In Vivo Modulation of FGF Biological Activity Alters Cranial Suture Fate

Joshua A. Greenwald,* Babak J. Mehrara,*
Jason A. Spector,* Stephen M. Warren,[‡]
Peter J. Fagenholz,[‡] Lee P. Smith,*
Pierre J. Bouletreau,[‡] Francesca E. Crisera,[‡]
Hikaru Ueno,[†] and Michael T. Longaker[‡]

From the Laboratory of Developmental Biology and Repair,* The Institute of Reconstructive Plastic Surgery, and The Department of Surgery, New York University Medical Center, New York, New York; the Department of Cardiology,[†] Molecular Cardiology Unit, Kyushu University School of Medicine, Fukuoka, Japan; and the Department of Surgery,[‡] Stanford University School of Medicine, Stanford, California

Gain-of-function mutations in fibroblast growth factor receptors have been identified in numerous syndromes associated with premature cranial suture fusion. Murine models in which the posterior frontal suture undergoes programmed fusion after birth while all other sutures remain patent provide an ideal model to study the biomolecular mechanisms that govern cranial suture fusion. Using adenoviral vectors and targeted *in utero* injections in rats, we demonstrate that physiological posterior frontal suture fusion is inhibited using a dominant-negative fibroblast growth factor receptor-1 construct, whereas the normally patent coronal suture fuses when infected with a construct that increases basic fibroblast growth factor biological activity. Our data may facilitate the development of novel, less invasive treatment options for children with craniosynostosis. (*Am J Pathol* 2001, 158:441–452)

Gain-of-function mutations in the fibroblast growth factor receptors (FGF-Rs) have been identified in many syndromes that have craniosynostosis as their defining characteristic including Apert, Pfeiffer, and Crouzon syndromes. These syndromes are all characterized by craniosynostosis (ie, premature fusion of one or more cranial sutures) and varying degrees of extracranial skeletal abnormalities.¹ *In vitro* studies of these mutated FGF-Rs have identified at least two biomolecular mechanisms that mediate excess signaling by these receptors: 1) formation of receptor dimers in the absence of receptor ligand [ie, basic fibroblast growth factor (FGF), FGF-2] resulting in ligand-independent intracellular signaling; and 2) increased affinity of mutated receptors for ligand.^{2–6} Increased FGF-biological activity is thought

to lead to the premature fusion of cranial sutures and the dysmorphic craniofacial phenotype associated with these syndromes. To date, however, direct evidence supporting this hypothesis has been lacking.

To understand the events that lead to premature cranial suture fusion, numerous investigators have relied on animal models of cranial suture fusion. Murine suture development, in which the posterior frontal (PF) suture undergoes programmed sutural fusion shortly after birth, provides an ideal model to study the biomolecular mechanisms that occur before, during, and after cranial suture fusion.^{7,8} In these models, the PF cranial suture fuses between postnatal days 12 to 22 in the rat and days 25 to 45 in the mouse whereas all other sutures, including the coronal (COR) and sagittal (SAG), remain patent (Figure 1). Using these models, our group and others have demonstrated that the dura mater directly underlying a cranial suture regulates sutural fate (ie, fusion or patency).^{8–11} In addition, we have shown that FGF-2 mRNA and protein expression in the fusing PF suture is up-regulated in the suture-associated dura mater just before and during active sutural fusion.^{12,13} These findings implicate FGF biological activity in the regulation of cranial suture fusion.

In this study, replication-deficient adenoviruses encoding a truncated form of FGF-R1 (AdCAFGF-TR) or a secreted form of FGF-2 (AdCAsFGF-2) were used either to abrogate or increase FGF biological activity *in vivo* in the dura mater underlying a fusing or patent suture, respectively. Our findings demonstrate that *in utero* AdCAFGF-TR infection of the dural tissues underlying the PF cranial suture inhibits physiological cranial suture fusion *in vivo*, whereas *in utero* AdCAsFGF-2 infection of the dural tissues underlying the COR suture results in fusion of this normally patent suture. Through a variety of *in vitro* analyses, we demonstrate that these effects are mediated via alterations in cellular proliferation, extracellular matrix molecule gene expression, and transforming growth factor (TGF)- β 1 synthesis. These data provide direct support for the hypothesis that FGF biological activity is a critical regulator of both programmed and pathological cranial suture fusion. In addition, this report represents the first successful alteration of cranial suture fate *in vivo* in a nontransgenic model, and may facilitate the

Supported by National Institute of Dental and Craniofacial Research grant RO1-DE13194.

Accepted for publication October 13, 2000.

Address reprint requests to Michael T. Longaker, M.D., Stanford University School of Medicine, Department of Surgery, 269 Campus Drive, CCSR Room 1225-S, Stanford, CA 94305-5148. E-mail: longaker@stanford.edu.

Figure 1. **A:** Line drawing depicting murine calvarium, viewed from above. The PF suture fuses whereas all other sutures remain patent. **B:** Histological cross-section through SAG suture of postnatal day 50 mouse. Note patency of suture. **C:** Histological cross-section through PF suture of postnatal day 50 mouse. Note fusion of suture.

development of novel, less invasive treatment options for children with craniosynostosis.

Materials and Methods

Animals

All experiments were approved by the Institutional Use and Care Committee at New York University Medical Center. Time-dated pregnant Sprague-Dawley rats were purchased from Taconic Laboratories (Germantown, NY). CD-1 mice (24 days old) were purchased from Charles River Laboratories (Wilmington, MA). For all animals, the first day of life was considered the first day after birth.

Preparation of Adenoviral Vectors

A replication-deficient recombinant adenovirus encoding a truncated form of the chicken FGF-R1 gene (AdCAFGF-TR) was constructed as previously described.¹⁴ The truncated FGF-R1 was a 1.3-kb cDNA fragment lacking the sequence coding its cytoplasmic tyrosine kinase domain and tagged with an influenza virus hemagglutinin epitope at its C-terminus. This fragment was subcloned into an adenoviral expression vector (type 5 genome with deletions of E1 and E5 regions) containing a chicken β -actin promoter with a cytomegalovirus enhancer unit. This construct has been shown to inhibit signal transduction by FGF-R1, FGF-R2, and FGF-R3 *in vitro*.¹⁵

AdCAFGF-2 was prepared as previously described.¹⁶ Briefly, a recombinant cDNA for the secreted form of human FGF-2 was constructed by adding the signal sequence of FGF-4 to the 5' end of cDNA encoding the full-length human FGF-2. The control adenovirus encoding the *Escherichia coli* lacZ (β -galactosidase cDNA) was constructed in a similar manner.¹⁴ All adenoviruses were propagated in human 293 cells and purified by centrifugation in cesium chloride step gradients before dialysis against 10% glycerol in phosphate-buffered saline (PBS) as previously described.¹⁷

Tissue Harvest for PF-Dural Cell and Osteoblast-Enriched Cell Cultures

Dural cell cultures derived from PF-associated dural tissues (PFDCs) from postnatal day 6 rats were established as previously reported.¹⁸ Osteoblast-enriched cell cultures (NRCs) derived from neonatal rat calvaria were established as previously described by Frick and colleagues.¹⁹ First passage cells were used for all experiments.

Western Blot Analysis for FGF-TR, Phosphorylated ERK-1 and -2, and FGF-2

Western blot analyses were performed to assess transgene expression and phosphorylation of proteins involved in FGF signal transduction. One million first pas-

sage NRCs and PFDCs were plated on tissue culture plates. Once confluent, cells were infected with vehicle, AdCALacZ (100 pfu/cell), or AdCAFGF-TR (50 and 100 pfu/cell). Protein was collected 48 hours after infection and an immunoblot analysis of hemagglutinin protein expression (ie, marker on the FGF-TR transgene) was performed using a monoclonal hemagglutinin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Twenty-four hours after infection, an additional group of infected cells was placed in serum-free media. After 24 hours (ie, 48 hours after infection), 10 ng/ml of recombinant human basic FGF (rhFGF-2; R&D Systems, Minneapolis, MN) was added to the serum-free media. After 30 minutes, total cellular protein was collected for Western blot analysis. Immunoblot analysis was performed using an antibody to phosphoERK-1 and -2 (ERK = extracellular regulated protein kinase; New England BioLabs, Beverly, MA) and reprobing the same membrane with a nonphosphorylation-specific ERK-2 antibody that recognizes ERK-1 to a lesser extent (Santa Cruz).

To verify overexpression of FGF-2 by the AdCAsFGF-2 construct, cells were infected with vehicle, AdLacZ, and AdCAsFGF-2 as described above. Protein was collected 48 hours after infection. Western blot analysis for FGF-2 was performed using a monoclonal human FGF-2 antibody (clone no. 354F1; Texas Biotechnologies, Houston, Texas). Immunoblot analyses of phosphoERK-1 and -2 and total ERK were performed as described above without rhFGF-2 stimulation of vehicle and AdCALacZ-infected cells.

Cranial Suture Organ-Culture System

To analyze the effects of alterations in FGF biological activity, a cranial suture organ-culture system was established as previously described (see Figure 3A).^{7,8} In this model, the mouse PF suture fuses after 30 days in culture. Mouse calvarial explants are ideal for these studies because their small size and thin bone cortices allow prolonged survival in culture whereas the thicker, larger bone in rat calvaria precludes similar analysis *in vitro*. Briefly, after euthanizing 30 CD-1 mice (24 days old), a portion of the calvaria inclusive of the PF, SAG, and COR sutures and their underlying dural tissues (suture complex) was isolated, and washed copiously with PBS. The suture complexes were then divided into three groups ($n = 10$ per group). Dural tissues underlying the PF suture were infected with AdCAFGF-TR (1×10^9 pfu), AdCALacZ (1×10^9 pfu), or vehicle (PBS; 20 μ l). Infected suture complexes were then placed into organ culture wells consisting of an insert with a 3.0- μ m pore size (Millipore, Bedford, MA). After placement of the suture complex into the inserts, 500 μ l of serum-free suture media was placed (BGJb medium supplemented with 500 μ g/ml penicillin, 500 μ g/ml streptomycin, 0.25 μ g amphotericin, 100 μ g/ml ascorbic acid, and 0.3 mmol/L sodium phosphate, pH 7.4) into each well and changed every other day.

All sutures were harvested after 30 days in culture and immediately fixed in 10% formalin. Intact suture complexes

were analyzed to assess fusion or patency under a dissecting microscope before histological evaluation. X-gal staining of suture complexes infected with AdCALacZ was performed after formalin fixation as previously described.¹⁷ Forty 5.0- μ m sections were prepared for each PF suture after dividing the PF suture through its midpoint.

Targeted, in Utero Delivery of AdCAFGF-TR and AdCAsFGF-2

Pregnant Sprague-Dawley rats (embryonic day 18; E18) were purchased from Taconic Laboratories. Modulation of FGF biological activity at E18 was chosen as FGF-2 mRNA is highly expressed by the calvarial dural tissues at this time point.²⁰ After attainment of adequate anesthesia, a midline laparotomy incision was made. The fetal rat heads were exposed by making a hysterotomy. For experiments examining the effect of FGF biological activity abrogation, the fetal PF suture was identified, and 1×10^9 pfu of AdCALacZ, AdCAFGF-TR, or 20 μ l of PBS (ie, vehicle) was injected into the region of the PF dura after piercing the calvaria adjacent to the PF suture with a tuberculin syringe. For experiments in which FGF biological activity was increased, the fetal COR suture was identified, and 1×10^9 pfu of AdCALacZ, AdCAsFGF-2, or 20 μ l of PBS (ie, vehicle) was injected into the dural tissues underlying the left COR suture. After injection, the fetal head was returned to the uterus and the hysterotomy was repaired with fine suture material. Four of the 11 to 12 fetuses per pregnant rat were treated. The remaining fetuses received a 40- μ l injection of India Ink in the dorsal soft tissues of the thorax to facilitate their postnatal identification. After delivery, untreated neonatal rats injected with India Ink were sacrificed. Two pregnant rats (four treated fetuses/rat times two pregnant rats equals eight treated fetuses/group) were used for AdCAFGF-TR and AdCAsFGF-2 transfections. Four pregnant rats each were used for AdCALacZ and vehicle transfections (two in each group received injections targeting the PF dura; two in each group received injections targeting the COR suture).

Treated animals were sacrificed on postnatal day 30. Calvaria were harvested and immediately fixed in formalin. X-gal staining of AdCALacZ-treated animals was performed. After decalcification, 5- μ m sections were prepared from the midpoint of the PF and COR sutures. A total of 40 sections was analyzed from each PF and COR sutures and assessed histologically for fusion or patency. To assess the presence of the truncated FGF-R1 construct, immunohistochemistry was performed using a rabbit anti-hemagglutinin antibody. Immunohistochemical techniques were performed as previously described.¹³

RNA Isolation from NRCs and PFDCs

RNA isolation was performed as previously described.¹⁷ Briefly, one million first-passage NRCs or PFDCs were plated on 100-mm culture dishes. After reaching confluence, cells were infected with AdCALacZ (100 pfu/cell),

AdCAFGF-TR (50 and 100 pfu/cell), or vehicle. Twenty-four hours after infection, cells were placed in serum-free media for 24 hours and 10 ng/ml of rhFGF-2 (R&D Systems) was added to the media. RNA was isolated after 6 and 48 hours of rhFGF-2 (R&D Systems) stimulation and analyzed for expression of TGF- β 1 and collagen type I mRNA, respectively. RNA isolated from infected cells in serum-free media and not exposed to rhFGF-2 served as controls for all groups. RNA was isolated from an additional group of infected cells 48 hours after infection and probed for expression of the transgene encoded by AdCAFGF-TR (see below).

RNA isolation from cells infected with AdCasFGF-2, vehicle and AdCALacZ was performed 48 hours after infection (serum-free media $\times$ 24 hours) and analyzed for alterations in baseline TGF- β 1 mRNA expression.

Probe Preparation

Rat probes for TGF- β 1 and collagen I α 1 were 535- and 727-bp polymerase chain reaction-amplified fragments, respectively, as previously described.²¹ The murine FGF-R1 cDNA probe was a 1.2-kb *EcoRI-BamHI* fragment from the full-length FGF-R1 cDNA.²² Glycerol aldehyde phosphate dehydrogenase (GAPDH) was a 1-kb fragment complementary to the human GAPDH-coding region with strong cross-reactivity against rat GAPDH (Clontech, Palo Alto, CA). Approximately 100 ng of each probe were labeled with 50 μ Ci of [α -³²P]-dCTP (New England Biotech, Piscataway, NJ). Northern blot analysis was performed as previously described.²³ All experiments were performed in duplicate.

Cellular Proliferation: BrdU Incorporation and Nonradioactive Proliferation Assay

To assess the effects of AdCAFGF-TR infection on cellular proliferation, NRCs and PFDCs were infected with AdCALacZ (100 pfu/cell), AdCAFGF-TR (50 pfu and 100 pfu/cell), or vehicle. On the following day, 20,000 infected (vehicle, 100 pfu/cell AdCALacZ, 50 and 100 pfu/cell AdCAFGF-TR) NRCs were grown in 16 wells of a 24-well tissue-culture plate into which glass coverslips had been placed. After 24 hours, serum-free media was added to each well. On the following day, 10 ng/ml of rhFGF-2 was added to the media of all wells. All cells were labeled with BrdU (10 μ M/L final concentration) for 1 hour before fixation with 10% formalin. BrdU-positive cells were identified using a monoclonal BrdU antibody (Amersham Pharmacia Biotech, Uppsala, Sweden) and a fluorescein-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, Penn) followed by incubation with Hoechst stain (1 μ g/ml in deionized water) (Sigma, St. Louis, MO).^{24,25}

Proliferation as assessed by BrdU incorporation was corroborated by performing a nonradioactive proliferation assay. Proliferation of infected cells was assessed using the WST-1 cell proliferation reagent assay (Boehringer Mannheim, Indianapolis, IN) after 1, 3, and 5 days

in culture. All experiments were performed in triplicate for each time point.

Similar proliferation analysis was performed on cells infected with AdCasFGF-2, vehicle, and AdCALacZ. In these studies, vehicle and AdCALacZ cells were not treated with rhFGF-2. All experiments were performed on similarly treated PFDCs.

Statistical Analysis

Statistical analyses of BrdU incorporation and the nonradioactive proliferation assay were performed using two-way ANOVA with $*P \leq 0.05$ considered significant. Post hoc tests were performed using the Tukey-Kramer multiple comparison test. Statistical analysis of Northern blot and Western blot analyses were not performed because these studies represent semiquantitative measures. All Northern and Western blot analyses were performed in duplicate.

Results

Adenoviral Constructs Alter FGF Biological Activity in Vitro

To assess the efficacy of the adenoviral constructs to alter FGF biological activity in infected cells, osteoblast-enriched cultures (NRCs) were infected with AdCAFGF-TR or AdCasFGF-2. High-level, dose-dependent, transgene expression was detected by Northern and Western blot analysis (Figure 2A). Furthermore, transgene expression resulted in a biologically active product resulting in abrogation of ERK-1 and -2 phosphorylation in AdCAFGF-TR-infected NRCs stimulated with rhFGF-2 (Figure 2B). As expected, NRCs infected with vehicle and AdCALacZ demonstrated ERK-1 and -2 phosphorylation in response to rhFGF-2 stimulation.²⁶ Similarly, Western blot analysis of FGF-2 demonstrated that AdCasFGF-2-infected cells express greater amounts of FGF-2 than control or AdCALacZ-infected cells resulting in increased FGF biological activity as assessed by ERK-1 and -2 phosphorylation (Figure 2, C and D). Similar results were obtained on identically treated cells derived from PF-associated dural tissues (PFDCs) (data not shown).

Abrogation of FGF Biological Activity Prevents Programmed PF Suture Fusion in Vitro

To assess sutural development in response to alterations in FGF biological activity we chose first to use an *in vitro* organ culture system in which mouse cranial sutures, together with the underlying dura mater, are placed in serum-free culture conditions (Figure 3A). We chose to analyze cranial suture fusion *in vitro* as this system enables isolation of the dura mater-cranial suture complex from humoral or mechanical forces that may contribute to sutural fusion/patency. To examine the effects of abrogating FGF biological activity, the dural tissues of mouse calvarial explants inclusive of the PF and SAG sutures

Figure 2. A: Northern (left) and Western blot (right) analysis demonstrating high-level expression of FGF-TR. Note increase in FGF-TR mRNA and protein with increasing plaque-forming units. Compare AdCAFGF-TR 50 with AdCAFGF-TR 100. 50 = 50 plaque-forming units/cell; 100 = 100 plaque-forming units/cell. B: Western blot analysis demonstrating increased phosphorylation of ERK-1 and -2 after FGF-2 stimulation (+) in vehicle and AdCALacZ-infected NRCs (top immunoblot). In contrast, AdCAFGF-TR-infected NRCs demonstrate no increase in ERK-1 and -2 phosphorylation in response to rhFGF-2 stimulation. Total ERK-2 immunoblot of same blot (bottom immunoblot) demonstrating presence of similar levels of unphosphorylated protein in all lanes. C: Western blot analysis for FGF-2 demonstrates increased expression of FGF-2 protein in NRCs infected with AdCAsFGF-2. D: Western blot analysis demonstrating increased phosphorylation of ERK-1 and -2 after AdCAsFGF-2 infection of NRCs (top immunoblot). Total ERK-1 and -2 immunoblot of same membrane (bottom immunoblot) demonstrating presence of similar levels of unphosphorylated protein in all lanes. In this experiment, cells were maintained in serum-free media without the addition of rhFGF-2.

were infected with vehicle, AdCALacZ, or AdCAFGF-TR, and cultured for 30 days as previously described.^{7,8} X-gal staining of AdCALacZ-infected suture complexes demonstrated significant transgene expression within cells localized to the underlying dura mater of the suture at the end of the culture period (Figure 3, B and C). In addition, examination of PF sutures under $\times 25$ magnification demonstrated complete fusion of all vehicle or AdCALacZ-infected PF sutures ($n = 10$ in each group) after the 30-day culture period (Figure 3D). In contrast, all PF sutures infected with the AdCAFGF-TR ($n = 10$) were widely patent at the end of the culture period (Figure 3E). Histological examination corroborated these findings (Figure 3, F and G).

Abrogation of FGF Biological Activity in Utero Prevents Programmed PF Suture Fusion

Based on our *in vitro* organ culture findings, we evaluated the fate of PF suture complexes treated with the truncated FGF-R1 adenovirus *in utero*. Rat embryos (E18) in which the PF-associated dural tissues were infected with AdCAFGF-TR demonstrated no gross phenotypic abnormalities. Similar to our *in vitro* findings, transgene expression as assessed by X-gal or immunohistochemical staining of sutural complexes harvested on postnatal day 30

demonstrated high levels of transgene expression in the dura mater underlying the PF suture (Figure 4; A, B, D, G, and H). In addition, as expected, all PF sutures infected with vehicle or AdCALacZ demonstrated complete sutural obliteration on postnatal day 30 (Figure 4, B and C). In contrast, all PF sutures infected with AdCAFGF-TR were widely patent (Figure 4, E and F). Thus, targeted *in utero* delivery of the truncated FGF-R1 virus prevented programmed postnatal PF sutural fusion.

Increased FGF Biological Activity in the Normally Patent COR Suture Results in Sutural Fusion

To evaluate the effects of increased FGF biological activity on normally patent cranial sutures, the left COR suture complexes of embryonic rats (E18) were infected with control (vehicle or LacZ) or the FGF-2 overexpression adenovirus. This experiment was designed to mimic the presumed effects of FGF-R mutations in syndromal craniosynostoses (ie, increased FGF biological activity). X-gal staining of AdCALacZ-infected COR sutures localized high-level expression of the LacZ transgene along the dural aspect of the left COR (Figure 5B; and Figure 6, A and B). In addition, all COR sutures infected with vehicle or AdCALacZ demonstrated complete histological patency (Figure 6, A-C). In contrast, all COR sutures

Figure 3. *In vitro* infection of PF-associated dural tissues. **A:** Schematic of mouse organ-culture system. Postnatal day 24 suture explants are placed in serum-free medium. After a 30-day culture period, the PF suture fuses whereas all other sutures remain patent. **B:** Overhead view (original magnification, $\times 10$) demonstrating high-level expression of LacZ transgene after 30 days of culture. **C:** Histological cross-section of same suture demonstrating high-level LacZ transgene expression in dural tissues underlying PF suture. Note PF suture fusion. **D:** Overhead view of AdCALacZ-infected PF suture after 30 days in culture. Original magnification, $\times 25$. Note fusion of PF suture. **E:** Overhead view of AdCAFGF-TR-infected PF suture after 30 days in culture. Original magnification, $\times 25$. Note patency of PF suture. **F:** Histological cross-section through vehicle-infected PF suture demonstrating osseous fusion of this suture. **G:** Histological cross-section through AdCAFGF-TR-infected PF suture demonstrating patency of this normally fusing suture.

Figure 4. *In utero* infection of PF-associated dural tissues. **A:** Overhead view (original magnification, $\times 12$) of *in utero* calvarium after targeted injection of PF-suture associated dural tissues with AdCALacZ. Note high-level transgene expression of PF-associated dura mater on postnatal day 30 (black arrow). **B:** Histological cross-section of PF suture infected *in utero* with AdLacZ. Note fusion of PF suture and LacZ transgene expression in underlying dural tissues. **C:** Same as B with enhancement of osseous tissue highlighted in black clearly demonstrating fusion of PF suture. **D:** Higher power magnification of dural tissues underlying fused PF suture. Note expression of LacZ transgene in PF-associated dural tissues. **E:** Histological cross-section of PF-suture infected *in utero* with AdCAFGF-TR. Note this normally fusing suture is widely patent. **F:** Same as E with enhancement of osseous tissue highlighted in black to more clearly demonstrate patency of PF suture. **G:** Immunohistochemical localization of FGF-TR transgene using a hemagglutinin antibody localizing FGF-TR transgene to the dural tissues underlying the PF suture. **H:** Higher power view of G.

Figure 5. *In utero* infection of COR-associated dural tissues. **A:** Photograph of vehicle-treated calvaria. Note the transparent nature and symmetry of the calvaria and linear plane of PF and SAG sutures of the 30-day-old animals. **B:** Original magnification ($\times 12$) of postnatal day 30 calvarium after targeted injection of left COR suture-associated dural tissues with AdCALacZ. Note high-level transgene expression of left COR suture-associated dura mater on postnatal day 30. **C:** Photograph of AdCasFGF-2-infected left COR suture. Note overgrowth and thickness of calvarial plates perpendicular to the patent right COR suture. The left COR suture is fused. The linear plane normally formed by the PF and SAG sutures is shifted toward the side of the fused suture secondary to the compensatory or continued calvarial growth occurring perpendicular to the patent right coronal suture. **D:** Schematic representation of altered calvarial growth secondary to fusion of the AdCasFGF-2 COR suture. There is increased growth perpendicular to the patent right COR suture that deviates the normal linear plane of the PF and SAG sutures to the left (compare **A** with **C**).

infected with AdCasFGF-2 demonstrated marked thickening of both the parietal and frontal bones bordering the COR suture with fusion of the suture (Figure 6, D–F). In addition, animals with fused left COR sutures demonstrated the characteristic craniofacial morphology associated with unilateral coronal synostosis (ie, frontal plagiocephaly; Figure 5). Compensatory or continued growth perpendicular to the patent right COR suture secondary to left COR suture fusion resulted in a left-sided deviation of the normally linear plane made by the PF and SAG sutures (Figure 5, C and D).

Alterations of FGF Biological Activity Result in Changes in TGF- β 1 Expression by Isolated Osteoblast-Enriched Cultures and Cranial Suture-Derived Dural Cell Cultures

To investigate possible cellular mechanisms underlying our *in vitro* and *in vivo* cranial suture findings, effects of cellular infection with AdCAFGF-TR and AdCasFGF-2 on TGF- β 1 gene expression were explored. Similar to FGF-2, TGF- β s have been implicated in the regulation of

sutural fusion.^{12,27} Infected cells were stimulated with rhFGF-2 (10 ng/ml) for 6 hours and TGF- β 1 mRNA expression was assessed. Both vehicle and AdCALacZ-infected cells demonstrated increased TGF- β 1 mRNA expression in response to rhFGF-2 stimulation. In contrast, NRCs infected with AdCAFGF-TR (50 and 100 pfu/cell) failed to increase expression of TGF- β 1 mRNA in response to rhFGF-2 (untreated and β -gal-infected NRCs demonstrated a greater than fourfold increase in TGF- β 1 mRNA expression; Figure 7A).

Evaluation of NRCs treated with the FGF-2 overexpression virus (AdCasFGF-2) demonstrated potent up-regulation of TGF- β 1 mRNA expression as compared with control cells (5.7-fold increase in TGF- β 1 mRNA expression; Figure 7E). These data demonstrate that changes in FGF biological activity modulate TGF- β 1 expression and may contribute to the regulation of sutural fate. Northern blot analysis performed on similarly treated PFDCs demonstrated identical changes in TGF- β 1 gene expression (data not shown).

Alterations of FGF Biological Activity Result in Changes in Cellular Expression of Collagen I by Cranial Suture-Derived Dural Cell Cultures

Opperman and colleagues²⁸ have demonstrated increased collagen synthesis in fusing sutures as compared to nonfusing cranial sutures. In addition, FGF-2 has been shown to inhibit collagen type I mRNA synthesis in fibroblasts and osteoblasts and may thus represent a potential mechanism by which alterations in FGF biological activity may mediate sutural fate in our system.²⁹ Interestingly, abrogation or stimulation of FGF biological activity in NRCs failed to significantly alter the expression of collagen I mRNA (data not shown). In contrast, rhFGF-2 stimulation of PF suture-derived dural cell cultures was associated with an increase in collagen type I mRNA expression, whereas abrogation of FGF biological activity with the truncated FGF-R1 virus attenuated this response (2.3-fold increase in collagen I mRNA expression in untreated, FGF-stimulated PFDCs; Figure 7B). Thus, in our system, dural cell populations respond differently to FGF stimulation than osteoblast-enriched cultures to FGF stimulation.

Alterations of FGF Biological Activity Affect Osteoblast and Dural Cell Proliferation

FGF-2 is a potent mitogen for many cell types including osteoblasts.³⁰ To assess the ability of AdCAFGF-TR-infected cells to resist the mitogenic effects of rhFGF-2 stimulation, cellular proliferation of infected cells was assessed using BrdU incorporation and a nonradioactive proliferation assay. Results of cellular BrdU incorporation are depicted in Figure 7C. NRCs infected with AdCAFGF-TR ($14 \pm 7\%$ BrdU-positive cells/HPF; 100 pfu/cell) demonstrated significantly less BrdU incorporation than both NRCs infected with AdCALacZ ($36.2 \pm 4.2\%$) and vehicle ($33.6 \pm 6.7\%$). A nonradioactive pro-

Figure 6. **A:** Histological cross-section of left COR suture infected *in utero* with AdCALacZ. Note patency of COR suture and LacZ transgene expression in underlying dural tissues. **B:** Higher power magnification of left COR suture in **A**. **C:** Same as **B** with enhancement of osseous tissue highlighted in black clearly demonstrating patency of COR suture. **D:** Histological cross-section of COR suture infected *in utero* with AdCasFGF-2. Note increased thickness of frontal and parietal bones as well as area of fusion on dural aspect of this suture. Compare **bold double arrow** in **D** with same **arrow** in **A**. **E:** Higher power view of **D**. Note fusion of COR suture (*) as well as marked increase in thickness of calvarial bones. **F:** Same as **E** with enhancement of osseous tissue highlighted in black to more clearly demonstrate marked thickening and fusion of COR suture.

liferation assay demonstrated similar results with AdCAFGF-TR-infected cells proliferating significantly more slowly in response to rhFGF-2 compared to vehicle and AdCALacZ-infected cells (Figure 7D). In contrast, cells infected with AdCasFGF-2 proliferated significantly more rapidly than vehicle or AdCALacZ-infected cells (Figure 7, F and G). Similar results were obtained on identically treated PFDCs (data not shown).

Discussion

Craniosynostosis is a common congenital malformation of the craniofacial skeleton with a reported incidence as high as one in 2,500 live births.¹ Despite its prevalence, our understanding of the etiopathogenesis of premature suture fusion has remained elusive. Although craniosynostosis occurs most often as an isolated (ie, sporadic)

anomaly, it has been associated with more than 100 reported syndromes.¹ Within the last few years, numerous genetic mutations in the FGF-Rs have been identified in patients with these syndromes.^{31–35} *In vitro* analysis of these mutations has provided insight into the biomolecular processes that lead to abnormal craniofacial development in these syndromes.

There are four known FGF-Rs and mutations in three of them (FGF-R1, FGF-R2, and FGF-R3) have been identified in patients with syndromic craniosynostosis. The gain-of-function associated with the mutated FGF-Rs has been attributed to ligand-independent dimerization/activation, and more recently to increased affinity of the mutated FGF-Rs for ligand, specifically FGF-2.^{2–6} The mechanism for ligand-independent FGF-R dimerization and subsequent activation has been attributed to disruption of intramolecular disulfide bonds in the third immu-

Figure 7. **A:** Representative Northern blot analysis demonstrating an increase in TGF- β 1 mRNA expression in RNA isolated from vehicle and AdCALacZ-infected NRCs in response to a 6-hour stimulation with FGF-2 (10 ng/ml). This increase in TGF- β 1 mRNA in response to FGF-2 was not seen in NRCs infected with AdCAFGF-TR. A similar pattern of TGF- β 1 mRNA expression was seen in PFDCs after infection and stimulation (data not shown). 50 = 50 plaque forming units/cell; 100 = 100 plaque forming units/cell. **B:** Representative Northern blot analysis demonstrating an increase in collagen type I mRNA expression in RNA isolated from vehicle and AdCALacZ-infected PFDCs in response to 48-hour stimulation with FGF-2 (10 ng/ml). PFDCs infected with AdCAFGF-TR failed to demonstrate an increase in collagen type I mRNA. Interestingly, infected NRCs did not demonstrate differences in collagen type I gene expression in response to FGF-2 (data not shown). **C:** Cells infected with AdCAFGF-TR proliferated significantly slower than cells infected with vehicle and AdCALacZ. Graphic representation of BrdU incorporation by NRCs infected with vehicle, AdCALacZ, or AdCAFGF-TR. Note statistically significant decreased proliferation in NRCs infected with AdCAFGF-TR (*, $P < 0.001$). All cells were stimulated with rhFGF-2. **D:** Results of proliferation assay. Note significantly decreased proliferation of NRCs infected with AdCAFGF-TR (*, $P < 0.001$). All cells were stimulated with rhFGF-2. **E:** Representative Northern blot analysis demonstrating an increase in baseline TGF- β 1 mRNA expression in RNA isolated from AdCAsFGF-2-infected NRCs. Similar pattern of TGF- β 1 mRNA expression was seen in PFDCs after AdCAsFGF-2 infection (data not shown). **F:** Cells infected with AdCAsFGF-2 proliferated significantly faster than cells infected with vehicle and AdCALacZ. Graphic representation of BrdU incorporation by NRCs infected with vehicle, AdCALacZ, or AdCAsFGF-2. Note statistically significant increased proliferation in NRCs infected with AdCAsFGF-2 (*, $P < 0.001$). **G:** Results of proliferation assay. Note significantly increased proliferation of NRCs infected with AdCAsFGF-2 (*, $P < 0.001$).

noglobulin loop of the FGF-R as a result of a point mutation and loss of an IgIII-associated cysteine residue.²⁻⁵ Similarly, Robertson and colleagues³⁶ demonstrated that FGF-R mutations not involving cysteine substitutions may disrupt intramolecular disulfide bonds by altering the conformation of the IgIII domain. Recently, *in vitro* analysis of the most common Apert mutations (FGF-R2 mutations; Ser252Trp and Pro253Arg) demonstrated in-

creased affinity for FGF-2 ligand as compared to wild-type FGF-R2.⁶

Our findings demonstrate that AdCAFGF-TR infection of the dural tissues underlying the PF cranial suture inhibited physiological cranial suture fusion, whereas AdCAsFGF-2 infection of the dural tissues underlying the COR suture resulted in fusion of this normally patent suture. In addition, we show that alterations in FGF biological activity is associated with significant changes in cellular proliferation and TGF- β 1 expression by NRCs and PFDCs. Moreover, stimulation of PFDCs with rhFGF-2 results in significant up-regulation of collagen I mRNA. Thus, sutural patency or fusion secondary to alterations in FGF biological activity is likely because of changes in cellular proliferation, TGF- β 1 expression, and collagen I expression by the underlying dura mater, and sutural mesenchyme. This conclusion is supported by the fact that the osteogenic actions of TGF- β 1 and FGF-2 are closely related because: 1) each cytokine directly enhances the expression of the other; 2) TGF- β 1 potentiates the mitogenic effects of FGF-2 on osteoblast-like cells; and 3) TGF- β 1 potentiates the expression of type I collagen, the major component of the bone extracellular matrix.^{30,37-40} Moreover, we have shown that increased expression of TGF- β 1, similar to FGF-2, is temporally and spatially related to programmed sutural fusion and patency.^{12,27}

It is interesting to note that Mehrara and colleagues¹⁸ demonstrated increased type I collagen production by cells derived from PF-associated dural tissues as compared to cells derived from the dural tissues underlying a patent cranial suture. Additionally, Opperman et al²⁸ demonstrated increased collagen synthesis in fusing cranial sutures as compared to patent sutures *in vitro*. Finally, osteoblasts derived from human synostotic sutures (ie, isolated and syndromic sutures) demonstrate greater basal levels of type I collagen and noncollagenous matrix molecules than control osteoblasts.⁴¹⁻⁴³ Taken together, these studies suggest that alterations in the expression of osteogenic cytokines (ie, FGF-2 and TGF- β 1) and extracellular matrix molecules likely determine the fate of the overlying cranial sutures. These conclusions are supported by our current study.

Manipulations of FGF-dependent signaling in the cranial sutures have been previously described. After placement of FGF-4-soaked beads along the osteogenic fronts of embryonic day 15.5 mouse SAG suture explants, Kim and colleagues⁴⁴ demonstrated close apposition of the parietal bones with evidence of SAG sutural fusion. These same authors demonstrated increased proliferation in the cells of the osteogenic front in the region adjacent to the FGF-4 beads, suggesting that increased proliferation of osteoblasts lining the osteogenic front contributes to osseous fusion of the SAG suture. Placement of FGF-2-soaked beads on E15 mouse COR sutures induced osteopontin expression in the sutural mesenchyme (ie, normally not mineralizing) in the area of the bead.⁴⁵ These studies suggest that increased availability of FGF ligand leads to alterations in mineralization and proliferation, and ultimately may alter the histological fate (ie, fusion or patency) of the cranial sutures. It is likely that

similar alterations in dural cell and osteoblast proliferation and subsequent changes in gene expression secondary to modulation of FGF biological activity contributed to the alterations of cranial suture fate observed in our study.

Taken together with the identification of gain-of-function FGF-R mutations in syndromic craniosynostosis, these studies suggest that FGF biological activity critically regulates suture fusion and patency and that this regulation occurs, at least in part, via alterations in cellular proliferation, TGF- β 1 expression, and collagen expression. We are currently investigating alterations in the temporal and spatial expression of numerous osteoinductive and osteogenic cytokines as well as their inhibitors in adenovirally treated PF and COR sutures to further elucidate the biomolecular mechanisms that resulted in the phenotypes reported in this study. Using targeted, *in utero* delivery of transgenes, we successfully altered postnatal cranial suture fate. As prenatal diagnosis and fetal manipulation become a reality, our data may facilitate the development of less invasive treatment options for craniosynostosis.

References

1. Wilkie AO: Craniosynostosis: genes and mechanisms. *Hum Mol Genet* 1997, 6:1647–1656
2. Mangasarian K, Li Y, Mansukhani A, Basilico C: Mutation associated with Crouzon syndrome causes ligand-independent dimerization and activation of FGF receptor-2. *J Cell Physiol* 1997, 172:117–125
3. Galvin BD, Hart KC, Meyer AN, Webster MK, Donoghue DJ: Constitutive receptor activation by Crouzon syndrome mutations in fibroblast growth factor receptor (FGFR)2 and FGFR2/Neu chimeras. *Proc Natl Acad Sci USA* 1996, 93:7894–7899
4. Neilson KM, Friesel R: Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. *J Biol Chem* 1996, 271:25049–25057
5. Neilson KM, Friesel RE: Constitutive activation of fibroblast growth factor receptor-2 by a point mutation associated with Crouzon syndrome. *J Biol Chem* 1995, 270:26037–26040
6. Anderson J, Burns HD, Enriquez-Harris P, Wilkie AOM, Heath JK: Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand. *Hum Mol Genet* 1998, 7:1475–1483
7. Bradley JP, Levine JP, Roth DA, McCarthy JG, Longaker MT: Studies in cranial suture biology: IV. Temporal sequence of posterior frontal cranial suture fusion in the mouse. *Plast Reconstr Surg* 1996, 98:1039–1045
8. Bradley JP, Levine JP, McCarthy JG, Longaker MT: Studies in cranial suture biology: regional dura mater determines *in vitro* cranial suture fusion. *Plast Reconstr Surg* 1997, 100:1091–1099
9. Levine JP, Bradley JP, Roth DA, McCarthy JG, Longaker MT: Studies in cranial suture biology: regional dura mater determines overlying suture biology. *Plast Reconstr Surg* 1998, 101:1441–1447
10. Opperman LA, Passarelli RW, Morgan EP, Reintjes M, Ogle RC: Cranial sutures require tissue interactions with dura mater to resist osseous obliteration *in vitro*. *J Bone Miner Res* 1995, 10:1978–1987
11. Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC: Tissue interactions with underlying dura mater inhibit osseous obliteration of developing cranial sutures. *Dev Dyn* 1993, 198:312–322
12. Most D, Levine JP, Chang J, Sung J, McCarthy JG, Schendel SA, Longaker MT: 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
13. Mehrara BJ, Mackool RJ, McCarthy JG, Gittes GK, Longaker MT: Immunolocalization of basic fibroblast growth factor and fibroblast growth factor receptor-1 and receptor-2 in rat cranial sutures. *Plast Reconstr Surg* 1998;102:1805–1820
14. Yukawa H, Miyatake SI, Saiki M, Takahashi JC, Mima T, Ueno H, Nagata I, Kikuchi H, Hashimoto N: *In vitro* growth suppression of vascular smooth muscle cells using adenovirus-mediated gene transfer of a truncated form of fibroblast growth factor receptor. *Atherosclerosis* 1998, 141:125–132
15. Ueno H, Gunn M, Dell K, Tseng Jr A, Williams L: A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J Biol Chem* 1992, 267:1470–1476
16. Ueno H, Li JJ, Masuda S, Qi Z, Yamamoto H, Takeshita A: Adenovirus-mediated expression of the secreted form of basic fibroblast growth factor (FGF-2) induces cellular proliferation and angiogenesis *in vivo*. *Arterioscler Thromb Vasc Biol* 1997, 17:2453–2460
17. Mehrara BJ, Saadeh PB, Steinbrech DS, Dudziak M, Spector JA, Greenwald JA, Gittes GK, Longaker MT: Adenovirus-mediated gene therapy of osteoblasts *in vitro* and *in vivo*. *J Bone Miner Res* 1999, 14:1290–1301
18. Mehrara BJ, Greenwald J, Chin GS, Dudziak M, Sagrioglu J, Steinbrech DS, Saadeh PB, Gittes GK, Longaker MT: Regional differentiation of rat cranial suture-derived dural cells is dependent on association with fusing and patent cranial sutures. *Plast Reconstr Surg* 1999, 104:1003–1013
19. Frick KK, Bushinsky DA: Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. *Am J Physiol* 1998, 275:F840–F847
20. Mehrara BJ, Most D, Chang J, Bresnick S, Turk A, Schendel SA, Gittes GK, Longaker MT: Basic fibroblast growth factor and transforming growth factor beta-1 expression in the developing dura mater correlates with calvarial bone formation. *Plast Reconstr Surg* 1999, 104:435–444
21. Rowe NM, Mehrara BJ, Dudziak ME, Steinbrech DS, Mackool RJ, Gittes GK, McCarthy JG, Longaker MT: Rat mandibular distraction osteogenesis: Part I. Histologic and radiographic analysis. *Plast Reconstr Surg* 1998, 102:2022–2032
22. Li Y, Basilico C, Mansukhani A: Cell transformation by fibroblast growth factors can be suppressed by truncated fibroblast growth factor receptors. *Mol Cell Biol* 1994, 14:7660–7669
23. Saadeh PB, Mehrara BJ, Steinbrech DS, Dudziak ME, Greenwald JA, Luchs JS, Spector JA, Ueno H, Gittes GK, Longaker MT: Transforming growth factor-beta1 modulates the expression of vascular endothelial growth factor by osteoblasts. *Am J Physiol* 1999, 277:C628–C637
24. Ormerod MG: Analysis of cell proliferation using the bromodeoxyuridine/Hoechst-ethidium bromide method. *Methods Mol Biol* 1997, 75:357–365
25. Sahni M, Ambrosetti DC, Mansukhani A, Gertner R, Levy D, Basilico C: FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev* 1999, 13:1361–1366
26. LaBonne C, Burke B, Whitman M: Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. *Development* 1995, 121:1475–1486
27. Roth DA, Longaker MT, McCarthy JG, Rosen DM, McMullen HF, Levine JP, Sung J, Gold LI: Studies in cranial suture biology: Part I. Increased immunoreactivity for TGF-beta isoforms (beta 1, beta 2, and beta 3) during rat cranial suture fusion. *J Bone Miner Res* 1997, 12:311–321
28. Opperman LA, Chhabra A, Nolen AA, Bao Y, Ogle RC: Dura mater maintains rat cranial sutures *in vitro* by regulating suture cell proliferation and collagen production. *J Craniofac Genet Dev Biol* 1998, 18:150–158
29. Chaudhary LR, Avioli LV: Extracellular-signal regulated kinase signaling pathway mediates downregulation of type I procollagen gene expression by FGF-2, PDGF-BB, and okadaic acid in osteoblastic cells. *J Cell Biochem* 2000, 76:354–359
30. Globus RK, Patterson-Buckendahl P, Gospodarowicz D: Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor beta. *Endocrinology* 1988, 123:98–105
31. Robin NH: Molecular genetic advances in understanding craniosynostosis. *Plast Reconstr Surg* 1999, 103:1060–1070
32. Jabs EW: Toward understanding the pathogenesis of craniosynosto-

- sis through clinical and molecular correlates. *Clin Genet* 1998, 53: 79–86
33. Malcolm S, Reardon W: Fibroblast growth factor receptor-2 mutations in craniosynostosis. *Ann NY Acad Sci* 1996, 785:164–170
34. Lewanda AF, Meyers GA, Jabs EW: Craniosynostosis and skeletal dysplasias: fibroblast growth factor receptor defects. *Proc Assoc Am Physicians* 1996, 108:19–24
35. Reardon W, Winter RM: The molecular pathology of syndromic craniosynostosis. *Mol Med Today* 1995, 1:432–437
36. Robertson SC, Meyer AN, Hart KC, Galvin BD, Webster MK, Donoghue DJ: Activating mutations in the extracellular domain of the fibroblast growth factor receptor 2 function by disruption of the disulfide bond in the third immunoglobulin-like domain. *Proc Natl Acad Sci USA* 1998, 95:4567–4572
37. Hurley MM, Abreu C, Gronowicz G, Kawaguchi H, Lorenzo J: Expression and regulation of basic fibroblast growth factor mRNA levels in mouse osteoblastic MC3T3–E1 cells. *J Biol Chem* 1994, 269:9392–9396
38. Noda M, Vogel R: Fibroblast growth factor enhances type beta 1 transforming growth factor gene expression in osteoblast-like cells. *J Cell Biol* 1989, 109:2529–2535
39. Centrella M, Casinghino S, Igotz R, McCarthy TL: Multiple regulatory effects by transforming growth factor-beta on type I collagen levels in osteoblast-enriched cultures from fetal rat bone. *Endocrinology* 1992, 131:2863–2872
40. Wrana JL, Maeno M, Hawrylyshyn B, Yao KL, Domenicucci C, Sodek J: Differential effects of transforming growth factor-beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. *J Cell Biol* 1988, 106:915–924
41. De Pollack C, Renier D, Hott M, Marie PJ: Increased bone formation and osteoblastic cell phenotype in premature cranial suture ossification (craniosynostosis). *J Bone Miner Res* 1996, 11:401–407
42. Lomri A, Lemonnier J, Hott M, de Parseval N, Lajeunie E, Munnich A, Renier D, Marie PJ: Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. *J Clin Invest* 1998, 101:1310–1317
43. Fragale A, Tartaglia M, Bernardini S, Di Stasi AM, Di Rocco C, Velardi F, Teti A, Battaglia PA, Migliaccio S: Decreased proliferation and altered differentiation in osteoblasts from genetically and clinically distinct craniosynostotic disorders. *Am J Pathol* 1999, 154:1465–1477
44. Kim HJ, Rice DP, Kettunen PJ, Thesleff I: FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. *Development* 1998, 125:1241–1251
45. Iseki S, Wilkie AO, Morriss-Kay GM: Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in the developing mouse skull vault. *Development* 1999, 126:5611–5620