Fibroblast Growth Factor-2 Is an Immediate-Early Gene Induced by Mechanical Stress in Osteogenic Cells

Chai-Fei Li1 and Millie Hughes-Fulford1,2,3

ABSTRACT: Fifteen minutes of physiological MS induces FGF-2 in osteogenic cells. Here, we show that MS induced proliferation in both MC3T3-E1 and BMOp cells isolated from Fgf2+/- mice; Fgf2-/- BMOp cells required exogenous FGF-2 for a normal proliferation response. The induction of fgf-2 is mediated by PKA and ERK pathways.

Introduction: Mechanical stress (MS) induces gene expression and proliferation of osteogenic MC3T3-E1 cells. We have previously shown that physiological levels of MS in MC3T3-E1 cells causes extracellular signal–regulated kinase (ERK)1/2 phosphorylation. Here we evaluate the induction and importance of fibroblast growth factor-2 (FGF-2) for MS-induced proliferation.

Materials and Methods: We characterized the MS induction of fgf-2 using a 15-minute pulse of 120 μstrain and studied the stability of fgf-2 message using actinomycin D. Bone marrow stromal cells (BMOp) isolated from Fgf2−/− and Fgf2+/+ mice were used to study the importance of FGF-2 in MS-induced proliferation.

Results: We found that the induction of fgf-2 by MS is dependent on both protein kinase A (PKA) and ERK pathways. MS transiently induces fgf-2 within 30 minutes. FGF-2 receptor (FGFR2) was also significantly increased within 1 h. All three isoforms of FGF-2 (24, 22, and 18 kDa) were significantly increased by MS. The MS-mediated increase of fgf-2 mRNA was caused by new synthesis and not stabilization. Pretreatment of MC3T3-E1 cells with cycloheximide showed that the induction of fgf-2 did not require new protein synthesis. Pretreating MC3T3-E1 cells with the mitogen-activated protein kinase (MAPK)/ERK kinase 1/2 (MEK1/2) inhibitor, U0126, or H-89, a PKA inhibitor, significantly inhibited the induction of fgf-2, showing that mechanical induction of fgf-2 is dependent on ERK and PKA signaling pathways. The downstream consequence of a single 15-minute stress pulse was a 3.5-fold increase in cell number in MC3T3-E1 compared with growth in nonstressed control cells. In studies using bone marrow osteoprogenitor cells (BMOp) isolated from Fgf2+/+ and Fgf2−/− mice, we found that FGF-2 was necessary for a full proliferative response to MS.

Conclusions: These studies show that FGF-2 is an immediate-early gene induced by MS, and its expression is mediated by both the PKA and MAPK signal transduction pathways. FGF-2 was required for a full proliferative response.

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Key words: mechanical stress, osteoblast, signal transduction, Cox-2, growth factors

INTRODUCTION

OSTEOBLASTS AND OSTEOCLASTS are known regulators in bone remodeling. In the absence of mechanical stress (such as in bedrest), osteoblast function is significantly decreased.(1,2) In vitro studies during space flight with osteoblastic cells have shown altered differentiation and cell morphology.(3)

Gravity compression mechanical stress (MS; 80–270 μstrain) regulates c-fos, phosphorylated p44/42 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (pERK), fibroblast growth factor-2 (fgf-2), cyclooxygenase-2 (cox-2), DNA synthesis, and proliferation.(4,5) The frequency and duration of mechanical loading required to maintain bone mass in vivo and stimulate growth of osteogenic progenitors in vitro have been measured experimentally. Hind limb suspension, where the rat or mouse is suspended by the tail preventing the hind limbs from being loaded, is used as an animal model of bone disuse. Hind limb suspension of the C57BL/6J mouse strain results in decreased bone formation rates, reduced alkaline phosphatase activity, and decreased bone mass in the femur.(6) In chickens,(7) as little as 15 minutes of exercise per day was sufficient to maintain bone mass, and in a rat hind limb suspension model, 10 minutes per day of mechanical loading over a 28-day period was sufficient to maintain 50% of the bone mass compared with normally loaded animals.(8) Therefore, short duration (~15 minute) mechanical...
loading seems to be an effective stimulus of bone growth and maintenance in vivo. It has also been shown in vitro that 24 h after MS, there is an induction of DNA synthesis.\(^{10}\) We have previously shown that 15 minutes of 120 \(\mu\)strain of MS induces \(c-fos\), phosphorylation of ERK, and cell proliferation, suggesting that the MAPK ERK pathway plays an important role in MS signaling.\(^{14}\)

FGFs constitute a large family of polypeptide growth factors found in a variety of multicellular organisms.\(^{10,11}\) In vertebrates, the 23 members of the FGF family range in molecular mass from 17 to 45 kDa and share 13–71% amino acid homology.\(^{12}\) FGFs are involved in diverse cellular processes including chemotaxis, cell migration, differentiation, cell survival, and apoptosis.\(^{10,11}\) FGF-2 stimulates MC3T3-E1 cell proliferation and differentiation in vitro; these effects vary with the state of the cell.\(^{13}\) Studies have shown that an application of FGF-2 increases the rate of bone formation in humans and animals with fractures.\(^{14–18}\) In vivo studies have also shown that FGF-2 accelerates angiogenesis in necrotic bone.\(^{19}\) Recently, FGF-2 was shown to be involved in regulating sialoprotein expressions.\(^{20,21}\) Moreover, FGF-2 null mice have low bone mass,\(^{15}\) and FGF-2 injections increase bone formation and expression of bone matrix proteins in estrogen-deficient rodents.\(^{22}\)

We have previously shown \(fgf-2\) mRNA induction with MS and established that 15 minutes of exposure to 120 \(\mu\)strain of stress is similar to stress observed in human tibia during walking and light exercise.\(^{15,23}\) In this report, we elucidated the mechanism behind stress induction of \(fgf-2\). We show that 120 \(\mu\)strain of MS significantly induces \(fgf-2\) mRNA expression in MC3T3-E1 cells as well as bone marrow osteoprogenitor cells (BMOp) isolated from Fgf2\(^{-/-}\) mice, and that exogenous FGF-2 was required for normal response of Fgf2\(^{-/-}\) BMOp cells. We also show that the protein synthesis for all three isoforms of FGF-2 (24, 22, and 18 kDa) and receptor FGF2R are significantly induced by application of stress. We analyzed the kinetics of mecha-notransduction and found that the induction of \(fgf-2\) mRNA is temporal and not biphasic in MC3T3-E1 cells. We show that the protein kinase A (PKA) and ERK pathways are involved in \(fgf-2\) mRNA induction. Finally, we show that FGF-2 is necessary for a full proliferative response to MS.

**MATERIALS AND METHODS**

**Materials**

\(\alpha\)-MEM was purchased from Fisher Scientific (Pittsburgh, PA, USA). U0126 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Taq DNA polymerase, RNase inhibitor, and MuLV Reverse Transcriptase were purchased from Perkin Elmer (Branchburg, NJ, USA). Oligonucleotides were purchased from Operon Technologies (Alameda, CA, USA). Supersignal Pico Enhanced Chemiluminescence kits were purchased from Pierce (Rockford, IL, USA). CyQuant Kit was purchased from Molecular Probes (Eugene, OR, USA). Polyclonal FGF-2 and FGFFR2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St Louis, MO, USA). FBS was purchased from Hyclone (Logan, UT, USA). All other reagents were from Sigma-Aldrich.

**Animal protocols**

Fgf2\(^{-/-}\) and Fgf-2\(^{-/-}\) mice were developed by Zhou et al.\(^{24}\) Breeder pairs of FGF-2 knockout Fgf2\(^{-/-}\) and Fgf-2\(^{-/-}\) mice were a kind gift from Dr Regina Armstrong (University of the Health Sciences, Bethesda, MD, USA). C57BL6 wildtype (Fgf2\(^{+/+}\)) mice were purchased from Harlan UK (Bicester, Oxon, UK). Mice were fed standard laboratory rat chow (22% Rodent Diet [W]; Harlan Teklad, Madison, WI, USA) containing 1.13% calcium and 0.94% phosphorus, and maintained on a 12:12-h light-dark cycle. Six- to 8-week mature Fgf2\(^{-/-}\) and Fgf-2\(^{-/-}\) mice were bred to obtain Fgf2\(^{-/-}\) offspring. Genotyping was performed to identify the Fgf2\(^{-/-}\) mice. These studies were approved by the Animal Use Committee of the San Francisco Veterans Affairs Medical Center where the studies were performed.

**Genotype procedure**

A short segment of the tail from each mouse was removed with scissors and digested in lysis buffer, and DNA was isolated according to Greenberg et al.\(^{25}\) The sequences of the synthetic oligonucleotides used in PCRs were taken from The Jackson Laboratory (Bar Harbor, ME, USA); their sequences are as follows (5\'-3\')—\(fgf-2\) forward, CGAGAAGAGCGACCCACAC; \(fgf-2\) reverse, CCGAGTTCCGGGACCTATT; hypoxanthine phosphoribosyltransferase (\(Hprt\)) forward, CAAAGAAGCTTATAACCCC; and \(Hprt\) reverse, TAGCGATGATGAACCGG.

**BMOp cell culture**

Mice 8–12 weeks of age were anesthetized and killed by inhalation of isoflurane, followed by cervical dislocation. The tibia and femur were isolated. The tibial and femoral BMOp cells predominantly express mRNA-marking osteoblast differentiation such as alkaline phosphatase and osteocalcin.\(^{26}\) Thus, BMOp cells showing alkaline phosphatase enzyme activity and mineralization\(^{27}\) were used in our study. BMOp cells were harvested using techniques from Sakata et al.\(^{27}\) The marrow was collected in \(\alpha\)-MEM medium (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Hyclone), 100 U/ml penicillin/streptomycin (UCSF Cell Culture Facility, San Francisco, CA, USA), and l-glutamine (Mediatech, Herndon, VA, USA). A single cell suspension was obtained by repeated passage through an 18-gauge needle. The cells were plated at 6.0 × 10\(^6\) cells/well in 6-well plates. Nonadherent cells were removed by aspiration on day 3, and the medium was replaced every 2 days. On day 7, cells were downregulated with 0.7% FBS \(\alpha\)-MEM media for 24 h. MS experiments were performed on day 8.

**Cellular G-load apparatus**

A Damon/IEC model UV centrifuge was modiﬁed into a cellular G-load apparatus (CGA) as previously described.\(^{14}\)
Cell culture

In this study, we used an early passage osteogenic MC3T3-E1 cell line, which has the capacity to differentiate into osteoblasts and osteocytes and to form calcified bone tissue in vitro to compare with normal osteoblasts in vivo. Our laboratory has maintained the MC3T3-E1 cells in an early passage number to maintain its osteoblastic characteristics. Cells were plated and grown as previously described. MC3T3-E1 cells were pretreated for 60 minutes with various agents as described in the figure legends, before the 15-minute load period of mechanical stress with centrifugal forces of 120 µstrain, and then incubated for a further 15 minutes at 1g (unless otherwise indicated) before sample collection. Cell counts were performed in a ZBI Coulter Counter or by hemacytometer (Improved Neubauer, Reichert, NY, USA). Ten micromolar of U0126 or H-89 was added 1 h before stimulation.

Immunoblot analysis

Cells were lysed 15 minutes after the end of mechanical stress stimulation, using lysis buffer as previously described. Membranes were probed with either anti-FGF-2 or FGFR2 goat polyclonal antibodies (Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence (Supersignal Pico kit) and exposed on autoradiography film. Films were scanned, and bands were quantified with UNSCANIT densitometry software (Silk Scientific).

RNA isolation

RNA was isolated through the use of the RNasey Mini kit (Qiagen, Valencia, CA, USA) or TriReagent according to the manufacturer’s protocol. For RNasey Mini kit RNA isolation, cells were seeded in 6-well plates with α-MEM media supplemented with 10% FCS and downregulated and activated as indicated in the figure legends. Cells are lysed using 350 µl of buffer RLT (supplied in kit) containing 2-mercaptoethanol (Biorad, Hercules, CA, USA). The lysate is placed into QIAshredder homogenizer (Qiagen) and centrifuged at 12,500 rpm for 2 minutes. Three hundred fifty microliters of 70% ethanol was added to the flow through, mixed, and centrifuged in the RNasey Mini column (supplied in kit) for 15 s at 12,500 rpm. Flow through was discarded, and the column was washed with 700 µl of buffer RW1 (supplied in kit) for 15 s at 12,500 rpm. Two additional washes were performed with 500 µl of buffer RPE (supplied in kit) at 12,500 rpm for 15 s and 2 minutes, respectively. The flow through was discarded, and the column was placed in a sterile 1.5-ml collection tube. Depending on the expected yield, 20–50 µl RNAse-free water was pipetted into the column and centrifuged for 1 minute at 12,500 rpm. The samples were stored at −80°C until further analysis. For TriReagent RNA isolation, cells were lysed directly with TriReagent, and RNA was isolated according to the manufacturer’s directions.

Reverse transcription

RNA (1.5 µg) was added to 30 µl RT reaction buffer containing 5 mM MgCl₂, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1 mM dNTPs, 2.5 µM oligo d(T) primer, 2.5 U/µl of MuLV, and 1 U/µl of RNase inhibitor. The RT reaction was incubated at room temperature for 10 minutes, 42°C for 30 minutes, inactivated at 99°C for 5 minutes, and cooled at 5°C for 5 minutes.

Real-time quantitative RT-PCR reaction

2 µl of cDNA from the RT reaction is added to 20 µl real-time quantitative PCR (qPCR) mixture containing 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 12 pmol of oligonucleotide primers. PCRs were carried out in an Applied Biosystems (ABI) Prism 7900HT Sequence Detector System (SDS; Applied Biosystems). The thermal profile was 50°C for 2 minutes, 95°C for 10 minutes to activate the Taq polymerase, followed by 50 amplification cycles, consisting of denaturation at 95°C for 100 s, annealing at 63°C for 70 s, and elongation at 72°C for 100 s. Fluorescence was measured and used for quantitative purposes. At the end of the amplification period, melting curve analysis was performed to confirm the specificity of the amplicon. RNA samples were normalized to 18S rRNA internal standard. Relative quantification of gene expression was calculated by using the 2^−(Ct gene T−Ct 18S T)−(Ct gene 0hr−Ct 18S 0hr) equation, where Ct gene T represents the calculated threshold cycle (Ct) of a time-point of each sample other than 0 h or each treatment other than control. Primer sequence information has been previously published. All data derived using qRT-PCR was from independent biological samples (n = 3–4).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was previously described. Semi-quantitative RT-PCR was accomplished by varying the number of PCR cycles. PCR conditions were established so that the amplification reaction was stopped in the linear range. RT was performed using 1.5 µg RNA. The following PCR cycles were used: 18S, 24 cycles (1.5 or 3 µg RNA); fgf-2, 32 cycles (3 µg RNA). Primer information has been previously published. All data derived using semi-quantitative RT-PCR were from independent biological samples (n = 3–4).

Immunofluorescence analysis

Cells on coverslips were fixed in 3.7% formaldehyde in PBS and maintained at 4°C until subsequent processing. Incubation with primary antibodies were performed in PBS for 30 minutes, washed 10 times with ddH₂O, and incubated for 30 minutes with goat-anti-rabbit Alexa Fluor 488 secondary antibodies, also in PBS. Cells were also stained with Hoechst 33258 (Calbiochem, San Diego, CA, USA) for visualizing the nucleus. Cell-specific proteins were imaged using a Zeiss AxioScope Fluorescent Microscope (Carl Zeiss) and an Orca-ER CCD camera (Hamamatsu, Bridgewater, NJ, USA).

Cell proliferation assay

One thousand MC3T3-E1 cells were plated on 96-well plates in α-MEM medium containing 10% FCS. Once attached, the were grown in α-MEM medium containing 4%
FCS for 66 h. The cells were treated with 2 ng/ml FGF-2, FCS, or subjected to 15 minutes of 120-μstrain MS. Cells were incubated for 24 h at 37°C with 5% CO₂. Cells were frozen, and cell number was quantified using the CyQuant Kit according to the manufacturer’s protocol.

BMOp cells (6 × 10⁵/well) were added to 96-well plates. Nonadherent cells were removed by aspiration on day 3, and the medium was replaced every 2 days. On day 7, cells were downregulated with 0.7% FBS/H9251-MEM media for 24 h. On day 8, cells were treated with 2 ng/ml FGF-2 or subjected to 15 minutes of 120-μstrain MS. Cells were incubated for 48 h in a 37°C incubator with 5% CO₂. Cells were frozen, and cell number was quantified using the CyQuant Kit according to the manufacturer’s protocol.

**Statistical analysis**

All experiments were performed at least twice with independent biological samples (n = 3–4) unless otherwise specified. Results from a representative experiment are shown. All statistical p values are from either a two-tailed Student’s t-test or ANOVA depending on appropriateness.

**RESULTS**

Egr-1, fgf-2, cox-2, and c-fos are regulated by mechanical stress in a temporal, nonbiphasic manner

We have previously shown that a MS of 120 μstrain for 15 minutes induces egr-1, c-fos, cox-2, and fgf-2 at 30 minutes. In this study, we investigated the temporal induction of these four genes. We examined the gene expression of egr-1, fgf-2, cox-2, and c-fos at 0, 15, 30, 45, 60, and 120 minutes from the start of a 15-minute treatment of MC3T3-E1 cells with 120 μstrain (where t = 0 at beginning of 15-minute 120-μstrain treatment). qRT-PCR showed that egr-1 expression was induced within 30 minutes and peaked at 45 minutes. Fgf-2 expression peaked as early as 30 minutes and gradually decreased by 120 minutes. Cox-2 expression was induced within 30 minutes and peaked at 45 minutes. c-fos expression was induced at 30 minutes and peaked at 45 minutes (Fig. 1).

Mechanical stress induces all three isoforms of FGF-2

FGF-2 isoforms were analyzed from mechanically stressed and unstressed MC3T3-E1 cells, 1 h after stimulation (Fig. 2). Samples were analyzed for FGF-2 using specific antibody to FGF-2. All three isoforms were seen in the samples. It is noteworthy that all three isoforms were seen in the MS samples in a significantly higher concentration than the unstressed cells (p < 0.05). All three isoforms were increased by MS. The ratio between them did not vary under stressed or nonstressed condition; this was expected because they are all translated from the same mRNA sequence with different start codons.

Mechanical stress regulates protein expression of FGF-2 and its receptor FGFR2

There are four known receptors in the FGF family: FGFR1–FGFR4. To further study FGF-2 signaling by MS, protein expression analysis was performed by Western blot using anti-FGF-2 and anti-FGFR2 antibodies (Santa Cruz). The 22-kDa isoform of FGF-2 was measured. As
shown in Fig. 3, FGF-2 was significantly increased ($p < 0.05$) by 30 minutes, whereas FGFR2 was increased by 60 minutes after stress ($p < 0.0001$).

Fgf-2 mRNA upregulation is caused by new synthesis and not stability of mRNA half-life

The half-life as a function of stability of fgf-2 mRNA has been reported to be altered by some agonists.\(^{(33–35)}\) To determine whether the upregulation of fgf-2 mRNA observed after loading was caused by new synthesis of mRNA or increased stability of mRNA, we treated MC3T3-E1 osteogenic cells with actinomycin D (Act D), a RNA synthesis inhibitor, before MS. Fgf-2 mRNA was examined over several time-points. With the addition of Act D, the observed upregulation of fgf-2 mRNA was significantly reduced (Fig. 4A). This suggests that the upregulation of fgf-2 mRNA is caused by new synthesis and not altered stability of mRNA.

Fgf-2 mRNA induction does not require new protein synthesis

To further examine the induction of fgf-2 mRNA, cycloheximide (CHX), a protein synthesis inhibitor, was added to MC3T3-E1 cells 30 minutes before 15 minutes of 120-μstrain MS. Even in the absence of new protein synthesis, fgg-2 was significantly induced compared with controls (Fig. 4B). The observed higher levels of fgg-2 message with CHX treatment may be caused by absence of protease produc-

Egr-1, cox-2, c-fos, and fgg-2 are regulated by PKA and ERK

To study whether the induction of fgg-2, cox-2, egr-1, and c-fos is dependent on the MAPK or PKA pathway, we used the specific MAPK/ERK kinase 1/2 (MEK1/2) inhibitor,
Visualization of mechanically induced FGF-2

To further understand the induction of FGF-2 protein, the increase of FGF-2 protein in the cell was visualized using immunofluorescent microscopy. MC3T3-E1 cells were mechanically stressed for 15 minutes at 120 μstrain (12g). After 45–60 minutes, an increase amount of FGF-2 protein was seen (Fig. 6).

Mechanical stress induces proliferation; stress-induced proliferation is mediated in part by FGF-2

To study the proliferative effects of mechanical stress, MC3T3-E1 cells were stressed (15 minutes at 120 μstrain), and cell proliferation was analyzed 24 h later. We found that the consequence of a MS pulse was a 3.5-fold increase in additional cell number after MS compared with growth in nonstressed cells. It is also noteworthy that nonstressed controls treated with 2 ng/ml of FGF-2 resulted in a 2.2-fold increase in proliferation compared with nontreated control cells (Fig. 7).

To determine whether the proliferation response was present in other osteoprogenitor cells and to see if FGF-2 was required for stress-induced proliferation, we isolated BMOp cells from Fgf2+/− and Fgf2−/− mice and applied 120 μstrain. Our results show that Fgf2+/− BMOp cells have a very similar MS proliferative response compared with the MC3T3-E1 cells with a 1.7-fold increase in proliferation (Fig. 7C).

We further elucidated the role of FGF-2 in MS-induced proliferation in osteoblast-like cells using BMOp cells isolated from Fgf2−/− mice. Without endogenous FGF-2, MS caused a 13% induction of Fgf2−/− BMOp cell growth ($p = 0.02$). The addition of exogenous FGF-2 significantly increased the proliferation strikingly by 95% in stressed Fgf2−/− BMOp cells ($p < 0.0001$; Fig. 7B).

DISCUSSION

Bone undergoes a constant process of remodeling in which mass is retained or lost in response to the relative activity of osteoblasts and osteoclasts. Weight-bearing exercise is critical for retaining skeletal integrity and promotes osteoblast function, whereas the lack of mechanical stimulation, as seen during space flight or prolonged bedrest, can lead to osteoporosis. We have previously mathematically characterized a compression model of MS(5) and have extensively reviewed the field of in vitro mechanical stress models. We have compared the various models of mechanical stress and found that we are working at physiological levels as first described by Burr et al.(23) Burr et al. measured in vivo strains on the tibias of two subjects through implanted strain gauges under conditions similar to those experienced by Israeli infantry recruits. Principal compressive and shear strains were greatest for uphill and downhill zigzag running, reaching nearly 2000 μstrain in some cases, which is about three times higher than recorded during walking. In comparison, our compression model in this study never exceeded 15 minutes of 120 μstrain. At the cellular level, mechanical stress is primarily from compression, with fluid shear and hydrostatic pressure playing secondary roles as previously described. In our review of signal transduction and mechanical stress, we found that...
both fluid shear stress (800–2000 μstrain) and our gravity model (120 μstrain) have similar mechanisms of action. In the human femur, vigorous exercise generates six times higher forces than standing. The frequency and duration of mechanical loading required to maintain bone mass in vivo and stimulate osteoblast growth in vitro have been measured experimentally. Hind limb suspension, where the rat or mouse is suspended by the tail preventing the hind limbs from being loaded, is used as an animal model of bone disuse. Hind limb suspension of the C57BL/6 mouse strain resulted in decreased bone formation rates, decreased alkaline phosphatase activity, and decreased bone mass in the femur. In chickens, as little as 15 minutes of exercise per day was sufficient to maintain bone mass, whereas in a rat hind limb suspension model, 10 minutes per day of mechanical loading over a 28-day period was sufficient to maintain 50% of the bone mass compared with normally loaded animals. Therefore, short duration (~15 minute) mechanical loading seems to be an effective stimulus of bone growth in vivo, and this period of MS was used throughout our study.

A brief exposure to 120 μstrain of MS induces the expression of immediate-early genes, c-fos, egr-1, and cox-2, in a temporal, nonbiphasic process in MC3T3-E1 cells. Cell cycle progression and proliferation have been shown to be regulated by c-fos expression in some cell types, including osteogenic cells. The egr-1 transcription factor is induced by both EGF and PGE₂, and cyclic strain forces have been shown to induce expression of egr-1. Egr-1 is known to regulate fgf-2, which in turn induces expression of prostaglandin G/H synthase-2 (PGH-2), also known as Cox-2, an important enzyme involved in prostaglandin synthesis and bone growth. These genes promote proliferation of osteogenic precursors and are all induced by stress.

MS induces the expression of genes that promote cell growth. There are several pathways that respond to different types of MS. In this report, we found the induction of c-fos was totally blocked by U0126 and H-89. At the concentrations used in the experiment, U0126 inhibited MEK1 and MEK2, and the inhibitor prevented activation of both ERK1 and ERK2. These data suggest that a significant fraction of the mechanical signal occurs through the ERK1/2 and PKA pathways. As shown in Fig. 5, the two inhibitors H-89 and U0126 inhibited the genes egr-1, cox-2, fgf-2, and c-fos. Both inhibitors brought the expression of fgf-2 to baseline levels, indicating that fgf-2 is equally mediated by ERK1/2 and PKA. However, for cox-2, egr-1, and c-fos, our data show that the induction of these genes is inhibited by U0126 to a greater extent than H-89; this indicates that these genes are regulated to a greater extent by the ERK1/2 pathway than the PKA pathway. Similarly, studies done by Samoto et al. showed that sialoprotein expression induced by PGE₂ was mediated by both cAMP and FGF-2 response elements.

All three isoforms of FGF-2 are translated from the same mRNA sequence with different start codons. The high molecular weight FGF-2 isoform contains a nuclear localization signal (NLS) and is more abundant in the nucleus. All three isoforms were encoded from the same mRNA sequence with different start codons. The egr-1 transcription factor is induced by both EGF and PGE₂, and cyclic strain forces have been shown to induce expression of egr-1. Egr-1 is known to regulate fgf-2, which in turn induces expression of prostaglandin G/H synthase-2 (PGH-2), also known as Cox-2, an important enzyme involved in prostaglandin synthesis and bone growth. These genes promote proliferation of osteogenic precursors and are all induced by stress.
was less in Fgf2−/− BMOp cells, with only an overall 13% increase, compared with ∼170% increase in growth in Fgf2+/+ BMOp cells and a 350% increase in MC3T3-E1 cells. The addition of FGF-2 to Fgf2−/− BMOp cells (Fig. 7B, bar 7) increased the overall growth from ∼50% in un-stressed (Fig. 7B, bar 5) to 95% in stressed cells (Fig. 7B, bar 8). The observed difference of 44% increase from un-stressed FGF-2–treated Fgf2−/− cells (Fig. 7B, bar 7) versus stressed FGF-2–treated Fgf2−/− cells (Fig. 7B, bar 8) indicates that FGF-2 plays a key role in the MS stimulation of proliferation of osteoprogenitor cells mediated by the FGF-2, MAPK, and PKA pathways. The observation that 2 ng/ml of exogenous FGF-2 was not as effective as 120/H9262 strain MS indicates that there are multiple pathways responsible for the MS-induced proliferative response in addition to FGF-2. (5) Because these studies use physiological levels of MS, the results suggest that FGF-2 is a key factor for full proliferation of osteoprogenitor cells mediated by the FGF-2, MAPK, and PKA pathways. The observation that 2 ng/ml of exogenous FGF-2 was not as effective as 120/H9262 strain MS indicates that there are multiple pathways responsible for the MS-induced proliferative response in addition to FGF-2. (5)

**FIG. 6.** FGF-2 protein is found in cytosol and nucleus after stress. MC3T3-E1 cells were cultured on coverslips and subjected to a 15-minute, 120-μstrain MS. Cells were fixed 30 minutes after the start of MS. Coverslips were stained for FGF-2 and Hoechst 33258 dye and imaged. A constant exposure was used to permit comparison between un-stressed control samples and stressed samples. Images shown for both FGF-2–stained cells and Hoechst 33258 dye–stained cells are from same field.

**FIG. 7.** FGF-2 is required for full proliferative response to MS. (A) MC3T3-E1 cells were seeded and downregulated. After downregulation, 2 ng/ml of recombinant FGF-2 was added to selected wells (bar 3), FCS samples were treated with 5% FCS as a positive control (bar 4), and the stressed samples received 120 μstrain for 15 minutes (bar 2). (1) Nonstressed control, (2) 120 μstrain, (3) nonstressed plus 2 ng/ml FGF-2, (4) 5% FCS. Each bar represents cell number mean ± SD (n = 21) of independent biological samples. ***p < 0.0001 relative to control. (B) Fgf-2−/− BMOp was seeded and downregulated. Recombinant FGF-2 (2 ng/ml) was added to selected wells at the beginning of stress (bars 7 and 8), stressed samples received 120 μstrain for 15 minutes (bars 6 and 8). (5) Nonstressed control, (6) 120 μstrain, (7) nonstressed plus 2 ng/ml FGF-2, (8) 120 μstrain plus 2 ng/ml of FGF-2, (9) 5% FCS. Bars represent mean ± SD (n = 24) of independent biological samples. *p < 0.05; ***p < 0.0001 relative to nonstressed control with two-tailed Student’s t-test. (C) Fgf2+/+ wildtype BMOp were seeded and downregulated. Recombinant FGF-2 (2 ng/ml) was added to selected wells at the beginning of stress (bars 7 and 8), stressed samples received 120 μstrain for 15 minutes (bars 6 and 8). (5) Nonstressed control, (6) 120 μstrain, (7) nonstressed plus 2 ng/ml FGF-2, (8) 120 μstrain plus 2 ng/ml of FGF-2, (9) 5% FCS. Bars represent mean ± SD (n = 16) of independent biological samples. ***p < 0.0001 relative to nonstressed cells with two-tailed Student’s t-test. NS, not significant (p > 0.05).
proliferative response of osteogenic cells to MS and may be an important factor in treatment of osteoporosis caused by spaceflight or prolonged bedrest.

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Address reprint requests to:
Millie Hughes-Fulford, PhD
VA Medical Center
Mail Code 151F
4150 Clement Street
San Francisco, CA 94121, USA
E-mail: milliehf@aol.com

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