

## Original Article

# Bone morphogenetic protein is required for fibroblast growth factor 2-dependent later-stage osteoblastic differentiation in cranial suture cells

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**Abstract:** Background: Understanding the pathophysiological process of calvarial bones development is important for the treatments on relative diseases such as craniosynostosis. While, the role of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) and how they interacted in osteoblast differentiation remain unclear. Methods: we digested bone fragments around the coronal and sagittal sutures from newborn rats to harvest suture cells. Markers expression at different osteoblast differentiation stage was analyzed by increasing FGF2 concentration and BMP2 blocking in these cells. Results: BMP2 expression could be stimulated by FGF2 in a dose and time dependent manner. FGF2 stimulation may decrease early marker of osteoblast differentiation (*collagen type-1*, COL-1) and increase the expression of continuously-expressed or late markers (alkaline phosphatase, ALP; *osteocalcin*, OC and *bone sialoprotein*, BSP) to accelerate mineralization. Inhibition of BMP2 signaling by Noggin weakens the effect of FGF2 on induction of later-stage osteoblastic differentiation of cranial suture cells. Conclusion: Our data suggest that BMP2 signaling is required for FGF2-dependent induction of later-stage of cranial suture cell osteoblastic differentiation.

**Keywords:** Fibroblast growth factor 2 (FGF2), bone morphogenetic protein 2 (BMP2), suture cells, osteoblast differentiation

## Introduction

The majority of calvarial bones are formed by intramembranous ossification, which occurs without previous cartilage formation [1]. Mesenchymal cells enter the osteoblastic lineage and undergo spatial and temporal progression; early preosteoblasts proliferate and then mature to osteoblasts that can differentiate and produce a collagenous matrix. Subsequent mineralization of the matrix results in the formation of the calvarial flat bones [1]. Cranial sutures are a soft connective-tissue interface between mineralized calvarial bones that primarily function as an articulation to allow expansion of bone and brain growth. Sutures consist of cells with different functions, such as mesenchymal cells and fibroblast-like cells, which are sandwiched between two osteoblast-

lined bone formation fronts [2]. Any perturbation in these processes induces premature or delayed fusion of the sutures, and abnormal cranial bone formation [1, 2].

Craniosynostosis (CS), the premature fusion of cranial sutures, is the second most common craniofacial development abnormality that occurs in one in 2,500 live births [3]. Because the skull fails to expand normally, CS can have serious consequences for affected children, particularly when more than one suture is affected, including raised intracranial pressure, impaired vision and hearing, airway obstruction, intellectual disabilities, and long-term psychological effects associated with abnormal head shape [4]. Children with CS require long-term management and complex transcranial surgery, which may need to be repeated as chil-

dren develop. Due to difficulties with airway patency, these children are prone to higher incidences of postoperative complications [5]. It is therefore highly desirable to develop non-surgical therapies based on a better understanding of the molecular regulation of skull bone growth and suture fusion.

CS may be sporadic or occur within over 150 syndromes, including Saethre-Chotzen, Apert, Pfeiffer, and Crouzon syndromes [3-5]. These syndromes are commonly inherited as autosomal dominant traits and are characterized by high genetic and clinical heterogeneity [3-5]. The molecular etiology consist of gene mutations, including gain-of-function mutations in the fibroblast growth factor receptors (FGFRs), FGFR2 and FGFR3, and a bone morphogenetic protein (BMP) effector transcription factor, MSX2 [6, 7]. Haploinsufficiency of the transcription factor TWIST1 is associated with Saethre-Chotzen Syndrome and is manifested by craniosynostosis, which is the premature closure of the calvaria sutures [8]. Reports showed that twist1 homodimers enhanced FGF responsiveness of the cranial sutures and promoted suture closure [8]. These studies suggest that FGFs, FGFRs as well as BMP signaling pathway participated in the process of osteoblastic differentiation of calvarial bones. Recent studies have shown that, FGF/FGFR signaling enhances the intrinsic osteogenic potential by selectively expanding committed osteogenic cell populations as well as inversely regulating BMP-2 and noggin gene expression [9]; low-dose FGF-2 may facilitate BMP-2-induced ectopic bone formation by altering the expression of BMPRs on the surface of bone forming progenitor cells [10]. However, what the role of FGF and BMP and how they interacted in calvarial bone formation and cranial suture fusion is still unclear.

In this study, we digested bone fragments around the coronal and sagittal sutures from newborn rats to harvest suture cells. We increased the FGF2 concentration in cultures to mimic the activated signaling that occurs in vivo setting to investigate whether FGF2 can induce BMP2 expression in cranial suture cells or not. Additionally, we measured the markers expression of different osteoblast differentiation stage to analyze what the role of FGF and BMP and how they interacted in osteoblast differentiation of cranial suture cells.

## Materials and methods

### *Suture samples and cell culture*

All experimental protocols involving animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine. All animal experiments have been conducted according to the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research released by the American Psychological Association.

Calvarial bones from newborn rats were dissected from surrounding muscle and soft tissue, including dura mater and periosteum, and washed in PBS containing penicillin and streptomycin. Next, we used a dissecting microscope and cut the bone and harvested the suture complex (suture mesenchyme plus 3mm of bone on either side of the patency coronal suture or sagittal suture). Suture complexes were digested in 0.1% collagenase (Type IV; SERVA, Germany) for 3 h at 37°C. Collagenase-digested suture complexes were washed extensively with basic media [low-glucose Dulbecco's modified essential media (L-DMEM) supplemented with glutamine (292 mg/l), 10% heat inactivated fetal bovine serum (FBS), and 1% antibiotic suspension (100 IU/ml of penicillin and 100 µg/ml of streptomycin)]. After several washes, cells were collected by centrifugation, suspended in DMEM, plated in 10 cm culture plates, and cultured in basic media.

To study osteoblast differentiation, cells were cultured for up to 21 d in growth media containing ascorbic acid (AA; 50 µg/ml) and β-glycerophosphate (BGP; 10 mM). Media were changed every 3 days.

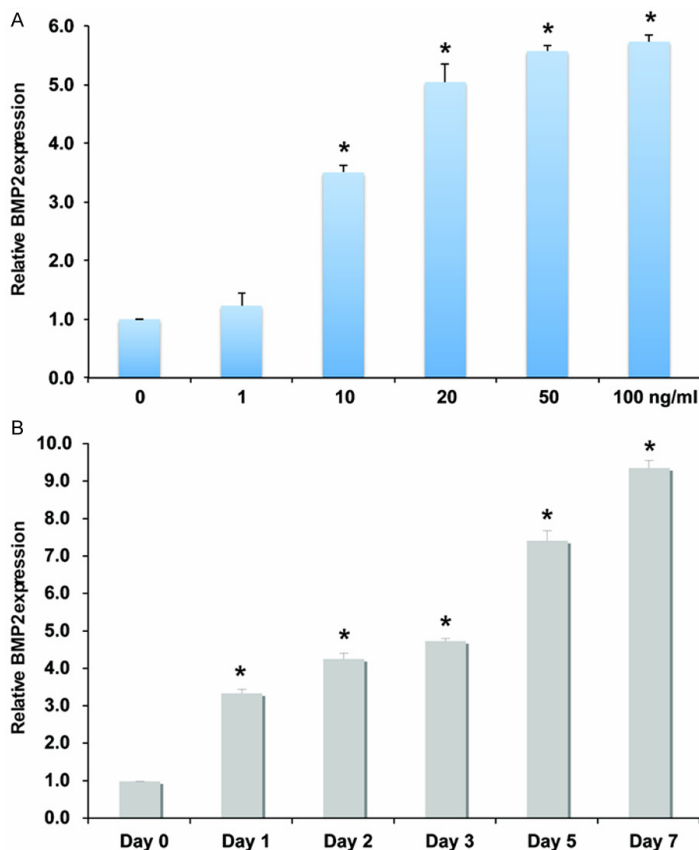
### *Cells treatment*

To study the role of FGF and BMP and how they interacted in osteoblast differentiation of cranial suture cells, the isolated cells were treated by various concentrations of FGF2 (Sino Biological Inc, Beijing, China), BMP2 (Sino Biological Inc, Beijing, China) and/or Noggin (Sino Biological Inc, Beijing, China).

### *Alkaline phosphatase staining and in vitro mineralization assays.*

Cells ( $1 \times 10^4$ ) were seeded in 24-well plates in basic media. After 24 h, the cells were induced

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**Figure 1.** FGF2 induces BMP2 expression in cranial suture cells. A: qRT-PCR analysis of BMP2 in cranial suture cells after treatment with different doses of FGF2. \*indicates the significance when compared with no FGF2. B: qRT-PCR analysis of BMP2 in cranial suture cells after treatment with 10 ng/ml FGF2 at different time points. \*indicates the significance when compared with day 0.

to differentiate with AA and BGP. Histochemical alkaline phosphatase staining was performed according to the manufacturer's instructions (Takara, Japan). To detect mineralized nodules formed *in vitro*, cultures were fixed in 4% paraformaldehyde for 10 min and rinsed with water. Cells were stained with 10% alizarin red solution and incubated at 37°C for 30 min. Mineralized nodules were identified as red spots on the culture dish.

### Total RNA isolation and quantitative reverse-transcription PCR

Total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (1 µg) was used to synthesize cDNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. Quantitative real-time PCR was performed on a

Light-Cycler machine (ABI, Foster City, CA, USA) using specific primers sets. The primers for rat genes are as follows: rat BMP2, Forward: 5' CGT CAA GCC AAA CAC AAA CA 3', Reverse: 5' AGT CAT TCC ACC CCA CAT CA 3'; rat COL1, Forward: 5' CGT GGA AAC CTG ATG TAT GCT 3', Reverse: 5' ACT CCT ATG ACT TCT GCG TCT G 3'; rat OC, Forward: 5' GAA CAG ACA AGT CCC ACA CAG 3', Reverse: 5' CAG GTC AGA GAG GCA GAA TG 3'; rat BSP, Forward: 5' TGA AGG GTC ATC GTA AAT CAG 3', Reverse: 5' CAG GGT ATG TTA GGG TGG TTA G 3'. GAPDH was used as internal control for reverse transcription. A series of quantified GAPDH plasmids (Biovisualab, Shanghai, China) were used as external controls and qualitative standards.

### Statistics

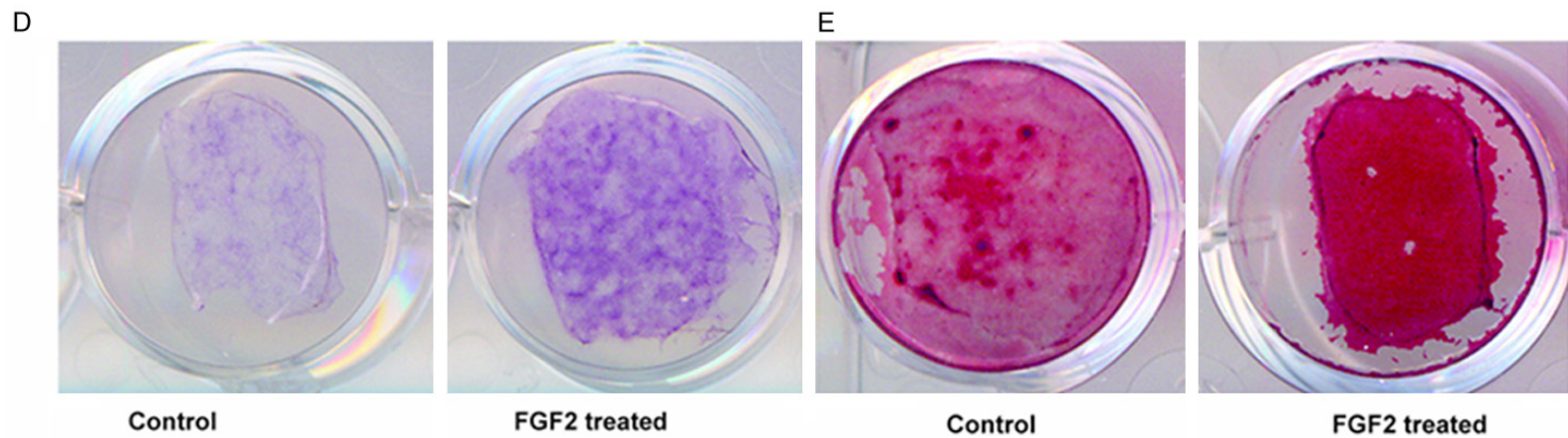
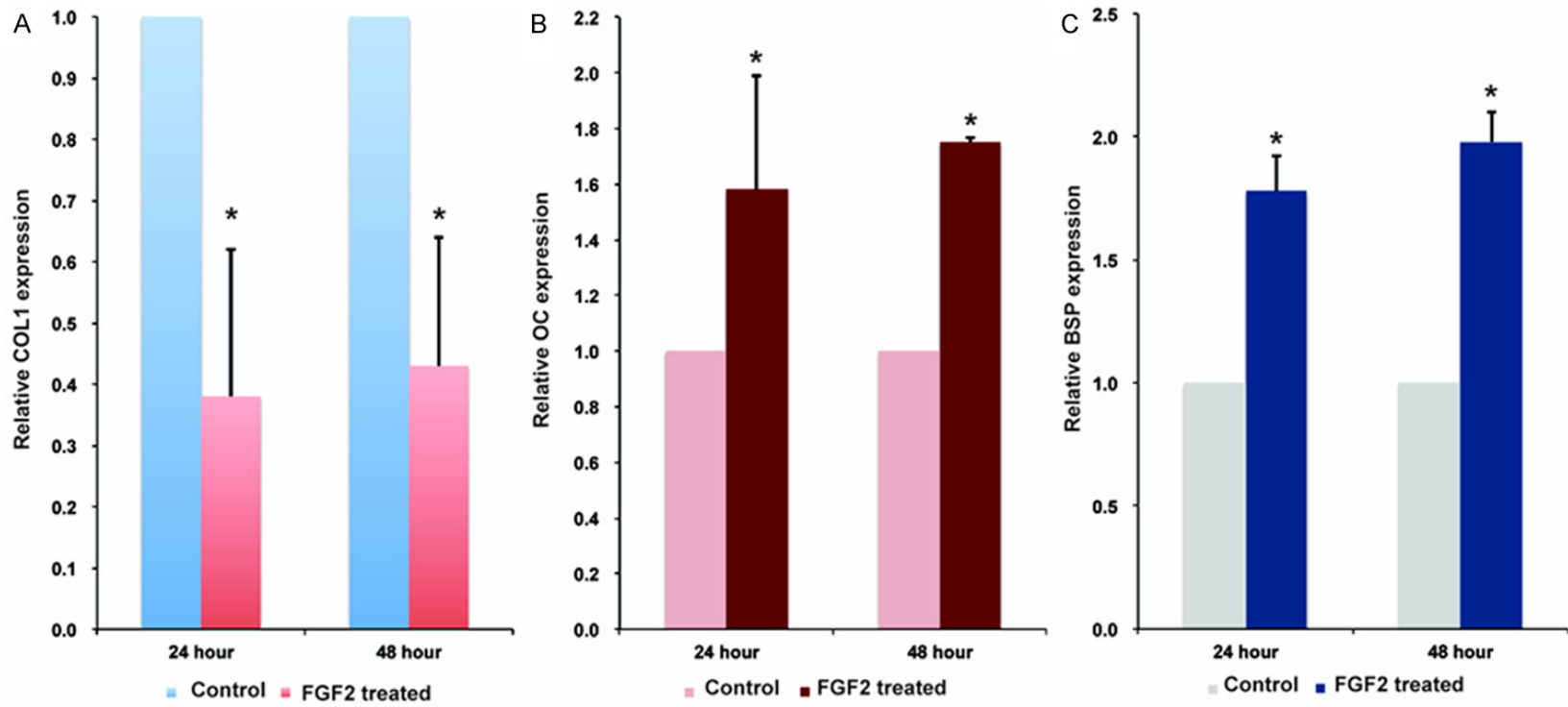
Continuous variables are presented as the means ± standard deviations. Differences were analyzed by student's t-test. A one-tailed *P* value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS ver. 17.0.0 (SPSS, Chicago, IL, USA).

### Results

#### *FGF2 induces BMP2 expression in a dose and time-dependent manner*

To examine the effect of FGF2 on BMP2 gene expression, cranial suture cells were treated with various concentrations of FGF2 (**Figure 1A**). After 24 h, BMP2 gene expression was analyzed using qRT-PCR. As showed in **Figure 1A**, BMP2 expression increased in a dose-dependent manner up to 50 ng/ml. when FGF2 concentration exceeded 50 ng/ml, BMP2 expression reached a plateau (**Figure 1A**). To evaluate the time course of FGF2 stimulation on BMP2 expression, cranial suture cells were treated with 10 ng/ml FGF2, cell RNA was harvested at various time points after treatment to examine BMP2 expression. We found that BMP2 expression increased in a time-dependent manner (**Figure 1B**). In contrast, BMP2 treatment had no effect on FGF2 expression (data not shown). In conclusion, these data

FGF2 controls osteoblastic differentiation via BMP2





## FGF2 controls osteoblastic differentiation via BMP2

**Figure 2.** Treatment of FGF2 promotes later-stage osteoblast differentiation and suppresses early markers of osteoblast differentiation. qRT-PCR analyses of osteoblast differentiation markers after cranial suture cells were treated with 10 ng/ml for 24 and 48 h. The mRNA level of control was defined as 1, mRNA levels of cells treated with FGF2 were converted by dividing by mRNA level of control. A: COL-1. B: OC. C: BSP. D: ALP staining. E: Mineralized nodule staining of cranial suture cells after treated with 10 ng/ml FGF2 for 7 days. \*Indicates the significance when compared with controls.

suggested that FGF2 might induce BMP2 expression, but BMP2 had no effect on FGF2 expression.

### *Treatment of FGF2 promotes later-stage osteoblast differentiation and suppresses early marker of osteoblast differentiation*

In order to determine whether FGF2 will alter osteoblast differentiation, we treated cranial suture cells with 10 ng/ml FGF2. After 24 or 48 h, the expression of osteoblast differentiation markers were analyzed. Treatment with FGF2 decreased the expression levels of *collagen type-1 (COL-1)*, a marker that is expressed early during osteoblast differentiation, at 24 and 48 h (**Figure 2A**). Expression of *osteocalcin (OC)*, a late osteogenic differentiation marker, increased upon the addition of FGF2 (**Figure 2B**). In addition, expression of *bone sialoprotein (BSP)*, a marker of mature osteoblasts, was also increased in the presence of FGF2 (**Figure 2C**). ALP, a marker that is expressed early in osteoblast differentiation but continues to be up-regulated at later stages, was also significantly enhanced after FGF2 treatment (**Figure 2D**). Finally, mineralized nodule staining showed that mineralized nodule was also increased upon the addition of FGF2 (**Figure 2E**). Thus, we conclude that FGF2 stimulation may decrease early marker of osteoblast differentiation (COL-1) and increase the expression of continuously-expressed and late markers (ALP, OC, and BSP) to accelerate mineralization.

### *BMP2 signals are required for FGF2-dependent induction of later-stage osteoblast differentiation*

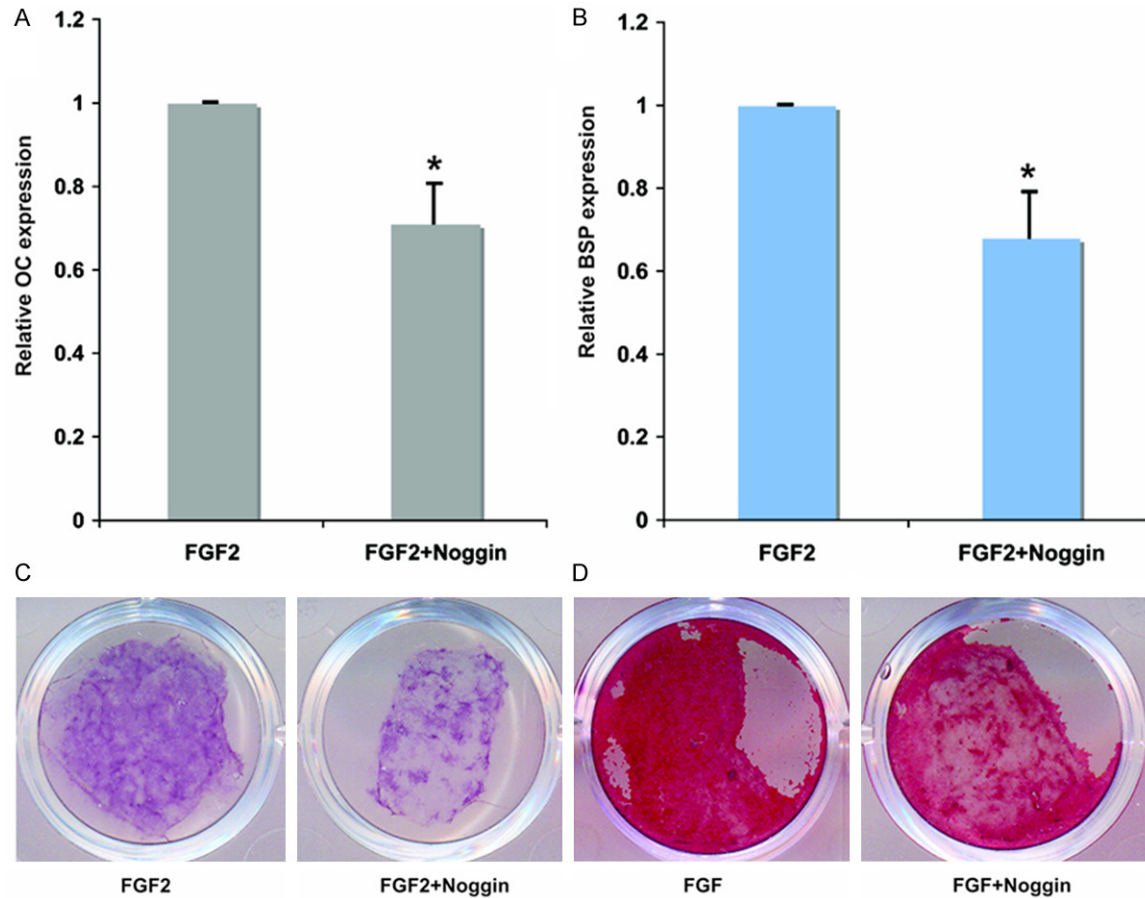
We have shown that FGF2 could stimulate BMP2 expression, decrease early marker of osteoblast differentiation (COL-1) and increase the expression of continuously-expressed and late markers (ALP, OC, and BSP) to accelerate mineralization. Therefore, we hypothesized that BMP2 signals are required for FGF2-dependent induction of later-stage osteoblast differentiation.

To evaluate this hypothesis, we used the natural BMP antagonist, Noggin, to inhibit BMP signals in cranial suture cells. Noggin is an extracellular BMP-binding protein that forms inactive complexes with BMP2, 4, and to a lesser extent, BMP7 [11]. As showed in **Figure 3**, when the cranial suture cells were treated with 10 ng/ml FGF2 and 1 µg/ml Noggin, 24 hours after the treatment, compared with the cells treated with FGF2 alone, OC and BSP expression was significantly decreased to  $71 \pm 9\%$  and  $68 \pm 11\%$  respectively (**Figure 3A** and **3B**). ALP staining and mineralized nodule staining was showed that ALP and mineralized nodule were also suppressed by Noggin (**Figure 3C** and **3D**). Taken together, these data suggest that BMP2 signals are required for FGF2-dependent induction of later-stage osteoblast differentiation in cranial suture cells.

## Discussion

Calvarial bone is formed by an intramembranous bone-forming process that involves many signaling molecules [1, 2]. Constitutive activation of the FGF signaling pathway accelerates osteoblast differentiation and results in premature cranial suture closure [1, 2]. BMP signaling pathways are also involved in the bone formation process [1-3]. However, the relationship between these two main signaling cascades is still unclear. In this study, we found that FGF2 treatment of cranial suture cells stimulated BMP2 gene expression. However, BMP2 treatment did not alter FGF2 expression. These observations indicate that BMP2 is downstream of FGF2 signaling. We further found that FGF2 stimulated BMP2 expression in a dose- and time-dependent manner. These findings are similar to the results of Choi et al. [12], but disagree with the data from Biver et al. [13]. Biver et al. found expression of BMPs and their receptors are up-regulated during human mesenchymal stem cell (HMSC) osteogenic differentiation, but FGF2 completely blocked the increase of BMP2, BMP4, BMPR1A, and BMPR1B. These differences may be caused by different cell types or different culture conditions.

## FGF2 controls osteoblastic differentiation via BMP2



**Figure 3.** Inhibition BMP2 signaling by Noggin weakens the effect of FGF2 on inducing later-stage cranial suture cell osteoblastic differentiation. qRT-PCR analyses of later-stage osteoblastic differentiation markers, the mRNA level of cells treated with FGF2 only was defined as 1; mRNA levels of cells treated with FGF2 plus Noggin were converted by dividing by mRNA level of cells treated with FGF2 only. A: The changes of OC expression after 24 hours treated with 10 ng/ml FGF2 or co-treated with 10 ng/ml FGF2 and 1  $\mu$ g/ml Noggin. B: The changes of BSP expression after 24 hours treated with 10 ng/ml FGF2 or co-treated with 10 ng/ml FGF2 and 1  $\mu$ g/ml Noggin. C: ALP staining of cranial suture cells treated for 7 days with 10 ng/ml FGF2 or co-treated with 10 ng/ml FGF2 and 1  $\mu$ g/ml Noggin. D: Mineralized nodule staining of cranial suture cells treated for 7 days with 10 ng/ml FGF2 or co-treated with 10 ng/ml FGF2 and 1  $\mu$ g/ml Noggin. \*Indicates the significance when compared with cells treated with FGF2 alone.

BMP2 has several roles in calvarial bone formation, acting at different stages to drive differentiation of mesenchymal osteoprogenitors into committed osteoblasts and promote mineralization of osteoblasts [14-17]. Recent studies have suggested that dysregulation of BMP2 activity is associated with suture fusion and craniosynostosis. Notably, Boston-type craniosynostosis appears to result from a gain-of-function mutation in *MSX2* [18, 19], a downstream transcriptional effector of BMP signaling. Animal models with high levels of exogenous BMP2 [20], or with constitutively active BMP type I receptors, cause premature suture fusion and craniosynostosis [21]. Therefore, we hypothesized that increased BMP2 expression

would enhance BMP signaling pathways and accelerate cranial suture cell osteoblast differentiation, resulting in premature cranial suture closure. This may be how constitutively activated FGFR mutations cause craniosynostosis.

There is evidence indicating that mutations in *FGFR1-FGFR3* cause craniosynostosis syndromes via ligand-independent constitutive activation of receptors [22]. In addition, analyses of the Bulgy-eye mouse, which is generated by an insertional mutation at the *FGF3/FGF4* locus, indicate that ligand overexpression may also cause a craniosynostotic phenotype [22-24]. There is considerable evidence indicating that FGF2 overexpression could also result in a

craniosynototic phenotype. Kim et al. applied FGF2 via beads to the osteogenic front and reported that suture closure is accelerated [22]. Greenwald et al. used a dominant-negative adenoviral FGFR1 construct injected into rats *in utero*. They found physiological posterior frontal suture fusion was inhibited, and injection of a construct that increased FGF2 biological activity resulted in the fusion of normal patent coronal suture [23]. Similarly, FGF2-treated posterior frontal sutures in rats show enhanced suture fusion [24].

BMPs were originally identified by their ability to induce ectopic bone formation [25]. They have subsequently been shown to be important signaling molecules in many developmental processes, including cranial suture fusion and patency [21]. A mutation in *MSX2*, a BMP effector transcription factor, accounts for Boston-type craniosynostosis. A recent study in a rabbit model system found that high levels of BMP2 causes premature suture fusion [20]. There is also considerable data suggesting that FGFs-FGFRs and BMPs interact during bone formation. When endogenous FGF signaling was blocked using a virally-transduced dominant-negative FGFR, *BMP-2* gene expression was drastically reduced, demonstrating that endogenous FGF/FGFR signaling is a positive upstream regulator of *BMP-2* in calvarial osteoblasts. Expression of the BMP antagonist Noggin was inhibited by FGF2 and FGF9 [9]. Nakamura et al. reported that FGF2 facilitates BMP-2-induced ectopic bone formation by altering BMP receptor expression on the surface of bone-forming progenitor cells [10].

We harvested primary cranial suture cells from the suture mesenchyme plus 3 mm of bone on either side of the patency coronal suture or sagittal suture. Thus, we harvested cells at all stages of osteoblast differentiation [13]. Bone was dissected free of dura mater to avoid the influence of growth factors secreted from dura mater [26]. We found that FGF2 stimulation decreased expression of early markers of osteoblast differentiation (COL-1) but increased expression of later markers (OC and BSP) and continuously-expressed markers (ALP). FGF2 stimulation also accelerated mineralization. Our data suggest that FGF2 and BMP2 cooperate during later-stage osteoblast differentiation. It has been shown that FGF-FGFR induces osteoblast differentiation through PKC signal-

ing and ERK-MAPK signaling [27, 28]. Because previous research has shown that FGF2 could induce BMP2 expression and co-treatment of FGF2 and BMP2 could induce later-stage osteoblast differentiation, we hypothesize that FGF2 induce osteoblast differentiation through BMP signaling. Our data showed that when BMP2 signaling was inhibited by Noggin, it weakened the effect of FGF2 on OC and BSP expression, suggesting that BMP2 is required for FGF-induction of later-stage osteoblast differentiation. Our work suggests that the FGF2/BMP2 axis controls osteoblast differentiation, with FGF2 enhancing BMP2 expression and BMP2 providing signals that enhance FGF2-dependent induction of later-stage differentiation.

This is an *in vitro* study, and we did not examine other influence factors (such as intracranial pressure) that are very important for osteoblast differentiation. Therefore, our results should be verified *in vivo*.

### Disclosure of conflict of interest

None.

### Abbreviations

ALP, Alkaline phosphatase; BMP2, Bone morphogenetic protein 2; BSP, Bone sialoprotein; COL-1, Collagen-1; FGF2, Fibroblast growth factor 2; OC, Osteocalcin; RT-PCR. Real-time quantitative polymerase chain reaction.

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