Differential spatial regulation of BMP molecules is associated with single-suture craniosynostosis

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OBJECTIVE The aim of this study was to examine messenger RNA (mRNA) levels of bone morphogenetic protein (BMP) ligands, receptors, and soluble inhibitors in cells isolated from single-suture synostoses from fused coronal, metopic, sagittal, and lambdoid sutures.

METHODS Cells were isolated from bone collected from patients undergoing craniotomies at Children's Healthcare of Atlanta. Real-time polymerase chain reaction was used to examine mRNA levels in cells isolated from fused sutures or patent sutures in comparison with levels in normal bone from the same patient.

RESULTS Cells isolated from fused sutures in cases of sagittal and coronal synostosis highly expressed *BMP2*, while cells isolated from fused metopic or lambdoid synostosis expressed high *BMP4*. *Noggin*, a BMP inhibitor, was lower in fused sutures and had high expression in patent sutures.

CONCLUSIONS These results suggest that BMPs and inhibitors play a significant role in the regulation of suture fusion as well in the maintenance of patency in the normal suture.

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KEY WORDS bone morphogenetic protein; craniosynostosis; bone; cranial sutures; craniofacial

CRANIOFACIAL patterning and development are regulated extensively. Craniosynostosis, defined as a premature fusion of 1 or more of the sutures that form the cranial vault or cranial base, affects 1 of every 2500 newborns.¹⁸ At birth, the skull contains fibrous sutures that allow bone expansion in the first years of life and accommodate brain growth. Sutures mineralize over time, allowing for complete skull mineralization. However, premature suture fusion can occur in utero or shortly after birth and cause skull deformities, increased intracranial pressure, vision loss, and mental retardation.^{16,17,38,39} These effects can be mitigated, and acceptable cosmetic outcomes obtained, if surgical intervention occurs early; however, there is a risk that the bone will fuse again prematurely.

Craniosynostosis can be classified by the number of sutures involved (single or multiple) and by the location of the sutures (metopic, coronal, sagittal, and lambdoid). Craniosynostosis that occurs as an isolated event where no gene defect has been identified ("nonsyndromic") usually affects a single suture. Alternatively, multiple sutures may be involved due to altered expression of 1 or more known genes ("syndromic"). Syndromic craniosynostoses are commonly observed with other deformities such as malformations of the limbs and other craniofacial features concurrent with the identified gene mutation and have a prevalence of approximately 20% of all craniosynostosis cases.²³ More than 150 genetic mutations have been associated⁸ with syndromic craniosynostosis.^{8,23,29,36}

Mutations in the fibroblast growth factor (FGF) recep-

ABBREVIATIONS BMP = bone morphogenetic protein; cDNA = complementary DNA; CHOA = Children's Healthcare of Atlanta; FGF = fibroblast growth factor; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; mRNA = messenger RNA; PCR = polymerase chain reaction; qPCR = quantitative PCR.

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tor family, which cause syndromes like Pfeiffer, Apert, Crouzon, Jackson-Weiss, Antley-Bixler, Beare-Stevenson, and Muenke,^{3,23} are the most commonly studied in premature suture fusion. Other mutations exist with varying frequencies, including in *TWIST1* (Saethre-Chotzen syndrome) and *MSX2* (Boston-type craniosynostosis).²³ Non-syndromic or single-suture craniosynostosis occurs more frequently than syndromic,⁴ but whether genetic mechanisms or environmental factors are involved in nonsyndromic craniosynostosis is not known.

Bone morphogenetic proteins (BMPs) were first identified as inducers of bone and cartilage formation. BMPs belong to the transforming growth factor β superfamily, and numerous inhibitors, receptors, and intracellular molecules (Fig. 1) regulate these soluble proteins.³² BMP molecules are critical players in skull morphogenesis¹³ and are abundant in both fusing and patent cranial sutures,⁴² suggesting that they have an important role in skull development and subsequent suture fusion. The potent BMP antagonist Noggin can inhibit osteoblast differentiation and its downregulation is involved in pathological suture fusion,⁴⁰ supporting the hypothesis that it is important in

suture patency. Genetic abnormalities in *MSX2*, a transcription factor activated by BMP signaling, can cause syndromic craniosynostosis.²⁹ Interestingly, FGF2, a molecule linked to syndromic craniosynostosis in murine models, downregulates expression of *Noggin* in coronal dura cells and osteoblasts.²⁹

These findings suggest that BMP signaling is regulated during normal suture fusion and syndromic craniosynostosis. The aim of this study was to elucidate how the messenger RNA (mRNA) expression patterns of BMP ligands, antagonists, and receptors in cells isolated from fused sutures in isolated or single-suture fusion differ from those in cells isolated from patent sutures.

Methods

Bone Collection

Bone samples were collected from donors undergoing corrective surgery for craniosynostosis at Children's Healthcare of Atlanta (CHOA) under Institutional Review Board approval from both CHOA and the Georgia Institute of Technology (Georgia Tech) with written consent

FIG. 1. Schematic of BMP signaling pathway. Dimers of BMP molecules (homodimers or heterodimers) bind to tetramers of BMP receptors, transducing downstream signaling that leads to osteogenic differentiation. BMP inhibitors like *Noggin*, *Gremlin*, and *NBL* bind to BMP ligands, preventing their dimerization. p = phosphorylated proteins. Figure is available in color online only.

from the patient's parent/guardian. The samples were obtained from 3 sites on the skull: open (patent) suture, the fused suture, and a piece of normal bone, i.e., bone harvested from cranial bone not associated with a suture. These samples were placed immediately in preservation media and transported to the Georgia Tech laboratory. To isolate suture cells, samples were trimmed so that only 2 mm of tissue remained on either side of the suture line (Fig. 2). Pieces of normal bone from several nonsuture sites were combined to minimize embryological origin as a variable. Excess soft tissues were removed, and the bone pieces were washed in Hank's balanced salt solution with penicillin-streptomycin. Bone was incubated at 37°C with 0.25% trypsin-ethylenediaminetetraacetic acid (Life Technologies) for 15 minutes to remove fibroblast contamination, and the digestion was discarded. The bone was then cut into 2-mm² fragments and placed in Dulbecco's modified Eagle medium (Corning Cellgro) with 1% penicillin-streptomycin (Life Technologies) and 10% fetal bovine serum (Life Technologies).

Cell Culture

Cells were grown from the 3 samples on the skull of each patient using the explant outgrowth method in which cells are allowed to grow from the explant to confluence. The cells were subpassaged, and first passage cells were used for the experiments.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (qPCR)

RNA was extracted from cell cultures by incubating them in TRIzol (Life Technologies) following the manufacturer's directions. RNA was quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher) and reverse transcribed into complementary DNA (cDNA) with a High Capacity cDNA Kit (Life Technologies). mRNA levels were measured using gene-specific primers (Table 1) in real-time PCR reactions in the StepOnePlus Real-Time PCR System and Power SYBR Green Master Mix (Life

TABLE 1. Primer sequences for mRNA sequences analyzed by real-time PCR

Gene	Primer Sequence
<i>BMP2</i>	F: GCG-TGA-AAA-GAG-AGA-CTG-C R: CCA-TTG-AAA-GAG-CGT-CCA-C
<i>BMP4</i>	F: ACG-GTG-GGA-AAC-TTT-TGA-TGT-G R: CGA-GTC-TGA-TGG-AGG-TGA-GTC
<i>BMPR1A</i>	F: CAA-GAG-GCA-TCT-CAA-GCA-GCA-G R: CAG-ACC-CAC-TAC-CAG-ACC-TTT-G
<i>BMPR1B</i>	F: AAG-GCT-CAG-ATT-TTC-AGT-GTC-G R: TTC-AAT-GGA-GGC-AGT-GTA-GGG
<i>GREM1</i>	F: GCA-GGG-TGG-GTG-AAC-TTT-ATT-G R: AGG-AGG-CTG-AGA-AGA-TAC-AAG-G
<i>NOG</i>	Hs_NOG_1_SG QuantiTect primer assay, Qiagen QT00210833
<i>GAPDH</i>	F: GCT-CTC-CAG-AAC-ATC-ATC-C R: TGC-TTC-ACC-ACC-TTC-TTG

F = forward; R = reverse.

Technologies). mRNA levels were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

mRNA data are presented as normalized to normal bone mRNA levels. Statistical significance was determined using a Mann-Whitney U-test with a significance threshold of $p < 0.05$.

Results

Samples were collected from 26 donors, ranging from 6 to 13 months old (mean 9.5 months; Table 2). Three of the 26 donors had syndromic synostosis as confirmed by genetic studies performed during the management of their clinical cases. Interestingly, 3 additional patients did not have a known syndrome, but did have other family members with synostosis. *BMP2* mRNA was significantly

FIG. 2. Schematic of cell isolation protocol. Cells were isolated from a 2-mm² area surrounding open or fused sutures, as well as from nonsuture normal bone. Figure is available in color online only.

TABLE 2. Summary of patient data

Metric	Synostosis			
	Coronal	Metopic	Lambdoid	Sagittal
No. of males	1	8	5	0
No. of females	7	0	1	4
Syndromic cases	2	1	0	0
Mean age at operation $\pm$ SD (mos)	8.88 $\pm$ 2.64	11.43 $\pm$ 8.09	6.17 $\pm$ 6.49	12.75 $\pm$ 5.62

higher in fused suture than in normal bone or patent suture in 7 of 8 patients with coronal synostosis (Fig. 3A), 1 of 4 lambdoid synostosis cases (Fig. 3B), 2 of 8 metopic cases (Fig. 3C), and 5 of 6 sagittal cases (Fig. 3D). In the remaining cases, patent sutures had similar or more *BMP2* mRNA than fused sutures from the same patient. *BMP4* mRNA was significantly higher in fused suture than in normal bone or patent suture in 2 of 8 patients with coronal synostosis (Fig. 3E), 3 of 4 lambdoid synostosis cases (Fig. 3F), 4 of 8 metopic cases (Fig. 3G), and 3 of 6 sagittal cases (Fig. 3H).

Noggin (*NOG*) mRNA was significantly lower in fused suture than in patent suture and normal bone in 6 of 8 instances of coronal synostosis (Fig. 4A), 2 of 4 cases of lambdoid synostosis (Fig. 4B), and 5 of 8 cases of metopic synostosis (Fig. 4C). Fused sagittal suture cells had less *NOG* than the patent suture cells in 5 of 6 donors but were higher than levels in normal bone in only 1 patient (Fig. 4D). mRNA for *GREM1* were lower in fused suture than patent suture in 4 of 8 coronal synostosis samples (Fig. 4E), higher than patent suture in 2 of 4 lambdoid synostosis samples (Fig. 4F), lower than patent in 5 of 8 metopic synostosis samples (Fig. 4G), and lower than patent in 3 of 6 sagittal synostosis samples (Fig. 4H). *GREM2* was lower in fused suture than patent suture in 4 of 8 cases of coronal synostosis (Fig. 4I), similar to normal bone and patent suture in 2 of 4 patients with lambdoid synostosis (Fig. 4J), and lower in fused sutures than in patent sutures in 5 of 8 metopic synostosis samples (Fig. 4K) and 3 of 6 sagittal samples (Fig. 4L). Neuroblastoma 1, DAN family BMP antagonist 1 (*NBL1*) was present at similar levels in the patent suture and normal bone for many of the samples examined (14/26; Fig. 4M–P). Levels of *NBL1* in fused sutures were the same as or less than levels in patent suture in 5 of 8 coronal specimens (Fig. 4M), but levels were higher in fused than patent sutures for 3 of 4 lambdoid (Fig. 4N), 5 of 8 metopic (Fig. 4O), and 4 of 6 sagittal (Fig. 4P) synostosis samples.

Levels of BMP receptor 1A (*BMPRIA*) were similar in the patent suture and normal bone in 14 of 26 patients (Fig. 5A–D). *BMPRIA* was lower in fused sutures than in patent sutures in 2 of 8 coronal samples (Fig. 5A), 3 of 4 lambdoid samples (Fig. 5B), and 2 of 8 metopic samples (Fig. 5C). Levels were higher in fused sutures than in patent sutures for 2 of 8 metopic samples (Fig. 5C) and 2 of 6 sagittal samples (Fig. 5D). *BMPR1B* was greater in fused sutures than in patent sutures in 2 of 8 coronal synostosis samples (Fig. 5E), 3 of 4 lambdoid synostosis samples (Fig. 5F), 2 of 8 metopic samples (Fig. 5G), and 2 of 6 sagittal samples (Fig. 5H). However, *BMPR1B* levels were lower

in fused sutures than in patent sutures in 6 of 8 metopic samples (Fig. 5G). *BMPR2* levels were higher in fused suture cells than in patent suture cells for 4 of 8 patients with coronal synostosis (Fig. 5I), 4 of 4 with lambdoid synostosis (Fig. 5J), and 4 of 6 with sagittal synostosis (Fig. 5L). In contrast, *BMPR2* was lower in fused suture than in patent suture in 6 of 8 with metopic synostosis (Fig. 5K).

Discussion

The purpose of this study was to compare molecules associated with BMP signaling in cells isolated from single fused sutures, open sutures, and normal bone of patients with craniosynostosis. Our results showed that cells isolated from fused sutures present a lower expression of *NOG* than cells isolated from the open suture, independent of suture location. BMP molecules were highly expressed in cells isolated from fused sutures. Our results showed an increase in *BMP2* expression in cells isolated from sagittal and coronal sutures, which originate from neural crest precursors and neural crest-paraxial mesoderm interactions, and higher *BMP4* expression in cells isolated from metopic and lambdoid sutures.

Craniofacial structures are the result of a complex, well-orchestrated migration and interaction of cells from the neural crest and adjacent mesenchyme. A complex genetic cascade involving multiple genes, including BMP molecules, tightly controls these developmental processes spatiotemporally. BMPs regulate a wide variety of biological functions including gastrulation, organogenesis, embryological development, postnatal growth, and tissue homeostasis.⁴¹ BMP signaling is critical for the induction and formation of the craniofacial structures. *BMP2*, *BMP4*, and *BMP7* are strongly expressed during early craniofacial development,^{2,14} and BMP signaling interacts with FGF, Hedgehog, and Wnt pathways to regulate the activity of key transcription factors in craniofacial skeletogenesis such as *SOX9*, *RUNX2*, and *MSX2*.²⁵

In this study, we isolated cells from single fused sutures and compared them with cells isolated from patent sutures or normal bone from the same patients to examine whether BMP molecules were highly regulated in the fused suture. Our results showed that *BMP2* is highly regulated in cells isolated from fused sagittal and coronal craniosynostosis, while *BMP4* was higher in fused suture cells isolated from metopic and lambdoid craniosynostosis. Coronal and sagittal sutures are major growth centers and are the result of neural crest and mesodermal tissue interactions.^{21,33,44} One hypothesis for these differences is related to the embryological origins of the bones in the suture. Transgenic

FIG. 3. mRNA expression of *BMP2* and *BMP4* in patients with craniosynostosis. Levels of mRNA for *BMP2* and *BMP4* were measured in confluent cultures of cranial suture cells isolated from patent or fused sutures of patients with coronal (**A and E**), lambdoid (**B and F**), metopic (**C and G**), or sagittal (**D and H**) synostosis and normalized to mRNA levels found in cells isolated from normal cranial bone of the same patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus normal bone; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus patent suture. The red dashed line in each panel represents the cutoff for $p < 0.05$; anything above/below this line was a significantly different fold-change from normal bone (NB). Figure is available in color online only.

mice used to trace the fate of cells originating from neural crest and mesoderm origin have shown that coronal and sagittal sutures are the primary boundaries between neural crest cells and mesodermal cells.^{21,22,44} These studies indicated that the frontal bone and the central part of the interparietal bone are neural crest-derived components,

while other portions of the parietal bones are derived from the mesoderm. The coronal suture is formed by the overlapping edges of the frontal and parietal bones, and the suture mesenchyme between those bones is derived from mesoderm. The same studies found that blood vessels in the frontal bone are derived from the mesoderm, and the

FIG. 4. mRNA expression of BMP inhibitors in patients with craniosynostosis. Levels of mRNA for *NOG*, *GREM1*, *GREM2*, and *NBL1* were measured in confluent cultures of cranial suture cells isolated from patent or fused sutures of patients with coronal (A, E, I, and M), lambdoid (B, F, J, and N), metopic (C, G, K, and O), or sagittal (D, H, L, and P) synostosis and normalized to mRNA levels found in cells isolated from normal cranial bone of the same patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus normal bone; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus patent suture. The red dashed line in each panel represents the cutoff for $p < 0.05$; anything above/below this line was a significantly different fold-change from normal bone (NB). Figure is available in color online only.

underlying meningeal layer of the parietal bone is derived from the neural crest, exemplifying the complexity and intimate interaction between these 2 tissues in skull and cranial suture formation.⁴⁴ While our results highlight the large differences in gene expression between the fused and open sutures, there is a risk that the other portions of the skull are also compromised and not completely normal. However, the only perfect control would be an identical twin sibling without craniosynostosis who would provide the genetic material, age, and environmental factors.

Studies of osteogenic induction and bone formation have mostly been conducted in appendicular skeletal models.^{6,25,26,43} BMP signaling is critical for skeletogenesis, regulating chondrogenesis and osteogenesis at early and late stages of bone formation, and is well conserved in craniofacial development and skeletogenesis, regulating both endochondral and intramembranous ossification.³⁴ BMPs are highly expressed in the migrating neural crest cells and are crucial for differentiation of progenitor cells that

later form cartilage and bone.²⁴ Dysregulation of BMPs during craniofacial development, either by overexpression or by a decrease in their levels, results in defects in skeletal patterning, altered skeletal size, and abnormal morphology.^{1,11,12}

It has been shown that BMP signaling regulates cranial suture morphogenesis and function.^{19,27} *BMP2* and *BMP4* have been localized in the osteogenic fronts as well as in the suture mesenchyme.^{27,42} BMP signaling increases expression of distal-less homeobox 5 (*DLX5*) and *Msh* homeobox 2 (*MSX2*), critical transcription factors involved in craniofacial development.¹⁹ Gain of function mutations in *MSX2* result in autosomal dominant Boston-type craniosynostosis, further emphasizing the importance of BMP signaling in suture patency.³¹ *BMP4* expression increases in the osteogenic fronts and suture mesenchyme of posterior frontal sutures in mice (analogous to the human metopic suture) just before the onset fusion, and this expression is correlated with suture fusion.⁴² Additionally,

FIG. 5. mRNA expression of BMP receptors in patients with craniosynostosis. Levels of mRNA for *BMPR1A*, *BMPR1B*, and *BMPR2* were measured in confluent cultures of cranial suture cells isolated from patent or fused sutures of patients with coronal (A, E, and I), lambdoid (B, F, and J), metopic (C, G, and K), or sagittal (D, H, and L) synostosis and normalized to mRNA levels found in cells isolated from normal cranial bone of the same patient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus normal bone; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus patent suture. The red dashed line in each panel represents the cutoff for $p < 0.05$; anything above/below this line was a significantly different fold-change from normal bone (NB). Figure is available in color online only.

application of BMP4 increases suture volume and induces expression of *MSX2*,²⁷ mutations of which are associated with craniosynostosis.

Several soluble inhibitors, receptors, and pseudo-receptors regulate BMP molecules. In our study, we measured mRNA levels of inhibitors *NOG*, *GREM1*, *GREM2*, and *NBL1*. Our results showed that *Noggin* was downregulated in the majority of the cells isolated from fused sutures in comparison with patent sutures, independently of suture

localization. *Noggin* expression has been reported to be higher in nonfusing sutures in mice, rats, and rabbits, and a decrease in its expression has been reported in fusing sutures and fused sutures.^{15,18,42} Interestingly, *Noggin* expression decreased in a model of craniosynostosis induced by fetal constraint in mice.²⁰ Another study found an abolishment of *Noggin* expression in patent and fusing sutures in rats after mechanical stress exposure, and an increase in *RUNX2* expression.¹⁸ The results of these studies sug-

gest the possibility of a mechanical stress-induced mechanism in craniosynostosis, and that these forces could be differentially distributed on the sutures according to their location. It is clear that BMP signaling modulates suture fusion, but recent reports have indicated that Noggin may be an essential molecule in controlling suture fusion and patency. Exogenous Noggin treatment has been successfully used to inhibit suture closure and postoperative resynostosis in rabbit, rat, and mouse models, highlighting its importance in suture fusion and patency.^{9,10,37}

Our study also found expression of BMP receptors *BMPRIA*, *BMPRIB*, and *BMPR2*, but the expression of these genes varied widely from patient to patient and from patent sutures and fused sutures without a clear pattern of expression. A transgenic model of craniosynostosis generated using a constitutively active form of *Bmpr1a* in neural crest-derived cells²⁸ found that the resulting mice displayed a short, broad snout and hypertelorism, and premature fusion of the anterior frontal suture. This study demonstrates the importance of BMP signaling and BMP receptors in controlling and modulating suture patency and fusion. Other studies have shown an increase in important BMP signaling molecules *Bmp2*, *Bmpr1a*, *Bmpr2*, *Dlx5*, and *Msx2* in bone when compared with suture mesenchyme.^{7,27}

One limitation of the present study is that we were not able to measure gene expression from cells in the adjacent dura in our human cohort. Several studies have reported that dura cells and the molecules they secrete play an important role in suture fusion and patency in animal models, and it is hypothesized that dura cells affect the phenotype of the mesenchyme in the patent suture.^{5,30,35} Dura cells from below patent mouse sutures express high levels of *Nog* while dura cells below fusing sutures have very low *Nog* expression.⁴² This suggests that cells from the dura may create and modulate distinct microenvironments that control suture patency. However, our study demonstrates that osteoblasts and cells from the suture mesenchyme can also produce microenvironments that may reciprocally affect dura cells.

Conclusions

Taken together, our data suggest BMP molecules, receptors, and inhibitors are highly regulated in the prematurely fused suture, independently of the suture localization. Our results suggest that BMPs, inhibitors, and receptors play a significant role in the regulation of suture fusion in craniosynostosis, as well as the maintenance of patency in the normal suture. Antagonists such as *Noggin* appear to have significant activity in both patent sutures and fused sutures.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Olivares-Navarrete. Acquisition of data: Olivares-Navarrete, Whitton, Hyzy, Britt. Analysis and interpretation of data: Olivares-Navarrete. Drafting the article: Olivares-Navarrete, Whitton, Hyzy, Britt. Critically revising the article: Olivares-Navarrete, Hyzy, Williams, Boyan. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Olivares-Navarrete. Statistical analysis: Olivares-Navarrete. Administrative/technical/material support: Olivares-Navarrete, Williams, Boyan. Study supervision: Olivares-Navarrete.

Supplemental Information

Previous Presentations

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