Journal of Cellular Biochemistry 103:1975–1988 (2008)

Reduced Expression and Function of Bone Morphogenetic Protein-2 in Bones of Fgf2 Null Mice

Takahiro Naganawa,[1] Liping Xiao,[1] J.D. Coffin,[2] Thomas Doetschman,[3] Maria Giovanna Sabbieti,[4] Dimitrios Agas,[4] and Marja M. Hurley[1]*

[1]Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut
[2]Department of Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, Montana
[3]BIO5 Institute and Department of Cell Biology and Anatomy, University of Arizona, Tucson, Arizona
[4]Department of Comparative Morphology and Biochemistry, University of Camerino, I-62032 Camerino (MC), Italy

Abstract Disruption of the fibroblast growth factor 2 (FGF-2) gene results in reduced bone mass in mice and impairs expression of bone morphogenic protein-2 (BMP-2) an important mediator of osteoblast and osteoclast differentiation. Since the relationship between FGF-2 and BMP-2 in bone remodeling has not been fully determined, in this study we examined whether endogenous FGF-2 was necessary for maximal effect of BMP-2 on periosteal bone formation in vivo and bone nodule formation and osteoclast formation in vitro in Fgf2−/− mice. We showed that BMP-2 significantly increased periosteal bone formation by 57% in Fgf2+/+ mice but the changes were not significant in Fgf2−/− littermates. In line with these results we found no significant increase in alkaline phosphatase positive (ALP) activity in calvarial osteoblasts or ALP mineralized colonies in stromal cultures from Fgf2−/− mice after BMP-2 treatment. Moreover, BMP-2 induced osteoclast formation was also impaired in marrow stromal cultures from Fgf2−/− mice. Interestingly, BMP-2 induced nuclear accumulation of the runt related transcription factor (Runx2) was markedly impaired in osteoblasts from Fgf2−/− mice. Examination of the effect of loss of FGF-2 on BMP-2 signaling pathway showed that BMP-2 caused a similar induction of phospho-Smad1/5/8 within 30 min in calvarial osteoblasts from both genotypes. In contrast BMP-2-induced p42/44 MAPK was reduced in Fgf2−/− mice. These findings strongly demonstrated that endogenous FGF-2 is important in the maximal responses of BMP-2 in bone and that this may be dependent on the p42/44 MAPK signaling pathway and downstream modulation of Runx2. J. Cell. Biochem. 103: 1975–1988, 2008.

Key words: BMP-2; Fgf2 null mice; osteoblast; MAPKinase

Members of the family of fibroblast growth factors and their receptors play important roles in skeletal development and bone homoeostasis [Gospodarowicz, 1990; Xu et al., 1999; Hurley et al., 2002]. Fibroblast growth factor (FGF-2) is expressed in osteoblasts and is stored in the extracellular matrix (ECM) [Gospodarowicz, 1990; Hurley et al., 2002]. FGF-2 stimulates osteoblast replication [Globus et al., 1998; Hurley et al., 2002] and has both inhibitory [McCarthy et al., 1989; Rodan et al., 1989; Hurley et al., 1993], and stimulatory effects on bone formation in vitro [Canalis et al., 1988] and in vivo [Mayahara et al., 1993; Nagai et al., 1995; Nakamura et al., 1995, 1998; Dunstan et al., 1999; Liang et al., 1999]. In addition, bone marrow stromal cells synthesize [Brunner et al., 1991] and responds to FGF-2 in an autocrine/paracrine manner [Oliver et al., 1990; Thomson et al., 1993; Locklin et al., 1995]. Previously, we reported that mice with disruption of the FGF-2 gene developed osteopenia with aging [Montero et al., 2000] demonstrating that FGF-2 is an important regulator of bone formation in vivo. In vitro studies also demonstrated that FGF-2 mutant mice had impaired osteoclast formation in response to parathyroid hormone [Okada et al., 2003].

FGF-2 signals through tyrosine kinase fibroblast growth factor receptors (FGFRs) that are

Grant sponsor: NIH; Grant number: AG021189.

*Correspondence to: Marja M. Hurley, MD, Division of Endocrinology & Metabolism, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06030. E-mail: hurley@exchange.uchc.edu

Received 10 July 2007; Accepted 30 August 2007
DOI 10.1002/jcb.21589

also important regulators of skeletal development [Xu et al., 1999; Hurley et al., 2002]. FGF-2/FGFRs activate several intracellular signaling pathways including mitogen activated protein kinases (MAPK), shown to be important in cell proliferation and initiation of differentiation [Hurley et al., 2002; Spector et al., 2004].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGFβ) super family that are major modulators of osteogenesis [Urist, 1965; Reddi and Huggins, 1972; Wozney, 1992]. Similar to FGF-2, BMPs play an important role in the commitment and differentiation of osteoprogenitors [Yamaguchi, 1995]. BMPs are synthesized, released by osteoblasts and stored in the bone matrix [Yamaguchi, 1995; Rosen and Wozney, 2002]. Since BMP-2 is a key regulator of osteoblast precursor differentiation [Yamaguchi, 1995], first we assessed whether BMP-2 regulated FGF-2 gene expression, then we examined whether endogenous FGF-2 was important in the maximal effects of BMP-2 in bone.

Studies have shown that Runt-related transcription factor 2 (Runx2) also known as cbfa1/pebp2aA/AML3, plays an important role in osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997]. Mice with disruption of Runx2 gene fail to develop mineralized bone [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997], while haploinsufficient mice develop cleidocranial dysplasia, which is characterized by delayed ossification [Mundlos et al., 1997; Otto et al., 1997]. Several studies showed that Runx2 regulated the expression of genes associated with osteoblast differentiation [Banergee et al., 1997; Ducy et al., 1997; Ji et al., 1998]. Of relevance to the present report, we previously reported that intermittent FGF-2 treatment increased Runx2 mRNA in osteoblast/stromal cells [Zhang et al., 2002] and that Runx2 mRNA expression is impaired in osteoblasts from Fgf2+/− and Fgf2−/− mice, Naganawa et al. [2006]. In addition, other studies showed that Runx2 protein is phosphorylated and activated by FGF2; [Xiao et al., 2002; Kim et al., 2003] and that induction of the osteocalcin gene by FGF-2 requires activation of MEK/ERK MAP kinase pathway as well as Runx2 phosphorylation. Since Runx2, as a transcription factor, act in the nucleus, we assessed whether BMP-2 induced nuclear accumulation of Runx2 was impaired in Fgf2−/− osteoblasts.

Although the major signaling pathway for BMP-2 action is via activation of SMADs, BMPs also activate the MAPK signaling pathway [Rosen and Wozney, 2002]. Both the p38MAPK and extracellular signal regulated kinases p44/42 were reported to be important in BMP-2 induced osteoblast differentiation [Kuo et al., 2005]. We therefore examined the role of modulation of SMAD/ MAPK signaling pathways in the impaired responses to BMP-2 in Fgf2−/− osteoblasts.

MATERIALS AND METHODS

Materials

Recombinant Human (rh)BMP-2 was purchased from R&D systems (Minneapolis, MN) and rhFGF-2 was a gift from Dr. Judith Abraham (Chiron Corp., CA). Anti-Phospho-ERK, anti-ERK, anti-Phospho-Smad1/5/8, and anti-phospho-p38 MAPKinase antibodies were purchased from (Cell Signaling Technology, Inc., Beverly, MA). Anti-Smad 1/5/8 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

The development of Fgf2 null mice was previously described [Zhou et al., 1998]. Heterozygote male and female Fgf2+/− mice that are maintained on a Black/Swiss/129 Sv background were bred and housed in the transgenic facility in the Center for Laboratory Animal Care at the University of Connecticut Health Center. Genotyping of littermate mice was performed using primers as previously described [Zhou et al., 1998]. Mice were sacrificed by CO_2 narcosis and cervical dislocation. The Institutional Animal Care and Use Committee of the University of Connecticut Health Center approved all animal protocols.

In Vivo Bone Formation Assay

Fgf2+/+ and Fgf2−/− male littermate mice at 3 months of age were weighed and injected s.c. over the calvariae with vehicle (20 μl PBS containing 0.1% BSA) or BMP-2 (20 μg/kg/day × 5 days) [Chen et al., 1997]. To analyze the changes in bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/day), mice were injected with calcein (0.6 mg/kg) on days 7 and 12 and were sacrificed on day 14. Calvariae were dissected

free of tissue and fixed in 70% ethanol at the time of sacrifice. The bones were dehydrated in increasing concentrations of ethanol, cleared in xylene, and embedded undecalcified in methyl methacrylate. Five-micron-thick longitudinal serial sections were cut on a Reichert-Jung Polycut S microtome (Reichert-Jung, Germany) with a D profile knife (Delaware Diamond Knives Corp., Wilmington, DE).

Primary Calvarial Osteoblast Cell Cultures

Primary calvarial osteoblasts were isolated from newborn (d1–d3) *Fgf2*+/+ and *Fgf2*−/− mice as previously described [Montero et al., 2000]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Alkaline Phosphatase Activity (ALP) and Stains

Calvarial osteoblasts were plated in 6-well plates and cultured for 7 days in differentiation media (DMEM, 10% FCS, 8 mM β-glycerophosphate, 50 mg/ml ascorbic acid) in the presence or absence of effectors. ALP activity in the lysate was measured by the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol. Absorbance was determined at 405 nm and compared with a *p*-nitrophenol (Sigma) standard titration curve. ALP activity was normalized to total proteins measured with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). ALP staining was performed with a commercial kit (Sigma).

Bone Marrow Stromal Cell Cultures

Stromal cells were isolated from bone marrow of 3-month-old Fgf2+/+ and Fgf2−/− mice as previously described [Montero et al., 2000]. Cells were plated at 2×10^6 or 10×10^6 cells/well, in 6-multiwell plates in αMEM containing 10% FCS; cultures were fed every 3 days with fresh differentiation media. Cells were fixed and stained for ALP positive colonies using a commercially available kit (Sigma). Dishes were scanned and then counterstained for mineral with Von Kossa reagent as previously described. Colony number and colony area was determined by NIH Image (version 1.61; National Institutes of Health, Bethesda, MD).

RNA Isolation and Northern Blotting

Total RNA was extracted from whole calvariae or cultured calvarial osteoblasts using TRizol-reagent (Invitrogen Life Technologies, Carlsbad, CA) as previously described [Okada et al., 2003]. For Northern analysis, 20 μg of total RNA was denatured and fractionated on 0.8% agarose/1.1 M formaldehyde gel, transferred to nylon membrane by positive pressure, and fixed to the filter by UV irradiation. After 4-h prehybridization, filters ware hybridized over night with a random primer deoxycytosine triphosphate (dCTP)-labeled-32p-cDNA probe for the mRNA of interest. Signals were quantitated by densitometry and normalized to the corresponding value for GAPDH.

Osteoclast Formation

Tibiae and femurs from 8- to 12-week-old littermate *Fgf2*+/+ and *Fgf2*−/− mice were dissected free of adhering tissue. Mouse bone marrow cells were isolated by a modification of previously published methods [Hurley et al., 1998]; and cells were plated in 24-multiwell plates (1×10^6 cells/well) in αMEM containing 10% FCS. Cultures were fed every 3 days with fresh medium. Effectors were added at the beginning of the culture and with each medium change. Cells for tartrate resistant acid phosphatase (TRAP) staining were fixed on days 5 and 6 of culture. TRAP staining was performed with a commercial kit (Sigma). Osteoclasts (OCL) were defined as TRAP positive multinucleated cells that contained greater than 3 nuclei.

Protein Isolation and Western Blotting

Total and phosphorylated Smad, total and P42/44 MAPK, total and p38 MAPK were determined by Western blotting [Xiao et al., 2004]. Calvarial osteoblasts were harvested in lysis buffer (Cell Signaling). Protein concentration of the cell lysates was measured using BCA protein assay (PIERCE, Rockford, IL). Protein samples were separated on SDS-PAGE (Ready Precast Gel, Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF membrane (Bio-Rad). Membranes were blocked at room temperature for 1 h in buffer containing (100 mM Tris 7.5, 100 mM NaCl, 0.1% polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma), and 5% nonfat dry milk (Bio-Rad) and incubated

with primary antibody overnight at 4C. Membranes were washed for 30 min with TBS-T then incubated with appropriate peroxidase-linked species-specific antibody at R/T for 1 h. Immunoreactive proteins were visualized with the Enhanced Chemiluminescence (ECL) system (Amersham Pharmacia Biotech) following the manufacturer's instructions. Band Density was quantified by densitometry.

Cell Cultures and Immunofluorescence

Fgf2−/− and Fgf2+/+ calvarial osteoblasts were plated at 5,000 cells/cm^{2,} on coverslips previously cleaned and sterilized, and grown for 4 days in DMEM medium containing 10% heat-inactivated FCS, penicillin and streptomycin. When at least 80% of confluence was reached, cells were serum deprived for 24 h and treated with BMP-2 (50 ng/ml) and FGF-2 (10 nM). Control cultures were pulsed with only vehicle. Then, cells were briefly rinsed with PBS 0.1 M, pH 7.4, and fixed in 4% PFA diluted in PBS for 25 min at room temperature. Cells were washed three times in PBS, permeabilized with 0.3% Triton X-100 for 30 min and incubated with 0.5% BSA diluted in PBS for 20 min at room temperature. Finally, cells were incubated with a rabbit anti-Pebp2aA/Runx2 (Santa Cruz Biotechnology) diluted 1:30 in PBS for 2 h at room temperature. After rinsing, cells were incubated with chicken anti-rabbit IgG Alexa Fluor 488 conjugated (Molecular Probe) for 1 h at room temperature. After washing, coverslips were mounted on slides with PBS/glycerol (1:1). Slides were imaged using fluorescent microscopy on a Zeiss Axioplan microscopy. Reaction controls were performed using a non-immune rabbit immunoglobulin IgG, by complexing the primary antibody with a relative blocking peptide or by omitting the primary antibody.

CMV/Fgf2/ires/eGFP Retroviral Vector Construction and Cell Infection

CMV/ires/eGFP (pMg1cla) vector containing the enhanced GFP (eGFP) gene driven from an internal cytomegalovirus (CMV) promoter was previously described [Dong et al., 2004]. The coding sequence of the Fgf2 was cloned into the *Eco*RI site of the CMV/ires/eGFP retroviral vector. eGFP was used to visualize the expression of protein mediated by CMV vectors in cells. Control virus was CMV/ires/eGFP vector without inserted Fgf2 cDNA. 293GPG packaging cell lines were infected by CMV/ires/eGFP or CMV/Fgf2/ires/eGFP vector using the Lipofectmine^{TM} 2000 Reagent (Invitrogen Life Technologies) following product protocol. Infected 293GPG packaging cell lines were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD) containing 10% heat-inactivated fetal bovine serum (HIFBS), 1 mg/ml of puromycin, 1 mg/ml of tetracycline and 0.4 mg/ml of G418. To produce vector virus, tetracycline and G418 were removed from the media, then conditioned media were collected after 24, 48, 72, and 96 h, filtered through a 0.2 mm Nalgene filter. Condition media was measured for reverse transcript activity by measurement of virus production and then frozen at −80°C.

Transduction of Mouse Primary Calvarial Ostoblasts

Primary calvarial osteoblasts from Fgf2−/− mice were plated in 6-well dishes and grown in DMEM (Sigma–Aldrich) containing 10% HIFBS plus 100 U/ml of penicillin and 100 mg/ml of streptomycin. Cells were transduced with virus when 50% confluent. Media including 5 ml of virus-containing media, 5 ml of DMEM containing 10% FBS and 8 mg/ml of protamine sulfate was added to the cell cultures for approximately 16 h, followed by fresh media replacement during 8 h. The cells were exposed to three cycles of virus infection. Expression of GFP was examined by a Zeiss Axioplan fluorescent microscopy.

Statistical Analysis

An unpaired two-tailed Student's *t*-test was used to test for significant differences between two groups with a minimum threshold of $P < 0.05$. Comparison among multiple groups were conducted using analysis of variance (ANOVA), and the significant differences were determined using the Bonferroni test with $P < 0.05$ (StatView 4.1J Abacus Concepts, Inc., Berkely, CA).

RESULTS

Reduced Expression of BMP-2 mRNA in Calvariae From Fgf2−/− and Fgf2+/− Mice

We compared the expression of BMP-2 mRNA in whole calvariae excised from 3-month-old Fgf2+/+, Fgf2−/− and Fgf2+/− mice. As shown in Figure 1a, there was a marked reduction in BMP-2 mRNA in calvariae from Fgf2−/− and

Fgf2+/− mice compared with Fgf2+/+ littermates. The result of pooled data for BMP-2/GAPDH mRNA ratio from three experiments is shown in Figure 1b.

BMP-2 Increases FGF-2 mRNA and Protein in Osteoblasts From Fgf2+/+ Mice

Primary osteoblast-enriched cells isolated from neonatal Fgf2+/+ mice were cultured for 7 days serum deprived for 24 h and treated with vehicle or rhBMP-2. Time course studies showed that BMP-2 (50 ng/ml) caused a maximal increase in FGF-2 mRNA at 3 h that was sustained for 24 h (Fig. 2a). Pooled results from three independent experiments (Fig. 2b) showed that BMP-2 treatment caused a maximal increase in FGF2 mRNA between 3 and 6 h. To assess whether new protein synthesis was necessary for the stimulatory effect of rhBMP-2 on FGF2 mRNA, cultures were pretreated with the protein synthesis inhibitor, cyclohexhimide (CHX 10 μg/ml, Sigma). As shown in Figure 2c, pretreatment with CHX did not block the stimulatory effect of BMP-2 on FGF-2 mRNA expression at 3 or 24 h. We, also, assessed whether BMP-2-induced increase in FGF-2 mRNA resulted in increased FGF-2 protein. As shown in Figure 2d, treatment with BMP-2 (50 ng/ml) from 3 to 48 h caused a 2-fold increase in FGF-2 protein at 3 h with a maximum 2.4-fold increase at 6 h.

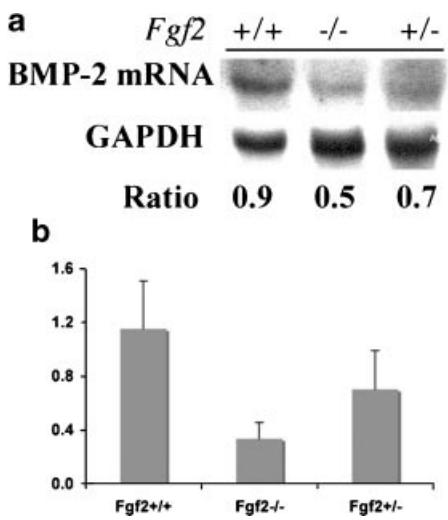

Fig. 1. **a**: Representative Northern Blot of BMP-2 mRNA expression in calvariae from 3-month-old Fgf2+/+, Fgf2−/−, and Fgf2+/− mice. Calvariae were isolated from all three genotypes and total RNA was extracted for Northern blot analysis as described in Materials and Methods Section. Filters were probed for BMP-2 mRNA and results were normalized to GAPDH ratio. **b**: BMP-2/GAPDH ratio represents pooled results from three independent experiments.

BMP-2-Induced Periosteal Bone Formation is Reduced in Fgf2−/− Mice

We examined in vivo periosteal bone formation in 3-month-old Fgf2+/+ and Fgf2−/− male mice that were treated with vehicle or BMP-2 (20 μg/kg body wt) for 5 days. There were no significant differences in body wt pre- and post-BMP-2 treatment in either genotype. As shown in Figure 3a, rhBMP-2 caused a greater increase in calvarial width in Fgf2+/+ mice. Quantitatative analysis (Fig. 3b) shows that rhBMP-2 significantly increased calvarial width by 13% ($P < 0.05$) in Fgf2+/+ but caused only a 5% increase in Fgf2−/− mice that was not significant. As shown in Figure 3c, periosteal bone formation rate (BFR/BS) was increased by 57% ($P < 0.05$) in Fgf2+/+ mice but the changes were smaller 15% and not significant in Fgf2−/− mice.

Impaired Ability of BMP-2 to Increase ALP Positive Colonies and ALP Activity in Calvarial Osteoblast From Neonatal Fgf2−/− Mice and Marrow Stromal Cells From Adult Fgf2−/− Mice

Based on our in vivo observation of reduced periosteal bone formation in response to BMP-2 in Fgf2−/− mice, we determined whether bone nodule formation was reduced in calvarial osteoblasts cultures from neonatal mice. As shown in Figure 4a, after 7 days of culture, there were fewer ALP positive colonies in vehicle treated Fgf2−/− osteoblasts. BMP-2 caused a greater increase in ALP colonies in Fgf2+/+ osteoblasts cultures. Similarly, ALP activity was reduced by 44% in vehicle treated Fgf2−/− calvarial osteoblasts cultures. BMP-2 caused a 90% increase in ALP activity in Fgf2+/+ cultures but only a 50% increase in Fgf2−/− calvarial osteoblasts cultures (Fig. 4b).

We also determined if BMP-2 induced bone formation was reduced in adult Fgf2−/− mice using marrow stromal cultures from 3-month-old Fgf2+/+ and Fgf2−/− male mice. We observed that there was a strong increase in ALP positive colonies in all groups containing Fgf2+/+ cells at 14 and 21 days. At 21 days, BMP-2 increased colony area fivefold in Fgf2+/+ cultures compared with vehicle (16.6 ± 4.9 vs. 3.2 ± 2.6), but had no effect in Fgf2−/− cultures (1.1 ± 0.4 vs. 0.0 ± 0.0). In contrast, FGF-2 increased colony area by 34-fold

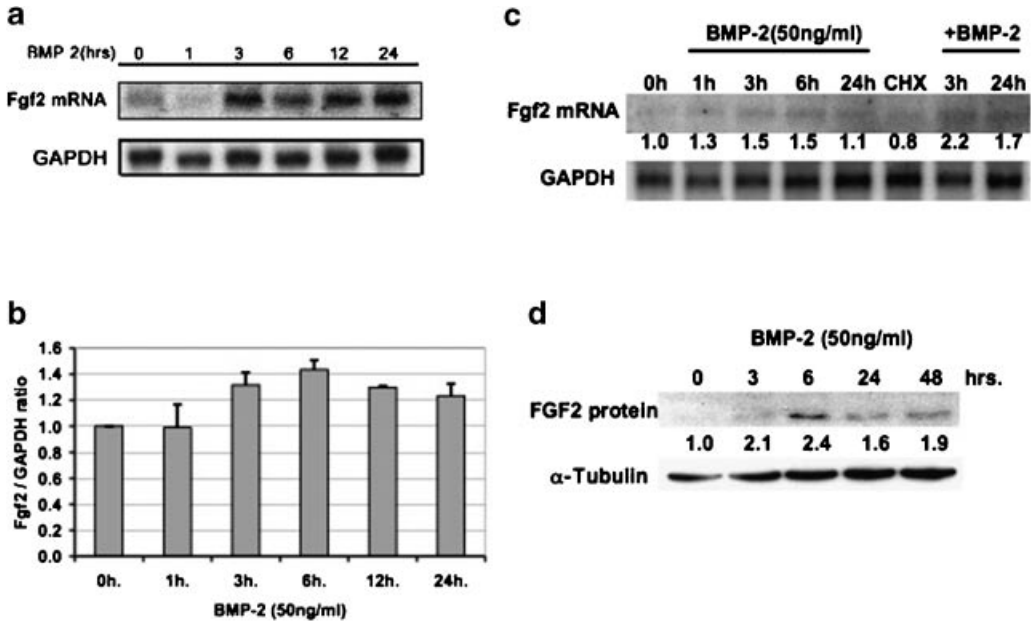

Fig. 2. Effect of BMP-2 on FGF-2 mRNA and protein expression in neonatal calvarial osteoblastic cells. Cells isolated from calvariae of 4-day-old Fgf2+/+ mice were cultured for 7 days serum deprived and treated with vehicle or rhBMP-2 (50 ng/ml) for the indicated times. Total RNA was extracted for Northern blot analysis or protein for Western blot analysis as described in Materials and Methods Section. Filters were probed for FGF-2 mRNA and results were normalized to GAPDH ratio. **a**: Representative time course of the effect of BMP-2 on FGF-2 mRNA. **b**: FGF-2/GAPDH ratio represents pooled results from three independent experiments. **c**: Demonstrates that pretreatment with CHX does not abrogate BMP-2 induced increase in FGF-2 mRNA. **d**: Western blot of cell lysates shows that BMP-2 increases FGF-2 protein.

(102+14.4) and 134-fold (134.4 + 7.5) in Fgf2+/+ and Fgf2−/− cultures, respectively. There was no additive or synergistic effect of the combination of FGF-2 plus BMP-2 in either genotype (data not shown).

BMP-2-Induced Osteoclast Formation is Impaired in Marrow Stromal Cultures From Fgf2−/− Mice

Since BMP-2 also increases the formation of osteoclasts (OCL), we examined its effect on OCL formation in *Fgf2*+/+ and *Fgf2*−/− marrow cultures. As shown in Figure 5a, few OCLs formed in vehicle treated marrow cultures from either genotype. BMP-2 (50 ng/ml) increased the number of TRAP positive OCL that formed per well in cultures from *Fgf2*+/+ mice at 5 and 6 days. In contrast, there were 50% fewer OCLs formed in response to BMP-2 in marrow cultures from Fgf2−/− mice. Quantitative results are shown in Figure 5b.

We further examined the effect of BMP-2 (50 ng/ml) singly or in combination with FGF-2 (10 nM). As shown in Figure 6, in the presence of FGF-2 the number of OCL in the cultures from the *Fgf2*−/− mice were similar to that observed in marrow cultures from *Fgf2*+/+ mice treated with BMP-2 alone. Interestingly, simultaneous treatment of marrow cultures with FGF-2 plus BMP-2 caused an additive effect in OCL formation in both Fgf2+/+ and Fgf2−/− cultures.

BMP2 Does Not Increase Runx2 Accumulation and Nuclear Localization in Fgf2−/− Osteoblasts

We examined the effect of 24 h treatment with BMP-2 (100 ng/ml) and FGF-2 (10 nM) on Runx2 protein synthesis and localization in cultures of osteoblasts from Fgf2+/+ and Fgf2−/− genotypes. Both BMP-2 and FGF-2 increased Runx2 labeling particularly evident in the nucleus of Fgf2+/+ calvarial osteoblasts (Fig. 7). In contrast, while FGF-2 increased nuclear labeling of Runx2 in Fgf2−/− osteoblasts, BMP-2 treatment did not.

BMP2 Increases Runx2 Accumulation and Nuclear Localization in Fgf2−/− Osteoblasts Transfected With a Retroviral Fgf2 Construct

To further confirm the above data (regarding the involvement of FGF-2 in BMP-2 regulating Runx2 protein accumulation and localization) Fgf2−/− COBs were transfected with vector or

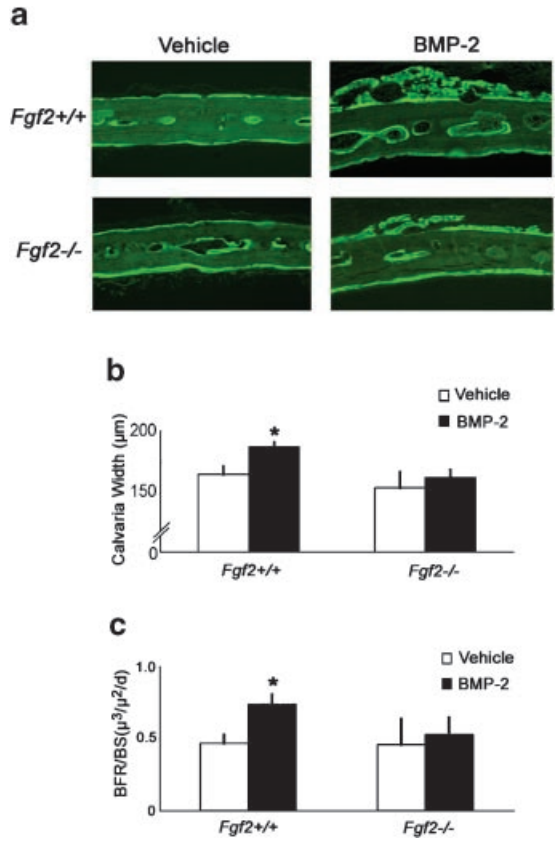

Fig. 3. Effects of BMP-2 injection on calvarial width and periosteal bone formation in Fgf2+/+ and Fgf2−/− mice. RhBMP-2 (20 μg/kg) was injected over the calvariae of 3-month-old male Fgf2+/+ and Fgf2−/− mice (n = 4 mice/group) as described in Materials and Methods Section. **a**: Histomorphometry shows increased calvarial width in BMP-2 treated Fgf2+/+ mice. Quantitative histomorphometry revealed (**b**) significant increased calvarial width and (**c**) periosteal bone formation rate (BFR/BS) in Fgf2+/+ but not Fgf2−/− mice. Results are expressed as the mean ± SEM.*$P < 0.05$ versus vehicle treated Fgf2+/+ mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

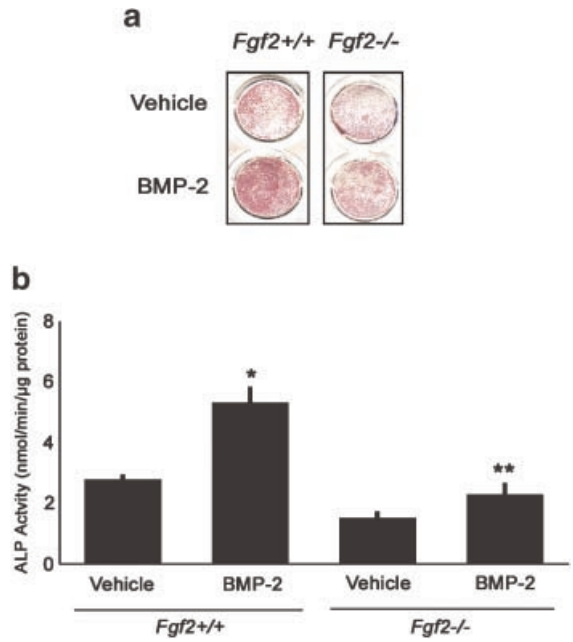

Fig. 4. **a**: Comparison of the ability of BMP-2 to form ALP positive colonies and (**b**) ALP activity in calvarial osteoblasts cultures from Fgf2+/+ and Fgf2−/− mice. Cells were cultured in the absence and presence of BMP-2 (50 ng/ml) in differentiation medium for 14 days as described in Materials and Methods Section. Results shown in panel b are expressed as the mean ± SEM. *$P < 0.01$ versus vehicle treated Fgf2+/+. **$P < 0.001$ versus BMP-2 treated Fgf2+/+. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fgf2 retrovirus. In situ experiments showed GFP labeling in COBs transfected with vector (Fig. 8a) or Fgf2 construct (Fig. 8b). BMP-2 did not increase Runx2 in cells transfected with vector construct (Fig. 8a) but increased Runx2 perinuclear and nuclear labeling in Fgf2−/− cells transfected with the Fgf2 construct (Fig. 8b). FGF-2 treatment increased Runx2 protein in cells transfected with either vector or Fgf2 construct (Fig. 8a,b).

BMP-2 Phosphorylates SMAD1/5/8 But Not p44/42 ERK in Osteoblast From Fgf2−/− Mice

To examine the integrity of BMP-2 signaling pathway, calvarial osteoblasts from Fgf2+/+ and Fgf2−/− mice were serum deprived for 24 h and treated with BMP-2 (50 ng/ml) or vehicle for 10–120 min and proteins were analyzed by Western blot. BMP-2 caused a marked and similar induction of phospho-Smad1/5/8 within 30 min in osteoblasts from both Fgf2+/+ and Fgf2−/− mice (Fig. 9).

Stimulation with BMP-2 (50 ng/ml) for 10 min increased phosphorylated ERK (phospho-p44/42) in Fgf2+/+ but not in Fgf2−/− osteoblasts cultures (Fig. 10a). In contrast, treatment with FGF-2 (10 nM) for 10 min caused a similar increase in phospho-p44/42 in osteoblasts from both genotypes that was still evident at 30 min (Fig. 10b). Figure 10c shows the quantitative densitometry data for phospho-p44/42 from three independent experiments in which osteoblasts from both genotypes were treated for 10 min with either vehicle, BMP-2 or FGF-2. BMP-2 caused a significant increase in phospho-p44/42 in Fgf2+/+ but not Fgf2−/− osteoblasts cultures. In contrast, FGF-2 treatment caused a similar significant increase in phospho-p44/42 in calvarial osteoblasts from both

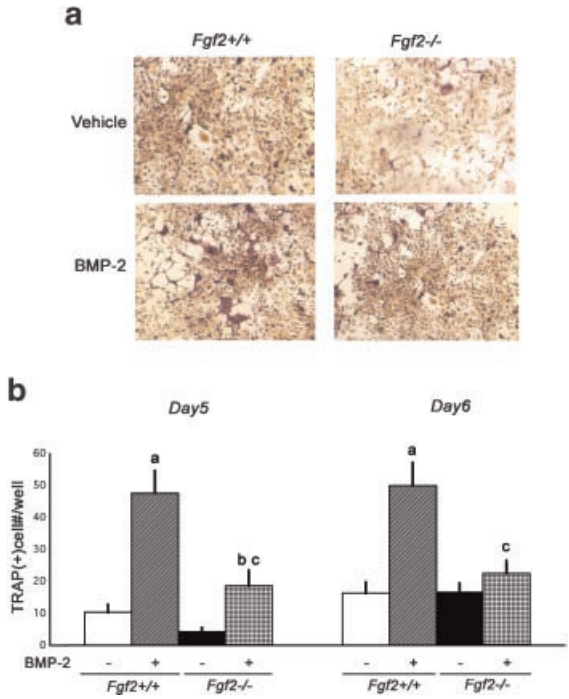

Fig. 5. Effect of BMP-2 on osteoclast formation in marrow cultures from Fgf2+/+ and Fgf2−/− mice. **a**: Representative experiment of TRAP-stained osteoclasts in bone marrow cultures from Fgf2+/+ and Fgf2−/− mice treated with vehicle or BMP-2 for 5 days. **b**: Time course effect of continuous treatment with BMP-2 (50 ng/ml) on osteoclast formation in bone marrow cultures from Fgf2 +/+ and Fgf2−/− mice. Results are expressed as the mean ± SEM. $^{a}P < 0.01$ versus vehicle (Fgf2+/+); $^{b}P < 0.05$ versus vehicle (Fgf2−/−); $^{c}P < 0.01$ versus BMP-2 (Fgf2+/+). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

genotypes. It should be noted that BMP-2 treatment for up to 120 min or treatment with a higher dose of BMP-2 (100 ng/ml) did not rescue the reduced induction of phospho-p44/42 observed in Fgf2−/− cells (data not shown).

We also examined the time course effect of BMP-2 on p-38 MAPK. BMP-2 caused a small but similar increase in p38 MAPK in osteoblasts from both Fgf2+/+ and Fgf2−/− mice (data not shown).

DISCUSSION

The present study extends our characterization of bone remodeling in mice with disruption of Fgf2 gene by examining the changes in endogenous BMP-2 expression and function of exogenous BMP-2 on periosteal bone formation in vivo as well as effects on in vitro osteoblast colony formation and osteoclastogenesis.

Our finding of reduced BMP-2 mRNA in osteoblasts from Fgf2 mutant mice supports a role for endogenous FGF-2 in regulating BMP-2 expression and is consistent with an earlier report that exogenous FGF-2 treatment increased BMP-2 mRNA expression in calvarial osteoblasts [Choi et al., 2005]. However, the present study also shows that BMP-2 increased FGF-2 mRNA and protein in osteoblasts from Fgf2+/+ mice. Thus, there is reciprocal regulation of FGF-2 and BMP-2 in osteoblasts. Interestingly FGF-2 and TGFβ were shown to reciprocally regulate each other's expression in osteoblasts [Hurley et al., 2002]. Such reciprocal regulation is probably of importance in the process of bone remodeling. It should be noted that our finding that BMP-2 regulates FGF-2 expression is in contrast to the earlier study of [Choi et al., 2005], who reported that 48 h of BMP-2 treatment did not increase the expression of FGF-2 mRNA in calvarial osteoblasts. The difference between the two studies could be due in part to the time points that were

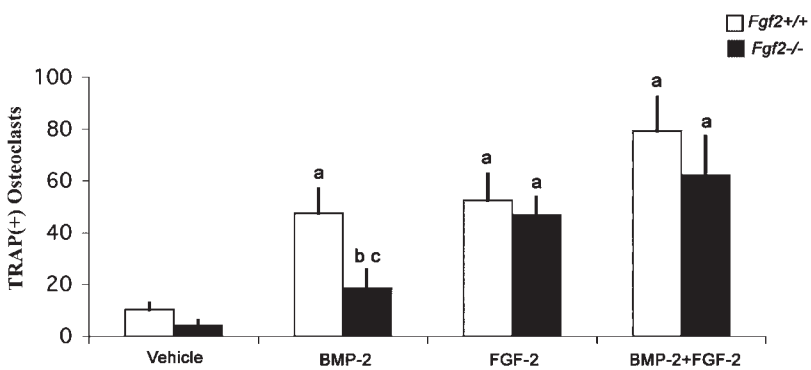

Fig. 6. Effect of continuous treatment with BMP-2 (50 ng/ml) alone or BMP-2 (50 ng/ml) + FGF-2 (10 nM) on osteoclast formation in bone marrow cultures from Fgf2+/+ and Fgf2−/− mice. Results are expressed as the mean ± SEM $^{a}P < 0.01$ versus vehicle. $^{b}P < 0.05$ versus vehicle; $^{c}P < 0.01$ versus BMP-2 (Fgf2+/+).

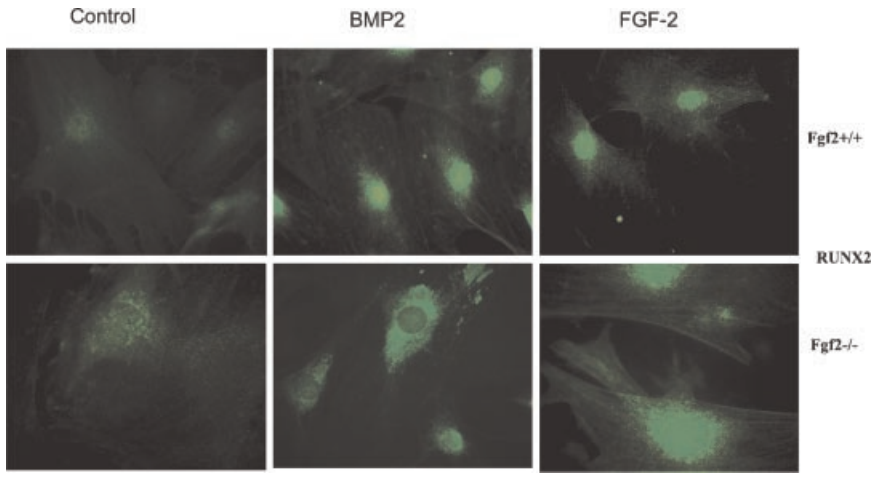

Fig. 7. Effect of BMP-2 and FGF2 on Runx2 localization in calvarial osteoblasts from Fgf2+/+ and Fgf2−/− mice. Localization of Runx2 was analyzed by immunocytochemical study. Micrographs, obtained by a Zeiss Axioplan fluorescent microscopy show cells stained with a polyclonal antibody to Runx2 (green: FITC staining). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

examined. A single time point in the earlier study versus the present report where time course studies were performed showing that BMP-2 increased FGF-2 mRNA within 3 h and FGF-2 protein within 6 h in osteoblasts. In support of our observations that BMP-2 does regulate FGF-2 expression, other investigators showed that BMP-2 treatment of premyoblast-C2C12 cell line, an established model to study osteoblast differentiation [Katagiri et al., 1994];

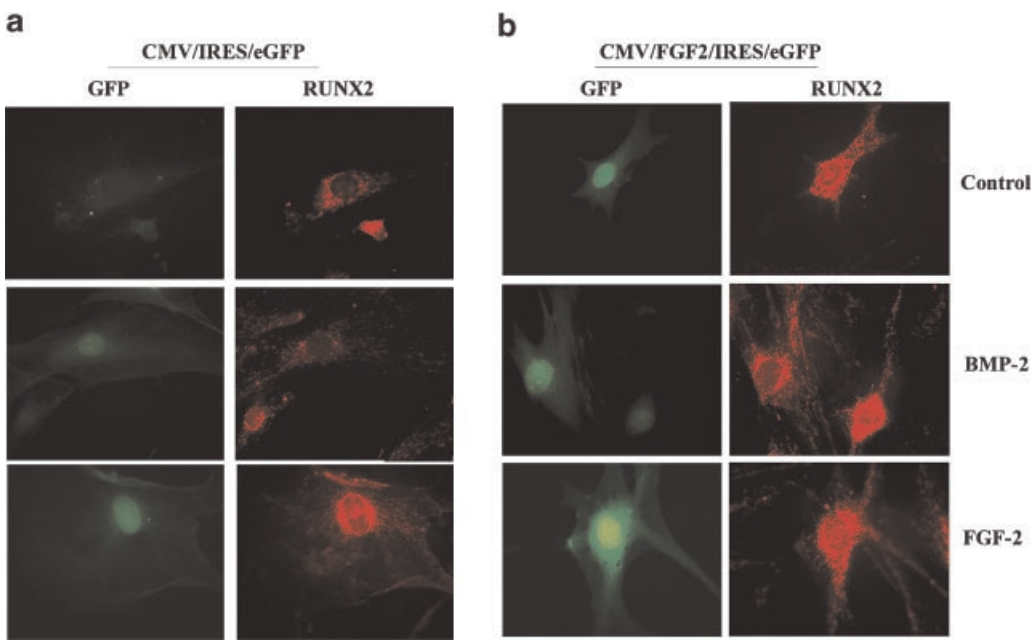

Fig. 8. Effect of BMP-2 and FGF-2 on Runx2 labeling in Fgf2−/− calvarial osteoblasts (COBs) transduced with either the vector or Fgf2 construct. COBs from Fgf2−/− mice were transduced with either CMV/ires/eGFP (Vector) or CMV/Fgf2/ires/eGFP construct containing the enhanced GFP (eGFP) gene used to visualize expression of protein mediated by CMV promoter. **a**: Micrographs, obtained by a Zeiss Axioplan fluorescent microscopy shows GFP labeling in cells transduced with only vector. Micrographs shows no increased labeling for Runx2 in cells transduced with only vector that were treated with BMP-2. In contrast increased Runx2 labeling was observed in cells transduced with vector that were treated with FGF-2 (red:Texas Red staining). **b**: Micrographs show GFP labeling in cells transduced with the Fgf2 construct. Micrographs show increased perinuclear and nuclear labeling for Runx2 in cells transduced with Fgf2 construct (red:Texas Red staining). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

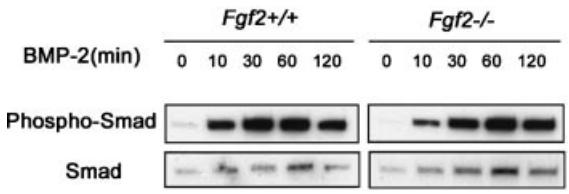

Fig. 9. Time course of the effect of BMP-2 on Smad 1/5/8 signal transduction in neonatal calvarial csteoblasts. Calvarial osteoblasts were isolated from 4-day-old Fgf2+/+ and Fgf2−/− mice cultured for 7 days serum deprived and treated with vehicle or rhBMP-2 (50 ng/ml) for the indicated times. Whole cell extracts were prepared for Western blot analysis using antibodies against phosphorylated Smad and total Smad as described in Materials and Methods Section. Representative Western blot of calvarial osteoblasts from both genotypes treated with vehicle or rhBMP-2 (50 ng/ml) shows similar induction of pSMAD1/5/8 in Fgf2+/+ and Fgf2−/− osteoblasts.

identified FGF-2 as one of several genes linked to BMP-2 mediated osteoblast differentiation [Balint et al., 2003].

Interestingly, pretreatment with the protein synthesis inhibitor cycloheximide did not block BMP-2 induced expression of FGF-2 suggesting that new protein synthesis was not required.

In view of the reciprocal regulation of BMP-2 and FGF-2 gene expression in osteoblasts as well as the marked decrease in BMP-2 mRNA in calvariae from Fgf2 mutant mice; we assessed whether treatment with BMP-2 could rescue reduced periosteal bone formation in the Fgf2 mutant mice. We chose the in vivo calvarial periosteal bone formation model since similar to FGF-2 [Hurley et al., 2002]. BMP-2 was previously reported to increase periosteal bone formation in rodents [Chen et al., 1997; Zhao et al., 2002]. Consistent with these reports, we observed a marked increase in BMP-2-induced periosteal bone formation in the Fgf2+/+ mice. In contrast, BMP-2 failed to significantly increase periosteal bone formation in the Fgf2−/− mice suggesting that FGF-2 is indeed important in mediating maximal effects of BMP-2 on periosteal bone formation.

BMP-2 promotes both the commitment and differentiation of marrow mesenchymal cells towards the osteoblast lineage [Gori et al., 1999]. In addition, BMP-2 was shown to synergize with FGF-2 to maintain the osteogenic differentiation of rat marrow-derived mesenchymal cells [Hanada et al., 1997; Hurley et al., 2002]. Consistently, exogenous BMP-2 did not rescue the decreased bone nodule formation in Fgf2−/− mice, strongly suggesting that in the absence of endogenous FGF-2, the ability of BMP-2 to induce maximal osteoblast differentiation is impaired consistent with the in vivo data.

The process of bone remodeling is coupled and FGF-2 is known to modulate both bone formation as well as resorption [Hurley et al., 2002]. Similar to FGF-2 [Hurley et al., 1998] in addition to its effect on osteoblast differentiation, BMP-2 was shown to increase OCL formation in murine models [Kanatani et al., 1995]. Consistent with previous studies we observed that BMP-2 significantly increased OCL formation in Fgf2+/+ marrow stromal cultures but did not significantly increase OCL formation in marrow stromal cultures from Fgf2−/− mice suggesting that endogenous FGF-2 is also important in BMP-2-induced OCL formation. These results are consistent with our previous observation that OCL formation in marrow stromal cultures from Fgf2−/− mice is impaired in response to parathyroid hormone (PTH), receptor activator of NFKB ligand (RANKL) and interleukin 11(IL-11) [Okada et al., 2003].

As previously stated Runx2 plays an important role in osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997] and several studies showed that Runx2 regulated the expression of genes associated with osteoblast differentiation [Banergee et al., 1997; Ducy et al., 1997; Ji et al., 1998]. We previously reported that intermittent FGF-2 treatment increased Runx2 mRNA in osteoblast/stromal cells [Zhang et al., 2002] and that Runx2 expression is impaired in osteoblasts from Fgf2+/− and Fgf2−/− mice [Naganawa et al., 2006]. Other studies showed that Runx2 is phosphorylated and activated by FGF-2 [Xiao et al., 2002; Kim et al., 2003] and that induction of the osteocalcin gene by FGF-2 requires Runx2 phosphorylation. Consistent with these reports, in addition to reduced Runx2 mRNA, we previously reported reduced osteocalcin mRNA expression in osteoblast/stromal cells from Fgf2−/− mice [Naganawa et al., 2006]. The regulation of Runx2 by members of the FGF superfamily has been particularly studied, as has the role of FGF in controlling the expression of multiple genes involved in osteoblast differentiation, however we report, for the first time, that the ability of BMP-2 to increase labeling and nuclear accumulation of Runx2 is impaired in calvarial osteoblasts from Fgf2−/− mice. In

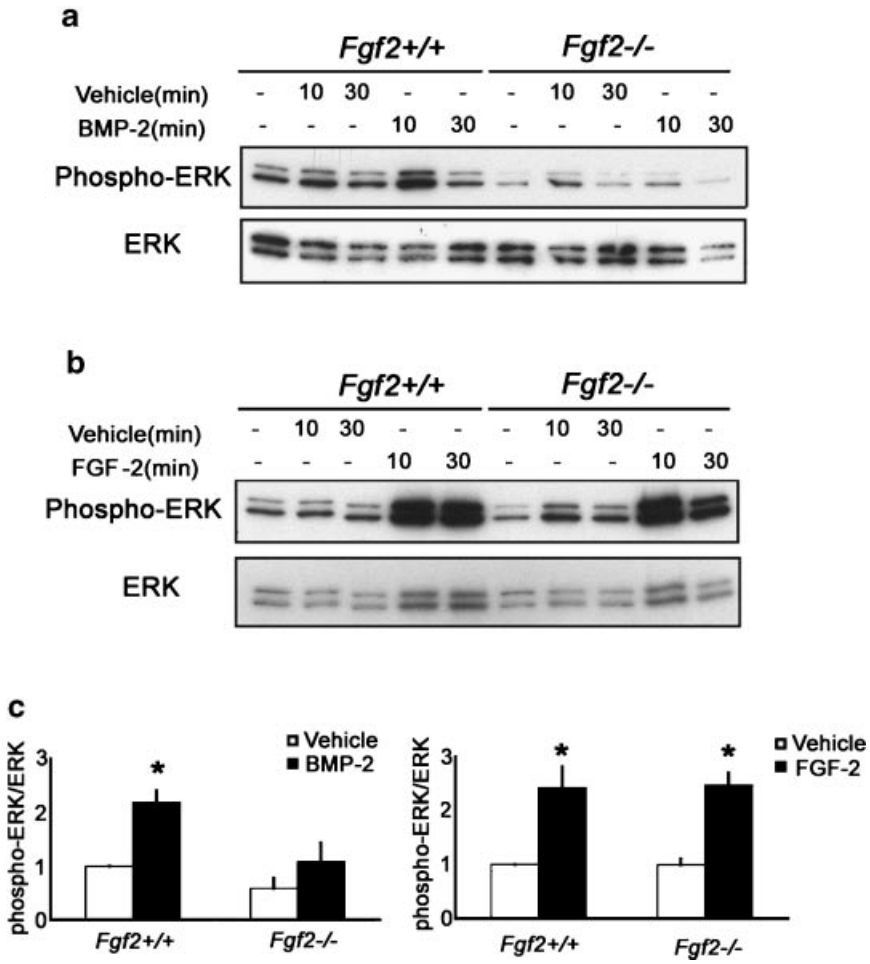

Fig. 10. Time course of the effect of BMP-2 and FGF-2 on ERK1/2 Phosphorylation in neonatal calvarial osteoblasts from Fgf2+/+ and Fgf2−/− mice. Calvarial osteoblasts were cultured for 7 days serum deprived and treated with effectors. Whole cell extracts were prepared for Western blot analysis using antibodies against total or phosphorylated ERK1/2 as described in Materials and Methods Section. **a**: Representative Western blot of calvarial osteoblasts from both genotypes treated with vehicle or rhBMP-2 (50 ng/ml). **b**: Representative Western blot of calvarial osteoblasts from both genotypes treated with vehicle or FGF-2 (10 nM) for the indicated times. **c**: Pooled results of Western blots of calvarial osteoblasts from both genotypes treated with vehicle or rhBMP-2 (50 ng/ml) expressed as the mean ± SEM for three independent experiments $^*P < 0.01$ versus vehicle (Fgf2+/+ cultures). Pooled results of Western blots of calvarial osteoblasts from both genotypes treated with vehicle or FGF-2 (10 nM) expressed as the mean ± SEM for three independent experiments $^*P < 0.01$ versus vehicle (Fgf2+/+ and Fgf2−/− cultures).

parallel, the observation that exogenous BMP-2 was only able to increase nuclear accumulation of FGF-2 protein in Fgf2−/− cells transduced with the Fgf2 construct, suggest either a direct requirement for endogenous FGF-2 or that endogenous FGF-2 is necessary for another factor to mediate this accumulation.

Similar to FGF-2, BMP-2 can activate multiple downstream signaling pathways. Classically, upon BMP-2 activation of cell surface serine/threonine kinase receptors, cytoplasmic signaling proteins Smads are phosphorylated [Canalis et al., 2003]. Smads 1, 5, and 8 are activated and transported to the nucleus where they interact with nuclear factors [Canalis et al., 2003]. Previous studies showed that overexpression of Smad 1 and Smad 5 in C2C12 mesenchymal cells resulted in osteoblastic differentiation. Therefore, we examined the activity of this pathway in Fgf2−/− calvarial osteoblasts. The results showed that BMP-2 phosphorylation of Smad 1, 5, 8 was active in Fgf2−/− osteoblasts, suggesting that failure of BMP-2 to induce OB differentiation was not

due to a defect in activating this signaling pathway.

BMP-2 also activates Ras/MAPK signaling resulting in activation of ERK/MAPK [Gallea et al., 2001]. Interestingly, in addition to increasing FGF-2 mRNA in the pluripotent C2C12 cells [Canalis et al., 2003]; BMP-2 treatment also increased p38 MAPK and ERK p42/P44 that are important down stream signaling pathways to mediate its effects on osteoblastic differentiation of these cells [Gallea et al., 2001]. These reports are of interest, since we [Hurley et al., 1996], and others [Spector et al., 2004], have shown that FGF-2 also signals via activation of MAPK in OBs. The present study shows that BMP-2 caused a similar increase in p38 MAP Kinase in both genotypes. However, BMP-2 phosphorylation of ERK was impaired in Fgf2−/− osteoblasts. In contrast FGF-2 increased phospho-ERK in osteoblasts from both genotypes.

As previously noted studies by Xiao et al. [2002] showed that induction of the osteocalcin gene by FGF-2 requires activation of MEK/ERK kinases pathway as well as Runx2 phosphorylation. These results identify the regulatory critical point in OB differentiation where the essential role of FGF-2 is its ability to allow other factors such as BMP-2 to modulate bone formation via the ERK signaling pathways and Runx2 activation.

ACKNOWLEDGMENTS

This work was supported by NIH grant AG021189 to M.M. Hurley.

REFERENCES

Balint E, Lapointe D, Drissi H, van derMeijden C, Young DW, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2003. Phenotype discovery by gene expression profiling of biological processes linked to mediated osteoblast differentiation. J Cell Biochem 89:401–426.

Banergee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. 1997. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 66:1–8.

Brunner G, Gabrilove J, Rifkin DB, Wilson EL. 1991. Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol anchored heparan sulfate proteoglycan. J Cell Biol 114:1275–1283.

Canalis E, Centrella M, McCarthy T. 1988. Effects of basic fibroblast growth factor on bone formation in vitro. J Clin Invest 8:1572–1577.

Canalis E, Economides AR, Gazzerro E. 2003. Bone morphogenetic proteins, their antagonist and the skeleton. Endocrine Rev 24:218–235.

Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, Mundy GR, Harris SE. 1997. Bone morphogenetic 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the Induction of mineralized matrix formation in cultures of fetal rat calvarial osteoblasts. Calcif Tissue Int 60:283–290.

Choi K, Kim H, Lee M, Kwon T, Nah H, Furuchi T, Komori T, Nam S, Kim Y, Kim H, Ryoo H. 2005. Runx2 regulates FGF-2-induced BMP-2 expression during cranial bone development. Dev Dyn 233:115–121.

Dong M, Guda K, Nambiar PR, Nakanishi M, Lichtler AC, Nishikawa M, Giardini C, Rosenberg DW. 2004. Azoxymethane-induced preadipocyte factor 1 (Pref-1) functions as a differentiation inhibitor in colonic epithelial cells. Carcinogenesis 25:2239–2246.

Ducy P, Zhang R, Geoffroy V, Ridall A, Karsenty G. 1997. Osf2/cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Dunstan CR, Boyce BF, Garrett IR, Izbicka E, Burgess WH, Mundy GR. 1999. Systemic administration of acidic fibroblast growth factor (FGF-1) prevents bone loss and increases new bone formation in ovariectomized rats. J Bone Miner Res 14:953–959.

Gallea S, Lallemand F, Atfi A, Rawadi SG, Ramez V, Spinella-Jaegle S, Kawai S, Faucheu C, Huet L, Baron R, Roman-Roman S. 2001. Activation of mitogen-activated protein kinase cascade in regulation of bone morphogenetic protein-2 induced osteoblast differentiation in pluripotent C2C12 cells. Bone 28:491–498.

Globus RK, Patterson-Buckendahl P, Gospodarowicz D. 1998. Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor. Endocrinology 123:98–105.

Gori F, Thomas T, Hicock KC, Spelsberg TC, Riggs BL. 1999. Differentiation of human marrow stromal precursor cells: Bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment and inhibits late adipocyte maturation. J Bone Miner Res 14:1522–1535.

Gospodarowicz D. 1990. Basic science and pathology fibroblast growth factor chemical structure and biologic function. Clin Orthop Rel Res 257:231–248.

Hanada K, Dennis JE, Caplan A. 1997. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein 2 on osteogenic differentiation of rat bone marrow derived mesenchymal stromal cells. J Bone Miner Res 12:1606–1614.

Hurley MM, Abreu C, Harrison JR, Lichtler A, Raisz LG, Kream BE. 1993. Basic fibroblast growth factor inhibits type I collagen gene expression in osteoblastic MC3T3-E1 cells. J Biol Chem 268:5588–5593.

Hurley MM, Marcello K, Abreu C, Kessler M. 1996. Signal transduction by basic fibroblast growth factor in rat osteoblastic Py1a cells. J Bone Miner Res 11:1256–1263.

Hurley MM, Lee SK, Marcello K, Abreu C, Raisz LG, Bernecker P. 1998. Basic fibroblast growth factor induces osteoclastogenesis in bone marrow cultures by a prostaglandin-mediated mechanism. Bone 22:309–316.

Hurley MM, Marie P, Florkiewicz R. 2002. Fibroblast growth factor and fibroblast growth factor receptor

families. In: Bilezikian JP, Raisz LG, Rodan G, editors. Principles of bone biology. San Diego: Academic Press. pp 825–852.

Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TG, Centrella M. 1998. CBFa (AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69:353–363.

Kanatani N, Sugimoto T, Kaji H, Kobayashi T, Nishiyama K, Fukase M, Kumegawa M, Chihara K. 1995. Stimulatory effect of bone morphogenetic 2 enhances osteoclast formation and bone resorbing activity. J Bone Miner Res 10:1681–1690.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kim HJ, Kim JH, Bae SC, Choi JY, Kim HJ, Ryoo HM. 2003. The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. J Biol Chem 278:319–326.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi Y, Shimizu R, Bronson T, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.

Kuo P, Hsu Y, Chang CH, Chang JK. 2005. Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase pathway in human osteoblast cells. J Pharm Exp Ther 314:1290–1299.

Liang H, Pun S, Wronski TJ. 1999. Bone anabolic effects of basic fibroblast growth factor in ovariectomized rats. Endocrinology 140:5780–5788.

Locklin RM, Williamson MC, Beresford JN, Triffitt JT, Owen ME. 1995. In vitro effects of growth factors and dexamethasone on rat marrow stromal cells. Clin Orthop Rel Res 313:27–35.

Mayahara H, Ito T, Nagai H, Miyajima H, Tsukada R, Takatomi S, Mizoguchi J, Kato K. 1993. In vivo stimulation of endosteal bone formation by basic fibroblast growth factor in rats. Growth Factors 9:73–80.

McCarthy TL, Centrella M, Canalis E. 1989. Effects of fibroblast growth factors on deoxyribonucleic acid and collagen synthesis in rat parietal bone cells. Endocrinology 125:2118–2126.

Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, Doetschman T, Coffin JD, Hurley MM. 2000. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. J Clin Invest. 105:1085–1093.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole, WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89:773–779.

Nagai H, Tsukuda R, Mayahara H. 1995. Effects of basic fibroblast growth factor (bFGF) on bone formation in growing rats. Bone 16:367–373.

Naganawa T, Xiao L, Abogunde E, Sobue T, Kalajzic I, Sabbieti M, Agas D, Hurley MM. 2006. In vivo and in vitro comparison of the effects of Fgf2 null and haploinsufficiency on bone formation in mice. Biochem Biophys Res Commun 339:490–498.

Nakamura T, Hanada K, Tamura M, Shibanushi T, Nigi H, Tagawa M, Fukumoto S, Matsumoto T. 1995. Stimulation of endosteal bone formation by systemic injections of recombinant basic fibroblast growth factor in rats. Endocrinology 136:1276–1284.

Nakamura K, Kawaguchi H, Aoyama I, Hanada K, Hiyami Y, Awa T, Tamura M, Kurokawa T. 1998. Stimulation of bone formation by intraosseous injection of basic fibroblast growth factor in ovariectomized rats. Int Orthop 22:49–54.

Okada Y, Montero A, Zhang X, Sobue T, Lorenzo J, Doetschman T, Coffin JD, Hurley MM. 2003. Impaired osteoclast formation in bone marrow cultures of Fgf2 null mice in response to parathyroid hormone. J Biol Chem 278:21258–21266.

Oliver LJ, Rifkin DB, Gabrilove J, Hannocks MJ, Wilson EL. 1990. Long-term culture of human bone marrow stromal cells in the presence of basic fibroblast growth factor. Growth Factors 3:231–236.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen DR, Selby PB, Ownen MG. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765–771.

Reddi AH, Huggins C. 1972. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci USA 69:1601–1605.

Rodan SB, Wesolowski G, Yoon K, Rodan GA. 1989. Opposing effects of fibroblast growth factor and pertussis toxin on alkaline phosphatase, osteopontin, osteocalcin and type I collagen mRNA levels in ROS 17/2.8 cells. J Biol Chem 264:19934–19941.

Rosen V, Wozney JM. 2002. Bone morphogenetic proteins. Belizikian J, Raisz LG, Rodan G, editors. Principles of bone biology. 2nd edition. San Diego: Academic Press. pp 919–928.

Spector JA, Mathy JA, Warren SM, Nacamuli RP, Song HM, Lenton K, Fong KD, Fand DT. 2004. FGF-2 acts through an ERK1/2 intracellular pathway to affect osteoblast differentiation. Plast Reconstr Surg 115:838–852.

Thomson BM, Bennett J, Dean V, Triffit J, Miekle MC, Loveridge N. 1993. Preliminary characterization of porcine bone marrow stromal cells: Skeletogenic potential, colony-forming activity, and response to dexamethasone, transforming growth factor beta and basic fibroblast factor. J Bone Miner Res 10:1173–1183.

Urist MR. 1965. Bone: Formation by autoinduction. Science 150:893–899.

Wozney JM. 1992. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 3:160–167.

Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. 2002. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx-2. J Biol Chem 277:36181–36187.

Xiao L, Naganawa T, Obugunde E, Gronowicz G, Ornitz DM, Coffin JD, Hurley MM. 2004. Stat1 controls postnatal bone formation by regulating fibroblast growth

factor signaling in osteoblasts. J Biol Chem 279:27743–27752.

Xu X, Weinstein M, Li C, Deng CX. 1999. Fibroblast growth factor receptors (FGFRs) and their role in limb development. Cell Tissue Res 296:33–43.

Yamaguchi A. 1995. Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-beta superfamily. Semin Cell Biol 6:165–173.

Zhang X, Sobue T, Hurley MM. 2002. FGF-2 increases colony formation, PTH receptor, and IGF-1 mRNA in mouse marrow stromal cells. Biochem Biophys Res Commun 290:526–531.

Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D. 2002. Bone morphogenetic protein receptor signaling is necessary for murine postnatal bone formation. J Cell Biol 157:1049–1060.

Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haundenschild CC, Moying Y, Coffin JD, Kong L, Kranias EG, Luo W, Boivin GP, Duffy JJ, Pawlowski SA, Doetschman T. 1998. Fibroblast growth factor 2 control of vascular tone. Nat Med 4:201–207.