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Fibroblast Growth Factor Downregulates Expression of a Basic Helix-Loop-Helix-type Transcription Factor, Scleraxis, in a Chondrocyte-Like Cell Line, TC6

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Abstract Scleraxis is a basic helix-loop-helix-type transcription factor that is expressed in sclerotome. Fibroblast growth factor (FGF) is one of the cytokines produced by the cells in skeletal tissues and is a potent modulator of skeletogenesis. The aim of this study was to examine the effects of FGF on the expression of scleraxis in chondrocyte-like cells, TC6. In these cells, scleraxis mRNA was constitutively expressed as a 1.2kb message at a high level in contrast to its low levels of expression in fibroblast-like cells or osteoblast-like cells. Upon treatment with FGF, scleraxis mRNA level was decreased within 12 h. This effect was at its nadir at 24 h and the scleraxis mRNA level returned to its base line level by 48 h. The FGF effect was maximal at 1 ng/ml. FGF effects on scleraxis were blocked by actinomycin D but not by cycloheximide, suggesting the involvement of transcriptional events that do not require new protein synthesis. The FGF effects on scleraxis were blocked by genistein, suggesting the involvement of tyrosine kinase in the post-receptor signaling. TGFβ treatment of TC6 cells enhanced scleraxis mRNA expression; however, combination of the saturation doses of FGF and TGFβ resulted in suppression of scleraxis mRNA level. BMP2 also suppressed scleraxis mRNA expression in TC6 cells and no further suppression was observed in combination with FGF. These results indicate that scleraxis is expressed in chondrocyte-like TC6 cells and it is one of the targets of FGF action in these cells. J. Cell. Biochem. 70:468–477.

Key words: scleraxis; transcription factor; FGF; chondrocyte; bHLH

Skeletal formation requires multiple steps in the commitment of mesenchymal cells to become chondrocytes or osteoblasts. During these pathways, cartilage and bone specific genes should be activated in an orderly fashion. Members of the basic helix-loop-helix transcription factors (bHLH-TFs) have been shown to play important roles in the control of cell-specific gene expression in a wide variety of cell types, including mesoderm-derived cells [Kadesch, 1993]. The myogenic bHLH-TFs, Myo D, myogenin, Myf5, and MRF4 [Olson and Klein, 1994] are among the most characterized bHLH-TFs. Neurogenin [Ma et al., 1996], Neuro D [Lee et al., 1995], and members of the Achaete Scute family in mammals and Drosophila [Jan and Jan, 1993] are also known as the neurogenic bHLH-TFs.

To date, no skeletal cell related HLH factors have been identified; however, one of the possible factors is scleraxis, a recently identified bHLH-TF that is expressed in sclerotome during mouse embryogenesis [Cserjesi et al., 1995]. Scleraxis expression is first detected at about day 10.5 p.c. in the sclerotome; then its expression is observed at high levels in intervertebral

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discs, forming vertebrae and rib primordia. In addition, scleraxis is expressed in chondrogenic precursors of the appendicular skeleton and in condensing cartilage of the nose and face and tendons. Scleraxis expression is downregulated in chondrogenic precursors before ossification is initiated, and it is not detected in cranial bones or their progenitors, which develop without a cartilaginous intermediate, suggesting that scleraxis is involved in relatively early skeletal cell formation [Cserjesi et al., 1995].

Skeletal cells regulate their proliferation and differentiation in an autocrine and/or paracrine manner by expressing a number of local factors including fibroblast growth factors (FGFs), which constitute a family of at least nine polypeptide growth factors [Mason, 1994]. These FGFs are the members of the heparin-binding family of growth factors and have been shown to be potent growth stimulators and differentiation modulators in a wide variety of cells derived from mesoderm, including myoblasts, osteoblasts, and chondrocytes [Gospodarowicz, 1990]. Among these, basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF), with a molecular size of ~16.5 kDa and 15.5kDa, respectively, are demonstrated to be expressed in cartilage and growth plate [Jingushi et al., 1995; Baird and Bohlen, 1991]. Both aFGF and bFGF promote repair of damaged cartilage [Cuevas et al., 1988; Jingushi et al., 1990], and bFGF is required for the complete chondrogenic differentiation of cultured mesenchymal cells derived from limb and developing optic capsule [Frenz et al., 1994].

The observations that mutations in human fibroblast growth factor receptor (fgfr) genes are associated with skeletal abnormalities indicate that FGF signaling pathways are important in skeletal development. Mutations in fgfr1, fgfr2, and fgfr3 result in Pfeiffer syndrome [Muenke et al., 1994], Crouzon syndrome [Reardon et al., 1994], achondroplasia, thanatophoric dysplasia, or hypochondroplasia [Rousseou et al., 1994; Shiang et al., 1994; Tavormina et al., 1995]. Recent observations on the FGF receptor 3-null mice suggest that FGF regulates endochondral ossification via a negative mechanism [Deng et al., 1996].

In this report, we examined whether FGF affects expression of certain types of bHLH-TF(s) can be observed in a chondrocyte-like cell line, TC6 [Mataga et al., 1996]. TC6 is a chondrocytic cell line recently established from the articular cartilage of the transgenic mice harboring a temperature-sensitive mutant of SV40 large T-antigen gene. TC6 cells constitutively express cartilage-specific genes encoding type II procollagen, link protein, aggrecan, and versican. Moreover, TC6 cells not only autonomously progress into more differentiated stage, but also form nodules and produce matrices stained for alcian blue and show metachromasia upon staining with toluidine blue. We have previously reported that scleraxis is expressed in osteoblast-like cells and mesenchymal precursor-like cells [Liu et al., 1996, 1997a, 1997b]. In this paper, we report that TC6 cells constitutively express scleraxis mRNA at relatively high levels and that FGF suppresses scleraxis mRNA expression in these cells.

MATERIALS AND METHODS

Recombinant human basic FGF (FGF), recombinant human acidic FGF (aFGF), and recombinant human TGFβ1 (TGFβ) were purchased from R&D Systems (Minneapolis, MN). Recombinant human bone morphogenetic protein-2 (BMP-2) was a kind gift from Dr. J. Wozney (Genetics Institute, Cambridge, MA). Cell culture reagents (media, serum, trypsin) were purchased from Gibco Life Technologies (Grand Island, NY). Tissue culture plastic wares were obtained from Costar Corporation (Cambridge, MA). [α-^{32}P]-dCTP and [γ-^{32}P]-dATP were purchased from NEN Research Products (Tokyo, Japan). Guanidinium thiocyanate, agarose, cycloheximide, actinomycin D, DRB(5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), genistein, and H-7 were obtained from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes were purchased from New England Bio Labs (Beverly, MA). Oligolabelling kit, MMLV reverse transcriptase and Taq DNA polymerase were obtained from Pharmacia Biotech Co. (Piscataway, NJ). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell Cultures

TC6 cells were cultured in a humidified atmosphere of 5% CO_2 at 33°C in *a*-modification of Eagle's medium (*a*-MEM) supplemented with 0.5% fetal bovine serum (FBS). C3H10T1/2 cells were obtained from Japan Health Science Foundation (Osaka, Japan). Murine osteoblastic MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Oh-U University, Japan) and were

cultured in α-MEM medium supplemented with 5% FBS. Rat osteoblastic osteosarcoma ROS17/2.8 cells were kindly provided by Dr. G. Rodan (Merck Research Laboratories, West Point, PA) and were cultured in modified Ham's F12 medium supplemented with 5% FBS [Noda et al., 1987]. All the cells used in this study except TC6 were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

RNA Extraction

Total cellular RNA was extracted according to AGPC (acid guanidinium thiocyanate-phenol-chloroform) method [Chomczynski and Sacchi, 1987]. Briefly, after discarding the medium, the cells were rinsed with PBS three times and were lysed in 2 ml of solution D (4 M guanidinium thiocyanate/0.1 M β-mercaptoethanol). After shearing DNA by passing through 23-gauge needles, the lysates were collected from each dish and transferred to polypropylene tubes. After addition of 0.2 ml of 2 M sodium acetate, 2 ml water-saturated-phenol (pH 4.0), and 0.4 ml of chloroform:isoamyl alcohol (49:1), the lysates were mixed by shaking vigorously for 10 sec cooled on ice for 15 min , and centrifuged at 10,000x*g* for 20 min at 4°C to separate aqueous phase, which were then mixed with 2 ml isopropanol and kept at −20°C for 2 h followed by centrifugation at 10,000x *g* for 20 min. The resulting RNA pellets were rinsed with 75% ethanol, vacuum dried (15 min), and dissolved in 30 µl TE (10mM Tris Cl, pH 7.6/1 mM EDTA, pH8.0).

Northern Blot Analysis

Total RNAs were electrophoresed in 1% agarose gel containing 0.22 M formaldehyde in 1x MOPS (20mM 3-(N-morphilino)propanesulfonic acid/15 mM sodium acetate/1 mM EDTA). Direct staining with ethidium bromide (EtBr) was performed to monitor RNA integrity as well as equality of loading [Noda, 1989]. Overnight transfer of the RNAs to Hybond N nylon filters (Amersham, Arlington Heights, IL) was carried out in 1x TAE (0.04 M Tris-acetate/0.001 M EDTA). The filters were prehybridized overnight in 50% formamide/5x Denhardt's solution/5x SSC/0.1 % sodium dodecyl sulphate (SDS)/50 µg/ml herring sperm DNA at room temperature. Murine scleraxis cDNA insert (1,200 base pairs, *Eco*RI/*Xho* I fragment) was gel purified and radiolabeled with [α-^{32}P]-dCTP according to the method described by Feinberg and Vogelstein [1983]. The specific activities of the probes were ~10^8 cpm/µg DNA. Overnight hybridization was performed at 42°C in a fresh buffer containing all the same ingredients in the prehybridization buffer in addition to the radiolabeled probes (~10^6 cpm/ml). Filters were washed three times at room temperature in 1x SSC/0.5% SDS, and once for 20 min at 65°C in 0.2x SSC/0.5% SDS, and were exposed to X-ray films for several days at −80°C using intensifying screens. The bands in the autoradiograms of the Northern blots were quantified by using a laser densitometer, Ultrascan XL (Pharmacia/LKB, Uppsala, Sweden).

RT-PCR Assay

Total RNAs from TC6 and random hexamers were denatured at 95°C. RT buffer, dNTPs, MMLV reverse transcriptase were added to each sample. Samples were incubated at 42°C for 50 min and 95°C for 5 min. After quenching the reactions on ice, the resulting cDNA was mixed with PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0% Triton X-100), 2.5 mM $MgCl_2$, 10 pmols single primer pair, which amplify conserved sequences in the tyrosine kinase domains of four FGFRs [primer-1(5′-TCNGAGA TGGAGRTGATGAA-3′), primer-2 (5′ CCAAAGTCHGCDATCTTCAT-3′), respectively] [McEwen and Ornitz, 1997]. The reactions were denatured while the following mixture (10x PCR buffer, 2.5 mM dNTPs, 0.2 ml(1 unit) Taq polymerase) was added. The reactions were then subjected to 30 cycles of 94°C x 1 min, 55°C x 2 min, and 72°C x 2 min, followed by a final extension at 72°C for 5 min. After confirming product amplification, the PCR reaction was digested with either Pvu II, Pst I, or EcoR I for 1 h at 37°C, then electrophoresed on a 10% nondenaturing PAGE in 1x TBE . The size of the PCR amplified fragment is 341 bp. In mice, FGFR1, 2 3 and 4 will give 195 /146 bp PvuII fragments, 233/108 PvuII fragments, 175/166 PstI fragments, and 216/125 EcoRI fragments, respectively.

RESULTS

Scleraxis mRNA Expression in TC6 Cell Line

We first examined whether scleraxis is expressed in TC6 cells. TC6 cells constitutively express scleraxis mRNA as a 1.2kb message at a level three- to fourfold higher than the levels

of expression in fibroblast-like C3H10T1/2 cells or osteoblast-like ROS17/2.8 cells (Fig. 1A). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 1.01, 0.20, 0.17, 0.31 for TC6 cultured in 5% FBS (lane 1), TC6 cultured in 0.5% FBS (lane 2), C3H10T1/2 (lane 3), MC3T3-E1(lane 4), and ROS17/2.8 (lane 5), respectively (Fig. 1A, B).

TC6 Expresses Genes Encoding FGF Receptors

The actions of FGFs are mediated via a family of closely related cell surface receptors, containing tyrosine kinase domain (FGFRs) [Burrus et al., 1992]. We examined whether these receptor genes are expressed in TC6. As shown in Figure 2, RT-PCR analysis indicated that TC6 cells express genes encoding FGFR-1 and-2, whereas the bands for FGFR 3 and 4 were barely detectable.

FGF Regulates Scleraxis mRNA Expression

We previously reported that FGF suppressed aggrecan mRNA level in TC6 cells [Mataga et al., 1996]. As aggrecan is the target gene of scleraxis [Liu et al., 1997], we examined whether bFGF affects scleraxis gene expression. Scleraxis mRNA level was decreased within 12 h of the initiation of the treatment with FGF by ~50% of the control. The effect lasted up to 24 h and then the scleraxis level returned to the control level by 48 h (Fig. 3A). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 0.98, 0.42, 1.03, 0.46, 1.19, 1.12 for time 0 (control), 12 h (control), 12 h (FGF), 24 h (control), 24 h (FGF), 48 h (control), and 48 h (FGF), respectively (Fig. 3B). Acidic FGF also downregulated scleraxis gene expression similarly in a time-dependent manner (data not shown). TC6 cells were also treated for 24 h with FGF at various concentrations ranging from 0.01 to 10 ng/ml. The FGF effect on scleraxis mRNA level was maximal at 1 ng/ml (Fig. 4). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 1.05, 1.03, 0.43, 0.74 for the FGF concentrations of 0, 0.01, 0.1, 1, 10 ng/ml, respectively (Fig. 4).

Transcriptional Suppression of Scleraxis mRNA Expression by FGF

The mode of FGF action in the regulation of the scleraxis gene expression was examined by treating the cells with FGF in the presence or absence of inhibitors for transcription or protein synthesis. Each inhibitor was added to the

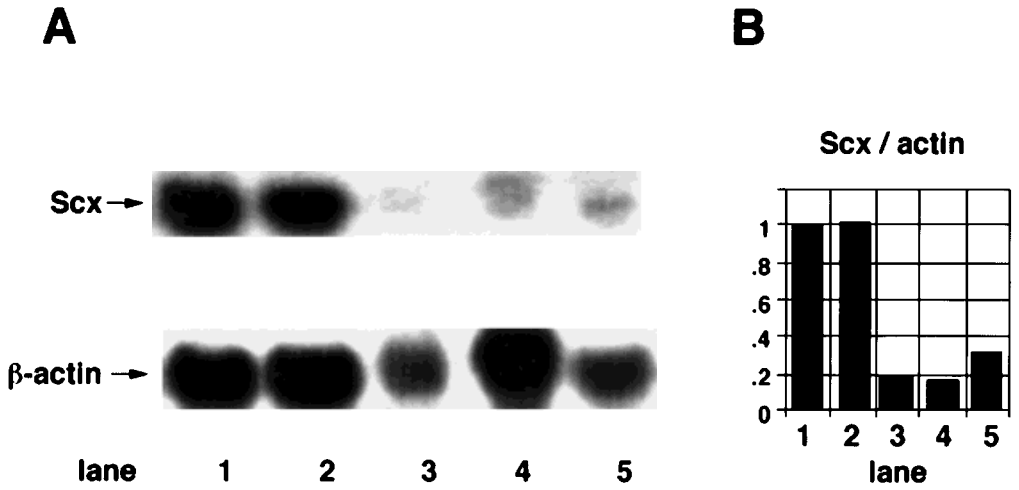
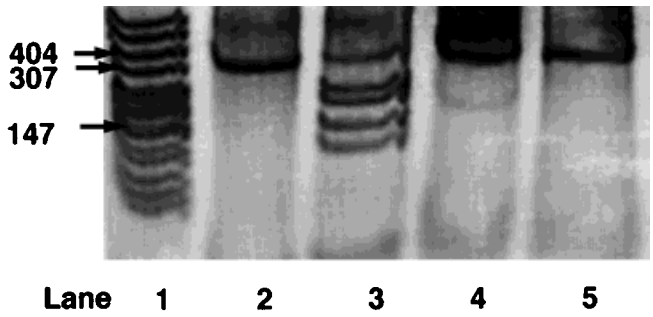


Fig. 1. High level expression of scleraxis (Scx) gene in **TC6** Murine chondrocytic(TC6)(**lane 1**, 5%FBS; **lane 2**, 0.5%FBS), murine fibroblastic (C3H10T1/2) (**lane 3**), murine osteoblastic (MC3T3-E1) (**lane 4**), and rat osteoblastic (ROS17/2.8) cells (**lane 5**) were cultured to confluence under the condition described in Materials and Methods. **A.** Total RNA was extracted according to AGPC method and Northern blot analysis was conducted as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. β-actin mRNA level is served as control. **B.** Quantitation of the bands in A. The ratios of Scleraxis(Scx) over actin are indicated. The data represent one of two independent experiments with similar results.



Lane 1. pBR322 /Msp I digest
2. RT- PCR product No digest
3. Pvu II digest
4. Pst I digest
5. EcoR I digest

Enzyme	FGFR1	FGFR2	FGFR3	FGFR4
Pvu II	195/146	233/108	-	-
Pst I	-	-	175/166	-
EcoR I	-	-	-	216/125

Fig. 2. RT-PCR analysis of FGF receptor expression in TC6 cells. RT-PCR analysis was conducted as described in Materials and Methods. List of enzymes and expected lengths of the fragments are shown. The data represent one of two independent experiments with similar results.

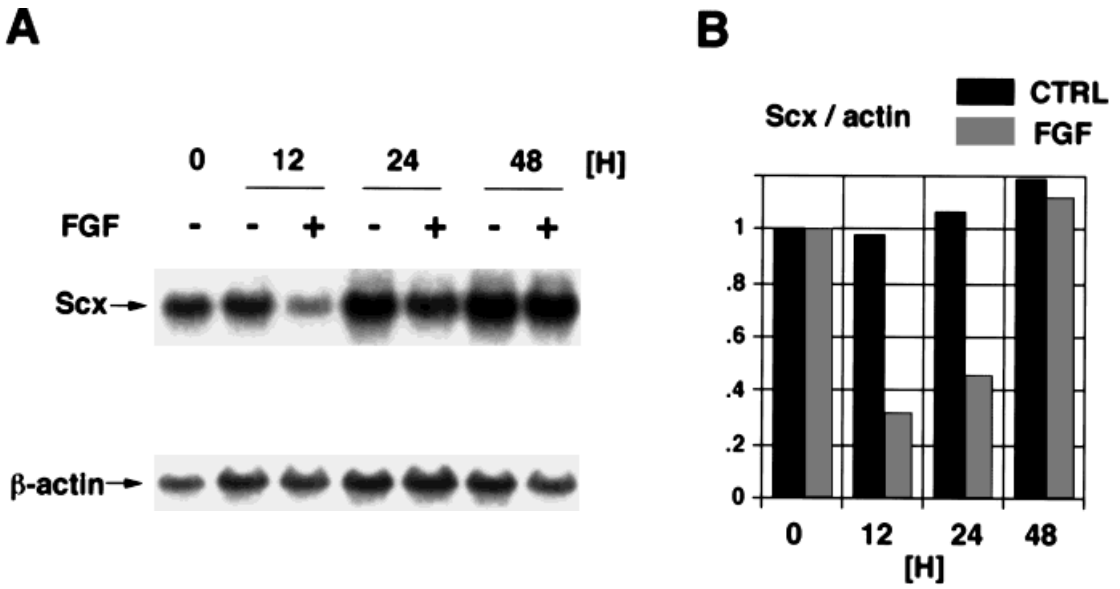


Fig. 3. Time course of FGF effect on scleraxis gene expression. Confluent TC6 cells were cultured in the absence or presence of FGF (2 ng/ml) for the indicated periods of time (h). Total RNA was extracted according to AGPC method and Northern blot analysis was conducted as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. β-actin mRNA level is served as control. **B.** Quantitation of the bands in **A.** The data represent one of two independent experiments with similar results.

medium 15 min before FGF treatment. The FGF effects on scleraxis were blocked by actinomycin D(AD) but not by cycloheximide (CX), indicating the involvement of transcriptional events, which does not require new protein synthesis (Fig. 5A). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 0.52, 0.9, 0.54, 0.88, 078 for control, FGF, CX, CX+FGF, AD, and AD+FGF, respectively (Fig. 5B). We also examined the FGF effect on stability of scleraxis mRNA using a transcription inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB was added to the medium 15 min before FGF treatment. Scre-

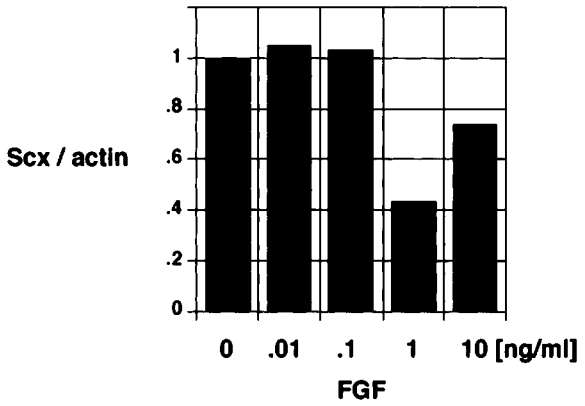


Fig. 4. FGF effect on scleraxis gene expression at various doses. Confluent TC6 cells were treated for 24 h with the indicated concentrations of FGF and Northern blot analysis was conducted as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. β-actin mRNA level is used as control. Quantitation was carried out by using laser densitometer. The data are the averaged values of two independent experiments with similar results.

laxis mRNA stability was not altered by FGF (Fig. 6A,B), suggesting that FGF would possibly exert its effect on scleraxis expression via transcriptional events.

Modulation by Kinase Inhibitors on Sleraxis mRNA Epression

Upon binding of the ligands, FGF receptors signal through their tyrosine kinase domains. To examine the involvement of tyrosine kinase-dependent signaling in FGF action on scleraxis gene expression, the cells were treated for 24 h with FGF in the presence or absence of protein kinase inhibitors. Each inhibitor was added to the medium 15 min before FGF treatment. The FGF effects on scleraxis was blocked by genistein but not by H7, suggesting the involvement of tyrosine kinase(s), at least in part, in mediating the post-receptor signaling (Fig. 7). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 0.68, 1.95, 1.95, 0.52, 0.25 for control, FGF, genistein, genistein+FGF, H7, and H7+FGF respectively (Fig. 7).

FGF Suppression of TGFβ Enhancement of Scleraxis mRNA Expression

Growth factors are known to act co-operatively to modulate cell functions [Noda & Vogel, 1989]. Therefore, we examined whether other calciotropic factors influence scleraxis gene expression in combination with FGF. As shown in Figure 8A, TGFβ treatment enhanced scleraxis mRNA expression in TC6 cells; however, combination of the saturation doses of FGF and TGFβ resulted in suppression of scleraxis mRNA level similar to that of the cells treated with FGF alone. BMP-2 also suppressed scleraxis gene expression in TC6 cells, whereas no further suppression was observed in the presence of FGF (Fig. 8A). The averaged ratios obtained by quantification of the scleraxis mRNA bands normalized against β-actin mRNA levels from the two independent experiments are 1, 0.52, 1.77, 0.52, 0.73, 0.63 for control, FGF, TGFβ, TGFβ+FGF,BMP-2, and BMP-2+FGF, respectively (Fig. 8B).

DISCUSSION

Mesenchymal cell differentiation and proliferation are under the control of certain sets of transcription factors, including basic helix-loop-helix transcription factors (bHLH-TFs). Scleraxis is a bHLH-TF cloned by two-hybrid screening and is expressed in sclerotome during mouse embryogenesis. Our study demonstrates that scleraxis is constitutively expressed at a relatively high level in the chondrocyte-like cell line, TC6. This is in contrast to the low levels of expression in fibroblast-like (C3H10T1/2) or osteoblast-like (MC3T3-E1, ROS17/2.8) cells.

We also found that FGF suppressed scleraxis expression. As we have previously shown, scleraxis regulates aggrecan gene expression [Liu et al., 1997] and FGF suppress aggrecan in TC6 cells [Mataga et al. 1996]. These observations in combination with the results presented here would be correlating the FGF effect on scleraxis gene expression to a chondrocytic phenotype regulation. In accordance with these observations, TC6 cells express at least FGFR1 and 2. FGF suppression of scleraxis suggests that FGF suppression of scleraxis mediates at least a part of FGF actions, although this point requires further experimental evidence. Basic FGF is present in the layers of resting, proliferating, and hypertrophic chondrocytes, but not in the layer of prehypertrophic chondrocytes [Twal et al., 1994]. The actions of FGFs are mediated via a family of four closely related FGF receptors (FGFR). Each of FGFR1, FGFR2, FGFR3, and FGFR4 shows a unique pattern of expression during embryogenesis, suggesting that these receptors mediate different functions of FGFs during development. In cartilage

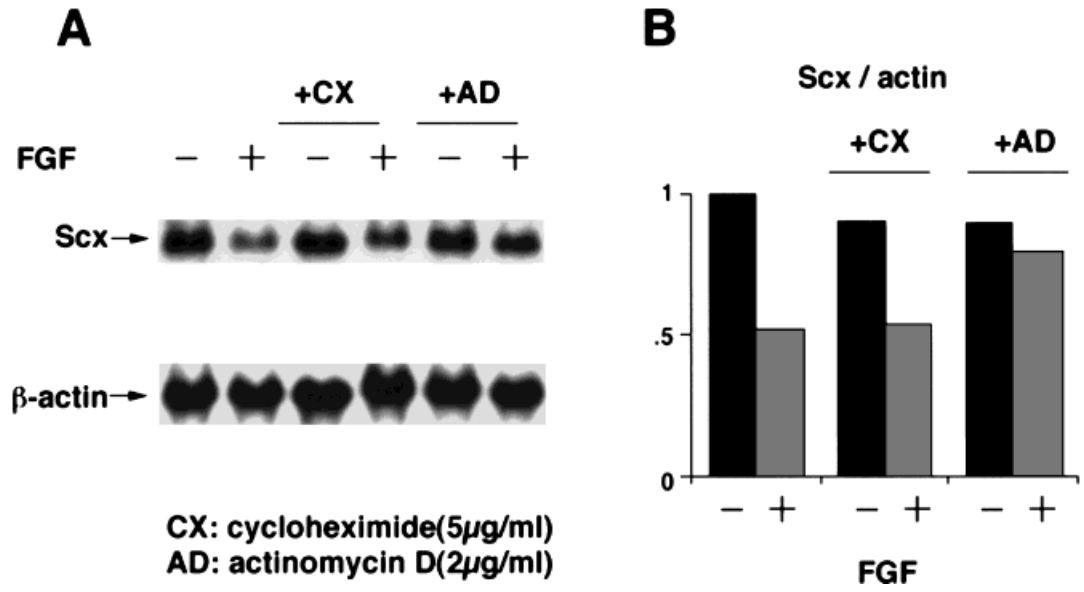


Fig. 5. Modulation by cycloheximide or actinomycin D of the FGF effect on scleraxis gene expression. Confluent TC6 cells were treated for 24 h in the presence or absence of 5 μg/ml cycloheximide[CX], or 2 μg/ml actinomycin D[AD]. **A**. Northern blot analysis was carried out as described in Materials and Methods. β-actin mRNA level served as control. **B**. Averaged values obtained by quantitation of the bands. The data represent one of two independent experiments with similar results.

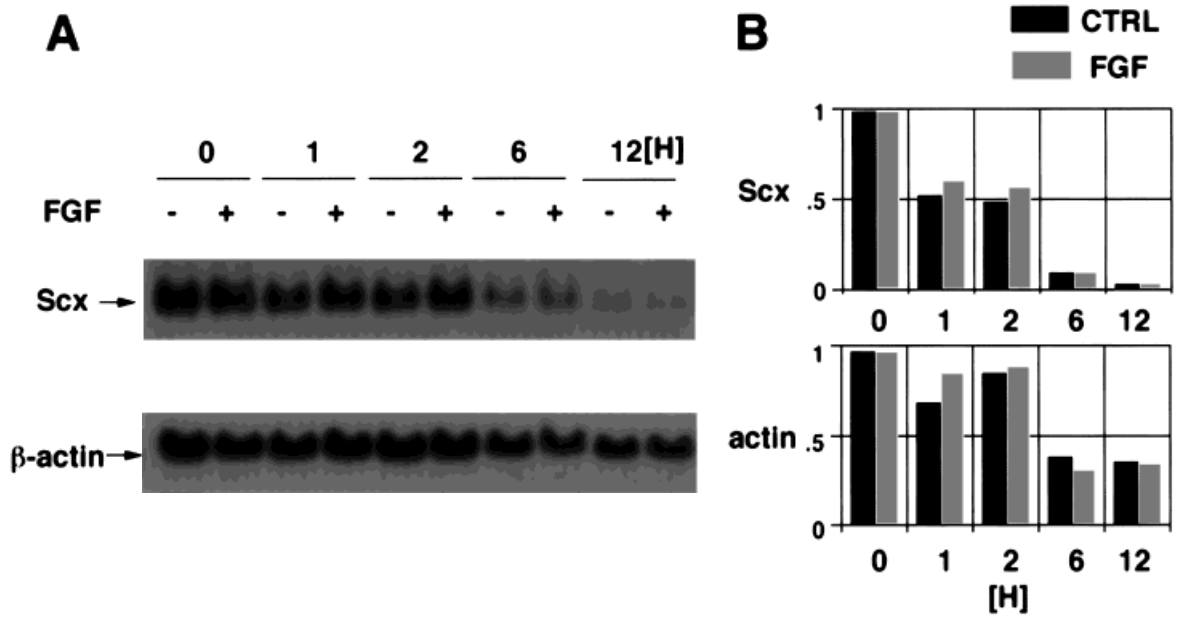


Fig. 6. Effects of FGF on scleraxis mRNA stability. Confluent TC6 cells were treated with 2 ng/ml FGF for 1, 2, 6, and 12 h in the presence of DRB (dichlororibofuranosyl benzimidazole). **A**. Northern blot analysis was carried out as described in Materials and Methods. **B**. Quantitation of the bands in A. The data represent one of two independent experiments with similar results.

development, the expression of FGFRs are intrinsic properties of differentiating cartilage. In somite differentiation, FGFR1 is expressed in both dermatome and sclerotome, whereas FGFR2 is preferentially expressed in sclerotome [Peters, 1992]. During endochondral ossification, FGFR1, FGFR2, and FGFR3 are expressed in precartilage blastema of vertebrae, long bones, and mandible. In the later stage, FGFR3 is detected exclusively in resting carti-

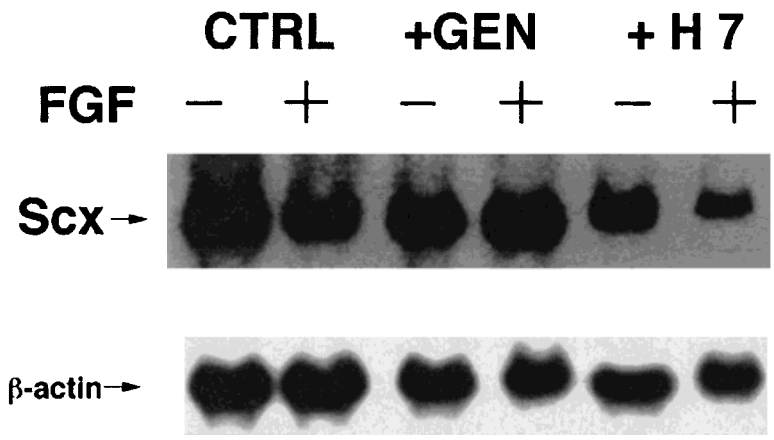


Fig. 7. Modulation by genistein or H-7 of the FGF effect on scleraxis gene expression. Confluent TC6 cells were treated with vehicle (CTRL) or FGF (2 ng/ml) for 24 h in the presence or absence of genistein [GEN] (100 ng/ml) or H-7 (100 μM). Northern blot analysis was carried out as described in Materials and Methods. β-actin mRNA level served as control. The data represent one of two independent experiments with similar results.

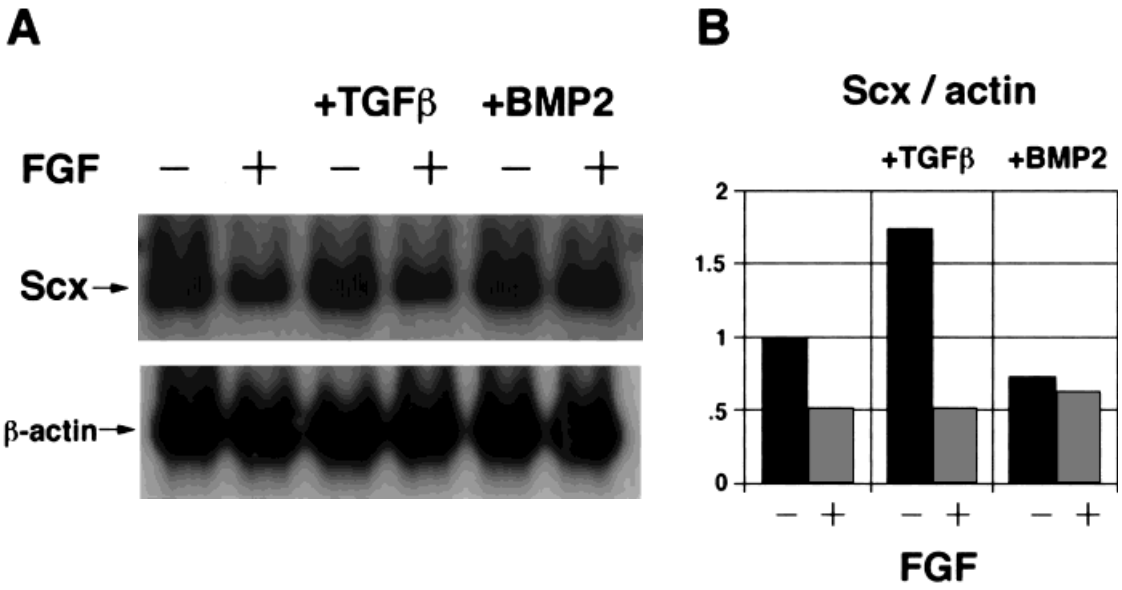


Fig. 8. Combinatory effects of FGF and calciotropic factors (TGFβ or BMP-2) on scleraxis gene expression. Confluent TC6 cells were treated for 24 h with vehicle alone, TGFβ1 (4ng/ml), or BMP-2 (200 ng/ml) in the presence or absence of 2 ng/ml FGF. **A.** Northern blot analysis was carried out as described in Materials and Methods. β-actin mRNA level served as control. **B.** Quantitation of the bands in two independent experiments.

lage [Peters et al., 1993] and FGFR1 is expressed predominantly in hypertrophic cartilage, osteocyte, and osteoblasts. In contrast, FGFR2 expression is concentrated in the perichondrium and periosteum and in cells within the degenerative matrix (presumptive marrow) between the hypertrophic cartilage and the newly forming bone [Peters, 1992]. These observations indicate importance of FGF in the regulation of chondrocytes while no critical transcription factors have been identified in chondrocytes to be under the control of FGF. Although FGFR3 expression was barely detectable in TC6 cells, very low levels of FGFR3 expression cannot be excluded since a faint band of FGFR3 was still observed in lane 4 of Figure 2. Whether our result is relevant to the observations in FGFR3 mutant animals [Deng et al., 1996] and patients [Tavormina et al., 1995] still needs further investigations.

Although our data suggest scleraxis could be one of the candidates for the chondrocyte-related transcription factors, further evidences by using primary cultures and other chondrocyte cell lines are also necessary to prove this issue.

Gene regulation could be controlled in several steps including transcriptional, translational, and posttranscriptional events. Among these, transcriptional control is the major event

in the regulation of many genes. Our data, although not proved, suggests that FGF transcriptionally downregulates scleraxis gene expression in a time- and dose-dependent manner via tyrosine kinase and that scleraxis gene is one of the targets of FGF actions in TC6. H7 by itself also suppressed to some extent the scleraxis expression in TC6 cells.This implies that certain basal levels of scleraxis expression are maintained based on the certain kinase-dependent activity. However, this suppression is only partial. The blocking of FGF suppression of scleraxis expression by genistein still indicates the involvement of a tyrosine kinase-dependent pathway, although it does not exclude the possibility that other pathways could exist.

Recently missense mutations in the FGFR3, the constitutive activation of the kinase domain [Bonaventure et al., 1996], were found in patients with achondroplasia, thanatophoric dysplasia, or hypochondroplasia. Furthermore, FGFR gene knock out mice showed accelerated enchondral bone formation [Deng et al., 1996]. These and other [Wroblewski and Edwall-Arvidsson, 1995] data suggest that FGF is inhibitory in cartilage formation. Ligand specificity to FGF receptors is not very tight [Amaya et al., 1991; Rapraeger et al., 1991; Yayon et al., 1991; Burrus et al., 1992; Kiefer et al., 1993; Ornitz et al., 1996] and therefore FGF2 used in our study could activate FGFR1, 2, or 3. As previously mentioned, the possibility for a very low level of FGFR3 expression is not excluded in the TC6 cells. It would be intriguing to examine scleraxis levels in the cells expressing constitutively active FGF receptors in achondroplasia or thanatophoric dysplasia patients.

FGF suppression of scleraxis gene expression would be a part of the regulatory network that is governing the function of chondrocytic function under the influence of local and systemic regulatory molecules. As shown in Figure 8, TGF beta enhancement of scleraxis expression is blocked by FGF, suggesting the dominance of the FGF action over that of TGFbeta. Such interactions of FGF and other local regulatory factors may play a role in vivo to determine the final levels of scleraxis. As observed in the other tissue, the local regulation of chondrocytic function is taking place in concert with the systemic regulation in vivo. It is still to be determined what the in vivo roles of scleraxis gene product would be, especially in cartilage as well as sclerotome, in the regulation of the chondrocytic differentiation and hence the maintenance of homeostasis of cartilage.

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