Original Article

Developmentally Regulated Expression of *Msx1*, *Msx2* and *Fgf*s in the Developing Mouse Cranial Base

Xuguang Nie[a]

ABSTRACT

Objective: To examine the expression pattern of the *Fgf* and *Msx* genes in cranial base development.

Materials and Methods: To detect the expression of these genes, antisense riboprobes were synthesized by in vitro transcription. Radioactive in situ hybridization was performed on parasagittal sections of embryonic mouse heads.

Results: *Msx2* was observed in the underlying perichondrium at restricted stages. *Msx1* was not observed in cranial base development. *Fgf1* was localized in osteogenic cells from the time of ossification; *Fgf10* was highly expressed in the occipital-vertebral joint during E13 to E14; *Fgf2*, *Fgf7*, and *Fgf18* were localized in the perichondria; *Fgf12* was transitorily expressed at early chondrocranium; *Fgf9* was seen in the hypertrophic chondrocytes.

Conclusions: The *Fgf* and *Msx* gene expression in the cranial base was different from that of other skeletons.

KEY WORDS: Cranial base; Signaling; *Msx*; *Fgf*

INTRODUCTION

The *FGF* and *MSX* genes are important for craniofacial development, particularly for the calvaria, as highlighted by the etiologic relationship between mutations of these genes and human craniosynostosis. However, the role of the *FGF* and *MSX* genes in the ventral part of the cranium, the cranial base, are largely unknown, and this structure is important for coordinated development and growth of craniofacial skeletons.

The cranial base, or basicranium, plays a key role in integrated craniofacial development. It is different from the facial bones that are formed through intramembranous ossification. The cranial base is formed through endochondral ossification and, in this process, a cartilage template is formed first and eventually replaced by bone via chondrocyte apoptosis and osteogenic cell migration. Well-organized cartilaginous structures analogous to long-bone growth plates, termed synchondroses, are formed among cranial base bones, acting as growth centers in advancing growth.[1] Cranial base synchondroses ossify at different growth stages in human beings, and are regulated by a mechanism that, thus far, is not completely understood.[2]

The cranial base is a unique structure that differs from other endochondral bones. Skeletogenic cells of the cranial base are derived from paraxial somites in the posterior part and from the neural crest in the anterior part.[3] These two different embryologic parts are distinguishable by distinct growth features. The posterior cranial base matures and reaches its final size earlier than the anterior cranial base. However, the anterior cranial base has a more active and more prolonged growth process that lasts to a very late growth stage.[4,5] This feature is a prerequisite for coordinated craniofacial development and growth. The basicranium also differs from other portions of the endochondral skeleton; to a great extent, the development and growth of the basicranium are under the influence of the brain, and the final shape and size of the basicranium follow that of the brain.[6,7] Cranial base angulation is formed by the relative flexion of its anterior and posterior parts, and is a unique feature of human beings and a reflection of brain evolution.[1]

[a] PhD Student, Department of Biomedicine, University of Bergen, Bergen, Norway.

Corresponding author: Dr Xuguang Nie, Section of Anatomy and Cell Biology, Department of Biomedicine, University of Bergen, Jonas Lies V91, 5009, Bergen, Norway
(e-mail: Xuguang.Nie@biomed.uib.no)

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In many human genetic and developmental disorders, the basicranium is also affected. Cranial base anomalies have been identified in Down syndrome, Turner syndrome, craniosynostosis syndromes, cleidocranial dysplasia, and many other pathologic conditions.[8–12] In some cases, the cranial base anomaly is believed to be the primary cause that leads to the overall abnormal craniofacial development.[9] Concurring with this, animal studies provide experimental evidence that cranial base fusion alone accounts for many craniofacial dysmorphic features.[13]

Although the genetic mechanisms of development and growth are starting to be elucidated in many structures, very little is known regarding these mechanisms in the cranial base. Cranial base development might be genetically determined.[1] Unique development and growth features also implicate unique signals or signal uniqueness in the regulation of development and growth. Deciphering cranial base development through a genetic approach has just started.[14–18] Studies on other structures, particularly the appendicular bones, provide some important clues for studies on the cranial base. In the present study, the author examined a set of genes that are important for craniofacial and skeletal development, including members of the *Fgf* family and *Msx1* and *Msx2.*

The homeobox genes *MSX1* and *MSX2* encode transcription factors that are critical for craniofacial development and are key participants in epithelial-mesenchymal interaction, which is an important mechanism of craniofacial organogenesis. *MSX1* has critical roles for tooth, alveolar bone, and palate development. Inactivation of *Msx1* in mice leads to anomaly or absence of most of these structures.[19,20] Mutation of *MSX1* is associated with tooth agenesis and orofacial cleft (OMIM, 106600 and 119530). *MSX2* is involved in a variety of craniofacial developmental processes. Mutation of *MSX2* is the cause of Boston-type craniosynostosis, in which the cranium shows obvious deformation caused by premature suture fusion (OMIM, 604757). Knockout of *Msx2* in mice leads to facial clefting and anomalies of facial bones.[20] Double knockout of *Msx1*/*Msx2* exacerbates the defects in the calvaria, implying their overlapping role in this structure.[20] *Msx1* and *Msx2* also have important roles in long-bone development.[20] The FGF signal pathway is critical for skeletogenesis and craniofacial development. Mutations of FGF receptors (*FGFR*) relate to a variety of human syndromes, characterized by skeletal deformities.[21] Some Fgfs, such as *Fgf2*, *Fgf10*, and *Fgf18*, are implicated as critical ligands at different sites during skeletogenesis.[22–24] I am interested in whether these important genes also regulate the development of the cranial base. If so, how are these genes regulated in the cranial base? To address this issue, a preliminary expression study was performed, using in situ hybridization.

MATERIALS AND METHODS

Preparation of Tissues

The use of mice from Naval Medical Research Institute (NMRI) was approved by the Animal Welfare Committee of the Department of Biomedicine, University of Bergen, Bergen, Norway. The stage of the embryos was determined by the day of appearance of a vaginal plug and confirmed by morphologic criteria. The appearance of a vaginal plug was taken as day 0 of embryogenesis (E0). NMRI mice are born on E19, which corresponds to the newborn stage, postnatal day 0 (P0). The mice were killed by cervical dislocation and decapitation. Mouse embryos from E10 to E18 were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde overnight at 4°C. Mouse embryos harvested at E14 or later were decalcified with 12.5% ethylenediamine tetraacetic acid and 2.5% paraformaldehyde in PBS. Paraffin-embedded parasagittal sections of 7 μm were obtained from the midline of the mouse heads and used for in situ hybridization.

In Situ Hybridization

Plasmids containing complementary DNA fragments were provided by Dr Keijo Luukko and Paivi Kettunen; most have been used in other studies.[25,26] Probes were prepared by in vitro transcription. The sections were deparaffinized in xylene, rehydrated through graded ethanol, washed in PBS, and treated with proteinase K for 30 minutes. The proteinase K activity was inhibited by washing with 2% glycine in PBS, followed by postfixation in 4% paraformaldehyde for 30 minutes. After washing in PBS for 25 minutes, sections were acetylated with freshly prepared 0.25% acetic anhydride in triethanolamine-HCl, pH 8.0, for 10 minutes, followed by two water washes. The sections were dehydrated by dipping in series of ethanol solutions for 30 seconds each and air-dried. Probe solutions were pipetted onto the sections.

Hybridization was carried out for approximately 15 hours at 55°C in a humid chamber. The sections were washed under high-stringency conditions with 20 mmol/L dithiothreitol in 50% formamide and 2× standard sodium citrate for 1 hour at 65°C. The slides were washed for 1 hour at 55°C in 0.1× standard sodium citrate, dehydrated in 70% ethanol, and air-dried. The slides were then dipped in NTB-2 emulsion and stored

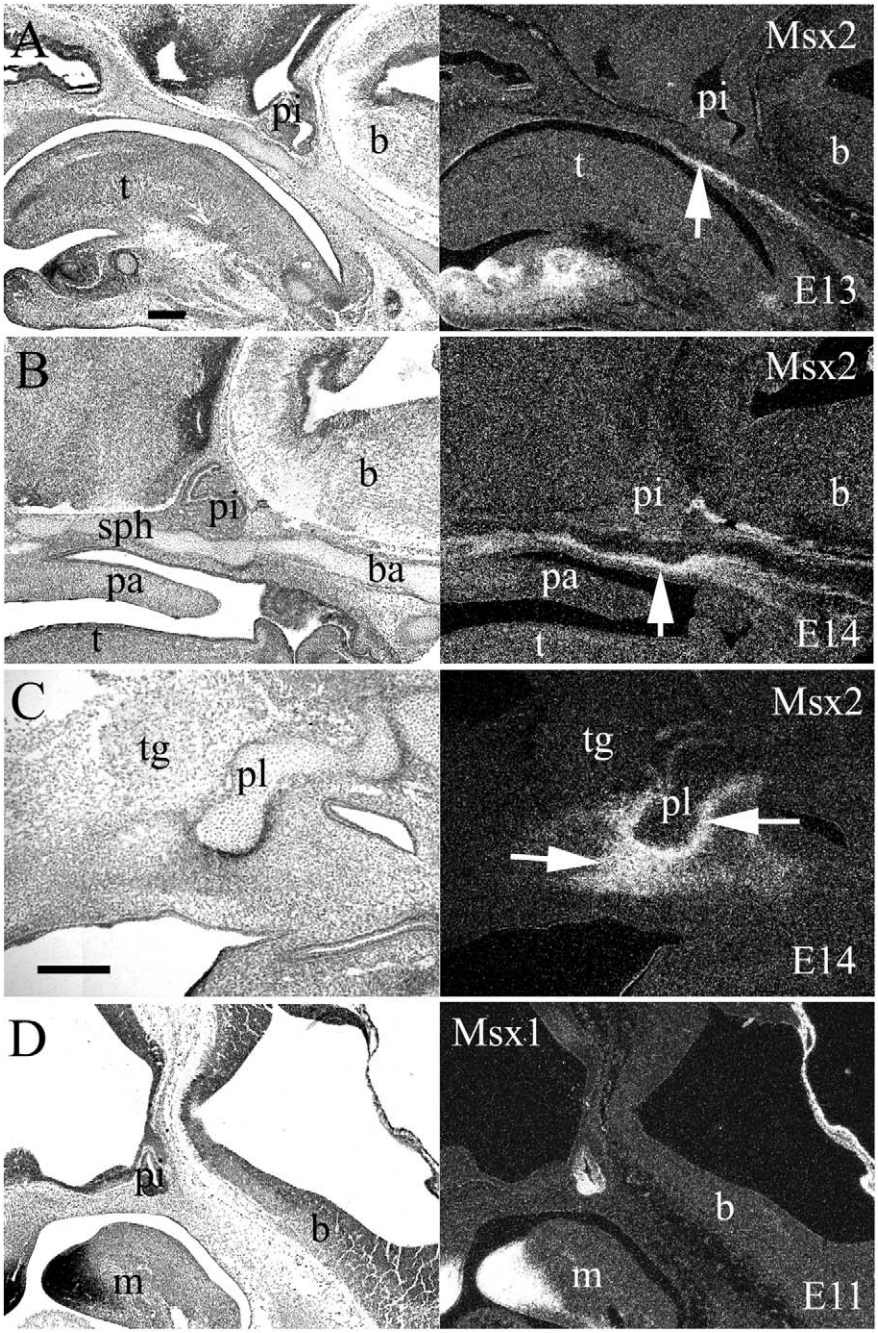

Figure 1. *Msx2* expression in the developing cranial base. *Msx2* was clear seen in the mesenchyme underlying the cranial base from E13 (A). At E14, this expression was intensified and extended to the sphenoid (B). *Msx2* was also intensely expressed around the pterygoid plate (C). *Msx1* was not detected in the developing cranial base (D). Scale bars represent 200 μm. The scale bar in (A) applies to (B and D). Arrows indicate expression; b, brain; ba, basioccipital; m, mandibular prominence; pa, palate; pi, pituitary gland; pl, pterygoid plate; sph, sphenoid; t, tongue; and tg, trigeminal ganglion.

in desiccated containers for autoradiography. After exposure for 3 weeks at 4°C, the slides were developed using Kodak D-19 developer and rapid fixer, counterstained with hematoxylin, and mounted with Distyrene, Tricresylphosphate and Xylene (DPX) mounting media. No specific signal was detected in sections hybridized with the control sense probes.

RESULTS

Msx1 and *Msx2* Expression in the Developing Cranial Base

Msx2 expression was seen in the underlying perichondrium from E13 to E16 (Figure 1A,B). *Msx2* was also intensely expressed around the pterygoid plate

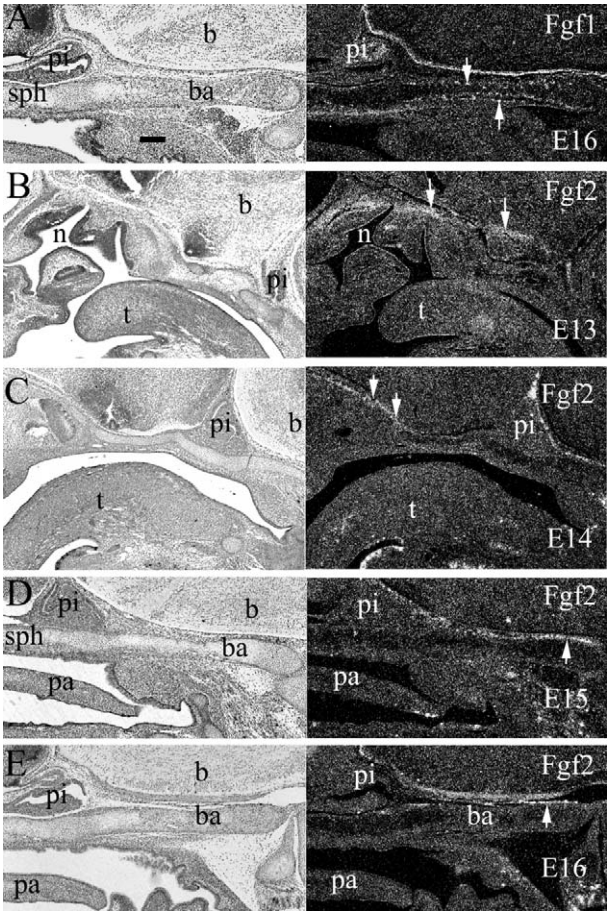

Figure 2. Expression of *Fgf1* and *Fgf2* in the developing cranial base. *Fgf1* was expressed in the perichondria, dura mater, and within the bone (A). *Fgf2* was first seen in the anterior chondrocranium during E13 and E14 (B, C), and was detected later in the perichondria and dura mater (D, E). The scale bar in (A) represents 200 μm and applies to (B, C, D). Arrows indicate the expression; b, brain; ba, basioccipital; n, nasal cavity; pa, palate; pi, pituitary gland; sph, sphenoid; t, tongue.

(Figure 1C). Surprisingly, *Msx1* was not observed in cranial base development, but had a high expression in the pituitary gland (Figure 1D).

Expression of *Fgf* Ligands in Cranial Base Development

To detect *Fgfs* that function during cranial base development, the expression of *Fgf1*, *Fgf2*, *Fgf7*, *Fgf9*, *Fgf10*, *Fgf12*, and *Fgf18* was examined. *Fgf1* was first seen in the dura mater and perichondria at E15. One day later, *Fgf1* transcripts were clustered inside the basioccipital bone, and subsequently also appeared in the anterior cranial base at a high level (Figure 2A). Later on, *Fgf1* was expressed in the osteoblasts throughout the basicranium. *Fgf2* expression was first seen in the perichondria of the anterior chondrocranium during E13 and E14 (Figure 2B,C). From E15, *Fgf2*

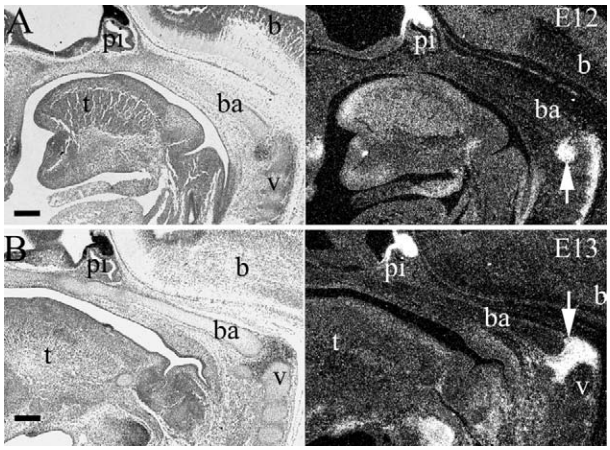

Figure 3. Expression of *Fgf10* in the developing cranial base. *Fgf10* was only detected in the occipital-vertebral joint during cranial base development (arrows in A, B). Scale bars represent 200 μm; b indicates brain; ba, basioccipital; pi, pituitary gland; t, tongue; v, vertebrae.

expression was seen in the dura mater and perichondria/periosteum of the basioccipital bone (Figure 2D,E). Later, *Fgf2* expression was no longer detectable in these areas. *Fgf10* was only localized in the occipital-vertebral joint at E12 and E13 at a high level (Figure 3A,B). *Fgf12* was detected in the basioccipital condensation at E11 and 1 day later in the chondrocranium (Figure 4A through C); thereafter, *Fgf12* was no longer detectable. *Fgf 18* was localized in the mesenchyme underneath the chondrocranium from E13, and, later, it was restricted to the perichondrium (Figure 5A,B). *Fgf7* was barely detectable in the perichondria during E12 to E13 (data not shown). *Fgf9* was detected in the hypertrophic chondrocytes from E16 (data not shown).

DISCUSSION

Among craniofacial skeletons, the midline cranial base is a major site of endochondral ossification, which intermediates transitional role from the brain to the craniofacial skeleton and, thus, integrates the craniofacial development. In the present study, the expression of *Msx* and some *Fgf* genes in the developing cranial base was examined.

Msx1 and *Msx2* are both critical transcription factors for craniofacial development, particularly for calvaria development. In skeletogenesis, Msx2 is an inhibitor of chondrogenesis and is involved in controlling bone morphogenesis.[27] In developing long bones, *Msx2* is present in the resting and proliferative chondrocytes of the growth plate and auricular cartilage, perichondria, and osteoblasts.[20] Unexpectedly, the *Msx* gene expression did not seem to have an eminent role in cranial base development. *Msx2* was only seen in the

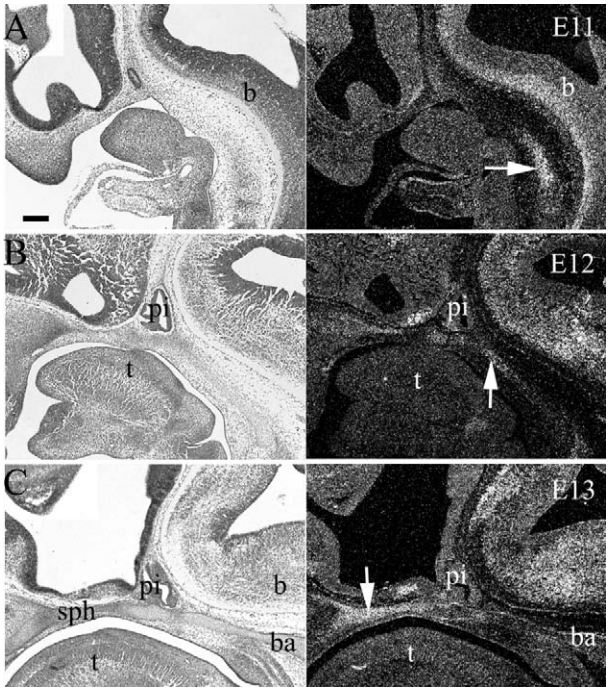

Figure 4. Expression of *Fgf12* in the developing cranial base. *Fgf12* was seen in the basioccipital condensation at E11 (arrow in A), and later in the chondrocranium (arrows in B and C). The scale bar in (A) represents 200 μm and applies to (B) and (C); b indicates brain; ba, basioccipital; pi, pituitary gland; sph, sphenoid; t, tongue.

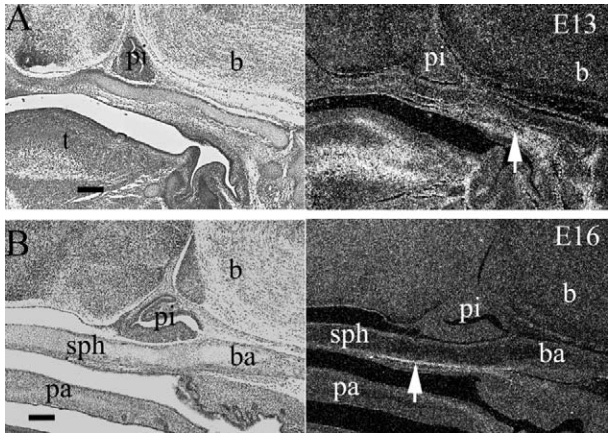

Figure 5. Expression of *Fgf18* in the developing cranial base. *Fgf18* was localized in the mesenchyme adjacent to the cranial base and subsequently in the perichondrium (arrows in A and B). The scale bars represent 200 μm; b indicates brain; ba, basioccipital; pa, palate; pi, pituitary gland; sph, sphenoid; t, tongue.

perichondria, and not observed in the chondrocytes. *Msx1*, on the other hand, was not observed during cranial base development. Additionally, no obvious defects in the cranial base have been reported in *Msx1*- and *Msx2*-null mice.[19,20] These data suggest that Msx1 and Msx2 are mainly regulators of intramembranous, but not endochondral, ossification in the skull, and that there are distinct molecular mechanisms between basicranium and long-bone development. Based on these results, it is logical to speculate that cranial anomaly in Boston-type craniosynostosis is primarily caused by malformation of the cranial calvaria, whereas other deformities might be secondary.

In the cranial base, *Fgf1*, *Fgf2*, *Fgf7*, *Fgf9*, *Fgf10*, *Fgf12*, and *Fgf18* were expressed at different stages of development: *Fgf2*, *Fgf7*, *Fgf9*, *Fgf12*, and *Fgf18* were mainly expressed in the cartilage and perichondria; *Fgf1* was observed in the bones. Expression of *Fgf1* indicates that it is among the principal ligands for cranial base osteogenesis.

An interesting finding of this study is the differential expression of *Fgf2* between the cranial vault and base. *Fgf2* was observed in the perichondria and dura mater overlying the cranial base, but not in the osteoblasts of the cranial base bones, whereas there was clear expression of *Fgf2* in the osteoblasts of the calvaria. This result suggests that *Fgf2* differentially regulates the intramembranous and endochondral bones of the cranium. Functional studies also support a critical roles of *Fgf2* in the cranial vault.[22,28] Neutralizing *Fgf2* blocks calvaria formation, whereas overexpression of *Fgf2* leads to delayed suture closing and macrocephaly.[22,28] *Fgf1* and *Fgf2* accumulations have been observed inside the targeted cell nucleus, suggesting that they might have additional modes of action in addition to binding and activation of cell-surface receptors.[29,30]

Expression of *Fgf9* in the hypertrophic chondrocytes suggests that it is an endogenous ligand at this site. *Fgf10* was intensely expressed in the occipital-vertebral joint, implying an important role at this site. However, phenotype examination of *Fgf10*-null mice did not report any defects at the occipital-vertebral joint.[31,32] *Fgf12*, first identified as *FHF1*, is involved in intracellular signaling.[33] The expression of *Fgf12* in embryonic chondrocranium implies that it regulates chondrogenesis at this site in an autocrine manner. Fgf18 has been suggested as a ligand for Fgfr3, which is a negative regulator of skeletogenesis.[23,34] The fact that *Fgf18*$^{-/-}$ mice have more severe defects than *Fgf3c*$^{-/-}$ mice suggests that Fgf18 has a broader role than Fgfr3c, and that Fgf18 must also be a ligand for other receptors.[23,34] In the developing cranial base, *Fgf18* was observed in the perichondrium throughout the cranial base, overlapping with *Fgfr1* and *Fgfr2*.

The present study systematically examined the expression domains of a set of important genes in the developing cranial base of the mouse. The results provide evidence that genetic control of the cranial base is distinct from that of other skeletals. Based on the cellular localization data and on other functional studies, it is proper to conclude that the molecular network involving *Msx* genes, a crucial mechanism for devel-

opment of the calvaria and the appendicular skeleton, is not critically used in cranial base development. Additionally, one can conclude that the Fgf-signaling in the cranial base is also characterized with different ligand expression in comparison to other skeletons.

Many important molecules remain to be tested at the basicranium. Gene expression profile will extend our understanding of the development of the basicranium. Particularly, the differential expression patterns between the basicranium and other bones will undoubtedly provide clues regarding specific signaling or signaling uniqueness at the basicranium. Further study is essential to unravel the genetic network of cranial base development, and, hence, its role in overall craniofacial development.

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