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Differential Effects of Transforming Growth Factor-beta1 and -beta3 on Chondrogenesis in Posterofrontal Cranial Suture-Derived Mesenchymal Cells *in Vitro*

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Abstract

BACKGROUND—Transforming growth factor- β 1 (TGF- β 1) has been associated with cranial suture fusion, while its isoform TGF- β 3 has been associated with suture patency *in vivo*. The mouse posterofrontal (PF) suture, analogous to the human metopic suture, fuses through endochondral ossification.

METHODS—TGF- β 1 and - β 3 protein expression in the PF suture was examined by immunohistochemistry. Next, we established cultures of mouse suture-derived mesenchymal cells (SMCs) from the PF suture, and examined the cellular and molecular responses of PF SMCs to TGF- β 1 and TGF- β 3. Proliferation of PF SMCs in response to TGF- β isoforms was examined by bromodeoxyuridine incorporation. High-density micromass culture of PF SMCs was employed to study the effects of TGF- β 1 and TGF- β 3 on chondrogenic differentiation.

RESULTS—TGF- β 1 but not - β 3 protein was highly expressed in chondrocytes within the PF suture by immunohistochemistry. Significant increases in PF SMC proliferation were observed with TGF- β 3 but not TGF- β 1 (N=6, $*P \leq 0.01$). TGF- β 1 led to significant increases in chondrogenic-specific gene expression (including *Sox9*, *Col II*, *Aggrecan*, *Col X*) as compared to moderate effects of TGF- β 3 (N=3, $*P \leq 0.01$). The chondrogenic progression induced by TGF- β 1 and TGF- β 3 was further temporally analyzed. TGF- β 1 increased cellular adhesion molecule expression (*N-Cadherin*, *Fibronectin*, N=3, $*P \leq 0.01$) and promoted cellular condensation, whereas TGF- β 3 increased cellular proliferation (*PCNA* expression, N=3, $*P \leq 0.01$). Finally, TGF- β 1 and to a lesser extent TGF- β 3 induced the expression of fibroblast growth factors (*FGF-2* and *FGF-18*), cytokines associated with PF suture fusion.

CONCLUSIONS—TGF- β 1 and - β 3 exhibit marked differences in their effects on chondrogenesis in PF SMCs. Both isoforms were observed to influence different stages of chondrogenic differentiation *in vitro*. TGF- β 3 significantly increased mesenchymal cell proliferation, while TGF- β 1 induced precartilaginous mesenchymal cell condensation, promoting chondrocyte differentiation.

Keywords

Craniosynostosis; Cranial suture fusion; Transforming growth factor- β isoforms; Endochondral ossification; Suture mesenchyme

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INTRODUCTION

Much remains to be understood regarding the cellular and molecular mechanisms of cranial suture fusion. Toward this goal, our laboratory has studied the murine posterofrontal suture as a model of physiologic suture fusion. The murine posterofrontal (PF) suture is unique in several regards: 1) it is the sole cranial suture to fuse, and 2) does so through a process of endochondral ossification (1). In both respects, the PF suture is analogous to the human metopic suture (2, 3). Dura mater directly underlying the PF suture has also been shown to be unique: PF suture-associated dura mater produces cytokines which promote suture fusion, among them fibroblast growth factor-2 (FGF-2) and transforming growth factor- β 1 (TGF- β 1) (4-6).

The TGF- β superfamily consists of a large set of structurally-related, pleiotropic proteins important in skeletogenesis, including TGF- β isoforms, and bone morphogenetic proteins (BMPs) (7). TGF- β isoforms (specifically TGF- β 1 and - β 3) have distinct, even opposing, effects *in vivo*; this has been shown in disparate fields of study, including embryogenesis, wound healing, and palatal suture fusion (8-10). Research by our group and others suggests that an antagonistic relationship may exist between TGF- β 1 and - β 3 in cranial suture biology as well (4-6,11-20). Collectively, data suggest that TGF- β 1 promotes whereas TGF- β 3 inhibits cranial suture fusion both *in vivo* and in organ culture. The mechanisms through which TGF- β 1 and - β 3 differentially influence suture fate have been poorly elucidated. Results from studies have varied, suggesting that TGF- β 1 and - β 3 may differentially influence suture proliferation (11-13), apoptosis (11,12), and/or osteodifferentiation (11,14).

TGF- β isoforms are involved in chondrogenic differentiation and endochondral ossification (15,16). TGF- β 1 induces chondrogenic differentiation of various mesenchymal cell types, while TGF- β 3 has mixed inductive and inhibitory effects (17-20). In this regard, we hypothesized that TGF- β 1 and TGF- β 3 may exert differential effects on PF suture mesenchyme through another mechanism: namely, by influencing the process of chondrocyte differentiation within the PF suture, and ultimately endochondral ossification.

Our laboratory has recently devised the isolation and culture of mouse suture-derived mesenchymal cells (SMCs) (21,22 2008, 23). This allows for the controlled exposure of SMCs to cytokines involved in suture biology, cytokines such as FGF-2 and TGF- β s (22 2008, 23). SMCs derived from the PF suture have previously been shown to display unique properties *in vitro*, including a significant capacity to differentiate into both osteogenic and chondrogenic lineages (23). In the current study, we utilized the culture of PF SMCs to delineate the isoform-specific effects of TGF- β 1 and - β 3 on chondrogenic differentiation.

MATERIALS AND METHODS

Preparation of tissues for histology

First, to examine the *in vivo* expression patterns of TGF- β isoforms in the developing posterofrontal (PF) suture, specimens were prepared for histological analysis. Whole calvaria denude of skin were fixed overnight in 0.4% paraformaldehyde in PBS at 4°C (postnatal days 5, 7, 10, N=3 per time point). PF sutures with flanking calvariae bones were dissected, tissue was decalcified in 19% ethylenediaminetetracetic acid for 2-3 days at 4°C, dehydrated through graded ethanol, and paraffin embedded. Coronal sections through PF sutures of 5-micron width were mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and dried overnight at 37°C. Approximately 100 slides were produced per PF suture.

Histological staining and Immunohistochemistry

PF sutures were stained with Movat's pentachrome to visualize both bone and cartilage, which appear yellow and blue, respectively. Immunohistochemistry was performed on adjacent sections for TGF- β 1 and TGF- β 3. Slides were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol, and slides were blocked with serum free protein block (Dakocytomation, Carpinteria, CA). Primary antibodies used include rabbit polyclonal anti-TGF- β 1, and anti-TGF β 3 (1:500 for each in block, Santa Cruz Laboratories, Santa Cruz, CA). Appropriate biotinylated secondary antibodies were used in 1:1000 dilution (Vector Laboratories, Burlingame, CA). The Vectastain ABC system (Vector Laboratories) was used for color according to the manufacturer's instructions. A 0.01% fast green counter-stain was employed to visualize bone. Probing without primary antibody was performed as a negative control.

Tissue harvest and primary cell culture

Next, suture-derived mesenchymal cells (SMCs) were harvested from 100 five-day-old FVB mice (Charles River Laboratories, Wilmington, MA) as previously described (22). Mice were euthanized by cervical dislocation after carbon dioxide sedation. PF sutures were meticulously dissected in sterile phosphate buffered saline (PBS) with 500 microns of bony margin on either side. Dissection was performed under 50x magnification (Carl Zeiss MicroImaging GmbH, Stemi 2000). Great care was taken to exclude pericranial and dural tissues. Next, explants of PF sutures were placed in 100mm tissue culture dishes, (approximately 10 explants per dish), with the endocranial surface flush to the plate. Explants were then cultured in standard growth medium, containing DMEM (GIBCO Life Technologies and Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA), and 100 IU/ml of penicillin and streptomycin (GIBCO Life Technologies and Invitrogen). Cells were maintained at 37 ° C in an atmosphere of 5% CO₂, and medium was replenished every two days. Over the course of five days in culture, PF SMCs had migrated from tissue explants. At seven days of primary culture, SMCs were passaged by trypsinization. On average, each suture explant yielded 50,000 SMCs upon trypsinization. PF SMCs of passage one only were used for the following *in vitro* assays.

Cellular proliferation assays

The growth of PF SMCs in response to TGF- β 1 and TGF- β 3 were compared by bromodeoxyuridine (BrdU) incorporation assays. PF SMCs were plated at 1,000 cells/well in 96-well plates (Corning). After attachment, cells were treated with growth medium supplemented with recombinant TGF- β 1 (Research Diagnostics, Inc., Concord MA), recombinant TGF- β 3 (Fitzgerald Industries, Concord, MA) (0.5-20 ng/ml), or with vehicle as a control (1 $\times$ 10⁻⁴% bovine serum albumin, N=6 per concentration TGF- β 1 or TGF- β 3). Medium was replenished every other day, continuing supplementation with TGF- β 1 or - β 3. At three and six days, BrdU incorporation assays were performed according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Means and standard derivation were calculated.

Cell viability was concurrently assessed by staining with Trypan blue. Staining was performed after three and six days treatment with vehicle control, TGF- β 1, or TGF- β 3. Cell counting was performed by hemocytometer after staining. The percentage positively stained cells was calculated by dividing the absolute number of trypan blue positive cells in the numerator by total cell number in the denominator. Less than 5% was considered normal cell turnover in culture.

Chondrogenic differentiation and assessments

To assess chondrogenic differentiation, cells were plated in high-density culture of 7×10^4 cells/well in 12-well dishes. After attachment, cells were treated with chondrogenic medium containing DMEM, 1% FBS, 1% penicillin/streptomycin, 37.5 ug/ml ascorbate-2-phosphate, and ITS premix (BD Biosciences, Franklin Lakes, NJ) supplemented with TGF- β 1, TGF- β 3 (both at 10 ng/ml), or with vehicle as a control. A single concentration of 10 ng/ml was chosen as it has been shown to enhance chondrogenic differentiation in other mesenchymal cell types (18). Medium was changed every three days. Expression of chondrogenic gene markers was examined by quantitative real-time polymerase chain reaction (PCR) at 0, 3, 6, and 9 days (*Sox9*, *Col II*, *Aggrecan*, *Col X*, *PCNA*).

Next, cells were cultured in three-dimensional micromass as previously described (24). Early changes in adhesion molecule expression were examined during active cellular condensation of micromasses. Total RNA was harvested at 0, 3, 6, 9, 12, 24, and 36 hours of differentiation, with 0 hrs considered the time of micromass seeding and 3 hrs the time of micromass treatment (N=2 per group). Expression of cell surface calcium-dependent adhesion molecules (*N-Cadherin*, *Ob-Cadherin*) and extracellular matrix glycoproteins (*Fibronectin*, *Tenascin-X*) was quantified by real-time PCR.

To further examine possible molecular mechanisms of TGF- β induced chondrogenesis, the effects of TGF- β on TGF- β isoforms, TGF- β receptors and fibroblast growth factor (FGF) expression was next examined. PF SMCs were plated at 20,000 cells/well in 12-well plates (Corning). After attachment, cells were treated with growth medium supplemented with recombinant TGF- β 1, TGF- β 3 (0.5-10 ng/ml), or vehicle as a control. After 48 hours in culture, total RNA was isolated to examine changes in specific gene expression by quantitative real-time PCR (*TGF- β 1*, *TGF- β 3*, *TGF- β R1*, *TGF- β R2*, *FGF-2*, *FGF-18*).

RNA isolation and quantitative real-time PCR

RNA was isolated with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Sciences, Maryland). DNase treatment was performed with the DNA-free kit according to the manufacturer's instructions (Ambion, Austin, TX). Reverse transcription was performed using Taqman Reverse Transcription Reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out using the Applied Biosystems Prism 7900HT Sequence Detection System and Power Sybr Green Master Mix. Specific primers for the genes examined were designed based on their PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>) sequence. Primer sequences are shown in Table 1. PCR products were first run on a 2% agarose gel to confirm the appropriate size and specificity. The levels of gene expression were determined by normalizing to the values of β -2-microglobulin. All reactions were performed in triplicate.

Statistical analysis

Means and standard deviations were calculated from numerical data, as presented in the text, figures, and figure legends. In graphs, points represent means whereas error bars represent one standard deviation. Statistical analysis was performed using the ANOVA two-factor with replication when more than two factors were compared. In addition, the Welch's two-tailed *t*-test was used when standard deviations between groups were unequal. $*P \leq 0.01$ was considered to be significant.

RESULTS

Spatiotemporal expression patterns of TGF- β 1 and TGF- β 3 in the developing posterofrontal suture

We first sought to examine the temporospatial expression patterns of TGF- β 1 and TGF- β 3 in the developing mouse posterofrontal (PF) suture. At day of life 5 (the time of SMC harvest), the growing osteogenic fronts of the PF suture are widely separated by an undifferentiated cellular mesenchyme, see Fig. 1A and A'. Over the next week, this suture mesenchyme differentiates into cartilage which undergoes hypertrophy and apoptosis, eventually giving rise to bone, see Fig. 1D, D', G and G'. We examined TGF- β 1 and - β 3 protein expression during this period of PF suture morphogenesis. Results are shown in Figure 1. At postnatal day 5, a time immediately preceding chondrogenic differentiation within the suture, both TGF- β 1 and - β 3 stained intensely in the suture mesenchyme, particularly on the endocranial or dural surface, see Fig. 1B, B', C, and C'. This is consistent with previous reports in the literature reporting an intensity of dural staining preceding PF suture fusion (25). Interestingly, at postnatal day 7, TGF- β 1 was strongly localized to hypertrophic chondrocytes within PF suture mesenchyme, see Fig. 1E and E'. In contrast, little staining was observed in chondrocytes with TGF- β 3 immunohistochemistry, see Fig. 1F and F'. Similar findings were observed at postnatal day 10, see Fig. 1G-I. Interestingly, not all chondrocytes showed evidence of staining for TGF- β 1 protein; a distribution of stained and unstained chondrocytes was observed on the edges and center of the chondrocytic mesenchyme, respectively, see Fig. 1E' and 1H'.

Cellular proliferation of PF SMCs with TGF- β 1 and TGF- β 3

Having observed distinct *in vivo* expression patterns for TGF- β isoforms, we next inquired as to the specific effects of TGF- β 1 and TGF- β 3 on PF SMC proliferation, determined by BrdU incorporation assays. After three days of growth, SMCs exposed to TGF- β 1 showed significant decreases in proliferation at a wide range of concentrations, see Fig. 2A (1-20 ng/ml, N=6, * $P \leq 0.01$). This data was congruent with our previously reported effects of TGF- β 1 on SMC proliferation *in vitro* (23). In contrast, no change was observed with TGF- β 3 treatment. After six days growth, differential effects of TGF- β 1 and - β 3 were significant, see Fig. 2B. Consistent with previously reported findings, TGF- β 1 significantly decreased BrdU incorporation in comparison to vehicle control (5-20 ng/ml) (23). In contrast, TGF- β 3 demonstrated a significant proliferative effect (1-20 ng/ml). Trypan blue staining showed no differences in cell viability caused by TGF- β 1 or - β 3 (data not shown).

Chondrogenic differentiation of PF SMCs with TGF- β 1 and TGF- β 3

Previously, we demonstrated that PF SMCs retain a significant capacity to undergo chondrogenic differentiation *in vitro* (23). We next focused on how TGF- β 1 and - β 3, well-known mediators of chondrogenesis and endochondral ossification, differentially influence chondrogenic differentiation of PF SMCs (7,26,27). SMCs seeded in high-density were cultured with chondrogenic medium alone, with recombinant TGF- β 1 (10 ng/ml), or TGF- β 3 (10 ng/ml). To elucidate the progression of chondrogenic differentiation, cultures were carried out over nine days. Chondrogenic specific gene expression of *Sox9*, a critical transcription factor of chondrogenesis, as well as extracellular matrix (ECM) components *Col II*, *Aggrecan*, and *Col X* was examined by real-time PCR, see Fig. 3A-D. Proliferative cell nuclear antigen (*PCNA*) was concurrently examined to evaluate proliferation during chondrogenesis, see Fig. 3E. Over nine days with chondrogenic medium alone, *Col II* expression slightly increased overtime, while *Sox9*, *Aggrecan*, and *Col X* showed no changes. Consistent with our previously reported findings, addition of TGF- β 1 to chondrogenic medium resulted in significant induction of chondrogenic markers over baseline expression: *Sox9*, *Col II*, *Aggrecan*, and *Col X* increased over 30-, 19-, 24-, and 13-fold, respectively (N=3) (23). In contrast, addition of TGF- β 3 to chondrogenic medium did not effect *Sox9*, *Aggrecan*, or *Col*

X expression, but increased *Col II* expression at 9 days only, see Fig 3B. Confirming our previous finding by BrdU incorporation, addition of TGF- β 3 to chondrogenic medium led to a significant increase in *PCNA* expression over baseline, see Fig. 3E (significance at 3 and 6 days, $N=3$, $*P\leq 0.01$).

Regulation of adhesion molecule gene expression by TGF- β 1 and TGF- β 3

We next employed high-density micromass culture to further investigate the isoform specific effects of TGF- β s in the early condensation stage of chondrogenic differentiation. TGF- β 1 was noted to grossly induce micromass condensation: within the first 24 hours, cell aggregates had adopted a compact, spherical shape (data not shown). In contrast, with chondrogenic medium alone or with TGF- β 3, PF SMC micromasses were not able to form condensed cell aggregates in culture (data not shown). Thus, these observations suggested possible differential regulation of cell:cell and cell:matrix adhesion proteins in micromass condensation.

Classic cadherins, calcium-dependent homotypic adhesion molecules, are integral to cellular condensation during early chondrogenesis (28,29). We investigated the effects of TGF- β 1 and - β 3 on expression of N-Cadherin and Ob-Cadherin (Cadherin-11) throughout the first 36 hours of the cellular condensation stage of chondrogenesis, see Fig 4A and B. With chondrogenic medium alone, *N-Cadherin* and *Ob-Cadherin* expression was limited to the first 24 hours of chondrogenesis, a finding similar to temporal cadherin expression patterns in other mesenchymal cell types (30). Addition of TGF- β 1 led to significant increases in cadherin expression: a maximum of 14- and 3-fold increase in *N-Cadherin* and *Ob-Cadherin*, respectively ($N=3$, $*P\leq 0.01$). In contrast, addition of TGF- β 3 abrogated expression of both cadherin family proteins.

Fibronectin and Tenascin-X are ECM glycoproteins important in cellular condensation and the regulation of chondrogenesis (31-33). Addition of TGF- β 1 to chondrogenic medium led to significant increases in gene expression of both *fibronectin* and *tenascin-X* over baseline: over 8- and 12-fold increases, respectively, see Fig. 4C and D ($N=3$). Conversely, TGF- β 3 blunted both *fibronectin* and *tenascin-X* expression in comparison to chondrogenic medium alone (significance at 3, 6, and 12 hrs, $N=3$, $*P\leq 0.01$).

Regulation of fibroblast growth factor gene expression by TGF- β 1 and TGF- β 3

To further examine the isoform specific effects of TGF- β s on PF SMCs, we next focused on changes in fibroblast growth factor (FGF) signaling, specifically FGF-2 and FGF-18, which have shown to be important in calvarial osteogenesis as well as PF suture fusion (1,34). Gene expression of *FGF-2* and *FGF-18* was examined after culture with different concentrations of TGF- β 1 or - β 3 (0.5-10 ng/ml), see Fig. 5. Interestingly, TGF- β 1 induced a significant upregulation of *Fgf2* across a wide range of concentrations, see Fig. 5A (1-10 ng/ml, $N=3$, $*P\leq 0.01$). In contrast, TGF- β 3 significantly increased *Fgf2* expression at higher concentrations only (5-10 ng/ml, $N=3$, $*P\leq 0.01$). TGF- β 1 was also noted to significantly increase *Fgf18* expression (10 ng/ml), see Fig. 5B. In contrast, TGF- β 3 resulted in a downregulation of *Fgf18* expression at lower concentrations (0.5-1 ng/ml), while at a high concentration *Fgf18* was upregulated (10 ng/ml). Collectively, TGF- β 1 and TGF- β 3 change FGF expression in a concentration dependent fashion. It appears that at low doses, TGF- β isoforms effect FGF expression differentially, while at higher doses TGF- β 1 and TGF- β 3 have similar effects.

Regulation of TGF- β /TGF- β R gene expression by TGF- β 1 and TGF- β 3

To further examine the differential effects of TGF- β isoforms on PF SMCs, we next examined the regulation of gene expression of TGF- β s and their receptors by recombinant TGF- β 1 or TGF- β 3. Results are shown in Fig. 5C-F. Results showed that addition of either TGF- β 1 or TGF- β 3 led to a significant, dose-dependent up-regulation of endogenous *Tgfb1* expression,

see Fig. 5C. Interestingly, TGF- β 1 only increased gene transcripts for *Tgfb3*, see Fig. 5D (1-5 ng/ml, N=3, * $P \leq 0.01$). Expression of TGF- β receptors was next examined (TGF- β RI and TGF- β RII). Both TGF- β 1 and TGF- β 3 increased *Tgfb1* expression in a dose-dependent manner, see Fig. 5E (N=3, * $P \leq 0.01$). In contrast, no change in *Tgfb2* expression was observed, see Fig. 5F.

DISCUSSION

The local production of growth and differentiation factors by suture-associated dural tissue has been considered a major driving force in physiologic cranial suture fusion (6). Both TGF- β 1 and - β 3 isoforms are expressed in dural tissue underlying the mouse PF suture, suggesting that TGF- β paracrine signaling plays a role in cranial suture fusion (6,35). Moreover, manipulation of TGF- β signaling has led to changes in suture fate. Indeed, *in vivo* experiments which involve applying different isoforms of TGF- β s to the PF suture have supported divergent roles for these isoforms: delivery of TGF- β 1 has resulted in ectopic PF suture fusion (25), in contrast, TGF- β 3 has resulted in persistent PF suture patency (36). The mechanisms, however, whereby these two cytokines act differentially in suture biology remain unclear.

Recently, the PF suture has been found to undergo a temporal sequence of endochondral ossification: a sequence including mesenchymal cell proliferation, condensation, chondrogenic differentiation and hypertrophy, apoptosis and invasion by bone forming cells (1). Signaling molecules proposed to regulate suture fusion, in particular TGF- β s, were re-evaluated for their contributions to chondrogenesis. In the present study, we first found that while TGF- β 1 stained intensely in chondrocytes within the PF suture, TGF- β 3 did not. Moreover, we found TGF- β 1 and TGF- β 3 to affect different stages of chondrogenic differentiation in PF SMCs. The predominantly observed effect of TGF- β 3 was an increase in cellular proliferation. This was observed by both elevated BrdU incorporation, as well as *PCNA* expression. Notably, SMCs isolated from the sagittal suture (a suture which does not undergo endochondral ossification) did not show a proliferative response in culture with TGF- β 3 (data not shown). Thus, the mitogenic effects of TGF- β 3 are not pervasive across all suture cell types, but rather are specific to PF SMCs. These data led us to speculate that culture with TGF- β 3 may predominately affect chondrogenesis by the expansion of chondroprogenitor cells within the PF SMC population.

Chondrogenesis is a well-orchestrated process driven by chondrogenic progenitors that undergo proliferation, mesenchymal condensation, and chondrocyte differentiation (33). Moreover, mesenchymal condensation is considered a prerequisite for progressive chondrogenic differentiation. A predominant effect of TGF- β 1 in PF SMCs was found to be the significant induction of expression of both cadherins and ECM glycoproteins important in the initiation of cellular condensation. This result has been demonstrated previously in other cell types (30), and suggests that TGF- β 1 functions to initiate PF SMC condensation. Moreover, TGF- β 1 was observed to have profound effects on production of ECM components (*Col II*, *Aggrecan*), as well as chondrocyte hypertrophy (*Col X*).

Finally, we examined TGF- β effects on fibroblast growth factor (FGF) expression. Expression of both *Fgf2* and *Fgf18* has been shown to be up-regulated during PF suture fusion (1), and both cytokines are integral to osteochondrogenic differentiation as demonstrated by mouse knockout models (34,37). Observations from clinical molecular genetics have identified FGF signaling as of central importance in pathologic suture fusion, as the majority of recognized craniosynostotic syndromes involve activating mutations in FGF receptors (FGFRs) (38,39). Moreover, studies have shown that *in vivo* manipulation of FGF signaling dramatically alters cranial suture fate: following over-expression of FGF-2 with an adenovirus, the normally patent coronal suture underwent pathologic fusion, whereas *in vivo* abrogation of FGF-2/FGFR signaling led to aberrant PF suture patency (40). Results showed that TGF- β 1 rather than TGF-

$\beta 3$ significantly enhanced *FGF* gene expression in PF SMCs, particularly at lower doses. However, at the highest concentrations of 10 ng/ml both TGF- $\beta 1$ and - $\beta 3$ showed equal, significant inductions. Thus, TGF- β and FGF signaling may function in a cooperative manner in PF SMCs to induce differentiation; further studies are planned to explore the possibility of FGF/TGF β interaction in PF SMCs.

To more fully differentiate the effects of TGF- β isoforms on PF SMCs, we examined expression patterns of TGF- β receptors upon addition of either TGF- $\beta 1$ or - $\beta 3$ at various concentrations. Interestingly, we found TGF- β RI expression to be dynamically affected by both TGF- β isoforms. Both TGF- $\beta 1$ and TGF- $\beta 3$ addition to medium resulted in a dose-dependent up-regulation of TGF- β RI. In contrast, TGF- β RII showed no change with addition of ligand. Additional studies are warranted to understand the relevance of this positive regulatory mechanism in PF SMCs as well as chondrogenic differentiation.

These data in PF SMCs have important limitations when considering the study of craniosynostosis. The posterofrontal suture is an example of physiologic suture fusion, and thus has parallels to the human metopic suture - in both there exists a cartilage intermediate preceding closure (1,2). However, chondrogenesis has not been shown to precede craniosynostosis. Moreover, a cartilaginous intermediate does not develop in any other sutures of the mouse skull. Thus, the roles of TGF- β isoforms in chondrogenic differentiation of SMCs may be largely restricted to a single suture. Moreover, the applicability of this data to clinical, pathologic suture fusion remains as yet unclear.

In aggregate, TGF- $\beta 1$ and - $\beta 3$ exhibit marked differences in their effects on chondrogenesis of PF SMCs. Both isoforms were observed to influence different stages of chondrogenic differentiation *in vitro*. TGF- $\beta 3$ significantly increased mesenchymal cell proliferation, while TGF- $\beta 1$ induced precartilaginous mesenchymal cell condensation, promoting chondrocyte differentiation. These *in vitro* findings suggest a coordinate role of TGF- β isoforms in PF SMC chondrogenesis. Experiments unraveling the mechanisms whereby *in vivo* dural-derived TGF- β s affect PF suture closure will be further explored.

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REFERENCES

1. Sahar DE, Longaker MT, Quarto N. Sox9 neural crest determinant gene controls patterning and closure of the posterior frontal cranial suture. *Dev Biol* 2005;280:344–361. [PubMed: 15882577]
2. Manzanares MC, Goret-Nicaise M, Dhem A. Metopic sutural closure in the human skull. *J Anat* 1988;161:203–215. [PubMed: 3254893]
3. Kokich, V. Craniosynostosis: diagnosis, evaluation, and management. Raven Press; New York: 1986. The Biology of Sutures.
4. Most D, Levine JP, Chang J, et al. Studies in cranial suture biology: up-regulation of transforming growth factor-beta1 and basic fibroblast growth factor mRNA correlates with posterior frontal cranial suture fusion in the rat. *Plast Reconstr Surg* 1998;101:1431–1440. [PubMed: 9583470]
5. Nacamuli RP, Song HM, Fang TD, et al. Quantitative transcriptional analysis of fusing and nonfusing cranial suture complexes in mice. *Plast Reconstr Surg* 2004;114:1818–1825. [PubMed: 15577353]

6. Greenwald JA, Mehrara BJ, Spector JA, et al. Regional differentiation of cranial suture-associated dura mater in vivo and in vitro: implications for suture fusion and patency. *J Bone Miner Res* 2000;15:2413–2430. [PubMed: 11127206]
7. Janssens K, ten Dijke P, Janssens S, et al. Transforming growth factor-beta1 to the bone. *Endocr Rev* 2005;26:743–774. [PubMed: 15901668]
8. Roberts AB, Sporn MB. Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Mol Reprod Dev* 1992;32:91–98. [PubMed: 1637557]
9. O'Kane S, Ferguson MW. Transforming growth factor beta s and wound healing. *Int J Biochem Cell Biol* 1997;29:63–78. [PubMed: 9076942]
10. Fitzpatrick DR, Denhez F, Kondaiah P, et al. Differential expression of TGF beta isoforms in murine palatogenesis. *Development* 1990;109:585–595. [PubMed: 2401212]
11. Cabiling DS, Kim E, Yan D, et al. Differential effects of TGF-beta isoforms on murine fetal dural cells and calvarial osteoblasts. *Plast Reconstr Surg* 2007;120:614–624. [PubMed: 17700112]
12. 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 Dyn* 2000;219:237–247. [PubMed: 11002343]
13. Song HM, Fong KD, Nacamuli RP, et al. Mechanisms of murine cranial suture patency mediated by a dominant negative transforming growth factor-beta receptor adenovirus. *Plast Reconstr Surg* 2004;113:1685–1697. [PubMed: 15114130]
14. Fagenholz PJ, Warren SM, Greenwald JA, et al. Osteoblast gene expression is differentially regulated by TGF-beta isoforms. *J Craniofac Surg* 2001;12:183–190. [PubMed: 11314630]
15. Leonard CM, Fuld HM, Frenz DA, et al. Role of transforming growth factor-beta in chondrogenic pattern formation in the embryonic limb: stimulation of mesenchymal condensation and fibronectin gene expression by exogenous TGF-beta and evidence for endogenous TGF-beta-like activity. *Dev Biol* 1991;145:99–109. [PubMed: 2019328]
16. Seo HS, Serra R. Deletion of *Tgfb2* in *Prx1*-cre expressing mesenchyme results in defects in development of the long bones and joints. *Dev Biol* 2007;310:304–316. [PubMed: 17822689]
17. Mehlhorn AT, Niemeyer P, Kaschte K, et al. Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. *Cell Prolif* 2007;40:809–823. [PubMed: 18021172]
18. Xu Y, Balooch G, Chiou M, et al. Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells (ASC) using an in vitro three-dimensional micromass culture system. *Biochem Biophys Res Commun* 2007;359:311–316. [PubMed: 17543281]
19. Jin EJ, Choi YA, Sonn JK, et al. Suppression of ADAM 10-induced Delta-1 shedding inhibits cell proliferation during the chondro-inhibitory action of TGF-beta3. *Mol Cells* 2007;24:139–147. [PubMed: 17846509]
20. Jin EJ, Lee SY, Jung JC, et al. TGF-beta3 inhibits chondrogenesis of cultured chick leg bud mesenchymal cells via downregulation of connexin 43 and integrin beta4. *J Cell Physiol* 2008;214:345–353. [PubMed: 17620312]
21. Xu Y, Malladi P, Chiou M, et al. Isolation and characterization of posterofrontal/sagittal suture mesenchymal cells in vitro. *Plast Reconstr Surg* 2007;119:819–829. [PubMed: 17312483]
22. James AW, Xu Y, Wang R, et al. Proliferation, Osteogenic Differentiation, and FGF-2 Modulation of Posterior-Frontal/Sagittal Suture-Derived Mesenchymal Cells in Vitro. *Plast Reconstr Surg*. 2008Accepted for publication
23. Xu Y, James AW, Longaker MT. Transforming growth factor beta-1 stimulates chondrogenic differentiation of posterofrontal suture-derived mesenchymal cells in vitro. *Plast Reconstr Surg*. 2008Accepted for publication
24. Malladi P, Xu Y, Chiou M, et al. Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. *Am J Physiol Cell Physiol* 2006;290:C1139–1146. [PubMed: 16291817]
25. Roth DA, Gold LI, Han VK, et al. Immunolocalization of transforming growth factor beta 1, beta 2, and beta 3 and insulin-like growth factor I in premature cranial suture fusion. *Plast Reconstr Surg* 1997;99:300–309. [PubMed: 9030135]discussion 310–306

26. Centrella M, McCarthy TL, Canalis E. Skeletal tissue and transforming growth factor beta. *Faseb J* 1988;2:3066–3073. [PubMed: 2903838]
27. Dangelo M, Sarment DP, Billings PC, et al. Activation of transforming growth factor beta in chondrocytes undergoing endochondral ossification. *J Bone Miner Res* 2001;16:2339–2347. [PubMed: 11760850]
28. Oberlender SA, Tuan RS. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development* 1994;120:177–187. [PubMed: 8119125]
29. Delise AM, Tuan RS. Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro. *Dev Dyn* 2002;225:195–204. [PubMed: 12242719]
30. Tuli R, Tuli S, Nandi S, et al. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. *J Biol Chem* 2003;278:41227–41236. [PubMed: 12893825]
31. Tavella S, Bellese G, Castagnola P, et al. Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. *J Cell Sci* 1997;110(Pt 18):2261–2270. [PubMed: 9378775]
32. Gehris AL, Stringa E, Spina J, et al. The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. *Dev Biol* 1997;190:191–205. [PubMed: 9344538]
33. Hall BK, Miyake T. All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* 2000;22:138–147. [PubMed: 10655033]
34. Ohbayashi N, Shibayama M, Kurotaki Y, et al. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes & development* 2002;16:870–879. [PubMed: 11937494]
35. 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* 1997;12:301–310. [PubMed: 9076572]
36. Opperman LA, Moursi AM, Sayne JR, et al. Transforming growth factor-beta 3(Tgf-beta3) in a collagen gel delays fusion of the rat posterior interfrontal suture in vivo. *Anat Rec* 2002;267:120–130. [PubMed: 11997880]
37. Naganawa T, Xiao L, Abogunde E, et al. In vivo and in vitro comparison of the effects of FGF-2 null and haplo-insufficiency on bone formation in mice. *Biochem Biophys Res Commun* 2006;339:490–498. [PubMed: 16298332]
38. Cohen MM Jr. Craniosynostoses: phenotypic/molecular correlations. *Am J Med Genet* 1995;56:334–339. [PubMed: 7778605]
39. Wilkie AO, Morriss-Kay GM. Genetics of craniofacial development and malformation. *Nat Rev Genet* 2001;2:458–468. [PubMed: 11389462]
40. Greenwald JA, Mehrara BJ, Spector JA, et al. In vivo modulation of FGF biological activity alters cranial suture fate. *Am J Pathol* 2001;158:441–452. [PubMed: 11159182]

Figure 1. Protein expression of TGF- β 1 and - β 3 in the developing PF suture

(A) Pentachrome staining of a postnatal day 5 PF suture. Osteogenic fronts are widely separated by a cellular suture mesenchyme without evidence of chondrogenesis. (A') Higher magnification, note highly cellular suture mesenchyme which lies between bony fronts (yellow). (B-B') TGF- β 1 immunohistochemistry of adjacent section. Intense staining was noted, particularly on the dural or endocranial surface (bottom of figure). (C-C') TGF- β 3 immunohistochemistry of adjacent section. A similar pattern of staining was observed, with the highest intensity noted on the dural surface of the suture. (D-D') Pentachrome staining of a postnatal day 7 PF suture. At this timepoint the previous undifferentiated suture mesenchyme is now populated by hypertrophic chondrocytes, seen as blue. (E) TGF- β 1 immunohistochemistry of adjacent section. TGF- β 1 shows staining in various portions of the suture, including dura mater, bone marrow cavity, and chondrocytes. (E') Higher magnification illustrates intense staining for TGF- β 1 in chondrocytes of PF suture mesenchyme, particularly on the upper and lower borders of the suture mesenchyme (F-F') In contrast, TGF- β 3 immunohistochemistry reveals a relative paucity of staining at this time point. (G-G') Pentachrome staining of a postnatal day 10 PF suture. Chondrocyte hypertrophy within the PF suture is still observable. (H-H') TGF- β 1 immunohistochemistry of adjacent section again demonstrates intense staining localized to hypertrophic chondrocytes, particularly on the lateral borders of the suture mesenchyme. (I-I') Again, in contrast, TGF- β 3 immunohistochemistry demonstrates little staining with chondrocytes of the suture mesenchyme.

Figure 2. Cellular proliferation in PF SMCs with TGF-β1 and -β3

(A) BrdU incorporation assay after three days with rTGF-β1 (blue) or rTGF-β3 (red). In comparison to control (far left), rTGF-β1 significantly decreased BrdU incorporation (1-20 ng/ml), while rTGF-β3 resulted in no change. (B) BrdU incorporation assay after six days growth with TGF-β1 or -β3. TGF-β1 significantly decreased BrdU incorporation (5-20 ng/ml). In contrast, TGF-β3 significantly increased BrdU uptake (1-20 ng/ml). Values are normalized to day 3 and day 6 vehicle control groups; error bars represent one standard deviation; all significance levels are determined in comparison to untreated controls (far left); N=6 per concentration TGF-β1 or TGF-β3; * $P \leq 0.01$.

Figure 3. Chondrogenic differentiation of PF SMCs with TGF-β1 and -β3

Quantitative real-time PCR examining *Sox9*, *Col II*, *Aggrecan*, *Col X* and *PCNA* expression at up to nine days of chondrogenic differentiation with rTGF-β1 (blue), rTGF-β3 (red) (10 ng/ml), or vehicle control (black). (A) *Sox9* expression. TGF-β1 led to significantly increased expression of *Sox9* at 3, 6 and 9 days in culture. (B) *Collagen II* expression. A slight baseline increase of *Col II* was observed in culture by 9 days (black). TGF-β1 significantly increased *Col II* expression at both 6 and 9 days differentiation as compared to baseline. TGF-β3 significantly increased *Col II* expression at 9 days only. (C and D) Expression of *Aggrecan* and *Col X*. TGF-β1 induced significant increases in both genes at 6 and 9 days of differentiation. (E) *PCNA* expression. Culture with TGF-β3 produced significant increases in *PCNA*

expression over baseline at days 3 and 6 of differentiation. Values are normalized to day 0 gene expression; error bars represent one standard deviation; N=3; * $P \leq 0.01$.

Figure 4. Expression of adhesion molecules during early condensation with TGF- β 1 and - β 3
 Temporal expression of adhesion molecules was evaluated during the first 36 hours of condensation in micromass culture of PF SMCs. Graphs indicate gene expression under conditions of vehicle control (black), with rTGF- β 1 (blue), or rTGF- β 3 (red) (10 ng/ml). (A) *N-cadherin* expression. An elevated baseline *N-Cadherin* expression was observed from six to 12 hrs in culture. In comparison to control, TGF- β 1 upregulated, whereas TGF- β 3 decreased *N-Cadherin* expression (significant from six to 12 hrs). (B-D) *OB-cadherin*, *Fibronectin*, and *Tenascin-X* expression. TGF- β 1 significantly induced the expression of each adhesion molecule over baseline, whereas TGF- β 3 either did not change or decreased expression levels. Values are normalized to gene expression at 0 hrs; error bars represent one standard deviation; N=3; * $P \leq 0.01$.

Figure 5. TGF- β mediated changes in FGF, TGF- β and TGF- β R expression

Quantitative real-time PCR was used to evaluate FGF, TGF- β and TGF- β R gene expression after 48 hrs in culture with rTGF- β 1 (blue), rTGF- β 3 (red, 0.5-10 ng/ml), or vehicle as a control (far left). (A) *Fgf2* expression. *Fgf2* mRNA levels were dose-dependently increased with TGF- β 1 (1-10 ng/ml), and was induced by TGF- β 3 at higher concentration only (5-10 ng/ml). (B) *Fgf18* expression. TGF- β 3 decreased *Fgf18* expression (0.5-1 ng/ml). At higher concentrations, both TGF- β 1 and - β 3 led to increased expression (10 ng/ml). (C-D) *Tgfb1* and *Tgfb3* expression. Both rTGF- β 1 and - β 3 led to a dose-dependent upregulation of *Tgfb1* (left). In contrast, rTGF- β 1 only (blue) increased *Tgfb3* expression (right). (E-F) *Tgfb1* and *Tgfb2* expression. Both rTGF- β 1 and - β 3 increased *Tgfb1* expression (left). In contrast, no

change in *Tgfr2* expression was observed (right). Values are normalized to control groups; error bars represent one standard deviation; N=3; * $P \leq 0.01$.

TABLE 1**Quantitative PCR Genes and Primer Sequences**

Gene Name	GenBank Accession Number	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
Aggrecan	NM_007424	CCTGCTACTTCATCGACCCC	AGATGCTGTTGACTCGAACCT
β -2 microglobulin	NM_009735	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTTCGGCTCCCATTC
Collagen II (Col II)	NM_031163	TCCAGATGACTTTCCTCCGTCTA	CAGGTAGGCGATGCTGTTCTTAC
Collagen X (Col X)	NM_009925	TTCTGCTGCTAATGTTCTTGACC	GGGATGAAGTATTGTGTCTTGGG
Fibronectin	AF_095690	GCAGTGACCACCATTCTG	GGTAGCCAGTGAGCTGAACAC
FGF-2	NM_008006	AACCCGAGGTATGCTTGATCT	CCAGTTCTTCATTGCATTGC
FGF-18	NM_008005	AGGAGTATATGCCCCACGTG	TCGTCCACATCCACACTGTT
N-Cadherin	NM_007664	CCGTGAATGGGCAGATCACT	TAGGCGGATTCCATTGTCA
Ob-Cadherin	NM_009866	GACACCAACAGACCACTGGAG	TCTCAGGCACATTGGCATGAT
Sox9	NM_011448	TACGACTGGACGCTGGTGC	TTCATGGGTCGCTTGACGT
Tenascin-X	NM_031176	CCCGTCTATTCTGACCATCCT	TCCTTCCACAGTATGCTCGTA
TGF- β 1	NM_011577	AACAATTCCTGGCGTACCTT	TCCTTCCACAGTATGCTCGTA
TGF- β 3	NM_009368	CCAAATCAGCCTCTCTCTGT	AATGGCTTCCACCCTCTTC
TGF- β R1	NM_009370	ATCTATGCAATGGGCTTAGTGTT	TAGTCTTCATGGATTCCACCAAT
TGF- β R2	NM_009371	TGTCTACTCCATGGCTCTGGTA	GGCTCGTAATCCTTCACTTCTC