Published in final edited form as:

Am J Med Genet C Semin Med Genet. 2018 June ; 178(2): 140–150. doi:10.1002/ajmg.c.31611.

Modeling the complex etiology of holoprosencephaly in mice

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Abstract

Holoprosencephaly (HPE) is a common developmental defect caused by failure to define the midline of the forebrain and/or midface. HPE is associated with heterozygous mutations in Nodal and Sonic hedgehog (SHH) pathway components, but clinical presentation is highly variable, and many mutation carriers are unaffected. It is therefore thought that such mutations interact with more common modifiers, genetic and/or environmental, to produce severe patterning defects. Modifiers are difficult to identify, as their effects are context-dependent and occur within the complex genetic and environmental landscapes that characterize human populations. This has made a full understanding of HPE etiology challenging. We discuss here the use of mice, a genetically tractable model sensitive to teratogens, as a system to address this challenge. Mice carrying mutations in human HPE genes often display wide variations in phenotypic penetrance and expressivity when placed on different genetic backgrounds, demonstrating the existence of silent HPE modifier genes. Studies with mouse lines carrying SHH pathway mutations on appropriate genetic backgrounds have led to identification of both genetic and environmental modifiers that synergize with the mutations to produce a spectrum of HPE phenotypes. These models favor a scenario in which multiple modifying influences – both genetic and environmental, sensitizing and protective – interact with bona fide HPE mutations to grade phenotypic outcomes. Despite the complex interplay of HPE risk factors, mouse models have helped establish some clear concepts in HPE etiology. A combination of mouse and human cohort studies should improve our understanding of this fascinating and medically important issue.

Keywords

Holoprosencephaly; mouse; Hedgehog signaling; Nodal signaling; mutation; modifier genes; teratogen; gene-environment interaction

1 INTRODUCTION

Holoprosencephaly (HPE; OMIM 236100) is a developmental defect in midline patterning of the forebrain and midface (Muenke & Beachy, 2001). It is remarkably common, occurring about once in 250 conceptions, but with ~97% succumbing in utero (Leoncini et al., 2008;

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

Shiota & Yamada, 2010). HPE encompasses a spectrum of anomalies. A phenotypic continuum of defects is broadly classified into three categories based on the degree of midline cleavage of the forebrain (Muenke & Beachy, 2001). Alobar HPE, the most severe form, is characterized by complete failure to partition the forebrain into left and right hemispheres; semilobar and lobar HPE are progressively less severe subtypes. The spectrum of craniofacial anomalies ranges from cyclopia in the most severe cases to single nostril, midface hypoplasia with midline cleft lip and/or palate, and single maxillary central incisor in progressively less affected individuals. The least severe facial midline anomalies may occur in the absence of brain malformations and are referred to as microforms of HPE.

The etiology of HPE is complex and heterogeneous (Krauss, 2007; Lipinski, Godin, O'leary-Moore, Parnell, & Sulik, 2010; Roessler & Muenke, 2010). It may be caused by large cytogenetic aberrations and/or as a feature of an identifiable syndrome. Isolated (non-chromosomal, non-syndromic) HPE occurs in both familial and sporadic forms, and is the focus of this review. Heterozygous, loss-of-function mutations in components and regulators of the Nodal and Hedgehog (Hh) signaling pathways are associated with HPE (Roessler & Muenke, 2010). However, in only a minority of isolated HPE cases have mutations been identified (Roessler & Muenke, 2010). Clinical presentation is extremely variable, with the full phenotypic spectrum seen in both sporadic and familial cases (Muenke & Beachy, 2001; Solomon et al., 2010). Furthermore, incomplete penetrance and variable expressivity are observed in pedigrees with heterozygous null mutations in *SHH* and *SIX3* (Lacbawan et al., 2009; Solomon et al., 2012; Stokes et al., 2018). Additionally, mutations in these genes identified in sporadic cases are most often inherited from a parent with little or no clinical manifestation (Lacbawan et al., 2009; Solomon et al., 2012). These observations are generally interpreted to indicate that most HPE-associated mutations are insufficient to cause severe, or possibly any, defects in forebrain patterning. Statistical analyses of this scenario led to a multifactorial, "autosomal dominant with modifier" model, in which the phenotypic outcome associated with a heterozygous mutation is influenced by more common genetic variants and/or environmental exposures (Roessler, Vélez, Zhou, & Muenke, 2012).

Non-genetic (environmental) risk factors are widely thought to be important in HPE etiology. In utero exposure to specific teratogens, including Hh pathway inhibitors, is sufficient to induce HPE in agricultural and experimental animals (Chen, 2016; Lipinski et al., 2010). However, epidemiological studies on HPE have been incomplete and inconclusive (Croen, Shaw, & Lammer, 2000; Johnson & Rasmussen, 2010; Miller et al., 2010; Stashinko et al., 2004). It is suspected that many structural birth defects are caused by a complex combination of genetic and environmental factors that interact to perturb morphogenetic processes (Krauss & Hong, 2016). The "autosomal dominant with modifier" model of HPE is wholly consistent with this likelihood, further complicating identification of environmental risk factors. Even though only a fraction of HPE cases currently have an identifiable genetic defect, it can be envisaged that future whole genome sequencing efforts will lead to a reasonably complete catalog of the genes that suffer true driver mutations in HPE. In contrast, modifiers, both genetic and environmental, are difficult to identify as their effects are context-dependent and occur within the complex genetic and environmental landscapes that characterize human populations.

One way to gain insight into these problems in studying HPE etiology is via animal models, particularly mice (Krauss, 2007; Schachter & Krauss, 2008). Several properties of laboratory mice, including genetic tractability, and conserved signaling and developmental processes, make them the current model of choice. In this review, we discuss the strengths and weaknesses of using mice to address and illuminate questions of HPE etiology.

2 RATIONALE FOR THE MOUSE AS A MODEL ORGANISM FOR STUDYING THE COMPLEX ETIOLOGY OF HPE

Development of the midline of the forebrain and midface is initiated by signals from the prechordal mesendoderm (PCM) (Kiecker & Niehrs, 2001; Lipinski, Godin, O'leary-Moore, Parnell, & Sulik, 2010; Muenke & Beachy, 2000; Rubenstein & Beachy, 1998). The PCM is specified from the anterior primitive streak in response to signaling by the Nodal pathway (Schier, 2009; Shen, 2007). Sonic Hh (Shh) produced by the PCM then initiates a developmental sequence which progressively patterns the rostroventral midline. *Shh* is expressed in a temporal cascade, first in the PCM, then in the developing ventral midline of the diencephalon and telencephalon, and induces ventral midline cell fates throughout this process (Aoto et al., 2009; Cordero et al., 2004; Geng et al., 2008; Marcucio, Cordero, Hu, & Helms, 2005; McMahon, Ingham, & Tabin, 2003; Rubenstein & Beachy, 1998). As embryogenesis proceeds, Shh produced by the forebrain induces *Shh* expression in the ectoderm of the frontonasal and maxillary processes of the developing face. Shh produced by these latter structures patterns neural crest-derived facial primordia to produce specific cartilaginous structures and bones of the craniofacial midline (Helms, Cordero, & Tapadia, 2005; Hu & Helms, 1999; Hu & Marcucio, 2009; Marcucio et al., 2005; Marcucio, Young, Hu, & Hallgrimsson, 2011).

Nodal and Shh activate evolutionarily conserved signal transduction pathways. Nodal is a TGF β superfamily member that signals via a receptor complex comprising TGF β type I and type II receptors (Alk4 and Acvr2A/B) and a GPI-linked coreceptor (Cripto/TDGF1) (Figure 1A) (Schier, 2009; Shen, 2007). Nodal binding leads to receptor-mediated phosphorylation of the transcription factors Smad2 and Smad3. Phosphorylated Smad2/3 then bind Smad4 and, in association with another transcription factor, FoxH1, activate target genes critical for PCM induction. Shh signals through an incompletely understood pathway (Figure 1B) (Briscoe & Thérond, 2013; Lee, Zhao, & Ingham, 2016). In the absence of Shh, its primary receptor Patched1 (Ptch1) functions to inhibit the signaling activity of a second membrane protein, Smoothened (Smo). In the absence of Smo activity, the transcription factors Gli2 and Gli3 undergo proteolysis to repressor forms, which inhibit expression of pathway-responsive genes (Gli3 is the major repressor). Binding of Shh to a receptor complex that contains Ptch1 and at least one of three co-receptors (Cdon, Boc, Gas1) relieves inhibition of Smo. Smo then signals to block Gli2/3 proteolysis, and they in turn activate expression of pathway target genes (Gli2 is the major activator).

Heterozygous mutations in a limited number of genes are implicated as driver events in isolated and/or microform human HPE. Almost all these genes are components or regulators (direct or indirect) of the Nodal or Shh signaling pathways. Those associated with Nodal

include: *NODAL*, *TDGF1* (human ortholog of mouse *Cripto*), *FOXH1*, and *ZIC2*; those associated with SHH include: *SHH*, *DISP1*, *PTCH1*, *CDON*, *GAS1*, and *SIX3* (Bae et al., 2011; Barratt & Arkell, 2018; Pineda-Alvarez et al., 2012; Ribeiro, Quiezi, Nascimento, Bertolacini, & Richieri-Costa, 2010; Roessler & Muenke, 2010). Mutation of *GLI2*, a key player in SHH signaling, results more commonly in pituitary deficits and mild facial anomalies, and only rarely in HPE (Bear et al., 2014; Roessler et al., 2003). *TGIF1* appears to regulate both Nodal and SHH pathways by at least partially independent mechanisms (Taniguchi et al., 2016). Mutations in FGF signaling pathway components are also found in human HPE (Arauz et al., 2010; Dubourg et al., 2016).

When tested in functional assays, virtually all such mutations confer a loss-of-function, indicating patients are haploinsufficient, or partially compromised (Geng & Oliver, 2009; Roessler & Muenke, 2010) (we note that *PTCH1* is a negative regulator of SHH signaling, so HPE-associated mutations presumably have a gain-of-function, but this has not been studied). Importantly, mutation of each of these genes (excluding *Ptch1*) produces HPE in mice, although the genetics are not identical between humans and mice. Most importantly, heterozygous mutations do not cause HPE in mice. We are aware of one exception: *Six3*^{+/-} mice on a C57BL/6 genetic background had HPE with low penetrance (Geng et al., 2008). We also note that while heterozygous mutants of *Nodal*, *Cripto*, and *FoxH1* are without obvious phenotype, homozygous mutants do not have HPE either, because they fail to complete gastrulation and die in utero at a very early stage of development. However, strong hypomorphs in the Nodal pathway display a range of phenotypes that includes HPE (Chu et al., 2005; Lowe, Yamada, & Kuehn, 2001; Nomura & Li, 1998; Song et al., 1999). Therefore, HPE is generally an autosomal recessive phenotype in mice, whereas it is autosomal dominant with partial penetrance and variable expressivity in human pedigrees. The phenomenon that mice require homozygous mutations to produce phenotypes associated with heterozygous mutations in humans is not restricted to HPE (Krauss, 2008). The underlying reasons are not fully clear, but likely include: 1) intrinsic differences between mice and humans; 2) that heterozygous mutations in humans likely combine with more common genetic and non-genetic modifiers to produce deleterious outcomes at high enough frequency to prevent accumulation of such mutations in the population (the range of modifiers experienced by gestating laboratory mice is likely to be much less complex); and 3) homozygous mutation of HPE genes in humans is likely to result in early embryonic lethality and pregnancy loss. Nevertheless, the fact that mutations in the same sets of genes in mice and humans is associated with HPE argues powerfully that the signaling and embryonic processes underlying development of the rostroventral midline are very similar between these species. This is hardly surprising, but serves as a critical basis for using mice to model the complex, multifactorial etiology of human HPE.

3 GENETIC INTERACTIONS IN HUMAN AND MOUSE MODELS OF HPE

In seeking potential explanations for the insufficiency of heterozygous mutations to result in severe HPE anomalies, one early proposal was a need for heterozygosity of two different HPE genes; i.e., digenic mutation (Ming & Muenke, 2002). However, double heterozygosity for known, bona fide HPE genes occurs infrequently in human HPE (Lacbawan et al., 2009; Roessler et al., 2012; Solomon et al., 2012). Furthermore, the likelihood of developing HPE

upon inheritance of a heterozygous mutation is too high to be explained by a second de novo mutation and therefore inconsistent with the analyses that led to the “autosomal dominant with modifier” model. Nevertheless, studies of doubly heterozygous mice have produced interesting results that should aid in understanding more common types of genetic interactions that may occur in human HPE. For example, several mouse lines with heterozygous mutations in two different genes that regulate Nodal signaling have HPE, suggesting that this pathway may be particularly dosage sensitive (Jin, Harpal, Ang, & Rossant, 2001; Nomura & Li, 1998; Varlet, Collignon, Norris, & Robertson, 1997). Within the Shh pathway, *Six3* is a transcription factor that regulates expression of *Shh* in the forebrain, and *Gli2* is a transcription factor whose activity is regulated by Shh signaling. On genetic backgrounds where single heterozygotes are normal, *Six3*^{+/-};*Shh*^{+/-} mice have HPE, but *Shh*^{+/-};*Gli2*^{+/-} mice do not (Geng et al., 2008; Heyne et al., 2016). Therefore, there is selectivity to which genes in the Shh network produce HPE as an outcome of double haploinsufficiency. Given that weak alleles of HPE genes might function as modifiers (see below), such studies could help illuminate which gene variant combinations might contribute to HPE, when studying these more subtly affected alleles.

An alternative possibility to digenic mutation is that loss-of-function driver mutations interact with modifier alleles, some of which may be relatively common in human populations. It seems likely that two types of HPE modifier gene exist. First, weak hypomorphic alleles of bona fide HPE genes may be present in the general population, but the degree of function retained is insufficient to act as a driver mutation for HPE. However, such alleles may act as modifiers of strong loss-of-function mutations in different HPE genes. Many variations of HPE genes are present in the human population but they will need to be assessed functionally to know whether they fit into this category (or act similarly to alleles considered as wild type). Very few studies of this type have been performed. One example is the work of Roessler et al. (Roessler et al., 2009), in which a hypomorphic *NODAL* variant was identified in normal individuals at high frequency. However, association of this allele with HPE or other Nodal pathway-related anomalies was not obvious.

A second type of modifier gene is the “silent” modifier. Silent modifier genes are those whose null mutation does not produce the phenotype in question, but variants of which can modify this same phenotype when it is induced by mutation of a different gene. Identification of silent modifier genes of human congenital anomalies is very difficult. The first evidence for such modifiers of HPE came from studies of mice in which null mutations of an HPE gene were placed on different genetic backgrounds, i.e., the mutations were crossed onto different inbred mouse strains. Inbred mouse strains are homozygous at all loci, and different strains have distinct alleles of many genes. Some of these loci may modify the phenotypic manifestation of unlinked mutant genes. For example, *Cdon*^{-/-} mice on a C57BL/6 genetic background had semi-lobar HPE and a single nostril with high penetrance, whereas these mice on a 129S6 background showed only HPE microforms with low penetrance (Cole & Krauss, 2003; Zhang, Kang, Cole, Yi, & Krauss, 2006) (Figure 2). Therefore, *Cdon*^{-/-} mice recapitulated a major portion of the HPE phenotypic spectrum simply by altering the strain background modifier genes. These results demonstrated that modifier genes are sufficient to grade HPE penetrance and expressivity in mice. Moreover,

they strongly suggest that such modifiers are one likely explanation for the phenotypic variability that characterizes human HPE. The C57BL/6 strain is a sensitized background for HPE induced by several mutations, including *Cdon*, *Six3*, *Gli2*, and *Tsg* (*Tsg* is a regulator of BMP signaling not found to be mutated in human HPE) (Geng et al., 2008; Heyne et al., 2016; Petryk et al., 2004; Zhang et al., 2006). The modifier loci in C57BL/6 mice that confer sensitivity to HPE by these various mutations are not known, and they may not be identical in each case.

The relatively HPE-resistant 129S6 *Cdon*^{-/-} mice offer an opportunity to identify candidate modifiers, both genetic and environmental, silent or otherwise. These mice have a largely subthreshold defect in Shh signaling that is subject to modification, as evidenced by the increased severity of the HPE phenotype associated with a transfer to the C57BL/6 background (Cole & Krauss, 2003; Zhang et al., 2006). *Cdon*, *Boc*, and *Gas1* are cell surface proteins that promote Hh pathway activity as ligand-binding coreceptors with *Ptch1* (Allen et al., 2011; Allen, Tenzen, & McMahon, 2007; Izzi et al., 2011; Martinelli & Fan, 2007; Okada et al., 2006; Tenzen et al., 2006; Zhang, Hong, Bae, Kang, & Krauss, 2011; Zhang et al., 2006). *Cdon* and *Boc* are closely related Ig superfamily members, whereas *Gas1* is a GPI-anchored protein unrelated to *Cdon* and *Boc*. Analysis of mice with targeted mutations in *Cdon*, *Boc*, and *Gas1*, singly or in combination, indicated that these three factors have overlapping and partially compensatory functions in regulating Shh signaling (Allen et al., 2011; Allen et al., 2007; Izzi et al., 2011; Tenzen et al., 2006; Zhang et al., 2011; Zhang et al., 2006). Mice lacking any two coreceptors have stronger phenotypes than mice lacking any single one, and mice lacking all three have a nearly complete loss of Hh pathway activity.

Heterozygous mutations of *CDON* and *GAS1* have been identified in human HPE patients (Bae et al., 2011; Pineda-Alvarez et al., 2012; Ribeiro et al., 2010). Consistent with this, *Cdon*^{-/-} and *Gas1*^{-/-} mice display a range of HPE phenotypes (Cole & Krauss, 2003; Seppala et al., 2007; Zhang et al., 2006). In contrast, *Boc*^{-/-} mice do not have HPE on any genetic background, and they are viable and fertile (Okada et al., 2006; Zhang et al., 2011). However, genetic removal of *Boc* from *Cdon*^{-/-} mice worsens their HPE phenotype, and this is seen with mice on both 129S6 or C57BL/6 backgrounds (Zhang et al., 2011) (Figure 2). Furthermore, this occurs in a *Boc* gene dosage sensitive manner, i.e., *Cdon*^{-/-};*Boc*^{+/-} mice show HPE phenotypes midway between *Cdon*^{-/-} mice and *Cdon*^{-/-};*Boc*^{-/-} mice (Zhang et al., 2011; and M.H., unpublished results). Removal of *Boc* from *Gas1*^{-/-} mice also exacerbates the latter's HPE, although in this case both copies of *Boc* must be lost for the effect to be manifest (Seppala, Xavier, Fan, & Cobourne, 2014).

From these results, it was concluded that *Boc* is a silent HPE modifier gene in mice. This naturally raised the possibility that *BOC* might also be a silent modifier in human HPE. To begin to assess this possibility, *BOC* was sequenced for 360 HPE patients and a similar number of unrelated individuals as controls (Hong et al., 2017). Several variants were identified in the regions of *BOC* that: 1) physically interact with SHH or PTCH1; and 2) were equivalent to regions of *CDON* subject to loss of function mutations in HPE patients. These *BOC* variants were tested by in vitro functional assays, and two (R533L and P828R) were associated with a loss of function. Importantly, some patients with these variants also

had pathogenic mutations in known HPE genes (*ZIC2* and *TGIF1*), consistent with the “autosomal dominant with modifier” model. Modifier alleles are classically categorized as phenotypic enhancers or suppressors (Kousi & Katsanis, 2015). Given that the R533L and P828R variants showed a loss of function, they would be predicted to further diminish a reduction of SHH signaling associated with heterozygosity for *ZIC2* or *TGIF1*, and are likely enhancers of the phenotypic outcome in these patients. Both these variants are also found in the general population as listed by the Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org>). R533L is rare, but P828R had a minor allele frequency of 0.0017. This is higher than the ~1:10,000 live birth frequency of HPE and also consistent with modifier function. One other *BOC* variant identified in this study (G556E) had never been reported before in any population and displayed a Shh-dependent *gain of function*. This is opposite what would be expected for causation in HPE. Ninety-seven percent of HPE cases succumb in utero and are not available for DNA sequence analyses. We speculate that this patient may have survived long enough for such an analysis because the G556E allele acted as a partial phenotypic suppressor of an unidentified insult that inhibited SHH signaling during development of the rostroventral midline. Similar protective effects have been seen in mouse HPE models by removal of one copy of the negative Hh pathway regulator *Ptch1* (Geng, Acosta, Lagutin, Gil, & Oliver, 2016; Hong & Krauss, 2013). Taken together, the combination of: 1) both loss- and gain-of-function properties (enhancer and suppressor, respectively); 2) co-occurrence of *BOC* variants with bona fide pathogenic variants of HPE genes; and 3) *BOC* allele frequencies, provided strong evidence that *BOC* is a modifier gene in HPE, the first to be identified (Hong & Krauss, 2017). Furthermore, this finding validates the use of mouse models as one foothold into the very difficult problem of identifying silent modifier genes in HPE patients.

4 GENE-ENVIRONMENT INTERACTIONS IN HPE

Small molecule inhibitors of Shh signaling, if sufficiently potent, are HPE-inducing teratogens in animals (Chen, 2016; Heyne et al., 2015). The first such molecule discovered is the naturally occurring teratogen and SMO antagonist, cyclopamine (Chen, 2016). Human exposure to naturally occurring or synthetic SMO inhibitors may therefore be a risk factor for HPE. Alternative teratogenic mechanisms also exist. Some teratogens may induce HPE by perturbing other signaling pathways, with consequent indirect inhibition of Shh signaling during the appropriate developmental period. Additionally, xenobiotics are not the only type of potential non-genetic risk factors for HPE. Pre-pregnancy maternal diabetes and infectious agents are associated with many birth defects, potentially including HPE (Johnson & Rasmussen, 2010; Miller et al., 2010). It must be emphasized that environmental risk factors need not act as complete teratogens to be involved etiologically in HPE, as they may serve as modifiers of germline mutations which predispose individuals to HPE. This type of scenario could arise either because a given environmental factor is intrinsically insufficient as a teratogen (analogous to silent modifier genes) or because the exposure dosage and/or timing are suboptimal.

Many structural birth defects are likely to arise from a complex combination of genetic and environmental influences (Krauss & Hong, 2016). Identifying gene-environment interactions in birth defect etiology is difficult, particularly if the interactions occur only between

specific subsets of all the genetic and non-genetic risk factors potentially involved. Mouse models are particularly well-suited to address this issue. The first reported example of gene-environment interaction in HPE concerned lowering of cholesterol levels (Lanoue et al., 1997). Cholesterol promotes Shh signaling in multiple ways, including covalent adduction of Hh ligands themselves and direct interaction with Smo (Byrne, Luchetti, Rohatgi, & Siebold, 2018; Mann & Beachy, 2004). 3 β -hydroxysterol-7-reductase catalyzes the final step in cholesterol biosynthesis and is encoded by *DHCR7*. Autosomal recessive mutations in *DHCR7* cause Smith-Lemli-Opitz syndrome (SLOS) (Kelley & Herman, 2001). Consistent with the need for sufficient levels of cholesterol for Shh signaling, some individuals with SLOS have HPE (Haas & Muenke, 2010). Lanoue et al. showed that when mice genetically deficient for apolipoprotein B (which have low cholesterol levels) were exposed in utero to the *DHCR7* inhibitor BM15.766, a fraction of the embryos were holoprosencephalic (Lanoue et al., 1997). While this represents an extreme lowering of cholesterol, the study demonstrated that mutations and chemicals affecting a similar process could synergize to produce HPE. Furthermore, the findings add weight to the recommendation that women who take statin drugs to lower cholesterol should not do so when pregnant (Haas & Muenke, 2010).

The notion that subthreshold combinations of genetic and chemical inhibition of Shh signaling can produce HPE spectrum phenotypes was specifically probed by Lipinski and colleagues. Vismodegib is a potent, FDA-approved SMO inhibitor (Sharpe, Wang, Hannoush, & de Sauvage, 2015). Single dose (40 mg/kg), in utero exposure to Vismodegib induced HPE in ~80% of C57BL/6 mice (Heyne et al., 2015). *Gli2*^{-/-} mice on this background also had HPE, but *Gli2*^{+/-} mice did not (Heyne et al., 2016). However, *Gli2*^{+/-} mice were sensitized to Vismodegib relative to wild type mice, displaying increased HPE penetrance and severity in response to doses of 2.5 and 10 mg/kg (Heyne et al., 2016). SMO is a complex protein, with multiple allosteric sites (Sharpe et al., 2015). It is targeted by many natural and man-made small molecule antagonists, and many more are likely to be discovered. Some of these are probably of a potency lower than that needed for sufficiency as an HPE-inducing teratogen (Lipinski & Bushman, 2010). However, individuals with heterozygous mutations in HH pathway genes may be sensitized to such compounds, making them potential modifiers in an “autosomal dominant with modifier” scenario.

Ethanol is a human teratogen, responsible for fetal alcohol spectrum disorders (FASD) (Mead & Sarkar, 2014). It has also been implicated in HPE, although epidemiological studies have been inconsistent on this point (Croen et al., 2000; Johnson & Rasmussen, 2010; Miller et al., 2010). Ethanol is teratogenic to mouse embryos, but this occurs in a strain-specific manner (Aoto, Shikata, Higashiyama, Shiota, & Motoyama, 2008; Downing, Balderrama-Durbin, Broncucia, Gilliam, & Johnson, 2009; M. Hong & Krauss, 2012). C57BL/6 mice are sensitive to fetal ethanol, developing FASD-like craniofacial phenotypes; additionally, ~20% of the embryos show HPE spectrum defects (Aoto et al., 2008; Sulik, Johnston, & Webb, 1981). In contrast, 129S6 mice are resistant to ethanol-induced teratogenesis (Downing et al., 2009; Hong & Krauss, 2012). As 129S6 *Cdon*^{-/-} mice are a sensitized line to identify genetic HPE modifiers, we tested the possibility that these animals were also sensitized to fetal alcohol-induced HPE. In 129S6 mice, *Cdon* mutation resulted in mild and infrequent HPE phenotypes and in utero ethanol exposure was without effect, but

together they produced the full HPE spectrum with ~75% penetrance (Hong & Krauss, 2012) (Figure 2). This included alobar HPE with cyclopia, which is not seen in any of the purely genetic HPE models that involve *Cdon* mutation. Moreover, certain phenotypes found in human HPE only infrequently, such as agnathia and ocular coloboma, arose at a similar low penetrance in these embryos. Finally, loss of *Cdon* and fetal ethanol combined to inhibit Shh signaling specifically in the developing rostroventral midline. Consistent with the central role of diminished Shh signaling in HPE, removal of one copy of the negative Hh pathway regulator *Ptch1* rescued both Shh signaling and normal development in ethanol-exposed *Cdon*^{-/-} embryos (Hong & Krauss, 2013). Taken together, this model of HPE: 1) produced the major features observed at the population level in human HPE; 2) displayed gene-environment interactions consistent with a mutation plus modifier etiology; and 3) required a critical level of Shh pathway inhibition to observe HPE phenotypes (Hong & Krauss, 2012, 2013). Importantly, HPE associated with fetal ethanol exposure in C57BL/6 mice was exacerbated in mice of this background that were heterozygous for either *Shh* or *Gli2* (Kietzman, Everson, Sulik, & Lipinski, 2014), indicating that the phenomenon is generalizable.

Mouse models offer a means to understand mechanistic underpinnings of teratogen action and gene-environment interactions. We have pursued such questions in the *Cdon* mutation plus fetal ethanol model. Many of ethanol's toxic effects are ascribed to its oxidative metabolism (Cederbaum, 2012). Ethanol is metabolized by alcohol dehydrogenase (ADH) to acetaldehyde, which is in turn metabolized by aldehyde dehydrogenase to harmless acetate. Acetaldehyde is a teratogen and a source of oxidative stress. Cytochrome P450 2E1 (Cyp2E1) metabolizes ethanol to acetaldehyde as well, and though less important quantitatively than ADH, it produces reactive oxygen species as part of its catalytic action (Cederbaum, 2012). A potential alternative, non-metabolism-based, teratogenic mechanism is that ethanol itself has solvent properties and perturbs cellular membranes (Goldstein, 1986; Klemm, 1990). To address whether oxidative metabolism is required for ethanol's action as an HPE teratogen, we tested whether *t*-butyl alcohol was teratogenic. *t*-butyl alcohol is neither a substrate nor an inhibitor of ADH or Cyp2E1; nevertheless, it induced HPE in *Cdon*^{-/-} mice similarly to ethanol, but at lower doses (Hong & Krauss, 2017). Because ethanol may generate oxidative stress through its metabolism as well as via a more general toxicity, we tested whether antioxidant treatment influenced its ability to induce HPE. Strikingly, an antioxidant regimen that completely blocked ethanol-induced production of reactive oxidant species had no effect on its teratogenic effects (M. Hong & Krauss, 2017). Together, these results are consistent with the conclusion that ethanol itself is the HPE-inducing teratogen, not consequences of its metabolism or oxidative stress.

This finding has practical consequences. Ethanol is rapidly metabolized, and is undetectable in the blood of female mice less than 24 hours after administration (Hong & Krauss, 2012). To determine a window of sensitivity, pregnant females were administered ethanol at several different time points during gestation. Ethanol was teratogenic to *Cdon*^{-/-} mice when given at embryonic day (E) 7.0 and E7.25, but not earlier and not at E7.5 or later (Aoto et al., 2008; Hong & Krauss, 2012; and M.H., unpublished results). This very narrow window (<12 hours) corresponds to the third week of human pregnancy, a time many women do not know they are pregnant.

Because ethanol itself appears to be the culprit, the developmental processes that are its direct targets must occur: 1) during the sensitive time window; and 2) before ethanol is metabolized. Shh signaling from the PCM begins at about E7.75, after almost all the ethanol has been metabolized in mice treated at E7.0. Additionally, the window of sensitivity for HPE induced by the direct Hh pathway inhibitor Vismodegib peaks at E7.5, later than for ethanol (Heyne et al., 2015). Therefore, Hh signaling is very unlikely to be the direct target of ethanol. We note that this is not inconsistent with a requirement for inhibition of Hh signaling during ethanol-induced HPE (Hong & Krauss, 2013); rather, this inhibition presumably occurs as a downstream consequence of ethanol's direct actions. The major relevant event occurring during the window of sensitivity to ethanol is Nodal pathway-dependent induction of the PCM. Consistent with the idea that Nodal signaling may be targeted by ethanol, expression of Nodal pathway target genes in the anterior primitive streak is reduced in ethanol-treated *Cdon*^{-/-} embryos (Hong & Krauss, 2012).

Combining these findings, a scenario consistent with production of a full spectrum of HPE phenotypes in ethanol-treated *Cdon*^{-/-} mice is as follows: *Cdon* mutation and fetal ethanol exposure synergize to transiently and partially inhibit Nodal signaling during gastrulation. We suspect that ethanol acts as a membrane perturbant, negatively affecting formation or localization of Nodal receptor complexes. This sets up a situation in which resultant embryos are susceptible to suboptimal Shh signaling associated with lack of *Cdon*. The consequent spectrum of HPE defects is postulated to arise because individual embryos fail to reach a threshold level of Shh signaling at specific developmental stages, the stage of the deficit occurring stochastically. The earlier during rostroventral midline patterning the failure to reach threshold occurs, the more severe the ensuing HPE phenotype (Cordero et al., 2004; Krauss, 2007; Marcucio et al., 2011).

5 CONCLUSIONS AND FUTURE DIRECTIONS

HPE arises from a complex interaction between germline mutations and modifier effects, both genetic and environmental. This has made a full understanding of HPE etiology challenging. We have argued here that the mouse, a genetically tractable system sensitive to teratogens, provides a model to address this challenge. Validation of this point of view is that *Boc* was initially found to be a silent HPE modifier gene in mice and was subsequently identified to be a modifier gene in human HPE. Similarly, ethanol is a suspect HPE teratogen, and studies with mice argue that this may be so in humans, but genetically predisposed individuals (e.g., those harboring SHH pathway mutations) may be particularly susceptible. Additionally, mice offer opportunities to decipher mechanisms in causation of HPE. Our finding that ethanol itself is the likely HPE teratogen, perhaps acting as a membrane-active agent, suggest that other environmental chemicals with solvent-like properties may have similar teratogenic effects.

Studies with human HPE cohorts and mouse models raise interesting questions that will be most easily addressed in mice, at least initially. One such question concerns the complexity of modifiers in HPE. Although the presence of more than one bona fide driver mutation is uncommon in human HPE, it may be that multiple modifying influences – both genetic and environmental, sensitizing and protective – interact to grade the phenotypic response to a

mutation. Another question is whether multiple HPE-enhancing modifiers could be sufficient to produce HPE in the absence of a driver mutation. Driver mutations have not been found in most isolated HPE cases. The percentage of cases that do have identified mutations will undoubtedly rise with the application of whole genome sequencing of triads. However, the notion that accumulation of many “smaller” insults might produce HPE is important to consider. As an initial test of this possibility we assessed whether 129S6 *Boc*^{-/-} mice were sensitive to ethanol-induced HPE (i.e., a genetic modifier plus an environmental modifier); these mice did not have HPE (Hong & Krauss, 2012). It may be that a strong driver insult is always necessary or that, in the absence of a bona fide mutation, several enhancing modifiers are required to produce HPE. The mouse will be a good model to explore this question. Finally, mutations in at least 14 genes have been identified in isolated HPE, with none accounting for a very high percentage of total cases. While it is still early days in the identification of modifiers, it can be envisaged that their number is higher. The heterogeneity of human genomes and the environments in which they live, plus the complexity of HPE causation and the wide range of its associated phenotypes, suggests that each case may be different. Although that is a daunting thought, some clear concepts have been established in HPE etiology. A combination of studies with human cohorts and increasingly complex mouse models should continue to improve our understanding of this fascinating and medically important issue.

Acknowledgments

The authors thank Rob Lipinski for critical reading of the manuscript, and Erich Roessler and Max Muenke for ongoing discussions and years of fruitful collaboration. Work in the authors' laboratory on this topic is currently funded by NIDCR grant DE024748.

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FIGURE 1.

Schematic of Nodal and Shh Signaling. (a) Simplified schematic of Nodal signaling. Nodal binds a receptor complex that includes ActRII, Alk4, and Cripto, leading to Smad2/3 phosphorylation. P-Smad2/3 binds Smad4 and, with FoxH1, activates pathway target genes. (b) Simplified schematic of Shh signaling. In the absence of Shh (far right cell), Ptch1 inhibits Smo activity, leading to proteolysis of Gli2 and Gli3 (Gli2/3) to transcriptional repressor forms (GliR), which inhibit pathway target gene expression. Shh is produced as a precursor product (Shh(pre); far left cell). Upon autoproteolysis, cholesterol adduction, and palmitoylation by Skn, mature Shh ligand is produced. Shh is released from producing cells in a Disp1-dependent manner. Upon binding of Shh to a complex of Ptch1 and at least one coreceptor (Cdon, Boc, or Gas1) (center cell), Smo inhibition by Ptch1 is relieved. Smo signals to block Gli2/3 proteolysis, allowing formation of transcriptional activator forms (GliA), which induce pathway target gene expression. Factors indicated in color have been identified as targets of mutation in HPE.

FIGURE 2.

129S6 *Cdon*^{-/-} mice display modifier-dependent HPE phenotypes. (a1–a3) *Cdon*^{-/-} mice on a 129S6 genetic background are largely normal, displaying two nostrils and normally partitioned upper lip (a1; arrow); two optic vesicles (a2; arrowheads); and normally partitioned forebrain (a3). We note that a minority of these mice show mild midfacial defects (not shown, see text). (b1) Genetic removal of *Boc* enhances this phenotype in a dosage sensitive manner, resulting in pronounced midfacial midline hypoplasia, fused upper lip, and single nostril (red arrow). (c1–c3) In utero exposure of 129S6 *Cdon*^{-/-} mice to alcohol leads to a full spectrum of HPE phenotypes, including in: (c1) single nostril (a mid-severity phenotype; arrow); (c2) unpartitioned forebrain plus cyclopia (the most severe phenotypes; arrow and arrowhead, respectively); and (c3) partitioned forebrain with ventral continuity (a mild phenotype; arrow). (d1) Genetic removal of one copy of the negative Shh pathway regulator *Ptch1* rescues HPE phenotypes in alcohol-treated 129S6 *Cdon*^{-/-} mice. (e1, e2) C57BL/6 *Cdon*^{-/-} have more severe HPE than 129S6 *Cdon*^{-/-} mice, and show single nostril (e1; arrow) and unpartitioned forebrain (e2; arrow). (f1, f2) Genetic removal of *Boc* enhances this phenotype in a dosage sensitive manner, resulting in severely hypoplastic midface and hypotelorism (f1) and cleft lip (f2; arrow). All whole mount embryos are at E14.5 or E15.5. Sections are at E10.5 (a2, c2, e2) and E14.5 (a3, c3).