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Tissue Origins and Interactions in the Mammalian Skull Vault

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During mammalian evolution, expansion of the cerebral hemispheres was accompanied by expansion of the frontal and parietal bones of the skull vault and deployment of the coronal (fronto-parietal) and sagittal (parietal–parietal) sutures as major growth centres. Using a transgenic mouse with a permanent neural crest cell lineage marker, *Wnt1-Cre/R26R,* we show that both sutures are formed at a neural crest–mesoderm interface: the frontal bones are neural crest-derived and the parietal bones mesodermal, with a tongue of neural crest between the two parietal bones. By detailed analysis of neural crest migration pathways using X-gal staining, and mesodermal tracing by DiI labelling, we show that the neural crest–mesodermal tissue juxtaposition that later forms the coronal suture is established at E9.5 as the caudal boundary of the frontonasal mesenchyme. As the cerebral hemispheres expand, they extend caudally, passing beneath the neural crest–mesodermal interface within the dermis, carrying with them a layer of neural crest cells that forms their meningeal covering. Exposure of embryos to retinoic acid at E10.0 reduces this meningeal neural crest and inhibits parietal ossification, suggesting that intramembranous ossification of this mesodermal bone requires interaction with neural crest-derived meninges, whereas ossification of the neural crest-derived frontal bone is autonomous. These observations provide new perspectives on skull evolution and on human genetic abnormalities of skull growth and ossification.

Key Words: **mouse development; neural crest; mesoderm; skull sutures; Wnt1; retinoic acid.**

INTRODUCTION

The vertebrate skull consists of the neurocranium (skull vault and base) and the viscerocranium (jaws and other branchial arch derivatives). It is formed from cranial skeletogenic mesenchyme derived from two distinct embryonic sources: mesoderm and neural crest. The neural crest cell origins of the viscerocranium and the anterior skull base are well established (Le Douarin and Kalcheim, 1999, and references therein). However, studies from two laboratories using the quail–chick chimaera technique have come to different conclusions on the origin of the skull vault, reporting that it is of mixed neural crest and mesodermal origin (Le Lièvre, 1978; Noden, 1978, 1984, 1988) or entirely neural crest-derived (Couly *et al.*, 1993). Skull bone origins have been difficult to analyse in mammals: cell lineage studies using embryo culture and/or short-term labelling techniques have successfully elucidated neural crest cell migration pathways but not their contributions to mature structures (Tan and Morriss-Kay, 1986; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994, 1996).

In tetrapods, the skull vault (roof of the neurocranium) is formed mainly by the paired frontal and parietal bones, with a lesser contribution from the postparietals (mammalian interparietal). During mammalian evolution, the enormous expansion of the cerebral hemispheres was accommodated by expansion of the frontal and parietal bones and incorporation of the squamosal and part of the alisphenoid into the neurocranium (Goodrich, 1958). Growth of the mammalian skull vault takes place mainly in the fibrous joints (sutures) between these bones, all of which differentiate directly within the skeletogenic mesenchyme that lies between the brain and surface ectoderm (intramembranous

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ossification). Two sutures, the sagittal (between the two parietal bones) and the coronal (between the frontal and parietal bones) form the main sites of growth in the human skull, so that premature loss of these growth centres has a major effect on skull size and shape (Wall, 1997). They are also major signalling centres, as indicated by the discovery that mutations of the human genes encoding FGF receptors and the transcription factors TWIST and MSX2 cause premature sutural fusion (Bellus *et al.*, 1996; El Ghouzzi *et al.*, 1997; Howard *et al.*, 1997; Wilkie and Morriss-Kay, 2001, and references therein). Developmental studies on the mouse skull have shown that these signalling systems control the balance between proliferation of osteogenic stem cells and their differentiation to form new bone (Iseki *et al.*, 1997, 1999; Kim *et al.*, 1997; Rice *et al.*, 2000; Zhou *et al.*, 2000).

Knowledge of the developmental tissue origins of the mammalian frontal and parietal bones, and hence of the sutural growth centres, is clearly of considerable scientific and clinical interest. It is now possible to identify neural crest cells in mouse embryos by using a compound transgenic mouse (Jiang *et al.*, 2000; Chai *et al.*, 2000). The mouse is doubly heterozygous for the constitutively expressed *R26R* allele, which encodes *β-galactosidase* only in cells (and their progeny) that express *Cre recombinase* (Soriano, 1999), and the *Wnt1-Cre* transgene, which is specific to neural crest and to some parts of the brain. The results of the previous studies demonstrated that all known neural crest cell lineages are efficiently and stably marked by combining these two components (Jiang *et al.*, 2000; Chai *et al.*, 2000). Here, we use the *Wnt1-Cre/R26R* construct to distinguish between neural crest-derived and mesodermal components of cranial skeletogenic mesenchyme and thus investigate the tissue origins of the skull vault. This method has advantages over previous methods of skull bone lineage analysis in that it does not require either surgical intervention or embryo culture, and the contributions made by neural crest cells can be analysed on whole specimens as well as sections, from early developmental stages to mature tissues.

The results show that both the sagittal and coronal sutures are formed as juxtapositions between neural crest-derived and mesodermal mesenchyme. Mesodermal tissue in the region from which the parietal bone is derived is identified by DiI labelling followed by embryo culture. Exposure to retinoic acid (RA) at E10.0 results in inhibition of ossification of the parietal bones and their partial replacement by a layer of ectopic cartilage. Analysis of RA-treated *Wnt1-Cre/R26R* mice reveals that this effect is correlated with defective formation of the neural crest-derived meningeal layer that underlies both frontal and parietal bones. These results are discussed in the context of the influence of tissue interactions on the differentiation, growth, and evolution of the skull vault.

MATERIALS AND METHODS

Wnt1-Cre/R26R transgenic mice were constructed as described previously (Jiang *et al.*, 2000). X-Gal staining was carried out on whole embryos from E8.5 to E10.5, and on E15.5 and E17.5 heads. For E17.5 heads, the brain was removed prior to X-gal staining; some specimens were further stained with Alizarin red S and subsequently cleared, dehydrated, and stored in glycerol. Sections (10 μm) were obtained from previously X-gal-stained E9.5 and E10.5 embryos following dehydration and paraffin embedding. Older stages (E11.5–E17.5) were embedded in OCT, cut on a cryostat at 10 μm, and X-gal stained after cutting. All sections were counter-stained with nuclear fast red and eosin. Retinoic acid (100 mg/kg suspension in sesame or peanut oil) was administered by gavage at 9 a.m. on E10.0 to both wild-type and transgenic pregnant female mice. Wild-type fetuses were double-stained with alcian blue and Alizarin red S for cartilage and bone at E17.5 as described previously (Morriss-Kay, 1999). Double-stained heads were embedded in paraffin and sectioned at 8 μm. DiI was injected into the cranial mesoderm under the convex neural folds of 3- or 4-somite-stage embryos, i.e., prior to the stage of neural crest cell migration (Nichols, 1981; Tan and Morriss-Kay, 1986; Echelard *et al.*, 1994). The DiI-filled micropipette was inserted through the yolk sac, amnion, and surface ectoderm just lateral to the heart. Embryos were subsequently cultured for up to 48 h then photographed by brightfield and fluorescence microscopy after removal of the fetal membranes, then embedded in OCT compound; frozen sections were cut and photographed.

RESULTS

Neural Crest and Mesodermal Contributions to the Skull Vault and Meninges

As described previously (Jiang *et al.*, 2000; Chai *et al.*, 2000), compound *Wnt1-Cre/R26R* transgenic embryos stably express β-galactosidase in migrating neural crest cells and in their mature derivatives. There is also expression in the midbrain and hindbrain regions of the cranial neural tube. Fetuses examined at E17.5 and stained with X-gal, alone or in combination with Alizarin, show the pattern illustrated in Figs. 1A–1C. The frontal bones, together with the squamosal and viscerocranial bones, are completely X-gal positive, and a tongue of X-gal-positive tissue extends in the midline from the frontal region to insert between the parietal bones. There is also an irregularly shaped patch of stained cells in the region of the interparietal bone, which coincides with the central part of the bone but also extends rostral to it.

Sections of E17.5 heads (Figs. 1D–1G) confirm that the frontal, but not the parietal, bones are X-gal-positive. The meninges that surround the cerebral hemispheres, but not the meningeal covering of the midbrain and hindbrain, are also X-gal-positive (Fig. 1D). The light blue staining visible through the unstained parietal bone in whole-mount preparations (Figs. 1A–1C) can be seen in sections to be meningeal (Figs. 1D and 1E). Sections through the coronal suture show that the boundary between crest and noncrest cells in the skeletogenic membrane is the caudal edge of the frontal

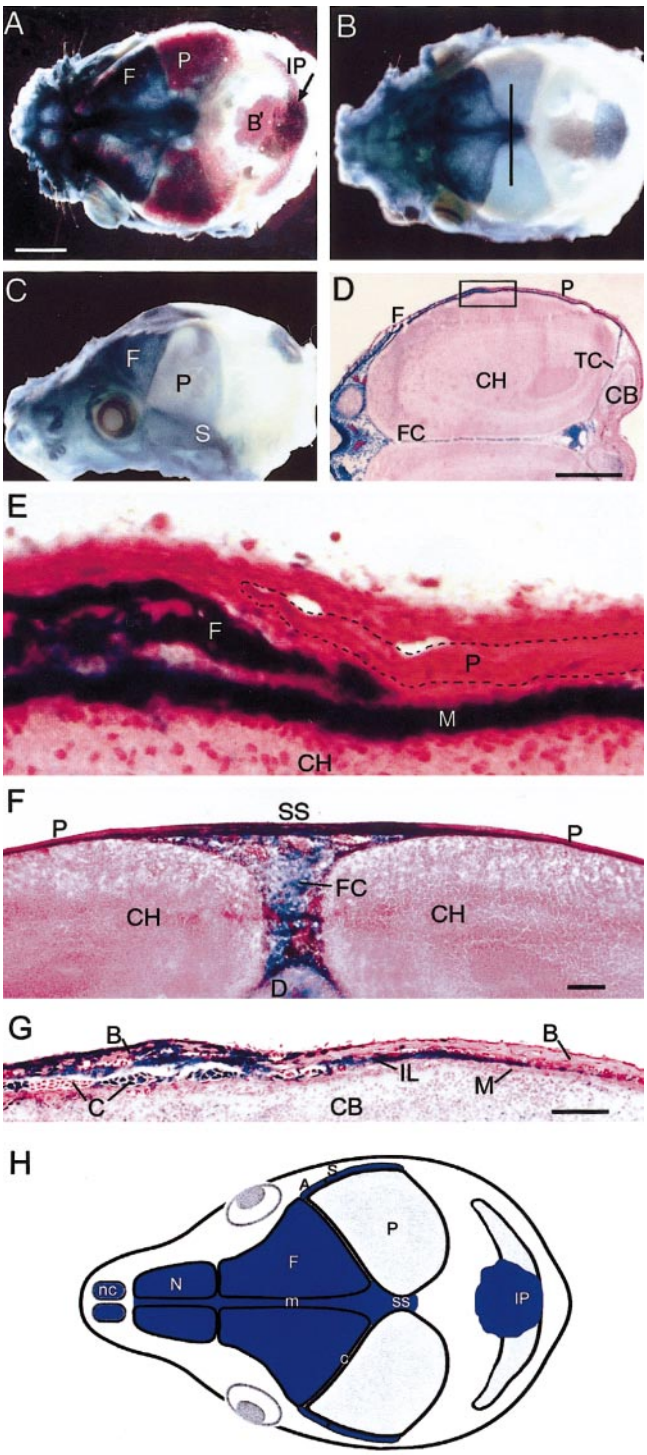


FIG. 1. Tissue origins of the mouse skull vault. X-Gal staining identifies neural crest-derived tissues in *Wnt1-Cre/R26R* mice. (A) Combined X-gal and Alizarin red S staining shows that the frontal bone (F) coincides with a strongly X-gal-stained area; in contrast, the parietal bone overlies a lightly stained area and extends just caudal to it. The entirely mesodermal basioccipital bone can be seen through the unossified part of the skull vault; the centre of the interparietal bone, and a patch of dermis rostral to it, is also

bone: the midsutural mesenchyme as well as the parietal bone are mesodermal. The characteristic overlap of the parietal over the frontal bone can be seen in the coronal suture at E17.5 (Fig. 1E). X-Gal staining of the skeletogenic membrane in the sagittal suture extends down into the meningeal layers, labelling the falx cerebri and tentorium cerebelli as well as the meningeal covering of the cerebral hemispheres (Figs. 1D and 1F). Although it cannot be assumed that all neural crest cells faithfully express *Wnt1*, there was no evidence of unlabelled cells in the frontal bone or labelled cells in the parietal bone; the gaps in frontal bone sections (Fig. 1E) are blood vessels, and the trabecular pattern shown here is characteristic of the frontal (but not the parietal) bone by E16.5 (Iseki *et al.*, 1999). Staining in the interparietal region is within the central area of the dermal bone and in a patch of cartilage beneath it; all of the osteoblasts are stained but some of the chondrocytes are not (Fig. 1G). The staining rostral to the bone is in unossified dermis (not shown).

These results, summarised in Fig. 1G, indicate that both the sagittal and coronal sutures are formed at interfaces between neural crest and mesodermal mesenchyme. The sagittal suture is formed as a sandwich of neural crest between the two mesodermal parietal bones, and the coronal suture is a juxtaposition between the neural crest-derived frontal bone and the mesodermal parietal bone.

X-gal-positive. (B) Similar specimen but without Alizarin staining, showing strong staining in the skeletogenic membrane between the parietal bones (sagittal suture); the orientation of the section shown in (F) is indicated by the line. (C) Side view, showing X-gal staining of the frontal, alisphenoid, and squamosal bones; the line shows the position and orientation of (E). (D) Low-power transverse section through the cerebral hemispheres at the level of the olfactory lobes (left) and upper cerebellum (right); neural crest-derived meninges surround the cerebral hemispheres and form the falx cerebri and tentorium cerebelli. The boxed area indicates the area shown at higher magnification in (E). (E) The coronal suture is formed between two bones of different origins, the neural crest-derived frontal bone and the mesodermal parietal bone (outlined); both overlie the neural crest-derived meninges covering the cerebral hemispheres. (F) Coronal section showing that the sagittal suture consists of a neural crest-derived membrane between the two mesodermal parietal bones. (G) Transverse section through part of the interparietal bone (midline is to the left), showing the neural crest-derived central area (left), mesodermal area (right), and underlying cartilage of mixed origin; noncartilaginous parts of the inner layer are also of neural crest origin in the central region, but the meningeal covering of the cerebellum is mesodermal. (H) Summary diagram showing neural crest contribution to the skeletal elements and sutures of the mouse skull vault. A, alisphenoid; B, mineralised bone; B′, basioccipital; C, cartilage; c, coronal suture; CB, cerebellum; CH, cerebral hemispheres; D, diencephalon; F, frontal; FC, falx cerebri; IL, inner layer of skeletogenic membrane; M, meninges; m, metopic suture; N, nasal; nc, nasal cartilage; P, parietal; S, squamosal; SS, sagittal suture; TC, tentorium cerebelli. Scale bars: A–D, 1 mm; E, F, 100 μm.

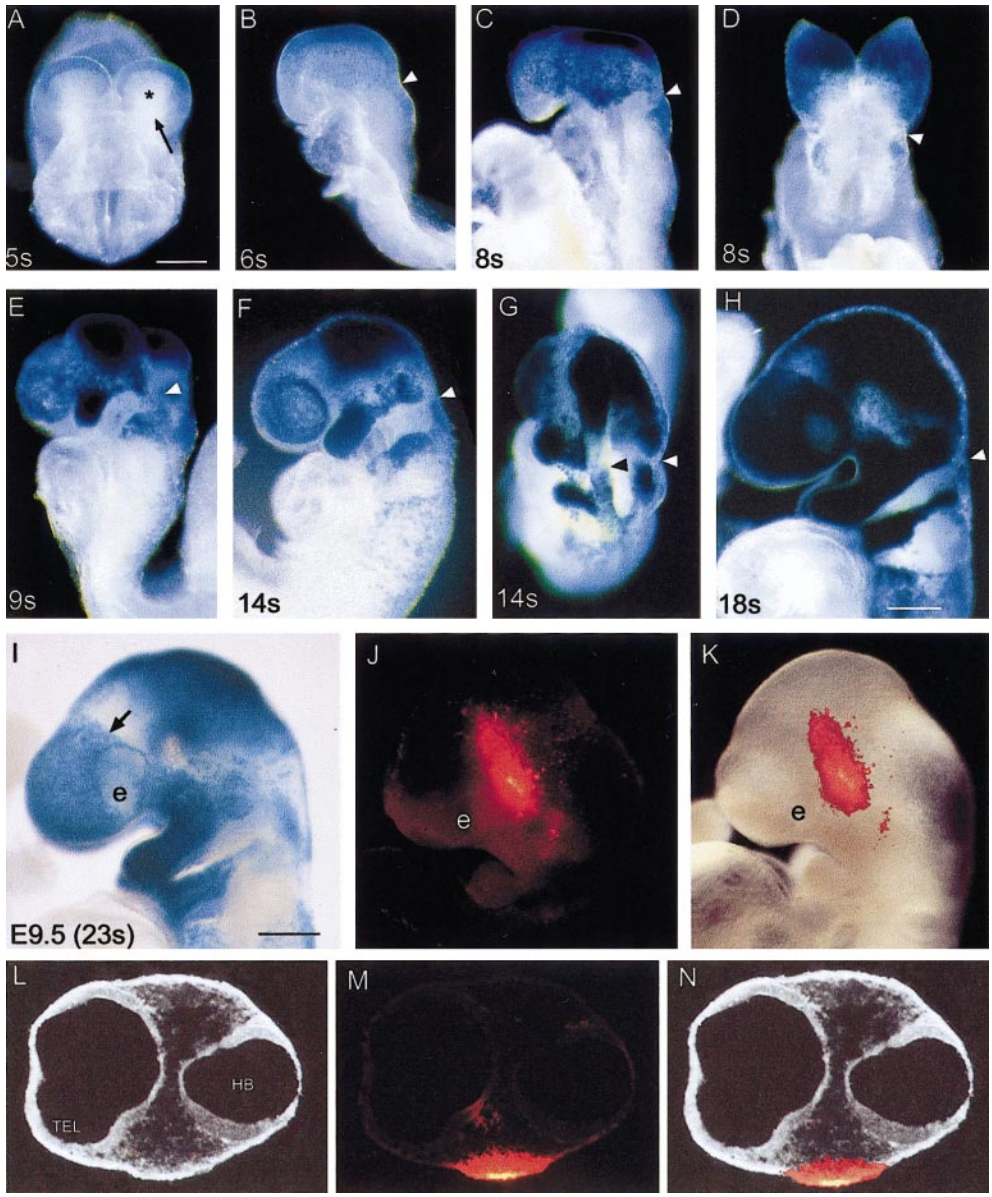


FIG. 2. The origin of the neural crest–mesoderm juxtaposition is revealed by X-gal staining of transgenic embryos during the period of neurulation/neural crest cell migration (A–I), and by DiI labelling of the cranial mesoderm at the 3-somite stage followed by embryo culture (J–L) (the site of injection is indicated by the asterisk in (A), and the trajectory of the micropipette by the arrow). Arrowheads indicate the position of the preotic sulcus (prorhombomere A/B boundary) in (B–E), and the equivalent rhombomere (r) 2/3 boundary in (F–H). (A) Five-somite (s) stage embryo, rostral view: expression in neural folds and neural crest cells, which are just starting to migrate from the lateral edge. (B) 6s embryo: neural crest cells migrate as a continuous population from the caudal forebrain to the preotic sulcus. (C) 8s embryo, lateral view: migrating crest cells are spreading over the surface of the expanding forebrain, and towards the mandibular arch; the hyoid population has started to migrate from prB. (D) 8s embryo, dorsal view of the open neural folds: expression in prA and prB is in the lateral part of the neuroepithelium only. (E) 9s embryo, lateral/dorsal view: crest cells have spread further over the forebrain and towards the mandibular arch; emigration of cells from prA results in some loss of expression in the neural folds. (F) 14s embryo, lateral view: the telencephalon is now completely covered by crest cells, and migration into the mandibular arch is complete; crest cells have moved away from prA, leaving an expression-free gap in the neuroepithelium. (G) 14s embryo, dorsolateral view: loss of both trigeminal and hyoid crest cells from the neural folds results in lower staining intensity in their regions of origin. (H) 18s embryo: migration is almost complete, and boundaries are becoming clear to delimit the neural crest from noncrest regions. (I) 23s embryo: the boundary of the frontonasal neural crest is well-defined, although some scattered crest cells are present in the trigeminal/squamosal region. Staining in the neural tube is mainly in the midbrain and the dorsal part of r1 + 2. (J–N) DiI localisation in wild type embryos cultured for 36–48 h after injection of DiI into the cranial mesoderm at the 3-somite stage: DiI fluorescence in a whole embryo (J) and frozen section of a different embryo (M); darkfield view of section (L); superimposed fluorescence and darkfield images (K, N); the DiI-labelled cells are positioned caudal to the frontonasal neural crest.

Growth at the edges of the parietal bones therefore occurs at a neural crest–mesodermal tissue interface in both coronal and sagittal sutures. In contrast, the metopic (interfrontal) suture is formed between two neural crest-derived bones without any mesodermal involvement. Only the interparietal bone is of mixed origin; it is still widely separated from the parietal bones at E17.5, so it is not clear how its two components contribute to the lambdoid suture.

Establishment of Distinct Neural Crest-Derived and Mesodermal Domains of Cranial Mesenchyme

The changing pattern of X-gal-positive cells during the period of cranial neurulation and neural crest cell migration was followed in a series of E8.5 to E9.5 transgenic embryos from the 5-somite (s) to the 23s stage (Figs. 2A–2I). At 5s, neural crest cells have just begun to migrate, as established previously (Nichols, 1981; Tan and Morriss-Kay, 1985, 1986). Cells emigrate from the caudal forebrain, midbrain, and prorhombomere (pr) A of the hindbrain as a continuous population. PrA is the area between the midbrain and the preotic sulcus; it later separates into rhombomeres (r) 1 and 2 (Bartelmez and Evans, 1926; Ruberte *et al.,* 1997). By the 8s stage, neural crest cells are spreading over the outer (basal) surface of the expanding forebrain neuroepithelium and also form a denser group migrating towards the mandibular arch (Fig. 2C; Tan and Morriss-Kay, 1985). At this stage, X-gal staining within the neuroepithelium has extended caudally from the midbrain domain into the lateral parts of prA; a second lateral population of X-gal-positive cells is present in the lateral parts of prB, from which the hyoid crest has just begun to migrate (Figs. 2C and 2D). As the X-gal-positive neural crest cells emigrate from prA, staining is lost from this region (Figs. 2F and 2G), suggesting that the X-gal-positive neuroepithelial cells of prA are presumptive neural crest. The trigeminal ganglion crest cell population emerges mainly from this region (Figs. 2E–2I; Osumi-Yamashita *et al.,* 1994). At the end of the migration period (Fig. 2I), the X-gal-positive cells form a domain of frontonasal plus first branchial arch mesenchyme that has also been observed in other studies using different techniques (Noden, 1984, 1988; Osumi-Yamashita *et al.,* 1994, 1996; Brault *et al.,* 2001). There is a clear boundary between the X-gal-stained frontonasal neural crest and the adjacent unstained mesenchyme. Evidence that the tissue caudal to this boundary is indeed mesodermal was provided by DiI labelling of the most rostral cephalic mesoderm at the 3- to 4-somite stage (just prior to neural crest cell emigration) in the position indicated by the asterisk in Fig. 2A, and subsequent embryo culture for up to 48 hours. The DiI-labelled cranial mesenchyme in the cultured embryos lies entirely caudal to the frontonasal neural crest and superior to the maxillary/squamosal/trigeminal crest (Figs. 2J and 2K). Sections confirm that the DiI labelling is confined to the area that is X-gal-negative in the transgenic embryos (compare Figs. 2L–2N and Figs. 3B and 3D).

Formation of the Positional Relationships between the Skull Vault Sutures and the Brain

The mammalian coronal suture is formed between the frontal and parietal bones and lies over the cerebral hemispheres. The position of this suture is not correlated with any anatomical feature of the underlying brain. This is in contrast to the sagittal and lambdoid sutures, which, respectively, overlie the tissue between the two cerebral hemispheres, and the tissue between the cerebral hemispheres and cerebellum. The neural crest–mesodermal tissue border observed at E9.5 is at the border of the telencephalon and diencephalon (Figs. 2I and 3A), but by E17.5 the coronal suture, which has by then formed between the bones derived from these two mesenchymal populations, lies over the cerebral hemisphere (Fig. 1D). In order to discover how this change in positional relationships develops, we examined *Wnt1-Cre/R26R* transgenic embryos at a sequence of developmental stages from E9.5 to E17.5, as both whole mounts and sections. The results are illustrated in Fig. 3.

Whole embryo preparations show that at E9.5 the frontonasal neural crest cell population surrounds the telencephalon and is continuous with the maxillary and mandibular populations (Figs. 2I and 3A). The maxillary crest cells are continuous proximally with the squamosal population, which partially covers the trigeminal ganglion. In sections, a demarcation between X-gal-positive and X-gal-negative cells can be seen in the cranial mesenchyme, although the trigeminal ganglion (which is derived partly from ectodermal placodal cells) is mixed (Fig. 3B).

By E10.5, the telencephalon has begun to expand to form the cerebral hemispheres (Figs. 3C–3F). The frontonasal crest layer covering them is thin except around the eye and within the nasal swellings, but the boundary between X-gal-positive and -negative cells remains in the same position as at E9.5 (Fig. 3C). Sections show that the subectodermal cranial mesenchymal cells are now condensed in both neural crest-derived and mesodermal domains (Figs. 3D–3F). Comparison with a sequence of later stages confirms identification of specific regions as frontal, parietal and squamosal domains, of which only the parietal domain is X-gal-negative, i.e., mesodermal. By this stage, there is some mixing of X-gal-positive and -negative cells in the deeper loose mesenchyme (fine arrows in Figs. 3D–3F) but not in the condensed dermal mesenchyme (broad arrows).

During the next 3 days, the cerebral hemispheres continue to expand. They extend towards the rostral hindbrain, passing between the diencephalon/midbrain and the dermal mesenchyme, displacing the deeper loose cranial mesenchyme (Figs. 3G–3J). As they extend, they take with them a thin layer of neural crest-derived cells, which later form the meninges as seen at E17.5 (Figs. 1E and 1F). The boundary between the neural crest-derived and mesoderm-derived dermis remains in the same position at all stages of growth of the cerebral hemispheres, which pass under it, together with their meningeal neural crest cell covering. By E13.5,

this boundary is situated over the surface of the cerebral hemispheres, having almost attained the position of the coronal suture as observed at E17.5 (compare Figs. 3I and 3J with Fig. 1D). The condensed dermal mesenchyme can now be identified as the skeletogenic membrane, since it lies immediately over the meninges. At E15.5, the caudal boundary of the neural crest domain is indented so that the most caudal part is in the midline of the vertex of the skull (Fig. 3K); the degree of indentation is increased by E17.5 (Figs. 1B and 1C), correlating with upwards growth of the parietal bone (Iseki *et al.*, 1997; Rice *et al.*, 2000).

The origin of the patch of X-gal-positive cells observed in the interparietal region at E17.5 (Figs. 1A–1C) was also traced in the sections. Its precursor cells emerge individually from the rostral hindbrain on E9.5 and insert into the surface ectoderm on E10.5 (not shown). They move from the surface ectoderm into the underlying dermal mesenchyme during the next two days, and by E13.5 (Fig. 3I) are located only in the dermis.

Effects of Retinoic Acid on Parietal Ossification

Administration of RA on E10.0 results in partial or complete failure of ossification of the parietal and interparietal bones, and the formation of patches of ectopic cartilage in their place (Figs. 4A–4D). The squamous part of the alisphenoid bone and the squamosal bone were also reduced, but no ectopic cartilage was formed in their place (Fig. 4D). In specimens in which some parietal bone is present, the ectopic cartilage can be seen to be deep to the bone, in the same tissue layer as the normal cartilage that is present in the temporal region at this stage (Figs. 4E and 4F) and which underlies part of the interparietal bone (Fig. 1G). Sections of E17.5 *Wnt1-Cre/R26R* fetuses show that the RA-affected frontal bone is less trabecular in structure than normal (Figs. 4G and 4H), but it is clearly mineralised, as shown by Alizarin staining of both transgenic and wild-type fetuses (Figs. 4B and 4D). The meningeal neural crest is thin and discontinuous in both frontal and parietal regions; like the parietal bone, the ectopic parietal cartilage is entirely of mesodermal origin (Figs. 4G and 4H). Comparing four sectioned specimens, there appears to be a correlation between the amount of reduction in thickness of the meningeal neural crest and the nature of parietal skeletal differentiation, the cartilage:bone ratio being highest in the specimen with the thinnest meningeal layer. Examination of sections from younger embryos showed that the neural crest deficiency is present early, the extending cerebral hemispheres having very few crest cells associated with them (Figs. 4I and 4J). Since neural crest cells already cover the telencephalon at E9.5 and the embryos were not exposed to RA until E10.0, the deficiency seen at E13.5 must have arisen during expansion of the cerebral hemispheres, possibly through decreased cell division specifically in the neural crest-derived tissue; this interpretation is consistent with the reduced size of the facial bones (Fig. 4D). The interparietal domain of neural crest was slightly reduced in RA-treated specimens (Fig. 4B), interparietal ossification was reduced or absent, and the thin layer of cartilage normally present caudal to the parietal bone was increased in extent and thickness (Fig. 4D).

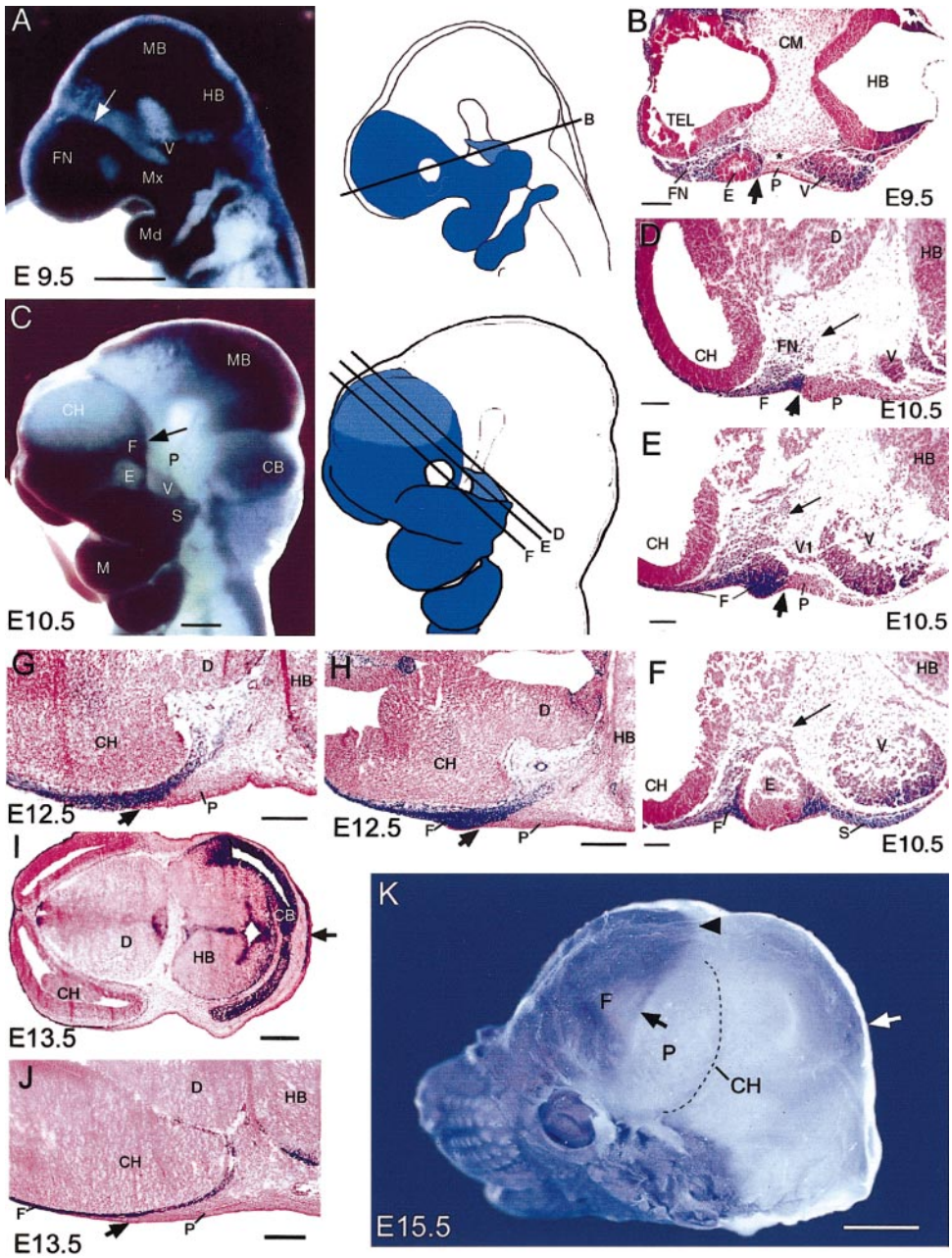
DISCUSSION

Tissue Origins of the Mammalian Skull Vault

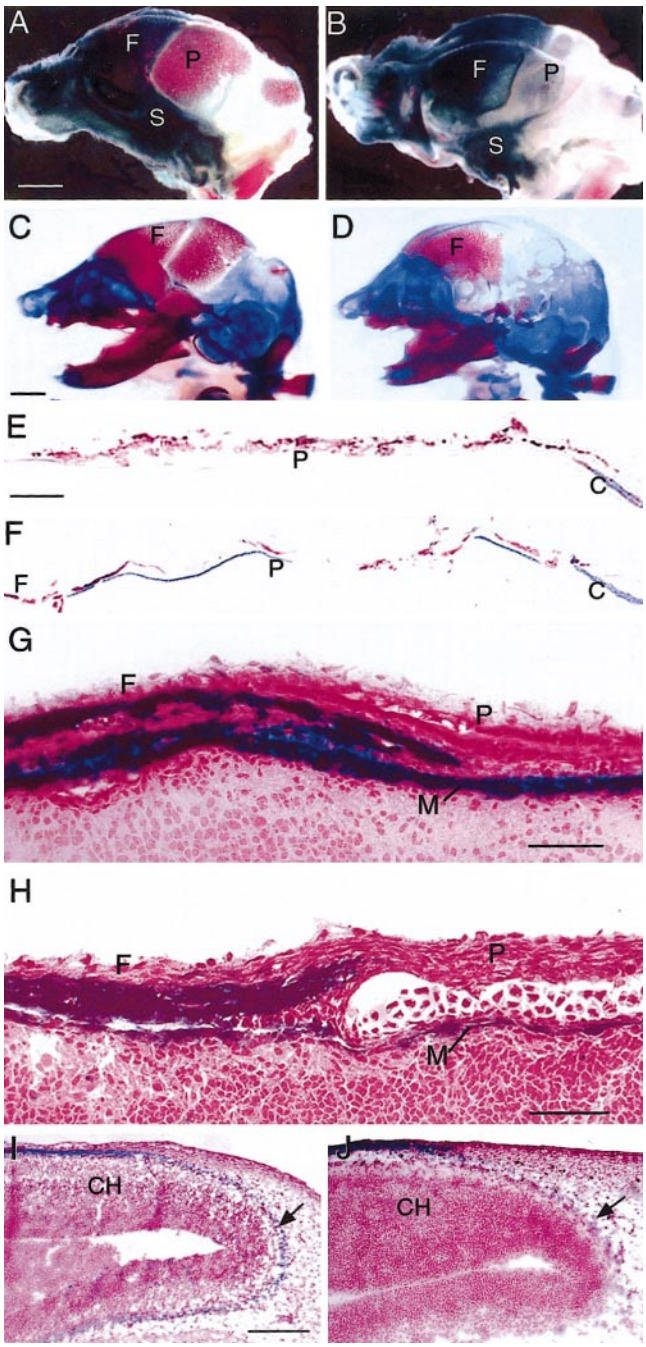
Using a transgenic embryo that enables detection of neural crest cells and their derivatives, as well as DiI labelling of cranial mesoderm, this study has demonstrated that the mammalian skull vault is constructed from embryonic tissues of two different origins, neural crest and mesoderm. The two sutures (sagittal and coronal) that make the greatest contributions to skull growth are formed as juxtapositions between these two tissues.

Analysis of a series of developmental stages from E8.5 to E17.5 revealed that the caudal boundary of the frontonasal neural crest observed at the end of neural crest cell migration (E9.5) later forms the caudal border of the frontal bone at the coronal suture. Initially the neural crest-derived and mesoderm-derived cranial mesenchyme is two-layered, being composed of a condensed dermal layer which subsequently forms the intramembranous (dermal) bones of the skull vault, and a loose mesenchymal layer deep to it. The loose tissue is displaced as the cerebral hemispheres expand caudally beneath the dermal skeletogenic layer. The frontonasal crest–mesoderm juxtaposition is initially situated over the telencephalon-diencephalon boundary. As the cerebral hemispheres expand caudally, they progressively change their positional relationship with the overlying dermis so that the neural crest–mesoderm boundary attains

FIG. 3. Development of the neural crest and mesodermal contributions to the skull vault. Whole embryos are stained with X-gal only; sections are counter-stained with eosin and nuclear fast red. In addition to the neural crest-derived tissues, X-gal stains some mid- and hindbrain areas. (A) E9.5 embryo showing the clearly defined junction (arrow) between the frontonasal neural crest, which overlies the telencephalon, and the unstained cranial mesoderm caudal to it. The drawing shows neural crest without the brain staining. (B) Transverse section of E9.5 head, as indicated in the drawing, showing frontonasal neural crest overlying the telencephalon and surrounding the eye. A condensed layer of dermal mesoderm forms the parietal anlage between the eye, the trigeminal ganglion and the primary head vein (asterisk). (C) E10.5 embryo: cerebral hemispheres have started to expand and the frontonasal neural crest is thinned over their upper surfaces. The parietal anlage lies immediately caudal to the edge of the frontonasal neural crest (arrowed) and dorsal to the maxillary/squamosal neural crest; it partially overlies the trigeminal ganglion. The drawing shows neural crest distribution only; the thin



layer over the cerebral hemispheres is indicated in lighter blue. (D–F) Transverse sections of an E10.5 head, as indicated in the drawing, showing the neural crest-mesoderm junction within the loose cranial mesenchyme (fine arrow) and at the fronto-parietal juxtaposition (broad arrow, D and E only); in (F) the continuity between frontal and squamosal neural crest can be seen. (G, H) E12.5: transverse sections at levels equivalent to those shown in (D) and (E), respectively; the cerebral hemispheres have extended back as far as the fronto-parietal junction (arrow), which now has the oblique orientation characteristic of the future coronal suture. (I) E13.5: transverse section through the cerebral hemispheres and cerebellum; X-gal-stained cells cover the cerebral hemispheres but not the diencephalon (all hindbrain staining is neural); a patch of stained cells is present in the dermis overlying the cerebellum (arrow). (J) E13.5: detail of the developing fronto-parietal area of a different specimen; the oblique junction between the X-gal-stained and unstained tissue (arrow) is now close to the position of the mature coronal suture. (K) E15.5: head, skin removed; the position of the caudal border of the cerebral hemisphere is indicated by a broken line. The caudal boundary of the neural crest domain is indented (arrow) except in the midline (arrowhead); the white arrow indicates the patch of interparietal staining (partially obscured by staining of the underlying brain). CB, cerebellum; CH, cerebral hemispheres; D, diencephalon; E, eye; F, frontal mesenchyme; FN, frontonasal neural crest; HB, hindbrain; MB, midbrain; Md, mandibular arch; P, parietal mesenchyme; S, squamosal mesenchyme; TEL, telencephalon; V, trigeminal ganglion; V1, ophthalmic branch of trigeminal nerve. Scale bars: A, C, G, H, J, 200 μm; B, D–F, 100 μm; I, 500 μm; K, 1 mm.



its mature position over the cerebral hemispheres at or soon after E13.5. Signals from the underlying cerebral hemispheres cannot therefore be involved in determining the site at which the suture forms, although they may play a role in initiating signalling activity; this is first detectable at E14, when *Twist* and *Fgfr2* expression are localised to the suture (Johnson *et al.*, 2000).

The neural crest–mesodermal tissue juxtaposition of the sagittal suture forms as the parietal mesoderm indents the caudal boundary of the frontal neural crest domain, leaving a strip of neural crest in the midline attached to the underlying falx cerebri. This observation is consistent with the pattern of growth of the parietal bone anlagen, which originate in a lateral position and grow up towards the vertex of the head (Iseki *et al.*, 1997; Rice *et al.*, 2000).

The effects of exposure to retinoic acid suggest that intramembranous ossification of the mesodermal parietal bone requires interaction with the underlying neural crest-derived meninges; in contrast, ossification of the neural crest-derived frontal bone appears to be autonomous. The mesodermal component of the skeletogenic membrane is revealed by these observations to consist of two layers: an outer osteogenic layer and an inner layer whose chondrogenic potential is normally expressed mainly in the areas between the bone anlagen. (These remarks do not apply to the endochondral bone of the occipital region and skull base.)

Validity of the Results

The above interpretation of our observations is based on the assumption that the *Wnt1-Cre/R26R* transgene combination is a reliable label for neural crest cells and their derivatives, as demonstrated by two previous studies (Jiang *et al.*, 2000; Chai *et al.*, 2000). In the second of these studies, a small but increasing number of X-gal-negative cells was observed to mingle with the X-gal-positive cells within the visceral cartilages; it was not clear whether these unstained cells were of nonneural crest origin, or whether a small subset of neural crest-derived cells fails to express the transgene. In our study of the lineage of the frontal and

FIG. 4. Effects of RA exposure at E10.0 on skeletogenic differentiation of the parietal mesenchyme. (A) Control, (B) RA-treated heads of E17.5 *Wnt1-Cre/R26R* fetuses stained with X-gal and Alizarin, showing decreased mineralisation of the parietal bone. (C) Control, (D) RA-treated E17.5 wild-type fetal heads double-stained with Alizarin and alcian blue, showing RA-induced decrease of parietal ossification and a lace-work pattern of ectopic cartilage in the parietal region. (E, F) Sections of the parietal bone of E17.5 control (E) and RA-exposed (F) skulls double-stained with Alizarin and alcian blue before sectioning: the normal parietal bone lies in a layer superficial to the cartilage of the temporal region, slightly overlapping it; in RA-treated fetuses the ectopic parietal cartilage lies beneath the dermal bone remnants, in the same tissue layer as the temporal and occipital cartilage. (G) Control, (H) RA-treated, transverse sections of the coronal suture region of E17.5 transgenic embryos: in the RA-treated specimen the ectopic cartilage lies beneath the layer of condensed parietal mesenchyme, and the meningeal neural crest is thin and discontinuous. (I) control, (J) RA-treated, E13.5 transgenic embryos, transverse sections of the caudal half of a cerebral hemisphere: migration of meningeal neural crest (arrowed) around the cerebral hemispheres is decreased in RA-exposed embryos. C, cartilage; CH, cerebral hemisphere; F, frontal; M, meninges; P, parietal; S, squamosal. Scale bars: A–D, 1 mm; E–H, 100 μm; I, J, 200 μm.

parietal bones no mixing of stained and unstained cells was observed in these two bones until the neural crest-derived frontal bone is invaded by blood vessels and develops a trabeculated form. However, the cartilage underlying the neural crest-derived component of the interparietal bone includes some unstained chondrocytes.

The tissue origin of the skull vault has been a matter of some controversy, following the publication of conflicting results using quail–chick grafting. Noden (1978, 1988) reported that the neural crest contributes only to the rostral part of the frontal bones, the remainder of the frontal bones, together with the parietal bones, being mesodermal. In contrast, Couly *et al.* (1993) described the frontal and parietal bones as being entirely neural crest-derived. However, the neural crest origin of the parietal bone is not clearly established in the report by Couly *et al.* (1993): the avian parietal bone is very small, and the histological section said to illustrate quail-derived cells in the parietal bone (their Fig. 5) does not in fact pass through that bone.

Neural crest cells arising from prA (r1 + 2) of the hindbrain do not express *Wnt1* or a *Wnt1-lacZ* transgene at presomite and post-neurulation stages (Echelard *et al.*, 1994; Brault *et al.*, 2001). Our results show that during early neurulation (early somite stages), the *Wnt1-Cre/R26R* transgene is expressed in the neural epithelium of prA, but only the lateral region. When these *lacZ*-positive crest cells emigrate, they leave a *lacZ* expression-free gap in the neuroepithelium between the midbrain and prB (r3 + 4) (Figs. 2G and 3C). This interesting observation confirms that crest cells from prA were not missed in our analysis of the skull vault origin, and suggests that *Wnt1* is expressed specifically in presumptive neural crest cells in prA.

The frontonasal and first branchial arch distribution of postmigratory neural crest cells at E9.5 shown by *Wnt1-Cre/R26R* reporter gene expression agrees with the results from other studies using a variety of methods, including quail–chick grafting (Noden, 1984, 1988), DiI labelling of mouse neural folds and subsequent embryo culture (Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994, 1996), and *AP2* gene expression (Brault *et al.*, 2001). Osumi-Yamashita *et al.* (1994, 1996) traced the prA neural crest to the most proximal region of the postmigratory domain. The quail–chick analysis of Köntges and Lumsden (1996) agrees with that result, and furthermore shows that the skeletogenic cells derived from this domain give rise to the squamosal bone and proximal elements of the jaws. Their study shows clear proximal boundaries of the squamosal neural crest tissue, with no contribution to the immediately adjacent parietal bone. These reports are all consistent with the interpretation that the *Wnt1-Cre/R26R* transgene-induced *lacZ* expression illustrated in our study is detecting all components of the skull derived from the forebrain, midbrain and rostral hindbrain neural crest. The evidence from our DiI results is complementary to these reports, tracing the lineage of the cephalic mesoderm that is present before neural crest cell emigration to the location that is *lacZ*-negative in the transgenic embryos at E9.5–E10.5, and which later forms the parietal bone.

The recent report that *Wnt1-Cre* conditional inactivation of *β-catenin* gene function results in a phenotype lacking neural crest derivatives (Brault *et al.*, 2001) appears at first sight to conflict with our observations, since both frontal and parietal bones fail to form in these mice. However, our RA experimental results indicate that parietal ossification depends on an interaction with the neural crest-derived meninges, so the absence of the parietal bones in these neural crest-deficient mice is consistent with our observations.

Sutural Tissue Origins and Organising Centre Function

Sutures are formed in the skull vault where the proliferating edges of two dermal bones are separated by a strip of mesenchyme. Our results show that in the coronal suture this mesenchymal strip is mesodermal, separating the neural crest-derived frontal bone from the mesodermal parietal bone. In the sagittal suture, the sutural mesenchyme is neural crest-derived, separating the two mesodermal parietal bones. Sutural growth and differentiation involves FGF signalling through FGFR1, -2, and -3, and transcription factors including TWIST and MSX2; mutations in each of these genes have been detected in human craniosynostosis, a condition characterised by premature loss of sutural growth centres (reviewed by Wilkie and Morriss-Kay, 2001). It is interesting to note that in mouse, *Twist* is expressed in the mesenchyme between and within the proliferating edges of the two bones in both the coronal and sagittal sutures (Johnson *et al.*, 2000; Rice *et al.*, 2000), i.e., in midsutural mesenchyme cells and osteogenic stem cells of both mesodermal and neural crest origins. These observations suggest that neural crest–mesodermal interfaces may play important roles in initiating these two sutures as signalling centres in which growth and differentiation are organised, but that their maintenance includes molecular components whose expression is not tissue origin-specific. Functional differences between the frontal and parietal bones are more clearly reflected in ossification defects of the skull vault, which most commonly (or most severely) affect the parietal bones (Gonzales-del Angel *et al.*, 1992; Wilkie *et al.*, 2000; Mavrogiannis *et al.*, 2001).

The dual tissue origins and molecular signalling characteristics of the coronal and sagittal sutures reflect Meinhardt's (1983) definition of an organising centre as a site where tissues of two different origins meet, and in which interaction between them results in the formation of a signalling system whose activity leads to developmental change.

Evolutionary Changes in the Neural Crest Component of the Skull Vault

The frontal and parietal bones show distinct evolutionary origins: the two frontal bones of tetrapods originated

by condensation of a group of small bones in the skull of lobe-finned fishes (sarcopterygians), but the parietals and postparietals have a much more ancient evolutionary lineage as large bones from early gnathostomes onwards (Ahlberg and Milner, 1994; Janvier, 1996). Application of the results of our study to the skull roof changes during the sarcopterygian-tetrapod transition suggests that these changes involved a major expansion of the neural crest territory in the skull roof (Morriss-Kay, 2001). If the avian frontal bones are of entirely neural crest origin as described by Couly *et al.* (1993), this process appears to have extended furthest in birds, in which the frontal bones extend as far back as the cerebellum. An alternative hypothesis for the origin of avian-mammalian skull vault differences is that the frontal and parietal bones fused in the reptilian ancestors of modern birds, as occurred in anuran amphibians (Goodrich, 1958). This idea is consistent with Noden's (1978, 1984) report of the dual neural crest/mesodermal origin of the frontal bones; it would also explain the apparent absence of avian postparietals, which are a general tetrapod feature, since the bones designated parietals in birds would then be postparietals.

Our observations may also shed light on the origin of the single mammalian interparietal bone from the paired postparietals of reptiles. Brault *et al.* (2001) observed that in the absence of neural crest, the interparietal bone was reduced to a narrow strip that was split into two in the midline. This raises the intriguing possibility that the patch of neural crest-derived dermal mesenchyme that forms the central area of the interparietal bone induces osteogenesis across the midline, thereby preventing formation of separate right and left post-parietal bones.

Relative to mammals, the avian skull undergoes late ossification and early sutural synostosis (Kardong, 1995). Late ossification would be essential in a skull vault in which one pair of bones (the frontals) forms the major roofing of the skull; no sutures are in a suitable position to form long-term growth centres contributing growth in the fronto-occipital plane, equivalent to the function of the mammalian coronal suture. The avian fronto-parietal suture is in the wrong position to play this role, and also differs from the mammalian coronal suture in not forming as a close juxtaposition between the two bones (illustrated in Bellairs and Osmond, 1998). These many differences between the avian and mammalian skull vaults suggest that different developmental mechanisms could have shaped the evolutionary changes generating their specific functional anatomical patterns.

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