Cellular Origin(s) of Congenital Diaphragmatic Hernia

Gabriëla G. Edel[1,2], Gerben Schaaf[3,4,5†], Rene M. H. Wijnen[1†], Dick Tibboel[1†], Gabrielle Kardon[6†] and Robbert J. Rottier[1,2†]*

[1] Department of Pediatric Surgery and Intensive Care, Erasmus MC-Sophia Children's Hospital, Rotterdam, Netherlands, [2] Department of Cell Biology, Erasmus MC, Rotterdam, Netherlands, [3] Department of Clinical Genetics, Erasmus MC, Rotterdam, Netherlands, [4] Department of Pediatrics, Erasmus MC-Sophia Children's Hospital, Rotterdam, Netherlands, [5] Center for Lysosomal and Metabolic Diseases, Erasmus MC, Rotterdam, Netherlands, [6] Department of Human Genetics, University of Utah, Salt Lake City, UT, United States

Congenital diaphragmatic hernia (CDH) is a structural birth defect characterized by a diaphragmatic defect, lung hypoplasia and structural vascular defects. In spite of recent developments, the pathogenesis of CDH is still poorly understood. CDH is a complex congenital disorder with multifactorial etiology consisting of genetic, cellular and mechanical factors. This review explores the cellular origin of CDH pathogenesis in the diaphragm and lungs and describes recent developments in basic and translational CDH research.

Keywords: congenital diaphragmatic hernia (CDH), diaphragm, pleuroperitoneal folds, perivascular cells, mesothelium

INTRODUCTION

In the past 25 years, the general mortality rate of CDH has decreased to approximately 25%, but the mortality rate remained 50% in patients who receive extracorporeal membrane oxygenation (ECMO) (1, 2). In a recent study, CDH patients were categorized pre-ECMO in a low-risk, moderate-risk or high-risk cohort by their risk score (RS) for mortality, which is based on multiple risk factors, like location of the hernia and weight before ECMO (1). Change in individual likelihood of death overtime was different for each cohort: it was increased in the low risk group, decreased in the moderate risk group and unchanged in the high-risk group. Although the average survival of CDH patients increased in the past decades as a result of advancements in prenatal diagnosis, CDH pathogenesis remains still poorly understood. The main reason is that CDH is a complex congenital disorder with multifactorial etiology including genetic, cellular and environmental factors (3–5). In this review, we will discuss different tissues and cell types that are implicated in CDH pathogenesis and review recent developments in basic and translational research in CDH.

DIAPHRAGM/PPF

The diaphragm is an essential muscle that is critical for proper respiration and forms a barrier between the thoracic and abdominal cavities (6). The diaphragm develops from multiple embryonic sources. Of primary importance are the pleuroperitoneal folds (PPFs). The PPFs are paired transient pyramidal-shaped structures located between the thoracic (pleural) and abdominal (peritoneal) cavities. The PPFs expand dorsally and ventrally across the cranial surface of the liver to give rise to the diaphragm's muscle connective tissue and central tendon (7–9). The somites, segmental structures lying adjacent to the neural tube, are the source of the diaphragm muscle.

Muscle progenitors emigrate from the cervical somites to the nascent PPFs and as the PPFs expand, the progenitors migrate and fuse into the radial array of costal myofibers (7–11). In addition, the diaphragm is innervated by nerves that arise from the C3-C5 segments of the neural tube and it is vascularized by endothelial cells derived from the somites and likely splanchnic lateral plate mesoderm (8–10).

The PPFs are essential for the morphogenesis of the diaphragm and defects in the PPFs lead to CDH. Experiments in mice genetically tracking the development of the PPFs have established that the expansion of the PPFs drives the overall morphogenesis of the diaphragm and guide diaphragm muscle development (7). Defects in the development of the PPFs are a significant source of CDH. Most CDH-implicated genes that have been examined are expressed in the PPF cells (7, 12–15). Mutations in CDH-implicated genes can lead to the incomplete expansion of the PPFs and thus lead to incompletely developed diaphragms that allow herniation of abdominal contents into thoracic cavity [e.g., (16)]. Alternatively, PPFs harboring mutations may be unable to signal to muscle progenitors [e.g., (7)], leading to defective progenitor migration to the PPFs, increased apoptosis, and/or decreased proliferation or differentiation into myofibers. In such scenarios, defects in the PPF cells lead to cell non-autonomous effects on neighboring muscle, resulting in weaker muscleless regions that allow herniation (7). To date, all evidence suggests that CDH arises from primary defects in the PPFs by either defective generation/migration of cell populations or impaired muscularization of the diaphragm, with little evidence to support a primary role for defects in muscle cells.

LUNG MESOTHELIUM

Pulmonary hypoplasia is another characteristic of CDH. Although a lower amount of alveolar type I cells have been identified in nitrofen-induced CDH (17, 18), the tissue that is primarily defective in CDH-associated hypoplasia is not yet clear. A potential cellular source in CDH pathogenesis is the lung mesothelium, which contributes to different cell populations in the lung and is important for proper mesenchymal growth. The pleural mesothelium is a monolayer of cells that forms a lining around the lungs and is derived from the embryonic mesoderm (19). Although mesothelial cells are mesenchymal in origin, they have epithelial characteristics (20). During development, pulmonary mesothelial cells (PMCs) undergo epithelial-mesenchymal transition and differentiate to contribute to different cell populations in the pulmonary mesenchyme under the influence of active hedgehog signaling (21).

Several studies reported conflicting data to what extent the embryonic mesothelium contributes to the pulmonary mesenchyme. These studies employed different *Wilm's tumor 1* (*Wt1*)-dependent driver lines, which is a gene encoding a zinc finger transcription factor expressed in mesothelial cells and diaphragm and that is associated with CDH (22). *Wt1*-dependent lines were used to trace WT1^{+} mesothelial cells and analyzed the fate of the progeny at several time points, which most likely explains the variation in their results. Que et al. (23) reported that WT1^{+} lung mesothelial cells contribute to vascular smooth muscle cells (SMCs) and to alveoli, which were potentially interstitial fibroblasts, alveolar myofibroblasts or endothelial cells. Dixit et al. chose a conditional strategy to lineage trace *Wt1*-expressing mesothelial cells and reported contribution of PMCs to vascular and bronchial smooth muscle cells, as well as fibroblasts (21). Cano et al. (24) focused on the contribution of PMCs to different lung cell types during embryonic lung development and observed contribution to a wider range of cell types including endothelial cells, airway and vascular smooth muscle cells, pulmonary cartilage and fibroblasts. Lastly, Von Gise et al. (25) found that labeling WT1^{+} cells at E10.5 resulted in a small contribution to bronchial- and vascular smooth muscle cells, while the majority differentiated into PDGFRα[+]-fibroblasts or PDGFRβ[+]/NG2^{+}-pericytes. Von Gise et al. (25) also showed that only fetal and not postnatal PMCs are capable of differentiating into pulmonary mesenchymal cell types. Postnatal PMCs remain in the mesothelial lining and do not migrate out or differentiate into other cell types. As such, postnatal PMCs do not seem to contribute to the lung parenchyma during normal lung homeostasis or after injury (25). In conclusion, mesothelial cells contribute to the bronchial- and vascular smooth muscle cell population as well as to fibroblasts and pericytes and that this only occurs during embryonic development and not postnatally (21, 25).

Besides being a progenitor source during lung development, the mesothelium also acts as a signaling source. One important pathway during development of the lungs is the FGF signaling pathway, in which FGF9 and FGF10 are essential (26). FGF10 is expressed in the lung mesenchyme, while FGF9 is expressed in the mesothelium and epithelium, signaling to the submesothelial- and subepithelial mesenchyme, respectively (27, 28). Mesothelial- and epithelial-produced FGFs have a different function: mesothelial-derived FGF9 is mainly responsible for mesenchymal growth by maintaining mesenchymal FGF-WNT/β-catenin signaling, whereas epithelial-derived FGF9 influences epithelial branching (29). One study reported reduced pulmonary FGF9 expression in the nitrofen-induced CDH rat model (30). However, recombinant co-cultures of fibroblasts and epithelial cells of nitrofen-treated- and control rats showed that not epithelial cells, but fibroblasts, are defective in nitrofen-induced hypoplastic lungs, showing decreased apoptosis and increased proliferation (31). Since the mesothelium is a source for mesenchymal fibroblasts (21, 23–25) and expresses FGF9 for the regulation of mesenchymal growth (29), the mesothelium is a potential source for CDH-associated lung hypoplasia.

Cano et al. (24) showed that homozygous *Wt1* knock-out (*Wt1*^{−/−}) mice had a CDH-like phenotype, abnormally fused lung lobes and reduced immunoreactivity for FGF9 in the pulmonary mesenchyme and mesothelium. A recent study reported similar findings and also reported an aberrant lung branching architecture already before closure of the diaphragm, when WT1 is expressed (32). When *Wt1*^{−/−} lungs were cultured *ex vivo*, lung branching was normal and any hypoplasia that had originated *in vivo*, was restored within 24 h *ex vivo* (32). Additional analyses showed that the space in the chest

cavity—that is usually present for the lungs to grow—was nearly absent, explaining why culturing the lungs *ex vivo* without physical constraints recovered branching (32). Aberrant WT1 expression in the lung mesothelium results in defective lung development and CDH as result of limited space in the chest cavity and potentially by defective signaling and migration of mesothelial cells.

In summary, the mesothelium acts as a progenitor source and signaling center for the pulmonary mesenchyme to facilitate proper mesenchymal growth and cellular differentiation. These results associate the lung mesothelium as a cellular contributor to CDH.

(PERI)VASCULAR CELLS

Besides a diaphragmatic defect and pulmonary hypoplasia, almost all CDH patients have pulmonary hypertension (33, 34), which is caused by an altered development of the pulmonary vasculature and pulmonary vascular remodeling (35). Changes in cell phenotypes, cellular proliferation and defective cell-cell communication have been proposed as underlying causes.

Previously, it was shown that CDH patients have higher abundance of contractile vascular SMCs, which were also more distributed along the proximo-distal axis of the lung vasculature (36). Although inhaled nitric oxide treatment is a successful treatment for preterm babies, it is only effective in a small number of CDH patients and even only beneficial in certain subsets of CDH patients (37). The pathological changes in vascular SMCs indicate a disturbed pulmonary vascular development and might explain the ineffectiveness of inhaled nitric oxide treatment in CDH patients. Another study by Acker et al. (38) showed increased proliferation of pulmonary arterial SMCs and pulmonary arterial SMC hyperplasia in a surgical CDH lamb model. This was not caused by an altered SMC phenotype, but by a disturbed interaction with pulmonary arterial endothelial cells (PAECs), indicating that defective endothelial signaling contributed to SMC hyperplasia and may therefore result in pulmonary hypertension (38). In the nitrofen-induced CDH mouse model, Kool et al. (39) observed an increased pericyte coverage in the large pulmonary vessels and pericytes had a more contractile phenotype. Furthermore, the basement membrane around the midsized vessels was discontinuous, indicating defective cross-talk between pericytes and endothelial cells (39). The impaired cross-talk between those two cell types and the altered pericyte phenotype may be the origin of pulmonary hypertension in CDH.

CDH patients show a decreased vascular growth that contributes to poor disease outcomes. Acker et al. (40) showed in the surgical CDH lamb model reduced proliferation and tube formation capacity of PAECs. They also found a marked reduction in high-proliferative PAECs, which is a progenitor subpopulation of endothelial cells (41, 42). A reduced capillary network was also observed by Kool et al. (39) in the nitrofen-induced CDH mouse model. These results suggest that reduced proliferation of endothelial cells contributes to the decreased vascular growth in CDH patients.

Altogether, several cell types may disturb the lung vascular network, leading to CDH-associated phenotypes. Abnormal phenotypes of endothelial cells, SMCs and pericytes and defective interactions between them may be the basis for the simplified vascular network and pulmonary hypertension in CDH.

CELLULAR MODELS IN CDH RESEARCH

CDH cannot be attributed to one single source or defect, which makes it hard to study its pathogenesis, but new cell culture models can aid in improving insights at the cellular level. Recently, a cell culture model was described where PPFs from mice were isolated and cultured to outgrow and expand PPF fibroblasts, that maintained expression of key diaphragm genes (43). Pharmacological inhibition or genetic manipulation that causes CDH resulted in reduced *in vitro* proliferation in PPF-derived fibroblasts (43). Also, lung organoids were recently derived from induced pluripotent stem cells (iPSC) from fetuses and infants with CDH and showed reduced generation of lung progenitor cells and impaired epithelial- and mesenchymal differentiation (44). Recently, it was shown that organoid cultures can be obtained with a low input material from clinical samples, like tracheal aspirates from preterm newborns, and this method could also be used to grow organoids from CDH patients without the need of *in vitro* differentiation from iPSCs (45). Furthermore, endothelial cell culture models could aid in understanding defective endothelial cell function (40) and endothelial cell-SMC interaction (38). A differentiation protocol from human iPSCs to endothelial progenitor cells that mimics *in vivo* embryonic vascular development was recently published (42). This method can be used to generate iPSC-derived endothelial cells from CDH patients to eventually use these cells in cell co-culture systems, like organoid cultures. Although CDH is a multifactorial disease, *in vivo* experiments are limited and these kind of culture models will help to understand the pathology of CDH by specific cells, the interaction between different cell types and the molecular mechanisms in patient specific cultures.

DISCUSSION

In this review, we discussed cellular origins that are associated with CDH pathogenesis, including the diaphragm and PPF, lung mesothelium and (peri)vascular cells. All can contribute to a CDH phenotype, but also extrinsic factors play a role. Because pulmonary defects and alterations in the space of the chest cavity occurred prior to diaphragm closure and may even be the cause of a diaphragm defect (32), it is interesting to study the space in the chest cavity in other CDH models and potentially in human fetuses to serve as an early predictor for CDH. To mimic limited chest space and compression, lung organoids have been subjected to mechanical pressure, which altered their development (44). These results show that extrinsic factors play a significant role in CDH pathogenesis and the influence of limited space and compression should be studied further, since *Wt1* knock-out lungs still had the capacity to develop normally *ex vivo*, which is promising for potential treatment strategies (32).

Intrapleural delivery of compounds has been suggested to target PMCs in idiopathic pulmonary fibrosis, since this may result in increased efficacy combined with reduced systemic toxicity of therapeutic agents (19, 46). This delivery strategy may also be interesting for targeting PMCs during lung development. Mesothelial mobilization is not only implicated in development of the lungs, but also in other organs, like the liver, heart and reproductive system (47). However, it has been shown that this process can differ between different organs and that mesothelial migration in developing lungs differs in timing and pathway dependency compared to other organs, like the heart (48). These features could be used for organ-specific targeting and makes the embryonic mesothelium an interesting therapeutic target.

In summary, defective development, communication or migration of cells from the diaphragm, the mesothelium and (peri)vascular cells of the lungs play an important role in CDH pathogenesis. Improved *in vitro* cell culture models and the generation of patient-specific cultures will provide more insights in cellular and molecular mechanisms that underlie these defects and their use may be beneficial for identification and testing of putative therapeutic agents.

AUTHOR CONTRIBUTIONS

GE and GK wrote the manuscript. GS, RR, and DT carefully edited and revised the various versions of the manuscript and approved the final manuscript. RW revised and approved the final manuscript. All authors approved the final manuscript as submitted.

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