Journal of Cellular Biochemistry 80:550–559 (2001)

Transcriptional Regulation of Alpha 2(I) Collagen Gene Expression by Fibroblast Growth Factor-2 in MC3T3-E1 Osteoblast-like Cells

Meika A. Fang,[1,2]* Carlotta A. Glackin,[3] Archana Sadhu,[1,2] and Skye McDougall[1,2]

[1]Geriatric Research, Education and Clinical Center, VA Greater Los Angeles Healthcare System, Los Angeles, California 90073
[2]University of California, Los Angeles, School of Medicine, Los Angeles, California 90095
[3]Division of Molecular Medicine, City of Hope National Medical Center, Duarte, California 91010

Abstract Fibroblast growth factor-2 (FGF-2) stimulates proliferation and inhibits differentiated function of osteoblasts by suppressing synthesis of type I collagen and other proteins. However, little is known regarding the molecular mechanisms regulating the suppressive effects of FGF-2 on type I collagen synthesis in osteoblasts. The zinc finger transcription factor Egr-1 and the basic helix-loop-helix (bHLH) family of proteins have been implicated in the regulation of genes crucial to mesodermal cell growth and differentiation. The aim of this study was to determine whether Egr-1 and *TWIST* might be potential transcriptional regulators of the inhibitory effects of FGF-2 on α2(I) collagen expression in MC3T3-E1 osteoblasts which undergo a developmental sequence in vitro. Upon treatment of undifferentiated MC3T3-E1 cells with 1 nM FGF-2, Egr-1 mRNA increased with the effect maximal after 30–60 min. *TWIST* mRNA also increased with the effect maximal at 2 h. We analyzed the transcriptional control of α2(I) collagen gene expression by FGF-2 by transient transfection of an α2(I) collagen-luciferase construct (pH5) into undifferentiated MC3T3-E1 cells. The activity of the pH5 luciferase promoter decreased in a dose-dependent manner following treatment with.01 and 1 nM FGF-2. We identified putative Egr-1 and *TWIST* recognition sequences in the proximal region of the promoter for the murine α2(I) collagen gene and a putative Egr-1 site in the 5′ region of the murine *TWIST* promoter. In gel mobility shift assays, potential Egr-1 response elements in the 5′ region of the murine *TWIST* and α2(I) collagen genes demonstrated specific Egr-1 binding activity with bFGF-treated nuclear extracts obtained from MC3T3-E1 cells. These results indicate that Egr-1 and *TWIST* are expressed in undifferentiated MC3T3-E1 osteoblast-like cells following treatment with FGF-2 and they may be potential transcriptional regulators of FGF-2s negative effects on α2(I) collagen gene expression. J. Cell. Biochem. 80:550–559, 2001. Published 2001 Wiley-Liss, Inc.†

Key words: FGF-2; transcription factor; helix-loop-helix; Egr-1; osteoblast

The osteoblast, the cell responsible for bone formation, arises from a postulated sequence of differentiation events that start with the multipotential stem cell and progress to the osteoprogenitor cell, preosteoblast, and finally, the mature osteoblast [Stein et al., 1996]. Our understanding of the extracellular factors that promote cell differentiation and the temporal expression of cell growth and osteoblast phenotype-related genes has greatly increased in recent years. Type I collagen, the most abundant collagen in the bone matrix, is deposited by preosteoblasts during a stage of active proliferation characterized by the expression of cell cycle and cell growth genes including type I collagen. Many cytokines, growth factors, and hormones regulate markers of osteoblast differentiation.

One such factor is fibroblast growth factor-2 (FGF-2), a heparin-binding growth factor, that is synthesized by osteoblasts, deposited in the extracellular matrix, and may exert its effects in an autocrine or paracrine manner. When administered continuously in vitro, this growth factor inhibits the expression of osteoblastic

Grant sponsor: NIH/NIA Geriatric Academic Program; Grant number: K12 AG-00489; Grant sponsor: American Federation for Aging Research.

*Correspondence to: Meika A. Fang, GRECC 11G, VA Greater Los Angeles Healthcare System, 11301 Wilshire Boulevard, Los Angeles, CA 90073.

Received 11 May 2000; Accepted 3 August 2000

differentiation markers including type I collagen, osteocalcin, alkaline phosphatase, and parathyroid-responsive adenylate cyclase activity [Hurley et al., 1994]. FGF-2 inhibits α1(I) collagen gene expression by a transcriptional mechanism and this is independent of cell replication [Hurley et al., 1993]. The effects of FGF-2 also appear to be differentiation stage specific as this growth factor slightly induces cell proliferation and inhibits expression of osteoblast markers in less mature human calvaria osteoblastic cells, while it stimulates osteocalcin production and matrix mineralization in more mature cells [Debiais et al., 1998]. The molecular mechanisms responsible for the negative action of FGF-2 on markers of osteoblastic differentiation have not been clearly defined, but most likely involve an orderly activation of intracellular biochemical signals and transcription factors that regulate expression of target genes involved in osteoblast differentiation.

One transcription factor that may control the effects of FGF-2 on the expression of type I collagen is Egr-1 (also known as Zif268, Krox-24, TIS8, and NGFI-A) [Gashler and Sukhatme, 1995], an immediate early response gene whose gene product binds with high affinity to the sequence 5′-CGCCCCCGC-3′. Egr-1 is induced by diverse mitogenic, hypertrophic, and differentiation signals in various cell types, including osteoblasts. Transcripts of Egr-1 are highly expressed in bone and cartilage during murine embryogenesis [McMahon et al., 1990]. They are also induced in osteoblasts by such stimuli as retinoic acid [Suva et al., 1991], hydrogen peroxide [Nose et al., 1991], epidermal growth factor [Fang et al., 1995], prostaglandin E2 [Fang et al., 1996], and prostacyclin [Glantschnig et al., 1996].

One physiologically relevant and potential target gene for Egr-1 in osteoblasts is type I collagen, the most abundant form of collagen, which is deposited in the extracellular bone matrix by normal diploid osteoblasts during a stage of active proliferation. Type I collagen is a triple helical supercoil composed of two identical α1(I) chains and an α2(I) chain. There is a potential Egr-1 recognition sequence which is perfectly conserved in the 5′ flanking region of the rat and human α2(I) collagen genes and highly conserved in mouse (eight out of nine nucleotides match) [Guenette et al., 1992].

Basic helix-loop-helix (bHLH) transcription factors have also been implicated in the molecular regulation of cell development and differentiation and are notable for binding to a core sequence, CANNTG, an E-box motif, as either homo- or hetero-dimers. Basic HLH proteins may be expressed in a wide range of tissues and cell types or be expressed in a tissue-restricted manner. Investigators have suggested that bHLH transcription factors may function as a nuclear regulator of osteoblastic cell growth and differentiation. The bHLH transcription factor HES-1, the mammalian counterpart of the Drosophila Hairy and Enhancer of split proteins, is expressed in rat osteosarcoma ROS17/2.8 cells and inhibited in a dose-dependent manner by $1,25(OH)_2$ vitamin D3 [Matsue et al., 1997]. Overexpression of scleraxis, another HLH transcription factor, in these osteoblastic cells results in increased type II collagen, aggrecan, and osteopontin mRNA and suppressed expression of osteoblast phenotype-related genes such as type I collagen and alkaline phosphatase [Liu et al., 1997]. The bHLH transcription factor DERMO-1 is likely involved in negative regulation of osteoblastic differentiation as Dermo-1 mRNA levels were lower in 21-day-old differentiated MC3T3-E1 cells compared to undifferentiated 3-day-old MC3T3-E1 cells [Tamura and Noda, 1999].

TWIST is a HLH transcription factor that, in vertebrates, regulates genes involved in spatial differentiation of mesoderm and in the development of cranial mesenchymal tissues. *TWIST* gene mutations or deletions are present in about 80% of patients with Saethre-Chotzen syndrome, an autosomal dominant craniosynostosis disorder [Gripp et al., 2000]. Interestingly, FGF and FGF receptor signaling are involved in the pathways regulating skull development, while *TWIST* interactions are important in the cascade of signals responsible for FGF action [Lajeunie et al., 1999]. *TWIST* may function as a transcriptional regulator of fibroblast growth factor receptors during mesodermal patterning of Drosophila [Shishido et al., 1993]. The level of *TWIST* mRNA is decreased following osteoblastic maturation of the MC3T3-E1 osteoblastic cell line [Murray et al., 1992], fetal bone development in both the mandible and hindfoot of the rat [Alborzi et al., 1996], and following exposure of C3H10T1/2 cells to BMP-2 [Tamura and Noda, 1999].

Studies with the human osteoblast HSaOS-2 cells have demonstrated that cells overexpressing *TWIST* de-differentiate and remain in an osteoprogenitor-like state while *TWIST* antisense expressing HsaOS-2 cells progress to a more differentiated mature osteoblast-like state [Lee et al., 1999].

The DNA–protein interactions and signal cascade mediating the effects of FGF-2 on osteoblast differentiation are not clearly defined. In this report, we hypothesize that the effects of FGF-2 on differentiation of phenotypically immature MC3T3-E1 osteoblasts involve expression of nuclear regulators Egr-1 and *TWIST*. A potential DNA binding site for Egr-1 is present in the regulatory region of *TWIST* and a putative DNA binding site for *TWIST* is present in the proximal region of the promoter for the α2(I) collagen gene. This suggests the possibility that FGF-2 downregulates expression of the α2(I) collagen gene via the Egr-1 and *TWIST* motifs and subsequently leads to suppression of osteoblast differentiation.

MATERIALS AND METHODS

Materials

All chemicals used were of reagent grade or molecular biology grade. Recombinant human fibroblast growth factor-2 was obtained from Bachem (Torrance, CA). The DNA probes included a 1.4 kb *Eco*RI murine Egr-1 cDNA fragment obtained from Dr. H. Herschman (University of California, Los Angeles) and a 1.3 kb *Pst*I rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment obtained from Dr. S. McDougall (University of California, Los Angeles). A mouse pH5-luc reporter construct (a 2054 base pair *Hind*III fragment of the α2(I) collagen DNA promoter sequence −2000 to +54 5′ ligated to the *Hind*III site of the pA3LUC vector) was generously provided by Dr. Benoit de Crombrugghe (M.D. Anderson Hospital, Houston, TX) [Goldberg et al., 1992].

Cell cultures

MC3T3-E1 cells [Quarles et al., 1992] were kindly provided by Dr. M. Kumegawa (Meikai University Dental School, Saitama, Japan). These cells were maintained in α-modified minimum essential medium (α-MEM; Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan UT), 26.2 mM $NaHCO_3$, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. Cells were grown in a humidified atmosphere of 10% CO_2/90% air at 37°C and subcultured every 3–4 days using 0.002% pronase/0.02% EDTA.

RNA Isolation and Northern Blot Analysis

Cells were plated at 275,000 cells per 10-cm diameter dish until 80% confluency was reached and then rendered quiescent by incubating cells for 24 h in α-MEM medium containing 0.1% bovine serum albumin (BSA). Cells were then treated with vehicle or FGF-2 for various periods in serum-free α-MEM medium containing 0.1% BSA. Total cytoplasmic RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction as described by Chomczynski and Sacchi [1987]. Fifteen micrograms each of total RNA was electrophoresed on 1.5% formaldehyde agarose gels. RNA was transferred onto UV-Duralon nylon membrane (Stratagene, La Jolla, CA) via capillary transfer and then crosslinked to the membrane by ultraviolet light (UV Stratalinker, Stratagene). cDNA probes were labeled with [^{32}P]-dCTP to a specific activity of about 10^8 cpm/μg DNA using a random primer kit from Pharmacia (Piscataway, NJ). Filters were prehybridized for 20 min and then hybridized to the radioactive probes for 1.5 h at 68°C with QuikHyb hybridization solution (Stratagene). Blots were then washed twice in 1 × SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 15 min at 25°C and then once in 0.1 × SSC and 0.1% SDS for 15 min at 37°C. Blots were exposed to Kodak XAR-5 film with intensifying screens for 16–24 h. Selected autoradiograms were scanned with an AMBIS Optical Imaging System (AMBIS, San Diego, CA) using the AMBIS Core software package (version 4.31). Equal loading of RNA in each lane was checked by hybridization with ^{32}P-labeled GAPDH cDNA.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from MC3T3-E1 cells was used to synthesize cDNAs with random oligonucleotide primers and reverse transcriptase (StrataScript RT-PCR kit, Stratagene). cDNAs were then amplified using a pair of oligonucleotide primers for the *TWIST* gene or for the GAPDH gene and GeneAmp PCR core reagents from

Applied Biosystems (Foster City, CA). The primer sequences for *TWIST* are shown below:

Helix A 5′-ACGCAGTCGCTGAACGAGGC-3′
Helix B 5′-GTCAGGGAAGTCGATGTACC-3′

A RT-PCR Amplimer Set for the mouse GAPDH 5′ and 3′ primers was obtained from Clontech (Palo Alto, CA). To determine the optimal amplification conditions, a series of pilot studies were conducted in which the amounts of reverse transcription products and cycle numbers of PCR amplification were varied. The linear portion of the amplification was determined for both *TWIST* and GAPDH on the basis of these initial experiments. *TWIST* and GAPDH samples were run simultaneously in PCR reactions that are carried out for 25–30 cycles with each cycle at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min in an Ericomp TwinBlock PCR machine. The amplified DNA (10 μl/lane) was fractionated by a 3% NuSieve 3:1 agarose gel electrophoresis and stained with ethidium bromide. The density of the bands was analyzed with an AMBIS Optical Imaging System (AMBIS, San Diego, CA) using the AMBIS Core software package (version 4.31). The amplified *TWIST* PCR product was normalized with the corresponding GAPDH PCR product by determining the ratio between the optical densities of the *TWIST* and GAPDH bands.

Transient Transfections

MC3T3-E1 cells were plated at $1.5 \times 10^3/cm^2$ in 60-mm tissue culture dishes in α-MEM containing 5% FBS. After 24 h, cells were cotransfected with 15 μg α2(I) collagen-luciferase DNA construct (pH5) and 10 μg pSV2βgal by the calcium phosphate precipitation method for 16 h [Graham and Van der Erb, 1973]. The transfected cells were then incubated in serum-free α-MEM medium containing 0.1% BSA for 16 h followed by the addition of FGF-2 for 24 h in serum-free α-MEM medium supplemented with 0.1% BSA. Cell lysates were prepared using a luciferase assay system with reporter lysis buffer (Promega, Madison, WI) and luciferase activity measured as light units in a Lumat LB9501 luminometer. β-Galactosidase activity in the cell lysates was measured using a Promega reagent kit. Luciferase activity of the transfected constructs was normalized for the amount of β-galactosidase activity present in the extracts. Preliminary experiments conducted with MC3T3-E1 cells transfected with empty vector followed by treatment with or without FGF-2 revealed minimal luciferase activity.

Gel Mobility Shift Assay

MC3T3-E1 cells were plated at 275,000 cells per 10-cm diameter dish until approximately 80% confluency was reached and then rendered quiescent by incubating cells for 24 h in α-MEM medium containing 0.1% BSA. Cells were treated with 1 nM FGF-2 or control for 2 h in serum-free α-MEM medium containing 0.1% BSA. Nuclear extracts were prepared by using the TKM/sucrose/NP40 method to obtain nuclei followed by removal of histones with salt extraction [Henderson et al., 1994]. Protein concentrations were determined by a dye-binding assay using bovine serum albumin as the standard (Bio-Rad Laboratories, Hercules, CA), and the nuclear extracts were stored in 50-μl aliquots at −70°C until use. α2(I) collagen and *TWIST* oligonucleotide probes containing the Egr-1 binding site were synthesized on a DNA synthesizer (Applied Biosystems International, Foster City, CA). The sequences and locations of these oligonucleotides (the putative Egr-1 binding domain is underlined) used in the binding reactions are as follows:

Murine α2(I)	5′-CTCCCCTGCTCCC CCGCAGTCTCCT-3′	−149/−125
Murine TWIST	5′-CGAGCCCA CGCCCCCGC GACCCGCG-3′	650/674 (reverse)

Gel shift oligonucleotides for the competition studies (wild-type Egr-1, mutant Egr-1, Sp1, AP-1, and CREB) and antibodies (p82 epitope of Egr-1, p88 epitope of Egr-1, Wilms tumor protein, and Sp1) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The oligonucleotide sequence for the wild-type Egr-1 is 5′-GGATCCAGC**GG**GGGCGAGC**GG**GGGCGA-3′ with the consensus binding sites underlined. The mutant Egr-1 oligonucleotide has a "GG" (in bold)-→"TA" substitution in the DNA binding regions.

Each oligonucleotide was annealed to its complementary strand and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Boehringer Mannheim). The unincorporated label was separated from the labeled oligonucleotide by centrifugation of the sample following addition to a Clontech TE-10 column.

Competition studies were performed with molar excesses of unlabeled double-stranded oligonucleotides. The DNA binding reactions were carried out at room temperature by incubating nuclear protein fractions (10 μg) from osteoblastic cells with 2 μg of double-stranded poly[d(I-C)] (Amersham Pharmacia Biotech, Piscataway, NJ), 20 mM HEPES, pH 7.9; 1 mM dithiothretol; 5% glycerol; 0.5 mM EDTA; and 1 mM $MgCl_2$ for 5 min followed by the addition of 1 μl of the radiolabeled oligonucleotide (100,000 cpm) for an additional 20 min. If antibodies were used in the reaction, they were added to the binding reaction for 15 min at room temperature followed by addition of the radiolabeled oligonucleotide for 20 min. Protein–DNA complexes were resolved from free probes in nondenaturing 5% polyacrylamide gels. Electrophoresis was performed in 0.5 × TBE (0.045 M Tris-borate, 0.001 M EDTA) at 20 mAmps for 45 min. The gels were then dried under vacuum and exposed to X-ray film with intensifying screens at −70°C for 16–48 h.

Statistical Analysis

Data were expressed as the means ± SE. The significance of differences between mean values was evaluated by the two-tailed Student's t test and differences were considered significant at the $P < 0.05$.

RESULTS

Continuous or chronic treatment of osteoblasts in vitro with FGF-2 results in suppression of type I collagen synthesis [McCarthy et al., 1989]. Type I collagen, the most abundant extracellular protein found in bone, is composed of two α1 chains and one α2 chain. To determine whether FGF-2 regulates α2(I) collagen gene expression at the transcriptional level, a promoter-luciferase construct containing the α2(I) collagen DNA promoter sequence −2000 to +54 5′ to the luciferase gene was tested in transient transfection studies. Following treatment of transfected MC3T3-E1 cells with 0.01 nM or 1 nM FGF-2 for 24 h, FGF-2 inhibited luciferase activity, with suppression greater with the higher concentration of FGF-2 (Fig. 1).

We carried out an analysis of the proximal region of the α2(I) collagen promoter to identify potential DNA sequence elements that may bind transcription factors and consequently

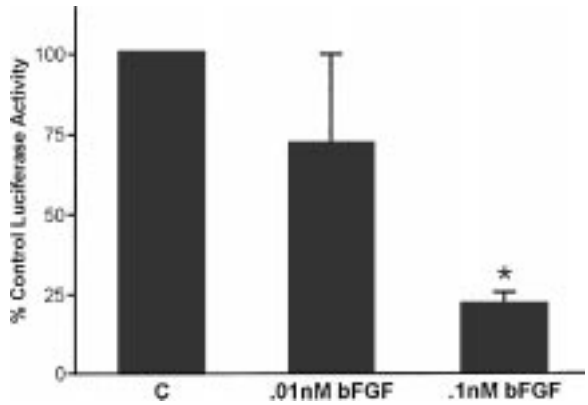

Fig. 1. Activity of α2(I) collagen-luciferase pH5 reporter gene construct following FGF-2 treatment of transfected MC3T3-E1 cells. Cells were transfected with 15 μg pH5 and 10 μg pSV2β2Gal for 16 h, then incubated for 16 h in 0.1% BSA, followed by the addition of serum-free media containing 0.1% BSA (control) or FGF-2 (bFGF) for 24 h. Luciferase values have been normalized for the amount of β-galactosidase activity present in the extracts. Values represent the mean ± SEM of three separate determinations. *Significantly different from control $P < 0.05$.

regulate the expression of the α2(I) collagen gene. Putative binding sites for the zinc finger protein Egr-1 and the basic helix-loop-helix factor *TWIST* were identified. A putative DNA binding site for Egr-1 was also identified in the promoter region for *TWIST*. We then examined whether FGF-2 affects Egr-1 and *TWIST* gene expression. Quiescent MC3T3-E1 preosteoblasts were treated with 1 nM FGF-2 and total RNA extracted at various time points. Northern blot hybridization was carried out using [^{32}P]-dCTP labeled Egr-1 and GAPDH cDNAs as probes. Treatment with FGF-2 induced Egr-1 mRNA as early as 15 min after addition, with a significant decrease by 120 min (Fig. 2). Peak stimulation occurred after treating the cells with 1 nM FGF-2 for 30–60 min. Dose-response experiments were next carried out to examine the effect of varying concentrations of FGF-2 on the induction of Egr-1 mRNA. Osteoblasts were incubated with 0.01–100 nM for 60 min before total cellular RNA was isolated for Northern blot analysis. Maximum induction of Egr-1 mRNA occurred when cells were treated with 1 nM FGF-2 (Fig. 3).

To determine if *TWIST* mRNA is expressed in undifferentiated MC3T3-E1 cells treated with 1 nM FGF-2 at various times ranging from 0.5 to 6 h, we performed reverse transcription (RT)-PCR analysis using total RNA from

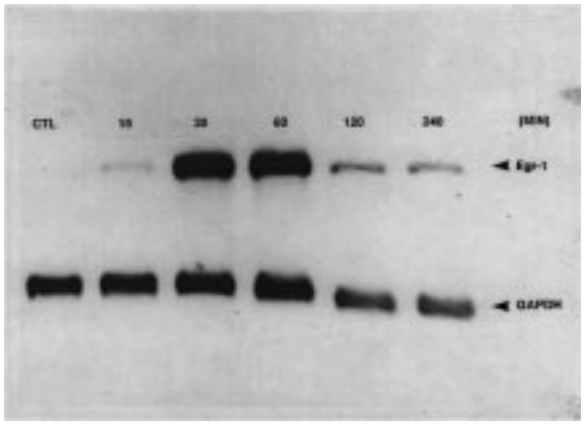

Fig. 2. Time course for induction of steady-state Egr-1 transcript levels by FGF-2. MC3T3-E1 cells were rendered quiescent by growing cells in α-MEM medium containing 0.1% BSA for 24 h and then exposing them to 1 nM FGF-2 for various times. Total cytoplasmic RNAs were isolated, fractionated on formaldehyde agarose gel, blotted onto nylon, and hybridized. Fifteen micrograms of RNA were loaded per lane. The pGAPDH probe was added to normalize RNA loading on the gel.

TABLE I. Time Course for Induction of *TWIST* Transcript Levels in MC3T3-E1 Cells Treated With 1 nM FGF-2 by the RT-PCR Assay[a]

	Time (h)	Control ± SEM (%)
FGF-2	0.5	84 ± 0.5
FGF-2	1	104 ± 1.2
FGF-2	2	469* ± 3.4
FGF-2	4	153 ± 1.7
FGF-2	6	133 ± 1.3

[a]MC3T3-E1 cells were rendered quiescent by growing cells in α-MEM medium containing 0.1% BSA for 24 h and then exposing them to 1 nM FGF-2 for various times. Total cytoplasmic RNAs were isolated and RT-PCR performed. PCR products were run on an agarose gel and results scanned with an AMBIS Optical Imaging System and normalized to GAPDH. Representative of three separate experiments.
*Significantly different from control $P < 0.05$.

MC3T3-E1 cells and *TWIST* and GAPDH primers. The predicted size of amplified products using the Helix A and Helix B primers was 125 bp. *TWIST* mRNA was increased in MC3T3-E1 cells following treatment with FGF-2 with expression maximal after 2 h of exposure to 1 nM FGF-2 (Table I).

DNA mobility shift assays were conducted to determine if Egr-1 and *TWIST* bind specifically to their respective sequences identified in the proximal promoter region of α2(I) collagen gene and the *TWIST* gene. The presence of Egr-1 protein in undifferentiated MC3T3-E1 cells treated with FGF-2 was investigated with oligonucleotides encoding potential Egr-1 binding sites in the α2(I) collagen promoter and *TWIST* promoter and an oligonucleotide containing a known Egr-1 binding site. We observed a top DNA–protein complex (band I) following treatment with 1 nM FGF-2 that was competed out by excess of unlabeled α2(I) collagen, *TWIST*, and Egr-1 (Fig. 4). The next series of experiments were carried out to determine the specificity of binding to the potential Egr-1 site in the murine α2(I) collagen promoter. As shown in Figure 5, the double-stranded oligonucleotide spanning the Egr-1 binding site of the murine α2(I) collagen promoter formed a specific nuclear protein–DNA complex (band I). This complex was readily removed by an excess of unlabeled oligonucleotide containing the Egr-1 binding site, but not by excess of unlabeled oligonucleotide for the unrelated transcription factors Sp1, AP-1, and CREB. Moreover, this complex was supershifted by antibody A (p82 epitope of Egr-1) but not by antibody B (p88 epitope of Egr-1), antibody C (Wilm's tumor nuclear protein), or antibody D (Sp1). Antibody D decreased the amount of complex formation. An excess of unlabeled mutant Egr-1 oligonucleotide does not eliminate this complex (data not shown).

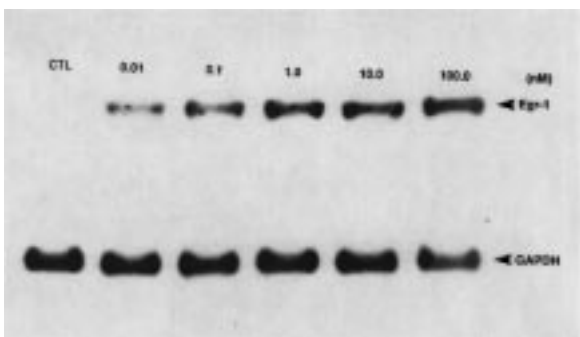

Fig. 3. Dose-curve for induction of steady-state Egr-1 transcript levels by FGF-2. MC3T3-E1 cells were rendered quiescent by growing cells in α-MEM medium containing 0.1% BSA for 24 h and then exposing them to varying concentration of FGF-2 for 60 min. Total cytoplasmic RNAs were isolated, fractionated on formaldehyde agarose gel, blotted onto nylon, and hybridized.

The specificity of binding to the potential Egr-1 site in the murine *TWIST* promoter was investigated in additional DNA mobility shift assays. The top DNA–protein complex formed

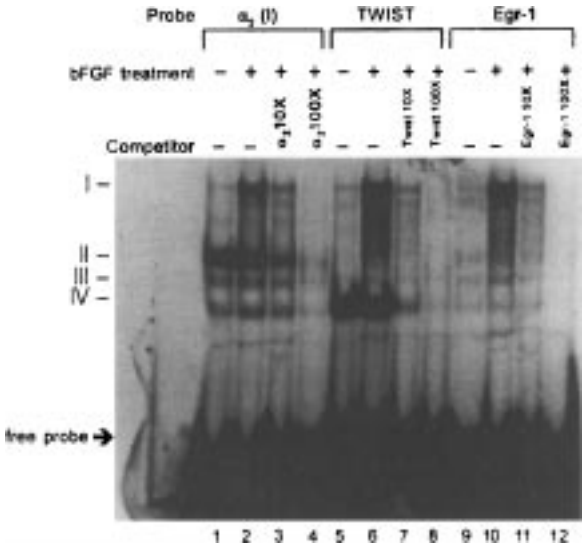

Fig. 4. Gel mobility shift assay of Egr-1 binding using nuclear extracts from MC3T3-E1 cells treated with FGF-2. Double-stranded, labeled oligonucleotides containing the DNA binding site for Egr-1 were incubated with nuclear extracts obtained from MC3T3-E1 cells treated for 2 h with 1 nM FGF-2. Increasing concentrations of unlabeled, double-stranded competitor oligonucleotides were included in the binding reactions.

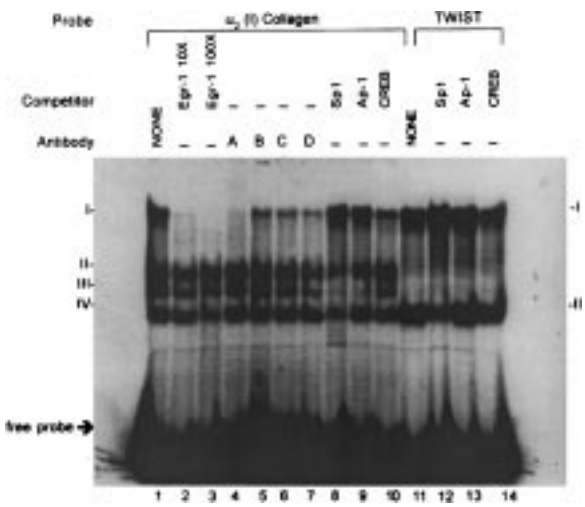

Fig. 5. Competition experiments and antibody analysis in gel shift assays. The murine α2(I) collagen or *TWIST* double-stranded, labeled oligonucleotide was incubated with nuclear extracts obtained from MC3T3-E1 cells treated for 2 h with 1 nM FGF-2. Excess concentrations of unlabeled, double-stranded oligonucleotides containing the Egr-1, Sp1, AP-1, or CREB binding site were added as competitors during the binding assays. The effect of various transcription factor antibodies on DNA–protein binding was studied by adding antibody A (p82 epitope of *Egr-1*), B (p88 epitope of *Egr-1*), C (Wilms tumor protein), or D (Sp1) to the nuclear extract prior to the binding reaction. Representative of three experiments.

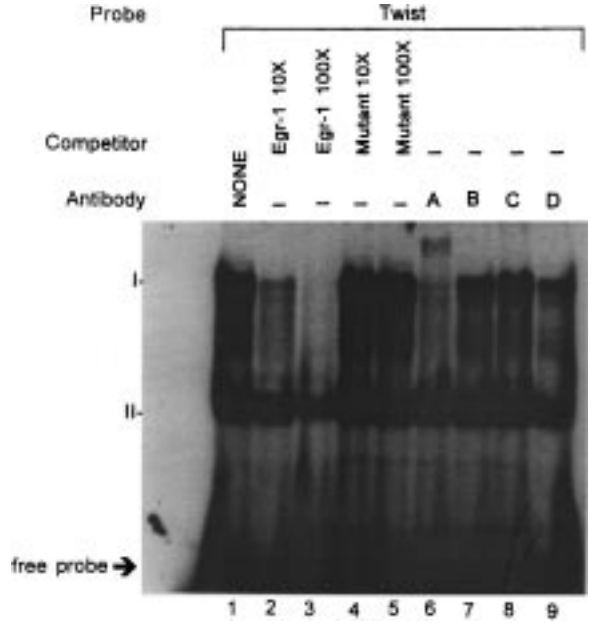

Fig. 6. Competition experiments and antibody analysis in gel shift assays. The murine *TWIST* double-stranded, labeled *TWIST* oligonucleotide was incubated with nuclear extracts prepared from MC3T3-E1 cells treated for 2 h with 1 nM FGF-2. Increasing concentrations of unlabeled, double-stranded Egr-1 or mutant oligonucleotides were added as competitors during the binding assays. The effect of various transcription factor antibodies on DNA–protein binding was studied by adding antibody A (p82 epitope of Egr-1), B (p88 epitope of Egr-1), C (Wilms tumor protein), or D (Sp1) to the nuclear extract prior to the binding reaction.

following treatment of MC3T3-E1 cells with 1 nM FGF-2 was readily removed by an excess of unlabeled oligonucleotide containing the Egr-1 binding site, but not by unlabeled oligonucleotide containing a mutant Egr-1 binding site (Fig. 6). In addition, this complex was supershifted by antibody A (p82 epitope of Egr-1) but not by antibody B (p88 epitope of Egr-1), antibody C (Wilm's tumor nuclear protein), or antibody D (Sp1). Antibody D decreased the amount of complex formation. This top complex (band I) was not removed by an excess of unlabeled oligonucleotide for the unrelated transcription factors Sp1, AP-1, and CREB (Fig. 5).

DISCUSSION

FGF-2 and its receptors are important clinically because of their roles in normal limb development, osteoblast function, fracture repair, and the inherited human skeletal dyspla-

sias [Xu et al., 1999; Boyce et al., 1999]. FGF-2 likely is important in regulating osteoblast differentiation and function in vivo since disruption of the FGF-2 gene in mice results in significant reduction in both bone mass and bone formation [Montero et al., 1999]. Continuous administration of exogeneous FGF-2 in vivo and in vitro inhibits markers of the osteoblast phenotype such as type I collagen, alkaline phosphatase, and parathyroid hormone responsive adenylate cyclase [Mundy, 1995].

Type I collagen, one of the markers of osteoblast differentiation, is the most abundant protein in the bone extracellular matrix and is the predominant protein synthesized and secreted by osteoblasts undergoing proliferation and differentiation. Type I collagen is a triple helical supercoil composed of two identical α1(1) chains and an α2(1) chain. In phenotypically immature MC3T3-E1 calvarial osteoblasts, FGF-2 inhibits collagen synthesis by a transcriptional mechanism and the inhibitory locus is located between −3.5 and −2.3 kb within the α1(1) collagen promoter [Hurley, et al., 1993]. A cascade of second messenger and nuclear mechanisms is initiated when FGF-2 binds to its receptors on the cell surface and then go on to inhibit type I collagen gene synthesis. The extracellular-signal regulated kinase (ERK) mitogen-activated protein kinase pathway has been implicated in the downregulation of type I collagen gene expression by FGF-2 in MC3T3-E1 cells [Chaudhary and Avioli, 2000]. The zinc finger transcription factor Egr-1 and the basic helix-loop-helix transcription factor *TWIST* have been identified as possible regulators of FGF-2s inhibitory effect on α2(1) collagen gene expression. The Egr-1 binding motif is present in the *TWIST* and α2(1) collagen promoters and the *TWIST* binding sequence has been identified in the α2(1) collagen promoter. The experiments described herein indicate that FGF-2 inhibits expression of α2(I) collagen by a transcriptional mechanism and induces mRNA expression of the transcription factors Egr-1 and *TWIST*. There is sequence-specific interaction of the Egr-1 recognition motif in both the *TWIST* and α2(1) collagen promoters.

Egr-1 is induced by diverse mitogenic, hypertrophic, and differentiation signals in various cell types, including osteoblasts. This zinc finger transcription factor appears to have a major role in the regulation of the growth and differentiation of osteoblasts since transcripts of Egr-1 are highly expressed in bone and cartilage during murine embryogenesis. The putative morphogen retinoic acid increases Egr-1 mRNA expression in rat preosteoblasts but not in more differentiated osteoblasts [Suva et al., 1991]. Hydrogen peroxide [Nose et al., 1991], hypergravity [Nose and Shibanuma, 1994], epidermal growth factor [Fang et al., 1995], prostaglandin E2 [Fang et al., 1996], prostacyclin [Glantschnig et al., 1996], mechanical deformation [Dolce et al., 1996], fluid flow [Ogata, 1997], and change in pH from 7.4 to 6.8 [Frick et al., 1997] will transiently induce Egr-1 mRNA in osteoblasts. Our results are the first to demonstrate downstream target genes for Egr-1, namely *TWIST* and α2(1) collagen promoters, in osteoprogenitor cells exposed to FGF-2.

FGF-2 likely inhibits collagen synthesis and other markers of osteoblast differentiation through complex molecular mechanisms involving transcription factors which coordinately regulate osteoblastic protein–DNA and protein–protein interactions. In vertebrates, *TWIST* appears to be involved in the negative regulation of cellular determination and differentiation of several lineages, including muscle and bone [Glackin et al., 1994; Hebrok et al., 1997; Spicer et al., 1996]. Levels of *TWIST* mRNA are decreased during fetal bone development in both the mandible and hindfoot of the rat [Alborzi et al., 1996]. Humans osteoblast HSaOS-2 cells which overexpress *TWIST* appear to dedifferentiate and remain in an osteoprogenitor state; conversely, those cells which underexpress *TWIST* progress to a more differentiated osteoprogenitor state [Lee et al., 1999].

Mice heterozygous for *TWIST* null mutations have a phenotype exhibiting minor skull and limb abnormalities resembling those seen in the Saethre-Chotzen syndrome, an autosomal dominant craniosynostosis syndrome [Bourgeois et al., 1998]. Mutations in the human *TWIST* gene have been identified in the Saethre-Chotzen syndrome that involve frameshift deletions/insertions and nonsense and missense mutations [El Ghouzzi et al., 1999]. There is genetic heterogeneity in the Saethre-Chotzen syndrome as some people have been identified with mutations of the FGF receptor rather than mutations within or upstream of *TWIST* [Paznekas et al., 1998]. These studies

suggest that *TWIST* and FGF receptors are part of the same molecular pathway modulating development of the limb and craniofacial bones.

Our data extends that hypothesis by demonstrating that FGF-2 induces expression of the zinc finger transcription factor Egr-1 and the basic helix-loop-helix factor *TWIST*, and that FGF-2 inhibits α2(I) collagen promoter activity. Further studies are needed to establish whether Egr-1 and *TWIST* have functional roles in mediating FGF-2 dependent inhibition of α2(I) collagen promoter activity and whether *TWIST* binds to its putative site in the α2(I) collagen promoter. Because putative Egr-1 sites were identified in both the *TWIST* and α2(I) collagen promoters, this suggests the possibility that multiple distinct signaling pathways are likely involved in the actions of FGF-2 on the α2(I) collagen promoter. Our experiments suggest that Egr-1 may mediate FGF-2 induction of *TWIST* since the time course of *TWIST* mRNA induction by FGF-2 was slower than FGF-2 induction of Egr-1 mRNA in the MC3T3-E1 cell line. Additional studies will help define whether (1) FGF-2 first stimulates Egr-1, which in turn enhances *TWIST* expression and subsequently regulates α2(I) collagen expression, (2) FGF-2 directly stimulates *TWIST*, without the assistance of Egr-1, or (3) a combination of the two alternatives.

In conclusion, our results presented in this report show that FGF-2 inhibits α2(1) collagen promoter activity and implicate nuclear factors Egr-1 and *TWIST* in downregulating α2(1) collagen gene transcription. FGF-2 induces expression of Egr-1 and *TWIST* mRNA and Egr-1 has sequence-specific interaction with its recognition motif in the α2(1) collagen promoter. Previous investigators have shown that Egr-1 and *TWIST* are expressed in undifferentiated osteoblasts and that the level of expression declines as the osteoblast differentiates [Murray et al., 1992; Suva et al., 1991]. Moreover, cells that overexpress *TWIST* de-differentiate and remain in an osteoprogenitor-like state, while anti-sense *TWIST* cells progress to a more differentiated mature osteoblast-like state [Lee et al., 1999]. Although we do not have direct evidence to support a role for Egr-1 and *TWIST* in regulating FGF-2 effects on osteoblast development, these observations suggest that defining the effects of Egr-1 and *TWIST* on osteoblast growth and differentiation will be important future directions.

REFERENCES

Alborzi A, Mac K, Glackin CA, Murray SS, Zernik JH. 1996. Endochondral and intramembranous fetal bone development: osteoblastic cell proliferation, and expression of alkaline phosphatase, m-twist, and histone H4. J Craniofacial Genetics Develop Biol 16:94–106.

Bourgeois P, Bolcato-Bellemin A-L, Danse J-M, Bloch-Zupan A, Yoshiba K, Stoetzel C, Perrin-Schmitt F. 1998. The variable expressivity and incomplete penetrance of the twist-null heterozygous mouse phenotype resemble those of human Saethre-Chotzen syndrome. Hum Molec Genet 7:945–957.

Boyce BF, Hughes DE, Wright KR, Xing L, Dai A. 1999. Recent advances in bone biology provide insight into the pathogenesis of bone diseases. Lab Invest 79:83–94.

Chaudhary LR, Avioli LV. 2000. Extracellular-signal regulated kinase signaling pathway mediates downregulation of type I procollagen gene expression by FGF-2, PDGF-BB, and okadaic acid in osteoblastic cells. J Cell Biochem 76:354–359.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Debiais F, Hott M, Graulet AM, Marie PJ. 1998. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific. J Bone Miner Res 13:645–654.

Dolce C, Kinniburgh AJ, Dziak R. 1996. Immediate early-gene induction in rat osteoblastic cells after mechanical deformation. Arch Oral Biol 41:1101–1108.

El Ghouzzi V, Lajeunie E, Le Merrer M, Cormier-Daire V, Renier D, Munnich A, Bonaventure J. 1999. Mutations within or upstream of the basic helix-loop-helix domain of the TWIST gene are specific to Saethre-Chotzen syndrome. Eur J Hum Genet 7:27–33.

Fang MA, Noguchi GM, McDougall S. 1995. Epidermal growth factor induces Egr-1 mRNA and protein in mouse osteoblastic cells. Calcif Tissue Int 57:450–455.

Fang MA, Noguchi GM, McDougall S. 1996. Prostaglandin E2 induces primary response gene Egr-1 in MC3T3-E1 osteoblastic cells by a protein kinase C-dependent pathway. Prostaglandins, Leukotrienes, and Essential Fatty Acids 54:109–114.

Frick KK, Jiang L, Bushinsky DA. 1997. Acute metabolic acidosis inhibits the induction of osteoblastic egr-1 and type I collagen. Am J Physiol 272:C1450–C1456.

Gashler A, Sukhatme VP. 1995. Early growth response protein 1(Egr-1): prototype of a zinc finger family of transcription factors. Prog Nucl Acid Res Mol Biol 50: 191–224.

Glackin CA, Winters KA, Murray EJB, Murray SS. 1994. Transcripts encoding the basic-helix-loop-helix factor *TWIST* are expressed in mouse embryos, cell lines, and adult tissues. Molecul Cell Different 2:309–328.

Glantschnig H, Varga F, Klaushofer K. 1996. The cellular protooncogenes c-fos and egr-1 are regulated by prostacyclin in rodent osteoblasts and fibroblasts. Endocrinology 137:4536–4541.

Goldberg H, Helaakoski T, Garrett LA, Karsenty G, Pelligrino A, Lozano G, Maity S, deCrombrugghe B.

1992. Tissue-specific expression of the mouse α2(1) collagen promoter—studies in transgenic mice and in tissue culture cells. J Biol Chem 267:19622–19630.

Graham FL, Van der Erb AJ. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.

Gripp KW, Zackai EH, Stolle CA. 2000. Mutations in the human TWIST gene. Hum Mutat 15:150–155.

Guenette DK, Ritzenthaler JD, Foley J, Jackson JD, Smith BD. 1992. DNA methylation inhibits transcription of procollagen alpha2(1) promoter. Biochem J 283:699–703.

Hebrok M, Fuchtbauer A, Fuchtbauer EM. 1997. Repression of muscle-specific gene activation by the murine Twist protein. Exp Cell Res 232:295–303.

Henderson AJ, McDougall S, Leiden J, Calame K. 1994. GATA elements are necessary for the activity and tissue specificity of the T-cell receptor beta chain enhancer. Mol Cell Biol 14:4286–4294.

Hurley MM, Abreu C, Harrison JR, Lichtler AC, 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, Abreu C, Gronowicz G, Kawaguchi H, Lorenzo J. 1994. Expression and regulation of basic fibroblast growth factor mRNA levels in mouse osteoblastic MC3T3-E1 cells. J Biol Chem 269:9392–9396.

Lajeunie E, Catala M, Renier D. 1999. Craniosynostosis: from a clinical description to an understanding of bone formation of the skull. Childs Nerv Syst 15:676–680.

Lee M-S, Lowe GN, Strong DD, Wergedal JE, Glackin CA. 1999. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J Cell Biochem 75:566–577.

Liu Y, Watanabe H, Nifuji A, Yamada Y, Olson EN, Noda M. 1997. Overexpression of a single helix-loop-helix-type transcription factor, scleraxis, enhances aggrecan gene expression in osteoblastic osteosarcoma ROS 17/2.8 cells. J Biol Chem 272:29880–29885.

Matsue M, Kageyama R, Denhardt DT, Noda M. 1997. Helix-loop-helix-type transcription factor (HES-1) is expressed in osteoblastic cells, suppressed by $1,25(OH)_2$ vitamin D3, and modulates $1,25(OH)_2$ vitamin D3 enhancement of osteopontin gene expression. Bone 20:329–334.

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.

McMahon AP, Champion JE, McMahon JA, Sukhatme VP. 1990. Developmental expression of the putative transcription factor Egr-1 and c-fos are coregulated in some tissues. Development 108:281–287.

Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, Doetchmann T, Coffin JD, Hurley MM. 1999. Disruption of the fibroblast growth factor-2 gene in mice results in decreased bone mass and bone formation. J Bone Miner Res: 14 (suppl 1); S161.

Mundy GR. 1995. Local control of bone formation by osteoblasts. 1995. Clin Ortho Rel Res 313:19–26.

Murray SS, Glackin CA, Winters KA, Gazit D, Kahn AJ, Murray EJ. 1992. Expression of helix-loop-helix regulatory genes during differentiation of mouse osteoblastic cells. J Bone Miner Res 3:53–61.

Nose K, Shibanuma M. 1994. Induction of early response genes by hypergravity in cultured mouse osteoblastic cells (MC3T3-E1). Exp Cell Research 211:168–170.

Nose K, Shibanuma M, Kikuchi K, Kageyama H, Sakiyama S, Kuroki T. 1991. Transcriptional activation of early response genes by hydrogen peroxide in a mouse osteoblastic cell line. Eur J Biochem 201:99–106.

Ogata T. 1997. Fluid flow induces enhancement of the egr-1 mRNA level in osteoblast-like cells: involvement of tyrosine kinase and serum. J Cell Physiol 170:27–34.

Paznekas WA, Cunningham ML, Howard TD, Korf BR, Lipson MH, Grix AW, Feingold M, Goldberg R, Borochowitz Z, Aleck K, Mulliken J, Yin M, Jabs EW. 1998. Genetic heterogeneity of Saethre-Chotzen Syndrome, due to TWIST and FGFR mutations. Am J Hum Genet 62:1370–1380.

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. 1992. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 7: 683–692.

Shishido E, Higashijima S, Emori Y, Saigo K. 1993. Two FGF-receptor homologues of Drosophila: one is expressed in mesodermal primordium in early embryos. Development 117:751–761.

Spicer DB, Rhee J, Cheung WL, Lassar AB. 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science 272:1476–1480.

Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. 1996. Transcriptional control of osteoblast growth and differentiation. Physiol Rev 76:593–629.

Suva LJ, Ernst M, Rodan GA. 1991. Retinoic acid increases zif268 early gene expression in rat preosteoblastic cells. Mol Cell Biol 11:2503–2510.

Tamura M, Noda M. 1999. Identification of DERMO-1 as a member of helix-loop-helix type transcription factors expressed in osteoblastic cells. J Cell Biochem 72:167–176.

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