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Effect of Overexpressing Fibroblast Growth Factor 2 Protein Isoforms in Osteoblastic ROS 17/2.8 Cells

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Abstract Fibroblast growth factor-2 (FGF-2) is made by osteoblasts and modulates their function. There are high molecular weight (HMW) protein isoforms of FGF-2 that have nuclear localization sequences and a low molecular weight (LMW) 18 kDa FGF-2 protein that is exported from cells. Since FGF-2 is a trophic factor and potent mitogen for osteoblasts, the goal of this study was to utilize targeted overexpression of FGF-2 as a novel means of assessing different FGF-2 isoforms on osteoblastic cell viability and proliferation. Either LMW or HMW human Fgf2 cDNAs were cloned downstream of 3.6 kb α1(I)—collagen 5′ regulatory elements (Col 3.6). A set of expression vectors, called Col3.6-Fgf2 isoforms-IRES-GFPsaph, capable of concurrently overexpressing either LMW or HMW FGF-2 isoforms concomitant with GFPsaph from a single bicistronic mRNA were built. Viable cell number in ROS 17/2.8 cells stably transfected with Vector (Col3.6-IRES-GFPsaph) versus each of the Col3.6-Fgf2-IRES-GFPsaph constructs were compared. In the presence of 1 or 10% serum, DNA synthesis was increased in cells expressing any isoform of FGF-2 compared with vector. However, cells transfected with HMW isoform had augmented DNA synthesis in 1 or 10% serum compared with cells expressing either ALL or LMW FGF-2 isoforms. A neutralizing FGF-2 antibody significantly reduced the mitogenic response in cells harboring ALL or the LMW FGF-2 isoforms but did not block the mitogenic effect of cells harboring the HMW isoforms. In summary, overexpression of any isoform of FGF-2 protein increased viable cell number and OB proliferation in the presence of low or high concentrations of serum. However, the HMW/nuclear isoforms preferentially mediate augmented OB proliferation. We conclude that differential expression of FGF-2 proteins isoforms is important in modulating OB function. J. Cell. Biochem. 89: 1291–1301, 2003.

Key words: osteoblasts; fibroblast growth factor 2 protein isoforms; survival; proliferation; green fluorescent protein

Signal transduction by FGF-2 occurs in an intracrine, paracrine, and/or autocrine manner. On the cell surface, at least two types of receptors interact with FGFs including low-affinity heparan sulfate, and high-affinity receptor tyrosine kinases. Interaction of ligand/receptor results in receptor dimerization, autophosphorylation, and activation of multiple second messengers including protein kinase C (PKC), MAP kinases, and extracellular signal-regulated kinase 2 (ERK2). FGF-2 also functions in an "intracrine" manner since high molecular weight (HMW) isoforms have nuclear localization sequences. FGF-2 has multiple effects on bone [Hurley et al., 2002]. It stimulates osteoblast replication decreases differentiation markers: including alkaline phosphatase and type 1 collagen and stimulates osteoclast formation and bone resorption [Globus et al., 1988; Rodan et al., 1989; Hurley et al., 1998; Kalajzic et al., 2003]. Intermittent treatment stimulates bone formation in vitro [Hurley et al., 2002], as well as in vivo [Mayahara et al., 1993; Nagai et al., 1995]. Fgf2 mRNA and protein levels in

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osteoblasts are regulated by parathyroid hormone (PTH), transforming growth factor beta (TGFβ), prostaglandins (PGs), and interleukin-1 (IL-1) [Hurley et al., 2002].

FGF-2 is encoded by a single copy gene, however, multiple isoforms of FGF-2 have been detected [Davis et al., 1997]. Each isoform is a primary translation product with no precursor–product relationship. Translation of each the three HMW human FGF-2 isoforms (22, 23, 24 kDa) is initiated with an unconventional CUG translation initiation codon. In contrast, translation of the low molecular weight (LMW, 18-kDa) isoform is initiated with a classical AUG codon located downstream of the CUG codons [Florkiewicz and Sommer, 1989; Florkiewicz et al., 1991; Quarto et al., 1991]. In humans, there are three nuclear isoforms of 22, 23, and 24 kDa and a LMW 18 kDa FGF-2 protein that is exported from cells [Florkiewicz and Sommer, 1989]. In rodents, there are two nuclear isoforms of 19, 21 kDa and a LMW, 17.5 kDa FGF-2 protein that is exported from cells. We previously reported that global deletion of all isoforms of FGF-2 protein resulted in decreased bone mass in older mice [Montero et al., 2000]. In contrast, nontargeted overexpression of all isoforms of human FGF-2 protein resulted in murine dwarfism [Coffin et al., 1995]. However, the differential effects of HMW/nuclear versus LMW extracellular FGF-2 on OB function has not been elucidated.

Our goal is to develop a system that will allow us to study the effects of FGF-2 isoforms in bone. The 3.6 kb Col1a1 5′ regulatory elements are active in a wide variety of type I collagen-producing tissues that include pre-osteoblasts and osteoblasts. They allow specific expression of candidate cDNAs of interest as well as marker genes in type I collagen-producing tissue. The same Col3.6 elements linked to green fluorescent protein (GFP) were previously utilized to identify subpopulations of cells at different stage in the osteoblast lineage and shown to be specifically expressed in Col1a1-producing tissues such as bone and skin [Dacic et al., 2001; Kalajzic et al., 2002]. Utility of the 3.6 kb Col1a1 regulatory elements should allow us to determine the role of endogenous FGF-2 on bone development and remodeling in a tissue-targeted manner. Therefore, the purpose of this study was to test whether HMW and LMW isoforms of FGF-2 have differential effects on osteoblast growth. The present study describes a novel approach that can be used to study the effect of FGF-2 isoforms specifically in osteoblasts or bone.

MATERIALS AND METHODS

Cloning of Expression Plasmids

To elucidate the role of endogenous FGF-2 isoforms in a bone specific manner, we generated a set of expression vectors, called Col3.6-Fgf2 isoforms-IRES-GFPsaph. Col3.6-Fgf2 isoforms-IRES-GFPsaph, were built by replacing a CAT fragment in previously made Col3.6-CAT-IRES-GFPsaph with individual human Fgf2 isoform cDNA between Afe I and Sca I sites. These individual expression vectors are capable of concurrently overexpressing individual FGF-2 isoforms [Florkiewicz and Sommer, 1989; Florkiewicz et al., 1991] and GFPsaph from a single bicistronic mRNA. The vector also harbor a neomycin selection gene. The Fgf2 cDNAs were kindly provided by Dr. Robert Florkiewicz. The following constructs were generated (Fig. 1): Col3.6-IRES/GFP (Vector); Col3.6-Fgf2-ALL-isoforms-IRES/GFP (ALL = 24, 23, 22, 18 kDa); Col3.6-Fgf2-LMW-isoform-IRES/GFP (18 kDa); Col3.6-Fgf2-24, 23, 22 kDa-isoforms-IRES/GFP (HMW); or Col3.6-Fgf2-24 kDa IRES/GFP (24 kDa).

Transient Transfection of Human 293GPG Cells and Stable Transfection of ROS 17/2.8 Cells

For transient tranfection, human kidney 293-GPG cells were plated in 6-well dishes (Costar, Cambridge, MA) at a density of 5×10^5/well 24 h before transfection. Cells were incubated over night at 37°C in 5% CO_2 with 10 μl LipofectAMINE™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA) and a total of 3 μg plasmid DNA/well in medium. The transfection medium was removed and replaced with DMEM Reagent (Invitrogen Life Technologies) containing 10% FBS (Life Technologies, Grand Island, NY). The experiments were performed 48 h later.

For stable transfections, ROS 17/2.8 cells were plated in 6-well dishes at a density of 5×10^5/well 24 h before transfection and were then incubated overnight at 37°C in 5% CO_2 with 10 μl LipofectAMINE 2000 Reagent. A total of 3 μg expression plasmid (Fig. 1) DNA was used. Cells were grown in 10% FBS/F-12 medium for 48 h, then switched to F-12 medium (Invitrogen Life Technologies, containing 10%

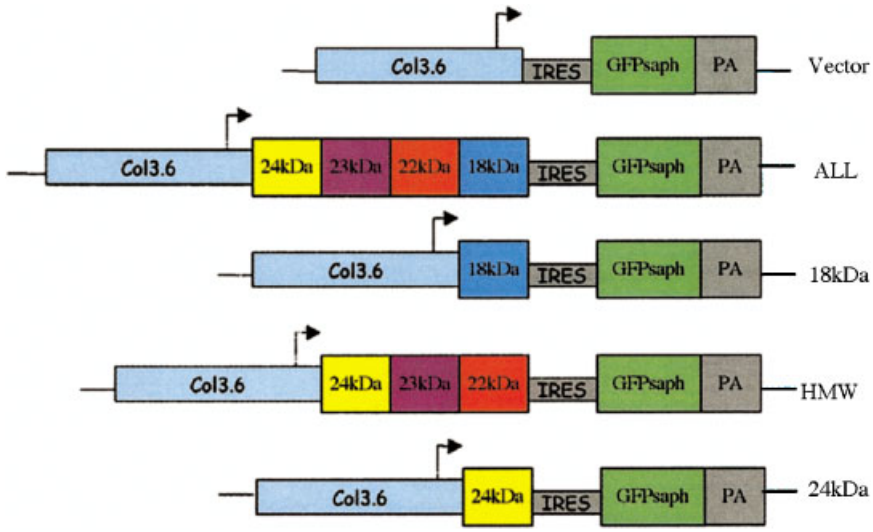


Fig. 1. Schematic diagram of plasmid constructs used in this study. The construction of 3.6Col-Fgf2isoforms-IRES-GFPsaph plasmids is detailed in Materials and Methods. The individual expression vectors are capable of concurrently overexpressing individual Fgf2 isoforms and GFPsaph from a single bicistronic mRNA. The vector also harbor a neomycin selection gene. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FBS, penicillin/streptomycin, and G418 (200 μg/ml; Invitrogen Life Technologies) as the selection antibiotic. Transfected cells were then selected with G418 (200 μg/ml) for a month. Stably transfected ROS 17/2.8 cells were then cultured in F-12 medium containing 10% non-heat-inactivated FBS, 200 μg/ml of G418, and a penicillin/streptomycin cocktail. Medium was changed every 3 days. Cells were trypsinized and passaged at a 1:4 ratio every 6 days.

Reporter Assays

The expression of GFP reporter gene in transfectants was detected under fluorescent microscopy. GFP expression in cell culture was visualized using an Olympus IX50 inverted system microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus America, Inc., Melville, NY). A specific excitation wavelength was obtained using filters for GFPsaph (exciter, D395/40; dichroic, 425DCLP; emitter, D510/40m) and recorded with an SPOT-camera (Diagnostic Instrument, Inc., Sterling Heights, MI). Fluorescent images were taken with equal exposure times applied to cells derived from different transgenic constructs.

Transgenes were confirmed by polymerase chain reaction (PCR). The presence of the *GFP* gene was detected by PCR using a set of primers from the middle region of the GFP: 5′-TCATCTGCACCACCGGCAAGC-3′ and 5′-AGCAGGACCATGTGATCGCGC-3′, which yield a fragment of 525 bp. DNA was isolated from stably transfected ROS 17/2.8 cells. PCR was performed in a final volume of 50 μl containing 0.2 μg of DNA and 1.25 U Taq DNA polymerase, 1.25 mM 4dNTP mix; 5 μl amplification buffer, 1.5 μl of 50 mM $CaCl_2$, 10 μl primers, 20–40 pmol of each. PCR was conducted using standard conditions on an GeneAmp PCR System 9700 (Brinkman Instruments Ltd., Westbury, NY): 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 40 s. Prior to the first cycle, initial denaturation was performed at 94°C for 3 min, and the last cycle was followed by an extension step of 5 min at 72°C.The amplification products were evidenced through 1.5% agarose gel electrophoresis. Gels were stained with ethidium bromide, and observed under UV light.

Regulation of Cell Growth and Gene Expression in ROS 17/2.8 Cells

To assess the effects of different FGF-2 isoforms on viable cell number, ROS 17/2.8 cells stably transfected with vector (Col3.6-IRES-GFPsaph) versus each of the Col3.6-Fgf2 isoform-IRES-GFPsaph constructs, were plated at 5,000/cm^2 in 96-well dishes in F-12 medium containing G418 (200 μg/ml) and either 1 or 10% FBS. After 24, 48, 72, or 96 h, cells were harvested. For the last 1 h of culture, 20 μl per well of MTT Reagent (Promega Corporation,

Madison, WI) was added and viable cell number was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance is directly proportional to the number of proliferating and living cells [Lucareli et al., 2002].

To assess the effects of overexpression of different FGF-2 isoforms on cell proliferation, thymidine incorporation into DNA in ROS 17/2.8 cells was measured. ROS 17/2.8 cells were plated at 4×10^4 cells/well in 6-well plates and cultured for 6 days in growth medium supplemented with 1 or 10% FBS. The cells were pulse-labeled with [^{3}H]thymidine 10 μCi/well for the last 2 h of the culture period, and the radioactivity in trichloroacetic acid-insoluble materials was counted [Ishimi et al., 1992]. Incorporation of [^{3}H]thymidine per milligram of DNA was calculated.

RNA Extraction and Northern Blot Analysis

For assessment of gene expression, stably transfected ROS 17/2.8 cells were plated at a density of 25,000 cells/cm^2 in 35-mm wells dish in complete culture medium (F-12 containing 10% FBS, 200 μg/ml G418, and a penicillin/streptomycin cocktail) and harvested at different time-point. Total RNA was prepared from cultured cells using TRIZOL reagent according to the manufacturer's instructions [Chomczynski and Sacchi, 1987; Kalajzic et al., 2002]. For Northern analysis, 20 μg of total RNA transferred onto nylon membrane [Towbin et al., 1979]. After a 4 h prehybridization, filters were hybridized overnight with a random primer deoxycytosine triphosphate (dCTP)-labeled [^{32}P]cDNA probe for the mRNA of interest. Then, membranes were exposed to photographic film (BioMax MR-1 film, Eastman Kodak Co., Rochester, NY) with an enhancing screen at −80°C. Prior hybridization signals were removed by washing in 0.5% SDS for 10 min at 80°C.The hybridization signal obtained with each cDNA was normalized to the signal obtained by hybridization with a GAPDH probe [Tso et al., 1985].

Western Blot Analysis

The expression of each FGF-2 isoform transgene in ROS 17/2.8 cells stably transfected with vector (Col3.6-IRES-GFPsaph) versus each of the Col3.6-FGF2isoforms-IRES-GFPsaph was compared by Western blot. Briefly, protein was extracted. After SDS–polyacrilamide gel electrophoresis on 12% gels, protein were transferred to Immobilon™ Transfer Membranes (Millipore; pore, 0.45 μm; Millipore Corporation, Bedford, MA). Membranes were blocked overnight in 1× TBS containing 5% nonfat dry milk (Carnation Co., Los Angeles, CA) and 0.1% Tween-20 (Sigma Chemical Co., St. Louis, MO). Incubation with primary antibody and followed by a rabbit antimouse secondary antibody (Amersham-Pharmacia Biotech, Piscataway, NJ) was performed in 1× TBS containing 1% nonfat milk and 0.1% Tween-20 for 1 h. After incubation with antibodies, membranes were washed 1 h in 1× TBS containing 0.1% Tween-20. The primary antibody generated from human FGF-2 was a mouse monoclonal antibody (BD Bioscience, San Diego, CA). This antiserum recognizes all FGF-2 forms. Western Lighting™ Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, MA) was used for detection. Signal was detected by KODAK X-OMAT autoradiography. Band density was quantitated densitometrically.

Indirect Immunofluorescence

Stably transfected ROS 17/2.8 cells were plated at a density of 8,000 cells/chamber in an eight-chamber slide system and grown for 24 h in 10% FBS medium. Cells were washed with PBS, fixed, and permeabilized with 4% paraformaldehyde and 0.18% Triton X-100 (Sigma Chemical Co.) in PBS for 10 min at room temperature. After washing the slides twice with PBS for 3 min each, the cells were blocked for 30 min with 1% BSA in PBS. Permeabilized cells were incubated for 1 h in a humid chamber at 37°C with monoclonal mouse antiserum against human FGF-2 (1:250 dilution). After three PBS washes, the cells were stained for 30 min in a humid chamber at room temperature with Alexa Fluor (Molecular Probes, Inc., Eugene, OR) of 1:250 dilution in PBS/1% BSA. After rinsing the samples several times in PBS, the slides were mounted by coverslide with Vectashield (Vector Laboratories, Burlingame, CA). The cells were observed and photographed in a Leitz Orthoplan microscope using epifluorescence illumination.

Statistical Analysis

Experiments were repeated at least once and representative data mean ± SEM are shown. Means of groups were compared using analysis of variance (ANOVA) and the Bonferroni

posthoc test when ANOVA showed significant differences. $P < 0.05$ was considered statistically significant.

RESULTS

To study the differential effects of LMW and HMW FGF-2 on OB, it was necessary to construct a set of expression vectors, called Col3.6-Fgf2 isoforms-IRES-GFPsaph, capable of concurrently overexpressing individual FGF-2 isoforms and GFPsaph from a single bicistronic mRNA in a tissue and lineage stage specific manner. Col3.6-Fgf2 isoforms-IRES-GFPsaph, were constructed by replacing a CAT fragment in previously made Col3.6-CAT-IRES-GFPsaph with individual human Fgf2 isoform cDNA between Afe I and Sca I sites. These individual expression vectors are capable of concurrently overexpressing individual FGF-2 isoforms and GFPsaph from a single bicistronic mRNA. As shown in (Fig. 1): the following constructs were generated Col3.6-IRES/GFP (Vector); Col3.6-Fgf2-ALL-isoforms-IRES/GFP (ALL = 24, 23, 22, 18 kDa); Col3.6-Fgf2-low-molecular-WT-isoform-IRES/GFP (18 kDa); Col3.6-Fgf2-24, 23, 22 kDa isoforms-IRES/GFP (HMW) or Col3.6-Fgf2-24 kDa IRES/GFP (24 kDa).

Analysis of Transgene Expression

In order to examine expression of GFP, cells were harvested at 5, 7, 10, and 14 days after plating and examined under a fluorescent microscope. GFP was expressed in 100% of cells derived from a single clone (Fig. 2A). GFP expression was confirmed by PCR. Figure 2B shows the presence of GFP transgene. ROS 17/2.8 cells stably transfected with Vector, ALL, 18 kDa, HMW, or 24 kDa expressed a 525 bp GFP transgene band and no band was detected in mock transfected ROS 17/2.8 cells.

Fgf2 mRNA Expression

We determined the time course of Fgf2 mRNA expression in stably transfected ROS 17/2.8 cells. A single Fgf2 mRNA transcript of about 4 kb was detected in stably transfected cells (Fig. 3A,B). Northern blot analysis showed a high level of expression of the Fgf2 transgene in all stably transfected ROS 17/2.8 cells expressing any isoform of FGF-2 at 4 and 21 days versus cells stably transfected with the control vector. At 4 days Fgf2 mRNA transcripts was increased 123, 93, 48, and 68 in cells transfected with ALL, 18 kDa, HMW, or 24 kDa Fgf2/GFP, respectively (Fig. 3A). At 21 days Fgf2 mRNA was increased 117, 48, 89, and 128% in cells transfected with ALL; 18 kDa, HMW; or 24 kDa Fgf2/GFP, respectively (Fig. 3B).

To investigate whether FGF receptors were modulated [Bikfalvi et al., 1995] differently in ROS 17/2.8 cells expressing the different isoforms of FGF-2, we examined FGFRs mRNA expression in stably transfected ROS 17/2.8 cells. There was no significant change in FGFR1, FGFR2, or FGFR3 expression (data not shown). There was a small reduction in

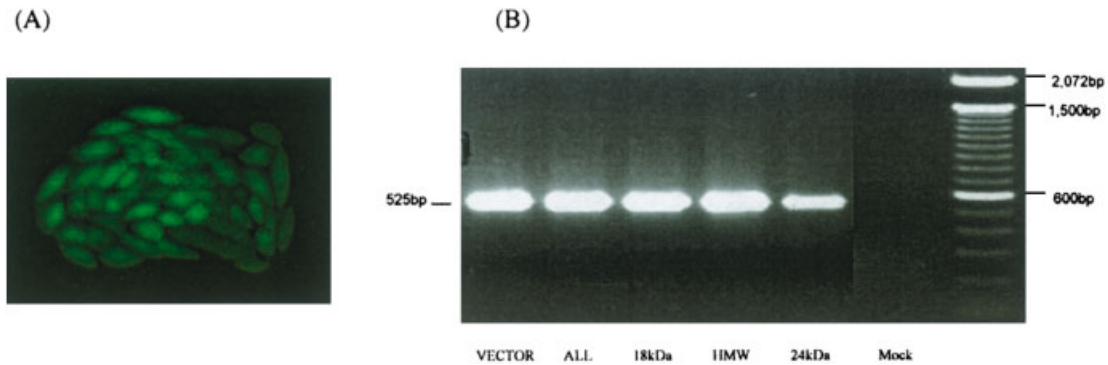


Fig. 2. Expression of GFP in transfected ROS 17/2.8 cells. **A**: GFP expression in a single clone of ROS 17/2.8 cells was monitored by fluorescent microscopy. Cells transfected with 3.6Col/Fgf2/GFP were examined 10 days after plating. **B**: Polymerase chain reaction (PCR) for presence of green fluorescent protein (GFP) transgene. ROS 17/2.8 cells were mock transfected or stably transfected with Col3.6/GFP (Vector); Col3.6/ALL-isoforms-Fgf2/GFP-(ALL: 24, 23, 22, 18 kDa); Col3.6/low molecular weight-isoform-Fgf2/GFP (18 kDa); Col3.6/high molecular weight isoforms Fgf2/GFP (HMW: 24, 23, 22 kDa), or Col3.6/24 kDa-Fgf2/GFP (24 kDa). Note that a 525 bp GFP transgene band was detected in all cell lines except for mock transfected ROS 17/2.8 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

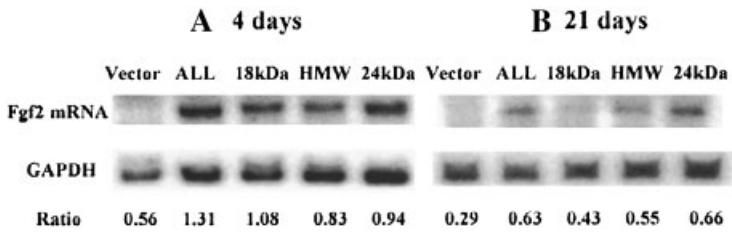


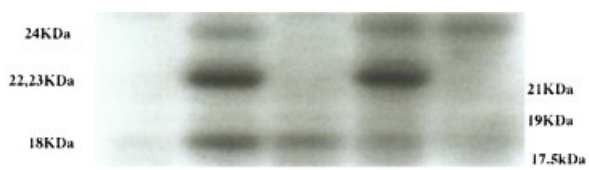
Fig. 3. Time course of the effect of overexpressing FGF-2 transgene on FGF-2 mRNA expression in ROS 17/2.8 cells. Cells were stably transfected with Vector; ALL-isoforms; 18 kDa; HMW; or 24 kDa isoform. Total RNA was extracted from cells at 4 days (**A**) and 21 days (**B**) for Northern blot analysis as described in Materials and Methods. Filters were probed for Fgf2 mRNA and results were normalized to GAPDH ratio.

FGFR4 mRNA expression in cells harboring the HMW and 24 kDa isoforms, This result, however, was not consistently observed.

FGF-2 Protein Isoform Expression

To confirm that human FGF-2 proteins were being overexpressed, we assessed transgene expression in 293GPG, a cell line developed from adenovirus 5-transformed human embryonic kidney 293 cells [Ory et al., 1996]. Expression of transgenic FGF-2 protein isoforms was higher in 293 GPG cells transiently transfected with ALL isoforms compared with control vector that expressed low levels of native FGF-2 protein isoforms (data not shown).

Transgenic FGF-2 protein isoforms were also detected in stably transfected ROS 17/2.8 cells (Fig. 4) by Western blot using human FGF-2 antibody. ROS 17/2.8 cells stably transfected with control vector (3.6COL/GFP) expressed low levels of native FGF-2 protein isoforms of 17.5, 19, and 21 kDa. Western blot analysis also showed overexpression of the human FGF-2 isoforms (ALL, 18 kDa, HMW, or 24 kDa) encoded by the respective transgenes compared to cells transfected with vector.



	Vector	ALL	18kD	HMW	24kD
24KDa	19.6	36.7	24.0	39.8	38.5
22,23KDa	21.9	47.5	25.9	48.2	24.6
18KDa	24.0	37.9	32.1	26.8	22.1

Fig. 4. Specific FGF-2 protein isoform expression in stably transfected ROS 17/2.8 cells. Cells were harvested 7 days after plating and Western blot analysis for FGF-2 protein was performed as described in Materials and Methods. Western blot analysis shows overexpression of the human FGF-2 isoforms encoded by the respective transgenes compared to cells transfected with Col3.6/GFP (Vector).

FGF-2 Protein Localization

Several investigators reported that the HMW isoforms of FGF-2 preferentially localized to the nucleus, in contrast to the 18 kDa isoform that was usually found in the cytoplasm or on the cell surface [Biro et al., 1994; Bikfalvi et al., 1995]. We, therefore, examined the cellular compartment for localization of FGF-2 protein isoforms by indirect immunofluorescence with an antibody against a sequence common to all isoforms of FGF-2. As shown in Figure 5, HMW FGF-2 isoforms localized to the nucleus while cells overexpressing 18 kDa FGF-2 displayed only cytoplasmic staining [Arese et al., 1999]. The nuclear staining varied both in intensity and homogeneity. This may relate to the position of the cell in the cell cycle and the overall degree of synchronization of each culture.

Cell Proliferation

Because FGF-2 has been shown to increase cell proliferation in several types of cells, we studied whether a 5′ regulatory fragment of the Col3.6 gene regulating Fgf2 expression could induce cell proliferation in osteoblastic cell line (ROS 17/2.8). An MTT assay was used as an initial screening technique [Lucareli et al., 2002]. ROS 17/2.8 cells were plated in 96-well plates at a density of 5,000 cells/well in F-12 medium containing 200 μg/ml of G418 and either 1 or 10% FBS. After 24, 48, 72, and 96 h cells were harvested and viable cell number was measured by MTT proliferation assay. Absorbance is directly proportional to the number of proliferating and living cells. Ninety-six hours after plating, in the presence of 1% serum, viable cell number was increased between 39

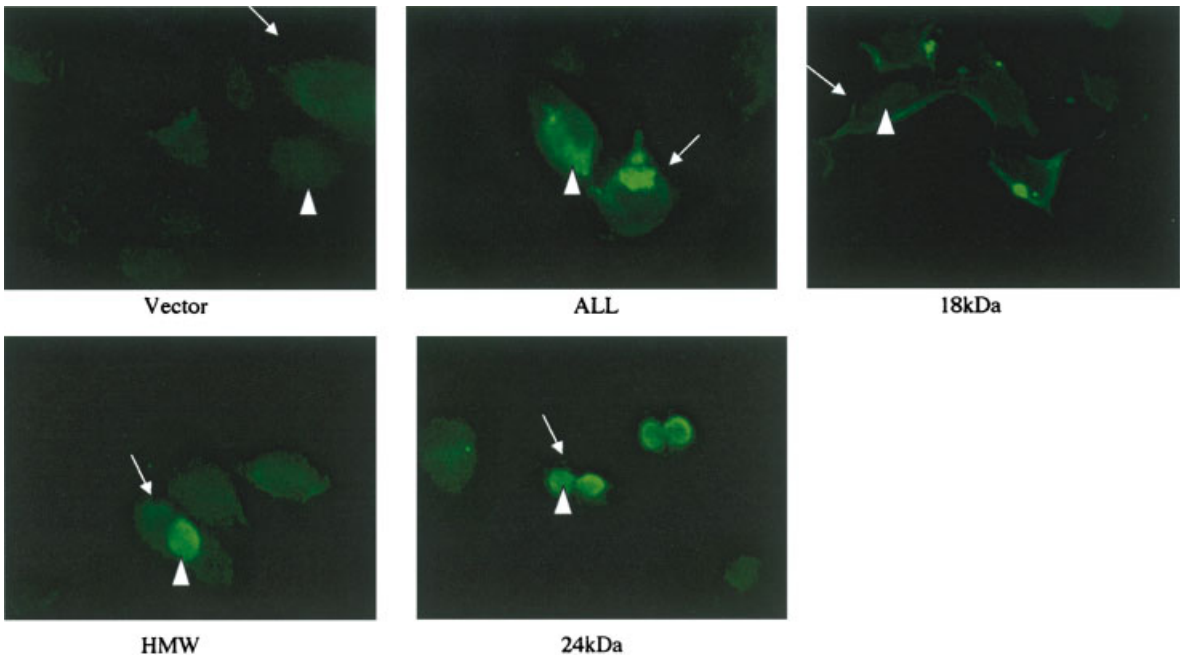


Fig. 5. Immunofluorescence localization of FGF-2 isoforms. Cells were plated at 8,000 cells/chamber in eight-chamber slide and grown for 24 h in 10% FBS medium. Indirect immunofluorescence was performed as described in Materials and Methods using a monoclonal antibody against human FGF-2 and Alexa Fluor. In cells expressing ALL isoforms, FGF-2 appears to be present in both the nuclei and cytoplasm. In cells expressing the 18 kDa isoform, FGF-2 appears to be mainly cytoplasmic. In cells overexpressing HMW or 24 kDa isoform, nuclear labeling is increased. Note weak fluorescence of Vector. Arrowheads denote the nuclei of ROS 17/2.8 cells. Arrows indicate cytoplasm (magnification ×400). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and 68% in cells harboring the different Fgf2 transgenes (Fig. 6A). At 96 h in the presence of 10% serum, viable cell number was increased between 16 and 72% in cells harboring the different Fgf2 transgenes (Fig. 6B). Thus, in the presence of a low or high concentration of serum viable cell number was higher in FGF-2 overexpressing cells compared with cells harboring the vector alone, suggesting that any FGF-2 isoforms stimulated cell growth. However, overexpression of the nuclear isoforms caused a greater increase in viable cell number than the 18 kDa isoform in both low and high serum.

In order to verify that the increased cell number observed in the cells overexpressing nuclear isoforms FGF-2 was due to intracellular action of FGF-2, we repeated the MTT studies in the presence of a neutralizing FGF-2 antibody. In the presence of the FGF-2 antibody, viable cell number decreased 1, 26, 34, 10, and 3% in vector, ALL, 18 kDa, HMW, and 24 kDa, respectively. The neutralizing antibody significantly reduced cell number only in cells overexpressing the exported 18 kDa isoform (Fig. 6C).

The difference in cell number that was observed between the cells overexpressing FGF-2 and vector could be due to an increase in the mitogenic stimulus, a decreased level of apoptosis, or a combination of the two. In order to verify that there was increased proliferation of cells overexpressing FGF-2 [^{3}H]thymidine incorporation into DNA was determined. In the presence of 1% FBS, DNA synthesis in cells expressing HMW, or 24 kDa was much higher than vector (Fig. 7A). Similarly, in the presence of 10% serum, cells expressing high levels of ALL, HMW, or 24 kDa had a higher rate of DNA synthesis compared to control cells transfected with vector (Fig. 7B). Consistent with the MTT results, cell lines that expressed only HMW or 24 kDa isoforms had augmented DNA synthesis in 1 or 10% serum compared to cells expressing either ALL or 18 kDa FGF-2 isoform. These results suggest that Fgf2 transgenes increased cell proliferation.

DISCUSSION

The rat osteoblast-like, osteosarcoma cell line, ROS 17/2.8, is a well-established cell line that has been extensively used experimentally [Motomura et al., 1996; Onyia et al., 1999;

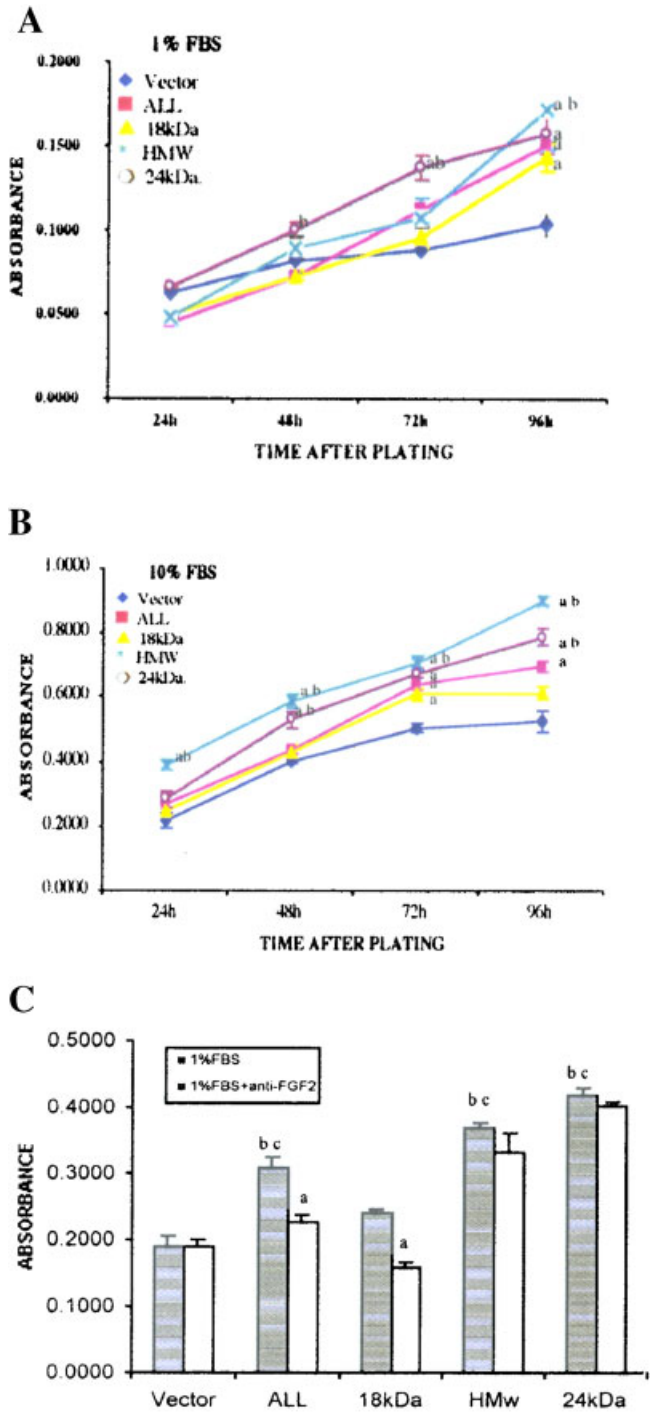


Fig. 6. Time course effect of overexpression of FGF-2 protein isoforms on cell viability of ROS 17/2.8 cells cultured in (**A**) 1% or (**B**) 10% FBS. To test cell viability after 24, 48, 72, 96 h, MTT assayed was performed as described in Materials and Methods. Absorbance is directly proportional to the number of proliferating and living cells. Data are presented as the mean ± SE, n = 6. Different from Vector: a, $P < 0.05$. Different from 18 kDa: b, $P < 0.05$. **C**: Viable cell number assayed by MTT in the absence or presence of neutralizing FGF-2 antibody. Result show suppression of increased cell number in cells expressing ALL or the 18 kDa isoform, and no effect on cells which only express the HMW isoforms. a: Compared with 1% FBS $P < 0.05$; (b) compared with 18 kDa $P < 0.05$; (c) compared with vector $P < 0.05$; n = 5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

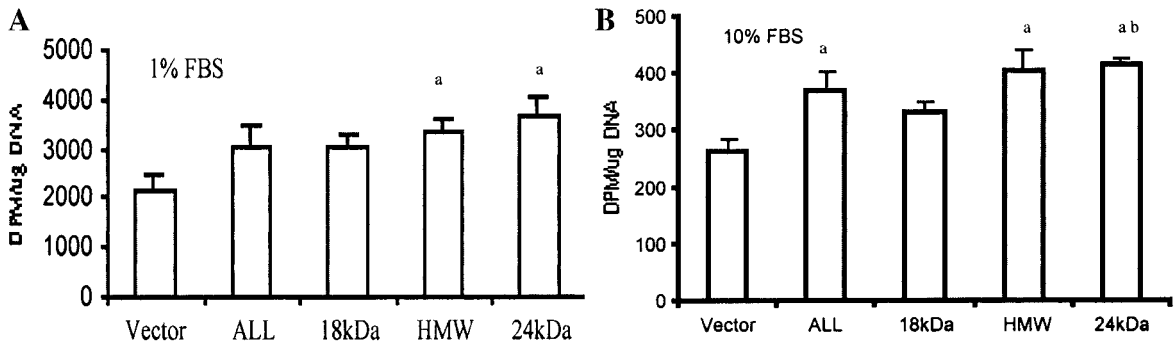


Fig. 7. Effect of overexpression of FGF-2 protein isoforms on [^{3}H]thymidine incorporation into DNA in ROS 17/2.8 cells. Cells were plated at 5,000 cells/cm^2 in (**A**) 1% or (**B**) 10% FBS and was harvested when 70–80% confluence at 6–7 days. Data are presented as the mean ± SE, n = 12. Different from Vector (a) $P < 0.05$. Different from 18 kDa (b) $P < 0.05$.

Woitge et al., 2001]. ROS 17/2.8 cells possess a proliferative capacity that characterizes immature osteoblasts and also express differentiated proteins that characterize mature osteoblasts such as alkaline phosphatase and bone morphogenetic protein [Rodan et al., 1989; Ogawa et al., 1998; Du et al., 2000]. In this study, we established clonal lines of ROS 17/2.8 cells stably transfected with individual or functionally similar subsets of Fgf2 cDNA to alter the endogenous production of FGF-2 protein isoforms. These cell lines were utilized to study the potential regulatory effects of endogenous FGF-2 on proliferation and survival ability in this osteoblast model.

In order to determine whether overexpression of different FGF-2 isoforms contributed to OB function, we cloned human Fgf2 isoforms-IRES-GFPsaph cDNA downstream of a 3.6Col1a1 5′ regulatory fragment to produce Col3.6-Fgf2 isoforms-IRES-GFPsaph, respectively. These individual expression vectors are capable of concurrently overexpressing FGF-2 isoforms and GFPsaph from a single bicistronic mRNA. Transfection of these constructs into osteoblastic ROS 17/2.8 cells produced strong GFP expression, Fgf2 mRNA and FGF-2 protein isoforms expression. Taken together, these data demonstrate that Col3.6 regulated expression of FGF-2 isoform and GFP in transfected osteoblastic cells.

To assess the effects of overexpression of different FGF-2 isoforms on OB proliferation, we compared cell number in ROS 17/2.8 cells stably transfected with control (Col3.6-IRES-GFPsaph) versus each of the Col3.6-Fgf-IRES-GFPsaph constructs. The results show distinct growth modifying effects of FGF-2 isoforms in ROS 17/2.8 cells, and are the first to use transgenic cell models to characterize FGF-2 isoform function in a bone specific fashion. We characterized the growth of ROS 17/2.8 cells overexpressing combinations of human FGF-2 isoforms and observed that transgenic cells expressed high levels of Fgf2 gene mRNA transcripts and expressed levels of FGF-2 approximately 0.5- to 1.3-fold greater than nontransgenic control. Though a 0.5- to 1.3-fold increase in FGF-2 may not appear significant, only a twofold increase is capable of producing a full dwarf phenotype in transgenic mice [Coffin et al., 1995]. Davis et al. [1997] previously reported that the FGF-2 protein expressed in transgenic vascular smooth muscle cells is biologically active and did not induce unrestrained growth of serum-deprived vascular smooth muscle cells. In this study, we show that overexpression of any FGF-2 isoform increased the growth potential of ROS 17/2.8. Overexpression of any subset of FGF-2 isoform significantly increased viable cell number partially due to augmented DNA synthesis in low and high concentration of serum.

We also observed that overexpression of one or more nuclear-targeted isoform of FGF-2 was significantly more effective in stimulating ROS 17/2.8 cell DNA synthesis or viable cell number than overexpression of the nonnuclear targeted FGF-2 isoform alone. This result is consistent with the result found in vascular smooth muscle cells [Davis et al., 1997]. Because [^{3}H]thymidine incorporation into DNA and viable cell number in cells that overexpressed only one nuclear targeted FGF-2 (24 kDa) was similar to that observed in cells which expressed all three nuclear targeted isoforms (HMW), the enhanced

DNA synthesis or viable cell number in nuclear targeted FGF-2 transgenic cells was not simply a concentration effect.

As previously noted, LMW FGF-2 is primarily cytoplasmic and function through a paracrine/autocrine manner, whereas HMW FGF-2 are nuclear and exert activities through an intracrine, nuclear pathway [Arese et al., 1999]. The former modulates cell proliferation through the interaction with its cell surface receptor. The latter modulates cell growth by a mechanism independent of its cell surface receptor. We, therefore, examined whether a neutralizing FGF-2 antibody could block growth of cells harboring the various FGF-2 isoforms. The neutralizing FGF-2 antibody failed to inhibit growth of HMW FGF-2 isoform transgenic cells, but blocked cell growth in cells overexpressing the exported 18 kDa/LMW isoform. These finding suggest that the 18 kDa and HMW FGF-2 can have distinct mechanisms of action. In cells harboring HMW isoforms, increased viable cell number is most likely the result of intracellular FGF-2 effects. A possible mechanism for the distinct effects of nuclear targeted FGF-2 is that the arginine/glycine rich nuclear targeting peptide of the 24, 23, and 22 kDa FGF-2 could facilitate interactions with specific nuclear transcription factors which regulate growth modifying genes [Nakanishi et al., 1992]. Other reports also related cell growth to nuclear localization of FGF-2 [Dono et al., 1998; Arese et al., 1999] by presenting evidence that the overexpression of HMW FGF-2 isoforms have a greater potential to promote growth than overexpression of LMW FGF-2 isoform through an intracellular mechanism [Delrieu, 2000].

In cells overexpressing FGF-2 isoforms, we observed that the mRNAs for FGF-receptors was similar to cells harboring the vector. However, we cannot rule out a change in their affinity or receptor activation. Since the amount of FGFR mRNA was similar in cells harboring the nuclear isoform and vector, these results suggest that nuclear isoform FGF-2 stimulates cell growth by a mechanism independent of its cell surface receptor [Bikfalvi et al., 1995].

In conclusion, we have demonstrated a role of overexpression of FGF-2 isoforms in osteoblast function using Col3.6-Fgf2 isoform-IRES-GFPsaph transgenes cell in which FGF-2 isoforms are targeted to osteoblasts. Cells overexpressing FGF-2 exhibited increased cell growth compared to nontransgenic cells. Furthermore overexpressing nuclear targeted FGF-2 isoforms has additional growth stimulating effects compared to overexpressing the nonnuclear targeted isoform alone. Differential expression of FGF-2 proteins may be important in modulating OB function.

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