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Recent Developments in the Genetic Factors Underlying Congenital Diaphragmatic Hernia

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Abstract
Congenital diaphragmatic hernia (CDH) is a birth defect affecting around 1 in 3,000 births and is associated with high mortality and morbidity. It has become increasingly apparent that genetic factors underlie many forms of CDH. We review the recent developments in the area of the genetics of CDH, including potential candidate genes supported by evidence from animal models. We also discuss the possible role in the pathogenesis of CDH of defective retinoid signalling and abnormal mesenchymal cell function.

Introduction

Congenital diaphragmatic hernia (CDH) is a defect in the development of the diaphragm with an incidence of around 1/3,000 births [1, 2]. CDH is associated with variable degrees of pulmonary hypoplasia and postnatal pulmonary hypertension which account for the high mortality and most of the morbidity observed for this severe birth defect. CDH can be anatomically divided into three main subtypes: a posterolateral 'Bochdalek' hernia representing around 70% of cases, an anterior 'Morgagni' type, accounting for around 27% of cases, and a central septum transversum hernia which totals around 3% of cases. The vast majority of hernias are left-sided (85%), whilst the remainder are right-sided (13%) or bilateral (2%) [1, 3, 4]. CDH occurs as an isolated defect in around 50% of cases, or as non-isolated CDH for the remainder in which additional congenital malformations are present [5]. Non-isolated CDH is associated with abnormalities in a number of other systems including the cardiovascular system (27.5%), urogenital system (17.7%), musculoskeletal system (15.7%), and central nervous system (9.8%) [5]. Non-isolated CDH may occur as part of a recognised syndrome for which a single causal gene may be identified, for example STRA6 in Mathew-Wood syndrome [6–9],

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WT1 in Denys-Drash syndrome [10, 11], LRP2 in Donnai-Barrow syndrome [12, 13], or EFNB1 in craniofrontonasal syndrome [14, 15]. Alternatively, non-isolated CDH may be associated with rearrangements in specific genetic loci, including 8p23.1 [16, 17] and 15q26 [18–20], or with a clinically recognised syndrome of currently unknown genetic cause such as Fryns syndrome [21–24]. Slavotinek [25] recently provided an excellent review of single gene disorders associated with CDH, and Bielinska et al. [26] have previously reviewed the molecular genetics of CDH.

It has become increasingly evident that genetic factors play an important role in the aetiology of CDH. However, it is still uncertain whether complex genetic factors are the sole cause of CDH. More research is essential to progress our understanding of both normal and abnormal diaphragm development. The evidence from teratogen- and dietary-induced CDH animal models is suggestive of environmental and/or nutritional factors also playing a role [reviewed in 27]. A recent study which investigated the vitamin A status of mothers and their newborns in association with CDH found a strong association with low retinol and retinol-binding protein (RBP) independent of maternal retinol levels [28]. These findings are more consistent with there being an abnormality in embryological retinoid homeostasis in the fetus, rather than maternal vitamin A deficiency being a risk factor. More research in this area is certainly warranted since maternal malnutrition and lifestyle factors are easily modified, which may potentially reduce the risk of CDH and other congenital malformations.

The exact genetic causes of isolated CDH remain elusive despite extensive research. Given the high mortality and morbidity observed in CDH, the current fetal and/or neonatal therapies are understandably focused on improvement of lung growth and/or function, with surgical correction of the diaphragmatic defect being a relatively 'simple' procedure in comparison. It is important to understand the role genetics has to play in cases that are today considered as 'isolated' CDH. Since these patients, when they also have a poor prognosis, are the focus of fetal therapy by tracheal occlusion [29], it is this cohort of isolated CDH patients who are likely to benefit most from future therapeutic targets. Improving our understanding of the pathogenesis of CDH will reveal novel targets and pathways for future fetal therapies. In this review we will focus on isolated CDH and the recent developments and approaches which can improve our understanding of the aetiology of isolated CDH.

Genetics of Congenital Diaphragmatic Hernia

There are more than 70 syndromes in which diaphragmatic hernias have been observed [25]. However, whether CDH has a genetic cause for all of these disorders or is merely an incidental occurrence with unique genetic aetiology in addition to the other observed malformations is uncertain. For a number of these disorders a single gene has been identified. These include genes for transcription factors, molecules involved in cell migration, and extracellular matrix components [26]. The elucidation of the genetic mechanisms by which these genes exert their phenotypic effects may shed light on the cause of isolated diaphragmatic defects and the specific role that these genes play in diaphragm development.

The variable penetrance of CDH in teratogen-induced and genetic models of different strains [30] is suggestive of a genetic background which confers a susceptibility to the development of CDH. However, the observation that CDH in humans is strongly associated with a number of genomic regions, particularly loci at 15q26 (OMIM 142340, DIH1), and 8p23.1 (OMIM 222400, DIH2), as well as numerous genetic disorders provides support to genetic factors being the main underlying cause of syndromic or non-isolated CDH. Still, the observation that CDH does not display full penetrance for genetic disorders or genomic loci adds further weight to as yet unidentified regulatory factors or environmental influences also playing an important role.

The number of loci associated with CDH is extensive and scattered across the genome [reviewed in 31]. This is suggestive of common developmental pathways being disrupted by numerous genes at locations spread across the genome. Interestingly, some of the loci which share a strong association with CDH, such as 15q26, 8p23.1, and 8q23, harbour genes involved in the retinoic acid (RA) pathway. It is proposed that defects in a number of genes involved in the RA pathway result in the similar phenotypic outcome of CDH (the retinoid hypothesis) [32].

One approach to identify the genes involved in diaphragm development is to study genomic regions recurrently associated with CDH patients, to identify genes responsible for diaphragm and lung development. The technique of array comparative genomic hybridisation is one such method which has demonstrated the ability to refine the critical regions associated with various disorders, including CDH [18, 19, 33–35]. Further use of this technique for both isolated and non-isolated CDH patients will help to determine whether loci such as that at 15q26, 8p23.1 and 1q41–42 truly represent contiguous

gene deletion syndromes causal for non-isolated CDH, or if we can further refine the critical regions and identify single genes in these regions responsible for cases of isolated CDH. There are a number of software applications which can be used to prioritise and rank potential candidate genes based upon factors such as expression, interaction information, protein structure, sequence similarity, and other available information. Many of these are freely available as web applications, including Endeavour (www.esat.kuleuven.be/endeavour/) [36, 37]. This type of bioinformatics approach may complement current research findings and assist in identifying the most plausible candidates for further research.

Animal Models

A wealth of information has been obtained from teratogen- or surgically-induced animal models, as well as from genetic and transgenic models of CDH. Many research efforts, particularly those using surgically-induced CDH models, have focused on the pathogenesis of the altered pulmonary airway and vascular development. This is understandable since it is the lung hypoplasia and pulmonary hypertension which are the factors responsible for the immediate mortality and contribute largely to the long-term morbidity often seen in CDH patients. Aside from the disadvantages of using teratogen-induced models to study the genetics of CDH, the nitrofen-induced CDH model has provided a better understanding of normal and abnormal diaphragm development at the anatomical level. Furthermore, the nitrofen model has also highlighted differences in gene expression in the diaphragm and lungs, as well as providing further evidence of the RA pathway as having a causative role in the pathogenesis of CDH. The mechanism of action of nitrofen which was for long uncertain has now been demonstrated to occur by inhibition of the rate-limiting enzymes involved in RA synthesis, most likely by suppression of RALDH2 activity and thus retinoid signalling [38–40].

Among the most interesting research developments using teratogen-induced animal models is the recent investigation of abnormal retinoid signalling from Clugston et al. [40]. In this study, a number of teratogens were used to induce CDH in RARE-lacZ transgenic mice providing further evidence that disruption of retinoid signalling causes CDH, and is not specific to the nitrofen model alone. In common with other studies, dietary supplementation of RA was shown to reduce the incidence of diaphragm defects from 58.7 to 1.3% supporting the concept of possible therapeutic intervention through maintaining normal retinoid levels, and thus retinoid-dependent signalling, during development. Expression analysis in the pleuroperitoneal folds (PPFs) showed that proteins involved in the retinoid signalling pathway are expressed within this structure which will be discussed later. Of particular interest, this study generated a novel CDH model using the pan-RAR antagonist BMS493 to pharmacologically block retinoic acid receptor (RAR) signalling. This model produced a Bochdalek CDH phenotype, which is the most common type observed in humans. Furthermore, the timing of teratogen exposure correlated with the side of the diaphragm defect, i.e. early administration (E8-E9) generated predominantly left-sided CDH, whilst late administration (E11-E13) generated predominantly right-sided CDH, and exposure on E10 produced left- and right-sided CDH as well as bilateral CDH. These results are suggestive of different critical time periods in development of the left or right side of the diaphragm.

Historical evidence from vitamin A-deficient (VAD) models have long implicated the RA pathway as having an important role in diaphragm development and therefore being a potential target for fetal therapy. More recently, genetic models have demonstrated the phenotypic effects of faults in specific genes, and further research in these models and the development of novel genetic models has the potential to pinpoint the roles that individual candidate genes play in diaphragm and lung development. Table 1 summarises the genetic models which have been generated to study CDH, or in which diaphragm and/or lung defects have been observed.

The Retinoid Signalling Pathway and the Retinoid Hypothesis

Vitamin A is essential for various aspects of early embryonic development, as demonstrated by the spectrum of abnormalities observed in VAD animal models [78]. The observation that CDH is associated with VAD models, and with teratogen-induced models such as nitrofen, which disrupt the RA, or retinoid signalling pathway, provides strong evidence for a link with diaphragm development and this pathway [39, 40, 79–83]. More recently, genetic models for various genes known to be involved in the RA pathway have provided further evidence of a link to CDH. These models include the FOG2 ENU mutant, the COUP-TFII knockout model, the heterozygous $GATA4^{+/\Delta ex2}$ model, and the RAR double knockout mod-

Table 1. Mouse models generated to study CDH, or in which diaphragm and/or lung developmental abnormalities were observed incidentally

Gene/model	Type of defect	Gene function (OMIM)	Ref.
COUP-TF II/Tissue-specific null mutant mice	Bochdalek-type CDH	Orphan nuclear receptor, transcription factor. Involved in mesenchymal-epithelial interactions for organogenesis	41
FOG2 (ENU) mutant mice	Posterolateral muscularisation defect & severe pulmonary hypertension	Zinc finger transcription co-factor. Modulation of GATA4 function during cardiac development	42
c-met (–/–) mutant mice embryo	Amuscular diaphragm	Cell-surface receptor for hepatocyte growth factor	43
Gab1 (–/–) mice embryo	Amuscular diaphragm	Tyrosine phosphorylated upon stimulation of various cytokines, growth factors, and antigen receptors in cell lines	44
Gata4 (+/–) C57Bl/6 mice	Ventral hernia covered with sac	Zinc finger transcription factor, involved in cardiogenesis during foetal development	30
Lox (–/–) mice	Central diaphragmatic rupture, aortic defects	Extracellular copper enzyme, initiates cross-linking of collagens & elastin	45
Lox (–/–) mice embryos	Primary developmental defect of airways, independent diaphragm rupture		46
Wt1 (–/–) mice	Bochdalek hernia & cardiac anomaly	Zinc finger transcription factor, acts as transcriptional activator or repressor	47, 48
Wnt7b (–/–) mice	Severe lung hypoplasia	Wnt signalling pathway	49, 50
Myogenin null mice	Lung hypoplasia, intact but amuscular diaphragm	Muscle-specific transcription factor, can induce myogenesis	51
mdx:MyoD$^{-/-}$ 9th mice	Abnormal muscularisation of diaphragm	bHLH transcription factor, regulates skeletal muscle differentiation	52
Capsulin null mice	Abnormal pulmonary development – absence of lung alveoli	bHLH transcription factor, involved in epithelial-mesenchymal interaction	53
MyoR$^{-/-}$ & capsulin double mutant mice	Posterior CDH, facial muscle defects	bHLH transcription factor, expressed in undifferentiated myoblasts, possible role as a lineage-restricted transcriptional repressor of the muscle differentiation program	54
Pax3-deficient transgenic Splotch mice	Absence of muscular diaphragm, thin fibrous membrane present	Transcription factor, involved in skeletal myogenesis	55
RARα/β2 double knockout mice	CDH, pulmonary hypoplasia, lung agenesis	Nuclear receptors, retinoic acid signalling is transduced by RAR & RXR heterodimers to regulate gene transcription	56, 57
RARα/β2 (+/–) double knockout mice			56
Single RAR null mutant mice	No diaphragm or lung abnormalities		58–60
Slit3 (–/–) mice	Central midline hernia with sac	Central nervous system midline formation	61, 62
SHH null mutant mice	Lung hypoplasia, defective lung branching morphogenesis	A morphogen with key roles in vertebrate organogenesis	63–66
Gli2 (–/–) Gli3 (–/–) double knockout mice	No lung, trachea, oesophagus	Zinc finger transcription factors, mediators of sonic hedgehog signal transduction	67
Gli2 (–/–) mice, Gli3 (–/–) mice, Gli2 (–/–) Gli3(+/–) double knockout mice	Left-sided CDH		65
Pdgfrα homozygous null mice	Posterolateral diaphragmatic defect & pulmonary hypoplasia	Growth factor receptor	68, 69
Fgfrl1 homozygous null mice	Thin amuscular diaphragm	Growth factor receptor	70, 71
Fgf9 (–/–) mice	Lung hypoplasia, normal diaphragm	Fibroblast growth factor	72

Table 1 (continued)

Gene/model	Type of defect	Gene function (OMIM)	Ref.
FGF10 null mice	Complete lack of lung tissue, normal diaphragm	Encoding member of fibroblast growth factor family from lung	43, 73–75
NEDD4 knockout mice	Muscularisation defect of diaphragm	Potentiate hormone-dependent activation of transcription	
Sim2	Diaphragm hypoplasia	Transcription factor, involved in central nervous system development	76
Robo1 (–/–) mice	CDH, lung defects – increased mesenchyme	Encodes receptor that is a member of neural cell adhesion molecule family receptors	77

bHLH = Basic helix-loop-helix; RAR = retinoic acid receptor; RXR = retinoid X receptor.

els (table 1). However, it is of interest that the Bochdalek-type hernias, which account for the vast majority of human CDH cases, have not been observed in the FOG2 or GATA4 mutants. The RA pathway has been described in a number of publications [including 84–89] and an overview is presented in figure 1.

FOG2 (also known as ZFPM2) is a zinc finger transcription cofactor which modulates the activity of GATA transcription factors, in particular GATA4, and is located on 8q23, a region associated with CDH in humans (OMIM 610187, DIH3). Using mice treated with N-ethyl-N-nitrosourea (ENU), Ackerman et al. [42] identified a FOG2 mutation generating a truncated protein and causing pulmonary hypoplasia and abnormal diaphragmatic development. Furthermore, the authors identified a de novo mutation in 1 of 30 deceased children with diaphragm defects, in which severe bilateral pulmonary hypoplasia and an abnormally muscularised diaphragm were observed. Bleyl et al. [69] later identified novel sequence alterations in 2 of 96 patients with isolated CDH, however were unable to confidently determine the variants as causal mutations due to the lack of parental samples.

COUP-TFII is a transcription factor and orphan nuclear receptor which can interact with FOG2. COUP-TFII can inhibit gene transcription by preventing heterodimerization of retinoic acid receptors (RARs) and retinoid X receptors (RXRs), as well as modulate the transcriptional activity of GATA proteins, particularly GATA4. You et al. [41] generated tissue-specific null mutants of COUP-TFII which displayed the common Bochdalek type of CDH. Targeted ablation of COUP-TFII in the foregut mesenchyme, including the posthepatic mesenchymal plate which is also referred to as the PPF, resulted in malformation of the diaphragm and the failure of the posthepatic mesenchymal plate to attach to the body wall. The location of COUP-TFII, also known as NR2F2, on chromosome 15q26, a region recurrently associated with CDH patients, makes this gene an interesting candidate for human CDH [18, 19]. However, the study of Scott et al. [34] was unable to identify COUP-TFII mutations in 73 CDH samples. In another study, Slavotinek et al. [19] studied 6 candidate genes in the 15q26 critical region in over 100 CDH patients. However, although mis-sense changes were identified, none of these alterations could be assigned as definitively causal for CDH. This data may be suggestive of chromosome deletions at 15q26 being a contiguous gene deletion syndrome, or may be due to the suggested multifactorial aetiology of CDH.

GATA4 is a zinc finger transcription factor known to interact with FOG2 [90] and involved in regulating gene expression [91]. GATA4 null mutant mice are embryonic lethal due to the essential role of this gene in heart development [92, 93]. However, Jay et al. [30] generated a mouse model with a heterozygous deletion in exon 2 of GATA4 which displayed CDH and primary lung abnormalities. GATA4 is located in the critical region of the 8p23.1 deletion syndrome which shows a strong association with CDH [94] as well as heart defects. However, in humans, GATA4 mutations are typically associated with heart abnormalities and not with CDH [95–100].

Several studies have not observed defects in embryological development for individual RAR knockout models [60, 101], indicating a degree of overlapping function and compensation by the α, β, and γ forms of receptor. However, generation of compound null RAR mutants were shown to have a spectrum of VAD-like defects including diaphragm defects [56, 57, 102], confirming the importance of these receptors for early development in a

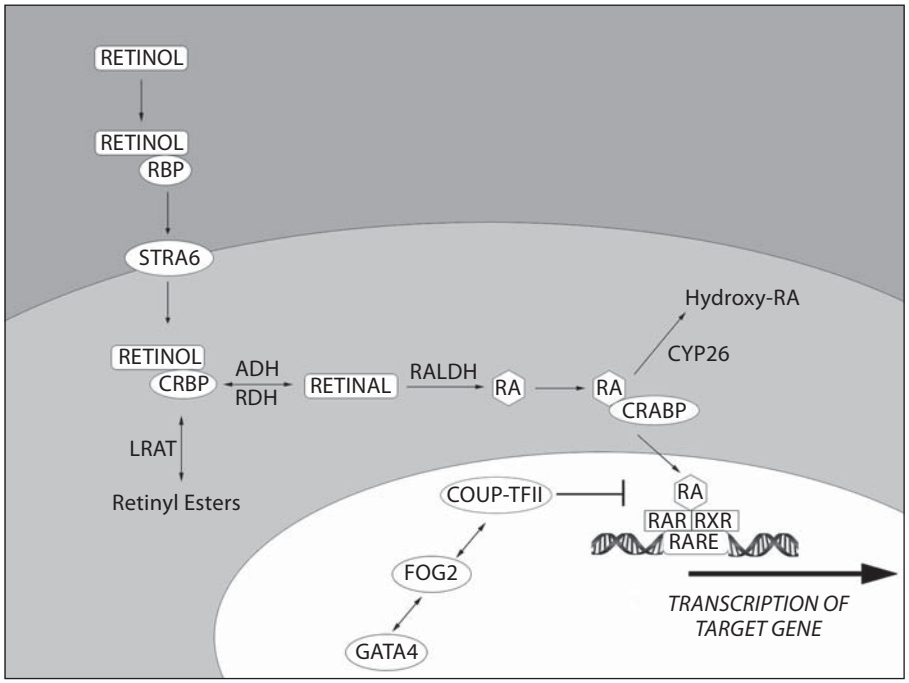

Fig. 1. An overview of the RA pathway is presented. Retinol is transported to target cells in a complex with RBP, with transfer into the cell occurring by the cell surface receptor STRA6. Within the target cell, retinol is bound to CRBP, and can be converted to retinal by ADH (in a reversible reaction), or to retinyl esters by LRAT. Retinal is oxidised to RA by RALDH in an irreversible reaction, whereby RA can complex with CRABP which enables transport to the nucleus. Within the nucleus RA forms a complex with RAR/RXR heterodimers, which in turn bind to a short DNA sequence, the RARE thereby activating transcription of the target gene. COUP-TFII can inhibit the heterodimerisation of RAR/RXR, thus inhibiting gene transcription. FOG2 interacts with both COUP-TFII and GATA4, thereby modulating the activity of GATA4 transcription factors. Excess cellular RA can be presented to CYP26 enzymes for degradation to Hydroxy-RA. Nitrofen is believed to exert its effects through inhibition of RALDH activity. RBP = Retinol-binding proteins; STRA6 = stimulated by retinoic acid 6; CRBP = cellular retinol-binding proteins; ADH = alcohol dehydrogenases; RDH = retinol dehydrogenases; RALDH = retinaldehyde dehydrogenases; RA = retinoic acid; CRABP = cellular retinoic acid-binding proteins; RAR = retinoic acid receptors; RXR = retinoid X receptors; RARE = retinoic acid response element; LRAT = lecithin: retinol acetyltransferase; CYP26 = cytochrome P450 family 26.

number of systems. It has recently been proposed that the unique expression pattern of RARα in the PPF may be of particular importance for diaphragm development [40].

In humans, mutations in STRA6 (stimulated by retinoic acid 6), a RBP receptor, have recently been linked to a spectrum of defects including diaphragmatic and pulmonary defects known collectively as Mathew-Wood syndrome [6–8], providing evidence for the involvement of this pathway in human CDH.

Collectively there is an increasing amount of evidence which supports the hypothesis that abnormal retinoid signalling plays an important role in the development of CDH. Further investigation and the generation of additional novel models which are targeted to specific components of the retinoid signalling pathway will increase our understanding of the pathogenesis of CDH and of the role that retinoid signalling and the downstream targets of this pathway play in diaphragm development. However, given the spectrum of defects seen in humans with STRA6 mutations, as well as the evidence for the RSP having roles in various aspects of embryological development [reviewed in 86], it remains to be determined whether defects at the higher levels of this pathway are responsible for isolated CDH.

PPF Development and the Mesenchymal-Hit Hypothesis

There is a body of evidence from animal models that CDH originates from a malformation of the PPFs, which are a key structure in the embryogenesis of the diaphragm

being the target for muscle precursor cells and for the phrenic nerve. It has been hypothesised that abnormal PPF development may underlie Bochdalek CDH [103–105]. A number of histological studies have compared this structure in both normal and abnormal situations, and three-dimensional reconstructions have also been generated by some groups [48, 105, 106]. Through the use of histological investigations, Clugston et al. [48] have demonstrated that teratogen-induced, dietary and genetic models of CDH appear to share a common mechanism of pathogenesis originating from defects in the PPF. More recently, the same group investigated the timeframe of PPF formation in rat embryos and human embryos, identifying a critical period of normal diaphragm development at 4–6 weeks' gestation. Importantly, this time period is earlier in gestation than conventionally thought and occurs before the typical time-point of pregnancy determination. Through additional investigations using nitrofen-exposed NIH 3T3 cells in vitro, it is proposed that impaired cell proliferation, and not increased apoptosis, contributes to abnormal diaphragm development in the nitrofen model of CDH [105].

Expression analysis in the diaphragm has revealed interesting results supporting the mesenchymal-hit hypothesis. Analysis of wild-type mouse embryos demonstrated co-expression of GATA4 and FOG2 in mesenchymal cells of the developing diaphragm, lungs and heart [30]. Double immunolabelling for COUP-TFII and WT1 has shown that these proteins co-localise within the non-muscular mesenchymal cells of the developing PPF [48]. These co-expression data indicate that the COUP-TFII, FOG2, GATA4 and WT1 models provide support for the mesenchymal-hit hypothesis.

Clugston et al. [40] found that RALDH2 is the only retinal dehydrogenase isoform expressed in the developing PPF. Examination of retinoid receptor expression in the PPF for the α, β, and γ isoforms of both the RARs and RXRs revealed positive expression for only RARα, RARγ, and RXRα. These receptors were shown to be strongly expressed within the non-muscular mesenchymal cells (WT1-positive), with weak expression in the muscle precursor cells (PAX3-positive). Interestingly, whilst the RARγ and RXRα expression was relatively uniform and widespread, the expression pattern of RARα was largely restricted to the caudal region of the PPF, corresponding to the affected region of the PPF in nitrofen-exposed embryos. It is therefore now proposed that RARα signalling in the developing PPF may be of particular importance for normal diaphragm development. The finding that the PPF is a centre for retinoid signalling further implicates this pathway and provides a potential link to defective retinoid signalling causing abnormal PPF development and thus CDH by affecting the mesenchymal component of this tissue early in diaphragm development [40].

Additional research has shown that the 15q26 region contains a cluster of genes that are expressed in the developing diaphragm in rodents, supporting the association of CDH with deletions of 15q26. Further examination of protein expression within the developing diaphragm has shown that genes strongly associated with CDH (COUP-TFII, FOG2, GATA4, and WT1) are expressed only in the non-muscular mesenchymal component of the diaphragm, supporting the mesenchymal-hit hypothesis, and well as demonstrating that these factors are all co-expressed in the same cells. This is suggestive of different genetic causes leading to a common defect in the mesenchymal cells of the PPF which causes CDH, supporting the theory that unique genetic defects affecting a common pathway can lead to CDH [107].

Given the apparent defective development of the PPF in multiple animal models of Bochdalek-type CDH, as well as in archived human embryo specimens [105], further investigation is needed of the role that this structure plays and in particular the role of the non-muscular mesenchymal cells in the pathogenesis of isolated CDH. It will also provide an improved understanding of the embryogenesis of the diaphragm, and the influence that defects in the PPFs have on the development of CDH in humans. It is essential for us to understand the genetic factors involved in normal PPF development and how genetic defects can affect the PPF and further development of the diaphragm.

Diaphragm and Lungs – the Dual-Hit Hypothesis

Keijzer et al. [108] demonstrated using lung explants that nitrofen interferes with early lung development before and separate from aberrant diaphragm development. The authors postulate the dual-hit hypothesis to explain pulmonary hypoplasia in CDH by two mechanisms, the first affecting both lungs before diaphragm development and a second affecting the ipsilateral lung after defective diaphragm development. Nitrofen is however damaging at high doses to a number of developing organs which are dependent on retinoid signalling. It is possible that the diaphragm may be particularly sensitive to disruption of retinoid signalling in the case of CDH.

The FOG2 ENU mouse model implicates the role of this gene in the pathogenesis of isolated CDH, and its ne-

cessity for pulmonary development validates the hypothesis that neonates with CDH may also have primary pulmonary developmental abnormalities [42]. Further evidence supporting the dual-hit hypothesis comes from the investigation of FGF10 null mice which completely lack lung tissue but were shown to have phenotypically normal diaphragms. This finding demonstrates that normal diaphragm development can occur independently of lung development [74]. Furthermore, expression analysis of Gata4 and Fog2 in wild-type mouse embryos has demonstrated co-expression in the mesenchymal cells of the developing diaphragm, lungs and heart [30].

The overlapping function of certain genes and pathways in both diaphragm and lung development supports the dual-hit model, however, exactly how defects in retinoid signalling and mesenchymal cell function may contribute to cases of isolated CDH remains to be determined.

Alternative Pathways

A number of CDH candidate genes are involved in neural crest cell development. In a recent review, Klaassens et al. [87] propose the potential involvement of the neural crest cell developmental pathway in CDH. Recently, the thyroid hormone signalling pathway has also been suggested to have a potential role in the pathogenesis of CDH [3]. Whilst there is limited evidence to confirm this role, it is important for researchers to maintain an open mind to the involvement of this and other unidentified pathways and mechanisms in the development of both isolated and non-isolated CDH, or in the different types of CDH.

Table 2 lists evidence from a number of expression studies indicating dysregulated genes and highlighting potential pathways and mechanisms which may be involved in CDH. Much of the data comes from pulmonary gene expression and more research is certainly needed to focus on differences in gene expression in the diaphragm, and at different time-points in development. The difficulty in obtaining post-mortem tissue from CDH patients requires the further use of animal models, novel cellular models, or novel investigative techniques to further our knowledge of gene expression in the developing diaphragm. It remains to be fully determined exactly which differences in expression are directly linked to the genetic causes of CDH, and which are merely downstream effects of abnormal diaphragm and/or lung development.

Future Perspectives

Sato et al. [124, 125] describe the use of optical projection tomography, a non-invasive technique which allows three-dimensional imaging of small biological tissue specimens to visualise both the anatomy of developing organs and gene expression. Optical projection tomography therefore offers the possibility to visualise in vivo gene expression patterns in genetic or teratogenic models of congenital malformations such as CDH. This technique was applied to SHH gene expression patterns in the nitrofen-treated and control mouse lung buds at early stages of lung development. However, this study did not reveal any significant alterations in pulmonary SHH transcript distribution or gene expression levels during early gestation. Further applications to alternative gene expression studies in the diaphragm and lungs at different time-points during development have the potential to reveal important information on the pathogenesis of CDH.

In a recent study, Goumy et al. [126] studied fetal skin fibroblasts as a model to investigate CDH. It was shown that these fetal cells expressed enzymes involved in the RA pathway. Expression analysis in CDH patients revealed altered levels of RARs, RALDH2, and CYP26 in 2 of 7 fetuses investigated. This study identifies a potential model to further investigate human CDH. Due to the difficulties of obtaining diaphragm and lung tissue in humans, further studies of global expression in amniotic fluid cells has the potential to reveal important differences between CDH and non-CDH patients.

It is apparent from humans that there are different types of CDH, and animal models have also revealed certain genes involved in different types of CDH. Expression analysis and comparison of subtypes of CDH may reveal differences between different cohorts of patients, or suggest common mechanisms for CDH. Clinically, it would be useful to timely identify good responders and poor responders to currently available fetal therapy, and the genetic factors influencing this response. The identification of genetic markers, ideally from AF or from maternal blood or plasma, offers the possibility of a more personalised fetal therapy dependent upon the presence or absence of particular markers.

Over recent years, genome-wide association studies have shown success in identifying common risk loci for a number of complex diseases. Whilst the costs and availability of large CDH patient cohorts may restrict the immediate use of this approach, this technique could have potential applications in the future as costs reduce. Simi-

Table 2. Recent findings from expression analysis in CDH animal models

Gene(s)	Description	Potential mechanisms	Technique used	Ref.
Up-regulation				
COUP-TFII, FOG2, GATA4	Up-regulation of pulmonary gene expression after prenatal treatment with RA in nitrofen model	RA may have therapeutic potential in modulating lung growth	Real-time RT-PCR on RNA isolated from lung	109
COUP-TFII	Pulmonary gene expression of COUP-TFII is up-regulated in the early stages of lung development in the nitrofen-induced hypoplastic lung	Up-regulation of COUP-TFII gene expression during the stage of branching lung morphogenesis may cause pulmonary hypoplasia by repressing RSP	Real-time RT-PCR on RNA isolated from lung	110
EDNRA, EDNRB	Increased pulmonary vascular expression in nitrofen model	Altered expression of EDNRA and EDNRB occurs early in lung morphogenesis. Pulmonary arteries in CDH may become excessively muscularised and unable to adapt at birth	Real-time RT-PCR on RNA isolated from lung	111
Wnt5a	Pulmonary Wnt5a gene expression in the late lung morphogenesis	Wnt5a controls late lung morphogenesis, including patterning of distal airway and vascular tubulogenesis (alveolarisation). Up-regulation of pulmonary Wnt5a gene expression in late lung morphogenesis may interfere with patterning of alveolarisation, causing pulmonary hypoplasia	Real-time RT-PCR on RNA isolated from lung. Immunohistochemistry on lung tissue	112
Slit-2, Slit-3	Slit genes are up-regulated in nitrofen-induced hypoplastic lungs in both early and late stages of lung development	Altered pulmonary Slit gene expression may disrupt branching lung morphogenesis resulting in pulmonary hypoplasia (no change observed in Robo1 or Robo2)	Real-time RT-PCR on RNA isolated from lung	113
TGF-β_1	Increased expression of TGF-β_1	Increased expression of TGF-β_1 in the lung of CDH rat model may suppress lung growth and development	Immunohistochemistry on lung tissue	114
CRBPI, Aldh1a3, RARα, RARβ, RXRα	Significantly up-regulated in the lungs of the nitrofen with CDH group	Lung retinol storage is decreased in the nitrofen model of CDH. The increase in gene expressions of downstream components of the RSP may be a feedback reaction to lung retinol deficiency. Nitrofen may act by interfering with the cellular uptake of retinol during lung morphogenesis resulting in pulmonary hypoplasia	Retinol levels in serum, lungs & liver using HPLC. Real-time RT-PCR on RNA isolated from lung	83
pVHL	pVHL was expressed more frequently in the arterial smooth muscle cells of CDH lungs compared with both other groups	A possible role for pVHL and HIF-1a in normal and abnormal pulmonary angiogenesis. The differential expression of these proteins may provide a molecular basis for the histological differences observed in the lung vessels of patients with CDH	Immunohistochemistry on lung tissue	115
Down-regulation				
PTHrP, PTHrP-R	Down-regulation of gene expression during late lung morphogenesis	May disrupt alveolar maturation and surfactant production by interfering with mesenchymal-epithelial interactions causing pulmonary hypoplasia in the nitrofen CDH model	Real-time RT-PCR on RNA isolated from lung. Immunohistochemistry on lung tissue	116
IGFBP-3	IGFBP-3 mRNA were significantly decreased in the nitrofen group	Down-regulation of IGFBP-3 and IGFBP-5 gene expression may cause pulmonary hypoplasia in the nitrofen-induced CDH model by interfering with the RSP	Real-time RT-PCR on RNA isolated from lung. Immunohistochemistry on lung tissue	117

Table 2 (continued)

Gene(s)	Description	Potential mechanisms	Technique used	Ref.
Tpm3, Fgfrl1, Myl2, Lrtm1, Myh4, Myl3, Myh7, Hephl1	Significantly reduced expression levels in the diaphragms of Fgfrl1 homozygous null mice. Lrtm1 is closely related to Slit3	Slit proteins are known to regulate axon branching and cell migration, and inhibition of Slit3 reduces cell motility and decreases the expression of Rac and Cdc42, two genes that are essential for myoblast fusion. Reduced Fgfrl1 expression may cause diaphragm hypoplasia through a mechanism involving decreased myoblast motility and/or myoblast fusion	Gene expression arrays on diaphragm tissue. Real-time RT-PCR on RNA isolated from diaphragm	118
ClC-2, ClC-3	Developmental changes in ClC-2 and ClC-3 protein expression are negatively affected in hypoplastic CDH lungs	Fetal lung growth is dependent on the secretion of lung liquid, in which Cl(–) secretion by the pulmonary epithelium plays a crucial role. Lung hyperplasia created by TO up-regulates the expression of ClC-2, and is therefore an interesting potential therapeutic target for CDH treatment	Western blot on protein isolated from lung tissue	119
FGF18	Impaired septation in CDH is associated with decreased FGF18 expression and elastic fibre deposition	Simultaneous correction of FGF18 and elastin defects by TO and vitamin A suggests that defective elastogenesis may result, at least partly, from FGF18 deficiency	Western blot on human & sheep lungs. Elastin staining in human & sheep lungs. Real-time RT-PCR on sheep lungs. Northern blot on rat lungs. Immunolocalisation on lungs of human, sheep & rat	120
Wnt7b, Wnt2, BMP4, GATA6	Wnt signalling pathway is down-regulated in nitrofen-induced hypoplastic lungs in the early stages of lung development	Decreased expression of GATA6 may account for the down-regulation of Wnt signal pathway the down-regulation of Wnt signalling pathway may disrupt branching lung morphogenesis, resulting in pulmonary hypoplasia in the nitrofen rat model of congenital diaphragmatic hernia	Real-time RT-PCR on RNA isolated from lung	121
AQP5	Expression of AQP5 is down-regulated in hypoplastic lungs with CDH	Down-regulation of AQP5 may result in abnormal pulmonary fluid metabolism in perinatal period and may be one of the mechanisms disturbing the pulmonary development in late stage in the CDH model	Real-time RT-PCR on RNA isolated from lung. Immunohistochemistry on lung tissue	122
HIF-1a	HIF-1a was expressed less frequently in the endothelium of arteries, veins, and capillaries of CDH lungs as compared with both other groups	A possible role for pVHL and HIF-1a in normal and abnormal pulmonary angiogenesis. The differential expression of these proteins may provide a molecular basis for the histological differences observed in the lung vessels of patients with CDH	Immunohistochemistry for HIF-1a in lung tissue	115
Cyp26b1, LRAT	Down-regulation of Cyp26b1 and LRAT in nitrofen-induced hypoplastic lungs	Down-regulation of Cyp26b1 and LRAT demonstrates that RA content is decreased in nitrofen-induced hypoplastic lungs	Real-time RT-PCR on RNA isolated from lung	82
GATA4, GATA6, Wnt2, BMP4, MEF2C	Gene expression of GATA4, GATA6, Wnt2, BMP4, and MEF2C on embryonic day 13 were significantly reduced in the hearts of nitrofen-treated animals compared with normal hearts of equivalent age	Decreased expression of GATA4 and GATA6 and their target genes in the developing fetal heart may perturb the delicate regulation of cardiovascular development, resulting in cardiovascular malformations in the nitrofen rat model	Real-time RT-PCR on RNA isolated from heart	123

RA = Retinoic acid; RSP = retinoid signalling pathway; pVHL = pulmonary von Hippel-Lindau; HPLC = high-performance liquid chromatography; Real-time RT-PCR = real-time reverse transcription polymerase chain reaction.

larly, next-generation sequencing is beginning to move from research applications to the clinic as costs reduce and the availability of these technologies widens. It is foreseeable that these new sequencing technologies have the potential for both whole genome sequencing, and targeted sequencing following sequence capture, for CDH patients. In addition, this technology offers the possibility for global transcriptome studies. However, at this moment in time the costs remain high, although we hope to see the use of these techniques in the near future as part of large, well-funded, international collaborations into the pathogenesis of CDH.

Conclusions

It is apparent that evidence from a variety of disciplines is increasing our understanding of normal diaphragm and lung development and of the pathogenesis of CDH. Histological data has been important in identifying the anatomical origins of abnormal diaphragm and lung development and the specific timing of these abnormalities in embryological development. Immunohistochemistry and molecular techniques are revealing differences in gene expression between a number of normal and CDH models, highlighting potential candidate genes and genetic pathways for further research. Advances in technology, such as the use of array comparative genomic hybridisation, have allowed to refine certain critical regions which display strong associations with CDH in humans. The continued collaborative approach from numerous scientific fields is essential to improve our understanding of normal diaphragm development and of CDH in animal models, as well as building a more detailed picture of CDH in humans. This will eventually allow for the development of novel therapies which will improve the survival rate for CDH patients and reduce the long-term morbidity commonly observed for these patients.

Three main concepts have emerged over recent years: the retinoid hypothesis, the mesenchymal-hit hypothesis, and the dual-hit hypothesis. These three hypotheses are supported by growing evidence, mainly from animal models. It is tempting to combine these to create a more general hypothesis for isolated cases of CDH. We may speculate that, in humans, genetic defects affecting retinoid signalling, and the downstream target genes and pathways, may cause abnormalities in the developing PPFs by affecting mesenchymal cell function leading to CDH. In some isolated CDH cases, this interference with retinoid signalling may disrupt only the diaphragm which may be much more susceptible. However, the importance of retinoid signalling in many aspects of embryological development may also result in some CDH cases for which the lungs, and even other tissues or organs, may also be affected to varying degrees. There is a lack of evidence to confirm this and more research is required to identify the exact influence these three hypotheses have in cases of isolated CDH in humans.

We still do not fully understand the normal embryogenesis of the diaphragm, however it is encouraging to see that much progress has been made in this area. In understanding diaphragm development in the normal situation, we will achieve an insight into how genetic defects contribute to abnormal diaphragm development leading to CDH. However, we must remain open-minded to alternative pathways and the role these may play in the development of CDH. The continued application of novel technologies and models to the study of CDH is essential in our effort to fully understand the pathogenesis of isolated CDH and the exact role that individual genes play in the development of the diaphragm and lungs.

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