The Relationship of Gender and Disorders of Sex Development with Congenital Diaphragmatic Hernia in the Rat Nitrofen Model

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by

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Figures

*Abbreviation congenital diaphragmatic hernia (CDH)

Tables

Declaration

This thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

The research was carried out at the Institute of Child Health, Royal Liverpool Children's Hospital (Alder Hey), Eaton Road, Liverpool, L12 2AP and in the Bio-surgical Unit of the University of Liverpool.

Abstract

Research Questions

Is there a gender susceptibility to congenital diaphragmatic hernia in the nitrofen rat model and is it associated with disordered sexual development?

Background

Congenital diaphragmatic hernia is a frequently fatal birth defect, mostly due to associated pulmonary problems, affecting 1 in 3000 live births. About 40% of cases have associated anomalies of which 20% affect the sexual phenotype. Human cases note sex reversal syndromes, ambiguous genitalia and maldevelopment of genitalia accompanying congenital diaphragmatic hernia. Human studies have also noted sex difference (s) in the incidence of congenital diaphragmatic hernia but the reports are ambiguous, some epidemiology surveys recording a higher incidence of congenital diaphragmatic hernia in females, whilst other reports cite a higher frequency in males. Nitrofen, a teratogenic substance, produces experimental congenital diaphragmatic hernia in rodents. Nitrofen is speculated to interfere with retinoic acid - steroid signalling pathways and these may also be linked with sexual differentiation. Development of the diaphragm and lungs has been studied using animal models. This study was designed to test the hypothesis that nitrofen may influence sexual phenotype and gender frequency of congenital diaphragmatic hernia in a well characterised animal model of this condition.

Methods

Time mated Sprague Dawley rats were gavage fed nitrofen at day 9.5 to generate predominantly left sided congenital diaphragmatic hernia. Fetuses were delivered by caesarean section on days 20 or 21 of gestation (term = day 22). External genitalia were examined to define external genital phenotype. The abdominal cavity was opened and the genito-urinary system examined. The internal genital organs were assigned a phenotype and findings correlated with external appearances. The diaphragm of each fetus was studied for the absence or presence of congenital diaphragmatic hernia and the laterality of the defect recorded. Controls (non nitrofen fed) were used for all comparative analysis.

Results

Control (n = 600) and nitrofen exposed offspring (n = 518) had equal frequencies of males and females. There was no significant difference in the frequency of congenital diaphragmatic hernia occurring in male and female nitrofen treated pups. In all nitrofen exposed fetuses and normal controls, internal and external genitalia concorded without evidence of significant genital tract malformations or intersex states.

Conclusions

Prenatal nitrofen exposure is not associated with significant gender differences (or prenatal loss) in the risk of congenital diaphragmatic hernia. Genital tract malformations do not appear to accompany congenital diaphragmatic hernia in the nitrofen rat model.

Key Words

Sexual phenotype, congenital diaphragmatic hernia, nitrofen,

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CHAPTER 1

Introduction, the Development of the Diaphragm, Lung and Reproductive System

1.1 Congenital Diaphragmatic Hernia

1.1.1 Background

Congenital diaphragmatic hernia is a developmental defect resulting in disordered diaphragmatic growth in the embryo causing an abnormality in the integrity of the diaphragm.

Congenital diaphragmatic hernia is a frequently fatal birth defect affecting 1 in 3000 live births.

1.1.2 Features and Definition of Congenital Diaphragmatic Hernia

1.1.2.1 Definition of Congenital Diaphragmatic Hernia

The diaphragm is a muscular structure that separates the abdominal cavity from the thoracic cavity and this maintains the pressure differentials between the two cavities. Contraction of the diaphragm contributes to ventilation. Congenital diaphragmatic hernia forms a spectrum of birth defects characterised by incomplete formation of the diaphragm allowing a communication between the thoracic and abdominal cavities. Also in this spectrum is a thinning of the diaphragm, known as an eventration. This is the result of incomplete muscularisation of the diaphragm resulting in the elevation of the thinned portion of the diaphragm. Congenital diaphragmatic hernia can be classified as isolated or complex. Isolated congenital diaphragmatic hernia is diagnosed when the hernia is the only apparent major malformation. Pulmonary hypoplasia and pulmonary hypertension, intestinal rotation and left heart hypoplasia due to compression are considered to be part of the primary anomaly sequence (Pober 2007).

Complex congenital diaphragmatic hernia is diagnosed when additional abnormalities occur either as part of a recognised syndrome, a chromosomal abnormality or with accompanying major anomalies of other organs that do not form part of a recognised syndrome (Pober 2007). The frequency of complex congenital diaphragmatic hernia ranges from as low as 10% to as high as 50% which could be

due to the difference in the methods of data collection (Skari, Bjornland et al. 2000). Other more recent studies classify approximately 40% of cases as complex (Torfs, Curry et al. 1992; Dott, Wong et al. 2003; Colvin, Bower et al. 2005).

1.1.2.2 Types of Congenital Diaphragmatic Hernia

The most common type of hernia is the posterolateral, Bochdalek hernia. These form about 80% to 90% of all cases of congenital diaphragmatic hernia. 85% of posterolateral hernia cases are on the left side, 10% are right sided and 5% are bilateral. Congenital diaphragmatic hernia is often accompanied by herniation of the abdominal organs into the chest cavity.

Apparent absence or an extremely large hernia of the diaphragm is termed agenesis but this defect probably represents the severe end of the Bochdalek hernia spectrum and is not likely to be a distinct entity. These hernias can be associated with absent or extremely deficient rim of posterior and lateral muscle or have a complete rim of the muscle (Pober, Russell et al. 1993).

A less common defect is the Morgagni hernia, an anterior hernia. This type comprises approximately 2% of all congenital diaphragmatic hernia cases and is generally associated with a hernia sac. These hernias may be undetected in the newborn as they may be asymptomatic. Other rare hernias include central hernias and rare anterior types of hernia. Anterior hernias tend to be severe and are found in individuals with associated midline anomalies, termed the Pentalogy of Cantrell (Cantrell, Haller et al. 1958; Milne, Morosin et al. 1990)

It is difficult to estimate the frequency of eventration because it may coexist with and/or be misdiagnosed as a Bochdalek hernia. Severe diaphragmatic eventration is associated with pulmonary hypoplasia and respiratory distress during infancy. Milder degrees of diaphragmatic eventration can present later in life with respiratory symptoms such as cough and pneumonias, or without symptoms so that the diagnosis is made incidentally on chest x-ray. It has been observed that eventration of the diaphragm and "true" congenital diaphragmatic hernia can occur in the same individual, suggesting that they may share a common aetiology (Mertins 1952; Thomas, Stern et al. 1976; Rodgers and Hawks 1986).

The following diagrams show a normal diaphragm and the different types of hernia from an overview by Barbara J Pober (Pober, Russell et al. 1993) updated 2010.

Figure 1 Normal Diaphragm

The following figures show diaphragms as seen from below from an overview by Barbara J Pober (Pober, Russell et al. 1993) updated 2010 as shown in diagram A. Diagram B shows a normal diaphragm

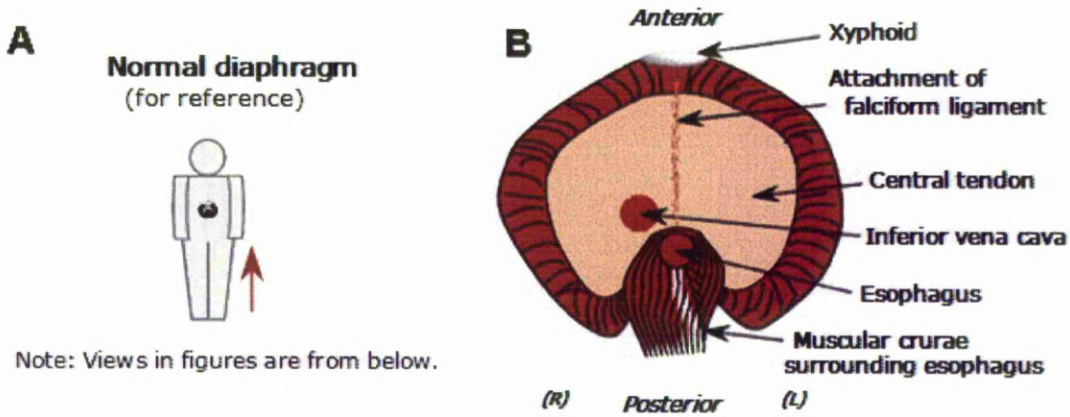

Figure 2 Different types of congenital diaphragmatic hernias

Diagrams showing different types of congenital diaphragmatic hernia. Diagram A demonstrates the posterior position of the Bochdalek hernia. Diagram B shows the position of a Morgagni hernia and other anterior hernias. Diagram C shows the position of a central hernia

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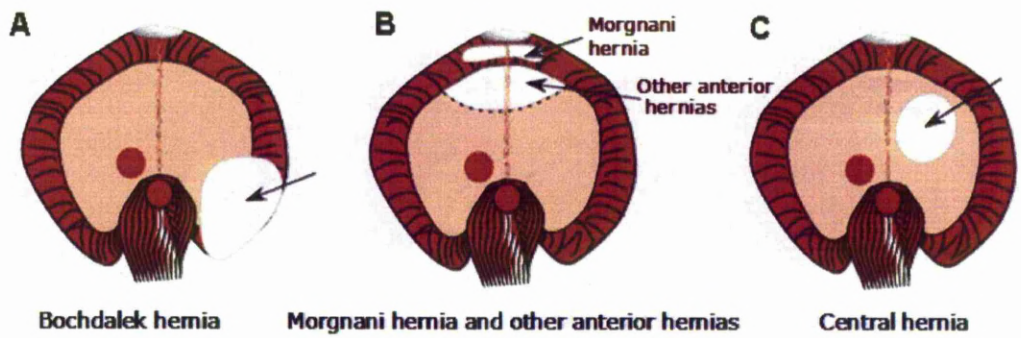


1.1.2.3 Incidence of Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia is identified in approximately 1 in 3000 live births but the true incidence of this defect is difficult to ascertain because:-

1 It is difficult to establish how many pregnancies are lost due to congenital diaphragmatic hernia prior to term (Harrison, Adzick et al. 1994; Skari, Bjornland et al. 1998).

2 Also newborns with congenital diaphragmatic hernia may die prior to transfer to units that collect data for this disease (Harrison, Bjordal et al. 1978; Jaffray and MacKinlay 1996).

3 Cases of congenital diaphragmatic hernia that present outside the neonatal period may never be included in the estimates of frequency (McCue, Ball et al. 1985; Berman, Stringer et al. 1988; Berman, Stringer et al. 1988).

4 The facilities for collecting accurate details of all live and still births may vary from country to country.

Given these caveats the frequency estimation can only be an approximation.

1.1.2.4 Epidemiology

Robert and colleagues collected data from 1973 until 1993 on postero-lateral congenital diaphragmatic hernia. These data were from three population based studies looking at the epidemiology of birth defects. This study did not include induced or spontaneous abortions, but included all infants born and still births from 28 weeks. The total number of births included in this study was 5,971,525 which were from France, Sweden and California. The study was based on a total of 1439 cases of congenital diaphragmatic hernia of which 875 cases were isolated hernias, 486 had associated malformations with no recognised chromosomal anomalies and 78 with chromosomal anomalies. Maternal age had no significant effect. Right sided congenital diaphragmatic was found in 20% of unilateral cases in both isolated and complex cases and there was no difference in the sex ratio (Robert, Kallen et al. 1997) as had been previously suggested (Benjamin, Juul et al. 1988). Bilateral cases of congenital diaphragmatic hernia were found more frequently in the complex cases than in the isolated cases. The risk of congenital diaphragmatic hernia was significantly increased in the case of twinning (Torfs, Curry et al. 1992). There was no evidence for geographical or racial variations in the frequency of congenital diaphragmatic hernia (Robert, Kallen et al. 1997; Yang, Carmichael et al. 2006).

Using the National Birth Defects Prevention study and a telephone interview for births between 1989 and 1997, Yang and colleagues surmised that maternal uptake

of nutrients and vitamins in the year before pregnancy reduced the incidence of congenital diaphragmatic hernia, and that in many cases this may be involved with the retinoid signalling pathway. This was a substantial study with 377 cases of mothers of babies born with congenital diaphragmatic hernia and 5008 control mothers (Yang, Shaw et al. 2008).

The Eurocat statistatical monitoring of trends of congenital anomalies in Europe reported a 4% decreasing incidence in the frequency of congenital diaphragmatic hernia (Loane, Dolk et al. 2011).

1.1.2.5 Impact of Gender on Incidence

Birth registries and epidemiology surveys record conflicting data on gender and congenital diaphragmatic hernia. Slightly more males are born than females; this has long been documented but remains unexplained. The ratio of males to females is approximately 1.06-1.04 with small fluctuations (Ohmi, Hirooka et al. 1999) obtained from the statistics of pregnancy and childbirth from the London Stationary office.

In a subset of newborns with congenital anomalies some are much more prevalent in males, such as certain heart defects, transposition of the great arteries or hypoplastic left heart (Botto, Correa et al. 2001; Harris, Francannet et al. 2003; Pradat, Francannet et al. 2003; Shaw, Carmichael et al. 2003) with up to two out of every three cases being male. For spina bifida and anencephaly there is a female excess (Bamforth and Baird 1989; Kallen, Cocchi et al. 1994). This sex related susceptibility could be related to a sex specific sensitivity to environmental teratogens or sex specific survival during pregnancy (Lisi, Botto et al. 2005).

David and colleagues reported in 1976 a higher proportion of females with congenital diaphragmatic hernia from cases between 1943 and 1974, 81 female and 62 male. This study included all cases of congenital diaphragmatic hernia born in Bristol, UK, during this time and includes cases of eventration of the diaphragm and stillbirths (David and Illingworth 1976) but the numbers from this study were small. In a later study from Avon a further 26 cases were added to this study, collected by the same methods giving sex totals of 90 females and 79 males (David, Parker et al. 1980), but these later figures included a higher proportion of males with congenital diaphragmatic hernia but this was still a small number of total cases.

A study reported in 2005 including 18 registries from 24 countries reported a higher incidence in males with a ratio of 1.42 to 1.0; the numbers involved being 1,730

males and 1,219 females with congenital diaphragmatic hernia. This study also reported that as well as defects of the diaphragm, defects of the abdominal wall are also more frequent in males (Lisi, Botto et al. 2005).

The California birth defects registry reported by Robert and colleagues found a change in distribution in the gender prevalence of complex congenital diaphragmatic hernia from 1988 onwards. This was a shift to a higher prevalence in males in cases of complex congenital diaphragmatic hernia that was statistically significant but the reasons for this have not yet been explained (Robert, Kallen et al. 1997). This same study found a higher incidence of congenital diaphragmatic hernia in males in studies from France and Sweden, also as reported previously (Robert, Kallen et al. 1997).

1.2 The Normal Embryonic Development of the Diaphragm

1.2.1 Morphological Development of the Diaphragm.

The human diaphragm develops between the 4th and 12th week of gestation and in the mouse between 10.5 and 15.5 days post coitum (dpc). The gestation period in the mouse is 18.5 days. The tissues that contribute to the muscular diaphragm development are referred to as the pleuroperitoneal folds and the post hepatic mesenchymal plate tissue. These structures may be different sections of the same mesodermal tissue and the terms are used interchangeably by different investigators to describe tissue contributions to the diaphragm (Greer, Allan et al. 2000; Ackerman and Greer 2007). The pleuroperitoneal folds are paired pyramidal shaped structures most evident in the embryonic thorax just before the formation of the diaphragm at the level of the forelimb, 11.5dpc in the mouse 13.5dpc in the rat and 4-6 weeks in the human. The diaphragm is formed by the closure of the pleuroperitoneal fold, occurring at 12.5dpc in mice, to form the membranous scaffold of the diaphragm (Babiuk, Zhang et al. 2003), a mesenchymal substratum that is derived from the somatopleure (Tosney 1988). This creates a continuous sheet that completely separates the abdominal and thoracic cavities. The muscular component of the diaphragm forms on this substratum (Babiuk, Zhang et al. 2003). In mice with a mutation *(c-met* null mice*)* that prevents muscle precursors from migrating to the peripheral muscles including the pleuroperitoneal fold thus preventing the muscularisation the diaphragm the membranous scaffold of the diaphragm is complete (Babiuk and Greer 2002).

It was the traditional view that the diaphragm developed from four different structures, the central portion of the diaphragm came from the septum transversum, the posterolateral section from the pleuroperitoneal folds, the portion of the diaphragm posterior to the oesophagus came from the oesophageal mesentery and a rim of musculature around the periphery of the diaphragm arose from elements of the thoracic body wall.

Defects in the diaphragm of multiple animal models of congenital diaphragmatic hernia are shown to originate from abnormal pleuroperitoneal fold development (Clugston, Klattig et al. 2006; Clugston, Zhang et al. 2010).

The study of rodent diaphragm development has failed to identify contribution from the elements traditionally considered to be involved in human diaphragmatic development, apart from the pleuroperitoneal fold which contributes myogenic cells

and axons which expand to form the neuromuscular component of the diaphragm (Babiuk, Zhang et al. 2003). If this model is also applicable to diaphragm development in humans then the traditional view of diaphragmatic development needs revising (Clugston, Klattig et al. 2006).

1.2.1.1 The Pleuroperitoneal Fold

The pleuroperitoneal fold in human embryos was observed to have the same pyramidal structure, triangular cross section and anatomic location as the pleuroperitoneal fold seen in animal models. The observation that abnormal pleuroperitoneal fold development underlies diaphragm defects in numerous animal models has lead to the hypothesis that a defect in the human pleuroperitoneal fold leads to diaphragmatic maldevelopment in humans (Clugston, Klattig et al. 2006). If this were to be the case, the critical time for congenital diaphragmatic hernia development would be between four and six weeks gestation, before the pregnancy has been positively confirmed (Torfs, Curry et al. 1992).

Myogenic cells, the diaphragmatic muscle precursor cells, and phrenic axons migrate to the pleuroperitoneal fold reaching the pleuroperitoneal fold by 12.5dpc in the rat (Babiuk, Zhang et al. 2003) where they begin to differentiate and proliferate. From 13.5dpc in the rat muscle precursor cells radiate across the mesenchymal substratum from the pleuroperitoneal fold migrating and differentiating towards the dorsolateral costal, sterna-costal, and crural regions of the developing diaphragm. By 17dpc in the rat they will have spread to all the regions of the diaphragm that are destined to be muscularised (Babiuk, Zhang et al. 2003).

This development of the mesenchymal substratum is not well understood but there is evidence to suggest that the mesenchymal substratum is derived from the somatopleure (Tosney 1988). The mesenchymal substratum forms a continuous sheet that completely separates the thoracic and abdominal cavities and it may be a separate anatomical entity in itself (Babiuk, Zhang et al. 2003). It appears that the muscle forms on top of the mesenchymal substratum leaving the central portion, the central tendon, amuscular. Babiuk and colleagues found no contribution to the diaphragm musculature from the body wall and other areas as was previously described (Babiuk, Zhang et al. 2003). The identity of the guidance cues directing the migration and differentiation of these muscle cells destined to populate the diaphragm muscle layer is largely unknown, the embryology of this aspect of diaphragmatic development being not completely understood (Babiuk, Zhang et al. 2003).

The phrenic axons from the brachial plexus migrate towards the pleuroperitoneal fold after the muscle precursor cells. Once the phrenic axons reach the pleuroperitoneal fold they extend to the same three axis of the diaphragm as the muscle precursors, lagging slightly behind the muscle precursors. The muscle precursors may be providing guidance cues to these phrenic axons (Allan and Greer 1997; Allan and Greer 1998).

Congenital diaphragmatic hernia can form in the absence of lung tissue, the embryological origin of the defect is hypothesised to originate from a failure of the mesenchymal substratum to form properly (Babiuk and Greer 2002; Clugston, Klattig et al. 2006; Clugston, Zhang et al. 2010). The presence of a thickening of muscle fibres around the diaphragmatic hernia is consistent with the hypothesis that the number of migrating muscle precursor cells is not reduced. Those muscle precursor cells destined to populate the area of the defect migrate round the defect and differentiate causing the thickened muscular rim (Allan and Greer 1997; Gallot, Marceau et al. 2005). This thickening surrounding the defect has also been identified in human cases of congenital diaphragmatic hernia at post mortem and on magnetic resonance imaging on infants surviving the perinatal period (Clugston, Klattig et al. 2006).

1.2.2 Transcription Factors and Pathways Implicated in Diaphragm Development

1.2.2.1 Retinoic Acid Signalling Pathway

Retinoic acid is a small lipophilic molecule derived from Vitamin A. Unlike protein factors such as the fibroblast growth factors or the transforming growth factors that bind to cell surface receptors and initiate cell signalling pathways, retinoic acid enters the cell nucleus and directly binds to target genes via nuclear receptors (Duester 2008). The first connection between retinoids, diaphragm development and congenital diaphragmatic hernia resulted from the observation that rats fed a diet deficient in vitamin A had offspring with a 25% to 40% incidence of congenital diaphragmatic hernia. The incidence of congenital diaphragmatic hernia in these offspring reduced if vitamin A was reintroduced into the diet mid-gestation (Warkany and Schraffenberger 1946; Wilson, Roth et al. 1953).

The conversion of retinoic acid from vitamin A is an enzymatic conversion of the alcohol form of vitamin A (retinol) to an aldehyde (retinaldehyde) and then to a carboxylic acid (retinoic acid). This conversion is catalysed by several alcohol dehydrogenases and retinol dehydrogenases whose expression is widespread and overlapping (Ang, Deltour et al. 1996; Zhang, Chen et al. 2001; Sandell, Sanderson et al. 2007). The developing diaphragm expresses proteins associated with retinoids (Bavik, Ward et al. 1997; Mey, Babiuk et al. 2003). Retinoic acid receptor α and β double null mutant mice have congenital diaphragmatic hernias similar to those observed after nitrofen exposure (Mendelsohn, Lohnes et al. 1994; Lohnes, Mark et al. 1995).

The concentration of dietary vitamin A must be within a very narrow range to avoid either toxicity or deficiency. Early studies indicated that retinoic acid was essential for the embryological development of several organs including the hindbrain, spinal cord, heart, forelimb buds, lung, pancreas, diaphragm and genitourinary tract (Dersch and Zile 1993; Lohnes, Mark et al. 1994; Mendelsohn, Lohnes et al. 1994; Dickman, Thaller et al. 1997; Clagett-Dame and DeLuca 2002). In a small hospital based study human infants with congenital diaphragmatic hernia were demonstrated to have reduced levels of retinol and of retinol binding protein compared to infants without anomalies (Major, Cadenas et al. 1998). Maternal retinol and retinol binding protein status was no different between the mothers of affected or normal infants in a later study (Beurskens, Tibboel et al. 2010). This

study demonstrates that a perturbation of retinoid signalling and congenital diaphragmatic hernia observed in animal models is also implicated in the pathogenesis of human congenital diaphragmatic hernia (Beurskens, Tibboel et al. 2010).

Goumy and colleagues proposed that Stimulated by retinoic acid gene 6 homolog (*STRA6),* Lecithin retinol acyltransferase (*LRAT),* Cellular retinol binding protein-1 (*CRBP1),* Cellular retinol binding protein-2 *CRBP2* and Cellular retinoic acid binding protein-1 (*CRABP1)* are directly implicated in retinoic acid metabolism and are potential candidate genes for cases of congenital diaphragmatic hernia detailed below. There has been a total of more than twenty retinoic acid pathway genes proposed, including the well studied *COUP-TF11, FOG2* and *GATA4* detailed below. They are implicated in diaphragmatic development and are given in more detail later (Gremeau, Coste et al. 2009; Goumy, Gouas et al. 2010).

Stimulated by retinoic acid gene 6 homolog *(STRA6)* is the receptor for retinol binding protein and is responsible for the transport of retinol to specific sites (Kawaguchi, Yu et al. 2007). Mutations in *STRA6* are associated with Matthew-Wood syndrome (Golzio, Martinovic-Bouriel et al. 2007; Pasutto, Sticht et al. 2007).

Lecithin retinol acyltransferase *(LRAT)* is an enzyme that catalyses the esterification of all-trans-retinol into all-trans retinyl ester for storage in the cytoplasm (Ruiz, Winston et al. 1999).

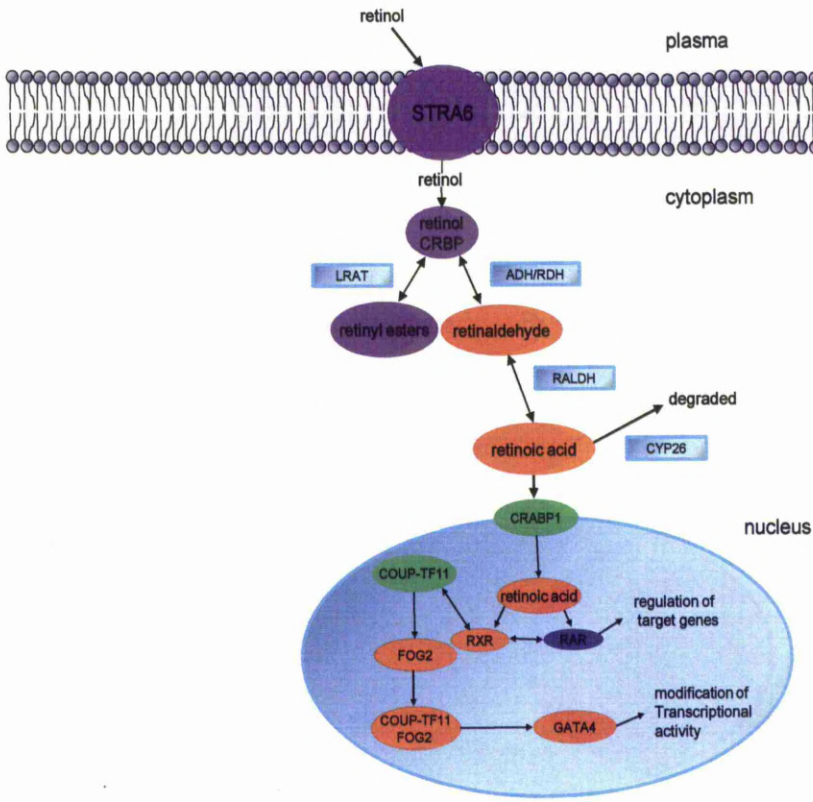
Cellular retinol binding proteins 1 and 2 *(CRPB1 and CRPB2)* are the carrier proteins involved in the transport of retinol (Herr and Ong 1992; Noy 2000).

Cellular retinoic acid binding protein 1 *(CRAPB1)* assists the entry of retinoic acid into the nucleus. Retinoic acid binds to the nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR), and this binding activates the transcription of many target genes (Goumy, Gouas et al. 2010).

Chicken ovalbumin upstream promoter factor 11 *(COUP-TF11)* inhibits the retinoic acid pathway by affecting the RAR/RXR heterodimer, inhibiting gene expression. *COUPTF-11* interacts with friend of *GATA2 (FOG2)* modulating the activity of *GATA4* proteins a transcription factor that has an important role in the embryological development of many organs (Goumy, Gouas et al. 2010).

Figure 3 Retinoic acid pathway

The alcoholic form of Vitamin A, retinol is carried in the plasma by retinol binding protein (RBP). Retinol enters the cell via a specific receptor, Stimulated by retinoic acid gene 6 homolog (STRA6) which is responsible for the transport of retinol to specific sites. In the cell cytoplasm retinol may be converted to retinyl esters for storage facilitated by cellular retinol binding protein (CRPB) or oxidised to retinaldehyde by either alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH). Retinaldehyde is oxidised to retinoic acid (RA) by retinaldehyde dehydrogenase (RALDH). In cells that are not RA target cells RA is degraded by CYP26 to hydroxy RA and excreted. RA target cells express cellular retinoic acid binding protein 1 (CRABP1) that transports the RA into the cell nucleus. In the nucleus RA binds with the retinoid X receptor (RXR) and the retinoic acid receptor (RAR) heterodimer regulating the transcription of RA target genes. This transcription is modulated by chicken ovalbumin upstream promoter factor 11 (COUPTF11) by repressing RXR thereby inhibiting the RAR/RXR heterodimer. COUPTF11 interacts with FOG2 modulating the transcriptional activity of GATA4 proteins which plays an important role in early embryogenesis



1.2.2.2 Paired box 3 *(PAX3)* Formally Known as Splotch

Paired box 3 *(pax3)* deficient splotch mice display an array of neural crest related abnormalities including congenital heart disease. Transgenic Splotch mice have an absence of a muscular diaphragm, having only a thin fibrous membrane which prevented the abdominal organs from entering the thorax (Li, Liu et al. 1999). *Pax3* is expressed in the muscle precursor cells in the pleuroperitoneal fold.

1.2.2.3 Friend of GATA Binding Protein 2, (*FOG2)*

GATA binding protein transcription factors are a family of transcription factors characterised by their ability to bind to the DNA sequence GATA (Ko and Engel 1993). Friend of *GATA2 (FOG2)* is a transcription cofactor for the GATA family of transcription factors. The interactions of *fog2* with *GATA4* in vivo are necessary for normal lung, diaphragm, cardiac and gonadal development (Crispino, Lodish et al. 1999; Tevosian, Albrecht et al. 2002; Ackerman, Wang et al. 2007; Jay, Bielinska et al. 2007). *Fog2* is a zinc finger transcription factor and these are small protein structural motifs that include one or more zinc ions to help stabilise their folds. *FOG2* is the first gene implicated in the pathogenesis of non-syndromic congenital diaphragmatic hernia (Ackerman, Herron et al. 2005), and the first gene recognised to be necessary for the primary development of both lung and diaphragm. *Fog2* is expressed in the pleuroperitoneal tissue and mice carrying a homozygote hypomorphic mutation in *fog2* have abnormal patterns of muscularisation and no delineated central tendon. These mice may survive until late gestation (Ackerman, Herron et al. 2005) but *fog2* null mice die from cardiac defects before diaphragm development (Tevosian, Deconinck et al. 2000).

A *fog2* mutation was created in mice by Ackerman and colleagues (Ackerman, Herron et al. 2005) that displayed pulmonary hypoplasia and a thin diaphragm. They called the mutation little lung *(lil)*. Diaphragms from the *lil* mice have a defect in the muscular patterning of the diaphragm similar to mice with defects in hepatocyte growth factor expression, which is a candidate factor responsible for the guidance of the muscle precursor cells (Dietrich, Abou-Rebyeh et al. 1999). *Fog2* does not colocalise with *pax3*, as it is expressed in the mesenchyme of the pleuroperitoneal fold, and *pax3* is expressed in the muscle precursor cells. *Fog2* does however colocalise with *GATA4* (Clugston, Zhang et al. 2008) Both *fog2* and *GATA4* expression has been observed in the nuclei of cells throughout the pleuroperitoneal fold of rat embryos at 13.5dpc. This pattern resembles that previously observed in mice (Ackerman and Greer 2007). *GATA4* is a member of the zinc finger

transcription factors and regulates genes involved in embryogenesis and myocardial differentiation and function. Mutations of this gene have been associated with congenital heart defects and reproductive defects (Crispino, Lodish et al. 2001; Tevosian, Albrecht et al. 2002; Ackerman, Wang et al. 2007).

1.2.2.4 GATA Binding Protein 4 *(GATA4)*

The GATA binding proteins are a family of zinc finger domain containing transcription factors which recognise the DNA sequence (A/T)GATA(A/G) in target genes (Patient and McGhee 2002). *GATA4* co-localises with *fog2* (Clugston, Zhang et al. 2008), and is a transcription factor that interacts with *fog2* and this interaction is necessary for the development of many organs including the heart, gonads and lungs (Crispino, Lodish et al. 2001; Tevosian, Albrecht et al. 2002; Ackerman, Wang et al. 2007). 30% of mice heterozygous for a mutant allele of *GATA4* have herniation of liver through a central diaphragmatic defect (Jay, Bielinska et al. 2007). Whether *GATA4* mediated diaphragm development is *fog2* dependent as in other organs is not as yet known, as early embryonic lethality makes the role of these factors difficult to establish (Ackerman and Greer 2007). Mutations with *GATA4* have been associated with congenital heart malformations in humans but to date an association with human congenital diaphragmatic hernia has not been proved (Pober, Russell et al. 1993) updated 2010.

1.2.2.5 Wilms Tumour 1 (*WT1*)

The Wilms Tumour 1 *(WT1)* mouse model was developed to study the role of *WT1* in urogenital development and the diaphragmatic defects were an incidental finding (Kreidberg, Sariola et al. 1993). *WT1* is expressed in the pleuroperitoneal fold of humans (Pritchard-Jones, Fleming et al. 1990) and is known to be essential for normal diaphragm development (Kreidberg, Sariola et al. 1993; Clugston, Klattig et al. 2006). Posterior diaphragmatic defects and other multiple anomalies associated with mutations of *WT1* and were first reported in 1993 in mice confirming a requirement of *WT1* for the development of multiple organs (Kreidberg, Sariola et al. 1993). Clugston and colleagues also reported that the structure of the pleuroperitoneal fold in *WT1* null mice was found to be abnormal (Clugston, Klattig et al. 2006), but early embryonic lethality has prevented detailed analysis of the diaphragmatic phenotype.

1.2.2.6 Slit3

Slit3 is one of the three human homologs of the drosophila Slit gene. During embryonic development in mice *slit3* is expressed in the mesothelium of the diaphragm (Yuan, Rao et al. 2003). The *Slit3* knockout mouse models demonstrate anterior hernias which extend into the anterior portion of the central tendon; this may be due to a defect in the septum transversum (Liu, Zhang et al. 2003; Yuan, Rao et al. 2003). This type of congenital diaphragmatic hernia is relatively rare and no human cases have been identified with *Slit3* mutations (Holder, Klaassens et al. 2007).

1.2.2.7 Chicken Ovalbumin Upstream Promoter Factor 11 *(COUP-TF11)*

Chicken ovalbumin upstream promoter factor 11 *(COUP-TF11)* is a member of the nuclear receptor super family and essential for normal embryonic development. It is a transcription factor in the retinoic acid / thyroid hormone / steroid receptor pathway and its expression has been shown to be regulated by retinoids. *COUP-TF11* regulates gene transcription by regulating retinoic acid receptors RAR and RXR (Qiu, Krishnan et al. 1996; Shibata, Nawaz et al. 1997; Tsai and Tsai 1997).

In mice *coup-tf11* is found in the nuclei of cells throughout the pleuroperitoneal fold and in the mesenchyme of the adjacent lung. *Coup-tf11* does not co-localise with *pax3* in the pleuroperitoneal fold but does co-localise with *WT1* in the mesenchyme of the pleuroperitoneal fold (Clugston, Klattig et al. 2006; Clugston, Zhang et al. 2008). Mouse embryos null for *coup-tf11* die before diaphragm formation (Pereira, Qiu et al. 1999) but a conditional deletion mouse model has allowed for the discovery of a role in posterior diaphragm development (You, Takamoto et al. 2005). The proteins *fog2* and *coup-tf11* have been shown interact physically (Huggins, Bacani et al. 2001) and it is possible these proteins work together to influence the expression of downstream genes important in diaphragm development (Scott 2007).

The gene *COUP-TF11* is located on chromosome15q26.2 which has recently been discovered to be a cytogenetic hotspot for congenital diaphragmatic hernia (Pober 2007; Pober 2008). *COUP TF11* is believed to play a role in human diaphragmatic defects associated with multiple birth defects, syndromic congenital diaphragmatic hernia.

1.2.2.8 Basic Helix-Loop-Helix *(bHLH)*

Numerous basic helix-loop-helix *(bHLH)* transcription factors have been identified which control cell fate, differentiation and morphogenesis during development (Jan and Jan 1993; Kadesch 1993). Several *bhlh* factors are implicated in the development of the diaphragm in mice, including capsulin, (also known as pod-1 and epicardin) which is expressed in the primordial diaphragm and in the mesenchymal cells of other developing organs including the lung and testis (Lu, Richardson et al. 1998). Capsulin appears to function as a negative regulator of differentiation by repressing transcription (Funato, Ohyama et al. 2003). Mice homozygous for a capsulin null mutation have pulmonary, cardiac, splenic and renal anomalies and male to female sex reversal (Lu, Chang et al. 2000; Cui, Ross et al. 2004). Mice homozygous for both capsulin and another *bhlh* factor *myoR* (myogenic repressor) mutation also have congenital diaphragmatic hernia (Lu, Bassel-Duby et al. 2002). Mice homozygous for the myogenic differentiation factor 1 *(MyoD)* mutation, another *bhlh* factor have abnormal muscularisation of the diaphragm (Inanlou, Dhillon et al. 2003). *MyoR* antagonises the actions of *myoD.* (Lu, Webb et al. 1999). No human cases of congenital diaphragmatic hernia have been reported with these mutations.

1.2.2.9 Slit2 and Roundabout *(ROBO) 1, 3 and 4*

Slit2 and *Robo1* are a ligand receptor pair involved in cell migration. Congenital diaphragmatic hernia, cardiac and renal defects are associated with *Slit* deficiency in mice (Liu, Zhang et al. 2003). *Slit-Robo* signalling complex involves heparan sulphate (Hussain, Piper et al. 2006). Mutations in the heparan sulphate proteoglycan, glypican-3 cause congenital diaphragmatic hernia in humans as does duplication of a region of chromosome, which includes *ROBO3* and *ROBO4* (Klaassens, Scott et al. 2006).

1.2.2.10 Glypican-3

Glypican-3 regulates the activities of many growth factors that play a critical role in morphogenesis including fibroblast growth factors, bone morphogenic proteins, guidance molecules and growth factor antagonists (Paine-Saunders, Viviano et al. 2000; Paine-Saunders, Viviano et al. 2002; Hussain, Piper et al. 2006). Loss of function mutations in the glypican-3 gene causes congenital diaphragmatic hernia, facial, skeletal and cardiac abnormalities and a predisposition to embryonal tumours including Wilms tumour (Enns, Cox et al. 1998; DeBaun, Ess et al. 2001). Loss of

function mutations in the gene encoding glypican-3 causes most cases of Simpson-Golabi-Behmel syndrome (Pober, Russell et al. 1993; Enns, Cox et al. 1998; Slavotinek 2007).

1.2.2.11 Notch

Notch signalling is involved in boundary formation and cell fate by mediating short range interactions (Bielinska, Jay et al. 2007). Defects in the Notch signalling pathway cause spondylocostal dysostosis, a syndrome with aberrant mesodermal patterning. Congenital diaphragmatic hernia is one of the anomalies accompanying this syndrome (Lam, Eik-Nes et al. 1999; Sparrow, Clements et al. 2002). Nipped-b homolog *(NIPBL)* is a gene implicated in the Notch signalling pathway and mutations of this gene cause Cornelia de Lange syndrome, a developmental disorder with genito-urinary anomalies, congenital diaphragmatic hernia and other developmental anomalies (Krantz, McCallum et al. 2004).

1.2.2.12 Ephrin-B1

Ephrins play a critical role in cell sorting during a number of developmental processes including diaphragmatic development (Davy and Soriano 2005). Mutations in the ephrin-B1 gene cause craniofacial syndrome of which congenital diaphragmatic hernia is one of the anomalies and although it is an X-linked disorder the phenotype is more severe in females (Morris, Palumbos et al. 1987; Vasudevan, Twigg et al. 2006). Ephrin-B1 is involved in the process of forming normal tissue boundaries (Twigg, Kan et al. 2004; Twigg, Matsumoto et al. 2006).

1.2.2.13 Hepatocyte Growth Factor *(HGF)*

Hepatocyte growth factor *(HGF)* is produced in the pleuroperitoneal fold and mesenchymal cells including those of the lung, and its tyrosine kinase receptor *c-Met* is expressed in myoblast precursors. In both *hgf* and *c-met* null mice the diaphragmatic substratum forms normally but due to defects in myoblast migration the muscularisation of the diaphragm is impaired (Bladt, Riethmacher et al. 1995; Dietrich, Abou-Rebyeh et al. 1999; Sachs, Brohmann et al. 2000). *Hgf* may be one of the target genes for *fog2* as *fog2* null mice have reduced expression of *hgf* in the pleuroperitoneal fold. (Ackerman, Herron et al. 2005). In the mouse *hgf* signalling has also been linked to testicular differentiation (Ricci, Catizone et al. 1999) but it is unclear if genes encoding *HGF* or *c-Met* cause congenital diaphragmatic hernia or testicular defects in humans (Bielinska, Jay et al. 2007).

1.2.3 Extra Cellular Matrix Proteins Involved in Diaphragm Development

The extracellular matrix (ECM) includes the interstitial matrix and the basement membrane, and is the scaffold providing structural support for the cells, segregating tissues from one another, regulating intercellular communication and dynamic behaviour. The components of the ECM are mainly secreted by fibroblasts, the major component of the ECM scaffold being collagen of which there are many isoforms. Glycoproteins such as tenascin, laminin, fibronectin and proteoglycans are attached and interwoven with fibrinous proteins such as fibrillins and elastin.

Extra cellular matrix proteins are critical for development and abnormalities in several extra cellular proteins have been linked to global abnormalities including congenital diaphragmatic hernia and pulmonary complications (Bielinska, Jay et al. 2007).

1.2.3.1 Elastin

Human mutations in the elastin gene cause congenital diaphragmatic hernia, vascular problems, inelastic skin and joint laxity, these are all connective tissue defects and mice with homozygous mutations of the elastin gene have similar anomalies (Li, Brooke et al. 1998; Wendel, Taylor et al. 2000; Urban, Zhang et al. 2001; Szabo, Crepeau et al. 2006).

1.2.3.2 Collagen

Ehlers-Danlos syndrome is characterised by abnormal collagen metabolism resulting in skin hyper-extensibility, joint laxity, vascular problems and congenital diaphragmatic hernia (Abdul Wahab, Janahi et al. 2003; Lin, Ko et al. 2006).

1.2.3.3 Lysil Oxidase

Lysil oxidase is an enzyme that catalyzes the linking of collagen and elastin required for the structural integrity of the extra cellular matrix. Lysil oxidase null mice die from vascular problems, cardiovascular anomalies and congenital diaphragmatic hernia (Maki, Rasanen et al. 2002; Hornstra, Birge et al. 2003; Maki, Sormunen et al. 2005). No human cases of congenital diaphragmatic hernia have been reported with mutations of the lysil oxidase gene. However, a mutation in the human Copper-transporting ATPase 1 protein encoded by the ATP7A gene (involved in copper transport during the biosynthesis of lysil oxidase) causes Menkes disease. Menkes

disease is a lethal X linked disorder that has congenital diaphragmatic hernia, skin and vascular problems amongst its symptoms (Schaefer and Gitlin 1999).

1.2.3.4 Fibulins

Fibulins are proteins that serve to link elastic fibres to the surface of cells (Bielinska, Jay et al. 2007). Mutations in the human fibulin genes cause recessive cutis laxa with vascular and joint problems, congenital diaphragmatic hernia and emphysema (Loeys, Van Maldergem et al. 2002; Hucthagowder, Sausgruber et al. 2006). Mice with a mutation in the fibulin genes have a similar phenotype (Yanagisawa, Davis et al. 2002; McLaughlin, Chen et al. 2006).

1.2.3.5 Fibrillin

Mutations in the fibrillin 1 gene cause Marfan syndrome which includes tall stature, cardiovascular anomalies, joint laxity, lung abnormalities and diaphragmatic eventration amongst its features (Neptune, Frischmeyer et al. 2003; Revencu, Quenum et al. 2004; Robinson, Arteaga-Solis et al. 2006).

1.3 Pulmonary Development and Pulmonary Complications

Congenital Diaphragmatic Hernia is inextricably linked to pulmonary hypoplasia, pulmonary hypertension and lung development.

1.3.1 Normal lung development

1.3.1.1 Stages of Lung Development

Normal lung development in the human begins during the fourth week of gestation when the lung bud forms as a ventral outgrowth of the foregut and continues until early childhood when it is considered to be complete (Boyden 1977). This can be divided into five stages (Zeltner and Burri 1987).

1. The embryonic stage, from fertilization until approximately 7 weeks.

2. The pseudoglandular stage, 5-17 weeks, by the end of this stage all major elements have formed except those involved with gas exchange.

3. The canalicular stage, (Zeltner, Caduff et al. 1987)16-25 weeks, the lumens of the bronchi and terminal bronchioles have become larger and the lungs vascularised. By week 24 respiratory airways have formed and respiration is possible, although the chances of survival are slim.

4. The saccular stage, more terminal sacs appear and the capillaries develop a close relationship with them. The sacs are lined with type 1 alveolar cells or type 2 pneumocytes which secrete surfactant counteracting the surface tension forces and facilitating expansions of the terminal sacs. The surfactant usually reaches adequate levels two weeks before birth.

5. The alveolar stage, late fetal period until a few years after birth, a newborn infant has only a small proportion of the alveoli of an adult. 95% of alveoli develop after birth.

Among different species the timing and duration of these stages may overlap.

1.3.1.2 Early Embryonic Development of the Lung and the Relationship of Animal Models to Human Lung Development

Although the development of the human lung has been studied, most of our understanding of lung embryology and growth has come from animal models due to their accessibility and availability. The creation of knock out, knock in and

teratogenic models has permitted the study of the roles of genes, transcription factors and other factors such as the retinoids involved in the development and branching morphogenesis of normal lung. The expression patterns of these factors are well preserved between species including invertebrates, even though the timing of the mechanisms may differ slightly (Ghabrial, Luschnig et al. 2003). This allows for a cautious translation of these factors to study the development of the normal human lung, as required by the organism for a continuous and adequate oxygen supply after birth.

The lung is an appendage of the foregut; some of the key transcription factors essential for the formation and maintenance of the foregut are also important in lung development. These include Forkhead box gene A1 *(FoxA1),* Forkhead box gene A2 *(FoxA2), GATA4* and *GATA6* (Wan, Xu et al. 2004; Capo-Chichi, Rula et al. 2005; Wan, Dingle et al. 2005). As a number of organs bud at specific times from the foregut there must be some signalling mechanism that allows each cell to know its relative position. This could include signals from the surrounding structures or a morphogenic gradient along the foregut tube such as bone morphogenic protein 2 (Tiso, Filippi et al. 2002) and retinoic acid (Stafford and Prince 2002; Stafford, White et al. 2006). There is evidence that both signalling mechanisms and gradients may be involved in this process. Embryos treated with BMS493, an inverse pan-retinoic acid receptor agonist, had absent pancreas and liver markers. However, the structures derived from the anterior region of the foregut, the thyroid and lungs were unaffected (Stafford and Prince 2002) indicating a gradient (Kinane 2007). Bone morphogenic proteins via *GATA4* and fibroblast growth factors from the primitive heart are critical for the localisation of foregut derived organs (Rossi, Dunn et al. 2001). Using lung organ culture Serle and colleagues found that lung specification is induced by adjacent cardiac mesoderm but that it is replaceable by fibroblast growth factors 1 and 2 (Serls, Doherty et al. 2005). Morphogen gradients are critical for the induction of the lung. Retinoic acid, sonic hedgehog, fibroblast growth factors 1 and 2 and bone morphogenic protein 4 are known to define cell position in the foregut. These signalling molecules are highly conserved and are used frequently during fetal development with different effects. The understanding of the signals required for lung bud initiation are incomplete and involve many factors but Sonic hedgehog is likely to be critical for the lung cascade and fibroblast growth factors are a probability (Kinane 2007).

1.3.2 Factors Involved in Lung Development

1.3.2.1 Retinoic Acid Signalling Pathway

Mechanisms controlling retinoic acid signalling are critical for normal branching morphogenesis of the embryonic lung, and retinoic acid signaling is detectable in the lung from the very beginning of organogenesis (Wilson, Roth et al. 1953; Malpel, Mendelsohn et al. 2000).

The lung requires retinoic acid for organogenesis (Chytil 1996; Morriss-Kay and Sokolova 1996; Massaro and Massaro 1997). Retinoic acid from the splanchnic mesoderm surrounding the endoderm is required for stimulating the posterior foregut endoderm to a lung fate in the mouse (Malpel, Mendelsohn et al. 2000). In retinoic acid deficient mouse embryos the primary lung bud is specified but does not express the retinoic acid inducible homeobox A5 *(hoxa5)* gene. It is also unable to achieve outgrowth or branching morphogenesis. This results in a lack of fibroblast growth factor 10 *(fgf10)* expression and signalling in the lung epithelium. However lung budding and branching can be restored by treatment with *fgf10* in these retinoic acid deficient embryos (Wang, Dolle et al. 2006).

At the onset of lung development abundant retinoic acid signalling is activated in primary lung buds. Further airway branching requires downregulation of retinoic pathways in a proximal distal gradient with less retinoic acid expression in the mesenchyme near the sites of terminal budding (Mollard, Ghyselinck et al. 2000). This decreased response can be ascribed to decreased levels of retinaldehyde dehydrogenase 2 limiting ligand availability, and an increase in chicken ovalbumin upstream promotor-transcription factor 11 *(coup-tf11)* ((Jonk, de Jonge et al. 1994) that antagonises retinoid signalling (Malpel, Mendelsohn et al. 2000). Manipulating retinoic acid levels to maintain the high levels of retinoic acid signalling present during the onset of lung development, leads to an immature lung phenotype with absent formation of typical distal buds (Cardoso, Williams et al. 1995). This alters the levels and distribution of *fgf10* and bone morphogenic protein 4 *(bmp4)* genes that are essential for distal lung formation (Malpel, Mendelsohn et al. 2000). High levels of retinoic acid stabilise proximal tubule formation (Chazaud, Dolle et al. 2003). If retinoic acid signalling is blocked by BMS483 a pan retinoic acid receptor antagonist, *fgf10, bmp4,* Sonic hedgehog *(Shh)*, thyroid transcription factor *1 (ttf1) and GATA6* expression are all increased. This results in increased branching (Chazaud, Dolle et al. 2003; Wongtrakool, Malpel et al. 2003).

Loss of retinoic acid signalling in the foregut results in upregulation of transforming growth factors *(tgf) β1* and *β2* target genes. Exogenous administration of retinoic acid in vitro upregulates forkhead box gene A2 *(foxa2)* and *tgfβ* both inhibitors of branching (Chazaud, Dolle et al. 2003; Wongtrakool, Malpel et al. 2003). Treatment of wild type embryos with exogenous *tgfβ1* reproduces the lung bud defect seen in the retinoic acid deficient embryos (Chen, Desai et al. 2007). These data support a novel mechanism of *tgfβ, foxa2,* retinoic acid and *fgf10* interactions in the developing foregut. Endogenous retinoic acid controls *tgfβ* activity in the prospective lung field, allowing the *fgf10* expression necessary for the induction of lung bud formation and subsequent branching morphogenesis. (Chen, Desai et al. 2007).

Retinaldehyde dehydrogenase-2 deficient embryos can be rescued from early lethality by maternal dietary retinoic acid between 7.5 to 8.5 dpc but they fail to develop lungs and lack retinoic acid signalling in the foregut. (Chen, Desai et al. 2007).

1.3.2.2 Fibroblast Growth Factor 9 *(FGF9)*

Fibroblast growth factor 9 *(FGF9)* has roles in both the growth and branching of the lung and the development of the vascular system. Vascular development matches branching morphogenesis from 10.5dpc in the mouse forming a dense capillary network surrounding the distal epithelium (Gebb and Shannon 2000; Schachtner, Wang et al. 2000; Parera, van Dooren et al. 2005). The mesothelial cells are the source of the vascular smooth muscle cells in the developing lung (Que, Wilm et al. 2008). *Fgf9* and *shh* regulate vascular endothelial growth factor A *(vegfa)* expression, essential for the formation of the pulmonary vasculature and for epithelial branching morphogenesis; *Fgf9* null mice embryos have defective blood vessel development (White, Lavine et al. 2007). *Fgf9* has a role in suppressing cell differentiation; embryonic lung explants treated with *fgf9* inhibit *shh* induced smooth muscle differentiation (Weaver, Batts et al. 2003; Yi, Domyan et al. 2009). *Fgf9* null mice have severe lung hypoplasia and reduced mesenchyme caused by a reduction in mesenchymal proliferation at 10.5 to 11.5dpc. They also have reduced branching of epithelial tubules This may be due to *fgf9* interacting with *fgf10* leading to reduced *fgf10* expression in the mesenchyme of the actively branching areas of the lung, the terminal buds (Colvin, White et al. 2001).

1.3.2.3 Fibroblast Growth Factor 10 *(FGF10)*

As well as early lung bud specification as previously discussed *fgf10* has a significant role in the branching and development of the lung. *FGF10* is required by the lung for the formation of lung buds and therefore for branching morphogenesis, *fgf10* null mice have no lung development but do have a diaphragm. Retinoic acid acts upstream of *fgf10* signalling in the developing lung and stomach (Wang, Dolle et al. 2006). *Fgf10* is expressed in the distal mesenchyme at the budding tip and acts as a chemoattractant for the development of a new bud (Park, Miranda et al. 1998).

From lung culture studies it has been shown that *fgf10, fgf1* and *fgf7* have overlapping capacities to stimulate lung growth and branching morphogenesis (Bellusci, Grindley et al. 1997; Cardoso, Itoh et al. 1997).

1.3.2.4 Sonic Hedgehog *(SHH)*

Sonic hedgehog *(SHH)* is essential for functional lung formation and is expressed in all pulmonary epithelial cells, the highest expression at the tips of the endbuds (Bellusci, Furuta et al. 1997; van Tuyl and Post 2000; Shannon and Hyatt 2004). With its receptor patched *(ptc) shh* acts as a morphogen and is one of the factors involved in region specific morphogenesis and initiation of organ specific developmental programs (Shannon and Hyatt 2004; Lu, Izvolsky et al. 2005). Overexpression of *Shh* shows increased mesenchymal and epithelial proliferation leading to an excess of mesenchyme (Chuang, Kawcak et al. 2003), (Bellusci, Furuta et al. 1997).

Shh is a regulatory factor in branching morphogenesis and affects the differentiation of the peripheral lung mesenchyme towards the smooth muscle lineage (Miller, Wert et al. 2004). *Shh* null mice have abnormal lung phenotype, lack of asymmetry, hypoplastic lobes, undivided oesophagus and trachea, reduced mesenchymal proliferation and greatly reduced branching (Pepicelli, Lewis et al. 1998).

1.3.2.5 Bone Morphogenic Protein 4 *(BMP4)*

Bone morphogenic protein 4 *(BMP4)* is expressed in the distal epithelium where it promotes distal epithelial and mesenchymal development. It also has a role in specifying smooth muscle precursors and antagonising *FGF10* mediated epithelial budding (Bitgood and McMahon 1995; Bellusci, Henderson et al. 1996; Weaver, Dunn et al. 2000; Mailleux, Kelly et al. 2005). Interactions between mainly *FGF9,*

FGF10, SHH and BMP4 control the budding necessary for branching morphogenesis (Weaver, Yingling et al. 1999; Malpel, Mendelsohn et al. 2000).

1.3.2.6 Thyroid Transcription Factor1 *(TTF1)* or (more recently known as *NKX-2-1*)

Thyroid transcription factor-1 *(TTF1)* is a protein which is encoded by the NK2 homeobox 1 *(NKX2-1)* gene and expressed in the thyroid and forebrain during development (Lazzaro, Price et al. 1991; Lee, Cho et al. 2001; DeFelice, Silberschmidt et al. 2003). *Ttf1* is detected early in the endodermal cells of the lung anlage and may have a role in cell determination (Lazzaro, Price et al. 1991). During development *ttf1* expression is localised to the epithelial cells of the branching lung buds and the expression decreases in the proximal airways as the lung grows. It is almost absent in the post natal lung apart from type 11 cells (Stahlman, Gray et al. 1996; Morotti, Gutierrez et al. 2000). *Ttf1* knockout mice have only a rudimentary bronchial tree without branching and no distal parenchyma but overexpression of *ttf1* has no effect on branching morphogenesis. It does however reduce alveolarisation (Wert, Dey et al. 2002).

1.3.2.7 Transforming Growth Factor β *(TGFβ)*

The transforming growth factor β *(Tgfβ)* family both influence lung development and are involved in lung disease. There are three isoforms of *Tgfβ* all expressed at high levels during lung development and are particularly important for branching morphogenesis, epithelial cell differentiation and surfactant synthesis (Bartram and Speer 2004). *Tgfβ* is also involved in tissue repair following lung injury, chronic lung disease of prematurity and vascular remodelling in pulmonary hypertension (Bartram and Speer 2004).

Tgfβ has an inhibitory effect on branching morphogenesis by influencing the proteoglycans and metalloproteinases implicated in branching (Hildebrand, Romaris et al. 1994; Sakurai and Nigam 1997; J, Tefft et al. 1998). It also hampers the activity of *ttf1* and *foxa2* by trapping them in the cytoplasm of cells (Kumar, Gonzales et al. 2000) and interferes with other peptide growth factors implicated in branching morphogenesis (Zhao, Sime et al. 1998).

1.3.2.8 Friend of GATA2, (*FOG2)* and GATA 4

Friend of GATA2 *(FOG2)* is the first gene implicated in the pathogenesis of nonsyndromic human diaphragmatic defects and is also necessary for pulmonary development indicating this gene could be responsible for the primary pulmonary developmental abnormalities in neonates with diaphragmatic defects (Ackerman, Herron et al. 2005).

In a screen of fetal mice carrying chemically induced genetic mutations in the *fog2 gene,* abnormal diaphragm development was present and small lungs were evident. The lungs had primary morphological defects such as the specific loss of the accessory lobe on the right lung and small underdeveloped right anterior middle lobe (Ackerman, Herron et al. 2005). In normal mouse development at 11.5dpc there was an increase in expression of *fog2.* The zinc finger domain containing transcription factor *GATA4* is expressed in early mesenchymal and mesothelial cells of the lungs, great vessels, heart and diaphragm and like its co-regulator *FOG2* is expressed in the early embryonic development of these organs. The expression and activity of *GATA4* is influenced by retinoids (Arceci, King et al. 1993; Kostetskii, Jiang et al. 1999; Clabby, Robison et al. 2003; Ghatpande, Brand et al. 2006). Expression pattern indicates *GATA4* plays a functional role in organogenesis in the organs in which it is expressed (Crispino, Lodish et al. 2001; Jay, Bielinska et al. 2007). Pulmonary expression of *GATA4* is restricted to mesodermal derivatives and the airway defects are probably the result of impaired signaling from the mesenchyme or mesothelium to endoderm (Jay, Bielinska et al. 2007). While *GATA4* heterozygous mice develop hernias in the ventral midline, gross inspection of the lungs mostly resembled those of the wild type mice. Some of the heterozygote mice had some dilated distal airways and thickened mesenchyme particularly around the accessory and middle lobes of the right lung (Jay, Bielinska et al. 2007). This is also the area from where the majority of lung peristaltic waves originate (Jesudason, Smith et al. 2005). In these *GATA4* heterozygous mice the production of surfactant protein and clara cell secretory protein was found to be delayed (Jay, Bielinska et al. 2007).

1.3.2.9 Slit 2, Slit 3 and Roundabout *(ROBO) 1 and 2*

Two Slit genes, *Slit-2* and *Slit-3* and two Roundabout genes *ROBO1* and *ROBO2* are expressed in the central nervous system where they act as a guidance mechanism (Whitford, Marillat et al. 2002). *Slit-Robo* interactions repulse cells away from or attract cells to specific targets (Kramer, Kidd et al. 2001). *Slit* and *Robo*

expression has been documented in non neural tissue, the developing kidneys and limbs (Piper, Georgas et al. 2000; Vargesson, Luria et al. 2001). *Slit* and *Robo* are expressed in the developing mouse lung and mostly restricted to mesodermal derivatives where they have a role in the positioning and alignment of the early vascular network and airways (Greenberg, Thompson et al. 2004). Production of smooth muscle alpha actin colocalises with *Robo2* expressing mesenchyme and the interactions may determine the position of the airway smooth cell precursors (Greenberg, Thompson et al. 2004).

1.3.2.10 Forkhead Box Gene A2 *(FOXA2)* formally Hepatocyte Nuclear Factor-3β

Members of the forkhead box family of transcription factors share homology in the winged helix binding domain and play an important role in the expression of genes (Tuteja and Kaestner 2007; Tuteja and Kaestner 2007). Early in development of the lung the expression of *foxa2* is mainly in the trachea and later restricted to distal airways, the expression pattern being very similar to that of *ttf1* (Zhou, Lim et al. 1996). *Foxa2* modulates the expression of *ttf1* and *foxa2* and also inhibits branching in the developing lung (Zhou, Dey et al. 1997; Chazaud, Dolle et al. 2003).

1.3.2.11 Transforming Growth Factor α (*TGFα)* and Epidermal Growth Factor (EGF)

These are both members of the epidermal family of growth factors and are present in bronchial and bronchiolar epithelium (Ruocco, Lallemand et al. 1996). They have a role in stimulating branching in a dose dependant manner (Warburton, Seth et al. 1992).

1.3.2.12 Wingless-Related MMTV Integration Site Family Members *(WNT)*

Wingless-related MMTV integration site family members *(WNT)* growth factor family are secreted glucoproteins which bind to a cell surface receptor and initiate signaling. *Wnt* signaling controls many embryonic processes including cell growth, migration, differentiation and fate (Cadigan and Nusse 1997; Wodarz and Nusse 1998; Roth-Kleiner and Post 2005).

Several members of the *wnt* family are expressed in the developing lung and three have been linked to airway branching (Lako, Strachan et al. 1998; Pepicelli, Lewis et al. 1998; Weidenfeld, Shu et al. 2002) as detailed below.

Wnt2 is expressed in the airway bud tip mesenchyme and may have a relationship with *Shh* but *wnt2* null mice display no lung phenotype (Bellusci, Henderson et al. 1996).

Wnt5a expression has a gradient along the airways, maximum expression is in the epithelium of the airway endbud (Li, Xiao et al. 2002). Inactivation of *wnt5a* causes shortened limbs and trachea, overexpansion of distal airways and an immature lung. Absence of *wnt5a* leads to increased expression of *fgf10, bmp4, shh* and its receptor patched *(ptc)* indicating that *wnt5a, fgf10, bmp4* and *shh* are functionally interactive (Yamaguchi, Bradley et al. 1999; Li, Xiao et al. 2002).

Wnt7b is exclusively expressed in the epithelium, maximum expression at the tip of the branching endbuds (Pepicelli, Lewis et al. 1998) and reduced function of *wnt7b* leads to reduced mesenchyme surrounding the distal airways and lung hypoplasia incompatible with life (Shu, Jiang et al. 2002). *Wnt7b* expression is regulated by *ttf1, GATA6* and *foxa2* (Weidenfeld, Shu et al. 2002). Deficiency in *wnt7b* expression also leads to severe defects in the smooth muscle component of the major pulmonary vessels. This leads these vessels to rupture after birth indicating its requirement for proper mesenchymal and vascular development (Shu, Jiang et al. 2002).

1.3.2.13 Insulin Like Growth Factors *(IGF)*, their Receptors *(IGFR)* and their Binding Proteins *(IGFBP)*

Insulin like growth factors are peptide growth factors with a high sequence similarity to insulin and are expressed in the lungs of humans, rodents and other species (Maitre, Clement et al. 1995; Schuller, van Neck et al. 1995). This system consists of two cell surface receptors, Insulin-like growth factor 1 receptor *(IGF1R)* insulin-like growth factor 2 receptor *(IGF2R),* two ligands insulin-like growth factor 1 *(IGF1)* and insulin-like growth factor 2 *(IGF2)* and a family of insulin-like growth factor binding proteins *(IGFBP)1* to *6. IGFs* and their binding proteins play a role in abnormal and normal fetal growth (Chard 1994). They are involved in growth hormone stimulation and bone development (Baker, Liu et al. 1993).

The insulin like growth factor system plays a key role in lung development by the interaction of *IGFBP3* and *IGFBP5* with the retinoid signaling pathway (Liu, Lee et al. 2000). Both *IGFBP3* and *IGFBP5* have an effect on cell growth by modulating gene expression (Ricort 2004). They are expressed in the distal airway epithelium

and mesenchyme of the developing lung, their expression increasing as gestation advances (Ruttenstock, Doi et al. 2010).

During fetal lung development insulin modulates the cellular uptake of glucose a substrate for surfactant phospholipids and may be a bioregulator of surfactant synthesis (Felts 1964).

Insulin-like growth factor binding protein 3 *(IGFBP3)* regulates apoptosis (Liu, Lee et al. 2000).

Deletions of the chromosomal site of the *IGF1R* gene in humans has been reported in several cases of congenital diaphragmatic hernia (Rubin and Baserga 1995)

1.3.2.14 Basic Helix Loop Helix *(bHLH)*

Capsulin *(Pod-1)* is a basic helix loop helix factor that is expressed in the developing lung mesenchyme. *Pod-1* knockout mice have no alveoli, hypoplastic lungs and defective branching morphogenesis (Quaggin, Schwartz et al. 1999; Maeda, Dave et al. 2007).

1.3.3 Extracellular Matrix (ECM)

As discussed previously the extracellular matrix includes the interstitial matrix and the basement membrane and is the scaffold providing structural support for the cells, segregating tissues from one another, regulating intercellular communication and dynamic behaviour. Several extra matrix molecules are involved in the process of branching morphogenesis of the lung.

1.3.3.1 Proteoglycans

Proteoglycans are proteins that have glycosaminoglycans attached to their serine residues. The proteoglycan heparan sulphate is a major component of the extra cellular matrix and is on the surface of most cells. It plays a pivotal role in cell to cell communications (Ori, Wilkinson et al. 2008). Heparan sulphate proteoglycans form tertiary structures with growth factors and their receptors, modulating the signaling of the growth factors and their receptors (Tumova, Woods et al. 2000; Kresse and Schonherr 2001).

Most extracellular proteins involved in embryonic development interact with heparan sulphate/heparin (Ori, Wilkinson et al. 2008), included are the proteins important for

lung morphogenesis (Warburton, Schwarz et al. 2000; Thompson, Jesudason et al. 2010).

Chondroitin sulphate proteoglycans provide tissue rigidity and stimulate fibroblast proliferation (Zhang, Cao et al. 1998). In vitro reduced sulfation of glycosaminoglycan reduces lung branching (Shannon, McCormick-Shannon et al. 2003). This is caused by the reduction of the binding of *fgf10* causing a loss of *fgf10* signaling, therefore reducing *fgf10* dependant branching morphogenesis (Powers, McLeskey et al. 2000). *Fgf9* and *tgfβ* and other growth factors are also similarly affected by the reduced sulfation of glycosaminoglycan (Zhao, Lee et al. 1998).

1.3.3.2 Elastin

Elastin is a major component of the mammalian lung and localises in the mesenchyme next to the airway indicating a role in airway formation (Wendel, Taylor et al. 2000). Elastic fibres are required for the proper expansion and recoil of the lungs (Schellenberg, Liggins et al. 1987). Elastin is first expressed in the extracellular matrix and associated with sites of airway branching (Wendel, Taylor et al. 2000; Mychaliska, Officer et al. 2004)

Early branching is not affected in elastin deficient mice but distal branching is impaired later in fetal development. At 18.5dpc elastin deficient mice have dilated distal air sacs. This mechanism is not at present fully understood (Wendel, Taylor et al. 2000). Elastin therefore has roles in the structure and function of the lung, essential for pulmonary development and terminal airway branching. Lungs of mice with deleted retinoic acid receptor have reduced elastin content (McGowan, Jackson et al. 2000).

1.3.3.3 Tenascin

Tenascin is a multifunctional protein located at the epithelial-mesenchymal interface of the developing lung (Zhao and Young 1995; Kaarteenaho-Wiik, Kinnula et al. 2001) and accumulates at sites where new airway branches form indicating a role in airway division (Koch, Wehrle-Haller et al. 1991). Both tenascin null mice and fetal lungs cultured with tenascin antiserum have a simplified branching pattern (Koch, Wehrle-Haller et al. 1991; Young, Chang et al. 1994).

1.3.3.4 Fibronectin

Fibronectin is also a major component of the pulmonary ECM. Inhibition of fibronectin reduces branching morphogenesis in the lungs and addition of fibronectin to lung explants increases branching (Roman, Crouch et al. 1991; Sakai, Larsen et al. 2003). Fibronectin is required for cleft formation in branching morphogenesis of the lung and induces cell to cell adhesions and cell to matrix adhesions (Sakai, Larsen et al. 2003).

1.3.3,5 Laminin

Laminins and collagen 4 together with nidogen, entactin and heparan sulphate proteoglycans build the scaffold of the basement membrane (Sannes and Wang 1997). The basement membrane acts as a barrier to some cells and molecules and serves as attachment points and binding sites for others.(Sannes and Wang 1997; Miosge 2001). The nidogen proteins may serve as a link between collagen 4 and laminin 1 stabilising the basement membrane during embryogenesis (Miosge, Holzhausen et al. 2001).

1.3.3.6 Collagen

As previously discussed collagen is the major component of the extra cellular matrix scaffold. Ehlers-Danlos syndrome, a connective tissue disorder with abnormal collagen synthesis, has been reported with pulmonary hypoplasia but this is an infrequent finding (Pradhan, Deb et al. 2009).

1.3.4 Pulmonary Complications of Congenital Diaphragmatic Hernia

1.3.4.1 Pulmonary Hypoplasia and Pulmonary Hypertension

Respiratory failure is the main cause of death in the neonatal infant with congenital diaphragmatic hernia. Lung hypoplasia is a leading contributor to the lethality of congenital diaphragmatic hernia (Clark, Hardin et al. 1998). Traditionally pulmonary hypoplasia was thought to develop after the failure of diaphragmatic closure. Proposed mechanisms for this include compression of the fetal lung by herniated abdominal viscera (Harrison, Jester et al. 1980). Pulmonary hypoplasia, vascular remodelling and pulmonary hypertension are characteristic of the surgically created fetal sheep model of congenital diaphragmatic hernia indicating compression of the lung does play a role in the pulmonary problems. The ipsilateral lung suffers more compression therefore becoming more hypoplastic (Guilbert, Gebb et al. 2000;

Jesudason, Connell et al. 2000; Keijzer, Liu et al. 2000; Acosta, Thebaud et al. 2001).

Growth competition between liver and lung (Kluth, Tenbrinck et al. 1993) and failure of fetal diaphragmatic activity to maintain lung expansion (Harding and Hooper 1996) are also implicated in these pulmonary problems. However myogenic differentiation factor1 *(MyoD)* knockout mice have a markedly thinned and none functioning diaphragm. This results in pulmonary hypoplasia due to the lack of fetal breathing movements (Inanlou, Dhillon et al. 2003; Inanlou and Kablar 2003). MyoD and myogenic factor5 *(Myf5)* play a crucial role in muscularisation More recently findings from experimental models have suggested lung hypoplasia may also precede diaphragmatic closure (Iritani 1984; Kluth, Tenbrinck et al. 1993; Jesudason, Connell et al. 2000; Keijzer, Liu et al. 2000).

Unlike most other causes of respiratory failure in the newborn a significant proportion of infants with congenital diaphragmatic hernia do not respond to modern therapeutic interventions such as exogenous surfactant, high frequency oscillatory ventilation and inhaled nitric oxide. With the failure of conventional ventilator support extra-corporeal membrane oxygenation may be applied in some centres but this is highly invasive, labour intensive and expensive. Extra-corporeal membrane oxygenation bypasses the lung allowing pulmonary vascular resistance to lower but does not overcome the basic problem of lung hypoplasia and studies do not suggest benefit in survival. The mortality rate amongst children with congenital diaphragmatic hernia is 40% to 50%, mainly caused by the pulmonary hypoplasia and pulmonary hypertension. Morbidity remains significant in many cases, with chronic oxygen dependence, gastroesophageal reflux, poor growth, developmental delay, prolonged and frequent hospitalisation are all features of infants surviving with congenital diaphragmatic hernia (Smith, Jesudason et al. 2002).

The hypoplastic lungs of newborns with congenital diaphragmatic hernia show variable defects in airway branching, epithelial differentiation and alveogenesis (Kitagawa, Hislop et al. 1971). Fibroblast growth factors, transcription factors and heparin sulphate proteoglycans are morphogens involved in these specific stages of lung development (Warburton and Lee 1999).

It is well established that increased distension of the fetal lung accelerates lung growth and that reduced distension of the fetal lung decreases lung growth (Alcorn, Adamson et al. 1977).

This consequence of impaired lung development in these infants has focused research on the developmental biology of the lungs and diaphragm to identify early therapeutic interventions. Pulmonary hypoplasia is now considered to be a primary defect and not secondary to the congenital diaphragmatic hernia (Keijzer, Liu et al. 2000), therefore it is important that the interplay of factors involved in lung development are characterised. A fundamental intervention to correct the pulmonary hypoplasia and pulmonary hypertension may be necessary to have a significant input on the outlook for these infants (Smith, Jesudason et al. 2002).

1.3.5 Management of Congenital Diaphragmatic Hernia and Pulmonary Complications

1.3.5.1 Prognosis of Congenital Diaphragmatic Hernia

The prognosis of neonates born with congenital diaphragmatic hernia remains unsatisfactory despite advances in surgical and medical treatment. The degree of pulmonary hypoplasia and persistent pulmonary hypertension are the main factors that influence prognosis in isolated congenital diaphragmatic hernia (Boloker, Bateman et al. 2002) and are a significant factor in complex congenital diaphragmatic hernia that is with associated anomalies. The outcome for infants with accompanying cardiovascular malformations, comprising between 10-35% of cases, is very poor (Fauza and Wilson 1994; Graziano 2005).

For those infants with genetic associations include trisomies 13 and 18, syndromes such as Fryns, Coffin-Siris and Denys-Drash, survival is very poor with mortality as high as 93%, many succumbing during the antenatal period (Witters, Legius et al. 2001).

1.3.5.2 Antenatal Diagnosis of Congenital Diaphragmatic Hernia

Diagnosis of congenital diaphragmatic is increasingly reported on antenatal ultrasound scans, confirmed by the presence of loops of bowel or stomach within the thoracic cavity. This diagnosis should lead to a careful search for associated anomalies such as cardiac and neural tube defects (Smith, Jesudason et al. 2002). However it has been shown that antenatal ultrasound diagnoses only approximately 50% of cases (Adzick, Vacanti et al. 1989; Dillon, Renwick et al. 2000; Detti, Mari et al. 2001; Garne, Haeusler et al. 2002; Thilaganathan 2002). Early diagnosis provides an opportunity for parental counselling by the clinical team to discuss

prognosis and if required formulate a plan for delivery, preferably elective at an obstetric centre with available surgical expertise and medical support.

For isolated congenital diaphragmatic hernia defining accurate antenatal prognostic features has proved challenging. The major factor determining outcome is the degree of pulmonary hypoplasia, therefore estimation of lung growth would seem the logical prognostic indicator. Prognostic features including early diagnosis, polyhydramnios and the presence of an intrathoracic stomach bubble have been equated with poor prognosis but have not been found to be consistently reliable (Adzick, Harrison et al. 1985; Nakayama, Harrison et al. 1985; Adzick, Vacanti et al. 1989; Burge, Atwell et al. 1989). Lung head ratio has been used as a prognostic indicator, using a ratio of right lung diameter to head circumference and can predict outcomes at the extreme end of the scale, a value of <0.6 correlating with 100% mortality (Lipshutz, Albanese et al. 1997; Sbragia, Paek et al. 2000). The lung head ratio is a less reliable prognostic indicator in the mid ranges. Fetal magnetic resonance imaging has been used as a three dimensional estimation of lung growth and initial reports are encouraging (Paek, Coakley et al. 2001).

1.3.5.3 Postnatal Diagnosis of Congenital Diaphragmatic Hernia

Postnatal diagnosis is usually made during the first few minutes of life, signs of congenital hernia include respiratory distress, a scaphoid abdomen and a mediastinal shift away from the side of the lesion.

1.3.5.4 Surgical Repair of Congenital Diaphragmatic Hernia

Surgical repair of neonatal congenital diaphragmatic hernia has remained with little variation in operative techniques. The timing of surgery has now changed favouring delayed surgery.

Failure of postnatal therapies to significantly improve the prognosis for children with congenital diaphragmatic hernia prompted paediatric surgeons to consider fetal surgery to repair the hernia. To address these hypothesis surgeons created a lamb model of congenital diaphragmatic hernia simulating antenatal closure of the defect improving survival at birth (Harrison, Bressack et al. 1980; Harrison, Jester et al. 1980). This led to the experimental fetal surgery for human foetuses diagnosed with a severe congenital diaphragmatic hernia but without survival benefit over postnatal surgery suggesting at that time the antenatal risks of surgery were not justified (Harrison, Adzick et al. 1993; Harrison, Adzick et al. 1997). However more recently antenatal surgery has been performed more successfully on cases with a poor prognosis as detailed in the following section.

1.3.5.5 Antenatal Management of Congenital Diaphragmatic Hernia

Antenatal steroid therapy has been shown to improve morphological maturity and reduce excessive muscularisation of the pulmonary vasculature in animal models of congenital diaphragmatic hernia. A report has shown that antenatal steroid therapy has improved the outcome in the case of three fetuses with a poor prognosis (Van Tuyl, Hosgor et al. 2001; Ford, Kirby et al. 2002).

Prenatal intervention is now offered to cases where the predicted prognosis is poor consisting of percutaneous fetoscopic endoluminal tracheal occlusion (FETO) (Deprest and De Coppi 2012). This is believed to work by preventing egress of lung fluid therefore increasing airway pressure and inducing cellular proliferation. FETO also increases alveolar airspace and helps maturation of pulmonary vasculature (Khan, Cloutier et al. 2007). This procedure can either be reversed by removal of the FETO either later in utero or immediately after birth but removal in utero is preferred (Deprest and De Coppi 2012). Deprest and colleagues reported outcomes showing considerable increase in survival in severe cases by using FETO, from 24% to 49% in cases of left sided congenital diaphragmatic hernia and from 0% to 35% in cases of right sided congenital diaphragmatic hernia (Deprest and De Coppi 2012).

1.3.5.6 Respiratory Management of Congenital Diaphragmatic Hernia

Early postnatal management is aimed at providing adequate oxygenation, avoiding high ventilator pressures to further damage the lungs aggravating the pulmonary hypertension accompanying the pulmonary hypoplasia.

Inhaled nitric oxide given to neonates to treat pulmonary hypertension has shown a variable response. A Cochrane review has shown an improvement in neonates with pulmonary hypertension from inhaled nitric oxide only if they do not have congenital diaphragmatic hernia (Oliveira, Troster et al. 2000; Finer and Barrington 2001; Finer and Barrington 2001).

High frequency oscillatory ventilation (HFOV) has been widely adopted; this method increases the ventilator rate to improve gas exchange and decrease barotraumas. A multicentre trial in North America indicated no benefit to preterm neonates with respiratory failure but several anecdotal reports indicate improvement in cases of congenital diaphragmatic hernia (Miguet, Claris et al. 1994). An Italian study reported outcomes over a ten year period showed an increase in survival from 67% to 94% by employing HFOV in the preoperative management. Surgical repair was also performed under HFOV (Cacciari, Ruggeri et al. 2001).

Extra corporal membrane oxygenation (ECMO) was introduced in the late 1970s; it enables a period of lung rest by placing the infant on cardiopulmonary support. This allows a reduction in pulmonary vascular resistance whilst providing oxygenated blood from an artificial circuit. Evidence supporting the use of ECMO is conflicting, early favourable reports were flawed by the use of historical data (Johnston, Bashner et al. 1988; Atkinson, Ford et al. 1991; Finer, Tierney et al. 1992). A multicentre trial in the UK showed no benefit for infants with congenital diaphragmatic hernia but showed benefit for infants with respiratory failure. A Cochrane review however showed a survival advantage for all infants but less for infants with congenital diaphragmatic hernia (Al-Shanafey, Giacomantonio et al. 2002; Elbourne, Field et al. 2002). Despite the lack of clear evidence ECMO is likely to remain a treatment for congenital diaphragmatic hernia (Smith, Jesudason et al. 2002).

Permissive hypercapnia was first suggested by Wung in 1985 (Wung, James et al. 1985). This technique allows infants to breathe normally on the ventilator which removes the necessity of sedation and avoids baratrauma. The risk of ensuing acidosis is controlled medically and impressive results have been reported using this technique (Moffitt, Schulze et al. 1995; Wung, Sahni et al. 1995; Boloker, Bateman et al. 2002).

Exogenous surfactant therapy is very effective at improving ventilation in premature infants but the benefits in cases of congenital diaphragmatic hernia are less clear (Jobe 1993; Yost and Soll 2000). Both lungs from the congenital diaphragmatic hernia lamb model and the nitrofen rat model have been shown to have reduced surfactant proteins (Glick, Stannard et al. 1992; Suen, Catlin et al. 1993; Hedrick, Kaban et al. 1997; Mysore, Margraf et al. 1998). The nitrofen rat model of congenital diaphragmatic hernia showed an early increase in pulmonary compliance but this was short-lived with no overall benefit. A small randomised trial in human infants also failed to demonstrate any sustained benefit (Lotze, Knight et al. 1994).

Liquid ventilation using fluorocarbons has increased lung volume and ventilator mechanics in congenital diaphragmatic hernia neonates with a dismal prognosis. Liquid ventilation may stimulate lung growth and increase pulmonary compliance (Major, Cadenas et al. 1995; Pranikoff, Gauger et al. 1996; Fauza, Hirschl et al. 2001).

1.4 Normal and Abnormal Sex Differentiation in the Embryo

1.4.1 Background

Sex reversal and indeterminate sex states are defined as the observed morphology of the genitalia not corresponding with the chromosomal sex state. It is estimated that 1 in 4600 live births have ambiguous genitalia (Gillam, Hewitt et al. 2010) and 1 in 1000 live births show some form of disorder of sex development (Cui, Ross et al. 2004). Severe disorders of sex development present a unique challenge, both diagnostically and in terms of acute and longer-term management. These are relatively rare conditions usually requiring a multidisciplinary approach from the outset and the involvement of a tertiary centre for assessment and management recommendations.

Disorders of sexual development can be considered to be divided into three main groups:-

- Virilised 46XX disorders
- Under-virilised 46XY disorders
- Chromosomic problems represented by mixed gonadal dysgenesis

1.4.2 Clinical Aspects of Disordered Sex Development

1.4.2.1 The Clinical Team

Infants presenting with ambiguous genitalia should be evaluated by a multidisciplinary team that is dedicated to the evaluation and management of children and adults with suspected and confirmed disorders of sex development. A thorough knowledge of the underlying pathophysiology and the strengths and weaknesses of the investigative tools that are available for reaching a diagnosis are crucial (Brain, Creighton et al. 2010). In particular gender assignment remains a difficult decision involving various indicators and long term support. (Gillam, Hewitt et al. 2010; Gillam, Hewitt et al. 2011).

1.4.2.2 Increasing Incidence of Male Sex Developmental Disorders

The prevalence of male reproductive disorders, such as testicular cancer and impaired semen quality, is increasing in many, albeit in not all, countries (Main, Skakkebaek et al. 2010). These disorders are aetiologically linked with congenital cryptorchidism and hypospadias by common factors leading to perinatal disruption

of normal testis differentiation, referred to as the testicular dysgenesis syndrome (Main, Skakkebaek et al. 2010). This could possibly be due to lifestyle and environmental factors (Brain, Creighton et al. 2010).

1.4.2.3 Malignancy

Disorders of sex development have been recognised as one of the main risk factors for development of type II germ cell tumours. Any disorder of sex development associated with a Y chromosome has an increased risk of germ cell tumours, such as seminomas/dysgerminomas and non-seminomas (e.g., embryonal carcinoma, yolk sac tumour, choriocarcinoma and teratoma) (Warne and Hewitt 2009). This type of tumour is also the most frequent malignancy in adolescent and young Caucasian males without any evidence of dysgenic gonads. This suggests there may be common aetiological mechanisms, either genetic, environmental or a combination of the two (Looijenga, Hersmus et al. 2010).

1.4.2.4 Psychological Aspects

There are psychological aspects of disorders of sexual development to be considered which will have effects on the patient's quality of life. Clinical decisions such as gender assignment are often made with long lasting effects on quality of life. Timing of surgery and communication should be based on empirical evidence but this evidence is largely not available. Protocols need to be developed to evaluate interventions to facilitate the decision making of professionals and individuals to enhance the psychological outcome of the cases (Gorduza, Vidal et al. 2010; Vidal, Gorduza et al. 2010).

1.4.3 Outline of Sex Differentiation

Mammalian sex determination involves complex interacting networks of gene expression, hormonal and cellular signals leading to the development of male or female phenotype. These processes can be divided into three main areas:-

1. The genetic sex of the embryo is decided at fertilisation, depending on whether an X or Y bearing sperm fertilises the oocyte. This inheritance of the X or Y chromosome from the father starts the profoundly different journeys of male or female life.
2. The next stage of sexual differentiation occurs when the fate of the bipotential gonad is determined by the expression of the Y linked genic switch in the XY embryo or no expression in the XX embryo, determining the

gonadal phenotype. The male and female journeys unfold during embryonic life when this switch on the Y chromosome is activated to start the undifferentiated gonadal primordium on the pathway towards testes development,

3. The third stage is governed by hormonal secretions from the developing testes, setting in train a cascade of morphological changes, gene regulation and molecular interactions to direct the characteristics of male differentiation. If this mechanism fails to occur alternative cellular and molecular events take place to direct the gonad towards the characteristics of female differentiation.

Thus one common primordium, part of the urogenital ridge, has the potential to develop into either one of two functionally and morphologically different organs, the ovaries or testes giving phenotypic sex.

1.4.4 Mechanisms that Underpin Sex Differentiation

1.4.4.1 Early Research

In 1947 Alfred Jost reported that when male rabbit embryos were castrated in utero before gonadal sexual differentiation all embryos developed as phenotypic females. He suggested that the default sexual phenotype is female and there must be a factor expressed before male phenotype could develop, he called this testis determining factor *(TDF)* (Jost 1947). He also transplanted fetal testes into female embryos and he found they developed as phenotypic males, therefore demonstrating that females have all factors necessary for the differentiation of a phenotypical male. His hypothesis was confirmed in 1990 (Berta, Hawkins et al. 1990; Koopman, Munsterberg et al. 1990; Sinclair, Berta et al. 1990). This factor *TDF* was then defined as Sex determining region on the Y chromosome *(SRY)*. Since then an increasing list of genes and cell lineages expressed in the genital ridges has been elucidated and the genetic pathways and interactions necessary for genetic phenotype are being understood. Jost also performed innovative experiments to study the differentiation of the reproductive tract based on the fact that the female reproductive system develops in the absence of any gonad (Jost 1970).

1.4.4.2 Development of the Gonads

The gonads, kidneys and adrenals derive from the urogenital ridge. These first stage early mammalian genital ridges are a paired undifferentiated primordium which can be divided into three segments.

1) The pronephros, near the caudal end which includes the adrenal primordium.

2) The mesonephros, the central region from which the gonad arises.

3) The metanephros, the most posterior region from which the kidney arises.

The urogenital ridge derives from the intermediate mesoderm, which contains the cell precursors that help form the mesonephros, kidneys, adrenal and gonads. Both testes and ovaries arise from the genital ridges in the mammalian embryo. They are both highly specialised and different organs but from the same origin. The development of the gonads can be divided into two stages. The first stage is the genital ridge, a bipotential gonad which is identical in both male and female. The second phase is the development of the testes or ovary.

The main steps of phenotypic human sex differentiation are as follows:-

1) The paramesonephric duct (Müllerian duct) develops next to the mesonephric duct (Wolffian duct) at gestational age six to seven weeks.

2) For male phenotypic development the Leydig cells in the developing testes secrete testosterone which is converted to the more active dihydrotestosterone (Wilson, Griffin et al. 1993) which is required for the development of male external genitalia. The Wolffian duct increases in size and differentiates into epididymis, vas deferens and prostate. Recently a secondary pathway for dihydrotestosterone production has been discovered. A family was discovered to have a mutation of an enzyme involved in this alternative pathway of dihydrotestosterone production causing disorders of sex development (Fluck, Meyer-Boni et al. 2011). This was the first case of this alternative pathway known in the human population, this pathway had only previously been identified in a marsupial (Fluck, Meyer-Boni et al. 2011).

   Sertoli cells in the testes secrete Anti-Müllerian Hormone resulting in the regression of the Müllerian duct.

3) For female phenotypic development there is no development of the testes and without the hormonal influence of testosterone the Wolffian

duct eventually degenerates. The Müllerian duct proliferates and forms the fallopian tube, uterus and the upper third of the vagina.

This development is under the control of many factors.

1.4.5 Factors Involved in the Bipotential Stage of Sex Determination

There are a number of cell progenitor lineages within the bipotential genital ridges each having the potential to differentiate into testicular or ovarian cell types under the influence of transcription factors, hormones and enzymes. The supporting cell precursor lineage, so named for their role in sustaining germ cells, are believed to be present in the early gonad. These cells have the potential to differentiate into either Sertoli cells in the testis or granulosa cells in the ovary (Albrecht and Eicher 2001).

Sertoli cells are the first cell type known to differentiate within the gonad from bipotential precursors of the supporting cell lineage. They express *SRY* and are the first indication of testis development of the bipotential gonad. Within this area there is a single cell population of Steroidogenic Factor 1 (*SF-1)* immunoreactive cells named 'adrenogenital primordia' (Hatano, Takakusu et al. 1996; Morohashi 1999). Steroidogenic precursor cells have the potential to differentiate into either Leydig cells in the testis or theca cells in the ovary. These are also present in the early gonad (Merchant-Larios, Moreno-Mendoza et al. 1993).

Germ cells have the potential to follow the spermatogenic pathway or the oogenic pathway depending in which developing gonad they find themselves. The primordial germ cells do not arise in the genital ridges but originate at the base of the allointois at the posterior end of the primitive streak but at this point are not restricted to a germ cell fate as they can form extraembryonic mesoderm. When first seen in the developing mouse embryo at 7dpc they are in the region of the developing hindgut and enter the embryo with the hindgut as it invaginates where they leave the hindgut and pass into the forming urogenital ridges at 9.5dpc in the mouse.
The most important factors involved in the molecular processes controlling this phase are described below.

1.4.5.1 Paired Homeobox 2 *(PAX2)*

The transcription factor paired homeobox 2 (*PAX2)* is widely expressed during the development of both ductal and mesenchymal components of the urogenital

system. *Pax2* homozygous mice lack genital tracts, kidneys and ureters. These defects could be attributed to both mesechymal and ductal components of the urogenital system. However, human genetic defects in *PAX2* result in renal-coloboma syndrome (hypodysplastic kidneys and optic nerve abnormalities) but they do not have genitourinary abnormalities (Torres, Gomez-Pardo et al. 1995).

1.4.5.2 Empty Spiracle Homolog 2 (*EMX2*)

The homeobox gene Empty spiracle homolog 2 *(emx2)* is a mouse homologue of a Drosophila head gap gene empty spiracles *(ems)* and is essential for the development of dorsal telencephalon (Yoshida, Suda et al. 1997).

Emx2 is expressed in the epithelial components of the developing urogenital system. In *emx2* mutant mice the kidneys, ureters, gonads and genital tracts were absent and there was accelerated degeneration of the Wolffian duct and mesonephric tubules without the formation of the Müllerian duct. These mutants completely lacked gonads and genital tracts (Miyamoto, Yoshida et al. 1997). No human sex development defects in human cases of *EMX2* have been described. Somatic mutations have been described in endometrial carcinomas and are a rare cause of schizencephaly (Noonan, Mutch et al. 2001). The function of *emx2* has been linked to the wingless related MMTV integration site family members *(wnt)* signalling pathway during embryonic patterning in mice (Okamoto, Hirata et al. 2010).

1.4.5.3 Wilms Tumour 1 *(WT1)*

Wilms Tumour 1 *(WT1),* a zinc finger transcription factor, is the product of the Wilms tumour suppressor gene. Zinc finger transcription factors are small protein structural motifs that can include one or more zinc ions to help stabilise their folds. Mutation analysis in humans and genetic experiments in mice revealed that *WT1* has a role in development as well as tumour suppression. *WT1* has at least 4 splicing variants and post transcriptional modifications of the *WT1* pre-mRNA leading to the production of at least 24 isoforms increasing the complexity of *WT1* action. *WT1* is expressed widely in the human undifferentiated gonad, mesonephros and kidney (Armstrong, Pritchard-Jones et al. 1993). In *WT1* null mice the gonads undergo apoptosis and the adrenal glands are also affected as they share a common primordium (Hatano, Takakusu et al. 1996; Moore, McInnes et al. 1999). *WT1* also plays a role in the growth of the ureteric bud during kidney development (Moore, McInnes et al. 1999). Experiments using transgenic mice suggest that *WT1*

activates a number of genes, and steroidogenic factor 1 *(SF1)* is believed to be a *WT1* target (Wilhelm and Englert 2002).

During gonad formation *WT1* has several potential targets, it controls *SRY* and wingless related MMTV integration site family member 4 *(WNT4)* expression, but the control of *WNT4,* may be an indirect effect (Sim, Smith et al. 2002). *WT1* also controls dosage sensitive sex reversal, Adrenal hypoplasia congenital critical region on the X chromosome gene 1 *(DAX1). WT1* expression precedes that of *DAX1* and can activate the *DAX1* promoter, the *WT1-DAX1* pathway is an early event in mammalian sex differentiation and anti Müllerian hormone also seems to be activated by *WT1* variants (Kim, Prawitt et al. 1999).

1.4.5.4 Steroidogenic Factor 1 *(SF1)*

Steroidogenic factor 1 *(SF1)* is expressed in the bipotential urogenital ridge and pituitary gland indicating a role in the hypothalamic- pituitary-gonadal axis (Wilhelm, Palmer et al. 2007). Lack of a functional *SF1* gene in mice causes failure of adrenal and gonadal development and also pituitary and hypothalamus gonadotrope abnormalities. XY mice lacking a functional *SF1* gene also show male to female sex reversal (Luo, Ikeda et al. 1994; Shinoda, Lei et al. 1995; Sadovsky and Dorn 2000).

1.4.5.5 Lim Homeobox Protein 9 *(LHX9)*

Transcripts of the LIM homeobox gene *(lhx9)* are present in the urogenital ridges of mice at 9.5dpc, they then localise in the interstitial cells as morphological differentiation occurs. In mice lacking *lhx9* function germ cells migrate normally but the somatic cells of the genital ridge fail to proliferate and there is no formation of a discrete gonad. In the absence of testosterone and anti Müllerian hormone genetically male mice are phenotypically female. The expression of *SF1,* a nuclear receptor essential for gonadogenesis is reduced to minimal levels in the *lhx9* deficient genital ridge. This indicates that *lhx9* may lie upstream of *SF1* in a developmental cascade. *Lhx9* null mice gonadal phenotype is similar to that of *SF1* and *WT1* null mice, *lhx9* mutants have not been found to exhibit any additional developmental defects (Birk, Casiano et al. 2000). Although *LHX9* mutations may underlie certain forms of isolated gonadal agenesis in humans as to date such mutations have not been found (Biason-Lauber 2010).

1.4.5.6 GATA Binding Protein 4 *(GATA4)*

GATA Binding Protein 4 is a member of the *GATA* family of transcription factors. It is present in the gonads and may be a regulator of gene expression. In mice it marked the developing somatic lineages, Sertoli (testes) and granulosa (ovary) but did not mark the primordial germ cells. *GATA4* expression remained abundant in Sertoli cells throughout embryonic development but shortly after the histological differentiation of the ovary at 13.3dpc *GATA4* was downregulated. This pattern of expression indicates that *GATA4* may be involved in early gonadal development and possibly sexual dimorphism (Viger, Mertineit et al. 1998). In humans *GATA4* has only been related to a specific heart defect, an atrial septal defect (Liu, Wang et al. 2011).

1.4.5.7 Chromobox Homologue 2 *(CBX2* also known as *M33)*

It has been suggested that Chromobox Homologue 2 *(CBX2)* compacts chromatin thereby preventing the binding of transcriptional activators. Homozygous XY *cbx2 (M33)* knockout mice have male to female sex reversal and XX *cbx2 (M33)* mice have absent or small ovaries (Katoh-Fukui, Tsuchiya et al. 1998). As *CBX2 (M33)* has a role in early gonad development of both sexes it indicates a role before the time of sex determination (Katoh-Fukui, Tsuchiya et al. 1998). *Cbx2 (M33)* has recently been implicated in *SF1* regulation in the spleen and adrenal gland (Katoh-Fukui, Owaki et al. 2005) therefore it may have a similar function in gonad development but as yet this has not been confirmed (Wilhelm, Palmer et al. 2007). Cbx2 null mice also have other development defects and exhibit male to female sex reversal, retarded growth, skeletal anomalies and a failure of some cell types to expand. When these mutants are treated with retinoic acid the skeletal anomalies are aggravated indicating the *cbx2* protein may have a role in defining access to retinoic acid response elements affecting the regulating region of several Hox genes which are genes determining the structure and orientation of the developing embryo (McGinnis and Krumlauf 1992; Core, Bel et al. 1997).

1.4.6 Overview of Development of Sexual Phenotype

Male and female embryos possess initially a bipotential gonad that becomes committed to ovary or testis development as a result of interactions between the mesenchymal cells. These cells are derived from the mesonephros and the epithelial cells of the genital ridge (Schmahl and Capel 2003; Brennan and Capel 2004).

Sertoli cells are considered to be generated from the epithelium of the genital ridge, whereas many of the other somatic cell types, including endothelial cells and peritubular myoid cells, are derived from the mesonephros by migration into the XY gonad (Merchant-Larios, Moreno-Mendoza et al. 1993). The extragonadal germ cells are encapsulated into the seminiferous tubules encircled by the peritubular myoid cells

Desert hedgehog *(DHH)* is produced by Sertoli cells and regulates the proliferation and differentiation of Leydig cells (Yao, Whoriskey et al. 2002; Park, Tong et al. 2007; Zou, Li et al. 2012). One of the important steps in male sex organogenesis occurs when the differentiating Sertoli cells start to secrete anti-Müllerian hormone. This induces regression of the female Müllerian duct, while the differentiating Leydig cells initiate the production of testosterone. Testosterone promotes the development of the other sex duct, the male Wolffian duct, into the epididymis, vas deferens, and seminal vesicles. Insulin-like factor 3, a hormone produced by the Leydig cells, is also implicated in Wolffian duct development because its deficiency leads to bilateral cryptorchidism (Hutson and Donahoe 1986; Nef and Parada 1999).

In females, the absence of anti-Müllerian hormone and testosterone and the presence of *wnt-4* and *wnt-7a* signaling leads to development of the Müllerian duct. This forms the oviduct, uterus, and upper part of the vagina, and the degeneration of the Wolffian duct (Parr and McMahon 1998; Vainio, Heikkila et al. 1999; Heikkila, Peltoketo et al. 2002; Heikkila, Prunskaite et al. 2005).

1.4.7 Factors Involved in the Development of Male Sexual Phenotype

1.4.7.1 Sex Determining Region on the Y Chromosome *(SRY)*

The default pathway in the genital ridges is considered to be ovarian development. In males the Y chromosomal testis determining gene *(SRY)* pre-empts the ovarian pathway diverting the genital ridges towards testicular development (Koopman 2010). The presence of a Y chromosome results in male development regardless of the number of X chromosomes (Ford, Jones et al. 1959; Jacobs and Strong 1959).

Early studies in mice showed that *SRY* is first expressed around 10.5dpc shortly after the emergence of the genital ridges. Peak levels of expression are reached at 11.5dpc and *SRY* expression is no longer detected shortly after 12.5dpc in the mouse (Koopman, Munsterberg et al. 1990; Hacker, Capel et al. 1995; Jeske, Bowles et al. 1995). The function of *SRY* seems to cause the supporting cell

precursors to differentiate into Sertoli cells (Burgoyne, Buehr et al. 1988; Sekido, Bar et al. 2004; Wilhelm, Martinson et al. 2005). This was proved in 1991 using XX female transgenic mice expressing *SRY* and they developed testes as a result (Koopman, Gubbay et al. 1991). *SRY* triggers differentiation of Sertoli cells from supporting cell precursors which would otherwise have given rise to follicle cells. Sertoli cell differentiation is the pivotal event essential for male sex determination and testicular morphogenesis. There is evidence that Sertoli cells are the organising centres of the developing testes communicating with cells around them and secreting factors that bind to receptors on the receiving cells (Brennan and Capel 2004).

Many mutations have been identified in human *SRY*, typically affecting the structure of the *SRY* protein therefore affecting male sex development. Such mutations result in disorders of sex development including sex reversal (Harley, Jackson et al. 1992). The regulatory sequences required for directing the expression of *SRY* are also vulnerable to mutation as *SRY* is only expressed for a few days during the differentiation in the genital ridges. The *SRY* sequences have become so degraded that the level of expression is very low leaving only a minimum amount of expression to initiate testes development, leaving male sex determination vulnerable to perturbation. As the *SRY* gene is vulnerable to mutation or reduced expression, it is not a very robust sex determining mechanism. As it resides on the Y chromosome, if *SRY* becomes mutated some repair mechanisms are unavailable. This is due to a lack of a meiotic pairing partner, such as duplex repair which requires exchange of a DNA strand from one duplex to another (Bullejos and Koopman 2001). However not all mammals rely on *SRY* as the sex determining switch; in some species of spiny rats the males have no Y chromosome, they have XO/XO chromosome composition. The males have extra copies of chromobox homologue 2 *(cbx2)* gene and it suggests that these spiny rats have acquired a new sex determining gene that has superseded SRY, and SRY was lost with the disappearance of the Y chromosome (Kuroiwa, Handa et al. 2011). A girl with a Y chromosome having completely normal genitalia is considered to have mutation of gene *CBX2* on chromosome 17 which has shut off the *SRY* gene. It is too early to assess her fertility (Biason-Lauber, Konrad et al. 2009).

SRY triggers the expression of a related gene SRY (sex determining region Y)-box9 *(SOX9)* by binding to an enhancer sequence upstream of *SOX9.* Once *SRY* has managed to initiate the *Sox9* feedback loop its expression is no longer required (Sekido and Lovell-Badge 2008). Up regulation of *Sox9* in pre-Sertoli cells is one of

the earliest markers of Sertoli cell differentiation (Kent, Wheatley et al. 1996; Morais da Silva, Hacker et al. 1996). *Sox9* expression is then able to stimulate and maintain its own transcription through auto regulation, the *Sox9* protein also being a transcription factor. This creates a positive feedback loop for the continued production of *Sox9* protein independent of continued *SRY* expression ensuring continued testis development. If *SRY* is absent or expression fails at the critical time *Sox9* is silenced and development of the follicle and ovary ensues.

1.4.7.2 SRY (Sex Determining Region Y)-Box9 *(SOX9)*

SRY(sex determining region Y)-box9 *(SOX9)* is considered the best candidate for a direct *SRY* target gene (Sekido, Bar et al. 2004; Wilhelm, Martinson et al. 2005; Sekido and Lovell-Badge 2008). *SOX9* expression is upregulated soon after the expression of *SRY* begins. Cell mapping experiments indicate that *SRY* positive cells exclusively become *SOX9* positive Sertoli cells (Chaboissier, Kobayashi et al. 2004). Heterozygous mutations in *SOX9* are responsible for camptomelic dysplasia, a condition in which most XY patients have male to female sex reversal. They also possess skeletal malformations due to the reduction of *SOX9* activity which is required for type2 collagen formation, a major structural component of cartilage (Bell, Leung et al. 1997; Ng, Wheatley et al. 1997).

Targeted *sox9* ablation in mice also leads to ovary development in XY embryos (Barrionuevo, Bagheri-Fam et al. 2006).

Sox9 is abolished in steroidogenic factor 1 *(SF1)* null mutant mice gonads suggesting *SF1* to be a good candidate for initiating or sensitizing *sox9* (Lovell-Badge, Canning et al. 2002; Sekido, Bar et al. 2004). *Sox9* is expressed at a low level in the genital ridges of both sexes at 10.5dpc in the mouse, expression of which is sensitised by *SF1*. In the human male *SF1,* probably in conjunction with other factors such as *WT1,* also activates *SRY* expression. Therefore *SOX9* expression is upregulated by the action of *SRY* together with *SFI.* After the transient high levels of *SRY* have ceased high levels of *SOX9* are maintained by its direct autoregulation and by Fibroblast Growth Factor 9.

1.4.7.3 Fibroblast Growth Factor 9 *(FGF9)* and its Receptor Fibroblast Growth Factor Receptor 2 *(FGFR2)*

In vivo and *in vitro* studies have suggested that Fibroblast Growth Factor 9 *(FGF9)* has several direct and indirect roles in male sex determination and testicular development (Colvin, Green et al. 2001; Schmahl, Kim et al. 2004; Willerton, Smith

et al. 2004; El Ramy, Verot et al. 2005; Yoshioka, Ishimaru et al. 2005; Chi, Itaranta et al. 2006; Kim, Kobayashi et al. 2006)

Fgf9 is also implicated in Sertoli cell specification maintaining high levels of *sox9*. It is broadly expressed in the mouse embryo with a sex specific pattern in the developing gonad, in the Wolffian duct, the mesonephric tubules and the Sertoli cells of the developing testis (Colvin, Feldman et al. 1999). It can be detected from 11.5dpc in both sexes (Schmahl, Kim et al. 2004) but later restricted to the testes cords of the XY gonad. *Fgf9* null mice show male to female sex reversal most likely due to a reduced proliferation rate and possibly impaired differentiation rate of pre Sertoli cells. As a consequence the threshold number of required cells for testes differentiation is not met (Schmahl and Capel 2003). *Fgf9* signaling stimulates mesenchymal cell proliferation and the migration of mesonephric cells into the testis (Colvin, Green et al. 2001), which is one critical cellular consequence of *SRY* activation, contributing to the formation of the interstitial compartment of the testis (Karl and Capel 1998).

Following *SRY* mediated upregulation of *sox9* in the mouse at 11.5dpc, fibroblast growth factor receptor 2 **(***fgfr2)* accumulates in the nuclei of Sertoli cells of the XY gonads (Schmahl, Kim et al. 2004). Deletion of *fgfr2* in embryonic gonads has the same phenotype as deletion of *fgf9* leading to male to female sex reversal. This indicates a distinct role for fgfr2 in proliferation and Sertoli cell differentiation during testis development in mice. Kim and colleagues were the first to report this genetic evidence (Kim, Bingham et al. 2007).

1.4.7.4 Steroidogenic Factor 1 *(SF1)* and Nuclear Receptor Subfamily 5 Group A Member 1, *(NR5A1)*

Steroidogenic factor 1 *(SF1)* is a member of the nuclear receptor family of intracellular transcription factors and is encoded by the Nuclear Receptor subfamily 5 group A member 1 *(NR5A1)* gene (Taketo, Parker et al. 1995). The central role of *SF1* is regulating adrenal development, gonad determination and differentiation, and in the hypothalamic-pituitary control of metabolism and reproduction. In the mouse *SF1* is expressed in the early adrenogonadal primordium from 9dpc and thereafter in the developing adrenal gland and gonad (Ikeda, Shen et al. 1994; Nef, Schaad et al. 2005; Val, Martinez-Barbera et al. 2007). In humans *SF1* expression has been shown in the bipotential gonad and developing adrenal gland at 32-33 days post conception (Ramayya, Zhou et al. 1997; Hanley, Ball et al. 1999). From around 42

days post conception onwards in humans after testes determination *SF1* expression is maintained in the somatic cells of the early testes where it may play a crucial role with *SRY* in supporting *SOX9* expression (Sekido and Lovell-Badge 2008).

From around 7 weeks gestation in the human male fetus the Sertoli cells expression of *SF1* activates expression of anti-Müllerian hormone leading to the regression of the Müllerian ducts. Leydig cells differentiate later than Sertoli cells and their differentiation may be controlled by Sertoli cell secreted factors (Habert, Lejeune et al. 2001). From 8 weeks gestation *SF1* activates the expression of Steroidogenic enzyme systems in the Leydig cells resulting in androgenisation of the external genitalia.

In XY mice deletion of the gene *Nr5a1* encoding *SF1* results in female external genitalia, Müllerian structures, complete testicular dysgenesis and impaired adrenal development. This prompted a search for a human counterpart focusing on patients with primary adrenal failure, 46XY gonadal dysgenesis and Müllerian structures. The first patient with this phenotype was identified in 1999 by Achermann and colleagues (Achermann, Ito et al. 1999). The phenotypic human anomalies with *SF1/NR5A1* gene mutation range from complete testicular dysgenesis with Müllerian structures, severe penoscrotal or anorchia to cases of mild clitoromegaly or genital ambiguity (Lin and Achermann 2008), Heterozygote mutations in *NR5A1* have recently been linked to ovarian insufficiency although it is not essential for ovarian determination (Biason-Lauber and Schoenle 2000; Lourenco, Brauner et al. 2009).

1.4.7.5 Basic Helix Loop Helix *(bHLH)*

Pod-1 (Capsulin), a member of the basic helix loop helix transcription factors is first expressed in the bipotential gonad at 11.5dpc in the mouse embryo. In the *pod-1* knockout mouse abnormalities are seen in the bipotential gonad at 11.5dpc. During male sex development the expression pattern of *pod-1* is closely related to the pattern of expression of *SF1*. *Pod-1* may repress *SF1* expression and its absence may cause premature differentiation disrupting testicular structure and development. Both XX and XY *pod1* knockout mice have hypoplastic gonads with vascular abnormalities. XY *pod-1* knockout mice also have male to female sex reversal (Cui, Ross et al. 2004).

1.4.7.6 Dosage Sensitive Sex Reversal and Adrenal Hypoplasia Congenital Critical Region on the X Chromosome Gene 1 *(DAX1)*

Dosage Sensitive Sex Reversal and Adrenal Hypoplasia Congenital Critical Region on the X Chromosome Gene 1 *(DAX1)* is encoded by the gene *NROB1* and is expressed in the developing adrenal gland, the hypothalamus, the pituitary and in the somatic cells before the expression of *SRY*. During gonadal differentiation *dax1* expression is regulated by *WT1* (Kim, Prawitt et al. 1999). Anti Müllerian Hormone promoter can be repressed by DAX1 and can displace Wilms tumour1 *(WT1)* from its complex with *SF1*. (Wagner, Wagner et al. 2003). Nuclear receptors *DAX1* and *SF1* mediate somatic cell differentiation during testis development, *DAX1* being an antagonist of SF1 (Achermann, Meeks et al. 2001; Iyer and McCabe 2004; Park, Meeks et al. 2005).

Loss of function of *WT1* in male development causes impaired testicular development from reduced testicular size to complete sex reversal and *dax1* is inhibited by *coup-tf11* (Yu, Achermann et al. 1998; Yu, Ito et al. 1998; Yu, Ito et al. 1998; Achermann, Meeks et al. 2001; Meeks, Crawford et al. 2003; Meeks, Weiss et al. 2003; Bouma, Albrecht et al. 2005). In mice and humans, sensitivity to levels of *DAX1* is important and may have a window of activity (Ludbrook and Harley 2004). Loss of function of *dax1* and *SF1* in mice reduced desert hedgehog *(dhh)* expression, *dhh* is a paracrine signalling factor regulating fetal Leydig cell development (Yao, Whoriskey et al. 2002).

1.4.7.7 GATA Binding Protein 4 *(GATA4)* and Friend of GATA2 (*FOG2)*

In mice of both sexes *GATA4* is present in the gonads and marks the developing somatic cell lineages, Sertoli cells in the XY gonads and granulosa cell in the XX gonad. Both *GATA4* and *fog2* are required for fetal testes development in mice (Bouma, Affourtit et al. 2007; Bouma, Washburn et al. 2007). Mice having a homozygous mutation disrupting *GATA4/ fog2* interactions have male to female sex reversal (Tevosian, Albrecht et al. 2002; Barber, Maloney et al. 2005) In the absence of *fog2* in mice the function of SRY was impaired leading to sex reversal (Tevosian, Albrecht et al. 2002). In humans mutations of *GATA4* have been linked to heart defects only (Biason-Lauber 2010).

1.4.7.8 Paired Homeobox 2 *(PAX2)*

Paired homeobox 2 *(PAX2)* as well as having a role in the bipotential development of the urogenital ridge also continues to have a role in the development of sex

differentiation and kidney development. *Pax2* homozygous mutant mice lack kidneys, ureters and genital tracts due to dysgenesis of both ductal and mesenchymal components of the urogenital system. The wolffian and Müllerian ducts, precursors of the male and female genital tracts develop only partially and degenerate during embryogenesis (Torres, Gomez-Pardo et al. 1995).

1.4.7.9 Double Sex and MAB3-Related Transcription Factor 1 *(DMRT1)*

Human *DMRT* encodes a protein that is expressed only in testes. The *DMRT* genes are carried on chromosome 9p and monosomy of this area has been linked to sex reversal. Deletion of the short arm of chromosome 9 (9p) is linked to cases of gonadal dysgenesis and XY sex reversal. This suggests this region contains genes requiring two copies for normal testis development (Raymond, Parker et al. 1999; Muroya, Okuyama et al. 2000).

Mouse *dmrt1* is expressed in the developing urogenital ridges of XX and XY embryos and in the Sertoli cells and germ cells of the developing testes (Raymond, Kettlewell et al. 1999). Thus *dmrt1* and SRY are the only regulatory genes expressed exclusively in the urogenital ridge prior to sexual differentiation (Raymond, Kettlewell et al. 1999).

1.4.7.10Retinoic Acid, Retinaldehyde Dehydrogenase and Aldehyde Dehydrogenase Family 1, Subfamily A1

Vitamin A given to pregnant rats caused genitourinary anomalies in the offspring (Wilson and Warkany 1947). Retinoic acid controls some aspects of the development of the urogenital tract as retinaldehyde dehydrogenase isoforms are expressed in the mesenchymal cells of the mesonephros, stromal cells of the developing kidney and the ureteric bud. Here they control epithelial mesenchymal interactions during kidney development and for the development of connections between the ureters and bladder (Batourina, Gim et al. 2001; Mic, Haselbeck et al. 2002; Batourina, Tsai et al. 2005).

When added to rat fetal testis in culture, exogenous retinoic acid inhibits Sertoli cell differentiation, mesonephric cell migration, seminiferous cord formation and gonocyte survival thus inhibiting XY gonadal development (Cupp, Dufour et al. 1999; Livera, Rouiller-Fabre et al. 2000; Li and Kim 2004). Aldehyde Dehydrogenase Family 1, Subfamily A1 is an enzyme involved in retinoid metabolism and is expressed at high levels in mouse Somatic cells shortly after *SRY* expression is first detected and is dependent on *sox9* expression (Bowles,

Feng et al. 2009). A low level of retinoic acid may be necessary for male gonad development (Bowles, Feng et al. 2009).

1.4.8 Molecular Development of Sexual Phenotype (Female)

1.4.8.1 Wingless-Related MMTV Integration Site Family Member 4 (WNT4)

The wingless-related MMTV integration site family members *(WNT)* family of genes are structurally related genes which encode secreted signalling proteins from the extra cellular matrix. These proteins have been implicated in several development processes including regulation of cell fate and patterning during embryogenesis and also in oncogenesis. *WNT4* is the first signalling molecule shown to influence the female sex determining cascade. The human *WNT4* shows a 98% homology to the amino acid identity of mouse and rat *WNT4* (Jordan, Mohammed et al. 2001).

WNT4 is the first human gene to be identified that directed the developmental of the bipotential gonad towards ovarian development. Ovarian development was considered to be the default pathway, but has been shown not to be a passive default pathway. *WNT4* null XX mice show partial female to male sex reversal and reduced expression of follistatin limited to early ovarian development. This indicates a role in the initiation but not the maintenance of follistatin expression (Kashimada, Pelosi et al. 2011). *WNT4* acts through follistatin to inhibit male specific vascularisation and maintain germ cell survival in the developing ovary (Tomizuka, Horikoshi et al. 2008).

WNT4 is produced in ovarian somatic cells and it upregulates dosage sensitive sex reversal, adrenal hypoplasia congenital region on the X chromosome gene1 *(dax1),* a gene known to antagonise the nuclear-receptor *SF1* and SRY therefore preventing testicular development (Jordan, Mohammed et al. 2001). Also in the early stages of ovarian development *WNT4* suppresses the migration of mesonephric endothelial and steroidogenic cells preventing the formation of male specific coelomic blood vessels and the production of steroids (Jeays-Ward, Hoyle et al. 2003; Yao, Matzuk et al. 2004). *WNT4* It is implicated in Müllerian duct formation being required for the initial Müllerian duct formation in both sexes (Vainio, Heikkila et al. 1999).

A lack of *WNT4* in the XX female mouse embryo leads to the mascularisation of the embryo, having no Müllerian structures but having a wolffian duct. However no Sertoli cell markers are expressed and no testicular tissue is formed. The external

genitalia is phenotypically female indicating that *WNT4* is not a primary sex determining gene (Bernard and Harley 2007). This has been corroborated by a single point mutation of the *WNT4* gene in a human patient with similar phenotypical anomalies to the mouse model (Biason-Lauber, Konrad et al. 2004). XY mice generated to overexpress *WNT4* showed no male to female sex reversal, (Jeays-Ward, Hoyle et al. 2003; Jordan, Shen et al. 2003), however in humans duplications of chromosome 1p including the *WNT4* gene do show gonadal anomalies (Cousineau, Higgins et al. 1981; Elejalde, Opitz et al. 1984).

In the presence of Wolffian ducts in *WNT4* knockout female mouse embryos steroidogenic enzymes that are required for the production of testosterone are expressed. These enzymes are normally suppressed in the developing ovary and the ovaries of these mice have less oocytes suggesting a role for *WNT4* in germ cell development. Germ cells have a role in the organisation of ovarian structure and maintenance (Merchant 1975; McLaren 1984). *WNT4* appears to protect germ cells in mice (Vainio, Heikkila et al. 1999) and probably in humans (Philibert, Biason-Lauber et al. 2008).

1.4.8.2 Follistatin *(Fst)*

Follistatin, *(Fst)* a secreted glycoprotein required for early female sex determination and ovarian development acts downstream from *WNT4*. It binds to the *tgfβ*-superfamily molecules such as activin and neutralises their activity (Phillips and de Kretser 1998). The expression of *Fst* is restricted to the developing ovary and is not present in the developing testis (Menke and Page 2002; Yao, Matzuk et al. 2004). *Fst* is however expressed in many other organs, neuronal tissue, kidney, liver, bone, heart, muscle and skin. *Fst* null mice die soon after birth due to a wide range of affected organs confirming a broad range of biological roles (Matzuk, Lu et al. 1995).

Follistatin null mice also displayed partial sex reversal during embryogenesis suggesting an earlier role in ovarian development. A deficiency results in the development of coelomic blood vessels in an XX gonad, coelomic blood vessels being a normal feature of testis development. (Yao, Matzuk et al. 2004).

1.4.8.3 Roof Plate Specific Spondin 1 *(RSPO1)*

The R-spondin *(RSPO)* protein family is a recently described group of secreted proteins (Kim, Zhao et al. 2006), their activities are similar to those of the *wnt* ligands (Kazanskaya, Glinka et al. 2004; Kim, Kakitani et al. 2005). The expression

pattern of *Rspo* mRNAs was shown to partially overlap that of *wnt* genes (Kamata, Katsube et al. 2004; Nam, Turcotte et al. 2007).

Roof plate specific spondin1 (rspo1) was first identified in the dorsal neural tube of mice (Kamata, Katsube et al. 2004). *Rspo1* is expressed in the developing gonads of 11.5dpc mice, in the XX gonads it is expressed in the somatic cells but in the XY gonads expression is mostly restricted to the coelomic epithelium and weak expression showing in some interstitial cells from 12.5dpc (Parma, Radi et al. 2006; Chassot, Ranc et al. 2008).

Loss of function studies demonstrated that ablation of *Rspo1* triggers sex reversal of XX mice with the formation of ovatestis and hermaphroditism of male and female internal genitalia. The left gonad seems to be more severely affected suggesting possible mechanisms in left/right asymmetry as in birds (Guioli and Lovell-Badge 2007). The phenotype of *rspo1* null mice is similar to *WNT4* null mice suggesting that these genes act in the same molecular pathway. *Rspo1* is required for the upregulation of *WNT4* in XX gonads (Chassot, Gregoire et al. 2008).

Rspo1 also activates the β-catenin signalling pathway required for female somatic cell differentiation and germ cell commitment into meiosis (Chassot, Gregoire et al. 2008).

1.4.8.4 Dosage Sensitive Sex Reversal, Adrenal Hypoplasia Congenital Critical Region on the X Chromosome Gene 1 *(DAX1)*

Originally *DAX1* was considered to be an ovarian determining or anti testis gene as it is a *SF1* and *SRY* antagonist (Iyer and McCabe 2004), but loss of function had no developmental consequences (Bardoni, Zanaria et al. 1994; Swain, Zanaria et al. 1996).

1.4.8.5 Forkhead Box Gene L2 *(FOXL2)*

Despite their importance in female genital development neither *dax1* or *WNT4* has been proved to be the ovarian determining factor (Wilhelm, Palmer et al. 2007). Another possible candidate for this role is *foxl2* which is expressed in a female specific manner in the gonads in mesenchymal pre-granulosa cells and then in granulosa cells. (Schmidt, Ovitt et al. 2004). However *foxl2* null mutations do not result in early ovarian defects thus excluding *foxl2* as the ovary determining factor (Schmidt, Ovitt et al. 2004; Uda, Ottolenghi et al. 2004).

Kashmada and colleagues reported that *foxl2* and bone morphogenic protein 2 (*bmp2)* cooperate to regulate follistatin expression during ovarian development. *Foxl2* null mice have reduced follistatin expression throughout fetal ovarian development and reduced *bmp2* expression (Kashimada, Pelosi et al. 2011).

Foxl2 is the only female specific gene that is active continuously throughout female sex development and the *foxl2* pathway both initiates and maintains sex differentiation in the somatic cells during this development (Ottolenghi, Uda et al. 2007; Garcia-Ortiz, Pelosi et al. 2009). Loss of *foxl2* and *WNT4* lead to partial female to male sex reversal resulting in testis differentiation (Ottolenghi, Pelosi et al. 2007).

1.4.8.6 Basic Helix Loop Helix *(bHLH)*

Pod-1 a basic helix loop helix transcription factor is required for correct ovarian development and as discussed previously the bipotential genital ridge is abnormal in *pod-1* knockout mice with impaired vascular development (Cui, Ross et al. 2004).

1.4.9 Germ Cell Development

During the early development stages a germ cells fate rests on which differentiating gonad it resides. If it resides in an ovary it commits to oogenesis and enters meiosis. If it resides in the testes it commits to spermatogenesis and enters mitotic arrest until puberty (McLaren 1984; Rolland, Lehmann et al. 2010). Towards the end of the embryonic period both XX and XY germ cells are capable of meiosis, expressing 'deleted in azoospermia-like' (*Dazl*) which are RNA binding proteins involved in fertility (Lin, Gill et al. 2008).

Retinoic acid stimulates the onset of meiosis of germ cells in the ovary, at 13.5dpc in the mouse. The testes are prevented from developing germ cells initially by expressing a retinoic acid degrading enzyme CYP26 (Bowles, Knight et al. 2006; Koubova, Menke et al. 2006). For XY germ cells in the testes, to commit to the male pathway it appears necessary for there to be an absence of retinoic acid and may also rely on additional male specific factors. Sertoli cells express CYP26B1 which is a retinoic acid metabolizing enzyme and this reduces the exposure to retinoic acid (Rolland, Lehmann et al. 2010).

1.5 Possible Association of Sex Determining Processes with Congenital Diaphragmatic Hernia

Approximately 30-40% of cases of congenital diaphragmatic hernia including pulmonary hypoplasia and pulmonary hypertension have additional major malformations including cardiovascular, gonadal, renal, skeletal, digestive system and central nervous system anomalies (Benjamin, Juul et al. 1988). There may be potential mechanisms underlying the seemingly random combination of diaphragmatic, pulmonary, cardiovascular, gonadal and other defects (Benjamin, Juul et al. 1988; Losty, Vanamo et al. 1998; van Dooren, Goemaere et al. 2004). In both animal models and humans a single gene defect can cause a global embryopathy, the result of a disruption of fundamental developmental processes during organogenesis. Congenital diaphragmatic hernia has been described as part of genetic syndromes (Slavotinek 2005) and also non-syndromic birth defects when a chromosomal or gene abnormality cannot be established. It is possible to hypothesise that a mutant gene as yet unidentified is responsible for the range of anomalies in these non-syndromic cases including congenital diaphragmatic hernia and disorders of sex development (Pober 2007).

In humans, mutations in *SRY, SOX9, SF1, WT1 or DHH* as well as duplications of *DAX1* or *WNT4* can disrupt the balanced network of gene expression causing XY gonadal dysgenesis or XY sex reversal (Fleming and Vilain 2005). These together explain 20% of the cases of disorders of sex development, as previously stated most cases remain unexplained implying that a number of sex determining genes remain undiscovered (Bagheri-Fam, Sim et al. 2008).

One mechanism for this embryopathy of diaphragmatic and extradiaphragmatic defects including disorders of sex development is a mesenchymal hit hypothesis. There are similar signalling pathways in all the affected organs and the function of the mesenchymal cells may be perturbed by environmental or genetic factors (Keijzer, Liu et al. 2000; Featherstone, Connell et al. 2006; Jesudason 2006; Jesudason, Smith et al. 2006). Germline mutations in transcription factors lead to impaired structural integrity and development of the affected organs. Several factors have been identified that have a multi organ developmental role including *WT1, FGF9*, Retinoids, *FGFR2, FGF10, FOG2* and *GATA4,* (Lin, Tsai et al. 2010).

Correct dosage of *fog2* and *GATA4* transcription factors are required for fetal testes development in mice (Bouma, Affourtit et al. 2007; Bouma, Washburn et al. 2007).

FOG2, a zinc fingered transcription factor is expressed in the embryonic diaphragm, lung mesenchyme, epicardium, myocardium and testicular somatic cells (Tevosian, Deconinck et al. 1999; Ketola, Anttonen et al. 2002). The *FOG2* gene is on chromosome 8q23 and rearrangements on 8q22-23 have been observed in patients with congenital diaphragmatic hernia. Patients who are heterozygous for loss of function point mutations of *FOG2* have diaphragm anomalies, (usually eventration), pulmonary hypoplasia, and may have cardiac anomalies (Ketola, Anttonen et al. 2002; Anttonen, Ketola et al. 2003; Ackerman, Herron et al. 2005). *Fog2* null mice also have male to female sex reversal (Tevosian, Albrecht et al. 2002; Ackerman, Herron et al. 2005). During the initiation program of testes development the primary function of *SRY* in mouse is impaired in the absence of *fog2* (Tevosian, Albrecht et al. 2002).

GATA4, also a zinc fingered transcription factor, interacts with *FOG2* during embryonic development and has also been implicated in congenital diaphragmatic hernia and associated anomalies. The human *GATA4* gene is located on chromosome 8p23.1 and microdeletion of this region is a recurring finding in patients with congenital diaphragmatic hernia (Pecile, Petroni et al. 1990; Faivre, Morichon-Delvallez et al. 1998; Lurie 2003; Barber, Maloney et al. 2005; Shimokawa, Miyake et al. 2005). *GATA4* and *Fog2* interactions for normal organogenesis are confirmed by the fact that homozygous mice that have a *GATA4* mutation disrupting the *GATA4 / fog2* interactions have male to female sex reversal as well as pulmonary and cardiac malformations (Tevosian, Albrecht et al. 2002; Barber, Maloney et al. 2005). Patients heterozygous for *GATA4* loss of function mutation have cardiac malformations but it not known whether point mutations or small deletions can cause defects (Garg, Kathiriya et al. 2003; Okubo, Miyoshi et al. 2004; Nemer, Fadlalah et al. 2006).

Basic helix loop helix proteins govern cell fate and differentiation in a variety of tissues. Subgroups of these factors have an essential role in the embryonic development of mesodermal tissue, and *pod-1* was one of the first of these subgroups to be described. It was found to be expressed at sites of epithelial mesenchyme interaction in the developing kidney, lung, intestine and pancreas (Quaggin, Vanden Heuvel et al. 1998).

There are several syndromes which include congenital diaphragmatic hernia and disorders of sex development amongst its recognised features. In some of these

syndromes the causative gene is known but in other cases the causative gene has yet to be discovered.

1.5.1 Syndromes with Features Including Disorders of Sex Development and Congenital Diaphragmatic Hernia

1.5.1.1 Denys Drash, Frazier, WAGR and Meacham Syndromes, Mutations in Wilms Tumour 1 gene

Wilms Tumour 1 *(WT1),* a zinc finger transcription factor, is the product of the Wilms tumour suppressor gene and expressed in the amuscular diaphragm, pleural and abdominal mesothelial cells, epicardium, testicular somatic cells and the developing kidney (Kreidberg, Sariola et al. 1993; Moore, McInnes et al. 1999; Natoli, Alberta et al. 2004). Heterozygous *WT1* losses of function mutations cause a range of overlapping clinical syndromes for example Denys-Drash syndrome, Meacham syndrome, Frasier syndrome and WAGR (Wilms tumour-Aniridia-Genitourinary anomalies-Mental retardation). These syndromes have clinical features of congenital diaphragmatic hernia, cardiac malformations and genitourinary defects.

The *WT1* gene has many isoforms that are critical for the formation of mesenchymal tissues, kidney and gonads. WT1 null mice have defects in the pleuroperitoneal fold (Clugston, Klattig et al. 2006) however not every case of Denys Drash or Frazier syndrome has a congenital diaphragmatic hernia. This indicates loss of function of *WT1* is a predisposing factor but other factors, possibly genetic or environmental are required for the expression of the diaphragmatic defect (Little and Wells 1997).

1.5.1.2 Fryns Syndrome

The multiple developmental anomalies of Fryns syndrome were first described in 1978 and 1979 (Fitch, Srolovitz et al. 1978; Fryns, Moerman et al. 1979). The features of Fryns syndrome include congenital diaphragmatic hernia, pulmonary hypoplasia, genitourinary anomalies, cardiovascular malformations and several other anomalies. Fryns syndrome is the most common multiple congenital anomaly syndrome associated with congenital diaphragmatic hernia and is thought to be inherited in an autosomal recessive manner (Slavotinek 2004; Gremeau, Coste et al. 2009).

The cause of Fryns Syndrome is at present unknown (Slavotinek 2004).

1.5.1.3 Swyer Syndrome

Children with Swyer syndrome have XY gonadal dysgenesis. There is male to female sex reversal and occasionally congenital diaphragmatic hernia. The gene involved is at present unknown but this syndrome may be caused by a mutation or deletion of *SRY* as 30% of human Swyer syndrome cases have some mutation of the *SRY* gene. However these human cases with a mutation of the *SRY* gene do not include any cases in which a congenital diaphragmatic hernia is present (Pober, Russell et al. 1993).

1.5.1.4 CHARGE Syndrome

CHARGE is an acronym for a syndrome summarised by the main features, coloboma, heart defect, atresia chonae, retarded growth and development, genital anomalies and ear anomalies. Congenital diaphragmatic hernia is an occasional finding (Casaccia, Digilio et al. 2008).

A mutation on the Chromodomain-helicase-DNA-binding protein 7 (*CHD7)* gene located on chromosome 8 has been identified in a high number of CHARGE syndrome cases (Vissers, van Ravenswaaij et al. 2004; Lalani, Safiullah et al. 2006). *CHD7* is chromatin remodeler and is essential for the formation of migratory neural crest cells that give rise to many structures throughout the embryo. It is essential for activation of *sox9* in the otic placode and neural crest (Bajpai, Chen et al. 2010) and thus could be necessary also in other organs as not yet identified. Kallman syndrome a mild variant of CHARGE syndrome is also caused by *CHD7* mutations but these mutations are less severe (Kim, Kurth et al. 2008).

1.5.1.5 Spondylocostal Dysostosis and Spondylothoric Dysplasia

Spondylocostal dysostosis is characterised by defective development of the axial skeleton with abnormal rib and spinal development, cardiac defects, vascular defects, disorders of sex development and in some cases congenital diaphragmatic hernia. It has been speculated that the cases of congenital diaphragmatic hernia may have been caused by the chest abnormalities interfering mechanically with diaphragm development (Slavotinek 2007).

All genes identified in cases of Spondylocostal dysostosis are involved in Notch signalling, early embryonic patterning and somite formation in the mouse (Bulman, Kusumi et al. 2000; Whittock, Ellard et al. 2004; Whittock, Sparrow et al. 2004;

Sparrow, Chapman et al. 2006) but the expression of these genes in human somite formation has not been determined (Sparrow, Chapman et al. 2006).

Spondylothoric dysplasia has phenotypic similarities with Spondylocostal dysostosis but with a different rib maldevelopment, disorders of sex development, malformed vertebrae (Day and Fryer 2003) and congenital diaphragmatic hernia has been reported in some cases (Shehata, El-Banna et al. 2000). The genes involved in Spondylothoric dysplasia have not been identified and the diaphragmatic hernia may also be due to the chest abnormalities (Slavotinek 2007).

1.5.1.6 Craniofrontonasal Dysplasia

Craniofrontonasal dysplasia is caused by a mutation in the Ephrin B1 gene (Twigg, Kan et al. 2004; Wieland, Reardon et al. 2005) and is more severe in females. Ephrin B1 is involved in the migration of neural crest cells (Wieacker and Wieland 2005).

Congenital diaphragmatic hernia and sacrococygeal teratoma, a germ cell tumour are features that may occur with Craniofrontonasal dysplasia (Vasudevan, Twigg et al. 2006).

1.5.1.7 Serkal Syndrome

A mutation in the *WNT4* human gene is the cause of Serkal syndrome. This causes downregulation in the expression of *WNT4* mRNA levels and thus affecting the many *WNT4* pathways involved in human embryogenesis (Logan and Nusse 2004). *WNT4* deficiency results in human female to male sex reversal and cases including congenital diaphragmatic have been reported (Mandel, Shemer et al. 2008) but the *WNT4* deficient mouse has only partial sex reversal (Chassot, Ranc et al. 2008; Tomizuka, Horikoshi et al. 2008).

1.5.1.8 Cornelia- de-Lange Syndrome

Congenital diaphragmatic hernia is an uncommon but recognised clinical feature of Cornelia de Lange syndrome (Martinez-Frias, Bermejo et al. 1998; Slavotinek 2007), caused by a mutation in the Nipped-B-like *(NIPBL)* gene. The *NIPBL* gene provides the codes for delangin, a protein which helps control the activity of chromosomes during cell division. Delangin is also involved in DNA repair and regulates the activity of some of the genes involved in development. This mutation

leads to the production of a small non functioning version of delangin (Dorsett and Krantz 2009).

1.5.1.9 Turner syndrome

Turner syndrome is a condition in which a part of one of the X chromosomes is missing in females. Multiple anomalies are a feature of this syndrome which include gonadal dysgenesis and sometimes congenital diaphragmatic hernia (Cigdem, Onen et al. 2007).

1.5.1.10 Emanual syndrome

Emanuel syndrome is caused by a chromosome imbalance affecting chromosome 22 (Zackai and Emanuel 1980). In most cases one of the parents is a carrier with a balanced defect on chromosome 22 and is phenotypicaly normal. This syndrome has multiple anomalies including mental retardation, ear, cardiac, renal, diaphragmatic, anal and genital anomalies in males (Zackai and Emanuel 1980). This syndrome was named Emanuel syndrome in 2004 and was previously reported as 'partial trisomy 22' or 'partial trisomy 11' (Gremeau, Coste et al. 2009).

1.5.1.11 Simpson-Golabi-Behmel Syndrome

Simpson-Golabi-Behmel syndrome can affect many developing organs including the diaphragm and the genitourinary system (Iglesias, Centeno et al. 2008). Mutations of the glypican-3 gene *(GPC3)* which is involved in cell growth and proliferation are responsible for some cases of Simpson-Golabi-Behmel syndrome but other cases do not have identifiable mutations of the *GPC3* gene and the cause of this anomaly is at present unidentified.

1.6 Nitrofen, (2,4-dichloro-4'-nitrodiphynel ether)

1.6.1 Nitrofen Rat Model of Congenital Diaphragmatic Hernia

1.6.1.1 Suitability for research

Congenital diaphragmatic hernia is an abnormality affecting approximately 1 in 3000 live births with considerable morbidity and mortality. The development of animal models has enabled the detailed study of congenital diaphragmatic hernia and accompanying anomalies. In particular the nitrofen model has greatly increased our understanding of the development of this anomaly.

Nitrofen, (2,4-dichloro-4'-nitrodiphynel ether) is a toxic herbicide that was first reported by toxicologists in 1971 to cause congenital anomalies in rodents following oral administration to pregnant rodents, (for example skeletal, craniofacial, renal, cardiac, pulmonary and congenital diaphragmatic hernia). The developing lung anlage, heart and diaphragm are believed to be the principle target sites for nitrofen induced malformations (Ambrose, Larson et al. 1971; Costlow and Manson 1981; Nakao, Miura et al. 1981; Lau, Cameron et al. 1988; Kluth, Kangah et al. 1990; Kluth, Tenbrinck et al. 1993; Wickman, Siebert et al. 1993). These malformations are induced at an early embryonic stage of development in rodents and are strikingly similar to the pattern of anomalies associated with congenital diaphragmatic hernia in humans (Sweed and Puri 1993; Fauza and Wilson 1994; Losty, Connell et al. 1999). These results support the use of this animal model in the study the pathogenesis of congenital diaphragmatic hernia. Environmental agents have been implicated in the aetiology of human malformations.

1.6.1.2 Toxicology of Nitrofen

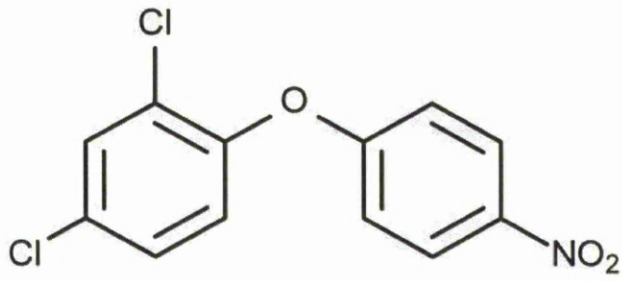
Toxicology studies have demonstrated that the teratogenicity of nitrofen is dose dependent and its effects are variable depending on the gestational age of the embryo. In the case of the rat model the most susceptible age for dosing of the dam to create congenital diaphragmatic hernia is between 9dpc and 11dpc, (term = day22), (Manson JM 1986, Costlow R 1981, Kluth D 1990, Tenbrinck R 1990). Maternal dosing by gavage on day 9.5dpc produces mostly left sided congenital diaphragmatic hernia, whilst dosing 10-11dpc produces mostly right sided congenital diaphragmatic hernia, (Kluth, Kangah et al. 1990). Dosing with nitrofen at 9.5dpc to create mainly left sided congenital diaphragmatic hernia corresponds closely with the time 24 hours before the lung anlage branches from the

oesophagus. Dosing at 11.5dpc is 24 hours after the primitive lung bud has developed and undergoing early development creates mainly right sided congenital diaphragmatic hernia. The optimum dosage of nitrofen chosen by Kluth and colleagues (Kluth, Kangah et al. 1990) to induce congenital diaphragmatic hernia in rat embryos was 100mg. During the period of susceptibility to nitrofen administration in the rat model (9 – 11dpc) there is a reduction in retinol levels in the rat due to increases in retinol utilisation (Takahashi, Smith et al. 1977). This may leave the embryo susceptible to perturbations of retinoid levels or function during this period. Also the developing lung which expresses retinal dehydrogenase and relies on retinoid mediated signalling for proper development (Malpel, Mendelsohn et al. 2000) is also compromised by nitrofen (Jesudason, Connell et al. 2000; Keijzer, Liu et al. 2000; Acosta, Thebaud et al. 2001).

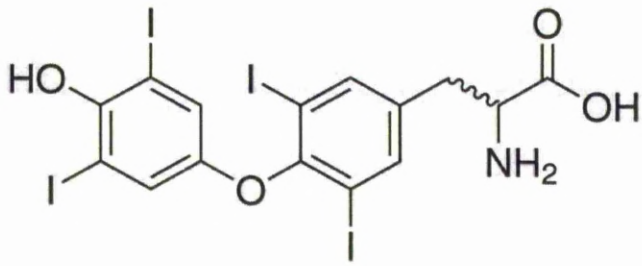
1.6.1.3 The Nitrofen Molecule ($C_{12}H_7Cl_2NO_3$)

Nitrofen is a structural analogue of thyroid hormones T3 and T4 (tridothyronin and thyroxine). It was believed to exert its teratogenic effects on maternal and or fetal thyroid hormone metabolism (Gray, Kavlock et al. 1982; Manson 1986). However more recently it is considered that nitrofen has no substantial role in the perturbations of thyroid hormone signaling or receptor function (Noble, Babiuk et al. 2007).

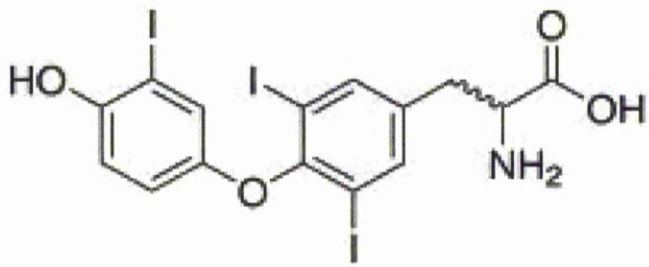
Figure 4 Nitrofen and thyroid hormone molecules



The Nitrofen molecule



T4 Thyroxine molecule



T3 Triiodothyronin molecule

Administering radio labelled nitrofen to pregnant rats demonstrated that only 1-5% of the product crosses the placenta. Radioactivity is detected in embryonic rat tissues within three hours of gavage administration to the dam reaching its maximum concentration at 72 hours. Nitrofen is degraded to hydroxylation, nitroreduction and acetylation products. In the embryo only the native nitrofen can be detected, therefore intermediate metabolites do not by themselves exert any teratogenic potential on the embryo (Manson JM 1986).

1.6.2 Impact of Nitrofen on the Molecular Processes of Development in the Rat Model of Congenital Diaphragmatic Hernia

1.6.2.1 Impact of Nitrofen on the Retinoic Acid Pathway

Nitrofen suppresses the action of retinaldehyde dehydrogenase-2, the key enzyme involved in retinoic acid production (Greer, Cote et al. 2000; Greer, Babiuk et al. 2003). The administration of retinoic acid to pregnant rats reduces the incidence of teratogenic effects of nitrofen on the diaphragm lung, thymus and heart (Thebaud,

Tibboel et al. 1999; Thebaud, Barlier-Mur et al. 2001; Yu, Gonzalez et al. 2002; Babiuk, Thebaud et al. 2004).

Genes associated with the retinoic acid pathway including Chicken ovalbumin upstream promoter-transcription factor 11 *(COUP-TF11),* GATA binding protein 4 *(GATA4)* and friend of *GATA 2 (FOG2).* These genes are all located on an area of chromosome that is disrupted in some cases of congenital diaphragmatic hernia, (a chromosomal 'hot spot' for congenital diaphragmatic hernia) (Doi, Sugimoto et al. 2009). It is speculated that the expression of these factors is altered after nitrofen exposure disrupting downstream genes in lung and diaphragm development (Doi, Sugimoto et al. 2009). The pulmonary gene expression of *coup-tf11* is upregulated in the early stages of development, 9.5dpc to 15dpc, of the nitrofen induced hypoplastic rat lung and this increased expression of *coup-tf11* may inhibit the retinoid signaling pathway (Doi, Sugimoto et al. 2009). Also treating pregnant nitrofen exposed rats with retinoic acid upregulates the pulmonary gene expression of *coup-tf11, GATA4*, and *fog2* in the offspring, but did not increase the expression of these genes in non nitrofen exposed offspring. This suggests that retinoic acid may have a role in modulating lung growth (Doi, Sugimoto et al. 2009).

Wilms tumour gene 1 *(WT1)* is also involved in the retinoid signaling pathway. Dingeman and colleagues provided evidence that *WT1* expression is reduced in nitrofen exposed rat fetuses. It is significantly downregulated in the pleuroperitoneal fold at 13dpc and in the developing diaphragm at 18 and 21dpc (Dingemann, Doi et al. 2011).

1.6.2.2 Impact of Nitrofen on the Myogenic Factors Involved in Diaphragm Development

Myogenic differentiation 1 *(MyoD)* and myogenic factor 5 *(Myf5)* have a critical role in muscularisation and mutants of *MyoD* have a reduced size diaphragm (Inanlou, Dhillon et al. 2003). However *Myf5* mutants do not have diaphragmatic defects. In the nitrofen exposed fetuses *MyoD* gene expression was reduced during the muscularisation process of diaphragm formation impairing the migration and proliferation of the muscle precursor cells (Dingemann, Doi et al. 2011).

1.6.2.3 Impact of Nitrofen on Heparan Sulphate Proteoglycans

Nitrofen has been shown to cause abnormalities in heparan sulphate fine structures and spatiotemporal distribution of heparan sulphate epitopes in the hypoplastic lungs of the nitrofen rat model of congenital diaphragmatic hernia. Fibroblast growth

factors *(FGF)s* are essential for lung morphogenesis, particularly *FGF2, FGF9* and *FGF10* and require heparan sulphate for receptor activation and subsequent signalling (Rapraeger, Krufka et al. 1991; Yayon, Klagsbrun et al. 1991; Ornitz, Yayon et al. 1992). The changes in the heparan sulphate structures were identified as those recognised by *FGF2* and *FGF9* (Thompson, Connell et al. 2011).

1.6.2.4 Impact of Nitrofen on Wingless-Related MMTV Integration Site Family Members *(WNT)*

The expression of several of the Wingless-Related MMTV Integration Site Family Members *(wnt)* is altered in the offspring of nitrofen exposed dams. The gene expression of *wnt5* is upregulated in the lungs of nitrofen exposed offspring during the mid to late stages of lung development (Doi and Puri 2009). *Wnt5* regulates sonic hedgehog *(Shh),* bone morphogenic protein 4 *(bmp4)* and *fgf10* expression in the developing lung and the upregulation of *wnt5* reduces the expression of particularly *fgf10* leading to reduced branching morphogenesis and pulmonary hypoplasia (Bellusci, Grindley et al. 1997; Cardoso, Itoh et al. 1997). Also *wnt2* and *wnt7* gene expression is downregulated in nitrofen exposed fetuses, both these genes are also involved with branching morphogenesis (Takayasu, Murphy et al. 2010).

1.6.2.5 Impact of Nitrofen on Fibroblast Growth Factors and their Receptors

Fibroblast growth factor receptor-like 1 is downregulated in the nitrofen model of congenital diaphragmatic hernia and this may contribute to the diaphragmatic defect in the nitrofen model (Dingemann, Doi et al. 2010; Dingemann, Doi et al. 2011).

Fibroblast growth factors 10 and 7 are also downregulated in the nitrofen model of congenital diaphragmatic hernia (Teramoto, Yoneda et al. 2003). *Fgf10* is critical for branching morphogenesis at the lung bud tip (Park, Miranda et al. 1998) as discussed in the previous section interacting with *Shh* and *bmp4* regulated by *wnt5* (Bellusci, Grindley et al. 1997; Cardoso, Itoh et al. 1997).

1.6.2.6 Impact of Nitrofen on Insulin Like Growth Factors, Receptors and Binding Proteins 3, 4 and 5

As previously discussed Insulin-like growth factor family is essential for lung development. They are involved with DNA synthesis, cell differentiation and survival (Rubin and Baserga 1995).

The gene expression of insulin-like growth factor binding proteins *(IGFBP)* 3 and 5 are downregulated in nitrofen induced pulmonary hypoplasia and may play a role in the development of the pulmonary hypoplasia by interfering with the retinoid signaling pathway (Dingemann, Doi et al. 2010; Ruttenstock, Doi et al. 2011; Ruttenstock, Doi et al. 2011). Insulin-like growth factor receptors 1 and 2 are downregulated in this model particularly on 21dpc in the rat nitrofen model, a critical time for alveogenesis (Ruttenstock, Doi et al. 2010). It can however be upregulated by prenatal administration of retinoic acid indicating that retinoic acid may promote lung growth by stimulating insulin like growth factor receptor mediated alveologenesis (Ruttenstock, Doi et al. 2011).

The insulin like receptor gene and protein expression is involved in surfactant synthesis (Felts 1964), and these are downregulated in the rat nitrofen model of congenital diaphragmatic hernia. This may interfere with normal surfactant synthesis during the later stages of lung development contributing to the pulmonary hypoplasia (Ruttenstock, Doi et al. 2010).

Overexpression of *IGFBP4* is reported in the nitrofen exposed fetal lungs during the later stages of development and may contribute to the pulmonary hypoplasia by inhibiting insulin like growth factor mediated cell proliferation (Ruttenstock, Doi et al. 2011).

1.6.2.7 Impact of Nitrofen on Slit2 and Slit3

Slit2 and *Slit3* and have a role in the differentiation and organisation of lung mesenchyme during branching morphogenesis and vasculargenesis (Greenberg, Thompson et al. 2004). *Slit2* and *Slit3* gene expression is upregulated in the nitrofen model of congenital diaphragmatic hernia and Doi and colleagues hypothesised that this would disrupt the organisation and differentiation of pulmonary mesenchyme leading to pulmonary hypoplasia (Doi, Hajduk et al. 2009).

1.6.2.8 Impact of Nitrofen on Parathyroid Hormone Signaling

Nitrofen downregulates the gene expression of parathyroid hormone in the nitrofen exposed fetus inhibiting the related protein signalling disrupting alveolar maturation and surfactant production (Doi, Lukosiute et al. 2010; Doi, Sugimoto et al. 2011). This downregulation of parathyroid hormone expression can be upregulated by treatment with retinoic acid in the lungs of the offspring of nitrofen exposed dams stimulating alveolar maturation and surfactant production (Doi, Sugimoto et al. 2011).

1.6.2.9 Impact of Nitrofen on Vascular Endothelial Growth Factor, Endothelin 1 and Endothelin Receptors A and B

Endothelin 1 is produced in vascular endothelial cells and affects vascular smooth muscle cells acting through its receptors, endothelin receptors A and B *(EDNRA* and *EDNRB)* (Boscoe, Goodwin et al. 2000) which control vasoconstriction, vasodilation and regulate vascular smooth muscle proliferation (Michael and Markewitz 1996; McCulloch, Docherty et al. 1998; Okazaki, Sharma et al. 1998). In the nitrofen induced hypoplastic lung the expression of *EDNRA* and *EDNRB* are upregulated leading to the pulmonary arteries becoming excessively muscularised therefore causing pulmonary hypertension (Dingemann, Doi et al. 2010).

Vascular endothelial growth factor *(VEGF)* also plays a key role in the development of the pulmonary vasculature (Shifren, Doldi et al. 1994; Patan 2000) and is downregulated in nitrofen induced congenital diaphragmatic hernia (Chinoy, Graybill et al. 2002; Chang, Andreoli et al. 2004). *VEGF* added to lung buds in culture from nitrofen exposed offspring and normal offspring accelerated airway branching (Shinkai, Shinkai et al. 2006).

1.6.2.10 Impact of Nitrofen on Sonic Hedgehog

Sonic hedgehog *(shh)* is essential for lung development and branching morphogenesis and its expression was reported to be unaltered in one report of nitrofen exposed offspring (Sato, Murphy et al. 2009). Previously Unger and colleagues reported that *shh* expression in nitrofen exposed offspring was initially downregulated and its expression peaked at a later stage than in non nitrofen exposed offspring (Unger, Copland et al. 2003). *Shh* was also seen to inhibit fetal lung fibroblasts in vitro therefore also interfering with extra cellular matrix formation (Unger, Copland et al. 2003).

1.6.2.11 Impact of Nitrofen on Extra Cellular Matrix Proteins

Lungs of mice with deleted retinoic acid receptor have reduced elastin content (McGowan, Jackson et al. 2000) and pulmonary elastin expression is reduced and the elastic fibres disorganised in the lungs of offspring of nitrofen exposed dams. The expression of elastin appears to be regulated transcriptionaly and the altered mechanical forces of the nitrofen model may have a role in the elastin anomalies (Mychaliska, Officer et al. 2004). The lungs of this model also had increased expression of tropoelastin and procollagen 1 leading to decreased compliance in the lung (Taira, Oue et al. 1999).

1.6.3 Impact of Nitrofen on Diaphragmatic Development

The pleuroperitoneal fold has become a focus of attention for studies into the pathogenesis of congenital diaphragmatic hernia in the rat nitrofen model. The pleuroperitoneal fold is a pyramid shaped tissue that extends medially from the lateral cervical wall to the oesophageal mesentery and fuses ventrally with the septum transversum. The substructure of the pleuroperitoneal fold is derived from mesenchymal cells migrating from the somatic mesoderm, followed by muscle precursors migrating from the dermomyotome of cervical somites following guidance clues provided by the somatopleure substructure (Greer, Cote et al. 2000). Greer and colleagues generated 3D reconstructions of the pleuroperitoneal fold of control and nitrofen exposed 13.5dpc rat embryos. They showed that in both left and right sided defects the malformations were always limited to the dorsolateral portions of the caudal regions of the pleuroperitoneal fold. This accounts for the fact that the diaphragmatic defects in nitrofen models are located in the dorsolateral regions on either the left side, right side or both sides (Greer, Babiuk et al. 2003).

The pleuroperitoneal fold in nitrofen exposed rats only showed gross abnormalities at 13 to 13.5dpc but the contribution of muscle precursors and the phrenic nerve were largely preserved in the abnormal pleuroperitoneal fold of nitrofen exposed embryos. This is consistent with the hypothesis that the pleuroperitoneal fold abnormalities arise from a defect in the nonmuscular mesenchyme of the pleuroperitoneal fold (Babiuk and Greer 2002). The muscle precursors in the developing diaphragm and the pleuroperitoneal fold of normal and nitrofen exposed embryos were tracked and muscle precursors appear to congregate, proliferate and differentiate normally in animals with diaphragmatic defects. There is a lack of regions of an underlying mesenchymal substratum in the pleuroperitoneal fold of the nitrofen exposed embryos (Babiuk and Greer 2002). This subsequently contributes to the defective herniated region thus this is consistent with the hypothesis that the underlying amuscular component specific to the diaphragmatic anlagen is defective in congenital diaphragmatic hernia. Babiuk and colleagues treated their *c-met* null mice, discussed previously, with the teratogens nitrofen and bis-diamine (also known to cause diaphragmatic defects) causing a defect in the mesenchymal scaffold also adding to the hypothesis that in congenital diaphragmatic hernia it is the membranous scaffold that is defective (Babiuk and Greer 2002). As there is no malregulation of myogenesis of the diaphragmatic muscle this explains why diaphragmatic muscle is affected but other skeletal muscle is not and also explains the muscular rim observed surrounding the diaphragmatic defect. Nitrofen exposed

rats have decreased rates of cell proliferation in the pleuroperitoneal fold, indicating this to be a primary mechanism of nitrofen teratogenesis leading to abnormal pleuroperitoneal fold development leading to congenital diaphragmatic hernia (Clugston, Zhang et al. 2010).

1.6.4 Impact of Nitrofen on the Lung

Newborns with congenital diaphragmatic hernia have a high mortality rate mainly caused by pulmonary hypoplasia and pulmonary hypertension. Lung hypoplasia had traditionally been considered a consequence of the diaphragmatic hernia due to compression of the lungs by the abdominal organs that had herniated into the thoracic cavity (Harrison, Bressack et al. 1980; Harrison, Jester et al. 1980) or that there was growth competition between the lung and the herniated liver (Kluth, Tenbrinck et al. 1993). With the advent of the nitrofen rat model of congenital diaphragmatic hernia model this causal relationship had to be reappraised as there were pulmonary abnormalities before the development of the diaphragm (Iritani 1984; Kluth, Tenbrinck et al. 1993; Jesudason, Connell et al. 2000).

As discussed previously the correct cascade of signals is required for initial lung bud formation followed by the development of the lung itself. Nitrofen is given to rats at day 9.5dpc in the case of this study, to create mainly left sided congenital diaphragmatic hernia at a time preceding foregut or lung development and therefore nitrofen has the potential to interfere with early growth and transcription factors (Whitsett 1998; Warburton and Lee 1999; Warburton, Zhao et al. 1999). Thus the nitrofen model enables the investigation of congenital diaphragmatic hernia before diaphragmatic abnormalities and this system therefore may be used to screen promoters of early pulmonary growth (Kluth, Kangah et al. 1990; Kluth, Tenbrinck et al. 1993; Kluth, Keijzer et al. 1996).

During the period of susceptibility to nitrofen administration in the rat model (9 – 11dpc) there is a reduction in retinol levels in the rat due to increases in retinol utilisation (Takahashi, Smith et al. 1977). This may leave the embryo susceptible to perturbations of retinoid levels or function during this period. Also the developing lung which expresses retinal dehydrogenase-2 and relies on retinoid mediated signalling for proper development (Malpel, Mendelsohn et al. 2000) is also compromised by nitrofen (Jesudason, Connell et al. 2000; Keijzer, Liu et al. 2000; Acosta, Thebaud et al. 2001). As previously discussed human cases of congenital

diaphragmatic hernia have also been shown to have reduced retinoid levels (Beurskens, Tibboel et al. 2010).

1.6.4.1 Lung Growth and Branching Morphogenesis

Using a terminal bud count as a measurement of branching morphogenesis in control and nitrofen rat lungs Jesudason and colleagues found that the nitrofen exposed lungs had a significantly reduced number of buds. A significant number of the nitrofen exposed lungs (36%) had an even more diminished lobar bronchi. This incidence of further stunted growth parallels the incidence of left sided congenital diaphragmatic hernia in this rat nitrofen model of congenital diaphragmatic hernia. They proposed that this dramatic disturbance of basic airway patterning specifically identified those embryos that would have developed a congenital diaphragmatic hernia (Jesudason, Connell et al. 2000). Fibroblast growth factors *(FGF)s* 1 and 2 added to culture media of control and nitrofen lungs demonstrated different effects on growth and branching. Normal 13.5dpc embryonic lungs cultured with *FGF1* showed enhanced growth but nitrofen exposed lungs showed decreased growth in the presence of *FGF1*. Conversely *FGF2* had minimal effect on the growth of normal lungs but the nitrofen exposed lungs showed an increase in lung area and the lungs were cystic and dilated (Jesudason, Connell et al. 2000).

1.6.4.2 Lung Growth and Cell Proliferation

Apoptosis and proliferation studies were performed in nitrofen exposed rat embryos from 13.5dpc until 16.5dpc. High levels of proliferation are detected in both normal and nitrofen exposed lungs at 13.5dpc but by 15.5dpc the nitrofen exposed lungs had a significant reduction in proliferation. This reduction was proportionately higher in the mesenchyme than in the airway epithelium (unpublished data). Both control and nitrofen exposed lungs showed low levels of apoptosis comparable to murine studies (Litingtung, Lei et al. 1998; Motoyama, Liu et al. 1998; Jesudason, Connell et al. 2000). These studies showed that reduced proliferation may play a role in lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia, increased apoptosis in the lung does not (Jesudason, Connell et al. 2000). Enhanced apoptosis in the cervical somites of nitrofen exposed rat embryos has been implicated as a mechanism for diaphragmatic maldevelopment (Alles, Losty et al. 1995). However with the somatic cell death in the cervical somites of nitrofen exposed rat embryos at 10.5dpc it remains possible that this enhanced apoptosis may induce embryonic lung hypoplasia by the removal of precursor cells earlier in development (Alles, Losty et al. 1995; Jesudason, Connell et al. 2000).

1.6.4.3 Lung Growth, Peristalsis and Fetal Breathing Movements

Prenatal airways from diverse species are capable of spontaneous peristaltic contractions in each trimester and were observed in chick and guinea pig over 80 years ago, reported by Lewis in the American Journal of Physiology in 1924. Airway smooth muscle has been described as the 'appendix of the lung' considered an evolutionary remnant that could cause lung disease in the future (Mitzner 2004). Another view suggests that airway smooth muscle is important in mammalian lung development driving airway peristalsis (Schittny, Miserocchi et al. 2000; Featherstone, Connell et al. 2006; Jesudason, Smith et al. 2006). The airway contractions produce intraluminal fluid flux in the developing lung distending the terminal buds of the lung and mechanical factors are known to regulate diverse aspects of morphogenesis including pre and postnatal lung development.

Airway smooth muscle dysfunction has been identified in the nitrofen model of congenital diaphragmatic hernia and pulmonary hypoplasia (Belik, Davidge et al. 2003). The contraction of airway smooth muscle cells is propagated by spontaneous calcium waves (Featherstone, Jesudason et al. 2005). In the nitrofen model of congenital diaphragmatic hernia the airway smooth muscle calcium transients were abnormal at an embryonic stage before diaphragmatic development leading to abnormal peristaltic patterns (Featherstone, Connell et al. 2006).

1.6.5 Impact of Nitrofen on Different Organs

1.6.5.1 Impact of Nitrofen on Cardiac Development

Cardiac anomalies are the most common associated defect in infants with congenital diaphragmatic hernia (Fauza and Wilson 1994) and are observed in the nitrofen rat model of congenital diaphragmatic hernia (Losty, Connell et al. 1999; Migliazza, Otten et al. 1999). The heart, lung and diaphragm may be particularly susceptible to nitrofen as the safety margin for retinoic acid mediated regulation of these organs is relatively low and thus more susceptible to perturbations than some other organs.

GATA4 function is first required in the visceral endoderm for normal heart development (Heikinheimo, Ermolaeva et al. 1997; Narita, Bielinska et al. 1997) and *GATA4* and its coregulator *GATA6* are downregulated in the hearts of nitrofen exposed embryos, also reduced were their target genes *wnt2, bmp4* and Myocyte specific enhancer factor 2c a gene involved in cardiac morphogenesis, myogenesis

and vascular development (Bi, Drake et al. 1999; Takayasu, Sato et al. 2008). *GATA4* has been reported to be regulated by retinoic acid signalling in cardiogenesis (Kostetskii, Jiang et al. 1999) and the nitrofen mediated perturbed retinoic acid and *GATA4* expression may contribute to the cardiac maldevelopment (Takayasu, Sato et al. 2008).

1.6.5.2 Impact of Nitrofen on Renal Development

There is considered to be an association between renal hypoplasia, lung hypoplasia and congenital diaphragmatic hernia (Glick, Siebert et al. 1990; Hosoda, Rossman et al. 1993). The kidneys in the nitrofen model of congenital diaphragmatic hernia have been found to be hypoplastic and this was the result of altered rennin-angiotensin system gene expression (Chertin, Nakazawa et al. 2006; Montedonico, Nakazawa et al. 2007). However, Montedonico and colleagues also found kidney weights and retinol expression reduced in this model but disputed the association between renal and lung hypoplasia reported by Glick (Hosoda, Rossman et al. 1993). Retinoids control kidney development through Ret expression (Gilbert 2002) and they suggested that nitrofen interfered with the retinoid pathway in kidney development and that the concentration of retinol is reduced in these kidneys (Montedonico, Nakazawa et al. 2007).

1.6.5.3 Impact of Nitrofen on Enteric Development

Intestinal rotation is often seen in human cases of congenital diaphragmatic hernia and it is assumed that this is caused by the abnormal thoraco-abdominal space that distorts the positioning of the gut during the reintegration of the umbilical hernia. The presence of a congenital diaphragmatic hernia increased the incidence of intestinal malrotation in the nitrofen model (Berman, Stringer et al. 1988; Baoquan, Diez-Pardo et al. 1995).

1.6.5.4 Impact of Nitrofen on Skeletal Development

Newborn human infants born with congenital diaphragmatic hernia may have a cluster of anomalies including skeletal defects. In a study by Migaliazza and colleagues to study the skeleton of the nitrofen exposed fetus they found abnormalities of the vertebrae, ribs and sternum (Migliazza, Xia et al. 1999) similar to those found in human cases.

CHAPTER 2

Hypothesis, Aims and Methods

2.1 Hypothesis and Aims

Human studies note disorders of sex development and sex differences in the incidence of congenital diaphragmatic hernia. Nitrofen is a teratogen that produces experimental congenital diaphragmatic hernia in rodents. The action of nitrofen has been speculated to perturb the retinoic acid pathway. Transcription factors linked to the retinoic acid pathway are involved with sexual differentiation.

This study was designed to test the hypothesis that nitrofen may influence the sexual phenotype and frequency of congenital diaphragmatic hernia in the rat model.

2.2 Methods

2.2.1 Purchase and Housing

Sexually mature Sprague Dawley female rats (dams) and male rats (seven to nine weeks) were purchased from Charles River UK Ltd and kept in standard conditions and fed a standard diet with permanent access to food and drinking water. These formed the basis of a breeding colony. The rats were weighed at regular intervals and prior to mating.

2.2.2 Experimental Methods

Sprague Dawley rats were mated overnight in wire bottomed cages, one male and one female to a cage. The presence of a vaginal plug the following morning indicating positive pregnancy and recorded as day zero. Full term of pregnancy of Sprague Dawley rats was considered to be 22 days. The dams, both those with identifiable vaginal plug and also those with no detectable plug were regularly weighed during the next days to check for expected weight gain results. Occasionally a dam without a detectable vaginal plug would be pregnant and a dam with a vaginal plug would not be pregnant. This was an infrequent occurrence but was checked for. Pregnant rats were kept two or three to a cage using one, two or three bands drawn round the tail with a permanent marker pen to differentiate individuals.

All procedures involving nitrofen were performed in a fume cupboard wearing protective equipment, gloves, face-mask and laboratory coat.

The concentration of nitrofen for dosing of rats was made up to 50milligrams (mg) nitrofen (Zhejiang Chemicals, Peoples Republic of China) dissolved in 1millilitre (ml) of olive oil.

The nitrofen solution was made by dissolving nitrofen into olive oil. To make 50ml of nitrofen solution, nitrofen (weight 2.5 grams) was added to about 20 mls of olive oil in a Schott 100 ml bottle and then the volume made up to 50 mls with olive oil. This was dissolved by placing the bottle of olive oil and nitrofen in a water bath on a heater stirrer and heating to 70°C stirring continuously until the nitrofen was dissolved. This was then cooled and labelled with the appropriate safety labels for mutatagen. The dissolved nitrofen was then kept at the Biomedical Services Unit in a fume hood which was also used for the dosing of pregnant dams.

Pregnant dams were gavage fed 2ml of the nitrofen solution on day 9.5 of gestation in the fume hood, this giving a dose of 100mg nitrofen dissolved in 2ml of olive oil. This was to produce predominantly left sided congenital diaphragmatic hernia. Controls (non nitrofen fed dams) were utilized for all comparative analysis. The nitrofen dosed dams were then kept in filter cages in a separate room. All waste bedding was double bagged and incinerated. A total of 39 nitrofen exposed pregnant dams were used for this study.

At day 20 or 21 of gestation pregnant dams were euthanized by an abdominal injection of pentabarbitone. The uterus was exposed and opened along its horns by cutting carefully with a pair of spring loaded scissors and was examined for evidence of fetal resorptions indicated by an area of blood stained clot of tissue. The fetuses were then recovered by opening the sac, exposing the fetal abdomen and giving an abdominal injection of pentabarbitone to confirm loss of life. The fetuses were then removed from the uterus by cutting the umbilicus.

All procedures were performed in accordance with UK Home Office legislation and regulations.

2.2.3 Photography

All photography was done using a Leica DFC300 digital camera mounted on a Leica WILD M8 stereo microscope. Lighting was from a Schott fibre optic light source.

Specimens were placed on thick black cotton velvet for photography to obtain a non reflective black background.

2.2.4 Examination of External Genitalia

The external genitalia of each pup were examined to determine sexual phenotype. The distance between the anus and genital organ was measured and studied. In females the distance between the anus and genital organ was shorter than in males. The males also had a crease in the perineum, the tissue between the anus and genital organ.

To photograph the external genitalia the fetuses were placed head down in a small beaker lined with black velvet for support and the tail held away using forceps exposing the external genitalia.

2.2.5 Examination of Internal Genitalia

To examine the internal genitalia of the fetuses they were pinned down on a cork board, pins holding the limbs and head clear of the abdomen and thorax.

The abdomen was opened using small springbow dissecting scissors (TAAB laboratories catalogue number S213). Starting at the umbilical cord cutting towards the pelvic region and then cutting towards the thorax being very careful to not cut sufficiently high to damage the diaphragm.

Using the curved edge of curved fine forceps TAAB laboratories fine tweezers number 7, the liver and gastro-intestinal tract was carefully removed to reveal the internal genito-urinary system and the diaphragm. Special care was needed to avoid damage to the ovaries as these were tucked behind the kidneys. Organs that had penetrated the thoracic cavity through the diaphragmatic hernia were carefully extracted exposing the thoracic cavity area that had been occupied by them. The liver was usually the organ that had invaded the thoracic cavity in all the different types of hernia observed. By using the curve of the forceps it avoided possible damage to the diaphragm from the points of the tweezers.

Internal genitalia were examined for the presence/absence of testes, ovaries and uterus with fallopian tubes. Findings of internal genitalia were checked with the findings of external genitalia for correlation.

For photographing the internal genitalia the fetuses were just laid on black velvet after the internal genitalia had been exposed.

2.2.6 Examination of the Diaphragm

The diaphragm was examined from the abdominal side and the site and position of a hernia was recorded if present or complete diaphragm examined.

Specimens were fixed in 10% buffered formalin and archived for photography. To obtain photographs of the abdominal surface of the diaphragm the abdominal organs were removed and the body wall was cut away to allow a clear view of the diaphragm and then the fetus was also placed head down in a small beaker lined with the black velvet to support it.

2.2.7 Recording of Results

All results from the examination of external and internal genitalia to define gender and from the examination of the diaphragm were recorded on a Microsoft excel spreadsheet.

Three separate tables were drawn from these results for analysis and a graph showing diaphragm morphology detailed below.

1 Table of gender and absence or presence and type of a hernia in nitrofen exposed offspring.

2 Table of results given as a percentage of the diaphragm examination of nitrofen exposed offspring.

3 Table of total numbers of hernias in nitrofen exposed offspring.

4 Graph of hernia distribution given as a percentage.

2.2.8 Statistics

The potential difference between susceptibility to congenital diaphragmatic hernia in male and female offspring of nitrofen exposed rat dams was calculated by two methods:-

1) Chi-squared test.
2) Odds ratio.

Both are recognised methods for analysing binomial discrete data.

CHAPTER 3

Results

3.1 Overview

In all Nitrofen exposed pups (n=518) internal and external genitalia concorded without evidence of significant genital tract malformations or intersex anomalies. For control non nitrofen exposed pups (n=300) diaphragm and genitalia were examined and no anomalies were found. To add to the figures for sex distribution the birth figures from the breeding colony were added to the total (n=300) By including the breeding colony figures an accurate figure of sex distribution could be obtained and this was an equal number of male and female pups. There was no evidence of excess foetal loss (i.e. prenatal resorptions) of either sex after nitrofen exposure as the ratios of male to female pups matched the control birth population.

Tables of distribution of diaphragmatic findings were created showing the numbers of nitrofen exposed fetuses and diaphragmatic defects in male and female offspring and graph of congenital diaphragmatic hernia distribution.

3.2 Gender

We were able to examine the external and internal genitalia in all the rat fetuses delivered from the dams. The morphology of the external genitalia was clearly defined in all cases. The internal genital morphology was also clearly defined and confirmed the findings of the external genitalia in all cases.

The following images show the morphology of the male and female external and internal genitalia.

3.3 External Genitalia of Male and Female 21dpc Rat Fetus

Figure 5 Male external genitalia

.

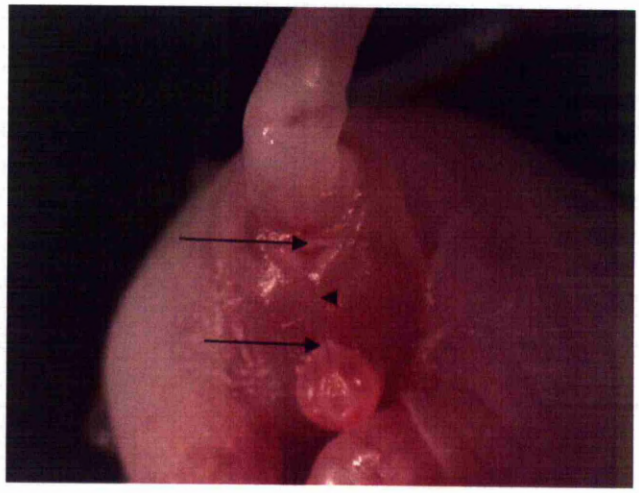


Figure 5

External genitalia of a 21dpc male rat fetus. The distance between the anus and the genital organ is greater than in the female indicated by the black arrows. A prominent crease is readily apparent in the perineum indicated by the short arrow.

Figure 6 Female external genitalia

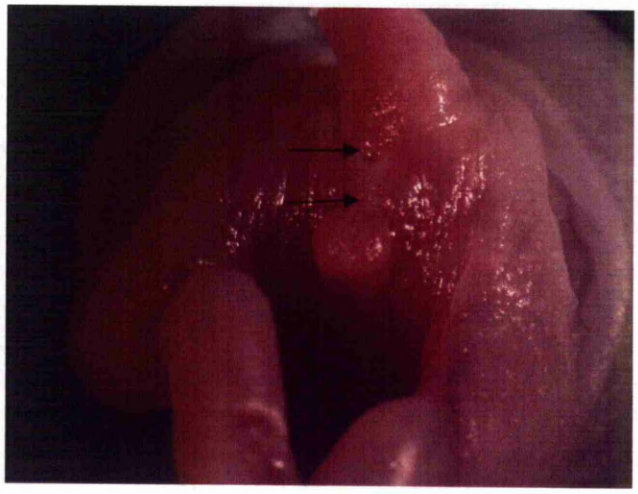


Figure 6

External genitalia of a 21dpc female rat fetus. The black arrows show the shorter distance between the anus and the genital organ compared to the male

Internal Genitalia of Male and Female 21dpc Rat Fetus

Figure 7 Male internal genitalia.

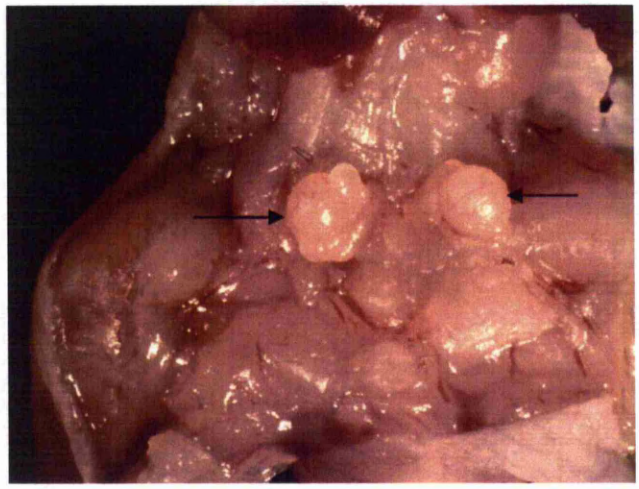


Figure 7

Internal genitalia of a male 21dpc rat fetus. The testes in the male are undescended at this stage indicated by the black arrows.

Figure 8 Female internal genitalia

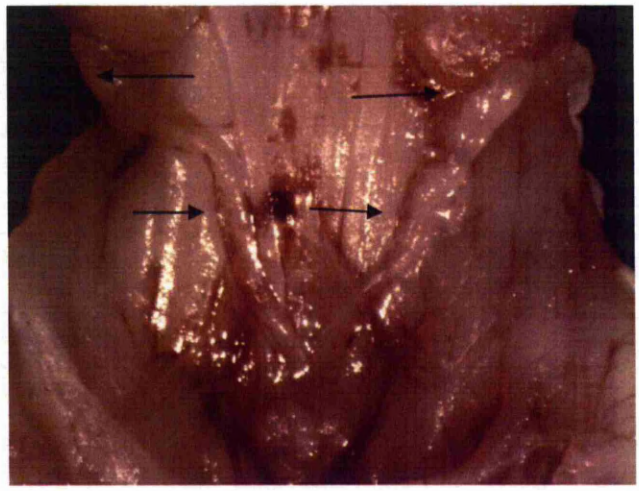


Figure 8

Internal genitalia of a female 21dpc rat fetus. The horns of the bicornuate female uterus and the ovaries are indicated by the black arrows

3.4 Diaphragm Images from Non Nitrofen Exposed (Control) and Nitrofen Exposed 21dpc (Without a Hernia) Rat Fetuses

Below is a series of images of the typical diaphragm findings

Figure 9 Control (non nitrofen exposed diaphragm)

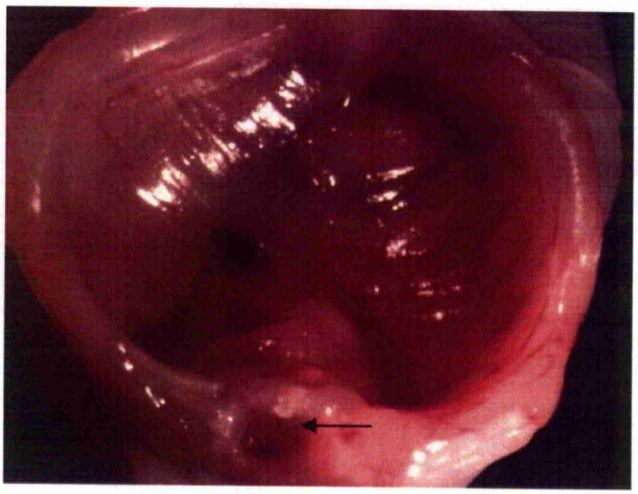


Figure 9

Diaphragm from a 21dpc non nitrofen exposed rat fetus. This shows a well muscularised diaphragm. The arrow indicates the position of the spine

The black arrow indicates the position of the spine in this figure and all subsequent diaphragmatic figures are in the same orientation

Figure 10 Diaphragm from a nitrofen exposed fetus without a hernia

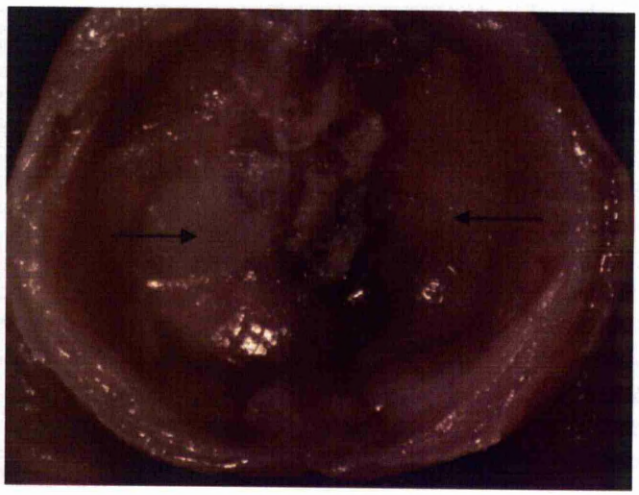


Figure 10

Diaphragm from 21dpc nitrofen exposed fetus without a hernia. The diaphragm is thinner than that of the control and the outline of the lungs are readily visible through it as marked by the black arrows. The diaphragm was also domed protuding into the area of the thoracic cavity, an eventration of the diaphragm

In the following figures of congenital diaphragmatic hernias the thickened rim of muscle surrounding the hernia can be seen.

Figure 11 Diaphragm showing left sided congenital diaphragmatic hernia

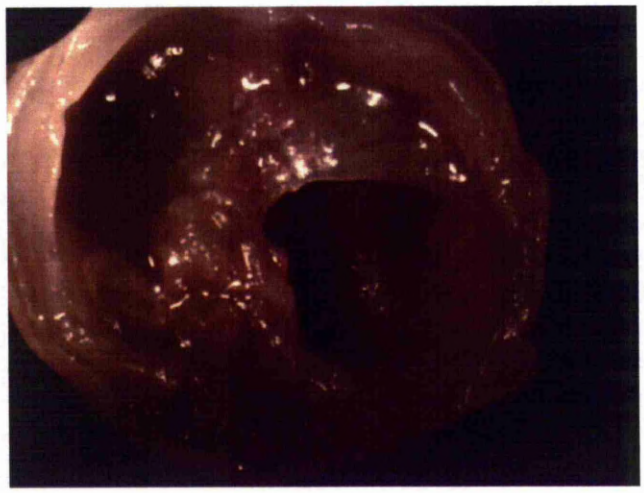


Figure 11

Diaphragm from 21dpc nitrofen exposed fetus showing a left sided congenital diaphragmatic hernia. The ribs can be seen through the diaphragmatic defect in the area of the thoracic cavity from which abdominal organs have been removed

Figure 12 Diaphragm showing right sided congenital diaphragmatic hernia

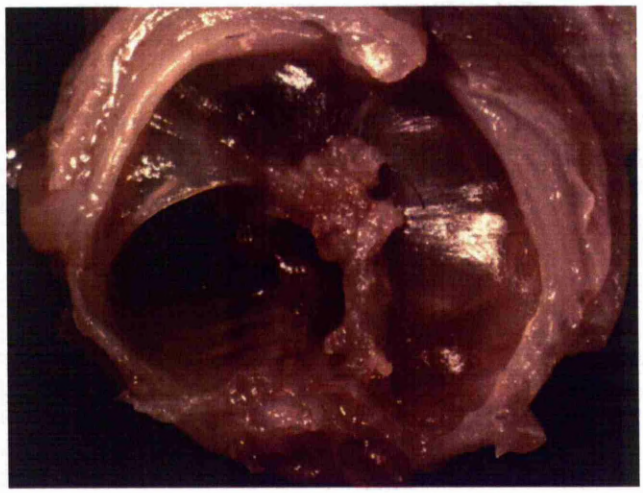


Figure 12

Diaphragm from a 21dpc nitrofen exposed fetus showing a right sided congenital diaphragmatic hernia. The ribs of the thoracic cavity can also be clearly seen through the hernia from where abdominal organs were extracted.

Figure 13 Diaphragm showing bilateral congenital diaphragmatic hernias

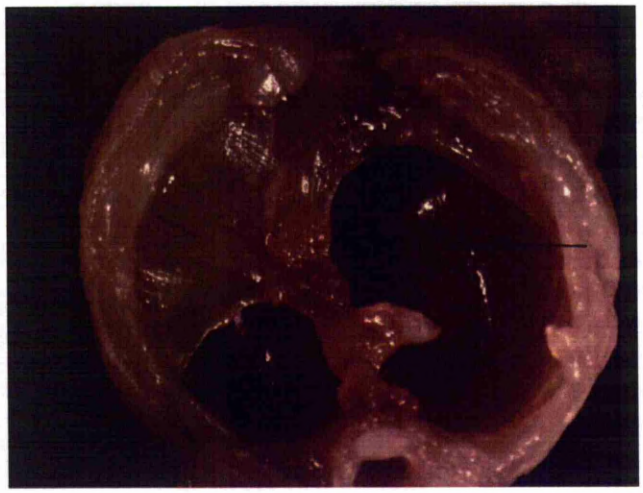


Figure 13

Diaphragm from a 21dpc nitrofen exposed fetus showing bilateral congenital diaphragmatic hernias. The ribs of the thoracic cavity can be seen through the hernias and the hypoplastic lung can be seen high in the thoracic cavity through the left sided hernia, marked by a black arrow.

Figure 14

Gender and results of examination for CDH from Excel spreadsheet

Male = M Female = F

	M	M	M	M	M		F	F	F	F	F
Dam no.	No CDH	L CDH	R CDH	Bil CDH	Total		No CDH	L CDH	R CDH	Bil CDH	Total
1	2	5	0	0	7		3	3	0	2	8
2	7	1	2	0	10		3	1	2	0	6
3	10	1	3	0	14		3	0	0	0	3
4	1	1	0	0	2		2	0	0	0	2
5	2	2	0	1	5		2	4	0	0	6
6	2	2	1	0	5		4	0	2	0	6
7	3	5	1	0	9		3	3	1	0	7
8	4	3	2	0	9		1	2	2	1	6
9	2	1	0	0	3		1	0	0	0	1
10	1	3	1	0	5		0	10	0	0	10
11	2	0	0	3	5		0	1	1	0	2
12	6	2	0	0	8		4	2	0	0	6
13	1	4	0	0	5		2	9	0	0	11
14	5	1	0	0	6		8	2	3	0	13
15	5	1	0	0	6		4	1	0	0	5
16	2	2	0	0	4		7	2	0	0	9
17	3	0	1	0	4		5	3	1	0	9
18	1	4	0	0	5		2	6	0	2	10
19	2	4	1	0	7		2	4	0	0	6
20	5	1	2	0	8		3	0	2	0	5
21	5	1	0	2	8		2	2	1	0	5
22	4	2	0	0	6		0	5	0	0	5
23	3	0	3	0	6		6	0	0	0	6
24	2	1	0	0	3		8	0	0	0	8
25	4	1	0	0	5		7	1	1	0	9
26	3	6	0	0	9		1	4	0	0	5
27	7	1	0	0	8		0	6	0	1	7
28	4	0	2	0	6		4	1	2	0	7
29	8	1	0	0	9		4	1	0	0	5
30	8	4	0	0	12		2	3	0	0	5
31	4	7	0	0	11		1	6	0	0	7
32	5	1	1	0	7		4	0	1	0	5
33	3	1	0	0	4		4	4	2	0	10
34	1	4	0	1	6		7	5	0	0	12
35	6	6	0	0	12		0	2	0	0	2
36	4	0	1	0	5		8	1	0	0	9
37	1	3	0	0	4		2	8	0	0	10
38	2	2	0	0	4		2	2	0	0	4
39	4	3	0	1	8		3	3	0	0	6
Totals	144	87	21	8	260		124	107	21	6	258

Table 1

Table showing numbers of male and female nitrofen exposed offspring with and without CDH

	Male	Female	TOTALS
No CDH	144	124	268
Left CDH	87	107	194
Right CDH	21	21	42
Bilateral CDH	8	6	14
TOTALS	260	258	518

Table 2

Table showing proportion (as percentages) of nitrofen exposed offspring with and without CDH

	Male	Female
No CDH	55.3%	48.1%
Left CDH	33.5%	41.5%
Right CDH	8.1%	8.1%
Bilateral CDH	3.1%	2.3%

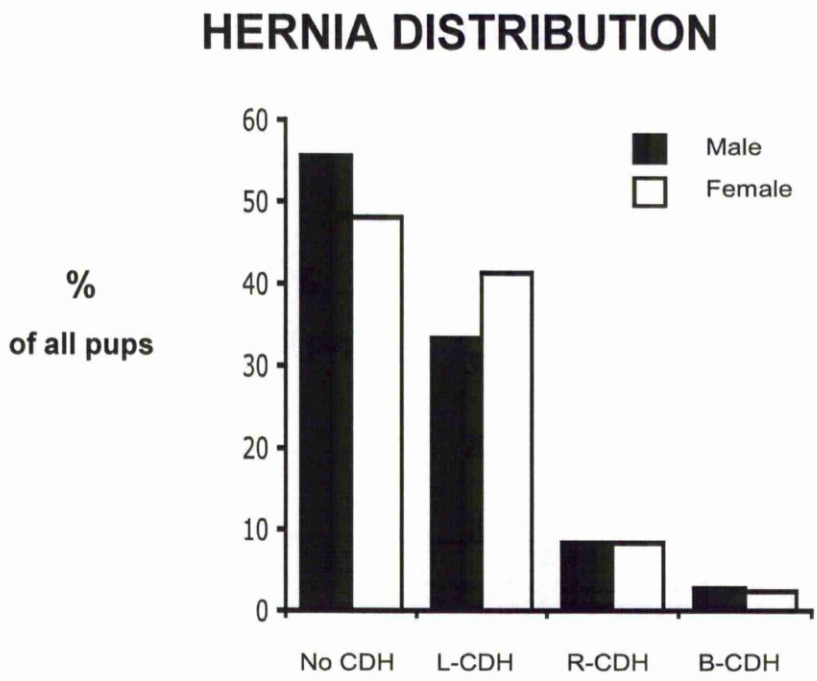
Table 3

Table showing the number of cases of CDH (as a total) in nitrofen exposed offspring

	Male	Female	TOTAL
No CDH	144	124	268
CDH	116	134	250
TOTAL	260	258	518

Figure 15

Graph showing CDH distribution as a percentage in nitrofen exposed offspring



Although there were more cases of congenital diaphragmatic hernia in the female offspring of nitrofen exposed dams this was not found to be significant. Chi squared test was performed to ascertain the significance of the results p=0.113.

An odds ratio was also calculated. The odds of a male rat fetus having a congenital diaphragmatic hernia is 0.75 of a female rat fetus. The confidence intervals recorded were 0.53 – 1.06. The confidence intervals span 1.0 thus indicating non-significance.

CHAPTER 4

Is There a Gender Susceptibility to Congenital Diaphragmatic Hernia in the Nitrofen Model of Congenital Diaphragmatic Hernia and does it have Disorders of Sexual Development? Discussion and Conclusions

Human congenital diaphragmatic hernia is frequently encountered with multiple co-existing anomalies, including cardiac, renal, skeletal, respiratory and genital tract anomalies (Sweed and Puri 1993; Fauza and Wilson 1994; Losty, Vanamo et al. 1998). Environmental agents are implicated in the aetiology of human malformations. Evidence from animal models suggest that it is a malformation of the mesenchymal substratum and the pleuroperitoneal fold that underlies the defect in the mature diaphragm and there is indirect evidence to suggest that this may also apply in human cases of Bochdalek congenital diaphragmatic hernia (Greer, Cote et al. 2000). Congenital diaphragmatic hernia can be produced experimentally in the offspring of nitrofen exposed rodents. Greer and colleagues have demonstrated that nitrofen interferes with retinoic acid signaling (Noble, Babiuk et al. 2007). Moreover, human infants with congenital diaphragmatic hernia have been demonstrated to have reduced levels of retinol binding protein (Beurskens, Tibboel et al. 2010). Retinoid signaling links with steroidogenesis and may influence downstream sexual differentiation pathways. This study tested the hypothesis that there may be gender susceptibility to nitrofen exposure and that intersex states may be present in the nitrofen model of congenital diaphragmatic hernia.

Congenital diaphragmatic hernia is a life threatening condition affecting approximately 1 in 3000 live births with a high mortality. It can be classed as isolated or complex and disorders of sex development can often accompany congenital diaphragmatic hernia. Currently congenital diaphragmatic hernia is considered to be found more frequent in human males (Robert, Kallen et al. 1997; Lisi, Botto et al. 2005) but historically the gender distribution reports have been ambiguous (David and Illingworth 1976; David 1980; David, Parker et al. 1980).

Although the results are not significant in this study more cases of congenital diaphragmatic were found in the female fetal rat population. The human studies concerning gender and the distribution of congenital diaphragmatic hernia included all cases whatever the causative mechanism may have been. In the rat model of

congenital diaphragmatic hernia normal embryological development occurs until the time of dosing with nitrofen at 9.5dpc. This would lead to a supposition that this model is more applicable to the human condition where the congenital diaphragmatic hernia may have been caused by environmental factors.

Disorders of sex development affect approximately 1 in 1000 live births and 1 in 4600 have ambiguous genitalia (Gillam, Hewitt et al. 2010). Disorders of sex development cause considerable problems to the patient, the patient's family and the clinical team deciding the best course for the long term welfare of the patient (Gillam, Hewitt et al. 2010; Gillam, Hewitt et al. 2011).

In both congenital diaphragmatic hernia and disorders of sex development, many cases have no known cause and also in some recognised syndromes including both of these anomalies amongst their features the causative agent is still unidentified. Research is continually adding to our understanding of these problems as shown by a recently published case concerning a mutation in a previously unidentified alternative pathway of dihydrotestosterone production. This mutation has been identified in a family and is the first case of this alternative pathway to be discovered in the human population, previously this pathway had only been identified in a marsupial (Fluck, Meyer-Boni et al. 2011).

4.1 Role of Animal Models in Understanding Organ Development

The advent of knock in, knock out and teratogenic animal models has greatly increased our understanding of the development of the embryo. These have enabled us to understand the relationship and interactions between genes, transcription factors, proteins and various pathways during development. The expression patterns of these factors is well preserved between species including invertebrates although the timing of the mechanisms involved may differ slightly (Ghabrial, Luschnig et al. 2003). Many mutant animal models have congenital diaphragmatic hernia, pulmonary hypoplasia and pulmonary hypertension combined with disorders of sex development allowing for a cautious translation of the role of these factors to the embryological development of these problems in the human population.

Disruption in the development of the mesenchyme, 'the mesenchymal hit hypothesis', is one mechanism describing the embryopathy of diaphragmatic and extradiaphragmatic defects including disorders of sex development. Signalling pathways are similar in all the affected organs and environmental or genetic factors

may cause a perturbation of these pathways disrupting the function of the mesenchymal cells (Keijzer, Liu et al. 2000; Featherstone, Connell et al. 2006; Jesudason 2006; Jesudason, Smith et al. 2006).

Germline mutations in transcription factors may lead to impaired structural integrity and development of the affected organs. Several factors have been identified that have a multi organ developmental role including *WT1, FGF9*, The retinoic acid pathway, *FGFR2, FGF10, FOG2* and *GATA4,* (Lin, Tsai et al. 2010).

One of the *bhlh* transcription factors capsulin *(pod1)* has been identified as causing congenital diaphragmatic hernia, disorders of sex development and other anomalies in mice but as yet no human cases have been identified (Lu, Chang et al. 2000; Lu, Bassel-Duby et al. 2002; Cui, Ross et al. 2004).

4.2 The Nitrofen Rat Model

The nitrofen rat model shows disruption in the expression of many of the factors involved in multi organ development. Given below are the factors involved particularly with the development of the diaphragm, lungs and the reproductive systems and the recognised syndromes including these anomalies.

Nitrofen suppresses the action of retinaldehyde dehydrogenase-2, the key enzyme involved in retinoic acid production (Greer, Cote et al. 2000; Greer, Babiuk et al. 2003).

Wilms tumour gene 1 *(WT1)* expression is reduced in nitrofen exposed rat foetuses. Mutations in *WT1* cause a range of overlapping clinical syndromes, Denys Drash, Frazier, WAGR and Meacham syndromes all of which may have clinical features of disorders of sex development and congenital diaphragmatic hernia. *WT1* is expressed in the amuscular diaphragm, pleural and abdominal mesothelial cells, the epicardium , testicular somatic cells and the developing kidney (Kreidberg, Sariola et al. 1993; Moore, McInnes et al. 1999; Natoli, Alberta et al. 2004).

Fryns syndrome is the most common multiple congenital anomaly syndrome associated with congenital diaphragmatic hernia although at present its cause is unknown (Slavotinek 2004; Gremeau, Coste et al. 2009). Acosta and colleagues created a Fryns syndrome type anomaly in a mouse by the administration of nitrofen. Although many of the anomalies present were typical of those caused by nitrofen administration and resembled some clinical features of Fryns syndrome no mention was made of disorders of sex development (Acosta, Chai et al. 2001).

Several genes associated with the retinoic acid pathway including Chicken ovalbumin upstream promoter-transcription factor 11 *(COUP-TF11),* GATA binding protein 4 *(GATA4)* and friend of *GATA 2 (FOG2.)* are all located on a chromosomal 'hot spot' for congenital diaphragmatic hernia. It is speculated that the expression of these factors is altered after nitrofen exposure disrupting downstream genes in lung and diaphragm development (Doi, Sugimoto et al. 2009). The pulmonary gene expression of *coup-tf11* is upregulated in the early stages of development, 9.5dpc to 15dpc of the nitrofen induced hypoplastic rat lung and this increased expression of *coup-tf11* may inhibit the retinoid signaling pathway (Doi, Sugimoto et al. 2009). Also treating pregnant nitrofen exposed rats with retinoic acid upregulates the pulmonary gene expression of *coup-tf11, GATA4*, and *fog2* in the offspring, (Doi, Sugimoto et al. 2009). The interactions and expressions of *GATA4* and *FOG2* are present in the early gonads of both sexes and mark the developing somatic cell lineage, Sertoli cells in the XY gonad and the granulosa cells in the XX gonad (Biason-Lauber 2010).

4.3 Nitrofen and Disorders of Sex Development

There are no references in the literature of nitrofen causing disorders of sex development. The only reference to nitrofen administration and internal and external sexual phenotype is this study (Connell, Corbett et al. 2006). However in this study only gross sexual phenotype was studied and possibly minor disturbances of sexual development may not have been detected, or that a study comprising larger numbers may have revealed some disorders of sex development. This study involved the administering of nitrofen at a specific time, 9.5dpc, and concentration, 100mg to cause predominantly left sided congenital diaphragmatic hernia. Therefore it cannot be assumed that nitrofen exposure at a different time or concentration would not produce disorders of sex development. Nitrofen perturbs factors involved in sex development and these factors may be expressed at a different time in sex development. The concentration of nitrofen in the embryo may be insufficient to interfere with sex development although it has been reported to affect renal development (Chertin, Nakazawa et al. 2006; Montedonico, Nakazawa et al. 2007).

4.4 Conclusions

From the paper published and presentation from this study, congenital diaphragmatic hernia was encountered with equal incidence in male and female

offspring using the current nitrofen dosing schedule. In conclusion, as reported in the paper, there is no sex risk for nitrofen induced congenital diaphragmatic hernia unlike conflicting human data (p=0.085) (Connell, Corbett et al. 2006). This thesis with increased numbers to the original publication also has no significant sex risk for nitrofen induced congenital diaphragmatic hernia (p=.113). This raises the possibility of a type 2 error, (i.e. if the numbers were increased a significant difference may be apparent). As this study could be considered to be more applicable to environmental causative agents it may be speculated that this may have a bearing on the difference in sex risk for congenital diaphragmatic hernia between this rat model and the human data. This may reflect the nature of this model, in that the offspring have no greater susceptibility prior to exposure with nitrofen.

The nitrofen litters did not show evidence of excessive prenatal resorptions of one sex or the other, (i.e. hidden mortality) as the ratio of males to females in the nitrofen exposed litters equalled the male to female ratio in the control population.

It is interesting to reflect on the possible mechanisms for no significant difference in the incidence of left congenital diaphragmatic hernia in the nitrofen exposed offspring especially when recent data from a number of human studies suggest an increased incidence in males (David and Illingworth 1976; David, Parker et al. 1980; Robert, Kallen et al. 1997; Lisi, Botto et al. 2005).

No evidence of gross morphological disorders of sex development were encountered in this study however minor disorders may have been present undetectable by the methods involved. The fertility of the offspring could not be assessed under the terms of the licence.

4.5 Future Work

This study looked at the relationship of congenital diaphragmatic hernia with disorders of sex development in the nitrofen rat model of predominantly left sided hernia produced by a specific dosing schedule of nitrofen at 9.5dpc. To create predominately right sided congenital diaphragmatic hernia dosing is carried out at 11.5dpc (Kluth, Kangah et al. 1990). A study using this dosing protocol to create predominantly right sided congenital diaphragmatic hernia was started and fetuses examined for diaphragmatic defects, gender and internal and external genitalia. This study was incomplete and insufficient data were obtained to draw conclusions. To

add to these numbers and check for disorders of sex development would complete this study.

To ascertain if nitrofen had an effect on sex development a timed series of dosing schedules with different concentrations of nitrofen would be required. A more detailed study of the internal and external genitalia than the gross morphology used in this study would be useful. Chromosomal analysis would improve the validity of sex determination.

Disorders of sex development and congenital diaphragmatic hernia with associated pulmonary problems cause considerable lifelong morbidity. These conditions place a drain on health resources. As understanding of developmental biology increases, so the mechanisms that underpin these processes will become more apparent. This thesis highlights potential linkage between certain developmental pathways, explaining to some degree co-morbidities and further research will hopefully clarify these relationships.

Publications and Presentations Arising from this Study

Sex and Congenital Diaphragmatic Hernia. **Connell MG**, Corbett HJ, Purvis A, Losty PD and Jesudason EC. Pediatr Surg Int 2006 Jan;22(1):95-8

Copy included after references

Sex and Congenital Diaphragmatic Hernia. **Connell MG**, Corbett HJ, Purvis A, Losty PD and Jesudason EC. Abstract presented to 18th International Symposium of Paediatric Surgical Research, Stockholm, September 29-October 1, 2005.

MG Connell Publications

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ORIGINAL ARTICLE

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Sex and congenital diaphragmatic hernia

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Abstract *Background and purpose*: Human studies note sex reversal syndromes and sex difference(s) in the incidence of congenital diaphragmatic hernia (CDH). Epidemiology surveys record a higher incidence of CDH in females, whilst other reports cite a higher frequency in males. Nitrofen, a teratogen, produces experimental CDH. This agent is speculated to interfere with retinoid acid–steroid signalling pathways and may also be linked with sexual differentiation. This study was designed therefore to test the hypothesis that nitrofen may influence sexual phenotype and frequency of CDH. *Methods*: Time mated Sprague Dawley rats were dosed with nitrofen at day 9.5 to generate predominantly left sided CDH. Fetuses were delivered by caesarean section on days 20 or 21 of gestation (term = day 22). External genitalia were examined to define external genital phenotype. The abdominal cavity was opened and the genito-urinary system carefully examined. The internal genital organs were assigned a phenotype and findings correlated with external appearances. The diaphragm of each fetus was studied for the absence or presence of CDH and the laterality of defect recorded. Controls (non nitrofen fed) were used for all comparative analysis. *Results*: Control ($n = 600$) and nitrofen exposed offspring ($n = 504$) had equal frequencies of males and females. CDH occurred with similar incidence in male and female nitrofen treated pups. In all nitrofen exposed fetuses and normal controls, internal and external genitalia concorded without evidence of significant genital tract malformations or intersex states. *Conclusions*: Prenatal nitrofen exposure is not associated with significant gender differences (or prenatal loss) in the risk of CDH. Genital tract malformations do not appear to accompany CDH in the nitrofen model.

Keywords Sexual phenotype · Congenital diaphragmatic hernia · Nitrofen

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Introduction

Congenital diaphragmatic hernia (CDH) is a birth defect affecting one in 3,000 live births with high mortality. Since 1971, nitrofen, an herbicide teratogen with homology to thyroid hormone, has been known to induce multiple abnormalities in rodents [1]. These include cardiac, renal, central nervous system, gastrointestinal, lung hypoplasia and CDH. Nitrofen dosing at varying gestational time schedules (e.g. days 9.5 and 10.5) yields a spectrum of malformations and diaphragmatic defects [2]. Studies have recently shown that nitrofen interferes with retinoid acid signalling [3–6]. This pathway links with steroidogenesis and may perturb sexual differentiation and phenotype. Whilst the cause of sporadic (non familial) CDH is unknown, it is of interest that sex reversal states (e.g. Meacham's and Swyer's syndrome) are reported with CDH [7, 8] Epidemiology surveys and human population registries note conflicting data on CDH and sex. Some early reports suggest a female predominance [9, 10] but latterly CDH has been cited as being more common in males [11, 12]. Is there gender susceptibility to CDH? This study was designed to examine whether nitrofen administration influences gender prevalence of experimental CDH and intersex anomaly states.

Materials and methods

Sprague Dawley rats (Charles River UK Ltd) were time-mated to generate offspring. Pregnant dams were gavage fed 100 mg Nitrofen (Zhejiang Chemicals,

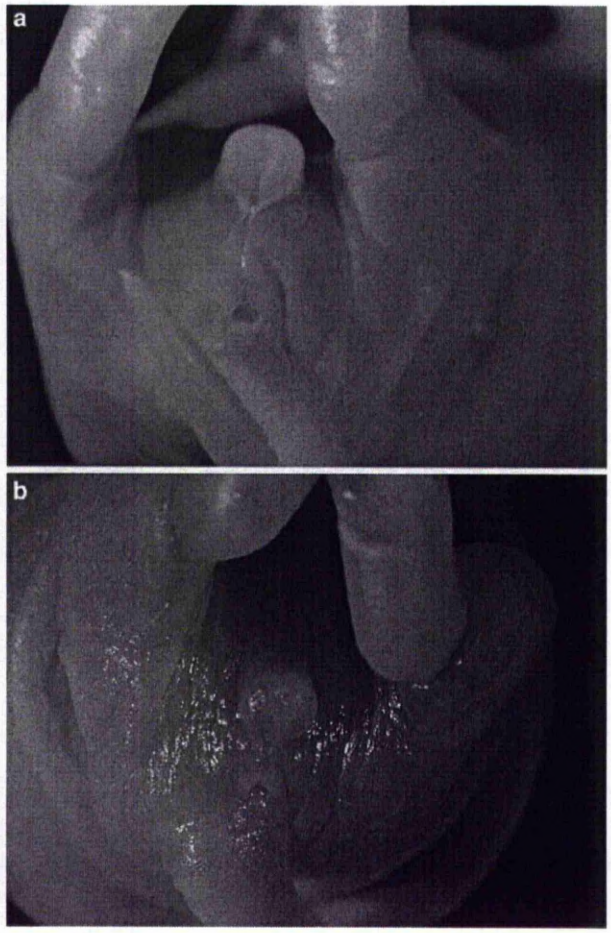

Fig. 1 External genitalia of a 21 days male (**a**) and female (**b**) rat fetus. Note the male has a greater distance between the base of the genitalia and the anal orifice versus female. A prominent skin crease is readily apparent in the male perineum

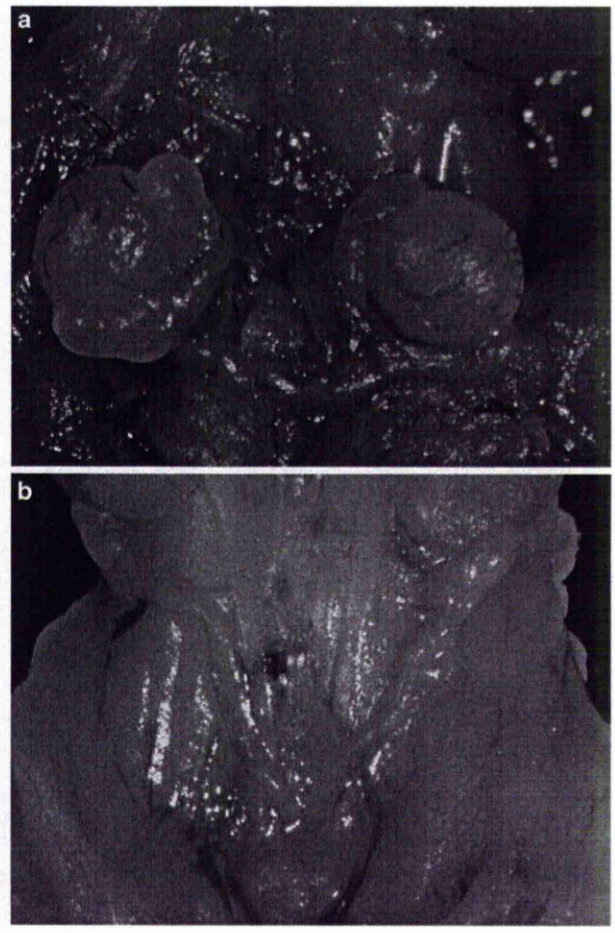

Fig. 2 Appearance of the internal genitalia of a 21 days male (**a**) and female (**b**) rat fetus. The testes in the male are undescended at this stage. The horns of the bicornuate female uterus are apparent. The ovaries are adjacent to the uterus/tubes

Peoples Republic of China) dissolved in 2 ml of olive oil on gestation day 9.5 to induce predominately left sided CDH (LCDH)—Term = day 22.5. Controls (non nitrofen fed) were utilised for all comparative analysis. Euthanised fetuses were recovered by elective caesarean section on days 20 and 21. The external genitalia of each pup was examined to determine sexual phenotype (Fig. 1). The abdomen was opened and the liver and gastro-intestinal tract carefully removed to reveal the internal genito-urinary system and the diaphragm. Internal genitalia were examined for the presence/absence of testes, ovaries and uterus with fallopian tubes (Fig. 2). Findings were correlated with external genitalia for intersex anomalies. The site and position of CDH was recorded (Figs. 3, 4). Fixed specimens (10% buffered formalin) were archived for clinical photography. All procedures were performed in accordance with the UK Home Office legislation and regulations.

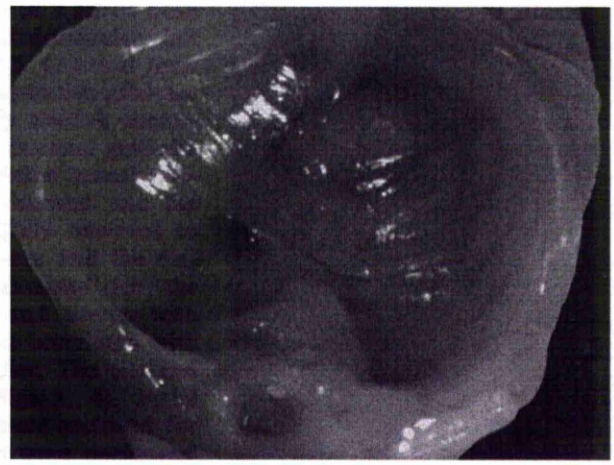

Fig. 3 Normal intact diaphragm (term control) viewed on cross section

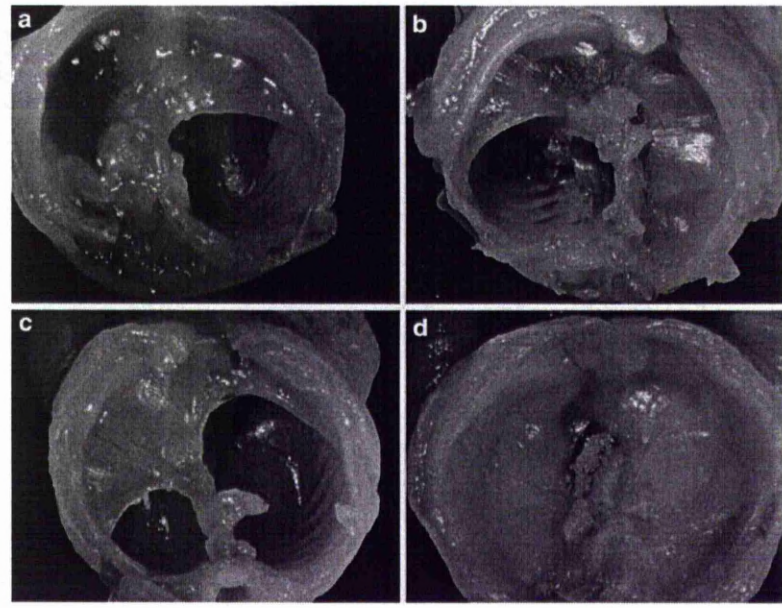

Fig. 4 Diaphragm anomalies from a selection of nitrofen exposed term rat fetuses. Panels demonstrate (**a**) Left CDH, (**b**) Right CDH, (**c**) Bilateral CDH and (**d**) Non-CDH

Results

Controls ($n = 600$) and nitrofen exposed pups ($n = 504$) had equal frequencies of males and females. CDH occurred with a similar incidence in male and female nitrofen treated pups. In all nitrofen exposed fetuses and normal controls, internal and external genitalia concorded without evidence of significant genital tract malformations or intersex states Findings are summarised in Fig. 5. There was no evidence of excess fetal loss (i.e. prenatal resorptions) in either sex after nitrofen exposure as the ratios of male: female pups matched the control birth population.

Discussion

Human CDH is frequently encountered with multiple co-existent anomalies indicating a major disturbance in embryological development [13–15]. The aetiology of this lethal birth defect remains largely unknown. Chromosomal defects and genetic disorders are reported whilst rare sex reversal states (e.g. Meacham's and Swyer's syndrome) [7, 8] are also noted in CDH. Birth registries and epidemiology surveys record conflicting data on gender and CDH with early reports from the 1960's suggesting female predominance. Later studies challenge these findings [9–12].

Environmental agents are implicated in the aetiology of human malformations. CDH can be produced experimentally in timed pregnant rodents with the herbicide nitrofen. Greer and colleagues have recently demonstrated that nitrofen interferes with retinoid acid signalling. Moreover, human infants with CDH have been demonstrated to have reduced levels of retinol binding protein [3–6]. Retinoid signalling links with steroidogenesis and may influence downstream sexual differentiation pathways. We therefore tested the hypothesis that there may be gender susceptibility and intersex states in the nitrofen CDH model.

Our findings demonstrate no sex at greater risk for CDH. Exposure to the teratogen—nitrofen at the

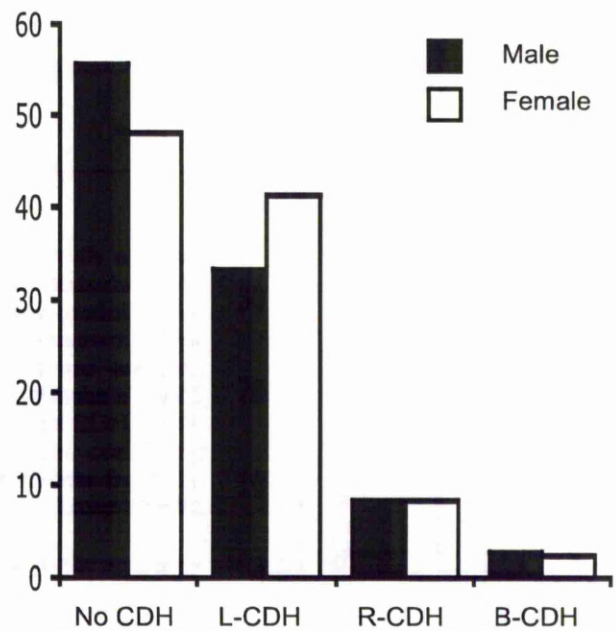

Fig. 5 Diaphragm anomalies (expressed as a percentage) in nitrofen exposed fetuses. There are no significant differences in incidence males versus females (Chi-square)

indifferent sexual stage (day 9.5) in the developing rat embryo does not perturb gross sexual differentiation. Intersex anomalies such as those encountered in humans (Meacham's, Swyer's syndrome) [7, 8] were not readily detected. Furthermore, nitrofen exposed litters did not show evidence of excessive prenatal resorptions in one sex or other (i.e. 'hidden mortality') [16]. CDH was encountered with equal incidence in male and female offspring using the current nitrofen dosing schedule. In conclusion, it appears from this experimental study that there is no sex risk for nitrofen-induced CDH (unlike conflicting human data).

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