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Medical treatment of craniosynostosis: Recombinant Noggin inhibits coronal suture closure in the rat craniosynostosis model

K Shen[1], **SM Krakora**[1], **M Cunningham**[2], **M Singh**[1], **X Wang**[1], **FZ Hu**[1,3], **JC Post**[1,3], and **GD Ehrlich**[1,3]

[1]Center for Genomic Sciences, Allegheny Singer Research Institute, Pittsburgh, PA, USA

[2]Children's Craniofacial Center, Children's Hospital, University of Washington, Seattle, WA, USA

[3]Departments of Otolaryngology-Head and Neck Surgery, and Microbiology and Immunology, Drexel University College of Medicine, Allegheny Campus, Pittsburgh, PA USA.

Abstract

Introduction—The mechanisms underlying craniosynostosis remains unknown. However, mutations in *FGFR2* are associated with craniosynostotic syndromes. We previously compared gene expression patterns of patent and synostosing coronal sutures in the nude rat and demonstrated down regulation of Noggin in synostosing sutures. Noggin expression is also suppressed by FGF2 and constitutive FGFR2 signaling.(1–2) Thus, we therefore hypothesized that the addition of rhNoggin to prematurely fusing sutures should prevent synostosis.

Materials and Methods—Cohorts of nude rats were subjected to: 1) surgical elevation of the coronal suture (shams); 2) surgical elevation and placement of normal or *FGFR2* mutant human osteoblasts onto the underlying dura (xenotranplants); or 3) xenotransplantation with co-application of heparin acrylic beads soaked with recombinant human (rh) Noggin. Eleven days post surgery the sutures were harvested, stained, and histologically examined.

Results—Animals that received control osteoblasts, sham surgery, or no surgery demonstrated normal skull growth and coronal suture histology, whereas animals transplanted only with *FGFR2* mutant osteoblasts showed evidence of bridging synostosis on the calvarial dural surface. Sutures treated with FGFR2 mutant osteoblasts and rhNoggin remained patent.

Conclusion—The chimeric nude rate model is a viable model of craniosynostosis. *FGFR2* mutations in osteoblasts induce bridging osteosynthesis demonstrating one of the mechanisms for premature suture fusion. Topical application of rhNoggin protein prevents craniosynostosis in the weanling nude rat xenotransplantation model of syndromic craniosynostosis.

Keywords

Craniosynostosis; FGFR2; noggin; tissue engineering; xenotransplant

Introduction

Craniosynostosis is the pathologic condition associated with premature fusion of calvarial sutures. The overall incidence of craniosynostosis is one out of every 1700 to 2500 live births.(3) In humans, premature suture fusion results in abnormalities in calvarial shape due to restriction of growth in the region of a fused suture. These changes of head shape can be

Correspondence to: Garth D. Ehrlich Center for Genomic Sciences, Allegheny Singer Research Institute, 320 East North Ave., Pittsburgh, PA, USA 15212, gehrlich@wpahs.org.

associated with increased intracranial pressure that may result in permanent brain injury.(4) In addition to the risks of brain injury, craniosynostoses are associated with: alteration of craniofacial growth leading to mid-facial hypoplasia; abnormalities in dental alignment; and orbital deformation.(5) The combination of craniosynostosis and its associated facial malformations leads to significant morbidity and occasional mortality. Patients with complex syndromic craniosynostoses require the skills of a large interdisciplinary medical and surgical team and often undergo multiple reconstructive surgeries to correct the functional deficits associated with their malformations. These conditions place a great burden on the patients, their families, and the health care system.

Crouzon craniofacial dysostosis (CFD) is an autosomal dominant disorder of craniofacial development characterized by premature craniosynostosis, exophthalmos with shallow orbits, and maxillary hypoplasia. Crouzon noted the familial occurrence when he described the syndrome.(6) The incidence has been estimated to be 1/25,000 and represents approximately 4.8% of craniosynostoses at birth.(7) There is complete penetrance with variable expressivity, and *de novo* mutations represent up to 50% of reported cases.(8) Defects of the hands and feet are not present, which clinically differentiates CFD from many other craniosynostotic syndromes, such as Apert (acrocephalosyndactyly), Pfeiffer, Saethre-Chotzen, and Jackson-Weiss syndromes. The gene for CFD was mapped by our laboratory (9) in 1994 which quickly led to the discovery that mutations in *FGFR2* were associated with some cases of CFD. (10,11)

The genetic etiology of human craniosynostoses is, however, only partially understood. Hereditary synostoses have been found to be associated with mutations in several of the fibroblast growth factor receptor genes (*FGFR*s), *TWIST*, and *Msx2*. Apert, Pfeiffer, CFD, and Jackson-Weiss syndromes are due to specific point mutations of *FGFR2*. (10–13) This is in distinction to the point mutations of *FGFR1* and *FGFR3* which result in Pfeiffer and "Muenke Type" craniosynostosis, respectively. (14–15) Several mutations in the DNA binding and loop domains of the TWIST protein have been found to be responsible for the Saethre-Chotzen phenotype. (16)

Although many mutations have been catalogued as being associated with the various syndromic craniosynostosis, the biology behind the development of these conditions is incomplete. However, *in vitro* experiments demonstrate that *FGFR* mutations in humans likely cause craniosynostosis by constitutive signaling without the need to bind ligand. Fused human sutures derived from patients with CFD also demonstrate a reduction in *FGFR2* expression most probably due to down regulation of receptor expression in response to constitutive activation.(17) Most likely a secondary event downstream of these mutations (e.g., cell signaling) is the proximal event leading to abnormal sutural development. Examination of the biology of hereditary craniosynostosis, downstream of the causative mutations, should provide for the elucidation of the mechanisms underlying synostosis. It is hoped that from this understanding that key signaling systems can be identified that are most suited for primary prevention and/or treatment of this disabling condition. The etiology of the more common forms of sporadic synostosis (e.g., isolated sagittal and metopic synostosis) remains elusive. By investigating the pathogenesis of syndromic synostoses we hope to be able to shed light on the etiology of these more common forms of synostosis.

Noggin is known to be required for embryonic neural tube development, as well as for somite and skeleton patterning. (18–19) In addition, noggin has been shown to be expressed postnatally in the sutural mesenchyme of patent, but not fusing, cranial sutures, and its expression is suppressed by FGF2 and syndromic FGFR signaling. Since Noggin mis-expression prevents cranial suture fusion *in vitro* and *in vivo*, it has been suggested that

syndromic FGFR mediated craniosynostoses may be the result of inappropriate down regulation of Noggin expression.

The effects of BMP4 on Noggin expression in calvarial osteoblasts have also been examined. (20) The BMP4 protein has been localized immunohistochemically to the sutural mesenchyme and to the osteogenic fronts of the posterior frontal sutures of mice one week before the onset of fusion, as well as to patent sutures. The presence of BMP4 in fusing and non-fusing sutures suggest that there might be suture-specific regulation of BMP activity by secreted BMP antagonists. (18–19) Osteoblasts treated with BMP4 expressed Noggin in a dose dependent manner. When FGF2 was added, the expression of Noggin was disrupted also in a dose dependent manner. This suggests that an environment with a low FGF2 concentration might not suppress BMP-induced Noggin expression, but environments high in FGF2 reduce Noggin expression and enable suture fusion.

While blocking FGF signaling, or activity, prevents cranial suture fusion, or osteogenesis, studies have shown that exogenous FGF signaling is capable of suppressing Noggin expression during cranial suture fusion. (21) These findings suggest that FGF2 guides suture fate by regulating suture-specific Noggin production in osteoblasts and in turn, suture-specific BMP activity. Moreover, these data suggest a possible mechanism for syndromic craniosynostoses arising from *FGFR2* gain-of-function mutations. Because constitutive FGFR signaling is associated with syndromic forms of premature cranial suture fusion, the role of Noggin in an established model of FGF-mediated coronal synostosis has been investigated. (21) In this model, injection of an FGF2-expressing adenovirus into the perinatal coronal dura mater led to FGF2 over expression and pathological osteogenesis and suture fusion within 30 days. Additionally, injection of this FGF2 expressing adenovirus into the coronal dura mater of neonatal *lacZ/noggin* transgenic mice led the suppression of Noggin and pathological coronal suture fusion. These studies taken together with the cell culture data suggest that increased FGF signaling might lead to suture fusion by suppressing Noggin production in the dura mater and osteoblasts of normally patent cranial sutures.

Because FGF2 misexpression led to Noggin suppression in coronal sutures, the effects of Apert (S252W) and Crouzon (C342Y) syndrome *FGFR2* gain-of-function mutations on Noggin production in dural cell and osteoblast cultures was investigated. (22) Both Apert and Crouzon *FGFR2* constructs markedly down regulated Noggin protein production in dura mater and also down regulated BMP4-induced Noggin expression in calvarial osteoblasts. Because both Apert and Crouzon syndrome *FGFR2* gain-of-function mutations promote pathological suture fusion, these findings provide an important link between the murine models and the gain-of-function *FGFR* mutations associated with syndromic forms of human craniosynostosis.

With multiple studies demonstrating the normal expression of Noggin in the patent suture complex, an *in vitro* organ culture model was used to demonstrate that the forced expression of *Noggin* would maintain frontal suture patency. (20) Using a Noggin-expressing adenovirus, 22-day-old frontal sutures were infected and placed in organ culture. After 30 days, all frontal suture negative controls, infected with *lacZ* virus, were fused. In marked contrast, all frontal sutures infected with the *Noggin* virus were widely patent.

In vivo studies have been done to demonstrate the effects of Noggin misexpression. (20) The frontal sutures of 3-day-old CD-1 mice were injected with PBS, a *lacZ* adenovirus (*AdlacZ*) or a *Noggin* adenovirus (*Adnoggi*n). After 50 days, the *Adnoggin*-infected mice had short broad snouts and widely spaced eyes due to increased frontal bone growth perpendicular to the frontal suture. All mice injected with PBS and *AdlacZ* virus had no fused frontal sutures, whereas the frontal sutures of all *Adnoggin* injected mice were widely patent. This result

demonstrates that *Noggin* misexpression at an early stage of suture development has profound consequences on cranial suture fate. Moreover, homozygous *noggin*-negative mice die at birth with multiple defects that include joint fusion of the appendicular skeleton, and human mutations have been linked to the fusion of finger joints. (18)

It has been clearly demonstrated that Noggin, a high-affinity secreted BMP antagonist, is present in patent sutures and is regulated by FGF-based signaling. Because Noggin promotes sutural patency, and ectopic noggin expression prevents the fusion of mouse frontal sutures, we hypothesized that syndromic *FGFR*-mediated craniosynostoses might result from inappropriate Noggin suppression. This raises the possibility that Noggin might be exploited as a novel therapeutic agent to control postnatal skeletal development.

Materials and Methods

Preparation of Noggin Protein

20μg of recombinant human (rh) Noggin protein (Alfa Diagnostic International, San Antonio, Texas) were diluted to a concentration of 0.5μg/μl in sterile water. The rhNoggin (either 2μg or 6μg) was then mixed with heparin-coated acrylic beads (125–150um in diameter, Sigma Corp.), which had been washed twice in phosphate buffered saline (PBS) prior to use, and incubated at 37 °C for 2 hours.

Establishment and Characterization of Human Osteoblastic Cell Lines

Human osteoblastic cell lines were established from residual patient material obtained intraoperatively during craniosynostotic suture osteotomies. Control osteoblast lines were derived from patients undergoing craniofacial surgeries for reasons other than craniosynostosis (e.g., trauma patients, or patients having calvarial bone harvest for non-synostosis craniofacial reconstruction). All osteoblast cell lines were established from the excised tissue using routine primary tissue culture techniques. The resultant primary cell lines were trypsinized, frozen in fetal calf serum/DMSO (9:1), and stored in liquid nitrogen for future use.

Each cell line was subjected to endogenous alkaline phosphatase and Von Kossa mineralization assays as indicators of osteoblast lineage. (23) In addition, gene expression studies were performed to verify that the primary cell lines expressed appropriate bone-specific markers. This was accomplished by the extraction of RNA from aliquots of the calvarial-derived cell lines for RT-PCR-based analyses for FGFR2 and BMP expression.

Chimeric Nude Rat Xenotransplant Model of Craniosynostosis—We developed a chimeric human/nude (athymic) rat xenotranplant model of craniosynostosis in which *FGFR2* mutant human osteoblasts were placed under the coronal suture of the rat. This procedure induced bridging synostosis within 11 days only in those cases receiving mutant osteoblasts; control animals did not evidence new bone formation. The model was created using a surgical approach in which 6–8 week old weanling nude rats (Harlan Labs) were first anesthetized and then had their coronal suture and adjacent 6 mm of the surrounding calvaria elevated (Fig. 1). The underlying dura was carefully dissected free from the calvaria and 20 ml of concentrated osteoblasts ($1–2 \times 10^6$ cells) were pipetted on top of the dura. The calvaria were then returned to their original position and the pericranium and skin incisions closed. The animals were then returned to germ-free housing. After an 11-day outgrowth period the animals were sacrificed for gross and histological examination. Rats were implanted with either normal human osteoblasts or osteoblasts obtained from Crouzon and Apert (*FGFR2* mutations) patients. Additional control groups included unoperated calvaria, and sham operations with elevation of the calvaria followed by a return to normal position.

Table 1 displays the surgical schedule. The rats were sacrificed 11 days post-operatively and the coronal sutures were harvested, incubated in 2% paraformaldehyde overnight at room temperature, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

To confirm the persistence of human osteoblasts in the rats 11 days after transplantation we subjected histologic samples from transplanted animals to fluorescence in-situ hybridization utilizing a commercial molecular probe that specifically labels human centromeres (Oncor Inc. Gaithersburg, MD).

Results

Phenotypic characterization of cell lines

Osteoblastic cell lines were developed from residual clinical material from patients undergoing craniofacial surgery for syndromic craniosynostoses (Apert and Crouzon syndromes: *FGFR2* mutations), and for nonsynostotic craniofacial reconstructions (control WT cell lines). The osteoblast lineage for each of these cell lines was confirmed by testing for endogenous alkaline phosphatase and mineralization activities (Fig. 2).

Confirmation of the osteoblastic lineage of these cell lines was accomplished through the use of by RT-PCR-based assays designed to test for expression of the following markers: CBFa1, bone sialoprotein (BSP), GP130, LIF receptor, LIF, and IGF I. CBFa1 and bone sialoprotein represent bone specific markers (23) and the expression of GP130, LIF receptor, LIF, and IGF I have been previously documented in primary cultured osteoblasts. All cell lines gave positive results for these markers (Fig. 3). *FGFR2* gene expression in these primary osteoblast cell lines was also confirmed using RT-PCR-based transcript detection. Amplified bands of the expected sizes (*FGFR2*=230bp, *TWIST*=557bp) were observed, thus confirming that the transplanted mutant osteoblasts did indeed express FGFR2 (Fig. 4).

The persistence of human osteoblasts in the xenotransplanted rats 11 days after transplantation was examined using fluorescence in-situ hybridization (FISH). Histologic sections were cut from transplanted animals and subjected to FISH using a human centromere-specific probe; in all cases human osteoblasts were identified in the region of transplantation throughout the dura and sutural tissues beneath the regions of new bone formation (Fig. 5).

Histological analysis of nude rat craniosynostosis model

A total of 32 animals were transplanted or sham transplanted with human osteoblast cell lines: 1) seven with the Apert type craniosynostotic mutations {*FGFR2*, C(767)G, ser(252)trp}; 2) two with CFD; 3) four with a KBS cell line; 3) two with a Saethre Chotzen (*TWIST*) cell line; 4) five with rapidly growing ‘control’ cell lines from craniofacial dysplasias and McCune Albright Syndrome patients; 5) nine with normal controls; 6) one with ink; and 7) two with media. All of the animals that received control osteoblasts, sham surgery, or no surgery demonstrated normal skull growth and coronal suture histology (Fig. 6a–c).

In contrast, all of the animals transplanted with osteoblasts harboring an *FGFR2* mutation demonstrated evidence of new bone formation on the dural surface of the calvaria (Fig. 6d–h). In sections of the coronal suture, new bone formation bridged the frontal and parietal components of the coronal suture (Fig. 6f, h). This data demonstrates that mutant osteoblasts, as compared to control osteoblasts, have the potential to “induce” new bone formation and bridging synostosis in the coronal suture. Thus, this chimeric xenotransplantation system has been demonstrated to be a faithful *in vivo* model of induced craniosynostosis.

To test the ability of RhNoggin to prevent the bridging synostosis that is induced in the rats transplanted with mutant FGFR2 osteoblasts we performed the following experiment. Twelve rats underwent sham surgery (n =4), transplantation with beads soaked with RhNoggin, or transplantation with synostosis inducing osteoblast in addition to RhNoggin soaked beads. Histological examination reveals the lack of bridging synostosis seen in positive controls (Fig. 7 C, D, E, F). Acrylic beads or RhNoggin alone demonstrate patent sutures as well (Fig. 7 A, B).

Discussion

In this study we analyzed the effects of human *FGFR2* mutant osteoblasts on the coronal sutural fate of xenotransplanted weanling nude rats by placing the human cells under a reflected portion of the rat calvaria spanning the parietal and frontal bones. These experiments establishing that human mutant osteoblasts from Crouzon and Apert craniosynostotic syndromes, but not those from craniofacially normal humans, could consistently induce bridging synostosis within 11 days in the chimeric rat model. These data suggest that osteoblasts, rather than the dura, are the key elements leading to craniosynostosis in mutant *FGFR2*-associated syndromes.

After establishing that the rat xenotransplant model could faithfully recapitulate the cardinal features of the human craniosynostotic syndromes we then examined whether it was possible to prevent this ectopic osteogenesis using recombinant human Noggin as our unpublished studies had demonstrated that noggin is down-regulated in the fusing sutures of the weanling rat following the human osteoblast xenotransplant. In addition, others have shown that Noggin expression is suppressed by FGF2 and syndromic FGFR2 signaling (1–2), as well as demonstrating that Noggin is an antagonist of BMP4. Since Noggin misexpression prevents cranial suture fusion *in vitro* and *in vivo*, it is likely that syndromic FGFR2-mediated craniosynostosis may, in part, be the result of inappropriate down regulation of Noggin expression. Many studies suggest that FGFR2 guides suture fate by regulating suture specific Noggin production in osteoblasts and therefore suture specific BMP activity. These data therefore suggest a possible mechanism for syndromic craniosynostosis arising from FGFR2 mutations.

In light of these observations we examined the ability of heparin acrylic beads soaked in rhNoggin and placed under the reflected coronal suture to counteract the FGFR2-based signaling of the human mutant osteoblasts simultaneously introduced into the same site. Histologic examination of the harvested coronal sutures 11 days post transplant/treatment revealed that the presence of the noggin resulted in the maintenance of patent sutures. The beads by themselves had no affect on suture morphology. Thus, these data suggest that Noggin can counteract the affect of constitutive FGFR2 signaling in this xenotransplant model.

Conclusions

By using a combination of advanced microsurgical, cell biological, and molecular techniques we have been able to gain substantial insight into the biology of cranial suture biology. These studies support our hypothesis that normal craniofacial sutural biology results from the development of a complex override of the default metazoan wound-healing response to permit the continued growth of the brain postnatally. Ultimately we hope to apply this understanding will provide the basis for the development of targeted therapeutic interventions for the treatment of craniosynostosis which may someday limit the need for major reconstructive surgery. Future studies are required to investigate the role of other signal molecules in the treatment of craniosynostosis.

Clinical Relevance

The studies described herein describe a human FGFR2 mutant osteoblast-nude rat xenotransplant model. This model faithfully recapitulates the cardinal features of human craniosynostosis. Preliminary findings using recombinant human noggin protein placed in the bony sutures prior to synostosis suggest that this procedure may be able to reduce the amount of premature sutural synostosis associated with FGFR2-associated craniofacial genetic disorders. Thus, recombinant human noggin protein may be a useful medical alternative to reconstructive surgery for the treatment of FGFR2-associated craniofacial syndromes.

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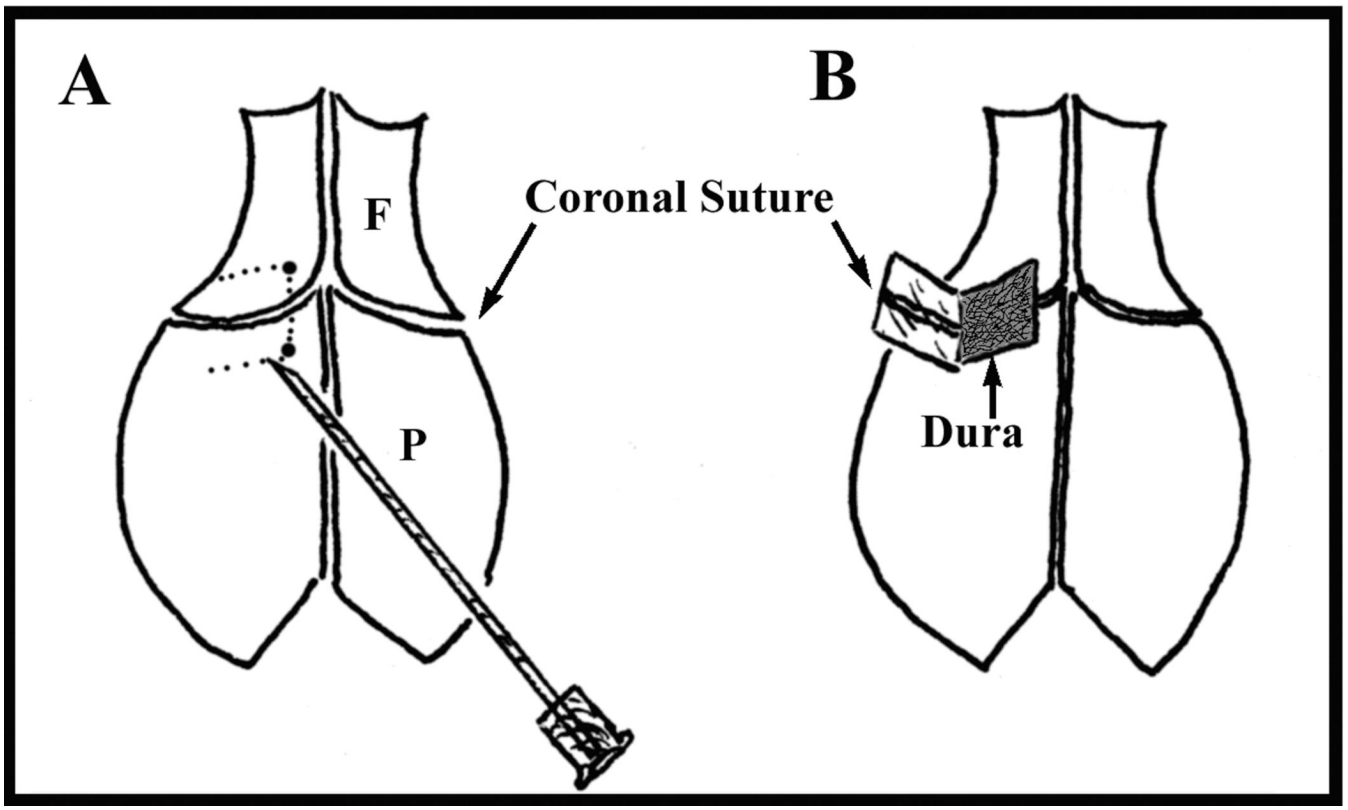

Figure 1.
Surgical exposure of the dura for human osteoblast xenotransplantation. Elevation of the weanling rat coronal suture and flanking regions of the frontal (F) and parietal (P) bones exposes the underlying dura for direct transplantation of human osteoblasts.

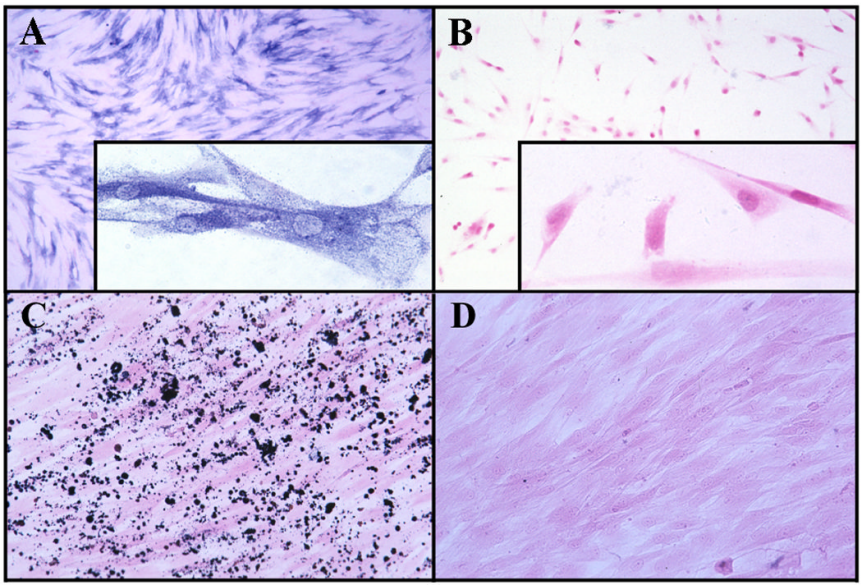

Figure 2.

Endogenous alkaline phosphatase and mineralization activity of cultured calvarial osteoblasts. Calvarial osteoblasts (A) and skin fibroblasts (B) from a patient with an *FGFR2* mutation and the clinical diagnosis of Crouzon syndrome were cultured using routine techniques. Endogenous alkaline phosphatase activity is obvious by the precipitation of the BCIP/NBT substrate (blue) in the osteoblasts derived from the calvaria (A). Calvarial osteoblasts (c) and skin fibroblasts subjected to the von Kossa mineralization assay. Black silver granules in (C) demonstrate matrix mineralization by osteoblasts. Skin fibroblasts (D) were processed in the same manner but do not demonstrate evidence of mineralization. In all lines examined, cells obtained from primary calvarial cultures demonstrated this high level of endogenous enzyme activity and matrix mineralization, supporting an osteoblast lineage.

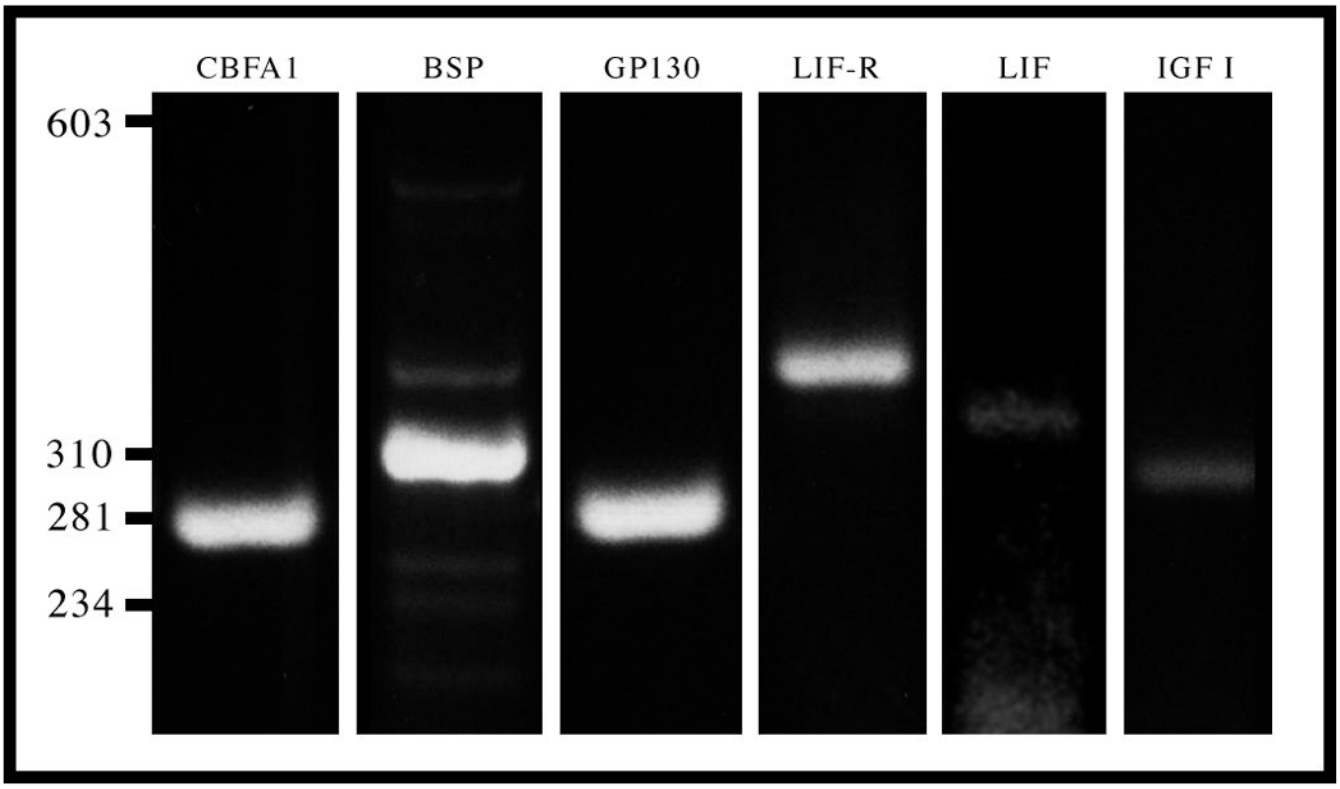

Figure 3.
Expression of bone-specific markers in primary osteoblast lines by RT-PCR. RT-PCR primers specific for each gene of interest were engineered to selectively amplify CBFa1, bone sialoprotein, GP130, LIF receptor, LIF, and IGF I. Each primer set was chosen to span one or more introns in order to control for possible genomic DNA contamination of RNA samples. Resultant RT-PCR products yielded bands of the expected size (CBFa1= 283bp, BSP=304bp, GP130=289bp, LIF receptor=376bp, LIF=376bp, and IGF I=310bp) confirming the expression of each of these transcripts.

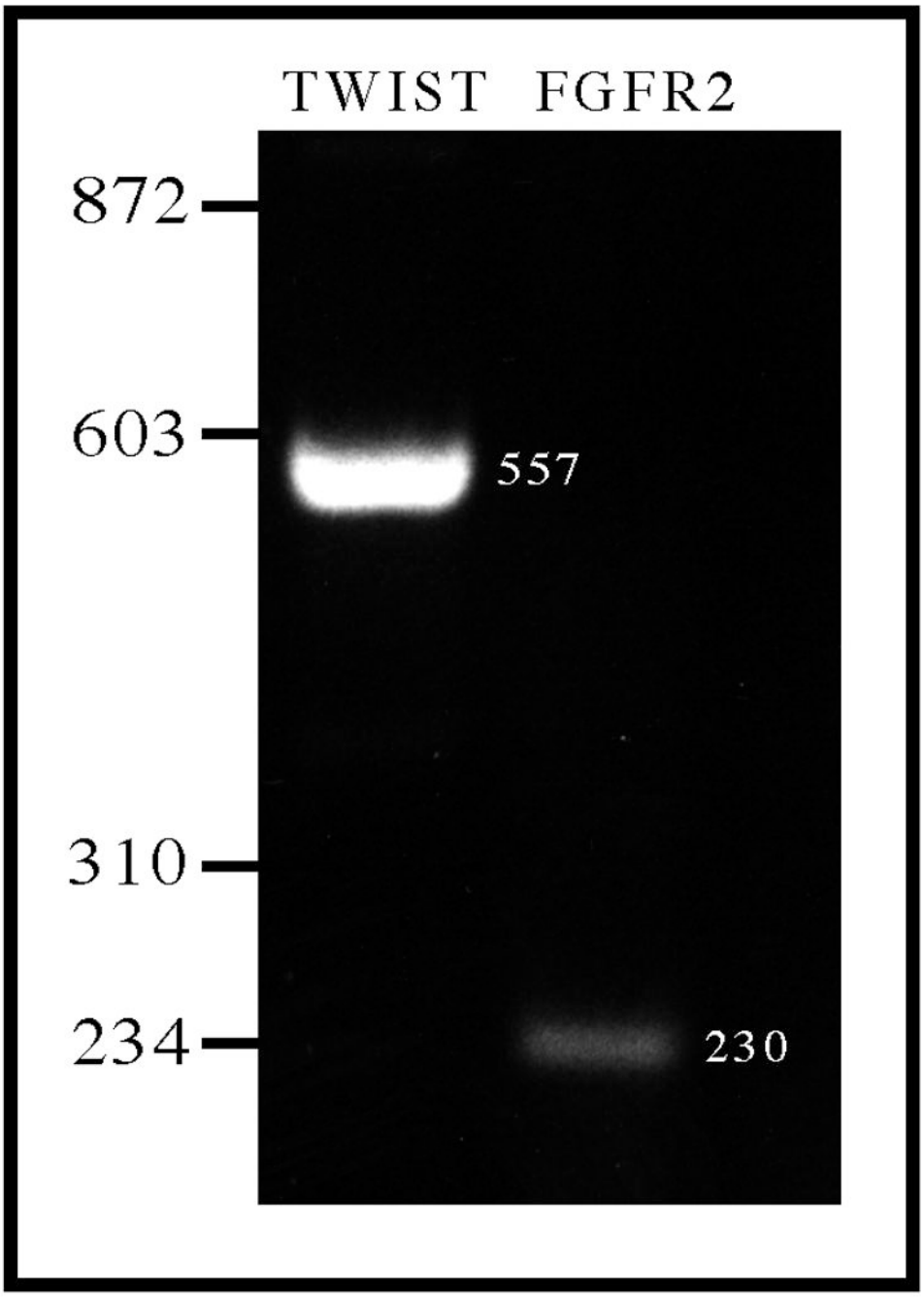

Figure 4.
Expression of *FGFR2* and *TWIST* by primary calvarial osteoblasts. RT-PCR primers were designed to selectively amplify *FGFR2* and *TWIST*. Each primer set spanned at least one intron to distinguish products derived from contaminating genomic DNA. The resultant products were of the expected size (*FGFR2*=230bp, *TWIST*=557bp).

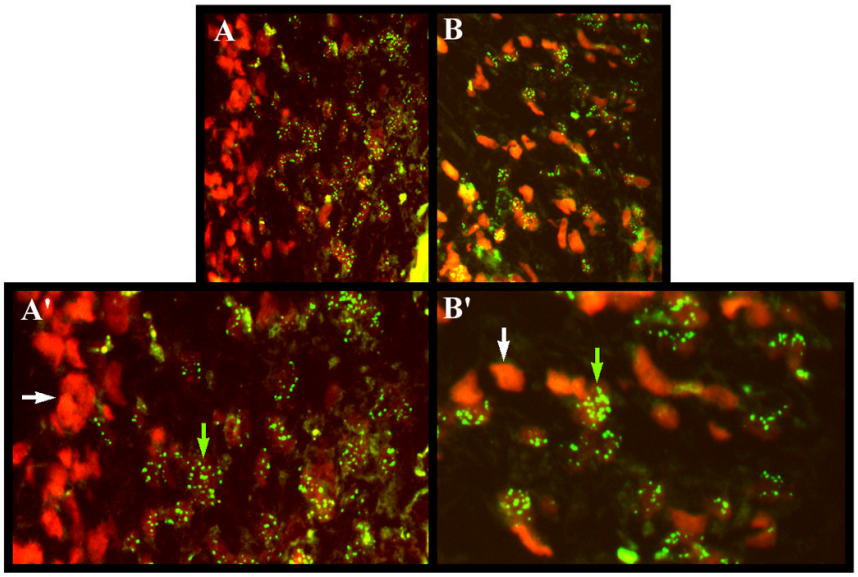

Figure 5.

Detection of human osteoblasts in athymic rats after transplantation. Four μm sections of the coronal sutures and surrounding tissues from the experimental animals were double labeled with propidium iodide (PI, red) labeling all human and rat nuclei and the FITC (green) labeled all human centromeric probe. A distinct positive FITC signal (green arrows) can be seen in both control osteoblast (A, A′) and mutant human osteoblasts (B and B′) chimeras representing hybridization of the human centromeric probe with transplanted human cells. The surrounding FITC negative, PI positive cells represent host (rat) cells in the region (white arrows). These data confirm successful engraftment and persistence of human cells in the transplanted host for at least 11 days in chimeras generated with control and mutant human osteoblast.

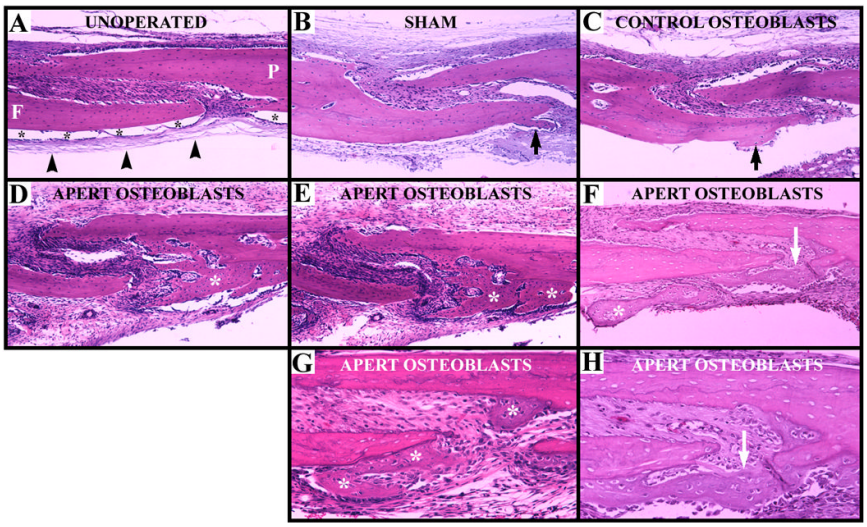

Figure 6.

Induction of new bone formation and bridging synostosis in the nude rat weanling with osteoblasts harboring an *FGFR2* mutation. Representative control coronal suture sectioned at 4 μm depicts normal suture configuration without surgical intervention (A), after sham operation without osteoblast transplantation (B), and after transplantation of control and mutant human osteoblasts (C–H). PanelA depicts the positions of the frontal (F) and parietal (P) bones flanking the coronal suture, the dura (black arrowheads) and potential space between the dura and endosteum into which human osteoblasts are transplanted (black asterisks). Panels 6B and 6C demonstrate minor alteration of sutural contours after surgical manipulation and potential regions of minimal new bone formation (black arrows). In contrast, 6D–H depict abundant new bone formation in the region of the coronal suture after transplantation of mutant human osteoblasts from a patient with Apert syndrome (*FGFR2* mutation) (white asterisks) and bridging synostosis (white arrows, 6F & H). Similar patterns of bridging synostosis have not been observed in any of control samples including those transplanted with control osteoblasts. All sutures were harvested and processed 11 days after osteoblast transplantation. (Relative magnification A–F 10x, G–H 20x).

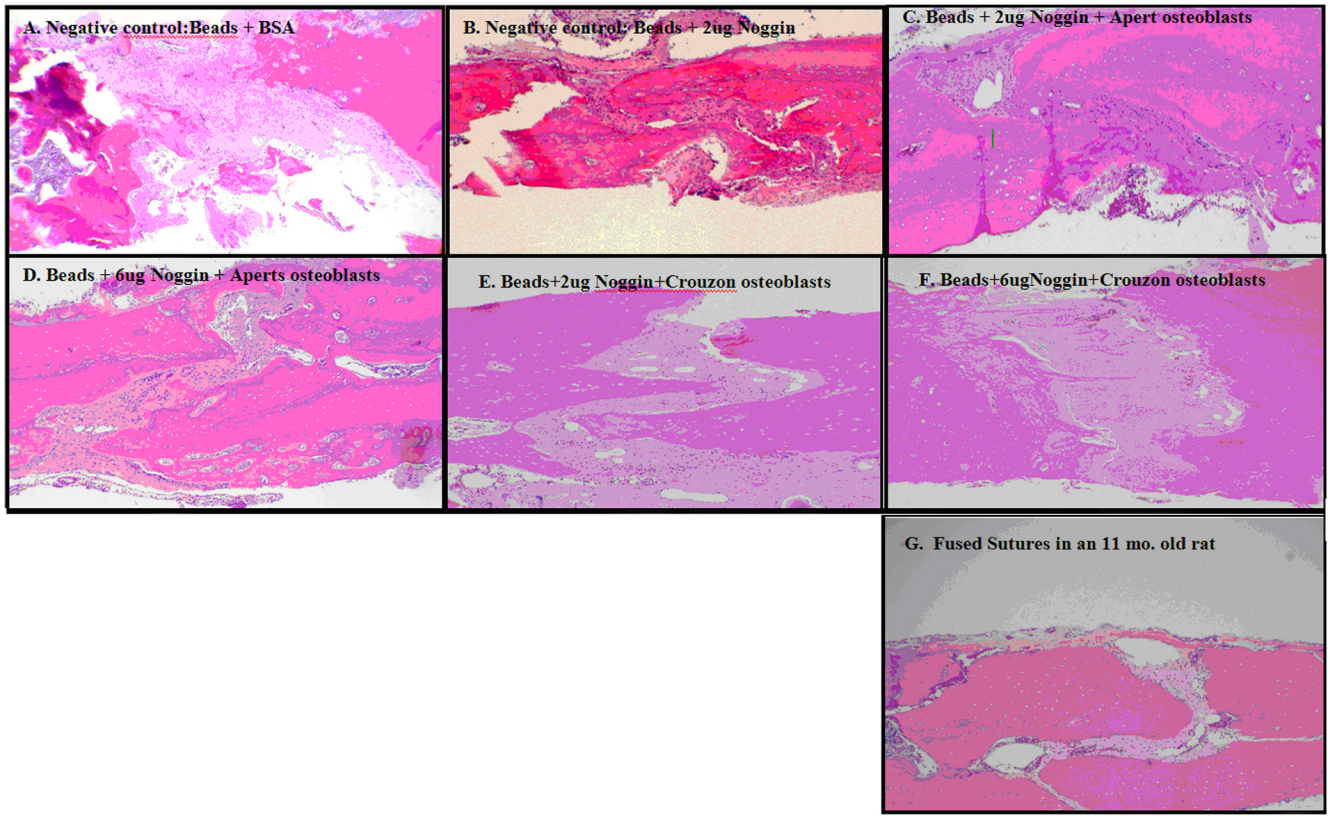

Figure 7.

A – F demonstrate minor alteration of sutural contours after surgical manipulation and lack of new bone formation and bridging synostosis in the nude rat weanling with osteoblasts harboring an *FGFR2* mutation. Representative control coronal suture sectioned at 4 μm depicts altered suture configuration secondary to surgical intervention, with acrylic beads and without osteoblast transplantation (A), after surgical manipulation, with control beads and Noggin, without osteoblast transplantation (B). C – F demonstrate patent suture with minimal evidence of bridging osteosynthesis after transplantation of mutant human osteoblasts, beads, and Noggin. G demonstrates a fused suture in a 11 month old nude rat. (Relative magnification A–G 10x)

Table 1

Experimental Surgical Procedures

Rat	Condition	Rat	Condition	
1	Sham	7	Beads+2ugNoggin+Apert OB	
2	Sham	8	Beads+2ugNoggin+Crouzon OB	
3	Beads+BSA	9	Beads+6ugNoggin+Apert OB	
4	Beads+BSA	10	Beads+6ugNoggin+CrouzonOB	
5	Beads+2ugNoggin	11	Control Osteoblasts - Died	
6	Beads+2ugNoggin	12	Control Osteoblasts	
		13	Unoperated	