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Congenital Diaphragmatic Hernia: Comparison of Animal Models and Relevance to the Human Situation

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CDH · Pulmonary hypoplasia · Transgenics · Nitrofen · Retinoids · Surgery

Abstract

Congenital diaphragmatic hernia (CDH) occurs in 1 in 3,000 newborns. Mortality and morbidity are due to the amount of pulmonary hypoplasia (PH), the response on artificial ventilation and the presence of therapy-resistant pulmonary hypertension. The pathogenesis and etiology of CDH and its associated anomalies are still largely unknown despite all research efforts over the past years. Several animal models have been proposed to study CDH. In this review we compare surgical, pharmacological and transgenic models, and discuss their strengths and limitations to study PH.

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Introduction

Congenital diaphragmatic hernia (CDH), a developmental defect of the diaphragm, has a prevalence of 1 in 2,000–3,000 newborns and accounts for approximately 8% of the known major congenital anomalies [1]. In humans, three different types of hernia can be distinguished: a posterolateral Bochdalek type (about 70% of the cases),

an anterior Morgagni type (about 27% of the cases) and a central hernia, septum transversum type (about 2–3% of the cases). Eighty-five percent of the hernias occur on the left side, 13% on the right and only 2% are bilateral [reviewed in 2–4]. Children with a CDH suffer from a substantial amount of morbidity and mortality due to the associated abnormal pulmonary development resulting in two clinical problems: pulmonary hypoplasia (PH) and persistent pulmonary hypertension. Both conditions are present to a variable extent in patients with CDH and despite the fact that recent progress in the care of these children has resulted in survival rates of up to 90% in some tertiary-care centers, these measures have not led to a lower morbidity [5]. However, due to the absence of sufficient lung-protective strategies, most of the newer treatment modalities have replaced mortality with a higher morbidity. The problem with these new treatment modalities such as high-frequency oscillation and/or inhaled nitric oxide and extracorporeal membrane oxygenation, is that they are designed for treating the sequelae of CDH, PH and persistent pulmonary hypertension and do not contribute to the prevention of these conditions. Moreover, prevention is not possible without a sound understanding of the etiology and pathogenesis of CDH. Essential elements required for a better understanding of this anomaly, notably how the different clinical problems relate to each other, are still lacking. A basic understand-

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ing of how a CDH arises together with PH and persistent pulmonary hypertension is fundamental in our quest for new answers on how to protect these children from the sequelae of this anomaly. Furthermore, it may aid in finding ways to modulate the natural course in a prenatally diagnosed baby in the near future [6].

Animal models available to study CDH include a surgical model in the rabbit and sheep, a pharmacological (nitrofen) model in the rat and mouse, and genetic (knock-out) mouse models. In this review we will describe these three models and specifically focus on PH, address their differences and discuss their relevance to human CDH.

Surgical Models

One of the hypotheses concerning the pathogenesis of PH in CDH is that it results from the intrathoracic herniation of the abdominal viscera, thereby compromising pulmonary development. Fetal breathing movements are impaired and, therefore, normal development of the lungs is hampered. Based on this idea, the first surgical animal models were created to study both lung pathogenesis and rescue options. The most commonly used surgical models use sheep and rabbits.

The sheep model was introduced by Delorimier et al. [7] in 1967. The hernia is surgically created at gestational days 72–75 (term is 145–149 days). The abdominal bowel is positioned into the chest to optimally mimic human CDH. Gestational days 72–75 in sheep are equivalent to a gestational age of 10 weeks in humans. This is the pseudoglandular stage of lung development: the moment of pleuroperitoneal canal fusion during diaphragmatic development [8]. Later, a similar surgical model was developed in rabbits. Advantages of the rabbit model over the sheep model are its shorter gestational period (term is 31 days with the hernia created at day 23), the larger litter size, easy availability and lower costs [9, 10].

Surgical models are mainly suitable to investigate interventional strategies in CDH. Examples of investigated interventions are administration of corticosteroids, in utero repair of the diaphragmatic defect and fetal tracheal occlusion or a combination of the two [11, 12]. In utero repair has been attempted with either primary closure of the defect by using a patch (immediate reduction) or secondary closure by using the slow 'silo' reduction technique in which the opening gradually reduces as the fetus grows [13–15]. After successful in utero repair of induced CDHs in animal models, including nonhuman primates, Harrison et al. [14] performed the first human surgical

repair in utero. Unfortunately, it quickly became clear that there was no improvement in survival and, moreover, an increase in premature delivery was observed.

Later, tracheal ligation or clipping was developed with the aim to gradually reposition the abdominal viscera back into the abdomen [16–18]. The rationale was based on the observation that children with a prenatal airway obstruction have hyperplastic lungs [19]. Preventing lung fluid efflux exerts a build-up pressure in the thoracic cavity that repositions the abdominal viscera back in the abdomen. Di Fiori et al. [17] demonstrated that tracheal ligation reversed the effects of surgically induced PH in fetal sheep. Unfortunately, the results of human trials on this ex-utero intrapartum treatment technique with clipping were disappointing even when a minimally invasive approach was used. Again premature delivery appeared to be the problem [20–22]. Later the 'plug the lung until it grows' method was developed in lambs. Endoscopically, an inflatable balloon is inserted through the fetal mouth in the trachea through a catheter, which then is filled with saline and kept in place for several days [23–28]. A tracheal occlusion trial in humans in North-America demonstrated no differences in survival when compared to controls. The authors blamed this on an improvement in survival in the control group due to increased care in a specialized center [22]. However, Deprest et al. [29, 30] stated that a great part of the enrolled patients were likely to have survived without treatment based on the 'lung area to head circumference ratio' risk assessment. Exclusively, the most severe CDH cases were enrolled in a European study and underwent the fetoscopic endoluminal tracheal occlusion (FETO) procedure. This study demonstrated up to 64% survival in comparison to 8% survival of nontreated comparable CDH patients, and thus appeared to be very promising [29, 31–33]. The same group demonstrated similar results in a more homogeneous group a year later [34]. Premature rupture of the membranes, with the risk of premature delivery, appeared to be a common complication, but in a more recent study it was shown that there was an increase to 75% of deliveries after 34 weeks when FETO was performed due to improved experience. The most recent publication demonstrated a survival rate of 50%, with a higher neonatal survival with prenatal balloon removal in comparison to perinatal removal [33]. It was also demonstrated that lung volume before and lung response after FETO are important survival predictors [35, 36]. At this moment, the FETO task force is working on permission to start a randomized controlled trial to better validate these preliminary results [33, 37].

Tracheal occlusion in surgical models has not only been shown to improve PH, but pulmonary vascular abnormalities also benefit from this procedure. For instance, tracheal occlusion studies in fetal sheep with a surgically induced diaphragmatic hernia demonstrated thinning of the pulmonary artery, correction of the abnormal muscularization of pulmonary arterioles, and a decrease in vessel resistance in the left pulmonary artery with maternal hyperoxia as seen in normal fetal sheep at term [38–40]. Similar positive effects of tracheal occlusion on pulmonary vascular development have been demonstrated in the surgical rabbit model [28]. Both surgical models are useful to study pulmonary vascular abnormalities in CDH [41–43].

Surgical models are based on a surgical intervention making a diaphragmatic defect in fetal rabbits and sheep [44, 45]. This CDH model has proven especially useful in investigating interventional therapies such as the administration of corticosteroids, in utero repair of the diaphragmatic defect and tracheal occlusion [46, 47]. Unfortunately, the diaphragmatic defect is created relatively late in gestation and certain pulmonary changes seen in human CDH might have occurred prior to this time. Therefore, no information about the cause and early pathogenesis of the lung hypoplasia can be obtained with this model. Moreover, it is a uni-hit lung hypoplasia model while evidence suggests that a dual-hit might be responsible for the hypoplasia [48]. On top of that, a lot of the other associated anomalies (such as cardiac anomalies) cannot be studied in this model.

In summary, surgical animal models are useful in investigating interventional therapies, but are less instructive in studying the etiology and pathogenesis of CDH. Interventional studies in this model have resulted in the incorporation of new prenatal techniques in human fetuses, with very promising results so far.

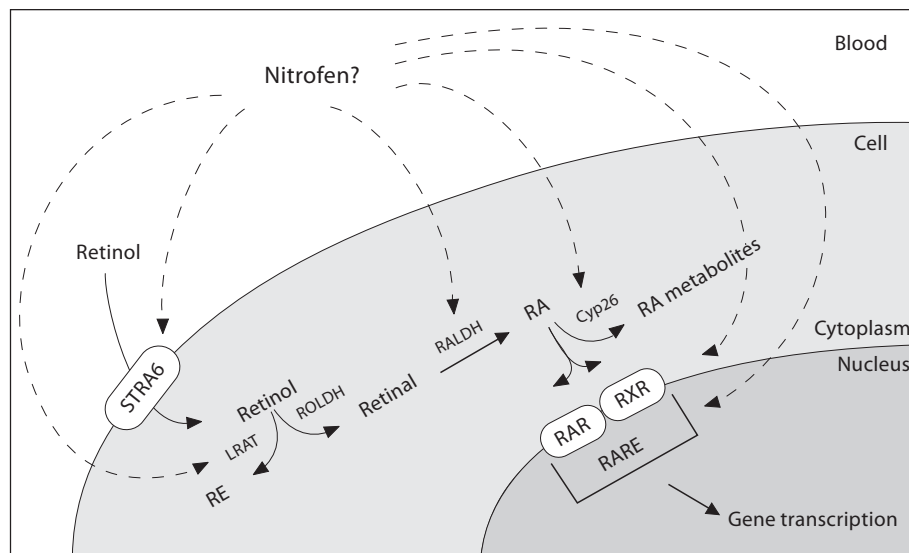
Nitrofen Model

The nitrofen model has been used for the past two decades to investigate the anomalies in CDH. Originally, nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) was used as a herbicide. In toxicology screens in adult rats, no apparent problems were observed, though administration during midgestation to pregnant dams appeared to cause developmental anomalies of the heart, lungs, diaphragm, and skeleton of the embryos [49, 50]. Based on the latter findings, nitrofen has been investigated for its usefulness to simulate the anomalies of CDH in rodents.

Numerous groups including ours demonstrated that nitrofen induced diaphragmatic hernias that were strikingly similar to the human condition. The specific location and extent of the diaphragmatic defects were very comparable, but the similarities in the CDH-associated anomalies, including PH and persistent pulmonary hypertension, and cardiovascular and skeletal defects, were impressive too [51–54]. When nitrofen is administered to pregnant rat dams on day 9 of gestation when normal lung (day 11 of gestation) and diaphragm development (day 13 of gestation) are just about to begin, approximately 70% of the offspring will develop CDHs and 100%, PH. Therefore, the nitrofen animal model, taking into account the obvious disadvantages of being a toxicological (animal) model, can serve as a good tool to investigate the pathogenesis and therapeutic options in CDH and its anomalies in rodents. Despite the extensive use of nitrofen as a herbicide in agriculture, its possible teratogenic effects have never been shown to play a role in human CDH.

The etiology of both human CDH and nitrofen-induced CDH in rodents has been connected to perturbations in the retinoid signaling pathway (fig. 1), although the exact underlying mechanism remains to be elucidated. The first evidence that CDH could be connected to perturbations in the retinoid signaling pathway was obtained already in 1941 by Andersen [55], who noted diaphragmatic hernias in embryos of pregnant rats on a vitamin-A-deficient diet. This effect of maternal vitamin A deficiency was confirmed by Wilson et al. [56] in 1953. More modern approaches using genetic manipulation in mice have shown that ablation of retinoic acid receptor (RAR) signaling during development indeed results in diaphragmatic hernias, PH and/or lung agenesis [57]. In humans, only one small clinical study (n = 7) demonstrated that newborns with CDH had lower levels of plasma retinol in cord blood than controls [58]. Subsequently, CDH has been observed in patients with deletions on the 15q chromosome, which contains the encoding gene for a cellular retinoic acid binding protein (CRABP1), although so far mutation analysis in isolated CDH cases are negative [59–61]. In 2000, the dual-hit hypothesis was introduced. It explained PH in CDH as a result of two insults [48]. There is an early bilateral nitrofen-induced PH observed prior to closure of the diaphragm (first insult) [62, 63]. The second insult is caused by herniation of the abdominal viscera into the thorax due to disrupted closure of the diaphragm and affects the ipsilateral lung only by interference with fetal breathing movements. Administration of retinoic acid (RA) to nitrofen-treated lung ex-

Fig. 1. Schematic representation of the retinoid signaling pathway. Retinol binds STRA6 and is transferred into the cytoplasm. Retinol can either be stored as retinyl ester (RE) by LRAT or be converted into retinal by retinol dehydrogenase (RALDH). Retinal is converted into RA by RALDH. RA can either remain in the cytoplasm to be metabolized by cytochrome p450 (Cyp) 26 enzymes, or bind to RAR or retinoid X receptor (RXR) to activate the RA response element (RARE) and thereby alter gene transcription. Pathways that might be influenced by the effects of nitrofen are indicated by arrows.



plants demonstrated an increase in lung growth and partially rescued the hypoplasia [64]. This observation was supported by Thebaud et al. [65, 66], who demonstrated an improvement in lung maturation and growth in nitrofen-treated embryos when the pregnant dam was treated with vitamin A before, during or after nitrofen administration. In addition, survival of the fetuses improved in the vitamin-A-treated group [66]. Subsequently, administration of RA and vitamin A were compared in their effectiveness to reduce the number of hernias. In the untreated group the incidence of hernia was 54%. With vitamin A treatment, the number of hernias was reduced to 32%. RA demonstrated a reduction to 15% and with continuation of the RA feedings for up to 5 days, even a percentage of less than 10% was reached [67]. This supports the concept that both the diaphragmatic hernia and the PH result from a disruption in the retinoid signaling pathway.

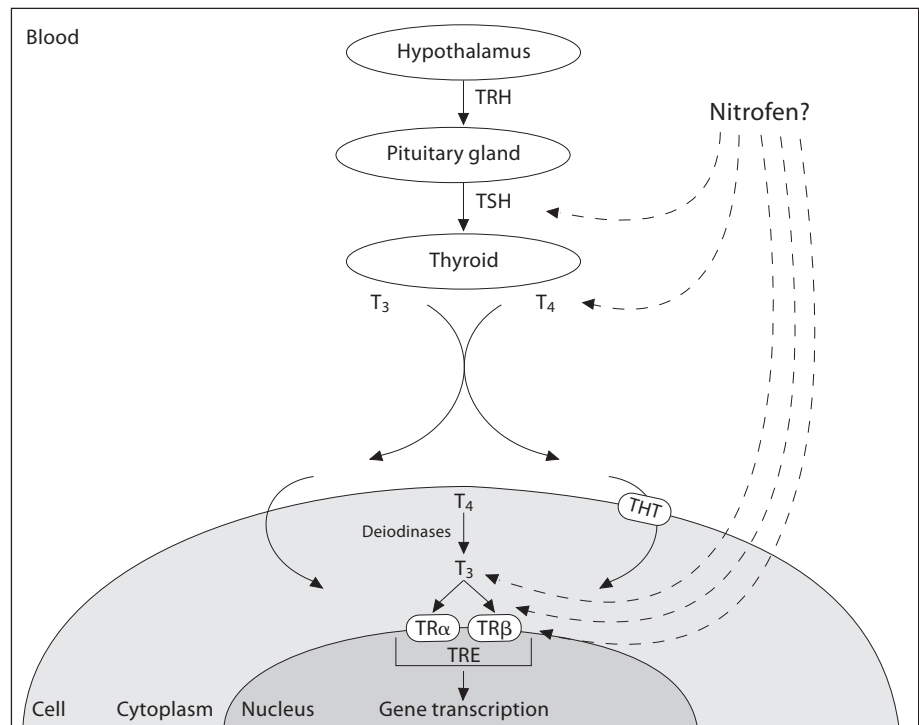
Retinal dehydrogenase (RALDH) 2 is perceived to be the key enzyme in the RA synthetic pathway [68–70]. In vitro experiments have demonstrated that several agents (including nitrofen) responsible for the induction of diaphragmatic hernias, inhibit RALDH2 activity [71, 72]. Others have proposed that nitrofen interferes with the uptake of retinol by lung cells. Nitrofen-treated lungs have lower retinol levels while circulating retinol levels are increased in comparison to controls, in agreement with the idea of a disturbed retinol uptake [73]. Recently, STRA6 has been identified as the membrane receptor for serum retinol, and mutations in STRA6 result in dia-

phragmatic hernias and PH amongst a variety of other anomalies [74–76]. However, nitrofen does not block the uptake of retinol by STRA6 [72]. Nitrofen treatment has been reported to downregulate the pulmonary retinol storage enzyme, lecithin:retinol acyltransferase (LRAT), and the RA-degrading enzyme Cyp26, while not affecting RALDH2 [42]. Since vitamin A deficiency experiments have shown similar decreases in RA-degrading enzymes and storage enzymes [77, 78], it is thought that this downregulation is due to low pulmonary retinol levels. Evidently, the exact mechanism by which nitrofen affects RA synthesis remains to be elucidated. It has also been suggested that nitrofen may compete with RA to bind to the RA receptor during embryogenesis, thereby impairing lung and diaphragm development [66, 79]. In a two-hybrid yeast assay nitrofen inhibited RAR and retinoid X receptor association only when very high embryonic lethal dosages were used [72]. In contrast, Chen et al. [80] demonstrated that nitrofen inhibits the activation of RA response elements in lacZ mice. RAR expression is not affected in CDH. Rajatapiti et al. [81] reported that it was normal in human CDH lungs and in nitrofen-induced rat CDH lung tissue.

In summary, the retinoid signaling pathway is complex and it appears that a disruption anywhere in the pathway might be responsible for the morphological changes including PH seen in CDH.

Besides the retinoid signaling pathway, another pathway implicated in CDH is the thyroid hormone signaling pathway (fig. 2) [82]. Nitrofen, triiodothyronine (T_3) and

Fig. 2. Schematic representation of the thyroid signaling pathway. Thyroid hormones T_4 and T_3 are produced in the thyroid gland. The hypothalamus produces thyrotropin-releasing hormone (TRH) which stimulates the pituitary gland to release thyroid-stimulating hormone (TSH) which for its part directly acts on the thyroid gland to stimulate T_4 and T_3 synthesis. At the target cells membrane passage is either carrier-mediated by thyroid hormone transporters (THT) or by diffusion. In the cell T_4 is converted to T_3 by deiodinases and binds to the nuclear TRs $TR\alpha$ and $TR\beta$. Through activation of the thyroid response element (TRE), gene expression is altered. Pathways that might be influenced by the effects of nitrofen are indicated by arrows.



thyroxine (T_4) have similar chemical structures. All three are halogenated diphenyl ethers [83–85]. Thyroid hormones are important in lung morphogenesis [86–88]. Thyroid hormone receptors (TRs) are mostly expressed after gestational day 13 in the rat [89], but it has been demonstrated that very low levels of message are present at day 11 in the embryo [90]. Both T_3 and T_4 can cross the placenta during embryo morphogenesis in the rat from gestational day 9 onwards [91–93]. Therefore, it is possible that nitrofen influences both diaphragm formation and lung development by interfering with the thyroid hormone signaling pathway [72]. However, nitrofen-treated adult mice have decreased T_4 levels while T_3 levels remain normal [83]. In addition, fetuses of pregnant rats treated with nitrofen have lower circulating T_3 and T_4 levels, but pulmonary levels of T_3 and T_4 are not changed when compared to control fetuses [84, 94, 95]. If nitrofen exerts its action due to structural similarities with thyroid hormones (thyromimetics), it would be in a competitive manner. Nonetheless, Brandsma et al. [85] demonstrated that nitrofen inhibits binding of T_3 to $TR\alpha 1$ and $TR\beta 1$ in a noncompetitive manner by reducing the maximal binding capacity in vitro. In contrast, Noble et al. [72] found no perturbation in TR binding in the presence of nitrofen. When nitrofen and T_4 were administered simultane-

ously to thyroidectomized pregnant rats, the incidence of congenital anomalies in embryos dropped by 70% [84]. Despite this observation, coadministration of T_4 and nitrofen did not reduce the percentage of CDHs [72].

Similar to RAR, $TR\alpha 1$ and $TR\beta 1$ belong to the steroid/thyroid/retinoid receptor superfamily. Noble et al. [72] demonstrated that $TR\alpha 1$ and thyroid response element activity were not influenced by nitrofen in vitro. In vivo, however, nitrofen reduced the expression of $TR\alpha 1$ and $TR\beta 1$ in CDH rat lungs [96] without changing their cellular localization [81]. A similar decrease in $TR\alpha 1$ expression was noted in human CDH-related hypoplastic lungs [81]. This decrease in $TR\alpha 1$ could lead to a diminished response to maternal thyroid hormone and later on (from gestational day 18 in rats) to the hormones produced by the fetus itself. In this way, lung morphogenesis might be affected by nitrofen [96]. As described earlier, Montedonico et al. [64] demonstrated a partial rescue by RA treatment in nitrofen-induced hypoplastic lung explants. The authors suggested that this might be only partial because downregulation in the thyroid hormone signaling pathway might also contribute to the PH. However, we should bear in mind that the role of thyroid hormones appears to be limited since in TR null mutant mice no apparent lung and diaphragm problems were observed and, there-

fore, antenatal lung growth does not seem to be impaired by a lack of thyroid hormones [97]. In addition, van Tuyl et al. [98] demonstrated that both maternal and fetal hypothyroidism in transgenic mice did not alter prenatal lung development. Furthermore, there is great redundancy between the different TRs. Thus, while TR α 1 levels are reduced in nitrofen-induced CDH rat lungs as well as human hypoplastic CDH lungs, the lack of both lung and diaphragm defects in TR null mice makes its role in the pathogenesis of CDH less likely. Even though nitrofen and thyroid hormones have similar structures, a clear relation between the thyroid signaling pathway and lung or diaphragm defects has not been demonstrated so far.

Similar to the surgical CDH models, the nitrofen model has been used to study vascular defects in CDH [99]. However, a detailed discussion of vascular abnormalities associated with CDH is beyond the scope of this review.

The nitrofen model is based on the administration of the herbicide before the onset of lung and diaphragm formation. Although this model appears to be the best model available since the timing of the developmental insult is similar to that in humans, a large disadvantage is that the significance of the potential teratogenic effects of nitrofen in rodents has never been demonstrated in humans. Although increasing evidence of the etiology of CDH points towards a disturbance in the retinoid signaling and/or thyroid signaling pathways, the nitrofen model has not resolved the pathogenesis of CDH and the associated PH.

Genetic Models

Since the first knockout mice were produced at the end of the eighties, they have been widely employed by molecular biologists to investigate the function of the gene that is made inoperable. In this way, several expected and unexpected genes have been linked to CDH.

Wilm's Tumor 1 (wt1)

The original paper describing the phenotype of Wilm's tumor 1 (*wt1*) null mutant mice focused on the role of this tumor-suppressor gene in urogenital development [100]. In the same paper, the authors briefly describe the incomplete formation of the diaphragm in the mutants resulting in the herniation of the lungs into the abdominal cavity, whereas in human CDH, abdominal contents normally herniate into the thorax. However, in a recent publication, Clugston et al. [101] describe a more classical picture of abdominal contents herniating into the thorax

in a comparative study on diaphragm development in three animal models for CDH including *wt1* null mutant mice. In addition, the authors observe a 'real' posterolateral (Bochdalek) hernia based on the malformation of the pleuroperitoneal folds as opposed to different diaphragmatic defects observed in other knockout mice. An accurate indication of the incidence of CDH could not be calculated because of the small numbers of fetuses investigated. Despite the 'true' Bochdalek phenotype of the *wt1* null mutant mice, a translation to the human situation of CDH has not been made. Besides a few reports of mutations of WT1 in human case reports on syndromic CDH such as WAGR and Denys-Drash, no relationship between the presence of the WT1 mutation and isolated CDH was found [102–105]. In a Swedish series of 27 children with isolated CDH no WT1 gene mutations could be detected [106].

Sonic Hedgehog (Shh) and Gli2/Gli3

Sonic Hedgehog (Shh) and *Gli2* and *Gli3* are members of a highly conserved morphogenetic family known as the *Shh*-signaling pathway [107]. In the original publications on the functions of *Shh* and *Gli2* and *Gli3*, no mention was made on the diaphragmatic defects some of the null mutant mice displayed. These papers focused on the foregut anomalies such as abnormal branching morphogenesis of the lungs, tracheal-esophageal fistula and esophageal atresia, respectively [108–110]. In *Shh* null mutants, there is a failure of tracheo-esophageal separation and, in addition, the lungs have undergone less branching morphogenesis, making them hypoplastic [108, 110]. Interestingly, *Shh* expression is decreased in human hypoplastic lungs of CDH patients [111].

Studies from *Gli2* and *Gli3* double-knockout mice demonstrated a similar phenotype of foregut abnormalities, but a more severe phenotype of disturbed branching morphogenesis. *Gli2*^{-/-} mice have only one lobe on the right side (instead of four) indicating that lungs are formed, but primary branching is affected. In *Gli2*^{-/-}/*Gli3*^{-/-} mice, no lungs are formed at all. In *Gli2*^{-/-}/*Gli3*^{+/-} mice there is ectopic branching and fusion of lung lobes [109]. However, none of the first studies described a CDH in these mice. In a more recent publication, the same group demonstrated that the single null mutant mice for both *Gli2* and *Gli3* as well as the double mutant *Gli2*^{-/-}/*Gli3*^{+/-} mice have diaphragmatic defects [112]. No description of the type of hernia was given for either *Shh* or *Gli* null mutant mice. No relationship between a mutation in GLI genes and CDH has been demonstrated in humans so far.

Slit3

Slit3 belongs to the family of Slit guiding proteins that are highly conserved throughout evolution. Especially *Slit1* and *Slit2* have been investigated for their role in axon guidance and cell migration [reviewed in 113]. Approximately 70% of *Slit3* null mutant mice have a CDH [114, 115]. In contrast to other animal models for CDH, most of these mice do not die after birth. The mice display a defect in the central tendon of the diaphragm, which fails to detach from the liver on the right side, thereby making it a good model of the human central (septum transversum) type of hernia. The origin of the defect lies in a defective connective tissue formation in the central septum transversum. The innervation of the phrenic nerve to the diaphragm was found to be normal in the knockout mice. No primary lung phenotype was observed in the mice. However, mice with end-stage CDH were short of breath due to atelectasis and intrapulmonary hemorrhage. Altogether, these data suggest that *Slit3* might play a role in a small subgroup of CDH cases. Recently, a case report of a newborn with CDH with a hernia sac attached to the liver was published [116]. The left side of the liver formed a part of the sac of the CDH. Liver tissue including vessels was seen in the sac, as seen in *Slit3*^{-/-} mice [115]. This is the first time that presence of hepatocytes within the sac itself was observed in a patient with CDH.

Fog2 (Friend of GATA)

Employing an elegant approach of high-throughput mutagenesis analysis using the chemical mutagen N-ethyl-N-nitrosourea, Ackerman et al. [117] identified a mouse line with respiratory failure at birth. This phenotype was based on PH, disturbed heart development and a diaphragmatic defect and could be linked to a mutation in the gene *Fog2 (Friend of GATA)*. In contrast to the other models, these mice displayed severe PH in the absence of a hole in the diaphragm. Instead, a posterolateral muscularization defect was observed. As such, this phenotype correlates better to the human situation of CDH with regard to the existence of PH. Interestingly, the authors demonstrated that PH occurs independently of deficient diaphragm development. This is a situation that can also be observed in unilateral agenesis of the lung with an intact diaphragm. Others and we have demonstrated that the same holds true for lung development in the nitrofen model of CDH [48, 118, 119]. However, a clear relationship between disturbed lung development and diaphragmatic development has not been shown. It is more likely that both lungs and diaphragm are disturbed in their development separately.

Subsequently, the authors searched for FOG2 mutations in a series of 30 autopsy specimens of CDH patients and found a nonsense mutation in a female patient with severe bilateral PH and a posterior diaphragmatic eventration on the left side. The authors suggested that this was the first reported gene mutation in a patient with CDH and PH. More recently, Bleyl et al. [120] reported novel FOG2 sequence variants in 2 isolated CDH patients, but could not identify them as mutations.

Gata4 and Gata6

Fog2 can interact with many different transcription factors such as the *Gata* zinc finger transcription factors *Gata4* and *Gata6*. Null mutant mice for both *Gata4* and *Gata6* die early in embryonic development because of the essential roles of these factors in ventral morphogenesis (including heart development) and differentiation of visceral endoderm, respectively [121–123]. Therefore, these models could not be used to evaluate their roles in lung or diaphragm development that occur later in gestation. However, others and we demonstrated that *Gata6* is essential for normal branching morphogenesis of the lung and late epithelial cell differentiation using a chimeric mouse mutagenesis approach [124–126]. So far *Gata6* has not been implicated in diaphragm development. A role for *Gata4* in lung or diaphragm development was recently observed by Jay et al. [127]. They noticed disturbed heart, lung and diaphragm development in approximately 70% of heterozygous *Gata4* knockout mice that were generated in a different genetic background [127]. The mutation resulted in a mortality of up to 40%. The defect in the diaphragms consisted of a ventral hernia covered with a sac that was not attached to the liver, but allowed abdominal viscera to protrude. The incidence of CDH was approximately 30%. Pulmonary development in the mutant mice was not very disturbed although the authors describe some airway dilatation and altered expression of certain genes in the most affected mice. In addition, another study recently demonstrated that *Gata4* is important for normal pulmonary lobar development [128]. Interestingly, a microdeletion on human chromosome 8p23.1 that includes the GATA4 gene, has been linked to isolated human cases of CDH especially in combination with cardiac anomalies [129–132].

COUP-TFII

Another transcription factor that is a binding partner of *Fog2* is *COUP-TFII*, which belongs to a nuclear steroid/thyroid/retinoid hormone receptor superfamily and has been shown to be essential for embryonic mouse develop-

ment [133]. Mice lacking *COUP-TFII* show defects in cardiovascular development and die around day 10.5 of gestation. Conditional mutagenesis of *COUP-TFII* in mice using the Cre-lox conditional knockout system to ablate *COUP-TFII* function in the mesenchyme only resulted in a Bochdalek-type diaphragmatic defect on the left side [134]. However, the authors did not find a deficient lung phenotype in these mice, although this might be due to the tissue-specific ablation of the gene in the mesenchyme. In line with the latter idea, the authors state that *COUP-TFII* expression is markedly decreased in the structures contributing to the developing diaphragm such as the pleuroperitoneal folds, but the expression was only slightly reduced in the developing lung. These results are even more interesting in the light of the location of *COUP-TFII* on human chromosome 15q26. Our group has identified this region as a potential candidate region for human patients with isolated CDH [135, 136]. However, following an evaluation of over 130 cases of isolated CDH from different hospitals, no mutations in the coding regions of *COUP-TFII* have yet been identified.

Platelet-Derived Growth Factor Receptor- α

Very recently, the platelet-derived growth factor receptor- α (PDGFR α) gene has been identified as an important factor in the formation of the diaphragm and lung development [120]. This gene is known for its role in tumorigenesis of gastrointestinal and neural tumors [137–140]. In *pdgfra* null mice, Bleyl et al. [120] observed PH and a range of diaphragmatic defects including posterolateral diaphragmatic hernias. This and the other phenotypical characteristics observed are similar to the human Fryns syndrome (nonisolated CDH) [141]. Hence PDGFR α might be a candidate gene for nonisolated CDH. Moreover, in one patient with nonisolated CDH a novel sequence variant of PDGFR α was identified. The authors did not prove the variant to be a mutation [120].

Retinoid Signaling Pathway in Knockout Mice

Increasing evidence from data obtained with the nitrofen model and knockout mice points towards perturbations in the retinoid signaling pathway. For example, *COUP-TFII* has been shown to be a downstream target of retinoid signaling [reviewed in 142]. In addition, different *Gata* transcription factors have been demonstrated to interact with RARs [143]. Therefore, we also want to review the role of members of the retinoid signaling pathway in knockout mice. The first evidence from knockout mice that RA is involved in the pathogenesis of CDH came from RAR double-knockout mice, as described earlier.

Single RAR null mutant mice did not show the expected anomalies that were observed in the vitamin-A-deficient rats, indicating that the different types of receptors are highly redundant [144–147]. However, when the function of multiple receptors was abolished, multiple congenital abnormalities were observed including right-sided CDH in RAR α 2 and left-sided CDH in RAR α 2^{+/-}. In addition, these mice displayed severe PH [57]. Despite the convincing data from animal studies, the results in humans have been limited. Until now the only described mutations in CDH patients related to the RA pathway are in *STRA6* and *CRABP1* on chromosome 15 [59–61, 74–76]. A study evaluating the RA status of CDH cases, their mothers and age-matched controls is currently under way.

Despite the ample evidence that certain genes are involved in the pathogenesis of different types of CDH, only a mutation in *FOG2* has so far been demonstrated in a single patient with nonsyndromic CDH. This might be due to several factors. First, as described for the *Gata4* gene, the genetic background of the species carrying the mutation is of importance for the phenotype that is related to the mutation. The diaphragmatic defect was only observed in heterozygous C57Bl/6 *Gata4* mutant mice. This is also true for the rodent model based on the teratogenic effects of nitrofen. When nitrofen is administered to Sprague-Dawley rats, the percentage of the offspring having CDH is higher than following administration to Wistar rats. The same phenomenon has been observed in different mouse strains. Second, the pathogenesis of CDH might be explained by the necessity of multiple developmental insults to happen during development of the diaphragm and the lung. We and others demonstrated this scenario for PH in the nitrofen model for CDH [48]. We named this the dual-hit hypothesis. Finally, the observed phenotype in CDH is so variable that it is potentially not due to a single gene mutation, but the result of multiple gene mutations. Different genes involved in different signaling pathways that have been shown to be important for normal embryonic development might be involved. In addition, as has been suggested for the nitrofen model, there may be a disturbed interaction of certain genes with environmental factors.

Concluding Remarks

Surgical models have been of great importance for validating new interventions in CDH. Many new approaches used today in CDH patients, as for example FETO,

were first optimized in surgical animal models. However, these models are less suitable than either nitrofen or genetic models for elucidating the pathogenesis of CDH, since the diaphragmatic defect is created rather late in fetal development. A combination of two models might further improve our understanding of CDH-associated PH [148]. Nitrofen has never been demonstrated to cause CDH in humans, despite its massive use as a herbicide. Nevertheless, the nitrofen rodent model has great similarities with human CDH. The most plausible pathogenetic explanation for CDH and its associated anomalies is a general genetic defect that causes cardiovascular, lung and diaphragm defects. However, this particular genetic defect has not been found, although some mouse models resemble human CDH. A new approach to discover changes in the genetics of CDH in human cases is the genome-wide array. Genome-wide arrays are useful to compare the genetic changes between CDH patients and to search for the existence of CDH-related genes. Up to now, such an approach has only been attempted in nonisolated (syndromic) human cases, mainly for Fryns syndrome [136, 149, 150].

Although none of the animal models perfectly mimic human CDH and its associated anomalies, they all have shed light on the underlying pathogenesis of the disease.

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Increasing evidence from studies in both human CDH and animal models of CDH (nitrofen and knockout mice) suggest that a disturbance in the retinoid signaling pathway might be responsible for the anomaly. However, not all findings can solely be explained by disturbances in this pathway. Based on the spectrum of defects in heart, lungs and diaphragm, it is likely that there is a general defect in mesenchymal signaling in all organs involved in CDH. More research is warranted to improve our understanding of normal and abnormal diaphragm and lung development in relation to CDH. Eventually, such investigations will help in the design of new treatment modalities to improve the natural course or even to prevent this anomaly.

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