Metabolic disturbances of the vitamin A pathway in human diaphragmatic hernia

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CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a life-threatening birth defect with a worldwide incidence ranging from 1 in 2,000 to 1 in 4,000 newborns. The protrusion of abdominal viscera into the chest through the diaphragm defect is associated with the pulmonary hypoplasia and pulmonary hypertension that mainly account for the high mortality and morbidity rates (20). CDH occurs as either an isolated congenital anomaly or as a nonisolated defect (also called complex or syndromic CDH). A small subset of nonisolated CDH is recognized as composed of Mendelian disorders caused by single-gene mutations or genomic rearrangements (3). By contrast, most isolated CDH cases are sporadic and thought to stem from multifactorial inheritance. Despite recent genome-wide screenings (3, 42), the genetic alterations that contribute to isolated CDH etiology. Here we used time series of normal and CDH lungs in humans, in nitrofen-exposed development, and it has been hypothesized that subtle disruptions of this pathway could contribute to isolated CDH etiology. Here we used time series of normal and CDH lungs in humans, in nitrofen-exposed rats, and in surgically induced hernia in rabbits to perform a systematic transcriptional analysis of the RA pathway key components. The results point to CRBP2, CY26B1, and ALDH1A2 as deregulated RA signaling genes in human CDH. Furthermore, the expression profile comparisons suggest that ALDH1A2 overexpression is not a primary event, but rather a consequence of the CDH-induced lung injury. Taken together, these data show that RA signaling disruption is part of CDH pathogenesis, and also that dysregulation of this pathway should be considered organ specifically.

Nonisolated CDH. Through the canonical RA signaling pathway, retinol (Rol) is converted into retinal (Ral) by several enzymes [alcohol dehydrogenases (ADH), short-chain dehydrogenase reductase (DHRS), and retinol dehydrogenase (RDH)] and into the active component retinoic acid (RA) by aldehyde dehydrogenases (ALDH), of which ALDH1A2, also known as retinaldehyde dehydrogenase 2 (RALDH2), is the main one during development (Fig. 1) (30). The two main isoforms of RA (all-trans and 9-cis RA) are the ligands for the nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs), acting in heterodimeric combinations to regulate transcription of target genes containing RA response elements (RARE) (Fig. 1) (24). RA is degraded by members of the cytochrome P-450 family: CYP26A1, CYP26B1, and CYP26C1 (39). Intracellular retinoids are bound to specific proteins: cellular retinol-binding proteins (CRBP1 and CRBP2) and cellular retinoic acid binding proteins (CRABP1 and CRABP2). A balance between RA synthesis and degradation determines the intracellular RA concentration.

The possibility that the disruption of any of these multiple steps could be involved in isolated CDH is the “retinoid hypothesis.” This hypothesis has been extensively reviewed over the past decade (4, 12, 13, 17, 28, 41) and is strongly supported by several lines of evidence.

1) Fetuses exposed to maternal vitamin A deficient diet (VAD) display CDH (31%) and pulmonary hypoplasia (4%) (44). This phenotype is present in 100% of the fetuses if the deficiency concerns RA (37). In both VAD and teratogenic RA models, CDH incidence is reduced by vitamin A intake or reintroduction (1, 29, 31, 40, 44).

2) The phenotype of the RAR alpha/beta knockout mice recapitulates most of the lung defects observed in VAD animal models (6, 26).

3) RA-induced genes (such as Gata4 and Coup-tfl1) are expressed in the pleuroperitoneal folds (7), and pharmacological ALDH1A2 inhibition results in posterolateral diaphragm defect (27). In the prospective lung mesoderm, RA signaling provides a Fgf10-inductive signal through Tgfb1 repression (8).

4) In human nonisolated CDH, chromosomal rearrangements containing RA pathway-related genes have been reported (12, 36). Mutations in RA pathway genes have also been identified in Mendelian conditions featuring CDH. This is especially the case for Matthew-Wood syndrome (MIM no. 601186), in which STRA6, a gene coding the retinol binding protein receptor, is mutated (11, 32). Recently, mutations in LR2P have also been shown to cause Donnai-Barrow syndrome (MIM no. 222448). LR2P encodes the multiligand receptor megalin. Retinol binding protein (RBP) is known to
be one of the megalin ligands, and megalin deficient patients have increased RBP urine spillage (14).

5 Finally, CDH has been demonstrated to be strongly associated with low retinol and RBP levels in newborns, independently of maternal retinol status (2, 22).

Even with all this attractive evidence of RA signaling involvement in nonisolated CDH, the role of this pathway in the isolated forms remains elusive. One possibility is that at least a subset of cases is promoted by low vitamin A intake. Retrospective questionnaires about maternal periconceptional nutrient intakes yields conflicting results (45). In the developing world, where vitamin A intake could be expected to be low, CDH is not reported to occur at higher rates (43). However, lack of birth defect registries may cause CDH incidence to be underestimated. Another possibility is that the RA pathway is intrinsically affected by subtle functional defects of one or more signaling components. Previously, a temporal and spatial expression study of the nuclear receptor genes RAR (\(\alpha,\beta,\gamma\)) and RXR (\(\alpha,\beta,\gamma\)) failed to demonstrate any difference between human CDH lungs, hypoplastic lungs due to other causes, and normal lungs (33). Here we extended this first line of analysis using transcriptional screening from the retinol binding protein receptor gene (\(\text{STRA}6\)) to the downstream actors of the RA pathway throughout the lung development. Comparing different conditions (human normal lungs, human CDH lungs, wild-type and nitrogen-exposed lungs in the rat, and wild-type and hernia-injured lungs in the rabbit), we show that RA signaling is affected by several transcriptional alterations in human CDH, of which \(\text{ALDH}1\alpha2\) overexpression is probably the most striking. The results from the rabbit CDH surgical model suggest that this dysregulation may be secondary to the lung injury process.

MATERIALS AND METHODS

Chemicals. All-trans retinol, all-trans RA (ATRA) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), culture medium and additives from Invitrogen (Cergy-Pontoise, France), and the transfection reagent GeneJammer from Stratagene (Amsterdam, The Netherlands).

Human tissue collection. With the approval of the Erasmus MC Medical Ethical Committee and the informed consent of the parents, human lung samples were obtained from the tissue bank of the Department of Pathology, Erasmus MC (Rotterdam). The selected lung tissues were collected after elective termination of pregnancy or at autopsy. The characteristics of the nine CDH patients are reported in Table 1. Lung tissues from 10 stage-matched fetuses without pulmonary abnormalities were used as controls (Table 1).
Table 1. Characteristics of the nine congenital diaphragmatic hernia patients

<table>
<thead>
<tr>
<th>Gestational Age (weeks)/Lung Developmental Stage</th>
<th>Sex</th>
<th>Lung Weight-to-Body Weight Ratio</th>
<th>Phenotype/Malformations</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5 (pseudoglandular)</td>
<td>M</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>14 (pseudoglandular)</td>
<td>F</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>15 (pseudoglandular)</td>
<td>F</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>17 (pseudoglandular)</td>
<td>M</td>
<td>normal</td>
<td>Trisomy 13</td>
<td>47, XY, +13</td>
</tr>
<tr>
<td>17.5 (canalicular)</td>
<td>M</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>26 (canalicular)</td>
<td>F</td>
<td>normal</td>
<td>HLHS</td>
<td>46, XX</td>
</tr>
<tr>
<td>26 (canalicular)</td>
<td>M</td>
<td>normal</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>28.5 (saccular)</td>
<td>M</td>
<td>normal</td>
<td>ASD, RDS</td>
<td>46, XY</td>
</tr>
<tr>
<td>31 (saccular)</td>
<td>F</td>
<td>normal</td>
<td>hydrothorax</td>
<td>46, XX</td>
</tr>
<tr>
<td>34 (saccular)</td>
<td>M</td>
<td>normal</td>
<td>EA, hydrocephalus</td>
<td>46, XY</td>
</tr>
<tr>
<td>CDH-injured lungs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 (canalicular)</td>
<td>M</td>
<td>0.02</td>
<td>isolated CDH</td>
<td>46, XY</td>
</tr>
<tr>
<td>22 (canalicular)</td>
<td>M</td>
<td>n.a.</td>
<td>isolated CDH</td>
<td>46, XY</td>
</tr>
<tr>
<td>25 (canalicular)</td>
<td>M</td>
<td>0.012</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>34 (saccular)</td>
<td>M</td>
<td>0.005</td>
<td>PCH</td>
<td>46, XY</td>
</tr>
<tr>
<td>34 (saccular)</td>
<td>M</td>
<td>0.008</td>
<td>testes and kidney cysts</td>
<td>46, XY</td>
</tr>
<tr>
<td>37 (saccular)</td>
<td>M</td>
<td>0.005</td>
<td>isolated CDH</td>
<td>46, XY</td>
</tr>
<tr>
<td>38 (saccular-alveolar)</td>
<td>F</td>
<td>0.011</td>
<td>isolated CDH</td>
<td>n.a.</td>
</tr>
<tr>
<td>40 (alveolar)</td>
<td>M</td>
<td>&lt;0.001</td>
<td>Meckel’s diverticulum</td>
<td>46, XY</td>
</tr>
<tr>
<td>41 (alveolar)</td>
<td>M</td>
<td>0.006</td>
<td>isolated CDH</td>
<td>46, XY</td>
</tr>
</tbody>
</table>

ASD, atrial septal defect; CDH, congenital diaphragmatic hernia; EA, esophagus atresia; HLHS, hypoplastic left heart syndrome; n.a., not available; PCH, pontocerebellar hypoplasia; RDS, respiratory distress syndrome.

Animal CDH induction: rabbit surgical and rat nitrofen models. New Zealand rabbits (Charles River, l’Arbresle, France) and pregnant Sprague-Dawley rats (Harlan Laboratories, Horst, The Netherlands) were respectively shipped at day 17 and day 7 of gestation. The Ethics Committees for Animal Experimentation of the Faculties of Medicine at Clermont-Ferrand and Rotterdam approved the experiments (they conformed to NIH guidelines). For rabbit spatiotemporal expression studies, lung tissue was collected at embryonic day 21 (pseudoglandular stage), day 26 (canalicular stage), day 28 (saccular stage) and day 31 (alveolar stage). Diaphragmatic hernia was created by surgery at embryonic day 23 (pseudoglandular stage), and lungs were collected at embryonic day 29 (saccular stage) as previously described (9, 10). For the teratogenic model, rats received 100 mg of nitrofen at embryonic day 9 as previously described (33).

Alveolar epithelial cell line A549 culture and treatment. The A549 cell line, purchased from the American Tissue Culture Collection (LGC Standart, Molsheim, France), was treated with ATRA (Sigma-Aldrich) in DMSO (vehicle) or with DMSO alone for 6, 12, 24, and 48 h. Cells were transfected using GeneJammer with 1 µg of reporter DR5-tk-CAT plasmid and 1 µg of cytomegalovirus (CMV)-beta-galactosidase vector serving as internal control to normalize variations in transfection efficiency. The CAT measurements were performed as previously described (23).

RNA extraction and RT-PCR experiments. TRIzol (Invitrogen) reagent was used to extract total RNA from all samples and from the A549 cell line. Complementary DNA (cDNA) was generated using Superscript III First-Strand Synthesis System (Invitrogen). Specific oligonucleotide primers were originally generated using the web program Primer3 and PerlPrimer (Table 2 and Ref. 23 for human oligonucleotide primers were originally generated using the web program Primer3 and PerlPrimer). PCR amplification was carried out in a DNA-Engine PTC-200 (Bio-Rad, Marne-la-Coquette, France). Only high-quality RNA samples (RNA integrity number less than or equal to 9 on a scale up to 10) checked using Bioanalyzer (Agilent) were included in the analysis.

Real-time quantitative PCR assays. Real-time PCR was performed by using Lightcycler Syber Green technology (Roche Diagnostics, Meylan, France) and by using the same couples of primers and cDNA generation as described above. All the experimental steps followed the MIQE guidelines (5). Quantitative data were corrected for three housekeeping genes: RPLP0, 18S rRNA, and ß-actin.

ALDH1A2 immunohistochemistry protocol. Sections of normal human lungs, wild-type rabbit lungs, and rabbit CDH lungs were stained by incubation with anti-ALDH1A2 rabbit polyclonal primary antibody [ALDH1A2 (N-20): sc-22591; Santa Cruz Biotechnology] diluted 1:200 in PBS (Tebu, Le Perray-en-Yvelines, France). The samples were then examined, after Hoechst nuclear counterstaining, under a Zeiss Axiohot microscope.

ALDH1A2 Western blotting protocol. The extracts of total proteins were loaded (40 µg/lane) and separated on SDS-PAGE gel, and incubated overnight with goat anti-ALDH1A2 antibody (sc-22591, Santa Cruz Biotechnology) and anti-GAPDH antibody (sc-20357, Santa Cruz Biotechnology) diluted, respectively, 1:200 and 1:100. The blots were incubated with peroxidase conjugated secondary anti-IgG antibody (Abcam) diluted 1:5,000. Blots were stained using a chemiluminescence procedure (ECL Plus kit; Amersham). Band quantification was then performed using Scion software.

Statistical analysis. Results are expressed as means ± SD. Given the small size of the samples, Mann-Whitney U-test or alternatively Kruskal-Wallis one-way analysis of variance were performed to determine significance. A P value of <0.05 was considered statistically significant.

RESULTS

Expression pattern of RA signaling genes during human lung development. Twenty-five critical RA signaling genes were screened by qualitative RT-PCR at the pseudoglandular, canalicular, and saccular stages in a series of 10 whole human lungs (Fig. 2A and Table 1). Most of the genes were expressed from the pseudoglandular to the saccular stages. This was the case in particular for genes encoding 1) the main components of Rol uptake and intracellular transport (STRA6, CRBP1, and CRBP2), 2) the Rol/β-carotene-to-Ral converting enzymes (ADH3, ADH4, DHRS4, DHRS9, RDH10 and epimerase, BCMO1, and BCO2), 3) the Rol-to-Rol aldoketoreductases (AKR1B1 and AKR1B10), 4) the Rol-to-RA converting enzymes (ALDH1A1 and ALDH1A2), 5) the cellular retinoic acid binding proteins (CRABP1 and CRABP2), 6) the RA degrading enzyme (CYP26B1), and 7) the enzymes for storage and metabolism of retinoids.
Changes in RA signaling gene expression in human CDH lungs. Next we examined the expression levels of the above RA signaling genes in human CDH-injured lungs. The 9 samples originated mainly from isolated CDH (n = 6) or alternatively from nonisolated CDH (n = 3), with a gestational age ranging from 21 to 41 wk (Table 1). The nonisolated CDH did not display classical RA signaling-related malformations. The relative expression of each gene of the panel was measured by quantitative PCR (qPCR), and the means of human CDH samples (n = 9) and normal human lungs (n = 10) were compared (Fig. 3A). As described above for normal human lungs, LRAT, CYP26A1, and CYP26C1 were not expressed in CDH lungs. None of the other RA signaling genes showed any statistically significant difference of expression between CDH and normal lungs, apart from three striking exceptions (Fig. 3A). Two genes, CRBP2 and CYP26B1, showed a complete transcriptional extinction in CDH-injured lungs. Because both of them were known to be regulated by retinoic acid levels in other conditions, we tested their potential regulation by ATRA in the A549 human alveolar cell line. We showed that 1 μmol/l RA increased CYP26B1 expression by the 6th hour (1.8-fold,

### Table 2. Primers sequences used for PCR genes amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol dehydrogenase 3 (ADH3)</td>
<td>ATGAAGTTCGCATTAAGATG</td>
<td>TTTCAACGATACTGATGCTCT</td>
<td>239</td>
</tr>
<tr>
<td>Alcohol dehydrogenase 4 (ADH4)</td>
<td>AAGGCTTGTATTCAAGAAC</td>
<td>AGTGCAGTGCGAGTCTGCTCT</td>
<td>231</td>
</tr>
<tr>
<td>Short chain dehydrogenase 4 (DHRS4)</td>
<td>CAAAGCTCTATTCACTGACAT</td>
<td>TATTCACTGCTCTGATGCTG</td>
<td>235</td>
</tr>
<tr>
<td>Short chain dehydrogenase 9 (DHRS9)</td>
<td>AACCTTGAAGAAGCTGCTCT</td>
<td>TCTAGTGAGGACAGTCAGT</td>
<td>169</td>
</tr>
<tr>
<td>Retinol dehydrogenase 10 (RHDS10)</td>
<td>CATATGCTGAGTAACTGACT</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>278</td>
</tr>
<tr>
<td>Epimerase (EPIM)</td>
<td>TGACAGATGAGAAGCTGCTCT</td>
<td>TTTCAAGTCTGCTGATGCTG</td>
<td>242</td>
</tr>
<tr>
<td>Aldo-keto reductase family 1, member 1 (AKR1B1)</td>
<td>CCAAATGAGAAGCTGCTCT</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>235</td>
</tr>
<tr>
<td>Aldo-keto reductase family 1, member 10 (AKR1B10)</td>
<td>CAAATGAGAAGCTGCTCT</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>235</td>
</tr>
<tr>
<td>Aldo-dehydrogenase 1 family, member A1 (ALDH1A1)</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>151</td>
</tr>
<tr>
<td>Aldo-dehydrogenase 1 family, member A2 (ALDH1A2)</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>179</td>
</tr>
<tr>
<td>Aldo-dehydrogenase 1 family, member A3 (ALDH1A3)</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>291</td>
</tr>
<tr>
<td>Aldo-dehydrogenase 8 family, member A1 (ALDH8A1)</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>TACAGAGATATTTTAAATGAG</td>
<td>224</td>
</tr>
<tr>
<td>Cytochrome P-450 family 26 subfamily A polypeptide 1 (CYP 26A1)</td>
<td>AAGCTTGTATTCAAGAAC</td>
<td>AGTGCAGTGCGAGTCTGCTCT</td>
<td>163</td>
</tr>
<tr>
<td>Cytochrome P-450 family 26 B1 (CYP 26B1)</td>
<td>AGCAGATGAGAAGCTGCTCT</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>194</td>
</tr>
<tr>
<td>Cytochrome P-450 family 26 C1 (CYP 26C1)</td>
<td>GAGAATGAGAAGCTGCTCT</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>236</td>
</tr>
<tr>
<td>Diacylglycerol acyl transferase (DGAT)</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>ATGTCAGTACATTTGTTTACCT</td>
<td>171</td>
</tr>
<tr>
<td>Retinyl esters hydrolase (REH)</td>
<td>GAGCTTGCTCATTGGTGGAG</td>
<td>GATTGAAGTCGGGTTTTCGCTG</td>
<td>206</td>
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<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>180</td>
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<tr>
<td>Cellular retinol binding protein 2 (CRBP2)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>155</td>
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<tr>
<td>Cellular retinol-binding protein gene (CRBP)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>234</td>
</tr>
<tr>
<td>Cellular retinol-binding protein gene (CRBP2)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>243</td>
</tr>
<tr>
<td>Stimulated by retinoic acid 6 (STRA6)</td>
<td>GAGCTTGCTCATTGGTGGAG</td>
<td>GATTGAAGTCGGGTTTTCGCTG</td>
<td>253</td>
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<tr>
<td>Beta-carotene 15,15 -monooxygenase 1 (BCMO1)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>218</td>
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<tr>
<td>Beta-carotene oxygenase 2 (BCO2)</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>ATGAGCAAGAGCATGATGAG</td>
<td>293</td>
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<tr>
<td>Ribosomal protein, large, P0 (RPLP0)</td>
<td>GAGCTTGCTCATTGGTGGAG</td>
<td>GATTGAAGTCGGGTTTTCGCTG</td>
<td>219</td>
</tr>
<tr>
<td>Rabbit</td>
<td>TAAAGATGGAGAAGCATGATGAG</td>
<td>TAAAGATGGAGAAGCATGATGAG</td>
<td>156</td>
</tr>
<tr>
<td>Ribosomal protein, large, P0 (RPLP0)</td>
<td>GAGCTTGCTCATTGGTGGAG</td>
<td>GATTGAAGTCGGGTTTTCGCTG</td>
<td>256</td>
</tr>
<tr>
<td>Rat</td>
<td>TAAAGATGGAGAAGCATGATGAG</td>
<td>TAAAGATGGAGAAGCATGATGAG</td>
<td>169</td>
</tr>
<tr>
<td>Microosomal short chain dehydrogenase 4 (DHRS4)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
<td>TCTGGCTCATTGGTGGAGAG</td>
<td>89</td>
</tr>
<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
<td>TCTGGCTCATTGGTGGAGAG</td>
<td>71</td>
</tr>
<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
<td>TCTGGCTCATTGGTGGAGAG</td>
<td>90</td>
</tr>
<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
<td>TCTGGCTCATTGGTGGAGAG</td>
<td>77</td>
</tr>
<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
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<td>145</td>
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<td>TCTGGCTCATTGGTGGAGAG</td>
<td>110</td>
</tr>
<tr>
<td>Cellular retinol binding protein 1 (CRBP1)</td>
<td>GCAATGCAGTGGTGGAGAG</td>
<td>TCTGGCTCATTGGTGGAGAG</td>
<td>189</td>
</tr>
</tbody>
</table>

The hydrolysis of retinyl esters (DGAT and REH). Consistent with animal models, this result suggests that RA pathways play a critical role throughout lung development in humans. By contrast, ALDH1A3 and ALDH8A1 expression was found to be restricted to the canalicular stage, whereas CYP26A1, CYP26C1, and LRAT were not expressed. Pooling the 10 human lung samples and matching the results with positive controls, we confirmed that CYP26A1, CYP26C1, and LRAT were not expressed. Since the endodermal and mesodermal compartments were not discriminated by this whole lung screening, we also performed qualitative RT-PCR on the human alveolar epithelial cell line A549. The same expression pattern was found for all the genes (Fig. 2A). To further analyze the functional significance of this metabolic expression pattern, we performed an RA-dependent CAT gene reporter assay in A549 cells. After 24-h incubation with 1 μmol/l of retinol or RA, we observed, respectively, a 2.4-fold (P < 0.05) and 4.1-fold (P < 0.05) induction of CAT expression (Fig. 2C), demonstrating that the RA signaling gene expression pattern in this lung epithelial cell line is consistent with the metabolic activity of the pathway.
P = 0.03) with highest induction level (5.3-fold, \( P = 0.019 \)) at the 24th hour (Fig. 3B). CRBP2 was also induced by RA treatment, but later (48 h) and to a lesser degree (2-fold, \( P = 0.03 \)), with a progressive increase between 6 and 48 h of treatment (Fig. 3C). Together, these data were strongly suggestive of intracellular RA depletion in human CDH lungs. Because ALDH1A2 is mainly responsible for Ral-to-RA conversion, such loss of CRBP2 and CYP26B1 expression could result from a primitive decrease in ALDH1A2 transcription. On the contrary, we found that ALDH1A2 was strongly overexpressed (5.9-fold, \( P = 0.03 \)) in human CDH lungs (Fig. 3A). Consistent with the ALDH1A2 mRNA overexpression, the respective protein level showed a 1.6-fold increase (\( P = 0.028 \)) (Fig. 3, D and E).

Pulmonary RA pathway gene expression in teratogenic and surgical CDH models. To gain further insight into the RA pathway transcriptional signature of human CDH-affected lungs, we performed a second round of qRT-PCR in two CDH animal models.

First we screened the expression of RA signaling genes in the rat nitrofen-induced CDH model (7, 13, 31). This model has been extensively investigated over the past decade. Nitrofen toxicity is now thought to act through aldehyde dehydrogenase inhibition—mostly of ALDH1A2—which leads to a decrease in RA levels. Over the RA signaling gene panel we tested, the expression of Aldh1a1 and Aldh1a2 mRNA was not significantly affected in nitrofen-exposed lungs (Fig. 4A). Cyp26a1 expression appeared to be only slightly and not
Fig. 3. Diaphragmatic hernia disrupts RA signaling gene expression in human lung. A: relative mRNA expression of the RA signaling genes panel [congenital diaphragmatic hernia (CDH)/normal, respectively, \( n = 9 \) and \( n = 10 \)] as measured by qPCR. CYP26B1 and CRBP2 are not expressed in CDH-injured lungs. B and C: bar chart showing, respectively, CYP26B1 and CRBP2 relative induction in A549 cells as measured by qPCR (\( n = 4 \)) after treatment (6th, 24th, and 48th hours). White bar: no treatment (NT); gray bar: vehicle (DMSO); black bar (ATRA, 1 \( \mu \)mol/l). D: representative image of Western blotting with ALDH1A2 and GAPDH specific antibodies in one human CDH-injured lung and one normal human lung. According to the manufacturer’s technical information, the ALDH1A2 antibody (sc-22591) detects two ALDH1A2 isoforms around 55 kDa. E: relative ALDH1A2 protein levels in normal human lungs (\( n = 4 \)) and human CDH-injured lungs (\( n = 4 \)). Results are corrected for GAPDH protein levels. *\( P < 0.05 \).

statistically reduced, whereas Cyp26b1 and Lrat mRNA levels were halved (\( P < 0.05 \)). Furthermore, Dhrs4 mRNA level showed a 1.5-fold increase (\( P < 0.05 \)). Together, these data were consistent with a pattern of intracellular RA decrease. However, the transcription level of aldehyde dehydrogenase genes—especially Aldh1a2—remained unchanged, supporting the view, in parallel, that the ALDH1A2 overexpression in human CDH was not primarily promoted by a decrease in RA levels and positive feedback.

We thus went on to consider the rabbit CDH surgical model (10). In this model, diaphragmatic hernia results only from the surgical procedure on a wild-type background. Thus no genetic or toxic RA signaling disruption occurs, and any mRNA level variation in RA signaling genes is expected to be secondary to lung CDH-related injury. The hernia was created at day 23 (pseudoglandular stage), and the qRT-PCRs were performed at day 29 (saccular stage) on the ipsilateral lung. Crbp2 and Cyp26b1 expression levels remained unchanged. By contrast, we detected a dramatic 10.1-fold increase (\( P < 0.001 \)) in Aldh1a2 transcript level (Fig. 4B). This mRNA increase correlated with a 3.2-fold increase (\( P = 0.029 \)) in the ALDH1A2 protein level (Fig. 4, C and D). Because this induction could result from a putative systemic transregulatory factor, we compared Aldh1a2 mRNA expression in brains from CDH-affected and control rabbit fetuses. The surgical procedure did not affect the mRNA expression of Aldh1a2 in brain tissues (data not shown). During rabbit lung development, the retinaldehyde dehydrogenase ALDH1A2 was found to be specifically localized in the epithelial compartment from bronchi down to saccules (Fig. 5, E–G) and later down to alveoli (Fig. 5, M–O). A similar pattern was evidenced in human fetal lungs at the saccular and alveolar stages (Fig. 5, A–D and I–K). The surgically induced CDH in the rabbit did not modify the site-specific ALDH1A2 localization in epithelial cells along the airways (Fig. 5, Q–T). Further quantitative analysis of Aldh1a2 mRNA level throughout the rabbit lung development showed two bursts of expression, respectively, at the pseudoglandular and saccular stages (Fig. 6A). Comparing this developmental sequence with ALDH1A2 expression in human fetal lung, we observed similar timing (Fig. 6B). In particular, Aldh1a2 mRNA levels appeared to be consistently high at the saccular stage, in both rabbits and
humans. Taken together, these data suggest that Aldh1a2 is strongly overexpressed at the saccular stage in surgically induced CDH and human CDH.

**DISCUSSION**

Multifactorial diseases result from multiple common variants with small effect sizes and environmental factors. Unlike syndromic CDH, isolated CDH etiology in humans is thought to arise from a complex inheritance (41). In this case, identifying small size variants or subtle transcriptional shifts requires huge cohorts of cases and controls, far more than is technically feasible in congenital defects. Therefore, by analyzing the expression of 25 genes of the candidate RA signaling pathway in 10 human normal lungs and 9 human CDH lungs, we expected to detect only significant variations of expression.

One striking feature of this stringent transcriptional screening was the overexpression of ALDH1A2 mRNA in human CDH lungs, subsequently confirmed at the protein level. ALDH1A2 is known to be the functionally most important aldehyde dehydrogenase during mammalian development (30). During human lung development, ALDH1A1 and ALDH1A2 transcripts were detected from the pseudoglandular to the saccular stages, whereas ALDH1A3 and ALDH8A1 were expressed only at the canalicular stage. The ALDH1A2 expression levels were higher at the pseudoglandular and saccular stages, with a maximum at the saccular stage. Together, these data strongly support the idea that ALDH1A2 expression is critical along these two distinct time windows to supply appropriate intracellular RA levels. This critical expression pattern of aldehyde dehydrogenases during human lung development raises the question of how ALDH1A2 is overexpressed in

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**Fig. 4. RA signaling genes are deregulated in animal CDH models.** A: rat nitrofen CDH model; relative mRNA expression in lungs from nitrofen-exposed rats (n = 5) as measured by qPCR. B: rabbit surgical CDH model. Relative Aldh2a1 expression in control lungs (n = 4) and CDH-injured lungs (n = 4) as measured by qPCR. C: rabbit surgical CDH model; representative image of Western blotting with ALDH1A2 and GAPDH specific antibodies in one rabbit CDH-injured lung and one normal rabbit lung. D: rabbit surgical CDH model; relative ALDH1A2 lung protein levels in sham group (n = 4) and CDH group (n = 4). *P < 0.05; **P < 0.001.
Fig. 5. Lung epithelial ALDH1A2 localization is not modified by rabbit surgically induced diaphragmatic hernia. Immunostained sections of human lung (A–D: canalicular stage; I–L: saccular stage), rabbit lung (E–H: saccular stage; M–P: alveolar stage), and CDH-injured lung in rabbit (Q–T: saccular stage) showing the temporal and site-specific expression of ALDH1A2 in the epithelial cells from bronchi down to alveoli. Q–T: CDH-injured lungs in rabbits showing increased ALDH1A2 staining in epithelial cells, without modification of localization. Right: higher magnifications. Counterstaining: Hoechst; b, bronchi; s, saccule; a, alveoli. Scale bar: 100 μm.
human CHD lungs. This transcrip
tional shift could reflect either a molecular signature of intracellular retinoid depletion or an intrinsic disruption of the pathway.

The former hypothesis was supported by the dramatic ex-
tinction of CRBP2 and CYP26B1 expression in the human

CHD lungs. Yet both of these genes are known to be retinoi-
duced in vivo and in vitro (29, 31, 34, 46). Furthermore, Lrat

and Cyp26b1 were found to be expressed at low levels in the

rat nitrofen model, whereas Cyp26a1 expression remains un-
affected, as previously described (31). Conversely, Dhrs4 was

upregulated. These transcriptional modifications can be inter-

preted as cellular metabolic adaptations to nitrofen-induced RA
depletion. Retinol storage and retinoic acid catabolism de-
crease, whereas the retinol-to-retinal conversion increases to

overcome the intracellular RA depletion. Consistent with this

picture, Lrat and Cyp26b1 expression levels are also decreased in

the mouse VAD model (34, 35). Together, these data

support the idea that CRBP2 and CYP26B1 mRNA decreases

in human CHD lungs are indirect marks of RA depletion.

However, the transcription levels of DGAT, REH (the human

retinyl ester storage enzyme), and DHR54 remains stable.

These contrasting data suggest that, if present, the intracellular

RA deficiency is not a primary event. Rather, the low retinol

and RBP levels found in human newborns (2) probably induce

chronic intracellular retinol deficiency and secondary RA de-
crease. Consistent with previous data (31), another finding from

our screening in the rat nitrofen model is that Aldh1a2 expres-
sion is not sensitive to RA decrease. Thus ALDH1A2 overexpres-
sion in human CHD lungs is unlikely to result from low intracellular RA levels.

An important feature of the nitrofen model is that aldehyde
dehydrogenase inhibition impairs lung development, dia-

phragm development, or both (15, 19). RA signaling is in-

volved in both lung and diaphragm development, and it is

usually difficult to parse out the respective effects of the hernia

and the RA signal disruption on lung development. This fact is

further illustrated by the Matthew-Wood syndrome (MIM no.

601186), where lung hypoplasia can occur independently of

the diaphragmatic hernia (11). Thus a critical issue in under-

standing the cause of ALDH1A2 overexpression in human

CDH lung was to study the transcriptional consequences of

diaphragmatic hernia on a wild-type background. In the light of

this concern, we took advantage of the rabbit model to inves-
tigate this last hypothesis. The temporal ALDH1A2 and spatial

ALDH1A2 expression patterns were very similar during rabbit

and human lung development. In particular, ALDH1A2 showed

a burst of expression at the saccular stage both in humans and

rabbits. It has been previously proposed that this specific

sequence plays a critical role in RA-dependent alveolization

(21). But most importantly, just as in human CDH, we found

that both Aldh1a2 mRNA and ALDH1A2 protein levels were

strongly increased in rabbit hernia-injured lungs. Therefore, we

concluded that ALDH1A2 overexpression in human CDH is

likely to be a mechanistic side effect of the hernia rather than

a constitutive disruption of the RA pathway. The retinoid

pathway regulates a wide range of cellular behaviors during

embryonic development and adult homeostasis (6, 38). Of

note, RA signaling is involved in tissue regeneration and

repair. Consistent with our findings in the rabbit CDH surgical

model, ALDH1A2 increases in the first week after rat spinal

cord injury, suggesting that retinoid signaling is not involved in

the early phase of inflammation in vivo, but rather promotes

tissue regeneration in subsequent phases of the inflammatory

reaction (16). Furthermore, in situ hybridization experiments

showed that Aldh1a2 is strongly expressed around the wound

at day 2 during caudal fin regeneration in zebrafish (25). Thus

we consider that the Aldh1a2/ALDH1A2 overexpression in

rabbit and human CDH could result from the hernia-mediated

lung injury. The herniated organs not only reduce the lung

expansion space in the chest, but also compress lung tissues.

How tissue compression intrinsically impairs the lung devel-

opmental program is still unclear. Moreover, lung volume

measurements in human fetuses with CDH demonstrate that

lung volume can vary dramatically and is of major interest for

![Graph A](http://ajplung.physiology.org/)

![Graph B](http://ajplung.physiology.org/)

**Fig. 6.** Aldh1a2 is strongly expressed at the saccular stage, in both rabbit and human lungs. Relative Aldh1a2 mRNA expression in rabbit (A) and human lung (B), as measured by qPCR from the pseudoglandular to the alveolar or alternatively saccular stage (pseudoglandular stage: Aldh1a2 mRNA relative expression (rabbit lung) n = 4; the following stages: n = 3). *p < 0.05.
the fetal prognosis and postnatal outcome (18). Beside the volume of the hernia and the intensity of the compression, the timing of the compression could also modulate the tissue injury. In the rabbit CDH surgical model, the homolateral lung experiences acute compression. Conversely, in human CDH, tissue compression is expected to be more progressive, and lung hypoplasia occurs or is aggravated concomitant to the hernia. Although the timing and the extent of the hernia are controlled in our surgical model, the human CDH samples we analyzed may represent heterogeneous conditions, especially in hernia severity. Therefore, determining the etiology of the lung developmental changes in CDH remains a critical issue. Comparisons can be made only if appropriate precautions are taken, and we can only suppose from the above discussion that rabbit Aldh1a2 and human ALDH1A2 overexpression is related to lung tissue injury.

In conclusion, although metabolic retinoid signaling disruption does probably not recapitulate the complete pathogenesis of CDH, our systematic screening in a human CDH cohort identified a molecular signature for the retinoid pathway disruption. On the one hand, CYP26B1 and CRBP2 downregulation suggests that intracellular fetal lung levels of RA are low, consistent with low retinol and RBP levels found in cord fetal blood. On the other hand, ALDH1A2 upregulation is not expected to be secondary to low RA levels. Rather the CDH rabbit model we used suggests it could be due to lung tissue injury per se. Thus this study completes the overall CDH mechanistic picture where the retinoid signaling can be primarily disrupted by single-gene mutations or large genomic alterations in the nonisolated forms, or only be affected by subtle primary or secondary modifications in the isolated forms. In the second case, how these modifications contribute to lung damage requires further investigation.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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