

ORIGINAL ARTICLE

Katayoun Adab · Jennifer R. Sayne · David S. Carlson ·
Lynne A. Opperman

Nasal capsular cartilage is required for rat transpalatal suture morphogenesis

Received October 12, 2002; accepted in revised form August 7, 2003

Abstract In the cranial vault, suture morphogenesis occurs when the growing cranial bones approximate and overlap or abut one another. Patency of developing sutures is regulated by the underlying dura mater. Once cranial sutures form, bone growth proceeds from the sutures in response to growth signals from the rapidly expanding neurocranium. Facial sutures do not develop in contact with the dura mater. It was therefore hypothesized that facial suture morphogenesis and bone growth from facial sutures are regulated by tissues with an equivalent role to the dura mater. The present study was designed to test this hypothesis by characterizing the morphology and growth factor expression in developing transpalatal (TP) sutures and their surrounding tissues, and then assessing the role of the overlying nasal capsular (NC) cartilages in maintaining suture patency. TP sutures develop as overlapping sutures, similar to cranial coronal sutures, and expression of Tgf- β s in TP sutures was similar to their distribution in cranial coronal sutures. To establish whether NC cartilages play a role in regulating TP suture morphogenesis, fetal rat TP sutures were cultured with associated attached NC cartilages or with NC cartilages removed. Sutures cultured for upward of 5 days with intact NC cartilages remained patent and maintained

their cellular and fibrous components. However, in the absence of NC cartilages, the cellular nature of the sutures was not maintained and they became progressively acellular, with bony bridging across the suture. This finding is similar to that for cranial vault sutures cultured in the absence of dura mater, indicating that NC cartilages play an equivalent role to dura mater in maintaining the patency of developing sutures. These studies indicate that tissue interactions likely regulate morphogenesis of all cranial and facial sutures.

Key words transpalatal suture morphogenesis · nasal capsular cartilage · bone growth · craniofacial anomalies · craniosynostosis

Introduction

Except for the mid-palatal suture, the facial sutures do not fuse before the seventh decade (Cohen, 2000). Several syndromes with bone anomalies present with abnormal cranial vault growth and midface hypoplasia. However, while the bony obliteration of cranial vault sutures (craniosynostosis) that produces cranial anomalies is well described, premature obliteration of facial sutures is very uncommon (Cohen, 2000). Only brief reference is made to postnatal fusion of facial sutures in Crouzon and Apert syndromes (Kreiborg, 2000), and there is no mention in the literature of facial suture fusion in any other syndromes.

Bone growth from both cranial and facial sutures is thought to be a passive process in response to extrinsic forces (Moss, 1954; Scott, 1956; Babler, 1989). Various extraneous functional stimuli driving craniofacial bone growth have been proposed, including the possibility that the cartilaginous nasal septum provides the primary stimulus in the face (Scott, 1953a, 1953b, 1956; Moss, 1962; Sarnat, 1963) and the neurocranium

Katayoun Adab · Jennifer R. Sayne · David S. Carlson ·
Lynne A. Opperman
Department of Biomedical Sciences
Baylor College of Dentistry
Texas A&M University System Health Science Center
Dallas, TX 75246, USA

David S. Carlson · Lynne A. Opperman (✉)
Center for Craniofacial Research and Diagnosis
Baylor College of Dentistry
Texas A&M University System Health Science Center
Dallas, TX 75246, USA
Tel: (214) 828-8134, Fax: (214) 828-8951
e-mail: opperman@tambcd.edu

is the driving force in the cranial vault (Babler, 1989). However, dispute over the nature of this stimulus occurs to this day.

It is well established that the dura mater is required to preserve cranial suture patency (Opperman et al., 1995; Roth et al., 1996; Kim et al., 1998). This makes the dura mater the likely candidate for processing signals derived from the neurocranium and brain, because the dura mater secretes soluble heparin-binding factors that regulate suture patency (Opperman et al., 1996), including fibroblast growth factors (Fgfs) and transforming growth factors β (Tgf- β s).

Several mutations in Fgf receptors (*FGFR* 1, *FGFR2*, and *FGFR3*) and transcription factors (*TWIST* and *MSX2*) are associated with syndromes presenting with craniosynostosis (Jabs et al., 1993, 1994; Muenke et al., 1994; Reardon et al., 1994; Myers et al., 1995; Wilkie et al., 1995; Bellus et al., 1996). Furthermore, Tgf- β -related *NOGGIN*, *CDMP1*, and *TGIF* are associated with multiple synostoses syndrome, chondrodysplasia, and holoprosencephaly (Massague et al., 2000; Muenke and Beachy, 2000; Warren et al., 2003). A variety of growth factors have also been identified that regulate cranial suture morphogenesis. To date, *in vitro* experiments have identified Bmp2, Bmp4, Fgf2, Fgf4, Tgf- β 2, and Tgf- β 3 (Jabs et al., 1993; Roth et al., 1997; Kim et al., 1998; Opperman et al., 1999, 2000; Moursi et al., 2002; Ignelzi et al., 2003), and *in vivo* experiments have identified Fgf2, Tgf- β 2, and Tgf- β 3 (Chong et al., 2003; Opperman et al., 2002).

It is generally believed that facial sutures function in the same manner as cranial sutures during craniofacial bone growth (Kokich, 1986). However, most facial sutures do not contact the dura mater, and the equivalent tissue to the dura mater in the facial complex is unknown. It is therefore thought that facial and cranial sutures are regulated differently. In support of that idea, frontonasal sutures were found to have some of the same growth factors present as cranial sutures (Tgf- β 1, Tgf- β 2, and Tgf- β 3), but their localization during suture morphogenesis and postnatal development were different (Adab et al., 2002).

This study was designed to determine whether facial suture morphogenesis is regulated by tissue interactions, similar to dural regulation of cranial suture morphogenesis. Rat transpalatal (TP) sutures were examined *in vivo* for the distribution of *Tgf- β 1*, *Tgf- β 2*, *Tgf- β 3*, and *Msx2* mRNA and protein during embryonic and early postnatal development. These growth and transcription factors were chosen, as they play an integral role in suture morphogenesis and maintenance and could serve as possible pathways for intervention when mutations in other pathways result in sutural pathology. To test whether nasal capsular (NC) cartilages regulate transpalatal suture morphogenesis, fetal rat TP sutures were cultured in the presence or absence of the overlying NC cartilages and examined for suture

patency and associated changes in the distribution of Tgf- β s and *Msx2* over time.

Methods

Preparation of *in vivo* tissues for histology, immunohistochemistry, and RT-PCR

Fifty Sprague-Dawley rats (Harlan, Houston, TX) between the ages of embryonic day 16 (E16) and postnatal day 20 (P20) were used to examine the developmental morphology of TP sutures *in vivo*. The date of mating plug was considered day 0 and the day of birth as P0. All animals were euthanized by halothane overdose (Butler, Grand Prairie, TX).

Whole heads were used for histology and immunohistochemistry. Animals were decapitated, scalp tissues were removed, and specimens were fixed in 4% paraformaldehyde (Fisher Scientific, Houston, TX) at 4°C for 1 to 3 days. Larger postnatal specimens were perfused with 4% paraformaldehyde. After fixation, specimens were decalcified in 0.5 M EDTA (Sigma, St. Louis, MO), cut in a sagittal plane into right and left halves, dehydrated, cleared, and embedded in paraffin (Fisher Scientific). Between two and seven animals were used for each time point. Embedded samples were sectioned at 5–6 μ m thickness and mounted on glass slides prior to histological staining or immunohistochemistry.

For RT-PCR procedures, heads were dissected under RNase-free conditions and placed on ice in diethylpyrocarbonate (Sigma)-treated dishes containing Hank's balanced salt solution (GIBCO, Carlsbad, CA). The TP sutures with their associated palatal and maxillary bones and NC cartilages were dissected from the surrounding tissues. The bones were then dissected from the suture, leaving only a thin rim of the bony fronts attached to the suture. The dissected bone and sutures were placed on RNase-free glass slides on dry ice to rapidly freeze, wrapped in aluminum foil, and stored at -80°C prior to RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR).

Preparation and culture of fetal tissues

Twenty-day pregnant rats (plug day: day 0) were euthanized by overdose inhalation of halothane and the uteri removed and placed on ice. E20 fetal palates were dissected free of all surrounding tissues as described above. The prepared explants comprised palatal tissues from the middle of the maxilla to the presphenoid synchondrosis as the most posterior landmark of the rudiment. In control explants, the NC cartilages comprising the nasal septum and its peripheral attachments were left intact, except for minor trimming to reduce the bulk of cartilage. In explants randomly selected for the experimental group, all of the NC cartilages were removed, with care taken not to damage the underlying bone and presumptive suture. Explants were placed in 24-well culture dishes (Costar, VWR, Houston, TX), with the oral mucosa toward the bottom of the well. Explants were covered with 600 μ l serum-free medium (Opperman et al., 1995), supplemented daily with 6 μ l of vitamin C (100 μ g/ml; Sigma) and placed in a high-humidity 37°C incubator with 5% CO₂ in air. Medium was replaced every 2 days and explants were harvested after 3 and 5 days in culture.

After harvesting, explants were fixed and prepared for histology, immunohistochemistry, or RT-PCR. All organ culture experiments were repeated four times.

Histological staining

For histological analysis, paraffin was removed with xylene, and sections were rehydrated through a decreasing ethanol series and stained with hematoxylin and eosin (Fisher Scientific). Sections

were then dehydrated through an increasing ethanol series and covered with Permount (Fisher Scientific) and glass cover slips.

Immunohistochemistry

Immunohistochemical procedures were carried out as described previously (Opperman et al., 1997), with primary antibodies against Tgf- β 1, Tgf- β 2, and Tgf- β 3 used at 1:250 (polyclonal antibody raised in rabbit; R&D Systems, Minneapolis, MN) and secondary antibodies at 1:500 (peroxidase-conjugated mouse anti-rabbit IgG; Jackson Immunochemicals, West Grove, PA). Diaminobenzidine-tetrahydrochloride (Sigma) was used as chromagen (750 μ g/ml in PBS/ethanol at 1:7 v/v) plus hydrogen peroxide as substrate (1 μ l/ml). All sections were counterstained with Harris's hematoxylin (Butler) and cover slips secured with Permount.

RNA isolation and RT-PCR

Numbers of TP suture specimens collected for the RT-PCR procedure varied between four and six and each specimen was kept separate except for E18 sutures, where two samples were homogenized at the same time to ensure adequate yield of RNA. RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) and converted to first-strand cDNA as described (Adab et al., 2002) and stored at -20°C . One negative control (no reverse transcriptase added) was included in each experiment. Amplification of target cDNA (Tgf- β 2, Tgf- β 3, and *Msx2*), with glyceraldehyde 3-phosphate dehydrogenase (*Gpdh*) used as an internal control was completed with primers and PCR cycles and the product was run out on a 1.5% agarose gel with a DNA ladder as described (Adab et al., 2002).

Scoring immunohistochemistry, densitometry, and data analysis

Individual bands on digital micrographs of RT-PCR products were read by densitometer. *Gpdh* levels were expected to remain constant

in cells and this gene product was used as a loading control on gels. Densitometry numbers for *Gpdh* for each sample were set as 100% and intensity for experimental bands (Tgf- β 2, Tgf- β 3, and *Msx2*) was calculated as a percentage thereof.

For immunohistochemical scoring, the suture region was scored as osteoblasts lining the bone fronts, suture fibroblasts, suture matrix, and bone matrix on either side of the suture. The scores generated were expressed as (+) in Tables 1 and 2. In descriptions, 1+ to 2+ immunoreactivity were considered low, 3+ to 4+ moderate, and 5+ to 6+ as high expression. The median of the scores for at least six slides (two sections per slide) for each specimen was calculated.

Results

In vivo morphogenesis and growth of TP sutures

Histology using hematoxylin and eosin-stained sections was done to establish when and how TP sutures develop. At E16, the NC cartilages could be observed in the upper facial region overlying the presumptive palatal structures (Fig. 1B). Small islands of ossification representing the future palatal plate of the maxilla and horizontal plate of the palatal bone could be seen (Fig. 1C). The mesenchyme in the position of the future TP suture separated more cellular areas around the edges of the bone islands, resembling laterally polarized blastomas. By E18, the palatal process of the maxilla and the horizontal plate of the palatal bone were mineralized into plates of bone and the transpalatal suture had begun to develop (Fig. 1D). The bones proceeded to grow and overlap each other, with an elongated transpalatal suture continuing to form between them by E20 (Fig. 1E). By 5

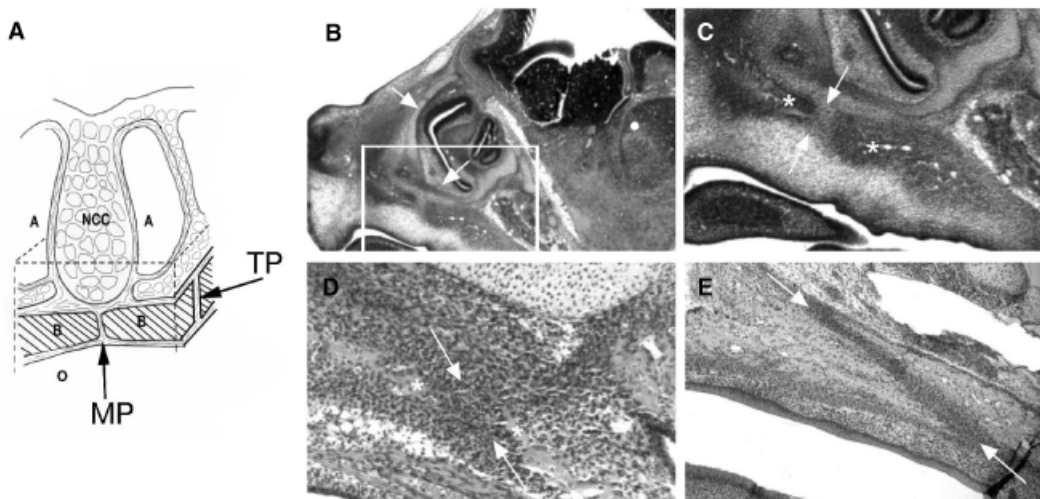


Fig. 1 (A) Diagram showing the relationship between the NC cartilages and the transpalatal suture. Dotted lines indicate cut lines for removing the palate from the embryo and the NC cartilage from above the sutures. (B–E) Micrographs of parasagittal sections through fetal rat heads, showing the prenatal development of TP sutures. (B) At E16, NC cartilages (arrows) can be seen directly above the presumptive TP suture region (in box). (C) High-power micrograph of the region in the box, showing the advancing palatal plate of the maxilla and horizontal plate of the palatal bone

(asterisks) on either side of the presumptive TP suture (between arrows). (D) At E18, the advancing bone fronts (asterisks) begin to overlap one another, creating a highly cellular suture blastema (between arrows). (E) By E20, an elongated TP suture (between arrows) continues to form as the bone fronts proceed to overlap one another. Magnification: (B) $4\times$, (C) $10\times$, (D) $40\times$, (E) $20\times$. A, airway; B, shelves of maxillary bones; MP, mid-palatal suture; NCC, nasal capsular cartilage; O, oral cavity; TP, transpalatal suture.

days after birth, the TP suture was well developed, with the bone fronts distinctly overlapping (not shown). As development progressed, the TP suture increased in size and became more complex in shape until by P20, the TP suture was highly complex and interdigitated (not shown).

Tgf-β2, *Tgf-β3*, and *Msx2* mRNA expression in TP sutures *in vivo*

RT-PCR using RNA isolated from E18 to P20 TP sutures was used to establish when mRNAs for growth and transcription factors appear and their relative levels within TP sutures. Gels run with *Tgf-β2*, *Tgf-β3*, *Msx2*, and *Gpdh* were digitized (Fig. 2A), subjected to densitometry, and data for experimental mRNAs expressed as a percentage of *Gpdh* (Fig. 2B). At E18, *Tgf-β2* expression was 90% of *Gpdh*, after which levels declined to 50% by P0. From P1 to P11 *Tgf-β2* expression remained relatively unchanged, and then declined to 40% that of *Gpdh* by P21.

At E18 and E20, *Tgf-β3* expression levels were 58%–60% of *Gpdh* levels, increasing to 80% by P1 and declining gradually to 20% of *Gpdh* levels by P21.

Msx2 expression levels increased from 28% of *Gpdh* levels at E18 to 60% at E20, and then leveled off at 30%–40% of *Gpdh* expression from P0 to P15, declining to 20% expression by P21.

Immunohistochemical localization of Tgf-β1, Tgf-β2, and Tgf-β3 in TP sutures *in vivo*

Immunohistochemistry was done to establish appearance and distribution of Tgf-βs during TP suture development. Tgf-β1 expression in TP sutures was concentrated in the osteoblasts lining the bone fronts of the sutures, with increased immunoreactivity from E20 to P20 (Table 1;

Figs. 3A,3D). Suture matrices lacked immunoreactivity until P20, when low levels of immunoreactivity were detected. Bone matrices showed sporadic low levels of immunoreactivity at E20 and P5, with no immunoreactivity present at other time points examined. No Tgf-β1 immunoreactivity was detected in suture cells. Sections used as negative controls showed a complete absence of chromagen in all tissues at all time points (see below).

Tgf-β2 immunoreactivity was absent in osteoblasts surrounding the bone fronts at E20 through P5, but was detected at low levels in osteoblasts from P11 to P20 (Table 1; Figs. 3B, 3E). Low levels of Tgf-β2 immunoreactivity were detected in the bone matrix at E20, but not at any other time points examined. Suture matrices had low levels of Tgf-β2 immunoreactivity at P11 and P15, with immunoreactivity absent at all other time points examined. Suture cells were immunoreactive for Tgf-β2 at P11, but were negative for Tgf-β2 immunoreactivity at all other time points examined.

Immunoreactivity for Tgf-β3 is shown in Table 1 and Figures 3C and 3F. Osteoblasts showed low levels of immunoreactivity at E20, increasing to moderate levels of expression at P1 and high levels by P5, remaining high through P20. In contrast, bone matrices had low levels of Tgf-β3 immunoreactivity at E20, with no immunoreactivity at any other time points examined. Both suture matrices and cells were negative for Tgf-β3 immunoreactivity until P5, with moderate levels of immunoreactivity noted in both the matrices and cells from P11 to P20.

In vitro morphogenesis and growth of E20 TP sutures in the presence and absence of NC cartilages

To establish the role of NC cartilages in TP suture morphogenesis, E20 TP sutures were cultured in the

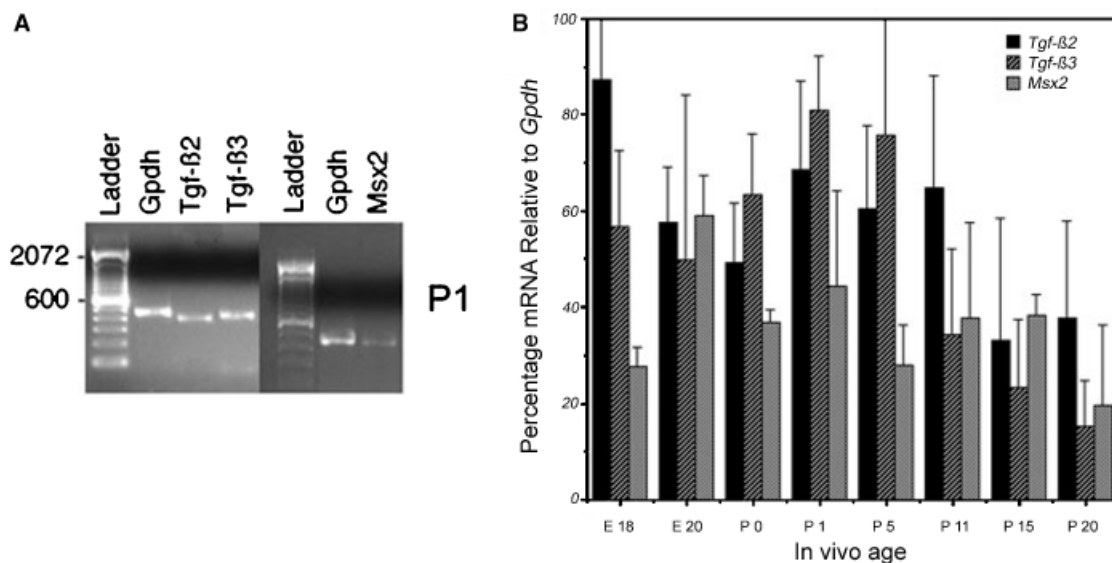


Fig. 2 (A) Photograph of RT-PCR products from a representative sample (P1) of TP sutures *in vivo* run on 1.5% agarose gels. (B) Graph of PCR products calculated as a percentage of *Gpdh*.

Table 1 Tgf- β 1, Tgf- β 2, and Tgf- β 3 immunoreactivity in TP suture of rat from E20 to P20

Growth factor	Age	Suture cells	Osteoblasts	Suture matrix	Bone matrix	n
Tgf- β 1	E20	–	++	–	+	4
	P1	–	+	–	–	4
	P5	–	+++	–	+	4
	P11	–	++++	–	–	4
	P15	–	++++	–	–	4
	P20	–	++++	++++	+	4
Tgf- β 2	E20	–	–	–	++	3
	P1	–	–	–	–	3
	P5	–	–	–	–	3
	P11	++	++	++	–	3
	P15	–	++	+	–	4
	P20	–	++	–	–	4
Tgf- β 3	E20	–	++	–	++	4
	P1	–	++++	–	–	4
	P5	++	+++++	++	–	3
	P11	++++	+++++	++++	–	4
	P15	+++	+++++	++++	–	4
	P20	++++	+++++	++++	–	4

n, number of sutures.

presence and absence of NC cartilages. After 3 days in culture, the thin palatal shelves and processes had grown toward each other and had abutted against each other (Figs. 4A, 4C). Many cuboidal osteoblast-like cells were densely packed around the bone fronts and on the outer and inner surfaces of the growing bone. Mesenchymal cells filled the suture matrix between the bone fronts. Fibroblastic cells were seen in the periosteal envelope surrounding the suture. In experi-

mental tissues cultured with NC cartilages removed, the palatal bones had grown toward each other, similar to that seen in the control cultures (Figs. 4B, 4D). However, the suture space between them was much more limited and the bone plates were thicker. Fewer osteoblastic and mesenchymal cells lined the bone fronts on either side of the suture. The periosteum surrounding the bones and sutures appeared thicker but less cellular than the periosteum surrounding control sutures.

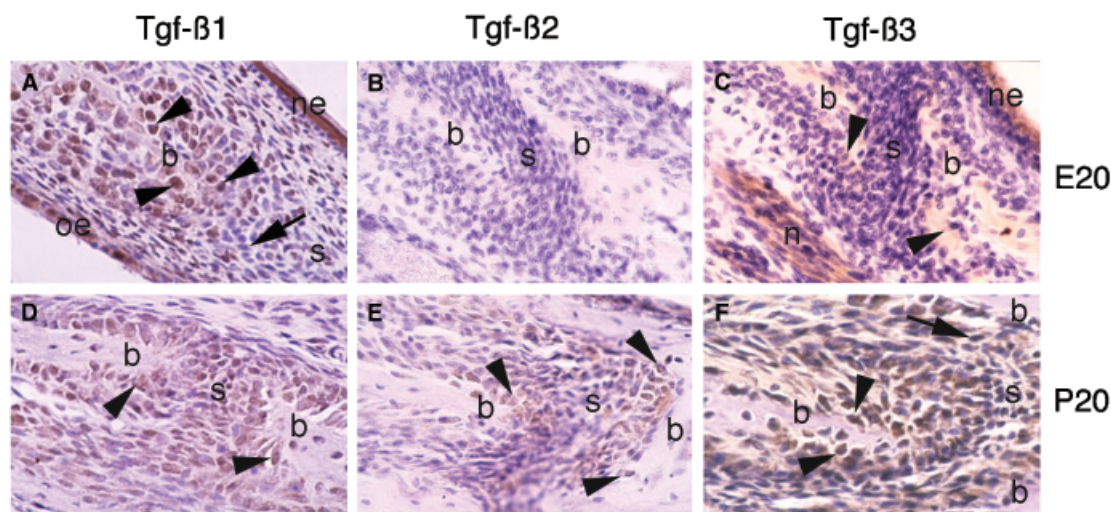
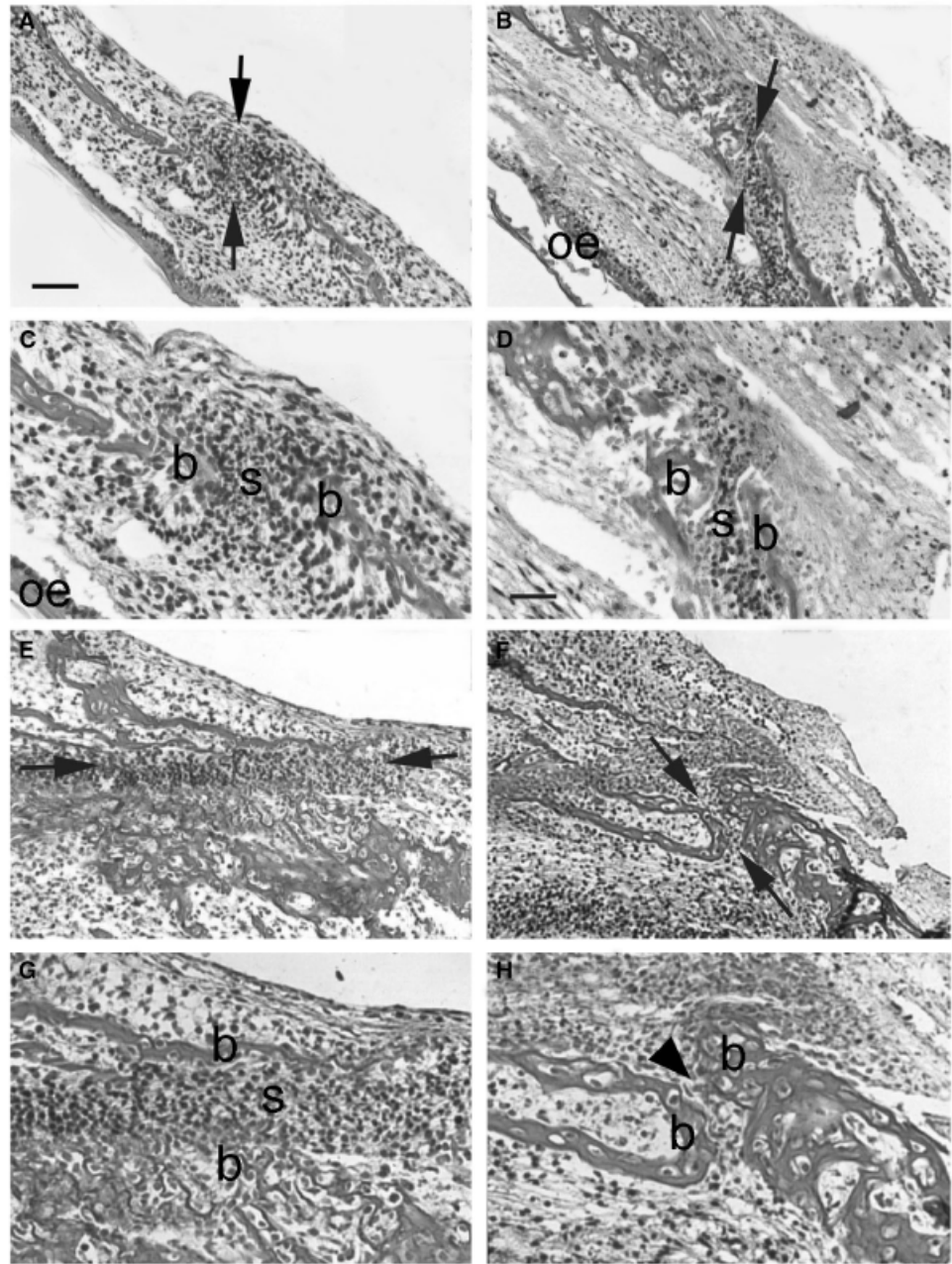


Fig. 3 Micrographs showing immunoreactivity for Tgf- β 1, Tgf- β 2, and Tgf- β 3 in TP sutures of E20 and P20 rats. (A) In E20 sutures, immunoreactivity for Tgf- β 1 is present in osteoblasts (arrowheads), but not in suture cells (arrows), and is absent in bone (b) and suture (s) matrices. Tgf- β 1 immunoreactivity can also be seen in the nasal epithelium (ne) above the suture and the oral epithelium (oe) below the suture. (B) Tgf- β 2 immunoreactivity is completely absent from all cells and matrices of E20 sutures. (C) Tgf- β 3 immunoreactivity in E20 sutures is low (arrowheads) to absent in all cells and

tissues, except in nasal epithelium (ne) and a small nerve (n). (D) At P20, large numbers of Tgf- β 1 immunoreactive osteoblasts can be seen (arrowheads). (E) Low levels of Tgf- β 2 immunoreactivity are present in osteoblasts (arrowheads), with no immunoreactivity in the suture (s). (F) Intense immunoreactivity for Tgf- β 3 can be seen in the osteoblasts lining unstained bone matrix (arrowheads). Low Tgf- β 3 immunoreactivity is present in the suture cells and matrix (s). Magnification 60 \times ; scale bar represents 150 μ m.

Fig. 4 (A and B) Low-power micrograph of a control culture (A) and an experimental culture (B) after 3 days, showing TP sutures (between arrows) forming between bone fronts that are beginning to overlap one another. (C and D) High-power micrographs of (A) and (B), respectively. (E) Low-power micrograph of a control culture showing highly overlapping bone fronts separated by an elongated TP suture (between arrows). (F) Low-power micrograph of an experimental culture after 5 days, showing the region between abutting bone fronts where the suture is missing (between arrows). (G and H) High-power micrographs of (E) and (F), respectively. Arrowhead in (H) points to the region where the suture has been obliterated. b, bone; s, suture; oe, oral epithelium. Magnification (A, B, E, and F) 50 \times ; scale bar represents 125 μ m. Magnification (C, D, G, and H) 100 \times ; scale bar represents 250 μ m.



After 5 days in culture, the palatal bones of control tissues had grown in thickness and the bone fronts had overlapped one other, and abundant suture matrix and cells were seen between the bones (Figs. 4E, 4G). In experimental cultures, the bone fronts did not overlap each other, but became thickened (Figs. 4F, 4H). The suture space between the bone fronts was markedly narrowed, with occasional bony bridges spanning the suture. Where intact sutures remained, the cellularity of the bone fronts and the sutures was reduced, producing a more fibrous suture than that seen in control cultures. Large osteocytes were visible embedded in their own calcified matrix, and bone marrow spaces could be seen in the center of the thickened bony plates.

Tgf- β 2, *Tgf- β 3*, and *Msx2* mRNA expression in TP sutures *in vitro*

RT-PCR was done on RNA isolated from E20 TP sutures after 3 or 5 days in culture, to establish whether growth factor and transcription factor mRNA levels were altered in TP sutures in culture and whether they were altered when sutures were cultured without NC cartilages. Gels run with *Tgf- β 2*, *Tgf- β 3*, *Msx2*, and *Gpdh* were digitized (Fig. 5A), subjected to densitometry, and data for experimental mRNAs expressed as a percentage of *Gpdh* (Fig. 5B). After 3 days in culture, *Tgf- β 2* expression levels in control tissues had increased from 58% (at E20 prior to culture) to 110% of *Gpdh*,

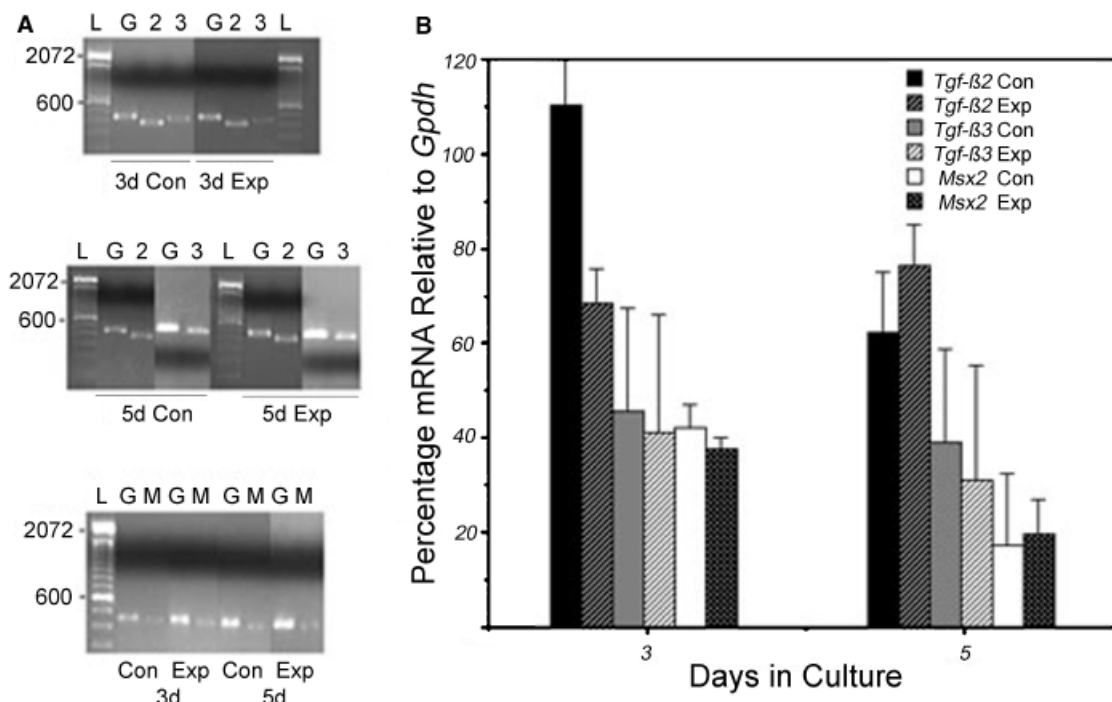


Fig. 5 (A) Photograph of RT-PCR products for *Gpdh* (G), *Tgf-β2* (2), *Tgf-β3* (3), *Msx2* (M), and DNA ladder (L) from a representative sample of TP sutures cultured for 3 (3d) and 5 (5d) days in the presence (Con) or absence (Exp) of NC cartilages, run on 1.5% agarose gels. (B) Graph of PCR products calculated as a percentage of *Gpdh*.

and then declined to 62% of *Gpdh* by 5 days in culture (Figs. 2B, 5B). Experimental tissues increased *Tgf-β2* expression to 68% of *Gpdh* by 3 days in culture, and continued to increase levels of expression to 76% of *Gpdh* by 5 days in culture.

After 3 days, *Tgf-β3* expression levels in control cultures were similar to those seen in comparable aged E20 tissues (46% and 50% of *Gpdh*, respectively), and declined to 39% of *Gpdh* by 5 days in culture. In experimental tissues, *Tgf-β3* expression levels declined to 41% of *Gpdh* after 3 days in culture and declined further to 31% of *Gpdh* by 5 days in culture.

In both control and experimental tissues, *Msx2* expression levels declined after 3 days in culture (from 59% of *Gpdh* to 42% and 39%, respectively) and declined further to 17% and 20%, respectively, of *Gpdh* after 5 days in culture.

Immunohistochemical localization of Tgf-β1, Tgf-β2, and Tgf-β3 in TP sutures *in vitro*

Immunohistochemistry was done to establish whether growth factor levels were altered in TP sutures in culture and whether they were altered when sutures were cultured without NC cartilages. Tgf-β1 immunoreactivity in control tissues after 3 days in culture was only detected at low levels in the osteoblast-like cells at the edges of the bone fronts on either side of the suture, and remained unchanged after 2 more days of culture

(Table 2; Fig. 6A). In experimental tissues after 3 days in culture in the absence of NC cartilages, Tgf-β1 immunoreactivity was similar to control tissues, with only the osteoblasts being immunoreactive. After 5 days, both suture cells and suture matrix of experimental tissues showed low levels of immunoreactivity for Tgf-β1, in addition to that seen in osteoblasts (not shown).

Tgf-β2 immunoreactivity in the control group after 3 days or 5 days in culture was confined to the osteoblasts, with no immunoreactivity in the bone or suture matrices (Table 2; Fig. 6B). In the experimental group, osteoblasts, suture cells, and suture matrix were all immunoreactive for Tgf-β2 after both 3 and 5 days in culture (Table 2; Fig. 6E).

Immunoreactivity for Tgf-β3 was present at high levels in all cells and in the suture matrices of control and experimental tissues at both 3 and 5 days in culture (Table 2; Figs. 6C, 6F). All tissues used as negative controls for immunohistochemistry were incubated in the absence of primary antibodies and showed no positive immunoreactivity (Fig. 6D).

Discussion

In this study, it was determined that the NC cartilages are necessary for maintaining TP sutures *in vitro*. In the presence of NC cartilages, a suture similar to that seen *in vivo* was formed, while in the absence of NC

Table 2 Tgf- β 1, Tgf- β 2, and Tgf- β 3 immunoreactivity in cultured E20 rat TP sutures

Growth factor	Days in culture	Plus (+) or minus (-) NC cartilages	Suture cells	Osteoblasts	Suture matrix	Bone matrix	n
Tgf- β 1	3	+	-	++	-	-	4
	5	+	-	++	-	-	4
	3	-	-	++	-	-	4
	5	-	+	++	+	-	4
Tgf- β 2	3	+	-	+	-	-	4
	5	+	-	++	-	-	3
	3	-	++	++	++	-	4
	5	-	+	+++	++	-	4
Tgf- β 3	3	+	+++++	+++++	++++	++	4
	5	+	+++++	+++++	++++	++	3
	3	-	+++++	+++++	++++	++	4
	5	-	+++++	+++++	++++	++	4

n, number of sutures.

cartilages, the bone fronts overlapped only marginally and in many places became fused to one another, obliterating the suture. This is similar to the obliteration of sagittal and coronal sutures seen *in vitro* when they were cultured in the absence of dura mater (Opperman et al., 1995; Kim et al., 1998). Hence, it is likely the NC cartilages play a similar role in TP suture morphogenesis to that of the dura mater during coronal or sagittal suture morphogenesis. The dura mater secretes soluble signals capable of keeping the suture open and the NC cartilages may secrete similar signals. However, it remains to be seen whether the NC cartilages rescue TP sutures from obliteration by secreting soluble factors, using co-culture experiments like those done with coronal sutures after removing dura mater, or using conditioned medium (Opperman et al., 1995,

1996). Cells within the dura mater express *Tgf- β 1*, *Tgf- β 2*, and *Tgf- β 3* (Opperman et al., 1997), making these candidate growth factors for regulating suture morphogenesis. Evidence that they do play a role in developing sutures is provided by experiments where obliteration of coronal sutures was induced when calvaria were cultured in the presence of neutralizing antibodies to Tgf- β 3 (Opperman et al., 1998) or in the presence of Tgf- β 2 (Opperman et al., 2000). In addition, sutures can be rescued from obliteration with Tgf- β 3, both *in vitro* (Opperman et al., 2000) and *in vivo* (Chong et al., 2003; Opperman et al., 2002).

Prenatal expression levels of *Tgf- β 2* and *Tgf- β 3* in TP sutures *in vivo* were similar to those seen in FN sutures (Adab et al., 2002). High expression of these mRNAs was seen before birth during the period of suture

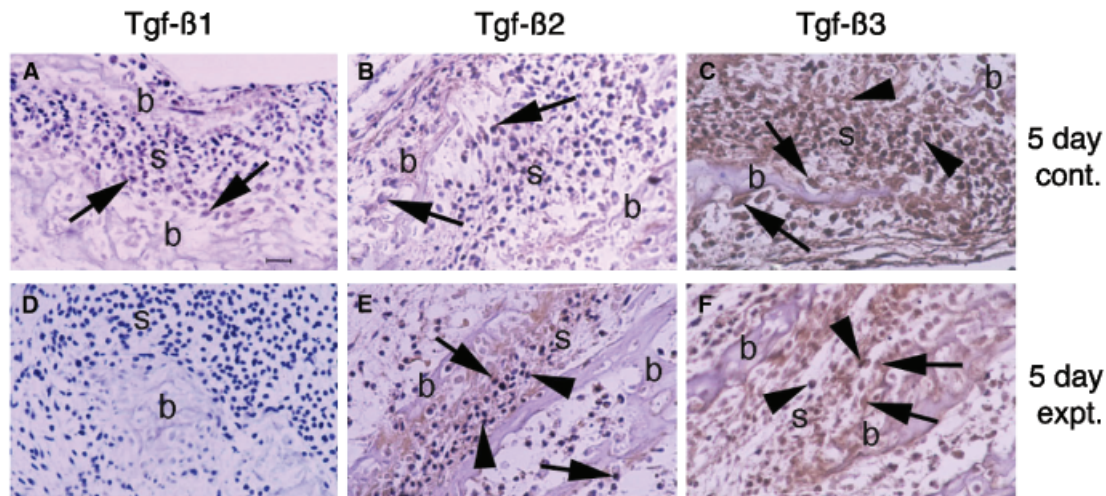


Fig. 6 Micrographs of immunohistochemical immunoreactivity for Tgf- β 1, Tgf- β 2, and Tgf- β 3 in TP sutures of E20 rats cultured for 5 days in the presence or absence of NC cartilages. (A) Pale Tgf- β 1 immunoreactivity (brown) is only seen in osteoblasts lining the bone fronts (arrows). (B and E) Immunoreactivity for Tgf- β 2 is only seen in osteoblasts after 3 days in culture (B, arrows) and in osteoblasts (arrows), suture cells (arrowheads), and suture matrix

after 5 days in culture (E). (C and F) Intense immunoreactivity for Tgf- β 3 is seen in the osteoblasts (arrows), suture cells (arrowheads), and suture matrices and low immunoreactivity in bone matrices of TP sutures cultured for 3 (C) and 5 (F) days. (D) Negative control tissue incubated in the absence of primary antibodies shows a complete absence of reaction product. b, bone; s, suture. Magnification 60 \times ; scale bar represents 150 μ m.

morphogenesis, and in the postnatal period during active growth with elongation of the snout. Interestingly, the decline in mRNA expression seen at birth in FN sutures was not seen in TP sutures. It is possible that the decline seen in FN sutures is due to birth trauma, when the head is deformed through the birth canal, as the FN sutures occur superficially under the epidermis. On the other hand, the TP sutures are found deep within the craniofacial tissues near the cranial base and would be protected from the forces exerted on the head at birth.

Protein expression in FN sutures *in vivo* reflected the pattern of mRNA expression seen in these sutures (Adab et al., 2002), but this correlation was not seen in TP sutures. This is not surprising, as a lack of correlation between protein and mRNA abundance has been reported previously (Gygi et al., 1999). In TP sutures, mRNA levels were elevated in the perinatal period, while protein expression was low to absent. Tgf- β 3 protein levels then increased in sutural and perisutural cells and tissues, being maintained at high levels up to the age of weaning. This is more similar to the protein expression levels noted in coronal and sagittal sutures (Opperman et al., 1997; Roth et al., 1997), although in these sutures, the expression of Tgf- β 1 and Tgf- β 2 was more elevated in the prenatal and early postnatal period than that seen in TP sutures. Differences in protein expression between the FN sutures and the TP, coronal, and sagittal sutures may reflect different growth patterns and rates of bone growth within the face and head. The bulk of the bone of the rat cranial vault is laid down postnatally, as brain growth is completed prior to weaning, similar to the rapid postnatal growth of the cranial vault seen in humans (Massler and Schour, 1951; Moss, 1954; Enlow, 2000). However, the facial bones of both humans and rats grow at a slower rate (Moss, 1954; Enlow, 2000). Because the palate has to grow rapidly soon after birth to accommodate the teeth, its growth rate would be more similar to the cranial vault growing to accommodate the expanding neurocranium, rather than the slower growing face. Hence, the appearance and distribution of growth factors within the TP sutures would be expected to be more similar to that seen in coronal and sagittal sutures than in FN sutures.

The Tgf- β 2 levels in cultured intact TP sutures were greatly elevated when compared to levels in sutures cultured without NC cartilages. However, the high level of Tgf- β 2 expression was not reflected by elevated Tgf- β 2 immunoreactivity in these tissues. As stated above, this result is not surprising, because Gygi et al. (1999) have shown that RNA and protein levels do not necessarily correlate. The reduced levels of protein may also reflect post-transcriptional regulation of protein expression in these growth factors. These data demonstrate the importance of looking at both RNA and protein expression, because the presence of RNA

does not necessarily reflect the presence of protein. Making assumptions about protein activity based on looking at RNA expression only may therefore be misleading.

Expression levels of Tgf- β 3 *in vitro* were lower than those seen *in vivo*. The culture environment appears to accelerate the morphogenetic process, so that after 5 days in culture, RNA levels have declined to levels comparable to those seen in more mature sutures. In contrast, Tgf- β 3 immunoreactivity was high in all cultured TP suture tissues and cells, while only control coronal sutures *in vitro* had high levels of Tgf- β 3 in their tissues. This finding could indicate that the regulation of suture patency by Tgf- β s depends less on high levels of Tgf- β 3 being present than it does on low levels of Tgf- β 2 being present. However, the effect of normal or high levels of Tgf- β 2 could be overcome by adding exogenous Tgf- β 3, rescuing coronal sutures from obliteration as has been shown both *in vitro* and *in vivo* (Opperman et al., 2000, 2002). The rescue of transpalatal sutures from obliteration by Tgf- β 3 has yet to be demonstrated for TP or FN sutures.

In conclusion, data presented here provide evidence that NC cartilages are required for the maintenance of TP sutures as bone growth sites. This strongly supports the theory that the NC cartilages regulate mid-facial bone growth. However, it does not provide evidence that the NC cartilages produce the extrinsic forces postulated to drive bone formation at the suture site (Moss, 1954; Scott, 1956). Furthermore, it appears that similarity between sutures is based on the growth rate of the bones surrounding the sutures, with sutures of rapidly expanding bones sharing distinct growth factor distributions that differ from sutures of slower growing bones. Hence, it can be predicted that not all facial sutures will respond the same to similar external stimuli, and sutures from different aged individuals will likely also respond differently. Interestingly, this implies that more accessible sutures like those of the cranial vault can be used to determine suture responses to various stimuli applied to similar but less accessible facial sutures supporting rapidly expanding bones.

Acknowledgments This research was funded in part by a grant from the National Institutes of Health National Institute of Dental and Craniofacial Research, grant number DE11978.

References

- Adab, K., Sayne, J.R., Carlson, D.S. and Opperman, L.A. (2002) Tgf- β 1, Tgf- β 2, Tgf- β 3 and Msx2 expression during rat frontonasal suture morphogenesis and postnatal facial growth. *Orthod Craniofac Res* 5:227–237.
- Babler, W. (1989) Relationship of altered cranial suture growth to cranial base and midface. In: Persing, J.A., Edgerton, M.T. and Jane, J.A. (eds) *Scientific foundations and surgical treatment of craniosynostosis*. Williams and Wilkins, Baltimore, pp 87–95.

- Bellus, G.A., Gaudenz, K., Zackai, E.H., Clarke, L.A., Szabo, J., Francomano, C.A. and Muenke, M. (1996) Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. *Nat Genet* 14:174–176.
- Chong, S.L., Mitchell, R., Moursi, A.M., Winnard, P., Losken, H.W., Bradley, J., Ozerdem, O., Azari, K., Acarturk, O. and Opperman, L.A., et al (2003) Rescue of coronal suture fusion using transforming growth factor- β 3 (Tgf- β 3) in rabbits with delayed-onset craniosynostosis. *Anat Rec* 274:962–971.
- Cohen, M.M.J. (2000) Craniosynostosis: diagnosis, evaluation, and management. Oxford University Press, New York.
- Enlow, D.H. (2000) Normal craniofacial growth. In: Cohen, M.M.J. and MacLean, R.E. (eds) Craniosynostosis: diagnosis, evaluation, and management. Oxford University Press, New York, pp 35–50.
- Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720–1730.
- Igelzli, M.A. Jr., Wang, W. and Young, A.T. (2003) Fibroblast growth factors lead to increased *Msx2* expression and fusion in calvarial sutures. *J Bone Miner Res* 18:751–759.
- Jabs, E.W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I.S., Klisak, I., Sparkes, R., Warman, M.L. and Mulliken, J.B., et al. (1993) A mutation in the homeodomain of the human *MSX2* gene in a family affected with autosomal dominant craniosynostosis. *Cell* 75:443–450.
- Jabs, E.W., Li, X., Scott, A.F., Meyers, G., Chen, W., Eccles, M., Mao, J.I., Charnas, L.R., Jackson, C.E. and Jaye, M. (1994) Jackson-Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. *Nat Genet* 8:275–279; erratum, 1995, *Nat Genet* 9:451.
- Kim, H.J., Rice, D.P., Kettunen, P.J. and Thesleff, I. (1998) FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. *Development* 125:1241–1251.
- Kokich, V.G. (1986) The biology of sutures. In: Cohen, M.M.J. (ed) Craniosynostosis: diagnosis, evaluation and management. Raven Press, New York, pp 81–103.
- Kreiborg, S. (2000) Postnatal growth and development of the craniofacial complex in premature craniosynostosis. In: Cohen, M.M.J. (ed) Craniosynostosis: diagnosis, evaluation and management. Oxford University Press, New York, pp 158–174.
- Massague, J., Blain, S.W. and Lo, R.S. (2000) Tgf β signaling in growth control, cancer, and heritable disorders. *Cell* 103:295–309.
- Massler, M. and Schour, I. (1951) The growth pattern of the cranial vault in the albino rat as measured by vital staining with alizarin red "S". *Anat Rec* 110:83–101.
- Moss, M.L. (1954) Growth of the calvaria in the rat. The determination of osseous morphology. *Am J Anat* 94:333–362.
- Moss, M.L. (1962) The functional matrix. In: Kraus, B.S. and Riedel, R.A. (eds) *Vistas in orthodontics*. Lea and Febiger, Philadelphia, pp 85–98.
- Moursi, A.M., Winnard, P.L., Winnard, A.V., Rubenstrunk, J.M. and Mooney, M.P. (2002) Fibroblast growth factor 2 induces increased calvarial osteoblast proliferation and cranial suture fusion. *Cleft Palate Craniofac J* 39:487–496.
- Muenke, M. and Beachy, P.A. (2000) Genetics of ventral forebrain development and holoprosencephaly. *Curr Opin Genet Dev* 10:262–269.
- Muenke, M., Schell, U., Hehr, A., Robin, N.H., Losken, H.W., Schinzel, A., Pulleyn, L.J., Rutland, P., Reardon, W. and Malcolm, S., et al. (1994) A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome. *Nat Genet* 8:269–274.
- Myers, G.A., Orlow, S.J., Munro, J.R., Przylepa, K.A. and Jabs, E.W. (1995) Fibroblast growth factor receptor 3 (FGFR3) transmembrane mutation in Crouzon syndrome with acanthosis nigricans. *Nat Genet* 11:462–464.
- Opperman, L.A., Passarelli, R.W., Morgan, E.P., Reintjes, M. and Ogle, R.C. (1995) Cranial sutures require tissue interactions with dura mater to resist osseous obliteration in vitro. *J Bone Miner Res* 10:1978–1987.
- Opperman, L.A., Passarelli, R.W., Nolen, A.A., Gampper, T.J., Lin, K.Y. and Ogle, R.C. (1996) Dura mater secretes soluble heparin-binding factors required for cranial suture morphogenesis. *In Vitro Cell Dev Biol Anim* 32:627–632.
- Opperman, L.A., Nolen, A.A. and Ogle, R.C. (1997) TGF- β 1, TGF- β 2, and TGF- β 3 exhibit distinct patterns of expression during cranial suture formation and obliteration in vivo and in vitro. *J Bone Miner Res* 12:301–310.
- Opperman, L.A., Chhabra, A., Nolen, A.A., Bao, Y. and Ogle, R.C. (1998) Dura mater maintains rat cranial sutures in vitro by regulating suture cell proliferation and collagen production. *J Craniofac Genet Dev Biol* 18:150–158.
- Opperman, L.A., Chhabra, A., Cho, R.W. and Ogle, R.C. (1999) Cranial suture obliteration is induced by removal of transforming growth factor (TGF)- β 3 activity and prevented by removal of TGF- β 2 activity from fetal rat calvaria in vitro. *J Craniofac Genet Dev Biol* 19:164–173.
- Opperman, L.A., Adab, K. and Gakunga, P.T. (2000) TGF- β 2 and TGF- β 3 regulate fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis. *Dev Dyn* 219:237–247.
- Opperman, L.A., Moursi, A., Sayne, J.R. and Wintergerst, A.M. (2002) Transforming growth factor- β 3 (Tgf- β 3) in a collagen gel delays fusion of the rat posterior interfrontal suture in vivo. *Anat Rec* 267:120–130.
- Reardon, W., Winter, R.M., Rutland, P., Pulleyn, L.J., Jones, B.M. and Malcolm, S. (1994) Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. *Nat Genet* 8:98–103.
- Roth, D.A., Bradley, J.P., Levine, J.P., McMullen, H.F., McCarthy, J.G. and Longaker, M.T. (1996) Studies in cranial suture biology: part II. Role of the dura in cranial suture fusion. *Plast Reconstr Surg* 97:693–699.
- Roth, D.A., Gold, L.I., Han, V.K., McCarthy, J.G., Sung, J.J., Wisoff, J.H. and Longaker, M.T. (1997) Immunolocalization of transforming growth factor β 1, β 2, and β 3 and insulin-like growth factor I in premature cranial suture fusion. *Plast Reconstr Surg* 99:300–309; discussion 310–316.
- Sarnat, B.G. (1963) Postnatal growth of the upper face. *Angle Orthod* 33:139.
- Scott, J.H. (1953a) The cartilage of the nasal septum. *British Dent J* 95:37–40.
- Scott, J.H. (1953b) Growth of the human face. *Proc Royal Soc Med* 47:91–100.
- Scott, J.H. (1956) Growth at the facial sutures. *Am J Orthod* 42:381–387.
- Warren, S.M., Brunet, L.J., Harland, R.M., Economides, A.N. and Longaker, M.T. (2003) The BMP antagonist noggin regulates cranial suture fusion. *Nature* 422:625–629.
- Wilkie, A.O., Slaney, S.F., Oldridge, M., Poole, M.D., Ashworth, G.J., Hockley, A.D., Hayward, R.D., David, D.J., Pulleyn, L.J. and Rutland, P., et al. (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nat Genet* 9:165–172.