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## Age and Skeletal Sites Affect BMP-2 Responsiveness of Human Bone Marrow Stromal Cells

### Abstract

Bone marrow stromal cells (BMSCs) contain osteoprogenitors responsive to stimulation by osteogenic growth factors like bone morphogenetic proteins (BMPs). When used as grafts, BMSCs can be harvested from different skeletal sites such as axial, appendicular and orofacial bones, but the lower therapeutic efficacy of BMPs on BMSCs-responsiveness in humans compared to animal models may be partly due to effects of skeletal site and age of donor. We previously reported superior differentiation capacity and osteogenic properties of orofacial BMSCs relative to iliac crest BMSCs in same individuals. This study tested the hypothesis that recombinant human BMP-2 (rhBMP-2) stimulates human BMSCs differently based on age and skeletal site of harvest. Adult maxilla, mandible and iliac crest BMSCs from same individuals and pediatric iliac crest BMSCs were comparatively assessed for BMP-2 responsiveness under serum-containing and serum-free insulin-supplemented culture conditions. Adult orofacial BMSCs were more BMP-2-responsive than iliac crest BMSCs based on higher gene transcripts of alkaline phosphatase, osteopontin and osteogenic transcription factors MSX-2 and Osterix in serum-free insulin-containing medium. Pediatric iliac crest BMSCs were more responsive to rhBMP-2 than adult iliac crest BMSCs based on higher expression of alkaline phosphatase and osteopontin in serum-containing medium. Unlike orofacial BMSCs, MSX-2 and Osterix transcripts were similarly expressed by adult and pediatric iliac crest BMSCs in response to rhBMP-2. These data demonstrate that age and skeletal site-specific differences exist in BMSC osteogenic responsiveness to BMP-2 stimulation and suggest that MSX-2 and Osterix may be potential regulatory transcription factors in BMP-mediated osteogenesis of adult orofacial cells.

### Keywords

Bone morphogenic proteins, age, skeletal site, stem cells

### Disciplines

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## Age and Skeletal Sites Affect BMP-2 Responsiveness of Human Bone Marrow Stromal Cells

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### Abstract

Bone marrow stromal cells (BMSCs) contain osteoprogenitors responsive to stimulation by osteogenic growth factors like bone morphogenetic proteins (BMPs). When used as grafts, BMSCs can be harvested from different skeletal sites such as axial, appendicular and orofacial bones, but the lower therapeutic efficacy of BMPs on BMSCs-responsiveness in humans compared to animal models may be partly due to effects of skeletal site and age of donor. We previously reported superior differentiation capacity and osteogenic properties of orofacial BMSCs relative to iliac crest BMSCs in same individuals. This study tested the hypothesis that recombinant human BMP-2 (rhBMP-2) stimulates human BMSCs differently based on age and skeletal site of harvest. Adult maxilla, mandible and iliac crest BMSCs from same individuals and pediatric iliac crest BMSCs were comparatively assessed for BMP-2 responsiveness under serum-containing and serum-free insulin-supplemented culture conditions. Adult orofacial BMSCs were more BMP-2-responsive than iliac crest BMSCs based on higher gene transcripts of alkaline phosphatase, osteopontin and osteogenic transcription factors MSX-2 and Osterix in serum-free insulin-containing medium. Pediatric iliac crest BMSCs were more responsive to rhBMP-2 than adult iliac crest BMSCs based on higher expression of alkaline phosphatase and osteopontin in serum-containing medium. Unlike orofacial BMSCs, MSX-2 and Osterix transcripts were similarly expressed by adult and pediatric iliac crest BMSCs in response to rhBMP-2. These data demonstrate that age and skeletal site-specific differences exist in BMSC osteogenic responsiveness to BMP-2 stimulation and suggest that MSX-2 and Osterix may be potential regulatory transcription factors in BMP-mediated osteogenesis of adult orofacial cells.

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## Introduction

Skeletal homeostasis is maintained by bone marrow stromal cells (BMSCs), a major source of osteoprogenitor cells for bone renewal based on their multipotent properties and capacity to respond to stimulation by hormones and growth factors [1–3]. Bone morphogenetic proteins (BMPs) are potent osteogenic stimulants; they belong to the transforming growth factor- $\beta$  (TGF- $\beta$ ) super family that promote osteoblast differentiation *in vitro* and play key roles in bone formation *in vivo* [4–6].

In adults, BMP-2 and BMP-7 have broadest orthopedic and dental applications because *in vitro* studies have shown they stimulate the entire process of stem cell differentiation into mature osteoblasts. Specifically, BMP-2 increases *in vitro* alkaline phosphatase activity and gene expression of osteopontin and osteocalcin in human, rat and mouse BMSCs [6–8]. *In vivo* studies have also shown that a combination of BMP-2 and retinoic acid significantly increased healing of mouse calvarial defects within a short time [9] and *ex vivo*-expanded human BMSCs isolated during pelvic or femoral osteotomy exhibited higher *in vivo* bone forming capacity after exposure to BMP-2 [10–12].

BMPs appear to predominantly control growth and differentiation of cartilage and bone during embryonic development [4,13], and age-related differences in response of canine BMSCs to BMP-2 have been described [14]. Using cells isolated from human iliac crest and rodent femurs, we have previously shown that human BMSCs exhibit lower *in vitro* osteogenic response to BMP-2 compared to rodents [15]. Additionally, we have identified skeletal site-specific differences in osteogenic properties of human orofacial and axial BMSCs [16]. However, it is still unclear whether disparate BMSC responses to BMP are specie, age or skeletal site dependent. Also, the underlying mechanisms of disparate response of human BMSCs to BMP-2 stimulation have not been clearly defined.

The present study tested the hypothesis that BMP-2 stimulation of human BMSCs is dependent on age and skeletal site of harvest. Using human BMSCs isolated from pediatric iliac crest and adult maxilla, mandible and iliac crest, we provide evidence that age and skeletal site-specific differences exist in BMP-2-induced osteogenesis of BMSCs.

## Materials and Methods

### Bone Marrow Stromal Cell Isolation and Culture Conditions

Trabecular bone samples were collected from four healthy adult volunteers at the Hospital of the University of Pennsylvania, and marrow aspirates from four pediatric patients at Children's Hospital of Philadelphia, Philadelphia PA. Written informed consent was obtained under two Institutional Review Board-approved protocols. Mononucleated cells were harvested from maxilla and mandible (orofacial) trabecular bone samples and iliac crest (axial) marrow of adult donors; and also from iliac crest marrow aspirates of pediatric donors. Primary cultures of BMSCs were established from all samples in  $\alpha$ -modified minimum essential medium ( $\alpha$ -MEM) supplemented with 20% fetal bovine serum (FBS) (Equitech Bio, Kerville, TX), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate and 2 mM glutamine (all reagents from Gibco, Invitrogen, Carlsbad, CA unless otherwise stated) incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and air as previously described [16]. Sub-confluent primary BMSCs were either stored in liquid nitrogen or further expanded in culture so that lower passage BMSCs (passages 2 to 4) were used for all experiments.

### Treatment of Bone Marrow Stromal Cells With Recombinant Human BMP-2 (rhBMP-2)

In separate 6-well plates, early passage maxilla and mandible (orofacial) and iliac crest (axial) BMSCs were seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> in growth medium as described above. Twenty four

hours after seeding, growth medium was changed to osteogenic medium consisting of  $\alpha$ -MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, 2 mM glutamine and 0.35 mM L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Ltd., Japan). On day 4, BMSCs were exposed to six different culture conditions as follows: 1) osteogenic medium supplemented with or 2) without 100 ng/ml of rhBMP-2 (R&D Systems, Minneapolis, MN); 3) serum-free insulin containing osteogenic medium in which FBS was replaced with 0.1 % ITS Universal Culture Supplement Premix (BD Biosciences, San Jose, CA) with or 4) without 100 ng/ml rhBMP-2; and 5) serum-free osteogenic medium in which FBS was replaced with 1.25 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO) with or 6) without 100 ng/ml rhBMP-2. The cells were maintained in their respective culture conditions with daily medium changes until analyzed on day 7 (i.e. 3 days after exposure to rhBMP-2).

### **Analysis of Effect of Culture Conditions on rhBMP-2 –induced Proliferation of Bone Marrow Stromal Cells**

After 3-day BMP-2 treatment under the six culture parameters listed above, actively proliferating cells were assessed using WST-1 cell viability assay (Roche Applied Science, Indianapolis IN) following manufacturer's protocol as previously described [17]. Number of actively proliferating cells was directly proportional to absorbance at 450 nm measured with Multiskan MCC microplate reader (Thermo Fisher Scientific, Waltham, MA). Based on this preliminary WST-1 cell survival results that showed no BMP-2 –associated differences among the culture conditions, only serum-containing and insulin-containing culture parameters (i.e. #1 to 4 above) were further evaluated in this study.

### **Analysis of Live Bone Marrow Stromal Cells and Alkaline Phosphatase Activity**

In separate experiments for assessment of alkaline phosphatase activity, numbers of viable cells were estimated using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS, Promega Corp., Madison, WI) before harvesting the cells for alkaline phosphatase analysis. Briefly, BMSCs were washed once with Hank's Buffered Salt Solution (HBSS), before adding 200  $\mu$ l MTS reagent (1:10 volume/volume in phenol red-free  $\alpha$ -MEM). Cells were incubated for 15 min at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere. MTS-containing medium from each well was transferred to 96-well plates to measure absorbance at 490 nm. Subsequently, BMSCs were washed twice with HBSS, lysed in 10% Triton X-100 and kept at 4°C. Alkaline phosphatase activity was determined kinetically by monitoring conversion of p-nitrophenyl phosphate to p-nitrophenol over 10-minutes as previously described [7]. Alkaline phosphatase activity was calculated as nM p-nitrophenol released per minute, further normalized to live cell number to obtain specific alkaline phosphatase activity/cell.

### **Real-Time PCR Analysis of rhBMP-2-Treated Bone Marrow Stromal Cells**

BMSCs from the different culture conditions were harvested using Trizol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated following manufacturer's protocol. 2  $\mu$ g of total mRNA was converted into cDNA using oligo (dT) and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Real-time PCR reaction was carried out in ABI 7300 Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA). The following primer sequences were used: human alkaline phosphatase, forward, 5'-CCGTGGCAACTCTATCTTTGG-3', reverse, 5'-GATGGCAGTGAAGGGCTTCTT-3'; human osteopontin, forward, 5'-TGGAAAGCGAGGAGTTGAATG-3', reverse, 5'-CATCCAGCTGACTCGTTTCATAA-3'; human RUNX2/CBFA1, forward, 5'-TCAGCACCACAAGCCACTTC-3', reverse, 5'-GGTCGGAGAATGGGTTTCAGTT-3'; human Osterix, forward, 5'-ACTCACACCCGGGAGAAGAA-3', reverse, 5'-

GGTGGTCGCTTCGGGTAAA-3' and human MSX-2, forward, 5'-GCACCCTGAGGAAACACAAG-3', reverse, 5'-CGAGGAGCTGGGATGTGGTA-3'. TATA binding protein (TBP), forward, 5'-GGAGCTGTGATGTGAAGTTTCTTA-3', reverse, 5'-CCAGGAAATAACTCTGGCTCATAAC-3' was used as normalizing control. Results were expressed as relative fold change using the  $\Delta\Delta C_t$  method (Applied Biosystems, Foster City CA).

### Statistical Analysis

Results from triplicate experiments from each subject were expressed as mean  $\pm$  standard deviation. BMP-2 effects on BMSCs were presented as fold change or percent change relative to control untreated BMSCs. Data analysis was by two-way analysis of variance (ANOVA) followed by post-hoc comparisons with Holm-Sidak test using SigmaStat 3.1 statistical package (Systat Software, Inc., Chicago, IL). Statistical significance was set at  $P < 0.05$ .

## Results

### Skeletal site and age-related differences in rhBMP-2-induced alkaline phosphatase expression

BMSCs isolated from four pediatric (ages 6 to 9 years) and four adult (ages 17 to 25 years) patients (Table 1) were tested for age and skeletal site-dependent response to BMP-2 stimulation under different culture conditions. There were no significant differences in proliferation of adult (Figure 1) or pediatric (data not shown) BMSCs among the different culture conditions when rhBMP-2 was added to the culture media. This confirmed that 100 ng/ml rhBMP-2 was conducive to cell survival irrespective of culture condition and the BMSCs continued proliferating to confluent monolayer by day 7 with or without BMP-2 stimulation (data not shown). Adult BMSCs from maxilla and mandible were more responsive to BMP-2 stimulation based on higher alkaline phosphatase mRNA in 2 of 4 patients while iliac crest BMSCs from all 4 adult patients were unresponsive (Figure 2A). In contrast, 3 of 4 pediatric iliac crest BMSCs responded to BMP-2 stimulation like adult maxilla and mandible BMSCs by displaying comparatively similar increase in alkaline phosphatase mRNA (Figure 2B) and activity (data not shown). Further testing of additional pediatric BMSCs ( $n = 12$ ) in our cell library confirmed a statistically significant increase in alkaline phosphatase activity by pediatric BMSCs in response to BMP-2 ( $p < 0.01$ ) (Figure 2C). These alkaline phosphatase results indicate skeletal site and age-related differences in BMSCs responsiveness to BMP-2 stimulation. Adult maxilla, mandible and pediatric iliac crest BMSCs demonstrated similar BMP-2 responsiveness while adult iliac crest BMSCs were less responsive.

### Insulin-Containing Culture Affects Alkaline Phosphatase Levels in rhBMP-2-Treated Bone Marrow Stromal Cells

We have previously reported that switching culture conditions to serum-free insulin-containing medium enhanced BMP-induced early osteogenic response of adult human femoral BMSCs (4). Similarly, insulin-mediated increase in alkaline phosphatase activity in response to BMP-2 was recapitulated in pediatric iliac crest BMSCs (Figure 3A). To further define this response, we evaluated effect of age and skeletal sites on insulin-mediated BMP-2 responsiveness of adult BMSCs by real time PCR. Higher levels of alkaline phosphatase mRNA transcripts were displayed by 3 of 4 adult orofacial BMSCs compared to iliac crest cells (Figure 3B). Despite individual variability, insulin effect on adult BMSCs response to BMP-2 appeared to be skeletal site-dependent. Adult iliac crest BMSCs from 3 of 4 donors (Figure 3B) did not respond as pediatric iliac crest cells, which further suggests that insulin effect on BMP-2 response may be age-dependent.



### Skeletal Site and Age-Related Differences in BMP-Induced Osteopontin Expression

In serum-containing medium, osteopontin gene was generally poorly expressed by adult iliac crest and 3 of 4 maxilla and mandible BMSCs in response to BMP-2 stimulation (Figure 4A). However, pediatric iliac crest unlike adult iliac crest BMSCs were slightly more BMP-2-responsive (Figure 4B); which further supports possible age-related effects. Additionally, switching to insulin-containing medium enhanced osteopontin gene expression in adult maxilla and mandible BMSCs (Figure 4C) similar to the alkaline phosphatase response presented in Figure 3B. This further confirms that insulin effect on BMSC BMP-2 responsiveness is apparently skeletal site-dependent.

### Effect of rhBMP-2 on Activation of Osterix and MSX-2

Assessment of BMP-2-mediated osteogenesis based on gene expression of RUNX2, the master osteogenic transcription factor, did not show any difference between control and BMP-2 stimulated BMSCs in either adult or pediatric BMSCs (data not shown) as previously described in earlier reports [3]. We further assessed gene expression levels of Osterix and MSX-2 that can be activated independent of RUNX2. In serum-free insulin-containing medium, BMP-2 enhanced MSX-2 (Figure 5A) and Osterix (Figure 5B) gene expression in adult maxilla, mandible and iliac crest BMSCs with mandible showing the highest response and Osterix mRNA much more elevated than MSX-2. In contrast, Osterix and MSX-2 mRNA levels were much lower in adult maxilla and mandible BMSCs under serum-containing culture conditions (data not shown) and there were no significant differences between expression levels of Osterix and MSX-2 in either adult or pediatric iliac crest BMSCs (Figure 5C and D).

### Discussion

We evaluated the effects of age and skeletal site-specificity on BMP-2-mediated osteogenic differentiation of human BMSCs from adult maxilla, mandible and iliac crest and pediatric iliac crest. Previously, we have established the existence of site-specific variations in the response of orofacial (maxilla and mandible) and iliac crest human BMSCs to osteogenic and adipogenic inducers *in vitro* as well as their *in vivo* bone regenerative capacity [16]. Adult orofacial BMSCs were reported to be more responsive to osteogenic inducers *in vitro* and regenerated quantitatively more bone *in vivo*. Those initial studies were confined to the use of osteogenic medium that contained ascorbic acid and dexamethasone while BMPs and other osteogenic growth factors were not tested. The current study advanced the previous work by further testing age and skeletal site-specific osteogenic response of BMSCs to BMP-2 stimulation.

The osteogenic effects and therapeutic potential of rhBMP-2 is well recognized using animal and human BMSC cultures [18]. But human BMSCs are apparently less responsive to BMP stimulation than animal cells. Our previous studies demonstrated that adult human femoral BMSCs treated with rhBMP-2 did not exhibit higher expression of either alkaline phosphatase or osteopontin under standard culture conditions but needed modifications of culture conditions before undergoing osteogenic differentiation [3]. Consistent with this report, our present study indicated that adult human iliac crest BMSCs responded like femoral cells and did not display appreciable osteogenic response to rhBMP-2. Although insulin/Insulin Growth Factor signaling pathway acts synergistically with BMP pathway in adult human mesenchymal stem cells to induce gene expression [19], adult iliac crest BMSCs were still osteogenically less responsive to rhBMP-2 stimulation when cultured in serum-free insulin-containing medium. This is consistent with similar reports on vertebral body-derived adult human BMSCs that also displayed limited osteogenic response to rhBMP-2 in a defined serum-free culture condition [20].

The minimal response of adult iliac crest BMSCs prompted further evaluation of age-related effects on BMP-2 response. The impact of age is relevant in rodents considering that BMSCs usually isolated from young animals are readily responsive to BMP stimulation. In humans, these age-related effects still need to be further clarified [21–23]. The human pediatric iliac crest BMSCs evaluated in this study were more osteogenically responsive to BMP-2 stimulation than adult cells based on elevated alkaline phosphatase and osteopontin mRNA levels. These observations support the existence of age-related BMP-2 responsiveness of iliac crest BMSCs and are consistent with earlier reports that rhBMP-2 treated BMSCs from young subjects have high bone forming capacity [12,14,24]. However, further studies on long-term osteogenesis, effects on bone remodeling and osteoblast-osteoclast balance are still needed to fully understand the underlying mechanisms.

In contrast to the low response of adult iliac crest BMSCs, orofacial (maxilla and mandible) BMSCs displayed high expression of alkaline phosphatase and osteopontin in response to rhBMP-2. This is consistent with our previous report that orofacial BMSCs have greater osteogenic potential in culture [16] and underscores the existence of skeletal site-specificity of BMSC osteogenesis. Skeletal site-specificity of BMSCs may be due to their different embryological origins because craniofacial bones arise from neural crest while axial and appendicular bones arise from mesoderm. Similarly, craniofacial bones have dual intramembranous and endochondral ossification patterns while axial and appendicular bones undergo only endochondral ossification. During endochondral ossification, BMPs are thought to serve more as inducers of mesenchymal condensation and chondrogenic differentiation rather than bone inducers [25,26]. On the other hand, recent studies have shown that BMPs influence differentiation of neural crest-derived mesenchyme into bone [27] and exogenous treatment of avian mandible with BMP resulted in accelerated osteogenesis [28]. It is noteworthy that BMP-2 did not alter proliferative capacity of BMSCs tested in this study (Figure 1), further highlighting that these disparate BMP-2 mediated responses were more related to BMSC differentiation and not cell number.

We assessed the possibility that disparate BMP-induced expressions of early osteogenic genes like alkaline phosphatase and osteopontin indicate age and skeletal site-specific variations in expression of three osteogenic transcription factors, namely RUNX2, MSX-2 and Osterix. BMSCs were kept in culture for 7 days in this study, so it was not surprising that mRNA levels of RUNX2, an early marker of osteoblast differentiation, remained unchanged in both pediatric and adult BMP-treated cells (data not shown) because RUNX2 expression is usually transiently elevated within the first hours of BMP treatment in adult BMSCs [25,29]. Since actions of Osterix and MSX-2 are interrelated [30–32] and BMP-2 directly regulates MSX-2 gene expression through Smad binding elements in a promoter region [33], it is possible that BMPs can indirectly activate Osterix expression through MSX-2 [32] or DLX-5 [34] without involving RUNX2, the master osteogenic transcription factor [25]. Therefore, we focused on Osterix and MSX-2 expression levels in assessing skeletal site and age-related effects on BMSCs response to BMP-2. In contrast to unchanged expression of RUNX2, induction of MSX-2 and Osterix mRNAs was sustained in response to BMP-2 stimulation. The higher levels of Osterix and MSX-2 mRNA displayed by maxilla and mandible relative to iliac crest BMSC were consistent with similarly high expressions of alkaline phosphatase and osteopontin. Therefore, adult maxilla and mandible may have responded by activation of MSX-2 and Osterix while iliac crest cells probably responded to BMP-2 by a different mechanism. Understanding these mechanisms will further illuminate age-related differences between pediatric and adult iliac crest BMSCs that displayed similar levels of MSX-2 and Osterix despite pediatric cells being more responsive to BMP-2. The small number of BMSC samples tested is one limitation of this study. But testing BMSCs from four adult and four pediatric subjects was in accordance with earlier studies that showed sample size of 4 was adequate to demonstrate statistically significant cellular response in mesenchymal stem cells obtained from different skeletal sites



in same individuals [16,17]. To minimize individual variability, maxilla, mandible and iliac crest BMSCs from each adult subject were matched so that cells from same individuals were evaluated together. Another limitation was that only pediatric iliac crest BMSCs were tested, but this decision was based on ethical guidelines of the experimental design.

In summary, this study showed that age and skeletal site-specific differences exist in BMP-2-induced BMSC osteogenesis. BMP-mediated osteogenesis of pediatric iliac crest and adult orofacial BMSCs were generally higher than adult iliac crest cells. Our results suggest that Osterix and MSX-2 are potential regulatory transcription factors in BMP-mediated osteogenesis of adult orofacial cells. Further elucidation of underlying mechanisms of this disparate BMP-2 response is still needed.

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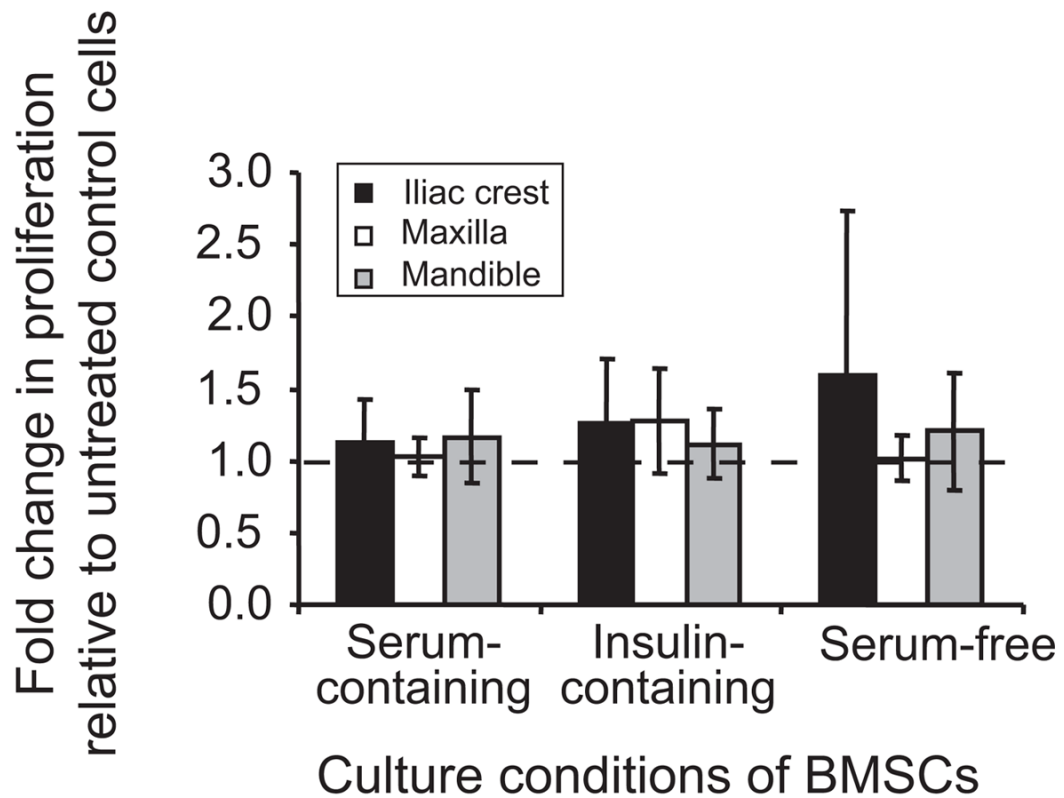
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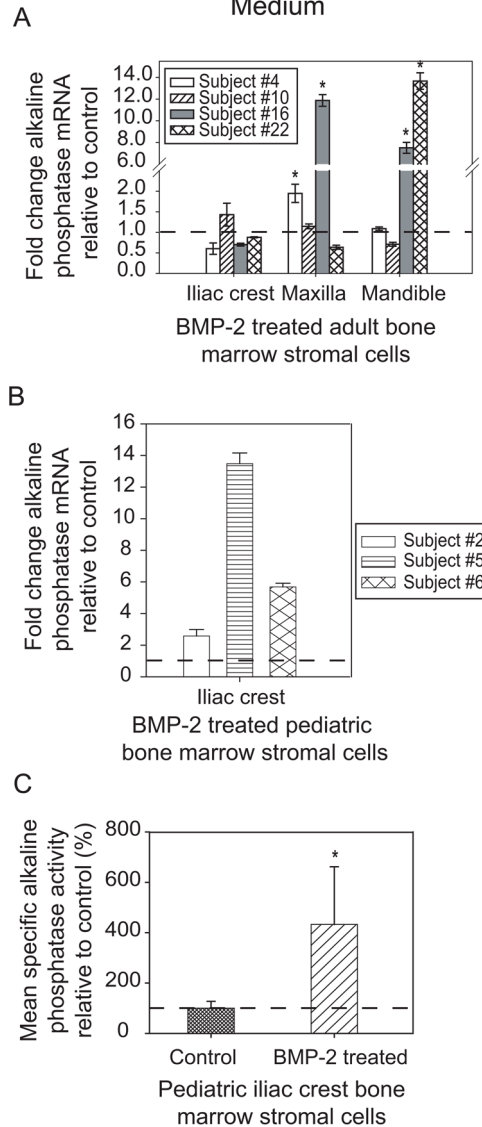
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## BMP-2 Effects on BMSC Proliferation



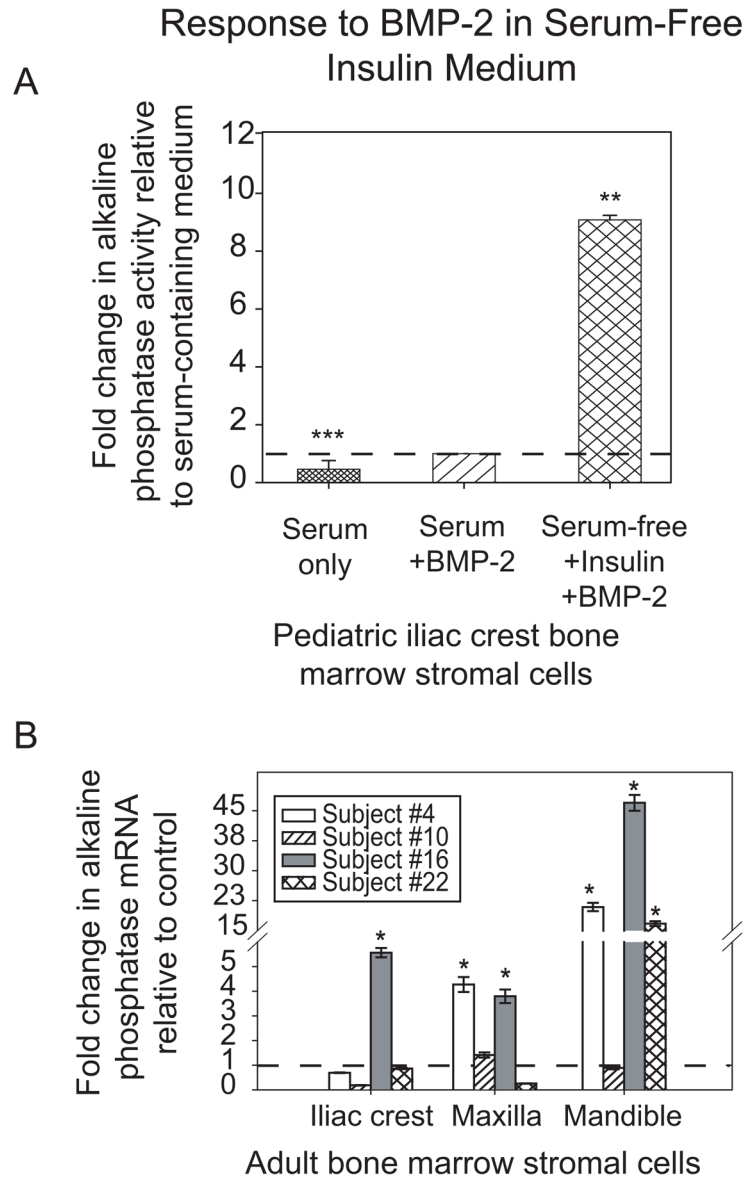
**Figure 1. BMP-2 effects on adult BMSC proliferation unaffected by variable culture conditions** BMSCs from different skeletal sites were seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> were stimulated with and without rhBMP-2 in medium containing serum, or serum-free  $\alpha$ -MEM with or without insulin followed by WST-1 cell viability assay at day 7. BMSC proliferation was unaffected by BMP-2 relative to controls (expressed as 1) and similar survival pattern was displayed irrespective of culture conditions. [Error bars are standard deviation of mean of triplicate experiments from each subject (n = 4)].

### Response to BMP-2 in Serum-Containing Medium



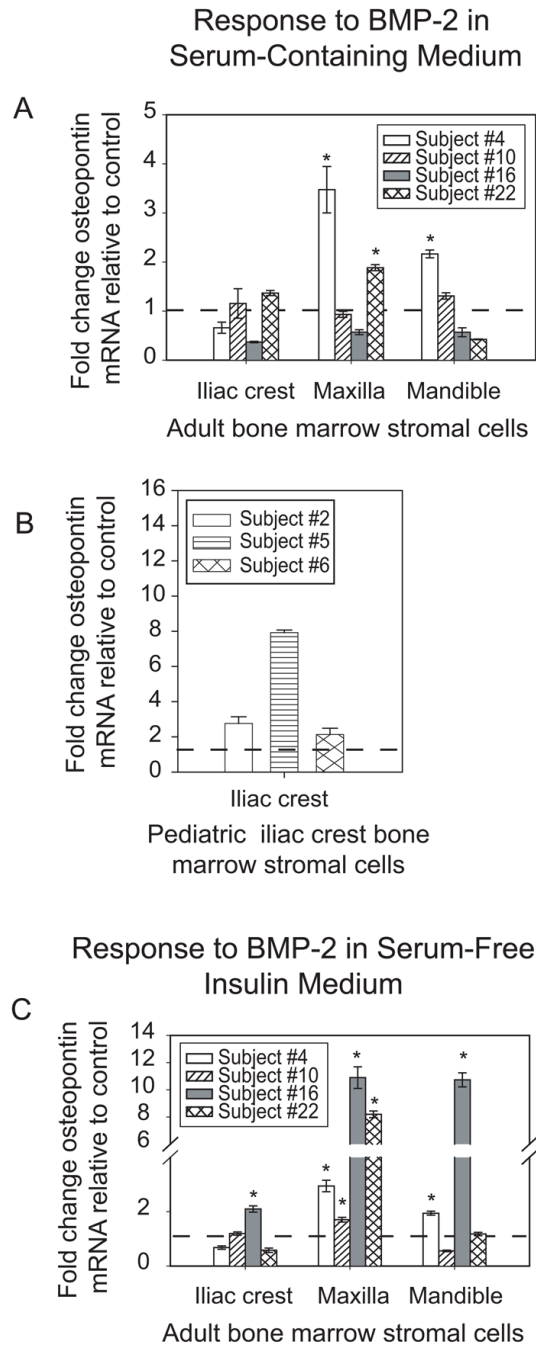
**Figure 2. BMP-2 stimulated alkaline phosphatase expression in adult orofacial and pediatric iliac crest BMSCs in serum-containing medium**

BMSCs seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> were stimulated with and without rhBMP-2 in serum-containing medium for 7 days followed by analysis of alkaline phosphate mRNA by real-time PCR in (A) adult BMSCs from different skeletal sites and (B) pediatric iliac crest BMSCs. There was high BMP-2 responsiveness of adult maxilla and mandible cells in 2 of 4 subjects similar to pediatric iliac crest cells, while adult iliac crest cells were unresponsive. (C) Similarly treated iliac crest BMSCs from additional pediatric subjects (n = 12; demographic information not provided in table 1) were analyzed for BMP-2 induced change in alkaline phosphatase activity per cell. There was a statistically significant increase in BMP-responsiveness of pediatric iliac crest BMSCs further confirming age-related differences in iliac crest BMSCs. [Error bars are standard deviation of mean of triplicate experiments; \* = P < 0.05].



**Figure 3. Serum-free insulin-containing culture condition enhanced BMP-2-induced expression and activity of alkaline phosphatase in pediatric iliac crest and adult orofacial BMSCs**  
 BMSCs seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> were stimulated with and without rhBMP-2 in serum-free insulin-containing medium for 7 days followed by analysis of (A) alkaline phosphatase activity in pediatric iliac crest BMSCs (n = 4) and (B) alkaline phosphatase mRNA by real-time PCR in adult BMSCs from different skeletal sites. Insulin increased alkaline phosphatase activity in pediatric iliac crest cells and high mRNA increase in adult maxilla and mandible cells while 3 of 4 adult iliac crest cells were unresponsive. [Error bars are standard deviation of mean of triplicate experiments; \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.0001].

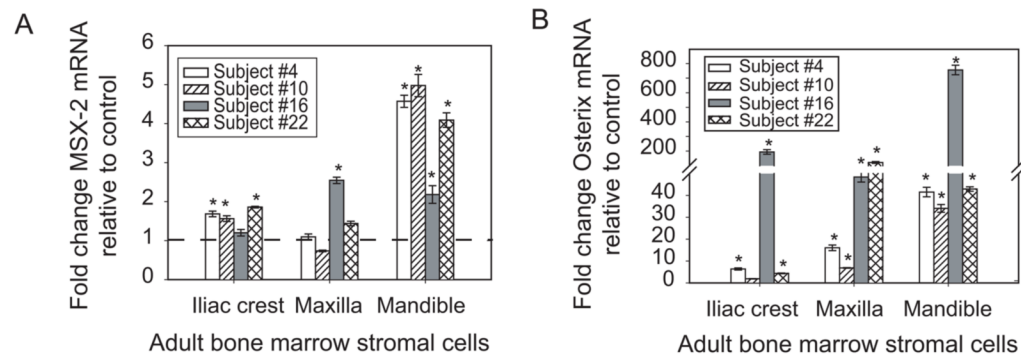




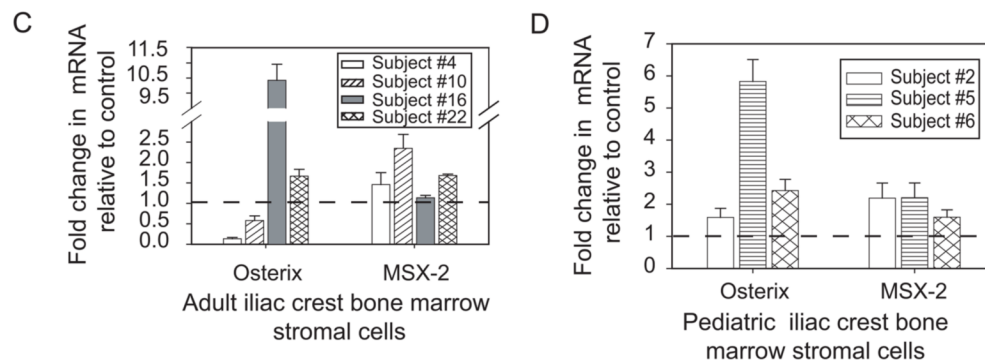
**Figure 4. BMP-2-induced osteopontin expression in orofacial adult BMSCs is affected by culture conditions**

BMSCs seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> were stimulated with and without rhBMP-2 in serum-containing medium for 7 days followed by analysis of osteopontin mRNA from (A) adult BMSCs from different skeletal sites and (B) pediatric iliac crest BMSCs. Osteopontin mRNA was generally poorly expressed by BMP-treated adult BMSCs (except subject 4) unlike pediatric iliac crest BMSCs that were generally more responsive. (C) Switching to insulin-containing medium however supported higher osteopontin expression in maxilla and mandible BMSCs while adult iliac crest cells were still less responsive. [Error bars are standard deviation of mean of triplicate experiments; \* =  $P < 0.05$ ].

## Response to BMP-2 in Serum-Free Insulin Containing Medium



## Response to BMP-2 in Serum-Containing Medium

**Figure 5. BMP-2 disparately increased Osterix and MSX-1 expression in orofacial BMSCs cultured in serum-free insulin-containing medium**

BMSCs seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> were stimulated with and without rhBMP-2 in serum-free insulin-containing medium for 7 days followed by analysis of (A) MSX-2 and (B) Osterix mRNA by real-time PCR. Moderate expression of MSX-2 and high expression of Osterix mRNAs were induced by BMP-2 in orofacial cells (especially mandible) while iliac crest cells were less responsive (except subject 16 in B). In the presence of serum, expression of both MSX-2 and Osterix mRNAs were low in orofacial BMSCs (not shown) but there were no age-related differences based on similar expression levels of MSX-2 and Osterix in both (C) adult and (D) pediatric iliac crest BMSCs. [Error bars are standard deviation of mean of triplicate experiments; \* =  $P < 0.05$ ].

**Table 1**

## Characteristics of BMSC donors

<b>Subject #</b>	<b>Age (Years)</b>	<b>Sex</b>
6	6	Male
7	6	Male
2	8	Male
5	9	Male
22	17	Male
4	18	Female
10	20	Male
16	25	Female