CHAPTER FOUR

The role of genes and environment in the etiology of congenital diaphragmatic hernias

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Contents

Abstract

Structural birth defects are a common cause of abnormalities in newborns. While there are cases of structural birth defects arising due to monogenic defects or environmental exposures, many birth defects are likely caused by a complex interaction between genes and the environment. A structural birth defect with complex etiology is congenital diaphragmatic hernias (CDH), a common and often lethal disruption in diaphragm development. Mutations in more than 150 genes have been implicated in CDH pathogenesis. Although there is generally less evidence for a role for environmental factors in the etiology of CDH, deficiencies in maternal vitamin A and its derivative embryonic retinoic acid are strongly associated with CDH. However, the incomplete penetrance of CDH-implicated genes and environmental factors such as vitamin A deficiency suggest that interactions between genes and environment may be necessary to cause CDH. In this review, we examine the genetic and environmental factors implicated in diaphragm and CDH development. In addition, we evaluate the potential for gene-environment

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interactions in CDH etiology, focusing on the potential interactions between the CDH-implicated gene, *Gata4*, and maternal vitamin A deficiency.

1. Introduction

One of every 33 babies suffers from serious structural birth defects such as congenital heart defects, cleft palate, urogenital and limb malformations, and congenital diaphragmatic hernias (Centers for Disease Control and Prevention, 2008). Birth defects can be caused by both genetic and environmental factors. Some structural birth defects, such as achondroplasia, craniofrontonasal syndrome, and focal dermal hypoplasia, are monogenic defects (Brady et al., 2015; Colvin, Bohne, Harding, McEwen, & Ornitz, 1996; Harmsen et al., 2009; Niethamer et al., 2020; Twigg et al., 2006). Other defects arise from environmental insults, such as teratogen exposure. For example, defects in limb, heart, and vertebrae development are associated with exposure to thalidomide, alcohol, tobacco, and antiepileptic drugs (Alexander, Clark, & Tuan, 2016; Caputo, Wood, & Jabbour, 2016; Rogers, 2009). Overall, chromosomal and genetic defects are estimated to account for 20–25% of birth defects, while environmental insults cause 10–12% birth defects; thus, the remaining 63–70% are presumed to result from a combination of genetic mutation(s) and environmental exposure(s) (Alexander et al., 2016).

A relatively small number of structural birth defects have been formally demonstrated to be caused by the interaction of genetic and environmental factors. Craniofacial defects are an excellent example where such interactions have been experimentally tested and shown using zebrafish and mouse models (Hong, Christ, Christa, Willnow, & Krauss, 2020; Hong & Krauss, 2012; Kietzman, Everson, Sulik, & Lipinski, 2014; McCarthy et al., 2013). Mutations in *Cdon*, *Gli2*, or *Shh* coupled with maternal alcohol exposure during organogenesis lead to a higher incidence of holoprosencephaly (a defect in midline pattering of the forebrain and midface) than those with only alcohol exposure (Hong et al., 2020; Hong & Krauss, 2012; Kietzman et al., 2014), showing that alcohol exacerbates the effect of genetically sensitized individuals. Another example is congenital scoliosis, a lateral curvature of the spine, caused by defects in vertebral development. Based on human genetic studies, deleterious mutations in *HES7* were found to be a dominant, but weakly penetrant cause of scoliosis (Sparrow et al., 2012). Using mice that were *Hes7*+/−, the authors found that short-term gestational hypoxia

increased the severity and penetrance of vertebral defects and thus demonstrated a synergistic interaction between genes and environment. Finally, congenital heart defects are the most common type of structural birth defect, and at least 100 genes have been implicated as well as a multitude of environmental factors, including environmental teratogens and maternal alcohol exposure (Moreau et al., 2019). Recently, short-term gestational hypoxia has been shown to increase the incidence and severity of heart defects of mouse embryos harboring heterozygous null mutations for several genes (*Fgfr1*, *Fgfr2*, and *Tbx1*) implicated in heart development (Moreau et al., 2019).

Another structural birth defect in which both genetic and environmental factors have been implicated in its etiology is Congenital Diaphragmatic Hernia (CDH). The diaphragm is a skeletal muscle that is essential for respiration and acts as a barrier separating the abdominal and thoracic cavities. CDH is a common birth defect (1/3000 births; Torfs, Curry, Bateson, & Honoré, 1992) in which the diaphragm does not develop completely, its barrier function is lost, and abdominal contents herniate into the thoracic cavity (Pober, 2007). The resulting lung hypoplasia is the cause of the 50% mortality rate in CDH patients (Pober, 2007). The etiology of CDH is complex with over 100 genes implicated in its pathogenesis (Kardon et al., 2017). Correlative studies suggest that environmental factors may also contribute to the etiology of CDH, although there is currently no direct evidence linking an environmental insult with development of CDH in humans. The prevalence of CDH and the lack of common, highly penetrant genetic or environmental causes of CDH suggest that interactions between genetic variants and environmental factors may contribute to CDH etiology (Clugston, Zhang, Lvarez, De Lera, & Greer, 2010; Rocke et al., 2021; Warkany & Roth, 1948; Wilson, Roth, & Warkany, 1953). In this review, we will explore the role of genetic, environmental, and gene-environment interactions in the development of CDH.

2. Diaphragm development

The diaphragm is composed of costal and crural domains (Fig. 1A; Merrell & Kardon, 2013). The crural muscle surrounds the esophagus and aorta, while the costal diaphragm is composed of a radial array of muscle fibers surrounded by muscle connective tissue that connect medially to a central tendon and laterally to the ribs. It is the costal diaphragm that primarily acts as a barrier between the abdominal and thoracic cavities and is breached in CDH. The muscle, muscle connective tissue, and central

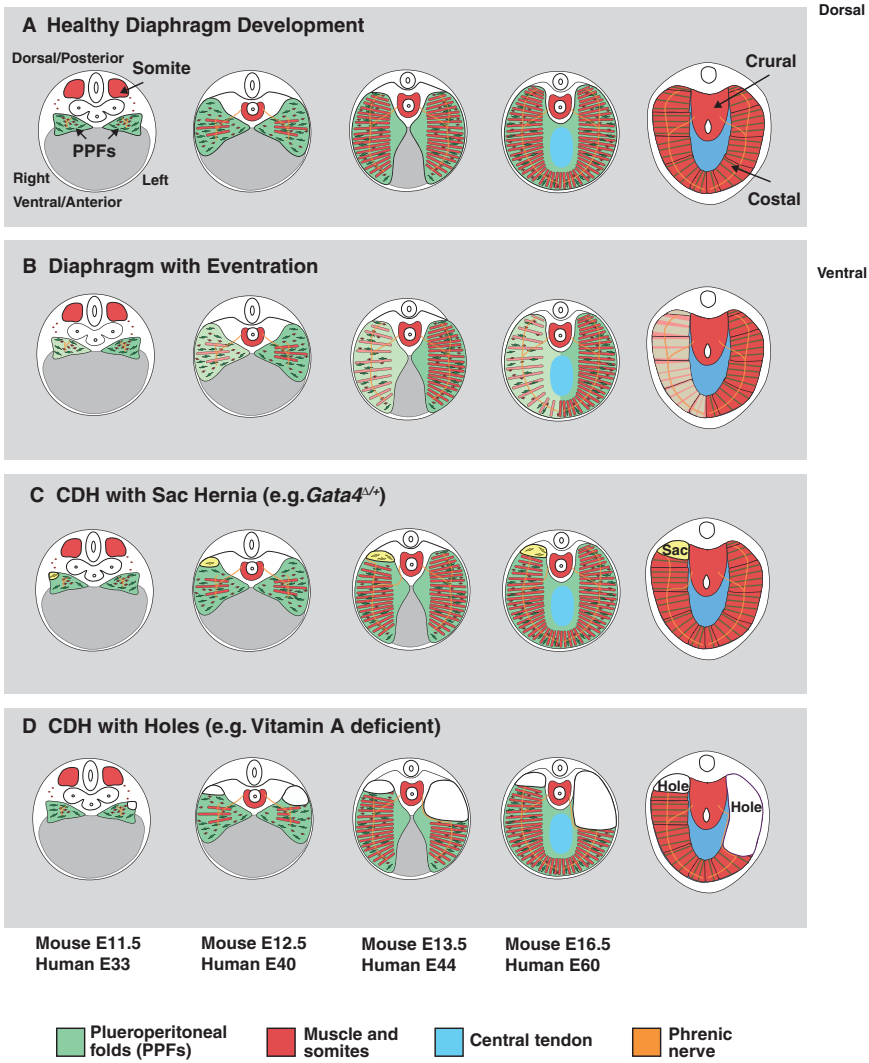

Fig. 1 Diaphragm and CDH development. (A) In a healthy diaphragm, muscle progenitors (red) migrate from the cranial somites toward the pleuroperitoneal folds (PPFs, green). The PPFs, along with the myogenic progenitors then expand dorsally and ventrally along the surface of the liver and differentiate into connective tissue and central tendon or myofibers. (B) Eventration arises when the diaphragm is poorly muscularized. The eventrated region bulges into the thoracic cavity, however, there is no overt sac or hole. (C) Sac hernias (yellow), such as in *Gata4*$^{\Delta/+}$ diaphragms, arise early due to a localized loss of muscle tissue. The loss of muscle tissue provides a weak point through which the liver herniates into the thoracic cavity and is surrounded by a connective tissue sac. (D) Hernias with holes (white), such as in vitamin A deficient diaphragms, arise due to a total loss of both connective tissue and muscle in a localized region of the diaphragm, which allows the liver to herniate into the thoracic cavity.

tendon are derived from two embryonic sources (Babiuk, Zhang, Clugston, Allan, & Greer, 2003; Merrell et al., 2015). The connective tissue and central tendon are derived from the pleuroperitoneal folds (PPFs), two pyramidal structures lying on either side of the esophagus (Merrell et al., 2015; Sefton, Gallardo, & Kardon, 2018). The PPFs arise from the lateral plate mesoderm and begin to thicken by embryonic day (E) 10.5 of mouse development (human E28) and are distinct structures by E11.5 (human E33; Sefton et al., 2018). The PPFs expand both dorsally and ventrally to cover the surface of the liver and differentiate into the muscle connective tissue and central tendon by E16.5 (human E60; Merrell et al., 2015; Sefton et al., 2018). The muscle progenitors, which ultimately give rise to the diaphragm's myofibers, are derived from a different embryonic structure, the cranial somites (Fig. 1A; Merrell et al., 2015; Sefton et al., 2018). Beginning at E9.5, the muscle progenitors delaminate from the cranial somites and migrate toward the PPFs at E9.5 and then reside within the core of the PPFs by E11.5 (Allan & Greer, 1997; Babiuk et al., 2003; Sefton et al., 2018). As the PPFs expand, the muscle precursors migrate with them and differentiate into a radial array of muscle fibers by E16.5 (Fig. 1A; Merrell et al., 2015; Sefton et al., 2018). Thus diaphragm development is an early embryonic event, largely complete during the first 3 months of human development.

3. Congenital diaphragmatic hernia (CDH)

Defects in the development of the costal diaphragm lead to CDH (Torfs et al., 1992). In the majority (80–86%) of cases the defect is a hole in the diaphragm, allowing abdominal contents to directly herniate into the thoracic cavity (Fig. 1D; Heiwegen et al., 2020). In other cases, there is no overt hole in the diaphragm. Instead the diaphragm may be poorly muscularized and this weaker area bulges into the thoracic cavity (termed an eventration; Fig. 1B; Pober, 2007). Alternatively, the diaphragm may contain localized connective tissue regions that completely lack muscle, and these weaker regions balloon into the thoracic cavity, leading to hernias covered with a connective tissue sac (Fig. 1C; Pober, 2007). Hernias in the dorsal (referred to as posterior in medical parlance) region, called Bochdalek hernias, comprise the vast majority (90%) of diagnosed and symptomatic CDH cases (Pober, 2007; Torfs et al., 1992). Hernias within the ventral (anterior) region of the diaphragm, termed Morgagni hernias, are less often diagnosed; however, this may be due to an ascertainment bias, as hernias in

this region generally do not lead to lung defects. Interestingly, CDH more commonly (~80%) occurs on the left side, as opposed to the right side, of the diaphragm (Pober, 2007; Schulz et al., 2021; Yang et al., 2008).

4. Development of CDH

The phenotypic heterogeneity of diaphragmatic hernias suggests that CDH develops by different mechanisms depending on the type of hernia. The formation of a hole in the diaphragm, allowing communication between the abdominal and thoracic cavities, has generally been attributed to defects in the development of the PPFs (Fig. 1D; Babiuk & Greer, 2002; Clugston et al., 2006). Decreased proliferation, increased apoptosis, defective migration, and altered differentiation of PPF fibroblasts have all been implicated in the development of holes in the diaphragm (Cleal et al., 2021; Clugston, Zhang, & Greer, 2009; Coles & Ackerman, 2013; Paris, Coles, & Ackerman, 2015). Analysis of gene expression, mouse genetic mutants, and embryonic mice subject to pharmacological inhibitors have shown that defects in several molecular pathways and transcription factors cause PPF defects and lead to hernias with holes: signaling pathways include retinoic acid (Clugston et al., 2006, 2009, 2010), Wnt/β-catenin (Paris et al., 2015), Hedgehog (Coles & Ackerman, 2013), and PDGF (Bleyl et al., 2007) and transcription factors include WT1 (Carmona et al., 2016; Cleal et al., 2021; Clugston et al., 2006; Paris et al., 2015). Defects in other embryonic tissues, such as the posthepatic mesenchymal plate and the septum transversum, have also been suggested to cause diaphragmatic hernias with holes (Carmona et al., 2016; Cleal et al., 2021; Iritani, 1984).

The development of hernias covered with sacs is also closely tied to defects in the PPF fibroblasts. The most detailed analysis of the etiology of this type of hernia comes from studies from our lab analyzing the role of the transcription factor *Gata4* (Fig. 1C; Merrell et al., 2015). GATA4 is expressed in the PPF fibroblasts and conditional deletion of *Gata4* in these cells in mice leads to diaphragmatic hernias covered by a connective tissue sac. Unlike hernias with a hole, these hernias with a sac have no defect in the migration or differentiation of the PPF fibroblasts. Instead, loss of *Gata4* leads to localized muscleless regions that are biomechanically weaker and allow abdominal contents to herniate. Although we originally thought that the development of such muscleless regions was sufficient to initiate

herniation, our recent analysis of *Hgf* mutants now shows that additional defects in the connective tissue extracellular matrix or vascularization are also necessary (Sefton et al., 2022).

Finally, there is the least amount of data concerning the origin of poorly muscularized, eventrated diaphragms (Fig. 1B). Defects in the muscularization of the diaphragm may arise from loss of PPF-derived signals necessary to recruit and support muscle progenitors in the nascent diaphragm (Sefton et al., 2022). Alternatively, there may be cell-autonomous defects in muscle cells that prevent full muscularization of the diaphragm.

Overall, the preponderance of evidence suggests that the PPFs are critical for normal morphogenesis of the diaphragm and defects in the PPFs are central to the formation of diaphragmatic hernias (Kardon et al., 2017). Therefore, the PPFs are likely the focal region in which genetic and/or environmental factors act and interact to produce CDH.

5. Genetic etiology of CDH

CDH is generally thought to be caused by genetic factors, although its genetic etiology is highly heterogeneous (Kardon et al., 2017; Pober, 2007). Currently, genetic causes have been identified in about 30% of cases (Kardon et al., 2017; Russell et al., 2012; Yu et al., 2015) and these causes include aneuploidies, cytogenetic rearrangements, copy number variants, and single-gene mutations (see references in Kardon et al., 2017). While there are familial cases of CDH (e.g., Longoni et al., 2015; Yu et al., 2013), the sibling recurrence rate of CDH is rare, at 0.7% (Pober et al., 2005). Instead, most CDH cases arise sporadically without a family history of CDH, and so *de novo* mutations are expected to contribute significantly to the etiology of these cases. A focus of current research is the identification of gene-disrupting variants in trios of CDH-affected child and unaffected parents (Longoni et al., 2017, 2014; Qi et al., 2018; Yu et al., 2015, 2012).

More than 150 genes have been implicated in CDH (Bogenschutz, Fox, et al., 2020), many of which have been identified by trio studies. The CDH genes with the strongest level of support are those genes in which gene-disrupting variants have been identified in CDH individuals and functional studies of these genes have been conducted in human fibroblast cultures or *in vivo* in mice. Three genes with particularly high levels of support are the zinc finger transcription factor *GATA4*, the zinc finger transcriptional co-factor *ZFPM2*, and the membrane-associated transcription factor *MYRF*.

The transcription factor *GATA4* is one of the genes most strongly implicated in CDH. *GATA4* is located in a chromosomal region, 8p23.1, that is associated with recurrent CDH-associated copy number variants (Holder et al., 2007; Longoni et al., 2012), and *GATA4* variants are found in two cases of familial CDH (Arrington et al., 2012; Yu et al., 2013). While mice null for *Gata4* die prior to diaphragm development (Kuo et al., 1997; Molkentin, Lin, Duncan, & Olson, 1997), mice heterozygous for *Gata4* exhibit CDH (Jay et al., 2007). Furthermore, as discussed above, conditional null deletion of *Gata4* specifically in PPF fibroblasts leads to CDH with complete penetrance and shows that *Gata4* in these fibroblasts is required to support the survival and proliferation of neighboring myogenic cells (Merrell et al., 2015). Thus these experiments demonstrate conclusively that loss of *Gata4* in the PPFs leads to CDH. Yet patients with *GATA4*-associated CDH are, at most, missing one allele of *GATA4* (as *GATA4* null mutations would lead to early embryonic lethality). In humans, *GATA4* haploinsufficiency is incompletely penetrant for CDH (Longoni et al., 2012; Wat et al., 2009), and similarly mice with heterozygous null mutations in *Gata4* cause CDH in 29–50% of pups (our unpublished data and Jay et al., 2007). In addition, individuals with shared missense (Yu et al., 2013) or intronic (Arrington et al., 2012) *GATA4* variants exhibit hernias that differ in location and severity. The incomplete penetrance and variable expressivity of *GATA4* suggests that *GATA4* haploinsufficiency is not sufficient, but rather sensitizes individuals to develop CDH. Given the noted dosage sensitivity of GATA4 protein in regulating morphogenesis (Pu, Ishiwata, Juraszek, Ma, & Izumo, 2004), other genetic mutations or environmental modifiers may be necessary to lower GATA4 protein expression or function below a critical threshold that then induces CDH.

ZFPM2 is another gene strongly implicated in CDH. Like *GATA4*, *ZFPM2* is located in a chromosomal region, 8q22-23, in which copy number variants are associated with CDH (Holder et al., 2007; Temple, Barber, James, & Burge, 1994; Wat et al., 2011). In addition, CDH patients have been identified with gene-disrupting mutations in *ZFPM2* (Ackerman et al., 2005; Bleyl et al., 2007; Brady et al., 2014; Longoni et al., 2015). Null mutations in *Zfpm2* in mice lead to defective diaphragms in which regions develop that contain connective tissue, but no muscle (Ackerman et al., 2005). The ZFPM2 protein is a zinc finger protein that does not directly interact with DNA, but rather functionally interacts with GATA proteins to regulate GATA4-mediated transcription (Chlon & Crispino, 2012). Given the similarity in diaphragm defects in mice with mutations in

Gata4 or *Zfpm2*, it is likely that ZFPM2 synergistically regulates GATA4-mediated transcription in PPF fibroblasts. Also, like CDH patients with *GATA4* mutations, individuals harboring pathogenic mutations in *ZFPM2* dominantly exhibit CDH with a penetrance estimated at only 37.5% (Longoni et al., 2015; Wat et al., 2011).

Finally, an analysis of 362 proband-parent trios identified damaging *de novo* variants in *MYRF* in 4 unrelated individuals with CDH (Qi et al., 2018). A subsequent review of a large database of exome sequencing studies identified three more unrelated CDH patients harboring deleterious *MYRF* variants (Rossetti et al., 2019). Although there is less experimental support for the functional significance of *MYRF* in CDH, RNA-seq on diaphragm fibroblast cell cultures derived from neonatal CDH patients revealed that a large number of genes, including *GATA4*, are differentially expressed in *MYRF* mutant fibroblasts (Qi et al., 2018). Although these data strongly link *MYRF* to CDH, nevertheless like *GATA4* and *ZFPM2*, mutations in *MYRF* are incompletely penetrant for CDH (Qi et al., 2018).

Altogether the literature indicates that mutations in particular genes or chromosomal regions are critical for the development of CDH. However, the etiology of CDH is complex. Even for genes (such as *GATA4*, *ZFPM2*, and *MYRF*) strongly implicated in CDH, nevertheless mutations in these genes exhibit incomplete penetrance and variable expressivity for CDH. Thus, while mutations in these genes are critical for the development of CDH, they are not by themselves sufficient. Instead, mutations in other genes, epigenetic modifications, or environmental factors must also be required for CDH to actually arise.

6. Environmental factors contributing to CDH

Environmental factors have been postulated to also contribute to CDH etiology (Kardon et al., 2017; Pober, 2007; Torfs et al., 1992). Most data on the role of environmental factors comes from analyses of CDH patient records coupled with parent questionnaires. Some studies have shown that nicotine may be associated with developing CDH (Balayla & Abenhaim, 2014; Finn et al., 2022), although other studies have either found the opposite effect or no association at all (Caspers et al., 2010; Felix et al., 2008; McAteer, Hecht, De Roos, & Goldin, 2014; Perry, Mulcahy, & DeFranco, 2019). Similar conflicting results have been found regarding maternal alcohol intake, with some studies finding that it is correlated with CDH (Balayla & Abenhaim, 2014; Felix et al., 2008; McAteer et al., 2014)

and others finding that it is not (Caspers et al., 2010; Finn et al., 2022). Thus there appears to be no consensus on whether nicotine or alcohol contribute to CDH etiology. However, it should be noted that differences in study design, including time during which nicotine or alcohol intake was investigated (such as periconception, 1st trimester, or anytime throughout pregnancy) may lead to these inconclusive results. An environmental factor for which there is stronger support for its role in CDH is maternal vitamin A deficiency (Greer, Babiuk, & Thebaud, 2003). Vitamin A is processed into retinoic acid (RA), which is essential for diaphragm development. Below, we will discuss in more detail the evidence that vitamin A and the retinoic acid signaling pathway are critical for development of the diaphragm and CDH. Because Vitamin A and retinoic acid signaling also interact with CDH-implicated genes, we also explore the potential for gene-environment interactions in the etiology of CDH.

7. Vitamin A in CDH development

Vitamin A (retinol) is an essential nutrient obtained through the diet and converted, through a series of oxidative steps, to its active metabolite retinoic acid, which in turn regulates gene transcription (see Duester, 2008; Rhinn & Dollé, 2012; Shannon, Moise, & Trainor, 2017). Retinol can be obtained in two forms: preformed vitamin A, primarily coming from animal sources such as meats and dairy; or as provitamin A carotenoids, primarily derived from plants including carrots, sweet potatoes, and leafy greens (Harrison, 2012). During pregnancy, retinol must be obtained through the maternal diet and passed to the embryo through the placenta. Cellular retinol uptake is mediated by the STRA6 transmembrane receptor and oxidized to retinal, primarily by retinal dehydrogenase 10 (RDH10) in the embryo (Fig. 2; Duester, 2008; Rhinn & Dollé, 2012; Sandell, Lynn, Inman, McDowell, & Trainor, 2012; Sandell et al., 2007). Retinal is then irreversibly oxidized to all-*trans*-retinoic acid (ATRA) by the ALDH1A (formerly called RALDH) family of enzymes and translocates to the nucleus. ALDH1A2 is the most broadly expressed ALDH1A enzyme in the embryo, suggesting it is the critical ALDH1A enzyme needed for RA synthesis (Duester, 2008; Niederreither, McCaffery, Drager, Chambon, & Dolle, 1997; Rhinn & Dollé, 2012; Shannon et al., 2017). Once inside the nucleus, ATRA binds to the retinoic acid receptor (RAR) on the retinoic acid response element (RARE) in the promoter region of a target gene to initiate transcription (Fig. 2; Al Tanoury, Piskunov, & Rochette-Egly, 2013; Delva et al., 1999; Mic, Molotkov, Benbrook, & Duester, 2003).

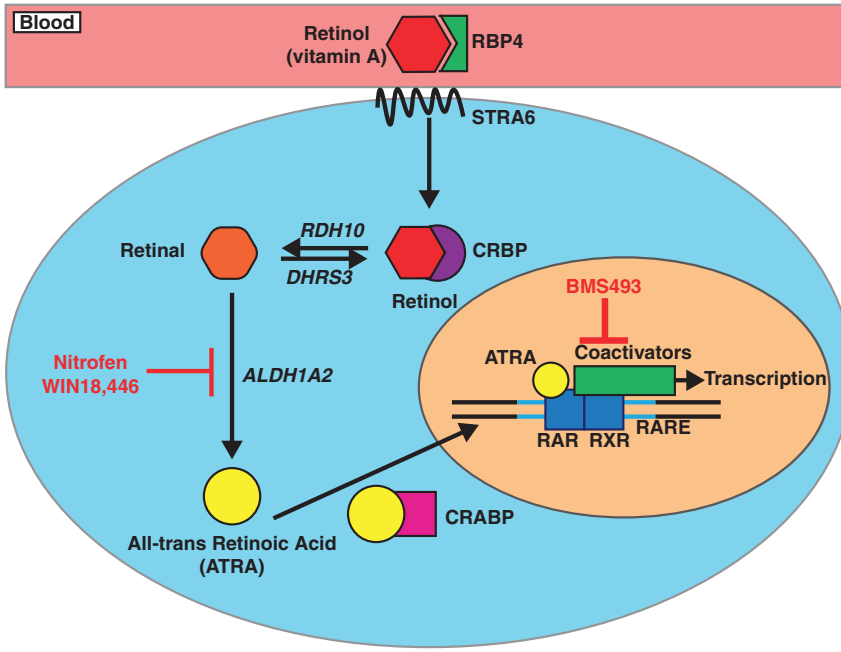

Fig. 2 The RA biosynthesis pathway. Circulating vitamin A (retinol, red) is brought into the cell by STRA6 and is bound by the cellular retinoic acid-binding protein (CRBP, purple) until it is oxidized to retinal (orange) by RDH10. Retinal is further irreversibly oxidized to all-*trans*-retinoic acid (ATRA, yellow) by ALDH1A2 and translocates to the nucleus, mediated by the cellular retinoic acid-binding protein (CRABP, pink). Once inside the nucleus, ATRA binds to the retinoic acid receptor (RAR, blue) bound to a retinoic acid response element (RARE), leading to recruitment of a transcription co-activator complex and subsequent transcription of a gene. ALDH1A2, can be pharmacologically inhibited with nitrofen and WIN18,446 and the transcriptional activity of ATRA can be inhibited *via* BMS493.

Vitamin A deficiency during pregnancy causes multiple birth defects, including CDH. The importance of vitamin A consumption during pregnancy was first demonstrated in animal studies as early as the 1930s. When kept on a vitamin A deficient (VAD) diet, female pigs gave birth to piglets with a variety of developmental anomalies including craniofacial defects and missing or malformed eyes (Hale, 1935). Subsequent studies in rodents found that females on a VAD diet gave birth to pups with similar malformations as well as defects in heart and lung development, vertebral defects, and CDH (Kostetskii et al., 1999; See, Kaiser, White, & Clagett-Dame, 2008; Warkany & Roth, 1948; Wilson et al., 1953), which can be rescued by exogenous vitamin A supplementation (See et al., 2008; Warkany & Roth, 1948; Wilson et al., 1953). As a consequence of these

animal studies, researchers have analyzed whether vitamin A deficiency is associated with babies born with CDH. Reduced maternal dietary intake of vitamin A is associated with an increased risk of babies with CDH (Beurskens et al., 2013; Michikawa et al., 2019; Yang et al., 2008). In addition, CDH infants have been found to have significantly lower levels of circulating retinol than healthy newborns (Beurskens et al., 2010; Major et al., 1998).

Further evidence that defects in RA signaling cause CDH comes from experiments pharmacologically blocking RA signaling or synthesis. BMS493 is a pan-RAR antagonist that stabilizes co-repressors bound to RAR (Germain et al., 2009) and thus inhibits RA signaling. Treatment with BMS493 of rat embryos between E8-E11 leads to CDH (Clugston et al., 2010). Similarly, treatment of pregnant dams with nitrofen or WIN18,446, teratogens that inhibit ALDH1A2 and RA synthesis (Chen et al., 2018; Noble et al., 2007), leads to CDH in mice and rats (Babiuk, Thébaud, & Greer, 2004; Kilburn, Hess, Lesser, & Oster, 1982; Kluth et al., 1990; Momma, Ando, Mori, & Ito, 1992; Rocke et al., 2021; Taleporos, Salgo, & Oster, 1978). Experiments with these pharmacological inhibitors have also provided important mechanistic insights into how defects in RA signaling lead to CDH. Analysis of nitrofen-treated rat embryos or mouse PPF explants shows that hernias are caused by a failure of the PPFs to expand, due to inhibition of proliferation of PPF fibroblasts (Bogenschutz, Sefton, & Kardon, 2020; Clugston et al., 2006, 2009).

Altogether these studies strongly support a role for vitamin A and RA signaling deficiencies in the etiology of CDH. However, maternal vitamin A deficiency in rodents generally does not lead to CDH with complete penetrance (See et al., 2008; Wilson et al., 1953). Similarly, pharmacological perturbation of RA signaling pathway only leads to CDH with 50–59% penetrance (Babiuk et al., 2004; Clugston et al., 2010; Rocke et al., 2021). Therefore, it is likely that low maternal levels of vitamin A and deficient embryonic RA signaling are generally not completely penetrant and suggests that other collaborating factors to may be necessary to cause diaphragmatic hernias.

8. Gene–environment interactions in etiology of CDH

While mutations in particular genes, such as *Gata4*, and environmental perturbations, such as vitamin A deficiency, cause CDH, all are notably incompletely penetrant for development of diaphragmatic hernias.

This suggests that the etiology of CDH is more complex and involves a combination of different genetic mutations, several environmental perturbations, or CDH-inducing gene-environment interactions. While gene-environment interactions are likely to play a role in CDH, establishing that such generally complex interactions are indeed taking place and the molecular and cellular mechanisms underlying these interactions is challenging.

To test whether gene-environment interactions are important in CDH etiology, it is important to define what is meant by gene-environment interactions and establish the criteria that must be met to demonstrate that such interactions are functioning. A gene-environment interaction is defined as "a different effect of an environmental exposure on disease risks in persons with different genotypes" or conversely as "a different effect of a genotype on disease risk in persons with different environmental exposures" (Ottman, 1996). A useful concept for designing studies to test gene-interactions is the epidemiological analysis of risk, *via* the calculation of an odds ratio (OR). An OR is the odds of an outcome, e.g., CDH, occurring after an exposure (genetic or environmental) compared to the odds of the same outcome occurring in the absence of the exposure (Szumilas, 2010). For example, if an OR is calculated to be 1, this indicates that the exposure does not affect the odds of CDH. If the OR is greater or less than 1, however, it indicates that exposure is associated with higher or lower odds of CDH, respectively (Szumilas, 2010).

To test, using the concept of odds ratios, whether gene-environment interactions contribute to CDH it is necessary to determine the OR of CDH with a genetic mutation (OR_G), the OR of CDH with an environmental perturbation (OR_E), and then the OR of CDH with both a genetic mutation and an environmental perturbation (OR_{GE}). To illustrate such a series of experiments in mice, we will use the example of the effects of a genetic mutation in *Gata4* and maternal vitamin A deficiency (Fig. 3). In our lab, we found that mouse embryos heterozygous for a null mutation in *Gata4* (*Gata4Δ/+* on a C57BL/6J background) exhibit small dorsal sac hernias generally on the right side with an $OR_G = 4.0$ (see calculation in Fig. 3B). Several labs have found that embryos developing in mothers on a VAD diet develop hernias with holes in left and right dorsal regions of the diaphragm (Fig. 3C; See et al., 2008; Wilson et al., 1953). The frequency of CDH depends on the level of vitamin A deficiency, but one epidemiological study estimated an adjusted OR_E of 1.8 for risk of CDH in babies born to mothers with low vitamin A (Yang et al., 2008). To test in mice whether *Gata4* mutations genetically interact with low maternal vitamin

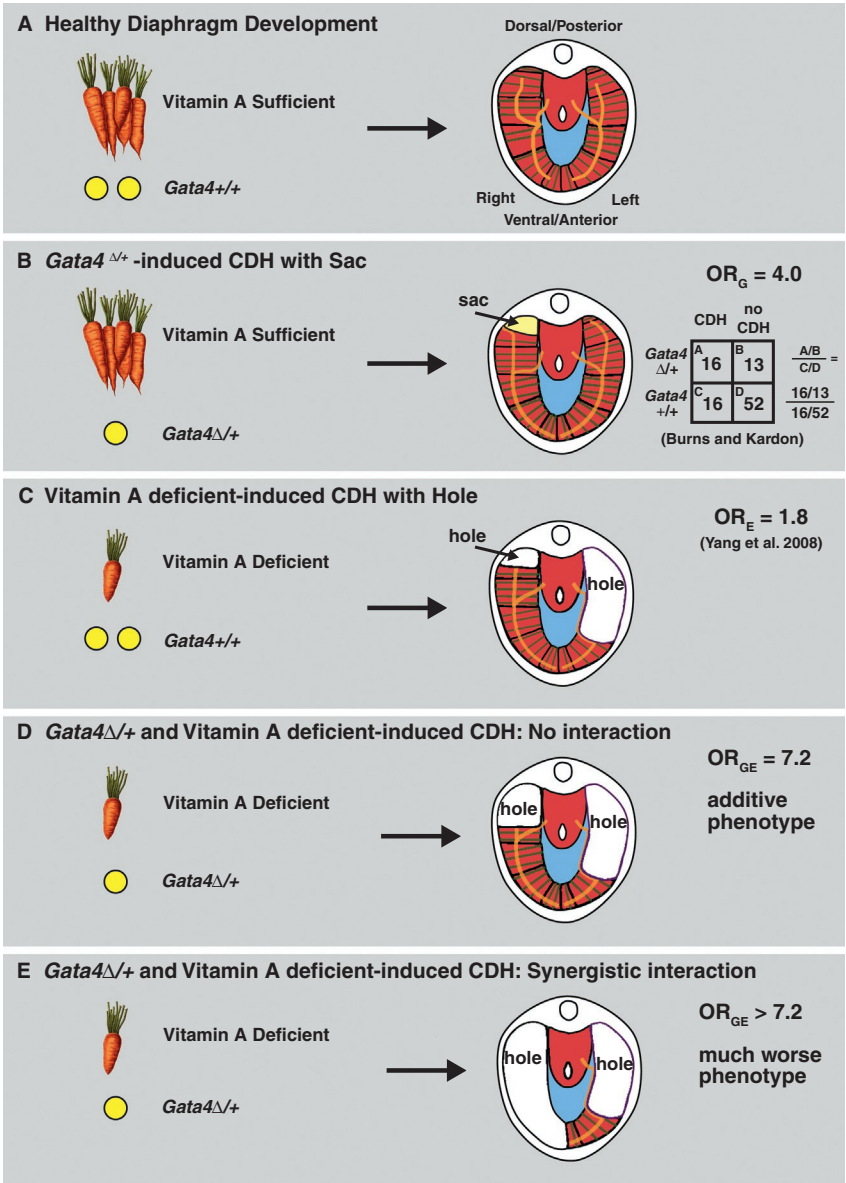

Fig. 3 Gene–environment interactions in diaphragm development. (A) A normal level of vitamin A (full bunch of carrots) coupled with two alleles of *Gata4* (yellow circles) leads to healthy diaphragm development. (B) Loss of one copy of *Gata4* under normal levels of vitamin A induces a sac hernia (yellow). Sac hernias arise in $Gata4^{\Delta/+}$ mice with an OR = 4. (C) Vitamin A deficiency (one carrot) in an individual with both copies of *Gata4* leads to hole hernias (white) with an OR = 1.8. (D) If there is no interaction between vitamin A deficiency and loss of one copy of *Gata4*, the diaphragm will develop a slightly larger hole hernia on the right due to the addition of the sac and hole hernias with an OR = 7.2. (E) If there is a synergistic interaction between vitamin A deficiency and loss of one copy of *Gata4*, a large hole hernia on the right will develop on both sides of the diaphragm with an OR > 7.2.

A, *Gata4Δ/+* pups would be generated in VAD dams. If the OR_{GE} of CDH in these pups is 7.2 (Fig. 3D), this would indicate that there is no gene-environment interaction because the OR_{GE} is simply equivalent to OR_G X OR_E (assuming a multiplicative model; see discussion in Ottman, 1996). However, if $OR_{GE} > 7.2$ (Fig. 3E), this would indicate that there is a greater than expected odds of developing CDH when *Gata4Δ/+* pups develop with low vitamin A and thus there is a synergistic gene-environment interaction. Likewise, if $OR_{GE} < 7.2$, this would indicate there is an antagonistic gene-environment interaction. In addition to the frequency of CDH, gene-environment interaction can also be revealed by the nature of the hernias that develop. If there is no interaction between *Gata4* and vitamin A, then *Gata4Δ/+* pups born to dams on a VAD diet would be expected to show the additive phenotype of moderate-sized hernias with holes on right and left sides of the diaphragm (Fig. 3D). However, if there is a gene-environment synergistic interaction, the pups might be expected to have much larger hernias on the right side than expected with a simple additive phenotype (Fig. 3E).

Previous studies in rodents suggest mechanistically how genetic mutations and environmental factors may act synergistically to increase the incidence and severity of CDH. To date, the preponderance of evidence points to the PPFs as a major target of CDH. In *Gata4* mutants (Bogenschutz, Sefton, et al., 2020; Merrell et al., 2015) and embryos in which RA signaling is pharmacologically inhibited (Bogenschutz, Sefton, et al., 2020; Clugston et al., 2006, 2009), PPF fibroblast proliferation is decreased and overall PPF morphogenic expansion is defective in mice; thus, it is plausible that *Gata4* mutants developing on a vitamin A deficient diet could synergistically interact to inhibit PPF development and lead to a higher incidence of CDH with more severe hernias. The timing of the effects of genetic and environmental factors may also be critical. Rodent studies indicate that CDH initiates early in development, likely prior to E11.5 in mouse. Mutations in *Gata4* cause visible right-sided defects in diaphragm development by E12.5 (Merrell et al., 2015). VAD diet or teratogen inhibition of RA experiments indicate that development of CDH is extraordinarily sensitive to timing of reduced RA signaling (Clugston et al., 2010; See et al., 2008); RA is required before E11.5 of mouse development, with RA inhibition prior to E9.5 leading preferentially to left hernias and inhibition E9.5–11.5 leading to right hernias (Clugston et al., 2010). The likely temporal overlap of GATA4 and RA function suggests that they may act synergistically to regulate the formation of right-sided hernias. On a molecular level, RA may either directly or

indirectly regulate *Gata4* expression in the diaphragm similar to results in heart development, in which a VAD diet (Kostetskii et al., 1999) or nitrofen exposure (Takayasu, Sato, Sugimoto, & Puri, 2008) decreases *Gata4* expression.

Other scenarios in which genetic mutations and environmental factors synergistically interact are also possible. For instance, deficiencies in vitamin A may interact with mutations in several genes. In addition to *Gata4*, RA signaling also regulates the CDH-implicated receptors *COUP-TFII* (Doi, Sugimoto, & Puri, 2009; Jonk et al., 1994; Kruse et al., 2008) and *Stra6* (Zheng et al., 2020). The effects of genetic mutations and environmental perturbations may be temporally separated. Genetic mutations may act early in development to produce muscleless regions, but these regions may only herniate in the presence of later environmental perturbations, such as transient hypoxia that could reduce vascularization, biomechanically weaken the diaphragm, and permit the liver to herniate. Finally, the clinical severity of CDH is largely determined by the magnitude of the effects on lung development, and a "dual-hit" hypothesis has been proposed (Keijzer, Liu, Deimling, Tibboel, & Post, 2000), whereby both the developing diaphragm and lungs are affected by genetic mutations and/or environmental perturbations. Therefore, a complex scenario may be at work, in which genetic mutations disrupt diaphragm development, environmental perturbations affect lung development, and these effects interact synergistically to produce clinically severe CDH.

While gene–environment interactions are likely critical to the etiology of CDH, it is important to acknowledge the challenges of designing experiments to test such interactions. As the diaphragm is only present in mammals (Perry, Similowski, Klein, & Codd, 2010) and currently mice are the most tractable mammal for genetic manipulation, this limits such gene-environment experiments to mice. Engineering mutations in CDH candidate genes and characterizing the prevalence and phenotype of hernias that arise is relatively straightforward in mice. However, designing and testing environmental perturbations is more difficult. For example, testing the effects of vitamin A deficiency has been a central focus of CDH research, but designing appropriate VAD diets for mice that do not cause embryonic lethality but lead to reproducible levels of CDH is complex (see discussion in Rocke et al., 2021). The challenges are further compounded when trying to test for gene–environment interactions. Detecting a statistically significant increase between observed OR_{GE} and OR_{GE} expected from $OR_G \times w$ OR_E will generally require hundreds of embryos and so is often prohibitively time-consuming and expensive. An important strategy to maximize

the chance to detect gene–environment interactions is to design alleles of CDH-implicated genes or environmental perturbations (such as VAD diets; Rocke et al., 2021) that sensitize embryos to develop CDH, but by themselves do not cause hernias.

9. Concluding remarks

CDH is a common and often devastating birth defect associated with high rates of morbidity and mortality. Its etiology is notably complex, involving genetic and environmental factors. Recent research has largely concentrated on the role of genetic mutations and has revealed more than 150 candidate CDH-implicated genes. Yet, even genes for which there is strong experimental evidence in mice for their role in CDH are incompletely penetrant for CDH. This suggests that single genetic mutations are rarely sufficient to induce CDH. At present, there is limited evidence for the role of environmental factors, but maternal vitamin A insufficiency is one environmental perturbation for which there is strong support for its role as a sensitizing factor for development of CDH. Altogether, the lack of common, highly penetrant genetic or environmental causes of CDH suggests that gene–environment interactions may also contribute to CDH etiology. A future challenge is designing sensitive and cost-effective experiments to test such gene–environment interactions.

Are there opportunities to reduce the risk of CDH? Because CDH most often arises spontaneously by *de novo* mutations and CDH-implicated genes are incompletely penetrant for CDH, prenatal screening for potential genetic risk factors is less likely to be beneficial. However, vitamin A insufficiency is an environmental risk factor that is preventable. Vitamin A deficiency is a major global health problem, particularly in low- and middle-income countries, most notably in sub-Saharan Africa and southern Asia (Stevens et al., 2015). Even in the United States, as many as 15% of pregnant women in the United States consume insufficient vitamin A (Bailey, Pac, Fulgoni, Reidy, & Catalano, 2019). Efforts to reduce maternal vitamin A insufficiency, particularly during the first trimester of pregnancy, would lower the risk of CDH and other birth defects associated with low vitamin A intake.

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