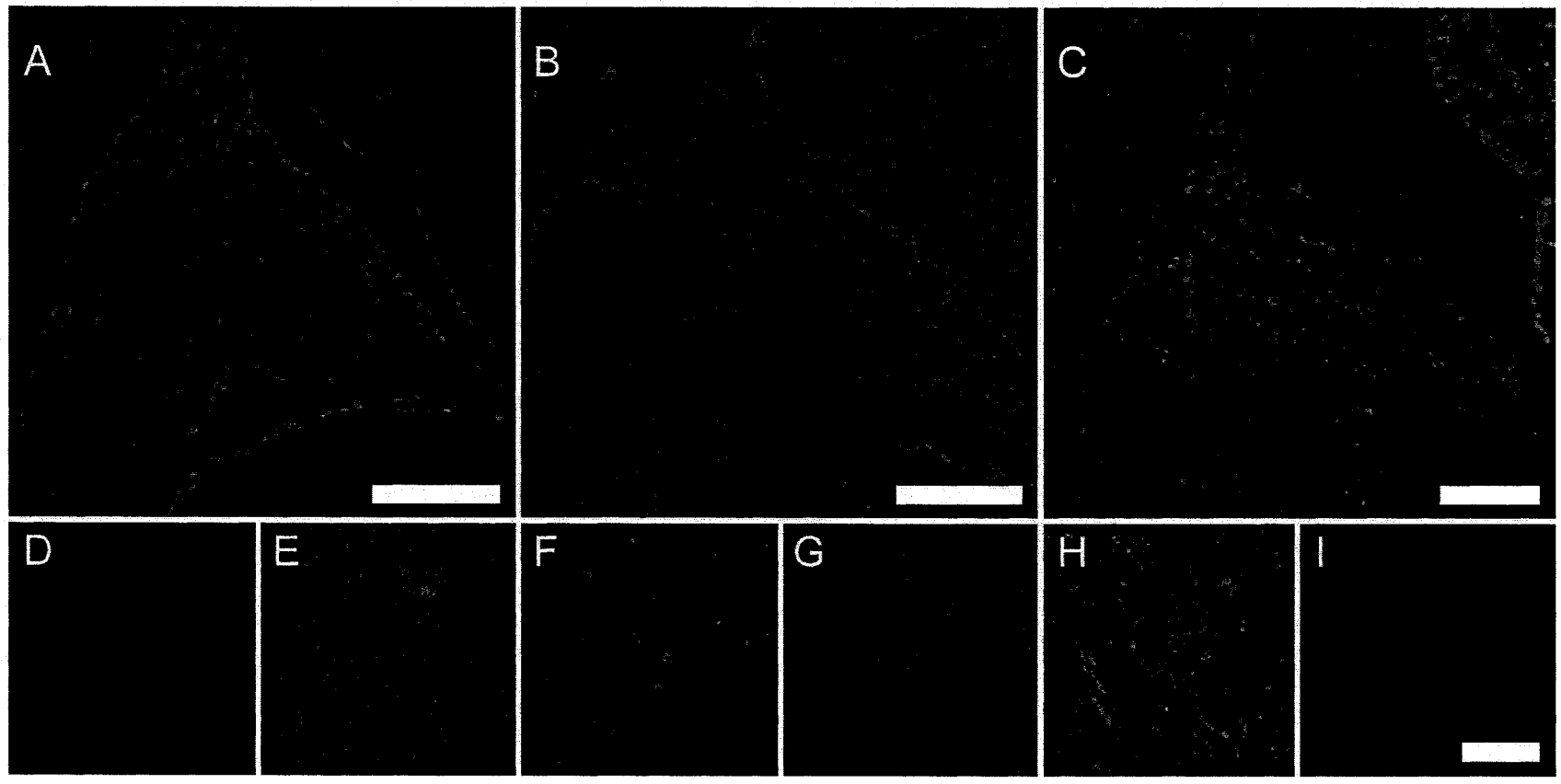

Figure 4.2 Coup-tfII, Igf1r and Rgma expression in the PPF (figure legend overleaf).

Figure 4.2 A: Single channel confocal image showing Coup-tfII immuno-positive cells in the PPF. B: IgfIr immuno-staining in the PPF. C: Rgma immuno-staining in the PPF. D: High magnification images of double labelling for Pax3 (red) and Coup-tfII (green). E: Pax3 (red) and Igf1r (green) expression. F: Pax3 (red) and Rgma (green) expression. G: Double labelling for Igf1r (red) and CouptfII (green). H: Double labelling for Igf1r (red) and Rgma (green). I: Double labelling for Rgma (red) and Coup-tfII (green). Scale bars: A, B and C = 75 μm; D-I = 25 μm.

these proteins. The expression pattern of Coup-tfII within the PPF has been partially described before and is extended upon here (Clugston et al., 2006). In agreement with previous data, Coup-tfII expression is found in the nuclei of cells throughout the PPF, and in the mesenchyme of the adjacent lung (Fig. 4.2A). Igf1r is expressed in the cytoplasm of every cell within the PPF, as well as in the adjacent lung mesenchyme and bronchi (Fig. 4.2B). The expression of Rgma was more restricted in the PPF (Fig. 4.2C); punctate staining for this protein was seen throughout the PPF, though a smaller proportion of cells were labelled compared to CouptfII and Igf1r. There was also obvious Rgma staining in the lung bronchi. The transcription factor Pax3 is a marker of muscle precursor cells within the PPF (Babiuk et al., 2003). Double labelling experiments with Pax3 were performed to determine if Coup-tfII, Igf1r and Rgma were expressed in the mesenchyme of the PPF or in its muscular component. We found no co-localisation between Coup-tfII positive nuclei and Pax3 positive nuclei, indicating that Coup-tfII is only expressed in the mesenchyme of the PPF (Fig. 4.2D). Contrastingly, Igf1r expression was found in the cytoplasm of Pax3 positive muscle precursors and mesenchyme cells throughout the PPF (Fig. 4.2E). Rgma was not expressed in association with Pax3 positive muscle precursors, suggesting that this protein is only associated with the mesenchymal cells of the PPF (Fig. 4.2F). A second series of double labelling experiments were performed in order to determine if Coup-tfII, Igf1r and Rgma were co-expressed within the same cells. Consistent with the widespread expression of Igf1r in the PPF we found that it was co-expressed in Coup-tfII (Fig. 4.2G) and Rgma (Fig. 4.2H) immuno-positive cells. With regards to the co-expression of Rgma

and Coup-tfII, cells that expressed both proteins were observed, however singly labelled cells for both proteins were also observed (Fig 4.2I). Though one of the functions ascribed to Rgma is in axon guidance, we found no association between Rgma staining and the phrenic nerve within the PPF (visualized by immune-staining for neurofilament; data not shown).

4.3.3 Immunohistochemical expression of Fog2 and Gata4 in the PPF

We performed a systematic immunohistochemical evaluation of Fog2 and Gata4 expression in the PPF at E13.5, finding that both proteins are expressed in the nuclei of cells through-out this structure (Fig. 4.3A and 4.3B respectively). The basic pattern of Fog2 expression observed closely resembled that described previously in mice (Ackerman et al., 2007b). In order to determine if either Fog2 or Gata4 was expressed in the pool of diaphragmatic MPCs that are localized within the PPF, double labelling experiments with Pax3 were carried out. Neither Fog2 (Fig. 4.3C) or Gata4 (Fig. 4.3D) were found to co-localise with Pax3 expressing cells within the PPF, thus the expression of these genes is restricted to the mesenchymal component of this structure. However, as could be expected from their partnership in gene regulation, double labelling for Fog2 and Gata4 revealed almost complete co-localisation within the PPF (Fig. 4.3E; Cantor and Orkin, 2005). Expression of both these proteins within the lung was observed at E13.5, however a detailed description of their expression in this tissue has been described elsewhere (Ackerman et al., 2007; Jay et al., 2007)

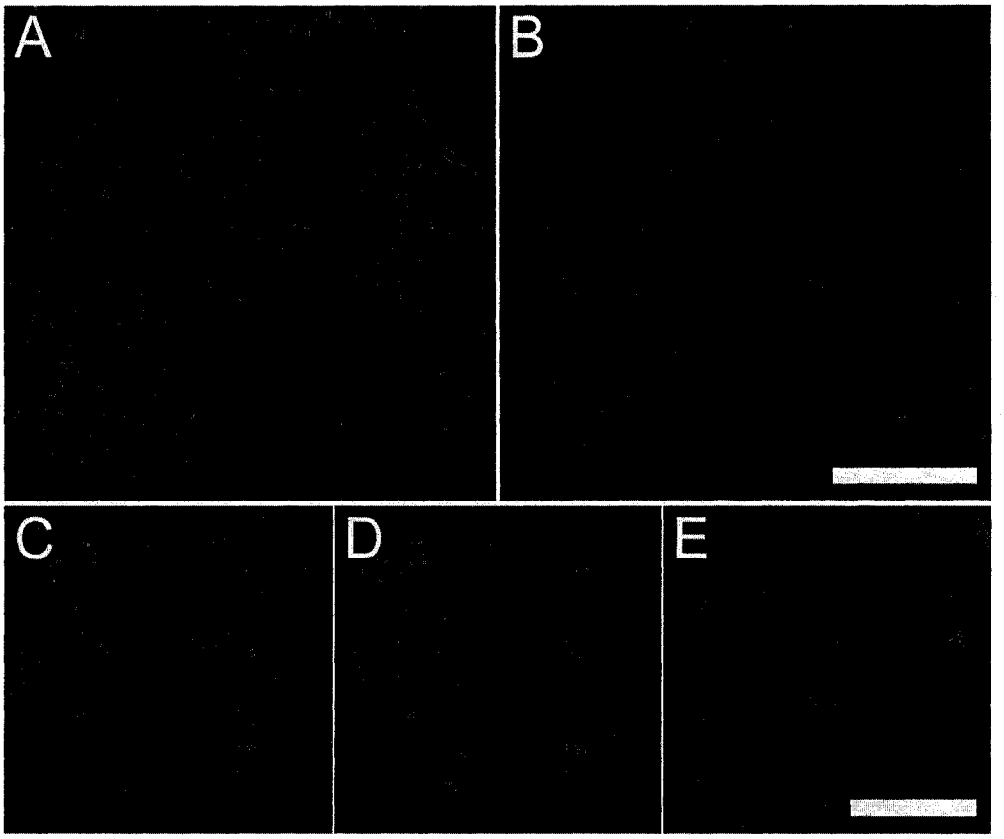

Figure 4.3 Fog2 and Gata4 expression in the PPF.

A: Fog2 immuno-positive cells in the PPF. B: Gata4 immuno-positive cells in the PPF. C: Fog2 (red) and Pax3 (green) expression does not co-localise in the PPF. D: Gata4 (red) and Pax3 (green) expression does not co-localise in the PPF. E: Fog2 (red) and Gata4 (green) immuno-staining colocalises in the PPF (yellow staining). Scale bars: A and B = 100 μm; C-E = 25 μm.

4.3.4 Expression of Fog2 and Gata4 within the whole diaphragm

In addition to their expression pattern in the PPF, Fog2 and Gata4 expression was also studied in the whole diaphragm at E16.5 (Fig. 4.4). Whole diaphragm immunohistochemistry performed at this stage was used to determine the expression of these factors in the muscularised part of the diaphragm and the central tendon. Fog2 and Gata4 were both found to be expressed in the muscularised regions of the diaphragm (Fig. 4.4C and 4.4B respectively); however they had a different cellular localisation. Gata4 staining was localized to nuclei, but Fog2 expression appeared to be cytoplasmic, representing an unexpected shift from the nuclear expression observed in the PPF. The cytoplasmic expression of Fog2 is paradoxical given its widely recognized function as a co-factor for Gata4 controlling gene transcription in the nucleus. We found only one reference to cytoplasmic Fog2 expression in the literature, which the authors described as having "unclear significance" (Bielinska et al., 2005). By focusing on the boundary between the central tendon and the muscularised region of the diaphragm we observed that while Gata4 is expressed throughout the entire diaphragm (Fig. 4.4D); Fog2 expression was restricted to the muscularised part of the diaphragm and not the central tendon (Fig. 4.4E). This result is in contrast to a previous report which noted that Fog2 expression overlapped with Gata4 in the central tendon of foetal mice (Jay et al., 2007). Double labelling studies in diaphragm cross-sections demonstrated that Gata4 and MyoD are not co-expressed in the same cells, thus Gata4 expression is restricted to the mesenchymal substrate of the diaphragm (Fig. 4.4F). No over-lapping expression between Fog2 and MyoD was observed (Fig. 4.4G), however when viewed at higher

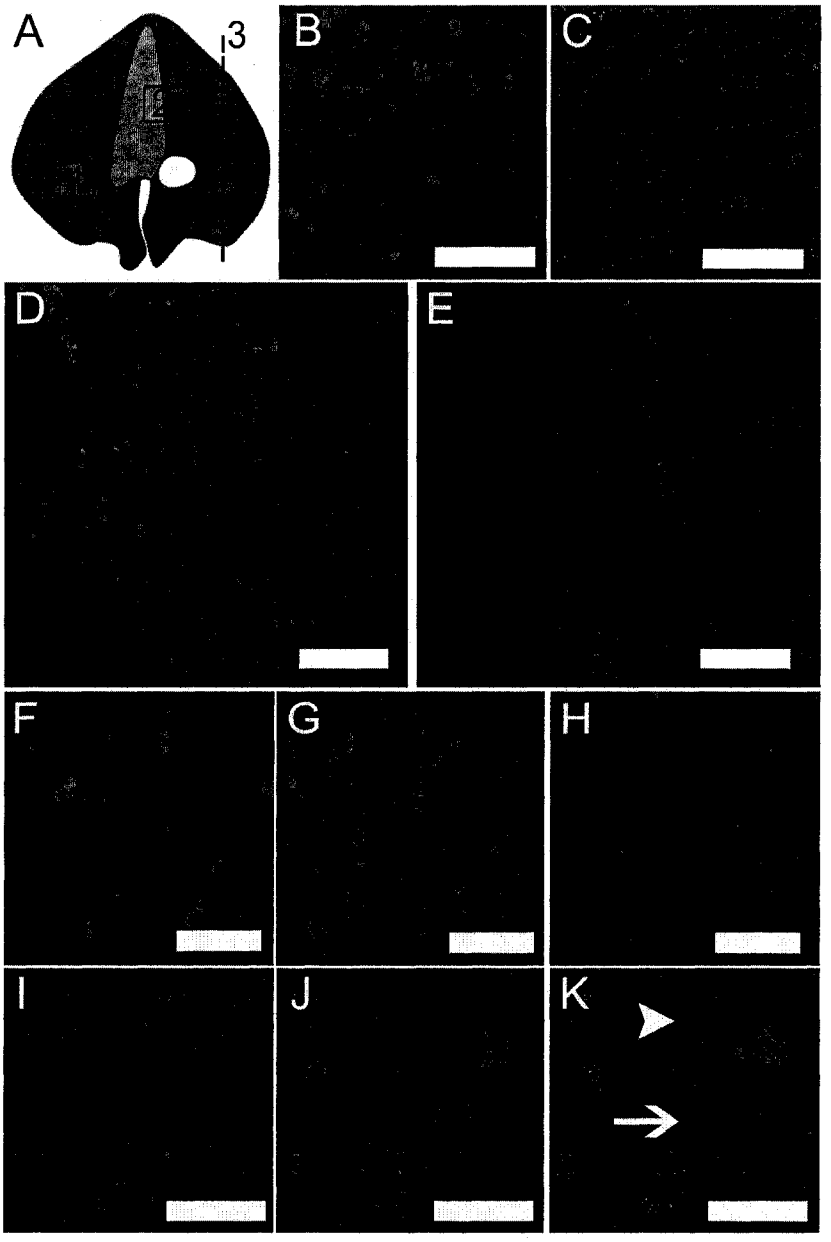

Figure 4.4 Fog2 and Gata4 expression in the diaphragm (figure legend overleaf).

Figure 4.4 A: Schematic diagram of the diaphragm (plan view); box 1 indicates the region represented in B and C, detailing the muscularised part of the diaphragm; box 2 indicates the region represented in D and E, detailing the boundary between the diaphragm muscle and the central tendon; line 3 indicates the plane of section through the diaphragm used in F-K. B: Gata4 immunopositive cells within the muscularised part of the diaphragm. C: Fog2 immuno positive cells within the muscularised part of the diaphragm. D: Gata4 immuno-positive cells are found in the central tendon and the muscularised part of the diaphragm. E: Fog2 immuno-staining is restricted to the muscularised part of the diaphragm, and is not seen in the central tendon. F: Gata4 (red) and MyoD (green) immuno-staining does not colocalise. G: Double labelling for Fog2 (red) and MyoD (green) shows no co-localised staining. H: Double labelling for Gata4 (green) and Fog2 (red) does not co-localise. I: High magnification image of Fog2 expression showing cytoplasmic labelling. J: High magnification image of nuclear MyoD expression. K: Merged image of Fog2 (red) and MyoD (green) expression; Fog2 expression is found in MyoD-negative (arrowhead) and MyoD-positive cells (arrow). Scale bars: B, C = 50 μm, D, E = 200 μm, F-H = 20 μm, I-K = 10μm.

power (Fig. 4.4I-K) it became obvious that Fog2 was expressed in the cytoplasm of MyoD-positive muscle-precursors and MyoD-negative mesenchymal cells of the diaphragm. Double-labelling for Fog2 and Gata4 revealed no over-lapping expression of these transcription factors; careful study of high magnification images failed to find any obvious Fog2 staining in the cytoplasm of Gata4-positive cells (not shown).

4.3.5 Relative expression of proteins implicated in CDH in the PPF

In the previous sections of this paper we described the expression pattern of several genes that have been implicated in the development of CDH and how they relate to each other and the myogenic cells of the PPF. Here we present further data describing the relative expression of Coup-tfII, Fog2, Gata4 and Wt1 to each other. Through our double labelling experiments we found that Coup-tfII strongly co-localises with Gata4 and Fog2 within the PPF (Fig. 4.5A and 4.5B respectively). Further, we found that Wt1 also strongly co-localises with Gata4 and Fog2 within this structure (Fig. 4.5C and 4.5D respectively). In addition to the data presented here, it has previously been reported that Coup-tfII and Wt1 also co-localise within the PPF (Clugston et al., 2006). The extent of co-localisation throughout the PPF in all cases is such that there is an almost complete overlap in expression of all these factors.

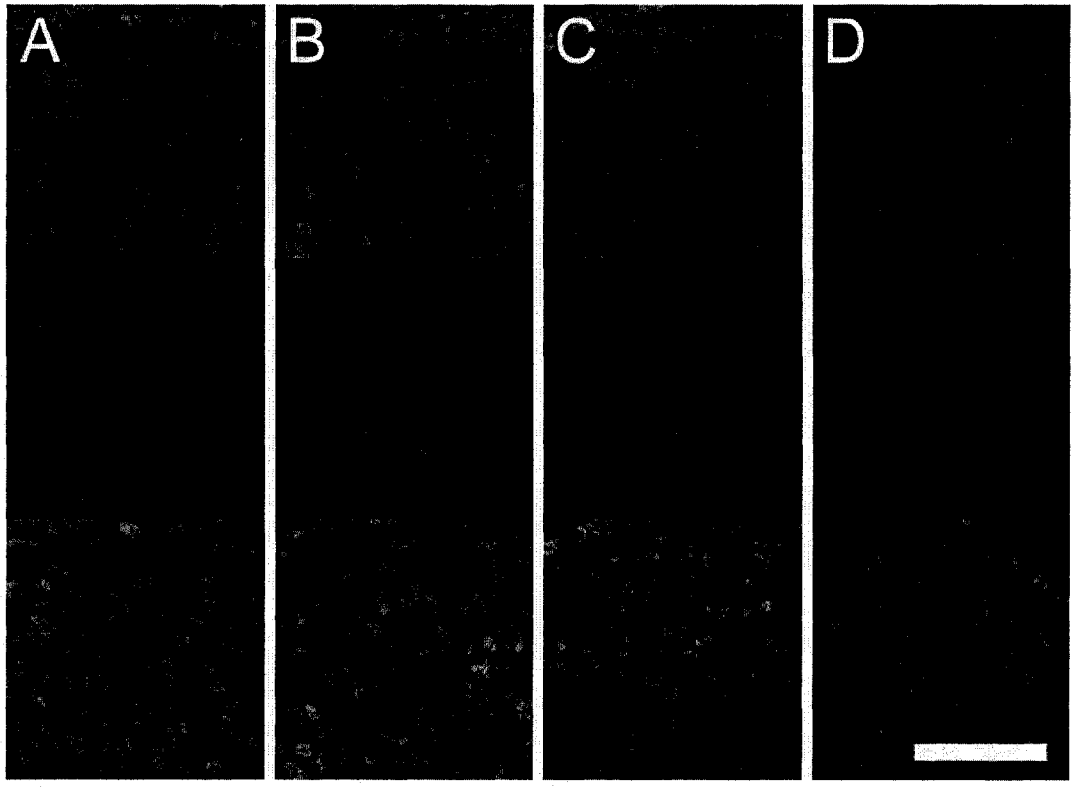

Figure 4.5 Relative expression of Coup-tfII, Fog2, Gata4 and Fog2 in the PPF

Each section shows a column of three confocal microscope images obtained from the same section, the upper panel shows the green channel, the middle panel shows the red channel, and the lower panel shows a merged image (yellow staining represents colocalisation). A: Coup-tfII (green) and Gata4 (red). B: Coup-tfII (green) and Fog2 (red). C: Wt1 (green) and Gata4 (red). D: Wt1 (green) and Fog2 (red). Scale bar: A-D = 50 μm.

4.4 Discussion

4.4.1 15q26 contains a cluster of genes expressed in the developing diaphragm

In the first part of this study we tested the hypothesis that genes from region 15q26 are expressed in the developing diaphragm. Several different groups have reported subtly different boundaries of the minimally deleted region at 15q26 associated with CDH and there is currently no consensus on its exact boundary (Castiglia et al., 2005; Klaassens et al., 2005; Scott et al., 2007). Therefore, for the purpose of this study we considered the largest deleted area so as not to exclude any genes unnecessarily. Our RT-PCR analysis identified six genes from 15q26 that were expressed in the PPF and seven genes in the more developed diaphragm. Thus we can conclude that region 15q26 contains a cluster of genes that are expressed in the developing diaphragm. This finding may help to explain why chromosomal deletions encompassing this region frequently result in CDH.

In the next step we determined the expression pattern of positively identified genes from 15q26 in the PPF. This was critical for determining the type of PPF cell in which the proteins were expressed and whether there was a common pattern amongst the gene products. Commercial antibodies were unavailable for three of the genes (*ST8SIA2*, *ARRDC4*, and *LRRC28*) and they were excluded from further analysis in this study. However future studies examining the expression of these genes by in situ hybridization are planned. The function of these excluded genes and the proteins they encode are not fully understood, therefore it is difficult to speculate on their role in diaphragm development although they certainly require further investigation. *ARRDC4* may be of particular interest: by including data from one

infant with a small 15q26 deletion, a minimally deleted region that only contained this gene was defined. Further, the same authors also identified an infant with CDH who had a unique nucleotide substitution in *ARRDC4*, though they were unable to conclude that this was causal (Slavotinek et al., 2006). *ST8SIA2* is also an interesting candidate; this gene encodes a polysialyltransferase enzyme that is thought to play a role in the polysialylation of the neural cell adhesion molecule (NCAM; Close et al., 2001). This is of interest because polysialylated-NCAM is thought to play a role in the guidance of the phrenic nerve to the PPF and has been shown to be differentially expressed during myotube separation in the diaphragm, though the significance of this with regard to the development of CDH is unclear as the pathogenesis of Bochdalek CDH does not appear to be related to diaphragm myogenesis and innervation (Allan and Greer, 1997b; Allan and Greer 1998; Babiuk et al., 2002). Below we discuss the expression pattern of *Igf1r*, *Rgma* and *Coup-tfII* in the PPF and the significance of this with regard to CDH.

The full name for *Igf1r* is insulin-like growth factor one receptor; this gene encodes a membrane-bound receptor; the primary ligand for which is insulin-like growth factor one (Igf1). Signalling by Igf1 through Igf1r mediates many of the effects of growth hormone during development and post-natal life (Jones et al., 1995). Igf1r is widely expressed in the embryo throughout gestation including, as our results show, the PPF (Bondy et al., 1990). With respect to CDH, mice with a targeted deletion of *Igf1r* do not have diaphragmatic hernia however the majority of these animals do die at birth from respiratory failure. The explanation for this respiratory failure was not determined and no lung abnormality could be found. The

diaphragm in these mice was hypoplastic, though this is set in the context of widespread muscular dystrophy and is not specific to the diaphragm (Liu et al., 1993; Powell-Braxton et al., 1993). *Igf1r* is clearly important in the differentiation of skeletal muscle including the diaphragm; however the phenotype of null-mutant mice suggests that its deletion is not directly related to the diaphragmatic hernia observed in infants with 15q26 deletion. Rather, deletion of *IGF1R* is thought to contribute to the intra-uterine growth retardation that is a common clinical feature associated with this deletion (Schlembach et al., 2001; Klaassens et al., 2007).

Repulsive guidance molecule A (Rgma) is a membrane bound signalling molecule that binds to the neogenin receptor mediating a chemorepulsive axon guidance response. It is also thought to have further roles in neuronal differentiation and survival (Matsunaga et al., 2004; Matsunaga et al., 2006; Cole et al., 2007). Recently, the family of repulsive guidance molecule proteins has also been identified as co-receptors in the bone morphogenetic protein signalling pathway (Halbrooks et al., 2007). Sequencing of critical region genes in infants with CDH revealed two instances of unique sequence variations in *RGMA*, though these were not conclusively proven to be causal in the formation of diaphragmatic hernia (Slavotinek et al., 2006). However, *Rgma* knock-out mice die at birth due to early failure of cephalic neural tube closure leading to exencephaly, there were no reports of diaphragm abnormalities and 50% of mutants are viable (Niederkofler et al., 2004). *Rgma* is not essential for diaphragm formation in mice, although it is expressed in the rodent PPF and its association with CDH in humans justifies further clarification of its role in diaphragm development.

Chicken ovalbumin upstream promoter-transcription factor II (*Coup-tfII*) was the third gene whose expression pattern we studied in the PPF. *Coup-tfII* encodes a nuclear transcription factor with a critical role in mouse development. *Coup-tfII* null mutant mice die at mid-gestation, exhibiting defects in angiogenesis and heart development (Pereira et al., 1999). Specific ablation of *Coup-tfII* in tissues expressing *Nkx3-2* induces diaphragmatic hernia phenotypically identical to Bochdalek CDH (You et al., 2005). However, unlike some of the other genes from this region, no specific CDH-causing mutation in the coding region of this gene has been identified (Slavotinek et al., 2006). Its chromosomal location in the region of 15q26 associated with CDH and the phenotype of mice lacking *Coup-tfII*, provide strong evidence that this gene is essential for diaphragm development.

4.4.2 Fog2 and Gata4 expression in the developing diaphragm

In the second part of our study we shifted our focus from the genes of 15q26 to the expression of two other genes, friend of GATA (FOG)-2 and GATA-family transcription factor (GATA)-4, both of which have been linked with CDH in humans and animal models. It is important to point out here that FOG2 is a transcriptional co-regulator of GATA4 transcriptional activity, such that FOG2 can enhance/inhibit GATA4 activity depending on the cellular context (Cantor and Orkin, 2005). *FOG2* is considered a strong candidate responsible for the development of Bochdalek hernia in infants harbouring micro-deletions encompassing 8q22-23, this hypothesis is supported by the recent identification of 2 infants with unique sequence variations in *FOG2* who had isolated Bochdalek CDH (Lurie et al., 2003; Bleyl et al., 2006; Holder et al., 2007). *GATA4* has also been singled out as an important gene in the

context of infants harbouring micro-deletions in the region of 8p23.1 (Lurie et al., 2003; Holder et al., 2007). If we interpret our PPF expression studies in the context of Bochdalek CDH in humans then our data supports a role for these genes in the basic development of the diaphragm and the etiology of CDH.

In addition to their expression pattern in the PPF and their association with Bochdalek CDH, we were also interested in looking at rodent FOG2 and GATA4 protein expression later in diaphragm development because of an association between mutations in these genes and other types of diaphragm defects. With regard to *FOG2*, there has been a case report of an infant with a specific mutation in the *FOG2* gene who had diaphragm eventration. This infant harboured a de novo mutation in exon 4 of *FOG2*, leading to the production of a truncated protein that contains none of the zinc finger DNA-binding domains essential for normal protein function (Svensson et al., 2000; Ackerman et al., 2005). Mutant mice carrying a point mutation in *Fog2* produce a similar truncated protein and, significantly, they also have abnormal muscularisation of the diaphragm. Thus, not only is deletion and mutation of *FOG2* in humans associated with Bochdalek CDH, but mutation of the gene leading to the expression of a truncated peptide is associated with diaphragm eventration. This finding can be rationalized when the expression pattern of FOG2 described in this paper is considered. In rodents, Fog2 expression in the PPF is exclusive to its non-muscular component, which is consistent with a role in early diaphragm development and the hypothesis that a malformation of the PPF precedes Bochdalek CDH. However, the expression of Fog2 in the fully formed diaphragm is intimately linked to the developing musculature, the disruption of which leads to

diaphragm eventration. This hypothesis suggests that Fog2 may have two roles in diaphragm development, an early role in helping to establish the basic structure of the diaphragm and a later role linked to its muscularisation. Therefore, genetic redundancy or residual function of the truncated Fog2 protein could be enough to support early diaphragm development, with the mutation only manifesting itself during diaphragm muscularisation. The fact that these mice do not have as severe a phenotype as *Fog2* null mutant mice suggests that some function of this protein is preserved, or compensated for, which may be able to support early diaphragm development (Svensson et al., 2000b; Tevosian et al., 2000; Ackerman et al., 2005). While *Fog2* certainly has an important role in diaphragm development, the generation of conditional *Fog2* mutant mice will be helpful in establishing the exact role of this gene in diaphragm formation.

Similarly, in addition to *GATA4's* association with Bochdalek CDH in humans, a low percentage (~30%) of mice heterozygous for a deletion in this gene (*Gata4*$^{+/-}$) have recently been reported to have diaphragm abnormalities which are phenotypically consistent with the rare central tendon defect seen in humans. However, the literature contains no link between babies with central tendon defects and *GATA4* mutations (Jay et al., 2007). The appearance of an abnormal phenotype in these mice with only one copy of *Gata4* is consistent with the observation that there is a threshold of Gata4 activity required for normal control of gene expression, below which abnormal development can occur (Xin et al., 2006). Other than establishing that Gata4 is expressed in the central tendon our results cannot explain the occurrence of central tendon defects in these mice, however review of the

literature raises one possibility. Though they do not survive long enough to study the diaphragm, it is known that *Gata4-/-* mice lack an epicardium; interestingly it is this structure that forms part of the pericardium which adheres to the central tendon of the diaphragm, thus the central tendon defects seen in *Gata4+/-* mice could result from a congenital weakness of the central tendon associated with abnormal epicardial development, rather than a primary defect in diaphragm formation (Watt et al., 2004). This possibility suggests that development of central tendon defects in *Gata4+/-* mice have distinct embryonic origins than the posterior defects seen in humans with 8p23.1 micro-deletions. A thorough examination of the central tendon defects of *Gata4+/-* mice beyond that of the original manuscript are required to address this issue. What is clear, is that the development of central tendon defects in *Gata4+/-* mice is independent from *Fog2*; not only have we shown that *Fog2* is not expressed in the central tendon but mice heterozygous for a mutant copy of *Gata4* that cannot physically interact with *Fog2*, and therefore can only function independently from it, were born at the expected ratio and were morphologically normal (Crispino et al., 2001). Despite Fog2 and Gata4's known partnership in gene regulation, the differing phenotype of mice mutant for these genes, coupled with our expression data, suggest they may function independently in the later stages of diaphragm development.

4.4.3 Mesenchymal expression of genes associated with CDH in the PPF

It has been hypothesized that the diaphragm abnormality in Bochdalek CDH arises from a defect in the non-muscular, mesenchymal cells of the PPF, and is independent of myogenesis (Babiuk et al., 2003; Clugston et al., 2006; Clugston and

Greer, 2007). Therefore, we were interested in determining if the genes associated with CDH described above were mesenchymally expressed. Insightfully, our data from this and a previous study indicates that neither Coup-tfII, Fog2, Gata4 or Wt1 expression colocalises with Pax-3 positive muscle precursor cells within the PPF, and are therefore only expressed in the non-muscular, mesenchymal component of the PPF (Clugston et al., 2006). Given the association between these factors and CDH, coupled with their exclusive expression in the mesenchyme of the PPF, we interpret the current data as supporting the hypothesis that Bochdalek CDH arises from a defect in the mesenchyme of the PPF and is independent of myogenesis. In this regard, future studies into the embryological origins of Bochdalek diaphragmatic hernia in our lab are focused on the non-muscular, mesenchymal cells of this structure.

4.4.4 Genes associated with CDH are co-expressed in the PPF

With regard to understanding how aberrant expression of certain key genes contributes to the development of an abnormal diaphragm we were also interested in examining how the expression of Coup-tfII, Fog2, Gata4 and Wt1 related to each other. Wt1 was included in this analysis because it is already known to be expressed in the PPF, *Wt1* null mutant mice have Bochdalek CDH and there are several syndromes that include CDH within their spectrum of abnormalities that are caused by a *WT1* mutation (Pritchard-Jones et al., 1990; Kreidberg et al., 1993; Devriendt et al., 1995; Reardon et al., 2004; Scott et al., 2005; Clugston et al., 2006). Significantly, we found that all of these factors are co-expressed within the same mesenchymal cells of the PPF. This is interesting with regards to the etiology of

CDH as it suggests that different genetic mutations affect the same cells within the PPF, representing a point of convergence for distinct genetic insults, i.e. the same cells within the developing diaphragm are being affected in individuals with distinct genetic etiologies. With regard to the clinical management of CDH, it is also important to observe that several of these genes are also expressed in the developing lung, thus their mutation may not only affect diaphragm development, but also that of the lung. Interestingly, Coup-tfII, Fog2, Gata4 and Wt1 all regulate gene transcription. In this regard it is possible to hypothesise that they are members of the same gene regulatory network. In support of this hypothesis, it has previously been shown that Fog2 can act as a co-repressor for Coup-tfII, and that Fog2, Gata4 and Wt1 act together to regulate the expression of some genes (Huggins et al., 2001; Rey et al., 2003). If these factors really are members of a common or overlapping gene regulatory network in the developing diaphragm it will be important to identify what the downstream genes are, and how their dysregulation might contribute to the development of diaphragm abnormalities. Further, it will be intriguing to explore any potential links with the retinoid signalling pathway, perturbation of which has been hypothesized to cause CDH (Greer et al., 2003). In this regard, it is known that retinoids are involved in controlling the expression of Gata4, Wt1 and Coup-tfII (Arceci et al., 1993; Balmer and Blomhoff, 2002). There is also a precedent for a model which combines these two hypotheses. The expression of phosphoenolpyruavte carboxykinase is sensitive to retinoids and its promoter contains composite binding sites for various transcription factors, most notably Coup-tfII and the retinoid receptors (Scribner et al., 2007). Further, retinoids can

regulate the expression of cardiogenic genes by directly interacting with Fog2 and Gata4 via the retinoid X receptor alpha (Clabby et al., 2003). The hypothesis that distinct genetic insults might contribute to the development of CDH by disrupting different components of a common pathway is attractive because it provides a unified model to understand the complex etiology of CDH, and may allow therapeutic targets to be identified (Kantarci et al., 2007).

4.4.5 Summary

It is implied from genetic studies in humans and mice that the loss of function of critical genes can lead to CDH. Our analysis of the candidate genes from the critical region at 15q26 revealed that this region contains a cluster of genes that are expressed in the developing rodent diaphragm. While there is compelling evidence to suggest that Coup-tfII is essential for normal diaphragm development, our data also indicate that there are other genes in this region which may contribute to the formation of this structure. The significance of our study into the protein expression pattern of specific candidate genes is twofold. First, we have shown that Coup-tfII, Fog2, Gata4 and Wt1 are all expressed in the non-muscular mesenchyme of the PPF, therefore supporting the hypothesis that it is abnormalities in the mesenchymal cells of the developing diaphragm that lead to the formation of Bochdalek CDH. Second, we have demonstrated that these genes are all co-expressed within the same population of cells, supporting the hypothesis that occurrences of CDH with distinct genetic etiologies arise from abnormalities in the same cell population, and possibly the same pathway within these cells. To conclude, the genetic origins of CDH are diverse and complex. However, our

analysis has revealed unifying patterns of expression that point to a common pathogenic mechanism for the development of diaphragmatic hernia.

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Chapter 5: Early development of the diaphragm and cellular mechanisms of nitrofen-induced congenital diaphragmatic hernia in the rat

Contributions: The majority of the experimental work described in this paper was performed by RDC, with technical assistance from Wei Zhang. The manuscript was written by RDC and edited by JJG.

5.1 Introduction

Congenital diaphragmatic hernia (CDH) is a poorly understood birth defect which can cause severe neonatal respiratory distress. CDH occurs in approximately 1:3000 births and is associated with a high mortality rate and long-term morbidity in survivors (Torfs et al., 1992). One of the most commonly used animal models to study the pathophysiology of CDH is the nitrofen model. Nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether) is an herbicide; administration of this compound to pregnant rats is tolerated well by the adult females but their off-spring display several congenital anomalies, including CDH (Ambrose et al., 1971; Costlow and Manson, 1981). The development of diaphragm defects in nitrofen exposed rats has been traced back to an abnormality in the pleuro-peritoneal fold (PPF; Allan and Greer, 1997). The PPF is of particular importance in diaphragm development because it is this structure that is the target for migrating muscle precursor cells (MPCs) that populate the diaphragm, and pioneer axons of the phrenic nerve. It is from the relative position of the PPF that these components spread out to form the complete neuro-musculature of the diaphragm (Clugston and Greer, 2007). Abnormalities in the PPF have been identified in nitrofen-exposed, vitamin A deficient, and *Wt1* null-mutant models of CDH (Allan and Greer, 1997; Clugston et al., 2006). The mechanism that leads to this abnormality is unknown; in fact, even the normal formation of the PPF has not been previously studied in detail. The purpose of this study was to describe the normal development of the PPF in the rat, as well as trace the origins of the PPF defect in nitrofen exposed embryos, with particular regard to the pathogenic mechanism by which it arises.

Normal formation of the PPF was examined using a carefully collected series of rat embryos. Embryonic sections were processed for basic histology to record the morphologic development of the PPF, and immunohistochemistry was performed to observe MPC and phrenic nerve distribution within this structure. In a unique opportunity we were able to compare the developing rat PPF with a series of archived histological sections from human embryos, allowing us to delineate the period of PPF development in humans as well.

Following our morphologic examination of the developing PPF we became interested in determining the cellular mechanism through which nitrofen acts to cause abnormal PPF formation. In order to achieve this goal we first explored the effect of nitrofen exposure on fibroblast cells growing in culture. This was followed by *in vivo* experiments examining nitrofen exposure in the offspring of pregnant rats. We carried out our cell culture experiments using NIH 3T3 cells. These murine fibroblast cells are embryonic in origin and we felt were an appropriate proxy for the mesenchymal cells of the developing diaphragm (Torado and Green 1963). Specifically, using NIH 3T3 cells *in vitro* we generated growth curves of control and nitrofen exposed cells. Based on the results of these experiments we also tested the effect of nitrofen exposure on cell proliferation and apoptosis. Our *in vitro* experiments led us to hypothesize that nitrofen may cause diaphragmatic hernia *in vivo* by two possible mechanisms; 1) by decreasing cell proliferation within the PPF, and/or 2) by increasing apoptosis within this structure. These hypotheses were tested using the nitrofen model of CDH. Nitrofen was administered to pregnant rats during the critical period of diaphragm development and tissue was collected at a

later stage to assess the number of actively dividing cells in the PPF, as well as to identify those cells that might be apoptotic.

The results presented in this paper are the first to systematically describe the development of the PPF in rats, as well as make unique observations from human embryos. We also report the first experiments examining the effect of nitrofen on NIH 3T3 cells grown in culture, and significantly, the cellular mechanisms of abnormal PPF development in the nitrofen model of CDH. We conclude that decreased cell proliferation contributes to the development of CDH in nitrofen exposed rats and that there is no clear evidence to support the hypothesis that increased apoptosis also contributes to this defect. This study provides an improved understanding of the pathogenesis of diaphragm defects in the nitrofen model of CDH.

5. 2 Methods

A description of the basic methodologies concerning rat breeding, tissue collection, basic histology, immunohistochemistry, and microscopy can be found in the relevant section of chapter 2. The following sections concern methods only employed in this study and are not described elsewhere in this thesis.

5.2.1 Human embryo archive

To study PPF development in humans we examined archived slides of human embryos drawn from the Shaner collection, housed at the University of Alberta under the custodianship of Dr Keith Bagnall. A complete series of transverse sections from embryos ranging in crown rump length (CRL) from 5 to 15 mm, stained with haematoxylin and eosin, were available for study.

5.2.2 Immunohistochemistry

A list of antibodies used in this study can be found in table 5.1, a description of the basic immunohistochemistry protocol used can be found in section 2.4. The protocol for BrdU immunocytochemistry is provided below (section 5.2.4).

5.2.3 NIH 3T3 cell growth curve generation

NIH 3T3 cells were obtained from the American type culture collection (Manassas, VA) and grown using Dulbecco's modified essential medium (DMEM; Invitrogen, Burlington, ON) with 10% bovine calf serum, 100 units of penicillin and 100 μg of streptomycin under standard conditions (37°C, 5% CO_2, 95% relative humidity). To generate growth curves, cells were plated in 60 mm culture dishes at a seeding density of 1 x 10^5 cells/ml. There were 5 experimental groups; control (0 μM), 10 μM, 25 μM, 50 μM, and 100 μM nitrofen. Stock solutions of nitrofen were

Table 5.1 Details of primary antibodies used

Antibody	Dilution	Source (Catalogue number)
Mouse monoclonal antibodies:		
Neurofilament	1:200	DSHB (2H3)
BrdU	1:50	BD (555627)
Goat polyclonal antibodies:		
Pax3/7 (C-20)	1:100	SCBT (sc-7748)

DSHB: Developmental studies hybridoma bank, University of Iowa; BD: Becton-Dickson Biosciences, Mississauga, ON; SCBT: Santa Cruz Biotechnology, Santa Cruz, CA.

made up in a 10% ethanol solution at a concentration of 10 mM, this was then diluted to the final concentration used in the experiment with DMEM. The highest ethanol concentration in any group was 0.1%; control experiments comparing untreated cells with the ethanol vehicle found no difference (data not shown). Once cells were plated they were incubated over a period of 144 h; at 24 h intervals a subset of plates were removed for cell counting. To count the number of cells first the DMEM was removed and the plate was rinsed with 0.1M phosphate buffered saline (PBS). Cells were then trypsinised using 0.25% trypsin (5 min at 37°C). Following trypsinisation, the cells were then diluted to a fixed volume using DMEM and a sample was counted using a haemocytometer (Sigma-Aldrich Inc., St Louis, MO). Data is presented as the mean ± SEM, n = 4. Data from each time point was analyzed using a one-way ANOVA with Tukey's multiple comparisons test as a post-hoc analysis. In all experiments a p-value < 0.05 was considered statistically significant. In all experiments statistical analysis was performed using Prism v4.0 (GraphPad software Inc., San Diego, CA).

5.2.4 In vitro cell proliferation assay

Bromodeoxyuridine (BrdU) is an analogue of the nucleoside thymidine and is commonly used to detect proliferating cells (Hardonk et al., 1990). BrdU is incorporated into the newly synthesized DNA of proliferating cells and can be detected via immunocytochemistry to indicate which cells are proliferating. In this experiment cells were plated at a seeding density of 1 x 10^5 cells/ml and grown in 8-chambered slides (Nalge Nunc Int., Rochester, NY) using DMEM as a culture medium. In order to assay the cells while they were actively dividing, they were

grown for a period of 24 h before nitrofen was added to the culture medium. Separate experiments, with matched controls, were carried out for each dose of nitrofen tested at 24 h and 48 h time points. One hour before the end of the incubation period, BrdU (F. Hoffmann-La Roche Ltd, Basel, Switzerland) was added to the culture medium (10 μM final concentration). BrdU incorporation was visualized by immunocytochemistry described here in brief. Initially, DMEM was removed and the cells were rinsed with PBS before being fixed with 2% para-formaldehyde at room temperature for 20 min. Cells were then incubated for 60 min with 2N HCl, which was subsequently removed and replaced with 0.1 M sodium borate for 10 min. Following thorough washing with PBS a blocking step was carried out using 1% bovine serum albumin (BSA) and 0.4% Triton-100 in PBS for 30 min. Following this blocking step the cells were incubated with a mouse monoclonal anti-BrdU antibody (BD Biosciences, Mississauga, ON; 1:500 dilution in 0.1% BSA, 0.4% Triton-100, and PBS) for 2 h at room temperature. Following incubation with the primary antibody, cells were thoroughly washed with PBS and incubated with a biotin conjugated Goat anti-mouse secondary antibody (Sigma-Aldrich, Inc.) for 60 min at a 1:200 dilution. The slides were then washed in PBS and incubated with a 1:100 avidin-biotinylated peroxidase complex solution (ABC kit, PK4000, Vector Laboratories, Burlingame, CA) for 60 min. A final wash step was followed by antigen visualization via Nickel intensified 3,3-Diaminobenzidine tetrahydrochloride labelling (0.04% 3,3-Diaminobenzidine tetrahydrochloride, 0.04% hydrogen peroxide, 0.6% ammonium nickel sulphate, in 0.1 M Tris buffer). Following immunocytochemistry, stained cells were viewed using an inverted

microscope (Leica DM IRB; Leica Microsystems; Wetzlar, Germany) and digital photographs were taken of three randomly assigned fields in each well. These images were then manually analyzed to determine the total number of cells and the number of BrdU-positive cells. Data is presented as mean ± SEM, n = 4 to 8. As each experimental group was matched with its own control, statistical analysis was performed by Student's unpaired *t*-test.

5.2.5 In vitro cell death assay

Nitrofen's possible effect on cell death was determined using a cell death detection ELISA kit (Roche). This assay works on the principle that during apoptosis DNA is degraded by endogenous endonucleases generating apoptosis-associated nucleosomal fragments that can be detected in the cytoplasm of lysed cells. Further, by analysing the culture medium supernatant and cell lysates separately, it is possible to distinguish between necrotic cell death and apoptotic cell death. For this experiment NIH 3T3 cells were grown in 96-well plates using DMEM at a seeding density of 1 x 10^5 cells/ml. There were 5 experimental groups, control (0 μM), 10 μM, 25 μM, 50 μM, and 100 μM nitrofen. In this experimental series, 3 time points were used; 4 h, 24 h and 48 h. The ELISA was carried out according to the manufacturers protocol as briefly described below. Following the defined incubation period, culture plates were centrifuged at 200 g for 10 min. After centrifugation the supernatant was removed from each well and a portion stored at 4°C for later analysis. Subsequently, 200 μl of lysis buffer was added to each well and incubated at room temperature for 30 min and then the plate was centrifuged at 200 g for 10 min. After this step three 20 μl samples of supernatant were taken from

each well and added to separate wells on the ELISA plate; three 20 µl samples were also distributed from the culture medium supernatant collected previously. To each well 80 µl of immune-reagent were added (this reagent contained anti-DNA-peroxidase and anti-histone-biotin antibodies). The ELISA plate was subsequently incubated at room temperature for 2 h on a shaker (at 300 rpm). Following incubation with the immune-reagent, each well was thoroughly rinsed with incubation buffer, then 100 µl of ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt) solution was added to each well and incubated for ~15 min. After this time, 100 µl ABTS-stop solution was added to each well and the absorbance was measured at 405 nm using a plate reader (references wavelength = 490 nm; BioTek EL808; BioTek Instruments, Inc., Winooski, VT). Data is presented as mean ± SEM, n = 3 to 4. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons test as a post-hoc analysis.

5.2.6 BrdU administration and labelling in vivo

Cell proliferation *in vivo* was determined by relative BrdU incorporation in control and nitrofen exposed embryos. One hour prior to tissue collection on E13.5 pregnant dams were given an intra-peritoneal injection of 50mg/kg BrdU dissolved in sterile saline solution (0.9%). Tissue was then collected and processed as described above. BrdU incorporation was visualized by immunohistochemistry using a methodology identical to that described for the *in vitro* cell proliferation assay except that slides were incubated with streptavidin-488 (Molecular Probes, Eugene, OR; 1:200 dilution) instead of the avidin-biotinylated peroxidase complex described above, and the cells were cover-slipped with a DAPI containing mounting

medium. This approach allowed fluorescently labelled cells to be visualized by confocal microscopy. On each section analyzed we focused on three areas of the embryo to assess BrdU incorporation; the PPF, the lung bud, and the distal limb bud. Sections were randomly assigned for manual cell counting. All the cells were counted within the triangular structure of the PPF; in the lung and limb buds a randomly assigned square was assigned within which the cells were counted. In each case the number of BrdU-positive nuclei and the number of DAPI-positive nuclei was counted to produce a percentage of BrdU-positive cells within the structure. Data is presented as mean ± SEM; statistical analysis was performed using Student's unpaired t-test to compare means from control and nitrofen exposed animals for each structure.

5.2.7 TUNEL labelling in vivo

Apoptotic cells were detected *in vivo* using an *in situ* cell death detection kit (Roche) which is based on terminal uridine deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). TUNEL labelling was carried out according to the manufacturer's protocol with the appropriate positive and negative controls and is described here only in brief. Slides of paraffin-embedded tissue sections were dewaxed in xylene and rehydrated in a graded series of alcohol. Sections were then incubate with proteinase K (10 μg/ml) for 30 min at room temperature. Following a thorough rinse with PBS the sections were incubated with the TUNEL reaction mixture for 60 min at 37°C, then given a final rinse in PBS and cover-slipped with DAPI-containing mounting medium. TUNEL labelling was visualized in the PPF using confocal microscopy. TUNEL-positive cells within the PPF were counted in

randomly selected sections from 6 controls and 6 nitrofen exposed embryos. Due to the low number of TUNEL-positive cells observed in both groups only the absolute number of cells was counted; this data is presented as the mean ± SEM. Statistical analysis was performed by Student's unpaired *t*-test.

5.2.8 Administration of pifithrin-α

The apoptosis-inhibitor pifithrin-α (Sigma-Aldrich) was dissolved in DMSO at a concentration of 1 mg/ml, it was administered by intra-peritoneal injection using a 26 G ½ needle. Nitrofen treated rats were administered two 2.2 mg/kg doses of pifithrin-α, 6 h and 18 h after nitrofen administration; this timing and dose was selected based on the rate by which nitrofen is taken up by the embryo and the possible embryo-toxic effects of pifithrin-α (Komarov et al., 1999). The administration of DMSO only had no effect on the incidence of diaphragmatic hernia (data not shown). At E17 tissue was collected by caesarean section and dissected to determine the incidence of diaphragm defects. Data is presented as the mean ± SEM; statistical analysis was performed using a Student's *t*-test.

5.3 Results

5.3.1 Development of the PPF in rats

A series of rat embryos were collected ranging in CRL from 5 mm to 9 mm. This covers an approximately 48 hour period from embryonic day (E)12 to E14. Embryonic tissue was processed and split into two groups: one for haematoxylin and eosin staining and the second for immunohistochemistry. No structure equivalent to the PPF could be visualized in 5 mm embryos (data not shown). The PPF first becomes apparent in 6 mm embryos and grows rapidly over the next ~36 hours (figure 5.1A-D). Immunohistochemical staining for MPCs (Pax3) and the phrenic nerve (neurofilament) revealed the relative chronology of these components arrival into the PPF (figure 5.1E-H). In 6 mm embryos (figure 5.1E), MPCs can be observed in the body wall adjacent to the nascent PPF, although only one or two actual MPCs were observed in the PPF itself. At 7 mm (figure 5.1F), the number of MPCs in the growing PPF has increased, however the phrenic nerve is not yet visible in this structure. At 8 mm (figure 5.1G) the PPF has grown in size again and this is matched by a further increase in the number of MPCs, a similar picture was observed at 9 mm (figure 5.1H). At 8 mm (figure 5.1G), the phrenic nerve first becomes obvious in the PPF as a bundle of neurofilament-positive fibres in the medial angle of the triangular PPF.

5.3.2 Human versus rat PPF development

Archived sections of human embryos from the Shaner collection were examined. The classic triangular outline of the PPF was first discernible in human embryos with a CRL of 6 mm. As embryonic development continues the PPF can

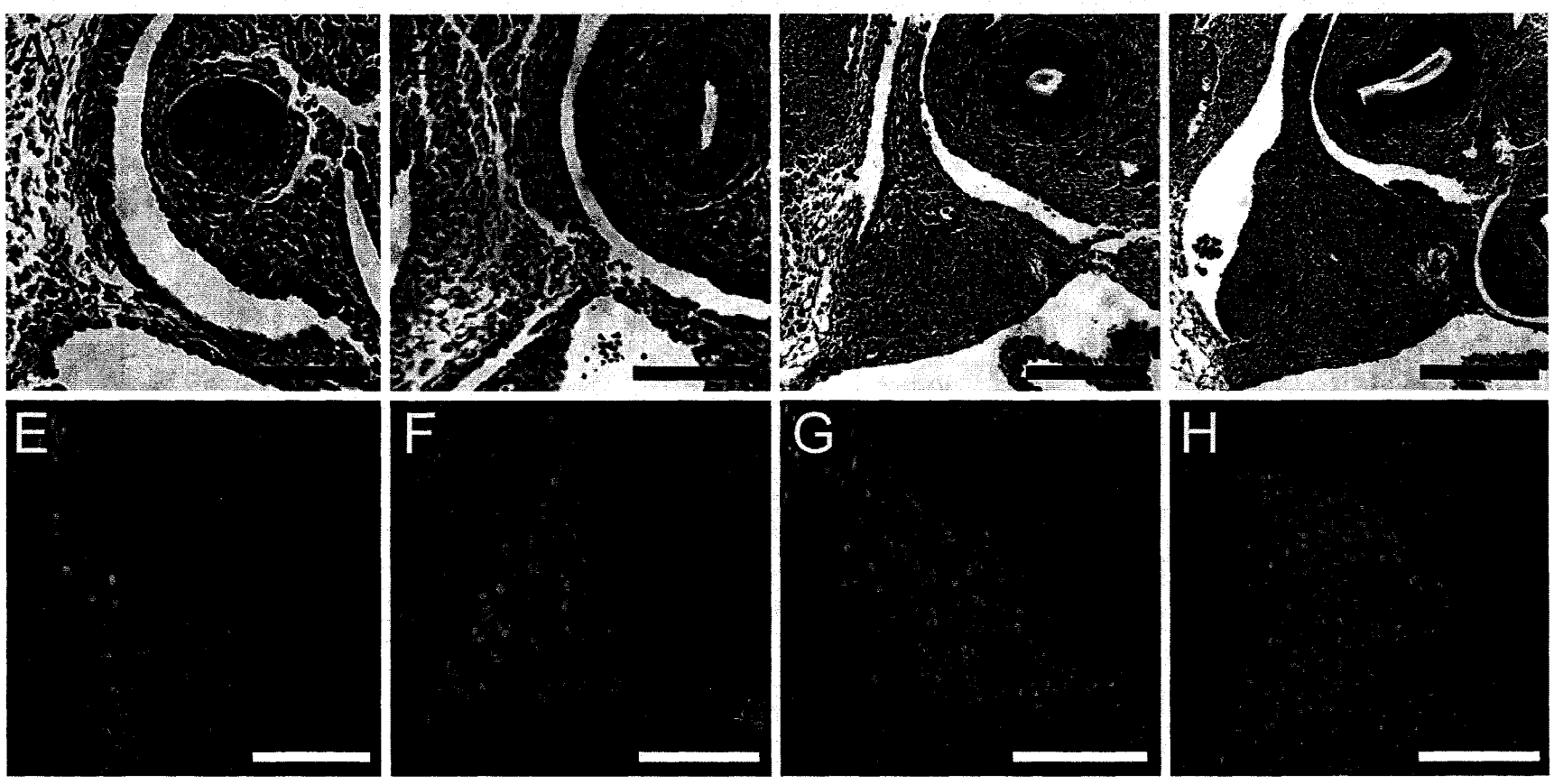

Figure 5.1 Timeline of PPF development in the rat embryo (figure legend overleaf).

Figure 5.1 Haematoxylin stained transverse sections of the developing PPF from embryos with a CRL of 6 (A), 7 (B), 8 (C) and 9 (D) mm. The lower row shows a series of confocal microscope images showing fluorescence for muscle precursor cells (Pax3, green) and the phrenic nerve (neurofilament, red) from embryos with a CRL of 6 (E), 7 (F), 8 (G) and 9 (H) mm. Scale bars: A, E = 100 μm; B, C = 150 μm; D = 200 μm; F, G, H = 125 μm.

be in seen in embryos up to a CRL of 14 mm. At this later stage, the structure of the PPF is relatively smaller and cannot be easily distinguished from the diaphragm itself. Thus the transient structure of the PPF exists in human embryos with a CRL between 6 and 14 mm, this corresponds to Carnegie stages 13 to 17 and spans the 4^{th} to 6^{th} week of human gestation. In figure 5.2 we present comparison images between human and rat embryos; these images clearly show the triangular profile of the PPF adjacent to the developing lung bud in both species.

In human embryos a structure which we identified as the phrenic nerve (based on its location and different uptake of the counter-stain) was visible in the same location as it is in the embryonic rat. However, as we could not perform immunohistochemistry for neurofilament on these sections this identification could not be confirmed.

5.3.3 NIH 3T3 cell growth curve analysis

To determine if nitrofen has any effect on NIH 3T3 cells grown in culture we generated growth curves of proliferating cells at concentrations of 0 μM (control), 10 μM, 25 μM, 50 μM, and 100 μM nitrofen (figure 5.3). A count of cells grown in the absence of nitrofen produces a typical sigmoidal growth curve with an obvious lag phase, exponential growth phase and a plateau when the culture is saturated. As can be seen in figure 5.3 the addition of nitrofen to the culture medium shifted the growth curve to the right, indicating delayed growth in these cells. Moreover this effect was dose-dependent, with increasing concentrations of nitrofen having a greater inhibitory effect. This effect was confirmed by statistical analysis of cell number as presented in table 5.2.

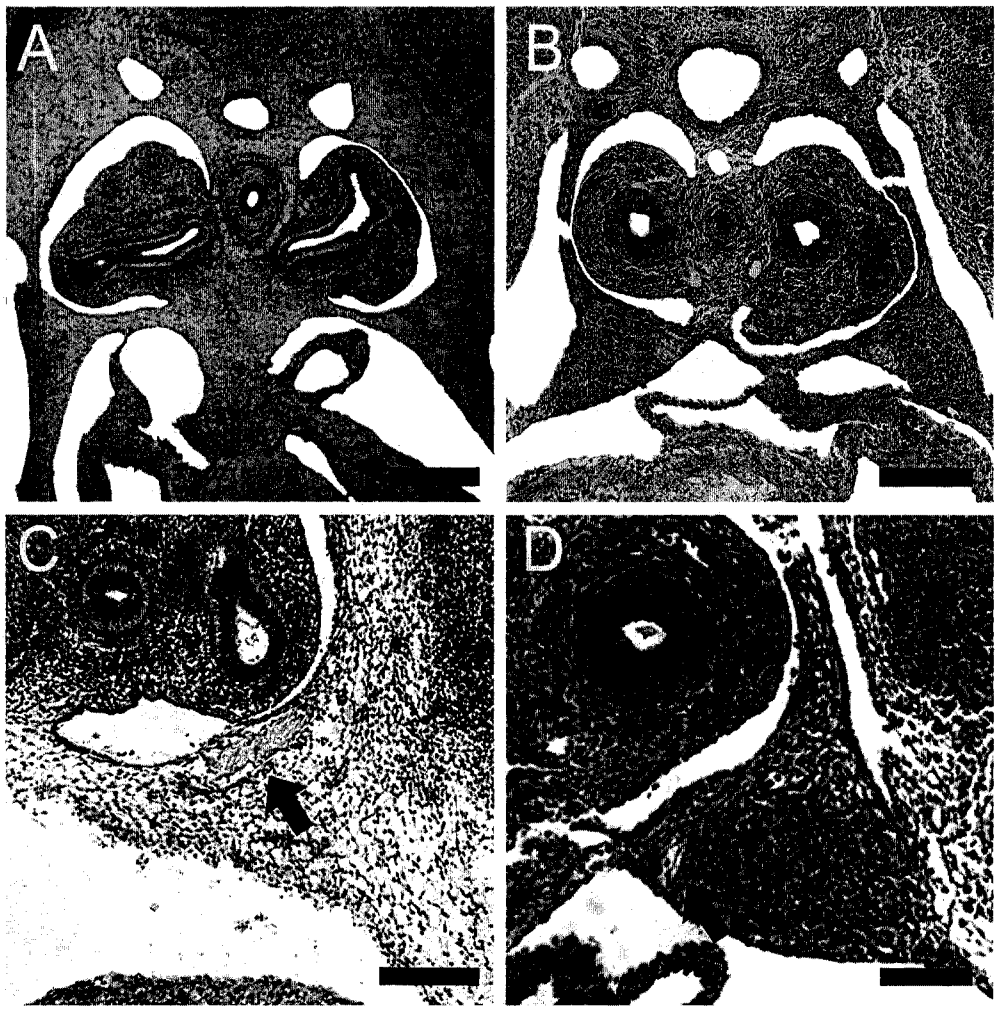

Figure 5.2 Comparison of the PPF in rats and humans.

A) Transverse section through a 10 mm human embryo, showing the typical triangular profile of the PPFs on either side of the body, adjacent to the developing lung buds. Compare with B, a transverse section through a rat embryo (8-9 mm). C) High power view of a PPF from an 11 mm human embryo, compare with the similar anatomy of the rodent PPF (D). The black arrows in C and D indicate the location of the phrenic nerve in the PPF. Scale bars: A = 400 μm, B = 250 μm, C = 200 μm, D = 100 μm.

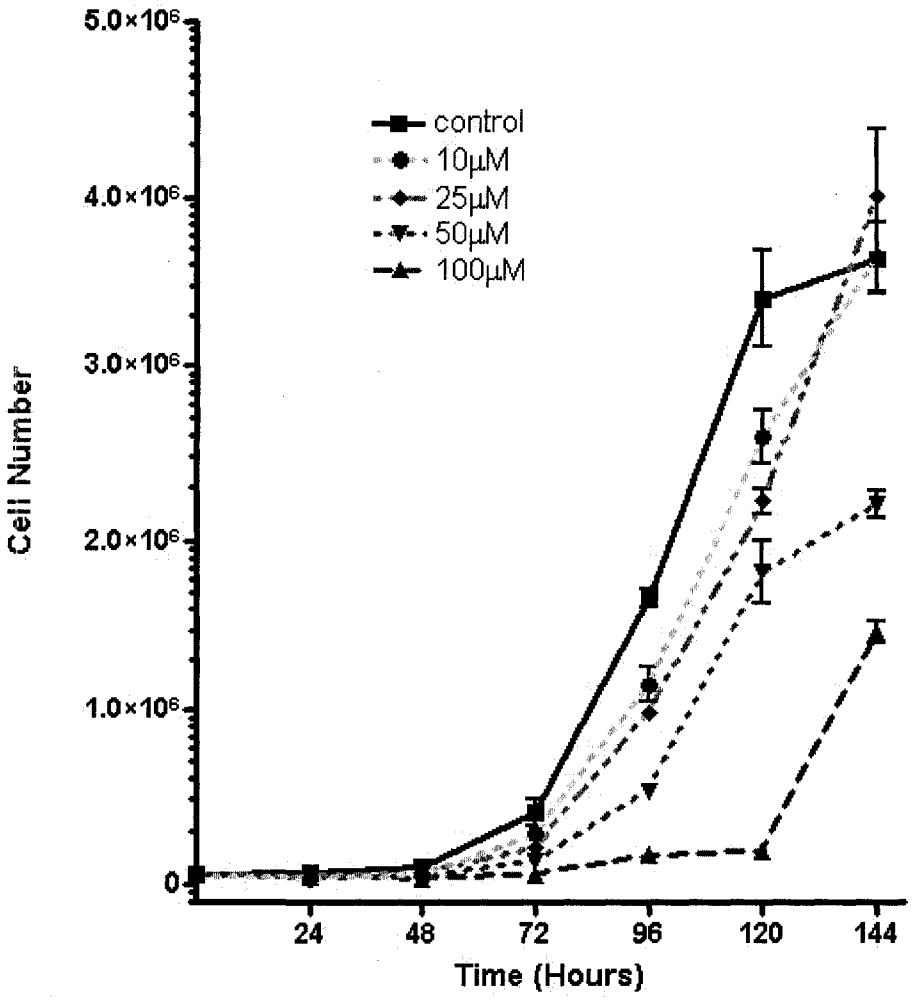

Figure 5.3 Growth curve of NIH 3T3 cells grown *in vitro*.

Representative growth curve of NIH 3T3 cells grown in culture with increasing concentrations of the CDH-inducing teratogen, nitrofen, over a 144 h time period. Nitrofen exposure decreases the number of cells observed in a dose-dependent manner. Each data point is the mean cell number ± SEM; n = 4.

Table 5.2 NIH 3T3 Cell counts following nitrofen exposure

		Nitrofen			
	Control	10μM	25μM	50μM	100μM
24 Hours	6.5 ± 0.8	3.6 ± 0.7*	4.7 ± 0.3	4.4 ± 0.4	4.7 ± 0.4
48 Hours	10.2 ± 1.8	5.3 ± 0.3*	7.0 ± 0.8	4.6 ± 1.1*	3.6 ± 0.2*
72 Hours	40.1 ± 7.8	28.4 ± 2.7	20.7 ± 1.5*	13.2 ± 3.1*	5.9 ± 1.2*
96 Hours	167.4 ± 5.5	115.2 ± 10.5*	98.3 ± 2.0*	52.9 ± 1.9*	16.3 ± 1.0*
120 Hours	340.1 ± 29.2	258.5 ± 15.2*	222.5 ± 7.1*	181.9 ± 18.5*	18.8 ± 1.4*
144 Hours	364.2 ± 21.3	365 ± 20.8	400.9 ± 38.1	221 ± 7.6*	146.7 ± 6.4*

All values are n = 4; mean ± SEM (values are all $x10^4$); * significant difference from control, $P < 0.05$, one-way ANOVA.

5.3.4 BrdU incorporation in vitro

In order to determine if nitrofen had an inhibitory effect on NIH 3T3 cell proliferation we quantified BrdU incorporation in actively dividing cells exposed to 0 μM (control), 10 μM, 25 μM, 50 μM and 100 μM nitrofen (figure 5.4). As a metric of cell proliferation we used the percentage of BrdU-positive cells, e.g. cultures with an elevated fraction of BrdU cells can be considered to be proliferating faster than cells with less BrdU incorporation. As can be seen in figure 5.4A, the presence of nitrofen in the culture medium had no effect on BrdU incorporation at any dose after a 24 hour exposure. However, after an incubation period of 48 hours (Figure 5.4B) a clear effect on cell proliferation emerges. At this time point nitrofen exposed cells incorporated significantly less BrdU compared to control cells, further there appeared to be a dose-dependent trend in nitrofen's effect, such that 100 μM > 50 μM > 25 μM > 10 μM at inhibiting cell proliferation.

5.3.5 Detection of cell death in vitro

To determine if nitrofen exposure caused increased cell death in cultures of NIH 3T3 cells we carried out a cell death detection ELISA. We examined cell death at three time points (4, 24 and 48 hours) using 10 μM, 25 μM, 50 μM, 100 μM nitrofen. For each time point two separate ELISA experiments were performed, with each group repeated in triplicate. Examination of culture medium supernatants in all experiments found no evidence of cell death by necrosis (data not shown). Cytoplasmic fractions of cells exposed to nitrofen were also assayed (Figure 5.5); after incubation periods of 4 h and 24 h we found no evidence of increased apoptotic cell death following nitrofen exposure (figure 5.5A and 5.5B).

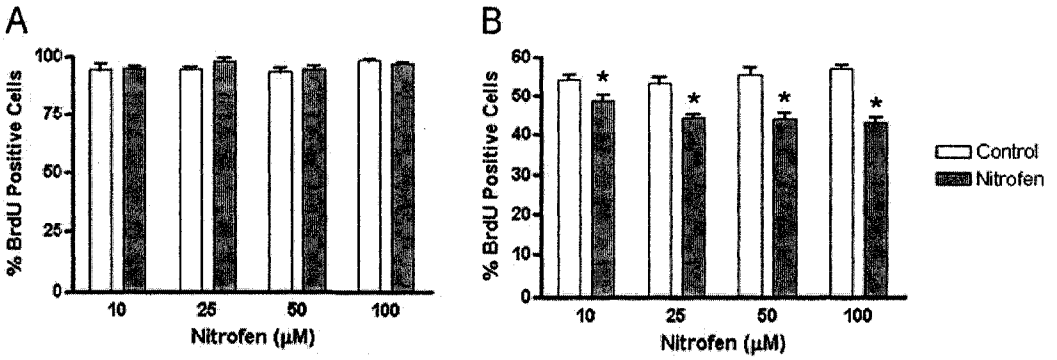

Figure 5.4 NIH 3T3 cell proliferation *in vitro*.

The percentage of BrdU-positive cells following exposure to increasing concentrations of nitrofen over a 24 h (A) and 48 h (B) time period is shown. Nitrofen decreases cell proliferation after a 48 h incubation period at all concentrations, but not after 24 h. * $P < 0.05$; mean ± SEM; n = 4-8.

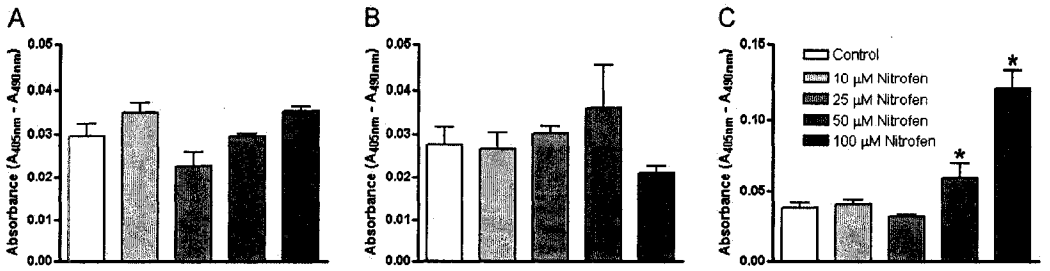

Figure 5.5 Analysis of nitrofen induced apoptotic cell death in NIH 3T3 cells.

Data generated from ELISAs examining apoptosis-associated nucleosomal fragments in nitrofen exposed cells after incubation periods of 4 h (A), 24 h (B) and 48 h (C) is shown. Nitrofen only induces apoptosis at the highest doses tested after a 48 h time period. * $P < 0.05$, mean ± SEM; n = 3-4.

On the other hand, after a 48 h incubation period we consistently saw increased absorbance in cytoplasmic fractions of cell exposed to 100 μM and, to a lesser extent, 50 μM nitrofen (figure 5.5C). There was no such increase observed in cells exposed to 10 μM and 25 μM nitrofen.

5.3.6 Cell proliferation in nitrofen-exposed embryos

To determine nitrofen's effect on cell proliferation, the percentage of BrdU-positive cells in the PPF, lung, and limb bud was determined for control and nitrofen exposed animals (figure 5.6). Dams in the nitrofen exposed group were administered 150 mg of nitrofen by oral gavage on E8 of gestation and tissue was collected at ~E13 (average CRL = 8 mm). For each group 6 to 11 embryos were studied, with cell counts taken from 3 randomly selected sections, and the data pooled to provide an average value of percent BrdU incorporation. A representative example of BrdU staining in the PPF of control and nitrofen exposed embryos is shown in figure 5.6A. Statistical analysis revealed a significant reduction in cell proliferation within the PPF of nitrofen exposed embryos compared to control (figure 5.6B); the lung buds of embryos in the nitrofen group also had a significantly lower percentage of BrdU incorporation (figure 5.6C). While both the lung and diaphragm are recognized as targets of nitrofen exposure we also counted BrdU incorporation in the limb bud, a structure not typically associated with nitrofen teratogenesis. This was to determine if the decreased cell proliferation seen in the PPF and lung was a general effect or specific to these structures. In this regard we found no significant difference in the mean number of BrdU-positive cells in the limb bud of control and nitrofen exposed embryos (figure 5.6D).

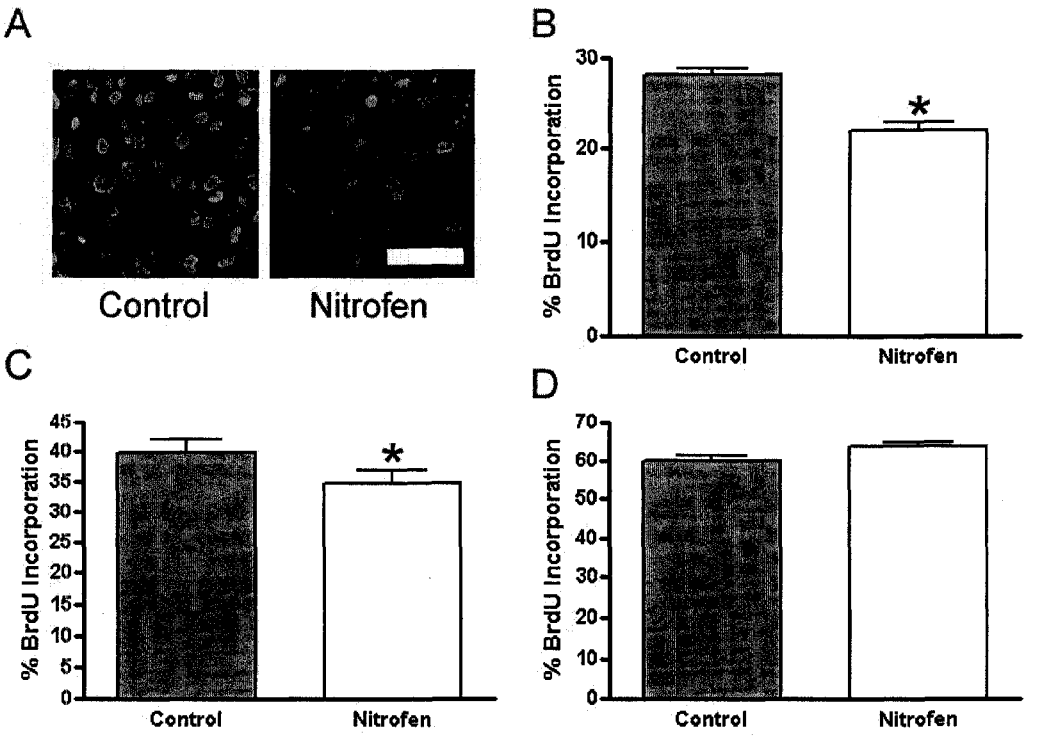

Figure 5.6 Cell proliferation in nitrofen exposed embryos.

Representative immuno-staining in the PPF of control and nitrofen exposed embryos (A); BrdU-positive cells are labelled green and DAPI-positive nuclei are labelled blue. The percentage of BrdU-positive cells was determined for the PPF (B), lung (C) and limb bud mesenchyme (D) in control and nitrofen exposed tissue. * $P < 0.05$, mean ± SEM; n = 17-33. Scale bar = 50 μm.

5.3.7 TUNEL labelling in the PPF of nitrofen-exposed rat embryos

TUNEL labelling was carried out in the PPF of nitrofen exposed rat embryos in order to determine if exposure to this teratogen induced cell apoptosis. A previous study in our laboratory examined cell death in the PPF following nitrofen exposure on E8.5 and was unable to observe an increase in apoptosis at E12.5 (D. Allan, unpublished observations). Therefore, in this series of experiments 150 mg of nitrofen was administered at E11.5 and tissue was collected at E12.5 (average CRL = 6-7 mm), thus reducing the time period between nitrofen administration and the assay for apoptotic cells; we considered this approach to increase our chance of observing any effect of nitrofen on apoptosis within the PPF. Serial sections of the PPF were labelled with TUNEL and searched for evidence of apoptotic cells in control and nitrofen-exposed embryos. We scanned through the rostral to caudal extent of PPFs in both groups and could find no evidence of increased apoptosis in nitrofen-exposed tissue. A small number of apoptotic cells were observed in the PPF of either groups of animals, a count of these cells in a series of randomly selected PPFs from 6 control and 6 nitrofen exposed animals revealed no significant difference in the average number of apoptotic cells observed (figure 5.7A). Representative TUNEL labelling within the PPF is shown for positive control tissue (DNase treated; figure 5.7B), untreated control tissue (figure 5.7C), and nitrofen exposed tissue (figure 5.7D).

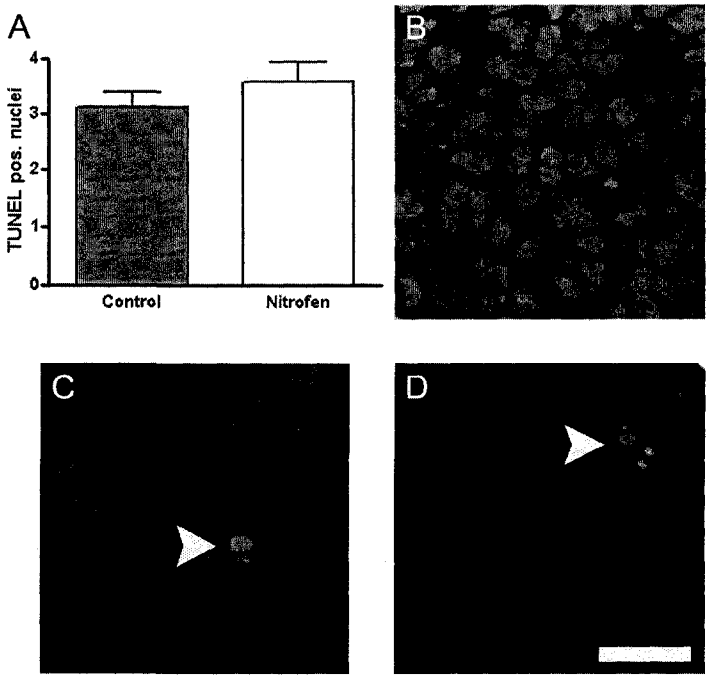

Figure 5.7 Apoptotic cell death in nitrofen-exposed embryos.

A) Bar chart comparing the number of apoptotic cells in the PPF of control and nitrofen-exposed embryos, no statistical difference was found ($P > 0.05$; mean ± SEM; n = 17-19). Representative immuno-staining in the PPF of control and nitrofen-exposed embryos are also shown; in each image cell nuclei are coloured blue (DAPI staining) and TUNEL-positive labelling is green: B) DNase treated positive control tissue, C) control embryo, and D) nitrofen-exposed embryo. Scale bar = 20 μm.

5.3.8 Pifithrin-α has no effect on the incidence of CDH

In order to determine if maternal nitrofen administration induces apoptosis in the developing embryo within 24 hours of exposure, we compared the incidence of diaphragmatic hernia in nitrofen exposed rats against nitrofen exposed rats also treated with the apoptosis-inhibitor pifithrin-α (figure 5.8). The incidence of CDH in the nitrofen only group was 50.93% ± 6.98 (n=4), and the incidence of CDH in the nitrofen plus pifithrin-α group was 58.57% ± 13.19 (n=5). These values were not significantly different ($p > 0.05$), thus administration of the apoptosis-inhibitor pifithrin-α had no effect on the incidence of nitrofen induced CDH.

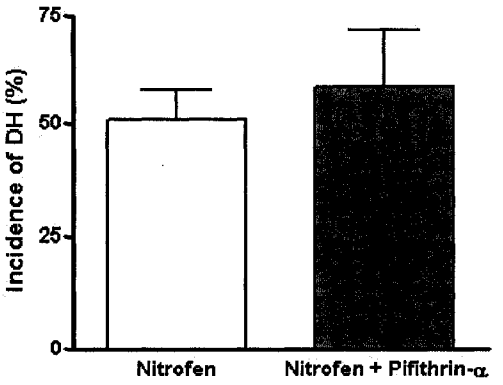

Figure 5.8 Pifithrin-α has no effect on the incidence of nitrofen-induced CDH.

Bar chart comparing the incidence of CDH amongst the off-spring of nitrofen-exposed mothers compared to those who were exposed to nitrofen and the apoptosis inhibitor pifithrin-α ($P > 0.05$; mean ± SEM; n = 4-5).

5.4 Discussion

The purpose of this study was to investigate the embryonic origins of the PPF and to study the mechanism of the teratogen nitrofen, *in vitro* and in the cells of the developing diaphragm *in vivo*, which leads to malformation of the PPF and subsequent CDH.

5.4.1 Development of the PPF in rats and humans

The PPF is an essential part of the developing diaphragm and has shown to be malformed in multiple animal models of CDH (Clugston et al., 2006). Our studies into PPF development in rat embryos show that this structure rapidly develops in embryos with a CRL of 6 to 9 mm which is equivalent to ~ E12 to E14. As has been previously shown, the growing PPF contributes to a region of the postero-lateral diaphragm. Further, at E15 MPCs and the phrenic nerve from the relative position of the PPF spread out to populate the entire diaphragm (Clugston and Greer, 2007). MPCs migrate to the PPF from the lateral dermo-myotomal lip (Bladt et al., 1995; Birchmeier and Brohman, 2000; Babiuk et al., 2003), the data presented here shows that they do not first arrive at the PPF until the 6 mm stage (equivalent to ~E12.5). Interestingly, we found that the MPCs that populate the PPF arrive ~12 hours in advance of the phrenic nerve. The relationship between MPC migration to the PPF and innervation by the phrenic nerve is the subject of continuing research.

In human embryos we observed a structure with the same triangular cross-section and anatomical location as the PPF in rats, supporting the assumption that early development of the diaphragm is similar in humans and rats, and justifying the

use of rodents as a model of human diaphragm development. By examining an archived series of human embryos we were able to delineate the period of PPF development in humans which takes place in embryos with a CRL between 6 and 14 mm, this is equivalent to the 4^{th} to 6^{th} week of gestation.

The question remains whether CDH in humans arises from a defect in the PPF. It has previously been shown that abnormal development of the PPF underlies diaphragm defects in three distinct animal models of CDH; the nitrofen model, vitamin A deficient rats and Wt1 null-mutant mice, thus this type of defect seems generally applicable (Clugston et al., 2006). If we assume that CDH in humans does have its origins in a malformation of the PPF, that means a critical period of diaphragm development for CDH exists between 4 and 6 weeks of gestation. This is of significance because this critical period is very early in gestation and occurs before the mother may know she is pregnant.

Further to our studies into early development of the PPF and nitrofen's pathogenic mechanism, continuing research is focusing on the earliest age at which a malformation in the PPF can be discerned. Based on preliminary data this certainly occurs prior to the 8 mm stage of rodent development (E13.0).

5.4.2 Nitrofen's effect on NIH 3T3 cell proliferation and apoptosis

In the second part of this study we utilized an *in vitro* cell culture model to probe nitrofen's effects in this system and to guide our *in vivo* experiments. Growth curve analysis demonstrated that nitrofen has a dose-dependent effect on NIH 3T3 cells growing in culture. The reduction in cell number associated with nitrofen exposure indicated that this compound could be having two possible effects: first it

could be inhibiting cell proliferation, or second it could be inducing cell death. We first tested the hypothesis that nitrofen could inhibit NIH 3T3 cell proliferation. By using BrdU incorporation as a metric for cell proliferation we found no effect of nitrofen on cell proliferation after a period of 24 hours. However, after an exposure period of 48 hours we found that nitrofen-exposed cells incorporated significantly less BrdU than control cells (in a dose-dependent manner), therefore we conclude that nitrofen has an inhibitory effect on NIH 3T3 cell proliferation.

With regard to cell death, we saw no evidence of increased apoptosis following nitrofen exposure after 4 and 24 hours. However, after an exposure period of 48 hours we saw increased levels of apoptosis with 50 μM and 100 μM nitrofen, but not 10 and 25 μM concentrations. Thus the effects of nitrofen *in vitro* appear to be dose dependent and the duration of exposure also seems to be an important factor, as we saw no effect after 24 hours exposure, but did see one after 48 hours. This suggests that the cytotoxic effects of nitrofen may be indirect and that detectable changes only accumulate over time.

This is the first report to describe the effect of nitrofen exposure on NIH 3T3 cells grown in culture. Our results show that nitrofen can inhibit NIH 3T3 cell proliferation and, at high doses, induce apoptosis. These data are consistent with previous reports studying the effect of nitrofen on different cell lines; for example the proliferation of pneumocytes and cardiac fibroblasts has been previously shown to be inhibited by nitrofen exposure (Borck et al., 2004; Gonzalez-Reyes et al., 2006). With regard to apoptosis, no cell death was observed in these cell types when they were exposed to nitrofen however the maximum dose tested in these

experiments was only 20 μM nitrofen (Borck et al., 2004; Gonzalez-Reyes et al., 2006). One study using P19 teratcarcinoma cells did observe increased numbers of apoptotic cells following exposure to a high dose of nitrofen (~120 μM; Kling et al., 2005). Again, these results are consistent with our findings in NIH 3T3 cells; we observed no apoptosis at low doses of nitrofen but saw a significant increase in cell death with high levels of this compound. This is significant because the level of nitrofen estimated to reach the embryo and cause CDH is only ~10-20 μM (Manson, 1986; Chen et al., 2003). Based upon our *in vitro* data, this would be too low to induce apoptosis. Conversely, we observed decreased cell proliferation in cells exposed to 10 μM nitrofen, the lowest concentration we tested. Further, decreased cell proliferation has been observed in other cell lines at doses as low as ~200 nM (Borck et al., 2004). Similar to these cell culture experiments, decreased cell proliferation, but no abnormal apoptosis, was observed in rat lung explants grown in culture and exposed to nitrofen (Keijzer et al., 2000). Thus, collective evidence from these *in vitro* studies suggests that teratogenic doses of nitrofen, sufficient to induce CDH *in vivo*, inhibit cell proliferation but do not reach high enough levels to induce apoptosis.

5.4.3 Nitrofen inhibits cell proliferation in vivo, but does not induce apoptosis

The effect of nitrofen exposure on cell proliferation and apoptosis within the cells of the PPF was also examined *in vivo*. Examination of BrdU incorporation following nitrofen exposure revealed a significant decrease in cell proliferation in the PPF, as well as in developing lung tissue. The diaphragm and lungs are well recognized targets of nitrofen teratogenesis (Costlow and Manson, 1981; Keijzer et

al., 2000), however in order to establish that nitrofen's effect on cell proliferation was specific to these structures and not a general effect, we also counted BrdU-positive cells within the limb bud. In this structure we found no inhibitory effect of nitrofen on cell proliferation. From this experiment we conclude that nitrofen's ability to inhibit cell proliferation in the PPF and developing lung directly contributes to the teratogenic effect of this compound, which ultimately manifests in CDH.

In addition to examining cell proliferation we also tested the hypothesis that nitrofen could induce increased apoptosis in the PPF and contribute to CDH. Despite extensive searching we could find no evidence of increased apoptosis in the PPF following nitrofen exposure, thus we are lead to conclude that nitrofen does not cause CDH by inducing a wave of apoptosis in the PPF at the time-point studied. One limitation of our approach to detect apoptotic cells in the developing diaphragm is that we may have missed an early period of cell death immediately following nitrofen exposure. Increased cell death was observed *in vitro* 4 hours after exposure to a high dose of nitrofen (~120 μM) and dead cells were observed in rat embryos 24 hours after nitrofen exposure, supporting this possibility (Alles et al., 1995; Kling et al., 2005). In order to overcome this limitation, we used a pharmacologic approach to inhibit apoptosis immediately following nitrofen exposure. The incidence of diaphragmatic hernia was unchanged in the off-spring of rats exposed to nitrofen and the apoptosis inhibitor pifithrin-α, compared to rats only exposed to nitrofen. This data suggests that nitrofen does not induce apoptosis in the developing diaphragm within the 24 hour period following maternal administration, and supports the

overall conclusion that nitrofen does not primarily cause CDH by inducing apoptosis.

With regard to other studies examining the cellular mechanism of nitrofen pathogenesis *in vivo*, we could only find one published study in the literature. Alles and colleagues used Nile blue sulphate staining to visualize cell death patterns following nitrofen exposure. Nitrofen treatment on embryonic day 9.5 and day 10.5 was followed by observations of excessive cell death in the cervical somites, leading the authors to conclude that a reduction in an unspecified somitic contribution to the primordial diaphragm manifested in CDH (Alles et al., 1995). With regard to the identity of this 'somitic contribution', it is known that MPCs which populate the diaphragm migrate from the dermomyotome, a derivative of the somite (Bladt et al., 1995; Birchmeier and Brohman, 2000). However, the pattern of Nile Blue sulphate staining observed is inconsistent with cell death within this population of cells and previous work has shown that CDH develops independently of myogenesis (Babiuk et al., 2002). We feel that to conclude that the cell death observed by these authors is associated with abnormal diaphragm development is unjustified given the data presented. It is equally plausible that the cell death patterns observed may be associated with the myriad of other defects associated with nitrofen exposure.

As mentioned in the introduction, it is known that the PPF is abnormally formed in nitrofen-exposed rat embryos and it is this defect that underlies diaphragmatic hernia in this model (Allan and Greer 1997). Until now, the mechanism through which the PPF defect arises has not been directly studied however, as discussed above, our results clearly indicate that nitrofen acts, at least in

part, by inhibiting cell proliferation. While its occurrence cannot be excluded, we found no evidence in this study to support the hypothesis that PPF defects arise from excessive apoptosis. This conclusion is not only supported by our *in vitro* data and study of the PPF in the nitrofen model *in vivo* but also by review of the extant data, albeit the majority of which comes from *in vitro* models, which indicates that the primary result of nitrofen exposure is a decrease in cell proliferation. Although we focused on apoptosis and cell proliferation in this study, an alternative mechanism may also contribute to abnormal PPF formation, for example migration/adhesion of cells within this region may be affected.

Our observation of reduced cell proliferation in the developing lung confirms the observation by Keijzer and colleagues, that nitrofen has a direct effect on the lung, which precedes invasion of the abdominal organs into the thoracic cavity (Keijzer et al., 2000). It is important here to emphasise the significance of the so-called dual-hit hypothesis with regard to the nitrofen model of CDH and studies of pulmonary hypoplasia using this model. This hypothesis posits that hypoplasia of the lung arises from a direct *hit* from the teratogenic effects of nitrofen and a second, later *hit,* from compression of the lungs by abdominal organs herniating through a malformed diaphragm (Keijzer et al., 2000). The implications of this hypothesis are that studies into pulmonary hypoplasia using the nitrofen model cannot easily separate the direct effects of nitrofen upon lung development (which is an artefact of the model) and lung compression (which is a cardinal feature of CDH), thus limiting the interpretation of results regarding the cause and effects of lung hypoplasia in this particular model. A tissue specific gene knock-out model of CDH, such as the

Coup-tfII:Nkx3-2^{cre} strain may be a preferable model to study lung hypoplasia in rodents, as Coup-tfII is only knocked out in the diaphragm of these mice and the lungs are only affected by compression (You et al., 2005).

5.4.4 Summary

In summary, in this study we have characterized the formation of the PPF, it being a rapidly growing and transient structure which is a key component of the developing diaphragm in rodents and humans. In the nitrofen model of CDH, we conclude that decreased cell proliferation is the primary mechanism of nitrofen's action *in vitro*. Abnormal apoptosis was observed *in vitro*, but at doses higher than developing rats are exposed to following maternal nitrofen administration. Our *in vivo* data obtained from nitrofen-exposed rat embryos leads us to conclude that the primary mechanism of nitrofen teratogenesis in the PPF is a decrease in the rate of cell proliferation. Based upon our results we propose a model of nitrofen teratogenesis in which exposure to this compound inhibits cell proliferation within the developing PPF, leading to the formation of an abnormal PPF, which translates to a hole in the fully formed diaphragm later in gestation. These experiments represent a significant advance in our understanding of normal PPF development and nitrofen's pathogenic mechanism within the cells of the developing diaphragm, providing insight into abnormal PPF development within this model.

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Chapter 6: Retinoid Signalling in the Developing Diaphragm: Significance for Congenital Diaphragmatic Hernia

Contributions: The majority of the experimental work described in this paper was performed by RDC, with technical assistance from Wei Zhang. The manuscript was written by RDC and edited by JJG.

6.1 Introduction

Congenital diaphragmatic hernia (CDH) is a frequently occurring birth defect which can cause severe neonatal respiratory distress and is associated with high mortality and morbidity. The etiology of CDH is poorly understood; although 10 to 20% of cases have an identifiable genetic cause the remaining cases are idiopathic (Torfs et al., 1992; Yang et al., 2006). One hypothesis forwarded to explain the cause of idiopathic CDH is the "retinoid hypothesis". This hypothesis posits that abnormalities in retinoid signalling, or related pathways, contribute to abnormal development of the diaphragm in CDH (Greer et al., 2003).

The retinoid hypothesis was founded on convincing data from studies using animal models and some clinical observations. In brief, these studies found that i) the off-spring of vitamin A deficient (VAD) rats have diaphragmatic hernia, ii) retinoid receptor knock-out mice have diaphragmatic hernia, iii) the CDH-inducing teratogen, nitrofen, suppresses retinoic acid response element (RARE) activation, iv) co-administration of nitrofen and exogenous retinoids lowers the expected incidence of CDH in rats, v) CDH-inducing teratogens can inhibit retinoic acid (RA) synthesis *in vitro*, and vi) infants with CDH have lower levels of circulating retinoids compared to age-matched controls (see review by Greer et al., 2003 and references therein). Recent studies continue to provide supporting evidence for the retinoid hypothesis. For example, the CDH-inducing teratogen nitrofen has been shown to inhibit the RA synthesizing enzymes retinal dehydrogenase-1, -2, and -3, but has no effect on retinoic acid receptor (RAR) activity *in vitro*. This suggests that inhibition of RA synthesis is the primary mechanism of nitrofen teratogenesis. This is

supported by *in vivo* data showing a persistent decrease in RA levels following nitrofen exposure (Noble et al., 2007). With regard to CDH in humans, mutations in the recently identified retinol bind protein (RBP) receptor, *STRA6*, have been linked with multiple congenital anomalies, including diaphragmatic hernia (Kawaguchi et al., 2007; Pasutto et al., 2007). In addition to links with diaphragm abnormalities, altered retinoid signalling may also contribute to abnormal lung, heart and kidney development in the nitrofen model of CDH (Gonzalez-Reyes et al., 2006; Nakazawa et al., 2007; Montedonico et al., 2007).

The general purpose of this series of experiments was to further examine the importance of retinoid signalling in the etiology of CDH. In the first part of the study we systematically examined the effect of CDH-inducing teratogens on retinoid signalling *in vivo*. To achieve this goal we utilized a strain of transgenic mice which constitutively express a construct with a RARE driving the expression of a β-galactosidase reporter gene (RARE-lacZ). Specific spatial and temporal activation of the transgene in these mice has been shown to reflect transcriptional activity by RARs, and is considered a proxy for retinoid signalling (Rossant et al., 1991). The same strain of mice has previously been used to show that nitrofen causes suppression of transgene activity 24 hours after exposure on embryonic day (E) 8.5 of gestation (Chen et al., 2003). It is unknown whether decreased retinoid signalling is a unique result of nitrofen exposure or if it is caused by other teratogens. Therefore the first question we asked was, do the compounds Bisdiamine [N,N'-octamethylenebis (dichloroacetamide)], SB-210661, and BPCA (4-

Biphenylcarboxylic acid), all of which are known to induce CDH in rats (Mey et al., 2003), suppress retinoid signalling *in vivo*?

As previously mentioned, 24 hours after nitrofen exposure on E8.5 retinoid signalling is decreased in RARE-lacZ mice (Chen et al., 2003). Studies in the rat show that following nitrofen exposure on E8.5, abnormalities in a part of the primordial diaphragm called the pleuro-peritoneal fold (PPF) can be observed at E13.5 (Allan and Greer, 1997). Based on the retinoid hypothesis it is assumed that nitrofen suppresses retinoid signalling within this time-frame. Therefore, in our second series of experiments we tested the hypothesis that nitrofen has a sustained effect on RARE-lacZ activation.

In addition to examining the effect of CDH-inducing teratogens on retinoid signalling we wanted to establish the identity of the members of the retinoid signalling pathway expressed in the PPF; indeed as Beurskens and colleagues (2007) recently pointed out, the contribution of retinoid signalling to the developing diaphragm has not been extensively studied. It is implied from the retinoid hypothesis that the PPF is a centre for retinoid signalling, however little data is available regarding the expression of proteins related to RA synthesis and signalling in this specific structure. As mentioned above, the PPF is an important structure in early diaphragm development; not only is it the target for the complete neuromuscular component of the diaphragm but it is also malformed in the nitrofen, vitamin A deficient, and *Wt1* null-mutant models of CDH (Allan and Greer 1997, Babiuk et al., 2003; Clugston et al., 2006). With regard to retinoid signalling, the pathway for the synthesis of retinoic acid and its signalling through the RARs is

generally well understood (see the relevant section in chapter 1 and reviews by Ruberte, 1994; Napoli, 1996). In order to provide support for the retinoid hypothesis of CDH and better understand the role of retinoid signalling in diaphragm development, in this study we wanted to confirm that the PPF in the developing rat expressed the proteins necessary for successful retinoic acid signalling, with specific regard to cellular retinoic acid binding proteins (Crabps), retinal dehydrogenase (Raldh) enzymes and the retinoid receptors.

Finally, the last part of this study was aimed at testing the importance of signalling through RARs for diaphragm development. A low incidence of diaphragmatic hernia has previously been reported in double knock-out mice for RARα and RARβ, suggesting that signalling through these receptors is required for normal diaphragm development (Mendelsohn et al., 1994; Ghyselinck et al., 1997). In this study we used the high-affinity pan-RAR antagonist BMS493 to block signalling through the RARs during the critical period of diaphragm development in rats to test the hypothesis that this compound can induce CDH (Johnson et al., 1995; Agarwal et al., 1996).

The results reported in this paper are consistent with the retinoid hypothesis of CDH. We have clearly shown that nitrofen has a sustained inhibitory effect on retinoid signalling and that the ability to suppress RARE-lacZ transgene activation is common to several CDH-inducing teratogens. Further, our analysis of protein expression within the rodent PPF has shown this structure to be a centre for retinoid signalling and we have identified the expression of specific retinoid receptors within this structure, signalling through which is likely essential for normal diaphragm

development. This latter conclusion is highlighted by the effectiveness of maternal administration of BMS493 in causing CDH.

6.2 Methods

A description of the basic methodologies concerning rat breeding, tissue collection, basic histology, immunohistochemistry, and microscopy can be found in the relevant section of chapter 2. The following sections concern methods only employed in this study and are not described elsewhere in this thesis.

6.2.1 Teratogen administration

On E8.5 pregnant mice were lightly anesthetized with isoflurane and teratogens were administered by oral gavage. Each compound was dissolved in 0.5 ml of olive oil by sonication at the doses specified below. The following teratogens were administered; Nitrofen (25 mg; China national chemical construction company, Nanjing, China), SB-210661 (25 mg, generously provided by Dr HM Solomon, GlaxoSmithKline Pharmaceuticals, King of Prussia, PA), Bisdimaine (25 mg, [N,N_-octamethylenebis (dichloroacetamide)], ARCOS Organics, Geel, Belgium), and BPCA (25 mg, 4-Biphenylcarboxylic acid, Sigma, St Louis, MO).

BMS493, a gift from Dr A.R. de Lera (University of Vigo, Spain), is a potent antagonist of RARs (Johnson et al., 1995; Agarwal et al., 1996). A 0.01 M solution of BMS493 was made up in ethanol and diluted 1:2.5 in olive oil for administration by oral gavage at a final concentration of 5 mg/kg. Several different administration regimes were used and are described in the results section.

6.2.2 Detection of β-galactosidase expression

On the required day of gestation embryos were collected by caesarean section. Embryos were immediately isolated from their extra-embryonic membranes and rinsed in ice cold X-rinse (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02%

Nonidet P-40 in 0.1 M phosphate buffered saline [pH 7.3]) for 5-10 minutes. Rinsed embryos were then incubated for 4-6 hours (dependent on gestational age) in X-gal staining solution (1.0 mg/ml X-gal [5-bromo-4-chloro-3-indyl-β-*D*-galactopyranoside], 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20mM Tris, in 0.1 M phosphate buffered saline [pH 7.3]); following staining each embryo was then fixed overnight in 4% paraformaldehyde. The following morning embryos were photographed with a digital camera (Nikon Coolpix 990, Japan) mounted on a dissecting microscope (Leica Wild M3C, Wetzlar, Germany). Each image was individually scaled with a 1 mm grid. Staining intensity and surface area was quantified by digital image analysis using ImagePro software (Media Cybernetics, Bethesda, MD). Staining conditions were kept uniform throughout the experimental series and where possible multiple litters were processed simultaneously.

6.2.3 Immunohistochemistry

A list of antibodies used in this study can be found in table 6.1, a description of the basic immunohistochemistry protocol used can found in section 2.4.

6.2.4 Wnt1-cre/R26R-lacZ mice

Wnt1-cre/R26R embryos were generously provided by Dr H.M. Sucov (University of Southern California). Immunohistochemical localisation of β-galactosidase (β-gal) in these mice was used to determine the contribution of neural crest cells to the PPF. Neural crest cells formed within embryos of this strain of mice are efficiently labelled with β-gal, allowing the entire mouse neural crest cell population to be tracked throughout gestation (Jiang X et al., 2000).

Table 6.1 Details of primary antibodies used

Antibody	Dilution	Source (Catalogue number)
Mouse monoclonal antibodies:		
CrabpI (C-1)	1:250	Sigma (C 1608)
CrabpII	1:100	C. Bavik (gift)
Rarα	1:600	PPMX (H1920)
Rarβ	1:150	PPMX (H4338)
Rarγ (G-1)	1:10	SCBT (sc-7387)
Rxrα	1:400	PPMX (K8508)
Wt1 (6F-H2)	1:50	Dako (M3561)
Neurofilament	1:200	DSHB (2H3)
Goat polyclonal antibodies:		
Raldh3 (aldh1a3: C-13)	1:50	SCBT (sc-26713)
Pax3/7 (C-20)	1:100	SCBT (sc-7748)
Rabbit polyclonal antibodies:		
Raldh1 (aldh1a1)	1:2000	Abcam (ab24343)
Raldh2	1:1500	P. McCaffery (gift)
Rarα (C-20)	1:75	SCBT (sc-551)
Rxrβ (C-20)	1:300	SCBT (sc-831)
Rxrγ (Y-20)	1:1200	SCBT (sc-555)

PPMX: Perseus Proteomics, Tokyo, Japan; SCBT: Santa Cruz Biotechnology, Santa Cruz, CA; Dako Canada, Mississauga, ON; Abcam: Abcam plc. Cambridge, UK; DSHB: Developmental studies hybridoma bank, University of Iowa.

6.3 Results

6.3.1 Decreased RARE-lacZ activation following teratogen exposure

The effect of nitrofen, SB-210661, Bisdiamine, and BPCA on retinoid signalling *in vivo* was determined using RARE-lacZ mice. Following maternal administration on E8.5 of gestation, exposed embryos were collected 24 hours later (at E9.5) and stained with X-gal. As can be seen in figure 6.1, relative to age-matched untreated control embryos, all four compounds tested produced a visible decrease in β-gal staining. Quantitative image analysis supported this finding; both the area of β-gal staining (Figure 6.2A) and the intensity of staining (Figure 6.2B) were significantly reduced relative to control for all three compounds studied (one-way ANOVA; p-value < 0.05).

6.3.2 Nitrofen has a sustained effect on RARE-lacZ activation

In order to determine the duration of nitrofen's effect on retinoid signalling, litters of embryos that were exposed to nitrofen on E8.5 were collected at E9.5, E10.5, E11.5, and E12.5. Visual inspection of embryos revealed an apparent decrease in β-gal staining (data not shown). Quantitative image analysis of photographed embryos revealed that at all ages studied the area of β-gal staining was significantly reduced relative to age-matched control tissue (figure 6.3A). The greatest reduction was observed at E10.5, and by E11.5 and E12.5 the degree of reduced staining was diminished, but remained significantly lower than control levels. With regard to staining intensity (figure 6.3B), a significant decrease was seen at ages E9.5 and E10.5, but not at E11.5 and E12.5. As was seen with measurements of staining area, the greatest reduction in staining intensity was

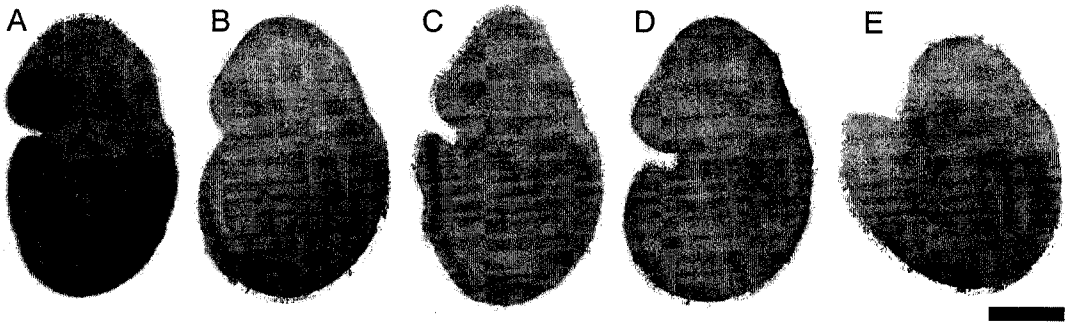

Figure 6.1 Teratogen-exposed RARE-lacZ embryos.

Images of whole RARE-lacZ embryos at E9.5 and stained with X-gal are shown. A) Control embryo showing distinct staining in the forebrain region and throughout the trunk of the embryo. Representative examples of teratogen exposed embryos are shown, in each case there is a marked reduction in the amount of staining observed. B) Nitrofen exposed embryo. C) Bisdiamine exposed embryo. D) SB-210661 exposed embryo. E) BPCA exposed embryo. Scale bar = 1 mm.

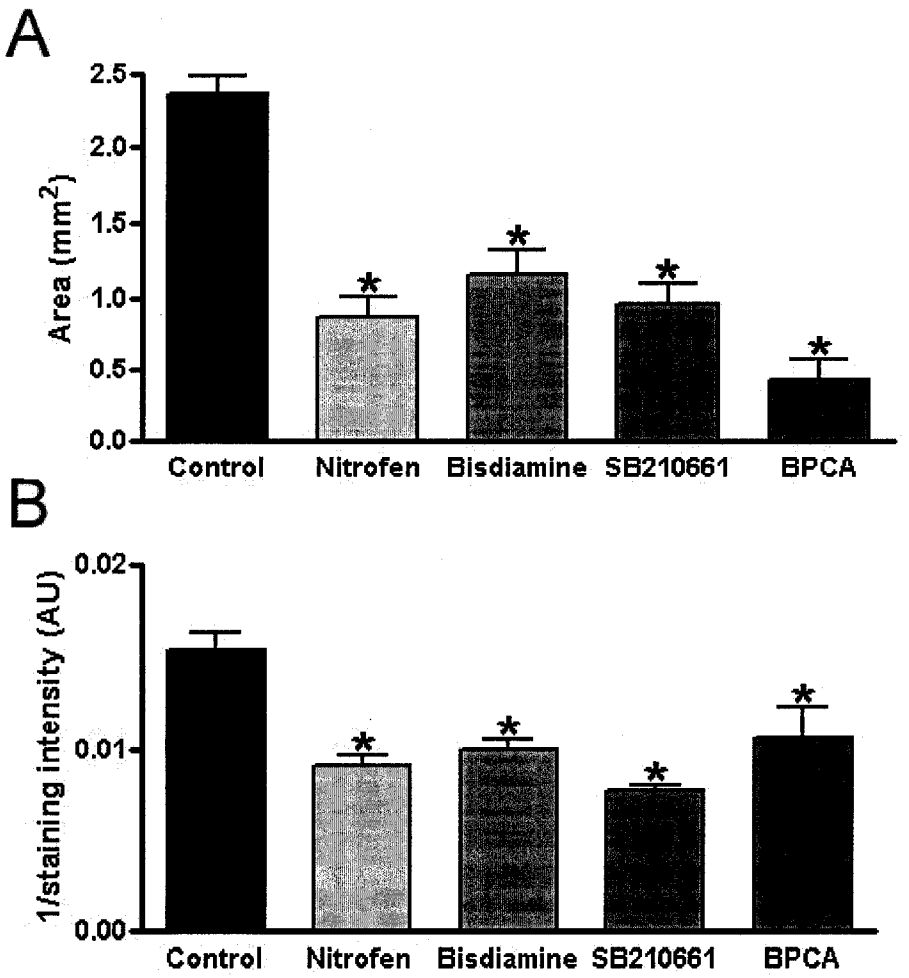

Figure 6.2 Reduced RARE-lacZ expression following teratogen exposure.

Bar graphs showing quantitative data gathered by image analysis from teratogen-exposed RARE-lacZ embryos are presented. A) The area of staining in embryos exposed to nitrofen, bisdiamine, SB-210661, and BPCA is significantly decreased relative to control embryos. B) The intensity of staining in embryos exposed to CDH-inducing teratogens is also significantly decreased compared to control embryos.

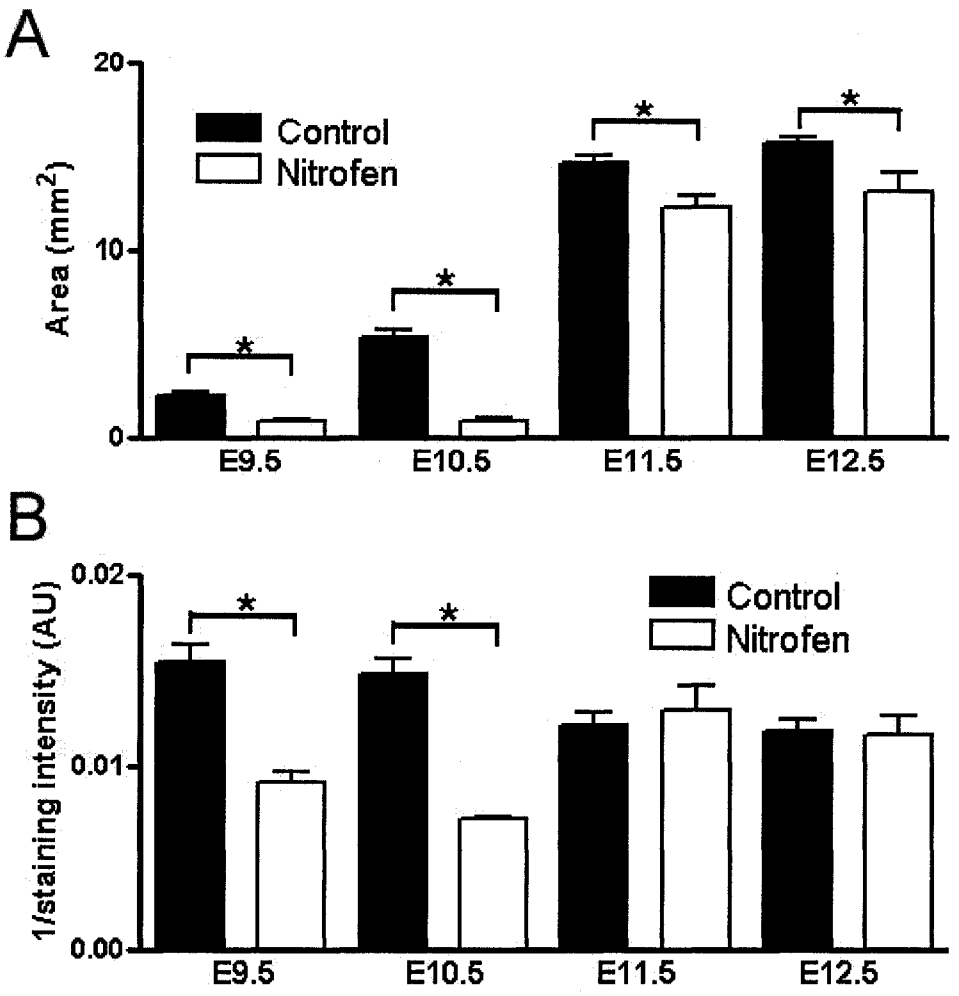

Figure 6.3 Timeline of RARE-lacZ expression.

Bar graphs showing quantitative data gathered from control and nitrofen-exposed RARE-lacZ embryos over a 4-day time period are presented. A) At all of the ages studied, the area of staining in nitrofen-exposed embryos was significantly lower compared to age-matched controls. B) The intensity of staining in nitrofen-exposed embryos was significantly lower at E9.5 and E10.5, but not E11.5 and E12.5.

observed at E10.5. Please note, the data plotted in figure 6.2 and 6.3 is presented together in table 6.2.

6.3.3 Crabp expression in the PPF

Immunostaining for CrabpI and CrabpII was carried out in the PPF at E13.5 (Figure 6.4). The expression pattern of CrabpI within the PPF was very restricted and was largely limited to the cytoplasm of cells in the region of the phrenic nerve (Figure 6.4A). However, double-labelling for CrabpI and neurofilament (figure 6.4B) did not reveal any direct co-localisation between these two proteins, suggesting they are expressed in intimately associated but distinct cells. Interestingly, the staining pattern of Crabp1 closely mirrored that of neural crest cells within the PPF (figure 6.4C) raising the possibility that these cells are primordial Schwann cells (see discussion).

In contrast to the limited expression of CrabpI, CrabpII is widely expressed in the cytoplasm of cells throughout the entire PPF (figure 6.4D). Double-labelling for CrabpII and Pax3 shows that this protein is expressed in the muscular and non-muscular component of the PPF (figure 6.4E). Double-labelling for CrabpI and CrabpII reveals closely associated staining in the region of the phrenic nerve, however there is no strong pattern of co-localisation (figure 6.4F).

6.3.4 Raldh expression in the PPF

Immunostaining for Raldh1, -2, and -3 was carried out in the PPF at E13.5 (figure 6.5). Consistent with previously published data, Raldh1 was observed in the developing lung (Chazaud et al., 2003), however in our examination of expression in the PPF, Raldh1 staining was absent (figure 6.5A). In contrast to this, Raldh2 was

Table 6.2 Quantitative data from RARE-lacZ embryo image analysis

		N	Area (mm^2)	1/Intensity (AU)
RARE-LacZ teratogen exposure data				
Control		32	2.359 ± 0.13	0.016 ± 0.001
Nitrofen		19	0.876 ± 0.14	0.009 ± 0.001
Bisdiamine		24	1.167 ± 0.17	0.01 ± 0.001
SB-210661		13	0.963 ± 0.14	0.008 ± 0.004
BPCA		6	0.432 ± 0.14	0.011 ± 0.002
RARE-LacZ time-line data				
E9.5	Control	32	2.36 ± 0.13	0.016 ± 0.001
	Nitrofen	19	0.876 ± 0.14	0.009 ± 0.001
E10.5	Control	21	5.55 ± 0.32	0.015 ± 0.001
	Nitrofen	9	0.89 ± 0.2	0.007 ± 0.0001
E11.5	Control	13	14.54 ± 0.32	0.012 ± 0.001
	Nitrofen	7	12.17 ± 0.64	0.013 ± 0.001
E12.5	Control	15	15.61 ± 0.36	0.012 ± 0.001
	Nitrofen	8	13.03 ± 0.98	0.012 ± 0.001

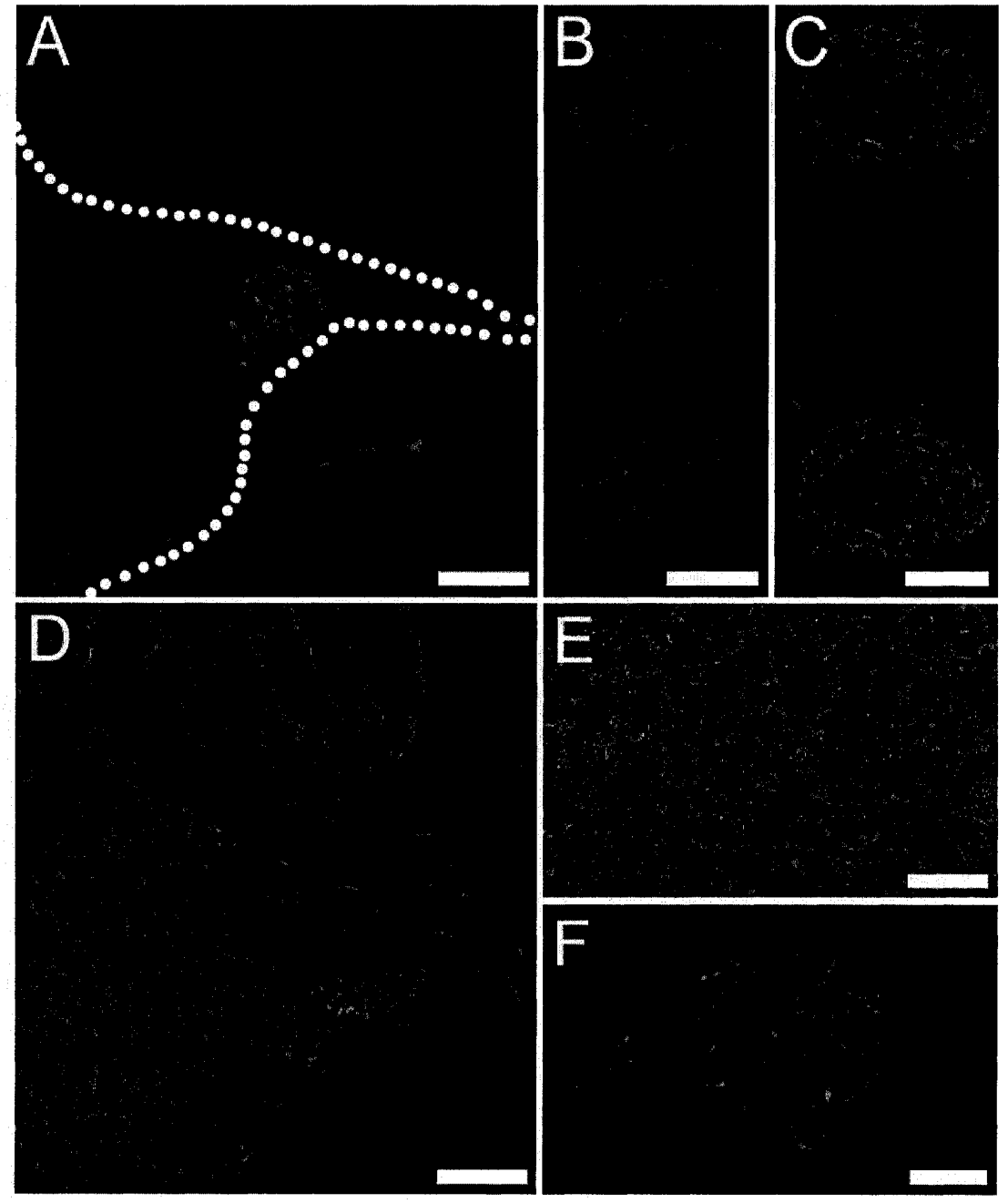

Figure 6.4 Crabp expression in the developing diaphragm at E13.5.

A) CrabpI expression is restricted in the PPF (dotted line represents the boundary of the PPF). B) Upper panel, single channel confocal microscope image of CrabpI-positive cells (green); middle panel, single channel image of the phrenic nerve (neurofilament-positive, red); lower panel, merged image showing close

association between CrabpI and neurofilament expression. C) Upper panel, neural crest cells within the PPF (Wnt1-cre/R26R-LacZ expression, green); middle panel, single channel image of the phrenic nerve (neurofilament, red); lower panel, merged image showing the close association between neural crest cells and the phrenic nerve within the PPF. D) CrabpII expression in found through-out the PPF and adjacent lung tissue. E) Merged image of CrabpII-positive cells (green) and Pax3-positive muscle precursor cells (red). F) Merged image of CrabpI (green) and CrabpII (red) expression in the PPF, in the region of the phrenic nerve. Scale bars: A, D = 100 μm, B = 50 μm, C = 40 μm, E = 25 μm, F = 30 μm.

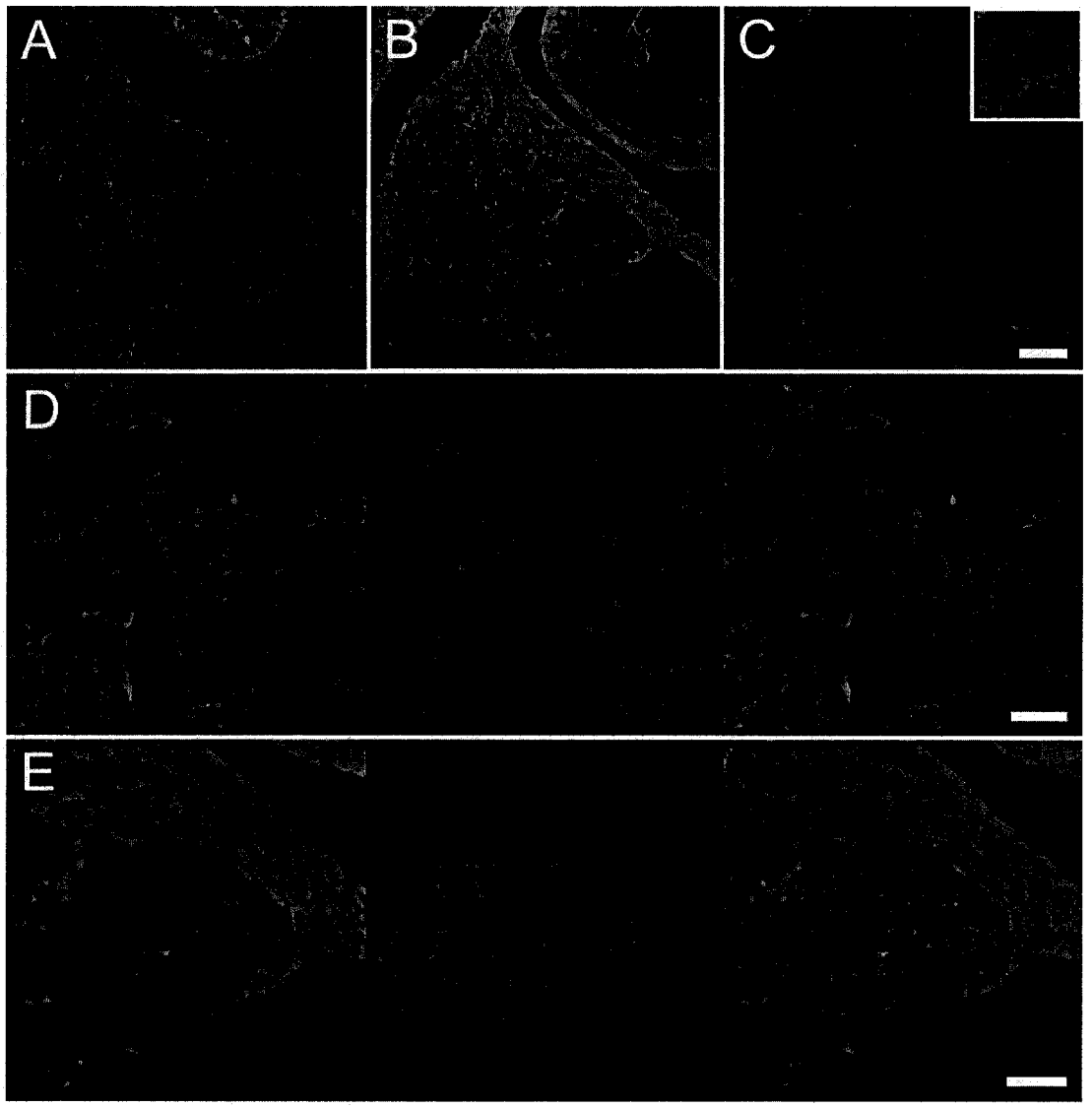

Figure 6.5 Raldh expression in the developing diaphragm at E13.5.

A) Raldh1 is not expressed in the PPF, though positive staining is visible in the adjacent lung tissue. B) Raldh2 is strongly expressed in the cytoplasm of cells through-out the PPF. C) Raldh3 is not expressed in the PPF; inset image shows Raldh3 expression in the developing eye. D) Left panel, high magnification image of Raldh2-positive cells in the PPF (green); middle panel, Pax3-positive muscle-

precursor cells (red); right panel, merged image of Raldh2 and Pax3 expression. E) Left panel, high magnification image of Raldh2 positive cells in the region of the phrenic nerve in the PPF (green); middle panel, neurofilament staining showing the phrenic nerve (red); right panel, merged image of Raldh2 and neurofilament expression. Scale bars: A, B, and C = 50 μm; D, and E = 25 μm; inset image not to scale.

strongly expressed in the cytoplasm of cells throughout the PPF (figure 6.5B). Similar to Raldh1, Raldh3 expression was absent in the PPF though positive staining was observed, as expected, in the developing eye (figure 6.5C; Li et al., 2000), therefore the only Raldh isoform expressed in the developing PPF is Raldh2.

Double-labelling for Raldh2 and Pax3 in the PPF shows that a small proportion of Raldh2-positive cells also have a Pax3-positive nuclei (figure 6.5D); this suggests that Raldh2 is expressed in the cytoplasm of MPCs and non-muscular, mesenchymal cells alike. Double-labelling for Raldh2 and neurofilament suggests that is enzyme is not strongly expressed along the tract of the phrenic nerve (figure 6.5E).

6.3.5 Retinoid receptor expression in the PPF

Immunohistochemistry for the α, β, and γ isoforms of RAR and RXR was carried out in the PPF at E13.5 (figure 6.6). Of the six receptors tested for, we could only find positive nuclear immuno-staining for RARα, RARγ, and RXRα. Despite the absence of RARβ staining in the PPF we did observe RARβ immuno-positive cells in the ventral neural tube suggesting that our immunohistochemical protocol was working (figure 6.6B; Dolle et al., 1994). Similarly, consistent with previous reports, RXRβ and RXRγ immuno-positive cells were observed in the developing lung and body-wall mesenchyme respectively (figure 6.6E and 6.6F; Dolle et al., 1994; Coste et al., 2007). Thus, in the presence of this positive staining we feel that the absence of immuno-staining in the cells of the PPF for RARβ, RXRβ, and RXRγ reflect a true lack of expression of these receptors in this tissue.

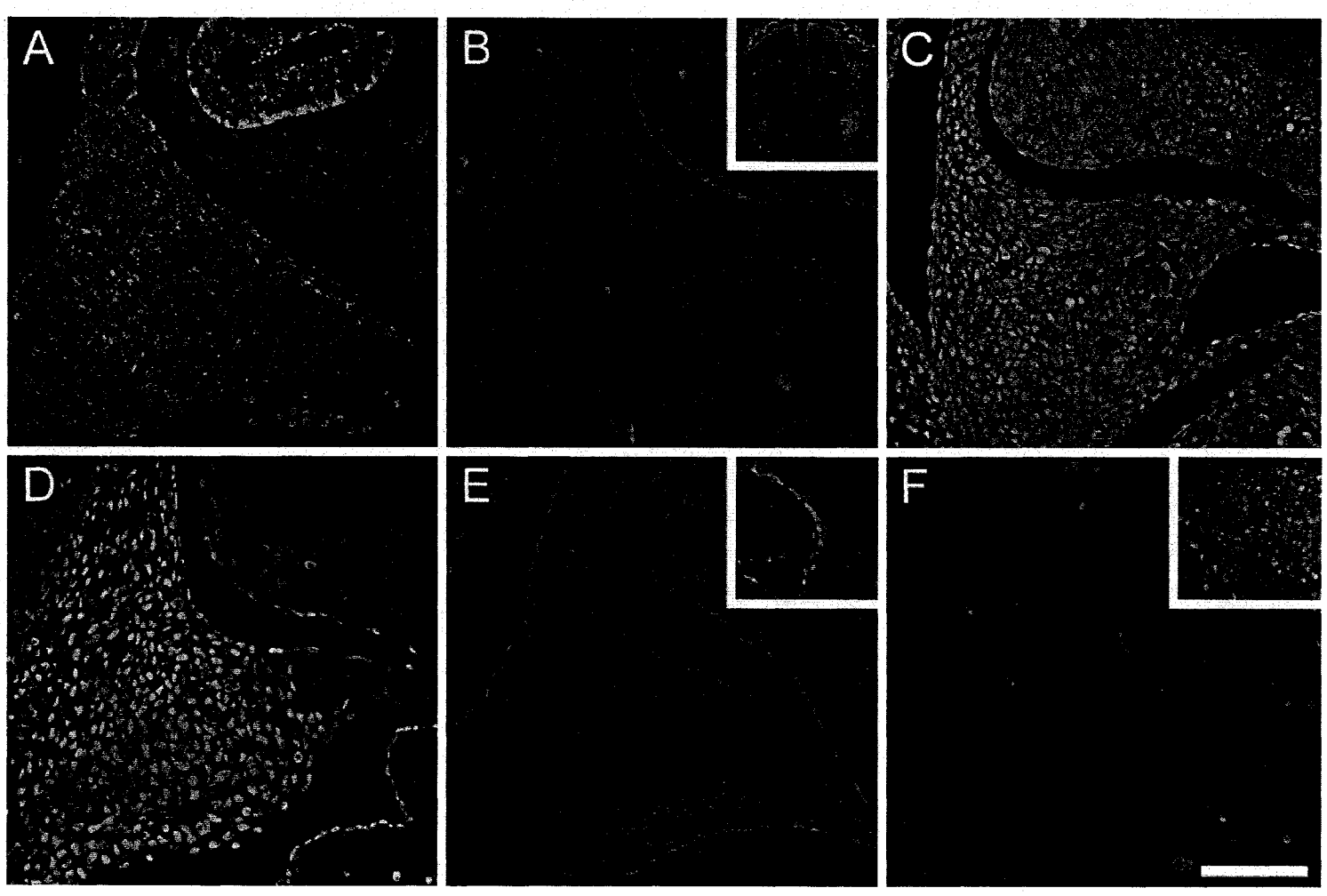

Figure 6.6 **Retinoid receptor expression in the developing diaphragm at E13.5 (figure legend overleaf).**

Figure 6.6 A) RARα is expressed in the PPF. B) RARβ is not expressed in the PPF; inset image shows RARβ-positive cells in the neural tube. C) RARγ is expressed through-out the PPF and the adjacent lung mesenchyme. D) RXRα is expressed through-out the PPF and can also be seen in the adjacent lung tissue. E) RXRβ is not expressed in the PPF, though positive staining was observed in the developing lung (inset image). F) RXRγ is not expressed in the PPF, though positive staining was observed in the body wall (inset image). Scale bar: 100 μm, inset images not to scale.

6.3.6 RARα expression is spatially-restricted in the PPF

Our examination of retinoid receptor expression in the PPF revealed an unexpected and unique pattern of RARα immuno-staining (figure 6.7A). The most rostral extent of the PPF appeared to be almost completely devoid of RARα-staining, sections through the middle of the PPF show a bias towards RARα expression in the dorsally-projecting angle of the PPF, and the most caudal extent of the PPF shows widespread expression of this receptor (note – RARγ and RXRα staining was uniform throughout the PPF). Interestingly, as illustrated in figure 6.7B, we feel that the pattern of RARα expression throughout the PPF closely mirrors the general region of the PPF that is missing following nitrofen exposure. This restricted pattern of RARα expression within the PPF is further evident when this structure is viewed in sagittal section, with a clear bias towards expression in the caudal region of the PPF (figure 6.8).

6.3.7 Co-expression of retinoid receptors with Pax3 and Wt1 in the PPF

We were interested in determining which cells of the PPF were positive for the retinoid receptors detected, therefore we carried out separate double-labelling experiments for RARα, RARγ, and RXRα receptors and two other proteins: 1) Pax3, which labels muscle precursor cells within the PPF (Babiuk et al., 2003), and 2) Wt1, which labels non-muscular mesenchymal cells within the PPF (figure 6.9; Clugston et al., 2006). Within the PPF, there is no strong pattern of co-localisation between RARα and Pax3 (figure 6.9A); RARα does not appear above background levels in Pax3-positive cells. In contrast to this, RARα and Wt1 are frequently co-

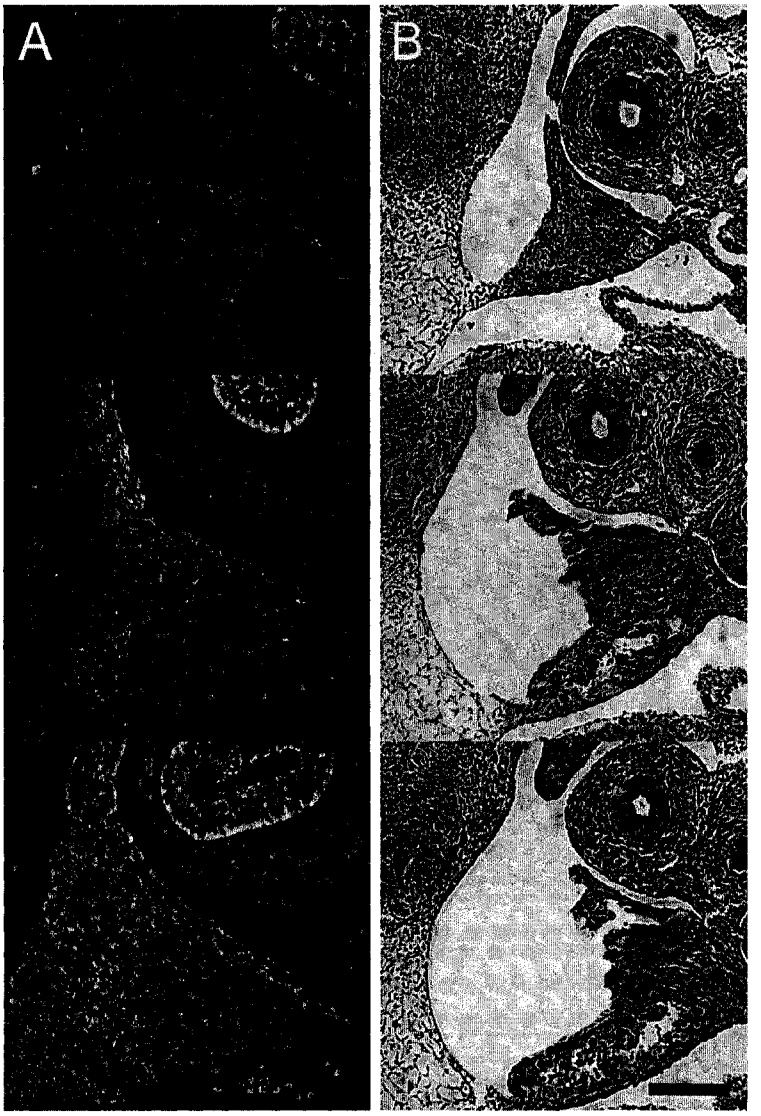

Figure 6.7 RARα expression is restricted in the developing diaphragm at E13.5.

A) Rostral to caudal series of sections through the PPF (upper to lower panel) illustrating the restricted pattern of RARα expression observed. B) Similar rostral to caudal series of sections through the PPF of a nitrofen-exposed embryo highlighting the missing region of tissue for comparison to the area of RARα expression shown opposite. Scale bar = 100 μm.

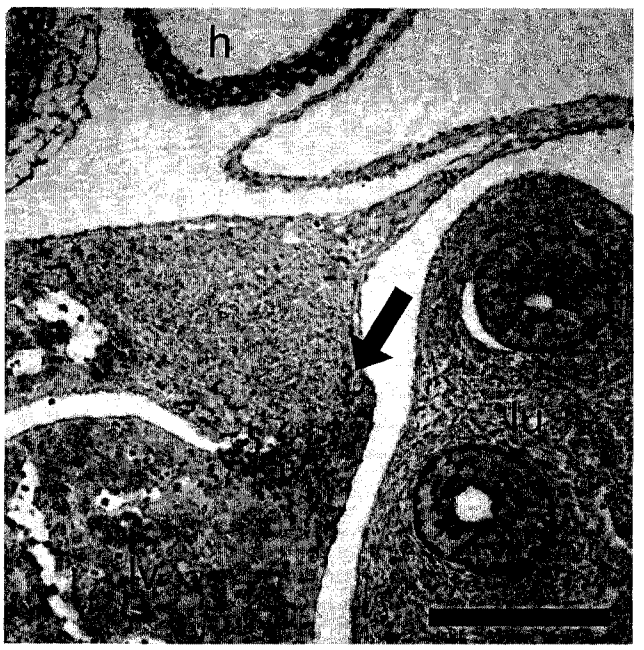

Figure 6.8 RARα expression is restricted in the developing diaphragm at E13.5 (sagittal view).

Sagittal section of the PPF highlighting the spatially-restricted pattern of RARα expression in this structure (arrow). Heart (h), liver (lv), lung (lu). Scale bar = 200 μm.

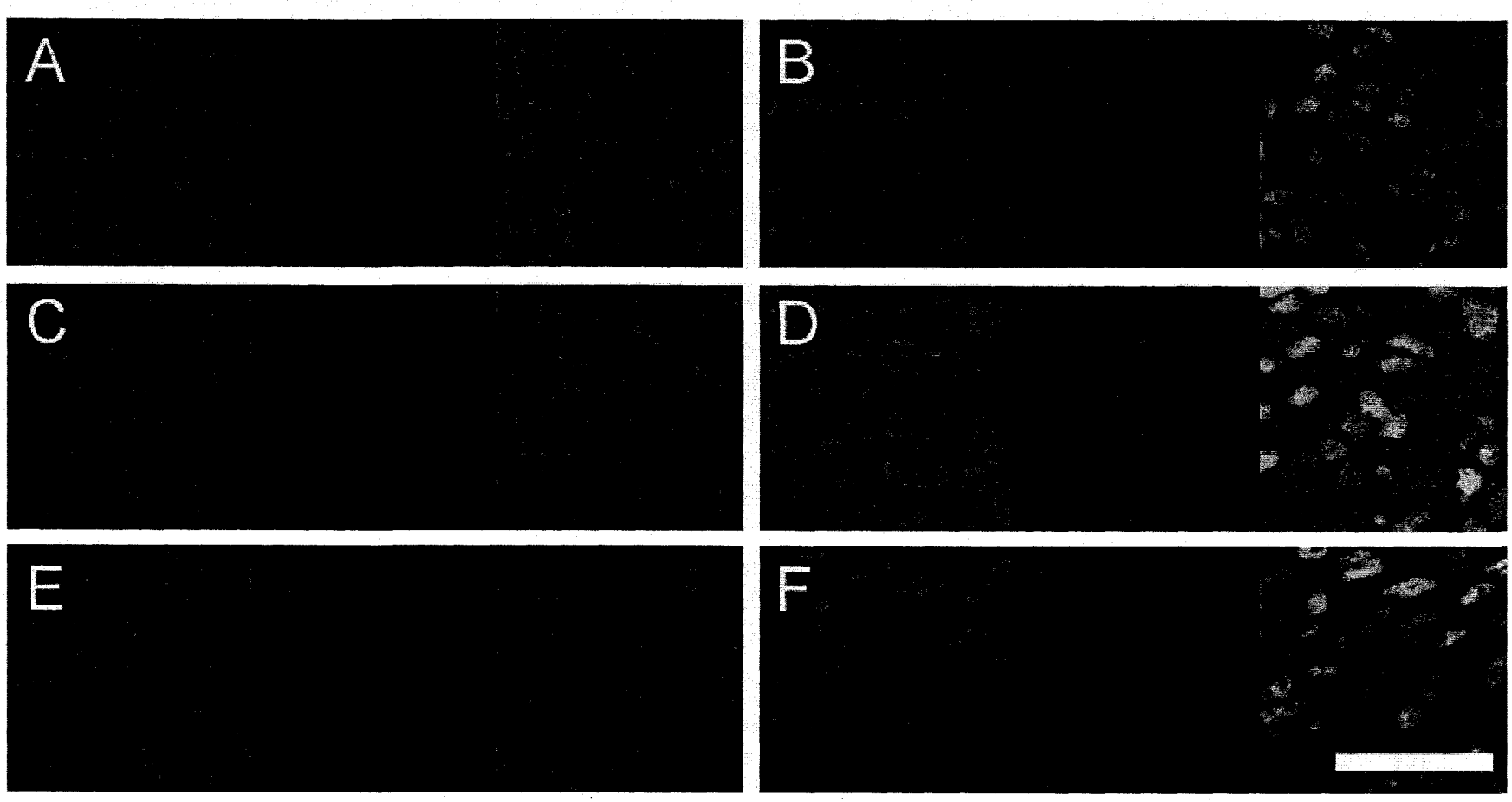

Figure 6.9 **Retinoid receptor double-labeling in the developing diaphragm at E13.5 (figure legend overleaf).**

Figure 6.9 In each section of the figure, the left panel shows cells expressing the specified retinoid receptor (green), the middle panel shows either Pax3-positive muscle precursor cells (A, C, E) or Wt1-positive mesenchymal cells (B, D, F; red), and the right panel shows a merged image. A) RARα does not co-localise with Pax3 in the PPF. B) RARα and Wt1 are co-expressed in the same cells of the PPF. C) RARγ and Pax3 are co-expressed within the PPF. D) RARγ and Wt1 are co-expressed within the PPF. E) RXRα and Pax3 are partially co-expressed in the PPF. F) RXRα and Wt1 are co-expressed in the PPF. Scale bar = 40 μm.

expressed within the same cells (figure 6.9B). With regard to RARγ, expression of this receptor in Pax3-positive cells appears to be more consistent relative to RARα (figure 6.9C), and RARγ also shows a strong pattern of co-localisation with Wt1 (figure 6.9D). RXRα appears to be weakly expressed in Pax3-positive cells (figure 6.9E) and is strongly expressed in Wt1-positive cells (figure 6.9F).

6.3.8 The pan-RAR antagonist BMS493 can induce CDH

The pan-RAR antagonist BMS493 was used to test the hypothesis that signalling through RARs is essential for normal diaphragm development. In the first trial, BMS493 was administered between E8 and E11 (5 mg/kg; every 12 hours). This treatment regime successfully produced diaphragm defects in 14/16 foetuses examined. The defects observed were typical of Bochdalek-type CDH, as well as the diaphragmatic hernia observed in the nitrofen model of CDH. In an attempt to refine the BMS493 treatment protocol, several other trials were carried out with drug administration occurring on different days of gestation within the critical period of diaphragm development (summarized in table 6.3). Treatment with BMS493 on E8 and E9 did not produce any diaphragm defects however treatment on E9 and E10, and E10 and E11, produced a high incidence of CDH (~100%). A wide spectrum of diaphragm defects was produced in the litters of BMS493 treated animals, with a range in size from small holes to almost complete agenesis of the hemi-diaphragm. The sidedness of the hernia produced was also varied, including left, right and bilateral defects (see figure 6.10). In all cases of diaphragmatic hernia observed, a large portion of liver tissue was present in the thoracic cavity. Further, although no quantitative analysis has been carried out yet, the lung ipsilateral to the side of the

diaphragm defect appeared noticeably smaller compared to the contralateral lung. In further addition to diaphragm defects, loose puffy skin and obvious facial dysmorphologies (blunt snout, facial clefting; not shown) were observed in BMS493 exposed foetuses. Some litters also had a high rate of resorption, suggesting some underlying pathology which currently remains unidentified. Experiments into the use of BMS493 as a new model of CDH are on-going; in addition to refining the treatment protocol, the pathogenesis of diaphragm defects in these animals is also being studied in detail.

Table 6.3 The pan-RAR antagonist BMS493 induces CDH

E8	E9	E10	E11	CDH
+	+	+	+	YES
+	+	-	-	NO
-	+	+	-	YES
-	-	+	+	YES

(+ indicates a day on which BMS493 was administered, - indicates a day when no drug was administered)

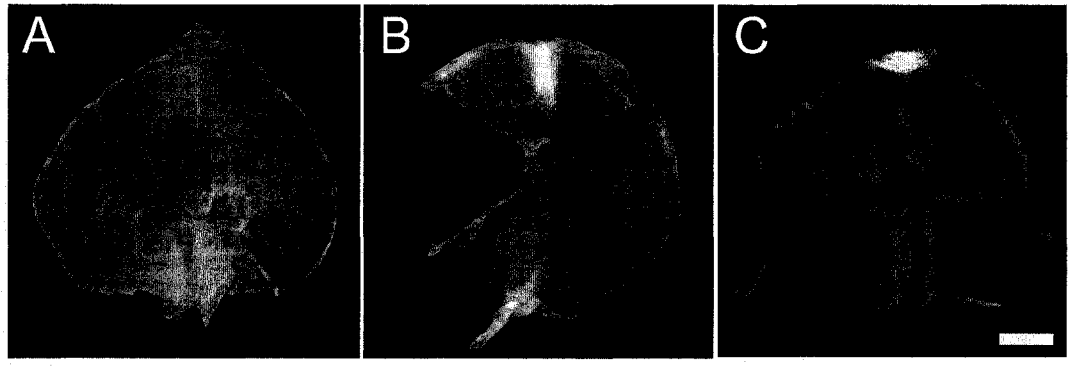

Figure 6.10 The pan-RAR antagonist BMS493 induces CDH.

A) E17 diaphragm from an untreated control animal (viewed from above). B) An example of a large left-sided diaphragm defect from a foetus exposed to BMS493. C) An example of bilateral diaphragmatic hernia from a BMS493 exposed foetus. Scale bar = 100 μm.

6.4 Discussion

6.4.1 Suppressed RARE-lacZ activation by CDH-inducing teratogens

It has previously been reported that RARE-lacZ embryos have decreased levels of β-gal 24 hours after nitrofen exposure (Chen et al., 2003). In this study we confirmed this observation and also found that three other CDH-inducing teratogens, SB-210661, Bisdiamine, and BPCA can also suppress RARE-lacZ activation 24 hours after exposure on E8.5. This result seems to suggest that all of these compounds induce diaphragmatic hernia by inhibiting retinoid signalling. Further, it has previously been shown that these compounds can inhibit Raldh2 *in vitro* (Mey et al., 2003; Noble et al., 2007); therefore we suggest that the mechanism for the reduced RARE-lacZ activation in teratogen exposed embryos is a decrease in retinoic acid production. This experiment serves to highlight the importance of retinoid signalling in diaphragm development, and demonstrates that a disruption in retinoid signalling as a mechanism which leads to diaphragmatic hernia is not unique to the nitrofen model of CDH.

6.4.2 Nitrofen has a sustained effect on RARE-lacZ activation

Nitrofen exposure between day 8 and 11 of rodent gestation can cause CDH; in our laboratory we prefer to administer nitrofen at E8.5 and have observed abnormalities in the PPF at E13.5 (Costlow and Manson 1981; Allan and Greer et al., 1997). It is unclear how nitrofen acts to produce PPF defects within this timeframe; in this study we show that nitrofen has a sustained inhibitory effect on RARE-lacZ activation. By administering nitrofen at E8.5 and collecting embryonic tissue in the subsequent days a clear picture emerged of nitrofen's effect on RARE-

lacZ activation. The intensity of RARE-lacZ activation was significantly reduced for at least 48 hours (up to E10.5) after nitrofen exposure and the area of staining was lower up to 96 hours later (up to E12.5). These results demonstrate that nitrofen has a sustained effect on retinoid signalling, this compliments other data from our laboratory which shows that RA levels are also significantly decreased several days after nitrofen treatment (Noble et al., 2007). Interestingly, when nitrofen is administered at E8.5, the period of decreased retinoid signalling produced corresponds to a time when the developing embryo utilizes increased amounts of retinol, such that between E9 and E14 retinol levels are at relatively low concentrations (Takahashi et al., 1977). Therefore, nitrofen exposure decreases RA production and signalling at a time when the developing embryo is most susceptible to disruptions in retinoid homeostasis, which likely enhances the teratogenic effects of nitrofen.

In this study, we assume that the decrease in RARE-lacZ expression observed is a result of Raldh inhibition. However there is an alternate hypothesis which could explain this decrease, such that nitrofen acts by inhibiting the cellular uptake of retinol (Nakazawa et al., 2007). With regard to this second hypothesis, studies by Dr H Sun (who recently identified the RBP-receptor, STRA6, which is responsible for cellular uptake of retinol; Kawaguchi et al., 2007) carried out on our behalf, found no evidence of decreased retinol uptake in the presence of nitrofen. Further, two distinct *in vitro* assays have demonstrated that this compound can inhibit Raldh function (Mey et al., 2003; Noble et al., 2007). Therefore we feel that

a decrease in RA production, via inhibition of Raldh, as the cause of decreased RARE-lacZ activation is a more plausible explanation.

One limitation with using the RARE-lacZ strain of mice is that transgene expression does not exactly reflect all sites of RAs action (Rossant et al., 1991). Thus, while we observed a global decrease in RARE-lacZ activation following nitrofen exposure we could not directly visualize retinoid signalling within the PPF, however as discussed below the expression of retinoid receptors within this structure indicate that it is indeed a centre for retinoid signalling.

6.4.3 Crabp expression in the PPF

During embryogenesis the expression of CrabpI and CrabpII is generally complementary and does not overlap; the PPF is no exception (Ruberte et al., 1992). CrabpI is only expressed in a very well defined population of cells in the PPF, surrounding the phrenic nerve. Further, this pattern of expression is mirrored by neural crest cells within this structure. Given that Schwann cells are of neural crest origin and are known to express CrabpI (Maden et al., 1992), we conclude that the population of CrabpI-positive cells within the PPF are Schwann cells ensheathing the phrenic nerve. Interestingly, it has been proposed that nitrofen has its teratogenic effect by specifically targeting neural crest cells (Yu et al., 2001). However, while this is a plausible explanation for nitrofen-induced heart defects we feel that the restricted pattern of neural crest cells within the PPF is inconsistent with a major role in diaphragm development and CDH. In contrast to CrabpI, CrabpII is expressed in cells throughout the PPF, given the recognized role of CrabpII in transporting RA to the nucleus and delivering it to RARs (Delva et al., 1999; Dong

et al., 1999), this pattern of expression is indicative of the importance of retinoid signalling within the PPF.

6.4.4 Raldh2 is the primary source of RA in the PPF

RA is synthesized from retinal by enzymes of the retinal dehydrogenase family. Of the three primary Raldh isoforms, only Raldh2 is expressed in the PPF. This finding indicates that Raldh2 is the sole source of RA within the developing diaphragm. Importantly, the CDH-inducing teratogens nitrofen, SB-210661, and Bisdiamine have all been shown to inhibit Raldh2 activity *in vitro* (Mey et al., 2003), supporting the concept that synthesis of RA by Raldh2 in the PPF is important for normal diaphragm development.

6.4.5 Retinoid receptor expression in the PPF

There are three basic isoforms of RARs and RXRs: α, β, and γ. Immuno-histochemistry for all of these receptor isoforms only found positive staining for RARα, RARγ, and RXRα in the cells of the PPF. Expression of the latter two receptors was uniform and widespread throughout this structure, whereas RARα expression was largely restricted to its caudal region. Interestingly, the population of RARα-positive cells in the PPF closely matches the population of cells that are absent in the PPF of nitrofen exposed embryos, this suggests that signalling through this specific receptor may be of particular importance in diaphragm development.

With regard to the basic type of cells that the retinoid receptors are expressed in within the PPF, a general theme emerges. All receptors are strongly expressed in the non-muscular mesenchymal cells of the PPF (Wt1-positive) and have weak expression in muscle precursor cells within this structure (Pax3-positive). While

RARγ and RXRα appear to be weakly expressed within muscle precursor cells, the evidence for RARα is less convincing with expression of this receptor within these cells never reaching above background levels. This separation between RARα and the other two retinoid receptors expressed within the PPF is of interest because it is thought that specific abnormalities in the non-muscular cells of the PPF contribute to the development of diaphragm defects in CDH, this further hints at the importance of RARα signalling in PPF development (Clugston et al., 2006; Clugston et al., 2008).

It is possible to interpret our retinoid receptor expression data with regard to the phenotype of retinoid receptor mutant mice. Indeed, diaphragmatic hernia has been recorded amongst the spectrum of abnormalities observed in several strains of retinoid receptor mutants. While RARα and RARβ single knock-out mice are all viable and relatively normal, RARα:RARβ compound mutants have a wide-array of defects consistent with vitamin A deficiency syndrome, including CDH (Li et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994; Ghyselinck et al., 1997). The absence of a phenotype in single mutants indicates that either of these receptors can compensate for the loss of the other. Though the phenotype of these mice indicates the importance of RARα in diaphragm development, it also implicates RARβ.

This latter comment is difficult to rationalize in the context of our results because we did not observe any RARβ-positive immunostaining in the PPF (please note that positive staining was observed elsewhere in our embryo sections suggesting that this result was not a false-negative). In fact, the absence of RARβ expression within the PPF is difficult to rationalize with regard to *two* previous

observations: not only do RARα:RARβ knock-out mice have CDH, but RARβ mRNA has been reported in the developing diaphragm (though no figure was presented; Mendelsohn et al., 1994). One explanation for these observations would be that as part of a compensatory mechanism, RARβ mRNA is only translated into protein in the absence of normal RARα expression. A similar phenomena has been reported in transgenic RARα knock-down mice (30 to 80% reduction in RARα expression), which show a compensatory increase in both RARβ and RARγ protein expression (Manshouri et al., 1997). A large degree of genetic redundancy has certainly evolved amongst the retinoid receptors (as demonstrated by the lack of phenotype in single knock-out mice), however further experiments are required to resolve the role of RARβ in relation to CDH (see below).

With regard to the other retinoid receptors whose expression was observed in the PPF, no diaphragm defects have been reported in RARγ knock-out mice, either singly or in combination with any of the other retinoid receptors, suggesting that signalling through this receptor is not essential for normal diaphragm development (Lohnes, et al., 1993). Further, although it is expressed through-out the PPF it is not sufficient to support normal diaphragm development, as evidenced by the occurrence of diaphragmatic hernia in RARα:RARβ knock-out mice (Mendelsohn et al.,1994; Ghyeslinck et al., 1997). Thus, signalling through RARγ is not essential or sufficient for normal diaphragm development, and its function in this developing tissue remains unclear.

Conversely, RXRα expression within the PPF may be of particular importance in diaphragm development. Complete null-mutation of the RXRα locus

is embryonic lethal; with mutant embryos displaying severe cardiac and ocular abnormalities, though no diaphragm defects were reported in these mice (Kastner et al., 1994). However, mice expressing a truncated form of RXRα with a mutation in the ligand-dependent transactivation domain do not suffer the same degree of embryonic lethality associated with null-mutation of this gene and diaphragm defects have been reported in these mice (Mascrez et al., 1999). This indicates that transcriptional activity of RXRα is essential for normal diaphragm development. Further, based upon our expression data it would seem that RARα:RXRα and RARγ:RXRα heterodimers would be the predominant receptor complexes mediating retinoid signalling in the PPF. Again, further studies dissecting the contribution of the different retinoid receptors in PPF development are required.

6.4.6 Pharmacologic blockade of RAR signalling

Further experiments into which retinoid receptors are essential for diaphragm embryogenesis are limited in genetically engineered mice by the large degree of genetic redundancy and compensation amongst the retinoid receptors, as well as the embryonic lethality that is frequently found in compound retinoid receptor mutants. In order to circumvent these limitations we tried a pharmacologic approach to specifically block retinoid signalling in the developing rat embryo. Previous experiments using RAR antagonists to produce congenital anomalies *in vivo* have met with mixed success; Kochhar and colleagues were unable to produce limb anomalies following antagonist exposure and concluded that some of the abnormalities observed in retinoid receptor mutant mice may be secondary to a

generalized growth retardation and therefore presumably independent of retinoid signalling (Kochhar et al., 1998).

In the experiments reported here we attempted to induce CDH in developing rats following exposure with the potent pan-RAR antagonist BMS493 (Johnson et al., 1995; Agarwal et al., 1996). By using an increased dose of this compound and specifically targeting the critical period of diaphragm development we were able to induce a large number of diaphragmatic hernias in exposed off-spring. The diaphragm defects produced were identical in phenotype to those observed in nitrofen exposed and vitamin A deficient rats, as well as human cases of Bochdalek CDH. These experiments highlight that signalling through the retinoid receptors is essential for normal diaphragm and provide proof of principle that CDH can be produced by the pharmacologic blockade of retinoid signalling. Although BMS493 is a pan-RAR antagonist, receptor subtype specific antagonists are available and future studies using these compounds will be used to clarify which specific retinoid receptor subtypes are essential for normal diaphragm development.

The finding that BMS493 can induce CDH represents a new and exciting model of CDH, with the advantage that it produces a high incidence of CDH and seems to lack some of the non-specific effects associated with nitrofen exposure. Further research into this compound and other RAR antagonists will be invaluable to our understanding of retinoid signalling in the normal and pathological development of the diaphragm.

6.4.7 Summary

In the first half of this study we demonstrated that several compounds which can induce CDH all inhibit retinoid signalling in the developing embryo. Further, in the case of nitrofen we have shown that a single bolus of this compound has a sustained inhibitory effect on retinoid signalling, which extends through a period in gestation when the embryo is particularly sensitive to disruptions in retinoid homeostasis. These studies indicate that the etiologic origins of diaphragmatic hernia in these teratogenic models of CDH is an inhibition of retinoid signalling secondary to decreased RA production.

In the second half of this study we show that all the components necessary for the synthesis of RA and its signalling are expressed during early diaphragm development. Specifically, our results indicate that Raldh2 is responsible for the synthesis of RA in the PPF. Newly synthesized RA would then be shuttled to the nucleus by CrabpII, where it can modulate gene expression via binding to heterodimer complexes of retinoid receptors (RARα:RXRα and/or RARγ:RXRα heterodimers). Review of reports describing retinoid receptor knock-out mice indicate that the retinoid receptors RARα and RXRα are important in diaphragm development, and will be the focus of future studies. The unique expression pattern of RARα indicates that it may be of particular importance.

A general theme that emerges from these experiments is the importance of retinoid signalling in diaphragm embryogenesis. The retinoid hypothesis of CDH etiology posits that abnormal retinoid signalling contributes to the development of CDH; this hypothesis is corroborated by the presented data. Future experiments in

our laboratory will employ retinoid receptor antagonists to dissect this signalling pathway and also focus on the expression of retinoid responsive genes in the developing diaphragm in order to identify which genes, controlled by retinoid signalling, are essential for normal diaphragm embryogenesis.

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Chapter 7: General Discussion

7.1 Introductory remarks

The general aim of this thesis was to provide a better understanding of the normal and pathological development of the diaphragm, particularly in the context of CDH. This is a frequently occurring birth defect that causes severe neonatal respiratory distress often with fatal consequences. While advances are being made into the treatment of CDH; how the diaphragm defect develops and its root cause remain unclear. The research described in this thesis not only provides an improved understanding of normal diaphragm embryogenesis and the formation of CDH, but also provides a foundation for better treatment strategies, improved identification of cases and risk stratification, and possibly the prevention of some cases.

The major findings of this thesis are:

1. Teratogen-induced, dietary, and genetic models of CDH have a common mechanism of pathogenesis, with parallel features found in human cases of CDH (Chapter 3).
2. The CDH-critical region 15q26 was found to contain several genes associated with the development of the diaphragm (Chapter 4).
3. Genes with a strong association with CDH were found to be preferentially expressed in the non-muscular, mesenchymal cells of the PPF (Chapter 4).
4. Development of the PPF in humans and rats was defined (Chapter 5).
5. Decreased cell proliferation was indicated as a putative causative factor in abnormal PPF development in the nitrofen model of CDH (Chapter 5).
6. CDH-inducing teratogens were shown to suppress retinoid signalling and, in the case of nitrofen, this suppression was shown to be sustained (Chapter 6).

7. The PPF was identified as a centre of retinoid signalling (Chapter 6).
8. A new model to study CDH, using a pan-RAR antagonist, was identified in rats (Chapter 6).

This general discussion integrates the results obtained into an improved and more unified understanding of how CDH develops. Importantly, an emphasis has been placed on future directions of the research in order to test new hypotheses generated regarding the pathogenesis and etiology of CDH. Several of the avenues of future research mentioned below are being actively pursued in the Greer laboratory. The majority of these studies will be carried out by Darine Roum (graduate student) with technical assistance from Wei Zhang.

7.2 Insights into the pathogenesis of CDH

The prevailing explanation for the occurrence of CDH is failure of the pleuro-peritoneal canal (PPC) to close (Harrington, 1948). Previous work from the Greer laboratory refuted this hypothesis and demonstrated that diaphragm defects in the nitrofen model of CDH were anatomically distinct from the PPC and actually originated from an abnormally formed pleuro-peritoneal fold (PPF; Allan and Greer, 1997). One of the significant questions raised by this research was whether PPF defects underlie CDH in humans or are they an artefact unique to the nitrofen model? Although the first part of this question cannot practically be answered, the second part was addressed in chapter 3 of this thesis. By examining the pathogenesis of CDH in vitamin A deficient rats and *Wt1* null-mutant mice we established that malformation of the PPF is not unique to the nitrofen model and may be generally applicable to CDH in humans. The latter half of this statement is supported by our examination of diaphragms collected at autopsy from infants who had succumbed to CDH. We observed that the musculature of the collected diaphragms was thickened around the rim of the defect, a characteristic feature of animal models of CDH, which indicates a common pathogenic origin in humans and animal models of CDH. Our analysis of different animal models of CDH and human diaphragms lead us to hypothesize that CDH in humans originates from abnormal development of the PPF.

A more refined version of the hypothesis that CDH has its embryologic origins in a malformed PPF states that it is the non-muscular, mesenchymal cells of the PPF that are specifically affected in this structure. This hypothesis was based on

the observation that myogenesis does not seem affected in humans with CDH, or in animal models, and that CDH can be induced independently of myogenesis (Allan and Greer, 1997; Babiuk et al., 2002; Clugston et al., 2006). In our study of CDH-critical genes (chapter 4) we found that several transcription factors which are essential for normal diaphragm development (*COUP-TFII, WT1, FOG2*, and *GATA4*) are all specifically expressed in the non-muscular, mesenchymal cells of the PPF. We interpret this result as further evidence to support the concept that it is this specific population of cells that are affected in the developing PPF.

In a further extension and refinement of this hypothesis, we became interested in the mechanism responsible for structural defects in the PPF. As described in chapter 5, we found no evidence to indicate that nitrofen induces apoptosis in the PPF; rather we observed a decrease in cell proliferation in this structure. The earliest stage at which PPF defects appear has yet to be determined and is the focus of continuing studies in the Greer laboratory. While abnormal cell proliferation appears to contribute to malformation of the PPF, other factors such as cell adhesion and migration of cells into the PPF have yet to be studied.

If we integrate the data gathered from the animal studies presented in this thesis, as well as the limited human tissue available for examination, we can hypothesize that CDH in humans arises from abnormal cell proliferation in the non-muscular, mesenchymal cells of the PPF which leads to the formation of an abnormal PPF. This defect in the PPF translates to the hole in the postero-lateral region of the fully-formed diaphragm which is characteristic of CDH. This experimentally-derived hypothesis is in contrast to the current dogma surrounding

the pathogenesis of diaphragm defects in CDH and, if accepted, could lead to a revision of the standard textbook explanation of CDH's origins. As such, this model represents a significant advance in our understanding of the embryologic origins of CDH.

7.2.1 Future studies on the pathogenesis of CDH

The experiments described in this thesis have presented a more refined model of the pathogenesis of CDH. As indicated above, the hypothesis that CDH in humans originates from a PPF defect cannot be directly tested, however future studies describing the pathogenesis of CDH in animal models should address this hypothesis to determine its veracity (see below). If malformations of the PPF are found to underlie diaphragmatic hernia in the majority of animal models investigated, that will certainly bolster the hypothesis that PPF defects underlie CDH in humans.

With regard to the mechanism of PPF malformation, there are still many unknowns that have to be addressed. Previous studies implicate an abnormality in the non-muscular, mesenchymal cells of the PPF as a foundation for PPF malformation (see chapter 4; Babiuk et al., 2002). As such, research in the Greer laboratory is now focused on this population of cells. As their contribution to the developing diaphragm is better known an improved understanding of how PPF defects arise will follow. The following questions are to be addressed:

1) What is the early embryonic origin of this cell population?
2) Is this population of cells homogenous in the PPF, or are they composed of different sub-populations?

Transcription factor expression within the PPF was shown to be uniform throughout the PPF (chapter 4), however RARα has a restricted pattern of expression (chapter 6). Further characterization of what proteins are expressed in the mesenchymal cells of the PPF by immunohistochemistry will address this question.

3) How do these cells contribute to the whole diaphragm?

   It is known that muscle precursor cells populate the entire diaphragm by migrating out from the PPF (see review by Clugston and Greer, 2007), however it is unknown if the mesenchymal cells of the PPF make a similar contribution. If a suitable cell marker within the PPF is identified, its expression could be tracked as the complete diaphragm forms. For example we have recently obtained frozen embryos to start a colony of *Wt1*-LacZ; these mice will allow us to observe *Wt1*-positive mesenchymal cells in the developing diaphragm.

Answering these questions will be complex, but the knowledge gained can be applied to determine the complete mechanism of PPF malformation in animal models of CDH. Identifying this mechanism is essential if it is to be addressed therapeutically.

7.2.2 Characterizing new animal models of CDH

There are several instances in the literature describing diaphragm abnormalities in a variety of transgenic mice, however the description of the diaphragm phenotype is often inadequate and an attempt to identify the embryologic origin of the diaphragm defect is rarely made (Oh et al., 2004; Bleyl et al., 2007).

Although these strains of mice indicate genes essential for diaphragm development, they also represent a missed opportunity to learn more about the pathogenesis of CDH. Based upon the studies described in this thesis and our hypotheses concerning the pathogenesis of CDH, we suggest that the following observations and experiments be performed to obtain the maximum amount of useful information from these mice, and future models of CDH:

1. Is the diaphragm defect typical of Bochdalek CDH, or is it a muscularisation or central tendon defect?
2. If there is a hole in the diaphragm, is the musculature surrounding it thickened?
3. Is the PPF abnormally developed? What is the cause of PPF malformation?
4. How is the gene of interest expressed in the developing diaphragm of control animals? Is it restricted to the non-muscular, mesenchymal cells of the PPF?
5. How is the expression of the gene of interest regulated? Is it controlled by retinoid signalling?

7.3 New perspectives on the etiology of CDH

Our current understanding of the etiology of CDH is incomplete. In animal models it is known that CDH can be triggered by a purely environmental manipulation, such as vitamin A deficiency (VAD), or a purely genetic insult, such as *Wt1* mutation (Clugston et al., 2006). With regard to CDH in humans, the triggers are not quite so clear. While neonates with CDH do have decreased levels of circulating retinoids (Major et al., 98), it seems unlikely that VAD alone is likely to precipitate CDH. For example, there are several populations around the globe in which VAD is endemic, yet the incidence of CDH among these populations is not reported to be abnormally high (West KP Jr, 2002; Nandi et al., 2008). Similarly, with regard to the genetic etiology of CDH, mutations in CDH-critical loci do not always leads to the development of diaphragm defects suggesting some other modifying factor, genetic or otherwise. As such, it seems that the etiology of CDH is multifactorial, likely involving a complex interplay between genetic and environmental factors. The sections below will discuss the genetic etiology of CDH and the retinoid hypothesis separately, and then an attempt will be made to unify these apparently distinct etiologies into a broader understanding of what causes CDH.

7.3.1 The genetics of CDH

The genetics of CDH is complex; review of the literature yields case reports linking genetic abnormalities in association with CDH in almost every human chromosome (Lurie et al., 2003). Recent review on the subject illustrates the shortfall between linking genotype with phenotype in CDH, i.e. while several CDH-

critical regions have been identified, how mutations in these regions lead to CDH is unknown (Pober et al., 2008). If we are to truly begin to understand the genetic etiology of CDH it will not be enough to identify specific genes, we must also understand how these genes actually function in diaphragm development.

The study described in chapter 4 of this thesis was designed to address this issue. Using the CDH critical region 15q26 as an example, we show that regions of the human genome recurrently associated with CDH are likely to contain multiple genes expressed in the developing diaphragm. Thus, a reductionist *one-gene-one-defect* mind-set may not be appropriate and a more holistic perspective preferable. In this regard, accurate definition of CDH-critical regions is required to determine which genes are encompassed within them. Subsequent analysis of gene expression, similar to that described in chapter 4, should also be performed to determine which of these genes may be involved in diaphragm development.

It is obvious from the number of CDH-critical regions that have been identified that CDH is not caused by a mutation in a single gene or loci, this presents the question: are CDH-associated genes linked by a common factor? The data collected in chapter 4 indicates the answer may be yes. In this chapter we focused on four transcription factors (*WT1*, *COUP-TFII, GATA4,* and *FOG2)*, which have all been strongly linked with CDH. Our expression studies revealed that all of these genes were expressed in the same population of cells within the PPF. Further, review of the literature revealed several instances where these genes were shown to interact together to control the expression of another gene. With regard to CDH, it would appear that these transcription factors may act together to control the

expression of genes essential for normal diaphragm development. This supposition raises two future research questions: 1) Are *WT1*, *COUP-TFII, GATA4,* and *FOG2* part of a common gene regulatory network in the developing diaphragm, and 2) What are the downstream targets of these transcription factors?

To address the first question, biochemical experiments carried out *in vitro* may be the only way to definitively illustrate the link between these genes. However a link between them, be it direct or otherwise, may be shown using animal models. For example, is the expression of *Coup-tfII* changed in *Wt1* null-mutant mice? Gene expression analysis would also be required to address the second question, but on a larger scale. In order to determine the downstream targets of a particular transcription factor, gene array analysis could be performed in wild type versus transgenic mice to determine what genes are disrupted in the absence of the target gene.

7.3.2 The retinoid hypothesis of CDH: an environmental etiology?

The retinoid hypothesis states that abnormal retinoid signalling contributes to the development of CDH (Greer et al., 2003). When we consider the retinoid signalling pathway as a whole, several points can be identified where perturbation has been linked with CDH, i.e. i) dietary intake of vitamin A, ii) cellular uptake of RBP, iii) conversion of retinal to retinoic acid, and iv) signalling through retinoid receptors. In the context of diaphragm embryogenesis, it appears that the retinoid signalling pathway is sensitive to perturbations which manifest in CDH. In animal models of CDH these perturbations can be environmental in origin; be it vitamin A deficiency or maternal exposure to teratogenic agents. Such insults are difficult to

assess in humans. As indicated above, endemic vitamin A deficiency is not associated with an increased incidence of CDH, and while newborns with CDH were shown to have reduced levels of circulating retinoids, the cause of this and how it relates to diaphragm development early in gestation is unclear (Major et al., 1998; West KP, 2002). It is hoped that a large international study examining retinoid levels in neonates with CDH, which the Greer laboratory initiated, will confirm the link between CDH and abnormal retinoid status. Further, through the use of maternal questionnaires specific risk factors or environmental exposures may be identified.

It should be noted here that perturbations to the retinoid signalling pathway in the context of CDH are not solely environmental in origin. Retinoid receptor knock-out mice have a low incidence of CDH, and mutation of the *STRA6* gene is associated with CDH in humans (Mendelsohn et al., 1994; Pasutto et al., 2007). As such, it is clear that regardless of the nature of the perturbation, retinoid signalling is important in normal diaphragm development and that an improved understanding of how retinoid signalling contributes to the normal and pathological development of diaphragm development will be beneficial. In this regard, Chapter 6 of this thesis described a series of experiments which provide further support to the retinoid hypothesis and how this signalling pathway is involved in the embryogenesis of the diaphragm.

Specifically, in chapter 6 we were able to show that four different CDH-inducing teratogens all decrease retinoid signalling in exposed mouse embryos. This *in vivo* study provides an important link between *in vitro* studies showing these

compounds can inhibit retinoic acid synthesis, and the observation that they all induce CDH. In this chapter we also identified the specific components of the retinoid signalling pathway expressed in the PPF, allowing an improved understanding of how retinoid signalling functions in the development of this structure. Perhaps most significantly, in Chapter 6 we used a pan-RAR antagonist to induce CDH in the off-spring of exposed pregnant rats. Not only does this finding highlight the importance of retinoid signalling in diaphragm development, but it also provides proof of principle that pharmacologic antagonists can be used to induce CDH. In future studies, antagonists for each of the three RAR sub-types will be tested to determine signalling through which of these receptors is essential for normal diaphragm development.

7.3.3 Toward a unified understanding of CDH etiology

As discussed earlier, some cases of CDH have an obvious genetic abnormality at their origin, while some other cases are idiopathic. There is convincing evidence from animal studies and some human data to suggest that retinoid signalling is important in diaphragm development. Given the increasing support for the retinoid hypothesis and an improved understanding of the genetics of CDH, can a more unified understanding of the etiology of CDH be developed?

As discussed in chapter 4, when we consider some of the genes that have been strongly associated with CDH (i.e. *WT1*, *COUP-TFII, GATA4,* and *FOG2*), evidence exists that their expression is controlled by retinoid signalling, indicating that links can be made between the retinoid signalling pathway and genes essential for diaphragm development. To provide another example, consider the gene *PDGRF-alpha* (platelet derived growth factor alpha receptor). The expression of this gene has been shown to be controlled by retinoic acid, mice with a null-mutation in *Pdgrf-alpha* develop diaphragm defects, and at least one human infant with CDH has been identified carrying a mutation in this gene (Wang et al., 1999; Bleyl et al., 2007). As such, we can state that this gene is essential for diaphragm development and its expression is controlled by retinoic acid.

Review of these facts suggests a model where retinoid signalling controls the expression of several genes which are essential for normal diaphragm development. In the context of CDH, this implies that an abnormality in the retinoid signalling pathway could affect the expression of genes essential for diaphragm embryogenesis and lead to CDH. Further, direct genetic disruption of these genes would also be

causative. Therefore there appears to be several inter-related cellular signalling events which are required for normal diaphragm development, disruption of which at any point can lead to CDH.

The concept described above represents a significant advance in our understanding of the etiology of CDH. Until recently the cause of this birth defect was poorly understood, however simultaneous improvements in our genetic understanding of CDH and the derivation of the retinoid hypothesis have provided novel insight into its origins. These advances have created a new perspective on the etiology of CDH and act as a guide for future research in this area.

Experiments aimed at testing a link between abnormal retinoid signalling and CDH associated genes will be performed in the Greer Laboratory. Using the nitrofen model of CDH, which is known to disrupt retinoid signalling, the expression of the CDH-critical genes *WT1*, *COUP-TFII, GATA4,* and *FOG2* will be assessed in control and nitrofen exposed embryos. Quantitative analysis of mRNA and protein expression will be assayed to determine if their levels change in response to nitrofen exposure. If a change in the expression of these genes in response to nitrofen is established, this will indicate that during embryogenesis their expression is controlled by retinoic acid. Results from the nitrofen model of CDH could be corroborated in the Vitamin A deficient rat model of CDH, or through the use of BMS493.

In a related series of experiments the concept that genetic and environmental triggers combine to induce CDH will be further explored by asking the question: does haplo-insufficiency in CDH-associated genes predispose to CDH? To give a

specific example: it is known that *Wt1* null-mutant mice develop diaphragmatic hernia and *Wt1* heterozygous mice are essentially normal (Kreidberg et al., 1993), but are heterozygous mice more susceptible to developing CDH? An increase in the incidence of nitrofen-induced CDH in *Wt1* heterozygous mice compared to wild-type littermates would support the hypothesis that haploinsufficiency for CDH-associated genes could leave individuals more sensitive to an environmental insult which in turn could precipitate the development of CDH.

7.5 Closing comment

The research described in this thesis has addressed various aspects of the pathogenesis and etiology of CDH. In an attempt to answer some of the outstanding questions in the field of CDH, it has inevitably generated new hypotheses and questions to be answered. However, to paraphrase and extend upon a thought by the physicist Alan Lightman, these will be *well-posed* questions and, guided by the research presented herein, should provide more insightful answers (Lightman A, 2005).

7.4 References

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