Timing of Egf Treatment Differentially Affects Tgf-β2 Induced Cranial Suture Closure

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Premature suture obliteration results in an inability of cranial and facial bones to grow, with craniofacial dysmorphology requiring surgical correction as a consequence. Understanding signaling pathways associated with suture morphogenesis might enable non-invasive treatment of patients with fused sutures. Tgf-β2 induces premature suture fusion associated with increased cell proliferation both *in vitro* and *in vivo*. Tgf-β2 and Egf signal transduction pathways use some signaling proteins in common to regulate proliferation and differentiation, leading to speculation that these two pathways converge to regulate normal suture development. It was therefore hypothesized that Egf could induce suture fusion, and that Tgf-β2-induced suture closure occurred via an Egf-dependent pathway. A well-established fetal calvarial organ culture system was used to expose developing E19.5 fetal rat coronal sutures to Egf, Tgf-β2 and SC-120, a blocker of Egf receptor activity. Co-culture experiments examined the effect of Egf on Tgf-β2-induced suture closure when Egf was given either prior to or after Tgf-β2 treatment. Histomorphometric measurement of suture width was done on sagittal sections through coronal sutures harvested after 5 days in culture. Western blotting using phospho-antibodies against Egf receptors was used to confirm Egf receptor activity. Suture width increased with increasing concentrations of Egf, demonstrating that Egf-induced cell activity alone was not sufficient to cause premature suture obliteration. Egf administered prior to Tgf-β2 treatment rescued sutures from Tgf-β2-induced suture obliteration, demonstrating that pre-exposure of cells to this powerful mitogen prevented their response to signals induced by Tgf-β2. However, Egf added after Tgf-β2 treatment had no effect on Tgf-β2-induced suture closure. Blocking Egf activity after Tgf-β2 treatment rescued sutures from Tgf-β2-induced obliteration, indicating that Tgf-β2 required Egf activity to induce suture obliteration. Appropriate timing of signal generation by Egf and Tgf-β2 is critical for normal suture development and maintenance of suture patency.
Exp Biol Med 233:1518–1526, 2008

Key words: craniosynostosis; cranial sutures; Tgf-β2; Egf; intramembranous bone growth

Introduction

Cranial sutures have been increasingly studied as the major intramembranous bone growth sites of the cranial vault (1). During normal development, these sutures remain patent, allowing the cranial vault to expand to accommodate the growing brain. Craniosynostosis is a disease that results in the premature closure of sutures. Premature suture obliteration results in an inability of cranial and facial bones to grow, with craniofacial dysmorphology requiring surgical correction (2). Craniosynostosis is a frequently occurring condition, whose etiology is multifactorial and which exhibits genetic heterogeneity (2). Maintenance of suture patency depends on the regulation of a complex array of factors that include tissue interactions, mechanical influences, and biochemical signaling. For this reason, it is important to understand the links between different transcription factors, growth factors, and their receptors so that molecular intervention could limit the need for gross surgical repair.

Calvarial suture obliteration is associated with increased osteoblast proliferation and reduced suture cell apoptosis, induced by growth factors such as fibroblast growth factor 2 (Fgf2), bone morphogenic protein 4 (Bmp4), and transforming growth factor β2 (Tgf-β2) (3–6). Intracellular signaling pathways downstream of Fgf and Tgf-β have not been well studied in craniofacial suture development and maintenance. Fgf2 treatment has been shown to induce premature suture closure (3, 7, 8), and the

This research was supported by grant F30 DE014657 (JTR) and an intramural grant from the Office of the Vice-President for Research and Graduate Studies (LAO).

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Received May 7, 2008.
Accepted July 24, 2008.

DOI: 10.3181/0805-RM-151
1535-3702/08/23312-1518$15.00

Map kinase Erk1/2 signaling pathway was demonstrated to be required for Fgf-stimulated suture closure (7). Tgf-β2 has also recently been shown to induce suture closure by phosphorylating Erk1/2 (9). Further, Tgf-β2 increases *Egf* expression greater than 3-fold in gene array analyses (Rawlins, unpublished data).

Several cell proliferation and differentiation pathways involve stimulation of tyrosyl phosphorylation of Map kinases. Epidermal growth factor (Egf) is a potent mitogen known to use the Map kinase Erk1/2 pathway (10). Egf was shown to stimulate proliferation but inhibited differentiation in a number of cell types (11). Egf expression was found to be uniform across human osteoblast cell lines (12), and greatly increased thymidine uptake in primary cultures of rat osteoblasts (13). Additionally, Matsuda *et al*. found that Erk1/2 selectively mediated Egf proliferation of human osteoblasts (14).

Transgenic mice over expressing *Egf* showed greatly increased numbers of osteoblasts in the periosteum and endosteum of bones, but interestingly, cortical bone thickness was reduced in the transgenic mice, compared to wild-type mice (15). Osteoblasts derived from sagittal sutures of patients with craniosynostosis showed differences in Egf receptor (Egfr) distribution compared to osteoblasts from normal bone (16), indicating different responsiveness to Egf of cells from synostotic and non-synostotic sutures.

Tgf-β2 has been shown to increase cell proliferation and was associated with premature suture closure (4). Interactions between Tgf-β2 and other growth factors that regulate cell proliferation, such as Egf, may alter the equilibrium between proliferation, differentiation, and apoptosis, resulting in suture obliteration. Because Tgf-β2 and Egf signal transduction pathways can use similar signaling proteins to regulate proliferation and differentiation, it is reasonable to speculate that these two pathways may converge to regulate the development of sutures. Disregulation of this interaction could then result in abnormal or fused sutures. The hypothesis that was tested was that Egf induces suture obliteration, and that Tgf-β2-induced suture closure occurs via an Egf-dependent pathway.

Material and Methods

Animals and Calvarial Culture. Sprague-Dawley rats (Harlan, Houston, TX) were used for experiments using histology and western blot analysis. All animals were handled according to the institutional animal care and use committee guidelines at Baylor College of Dentistry. Due to the large numbers of fetal calvariae required for all experiments, each experiment was conducted independently and serially. Multiple pregnant females were needed, and each breeding batch produced slight variability in the ages of the fetuses at sacrifice. As little as 6–12 hours difference in gestation led to more highly developed sutures, resulting in slightly narrower sutures, or underdeveloped sutures, resulting in slightly enlarged suture widths. However, we noted that as long as we did not exceed 12 hours difference in gestation, the suture responses to the treatment regimens were the same. It is for this reason that we specifically reported the controls for each experiment separately, rather than averaging the numbers between experiments. Embryonic day 19.5 (E19.5) calvarial tissues were harvested from pregnant females (day of mating plug = day 0.5). Female rats were sacrificed on day 19.5 of pregnancy by inhalation overdose of Isofluorane anesthetic (Baxter, Grand Prairie, TX). The embryos were placed on ice, decapitated, and calvariae dissected as previously described (17). Briefly, calvariae were prepared to include frontal and parietal bones with intervening intrafrontal, coronal and sagittal sutures, and with intact dura mater. Following organ culture, tissues were harvested and used for histology, immunohistochemistry, and protein isolation.

Calvarial Organ Culture. E19.5 rat calvariae were placed in tissue culture with 400 μl serum free medium (SFM) for 5 days as previously described (17).

Effect of Egf on Suture Width. Calvariae with intact dura mater were cultured in the presence of 0, 10, 20, and 40 ng/ml Egf (R&D Systems, Minneapolis, MN). After 5 days in culture, calvariae were harvested from organ culture and prepared for histology and histomorphometry. After establishing the appropriate Egf concentration from this experiment, three separate experiments were performed using E19.5 rat calvariae.

Effect of Tgf-ß2 Treatment Followed by Addition of Egf. Calvariae were cultured in SFM. Group 1 (control calvariae) remained unsupplemented. Group 2 was supplemented with 20 ng/ml Egf, and Group 3 with 3 ng/ml Tgf-β2 for the duration of culture. Group 4 was supplemented with 3 ng/ml Tgf-β2, with 20 ng/ml Egf added to the cultures at 48 hours ($n = 5$–7 calvariae per group). After 5 days in culture, calvariae were harvested from tissue culture and prepared for histology and histomorphometry.

Effect of Egf Treatment Followed by Addition of Tgf-ß2. Calvariae were cultured in SFM. Group 5 (controls) remained unsupplemented. Group 6 was supplemented with 20 ng/ml Egf, and Group 7 with 3 ng/ml Tgf-β2 for the duration of culture. In Group 8, 20 ng/ml Egf was added for the duration of the culture period, and 3 ng/ml Tgf-β2 was added to the cultures after 48 hours ($n = 5$–7 calvariae per group). After 5 days in culture, calvariae were harvested from tissue culture and prepared for histology and histomorphometry.

Effect of Egf Receptor Inhibitor sc-120 on Tgf-ß2 Activity in Sutures. Egfr blocker (sc-120; Santa Cruz Biotechnology, Santa Cruz, CA) was used to test the effect of blocking Egfr signaling before or after Tgf-β2 treatment. Calvariae were cultured in SFM alone (control, Group 9), 50 mM sc-120 (Group 10), 3 ng/ml Tgf-β2, with 50 mM sc-120 plus 20 ng/ml Egf added at 48 hours (Group 11), 50 mM sc-120 with 3 ng/ml Tgf-β2 plus 20 ng/ml Egf added at 48 hours (Group 12), and 3 ng/ml Tgf-β2 plus 50 mM sc-

120 with 20 ng/ml Egf added after 48 hours (Group 13). $N =$ 5–7 calvariae per group. After 5 days in culture, calvariae were harvested from tissue culture and prepared for histology and histomorphometry.

Histology and Histomorphometry. Calvariae harvested from culture were fixed in 4% paraformaldehyde (Fisher Scientific). After fixation, specimens were decalcified in 0.5 M EDTA (Sigma). Specimens were then cut into right and left halves through the sagittal and intrafrontal sutures, dehydrated, cleared, and embedded in paraffin (Fisher Scientific) for coronal sectioning ($n = 10$–14 coronal sutures per experiment). Samples were sectioned at 6 μm thickness, mounted on glass slides and stained with hematoxylin and eosin. Microscope images were captured using a Kodak SPOT camera, and the digitized images were transferred to a Metamorph® program, which was used to measure suture width. Suture fusion does not occur as an all-or-nothing phenomenon, but rather appears as "spot welds" of bone across the suture, spread out throughout the suture. When measurements of suture width are taken, these areas of bony fusion give values of 0, but in between the regions of fusion, sutures can be several micrometers wide. Sutures with more regions of fusion also tend to have narrower sutures. While it is therefore difficult to quantify degree of fusion, a numerically reported suture width accurately reflects the degree of fusion. Suture width was measured at the shortest distance between the overlapping bone fronts. Data is presented as means plus/minus standard deviations. A two-tailed, unpaired t test using GraphPad software was used to establish statistical significance. Alpha $= 0.001$ was considered significant.

Western Blotting. Fetal day E19.5 rat calvariae were cultured in SFM supplemented with 0 or 20 ng/ml Egf (R&D Systems, Minneapolis, MN), in the presence of 0 or 50 mM sc-120 (Santa Cruz Biotechnology, Santa Cruz, CA), and in the presence of 20 ng/ml Egf plus 50 mM sc-120. These concentrations were previously determined to be functional (18).

Calvariae were harvested immediately (0 minutes), or after 15, and 30 minutes in culture, with 3–4 calvariae per time point. Whole calvariae were homogenized in PBS containing 1 μg/ml protease inhibitor cocktail and 1 μl/ml phosphatase inhibitor (Sigma, St. Louis, MO). Protein concentrations were calculated, and 10 μg/ml protein was loaded onto commercially available 12% Tris-glycine gels (Invitrogen, Carlsbad, CA), and gels run for 2.5 hours at 100 Volts on an XCell Sure-Lock® apparatus (Invitrogen), prior to blotting onto PVDF Immobilon®-P membranes (Millipore, Bedford, MA) using a semi-dry blotter (Buchler Instruments, Wrightsville, PA).

Nitrocellulose blots were immediately placed into blocking solution (GenoTechnology, Inc., St. Louis, MO) overnight, after which primary antibodies for phospho-Egfr (sc-12351; Santa Cruz Technologies, Santa Cruz, CA) were added for 24 hours at 1:100. Primary antibodies were then washed off three times with seven-minute washes with TTBS (1 mM TBS plus 20% Tween), prior to addition of secondary antibodies—peroxidase-conjugated rabbit anti goat (1:5000; Jackson ImmunoResearch, West Grove, PA) for phospho-Egfr for 2.5 hours. Secondary antibodies were washed off three times with seven-minute washes with TTBS, and the color reaction developed using a Vector DAB staining kit (Vector Laboratories Inc., Burlingame, CA). Blots were allowed to air dry prior to digitizing, and densitometry was performed using Scion Image (Scion Corporation, Frederick, MD). Densitometry was used for descriptive purposes only, and no statistical analysis was performed.

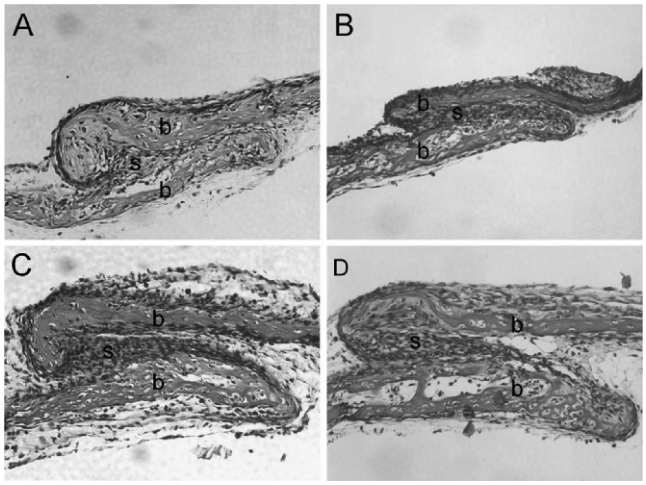

Figure 1. Photomicrographs of H & E stained sagittal sections through E19.5 fetal rat coronal sutures after 5 days in culture. A, Control coronal suture cultured in the absence of Egf. B, Coronal suture cultured in the presence of 10 ng/ml Egf, showing widening and increased cellularity of the suture. C, Coronal suture cultured in the presence of 20 ng/ml Egf showing greater width and cellularity of the suture when compared to sutures cultured with 0 or 10 ng/ml Egf. D, Coronal suture cultured in the presence of 40 ng/ml Egf showing little difference from sutures cultured with 20 ng/ml Egf. (Original magnification ×20; s, suture; b, bone.)

Results

Increasing Concentrations of Egf Induce Increased Suture Width. To examine whether Egf levels were responsible for accelerating suture closure seen in Tgf-β2 treated calvariae, E19.5 rat calvariae were cultured in the presence of increasing concentrations of Egf. Sutures cultured in all concentrations of Egf remained patent throughout the culture period. In control sutures, a narrow suture was seen situated between thickened overlapping bone fronts (Fig. 1). The cellularity and width of the suture increased as increasing amounts of Egf were added to cultures, and reached a plateau between 20 and 40 ng/ml. The thickness of both the periosteum and dura mater also increased with increasing amounts of Egf, especially the osteogenic layer closest to the bones. However, the bones did not appear to increase in thickness with increasing concentrations of Egf. Analysis of photomicrographs showed increased numbers of cells within the sutures and

Figure 2. Graph showing changes in coronal suture width following 5 days in culture with and without Egf. Note increased width of sutures induced by increasing concentrations of Egf.

lining the bone fronts in all calvariae treated with Egf; however, these differences were not quantified.

Suture width was measured as the shortest distance between the overlapping bone fronts. Suture width showed a dose dependent increase with increasing concentrations of Egf—53% from 10 ng (112.5 ± 63.4 μm), to 20 ng (239.2 ± 156.7 μm), with a plateau at 40 ng (242.4 ± 139.8 μm; Fig. 2). There was a significant increase in suture width from 0 ng to 20 ng/ml Egf ($P < 0.001$), but no significant differences in width between 0 ng and 10 ng, or 10 ng and 20 ng/ml Egf.

Tgf-β2 Treatment Followed by Egf Induces Suture Fusion. To test whether the effect of Egf on sutures is altered by Tgf-β2 treatment, E19.5 rat calvariae were cultured with Tgf-β2, and 20 ng/ml Egf was added at 48 hours in culture. In Group 1 (control) cultures with no Tgf-β2 or Egf added, a narrow but uniformly patent suture was seen situated between thickened overlapping bone fronts (Fig. 3A). In the presence of Egf alone (Group 2), the coronal suture widened with increased cellularity within the suture matrix and along the overlapping bone fronts as described above (Fig. 3B). In Group 3, the addition of Tgf-β2 alone narrowed the suture compared to controls or induced suture fusion (Fig. 3C). In Group 4, when Egf was added at 48 hours to samples that had been cultured in the continuous presence of Tgf-β2, sutures appeared similar in width to sutures cultured with Tgf-β2 alone (Fig. 3D). However, a consistent finding was a marked increase in the thickness and cellularity of the dura mater and the periosteum in samples treated with Tgf-β2 followed by Egf when compared to the other treatment groups.

Measurements of suture widths confirmed that addition of Egf significantly ($P < 0.0002$) increased suture width by 42% when compared to control sutures (154.2 ± 29.2 μm vs. 88.6 ± 15.4 μm) (Fig. 4). The addition of Tgf-β2 significantly ($P < 0.0001$) narrowed coronal sutures by 60% (to 35.1 ± 11.2 μm) compared to controls, and the combination treatment of Tgf-β2 followed by Egf resulted in sutures significantly ($P < 0.0001$) narrowed by 70% (to 26.6 ± 11.8 μm) when compared to control suture width (Fig. 4). Coronal suture fusion rates were 70% with the combination treatment compared to 60% when treated with Tgf-β2 alone, but this difference was not significant.

Egf Treatment Followed by Tgf-β2 Rescues Sutures from Obliteration. Increasing concentrations of Egf increased suture width, but Egf added after Tgf-β2 treatment exacerbated Tgf-β2 induced suture closure. Therefore, the ability of Egf to prevent Tgf-β2 induced obliteration was tested by culturing calvariae with Egf for 48 hours before addition of Tgf-β2. Compared to Group 5 controls, suture width increased with the addition of Egf (Group 6) as shown previously, and Tgf-β2 treatment (Group 7) induced fusion or accelerated suture narrowing in all of the samples (Fig. 3B, C). Analysis of photomicrographs showed increased cellularity within the sutures and lining the bone fronts in all calvariae treated with Egf compared to the other treatment groups (Fig. 3B, D, E). In Group 8, no thickening or increased cellularity was observed in the dura mater or periosteum when Egf was added prior to Tgf-β2 stimulation at 48 hours.

Confirming the histological assessment, and similar in significance levels to the previous experiment, suture width decreased 52% with Tgf-β2 treatment compared to control sutures (28.9 ± 8.3 μm vs. 61.1 ± 27.7 μm), while Egf increased suture width by 57% (to 142 ± 47.1 μm) (Fig. 5). The width of sutures treated with Egf prior to addition of Tgf-β2 was not significantly different from the width of control sutures.

Egf Receptor Inhibitor sc-120 Disrupts Tgf-β2 Activity in Sutures. To confirm sc-120 blocked Egfr signaling, calvariae were cultured with and without Egf and with and without sc-120. Egf stimulated cultures showed upregulation of Egfr-phosphorylation (Egfr-P) after 30 minutes exposure to Egf (Fig. 6). Upregulation of Egfr-P was completely blocked by the addition of sc-120.

Since timing of Egf treatment differentially regulated Tgf-β2 activity in sutures, fetal day 19.5 rat calvariae were cultured with sc-120 either before or after treatment with Tgf-β2. Measurements of suture width showed that the addition of sc-120 alone (Group 10) to calvarial cultures did not have an effect on suture width compared to Group 9 control untreated sutures (Fig. 7). Treatment of sutures with sc-120 either before or concurrent with treatment with Tgf-β2 (Groups 11, 12 and 13) prevented Tgf-β2 induced suture narrowing (Fig. 7). There were no significant differences in suture width between any of the groups.

Discussion

The Tgf-β family of growth factors is part of a complex signaling network that is involved in cranial suture morphogenesis (6, 8, 19). Recently, mutations in the human *TGF-β TYPE 1* (*TGFβRI*) and *TYPE 2* (*TGFβRII*) receptors were shown to produce defects phenotypically resembling

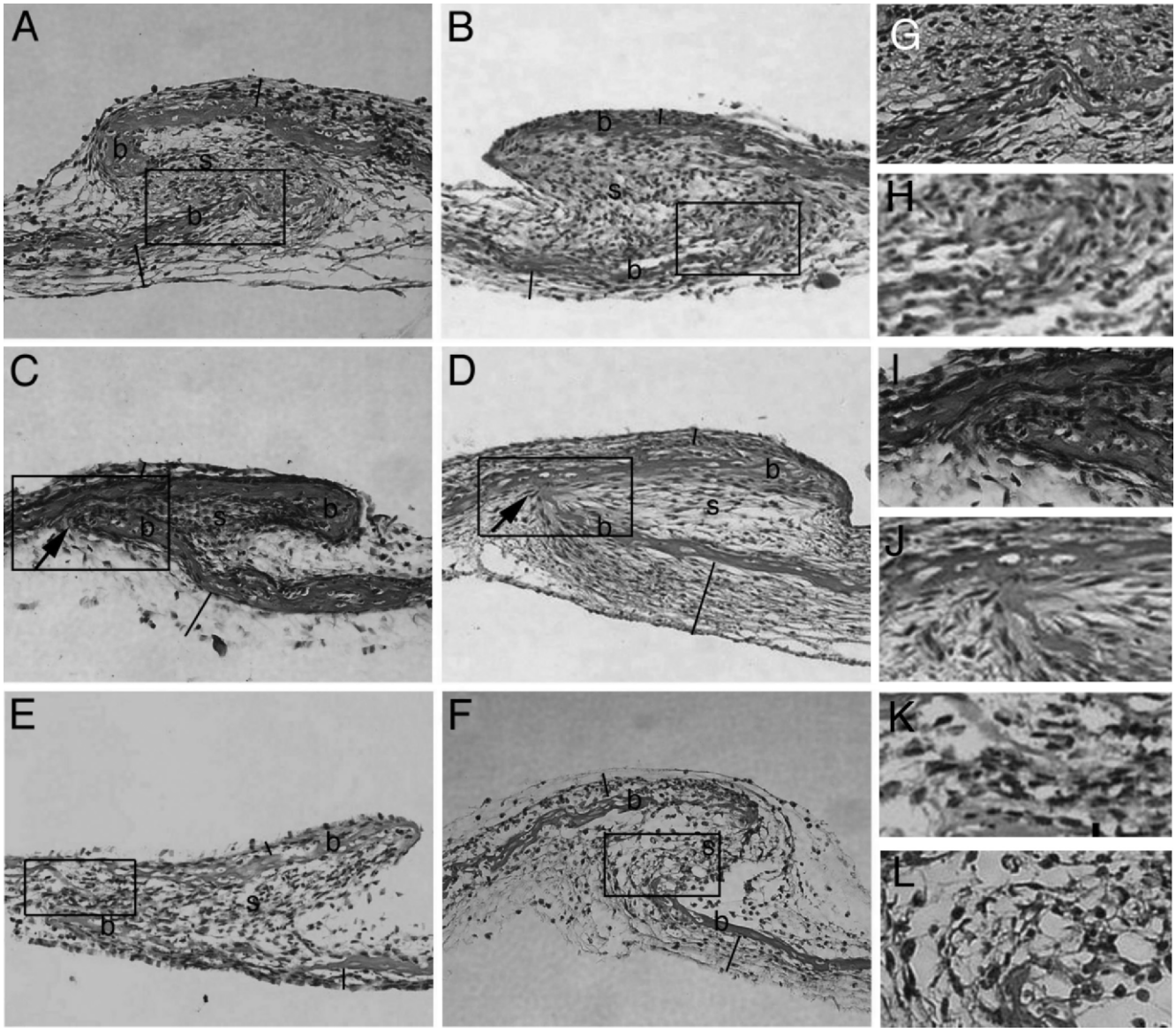

Figure 3. Photomicrographs of H & E stained sagittal sections through E19.5 fetal rat coronal sutures after 5 days in culture. A, Group 1 control coronal suture of calvaria cultured without Tgf-β2, Egf, and sc-120. B, Group 2 coronal suture of calvaria cultured in the presence of 20 ng/ml Egf, showing greater width and cellularity of the suture when compared to control sutures. C, Group 3 coronal suture of calvaria cultured in the presence of 3 ng/ml Tgf-β2 showing bony fusion across the suture (arrow). D, Group 4 coronal suture cultured in the presence of 3 ng/ml Tgf-β2, with 20 ng/ml Egf added after 48 hours in culture. Note fusion of bone fronts across the coronal suture (arrow), and increased thickness of the dura mater and periosteum compared to all other treatment conditions. E, Group 8 coronal suture of calvaria cultured in the presence of 20 ng/ml Egf, with 3 ng/ml Tgf-β2 added after 48 hours in culture, showing the prevention of Tgf-β2 induced coronal suture closure by Egf. F, Group 13 coronal suture of calvaria cultured in the presence of 3 ng/ml Tgf-β2 and 50 mM sc-120, with 20 ng/ml Egf added after 48 hours in culture. Sutures cultured with Tgf-β2 and sc-120 showed lack of overlap of the bone fronts, and similar cellularity and bone quality to sutures in (E). All calvariae are shown with the dura side on the bottom, and periosteal surface at the top. A bar delineates the thickness of each in each panel. G–K represent high power images of the areas in the boxes of A–F respectively. (Original low power magnification ×20; high power magnification ×40; s, suture; b, bone.)

Marfanoid craniosynostosis syndrome (20). It has been shown that differential expression of various Tgf-β isoforms plays a crucial role in regulating suture patency once the sutures have formed (6, 21–23). It has also been shown that differential expression of Tgf-β isoforms may also play a role in craniosynostosis by altering proliferation, differentiation, and apoptosis within the suture (4). Tgf-β2 has been demonstrated to induce premature suture closure both in vitro and in vivo, associated with increased proliferation (24–26). Both Tgf-β2 and Fgf2 have also been shown to require upregulated Erk1/2 signaling to induce narrowing or closure of sutures (7, 9, 27). Egf is a mitogen that can also signal via Erk1/2 phosphorylation, and gene arrays showed upregulation of *Egf* expression in Tgf-β2-treated sutures (Rawlins, unpublished data). This latter observation led to the investigation of the role played by Egf in Tgf-β2

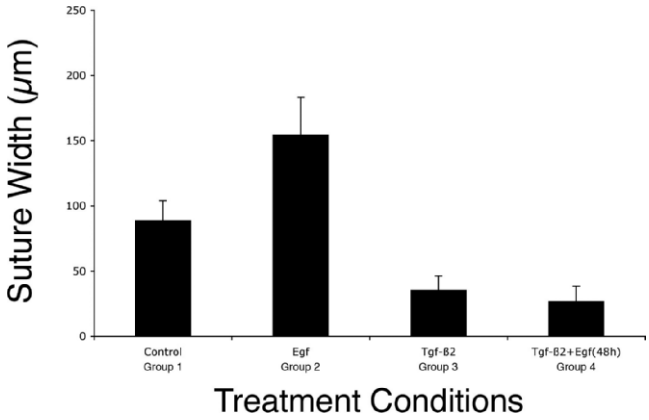

Figure 4. Graph showing coronal suture width following 5 days in culture in the presence of Egf, Tgf-β2, and Tgf-β2 followed by Egf treatment at 48 hours in culture. Note the decreased suture width in the group pretreated with Tgf-β2 followed by Egf at 48 hours in culture decreases suture, similarly to that seen with Tgf-β2 treatment alone.

regulation of suture width. Interestingly, addition of Egf prior to stimulation with Tgf-β2 prevented Tgf-β2 induced suture fusion. However, Egf added after Tgf-β2 stimulation was unable to prevent Tgf-β2 induced suture closure and appeared to accelerate the rate of fusion induced by Tgf-β2.

Previous experiments showed that Tgf-β2 induced cell proliferation and decreased apoptosis in sutures, and it was postulated that the increased numbers of cells disrupted the balance between proliferation and differentiation, accelerating differentiation of suture cells into osteoblasts (4). Because Tgf-β2 upregulated *Egf* expression, it was hypothesized that cell proliferation induced by Egf could induce suture obliteration similar to that seen with Tgf-β2 alone. Contrary to this expectation, Egf resulted in thickening of the sutures, increasing the width between bone fronts. This increased thickening was accompanied by dense cellularity within the suture and surrounding tissues

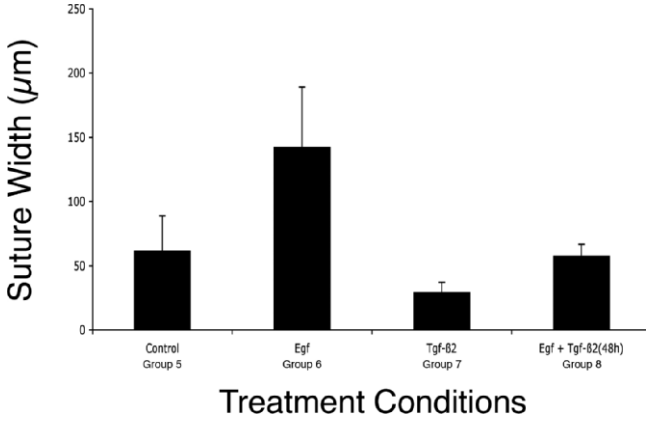

Figure 5. Graph showing coronal suture width following 5 days in culture in the presence of Egf, Tgf-β2, and Egf followed by Tgf-β2 treatment at 48 hours in culture. Note that sutures pretreated with Egf followed by the addition of Tgf-β2 at 48 hours in culture rescues coronal sutures from Tgf-β2 induced suture obliteration.

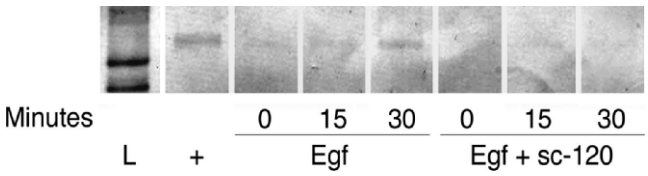

Figure 6. Western blot for phosphorylated Egfr (Egfr-P) in E19.5 whole rat calvaria. Note upregulation of Egfr-P following stimulation with Egf at 30 minutes, and down regulation of Egfr-P after blocking with sc-120. (+) indicates commercially bought positive control for Egfr-P.

of cultured fetal rat calvariae. This finding suggested that increased cellularity on its own was not sufficient to induce suture obliteration.

In contrast to the finding of wide-open sutures with Egf pretreatment, most sutures fused when calvariae were pretreated with Tgf-β2 prior to the application of Egf. This finding was not unexpected, given that Tgf-β2 treatment alone narrowed sutures and increased Egf expression in sutures. These findings demonstrate that the timing of gene upregulation or down regulation is a critical element that determines the fate of cranial sutures. By reversing the order of Egf and Tgf-β2 treatment, coronal sutures can be induced to fuse or remain patent *in vitro*. It would be interesting to test if exposure of calvariae to Egf in vitro for longer periods of time ultimately resulted in suture obliteration, or if Tgf-β2 is the crucial signal needed for suture osteoblast differentiation.

Since Egf exposure before but not after Tgf-β2 treatment can reverse Tgf-β2 induced suture narrowing, it was postulated that blocking Egf receptor activity before Tgf-β2 treatment would reverse the Egf rescue effect. Surprisingly, blocking Egfr prior to Tgf-β2 treatment did not reverse Egf rescue of sutures from Tgf-β2-induced

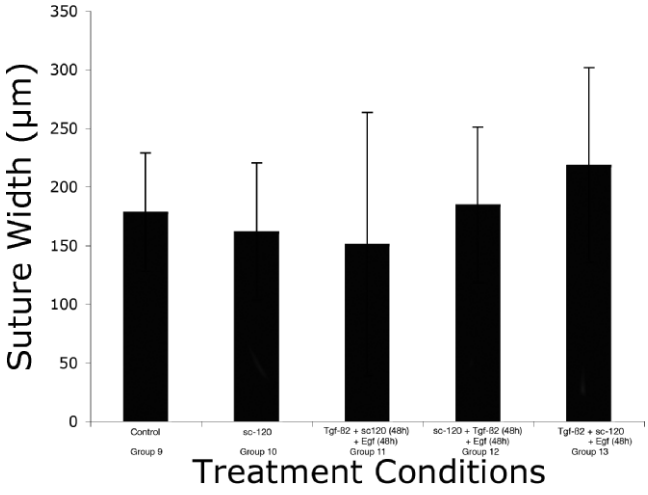

Figure 7. Graph showing coronal suture width following 5 days in culture in the presence of Egf, Tgf-β2, and sc-120. Note lack of effect of sc-120 alone on sutures (Group 10), and reversal of Tgf-β2-induced suture narrowing in the presence of sc-120 given either concurrently or before Tgf-β2 treatment (Groups 11–13).

suture narrowing. However, this result may be due to the fact that Egfr activity was blocked for the duration of the culture period in calvariae treated with Tgf-β2. This was done since the differential effect of Egf given pre- and post-Tgf-β2 treatment was unexpected. Ideally, Egf activity should have been blocked only prior to Tgf-β2 treatment, then removed when Tgf-β2 was added. As expected, when blocker was added after Tgf-β2 treatment, sutures were rescued from Tgf-β2-induced narrowing, indicating that Egf is required for Tgf-β2 activity in sutures.

In mice overexpressing *Egf,* there are greatly increased numbers of osteoblasts in the periosteum and endosteum of bones, but interestingly, cortical bone thickness was reduced compared to wild-type mice (15). These results seem to imply that osteoblastic cells responding to Egf become insensitive to differentiation signals like those from Tgf-β2, but rather accumulate in the surrounding connective tissue. This idea is supported by the high cellularity noted at the edges of the bone fronts of Egf-treated sutures, and these sutures remaining patent. Raisz *et al*. showed that at high concentrations Egf induced bone resorption, implying additional mechanisms for keeping the suture open (28). Interestingly, Lana-Elola *et al*. demonstrated that cells at the osteogenic fronts contributed to the osteoblastic lineage while those in the center of the suture did not. It therefore appears that the Egf-responsive cells in this study correspond to the osteoprogenitors present in the osteogenic fronts reported by Lana-Elola *et al*. (29).

When sutures were exposed to Tgf-β2 prior to Egf exposure, they were exposed to a factor that affects both proliferation and differentiation, and which resulted in Egf expression. Egf added after Tgf-β2 treatment had no effect on sutures, likely because cells were already maximally responsive to the Egf produced in response to Tgf-β2 treatment. Since Tgf-β2 on its own can induce suture obliteration, and addition of Egf before but not after Tgf-β2 treatment reverses Tgf-β2 induced obliteration, the following model is proposed. The continuous presence of Egf keeps cells in an undifferentiated, proliferative state, preventing cells from responding to inducers of accelerated differentiation such as Tgf-β2. However, cells already primed to differentiate by the presence of Tgf-β2 are pushed rapidly through their final proliferative cycles by Egf produced in response to Tgf-β2, accelerating the differentiative process initiated by Tgf-β2, and resulting in suture fusion seen in the presence of Tgf-β2.

This mechanism is reminiscent of the effect of Axin2 modulating Wnt/beta-catenin signaling in cranial sutures, demonstrated by Hsu's group in Rochester (30, 31). This group showed that removal of Axin2—a negative regulator of the canonical Wnt pathway—resulted in expansion of the osteoprogenitor lineage, followed by accelerated ossification and premature fusion of sutures (30). Similar to Lana-Enola *et al*. (29), they provide evidence supporting a region-specific effect of skeletogenic factors within the suture. This group goes on to show that beta-catenin promotes cell proliferation, followed by differentiation accompanied by elevated BMP signaling, accelerating the process in a positive feedback mechanism (31). Significantly, Tgf-β1 suppresses Axin1 and Axin2 expression, while Axin1 and Axin2 stimulate Tgf-β signaling during endochondral bone formation (32), pointing to a possible integration of the canonical Wnt and Tgf-β/Smad pathways in regulating cranial bone growth and suture development.

Recent data showed that Tgf-β2 phosphorylation of Erk1/2 inhibited Smad2/3 protein expression (9). This is possibly a similar mechanism by which Egf was shown to disrupt Smad signaling via an Erk1/2-P dependent mechanism (33). In contrast to Tgf-β2, Tgf-β3 rescues suture from obliteration (5, 24, 34, 35). Since Tgf-β2 appears to signal via Erk1/2 to regulate suture patency, it is possible that Tgf-β3 uses the Smad2/3 signaling pathway to keep sutures patent, possibly by inhibiting Tgf-β2 signaling via Erk1/2. However, in contrast to Tgf-β2, Tgf-β3 was shown by gene arrays to inhibit Egf expression (Rawlins, unpublished data), so another mechanism could be the control of Egf-induced cell proliferation, to regulate the number of cells available to respond to Tgf-β2.

Several research groups have posited that maintenance of suture patency requires a delicate balance between cell proliferation, differentiation and apoptosis (27, 36–43). It appears that the timing of exposure of cells to proliferative versus differentiative signals is critical for maintenance of suture patency.

The authors would like to thank Ms. Jo Taylor for processing and sectioning all tissues for histological and histomorphometric analysis.

1. Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dynam 219:472–485, 2000.
2. Cohen MM Jr. Craniofacial disorders caused by mutations in homeobox genes MSX1 and MSX2. J Craniofac Genet Dev Biol 20:19–25, 2000.
3. Kim HJ, Rice DP, Kettunen PJ, Thesleff I. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 125: 1241–1251, 1998.
4. Opperman LA, Adab K, Gakunga PT. Transforming growth factor-beta 2 and TGF-beta 3 regulate fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis. Dev Dynam 219: 237–247, 2000.
5. Opperman LA, Chhabra A, Cho RW, Ogle RC. Cranial suture obliteration is induced by removal of transforming growth factor (TGF)-beta 3 activity and prevented by removal of TGF-beta 2 activity from fetal rat calvaria in vitro. J Craniofac Genet Dev Biol 19:164–173, 1999.
6. Roth DA, Longaker MT, McCarthy JG, Rosen DM, McMullen HF, Levine JP, Sung J, Gold LI. Studies in cranial suture biology: part I. Increased immunoreactivity for TGF-beta isoforms (beta 1, beta 2, and beta 3) during rat cranial suture fusion. J Bone Miner Res 12:311–321, 1997.
7. Kim HJ, Lee MH, Park HS, Park MH, Lee SW, Kim SY, Choi JY, Shin HI, Kim HJ, Ryoo HM. Erk pathway and activator protein 1 play

crucial roles in FGF2-stimulated premature cranial suture closure. Dev Dynam 227:335–346, 2003.

8. Ignelzi MA Jr, Wang W, Young AT, Carinci F, Bodo M, Tosi L, Francioso F, Evangelisti R, Pezzetti F, Scapoli L, Martinelli M, Baroni T, Stabellini G, Carinci P, Bellucci C, Lilli C, Volinia S. Fibroblast growth factors lead to increased Msx2 expression and fusion in calvarial sutures: expression profiles of craniosynostosis-derived fibroblasts. J Bone Miner Res 18:751–759, 2003.
9. Opperman LA, Fernandez CR, So S, Rawlins JT. Erk1/2 signaling is required for Tgf-beta 2-induced suture closure. Dev Dynam 235:1292–1299, 2006.
10. Mahimainathan L, Ghosh-Choudhury N, Venkatesan BA, Danda RS, Choudhury GG. EGF stimulates mesangial cell mitogenesis via PI3-kinase-mediated MAPK-dependent and AKT kinase-independent manner: involvement of c-fos and p27Kip1. Am J Physiol Renal Physiol 289:F72–82, 2005.
11. Yoon YM, Oh CD, Kim DY, Lee YS, Park JW, Huh TL, Kang SS, Chun JS. Epidermal growth factor negatively regulates chondrogenesis of mesenchymal cells by modulating the protein kinase C-alpha, Erk-1, and p38 MAPK signaling pathways. J Biol Chem 275:12353–12359, 2000.
12. Bilbe G, Roberts E, Birch M, Evans DB. PCR phenotyping of cytokines, growth factors and their receptors and bone matrix proteins in human osteoblast-like cell lines. Bone 19:437–445, 1996.
13. Damien E, Price JS, Lanyon LE. The estrogen receptor's involvement in osteoblasts' adaptive response to mechanical strain. J Bone Miner Res 13:1275–1282, 1998.
14. Matsuda N, Morita N, Matsuda K, Watanabe M. Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP kinases in response to epidermal growth factor, hypoxia, and mechanical stress in vitro. Biochem Biophys Res Comm 249:350–354, 1998.
15. Chan SY, Wong RW. Expression of epidermal growth factor in transgenic mice causes growth retardation. J Biol Chem 275:38693–38698, 2000.
16. Pensler JM, Ivescu AS, Radosevich JA. Scaphocephaly: premature closure of the sagittal suture: a localized disorder of cellular metabolism? Ann Plast Surg 40:48–52, 1998.
17. Opperman LA, Passarelli RW, Morgan EP, Reintjes M, Ogle RC. Cranial sutures require tissue interactions with dura mater to resist osseous obliteration in vitro. J Bone Miner Res 10:1978–1987, 1995.
18. Chu CT, Everiss KD, Wikstrand CJ, Batra SK, Kung HJ, Bigner DD. Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRvIII). Biochem J 324:855–861, 1997.
19. Opperman LA, Chhabra A, Nolen AA, Bao Y, Ogle RC. Increased cell proliferation and altered collagen production precede rat cranial suture fusion in vitro. Mol Biol Cell 8:329a, 1997.
20. Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N, Xu FL, Myers LA, Spevak PJ, Cameron DE, De Backer J, Hellemans J, Chen Y, Davis EC, Webb CL, Kress W, Coucke P, Rifkin DB, De Paepe AM, Dietz HC. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. Nat Genet 37:275–281, 2005.
21. Bradley JP, Levine JP, McCarthy JG, Longaker MT. Studies in cranial suture biology: regional dura mater determines in vitro cranial suture fusion. Plast Recon Surg 100:1091–1099, 1997.
22. Opperman LA, Nolen AA, Ogle RC. TGF-beta 1, TGF-beta 2, and TGF-beta 3 exhibit distinct patterns of expression during cranial suture formation and obliteration in vivo and in vitro. J Bone Miner Res 12: 301–310, 1997.
23. Roth DA, Gold LI, Han VK, McCarthy JG, Sung JJ, Wisoff JH, Longaker MT. Immunolocalization of transforming growth factor beta 1, beta 2, and beta 3 and insulin-like growth factor I in premature cranial suture fusion. Plast Recon Surg 99:300–309, 1997.
24. Opperman LA, Adab K, Gakunga PT. TGF-*B*2 and TGF-*B*3 regulate fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis. Dev Dynam 219:237–247, 2000.
25. Moursi AM, Winnard PL, Fryer D, Mooney MP. Delivery of transforming growth factor-beta2-perturbing antibody in a collagen vehicle inhibits cranial suture fusion in calvarial organ culture. Cleft Palate Craniofac J 40:225–232, 2003.
26. Mooney MP, Losken HW, Moursi AM, Shand JM, Cooper GM, Curry C, Ho L, Burrows AM, Stelnicki EJ, Losee JE, Opperman LA, Siegel MI. Postoperative anti-Tgf-beta2 antibody therapy improves intracranial volume and craniofacial growth in craniosynostotic rabbits. J Craniofac Surg 18:336–346, 2007.
27. Lee SW, Choi KY, Cho JY, Jung SH, Song KB, Park EK, Choi JY, Shin HI, Kim SY, Woo KM, Baek JH, Nam SH, Kim YJ, Kim HJ, Ryoo HM. TGF-beta2 stimulates cranial suture closure through activation of the Erk-MAPK pathway. J Cell Biochem 98:981–991, 2006.
28. Raisz LG, Simmons HA, Sandberg AL, Canalis E. Direct stimulation of bone resorption by epidermal growth factor. Endocrinology 107:270–273, 1980.
29. Lana-Elola E, Rice R, Grigoriadis AE, Rice DP. Cell fate specification during calvarial bone and suture development. Dev Biol 311:335–346, 2007.
30. Yu HM, Jerchow B, Sheu TJ, Liu B, Costantini F, Puzas JE, Birchmeier W, Hsu W. The role of Axin2 in calvarial morphogenesis and craniosynostosis. Development 132:1995–2005, 2005.
31. Liu B, Yu HM, Hsu W. Craniosynostosis caused by Axin2 deficiency is mediated through distinct functions of beta-catenin in proliferation and differentiation. Dev Biol 301:298–308, 2007.
32. Dao DY, Yang X, Chen D, Zuscik M, O'Keefe RJ. Axin1 and Axin2 are regulated by TGF- and mediate cross-talk between TGF- and Wnt signaling pathways. Ann N Y Acad Sci 1116:82–99, 2007.
33. Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389:618–622, 1997.
34. Opperman LA, Moursi A, Sayne JR, Wintergerst AM. Transforming growth factor-beta 3 (Tgf-β3) in a collagen gel delays fusion of the rat posterior interfrontal suture in vivo. Anat Rec 267:120–130, 2002.
35. Chong S, Mitchell R, Moursi AM, Winnard P, Wolfgang Losken H, Bradley J, Ozerdem OR, Azari K, Acarturk O, Opperman LA, Siegel MI, Mooney MP. Rescue of coronal suture fusion using transforming growth factor-beta 3 (Tgf-beta3) in rabbits with delayed-onset craniosynostosis. Anat Rec 274:962–971, 2003.
36. Iseki S, Wilkie AO, Heath JK, Ishimaru T, Eto K, Morriss-Kay GM. Fgfr2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2. Development 124:3375–3384, 1997.
37. Morriss-Kay GM, Iseki S, Johnson D. Genetic control of the cell proliferation-differentiation balance in the developing skull vault: roles of fibroblast growth factor receptor signalling pathways. Novartis Found Symp 232:102–116, 2001.
38. Mansukhani A, Bellosta P, Sahni M, Basilico C. Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. J Cell Biol 149:1297–1308, 2000.
39. Johnson D, Iseki S, Wilkie AO, Morriss-Kay GM. Expression patterns of Twist and Fgfr1, –2 and –3 in the developing mouse coronal suture suggest a key role for twist in suture initiation and biogenesis. Mech Dev 91:341–345, 2000.
40. Iseki S, Wilkie AO, Morriss-Kay GM. Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. Development 126:5611–5620, 1999.
41. Greenwald JA, Mehrara BJ, Spector JA, Warren SM, Crisera FE,

Fagenholz PJ, Bouletreau PJ, Longaker MT. Regional differentiation of cranial suture-associated dura mater in vivo and in vitro: implications for suture fusion and patency. J Bone Miner Res 15:2413–2430, 2000.

42. Fragale A, Tartaglia M, Bernardini S, Di Stasi AM, Di Rocco C, Velardi F, Teti A, Battaglia PA, Migliaccio S. Decreased proliferation and altered differentiation in osteoblasts from genetically and clinically distinct craniosynostotic disorders. Am J Pathol 154:1465–1477, 1999.
43. Ito Y, Yeo JY, Chytil A, Han J, Bringas P Jr, Nakajima A, Shuler CF, Moses HL, Chai Y. Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. Development 130:5269–5280, 2003.