Noggin null allele mice exhibit a microform of holoprosencephaly

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Holoprosencephaly (HPE) is a heterogeneous craniofacial and neural developmental anomaly characterized in its most severe form by the failure of the forebrain to divide. In humans, HPE is associated with disruption of Sonic hedgehog and Nodal signaling pathways, but the role of other signaling pathways has not yet been determined. In this study, we analyzed mice which, due to the lack of the Bmp antagonist *Noggin*, exhibit elevated Bmp signaling. *Noggin*$^{-/-}$ mice exhibited a solitary median maxillary incisor that developed from a single dental placode, early midfacial narrowing as well as abnormalities in the developing hyoid bone, pituitary gland and vomeronasal organ. In *Noggin*$^{-/-}$ mice, the expression domains of *Shh*, as well as the Shh target genes *Ptch1* and *Gli1*, were reduced in the frontonasal region at key stages of early facial development. Using E10.5 facial cultures, we show that excessive BMP4 results in reduced *Fgf8* and *Ptch1* expression. These data suggest that increased Bmp signaling in *Noggin*$^{-/-}$ mice results in downregulation of the hedgehog pathway at a critical stage when the midline craniofacial structures are developing, which leads to a phenotype consistent with a microform of HPE.

INTRODUCTION

Holoprosencephaly (HPE) is a developmental anomaly that can present as a range of midline craniofacial and neural malformations. HPE is the most common developmental defect of the forebrain and is characterized by the abnormal separation of the telencephalon into left and right hemispheres and the associated abnormal development of midline neural structures. Alobar HPE is the most severe form of this developmental defect, in which the prosencephalon does not separate along both its medial–lateral and anterior–posterior axes which result in a single uncleaved cerebral lobe, and the telencephalon and diencephalon remain unsegmented. There is a spectrum of clinical facial features including the presence of only one upper central incisor tooth with an absent maxillary midline frenum, cleft of the lip and or palate, nasal defects including single

nostril and nasal agenesis, central proboscis, hypotelorism and cyclopia (1,2). When these features appear without defects in the central nervous system, they are termed 'microforms'. HPE occurs in 1 in 7600 live births (3). However, the incidence is much higher, 1 in 240, during early embryogenesis with most embryos undergoing spontaneous abortion (4).

The etiology of HPE is heterogeneous with both genetic and environmental factors. Environmental factors include poorly controlled maternal diabetes, alcohol, vitamin A analogues and alteration in cholesterol metabolism (5). Approximately 25% of HPE cases are syndromic; these syndromes include Pallister–Hall, Rubinstein–Taybi and Smith–Lemli–Opitz. In non-syndromic cases, 12 different chromosomal loci have been implicated in HPE and mutations in 7 genes have been found to cause HPE, and these are all members of the Hedgehog and Nodal signaling pathways. Genes with mutations

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known to cause HPE are *SHH* (HPE3), the Hedgehog receptor *Patched 1* (*PTCH1*) (HPE7), the transcription factors *GLI2*, *SIX3* (HPE2) and *ZIC2* (HPE5), the Hedgehog regulator *GAS1*, as well as *Transforming growth factor interacting factor* (*TGIF*) and *Teratocarcinoma-derived growth factor 1* (*TDGF1/CRIPTO*) (6–8).

Evidence from animal forebrain developmental studies and the characterization of some of the genes causing HPE in humans suggest that distinct mechanisms underlie the two major classes of HPE (9). First, 'classic' HPE in which the ventral telencephalon is most severely affected and where the ventralizing effects of Shh signaling are disrupted. Second, midline interhemispheric HPE or syntelencephaly in which the dorsal telencephalon fails to split while the ventral telencephalon may be normal, and the dorsalizing effect of Bone morphogenetic protein (Bmp) signaling is disrupted. Evidence for the role of Bmp signaling in the pathogenesis of midline interhemispheric HPE comes from the analysis of *Bmpr1a*$^{-/-}$; *Bmpr1b*$^{-/-}$ compound mutant mice which exhibit a loss of all dorsal midline cell types without affecting the specification of cortical and ventral precursors in the telencephalon (10).

Bmps regulate many aspects of craniofacial morphogenesis (11). Variations in Bmp signaling create morphological species-specific diversification of the middle and upper face (12,13) and Bmps together with retinoid acid specify the identity and regulate the growth of the frontonasal mass and maxillary prominences (14,15). Noggin is an extracellular Bmp antagonist which binds, with picomolar affinity, to Bmp2, 4, 6 and 7 (16). This high-binding affinity prevents Bmps from binding to their receptors and subsequent signal transduction. *Noggin* null allele mice exhibit elevated Bmp signaling which results in patterning and growth defects of the neural tube and somites. In the appendicular and axial skeleton cartilage is hyperplastic and there is a failure to initiate joint formation (17–20).

In this study, we show that *Noggin* is expressed in the midline of the developing upper face and that *Noggin*$^{-/-}$ mouse embryos exhibit craniofacial abnormalities which equate to a microform HPE. More specifically, the mutant mice display a solitary median maxillary incisor, midfacial narrowing and abnormalities in the developing hyoid bone, pituitary gland and vomeronasal organ (VO). The Shh signaling pathway regulates midface development and has also been implicated in HPE. We found smaller expression domains of *Shh*, and the Shh target genes *Ptch1* and *Gli1* in the frontonasal region of *Noggin*$^{-/-}$ mice compared with their wild-type (WT) littermates. Using bead implantation assays on E10.5 WT upper facial explants, we show that excessive BMP4 protein results in reduced expression of both *Fgf8* and *Ptch1*.

Taken together, the present data suggest that disrupted Bmp signaling can result in HPE by regulating the Fgf8-Shh pathway and we put forward the *Noggin*$^{-/-}$ mouse as a model of microform HPE.

RESULTS

Noggin$^{-/-}$ mice exhibit a narrowing of the frontonasal process and a solitary median upper incisor

Noggin expression, revealed by LacZ staining, was analyzed in mice at keys stages when the midline of the upper face is
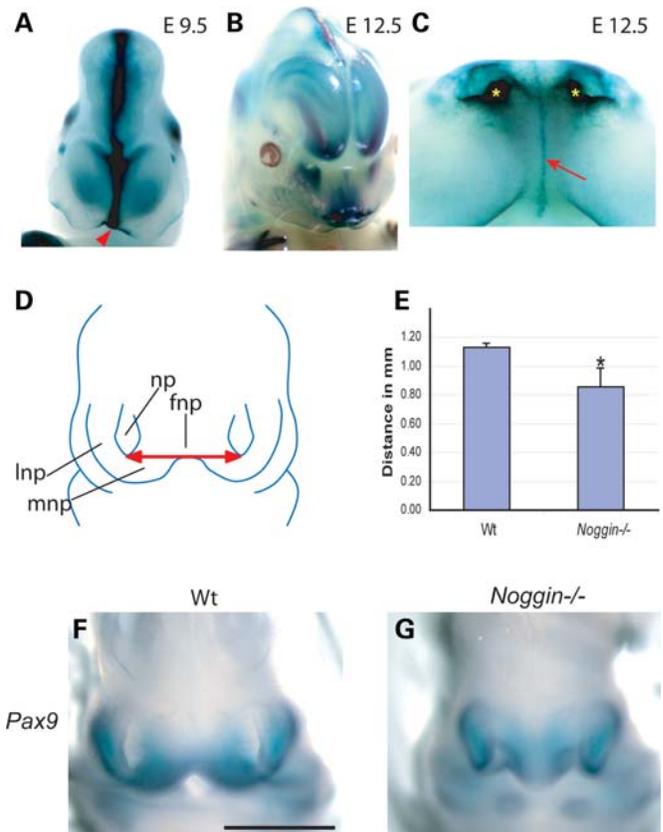

Figure 1. *Noggin* is expressed in the developing face and *Noggin*$^{-/-}$ mice exhibit a narrow frontonasal process. (**A**–**C**) Whole-mount LacZ staining of E9.5 and E12.5 mice. At E9.5, *Noggin* is expressed in the midline of the developing frontonasal process (arrowhead). Later *Noggin* expression is detected in the midline of the frontonasal process as fine line which extends into the oral cavity (arrow) and in the developing nasal pits (asterisks). (**D** and **E**) Schematic diagram illustrates the upper face of an E11.5 mouse embryo. The red arrow indicates the distance between the nasal pits. *Noggin*$^{-/-}$ mice have a shorter distance between the nasal pits. (**F** and **G**) Localization of *Pax9* in the developing upper face of E11.5 WT and *Noggin*$^{-/-}$ mice. Using *Pax9* as a marker of the lateral and medial nasal processes, we were able to record a reduction in the distance between the nasal pits in *Noggin*$^{-/-}$ heads compared with their WT littermates. fnp, frontonasal process; lnp, lateral nasal process; mnp, medial nasal process; np, nasal pit. Scale bar: 1 mm. (F and G, same magnification). Error bars: standard deviation.

patterned and undergoing major morphogenesis. At E9.5 and E12.5, *Noggin* was detected in the midline of the developing frontonasal process and in the developing nasal pits (Fig. 1). E11.5 *Noggin*$^{-/-}$ mouse embryos exhibited a shorter distance between the lateral nasal processes compared with their WT littermates. Furthermore, the morphology of the frontonasal mass was abnormal, with *Noggin* mutant mice lacking the characteristic invagination of the anterior/oral surface (Fig. 1). To quantify these changes, we have carried out measurements on tissue sections from WT and noggin null mice stained for *Pax9* expression, a transcription factor used as a marker of the nasal processes. In the absence of *Noggin*, the distance between the nasal pits was found to be significantly reduced at E11.5 ($P = 0.02$; WT = 1.14 mm, $n = 12$; *Noggin*$^{-/-}$ 0.86 mm, $n = 9$, non-paired t-test) (Fig. 1).

WT mice have two upper incisor teeth, each within separate premaxillary bones. *Noggin*$^{-/-}$ mice had a single upper incisor
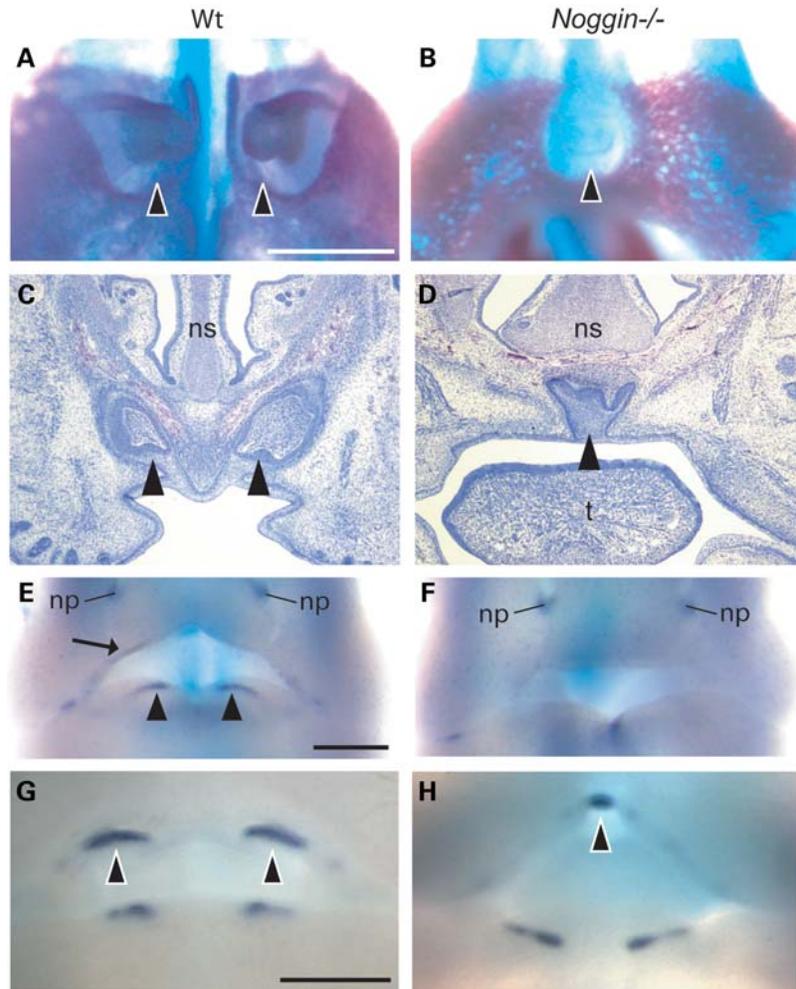
Figure 2. $Noggin^{-/-}$ mice have a solitary median upper incisor tooth. (**A** and **B**) Premaxillary region of new born WT and $Noggin^{-/-}$ mice stained with alcian blue (cartilage) and alizarin red (bone). WT mice have two upper incisor teeth (A, arrowheads), whereas $Noggin^{-/-}$ mice have only one (B, arrowhead). (**C** and **D**) Hematoxylin and eosin stained frontal sections from E16.5 WT and $Noggin^{-/-}$ heads confirming the presence of two upper incisors in WT mice and a single upper incisor in $Noggin^{-/-}$ mice (arrowheads). The developing upper incisor in $Noggin^{-/-}$ mice has an abnormal thick stalk attachment to the oral epithelium and abnormal epithelial convolutions (D). Associated with the dental epithelium is a condensed dental mesenchyme. (**E–H**) *Shh* whole-mount *in situ* hybridization of E11.5 and E12.5 WT and $Noggin^{-/-}$ oral region. (E) In WT mice, at E11.5, *Shh* expression is seen in the two mandibular incisors (arrowheads) and in the continuous maxillary dental lamina (arrow). (F) In $Noggin^{-/-}$ mice, dental development is delayed in that *Shh* mRNA is not detected in the oral region. (G and H) At E12.5, WT mice have two upper incisor primordia (G, arrowheads) and two lower incisor primordia which express *Shh*. $Noggin^{-/-}$ exhibit two lower primordia which express *Shh* but have only one centrally positioned incisor primordium which is smaller than its WT counterparts (F, arrowhead). np, nasal pit; ns, nasal septum; t, tongue. Scale bars: 0.5 mm. Images on the left of the panel are the same magnification as the images on the right.

located in the midline in a single premaxillary bone (Fig. 2). The morphology of the incisor was analyzed in histological sections at E16.5 (Fig. 2C and D). Instead of two separated tooth germs positioned adjacently to the nasal cavity and separated by the inter-premaxillary suture, $Noggin^{-/-}$ mice had a single midline incisor situated below the nasal septum.

In order to establish whether this tooth had developed from a single primordium or from the fusion of two well-defined primordia, we analyzed early tooth development in $Noggin^{-/-}$ mice and their WT littermates. During tooth development, *Shh* is expressed in dental placodes, which are thickenings of the oral epithelium and represent the earliest stages of tooth development. We performed whole-mount *in situ* hybridization for *Shh* mRNA at E11.5 and E12.5 and found that $Noggin^{-/-}$ mice exhibit a single *Shh* expression domain in the midline of the frontonasal process (9/10 mice)

(Fig. 2). This expression domain had not formed from two placodes, as occurs in WT mice. In WT mice, two maxillary incisor primordia were seen at E12.5; these had formed from a continuous dental lamina. Also, we noted that the *Shh* expression domain in the incisor placode was smaller in $Noggin^{-/-}$ mice compared with WT littermates (Fig. 2).

Consistent with previous reports, we found that the cranial neural tube of most $Noggin^{-/-}$ mice (18/22 E17.5 and E18.5 mice) failed to close leaving the mutants with an exencephalic phenotype (19).

Shh signal transduction is decreased in the oral region in $Noggin^{-/-}$ mice

As *Shh* expression domains in the incisor placode were smaller in $Noggin^{-/-}$ mice compared with the WT and because Shh
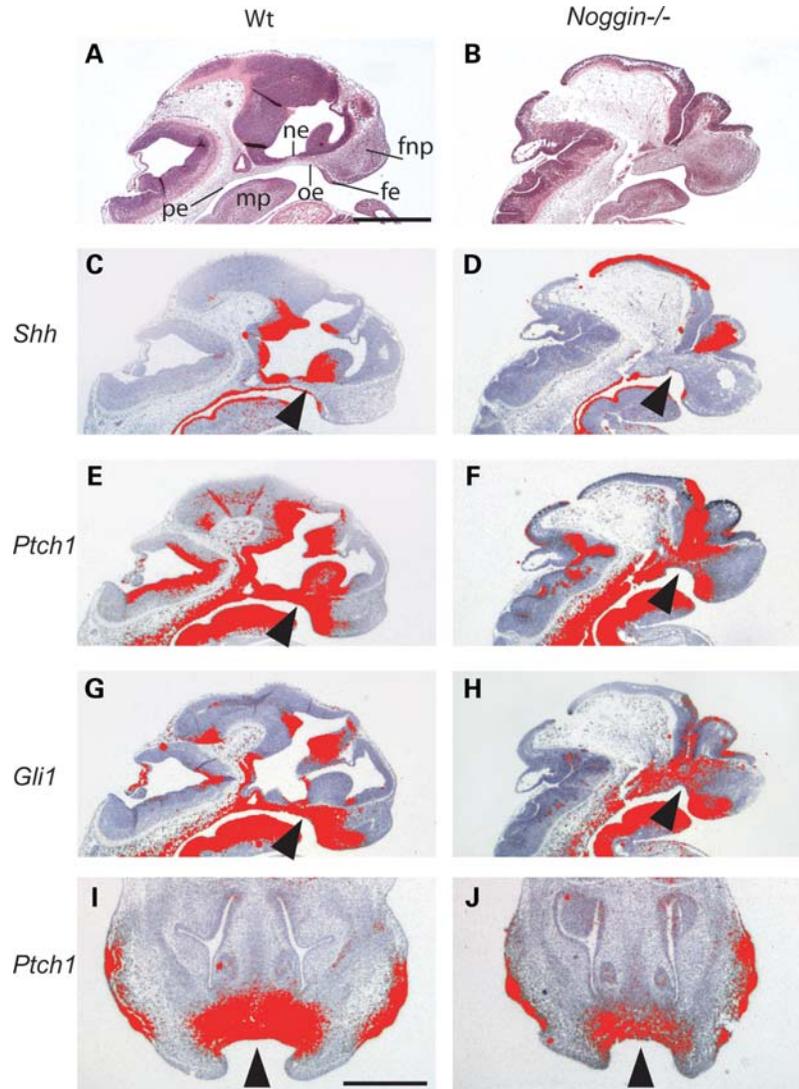
Figure 3. Decreased Shh signal transduction in the oral region of *Noggin*^{−/−} mice. (**A**–**H**) Sagittal sections through the midline region of WT and *Noggin*^{−/−} mice. (A–H) E11.5 (A and B) hematoxylin and eosin stained sections. (C–**J**) [35]S-*in situ* hybridization for Shh signaling pathway members. (C and D) *Shh* is expressed in the oral epithelium (C, arrowhead), pharyngeal endoderm and neuro epithelium of the diencephalon. In *Noggin*^{−/−} mutant mice, the expression of *Shh* is missing from the oral epithelium (D, arrowhead). (E and G) *Ptch1* and *Gli1* are expressed in the oral epithelium and in the facial mesenchyme (arrowheads). They show a gradient of transcriptional activity across these regions away from the epithelium, the source of hedgehog ligand. (E–H) In *Noggin*^{−/−} embryos, there is a reduction in the expression of *Ptch1* and *Gli1* in facial region compared with WT littermates (arrowheads). (I and J) E12.5 frontal sections. *Ptch1* expression domain smaller in *Noggin*^{−/−} mice compared with WT littermates (arrowheads). fe, facial ectoderm; fnp, frontonasal process; mp, mandibular process; ne, neural ectoderm; oe, oral epithelium; pe, pharyngeal endoderm. Scale bars: (A) 200 μm; (I) 500 μm. (A–H, same magnification) (I and J, same magnification).

signaling has been shown to developmentally regulate midfacial width, we compared the expression of several genes involved in the *Shh* pathway in the midline facial regions of WT and *Noggin*^{−/−} mice during early embryogenesis (E11.5 and E12.5) (Fig. 3). In WT mice, *Shh* was expressed in the oral epithelium and facial ectoderm, as well as the pharyngeal endoderm and diencephalic epithelium. In *Noggin*^{−/−} mice, *Shh* was absent from the oral epithelium (Fig. 3D). The transmembrane receptor *Ptch1* and the transcription factor *Gli1* are both targets of Shh. Consistent with previous reports that there is a gradient of Hh signaling activity across the craniofacial region (21), we found that in WT mice the expression of *Ptch1* and *Gli1* was strongest near to the expression domains

of *Shh* and that this mesenchymal expression reduced with increasing distance from the epithelium (Fig. 3C–J). In *Noggin*^{−/−} mice, the expression levels of *Ptch1* and *Gli1* in the mesenchyme adjacent to the oral epithelium were reduced, consistent with the reduction in *Shh* expression (Fig. 3F, H, J).

As Bmp2, -4 and -7 are binding partners of Noggin and because the *Noggin* null phenotype is caused by the deregulation of Bmp signaling (17,22), we analyzed the expression of *Bmp2*, *-4* and *-7* during early facial development. *Noggin* is expressed in the epithelium of the developing frontonasal mass (Fig. 1A–C) (23). At E10.5, *Bmp2* was expressed in the nasal epithelium, then at E12.5 in the pharyngeal
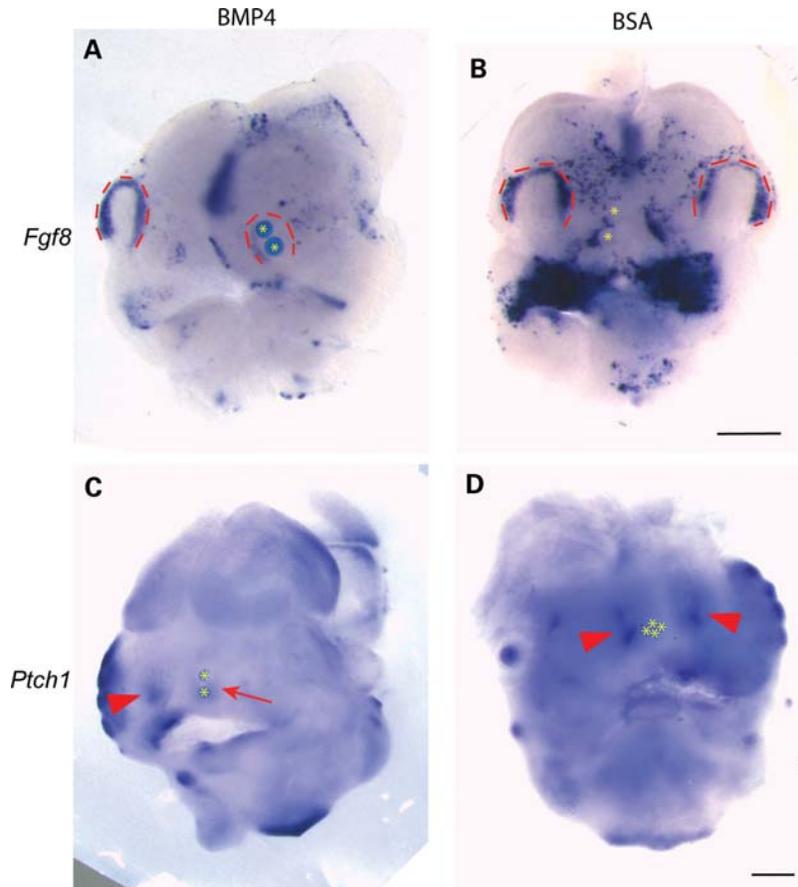
Figure 4. BMP4 downregulates *Fgf8* and *Ptch1* in the developing mouse face. (**A**−**D**) WT mouse heads cultured overnight with beads impregnated with BMP4 or BSA. (A and B) E10.5 whole-mount *in situ* hybridization for *Fgf8*. *Fgf8* is downregulated in the nasal pit in response to BMP4. (C and D) E11.5 whole-mount *in situ* hybridization for *Ptch1*. *Ptch1* is downregulated in mesenchyme underlying the nasal pit in response to BMP4 (arrow), but *Ptch1* expression is maintained in the contralateral side (C, arrowhead) and in response to BSA (negative control, D arrowheads). Nasal pits demarcated with red dotted lines. Asterisks indicate bead locations. Scale bars 500 μm. (A and B, same magnification) (C and D, same magnification).

endoderm and in the mandibular process. *Bmp4* and *Bmp7* were expressed in the facial, oral and mandibular epithelia as well as in the mesenchyme of the frontonasal mass and the first branchial arch (mandible) at both E10.5 and E12.5 (Supplementary Material, Fig. S1).

It has been reported that *Bmp4* expression is more intense and has an expanded domain in the first branchial arch of *Noggin*$^{-/-}$ mice compared with WT littermates (24). Using *in situ* hybridization, we tested whether the expression of *Bmp4* and *Bmp7* were changed in the midline of the developing face in *Noggin*$^{-/-}$ mice. Similar to WT littermates, *Bmp4* and *Bmp7* were expressed in the epithelium and mesenchyme of the frontonasal mass and in the first branchial arch. Levels of intensity were comparable to those found in WT littermates (Supplementary Material, Fig. S1).

BMP4 inhibits *Fgf8* and *Ptch1* in the developing midface

In the developing chick face, it has been shown that increasing levels of Bmp leads to a downregulation of *Fgf8* and *Shh* (23). We postulated that increased Bmp signaling could, via Fgf8, reduce Shh signaling in the developing frontonasal process and thereby result in the *Noggin*$^{-/-}$

phenotype. Using bead assays, we tested the effects of adding exogenous BMP4 to E10.5 mouse facial organ cultures. BMP4 downregulated *Fgf8* in the nasal pit [8/9 explants, compared with 2/9 with bovine serum albumin (BSA)] (Fig. 4). *Fgf8* and *Shh* are expressed in the facial ectoderm; however, Shh signals to its receptor Ptch1 which is located in the facial mesenchyme to regulate facial morphogenesis. As ligand binding to Ptch1 initiates signal transduction and as Ptch1 is also upregulated by the binding of Hh, *Ptch1* expression is regarded as a readout of Hh signaling. We therefore investigated the effects of BMP4 on *Ptch1* expression in the developing mouse frontonasal process and found that BMP4 downregulated Ptch1 expression (9/11 explants, compared with 2/11 with BSA). Thus, in *Noggin* null allele mice, increased Bmp activity may inhibit Fgf 8/ Shh-dependent facial development.

Normal palate formation despite abnormal early shelf morphogenesis

Cleft lip and/or palate are part of the spectrum of features in human HPE. We examined the developing lips and palate of *Noggin*$^{-/-}$ and WT mice from the initial stages of
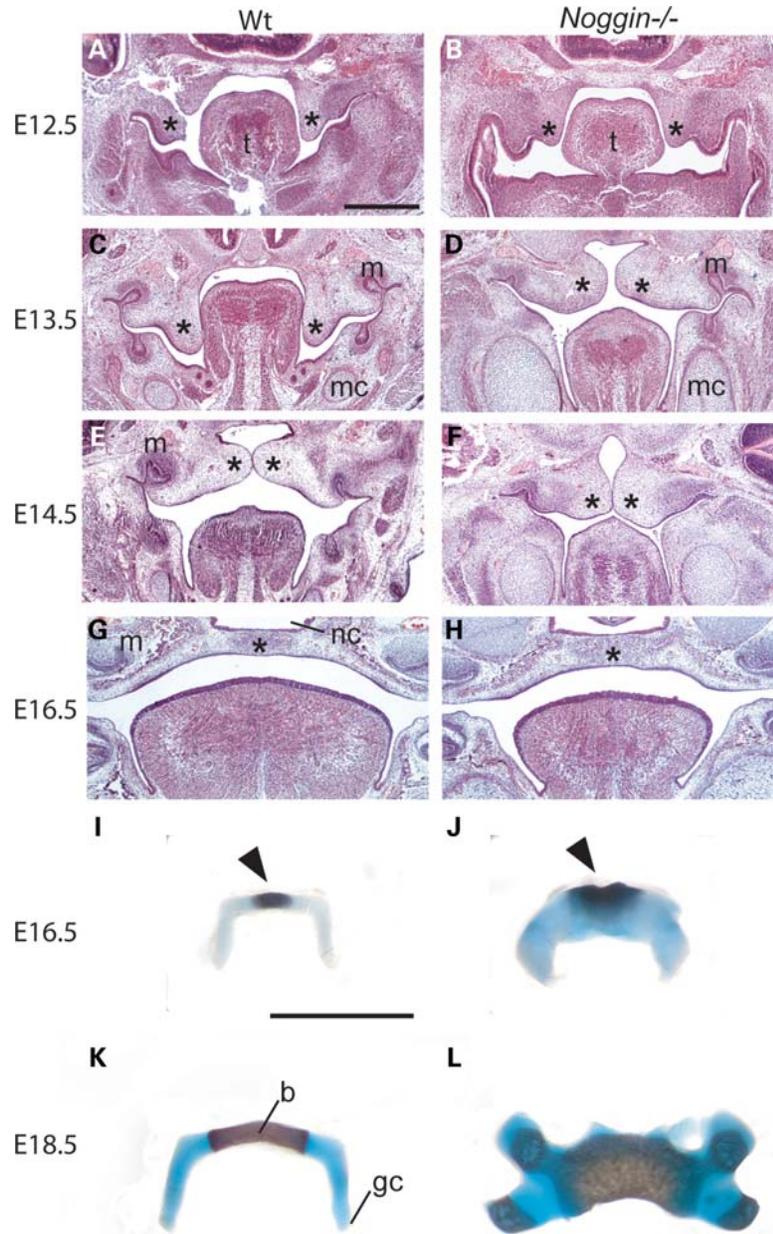
Figure 5. Palate and hyoid bone development in *Noggin*$^{-/-}$ mice. (**A–H**) Hematoxylin and eosin stained frontal sections of WT heads and *Noggin*$^{-/-}$ heads E12.5–E16.5. In WT mice, the palatal shelf primordia bud from the maxillary processes (A), they extend into a position between the tongue and the floor of the mouth, (C) then elevate to a position between the tongue dorsum and the nasal capsule (E) and fuse in the midline. (D) In contrast, the palatal shelves of *Noggin*$^{-/-}$ mice were in a position above the tongue, already at E13.5. (D and F) Also, the shape of the *Noggin*$^{-/-}$ palatal shelves was not 'finger-like' but more bulbous. (H) At E16.5, the palatal shelves of *Noggin*$^{-/-}$ mice appeared normal having fused together. (**I–L**) Alcian blue and alizarin red staining of hyoid bones from WT and *Noggin*$^{-/-}$ mice. (I and J) At E16.5, the *Noggin*$^{-/-}$ hyoid is bigger than its WT counterpart. In both WT and *Noggin*$^{-/-}$ mice, the body of the hyoid has started to mineralize (arrowheads). (K and L) At E18.5, the body of the *Noggin*$^{-/-}$ hyoid bone is larger. It has four instead of two cornua which are thicker, shorter and prematurely mineralized compared with their WT littermates. b, body of the hyoid bone; gc, greater cornua of hyoid bone; m, upper molar tooth; mc, Meckel's cartilage; nc, nasal cavity; np, nasal projection of the maxillary bone; ns, cartilage primordium of the nasal septum; oc, oral cavity; t, tongue; vc, vomeronasal cartilage; *palatal shelves. Scale bars: (A) 500 μm, (I) 1 mm (A–H, same magnification) (I–L, same magnification).

development at E12.5 until after palatal shelf fusion is complete at E16.5 and E18.5. Normally, the palatal shelves develop as buds from maxillary processes and then start to grow downwards between the tongue and the floor of the mouth. The palatal shelves elevate rapidly between E14.0 and E14.5 to a position above the tongue, where after they approximate in the midline and fuse. In *Noggin*$^{-/-}$ mice,

the palatal shelves appeared morphologically normal at E12.5; however at E13.5, they were thick and bulbous in shape and had failed to grow down between the tongue and floor of mouth instead remaining above the dorsum of the tongue (Fig. 5). *Noggin*$^{-/-}$ palatal shelves met in the midline and fused normally so that by E16.5 a normal palate had formed (20/21 mice) (Fig. 5G and H).
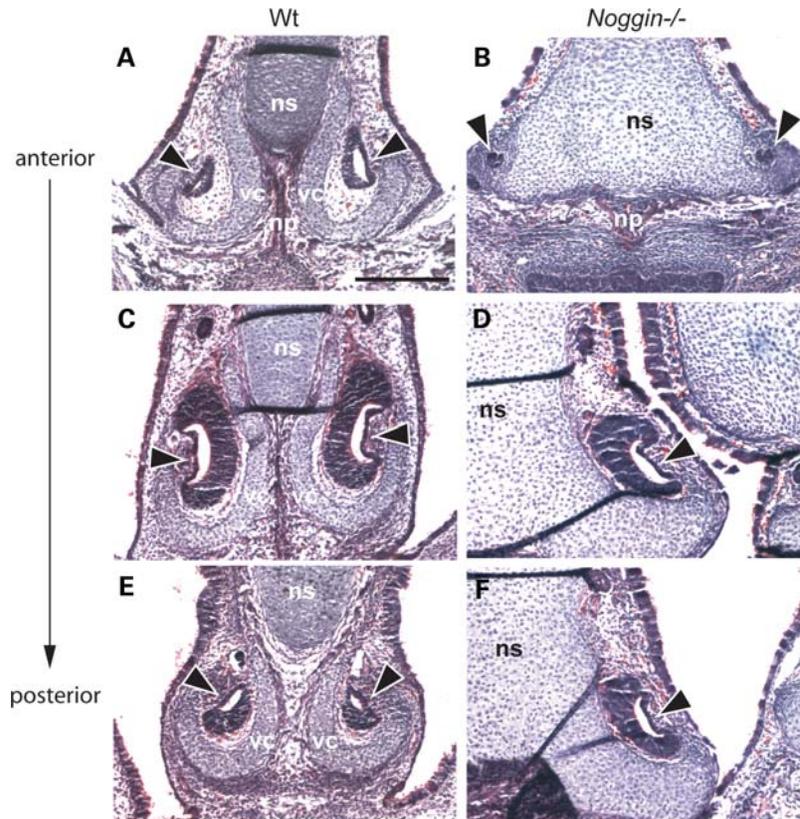
Figure 6. Abnormalities of the VO in *Noggin*$^{-/-}$ mice. (**A**–**F**) E16.5 hematoxylin and eosin stained frontal sections of WT and *Noggin*$^{-/-}$ heads showing the developing nasal septum and VO. The VO is similar in WT and *Noggin*$^{-/-}$ mice (arrowheads), however the vomeronasal cartilage (vc) which cups the WT VO is missing in *Noggin*$^{-/-}$ mice, the mesenchyme surrounding the VO is reduced as is the developing nasal projection of the maxillary bone. The cartilage of the nasal septum is greatly enlarged in *Noggin*$^{-/-}$ mice. np, nasal projection of the maxillary bone; ns; cartilage primordium of the nasal septum; vc, vomeronasal cartilage. Scale bar: (A) 200 μm (A–F, same magnification).

It seems likely that this phenotype is, at least in part, secondary to abnormalities causing the mandible and tongue to be held down so that the palatal shelves remain above the tongue. Meckel's cartilages in *Noggin*$^{-/-}$ mice were greatly enlarged holding the oral cavity open (Fig. 5). In addition, there were developmental abnormalities in the hyoid bone. The hyoid bone is a U-shaped bone positioned below the mandible in the anterior triangle of the neck. It gives attachment to muscles controlling the function of the tongue and mandible. The hyoid comprises a body, two greater cornua and two lesser cornua. It develops from cartilages from the pharyngeal arches (PA), the body from both PA II and III, the lesser cornua from PA II and the greater cornua from PA III. We examined the hyoid bones by alizarin red (bone) and alcian blue (cartilage) staining at E16.5, E17.5 and E18.5. Already at E16.5, the mutant hyoid bones/cartilages were much larger than in their WT littermates (Fig. 5I and J). In WT E18.5 mice, the hyoid was composed of a body and greater cornua with the lesser cornua developing later, while the hyoid of *Noggin*$^{-/-}$ mice was abnormal in size, shape and number of cornua (Fig. 5K and L) (5/5 mice). The body of the hyoid bone was enlarged greatly and its shape changed with four instead of two cornua. These cornua were shorter, wider and mineralized distally compared with the cornua of WT littermates.

In WT mice, the palatal shelves develop in an oral cavity that is expanding in all directions. As *Noggin*$^{-/-}$ mice exhibit a lack of midfacial transverse growth, the oral cavity is narrow and this may allow the palatal shelves to touch and fuse so that a palate could form. That said, 2 out of 31 *Noggin*$^{-/-}$ mice exhibited facial clefts and 1 out of 21 mice exhibited a cleft palate (data not shown).

The vomeronasal capsule is absent in *Noggin*$^{-/-}$ mice

The VO (also known as Jacobson's organ) is an auxiliary olfactory sense organ situated adjacent to the cartilage of the nasal septum, mainly used to detect pheromones. The VO is a c-shaped structure surrounded by a cartilaginous capsule which opens into the base of the nasal cavity. The vomeronasal capsule is a structure distinct from the nasal septum, which it neighbors. Histological analysis of the frontal section of *Noggin*$^{-/-}$ E16.5 heads revealed that the epithelial section of the VO appeared normal (Fig. 6). However, the vomeronasal capsule was missing in the *Noggin*$^{-/-}$ mutants and the mesenchyme around the VO was reduced in size (in all animals examined).

Pituitary gland abnormalities in *Noggin*$^{-/-}$ mice

The developing pituitary gland was analyzed in sagittal and coronal sections at E13.5, E14.5 and E16.5 (Supplementary Material, Fig. S2, data not shown). The most notable feature in *Noggin*$^{-/-}$ mice was that the developing pituitary was smaller when compared with WT mice, especially the pars anterior. The pars anterior (pars distalis) ultimately produces the most of the pituitary hormones. Other defects that have previously been reported in *Noggin*$^{-/-}$ mice include a rostral displacement of Rathke's pouch and the induction of secondary pituitary tissue (25).

DISCUSSION

Noggin null allele mice exhibit several craniofacial abnormalities including a narrowed frontonasal process, a solitary median maxillary incisor and defects in the hyoid bone, VO and pituitary gland. All these Noggin-related facial anomalies are characteristic of a microform of HPE and are associated with reduced expression of hedgehog target genes. Our findings are consistent with the hypothesis that Bmps have a role in midline patterning and the regulation of midface width by inhibiting Shh signaling.

Solitary median upper incisor

WT mice have two upper incisors. We found that *Noggin*$^{-/-}$ mice have a solitary median upper incisor (100% penetrant), which develops from single expression domain in the midline of a single premaxillary bone. We cannot rule out that the single *Shh* expression domain is a consequence of a lack midline tissue intervening between two anlagen which have subsequently fused together, although this is relatively unlikely as the centrally located Shh domain in the *Noggin*$^{-/-}$ mice is smaller than the individual domain of WT upper incisors. Alterations in the number of teeth can be caused by irregularities in the tooth initiation process (26). In *Noggin*$^{-/-}$ mice, the single incisor is part of a HPE midfacial phenotype where the lack of tissue in the midline and a patterning defect are responsible for the tooth phenotype. Overactivation of *Noggin* in the oral epithelium using transgenic tools (K14 promoter) does not result in a change in the number of incisors. However, the morphology and structure of the K14-Noggin incisors are altered: incisors are large with abnormalities in enamel and dentine formation. In addition, all mandibular and maxillary third molars are lost due to developmental arrest at the early bud stage (27).

Humans normally have two deciduous and two permanent central incisors and the development of a solitary median maxillary central incisor (SMMCI) in the primary and/or secondary dentition is rare. SMMCI can occur as part of a holoprosencephalic phenotype, in association with abnormalities not related to HPE or as an apparently isolated finding. Missense mutations in *SHH* have been reported in patients with SMMCI that do not have other features of HPE (28,29). The I111F mutation has been found in eight members of the same family and it has been suggested that this mutation may be specific for SMMCI as it has not been found in either HPE patients or in a normal population (28).

Hypopituitarism

Hypopituitarism due to pituitary hypoplasia has been described in patients with SMMCI together with other HPE-like features and has been reported in patients with loss of function mutations in the HH signaling transcription factor *GLI2* (30,31). In zebrafish, it has been shown that Gli1 and Gli2 regulate Hh signaling to induce and pattern the developing adenohypophysis (32). And in mice, 50% of *Gli2*$^{-/-}$ mutants and 100% of *Gli2*$^{-/-}$;*Gli1*$^{-/-}$ mutants fail to develop a pituitary gland (33). Whether Bmp and Hh signaling interact during pituitary development is not known. In *Noggin*$^{-/-}$ mice, the pituitary abnormalities appear to be due to an expanded domain of Bmp4 activity that results in *Fgf10* repression and rostral shift of the *Bmp4* and *Shh* boundary (25).

Midfacial width is regulated by Noggin

Several studies have analyzed the role of Bmps and other growth factors in the control of facial proximal/distal outgrowth, but very few have addressed what controls facial width. Here we show that Bmp, by modulating Shh signaling, plays a fundamental role in regulating facial width. Although the facial features of *Noggin*$^{-/-}$ mice have not been previously reported, there is evidence that Bmps control facial patterning and growth and that alteration of Bmp signaling can lead to HPE. For instance, compound mouse mutants for *Chordin* and *Noggin* (34,35) or chick embryos that have had BMP4-soaked beads implanted into their forebrains (36) exhibit holoprosencephalic facial features including cyclopia, a central proboscis and orofacial clefting. With regard to the proximal/distal outgrowth of the facial processes, Noggin-soaked beads implanted into the developing chick face at different developmental stages results in decreased cell proliferation and subsequently decreased outgrowth, smaller frontonasal and maxillary processes and deletion of the maxillary and palatine bones (14,15,23).

We show that in *Noggin*$^{-/-}$ mice, *Shh* and the Shh target genes *Ptch1* and *Gli1* are misexpressed at E11.5 and E12.5 in the developing face. This suggests that disrupted Bmp signaling in *Noggin*$^{-/-}$ mice results in downregulation of the hedgehog pathway at the critical time when the midline craniofacial structures are developing. This is supported by experiments in the developing chick that demonstrate that excessive exogenous BMP2 (micro bead implantation) downregulates *Shh* expression (23), and transient loss of SHH signaling by either excision of the frontonasal epithelium or by the introduction of Shh neutralizing antibodies inhibits growth of the facial primordia and results in a narrowing of the mid and upper face and subsequent hypotelorism (37). Also, mice lacking the transmembrane protein Cdo, which positively regulates Shh signaling, exhibit HPE with a midface hypoplasia and hypotelorism (38).

Disruption in Shh signal transduction by exposing chick embryos, at Hamilton Hamburger stages 15 and 17, to the Smoothened inhibitor cyclopamine, results in a continuum of HPE-related defects including hypotelorism (39). The time when Shh signaling, and presumably Bmp signaling, is blocked is critical. Early blockade of Shh (before the division

of the eye field into two) (stage 4) causes cyclopia, Shh blockage later (stages 15 and 17) results hypotelorism, midfacial hypoplasia and orofacial clefting, while blockage later in development has no effect (39). Conversely, excessive SHH in the embryonic face leads to increased proliferation in the frontonasal process, increased width of mid and upper facial processes and consequently hypertelorism (37). When considering facial development, it is important to differentiate between early abnormal *Shh* signaling from the developing brain and signaling later from the developing facial structures.

In this study, we show that exogenous BMP4 inhibits *Fgf8* and *Ptch1* in the developing mouse frontonasal process. Fgf8 is a key regulator of facial development which is known to regulate Shh signaling in several embryonic locations, including the limb and genital tubercle. Blocking Fgf signaling in the developing chick face results in a narrowing of the upper face, approximation of the maxillary processes and a coalescence of the nasal pits into a single central pit (40). There is good evidence that during very early chick facial development Bmp signaling acts to restrict *Fgf8* expression (41). Equally, blocking endogenous Bmp signaling in the developing chick frontonasal prominence by overexpressing *Noggin* results in an expansion of the *Fgf8* domain (40). Taken together, the downregulation of Fgf8 and Hedgehog signaling by excessive Bmp signaling provides a mechanism underlying the HPE in the *Noggin* null allele mouse.

Mutations in *SHH*/*Shh*, as well as mutations in several hedgehog signaling pathway members, result in HPE in both humans and mice. Human and mouse mutations of *GLI2*/*Gli2* have been shown to cause HPE-like phenotypes, including midfacial hypoplasia with a single maxillary incisor (31,42). A *SHH* missense mutation has been associated with HPE and shown to cause defective binding to the HH regulator GAS1 (43). Indeed, mutations in *GAS1* alone or in addition to mutations in *SHH* can result in HPE in humans (8). Similar to *Noggin*$^{-/-}$ mutants, 40–50% of *Gas1*$^{-/-}$ mice exhibit several features consistent with a microform of HPE including a single maxillary incisor, a narrow frontonasal process and abnormalities in the pituitary, palate and VO (21). The *Gas1*$^{-/-}$ phenotype appears to be due to a reduction in Shh signaling as loss of a single allele of *Shh* in the *Gas1*$^{-/-}$ background worsened the craniofacial phenotype and *Gas1*$^{-/-}$ mice exhibit a reduced *Ptch1* expression domain in the developing frontonasal process (21,44).

Noggin$^{-/-}$ mice have a narrow facial width and malformed neural crest cell derivatives, namely the upper incisors, premaxillary bones, nasal septum and the palate. There is evidence that Bmp signaling is important in neural crest induction, delamination and migration and it has been suggested that elevated Bmp signaling disrupts the development of postmigratory, differentiating skeletal neural crest cells (18,24,34). Thus, overexpression of Noggin in the second branchial arch (Hoxa2) results in reduced numbers of neural crest cells and consequently hypomorphic skeletal and neural elements (45). Also, Noggin may protect neural crest cells from apoptosis induced directly by elevated Bmp signaling, and indirectly by maintaining Shh signaling as Noggin and also Chordin promote the rostral expression of *Shh* (34). Noggin and Chordin are Bmp antagonists that have similar biochemical activity and expression domains and may well compensate

functionally for each other. Compound mutant mice for *Noggin* and *Chordin* display a variety of HPE craniofacial defects (24,34,35). Consistent with our data, disruption of Bmp and Follistatin signaling in the chick results in a holoprosencephalic phenotype through a mechanism which involves modulating midline Shh signaling (46).

Despite multiple regulators controlling the Bmp pathway, disruption of *Noggin* alone is sufficient to result in the midfacial phenotype described in this paper. In conclusion, we demonstrate the importance of Noggin in the correct patterning of the face and in the etiology of HPE.

MATERIALS AND METHODS

Mice

The generation, breeding and genotyping of the *Noggin* mutant mice used in this study have been described previously (17,22). The initial inactivation of the *Noggin* gene was done in an inbred 129SvJ genetic background. Heterozygote males have been serially backcrossed with CD1 females for at least 10 generations. *NogginLacZ* reporter mice were backcrossed to C57BL/6 and used as heterozygotes.

Skeletal and LacZ staining

Alcian blue/alizarin red and LacZ staining were performed according to Rice *et al.* (2010) and Zouvelou *et al.* (2009) (47,48).

Organ culture

E10.5 and E11.5 WT mouse heads were dissected and placed on Nuclepore filters in a Trowell-type organ culture system as described previously (49). Affi-gel agarose beads (Biorad) were incubated with recombinant human BMP4 (100 ng/μl, R&D Systems) or BSA at 37°C for 1 h and stored at 4°C before being placed on the explants. Bead assays were cultured overnight.

Preparation of probes and *in situ* hybridization

^{35}S *in situ* hybridization on paraffin sections was performed as previously described (50). Both bright and dark field images were taken of hybridized sections. Silver grains were selected from the dark field images, colored red and then superimposed onto the identical bright field image using Adobe Photoshop 6.0 software. Whole-mount *in situ* hybridization was performed using digoxigenin-UTP-labelled riboprobes as previously described (51). The preparation of the *Bmp*, *Fgf8*, *Gli1*, *Pax9*, *Ptch1* and *Shh* RNA probes has been described previously (52–54).

Statistical analysis

An independent samples *t*-test was used for the statistical analysis of normally distributed samples. A *P*-value of <0.05 was considered statistically significant. SPSS 15.0 was used for the statistical analysis of the data.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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