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Pathogenesis of holoprosencephaly

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Holoprosencephaly (HPE), the most common human forebrain malformation, occurs in 1 in 250 fetuses and 1 in 16,000 live births. HPE is etiologically heterogeneous, and its pathology is variable. Several mouse models of HPE have been generated, and some of the molecular causes of different forms of HPE and the mechanisms underlying its variable pathology have been revealed by these models. Herein, we summarize the current knowledge on the genetic alterations that cause HPE and discuss some important questions about this disease that remain to be answered.

Development of the forebrain

Holoprosencephaly (HPE) is a developmental disorder, and interpretation of its pathogenesis requires a clear understanding of normal forebrain development (Figure 1). During early embryogenesis, the mouse blastocyst develops into a bilayered conical structure, with the epiblast inside and the visceral endoderm outside (Figure 1A). As development progresses, a group of visceral endodermal cells located at the distal tip of the egg cylinder moves anteriorly to form the anterior visceral endoderm, while the primitive streak forms at the posterior epiblast (Figure 1A). Subsequently, gastrulation occurs and epiblast cells ingress through the primitive streak (1, 2) (Figure 1A). As gastrulation progresses, neural specification results in the formation of the neural plate (3–5). The neural plate initially has an anterior identity (3–5), but signaling molecules from the posterior epiblast and lateral mesoderm subsequently induce posterior characteristics in this structure. Antagonists secreted mainly by the anterior visceral endoderm maintain and stabilize the fate of the anterior neuroectoderm (ANE), from which the forebrain arises (3, 4) (Figure 1, A and D).

Unlike the conical structure of the mouse blastocyst, that of the human blastocyst is a flat, bilayered disc (6) (Figure 1, B and C). Despite these differences in shape, the epiblast cells of both organisms undergo similar cell movements, and gastrulation initiates at the posterior end of both embryos (6) (Figure 1, B and C). The anterior hypoblast of the rabbit embryo, which is also a flat, disc-shaped structure, has head-inducing activity (7); thus, it is possible that the anterior hypoblast of human embryos is the functional equivalent of the mouse anterior visceral endoderm.

Toward the end of gastrulation, the embryo contains the three primary germ layers: the ectoderm, mesoderm, and endoderm. The axial mesoderm consists of the prechordal plate (PrCP) anteriorly and the notochord posteriorly (Figure 1D). The neural plate folds upon itself to form the neural tube, the anterior end of which subsequently expands and bifurcates to form the telencephalon (8, 9) (Figure 1, E and F). By the end of somatogenesis, the forebrain is comprised by the telencephalon, diencephalon, and hypothalamus (3, 4, 10, 11) (Figure 1F). The dorsal telencephalon will develop into the cerebral cortex; the ventral telencephalon will develop into the basal ganglia; and the olfactory bulbs will form the most anterior portion of the cerebrum in mouse and in human it lies underneath the frontal lobe (Figure 1, G–J).

Overview of HPE

HPE is defined as the incomplete separation of the two cerebral hemispheres. Based on the severity of the defect, HPE is subgrouped into three forms (12–16): alobar, semilobar, and lobar HPE. Alobar HPE, the most severe form, is characterized by the presence of a small single cerebral ventricle that lacks interhemispheric division, corpus callosum, and olfactory bulbs (17, 18) (Figure 2, compare A–C and D–F). In semilobar HPE, the moderate form, the frontoparietal lobes fail to separate; however, the interhemispheric fissure is present posteriorly, and the olfactory bulbs and corpus callosum are either absent or hypoplastic (underdeveloped) (17) (Figure 2, G–I). In lobar HPE, the mild form, a distinct interhemispheric fissure is present; however, some midline continuity of the cingulate gyrus persists (Figure 2, J and K).

Eighty percent of HPE cases are associated with facial abnormalities. Cyclopia, proboscis (a tubular appendage located above the eye), and cleft lip/palate are associated with severe forms of HPE (19, 20) (Figure 2, M and N). Ocular hypotelorism (an abnormal decrease in the distance between both eyes), nasal abnormalities, and a single central maxillary incisor are associated with minor forms of HPE (19–21) (Figure 2, O–Q). Milder craniofacial features that occur in the absence of forebrain defects are called microforms (12–16, 19, 20) (Figure 2, P and Q). A middle interhemispheric variant of HPE (MIH), also known as syntelencephaly, is also observed in humans. In MIH, the defects in cerebral hemisphere separation occur only at the posterior frontal and parietal regions; the anterior frontal and occipital lobes separate normally (22–24) (Figure 2, L and R).

Etiology of HPE. The etiology of HPE includes genetic and environmental causes. The environmental risk factors include maternal diabetes, maternal alcoholism, and prenatal exposure to drugs (e.g., retinoic acid, cholesterol biosynthesis inhibitors) (25). HPE is inherited as an autosomal-dominant disease. Mutations in the following nine genes have been identified in patients with HPE: sonic hedgehog (SHH), patched homolog 1 (PCH1), glioma-associated oncogene family zinc finger 2 (GLI2), twist (TWIST), transcriptional regulator 1 (TFR1), teratocarcinoma-derived growth factor 1 (TDGF1), teratocarcinoma-derived growth factor 2 (TDGF2), teratocarcinoma-derived growth factor 3 (TDGF3), forkhead box H1 (FOXH1), and forkhead box H2 (FOXH2).
dispatched homolog 1 (DISP1) (13–16, 26–28). Only about 28% of HPE cases are caused by mutations of these genes; thus, other genetic and/or environmental factors probably contribute to this malformation (15). Mild HPE is observed in a few (2%–4%) patients with Smith-Lemli-Opitz syndrome, which is caused by defects in the 7-dehydrocholesterol reductase (DHCR7) gene (14, 27).

Pathology of HPE. HPE phenotypes vary greatly, even across familial forms. Among consanguineous obligate mutation carriers, about 37% have HPE, 27% display microforms, and 36% have no clinical manifestation (16). The cause(s) of this variability remains unknown.

Diagnosis of HPE. Prenatal diagnosis of HPE is primarily based on ultrasound and MRI. Ultrasound can detect severe HPE as early as the first trimester but is less sensitive to milder forms of HPE. Fetal MRI better characterizes HPE malformations during the third trimester (15). Genetic diagnosis is currently not practical because of the etiologic heterogeneity and pathologic variability of HPE (15).

Current mouse models of HPE
Several mouse models of HPE have been generated during the last decade. Most were generated by genetic alterations, though some were obtained through the use of chemicals (29–33). In this Review, we will focus on only those mouse models generated using genetic approaches (Table 1 and Figure 3). Based on phenotypic criteria established in humans, the available mouse models can be separated into those that exhibit alobar HPE– or semilobar HPE–like phenotypes and those that exhibit microforms or MIH.

Mouse models exhibiting an alobar HPE–like phenotype
Nodal signaling pathway. Nodal is a member of the TGF–β superfamily. It signals through a heterodimer of type I serine-threonine kinase receptor, activin A receptor, type 1B (ActR1B) and type 2 receptors ActR2A or ActR2B in the presence of the coreceptor Cripto/Tdgf1 (Figure 4) (34). The binding of Nodal to the receptor complex leads to the phosphorylation of ActR1B by ActR2A/B. In

Figure 1
Development of the mammalian forebrain. (A–C) At early primitive-streak stage, epiblast cells ingress through the primitive streak (PS) to form the mesoderm. Medial sagittal section of E6.5 mouse (A) and Carnegie Stage 7 (CS7) human (B) embryos with anterior to the left. (C) Dorsal sagittal section of a CS7 human embryo. AVE, anterior visceral endoderm; VE, visceral endoderm. (D) At early somite stage (E8.5 for mouse; CS10 for human), the neural ectoderm has been specified into different regions along the anteroposterior axis and the axial mesoderm is underlyin the midline of the neural ectoderm. ANC, anterior notochord; PFB, prospective forebrain (or ANE); PH, prospective hindbrain; PM, prospective midbrain; PNC, posterior notochord; PSC, prospective spinal chord. (E) Neural tube closure occurs at around the 15-somite stage (E9.0 for mouse; CS11 for human). The forebrain gets further regionalized into telencephalon, diencephalon, and prospective hypothalamus (PH). OV, optic vesicle. (F) Approximately at E10.5 in the mouse or at CS14 in human embryos, the expanding telencephalon bifurcates dorsally to form the two hemispheres and gets patterned into dorsal telencephalon (DT) and ventral telencephalon (VT). (G and I) Lateral views of adult mouse (G) and human brain (I). OB, olfactory bulb. Black dashed lines in G and I indicate the location of coronal sections shown in H and J. (H and J) Coronal sections of adult mouse (H) and human brain (J). BG, basal ganglia; CiC, cingulate cortex; CoC, corpus callosum; LV, lateral ventricle.

genes encoding key components of the Nodal signaling pathway, such as Cripto/Tdgf1 and FoxH1, have been identified in patients. The analysis of Nodal-insufficient embryos revealed that although inactivation of the Nodal signaling pathway by genetic deletion of one Nodal allele causes early embryonic lethality due to defects in mesendoderm specification and gastrulation (38–42). However, reduced Nodal signaling, either by introducing a hypomorphic allele into the null background (Nodal<sup>fluc<sup>-/-</sup></sup> or Cripto<sup>fluc<sup>-/-</sup></sup> mice) or by mutating two components of the pathway (Nodal<sup>−/−</sup>/Smad2<sup>-/-</sup> or Nodal<sup>−/−</sup>/ActR2A<sup>-/-</sup>), results in mutant embryos that undergo gastrulation but exhibit the alobar HPE–like phenotype (39, 43–45). Further analysis of Nodal-insufficient embryos revealed that although the mesendoderm is specified and gastrulation takes place, mesendodermal cells fail to migrate anteriorly and form the PrCP (Figure 5A) (39, 43–45). Interestingly, physical ablation of the PrCP in amphibian or chicken embryos also causes cyclopia (46–48). Furthermore, transplantation of a donor PrCP laterally to that of the recipient PrCP activates expression of the ventral midline gene NK2 homeobox 1 (Nkx2.1) and represses the expression of paired box gene 6 (Pax6), a marker for the dorsal telencephalon and eye field in the overlying neuroectoderm (49). These results indicate that the PrCP is required for eye field separation and forebrain patterning.

Nodal also collaborates with growth differentiation factor 1 (Gdf1), a member of the TGF-β superfamily that signals through the Nodal signaling pathway to regulate gastrulation and PrCP formation. Functional inactivation of Gdf1 and genetic deletion of one Nodal allele (Nodal<sup>−/−</sup>/Gdf1<sup>-/-</sup>) results in a defective PrCP and alobar HPE–like phenotype (50).
Table 1
Mouse models of HPE

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* Alobar HPE–like phenotypes include single telencephalic vesicle, loss of MGE and most of LGE, and cyclopia. Mouse models that only exhibit HPE on the C57BL/6 background. Semilobar HPE–like phenotypes include loss of septum, loss or hypoplasia of OB, loss of MGE, and midfacial defects. Microforms of HPE include midfacial defects. MIH-like phenotypes include loss of dorsal midline structures of telencephalon. HPE–like phenotypes include the presence of a single telencephalic vesicle due to abnormal folding of the neural epithelium or to failure in expansion and bifurcation of the cerebral hemispheres. Genes whose mutations have been identified in HPE patients are in bold. ACTB, actin, β; ActR2A, activin A receptor, type 2A; Bmp1α, Bmp receptor, type 1α; Cdc42, cell division cycle 42 homolog; Disp1, dispatched homolog 1; DTA, diphtheria toxin A subunit; f/f, flox/flox; FNP, frontonasal process; Hnf3b, hepatocyte nuclear factor 3β; OB, olfactory bulb; Otx2, orthodenticle homolog 2; Smo, smoothened homolog.

Zic2. The zinc finger protein Zic2 shares sequence homology with Gli proteins and physically interacts with Gli2 (51). During gastrulation, Zic2 is expressed in the mesoderm and head-fold region. At later stages, its expression is restricted to the dorsal neural tube and somites (52). ZIC2 mutations account for 9.2% of all HPE cases (15). A missense mutation has been introduced into the fourth zinc finger domain of Zic2 by ENU, which abolishes Zic2’s DNA binding and transcriptional activation abilities. Mouse embryos homozygous for this mutated Zic2 allele (kumba [ku] allele) exhibit alobar HPE–like phenotype (53–55). Further studies revealed defects in the PrCP and anterior notochord of Zic2+/–/ku embryos (55). Interestingly, injection of an N-terminal truncated form of zic2 that may function as a dominant negative lead to microcephaly (in which the circumference of the head is markedly smaller than average) and cyclopia in Xenopus embryos. Furthermore, depletion of maternal zic2, resulting in the upregulation of Nodal signaling via the activation of Xenopus nodal–related (xnr) genes, leads to abnormal gastrulation and anterior truncation of the embryo (56). In mice, elevated Nodal signaling causes abnormal gastrulation; mesoderm cells arrest in/near the primitive streak and fail to move anteriorly (Figure 5A) (57). These studies raise interesting questions. Does Zic2 regulate gastrulation via modulating Nodal activity in mice (Figure 5A)? Does increased Nodal activity cause HPE via abnormal gastrulation and PrCP defects?

Bone morphogenetic protein signaling pathway. Bone morphogenetic proteins (Bmps) belong to another branch of the TGF-β superfamily. They bind to type 1/2 Bmp receptors (BmpR1 and BmpR2) and signal through Smad1/5/8 and Smad4 complexes (Figure 4B) (58). Chordin (Chd) and noggin (Nog) act as secreted antagonists of Bmp. By directly interacting with Bmp ligands, these proteins are
signaling pathway components have been identified in patients with HPE, inactivation of the formation of a stable Bmp/Chd/Tsg complex that prevents the in this setting (Figure 4B) (59). Although no mutations in Bmp effectors (Figure 4B) (59). Twisted gastrulation (Tsg) is a secreted protein that directly interacts with Bmp and Chd (Figure 4B). The function of Tsg in regulating the Bmp signaling pathway is complex. On one hand, it behaves as a Bmp antagonist by promoting the formation of a stable Bmp/Chd/Tsg complex that prevents the binding of Bmps to their receptors (Figure 4B) (59). On the other, Tsg enhances Chd as a substrate for the metalloprotease tollloid, which cleaves Chd at specific sites. By promoting Chd degradation, Tsg releases Bmp from the inhibition of Chd and allows it to signal through its receptors (59). Therefore, Tsg promotes Bmp signaling in this setting (Figure 4B) (59). Although no mutations in Bmp signaling pathway components have been identified in patients with HPE, inactivation of Chd and Nog (Chd–/– Nog–/– or Tsg (Tsg–/–) causes PrCP defects and alobar HPE–like phenotype in mice (Figure 3, A and B) (60, 61). Chd, Nog, Tsg, and Bmp7 are expressed in the axial mesoderm during gastrulation; thus, repression of Bmp signaling activity is required for the normal development of the PrCP, and Tsg represses Bmp signaling (Figure 5A) (60–62). Interestingly, the alobar HPE–like phenotype reported in Tsg–/– embryos can be observed only on the C57BL/6 background, suggesting that other genetic modifiers contribute to the phenotype (61, 63).

In summary, studies from genetic mouse models confirm the results obtained using ablation and transplantation experiments in several species and reveal that signaling from the PrCP is essential for the separation of the single eye field and the patterning and morphogenesis of the forebrain. Defects in the PrCP promote alobar HPE. This then led to the obvious question: What is the molecular identity of the PrCP signal?

Shh signaling pathway. One signal from the PrCP is Shh, a member of the Hedgehog family of secreted proteins. During early embryogenesis, Sbb is expressed in the axial mesoderm, including the PrCP (64, 65). Shh null mouse embryos null for Disp1 or Smo exhibit a single telencephalic vesicle and cyclopia (66). These features are characteristic of alobar HPE and resemble the effects reported after physical ablation of the PrCP in chicken and amphibian embryos (46–48). These results demonstrate that Shh constitutes or contributes to the PrCP signal involved in the patterning of the forebrain (Figure 5A).

Shh signals through members of the Gli family of transcription factors. Gli1–3 are bifunctional transcriptional factors with repressor and activator domains that flank a central DNA-binding zinc finger region. In the absence of Shh signaling, Gli proteins are cleaved into truncated forms that function exclusively as transcriptional repressors (Figure 4C) (67, 68). Once Shh binds its receptor, patched (Ptc), and releases the serpentine protein smooothed (Smo) from the inhibition of Ptc, activated Smo prevents the cleavage of Gli. Unprocessed, full-length Gli accumulates in nuclei and activates downstream genes (Figure 4C) (67, 68). The secretion and long-range activity of Shh require the function of dispatched (Disp), a family of transmembrane proteins that shares sequence homology with and is structurally similar to Ptc (Figure 4C) (69). Shh null mouse embryos null for Disp1 or Smo exhibit a single telencephalic vesicle and cyclopia (70, 71).

Mutations in SHH, GLI2, DISP1, and PTCH1 have been identified in HPE-affected individuals (14–16). Mutations in SHH alone account for 12.7% of HPE cases (15).

Similar to Nog–/– Chd–/– embryos, Nog–/– Chd–/– embryos exhibit alobar HPE and Shh expression in the PrCP is lost (62). Chd, Nog, Bmp7, and Shh are coexpressed in the PrCP, and ectopic Bmp activity represses Shh expression in mouse cephalic explants; thus, Bmp signaling may repress Shh expression in the PrCP (62). However, in Nog–/– Chd–/– embryos, excessive Bmp signaling also results in a defective PrCP (60); therefore, Nog–/– Chd–/– embryos should be stained with other PrCP markers to conclusively show that Shh expression in the PrCP, rather than the PrCP itself, is defective.

In mice, targeted deletion of the homeobox gene orthodenticle homolog 2 (Otx2) results in loss of the forebrain and midbrain (72), and deletion of the wing-helix hepatocyte nuclear factor 3β (Hnf3b, also known as Foxa2) results in the absence of midline structures (axial mesoderm and floor plate) (73). Alobal HPE–like phenotype and loss of Shh expression in the ventral forebrain were observed in Otx2–/– Hnf3b–/– mouse embryos (74). Although Shh expression in the PrCP of earlier-stage mutant embryos was not reported by

Figure 3
Mouse models of HPE. (A and B) Chd+/– Nog+/– embryo exhibiting alobar HPE–like phenotype: cyclopia (arrow in A) and proboscis (arrowhead in A). (B) Coronal section of Chd+/– Nog+/– embryo highlighting the single cerebral ventricle (arrow). (C and D) Six3+–/– Shh+/– embryos exhibit semilobar HPE–like phenotype: agenesis of philtrum (arrow in C), lack of corpus callosum (arrowhead in D), and a single telencephalic ventricle anteriorly (arrow in D). (D) Coronal section of a Six3+–/– Shh+/– embryo. (E) Image of an adult Cdo+/– mouse exhibiting microforms of HPE: dysgenesis of philtrum (arrow) and single central maxillary incisor (arrowhead). (F) Coronal section of an ShhN+/– embryo exhibiting MIH–like phenotype: lack of dorsal telencephalic midline structures (arrow in F) and relatively normal ventral telencephalic structures. A and B are reprinted with permission from Nature (60); C and D are reprinted with permission from Developmental Cell (65); E is reprinted with permission from Human Molecular Genetics (101).
the authors, based on the severity of the phenotype and the coexpression of Otx2 and Hnf3b in the PrCP, it is likely that both genes coregulate Shh expression in the PrCP (Figure 5A).

In summary, detailed analyses of these mouse models of alobar HPE consistently demonstrate that Shh is the most important signaling molecule from the PrCP involved in the development of the forebrain and the pathogenesis of alobar HPE.

Mouse models exhibiting the semilobar HPE–like phenotype

Six3. During mouse embryogenesis, expression of the homeobox gene Six3 starts as early as E7.0 in the ANE, which eventually gives rise to the telencephalon and eye field (65, 75). Around the 8-somite stage, Six3 expression is restricted to the ventral forebrain and developing eyes. In patients with HPE, 46 mutations in SIX3 have been identified and account for approximately 4% of HPE cases (15, 76).

Functional inactivation of Six3 in mice causes the ectopic anterior expansion of wingless-related MMTV integration site 1 (Wnt1) expression and posteriorization of the mutant head (77, 78). As a consequence, the homozygous mutant heads lack the telencephalon and anterior diencephalon (77, 78). Six3+/– mice appeared typically normal in the outbred background used for these studies (77). However, HPE-like phenotypes were observed in approximately 16% of Six3+/– embryos when backcrossed onto the inbred C57BL/6 background; this percentage increased to almost 85% in the third generation (65). Interestingly, 10% of Six3 heterozygous embryos from a knockin mouse line (Six3+/ki), generated by replacing WT Six3 with the Six3 mutant Six3V250A, which is identified in patients with HPE, exhibited a similar HPE-like phenotype in a mixed genetic background (65). Upon crossing this strain with Shh+/– mice, 75% of the generated Six3+/–/Shh+/– embryos exhibited an HPE-like phenotype, and upon backcrossing into the C57BL/6 background, 100% exhibited HPE (Figure 3, C and D) (65).

Six3V250A has a valine-to-alanine substitution caused by a single nucleotide mutation (T749C) in the third helix of the homeodomain (21, 79). This amino acid substitution greatly reduced the DNA-binding ability of the mutant Six3, such that it behaved as a hypomorph (showing partial loss of gene function) (65, 79). These results demonstrated the hypomorphic nature of the HPE-promoting Six3 mutations and uncovered the cooperation between Six3 and Shh in the pathogenesis of HPE. Detailed phenotypic analysis of the HPE-like mutant embryos revealed the lack of the nasal septum, hypoplasia of the olfactory bulbs, and the presence of an anterior single telencephalic vesicle, resulting from the absence of the septum and the presence of two posteriorly separated cerebral hemispheres (65). All of these features resemble the characteristics typical of semilobar HPE.

Molecular marker analysis revealed the following ventral telencephalic defects: the presence of a single ganglionic eminence with molecular features of the lateral ganglionic eminence (LGE) and the presence of posterior dorsal telencephalic midline structures, such as hippocampus, cortical hem, and choroid plexus (65). Unlike the abnormal Shh expression observed in embryos exhibiting alobar HPE–like phenotype, Shh expression in the PrCP is not affected in embryos exhibiting semilobar HPE–like phenotype (65). However, Shh expression in the midline of the ventral forebrain (prospective hypothalamus) was missing (65). Interestingly, Nkx2.1, a marker for the prospective hypothalamus and a downstream target of Shh signaling from the PrCP, was still detected in these mutant embryos, suggesting that the absence of Shh expression in the ventral forebrain is not caused by a defective ANE (65). Further studies revealed that Six3 is a direct activator of Shh expression in the ventral forebrain and, in turn, Shh maintains Six3 expression in this region (Figure 5A) (65, 79).

Cell adhesion molecule–related/downregulated by oncogenes. Cell adhesion molecule–related/downregulated by oncogenes (Cdo) is a
Mechanistic model of telencephalon development in normal and HPE conditions. (A) Model of normal mammalian telencephalic development. On the left side, the PrCP is represented by a white rectangle. The blue square around it highlights those steps known to be critical in the pathogenesis of alobar HPE. Toward the right side of the diagram, genes known to be important during subsequent steps of forebrain development are indicated. The orange rectangle highlights steps that are important for semilobar HPE, the green rectangle highlights those important for MIH, and the gray rectangle highlights those important for microforms of HPE. Solid lines represent those processes that have been demonstrated and dashed lines represent those processes that have not yet been directly proved. To better understand the regional relationships between some of those critical genes, their normal expression patterns in the telencephalon at E9.0 and E10.5 are illustrated in Figure 5A (81). Therefore, the loss of Shh expression in the ventral forebrain is probably not sufficient to cause HPE (81).

Fgf signaling pathway. The semilobar HPE–like phenotype was reported in embryos with Fgf signaling reduced by the conditional deletion of Fgf8 or Fgf receptors (Fgfrs) in the developing telencephalon (Fgf8fl/fl;Foxg1Cre, Fgf10fl/fl;Foxg1Cre, and Fgf11fl/fl;Foxg1Cre, and Fgf18fl/fl;Foxg1Cre) or by the generation of the Fgf8 hypomorphic allele Fgf8−/−m (82, 83). During telencephalon development, several Fgf-encoding genes (Fgf8, Fgf14, Fgf15, Fgf17, and Fgf18) are expressed in the rostral midline of the telencephalon (commissural plate) and three Fgfr genes (Fgfr1, Fgfr2, and Fgfr3) are expressed in neural progenitor cells (83). In chicken embryos, ectopic Fgf8 activity in the telencephalon frequently induces a sulcus (a depression or fissure in the brain surface) that resembles an ectopic rostral midline (84). Consistent with these results, reduced Fgf signaling in mouse embryos prevents the separation of the anterior cerebral hemispheres (82, 83). In the ventral telencephalon, the defects are similar to those observed in Six3-promoted HPE (i.e., loss of median ganglionic eminence [MGE] and presence of a single ganglionic eminence with LGE identity) (82).

Fgf8 expression is downregulated in the commissural plate of Shh-null embryos (85). This finding supports the hypothesis that Fgf8 functions downstream of Shh during telencephalon patterning. However, the source of Shh activity required for maintaining Fgf8 expression in the commissural plate is unknown. Is the Shh signaling activity from the PrCP or the ventral forebrain? Fgf8 expression in Shh-null embryos is downregulated after the 10-somite stage, shortly after the normal initiation of Shh expression in the ventral forebrain; thus, the ventral forebrain may be the source of Shh that maintains Fgf8 expression in the commissural plate. Further support of this hypothesis is provided by the fact that Fgf8 expression is downregulated after the 10-somite stage in the commissural plate of Six3-haploinsufficient or Cdo-null embryos, in which Shh expression is absent from the ventral forebrain but remains normal in the PrCP (65, 80). Interestingly, Fgf8 expression in the commissural plate is restored in ShhGli3 double-null embryos, in which the MGE and LGE are rescued (86). However, deletion of Gli3 could not rescue the ventral telencephalic defects resulting from reduced Fgf signaling (83). These results suggest that Fgf signaling functions downstream of Shh signaling activity in the regulation of ventral telencephalic patterning (Figure 5A).

Megalin. Megalin is a member of the LDL receptor–related protein family (87). Megalin (also known as Lrp2) is expressed in the yolk sac and the apical side of the neuroepithelium of early embryos, and its functional inactivation causes semilobar HPE–like phenotypes (87, 88). Megalin is an endocytic receptor that binds to the amino-terminal of Shh and internalizes it; thus, Megalin may...
regulate the Shh signaling pathway (89, 90), and inactivation of *Megalin* may cause HPE by affecting Shh signaling. Megalin is also an endocytic receptor for Bmp4 and negatively regulates Bmp4 activity (Figure 5A) (88). Loss of *Megalin* leads to an increase in Bmp4 expression and signaling in the dorsal telencephalon of mutant embryos and the subsequent loss of Shh expression in the ventral telencephalon, which ultimately leads to HPE (88). Ectopic Bmp4 activity in the ventral telencephalon downregulates Shh and causes HPE in chicken embryos (84). Interestingly, reducing Bmp signaling by deleting one copy of *Bmp4* in a *Tgf*-null background in the B6SJ/F1 strain also causes the loss of Shh expression in the ventral forebrain and that of *Fgf8* in the commissural plate (Figure 5A). These alterations ultimately lead to the semilobar HPE–like phenotype (63). The fact that this phenotype is observed only in *Tgf*-/- embryos upon deleting a *Bmp4* allele supports the argument that during telencephalon patterning and morphogenesis *Tgf* functions as an agonist of Bmp signaling (Figure 5A). These alterations ultimately lead to the semilobar HPE–like phenotype (63). These results suggest that excessive or insufficient Bmp signaling causes similar telencephalic defects.

**Mouse models of lobar HPE**

No mouse models of lobar HPE have yet been reported, probably because the relatively mild forebrain defects defining this alteration can be easily overlooked. A more detailed and thorough analysis of the generated mouse models will be required to uncover these phenotypic alterations.

**Mouse models exhibiting microform HPE**

The pathologic variability of HPE includes milder forms of the disease, such as microforms, in which affected individuals exhibit a normal brain but have mild facial midline defects.

*Cdo*. As mentioned above, *Cdo*-null mouse embryos exhibit semilobar HPE in the C57BL/6 background (80). However, in a mixed 129/Sv/C57BL/6 background, *Cdo*-null pups exhibit no malformation of the forebrain but mild facial defects reminiscent of those in patients with microform HPE. These include craniofacial defects, dysgenesis of the pharynx (the vertebrae groove in the upper lip), lack of hypoplasia of the cartilage of the nasal septum, and a lack of primary palate (Figure 3E) (91). During facial development, cranial neural crest cells migrate from the dorsal midline of the posterior diencephalon to form several facial primordia, including the frontonasal process (FNP). The FNP later splits at the midline into 2 medial nasal processes that fuse to form the nasal septum, pharynx, premaxilla, upper incisors, and primary palate (92). *Shh* is expressed in the facial ectoderm of the FNP, and Shh signaling is important for normal development of the facial midline (93, 94). *Cdo* is also highly expressed in the FNP and, as mentioned above, it positively regulates Shh activity. These results suggest that *Cdo* may regulate facial midline development by modulating Shh signaling (80, 91).

**Growth arrest–specific 1**. Microform HPE has been described in mice lacking the activity of the growth arrest–specific 1 (*Gli1*) gene (95). In certain genetic backgrounds, these mice exhibit midfacial hypoplasia, solitary central maxillary incisors, and cleft palate (95). *Gas1* encodes a membrane glycoprotein, and like Cdo, it functions as an agonist of Shh signaling during facial midline development (95, 96). In addition, *Gas1* and Cdo cooperate to promote Shh signaling during neural tube patterning and craniofacial and vertebral development (96). Although *Gas1* and Cdo have similar, cooperative functions during neural and craniofacial development, whether *Gas1*-null or *Gas1/Cdo* double-heterozygous embryos phenocopy *Cdo*-null embryos and exhibit semilobar HPE in a C57BL/6 background remains unknown.

**Gli2**. As previously mentioned, *Gli2* is a component of the Shh signaling pathway, and mutations in *Gli2* have been identified in patients with HPE (14–16, 67, 68). However, functional inactivation of *Gli2* in mice causes only mild defects (e.g., variable loss of pituitary and lack or partial fusion of the maxillary central incisors) (97, 98). *Gli2* inactivation downregulates *Gli1* and *Pch1* from the epithelium of the tooth germ, a result suggesting that reduced Shh signaling contributes to the defects observed in the maxillary central incisors (98).

**Mouse models exhibiting MIH**

Patients with MIH generally lack the craniofacial and ventral telencephalic defects normally associated with HPE (22, 23). *ZIC2* is the only gene whose mutations have been identified in patients with MIH (24). As mentioned above, removal of Zic2 activity causes alobar HPE in mice (55). However, reduced Zic2 results in normal telencephalic patterning and craniofacial development but a defective telencephalon roof plate that affects the separation of the cerebral hemispheres (Figure 5A) (52). These phenotypes are some of the key features of MIH. Ablation of the roof plate or telencephalic deletion of Bmp receptors also causes dorsal telencephalic midline defects and MIH-like phenotypes (99, 100). Similar MIH-like phenotypes have been observed in *ShhN/+* embryos in which expression of noncholesterol-modified Shh is ectopically expanded into the dorsal telencephalon (Figure 3F) (101). This ectopic activity upregulates *Fgf8* expression in the commissural plate and downregulates that of *Bmp* and *Wnt* genes in the roof plate (101). As a consequence, the roof plate fails to invaginate, and dorsal midline structures fail to develop (101). Results from these mouse models highlight the critical role of roof plate–derived Bmp and Wnt signaling activity in the pathogenesis of MIH.

**Clinical implications**

The knowledge we have gained from the animal models of HPE described in this Review has significantly improved our understanding of normal telencephalic development and the pathogenesis and clinical variability of HPE. This understanding should foster better tools for genetic screening for HPE and, therefore, improve the prevention, diagnosis, and treatment of patients with HPE.

**Mechanistic model of telencephalon development in normal and HPE conditions**

To better interpret the pathogenesis and phenotypic variability of HPE, we have now incorporated into the current model of normal telencephalic development, data generated from the analysis of animal models of HPE (Figure 5). The current view indicates that starting at pre-gastrula stages, Nodal activity regulates the formation of the primitive streak (Figure 5A) (2, 38, 57). Upon the initiation of gastrulation, the development and maintenance of the PrCP and anterior notochord requires the activity of Bmp signaling antagonists such as Nog, Chd and Tsg (Figure 5A) (60). Toward the end of gastrulation, the Shh-expressing axial mesoderm is formed along the midline of mouse embryos (10). Then Shh signaling originating in the PrCP acts on the overlying ANE to separate the single eye field, induce optic stalk specification, and activate downstream genes such as *Nkx2.1* and probably *Six3* in the midline of the ventral forebrain (Figure 5A) (66, 102).

Next, the developing forebrain becomes patterned along the ventrodorsal and anteroposterior axes. On the ventral side, *Six3*
and Nkx2.1 are required to activate Shh expression in the ventral forebrain (65, 79, 81) (Figure 5, A and B). In this region and by antagonizing the repressor activity of Gli3, Shh activity maintains Fgf8 expression in the commissural plate (Figure 5, A and B) (85, 86). In turn, Fgf8 activates Nkx2.1 expression in the ventral telencephalon, which in turn, induces Shh expression and specifies the MGE fate (Figure 5A) (65, 81, 82, 103).

In the dorsal telencephalon, Fgf8 activity restricts Wnt8b to the dorsal midline and regulates Bmp4 expression in a dosage-dependent manner (Figure 5, A and C) (82, 104). Bmp4 activity also appears necessary to restrict Fgf8 and Shh expression (63, 85). In addition and as shown in telencephalic explants maintained in culture, Fgf8 appears to regulate the dorsal midline and regulates midline (105). As mentioned above, Zic2 activity is necessary for maintaining an anterior neural identity. Removal of one or two copies of another gene in the same signaling pathway (e.g., Bmp and Wnt), i.e., dorsal midline structures fail to develop but do not affect dorsoventral patterning of the telencephalon (Figure 5A). Alterations occurring during or after the closure of the neural tube (E8.5–10.5 in mice; CS10–14 in humans) disturb rostral (Fgf8) and ventral (Shh) telencephalic patterning centers, thereby impairing ventral specification and rostral midline development and causing semilobar HPE (Figure 5A).

In MIH, the dorsal telencephalic patterning center is defective (Bmp and Wnt), i.e., dorsal midline structures fail to develop but do not affect dorsoventral patterning of the telencephalon (Figure 5A). In microform HPE, Shh signaling is impaired in the facial ectoderm; therefore, facial midline structures develop abnormally but forebrain development is not affected (Figure 5A).

Variable pathology of HPE

The clinical manifestation of HPE is highly variable; the whole spectrum of HPE is observed in family members carrying the same mutation (16). The explanation for this variability is the multi-hit model: HPE is not a monogenic disease; more than two genetic mutations are most likely to promote alobar HPE (Figure 5A). Alterations occurring during or after the closure of the neural tube (E8.5–10.5 in mice; CS10–14 in humans) disturb rostral (Fgf8) and ventral (Shh) telencephalic patterning centers, thereby impairing ventral specification and rostral midline development and causing semilobar HPE (Figure 5A).

Forebrain-specific defects associated with HPE

Except for those individuals with syndromic disease (e.g., Smith-Lemli-Opitz syndrome), most patients with HPE exhibit forebrain malformations only; no defects occur in any other organ. HPE is an autosomal-dominant disease in humans; however, most mouse models of HPE are autosomal recessive. One possible explanation for the forebrain-specific phenotype is that HPE is not monogenic, and another factor(s) contributing to the phenotype specifically affects the expression or function of HPE-associated genes in the forebrain. For example, Shh-null mouse embryos exhibit the alobar HPE–like phenotype and defects in other organs whose development requires Shh signaling (66). However, removal of one copy of Shh causes semilobar HPE only if a copy of Six3 is also deleted (65).

Another explanation for the forebrain-specific phenotype is that HPE-promoting mutations occur in the regulatory regions driving the expression of HPE-associated genes in the forebrain. Although no mouse models of HPE addressing this possibility have been generated, mutations in a conserved regulatory element of SHH have been identified in a patient with semilobar HPE (79). It will be interesting to determine whether deletion of this conserved regulatory element in mice will also result in semilobar HPE.

Finally, the forebrain may be more sensitive to changes in the activity of HPE-associated genes. For instance, Shh signaling is essential for forebrain and limb development, and its positive regulator, Cdo, is expressed along the neural tube and in developing limbs. However, Cdo-null embryos exhibit semilobar HPE without limb defects (80). Within the forebrain, some developmental processes are more sensitive to gene dosage than others. For example, at the head-fold stage, Six3 is expressed in the ANE and is responsible for maintaining anterior neural identity. Removal of one Six3 allele does not affect anteroposterior patterning of the neural tube; however, dorsoventral patterning of the telencephalon is impaired and HPE arises (65). This result argues that dorsoventral patterning of the telencephalon is more sensitive to Six3 dosage than is the specification of the telencephalon.

Screening for HPE-associated mutations

The identified HPE-associated genes have been related to two signaling pathways, Nodal and Shh; however, these mutations account for only about 28% of all HPE cases. As most sequence analyses are focused on the coding regions of candidate genes, mutations in the regulatory regions that may downregulate the expression of the gene are bound to be overlooked. Therefore, the actual percentage of mutations in any identified gene could be higher. However, other HPE-associated genes eventually may be identified. Studies of animal models of HPE have identified some
putative candidates (e.g., genes related to Bmp and Fgf signaling pathways). As discussed above, HPE is not a monogenic disease; therefore, single mutations identified in patients with HPE may not be enough to cause the phenotype. Therefore, identification of new HPE-associated genes is crucial for accurate molecular diagnosis and further understanding of the pathogenesis of HPE.

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