

Erk1/2 Signaling Is Required for Tgf- β 2-Induced Suture Closure

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Transforming growth factors β (Tgf- β s) act by means of Smad signaling pathways and may also interact with the mitogen-activated protein kinase pathway. The hypothesis was tested that Erk1/2 signaling is required for Tgf- β 2-induced suture closure, by culturing embryonic mouse calvariae in the presence of Tgf- β 2 with or without Erk1/2 inhibitor PD98059 (PD). Suture widths were measured daily, and microdissected sutures and bones were homogenized and protein analyzed by Western blots. Tgf- β 2 induced narrowing of the sutures after 72 hr, an effect inhibited by treatment with PD. Erk1/2 and Egf but not Smad2/3 protein expression was up-regulated by Tgf- β 2 calvarial tissues at 72 hr. PD inhibited endogenous and Tgf- β 2-stimulated Erk1/2 protein as well as Tgf- β 2-stimulated Egf, but increased Smad2/3 protein expression. In tissues harvested 0, 15, and 30 min after exposure to Tgf- β 2, Erk1/2 phosphorylation was up-regulated after 15 min, an effect abrogated by the simultaneous addition of PD. In summary, Tgf- β 2 stimulated Erk1/2 phosphorylation and induced Egf and Erk1/2 expression, associated with suture closure after 72 hr. Blocking Erk1/2 activity with PD inhibited these effects but increased Smad2/3 expression. We postulate that Tgf- β 2 regulates suture closure directly by means of phosphorylation of Erk1/2 and indirectly by up-regulating Erk1/2, a substrate for Fgf receptor signaling required for Fgf induction of premature suture obliteration. *Developmental Dynamics* 235:1292–1299, 2006. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Several growth factor signaling pathways are implicated in the development and maintenance of craniofacial sutures. Of these, mutations in the genes for several factors, their receptors, or downstream signaling molecules have been implicated in craniosynostosis, or the premature obliteration of sutures. The first identified mutations were in genes for transcription factors *MSX2* and *TWIST* (Jabs et al., 1993, 1994; Liu et al., 1994, 1995; el Ghouzzi et al., 1997; Howard et al., 1997). Mutations in genes for *fibroblast growth factor receptor (FGFR)* and *transforming growth factor β (TGF- β) receptor II*

(*T β R-II*) have also been associated with craniosynostotic disorders (Muenke et al., 1994; Wilkie et al., 1995; Bellus et al., 1996; Loeys et al., 2005).

Intracellular signaling pathways downstream of Fgf and Tgf- β receptors have not been well studied in craniofacial suture development and maintenance. Fgf2 treatment has been shown to induce premature suture closure (Kim et al., 1998, 2003; Ignelzi et al., 2003), and the map kinase Erk1/2 signaling pathway was required for Fgf-stimulated suture closure (Kim et al., 2003). In both in vitro and in vivo experiments, Tgf- β 3 rescued sutures from obliteration,

whereas Tgf- β 2 induced suture obliteration (Opperman et al., 2000, 2002; Chong et al., 2003; Moursi et al., 2003). Tgf- β 2 and Tgf- β 3 use the serine/threonine kinase Smad2/Smad3 signaling pathway (Centrella et al., 1996; Massague and Wotton, 2000). Smad3 signaling is reported to be central in regulating the mucosal immune response, whereas Smad2 signaling is central in craniofacial development (Weinstein et al., 2000). If Smads have differential roles for regulating tissue responses to Tgf- β signaling, then it is possible that other signaling pathways are required to regulate differential responses to Tgf-

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β 1, Tgf- β 2, and Tgf- β 3 by the same tissues.

There is now substantial evidence that Tgf- β family members—both Tgf- β s and bone morphogenetic proteins (Bmps)—signal by means of tyrosine kinase mitogen-activated protein kinase (MAPK) pathways. This signaling can occur in both Smad-dependent and Smad-independent ways. For Smad-dependent signaling, Smads can be either upstream or downstream of the MAPK pathways. For example, (Yue et al., 1999) showed that Tgf- β 1 phosphorylated Smad1 by signaling by means of the Ras/Mek pathway, whereas Tgf- β 1 activation of Atf2 was dependent upon Smad4 activation of p38 (Simeone et al., 2001). Furthermore, Erk and p38 differentially regulate Bmp2-mediated and Tgf- β 1-mediated osteoblast function in a Smad4-dependent manner (Lai and Cheng, 2002). In Smad-independent signaling, Tgf- β activated Jnk signaling by means of a Rho-dependent pathway to activate AP-1 (Engel et al., 1999), c-Jun, and Atf-2 function (Hocevar et al., 1999; Rouse et al., 2001), and mobilization of the actin cytoskeleton (Edlund et al., 2002). This Smad-independent signaling was pathway specific, as Bmp2-induced apoptosis was protein kinase C-dependent but protein kinase A-, p38-, and Erk/Mek-independent (Hay et al., 2001).

Smad2 but not Smad3 is required for early craniofacial development (Weinstein et al., 2000); however, the signaling pathways required for Tgf- β 2 regulation of later events such as suture development and growth is unclear. Alterations in T β R-II signaling induced by gene mutations have been associated with cranial suture defects, and several pathways could be responsible for transducing the signal initiated by Tgf- β 2 binding to this receptor. Erk1/2 signaling is critical for normal expression of the osteoblast phenotype (Lai and Cheng, 2002). The Erk1/2 pathway is downstream to growth factor signaling such as Egf and Fgf and is critical for Fgf2-stimulated premature cranial suture closure, as blocking Erk1/2 phosphorylation prevents Fgf2-induced suture closure (Kim et al., 2003). It was hypothesized, therefore, that Tgf- β 2-induced suture closure occurred by

means of an Erk1/2-dependent signaling pathway. In this study, using a calvarial explant assay, it was demonstrated that Tgf- β 2 both phosphorylated Erk1/2 and up-regulated Erk1/2 protein expression. Blocking Erk1/2 signaling prevented Tgf- β 2-induced suture closure, inhibited Erk1/2 protein expression, and induced Smad2/3 expression. Based on these findings, a model for Tgf- β 2 responsive signaling pathways responsible for Tgf- β regulation of suture patency is proposed.

RESULTS

Erk Inhibitor PD98059 (PD) Prevents Tgf- β 2-Induced Suture Closure

To test whether Tgf- β 2 induced suture closure is dependent on Erk1/2 signaling, fetal day 17.5 (E17.5) mouse calvariae were cultured in a well-established explant model system (Opperman et al., 1995, 1999, 2000; Kim et al., 1998, 2003), with

Tgf- β 2 and PD, an inhibitor of Erk1/2 activity. Calvariae were photographed during culture (Fig. 1), and the widths of the posterior frontal sutures (PFS) measured (Fig. 2). In control cultures with no Tgf- β 2 or PD added, widely patent sutures could be seen at both 0 and 72 hr (Fig. 1A,B). In the presence of Tgf- β 2 (Fig. 1C,D), the suture at 72 hr appeared narrowed compared with sutures seen at 0 hr, and this finding was confirmed by digital measurements of the suture width (Fig. 2). When PD was added with Tgf- β 2 to the cultures, the PD inhibited narrowing of the sutures induced by Tgf- β 2 (Fig. 1E,F); PD without Tgf- β 2 had little effect on suture width (Fig. 1G,H), as did dimethyl sulfoxide (DMSO) used as a carrier for PD (not shown).

Measurements of suture widths showed that all sutures gradually narrowed over time in culture (Fig. 2). Control sutures in culture medium alone narrowed by 22% of the original

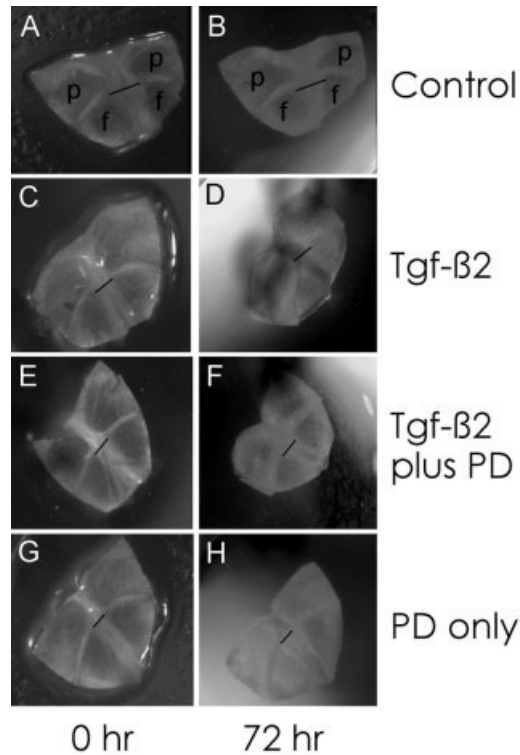


Fig. 1. Digital photographs taken of representative fetal day 17.5 (E17.5) mouse calvariae in serum-free culture medium. **A,B:** Control calvariae cultured in medium alone. **C,D:** Calvariae cultured with 3 ng/ml transforming growth factor β 2 (Tgf- β 2) added to the culture medium. **E,F:** Calvariae cultured with 3 ng/ml Tgf- β 2 plus 50 μ M PD98059 added to the culture medium. **G,H:** Calvariae cultured with 50 μ M PD98059 added to the culture medium. The black line indicates the position across the posterior frontal suture (PFS), at which daily measurements were taken. f, frontal bone; p, parietal bone.

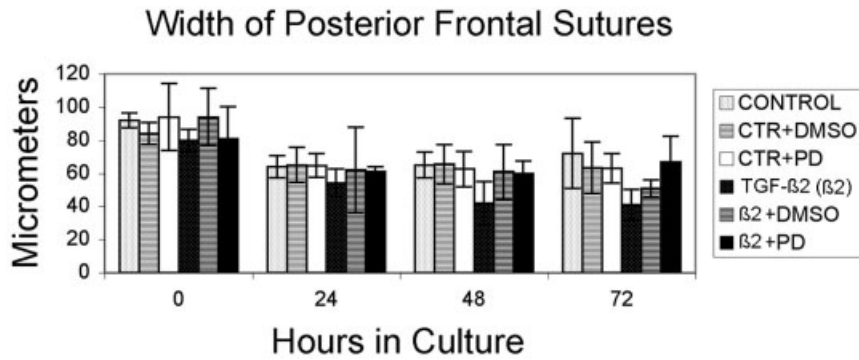


Fig. 2. Graph showing means and standard deviations of posterior frontal suture (PFS) measurements taken at various times throughout culture. Note the gradual narrowing of all sutures with time. Sutures cultured with 3 ng/ml transforming growth factor β 2 (Tgf- β 2, spotted black columns) show markedly greater narrowing than control sutures (lighter columns), or sutures cultured with Tgf- β 2 plus PD98059 (PD, black column). Addition of dimethyl sulfoxide (DMSO) used as a carrier for PD had no effect on suture width (barred columns).

width. The addition of Tgf- β 2 induced sutures to narrow by 49% of their original widths, which was similar to the 46% narrowing noted with Tgf- β 2 and DMSO. The addition of PD with Tgf- β 2 reversed Tgf- β 2-induced suture closure, with sutures narrowing by 17%, comparable to control sutures. The addition of PD alone to sutures resulted in sutures narrowed by 33%.

Tgf- β 2 Induced Phosphorylation of Erk1/2 but Not p38 and Up-Regulated Erk1/2 Protein Synthesis

Blocking Erk1/2 activity with PD blocked Tgf- β 2-induced suture closure; therefore, the effect of Tgf- β 2 on Erk1/2 phosphorylation and protein synthesis was examined. The E17.5 mouse calvariae were treated with Tgf- β 2 in the presence and absence of PD, and the calvariae were harvested after 0, 15, and 30 min exposure to Tgf- β 2, or after 3 days in culture. Bones and sutures were separated by microdissection, and Western blotting was performed for Erk1/2 activity (phosphorylated Erk1/2; Erk1/2-P) and for Erk1/2 protein expression. Control calvarial bones did not up-regulate Erk1/2-P up to 30 min in culture, whereas control calvarial sutures showed a transient up-regulation of Erk1/2-P after 15 min in culture (Fig. 3A,B). Both calvarial bones and sutures showed greater than threefold up-regulation of Erk1/2-

2-P 15 min after treatment with Tgf- β 2, and Erk1/2-P was sustained up to 30 min in culture after Tgf- β 2 treatment. All Erk1/2-P was blocked when PD was added to cultured calvariae (not shown). In contrast to Erk1/2 phosphorylation, no p38 phosphorylation was noted in response to Tgf- β 2 stimulation (not shown).

After 72 hr in culture, control calvarial bones and sutures expressed Erk1/2 protein; this expression was reduced in bones and sutures when PD was added to calvarial cultures (Fig. 3C,D). When calvariae were cultured for 72 hr in the presence of Tgf- β 2, Erk1/2 expression was up-regulated 2- and 2.5-fold in sutures and bones, respectively. PD abrogated Tgf- β 2 up-regulation of Erk1/2 in both bone and sutures. No detectable Erk1/2-P was present in either control or Tgf- β 2-treated calvarial bones and sutures after 72 hr in culture (not shown).

Fgf2 Phosphorylation of Erk1/2 Is Accelerated by Tgf- β 2 Treatment

Calvarial bones and sutures continuously treated with Tgf- β 2 for 72 hr do not continue to phosphorylate Erk1/2-P. Therefore, calvariae were treated for 72 hr with Tgf- β 2 and then stimulated with Fgf2, and calvariae were harvested at 0, 15, and 30 min. Calvarial bones and sutures were microdissected and analyzed using Western blots for Erk1/2-P. In control cultures, calvarial sutures had transient up-

regulation of Erk1/2-P after 15 min, whereas calvarial bones up-regulated Erk1/2-P 30 min after exposure to Fgf2 (Fig. 4A,B). PD completely blocked Erk1/2-P in sutures but did not completely down-regulate phosphorylation in bone samples. In cultures exposed to Tgf- β 2 for 72 hr before Fgf2 administration, both calvarial bones and sutures showed up-regulation of Erk1/2-P immediately after exposure to Fgf2. This up-regulation was sustained at elevated levels up to 30 min in sutures, but declined over time in bones (Fig. 4B). PD blocked Tgf- β 2-up-regulated Erk1/2-P expression similar to that seen in control cultures.

PD and Tgf- β 2 Act Synergistically to Increase Smad2/3 but Not Egf Protein Synthesis

The effect of Tgf- β 2 and Erk1/2 on Smad2/3 expression was examined. After 3 days in culture, control calvarial bones and sutures expressed Smad2/3 (Fig. 5A,B). When Erk1/2 activity was blocked, Smad2/3 expression was up-regulated 10-fold in control calvarial bones, but not in sutures. Tgf- β 2 increased Smad2/3 expression 2-fold in calvarial bones and 1.5-fold in calvarial sutures (Fig. 5B). Blocking Erk1/2 expression potentiated a 12-fold increase in Smad2/3 expression in calvarial bones and a 2-fold increase in sutures compared with untreated controls.

In contrast to Smad2/3 protein expression, Egf expression in control calvarial bones was higher than the expression seen in sutures, and Egf expression in both bones and sutures was unaffected by adding PD to cultures (Fig. 5C,D). Egf expression in calvarial bones was unaffected by Tgf- β 2 treatment; however, Egf in sutures was up-regulated twofold. Blocking Erk1/2-P with PD resulted in almost 50% reduction of Egf expression in bones treated with Tgf- β 2, and abrogated the Tgf- β 2-stimulated increase in Egf expression by sutures.

DISCUSSION

The Erk1/2 signaling pathway is one of many signaling pathways critical for Fgf receptor-mediated signaling.

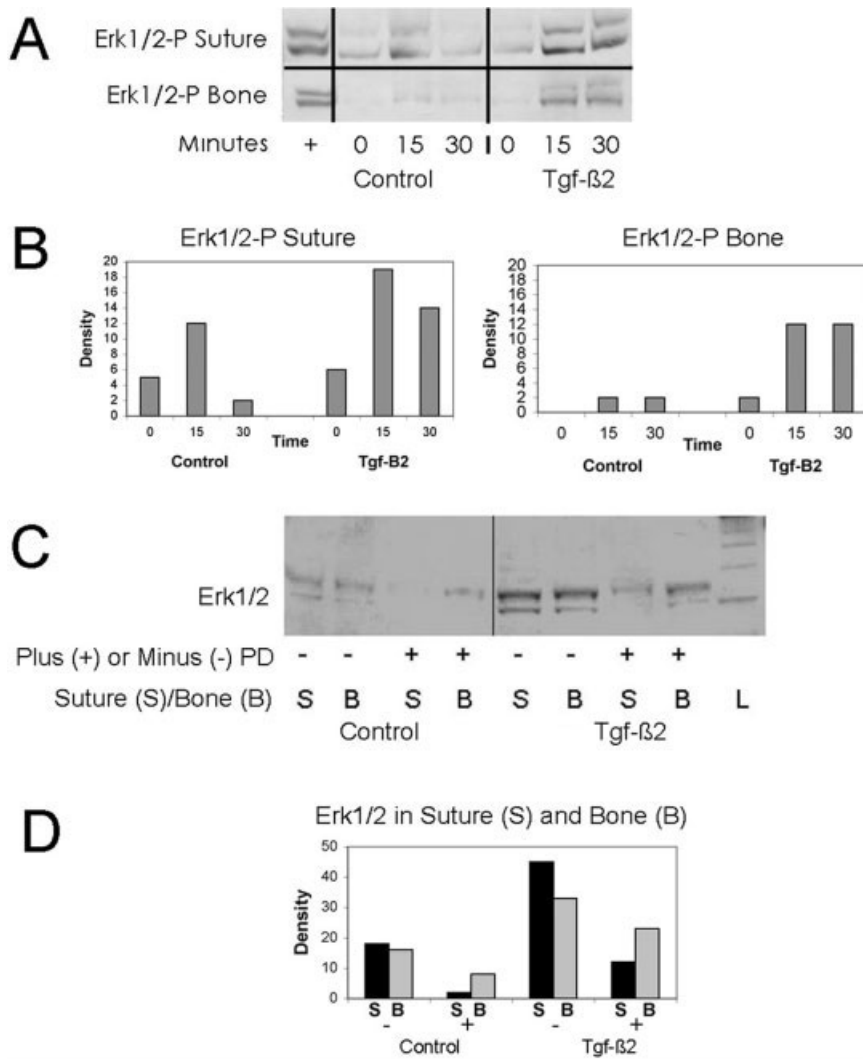


Fig. 3. A–D: Representative Western blots (A,C) and accompanying densitometry (B,D) of fetal day 17.5 (E17.5) mouse calvarial sutures and bones. **A:** Western blot with primary antibodies to phosphorylated Erk1/2 (Erk1/2-P). Note transient expression of Erk1/2-P at 15 min in control sutures and sustained up-regulation of Erk1/2-P in transforming growth factor β 2 (Tgf- β 2)-treated sutures and bones. The plus sign indicates positive control for Erk1/2-P. **B:** Densitometry of Western blot shown in A. **C:** Western blot with primary antibodies to Erk1/2. Note up-regulation of Erk1/2 expression in response to Tgf- β 2 treatment and down-regulation of Erk1/2 after blocking with PD. **D:** Densitometry of Western blot shown in (C).

Because Fgf receptor-mediated signaling has been associated with multiple craniosynostotic syndromes, it was possible that the Erk1/2 signaling pathway regulates Fgf receptor-mediated signaling during induced suture closure. Erk1/2 activity is a critical regulator of Fgf2 signaling during suture closure, as blocking Erk1/2-P abrogated the effect of Fgf2 on suture closure (Kim et al., 2003). Tgf- β 2 also induced premature suture closure both in vitro and in vivo (Opperman et al., 2000; Moursi et al., 2003; Mooney et al., manuscript submitted for publication). The present study showed

that Erk1/2-P was also required for Tgf- β 2 regulation of suture patency, suggesting that signaling by means of Erk1/2 is required for the induction of suture closure. Tgf- β 2-induced suture closure by means of Erk1/2-P may occur as a secondary event, perhaps in response to Tgf- β 2-induced protein synthesis of Fgf-related signaling molecules. However, Tgf- β 2 induced phosphorylation of Erk1/2, supporting the theory that signaling by means of Erk1/2-P was required for Tgf- β 2 regulation of suture closure. Whereas Tgf- β 2 phosphorylation of Erk1/2 may require prior phosphorylation of

Smad2/3, this was not a necessary event, as Sowa et al. (2002) showed that Tgf- β activation of Erk1/2 was independent of Smad phosphorylation and that Smads have antagonistic effects to Erk1/2 activity stimulated by Tgf- β .

Of interest, control calvarial sutures showed transient up-regulation of Erk1/2-P in culture, although control calvarial bones did not. Erk1/2-P up-regulation in Tgf- β 2 treated sutures and bones occurred at a similar time after stimulation to control sutures; however, in Tgf- β 2-treated tissues Erk1/2-P was sustained. Up-regulation of Erk1/2-P in control cultures was unexpected, because calvariae were cultured in serum-free conditions. Because the DMEM culture medium was supplemented with several factors, including glutamine, insulin, selenium, and transferrin, the culture medium itself or one or more of these molecules may have been responsible for transient stimulation of Erk1/2-P. Up-regulation of Erk1/2-P by the culture conditions could have been responsible for the gradual narrowing of control sutures in culture; however, it was not sufficient to induce suture closure comparable to Tgf- β 2 exposure. Similar gradual narrowing of control sutures was seen in other culture experiments (Kim et al., 2003). Suture narrowing could have been due to sutural cells behaving like fibroblastic cells, which contract in culture medium, a process that is Erk1/2-dependent (Langholz et al., 1997; Hirano et al., 2002). No transient up-regulation of p38 phosphorylation was noted in control cultures, and no phosphorylation of p38 by Tgf- β 2 was detected in either calvarial bones or sutures, indicating that Erk1/2 phosphorylation in response to Tgf- β 2 treatment was specific.

After Tgf- β 2 treatment for 72 hr, sutures narrowed substantially compared with controls. Calvarial bones and sutures up-regulated Erk1/2 protein expression, which was blocked if Erk1/2 phosphorylation was inhibited. This finding provides evidence that several events in addition to sutural width were regulated by Tgf- β 2 by means of Erk1/2-P. Of interest, Tgf- β 2 had little effect on Smad2/3 protein expression, but when Erk1/2 phosphorylation was blocked, Smad2/3

Fgf2 phosphorylation of Erk1/2 is accelerated by Tgf- β 2 treatment

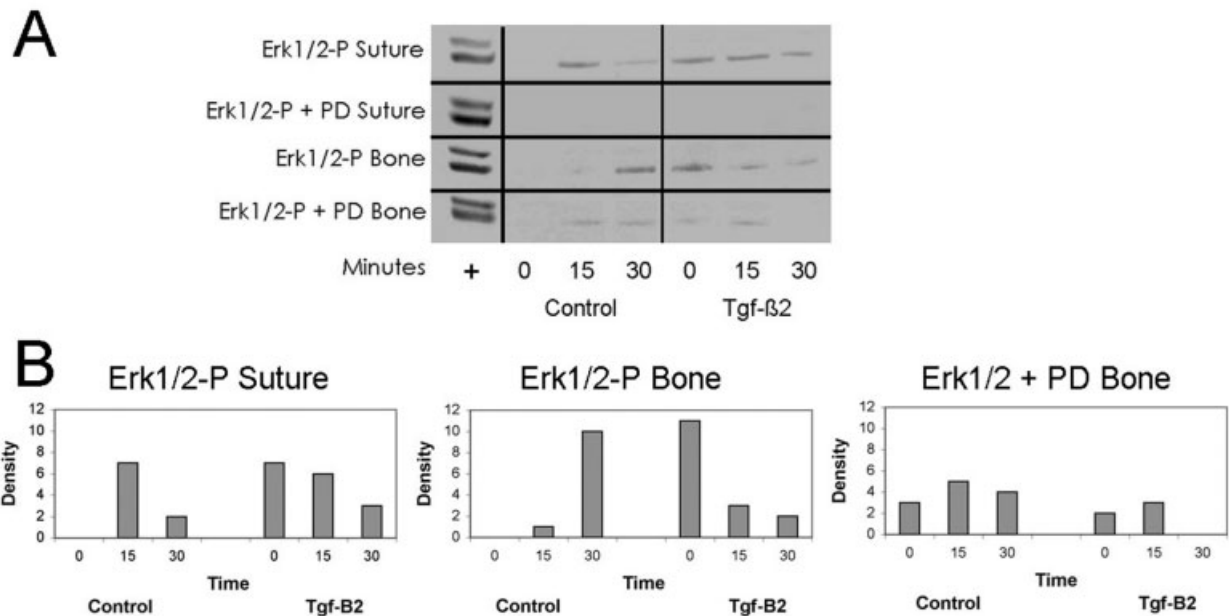


Fig. 4. **A:** Representative Western blot for Erk1/2-P in fetal day 17.5 (E17.5) mouse calvarial sutures and bones pretreated with transforming growth factor β 2 (Tgf- β 2) for 72 hr before stimulation with fibroblast growth factor 2 (Fgf2). Note transient Fgf2-induced expression of Erk1/2 in sutures and later up-regulation of Erk1/2-P in bones in control cultures not pretreated with Tgf- β 2. Note rapid and sustained up-regulation of Erk1/2-P after stimulation with Fgf2, in cultures pretreated with Tgf- β 2. Note inhibition of Erk1/2-P in sutures and bones with PD added to the culture medium. **B:** Densitometry of Western blot shown in A.

expression in bone was increased, an effect synergized by the addition of Tgf- β 2. No effect of blocking Erk1/2 was noted in control sutures, although a combination of Tgf- β 2 treatment and blocking Erk1/2 resulted in increased Smad2/3 expression. In contrast, Egf expression was elevated in control bones compared with sutures and was increased in sutures after Tgf- β 2 treatment. However, this increase in Egf in response to Tgf- β 2 was abrogated if Erk1/2 phosphorylation was inhibited. These differences in Smad2/3 and Egf expression between bones and sutures may speak to the relative differences in the differentiated states of suture tissues compared with bone.

Erk1/2-P activity inhibited Tgf- β 2-induced Smad2/3 expression, and Tgf- β 2 increased Smad2/3 expression by means of an Erk1/2-P-independent mechanism. This process could occur by means of a mechanism described by Lo et al. (2001). These researchers showed that Egf phosphorylated a

Smad2 corepressor transcription factor called Tgif by means of an Erk1/2-P-dependent pathway, and that Tgif-P formed a complex with Smad2-P in response to Tgf- β 1 treatment. This finding leads to the prediction that Egf or other molecules signaling by means of Erk1/2-P (such as Fgfs) could inhibit Smad2/3 protein expression by means of an Erk1/2-P signaling mechanism. In this manner, molecules signaling by means of Erk1/2 can indirectly regulate Tgf- β Smad signaling pathways. Because Egf was elevated in bones but not sutures, it is possible that Erk1/2 signaling after Egf stimulation was responsible for inhibiting Smad2/3 expression in bones.

Tgf- β 2 was shown to up-regulate Erk1/2 protein expression in calvarial tissues after 3 days in culture. Furthermore, pretreating calvarial tissues with Tgf- β 2 before stimulation with Fgf2 accelerated Fgf2 phosphorylation of Erk1/2. These data support the idea that molecules such as Fgf2

that signal by means of Erk1/2 could be responsible for the inhibition of Smad2/3 protein expression seen in response to Erk1/2 signaling (Fig. 6) and could be partly responsible for Tgf- β 2-induced suture closure, which occurs in a manner similar to Fgf2-induced suture closure described by Kim et al. (2003). Egf also uses Erk1/2-P as one of its primary signaling pathways, and blocks Smad1 phosphorylation by means of an Erk1/2-P-dependent mechanism, resulting in less Smad1 translocation to the nucleus (Kretzschmar et al., 1997). Egf is, therefore, a further candidate molecule for signaling by means of Erk1/2-P to inhibit Tgf- β 2-induced Smad2/3 expression and possibly exacerbate Erk1/2-induced suture closure.

Because Erk1/2-P activity inhibited Smad2/3 protein expression, it is possible that Erk1/2 and Smad2/3 have counter-regulatory effects on cell activity. In support of this theory, other investigators have shown that Tgf- β 1 inhibited alkaline phosphatase (ALP)

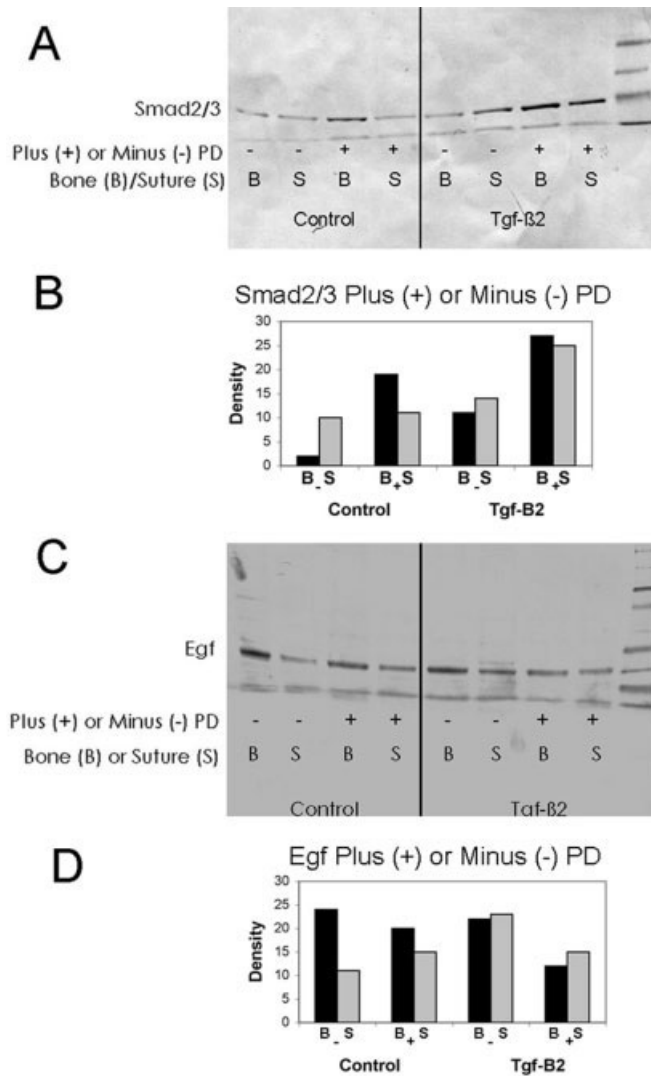


Fig. 5. A–D: Representative Western blots and densitometry of Smad2/3 (A,B) and EGF (C,D) in fetal day 17.5 (E17.5) mouse calvarial sutures and bones treated with and without transforming growth factor β 2 (Tgf- β 2) and PD98059 (PD). **A:** Note increased Smad2/3 expression in bone when Erk1/2-P is blocked with PD, and how Tgf- β 2 provides an additive effect to that of PD. Note also that Smad2/3 expression in sutures is up-regulated after treatment with both Tgf- β 2 and PD. **B:** Densitometry of gel shown in A. **C:** Note higher levels of EGF in bone compared with suture in control cultures. EGF expression in sutures becomes similar to levels in bone after treatment with Tgf- β 2. Addition of PD has no effect on EGF expression in control tissues but decreases EGF expression in bones and sutures exposed to Tgf- β 2. **D:** Densitometry of Western blot shown in C.

activity by means of Erk1/2-P and that Tgf- β 1 up-regulated ALP by means of Smad3-P (Sowa et al., 2002). Similarly, it was demonstrated that a counter-regulatory function of Erk1/2 and Smads operated in the inhibition of ALP activity by Bmp2 (Nakayama et al., 2003). Combining current findings with the demonstration of counter-regulatory effects of Erk1/2 and Smad signaling by Sowa et al. (2002) and Nakayama et al. (2003), a model is proposed that incorporates Erk1/2-P signaling mediating Tgf- β 2–

induced suture closure by up-regulating Erk1/2, and inhibiting Smad2/3 protein expression (Fig. 6). Up-regulation of Erk1/2 protein also provided increased substrate for signaling by means of Fgfr, a known pathway for inducing premature suture closure (Muenke et al., 1994; Wilkie et al., 1995; Bellus et al., 1996; Kim et al., 2003). This increase in Erk1/2 protein expression was demonstrated in calvarial tissues pretreated with Tgf- β 2, and these tissues had greater and more sustained Erk1/2 phosphoryla-

tion in response to Fgf2 than tissues not treated with Tgf- β 2.

If Smad2/3 expression is inhibited by Erk1/2-P, this inhibition would decrease one of the substrate molecules important for signaling by Tgf- β 3, a factor known to rescue sutures from obliteration (Opperman et al., 1999, 2000, 2002; Chong et al., 2003). Hence, Tgf- β 3 is hypothesized to rescue sutures from obliteration through Smad-mediated signaling, using this pathway to decrease expression of molecules involved in the Erk1/2 signaling pathway. Precedent for the use of alternate signaling pathways by Tgf- β 1, Tgf- β 2, and Tgf- β 3 in regulating morphogenetic processes is demonstrated by Potchinsky et al. (1997) who showed that, in contrast to Tgf- β 2, Tgf- β 1 and Tgf- β 3 do not phosphorylate Erk1/2. Ongoing experiments are being done to determine whether Tgf- β 2 activates the Rac-mediated Jnk1/2 pathway and to determine which molecules upstream of Erk1/2 (e.g., Ras, Raf) are responsible for Tgf- β 2 activation of Erk1/2. Experiments looking at the effect of non-Smad pathways—specifically Erk1/2 and p38—on Tgf- β 3 regulation of suture patency are also under way.

It is increasingly evident that several growth factor signaling pathways previously thought to be discrete pathways interact extensively with one another in the regulation of complex tissue interactions, such as dural regulation of cranial suture development. It is, therefore, hypothesized that common intracellular signaling pathways are the shared link in growth factor regulation of these processes. This linkage would explain the similar phenotypic outcomes induced by different growth factors.

EXPERIMENTAL PROCEDURES

Animals and Calvarial Culture

Pregnant female mice were obtained from Harlan (Indianapolis, IN) and housed in the Texas A&M University System HSC, Baylor College of Dentistry animal facilities under the guidelines dictated by NIH and IACUC. Female mice were killed on day 17.5 of pregnancy (vaginal plug

date = day 0.5) by inhalation overdose of Isoflurane anesthetic (Baxter, Grand Prairie, TX), and embryos were placed on ice, decapitated, and calvariae dissected and placed in tissue culture in 400 μ l of serum-free medium as previously described (Opperman et al., 1995). Calvariae were cultured in the presence of 0 and 3 ng/ml Tgf- β 2 (R&D Systems, Minneapolis, MN), and in the presence of 0 and 50 μ M PD98059 (PD, Calbiochem, La Jolla, CA). These concentrations previously were determined to be functional (Chu et al., 2000; Opperman et al., 2000). Calvariae were photographed daily using a digital camera mounted on a dissecting microscope, and the width of the PFS was measured using Metamorph software (Universal Imaging Corp., West Chester, PA). Each experiment was repeated twice.

In a second set of experiments, calvariae were pretreated with 0 (control) and 3 (experimental) ng/ml Tgf- β 2 for 72 hr, and then stimulated with 3 ng/ml Fgf2. Tissues were harvested immediately (0 min), 15 min, and 30 min after addition of Fgf2 and prepared for Western blotting as described below.

Western Blotting

Calvariae were harvested at 0 min, 15 min, 30 min, and 72 hr in culture, with three to six calvariae per time point. Sutures were microdissected from bones, and tissues from three calvariae were homogenized in phosphate buffered saline (PBS) containing 1 μ g/ml protease inhibitor cocktail and 1 μ l/ml phosphatase inhibitor (Sigma, St. Louis, MO). Protein concentrations were calculated, and 10 μ g/ml protein was loaded onto commercially available 12% Tris-glycine gels (Invitrogen, Carlsbad, CA); gels were run for 2.5 hr at 100 volts on a XCell Sure-Lock apparatus (Invitrogen), before blotting onto polyvinylidene difluoride 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—phospho-Erk1/2 and phos-

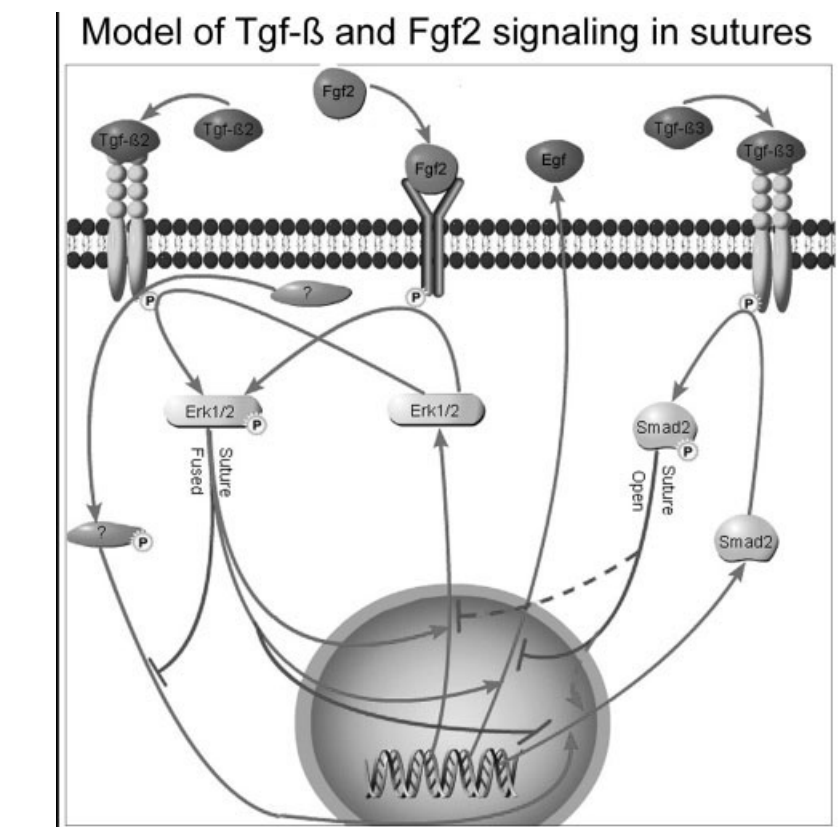


Fig. 6. Diagram of a model showing cell signaling pathways regulating suture patency. Question mark indicates unidentified signaling molecules involved in a pathway identified by experimental data. Solid lines indicate known signaling events. Dashed lines indicate predicted signaling events based on the model. For clarity, molecules upstream and downstream of Erk1/2 and Smad2 have not been included in the model. Nuclear events shown in this model are for expression or inhibition of Erk1/2, Egf, and Smad2 proteins. Phosphorylation events are shown in response to growth factor receptor binding.

pho-p38 (Cell Signaling Technologies, Beverly, MA), Smad2/3 and Egf (R&D Systems, Minneapolis, MN), and Erk1/2 (Santa Cruz Technologies, Santa Cruz, CA)—were added for 24 hr at 1:100. Primary antibodies were then washed off in three times 7-min washes with TTBS (1 mM Tris buffered saline [TBS] plus 20% Tween) before the addition of secondary antibodies—peroxidase-conjugated goat anti rabbit (1:500, Bio-Rad, Hercules, CA) for Erk1/2 and phospho-Erk1/2, and rabbit anti goat (1:1,000; Jackson ImmunoResearch, West Grove, PA) for Smad2/3 and Egf for 2.5 hr. Secondary antibodies were washed off with three times 7-min washes with TTBS, and the color reaction was developed using a Vector diaminobenzidine staining kit (Vector Laboratories, Inc., Burlingame, CA).

Blots were allowed to air-dry before digitizing, and densitometry was performed using Scion Image (Scion Cor-

poration, Frederick, MD). Western blots are notoriously variable, so densitometry was used for descriptive purposes only, and no statistical analyses were done.

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