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Holoprosencephaly: signalling interactions between the brain and the face, the environment and the genes, and the phenotypic variability in animal models and humans

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Abstract

Holoprosencephaly (HPE) is the most common developmental defect of the forebrain characterized by inadequate or absent midline division of the forebrain into cerebral hemispheres, with concomitant midline facial defects in the majority of cases. Understanding the pathogenesis of HPE requires knowledge of the relationship between the developing brain and the facial structures during embryogenesis. A number of signalling pathways control and coordinate the development of the brain and face, including Sonic hedgehog (SHH), Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), and Nodal signalling. Mutations in these pathways have been identified in animal models of HPE and human patients. Due to incomplete penetrance and variable expressivity of HPE, patients carrying defined mutations may not manifest the disease at all, or have a spectrum of defects. It is currently unknown what drives manifestation of HPE in genetically at risk individuals, but it has been speculated that other gene mutations and environmental factors may combine as cumulative insults. HPE can be diagnosed *in utero* by a high-resolution prenatal ultrasound or a fetal magnetic resonance imaging, sometimes in combination with molecular testing from chorionic villi or amniotic fluid sampling. Currently, there are no effective preventive methods for HPE. Better understanding of the mechanisms of gene-environment interactions in HPE would provide avenues for such interventions.

Introduction

Holoprosencephaly (HPE; OMIM 236100) is characterized by inadequate or absent midline division of the developing forebrain into cerebral hemispheres (Fig. 1), with concomitant

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midline facial defects in about 80% of the cases.[1] In 1–10% of cases, HPE may be associated with jaw defects.[2–4] HPE is the most common developmental defect of the forebrain with an incidence of 1 in 250 conceptuses and about 1 in every 10,000 at term.[5, 6] The etiology includes both genetic and environmental causes (e.g. maternal diabetes, prenatal exposure to ethyl alcohol, retinoic acid).[7–9] An important feature of HPE is incomplete penetrance and variable expressivity. Patients carrying defined mutations may not manifest the disease at all, or have a spectrum of defects ranging from mild defects referred to as microforms (hypotelorism, midfacial hypoplasia, a single maxillary central incisor) that are generally non-lethal, to severe (cyclopia, proboscis), which are usually lethal.[6, 10] It is currently unknown what drives manifestation of HPE in genetically at risk individuals, but it has been speculated that other gene mutations and environmental factors may combine as cumulative insults.[11] Currently, there are no effective preventive methods for HPE, and children who survive require long-term multidisciplinary care at a significant financial and emotional cost.

Much of the understanding about the embryological and genetic basis of HPE has been gained by studying mouse and other animal models (chicken, zebrafish) of HPE.[12] Mouse models offer an experimental advantage because of a similar embryonic development of the forebrain and face between mice and humans, the ability to engineer disease-causing mutations, and because variables like environment and genetic background can be controlled.[1, 13]

Morphogenetic events during brain and face development are controlled and coordinated by what appears to be only a handful of extracellular signalling networks, such as Sonic hedgehog (SHH), Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Nodal, and retinoid signalling. All these signalling pathways are used in a reiterated fashion not only during brain and face development, but also during embryonic development as a whole. These factors are often produced together in signalling centers from where they specify and pattern surrounding cells and tissues. Numerous regulatory loops and crosstalk between the different signalling pathways buffer against perturbations, making the system somewhat resilient to genetically or environmentally caused fluctuations. Place, time, and duration of the signalling activity are all important. Signal strength is often fine-tuned by accessory molecules (co-receptors, agonist, antagonists). Once signalling falls outside the limits of tolerance, a phenotypic change may become manifest.

Signalling from the ventral midline is critical for normal midface development, a process in which SHH plays a key role.[8, 14, 15] Mutations in *SHH* are the most common genetic cause of HPE in humans[6] and many of the HPE genes encode proteins that either directly or indirectly regulate SHH expression or signalling. Some of the environmental factors, such as retinoic acid[16, 17] or ethyl alcohol[18] also converge on SHH signalling. Nevertheless, *SHH* mutations account for only a minority of overall HPE cases and carrying a mutation alone does not mean that an individual will manifest HPE. Only about 37% of human carriers of *SHH* mutations develop HPE, while others display mild signs or no signs at all[19], highlighting the complexity of HPE pathogenesis and complicating the process of genetic counselling.

PHENOTYPIC VARIABILITY OF HPE

Both brain and facial manifestations of HPE are highly variable. A generally accepted classification of cerebral defects in humans with HPE incudes four main subtypes from the most severe to the least severe based on the degree of separation of the cerebral hemispheres: alobar, semilobar, lobar HPE and middle interhemispheric variant MIHV) HPE.[20, 21] In alobar HPE, there is lack of midline separation of the cerebral hemispheres with a single large ventricle (monoventricle, Fig. 1). In semilobar HPE, interhemispheric fissure is incomplete with a partial separation of the hemispheres posteriorly. In lobar HPE, interhemispheric fissure is mostly present except for the most rostral portion of the frontal lobes (frontal neocortex). Finally, in MIHV HPE, the interhemispheric fissure is formed anteriorly and posteriorly, with fusion of the cerebral hemispheres centrally.[22, 23] In addition, the so called septopreoptic HPE, in which the nonseparation is restricted to the septal and/or preoptic regions is considered by some authors a very mild subtype within the HPE spectrum.[24] Inclusion of other less severe defects, such as agenesis of the corpus callosum or arhinencephaly (absence of the olfactory tracts and bulbs), as mild forms of HPE remains controversial.[21] It should be noted that a precise distinction between these subtypes is not always possible because HPE represents a continuum of hemispheric nonseparation.[21, 24]

Facial defects also show various degrees of severity and involve both upper and lower face (Fig. 2 A–E). Separation of the eyes may range from normal to closely spaced (hypotelorism) to the most severe cyclopia. Failure of proper separation of the frontonasal region results in proboscis (elongated nose-like structure, usually above the cyclopic eye in humans and either above or below the cyclopic eye in mice), reduced distance between the nares, or a single nostril. Other defects include midline cleft lip with or without cleft palate, midface hypoplasia, a single maxillary central incisor, micrognathia or agnathia.[25, 26] Milder defects (hypotelorism, midfacial hypoplasia, a single maxillary central incisor) are also referred to as microforms.[27] In the majority of cases (60–90%) there is a correlation between the severity of midline facial defects and the severity of brain defects.[5, 28] HPE microforms are usually associated with normal hemispheric separation, although mild midline defects, such as agenesis of corpus callosum may be present.

This phenotypic variability can also be reproduced experimentally in animal models. As described above, mice deficient in BMP binding proteins, including *Twsg1*$^{-/-}$ mice[29] and *Chordin*$^{-/-}$;*Noggin*$^{+/-}$ double mutants[30] show a spectrum of craniofacial defects similar to human HPE. While these mutants are not considered traditional HPE mouse models, their phenotype more faithfully reflects a human HPE than other mouse models of classical human mutations (e.g. *SHH* mutations) because of incomplete penetrance and a range of defects. In contrast, HPE is fully penetrant in *Shh* null embryos with all of them exhibiting severe HPE.[31] While homozygosity for *Shh* has profound effects on craniofacial development in mice, intermediate levels of SHH signaling obtained by pharmacologic intervention do result in phenotypic variability in avian embryos.[32, 33]

The mechanisms of the incomplete penetrance and variable expressivity remain poorly understood. The examples mentioned above offer some possible explanations. Most

importantly, there appears to be a gene dosage effect with resultant variable functional thresholds of encoded proteins.[11, 34] For example, in mice, haploinsufficiency for *Shh* does not lead to HPE, while homozygosity for *Shh* mutation results in severe HPE. This scenario illustrates a threshold for SHH signalling below which the phenotype is always severe. In humans, however, haploinsufficiency may lead to HPE, but in the majority of cases it is either not enough to evoke the HPE phenotype or leads to only mild defects[19], presumably due to variable levels of SHH signalling. Additional genetic or environmental factors (e.g. *in utero* exposure to teratogens) can lower or raise the threshold below which the disease manifests itself. Several models for modulating HPE severity have been proposed. First, two or more HPE genes may interact in generating the phenotype, for example *SHH* and *TGIF* or *SHH* and *ZIC2*[35]. *Chordin*$^{-/-}$;*Noggin*$^{+/-}$ double mutants[30] are an example of digenic inheritance in mice. Second, mutations may involve other genes that are not classical HPE genes, but are nonetheless important for normal forebrain development through other signaling pathways that act either concurrently or at different developmental times, for example by altering proliferation and/or apoptosis of critical cell types.[11] The effect of concurrent partial defects in more than one pathway, or at multiple steps in one pathway on variable manifestation of disease has been shown in other diseases in humans.[36] Third, there is a clear effect of a genetic background on the penetrance of HPE phenotype in mice carrying a predisposing mutation with a C57BL/6 background conferring an increased susceptibility[29], suggesting a role for genetic modifiers.[37] Finally, presence of phenotypic variability in the same genetic background and under the same environmental conditions in mice suggests stochastic and/or epigenetic contribution.[38] All of these factors support a "multiple hit" hypothesis[11] in the pathogenesis of HPE.

ETIOLOGY OF HPE

GENETIC CAUSES

Genetic causes include chromosomal aberrations and single gene mutations. Chromosomal abnormalities, such as trisomy 13 (most prevalent), trisomy 18, triploidy are the most common cause of HPE (24–45% of cases)[4, 8, 39, 40] and frequently result in embryonic or perinatal mortality. HPE that is due to gene mutations can be classified into syndromic and nonsyndromic. Syndromic HPE is part of multiple malformation syndromes, for example Smith-Lemli-Opitz syndrome (OMIM 270400) caused by mutation in the gene encoding sterol delta-7-reductase, Pallister–Hall syndrome (OMIM 146510) due to mutation in the GLI3 gene, or Rubinstein–Taybi syndrome (OMIM 180849) caused by mutation in the gene encoding the transcriptional coactivator CREB-binding protein. At least 13 chromosomal loci have been associated with nonsyndromic HPE[4], 9 of them including known HPE genes (Table 1). About 25% of these point mutations or microdeletions involve *SHH* (most common), zinc finger protein of the cerebellum 2 (*ZIC2*), SIX homeobox 3 (*SIX3)*, and *TGIF*.[41] Other genes include glioma-associated oncogene family zinc finger 2 (*GLI2*), patched homolog 1 (*PTCH1*), NODAL, forkhead box H1 (*FOXH1*), teratocarcinoma-derived growth factor 1 (*TDGF1*, also known as *CRIPTO*), dispatched homolog 1 (DISP1).[4] Mutations in these genes account for about 25% of HPE cases, with point mutations being more common in live born children than in fetuses who are more likely to have microdeletions.[1, 4, 9] New candidate HPE loci continue to be identified by high resolution

cytogenetic techniques such as microarray-based comparative genomic hybridization (array CGH) and subtelomeric multiplex ligation-dependent probe amplification (MLPA).[9] HPE has been reported to be about twice as common in females than in males.[40, 42, 43]

ENVIRONMENTAL FACTORS

Animal studies have shown that exposure to teratogens can lead to HPE if it occurs during a specific developmental stage. In mice, E7.5 is the most sensitive window for teratogen-induced HPE[16, 44] (3rd to 4th week post-fertilization in humans).[27] Environmental risk factors have been reviewed in detail elsewhere[7, 45] The most extensively studied risk factor for HPE (as well as other birth defects) is maternal diabetes, particularly with pregestational onset.[46] The incidence of HPE among infants of diabetic mothers is about 1–2%[47], which is very significant on a population scale given the high prevalence of diabetes. The association between *in utero* exposure to ethanol and HPE has been demonstrated in a number of animal studies[44, 48, 49] and some human studies[50, 51], although a frequent confounding variable in humans is cigarette smoking during pregnancy.[43] Similar association has been shown for retinoic acid[16, 52], which continues to be prescribed for the treatment of acne, sun-damaged skin, psoriasis, prevention of nonmelanoma skin cancer, and for cancer chemotherapy. Since cholesterol is important for SHH processing and signaling[53], and marked reduction in cholesterol levels in mice can produce HPE[54], it has been speculated that cholesterol-lowering drugs, such as statins, could put offspring at risk for HPE.[55] However, human data are inconclusive. The link between HPE and other risk factors, including Infections during pregnancy (e.g. cytomegalovirus infection), medications (e.g. antiepileptics, salicylates, antibiotics), the use of assisted reproductive technologies[56], among others, remains tenuous and is based mostly on either case reports or animal studies.

It has been hypothesized that even subteratogenic doses of some of these agents may cause HPE when acting in concert with other environmental or genetic variables.[11] For example mice homozygous for *Tgif* mutation have a normal phenotype, but show greater sensitivity to RA-induced teratogenesis than wild type mice even in a heterozygous state.[57] Likewise, haploinsufficiency for *Shh* or *Gli2* in mice predisposes them to teratogenic effects of ethanol.[49] It is conceivable that individuals carrying mutations that predispose to HPE would show a similar heightened sensitivity to environmental influences. The exact mechanism of teratogenic effects is unknown. Hyperglycemia in diabetes[58], reduced SHH signaling after alcohol[18, 49] or RA exposure[17], increased apoptosis[59], or oxidative stress, which is increased after each of these exposures[60–62], have been proposed as possible mechanisms.

DEVELOPMENT OF FOREBRAIN AND MIDLINE FACIAL STRUCTURES

Understanding the pathogenesis of HPE requires knowledge of the relationship between the developing brain and the developing facial structures. During gastrulation, the three germ layers are established in the head. The neural tube occupies the midline of the embryo and is flanked by paraxial mesoderm, underlain by prechordal mesoderm and endoderm, and covered by surface ectoderm (Fig. 3). These tissues play important roles during development of the craniofacial complex, and aberrant structural or signalling interactions among some of them may contribute to the phenotypic presentation of patients with HPE. The neural

ectoderm is initially continuous with the sheet of surface ectoderm. During gastrulation, the boundary of the neural ectoderm and surface ectoderm is established by the signalling interactions among the FGF, WNT, and BMP pathways (reviewed by Groves *et al*.[63]) and then through a series of morphogenetic movements the neural tube closes and detaches from the surface ectoderm.[64] The progenitors of the facial skeleton and portions of the calvarium arise from the neural crest cells. This is a migratory population of cells that are derived from the cephalic ectoderm that is situated at the border between the presumptive neural and surface ectoderm.[63, 65] Cells located at the border undergo an epithelial-to-mesenchymal transformation and migrate ventro-laterally to form the facial primordia.[66] Eventually, these cells will give rise to the connective tissues of the face.[67]

PRIMARY PATTERNING AND GROWTH OF THE BRAIN

The developing brain is divided into regions along the anterior-posterior axis, exhibits bilateral symmetry and has a dorsal-ventral polarity.[68, 69] Regionalization of the brain into the prosencephalon, mesencephalon, and rhombencephalon represents the earliest partitioning of the brain. Signalling from the mesendoderm establishes the bilateral symmetry of the prosencephalon.[70] The prosencephalon is further divided into telencephalon at its most anterior end and diencephalon caudally.[71] While the mesencephalon and rhombencephalon are also further divided, these regions are less relevant for our understanding of the pathogenesis of HPE. For the correct specification of the telencephalon, the balance between SHH, FGF, and BMP signalling is critical.[72, 73] *Shh* is expressed in the ventral signalling center, BMP/WNT in the dorsal signalling center, and FGF8 in the anlage of the septum (Fig. 4). Signalling by SHH separates the single eye field into a left and right eye field that will form the optic cups.[74]

Expansion of the brain has a direct effect on the morphogenesis of the face. As the brain grows, the position of the facial prominences is affected.[75] Furthermore, the rate of brain growth during early stages of skull development impacts facial morphogenesis. Mice with slower growing brains have faces that appear more advanced and are longer, presumably because growth of the facial anlagen on a smaller platform produces a larger and apparently more advanced face.[76] While these early effects of growth on facial development may have an effect on the facial features of patients with HPE, the specific contributions are unknown. The molecular interactions between the brain and face may be more relevant for understanding the facial phenotype in these individuals.

SIGNALING INTERACTIONS BETWEEN THE BRAIN AND THE FACE

In addition to the physical effect that the growing brain has on facial development, the brain participates in a reciprocal series of signalling interactions along with the surface ectoderm and adjacent neural crest cells.[77] Our research has revealed a signalling relay system that operates in the brain to control *Shh* expression in the telencephalon and adjacent surface cephalic ectoderm.[14, 78] Blocking SHH signalling in the brain alters the dorso-ventral polarity of the forebrain, prevents the onset of *Shh* expression in the basal telencephalon, and inhibits the subsequent induction of *Shh* expression in the Frontonasal Ectodermal Zone (FEZ).[78] In contrast, activating SHH signalling in the brain ventralizes the forebrain, expands *Shh* expression in the basal telencephalon, and alters the pattern of *Shh* expression

in the FEZ.[14] Both of these treatments lead to significant changes in facial morphology. Further, by varying the level of SHH signal activation in the brain, phenotypic variation is produced that is continuous with normal variation.[79] Blocking SHH to varying degrees in the brain produces phenotypes that are consistent with the range of phenotypes that appear in patients with loss-of-function mutations in *Shh*[80–82], and activating SHH in the brain to varying degrees produces phenotypes that span the range observed in patients with gain-of-function mutations in the *Shh* pathway.[83] Moreover, all of these phenotypes are associated with changes in the level and pattern of expression of *Shh* in the FEZ. Thus, SHH signalling in the brain directly affects facial development by controlling the induction and spatial organization of the FEZ, a signalling center that regulates facial development.[15] However, most importantly, these experiments revealed a fundamental non-linear relationship between SHH signalling and phenotypic outcome that may help explain the extreme phenotypic variability that is observed among patients with mutations that cause HPE (Fig. 5). For example, by using a doubling dilution of the SHH inhibitor we observed a very large change in facial shape over small changes in the concentration of available ligand suggesting that for very small changes in pathway activation large variation can be produced.[77, 79]

GROWTH OF THE FACE

The stereotypical changes in the facial phenotype of patients with mild forms of HPE (hypotelorism, midfacial hypoplasia, cleft lip with or without cleft palate) may be a direct result of the signalling interactions among the brain, surface ectoderm, and adjacent neural crest cells. The FEZ is an important signalling center that regulates patterning and growth of the upper jaw in amniotes.[15, 84, 85] The spatial organization of the gene expression patterns in the FEZ is highly associated with facial shape[79, 85], and changes to the shape of the expression domains may directly result in the facial phenotypes observed in patients with HPE. For example, reduced SHH signalling in the brain of patients may lead to midfacial hypoplasia and clefting as a result of alterations to the initial growth patterns of the facial primordia prior to fusion of the primary palate anlagen. The surface ectoderm that covers the midline of the developing upper jaw expresses a series of signalling molecules including: *Shh*, *Fgf8*, *Wnt9b*, *Bmp2*, *Bmp4*, and *Bmp7* among others.[86–88] *Shh* is expressed in the ectoderm that will form the roof of the mouth and forms a boundary with cells expressing *Fgf8* and *Wnt9b* in more dorsal ectoderm.[15, 84] This ectoderm has the ability to promote growth and patterning of the upper jaw[15], in part by regulating expression of *Bmp2*, *-4*, and *-7* in the adjacent mesenchyme that then controls growth of the upper jaw anlagen.[84, 89] Thus, the changes that occur in the brains of patients with HPE may have a direct impact on the developing face through the regulation of the signalling interactions that occur among these adjacent tissues during the earliest periods of their development.

KEY SIGNALING PATHWAYS IMPLICATED IN HPE - EXPERIENCE FROM ANIMAL STUDIES

NODAL SIGNALLING

Studies in zebrafish and mouse have shown that signalling by the Transforming Growth Factor-β (TGF-β) superfamily member Nodal is critical for the initial specification of the prechordal plate.[90, 91] Nodal signals through a receptor complex consisting of type I serine-

threonine kinase receptors (Activin receptor-like kinase (Alk)4/ActrIb/Acvr1b or Alk7) and type II receptors (ActRIIA and ActRIIB) (Fig. 6). Their activation leads to the phosphorylation of Smad2 and Smad3, which following binding to Smad4 allows their translocation to the nucleus, where the complex interacts with transcriptional regulators such as the helix-loop-helix transcription factor FoxH1/FAST1[92] to activate transcription of prechordal-plate specific genes such has Goosecoid. Nodal signalling is regulated by the membrane-bound extracellular co-factors Tdgf1 (Cripto) and Cfc1 (Cryptic), which are members of the epidermal growth factor (EGF)-cysteine rich (CFC) family. Nodal is critical for mesoderm formation at the beginning of gastrulation and mutant mice die prior to the onset of brain development. Mice deficient in ActRIIA, Smad2 or FoxH1 exhibit rather severe forms of HPE due to failure to properly form prechordal mesendoderm.[93–96] Allelic series with Nodal[91] or Cripto hypmorph alleles[97] support the concept of signalling thresholds in HPE and genetic combinations of haploinsufficient mice (Nodal, Alk4, Gdf1) illustrate how several heterozygote mutations within one pathway suffice for the development of HPE.[98] A common feature in all these mutants is that expression of *Shh* is reduced or missing. Mutations in several components of the Nodal pathway or downstream targets (TDGF1, TGIF, FAST1) have been identified in human cases of HPE.[99]

HEDGEHOG SIGNALLING

Hedgehog (HH) signalling is essential for the development of the ventral forebrain.[31] Proteins of the HH family undergo extensive posttranslational modification before they are secreted as active signalling molecules. The insoluble and inactive precursor molecule is autocatalytically cleaved and is subject to two covalent lipid modifications. First, a cholesterol moiety is added at its N-terminus[100] followed by a palmitate moiety at its C-terminus that allows it to associate with lipid rafts and to be transported to the cell surface.[101] Perturbations in either lipid modification have been associated with HPE in humans (mutations in DHCR7[102]) or mice (loss of function in the Hedgehog acyltranferase (Hhat) required for palmitoylation[103]). The now active multimeric HH protein remains lipid anchored at the cell membrane and requires the transmembrane protein Dispatched1 (DISP1) for its release. The HH receptor Patched (PTCH) functions as inhibitor of Smoothened (SMO). Binding of HH to PTCH releases SMO and the signal can be transduced intracellularly. Loss of *Disp1* in mice leads to HPE including cyclopia.[103–105] A similar phenotype is seen in *Smo*-deficient mice[106], whereas *Ptch*-deficient mice show a constitutive HH pathway activation and dorsal expansion of the *Shh* expression domain.[107] Canonical HH signalling proceeds through the glioblastoma gene products (Gli) family of transcription factors (Gli1, Gli2, Gli3), which are orthologues of Drosophila cubitus interruptus (Ci) and can either act as transcriptional activators or repressors. Gli independent signalling pathways (non-canonical signalling) have also been described.[108] Loss of Gli2, which appears to be responsible for the majority of the HH-dependent transcriptional response, is neonatal lethal in the mouse with defects in early brain and spinal cord development[109, 110] and several mutations in *GLI2* have been reported in HPE cases.[111] Deficiency of *Gli3*, which in the absence of HH is proteolytically processed into a transcriptional repressor, thus acting as HH pathway antagonist, leads to a ventralized forebrain and exencephaly.[112] An increase in *Gli3* is observed in mice deficient for both *Tgif1* and its homologue *Tgif2* resulting in HPE with features similar to loss of *Shh*.[113] There

are several HH-binding proteins, such as Cdo, Boc, Gas1, which may function as coreceptors with PTCH1 and enhance HH signaling.[114] Loss of these proteins leads to a reduction of HH signalling that dependent on the genetic background leads to HPE of variable severity.[115–117] Crosses to *Shh*-haploinsufficent mice (*Gas1*$^{-/-}$;*Shh*$^{+/-}$, *Cdo*$^{-/-}$;*Shh*$^{+/-}$) result in more severe phenotypes, establishing a genetic interaction between these molecules, and exemplify that amount and location of the SHH ligand is important for ventral forebrain patterning.[115, 118] A lack of the cholesterol moiety in SHH leads to ectopic SHH signalling, which impairs correct forebrain development as well.[119]

RETINOID SIGNALLING

TG-interacting factor (TGIF) was originally discovered through its ability to bind a retinoic acid response element[120] indicating a link to retinoid signalling. Retinoic acid (RA) is the active derivative of vitamin A, which is exclusively provided through the diet. RA plays a critical role in anterior-posterior patterning of the central nervous system. RA is produced by retinal dehydrogenases that convert vitamin A to the main biologically active metabolite, all-trans RA. All-trans RA mobilizes to the nucleus and binds to nuclear retinoic acid receptors, which act as transcription factors. Levels of RA are under tight regulation. Though Vitamin A is hydrolyzed by ubiquitous alcohol deyhdrogenases, the following irreversible conversion to all-trans RA catalyzed by retinaldehyde dehydrogenases is rate limiting. At the same time, RA-degrading enzymes such as the cytochrome P450 family member CYP26A1 tightly control RA levels. TGIF regulates the expression of genes controlling both RA synthesis and degradation.[121]

FIBROBLAST GROWTH FACTOR SIGNALLING

FGF8 is a member of the FGF family of secreted signaling proteins. FGF receptors are receptor tyrosine kinases that can activate several signalling cascades, most notably the MAP kinase pathway through Ras/Raf/Erk, which can lead to the phosphorylation and activation of key transcription factors.[122] Reduction in *Fgf8* in the mouse results in hypoplasia of the developing forebrain due to reduced cell proliferation and increased cell death, and midline anomalies.[72] FGF8 can induce *Zic2* expression in the dorsal signalling center.[123] Both mutations in *ZIC2*[124] and *FGF8*[125] can cause HPE in humans.

BONE MORPHOGENETIC PROTEIN SIGNALLING

Members of the BMP subgroup of the TGF-β family of secreted signalling proteins are involved in the organization of the medial-lateral axis of the developing telencephalon and are required for formation of a morphologically defined dorsal midline. BMPs signal similar to Nodal through a receptor complex consisting of type I serine-threonine kinase receptors (Alk1, Alk2, Alk3 (also known as BmprIa) or Alk6 (BmprIb)) and type II receptors (BmprII or ActRII)[126]. Transduction of the signal is either via the BMP-specific Smad1, 5 and 8 effector proteins or via non-BMP specific signal transduction pathways such as MAPK/PI3K/Akt.[126] BMP signaling activity *in vivo* is highly regulated at several levels of the pathway; in the intracellular space, where SMAD1 serves as a signaling hub integrating BMP, WNT, and FGF signals[127], at the receptor level, and in the extracellular space, where secreted BMP-binding proteins like Noggin, Chordin, Twisted gastrulation (TWSG1) or

Gremlin control BMP activity.[128, 129] Dosage and timing of BMP signalling are important. Both a reduction in BMP signalling, as seen in *Bmpr1a:Bmpr1b* double mutant mice[130], or increase, as seen in *Chordin/Noggin* mutant mice[131] or *Noggin* mutant mice[132], can affect forebrain and craniofacial development. Chordin and Noggin appear to antagonize the inhibitory effects of BMP signalling on the *Fgf8* and *Shh* expression domains. Mutant mice display phenotypes remarkably analogous to the wide range of malformations seen in human HPE[131, 132]. In contrast, reduction of BMP signalling in *Bmpr1a:Bmpr1b* double mutant mice affects the development of the dorsal midline including the choroid plexus and cortical hem.[130] This phenotype resembles the midline interhemispheric (MIH) subclass of HPE, which is rarer and generally milder than other types of HPE. TWSG1 can act as a BMP agonist[133] or antagonist.[129] Mice deficient in TWSG1 display a range of forebrain defects resembling human HPE[29] reminiscent of a gain of BMP activity in this mutant. In contrast to this a study, Zakin and De Robertis 2004[134] showed that removal of one copy of *Bmp4* in a TWSG1-deficient mouse without overt phenotype on its own resulted in HPE with anophtalmia, reminiscent of a loss of BMP activity, supporting TWSG1's role as a context dependent modulator of BMP signaling. Interestingly, human *TWSG1* maps to the HPE4 locus on Chromosome 18p11.3 within 5 Mbp of TG-interacting factor (*TGIF*)[135]. Whereas mutations in *TGIF* have been described, minimal evidence for alterations in *TWSG1* was found in humans, suggesting that coding sequence alterations of *TWSG1* are not a common direct cause of human HPE.[136]

DIAGNOSIS AND CLINICAL MANAGEMENT

HPE can be diagnosed *in utero* by a high-resolution prenatal ultrasound[137] or a fetal magnetic resonance imaging[24], sometimes in combination with molecular testing from chorionic villi or amniotic fluid sampling if a mutation has been previously identified in a probant.[138] Postnatally, presence of midline facial defects usually prompts neuroimaging for diagnostic confirmation. Recommendations for the molecular evaluation of newly diagnosed HPE patients (and their parents) have been summarized by Pineda-Alvarez *et al*.[139] Molecular testing includes a high-resolution karyotype as first tier approach since cytogenetic abnormalities are the most common cause of HPE, DNA sequencing of most common HPE genes (*SHH, ZIC2, SIX3*) as second tier, followed by other complementary studies, including array CGH, MLPA, and next generation sequencing. In patients who show features of Smith-Lemli-Opitz syndrome[140] (microcephaly, micrognathia, mental retardation, syndactyly of the second and third toes, incomplete development of the male genitalia), biochemical testing includes measurement of 7-dehydro-cholesterol level, which is elevated in this syndrome.

Patients with HPE have a number of medical problems, including seizures, developmental delay, hormonal deficiencies due to hypothalamic and pituitary dysfunction (diabetes insipidus being most common), abnormalities of tone and movement, temperature instability, abnormal sleep pattern, feeding difficulties, chronic lung disease, hydrocephalus and others that require a long-term follow-up and interdisciplinary care.[20, 141] In addition to reconstructive surgeries, patients require other therapeutic interventions that address specific medical problems mentioned above (e.g. antiepileptics, hormonal replacement, physical therapy). Survival depends on the severity of brain and facial malformations, presence of

chromosomal abnormalities, other congenital anomalies, and organ involvement (e.g. respiratory problems). Patients with mild forms of HPE may have a normal life expectancy.

Genetic counseling should be offered with a caveat that the presence of a mutation in one of the HPE-associated genes does not predict the severity of the disease due to remarkable variability in expressivity and overall poor genotype-phenotype correlation, which reflects a multifactorial etiology of the disease.[138, 142] Gene mutations or chromosomal aberrations usually occur *de novo*, although familial HPE has been reported, usually with an autosomal dominant mode of inheritance (in which case the risk may be as high as 50%). For sporadic cases, the recurrence risk after an isolated case is 13–14%.[26]

Conclusion

In conclusion, HPE is a developmental disorder of the forebrain and midline facial structures that epitomizes the complexity of interactions between different developmental pathways and environmental influences in defining the pathology and its severity. Remarkable progress has already been made in identifying genetic basis of a significant proportion of cases and, with advances in molecular techniques, new candidate genes as well as mutations outside of the coding regions are likely to be found. Large epidemiological studies and animal models have facilitated detection of a number of environmental factors. However, our understanding of the mechanisms of gene-environment interactions remains rudimentary. Although the role of the modifier genes is implied, their identification has not been successful so far. Finally, future efforts should focus on designing preventive strategies. While supplementation of women of childbearing age with folic acid has been effective in preventing neural tube defects[143], this has not been the case in HPE. Recent findings from the National Birth Defects Prevention Study suggested that folic acid supplementation during the periconceptional period was somewhat protective, but the association was of borderline statistical significance.[56] Likewise, maternal diet supplementation with methyl donors did not reduce the incidence of midline facial defects in *Twsg1*$^{-/-}$ mice.[144] Unlike genetic aberrations, environmental factors or a biological response they evoke are modifiable as long as the underlying mechanisms can be identified. While avoidance of teratogens is the most effective primary prevention method, it is not always feasible. Further, individual susceptibility may vary due to other intrinsic factors (e.g. silent gene mutations) that act in combination to cause HPE in at risk individuals. Therefore, further research into the mechanisms of gene-environment interactions and development of suitable animal models (chicken, zebrafish, mouse) will be critical for gaining insight into such interactions and providing avenues for future preventive interventions.

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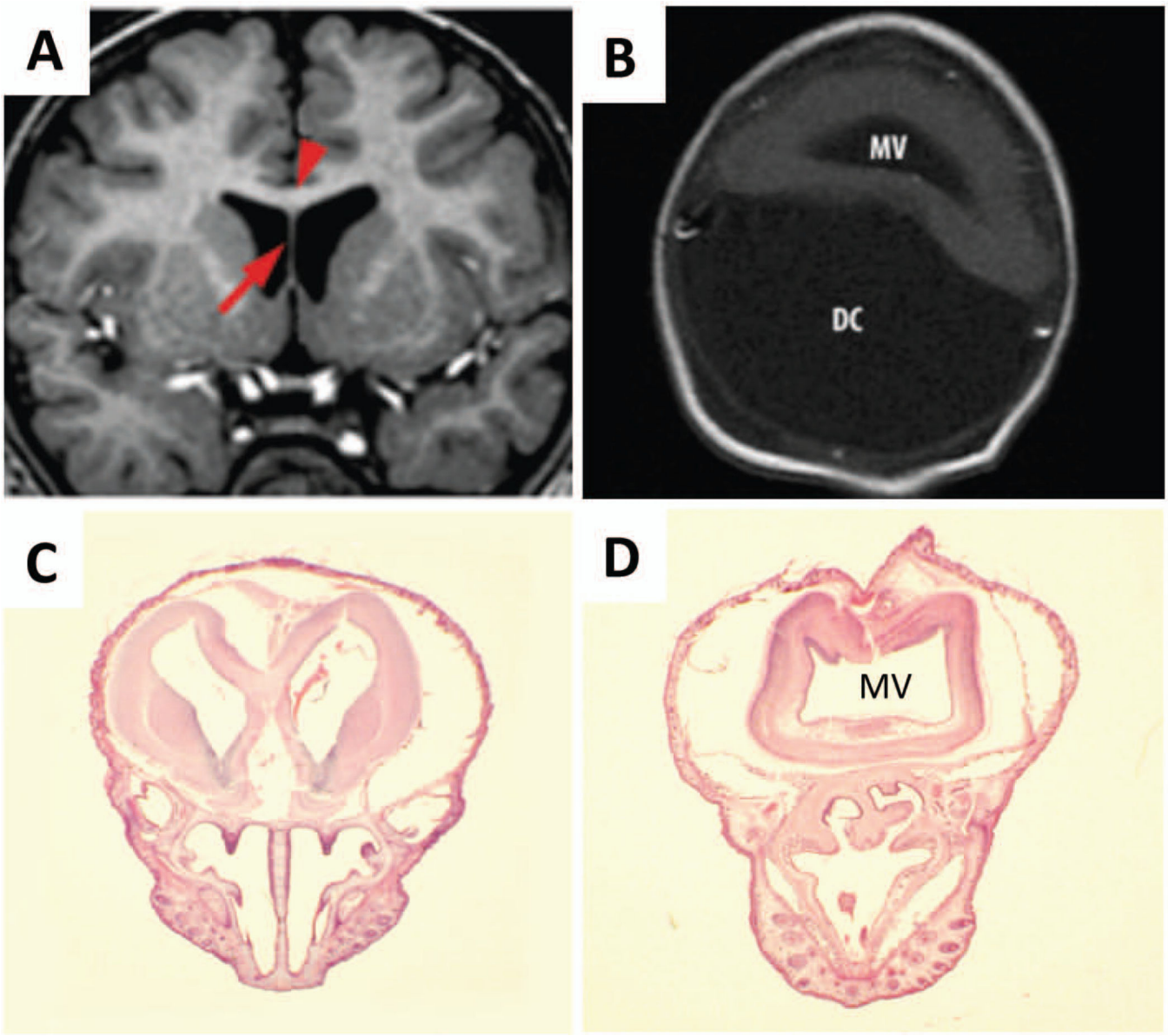

Fig. 1. Holoprosencephaly in humans and mice

(A) Normal human brain MRI. The two cerebral hemispheres are completely separated. The septum (arrow) and the corpus callosum (arrowhead) are present (reprinted with permission from Geng et al. 2009[1], American Society for Clinical Investigation). (B) MRI of a neonate with alobar HPE showing a monoventricle (MV) and a large dorsal cyst (DC) posteriorly (reprinted with permission from Hahn et al. 2010[24], John Wiley and Sons). (C) Transverse section of wild type mouse brain at birth. (D) Nonseparation of the brain and a large monoventricle in *Twsg1*$^{-/-}$ mouse at birth (C and D reprinted with permission from Petryk et al. 2004[29], Elsevier).

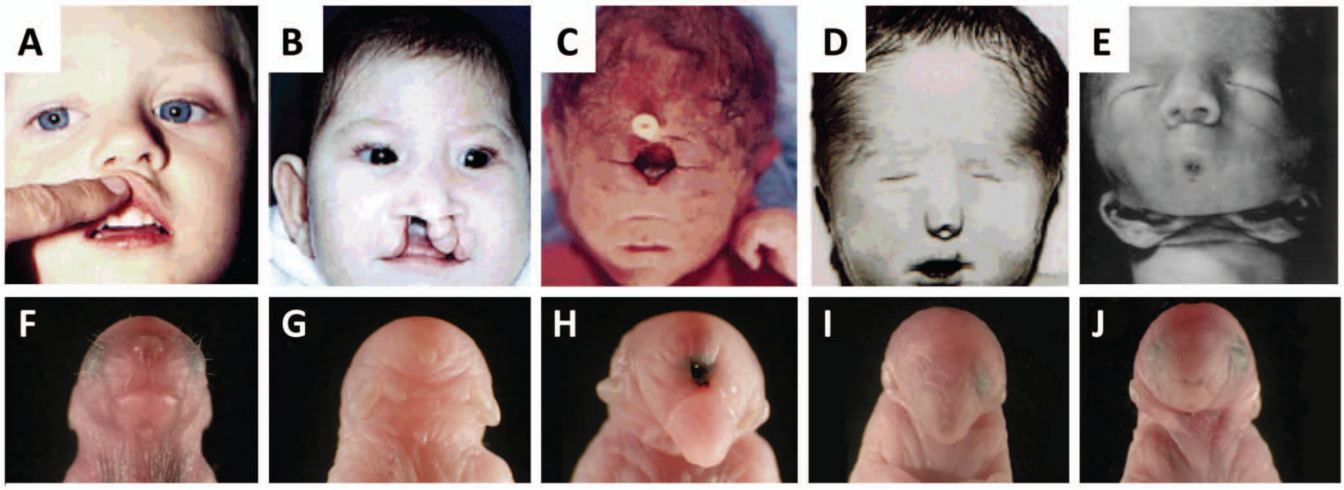

Fig. 2. Spectrum of craniofacial phenotypes in humans (A–E) and *Twsg1*$^{-/-}$ mice (F–J)

(A) Single central incisor; (B) Microcephaly, midface hypoplasia with bilateral cleft lip and palate; (C) Cyclopia with proboscis above the fused eye; (D) Hypotelorism and a single nostril. Images A-D are reprinted with permission from Muenke and Cohen 2000[81], John Wiley and Sons. (E) Agnathia with downward displacement of the ears and microstomia (reprinted with permission from Schiffer et al, 2002[149], John Wiley and Sons); (F) Wild type; (G) severe anterior truncation; (H) cyclopia with proboscis); (I) single nostril with agnathia, (J) agnathia. Images F–J are reprinted with permission from Petryk et al., 2004[29], Elsevier).

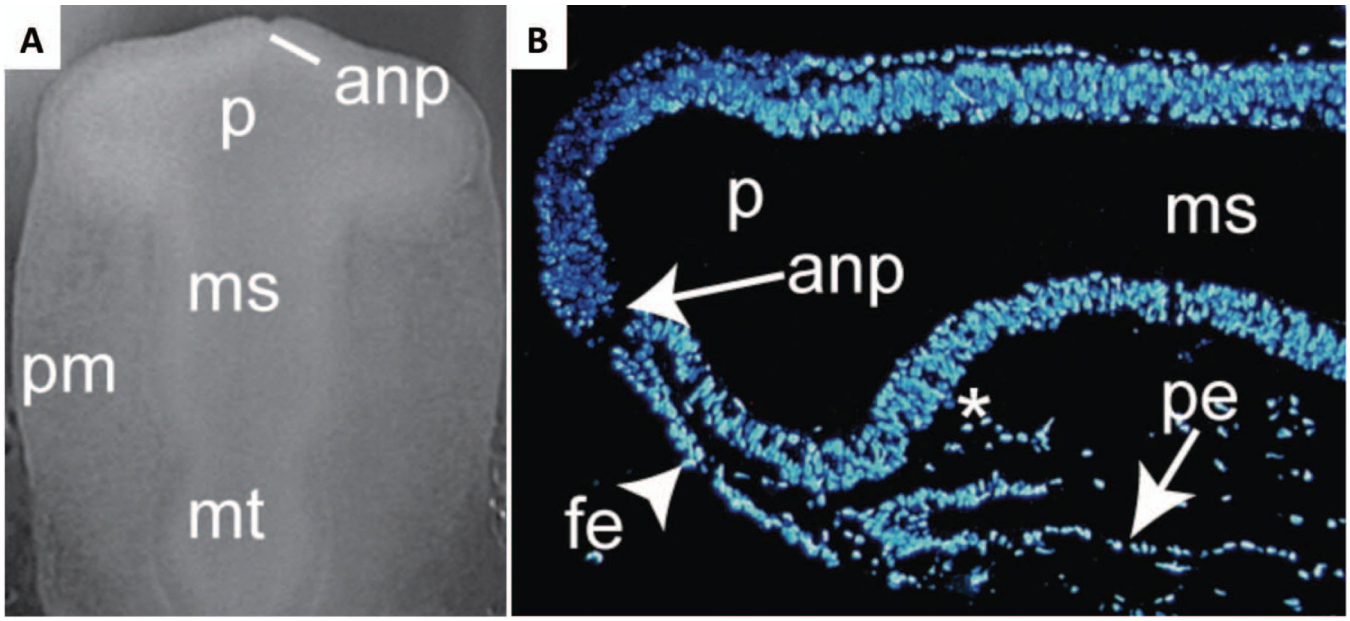

Fig. 3. Spatial relationships during development of the head

(A) A bright field image of a HH10[145] chicken embryo *in ovo*. Dorsal view showing regionalization of the neural tube into the prosencephalon (p), mesencephalon (ms), and metencephalon (mt). The anterior neural pore (anp) is located at the anterior end of the neural tube, and the neural tube is flanked by paraxial mesoderm (pm). (B) A sagittal section of a HH10 embryo that has been stained with bis-benzimide and imaged using an epifluorescent microscope (Leica) showing the prosencephalon (p), mesencephalon (ms), the anterior neural pore (anp), the pharyngeal endoderm (pe), the facial ectoderm (fe), and the prechordal mesoderm (asterisk).

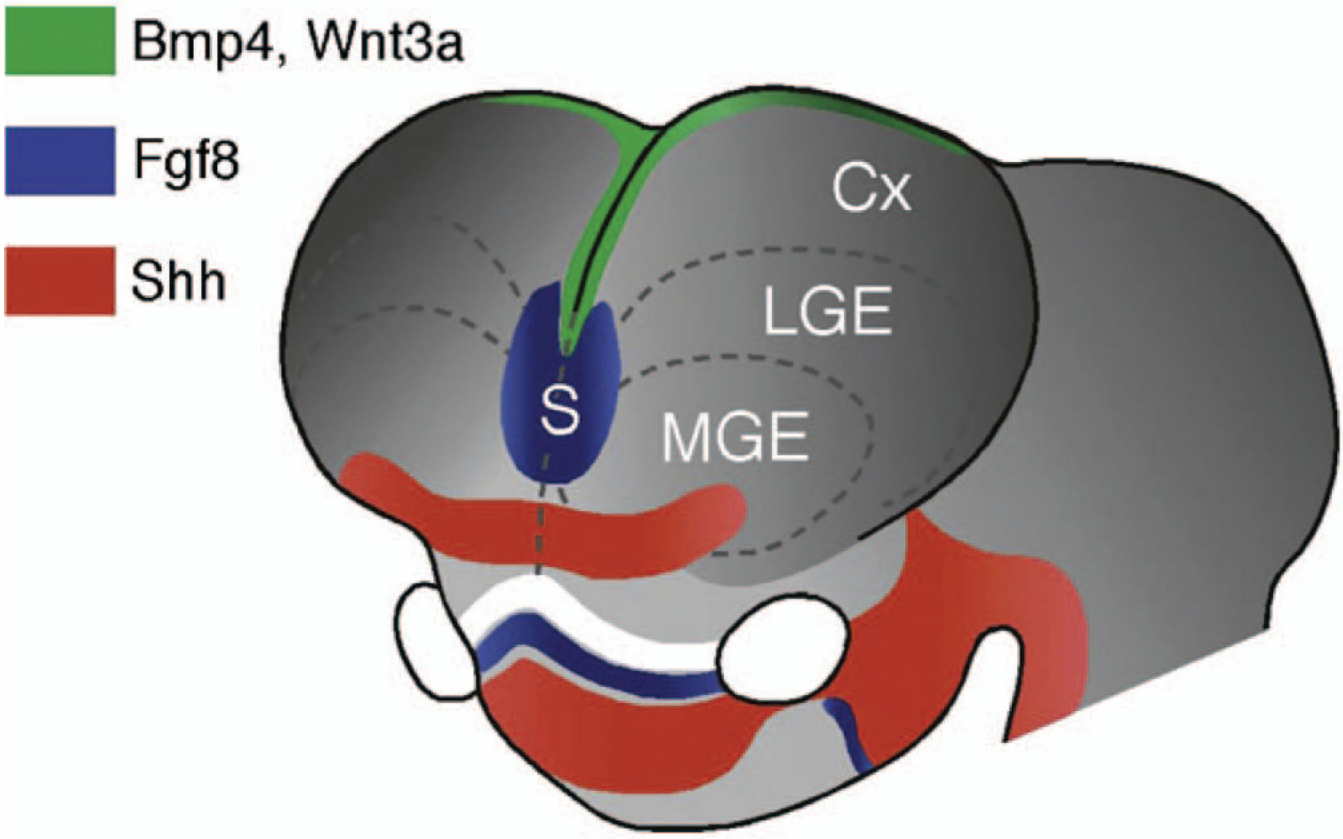

Fig. 4. Signalling centers instructing telencephalon

Frontolateral view of the telencephalon indicating its distinct signalling centers important for regional differentiation in relation to other developing head structures: the ventral center secreting SHH (red), the rostral center secreting FGF8 (blue), and the dorsal center secreting BMPs and Wnt (green). Cross-regulation between the rostral, dorsal, and ventral signalling centres plays an essential role in patterning the early telencephalon. Cx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; S, septum (reprinted with permission from Hoch et al, 2009[146], Elsevier).

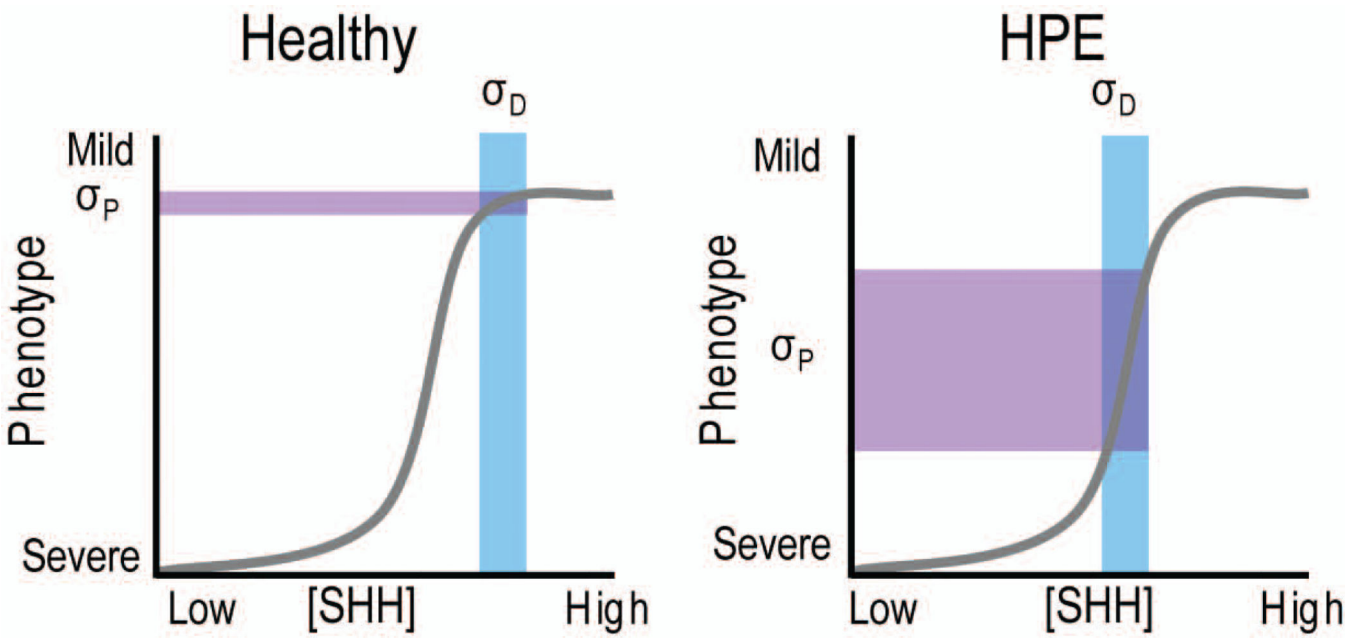

Fig. 5. Non-linearities in signaling may produce phenotypic variation

A model illustrating how non-linear properties of a signalling pathway can produce phenotypic variation. By reducing SHH signalling a large amount of phenotypic variance could be produced due to the non-linear nature of the SHH pathway (adapted with permission from Hallgrimsson et al, 2009[147], Springer).

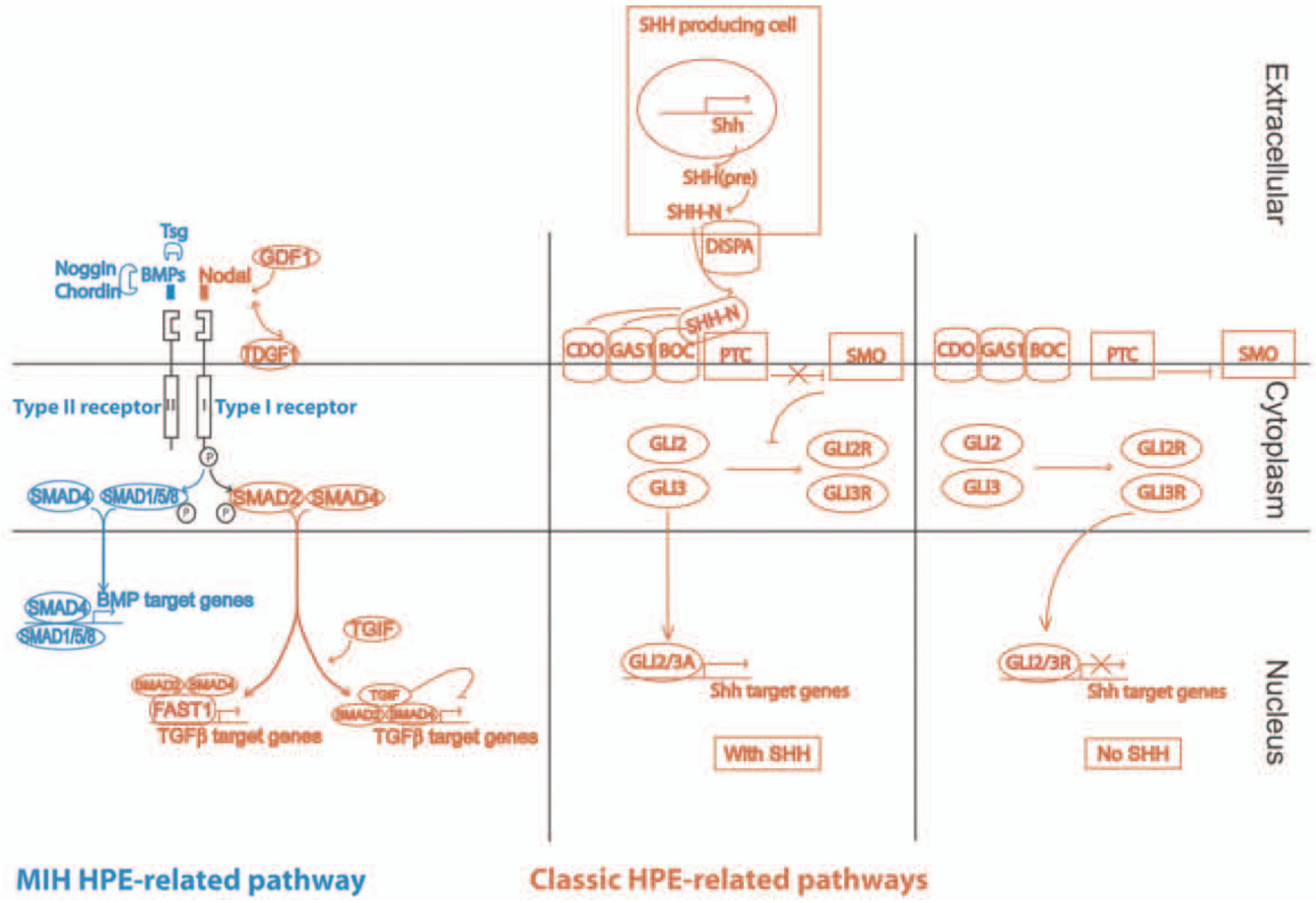

Fig. 6. Schematic representation of core pathways involved in forebrain development and HPE

Classic HPE-related pathways that signal predominantly at the ventral midline are shown in orange. The BMP pathway shown in blue signals predominantly at the dorsal midline and is involved in MIH (reprinted with permission from Fernandes and Hébert, 2008[148], John Wiley and Sons).

Table 1

Genes (loci) contributing to HPE reprinted with permission from Roessler and Muenke 2010[4], John Wiley and Sons).

Human gene	(Human locus)	Chromosome	Molecular function
—	*HPE1*	21q22.3	(unknown)
SIX3	*HPE2*	2p21	Forebrain and eye development
SHH	*HPE3*	7q36	Ventral CNS patterning
TGIF	*HPE4*	18p11.3	Transcriptional repressor including retinoids
ZIC2	*HPE5*	13q32	Axis formation and dorsal brain development
—	*HPE6*	2q37.1-q37.3	(unknown)
PTCH1	*HPE7*	9q22.3	Receptor for hedgehog ligands
—	*HPE8*	14q13	(unknown)
GLI2	*HPE9*	2q14	Transcription factor mediating hedgehog signaling
—	*HPE10*	--	(unknown)
DISP1	—	1q42	Release of hedgehog ligands
NODAL	—	10q	TGFβ-like ligand involved in midline and laterality establishment
FOXH1	—	8q24.3	Transcription factor for NODAL signaling