REVIEW

Polygenic Causes of Congenital Diaphragmatic Hernia Produce Common Lung Pathologies

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Congenital diaphragmatic hernia (CDH) is one of the most common and lethal congenital anomalies, and significant evidence is available in support of a genetic contribution to its etiology, including single-gene knockout mice associated with diaphragmatic defects, rare monogenetic disorders in humans, familial aggregation, and association of CDH with chromosomal abnormalities. Structural lung defects in the form of lung hypoplasia are almost invariably seen in patients with CDH and frequently in animal models of this condition. Better understanding of the mechanisms of pulmonary defects in CDH has the potential for creating targeted therapies, particularly in postnatal stages, when therapeutics can have maximum clinical impact on the surviving cohorts. Successful treatment of CDH is dependent on the integration of human genomic and genetic data with developmental expression profiling, mouse knockouts, and gene network and pathway modeling, which have generated a large number of candidate genes and pathways for follow-up studies. In particular, defective alveolarization appears to be a common and potentially actionable phenotype in both patients and animal models. (*Am J Pathol* 2016, 186: 2532–2543; <http://dx.doi.org/10.1016/j.ajpath.2016.07.006>)

A Multimodal War on Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia (CDH) is a common and severe birth defect that affects 1:3000 live births.^{1,2} It is characterized by defective formation and/or muscularization of the diaphragm, often with displacement of abdominal contents into the chest cavity. CDH is almost invariably accompanied by pulmonary hypoplasia, which is the major cause of morbidity and mortality in patients and requires immediate and complex care.³ The clinical importance of CDH relates to its high frequency among birth defects, its severity, and the challenges related to its treatment, which is intense and expensive, placing a burden on the affected families and the health care system. Approximately 60% of cases are isolated defects that affect otherwise healthy term infants, indicating a good clinical prognosis if lung hypoplasia can be improved.

Significant progress has been made in the care of these patients in the past 2 decades, although mortality rates remain at 30% to 50% worldwide.¹ Currently, the main therapeutic goal is to stabilize the cardiac and respiratory systems in infants with CDH, while attempting to minimize iatrogenic (therapy-induced) injury to the delicate pulmonary tissue, before and after surgical repair of the diaphragm. Gentle ventilation techniques, high-frequency ventilation, cardiovascular pharmacologic support, and extracorporeal membrane oxygenation^{3–6} have improved survival rates, although these interventions have their own early and long-term complications. Lengthy and expensive

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stays in neonatal intensive care units are often necessary.⁷ A significant number of infants still succumb to the pulmonary complications of CDH, with late deaths mostly attributable to pulmonary hypertension and pulmonary insufficiency. Furthermore, many CDH survivors experience adverse long-term outcomes, including residual cardiopulmonary disease, neurodevelopmental defects, sensorineural hearing loss, and feeding difficulties.^{8–10}

The lungs in infants with CDH have reduced volumes and weight with histologic features of developmental immaturity, manifested by a reduction in the number and size of alveoli. Several mechanisms have been hypothesized to cause CDH-associated lung hypoplasia. Classic teaching indicated that compression of the lung by herniated viscera and the lack of respiratory movements were the root causes of the hypoplasia phenotype; however, other studies suggest that abnormal lung development precedes the herniation of abdominal contents into the thoracic cavity, and it is therefore a primary, not secondary, defect.^{11–13} These observations have led to the conclusion that the lung defects are at least partially independent of the diaphragm defect and pose many questions about what links the abnormalities of these two organs at the molecular or mechanistic level.

Whereas others have reviewed the embryology of the diaphragm,¹⁴ this review focuses on our current understanding of the mechanisms of pulmonary defects in CDH and on the potential for targeting postnatal stages of lung development, when therapeutics can have maximum clinical impact on the surviving cohorts.

Overview of Diaphragm and Lung Development

The diaphragm forms from multiple tissue sources, including transient embryonic structures of mesenchymal tissue that make up the septum transversum and pleuroperitoneal folds (PPFs). The muscles lying at the periphery of the diaphragm derive from migrating cells delaminating from the somitic dermatomyotome, which also contributes to the skeletal muscle of the limbs and body wall.^{15–18} The fundamental role of connective tissue in the PPFs was demonstrated by analysis of conditional *Prx1-cre^{Tg/+};Gata4^{Δ/f}* mice, with complete *Gata4* ablation in the lateral plate mesoderm—derived PPF cells. Abnormalities in the PPF connective tissue result in failure of migration and differentiation of muscle precursor cells, leading to subsequent hernia formation.¹⁹ Eighty percent of hernias occur in the left posterolateral diaphragm, begging the questions of why this position is so often targeted for mishap.

Development of the lungs is a complex, multistep process, and perturbation of any of these steps can lead to pulmonary defects (Figure 1). The process initiates with ventral budding from the ventral foregut endoderm. This lung bud then undergoes a process of elongation and branching morphogenesis, which follows a characteristic reproducible pattern based on the Fibonacci sequence, together with subsequent sequential multiplanar rotations that are regulated with a periodicity²⁰ and is regulated by multiple pathways, including fibroblast growth factor, Wnt, sonic hedgehog, transforming growth factor- β , and bone

Figure 1 Timeline of pulmonary development. Human lung development is divided into prenatal and postnatal phases, separated by the dotted line indicating birth. Developmental stages are plotted against gestational weeks (below) and are divided into the partially overlapping embryonic, pseudoglandular, canaliculal, saccular, and alveolar stages. Development of the alveolus continues with increased septation for several years during childhood. A number of CDH-associated genes affect branching morphogenesis in the lungs, but alveolarization and vascular defects are also reported in patients with CDH. The most practical window for therapeutic intervention is indicated by the pink box.

morphogenetic protein.^{21–26} Primary buds undergo additional branching to form multiple distal tubules during the pseudoglandular stage, and the tubules elongate during the canalicular phase. In the later stages of lung development, the distal airways form the critical interface for gas exchange. During middle to late gestation, the lungs enter the saccular phase, where the distal airways form rudimentary saccules, and the airway epithelium begins to secrete surfactant.

The final stage of lung development, alveolarization, begins before birth and continues through the newborn period and much of childhood in humans, commensurate with overall body growth. During the first phase of alveolarization, known as primary septation, the airway saccules become thinner, bringing the airway epithelium in close contact with the lung vasculature, thereby forming a basic unit for gas exchange. Subsequently, the alveoli mature through the process of secondary septation, in which multiple septa grow inward from the walls of each alveolus. Each septum contains two epithelial layers with a central core that contains a single capillary and mesenchymal components that include myofibroblasts and elastin, as well as other extracellular matrix components. The migration and differentiation of

myofibroblasts in the alveolar septa are critical,²⁷ although it remains a poorly understood aspect of lung development, as does the formation of lipofibroblasts.²⁸ The process of secondary septation greatly increases the surface area for gas exchange within the lung, and because it occurs immediately prenatally and continues postnatally, it provides an attractive target for designing therapeutic interventions for patients with CDH and other neonatal lung diseases (Figure 1).

Pulmonary Hypoplasia and Alveolarization Defects in CDH

The eponymous hernia or hole in the diaphragm is accompanied by lung hypoplasia and pulmonary hypertension whose severity determines early mortality or survival. The lungs in patients with CDH appear immature, with thickened alveolar walls and increased interstitial tissue, effectively resulting in reduced alveolar spaces and gas-exchange surface area²⁹ (Figure 2). Incomplete pulmonary mesenchymal thinning attributable to defective apoptosis, rather than true immaturity, has been proposed as a mechanism based on cellular models.^{13,30}

Figure 2 CDH lung abnormalities. An alveolar block is hypothesized as a late phenotype in CDH, resulting in alveolar simplification. Smaller alveoli, lined by increased type 2 and reduced type 1 pneumocytes, contain stunted septa linked with defective myofibroblast proliferation and migration. Reduced Sox9-positive progenitors indicate a defect in epithelial differentiation. The thickened interstitium suggests a delay in pulmonary maturation or abnormal resorption and results in poor oxygen exchange. α -SMA, α -smooth muscle actin; PDGFR, platelet-derived growth factor receptor; PDPL, podoplanin; SPC, surfactant protein C.

On a finer structural level, the lungs of patients with CDH are characterized by a failure to establish the necessary close association of the air-exposed epithelium to the abnormally differentiated and diminished mesenchymal vessels carrying the blood that must be oxygenated for survival.^{31,32} Failure of co-differentiation of the lung epithelium and mesenchymal compartment components can lead to insufficient gas exchange, the final common pathway of lethality in CDH. This can result from mutations in any number of genes, each critical to the linear and parallel morphologic steps necessary to achieve a normally differentiated lung.

Much of our mechanistic understanding of the pulmonary defects in CDH has come from the study of animal models, particularly genetic models with both diaphragm and lung abnormalities (Table 1).³³ In addition, surgical models on sheep and rabbits provide valuable information to study the mechanical effects of herniated viscera on the lung and to test interventional strategies, whereas the teratogenic nitrofen model mimics the human condition to a large extent, even though its applications are limited by the fact that nitrofen is not considered a cause of CDH in patients.³⁴ Deficient alveolarization has been observed in CDH, resulting from nitrofen administration, but can be improved by antenatal treatment with corticosteroids.³⁵ Human studies have proven more challenging, likely because of pragmatic clinical difficulties in obtaining appropriate pathology specimens, because most infants with CDH undergo extensive ventilatory interventions that disrupt the natural architecture of the lung. Major bronchial subdivisions are normal in human autopsy specimens of hypoplastic CDH lungs; however, the number of intermediate bronchial branches is often reduced severely in the ipsilateral lung and less so in the contralateral lung, which comparatively maintains the potential of faster growth and complete differentiation during gestation.^{36,37} The prevalence of left-sided hernias suggests that left-right asymmetry may also be an important component of the pathophysiology of the hypoplasia.

Few of the extant alveoli have a normal structure. Pulmonary developmental studies of alveolarization assessed by such measures as mean linear intercepts and septal density^{38–41} confirm that patients with CDH never achieve a full number of airway branches or spaces, despite the alveolar multiplication that occurs after birth.^{42,43} Vascular growth is even more compromised than alveolar growth. However, because alveolar maturation continues throughout infancy and childhood, there is a long actionable window during which to improve differentiation and pulmonary function in CDH survivors. The question is whether differentiation can be improved postnatally by targeting the developmental pathways found to be involved in the process of alveolarization.

Several homozygous knockout mouse models of CDH result in severe phenotypes with embryonic lethality.^{44,45} Many genes involved in the formation of the diaphragm are also likely to play important roles in the early stages of lung development (Table 1). Examples include *ZFPM2* (*FOG2*)⁴⁶

and members of the fibroblast growth factor signaling pathway, which result in pulmonary hypoplasia attributable to defects in branching morphogenesis.^{47,48} The effects of complete ablation of these early-acting genes in mice are especially severe and irreversible.³² However, the pathologic features in humans, who usually have only one nonfunctional copy of the gene, may be less severe and addressable in the immediate postnatal period. An increasing number of mouse models have implicated a role for CDH genes in the later stages of lung development, where early molecular correction or rescue can have potentially maximum clinical benefit and complications of barotrauma (eg, bronchopulmonary dysplasia and pulmonary fibrosis) are minimized or averted. CDH and bronchopulmonary dysplasia share structural and functional abnormalities in alveolar formation and pulmonary vasculature, possibly related to common disturbances in the retinoid acid pathway genes,⁴⁹ which result in impaired gas exchange.

A complementary set of mammalian genetic, genomic, physiologic, and three-dimensional morphologic approaches have pointed to myofibroblasts and extracellular matrix constituents, such as elastin, as key players in these late-stage CDH-associated pulmonary abnormalities.^{27,50} Lipofibroblasts, a lipid-containing population of interstitial fibroblasts first identified during the canalicular phase of lung development, were also reduced in rats treated with the CDH-inducing teratogen nitrofen.^{51,52} Lipofibroblast signaling is critical for the completion of alveologenesis, possibly in directing the production of surfactant phospholipids in type 2 pneumocytes.²⁸

Pulmonary neuroendocrine cells (PNECs), detected by acetylcholine esterase staining in earlier studies,^{53,54} differentiate from distal progenitors and migrate proximally to form neuroepithelial bodies or clusters. Neuroepithelial bodies were reported to be increased in number and size in neonatal rats with diaphragmatic defects.⁵⁵ The PNEC-produced factors bombesin and ghrelin, which are likely to play important roles in lung development and regulating vascular tone, are expressed at higher levels in patients with CDH.⁵⁶ PNEC clusters normally function *in vivo* as airway sensors for hypoxia, hypercarbia, and other toxic environmental changes. Excess secreted neuropeptides were linked to lung matrix remodeling and alveolar simplification or failure of septation,⁵⁷ and PNEC factors have been proposed to sensitize the lungs to the effects of retinoic acid by regulating retinoic acid receptors α and γ expression.⁵⁸

Pulmonary Hypertension in CDH

In addition to the defective gas exchange mechanisms discussed above, another major contributor to the mortality of infants with CDH is pulmonary hypertension, which is characterized by abnormal endothelial and smooth muscle development, associated with failure of the vascular smooth muscle to relax normally after birth.⁵⁹ The cause of pulmonary hypertension in patients with CDH is multifactorial,

Table 1 Mouse Models with Both Diaphragm and Lung Abnormalities

Symbol	Name	Diaphragmatic phenotype	Lung phenotype
<i>Atp2a1</i>	ATPase, Ca ⁺⁺ transporting, cardiac muscle, fast twitch 1	Abnormal diaphragm muscle	Abnormal alveoli (failure to expand, hypercellularity)
<i>Ctnnb1</i>	Catenin (cadherin associated protein), β 1	Diaphragmatic hernia (Wt1-Cre conditional knockout)	Absent lung buds (Shh-Cre conditional knockout)
<i>Efemp2</i>	Epidermal growth factor—containing fibulin-like extracellular matrix protein 2	Diaphragmatic hernia	Abnormal elastic fibers
<i>Eya1</i>	EYA transcriptional co-activator and phosphatase 1 and 2	Amuscular diaphragm (double Eya1; Eya2 knockout)	Lung hypoplasia, abnormal epithelium morphologic features
<i>Eya2</i>	EYA transcriptional co-activator and phosphatase 1 and 2	Amuscular diaphragm (double Eya1; Eya2 knockout)	Lung hypoplasia, abnormal epithelium morphologic features
<i>Frem1</i>	Fras1 related extracellular matrix protein 1	Diaphragmatic hernia	Fused pulmonary lobes
<i>Frem2</i>	Fras1-related extracellular matrix protein 2	Diaphragmatic hernia	Fused pulmonary lobes
<i>Fuz</i>	Fuzzy planar cell polarity protein	Diaphragmatic hernia	Lung hypoplasia
<i>Gata4</i>	GATA-binding protein 4	Diaphragmatic hernia	Abnormal sacculle morphologic features, abnormal vasculature
<i>Gli2, Gli3</i>	GLI-Kruppel family member 2 and 3	Diaphragmatic hernia (double Gli2; Gli3 knockout)	Lung hypoplasia, absent right lung accessory lobe, thick mesenchyme
<i>Hlx</i>	H2.0-like homeobox	Diaphragmatic hernia	Enlarged lungs with normal structure
<i>Igf2</i>	Insulin-like growth factor 2	Thin diaphragm muscle (double Igf2; Myod1 knockout)	Abnormal epithelial proliferation/differentiation (organ culture)
<i>Kif7</i>	Kinesin family member 7	Diaphragmatic hernia, thick diaphragm muscle	Lung hypoplasia
<i>Lmnb1</i>	Lamin B1	Thin diaphragm muscle, abnormal phrenic nerve	Abnormal alveoli
<i>Lmnb2</i>	Lamin B2	Thin diaphragm muscle, abnormal phrenic nerve (double Lmnb1; Lmnb2 knockout)	Abnormal alveoli
<i>Lox</i>	Lysyl oxidase	Diaphragmatic hernia, thin diaphragm muscle	Lung hypoplasia, abnormal acini, abnormal elastic fibers
<i>Met</i>	Met proto-oncogene	Diaphragmatic hernia, thin diaphragm muscle	Abnormal sacculle morphologic features (conditional knockout in the respiratory epithelium)
<i>Mmp2, Mmp14</i>	Matrix metalloproteinase 2, and 14	Thin diaphragm muscle (double Mmp14; Mmp2 knockout)	Lung hypoplasia, abnormal alveoli, dilated alveolar ducts, abnormal elastic fibers
<i>Myod1</i>	Myogenic differentiation 1	Thin diaphragm muscle (MyoD:mdx)	Pulmonary hypoplasia (MyoD:mdx)
<i>Myog</i>	Myogenin	Thin diaphragm muscle	Lung hypoplasia
<i>Msc</i>	Musculin	Diaphragmatic hernia (double Msc; Tcf21 knockout)	Lung hypoplasia, abnormal branching, abnormal vasculature
<i>Ndst1</i>	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	Diaphragmatic hernia, thin diaphragm muscle (conditional knockout)	Lung hypoplasia, thick interalveolar septa
<i>Pbx1</i>	Pre-B-cell leukemia homeobox 1	Diaphragmatic hernia	Lung hypoplasia
<i>Pdgfra</i>	Platelet-derived growth factor receptor, α -polypeptide	Diaphragmatic hernia	Lung hypoplasia, abnormal alveoli, increased cell proliferation
<i>Rara, Rarb</i>	Retinoic acid receptor, α and β	Diaphragmatic hernia (double Rara; Rarb knockout)	Lung hypoplasia, abnormal alveoli (double Rara; Rarb knockout)
<i>Robo1, Robo 2</i>	Roundabout guidance receptor 1	Diaphragmatic hernia (double Robo1; Robo2 knockout)	Abnormal alveoli, thick septa
<i>Six1</i>	Sine oculis homeobox, <i>Drosophila</i> , homolog of, 1	Amuscular diaphragm (double Six1; Six4 knockout)	Lung hypoplasia
<i>Tcf21</i>	Transcription factor 21	Diaphragmatic hernia (double Msc; Tcf21 knockout)	Lung hypoplasia, abnormal branching, abnormal vasculature
<i>Wdr35</i>	WD repeat domain 35	Diaphragmatic hernia	Pulmonary hypoplasia
<i>Wt1</i>	Wilms tumor 1 homolog	Diaphragmatic hernia	Lung hypoplasia
<i>Zfp2</i>	Zinc finger protein, multitype 2	Abnormal diaphragm morphologic features	Lung hypoplasia, absent right lung accessory lobe

The Mouse Genome Database Group 2015.³³

with defects that affect lung vessel number and size. Autopsy specimens from patients with CDH reveal that lung vessel density is decreased.⁵⁹ Also characteristic are the reduced complexity of proximal pulmonary artery branching and the paucity of microvascular or capillary networks, which should occur in parallel to the alveolarization of the distal lung. Diminished lung vessel size is also accompanied in patients by malposition and hypertrophy of vascular smooth muscle, as well as failure of the vascular smooth muscle to respond by relaxation in response to physiologic oxygen or nitrogen levels present at birth, compounded to the reduced response to treatment. It is unclear which of these abnormalities is the major contributing factor to pulmonary hypertension in patients with CDH.

Genomic studies have found little overlap with gene mutations seen in hereditary forms of primary pulmonary hypertension, suggesting that CDH-associated lung hypertension is likely dependent partially on different pathophysiologic mechanisms.⁶⁰ Pulmonary antihypertensive therapy with inhaled nitric oxide or the use of phosphodiesterase inhibitors is suboptimal in patients with CDH,⁶¹ as has been the use of phosphodiesterase inhibitors attempted in clinical practice. In fact, no current pharmacologic regimen improves the pulmonary hypertension associated with CDH.^{3,62} Improved understanding of how genetic defects contribute to CDH-associated lung pathologies may lead to the discovery of pathways that affect alveolar septation and pulmonary vascular hemodynamics. This will help generate more accurate models of the pulmonary and vascular pathophysiology associated with CDH and ultimately improve short-term survival and ameliorate long-term morbidity.

Genomic Studies in CDH

Genetic studies on patients with CDH are providing important insight into the molecular mechanisms of both diaphragm and lung development. Several observations point toward genetic causation in CDH.^{1,2,63–66} Because of the clinical burden of CDH, we and others have invested in genetic studies of rare families with mendelian patterns of inheritance^{67–71} and of large patient cohorts.^{68,72}

Familial studies revealed monogenic causes of CDH, including the critical CDH genes *ZFPM2* or *FOG2*,^{69,73} *GATA4*,⁷¹ and *GATA6*.⁷⁰ These families are rare, in part because of the early high mortality and reproductive disadvantage in CDH. This is particularly true in complex CDH cases, which make up approximately 40% of all CDH cases. Isolated CDH (60%), without additional birth defects, can be familial, but pedigrees can be difficult to recognize because of reduced penetrance.^{69,71} On the other hand, systematic analysis of pedigrees contained in the Utah Population Database revealed distantly related patients with shared genomic regions from several extended families characterized by a high incidence of isolated CDH,⁷⁴ supporting the notion of heritability in isolated CDH.

CDH with associated lung hypoplasia and pulmonary hypertension is seen in a number of complex monogenic syndromes, including *LRP2* (Donnai-Barrow syndrome),⁷⁵ *CHD7* (CHARGE syndrome),⁷⁶ *STRA6* (Matthew-Wood syndrome),⁷⁷ *GPC6* (Simpson-Golabi-Behmel syndrome),⁷⁸ and *NIPBL* (Cornelia de Lange syndrome),⁷⁹ among others.^{66,80} The importance of these genes is illustrated by the fact that we identified heterozygous predicted pathogenic variants, possibly risk alleles, in these genes in patients with both isolated and complex CDH.⁶⁸

Copy number variation studies using array-based comparative genome hybridization have allowed for more detailed delineation of chromosome regions and have provided higher resolution to detect smaller deletions and duplications.^{81,82} In addition, recurrent chromosomal anomalies have been described in association with human CDH cases, and these have pointed toward several known and putative CDH genes. Chromosome studies identified several recurrent deletions in patients with CDH, so-called hotspots, including 1q41-42, 8p23.1, and 15q26.^{83–87}

Recently, next-generation sequencing technology, including whole exome sequencing and whole genome sequencing, allowed detection of single-nucleotide variants; however, the major challenge is how to prioritize the overwhelming number of potentially damaging variants that are detected.^{68,72} Bioinformatics can be used to enhance interpretation of individual gene variants by identifying their participation in molecular pathways and by documenting normal gene expression in the developing diaphragms and lungs, first in wild-type mice,⁸⁸ then in mutant mice, and eventually in diaphragms of patients with CDH for comparison. The underlying hypothesis of these systems biology approaches is that CDH causative genes, however disparate, will fall into a limited number of key developmental pathways,^{68,88–90} as exemplified in a recent study of a cohort of 275 patients with CDH by whole exome sequencing.⁶⁸

Mouse Models of CDH and Lung Hypoplasia

Because diaphragm and lung development in the mouse closely parallels that of the human, mouse models have played an invaluable role in enhancing our understanding of the genetic mechanisms underlying CDH and CDH-associated lung disease. To date, >70 genes have been found to cause diaphragm defects in existing mouse models, many of which have comorbid lung hypoplasia^{63,91} (Table 1). Many of these critical genes, discovered by mouse models, were later found to harbor rare and predicted pathogenic mutations in patients with CDH, thereby implicating these genes in human CDH.⁶⁸

Unbiased genetics screens can be used to identify further genes that cause CDH and lung defects, for example, through such approaches as the second phase of the Knockout Mouse Project, a multi-institutional initiative to produce a knockout for every known protein-coding gene in

the mouse genome.^{92,93} Gross phenotyping of pups and adults can be supplemented by high-throughput screening of animal repositories by X-ray microtomography for identifying diaphragm defects in embryonic lethal strains.⁹⁴

Globally, mouse models have been useful for the characterization of developmental mechanisms (Mouse Genome Informatics, <http://www.informatics.jax.org>, last accessed February 2016) and provide models for future *in vivo* rescue experiments after drug discovery screens. CRISPR/Cas9-mediated genome editing has been useful in enhancing the efficiency and specificity of generating targeted mutations in mice.^{95–98} When coupled with Cre-directed strategies,²⁷ it has been possible to dissect tissue- and cell-specific roles (eg, epithelial versus mesenchymal) for each gene in the developing lung.

Studies of Alveolar Septation in CDH Models

CDH lungs have aberrant morphologic features characterized by reduced radial alveolar count and thicker alveolar septa. Defective morphogenesis of the distal lung in the form of alveolar simplification is common.^{99,100} Late creation of the hernia in surgical models can produce alveolar hypoplasia without obvious branching defects.¹⁰¹

A number of mechanisms have been hypothesized to lead to abnormal septation in these models. Epithelial and endothelial approximation, often referred to as blood-gas barrier, is the functional unit of the alveoli, and collagens together with other extracellular matrix proteins are the major components of the basement membranes that support the architecture and function of the epithelium and endothelium. Reduced elastic fiber deposition may be responsible for the abnormal alveolar walls in patients with CDH,¹⁰² more specifically, defects of secondary alveologenesis possibly linked to insufficient fibroblast growth factor-18 expression.¹⁰³ Reduced tropoelastin and elastin fibers result in stunted secondary septa with immature alveolar myofibroblasts ectopically displaced into the lung interstitium,^{50,103} where they disrupt oxygen exchange.²⁷ Because this phenotype mimics the pathologic findings in humans surviving with CDH, it offers a good model in which to test for therapeutic rescue.

Although several techniques have been used to investigate postnatal lung development, few provide the means to study secondary alveolarization directly. In fact, advancements in our understanding of pediatric pulmonary pathophysiology have been hindered by the lack of reproducible *in vitro* models. To this end, Kelleher and colleagues¹⁰⁴ developed an assay based on *ex vivo* organ culture of P4 murine lungs that overcomes the limitations of substrate diffusion by preinflating the organ with 0.4% low-melt agarose at 20-cm H₂O pressure. Organ maturation progresses for up to 4 days in culture, maintaining alveolar architecture, histologic features, and morphometrics (septal density and mean linear intercept). Furthermore, this *ex vivo* model is amenable to

experimental manipulations, which will be useful to validate candidates emerging from drug discovery.

Developmental Expression Profiling

A useful strategy that we and others have used to identify CDH candidate genes is to compare human genetic data against expression profiles of the developing diaphragm, with the assumption that genes expressed at high levels during development are most likely to cause disease. We generated gene expression profiles from laser dissected PPFs, the anlage of the primordial diaphragm, prominent at E11.5 to E12.5 in the mouse embryo. This study identified a group of genes highly expressed in the PPFs but downregulated in the mature and fully muscularized diaphragm.⁸⁸ As proof of principle, we validated this approach by detecting the presence of diaphragm and lung defects in the mouse knockout of one of the prioritized gene candidates, *Pbx1*⁸⁸; furthermore, pathogenic variants in its paralog gene *Pbx3* were subsequently identified in exomes of a cohort of 275 patients with CDH while being absent in controls,⁶⁸ thus adding credence to this integrative approach for identifying novel CDH genes. This developmental data set has also helped in the interpretation of *de novo* variants from sequencing studies in trios and familial cases.⁷²

Because developmental gene expression profiling in the diaphragm has proven to be a useful tool for identifying CDH genes, the search for candidate genes is being expanded by examining genes expressed in the developing lung,¹⁰⁵ with a focus on genes that play a role in the later stages of lung development that may be amenable to therapeutic intervention.¹⁰⁴

In addition, RNA sequencing was used to elucidate mRNA and miRNA expression changes in nitrofen-induced rat hypoplastic lungs¹⁰⁶ that are evident even before the diaphragmatic defect could be observed. KEGG pathway analysis of significantly differentially expressed transcripts suggested that Wnt, transforming growth factor- β signaling, and retinol metabolism pathways are involved in nitrofen-induced hypoplastic lungs, whereas differentially expressed miRNAs appeared to mediate nitrofen-induced cell cycle arrest and inhibition of proliferation, as well as smooth muscle cell dysfunction.¹⁰⁶ Furthermore, miR449a, which functions to control *Myc*-directed normal lung proliferation, was elevated in mouse and human CDH lungs.¹⁰⁷

Drug Discovery and Novel Therapeutic Approaches

Human and animal genetics held the promise of uncovering novel therapeutic targets because current treatment options for infants with CDH are limited to supportive ventilatory care and surgical repair. Attempts to increase pulmonary function with the use of prenatal and perinatal glucocorticoids, surfactants, and a number of pulmonary vasodilators

have not been entirely successful.^{3,62} In addition, the development of therapeutics for CDH-associated lung hypoplasia and pulmonary hypertension has been hindered by lack of standardization in methods and validated outcomes in preclinical research.¹⁰⁸

Recent experimental approaches in nitrofen-treated rats with simvastatin revealed improvement in lung-to-body weight ratios and lung parenchyma structure,¹⁰⁹ with effects attributed to changes in the endothelin, nitric oxide, and bone morphogenetic protein receptor type 2 signaling pathways, and activation of epithelial apoptosis for tissue remodeling.¹⁰⁹

Fetal endotracheal occlusion^{110,111} was developed on the premise that increased intrapulmonary pressure would improve lung maturation. In the fetal endotracheal occlusion procedure, an inflatable balloon^{110,112,113} or, more recently, a dissolving gel bolus¹¹⁴ is inserted in the trachea of late-stage fetuses. A fetal endotracheal occlusion study is currently recruiting patients with CDH in the United States (ClinicalTrials.gov identifier: NCT00881660), but the clinical utility of this procedure is still unclear. Stem cell therapy by transplantation of human amniotic fluid stem cells has also been attempted in fetal endotracheal occlusion rabbit models with promising effects on lung-to-body weight ratio, mean terminal bronchiole density, and interstitial thickness.^{115,116}

Retinoid signaling defects have been associated with diaphragm abnormalities and lung defects.¹¹⁷ Because of its poorly understood therapeutic window, retinoids and their derivatives have been avoided in the treatment of CDH and its related complications. However, improved knowledge of the mechanism of action of retinoic acid in the developing lung, such as through its effect on PNECs,^{57,58} may revive its consideration as a treatment option. In fact, the PNEC-secreted hormone ghrelin alleviates the phenotype of pulmonary hypoplasia in a CDH animal model.¹¹⁸

Among molecules currently being tested for their ability to rescue the pulmonary phenotype of CDH is the glucagon-like peptide-1. When administered transplacentally in a surgically induced rabbit model, glucagon-like peptide-1 improved peripheral pulmonary vessel morphologic features without changes in alveolarization. However, its administration was associated with significant maternal and fetal adverse effects.¹¹⁹

Drug discovery should be designed to target the basic developmental processes of alveologenesis in mice with conditional knockouts and directed toward specific cell types in the developing lung as potential therapeutics to rescue animal models *in vivo*. Similar approaches can be used to uncover pharmacologic agents to correct vascular changes in CDH-associated pulmonary hypertension and in the hyperoxia model of bronchopulmonary dysplasia.⁴⁹ Molecules that have a successful outcome *in vivo* could be advanced, in due time, to the design and implementation of clinical trials. To this end, a clinical translational infrastructure already established to study the genetics of CDH,

through collaborative efforts among neonatal care centers in the United States and abroad in the form of multicenter consortia, can be used to conduct clinical trials and to carry forward promising candidates as effective therapies for wider use in the clinic.

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