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TGF-β2 Stimulates Cranial Suture Closure Through Activation of the Erk-MAPK Pathway

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Abstract Cranial sutures are important growth sites of the skull. During suture closure, the dura mater is one of the most important sources of various positive and negative regulatory signals. Previous results indicate that TGF-β2 from dura mater strongly accelerates suture closure, however, its exact regulatory mechanism is still unclear. In this study, we confirmed that removal of dura mater in calvarial organ culture strongly accelerates sagittal suture closure and that this effect is further enhanced by TGF-β2 treatment. TGF-β2 stimulated cell proliferation in the MC3T3-E1 cell line. Similarly, it stimulated the proliferation of cells in the sutural space in calvarial organ culture. Furthermore, TGF-β2-mediated enhanced cell proliferation and suture closure were almost completely inhibited by an Erk-MAPK blocker, PD98059. These results indicate that TGF-β2-induced activation of Erk-MAPK is an important signaling component that stimulates cell proliferation to enrich osteoprogenitor cells, thereby promoting their differentiation into osteoblasts to achieve a rapid calvarial bone expansion. J. Cell. Biochem. 98: 981–991, 2006.

Cranial sutures are important growth sites in craniofacial development [Baer, 1954]. The maintenance of suture space is critical in early cranial development as it facilitates cranial adjustment to accommodate brain and facial growth. The importance of this effect is clearly demonstrated in craniosynostosis, in which premature obliteration of one or two sutures leads to abnormal compensatory morphogenesis throughout the whole head. Mutations in genes encoding fibroblast growth factor receptor (FGFR) 1, 2, and 3 [Jabs et al., 1993, 1994; Reardon et al., 1994; Wilkie et al., 1995; Bellus et al., 1996; Meyers et al., 1996; Muenke and Schell, 1995; Muenke et al., 1997], MSX2 [Jabs et al., 1993; Ma et al., 1996], and TWIST [Howard et al., 1997; el Ghouzzi et al., 1997] are reported to be associated with craniosynostosis. Coincidentally, the expression patterns of these genes, as well as their upstream regulatory genes, in the developing suture area further support the hypothesis that they are implicated in the pathogenesis of the disease [Kim et al., 1998; Rice et al., 2000].

The meninges comprise three layers of membrane (from the innermost layer; pia mater, arachnoid mater, and dura mater) that cover the brain on one side and make contact with bones of the skull on the other side. The dura mater, the outermost layer, is tightly bound to

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developing calvarial bones and generates signals that regulate intramembranous bone formation and cranial suture patency. Several lines of evidence indicate that the dura mater expresses a plethora of signaling molecules, including FGF, TGF-β, BMP, and some of these proteins are known to accelerate cranial suture closure while others maintain suture patency [Opperman et al., 1996, 2000; Iseki et al., 1997; Kim et al., 1998]. A previous report indicated that the removal of dura mater strongly accelerated cranial suture closure in organ culture [Kim et al., 1998]. That study suggested that the overall effect of signals emanating from dura mater is to maintain the patency of cranial sutures, despite the fact that many of the individual signaling components have opposing effects on this process. From this perspective, generating a more comprehensive understanding of the role of the dura mater in mediating cranial suture patency would be a first step towards elucidating the role of individual signaling molecules, and their mutual regulatory relationships, in this process. Many of these gene products inter-regulate each other expression through tissue interactions between the dura mater, bone fronts, and sutures [Opperman et al., 1993, 1995; Iseki et al., 1997; Kim et al., 1998]. Although there are no reports of mutations in TGF-β subtypes or their receptors being associated with human cranial suture pathology, however, expression levels of members of the TGF-β family are altered during normal and abnormal suture closure [Lin et al., 1997; Opperman et al., 1997, Roth et al., 1997]. Among the TGF-β family members, TGF-β2 has known to play an important role in cranial suture closure [Moursi et al., 2003].

TGF-β has a broad array of biological activities including cell proliferation, lineage determination, differentiation, mobility, adhesion, and death. It initially binds to a cell surface complex comprising two types (I and II) of transmembrane serine/threonine kinase receptor. Subsequently, intracellular Smads are directly phosphorylated at the carboxy-terminal consensus site by type I receptors [Massague and Wotton, 2000]. The receptor-activated Smads form a stable complex with Smad4, and this complex translocates to the nucleus where it regulates transcriptional responses to TGF-β [Miyazono et al., 2001]. Besides Smad-mediated signal transduction, TGF-β also activates other signaling cascades, such as MAPK pathways. The mechanisms of Erk, JNK, and p38 MAPK activation by TGF-β, and their biological consequences, have been poorly characterized. TGF-β-activated receptors stimulate TGF-β-activated kinase 1 (TAK1), a MAPKKK family member. It subsequently phosphorylates and activates MAPK pathways [Yamaguchi et al., 1995]. Thus, activation of MAPK pathways by TGF-β may regulate diverse transcriptional responses [Yu et al., 2002; Zavadil et al., 2001].

MAPK pathways are a key link between membrane-bound receptors and the machinery that regulates gene expression. The three known MAPK cascades in mammalian cells have as their central players the extracellular signal-regulated kinase (Erk), the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), and p38 MAPK. The Erk pathway appears to be particularly associated with osteoblast proliferation and differentiation. Studies supporting this notion include that of Lai et al. [2001], who demonstrated that Erk is essential for osteoblast growth and proliferation, as well as for osteoblast adhesion, spreading, migration, and integrin expression. The Erk pathway also appears to be involved in the stimulation of osteoblast-related gene expression that arises from an interaction between the extracellular matrix (ECM) and a cell surface integrin receptor [Takeuchi et al., 1997]. Furthermore, the Erk pathway and activator protein 1 play crucial roles in FGF2-stimulated premature cranial suture closure [Kim et al., 2003]. Moreover, TGF-β regulates fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis [Opperman et al., 2000]. Thus, taken together, these studies may infer that TGF-β2 is an important signaling component of the dura mater that stimulates cranial suture closure through activation of osteogenic cell proliferation via Erk-MAPK signaling.

The specific aims of this study were to confirm the role of the dura mater in cranial suture obliteration by removing the tissue in organ culture. In addition, we analyzed function of an individual signaling molecule, for example TGF-β2, by application of the protein in the absence of dura mater in the organ culture system. Furthermore, we tried to elucidate the signaling cascades involved in TGF-β2-mediated cranial suture closure.

MATERIALS AND METHODS

Materials

Recombinant human TGF-β1, -2, and -3 were purchased from R&D systems (Minneapolis, MN). MTT (Microculture Tetrazolium), and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and α-MEM were purchased from GibcoBRL (Grand Island, NY). Fetal bovine serum was obtained from HyClone (Logan, UT). SB203580, PD98059, and SP600125 were purchased from Tocris (Ballwin, MO), and stock solutions were prepared in DMSO. The bromodeoxyuridine (BrdU) labeling and detection kit was purchased from Roche (Indianapolis, IN).

Cell Culture and Cell Proliferation Assay

The mouse osteoblastic cell line, MC3T3-E1, was cultured as described previously [Kim et al., 2003]. Briefly, confluent cells were washed twice with phosphate-buffered saline (PBS) and then treated for 24 h with 2.5 ng/ml TGF-β2 in serum-free medium supplemented with 0.2% BSA. Inhibitors of MAPK were used at a final concentration of 50 μM PD98059 (a MEK1/2 specific blocker), 25 μM SP600125 (a JNK inhibitor), or 25 μM SB203580 (a p38-MAPK-specific inhibitor). The increase of vital cell number was determined by MTT assay on the basis of mitochondrial metabolic reduction, as described previously [Kim et al., 2003]. Briefly, 5×10^3 cells/well were plated on 96-well microplates and cultured for 24 h in a humidified atmosphere of 5% CO_2 in air at 37°C. Inhibitors of MAPK were dosed into serum free medium for 1 h prior to treatment with TGF-β2 and the cells were cultured for an additional 24 h after TGF-β2 treatment. Medium was replaced with 50 μl of MTT solution (5 mg/ml in PBS) and 200 μl of culture medium containing 10% FBS, and cells were cultured for an additional 3 h at 37°C. The culture medium containing MTT was removed and 200 μl of DMSO and 50 μl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5 with NaOH) were added. Optical density was measured at 570 nm using an ELISA plate reader (E_{max}, Molecular Device).

Organ Culture of Calvaria

Calvaria of E15 mice were dissected, skin was removed and cultured in a Trowell-type culture dish. To evaluate the effect of the dura mater on suture closure, the dura mater beneath the sagittal suture area was removed. Explants were placed on 0.1-μm pore size Nucleopore filters, supported by metal grids and cultured for 48 h in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. Ascorbic acid, 100 μg/ml, was supplemented daily and the culture medium was changed every other day. The same concentration of MAPK blockers that were used in cell culture experiments was applied for the organ culture. Higher concentration of TGF-β proteins (10 ng/ml) were treated for organ culture. After 48 h in culture, calvaria were harvested, fixed in 4% paraformaldehyde (PFA) and dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned to a thickness of 5 μm. The final numbers of explants used for each experimental condition are presented in Table I.

Fluorescent Labeling in New Bone

For the determination of new bone growth in culture by fluorescent labeling, pregnant mice were injected intraperitoneally with calcein (1 mg/ml, Sigma Chemical Co.) in 2% sodium bicarbonate solution 1 day prior to sacrifice, following which calvaria were dissected from each embryo for organ culture. Alizarin complexone (0.09 mg/ml, Aldrich Chemical Co.) was added to the culture medium for secondary labeling. Using this technique, calcein-labeled tissue (green fluorescence) indicates embryonic mineralization, while alizarin complexone-labeled tissue (red fluorescence) represents mineralization during the organ culture. The labeled fluorescence was investigated under the confocal microscope (Leica, TCS-ST2).

BrdU Labeling

Cell proliferation in sagittal sutures was monitored by deoxybromouridine (BrdU) labeling. The calvaria separated from E16 mice without dura mater were labeled for 24 h in DMEM supplemented with 10% fetal bovine serum and 10 μM BrdU, as per the organ culture method described above. After 24 h of labeling, samples were fixed in 4% PFA, dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned to a thickness of 5 μm. BrdU incorporation was determined by immunostaining (Boehringer Mannheim), according to the manufacturer's

TABLE I. The Effect of TGF-β Treatment on Sagittal Suture Closure[a]

	Group (number of explants)							
	Without treatment (88)		TGF-β1 (42)		TFG-β2 (74)		TGF-β3 (43)	
Time (hour)	Mean (range)	%[d]	Mean (range)	%	Mean (range)	%	Mean (range)	%
0	0.535 ± 0.194[b] (0.320–0.928)		0.491 ± 0.178 (0.448–0.704)		0.516 ± 0.169 (0.317–0.740)		0.499 ± 0.194 (0.256–0.608)	
24	0.238 ± 0.146 (0.000–0.544)	55.6	0.301 ± 0.126 (0.096–0.448)	30.7	0.152 ± 0.160 (0.079–0.402)	70.6[c]	0.288 ± 0.114 (0.096–0.448)	42.3
48	0.098 ± 0.117 (0.000–0.192)	81.7	0.205 ± 0.128 (0.000–0.288)	58.2	0.020 ± 0.151 (0.000–0.192)	92.1[c]	0.190 ± 0.142 (0.000–0.416)	61.3

Dura mater-removed calvarial explants of E15.5 were cultured in Trowell-type organ culture dish with or without treatment of TGF-β protein in the media.
[a]The calvarial explants were magnified (70×) under a stereomicroscope and changes of suture width were statistically analyzed by repeated measures ANOVA.
[b]The suture width was measured from the pictures of each explant at each time point with the same magnification. The value indicates width between two nearest tangential line of opposing osteogenic fronts of parietal bones (mean ± SD, mm).
[c]The suture closure in TGF-β2 treated explants at each time point was significantly faster ($P < 0.0001$) than that of other groups.
[d]% decrease from initial width.

protocol. BrdU-positive and total cell numbers within the suture were counted from five randomly selected sections per sample. Five calvaria samples were evaluated from each experimental group.

Measurement of Suture Width and Statistical Analysis

For statistical analysis of the effect of TGF-β treatment on cranial suture closure, we took three consecutive images from each explant under a stereomicroscope (70×, Olympus, Japan) at 0, 24, and 48 h after the commencement of treatment. In images of the same magnification, two tangential lines parallel to the midline of the sagittal suture were drawn from the nearest points of the opposing OFs of the parietal bones. The width between the two lines was then measured and data were analyzed with SAS 10.2 (SAS Institute, Inc., Cary, NC). The differences between groups were compared by repeated measures ANOVA. $P < 0.05$ was considered significant.

RESULTS

Effect of the Dura Mater on Sagittal Suture Development

To confirm the role of the dura mater in mouse cranial suture development, we dissected calvaria from ICR mice at embryonic day 15.5 (E15.5), removed the overlying skin, and cultured them with (Fig. 1A–C) or without (Fig. 1D–O) the underlying dura mater in a Trowell-type organ culture system. After 24 h in culture, the osteogenic fronts of the parietal bones approximated each other, but no significant differences in suture width could be detected between the explants cultured in the presence or absence of the dura mater. At 48 h in culture, the osteogenic fronts of the parietal bones approximated each other much more closely under both culture conditions. However, the sagittal suture of the explants cultured without dura mater appeared to be closed over (Fig. 1F), whereas in the calvaria with the intact dura mater, the suture was still patent (Fig. 1C). These results further confirm the importance of the embryonic dura mater in cranial suture closure.

Effect of TGF-β Subtypes in Sagittal Suture Closure

To study the effect of individual TGF-β signals generated from the dura mater on suture closure, we removed dura mater from calvarial explants of E15.5 mice and applied the individual TGF-β proteins to the organ culture medium. After 24 h in culture, the widths of the sagittal sutures in explants treated with TGF-β1, and -β3 (Fig. 1H,N) were similar to those of controls (Fig. 1E). However, in explants treated with TGF-β2 (Fig. 1K), the sagittal sutures closed more rapidly, with this difference being much more prominent after 48 h of treatment (Fig. 1F, I, L, and O). TGF-β2-treatment (Fig. 1L) further accelerated the rate of suture closure over that observed with dura mater

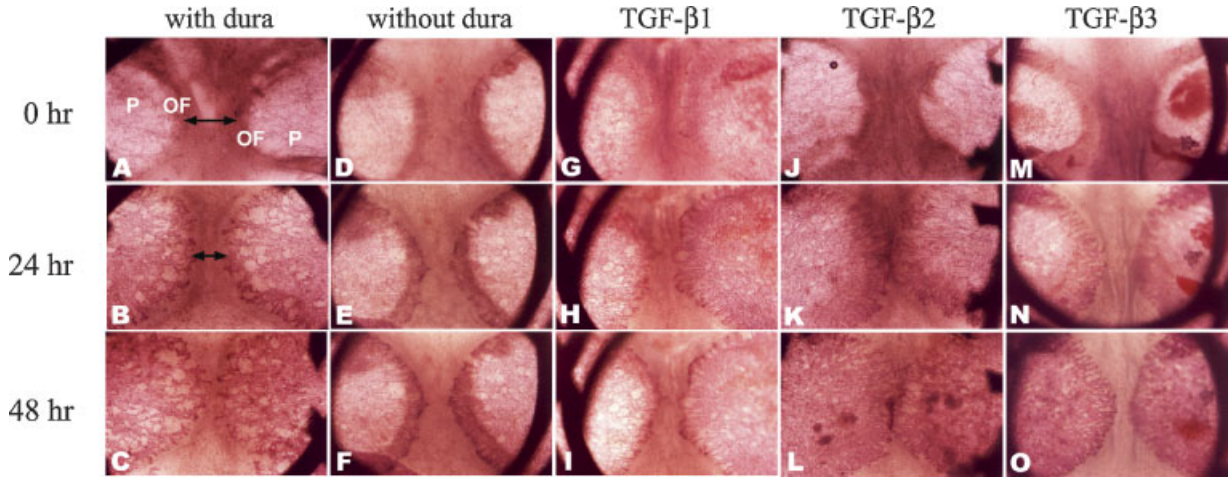


Fig. 1. Removal of the dura mater accelerates sagittal suture closure and this process is modulated by treatment with TGF-β1,-β2, and -β3. Mouse embryonic calvaria (E15.5) were cultured in Trowell-type organ culture dishes for 48 h with (**A**–**C**) or without dura mater (**D**–**O**). The osteogenic fronts (OF) of the parietal bones cultured in the absence of dura mater have almost approximated after 48 h (F, indicated by asterisk), however, there was a definite gap (arrow) between osteogenic fronts (OF) of parietal bones in the presence of dura mater (C). The calvarial explants from mice at E15.5 cultured in the absence of dura mater were treated with 10 ng/ml of each TGF-β protein, respectively, for 48 h. Photograph was taken under a stereomicroscope at 0 h (A, D, G, J, and M), 24 h (B, E, H, K and N) and 48 h (C, F, I, L, and O) of culture for each explant, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

removal alone (Fig. 1F). In contrast, TGF-β1 and β3 (Fig. 1I,O) delayed the suture closure that was accelerated by dura mater removal, so that the overall rate of suture closure appeared similar to that observed with intact dura mater (Fig. 1C). In order to analyze these differences in suture closure statistically, we made a total of 247 calvarial explant cultures. Since even a slight difference in mating time markedly affected the initial sagittal suture width, littermates from an individual pregnant mouse were divided into four groups; without dura, TGF-β1, TGF-β2, and TGF-β3. Although variations in the rate of the suture closure within a group were still observed (Table I), the acceleration of suture closure induced by TGF-β2 ($P < 0.005$), and the delay induced by TGF-β1 or β3 ($P < 0.001$), were statistically significant when compared to the control group without dura mater. The difference between TGF-β2 and the other TGF-β groups was also statistically significant ($P < 0.0001$). To account for and overcome the intra-explant/inter-explant variation, we analyzed the data by repeated measures ANOVA.

Capturing images of each individual explant three times (0, 24, and 48 h) and measuring the distance between two tangential lines on each picture was rather laborious and time-consuming. To surmount this issue, we labeled mineralized tissue with fluorescent dyes. In the system employed, calcein-labeled bone, which was mineralized in utero, fluoresced green while alizarin complexone-labeled bone was mineralized during organ culture and fluoresced red. Thus, the width between green fluorescent bones was measured as the starting width and the width between red fluorescent bones represented the final width. Linear regression analysis of fluorescently labeled and unlabeled samples indicated that these two measuring techniques produce data that correlate very well (correlation coefficient = 0.82), with this correlation being statistically significant ($P < 0.001$).

Under confocal microscopy, the effects of TGF-β subtypes on suture closure (Fig. 2) were comparable to those determined under stereomicroscopy (Fig. 1). In cross-sectional view of explants treated with TGF-β2, the osteogenic fronts of parietal bones overlapped partially (Fig. 2F; asterisk), whereas in similar sectional views taken from explants treated with TGF-β1 or -β3 the sutures remained open (Fig. 2D,H). It is notable that TGF-β2 treatment stimulates mineralization most prominently in the periphery of the expanding bone fronts, as indicated by red fluorescence in that region. This effect is primarily localized to a newly mineralized area between the two parietal bones (Fig. 2E). TGF-β1 or β3 group showed weaker degree of red fluorescence in the periphery of the expending

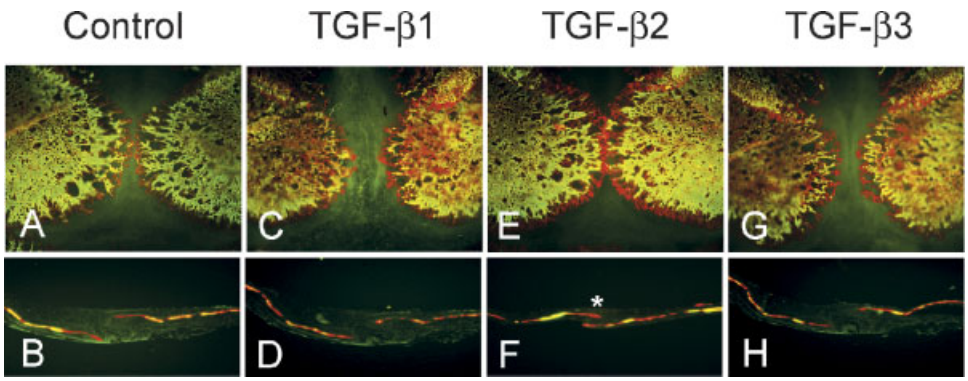


Fig. 2. Determination of new bone growth in organ culture by fluorescent labeling. Calcein (green fluorescence) was injected intraperitonially to the pregnant mouse at 24 h prior to the removal of embryonic calvarial tissue. E15.5 mouse calvarial tissue explants were cultured in Trowell-type tissue culture dishes in the presence of alizarin complexone (red fluorescence) in the medium for 48 h. The calvaria were observed under the fluorescence microscope as whole tissue (**A**, **C**, **E**, and **G**) or as a coronal section (**B**, **D**, **F**, and **H**). The edges of parietal bones are partially overlapping in TGF-β2-treated (10 ng/ml) explants (E&F, asterisk). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bone fronts compare to TGF-β2 group. On the contrary, TGF-β1 or β3 appear to stimulate bone remodeling of already mineralized tissue, as evidenced by the large degree of replacement of fluorescence dye from green to red in the area (Fig. 2C,G).

Blocking the Erk Pathway Retards TGF-β2-Stimulated Cell Proliferation and Cranial Suture Closure

Next, we investigated which of the TGF-β2 signaling pathways is/are specifically involved in cranial suture closure. Binding of TGF-β to its receptor (TGF-βR) is known to stimulate receptor dimerization, receptor serine/threonine phosphorylation, and the activation of multiple signal transduction pathways, including those involving Smads and the MAPK, Erk, and p38 MAP kinases. Of these multiple signaling pathways, we focused on the MAPK cascade because our previous study indicated that Erk-MAPK plays a very important role in cranial suture closure by activating FGF/FGFR signaling [Kim et al., 2003]. To investigate this issue, we treated MC3T3-E1 cells with pathway-specific inhibitors of several MAPKs prior to TGF-β2 treatment. Notably, inhibition of the Erk pathway by PD98059 significantly blocked cell proliferation stimulated by TGF-β2 (Fig. 3). In contrast, inhibition of the p38 MAPK and JNK pathways by SB203580 and SP600125, respectively, did not influence TGF-β2-mediated proliferation (Fig. 3).

Based on these proliferation results, we introduced the MAPK blockers to the calvarial organ culture system. Dura mater-stripped calvarial explants were grown in the presence or absence of PD98059 for 6 h prior to TGF-β2 (2.5 ng/ml) treatment. By itself, TGF-β2 treatment (Fig. 4E–H) significantly accelerated ($P < 0.0001$) cranial suture closure. Notably, only the Erk blocker (Fig. 4I–L) reversed the effect of TGF-β2 on suture closure, resulting in values comparable to the control (Fig. 4A–D). The statistical analysis of the organ cultures that are represented by pictures (Fig. 4A–L) is summarized in Figure 4M. This effect was reminiscent of that observed in the cell proliferation assay (Fig. 3).

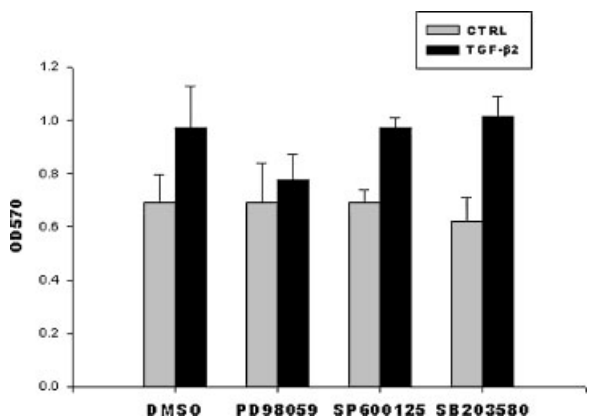
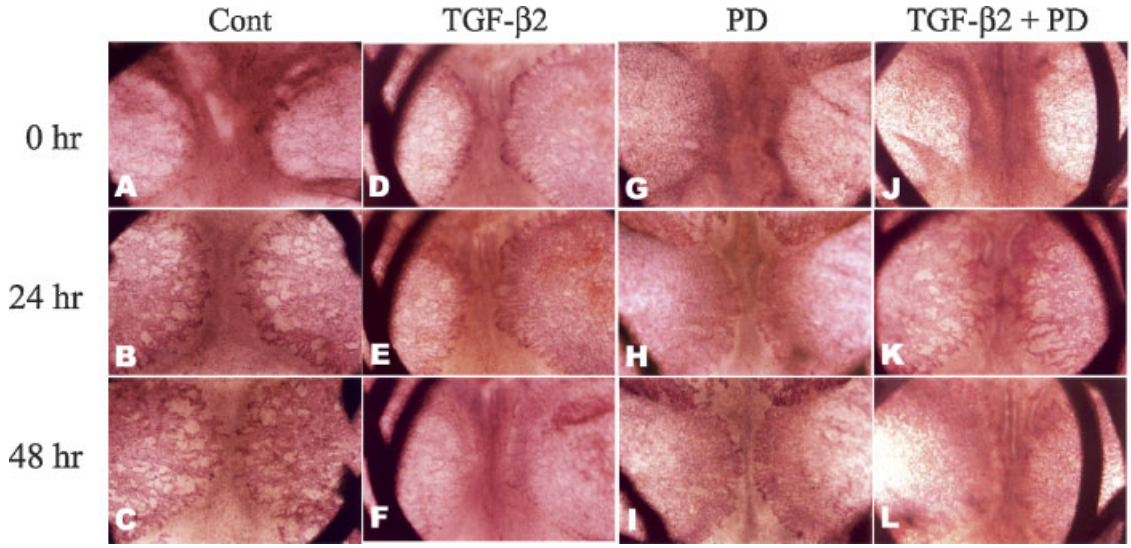


Fig. 3. The effect of MAPK inhibitors on cell proliferation. MC3T3-E1 cells were treated with the indicated inhibitor for 1 h prior to TGF-β2 (2.5 ng/ml) treatment in 96-well plates. Cell proliferation was assessed by MTT assay after 24 h of TGF-β2 treatment. Each inhibitor was dissolved in DMSO as a vehicle. The final concentration of each inhibitor was PD98059 (50 μM), SP600125 (25 μM), and SB203580 (25 μM).



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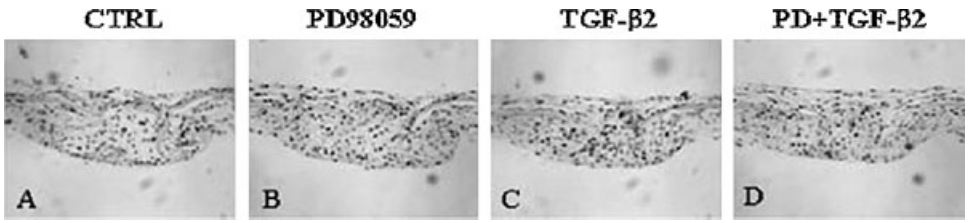
	Group (number of explants)			
	Without Tx. (48)	TGF-β2 (42)	PD98059 (44)	PD + TGF-β2 (43)
Time (hour)	Mean ± SD%	Mean ± SD%	Mean ± SD%	Mean ± SD%
0	0.569 ± 0.187	0.593 ± 0.195	0.583 ± 0.172	0.575 ± 0.183
24	0.241 ± 0.137 57.6	0.167 ± 0.163 71.8[a]	0.254 ± 0.183 56.4	0.238 ± 0.159 58.6
48	0.101 ± 0.116 82.2	0.019 ± 0.125 96.8[a]	0.138 ± 0.174 76.3	0.096 ± 0.128 83.3

Fig. 4. Accelerated cranial suture closure by TGF-β2 is inhibited by an Erk-MAPK blocker. The dura mater-stripped calvarial tissue of an E16 mouse was pretreated with or without PD98059 (50 μM) for 6 h, then TGF-β2 (10 ng/ml) was treated for an additional 24 and 48 h. The suture closure was assessed under a stereomicroscope as described in Figure 1 and Table I. Representative pictures are shown for each condition at each time point (**A**–**L**). The statistical analysis of the organ culture study was summarized in (**M**). The calvarial explants were magnified (70×) under a stereomicroscope and changes of suture width were statistically analyzed by repeated measures ANOVA. The value indicates width between two nearest tangential line of opposing osteogenic fronts of parietal bones (mean ± SD, mm). [a]The suture closure in TGF-β2 treated explants at each time point was significantly faster ($P < 0.001$) than that of other groups. Percent decrease from initial width. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

To confirm the abrogation of TGF-β2-induced cell proliferation by an Erk-MAPK blocker is responsible for premature suture closure, we quantified bromodeoxyuridine (BrdU) incorporation in calvarial organ culture. Consistent with our earlier results (Figs. 3 and 4), BrdU incorporation (dark brown dots) in the suture area was significantly ($P < 0.01$; Fig. 5E) increased by TGF-β2 treatment, but was decreased to almost control levels by the Erk-MAPK blocker/TGF-β2 combination (Fig. 5C–D). TGF-β2 treatment increased total cell numbers, too, but was not statistically significant. Thus, taken together it appears that Erk-MAPK mediates the effect of TGF-β2 and that the main downstream target of its activation is



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	Group (number of explants)			
	Without Tx. (25)	PD98059 (25)	TGF-β2 (25)	PD + TGF-β2 (25)
	Mean ± SD%[b]	Mean ± SD%	Mean ± SD%	Mean ± SD%
Total	82.5 ± 13.4	78.9 ± 14.2	98.3 ± 15.2	86.6 ± 14.8
BrdU labeled	18.4 ± 4.3 22.3	16.7 ± 3.7 21.2	36.4 ± 5.8 37.0[a]	23.5 ± 4.3 27.3

Fig. 5. Increased cell proliferation by TGF-β2 is inhibited by an Erk-MAPK inhibitor (PD98059). Dura mater-stripped calvarial tissue of an E16.5 mouse was cultured in the presence or absence of the Erk-MAPK blocker (50 μM) and TGF-β2 (10 ng/ml) for 48 h. Cell proliferation was assessed by BrdU labeling (brown spots; **A**–**D**). **E**: BrdU-positive and total cell numbers in the suture were counted from five randomly selected sections per sample. Five calvaria samples were evaluated from each experimental group (total 25 fields per each group). Differences of total and BrdU positive cell number were statistically analyzed by repeated measures ANOVA. [a]Portion of BrdU positive cells are significantly increased ($P < 0.01$) when compared to the other groups. [b]Values are percent of the BrdU positive cells among the total cells.

the stimulation of osteoprogenitor cell proliferation in the suture area, thereby resulting in the expansion of calvaria to obtain suture closure.

DISCUSSION

The dura mater is thought to play essential roles in cranial sutures closure, including calvarial morphogenesis [Ito et al., 2003], postnatal suture fusion [Levine et al., 1998], and osseous repair of calvarial defects [Gosain et al., 2003]. Previous studies have shown that sagittal sutures from mouse calvaria, with intact dura mater, could be maintained in culture for 2 days, and that the removal of the dura mater resulted in sutural obliteration after 2 days in culture [Kim et al., 1998]. Here, we adopted this mouse calvaria organ culture model and applied TGF-β1, TGF-β2, and TGF-β3, to examine the mechanisms by which these growth factors modulate suture closure. In addition, for a simpler analysis of calvarial growth, we applied fluorescent dyes before and during organ culture.

Fluorescence Labeling Could Substitute for Direct Measurement of Suture Space

Cranial suture closure in organ culture has traditionally been determined by direct measurement of suture space from staggered images captured after 0, 24, and 48 h of organ culture. Although we found this method to be very reproducible and reliable, it is time-consuming and laborious. Thus, to circumvent the tedious nature of this task, we developed a method whereby we introduced two different fluorescent dyes before and during organ culture. According to our analysis these two measurement techniques exhibited a very high correlation coefficient ($r > 0.82$). We speculate that the mismatch arose from differences in processing; we capture steromicroscopy images during organ culture without any fixative but the fluorescent technique requires fixation just prior to image capture. Moreover, the

delineation of osteogenic fronts from confocal images is not as clean as from stereomicroscopy images. Overall, we found that direct measurement from stereomicroscopy images is more reliable than using confocal fluorescent images. Nevertheless, the confocal technique has several advantages; first, it is quite comparable to the direct measurement data. Second, it circumvents a series of tedious, time-consuming procedures. Third, it provides some additional information that is not obtainable from stereomicroscopy images, such as the definition of an active mineralization area evidenced by higher intensity of second dye labeling.

TGF-β2 Enhanced Sagittal Suture Closure by Stimulating Cell Proliferation

In this study, we confirmed that removal of the dura mater strongly accelerates sagittal suture closure. In order to define which component of TGF-β signaling from the dura mater controls suture closure, we exposed organ cultures, in which the dura mater was removed, to TGF-β1, -β2, or -β3. Notably, TGF-β1 and -β3 suppressed cranial suture closure while TGF-β2 further enhanced suture closure. These results agree with previous studies of rat coronal suture closure [Opperman et al., 2000]. Furthermore, the fluorescent labeling experiment indicated that TGF-β1 and -β3 primarily stimulate remodeling of already mineralized parietal bones. In contrast, TGF-β2 mainly acts to expand the mineralization of osteogenic fronts, but acts much less on the remodeling of parietal bones. In other words, it appears that TGF-β2 is involved in primary bone expansion, which is accompanied by brain growth, whereas TGF-β1 and -β3 are involved in secondary growth events that include bone remodeling.

As our previous report indicated that acceleration of sagittal suture closure by FGF2 is caused by increased cell proliferation in the suture area [Kim et al., 2003]. In this study, TGF-β2 stimulated the proliferation of MC3T3-E1 cells, and also stimulated cell proliferation in the sutural mesenchyme and osteogenic fronts, as determined by BrdU incorporation in organ culture. It strongly suggests that TGF-β2 and FGF2 accelerate suture closure by stimulating cell proliferation in the suture and osteogenic fronts. These data provided good evidence for abnormally elevated cell numbers being a contributory factor to premature obliteration of cranial sutures by producing a critical density at which cells are induced to differentiate [Frenkel et al., 1992; Mayahara et al., 1993]. Further support for this notion is provided by the observation that addition of FGF-2 to cultured fetal mouse calvaria induces premature suture fusion, associated with elevated levels of cell proliferation [Kim et al., 2003], as does overexpression of MSX2 [Liu et al., 1999]. In this context, it is noteworthy a previous report [Opperman et al., 2000] that blocking endogenous TGF-β2 with neutralizing antibody did not reduce cell proliferation, but still prevented suture fusion, suggesting a possibility that endogenous TGF-β2 might have a role in osteoblast differentiation. In this study, we mainly focused on the cell proliferation by TGF-β2, however, the enhanced mineralization of TGF-β2 treatment group in Figures 1 and 2 strongly indicates dual activity of TGF-β2 on proliferation and differentiation even in the exogenous applied TGF-β2 as well.

According to previous report [Roth et al., 1997], in vivo injection of TGF-β2 in suture area generally stimulated in suture fusion but inconsistency was generated by different dosage or injection sites. These results suggest that it is not simple to interpret the role of a single molecule in biosystem; for example, different vasculature may influence on the degradation or sequestration of the injected protein. In order to analyze role of an individual signaling molecule, we strongly advocate our system in this study is a simpler and consistent analytical model because complexity is eliminated by removing dura mater, a complex signal generator. Applying an individual signaling molecule over this system will provide us an analytical insight for the role individual molecules.

TGF-β2-Induced Cell Proliferation and Cranial Suture Closure Are Mediated by Erk- MAPK

Extracellular signals are transmitted to the nucleus in a variety of ways, including the activation of receptor associated kinases or intracellular kinases. TGF-β binding to TGF-βR induces receptor hetero-dimerization, phosphorylation of receptor serine/threonine and Smads, and the activation of multiple signal transduction pathways [Derynck and Zhang, 2003]. Recent studies demonstrated that MAPK pathways mediate FGF2 stimulation in proliferation, differentiation, and apoptosis of osteoblasts [Lemonnier et al., 2000; Debiais et al., 2001; Marie et al., 2002; Kim et al., 2003].

TGF-β2-stimulated cell proliferation was reduced by the selective Erk inhibitor, PD98059, resulting in inhibition of suture closure. However, inhibitors of the other MAPK pathways, p38 MAPK and JNK-MAPK, were without effect.

Different from the immediate early activation of Erk in FGF signaling [Kim et al., 2003], Erk activation by TGF-β occurs somewhat delayed in osteoblast [6 h after TGF-β treatment; Lai and Cheng, 2002]. Moreover, Ghayor et al. [2005] further demonstrated that TGF-β-induced cell proliferation is mediated by prostaglandin E2 (PGE2) generation and this prostaglandin in turn activates Erk through the mediation by protein kinase C. Based on these previous results, we may not rule out the possibility that TGF-β2-induced cell proliferation, Erk activation and suture obliteration are not an immediate early response but a delayed secondary effect mediated by PGE2 generation. Thus, TGF-β2-induced responses should be interpreted and further clarified more carefully.

Collectively, it can be summarized as follow; first, in sum the cumulative effect of individual signaling molecules on cranial suture closure is negative because the existence of dura mater slows down the suture closure. Second, TGF-β2 generated from dura mater strongly accelerated the suture closure. Third, the TGF-β2-induced acceleration of suture closure is resulted from increased cell proliferation, which is mediated by Erk MAPK activation either directly or indirectly.

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