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FGF-2 Increases Colony Formation, PTH Receptor, and IGF-1 mRNA in Mouse Marrow Stromal Cells

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FGF-2 stimulates bone formation *in vitro* and *in vivo* in rats. However, there are limited studies in mice and no data on the mechanism(s) by which FGF-2 induces bone formation. We assessed whether short-term FGF-2 treatment of marrow stromal cells from young mice would increase alkaline phosphatase-positive (ALP), mineralized colony formation and expression of genes important in osteoblast maturation. Short-term treatment with FGF-2 (0.01–1.0 nM) for the first 3 days of a 14- or 21-day culture period increased the number of ALP mineralized colonies in bone marrow stromal cells. FGF-2 (0.1 nM) increased the mRNAs for type 1 collagen: osteocalcin, runt domain/core binding factor, PTH/PTHR receptor, and insulin-like growth factor 1 (IGF-1) at 14 and 21 days. We conclude that short-term FGF-2 treatment enhances osteoblast maturation *in vitro*. Furthermore, the anabolic effect of FGF-2 may be attributed in part to regulation of IGF-1 in osteoblasts. © 2002 Elsevier Science

Key Words: **FGF-2; IGF-1; PTH/PTHrP receptor; Runx2/Cbfa1; bone nodules.**

FGF-2 a potent regulator of cellular proliferation and differentiation *in vitro* (1–5) is stored in the extracellular matrix (ECM) (3, 6, 7) and is expressed in bone cells (7). FGF-2 is an important modulator of cartilage and bone growth and differentiation (2, 8–11). Fibroblast growth factor receptors (FGFR) are also important regulators of bone growth and development (2, 12, 13) and a number of human dysmorphic syndromes have been genetically linked to mutations in different FGF receptors (2, 14, 15). Studies have shown that disruption of the FGFR3 gene in mice resulted in increased endochondral bone growth (16) while mutations in FGFR2 increased expression of osteoblast differentiation markers in calvarial osteoblasts (17). We recently reported that disruption of the fibroblast growth factor gene in mice results in decreased bone mass and bone formation (18) confirming a role for endogenous FGF-2 in maintaining bone mass.

Chronic FGF-2 treatment stimulates bone cell replication (19) and reduces differentiation markers such as alkaline phosphatase and Type 1 collagen *in vitro* (20–23). Although, intermittent FGF-2 treatment can stimulate bone formation *in vitro* (22, 24–30), as well as *in vivo* (2, 31), there is little data on the mechanism by which this occurs. FGF-2 may also play an important role in fracture repair in rats (32) and in humans (33) since the FGF-2 gene is expressed from the early stage of fracture repair in the granulation tissue at the fracture site (34). FGF-2 can stimulate osteoclast formation, as well as, increase bone resorption (5, 35, 36). Since FGF-2 has effects on both osteoblasts and osteoclasts it may be involved in the coupling of bone formation and bone resorption.

We previously reported that the expression of FGF-2 mRNA and protein in osteoblasts (37) is increased by transforming growth factor beta (TGFβ) (37), prostaglandins (PG) (38), 17β estradiol (39), parathyroid hormone (PTH) (40) and interleukin-1 (41). Thus, hormones and local factors which have important functions in bone homeostasis, regulate FGF-2 production.

In this report we show that short-term FGF-2 treatment significantly increased ALP colony number, size and mineralization of bone marrow stromal cultures derived from young mice. Since these cultures showed increased expression of type 1 collagen (COL1A1), osteocalcin (OCN), runt domain factor/core binding factor (Runx2/Cbfa1) and parathyroid hormone/parathyroid related protein receptor (PTH/PTHrP receptor) mRNAs, we conclude that short-term FGF-2 treatment can also enhance osteoblast maturation *in vitro*. These studies are also the first to report that FGF-2 increases PTH/PTHrP receptor and IGF-1 mRNAs in osteoblasts and suggest a role for IGF-1 in the anabolic response to FGF-2 in bone.

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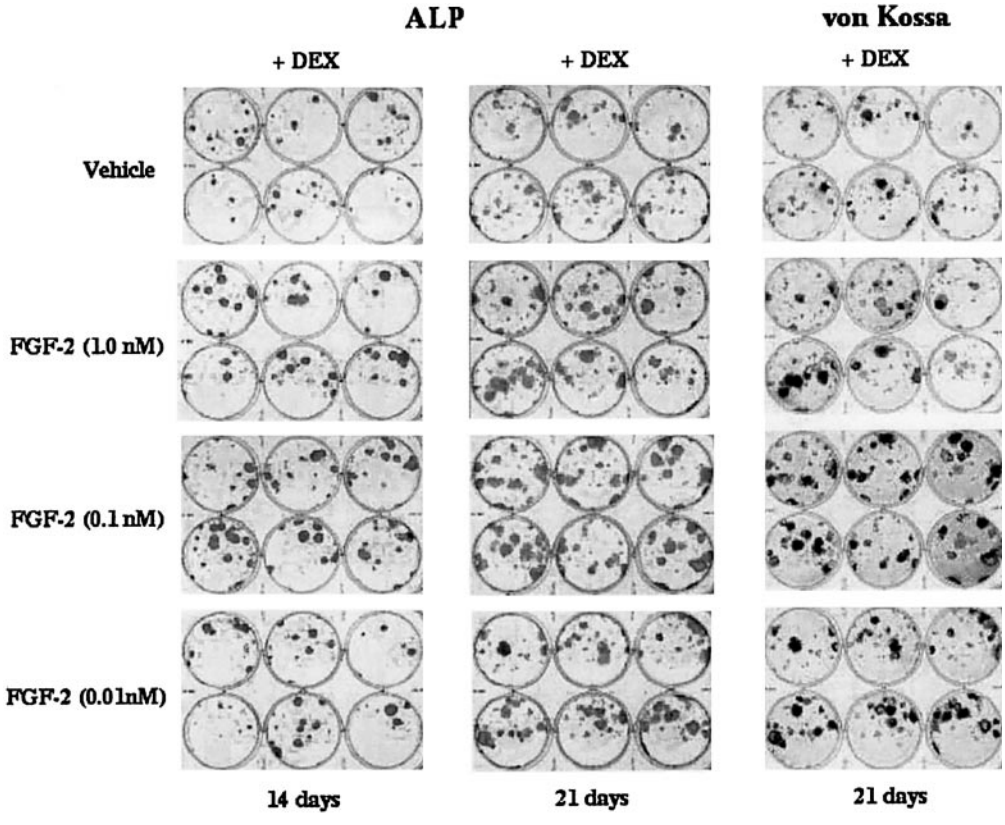

FIG. 1. Time-course, dose–response effect of short-term treatment with FGF-2 in the presence of DEX on ALP mineralized colony formation in mouse bone marrow stromal cells. Marrow stromal cells were treated with FGF-2 for the first 3 days only of the 14- or 21-day culture period. DEX (10 nM) was added to the culture during each media change. ALP staining and von Kossa staining for mineral was performed as described under Materials and Methods.

MATERIALS AND METHODS

Cell cultures. Mouse bone marrow cells: were isolated as previously described (42). Briefly, tibiae, and femur from 2 month-old C57/BL6 mice were dissected free of adhering tissue. The bone ends were removed and the marrow cavity flushed with alpha minimal essential medium (αMEM, GIBCO-BRL, Grand Island, NY). Bone marrow cells were plated at 1×10^6 cell/cm^2 in complete media consisting of [αMEM, with 10% heat inactivated fetal calf serum (FCS), ascorbic acid (50 μg/ml); and beta-glycerophosphate (BGP 8 mM)] in the absence or presence of varying concentrations of FGF-2 (0.01, 0.1, 1.0 nM). Some experiments were conducted in the presence or absence of dexamethasone (DEX 10^{-8} M) (43). After the first 3 days of culture, media was changed to complete media without further addition of FGF-2. Media were changed every 3 days for the remainder of the culture period. Cells were fixed and stained for alkaline phosphatase using a commercially available kit (Sigma, St. Louis, MO). Colony area was measured using NIH IMAGE-1-61. Cultures were restained with von-Kossa to detect mineralization.

Measurement of mRNA levels. In some experiments total RNA was extracted from cells by the method of Chomczynski and Sacchi (44). For Northern analysis, 20 μg of total RNA was denatured and fractionated on a 0.8% agarose/1.1 M formaldehyde gel, transferred to filters by capillary blotting or positive pressure (Posiblot), and fixed to the filter by UV irradiation (Stratalinker) (45). After a 4-h prehybridization, filters were hybridized overnight with a [^{32}P]cDNA probe for the mRNA of interest. Bands were normalized to GAPDH and quantitated by autoradiography and densitometry.

cDNA probes. The following cDNA probes were utilized in this study. The mouse FGF-2 cDNA was obtained from Dr. Gail Martin, University of San Francisco, California. A cDNA for rat COL1A1 was provided by Dr. Barbara Kream, University of Connecticut Health Center (Farmington, CT). A cDNA for rat IGF-1 was obtained from Dr. Liam Murphy, University of Manitoba, Winnipeg, Manitoba. Mouse PTH/PTHrP Receptor cDNA was provided by Dr. William Philbrick Yale School of Medicine (New Haven, CT). Mouse OCN and Runx2/Cbfa1 cDNAs were obtained from Dr. David Rowe (University of Connecticut Health Center, Farmington, CT). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was utilized as a control for normalization of data.

RESULTS

Dose–Response Effect of FGF-2 on Alkaline Phosphatase Positive Colonies in Bone Marrow Cultures of 2-Month-Old Mice

We assessed whether short-term treatment with FGF-2 for the first 3 days of culture would result in increased alkaline phosphatase positive (ALP) colonies at 14 and 21 days. As shown in Fig. 1, short-term treatment with FGF-2 (0.01, 0.1, 1.0 nM) in the presence of DEX (10 nM) increased the number and area of ALP colonies at 14 and 21 days in marrow stromal

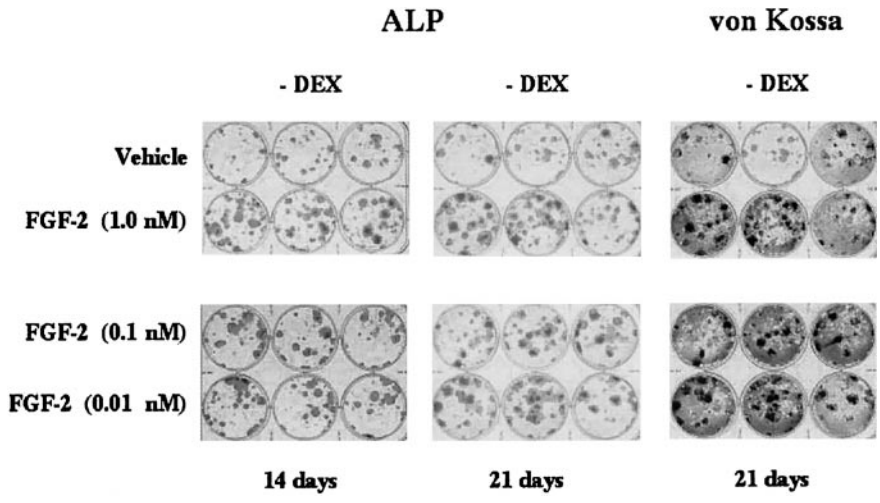

FIG. 2. Time-course, dose–response effect of short-term treatment with FGF-2 in the absence of DEX on ALP mineralized colony formation in mouse bone marrow stromal cells. Marrow stromal cells were treated with FGF-2 for the first 3 days only of the 14- or 21-day culture period. DEX was not utilized in these cultures. ALP staining and von Kossa staining for mineral was performed as described under Materials and Methods.

cultures from 2 month old mice. At 14 days, the maximum increase in colony number (14.7 ± 1.4 vs 7.0 ± 1.5) and colony area (0.26 ± 0.03 vs 0.09 ± 0.02) was observed in bone marrow stromal cells treated with FGF-2, 0.1 nM. To examine whether DEX was a prerequisite for mineralized colony formation, bone marrow stromal cells were treated with FGF-2 for the first 3 days of the culture period in the absence of DEX. As shown in Fig. 2, in the absence of DEX, short-term treatment with FGF-2 (0.01–1 nM) increased ALP colonies at 14 days and the number of mineralized colonies at 21 days.

Dose–Response Effect of FGF-2 on Gene Expression in Bone Marrow Stromal Cultures

In parallel experiments, total RNA was extracted from bone marrow stromal cultures from mice that were treated with FGF-2 (0.01–1.0 nM) for the first 3 days of a 14- or 21-day culture period. Northern analysis was performed to examine the expression of COL1A1; OCN, Runx2/Cbfa1; PTH/PTHrP receptor; and IGF-1 mRNA levels. As shown in Fig. 3, in vehicle-treated cultures COL1A1 mRNA was expressed as a doublet, while a single transcript was observed for OCN, Runx2/Cbfa1, and PTH/PTHrP receptor respectively. In vehicle treated cultures, we observed multiple IGF-1 mRNA transcripts including a major 7.5 kb band. Quantitation of the expression of each mRNA normalized to GAPDH is shown in Table 1. At 14 days, marrow stromal cells that had been treated with 1.0 nM of FGF-2 for the first 3 days of the culture period, showed decreased expression of COLIA1, OCN and IGF-1 mRNA, although there was a slight increase in Runx2/Cbfa1 expression, while PTH/PTHrP mRNA expression was similar to that observed in vehicle-treated cultures. In contrast, lower concentrations of FGF-2 (0.1 and 0.01 nM) increased all mRNAs at 14 days. At 21 days, maximum increases in COL1A1, OCN, Runx2/Cbfa1, PTH/PTHrP receptor and IGF-1 mRNAs was observed in marrow stromal cells that

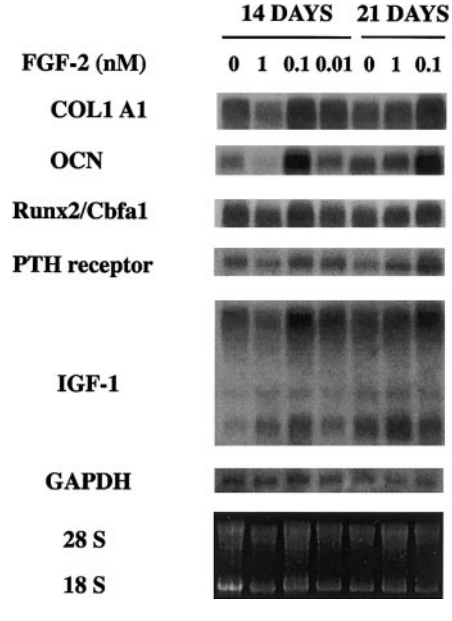

FIG. 3. Time-course, dose–response effect of short-term treatment with FGF-2 on gene expression in mouse bone marrow stromal cells at 14 and 21 days. Bone marrow stromal cells were treated with FGF-2 for the first 3 days only of the 14- or 21-day culture period. Cells were harvested for Northern analysis as described under Materials and Methods.

TABLE 1

Dose–Response Effect of Short-Term FGF-2 Treatment (Days 1–3) on mRNA Expression in Mouse Bone Marrow Stromal Cells Harvested after 14 and 21 Days of Culture

	mRNA/GAPDH ratio				
	COL1A1	OCN	PTH/ PTHrP	Cbfa1/ RUNX2	IGF-1
14 Days					
Vehicle	1.10	0.54	0.34	1.26	1.08
FGF-2 (1.0 nM)	0.75	0.18	0.34	1.41	0.80
FGF-2 (0.1 nM)	2.26	3.35	0.50	1.81	2.52
FGF-2 (0.01 nM)	2.07	1.12	0.57	1.55	2.22
21 Days					
Vehicle	1.51	1.58	0.36	1.66	1.48
FGF-2 (1.0 nM)	1.99	2.07	0.65	2.48	2.23
FGF-2 (0.1 nM)	3.38	5.61	1.11	3.05	3.89

Note. Total RNA was extracted from cells after 14 or 21 days for Northern blot analysis as described under Materials and Methods. Filters were hybridized to the cDNA of interest and then reprobed for GAPDH. Signals were quantitated by densitometry and normalized to the corresponding value for GAPDH.

were treated with 0.1 nM FGF-2. COL1A1 mRNA was increased by 205 and 223%; OCN by 620 and 355%; Runx2/Cbfa1 by 144 and 184%; PTH/PTHrP receptor by 147 and 308%; and IGF-1 by 233 and 263% at 14 and 21 days, respectively.

DISCUSSION

Previous studies have shown that bone marrow stroma contains osteoprogenitor cells that are capable of differentiating into mature osteoblasts capable of forming mineralized bone nodules (46–49). This study shows that short-term FGF-2 treatment of bone marrow stromal cultures derived from young and adult mice significantly increased ALP colony number and size, as well as their mineralization. Similar to these observations in mice, previous studies showed that FGF-2 also increased bone nodule formation in rat and human bone marrow stromal cells (25–30). As shown in Figs. 1 and 2 we observed an increase in colony number that represents enhanced recruitment of osteoblast progenitors by FGF-2, as well as an increase in colony size due to expansion of clones in response to FGF-2. It is believed that FGF-2 enhances bone formation due to its stimulatory effect on osteoblast proliferation to increase the precursor pool, as well as effects on their differentiation (25–30).

Since DEX was found to be a prerequisite for the recruitment of progenitor cells and their terminal differentiation in cultures of rat stromal bone marrow cells (51), we examined the effect of FGF-2 in the presence (Fig. 1) and absence (Fig. 2) of DEX. Under both culture conditions, we observed that FGF-2 increased the number of ALP mineralized colonies. These results suggest that DEX is not absolutely required for FGF-2 to enhance murine marrow stromal cell proliferation and differentiation.

As shown in Fig. 3 and Table 1, we examined the dose response effect of short-term exposure to FGF-2 on osteoblast gene expression in bone marrow stromal cells from 2 month old mice. We examined the expression of OCN since previous studies showed that OCN, is a specific marker of differentiated osteoblast (52) and Runx2/Cbfa1 that has been shown to regulate the onset of mineralization (53). We observed a greater increase in the expression of mRNAs for OCN, and Runx2/Cbfa1 in FGF-2 treated cultures than vehicle treated cultures. We conclude from these results that short-term FGF-2 treatment can also enhance osteoblast maturation and differentiation.

Another phenotypic characteristic of mature osteoblasts is the expression of PTH/PTHrP receptor (54). Since previous studies in transgenic mice demonstrated an important role for PTH/PTHrP receptor in bone formation (55), we therefore examined whether short-term FGF-2 treatment modulated PTH/PTHrP mRNA expression in these cultures. As shown in Fig. 3 and Table 1, short-term exposure to FGF-2 increased PTH/PTHrP mRNA expression in bone marrow stromal cells from 2 month old mice. Although, FGF-2 at 0.1 and 1. 0 nM caused only a slight increase in PTH/PTHrP mRNA expression at 14 days, both concentrations of FGF-2 caused a greater increase at 21 days, consistent with a higher degree of PTH/PTHrP receptor expression in more mature osteoblasts. The present study provides the first evidence that short-term FGF-2 treatment upregulates PTH/PTHrP receptor mRNA expression in osteoblastic cells.

Growth factors such as IGF-1 that are made by bone cells and stored in bone matrix (3) can modulate the proliferation and differentiation of osteoblast progenitors (56–58). IGF-1 has anabolic effects in humans and rodents (59) and was previously shown to mediate selective anabolic effects of PTH *in vitro* (60). We therefore examined whether similar to PTH, short-term FGF-2 treatment would increase IGF-1 mRNA expression in bone marrow stromal cells. As shown in Fig. 3 and Table 1, short-term treatment with FGF-2 increased IGF-1 mRNA expression in bone marrow stromal cells from young mice. These studies are the first to show that FGF-2 can increase IGF-1 mRNA levels in bone marrow stromal cells and suggest a role for IGF-1 in the anabolic response to FGF-2 *in vitro.*

In summary, we have shown that FGF-2 not only increased bone nodule formation in murine bone marrow stromal cells but also increased the expression of PTH/PTHrP and IGF-1 mRNA that play critical roles in bone formation.

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