Fibroblast Growth Factor-2 and Bone Morphogenetic Protein-2 Have a Synergistic Stimulatory Effect on Bone Formation in Cell Cultures From Elderly Mouse and Human Bone

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Combined regimens of fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein-2 (BMP-2) were investigated to stimulate osteogenic differentiation. In young mouse calvaria-derived cells, FGF-2 (0.16 ng/mL) in combination with BMP-2 (50 ng/mL) did not enhance mineralization, but in old mouse cells it resulted in more mineralization than BMP-2 alone. In young long bone mouse cultures, FGF-2 enhanced mineralization relative to BMP-2 alone, but in old cultures, lower dose of FGF-2 (0.016 ng/mL) was necessary. In neonatal mouse calvarial cells, sequential delivery of low-dose FGF-2 and low-dose BMP-2 (5 ng/mL) was more stimulatory than co-delivery. In young human cultures, 0.016 ng/mL of FGF-2 did not enhance mineralization, in combination with 5 ng/mL of BMP-2, but in older cultures, codelivery of FGF-2 and BMP-2 was superior to BMP-2 alone. In conclusion, BMP-2 treatment alone was sufficient for maximal mineralization in young osteoblast cultures. However, coadministration of FGF-2 and BMP-2 increases mineralization more than BMP-2 alone in cultures from old and young mouse long bones and old humans but not in young mouse calvarial mouse cultures.

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Bone regeneration involves a series of osteoinductive cellular events, including commitment of undifferentiated mesenchymal stem cells (MSCs) to the osteogenic lineage, cell proliferation, sequential activation, and upregulation of genes for bone formation and mineralization. An unsolved problem is how to enable a biomaterial bone substitute to enhance the proliferation and mineralization cascade of MSCs, especially in elderly patients, who have less active mesenchymal progenitors (1) and reduced osteogenic potential (2–4). To enhance the osteoinductive property of the biomaterial, a number of regulatory molecules, such as growth factors, hormones, transcription factors, and vitamins, have been applied to the biomaterial for use in bone defects.

Bone morphogenetic protein-2 (BMP-2) has been shown to be an active inducer of osteoblast differentiation of both the immature osteoblast and less-committed cells (5). A recently published randomized prospective study of older cleft palate patients showed that patients receiving BMP-2 in a resorbable collagen matrix had better alveolar filling, less pain, shorter hospital stay, and lower total costs than patients receiving a traditional iliac graft (6). Additional clinical studies showed that recombinant BMP-2 augmented the maxillary sinus floor (7) and preserved the alveolar ridge (8). Although both of these clinical studies were successful in generating new bone formation, large doses of BMP-2 (1.7–3.4 mg) were used. Strategies to reduce the high levels of BMP-2 used clinically are needed due to cost and safety reasons—BMP-2 can cause unwanted calcification (9) and may stimulate cancerous cell growth (10). BMP-2 must also be given at even higher doses in elderly patients due to reduced efficacy (11), thus providing further motivation for additional research on increasing efficacy of BMP-2 in elderly patients at reduced doses.

We postulated that the use of low doses of basic fibroblast growth factor-2 (FGF-2) in combination with BMP-2 would be efficacious in overcoming age-related decline of in vitro osteogenic performance by bone-derived MSCs based on the proliferative and osteogenic effects of FGF-2 (12,13). FGF-2 is a strong mitogen for bone-derived cells (14). In in vivo studies, systemic and local administration of FGFs increases bone formation and accelerates callus remodeling and fracture healing (15–17). In vitro studies...
with human and rat calvarial cells and osteoblast cell lines have demonstrated the importance of FGF-2’s dose and that the responses to FGF-2 may be anabolic or catabolic depending on the dose. Low doses of FGF-2 were shown to promote differentiation, in contrast, high doses inhibited the differentiation of MSCs (12,13,18–21). We have reported that low-dose FGF-2 enhanced calvarial cell proliferation in vitro (22), and the disruption of the FGF-2 gene in mice led to significant loss of bone mass and bone formation, as well as downregulation of BMP-2 (23,24).

Previously published in vitro and in vivo studies support the combined use of relatively high doses of FGF-2 and BMP-2 for osteogenesis by MSCs from young mice or humans (25,26); however, a confounding issue for bone tissue engineering is the age-dependent responsiveness of MSCs to growth factors. In a study of FGF-2 effects on bone marrow stromal cultures derived from both young and adult rats, FGF-2 (2 ng/mL) was not required for maximal mineralization of cells from 6-week-old rats, but in cells from 9-month-old adult rats, FGF-2 was needed to obtain optimal mineralization (27). In another aging study with FGF-2 and BMP-2, FGF-2 had no significant additive effect on BMP-2-induced mineralization in bone marrow cell cultures obtained from young or adult rats (28). Our previous work showed a decrease in mineralization associated with age in human MSC cultures from elderly female patients (3) and that the transforming growth factor-β could stimulate mineralization, but did not increase the number of osteoprogenitors in these cell cultures (29). In another study, we found that FGF-2 doses, 10 times lower than those tested previously (0.16 ng/mL), significantly stimulated, in a dose-dependent manner, proliferation of MSCs from bones of young and old mice and humans, and we also found that FGF-2 prevented the decline with time of early progenitor markers, but proliferation was still less in cells from older bones (22).

Knowing that initial dosing with FGF-2 could increase the pool of committed progenitor cells, that continuous FGF-2 might block later phases of osteoblast differentiation in vitro, and that later stages of osteoblast differentiation in vivo seem to be regulated by other growth factors such as BMP-2 (30,31), we hypothesized that sequential addition of FGF-2 followed by BMP-2, rather than continuous dosing would optimize in vitro osteogenesis. In preparation for further studies with growth factor delivering biomaterial scaffolds and to further pave the way for future clinical use of low-dose combinations of FGF-2 and BMP-2, the following two aims were addressed in these studies: (a) to determine an optimal dosing regimen to obtain a synergistic effect of low doses of FGF-2 and BMP-2 on osteoblast differentiation, and matrix deposition and mineralization as a function of age of the donors used to obtain progenitors and (b) to compare mouse versus human responsiveness to combinations of FGF-2 and BMP-2 as a function of age.

**Materials and Methods**

**Materials**

Dulbecco’s Modified Eagle Medium/F-12, penicillin (10,000 U/mL), streptomycin (10,000 µg/mL), and heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY) were used for cell cultures. All tissue culture plasticware was from Corning (Corning, NY). Recombinant FGF-2 was obtained from Peprotech Inc (Rocky Hill, NJ). *Escherichia coli* expressed nonglycosylated recombinant human BMP-2 was used in these studies and was prepared as described previously (32). All tissue culture reagents such as β-glycerol phosphate and ascorbic acid were from Sigma (St. Louis, MO).

**Isolation and Culture of Bone-Derived Osteoprogenitors**

All animal protocols were approved by the Animal Care and Use Committee of the University of Connecticut Health Center, Farmington, Connecticut.

**Adult mice.**—Three-month-old (young) and 19- to 22-month-old (old), female BALB/c mice were obtained from National Institute of Aging (Bethesda, MD). The animals were housed for an additional 2 weeks for acclimatization to laboratory conditions. The BALB/c mice were sacrificed by CO₂ asphyxiation and its calvaria and femurs were removed using aseptic technique in a biological safety cabinet. Briefly, soft tissues were removed, epiphyses of the femurs were cutoff and the bone marrow flushed out with phosphate-buffered saline using a syringe and needle. The femoral diaphysis was chopped into 1 mm² fragments. Bone fragments were extensively and repeatedly washed with cold phosphate-buffered saline to remove adherent marrow cells. Calvarial cells were obtained from frontal and parietal bones of the calvariae. The interior and exterior surfaces of the calvariae were scraped, the sutures were removed, and the remaining bone was chopped into 1 mm³ fragments.

Bone fragments were transferred to 100-mm dishes and cultured in Dulbecco’s minimum essential medium/F-12 supplemented with 10% FBS (Gibco/Invitrogen, Rockville, MD), 100 U/mL of penicillin-G (Sigma-Aldrich, St. Louis, MO), and 100 mg/mL of streptomycin (Sigma) at 37°C in 95% humidified air and 5% CO₂. Culture medium was replaced two times per week without disturbing bone fragments. Bone cells started to migrate from the bone chips after 4–5 days. At 12–14 days, cells growing from the bone fragments reached confluence and were trypsinized using 0.25% trypsin and 0.1% EDTA (Gibco) in phosphate-buffered saline. The cells were plated into 100-mm dish in 10% FBS medium for 3 days to expand the cells. Then, cells were trypsinized, centrifuged, resuspended, and seeded at 10,000/cm² in 24-well plates for experiments.

**Neonatal mice.**—Previously, we developed transgenic mice that have a green fluorescent protein (GFP)-driven...
2.3-kb Col1a1 promoter fragment of the type I collagen gene (pOBCol2.3GFP, referred to as 2.3GFP) that is expressed in mature osteoblasts when the nodules start to mineralize (33). A 3.6-kb Col1a1 promoter fragment driving a cyan variant of GFP (pOBCol3.6GFP, referred to as 3.6GFP) is expressed in preosteoblast cultures 5–7 days before the colonies develop into mineralizing multilayered nodules (34). Cells harvested from these mice provide a simple means of evaluating osteogenic responses of progenitor cells to biomaterial and growth factor stimulus (35). Primary mouse calvarial osteoblast progenitor cells were harvested from the calvaria of 5- to 8-day-old neonatal transgenic mice carrying these transgenes, following a published protocol with modifications (33). Briefly, after removal of sutures, calvaria were subjected to four sequential 15-minute digestions in a mixture containing 0.25% trypsin, EDTA (Gibco BRL), and 2 U/mL collagenase P (Boehringer Mannheim, Germany) at 37°C on a rocking platform. Cell fractions 2–4 were collected and enzyme activity was stopped by the addition of an equal volume of Dulbecco’s Modified Eagle’s Medium (#11885; Gibco), 10% FBS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.1 mM of nonessential amino acids (Gibco BRL). The fractions were pooled, centrifuged, and resuspended in the culture medium and were filtered through a 70-mm cell strainer. Then, cells were seeded at 10,000/cm² in 24-well plates for experiments.

Human.—Bone discarded from orthopedic procedures was obtained from young (19, 21, 29, and 35 years old) and older (56, 68, 72, 74, and two 58 years old) female patients. The Institutional Review Board at the University of Connecticut Health Center determined, per Department of Health and Human Services (DHHS) criteria, that this work did not constitute human participant research. The soft tissues were scraped off the bone and the bone was chopped into 1 mm² fragments. Human bone fragments were transferred to 100-mm dishes. Only one patient’s bone was used for each experiment. Multiple experiments were conducted using bone cells from different patients. Human bone fragments were cultured in Dulbecco’s Modified Eagle Medium/F-12 supplemented with 10% FBS and antibiotics. MSCs started to migrate from the bone chips after 1–2 weeks. At near confluence, cells were trypsinized and plated in 100-mm dishes. After the cells were expanded, the cells were trypsinized and plated at 10,000 cells/cm² in 24-well plates for experiments.

Mineralization in Response to FGF-2 and BMP-2

For the first 4 hours, cells were allowed to attach to the 24-well plates in Dulbecco’s Modified Eagle Medium/F-12 medium with 10% heat-inactivated FBS and antibiotics. The cells were then treated with vehicle and/or FGF-2 and/or BMP-2 in medium for 3 days with 0.5% heat-inactivated FBS for bone chip–derived cells and 7 days in 10% heat-inactivated FBS for neonatal fluorescent reporter cells (ie, 2.3GFP or 3.6GFP). Varying concentrations of FGF-2 (0.016, 0.16, and 1.6 ng/mL) and BMP-2 (5, 10, 25, and 50 ng/mL) were used. After 7 days, the media was changed to osteogenic media (4 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, and 10% heat-inactivated FBS in α-minimum essential medium) with or without FGF-2 and/or BMP-2 or vehicle. The osteogenic medium was changed every 3 days with fresh addition of the growth factors for a period of 21 days (neonatal calvarial cells and human osteoblast cultures) and 24 days (young and adult mouse calvaria– and long bone–derived cells). The bone chip outgrowth cells of mouse origin required additional culturing time due to slower growth than the neonatal cells.

Bone Nodule Assay

To assay bone nodule formation, mineralized nodules were stained with the fluorochrome, xylene orange (XO), which is advantageous over the von Kossa technique in that it is more specific to calcium (as opposed to its salt anions) and does not require termination of the culture (36). A 20 mM stock solution of XO (Sigma, St. Louis, MO) was made and an aliquot was added to medium overnight for a final 20 μM concentration. Prior to microscopic examination, cultures received fresh medium without XO to avoid nonspecific fluorescent background. The plates were evaluated for the XO-stained bone nodules under a fluorescence microscope. The cell cultures were continued until the end of the experiment.

Determination of Calcium Content

The calcium content was measured using a Calcium Reagent Set Kit (Eagle Diagnostics, DeSoto, TX). Briefly, the cells were decalcified with 5% trichloroacetic acid, 30 minutes two times, and the pooled supernatants were collected. A 10 μL aliquot of each sample was transferred to 96-well plates. Thereafter, reagents from the commercial calcium assay kit were added to the samples and the absorbance was read at 570 nm. Reference solutions were supplied by the manufacturer for a calcium standard curve.

Statistical Analyses

Experimental data and error bars represent a mean ± standard error of the mean for each group. The statistical analysis of the results was performed by two-way analysis of variance with multiple comparison procedures to isolate group mean differences followed by the Bonferroni posttest with the conventional p < .05 level considered to reflect statistical significance. All experiments were repeated at least three times from different isolations of MSCs from young and old mouse bones and human cell cultures from different patients.
**Results**

Our main interest is in the response of aging bone to low doses of FGF-2 and BMP-2, therefore, we studied the responses of bone-derived osteoprogenitor cells from calvarial and long bones of young (3-month-old) and old (19- to 22-month-old) mice in our first set of experiments (Figure 1). For these studies, we initially chose a BMP-2 concentration of 50 ng/mL based on previously published literature: 50 ng/mL of BMP-2 in rat bone marrow (25), 50 ng/mL of recombinant BMP-2 in chicken calvarial bone cell cultures (31), 100 ng/mL of BMP-2 in C2C12 myoblast cultures to produce ectopic bone formation (37), and 50–500 ng/mL of BMP-2 for rat bone marrow cells (28). The present studies were conducted with E. coli-derived BMP-2, which is nonglycosylated and less soluble than the glycosylated BMP-2 that is used clinically. The concentration of FGF-2 for the young versus old experiments was 0.16 ng/mL since our previous work showed that this concentration was the most anabolic in stimulating osteoprogenitor proliferation in cell cultures from young and old mouse calvarial and long bones (22).

In cultures of cells derived from calvarial bones of young mice, BMP-2 alone (administered after 7 days of vehicle, hence denoted as V/B), and the combinations of FGF-2 and BMP-2 (denoted as F/B for sequential dosing and FB/FB for continuous dosing of both factors), produced comparable mineralization, which was significantly greater than vehicle or pretreatment with FGF-2 for 3 days followed by vehicle (F/V) for 24 days of culture (Figure 1A). The addition of FGF-2 did not improve the amount of mineralization in the young calvarial cultures. However, the cells from old calvaria had significantly more mineralization when treated with FGF-2 and BMP-2 together for the entire period than with sequential growth factor treatment (F/B) or BMP-2 alone (V/B: Figure 1B). Cells derived from young long bones showed a similar trend to cells from old calvaria; FB/FB had greater mineralization than sequential growth factor delivery or BMP-2 alone (Figure 1C). In old long bone cultures, there was no increase of mineral deposition with addition of FGF-2 when administered alone or in combination with BMP-2 at 0.16 ng/mL (Figure 1D). Decreasing the dose of FGF-2 by 10-fold, however, was effective at increasing mineralization of young and long bone cultures when codelivered (Figure 1E and F). In Figure 1G, the results from XO-stained cell cultures from young mouse long bones are shown to complement the calcium data presented in Figure 1C and D. The type of mineral in the FB/FB-treated cell cultures covers a greater area and is less punctate than that from cultures exposed to only BMP-2.

To further investigate the optimal concentrations of FGF-2 and BMP-2 for in vitro mineralization in combination with this type of nonglycosylated BMP-2, dose-response studies of single factors were completed using collagen reporter cells from bones of transgenic mice enabling a discriminating nondestructive evaluation of osteoblast differentiation at various time points. Cells from calvaria of 5-day-old transgenic mice, 2.3GFP, were used to detect the development of mature osteoblasts expressing type I collagen (2.3-kb Coll1 promoter fragment driving GFP) and XO staining was used to detect mineralization after exposure to FGF-2 at doses ranging from 0.016 to 1.6 ng/mL for 21 days of culture. The microscopy images (Figure 2A) of confluent cultures demonstrate that untreated confluent 2.3GFP cell cultures express small areas of type I collagen expression (green), reflecting mature osteoblasts in cell nodules, which subsequently mineralize. The lowest concentration of FGF-2 tested, 0.016 ng/mL, significantly increased osteoblast differentiation and mineralization, as seen by increased percent GFP-positive area (Figure 2A and B) and percent XO-stained mineralized nodules (Figure 2A and C). The areas of Col2.3GFP-positive mature osteoblasts coincided with the regions of mineralized matrix indicating that there was no dystrophic or nonphysiological mineral. Both vehicle control (no FGF-2) and 0.16 ng/mL of FGF-2-treated cultures had similar levels of percent GFP-positive and percent XO-stained areas. However, continuous 1.6 ng/mL of FGF-2 inhibited osteogenesis as shown by complete reduction of GFP expression and associated mineralization. To confirm the GFP and XO results, calcium content was assayed biochemically, and it demonstrated that 0.016 ng/mL of FGF-2 stimulates mineralization, and higher concentrations of FGF-2 inhibit mineralization compared with control cultures (Figure 2D).

Next, varying concentrations of BMP-2 (5, 10, 25, and 50 ng/mL) were examined for their ability to stimulate mineralization of cells from calcifying 5-day-old neonatal transgenic mice at 21 days of culture (Figure 3). Col3.6GFP cells were used to detect the development of preosteoblasts expressing type I collagen, and XO staining of the cell cultures was used to detect mineralization. Both 5 and 10 ng/mL of BMP-2 increased the areas of 3.6GFP-positive preosteoblasts and XO-stained mineral compared with vehicle-treated cultures (Figure 3A, B, and C). However, 25 and 50 ng/mL of BMP-2 decreased mineralization, and the percent area of GFP-positive (cyan) neonatal preosteoblasts was less than vehicle-treated control. These results were confirmed by measuring the calcium content of the cultures (Figure 3D). When the 2.3GFP-positive cultures are compared with the 3.6GFP-positive cultures, there are larger fluorescent areas in the 3.6GFP cultures because the fluorescence is associated with less-differentiated preosteoblasts that extend into unmineralized regions that will subsequently calcify into the 2.3GFP-positive stage. In the 2.3GFP-positive cultures, only mature osteoblasts coinciding with mineralizing regions are fluorescent and consist of a smaller population of cells compared with all cells in the osteoblast lineage in these cultures.
Figure 1. The effect of combinations of vehicle (V), fibroblast growth factor-2 (FGF-2; F), and bone morphogenetic protein-2 (BMP-2; B) on mineralization of 24-day osteogenic cultures of calvarial and long bone chip outgrowth cells from young (3-month-old) and old (19- to 22-month-old) mice. The addition of 0.16 ng/mL of FGF-2 to a 50 ng/mL of BMP-2 dose did not increase the calcium content of (A) young calvarial cultures for all patterns of BMP-2 treatment; however, in (B) old calvarial cultures, 0.16 ng/mL of FGF-2 addition caused a significant increase of calcium content above that of BMP-2 alone. (C) Osteoblast cultures from young long bones responded in a similar manner as old calvarial cultures, but sequential delivery of the two growth factors, in addition to codelivery, also stimulated mineralization more than BMP-2 alone. (D) In older long bone cultures, the addition of 0.16 ng/mL of FGF-2 to BMP-2 did not enhance mineralization. (E) A 10× lower dose of FGF-2 (0.016 ng/mL) combined with 50 ng/mL of BMP-2 also enhanced mineralization of young mouse long bone cultures and (F) long bone, old mouse cultures. These studies illustrate the need to optimize growth factor delivery for cells of various tissues and ages. (G) Phase contrast and xylene orange (XO)-stained cultures demonstrating the extent of mineralization in young and old long bone cultures for the high-dose combination. Scale bar = 100 μm. *p < .05 compared with vehicle treatment and #p < .05 compared with BMP-2 treatment.
Cultures of 2.3GFP cells were also used to compare the osteogenic effects of combinations of the lower doses identified by the dose–response studies to the original higher doses selected from the literature (Figure 4). Sequential and continuous dosing of FGF-2 and BMP-2, for example, FGF-2 (0.016 and 0.16 ng/mL) and BMP-2 (5 and 50 ng/mL), as well as single factors and vehicle for 21 days of culture, were evaluated. The lower concentrations of FGF-2 and BMP-2 were most effective at increasing osteoblast differentiation and mineralization, as seen by increased percent GFP-positive area (Figure 4A and B) and percent XO-stained mineral area (Figure 4A and C) and calcium content (Figure 4D). The higher dose combinations of FGF-2 and BMP-2 decreased these parameters compared with vehicle alone. In these neonatal progenitor cells, more osteoblast differentiation and mineralization was found when low-dose FGF-2 (0.016 ng/mL) was given for the first 7 days and then replaced by low-dose BMP-2 (5 ng/mL; F/B) than when both growth factors were administered continuously in combination (FB/FB). Overall, osteoblast differentiation or mineralization was not increased by the addition of FGF-2 over that of BMP-2 alone in these cells from very young mice. Together, these techniques provided consistent results showing the same effects for the different growth factor delivery patterns.

Our ultimate goal is to provide better therapies for stimulating bone formation in older human patients; therefore, we studied the response of human MSCs derived from the bones of young and old patients. Our previous work had shown that MSCs from human bone responded best to 0.016 ng/mL of FGF-2 in terms of proliferation compared with the optimal dose of 0.16 ng/mL of FGF-2 for mouse MSCs (22). Because 5 ng/mL of BMP-2 produced the most mineralization in the dose–response study (Figure 3), this concentration was used on the human MSCs. In cultures of human cells derived from bones of young patients, BMP-2 alone (given at either the later stage of culture [V/B] or continuously [B/B]) produced significantly more mineralization than vehicle-treated cultures at 21 days of culture. For the cell cultures from older patients’ bones, only continuous administration of both factors (FB/FB) produced significantly more mineralization than vehicle or any of the other treatment groups. In MSCs from young human bones, continuous FGF-2 treatment (F/F) did not significantly increase calcium content more than F/V or V/V
KUHN ET AL. 

1176

(Figure 5B); however, in cells from old patients, continuous FGF-2 had greater calcium content than F/V and was comparable to V/B and B/B (Figure 5C), but there was no positive XO staining. In addition, light micrographs showed that the cells were not confluent and individual cells had poor intracellular morphology with many vacuoles, suggesting that the human cells were undergoing apoptosis after continuous FGF-2 administration (data not shown). Cells derived from younger patients had higher calcium content in the BMP-2-treated groups, than cells from older patients. In fact, in cells from older patients, there were no significant increases in calcium content after BMP-2 treatment compared with vehicle, but the application of continuous FGF-2 in combination with BMP-2 (FB/FB) significantly increased calcium content, more than the sequential combination (F/B). Visually, mineralized nodule formation in the young and old human cell cultures was more sporadic and smaller (Figure 5) than in mouse cell cultures (Figure 1).

**Discussion**

Our studies demonstrate that low doses of FGF-2 (0.016 ng/mL) can effectively increase in vitro mineralization of BMP-2-stimulated cells derived from elderly mouse and human bones. This dose is hundreds of times less than the doses used previously by others when investigating the possibility of FGF-2 enhancement of BMP-2-stimulated mineralization (25,26,28,31). That BMP-2 activity can be enhanced by these low doses of FGF-2 is an important finding given the complications of high doses of BMP-2 (9,10) and that increasing the dose of BMP-2 has previously been the accepted means for overcoming reduced activity of BMP-2 in elderly patients (11).

Both calvaria-derived and long bone–derived mesenchymal progenitor cell cultures from mice were tested, and bone site–dependent growth factor responsiveness, as well as the expected age-dependent responsiveness, was demonstrated. Most mouse osteoblast cell cultures are derived from the calvaria, and our study demonstrates that osteoblasts taken from mouse long bones differ in their ability to form bone and to respond to growth factors. It is particularly important to study the response of osteoblasts from long bone if we are to compare human osteoblast responses because osteoblasts are isolated from the bone of the appendicular skeleton in humans and not from human calvaria. These primary cell cultures from mouse and human have been previously shown to contain more than 95% osteoblasts (3). In addition, most injuries and orthopedic injuries are performed on the bone of the appendicular skeleton. BMP-2 treatment of cells from the calvaria of young mice in the presence or absence of FGF-2 produced comparable mineralization. However, in calvarial osteoblast cultures from old mice,
the simultaneous administration of both FGF-2 and BMP-2 gave the greatest mineralization than BMP-2 alone. Osteoblast cultures from young long bones responded in a similar manner as old calvaria but sequential delivery of FGF-2 and BMP-2 also stimulated calcification more than BMP-2 alone. In older long bone cultures, only codelivery of FGF-2 and BMP-2 produced more mineralized matrix compared with other groups including vehicle. In fact, BMP-2 administration alone did not enhance mineralization significantly. Therefore, the mouse studies demonstrate that osteoblast cultures from older mice form more bone with the simultaneous administration of both growth factors than BMP-2 alone, suggesting that FGF-2 is needed for a maximal bone anabolic effects during aging, whereas this FGF-2 dependency is not as critical for bone cells from younger animals. These results can be partially explained by the age-dependent, stimulatory effect that FGF-2 has on osteoblast progenitor proliferation (22) that is not seen in cell cultures from younger animals. This finding also explains why there is less mineralization in the cell cultures from older mice and humans compared with osteoblast cultures from younger mouse or human osteoblast cultures.

Identifying the optimal doses and delivery profiles of two growth factors can be daunting particularly when including age as a variable. Therefore, we used a unique and efficient biological tool that relies on using primary cells from transgenic mice that contain a built-in fluorescent reporter for mature osteoblasts. Because the production of fluorescence is nondestructive to the cells, we could efficiently screen for proliferative and differentiation effects in the same cultures, at all time points, using image analysis. Importantly, we are able to confirm colocalization of collagen type I expression with mineral deposition in the growth factor–stimulated cultures. This technique avoids identifying dystrophic mineralization that can occur in dying cell cultures as a positive response to a growth factor. Various doses of FGF-2 and BMP-2 were tested in cells from neonatal transgenic mice to determine the optimal concentration for mouse osteoblast differentiation assayed by fluorescently tagged Collal promoter transgenes, and mineralization, by both staining for calcified nodules and measuring calcium content. Both FGF-2 and BMP-2 were shown to stimulate osteoblast differentiation and mineralization at low concentrations of 0.016 ng/mL of FGF-2 and 5–10 ng/mL of BMP-2. Higher concentrations

Figure 4. The effect of vehicle (V) and combinations of 0.016 ng/mL fibroblast growth factor-2 (FGF-2, F; Low) or 0.16 ng/mL FGF-2 (F, Hi) and 5 ng/mL of bone morphogenetic protein-2 (BMP-2; B, Low) or 50 ng/mL (B, Hi) on mineralization of mouse calvarial cells from 5-day-old neonatal Col2.3GFP reporter mice at 21 days of culture. (A) Fluorescence micrographs of calvarial progenitor cell cultures after treatment with various dose combinations of FGF-2 and BMP-2 and staining with xylene orange (XO) to identify mineral. (B) Percent culture area of Col2.3GFP-positive cells quantified by image analysis. (C) Percent culture area of positive XO-stained mineralized nodules and (D) calcium content. Low concentrations (Low) of FGF-2 and BMP-2 increased osteoblast differentiation and mineralization when administered sequentially rather than in combination, as seen by increased percent GFP-positive cells (A and B) and percent XO-stained mineralized nodules (A and C) and calcium content (D), significantly more than vehicle or higher doses of FGF-2 and BMP-2. Higher doses of FGF-2 and BMP-2 decreased differentiation and mineralization compared with vehicle. Scale bar = 1,000 μm. *p < .05 compared with vehicle treatment.
Figure 5. The effect of vehicle (V) and combinations of 0.016 ng/mL fibroblast growth factor-2 (FGF-2, F) and 5 ng/mL of bone morphogenetic protein-2 (BMP-2; B) on mineralization of human cells cultured from bone chips of young (29-year-old) and old (59-year-old) human patients. This figure shows a representative experiment from three experiments with separate patients. (A) Phase contrast micrographs and fluorescence micrographs of xylenol orange (XO)-stained cultures at Day 21. (B) Calcium content for young human bone chip outgrowth cultures as a function of BMP-2 and FGF-2 dose combinations. (C) Calcium content for old human bone cultures. In cell cultures from young patients, BMP-2 alone (either V/B or B/B) produced significantly more calcification than vehicle-treated cultures and FGF-2 did not increase mineralization over that of BMP-2 alone. In osteoblast cultures from older patients, the addition of FGF-2 to BMP-2 (FB/FB) throughout the culture time period was necessary to significantly increase mineralization above vehicle. Cells from younger patient responded to both growth factors more than cells from older patients. Scale bar = 20 μm. *p < .05 compared with vehicle treatment.
of either growth factor inhibited these processes. This opposite effect of low and high concentrations of FGF-2 has been shown by other investigators (12,13,18–21). Our laboratory has previously shown an inhibition of osteoblast differentiation markers and mineralization in primary murine marrow stromal osteoblast cultures in which the Col3.6 and Col2.3 constructs were utilized with continuous FGF-2 (38). In a mouse osteoblastlike cell line, MC3T3-E1, collagen synthesis, critical for bone formation, was inhibited by continuous FGF-2 (39); therefore, we did not fully evaluate continuous FGF-2 treatment in all groups of this study. Although the sequential dosing profile was most effective in the neonatal mice, it was continuous dosing of both factors that was most effective in the adult and elderly cultures of the mouse and human bone chip studies. This further emphasizes the importance of completing studies in appropriately aged model systems.

A few in vivo studies have investigated the combined actions of FGF-2 and BMP-2 on bone formation and have demonstrated synergistic bone-enhancing effects depending on the doses used in young animals (40,41). For example, the concurrent codelivery of FGF-2 and BMP-2 from a collagen sponge in the muscle of young mice caused a modest, but significant increase in ectopic bone growth, compared with treatment with either growth factor individually (41). Too much FGF-2, however, reduced the amount of bone formed in those studies. As an alternative to direct growth factor delivery in vivo, other strategies for synergistic use of FGF-2 with BMP-2 have also been demonstrated. When rabbit MSCs were precultured with FGF-2, BMP-2, and dexamethsone for 2 weeks on porous hydroxyapatite particles and then transplanted into rabbits to assess spinal fusion, more adult rabbits had successful spinal fusion on radiographic analysis than those animals with each growth factor alone (42). Our studies suggest that the addition of low doses of FGF-2 to BMP-2 in elderly animals and administered in a continuous fashion may prove even more stimulatory than the results in the young adult animals used in these previous studies would suggest. As seen in the present studies, it is important to complete studies in truly aged animals to make a definitive conclusion regarding synergistic effects of FGF-2 to BMP-2. Our studies were completed in 18- to 22-month-old mice as per the suggestions from the National Institute on Aging rodent colony for aging research guidelines because they represent a truly elderly animal model.

Our work is novel in that both mouse and human MSC cultures have been compared for their responses to combinations of FGF-2 and BMP-2, in particular for stimulating mineralization. It is critical to understand species-specific biological responses to develop appropriate models for the study of human diseases and biological responses. Surprisingly, there is very little literature on comparison of the responses of mouse and human cell cultures. We assume that their biological responses are similar probably due to the fact that many antibodies to mouse proteins recognize human proteins, and transgenic and gene knockout mice often simulate human diseases. However, side-by-side studies of human and mouse cells are rarely found. From our studies, MSCs from young mice and humans, respond well to BMP-2 by demonstrating significantly increased mineralization compared with vehicle-treated cultures. This is also true for MSCs from the calvaria of old mice. However, only simultaneous administration of FGF-2 and BMP-2 to MSCs from old mouse long bones and old patients was required to stimulate mineralized matrix formation above vehicle-treated cultures; in general, these studies do not demonstrate a significant stimulatory effect of BMP-2 alone on MSC differentiation and mineralization compared with vehicle-treated cultures.

Our cell studies have shown site- and age-dependent effects of FGF-2 and BMP-2 on mineralization and have provided us with a framework in which to complete mechanistic studies and to start in vivo studies to enhance bone formation in old animals. Codelivery of low doses of FGF-2 and BMP-2 appears to be the most osteoinductive and osteogenic strategy for bone formation in aging organisms. The development of biomaterials that can deliver these low doses of growth factors for a prolonged period of time during bone formation will be a future direction of this research.

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