Genetics of ventral forebrain development and holoprosencephaly

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The disease holoprosencephaly is the basis of the most common structural anomaly of the developing forebrain in humans. Numerous teratogens when administered during early gastrulation, have been associated with this condition. Recent studies have characterized molecules expressed in the prechordal plate which are critical for normal brain formation. Perturbation of signaling pathways involving these molecules have been shown to cause holoprosencephaly in humans and other organisms.

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Abbreviations

Hh	Hegdehog
HPE	holoprosencephaly
PHS	Pallister–Hall syndrome
PTC	Patched
RTS	Rubenstein–Taybi syndrome
RXR	retinoid X receptor
SHH	*Sonic Hedgehog*
SLOS	Smith–Lemli–Opitz syndrome
SMO	Smoothened
SO	*Sine oculis*
TGIF	TG-interacting factor

Introduction

Cyclopia has been known since ancient times with the descriptions of mythological single-eyed creatures living on the coast of Sicily. In humans, the comparable malformation is holoprosencephaly (HPE), which has a prevalence of 1 in 10,000–20,000 livebirths and 1 in 250 during early embryogenesis, making it the most common brain anomaly in humans (reviewed in [1,2]). The primary brain malformations comprise incomplete cleavage of the forebrain (prosencephalon) into right and left hemispheres, into telencephalon and diencephalon, and into olfactory and optic bulbs and tracts. In the most severe form, a single brain ventricle is present without any evidence of an interhemispheric fissure [3,4]. The spectrum of brain malformations in HPE extends from most to least severe in unbroken continuity. These brain malformations are frequently accompanied by facial anomalies including cyclopia with a proboscis (nose-like structure) above the eye, a single-nostril nose, median cleft lip, and others [5]. Developmental delay is present in virtually all affected individuals with central nervous system anomalies, although the degree of delay is variable, correlating with the severity of the brain malformation. Likewise, survival of children with HPE depends on the severity of the central nervous system anomalies. The majority of children with severe HPE die during the first year of life [6].

Embryology of forebrain development

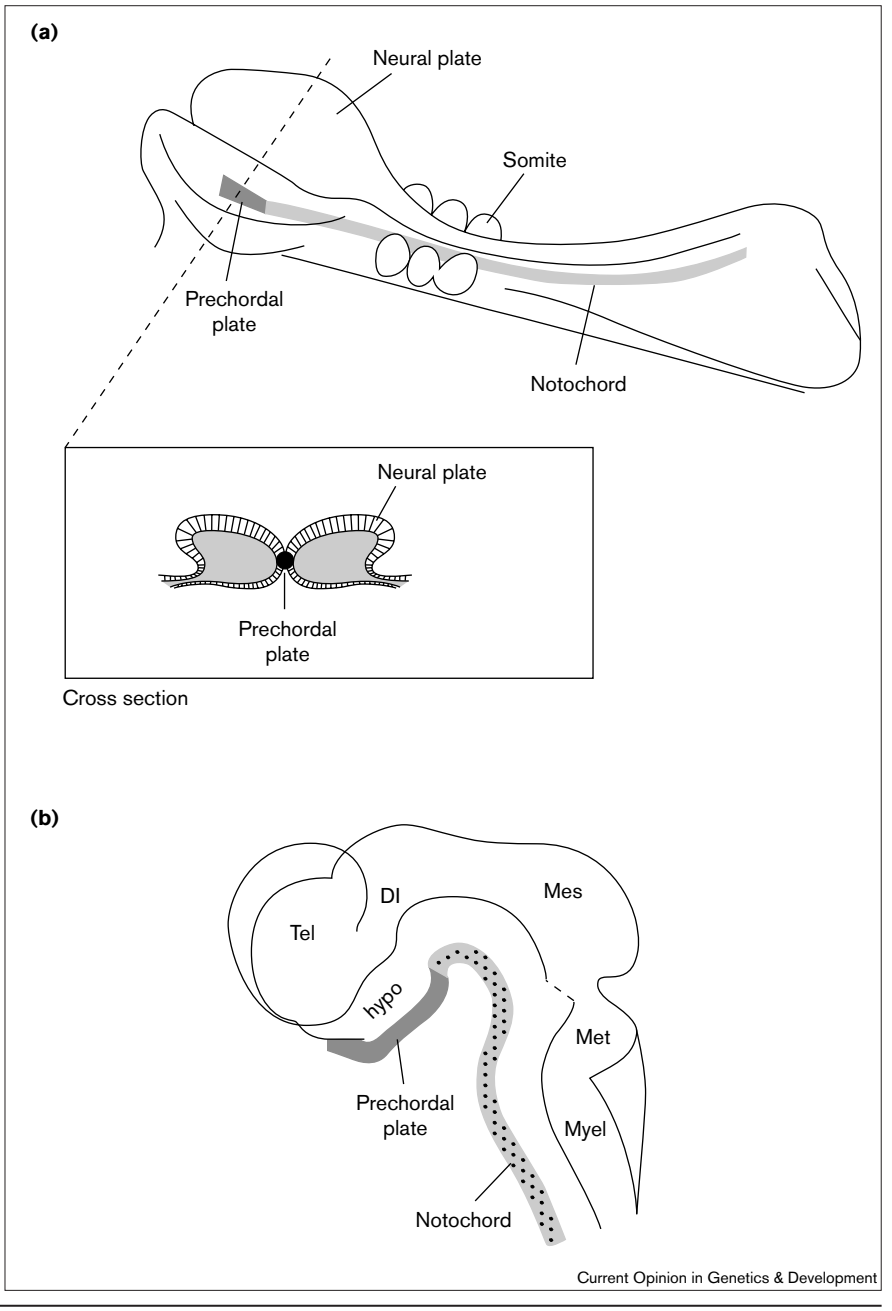
Much of what we know about the pathogenesis of HPE is derived from observations in animals. Embryologically, HPE can be traced to varying degrees of loss or disruption in the development of ventral forebrain and midline facial structures. In normal development, the optic vesicles evaginate from the lateral walls of the forebrain, at locations separated by the developing structures of the ventral forebrain. In severe HPE, ventral forebrain structures are absent and the optic primordia consequently develop as a single unpaired evagination from the floor of the forebrain. The resulting cyclopic eye protrudes into the developing face, thus displacing the fused nasal structures superiorly and accounting for the appearance and placement of the eye and proboscis [7].

Beginning in the late nineteenth century, experimental embryologists learned how to induce cyclopia in diverse vertebrate species by subjecting embryos to various treatments — radiation, heat, cold, hypoxia, salts, alcohols and other solvents, alkali, vitamin A, and certain plant alkaloids and synthetic compounds — during early gastrulation (reviewed in [8]). In the early twentieth century, Otto Mangold and Howard Adelmann independently carried out surgical manipulations in amphibians. These experiments demonstrated that removal of prechordal plate mesoderm (also known as prechordal mesendoderm; Figure 1) caused a failure in formation of structures such as the chiasmatic plate and optic stalks and resulted in cyclopia. The prechordal plate, thus, was postulated to supply an influence essential for induction of ventral forebrain structures and for bilateral subdivision of an otherwise continuous eye field within the overlying prosencephalic plate. The importance of prechordal mesendoderm notwithstanding, later disturbances of the prosencephalic neural plate can also cause HPE. In the chick, for example, late exposure to high concentrations of the bone morphogenetic proteins BMP4 and BMP5 produces cyclopia and HPE by inducing abnormal cell death in the ventral forebrain [9••].

Etiology of HPE

HPE is etiologically extremely heterogeneous, with both environmental and genetic bases. Its formation may depend on an interaction of both genetic and environmental factors in at least some cases. Specific teratogens such as maternal diabetes have been shown to increase the risk for HPE 200-fold. Numerous other teratogens are known to cause HPE in various animal models (see above).

Figure 1

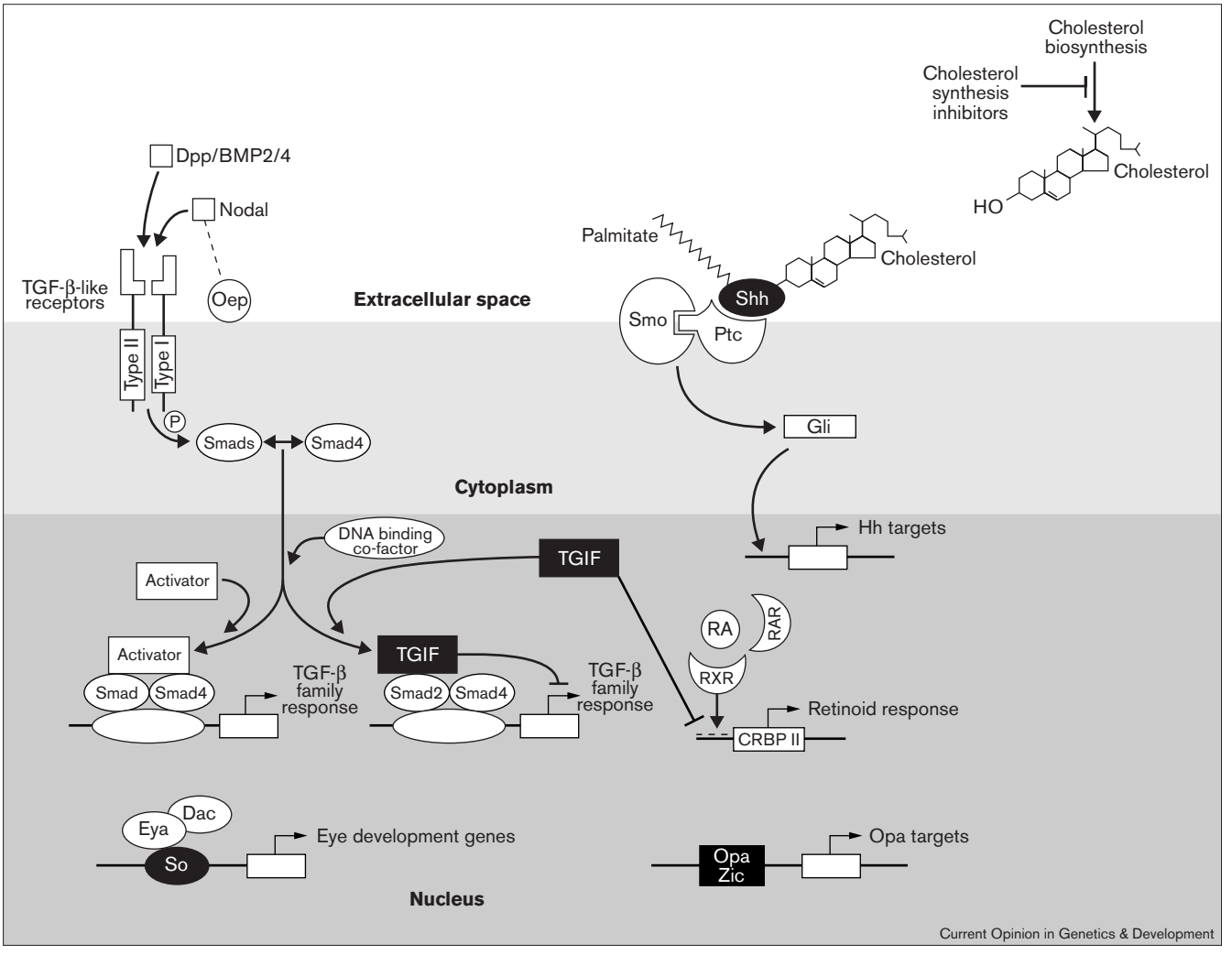


Prechordal plate mesoderm underlies the embryonic ventral forebrain. **(a)** Prechordal plate mesoderm (dark shading) is located beneath the midline of the neural plate at the level of the forebrain, analogous to the location of the notochord (light shading) at more caudal levels of the neural plate. The inset shows a cross section through the neural plate at the level of the forebrain. **(b)** Schematic diagram showing the relationship of the prechordal plate to the structures of the brain. Abbreviations: Dl, diencephalon; hypo, hypothalamus; Mes, mesencephalon; Met, metencephalon; Myel, myelencephalon; Tel, telencephalon (modified from [7]).

Evidence for genetic causes of HPE as well as for its heterogeneity comes from, first, familial occurrence of HPE, second, known genetic syndromes or associations with HPE, and third, non-random chromosome anomalies in individuals with HPE (reviewed in [10]). Although the majority of HPE cases are sporadic, familial HPE has been described in pedigrees suggesting autosomal dominant, autosomal recessive, and possibly X-linked inheritance. The clinical variability can be quite striking even within a single pedigree [5,11]. Reported pedigrees with clinically unaffected parents and multiple affected siblings suggest autosomal recessive inheritance but because abnormal HPE genes are not fully penetrant and there is also the possibility of germline mosaicism, some of these cases may actually be autosomal dominant [12••]. This could be demonstrated for all known HPE-associated genes: *Sonic Hedgehog* (*SHH*), *ZIC2*, *SIX3*, and *TGIF* (see below). Thus, the true inheritance pattern of these pedigrees will be determined by molecular analysis once the genes responsible for HPE in these kindreds are identified.

HPE can also be seen in several defined multiple malformation syndromes with different modes of inheritance. It is estimated that ~18–25% of HPE cases have a recognizable monogenic syndrome. There are at least 25 different conditions in which HPE has been described as an occasional

Figure 2



Association of holoprosencephaly with abnormal function of signaling pathways, cholesterol biosynthesis, and of odd-paired/ZIC2 and So/SIX3 transcription factors. Some aspects of the interactions are speculative because not all of the links have been explicitly demonstrated experimentally and many of the links are only suggestive. In addition, results have been synthesized from several species, and each part of the pathway may not have been shown for all species. To date, the following genes (highlighted) have been implicated in HPE in humans: *SHH*, *ZIC2*, *SIX3*, and *TGIF* (modified from [31]).

finding, although the majority of these disorders are rare. The molecular defect has been identified in three of these disorders: Smith–Lemli–Opitz syndrome (SLOS), Pallister–Hall syndrome (PHS), and Rubenstein–Taybi syndrome (RTS). It is of interest that the underlying bases in these three disorders can plausibly be connected to the Hedgehog signaling pathway. SLOS is caused by a biochemical block of the last step in cholesterol biosynthesis. The formation of cholesterol from 7-dehydrocholesterol is blocked because 7-dehydrocholesterol reductase is altered; this results in decreased cholesterol and a 1000-fold increase in 7-dehydrocholesterol [13]. This block is similar to the one caused by AY9944 that has been shown to cause HPE by blocking Shh signaling in the animal model [14,15]. PHS, a multiple congenital anomaly with autosomal dominant transmission, is caused by mutations in the *GLI3* gene [16]. These mutations predict premature truncation of the zinc-finger-containing portion of the GLI3 protein and suggest a suppression of the SHH pathway. RTS is a multiple congenital anomaly with mutations in the gene encoding the CREB-binding protein (CBP) [17]. In *Drosophila*, CBP appears to be necessary for *cubitus interruptus* activation. Furthermore, on the basis of RTS defects in humans, CBP appears to be a crucial cofactor for GLI proteins. Thus, the defects in PHS and RTS can potentially be explained by a reduction in activity of the SHH signaling pathway.

Chromosomal anomalies in live births with HPE range from 24% to 45%. Most commonly, these involve chromosomes 13 and 18. Regions on these and other chromosomes have been shown to be non-randomly associated with this disease. On the basis of these non-random cytogenetic

Table 1

Summary of known and potential molecular mechanisms of holoprosencephaly.*

Genetic factors		Functions
Drosophila	**Vertebrate**	
Hedgehog	*Shh*	Secreted signaling factor involved in embryonic patterning; mouse mutants have HPE phenotype; SHH mutations cause HPE
Patched	*Patched*	Transmembrane protein; Shh receptor; *PTC* mutations in human HPE
Smoothened	*Smoothened*	Transmembrane protein that interacts with patched
	Hip	Transmembrane protein interacts with all vertebrate hedgehogs; may negatively regulate vertebrate Hedgehog signaling
Cubitus interruptus	*Gli* family	Zinc finger transcription factor, mediates Hedgehog signaling; ectopic Gli-1 activates HNF-3β, Ptc, and Shh; Gli-2 mouse mutants have HPE microsigns (e.g. single central incisor); GLI3 is associated with human disease
–	*HNF-3β*	Transcription factor; required for the development of axial structures; regulates Shh with Goosecoid; expressed in the head process and floor plate of the neural tube; contains Gli-binding site in its enhancer
Dpp	*BMP2/4*	TGF-β family secreted protein; target of Hedgehog signaling; Dpp regulates So and Eya (*Eyes absent*) expression; Dpp negatively regulates *Odd Paired* expression; BMP4 antagonizes opl expression; ectopic BMP4 or 5 in chick results in HPE phenotype
	nodal	TGF-β signaling factor; mutations cause cyclopia in animal models
	oep	Extracellular membrane associated ligand required for nodal signaling; mutations cause cyclopia in zebrafish
	TGF-β-like receptors	Transduce TGF-β family signals by phosphorylating Smads; activin receptor IB rescues *one-eyed pinhead* phenotype
mad	*Smad*	MediateTGF-β-like signals from cell surface to nucleus; Smad2/nodal heterozygous mouse mutants have cyclopia; rescues *one-eyed pinhead* phenotype
	TGIF	Homeodomain protein that interacts with Smad2; binds CRBPII promotor and competes with RXR for binding sites; TGIF mutations in human HPE
	Cerberus	Secreted factor that specifies anterior CNS properties; binds and represses nodal, BMP, and Wnt
Odd paired	*Zic* family	Zinc finger transcription factor; required for Engrailed and Wingless activity; ZIC2 mutations cause HPE
So/Optix	*Six* family	Homeoprotein important for eye and forebrain development; So interacts with Eya; SIX3 mutations cause HPE
	Dkk-1	Secreted protein required for head formation; Wnt agonist; expressed in Spemann organizer and prechordal plate; antibody inhibition of Dkk-1 results in microcephaly and cyclopia
	Floating head	Homeobox gene *Not*; mutants lack prechordal plate; synergistic with cyclops in midline development
	Masterblind	Required for anterior structures and floatinghead expression
	Bozozok	Homeoprotein required for forebrain specification; may be downstream of Wnt and upstream of TGF-β signaling
Other factors		
Cholesterol		May be required for proper spatial restriction of Shh signaling; inhibitors of cholesterol synthesis in mice result in HPE phenotypes; mutations in cholesterol synthesis genes may result in human HPE; inhibitors of cholesterol synthesis inhibit Shh signaling; megalin mutations in mice lead to HPE phenotype
Retinoic acid		High doses inhibit *Shh* and *Patched* expression in craniofacial primordia; ectopic treatment induces Shh in the limb bud; acts synergistically with Activin RIIB mutation on vertebral patterning; Prenatal exposure can cause CNS abnormalities consistent with HPE

*Modified with permission from [31]. BMP, bone morphogenetic protein; CNS, central nervous system; *Dpp, Decapentaplegic.*

rearrangements involving at least 12 chromosomal regions on 11 chromosomes, it was hypothesized that these regions may contain genes critical for normal brain development, and abnormalities in these genes could result in HPE [18]. As discussed below, several genes located in these HPE minimal critical chromosomal regions were recently identified as causing HPE when mutated in individuals with normal chromosomes.

The *Sonic hedgehog* pathway in brain development and HPE

The Hedgehog (Hh) signaling pathway (Figure 2) is best defined in *Drosophila*, where the *hedgehog* gene was identified and isolated but also is well conserved in many vertebrate species (for recent reviews, see [2,7,14]). The biologically active form of the Hh class of proteins involves cleavage of a signal sequence upon entry of the Hh precursor protein into the secretory pathway, followed by an internal autocatalytic cleavage coupled with the addition of cholesterol to the 19 kD amino-terminal product of that internal cleavage. This amino-terminal product contains all of the known signaling activities [14], and the cholesterol modification appears to determine the pattern of signaling activity within developing tissues by limiting diffusion of the mature signaling protein from its site of synthesis. A second modification of the signaling domain by palmitate has recently been described; the occurrence of this second lipid modification is regulated by autoprocessing and may also influence the signaling activity and tissue distribution of the signaling domain [19].

Vertebrate homologues of the *hedgehog* gene constitute a multigene family, of which *Shh* is the best-studied member. The Shh protein is expressed throughout axial mesendoderm including prechordal plate (Figure 1), the tissue found by Mangold and Edelmann many years ago to function in induction of ventral forebrain structures and in bilateral subdivision of the developing eye field. Indeed, loss of *Shh* function in mouse embryos is associated with a loss of ventral structures and cell fates throughout the neuraxis, including an extreme form of HPE at rostral levels [20]. These deficits include an absence of ventral forebrain structures and an undivided eye field, resulting in cyclopia with an overlying proboscis. The remainder of the forebrain in these embryos develops as a single, undivided vesicle characteristic of severe HPE. The Shh protein thus either constitutes or contributes to the signal from prechordal mesendoderm that is responsible for the induction of ventral forebrain and subdivision of the eye field, and absence of this signal results in cyclopia and HPE in the mouse. The loss of function of *Gli2*, another component of the Shh signaling pathway (Figure 2), also results in significant ventral defects and lack of floorplate differentiation, although the full cyclopic phenotype of $Shh^{-/-}$ is not seen [21]. Interestingly, these mice do show craniofacial abnormalities, such as fused incisors [22], that resemble the mild end of the HPE spectrum in humans.

Abnormalities in several genes involved in the SHH pathway are known to cause a number of different diseases in humans: HPE, various forms of cancer including basal cell nevus carcinoma, and several multiple congenital anomaly syndromes, including Greig syndrome, PHS and others (reviewed in [23]). In HPE, SHH was deleted in all individuals with cytogenetic deletions involving chromosome 7q36 [24]. Most but not all large HPE families were linked to markers on chromosome 7q36 [11]. Mutations in SHH account for 37% families with autosomal dominant transmission of the disease spectrum, on the basis of structural anomalies, whereas the detection rate in sporadic cases is rare (<5%) [12••]. Mutations in SHH are distributed evenly throughout the entire coding region. They consist of base-pair changes that predict stop codons (n = 6), insertions or deletions resulting in a frameshift (n = 2), in-frame deletions (n = 4), and changes predicting missense mutations (n = 17) ([12••,25,26,27•]; L Nanni, M Muenke, unpublished data).

On the basis of known domains in the Hh family of proteins, we can speculate about the effects of the mutations observed in HPE patients. First, the association of chromosomal deletions that remove *SHH* with HPE is consistent with a loss of SHH function. Mutations could affect either the SHH amino signaling domain, leading to alterations in biological activity, or interfere with the processing reaction by affecting the carboxyl region, because an intact precursor molecule has little patterning activity. Of the eight mutations predicted to cause premature termination of the SHH protein, five cause truncation within the amino-terminal domain and three cause truncation within the carboxy-terminal domain. The functional significance of numerous missense mutations throughout the entire coding region of SHH is being analyzed at present.

Given the great intrafamilial clinical variability in kindreds carrying a *SHH* mutation, we speculate that other genes acting in the same or different developmental pathways might act as modifiers for the expression of the HPE spectrum. Interestingly, three HPE patients who were identified as having a *SHH* mutation also had an alteration in a second gene which acts in brain development [12••]. In addition to potential modifier genes, environmental factors may also modulate the clinical expression of HPE. The importance of cholesterol in normal SHH function suggests that cholesterol levels *in utero* may contribute to the variable phenotype of HPE caused by *SHH* mutations [28].

The autosomal dominant form of HPE associated with heterozygous loss-of-function mutations at the human *Shh* locus is less severe than that seen in the homozygous $Shh^{-/-}$ mouse. Such human heterozygotes apparently do not display deficits in ventral derivatives of the more caudal neural tube, nor in the many other tissues affected in homozygous mouse embryos. Midline facial and ventral forebrain structures thus appear to constitute particularly sensitive indicators of genetic deficits in midline signaling

pathways. This heightened sensitivity of ventral forebrain and midline facial development might account for the dosage-sensitive function of a number of other HPE genes whose non-lethal phenotypes and dominant patterns of inheritance facilitate their detection and study in human pedigrees. Human HPE thus constitutes a clinical entity particularly amenable to study by genetic approaches.

Other genes in the SHH-signaling pathway (Figure 2) have been analyzed in individuals with HPE. DNA sequence changes predicting amino acid substitutions with yet unknown functional significance have been identified in the following genes: *Patched* [29], *GLI1* and *GLI2* (E Roessler, Y Du, M Muenke, unpublished data). In contrast, no mutations have been detected in the genes for Hedgehog-interacting protein (L Huo, E Roessler, M Muenke, unpublished data) and Smoothened (Smo; JE Ming, M Muenke, unpublished data).

ZIC2 mutations in HPE

The second HPE-associated gene, *ZIC2*, was identified by positional cloning in the minimal critical region of human chromosome 13q32 [30]. *ZIC2* is a member of a family that includes the *Drosophila* gene *Odd-paired* and the zebrafish gene *Odd-paired like* which both contain zinc finger DNA binding motifs of specificity very closely related to that of the Gli proteins (reviewed in [31]). In *Drosophila*, the *Odd-paired* gene (*Opa*) appears to affect the expression of targets of *Hedgehog* signaling, and the gene expression of Zic2 in dorso-ventrally restricted stripes within mammalian neuroepithelium, including the ventral midline of the neural tube, suggests that it may have a role in mediating the response to Shh signaling. In the mouse, expression of *Zic2* begins during gastrulation and, later in development, is expressed in the dorsal neural tube, eye, and distal limb [32]. As the Zic proteins have similar binding sequences to the Gli proteins, in addition to similar patterns of expression [33], it seems plausible that functional interactions between them in interpreting the patterning effects of Shh exist and that these may lead to a better understanding of the mechanism of HPE. Further studies are necessary to clarify the functional effects of the loss of ZIC2 activity as correlated with HPE.

Heterozygous *ZIC2* mutations in patients with HPE were identified as falling into two categories: first, small insertions or deletions (1–56 bp) which lead to frameshifts and stop codons and predict ZIC2 protein truncations, and second, insertion of 30 bp which expands the alanine tract from normally 15 to 25 alanine residues. The first group of mutations predicts a loss of function of one of the *ZIC2* alleles, similar to deletions of *ZIC2* in individuals with del(13)(q32). Expansion of the alanine tract has not yet been described for any other HPE-associated genes, although they occur in several homeodomain transcription factors causing at least three developmental disorders (reviewed in [2]).

SIX3 mutations in HPE

The third known HPE-associated gene, *SIX3*, which was identified by a positional candidate gene approach, is on human chromosome 2p21 [34•]. The vertebrate *Six-3* genes have been shown to participate in midline forebrain and eye formation in several organisms ([35]; reviewed in [31]). *Six-3* message is present in the rostral, anterior region of the neural plate, optic recess, developing retina, and midline ventral forebrain. The *Sine oculis* (*So*)/*SIX* family of transcription factors form a distantly related subclass of homeobox-containing genes that are further characterized by the presence of a contiguous homology domain, the SIX domain — which is also thought to participate in transcriptional activation [36]. Sequence comparisons show extensive homology within the homeodomain in vertebrates and confirm that *SIX3* genes are more closely related to the *Drosophila* gene *Optix* than they are to *So* [37].

Heterozygous mutations in *SIX3* in HPE are rare — four found in 300 HPE patients. These four mutations in the *SIX3* homeodomain, cytogenetic deletions of 2p21 involving the deletion of one *SIX3* allele and HPE-associated translocation breakpoints in measurable distance (up to 120–200 kb from the 5′ end of the *SIX3* coding sequence, are compatible with a mechanism of haplo-insufficiency/loss-of-function of *SIX3* as a cause for HPE [34•].

TGIF mutations in HPE

The fourth human gene known to be associated with HPE, TG-interacting factor (TGIF) resides in the HPE minimal critical region on chromosome 18p11.3 [38]. TGIF was first identified as a homeodomain protein capable of binding a retinoid-responsive motif [39]. TGIF and RXR compete for binding to the promoter element by sterically hindering binding to overlapping sequences within the RXR response element [40]. Thus, TGIF is of interest as a repressor of retinoic acid regulated gene transcription. Mutations in *TGIF* could potentially lead to loss of function as a repressor resulting in overactivity for retinoic-acid-regulating genes, simulating the effect of excessive retinoic acid exposure. Prenatal retinoic acid exposure in mice and humans has been shown to cause abnormalities within the HPE spectrum [41,42]. Furthermore, a close interrelation between the Shh and retinoic acid pathways has been demonstrated in the chick embryo [43••]. In addition, teratogens such as retinoic acid are known to modulate the phenotypes of mutations in the TGF-β signaling pathway [44].

TGIF has been shown recently to act *in vivo* as a Smad2 transcriptional co-repressor [45,46••]. Smad2 is a key substrate of receptors for the TGF-β family of growth and differentiation factors. Mutants in the zebrafish genes *Cyclops* [47,48], *Squint* [49] and *One-eyed pinhead* [50] each produce cyclopia-related phenotypes which resemble closely the severe symptoms of human HPE. The TGF-β homologue, *Nodal*, which is required during early gastrulation to form the primitive streak and define the

anterior–posterior neuraxis, and related genes such as *Lefty* are expressed asymmetrically at early embryonic stages and participate in the definition of the left–right axis of the mouse embryo. The phenotype of the *One-eyed pinhead* mutation can be rescued by expression of *Smad2*, indicating that the former gene is part of the Nodal signaling pathway [50]. Mice doubly heterozygous for null alleles in *Nodal* and *Smad2* result in cyclopia in half of the embryos [51].

In humans, heterozygous missense mutations in *TGIF* predict amino acid substitutions in the amino-terminal transcription repression domain, the homeodomain between helix 1 and 2, in the Smad-interacting domain. Results of functional studies provide evidence that the *TGIF* mutations observed in human HPE impair different activities of TGIF and suggest that reduction of TGIF activity can influence the developmental program and lead to HPE by altering Nodal/ TGF-β signaling [38].

Conclusions

A better understanding of the underlying mechanisms of normal and abnormal brain morphogenesis has been derived mainly from the studies of various animal models. Cloning and analysis of genes in *Drosophila*, zebrafish, and the mouse has helped identify HPE-associated genes by a positional candidate approach. Although mutations in *SHH*, *ZIC2*, *SIX3*, and *TGIF* can result in HPE, alterations in these genes account only for a minority of both familial and sporadic instances of disease. Thus, the search for additional genes continues. The focus in future will be on HPE candidate genes with at least one of the following characteristics: first, genes that map to the minimal critical regions of HPE loci [18]; second, genes which, when altered, cause HPE in animal models (Table 1); third, components of signaling pathways such as SHH, retinoic acid, Nodal/TGF-β and others (Figure 2); fourth, ancillary components that may be involved in transcription regulation including transcription factors such as odd paired/ZIC2 or So/SIX3; fifth, components of the pathway of cholesterol biosynthesis and metabolism, and sixth, factors important in establishing dorsal–ventral patterning of the forebrain.

As the genetic factors involved in HPE are further identified, the complex relationships between these genes will be better appreciated. Elucidating how genetic and environmental influences interact to cause HPE will provide an understanding of the basis for the variation in phenotype and possibly even suggest interventions to improve outcome. Continued studies on the basis of HPE will improve our understanding of HPE on clinical, genetic, and molecular levels and provide a powerful tool for elucidating normal forebrain development in humans.

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